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Neuraminidase Virus-like Particles as Candidate Influenza A Virus Vaccines

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the Graduate Division of Biological and Biomedical Science
Microbiology and Molecular Genetics
2021

Abstract

Neuraminidase Virus-like Particles as Candidate Influenza A Virus Vaccines

By Zachary Menne

Influenza A viruses (IAV) are a seasonal cause of acute respiratory disease and a significant contributor to human morbidity and mortality worldwide. Current strategies to mitigate IAV risk focus on prophylactic seasonal vaccines manufactured primarily in embryonated chicken eggs, requiring long lead times and that vaccines be updated and re-administered on a yearly basis due to antigenic changes in the hemagglutinin (HA) protein. A clear need exists for influenza vaccines that can be rapidly manufactured and result in broader immunity and protection against disease caused by influenza virus infection. In this dissertation, I describe the production and characterization of novel IAV virus-like particle (VLP) vaccines containing neuraminidase (NA) from recent seasonal IAV strains produced using a recombinant baculovirus expression system in *Trichoplusia ni* insect cells. I demonstrate that NA virus-like particles produced using the NA sequence derived from the H1N1 virus A/California/04/2009 (NA1) and the H3N2 virus A/Perth/16/2009 (NA2) are immunogenic in a murine model. Additionally, I demonstrate that the bivalent administration of NA1 and NA2 VLPs is protective in a murine model against lethal challenge with both heterologous H3N2 mouse adapted A/Hong Kong/1/1968 virus and homologous H1N1 mouse adapted A/California/04/2009. Furthermore, I show that bivalent administration of NA1 and NA2 VLPs reduced viral lung replication after A/Hong Kong/1/1968 challenge compared to monovalent administration of NA2 VLPs.

I also investigate NA2 VLP vaccination in a swine infection model. I compared the immunogenicity and virological responses against a commercially available licensed swine influenza vaccine. Pigs vaccinated with NA2 VLPs demonstrated a superior anti-NA immune response compared to vaccination with the commercial vaccine or mock vaccination. Furthermore, pigs vaccinated with NA2 VLPs and the commercial vaccine had a similar and significant reduction in virus replication in lung tissue and pulmonary histopathology scores compared to mock vaccinated controls post challenge with the recently circulating H3N2 swine virus, A/swine/NC/KH15/2016.

This work has demonstrated that anti-NA immunity is capable of conferring protection against IAV challenge and could augment existing influenza virus vaccines. NA VLPs have the potential to supplement current influenza virus vaccines and should be considered in future vaccine development.

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Abbreviations

CDC – Centers for Disease Control
cRNA – Complimentary RNA
DNA – Deoxyribonucleic Acid
ER – Endoplasmic reticulum
FDA – Food and Drug Administration
GMP – Good manufacturing practices
HA – Hemagglutinin
HAI – Hemagglutination inhibition
HPAI – Highly pathogenic avian influenza
IAV – Influenza A virus
IFN – Interferon
IN – Intranasal
kb – kilobase
M1 – Matrix 1
M2 – Matrix 2
MDCK – Madin-Darby Canine Kidney
mRNA – messenger RNA
NA – Neuraminidase
NAI – Neuraminidase inhibition
NEP – Nuclear export protein
NP – Nucleoprotein
NS1 – Nonstructural protein 1
NS2 – Nonstructural protein 2
PA – Polymerase acidic
PB1 – Polymerase basic 1
PB2 – Polymerase basic 2
Pol II – DNA dependent RNA polymerase II
PR8 – A/Puerto Rico/8/1934
QWIV – Quadrivalent whole inactivated virus
rBV – recombinant baculovirus
RdRp – RNA dependent RNA polymerase
rHA – recombinant hemagglutinin
rNA – recombinant neuramindase
RNA – Ribonucleic acid
Sf9 – *Spodoptera frugiperda* 9
Tni – *Trichoplusia Ni*
UTR – Untranslated region
vRNA – viral RNA
vRNP – viral ribonucleoprotein
WHO – World Health Organization

Chapter I: Introduction

Influenza Disease

Influenza virus infection is a leading cause of acute respiratory disease and responsible for an estimated 290,000 to 645,000 deaths globally per annum during seasonal epidemics (Iuliano et al., 2018). In addition to mortality caused by influenza virus disease, the World Health Organization (WHO) estimates that influenza virus causes approximately 1 billion infections with 3-5 million of those infections resulting in severe illness, although reliable data is not always available for every region on a global scale (Krammer et al., 2018b). Influenza disease also poses a significant economic burden on health care systems and the general economy as a whole. In the United States specifically, it is estimated that influenza related disease is responsible for over 11 billion USD in total economic costs each year, with 3.2 billion USD in direct healthcare related costs and 8 billion in indirect costs due to lost economic activity (Putri et al., 2018).

Mild acute respiratory disease caused by influenza virus typically affects the upper respiratory tract causing symptoms such as sore throat, fever, cough, runny nose, headache, muscle pain and fatigue (Krammer et al., 2018b). However, more severe cases of influenza virus infection can cause lethal infections due to pneumonia or to secondary bacterial infections (Shirey et al., 2019). Influenza viruses are members of the *Orthomyxoviridae* family and divided into four major types: influenza A, influenza B, influenza C, and influenza D (Hause et al., 2013). Of these four types, influenza C virus typically causes mild or asymptomatic infections in humans with influenza D virus causing infections in cattle and swine (Su et al., 2017). Most clinical disease in humans is attributed to infection with influenza A and influenza B viruses, with influenza A virus causing the large majority of infections globally (Mosnier et al., 2015).

Influenza A Virus

Influenza A viruses (IAV) are enveloped viruses with an 8 segment negative-sense single-stranded RNA genome (Webster et al., 1992). The 8 gene segments contain approximately 14 kilobases (kb) of genetic information encoding for at least 10 proteins (Hao et al., 2020). Of the 10 proteins including hemagglutinin (HA), neuraminidase (NA), matrix 2 (M2), matrix 1 (M1), polymerase acidic (PA), polymerase basic 1 (PB1), polymerase basic 2 (PB2), nucleoprotein (NP), nonstructural protein 1 (NS1), and nonstructural protein 2 (NS2), all but NS1 and NS2 are present in the IAV virion. In the context of human host cell infection, the infection cycle is initiated by the attachment of the viral HA protein to N-acetylneuraminic acid (α2,6-linked sialic acid) attached to host cell glycoproteins (Van Riel et al., 2010). The infecting virion is the endocytosed through clathrin-mediated endocytosis (Matlin et al., 1981). The endocytosed vesicle is then acidified triggering a low-pH dependent conformation change in the HA homo-trimer resulting in membrane fusion of the viral and host cell endosomal membranes (Dou et al., 2018). Additionally, during the acidification of the endosome, the M2 homo-tetramer protein functions as an ion channel to facilitate proton entry into the infecting virion to equilibrate the pH inside the virion (Pielak and Chou, 2011). The acidification across the viral membrane functions to disrupt the interaction between the M1 protein and the incoming viral ribonucleoprotein (vRNP) complexes consisting of the viral RNA (vRNA) coated in NP folded in a hairpin-like structure based on 5' and 3' sequence complementarity in complex with PA, PB1, and PB2 thus facilitating delivery of the genetic material into the host cell cytoplasm (Rossman and Lamb, 2011). The viral RNPs proceed to the host cell nucleus where viral replication is organized. While unusual for RNA viruses, nuclear localization of the viral RNPs is necessary to gain access to the host cell mRNA splicing machinery to facilitate alternative splicing of the M and NS gene segments to produce M1

or M2 and NS1 or NS2 mRNAs, respectively (Dubois et al., 2014). Once inside the host cell nucleus, the PB2 protein binds nascent 5' caps of host cell mRNA transcripts recruiting PA to cleave these short, nascent transcripts via an endonuclease domain. The cleavage of these short 5'-capped transcripts with a short untranslated region (UTR) from the host cell RNA polymerase II (pol II) has been termed "cap snatching" (De Vlugt et al., 2018). Usurping the host cell 5' cap and short RNA transcript as a primer, PB1 then elongates the viral mRNA transcript starting at the promoter using the vRNA as a template for transcription followed by polyadenylation at the 3' end of nascent transcripts via a stuttering mechanism on five to seven uracil residues on the vRNA template strand (Li and Palese, 1994). The viral polymerase complex also transcribes full length, positive-sense, complimentary RNA (cRNA) to serve as the template for genomic vRNA replication. The mechanisms governing the switch from mRNA synthesis to cRNA and vRNA synthesis is not completely understood. High concentration of free, soluble NP has been suggested to serve as a temporal regulator to initiate the switch between mRNA synthesis and cRNA and subsequent vRNA synthesis (Barrett et al., 1979). Alternatively, it has also been suggested that cRNA is transcribed throughout all stages of infection, however it is stabilized and protected from host cell nuclease degradation through the presence of nascently translated NP and viral polymerase (Vreede et al., 2004). Upon transcription and translation of viral proteins, NP also functions to bind nascent vRNA and viral polymerase proteins to form vRNP complexes (Biswas et al., 1998). The NS2 protein, also referred to as the nuclear export protein (NEP), is responsible for interacting with the host cell nuclear pore to export nascent vRNP complexes out of the nucleus (O'Neill et al., 1998). After export from the host cell nucleus, vRNP complexes are trafficked to the cell membrane where they assemble with the M1 protein localized at the apical cell membrane in conjunction with the cytoplasmic tails of HA, NA, and M2 localized at lipid raft domains of the

host cell membrane (Nayak et al., 2004). The M1 protein serves to form the interior structure of the nascent virion driving budding at the host cell apical membrane surface (Rossman and Lamb, 2011). After budding, the function historically ascribed to the NA protein is the cleavage of neuraminic acids (sialic acids) to promote nascent virion release as well as prevent the aggregation of nascent virions with one another (Palese et al., 1974). Lastly, the alternatively spliced NS segment transcript, the NS1 protein, is a multifunctional protein responsible for disrupting multiple host cell innate immune signaling pathways, most notably inhibiting interferon (IFN) production, and has been suggested to temporally regulate the IAV replication cycle (Hale et al., 2008).

The viral glycoproteins present on the virion surface and available for recognition by host antibodies, the HA, NA, and M2 proteins, are translated at the rough endoplasmic reticulum (ER) and follow the cellular secretory pathway where they eventually adorn the viral membrane. Of the IAV virion surface glycoproteins, HA and NA proteins are the most abundant with HA outnumbering NA by a ratio of approximately 4 - 5 to 1, respectively (Harris et al., 2006). In contrast, HA outnumbers the M2 ion channel protein by a ratio of approximately 10 – 100 to 1, respectively (Zebedee and Lamb, 1988). Subsequently, the HA and NA proteins are under the greatest selective pressure from host immunity and show the greatest sequence diversity amongst IAV proteins (Zhuang et al., 2019).

Influenza Epidemiology

With respect to all IAV proteins, HA and NA show the greatest sequence diversity and are further classified into different subtypes based on HA and NA sequence homology (Shaw and Palese, 2013). Based on sequence divergence, HA and NA are subdivided into 18 (H1 - H18) and 11 (N1 – N11) different subtypes, respectively. Of the HA molecules, 16 of the subtypes are found in the

natural IAV reservoir host, aquatic waterfowl, while H17 and H18 have only been identified in new world bats (Tong et al., 2013). Similarly, NA subtypes N1-N9 have all been found in aquatic waterfowl, while N10 and N11 have only been recovered from new world bat species (Tong et al., 2013). Interestingly, the HA and NA sequences recovered from new world bats are evolutionarily and functionally distant from the other IAV HA and NA sequences, with the NA molecule lacking any sialidase activity (Li et al., 2012).

Of the 16 HA subtypes and 9 NA IAV subtypes found in wild waterfowl, other hosts include birds, pigs, domestic fowl, horses, dogs, whales, seals, and mink (Long et al., 2019). Given the wide host range and ability of IAV viruses to exchange genetic RNA segments with other IAV viruses (but not Influenza B, C, or D viruses), the risk of introduction of a novel HA or NA subtype with pandemic potential in the human population exists (Steel and Lowen, 2014). However, in the past 100 years, only H1N1, H2N2, and H3N2 viruses have readily circulated amongst the human population with efficient human-to-human transmission. There have been numerous instances of novel human zoonotic infections of IAVs, most notably recent infections with H5N1 viruses and H7N9 viruses, however these IAVs have not transmitted efficiently from human to human (Jernigan and Cox, 2015; Neumann et al., 2010). Alternatively, the novel 1918 “Spanish flu” pandemic caused by an H1N1 virus was responsible for an estimated 50-100 million deaths and is thought to have infected approximately one-third of the world’s population at the time (Taubenberger and Morens, 2006). H1N1 strains continued to circulate within the population until their replacement by the emergence of a pandemic H2N2 strain (colloquially referred to as the “Asian Flu”) in 1957 which infected an estimated 25% of the population in the United States (Henderson et al., 2009). Similarly, the H2N2 strain was replaced in 1968 by the emergence of a pandemic H3N2 strain (colloquially referred to as the “Hong Kong flu”) resulting in an estimated

500,000 deaths in the United States alone (Jester et al., 2020). In 1977, another global H1N1 pandemic occurred (colloquially referred to as the "Russian flu"), likely as a result of a laboratory accident originating in Russia as the genetic sequence of the 1977 virus was found to be virtually identical to H1N1 viruses circulating in the 1950's (Nakajima et al., 1978). Interestingly, the 1977 H1N1 virus only caused appreciable disease and demonstrated an attack rate in individuals under 26 years of age, and led to the co-circulation of both H1N1 and H3N2 viruses in the human population observed today (Zakstelskaja et al., 1978). Furthermore, the circulating H1N1 viruses that re-entered the human population in 1977 have since been completely replaced by the emergence of the 2009 H1N1 pandemic virus (colloquially known as the "Swine Flu") (Girard et al., 2010). The 2009 H1N1 pandemic virus was the result of an antigenic shift reassortment event in swine that then entered the human population and is a noteworthy example that antigenically novel strains of a currently circulating subtype can emerge to cause a pandemic with dire consequences for the human population (Fineberg, 2014).

The co-circulation of both H1N1 and H3N2 viruses in human populations over the past 50 years is underscored by the continuous antigenic drift observed within IAV subtypes. The IAV RNA dependent RNA polymerase (RdRp) lacks any proofreading capability, resulting in a mutation rate of greater than 10^{-4} (Pauly et al., 2017). Subsequently, this implies that one infected host cell can produce greater than 10,000 mutant IAVs capable of spreading to new hosts or infecting neighboring cells in the same host (Boivin et al., 2010). As single amino acid changes in the HA protein can have marked effects on the binding affinity of both broadly reactive and specific antibodies, the viral diversity generated in a single infection event leads to the selection of IAV mutants capable of evading current host immunity resulting in a rapidly evolving, diverse

population of IAVs circulating in the human population (Blackburne et al., 2008; Doud et al., 2018).

Influenza Treatment

Strategies for preventing adverse outcomes and complicated influenza cases after infection in susceptible human populations include small molecule inhibitors of IAV replication. The first small molecules licensed by the United States Food and Drug Administration (FDA) for the treatment of IAV infection were adamantane derivatives targeting the M2 ion channel inhibiting the transfer of protons from the acidified endosome into the virion during viral entry (Jalily et al., 2020). The FDA approved the first small molecule M2 ion channel inhibitor, amantadine, for the treatment specifically of "Asian influenza virus" infection and it was later approved for treatment of influenza A virus infection in 1976 (Hubsher et al., 2012). However, widespread IAV resistance to amantadine was reported as early as 1980 (Kumar et al., 2018). Another small molecule M2 ion channel inhibitor adamantane derivative, rimantadine, was approved by the FDA for use in 1994, however resistance to both adamantanes is conferred by a single amino acid substitution, S31N, in the M2 transmembrane domain and M2 ion channel inhibitors are no longer recommended by the CDC for use in treatment of IAV infection (Bright et al., 2006).

Other molecules used to treat IAV infection target the enzymatic activity of NA, commonly known as neuraminidase inhibitors. Neuraminidase inhibitors were among the first "rationally" designed drugs, engineered as sialic acid analogs to competitively bind the NA enzymatic active site and prevent the binding of host sialic acids in the respiratory tract (Hussain et al., 2017). The first FDA approved neuraminidase inhibitor was zanamivir (Relenza) as a powder for inhalation in July of 1999, followed by oseltamivir (Tamiflu) in oral pill form in October of 1999 (Moss et

al., 2010). However, wide spread resistance to both neuraminidase inhibitors has been reported, with single point mutations conferring resistance to oseltamivir and zanamivir with a several fold reduction in binding affinity of both analogs (Collins et al., 2009). Indeed, over 99% of circulating H1N1 seasonal viruses demonstrated resistance and reduced susceptibility to neuraminidase inhibitors prior to emergence of the 2009 H1N1 pandemic virus (Samson et al., 2013).

More recently, the FDA approved the small molecule baloxavir marboxil (Xofluza) in October of 2018 in oral form. Baloxavir is a cap-dependent endonuclease inhibitor responsible for inhibiting the PA subunit of the viral polymerase from "cap snatching" the 5' cap and UTR of nascent host cell transcripts, thereby inhibiting IAV viral gene expression (Shirley, 2020). However, resistance can be conferred by a single amino acid substitution after a single dose, with almost 10% of patients in the phase III clinical efficacy trial yielding virus with the resistant point mutation I38T/M/F (Hayden et al., 2018).

While several small molecules are available to treat complicated IAV infection, all modalities have demonstrated a challenging resistance profile to support their sustained use on a wide scale. None of the currently available molecules are approved for use in combination with one another, and a single IAV amino acid substitution can drastically alter their efficacy in a clinical setting after infection. In some cases, small molecule inhibitors have been used prophylactically in close contacts of index patients with varying efficacy and have also been subject to the same challenge of selecting for viral escape mutants (Doll et al., 2017; Ikematsu et al., 2020). While zanamivir and oseltamivir are currently approved by the FDA for chemoprophylaxis of IAV infection, the approved duration of treatment is 7 days for both

molecules which necessitates a longer-term prophylactic solution for the duration of seasonal influenza epidemics.

Prophylaxis and Current Vaccine Strategies

The current and historic strategy to prophylactically address the threat posed by IAV to the global population relies mainly on seasonal vaccination and non-pharmaceutical interventions (NPI) such as hand hygiene (Grohskopf et al., 2013). However, the development of IAV vaccines first required the successful isolation and cultivation of influenza viruses. Influenza virus was first reportedly isolated from swine in 1931 by Richard Shope at Rockefeller and then from the nasal secretions of infected human patients in 1933 at Mill Hill by Wilson Smith and colleagues (Shope, 1931; Smith et al., 1933). However, no methods existed to successfully passage and maintain IAV virus stocks outside of crude passage in animal hosts (Smith et al., 1933). It wasn't until 1940 that IAV was propagated in the amniotic sac of embryonated chicken eggs, allowing for the creation and titration of viral stocks (Burnet, 1940; Nigg et al., 1940). Prior to viral passage in embryonated eggs, vaccination experiments were conducted using crude extracts from infected tissues using both replication competent virus as well as inactivated virus, both of which induced measurable but highly variable antibody responses in humans and animal models (Francis and Magill, 1937). Other attempts at producing live attenuated IAV vaccines by passage in mice and ferrets were experimentally given to military members and factory workers, however these trials were poorly controlled and produced variable data on efficacy while also resulting in mild disease (Hannoun, 2013). Early attempts at vaccination using inactivated crude viral extracts were largely unsuccessful due to the abundance of foreign tissue protein antigens compared to IAV proteins present in the vaccines (Andrewes and Smith, 1939). As observed by Andrewes and Smith, and

later highlighted by Stanley, the appropriate purification and concentration of viral vaccine antigen are crucial to achieving a robust immune response when the vaccine is manufactured in cells from non-host species (Andrewes and Smith, 1939; Stanley, 1945). Indeed, concentration of IAV virus using viral agglutination to red blood cells led to higher antibody responses in humans in a dose dependent manner and higher antibody responses than crude allantoic fluid alone (Hirst et al., 1942). Due to the unsuitability of these methods for mass production of potential IAV vaccines, Stanley reported a method for concentrating and purifying IAVs grown in embryonated chicken eggs by differential centrifugation followed by inactivation in 1944 that is relatively similar to the process still in use for the majority of IAV vaccines produced today (Stanley, 1945).

The first licensed whole-virus inactivated influenza vaccine became available in the United States to the general public in 1945 and contained an H1N1 IAV virus strain and an influenza B virus strain (Weir and Gruber, 2016). Other than the inclusion of updated strains in response to antigenic drift detected via WHO surveillance or the inclusion of the H2N2 subtype in 1957, the manufacturing process remained largely unchanged through the 1960s (Barberis et al., 2016). In the 1960s concerns over adverse events in children spurred the development of split virus vaccines and subunit vaccines, disrupted using detergents or ethers followed by further purification of HA and NA via downstream processing (Krammer and Palese, 2015). The first detergent split vaccines were authorized for use in the United States in 1968 (Crovari et al., 2011). Indeed, studies involving children at higher risk for adverse outcomes due to influenza virus infections demonstrated acceptable safety profiles for split virus vaccines (Allison et al., 1977). The production and subsequent use of purified subunit vaccines containing predominantly HA and NA antigen were also successfully used as a strategy to mitigate adverse reactions in children in the 1970s (Laver and Webster, 1976). However, both subunit and split virus vaccines proved to be

less immunogenic than whole virus inactivated vaccines. The re-emergence of H1N1 strains during the 1977 H1N1 pandemic and their continued circulation in the human population led to the inclusion of two IAV strains and an influenza B virus strain in seasonal vaccines as trivalent vaccines during the 1978 influenza season and thereafter (Hannoun, 2013). Additional improvements in vaccine production were also realized in the 1970s, with the advent of the use of genetic recombinants in vaccine production resulting in the increased yield of virus grown in embryonated eggs (Crovari et al., 2011). Additionally, live attenuated influenza vaccines (LAIV) have been in use to varying degrees since their approval in the US in 2003 and in Russia in 1980 (Jin and Subbarao, 2015). LAIVs are based on cold temperature adapted virus mutants produced in eggs and administered via the intranasal (IN) route allowing for replication at the lower temperatures in the upper respiratory tract but not in the lower respiratory tract (Maassab, 1967). In 2012, the United States FDA approved the first quadrivalent influenza seasonal vaccine including two IAV strains and two influenza B virus strains as a LAIV, with quadrivalent inactivated influenza vaccines receiving approval shortly thereafter (Treanor, 2021). However, the clinical benefit of quadrivalent influenza virus vaccines is still unclear, with no apparent benefit in immunogenicity against the more common epidemiologically dominant IAV strains (Tinoco et al., 2014). Additionally in 2009, the FDA approved a high dose trivalent influenza A vaccine (Fluzone High-Dose, Sanofi Pasteur, Inc.) manufactured in embryonated chicken eggs for use in persons aged 65 and older containing four times the HA antigen (60 µg) from each vaccine strain per dose compared to standard dose vaccines (15 µg of each strain) (Robertson et al., 2016). This was followed by the FDA approval of a quadrivalent version (Fluzone High-Dose Quadrivalent, Sanofi Pasteur) also containing 60 µg of each HA antigen per dose (for a total of 240 µg of HA protein per dose) in 2019 (Grohskopf et al., 2020). However, the high dose egg based vaccine showed

only a modest 9% increase in vaccine effectiveness over standard dose quadrivalent egg based vaccines in persons aged 65 or older during the 2017-2018 influenza season in the United States (Izurieta et al., 2019). While any modest increase in vaccine efficacy is welcomed, the modest efficacy increase is overshadowed by the overall vaccine efficacy of 38% for the entire United States during the 2017-2018 season (Rolfes et al., 2019).

Currently, approximately 87% of seasonal influenza vaccines distributed in the United States were manufactured in embryonated chicken eggs (Schuchat et al., 2016), with only two influenza vaccines currently approved for use by the FDA not manufactured in embryonated chicken eggs. In 2012, the FDA approved a trivalent influenza vaccine manufactured in Madin-Darby Canine Kidney (MDCK) cells made by Sequiris marketed under the brand name Flucelvax (Moro et al., 2015) and the FDA subsequently approved a quadrivalent version (Flucelvax Quadrivalent, Sequiris) in 2016 (Lamb, 2019). The only other influenza virus vaccine currently licensed for distribution in the United States is a purified, recombinant HA (rHA) subunit vaccine (Flublok Quadrivalent, Sanofi Pasteur) manufactured using a recombinant baculovirus (rBV) expression system in Spodoptera frugiperda 9 (Sf9) insect cells containing three times the HA antigen compared to inactivated split virus vaccines (45 µg per subtype) (Cox et al., 2015; Cox and Karl Anderson, 2007).

While progress has been made in vaccine technology as previously described, currently available seasonal influenza vaccines routinely exhibit suboptimal vaccine efficacy. According to the CDC, vaccine effectiveness in the United States has ranged from 19% - 48% over the past six influenza seasons from 2014-2020 (Flannery et al., 2020, 2017; Rolfes et al., 2019; Tenforde et al., 2020). Furthermore, in select seasons the vaccine provides essentially no protection against

antigenically drifted circulating virus subtypes such as the H1N1pdm09 clade 6B.1A viruses that predominated in the second half of the 2019-2020 influenza season in the northern hemisphere (Tenforde et al., 2020).

While many factors can contribute to variable vaccine efficacy, the dominance of egg-based manufacturing practices have been implicated as contributors to low efficacy for several reasons. First, evolutionary pressures select for viral adaptation to enhance growth in embryonated eggs giving rise to changes in vaccine target antigens, most recently seen in the adaptation of the hemagglutinin protein of the H3N2 component of the 2016-2017 seasonal vaccine and subsequent low vaccine efficacy (Flannery et al., 2017). Second, the long production lead time necessary to produce an adequate number of vaccine doses in embryonated chicken eggs provides time for antigenic drift in currently circulating viral strains in response to human host immune pressures. Antigenic drift was responsible for the observed poor efficacy of the H1N1 component of the seasonal influenza vaccine during the 2014-2015 and 2019-2020 influenza seasons (Tenforde et al., 2020; Zimmerman et al., 2016). Furthermore, due to the long lead times required for egg-based vaccine production, the seasonal vaccine strains for technologies more amenable to rapid scale up and manufacture of adequate doses are also selected in February for the northern hemisphere. The regulatory constraints of WHO strain selection to coincide with lead times necessary for egg-based manufacture severely hamper the potential of more efficient manufacturing technologies and their ability to respond to potential circulating virus antigenic drift observed closer to the start of the influenza season.

As vaccine efficacy measurements are made at a population scale, it can be difficult to collect reliable data regarding efficacy differences between different vaccine platforms. However,

limited data is available stratified by vaccine manufacturer for select at risk populations, specifically patients over 65 years of age enrolled in Medicare in the United States. During the 2017-2018 influenza season, an analysis of over 13 million eligible Medicare beneficiaries demonstrated a 10% relative vaccine effectiveness for quadrivalent MDCK cell culture based vaccines (Flucelvax Quadrivalent, Sequiris) compared to quadrivalent egg based vaccines. In a separate randomized study of healthcare workers aged 18-64, study participants vaccinated with quadrivalent rHA vaccine (Flublok, Sanofi Pasteur) yielded an increased seroconversion rate and higher anti-HA geometric mean antibody titers than participants vaccinated with quadrivalent MDCK cell based (Flucelvax, Sequiris) or quadrivalent egg based vaccines (Dawood et al., 2021). In that same study, participants vaccinated with MDCK cell based vaccines and egg based vaccines had statistically indistinguishable seroconversion rates as well as no difference in anti-HA geometric mean antibody titers (Dawood et al., 2021). Additionally, a different study evaluating CD4 T cell responses post vaccination in a cohort of randomized human volunteers demonstrated that rHA vaccines (Flublok, Sanofi Pasteur) induced greater anti-HA antibody responses and CD4 T cell responses than licensed MDCK cell based or egg based influenza vaccines (Richards et al., 2020). While these limited data sets have shown cell culture based vaccines or rHA vaccines to be slightly more efficacious than egg produced split influenza vaccines, large opportunities for improvements in vaccine efficacy still exist.

Historically, seasonal influenza vaccines have targeted immune responses against only the HA glycoprotein as HA is responsible for viral attachment to target cells and entry into host cells allowing anti-HA neutralizing antibodies to prevent infection (Krammer et al., 2015). Indeed, the WHO only standardizes the HA content in current seasonal influenza vaccines and requires that the second major glycoprotein NA only be present (Wohlbold and Krammer, 2014). The lack of

standardization of NA in vaccines has led to large lot to lot variability in vaccine NA content with varying anti-NA responses among recipients (Couch et al., 2012a). Not surprisingly, recent evidence has indicated that current seasonal influenza vaccines induce a poor or nonexistent response to the NA glycoprotein, while natural infection with IAV results in a robust anti-NA immune response (Chen et al., 2018). This is troublesome as higher anti-neuraminidase antibody levels have been demonstrated to be better correlates of protection upon challenge with IAV (Chen et al., 2018; Walz et al., 2018a). It will be necessary to focus on incorporating additional antigens beyond HA and leveraging more agile production platforms to make meaningful improvements in influenza vaccine effectiveness to prepare for future influenza virus epidemics and pandemics.

Neuraminidase Function, Structure, and Immunogenicity

The NA glycoprotein is the second most abundant glycoprotein on the influenza virion surface, outnumbered only by HA at a ratio of 4 - 5 to 1 (Harris et al., 2006). An enzymatic "receptor destroying enzyme" activity was first described by George Hirst in 1942 in a report detailing the kinetics of chicken red cell agglutination by influenza virus and subsequent loss of agglutination and ability of the red cells to be re-agglutinated by fresh influenza virus preparations after an initial incubation with influenza virus preparation (Hirst, 1942). Hirst's early work also suggested this activity was likely mediated by a heat labile proteinaceous enzyme (Hirst, 1942). Work by Burnet and others demonstrated that this enzymatic activity was essential to overcome the hemagglutination inhibition of mucins and mucoids (Burnet, 1948, 1951). The enzymatic activity of influenza virus NA was further characterized by Alfred Gottschalk. NA was shown to function similarly to the soluble secreted receptor destroying enzyme of *Vibrio cholera* in its ability to remove the terminal neuraminic acid (sialic acid) unit from neuraminyl-lactose and inhibitory

mucoproteins and was subsequently named “neuraminidase” (Gottschalk, 1957). Early work using virus subjected to heat treatment for 30 minutes at 55˚C suggested that the receptor destroying activity of this enzyme was separate and distinct from the agglutinating activity (Briody, 1948). However, it wasn’t until 1963 when William Laver used sodium dodecyl sulphate and sodium deoxycholate to separate and purify NA from HA that it was conclusively demonstrated that the receptor destroying activity of influenza viruses was separate from the hemagglutination activity (Laver, 1963). Early attempts to purify NA via proteolytic removal from virus preparations using trypsin and chymotrypsin resulted in heterogenous preparations, however in 1966 a method was reported for purifying enzymatically active globular NA heads after cleavage using pronase (Seto JT Brzeniek and Rott, 1966). Morphology analysis of surface proteins purified from detergent disrupted influenza virions via electron microscopy (EM) revealed NA subunits with a 100 angstrom tail and a 40 angstrom globular head (Laver and Valentine, 1969). The RNA sequence, amino acid sequence, and N-terminal membrane orientation (type II membrane protein) of NA was first reported for NA1 in 1981 (Fields et al., 1981) followed by the sequence of NA2 in 1982 (Ward et al., 1982). It wasn’t until 1983 that the crystal structure of pronase solubilized NA2 was solved at 2.9 angstrom resolution revealing a homotetramer with a box shaped globular head composed of four monomers around a 4-fold axis of symmetry stabilized by bound metal ions (Varghese et al., 1983). While the enzymatic active site is present in each individual monomer of the homotetramer, previous work had suggested that the monomeric form lacked enzymatic activity (Bucher and Kilbourne, 1972). Activity and correct conformation of influenza virus neuraminidase had previously been shown to be dependent on the presence of the divalent cation Ca^{2+} (Dimmock, 1971), however the degree of instability and loss of activity appears to be subtype dependent (Air, 2012).

During early biochemical work characterizing NA, researchers had a distinct appreciation for the possible role of NA in influenza virus entry and in navigating the competitive role of highly glycosylated mucoproteins, with appreciation that the competitive inhibition of NA enzymatic activity could have therapeutic benefit (Burnet, 1951; Seto et al., 1967). Further information on the function of NA's role in the influenza virus infection was garnered via work with temperature sensitive WSN mutants suggesting an important role for NA in nascent virion release from infected cells as well as the prevention of virion to virion agglutination demonstrated using an NA activity deficient temperature sensitive mutant grown at 39.5˚C (Palese et al., 1974). In 2004, after the advent of widely available NA inhibitors, further confirmation of NA's important role in virus entry via enzymatic cleavage of sialic acid residues on heavily glycosylated host mucins to access host epithelial cells was further supported by work form Hans Dieter Klenk and colleagues (Matrosovich et al., 2004). The work by Klenk and colleagues demonstrated the necessity of NA enzymatic activity in navigating the labyrinth of glycosylation present on viral host mucoproteins to allow access to vial receptors on airway epithelial cells to initiate productive infection of host cells. Since this early experimental demonstration, the important role for NA in the early stages of influenza virus infection in penetrating host respiratory mucous has been experimentally repeated by additional investigators (Cohen et al., 2013; Yang et al., 2014).

Despite both early speculation and more recent experimental evidence of the important role of NA in early and late stages of infection, initial studies of anti-influenza virus immunity directed vaccine antigen focus toward HA and left NA largely ignored. Initial in vitro experiments using serum demonstrated that antigenically matched sera could reduce the overall number of influenza virus plaques when incorporated into plaque assays (Sugiura and Kilbourne, 1965). Also, vaccination of human military recruits with adjuvanted, detergent disassociated HA led to

measurable antibody responses capable of inhibiting hemagglutination in vitro and protection against influenza virus disease (Hennessy and Davenport, 1966). With the advent of antigenically defined recombinant viruses (Laver and Kilbourne, 1966), experiments using sera from rabbits vaccinated with recombinant IAVs demonstrated that antigenically matched sera towards the NA glycoprotein resulted in a reduction in overall plaque size, but not in an appreciable effect on plaque number when incorporated into plaque assays (Jahiel and Kilbourne, 1966). However, the use of specific sera derived from recombinant virus immunization demonstrated that antigenically matched sera towards the HA glycoprotein was responsible for the reduction in plaque number when incorporated into the assay (Jahiel and Kilbourne, 1966). Work using recombinant viruses as vaccine antigens demonstrated that vaccination produced antibodies capable of inhibiting the enzymatic activity of NA (Webster and Laver, 1967). Further work in a murine model showed that intraperitoneal vaccination with UV inactivated reassortant viruses reduced lung viral titers and lung lesions after challenge with IAV containing homologous NA (Schulman et al., 1968). While these experiments suggested an important role for anti-NA immunity in the control of viral replication in controlled experimental systems, the emergence of the 1968 H3N2 influenza virus pandemic and subsequent replacement of the H2N2 strain that emerged in 1957 provided for a natural experiment in human immunity to NA (Schulman and Kilbourne, 1969). The NA protein of the 1968 H3N2 virus was found to be essentially antigenically identical to the NA2 of H2N2 virus circulating from 1967-1968 and very closely related to the NA antigens in H2N2 viruses circulating from 1957-1964, and anti-NA2 antibody from passive transfer and from immunization led to reduction of viral dissemination and lung lesions in mice (Schulman, 1969). Both Schulman and Kilbourne hypothesized that pre-existing anti-NA immunity against the NA2 antigen of circulating H2N2 viruses would offer some protection against severe disease and subsequent

transmission of the 1968 H3N2 pandemic virus (Schulman, 1969; Schulman and Kilbourne, 1969). Indeed, Arnold Monto and Alan Kendal analyzed banked serum samples collected from adults in Michigan prior to the emergence of the 1968 H3N2 strain for anti-NA antibody titers and demonstrated that individuals with higher anti-NA2 titers prior to emergence of the pandemic experienced lower rates infection (Monto and Kendal, 1973). This finding was also experimentally replicated in a more controlled setting where anti-NA serum antibody titers were measured in Maryland prisoners negative for anti-HA3 antibodies, followed by experimental infection of the prisoners with wild-type A/Hong Kong/1968 H3N2 virus (Murphy et al., 1972). Prisoners with high levels of anti-NA2 antibodies were completely protected from clinical disease, while those with intermediate antibody levels experienced afebrile disease, and prisoners with low levels of anti-NA2 antibodies experienced full febrile influenza disease (Murphy et al., 1972). Furthermore, Murphy et al. showed that the magnitude and duration of virus shed was inversely correlated with pre-existing anti-NA2 antibodies (Murphy et al., 1972). Additional work in 1973 using Texas prison inmates as clinical subjects demonstrated that H3 seronegative volunteers who received a single immunization of a whole inactivated recombinant influenza virus containing the irrelevant HA7 from the equine strain A/equine/prague/56 virus and NA2 from the A/Hong Kong/1/1968 virus were protected from illness after challenge with A/Hong Kong/1/1968 and protected from subsequent infection with A/Hong Kong/1/1968 (Couch et al., 1974a). While these observations argued for an important role of anti-NA immunity in protection against disease caused by influenza virus, further studies suggested a diminished anti-NA response after administration and re-administration of vaccines containing HA antigen that had been previously encountered by the host (Johansson et al., 1987; Kendal et al., 1977; Kilbourne et al., 1987). Additional work demonstrated that vaccination of mice with inactivated whole virus presenting both HA and NA

on the same virion led to antigenic competition, with HA being immunodominant over NA in priming B cells and T cells (Johannsson et al., 1987). However, subsequent work by the same group demonstrated that the dissociation of HA and NA from the same viral particle via detergent disruption and subsequent purification eliminated this antigenic competition in mice upon vaccination (Johansson et al., 1989; Johansson and Kilbourne, 1993). Furthermore, subsequent work showed that immunization of mice with purified NA1 and NA2 antigen resulted in cross reactivity without any antigenic competition between NA1 and NA2 antigens, resulting in higher mean NA1 and NA2 titers after vaccination compared to monovalent NA1 or NA2 vaccination given separately (Johansson and Kilbourne, 1994). The same laboratory also showed that supplementation of a seasonal egg grown, inactivated split-virus vaccine with detergent disrupted, purified NA led to a balanced immune response against HA and NA in mice, with a greater reduction in viral replication after heterologous challenge compared to the non-supplemented vaccine (Johansson et al., 1998). Perhaps most importantly, monovalent NA2 vaccine purified from A/Beijing/32/92 (X-117) administered to healthy human volunteers in a clinical trial was well tolerated and induced robust anti-NA immune responses capable of inhibiting NA enzymatic activity (Kilbourne et al., 1995).

More contemporary work has leveraged novel expression systems and technologies to investigate the role of anti-NA immunity in protection from influenza virus disease. Recombinant NA (rNA) produced in using a recombinant baculovirus (rBV) infection model demonstrated that NA2 produced in *Spodoptera frugiperda* cells was the expected size, enzymatically active, recognized by anti-NA2 polyclonal sera and monoclonal antibodies, and the enzymatic activity was able to be inhibited by anti-NA2 antibodies in a similar manner to wild-type virus (Mather et al., 1992). It was also demonstrated that NA with similar activity and in vitro antigenicity could

be recovered and purified from lysates after rBV infection of *Trichoplusia ni* (Tni) larvae (Price et al., 1989). In a separate report, the same investigators demonstrated that purified recombinant NA2 from rBV infection of Tni larvae used to vaccinate mice was similar in immunogenicity to NA2 purified from influenza virions with respect to magnitude of anti-NA responses and ability to inhibit enzymatic function (Johansson et al., 1995). Later work described a 3-log reduction in lung viral titers and decrease in morbidity measured by weight loss in a murine model after vaccination with a commercially prepared recombinant NA2 (Kilbourne et al., 2004). Subsequent work in mice vaccinated with adjuvanted soluble rNA derived from H1N1 and H3N2 IAV sequences as well as influenza B virus sequences expressed in a rBV system in insect cells demonstrated protection from mortality against lethal heterologous homosubtypic challenge but not against heterosubtypic challenge (Wohlbold et al., 2015a)

The role of NA specific immunity has also been investigated using DNA vaccines encoding for the full-length NA protein. Work by Chen et al. demonstrated that while DNA vaccination with the sequence encoding for NA2 is capable of protecting mice against heterologous H3N2 viruses, vaccination with NA2 DNA was not protective upon lethal challenge with heterosubtypic H1N1 virus (Chen et al., 2000). Subsequent work investigating the potential for maternal antibody interference from inactivated influenza vaccines in a murine DNA vaccination model demonstrated that vaccination with DNA encoding for NA1 protein was capable of overcoming passively transferred maternal antibodies in pups and was protective against lethal H1N1 challenge (Chen et al., 2007). While DNA vaccines encoding for both HA and/or NA antigens of influenza virus initially showed promise, DNA vaccine technology did not scale well in larger animal models (Yang Lee et al., 2018). None the less, DNA vaccines are still a viable tool for mechanistic

investigation of anti-NA immunity in rodent influenza models and have the capacity to deliver antigen in a monovalent form.

Additional work at Wyeth-Lederle laboratories using recombinant baculoviruses demonstrated that the co-expression of HA, NA, M2, and the M1 protein in a Sf9 cell was sufficient to drive the budding process at the cell surface resulting in virus-like particles (VLP) displaying HA, NA, and M2 on the VLP surface without inclusion of IAV genetic material in the VLP (T Latham and Galarza, 2001). The same work also demonstrated that expression of the M1 protein alone was sufficient to drive budding and the release of vesicular particles from infected cells (T Latham and Galarza, 2001). In an important step for VLPs as influenza virus vaccines, the same group demonstrated that influenza virus VLPs containing HA and the M1 protein derived from the H3N2 strain A/Udorn/72 produced in a rBV expression system in Sf9 cells were protective against lethal challenge with heterologous H3N2 A/Hong Kong/1/1968 in a murine challenge model (Galarza et al., 2005). In 2010, it was reported that expression of NA alone was sufficient to drive budding of VLPs in the continuous HEK-293T human cell line (Lai et al., 2010). The first report investigating NA in a baculovirus expression system to evaluate NA VLPs in a murine model employed the NA1 and M1 sequence from A/Puerto Rico/8/1934 (PR8) in a co-infection production system (Quan et al., 2012). Intranasally administered PR8 NA1 VLPs were immunogenic and protective against morbidity and mortality in a lethal homologous virus challenge as well as protective against mortality but not morbidity in the form of weight loss against heterosubtypic challenge with the H3N2 virus A/Phillipines/1982 (Quan et al., 2012). Simultaneously, Easterbrook et al. demonstrated that NA1 VLPs containing M1 protein produced in HEK-293T cells using NA sequences from the A/California/04/2009 H1N1 virus and highly pathogenic avian influenza (HPAI) A/Vietnam/1203/2004 H5N1 virus were protective against

lethal challenge with A/Vietnam/1203/2004 virus in a murine model (Easterbrook et al., 2012). More work examining the efficacy of NA VLPs produced in insect cells against H5N1 highly pathogenic avian influenza (HPAI) was evaluated in a ferret model of influenza infection at the CDC (Smith et al., 2017). Vaccination of ferrets with N1 VLPs containing the NA1 and M1 sequence from the HPAI H5N1 A/Indonesia/05/2005 virus was protective against mortality in a lethal homologous challenge model and significantly reduced nasal wash viral titers post challenge (Smith et al., 2017). Further work examining NA VLPs in a murine model demonstrated that vaccination with NA1 VLPs produced in Sf9 cells using the sequence from A/California/04/2009 yielded superior protection compared to a detergent split PR8 vaccine after lethal challenge with A/California/04/2009 (Kim et al., 2019). Kim et al. also showed that vaccination with NA1 VLPs was protective against mortality but not morbidity in the form of weight loss against lethal challenge with A/Phillipines/1982 H3N2 virus (Kim et al., 2019). Taken together, these studies suggest that NA VLPs comprise a useful tool in investigating the contribution of anti-NA immunity with the NA antigen presented in the proper antigenic conformation employing the full-length NA protein sequence.

Repeated evidence has suggested that the inhibition of NA activity is a useful correlate of protection against influenza virus disease in humans, especially against heterologous virus strains. While the FDA and EMA have long considered a hemagglutination inhibition (HAI) titer of greater than or equal to 1:40 protective against disease caused by influenza virus, a human challenge study determined that pre-existing neuraminidase inhibition (NAI) titers were actually a more accurate correlate of disease severity post-challenge (Memoli et al., 2016). In the study, human volunteers were stratified into two groups based on pre-existing HAI titers of greater than or less that 1:40 and challenged with 10^7 $TCID_{50}$ of wild type H1N1 pandemic virus (Memoli et al., 2016). The

data analysis revealed that pre-existing HAI titers were not a reliable predictor of severity of disease post challenge, but pre-existing NAI titers greater than 1:40 reduced disease symptom severity, duration of symptoms, and the duration of viral shedding in volunteers post challenge (Memoli et al., 2016). A separate randomized controlled trial evaluating NAI titers as a correlate of protection post-vaccination with inactivated or live attenuated influenza virus vaccine determined that NAI titers were inversely correlated with frequency of infection (Monto et al., 2015). Additionally, a recent observational study found that with every 2-fold increase in pre-existing NAI titer among military recruits, there was a 32% decrease in odds of medically attended H3N2 infection during a localized outbreak (Weiss et al., 2020). While there is a clear correlation between pre-existing anti-NA immunity and improved outcomes in influenza disease, further research is warranted to define critical immunity thresholds to further inform seasonal vaccine design and potency.

Numerous studies have suggested that antibodies specifically capable of inhibiting NA enzymatic activity are a strong correlate of protection against influenza virus infection (Johansson et al., 1998; Monto et al., 2015; Rockman et al., 2013; Wohlbold et al., 2015a). The potential mechanism mediated by antibodies inhibiting the enzymatic activity is easy to conceptualize and likely mirrors protection afforded by small molecule NA inhibitors in the inhibition of viral ingress and egress. However, it is likely that other mechanisms contribute to the protective effect observed from anti-NA immunity as well. In relatively early investigations into anti-NA immunity, it was recognized that specific anti-NA antibody caused aggregation of virus particles and the specific antibodies were bound to different viral surface proteins than when virus particles were incubated with antibodies that prevented hemagglutination when visualized via EM (Kendal and Madeley, 1970). This direct evidence of aggregation of viral particles was the first experimental

demonstration of one mechanism by which anti-NA immunity can effectively reduce viral load by spatially restricting the dissemination of virions. Other work suggests that limiting viral spread within the host respiratory tract and preventing viral dissemination into the lower respiratory tract could limit opportunistic secondary bacterial infections (Shirey et al., 2019). Indeed, secondary bacterial infections have been attributed as the cause of nearly all deaths in the 1918 H1N1 pandemic (Morens et al., 2008) and as the cause in up to 34% of the deaths attributed to the 2009 H1N1 pandemic (Chertow and Memoli, 2013). Furthermore, the inhibition of NA activity in the respiratory tract and the subsequent aggregation of viral particles could effectively increase the total particle size and alter airborne transmission dynamics of aerosol droplets (Eichelberger et al., 2018). Additionally, inhibition of NA at the host cell surface and subsequent tethering of nascent progeny virions to host cells could potentially limit the amount of virus in aerosol droplets, having positive epidemiological effects from a public health perspective (Monto, 2019). Additional mechanisms have been proposed for non-inhibitory anti-NA antibodies including complement activation and antibody dependent cell cytotoxicity (ADCC) (Krammer et al., 2018a). However, NA1 VLP vaccination in mice deficient for Fc gamma chain receptor deficient mice was still protective in a lethal murine challenge model, suggesting that ADCC does not contribute significantly to the protection mediated by anti-NA immunity (Kim et al., 2019). Additionally, in the same study, Kim et al. used passive transfer of NA1 VLP immune sera to confer protection to naïve mice, suggesting that humoral anti-NA immunity is sufficient in the absence of ADCC activity when subjects are immunized with VLPs containing NA in the proper membrane bound conformation (Kim et al., 2019). Indeed, recent work has suggested that tetrameric NA is required to provide optimal NA enzymatic inhibition and protection against influenza challenge in a murine model (Deng et al., 2021). Based on available evidence, NA VLPs afford the opportunity to

maximize anti-NA immunity by presenting antigen to host immune cells in its native, tetrameric conformation allowing for an immune response capable of inhibiting NA activity (Deng et al., 2021).

Influenza virus HA and NA have been shown to coevolve together to preserve the balance between HA receptor affinity and NA enzymatic activity to permit virus mobility through host mucins and permit entry into host cells (Gaymard et al., 2016; Guo et al., 2018; Vries et al., 2019). The first experimental evidence suggesting a balanced co-evolution between HA and NA showed that genetic recombinants eluted faster during hemagglutination from erythrocytes than did the parent viruses (Laver and Kilbourne, 1966)**.** While there is a clear balanced co-evolution between HA and NA, evidence has shown that the glycoproteins do evolve independent from one another. Multiple studies have demonstrated that there is an independent and disparate evolution between HA1 and NA1 as well as between HA3 and NA2 viral antigens, with HA evolving at a much faster rate with changing antigenicity due to greater selective pressures at a population level (Kilbourne et al., 1990; Sandbulte et al., 2011). As NA shows greater sequence conservation, monoclonal antibodies (mAb) have been recently identified that recognize serval NA subtypes (Gravel et al., 2010; Rijal et al., 2019). Indeed, the enzymatic function of NA has led to the conservation of a linear amino acid sequence in all 9 IAV enzymatic subtypes, “ILRTQESEC” (residue 222-230, NA2 numbering), with a characterized mAb raised in rabbits specific for this epitope capable of inhibiting the enzymatic activity of multiple NA subtypes (Doyle et al., 2013). The relative antigenic stability of the NA protein compared to the HA protein suggests that NA could be an attractive antigenic target for augmentation of influenza virus vaccines.

Virus-like Particles as a Vaccine Platform

Virus-like particles (VLPs) represent an attractive vaccination platform for the delivery of antigens in a membrane bound form, mimicking the native structure of the target antigens (Kang et al., 2012). VLPs are virus-like in that viral antigen is displayed on a particle resembling a virion for the host immune system, yet they do not contain viral genetic information nor the ability to infect cells or replicate. Additionally, VLPs can be produced in insect cells using a recombinant baculovirus expression vector system (Theresa Latham and Galarza, 2001) and are immunogenic in animal models (Galarza et al., 2005). While VLPs can also be produced in continuous mammalian cell lines, an analysis demonstrated that production in Sf9 insect cells yielded 35 times more VLPs than production in HEK293 cells (Thompson et al., 2015). Furthermore, VLP platforms are currently used in approved human vaccines protecting against hepatitis B virus and human papilloma virus. VLPs also have the potential to overcome both the egg-adaptive mutation and long production lead time limitations of current seasonal influenza virus vaccines (Haynes, 2009; Kang et al., 2012). VLPs afford the opportunity to control both the incorporation of certain antigens as well as the omission of others, making VLPs an extremely flexible vaccine platform.

An additional advantage of VLP platforms is the ability to present specific membrane bound glycoprotein antigens to host immune cells in their native conformation without the need for membrane disruption and further downstream processing, potentially altering the antigenic conformation. Recent work investigating the stability of NA in seasonal vaccines identified that anti-NA response correlated with greater enzymatic activity (Sultana et al., 2014). Interestingly, the same study found that NA activity and subsequent conformation were subject to disruption after detergent exposure in a strain specific manner, with some vaccine strains being more

susceptible to disruption than others (Sultana et al., 2014). Furthermore, recombinant, soluble, subunit NA vaccines require truncation of the native NA stem domain to remove the transmembrane domain and the insertion of a soluble chimeric tetramerization domain to achieve proper tetramerization and enzymatic activity, with the most common domains derived from the tetrabrachion domain from *Staphylothermus marinus*, the human vasodilator stimulated phosphoprotein (VASP), or the artificial GCN4-pLI domain (Daniels et al., 2016). While these proteins do promote tetramerization of soluble NA protein, the resulting recombinant NA proteins lose over 50 - 75% NA activity compared to full length NA, suggesting changes to the protein structure and the requirement for the transmembrane domain to achieve proper NA tertiary and quaternary structure (Daniels et al., 2016). Characterization of several broadly protective anti-NA monoclonal antibodies has demonstrated specificity for conformational epitopes and further highlights the importance of enzymatic activity as a surrogate of proper NA tertiary and quaternary structure (Wohlbold et al., 2015a, 2017).

To date, several clinical trials have been conducted in humans using influenza VLPs produced using a recombinant baculovirus expression system in insect cells. In a study of 4,563 healthy adult volunteers in a hybrid phase I/II trial, volunteers were administered VLP vaccine containing HA, NA, and the M1 protein from A/California/04/2009 H1N1 pandemic strain in two vaccine doses 21 days apart (López-Macías et al., 2011). The VLP vaccine was well tolerated and induced HAI titers greater than 1:40 in 92% of participants, however anti-NA responses weren't evaluated (López-Macías et al., 2011). Another hybrid phase I/II study evaluating a H5N1 HPAI VLP vaccine containing HA, NA, and M1 proteins from A/Indonesia/05/2005, executed as a collaboration between the FDA and Novavax, demonstrated the vaccine was well tolerated and induced high serum antibody titers capable of inhibiting NA enzymatic activity in high dose groups

(Khurana et al., 2011). Additionally, healthy adult volunteers receiving H7N9 VLPs combined with a saponin based ISCOMATRIX adjuvant demonstrated a 97.2% seroconversion rate against the NA fraction of the vaccine and 71.9% seroconversion without adjuvant, with VLP vaccination including adjuvant only associated with mild local and systemic reactions (Fries et al., 2013). The high observed VLP anti-NA seroconversion rate is promising compared to the variable anti-NA response to seasonal vaccines of approximately 30% or less (Chen et al., 2018; Couch et al., 2012b). While rBV derived VLPs produced in insect cells have a challenging regulatory path to meet FDA licensure requirements, they have demonstrated the ability to elicit immune responses capable of inhibiting hemagglutination and NA enzymatic activity while presenting an agile and economical alternative to current influenza vaccines.

Model Systems for Influenza Vaccine Research

While many animal model systems have been used in influenza vaccine research including mice, rabbits, hamsters, ferrets, guinea pigs, pigs, and non-human primates, the most commonly used animal model in influenza vaccine research is the murine model, *Mus musculus* (Bouvier and Lowen, 2010). The genetic homogeneity of commercially available inbred mouse colonies and genetically altered knockout mice, small housing footprint, low experimental costs, and high availability of immunological reagents are a few of the many advantages leading to the dominance of murine models in influenza vaccine research (Starbæk et al., 2018). While the mouse model presents a cost-effective option for early pre-clinical development work as well as a tractable model for mechanistic investigations, murine models do not recapitulate human influenza virus pathology or disease symptoms due to the many differences between rodents and humans (Russell, 2021). Additionally, study of transmissibility of influenza virus in are not possible in modern

murine models of influenza infection in BALB/c mice as they fail to efficiently transmit disease to naïve contact mice (Lowen et al., 2006; Rajao and Vincent, 2015). Of note, the distribution of the virus receptor typically ascribed to avian influenzas (alpha-2,3 linked sialic acid) and human influenzas (alpha-2,6 linked sialic acid) differs in the respiratory tract between humans and BALB/c mice (Ning et al., 2009). Indeed, Ibricevic et al. failed to detect alpha-2,6 linked sialic acid receptors in the upper respiratory tract or lungs of C57BL/6J mice (Ibricevic et al., 2006). As a result, human influenza viruses often need to be adapted by serial passage to permit efficient replication in murine hosts, resulting in the selection for genetic changes in the virus affecting antigenicity and the subsequent loss of clinical relevance (Ilyushina et al., 2010; Narasaraju et al., 2009; Ping et al., 2011). Additionally, contemporary human H3N2 viruses do not replicate efficiently in murine models and have been recalcitrant to adaptation, creating an appreciable gap in the study of these viruses (Baz et al., 2019; Chen et al., 2008). While not without limitations, due to the relative cost efficiency, available murine genetics, and availability of immunologic reagents and assays, murine models of influenza are a valuable tool in the study of influenza virus vaccines.

With respect to modelling virus transmission, ferret, guinea pig, and swine influenza models all permit studies of Influenza virus transmission (Starbæk et al., 2018). While guinea pigs offer the flexibility of both inbred strains and outbred strains, guinea pigs do not typically show signs of overt disease and clinical signs do not mimic those observed in humans (Bouvier and Lowen, 2010). Conversely, clinical signs of disease in both ferret models and swine models mimic clinical signs observed in human influenza virus infection (Russell, 2021). While both models are economically resource intense, require a greater housing footprint, and require more specialized animal care, both model's responses to infection with influenza viruses better mimic the human

immune cell transcriptional profile post infection, likely due to a higher amount of sequence homology to humans in genes associated with antiviral responses compared to rodent models (Starbæk et al., 2018). However, the innate and adaptive immune responses of pigs and ferrets are not as well characterized as in mice, and, while improving, there is a paucity of reagents available for the investigation of immune responses in ferrets (Starbæk et al., 2018). Furthermore, domestic swine are a natural host for both swine specific viruses and viruses that are endemic in humans, with experimental 2009 H1N1 pandemic infections in swine models mimicking human seasonal influenza infections with respect to cell mediated T cell responses (Schwaiger et al., 2019). With endemic influenza A viruses readily circulating in domestic swine populations, swine influenza is an important economic pathogen in swine production responsible for decreased profitability and increased resource consumption and worthy of investigation in swine alone (Calderón Díaz et al., 2020). Influenza infection in swine also creates human public health risk with variant IAV viruses of swine-origin sporadically causing infection in humans, however typical variant IAV infections are not efficiently transmitted from human-to-human (Jhung et al., 2013). Of greater concern is the human public health risk for zoonotic infection from IAVs of human, swine, and avian origin upon co-infection in swine due to the presence of both alpha-2,6 and alpha-2,3 linked sialic acid receptors in the respiratory tract of pigs and their subsequent susceptibility to infection with IAVs endemic in multiple species (Trebbien et al., 2011). Indeed, the origin of the 2009 H1N1 “swine flu” pandemic was traced to a zoonotic spillover event from swine farms in central Mexico (Mena et al., 2016). The 2009 H1N1 pandemic virus was identified as a reassortant virus containing 6 viral gene segments (PA, PB1, PB2, HA, NP, and NS) from “triple-reassortant” viruses circulating in swine in North America since 1998 and the NA and matrix protein segments form a Eurasian “avian-like” lineage of swine viruses circulating in Europe since 1979 (Gibbs et al., 2009). The

H1N1 pandemic virus of 2009 quickly spread around the world and displaced previously circulating H1N1 seasonal viruses to become the dominant H1N1 circulating virus. While swine models of IAV infection are incredibly useful animal models due to their similarities to humans for studying IAV vaccines, vaccines capable of protecting multiple species and limiting the emergence of potential pandemic zoonotic IAVs warrant investigation in swine as part of a "One Health" approach to controlling influenza (Powdrill et al., 2010).

Introduction to Dissertation Research

In this dissertation, I investigated the immunogenicity and protective efficacy of novel influenza virus NA VLPs produced via coinfection with recombinant baculoviruses in *Trichoplusia ni* insect cells as candidate influenza virus vaccines in multiple animal models. In Chapter II, I first described the production, physical characterization, and enzymatic characterization of novel NA2 VLPs. NA2 VLPs were the initial focus, as VLPs containing only NA2 in the absence of HA as the surface antigen have not yet been investigated. I then investigated the bivalent administration of NA2 VLPs in combination with NA1 VLPs. These initial investigations into NA VLP immunogenicity and efficacy were conducted in a murine model using a lethal IAV challenge model. In Chapter III, I investigated the immunogenicity and protective efficacy of NA2 VLPs in a swine infection model using a heterologous virus challenge. In Chapter IV, I summarized the results of work investigating NA VLPs in both the murine model system and the swine infection model and the conclusions that can be made. I further compared the results to previous work investigating both VLPs and anti-NA immunity in general in the context of influenza virus vaccines. Lastly, I identified knowledge gaps that still exist surrounding anti-NA immunity and

propose future directions of research to further inform the development of future influenza virus vaccines.

Chapter II: Bivalent vaccination with NA1 and NA2 neuraminidase virus-like particles is protective against challenge with H1N1 and H3N2 influenza A viruses in a murine model.

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Published in Virology, volume 562 (2021)

Abstract

Neuraminidase (NA) is the second most abundant glycoprotein on the surface of influenza A viruses (IAV). Neuraminidase type 1 (NA1) based virus-like particles (VLPs) have previously been shown to protect against challenge with H1N1 and H3N2 IAV. In this study, we produced neuraminidase type 2 (NA2) VLPs derived from the sequence of the seasonal IAV A/Perth/16/2009. Intramuscular vaccination of mice with NA2 VLPs induced high anti-NA serum IgG levels capable of inhibiting NA activity. NA2 VLP vaccination protected against mortality in a lethal A/Hong Kong/1/1968 (H3N2) virus challenge model, but not against lethal challenge with A/California/04/2009 (H1N1) virus. However, bivalent vaccination with NA1 and NA2 VLPs demonstrated no antigenic competition in anti-NA IgG responses and protected against lethal challenge with H1N1 and H3N2 viruses. Here we demonstrate that vaccination with NA VLPs is protective against influenza challenge and supports focusing on anti-NA responses in the development of future vaccination strategies.

Keywords: Neuraminidase (NA), Influenza virus vaccine, virus-like particle (VLP), Influenza virus

Research Highlights

- Neuraminidase 2 virus-like particle vaccination protected mice against H3N2
- Vaccinating mice with neuraminidase 1 and 2 together improved responses in mice
- Influenza vaccines could benefit from a balanced neuraminidase response

Introduction

Influenza A virus (IAV) is a causative agent of acute respiratory disease responsible for an estimated 290,000-645,000 deaths globally per annum during seasonal epidemics (Iuliano et al., 2018). While several small molecule inhibitors are licensed for treatment of IAV infection, current strategies to proactively mitigate the threat posed by IAV to the global population focus on seasonal vaccination (Grohskopf et al., 2013). However, recent seasonal influenza vaccines have demonstrated a suboptimal overall efficacy, ranging from 29%-40% from 2016-2019, with only 9% vaccine efficacy against circulating H3N2 strains and 5% efficacy against the emergent A(H3N2) clade 3C.3a viruses in the latter part of the influenza season in 2019 (Flannery et al., 2020, 2019; Rolfes et al., 2019). The efficacy of seasonal IAV vaccines has historically depended on sequence homology between the hemagglutinin (HA), the most abundant surface glycoprotein on the virion, of the vaccine strain and the circulating strain during the influenza season (Krammer and Palese, 2015).

HA and neuraminidase (NA) are the two most abundant glycoproteins on the surface of IAV and are divided into 18 and 11 subtypes, respectively. Currently licensed seasonal influenza vaccines only standardize the antigenic content of HA, with no standard potency requirement for NA, often leading to variable or nonexistent anti-NA responses after seasonal vaccination (Chen et al., 2018; Couch et al., 2012b; Monto et al., 2015; Wohlbold and Krammer, 2014). The suboptimal anti-NA response post-vaccination is particularly problematic given that the antigenic evolution of NA has been suggested to be slower and discordant from that of HA (Kilbourne et al., 1990). Furthermore, cross-reactive anti-NA antibodies have been identified after infection with seasonal H1N1 influenza strains that inhibit NA activity of A/California/04/2009 H1N1 pandemic

virus as well as against H5N1 viruses (Chen et al., 2012; Marcelin et al., 2011). However, previous work has suggested that anti-NA immune responses are dampened when both HA and NA are presented on the same viral particle, with HA being immunodominant over NA (Johannsson et al., 1987; Johansson and Kilbourne, 1993). Strategies attempting to overcome the immunodominance of HA over NA have included both supplementing seasonal vaccines with purified NA and more recently by extending the NA stalk to potentially expose additional epitopes on the NA molecule itself (Broecker et al., 2019b; Johansson et al., 2002, 1998; Kim et al., 2017). Previous work has also shown that antigens delivered via VLPs in their native form within the context of a membrane demonstrated superior protection against heterologous IAV challenge compared with soluble recombinant antigen (Bright et al., 2008).

As anti-HA antibodies have been shown to be neutralizing and able to inhibit virus entry into cells (Krammer et al., 2015), IAV vaccine development has historically focused on HA responses. However, natural infection with IAV viruses induces a much more balanced immune response against both HA and NA (Chen et al., 2018). NA is present on the surface of virions at a ratio of 1 molecule of NA for every 4 molecules of HA (Harris et al., 2006). In the past 100 years, only N1 and N2 IAV subtypes have circulated in humans compared with H1, H2, and H3 IAV subtypes. Anti-NA based immunity and the contribution to protection against IAV infection has been appreciated since the 1960's. Previous work has demonstrated that both natural infection (Murphy et al., 1972) and vaccination (Couch et al., 1974b) with heterosubtypic HA but homosubtypic NA contributed to a reduction in illness and clinical signs upon infection with a homosubtypic NA. More recent work has demonstrated that anti-NA serum antibody titers were a stronger correlate of protection against mild to moderate influenza disease than anti-HA titers (Memoli et al., 2016). Historical work has shown that current IAV vaccination strategies targeting

HA in an effort to prevent IAV infection could be augmented by targeting anti-NA immune responses as well.

The efficacy of NA1 VLPs produced using a *Spodoptera frugiperda* (Sf9) insect cell expression system has been previously demonstrated against influenza A virus in murine challenge models (Kim et al., 2019; Quan et al., 2012) as well as in a ferret challenge model (Smith et al., 2017). Additionally, *Trichoplusia ni* (Tni) insect cell expression systems have demonstrated an increased yield of influenza HA VLPs with a reduced amount recombinant baculovirus in VLP preparations compared to Sf9 cells (Krammer et al., 2010). However, production of NA VLPs in Tni cells and their subsequent use as a vaccine in a murine influenza A challenge model has not yet been reported, nor has the use of VLPs using NA2 only or the combined administration of NA1 and NA2 VLPs as a bivalent vaccine against influenza A viruses. In this study, we produced NA2 VLPs using the NA sequence derived from A/Perth/16/2009 in a Tni insect cell expression system. We compared different routes of NA2 VLP vaccination in a murine model in a lethal heterologous challenge model against A/Hong Kong/1/1968 (H3N2). Using the same expression system, we also produced NA1 VLPs using the NA sequence derived from A/California/04/2009 (H1N1). Vaccination with both NA1 and NA2 VLPs induced high levels of anti-NA serum IgG capable of inhibiting NA activity with no evidence of antigenic competition between NA1 and NA2. The bivalent administration of NA1 and NA2 VLPs was protective against mortality in both lethal H3N2 and H1N1 challenge models. This work demonstrates the efficacy of bivalent vaccination with NA1 and NA2 VLPs in mice against both lethal H1N1 and H3N2 virus challenge.

Materials and Methods

Cells and virus stocks

Spodoptera frugiperda (Sf9) cells (IPLB-Sf-21-AE, Expression Systems, Davis, CA, USA) were maintained at 27˚C in suspension in shaker flasks using serum free SF900II media (Gibco/ThermoFisher Scientific, Waltham, MA, USA). *Trichoplusia ni* (Tni) cells (Expression Systems, Davis, CA, USA) were maintained at 27˚C in suspension in shaker flasks using serum free ESF 921 media (Expression Systems, Davis, CA, USA). Madin-Darby canine kidney (MDCK) cells (ATCC CCL 34, American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's media (DMEM) (Corning Life Sciences, Corning, NY, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Westborough, MA, USA). A/California/04/2009 (H1N1) virus (BEI Resources, Manassas, VA, USA) was expanded one time in MDCK cells and then serially passaged in the lungs of BALB/c mice. Mice were anesthetized and thirty microliters of virus suspension was instilled intranasally. Three days post infection, mice were humanely euthanized. Lungs were harvested and homogenized in sterile PBS followed by intranasal (IN) instillation in subsequent mice for a total of 10 passages. A/Hong Kong/1/1968-1 Mouse-Adapted 12A (H3N2) virus was obtained from BEI Resources (Manassas, VA, USA) and expanded one passage in MDCK cells. Sequence identity of mouse adapted viruses was confirmed by sequencing on an Illumina iSeq 100 platform as previously described (Kyriakis et al., 2017; Maljkovic Berry et al., 2020).

Animals

Six to eight week old BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were housed in the Emory University Division of Animal Resources biosafety level 1 animal facility at Emory University Whitehead animal facility. Mice were transported to biosafety level 2 containment rooms in the same facility 1 day prior to infection. All animal experiments and procedures were conducted in accordance with protocols approved by Emory University's Institutional Animal Care and Use Committee (IACUC) and in accordance with the United States Federal Animal Welfare Act (PL 89-544) and subsequent amendments.

VLP Production and Purification

The neuraminidase 1 (NA1) gene from A/California/04/2009, the neuraminidase 2 (NA2) gene from A/Perth/16/2009, and the matrix 1 gene from A/Michigan/73/2015 (M1) were synthesized with unique BamHI and HindIII restriction sites at the 5' and 3' ends, respectively (Integrated DNA Technologies, Coralville, IA, USA). Individual gene fragments were digested using BamHI and NotI restriction endonucleases and ligated into BamHI and NotI digested pFastBac1 vector from the Bac-to-Bac® Baculovirus Expression System kit (ThermoFisher Scientific, Waltham, MA, USA). Bacmid DNA was created and purified according to the Bac-to-Bac® manufacturer's instructions. Recovered high molecular weight DNA was then transfected into SF9 cells in SF900II media. The resulting recombinant baculovirus (rBV) stocks were amplified in SF9 according to the Bac-to-Bac® manufacturer's instructions.

Recombinant baculovirus stocks were used to coinfect Tni cells at a multiplicity of infection (MOI) of 5 for NA1 or NA2 rBVs and an MOI of 3 for M1 rBVs. VLPs recovered from the clarified supernatant were first pelleted at 136,000 x g at 4˚C for 90 min. Resulting pellets were resuspended in 1xPBS overnight for approximately 12 hours at 4˚C. Resuspended pellets were then subjected to purification via discontinuous sucrose density gradient centrifugation from 20%-60% sucrose at 190,000 x g for 16 hours. The resulting gradients were then fractionated followed by resolution via SDS-PAGE and western blotting.

VLP Characterization

Sucrose gradients were fractionated and analyzed via SDS-PAGE and Western Blot. Western Blots were probed with polyclonal anti-NA1 sera (NR-3136, BEI Resources, Manassas, VA, USA), polyclonal anti-NA2 sera (NR-3137, BEI Resources, Manassas, VA, USA), or polyclonal anti-M1 sera (NR-3134, BEI Resources, Manassas, VA, USA) where indicated. Blots were then probed with secondary rabbit anti-goat HRP conjugate antibody (Southern Biotech, Birmingham, AL, USA) and the resulting chemiluminescent signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, Waltham, MA, USA). The signal was read using the Bio-Rad ChemiDoc Imaging System (Hercules, CA, USA). The three sucrose fractions containing the maximum respective NA and M1 signal were combined. In the case of M1 only VLPs, the three sucrose fractions containing the maximum M1 signal were combined. Combined fractions were then diluted in 1xPBS and pelleted via centrifugation at 136,000 x g for 90 minutes at 4˚C. Pellets were resuspended in 1xPBS overnight for approximately 12 hours. Total protein VLP concentration was measured via BCA assay according to manufacturer's instructions

(Thermo Scientific, Waltham, MA, USA). Resuspended VLP preparations were then resolved via SDS-PAGE and followed by Coomassie staining or Western blotting. VLPs were visualized via transmission electron microscopy after negative staining with 1% phosphotungstic acid.

Neuraminidase Activity Assay

Purified VLPs preparations were tested for enzymatic neuraminidase activity using the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, VLP solutions were diluted in NA-Star Assay Buffer and then incubated with substrate for 30 min at room temperature. NA-Star accelerator solution was then injected followed by measurement of the chemiluminescent signal in relative luminescent units (RLU) via Modulus II Microplate Luminometer (Promega, Madison, WI, USA). Inhibition of neuraminidase activity was measured similarly, with collected sera diluted in NA-Star Assay Buffer followed by addition of 40 ng or 25 ng of NA2 or NA1 VLPs, respectively. Sera and VLPS were incubated at 37˚C for 30 minutes followed by addition of substrate and incubation at room temperature for 30 minutes. Plates were read after addition of accelerator using the Modulus II Microplate Luminometer. The highest sera dilution inhibiting at least 50% of NA activity was reported as the neuraminidase inhibition (NAI) titer.

Immunization and infection

Mice were immunized via either the IN or intramuscular (IM) route as indicated with the dose indicated for each experiment. Mice vaccinated via the IN route were lightly anesthetized via

isoflurane prior to instillation of NA VLPs in 50 µl total volume. Mice vaccinated via the IM route were injected with a total volume of 50 µl of VLP solution. Mice were prime-boost vaccinated 28 days apart with sera collected 14 days post-prime vaccination and 14 days post-boost vaccination. Mice were challenged 28 days post-boost vaccination via IN instillation of $5xLD_{50}$ of the indicated virus under isoflurane anesthesia. The LD_{50} of viruses were determined via the Reed-Muench method (Reed and Muench, 1938). For measurement of viral replication in vaccinated mice, 3-5 mice per group were humanely euthanized at day 4 post-challenge and lungs were collected in sterile 1xPBS. Mice were monitored daily for morbidity via weight loss and were humanely euthanized upon reaching the experimental endpoint of 25% total weight loss.

Measurement of NA-specific antibody using ELISA

Total immunoglobulin G (IgG) levels were measured as previously described (Esser et al., 2016) with modifications. Nunc Maxisorb (ThermoFisher Scientific, Waltham, MA, USA) plates were coated with 100 ng / well of recombinant neuraminidase (rNA) derived from A/Wisconsin/67/2005 (rN2) (BEI Resources #NR-19237, Manassas, VA, USA) or A/California/04/2009 (rN1) (BEI Resources #NR-19234, Manassas, VA, USA) where indicated. Purified recombinant NA2 derived from the homologous A/Perth/16/2009 sequence was not commercially during the execution of the study. Total murine anti-NA IgG was detected against a standard curve of murine IgG with HRP conjugated goat anti-mouse secondary antibody (Southern Biotech, Birmingham, AL, USA).

Plaque Assay for virus titration

Whole lungs were homogenized as previously described (Littauer et al., 2017). Briefly, lungs were homogenized and passed through a 40 µm cell strainer. Virus containing supernatants were clarified by centrifugation at 800 x g for 10 minutes at 4˚C. Clarified supernatants were added in 10-fold serial dilutions in DMEM media to confluent monolayers of MDCK cells in 6-well culture plates in a volume of 500 µl for 1 hour at room temperature with gentle rocking (Corning Life Sciences, Corning, NY, USA). Inoculations were aspirated followed by overlay with agar media as previously described (Littauer et al., 2018). Cells were incubated for 48-72 hours followed by visualization of plaques via crystal violet staining. The limit of detection of the assay was 20 plaque forming units (PFU) per 100 mg of lung tissue.

Statistics

Statistical comparisons between vaccinated groups were performed via one way ANOVA with post-hoc Bonferroni multiple comparisons using GraphPad Prism statistical software (GraphPad Software, San Diego, CA, USA) with an alpha of 0.05 ($\alpha \leq 0.05$).

Results

Characterization of NA VLPs

As production of NA2 VLPs had not been previously reported in a Tni insect cell expression system, we first set out to characterize NA2 VLPs produced by coinfection of Tni cells. Previous work with IAV HA VLPs indicated that a VLPs expressed in Tni cells migrated at a density of 40-50% sucrose in a sucrose gradient whereas HA VLPs expressed in an Sf9 expression system migrated at 35-45% sucrose (Krammer et al., 2010). To purify NA2 VLP preparations, NA2 VLPs produced by coinfection of Tni cells with NA2 and M1 rBVs were subjected to sucrose gradient ultracentrifugation followed by fractionation and Western blots probed with goat anti-NA2 and goat anti-M1 polyclonal sera. Similar to previous HA VLP work (Krammer et al., 2010), Western blots of sucrose fractions of NA2 VLPs expressed in Tni Cells revealed signals for NA2 and M1 that comigrated at an approximate density of 40-50% sucrose corresponding to fractions 5-7 after ultracentrifugation (Figure 1A). After fractions 5-7 were combined and washed, the VLP preparation revealed two defined bands upon SDS-PAGE electrophoresis followed by Coomassie staining that migrated at approximately 28 kDa and 55 kDa corresponding to M1 and NA2, respectively (Figure 1B). To confirm the identity of these bands, we performed a Western blot probing the membrane with polyclonal goat anti-NA2 sera and polyclonal goat anti-M1 sera. The resulting chemiluminescent signal revealed signals at the expected sizes of NA2 and M1 (Figure 1C). To examine both the size and morphology of the resulting VLPS, we visualized NA2 VLPs via electron microscopy. The resulting micrographs revealed spherical particles approximately 70-100 nm in size with morphology consistent with influenza VLPs and virions (Figure 1D)(Krammer et al., 2010). Lastly, we measured the neuraminidase activity of the NA2 VLPs to investigate the

functional activity of NA2 incorporated into NA2 VLPs. The NA2 VLPs displayed increasing NA activity with increasing total protein concentration (Figure 1E). VLP preparations treated identically but created via infection with only M1 rBVs did not show any NA activity above background levels at any concentration examined (Figure 1E). These results suggest that coinfection of Tni cells with rBVs expressing the NA2 and M1 gene from IAV produce enzymatically active NA2 VLPs of the expected size and morphology.

Intramuscular vaccination with NA2 VLPs is immunogenic and protective in a heterologous challenge model

Previous work with NA VLPs in a murine model has demonstrated that NA1 VLPs produced in Sf9 insect cells were immunogenic and effective against homologous challenge when administered via the IN route (Quan et al., 2012) and the IM route (Kim et al., 2019). To determine if NA2 VLPs produced in Tni cells were immunogenic, we prime-boost vaccinated groups of BALB/c mice with 5 µg NA2 VLPs via the IN route and the IM route 28 days apart. A separate group of mice were mock vaccinated IM with 1xPBS to serve as controls. Sera were collected 14 days post-prime and 14 days post-boost vaccination. Mice were challenged with 5xLD_{50} of homosubtypic but heterologous A/Hong Kong/1/1968 (H3N2) (85.9% neuraminidase amino acid homology between the NA2 VLP antigen A/Perth/16/2009 and A/Hong Kong/1/1968) virus 28 days post-boost vaccination with lung homogenates collected 4 days post-infection to evaluate viral lung titers. To evaluate the immunogenicity of NA2 VLPs, we measured total serum anti-NA2 VLP IgG levels via ELISA. In sera collected 14 days post-prime vaccination, IM vaccinated mice showed an approximate 4-fold increase over IN vaccination in total serum IgG levels (Figure 2A). The

magnitude of this difference increased 14 days post-boost vaccination, with IM vaccinated mice showing an approximate 9-fold increase over IN vaccinated mice in total serum IgG levels (Figure 2B). Higher total serum IgG levels correlated with an increased functional anti-NA2 immune response. Sera collected from mice post-boost vaccination via the IM route demonstrated a 2-fold increase in neuraminidase inhibition (NAI) titer compared to mice vaccinated IN (Figure 2C). Mice vaccinated via the IM route survived lethal infection with heterologous H3N2 virus despite a peak mean weight loss of 14.9% at day 5 post-challenge (Figure 2D). Fifty percent of mice vaccinated IN reached the experimental endpoint in the challenge experiment and were humanely euthanized (Figure 2E). All mock vaccinated mice reached the experimental endpoint by day 6 post-challenge and were humanely euthanized (Figure 2E). IM vaccinated mice demonstrated an approximate 2-log reduction in viral titers measured in lung lysates harvested 4 days post-infection (dpi) compared to mock vaccinated control mice (Figure 2F). Alternatively, the mean viral lung titer of IN vaccinated mice was reduced compared to mock vaccinated mice however this reduction was not statistically significant ($p = 0.063$) (Figure 2F). Taken together, these data suggest that IM vaccination with NA2 VLPs produced in Tni cells affords increased protection compared to IN vaccination in a lethal H3N2 IAV challenge model.

Vaccination with NA2 VLPs has a dose dependent effect on serum IgG levels and morbidity after challenge

Previous work has used varying doses of NA VLPs produced using Sf9 insect cell expression systems ranging from 5 µg – 10 µg per dose in murine models(Kim et al., 2019; Quan et al., 2012). In our IM model of vaccination, all mice displayed signs of morbidity in the form of weight loss

at a dose of 5 µg of total protein (Figure 2D). In an effort to improve upon the observed morbidity after challenge, we next sought to optimize the dose of NA VLPs in our IM vaccination regimen. Mice were prime-boost vaccinated 28 days apart followed by lethal challenge using 5xLD_{50} H3N2 virus 28 days after boost. Sera was collected 14 days post each vaccination and lung tissues were harvested 4 days post-infection. Four groups of mice were vaccinated IM with 1 µg, 5 µg, 10 µg, or 15 µg of total protein of NA2 VLPs. One group was mock vaccinated with 1xPBS to serve as controls.

Post-prime vaccination, all mice vaccinated with NA2 VLPs showed elevated anti-NA2 serum IgG levels compared to mock vaccinated control mice, with the 15 µg dose yielding a statistically significant total serum IgG concentration compared to both the 1 µg and 5 µg dose (Figure 3A). However, post-boost vaccination, serum IgG levels of mice vaccinated with 10 µg and 15 µg of NA2 VLPs were approximately 1.6 fold and 2.9 fold higher than the groups vaccinated with 5 µg and 1 µg of NA2 VLPs, respectively (Figure 3B). Interestingly, increasing the dose to 15 µg from 10 µg of NA2 VLPs did not result in a significant increase in mean serum anti-NA2 IgG levels ($p = 0.98$). Similar to the previous experiment investigating the route of administration, functional anti-NA2 antibody responses followed the same trend as the total serum anti-NA2 IgG levels. NAI titers increased with increasing doses of NA2 VLPs, however a plateau was observed at the 10 µg dose with no significant increase observed in total NAI titer at a 15 µg dose ($p = 0.8272$) (Figure 3C). Along these lines, observed morbidity decreased with an increasing dose of NA2 VLPs with a plateau observed when increasing from a 10 µg dose to a 15 µg dose of NA2 VLPs (Figure 3D). All mice experienced morbidity at all doses examined, however increasing the dose from 5 µg to 10 µg decreased the mean peak weight loss from 15.2% to 9.6%, respectively. No decrease in mean peak weight loss was observed when increasing the dose from

10 µg to 15 µg of total NA2 VLPs. While all groups experienced morbidity, mice vaccinated with a dose of 5 µg of total NA2 VLPs or higher were completely protected from mortality (Figure 3E). One individual animal vaccinated with a 1 µg dose did reach the experimental endpoint and was humanely euthanized 7 dpi, while all mice receiving the mock PBS vaccination reached the experimental endpoint and were humanely euthanized by 6 dpi (Figure 3E). Similar to the serology and challenge data, the 10 µg and 15 µg dose groups showed over a 1.5 log reduction in mean viral lung titers compared to mock vaccinated control mice, with no statistically significant difference between these groups, whereas the 5 µg dose group demonstrated an approximate 1 log reduction in viral titers (Figure 3F). While increasing the dose of NA2 VLPs resulted in a dose dependent reduction in mean viral titers from 1 µg up to a 10 µg dose, no statistical difference was observed between the vaccinated groups given the small sample size.

These data suggest that increasing the dose from 5 µg to 10 µg of total protein of NA2 VLP preparation reduces viral replication in lung tissues and reduces morbidity observed in our lethal H3N2 challenge model. Conversely, reducing the dose to 1 µg of NA2 VLPs resulted in a 0.5 log reduction in viral lung titers but only 80% survival after lethal challenge. Lastly, while positive effects were observed increasing the dose from 5 µg to 10 µg, no additional statistical benefit was observed by increasing the dose further to 15 µg of NA2 VLPs. These results suggest that a 10 µg dose of total protein of NA2 VLP preparation is the optimal dose in this challenge model.

Vaccination with NA2 VLPs was not protective in a heterosubtypic H1N1 lethal challenge model

It has been previously reported that vaccination with NA1 VLPs produced in an Sf9 insect cell expression system protected against mortality but not morbidity in a heterosubtypic, lethal H3N2 murine challenge model (Kim et al., 2019; Quan et al., 2012). However, the efficacy of NA2 VLPs against H1N1 viruses has not been previously investigated. To this end, we sought to investigate the ability of NA2 VLPs to protect mice at various doses against a lethal challenge using $5xLD_{50}$ of A/California/04/2009 (H1N1).

In this experiment, all mice were prime-boost vaccinated with 1 μg, 5 μg, 10 μg, 15 μg of NA2 VLPs or mock vaccinated with 1xPBS. All mice experienced morbidity in the form of rapid weight loss following lethal H1N1 virus infection (Figure 4A). Furthermore, all mice in all groups reached experimental endpoints by day 9 post infection and were humanely euthanized (Figure 4B). Based on these data, vaccination with NA2 VLPs did not provide protection against morbidity or mortality at any dose when challenged with a lethal dose of heterosubtypic H1N1 virus.

Multivalent vaccination with NA1 and NA2 VLPs efficiently induce both anti-N1 and anti-N2 antibodies

As vaccination with NA2 VLPs alone did not confer protection from morbidity or mortality in a heterosubtypic H1N1 challenge model, we next asked if simultaneous vaccination with NA1 and NA2 VLPs in a multivalent vaccine would afford to protection against both an H1N1 and H3N2 IAV. While attempting to coexpress both NA1 and NA2 antigens in a single host cell to create

multivalent VLPs is an attractive approach capable of potentially enriching cross reactive B cell epitopes in germinal center reactions, controlling for equivalent expression of both NA1 and NA2 anigens in a single preparation presents unique technical challenges. Along these lines, we blended NA1 and NA2 VLPs together after production to create a equal mixture of both VLP preparations. Previous work has identified potential intramolecular dominance of specific antigens and epitopes in multivalent vaccines with a biased response observed towards specific antigens in vaccine formulations (Schutze et al., 1989). To determine if inclusion of both NA1 and NA2 VLPs administered concomitantly in a multivalent form induced a biased immune response towards either group 1 or group 2 neuraminidases, we next examined sera from mice vaccinated with a multivalent NA1 + NA2 VLP vaccine. Groups of BALB/c mice were prime-boost vaccinated 28 days apart with 10 μg of a multivalent NA1 + NA2 vaccine (5 μg NA1 VLPs plus 5 μg NA2 VLPS), 10 μg of monovalent NA1 VLPs, 10 μg of monovalent NA2 VLPs, or mock vaccinated with 1xPBS. Mice were bled 14 days post-prime vaccination and 14 days post-boost vaccination to assess serological responses to both NA1 and NA2 antigen.

As expected, mock vaccination with 1xPBS produced minimal or undetectable serum IgG responses against rNA2 post-prime and post-boost vaccination (Figures 5A and 5B). While mean anti rNA2 serum IgG levels were slightly elevated post-boost vaccination with NA1 VLPs, this difference was not statistically significant ($p = 0.4245$) (Figure 5B). Furthermore, the commercially sourced rNA2 coating antigen was produced using a baculovirus expression system and purified via metal affinity chromatography, potentially leading to contaminating insect cell and baculovirus proteins contributing to background non-NA2 IgG reactivity in serum samples. Multivalent vaccination with NA1 + NA2 VLPs afforded an equivalent response against rNA2 to monovalent vaccination with NA2 VLPs alone (Figure 5A). Post-boost, vaccination with NA1 +

NA2 VLPs demonstrated an increased anti-NA2 IgG response compared to monovalent vaccination with NA2 VLPs (Figure 5B). Similar to previous experiments, total IgG levels correlated with an increased functional anti-NA titer. Specifically, mice receiving multivalent NA1 + NA2 VLPs had higher functional NAI titers against NA2 VLPs than mice vaccinated with monovalent NA2 VLPS (Figure 5C). Based on these data, multivalent vaccination with NA1 + NA2 VLPs induced a superior IgG response against group 2 NA.

Similarly, vaccination with 1xPBS or NA2 VLPs did not produce an appreciable serum IgG response against rNA1 post-prime or post-boost vaccination (Figures 5D and 5E). However, mean serum anti-N1 IgG levels were elevated post-boost vaccination in mice vaccinated with NA1+NA2 VLPs compared to vaccination with NA1 VLPs alone (Figure 5E). Likewise, sera collected post-boost vaccination from mice receiving either 1xPBS or NA2 VLPs did not exhibit any inhibition of NA1 neuraminidase (Figure 5F). Conversely, sera from mice vaccinated with NA1 + NA2 VLPs exhibited a significant increase in mean NAI titers compared to mice vaccinated with NA1 VLPs in a monovalent form. Similar to the serology results against group 2 NA, these data suggest that multivalent vaccination with NA1 + NA2 VLPs induced a superior IgG response against group 1 NA.

Multivalent vaccination with NA1 and NA2 VLPs is protective against lethal challenge with IAVs containing group 1 or group 2 neuraminidases

As vaccination with multivalent NA1 + NA2 VLPs appeared to have a positive effect on anti-NA IgG responses (Figure 5), we next sought to investigate the protection after lethal challenge with H1N1 and H3N2 IAVs. Mice were IM vaccinated in a prime-boost regimen with 10 µg total

protein of NA1 VLPs, NA2 VLPs, a combination of NA1 (5 μg) + NA2 (5 μg) VLPs, or mock vaccinated with 1xPBS. All groups were challenged 28 days post-boost vaccination with either 5xLD_{50} of H1N1 or H3N2 virus and monitored for morbidity in the form of weight loss, mortality, and for viral replication in lung tissues.

After challenge with H3N2 virus, all mice mock vaccinated with 1xPBS or vaccinated with NA1 VLPs showed severe morbidity (Figure 6A) and were humanely euthanized after reaching experimental endpoints by day 7 and day 8 post infection, respectively (Figure 6B). Similar to previous experiments, all mice vaccinated with either the monovalent NA2 VLPs or the multivalent NA1 + NA2 VLPs experienced minor morbidity with peak mean weight loss of approximately 8% and 5%, respectively (Figure 6A). However, all mice were completely protected from mortality when administered NA2 VLPs or NA1 + NA2 VLPs (Figure 6B). Notably, vaccination with NA1 + NA2 VLPs or NA2 VLPs alone resulted in an approximate 2.5 log and 1.5 log reduction in viral lung titers compared to mock PBS vaccination, respectively (Figure 6C). Vaccination with NA1 VLPs did not lead to a reduction in viral lung titers compared to the mock vaccinated group (Figure 6C). Interestingly, while vaccination with NA1 + NA2 VLPs did reduce the mean viral lung titer compared to monovalent vaccination with NA2 VLPs, this reduction was not statistically significant ($p = 0.0828$). Taken together, these results demonstrate that multivalent vaccination with NA1 + NA2 VLPs protected mice against mortality after lethal challenge with heterologous H3N2 IAV.

All mice vaccinated with 1xPBS or NA2 VLPs experienced weight loss (Figure 6D) and were humanely euthanized after reaching experimental endpoints after 9 and 10 days post infection with H1N1 virus, respectively (Figure 6E). Conversely, all mice vaccinated with monovalent NA1

VLPs or multivalent NA1 + NA2 VLPs were fully protected against homologous H1N1 IAV, with no signs of morbidity (Figure 6D) and no mortality (Figure 6E) throughout the observation period. Similarly, administration of both NA1 VLPs and NA1 + NA2 VLPs led to an equivalent approximate 3.5 log reduction in viral lung titers compared to mock vaccination with 1xPBS after homologous challenge (Figure 6F). Expectedly, viral lung titers in mice vaccinated with NA2 VLPs did not differ significantly from mice mock vaccinated with 1xPBS after a heterosubtypic challenge with H1N1 IAV (Figure 6F). Based on these data, the multivalent administration of NA1 + NA2 VLPs is protective against lethal challenge with homologous H1N1 IAV and equivalent to monovalent NA1 VLPs alone.

Discussion

Currently licensed influenza vaccines both target and predominantly induce antibody responses against HA glycoproteins with highly variable or non-existent anti-NA responses (Chen et al., 2018; Couch et al., 2012b; Laguio-Vila et al., 2015; Powers et al., 1996). However, anti-NA2 responses have been shown to contribute to protection after both infection and immunization in humans (Beutner et al., 1979; Monto and Kendal, 1973; Murphy et al., 1972). Previous works investigating the role of NA VLPs as candidate IAV vaccines have focused on using NA1 as the immunizing antigen (Easterbrook et al., 2012; Kim et al., 2019; Quan et al., 2012; Smith et al., 2017), however none have investigated the role of only NA2 as an immunizing antigen in an insect cell based NA VLP expression system. Here, we used a murine model to demonstrate that IM vaccination with NA2 VLPs containing the NA2 antigen from A/Perth/16/2009 provided protection against a distant heterologous mouse adapted H3N2 virus (A/Hong Kong/1/1968) but failed to protect mice in a lethal heterosubtypic challenge with H1N1 virus (A/California/04/2009). However, bivalent vaccination with NA1 and NA2 VLPs induced stronger humoral immune responses against both NA1 and NA2 antigens as well as an overall decrease in morbidity and viral lung titers in our H3N2 challenge model. High anti-NA serum levels measured by ELISA and NAI have correlated with protection in a ferret and murine models of infection (Quan et al., 2012; Smith et al., 2017; Walz et al., 2018b) and as an independent correlate of protection in humans (Monto et al., 2015). Additionally, passive transfer of anti-NA immune sera to naïve animals has been demonstrated to be protective in murine challenge models suggesting that humoral immunity is sufficient to confer protection (Walz et al., 2018b; Wohlbold et al., 2015a). In this study, total IgG levels and NAI titers correlated with protection of mice against morbidity and mortality as well as reducing viral replication after lethal challenge with IAV.

The majority of trivalent and quadrivalent inactivated split virus vaccines and recombinant purified HA protein vaccines currently licensed for use are administered intramuscularly. Additionally, a live attenuated influenza vaccine is licensed for IN administration. In this study, we compared both routes of administration for NA2 VLPs. Previous work has demonstrated protection against both homologous and heterosubtypic challenge using NA1 VLPs via both the IM and IN routes (Kim et al., 2019; Quan et al., 2012). However, IN vaccination did not offer complete protection against mortality in our heterologous challenge model. Of note, NA VLPs used in this study were produced in Tni insect cells where previous work with NA VLPs in insect cell expression systems used Sf9 expression systems (Kim et al., 2019; Quan et al., 2012; Smith et al., 2017). Vaccine production platforms require the ability to scale to produce adequate supply and Tni cells offer an attractive expression system for influenza VLPs with their increased VLP protein yields over Sf9 cells while minimizing contaminating baculovirus production (Krammer et al., 2010). However, local activation of the innate immune system by residual, contaminating baculovirus has been shown to increase the immunogenicity and subsequent survival after vaccination with influenza VLPs in a murine model (Heinimäki et al., 2017; Margine et al., 2012). Previous work has also shown that IN vaccination with a purified recombinant NA combined with an adjuvant afforded superior protection compared with IM vaccination using the same NA protein in a murine model (Wohlbold et al., 2015a), and that the incorporation of membrane bound adjuvants into VLPs has demonstrated increased immunogenicity after vaccination against influenza and other viruses (Liu et al., 2018; Skountzou et al., 2007). Further studies are needed to determine if the addition of adjuvants could improve the immunogenicity of both IN and IM administered NA VLPs produced in Tni cells.

Licensed influenza vaccines contain both an H1N1 and H3N2 IAV strain in addition to one or two Influenza B strains and, therefore, do not need to rely on heterosubtypic cross protection for efficacy. Multiple previous studies have shown that vaccination with NA1 VLPs was completely protective against mortality, but not morbidity, after challenge with a heterosubtypic H3N2 virus (Kim et al., 2019; Quan et al., 2012). Conversely, other studies vaccinating with purified recombinant NA have demonstrated homosubtypic but not heterosubtypic protection (Deroo et al., 1996; Wohlbold et al., 2015b). While the NA2 VLPs produced in the current study were protective against a heterologous homosubtypic H3N2 virus challenge, the NA2 VLPs afforded no protection against morbidity or mortality in a lethal heterosubtypic H1N1 virus challenge (Figure 4A and 4B). However, vaccination with a bivalent NA1 and NA2 VLPs preparation did offer protection against morbidity and mortality in both H3N2 and H1N1 challenge models. Similar to previous work examining the administration of a bivalent mixture of NA1 and NA2 purified proteins, we did not observe any antigenic competition between the NA1 or NA2 subtypes when both antigens were administered concomitantly (Johansson and Kilbourne, 1994). Conversely, we observed an increase in total mean IgG levels and NAI titers against each individual antigen similar to that observed by Johansson and Kilbourne, suggesting possible common B cell or T cell epitopes between subtypes. Indeed, previous work has identified shared B cell epitopes between NA subtypes and multiple monoclonal antibodies specific for epitopes that bind to and inhibit multiple NA subtypes (Doyle et al., 2013; Gravel et al., 2010; Rijal et al., 2019). Specifically, Doyle et al. identified a monoclonal antibody specific for the universally conserved influenza A NA active site epitope “ILRTQESEC” capable of inhibiting NA activity in 9 different subtypes. It is also possible that shared T cell epitopes between NA subtypes contribute to increased positive selection during B cell germinal center affinity maturation leading to

increased IgG levels observed in this study. Studies aimed at generating broadly neutralizing antibodies targeting the conserved HA stalk have taken the approach of repeatedly vaccinating with the same HA stalk with different chimeric irrelevant globular HA heads included in each subsequent vaccination (Krammer et al., 2013). This approach has yielded an increase in antibodies targeting the shared epitopes presented in each subsequent vaccination and afforded protection independent of CD8 T cells. The specific mechanisms responsible for enrichment of both total IgG and functional anti-NA antibodies warrant further investigation. Investigation of broadly reactive anti-NA antibodies capable of inhibiting multiple subtypes will require further research investigating antibody responses at a monoclonal level while the contribution of potential shared T cell epitopes will require further studies to map T cell epitopes for individual NA molecules. None the less, functional anti-NA immunity and its subsequent contribution to protection against morbidity caused by influenza virus infection appears to be a useful and underappreciated correlate of protection.

There is evidence that when HA and NA are presented together in the same virus particle, the immune response to HA is immunodominant over the NA response in priming both B and T lymphocytes (Johannsson et al., 1987). More recent work has improved the immunogenicity of the NA molecule by extending the length of the NA stalk by as few as 15 or 30 amino acids to potentially improve recognition by B cell receptors of infected hosts (Broecker et al., 2019b). Vaccination with NA VLPs where NA is presented on an individual particle in the absence of HA molecules has the potential to overcome the limitation of HA immunodominance. However, other work has shown that while both N1 VLPs and H5N1 VLPs protected against mortality in a homologous lethal H5N1 ferret challenge model, the experimental group vaccinated with H5N1 VLPs containing both the homologous HA and NA showed a reduction in morbidity and nasal

wash titers after challenge compared to the group vaccinated with N1 VLPs alone (Smith et al., 2017). While the role of anti-HA immunity and its ability to prevent virus infection has long been appreciated in homologous challenge models, the additional supplementation of seasonal influenza vaccines with purified NA has been shown augment the anti-NA immune response and increase the breadth of immunity against potentially drifted influenza viruses (Johansson et al., 2002, 1998; Kim et al., 2017). Indeed, individuals with higher anti-NA2 antibody titers developed after infection with circulating H2N2 viruses were less likely to be infected upon emergence of the 1968 Hong Kong H3N2 virus into the human population (Monto and Kendal, 1973; Murphy et al., 1972). Furthermore, anti-NA1 antibody titers have been previously credited with lessening the severity of the 2009 H1N1 pandemic (Marcelin et al., 2011). However, more recent work has shown that current seasonal vaccine responses are dominated by anti-HA recall responses with very poor anti-NA responses (Chen et al., 2018). NA VLPs offer a platform to supplement existing strategies already effective at eliciting anti-HA immune responses while presenting the NA molecule to the host immune system in its native, membrane bound conformation.

Taken together, the data from this study suggest that bivalent vaccination with NA1 and NA2 VLPs results in a balanced immune response against both group 1 and group 2 neuraminidases capable of protecting mice from mortality and reducing viral replication in both an H1N1 and H3N2 challenge model. Furthermore, these data show that there is no deleterious effect from antigenic competition between the two different NA subtypes in our murine model. While murine models of influenza infection are inexpensive and well established, mice are not natural hosts for IAVs. Expanding these studies to a ferret or swine model of IAV infection would be a logical next step and provide valuable insight into the applicability of these data in an outbred animal model.

Acknowledgements

Special thank you to Hong Yi of the Robert P. Apkarian Integrated Electron Microscopy Core of Emory University for technical assistance with electron microscopy in this publication.

Funding Support

This work was supported by the National Institutes of Health (1R01 AI110680-04)

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Figures

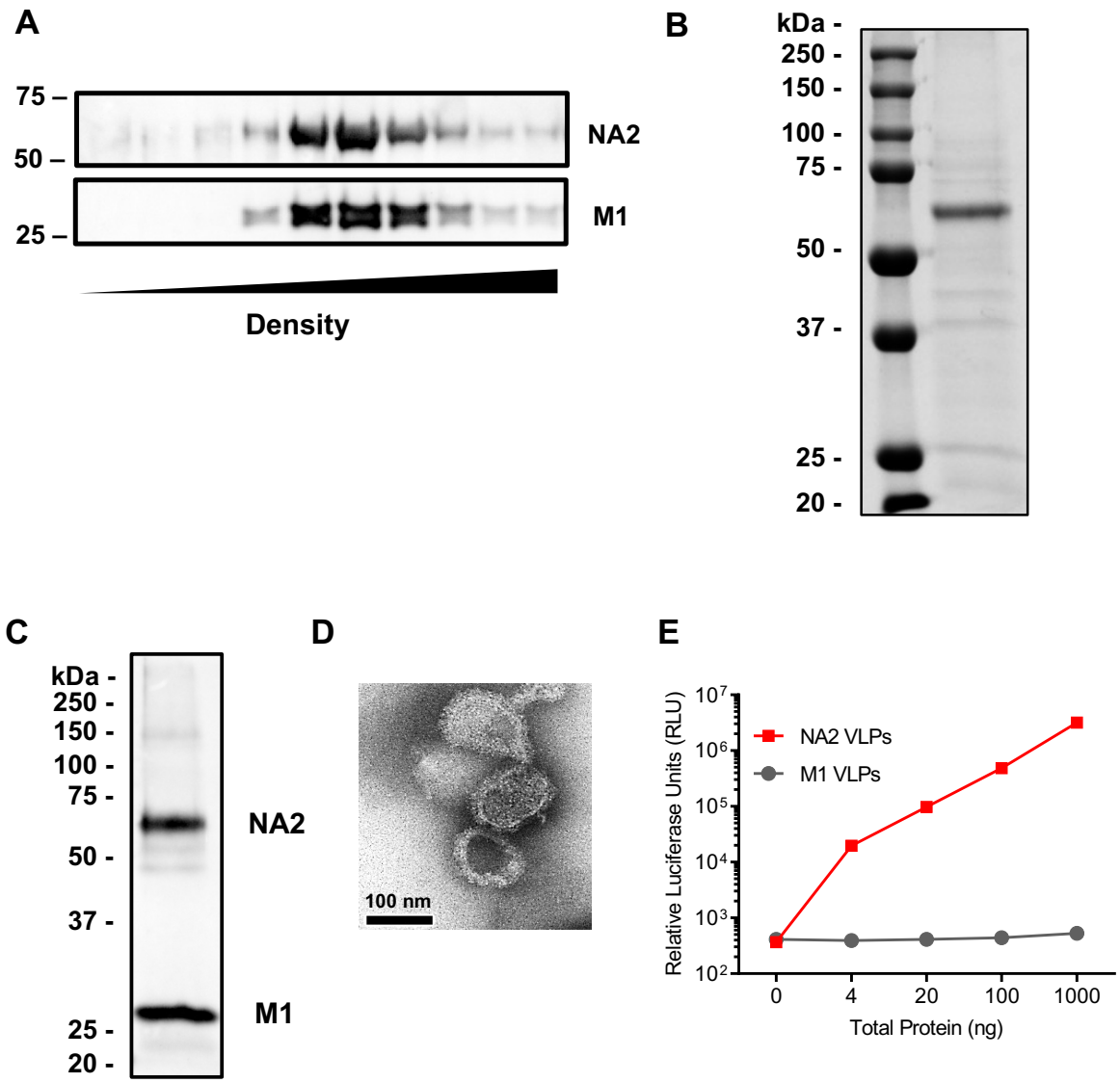

Figure 1: NA2 VLP purification and characterization. (A) Western blot analysis of the migration of NA2 VLPs in 20-60% sucrose gradients after ultracentrifugation. Lane 1 represents the fraction with the lowest density, lane 10 represents the fraction with the highest density. NA2 blots were probed with polyclonal goat anti-NA2 sera, while M1 blots were probed with polyclonal goat anti-M1 sera. (B) Coomassie stained SDS-PAGE gel loaded with 10 µg of NA2 VLP

preparation. Molecular weight in kilodaltons (kDa) is indicated to the left. (C) Western blot of 10 µg of NA2 VLP preparation. Blot was probed with polyclonal goat anti-NA2 and anti-M1 sera. Molecular weight in kilodaltons (kDa) is indicated to the left. (D) Negative stain electron micrograph of NA2 VLP preparation. Scale bar represents 100 nm. (E) Neuraminidase activity by NA-STAR assay. The NA2 VLP preparation is indicated by the black line and symbols, while M1 only VLPs are indicated by the grey line and symbols. Individual symbols represent the mean of 3 replicates. Error bars represent the standard error of the mean (SEM).

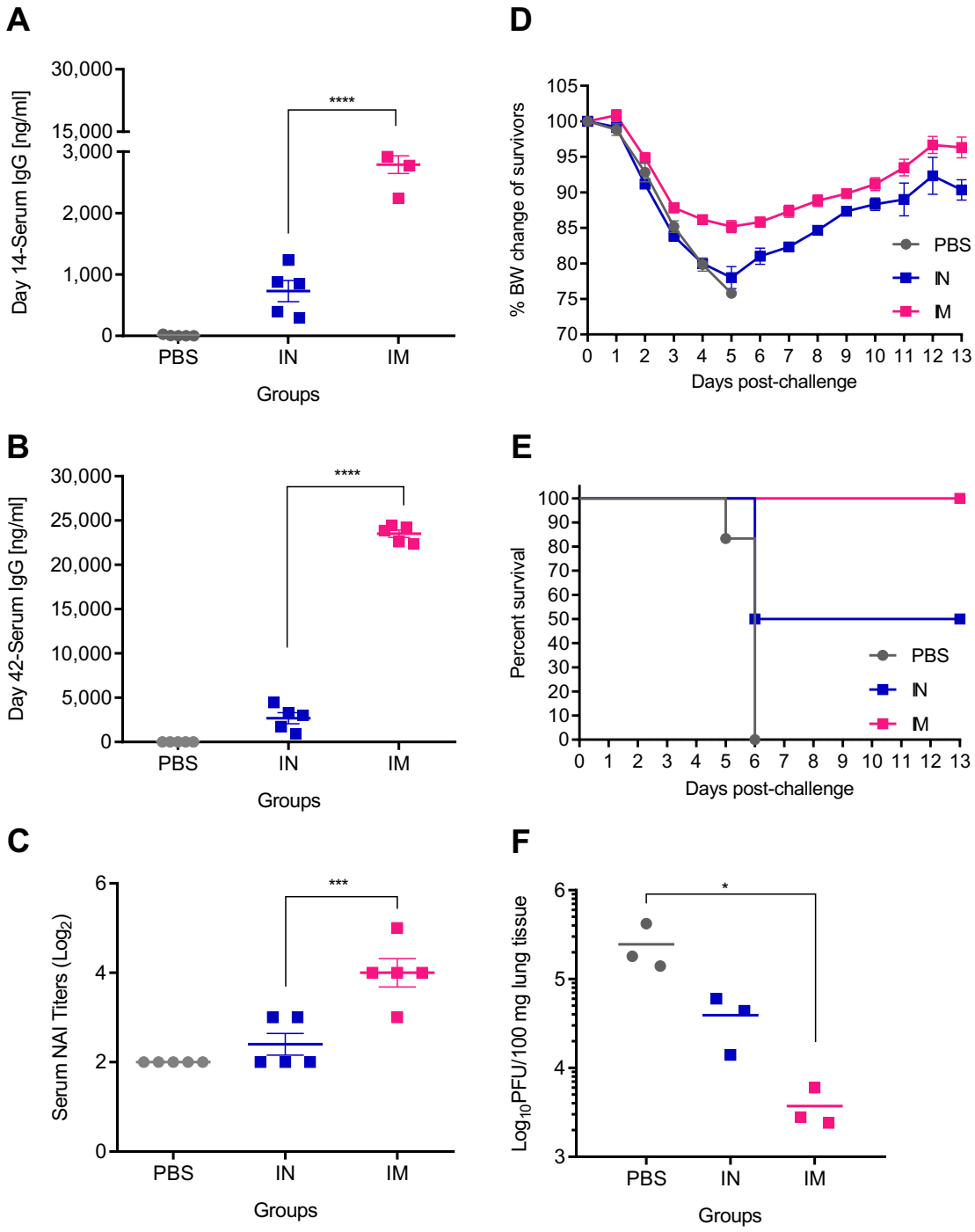

Figure 2: The route of administration affects immune responses to NA2 VLPs. Groups of BALB/c mice were vaccinated in a prime-boost regimen 28 days apart with 5 µg total protein of NA2 VLP preparation intramuscularly (IM), intranasally (IN), or mock vaccinated IM with PBS.

Mice were then challenged 28 days post-boost vaccination with 5xLD_{50} mouse adapted A/Hong Kong/1/1968 (H3N2) virus. (A) Total serum IgG responses measured by ELISA at 14 days post-prime vaccination (N=5). (B) Total serum IgG responses measured by ELISA at 14 days post-boost vaccination (N=5) (C) Neuraminidase inhibition (NAI) titers measured 14 days post-boost vaccination (N=5). (D) Body weight changes of mice after challenge with heterologous H3N2 virus (N=5). (E) Mortality of mice after challenge with heterologous H3N2 virus (N=5). (F) Viral titers of lung homogenates harvested at 4 days post infection (N=3). Error bars represent SEM. *= $p<0.05$; ***=$p<0.001$; ****=$p<0.0001$.

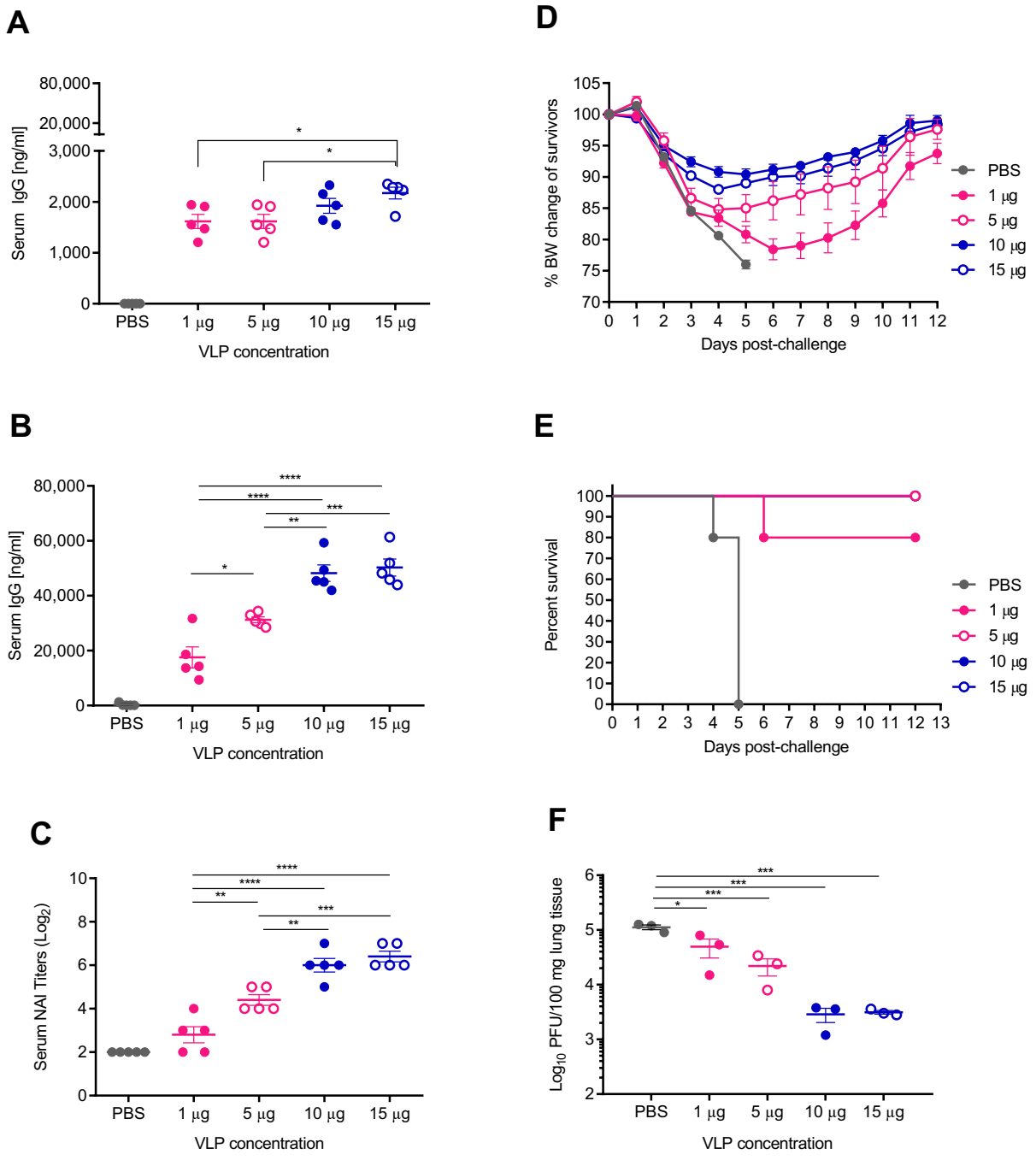

Figure 3: Dose response to intramuscular (IM) vaccination with differing amounts of NA2 VLPs. Groups of BALB/c mice were vaccinated IM in a prime-boost regimen 28 days apart with 1 µg, 5 µg, 10 µg, or 15 µg total protein of NA2 VLP preparation or mock vaccinated with PBS. Mice were then challenged 28 days post-boost vaccination with 5xLD_{50} mouse adapted A/Hong

Kong/1/1968 (H3N2) virus. (A) Total serum IgG responses measured by ELISA at 14 days post-prime vaccination (N=5). (B) Total serum IgG responses measured by ELISA at 14 days post-boost vaccination (N=5) (C) Neuraminidase inhibition (NAI) titers measured 14 days post-boost vaccination (N=5). (D) Body weight changes of mice after challenge with heterologous H3N2 virus (N=5). (E) Mortality of mice after challenge with heterologous H3N2 virus (N=5). (F) Viral titers of lung homogenates harvested at 4 days post infection (N=3). Error bars represent SEM. *= $p<0.05$; **=$p<0.01$; ***=$p<0.001$; ****=$p<0.0001$.

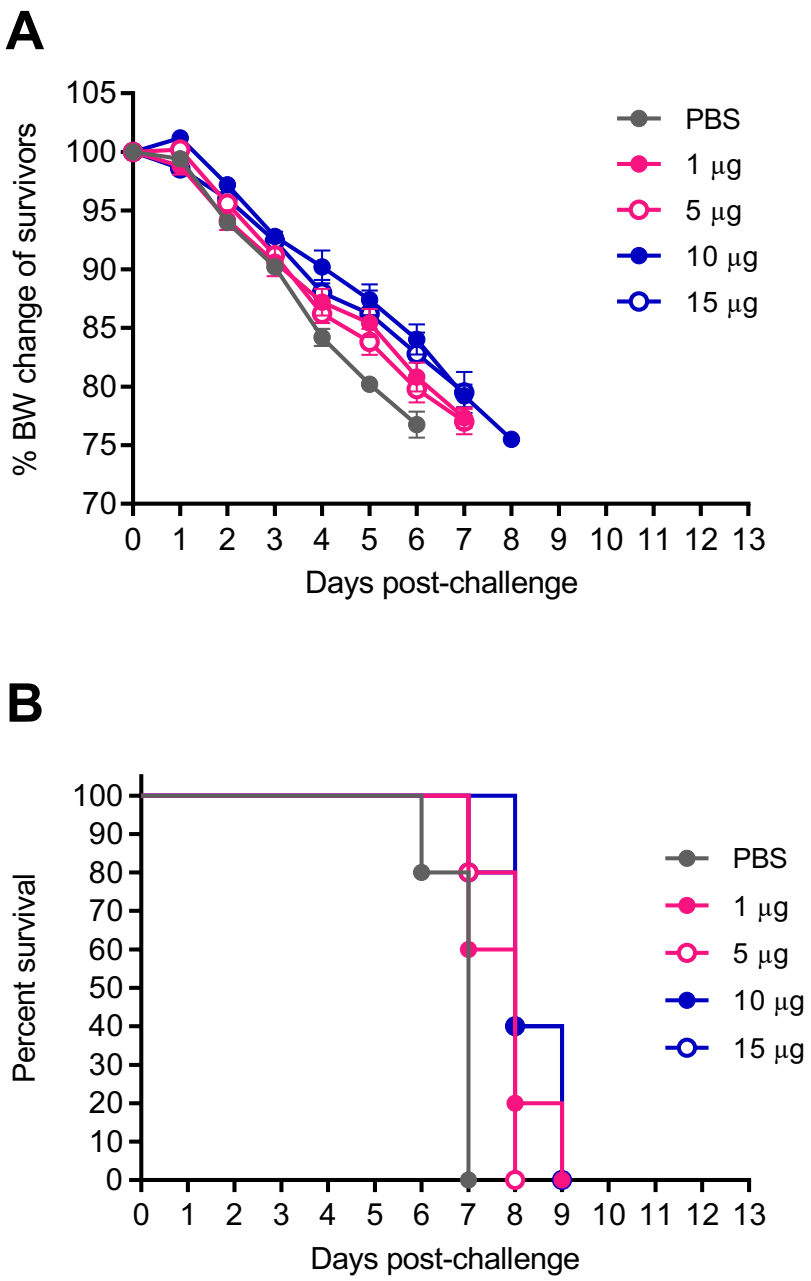

Figure 4: Intramuscular vaccination with differing amounts of NA2 VLPs in a heterosubtypic H1N1 challenge model. Groups of BALB/c mice were intramuscularly (IM) vaccinated in a prime-boost regimen 28 days apart with 1 µg, 5 µg, 10 µg, or 15 µg total protein

of NA2 VLP preparation or mock vaccinated with PBS. Mice were then challenged 28 days post-boost vaccination with 5xLD_{50} mouse adapted A/California/04/2009 (H1N1) virus. (A) Body weight changes of mice after challenge with heterosubtypic H1N1 virus. (B) Mortality of mice after challenge with heterosubtypic H1N1 virus. N=5 mice per group. Error bars represent SEM.

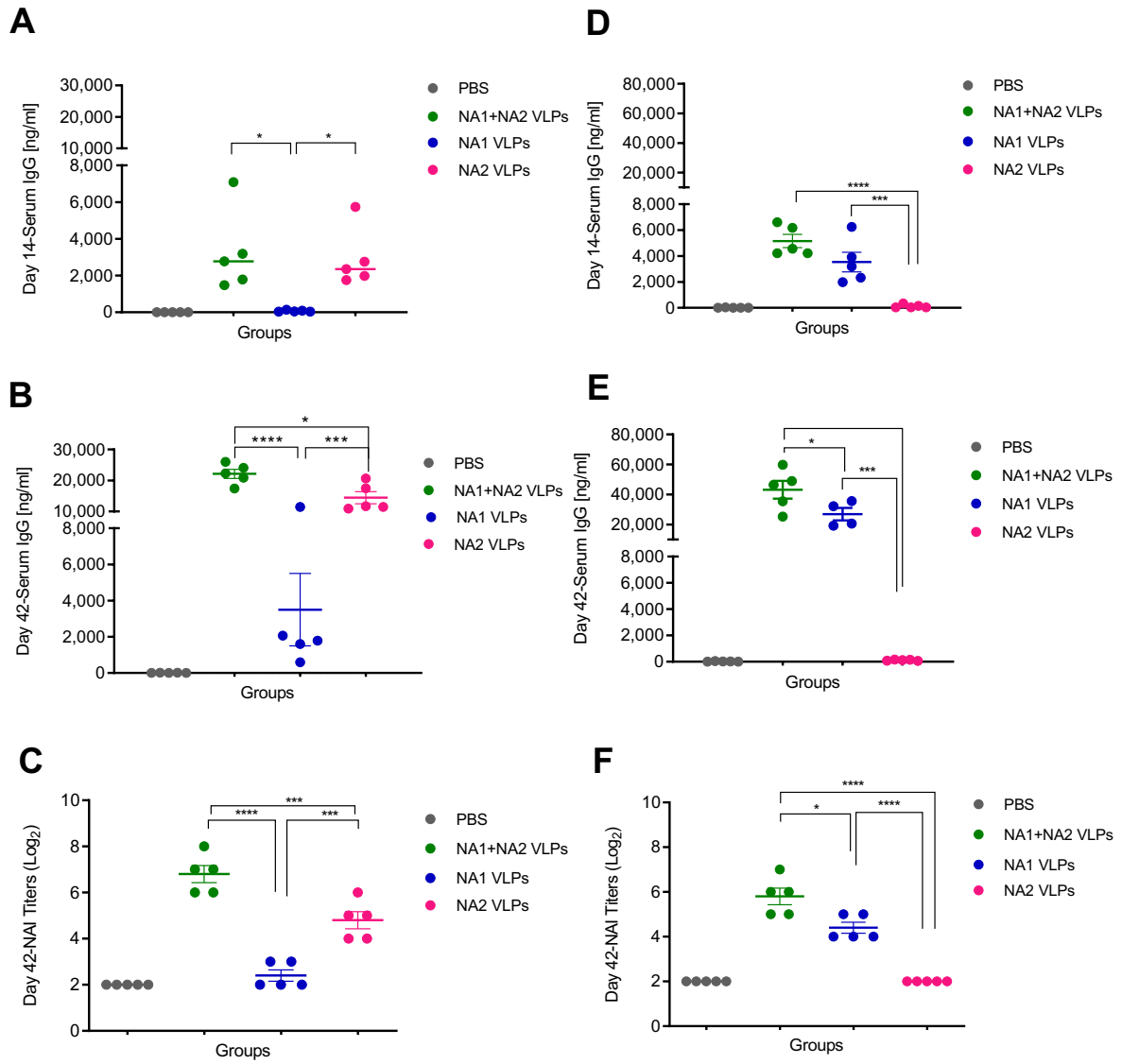

Figure 5: Serological responses to the combined administration of NA1 + NA2 VLPs. Groups of BALB/c mice were vaccinated IM in a prime-boost regimen 28 days apart with 10 µg NA1 + NA2 VLPs (5 µg NA1 VLPs + 5 µg NA2 VLPs), 10 µg NA1 VLPs, 10 µg NA2 VLPs or mock vaccinated with PBS. (A) Total serum anti NA2 IgG responses measured by ELISA at 14 days post-prime vaccination. (B) Total serum anti NA2 IgG responses measured by ELISA at 14 days post-boost vaccination. (C) Neuraminidase inhibition (NAI) titers against NA2 VLPs measured 14 days post-boost vaccination. (D) Total serum anti NA1 IgG responses measured by ELISA at 14

days post-prime vaccination. (E) Total serum anti NA1 IgG responses measured by ELISA at 14 days post-boost vaccination. (F) NAI titers against NA1 VLPs measured 14 days post-boost vaccination. N=5 mice per group. Error bars represent SEM. *= $p<0.05$; ***=$p<0.001$; ****=$p<0.0001$.

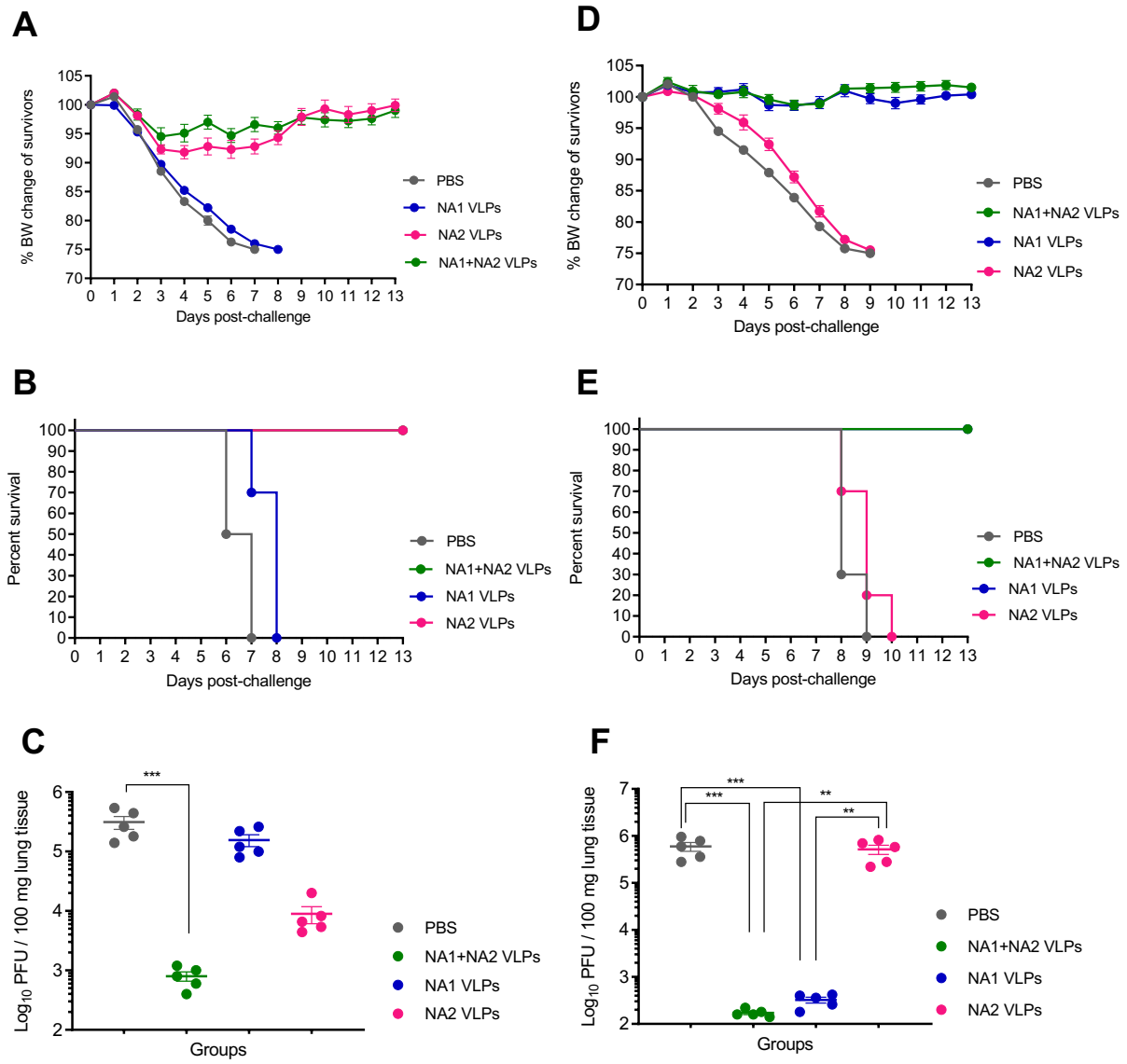

Figure 6: Lethal challenge of mice vaccinated with combined administration of NA1 + NA2 VLPs using H3N2 and H1N1 viruses. Groups of BALB/c mice were vaccinated IM in a prime-boost regimen 28 days apart with 10 µg NA1 + NA2 VLPs (5 µg NA1 VLPs + 5 µg NA2 VLPs), 10 µg NA1 VLPs, 10 µg NA2 VLPs or mock vaccinated with PBS. Mice were then challenged 28 days post-boost vaccination with either 5xLD_{50} mouse adapted A/Hong Kong/1/1968 (H3N2) virus or 5xLD_{50} mouse adapted A/California/04/2009 (H1N1) virus. (A) Body weight changes of

mice after challenge with H3N2 virus (N=10). (B) Mortality of mice after challenge with H3N2 virus (N=10). (C) Viral titers of lung homogenates harvested at 4 days post infection with H3N2 virus (N=5). (D) Body weight changes of mice after challenge with H1N1 virus (N=10). (E) Mortality of mice after challenge with H1N1 virus (N=10). (F) Viral titers of lung homogenates harvested at 4 days post-infection with H1N1 virus (N=5). Error bars represent SEM. **=$p<0.01$; ***=$p<0.001$.

Chapter III: Neuraminidase Virus-Like Particle vaccine: protective efficacy and immunogenicity in the swine model

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Keywords: neuraminidase, influenza A virus, virus like particles, vaccine, swine

Manuscript in Preparation / Submission

Abstract

Influenza A viruses (IAVs) pose a global health concern, contributing to hundreds of thousands of deaths and millions of hospitalizations annually. The two major surface glycoproteins of IAVs, hemagglutinin (HA) and neuraminidase (NA), are important antigens in eliciting neutralizing antibodies and subsequent protection against IAV disease. Immune responses directed against the NA protein have been shown to limit viral replication and protect against IAV disease in small animal models and human vaccination studies. However, the role of anti-NA immunity has not been investigated in a swine model of IAV infection. In this study, we evaluate influenza NA virus-like particles (VLPs) as a candidate IAV vaccine. We developed an NA2 VLP vaccine containing the NA protein from the A/Perth/16/2009 (H3N2) strain and the matrix 1 (M1) protein from the A/Michigan/73/2015 strain formulated with a water-in-oil-in-water adjuvant. Responses to NA2 VLPs were compared to a commercially available, quadrivalent whole inactivated virus (QWIV) swine IAV vaccine or pigs mock vaccinated with water-in-oil-in-water adjuvant. Pigs were prime boost vaccinated 21 days apart and challenged four weeks later with the H3N2 swine IAV field isolate A/swine/NC/HK1552516/2016. Pigs vaccinated with the commercial QWIV vaccine demonstrated high hemagglutination inhibition (HAI) titers but very weak anti-NA antibody titters and subsequently undetectable NA inhibition (NAI) titers. Conversely, NA2 VLP vaccinated pigs demonstrated undetectable HAI titers but high anti-NA antibody titers and functional NAI titers. Post challenge, NA2 VLPs and the commercial QWIV vaccine showed similar reductions in lung viral titers, pulmonary neutrophilic infiltration, and lung inflammation in the form of histopathology scores compared to mock vaccinated controls. Taken together, these data suggest that anti-NA immunity and NA2 VLP vaccination offer comparable protection to QWIV swine IAV vaccines inducing primarily anti-HA responses.

Introduction

Influenza A viruses (IAV) constitute a major public health concern and their pandemic potential highlights the need for vaccines that induce broadly protective immune responses against potential novel zoonotic IAV strains (Bailey et al., 2018; Rajão and Pérez, 2018). Currently available human influenza vaccines are standardized by hemagglutinin (HA) content only and, therefore, induce primarily anti-HA immune responses (Wohlbold and Krammer, 2014). Available influenza vaccines have historically demonstrated sub-optimal efficacy, especially against heterologous IAV viruses and newly emerged novel IAVs (De Jong et al., 2000; Flannery et al., 2019; Rolfes et al., 2019; Tricco et al., 2013; Zimmerman et al., 2016). The immunodominance of HA observed after vaccination with current vaccines has led to selection of escape mutants that result in antigenic mismatch between the HA in seasonal vaccines and the HA in circulating viruses at the time of infection which contributes to the observed low vaccine effectiveness of current seasonal vaccines (Das et al., 2013; Krammer, 2015).

Neuraminidase (NA) is the second most abundant glycoprotein on the surface of influenza virions and exists as a homotetramer typically present at a ratio of 1 to 4-5 with respect to HA (Harris et al., 2006). NA functions as a sialidase, cleaving terminal sialic acid residues from both host and viral glycosylated proteins to enable virion motility to receptor-dense regions of the epithelium and progression to the lower respiratory tract (Air, 2012; Gilbertson et al., 2017; McAuley et al., 2019). In addition to promoting viral uptake and infectivity, enzymatic removal of sialic acids prevents virion aggregation at the surface of infected cells, facilitating the efficient detachment and spread of nascent progeny virions (Air, 2012; McAuley et al., 2019; Palese et al., 1974). In the quest for improved influenza vaccines and conserved immunogenic epitopes which can induce broadly

reactive protection, neuraminidase has been identified as a candidate for use as a vaccine antigen that can potentially elicit broadly reactive NA-specific protective immune responses against heterologous viruses (Eichelberger and Monto, 2019; Jang and Seong, 2019; Johansson and Cox, 2011; Wohlbold and Krammer, 2014).

Contrary to HA specific immunity where the majority of HA-induced antibodies prevent viral infection by blocking HA receptor-binding or fusion activity, humoral responses directed against NA do not elicit a neutralizing immune response. Rather, anti-NA antibodies are characterized by inducing infection-permissive immunity (Couch et al., 1974a; Johansson et al., 1989, 1993). Rather than preventing viral infection, antibodies targeting neuraminidase inhibit nascent virion release through the inhibition of enzymatic activity by sterically blocking access of substrates to the NA catalytic pocket and preventing cleavage (Krammer et al., 2018a). In effect, these neuraminidase inhibiting antibodies limit viral replication and dissemination within the host by tethering newly budded virion clumps to the infected cell plasma membrane (Marcelin et al., 2012; Palese et al., 1974; Xiong et al., 2020). In addition to inhibiting NA enzymatic activity, anti-NA antibodies have also been reported to facilitate viral clearance by mediating recognition and killing of infected cells by immune effector cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (DiLillo et al., 2016). However, anti-NA immunity has also been shown to be protective in Fc-gamma receptor knock-out mice, suggesting ADCC effector functions are not necessary for NA mediated protection in murine models of IAV infection (Kim et al., 2019).

A number of vaccination-challenge studies have demonstrated that NA immunization using different types of vaccine platforms has conferred sufficient protective immunity to reduce both disease morbidity and mortality while limiting virus shedding (Kilbourne et al., 2004; Rockman

et al., 2013; Sandbulte et al., 2007; Sylte et al., 2007; Yang et al., 2012). As a platform, the use of virus like particles (VLPs) is an attractive option for NA presentation due to their resemblance in morphology, size, and structural conformation of viruses as well as their ability to present antigen in its native, membrane bound conformation (Kang et al., 2012). In addition to their immunogenicity, VLPs offer advantages with respect to safety as they are void of genetic material making VLPs non-replicating molecules without the risk of reversion to virulence (Haynes, 2009; Kang et al., 2012; Quan et al., 2016, 2007b). Additionally, VLP vaccine development is cost effective and offers the opportunity for a customizable vaccine approach allowing for the selective inclusion of specific antigens (Roldão et al., 2010). Previous reports of vaccination with HA VLP constructs demonstrated protective immune responses against homologous and heterologous seasonal influenza viruses as well as against potentially pandemic influenza viruses (Haynes, 2009; Liu et al., 2015; Quan et al., 2007a).

Recent studies have reported that intranasal and intramuscular vaccination of mice with NA1 VLPs produced in insect cells induce heterosubtypic immunity in a lethal murine infection model (Kim et al., 2019; Quan et al., 2012). An initial study demonstrated that intranasal immunization of mice with VLPs containing the NA1 and matrix 1 (M1) protein from the H1N1 PR8 strain was protective against a lethal challenge with the H3N2 A/Philippines/1982 influenza strain (Quan et al., 2012). A subsequent study by the same investigators reported that the NA and M1 proteins from the 2009 pandemic H1N1 strain (H1N1pdm09) conferred protection against lethal challenge with a A/Phillipines/1982 and a recombinant virus containing the HA and NA genes from the highly pathogenic avian influenza H5N1 A/Vietnam/1203/2004 strain, resulting in reduced pulmonary viral titers and reduced pathology (Kim et al., 2019). However, investigations

by our group and others using NA VLPs produced in insect cell expression systems have failed to show protection against heterosubtypic challenge (Menne et al., 2021; Smith et al., 2017).

Of the 9 subtypes of IAV neuraminidase (NA1-NA9) that have been identified in influenza viruses circulating in wild waterfowl, only the NA1 and NA2 subtypes currently circulate in human and swine populations (Rajao et al., 2019; Tong et al., 2013). Pigs are natural hosts of influenza A viruses and have comparable distribution of sialic acid receptors to humans throughout their respiratory tract (Trebbien et al., 2011). These shared receptor distributions enable influenza A viruses to cross species barriers, adapt, and establish endemic transmission in new populations resulting in the co-circulation of H1N1 and H3N2 subtypes in both humans and swine (Kyriakis et al., 2011). Additionally, similar clinical manifestations, pathogenesis, and immunological responses to influenza infection and vaccination in humans and pigs make the latter an ideal animal model for the study of vaccine immunogenicity, efficacy, and influenza A virus pathogenesis (Kyriakis et al., 2009; Rajao et al., 2019; Rajao and Vincent, 2015; Reeth et al., 1998).

Currently, no studies investigating the immunogenicity and breadth of protection of NA2 neuraminidase in large animal models have been reported. Investigation of anti-NA2 immunity in additional animal models is warranted as H3N2 influenza viruses have recently demonstrated increased pathogenicity compared to H1N1 viruses, resulting in higher rates of morbidity and mortality in the elderly and other at risk populations (Guarner and Falcón-Escobedo, 2009). Furthermore, the immunogenicity of NA VLPs has yet to be explored in the swine model. In the current study, we evaluated neuraminidase 2 virus-like particles (NA2 VLPs) as a candidate influenza vaccine in a vaccination-challenge model in swine. Specifically, we investigated the immunogenicity of NA2 (A/Perth/16/2009 H3N2 strain) and M1 (A/Michigan/73/2015 H1N1

strain) VLP formulation in swine and efficacy after challenge with the heterologous H3N2 swine field isolate A/Swine/NC/HK1552516/2016. This work demonstrates the immunogenicity and efficacy of NA2 VLPs in a swine model and highlights the contribution of anti-NA immunity in contrast to the anti-HA immunity provided by currently available swine vaccines.

Materials & Methods

Cells and virus stocks

Spodoptera frugiperda (Sf9) cells (IPLB-Sf-21-AE, Expression Systems, Davis, CA, USA) were maintained at 27˚C in suspension in shaker flasks using serum free SF900II media (Gibco/ThermoFisher Scientific, Waltham, MA, USA). Trichoplusia ni (Tni) cells (Expression Systems, Davis, CA, USA) were maintained at 27˚C in suspension in shaker flasks using serum free ESF 921 media (Expression Systems, Davis, CA, USA). Madin-Darby canine kidney (MDCK) cells (ATCC CCL 34, American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's media (DMEM) (Corning Life Sciences, Corning, NY, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Westborough, MA, USA), 1% antibiotic-antimycotic solution (Corning Life Sciences, Corning, NY, USA), in a 37°C incubator with 5% CO_2. Animals were challenged with A/swine/NC/HK1552516/2016, H3N2 swine IAV field isolate, propagated in MDCK cells. Virus titer was determined by 50% median tissue culture infectious dose ($TCID_{50}$) calculated using the Reed and Münch formula (Reed and Muench, 1938). Challenge virus stocks were aliquoted for single-use applications and stored at −80°C. On the day of challenge, virus was prepared for an inoculum of 10^6 $TCID_{50}$/ml. Inoculum was administered intranasally in 2 ml total volume (1 ml per nostril, total challenge does of $10^{6.3}$ $TCID_{50}$) using a mucosal atomization device (MAD Nasal™ Atomization Device, Teleflex, Wayne, PA).

Animals

Eighteen 6-week-old influenza-naive conventional Yorkshire/Hampshire cross pigs, were obtained from Auburn University's Swine Research Center (influenza virus and porcine reproductive and respiratory syndrome virus-seronegative farm). All study procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University. Until one week prior to challenge, animals were housed in industry-standard pens in groups of 10 at the Swine Research and Education Center and were provided food and water ad libitum. All eighteen pigs arrived at the age of 13 weeks into the BSL-2 facilities of the Sugg Laboratory for Animal Health Research. Pigs were randomly divided in four groups as outlined in the swine vaccination and virus challenge section. Each experimental group was housed in a separate isolation unit with HEPA filtered air. Food and water were provided ad libitum.

VLP Production and Purification

Neuraminidase 2 virus like particles (NA2 VLPs) were produced and purified as previously described (Menne et al., 2021). Briefly, sequence information for the NA2 gene from A/Perth/16/2009 and the M1 gene from A/Michigan/73/2015 were synthesized with the addition of a unique BamHI endonuclease restriction site at the 5' end and a unique HindIII endonuclease restriction site at the 3' end (Integrated DNA technologies, Coralville, IA, USA). Gene fragments were digested and directionally cloned into a pFastBac1 vector (ThermoFisher Scientific, Waltham, MA, USA). Baculovirus stocks were created according to the Bac-to-Bac® manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA). The resulting baculovirus stocks infected Tni cells at a multiplicity of infection (MOI) of 5 for NA2 expressing

recombinant baculoviruses (rBVs) and 3 for M1 rBVs. VLPs were purified from the culture supernatant as previously described (Menne et al., 2021)

Vaccine preparation

NA2 VLP antigen was co-formulated with the Water-in-Oil-in-Water (W/O/W) emulsion (Montanide^{TM} ISA 206 VG) which served as the adjuvant. Vaccine formulation was prepared under low shear rate and stored according to the manufacturer's instructions, one day prior each vaccination. For the commercial vaccine, we used FluSure XP® (Zoeitis Inc., Kalamazoo, MI, USA) which is a licensed swine quadrivalent whole inactivated virus (QWIV) influenza vaccine, that contains H1N1 Gamma cluster and H3N2 Cluster IV strains, A/swine/North Carolina/394/2012, which is a Cluster IV-A virus and A/swine/Minnesota/872/2012, which is a Cluster IV-B virus, and an H1N2 Delta-1 virus. Animals were vaccinated with either the recombinant NA2 VLP vaccine, the FluSure XP® commercial vaccine or a Phosphate-Buffered Saline (PBS) in adjuvant solution. Each pig received a deep intramuscular injection into the neck with 2 ml of the FluSure XP® commercial vaccine, 1 ml of the NA VLP vaccine (60 µg/mL) or 1 ml of phosphate-buffered saline with W/O/W adjuvant.

Swine Vaccination, Virus Challenge, and Sample Collection

Pigs were randomly allocated to one of three groups. Group 1 pigs (n = 6) were vaccinated with the NA2 VLP vaccine co-formulated with the water-in-oil-in-water (W/O/W) adjuvant (ISA 206, Seppic). Group 2 pigs (n = 6) were vaccinated with the QWIV vaccine (FluSure XP, Zoetis). Group

3 pigs (n=6) were mock vaccinated with W/O/W adjuvant (ISA 206, Seppic) only and served as controls. Prime immunization of the animals was conducted at the age of 6 weeks and booster immunization was administered 3 weeks later, at the age of 9 weeks. Throughout the course of the immunogenicity stage, the animals were housed at the Swine Research and Education Center.

Three weeks after the administration of the boost vaccination and one week prior to the onset of the animal challenge, all eighteen pigs were transferred into the BSL-2 facilities of the Sugg Laboratory for Animal Health Research. They were randomly divided into four groups: two groups of 6 pigs, including 2 NA2 VLP vaccinated animals, 2 QWIV vaccinated animals and 2 mock vaccinated controls; the other two groups were comprised of 3 animals; 1 NA2 VLP vaccinated animal, 1 QWIV vaccinated pig and 1 mock vaccinated control. The four groups of animals were housed in separate isolation units and were provided food and fresh water *ad libitum*. Serum was collected prior to prime and boost vaccination, at 2 weeks post-boost, and at 4 weeks post-boost prior to challenge. Four weeks after boost, the two groups of 6 and one group of 3 animals (n=15), were challenged with live virus ($10^{6.3}$ $TCID_{50}$ in 2 mL of PBS) by intranasal inoculation using a mucosal atomization device (MAD Nasal™ Atomization Device, Teleflex, Wayne, PA). The fourth group of 3 animals was not challenged and served as the non-infected control. However, one pig of the fourth group was removed from the study due to a medical issue unrelated to the experiment. Animals were clinically scored daily post challenge based on rectal temperature, respiratory rate and assessment of clinical demeanor. Specifically, rectal temperature score ranged from 0 to 3 (< 103°F = 0, 103-103.9°F = 1, 104 - 104.9°F = 2, > 105°F = 3), respiratory rate per minute score ranged from 0 to 2 (20-40 = 0, 41-59 = 1, > 60 = 2) and clinical behavior score, based on coughing (absent = 0, present = 1) and depression (absent = 0, present = 2) ranged from 0 to 3. Nasal swabs were collected daily starting two days prior to challenge until

day 5, for the valuation of viral shedding. All animals were humanely euthanized with a lethal dose of pentobarbital on day 5 post challenge. On the day of euthanasia, bronchoalveolar lavage fluid (BALF) was harvested for cytopathology and pigs were necropsied. During necropsy, nasal turbinates, trachea, right lung lobes (including accessory lobe) and left lung lobes were collected for histopathological evaluation and examination of viral replication.

Measurement of NA-specific IgG antibody titers

Sera from individual animals were evaluated for end-point dilution antibody titers as previously described with modifications (Masic et al., 2009; Pyo et al., 2012). Briefly, Nunc Maxisorb 96-well plates (ThermoFisher Scientific, Waltham, MA, USA) were coated overnight at 4˚C with 100 ng / well of purified recombinant N2 protein (rN2) produced using the sequence information from A/Wisconsin/67/2005 (BEI Resources #NR-19237, Manassas, VA, USA). After overnight coating, plates were blocked for 1 hour at room temperature with 200 µl of blocking solution (1x PBS with 0.1% Tween 20) followed by an automated wash step (4x with 1x PBS-T, 1x PBS with 0.1% Tween 20). Sera were then serially diluted and incubated as previously described followed by the same wash step. Plates were then incubated with goat anti-porcine IgG secondary antibody (Novus Biologicals, Centennial, CO, USA) followed by an additional wash step. Plates were then incubated with rabbit anti-goat-HRP detection antibody (Southern Biotech, Birmingham, AL, USA) followed by an additional wash step. Plates were developed for 20 minutes at room temperature after addition of OPD substrate (ThermoFisher Scientific, Waltham, MA, USA). Plate absorbance was read at 450 nm using an xMark Microplate Spectrophotometer (BioRad, Hercules, CA, USA). The end point dilution titer of a sample was defined as the highest reciprocal serum

dilution where the optical density (OD) measured greater than two times the mean of the blank sample.

Neuraminidase Activity and Inhibition Assay

Purified VLPs preparations were tested for enzymatic neuraminidase activity using the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, VLP solutions were diluted in NA-Star Assay Buffer and then incubated with substrate for 30 min at room temperature. NA-Star accelerator solution was then injected followed by measurement of the chemiluminescent signal in relative luminescent units (RLU) via Modulus II Microplate Luminometer (Promega, Madison, WI, USA). Inhibition of neuraminidase activity was measured similarly and as previously described with modifications (Chen et al., 2018). Briefly, 25 µl of processed sera diluted in NA-Star buffer was incubated with 40 ng of NA2 VLPs diluted in NA-Star buffer or $1.04x10^4$ $TCID_{50}$ of A/swine/NC/HK1552516/2016 diluted in NA-Star buffer in a volume of 25 µl for a total volume of 50 µl at 37˚C for 20 minutes. Diluted NA-Star Substrate was added in a volume of 10 µl and incubated at room temperature for 30 minutes. The chemiluminescent reaction was initiated upon injection of 60 µl of NA-Star Accelerator followed by measurement using the Modulus II Microplate Luminometer (Promega, Madison, WI, USA). The reciprocal of the highest serum dilution resulting in an inhibition of 50% of NA activity compared to NA-Star buffer alone was defined as the NAI titer.

Hemagglutination Inhibition (HAI) assay

Sera for hemagglutination inhibition titers were processed according to the WHO protocol (Who, 2011). Serum samples were treated for 1 hour at 37ºC with receptor destroying enzyme (RDE) (Denka Seiken Co Ltd) to remove nonspecific inhibitors. The residual RDE and the complement were inactivated at 56°C for 30 minutes. Sera were incubated with packed turkey RBC overnight at 4ºC, for removal of non-specific cryoglobulins interfering with RBC and antigen-antibody binding. Two-fold serially diluted sera were co-incubated with 4 HA units of MDCK grown A/swine/NC/HK1552516/2016 H3N2 stock for one hour followed by the addition of 0.5% turkey red blood cells for 45 minutes, all steps performed at room temperature (Esser et al., 2017; Kitikoon et al., 2014). The HAI titers were read as the reciprocal of the highest dilution of serum that conferred inhibition of hemagglutination.

Virus Titration ($TCID_{50}$) Assay

Virus titration assays were performed on nasal swabs and respiratory tissue homogenates to evaluate viral shedding and virus replication between vaccinated and unvaccinated animals. Nasal swabs collected daily post-challenge from each animal were first suspended with 2ml of phosphate-buffered saline (PBS) supplemented with 1% antibiotic-antimycotic solution, then mechanically agitated for 10 minutes at 4°C and stored as aliquots at -80°C for further use in virus titration assays. Additionally, respiratory tissue samples (nasal turbinates, trachea, right, left and accessory lung lobes) harvested after euthanasia were also processed prior to virological evaluation. Tissue samples were weighed and then suspended with cold PBS supplemented with 2% antibiotic-antimycotic solution cocktail to reach 10% gr of tissue dissolved in PBS. Tissue

suspensions were then homogenized and centrifuged. Subsequently, tissue homogenate supernatants were collected, aliquoted and stored at -80°C for $TCID_{50}$ assays.

Tested samples were initially serially tenfold diluted and transferred to confluent MDCK cell cultures in 96-flat bottomed well plates and were subsequently incubated at 37°C for 72 hours. After 72 hours, wells were subjected to microscopic evaluation for the presence of CPE and virus titers were determined by the Reed-Muench method (Reed and Muench, 1938).

Cytopathological Evaluation of Bronchoalveolar Lavage Fluid

After euthanasia, lungs were collected from all the (n=17) for tissue and bronchoalveolar lavage fluid (BALF) harvesting. The lungs were washed with Ca/Mg deficient PBS (Corning Life Sciences, Corning, NY, USA). Following BALF collection, samples were filtered through a cell strainer (70 µm pore size) (VWR International, Radnor, PA) to remove mucous and centrifuged at 1200 x g for 5 minutes. After centrifugation, the supernatant was collected and stored in aliquots at -80°C for further use. Before use, the pelleted cells were resuspended in 1X Lysis Buffer (BioLegend, San Diego, CA) and incubated for 5 minutes at room temperature. The cell suspension was then quenched with an equal volume of PBS and centrifuged at 1200 x g for 5 minutes. After supernatant was discarded, BAL cells were counted (Countess II Automated Cell Counter, Thermo Fisher Scientific, Carlsbad, CA) and resuspended with the appropriate volume of PBS to achieve a final density of 0.5-1 x 10^5 cells, added to the slide and cytocentrifuged at 1200 rpm for 5 minutes. The cytocentrifuged smear was stained with a three step Wright-Giemsa Stain (Quick Stain, VWR International, Radnor, PA) for cytological evaluation. A differential cell count was determined microscopically by evaluating 300 cells on a cytospin smear. Epithelial cells were not included in

the differential count and the cell differentials in BAL fluid were expressed as the percentage of the total number of cells counted (Jolie et al., 2000).

Macroscopic and microscopic evaluation of respiratory tissues

At necropsy, macroscopic scoring of lung lesions was performed to estimate the percentage of lung lobes affected (Halbur et al., 1995). Dark purple, depressed, firm areas of the lung lobes were considered a typical lesion for swine influenza virus infection. Additionally, sections of nasal turbinates, trachea and all seven lung lobes were collected from each individual pig for histopathological evaluation and were placed in 10% neutral buffered formalin for fixation. Tissues were routinely processed, embedded in paraffin, cut at 5 µm, stained with H&E, and examined by light microscopy (Olympus BX53; Olympus Scientific Solutions Technologies Inc., Waltham, MA). A modified version of a swine influenza grading system was applied to obtain histopathologic scores (Morgan et al., 2016). Histopathology scores considered the percent of lung affected, extent of bronchial and bronchiolar epithelial changes, degree of lymphocytic peribronchiolar cuffing, and the severity of interstitial pneumonia. Each criterion was scored from 0 to 4, with 4 being most severe, and added to obtain an overall score. Trachea was evaluated for epithelial changes and degree of tracheitis and was scored from 0 to 3. For the nasal turbinates, epithelial changes and the degree of rhinitis were examined and scored 0 to 3. Blinded microscopic evaluation was performed by two individuals, including a board-certified veterinary pathologist and the scores were averaged.

Statistical analysis

GraphPad Prism software (version 9, San Diego, CA, USA) was used for statistical analysis. Statistical comparisons between vaccinated groups were performed via one way ANOVA with post-hoc Bonferroni multiple comparisons with an alpha of 0.05 ($\alpha \leq 0.05$). The nonparametric Kruskal-Wallis test was used to test differences between groups for the macroscopic and microscopic lesion scoring.

Results

NA2 VLPs are immunogenic in swine model

In previous work, we demonstrated that NA2 VLPs produced using a recombinant baculovirus (rBV) co-infection expression model in *Trichoplusia ni* insect cells contained enzymatically active NA suggestive of proper tertiary and quaternary protein structure (Menne et al., 2021). Furthermore, the NA2 VLPs were immunogenic in a murine model and protective in a lethal murine H3N2 challenge model (Menne et al., 2021). To examine the applicability of NA2 VLP vaccination in a swine model of IAV infection, we first investigated the immunogenicity of adjuvanted NA2 VLPs in prime-boost vaccination regimen in pigs. Three groups of pigs (n=5) were vaccinated in a prime-boost regimen 21 days apart at day 0 and day 21 with a NA2 VLPs, a commercial swine influenza A licensed quadrivalent whole inactivated virus vaccine (QWIV) (FluSure XP, Zoetis), or mock vaccinated with water-in-oil-in-water adjuvant only. Serum was collected on day 0 before prime vaccination, on day 21 at boost vaccination, on day 35, and on day 48. Anti-NA IgG titers were measured via ELISA.

Prior to vaccination on day 0, all pigs were seronegative for NA specific IgG (Figure 1A). After prime vaccination on day 21, only pigs vaccinated with NA2 VLPs showed a measurable anti-NA IgG titer (Figure 1B). Interestingly, QWIV vaccinated pigs showed no anti-NA response above background after a single prime vaccination and, as expected, neither did the mock vaccinated group (Figure 1B). At 2 weeks post-boost vaccination, NA2 VLP vaccinated pigs demonstrated a statistically significant increase in NA specific serum IgG titers of approximately 50-fold compared to WIV vaccinated pigs ($p < 0.0001$) (Figure 1C). QWIV vaccinated pigs also showed a mean increase of approximately 4-fold in NA specific IgG titers over mock vaccinated

pigs at 2 weeks post-boost vaccination ($p < 0.01$) (Figure 1C). At four weeks post-boost, this trend continued with NA2 VLP vaccinated pigs maintaining an approximate 40-fold increase in anti-NA IgG titers compared to QWIV vaccinated pigs ($p < 0.0001$) and QWIV vaccinated pigs maintaining an approximate 3.5-fold increase in IgG titers over mock vaccinated pigs ($p < 0.05$) (Figure 1D).

These data suggest that NA2 VLPs are immunogenic and elicit high serum anti-NA IgG titers after vaccination. In addition, these data suggest that two doses of QWIV are required to elicit a modest but measurable anti-NA2 IgG response.

NA2 VLPs induce functional antibody responses directed at NA

While anti-NA antibody responses have historically been recognized as important in small animal models and in human IAV infection (Monto and Kendal, 1973; Murphy et al., 1972; Schulman et al., 1968), more recently the functional anti-NA antibody response capable of inhibiting NA enzymatic activity has been correlated with reduction of disease in humans (Memoli et al., 2016; Monto et al., 2015). Along these lines, we next measured the ability of sera from vaccinates to inhibit the NA enzymatic activity of the NA2 antigen contained in both the NA2 VLPs (A/Perth/16/2009) and the H3N2 challenge virus (A/Swine/NC/HK1552516/2016).

We first evaluated the ability of sera collected from vaccinates to inhibit enzymatic activity the A/Perth/16/2009 NA2 antigen contained in the NA2 VLPs. Interestingly, while the QWIV vaccine induced a measurable anti-NA IgG response post-boost vaccination (Figure 1C and 1D), sera collected from WIV vaccinated animals did show elevated neuraminidase inhibition (NAI)

titers above mock vaccinated pigs at any timepoint (Figure 2A-2D). As expected, sera from mock vaccinated animals also did not show any appreciable NAI titer at any timepoint sampled (Figure 2A-2D). However, NA2 VLP vaccinated pigs showed a statistically significant elevation in NAI titer compared to mock and QWIV vaccinated animals after prime vaccination ($p < 0.05$) (Figure 2B). This trend continued post-boost vaccination at day 35, with sera from all NA2 VLP vaccinates showing elevated NAI titers compared to mock and QWIV vaccinates ($p < 0.0001$) (Figure 2C). Finally, sera collected at day 48 from NA2 VLP vaccinates also showed elevated NAI titers compared to mock and QWIV vaccinates ($p < 0.0001$) (Figure 2D).

We next investigated the ability of sera collected from vaccinates to inhibit the NA activity of the NA2 antigen of the H3N2 challenge virus A/Swine/NC/HK1552516/2016. Similar to results obtained for the inhibition of the NA2 antigen used in the VLP vaccine, A/Perth/16/2009 (Figure 2), at no timepoint post-prime or boost vaccination did sera from mock vaccinated or QWIV vaccinated pigs demonstrate the ability to inhibit the NA activity of the challenge virus (Figure 3A – 3D). However, sera collected from NA2 VLP vaccinated animals demonstrated significantly elevated NAI titers compared to both mock and QWIV vaccinated groups post-prime vaccination ($p < 0.01$) (Figure 3B). As expected, this trend continued post-boost vaccination with NAI titers of sera from NA2 VLP vaccinates showing a significant increase compared to sera from QWIV and mock vaccinated groups ($p < 0.001$) (Figure 3C – 3D).

Appreciable NAI titers were observed in NA2 VLP vaccinated pigs both post-prime and post-boost vaccination against both antigens assayed, suggesting that NA2 VLP vaccination induced a broad anti-NA functional antibody response. Conversely, vaccination with QWIV did not significantly differ in ability to inhibit NA activity compared to mock vaccination at any

timepoint against either antigen, suggesting that QWIV vaccination either does not induce functional anti-NA antibody capable of inhibiting NA enzymatic activity or does so at a level below the limit of detection against NA2 antigen from A/Perth/16/2009 or A/Swine/NC/HK1552516/2016.

Vaccination with QWIV vaccine induces antibodies directed at HA capable of inhibiting hemagglutination of erythrocytes

As vaccination with QWIV did not induce functional antibodies capable inhibiting NA enzymatic activity in the form of measurable NAI titers, we next investigated the ability of vaccination with QWIV to induce antibodies capable of inhibiting hemagglutination. While the FDA and EMA consider serum hemagglutination inhibiting (HAI) titers greater that 1:40 to be a correlate of protection in humans (Memoli et al., 2016), no such specifically quantified HAI titer correlated to protection against disease exists in swine. An HAI assay was performed for the assessment of HA-specific serum antibodies induced by the QWIV vaccine. Serum samples collected at day 0, 21, 35 and 48 were analyzed by HAI assay to investigate the magnitude of functional anti-HA antibody levels against the heterologous A/SW/NC/KH1552516/2016 challenge virus. As expected, sera from NA2 VLP and mock vaccinated animals did not show a measurable HAI titer at any timepoint assayed (Figure 4). Conversely, QWIV vaccinated animals demonstrated elevated mean HAI titers 2 weeks post-boost vaccination (80 ± 48.99) and four weeks post-boost vaccination (96 ± 60.66) (Figure 4). Interestingly, while QWIV vaccinated animals had detectable HAI titers post-boost vaccination, all QWIV vaccinated individuals had an HAI titer below the limit of detection for the assay (1:20) after a single prime vaccination (Figure 4).

As suspected, NA2 VLP and mock vaccinated pigs did not demonstrate measurable HAI titers at any point post vaccination. However, QWIV vaccination induced appreciable HAI titers post-boost vaccination. While there is no reported HAI titer correlating with protection against IAV disease in swine, the human HAI correlate of protection may highlight the necessity of a second immunization with QWIV vaccine to elicit robust anti-HA functional antibodies in naïve pigs.

Challenge with A/Swine/NC/KH1552516/2016 did not induce significant clinical disease

A scoring system was applied for the evaluation of clinical disease after challenge with A/Swine/NC/KH1552516/2016. Recorded scores were used for monitoring the clinical status of the animals and for extrapolation of vaccine induced protection. From day -2 prior to challenge through day 5 post challenge, rectal temperature, respiratory rate and clinical behavior in the form of presence of coughing or weakness/depression were recorded daily. The range of scores of clinical disease ranged from 0 to 8, with 8 indicating the most sever clinical disease. Throughout the observation period post-challenge, no statistically significant differences were observed between the three groups of pigs at any timepoint (Figure 5). Overall, intranasal challenge resulted in mild clinical disease manifestation without clear distinctions in clinical signs or disease between vaccinated and unvaccinated animals.

QWIV vaccination reduced viral shedding post challenge

To evaluate virus shedding post-challenge, nasal swabs were collected daily through day 5 and virus was measured via titration in a $TCID_{50}$ assay. Overall, differences observed in mean virus titer collected from nasal swabs post-challenge were not statistically significant between the three vaccinated groups on days 1, 2, 4, and 5 post challenge (Figure 6). However, samples on day 3 post challenge demonstrated a significant reduction of greater than 1 Log of virus was observed in nasal swabs collected from QWIV vaccinated pigs compared to mock vaccinated pigs ($p=0.0099$) (Figure 6). Although a the difference wan not statistically significant, mean viral shedding recovered from nasal swabs trended lower in the NA2 VLP and QWIV vaccinated animals compared to mock vaccinated pigs (Figure 6).

Vaccination with NA2 VLPs and QWIV reduces viral replication in swine lung tissue

To investigate the ability of vaccination to inhibit viral replication throughout the respiratory tract, tissue samples were collected at necropsy, homogenized, and viral titers measured via $TCID_{50}$ assay. Pigs vaccinated with the QWIV vaccine demonstrated a statistically significant reduction in viral load in homogenized nasal turbinates compared to the unvaccinated animals ($p=0.014$) but not compared to NA2 VLP vaccinated pigs (Figure 7A). Vaccination with NA2 VLPs did not significantly affect virus replication in nasal turbinates relative to mock vaccination (Figure 7A). Additionally, mean tracheal viral loads appeared to be reduced in pigs vaccinated with either the QWIV vaccine or NA2 VLPs compared to mock vaccinated pigs, although the reduction was not significant (Figure 7B). In contrast to the upper respiratory tract where no clear differences were observed between our experimental vaccine and the mock group, in the lower respiratory tract both

vaccination with NA VLPs and the QWIV vaccine performed in a similar fashion resulting in statistically significant reduction of viral replication ($p < 0.0001$) (Figure 7C and 7D). In summary, vaccination of pigs with both NA VLPs and QWIV vaccine reduced viral replication after challenge with A/Swine/NC/KH1552516/2016.

Vaccination with NA2 VLPs and QWIV reduces pulmonary neutrophilic infiltration

To assess pulmonary infiltration of inflammatory cells, bronchoalveolar lavage fluid (BALF) was collected from all animals at necropsy 5 days post infection. In healthy uninfected pigs (n=2), the mean ratio between the cellular elements in BALF was 77.5% resident macrophages, 14.5% small sized lymphocytes and 8% non-degenerate neutrophilic granulocytes (Figure 8). The unvaccinated control animals exhibited statistically significant higher percentages of neutrophils (46.8 ± 21.16) and lower percentage of macrophages (47.6 ± 22.11) compared to the vaccinated groups (Figure 8). These findings support the presence of moderate to severe pulmonary neutrophilic inflammation, typical of uncomplicated influenza virus infection. In contrast to the neutrophilic accumulation observed in the BALF samples of unvaccinated pigs, the majority of BAL cells were macrophages in vaccinated animals (78 ± 5.339 and 71.8 ± 8.389, QWIV and NA2 VLP vaccine group, respectively) with the presence of low percentage of neutrophils (Figure 8). The QWIV and NA2 VLP vaccinated animals showed significant reduction ($p<0.0001$ and $p=0.0003$, respectively) in lung neutrophilic infiltration (11.4 ± 6.693 and 15.4 ± 9.45, respectively) compared to unvaccinated pigs (Figure 8). No significant differences were identified in the percentage of lymphocytes between the groups (Figure 8). These data suggest that vaccination of pigs with NA2 VLPs or QWIV vaccine reduces neutrophilic infiltration after IAV infection.

Vaccination with NA2 VLPs and QWIV reduces pulmonary histopathology scores

Gross pulmonary pathology was evaluated for the assessment of macroscopic lung lesion profiles. The majority of unvaccinated animals displayed dark red, multilobular to coalescing consolidation of the cranioventral regions of lungs consistent of uncomplicated influenza virus infection in swine. The macroscopic lung lesions and overall score of QWIV vaccine group were indistinguishable from those in the NA2 VLP group, and although the scores were lower compared to the unvaccinated control group, there was no statistically significant difference between the groups. Next, the severity of microscopic lesions in the lung lobes, trachea, and nasal turbinates were evaluated. The composite pulmonary histopathology score was significantly higher in the unvaccinated control animals (7.36 ± 2.35) compared to the NA2 VLP (4.01 ± 1.15, p=0.02) and QWIV vaccine group (3.98 ± 0.87, p=0.019) (Figure 9A). In particular, the unvaccinated control group demonstrated higher severity lesions regarding the interstitial thickening of the alveolar septa and lymphocytic peribronchiolar cuffing compared to the animals in the NA2 VLP vaccinated and QWIV vaccine group (Figure 9B). However, no statistically significant differences were observed in the microscopic nasal turbinate and trachea lesion scores, between vaccinated and unvaccinated control animals. These data demonstrate that vaccination of pigs with NA2 VLPs or QWIV reduces both macroscopic and microscopic pulmonary lesions while reducing lung histopathology scores.

Discussion

The swine origins of the 2009 H1N1 pandemic virus highlighted the importance of controlling IAV for human public health in addition to the economic impacts to swine producers (Calderón Díaz et al., 2020; Gibbs et al., 2009; Mena et al., 2016). While vaccine development has historically focused on anti-HA responses (Eichelberger and Monto, 2019; Johansson and Cox, 2011), anti-NA responses have recently been identified as a stronger correlate of protection against disease caused by IAV in humans (Memoli et al., 2016). While VLPs containing the only the NA1 surface glycoprotein have been previously studied in small animal models (Kim et al., 2019; Quan et al., 2012; Smith et al., 2017), only one study to date has investigated VLPs containing only the NA2 surface glycoprotein in a small animal model (Menne et al., 2021). Additionally, only one study to date has investigated VLPs containing HA and NA produced in insect cells in a swine model (Pyo et al., 2012). Here, we investigated the immunogenicity and efficacy of NA2 VLPs in an H3N2 infection model. Separating the relative contributions of anti-HA and anti-NA immunity to overall vaccine efficacy is difficult using traditional whole inactivated virus or split virus vaccines, and NA VLPs provide a useful tool to examine the contribution of NA specific immunity afforded through vaccination. In the current study, vaccination with NA2 VLPs offered similar protection against clinical signs of IAV disease, reduction in viral replication in lung tissues, and reduction in lung inflammation to a licensed, commercially available QWIV vaccine.

Interestingly, while vaccination with QWIV resulted in robust functional anti-HA immunity capable of inhibiting hemagglutination after boost vaccination (Figure 4), QWIV vaccination barely induced a measurable anti-NA2 response (Figure 1) and no measurable functional anti-NA2 response (Figure 2, Figure 3). Similarly, previous work evaluating HA and

NA immune responses in mice concluded that the presence of both HA and NA on the same inactivated virion led to antigenic competition, with HA being immunodominant over NA in priming B cells and T cells (Johannsson et al., 1987). However, subsequent work demonstrated that detergent disruption and disassociation of HA and NA from the same inactivated virion eliminated this antigenic competition (Johansson et al., 1989; Johansson and Kilbourne, 1993). Along these same lines, more recent work has suggested that extending the length of the NA stalk by 30 amino acids augments anti-NA responses in a murine vaccination model with whole inactivated virus without compromising anti-HA responses (Broecker et al., 2019a). Further research is needed to determine if such an approach would augment anti-NA responses in a swine model.

While vaccination with NA2 VLPs resulted in an equivalent reduction in inflammation and disease compared to QWIV vaccination, the equivalent reduction in viral replication in swine lungs compared to mock vaccinated pigs was notable (Figure 7C and 7D). However, with respect to virus shedding, while mean virus titers recovered from nasal swabs were consistently lower in NA2 VLP vaccinated pigs, this difference was not significant through daily sample collection up to day 5 (Figure 6). Reduced viral mobility and subsequent viral transmission have been proposed as a potential benefits of robust anti-NA functional immunity (Wohlbold and Krammer, 2014), however the absence of decreased viral shedding in NA2 VLP vaccinates does not support this proposal. Additional transmission studies employing cohoused naïve contact pigs could provide additional information on the effect of anti-NA immunity in virus dissemination and spread.

Ultimately, vaccination with novel NA2 VLPs was successful in preventing disease and controlling inflammation in this challenge study measured via clinical scoring (figure 5),

neutrophilic infiltration (Figure 8), and pulmonary histopathology scoring (Figure 9). As disease resulting from swine influenza is an important economic pathogen in swine production (Calderón Díaz et al., 2020), it will be interesting to determine if the control of disease, inflammation, and viral replication afforded by vaccination with NA2 VLPs positively affects and offsets the negative impacts caused by IAV on feed efficiency and average daily gain seen in disease outbreaks in market pigs. As an additional benefit to the swine production industry, NA2 VLPs offer the opportunity for the differentiation of infected from vaccinated individuals (DIVA) to support serological surveys and the global movement of pigs, the importance of which was demonstrated with the emergence of the 2009 H1N1 pandemic after infected pigs carrying Eurasian swine influenza virus strains were moved from Asia to Mexico (Mena et al., 2016).

In summary, vaccination with NA2 VLPs reduced viral replication in swine lungs and reduced pulmonary inflammation in a manner similar to a licensed, commercially available QWIV vaccine. Further studies are warranted to investigate the impact of NA2 VLP vaccination on virus transmission, both among swine and zoonotically, and the economic impact of NA2 VLP vaccination on swine production.

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Figures

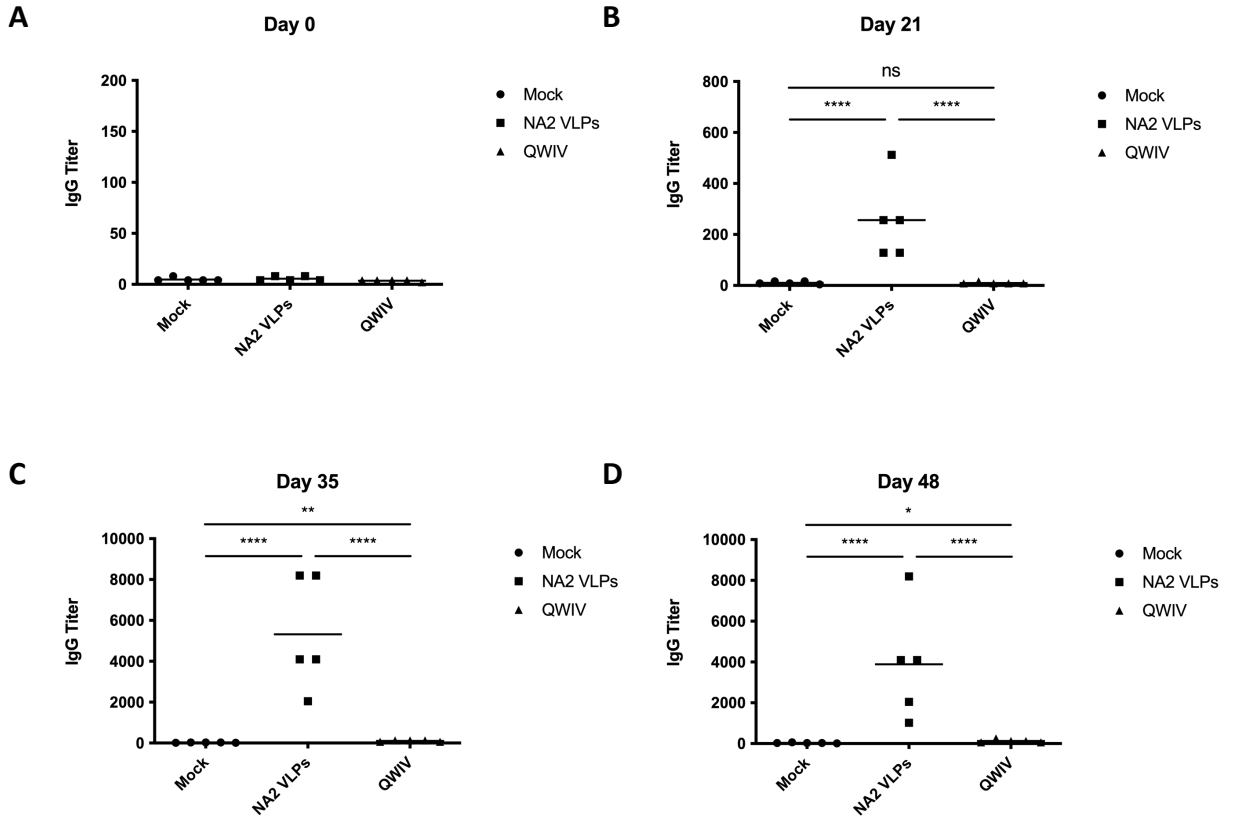

Figure 1: Vaccination with NA2 VLPS induces Anti-NA serum IgG titers in swine. Three groups of pigs (n=5) were vaccinated in a prime-boost regimen 21 days apart at day 0 and day 21 with a commercial quadrivalent whole inactivated virus vaccine (QWIV), NA2 VLPs (NA2 VLPs), or mock vaccinated with water-in-oil-in-water adjuvant only (Mock). Serum was collected on day 0 before prime vaccination, day 21 at boost vaccination, day 35, and at day 48. Anti-NA IgG titers were measured via ELISA. (A) Total anti-NA serum IgG titers at day 0 prior to prime vaccination. (B) Total anti-NA serum IgG titers at day 21 prior to boost vaccination. (C) Total anti-NA IgG titers two weeks post-boost vaccination on day 35. (D) Total anti-NA IgG titers prior

to virus challenge with A/swine/NC/HK1552516/2016 on study day 48. Horizontal bars represent the mean IgG titer for each respective group. * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$.

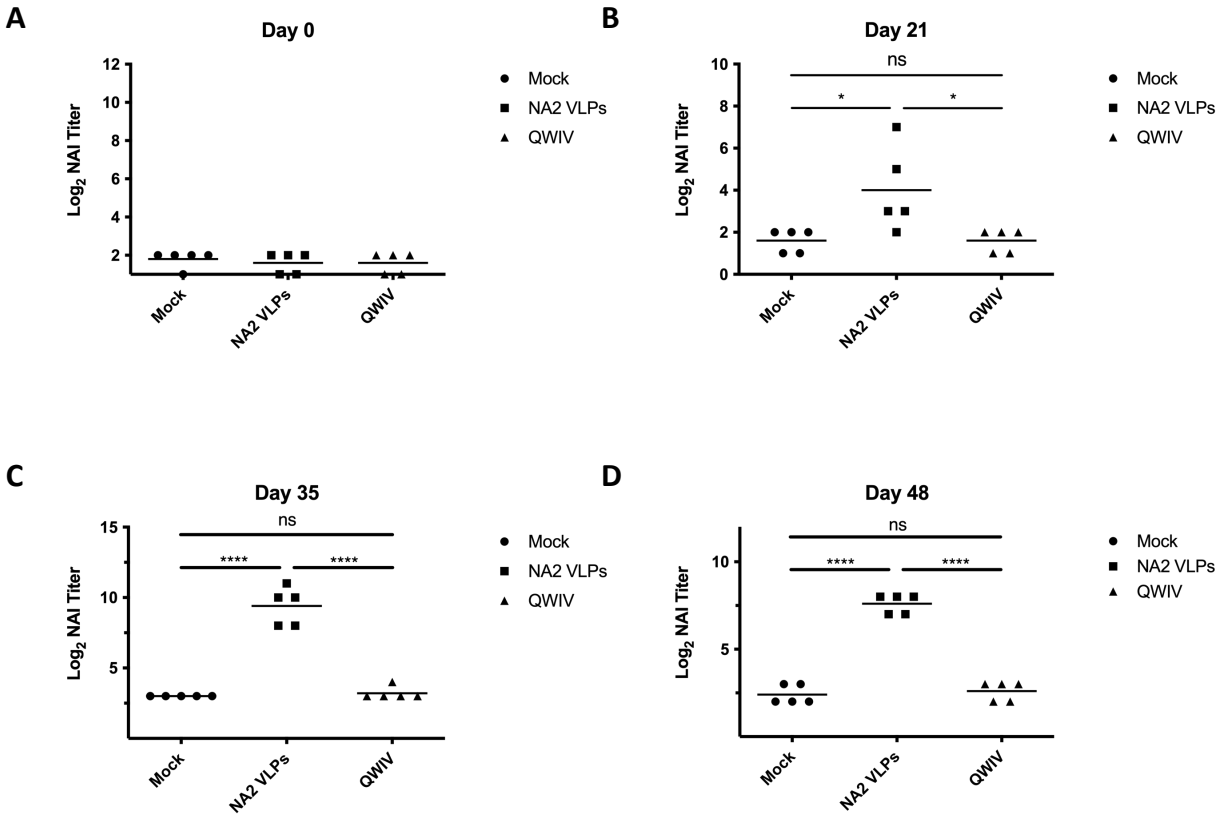

Figure 2: Vaccination with NA2 VLPS induces functional Anti-NA antibodies capable of inhibiting A/Perth/16/2009 NA activity in vitro. Three groups of pigs (n=5) were vaccinated in a prime-boost regimen 21 days apart at day 0 and day 21 with a commercial quadrivalent whole inactivated virus vaccine (QWIV), NA2 VLPs (NA2 VLPs), or mock vaccinated with water-in-oil-in-water adjuvant only (Mock). Serum was collected on day 0 before prime vaccination, day 21 at boost vaccination, day 35, and at day 48. Neuraminidase inhibition (NAI) titers were defined as the reciprocal of the dilution inhibiting at least 50% of the NA activity of A/Perth/16/2009 in NA2 VLPs. (A) NAI titers at day 0 prior to prime vaccination. (B) NAI titers at day 21 prior to boost vaccination. (C) NAI titers two weeks post-boost vaccination on day 35. (D) NAI titers prior

to virus challenge with A/swine/NC/HK1552516/2016 on study day 48. Horizontal bars represent the mean NAI titer for each respective group. * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$.

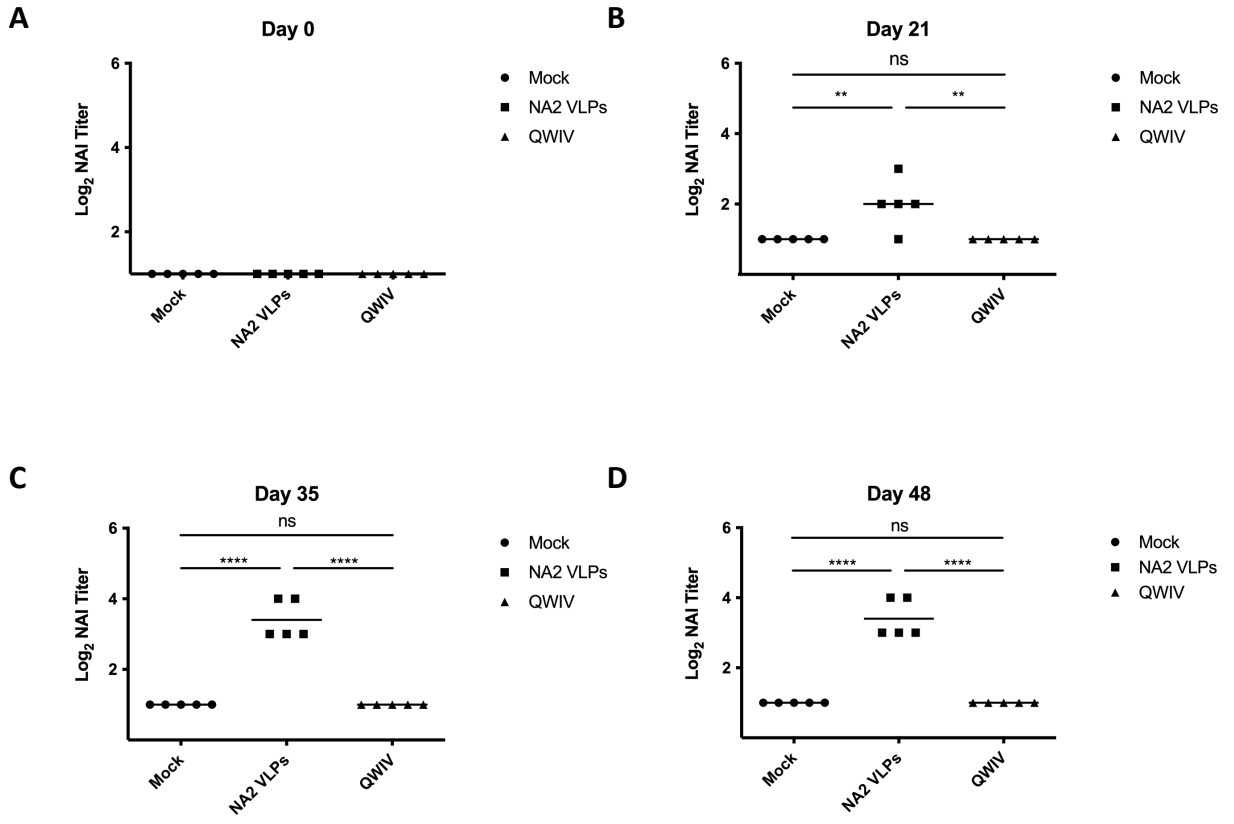

Figure 3: Vaccination with NA2 VLPS induces functional Anti-NA antibodies capable of inhibiting A/swine/NC/HK1552516/2016 NA activity in vitro. Three groups of pigs (n=5) were vaccinated in a prime-boost regimen 21 days apart at day 0 and day 21 with a commercial quadrivalent whole inactivated virus vaccine (QWIV), NA2 VLPs (NA2 VLPs), or mock vaccinated with water-in-oil-in-water adjuvant only (Mock). Serum was collected on day 0 before prime vaccination, day 21 at boost vaccination, day 35, and at day 48. Neuraminidase inhibition (NAI) titers were defined as the reciprocal of the dilution inhibiting at least 50% of the NA activity of A/swine/NC/HK1552516/2016 virus. (A) NAI titers at day 0 prior to prime vaccination. (B) NAI titers at day 21 prior to boost vaccination. (C) NAI titers two weeks post-boost vaccination

on day 35. (D) NAI titers prior to virus challenge with A/swine/NC/HK1552516/2016 on study day 48. Horizontal bars represent the mean NAI titer for each respective group. * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$.

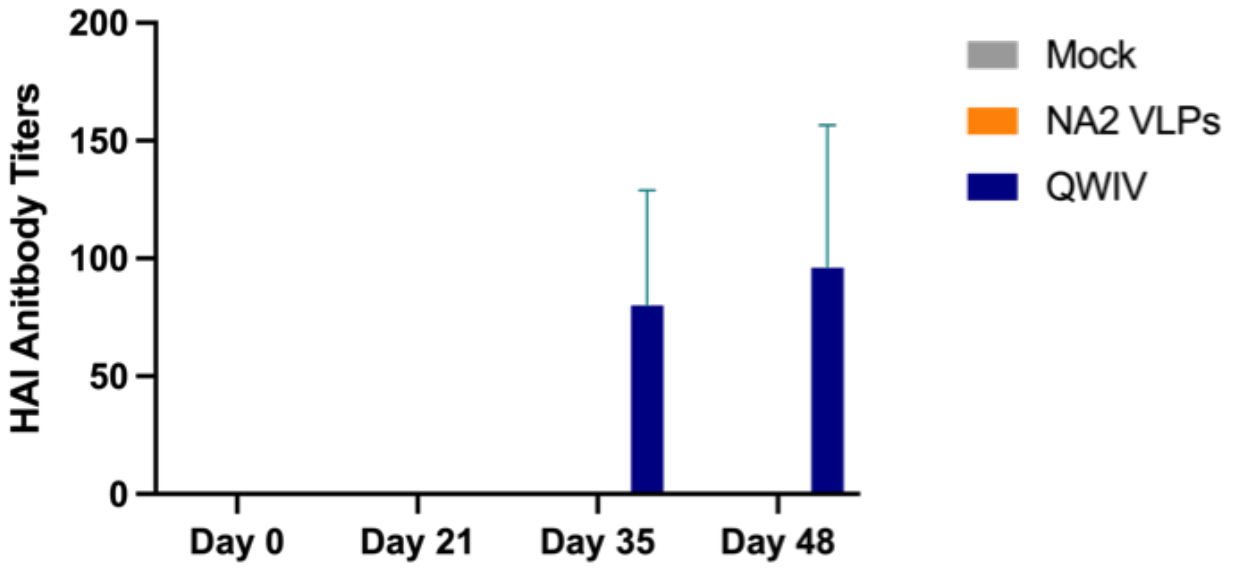

Figure 4: Prime boost vaccination with commercial QWIV induces serum antibody responses capable of inhibiting hemagglutination of A/Swine/NC/HK1552516/2016. Serum samples collected at day 0, 21, 35 and 48 were assayed for hemagglutination inhibition (HAI) titers against the heterologous H3N2 (A/SW/NC/KH15/2016) challenge virus. Grey bars represent mean HAI titers from sera collected from mock vaccinated animals (Mock), orange bars represent mean HAI titers from sera collected from NA2 VLP vaccinated animals (NA2 VLPs), and blue bars represent mean HAI titers from sera collected from animals vaccinated with commercial quadrivalent whole inactivated virus vaccine (QWIV). Error bars represent the standard deviation.

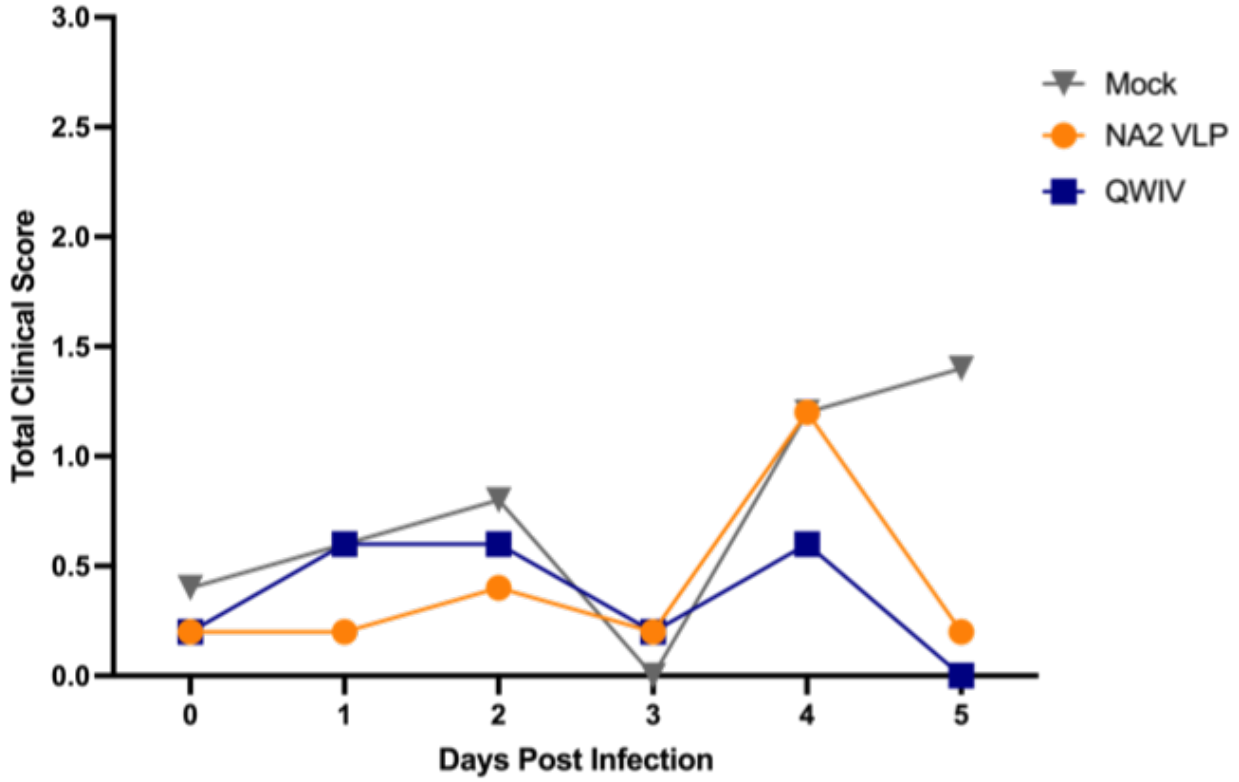

Figure 5: Swine clinical disease scores after challenge with A/swine/NC/HK1552516/2016. Clinical scores including rectal temperature, respiratory rate and assessment of clinical demeanor were recorded daily throughout the challenge. Specifically, rectal temperature score ranged from 0 to 3 (< 103°F = 0, 103-103.9°F = 1, 104 - 104.9°F = 2, > 105°F = 3), respiratory rate per minute score ranged from 0 to 2 (20-40 = 0, 41-59 = 1, > 60 = 2) and clinical behavior score, based on coughing (absenct = 0, present = 1) and depression (absent = 0, present = 2) ranged from 0 to 3. Grey upside down triangles represent the mean clinical score of mock vaccinated pigs (Mock), orange circles represent the mean clinical score of NA2 VLP vaccinated pigs (NA2 VLP), blue squares represent the mean clinical score of commercial whole inactivated virus vaccinated pigs (QWIV).

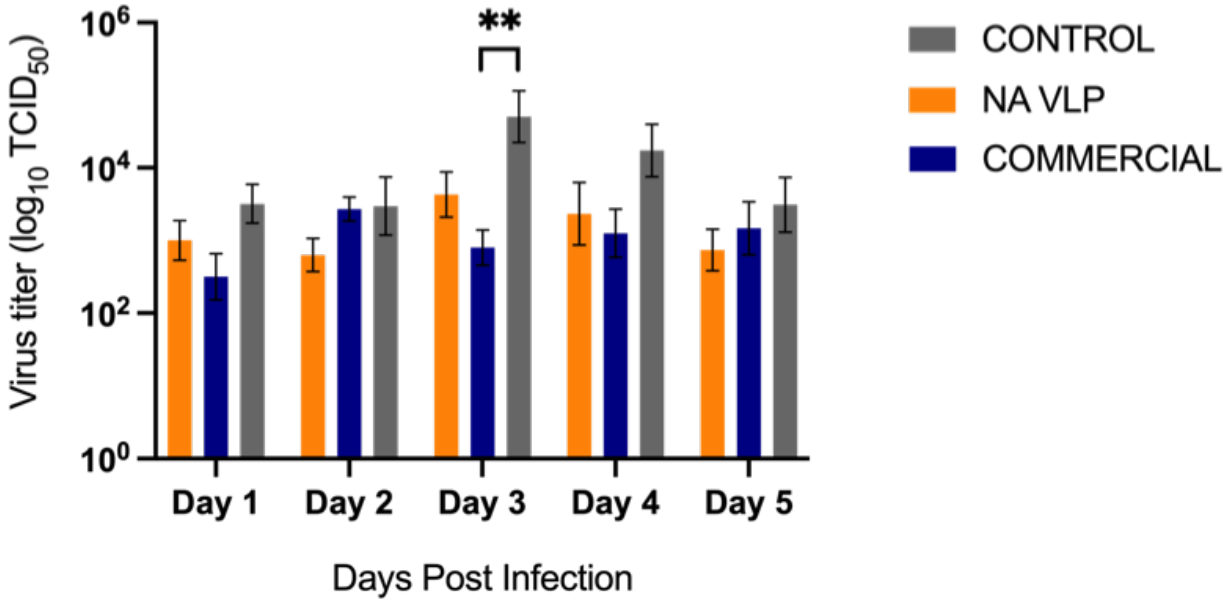

Figure 6: Nasal virus shedding after challenge with A/swine/NC/HK1552516/2016. Nasal swabs were collected from pigs daily post-challenge through necropsy on day 5 post challenge. Virus titers of nasal swabs were measured via $TCID_{50}$ assay. Each bar represents the mean viral titer per group each day, with the orange bars representing NA2 VLP vaccinated pigs (NA2 VLP), the blue bars representing pigs receiving the commercial quadrivalent whole inactivated virus vaccine (QWIV), and the grey bars representing the mock vaccinated pigs (Mock). Error bars represent the standard deviation. * = $p < 0.05$.

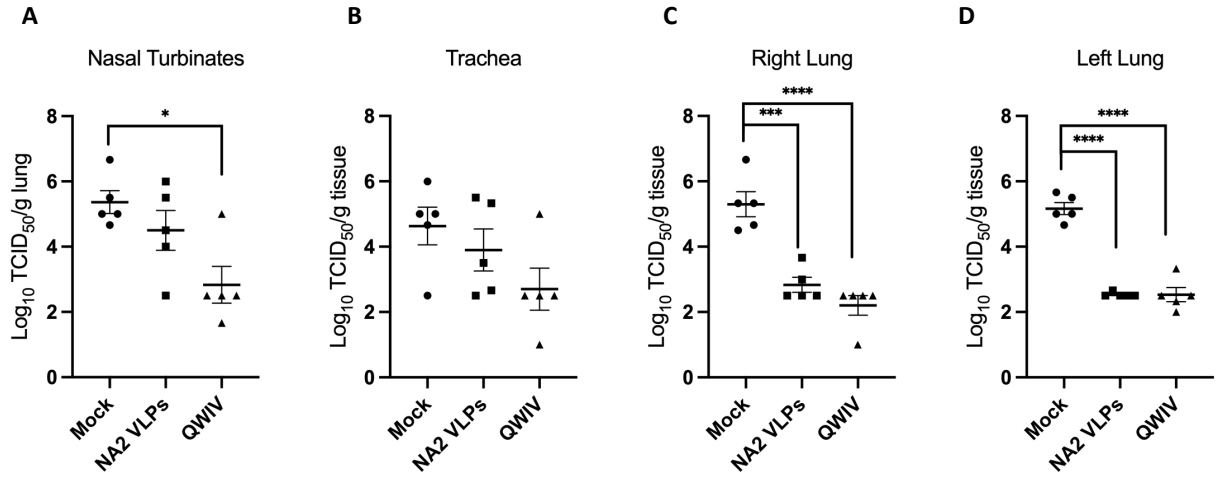

Figure 7: Vaccination with NA2 VLPs and the commercially available QWIV swine IAV vaccine reduced viral replication in pig lungs post challenge. Tissue samples from the respiratory tract including nasal turbinates, trachea, right lung lobes including the accessory lung lobe, and left lung lobes were harvested 5 days post challenge at necropsy. Following tissue homogenization, virus replication was measured in each respiratory compartment via $TCID_{50}$ assay of tissue homogenates. (A) Mean viral titers of homogenized nasal turbinates. (B) Mean viral titers of homogenized trachea tissue. (C) Mean viral titers of right lung lobe tissue homogenates. (D) Mean viral titers of left lung lobe tissue homogenates. Black circles represent mock vaccinated pigs (Mock), black squares represent NA2 VLP vaccinated pigs (NA2 VLPs), and black triangles represent commercial quadrivalent whole inactivated virus vaccinated pigs (QWIV). Horizontal lines represent the mean for each group. Error bars represent the standard deviation. * = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$.

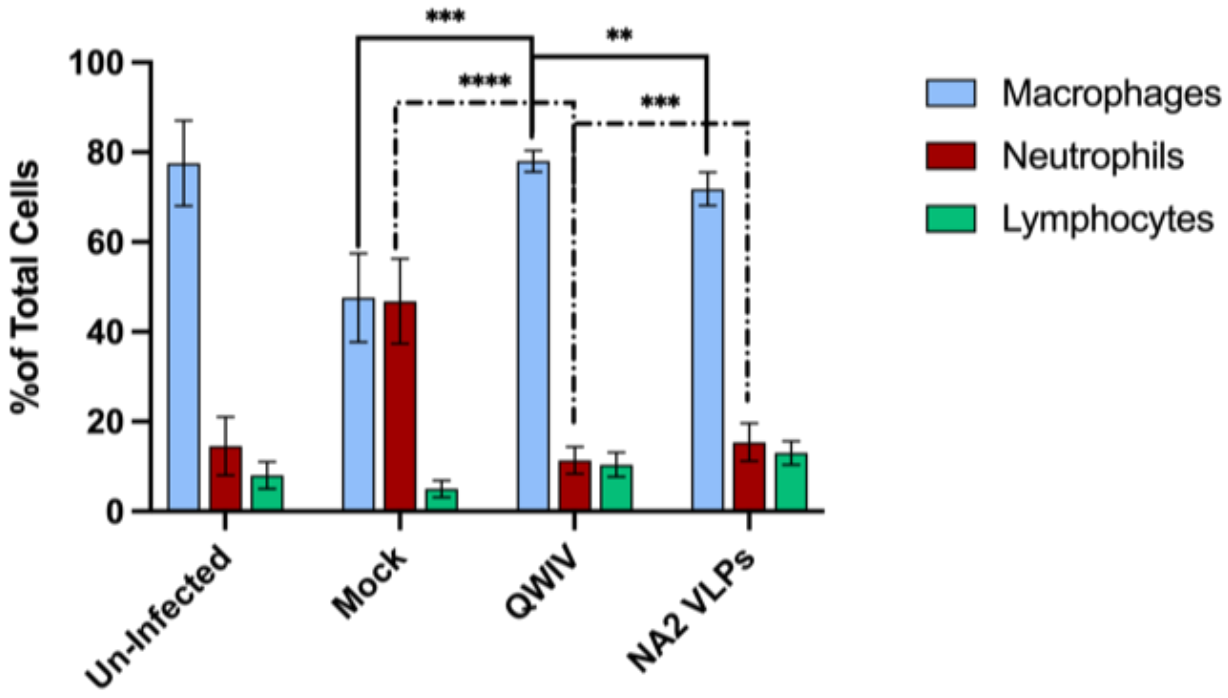

Figure 8: Vaccination with NA2 VLPs and the commercially available QWIV swine IAV vaccine reduced neutrophilic infiltration in bronchoalveolar lavage fluid collected from pig lungs post challenge. Bronchoalveolar lavage fluid (BALF) was collected from lungs at necropsy 5 days post challenge. Separated cells were added to slides and stained with Wright-Giemsa stain and differential cell counts were determined microscopically. Cell counts were expressed as a percentage of total cells. Blue bars represent the mean percentage of macrophages in each experimental group, red bars the mean percentage of neutrophils in each experimental group, and green bars represent the mean percentage of lymphocytes in each experimental group. Error bars represent the standard deviation. ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

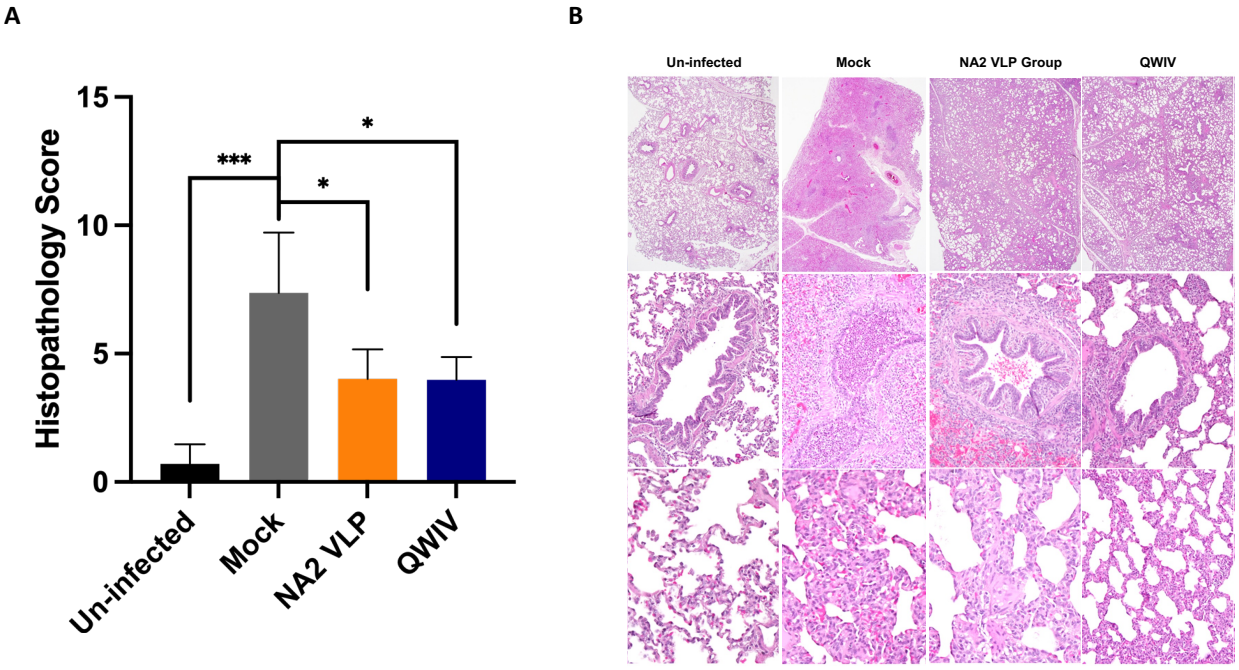

Figure 9: Figure 8: Vaccination with NA2 VLPs and the commercially available QWIV swine IAV vaccine reduced total histopathology scores in pig lungs post challenge. Lung tissues were collected at necropsy 5 days post challenge and fixed in formalin. Five µm sections were stained with H&E and examined by light microscopy. Lung sections were scored by a board-certified veterinary pathologist. (A) Mean lung histopathology scores from each vaccinated group. Black bars represent healthy un-infected pigs, grey bars represent mock vaccinated pigs after infection (Mock), orange bars represent NA2 VLP vaccinated pigs after infection (NA2 VLP), and blue bars represent commercial quadrivalent whole inactivated virus vaccinated pigs after infection (QWIV). (B) H&E stained representative lung tissue sections from each respective experimental group. Error bars represent the standard deviation. * = $p < 0.05$; *** = $p < 0.001$.

Chapter IV: Conclusions

Respiratory disease caused by influenza virus is significant cause of global morbidity and mortality and inflicts substantial economic losses during seasonal epidemics (Molinari et al., 2007; Reed et al., 2015). The disease burden can be much more severe with the emergence of novel IAV strains causing global pandemics than during seasonal epidemics and can drastically shift mortality demographics as observed during the 2009 H1N1 pandemic season (Shrestha et al., 2011). Indeed, the 1918 "Spanish" influenza pandemic is estimated to have caused between 50 and 100 million deaths globally (Johnson and Mueller, 2002). Since the advent of licensed influenza vaccines in the mid 20th century, seasonal vaccination has been the main prophylactic intervention to mitigate the burden caused by influenza disease. However, currently licensed influenza virus vaccines have shown poor efficacy in recent years with vaccine effectiveness estimated at approximately 50% in good years and well below 20% when the vaccine and seasonally circulating strains at the time of infection are not well antigenically matched (Flannery et al., 2017; Rolfes et al., 2019). Seasonal influenza vaccines are standardized by only their HA content and require that NA only be present in production lots (Wohlbold and Krammer, 2014). The lack of focus on NA in current vaccines has led to highly variable NA content between different lots of current seasonal vaccines and led to highly variable or non-existent anti-NA responses post vaccination (Couch et al., 2012b). However, more recent work has identified differential anti-NA immune responses in humans between vaccination and IAV infection (Chen et al., 2018) and also established anti-NA antibody responses as an important correlate of protection against disease caused by influenza virus (Gilbert et al., 2019; Memoli et al., 2016; Monto et al., 2015). This dissertation focused on evaluating the protective efficacy of NA VLPs against influenza virus disease in both murine and swine IAV vaccination-challenge models.

I first focused on the production and characterization of NA VLPs followed by the evaluation of their efficacy in a murine model (Chapter 2). While previous reports have demonstrated that co-infection of Sf9 insect cells with rBVs expressing NA1 and M1 from H1N1 yields spherical VLPs that are immunogenic and efficacious in murine and ferret models of IAV infection (Kim et al., 2019; Quan et al., 2012; Smith et al., 2017), production of NA VLPs has not been investigated using a co-infection model in Tni cells. In this dissertation, I demonstrated that co-infection of Tni cells with rBVs expressing the NA sequence from A/Perth/16/2009 or A/California/04/2009 and the M1 sequence from A/Michigan/73/2015 yielded characteristic VLPs of the expected size containing enzymatically active NA and, most importantly, were immunogenic and efficacious in both murine and swine vaccination-challenge models (Chapter 2 and Chapter 3). Tni cells have been used for recombinant protein expression using a baculovirus expression system for many years and are favored over *Spodoptera* cell lines as they have been demonstrated to produce as much as 10-fold to 20-fold more recombinant protein per individual cell (Davis et al., 1993). However, Tni cells have also been avoided by some laboratories as amplification of baculovirus consistently yields 100-fold less baculovirus (2 logs) compared to baculovirus amplification in *Spodoptera* cell lines, thus necessitating the maintenance of two separate cell lines with *Spodoptera* cell lines used for viral amplification and Tni cell lines used for protein production (Wilde et al., 2014). The reduction in amplification of baculovirus observed in Tni cell expression systems can be advantageous in IAV VLP production, as rBVs are of a similar density to IAV VLPs and unable to be resolved during purification in sucrose gradients resulting in rBV contamination of VLP preparations (Krammer et al., 2010; Margine et al., 2012). This increase in recombinant protein production and subsequent deficiency in baculovirus amplification has previously been leveraged to produce HA VLPs via co-infection with rBVs

encoding IAV HA and M1 proteins, resulting in a 10 fold increased yield in VLPs with between a 10-fold and 100-fold reduction in rBV viral particles in the resulting VLP preparation (Krammer et al., 2010). However, Tni cells have not been as well characterized as Sf9 cell lines and have not been qualified as a master seed cell line for Good Manufacturing Practices (GMP) used in commercial vaccine production. Conversely, Sf9 cell lines are currently in use by multiple vaccine manufacturers in licensed human and animal vaccines. Furthermore, several Tni cell lines, even those commercially available have been reported to be persistently infected with an alphanodavirus (Li et al., 2007). However, more recent work has extensively characterized a Tni cell line that is free of persistent alphanodavirus infection as well as other commonly recognized adventitious contaminants in production cell lines (Maghodia et al., 2020). While a well characterized cell line is necessary for any potential regulatory path forward for VLPs produced in Tni cells under GMP conditions, further research is also needed to develop standardized and cost-effective purification methods for removing residual adventitious baculovirus from VLP preparations for future commercialization. However, Tni cells still provide an exceptionally efficient platform for the production of VLPs in an academic setting and provide an expression system for the creation of tools to investigate NA and investigate basic research questions. This dissertation was the first work to describe the production and characterization of NA2 VLPs in an insect cell expression system, and the first report of production, characterization, immunogenicity, and efficacy of NA VLPs produced using a Tni cell expression system.

Intranasal vaccination with NA1 VLPs has previously been demonstrated to be immunogenic and efficacious in a lethal murine challenge model against both homologous and heterosubtypic virus challenge (Quan et al., 2012). As described in chapter 2, NA2 VLP vaccination via the IN route did not completely protect mice against mortality after lethal challenge

with heterologous but homosubtypic H3N2 A/Hong Kong/1/1968 virus. IN vaccination was also inferior to IM vaccination in production of anti-NA antibodies and controlling viral replication in mouse lungs post challenge. Vaccination via the IM route completely protected mice against mortality in this lethal challenge model. Of note, the NA1 VLPs described in the previous study by Quan et al. were produced using an Sf9 insect cell expression platform (Quan et al., 2012). Additionally, live baculovirus has been demonstrated to serve as a strong B cell and T cell adjuvant in previous studies (Heinimäki et al., 2017; Margine et al., 2012). A possible explanation for the observed lack of immunogenicity and efficacy observed in this dissertation when NA2 VLPs were administered via the IN route in mice could be the relative amounts of residual baculovirus contained in the VLP preparations produced using different insect cell lines, as VLP preparations produced in Tni insect cells have been shown to have 10-fold to 100-fold lower residual baculovirus titers (Krammer et al., 2010). Further research would be necessary using NA VLPs containing identical NA antigens produced in both Sf9 and Tni cell lines standardized by NA content to ultimately dissect any differences in VLP immunogenicity due to different production platforms. Alternatively, both mouse and human saliva have been demonstrated to contain inhibitors of influenza virus neuraminidase, with different influenza strains showing varying degrees of susceptibility to inhibition (Gilbertson et al., 2017; White et al., 2009). Another possible explanation for differing efficacy of NA VLPs derived from different strains and subtypes of IAV could be the differing susceptibility to inhibition by murine saliva and the subsequent ability to traverse the oropharynx and reach the murine lungs. However, IM vaccination of mice with NA2 VLPs resulted in complete protection against mortality and efficient control of lung viral replication in the lethal challenge model described in Chapter 2.

Vaccination with NA1 VLPs containing the NA antigen from A/Puerto Rico/8/1934 and, in a separate study, A/California/04/2009 previously demonstrated protection against mortality in a lethal challenge with heterosubtypic A/Philippines/1982 H3N2 virus (Kim et al., 2019; Quan et al., 2012). In Chapter 2, I demonstrated that vaccination with NA2 VLPs or vaccination with NA1 VLPs did not offer any protection against mortality when after a lethal challenge with heterosubtypic virus. Again, the adjuvant effect of residual baculovirus contained in VLP preparations produced in Sf9 cells described in the studies by Quan et al. and Kim et al. could have had an immunostimulatory effect responsible for the observed heterosubtypic protection (Heinimäki et al., 2017; Margine et al., 2012). However, other investigations using NA VLPs vaccines in a ferret model or purified rNA in murine models have not observed heterosubtypic cross-protection after challenge (Liu et al., 2015; Smith et al., 2017; Wohlbold et al., 2015a). As NA2 VLP vaccination did not provide heterosubtypic immunity against lethal challenge with A/California/04/2009 H1N1 virus, I next investigated the bivalent administration of NA2 VLPs combined with NA1 VLPs. While limited evidence has suggested that N2 may be more immunogenic than N1 in humans (Chen et al., 2018; Nachbagauer et al., 2017; Rajendran et al., 2017), it is difficult to draw any firm conclusions given the high variability in NA content of seasonal vaccines (Getie-Kebtie et al., 2013; Sultana et al., 2014). In this dissertation, no antigenic dominance or competition was observed when administering a bivalent vaccine containing both NA1 and NA2 VLPs to mice (Chapter 2). In fact, anti-NA antibody levels were slightly elevated and lung viral titers were slightly reduced after A/Hong Kong/1/1968 H3N2 challenge in mice vaccinated with the bivalent preparation compared to mice vaccinated with monovalent NA2 VLPs (Chapter 2). This finding was consistent with previous work examining the combined administration of N1 and N2 protein extracted and purified from whole virus in a murine model

(Johansson and Kilbourne, 1994). While the mechanism for this apparent increase in anti-NA antibody requires further investigation, many broadly reactive anti-NA monoclonals have been described that are capable of recognizing shared epitopes among different NA subtypes, suggesting that bivalent administration of NA1 and NA2 VLPs may enrich these epitopes. It has also been hypothesized that different NA subtypes may share common MHC epitopes leading to increased T cell activation after administration of NA1 and NA2 antigens (Johansson and Kilbourne, 1994). Ultimately, bivalent vaccination with NA1 and NA2 VLPs resulted in protection against mortality after lethal challenge with both homologous H1N1 virus and separate heterologous challenge with H3N2 virus.

In Chapter 2 of this dissertation, I demonstrated that mice are a cost-effective animal model for preliminary studies using novel candidate vaccines despite not being natural hosts of IAV. However, murine models of IAV have several limitations and care must be exercised in interpreting and applying conclusions obtained from work in murine models to other animal species and human IAV infection. In Chapter 3, I investigated the immunogenicity and efficacy of NA2 VLPs in a heterologous swine infection model using a challenge model with the circulating H3N2 field strain of swine influenza virus A/swine/NC/HK1552516/2016. Immunization with NA2 VLPs was also compared to vaccination with a licensed, commercially available quadrivalent whole inactivated virus (QWIV) swine influenza vaccine. Vaccination with NA2 VLPs induced robust anti-NA antibody titers capable of inhibiting NA enzymatic activity and reduced viral replication and inflammation in swine lungs post challenge. Conversely, despite containing two H3N2 strains, one H1N2 strain, and one H1N1 strain, the QWIV vaccine did not induce appreciable anti-NA responses or any measurable NAI titers. However, the robust anti-HA immunity induced by vaccination with the QWIV vaccine reduced viral replication and subsequent

inflammation in swine lungs in a manner similar to vaccination with NA2 VLP vaccination. Interestingly, the lack of robust anti-NA response in pigs vaccinated with QWIV is in alignment with previous work in a murine model suggesting that when HA and NA are presented on the same virion in close proximity to one another, the anti-HA response is immunodominant (Broecker et al., 2019a; Johannsson et al., 1987; Johansson and Kilbourne, 1993; Johansson et al., 1987). Broecker et al. employed a strategy of extending the stalk region of the NA to partially overcome this HA immunodominance in a murine model (Broecker et al., 2019a), it would be interesting to investigate if extending the NA stalk of whole inactivated virus vaccines had a measurable effect on anti-NA response in a swine model. Previous work has demonstrated that various methods of inactivation, including formalin inactivation of whole virus inactivated vaccines, affect immunogenicity of IAV vaccines and the subsequent anti-NA responses (Chen et al., 2020; Koskinen et al., 2010). Indeed, it was demonstrated as early as the 1940's that the use of formalin to inactivate IAV vaccine preparations can result in the loss of antigenic conformation and function of viral proteins (Stanley, 1945). While it is possible that the inactivation process used in the production of QWIV vaccine used in this work contributed to the poor anti-NA responses observed in Chapter 3, additional experiments using NA VLPs could easily investigate the effect of different inactivation techniques on the antigenicity of NA.

While it is tempting to conclude that vaccination experiments in the swine model described in Chapter 3 constituted a direct comparison between anti-HA and anti-NA immunity based on the relatively low anti-NA IgG responses observed after QWIV vaccination, QWIV vaccination did still induce measurable anti-NA serum IgG responses. While QWIV vaccination did not yield any measurable functional anti-NA antibody capable of inhibiting NA enzymatic activity, previous work has suggested a role for ADCC in anti-NA immunity in a murine model (Broecker et al.,

2019b). Conversely, other work has demonstrated an absence of ADCC activity directed at the NA antigen in a murine model. Kim et al. demonstrated that vaccination of Fc receptor gamma chain deficient mice with NA1 VLPs was still protective against homologous IAV challenge and that passive transfer of anti-NA sera was protective against homologous IAV challenge in Fc receptor gamma chain deficient mice (Kim et al., 2019). However, the role for ADCC in protection against IAV challenge in the absence of detectable anti-NA immunity capable of inhibiting NA enzymatic function has not been established. Future experiments could employ monoclonal antibodies specific for NA but incapable of inhibiting NA enzymatic activity in various animal models to investigate other potential mechanisms involved in anti-NA immunity.

While vaccination with NA VLPs inhibited viral replication in swine lungs post challenge, it did not have a significant effect on viral replication in the swine trachea or in homogenized nasal turbinates post challenge (Chapter 3). One proposed advantage of anti-NA immunity is the potential public health benefit from reduced viral transmission due to the inhibition of viral egress from infected host cells and the aggregation of nascent progeny virions at the surface of infected host cells (Palese et al., 1974; Wohlbold and Krammer, 2014). One distinct advantage of a swine model of IAV infection over murine models is the ability to conduct transmission studies using naïve contact pigs. While no statistically significant reductions in viral replication were observed in the upper respiratory tract after NA2 VLP vaccination, the mean virus titer from these tissue homogenates was lower than in mock vaccinated pigs. Our study may have been underpowered to statistically discern this effect given the genetic heterogeneity of the outbred swine model or, alternatively, protection against viral replication and dissemination afforded by intramuscular NA2 VLP vaccination may be limited to lung tissues and not observed at the mucosal surfaces of the upper respiratory tract in swine. Future research to discern if the reduction in pulmonary viral

replication observed in NA2 VLP vaccinated pigs is sufficient to limit viral transmission to naïve animals would be informative regarding the potential public health benefits of NA VLP vaccination and anti-NA immunity in general.

In conclusion, this dissertation has demonstrated the ability of NA2 VLPs to protect against IAV infection in both a murine and swine infection model as well as the benefits of bivalent administration of NA1 and NA2 VLPs in a murine model. While previous work in various models of influenza virus infection, including humans, has demonstrated the value of anti-NA immunity in protecting against disease caused by influenza virus, this is the first report of protection afforded by anti-NA immunity in a swine model of IAV infection. Overall, investigation of protection induced by immunity specific to NA as an antigen has been historically hampered by the relative paucity of NA specific reagents. The creation and distribution of widely available NA reagents will be paramount to continued mechanistic investigation of anti-NA immunity and its contribution to influenza virus immunity. Furthermore, the use of animal models of influenza virus infection amenable to both infection with contemporary H3N2 and potentially novel influenza virus strains needs to be prioritized from a funding perspective. The amenability of swine models to IAV transmission studies has the potential to inform vaccine design with respect to standardization of anti-NA responses with potential global public health impacts. While many mechanistic questions regarding anti-NA immunity remain to be answered, we cannot afford to ignore the contribution of anti-NA immunity to protection from influenza virus disease in future vaccine design.

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