Exploring Novel Influenza Vaccine Strategies in the Swine Model: Along With New Potential Come New Challenges

by

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Abstract

Influenza A virus (IAV) is a highly contagious respiratory pathogen that constitutes a significant threat to global public and animal health. Unremitting antigenic variation of IAV strains not only results in immune evasion but also has implications on vaccine development and efficacy. Indeed, current influenza vaccine technologies often induce poor immune responses and protection against antigenically distinct IAVs as they primarily target the immunodominant yet highly variable head domain of the hemagglutinin (HA) protein. To overcome the ever-increasing antigenic diversity, there is a need for developing novel vaccine strategies that target conserved influenza immunogens. The objective of this dissertation was to assess the immunogenicity and protective efficacy of two novel vaccine strategies in the swine model, a neuraminidase-based and a hemagglutinin-based vaccine approach as candidate broadly-protective influenza vaccine strategies.

Neuraminidase (NA), although commonly neglected in influenza vaccine formulations, is a major antigenic protein of influenza virions previously shown to confer broad heterologous protection. In Chapter II, we compared a novel NA2 virus-like particle (VLP) platform containing the NA protein from the human A/Perth/16/2009 H3N2 strain to a commercial quadrivalent whole inactivated virus (QWIV) influenza vaccine. The NA2 VLP construct, was highly immunogenic in the swine model and performed in a similar fashion to the QWIV vaccine in reducing pulmonary virus replication and lung pathology scores following heterologous challenge with A/swine/NorthCarolina/KH1552516/2016, an H3N2 swine field isolate. Overall, our study demonstrated that while neither vaccine elicited complete protection, the NA2 VLP platform induced substantial protection against heterologous challenge.

A novel vaccination strategy that deploys prime-boost immunization with antigenically mismatched whole inactivated virus (WIV) vaccines has been recently explored and has been shown to be highly effective in enhancing the immunogenicity of inactivated vaccines against antigenically divergent influenza viruses by presumably targeting conserved subdominant HA epitopes shared by the mismatched vaccine strains. In Chapter III, we investigated the immunogenic properties and protective efficacy of H1N1 heterologous prime-boost vaccination in the swine model compared to immunization with the corresponding mismatched monovalent and bivalent vaccines. Specifically, we prime immunized pigs with the A/California/07/2009 (CA09) H1N1 WIV and later boosted them with the heterologous A/sw/Minnesota/A02636116/2021 (MN21) H1N1 WIV. We demonstrated that heterologous boosting did not expand the scope of cross-reactive antibody responses against antigenically distinct swine and human IAVs compared to the corresponding monovalent and bivalent vaccination regimens. Furthermore, we observed that following challenge with the mismatched A/swine/Georgia/27480/2019 (GA19) H1N2 virus, heterologous prime-boosted pigs showed prolonged clinical disease, and increased pulmonary pathology scores compared to mock-vaccinated pigs. Our results showed that H1-specific heterologous prime-boost vaccination, rather than enhancing cross-protection, exacerbated the clinical outcome and pathology after challenge with the mismatched GA19 strain.

Overall the work presented here highlights the untapped potential of NA-based vaccination in conferring broad heterologous immunity and protection and underscores the concern for the development of candidate broadly protective influenza vaccine platforms that target conserved subdominant epitopes on the HA protein.

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Ad5v	Adenovirus serotype 5 vector
ADCC	Antibody-dependent cellular cytotoxicity
ADE	Antibody-dependent enhancement
APCs	Antibody presenting cells
BALF	Bronchoalveolar lavage fluid
BALT	Bronchus-associated lymphoid tissue
CA09	A/California/07/2009
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CEIRS	Centers of Excellence for Influenza Research and Surveillance
CMIR	Cell-mediated immune responses
CI	Confidence interval
CPE	Cytopathogenic effect
cRNA	Complimentary RNA
CTLs	Cytotoxic T lymphocytes
DAMPs	Damage associated molecular patterns

DC	Dendritic cells
DHF	Dengue hemorrhagic fever
DMEM	Dulbeco's modified Eagle's medium
ER	Endoplasmic reticulum
Fc	Fragment crystallizable
FcγR	Fc-gamma receptor
GA19	A/swine/Georgia/27480/2019
H&E	Hematoxylin and eosin
HA	Hemagglutinin
HAI	Hemagglutination inhibition
HA-SV	Hemagglutinin-subunit vaccine
IACUC	Institutional animal care and use committee
IAV	Influenza A virus
IFN-γ	Interferon-gamma
IFN-γ-STc	Interferon-γ secreting T-cells
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular

IN	Intranasal
IRF3	Interferon regulatory factor 3
IT	Intratracheal
LAIV	Live attenuated influenza vaccine
LNP	Lipid nanoparticles
M1	Matrix protein 1
M2	Matrix 2
M2e	M2 ectodomain
M2eNP	M2e and NP fusion protein
MDA	Maternally derived antibodies
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
MN21	A/swine/Minnesota/A02636116/2021
mRNAs	messenger RNAs
MUC5AC	Mucin receptor-5AC
MV/C	Mock-vaccinated/challenged
MV/NC	Mock-vaccinated/non-challenged
NA	Neuraminidase

NAI	Neuraminidase inhibition
NETs	Neutrophil extracellular traps
NF-κβ	Nuclear factor-kappa beta
NIAID	National Institute of Allergies and Infectious Diseases
NIH	National Institutes of Health
NK cells	Natural killer cells
nNAbs	Non-neutralizing antibodies
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
OD	Optical density
OPD	o-phenylenediamine dihydrochloride
OW	Oil-in-water
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline

pdmH1N1	pandemic H1N1
pi	Post infection
pp	Post-prime
PRRs	Pattern recognition receptors
RBC	Red blood cells
RBS	Receptor binding site
RDE	Receptor destroying enzyme
RdRp	RNA-dependent RNA polymerase
REU	Relative equivalent units
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription- polymerase chain reaction
SA	Sialic acid
SAα2,3Gal	Sialic acid-α2,3-galactose
SAα2,6Gal	Sialic acid-α2,6-galactose
SEM	Standard error of mean
swIAVs	Swine influenza A viruses

$TCID_{50}$	50% tissue culture infectious dose
TI	Thymus-independent
TIV	Trivalent inactivated influenza vaccines
TMB	3,3',5,5'-Tetramethylbenzidine
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor α
TNF-α-STc	TNF-α secreting T-cells
Tni cells	*Trichoplusia* ni cells
TRIG	Triple-reassortant internal gene
UIV	Universal influenza vaccine
US	United States
VAERD	Vaccine-associated enhanced respiratory disease
VERPD	Vaccine-enhanced RSV pulmonary disease
VLPs	Virus-like particles
vRNP	Viral ribonucleic protein complexes
VRP-NP	Virus replicon particles expressing NP
WIV	Whole-inactivated virus
WOW	Water-in-oil-in-water

α-clade	alpha-clade
β-clade	beta-clade
γ-clade	gamma-clade
δ-clade	delta-clade

1. Chapter I: Literature Review

1.1. Overview of influenza disease in human and animal health

Influenza viruses are important pathogens in public and animal health that can infect a wide variety of hosts. They belong to the family of *Orthomyxoviridae*, which is comprised of seven genera, including *Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus, Deltainfluenzavirus,, Isavirus, Thogotovirus*, and *Quaranjavirus* (Kibenge and Kibenge, 2016; Ma, 2022). *Alphainfluenzaviruses* are virulent pathogens that cause a highly contagious disease in humans and animals and are primarily responsible for seasonal epidemics and recurring pandemics (Javanian et al., 2021; Kapoor and Dhama, 2014). Betainfluenzaviruses are almost exclusive respiratory pathogens of humans and are implicated in seasonal flu illness, although less commonly than type A viruses (Koutsakos et al., 2016). Gammainfluenzaviruses primarily infect humans and occasionally swine, causing mild respiratory disease and sporadic small-scale outbreaks (Khanmohammadi and Rezaei, 2021; Matsuzaki et al., 2006). Deltainfluenzaviruses viruses were recently recognized as respiratory pathogens of cattle and pigs and are not known to cause illness in humans (Javanian et al., 2021; Su et al., 2017). Whereas influenza viruses infect various mammalian and avian hosts, Isaviruses (infectious salmon anemia viruses) cause a highly fatal disease in cold-water salmonids (Kibenge et al., 2004). Thogotoviruses and Quaranjaviruses are arboviruses (tick-borne and mosquito-borne viruses) of livestock and birds and have been reported to sporadically infect humans (Maclachlan and Dubovi, 2010; Popov et al., 2019).

While influenza A viruses (IAVs) have been detected in numerous avian and mammalian species, the primary natural reservoir of these viruses is wild aquatic birds *i.e.* waterfowl and shorebirds (Achenbach and Bowen, 2011; Parrish et al., 2015). IAVs have been isolated from a broad variety

of land and marine mammalian hosts, however the main mammalian species that serve as natural hosts and can sustain influenza virus infection and transmission within their respective populations, are humans, swine, horses and dogs (Joseph et al., 2017; Parrish et al., 2015; Runstadler and Puryear, 2020). Phylogenetic analyses have demonstrated that all mammalian IAVs are descendants of avian strains that crossed species barriers and adapted to various new hosts (Fields, 2007; Long et al., 2019; Taubenberger and Kash, 2010). Over the last century, there have been numerous instances where novel influenza subtypes, such as H5, H7, and H9, have been transmitted from waterfowl to domestic poultry and various mammalian species, including pigs and humans (Ma et al., 2009). The periodic zoonotic spillover of highly pathogenic avian influenza viruses, such as H5N1 and H7N9 strains, from poultry to humans during the past two decades has raised an alarm about a possible spread of these viruses with pandemic potential to human populations (Nandi and Allen, 2021; Song and Qin, 2020; Taubenberger and Morens, 2008).

Emergent influenza viruses that spill over from animal sources and contain a hemagglutinin to which most humans have no prior exposure and immunologic memory are considered to be the cause of all known influenza pandemics (Neumann and Kawaoka, 2006; Taubenberger and Morens, 2010). IAVs have caused one of the most devastating pandemics in recorded history, where the so-called "Spanish Flu" in 1918 resulted over 50 million deaths worldwide (Langford, 2002). Additionally, the 1957 H2N2 ("Asian flu") and 1968 H3N2 ("Hong Kong flu") pandemics and the most recent, 2009 H1N1 swine flu pandemic, which caused significant morbidity and mortality in children, pregnant women, and immunosuppressed individuals, are important reminders to the scientific and medical community to remain cautious for a potential new outbreak of an emerging IAV strain (Lapinsky, 2010; Lazzari and Stöhr, 2004; Lim and Mahmood, 2011; Smith et al., 2009).

Although influenza viruses with pandemic potential pose a serious threat to human populations, seasonal IAV infections remain an everlasting public health problem as they annually inflict a substantial disease burden and financial costs (Molinari et al., 2007). Work by Rolphes *et al.*, estimated that during six influenza seasons (2011-2016), seasonal IAVs were responsible for approximately 9.2 million to 35.6 million influenza-related illnesses and 140,000 to 710,000 influenza-related hospitalizations in the United States (US) (Rolfes et al., 2018). Additionally, it has been estimated that the annual economic burden of seasonal flu infections on the US healthcare system and society due to illness and loss of life remains very high, and approximates $87 billion (Molinari et al., 2007; Putri et al., 2018). IAVs are important pathogens in a variety of animal species, hence the overall economic impact of influenza disease is not solely derived from human infections. The annual financial losses in the US pork industry due to IAV infections are estimated to range between $360 million and $1 billion dollars (Kyriakis et al., 2017) while sporadic outbreaks of HPAIV in domestic poultry have been shown to negatively impact the industry up to 3 billion dollars per outbreak (Seeger et al., 2021; Swayne et al., 2017).

1.2. Influenza A Virus

1.2.1. Structure and Morphology

Influenza A viruses are infectious particles of pleomorphic shape (spherical or filamentous) and size, with a diameter varying from 80-120 nm (Badham and Rossman, 2016; Calder et al., 2010b). Influenza virions are covered by a lipid bilayer derived from the host plasma membrane, the viral envelope, which contains three surface glycoproteins that are consequential for the influenza virus

cycle, hemagglutinin (HA), neuraminidase (NA), and the matrix 2 (M2) ion channel (Figure 1.1.). The ratio of the HA and NA on the viral envelope is quite variable between different IAV strains and is estimated to be 4-5:1 (Harris et al., 2006). On the other hand, the M2 ion channel protein is significantly less abundant at the viral surface than the other two coat glycoproteins and is present at a ratio of one M2 ion channel for approximately every 10-40 molecules of the HA protein (Doherty et al., 2006). Beneath the envelope, an underlying coat is formed by the M1 protein, the matrix layer, which provides rigidity to the viral envelope and shapes virion morphology (Noda, 2012; Peukes et al., 2020). Internally, influenza virions contain eight helical nucleocapsids, which coat the IAV single-stranded negative-sense segmented RNA genome, thus forming viral ribonucleoprotein (vRNP) complexes, that are closely attached to the matrix layer (Bouvier and Palese, 2008). Each of the eight segments is bound to its own heterotrimeric RNA-dependent RNA polymerase (RdRp), which catalyzes the transcription and replication of that particular segment (Calder et al., 2010a). This gives each segment the ability to function as an autonomous transcription-replication unit (Bouvier and Palese, 2008; Stubbs and Te Velthuis, 2014b).

While IAVs contain eight genome segments, they can encode up to 22 viral proteins by taking advantage of the host cell splicing machinery (Dubois et al., 2014; Pinto et al., 2021). The first three and largest RNA segments encode the three RNA polymerase subunits of the RdRp complex; the polymerase basic protein 2 (PB2), the polymerase basic protein 1 (PB1), and the polymerase acidic protein (PA). The next three gene segments encode the envelope glycoprotein HA, the nucleoprotein (NP), and the second most abundant envelope glycoprotein, NA. Messenger RNAs (mRNAs) from genome segments 7 and 8 encode the matrix protein (M1) and the third envelope protein M2 ion channel, and the two non-structural proteins (NS1 and NS2) respectively. These ten viral proteins constitute the core proteome of all IAV virions (Bouvier and Palese, 2008; Vasin et al., 2014). However, it has been noted that several IAV strains utilize alternative reading frames of their eight

gene segments to encode a set of accessory short viral proteins (e.g. PB1-F2, PB1-N40, and PA-X). These proteins though non-essential for virus replication, can impact IAV virulence and/or transmission *in vivo* (Pinto et al., 2021; Suarez, 2016; Vasin et al., 2014).

1.2.2. IAV Infectious Cycle

IAV cycle is initiated by attachment and entry to susceptible cells of the respiratory tract through binding of the surface glycoprotein HA to host cell receptors. Hemagglutinin is a homotrimeric glycoprotein, and each monomer contains a receptor binding site for terminal sialic acid (SA) receptors. While SA receptors are an essential component for IAV infection, influenza virus binding and infectivity are also influenced by the type of galactose linkages present on the glycan structures. Terminal SA residues are linked to the penultimate galactose of the glycan receptor via α-2,3 or α-2,6 glycosidic bonds (SAα2,3Gal and SAα2,6Gal) (Viswanathan et al., 2010). Since the distribution of these polysaccharide residues varies among species and among tissues and cell types within the same host, these receptors are critical determinants of IAV tropism (Kumlin et al., 2008). Whereas avian- and equine-adapted IAV have a propensity for binding to SAα2,3Gal receptors, which are abundantly present in the respiratory tract of these species, human- and swine-adapted influenza viruses show preferential binding to SAα2,6Gal receptors and to a less extent to SAα2,3Gal receptors (Nelli et al., 2010; Sriwilaijaroen and Suzuki, 2012; Suzuki et al., 2000). SAα2,6Gal residues are predominant on swine and human nasal, tracheal and bronchial ciliated cells; however, SAα2,3Gal residues are also present on the bronchiolar and alveolar epithelium, albeit in lower quantity than SAα2,6Gal residues (Nicholls et al., 2007; Rajao et al., 2019; Shinya et al., 2006).

Following attachment to the host cell SA receptor, IAVs hijack the host cell endocytic machinery and are internalized to the cytoplasm through receptor-mediated endocytosis. In response to the acidic environment inside the endosomal lumen, HA undergoes a conformational change and mediates the fusion of the viral envelope with the endosomal membrane. Once in the late endosomes, the acidic endosomal environment triggers activation of the M2 ion channel resulting in the acidification of the viral core and the release of the vRNPs from the matrix layer (Manzoor et al., 2017; Samji, 2009). Viral RNPs are then able to escape to the host cell's cytoplasm through the fusion pore and move to the nucleus for the initiation of primary transcription. Nuclear localization sequences on the NP protein facilitate nuclear import of vRNPs from the cytoplasm (Ashour et al., 2015; Wu et al., 2007). Since influenza viruses have a negative-sense non-coding RNA genome, they take control of the host cell's transcription machinery to achieve RNA synthesis (Lakadamyali et al., 2004). Replication and transcription of the viral genome are two mechanistically distinct processes that use the same RNA template. On one hand, transcription requires modification of the initial sequence in order to synthesize 5' capped and 3' polyadenylated viral mRNAs that can be exported to the cytoplasm in order to be translated into the viral-encoded proteins. Replication, on the other hand, involves unaltered (*de novo*) synthesis of the positive-sense complementary RNA (cRNA). Specifically, the cRNA intermediate is used as a template for the generation of new copies of the viral negative-sense RNA genome that are eventually coated with newly-synthesized nucleoprotein. Both viral transcription and replication are catalyzed by the RdRp complex (Choi, 2012; Dou et al., 2018).

The RdRp heterotrimeric complex is comprised of three functionally distinct subunits. PB1 has the typical RNA polymerase structure and function, and catalyzes RNA synthesis while PB2 takes part in mRNA synthesis by binding to host mRNA caps. PA is necessary for a functional

polymerase complex, since it contains an endonuclease active site and catalyzes the cleavage of the 5' prime end from nascent host pre-mRNAs oligonucleotides (Dias et al., 2009; Stubbs and Te Velthuis, 2014a). The three subunits are linked head to tail in the order PA-PB1-PB2 and each one of the eight viral genome segments is bound through their 5' and 3' ends to the RdRp (Pflug et al., 2014; Sugiyama et al., 2009). This heterotrimeric complex transcribes the viral RNA gene sequences to generate 5' and 3' end-modified positive sense mRNAs that resemble host mRNAs in order to utilize the nuclear export pathway and evade host antiviral responses (Walker and Fodor, 2019).

Following export to the cytoplasm, viral mRNAs are then translated by the host ribosomes to the virally encoded proteins (York and Fodor, 2013). While surface glycoproteins are trafficked toward the plasma membrane, nuclear localization sequences import the newly synthesized PB2, PB1, PA, and NP proteins to the nucleus to assist in viral transcription, genome replication, and assembly of nascent vRNPs (Dou et al., 2018). After their assembly vRNPs are exported to the cytoplasm and trafficked to specific locations of the plasma membrane where the envelope glycoproteins have been incorporated. The genome-packaged particles bud off from the cell as they form enveloped infectious virions (Nayak et al., 2009; Samji, 2009). Finally, NA through its sialidase activity cleaves sialic residues to facilitate effective virus release and spread of progeny influenza virions in the airway lumen (Matrosovich et al., 2004).

1.2.3. Antigenic drift and shift

The two viral surface glycoproteins, HA and NA are the predominant antigenic molecules on influenza virions that induce humoral immune responses and based on their antigenicity, influenza

A viruses can be categorized into different subtypes (Calder et al., 2010a). Currently, 18 HA subtypes and 11 NA subtypes have been identified (Du et al., 2019). In nature, two primary strategies fuel the evolutionary dynamics of influenza viruses, the antigenic drift and antigenic shift mechanisms (Suarez, 2016).

Antigenic drift is characterized by a gradual accumulation of point mutations affecting the coat glycoproteins, HA and NA, steadily over time and the perpetual generation of antigenically divergent influenza viruses. IAVs have the ability to rapidly mutate and adapt to changes in new environments due to the high replication rate and the error-prone nature of the RNA-dependent RNA polymerase during viral replication, resulting in constant changes in the amino acid changes in the sequences of influenza-encoded proteins (Bouvier and Palese, 2008). Although most of the genetic changes have a deleterious effect on virus fitness by compromising functional and/or structural properties of the progeny virions and are eliminated by natural selection, mutations on the antigenic regions of HA and NA that enhance the replication capacity, transmissibility and immune evasion of the escape mutants are selected (Bouvier and Palese, 2008; Suarez, 2016). Antigenic changes that lead to immune evasion from virus neutralization by the host immune system are primarily responsible for the need for annual vaccine updates in order to confer adequate immune protection (Kim et al., 2018).

The segmented nature of IAV genome can facilitate the exchange and recombination of intact gene segments following concurrent infection of the host with two distinct influenza viruses. This phenomenon of genetic reassortment is termed antigenic shift. Co-infection of a cell with two IAVs can result in the formation of up to 256 (2^8) different progeny genotypes (Steel and Lowen, 2014). Gene segment swapping between parental viruses of different host origins potentiates the generation of diverse viruses that can overcome host restriction (Ganti et al., 2022; Zhang et al., 2021). While the natural reservoir of IAV viruses is wild aquatic birds such as waterfowl and shorebirds, periodic zoonotic and reverse zoonotic spillover events, primary between human, swine, and avian species

result in antigenic shift events and the generation and emergence of novel reassortant IAV strains that pose a potential public and animal health risk (Mostafa et al., 2018; Taubenberger and Kash, 2010).

Antigenic shift and drift drive constant viral evolution and circulation of genetically and antigenically distinct influenza viruses in human and animal populations (Shao et al., 2017). Overall, the poor-proofreading ability of the RdRp and the segmented RNA genome in addition to the existence of multiple natural reservoirs and intermediate hosts result in influenza disease being an ineradicable zoonosis (Yen and Webster, 2009).

1.3. IAV disease in swine and human hosts

1.3.1. Overview of influenza pathogenesis

IAVs demonstrate tropism toward epithelial cells that line the upper and lower respiratory tract from nasal mucosa to alveolar epithelium, due to the abundant distribution of primarily the SAα2,6Gal and to a lesser extent SAα2,3Gal receptors in the airways of humans and swine (Janke, 2014). Although influenza virus tropism is considered to be confined to respiratory tissues, there have been sparse reports of detection of viral antigen in extra-respiratory tissues, including ocular, brain, myocardial and gastrointestinal tissues in swine and humans (De Vleeschauwer et al., 2009; Filgueiras-Rama et al., 2021; Janke, 2013; Yoo et al., 2010).

Following influenza infection, five major innate immune mechanisms mediate respiratory tissue damage and pathology; apoptosis, necrosis, necroptosis, pyroptosis, and proinflammatory cytokine responses (Fujikura and Miyazaki, 2018; Janke, 2014). Apoptosis of influenza infected cells is instigated either by detection of viral pro-apoptotic components, such as vRNPs and NS1 by

intracellular pattern recognition receptors (PRRs) (intrinsic pathway) or activation of the extrinsic apoptotic pathway through the death receptors FasR and tumor necrosis factor receptor (TNFR) by recruited leukocytes (Gui and Chen, 2022; Janke, 2014; Othumpangat et al., 2018). Both of these pathways activate the caspase cascade. Apoptosis is the most common form of programmed cell death, and is characterized by cell shrinkage, chromatin condensation and formation of apoptotic bodies that can be easily cleared by phagocytic cells (Elmore, 2007). This highly orchestrated cellular process prevents inflammatory reactions and tissue damage that could be inflicted by exposing surrounding cells to toxic intracellular constituents (Elmore, 2007; Rock and Kono, 2008).

While apoptosis suppresses inflammatory responses, both necrosis and necroptosis induce inflammatory cell death in response to influenza virus infection. The main difference between necrosis or necroptosis following infection is that while the former is an uncontrolled and passive process, the latter is a form of regulated and programmed cell death mediated by the necrosome, mimicking features of both necrosis and apoptosis (Balachandran and Rall, 2020; Dhuriya and Sharma, 2018). However, both necrosis and necroptosis are mainly induced by caspase-independent pathways. Inhibition of host cell gene expression due to the 5’ cap removal and degradation of host mRNAs in concert with the initiation of virus replication induces direct viral damage to the infected host cells and triggers primarily necrotic cell death. On the other hand, necroptosis is instigated either by TNF-α binding to the cell-surface TNF receptor of the infected host cell (extrinsic pathway) or due to intracellular generation of reactive oxygen species (ROS) (intrinsic pathway) (Dhuriya and Sharma, 2018). Both of these types of cellular death result in cell swelling, cytolysis and release of intracellular molecules (including damage-associated

molecular patterns- DAMPs) that trigger strong inflammatory responses by the innate immune system (Fujikura and Miyazaki, 2018).

Another form of regulated inflammatory cell death that is implicated in influenza virus infection is pyroptosis. While necroptosis is associated with necrosome activation and induces a broad proinflammatory cytokine reaction, pyroptotic cell death is triggered by inflammasome activation initiated by the intracellular detection of viral RNAs and/or M2 ion channel activity. This type of inflammatory cell death results in membrane permeabilization and IL-1β and IL-18 cytokine release (Atkin-Smith et al., 2018). These proinflammatory cytokines chemoattract neutrophils to the site of inflammation that in turn degranulate and release digestive enzymes and neutrophil extracellular traps (NETs) to clear the pathogen from the airways, ultimately causing acute lung injury (Fujikura and Miyazaki, 2018; Gui and Chen, 2022).

Finally, cytokine responses, while they play an important role in virus clearance, have also been implicated in inducing pulmonary damage. Following infection, IAV is detected by PRRs within the infected epithelial cells or by sentinel cells (resident macrophages and inflammatory monocytes) resulting in the secretion of IFNα/β, IL-1, IL-6, and TNF-α. Although these primary cytokines have a protective role during the early stages of viral infection, extensive and prolonged production of these proinflammatory mediators in the later stages of infection can result in severe clinical disease and pulmonary immunopathology (D'Elia et al., 2013; McAuley et al., 2013; Sladkova and Kostolansky, 2006). In particular, TNF-α contributes to both localized and systemic hyperinflammatory responses. Specifically, TNF-α signaling stimulates increased vascular permeability and extensive recruitment of inflammatory cells to the site of infection. Chemoattracted resident and migratory leukocytes (macrophages, neutrophils, and natural killer-NK cells) eliminate virus-infected cells through phagocytosis, the release of ROS and granule-

associated serine proteases (e.g. myeloperoxidase) or by induction of apoptotic or inflammatory cell death. These activities can contribute to lung pathology due to tissue injury and destruction of parenchymal cells (Guo and Thomas, 2017; Janke, 2014; La Gruta et al., 2007; Murphy and Weaver, 2016).

Following infection, the first wave of cytokines is released within the first few hours or days and induces the activation and recruitment of group 2 innate lymphoid cells, NK cells, and different subsets of T- cells (Th1 $CD4^+$ and $CD8^+$ cells) to the site of viral replication. These cells in turn secrete the secondary wave of cytokines, such as interferon-γ (IFN-γ), amphiregulin, and IL-10, which have either an effector or anti-inflammatory function (Guo and Thomas, 2017; Tamura and Kurata, 2004). Specifically, INF-γ is responsible for prompting the production of IgG2a/c and IgG3 antibodies by B-cells and is also associated with the T-cell-mediated cytotoxic elimination of virus-infected cells (Karupiah et al., 1998; Moskophidis and Kioussis, 1998). Based on available evidence secondary cytokines mediate virus clearance, dampen inflammation, and restore lung function (Guo and Thomas, 2017).

1.3.2. Influenza-induced pathology

Swine and humans are natural hosts of IAVs and they show remarkable similarities with respect to influenza pathogenesis and clinicopathological features upon infection (Rajao and Vincent, 2015). In both species during the acute stage of influenza disease (days 1-3 post-infection), vacuolar degeneration and necrosis of the epithelial cells cause multifocal destruction and desquamation of the pseudostratified columnar epithelium of the respiratory tract (Taubenberger

and Morens, 2008). Repeated evidence has suggested that large noncartilaginous airways (primary bronchioles) are most frequently and severely damaged, potentially due to aerosol deposition dynamics affecting the uptake of viral droplets and SA receptor distribution (Janke, 2013; Thacker et al., 2001). In response to virus replication and primary cytokine release by infected cells, neutrophils migrate and infiltrate the bronchial and bronchiolar lumen. The release of their granule contents results in oxidative damage and acute lung tissue injury (Kellner et al., 2017). Extensive epithelial damage in concert with inflammatory cell infiltration in the submucosa and airway lumen generate the hallmark histopathological type of lesion of uncomplicated influenza infection, necrotizing to often necropurulent bronchitis and bronchiolitis (Janke, 2013; Landolt and Chambers, 2016).

By days 3 to 5 post-infection (pi), the transient neutrophilic infiltration detected at the early stages is then shifted to primarily mononuclear inflammation (Janke, 2013). At that point, mucin hypersecretion and extensive sloughing of necrotic epithelial cells into the airway lumen causes mechanical airway obstruction and respiratory distress, due to atelectasis (airway collapse) and/or lung consolidation (Landolt and Chambers, 2016; Li and Tang, 2021). While the lumen of affected alveoli is filled with cellular and inflammatory debris, the alveolar wall is thickened by vascular congestion and lymphatic dilation, primarily of perivascular and peribronchial distribution (Janke, 2013). Additionally, interstitial pneumonia characterized by the expansion of alveolar septa is intensified by swollen type II pneumocytes, interstitial edema, and monocyte accumulation (Landolt and Chambers, 2016). However, the degree of alveolar involvement and interstitial pneumonia is quite variable in severity and depends on IAV virulence, dose, or inoculation route in experimental challenge studies. By days 5 to 7 pi, while peribronchial lymphocytic cuffing remains prominent, epithelial and alveolar inflammation is slowly resolving and in most cases, the

immune system has cleared the virus infection. Epithelial hyperplasia (bronchiolar and alveolar) and mitotic figures, which characterize the pi lung sections, are inducted to restore airway lining and flow, and repair tissue damage inflicted by virus-induced or immune-mediated pathology (Janke, 2014; Landolt and Chambers, 2016; Taubenberger and Morens, 2008).

Regarding gross pathology in swine, the hallmark macroscopic manifestation of influenza virus infection is cranioventral broncho(interstitial) pneumonia. The gross lesions demonstrate a dark red, multifocal to coalescing pattern of consolidation that affects a variable percentage of the lung and have primarily lobular distribution (Janke, 2013). This is attributed to (i) the irregularities of airway branching of the bronchial tree affecting virus deposition and (ii) the high degree of lobulation in the swine lung. Collateral air ventilation is obstructed in pigs by a thick collagenous interlobular septum that separates individual lobules and prevents the spread of influenza viruses between adjacent lobules (Landolt and Chambers, 2016; Lopez and Martinson, 2017). In addition to pulmonary lesions, influenza infection in swine and humans causes tracheitis characterized by attenuation and/or squamous metaplasia of the tracheal epithelium and rhinitis characterized by epithelial flattening, squamous metaplasia and goblet cell hyperplasia (Janke, 2013; Kuiken and Taubenberger, 2008; Tian et al., 2018).

1.3.3. Clinical features of influenza infection

Uncomplicated influenza infection in swine and humans is characterized by a highly contagious acute and self-limited febrile respiratory disease with clinical symptoms varying from mild to severe (Javanian et al., 2021; Thangavel and Bouvier, 2014). The clinical features reflect the gross and histopathological lesions described above. Specifically, after natural infection, influenza

disease is typically of sudden onset and is characterized by high fever, anorexia, weakness, and nasal discharge. Additional symptoms in humans include headaches, myalgia, and sore throat (Eccles, 2005). In swine, tachypnea, labored breathing and expiratory dyspnea are often noticeable, especially in weaning and nursery influenza-naïve piglets. Following the establishment of necrotizing bronchitis and bronchiolitis, pigs demonstrate the characteristic clinical symptom of influenza infection, a harsh deep-dry "barking" cough. In the absence of secondary bacterial complications, clinical symptoms abate at day 5 to 7 pi and pigs return to normal body condition and weight gain at approximately 2 weeks pi (Gramer, 2006; Janke, 2013; Landolt and Chambers, 2016).

Influenza disease of swine in an experimental setting is quite variable and depends on multiple factors, including the age of the animal, route of administration, and infectious dose. Clinical disease in experimental virus challenges where pigs remain under controlled conditions varies from inapparent to moderate and rarely reaches the severity of natural on-farm infections (Janke, 2013, 2014). While a few clinical signs may develop to some degree, depending on the experimental protocol used, such as fever, depression, anorexia, and sneezing, the most common features observed in pigs post-virus inoculation are soft cough and tachypnea, at least when pigs are disturbed/agitated. These clinical signs are commonly observed between days 1-3 pi and they abate shortly, at days 3-7 pi. To achieve moderate to severe clinical disease, experimental studies use high virus titer in the inoculum [$>10^6$-10^7 50% tissue culture infectious dose - ($TCID_{50}$)] to challenge influenza-naïve neonatal and nursery piglets (3- to 7 weeks of age) via intratracheal (IT) inoculation. High titer IT inoculation regimens induce early-onset disease that resembles natural infection to some degree and often result in 10-30% lung involvement. In contrast, intranasal (IN) inoculation of older pigs (>10 weeks of age) with a low titer inoculum (10^3-10^6 $TCID_{50}$), often

delays the onset of symptoms, induces mild or inapparent clinical disease, and often results in less than 5-10% lung involvement (Janke, 2013, 2014; Keenliside, 2012; Landolt and Chambers, 2016; Takemae et al., 2018).

1.3.4. Shedding and transmission

Human and swine intraspecies transmission of influenza viruses occurs via four major routes; (i) direct physical contact with an infected host, (ii) through fomites (indirect contact), (iii) through inhalation of large respiratory droplets and/ or (iv) via airborne transmission (inhalation of small particle droplet nuclei-aerosolized virus) (Brankston et al., 2007; Cowling et al., 2013; Torremorell et al., 2012). In pigs and humans, upon IAV infection the incubation period averages 2 days and virus shedding is often first detected within 24-72 hours post-infection and from 24-48 hours before clinical onset (Carrat et al., 2008; Landolt and Chambers, 2016; Ng et al., 2016). Additionally, peak virus replication (nasal swab titer) is detected at 2-3 days pi (Baccam et al., 2006; Janke, 2013). Infected individuals and animals can remain contagious for up to 2 weeks after illness onset, however in most influenza cases virus becomes undetectable at days 6 to 8 pi (Janke, 2013; Subbarao, 2008; Torremorell et al., 2012).

The vast majority of swine herds in pig farms demonstrate a degree of immunity against influenza variants due to ongoing vaccination strategies or endemic infections (Janke, 2013; Torremorell et al., 2012). However, in most cases, the level of immunity achieved does not prevent virus shedding and transmission upon infection. In fact, viruses quickly establish an enzootic status in affected herds, following an influenza outbreak, and while infections show seasonal peaks, viruses are detected year-round (Myers et al., 2006; Rajao et al., 2014; Ryt-Hansen et al., 2019).

IAVs are maintained in swine populations by continued infections of susceptible animals that were introduced or bred on the farm (Torremorell et al., 2012). On endemically infected farms, viruses continue to circulate, often with a muted clinical expression that is indiscernible from other entrenched respiratory pathogen infections, and are transmitted from sick or subclinically infected animals to susceptible pigs, which are naive or of compromised immunity (Janke, 2013; Landolt and Chambers, 2016). Endemic infections in swine farms not only facilitate the continual emergence of vaccine escape mutants but importantly pose a significant risk of infection of swine workers with zoonotic influenza viruses that have pandemic potential (Hennig et al., 2022; Li et al., 2022; Ma et al., 2015; Myers et al., 2006).

1.4. History and epidemiology of North American swine influenza viruses

IAVs were first recognized as respiratory pathogens of swine in 1918 following the second and deadliest wave of the 1918 H1N1 Spanish flu pandemic in humans (Koen, 1919; Taubenberger, 2006). Contemporary investigators recognized this new disease entity in pigs by the remarkable similarities of the clinicopathological features between human and swine influenza disease (Knobler et al., 2005). While studies have debated the origins of IAV and raised questions whether the 1918 influenza virus spilled over from pigs to humans, genetic analyses have demonstrated that this pandemic virus most likely derived from an avian host and that is a common ancestor of all swine and human influenza viruses (Taubenberger, 2006; Taubenberger et al., 2005). Following the initial spread of this swine-adapted pandemic virus first in US Midwestern herds, IAVs established a long-term circulation in pigs and caused recurring epizootic outbreaks in the pork industry (Knobler et al., 2005; Nelson and Worobey, 2018). IAVs that evolved from the Spanish

flu pandemic strain are classified as "classical-swine" or α-clade (alpha) H1N1 viruses (1A.1). These alpha-clade H1N1 IAVs circulated in North American swine populations for approximately 70 years and demonstrated limited genetic and antigenic variability (Rajao et al., 2018; Vincent et al., 2008b). This is presumably due to the earlier pork industry practices, including the restricted domestic and international movement of pigs, small herd size, and limited exposure to humans (Fabrizio II, 2013).

Although H1 was the predominant subtype responsible for endemic infections of pigs until the late 1990s, H3 swine influenza A viruses (swIAVs) were continually circulating in the US swine albeit in a comparatively much lower frequency (Chambers et al., 1991; Lorusso et al., 2011). However, the epidemiology of North American swIAVs was radically changed in 1998 following the emergence of reassortant H3N2 viruses in swine populations. Outbreaks of severe influenza infections were first noted in August 1998 in North Carolina swine herds followed by similar epizootic cases, albeit of lower severity, in Texas and several Midwestern states from November to December of 1998 (Fabrizio II, 2013). Isolates from North Carolina corresponded to a novel double reassortant swIAV strain containing gene segments of human (PB1, HA, and NA) and swine-origin (PB2, PA, NP, M, and NS) viruses (Ma et al., 2010). The H3N2 isolates from Iowa, Minnesota and Texas were classified as triple-reassortant strains that contained the PB1, HA, and NA segments derived from contemporary seasonal human H3N2 viruses (as the double reassortant viruses), PB2 and PA segments were of avian origin, and NP, M, and NS gene segments from the classical-swine IAV H1N1 lineage. While double-reassortant strains failed to establish endemic status in swine, triple-reassortant strains rapidly swept across the US swine. The unique triple-reassortant internal gene (TRIG) cassette, conferred an apparent competitive advantage to these H3N2 influenza viruses and facilitated on one hand the preferential maintenance of TRIG H3N2

swIAV in US herds by replacing the classical-swine viruses, and on the other hand, the emergence of multiple novel IAV reassortants, dramatically altering the epidemiology of North American swIAVs (Gauger, 2012; Sandbulte et al., 2015; Webby et al., 2000).

From 1999 to 2003, subsequent reassortments between classical swine IAVs with the TRIG H3N2 viruses resulted in the generation of novel genotypes of H1N1 and H1N2, classified as β- (beta) (1A.2) and γ- (gamma) (1A.3.3.3) clade H1 swIAVs. These viruses contained HA and/or NA of classical swine origin and retained the internal segments from TRIG strains (Lorusso et al., 2011). An additional minor γ-clade (γ2 beta-like) (1A.3.2.) was later identified and concurrently circulated in US swine at a considerably lower frequency (Anderson et al., 2015; Mancera Gracia et al., 2020). To complicate the epidemiology of swIAVs even more, two separate introductions of human seasonal H1N1 viruses that subsequently reassorted with the contemporary circulating TRIG strains led to the establishment of a novel phylogenetic clade, that of δ (delta)- clade H1 viruses. These δ-clade H1N1 viruses contain a HA of human seasonal origin and are further divided into two distinct phylogenetic subclades, δ1 (1B.2.2) and δ2 (1B.2.1) corresponding to the two human flu variants that crossed species barrier and spread into the swine herds. From 2005 to 2008, gamma and delta clade viruses were predominantly isolated from US swine while beta and alpha clade swIAVs were detected at a much lower frequency (Lorusso et al., 2011; Mancera Gracia et al., 2020; Rajao et al., 2018; Vincent et al., 2017).

In early 2009, a novel reassortant H1N1 influenza virus that caused outbreaks of severe disease and pneumonia in humans was first detected in Mexico and quickly spread across the globe, prompting the world health organization (WHO) to declare a pandemic in the summer of 2009. The 2009 pandemic, became known as the “swine flu” pandemic due to the swine origins of the unique genome of the pandemic H1N1 (pdmH1N1) virus (1A.3.3.2). This novel reassortant virus

contained gene segments from both Eurasian and North American parental swine influenza viruses. Specifically, the NA and M gene segments were derived from a Eurasian avian-like swine influenza virus while the remaining gene segments (PB2, PB1, PA, HA, NP, and NS) were genetically and antigenically similar to the gene segments of North American TRIG strains (Garten et al., 2009; Lorusso et al., 2011). This novel pandemic human strain spilled over back into swine shortly after emerging in human populations, with the first case being detected in a pig farm in Canada in April 2009, and rapidly swept across the globe (Howden et al., 2009). Reassortment events between the pdmH1N1 with contemporary H1 and H3 swIAVs, resulted in the generation of novel viruses containing HA and NA gene segments from the endemic swIAV and/or the pdmH1N1 virus. Interestingly, the vast majority of reassortant progeny viruses, replaced the M gene segment of the original North American TRIG cassette with the Eurasian M gene segment. Previous reports presumed that acquisition of the Eurasian M gene segment rendered these progeny viruses increased transmissibility (Jhung et al., 2013; Ma, 2020). This pdmM gene-modified TRIG constellation became the backbone of the vast majority of influenza viruses currently circulating in the US and Canadian swine. The HA of the pdmH1N1 strain, although most closely related to the γ-clade swIAVs, demonstrated limited serologic cross-reactivity and failed to confer adequate protection against several γ-H1 viruses when used as a vaccine component, thus formed a separate unique subclade within the γ-phylogenetic clade (Gauger, 2012; Lorusso et al., 2011; Mancera Gracia et al., 2020; Vincent et al., 2010b). An overview of the history and epidemiology of North American H1 swine influenza viruses is shown in Figure 1.2..

Regarding the epidemiology of H3N2 swIAVs, following endemic circulation in US swine, between 1999 to 2003, TRIG H3N2 viruses acquired two additional phylogenetically distinct H3

hemagglutinin molecules from human seasonal IAVs, resulting in the establishment of the phylogenetic cluster I, II, and III H3N2 swIAVs (Lorusso et al., 2011; Vincent et al., 2008b; Webby et al., 2004). The continual evolution of cluster III H3N2 swIAVs resulted in the generation of the rapidly diversifying cluster IV H3N2 viruses that became predominant in North American swine. Overall, six distinct phylogenetic subclusters (A-F) have emerged from the cluster IV H3N2 viruses. Additionally, in the early 2010s a novel human reassortant H3N2 virus was spread in swine that contained the HA and NA of human seasonal origin and the pdmM gene modified TRIG cassette. Over the last decade, these human-like H3N2 viruses have been shown to periodically replace their HA gene, with that of more recent seasonal human origin. Human-like H3N2 viruses and cluster IV viruses containing the pdm M gene segment, are characterized as variant H3N2 viruses and demonstrate increased zoonotic potential as they have been isolated from more than 400 humans, raising significant public health concerns (Mancera Gracia et al., 2020; Rajao et al., 2014; Vandoorn et al., 2022; Winter et al., 2013; Wong et al., 2012).

Overall, the genetic and antigenic variability of North American swIAVs isolated over the last century is quite remarkable. The predominant subtypes isolated in swine are the H1N1, H1N2, and H3N2 subtypes. There are seven distinct H1 HA phylogenetic clades (α-H1, β-H1, γ-H1, γ2-H1, pdm-H1, δ1-H1, and δ2-H1) and at least ten H3 HA phylogenetic clusters (I, II, III, IV-A, IV-B, IV-C, IV-D, IV-E, IV-F, and human-like H3s). Regarding the NA gene segment, the vast majority of currently circulating N1 viruses contain a NA of classical-swine origin while most of the endemic N2 viruses retain the NA segment from a 2002 human seasonal H3N2 virus. However, a small but consistent percentage of N2 swIAVs are comprised of a NA from a 1998 human seasonal origin (Anderson et al., 2013; Lorusso et al., 2011; Mancera Gracia et al., 2020; Vincent et al., 2017).

1.5. Swine IAV vaccines

HA and NA serve as the major viral antigens utilized by current commercial and experimental novel vaccine technologies (Kosik and Yewdell, 2019; Sautto et al., 2018).

1.5.1. Functional, structural, and immunogenic properties of HA

HA serves a critical bifunctional role in the infectious cycle of the virus, as it facilitates receptor-binding to host SA residues and mediates the fusion of the viral and cellular membranes (Du et al., 2019). Structurally HA, is an envelope-associated type I transmembrane homotrimeric protein (Veit et al., 1991). Following translation and assembly into a trimeric precursor HA0, each HA subunit is subsequently proteolytically cleaved by cellular proteases into the two disulfide-linked components HA1 and HA2. Each monomer of the HA molecule is comprised of a globular head domain and a "rod-like" stem domain formed primarily by the HA1 subunit and HA2 subunits, respectively (Galloway et al., 2018). The HA1 surface subunit contains the sialic acid receptor binding site (RBS), which mediates attachment that ultimately enables endocytosis and penetration into the host cell, while the HA2 stalk domain is anchored in the virus envelope and contains a hydrophobic fusion peptide that is located near the lipid membrane of the virus envelope. This stem domain is required for viral infectivity since it facilitates the fusion of the lipid envelope and endosomal membranes and the consequent release of the viral ribonucleoproteins through the fusion pore to the host cytoplasm so that they eventually translocate to the nucleus for the initiation of primary transcription (Lakadamyali et al., 2004; Russell, 2016; Sriwilaijaroen and Suzuki, 2012).

The HA1 globular head domain contains five canonical antigenic sites (Sa, Sb, Ca1, Ca2, and Cb for H1 and A, B, C, D, and E for H3) recognized by the host immune system, and is the major influenza antigen inducing neutralizing antibody responses. It has been shown that there is a hierarchical order in the immunodominance of the five major sites on the H1 protein, with the anti-Cb antibodies predominating shortly after infection and being subsequently replaced by primarily anti-Sb antibodies by day 21 pi (Kosik and Yewdell, 2019). For the H3 protein, work by Broecker *et al*. demonstrated that antigenic site B shows immunodominance in mice and humans (Broecker et al., 2018). While the HA2 stem domain is somewhat obscured by the close proximity to the envelope bilayer, the globular head is highly exposed and easily accessible by antibodies, and displays a clear immunodominance pattern compared to other influenza antigens following influenza infection or vaccination. The vast majority of antibodies targeting the globular head restrict access of the HA to host SA receptors, thus neutralizing influenza virions by preventing them from attaching to and entering host cells and initiating virus infection (Angeletti et al., 2017; Broecker et al., 2018; Chen et al., 2018; Kosik and Yewdell, 2019; Padilla-Quirarte et al., 2019).

Selective pressure fuels the rapid genetic and antigenic evolution of influenza viruses to evade viral neutralization. The antigenic drift of HA is achieved by amino acid substitution primarily at the five main antigenic sites without compromising any of the structural and functional properties of the viral protein (Koel et al., 2013). This phenomenon drives the production of antigenically divergent progeny influenza viruses capable of escaping pre-existing host immunity. Due to antigenic drift, the vast majority of antibodies that target the globular head of HA are strain-specific and often fail to neutralize subsequent heterologous homosubtypic and heterosubtypic variants (Sangesland and Lingwood, 2021; Wang et al., 2022). While the globular head is the most variable region of HA, the stem domain, which carries out an essential step in virus replication, membrane

fusion, is highly conserved across several HA subtypes (Angeletti et al., 2017; Sautto et al., 2018). Antibodies that effectively target the conserved HA2 stem domain have been shown to be broadly cross-reactive across multiple antigenically distinct influenza viruses (Grødeland et al., 2020; Steel et al., 2010). Hence the HA2 domain remains one of the main antigenic targets for developing broadly protective influenza vaccine technologies to achieve the desired "universal" immune responses. However, epitopes on the stem domain are strongly selected to be subdominant considering that humoral responses are disproportionally skewed towards the globular head epitopes following influenza infection or vaccination (Tan et al., 2019). This could be attributed to humoral imprinting of non-conserved head domain epitopes, steric hindrance of HA2 epitopes by glycan shielding, poor intrinsic immunogenicity of the stalk domain (limited T follicular helper cell elicitation), and/or silencing of conserved potentially autoreactive epitopes by central tolerance mechanisms (Bajic et al., 2019; Glanville et al., 2020; Sautto et al., 2018; Tan et al., 2019).

1.5.2. Functional, structural and immunogenic properties of NA

NA is a homotetrameric envelope glycoprotein that accounts for approximately 10 to 20% of total influenza coat proteins. On the virus surface, NA proteins are either found localized in small clusters or individualized surrounded by HA spikes. Each NA monomer is comprised of four distinct structural elements, an N-terminal cytoplasmic tail, a membrane anchoring transmembrane region, a thin stalk of variable length, and a globular head domain that contains the active site. In contrast to hemagglutinin, NA does not require post-translational cleavage by host proteases, however its enzymatic function and structural stability are directly dependent on the tetrameric

conformation. While the stem domain of NA can accommodate multiple amino acid substitutions, the catalytic head demonstrates a highly conserved six-bladed propeller structure that is responsible for the enzymatic activity of the protein. Overall, between different NA subtypes amino acid sequence divergence varies from approximately 20 to 60% (Air, 2012; McAuley et al., 2019; Sylte and Suarez, 2009).

Regarding functional properties, NA plays multiple roles in the influenza virus cycle. Following entry into the respiratory tract, influenza virions are faced with a major host barrier, the respiratory mucous secretions, which are constantly cleared by the mucociliary escalator. Through its enzymatic activity as a sialidase, NA cleaves decoy mucin receptors (MUC5AC) and releases trapped influenza virions (Yang et al., 2014). Moreover, NA facilitates penetration through the respiratory mucus layer and allows virus movement over receptor-coated cell surfaces of the underlying epithelial cells (Guo et al., 2018; Ohuchi et al., 2006). Post-infection, NA prevents virion aggregation and facilitates viral budding. Specifically, NA cleaves SA from newly synthesized sialylated viral proteins that have been N-glycosylated in the endoplasmic reticulum (ER) and Golgi apparatus (Pandey et al., 2022). By thwarting virion aggregation, NA increases influenza virus transmissibility by allowing nascent virions to be distributed in small aerosol droplets (Krammer et al., 2018). Additionally, NA removes SA residues from the host cell plasma membrane preventing the binding of budding influenza virions to dying infected cells and enabling them to spread in the respiratory epithelium (Matrosovich et al., 2004; McAuley et al., 2019; Wohlbold and Krammer, 2014). In addition to cleaving SA, studies have demonstrated that NA can facilitate attachment to host cells by serving as the receptor-binding protein, replacing the role of HA while still retaining partial sialidase activity (Hooper and Bloom, 2013; Lin et al., 2010; Wen and Wan, 2019; Wohlbold and Krammer, 2014).

While the HA of human and swine influenza viruses demonstrate preferential binding to host SAα2,6Gal receptors, their neuraminidase cleaves SAα2,3Gal residues more efficiently than SAα2,6Gal. This substrate specificity towards α2,3 linkages could be attributed to the fact that swine and human bronchial mucus secretions contain large glycoproteins (MUC5AC) that primarily display SAα2,3Gal residues (Kobasa et al., 1999; Li et al., 2011). Since HA mediates viral entry and NA facilitates virion release, it is of utmost significance that influenza virions maintain an optimal functional balance in the activities of these proteins. Suboptimal NA enzymatic activity could result in enhanced capturing of influenza virions in the respiratory mucociliary apparatus and limited viral budding from infected cells. On the other hand, relative increased NA activity to the HA function, could impede virion entrance into susceptible host cells due to the removal of available host SA residues (Gaymard et al., 2016; McAuley et al., 2019; Mitnaul et al., 2000). In the context of suboptimal HA/NA balance, to restore viral fitness, selective pressure endows a competitive advantage to progeny virions with compensatory mutations. For example, sequence analysis of escape mutants after administration of an NA inhibitor demonstrated that resistant mutants, rather than showing substitutions in the NA protein, demonstrated numerous substitutions in the HA protein (Ilyushina et al., 2019). These mutations rendered HA to have a reduced SA affinity, thus allowing lower NA enzymatic activity to mediate efficient virion release (Ilyushina et al., 2019; McAuley et al., 2019).

NA is the second most immunogenic influenza protein after HA (Dou et al., 2018; Krammer, 2019). While anti-HA humoral immunity is mostly characterized as neutralizing since it prevents viral attachment and entry into the host cell, anti-NA responses induce infection-permissive immunity. Specifically, anti-NA antibodies interfere with virion release by blocking the sialidase's activity and virion budding, limit viral spread and reduce the severity of clinical disease

(Eichelberger and Monto, 2019; Johansson and Cox, 2011). Since SA residues are relatively large substrates, NA-targeting antibodies that bind even to epitopes located at some distance from the enzymatic pocket have been found to obstruct SA removal by the NA protein (Xiong et al., 2020). However, anti-NA antibodies have been also found to have virus-neutralizing activity either by sterically hindering access of HA to SA residues or by blocking a potential receptor-binding activity of NA (Gentles et al., 2020; Webster et al., 1984; Wohlbold and Krammer, 2014). Non-neutralizing antibodies targeting the NA mediate two crucial roles in viral clearance: they facilitate viral opsonization and uptake by phagocytic cells through Fc-receptor-mediated endocytosis, and they can mediate the elimination of infected cells by either antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (Wohlbold and Krammer, 2014).

HA is the most abundant glycoprotein on the virus coat (Dou et al., 2018). Consequently, B cells recognizing HA epitopes extensively capture influenza virions, depriving NA-targeting B cells of activation and subsequent proliferation. Hence, influenza infection or vaccination primarily induces HA-targeting antibodies with limited anti-NA humoral responses (ranging from 30 to 40% in human adults) (Ge et al., 2022; Krammer et al., 2018). The insufficient responses against NA following immunization with licensed vaccines are also attributed to the fact that while the HA antigen content is highly regulated and standardized (15 μg per subtype), commercial formulations have no specifications for NA antigen content, stability, and enzyme activity (Wohlbold and Krammer, 2014). Previous vaccination studies have demonstrated that the HA immunodominance over NA can be overcame either by immunizing animals with vaccine viruses containing heterologous HA and homosubtypic NA proteins or by administering HA and NA as separate purified antigens to remove intravirionic antigenic competition (Johansson and Kilbourne, 1993;

Johansson et al., 1987; Wohlbold and Krammer, 2014). Indeed, when HA and NA were administered in equal amounts in influenza vaccines were found to elicit similar antibody titers in mice, thus demonstrating similar immunogenicity (Johansson et al., 1989). Additionally, Johansson *et al*. revealed that supplementation of seasonal human influenza trivalent inactivated vaccines (TIV) with purified NA recombinant proteins broadened the vaccine-induced immunity against heterologous influenza strains than TIV alone and protected mice from heterosubtypic challenge (Johansson et al., 1998). This could be attributed to the fact that NA demonstrates a lower rate of antigenic drift over time and that NA epitopes are relatively more conserved compared to HA epitopes (Eichelberger and Monto, 2019; Eichelberger et al., 2018). Furthermore, work by Bosch *et al*. showed that NA supplementation can enhance the hemagglutination inhibition (HI) antibody titers induced by a recombinant trimeric HA recombinant protein vaccine (Bosch et al., 2010). While HA-specific vaccines have been found to primarily reduce virus replication following influenza infection, NA-specific vaccines mitigate multiple disease severity parameters including shedding duration, clinical disease, and lung pathology (Bosch et al., 2010; Memoli et al., 2016). Specifically, anti-NA antibodies have been found to limit pulmonary virus replication and induce cross-protection against homologous, heterologous intrasubtypic, and heterosubtypic viruses (Job et al., 2018; Kim et al., 2019; Liu et al., 2015; Menne et al., 2021; Quan et al., 2012; Smith et al., 2017; Walz et al., 2018; Wohlbold et al., 2015).

1.5.3. Commercial swine influenza vaccines

To date, vaccination constitutes the most effective prophylactic strategy to control influenza disease in the pork industry. The three main objectives of using influenza vaccines in swine herds

are to (i) reduce the severity of clinical disease, thus limiting any financial costs that could arise from influenza infections, (ii) limit shedding and transmission in order to mitigate the spread of the field viruses in the herd and (iii) reduce the risk of transmission of zoonotic influenza strains with pandemic potential to human populations. Three vaccine strategies are currently commercially available on the market: whole-inactivated virus vaccines, an NS1 truncated live-attenuated influenza vaccine, and an alphavirus RNA replicon vaccine.

1.5.3.1. Whole-inactivated virus vaccines

Whereas the first human influenza vaccine became available in 1945, almost 50 years passed before the licensure of the first swine influenza vaccine. A whole-inactivated virus (WIV) vaccine for swine that contained a classical-swine H1N1 became commercially available in North America in 1994. Currently, the vast majority of WIV or "killed" vaccines are produced in eggs or cell culture, are chemically inactivated by β-propiolactone or formalin, and are formulated with an oil-based adjuvant (Rajão and Pérez, 2018). In contrast to most human vaccines, which are standardized split-virus (disruption of viral lipid envelope by solvent/detergent) purified unadjuvanted vaccines, swine vaccine preparations are not standardized for antigen content and mass (Van Reeth and Ma, 2013). Additionally, most WIV vaccines currently available in the market are multivalent, containing 3 or 4 viral strains that are currently circulating in the swine populations. Regarding swine vaccination, WIV are administered intramuscularly (IM) to pigs with a 2-dose regimen at a 3 to 4-week interval. According to a US Department of Agriculture (USDA) survey conducted in 2016, over 80% of US pig farms vaccinate their breeding stock (USDA, 2016). The main vaccine strategies routinely followed in the pork industry is primarily

immunization of sows quarterly or at 3-6 weeks pre-farrowing to achieve protection during gestation and passive transfer of maternally derived antibodies (MDA) to suckling piglets and to less extent vaccination of feeder pigs at 12-16 weeks to avoid any interference with passive immunity (Mancera Gracia et al., 2020; Rajao et al., 2014; Van Reeth and Ma, 2013; Van Reeth et al., 2016; Vincent et al., 2008b).

The primary role of killed virus vaccines is the elicitation of serum-neutralizing HA-inhibiting (HAI) IgG antibodies. These antibodies are passively transferred to the epithelium of the lower respiratory tract resulting in a reduction of the lung virus load. Limited pulmonary virus replication corresponds to reduced levels of proinflammatory cytokines, lung pathology, and clinical disease. While high HAI titers can effectively neutralize virus infection, thus preventing pulmonary virus replication and mediating sterilizing lung immunity, antibody responses conferred by WIV in most cases only mitigate clinical symptoms. WIV-induced HA-targeting antibodies are predominantly strain-specific and induce optimal protection only in the context of homologous vaccination. However, they demonstrate suboptimal performance and confer partial or no protection against antigenically distinct influenza strains (Kitikoon et al., 2006; Lee et al., 2007; Macklin et al., 1998; Van Reeth and Ma, 2013). While killed influenza vaccines elicit strong serological responses against homologous viruses that often reach titers of ≥ 160- 1280 HAI units, long-term immunity is rarely achievable, as antibody titers fall rapidly following boost vaccination (Kyriakis et al., 2010). Another practical problem with the use of WIV is the potential interference with vaccine-induced responses by passive immunity. MDA can actively suppress adaptive humoral and cell-mediated immune responses conferred by influenza vaccination and severely impact WIV vaccine performance (Kitikoon et al., 2006; Vincent et al., 2012).

While WIV vaccines can reduce lung viral titers, they often fail to significantly reduce virus shedding. This is accounted for by the fact that WIV vaccines primarily elicit serological responses. Serum antibodies, although they can easily transudate via the alveolar epithelium and neutralize virus replication in the lower respiratory tract, are obstructed from being passively transferred in the nasal turbinates and trachea due to the presence of a thick mucosal layer at these sites (Graham et al., 2007; Janeway Jr et al., 2001). Additionally, several studies have demonstrated that IM vaccination of pigs fails to confer sufficient mucosal immunity and activation of IgA-secreting B-cells in the respiratory mucosa. Lack of mucosal immunity in concert with the limited transfer of serum antibodies to the nasal epithelium are the primary reasons why WIV vaccines are often ineffective in reducing nasal shedding and preventing influenza transmission to co-housed pigs (Heinen et al., 2001a; Van Reeth and Ma, 2013). Regarding cell-mediated immunity, WIV vaccines induce limited major histocompatibility complex-I (MHC-I)-mediated cytotoxic $CD8^+$ cellular responses since inactivated antigens are captured mainly by antigen-presenting cells (APCs) and are predominantly processed onto MHC-II complexes, resulting in primarily $CD4^+$ T-helper cell activation and proliferation (Aida et al., 2021).

The ever-changing nature of swine influenza viruses in concert with the remarkably complex epidemiology and immense antigenic diversity of swine viruses in North America are mounting important obstacles to maintaining a close antigenic match between vaccines and circulating field strains. To overcome these practical problems, over the last two decades, herd-specific autogenous inactivated influenza vaccines are popular for the swine producers. In 2008, over 50% of WIV doses given in the US pork industry corresponded to autogenous influenza vaccines (Vincent et al., 2010b). These vaccines are primarily used in the farm of origin or neighboring herds. While the main benefit of autogenous vaccines is that they are highly customizable, in terms of antigen

content, valency, and adjuvant formulation, there is often a lack of information regarding the immunogenicity, potency, and efficacy of autogenous influenza vaccines used in the field (Chen et al., 2012; Ma and Richt, 2010; Sandbulte et al., 2015).

Overall, large herd sizes, continuous transport of pigs to off-site nursery and finisher sites, and rapid turnover rates of animals impose great practical obstacles in maintaining sufficient herd immunity in pig farms by marketed or autogenous WIV vaccines in their mission to prevent virus transmission and spread. Mismatched influenza vaccinations and patchy herd immunity commonly result in clinically silent virus spread and continuous generation of vaccine escape mutants (Rahn et al., 2015; Van Reeth et al., 2016).

1.5.3.2. Live-attenuated influenza virus vaccines

Three main vaccine platforms have been utilized by various research groups to achieve attenuated virulence of influenza viruses via reverse genetic technologies: NS1 truncation, temperature-sensitive mutations in the polymerase genes (similar to the FluMist human-licensed live-attenuated virus vaccine), and modification of the HA cleavage site to attain an elastase-dependent motif (Vincent et al., 2017). An NS1-truncated bivalent live attenuated influenza virus (LAIV) vaccine became commercially available in 2017 in the swine market for use in pigs from 1 day of age (Vandoorn et al., 2022). NS1 is a viral protein expressed in infected host cells and is absent in influenza virions. Inside the host cell, the main responsibility of the NS1 protein is to overtake host defenses and establish efficient virus replication. Specifically, NS1 is an essential influenza virulence factor that blocks the interferon (IFN) α/β signaling pathway by thwarting the activation of the IFN regulatory factor 3 (IRF3) and nuclear factor-kappa beta (NF-κβ) transcription factors

thus limiting the expression of antiviral interferon-inducible proteins (Hale et al., 2008; Krug, 2015). Deletion of 126 amino acids from the NS1 protein sequence confers an attenuated phenotype to influenza virions. NS1-truncated viral mutants have been shown to retain their immunogenicity despite showing limited replication capacity in the respiratory tract (Mancera Gracia et al., 2020; Sandbulte et al., 2015; Van Reeth and Ma, 2013).

Intranasal administration with two doses of this LAIV platform mimics natural infection and induces balanced systemic and mucosal immune responses. Additionally, a single dose of an NS1 LAIV vaccine was found to be protective (Vincent et al., 2012). NS1 LAIV vaccines have been demonstrated to broaden cross-reactivity compared to WIV vaccines as they have been shown to protect from clinical disease and pathology not only against homologous viruses but also homosubtypic heterologous variants. However, they demonstrate limited efficacy against heterosubtypic viruses. Interestingly, the absence of serum HAI responses against heterologous viruses in LAIV-vaccinated animals indicates that alternative immune mechanisms such as mucosal immunity or cellular responses primarily mediate protection against antigenically distinct viruses (Kappes et al., 2012; Richt et al., 2006; Vincent et al., 2007). Additionally, LAIV vaccines have been shown to be protective, albeit suboptimally, against heterologous challenge in pigs with MDAs (Genzow et al., 2018; Vincent et al., 2008b; Vincent et al., 2012). However, a major concern with the use of LAIV vaccines in the field is concurrent infection and reassortment with endemic strains. A recent phylogenetic analysis study demonstrated that reassortment events have occurred in the field between the attenuated NS1-truncated vaccine virus and wild-type circulating strains (Sharma et al., 2020). Two additional weaknesses of LAIV vaccines is the potential evolutionary reversion of live-attenuated vaccine viruses to virulence over time through back-

mutation of attenuated mutations and the need for labor-intensive and time-consuming mucosal administration of LAIV vaccines via the intranasal route (Chen et al., 2012; Hanley, 2011).

1.5.3.3. Alphavirus RNA replicon vaccine

An alphavirus RNA replicon vaccine became licensed in 2012 in the US swine market and is the only third-generation vaccine technology commercialized in the pork industry. This technology employs an attenuated Venezuelan equine encephalitis virus where essential viral replication genes have been deleted and replaced with gene inserts expressing the protein(s) of interest. Although these vectors are single-cycle replication-defective constructs, they can still attach and enter into the host cell to deliver and express the inserted genetic material during the initial cycle, without producing any infectious progeny virions (Sandbulte et al., 2015; Van Reeth et al., 2016). RNA replicon vectors are highly immunogenic and have inherent adjuvant properties since they express RNA intermediates that stimulate endosomal PRRs during the single self-amplification cycle (Szurgot et al., 2021). HA-expressing RNA replicon vaccines have been shown to induce high HAI titers against homosubtypic H1 and H3 influenza viruses and confer robust protection against severe disease, virus shedding, and pulmonary pathology (Bosworth et al., 2010; Erdman et al., 2010; Vander Veen et al., 2012).

The main benefit of using this platform is that it is highly customizable and can allow quick strain updates to overcome the continuous rapid virus evolution and antigenic diversity observed in the field. In contrast to other vector vaccine technologies, RNA replicon vaccination induces limited anti-vector antibodies, thus preventing interference of such antibodies with subsequent booster immunization using the same vaccine platform (Pushko et al., 1997). However, work by Bosworth

et al. demonstrated that passive immunity can interfere with this platform and greatly limit the vaccine-induced immune responses in MDA-positive piglets (Bosworth et al., 2010). Due to the fact that this technology primarily focuses on the expression of the HA gene, self-replicating RNA vaccines elicit limited humoral and most importantly cellular responses against other conserved surface and internal viral proteins. However, this can be used as a diagnostic advantage in the field to differentiate vaccinated from infected animals (DIVA) strategy by assessing the presence of anti-NA or anti-NP antibodies in the sera of examined animals (Mancera Gracia et al., 2020; Sandbulte et al., 2015).

1.5.4. Platforms for universal influenza vaccine development

Although there is no clear consensus about the definition of a universal influenza vaccine (UIV), most research groups accept that an ideal UIV technology should elicit robust broadly cross-reactive humoral and cell-mediated immune responses against circulating antigenically drifted strains but also against emerging influenza variants with pandemic potential and can be utilized in both human and animal populations (Jang and Seong, 2019). Current candidate universal vaccine technologies utilize highly conserved influenza antigenic targets, including the stem domain of the hemagglutinin protein, the NA protein, the ectodomain of the M2 ion channel (M2e), the nucleoprotein, and M1 protein to elicit cross-reactive responses against antigenically diverse influenza strains. The three main platforms currently utilized by research groups around the world in pursuit of developing a universal influenza vaccine are (i) nucleic-acid vaccines (ii) virus-like particle vaccines and (iii) vector vaccines.

1.5.4.1. Nucleic-acid vaccines

There are two distinct categories of nucleic-acid vaccines: DNA-based and RNA-based vaccines. DNA-based vaccines utilize a naked DNA plasmid system containing the gene(s) of interest (downstream of a suitable eukaryotic promoter) to transiently express the encoded antigen once the DNA has entered the transfected host cells (Prather et al., 2003; Saade and Petrovsky, 2012). The main benefits of the nucleic-acid vaccine platforms such as DNA vaccination are that since plasmids are non-infectious constructs there is no safety concern for use in immunocompromised individuals, they can be quickly altered through genetic engineering and can express multiple DNA-encoded antigens (Aida et al., 2021; Lopes et al., 2019). DNA platforms have been explored experimentally in human and veterinary medicine against a variety of influenza antigens, such as HA, NA, M2e, and NP, and have been shown to confer strong serological and cell-mediated responses and protection against homologous and occasionally heterologous challenge (Gorres et al., 2011; Karlsson et al., 2018; Kim and Jacob, 2009; Sisteré-Oró et al., 2019). Interestingly, it has been demonstrated that priming with a DNA vaccine followed by boosting with WIV induces stronger protection compared to two doses of a DNA vaccine (Larsen et al., 2001). The main weaknesses of DNA vaccines are that they often require large quantities of expensive nucleic acid per dose in the vaccine inoculum and the administration of multiple boosters (three or more) to establish sufficient immunity. Another concern with the use of DNA vaccines especially in humans is that DNA could potentially integrate into the host genome leading to neoplastic or autoimmune diseases, although is extremely rare (Swayne and Kapczynski, 2008; Van Reeth and Ma, 2013).

In addition to DNA-based vaccines, RNA-based vaccines, including conventional mRNA and self-amplifying mRNA platforms, have emerged as promising constructs for developing universal

influenza vaccine technologies. Following delivery into the host cytoplasm, the inserted mRNA sequence is translated by the ribosomes into the viral protein of interest. Given that the proteins produced by RNA-based vaccines enter the endogenous pathway of antigen presentation (MHC-I pathway) and stimulate the activation of multiple cytoplasmic PRRs, they can induce robust humoral and cytotoxic $CD8^+$ T-cell responses (Aida et al., 2021).

There are multiple advantages that come with the use of mRNA vaccines against influenza disease: they can be easily and rapidly manufactured without the use of embryonated eggs and they provide a highly flexible and customizable platform, available for frequent updates to address continuous antigenic variation in the field. Additionally, they are short-lived (limited vector persistence) and allow precise control over antigen content and dosing, thus making less necessary the addition of reactogenic adjuvants in the vaccine formulation (Pilkington et al., 2021; Rahn et al., 2015; Vincent et al., 2017). Although naked mRNAs are susceptible to digestion by ubiquitous ribonucleases, delivery of this vaccine platform using lipid nanoparticles has been shown to protect from degradation (Ramachandran et al., 2022; Zhang et al., 2019). Work by Petsch *et al.* showed that vaccination of pigs and ferrets with mRNAs encoding HA, NA, and NP of H1N1, H3N2, and H5N1 viruses was highly immunogenic and mitigated clinical disease and virus shedding detected in nasal swabs (Petsch et al., 2012). Additionally, lipid nanoparticle (LNP)-formulated mRNA vaccines were shown to confer robust humoral immunity and protection from challenge in ferrets and non-human primates with zoonotic H10N8 and H7N9 avian influenza viruses (Bahl et al., 2017).

1.5.4.2. Virus-like particles vaccines

Virus-like particles (VLPs) is a novel vaccine technology that can revolutionize the field of vaccine development. The unique feature of VLPs is that they resemble the morphology, size, and structural conformation of native virions but they are devoid of viral genetic material (analogous to an empty cell). VLPs are non-infectious non-replicating macromolecules that are self-assembled in cell-culture systems and allow the incorporation of selected antigenic proteins on their envelope. Several expression systems have been utilized for the production of these constructs, including baculovirus vector systems, transient plasmid, or plant expression systems. Additionally, VLPs are highly immunogenic scaffolds as they allow a repetitive and high-density display of viral epitopes on their surface similar to native viral particles (Kang et al., 2009; Kushnir et al., 2012; Nooraei et al., 2021; Rahn et al., 2015; Syomin and Ilyin, 2019).

Following vaccination, these constructs are recognized by surface or endosomal PRRs of antigen-presenting cells. VLP uptake by dendritic cells (DC) prompts maturation and incorporation of the foreign coat-expressed antigens onto both MHC-I and MHC-II complexes. Peptides derived from VLPs are then presented to both $CD4^+$ and $CD8^+$ T cells (Zepeda-Cervantes et al., 2020). Primed $CD4^+$ T-helper cells stimulate the activation and proliferation of B-cells and induce robust humoral responses while cross-primed $CD8^+$ T-cells mediate antiviral cytotoxic responses against infected cells. Furthermore, VLPs have inherent adjuvant properties as they contain highly repetitive multimeric epitopes on their surface, allowing of cross-linking B-cell receptors, thus resulting in polyclonal activation of B-cells without requiring antigen-specific T-cell help [thymus independent(TI antigens)]. Specifically, during the early immune response, short-lived plasmablasts in the primary foci as well as B1 cells can recognize VLPs as TI antigens and secrete

natural non-specific IgM antibodies (Murphy and Weaver, 2016; Shirbaghaee and Bolhassani, 2016; Zepeda-Cervantes et al., 2020). Some other advantages of VLP vaccines are that they can be easily and rapidly produced on a large scale at a relatively low cost, and are considered inherently safe as there is no concern for incomplete inactivation, reversion to virulence, or inducing any anti-vector antibodies (Dai et al., 2018; Tariq et al., 2022).

Historically, influenza VLPs are traditionally generated by co-expressing surface glycoproteins (HA, NA or M2e) along with the M1 matrix protein. The latter protein is essential for VLP formation and structural stability (Gómez-Puertas et al., 2000). VLPs have been utilized by several research groups in the efforts to develop broadly-protective influenza vaccines. A study by Schwartzman *et al.* assessed a novel VLP cocktail vaccine containing HA molecules from multiple subtypes (H1, H3, H5, and H7) (Schwartzman et al., 2015). After intranasal vaccination, mice were found to be protected from lethal homologous, heterologous intrasubtypic, and heterosubtypic challenge (Schwartzman et al., 2015). Additionally, work by Steel *et al.* showed that VLPs vaccination of mice with constructs expressing the conserved HA stalk domain (headless HA molecules) elicited broadly cross-reactive humoral responses against heterologous strains and protected animals from lethal challenge (Steel et al., 2010).

Following natural infection or vaccination, due to the immunodominance of HA molecules, antibody responses against NA and M2e are quite limited. VLPs allow the selected expression of specific antigens on separate constructs, thus removing the concern of intravirionic antigenic competition between viral proteins. Studies by Quan *et al.* and Kim *et al.* demonstrated that VLP vaccines contain the NA protein from either the A/Puerto Rico/8/1934 (H1N1) virus or the A/California/2009 (pdmH1N1) were highly immunogenic and protected mice from heterosubtypic challenge with A/Philippines/82 (H3N2) virus (Kim et al., 2019; Quan et al., 2012). A recent study

by our group found no evidence of competition between different NA subtypes following multivalent NA (N1 and N2) VLP vaccination of mice. Interestingly, we demonstrated that N1-N2 VLPs induce higher IgG titers than N1 or N2 VLPs alone (Menne et al., 2021). A previous study in pigs demonstrated that vaccination with VLPs displaying HA, NA and M1 proteins derived from the 2009 pdmH1N1 strain reduced nasal shedding, pulmonary replication, and lung pathology following homologous challenge (Pyo et al., 2012). Regarding M2-specific immunization, work by Song *et al.* showed that M2 VLP vaccination can induce long-lasting humoral and cell-mediated immunity in mice against heterologous and heterosubtypic viruses. Interestingly however, VLPs comprised solely of the M1 protein failed to elicit detectable levels of anti-matrix antibodies and protection (Song et al., 2011).

1.5.4.3. Vector vaccines

Vector vaccines, primarily adenovirus, poxvirus, alphavirus, and paramyxovirus vectors, represent a highly promising vaccine platform in human and animal medicine. Several research groups have been exploiting these platforms for developing novel candidate universal influenza vaccines (Arunkumar et al., 2019; Goodman et al., 2011; Hassan et al., 2017; Hessel et al., 2014; Van Kampen et al., 2005). Following IM administration, vector vaccines target cells at the site of injection (primarily muscle cells) and display their encoded coat antigens on cell surfaces or MHC molecules, thus stimulating both humoral and cellular responses. The main benefits of this platform are that vectors are highly immunostimulatory constructs that do not require the addition of adjuvants and carry native tissue gene transduction capability (Singh et al., 2018; Ura et al., 2014). However, drawbacks associated with vector vaccines, including the risks of genomic

integration and induction of anti-vector immunity that could interfere with vaccine boosting, pose significant obstacles in the use of this platform for universal influenza vaccines (Li et al., 2020).

Several vector vaccines are currently licensed against viral pathogens of animals including an equine influenza vaccine (modified live canarypox vector- ProteqFlu) (Aida et al., 2021). In swine, a human replication-deficient adenovirus serotype 5 vector (Ad5v) expressing various influenza proteins has been extensively investigated in multiple vaccination-challenge studies by the Wesley group (Tang et al., 2002; Wesley and Lager, 2005, 2006; Wesley et al., 2004). A single dose of an Ad5v expressing HA and NP derived from H3N2 strains was found to elicit robust vaccine virus-specific HAI titers and protect pigs from a homologous challenge (Wesley et al., 2004). Additionally, this platform was used to prime MDA-positive piglets without showing any evidence of interference by passive immunity (Wesley and Lager, 2006). A subsequent study by Braucher *et al*. demonstrated that a single dose of an Ad5v construct expressing a codon-optimized HA of the pdmH1N1 strain conferred robust immunity against homologous challenge and partial protection against heterologous H1N2 challenge (Braucher et al., 2012).

Additional studies in swine exploited alternative recombinant vector systems, including an equine herpesvirus 1 platform and various pox-virus-based vector vaccines (canarypox, fowlpox, vaccinia, and swinepox vectors) to immunize pigs against the influenza HA protein (Kyriakis et al., 2009; Said et al., 2013; Xu et al., 2012). Although these vaccines were proven to be immunogenic in swine and confer partial (Xu et al., 2012) or complete protection following homologous challenge (Said et al., 2013) and heterologous challenge (Kyriakis et al., 2009), these studies utilized high viral vector antigen content (up to $10^{8.4}$ $TCID_{50}$) for vaccine dosing and did not assess the effect of passive immunity on vaccine performance.

1.5.5. Vaccine adjuvants

Adjuvants are molecular substances that are included in vaccine formulations to enhance and shape vaccine-induced immune responses. Although there is a big gap in our knowledge regarding their specific mechanistic function, most adjuvants strengthen the immunostimulatory potential of their vaccines by (i) retaining and slowly releasing the antigen at the site of injection (depot effect) (ii) inducing localized inflammatory responses that attract sentinel cells and (iii) enhancing the antigen uptake, activation, and maturation of APCs (DCs and macrophages) (Awate et al., 2013; De Gregorio et al., 2013). The addition of adjuvants in vaccine formulations can accelerate and significantly boost humoral and cell-mediated immune responses, especially in the elderly and immunocompromised individuals, and allows reduction of antigen content and vaccine doses, thus expanding vaccine supply which, could prove especially important in a pandemic situation (Pulendran et al., 2021; Reed et al., 2013; Tregoning et al., 2018).

Aluminum compounds/salts (such as aluminum hydroxide, aluminum phosphate, and aluminum potassium sulfate) were the first and only licensed adjuvants used in human vaccines for approximately 70 years (Di Pasquale et al., 2015). Currently, six types of adjuvants are licensed for use in human influenza vaccines: Aluminum salts, MF59, AS03, AF03, virosomes and heat-labile enterotoxin of Escherichia coli. In the US most commercial influenza vaccines are non-adjuvanted, and adjuvants are only approved to be added in seasonal vaccines for persons 65 years of age and older and for pandemic influenza vaccines (Tregoning et al., 2018). In veterinary medicine, the vast majority of influenza vaccines intended for use in the poultry and pork industries are formulated with oil-based adjuvants, such as oil-in-water (OW) emulsion and water-in-oil-in-water (WOW) emulsion adjuvants. Oil-based adjuvants are considered among the most potent

known adjuvants, as they are able to elicit robust long-lasting vaccine-induced immune responses, even following a single immunization (Heegaard et al., 2011; Jansen et al., 2005).

Despite the plethora of benefits that come with their use, adjuvants are frequently the primary suspects for inducing severe adverse effects following vaccine administration. In veterinary medicine, oil-based adjuvants are considered highly reactogenic and rapidly trigger strong local inflammation, which while enhancing the vaccine's immunogenicity, can lead to granuloma formation, and necro-ulcerative lesions at the injection site (Heegaard et al., 2011; Jones, 1996; Stewart-Tull, 1996). In humans during the 2009-2010 pandemic vaccine campaign, several studies reported that an AS03-adjuvanted vaccine increased the risk of sleeping disorders (narcolepsy) in immunized children (Ahmed and Steinman, 2016; Miller et al., 2013; Nohynek et al., 2012). Additionally, an intranasal heat-labile *Escherichia coli* toxin-adjuvanted influenza vaccine (NasalFlu) was withdrawn from the market after it was reported to cause Bell's palsy in a number of vaccinated children (Lewis et al., 2009; Mutsch et al., 2004).

1.6. Vaccine-associated enhanced respiratory disease (VAERD)

Antigenic shift and drift have resulted in constant viral evolution and circulation of genetically and antigenically distinct influenza viruses in swine and human populations (Shao et al., 2017). Due to this antigenic variation, influenza vaccination has not only been associated with often limited or no viral protection, but also with inducing vaccine-induced immune responses that can contribute to a severe disease outcome (Rajao et al., 2014). More specifically, Janjua *et al*. reported that during the 2009 pandemic prior immunization of humans with seasonal trivalent inactivated influenza vaccines was associated with an increased risk of pdmH1N1-related illness (Janjua et

al., 2010). Several epidemiologic investigations corroborated the association between receipt of 2008–09 TIV with a higher risk of medically-attended illness after infection with the heterologous pandemic 2009 H1N1 strain (Rosella et al., 2011; Skowronski et al., 2010). Additionally, during the early 2000s vaccination-challenge studies in pigs demonstrated that immunization with an adjuvanted whole-inactivated virus vaccine and subsequent challenge with a heterologous homosubtypic swine influenza A virus resulted in more severe respiratory disease compared to mock-vaccinated challenged animals (MV/C) (Gauger et al., 2011; Vincent et al., 2008a).

This phenomenon, described both in humans and swine, has been termed as vaccine-associated enhanced respiratory disease (VAERD). It has been reproduced in experimental studies in swine when there is an antigenic mismatch between the homosubtypic hemagglutinin protein of the WIV vaccine and challenge strains (Gauger et al., 2012; Gauger et al., 2011; Vincent et al., 2008a). A recent study demonstrated that ferrets are also susceptible to VAERD in the event of antigenically mismatched vaccine and challenge viruses (Kimble et al., 2022). Interestingly, VAERD-exhibiting animals often not only show virus replication titers comparable to non-vaccinated animals but also exhibit enhanced lung inflammation and exacerbated histopathological findings (Rajão et al., 2014). While there are various differences in the clinicopathological profile of VAERD-exhibiting animals between studies, the clinical manifestation of VAERD is often characterized by a prolonged febrile illness accompanied by increased coughing and dyspnea compared to MV/C animals. Macroscopic pneumonia is characterized by purple-red colored multifocal lung consolidation while histopathological lesions include necrosuppurative to proliferative bronchitis and bronchiolitis with prominent peribronchiolar lymphocytic cuffing and moderate lymphohistiocytic interstitial pneumonia (Gauger et al., 2012). Regarding the pathogenesis of this phenomenon, various immune and vaccine factors have been demonstrated to be critical for the

induction of lung immunopathology in the context of vaccine-associated enhanced respiratory disease following influenza A virus infection.

1.6.1. Humoral responses

The humoral responses elicited by influenza virus infection or vaccination are characterized either as neutralizing or non-neutralizing. While neutralizing antibodies that target the globular head domain prevent influenza virus infection by blocking the binding of hemagglutinin to the host sialic acid receptor and ultimately the viral entry, non-neutralizing antibodies targeting subdominant viral epitopes on the head and stalk domain of HA and other viral proteins are involved in a permissive type of immune responses, halting viral spread and reducing the severity of clinical disease (DiLillo et al., 2014; Halbherr et al., 2015; Wu and Wilson, 2020). This latter type of humoral response does not provide sterilizing immunity but instead confers various Fc-gamma receptor (FcγR)-mediated protective immune mechanisms like phagocytosis of opsonized pathogens, complement activation, or inducing antibody-dependent cellular cytotoxicity (Padilla-Quirarte et al., 2019).

However, non-neutralizing humoral responses do not always play a protective role and occasionally have been associated with cases of enhanced viral infectivity and severe disease (Kulkarni, 2020). A typical example of their involvement in pathogenic mechanisms is antibody-dependent enhancement (ADE), a phenomenon observed in a variety of viral infections, most notably in flaviviruses, when preexisting antibodies elicited after a primary dengue virus infection bind to the new virus serotype but are unable to neutralize the viral infection. Instead, immune complexes has the ability to attach to FcγR on circulating monocytes and efficiently infect them,

resulting in severe clinical disease in susceptible individuals, termed Dengue Hemorrhagic Fever (DHF) (Halstead and O'rourke, 1977; Katzelnick et al., 2017; Rothman, 2010).

Vaccination-induced non-neutralizing antibodies have also been implicated in the pathogenesis of severe respiratory viral infections (Smatti et al., 2018). The use of an aluminum-adjuvanted WIV formalin-inactivated whole virus vaccine against respiratory syncytial virus (RSV) in naive infants in 1969 resulted in a vaccine-enhanced RSV pulmonary disease (VERPD) upon subsequent natural RSV infection. Specifically, VERPD significantly increased the risk of a medically-attended illness in WIV-immunized infants compared to non-immunized infants following RSV infection (80% in RSV vaccine recipients versus 5% in controls) and caused the death of two vaccinated children (Acosta et al., 2016; Kim et al., 1969). Subsequent studies demonstrated that the enhanced severity of the VERPD phenomenon was associated with increased levels of non-neutralizing antibodies in WIV-immunized individuals and excessive T-helper 2 (Th2)-mediated pulmonary pathology (eosinophilic lung inflammation) (Ananaba and Anderson, 1991; Boelen et al., 2000; Murphy et al., 1986).

An antibody-dependent enhancement of influenza infection was originally described in the late 1980s when two studies demonstrated that preexisting cross-reactive antibodies could contribute to the formation of immune complexes with antigenically distinct viral strains and could facilitate binding to and virion entry into FcγR-positive macrophages (Ochiai et al., 1988, 1990). However, VAERD following influenza infection was first observed almost a decade later in a study that utilized a DNA vaccine construct that expressed a fusion protein comprised of the influenza M2e and NP (M2eNP). Specifically, M2eNP immunization of pigs and subsequent challenge with heterologous H1N1 viral strain not only failed to provide sufficient protection but instead resulted in severe pneumonia compared to MV/C animals and the death of three pigs in the M2eNP

vaccinated group. Regarding the pathogenesis of this phenomenon, it was suggested by Heinen *et al*. that M2eNP-induced non-neutralizing antibodies that enhanced FcγR-mediated cytotoxicity (ADCC and CDC) of infected cells by targeting M2e glycoproteins expressed on their cell-surface (Heinen et al., 2002).

Several subsequent vaccination-challenge animal studies demonstrated the impact of prior WIV immunization on exacerbated lung pathology and clinical outcome after infection with a homosubtypic antigenically distinct viral strain that shows limited serological HAI cross-reactivity with the vaccine strain (Gauger et al., 2012; Gauger et al., 2011; Kobinger et al., 2010; Skowronski et al., 2014; Vincent et al., 2008a). Multiple studies both in humans and animals demonstrated that serum non-neutralizing IgG antibodies are involved in the pathogenesis of VAERD and are associated with broader viral distribution and severe microscopic pneumonia (Braucher et al., 2012; Khurana et al., 2013; Rajão et al., 2014; To et al., 2012). Specifically, work by Khurana *et al*. demonstrated that VAERD-exhibiting animals showed high levels of non-neutralizing antibodies that targeted the HA2 stem domain of the 2009 pdmH1N1 challenge strain, following mismatched vaccination with a heterologous δ1-clade H1N2 virus. These cross-reactive antibodies were found to bind to a stem domain epitope that is in close proximity to the fusion peptide and is surface exposed in the pre-fusion conformation of the HA. By performing an *in vitro* infectivity assay, they demonstrated that HA2-targeting antibodies enhanced the fusion activity of the challenge virus up to 200% compared to pre-vaccination sera, ultimately resulting in increased viral infectivity due to more efficient membrane fusion. Additionally, they showed that H1N2-induced anti-HA2 non-neutralizing antibodies not only enhanced pdmH1N1 infection of Madin-Darby canine kidney (MDCK) cells but also had the ability to suppress the neutralizing activity of pdmHA1-targeting antibodies when added to the cell-culture system (Khurana et al., 2013).

Subsequently, Ramakrishnan and colleagues demonstrated, using structural and viral kinetics modeling, that non-neutralizing stem antibodies can bind to and induce a conformational change in HA that promotes the fusogenic activity of HA2 and ultimately increases the viral envelope-host endosomal membrane fusion efficiency, thus leading to enhanced influenza infection and lung pathology (Ramakrishnan et al., 2016).

1.6.2. Cytokine responses

While a limited number of studies have examined the effect of cytokine responses in the pathogenesis of VAERD and their exact role has not been clarified yet, it is clear that dysregulated and enhanced pro-inflammatory immune responses play an important part in VAERD-induced pulmonary pathology (Rajao et al., 2014). While VAERD-exhibiting animals often demonstrate comparable viral titers to non-vaccinated challenged pigs, they show elevated pro-inflammatory cytokines in their bronchoalveolar lavage fluid (BALF) at various time points throughout the infection (Braucher et al., 2012; Gauger et al., 2012; Gauger et al., 2011; Rajao et al., 2016a; Souza et al., 2016). Specifically, increased concentrations of IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12, IFN-γ, and TNF-α proteins were observed even from day 1 pi in VAERD-exhibiting pigs, while at day 5 pi IL-6 was additionally found to be elevated compared to MV/C animals. Paradoxically, the antiviral type I IFN response, and specifically the IFN-α protein levels were found to be reduced in VAERD-exhibiting animals, despite demonstrating high virus load in the respiratory tract (Gauger et al., 2012).

Excessive secretion of functionally redundant proinflammatory molecules can result in extensive activation of effector cells, increased migration to the lungs, and pulmonary immunopathology (La

Gruta et al., 2007; Newton et al., 2016). Specifically, severe clinical disease can be triggered by elevated concentrations of proinflammatory molecules in the lungs, such as TNF-α, IL-1β, and IL-6, which can induce hyperinflammatory responses, increased vascular permeability, and extensive recruitment of inflammatory cells, particularly neutrophils, at the site of infection. These activities contribute to the development of lung tissue pathology by causing vascular injury, mononuclear and suppurative infiltration, and destruction of the parenchymal cells (Liu et al., 2016; Sladkova and Kostolansky, 2006; Van Reeth, 2000). Hence, the exorbitant cytokine responses observed in VAERD-exhibiting animals are suggested to induce pulmonary injury by triggering an enhanced mixed-leukocyte inflammatory response resulting in necrosuppurative bronchiolitis accompanied by prominent peribronchiolar lymphocytic cuffing, a consistent lesion demonstrated in VAERD (Gauger et al., 2014; Gauger et al., 2012; Khurana et al., 2013).

1.6.3. Cell-mediated immune responses

The role of cell-mediated immune responses (CMIR) in the development or protection from VAERD has not been clarified yet. Even though it is widely accepted that CMIR are important for the successful control of influenza infection by facilitating viral clearance and providing cross-protection against heterologous viral strains, cellular responses have also been involved in the induction of pulmonary immunopathology (Crowe et al., 2009; Heinen et al., 2001b; Moskophidis and Kioussis, 1998; Peiris et al., 2010). Exacerbated clinical disease due to extensive lymphoproliferative responses in immunized pigs post-challenge was observed in a study that utilized an M2eNP DNA vaccine construct. While it was inferred by the authors that exacerbated disease was a consequence of a dysregulated non-neutralizing humoral and NP-specific T-cell

lymphoproliferative immune response, the specific mechanisms of disease enhancement were not further investigated (Heinen et al., 2002). A later study that utilized a virus replicon particle vaccine expressing the IAV nucleoprotein (VRP-NP) also resulted in enhanced disease in pigs after a heterologous challenge. VRP-NP immunization increased viral shedding and lung inflammation and induced NP-specific non-protective $CD4^+$ and $CD8^+$ T-cell responses, intensifying the activation of IFN-γ and TNF-α dual-secreting cells. These enhanced cellular responses may have played an important role in the induction of lung tissue injury by promoting cytolytic processes (Ricklin et al., 2017). Additionally, the cytokine profile of VAERD-exhibiting animals from several vaccination-challenge studies suggests enhanced priming and dysregulated activation of both Th1 and Th2 pathways by WIV, resulting in lymphocytic inflammatory responses that can potentially contribute to the VAERD-associated pulmonary immunopathology following heterologous challenge (Gauger, 2012; Souza et al., 2016).

1.6.4. Vaccination parameters

Several studies have attempted to elucidate the impact of various vaccination factors in the pathogenesis of VAERD. Multiple influenza vaccine platforms besides WIV vaccines have been tested for their ability to experimentally reproduce VAERD in swine in the case of antigenically mismatched vaccine and challenge viruses, including an NS1-truncated LAIV vaccine (Gauger et al., 2014; Vincent et al., 2012) and an Ad5v vaccine expressing influenza HA (Braucher et al., 2012). While intranasal immunization of pigs with either of these platforms elicited limited serological HAI cross-reactive antibodies against the challenge strains, unlike WIV vaccines they

were able to provide partial protection against the heterologous influenza virus, stimulate cross-reactive mucosal and IFN-γ-secreting T-cell immune responses and avoid VAERD development.

However, work by Rajao and colleagues demonstrated that a recombinant HA subunit vaccine (HA-SV) incited severe respiratory disease and pulmonary tissue injury consistent with VAERD following heterologous challenge (Rajão et al., 2014). The common denominator in the induction of VAERD between the HA-SV and WIV vaccines tested in additional VAERD studies besides IM administration was the use of an OW type of adjuvant. More specifically, work by Souza *et al.* demonstrated that out of four commercially used adjuvant formulations, including two OW emulsions, a squalene-based nano-emulsion, and a gel polymer, only OW adjuvanted WIV vaccines could elicit VAERD (Souza et al., 2018). While the specific role of OW adjuvants in VAERD pathogenesis is unclear, two potential mechanisms suggested in the literature may be worthy of note. OW formulations are potent adjuvants that can broaden the range of vaccine-induced humoral responses, thus enhancing the levels of non-neutralizing antibodies that recognize and bind to epitopes located on the HA2 stalk domain. Additionally, OW adjuvants could be involved in the dysregulated activation of T cells resulting in lymphoproliferative immune responses and pulmonary immunopathology (Dormitzer et al., 2011; Souza et al., 2018). It could be argued that the fact that the majority of commercial human influenza vaccines are unadjuvanted formulations, could explain why VAERD following influenza infection may not be a quite relevant phenomenon in human medicine.

While anti-HA responses are primarily responsible for the induction of VAERD, immune responses against NA (or the absence thereof) play an important role in this phenomenon. VAERD has been consistently reproduced by utilizing vaccine and challenge strains comprised of heterosubtypic neuraminidase proteins. Matched NA between the vaccine and challenge strains

not only abrogates VAERD but additionally confers protection against heterologous infection (Rajao et al., 2016a). Regarding the vaccination regimen, Souza *et al.* demonstrated that the age of pigs and the duration of the interval between boost vaccination and challenge did not substantially impact the development of enhanced disease (Souza et al., 2016). Furthermore, VAERD was also reproduced in passively immunized piglets from WIV-vaccinated sows that were carrying maternally-derived IgG antibodies that showed limited serologic reactivity to the antigenically divergent challenge strain. While MDA conferred significant protection and limited the viral replication in the homologous challenged piglets, heterologous infection was characterized by prolonged clinical symptoms, enhanced respiratory pathology, and mortality (Kitikoon et al., 2006; Rajao et al., 2016b).

1.7. Swine as an animal model for IAV research

Animal models for influenza research are fundamental experimental tools to overcome the many limitations imposed by *in vitro* studies. Several animal models are currently being used in influenza research to study virus pathogenesis and transmission and to investigate the safety, immunogenicity, and protective efficacy of vaccines and antiviral drugs. Mice, due to their small size, limited husbandry requirements, low cost, and decreased genetic variability are the most widely used experimental model for influenza research (Bouvier and Lowen, 2010). However, there are several practical drawbacks to the use of this model. Mice have a preponderance of SAα2,3Gal residues in their respiratory tract and they require prior adaptation of human isolates to cause clinical disease (Glaser et al., 2007; Thangavel and Bouvier, 2014). Since mice do not recapitulate the typical human clinical signs of influenza infection such as fever and coughing,

weight loss and survival rate are the most common parameters utilized to evaluate virus pathogenicity and vaccine efficacy in the murine model. Additionally, mice cannot be used for transmission experiments since sneezing and nasal shedding are not noted in this species (Bouvier, 2015; Bouvier and Lowen, 2010). Another benchmark animal model commonly used in influenza studies is ferrets. Contrary to mice, ferrets are susceptible to human IAVs without prior adaptation, demonstrate remarkably similar clinical disease to humans, and are widely used for transmission and vaccine/antiviral compound efficacy studies. However, their cost, husbandry requirements, and the lack of species-specific immunological reagents pose great limitations in the use of this model for experimental IAV studies (Bouvier and Lowen, 2010; Margine and Krammer, 2014; Nguyen et al., 2021).

Pigs and humans are natural hosts of influenza and they play an integral role in the ecology and epidemiology of IAVs. Pigs are susceptible to both human and avian influenza viruses as they express both SAα2,3Gal (avian receptors) and SAa2,6Gal (mammalian receptors) in their airways (Ito et al., 1998; Trebbien et al., 2011). Additionally, swine have been previously proposed to serve as a "mixing vessel" an intermediary mammalian host required for the adaptation of avian viruses to human populations, due to their propensity to generate and transmit novel reassortant viruses with pandemic potential to humans (Ma et al., 2009; Roubidoux and Schultz-Cherry, 2021). However, the evidence that (i) humans and swine show a similar distribution of both types of SA residues in their respiratory tract, (Nelli et al., 2010), (ii) avian viruses can directly infect humans without prior adaptation (Lai et al., 2016; Li et al., 2019; Zhong et al., 2022) and (iii) human viruses spill over to swine populations far more frequently than the reverse (Nelson et al., 2014), makes the "mixing vessel" hypothesis less likely to justify the host-jump and establishment of avian viruses into humans (Nelson and Worobey, 2018).

The remarkable similarities of the swine respiratory tract anatomy and histology with that of humans, along with the inherent ability of pigs to sustain infection by human, avian, and swine-origin viruses due to their SA receptor distribution, make pigs a unique model for IAV research (Rajao et al., 2019; Rajao and Vincent, 2015). Along with mice and non-human primates, the immune system of swine is one the most studied and characterized, thus offering a great variety of established methodologies and availability of commercial species-specific immunological reagents (Meurens et al., 2012). Additionally, work by Dawson *et al*. demonstrated that over 80% of assessed human immune markers closely resemble functional and structural properties of corresponding swine markers compared to 10% between human and murine parameters (Dawson, 2011). While not without limitations, pigs are an excellent model for the assessment of vaccine efficacy and immunogenicity since clinical manifestation and immunological responses to influenza infection and vaccination are similar to humans (Margine and Krammer, 2014; Rajao and Vincent, 2015).

1.8. Concluding remarks

Influenza A virus is one of the most important respiratory pathogens in numerous mammalian hosts including humans and swine. IAVs artfully utilize two distinct evolutionary strategies, antigenic drift and genetic reassortment, to evade host immunity and break species barriers, resulting in the continuous circulation of antigenically divergent strains in human and animal populations. Pigs are integral hosts in the ecology of IAVs and are currently utilized as an essential experimental tool for influenza research. Besides being an excellent animal model, pigs are important agricultural species of economic significance, with pork being the second most

consumed meat worldwide after poultry meat (OECD/FAO, 2022). Hence influenza infections in swine not only enhance the risk of generating zoonotic viruses with pandemic potential but pose an ongoing costly challenge for the pork industry. Influenza vaccination remains the greatest tool available to sufficiently control virus infections. While a wide variety of vaccine platforms are currently being explored for the development of "broadly reactive" or "universal" vaccine technologies, only a limited number of vaccine technologies are commercially available in humans and veterinary medicine. To date, multivalent whole-inactivated virus vaccines are the most commonly used platform both in human and swine throughout the world (Nuwarda et al., 2021). WIV vaccines, while proven to be optimally or partially protective against homologous challenge, show suboptimal or no protection against antigenically distinct IAVs (Vincent et al., 2010a). Interestingly, a vaccination challenge study by the van Reeth group that deployed a prime-boost immunization with antigenically mismatched WIV H3N2 vaccines, demonstrated that heterologous boosting has the potential to be highly effective in enhancing the immunogenicity of whole-inactivated virus vaccines and vaccine-induced protection against homosubtypic antigenically divergent H3N2 influenza strains (Van Reeth et al., 2017).

Constant viral evolution and antigenic diversity especially among North American H1 swIAVs along with the inability of WIV vaccines to induce protective neutralizing responses against heterologous viruses play a crucial role in the induction of VAERD following heterologous influenza infection. This phenomenon constitutes a severe vaccine-derived adverse effect and is considered to be one of the prime concerns for the development of influenza vaccine platforms that target conserved subdominant epitopes on the hemagglutinin and have a propensity to elicit primarily non-neutralizing serological responses.

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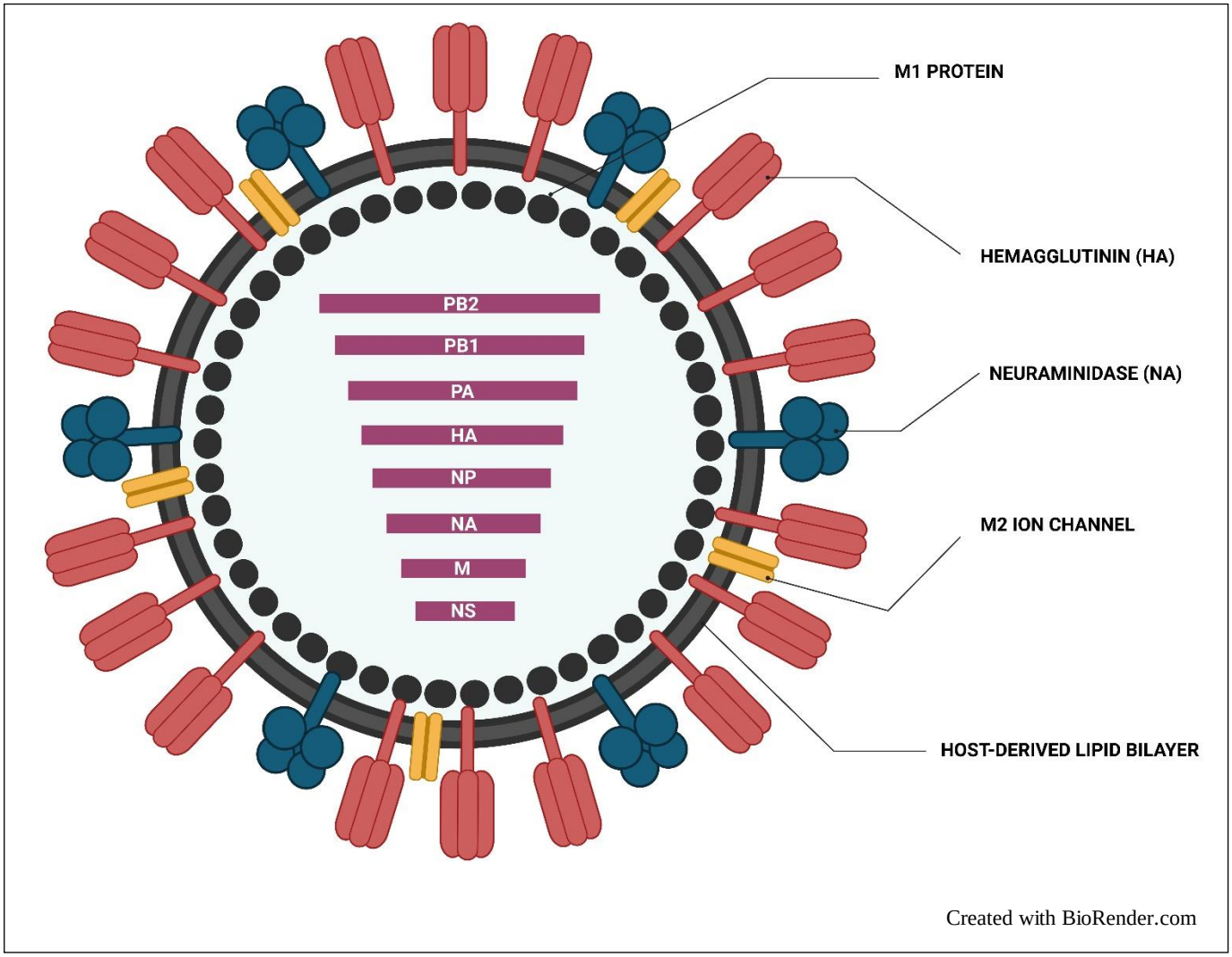
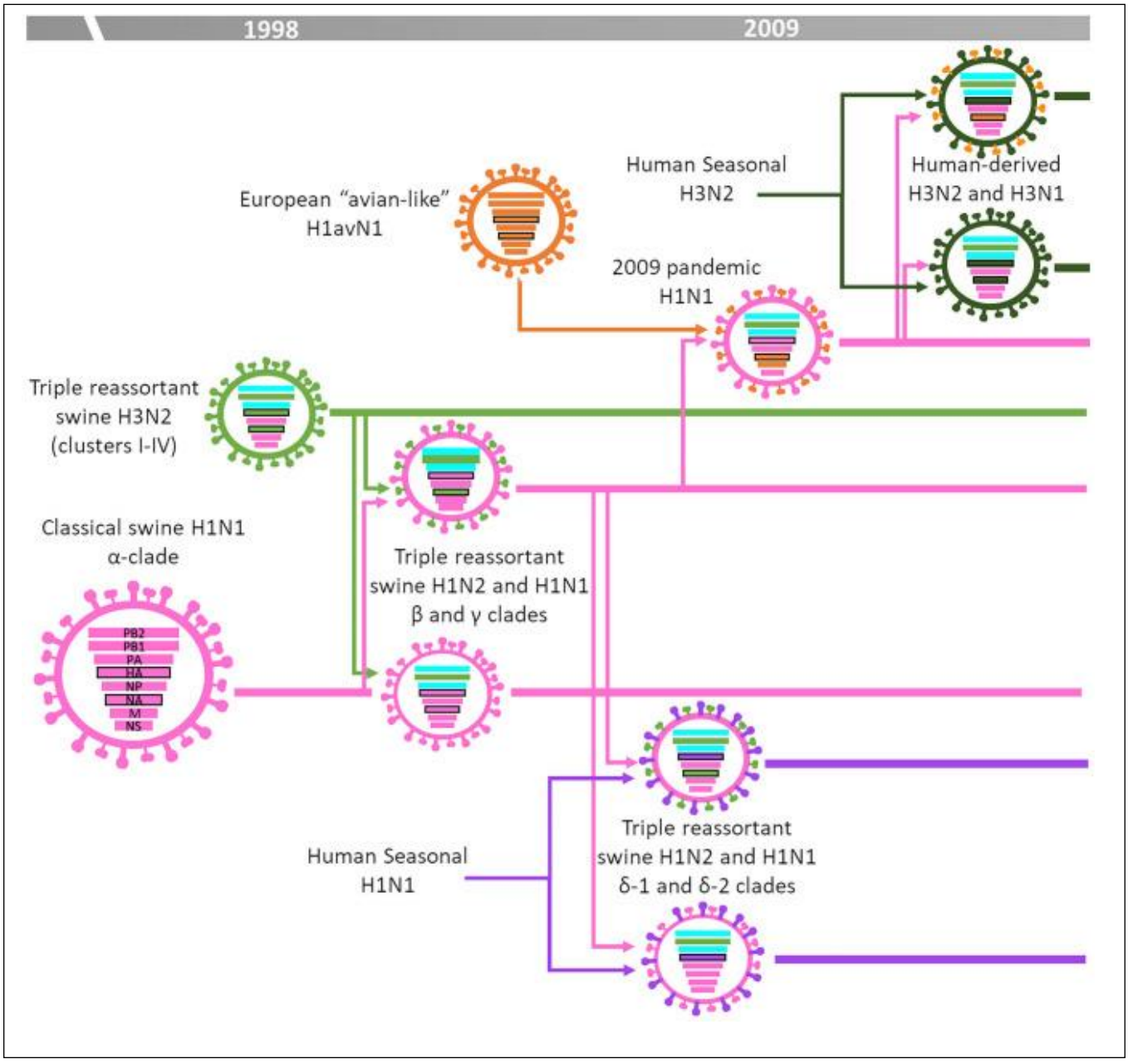
1.10. Figures

Figure 1.1. Structure of influenza A virus



PB2 Polymerase Basic Protein 2 gene segment, ***PB1*** Polymerase Basic Protein 1 gene segment, ***PA*** Polymerase Acidic Protein gene segment, ***HA*** Hemagglutinin gene segment, ***NP*** Nucleoprotein gene segment, ***NA*** Neuraminidase gene segment, ***M*** Matrix gene segment, ***NS*** Non-structural gene segment

Figure 1.2. Epidemiology of North American H1 swine influenza viruses



Adapted from (Mancera Gracia et al., 2020)

2. Chapter II: A novel neuraminidase virus-like particle vaccine offers protection against heterologous H3N2 influenza virus infection in the porcine model

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2.1. Abstract

Influenza A viruses (IAVs) pose a global health threat, 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 protection against disease. However, NA is generally ignored in the formulation and development of influenza vaccines. In this study, we evaluated the immunogenicity and efficacy against challenge of a novel NA virus-like particles (VLPs) vaccine in the porcine model. We developed an NA2 VLP vaccine containing the NA protein from A/Perth/16/2009 (H3N2) and the matrix 1 (M1) protein from A/Michigan/73/2015, formulated with a water-in-oil-in-water adjuvant. Responses to NA2 VLP were compared to commercial quadrivalent whole-inactivated virus (QWIV) swine IAV vaccine. Animals were prime-boost vaccinated 21 days apart and challenged four weeks later with an H3N2 swine IAV field isolate, A/swine/NorthCarolina/KH1552516/2016. Pigs vaccinated with the commercial QWIV vaccine demonstrated high hemagglutination inhibition (HAI) titers but very weak anti-NA antibody titers and subsequently undetectable NA inhibition (NAI) titers. Conversely, NA2 VLP vaccinated pigs demonstrated undetectable HAI titers but high anti-NA antibody titers and NAI titers. Post-challenge, NA2 VLPs, and the commercial QWIV vaccine showed similar reductions in virus replication, pulmonary neutrophilic infiltration, and lung inflammation compared to unvaccinated controls. These data suggest that anti-NA immunity following NA2 VLP vaccination offers comparable protection to QWIV swine influenza vaccines inducing primarily anti-HA responses.

2.2. Introduction

Influenza A viruses (IAVs) 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). Currently available seasonal 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 suboptimal efficacy, especially against heterologous IAV viruses and newly emerged novel IAVs (Flannery et al., 2019; Zimmerman et al., 2016). The immunodominance of HA observed after vaccination has led to selection of escape mutants that result in an 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).

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 (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 (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; 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. By contrast, anti-NA antibodies are characterized by inducing infection-permissive immunity (Couch et al., 1974; Johansson et al., 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 sialic acid cleavage (Krammer et al., 2018). 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). 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 (Rockman et al., 2013; Sandbulte 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. Additionally, VLP vaccine development is cost-effective and offers the opportunity for a customizable vaccine approach allowing for the selective inclusion of specific antigens (Roldao 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., 2007).

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 (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. 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/Philippines/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. 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., 2018). 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 and Vincent, 2015; Van 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 Falcon-Escobedo, 2009). While previous studies have demonstrated that NA inhibitory (NAI) antibody titers are associated with the induction of protective immunity in pigs, the full extent of anti-NA immunity, including humoral and cell-mediated immune responses, has not been properly investigated in the swine model (Holzer et al., 2019; Sandbulte et al., 2016; Van Reeth et al., 2003). Furthermore, the immunogenicity of NA VLPs has yet to be explored in the swine model.

In the current study, we evaluated a neuraminidase 2 virus-like particles (NA2 VLPs) construct as a candidate influenza vaccine in a vaccination-challenge study in the swine model. 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/NorthCarolina/KH1552516/2016. 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.

2.3. Materials & Methods

2.3.1. 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/NorthCarolina/KH1552516/2016, a Cluster IV-A H3N2 virus isolated from a recent outbreak of respiratory disease in a swine herd (Bakre et al., 2020). Its NA protein shared a 90.9% amino acid homology with the human NA of A/Perth/16/2009 strain, included in the NA VLP vaccine. The virus was propagated in MDCK cells, and the challenge titer was determined by 50% median tissue culture infectious dose ($TCID_{50}$) calculated using the Reed and Muench 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 dose of $10^{6.3}$ $TCID_{50}$) using a mucosal atomization device (MAD Nasal™ Atomization Device, Teleflex, Wayne, PA).

2.3.2. Animals

Eighteen 6-week-old influenza-naive conventional Yorkshire/Hampshire cross pigs were obtained from Auburn University's Swine Research and Education 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 in the BSL-2 facilities of the Sugg Laboratory for Animal Health Research. Pigs were randomly divided into 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.*

2.3.3. 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).

2.3.4. Vaccine preparation

NA2 VLP antigen was co-formulated with the Water-in-Oil-in-Water (W/O/W) emulsion (Montanide™ ISA 206 VG) which served as the adjuvant. Vaccine formulation was prepared under a low shear rate and stored according to the manufacturer's instructions, one day prior to each vaccination. For the commercial vaccine, we used FluSure XP® (Zoetis Inc., Kalamazoo, MI, USA) which is a licensed swine quadrivalent whole-inactivated virus (QWIV) influenza vaccine, that contains H1N1 gamma clade (virus) and H3N2 Cluster IV strains, A/swine/NorthCarolina/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 delta1 virus. The amino acid homology between the HA of A/swine/NorthCarolina/394/2012, included in the QWIV vaccine and the HA of the challenge virus was 97.78%. Animals were vaccinated with either the recombinant NA2 VLP vaccine, the FluSure XP® commercial vaccine, or a Phosphate-Buffered Saline (PBS) in an 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 NA2 VLPs vaccine (60 μg/mL), or 1 ml of phosphate-buffered saline with W/O/W adjuvant.

2.3.5. 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 mock-vaccinated 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 A/swine/NorthCarolina/KH1552516/2016 ($10^{6.3}$ $TCID_{50}$ 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 group. However, one pig from the fourth group was removed from the study due to a medical issue unrelated to the experiment. Animals were clinically scored daily post-infection based on rectal temperature, respiratory rate, and assessment of clinical demeanor. Specifically, rectal temperature score ranged from 0 to 3 (< 39.4°C = 0, 39.4-39.9°C = 1, 40-40.5°C = 2, > 40.6°C = 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 evaluation of viral shedding. All animals were humanely euthanized with a lethal dose of pentobarbital on day 5 post-infection. 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 in the airways.

2.3.6. Measurement of NA-specific IgG antibody titers

Sera from individual animals were evaluated for end-point dilution antibody titers as previously described with modifications (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 endpoint 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.

2.3.7. 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 (NAI) 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/NorthCarolina/KH1552516/2016 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.

2.3.8. 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 37°C 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 4°C, 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/NorthCarolina/KH1552516/2016 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 (Kitikoon et al., 2014). Sample analysis was performed in duplicates. The HAI titers were read as the reciprocal of the highest dilution of serum that conferred inhibition of hemagglutination.

2.3.9. Cytopathological evaluation of bronchoalveolar lavage fluid

After euthanasia, lungs were collected from the pigs (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 were 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 rcf 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 R et al., 2000).

2.3.10. 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 mock-vaccinated 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 a 2% antibiotic-antimycotic solution cocktail to reach 10% weight per volume (w/v) of tissue dissolved in PBS. Tissue suspensions were then homogenized and centrifuged at 4°C. 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 cytopathogenic

effect (CPE), and virus titers were determined by the Reed-Muench method (Reed and Muench, 1938).

2.3.11. Quantification of cytokine-secreting cells

Peripheral Blood Mononuclear Cells (PBMCs) were collected from the 15 animals that were challenged at: (a) 3 weeks post-prime vaccination (day 21) and (b) 2 weeks post-boost vaccination (day 35), as previously described (Greenbaum et al., 2009). Cryopreserved PBMC aliquots were thawed, washed with RMPI medium (Hyclone Laboratories Inc., Logan, UT), and resuspended in complete RPMI medium (10% FBS, 1% penicillin/streptomycin, Hepes buffer, and 0.1% beta-mercaptoethanol) for ELISPOT assays. The numbers of IAV-specific Interferon-γ (IFN-γ-STc) and TNF-α secreting T-cells (TNF-α-STc) were determined with swine IFN-γ and TNF-α ELISpot BASIC kit (Mabtech Inc., Cincinnati, OH), following the manufacturer's instructions with minor adjustments (Talker et al., 2015). Briefly, for IFN-γ ELISpot assay, $2x10^5$ cells/well from swine vaccinated with FluSure, NA2 VLPs or mock-vaccinated (adjuvant alone) were stimulated with either 10^6 $TCID_{50}$ of the challenge virus (A/swine/NorthCarolina/KH1552516/2016), 0.5 μg/ml PHA (ThermoFisher Scientific, Waltham, MA, USA) or with medium alone for 48 hours at 37°C. Regarding TNF-α ELISpot assay, 10^5 cells/well from swine vaccinated with QWIV, NA2 VLPs, or mock-vaccinated (adjuvant alone) were stimulated with either 10^4 $TCID_{50}$ of the challenge virus (A/swine/NorthCarolina/KH1552516/2016), 1 μg/ml PHA or with medium alone for 48 hours at 37°C. After incubation, the plates were washed with PBS and incubated with biotin-labeled detection antibody (0.5μg/ml) for 2 hours. Following the addition of streptavidin-horseradish

peroxidase for 1 hour, TMB substrate solution (Mabtech Inc., Cincinnati, OH) was then used for spot development until detection of distinct spots.

2.3.12. Quantification of cytokine secretion in lung homogenates

Cytokines were quantified in pulmonary lung homogenates from swine vaccinated with QWIV, NA2 VLPs or mock vaccinated (adjuvant alone) collected at day 5 post-infection, using the 13 analyte (GM-CSF, IFN-γ, IL-1α, IL-1ra, IL-1β, IL-2, IL4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α) MILLIPLEX MAP Porcine Cytokine/Chemokine Magnetic Bead Panel per manufacturer's instructions (EMD Millipore Corporation, Billerica, MA). Lung TNF-α was quantified using a Porcine TNF-a ELISA kit (R&D Systems Inc., Minneapolis, MN) according to manufacturer's instructions.

2.3.13. 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 histopathology scores (Morgan et al., 2016). Histopathology scores considered the percent of lungs affected, the extent of bronchial and bronchiolar epithelial changes, the severity of interstitial pneumonia, and the degree of lymphocytic peribronchiolar cuffing. The first three parameters were scored from 0 to 4 while the last from 0 to 3. All scores were 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 anatomic pathologist, and the scores were averaged.

2.3.14. 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 ($\alpha \leq 0.05$) for the comparison of anti-NA IgG ELISA titers, NAI titers, virus load and cytokine levels in respiratory tissue homogenates. The nonparametric Kruskal-Wallis test was used to test differences between groups for the microscopic lung lesion scoring. A two-way ANOVA with post-hoc Tukey's multiple comparison test was used for the comparison of clinical scores, cytokine-secreting cells (ELISPOT) and nasal swab virus titers and Dunnett's post-hoc multiple comparisons test for BALF cytology between groups with an alpha of 0.05 ($p<0.05$).

2.4. Results

2.4.1. NA2 VLPs demonstrated immunogenicity in the swine model

In previous work, we demonstrated that NA2 VLPs produced using a recombinant baculovirus (rBV) co-infection expression model in *Trichoplusia ni* insect (Tni) 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, we first investigated the immunogenicity of adjuvanted NA2 VLPs in a 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 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 prior to 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 2.1A). After prime vaccination on day 21, only pigs vaccinated with NA2 VLPs showed a measurable anti-NA IgG titer (Figure 2.1B). 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 2.1B). 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 QWIV-vaccinated pigs ($p<0.0001$) (Figure 2.1C). 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 2.1C). 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 2.1D).

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.

2.4.2. NA2 VLPs induced functional antibody responses directed at neuraminidase

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/NorthCarolina/KH1552516/2016).

We first evaluated the ability of sera collected from vaccinates to inhibit enzymatic activity of 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 2.1C and 2.1D), sera collected from QWIV-vaccinated animals did not show elevated neuraminidase inhibition (NAI)

titers above mock-vaccinated pigs at any timepoint (Figure 2.2A-2.2D). As expected, sera from mock vaccinated animals also did not show any appreciable NAI titer at any time point sampled (Figure 2.2A-2.2D). 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 2.2B). 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 2.2C). 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 2.2D).

Next, we investigated the ability of sera collected from vaccinates to inhibit the NA activity of the NA2 antigen of the H3N2 challenge virus A/swine/NorthCarolina/KH1552516/2016. Similar to results obtained for the inhibition of the NA2 antigen used in the VLP vaccine, A/Perth/16/2009 (Figure 2.2.), 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 2.3A – 2.3D). 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 2.3B). 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 2.3C – 2.3D).

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/NorthCarolina/KH1552516/2016.

2.4.3. Vaccination with QWIV vaccine induced HAI antibodies above protective levels after second vaccine dose

As vaccination with QWIV did not induce functional antibodies capable of 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 than 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 days 0, 21, 35, and 48 were analyzed by HAI assay to investigate the magnitude of functional anti-HA antibody levels against the heterologous A/swine/NorthCarolina/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 (Suppl. Figure 2.1.). QWIV-vaccinated animals demonstrated elevated mean HAI titers 2 weeks post-boost vaccination and four weeks post-boost vaccination (Suppl. Figure 2.1.). 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.

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 the QWIV vaccine to elicit robust anti-HA functional antibodies in naïve pigs.

2.4.4. NA2 VLP vaccination resulted in the generation of robust virus-specific cell-mediated responses

Cell-mediated immunity (CMI) plays an important role in the host immune response in conferring protection against virus-related illness and in the establishment of long-term immunological memory (Gianchecchi et al., 2019). The presence of pre-existing T-cell immunity in humans is critical for viral clearance from influenza-infected cells (Hayward et al., 2015; McMichael et al., 1983) and recovery from disease (Holzer et al., 2019; Wang et al., 2015). In the mouse model, T-cells correlate with reduced virus shedding even when antibodies are absent (Epstein and Price, 2010; Schulman and Kilbourne, 1965; Sridhar, 2016; Tchilian and Holzer, 2017). Studies analyzing in-depth T-cell immunity in response to immunization or infection against influenza virus in pigs are very limited (Khatri et al., 2010; Sinkora et al., 2014; Talker et al., 2016). There is evidence that some CMI responses occur in the lungs of pigs infected with swine influenza viruses (Heinen et al., 2001; Khatri et al., 2010; Lange et al., 2009) but these responses do not prevent infection (Grebe et al., 2008). Talker *et al* reported that at 44 days post-infection the IAV-specific response comprised primarily by IFN-γ single producing cells in the lung, TNF-α single

producers in the tracheobronchial lymph nodes (TBLN), and triple producers (IFN-γ, TNF-α, and IL-2) in the blood (Talker et al., 2015; Talker et al., 2016).

To determine whether pig immunization with NA2 VLPs leads to increased generation of NA2-specific activated T-cells, we enumerated vaccine-specific IFN-γ and TNF-α producing T-cells at 21 days post-prime and 14 days post-boost (day 35) and compared them to the QWIV vaccinated group following A/swine/NorthCarolina/KH1552516/2016 virus or PHA stimulation. A single vaccine dose did not increase the numbers of IFN-γ-secreting cells (Figure 2.4A, 2.4B). At two weeks post-boost though, we observed 2-fold differences between QWIV and NA2 VLP vaccinated pigs in virus-specific or PHA stimulated PBMCs, despite the lack of significant statistics that resulted in similar ratios of virus-specific *vs* polyclonally stimulated cells (V/P ratio) (Figure 2.4D). Swine virus and PHA stimulation increased at similar levels the numbers of TNF-α-secreting cells of NA2 VLP and QWIV vaccinated pigs, post-prime (Figure 2.4E, 2.4F), whereas booster vaccination led to a 4-fold increase of TNF-α secreting cells in the QWIV group with no changes in the NA2 VLP group following virus stimulation. At the same time point, we observed 10-fold and 2.5-fold increases in PHA-stimulated PBMCs from the QWIV and NA2 VLP groups respectively ($p<0.05$). These changes resulted in similar ratios of virus-specific *vs* polyclonally stimulated cells for both vaccinated groups whereas the mock-vaccinated pigs showed a 2-fold higher ratio suggesting similar effects of NA2 VLP and QWIV vaccine effect on cell-mediated immunity (Figure 2.4H).

2.4.5. Challenge with A/swine/NorthCarolina/KH1552516/2016 did not result in clinical disease in vaccinated animals

A scoring system was applied for the evaluation of clinical disease after challenge with A/Swine/NorthCarolina/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-infection, rectal temperature, respiratory rate, and disease signs, in the form of presence of coughing or weakness/depression, were recorded daily. Throughout the observation period, no statistically significant differences were observed between the three groups of pigs (Suppl. Figure 2.2.). Overall, intranasal challenge resulted in mild clinical disease manifestation. This was not unexpected, as an intratracheal inoculation with a high dose of IAV in the inoculum is required to replicate severe respiratory disease in the swine model (Kyriakis et al., 2010).

2.4.6. Vaccination with NA2 VLPs decreased pulmonary neutrophilic infiltration similarly to QWIV group

To assess pulmonary infiltration of inflammatory cells, which in swine correlates with the development or prevention of respiratory disease (Van Reeth and Nauwynck, 2000), bronchoalveolar lavage fluid (BALF) was collected from all animals at necropsy 5 days post-infection. In healthy uninfected pigs (n=2), the overwhelming majority of cells noted in BALF cytology were resident macrophages with the presence of sparse small-sized lymphocytes and non-degenerate neutrophilic granulocytes (Figure 2.5Ai, 2.5B). The unvaccinated control animals

exhibited statistically significant higher percentages of neutrophils and lower percentages of macrophages compared to the vaccinated groups (Figure 2.5Aii, 2.5B). 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 BALF cells were macrophages in vaccinated animals with the presence of low percentage of neutrophils (Figure 2.5Aiii, iv and 2.5B). The QWIV and NA2 VLP vaccinated animals showed significant reduction ($p<0.001$) in lung neutrophilic infiltration compared to mock-vaccinated pigs. No significant differences were identified in the percentage of lymphocytes between the groups. These data suggest that vaccination of pigs with NA2 VLP or QWIV vaccine reduces neutrophilic infiltration after IAV infection.

2.4.7. Vaccination with NA2 VLPs reduced viral replication in the lower respiratory tract of infected swine

To evaluate virus shedding post-challenge, nasal swabs were collected daily through day 5 and virus load 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-infection (Figure 2.6A). However, samples on day 3 post-infection demonstrated a significant reduction of greater than one log of virus load observed in nasal swabs collected from QWIV vaccinated pigs compared to mock-vaccinated pigs ($p<0.05$). Although the difference was 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.

Next, we investigated the ability of vaccination to inhibit viral replication throughout the respiratory tract. Tissue samples were collected at necropsy, and homogenized, and viral titers were 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 ($p<0.05$) but not compared to NA2 VLP vaccinated pigs (Figure 2.6B). Vaccination with NA2 VLPs did not significantly affect virus replication in nasal turbinates relative to mock vaccination. 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 2.6C). 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 (right and left lung) both vaccination with NA2 VLPs and the QWIV vaccine performed in a similar fashion resulting in a statistically significant reduction of virus replication ($p<0.0001$) (Figure 2.6D and 2.6E). In summary, vaccination of pigs with both NA2 VLPs and QWIV vaccine reduced pulmonary viral replication after challenge with the A/swine/NorthCarolina/KH1552516/2016 virus.

2.4.8. Low pulmonary histopathology scores were indistinguishable between NA2 VLP and QWIV vaccinated groups post-challenge

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 (Figure 2.7A iv-vi). The macroscopic lung lesions and overall score of QWIV vaccine group

(Figure 2.7A vii-ix) were indistinguishable from those in the NA2 VLP group (Figure 2.7A x-xii), and although the scores were lower compared to the unvaccinated control group, there was no statistical 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 compared to both the NA2 VLP and QWIV vaccine group ($p<0.05$) (Figure 2.7B-D). 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 groups. However, no statistically significant differences were observed in the microscopic nasal turbinate and trachea lesion scores, between vaccinated and unvaccinated control animals (Suppl. Figure 2.3.). These data demonstrate that vaccination of pigs with NA2 VLPs or QWIV reduced pulmonary pathology.

2.4.9. NA2 VLP vaccination reduces lung inflammation following exposure to influenza virus

Influenza infection induces the transcription of genes encoding cytokines such as IL-1β, IL-6, IL-8, IL-12, and TNF-α in endothelial cells and lung fibroblasts and IL-1β expression increases the inflammation of lung cells (Kim et al., 2015). Clinical studies in children infected by H1N1 virus demonstrated an early and significant upregulation of IL-1β and IL-6 plasma expressions indicating a proinflammatory role that contributes to airway inflammation and bronchial hyperactivity (Chiaretti et al., 2013). Interleukin-6 though has a pleiotropic nature leading to the upregulation of T-cells and IgG isotype switching, inflammatory resolution, tissue remodeling and

lung repair, migration and phagocytic activity of macrophages, prevention of viral-induced apoptosis in lung epithelial cells (Lauder et al., 2013; Yang et al., 2017). Van Reeth et al reported that swine influenza induced acute respiratory disease and lung damage by itself and that the outcome of the infection was tightly associated with the production of IFN-alpha, TNF-alpha, IL-1, and IL-6. In challenge studies of swine IAV-vaccinated pigs, levels of IFN-alpha, TNF-α, and IL-6, but not IL-1 were correlated with clinical and virological protection (Van Reeth et al., 2002). Lung lysates taken from NA2 VLP and QWIV-vaccinated swine 5 days after infection with A/swine/NorthCarolina/KH1552516/2016 and tested for 13 major Th1 and Th2 inflammatory cytokines, showed a statistically significant 3-fold increase of IL-1β ($p<0.01$) (Figure 2.8A), IL-6 ($p<0.05$) (Figure 2.8B) and IL-8 ($p<0.05$) (Figure 2.8C) in the infected mock vaccinated group as compared to challenged swine that had received NA2 VLP or QWIV vaccine. Interestingly, we did not see statistical differences in TNF-α levels between vaccinated swine following virus challenge despite an approximately 30% reduction in the NA2 VLP group (Figure 2.8D).

2.5. Discussion

While vaccine development has historically focused on anti-HA responses (Eichelberger and Monto, 2019), anti-NA responses have recently been identified as a stronger correlate of protection against disease caused by IAV in humans (Huber et al., 2010; Memoli et al., 2016; Monto et al., 2015). Although VLPs containing only the NA1 surface glycoprotein have been previously studied in small animal models, only one study to date has investigated VLPs containing only the NA2 surface glycoprotein in a small animal model. Additionally, only one study to date has investigated VLPs containing both HA and NA produced in insect cells in a swine model. 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 swine influenza vaccine.

Interestingly, while vaccination with QWIV resulted in robust functional anti-HA immunity, capable of inhibiting hemagglutination after boost vaccination (Suppl. Figure 2.1.), QWIV vaccination barely induced a measurable anti-NA2 response and no measurable functional anti-NA2 response. 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 (Johansson 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 and Kilbourne, 1993; Johansson et al., 1989). 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., 2019). 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. 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 2.6.). Reduced viral mobility and subsequent viral transmission have been proposed as 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.

Another factor that has to be taken into account regarding nasal shedding is that intramuscular vaccination predominantly induces serum antibodies and to a lesser degree mucosal antibodies (Van Reeth and Ma, 2012). While serum IgG antibodies can rapidly transudate through alveolar spaces and facilitate protection in pulmonary tissues, this process is generally impeded in the nasal mucosa due to the presence of a thicker mucosal layer (Brandtzaeg, 2009). The major isotype that mediates nasal immunity is secretory IgA (Renegar et al., 2004). In accordance with this, we presume that the intramuscular administered IAV vaccines examined in our report primarily conferred lung immunity, resulting in a significant reduction of pulmonary virus replication. While we did not examine in this study the extent of mucosal immunity elicited by our experimental NA VP or the commercial swine IAV vaccine, the non-significant reduction of nasal shedding could be correlated with a less than robust stimulation and activation of nasal secretory IgA. Future work is needed to determine the role of intranasal administration of our NA2 VLPs construct in the stimulation of mucosal immunity and reduction of virus shedding.

A main objective of broadly protective IAV vaccine strategies is the induction of CMI. Cell mediated-responses are not only associated with effective viral clearance after IAV infection but are also involved in protection against heterologous viruses, considering that they target conserved

epitopes present on viral proteins (Torremorell, 2016; Van Reeth et al., 2016). Previous studies have demonstrated that unlike naïve T-cells, primed memory CD4 T-cells secrete IFN-γ, as well as other antiviral cytokines (IL-6, IL-12, CXCL9, and CXCL10), rapidly after IAV infection (Lai et al., 2011; Zens and Farber, 2014). While the mechanism is not completely understood, these Th1-type immune responses producing IFN-γ, during the initial stages of infection, facilitate effective early viral clearance primarily through the activation and transmigration of CTL (cytotoxic T lymphocytes) and macrophages to the site of inflammation (Bot et al., 1998; Wilkinson et al., 2012). Consistent with this is the general notion that the presence of Th1 polarized IFN-γ secreting memory T cells is strongly correlated with positive clinical outcomes after IAV infection. In our study, both NA2 VLP and commercial swine IAV elicited adequate, although with no significant differences between these groups, levels of IFN-γ secreting T-cells after heterologous virus stimulation, in PBMCs collected at 2 weeks after boost vaccination, suggesting a competent Th1 response following infection with the heterologous A/sw/NorthCarolina/KH1552516/2016 challenge virus.

In addition to IFN-γ, we measured TNF-α-secreting cells in PBMCs collected post-prime and post-boost. TNF-α is a proinflammatory cytokine associated with systemic inflammation during the acute phase of IAV infection (Peiris et al., 2009; Tavares et al., 2017). TNF-α promotes increased vascular permeability and extensive recruitment of inflammatory cells to the site of inflammation (Biron, 1999; Neuzil and Graham, 1996; Peper and Van Campen, 1995). These activities can contribute to lung tissue pathology due to vascular injury and destruction of lung parenchymal cells (Wittekindt, 2017; Zhang et al., 2009). Previous studies have demonstrated that while TNF-α can induce hyper-inflammatory responses that result in severe clinical outcomes and lung tissue injury, this cytokine may have a limited impact in reducing virus replication and overall virus

clearance (Hussell et al., 2001; Peper and Van Campen, 1995; Perrone et al., 2010; Szretter et al., 2007). Interestingly, in our study we observed a two-fold higher ratio of virus-specific *vs* polyclonally stimulated TNF-α secreting cells, in PBMCs collected from naïve mock-vaccinated animals and QWIV vaccinated pigs, compared to PBMCs harvested from NA2 VLPs vaccinated animals post-prime vaccination. Additionally, we detected post-boost a two-fold decrease in the V/P ratio of TNF-α secreting T-cells, in both NA2 VLPs and QWIV vaccinated pigs compared to mock-vaccinated pigs. The ELISPOT results demonstrated that a single NA2 VLPs vaccination or two doses of QWIV vaccine were required to prime the pigs towards a sufficient, however less extensive, production of TNF-α by memory T-cells compared to naïve T-cells following virus stimulation.

While cell-mediated responses and particularly Th1 activation are required for effective virus clearance, a critical correlate of vaccine-induced protection is the elicitation of balanced pulmonary cytokine responses during IAV infection. Excessive production of proinflammatory cytokines, in the acute stages of viral infection, is associated with immunopathology and lung tissue injury (De Jong et al., 2006; Kobasa et al., 2007). In this report, the naïve mock vaccinated group demonstrated at necropsy elevated neutrophil levels in BALF, enhanced microscopic pneumonia, and proinflammatory lung cytokine profile, including IL-1β, IL-6, and IL-8 compared to both NA2 VLPs and QWIV group of animals. Previous studies have demonstrated that aberrant production of IL-1β and IL-6 cytokines are associated with exacerbation of inflammatory response and immunopathology (Hagau et al., 2010; Kaiser et al., 2001; Schmitz et al., 2005). In this respect, marked reduction of lung IL-1β and IL-6 levels may have contributed to the prevention of development of severe pulmonary lesions in the NA2 VLPs group. Interestingly the effect of IL-8 upregulation was impressively demonstrated in the neutrophilic recruitment in the lungs of mock-

vaccinated pigs after challenge (Bernhard et al., 2021). We detected a 4-fold increase of neutrophils post-infection compared to the NA2 VLP and QWIV groups and a concomitant 40% reduction of macrophages, resulting in equal representation of these populations in the BAL fluid of mock vaccinated animals (Figure 2.5B). In contrast, macrophages were the overwhelming majority in the QWIV (ratio 7:1) and NA2 VLP (ratio 5:1) vaccinated pigs similarly to the mock-vaccinated/uninfected control group (ratio 5:1). These findings suggest a strong anti-inflammatory role of vaccination by suppressing the neutrophil chemotactic effect of IL-8.

In summary, immunization with a novel NA2 VLP vaccine was successful in significantly reducing virus replication, mitigating clinical disease, and controlling inflammation measured via neutrophilic infiltration, and pulmonary histopathology scoring in the porcine model. These findings contribute to a better understanding of anti-NA immunity in preventing IAV infection and disease in swine, as an animal model for studying influenza in humans, but also in pork production, since swine influenza is an economically important pathogen in the pork industry (Díaz et al., 2020).

2.6. Acknowledgments

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2.8. Figures

Figure 2.1.

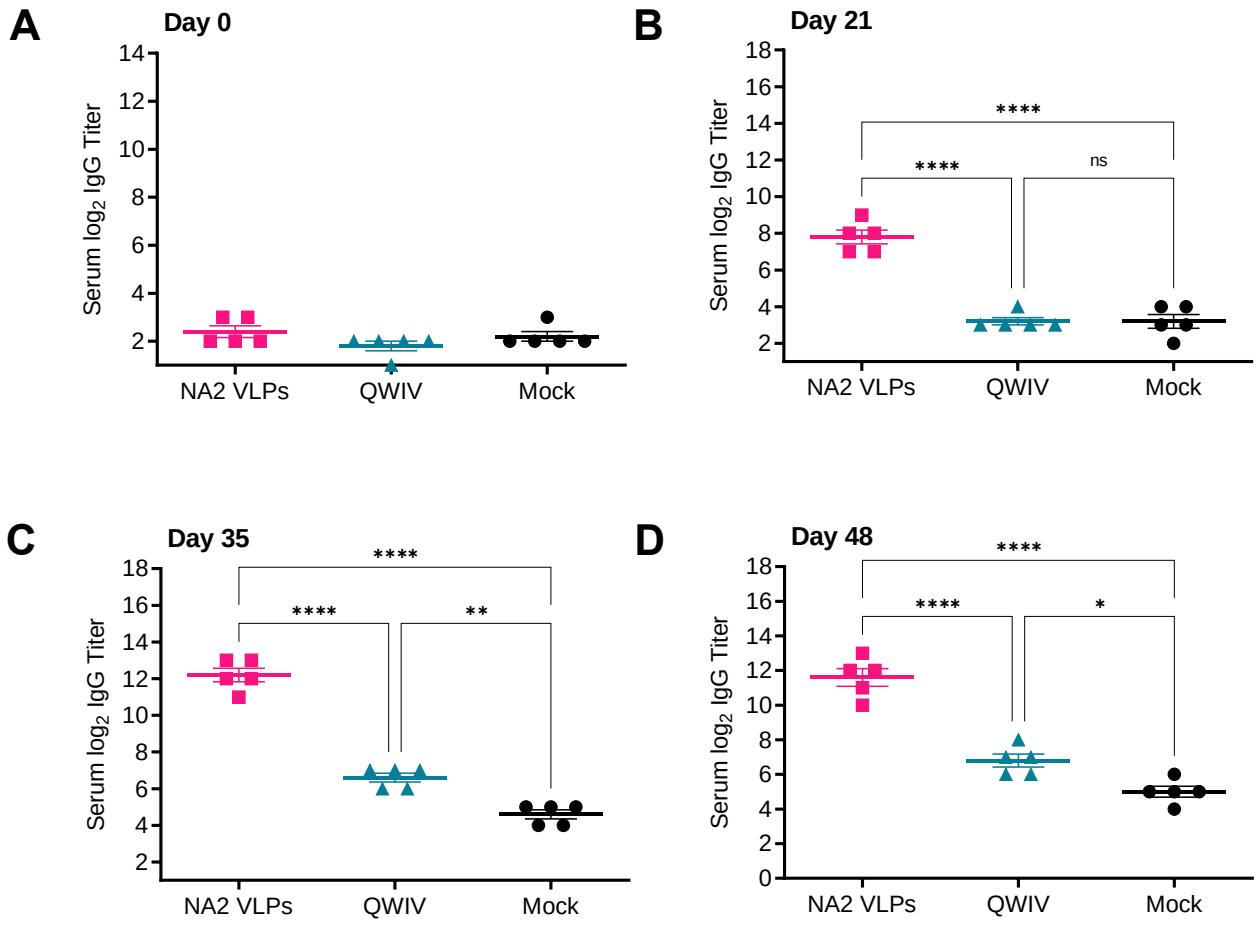


Figure 2.1. Vaccination with NA2 VLPs induces anti-NA serum IgG titers in swine. Three groups of pigs (n=5) were vaccinated in a prime-boost regimen 21 days apart at day 0 and day 21 with a commercial quadrivalent whole-inactivated virus vaccine (QWIV), NA2 VLPs, or mock vaccinated with water-in-oil-in-water adjuvant only (mock-vaccinated). 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/NorthCarolina/KH1552516/2016 on study day 48. Horizontal bars represent the mean IgG titer for each respective group. Statistical analysis was performed by one-way ANOVA test. * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$.

Figure 2.2.

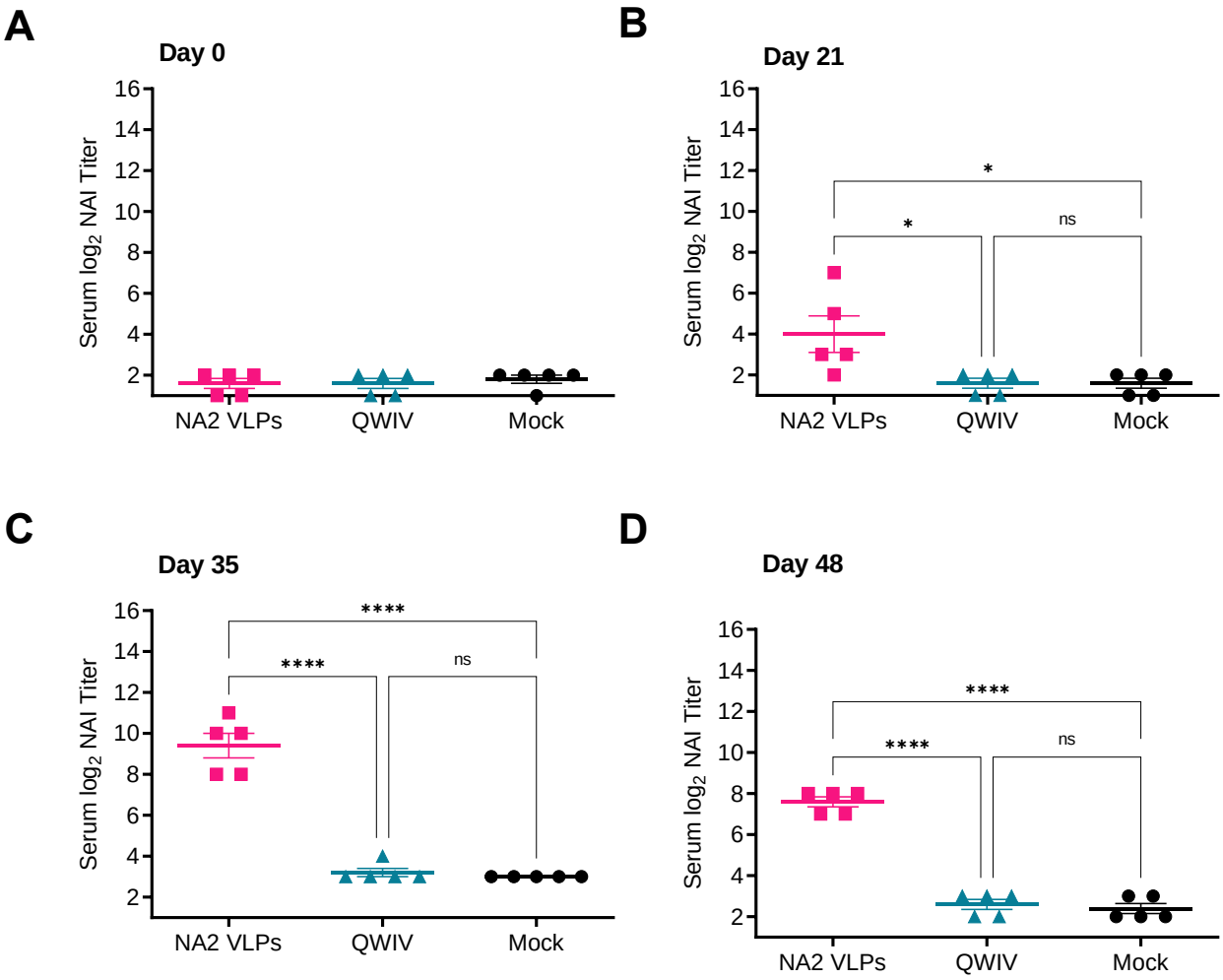


Figure 2.2. Vaccination with NA2 VLPs induces functional anti-NA antibodies capable of inhibiting A/Perth/16/2009 NA activity *in vitro*. Three groups of pigs (n=5) were vaccinated in a prime-boost regimen 21 days apart at day 0 and day 21 with a commercial QWIV, NA2 VLPs, or mock vaccinated with water-in-oil-in-water adjuvant only (mock-vaccinated). 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/NorthCarolina/KH1552516/2016 on study day 48. Horizontal bars represent the mean NAI titer for each respective group. Statistical analysis was performed by one-way ANOVA test. * = $p < 0.05$; **** = $p < 0.0001$.

Figure 2.3.

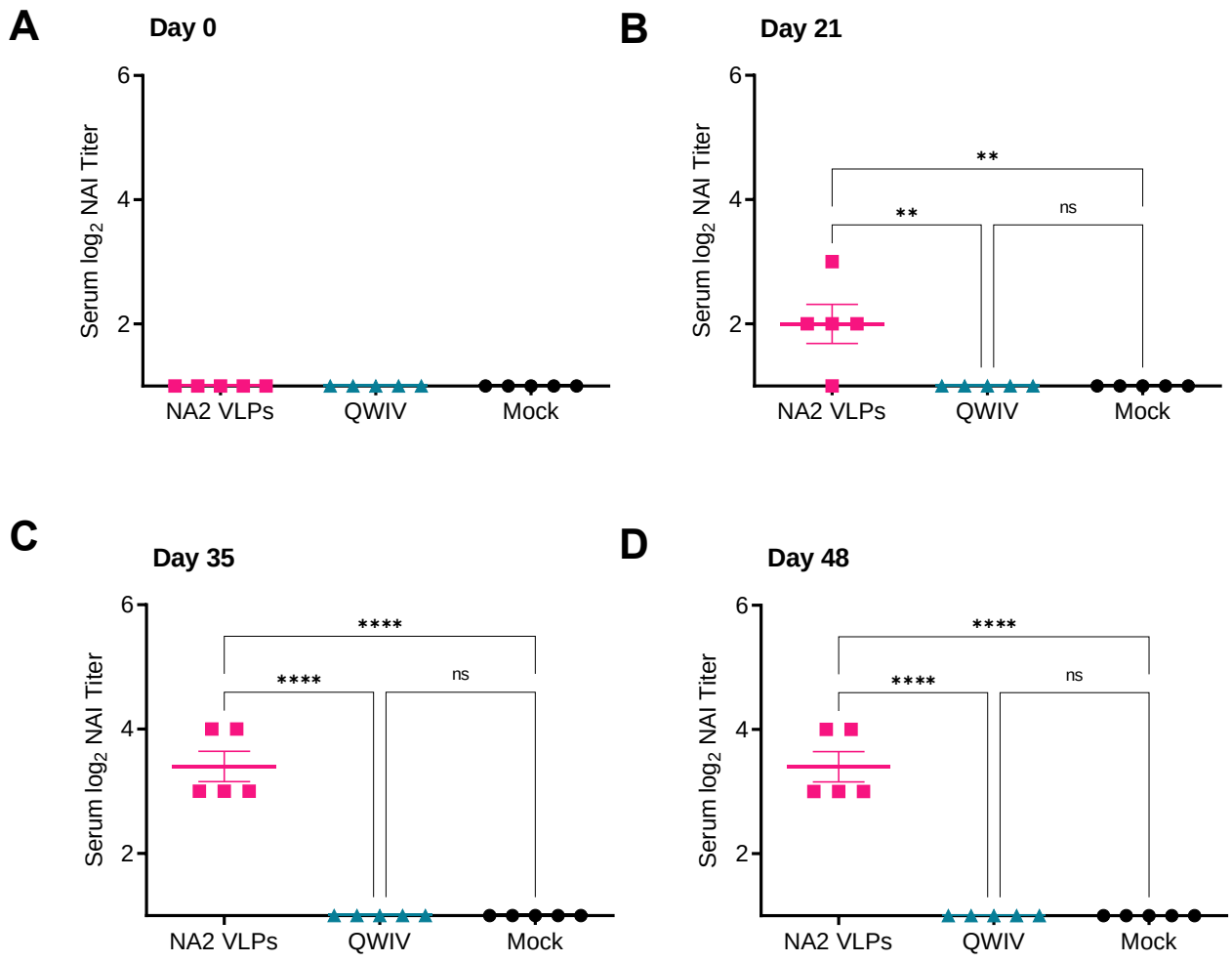
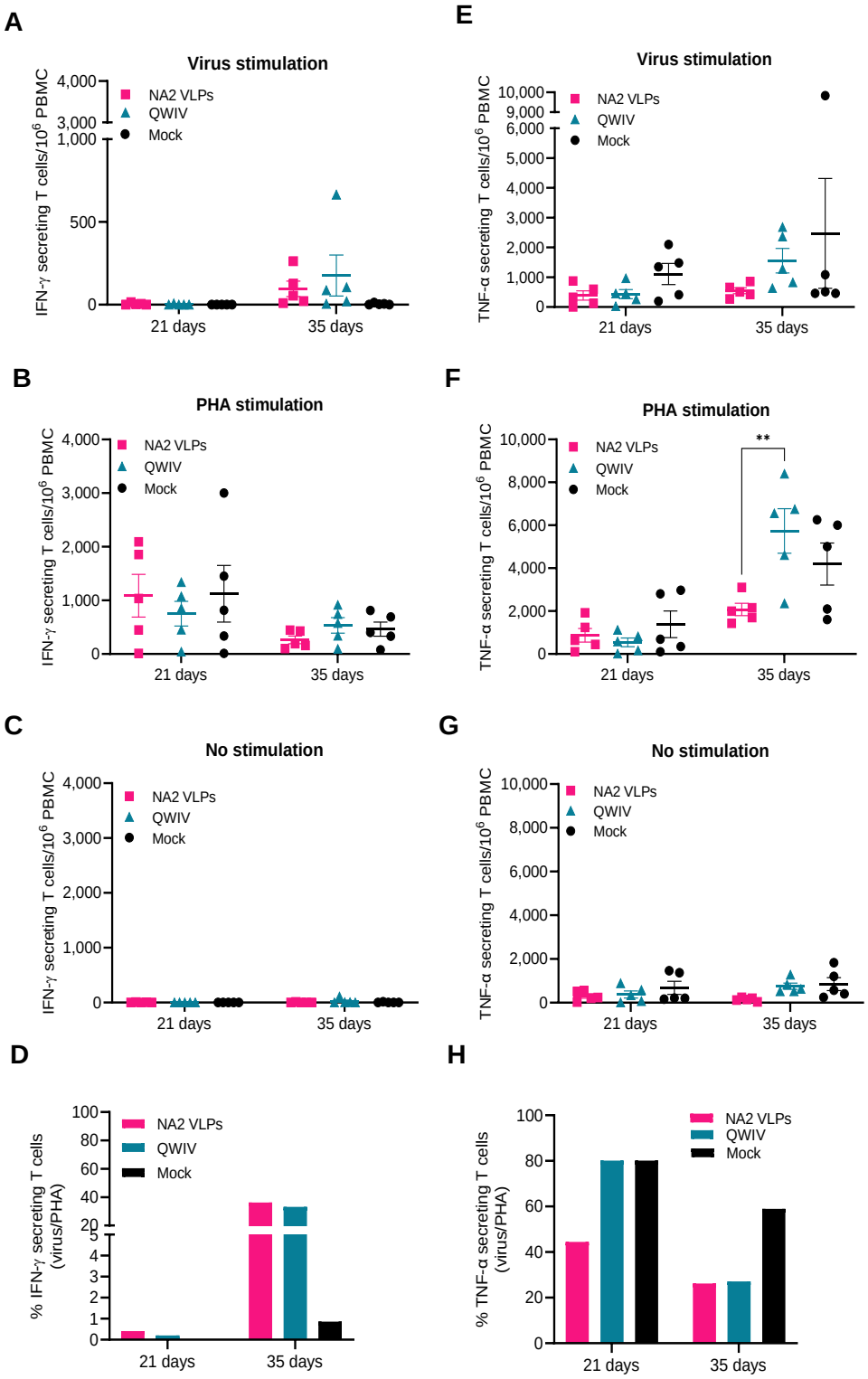


Figure 2.3. Vaccination with NA2 VLPs induces functional anti-NA antibodies capable of inhibiting A/swine/NorthCarolina/KH1552516/2016 NA activity *in vitro*. Three groups of pigs (n=5) were vaccinated in a prime-boost regimen 21 days apart at day 0 and day 21 with a commercial QWIV, NA2 VLPs, or mock vaccinated with water-in-oil-in-water adjuvant only (mock-vaccinated). 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/NorthCarolina/KH1552516/2016 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/NorthCarolina/KH1552516/2016 on study day 48. Horizontal bars represent the mean NAI titer for each respective group. Statistical analysis was performed by one-way ANOVA test. * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$.

Figure 2.4.


A
Virus stimulation
IFN-γ secreting T cells/10^6 PBMC
4,000
3,000
1,000
500
0
NA2 VLPs
QWIV
Mock
21 days
35 days
E
Virus stimulation
TNF-α secreting T cells/10^6 PBMC
10,000
9,000
6,000
5,000
4,000
3,000
2,000
1,000
0
NA2 VLPs
QWIV
Mock
21 days
35 days
B
PHA stimulation
IFN-γ secreting T cells/10^6 PBMC
4,000
3,000
2,000
1,000
0
NA2 VLPs
QWIV
Mock
21 days
35 days
F
PHA stimulation
TNF-α secreting T cells/10^6 PBMC
10,000
8,000
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4,000
2,000
0
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NA2 VLPs
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Mock
21 days
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No stimulation
IFN-γ secreting T cells/10^6 PBMC
4,000
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2,000
1,000
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NA2 VLPs
QWIV
Mock
21 days
35 days
G
No stimulation
TNF-α secreting T cells/10^6 PBMC
10,000
8,000
6,000
4,000
2,000
0
NA2 VLPs
QWIV
Mock
21 days
35 days
D
% IFN-γ secreting T cells (virus/PHA)
100
80
60
40
20
5
4
3
2
1
0
NA2 VLPs
QWIV
Mock
21 days
35 days
H
% TNF-α secreting T cells (virus/PHA)
100
80
60
40
20
0
NA2 VLPs
QWIV
Mock
21 days
35 days

Figure 2.4. Vaccination with NA2 VLPs decreases TNF-α secreting T cells compared to mock vaccination following infection with A/swine/NorthCarolina/KH1552516/2016 virus. PBMCs from pigs vaccinated in a prime-boost regimen 21 days apart with a QWIV, NA2 VLPs, or mock vaccinated with water-in-oil-in-water adjuvant were collected at 21 and 35 days and re-stimulated with A/swine/NorthCarolina/KH1552516/2016 virus or PHA for enumeration of cytokine secreting cells. They were tested for IFN-γ-secreting cells following (A) virus re-stimulation (B) PHA re-stimulation (C) no stimulation (D) ratio of virus *vs* PHA stimulated cells. Additionally, they were tested for TNF-α-secreting cells following **(**E) virus re-stimulation (F) PHA re-stimulation (G) no stimulation (H) ratio of virus *vs* PHA stimulated cells. Pink squares represent NA VLP vaccinated pigs, cyan triangles represent commercial quadrivalent whole-inactivated virus vaccinated pigs (QWIV), and black circles represent mock-vaccinated pigs. Horizontal lines represent the mean for each group. Statistical analysis was performed by two-way ANOVA test. Error bars represent SEM. ** = $p < 0.01$.

Figure 2.5.

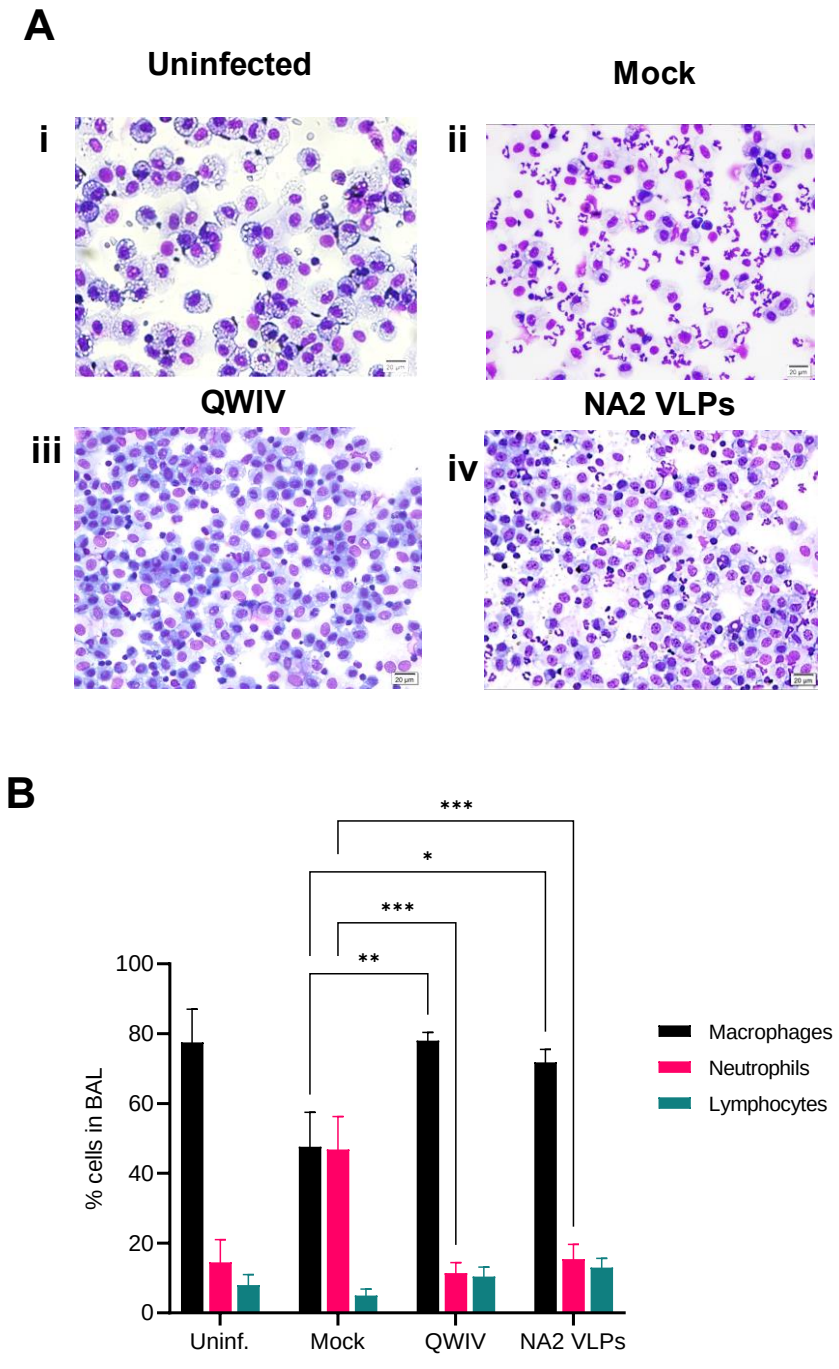

A
Uninfected
Mock
i
ii
QWIV
NA2 VLPs
iii
iv
B
***
*
***
**
100
80
60
40
20
0
% cells in BAL
Uninf.
Mock
QWIV
NA2 VLPs
Macrophages
Neutrophils
Lymphocytes

Figure 2.5. Vaccination with NA2 VLPs and swine QWIV vaccine reduces neutrophilic infiltration in bronchoalveolar lavage fluid collected from pig lungs post-infection. Bronchoalveolar lavage fluid (BALF) was collected from lungs at necropsy 5 days post-infection. (A) Separated cells were added to slides and stained with Wright-Giemsa stain and differential cell counts were determined microscopically in the different groups (i) uninfected unvaccinated group (ii) mock-vaccinated group (iii) QWIV vaccine group (iv) NA2 VLPs vaccine group (20 μm scale bar, 40x magnification). (B) Cell counts were expressed as a percentage of total cells. Black bars represent the mean percentage of macrophages, pink bars the mean percentage of neutrophils, and cyan bars represent the mean percentage of lymphocytes in each experimental group. Error bars represent the Standard Error of Mean (SEM). Statistical analysis was performed by two-way ANOVA test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure 2.6.

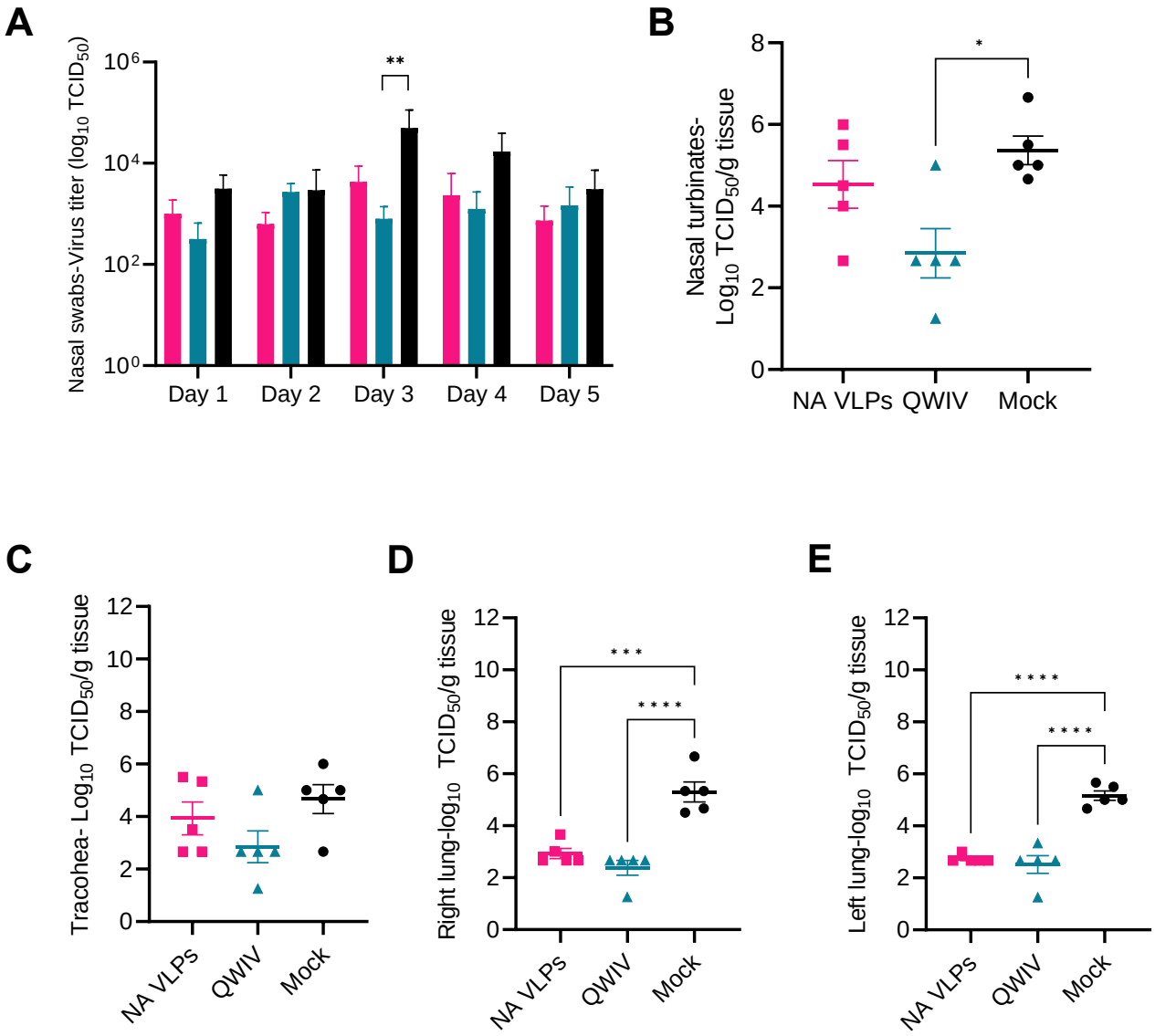


Figure 2.6. Vaccination with NA2 VLPs and the commercially available swine QWIV vaccine reduces viral replication in pig lungs post-infection. Nasal swabs were collected from pigs daily post-challenge through necropsy on day 5 and virus titers were measured via $TCID_{50}$ assay. Tissue

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-infection at necropsy. Following tissue homogenization, virus replication was measured in each respiratory compartment via $TCID_{50}$ assay on tissue homogenates. (A) Viral titers from nasal swabs. (B) Mean viral titers of homogenized nasal turbinates. (C) Mean viral titers of homogenized trachea tissue (D) Mean viral titers of right lung lobe tissue homogenates. (E) Mean viral titers of left lung lobe tissue homogenates. Pink squares represent NA2 VLP vaccinated pigs, cyan triangles represent commercial QWIV immunized pigs, and black circles represent mock vaccinated pigs. Horizontal lines represent the mean for each group. Error bars represent SEM. Statistical analysis was performed by two-way ANOVA test (A) and one-way ANOVA test (B-E). * = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$.

Figure 2.7.

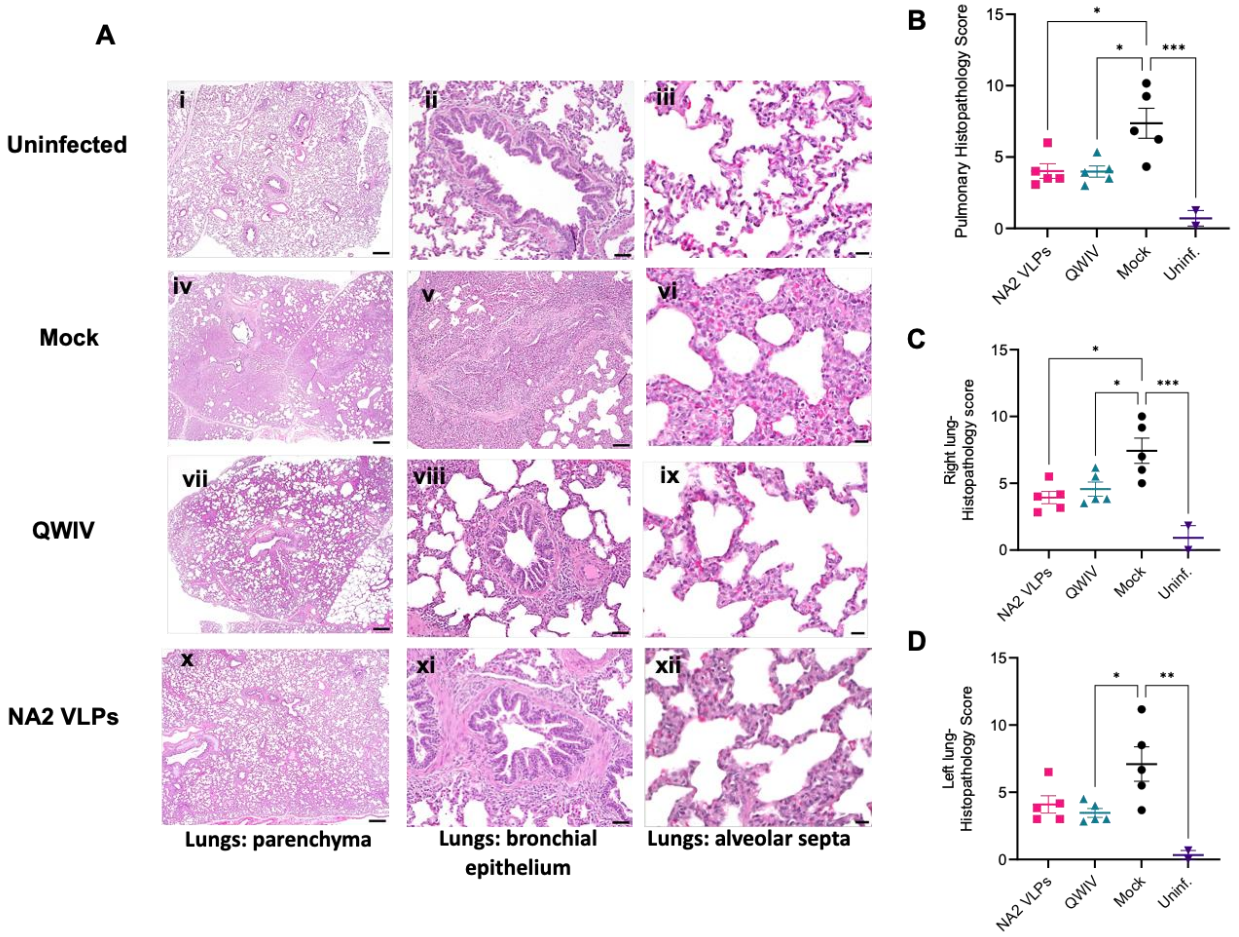


Figure 2.7. Vaccination with NA2 VLPs and the commercially available swine QWIV reduces total histopathology scores in pig lungs post-infection. Lung tissues were collected at necropsy 5 days post-infection and were fixed in formalin. Five µm sections were stained with H&E and were examined by light microscopy. Lung sections were scored by two individuals including a board-certified veterinary pathologist (A) H&E-stained representative lung tissue sections from each respective experimental group. Parenchyma 500 µm scale bar (2x

magnification), bronchial epithelium 50 μm scale bar (20x magnification); alveolar septa 20 μm scale bar (40x magnification). (B) Mean lung histopathology scores from each vaccinated group. (C) Right lung mean histopathology scores. (D) Left lung mean histopathology scores. Pink squares represent the NA2 VLP vaccinated pigs, cyan triangles the QWIV vaccinated pigs, black circles the mock-vaccinated pigs, and magenta triangles healthy uninfected pigs. Error bars represent SEM. Statistical analysis was performed by Kruskal-Wallis test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure 2.8.

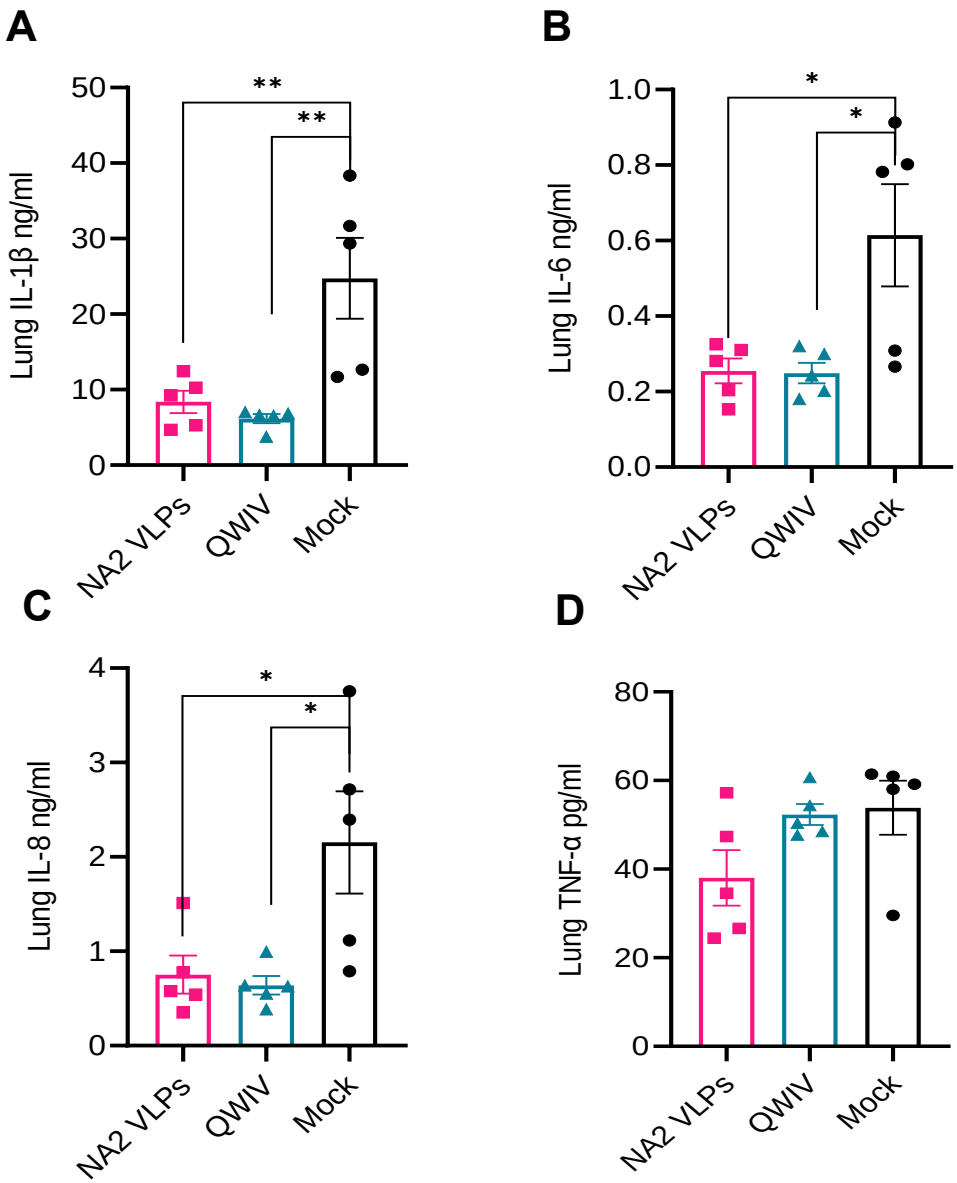
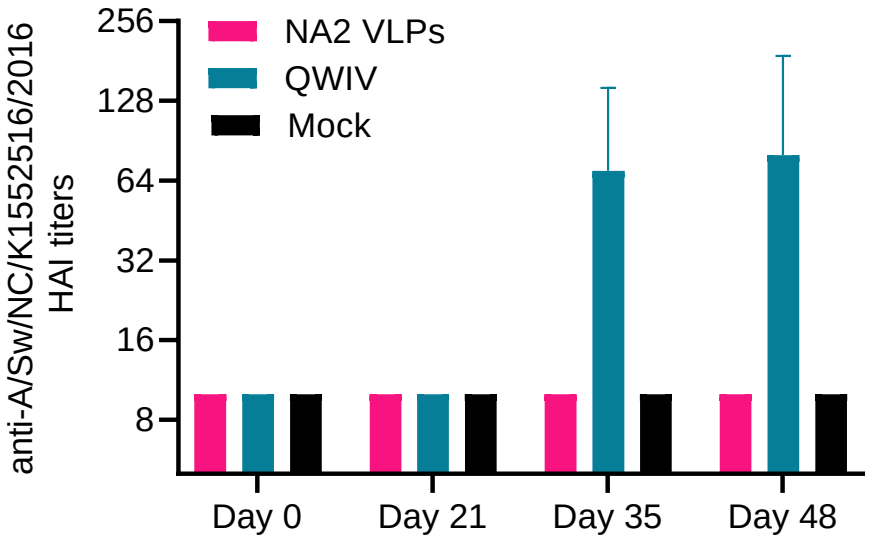


Figure 2.8. Vaccination with NA2 VLPs reduced lung inflammation in pig lungs post-infection. Lung homogenates prepared from tissues collected at necropsy of animals vaccinated

with QWIV, NA2 VLPs, or adjuvant alone 5 days post-infection, were tested for cytokine and chemokine secretion with MILLIPEX. (A) IL-1β (B) IL-6 (C) IL-8 (D) TNF-α. Pink squares represent NA2 VLP vaccinated pigs, cyan triangles QWIV vaccinated pigs, and black circles mock vaccinated pigs. Error bars represent SEM. Statistical analysis was performed by one-way ANOVA test. * = $p < 0.05$; ** = $p < 0.01$.

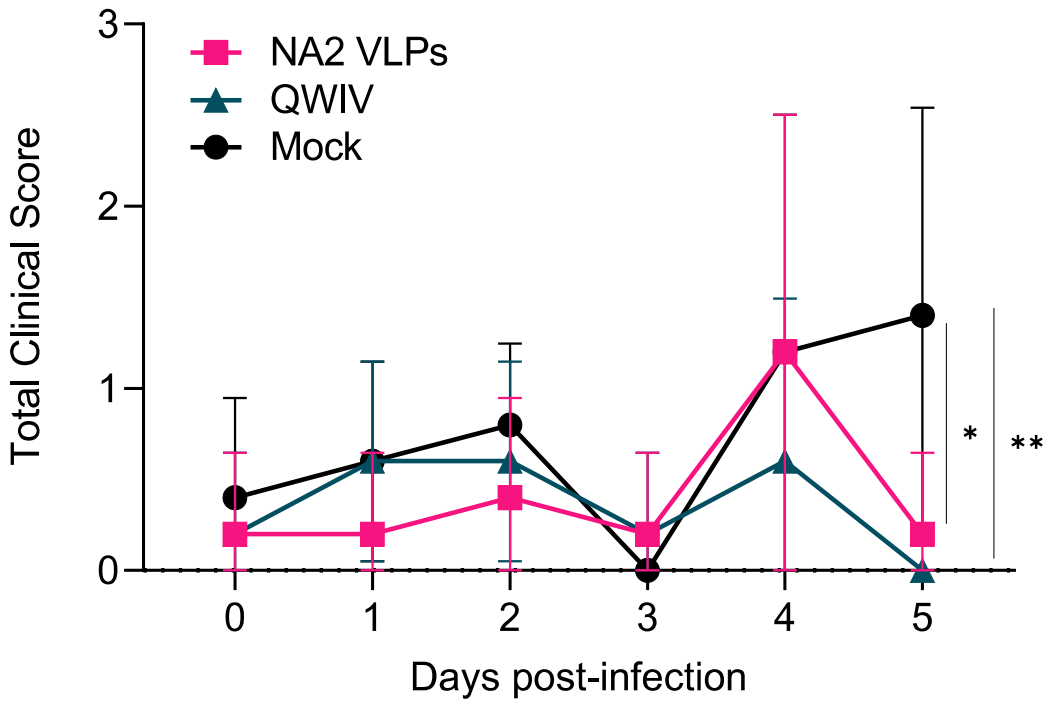
2.9. Supplemental Figures

Supplemental Figure 2.1.



Suppl. Figure 2.1. Prime boost vaccination with commercial QWIV induces serum antibody responses capable of inhibiting hemagglutination of A/swine/NorthCarolina/KH1552516/2016. Serum samples collected at day 0, 21, 35 and 48 were assayed for hemagglutination inhibition (HAI) titers against the heterologous H3N2 A/swine/NorthCarolina/KH15/2016 challenge virus. Bars represent geometric mean HAI titers and errors bars 95% CI. Sera were collected from mock vaccinated animals (black), NA2 VLPs vaccinated animals (pink), and QWIV vaccinated animals (cyan).

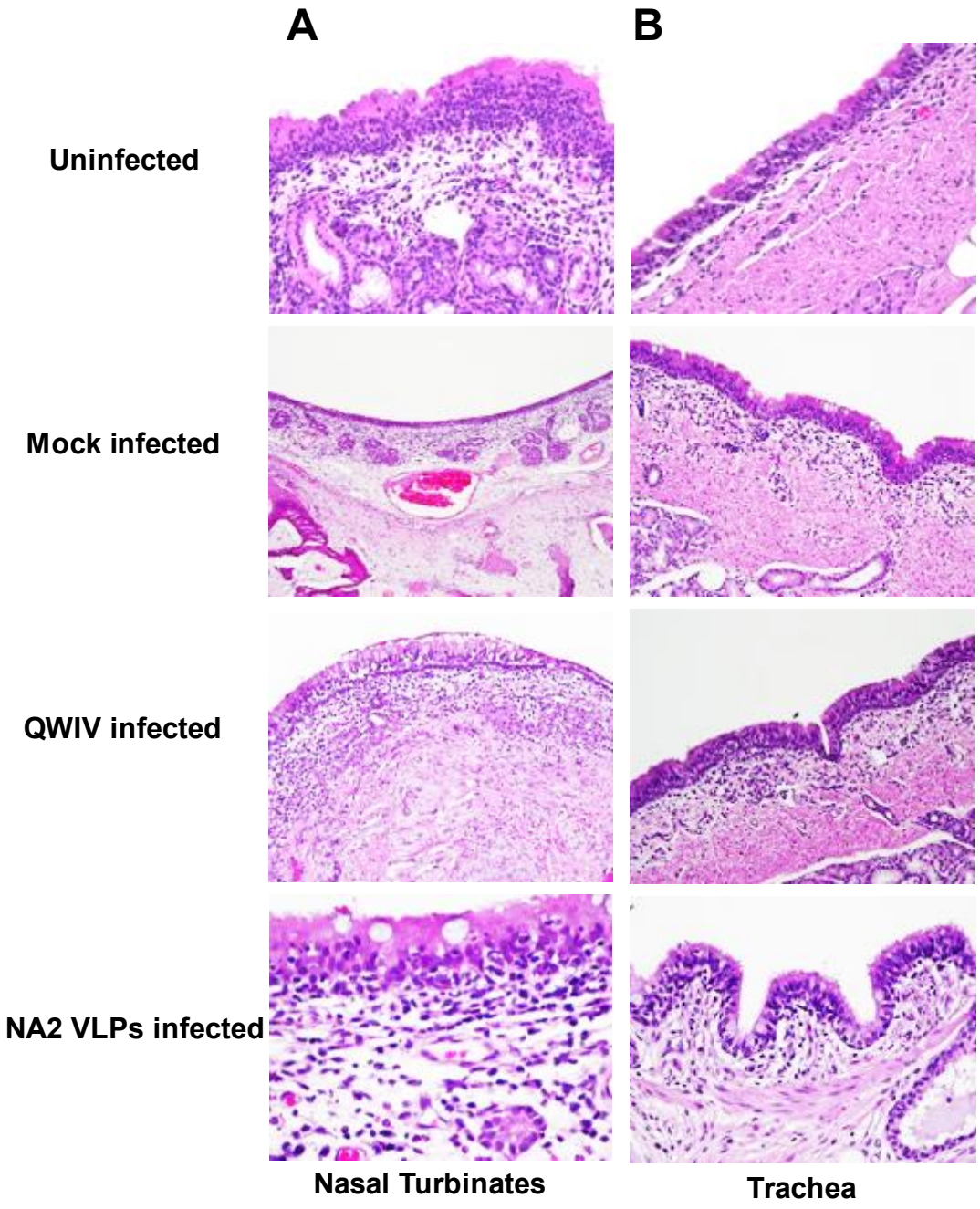
Supplemental Figure 2.2.



Suppl. Figure 2.2. Swine clinical disease scores after challenge with A/swine/NorthCarolina/KH1552516/2016. 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 (< 39.4°C = 0, 39.4-39.9°C = 1, 40-40.5°C = 2, > 40.6°C = 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. Black circles represent the mock vaccinated pigs (Mock) (n=5), pink squares the NA2 VLP vaccinated pigs (n=5) and cyan triangles the QWIV

vaccinated pigs (n=5). Statistical analysis was performed by two-way ANOVA test. * = $p < 0.05$; ** = $p < 0.01$.

Supplemental Figure 2.3.



Suppl. Figure 2.3. Vaccination with NA2 VLPs and the commercially available QWIV swine IAV vaccine did not affect total histopathology scores in pig nasal turbinates and trachea. Respiratory tissues were collected at necropsy 5 days post-infection and fixed in formalin. Five µm sections were stained with H&E and examined by light microscopy. Tissue sections were scored by a board-certified veterinary pathologist. H&E-stained representative upper respiratory tissue sections from each respective experimental group in (A) nasal turbinates and (B) trachea.

3. Chapter III: Heterologous prime-boost H1N1 vaccination exacerbates disease following challenge with a mismatched H1N2 influenza virus in the swine model

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Keywords: influenza A virus, vaccine, swine, hemagglutinin, heterologous prime-boost

3.1. Abstract

Influenza A viruses (IAVs) pose a significant threat to both human and animal health. Developing IAV vaccine strategies able to elicit broad heterologous protection against antigenically diverse IAV strains is pivotal in effectively controlling the disease. The goal of this study was to examine the immunogenicity and protective efficacy of diverse heterologous H1N1 influenza vaccine strategies including monovalent, bivalent, and heterologous prime-boost vaccination regimens, against a mismatched H1N2 swine influenza virus. Five groups were homologous prime-boost vaccinated with either an oil-adjuvanted whole-inactivated virus (WIV) monovalent A/swine/Georgia/27480/2019 (GA19) H1N2 vaccine, a WIV monovalent A/sw/Minnesota/A02636116/2021 (MN21) H1N1 vaccine, a WIV monovalent A/California/07/2009 (CA09) H1N1, a WIV bivalent vaccine comprised of CA09 and MN21, or adjuvant only (mock-vaccinated group). A sixth group was prime-vaccinated with CA09 WIV and boosted with MN21 WIV (heterologous prime-boost group). Four weeks post-boost pigs were intranasally and intratracheally challenged with A/swine/Georgia/27480/2019, an H1N2 swine IAV field isolate. Vaccine-induced protection was evaluated based on five critical parameters, (i) hemagglutination inhibiting (HAI) antibody responses, (ii) clinical scores, (iii) virus titers in nasal swabs and respiratory tissue homogenates, (iv) BALF cytology, and (v) pulmonary pathology. While all three type of vaccination regimens induced seroprotective titers against homologous viruses, heterologous prime-boost vaccination failed to enhance HAI responses against the homologous vaccine strains compared to monovalent vaccine regimens and did not expand the scope of cross-reactive antibody responses against antigenically distinct swine and human IAVs. Mismatched vaccination regimens not only failed to confer clinical and virological protection post-challenge, but instead exacerbated disease and pathology. In particular, heterologous-boosted pigs

showed prolonged clinical disease and increased pulmonary pathology compared to mock-vaccinated pigs. Our results demonstrated that H1-specific heterologous prime-boost vaccination, rather than enhancing cross-protection, worsened the clinical outcome and pathology after challenge with the antigenically distant A/swine/Georgia/27480/2019 strain.

3.2. Introduction

IAVs are considered important respiratory viral pathogens of both human and animals, causing substantial mortality and high economic losses. Influenza disease in swine and humans appears as an acute high-morbidity and low-mortality respiratory tract infection often characterized by anorexia, short-term fever, coughing, and breathing difficulties (Crisci et al., 2013; Eccles, 2005; Piroth et al., 2021). Influenza subtypes that are considered to be originally spilled over from humans into swine through reverse-zoonotic events, namely the H1N1, H1N2, and H3N2 variants are currently endemic in swine (Rajao et al., 2019; Rajao and Vincent, 2015). Although zoonotic and reverse-zoonotic infections are considered to be sporadic, the bidirectional transmission dynamics of influenza viruses contribute to the ever-increasing antigenic diversity and facilitates the potential of the emergence of a novel pandemic virus (Rajao et al., 2014; Van Reeth, 2007).

The epidemiology of IAV in the North American swine populations over the last 30 years is quite complex due to the rapid genetic evolution and immense antigenic variation, which are facilitated by frequent antigenic drift and shift events (Lewis et al., 2016; Rajao et al., 2014). While the alpha clade (1A.1.1.) or "classical" swine H1N1 viruses, which evolved from the Spanish flu 1918 pandemic strain, remained relatively genetically and antigenically stable in the US swine for approximately 70 years, the landscape of swine IAVs (swIAV) was dramatically changed by the emergence of a novel triple-reassortant H3N2 virus in 1998 (Lorusso et al., 2011; Webby et al., 2000). This H3N2 virus that contained the swine-human-avian triple-reassortant internal gene (TRIG) cassette, reassorted with the circulating previously established alpha clade H1N1 viruses, resulting in the formation of the beta clade (1A.2) and gamma clade (1A.3.3.3) lineages of H1N1 swIAVs (Kyriakis et al., 2017; Mancera Gracia et al., 2020).

During the early 2000s, following two separate introductions of human seasonal H1N1 viruses into swine, these human-origin viruses reassorted with the endemic strains at the time and resulted in the generation of the novel delta-clade swIAVs, which were further subclassified into delta1 (1B.2.2) and delta2 (1B.2.1) clades (Lorusso et al., 2011; Mancera Gracia et al., 2020). In contrast to alpha, beta, and gamma clade swIAVs, delta-H1 viruses are comprised of a hemagglutinin (HA) of human seasonal influenza virus origin (Rajao et al., 2018). Finally, the emergence of the 2009 pandemic H1N1 strain in humans and its subsequent spread into swine populations resulted in extensive reassortment events that drove the epidemiology of North American swIAVs to become even more complicated (Lorusso et al., 2011). The 2009 pandemic virus H1 (1A.3.3.2), while mostly related to the gamma clade viruses circulating in the US swine herds at that time, established a separate unique subclade within the gamma phylogenetic clade (Lorusso et al., 2011). In addition to the antigenic shift events, residual accumulation of point mutations on the external viral proteins, hemagglutinin and neuraminidase (NA), over time resulted in further diversification of endemic influenza viruses in the US swine populations (Rajao et al., 2014; Ryt-Hansen et al., 2020).

Developing effective prophylactic influenza vaccination strategies for both humans and animals is pivotal in controlling the disease. While several novel broadly protective vaccine approaches have been developed and tested for both human and animal health, multivalent whole-inactivated virus (WIV) vaccines are the most commonly used vaccine platform globally (Nuwarda et al., 2021). Numerous studies have demonstrated that although commercially available inactivated IAV vaccines elicit adequate protection against homologous viruses both in swine and humans, they often fail to establish sufficient cross-protection against antigenically divergent homosubtypic and heterosubtypic viruses (Kyriakis et al., 2010a; Vincent et al., 2010a). Thus, given the vast antigenic

diversity of viruses currently circulating in US swine populations, vaccination can only be used as a narrow measure aimed to control the disease burden. The most significant shortcomings of inactivated flu vaccines are that they elicit predominantly strain-specific serological responses primarily targeting the immunodominant globular head of the HA protein that decline rapidly following booster vaccination and that they fail to induce mucosal immunity and strong cell-mediated immune protection (Houser and Subbarao, 2015; Van Reeth and Ma, 2013).

A vaccination strategy that has been recently explored and has been shown to broaden the antibody responses of experimental or commercial WIV influenza vaccines is heterologous prime-boost vaccination, i.e. sequential immunization with antigenically mismatched homosubtypic vaccine strains (Chepkwony et al., 2020; Del Giudice et al., 2009; Li et al., 2020; Parys et al., 2022b; van den Brand et al., 2011; Van Reeth et al., 2017). In the current study, we utilized two H1N1 WIV vaccine strains, alpha clade A/swine/Minnesota/A02636116/2021 (1A.1.1) and pandemic-clade A/California/07/2009 (1A.3.3.2), mismatched to the challenge delta2 clade A/swine/Georgia/27480/2019 H1N2 virus (1B.2.1). The main objectives of this study were to examine the immunogenicity and protective efficacy of diverse heterologous WIV H1 vaccination strategies, including monovalent, bivalent, and heterologous prime-boost vaccination regimens, based on the three distinct US swine H1 lineages, against antigenically distinct homosubtypic US swIAVs. We report that heterologous H1 prime-boost vaccination rather, than enhancing cross-protection, exacerbated clinical disease and pathology following challenge with a virus antigenically distinct to the vaccine viruses strain.

3.3. Materials & methods

3.3.1. Cells and virus stocks

Madlin-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) and medium was 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. IAV strains that were used for vaccine and challenge purposes were propagated in MDCK cells. For swine vaccination, we utilized three swIAV strains: A/California/07/2009 H1N1 virus (CA09), which contains a pandemic (pdm)-clade HA, A/swine/Minnesota/A02636116/2021 H1N1 virus (MN21), which contains an alpha clade HA, and A/swine/Georgia/27480/2019 H1N2 virus (GA19), a swine IAV field isolate, which contains a delta2 clade HA. GA19 was also used for challenge. These strains were propagated in MDCK cells. The virus stock that was used for challenge was stored at −80°C and the virus titer [50% median tissue culture infectious dose (TCID50)] was calculated using the Reed and Muench formula (Reed and Muench, 1938).

3.3.2. Animals

Thirty-nine 7-week-old influenza virus-naïve and porcine reproductive and respiratory syndrome virus-naive conventional cross-bred Yorkshire/Hampshire male and female 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. Upon arrival (one week prior to vaccination), animals were housed in the BSL-2 facilities of the Sugg Laboratory for Animal Health Research and were prophylactically treated with enrofloxacin (Baytril, Elanco, Indianapolis, IN) and ceftiofur crystalline free acid (Excede, Zoetis Inc., Kalamazoo, MI) according to manufacturer's instructions. Pigs were randomly divided into six groups of six animals and one group of three animals, as outlined in the swine vaccination and virus challenge section. Each experimental group was housed in a separate isolation unit with a HEPA air filtration system. Animals were acclimated for one week prior to the start of the study. Food and water were provided ad libitum.

3.3.3. Vaccine preparation

Swine influenza vaccine strains (CA09, MN21, and GA19 viruses) grown in MDCK cells were inactivated by β-propiolactone (Sigma-Aldrich, St. Luis, MO) treatment as previously described (Jonges et al., 2010). Sufficient virus inactivation was demonstrated by failure of virus replication in two-serial passages in MDCK cells (Vincent et al., 2008). Following inactivation, vaccine preparations were formulated with a commercial mineral oil-in-water (O/W) emulsion (Montanide™ ISA 15 VG), which served as the adjuvant solution. Whole-inactivated virus (WIV) vaccine formulations were mixed at a volume-to-volume ratio of 85:15 virus suspension to adjuvant, prepared under a low shear rate, and stored at 4°C according to the manufacturer's instructions, one day prior to each vaccination. Monovalent vaccine formulations (CA09, MN21, and GA19) contained prior to adjuvant addition approximately 128 hemagglutination units

(HAU)/mL of WIV while bivalent vaccine formulations (CA09-MN21) contained approximately 256 HAU/mL (128 HAU of each WIV).

3.3.4. Swine vaccination, virus challenge, and sample collection

Pigs were randomly assigned to six groups of six animals and one group of three animals. Pigs were prime-vaccinated at the age of 8 weeks and booster immunization was administered 3 weeks later, at the age of 11 weeks. Animals were vaccinated with either the monovalent CA09, MN21, and GA19 WIV vaccines, the bivalent CA09-MN21 WIV vaccine, or a Phosphate-Buffered Saline (PBS) (Corning Life Sciences, Corning, NY, USA) in adjuvant solution. Each pig received a deep intramuscular injection into the neck with 1 ml of the monovalent or bivalent WIV vaccine or 1 ml of PBS-O/W adjuvant solution. Three homologous prime-boost vaccine groups (n=6) were each vaccinated with the monovalent CA09, MN21, and GA19 WIV vaccine. The heterologous prime-boost vaccine group (n=6) was prime-vaccinated with the monovalent CA09 WIV vaccine and was boosted on day 21 post-prime (pp) with the monovalent MN21 WIV vaccine. The bivalent vaccine group (n=6) was both prime and boost vaccinated with the bivalent CA09-MN21 WIV vaccine. Control groups included a mock-vaccinated /challenged group (MV/C) (n=6) and a mock-vaccinated/non-challenged group (MV/NC) (n=3). Four weeks after the boost vaccination, all pigs except the MV/NC control group were challenged with the GA19 H1N2 virus. On the day of challenge, GA19 virus was prepared for an inoculum of 10^5 $TCID_{50}$/ml. Swine were inoculated with 4 ml total volume, split into 2 ml intranasally (IN) (1 ml per nostril) using a mucosal atomization device (MAD Nasal™ Atomization Device, Teleflex, Wayne, PA) and 2 ml intratracheally (IT) (2 mL of 10^5 $TCID_{50}$/ml per route). Animals were clinically scored daily post-challenge (pc) based on rectal

temperature, respiratory rate, and assessment of clinical behavior. Specifically, rectal temperature scores ranged from 0 to 3 (< 39.4°C = 0, 39.4-39.9°C = 1, 40-40.5°C = 2, > 40.6°C = 3), respiratory rate scores assigned per minute ranged from 0 to 2 (20-40/minute = 0, 41-59/minute = 1, > 60/minute = 2) and clinical behavior score, based on coughing (absent = 0, present = 1) and depression (absent = 0, present = 2) ranged from 0 to 3, as previously described (Pliasas et al., 2022). To assess virus shedding, nasal swabs were collected daily starting two days prior to inoculation until day 5 pc. All challenged animals were humanely euthanized with a lethal dose of pentobarbital (intravenous administration) on day 5 pc. MV/NC animals were necropsied prior to challenge at day 49 pp. At necropsy, bronchoalveolar lavage fluid (BALF) was collected for cytopathology and respiratory tissue samples, including nasal turbinates, trachea, right lung lobes and left lung lobes, were harvested for further virological and histopathological evaluation. The experimental design of the vaccination-challenge study is summarized in Table 3.1.

3.3.5. Hemagglutination inhibition (HAI) assay

Serum samples were collected post-prime vaccination (day 0 pp), at boost vaccination (day 21 pp), at 2 weeks post-boost (day 35 pp), at 4 weeks post-boost (day 49 pp) prior to the challenge, and at day 5 pc. Sera for hemagglutination inhibition titers were processed according to the WHO protocol and as previously described (WHO, 2011). Briefly, serum samples were treated overnight at 37°C with a receptor-destroying enzyme (RDE) (Denka Seiken Co Ltd) to remove nonspecific inhibitors. Following overnight treatment, sodium citrate (1.5%) was added to the samples and the mixtures were heated at 56°C for 30 minutes to inactivate the residual RDE and complement factors. RDE-pretreated sera were incubated with packed 50% turkey red blood cells (RBC) for

one hour at 4°C, to remove any non-specific cryoglobulins interfering with RBC and antigen-antibody binding. Following centrifugation at 10,000 g for 3 minutes at 4°C, the supernatant (HAI-treated sera) were collected, aliquoted, and stored at -23°C for further use in HAI assays.

The following steps were carried out at room temperature. Two-fold serially diluted HAI-treated sera (50 µL/well) were incubated with 4 HA units of MDCK-grown swine or human IAVs that are listed in Supplemental Table 3.1 for one hour. Next 50 µL of 0.5% turkey RBC were added to each well and hemagglutination inhibition titers were recorded after 45-60 minutes (Esser et al., 2017; Kitikoon et al., 2014). North American swine IAV strains employed in the HAI assay that were not held in our repository were obtained from the U.S. Department of Agriculture (USDA) swine influenza repository at the National Veterinary Service Laboratories. Sample analysis was performed in duplicates and the HAI antibody titers were expressed as the reciprocal of the highest dilution of serum that conferred inhibition of hemagglutination.

3.3.6. Nasal swab reverse transcription polymerase chain reaction (RT-PCR) assay

To assess virus shedding, virus in nasal swabs collected daily post-challenge from each animal was suspended in 2 ml of PBS supplemented with 1% antibiotic-antimycotic solution by mechanically agitation for 10 minutes at 4°C and stored as aliquots at -80°C until RNA isolation for RT-PCR assays. Total RNA was extracted from 200 µl nasal swab samples harvested at necropsy using RNAzol-RT (Molecular Research Center, Cincinnati, OH - CAT #RN 190) according to manufacturer's instructions and resuspended in 20 µl. An RT-PCR reaction was performed using 2 µL of extracted total RNA, 6.25 µL Taqman® Fast Virus 1-Step Master Mix (Life Technologies- CAT # 4444434), 0.5 µL of forward primer (20 nmol) (5'-AGA TGA GTC TTC TAA CCG AGG TCG-3', Biosearch Technologies, Cat# INFA-F-20), 0.5 µL of reverse

primer (20 nmol) (5'-TGC AAA AAC ATC TTC AAG TCT CTG-3', Biosearch Technologies, Cat# INFA-R-20), 1 μL of probe (5 nmol) (FAM-TCA GGC CCC CTC AAA GCC GA-BHQ, Biosearch Technologies, Cat# INFA-P-5) and 10 μL of HyPure™ Molecular Biology Grade Water (GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT) in a total volume of 25 uL per reaction. Thermal cycling was performed under the following conditions: reverse transcription for 20 minutes at 50°C, inactivation of reverse transcriptase for 10 minutes at 95°C, followed by 45 cycles of denaturation at 95°C for 10 seconds and annealing/amplification at 55°C for 20 seconds. A standard curve was generated by using a ten-fold dilution series from the A/swine/Georgia/27480/2019 virus stock that was used for animal inoculation and was of known $TCID_{50}$ titer (10^7 $TCID_{50}$). Based on the standard curve, CT values from the extracted viral RNA of nasal swab samples were converted to Relative Equivalent Units (REU) as previously reported (Everett et al., 2021).

3.3.7. Virus titration ($TCID_{50}$) assay

To compare virus replication in the upper and lower respiratory tract between vaccinated and mock-vaccinated challenged animals we performed virus titration assays on respiratory tissue homogenates. Specifically, respiratory tissue samples (nasal turbinates, trachea, right lung lobes including accessory lung lobe, and left lung lobes) harvested after euthanasia were homogenized prior to virological evaluation, as previously described (Pliasas et al., 2022). Briefly, respiratory tissue samples were weighed to achieve a 10% weight (gr)-to-volume (w/v) of tissue suspended in PBS. Tissue suspensions were then homogenized and centrifuged. Following centrifugation, tissue homogenate supernatants were collected, aliquoted, and stored at -80°C for $TCID_{50}$ assays.

For the virus titration assay, following serial tenfold dilution, tissue homogenate samples were used to inoculate confluent MDCK cell monolayers in serum-free media. After 72-hour incubation at 37°C, wells were evaluated for the presence of cytopathogenic effect (CPE) and virus titers ($TCID_{50}$) were determined by the Reed-Muench method (Reed and Muench, 1938).

3.3.8. Cytopathological evaluation of bronchoalveolar lavage fluid

At necropsy time points (day 49 pp for MV/NC and day 5 pc for all other groups), lungs were harvested from all the animals (n=39) for virological and histopathological evaluation and bronchoalveolar lavage fluid (BALF) collection. BALF samples were processed as previously described (Pliasas et al., 2022). Briefly, BAL fluid was treated with 1X Lysis Buffer (BioLegend, San Diego, CA) for 5 minutes to lyse red blood cells. Following centrifugation, pelleted BALF 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 cell density of 0.5-1 x 10^5 cells. The cells were stained with a three-step Wright-Giemsa Stain (Quick Stain, VWR International, Radnor, PA) and the BAL fluid cell populations (macrophages, neutrophils, and lymphocytes) were expressed as the percentage of the total number of cells counted (Jolie et al., 2000).

3.3.9. Histopathologic evaluation of pulmonary tissues

Following necropsy, gross lung lesions were assessed to determine the macroscopic pulmonary pathology profiles between the different groups. Additionally, lung tissue samples (from the most

prominently affected right and left lung lobes) were collected, fixed in 10% neutral buffered formalin, sectioned, and stained with hematoxylin and eosin stains (H&E) for microscopic evaluation (Olympus BX53; Olympus Scientific Solutions Technologies Inc., Waltham, MA). Lung sections were examined by two individuals blinded to treatment groups, including a board-certified veterinary anatomic pathologist and the scores were averaged. Histopathology scores considered five parameters to reflect the severity of pulmonary pathology following influenza infection, as previously described (Pliasas et al., 2022). Specifically, pulmonary tissues were scored on a 17-point scale based on the composite scores of five parameters; (i) the percent of lung section affected, (ii) the extent of bronchial and bronchiolar epithelial changes, (iii) the severity of interstitial pneumonia, (iv) the degree of lymphocytic peribronchiolar cuffing, and (v) Bronchus-associated lymphoid tissue (BALT) hyperplasia. The first three parameters (i-iii) were scored from 0 to 4, with 4 being the most severe, parameter (iv) was scored from 0 to 3, with 3 being the most severe, and the last parameter (v) was scored from 0 to 2, with 2 being the most severe.

3.3.10. 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 test for the comparison of virus load in nasal turbinates and left lung homogenates and composite, right lung and left lung pathology scores between groups with an alpha of 0.05 ($p \leq 0.05$). A two-way ANOVA with post-hoc Tukey's multiple comparison test was used for the comparison of HAI titers, clinical scores, and nasal swab virus titers and Dunnett's post-hoc multiple comparisons test for BALF cytology between groups with

an alpha of 0.05 ($p<0.05$). The nonparametric Kruskal-Wallis test was used to test differences between groups for the trachea and right lung virus load.

3.4. Results

3.4.1. Heterologous prime-boost vaccination did not augment HAI antibody titers against vaccine constituents compared to homologous monovalent and bivalent prime-boost vaccination regimens

For vaccination purposes, we used three antigenically distinct contemporary US H1 swine influenza A viruses (swIAVs): an H1N1 swIAV [A/swine/Minnesota/A02636116/2021 (MN21)], which has a classical alpha-clade HA, an H1N1 swIAV [A/swine/California/07/2009 (CA09)], which has a HA of 2009 pandemic (pdm) origin, and an H1N2 swIAV [A/swine/Georgia/27480/2019 (GA19)] which has a human-like delta2 clade HA. The phylogenetic relationships of the HA genome segment between the vaccine viruses is shown in Supplemental Figure 3.1. While all three vaccine viruses are homosubtypic, the amino acid homology in their HA is quite variable as shown in Supplemental Table 3.1.

We vaccinated pigs at a 21-day interval with a homologous prime-boost vaccination regimen with either one of three monovalent WIV vaccines (GA19, MN21, and CA09) or a bivalent vaccine comprised of the CA09 and MN21 whole inactivated viruses. Additionally, a fifth group of pigs was prime immunized with the CA09 monovalent WIV vaccine and boosted with the MN21 monovalent WIV vaccine (heterologous-boosted group). Serum was collected at days 0, 21, 35, and 49 pp vaccination. Anti-HA antibody titers were determined by performing HAI assay. Our

first goal was to assess the HAI titers against the three vaccine viruses GA19, MN21, and CA09 (Figure 3.1.). According to previous literature, we consider HAI titers above 1:40 to be seroprotective (Coudeville et al., 2010; Sisteré-Oró et al., 2019; Wijnans and Voordouw, 2016; Wood and Levandowski, 2003).

Independent of the vaccination strategy used, prior to prime immunization all groups were seronegative for IAV. As expected, MV/C pigs did not show measurable HAI antibody titers at any time point assayed prior to the challenge. Notably, throughout the study vaccinated pigs demonstrated minimal or no detectable cross-reactive HAI titers against viruses that were not included in their respective vaccine formula. At day 21 pp, after a single vaccine dose, all vaccination regimens induced HAI antibodies below the positive cutoff reciprocal titer of 1:40 against the corresponding homologous viruses (Figure 3.1.). However, peak HAI antibody titers for all five vaccine groups were detected at 2 weeks post-boost against viruses included in the original vaccine. Homologous prime-boost monovalent WIV vaccines CA09, MN21, and GA19 generated approximately 25-fold, 28-fold, and 9-fold increases in HAI titers, respectively, from day 21 pp and these titers were found to be significantly higher than in MV/C pigs ($p<0.0001$). This trend continued at day 49 pp, with sera from all monovalent vaccinates showing elevated HAI titers compared to MV/C pigs ($p<0.0001$), although sustaining a mild decrease in their antibody titers of up to a half-log lower compared to day 35 pp (Figure 3.1.).

While both the bivalent and heterologous-boosted groups generated comparable HAI titers to the monovalent CA09 group against the A/swine/California/07/2009 virus at day 35 pp, two weeks later (day 49 pp) antibody titers were found to be significantly lower in the bivalent group compared to CA09 vaccinates ($p<0.05$). At that point, we detected no significant differences in the anti-CA09 antibody titer between the heterologous prime-boost group and either the bivalent or

monovalent CA09 vaccine group (Figure 3.1B). On the other hand at day 35 pp, anti-MN21 HAI titers were significantly higher in the MN21 group compared to both the bivalent and heterologous-boosted groups ($p<0.05$ and $p<0.0001$) with the former group demonstrating significantly elevated titers compared to the latter ($p<0.05$). Antibody titers against A/swine/Minnesota/A02636116/2021 maintained a similar pattern at day 49 pp (Figure 3.1C).

Overall, all three vaccination regimens induced seroprotective titers against viruses included in the vaccine formulation following two vaccine doses. However, these data demonstrate that monovalent vaccination was immunogenically superior in inducing HAI antibody titers against the respective homologous viruses compared to both the bivalent and heterologous prime-boost vaccination.

3.4.2. Heterologous-boosting did not expand cross-reactive HAI antibody responses against antigenically distinct swine and human IAVs

Our next goal was to compare the different vaccination approaches deployed in our study in their ability to broaden the immunological HAI cross-reactivity against antigenically distinct swine and human IAVs. In this regard, we included in our assay 15 contemporary representative swine IAVs (including the three vaccine viruses) of the main North American swIAV phylogenetic clades that were circulating in US swine populations over the past decade. According to Bakre and colleagues, the four dominant HA phylogenetic clades of US H1 swIAVs are gamma (1A.3.3.3) and delta 2 (1B.2.1) clade viruses followed by the pandemic (1A.3.3.3) and alpha (1A.1.1) clade viruses (Bakre et al., 2020). In addition to swIAVs, we examined HAI responses against 6 representative human IAVs isolated over the last century. The HA amino acid homology and phylogenetic

relationship of the viruses employed in this assay to the vaccine strains employed in our study are presented in Supplemental Table 3.1. and Supplemental Figure 3.1., respectively.

For the purpose of this assay, we tested sera collected at 2 weeks post-boost as it was demonstrated that peak antibody titers against the vaccine viruses were achieved at day 35 pp. As expected, sera from mock-vaccinated animals did not demonstrate any appreciable HAI titers measured at day 35 pp (results not shown). Previous studies have demonstrated that there is limited to no HAI cross-reactivity between viruses that belong to phylogenetically distinct swIAV clades (Shu et al., 2012; Venkatesh et al., 2022; Vincent et al., 2010b; Webby et al., 2004). Indeed, regardless of the WIV strains used, monovalent vaccination, with a few exceptions, failed to elicit cross-reactive responses against the heterologous vaccine viruses and viruses belonging to antigenically distant lineages to their corresponding WIV vaccine constituent(s) (Table 3.2.).

Specifically, the MN21 and GA19 vaccination elicited seroprotective antibody responses that were limited to swine alpha-clade viruses and delta-clade viruses and human delta-like viruses, respectively. Vaccination with the CA09 WIV however induced cross-reactive responses above the positive cutoff reciprocal HAI titer of 1:40, not only against homologous swine and human influenza viruses containing pdm-clade HA but also against 2 out of 3 gamma clade viruses and one beta clade virus tested in the assay. Heterologous-boosting and bivalent vaccination, although increased the breadth of HAI responses compared to the CA09 and MN21 monovalent vaccines by mounting titers against viruses antigenically related to those included in the original formulation, did not expand cross-reactivity against the antigenically distinct delta and delta-like clade viruses (Table 3.2.). Additionally, the bivalent and heterologous-boosted vaccine groups failed to evoke seroprotective responses against either beta clade virus tested in the assay. Overall, peak HAI antibody titers against each individual virus were only demonstrated in vaccinated pigs

immunized with the homologous monovalent prime-boost vaccination regimen, with bivalent and heterologous-boosting vaccination approaches often inducing antibody titers that were of multifold lower.

These data suggest that while heterologous-boosting and bivalent vaccination enhanced the breadth (but not the magnitude) of seroprotective HAI responses against antigenically related viruses compared to monovalent vaccines, they failed to expand serological responses against sufficiently distant swine and human H1 flu strains.

3.4.3. Bivalent and heterologous-boosting vaccination strategies enhanced and prolonged clinical disease following challenge with A/swine/Georgia/27480/2019

Clinical scores were recorded daily throughout the study to assess the clinical status of the animals following challenge with the GA19 virus. Starting from day -2 prior to challenge through day 5 pc, rectal temperature, respiratory rate, and disease signs, in the form of presence of coughing or weakness/depression, were recorded daily. Overall, clinical expression of influenza disease in MV/C was mild, with elevated rectal temperatures above normal detected in 3 pigs solely at day 4 pc. The delayed onset and mild course of clinical disease in the MV/C group could be attributed to the relatively low virus titer that was used in the inoculum (Janke, 2013). While mean clinical scores in the MN21 and CA09 vaccine groups were consistently higher compared to the MV/C group throughout the study, significant differences were only observed between MN21 and MV/C pigs at day 4 pc ($p<0.05$) (Figure 3.2.). Additionally, clinical disease in the bivalent and heterologous-boosted vaccine groups was more severe over a prolonged period of time in relation to all other challenged groups. Specifically, clinical scores were significantly increased in the

heterologous-boosted and bivalent vaccine groups compared to MV/C control pigs over a period of 3 and 4 consecutive days, respectively (Figure 3.2.). Respiratory distress was consistently observed in the majority of animals in these groups and soft coughing was noted at various times, particularly when pigs were aroused. Additionally, mild febrile responses were detected in the majority of the bivalent and heterologous-boosted vaccinates at days 3 and 4 pc. On the other hand, not unexpectedly, homologous GA19 vaccinated animals demonstrated minimal or no clinical signs following challenge with the identical virus (Figure 3.2.).

Overall, our results demonstrate that bivalent and heterologous prime-boost immunization induced prolonged clinical disease in vaccinated swine compared to the MV/C group.

3.4.4. Mismatched swine influenza vaccination elicited different inflammatory cytology profiles in BALF after influenza infection compared to the MV/C group

To further characterize the vaccine-induced clinical protection we harvested BAL fluid from all animals at necropsy 5 days pc to assess the infiltration of inflammatory cells in the lower respiratory tract and their population dynamics following infection with the GA19 virus. In healthy MV/NC pigs, the BALF cytology profile was characterized primarily by tissue-resident macrophages along with the presence of a few non-degenerate neutrophilic granulocytes and rare small-sized lymphocytes (Figure 3.3Ai). Interestingly, while we observed minimal clinical signs in MV/C animals, their BALF cytology profile was characterized by mild to moderate BALF neutrophilic infiltration, which is a typical sign of uncomplicated influenza virus infection (Figure 3.3Aii). Homologous challenge following GA19 vaccination resulted in a similar cytology status to that of healthy MV/NC animals, with statistically significant lower percentages of neutrophils

($p<0.0001$) and higher percentages of macrophages ($p<0.001$) compared to the MV/C group (Figure 3.3Aiii and 3.3B).

In contrast, all four heterologous vaccinated groups demonstrated mild to moderate lymphocytic pulmonary inflammation that was accompanied by a variable neutrophilic component that ranged from minimal in the bivalent vaccine group to mild to moderate in the MN21, CA09, and heterologous boosted vaccine groups (Figure 3.3Aiv-vii). Regarding lymphocytic infiltration, the monovalent MN21, bivalent, and heterologous boosted vaccine groups showed statistically significantly elevated percentages of lymphocytes in their BAL fluid compared to MV/C animals ($p<0.05$). While lymphocyte percentages trended higher in the monovalent CA09 vaccine group compared to MV/C, the difference did not reach statistical significance due to a large degree of inter-animal variability (Figure 3.3B).

In summary, these data suggest that mismatched vaccination of pigs to the challenge virus favors the induction of different inflammatory cytology profiles in the BAL fluid following challenge compared to non-vaccination, that are characterized by a primary lymphocytic or mixed lymphocytic-neutrophilic infiltration.

3.4.5. Heterologous vaccination strategies failed to reduce virus shedding and replication in the respiratory tract

To assess virus shedding following challenge with the GA19 virus, we collected nasal swabs daily through day 5 pc. Titers were measured via an RT-PCR assay as relative equivalent units of RNA by employing a standard 10-fold dilution series of RNA extracted from the stock of the inoculation

virus. Throughout the challenge, matched GA19 vaccinated pigs demonstrated statistically significant lower levels of virus shedding ($p<0.0001$) that approached or were greater than two logs of virus load lower compared to MV/C pigs (Figure 3.4A). Overall, mismatched vaccination strategies failed to reduce virus shedding in pigs with the exception of day 1 pc where the bivalent and the monovalent CA09 vaccine groups showed significantly lower levels of viral RNA than mock-vaccinated animals ($p<0.001$ and $p<0.0001$, respectively). Additionally, we observed that CA09 vaccination reduced virus shedding at day 3 ($p<0.05$) (Figure 3.4A). The monovalent MN21 and the heterologous-boosted vaccine groups consistently showed comparable virus shedding throughout the study to the MV/C pigs, with the virus load in the heterologous-boosted group trending higher than mock-vaccinated pigs from day 3 to day 5 (Figure 3.4A).

Next, we examined virus replication in the different compartments of the respiratory tract among the different experimental groups. Following tissue homogenization, viral titers were measured via a $TCID_{50}$ assay. While GA19 vaccination induced almost complete protection against the homologous challenge virus in all four respiratory tissue sections ($p<0.0001$), mismatched vaccination strategies did not offer any protection against virus replication of the GA19 virus (Figure 3.4B-3.4E). Mean virus load in respiratory tissue homogenate samples from the monovalent MN21 and CA09 vaccine groups appeared to be comparable or in some cases relatively reduced to that of mock-vaccinated animals, although differences were not statistical significant. On the other hand, the challenge virus replicated to high titers in the upper airways and lungs of the bivalent and the heterologous-boosted vaccine groups (Figure 3.4B-3.4E). Interestingly, we noted that heterologous prime-boost vaccination increased pulmonary virus replication resulting in a statistically significant increase of virus load in left lung tissue

homogenates compared to the MV/C group ($p<0.05$) while in right lung homogenates virus titer differences approached but did not reach statistical significance ($p= 0.076$) (Figure 3.4E and 3.4D).

Overall, we observed that while matched vaccination with the homologous virus offered almost sterilizing protection against virus shedding and replication in the respiratory tract, the mismatched vaccination strategies, and particularly heterologous-boosting not only were unsuccessful in controlling the infection but additionally demonstrated the potential of enhancing pulmonary virus replication after challenge with A/swine/Georgia/27480/2019.

3.4.6. Heterologous-boosting exacerbated pulmonary pathology following challenge with the A/swine/Georgia/27480/2019

Our next goal was to examine the heterologous H1N1 vaccination strategies employed in our study in preventing the development of pulmonary pathology after challenge with the antigenically distinct A/swine/Georgia/27480/2019 H1N2 virus. First, we grossly examined the lungs for the assessment of macroscopic pulmonary lesion profiles. Regardless of the immunization regimen used, the majority of heterologous vaccinated animals demonstrated multifocal to coalescing to lobular dark red discoloration and, to a lesser extent, consolidation in the hilar and cranioventral regions that ranged from approximately 5 to 30% lung involvement. Mock-vaccinated challenged pigs demonstrated minimal to mild gross lesions that represented less than 5-10% lung involvement. This is consistent with what has been previously observed following low virus titer inoculation in swine (Janke, 2013; Landolt et al., 2003).

Following necropsy we collected tissue sections from the most notably affected right and left lung lobes to examine the presence of histopathological changes in pulmonary tissues on a 17-point scale. In regards to histopathological scores, as expected the three MV/NC pigs did not show any microscopic lesions in the lungs, besides the occasional presence of minimal to mild bronchus-associated lymphoid tissue hyperplasia (Figure 3.5Di-iii). Similarly to gross lesions, the unvaccinated challenged control pigs showed mild microscopic pulmonary pathology (Figure 3.5Div-vi). Vaccination with GA19 displayed optimal performance and induced complete protection from the development of histopathological lesions following homologous challenge (Figure 3.5Dvii-ix).

On the other hand, we observed that all four mismatched vaccination regimens enhanced pulmonary pathology and demonstrated statistically significant increases in the composite histopathological score compared to MV/C animals ($p<0.05$ for CA09, $p<0.001$ for MN21 and heterologous-boosted group, and $p<0.0001$ for bivalent vaccine group) (Figure 3.5A). While the histopathology score measured in right lung lobes was significantly higher in all four heterologous vaccine groups compared to MV/C pigs, we noted that microscopic lesions in left lung sections were found significantly enhanced only in the bivalent and heterologous-boosted groups ($p<0.001$) but not in the MN21 and CA09 vaccine groups (Figure 3.5B and 3.5C, respectively). The difference in the left lung score in the MN21 group compared to MV/C group approached but did not reach statistical significance ($p=0.0643$).

Regarding histopathological lesions, MN21 and CA09 vaccinated pigs showed mild to moderate bronchial and bronchiolar epithelial attenuation and necrosis, along with moderate alveolar septal mononuclear inflammation (Figure 3.5Dx-xii and 3.5Dxii-xv, respectively). However, histopathological lesions noted in lung sections from the bivalent and heterologous-boosted groups

were more widespread and pronounced than the mismatched monovalent CA09 and MN21 vaccine groups. Specifically, we observed that bivalent vaccination and heterologous-boosting induced severe multifocal to locally extensive necrotizing bronchitis and bronchiolitis that was accompanied by broadly prominent lymphocytic peribronchiolar and perivascular cuffing (Figure 3.5Dxvi-xviii and 3.5Dxix-xxi, respectively). In addition, increased mild to moderate subepithelial mixed lymphoid and suppurative infiltration, predominantly of peribronchiolar distribution, was present. Alveolar septal thickening was found to be moderately to markedly higher in both groups and we occasionally noted large amounts of alveolar luminal and interlobular seroproteinasceous fluid (edema) and hemorrhage.

Overall, these data suggest that heterologous boosting and bivalent vaccination enhanced gross and microscopic pulmonary pathology to scores even higher than the monovalent heterologous vaccine groups compared to MV/C control pigs.

3.5. Discussion

Traditionally, influenza vaccination strategies both in human and animal sources have deployed homologous prime-boost vaccination platforms to control virus infections. A limited number of studies have explored heterologous prime-boost vaccination strategies against influenza infection in swine, and as of yet heterologous boosting approaches have been demonstrated to broaden immune responses against antigenically distinct influenza viruses (Chepkwony et al., 2020; Li et al., 2020; Parys et al., 2022b; Van Reeth et al., 2017). However, the vast majority of these studies have focused primarily on the assessment of monovalent experimental H3N2 (Chepkwony et al., 2020; Van Reeth et al., 2017) or multivalent commercial H1-H3 vaccines (Li et al., 2020; Parys et

al., 2022b). Only one study to date, a recently published study by Parys *et al*. has examined the immunogenicity of a heterologous boosting approach by utilizing H1 vaccines containing viruses circulating in European pig populations (Henritzi et al., 2020; Parys et al., 2022a).

Here, we examined the immune responses and protective efficacy induced by an H1-specific heterologous prime-boost vaccination approach based on contemporary North American swine influenza strains. In the current study, while we observed that heterologous-boosting increased the breadth of seroprotective HAI responses against antigenically related influenza variants compared to the monovalent vaccines, this approach failed to expand serological responses against the antigenically distant delta and delta-like H1 influenza strains. However, the most striking finding was that heterologous prime-boost vaccination exacerbated clinical disease and pathology following challenge with the delta2 GA19 virus.

First, we demonstrated that homologous prime-boost monovalent vaccination induced superior HAI titers against viruses that were included in the original vaccine formulation compared to both the bivalent and heterologous prime-boost vaccination (Figure 3.1. and Table 3.2.). In particular, the mean levels of antibodies measured in bivalent vaccinates post-day 35 pp, while still shown to be considerably above the seroprotective cutoff titer, were in most cases significantly lower compared to the corresponding monovalent vaccinated pigs against the homologous CA09 and MN21 vaccine viruses (Figure 3.1B-3.1C). On the other hand, we observed that heterologous boosting evoked antibody titers against the prime A/California/07/2009 virus comparable to monovalent CA09 prime-boosting at all sera collection time points (Figure 3.1B). Interestingly however, heterologous prime-boost vaccination failed to induce similar levels of antibodies against the boost vaccine virus A/swine/Minnesota/A02636116/2021 as compared to the prime CA09

virus and performed significantly inferiorly compared to both the monovalent MN21 and bivalent vaccine groups (Figure 3.1C).

These results are in line with previous prime-boost studies demonstrating that the sequential order of antigens administered in the regimen influences the immunogenicity and to an extent the protective efficacy of this approach against viruses that are antigenically distinct from the prime or boost vaccine strains (Ikeno et al., 2011; Li et al., 2020; Parys et al., 2022b; Van Reeth et al., 2017). This could be attributed to a phenomenon that has been primarily characterized in humans and to a lesser extent in swine called antigenic imprinting or back-boosting (Fonville et al., 2014; Henry et al., 2018; Kohler, 2015; Vincent et al., 2017; Zhang et al., 2019). The concept refers to the observation that secondary heterologous exposures often favor a memory recall and maintenance of enduring immunity preponderantly against influenza variants that are antigenically related to the priming antigen and at a lower level against subsequently encountered antigens (Fonville et al., 2016; Kyriakis et al., 2010b; Lessler et al., 2012; Van Reeth et al., 2017). Indeed, we subsequently observed in our study that heterologous prime-boost vaccination promoted the induction of superior HAI antibody responses against influenza strains that are closely related to the CA09 prime vaccine virus including representative human and swine pandemic HA strains (1A.3.3.2) and gamma clade (1A.3.3.3) swIAVs. Conversely, we detected an up to 4-fold and 2-fold decrease in the HAI titers in sera from heterologous-boosted vaccinates against homologous to the booster strain, alpha clade viruses (1A.1.1) as compared to pigs immunized with the monovalent MN21 WIV and bivalent vaccine, respectively (Table 3.2.).

Previous vaccination studies in swine have observed limited to no HAI cross-reactivity between isolates of different swIAV lineages (Kitikoon et al., 2013; Vincent et al., 2010b; Vincent et al.,

2009) A similar trend has been observed in the present study; serologic cross-reactivity was in most cases below the seroprotective cutoff value with viruses belonging to antigenically distant lineages to the WIVs included in the original vaccine formulations. However, we noted that sera from pigs vaccinated with the CA09 WIV showed mild cross-reactivity against contemporary gamma and beta clade H1 viruses. Additionally, both the bivalent and the heterologous prime-boost approach in our study failed to expand the scope of immune responses against the antigenically distant delta and delta-like swIAV isolates (Table 3.2.). This is in agreement with the recently published European H1 prime-boost study that demonstrated that WIV heterologous-boosting did not broaden the cross-reactivity of antibody responses against antigenically distinct swine influenza variants compared to the corresponding monovalent vaccine (Parys et al., 2022a).

While the HAI assay remains the gold standard method for assessing HA-targeting antibody responses following influenza vaccination or infection, a previous study by Leuwerke and colleagues noted that the erythrocyte agglutination ability of viruses tested may affect the sensitivity of the HAI assay (Kirchenbaum et al., 2021; Leuwerke et al., 2008). In addition to humoral immunity, vaccine-induced cell-mediated responses have been previously shown to be critical in mediating effective virus clearance (Hayward et al., 2015). Further investigation of serological and cell-mediated immune responses through serum neutralization assays, whole virus ELISA, and ELISPOT assays could provide valuable information that may prove pertinent in the interpretation of our results (Van Reeth and Ma, 2013).

The main objective of currently developing novel influenza vaccine technologies in human and animal health is the induction of broadly cross-reactive antibodies against circulating drifted seasonal or enzootic virus strains but also against emerging influenza variants with pandemic

potential (Crank et al., 2019). In order to overcome the ever-increasing antigenic diversity, numerous novel platforms have focused their attention on the HA stem domain. This region represents a promising antigenic target for broadly protective influenza vaccine development, given that is considered relatively conserved compared to the highly plastic and mutable globular head domain of the HA (Krammer and Palese, 2013; Krammer and Palese, 2019; Scorza et al., 2016). Along the same lines, previous prime-boost studies have presumed that heterologous-boosting has the potential of inducing broader cross-reactive immune responses against antigenically distinct IAVs by targeting conserved subdominant epitopes on the hemagglutinin protein that are shared between the heterologous vaccine strains (Van Reeth et al., 2017; Wang et al., 2021; Wu and Wilson, 2017). However, in our study, we observed that the prime-boost approach, rather than enhancing cross-protection, worsened the clinical and pathology outcome after challenge with the antigenically mismatched GA19 virus.

Interestingly, cross-reactive antibodies specific to the conserved epitopes of HA conferred by inactivated vaccines have been previously shown to be implicated in the induction of vaccine-associated enhanced respiratory disease (VAERD) in swine (Crowe Jr, 2013; Khurana et al., 2013). VAERD is most commonly observed in vaccinated pigs following a challenge with a homosubtypic heterologous strain to the vaccine virus and this phenomenon is characterized by an increased severity in clinical disease and pulmonary pathology (Gauger et al., 2012; Gauger et al., 2011; Rajao et al., 2016; Vincent et al., 2008). In addition to swine, inactivated influenza vaccine-induced immune responses have also been implicated as the cause of enhanced pulmonary disease in humans and other mammals (Janjua et al., 2010; Kimble et al., 2022; Skowronski et al., 2010).

Previous VAERD studies in swine have traditionally reproduced this phenomenon under experimental conditions by consistently deploying heterologous H1 swIAVs that have a

hemagglutinin of either of 2009 pandemic or delta1-lineage origin as their vaccine and challenge antigens. Additionally, these reports have routinely used monovalent WIV oil-adjuvanted vaccines to incite VAERD (Gauger et al., 2012; Gauger et al., 2011; Khurana et al., 2013; Rajao et al., 2016). Notably however, Parys and colleagues in their recently published study did not observe VAERD in animals immunized with a homologous prime-boost A/California/04/2009/H1N1 vaccination regimen that were later challenged with the heterologous delta1 A/swine/Illinois/A01047020/2010 H1N2 strain. Additionally, they did not observe VAERD in any of the heterologous-boosted animals included in their study (Parys et al., 2022a). To our knowledge, the VAERD phenomenon has never been reported before in prime-boost studies. This could be attributed to the fact that previous studies that investigated protection conferred by heterologous-boosting consistently conducted intranasal inoculation of pigs, which often causes mild respiratory disease and pathology, and fails to reproduce VAERD (Van Reeth et al., 2017).

In our study, we observed that both the bivalent and heterologous-boosted vaccine groups demonstrated increased mean clinical scores following challenge compared to MV/C animals and performed worse over a prolonged period of time than the mismatched monovalent vaccine groups (Figure 3.2.). Overall, our results are in agreement with previous findings that demonstrated prolonged clinical disease in VAERD-exhibiting swine (Gauger et al., 2014; Gauger et al., 2011; Kitikoon et al., 2006). To further characterize clinical protection, we evaluated the differential cytology profile in the BALF following challenge between pigs immunized with the different vaccination strategies. We have previously shown that non-vaccinated pigs following uncomplicated influenza infection demonstrate BALF neutrophilic infiltration (Pliasas et al., 2022). Interestingly in the current study, we noted that regardless of the regimen used, mismatched vaccinated pigs demonstrated cytology profiles that were different from those of MV/C animals

and were characterized by a primary lymphocytic or mixed lymphocytic-neutrophilic infiltration (Figure 3.3.). In contrast, we observed that the vast majority of cells in the BALF of GA19 vaccinates were macrophages, with the presence of only sparse neutrophils and lymphocytes, and the BALf of GA19 vaccinates demonstrated a similar cytology status to non-infected healthy animals. Increased percentages of lymphocytes in the BALF cytology of the mismatched vaccine groups compared to MV/C pigs could be ascribed to an intensified transmigration of these cells from peripheral organs to the inflamed lower respiratory tract, based on the observation that these animals also demonstrated increased mean pulmonary pathology scores (Lewis et al., 1986). Another potential explanation is enhanced activation of primed T-cells that recognize conserved epitopes shared with the mismatched vaccine strains (Greenbaum et al., 2009). Further research into the origin of this increased lymphocytic component is needed to delineate its role in disease enhancement.

Similarly to BALF cytology, we observed in lung histology that the majority of pigs from the mismatched vaccine groups showed moderate to marked submucosal lymphocytic infiltration, which was mild or absent in MV/C pigs (Figure 3.5.). Overall these results are suggestive of immunopathological pulmonary lymphocytosis, a phenomenon which has been previously described in humans as a consequence of immune complex-mediated severe 2009 pandemic influenza disease (Monsalvo et al., 2011). It remains to be determined whether a similar mechanism induces these types of phenomena in our study and if the large numbers of lymphocytes observed in BALF cytology and lung histology mediated a critical role in the exacerbation of lung pathology. In addition to increased accumulation of lymphocytes, we observed intensified alveolar septal thickening and necrotizing bronchitis and bronchiolitis that ranged from moderate to marked in the majority of animals in these groups. Interestingly, while we did not detect any substantial

differences in the mean composite and right lung scores between the different heterologous vaccine groups, we observed that only the bivalent and heterologous-boosted group demonstrated statistically significant differences in the left lung scores compared to the MV/C animals (Figure 3.5.).

Khurana *et al*. previously suggested that non-neutralizing antibodies that target the conserved stem domain of the HA can increase virus infectivity by enhancing the fusion kinetics of heterologous viruses, and are implicated in the pathogenic mechanism responsible for the induction of VAERD (Khurana et al., 2013). However, enhanced virus shedding and pulmonary virus replication is not a consistent finding in earlier VAERD studies, although it has been reported previously (Rajao et al., 2014; Rajão et al., 2014; Souza et al., 2018). In the current study, we noted that in addition to the severe pulmonary pathology observed in all heterologous vaccine groups, which is consistent with previous VAERD studies (Gauger et al., 2012; Gauger et al., 2011; Kimble et al., 2022; Souza et al., 2016; Vincent et al., 2008), mismatched regimens failed to reduce virus shedding following challenge with the GA19 virus (Figure 3.4A). Moreover, we observed that the virus load in respiratory tissue homogenates of these groups was comparable to that of MV/C pigs (Figure 4B-D). Interestingly we noted that virus titers in respiratory tissues from pigs immunized with the heterologous prime-boost regimen consistently trended higher and, particularly in left lung samples, were found significantly increased compared to mock-vaccinated pigs (Figure 3.4E).

In conclusion, we showed that heterologous prime-boost vaccination failed to extend the scope of HAI cross-reactivity against antigenically distinct influenza variants. Additionally, while the differences per parameter were not significant, overall heterologous-boosted pigs demonstrated higher clinical disease severity, pulmonary virus load, and lung pathology than the pigs immunized with the mismatched monovalent vaccines. Further research is needed to fill the gaps in existing

knowledge and to deepen our understanding of the deregulatory mechanisms that play a part in exacerbating influenza disease following vaccination, particularly mechanisms such as antibody dependent enhancement of influenza virus entry and cell-mediated and proinflammatory cytokine responses that can set off this phenomenon.

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3.8. Tables

Table 3.1.

Table 1: Experimental design of vaccination-challenge study

Groups	*Number of Animals*	*Vaccine Type*	*Prime (Day 0 pp)*	*Boost (Day 21 pp)*	*Challenge (Day 51 pp)*
MV/NC	3	-	ISA 15	ISA 15	-
MV/C	6	-	ISA 15	ISA 15	GA19 (H1N2)
mono GA19 (x2)	6	Monovalent	GA19 WIV (H1N2)	GA19 WIV (H1N2)	GA19 (H1N2)
mono MN21 (x2)	6	Monovalent	MN21 WIV (H1N1)	MN21 WIV (H1N1)	GA19 (H1N2)
mono CA09 (x2)	6	Monovalent	CA09 WIV (H1N1)	CA09 WIV (H1N1)	GA19 (H1N2)
biv CA09-MN21 (x2)	6	Bivalent	CA09 WIV (H1N1)+ MN21 WIV (H1N1)	CA09 WIV (H1N1)+ MN21 WIV (H1N1)	GA19 (H1N2)
mono CA09 → mono MN21	6	Monovalent Heterologous Prime-Boost	CA09 WIV (H1N1)	MN21 WIV (H1N1)	GA19 (H1N2)

MV/NC mock-vaccinated/non-challenged, ***MV/C*** mock-vaccinated/challenged, ***mono*** monovalent, ***biv*** bivalent, ***GA19*** A/swine/Georgia/27480/2019, ***MN21*** A/swine/Minnesota/A02636116/2021, ***CA09*** A/California/07/2009, ***WIV*** whole-inactivated virus, ***pp*** post-prime

Table 3.2.

Table 2: HAI cross-reactivity with representative swine and human IAVs						
		Vaccine Groups				
Tested Viruses	Designated Clade/ Global Nomenclature	mono GA19	mono MN21	mono CA09	biv CA09+MN21	mono CA09 → mono MN21
A/swine/Minnesota/A02636116/2021/H1N1	alpha/1A.1.1	<10	853	17	560	267
A/swine/North Carolina/A02246984/2021/H1N1	alpha/1A.1.1	<10	620	11	333	213
A/swine/South Dakota/A01823237/2015/H1N2	alpha/1A.1.1	<10	427	<10	240	103
A/swine/Kansas/A01785470/2018/H1N1	beta/1A.2	<10	12	27	19	17
A/swine/Missouri/A01432837/2013/H1N2	beta/1A.2	<10	18	50	32	28
A/swine/Indiana/A01732425/2016/H1N1	gamma/1A.3.3.3	<10	<10	73	40	53
A/swine/North Carolina/A02245704/2020/H1N1	gamma/1A.3.3.3	<10	<10	34	18	17
A/swine/South Dakota/A01349306/2013/H1N1	gamma2/1A.3.2	<10	<10	88	27	73
A/California/07/2009/H1N1	pdm/1A.3.3.2	<10	10	1120	720	880
A/swine/Colorado/A02635828/2021/H1N1	pdm/1A.3.3.2	<10	<10	667	427	533
A/swine/Oklahoma/A01290605/2013/H1N1	delta1b/1B.2.2.1	22	<10	<10	<10	11
A/swine/Iowa/A02431617/2019/H1N1	delta1a/1B.2.2.2	57	<10	11	<10	<10
A/swine/Illinois/A02214842/2017/H1N2	delta2/1B.2.1	187	<10	<10	<10	<10
A/swine/North Carolina/A02245213/2019/H1N1	delta2/1B.2.1	147	<10	<10	<10	<10
A/swine/Georgia/27480/2019/H1N2	delta2/1B.2.1	280	<10	<10	<10	<10
A/Puerto Rico/8/1934/H1N1	delta-like/1B.2	21	15	10	11	<10
A/New Caledonia/20/1999/H1N1	delta-like/1B.2	77	<10	<10	<10	<10
A/Brisbane/59/2007/H1N1	delta-like/1B.2	83	<10	<10	<10	<10
A/Michigan/45/2015/H1N1	pdm/1A.3.3.2	<10	15	613	227	427
A/Michigan/272/2017/H1N1	pdm/1A.3.3.2	<10	<10	480	187	313
A/Wisconsin/588/2019/H1N1	pdm/1A.3.3.2	<10	<10	297	173	153

mono monovalent, ***biv*** bivalent, ***GA19*** A/swine/Georgia/27480/2019, ***MN21*** A/swine/Minnesota/A02636116/2021, ***CA09*** A/California/07/2009, ***pdm*** pandemic

HAI Antibody Titer: □ **< 40** □ **40-80** □ **81-160** □ **161-320** □ **>320**

Table 3.2. Heterologous-boosting did not expand cross-reactive HAI antibody responses against antigenically distinct swine and human IAVs. Serum samples from all groups collected at day 35 pp (two-week post-boost) were assessed for hemagglutination inhibition antibody titers

against a broad panel of representative swine and human influenza viruses isolated over the last century. The initial dilution of serum used for the HAI assay was 1:10. Each row represents the mean HAI titers per vaccine group against the corresponding IAV strain listed in the table. HAI titers above 40 are considered seroprotective. Mean antibody titers are color-coded based on levels of cross-reactivity.

3.9. Figures

Figure 3.1.

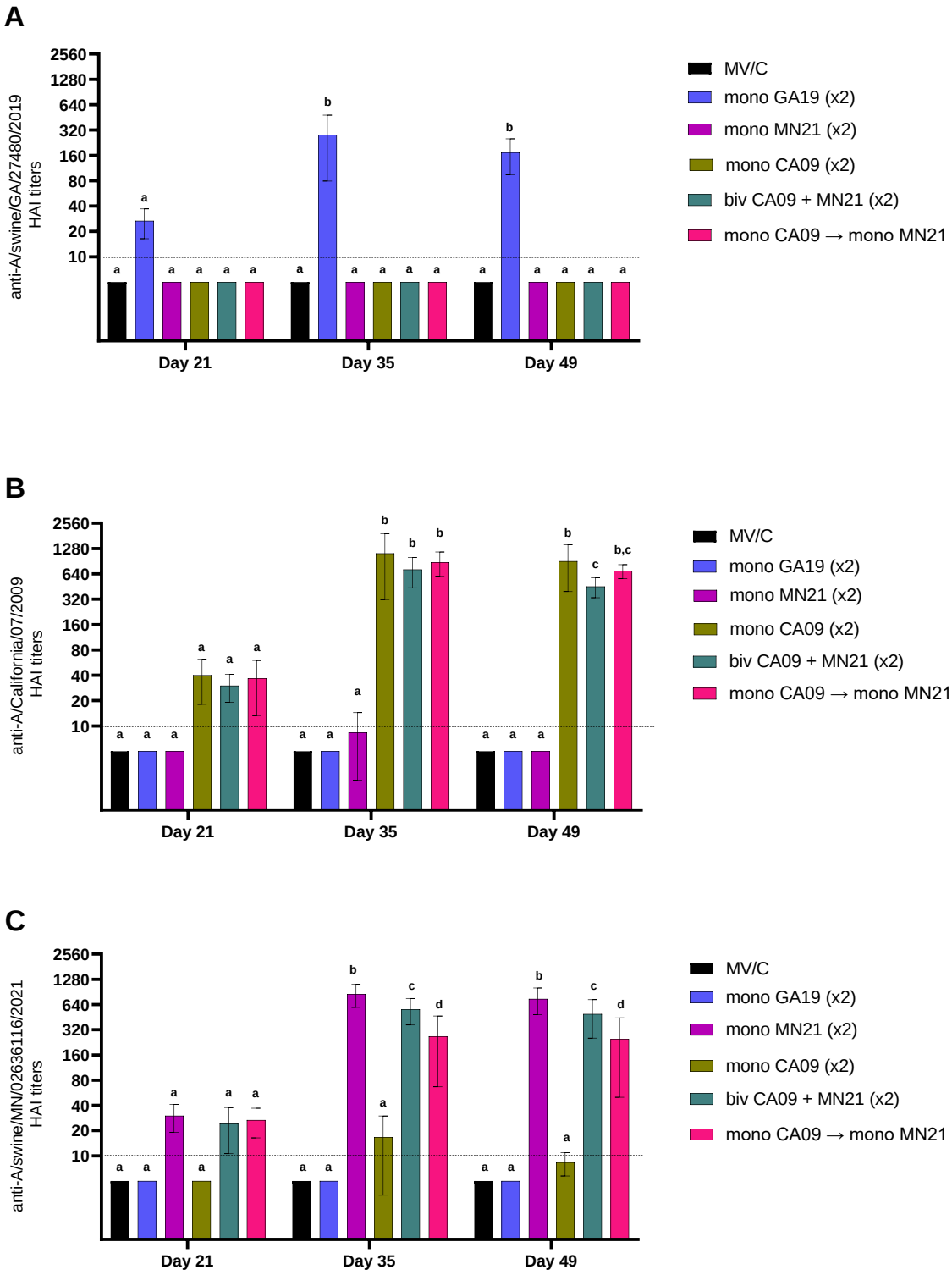


Figure 3.1. Heterologous prime-boost vaccination did not augment HAI antibody titers against vaccine constituents compared to homologous monovalent and bivalent prime-boost vaccination regimens. Five groups of pigs (n=6) were vaccinated in a homologous or a heterologous prime-boost regimen 3 weeks apart at day 0 and day 21. Serum samples from the groups were assessed on day 21 at boost vaccination, day 35, and day 49 pp (day -2 pre challenge) for hemagglutination inhibition titers against A/California/07/2009 H1N1, A/swine/Minnesota/A02636116/2021 H1N1 and A/swine/Georgia/27480/2019 H1N2 challenge virus. Antibody titers are compared between homologous-boosted monovalent GA19 (blue), MN21 (purple), and CA09 (olive) groups, bivalent CA09+MN21 vaccine group (cyan) and heterologous-boosted vaccine group (crimson red). Bars represent geometric mean HAI titers and error bars represent 95% confidence interval (CI). Statistical analysis was performed by two-way ANOVA test. Statistically significant differences ($p<0.05$) between vaccine group geometric means are noted by different lowercase letters.

Figure 3.2.

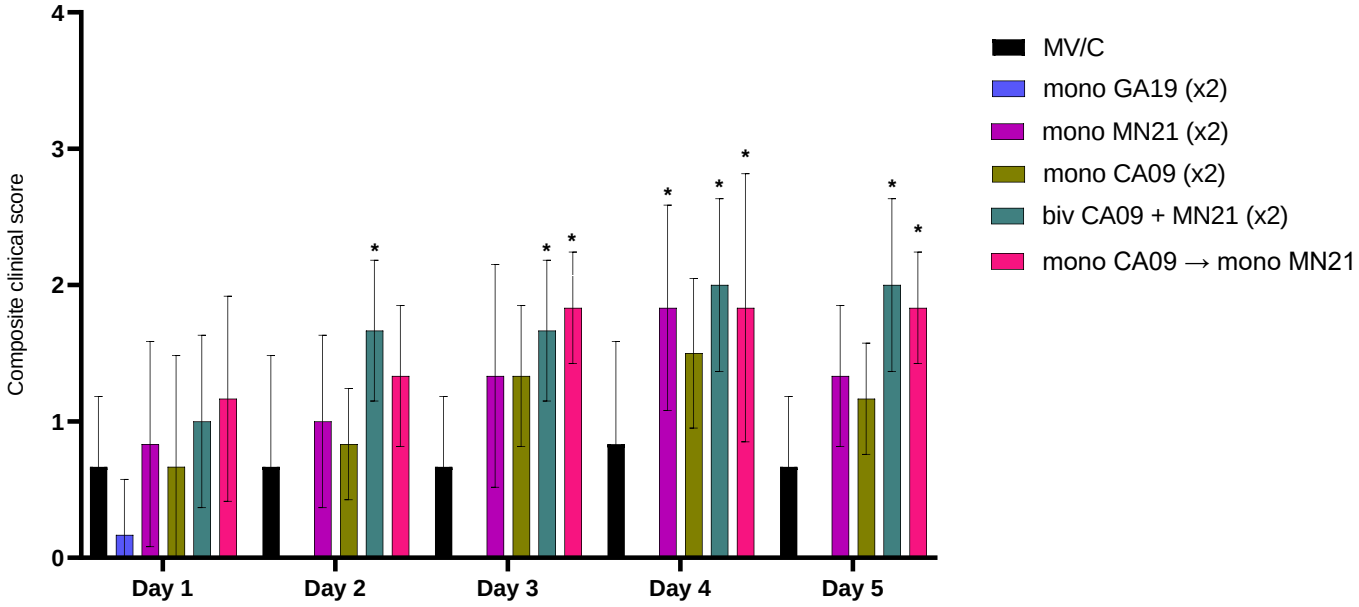


Figure 3.2. Bivalent and heterologous-boosting vaccination strategies enhanced and prolonged clinical disease following challenge with A/swine/Georgia/27480/2019. Clinical scores including rectal temperature, respiratory rate, and assessment of clinical demeanor (coughing and weakness/depression) were recorded daily following infection with the A/swine/Georgia/27480/2019 H1N2 challenge virus. Specifically, rectal temperature score ranged from 0 to 3 (< 39.4°C = 0, 39.4-39.9°C = 1, 40-40.5°C = 2, > 40.6°C = 3), respiratory rate per minute score ranged from 0 to 2 (20-40 = 0, 41-59 = 1, > 60 = 2) and clinical behavior scores, based on coughing (absent = 0, present = 1) and depression (absent = 0, present = 2) ranged from 0 to 3. Bars represent the geometric mean of clinical scores and error bars 95% CI. Statistical analysis was performed by a two-way ANOVA test. Statistically significant differences ($p<0.05$)

in clinical scores means between MV/C and experimental vaccine groups per day are indicated by an asterisk (*).

Figure 3.3.

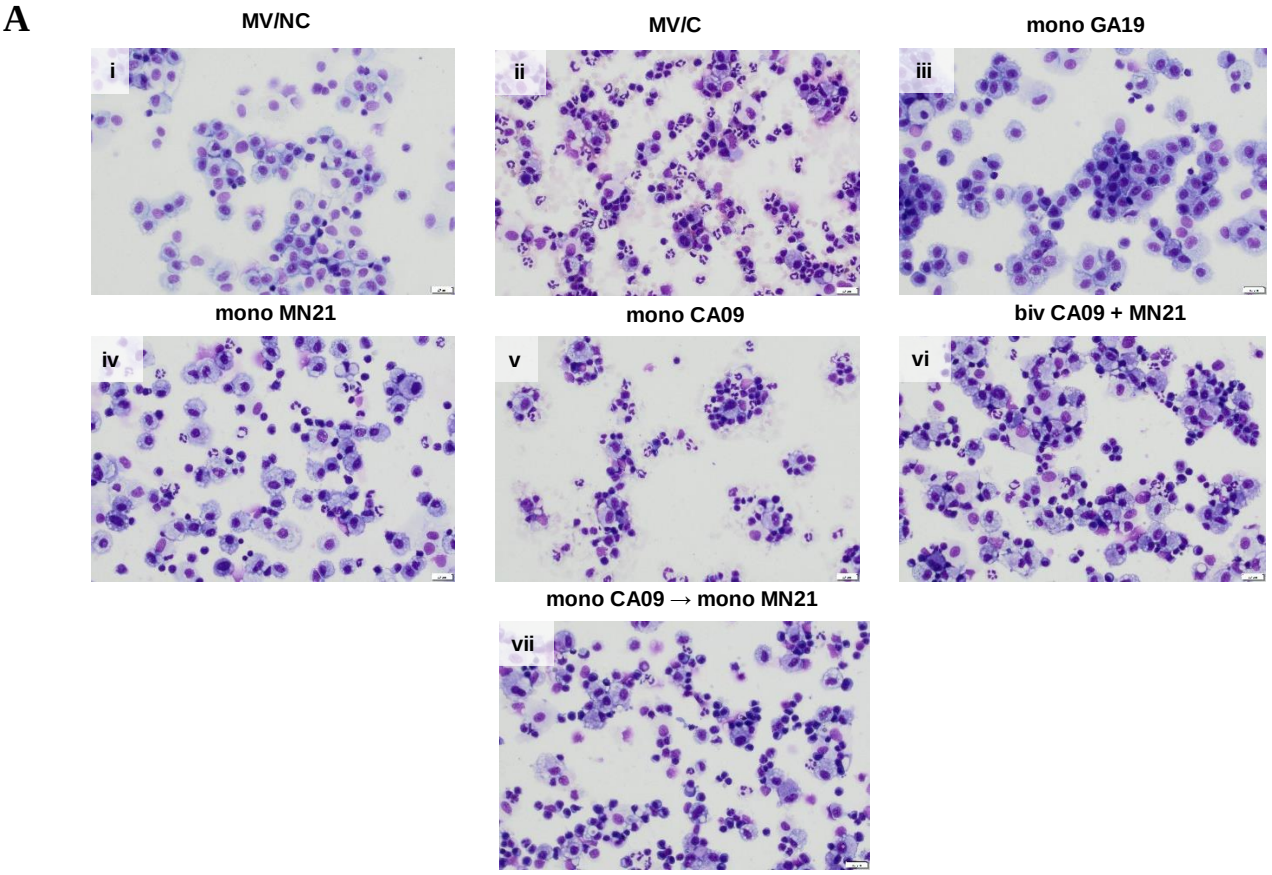

A
MV/NC
MV/C
mono GA19
i
ii
iii
mono MN21
mono CA09
biv CA09 + MN21
iv
v
vi
mono CA09 → mono MN21
vii
B
100
50
0
% cells in BALf
Macrophages
Neutrophils
Lymphocytes
MV/NC
MV/C
mono GA19 (x2)
mono MN21 (x2)
mono CA09 (x2)
biv CA09 + MN21 (x2)
mono CA09 → mono MN21

Figure 3.3. Mismatched swine influenza vaccination regimens elicited different inflammatory cytology profiles in BALF after influenza infection compared to the MV/C group. Bronchoalveolar lavage fluid (BALF) was harvested from lungs at day -2 pre-challenge for the MV/NC group and at day 5 pc from infected groups. (A) Concentrated cells were added to slides and stained with Wright-Giemsa stain and differential cell counts were determined microscopically (i) MV/NC group (ii) MV/C group (iii) mono GA19 vaccine group (iv) mono MN21 vaccine group (v) mono CA09 vaccine group (vi) bivalent CA09+MN21 bivalent vaccine group and (vii) mono CA09→ mono MN21 heterologous-boosted vaccine group (20 μm scale bar, 40x magnification) (B) Cell counts were expressed as a percentage of total cells. Black bars represent the mean percentage of macrophages, cyan bars the mean percentage of neutrophils, and pink bars represent the mean percentage of lymphocytes in each experimental group. Error bars represent the Standard Error of Mean (SEM). Statistical analysis was performed by two-way ANOVA test. Statistically significant differences ($p<0.05$) in population percentages between MV/C and experimental vaccine groups per cellular element are indicated by an asterisk (*).

Figure 3.4.

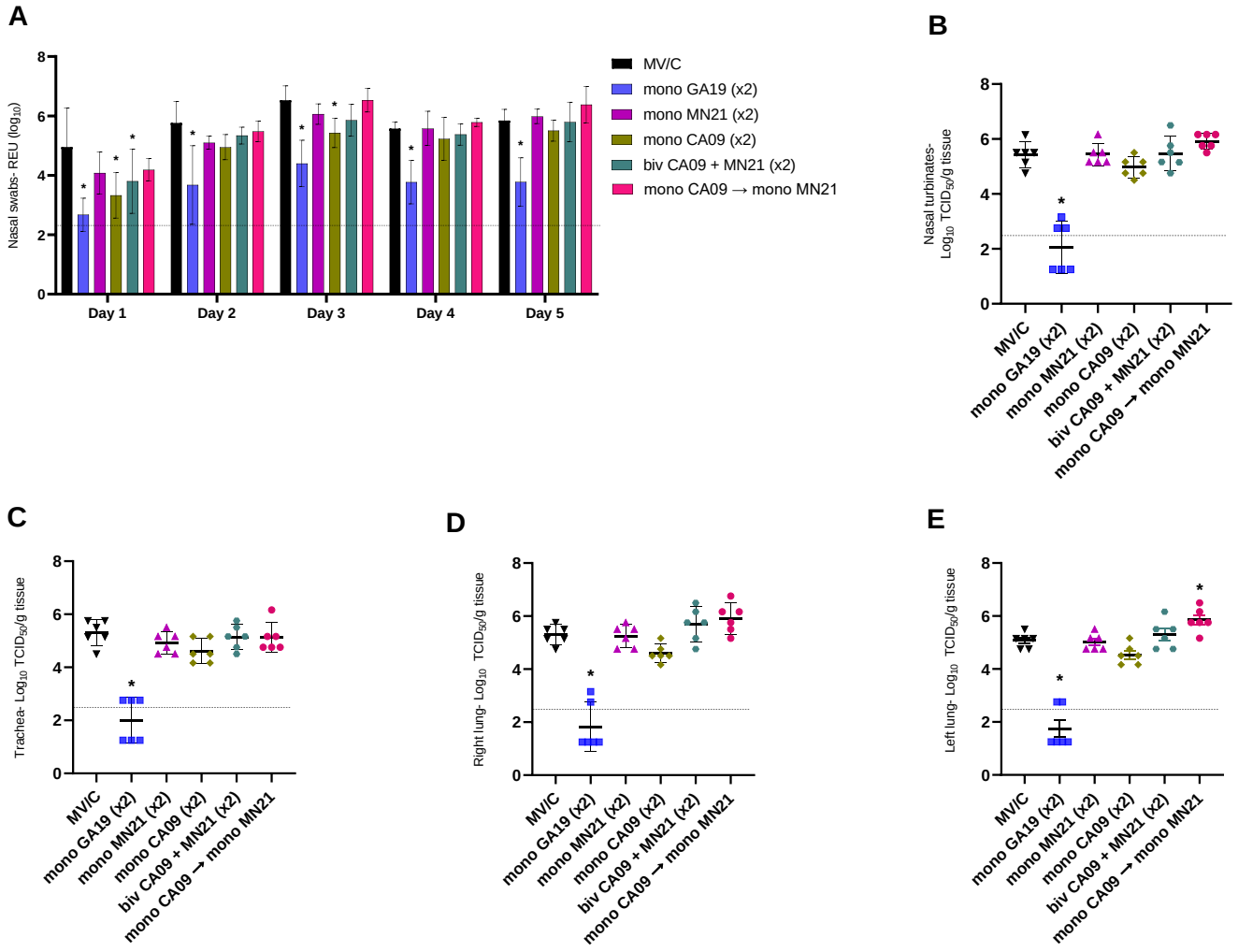


Figure 3.4. Heterologous vaccination strategies failed to reduce virus shedding and replication in the respiratory tract. Nasal swabs were collected from pigs daily post-challenge through euthanasia on day 5. Following necropsy, tissue samples from the respiratory tract including nasal turbinates, trachea, right lung lobes including the accessory lung lobe, and left lung lobes were harvested at day 5 post-challenge. Virus load in nasal swab samples was assessed by

an RT-PCR assay. Titers were measured as relative equivalent units (REU) of RNA corresponding to a 10-fold dilution series of RNA extracted from infective MDCK culture medium at a 10^7 $TCID_{50}$ of the A/swine/Georgia/27480/2019 H1N2 challenge virus. Virus replication in the respiratory tract was determined via $TCID_{50}$ assay in the respiratory tissue homogenates. (A) Mean viral titers from nasal swabs. (B) Mean viral titers of homogenized nasal turbinates. (C) Mean viral titers of homogenized trachea tissue. (D) Mean viral titers of right lung lobe tissue homogenates. (E) Mean viral titers of left lung lobe tissue homogenates. Black triangles represent MV/C pigs, blue squares represent GA19 vaccinated pigs, purple triangles represent MN21 vaccinated animals, olive rhombuses represent CA09 vaccinated pigs, cyan hexagons represent bivalent CA09+MN21 vaccinated pigs and crimson red circles represent heterologous-boosted pigs. For A, bars represent mean virus titers and errors bars represent 95% CI. For B to E, horizontal lines represent the mean virus titer per homogenate for each group. Error bars represent 95% CI. Dotted lines indicate the limit of detection. Statistical analysis was performed by two-way ANOVA test (A), one-way ANOVA test (B, E) and Kruskal-Wallis test (C, D). Statistically significant differences ($p<0.05$) in mean virus titer between MV/C and experimental vaccine groups are indicated by an asterisk (*).

Figure 3.5.

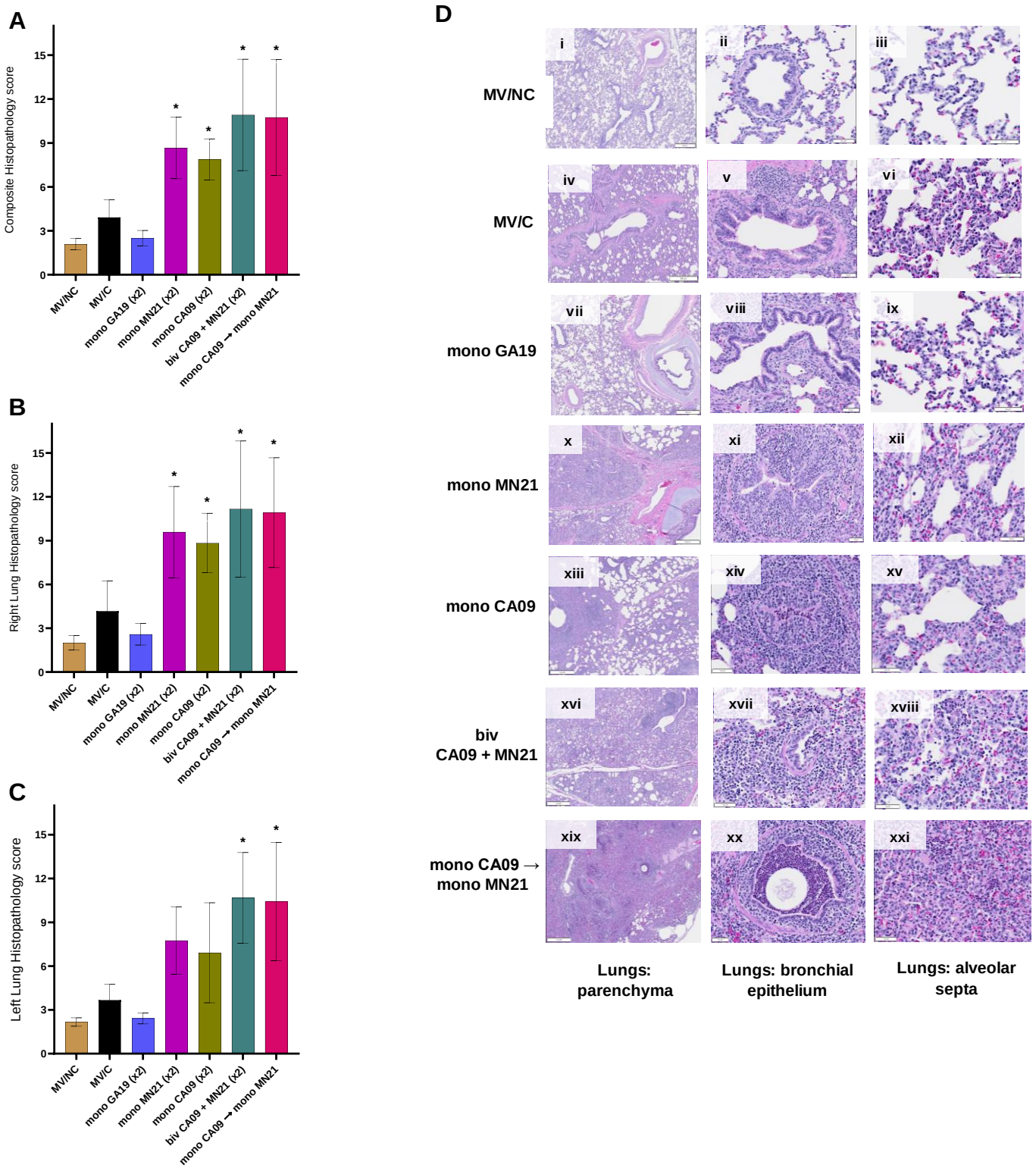

A
Composite Histopathology score
B
Right Lung Histopathology score
C
Left Lung Histopathology score
MV/NC
MV/C
mono GA19 (x2)
mono MN21 (x2)
mono CA09 (x2)
biv CA09 + MN21 (x2)
mono CA09 → mono MN21
D
MV/NC
MV/C
mono GA19
mono MN21
mono CA09
biv CA09 + MN21
mono CA09 → mono MN21
Lungs: parenchyma
Lungs: bronchial epithelium
Lungs: alveolar septa

Figure 3.5. Heterologous-boosting exacerbated pulmonary pathology following challenge with A/swine/Georgia/27480/2019. Lung tissues were collected at necropsy 5 days post-challenge and were fixed in formalin. Five µm sections were stained with H&E and examined by light microscopy. Lung sections were scored by two individuals including a board-certified veterinary pathologist. (A) Composite pulmonary histopathology scores from each experimental group. (B) Right lung mean histopathology scores. (C) Left lung mean histopathology scores. Bars represent mean histopathology scores and errors bars represent 95% CI. Statistical analysis was performed by one-way ANOVA test. Statistically significant differences ($p<0.05$) in mean histopathology score with MV/C and experimental vaccine groups are indicated by an asterisk (*). (D) H&E-stained representative lung tissue sections from each experimental group. Parenchyma 500 µm scale bar (2x magnification), bronchial epithelium 50 µm scale bar (20x magnification); alveolar septa 20 µm scale bar (40x magnification).

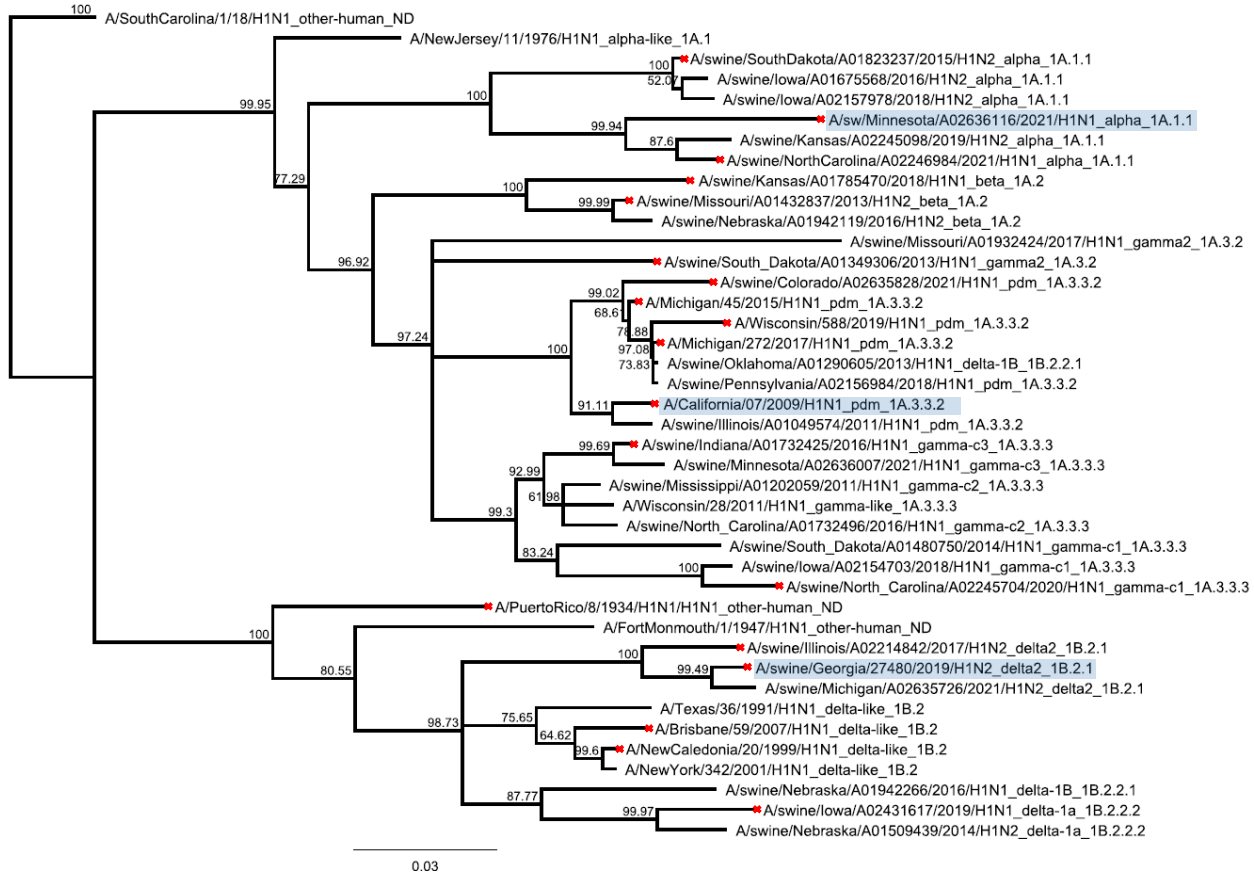
3.10. Supplemental Material

Supplemental Table 3.1.

Supplemental Table 1: Amino acid homology of swine and human influenza virus strains used in HAI assays with the HA of the vaccines strains

		Vaccine Strains		
	Designated Clade/ Global Nomenclature	A/California/07/2009 (H1N1)	A/sw/Minnesota/A02636116/2021 (H1N1)	A/swine/Georgia/A027480/2019 (H1N2)
A/swine/Minnesota/A02636116/2021/H1N1	alpha/1A.1.1	83.58%	100%	77.52%
A/swine/North Carolina/A02246984/2021/H1N1	alpha/1A.1.1	83.57%	97.16%	76.81%
A/swine/South Dakota/A01823237/2015/H1N2	alpha/1A.1.1	84.98%	93.44%	76.99%
A/swine/Kansas/A01785470/2018/H1N1	beta/1A.2	89.22%	82.16%	75.80%
A/swine/Missouri/A01432837/2013/H1N2	beta/1A.2	89.93%	83.04%	76.86%
A/swine/Indiana/A01732425/2016/H1N1	gamma/1A.3.3.3	92.05%	82.16%	78.62%
A/swine/North Carolina/A02245704/2020/H1N1	gamma/1A.3.3.3	89.75%	81.98%	76.50%
A/swine/South Dakota/A01349306/2013/H1N1	gamma2/1A.3.2	92.23%	82.51%	78.45%
A/California/07/2009/H1N1	pdm/1A.3.3.2	100%	83.58%	77.92%
A/swine/Colorado/A02635828/2021/H1N1	pdm/1A.3.3.2	95.23%	83.04%	77.39%
A/swine/Oklahoma/A01290605/2013/H1N1	delta1b/1B.2.2.1	77.74%	76.64%	77.74%
A/swine/Iowa/A02431617/2019/H1N1	delta1a/1B.2.2.2	78.27%	76.45%	87.61%
A/swine/Illinois/A02214842/2017/H1N2	delta2/1B.2.1	78.80%	76.28%	95.22%
A/swine/North Carolina/A02245213/2019/H1N1	delta2/1B.2.1	77.74%	76.28%	94.87%
A/swine/Georgia/27480/2019/H1N2	delta2/1B.2.1	77.52%	77.92%	100%
A/Puerto Rico/8/1934/H1N1	delta-like/1B.2	81.27%	79.47%	84.42%
A/New Caledonia/20/1999/H1N1	delta-like/1B.2	79.86%	78.41%	92.57%
A/Brisbane/59/2007/H1N1	delta-like/1B.2	79.33%	77.70%	92.04%
A/Michigan/45/2015/H1N1	pdm/1A.3.3.2	96.82%	83.57%	77.74%
A/Michigan/272/2017/H1N1	pdm/1A.3.3.2	96.29%	83.39%	77.74%
A/Wisconsin/588/2019/H1N1	pdm/1A.3.3.2	95.05%	83.39%	77.21%

Supplemental Figure 3.1.



ND non-defined, ***pdm*** pandemic

Supplemental Figure 3.1. Phylogenetic tree showing classification and antigenic relationship of the HA gene segment between the influenza strains employed in this study.

A phylogenetic tree was constructed based on amino acid sequences of representative H1 North American swine influenza viruses and human influenza viruses isolated over the last century. Amino acid sequences were aligned using MAFFT version 7 and edited using Geneious Software. In Geneious, we created a neighbor-joining tree using Jukes-Cantor genetic distance model and A/South Carolina/1/18/H1N1_other-human_ND as an outgroup. The tree was resampled

1,000,000 times using Bootstrap as a resampling method. All branch lengths are drawn to scale. The scale bar indicates the number of amino acid changes per site. Vaccine viruses are highlighted in blue color. Viruses employed in the HAI assays are labelled with a red mark.

4. Chapter IV: Concluding Remarks

4.1. General Discussion

The perennial threat posed by seasonal influenza epidemics and the emergence of zoonotic IAV strains with pandemic potential necessitate the development of broadly protective influenza vaccines for both human and animal health. Although significant progress has been made toward the advancement of influenza vaccines, rapid antigenic evolution and the continued diversification of IAVs pose a great challenge to the development of "broadly protective" or "universal" vaccine technologies and strategies (Angeletti and Yewdell, 2018; Chen et al., 2021). To date, multivalent inactivated vaccines are the most commonly used platform in both human and swine health throughout the world (Nuwarda et al., 2021). These vaccines only offer protection against homologous challenge, while they show limited or no protection against antigenically divergent viruses of the same subtype (Everett et al., 2019; Lorusso et al., 2011; Vincent et al., 2017). This reflects the fact that current commercial influenza vaccines both in humans and animals predominantly target non-conserved strain-specific epitopes present on the immunodominant globular head of the hemagglutinin (HA) (Andrews et al., 2018; Nachbagauer et al., 2021).

As a result of antigenic drift, seasonal influenza vaccines in humans need to be annually updated to reflect antigenic changes on the HA head and avoid a mismatch between the vaccine and circulating strains (Kim et al., 2018). To eliminate this necessity, novel influenza vaccine development has recently been focused on targeting conserved influenza antigens such as the neuraminidase (NA) protein and the stem domain of the HA protein (Nguyen and Choi, 2021). In the work described in this dissertation, we assessed the immunogenicity and protective efficacy of

two novel influenza vaccine strategies, an NA-based and an HA-based vaccine approach, against heterologous challenge in the swine model, a benchmark translational animal model for humans.

In Chapter II, we investigated a novel neuraminidase 2 (NA2) virus-like particle (VLP) platform in the swine model as a candidate broadly-protective influenza vaccine. Previous studies demonstrated that NA VLP vaccination is highly immunogenic in the murine and ferret animal models and has the potential of inducing broad protection against antigenically distinct influenza viruses even following lethal heterosubtypic challenge (Kim et al., 2019; Quan et al., 2012; Smith et al., 2017). To our knowledge, our study is the first one to explore NA-specific immunization in the swine model.

In our study, we utilized a VLP construct expressing the NA2 protein derived from the human A/Perth/16/2009 H3N2 virus. Following vaccination, we demonstrated by performing two NA-specific serological assays, IgG NA ELISA and neuraminidase inhibition (NAI) assay, that the NA2 VLP construct was highly immunogenic and induced functional NA-targeting antibody responses in the swine model. On the other hand, while pigs vaccinated with a commercial quadrivalent whole inactivated virus (QWIV) swine influenza vaccine elicited hemagglutination-inhibiting (HAI) antibodies, they did not elicit functional anti-NA antibodies capable of inhibiting the NA enzymatic activity. In addition to humoral immunity, we assessed cell-mediated immune responses and we observed that NA2 VLP vaccination generated robust challenge-virus specific INF-γ and TNF-α ELISPOT responses that were comparable to the commercial QWIV vaccine.

Next, to characterize clinical protection following challenge with the heterologous A/swine/NorthCarolina/HK1552516/2016 virus, an H3N2 swine field isolate, we evaluated the differential bronchoalveolar lavage fluid (BALF) cytology profiles between the groups. The BALF

cytology of the mock-vaccinated animals was characterized by a marked neutrophilic inflammation, which is a clear indication of more severe disease compared to NA2 VLP and QWIV vaccinated animals, where the vast majority of cells were resident macrophages with the presence of only rare non-degenerate neutrophils and small lymphocytes. In addition to clinical protection, we showed that the NA2 VLP vaccine significantly reduced not only pulmonary viral load but also lung inflammation, specifically the levels of several pro-inflammatory cytokines, in the lower respiratory tract. Lastly, we examined the vaccine's protective efficacy from the development of severe pulmonary lesions after heterologous challenge. Whereas mock-vaccinated animals exhibited high-severity lesions in regard to interstitial thickening of the alveolar septa and peribronchiolar and perivascular lymphocytic cuffing, QWIV and NA2 VLP immunized pigs demonstrated minimal to mild pulmonary pathology.

Overall, we demonstrated that while the NA2 VLP vaccine that we tested was comprised of a monovalent NA heterologous to the challenge strain without the presence of HA protein(s) in the vaccine formulation, it was able to confer comparable protection against disease and virus replication as the standardized commercial quadrivalent swine influenza vaccine. These results suggest that the novel NA2 VLP construct tested in our study is a promising platform for conferring immunity and protection against antigenically distinct homosubtypic IAVs. Additionally, in line with other studies, it highlights the outstanding potential of an NA-based vaccine either in supplementing current seasonal influenza vaccines or being utilized in combination with other platforms, arguably HA-specific vaccine approaches, towards the development of a universal influenza vaccine (Johansson et al., 1998; Kawai et al., 2021).

NA, the second most immunogenic influenza protein after hemagglutinin, has been widely neglected in commercial vaccine formulations, despite its antigenic potential to elicit broad

heterologous immune responses (Eichelberger and Monto, 2019; Giurgea et al., 2020). In contrast to anti-HA responses, which are mostly characterized as neutralizing, anti-NA responses induce infection-permissive immunity, which interferes with virion release, limits viral spread, and reduces the severity of clinical disease (Eichelberger and Wan, 2015; Johansson et al., 1993). In agreement with previous studies in mice, we observed that our NA2 VLPs vaccine reduced pulmonary virus replication and mitigated clinical disease and pathology (Easterbrook et al., 2012; Quan et al., 2012; Wohlbold et al., 2015). However, in contrast with the commercial QWIV vaccine tested, we did not detect any substantial decrease in virus titers in nasal turbinates and trachea tissue homogenates from pigs immunized with our experimental construct. While the fact that our NA2 VLP vaccine did not drastically reduce the virus load in the upper respiratory tract and achieve sterilizing immunity might appear as a major weakness of this platform, it might prove to be one of the biggest advantages of this vaccine technology.

Schotsaert and colleagues demonstrated the protective potential of NA-specific vaccine-induced responses against future pathogen encounters (Schotsaert et al., 2016). Specifically, mice priorly immunized with an intranasal H1-based or an N1-based platform were subsequently challenged with a sublethal dose of the pandemic A/Belgium/2009 H1N1 virus. While H1-immunized mice demonstrated sterilizing protection against the H1N1 virus, N1-based vaccination conferred infection-permissive immunity that resulted in curtailed but not absent virus replication in their airways. However, following a secondary challenge with a lethal dose of the heterosubtypic A/X47 H3N2 virus, whereas all N1-immunized mice survived the challenge and showed low virus titers in their lungs, mice vaccinated with the H1-based platform demonstrated approximately 90% lethality. The authors proposed that the infection-permissive immunity conferred by the NA-based platform allowed the development of $CD8^+$ responses following the H1N1 challenge that

ultimately protected mice from the subsequent lethal H3N2 exposure (Schotsaert et al., 2016). Besides cell-mediated protection, mucosal immune responses (IgA-secreting B cells) were not examined in that study, which could additionally have played an essential role in mediating cross-protection. These findings in combination with the results from our study suggest that NA-based vaccination may allow the induction of superior immunity and protection against antigenically divergent influenza viruses following boosting with future natural infections, compared to HA-based platforms, while still protecting the cohorts from experiencing severe clinical disease and pulmonary pathology. Future studies are needed to explore whether a similar protective mechanism could be established in NA-immunized swine following heterologous influenza infections.

VLPs are non-infectious non-replicating constructs that mimic the morphology and structural conformation of the native source viruses, but they lack genetic material (Nooraei et al., 2021; Zepeda-Cervantes et al., 2020). Their major advantage compared to other vaccine platforms is that they are highly immunogenic and customizable scaffolds that allow the incorporation of selected antigenic proteins in a high-density display on their surface (Mittal et al., 2022). This offers the opportunity of producing HA-based and NA-based VLPs separately to avoid any intravirionic antigenic competition between these two viral proteins. This phenomenon is commonly observed following influenza vaccination or infection, and often favors immune responses towards the immunodominant HA (Giurgea et al., 2020; Johansson and Kilbourne, 1993).

Based on the fact that HA is responsible for viral entry and NA for virion release, it is of pivotal importance for virus propagation to retain an optimal balance in the functional activities of these two proteins (Gaymard et al., 2016). Previous research has shown that influenza viruses achieve antiviral drug resistance or effective evasion of vaccine-induced immunity that is targeted against

HA or NA, through selecting compensatory mutations not exclusively in the targeted molecule but often in both viral proteins (Du et al., 2020; Mitnaul et al., 2000; Myers et al., 2013; Wang et al., 2021). This could be exploited by candidate universal influenza vaccine technologies that are utilizing combined HA and NA antigenic targets, on condition of immunogenic equivalence, to achieve balanced immune responses against these molecules. By putting antigenic pressure on both viral proteins, particularly against conserved functional epitopes on these antigens, novel vaccine technologies could curtail the rapid emergence of vaccine-escape mutants. That is because the effective restoration of the precarious HA/NA balance and viral fitness through synchronously selecting compensatory mutations on both viral proteins is more challenging (Gen et al., 2013; Johansson et al., 1998).

In addition to anti-NA antibodies, humoral responses targeting the HA stem domain have also been demonstrated to play an important role in the induction of cross-protection against antigenically distinct influenza strains (Krammer and Palese, 2013; Mallajosyula et al., 2014). Several novel candidate universal influenza vaccines strive to achieve durable antibody production towards the highly conserved stem domain of the HA protein (Krammer and Palese, 2019; Nachbagauer et al., 2017). A wide variety of stem-focused technologies have been developed during the last decade, including headless HA constructs, chimeric antigens with “exotic” globular heads on fixed stems, and computationally-derived consensus sequence HA structures (Giles and Ross, 2011; Impagliazzo et al., 2015; Krammer et al., 2013). However a major concern in targeting the HA stalk is the intrinsic poor immunogenicity of this domain compared to the immunodominant, yet highly variable globular head domain (Tan et al., 2019).

A recently explored vaccination strategy that employs sequential immunization with antigenically distinct influenza immunogens for prime and boost vaccinations, called heterologous prime-boost

vaccination, has previously shown interesting potential in expanding cross-reactivity against heterologous homosubtypic viruses (Chepkwony et al., 2020; Li et al., 2020; Parys et al., 2022b; Van Reeth et al., 2017). To our knowledge, only one study to date has explored H1-specific heterologous boosting in swine. This study utilized viruses circulating in European pig herd populations for their vaccine formulations (Parys et al., 2022a). Overall, heterologous boosting studies presumed that sequential vaccination with mismatched strains induces a memory recall to B cells that recognize conserved epitopes on the HA stem and head domains shared between the different immunogens, thus conferring broader cross-reactivity against antigenically divergent influenza strains (Pica et al., 2012).

In Chapter III, we examined the immunogenicity and protective efficacy of diverse heterologous H1 whole inactivated virus (WIV) vaccination strategies, including monovalent, bivalent, and heterologous prime-boost vaccination regimens, based on the three distinct contemporary North American swine H1 lineages against homosubtypic IAVs. We first demonstrated that while heterologous boosting and bivalent vaccination elicited seroprotective HAI titers against viruses included in the original vaccine formulation following two vaccine doses, these regimens were immunogenically inferior compared to the corresponding monovalent vaccines. To investigate whether heterologous boosting in swine could broaden humoral immune responses compared to the bivalent or monovalent vaccination, we performed HAI assays against a broad panel of representative heterologous swine and human H1 influenza viruses. Interestingly, we observed that heterologous prime-boost vaccination elicited HAI responses against viruses antigenically related to the prime vaccine virus A/California/07/2009 (CA09) H1N1 comparable to those of the monovalent CA09 vaccinated group. In contrast, serological responses against alpha-clade viruses that are antigenically related to the boost vaccine virus A/swine/Minnesota/A02636116/2021

H1N1 (MN21) dropped up to 4-fold compared to the monovalent MN21 vaccine group. Additionally, we did not observe any cross-reactivity to the antigenically-distant delta and delta-like swine and human IAVs in sera collected from the mismatched vaccinated groups.

Our findings are in agreement with a recently published study by Parys *et al*. that similarly did not observe an expansion of cross-reactivity in sera from pigs that were prime-boost immunized with heterologous whole-inactivated viruses (Vandoorn et al., 2022). Interestingly however, priming with an intranasally administered pseudorabies vectored vaccine followed by a mismatched WIV booster enhanced both humoral and cell-mediated immune responses and induced better protection against antigenically distinct to the vaccine viruses strains as compared to WIV-WIV heterologous-boosting (Parys et al., 2022a). Additionally, it would be interesting to examine whether a 3-dose heterologous prime-boost regimen would augment cross-reactive immunogenicity compared to the 2-dose regimen tested in our study. Further research is needed to determine if such an approach would enhance anti-HA responses and protection in the swine model.

Overall, the most interesting finding in Chapter III was that heterologous prime-boost vaccination, rather than inducing cross-protection, exacerbated several disease parameters following challenge with the mismatched A/swine/Georgia/27480/2019 H1N2 virus (GA19). Specifically, we noted that clinical signs of heterologous-boosted pigs were more severe over a prolonged period of time compared to the mock-vaccinated challenged (MV/C) pigs. Additionally, we showed that similarly to the other mismatched vaccination regimens included in our study, heterologous prime-boost vaccination induced an altered BALF cytology profile compared to that of mock-vaccinated animals that was characterized by lymphocytic inflammation. In contrast to homologous WIV GA19 vaccination which conferred sterilizing protection, heterologous-boosted pigs demonstrated

similar or even elevated nasal shedding and lung virus replication compared to MV/C animals. Furthermore, we observed that heterologous-boosting and bivalent vaccination regimens exacerbated macroscopic and microscopic pathology in both the right and left lung lobes compared to MV/C pigs.

Previous studies have shown that antibodies that target conserved subdominant epitopes on the HA stem can confer broader cross-reactive immune responses against antigenically distinct IAVs (Chiu et al., 2013; Dhar et al., 2020; Moin et al., 2022). However, cross-reactive antibodies specific to the conserved stem domain of the HA elicited by inactivated vaccines have also been implicated in the induction of vaccine-associated enhanced respiratory disease (VAERD) in swine (Khurana et al., 2013). VAERD-exhibiting animals typically demonstrate prolonged clinical disease and enhanced pulmonary pathology compared to non-vaccinated pigs following challenge (Rajao et al., 2014). This phenomenon has been most commonly observed in swine vaccinated with monovalent WIV vaccines co-formulated with an oil-in-water adjuvant and subsequently challenged with a heterologous homosubtypic swine IAV strain (Gauger, 2012; Gauger et al., 2012; Gauger et al., 2011; Rajao et al., 2016; Vincent et al., 2008). A similar vaccination-challenge regimen was used in the groups vaccinated with the monovalent CA09 and MN21 WIV constructs in our study. Notably however, in our study we observed exacerbated clinical disease and pathology not only in the mismatched monovalent vaccine groups but additionally in animals immunized with the mismatched bivalent and heterologous-boosting regimen.

While the exact mechanisms that induce vaccine-induced enhanced disease are still under investigation, it has been suggested that humoral immune responses are primarily responsible for this phenomenon. Specifically, it has been proposed that an antibody-dependent enhancement of virus infection and disease could be implicated in the induction of VAERD. Khurana *et al.*

demonstrated that vaccine-induced stem-domain targeting non-neutralizing antibodies (nNAbs) can enhance the fusion kinetics of the heterologous virus by binding to an epitope located in close proximity to the HA fusion peptide (Khurana et al., 2013). Hence, vaccine-induced humoral responses, rather than preventing viral entry, can enhance virus infectivity and exacerbate pulmonary disease following challenge with a virus antigenically distinct to the vaccine virus(es) strain.

A previous study in humans demonstrated that high levels of nNAbs can mediate immune complex formation resulting in exuberant inflammatory responses that could lead to severe clinical manifestation after heterologous infection (Monsalvo et al., 2011). A mechanistic pathway that would be interesting to investigate is whether the exacerbation of clinical disease and pulmonary injury exhibited in the mismatched vaccinated animals of our study is associated with an immune complex hypersensitivity reaction. This phenomenon could be characterized by immune complex formation and deposition in the alveolar tissues and extra-alveolar vascular walls leading to vasculitis and pulmonary immunothrombosis (Lee et al., 2020; Roncati et al., 2020). A schematic of this reaction is presented in Figure 4.1. It remains to be determined whether such responses may have contributed to the exacerbated disease and pathology observed in our study.

Vaccine-induced enhanced disease phenomena ought to be one of the prime concerns for the development of broadly protective human influenza vaccines, in particular “universal” vaccine platforms that consistently target conserved epitopes on the stem domain of the HA protein (Krammer and Palese, 2019). It is of critical importance to public health to investigate the pathogenic mechanisms that induce these phenomena and identify immunological correlates of protection or disease exacerbation in reliable translational animal models such as swine, for the development and evaluation of future vaccine interventions.

4.2. References

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4.3. Figures

Figure 4.1.

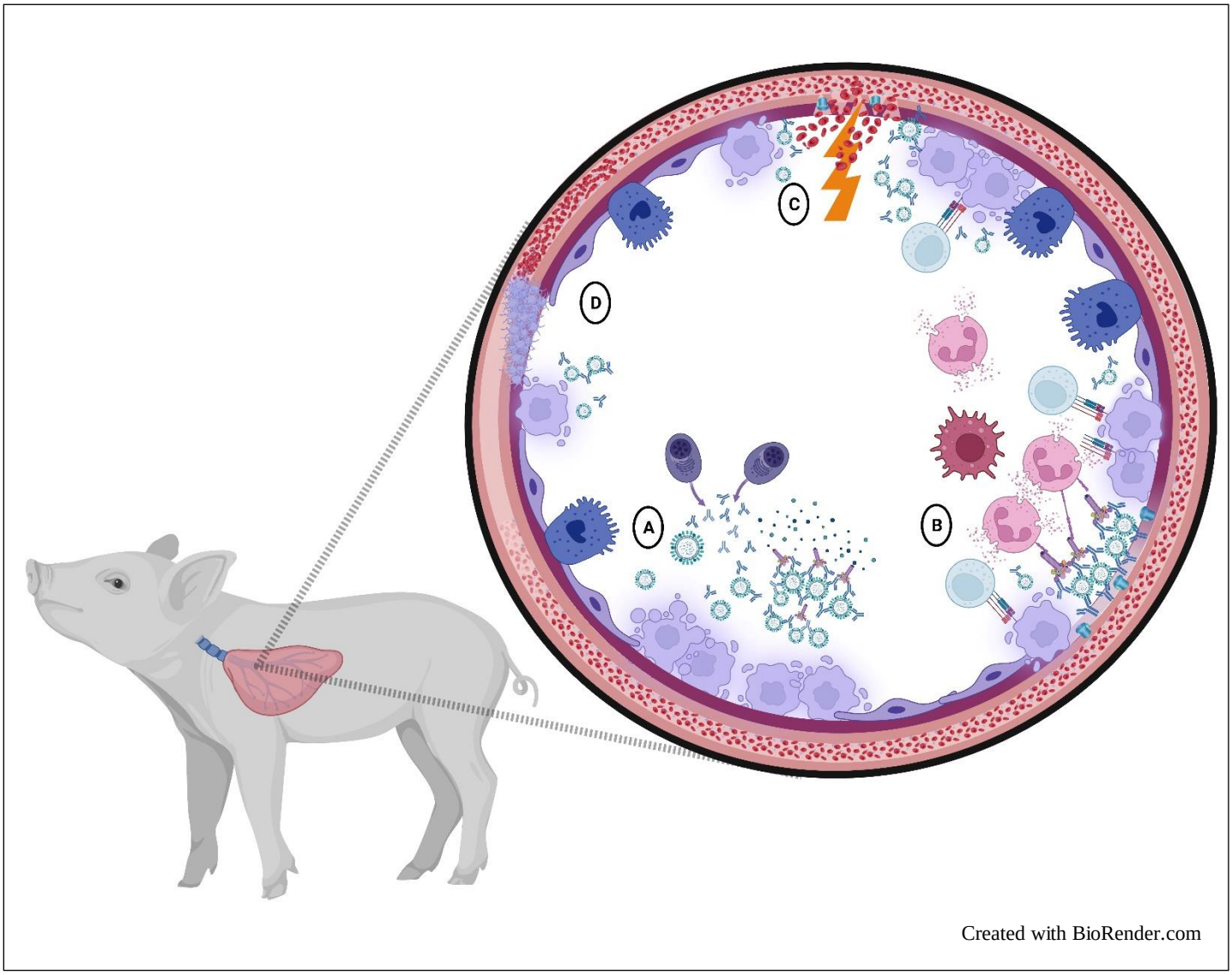

Figure 4.1. Proposed immune complex hypersensitivity reaction in the alveolar and extra-alveolar vascular spaces.

A) Following infection, influenza virus replication rapidly triggers humoral memory responses in vaccinated animals. The lack of shared epitopes on the globular head of the hemagglutinin (HA)

protein between the mismatched vaccine and challenge strains induces memory recall of B-cells that recognize conserved epitopes on the stem domain of the HA. Ample production of non-neutralizing antibodies can result in the generation of antibody-virion complexes in the alveolar spaces. Formation of immune complexes triggers complement activation, fixation of the C3b complement factor onto these compounds, and massive release of vasoactive amines and proinflammatory mediators such as the anaphylatoxins C3a and C5a. These inflammatory molecules have chemotactic activities that recruit effector cells to the site of injury. **B)** Neutrophil degranulation and generation of reactive oxygen species can exacerbate local inflammation and lung injury resulting in excessive tissue necrosis and sloughing of epithelial cells, exposing the underlying basal lamina. Accumulation of necrotic and inflammatory cells within bronchiolar and alveolar spaces could lead to airway obstruction. Excessive secretion of vasoactive amines causes endothelial cell retraction and increased vascular permeability, allowing the deposition of complement-antigen-antibody complexes not only in the lung tissues but additionally on the extra-alveolar vascular walls. **C)** The formation of the membrane attack complex and deposition of immune compounds onto the alveolar wall and subendothelial matrix could further exacerbate inflammatory lung injury, by disrupting the alveolar-capillary barrier thus inducing immune complex-mediated vasculitis-like lesions. **D)** Perturbations on the vessel wall and procoagulant complement factors trigger the activation of the coagulation cascade, promoting platelet adhesion and aggregation. In addition to the disruption of the pulmonary-endothelial barrier integrity, immune complexes have been previously shown to induce an Fcγ-mediated prothrombotic state. These conditions can favor the induction of localized intravascular coagulopathy and pulmonary immunothrombosis (Jevtic and Nazy, 2022; Kolb et al., 2022; Lee et al., 2020; Noris et al., 2020;

O’Donnell et al., 2021; Polack et al., 2003; Polack et al., 2002; Sweet et al., 2022; Yaugel-Novoa et al., 2022; Zanella et al., 2022).

This schematic figure illustrates potential mechanisms that could be involved in immune complex-mediated enhanced pulmonary pathology based on previous observations or conjectures from respiratory syncytial virus, measles virus, feline infectious peritonitis virus, and SARS-Cov2 infections. Shapes and figures are not drawn to scale.