PARADIGMS IN PORK: REASSESSING SWINE INFLUENZA PANDEMIC PREPAREDNESS AND VACCINE CONTROL MEASURES

by

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ABSTRACT

Swine influenza A viruses (swIAVs) pose a large economic burden on commercial swine production as well as a public health risk to human populations. Variant swIAVs annually infect humans in limited spillover events that indicate the potential for a future pandemic. Crucial to preempting these events is the establishment of a rigorous threat assessment framework for human populations. Utilizing animal models as well as primary cell culture systems our lab completed an assessment of a recent swIAV isolate, A/swine/GA/A27480/19. Our studies provided evidence that this virus may pose a risk to humans by demonstrating an ability to infect the human-derived Calu-3 cell line as well as naïve ferrets, which are widely used as a surrogate model for human infection and transmission. Critically, without adaptation to the ferret host the swine isolate was able to transmit to cohoused contact animals. Having established a health risk from the isolate we further investigated vaccine strategies within the weanling pig model. Vaccination with inactivated viruses related to the A/swine/GA/A27480/19 isolate has previously been found to result in vaccine-associated enhanced respiratory disease (VAERD) upon challenge with a heterologous virus. We developed a model of VAERD using the A/swine/GA/A27480/19 virus and A/California/04/2009 mismatch, further demonstrating a means of reducing pathology by mucosal priming with the virus. An intranasal route of inoculation was found to increase the number of tissue-resident T cells within the respiratory tract of animals compared to intramuscular vaccination. Characterization of the serological antibody responses after intramuscular or intranasal priming showed a non-neutralizing antibody response that may enhance viral replication within the host, however those

animals that were able to also mount a tissue-resident cell-mediated response at the mucosal interface were protected from VAERD. Together our studies provide evidence for a health risk posed to both humans and swine by an emerging lineage of swIAVs, and demonstrate the importance of mucosal immune responses in effective protection of weanling pigs from future influenza challenges.

INDEX WORDS: Pandemic Influenza, Vaccine, VAERD

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DEDICATION

This work is dedicated to my family and friends who have helped me through some of the most difficult and rewarding years of my life in pursuit of my doctorate. I also dedicate this work to my partner and first officer, Jed Dews, who pushes me to be the best version of myself.

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CHAPTER 1

INTRODUCTION

It is likely that influenza viruses have had repeated transmission events between human and swine populations for centuries, but the first well-documented instance of such was the infamous 1918 “Spanish flu” pandemic. Thought to have been initiated by a spillover from a sick pig to a soldier in Fort Funston, Kansas [1, 2], the virus was quickly transmitted across the country and then globally with the military transport lines. In fact the disease caused greater mortality than World War I, killing 25 million people within 25 weeks. As the pandemic subsided the etiological virus established stable lineages in both human and swine populations that would remain in circulation for decades, within swine becoming denoted as “classical” swine H1[3].

Contributing to the strains currently circulating in North American swine are those that have their lineages based in several human seasonal isolates. In the late 1990s human H3N2 contributed several genetic elements including surface HA and NA proteins as well as the polymerase basic 1 (PB1) to internal gene constellations that contained classical swine and avian segments[4]. This triple reassortant internal gene (TRIG) constellation quickly became a dominant isolate in North American swine herds. Further spillovers from human populations have served to drive a recent surge in swine influenza diversity[5]. In the early 2000s a human seasonal H1 reassorted with swine viruses, establishing the δ-clade of

human-origin swine influenza that has persisted to this day, and still causes severe disease in both swine and incidental human hosts [6]. The 2009 “swine flu” pandemic also has documented cases of spillback from human hosts into swine, again reassorting with circulating swine isolates to create another clade of swine H1[7, 8].

Disease pathogenesis in pigs mimics many of the symptoms seen in human disease, including coughing, sneezing, fever, anorexia and even death in severe cases[9]. This places a massive economic burden on the U.S. commercial swine industry, costing as much as $1 billion to producers [10, 11]. As such, research into understanding the constantly changing patterns of swine influenza evolution both within swine populations and at the interface with swine and people is a critical component for both public health and financial sectors. Recent research into swine vaccination practices have unveiled a troubling phenomenon. Common practice in commercial swine production includes vaccination with whole-inactivated virus against influenza, but as is the case with human influenza vaccine development, there’s frequent mismatch between the identity of the vaccine components and the challenge strains. This can lead to not only lowered efficacy of the vaccine, but even vaccine-associated enhanced respiratory disease (VAERD)[12-14]. The exact etiology of VAERD remains elusive, however a series of experiments investigating the role of HA-directed antibodies has demonstrated that these are crucial for recreating the VAERD phenotype[15]. A non-neutralizing serum antibody response with activity against the HA2-epitope serves to enhance fusion of the viral protein with the host cells and thereby increase viral replication[16]. A reduction in VAERD has been seen in use of a mucosal

administration of a live attenuated influenza virus, however the means by which this protection is mediated remains unknown.

The public health risk posed by swine influenza is seen annually in cases of variant swine influenza, in which limited spillovers from swine to people results in disease. While these have yet to become established pandemics, the historical documentation of influenza pandemics originating in swine would indicate that such an event is only a matter of time if given the opportunity. Among those variant swine influenza viruses, the δ-lineage constitutes a significant portion. With this in mind, the goal of our studies was to assess the pandemic risk potential of a contemporary swine influenza viruses belonging to this clade in a framework of animal infection and transmission models, as well as *in vitro* infections of human cell cultures[17-20]. We also recognized that recent isolate belongs to a lineage of influenza viruses that has been previously associated with VAERD within the swine host, and so investigated means by which this disease phenotype could be limited in vaccine development against the virus. This proposal examines these questions with the following specific aims:

Specific Aim 1: To assess the pandemic risk potential of the recently isolated swine influenza virus A/swine/Georgia/27480/2019 in the framework of phylogenetic analysis, animal infection and transmission models, and tissue culture systems as outlined in the CDC Influenza Risk Assessment tool[21]. Our hypothesis is that these models will illustrate the viruses capabilities to infect and transmit within its native host animal with in vivo and in vitro. We also believe that the models used as a surrogate for human infection, namely

ferrets and human primary respiratory epithelial cell cultures, will indicate whether this swine isolate poses a risk to humans.

Specific Aim 2: To investigate effective vaccination practices against the A/swine/Georgia/27480/ isolate in the weanling pig model. Our hypothesis is that the close genetic relationship of this isolate to other viruses used in the VAERD literature will allow us to develop a novel model of VAERD. By comparing immune responses elicited by either intramuscular or mucosal vaccination against the virus, we will demonstrate the importance of a tissue-resident cell-mediated immune response to protect animals from subsequent challenge to influenza, and prevention of the VAERD phenotype.

Knowledge obtained by the specific aims outlined above will contribute to improvement of pandemic preparedness against emerging swine influenza strains. Our vaccination studies will aid in the development of more effective influenza vaccine within swine as well as a universal influenza vaccine with increased activity against multiple viral subtypes.

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CHAPTER 2

LITERATURE REVIEW

Every year influenza infects millions of people around the globe, with an annual mortality of approximately 500,000 people. In the United States alone, there were an estimated 35 million flu-related illnesses and 20,000 flu-related deaths within the 2019-2020 flu season[1]. Seasonal influenza typically affects children <5 years old and adults 65 years or older with increased morbidity and mortality, accounting for more than 60% of influenza deaths in the 2019-2020 season. Uncomplicated influenza infection in people usually results in respiratory disease as well as symptoms such as fever, headache and myalgia[2]. The primary means by which human infection is controlled is by vaccination, however due to the virus's capability to mutate and exchange genetic information with related influenza viruses, there is a constant need to update vaccine formulations to better target emerging strains. The effectiveness of vaccines in people varies widely, dependent on a variety of factors including the age of the person vaccinated and the subtype and strain of the challenge virus. This leads to a vaccine effectiveness as low as 37% in some age groups[3].

Influenza viruses are enveloped viruses with a segmented, negative-sense RNA genome belonging to the family *Orthomyxoviridae*. This family encompasses influenza A, B, C, D as well as thogotovirus and isavirus[4]. Influenza A viruses can be further classified into subtypes based upon the expression of their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). The hemagglutinin protein decorates the surface of the viral envelope, and serves to both bind to sialic acid receptors on host cells as well as allow for subsequent release of viral contents from the endocytic vacuole into the host cell. The neuraminidase protein cleaves sialic acids present on both host cell

surfaces as well as nascent HA proteins, which allows maturing viral particles to be released from host cells[5, 6]. The hemagglutinin protein has been designated into 18 different subtypes while the neuraminidase protein has been categorized into 11 subtypes, which are displayed in various combinations with the HA protein. The majority of the combinations seen in nature are found among wild birds, however two of the most recently identified subtypes of both HA and NA proteins are found only in bat hosts[7, 8].

In order to initiate the viral life cycle, the influenza virus particle must first bind to a sialic acid receptor. These are found ubiquitously at the terminal end of cell surface glycoproteins. The viral HA protein binds to the sialic acid via its globular HA1 domain[9] triggering the process of clathrin-mediated endocytosis. Once within the endocytic vacuole the HA protein undergoes a conformational change with increased acidity of the vacuole environment. The transmembrane HA2 subunit of the HA protein unfolds, bringing together the host and viral membranes, and a fusion peptide located in the core of the HA protein is inserted into the host membrane which allows for release of the viral core into host cytoplasm[10, 11]. Critically, while there is a constant level of mutation among circulating strains within the HA1 protein, leading to antigenic drift and escape from neutralizing antibodies, there is a relative conservation of HA2 protein sequences among isolates, so that antibodies targeted to this region have activity not only within HA subtypes but also between subtypes[12-20].

In the initial binding step of the hemagglutinin protein to the host cell, the structure of the sialic acid receptor may act as a critical restriction factor to further viral replication. While influenza viruses are capable of infecting a remarkable range of host species, there is generally an adaptation of the hemagglutinin protein to bind to the specific sialic acids of a particular host species. Sialic acids are nine-carbon amino sugars in which the amino sugars are substituted with an acetyl or glycoyl group to produce N-acetylneuraminic (NeuAc) or N-glycolylneuraminic (NeuGc) acids. Each of these sialic acids can bind to the terminal galactose on cell surface glycoproteins via a α2, 6- or α2, 3-linked conformation.

The expression of either NeuAc or NeuGc can vary widely between influenza host species. Notably, humans and ferrets lack expression of NeuGc SAs [21, 22] while bovine, equine, porcine and avian tissues express variable amounts of both NeuGc and NeuAc SAs [23-26]. The expression of either a α2, 6- or α2, 3-linkages can also vary between species, and even within the tissues of a given host. For example, within the airways of humans the majority of sialic acid residues present in an α2, 6-linked conformation[27]. However in the digestive tract of waterfowl and poultry, where influenza virus replicates, there is a predominance of α2, 3-linked sialic acids [28].

The variability in expression of host sialic acids serves as a restriction factor in influenza's potential host range. Both the amino substitution as well as galactose linkage of these sialic acids has been seen to affect the ability of the influenza HA to bind to host cells. Studies using glycan screening technology with an array of sialoside analogs as the target substrate have highlighted key amino acids in the HA protein that limits its ability to bind to either the N-acetyl or N-glycolyl SAs, even considering a uniform a2,3 linkage[29]. A much deeper body of work has demonstrated the ability of some viruses to bind preferentially to either α2, 6- or α2, 3-linked SAs with increasing adaptation to a particular host species. For example the influenza strains circulating in wild birds are usually limited to tissues that express 2, 3-linked SAs, and cannot infect human-origin cells [30-33]. Conversely, human influenza viruses preferentially bind to α2, 6-linked SAs and have limited binding to the α2, 3-linked receptor [31, 33-37].

Despite this apparent restriction to a limited host range, there is ongoing movement of influenza strains across species boundaries, with countless spillover events allowing for viral adaptation to new host environments. While difficult to diagnose with certainty, a growing body of evidence that some of the earliest documented influenza epidemics in the modern period have their origins in animal reservoirs. The "Russian flu" of 1889, which emerged in December in St. Petersburg and within four months had swept across Europe and even North America, may have started in an equine host[38].

Serological studies from people born before 1891 have shown cross-reactivity to horse-origin H3N8 influenza A viruses[39-41]. The height of the pandemic in England was also found to align chronologically with a peak in "pink-eye" among horses in the region, a disease characterized by nasal discharge, cough, loss of appetite and lethargy[42]. This period also coincided with an unexplained deaths of cats, who can serve as competent hosts for influenza viruses.

The intricate lattice of influenza transmission events is far from unidirectional, with zoonotic spillovers aimed exclusively at human populations. Endemic equine influenza strains are believed to have originated in avian hosts, with subsequent spillover and adaptation to equine respiratory tissues[43, 44]. Expression of the sialic acid receptor is conserved between these two hosts, as tissues in the equine upper respiratory tract as well as avian gastrointestinal tract express almost exclusively α2, 3-linked sialic acids[34]. It is perhaps due in part to this shared aspect of physiology, as well as epidemiological considerations such as high contact rates of the two hosts in domestic animal settings, that have led to repeated spillovers of avian influenza into horses[45]. These avian-lineage viruses are then capable of jumping into a variety of other host species. The H3N8 virus that has become established within global equine populations seems to be particularly promiscuous, with separate spillover events into dogs in Australia, the United Kingdom, and the United States[46-48]. Surveillance efforts have even revealed outbreaks of the equine virus in Chinese swine herds, but while virulent never led to sustained transmission in the new host species[49].

Of great concern in modern public health is the looming threat of highly pathogenic avian influenza (HPAI), which may spill into human hosts causing a pandemic with the same high lethality as it presents in poultry. While there have been hundreds of human infections with HPAI H5N1 in the last couple decades, fortunately there is limited evidence for human-to-human transmission[50-54]. Those cases that are identified usually present with viral pneumonia and acute respiratory distress, with an overall case fatality around 60%[55, 56]. More elaborate chains of transmission can lead to spillover of

avian influenza viruses into people, including involvement of a feline intermediate host, as was the case with one individual that was diagnosed with avian H7N2 after prolonged contact with an infected shelter cat[57].

Pigs have been implicated as a key transitional host between humans and other species for a variety of reasons, one of them being their SA expression. While the influenza viruses that circulate in swine would seem to have enhanced binding to α2, 6-linked SAs [31, 35, 58] some also bind with high affinity to α2, 3-linked SAs. The successful replication of these latter viruses requires the presence of their receptor within the porcine host. Investigations into the distribution of SA receptors within the porcine respiratory tract have shown expression of both forms, with a proportionally greater amount of α2,6-linked SAs in the trachea and bronchi with a shift to α2,3-linked SAs in the bronchiole and alveolar tissues[59]. This trend has been supplemented by infectivity studies of both isolated primary epithelial cells from the respiratory tract as well as tissue explants, which have demonstrated a near equivocal ability of both human and swine viruses to infect those tissues of the upper respiratory tract [60-64]. These studies have also shown an increased ability of avian-origin viruses with a defined preference for α2, 3-linked SAs to infect those tissues in the lower respiratory tract.

The permeable barrier that prevents spillover of influenza between swine and human hosts has been crossed with regularity with ever growing commercial swine husbandry leading to increased contact between the two. While frequently limited in terms of disease severity and ongoing transmission after spillover, sometimes the virus adapts rapidly to the new host, causing a sweeping infection. The 1918 influenza pandemic was one such event, in which a soldier based in Camp Funston, Kansas contracted a strain with avian origins, leading to a pandemic that would claim millions of lives[65, 66]. The 2009 “swine flu” pandemic was another such case, albeit with less human mortality. While the greatest concern has been placed on how these viruses spilled over into people, what is less

appreciated is the subsequent spillover of both pandemic strains back into swine herds, establishing stable strains that would become a part of the porcine respiratory disease complex for decades.

Designated as “classical” swine flu, H1N1 established itself within commercial swine herds simultaneously with the 1918 pandemic. With the introduction of this isolate into swine, the maintenance of a single dominant strain held within global populations for a surprisingly long time. This may in part be due to lack of surveillance such as is performed in modern times, and even those efforts present through most of the 20th century relied on serological studies. Surprisingly, the human pandemic strains of 1957 and 1968 did not establish themselves in swine. This might be due to their composition, arising from reassortment events between human and avian strains[67], presenting a species restriction to swine hosts. Among the first documentation of disruption in the circulating swine strains is that of human-origin H3N2 within European herds[68]. Viruses isolated from Italian swine herds in 1977 and 1983 displayed external surface proteins that had their origins in human seasonal H3N2 influenza strains, later on further reassorting to incorporate avian-origin internal gene segments.

With the spillover of human seasonal H3N2 influenza into swine in Great Britain, the stable circulation of swine influenza strains of the past six decades was upset. In 1994 a novel H1N2 strain was detected in Scottish herds, responsible for an outbreak of respiratory disease within a herd and which had shown the ability to readily transmit to contact pigs within the same facility[69]. The influenza virus isolated from lungs of diseased piglets possessed a hemagglutinin gene similar to human seasonal strains circulating in the 1980’s, as evidenced by HAI studies in which there was negligible reactivity to contemporary swine viruses but strong reactivity to human seasonal strains. Further investigation into the genome of the Scottish isolate revealed evidence of a reassortment event between a human seasonal H1N1 with an endemic swine H3N2 strain to produce a virus that displays human like surface proteins with its internal gene segments (PB1, PB2, PA, M, NP and NS) belonging to a European avian-like lineage[70, 71]. This quickly became the dominant subtype among pigs in Great Britain, and within 5

years had spread over to continental Europe, in which it was detected from nasal swabs of pigs in West Flanders[72]. After initial detection of the virus a large effort was made to determine the extent of penetrance into Belgian herds. A serological study was performed in 74 herds, revealing that the virus already had spread widely with 68.8% of samples showing serological crossreactivity with one of two British H1N2 viruses.

A retrospective study examining samples taken in Brittany and Northern Italy pushes back the spread of human-origin H1N2 even further. While the isolates obtained from herds in Belgium had not caused any overt disease in infected animals, swabs and lung samples in this study were taken as a result of investigations into outbreaks of respiratory disease in pigs. As early as 1997 a “UK-like” virus was isolated from a case in Brittany[70] and showed strong serological crossreactivity compared to isolates obtained in 1999 and 2000 from both this region and Italy, demonstrating an establishment of the strain within herds from these areas. Researchers in this study performed phylogenetic analysis on the various gene segments of the invading H1N2 virus, which gave deeper insight than the previous Belgian study. This analysis provides further evidence that the HA and NA genes of the majority of H1N2 viruses found in France and Italy bear close relation to those found in Great Britain, rather than resulting from local reassortment between H1N1 and H3N2 circulating in the area. However, a single virus present in the study showed a similarity in the HA protein to a contemporary H1N1 variant in the area, as might be expected with the emergence of a dominant flu strain further reassorting with local strains.

Prior to 1998, the influenza subtype most commonly isolated from cases of diseased pigs in North America was H1N1, belonging to the classical swine lineage established at the beginning of the century. Sporadically, cases of H3N2 and H1N2 were detected in swine, but had not established themselves into wide circulation. Beginning in August of 1998, however, there was an explosion of cases of H3N2 among swine across major production centers in the United States. The most dramatic of the outbreaks occurred in a North Carolina pig farm, in which the virus was responsible for deaths of 2% of

the 2400 breeding sows, and abortions among 7% of those pregnant at the time[73]. Similar outbreaks were also seen in Texas, Minnesota and Iowa later that year. Genetic analyses of viruses obtained from all four outbreaks exhibited a strong phylogenetic similarity, the surface proteins HA and NA sharing 97-99% homology with contemporary human H3N2 viruses. Notably, three of the viruses from this outbreak contained internal gene segments from not only human and swine viruses, but also avian influenza viruses. Within this subset, viruses contained HA, NA and PB1 genes from a human-like background, NP, M and NS from a classical swine-like background (98% homology), and polymerase genes PB2 and PA from an avian-like background (93-98% homology).

This genetic constellation would continue to persist in North America, becoming one of the main circulating strains, and referred to as the internal triple reassortant gene (TRIG) cassette, indicating the human, swine, and avian origins of the genes. The TRIG cassette continued to reassort with other swine influenza viruses in North America, accumulating an array of HA / NA reassortant subtypes. By the early 2000s in addition to classical swine influenza there was also an endemic H1N2 reassortant with the HA gene from the classical swine background and the remainder of the gene segments belonging to the human-origin H3N2 group. Increasing complexity of circulating strains led to the development of a new nomenclature for H1 swine influenza circulating within North America. Broken down by the evolution of the HA gene, those viruses possessing a "classical" swine influenza background were designated H1α. Those viruses that possessed the classical H1 gene but had reasserted to contain the internal genes of the H3N2 viruses were designated H1β. Those that possessed the classical swine HA gene but had acquired both the NA and internal genes of the H3N2 viruses were designated H1γ.

The situation was to complicate further, as cases of respiratory disease in pigs led to the isolation of four more viruses in 2005 and 2007 by the Minnesota Veterinary Diagnostic Laboratory[74]. Sequence analysis of these viruses showed a close phylogenetic relationship in one sample for both HA and NA genes with human-like swine influenza viruses previously isolated in Canada. The remaining

three viruses showed a high degree of similarity with the Canadian swine viruses, but clustered on a separate branch, suggesting a second introduction of human influenza into American swine herds. The internal gene segments of all the viruses analyzed showed reassortment with the TRIG cassette, whereas as the original Canadian isolates had internal genes that were either wholly human or shared between human and swine backgrounds. The TRIG cassette, which contains a human PB1 gene, an avian PB2 and PA gene, and swine NP, M and NS genes, had become widespread across North America by the time of these outbreaks. The novel H1N2 virus was determined to be the result of a reassortment event between the Canadian human-like swine viruses and an American swine virus, the latter contributing its internal genes. These two distinct clusters would come to be identified as the swine H1 δ1- and δ2-lineages, which have rapidly spread to become two of the dominant lineages circulating in North American swine herds. This dominance may be in part explained by the lack of antigenic cross-reactivity between the δ-lineages and others previously circulating in U.S. swine[75], allowing the emerging viruses to sweep across an effectively naïve population.

In mid-April 2009, the CDC confirmed two febrile children in southern California to have been infected with a novel H1N1 influenza strain, with genes from both human and swine influenza viruses[76]. Although neither of the patients had contact with pigs, the virus possessed gene segments that had been in circulation among North American swine, including the TRIG cassette. These included an HA, NP and NS from a classical swine background, human PB1, avian PB2 and PA, and Eurasian avian-like swine genes NA and M[77]. Unlike most strains of influenza encountered by people, which have undergone moderate antigenic drift to give only partial protection to the new virus, this “swine flu” had undergone complete antigenic shift. In this case the immune system has little to no protection against the invading virus. Simultaneously with diagnosis of cases in the United States, the Mexican Ministry of Health was notified of clusters of severe pneumonia centered mainly in Mexico City and San Luis Potosi[78]. The virus was found to be identical to both cases. With clearly confirmed human-to-human

transmission in a naïve population, there was little to stop the rapid spread of this virus outside of North America, forcing the World Health Organization to declare a pandemic by June 11th.

Just as a simultaneous onset of human and swine respiratory disease was seen with the 1918 influenza pandemic, the situation was mirrored in the outbreak of the 2009 pandemic strain in North America. Only shortly after the virus was isolated from human patients, a commercial swine operation in Alberta, Canada notified his veterinarian of an outbreak of respiratory disease within his facility[79]. A worker at the site had recently returned from a trip to Mexico, and was documented to show signs of influenza-like-illness while working at the barn. The pandemic virus was diagnosed by RT-PCR, and the herd promptly quarantined to prevent transmission of the virus to other swine-processing facilities. Apart from the acute onset of cough little pathology or clinical manifestations were seen in the pigs. Mortality in the herd saw a mild increase, and affected pigs had a noted sneezing, mild conjunctivitis and labored breathing. Post-euthanasia necropsy findings were complicated by pathology of viral and bacterial coinfections, such as PRRSV and *Mycoplasma hypopneumoniae*, but showed signs consistent with severe influenza infection such as tracheitis, broncho-interstitial pneumonia, and multifocal necrotizing and suppurative alveolitis.

Despite efforts to limit spread of the new pandemic virus to other herds, the element of human transmission to naïve pigs remained an underlying problem. The reports of infection of global herds was staggered, in part due to lack of established surveillance systems in many affected countries, but was soon seen as far away as Thailand and Vietnam[80, 81]. Manitoba, Canada reported five additional swine herds in which nasal swabs taken from ill animals came back positive for the virus[82], among which two of the five reported contact between sick workers and animals and two more reported receiving pigs from a previously infected herd. As seen with the clinical presentations of the initial outbreaks, mild symptomology was seen however there was little that could be done to stop the spread

and establishment of the virus within North American swine herds, just as it has within human populations.

The pandemic 2009 lineage of swine influenza swiftly rose to become a dominant strain in the composition of U.S. endemic swine influenza. Surprisingly, retrospective analyses of the 2009 pandemic virus lineage has found little reassortment of those viruses possessing the pandemic HA with other structural proteins of different lineages, possibly indicating a fitness advantage of the gene constellation[83]. However this analysis also demonstrated that while the pandemic HA protein is not a promiscuous gene segment, the matrix gene quickly reassorted with the internal gene segments of other lineages to become one of the most prevalent in U.S. herds, present in >90% of viruses sampled in some surveys[84, 85]. The incorporation of the matrix gene into various swine lineages might be explained by enhanced transmissibility of the progeny viruses, as seen experimentally when the matrix gene is incorporated in the backbone of contemporary swine influenza viruses[86]. When combined with the pandemic lineage NA gene, viral infection also led to enhanced lung pathology and viral replication in the lung[87].

Once established within swine hosts, influenza viruses only infrequently transmit and cause disease to human hosts. Such was the case with the spillover of a H3N2 variant (H3N2v) from swine to humans in 2001, causing an outbreak of over 300 cases[88]. This illustrates the importance of surveillance among circulating swine influenza strains, as previously introduced viruses undergo genetic drift within a physiologically isolated host reservoir, and have the potential to return and cause severe disease in a susceptible human population. A rigorous study examining the antigenic and phylogenetic properties of swine H1 viruses from 1930 to 2013 saw the continued circulation of the 2009 pandemic lineage with alpha and delta lineages within the United States[89]. Within these strains, the δ-lineage in particular has undergone dramatic antigenic diversification. While the viruses belonging to the classical lineage evolved at a mean rate of 0.15 antigenic units (AU) per year, in the same period viruses from the

δ-lineage evolved at 0.63 to 0.85 AU per year. The distance between antigenic units represents the degree to which antiserum to a given virus is capable of neutralizing a comparative virus, and so from this rate of antigenic diversification it becomes clear that emerging δ-lineage viruses would more quickly be able to overcome neutralizing antibody activity even in individuals that had seen closely related viruses.

Of note is that while demonstrating an increase in antigenic diversity while adapting to the swine host, delta lineage viruses have maintained elements that allow for infection of human tissues. The binding pocket of the HA protein of these viruses is characterized by the conservation of "human-like" residues which may facilitate binding of the virus to human SA receptors[75]. In situations where humans are in close contact with infected animals for a prolonged period, such as agricultural fairs, this can lead to spillover events. Termed, "variant" influenza cases, there have been regular H1N2v infections of viruses belonging to δ-lineage[90, 91]. While these cases rarely present with severe disease without underlying conditions and no definite proof exists for continued human-to-human transmission exists, they should be considered with caution. Little serological cross-reactivity exists among surveyed human sera, possibly due to accelerated antigenic drift while in swine. Utilizing a ferret model as a surrogate for human transmission dynamics, delta viruses showed slightly decreased transmission potential compared to H1 variants belonging to other clades. While showing a clade-specific transmission ability, variant viruses also showed differential clinical disease manifestations and extrapulmonary spread after experimental infection of ferrets[92]. Within these challenge models it was only the delta viruses that possessed the ability to spread beyond the respiratory tract, with detectable virus in olfactory bulb and brain.

Apart from the growing risk to human populations in the event of a spillover, influenza places a huge economic burden on the commercial swine industry. Infected animals have less efficient conversion of feed to weight gain, requiring farmers to keep them longer before reaching market

weights[93, 94]. Additionally, the number of piglets produced from each sow is reduced in influenza-positive environments[95, 96]. While it is difficult to determine an exact dollar value to ascribe to these burdens, numbers as high as $10.31 per pig have been calculated in a wean-to-finish system[97]. Despite these costs, there has been a lack of consensus as to the most cost-effective means of controlling swine influenza. Indeed, there have been studies that both corroborate the reduced productivity of influenza-positive herds, but simultaneously show that given an influenza-positive status the administration of an influenza vaccine actually further decreases productivity compared to an unvaccinated herd[98].

The marked inefficacy of current swine influenza vaccination practices in the United States has its roots in a variety of factors, any single one of which is almost impossible to separate from the others. The previously mentioned study, which found the implementation of influenza vaccine in influenza positive herds to be ineffective, included the caveat that farmers were less likely to use a vaccine measure unless influenza cases were already particularly numerous or severe. This could skew the results of the study, but unfortunately is a very realistic scenario. There has been a shift in recent years to increased vaccine coverage regardless of herd status, with approximately half of sows being vaccinated prior to farrowing, and nearly all of weaned piglets receiving a vaccine[99]. Despite this increase in vaccination influenza persists in herds, with half of those which use vaccine interventions still reporting infections in nursery pigs.

Failure of vaccine interventions are again multifactorial. Vaccination of sows and passive transfer of maternally derived antibodies (MDAs) to nursing pigs has been proven to be ineffective in preventing influenza in piglets, and can even prolong viral shedding when challenged with a heterologous strain[100-102]. The strategy of vaccinating sows with the intent of reducing costs, each immune nursing sow providing immunity to an entire litter of piglets, does not prevent the onset of disease due to influenza even when serum neutralizing titers are high post-partum. In fact, among those

piglets delivered from sows vaccinated with whole inactivated virus formulations there is an additional suppression of the piglet immune response to subsequent influenza vaccination, as determined by presence of neutralizing antibodies[103-105]. Elevated MDA levels are also correlated so increased viral shedding after subsequent challenge with influenza[106]. This may be explained in part when taking into account the underdeveloped immune system of the piglet compared to that of an adult, with reduced numbers of peripheral lymphocytes as well as memory cell populations[105]. A greater antigenic stimulus would be needed to elicit a strong and durable antibody response in these animals, a response that may be stunted by an even partial neutralizing effect of extant maternally-derived antibodies. This phenomenon is not limited to influenza vaccinations, but has been noted for vaccines against Aujeszky's disease and PRRSV[107, 108].

Composition of commercially available swine influenza vaccines may play a large role in explaining the ineffectiveness of the vaccine within weanling pigs. Intramuscular administration of a whole killed virus with addition of an adjuvant can elicit a strong immune response in an adult pig, although the conferred protection is insufficient to prevent infection and ongoing transmission cycles. These formulations elicit strong antibody responses specific to the vaccine antigen, but are generally less useful in providing cross-protection against antigenically different strains the vaccinated animal might encounter. Additionally, the antibody responses are limited to serum IgG responses that would only be able to encounter and neutralize invading pathogens once they have reached the lower respiratory tract[109-111]. A far more effective strategy would be to prompt an IgA response in the upper respiratory tract where the influenza virus is first encountered. This type of immune response can be elicited by an intranasal vaccine, carrying either an attenuated virus or vector bearing influenza antigens. Studies examining intranasal vaccines in adult and weanling pigs have shown seroconversion, expression of IgA in upper and lower respiratory tracts, as well as protection from homologous and heterologous challenge[112-122]. This evidence extends to show such protection even in presence of

MDA[123]. Criticisms of intranasal vaccines concern the conflicting data that arise depending on the experimental formulation under consideration. Some have noted failure to seroconvert vaccinated animals, production of little to no antigen-specific IgA, and limited protection upon subsequent challenge[106, 124-128].

Natural infection with influenza prompts an immune response that protects the host at several levels. As the influenza virus initiates infection within epithelial cells proximal to the mucosal barrier, professional phagocytic cells such as macrophages and dendritic cells recognize those cells that are infected, phagocytize them and traffic to the lymphoid organs[129, 130]. It is in these tissues that the internal contents of the cells, including components of the influenza virus, are displayed to T and B cells. Naïve cells that are specific to the displayed viral antigens proliferate, creating germinal centers within the lymphoid organ. B cells that have been activated in such a response are specific for a variety of viral antigens, including the hemagglutinin and neuraminidase glycoproteins. These B cells differentiate further into memory B cells or plasmablasts that secrete gargantuan quantities of antibodies into the periphery that help neutralize progression of viral infection[131, 132]. Long-lived plasmablasts migrate to the bone marrow where they can live for upwards of a year, and memory B cell subsets persist in lymphoid and peripheral tissues that allow for a more rapid antibody response to future vaccinations or natural infections[133, 134].

While initially present in only IgM and IgG subclasses, virus-specific antibodies can also undergo class switching based upon activation status of their parental plasmablast. T cells in the lymphoid tissue express CD40 ligand, which engages CD40 on the proliferating B cell and allow for induction of alternative splicing of the genes regulating antibody classes so IgA can also be produced[135]. The secretion of the cytokines such as IL-2, -4, -5, -6 and -10 by T cells as well as TGFβ by CD4+ T cells has been found to further skew the class-switching of activated B cells towards an IgA phenotype. The resulting IgA antibodies are exceptional in that they are capable of translocation across the epithelial

boundary to effect the luminal space, neutralizing viral particles or opsonizing them for phagocytosis[136, 137]. Key to the neutralizing ability of these antibodies is a specificity for the globular head, or HA1 epitope, of the hemagglutinin protein. Antibodies that bind to this epitope prevent binding of the virion to cells and subsequent endocytosis and replication. Antibodies that target other antigenic sites, such as the stem of the HA protein or matrix protein, occur at lower frequencies than those directed toward the head, for reasons yet to be fully clarified[138].

In addition to the B cell responses within the lymphoid tissues, T cells are activated upon recognition of their cognate antigen displayed by antigen-presenting cells. In concert with activation of B cells, CD4+ T cells can differentiate into an effector phenotype which exits the lymphoid tissue and traffics to the respiratory tract. Here the CD4+ T cell exerts a protective effect via secretion of IFNγ as well as activation of another cytotoxic lymphocyte population, natural killer-cells[139-142]. CD8+ T cells can migrate to the periphery and at sites of infection release cytotoxic granules containing perforin or granzymes that destroy virus-infected cells, or induce apoptosis of cells via binding of the Fas receptor[143]. As seen in B cell population, a fraction of the activated T cells also differentiate into a memory phenotype. CD4+ memory T cells have been found to be critical in controlling secondary influenza infections, and also display heterosubtypic crossreactivity that can play a role in protecting a primed individual against a viral challenge to which they are naïve[140, 144-146]. The role of tissue-resident T cells, which are non-circulating cells that persist in the periphery and mediate protection at the mucosal interface, has become increasingly appreciated in protection from secondary influenza infections[147-149]. However, while these cells have been seen to show the same broad reactivity as traditional memory T cell subsets, their induction is dependent on a priming event through the respiratory tract and are not seen in traditional injected, inactivated influenza vaccines.

In comparative studies between intramuscular and mucosal administration of vaccines in swine, one of the greatest arguments in favor of the traditional intramuscular injection site has been the

production of a robust serum antibody response. While these vaccines normally provide a reliably detectable neutralizing antibody titer even after a single dose, antibodies produced are normally specific to the vaccine antigen with little cross-reactivity to heterologous strains[117, 123, 125, 128, 150-153]. In the case of human influenza vaccine studies, even multivalent vaccines have shown reduced cross-reactive antibody responses compared to those elicited by natural infection routes, possibly driven by the immunodominance of the variable HA1 domain of the influenza hemagglutinin protein[154-156]. Several vaccine studies in humans and mice have demonstrated that while neutralizing antibodies might not be produced after heterologous challenge of immunized individuals, there is a significant boost in antibody titers directed toward the HA2, or stalk, epitopes of the hemagglutinin protein[153, 157, 158]. These non-neutralizing antibodies engage several immune effector pathways to facilitate viral clearance. By binding to Fcγ receptors on NK cells, HA2-directed antibodies lead to activation of the cytotoxic cell and release of interferon and cytolytic vesicles that kill infected cells[159-161]. Opsonization of viral particles by these antibodies also enhances uptake by professional phagocytic cells, which can then go on to activate T and B cells[162, 163].

Spurred on by the discovery of stem-directed antibodies in humans that are cross reactive to heterologous and heterosubtypic influenza strains, this epitope has become a target for a variety of next generation influenza vaccine platforms that strive to provide “universal” protection[164]. Utilizing a multitude of strategies, including chimeric HA proteins, headless hemagglutinin antigen constructs, hyperglycosylated domains, or simply boosting with strains that possess minimal antigenic similarity, they all aim to direct an immune response to the conserved HA2 region[165-169]. Vaccine studies in mice and humans have found these strategies to be effective in creating serum antibody responses that indeed have reactive and even neutralizing activity beyond that of the vaccine viruses or antigens. However, these studies have been limited in their ability to investigate the corresponding effectiveness against natural infection.

There are cases in which an antibody response directed to a non-neutralizing epitope can have undesirable consequences. In recent years a prominent vaccine trial of the dengue fever vaccine, Dengvaxia, demonstrated the problems that can arise when a vaccine attempts to target multiple subtypes within a single vaccine construct. Antibody-dependent enhancement is a well-known problem in the context of dengue, where individuals previously infected with a single subtype can suffer severe and possibly fatal disease when challenged with a heterologous serotype[170]. The mechanisms behind this enhanced disease are still being illuminated, but one of the main issues is antibody-dependent enhancement (ADE). This phenomenon occurs when non-neutralizing antibodies bound to viral particles allow for enhanced uptake into phagocytic cells and potentially for enhanced replication once within host cells[171]. The Dengvaxia vaccine is composed of an attenuated yellow fever virus backbone (strain 17D) which has been modified to express the envelope and premembrane proteins of all four dengue serotypes. While effective in children that had prior exposure to dengue, naïve children had limited neutralizing antibody responses across serotypes[172]. In light of this, when 19 children in the trial due to dengue infection, vaccine-induced ADE was blamed and the trial halted[173].

To date, no human influenza vaccine has been shown to enhance disease upon subsequent challenge. However, this is the case in an environment where individuals are immunologically experienced against a wide range of influenza strains, and whose vaccines are multivalent and provide some degree of neutralizing protection. When considering food production animals, different factors must be considered. Vaccine strategies aimed at protecting weanling piglets against influenza infection involve either vaccinating a sow prior to farrowing, or vaccinating the newborn piglet. For reasons previously stated, this usually fails to provide protection to the piglet. The WIV vaccines used may even be detrimental to the animals, as seen by a growing body of literature describing vaccine associated enhanced respiratory disease (VAERD) in these animals. In the early 2000s, Vincent et al. described enhanced pneumonic lesions in lungs of weanling piglets vaccinated with a classical swine WIV, then

challenged with a heterologous H1 virus[174]. Subsequent studies would show enhanced clinical manifestation of influenza infection, as well as enhanced nasal shedding and inflammatory cytokine profile compared to naïve, challenged animals[175, 176]. While the exact cause of this enhanced disease and pneumonia is likely a combination of several factors, the presence of non-neutralizing antibodies may be a key mediator. Vaccination of animals with HA subunit vaccines alone was still sufficient to drive the phenotype, lending support to the idea that it is an immune response to this antigen in particular that is responsible for VAERD[177]. Immune sera from animals in this model has revealed a profile with startling activity, not only in that HA2-specific antibodies are present in large quantities, but that these antibodies function to enhance infectivity of the bound virus[178]. Crucial to alleviating the VAERD pathology was use of a live attenuated influenza virus, in which serum HA2-directed antibodies were still found in vaccinated animals, but clinical disease and lung pathology severely reduced[179].

Recent studies have extended the issue of VAERD outside a strictly porcine model of disease. Ferrets are widely considered the gold standard as a human surrogate model for influenza due to their shared SA receptor distribution which allows for infection experiments without adaptation, as well as a capability to transmit the virus to contact animals. Pathogenesis of influenza infection also mirrors that of human infections, resulting in fever, malaise, and respiratory signs[180]. With the virus mismatches that have been utilized in previous VAERD studies, a similar phenotype has been seen in ferrets, including enhanced clinical signs, viral shedding and lung pathology[181]. By increasing the scope of VAERD to the ferret, the concerns raised by VAERD might now be directed to people. While generally expressing a more complex antibody repertoire than is seen in the naïve animal models used in VAERD, there are instances in which an individual might become infected with an antigenically novel virus to which there is no neutralizing antibody. In this context VAERD might come to be a key factor in considerations for developing a “universal” influenza vaccine.

Few pathogens can claim the success of influenza. With a global reach that does not respect the boundaries that normally exist between potential host species, influenza will likely continue to remain a thorn in the side of public health for years to come. Far from hopeless, this fight has gained the advantages of global surveillance programs that have been able to trace the movement of influenza strains across continents with unprecedented accuracy, and allow for better prediction models in determination of vaccine strains and impact on commercial animal production. A growing understanding of immunology and molecular virology has put the target of a universal influenza vaccine with reach, but the various contexts in which such a vaccine is best implemented has to be considered. As a disease that affects birds, horses, dogs, humans and swine alike there will likely be a different approach for each species. Considering swine health, a sophisticated knowledge of strain phylodynamics, antibody- and cell-mediated immunology, and even commercial husbandry practices is required before an effective vaccine can developed. However, just as the burden of influenza in one host reservoir is felt by all, so too can the progress made in fighting infection extend to help the whole.

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CHAPTER 3

PANDEMIC RISK ASSESSMENT FOR A SWINE INFLUENZA A VIRUS IN COMPARATIVE HUMAN SUBSTRATES

Introduction

The ability of influenza viruses of various genetic backgrounds to infect swine is of great concern to public health. Swine have been proposed as key intermediate hosts in adaptation of avian influenza viruses to mammalian species, but they are also susceptible to infection with human influenza isolates. As multiple influenza viruses infect a single individual, they have the potential for reassortment of gene segments that can result in a novel virus to which the host has little or no immunity. This was exemplified in the 2009 H1N1 pandemic, in which gene segments from both human, avian and swine origins reassorted to create an antigenically shifted virus that rapidly spread across the globe[1, 2].

Currently endemic in swine flu populations are influenza strains belong to the H1N1, H1N2 and H3N2 subtypes[3]. In the U.S. human seasonal influenza spilled into commercial swine populations in the early 2000s, reassorting with circulating swine influenza strains to create a lineage of H1N2 that possesses external proteins of human seasonal origin with a swine internal gene cassette[4, 5]. This lineage, designated 1B.2.2 or δ-2, has continued to diversify within swine populations since this point of introduction. This has resulted in an increasingly dominant endemic virus within commercial herds as well as several human cases of H1N2 variant (H1N2v) viruses[6]. The ability of these swine viruses to spill back into human populations shows a waning protection due to cross-immunity with human seasonal influenza[7]. As such increased surveillance efforts have been focused on these viruses.

In order to properly assess the risk potential of emerging influenza strains, a framework has been established by the Center for Disease Control and Prevention, the Influenza Risk Assessment Tool (IRAT)[8]. The tool allows fort a systematic evaluation of emerging zoonotic influenza strains and a baseline comparison for prioritization and allocation of funding. By analyzing a given virus according to three main criteria, viral properties, host properties, and epidemiological factors, the tool seeks to answer two key questions. The first is that of emergence, that is what is the risk of a novel influenza strain being capable of sustained human-to-human transmission. The second question is that of impact of viral infection on public health if sustained transmission is possible. The three main criteria of the IRAT are broken down into a total of 10 elements, several relating to a virus's ability to transmit and cause disease within animal species as well as humans. The usage of animal models to assess risk to human populations is quite varied and involves additional cost and risk to investigators. Several *in vitro* model systems have also been used to determine susceptibility of human respiratory tissues, with increasingly complex culture systems allowing for growth and differentiation of primary epithelial cell cultures from host animals that approximate the *in vivo* environment[9-13].

We have isolated an influenza virus from a lethal case of swine influenza, A/swine/Georgia/A19-27480/2019 (H1N2; GA/19) that we then assessed in multiple models of infection to determine the extent of pathogenesis in its host species but also the transmission potential to humans. Phylogenetic analysis determined the isolate as belonging to the 1B.2.2 lineage, with genetic relatedness to Midwest swine influenza viruses but also H1N2v viruses. Animal infections in mice, swine and ferrets showed an ability of the virus to infect multiple animal species and transmit to naïve contact animals. We further investigated risk to humans through infections in tissue culture systems of Calu-3 cells as well as primary respiratory epithelial cells from human donors. Despite the human origins of both these substrates, results presented differing susceptibility of human tissues to the swine isolate that was dependent only on the model used.

Materials and Methods

Virus Isolation

A Hampshire cross market gilt was presented to the University of Georgia Department of Pathology after a sudden and severe illness of unknown etiology. Immunohistochemistry performed on lung tissue returned positive results for influenza A virus. Bronchiolar sections of lung tissue weighing approximately 100 grams were homogenized and the resulting homogenate passed through a 0.45 µm nylon mesh filter. The homogenate was then used to inoculate flasks of MDCK cells. The virus was passaged twice blindly, then plaque purified. The plaque-purified virus was used in further animal and *in vitro* infection studies.

Phylogenetic Analysis

Viral sequence data was generated using a MinION platform as previously described[14]. MinION reads were assembled using IRMA v 0.6.7 [15]. In order to investigate the potential for the isolates involvement in human-swine transmission and the virus's evolutionary history the consensus sequences for the HA protein (H1) and NA protein (N2) were used in phylogenetic analyses. All nucleotide sequence data was collected from the NCBI Influenza Virus Resource (date range 6/6/2014 through 7/2/2019) and grouped into separate datasets for human H1, swine H1, human N2, swine N2, human M, and swine M [16].

To determine potential human-swine transmission associated with the swine isolate *A/swine/Georgia/H1N2/2/7/2019 (H1N2)*, maximum likelihood trees were created using RAxML v 8.2.4 for each segment dataset [17]. The trees were created using a generalized time reversible nucleotide substitution model with gamma distributed rate variation among sites (GTR+ Γ4). A root-to-tip regression of the estimated trees performed to determine the 'clockliness" of the data and temporal outliers were identified and removed using TempEst v 1.5.3 [18].

Bayesian phylogenetic tree estimation was performed for a randomly sampled subset of n = 450 taxa for the swine HA, NA, and M segment datasets using BEAST v1.10.4 [19]. Six independent Markov Chain Monte Carlo (MCMC) runs for both proteins were preformed using a GTR+ Γ4 substitution model, lognormal uncorrelated relaxed clock, and a Gaussian Markov Random Field Skyride coalescent [20, 21]. Each MCMC run had a chain length of 100 million states, sampling every 10 thousand states. After removing appropriate burn-in from bringing of run (10%) a maximum clade credibility phylogenetic tree was generated for each segment from a sampling of 9000 trees.

Animal Infection Experiments

Five-week old female DBA/2 and BALB/c mice (Jackson Labs and Envigo, respectively; n=25 per strain) were divided into groups, infected (n=20) and mock (n=5). Mice were infected under isoflurane anesthesia then inoculated intranasally with $1x10^5$ pfu of GA/19 virus in a 30 µL volume. Mock infected mice were given 30 µL PBS intranasally. Animals were observed for clinical symptoms twice daily and weighed daily. At 2 and 4 days post-infection (dpi), 5 mice from each infected group were euthanized and lungs collected for virus titration. At 5 dpi, 3 mice from each infected group were humanely euthanized and lungs collected and perfused with formalin before submission for histopathological examination. Remaining mice were weighed daily until 13 dpi. All mice were euthanized at 25 dpi and serum samples collected.

Six weanling male and female Yorkshire pigs were obtained from a herd confirmed to be influenza A virus (IAV) and PRRSV-negative. One week prior to infection the study animals were treated with ceftiofur crystalline free acid (Zoetis). One day before infection animals were sedated with an intramuscular injection of ketamine (0.5 mg/kg), xylazine (0.5 mg/kg) and tiletamine-zolazepam (1 mg/kg) and baseline bronchoalveolar lavage (BAL) samples and blood samples taken. Animals were then separated into two groups, infected (n=3) and contact (n=3). Infected animals were inoculated

intranasally with 1 mL of $1x10^6$ pfu/mL GA/19 virus in each nostril. Temperatures and nasal swabs were taken daily after infection. At 2, 4 and 6 dpi infected animals were sedated and BAL and bloods samples taken. At 3 dpi contact animals were co-housed with infected animals. Nasal swabs were performed daily on infected animals and on contact animals after introduction. At 13 dpi blood samples were taken on all animals.

Ten 12-week old ferrets were divided into two groups, infected (n=6) and contact animals (n=4), with equal gender distribution between the two groups. Three days prior to infection, all animals were anesthetized under isolfurane and 3 mL venous blood draws performed, as well as placement of subdermal temperature transponders (BMDS) for animal identification and temperature monitoring. On day 0, six animals were anesthetized and inoculated intranasally with $1x10^6$ pfu of GA/19 virus in a 1 mL volume distributed equally between each nares. Nasal washes and weights were taken on infected animals one day post-infection and every other day subsequent. At 2 dpi, naïve contact animals were co-housed with infected animals in a 1:1 ratio and nasal washes and weighing performed as on infected animals. At 4 dpi, two infected animals were euthanized and tissue samples collected from the respiratory tract. At 7 and 14 dpi venous blood draws were performed, with animals humanely euthanized at 14 dpi.

Cell Culture and Infection

Calu-3 (ATCC), normal human bronchial epithelial (Lonza), and porcine primary nasal epithelial cells were cultured and differentiated at an air-liquid interface (ALI) in 12-well plates as previously described [9, 10]. At day 0 apical surfaces were washed and inoculated with GA/19 virus at an MOI of 0.01 in a 200 µL volume. All infections were run in triplicate wells. Cultures were then incubated in a humidified 5% CO_2 incubator at 37° C for 2 hours before inoculum was removed. At 12, 24, 48, 72, and

96 hours post-inoculation, 1 mL of sterile PBS was used to wash the apical cell surface, the wash was assayed for viral titers.

Sample Processing

Mouse lungs were individually placed in 1 mL PBS after collection and kept on ice until homogenization. Lungs were homogenized as previously described [22] clarified by centrifugation, aliquoted, and then stored at -80° C. BAL fluid was placed on ice immediately after collection and then passed through a 40 micron filter before aliquoting and freezing at -80° C. Nasal swabs were taken with polyester swabs and placed on ice immediately after collection. Samples were then sonicated for 10 minutes before aliquoting and storage at -80° C. Nasal washes were performed with sterile PBS, then samples were placed on ice after collection, clarified, aliquoted, and then stored at -80° C.

Virus Titration

Tenfold serial dilutions were prepared from thawed samples in serum-free MEM. An inoculum of 250 µL was placed into each well of a 12-well plate of confluent MDCK cells and incubated in a humidified chamber at 37° C for 1 hour before a 1.2% Avicel overlay supplemented with 1x TPCK trypsin was placed on the culture. Plates were then incubated for 72 hours in a 5% CO_2 humidified chamber at 37° C before overlay was removed and plates fixed with an 80% methanol 20% acetone solution. Fixed wells were then stained with crystal violet to visualize plaques that were counted, and then infectious virus titers calculated.

Viral RNA was extracted from nasal swab samples and tested for the matrix gene of IAV. Viral RNA was extracted using RNAzol RT. RT-PCR was performed using Taqman® Fast Virus 1-Step Master Mix. A 25 µL PCR mixture containing 6.25 µL 4X Fast Virus Master Mix, 14.75 µL DNase/RNase-free distilled water, 0.5 µL of each primer (forward: AGATGAGTC TTCTAACCGAGGTCG, reverse: TGCAAAAACATCTTCAAGTCTCTG), 1 µL of probe (FAM-TCAGGCCCCCTC AAAGCCGA-BHQ), and 2 µL of

sample RNA template was prepared. Reactions were run at 50° C for 30 minutes, followed by 95° C for 15 minutes, followed by 40 cycles at 95° C for 10 seconds then 60° C for 20 seconds. Data were acquired on the BioRad C1000 Touch Thermal Cycler and data analysis performed with BioRad CFX Manager (v3.1). Viral titers were calculated based upon titration of a stock of known concentration, given as relative expression units (REU) compared to a negative control sample.

Statistical analysis

Viral titers were analyzed using analysis of variance (ANOVA) using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. A p-value ≤ 0.05 was considered significant.

Results

Phylogenetic assessment

A purified viral stock was obtained from lung tissue of a diseased pig and then plaque purified before sequencing using a MinION platform. Obtained consensus sequence data identified the virus as belonging to the H1N2 subtype (1B.2.1) [23] and was confirmed by subsequent Illumina deep sequencing. We performed phylogenetic analysis of the isolate in the context of human and swine H1N2 viruses to determine genetic distance from recent human influenza viruses of similar genetic backgrounds. Maximum-likelihood trees assembled from sequences extending five years prior to the date of sample collection revealed a significant distance between the isolate and the closest human isolate. However, the isolate did demonstrate a close relationship to H1N2 variant (H1N2v) cases in which a swine influenza virus resulted in limited human infections (Figure 1)[24, 25].

Bayesian phylogenetic trees were created for the HA and NA genetic segments (Figure 1a-d). The resulting trees illustrate a close genetic distance between the GA/19 isolate and contemporary swine influenza viruses circulating in the Midwest United States. Interestingly, while phylogenies among the gene segments reveal a genetically drifted variant from otherwise unremarkable swine influenza isolates, they do not explain the virulence of the initial case presentation from which the isolate was obtained.

Viral replication in the murine model

Although mice do not shed influenza virus or exhibit symptomology correlating to swine or human disease, virulence of influenza infection in mice has been shown to correlate to severity of disease in the case of several human and swine influenza strains[22-24]. Intranasal infection with the GA/19 strain of swine influenza did not cause weight loss in mice in either DBA/2 or BALB/c mice, although there was a significant difference in weights between infected and naïve mice from each background, particularly among mice belonging to the DBA/2 group (Figure 2). Despite the lack of weight loss, there was significant viral replication at 2 days post infection, with average lung titers from DBA/2 mice of 5.28×10^5 pfu/mL and BALB/c mice at 2.64×10^4 pfu/mL of lung homogenate. At day 4 titers had reduced somewhat for DBA/2 mice to 8.32×10^4 pfu/mL, while BALB/c mice virus titers were not significantly reduced at 2.74×10^4 pfu/mL. Pathological findings at day 5 showed mild pathology in lung tissues, regardless of genetic background. Interestingly DBA/2 mice had a noted increased degree of necrotic epithelium and lymphocytic migration around vessels compared to BALB/c mice (data not shown).

Viral replication and transmission in swine

Clinical symptoms in infected pigs were mild, as seen in experimental infections with other human-like swine influenza viruses[25]. Viral titers in BAL fluid (BALF) averaged $10^{5.3}$ pfu/mL at day 2

post-infection, decreasing slightly at day 4 post-infection to $10^{5.1}$ pfu/mL (Figure 3). The virus transmitted to 2 out of 3 contact pigs, as seen by shedding in nasal swabs (Figure 3). Three days after contact animals averaged $10^{1.9}$ pfu/mL and remained positive for viral shedding until day 6 post-contact. Nasal swab samples were also assayed for influenza by qPCR, which showed REU levels corresponding to BAL titers among infected individuals (Supplementary Figure 1). However, by this assay all contact animals became positive by 3 days post-contact. Despite rapid recovery, contact animals showed mild symptoms, limited to lethargy and elevated temperature (data not shown).

Viral replication and transmission in ferrets

Ferrets infected with GA/19 showed greatest viral titers in nasal washes at 1 dpi, with most animals clearing virus after day 5, but one animal remaining positive until 7 dpi (Figure 4). Infection resulted in weight loss peaking at 3 dpi, however this was mild with average weights above baseline by 9 dpi (Supplementary Figure 2). Contact ferrets introduced at 2 dpi were all positive for viral shedding by 5 dpi (day 3 post-contact (dpc)). Contact animals displayed a pattern of viral shedding similar to infected animals, with all animals negative in nasal washes by 11 dpi (8 dpc). Weight loss within the contact group was also mild, but remained depressed through 11 dpi.

Viral replication in *in vitro* substrates

To further investigate capability of GA/19 to replicate in human respiratory tissues we cultured Calu-3 cells, normal human bronchial epithelial (NHBE) cells, and as a control, porcine nasal epithelial (PNE) cells at an air-liquid interface (ALI). Upon differentiation and culture at ALI, all three cell substrates produce mucous and reflect the airway surface. The airway cell substrates were infected on the apical surface with GA/19 (MOI of 0.01) and the apical surface sampled at 12 and every 24 hours to determine virus replication and differing levels of permissiveness to infection. Infection of PNE cells resulted in the highest titers of GA/19, rapidly increasing to $10^{6.21}$ at 24 hours post-inoculation and then peaking at $10^{7.6}$

pfu/mL by 48 hours post-inoculation. Calu-3 cells, derived from a human lung adenocarcinoma, are frequently used as a model of susceptibility to viral infection[26, 27]. Our infections with the Calu-3 substrate with GA/19 showed rapid viral replication, reaching peak titers of $10^{5.37}$ pfu/mL by 48 hours post-inoculation. Surprisingly, infection of NHBE cells with the swine isolate showed contrasting results to the other cell substrates. Viral titer remained depressed compared to Calu-3 and PNE substrates at equivalent timepoints, never exceeding 10^{3} pfu/mL. Timing of peak viral titer was also retarded, being seen at 72 hours post-inoculation as opposed to 48 hours observed for PNE and Calu-3 cells. GA/19 virus replication kinetics were confirmed in Calu-3 and NHBE cells of a different donor background with a duplicate experiment that demonstrated near identical virus titers over the 96-hour assay (Figure 5, dashed lines). While there was little to no virus replication, GA/19 was able to infect as demonstrated by confocal imaging of virus NP antigen staining of NHBE cells at 96 hours post-inoculation (Supplementary Figure 3).

To confirm the permissiveness of the human cell substrates to a human influenza virus we ran a complementary infection experiment with the 2009 pandemic virus, A/CA/07/09 (CA/09; MOI of 0.01). Calu-3 cells infected with CA/09 showed a pattern of replication kinetics markedly similar to that seen with GA/19, peaking at 48 hours post-infection at $10^{8.51}$ pfu/mL. NHBE cells infected with CA/09 also displayed a similar kinetics pattern to infections with GA/19, peaking at 72 hours, but resulted in much greater titers at 24, 48, 72, and 96 hours post-inoculation and a peak virus titer of $10^{6.54}$ pfu/mL by 72 hours post-infection compared to the minimal levels seen with the swine-origin virus.

Discussion

Our studies show a notable discrepancy in the spectrum of models used to assess pandemic risk of zoonotic influenza viruses to humans. Utilizing specific elements within each of the three main criteria of the IRAT tool, we assessed the potential of the A/swine/Georgia/A19-27480/2019 (GA/19) virus to

impact human health. To investigate viral characteristics we performed a phylogenetic analysis of sequences of both the HA and NA genes. This analysis demonstrated a close relationship of the GA/19 virus to contemporary swine H1-δ2 viruses within North America, as well as a surprisingly close relationship to human variant influenza isolates. Our studies further examined host interactions with the virus utilizing several animal models to assess pathogenicity as well as transmission potential.

Every animal model of infection used showed unequivocal replication of the GA/19 virus, as well as transmission potential in both swine and ferret hosts. Corroborating these results are infection experiments in human and swine-derived airway epithelial cell culture models. The virus displayed similar growth kinetics in Calu-3 and porcine nasal epithelial cells, peaking in both systems by 72 hours post-infection albeit with differing peak titers dependent on host background. Contradictory to these findings were the results of replication kinetics experiments in NHBE cells, a widely used model to ascertain permissivity of human cells to viral infection[29]. Within this latter culture model infection with the GA/19 virus resulted in little to no viral replication. Despite the lack of replication, confocal images confirmed viral invasion into the cell substrate by 96 hours post-infection suggesting the NHBEs were susceptible to infection, but failed to replicate the swine virus. Infection of the same NHBE cells confirmed them as permissive to IAV infection.

Of those animal models used in our studies, ferrets in particular are considered a gold standard for influenza A virus infection, virulence, and transmissibility in humans, and are thus used in assays to determine the risks posed by avian, porcine and emerging influenza A viruses [28-30]. The cellular ligand used by the influenza virus hemagglutinin to bind to and to enter human cells, an α2,6-linked sialic acid, shows similar distributions in ferret and human respiratory tissues[31]. Additionally, influenza infection in ferrets has a clinical disease phenotype very similar to what is seen in humans, including weight loss, sneezing, and lethargy[32]. Our infection studies in the ferret model provided clear evidence of replication and transmission, a strong indication that the isolate would pose a threat to a human host.

Two different human-origin cell culture systems were used in our *in vitro* infections. Calu-3 cells are a continuous human epithelial lung cell line derived from a pulmonary adenocarcinoma, and have been characterized extensively and determined to display a sialic acid receptor profile that allows for infection with human influenza virus isolates[26, 27]. Normal human bronchial epithelial cells are primary cells isolated from tracheobronchial tissue sections which have been used extensively in influenza research[33, 34]. When cultured at an air-liquid interface these cells display a mixed morphology, including ciliated and mucus-secreting goblet cells. Importantly, these cells share the same α2,6 sialic acid receptor profile as Calu-3 cells, a critical component in viral entry to the host cell. Despite these similarities, our infection studies showed strikingly different results between the two substrates. This hints at a subtler set of factors distinguishing the two cell substrates, creating a more permissive environment in Calu-3 cells for replication of a swine-origin influenza virus. While both models serve as useful tools in studying influenza infection in human respiratory tissues, this disparity must be taken into consideration for future risk assessments of emerging swine influenza isolates.

There were several limitations present in our study. First, in our ferret infection studies transmission was determined by placing naïve animals in direct contact with infected animals. More sophisticated housing systems have been created that would allow for determination of potential virus transmission by airborne droplets in addition to contact transmission. Additionally, our *in vitro* infections used primary human cells isolated from a single area of the respiratory tract. While the influenza virus is normally capable of infecting cells throughout the respiratory tract, the process normally begins in the nasal cavity and upper trachea. Including these tissues in future studies would give a greater insight into tissue-specific host restriction of swine influenza viruses.

In summary, we have confirmed the ability of GA/19 to replicate and transmit in multiple models of human influenza infection. Among these, our infection studies in mice and ferrets agree with the literature in their utility as models of infection with swine influenza A viruses. We have also

characterized the extent to which the isolate replicates in swine, its native host species. Importantly, our experiments we have highlighted considerations that must be taken in assessing pandemic risk of influenza strains *in vitro*, as commonly used models of human respiratory infections possess widely differing abilities to host zoonotic influenza viruses.

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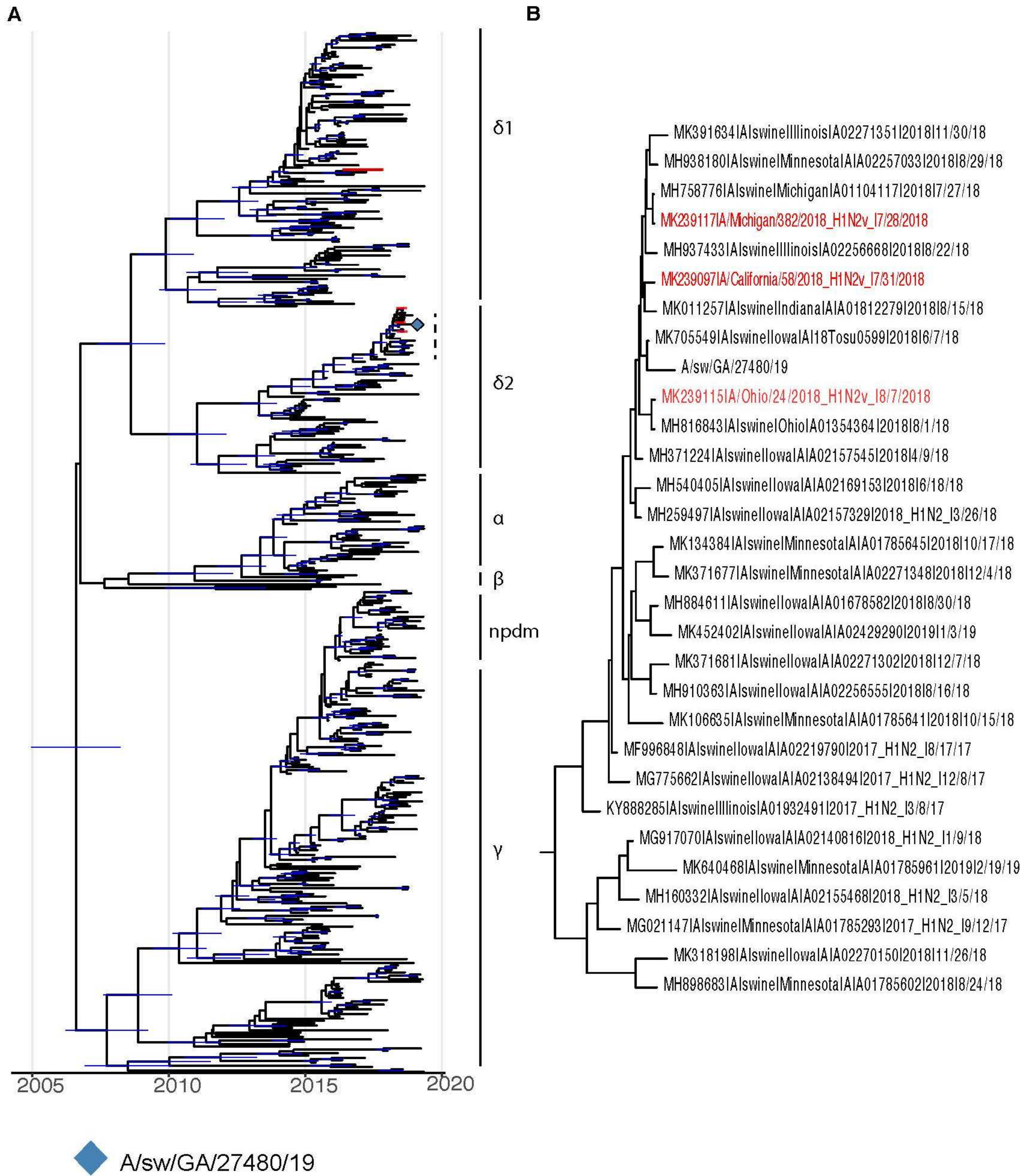
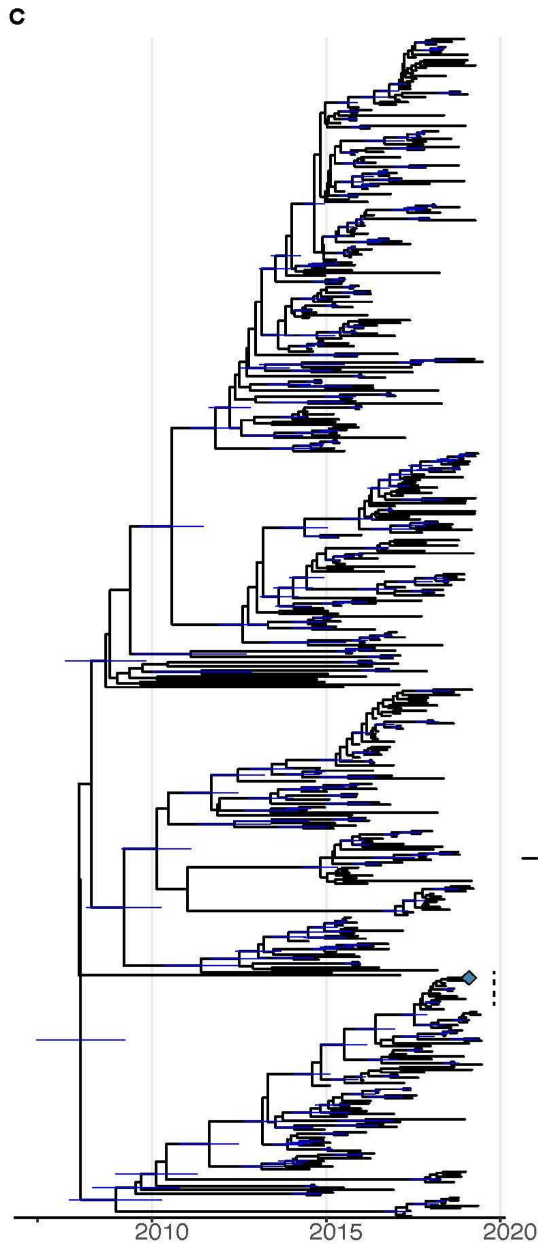
Figure 1





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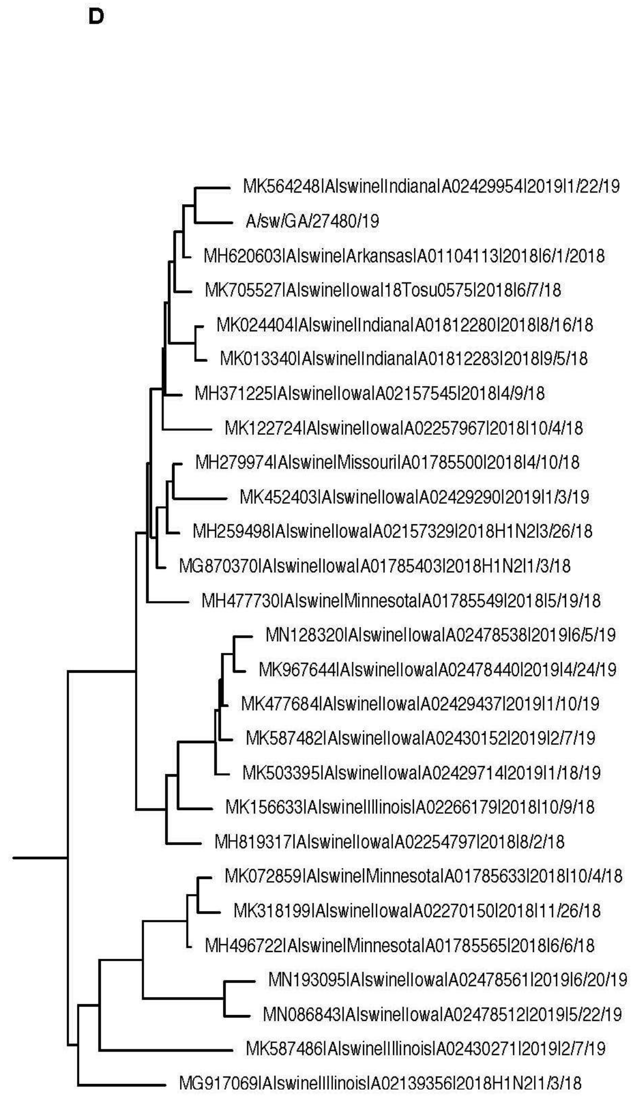
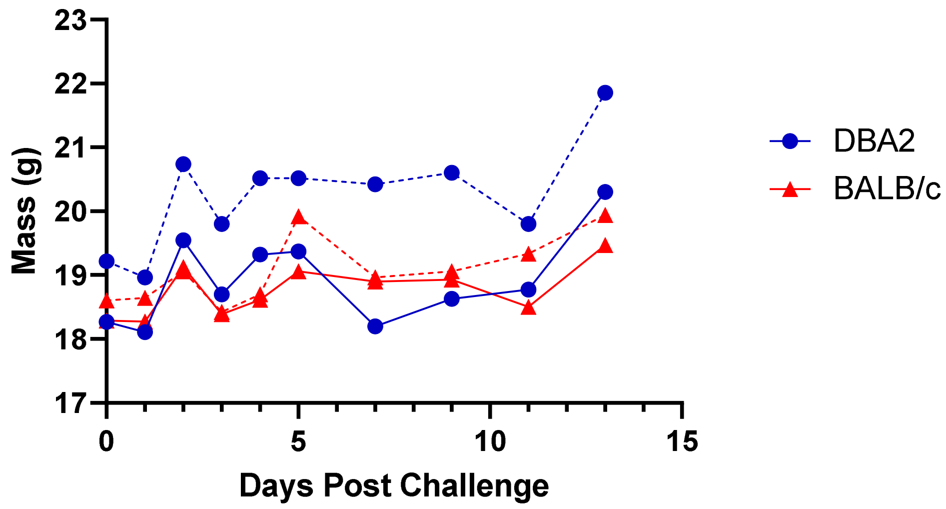


Figure 1. BEAST phylogenies for swine influenza A H1Nx and HxN2 isolates collected from 2014 to 2019. A, B) Phylogenetic reconstruction for hemagglutinin (HA) segment of swine H1Nx isolates. C, D) Phylogenetic reconstruction for neuraminidase (NA) segment of swine HxN2 isolates. Nodes with a posterior support of greater than 95% are annotated with a 95% Bayesian Credible Interval in blue. Taxa for H1N2 variant isolates are colored in red.

Figure 2

A.



B.

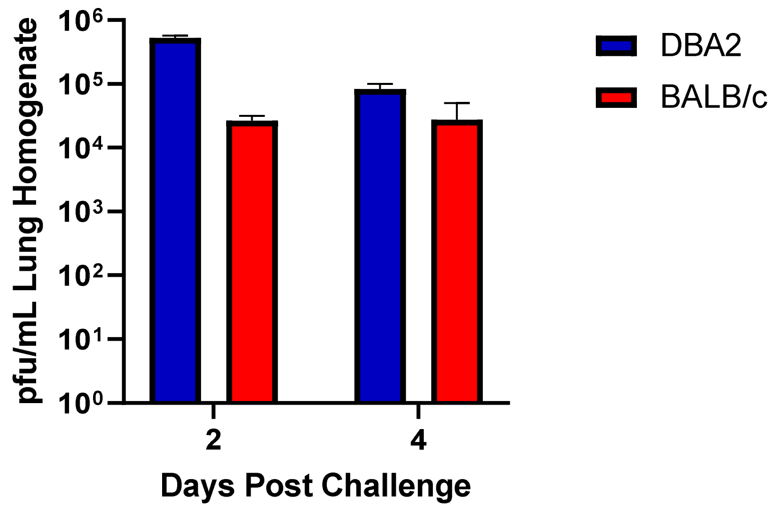


Figure 2. Replication and pathogenesis of A/sw/GA/27480/19 (H1N2) in DBA/2 and BALB/c mouse models. Six to eight week old BALB/c and DBA/2 mice (n=25 mice/group) were challenged with either 10^5 pfu of the virus in a 30 µL volume (n=20) or with PBS (n=5). Weight loss was tracked for 13 days post infection (dpi) (A), dashed lines represent mock-infected control groups. At 2 and 4 dpi, 5 mice from each infected group were euthanized and lungs collected. Viral titers, described a pfu/mL of lung homogenate, were determined by plaque assay (B).

Figure 3

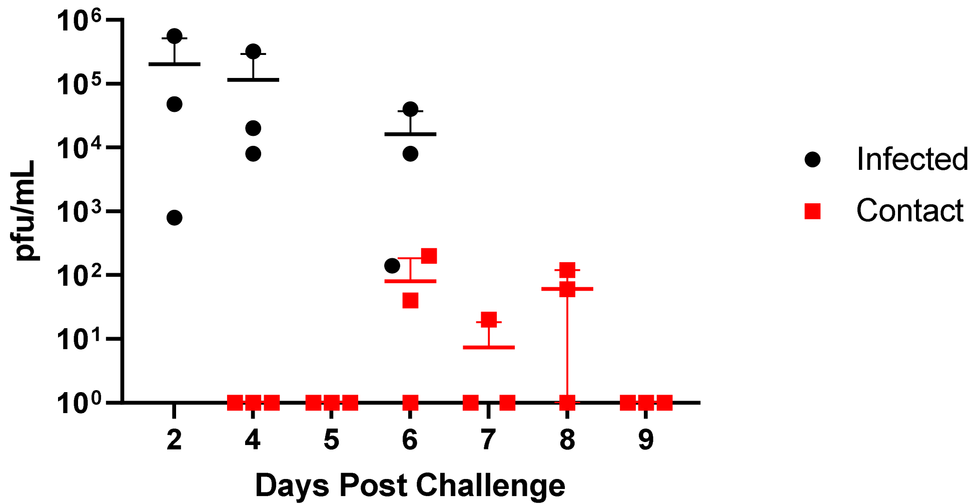


Figure 3. Replication and transmission of A/sw/GA/27480/19 (H1N2) in a swine model. Seven to eight week old pigs were inoculated intranasally with $2*10^6$ pfu of the isolate in a 2 mL volume (n=3). Nasal swabs were taken daily after infection. At 2, 4 and 6 dpi BAL samples were also taken (black data points). At 3 dpi, naïve contact animals (n=3) were co-housed with infected animals and nasal swabs taken daily (red data points). Viral titers were determined by plaque assay.

Figure 4

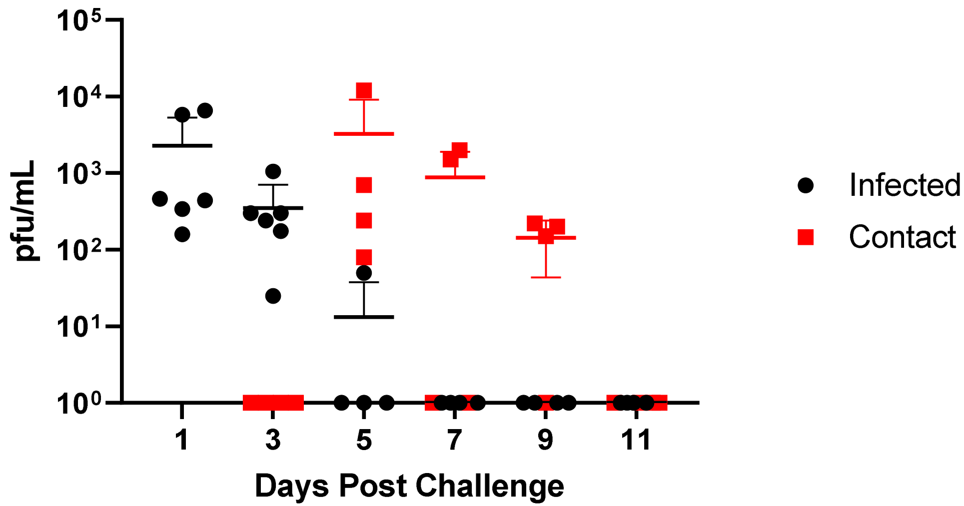
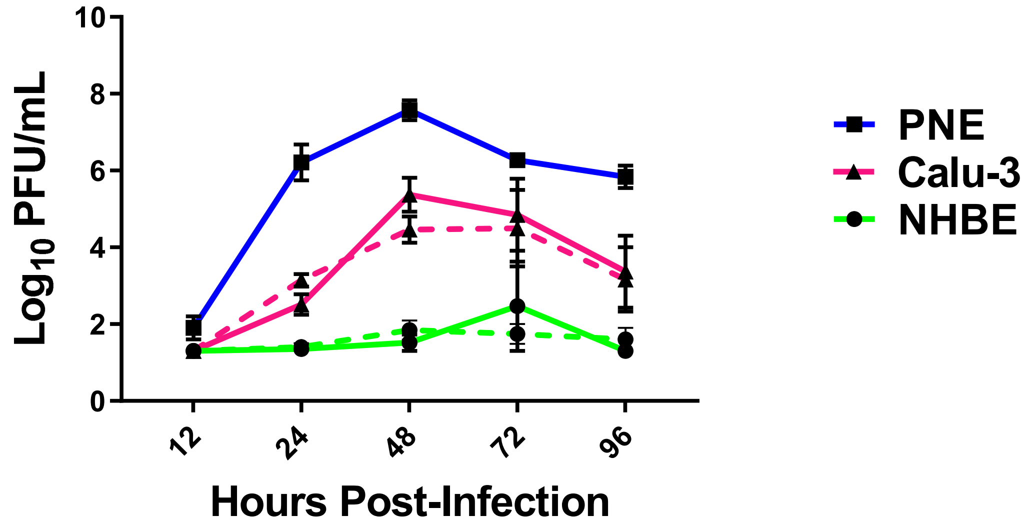


Figure 4. Replication and transmission of A/sw/GA/27480/19 (H1N2) in a ferret model. Twelve week-old ferrets were inoculated intranasally with $1*10^6$ pfu of the isolate in a 1 mL volume (n=6). At 1, 3, 5, 7, 9 and 11 dpi, nasal wash samples were taken and titers evaluated by plaque assay (black data points). At 2 dpi, naïve contact animals (n=4) were co-housed with infected animals (1:1) and nasal washes taken and evaluated for virus titer by plaque assay (red data points).

Figure 5

A.



B.

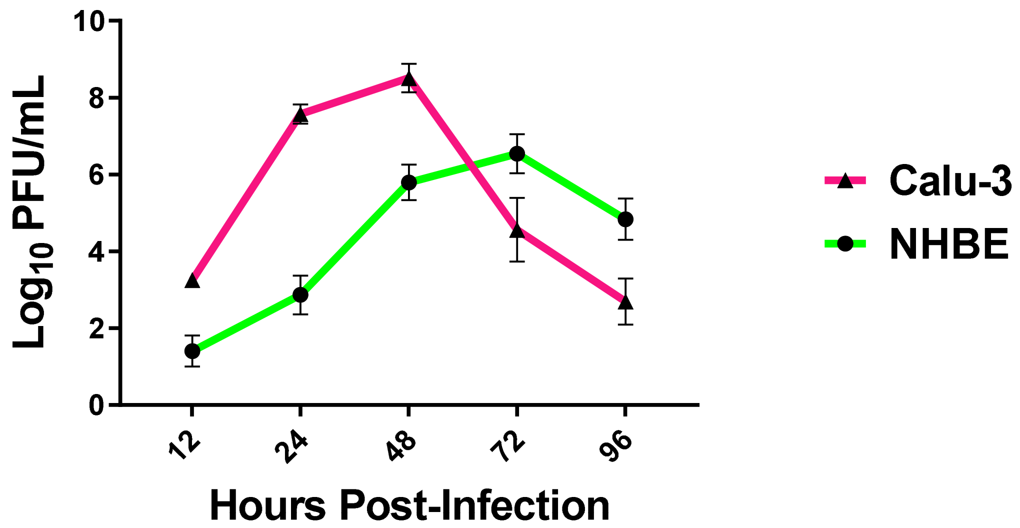
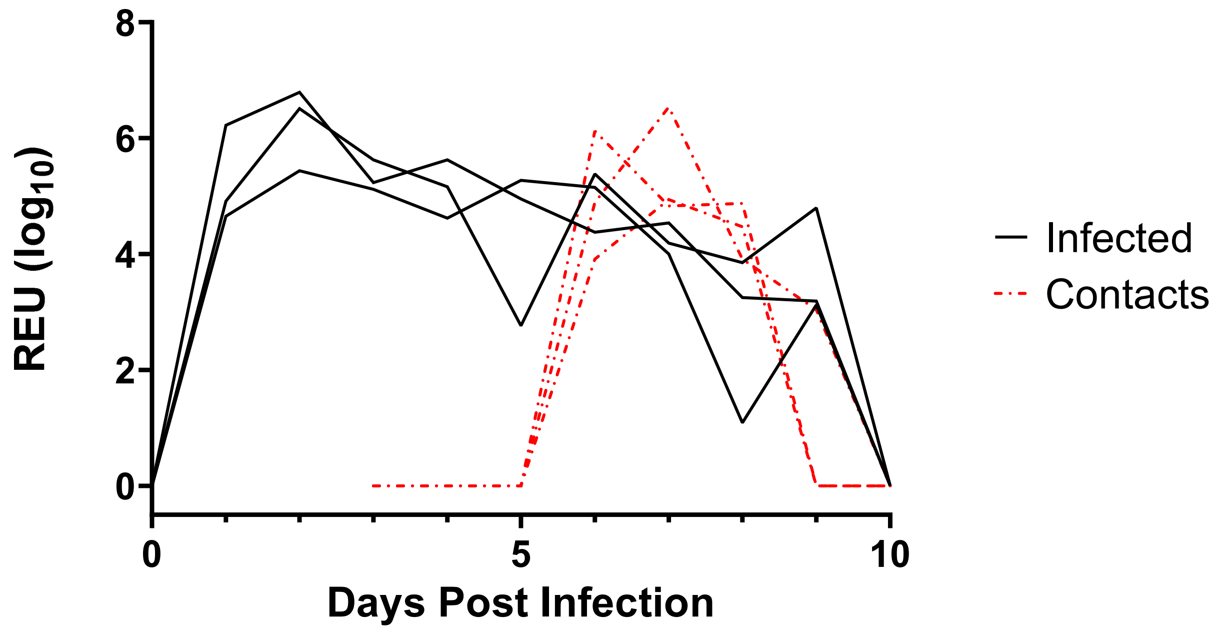


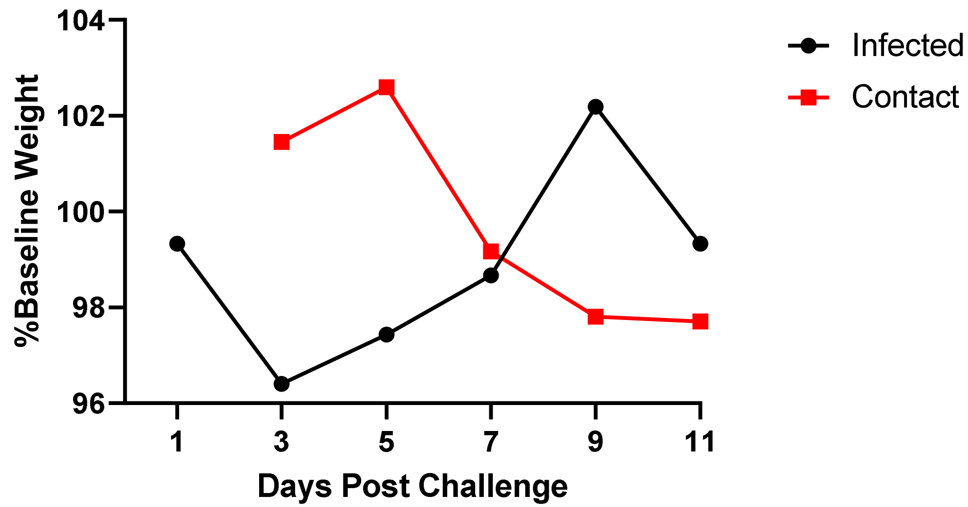
Figure 5. Replication of A/sw/GA/27480/19 (H1N2) *in vitro*. To investigate the permissivity of human and porcine tissues we cultured PNE, Calu-3, and NHBE cells at an air-liquid interface. Cultures were infected apically with either GA/19 (A) or A/CA/07/09 (B) at an MOI of 0.01. At 12, 24, 48, 72, and 96 hours post-infection the apical surface of cultures were washed and the fluid titered for virus by plaque assay. Dashed lines denote duplicate experiments.

Supplementary Figure 1



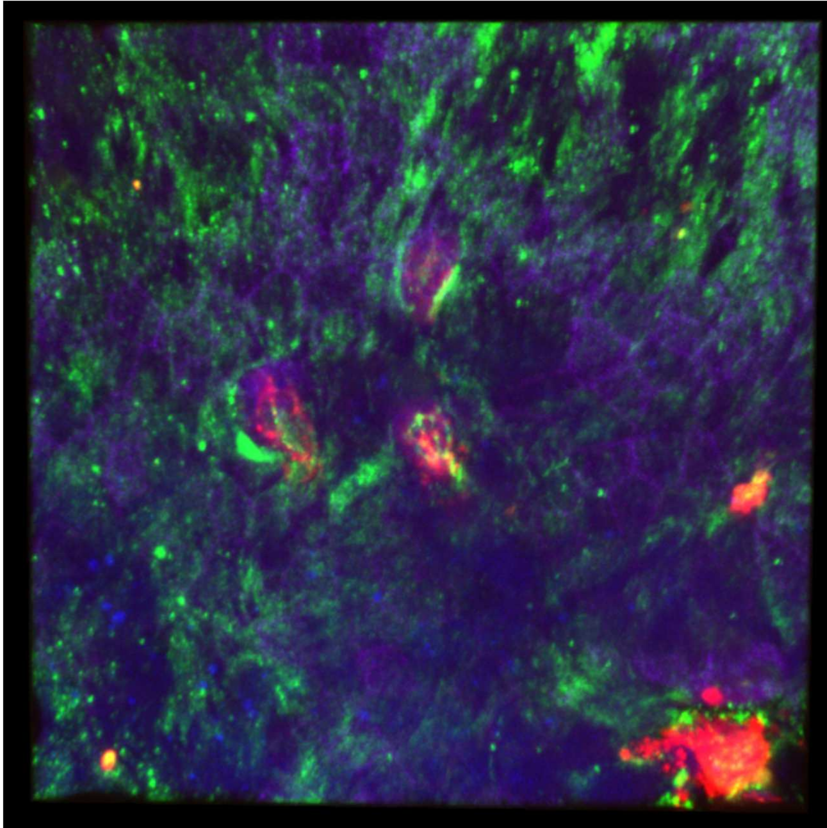
Supplementary Figure 1. Nasal shedding of A/sw/GA/27480/19 (H1N2) in swine as determined by qPCR. Infected animals showed peak viral loads by 2 dpi, remaining positive until day 9. All three contact animals became positive by 3 dpi, two remaining positive until day 8 and one until day 9.

Supplementary Figure 2



Supplementary Figure 2. Weight loss in ferrets post-challenge with A/sw/GA/27480/19 (H1N2). Weight measurements were taken until 11 dpi. Infected animals experienced an initial decrease in weight before returning to baseline levels. Contact animals experienced a comparable decrease in weight corresponding to an onset of viral shedding.

Supplementary Figure 3



Supplementary Figure 3. Confocal image of NHBE cells infected with GA/19 at 96 hours post-infection. Despite showing minimal viral replication, NHBE cultures infected with GA/19 showed clear evidence of viral invasion. Red foci indicate cells infected with the swine influenza isolate. Red: viral nucleoprotein, blue: nuclear stain, purple: F-actin, green: beta tubulin.

CHAPTER 4

THE ROLE OF CELL-MEDIATED IMMUNITY IN AMELIORATING VAERD IN WEANLING PIGS

Introduction

Influenza A virus is a pathogen of high concern in U.S. swine herds, responsible for substantial economic losses every year that continue despite advancements in husbandry and vaccination practices[1, 2]. Classified based upon the expression of surface proteins hemagglutinin (HA) and neuraminidase (NA), these key antigens constantly undergo antigenic drift that allows them to infect animals with incomplete immune protection[3]. Additionally, these viruses possess a segmented genome that facilitates the movement of gene segments within progeny viruses. Combined with antigenic drift, this antigenic shift allows for wide antigenic diversity among co-circulating strains of influenza. Swine influenza A virus (swIAV) in the U.S. exemplifies this, as while only H1 and H3 viruses are endemic, there are several clades within each that vary significantly enough that antibody responses do not provide homosubtypic protection[4-6].

The complex ecology of swine influenza viruses presents a challenge is development of a vaccine that adequately provides immunity across homosubtypic and heterosubtypic strains. Commercial vaccines are based upon whole inactivated viruses (WIV) that are combined in a single multivalent dosage that is designed to provide that maximum coverage based upon the selection of a few representative strains[7, 8]. Of increasing popularity is the use of autogenous vaccines, which allow for the rapid deployment of a vaccine using exactly the strain that has already been isolated from animals in a specific production facility[9]. These vaccines are normally administered to sows shortly before

farrowing so that maternal antibodies can be passively transferred to the neonate, or to weanling pigs shortly after farrowing[10]. Despite detectable serum antibodies in the weanling pig, this does not necessarily correlate with protection, and extant maternal antibodies can even interfere with the development of an immune response to a vaccine within the immature piglet[11, 12].

The use of WIV vaccines in swine has been associated with enhanced disease in weanling pigs[13, 14]. Vaccine-associated enhanced respiratory disease (VAERD) relies on an antigenic mismatch between the hemagglutinin head domain of the vaccine virus and that of a subsequent challenge swIAV. While animals vaccinated with WIV usually develop a strong serum antibody response that is capable of neutralizing homologous viruses, there can be a lack of neutralizing activity even within the same HA subtype due to the wide antigenic diversity of the influenza HA. A series of rigorous experiments have demonstrated the VAERD phenotype to be dependent on an antibody response to the more highly conserved HA2, or stem domain, of the hemagglutinin protein[15]. In a variety of influenza vaccination models, heterologous viral challenges have been found to increase the amount of stem-specific antibodies, and in humans stem-specific antibodies are thought to correlate with protection to emerging influenza strains[16, 17]. However, within the VAERD model these stem-directed, non-neutralizing serum antibodies may stabilize the virus and upon entry into host cells, enhance virus-cell membrane fusion leading to increased rates of infection.

In order to circumvent the stem-directed antibody response in VAERD, use of a live attenuated influenza virus vaccine delivered intranasally has been found to both reduce clinical disease and associated lung consolidation observed in VAERD[18]. This vaccine platform elicits a cross-reactive mucosal IgA response, as well as failing to elicit a non-neutralizing serum IgG response in vaccinated animals. While the role of cell-mediated immunity remains to be explored in LAIV prevention of VAERD, it is likely that this plays a role based upon investigations with other LAIV vaccines[19]. In our experiments we replicated VAERD using a contemporary swIAVs belonging to δ2- and 2009 pandemic H1

lineages, representative of an antigenic mismatch that is commonly seen in U.S swine herds. We further demonstrate that priming with an intranasal inoculation of live virus prevents VAERD pathology, as seen in previous LAIV studies. By investigating tissue-resident lymphocyte populations in the upper and lower respiratory tracts, we propose a potential role for both NK and CD4+CD8+ double-positive T cells in the airway that functions to reduce VAERD pathology despite systemic HA2 directed antibodies.

Methods

Vaccine and viruses

Influenza viruses A/California/04/2009 (pdmH1N1; CA/09) and A/Georgia/27480/2019 (H1N2 δ2; GA/19) were propagated on MDCK cells prior to use in animal studies. Whole-inactivated GA/19 virus was prepared by inactivation of viral stock by BPL and dilution to 128 HAU/mL before addition of an oil-in-water adjuvant in a 6:1 ratio of virus stock:adjuvant (Montanide ISA 15). Animals were vaccinated intramuscularly or infected at approximately 5 weeks of age.

Animals

Thirty-two male and female weanling Yorkshire pigs were obtained from a herd confirmed to be influenza- and porcine reproductive and respiratory syndrome virus-negative. One week prior to infection the study animals were treated with ceftiofur crystalline free acid (Zoetis). Animals were divided into groups of eight in equal numbers of either gender and housed in separate pens, acclimating for one week prior to infection. One day before infection animals were sedated with an intramuscular injection of ketamine (0.5 mg/kg), xylazine (0.5 mg/kg) and tiletamine-zolazepam (1 mg/kg) and baseline blood samples taken. All animal experiments were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee and complied with the National Institutes of Health guide for the care and use of Laboratory animals.

Experimental outline

At day 0 of the study animals were either vaccinated with inactivated GA/19 intramuscularly or intranasally infected with 1e6 pfu of GA/19 or CA/09 in a 1 mL volume using a mucosal atomization device (MAD, Teleflex). Control animals received an intranasal inoculation of phosphate buffered saline. Animals received daily nasal swabs post-challenge through day 7 of the study. At day 7 and at weekly timepoints afterwards animals received 2-3 mL venous bleeds. At day 20 of the study half of the animals in each experimental group were euthanized and respiratory tissues collected and examined for gross pathology and downstream tissue processing. At day 21 the remaining animals were sedated by an intramuscular injection of ketamine (0.5 mg/kg), xylazine (0.5 mg/kg) and tiletamine-zolazepam (1 mg/kg). While under sedation animals received 1 mL intranasally of 1e6 pfu of CA/09 delivered by MAD atomization device as well as 1e6 pfu in a 5 mL volume delivered intratracheally. After challenge animals were examined daily for clinical signs included respiratory rate and lethargy. Animals received daily nasal swabs as well as venous bleeds at days 2 and 4 post-challenge. At day 5 post-challenge remaining animals were euthanized and tissues collected from the respiratory tract and examined for gross pathology and processed for downstream analysis.

Pathological examination of the lungs

Immediately after euthanasia, plucks were dissected and imaged on both ventral and dorsal sides for later scoring. Scores were assigned by a veterinary pathologist blinded to experimental groups. The total percentage of atelectasis/consolidation was noted for each pair of lungs, and further divided into consolidation noted in the dorsal or ventral sides of the cranial, middle, caudal and accessory lobes.

Lung tissue sections were fixed in 10% buffered formalin before processing for histopathology. Prepared slides were stained with hematoxylin and eosin. Microscopic pathology was scored as previously described [14]. Slides were scored by a veterinary pathologist who was blinded to treatment

groups. In brief, pathological scoring was broken down into four categories: necrotizing bronchiolitis, suppurative bronchiolitis, peribronchiolar cuffing and alveolar inflammation. Depending on the severity of the associated pathology, each criteria could achieve a maximum score of 3, summed together to create a composite score for each individual animal.

Viral titration

Viral RNA was extracted from nasal swab samples and tested for the matrix gene of IAV. Viral RNA was extracted using RNAzol RT (MRC. RT-PCR was performed using Taqman® Fast Virus 1-Step Master Mix (Life Technologies). A 25 µL PCR mixture containing 6.25 µL 4X Fast Virus Master Mix, 14.75 µL DNase/RNase-free distilled water, 0.5 µL of each primer (forward: AGATGAGTC TTCTAACCGAGGTCG, reverse: TGCAAAAACATCTTCAAGTCTCTG), 1 µL of probe (FAM-TCAGGCCCCCTC AAAGCCGA-BHQ), and 2 µL of sample RNA template was prepared. Reactions were run at 50° C for 30 minutes, followed by 95° C for 15 minutes, followed by 40 cycles at 95° C for 10 seconds then 60° C for 20 seconds. Data were acquired on the BioRad C1000 Touch Thermal Cycler and data analysis performed with BioRad CFX Manager (v3.1). Titers were calculated based upon a standard curve generated by titration of viral stock of a known pfu/mL. Limit of detection was set at 10^1 pfu/mL.

Antibody detection assays

Hemagglutination inhibition (HI) assays on heat inactivated sera were performed with CA/09 as the antigen and turkey red blood cells following standard techniques[20]. Whole-virus ELISA assays were performed as previously described [15] with an adjustment made to plate 50 HAU/well of the virus. ELISA assays against recombinant chimeric HA H6/1[21] were performed with a concentration of 2 µg/well of the target antigen, and a serum dilution of 1:800 as previously described[15].

Flow cytometry

Prior to enzymatic digestion, dissected lung and tracheal tissues were briefly rinsed with sterile PBS, then minced and added to 25 mL of dissociation buffer. The dissociation buffer was composed of DMEM/Nutrient F-12 Ham (1:1) (Sigma-Aldrich), 40 U/mLDNase (Sigma-Aldrich), 1 mg/mL collagenase D (Sigma-Aldrich) and 0.1 mg/mL protease XIV from Streptomyces griseus (Sigma-Aldrich). Samples were then incubated at 37°C for 2 hours on a bacterial shaker. After enzymatic digestion tissue homogenates were then passed through a 100 µm nylon cell strainer then a 40 µm nylon cell strainer (Millipore-Sigma). The cell suspension was centrifuged at 1400 rpm for 5 minutes before resuspension for enumeration. Cells were diluted to 1e6 cells/mL and aliquoted into 1 mL volumes before staining with antibody. Cells were first blocked with 1 µL of rat serum IgG at 10 mg/mL (Millipore-Sigma) then incubated at 4° C for 30 minutes. Antibodies were then added to the cell solution specific for CD3ε (BD Biosciences 561476), CD4 (ThermoFisher MA5-28732), CD8α (BD Biosciences 559584), CD103 (ThermoFisher 62-1038-42), CD335/NKp46 (Bio-Rad MCA5972A488) and Ghost Red Dye 780 (Tonbo Biosciences 13-0865-T100). Cells were incubated for 1 hour at 4° C then centrifuged and resuspended in 200 µL of 4% paraformaldehyde (Fisher AAJ19943K2). Samples were run with a CytoFlex LX and analysis performed on FlowJo software v.10.8.1 (Tree Star, OR).

Statistical analysis

Statistical differences between lymphocyte populations were calculated by analysis of variance (ANOVA) using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA. Measurements of lymphocyte frequencies were performed with four biological replicates. A p-value $\leq$ 0.05 was considered significant.

Results

Mucosal versus systemic priming elicits an enhanced non-neutralizing antibody response in weanling pigs

After initial infection or vaccination, none of the animals in any experimental group displayed overt clinical disease. Animals that were intranasally infected with either GA/19 or CA/09 had productive viral infections, as determined by detection of virus in nasal swabs on days 2 and 4 post-infection (**Figure 1a**). One week after the initial infection, and at weekly timepoints afterwards venous blood draws were performed on animals in all experimental groups and sera screened for HI antibodies against the CA/09 virus. Hemagglutination inhibiting activity is shown in **Figure 1b**, where activity against the CA/09 virus was only present in those animals that had received an intranasal inoculation of the CA/09 virus. All other groups remained negative for neutralizing activity through day 21 post-primary infection.

Despite the lack of neutralizing activity, there were detectable serum antibody responses against whole CA/09 virus as measured by ELISA in all groups except control animals that had received a PBS at day 0 of the study (**Figure 1c**). Serum IgG levels specific for whole CA/09 virus were highest in animals that had been previously infected with CA/09, however both groups of animals that had been primed with GA/19 had detectable CA/09-specific antibody responses by day 14 post-priming. Animals that had been infected with GA/19 had approximately equivalent activity compared to animals that had received an intramuscular inoculation of inactivated GA/19. Surprisingly control animals had detectable activity against CA/09 by day 14, although at lower levels than all other experimental groups. We then measured HA2-specific serum antibody responses by ELISA, using a chimeric recombinant HA protein containing the HA1 domain from a heterologous H6 influenza virus and the HA2 domain of the CA/09 virus (cH6/1) (**Figure 1d**). Animals infected with the CA/09 virus had the highest stem-specific IgG

responses at days 14 and 21 post-infection, while animals infected with GA/19 had detectable but lower stem-specific IgG responses compared to the CA/09 infected pigs.

GA/19 infection or vaccination fails to prevent CA/09 infection and virus shedding

Twenty-one days after initial infection or vaccination, all animals were challenged intratracheally and intranasally with live CA/09 virus. Following infection, animals were observed for clinical signs but displayed no overt disease (data not shown). Daily nasal swabs post-challenge showed that animals that received a homologous virus priming (CA/09 primary infection followed by CA/09 challenge) had undetectable virus shedding (**Figure 2**). All other groups had detectable titers by day 1 post-challenge, peaking at days 2 or 3 post-infection. Animals that had been previously infected with GA/19 showed the greatest degree of viral clearance, but were still shedding low levels virus at 4 days post-challenge, whereas animals that had been vaccinated with GA/19 showed an extended period of virus shedding and titers comparable to control (unvaccinated and uninfected) animals at the end of the study.

Intramuscular vaccination with an antigenically dissimilar swIAV leads to enhanced lung pathology post-challenge

At day 5 post-challenge, all remaining animals were euthanized and lungs dissected for analysis of gross pathology. Animals that had been intramuscularly vaccinated with GA/19 WIV had higher levels of pneumonic lesions in the lung, compared to animals intranasally infected with GA/19. The GA/19 infected animals (GA/19 primary infection followed by CA/09 challenge) had limited lung consolidation comparable to control animals (mock (PNS) infection followed by CA/09 challenge; **Figure 3a**). Animals that had been first infected with CA/09 and then challenged with CA/09 were completely protected from lung pathology. The greatest degree of consolidation among GA/19 vaccinated animals was seen in the caudal left cranial lobe, and to a lesser degree in the right cranial and right middle lobes. In contrast,

the GA/19 infected animals showed mild consolidation in the right cranial and right middle lobes (**Figure 3b, c**).

Histopathological examination of lung tissue sections showed results consistent with previous VAERD studies[14]. Animals that had been vaccinated with GA/19 showed the highest composite scores, however animals in both groups that had been primed with GA/19 had low levels of necrotizing bronchiolitis (**Figure 3d**). Additionally, while no gross lesions were observable on lungs from animals previously infected with CA/09, microscopic examination of the lungs showed bronchiolitis, peribronchiolar cuffing and alveolar inflammation in equivalent levels to control animals.

Intranasal priming with live swIAV enhances cell-mediated immunity at the mucosal border

On day 20 of the study, half of the animals in each experimental group were euthanized and tracheal and lung tissues collected for analysis by flow cytometry. Single cell suspensions were stained against markers corresponding to T cell and natural killer (NK) cell populations, as well as the CD103 marker[22-24]. The latter is a dimer of in the integrins αE and β7, and has been used widely as a surrogate for determining tissue-residency of lymphocyte populations[25-27]. By utilizing this segregating marker in analysis of respiratory tissues, we observed clear differences in both resident and non-resident populations between animals that had received an intramuscular vaccination compared to those that had been intranasally infected at day 0.

Within the trachea there was a significantly higher number of CD4+ T cells among vaccinated and CA/09 infected animals prior to challenge at day 21 (**Figure 4a**). At this timepoint there were also higher frequencies of CD4+CD8+ cells in the vaccinated and control groups compared to either infected group. Conversely, CD8+ and CD8+CD103+ cells were highest in the GA/19 infected group. Within the infected groups, NK cell populations were greater in the CA/09 primed animals compared to the GA/19 primed group. Post-challenge with CA/09 there was a dramatic shift in T cell dynamics, with a decrease

in CD4+ and cD4+CD8+ cell frequencies in all groups and increases in CD8+ cells (**Figure 4b**). The highest frequencies of both CD8+ and CD4+CD8+ populations were found in the GA/19 infected group post-challenge.

Lung tissue showed a similar distribution of CD8+ and CD4+ T cells prior to challenge in all groups, but here both of the infected groups showed lower frequencies of both the single-positive and double-positive T cells prior to challenge (**Figure 4c**). There were also significantly higher numbers of NK cells in the lung among vaccinated animals compared to those that had received the same virus intranasally. Post-challenge the lower airways experienced a similar shift in T cell dynamics compared to the upper airways, with decreases in CD4+ cells in all groups and increases in CD8+ cells in all groups except for control animals (**Figure 4d**). Within the CD4+CD8+ compartment there was an increase within the GA/19 infected group, with both CD4+CD8+ and CD4+CD8+CD103+ populations significantly higher in these animals compared to vaccinated animals.

Discussion

The issue of antigenic mismatch in swIAV vaccines has been documented to lead to enhanced disease and lung pathology in the VAERD disease phenotype. However, previous studies have relied on limited sets of viruses, and none within the δ2-lineage which in recent years has become increasingly prevalent in U.S. herds[14, 15, 28]. Our study provides the first evidence of the potential for VAERD within a mismatch between a swIAV belonging to the δ2-lineage and the pandemic CA/09 virus. In addition to recreating VAERD with a novel antigenic mismatch, we sought to explore the mechanisms behind VAERD attenuation, as has been seen by use of an LAIV[18].

In order to verify the recreation of VAERD in the context of a mismatch with a δ2-lineage swIAV our study met several key criteria established by previous studies on the phenomenon. Firstly, after vaccination with a WIV preparation of the GA/19 virus, vaccinated animals failed to a neutralizing Ab response to the heterologous challenge virus (CA/09). While CA/09 neutralizing antibody responses were undetectable in animals primed with the GA/19 virus, both GA/19-specific IgG responses against whole virus and cross-reactive HA2-directed IgG antibodies were detected within 21 days of intranasal infection with the δ2-lineage swIAV. This correlates with previous studies that have found an intramuscular vaccination with δ1-lineage swIAV WIV can lead to the development of HA2-directed antibodies that enhance the fusion activity of the influenza HA protein and thereby increase viral infection and subsequent pathogenesis[15]. We did note a diminished HA2-directed antibody activity in animals vaccinated with WIV GA/19 compared to those given an intranasal infection with the GA/19 virus, however there was comparable serum antibody activity against whole virus between these two groups. Stem-directed antibodies comprise a relatively small portion of the antibody response to influenza[17], and so might be present within vaccinated animals at levels that enhance viral activity but are not detectable with the serum concentrations used in our assays.

In addition to the development of a non-neutralizing antibody response, critical to establishing VAERD was an increase in clinical disease post-challenge as well as the development of severe pneumonic lesions within the VAERD group. Contrary to other studies, our model never demonstrated enhanced clinical disease within animals that displayed the VAERD phenotype, however our study was limited in determination of several features of influenza pathogenesis in swine, such as fever and weight gain. Despite the lack of clinical signs,

we did note an extended window of viral shedding within vaccinated animals that was much longer than either group previously infected with either the pandemic 2009 virus or the δ2-lineage swIAV, which is consistent with previous studies on VAERD pathogenesis[13, 14]. Upon necropsy of animals at day 5 post-challenge we also observed lung pathology that was consistent with development of VAERD, with focal and diffuse pneumonic lesions distributed throughout the cranial and middle lobes within animals in the vaccinated group. In contrast, there was a complete abrogation of gross lung pathology in the CA/09 primed group, and only mild pathology seen within the GA/19 infected animals and controls.

To understand why the antigenic mismatch in priming resulted in diminished pathology within the GA/19 infected group, we analyzed tracheal and lung tissue homogenates to measure the T cell and NK cell populations resident within each site. The enhanced viral activity associated with HA2-specific antibodies in VAERD may have been counteracted by the early recruitment of CD4 and NK cell populations to limit viral spread in the respiratory epithelium. Moreover the role of tissue-resident T and NK cells has become increasingly appreciated in clearance of influenza infection[29-31]. The CD103 integrin marker, in addition to CD69, has become recognized as a surrogate marker for tissue residency within these cells, and so our flow cytometric analysis focused on co-expression of this marker with T cell and NK cell markers[32-34]. Surprisingly, prior to challenge with CA/09 these tissue-resident populations were somewhat lower in groups intranasally infected with live virus compared to vaccinated animals. However post-challenge, the tracheal tissues of these groups showed an increase in tissue-resident CD4+ cells and within the lower respiratory tract an increase in CD4+CD8+ cells. After challenge, the CD4+CD8+ population was consistently higher in GA/19 infected animals

compared to those that had received a WIV GA/19 vaccination, indicative of the importance of this lymphocyte population in amelioration of VAERD lung pathology. Notably, swine extrathymic CD4+CD8+ T cells have become recognized as an antigen-specific memory cell subset with increased antiviral activity upon challenge[24, 35, 36]. Animals vaccinated with WIV GA/19also had an increase in the NKp46+ cell populations compared to GA/19 infected animals, indicative of the role NK cells may play in pathology when the individual lacks a locally infection-primed cell-mediated immune response. In humans, NK cell lymphopenia has been associated with increased susceptibility to severe influenza infection, however within the weanling pig model with a relatively limited antibody repertoire this lymphocyte population might become an important factor in disease[37, 38].

Our study lends evidence to the continued relevance of VAERD when considering influenza vaccination strategies in commercial swine populations. By using a contemporary δ2-lineage swIAV, A/swine/Georgia/27480/2019, we were able to recapture the essential elements of the VAERD phenotype in a novel model. Increased viral shedding as well as enhanced macroscopic and microscopic lung pathology were seen in our WIV-vaccinated animals, as previously seen in δ1-lineage mismatches. Our study further examined the role played by cell-mediated immunity in the upper and lower respiratory tracts in order to better understand correlates of protection against VAERD, as well as identify a potential correlate of disease with the presence of NKp46+ cells in WIV-vaccinated animals. These findings should be placed in the context of commercial swine production. Current vaccination strategies for swine focus on an easily measurable antibody response, and so established interventions aim for as broad an antibody response as possible, even with mounting evidence of the negative impact of non-

neutralizing antibodies in swine. New correlates of protection should be established that take into consideration a cell-mediated response at the mucosal border in addition to a systemic antibody-mediated means of protecting animals from influenza infection.

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Figure 1.

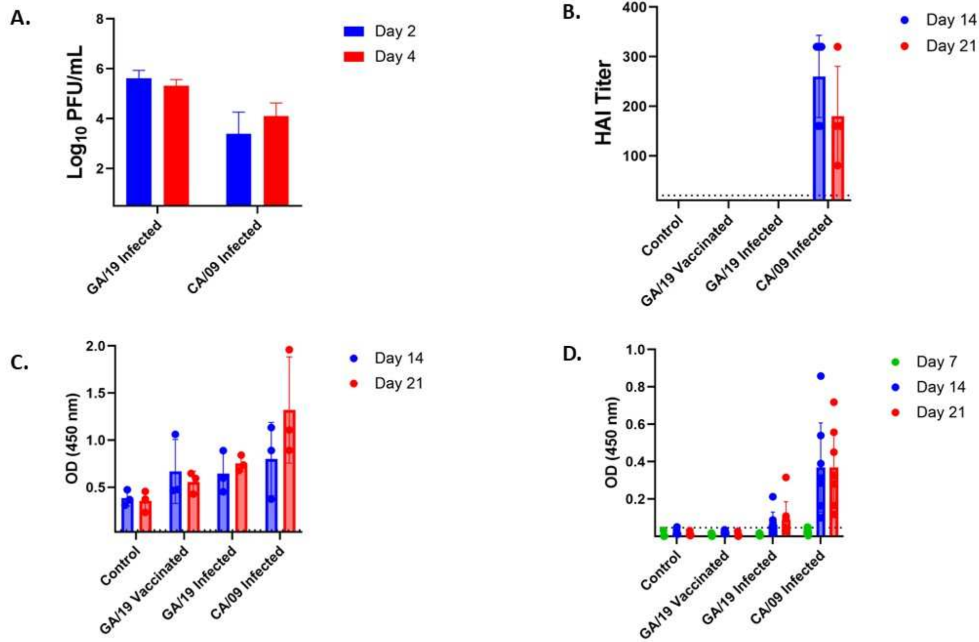


Figure 1. Vaccination with WIV leads to a differential serum antibody response compared to intranasal infection. A) Virus titers in nasal swabs on days 2 and 4 post-infection. B) Hemagglutination inhibiting serum antibody titers following vaccination or primary infection. C) Whole CA/09 specific antibody levels determined by IgG ELISA (serum dilution at 1:800). D) Stalk (HA2) specific antibody levels determined by ELISA using recombinant cH6/1 HA protein(serum dilution of 1:800).

Figure 2.

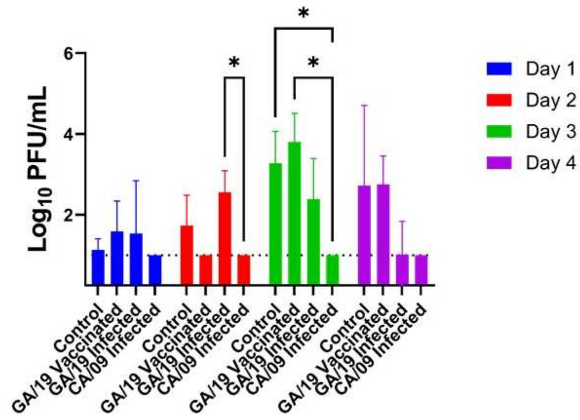


Figure 2. Priming with an antigenically distinct swIAV confers incomplete protection to CA/09 infection. Control, infected or vaccinated animals were challenged 21 days later with CA/09, nasal swabs collected daily, and assayed for infectious virus by plaque assay. Statistically different values represented as * for p-values ≤0.05.

Figure 3.

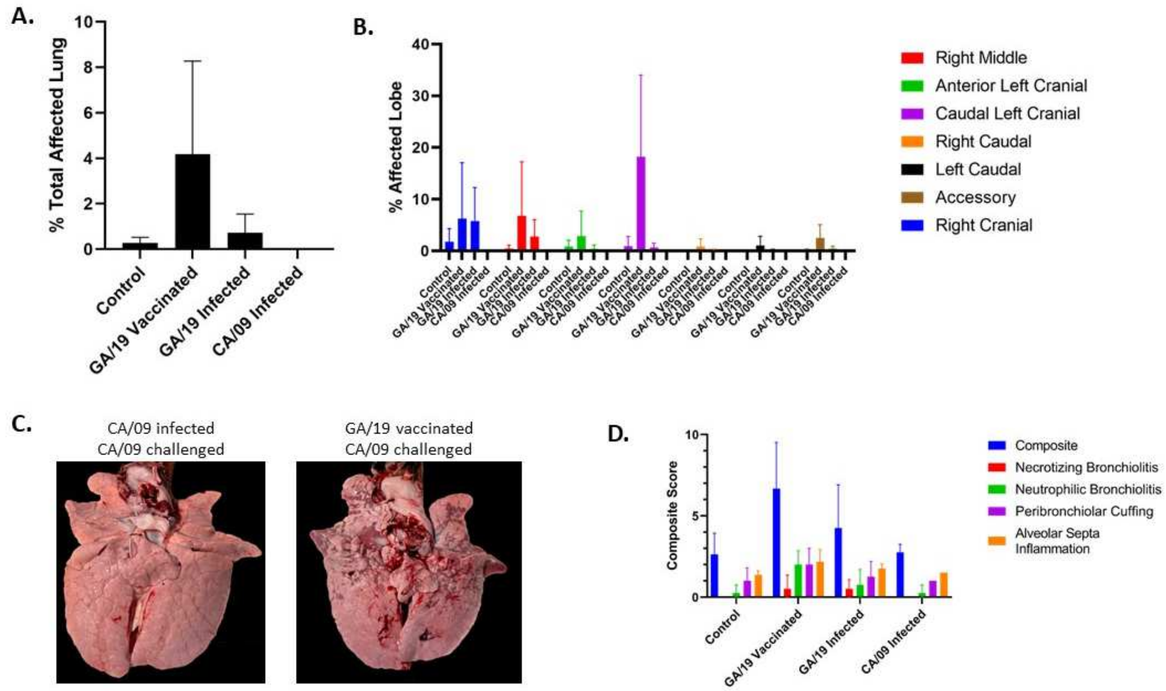
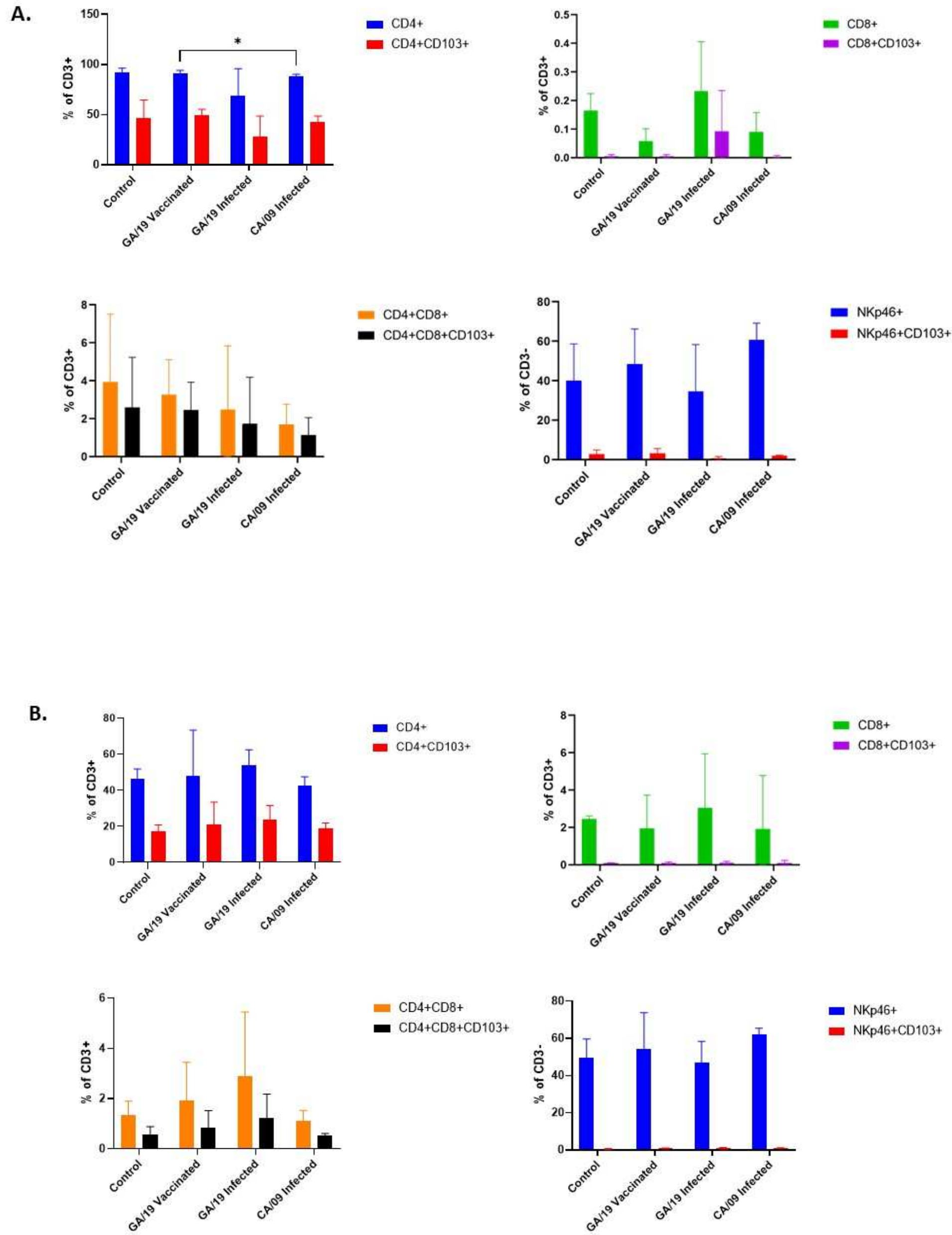


Figure 3. Vaccination with heterologous WIV leads to enhanced pathology compared to mucosal infection. A) Total percent consolidation score of the lungs. B) Lobular-specific gross pathology scores. C) Representative image of gross pathology between a GA/19 WIV-vaccinated animal and a CA/09 infected animal at day 5 post-CA/09 challenge. D) Average histopathology score.

Figure 4.



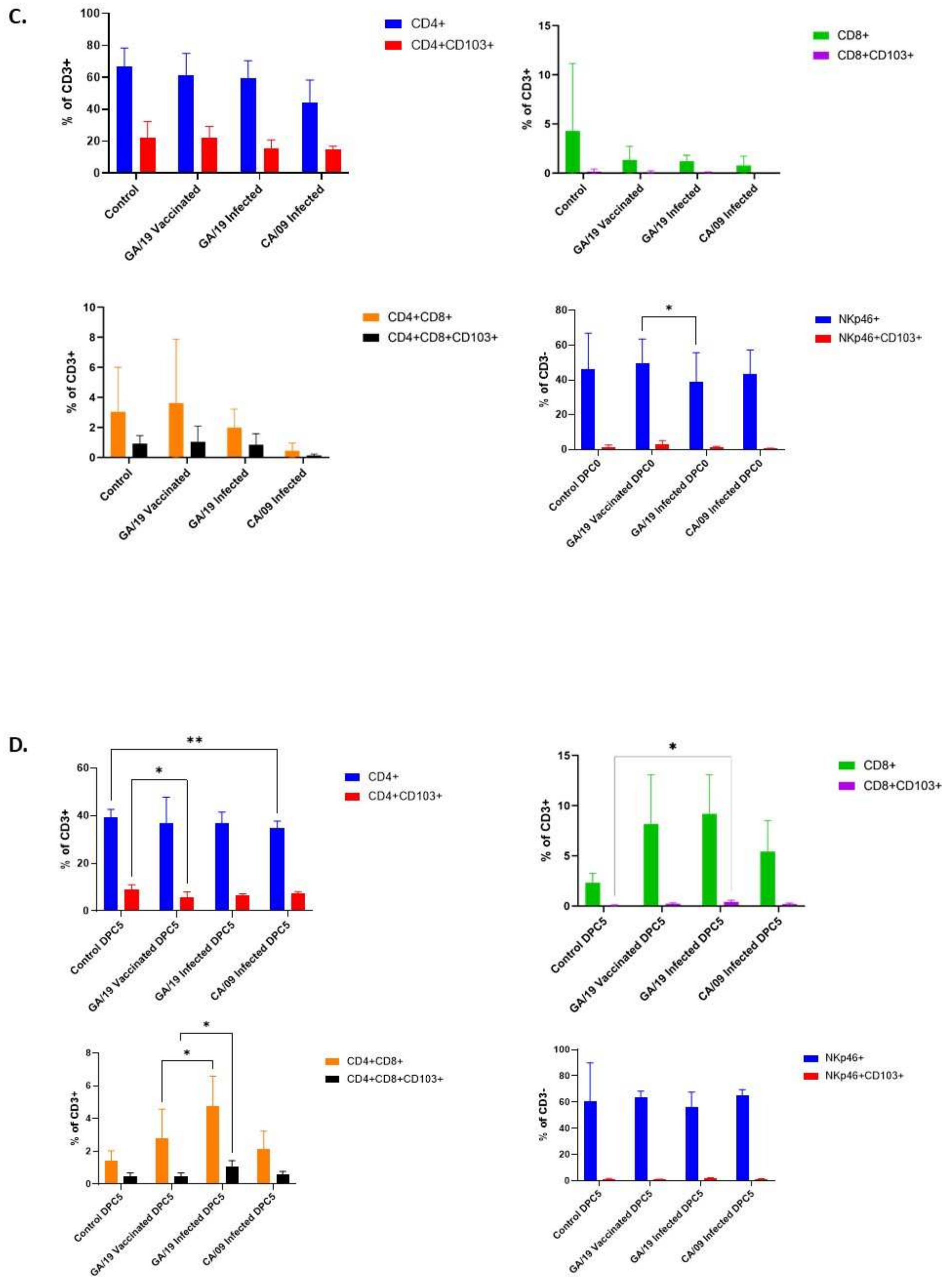


Figure 4. CD4+, CD8+, and CD4+CD8+ T cells and NK cells co-expressing tissue residency marker CD103 in the trachea and lung. A) Lymphocyte populations in the trachea at study day 20 (pre-challenge). B) Lymphocytes in the trachea at 5 days post-challenge. C) Lymphocyte populations in the lung at study day 20. D) Lymphocyte populations in the lung at day 5 post-challenge. Y axis descibes the percentage of either CD3+ or CD3- populations co-expressing the indicated markers. Data are representative of a total lymphocyte count of 50,000 events as determined by gating on FSC and SSC size exclusion. Statistically different values represented as * or ** for p-values ≤0.05 or 0.01, respectively.

CHAPTER 5

CONCLUSIONS

Swine influenza presents a continuing health risk to both commercial swine farms as well as human populations. Epidemiological features that serve to enhance the spread of influenza, such as increased overall herd count of animals, increased concentration of animals and specialization of facilities in North America have only increased in the last decade. This has been countered to a certain extent with increased biosecurity measures to control the spread of pathogens, however there are still periodic outbreaks not only of influenza but other common microbial pathogens of swine that highlight the shortcomings of current control measures. In order to control the spread of those influenza viruses of greatest concern, a rigorous framework first needs to be established that can reliably forecast which emerging strains pose the greatest risk to both humans and swine so preventative steps can be taken targeting them specifically. **Specific Aim 1** addressed this need by running a contemporary swine influenza isolate through a battery of tests used in current risk assessment tools to determine shortcomings in existing models of influenza risk assessment.

Despite a longstanding and recognized need for effective vaccines in both humans and swine, there are yet to be developed influenza vaccines with broadly protective capabilities. One of the barriers that might be overcome deals with the route of inoculation most commonly taken by commercial vaccine formulations. An intramuscular vaccination with whole inactivated influenza, which when paired with an adjuvant reliably induces a serum antibody response specific for the inoculating strain, regularly fails to protect individuals from slightly different influenza strains that have drifted at key antigenic epitopes. Even worse, in swine and ferrets this antigenic mismatch can be associated with enhanced disease. **Specific Aim 2** examined the relevance of the VAERD phenomenon by developing a novel model containing a viral mismatch outside the previous VAERD literature. Accomplishing this, we sought to

identify T and NK populations that had established residency in the respiratory epithelium that would confer heterosubtypic immunity upon challenge.

Part I. Pandemic Risk Assessment for a Swine Influenza A Virus in Comparative Human Substrates

Our study was able to rigorously examine several of the most commonly used models for assessment of influenza pandemic risk potential in the context of an emerging strain of influenza, the δ2-lineage swine influenza H1 viruses as represented by an isolate from a case of lethal influenza infection. Isolated from the lungs of the source animal, the virus was sequenced and assessed from pathogenicity and transmission potential in mouse, ferret and swine animal models as well as *in vitro* culture systems derived from human and swine respiratory tissues. Our results obtained a consensus in that the virus posed a risk to animal health by demonstrating both replication within inoculated animals as well as transmission potential to naïve contact animals. Further, our infection experiments within ferrets identify a potential risk of spillover to human populations, as this model is widely acknowledged as a gold standard for human influenza infection and the isolated swine virus possessed both replication and transmission potential within our ferret model.

One of the key findings of our study was an inconsistency in the replication of the swine influenza isolate in human primary epithelial cells compared to animal models and human-derived lab-adapted cell lines. The popularity of the NHBE model continues to grow as this is considered the closest approximation to the human respiratory epithelium, however there is an inherent heterogeneity in the model due to the variety of tissue donors from which the cells are derived as well as the diverse characterization of the cells within a given tissue biopsy. In some contexts, this disqualifies the NHBE model as a suitable surrogate for human respiratory epithelium in general, and assessment of pandemic risk potential might be one such instance. Our studies replicated our findings in NHBE cells from multiple donors, however a more rigorous methodology is needed before findings can be said to be applicable to

the human population as a whole. One means by which this standardization can be achieved is the immortalization of current donor stocks, which can eliminate the variation in cell cultures derived from donors that are continually depleted then restocked with new donor lots. However, the immortalization process itself comes with the caveat that it may reduce the relevance of future studies using the now lab-adapted cell line as it is no longer a truly primary cell culture. Even among more common laboratory strains such as Madin-Darby canine kidney (MDCK) or Vero cells, continued passage under different conditions results in differential viral susceptibility over time. Recent studies have characterized immortalized and primary NHBE cells in greater depth in regard to both physical characteristics as well as responses to influenza infection, but more work in the area is needed before a panel can be designed that captures the breadth of human bronchiolar tissues.

One feature of *in vitro* culture systems that makes them an attractive option for assessing risk potential of viruses in their ease and speed of use. Whereas animal models require established facilities with containment capabilities in which novel pathogens might be safely assessed, infection models in cell culture systems come with far fewer obstacles to containment. Infection of a cell culture may take place within hours of identification of clinical disease in a human or animal, compared to the days or even weeks that accompany the design and execution of animal models of influenza infection. Culture systems can also provide more nuanced information than an animal model regarding kinetics of viral replication, and even illuminate species barriers that prevent spillover as increasing numbers of primary and lab-adapted cell lines from multiple species backgrounds are developed. As is the case with NHBE cells, the origins and passaging of these cell cultures likely selects for certain features that distinguishes them from natural tissues, such as capability to grow differentiate under artificial culture conditions or increase permissivity to infection by a range of pathogens. Our infection studies using the Calu-3 cell line might be indicative of such a problem. Derived from a human lung adenocarcinoma, this cell line is widely used due to the ease with which it is cultured and its susceptibility to human respiratory

pathogens. While its origins from human lung tissue make it a more appropriate model for influenza infection than animal kidney-derived cell lines, the starkly contrasting capability of this cell line to become infected by the A/swine/Georgia/27480/2019 isolate compared to NHBE cells calls into question the usefulness of these cells in gauging a virus's ability to invade the human respiratory tract.

Part II. The Role of Cell-Mediated Immunity in Ameliorating VAERD in Weanling Pigs

By recapturing the VAERD disease phenotype using the A/swine/Georgia/27480/2019 virus we are the first lab to extend the issue of VAERD to the δ2-clade of swine influenza viruses. The increasing prevalence of this viral clade, as well as the δ1 viruses that have been previously used in VAERD mismatches, highlights a growing need to create an effective vaccine strategy that not only protects animals from viral shedding but doesn't enhance disease upon a heterologous challenge. Recent studies have further increased the scope of the VAERD phenotype, demonstrating that ferrets are also susceptible to increased clinical disease as well as lung pathology when established viral mismatches are used. With this increasing evidence of VAERD outside of swine and limited viral mismatches, it seems likely that antigenic mismatch in vaccination leads to enhanced pathology in humans. As seen in our studies, the increase in clinical sings might be sufficiently mild to go unnoticed, or more likely the wide influenza-specific antibody and T cell repertoire that exists in most people serves to mitigate viral infection before VAERD can be established. However, in the very young who are immunologically naïve or in the immunosuppressed upon new vaccination, VAERD may play a much more significant role in severity of influenza-related disease.

Key to reducing VAERD within the context of swine infection with influenza is mucosal inoculation with a live influenza virus. Previous studies have achieved this via priming of animals with a live attenuated influenza virus, and our study was able to accomplish this level of protection by infecting animals with a nonpathogenic dose of the A/swine/Georgia/27480/2019 virus. One of the key benefits

to an intranasal priming is that it elicits a localized immune response in the same way a natural infection might proceed. Intramuscular vaccination provides an influenza-specific serum IgG response, but fails to create an IgA response that allows for secretion of the antibody into the bronchiolar lumen that can quickly neutralize invading virus before infection foci become established. Intramuscular vaccination also fails to promote T cell trafficking to the respiratory tract, which is crucial for a rapid response upon subsequent challenge, as well as providing a broader scope of protection compared to neutralizing influenza-specific antibodies that target an antigenically variable head domain. A subset of the elicited T cells has been found to leave the vasculature and establish a tissue-resident phenotype, which in swine possess a combination of memory and effector cell functions that make them crucial in combatting future viral infections

The ease and widespread acceptance of intramuscular inoculation in a commercial swine setting, or a human clinic, has almost excluded the possibility of any alternative route of vaccination. While this may seem to also exclude the possibility of a tissue-resident T cell response, by better characterizing these cells in an intranasal vaccination, the first steps are being made in designing other vaccines that can redirect the immune system to shunt T cells to the respiratory epithelium, regardless of route of inoculation. Current work in the field has progressed immensely in our ability to track the development of these cells from naïve progenitors, and so manipulation of lineage determination of lymphocytes may be on the horizon. While not a fantasy, programming of lymphocytes to this extent may not be necessary so long as this arm of the immune response is considered when designing influenza vaccines, which currently use a durable and cross-reactive IgG response as the main correlate of protection. Rather than an afterthought, our studies have made it evident that a mucosal and cell-mediated response is crucial in effective protection of swine from influenza.