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# Dissecting Human Antibody Responses Against Influenza A Viruses And Antigenic Changes That Facilitate Immune Escape

Seth J. Zost

University of Pennsylvania, [szost@upenn.edu](mailto:szost@upenn.edu)

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# Dissecting Human Antibody Responses Against Influenza A Viruses And Antigenic Changes That Facilitate Immune Escape

## **Abstract**

Influenza A viruses pose a serious threat to public health, and seasonal circulation of influenza viruses causes substantial morbidity and mortality. Influenza viruses continuously acquire substitutions in the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). These substitutions prevent the binding of pre-existing antibodies, allowing the virus to escape population immunity in a process known as antigenic drift. Due to antigenic drift, individuals can be repeatedly infected by antigenically distinct influenza strains over the course of their life. Antigenic drift undermines the effectiveness of our seasonal influenza vaccines and our vaccine strains must be updated on an annual basis due to antigenic changes. In order to understand antigenic drift it is essential to know the sites of antibody binding as well as the substitutions that facilitate viral escape from immunity. In this dissertation, we explore both the epitopes targeted in human antibody responses and how influenza viruses evade these responses. We first demonstrate that prior exposure shapes the sites targeted in human antibody responses, and show that many middle-age adults mounted an antibody response against H1N1 viruses that is focused against sites on HA conserved between contemporary strains and strains that circulated in early childhood. In addition, we demonstrate that a viral substitution in this epitope allows influenza viruses to evade neutralizing antibody responses. We next demonstrate that an H3N2 HA substitution introducing a glycosylation site prevents the binding of neutralizing antibodies present in a large number of individuals. Importantly, our egg-based vaccines lack this glycosylation due to culture-adaptive substitutions, but a vaccine containing this glycosylation motif more potently induced antibody responses against circulating strains. Finally, we identify and characterize antibodies that target conserved residues in the receptor-binding site (RBS) of HA. We demonstrate that in some individuals RBS antibodies in sera contribute to neutralization of antigenically distinct strains, even in the case of an antigenically mismatched vaccine. Overall, the work presented here helps address the complex interaction of influenza viruses and human immunity. Importantly, our work identifies shortcomings with our current process of vaccine strain selection and production and investigates epitopes of interest for universal influenza vaccine efforts.

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DISSECTING HUMAN ANTIBODY RESPONSES AGAINST INFLUENZA A VIRUSES AND  
ANTIGENIC CHANGES THAT FACILITATE IMMUNE ESCAPE

Seth J. Zost

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Supervisor of Dissertation

---

Scott E. Hensley, PhD

Associate Professor of Microbiology

Graduate Group Chairperson

---

Daniel S. Kessler, PhD

Associate Professor of Cell and Developmental Biology

Dissertation Committee

Paul F. Bates, PhD, Professor of Microbiology

Frederic D. Bushman, PhD, Professor of Microbiology

Laurence C. Eisenlohr, PhD, VMD, Professor of Pathology and Laboratory Medicine

George M. Shaw, MD, PhD, Professor of Medicine



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## ABSTRACT

### DISSECTING HUMAN ANTIBODY RESPONSES AGAINST INFLUENZA A VIRUSES AND ANTIGENIC CHANGES THAT FACILITATE IMMUNE ESCAPE

Seth Julius Zost

Scott E. Hensley

Influenza A viruses pose a serious threat to public health, and seasonal circulation of influenza viruses causes substantial morbidity and mortality. Influenza viruses continuously acquire substitutions in the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). These substitutions prevent the binding of pre-existing antibodies, allowing the virus to escape population immunity in a process known as antigenic drift. Due to antigenic drift, individuals can be repeatedly infected by antigenically distinct influenza strains over the course of their life. Antigenic drift undermines the effectiveness of our seasonal influenza vaccines and our vaccine strains must be updated on an annual basis due to antigenic changes. In order to understand antigenic drift it is essential to know the sites of antibody binding as well as the substitutions that facilitate viral escape from immunity. In this dissertation, we explore both the epitopes targeted in human antibody responses and how influenza viruses evade these responses. We first demonstrate that prior exposure shapes the sites targeted in human antibody responses, and show that many middle-age adults mounted an antibody response against H1N1 viruses that is focused against sites on HA conserved between contemporary strains and strains that circulated in early childhood. In addition, we demonstrate that a viral substitution in this epitope allows influenza viruses to evade neutralizing antibody responses. We next demonstrate that an H3N2 HA substitution introducing a glycosylation site prevents the binding of neutralizing antibodies present in a large number of individuals. Importantly, our egg-based vaccines lack this glycosylation due to culture-adaptive substitutions, but a vaccine containing this glycosylation motif more potently induced antibody responses against circulating strains. Finally, we identify and characterize antibodies that target conserved residues in the receptor-binding site (RBS) of

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## LIST OF ABBREVIATIONS

Abs	Antibodies
BCR	B-cell receptor
BPL	B-propiolactone
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
HCDR	Heavy-chain complementarity determining region
CHOP	Children's Hospital of Philadelphia
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FFU	Focus forming unit
FRNT	Foci-reduction neutralization test
Fc	Immunoglobulin constant region
GISRS	Global Influenza Surveillance and Response System
HA	Hemagglutinin
HAI	Hemagglutination inhibition assay
HAU	Hemagglutination unit
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIV	Human immunodeficiency virus
HK/14	A/Hong Kong/4801/2014
i.p.	Intraperitoneal
i.v.	Intravenous
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
mAbs	Monoclonal antibodies
MDCK	Madin-Darby canine kidney
MEM	Minimum essential medium
MN	Micro-neutralization test
NA	Neuraminidase
NIH	National Institutes of Health
PBS	Phosphate-buffered saline
PFU	Plaque forming unit
pH1N1	Pandemic H1N1 A/California/07/2009
PNGase	Peptide:N-glycosidase F
PR8	A/Puerto Rico/8/1934
RBS	Receptor-binding site
RNA	Ribonucleic acid
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sH1N1	Seasonal H1N1
Switz/13	A/Switzerland/9715293/2013
TCID50	50% Tissue culture infectious dose
TMB	3,3',5,5'-tetramethylbenzidine
VLR	Variable lymphocyte receptor
Vic/09	A/Victoria/210/2009
WHO	World Health Organization
WT	Wild type

# CHAPTER 1: INTRODUCTION

Parts of this chapter have been submitted for publication:

**Seth J. Zost\***, Nicholas C. Wu\*, Scott E. Hensley+, and Ian A. Wilson+. Immunodominance and antigenic variation of influenza virus hemagglutinin: implications for design of universal vaccine immunogen

(\* = equal contribution)

(+ = co-corresponding author)

## **The 1918 Pandemic: one hundred years later**

One hundred years ago, as the First World War continued to rage, a novel influenza virus made the leap from an animal reservoir to human-to-human circulation. Two years later, after three pandemic waves, an estimated 20-50 million people worldwide had died following infection with this virus (Johnson and Mueller, 2002). Over the next century the descendants of this pandemic virus recombined with other influenza viruses, swapping gene segments and causing four other pandemics in addition to substantial morbidity and mortality from annual seasonal circulation. One hundred years later, both of the subtypes of influenza viruses that continue to circulate in humans have genes that can be traced back to the virus that emerged with such a devastating effect in 1918 (Taubenberger and Morens, 2006). The 1918 pandemic serves as a stark reminder of the dangers posed by infectious diseases and of the serious threat influenza viruses pose to public health.

Since 1918, we have learned much about how influenza viruses enter cells, replicate, and evade host immunity. Despite these advances, our influenza vaccines are at best 60-70% effective, and in many years vaccine effectiveness is much lower (Lewnard and Cobey, 2018). As a result of viral escape from vaccine-induced immunity, annual vaccination does not provide long-lived protection from influenza virus infection (Carrat and Flahault, 2007). In addition, novel

influenza subtypes pose constant pandemic threats. In this chapter, I will introduce influenza A viruses and the disease they cause, the response that the immune system makes upon infection or vaccination, how the virus evades pre-existing immunity, and the state of our current and future vaccines.

## **Influenza A virus structure and tropism**

Although influenza viruses have likely circulated in humans since well before the 1918 pandemic, influenza virus was first isolated from infected humans by Wilson Smith, Christopher Andrewes, and Patrick Laidlaw in 1933 (Smith et al., 1933). Influenza viruses are members of the family Orthomyxoviridae, composed of influenza A viruses, influenza B viruses, influenza C viruses, and influenza D viruses. Aquatic birds are the major natural reservoir for influenza A viruses as they harbor the greatest number of distinct influenza A strains (Olsen et al., 2006). However, influenza A viruses infect a wide range of non-human mammals. Two genetic lineages of influenza B viruses co-circulate in humans (Rota et al., 1990). Influenza C viruses circulate in humans as well, although human influenza C cases are generally much more mild (Matsuzaki et al., 2006). Influenza D viruses have recently been isolated from cattle and swine, although little is known about pathogenesis of these recently described viruses (Hause et al., 2014). Avian influenza A viruses infect epithelial cells lining the gastrointestinal tract of birds and the viruses are transmitted from bird to bird by fecal-oral transmission (Olsen et al., 2006). In humans however, influenza viruses are transmitted via the respiratory route and influenza viruses infect epithelial cells in the airway and lung (Krammer et al., 2018). In humans, influenza infection and immune cell infiltration as part of the immune response causes lung inflammation and serious respiratory distress that can sometimes be fatal.

Influenza A viruses are negative-sense, single-stranded RNA viruses with an approximately 14 kilobase genome. The influenza A genome is composed of eight different gene segments. Circulating influenza viruses are incredibly diverse, and this is due in large part to the

segmented genome of influenza viruses, which facilitates recombination between strains. Viruses from different lineages that co-infect the same cell can swap gene segments, resulting in the generation of new viral variants that can have altered virulence or antigenic properties (Steel and Lowen, 2014). Importantly, this can result in zoonotic spillover events where reassortant influenza strains make the jump from circulation in one species into another (Taubenberger and Kash, 2010).

The number of proteins encoded by influenza A viruses varies, and smaller proteins that result from alternative ribosomal start sites are still being identified (Machkovech et al., 2018; Muramoto et al., 2013). However, all influenza A viruses encode nine proteins essential for replication and infection. The membrane-bound viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) are present on the surface of the virion and function in attachment, cellular entry, and viral egress (Gamblin and Skehel, 2010). Influenza subtypes are denoted by the HA and NA proteins they encode. The M gene segment encodes two different proteins. Matrix protein 1 (M1) is the major component of the viral capsid and matrix protein 2 (M2) is a transmembrane ion channel. Both of these proteins play roles in viral entry as well as virion assembly and budding (Gómez-Puertas et al., 2000; Manzoor et al., 2017; Pinto et al., 1992). Given that influenza viruses are negative-strand RNA viruses, viral RNA-dependent RNA polymerases are packaged into virions. The viral polymerase is composed of three subunits – polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein 1 (PA), that form viral ribonucleoprotein complexes (vRNP) with the genome packaging protein nucleoprotein (NP) and the RNA genome (Eisfeld et al., 2015; Zheng and Tao, 2013). While all these proteins are present in influenza virions, influenza viruses also encode two nonstructural proteins that are produced within infected cells but are not packaged into virions. Nonstructural protein 1 (NS1) functions as an antagonist of the innate immune system in addition to performing other functions (Ayllon and García-Sastre, 2015), while nuclear export protein (NEP) mainly serves to facilitate the export of replication products from the nucleus (O'Neill et al., 1998).

## Hemagglutinin structure and function

As the viral attachment and entry factor, HA plays an essential role in the viral lifecycle. HA is a trimeric glycoprotein composed of a membrane-distal globular head domain and a membrane-proximal stalk domain (Gamblin and Skehel, 2010). After viral endocytosis, HA undergoes a conformational change that fuses the membrane of the virus and the membrane of the acidifying endosome (Skehel and Wiley, 2000). This conformational change requires the cleavage of the precursor protein HA0 by trypsin-like proteases into a fusion-competent form of HA composed of HA1 and HA2. This cleavage takes place during virion maturation (Skehel and Wiley, 2000). 18 different subtypes of HA have been described, and the globular head domains of these HA subtypes are highly divergent (Tong et al., 2012). These subtypes can be divided into two different phylogenetic groups, group 1 and group 2. Currently, two subtypes of influenza viruses, H1N1 (a group 1 HA) and H3N2 (a group 2 HA), circulate in humans. When circulating strains acquire novel HA or NA glycoproteins through genetic recombination, this dramatically changes the antigenic determinants of the strain. This particular type of reassortment in which the surface antigens change is known as antigenic shift. Due to the lack of population immunity against novel HA and NA proteins, antigenic shift variants pose a potential pandemic threat (Krammer et al., 2018; Taubenberger and Kash, 2010).

The receptor-binding site (RBS), located in the HA globular head domain, mediates the interaction of HA with sialic acid, and some residues that interact with sialic acid are completely conserved across different HA subtypes. Sialic acid is the terminal sugar moiety of many glycoproteins and glycolipids present at the cellular surface, and the affinity of HA for different sialic acid linkages plays an important role in determining viral tropism. Viruses that circulate in humans generally have high affinity for the  $\alpha$ 2-6 linkage of sialic acid, which is the predominant sialic acid linkage on human airway epithelial cells. In contrast, viruses that circulate in birds have high affinity for the  $\alpha$ 2-3 linkage of sialic acid, which is the major linkage on epithelial cells in the avian gut. Influenza viruses of avian origin that have entered human circulation generally acquire

changes near the RBS that shift the binding preference of HA from  $\alpha$ 2-3 to  $\alpha$ 2-6 (Taubenberger and Kash, 2010). Importantly, antigenic substitutions can also modulate receptor avidity (Hensley et al., 2009; Li et al., 2013a). Due to the importance of HA in both the viral lifecycle and the immune response to infection and vaccination, the rest of this introduction will focus on antibody responses against HA and the mechanisms by which influenza viruses evade these responses.

## **Antibody responses and antigenic drift**

Humoral adaptive immune responses play an essential role in protection from reinfection with viruses. Adaptive immune responses must make specific and protective responses against a dizzying array of different pathogens, each with vastly different molecular structures. In vertebrates, antibody responses address this daunting challenge by combating diversity with diversity. Antibodies, which are expressed by B-cells, are composed of heavy and light chains that are randomly assembled by genomic recombination in a process called VDJ recombination, generating tremendous structural diversity (Murphy et al., 2012) These rearranged antibody genes are expressed on the surface of mature B-cells as a membrane bound B-cell receptor (BCR). In an immune response to influenza virus, B-cells specific for influenza antigens are activated by the interaction of the BCR with antigen and begin secreting antibody (predominantly IgM) and begin to undergo class-switching to IgG isotypes. In the case of antibody responses against viral proteins, B-cells encounter antigen and are activated in specialized lymphoid structures called germinal centers that are present in the lymph nodes and spleen (De Silva and Klein, 2015). In germinal centers, B-cells proliferate with CD4<sup>+</sup> T-cell help and undergo somatic mutation of the antibody gene to generate variants. B-cells expressing these variants undergo clonal selection through competition for antigen binding (Victoria and Wilson, 2015). The end result of this process of affinity maturation is a high-affinity polyclonal antibody response against viral antigens. B-cells expressing these high-affinity, class-switched antibody specificities can become committed to long-lived plasmablasts or memory B-cells. In the case of a secondary immune response, memory B-cells can be recalled, and these B-cells can re-enter the germinal

center for another round of somatic hypermutation and affinity maturation (Kurosaki et al., 2015). The existence of memory B-cell populations that can be recruited in secondary responses to influenza exposure has important ramifications for antibody-mediated immunity to influenza, and will be discussed in further detail in this introduction.

Antibodies targeting epitopes in the HA globular head domain can protect animals and humans from influenza virus infections (Coudeville et al., 2010). The majority of these antibodies neutralize by blocking viral attachment, although other neutralization mechanisms can be at play for some of these antibodies, such as blocking the pH-induced conformational change of HA upon endosomal acidification or by inhibiting viral budding (Brandenburg et al., 2013; Krammer and Palese, 2015). Infection and vaccination typically elicit strain-specific HA-head antibodies that are often long-lived. In a particularly dramatic example, survivors of the 1918 pandemic had high levels of serum antibodies specific for the 1918 HA almost a century after being infected (Yu et al., 2008).

For many viruses, infection elicits neutralizing antibody responses that confer lifelong immunity. For example, infection with measles protects against reinfection, and many of our inactivated or live-attenuated vaccines also provide protection for life. Although we have vaccinated against measles for decades, the vaccine remains highly effective against circulating viruses, indicating that viral escape from vaccine-induced immunity has not occurred (Marin et al., 2006). This is not the case, however, for immunity to influenza viruses. Population immunity to influenza viruses selects for antigenic variants in HA and NA with substitutions that introduce substitutions abrogating the binding of pre-existing antibody responses. Due to the selective advantage of having escaped immunity, these variant viruses then dominate circulation, and these antigenic substitutions become fixed in circulating strains. This process of immune evasion through the acquisition of substitutions in antigenic sites is called antigenic drift. By allowing influenza viruses to escape population immunity, antigenic drift facilitates continuous circulation of



influenza viruses in the human population, resulting in considerable morbidity and mortality (Yewdell, 2011).

Antigenic drift requires pre-existing viral variants that can undergo positive selection, and these antigenic substitutions must also not compromise critical functions of HA required for the viral lifecycle, such as receptor binding or conformational change. The error-prone viral RNA polymerase plays an essential role in facilitating antigenic drift by generating antigenic variants that can be positively selected by immune pressure. However, other RNA viruses such as measles do not undergo antigenic drift, despite also having high mutation rates. This may be due, in part, to different functional constraints on viral glycoproteins that restrict possible substitutions. Supporting this hypothesis, influenza virus HA is much more tolerant of insertional mutagenesis than the glycoproteins of measles virus (Fulton et al., 2015). It is also important to note that genetic changes in HA do not necessarily correspond to antigenic change, as substitutions that become fixed in HA do not always result in substitutions that prevent the binding of antibodies. Teasing out the contribution of individual substitutions to antigenic drift is essential to understanding how influenza viruses evade host immune responses.

## **HA immunodominance of primary antibody responses**

Given that antibody responses drive selection of antigenic drift variants, a better understanding of the process of antigenic drift requires understanding the sites targeted in human antibody responses. Studies in the 1980s began to define the relationship between genetic and antigenic change by demonstrating that substitutions that abrogate the binding of most antibodies are clustered in antigenic sites that cover much of the globular head of HA, and that antigenic drift substitutions accumulate in these same sites during viral circulation (Caton et al., 1982; Wiley et al., 1981; Yewdell et al., 1979). These antibodies against highly exposed epitopes on the HA head usually dominate primary responses against influenza viruses, and the immunodominance of HA antibody responses is not a phenomenon unique to human antibody responses. Primary H3N2 infections in ferrets elicit high levels of antibodies that are directed towards HA antigenic

sites A and B, which are located in close proximity to the HA RBS (Koel et al., 2013). In particular, a study of H3N2 antigenic evolution that relied on ferret sera suggested that H3N2 antibody responses are narrowly focused, and that substitutions in only two antigenic sites are responsible for H3N2 antigenic drift (Koel et al., 2013). Similarly, H1N1-infected young children tend to mount antibody responses to epitopes in antigenic sites near the HA RBS (Koel et al., 2015). The observation that antigenically distinct strains have changes in multiple antigenic sites originally led to the proposal that acquisition of antigenic substitutions in multiple antigenic sites was required for antigenically drifted variants to circulate (Wiley et al., 1981). However, it is becoming clear that single substitutions in the HA head domain can have major effects on antigenicity. Such an example occurred during the 2014-2015 season when antigenically drifted H3N2 strains possessed new substitutions in the HA head (Chambers et al., 2015). The circulation of strains possessing these novel substitutions coincided with dramatically reduced vaccine effectiveness (Zimmerman et al., 2016).

In contrast to the immunodominant HA head, HA stalk antibody responses constitute a small fraction of total anti-influenza virus antibodies in most humans (Sui et al., 2011). While steric hindrance has been suggested to contribute to the immunosubdominance of HA stalk antibodies (Andrews et al., 2015), recombinant HA vaccines also fail to elicit high-titer HA stalk responses (Nachbagauer et al., 2016). In fact, cryoelectron tomography has shown that the majority of the HA on influenza virions are available to bind to stalk antibodies (Harris et al., 2013). HA stalk antibodies can be polyreactive (Andrews et al., 2015), and it is possible that due to an intrinsic property of antibodies recognizing stalk epitopes, B-cells specific for HA stalk epitopes are under negative selection. This could contribute to HA stalk antibody immunosubdominance. More recent work suggests that the fine-specificity of influenza virus antibody responses in mice change over time (Angeletti et al., 2017). Angeletti and colleagues found that antibodies against epitopes near the top of the HA head dominate the early response, and antibodies against other epitopes increase later in the response (Angeletti et al., 2017).

Given that most studies have only examined a limited range of timepoints, it is likely that shifts in antibody immunodominance dynamics have yet to be fully explored.

While there are likely antibody-intrinsic contributions to HA immunodominance, it is also possible that the observed immunodominance of HA is, in part, due to structural features of the HA protein itself. Almost all immunological studies of influenza virus have been carried out in organisms that make immunoglobulin-based humoral responses. To test whether some features of immunodominance are antigen-intrinsic, Altman and colleagues studied immune responses in lampreys that were immunized with influenza virus (Altman et al., 2015). Lampreys, a jawless fish, lack immunoglobulin genes but encode variable lymphocyte receptors (VLRs), which are an entirely different system of humoral adaptive immunity based on Leu-rich repeats (LRR) rather than immunoglobulin domains. Remarkably, lamprey VLR responses were found to be focused on the same HA epitopes as those that have been observed in mice (Altman et al., 2015). The similarity of antibody and VLR responses against HA in mice and lamprey suggest that properties of the HA protein itself, such as electrostatic or structural features, contribute to antibody immunodominance hierarchies.

### **Immune history shapes secondary immune responses to influenza viruses**

It has been known since the 1950s that antibodies elicited by primary influenza virus exposures are highly strain-specific, whereas antibodies elicited by secondary exposures with antigenically distinct viral strains tend to be highly cross-reactive with the first strain (Davenport and Hennessy, 1956; Davenport et al., 1953; Francis, 1960). This observation was originally referred to as 'original antigenic sin' (Francis, 1960), as the recalled antibody response that cross-reacted with the first strain appeared to come at the expense of a *de novo* response against the second strain. More recently, the effects of prior exposure have been referred to as 'antigenic seniority' (Lessler et al., 2012) or 'immune imprinting' (Gostic et al., 2016). Although the mechanisms behind 'original antigenic sin' have yet to be fully elucidated, it is thought that cross-

reactive B cells elicited by previous influenza virus exposures are preferentially recalled upon exposure with an antigenically distinct viral strain.

The HA head of the 2009 pandemic H1N1 strain was radically different compared with previously circulating seasonal H1N1 strains. Several studies suggest that prior seasonal H1N1 exposures influenced the fine-specificity of antibodies elicited against this antigenically distinct 2009 pandemic H1N1 virus in humans (Huang et al., 2015; Li et al., 2012; Li et al., 2013b; Wrammert et al., 2011; Xu et al., 2010). In many adults, the 2009 pandemic H1N1 strain preferentially boosted HA stalk antibodies (Li et al., 2012; Wrammert et al., 2011), likely because this strain possessed a radically different HA head but similar HA stalk compared to previously circulating seasonal H1N1 strains. Stalk antibodies, however, were not the only antibody type that was preferentially recalled in humans exposed to the 2009 pandemic H1N1. In some individuals, the 2009 pandemic H1N1 virus elicited antibody responses that were highly focused on rare HA head epitopes that were conserved in seasonal H1N1 strains that they were exposed to in childhood (Huang et al., 2015; Li et al., 2013b; Xu et al., 2010). Importantly, different aged individuals were found to mount antibodies of different specificities upon exposure to the 2009 pandemic H1N1 virus, due to differences in seasonal H1N1 exposure histories. Furthermore, age-specific antibody responses could be recapitulated in the ferret animal model by sequential infection with a seasonal H1N1 strain and the pandemic H1N1 strain (Li et al., 2013b). These studies of antibodies against the 2009 pandemic H1N1 illustrate how the recall pre-existing immune memory can shape the antibody response to antigenically drifted strains.

Although for decades it was assumed that H1 and H3 subtypes were completely antigenically distinct, it is becoming increasingly clear that human antibodies can target epitopes conserved across HA subtypes (Joyce et al., 2016; Lee et al., 2016; McCarthy et al., 2018). The existence of sites of cross-reactivity on HAs of different subtypes also means that prior immunity to one HA subtype likely shapes the response against other subtypes. This is consistent with a recent study by Gostic and colleagues who used epidemiological data to demonstrate a

correlation between the probability of first exposure in early childhood to either a group 1 or group 2 HA and susceptibility to avian H5N1 and H7N9 viral strains (Gostic et al., 2016). They found that individuals who were likely exposed to a virus with a group 1 HA in childhood appeared to be protected from H5N1 but susceptible to H7N9, while individuals who were likely exposed to a virus with a group 2 HA in childhood appeared to be protected from H7N9 but susceptible to H5N1. Given that all individuals were likely exposed to group 1 and group 2 strains through normal seasonal circulation, this finding suggests the first response to an influenza virus HA affects the immune response against different subtypes. While the mechanism mediating this heterosubtypic protection has not been determined, it is likely that stalk antibodies play a role. One possibility is that individuals exposed in childhood to group 1 HAs are more likely to respond well to group 1 HA stalk antigens, whereas individuals exposed in childhood to group 2 HAs are more likely to respond well to group 2 HA stalk antigens. The mechanism for this phenomenon of imprinting could in part be due to recall of pre-existing memory B-cells elicited by the first strain in the response to the second strain. These recalled B-cells could outcompete *de novo* anti-stalk responses in the response to the second strain, resulting in a lack of protective antibodies and increased susceptibility to infection with a pandemic strain.

### **Influenza vaccines, viral surveillance, and challenges with vaccine strain selection and production**

Some influenza vaccines are prepared by growing the virus in cell culture, expressing the HA as a recombinant protein in insect cells, or by generating a live-attenuated virus. However, the vast majority of influenza vaccines are produced by growing the virus in embryonated chicken eggs and chemically inactivating the virus (Grohskopf et al., 2018). This basic process has been used to prepare vaccines since the 1940s. After replication, the HA and NA from the vaccine strain are purified for inclusion in the vaccine. Our current licensed influenza vaccines contain one H1N1 strain and one H3N2 strain, and either one or two influenza B strains (Grohskopf et al., 2018). Due to antigenic drift however, circulating strains continuously acquire antigenic

substitutions that confer escape from antibody specificities boosted by vaccination. As a result, the strains included in the vaccine must be updated on a nearly annual basis in order for vaccines to be effective. As a part of this effort, the World Health Organization (WHO) has established the Global Influenza Surveillance and Response System (GISRS), a global network for influenza viral surveillance composed of collaborating centers throughout the world (Zhang and Wood, 2018). Through these centers circulating strains collected from infected individuals are isolated, sequenced, and antigenically characterized. As part of this effort, some of these isolated strains are passaged in embryonated chicken eggs or cell culture systems. The HA and NA genes from these isolates are recombined with the six internal gene segments of the culture-adapted virus A/Puerto Rico/8/1934 (PR8) in order to generate high-growth reassortant strains. Some of these high-growth reassortant strains are designated as candidate vaccine viruses (CVVs), and manufacturers are recommended to use these seed strains in their production of vaccine.

In order to determine whether circulating strains have antigenic changes that confer escape from vaccine-induced immunity, ferrets are infected with the vaccine strain. After the ferrets recover from infection, serum from these infected ferrets is collected. This standardized antiserum is then used to screen circulating strains in hemagglutination-inhibition (HAI) assays. The HAI assay measures the ability of antibodies to prevent hemagglutinin-mediated agglutination of red blood cells, and is used as a proxy for the ability of sera to inhibit viral receptor binding (Francis, 1947; Hirst, 1942). Vaccine strains are generally updated when this ferret antiserum poorly inhibits agglutination by circulating strains. In recent years, micro-neutralization (MN) assays, which measure the ability of serum antibodies to prevent viral infection and replication in cell culture, have also been used to measure the ability of serum antibodies to inhibit infection and human sera panels have also been incorporated into viral surveillance efforts (Beer et al., 2018).

Our current system of vaccine strain selection and production has several shortcomings. One weakness is the timeline of vaccine production. One dose of vaccine requires approximately

one embryonated chicken egg, and in the past five years, there have been approximately 140 million doses of vaccine produced and distributed in the United States alone (Sah et al., 2018). Producing such a large number of doses in time for the influenza season requires a demanding production schedule. Vaccine doses are distributed in September and October prior to the start of the influenza season, but in order to meet this production deadline, manufacturers of the Northern Hemisphere vaccine begin production immediately after the vaccine strain choices are announced in February. As a result, seasonal influenza vaccines are produced almost nine months prior to their use. Antigenically distinct strains with novel substitutions can emerge after it is too late to update the strain included in the vaccine (Houser and Subbarao, 2015). When these antigenically drifted strains circulate during an influenza season, vaccine effectiveness can be poor. This was the case during the 2014-2015 influenza season, when there was an antigenic mismatch between the strain in the vaccine and the strains that actually circulated (Zimmerman et al., 2016). This vulnerability can be especially serious in the event of an influenza pandemic. For example, in the case of the 2009 H1N1 pandemic, the vaccine arrived too late to prevent most of the infections that occurred (CDC, 2010).

In addition to the difficulties of producing so many vaccine doses, the process of adaptation to growth in chicken eggs often results in changes in the HA and NA glycoproteins. These changes often occur in or near antigenic sites around the RBS. The likely reason for many of these culture-adaptive changes is the selective pressure to improve receptor binding in chicken eggs. The end result of these adaptive substitutions is the introduction of potential antigenic mismatches between the vaccine strain and circulating strains, which has the potential to undermine vaccine effectiveness. Moreover, generation of CVVs is a labor-intensive and time-consuming process. Occasionally there will not be a CVV prepared from a clade that is predicted to circulate. This was the case during the 2015-2016 Northern Hemisphere influenza season, when a strain from a different genetic subgroup was included in the vaccine because there were no CVVs available from the clade that dominated circulation (Beer et al., 2018; Zimmerman et al., 2016).

Finally, the use of primary ferret antisera to determine antigenic mismatches is potentially problematic. As previously discussed for the 2009 pandemic H1N1, immune history can have a dramatic effect on the epitopes targeted by human antibody responses. However, reference antisera is prepared from ferrets that lack any prior influenza immunity. As a result, it is possible that viral substitutions that have antigenic effects in human populations might go undetected when relying solely on ferret sera to distinguish antigenic changes. Although human sera panels are used as part of viral surveillance efforts, the heavy reliance on ferret sera to distinguish antigenic changes could result in the vaccine strain not being updated, and could undermine vaccine effectiveness.

## **New universal vaccine approaches**

While most antibodies against the HA head are directed against epitopes adjacent to the conserved HA RBS (Koel et al., 2013; Popova et al., 2012), some antibodies are able to partially mimic the sialic acid receptor and bind to conserved residues within the HA RBS (Ekiert et al., 2012; McCarthy et al., 2018; Schmidt et al., 2015b; Whittle et al., 2011; Xu et al., 2013). While the HA RBS has been proposed as a universal vaccine target (Schmidt et al., 2015b), we know relatively little about how antibodies targeting the RBS are elicited and recalled.

In contrast to most epitopes on the HA head, the HA stalk is less tolerant of change (Doud and Bloom, 2016; Heaton et al., 2013; Thyagarajan and Bloom, 2014; Wu et al., 2014) and is much more highly conserved across subtypes. While some anti-HA stalk mAbs can directly neutralize viruses through inhibiting the proteolytic processing of HA by extracellular trypsin-like proteases, pH-induced conformational changes, and viral egress (Brandenburg et al., 2013; Krammer and Palese, 2015), many of the HA stalk antibodies that have been described require Fc-mediated effector functions for *in vivo* protection (DiLillo et al., 2014).

Several new universal vaccines are being developed to elicit antibodies against the immunosubdominant HA stalk. One approach is to generate stable “headless” HA constructs that



lack the head domain (Steel et al., 2010), and as a result, induce antibody responses exclusively directed against HA stalk epitopes. Historically, this has been a difficult task because the HA head domain serves to stabilize the HA stalk, and as a result, headless HA constructs were too unstable for use as immunogens. Recently, two groups have generated headless HAs by introducing stabilizing substitutions in the stalk domain (Impagliazzo et al., 2015; Yassine et al., 2015). Another approach to elicit stalk antibody responses is sequential immunization with chimeric HAs that express divergent head domains with the goal of refocusing antibody responses towards the HA stalk domain (Krammer et al., 2013). This approach is promising because chimeric HAs selectively recall subdominant HA stalk-reactive B-cells in the absence of HA head-reactive immunity. Due to the high degree of conservation between stalk domains of HA within the group 1 and group 2 subgroups, stalk-directed immunity against group 1 and group 2 HAs would have the additional benefit of protecting from infection by potential pandemic viruses (Ellebedy et al., 2014; Henry Dunand et al., 2015). Both of these approaches have shown protection in animal models, but their success in humans will likely depend on their ability to induce protective responses in the presence of different types of pre-existing immunity.

## **Experimental Questions**

In order to improve influenza vaccine strain selection and viral surveillance, we need a better understanding of how human antibody responses are shaped by prior exposure, how individual substitutions facilitate viral escape from antibody responses, and what factors might be undermining the effectiveness of our existing vaccines. Our work identifies several distinct sites on HAs of recent H3N2 and H1N1 viruses that are targeted by antibodies present in human individuals. Importantly, we also identify antigenic changes in HA that allow the virus to escape population immunity.

In Chapter 2 we first demonstrate the effects of prior immunity on responses against antigenically novel influenza strains. In particular, we identify a subset of individuals who mount a highly focused antibody response against an epitope in the 2009 pandemic H1N1 HA. We

demonstrate that this antibody specificity is likely a result of prior exposure with seasonal H1N1 strains that circulated during the early childhood of these individuals. Upon exposure to the 2009 HA, antibodies specific for an epitope on the HA head were selectively recalled by the 2009 HA because these antibodies cross-reacted between the 2009 HA and previous seasonal H1N1 HAs. Importantly, a single amino acid change in this epitope became fixed in circulating H1N1 strains and resulted in the abrogation of neutralizing antibody responses in a large number of middle-aged individuals. Due to this substitution, these middle-aged individuals were atypically susceptible to infection during the 2013-2014 influenza season. This case illustrates how prior exposure to influenza shapes subsequent responses and how influenza viruses can escape focused antibody responses. In addition, this work raises concerns with our reliance on ferret antisera to distinguish antigenic changes, as ferret antisera from primary infections does not recapitulate the full complexity of human immunity.

In Chapter 3 we explore the effects that culture-adaptive substitutions in our vaccines have on the antigenicity of the HA and the quality of the vaccine-induced antibody response. We first identify an antigenic substitution in H3N2 viruses that facilitated escape from neutralizing antibody responses present in a large number of individuals. This substitution introduced a glycosylation in a major HA antigenic site. As a result, pre-existing antibodies in humans could no longer bind to HA. Importantly, our egg-adapted vaccine strains lost this glycosylation site during adaptation of the virus to growth in chicken eggs, resulting in an antigenic mismatch between the vaccine strain and circulating strains. We demonstrate that as a result of this loss of a glycosylation, egg-adapted vaccines induced poor neutralizing antibody responses against circulating H3N2 strains. However, a vaccine lacking egg-adaptive changes more effectively induces neutralizing antibody responses against circulating strains. This work highlights how changes that occur as part of the vaccine manufacturing process may compromise the effectiveness of our vaccines. Furthermore, our findings suggest that producing vaccines in systems that are able to more faithfully recapitulate the HA structure of circulating strains would likely result in improvements in vaccine effectiveness.

In Chapter 4 we explore the prevalence and abundance of human antibodies recognizing epitopes involving residues in the highly conserved HA RBS. We first map the epitopes of monoclonal antibodies specific for the H3 HA that were elicited by seasonal vaccination. We show that while antigenic substitutions near the RBS abrogate the binding of many monoclonal antibodies, a subset of antibodies are able to tolerate antigenic changes due to their contacts with conserved residues in the HA RBS. We show that in some of the individuals who elicited these RBS antibodies, these antibodies arose from distinct lineages, are detectable in bulk sera, and bind to antigenic distinct strains that began circulating several years later. In human vaccination cohorts, we quantified the contribution of these RBS antibodies to neutralizing antibody responses against circulating strains. Remarkably, some individuals mount a neutralizing response that is focused on epitopes involving residues in the highly conserved RBS. This work represents the first quantification of the frequency of RBS-directed antibody responses and highlights how these antibodies have the potential to protect against circulating strains that have major antigenic differences.

Our work identifies shortcomings with our current process of vaccine strain selection and propagation and suggests steps we can take to improve the effectiveness of our current vaccines. In addition, this work helps address the feasibility of next-generation vaccines that might target more highly conserved epitopes such as the RBS.

## CHAPTER 2: POTENTIAL ANTIGENIC EXPLANATION FOR ATYPICAL H1N1 INFECTIONS AMONG MIDDLE-AGED ADULTS DURING THE 2013-2014 INFLUENZA SEASON

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(\* = equal contribution)

Contributions: I generated mutant viruses by reverse genetics, completed antigenic assays with ferret sera (Figure 2-8), and characterized the glycosylation status of HAs (Figures 2-5, 2-6). Co-authors Benjamin Chambers and Susanne Linderman completed antigenic assays with human sera samples. Susanne Linderman completed all murine experiments. We wrote the manuscript together with Scott Hensley.

### Summary

Influenza viruses typically cause the most severe disease in children and elderly individuals. However, H1N1 viruses disproportionately affected middle-aged adults during the 2013-2014 influenza season. Although H1N1 viruses recently acquired several substitutions in the gene of the hemagglutinin (HA) glycoprotein, classical serological tests utilized by surveillance laboratories indicate that these substitutions do not change antigenic properties of the virus. Here, we show that one of these substitutions is located in a region of HA targeted by antibodies elicited in many middle-aged adults. We find that over 42% of individuals born between 1965 and 1979 possess antibodies that recognize this region of HA. Our findings offer a possible antigenic explanation of why middle-aged adults were highly susceptible to H1N1 viruses during the 2013-

2014 influenza season. Our data further suggest that a drifted H1N1 strain should be included in future influenza vaccines to potentially reduce morbidity and mortality in this age group.

## **Introduction**

Seasonal H1N1 (sH1N1) viruses circulated in the human population for much of the last century, and as of 2009, most humans had been exposed to sH1N1 strains. In 2009, an antigenically distinct H1N1 strain began infecting humans and caused a pandemic (Dawood et al., 2009; Garten et al., 2009; Smith et al., 2009a). Elderly individuals were less susceptible to 2009 pandemic H1N1 (pH1N1) viruses due to cross-reactive Abs elicited by infections with older sH1N1 strains (Garten et al., 2009; Jacobs et al., 2012; Manicassamy et al., 2010; Skountzou et al., 2010; Xu et al., 2010). pH1N1 viruses have continued to circulate on a seasonal basis since 2009. Influenza viruses typically cause a higher disease burden in children and elderly individuals (Mertz et al., 2013) but pH1N1 viruses caused unusually high levels of disease in middle-aged adults during the 2013-2014 influenza season (Arriola et al., 2014; Ayscue et al., 2014; Davila et al., 2014; Epperson et al., 2014). For example, a significantly higher proportion of individuals aged 30-59 years old were hospitalized in Mexico with laboratory-confirmed pH1N1 cases in 2013-2014 relative to 2011-2012 (Davila et al., 2014).

Most neutralizing influenza Abs are directed against the hemagglutinin (HA) glycoprotein. International surveillance laboratories rely primarily on ferret anti-influenza sera for detecting HA antigenic changes (Stohr et al., 2012). For these assays, sera are isolated from ferrets recovering from primary influenza infections. Seasonal vaccine strains are typically updated when human influenza viruses acquire HA substitutions that prevent the binding of primary ferret anti-influenza sera. Our laboratory and others have demonstrated that sera isolated from ferrets recovering from primary pH1N1 infections are dominated by Abs that recognize an epitope involving residues 156, 157, and 158 of the Sa HA antigenic site (Chen et al., 2010a; Li et al., 2013b). The pH1N1 component of the seasonal influenza vaccine has not been updated since 2009, because very few pH1N1 isolates possess substitutions in residues 156, 157, and 158. The majority of

isolates from the 2013-2014 season have been labeled as antigenically similar to the A/California/7/2009 vaccine strain (Arriola et al., 2014).

It is potentially problematic that major antigenic changes of influenza viruses are mainly determined using anti-sera isolated from ferrets recovering from primary influenza infections. Unlike experimental ferrets, humans are typically re-infected with antigenically distinct influenza strains throughout their life (Miller et al., 2013). In the 1950s, it was noted that the human immune system preferentially mounts Ab responses that cross-react to previously circulating influenza strains, as opposed to new Ab responses that exclusively target newer viral strains (Davenport et al., 1953). This process, which Thomas Francis Jr. termed 'original antigenic sin', has been experimentally recapitulated in ferrets (Li et al., 2013b; Webster, 1966), mice (Kim et al., 2009; Virelizier et al., 1974a; Virelizier et al., 1974b), and rabbits (Fazekas de St and Webster, 1966b). Our group and others recently demonstrated that the specificity of pH1N1 Ab responses can be shaped by prior sH1N1 exposures (Carter et al., 2013; Li et al., 2012; Li et al., 2013b; Pica et al., 2012; Wrammert et al., 2011). We found that ferrets sequentially infected with sH1N1 and pH1N1 viruses mount Ab responses dominated against epitopes that are conserved between the viral strains (Li et al., 2013b). These studies indicate that primary ferret anti-sera may not be fully representative of human influenza immunity.

It has been proposed that increased morbidity and mortality of middle-aged adults during the 2013-2014 influenza season is primarily due to low vaccination rates within these populations (Catania et al., 2014). An alternative explanation is that recent pH1N1 strains have acquired a true antigenic substitution that has been mislabeled as 'antigenically neutral' by assays that rely on primary ferret anti-sera. Here we complete a series of experiments to determine if recent pH1N1 strains possess a substitution that prevents binding of Abs in middle-aged humans who have been previously exposed to different H1N1 strains.

## **Results**

## **Recent pH1N1 strains possess a substitution that prevents binding of human antibodies**

Anti-sera isolated from ferrets recovering from primary pH1N1 infections are highly specific for an epitope involving amino acids 156, 157, and 158 of the Sa HA antigenic site (Chen et al., 2010a; Li et al., 2013b). Very few pH1N1 isolates possess substitutions in these Sa residues (Figure 2-1), however, pH1N1 viruses recently acquired a K166Q HA substitution, which is located at the interface of the Sa/Ca (Caton et al., 1982) antigenic sites (Figure 2-2A). The K166Q HA substitution first arose during the 2012-2013 season and is now present in over 99% of pH1N1 isolates (Figure 2-2B, C). Based on experiments using primary anti-sera isolated from infected ferrets, surveillance laboratories have reported that pH1N1 viruses with the K166Q HA substitution are antigenically indistinguishable from the A/California/7/2009 pH1N1 vaccine strain (Arriola et al., 2014).

To address if human Abs are capable of recognizing pH1N1 viruses with the K166Q HA substitution, we performed hemagglutination-inhibition (HAI) assays using sera from healthy humans collected during the 2013-2014 influenza season in the United States. Remarkably, 27% of sera from individuals born between 1940-1984 possessed Abs specific for an epitope involving K166 (Figure 2-3A, Table 2-1). Over 42% of individuals born from 1965-1979 had K166 HA-specific Abs in their sera (n = 54 individuals). Sera isolated from individuals born between 1985-1997 (n = 49 individuals) did not have detectable levels of K166 HA-specific Abs. Differences in K166 HA-specificity were statistically significant between sera isolated from individuals born between 1965-1979 and individuals born after 1985 (Fisher's exact test;  $p < 0.0001$ ). Similar results were obtained when we analyzed sera from healthy humans collected during the 2013-2014 influenza season in Mexico (Figure 2-4 and Table 2-2).

It is remarkable that HAI assays, which are relatively insensitive, were able to reproducibly detect K166-specific Abs in so many individuals in our experiments. Figure 2-3A and Figure 2-4 show percentages of donors that had at least a 2-fold reduction in HAI titer using the K166Q HA mutant virus in 3 independent assays. It is worth pointing out that many sera samples had over 4-

fold reduced HAI titers using a pH1N1 virus engineered to possess the single K166Q HA substitution compared to the pH1N1 vaccine strain (Table 2-1). Age-related differences in K166-specificity among United States donors remained statistically significant using a 4-fold reduction in HAI titer as a cutoff (Fisher's exact test;  $p < 0.05$  comparing donors born between 1965-1979 and individuals born after 1985). Sera that had K166 HA-specificity based on HAI assays failed to efficiently neutralize K166Q-possessing viruses in *in vitro* neutralization assays (Table 2-3). K166 HA-specific sera were also unable to recognize a primary viral isolate collected in 2013 (A/CHOP/1/2013) that possesses a K166Q HA substitution (Table 2-3).

### **The K166 epitope is shielded by glycosylation sites present in sH1N1 viruses circulating after 1986**

'Original antigenic sin' Abs are originally primed by influenza strains that circulated in the past (Davenport et al., 1953; Fazekas de St and Webster, 1966a, b; Li et al., 2013b; Webster, 1966). We propose that K166-specific Ab responses were likely primed by sH1N1 viruses circulating in humans prior to 1985 and then boosted by the 2009 pH1N1 virus. Sera isolated from individuals born between 1965 and 1979 had the highest K166 HA-specificity (both in % and titer; Figure 2-3A and Table 2-1). sH1N1 viruses that circulated in the late 1970s and early 1980s share extensive homology with pH1N1 viruses in the vicinity of K166 (Figure 2-3B). sH1N1 viruses were absent from the human population from 1957-1976 and began infecting humans again in 1977. Therefore, humans born between 1957-1976 likely had their first H1N1 encounter with a sH1N1 virus that shared homology with pH1N1 viruses in the vicinity of K166.

In 1986, sH1N1 viruses acquired a new glycosylation site at HA amino acid 129 that is predicted to shield the epitope involving K166 (Figure 2-5, 2-6A). The absence of K166 HA-specific responses in individuals born after 1985 is likely because sH1N1 viruses glycosylated at HA amino acid 129 fail to prime K166-specific responses. The lower number of K166 HA-specific responders born in the 1950s might also be attributed to unique glycosylation sites in sH1N1 viruses that circulated during this time period (Figure 2-5), although precise glycosylation statuses of viruses circulating prior to 1977 are uncertain due to limited numbers of sequenced viruses.



Although we did not examine sera from very elderly individuals, it is possible that they also have immunodominant K166 HA responses, since a recent study reported that a mAb isolated from a survivor of the 1918 H1N1 pandemic binds to pH1N1 in an epitope involving K166 (Xu et al., 2010). There is considerable homology between the 1918 H1N1 and the 2009 H1N1 in the vicinity of K166 (Xu et al., 2010).

To experimentally address if glycosylation sites present in previous sH1N1 strains shield the epitope involving K166, we used reverse-genetics to produce pH1N1 viruses that had glycosylation sites that were either present in sH1N1 strains from 1977-1985 (sites 131+163) or 1986-2008 (sites 129+163). Western blot analysis revealed that residues 129 and 131, but not residue 163 were glycosylated in our reverse-genetics derived viruses (Figure 2-6B). Consistent with the hypothesis that the K166 epitope is shielded by glycosylation sites present in 1986-2008 sH1N1 viruses, K166-specific human sera had reduced titers to pH1N1 viruses with the 129 glycosylation site but normal titers to pH1N1 viruses with the 131 glycosylation site (Figure 2-6C). As a control, we also completed HAI assays with sera from donors that were born in the 1970s who did not have detectable levels of K166 sera Abs. As expected, these sera did not have reduced titers to pH1N1 viruses with the 129 glycosylation site, but interestingly, these sera did have reduced titers to pH1N1 viruses with the 131 glycosylation site (Figure 2-6C). We previously demonstrated that Ab responses focused on an epitope near the 131 glycosylation site can be elicited by sequential infections with a sH1N1 virus from the early 1990s and the pH1N1 virus (Li et al., 2013b). We speculate that donors in the 'non-K166 HA-specific' group were previously infected with antigenically distinct sH1N1 strains compared to donors in the 'K166 HA-specific group' (ie: A/Singapore/6/1986-like strain vs A/Chile/1/1983-like strain). Taken together, these data suggest that glycosylation sites on previously circulating sH1N1 viruses shield epitopes and influence the development of subsequent Ab responses against pH1N1 virus.

### **Vaccination with current pH1N1 vaccine strain elicits K166-specific Abs**

The pH1N1 vaccine strain has not been updated since 2009. We determined whether this vaccine strain, which possesses HA K166, elicits K166 HA-specific Abs in humans. First, we analyzed sera from individuals vaccinated in 2009. All of the individuals in this cohort were born prior to 1984 and most did not have pH1N1 Ab titers prior to vaccination (Table 2-4). Sera from 5 of 17 individuals possessed detectable levels of K166 HA-specific Abs following vaccination (Figure 2-7A, Table 2-4). Sera from all five of these individuals had < 1:40 HAI titers against the K166Q HA mutant pH1N1 virus (Table 2-4). One K166 HA-specific individual (subject #1) possessed K166 HA-specific Abs prior to vaccination (Figure 2-7A, Table 2-4). It is possible that this individual was naturally infected with pH1N1 prior to vaccination. All of the K166 HA-specific donors had detectable pre-vaccination Ab titers against sH1N1 viruses from 1977 and 1983; however, we also found titers against these strains in some donors that did not have detectable levels of K166 HA serum Abs (Table 2-4). We also measured binding of 42 HA head-specific mAbs isolated from 12 adult donors (born 1949-1985) that were vaccinated against the pH1N1 strain in 2009. Strikingly, 23% of these mAbs had reduced binding to pH1N1 engineered to have the K166Q substitution (Figure 2-7B). This is consistent with a previous report that identified several K166-specific mAbs derived from a donor that was born prior to 1977 (Krause et al., 2011b).

We passively transferred a K166 HA-specific mAb (SFV009-3F05) or a control mAb that binds equally to WT and K166Q-HA viruses (SFV015-1F02) to Balb/c mice 12 hours before infecting with a lethal dose of WT or K166Q-HA pH1N1 viruses. Control animals that did not receive a mAb prior to infection rapidly lost weight and died (Figure 2-7C). Mice receiving the control SFV015-1F02 mAb prior to infection with WT or K166Q-HA pH1N1 viruses all survived with minimal weight loss (Figure 2-7C). Mice receiving the K166 HA-specific SFV009-3F05 mAb survived following infection with WT pH1N1 but rapidly lost weight and died following infection with K166Q HA pH1N1 (Figure 2-7C). These data suggest that K166-specific Abs can be less

efficient at preventing disease in a mouse model following infection with a pH1N1 virus possessing K166Q HA.

### **Can K166 HA-specific immunity be recapitulated in ferrets for surveillance purposes?**

Current surveillance efforts rely heavily on anti-sera isolated from ferrets recovering from primary influenza virus infections. Ferret anti-sera could potentially be more reflective of human immunity if isolated from animals sequentially infected with antigenically distinct viral strains. We attempted to elicit K166 HA-specific Abs in ferrets by sequentially infecting animals with older sH1N1 strains and then the A/California/07/2009 pH1N1 strain.

We initially infected animals with a sH1N1 virus that circulated in 1977 (A/USSR/90/1977), a sH1N1 virus that circulated in 1983 (A/Chile/1/1983), or a sH1N1 virus that circulated in 1986 (A/Singapore/6/1986). After 84 days, we re-infected animals with the A/California/7/2009 pH1N1 strain. As controls, we infected some animals twice with A/California/7/2009 and other animals only once with A/California/7/2009. Three of 8 of the ferrets sequentially infected with A/Chile/1/1983 and A/California/7/2009 mounted K166 HA-specific Abs detectable in HAI assays (Figure 2-8, Table 2-5). The 22 ferrets in the other experimental groups did not mount detectable levels of K166 HA-specific Abs. The difference in K166 HA-specificity is statistically significant comparing the A/Chile/1/1983-A/California/7/2009 group with the rest of the groups (3 of 8 vs 0 of 22; Fisher's exact test  $p < 0.05$ ). K166 HA-specific Abs were likely not elicited in the A/Singapore/6/1986-A/California/7/2009 group because the K166 HA epitope is predicted to be shielded by a glycosylation site at residue 129 of A/Singapore/6/1986 (Figure 2-6). It is interesting that K166 HA-specific Abs were not elicited by A/USSR/90/1977-A/California/7/2009 sequential infections. This is likely due to variation at residue 125, which is close to residue 166 (Figure 2-9). A/Chile/1/1983 and A/California/7/2009 both possess S125, whereas A/USSR/1/1977 possesses R125 (Figure 2-9).

## Discussion

Our studies show that recent pH1N1 viruses have acquired a significant antigenic substitution that prevents binding of Abs elicited in a large number of middle-aged humans. For this reason, we propose that the pH1N1 vaccine strain should be updated. Conventional serological techniques utilized by most surveillance laboratories have failed to recognize the K166Q HA substitution as antigenically important (Arriola et al., 2014). HAI assays are based on serial sera dilutions and can only detect large antigenic changes. Many surveillance-based laboratories ignore 2-fold reductions in HAI titer since these laboratories typically process thousands of samples, which prohibit the experimental precision that is required to reliably detect 2-fold differences in these assays. However, a true 2-fold reduction in HAI titer against a mutated strain indicates an extremely immunodominant Ab response. While we reproducibly detected as low as 2-fold HAI differences using K166Q HA mutated viruses (Tables 1 and 2 show results from 3 independent HAI experiments), our HAI assays likely underestimate the number of individuals that possess K166Q Abs. For example, we were able to isolate K166-specific mAbs from pH1N1-vaccinated individuals whose sera yielded similar HAI titers using wild-type and K166Q mutated pH1N1 viruses. We also identified many human sera samples that had > 4-fold reductions in HAI titer using pH1N1 viruses with the K166Q HA substitution, and it is worth noting that these results would likely have been missed if we pooled human sera samples or simply compared overall geometric means of HAI data with mutant viruses.

We attempted to recapitulate K166 HA-specific immunity in ferrets by sequentially infecting with sH1N1 strains and the A/California/7/2009 pH1N1 strain. Only 3 of 8 ferrets sequentially infected with A/Chile/1/1983 and A/California/7/2009 mounted levels of K166 HA-specific Abs that could be detected by HAI assays. Outbred ferrets were used in these experiments, and the overall % of ferrets with K166 HA-specificity (Figure 2-8) is similar to the overall % of humans born in the 1970s with K166 HA-specificity (Figure 2-3). We speculate that variation in K166 HA-specificity in humans is due to variations in pre-exposure histories and genetic differences that

impact B cell repertoires. Studies are ongoing to determine if genetic differences in B cell repertoires among ferrets influence K166 HA-specificity.

Our results offer a possible antigenic explanation for the increased disease burden in middle-aged adults during the 2013-2014 influenza season. Given that the specificity of Ab responses is altered by pre-exposures, we propose that conventional serological techniques used to identify antigenically novel viruses should be re-evaluated. The usefulness of arbitrary HAI titer cutoffs and dependence on anti-sera generated in previously naïve ferrets (Koel et al., 2013; Smith et al., 2004) should be reconsidered. Although we believe that the pH1N1 vaccine should be updated immediately, it is not clear if a pH1N1 vaccine strain with Q166 HA will be able to break the 'original antigenic sin' that currently exists in some middle-aged individuals. Further studies should be designed to determine if an updated H1N1 vaccine strain with Q166 HA elicits more effective Ab responses in different aged humans with distinct sH1N1 exposure histories.

## **Materials and Methods**

Human donors: Studies involving human adults were approved by the Institutional Review Boards of Emory University, Vaccine and Gene Therapy Institute of Florida, the National Institute of Respiratory Diseases of Mexico, and the Wistar Institute. Informed consent was obtained. For all experiments, HAI and *in-vitro* neutralization assays were completed at the Wistar Institute using pre-existing and de-identified sera. We analyzed several sera panels in this study. We analyzed sera from healthy donors collected at the New York Blood Center in February of 2014. We analyzed sera from healthy donors collected at the Center for Research in Infectious Diseases at the National Institute of Respiratory Diseases in Mexico. We analyzed sera and mAbs derived from healthy donors vaccinated with a monovalent pH1N1 vaccine in 2009 as previously described (Li et al., 2012).

Viruses: Viruses possessing WT pH1N1 HA or K166Q pH1N1 HA were generated via reverse-genetics using HA and NA genes from A/California/07/2009 and internal genes from A/Puerto Rico/8/1934. All of these viruses were engineered to possess the antigenically neutral D225G HA

substitution (Chen et al., 2010a) which facilitates viral growth in fertilized chicken eggs. Viruses were grown in fertilized chicken eggs and the HA genes of each virus stock were sequenced to verify that additional substitutions did not arise during propagation. sH1N1 strains (A/USSR/90/1977, A/Chile/1/1983, A/Singapore/6/1986, A/Texas/36/1991, New Caledonia/20/1999, and A/Solomon Islands/3/2006) were also grown in fertilized chicken eggs. We isolated a pH1N1 virus from respiratory secretions obtained from a patient from the Children's Hospital of Philadelphia in 2013 (named A/CHOP/1/13 in this manuscript). For this, de-identified clinical material from the Children's Hospital of Philadelphia Clinical Virology Laboratory was added to MDCK cells (originally obtained from the National Institute of Health) in serum-free media with TPCK-treated trypsin, HEPES, and gentamicin. Virus was isolated from the MDCK-infected cells 3 days later. We extracted viral RNA and sequenced the HA gene of A/CHOP/1/13. We also used reverse-genetics to introduce glycosylation sites into A/California/7/2009 (pH1N1) HA. The consensus sequence for N-linked glycosylation (N-x-S/T) was added at HA residues 129, 131, and 163 by making the substitutions D131T, D131N and N133T, and K163N, respectively. Similar results were obtained in HAI assays when we used glycosylation mutants grown in eggs or MDCK cells. Glycosylation at residues 129 and 131 was confirmed by treating concentrated virus with PNGase-F (New England Biolabs) under denaturing conditions. The CM1-4 anti-HA1 antibody was used as a primary antibody, and a donkey anti-mouse fluorescent secondary antibody (Licor) was used. Blots were imaged using the Licor Odyssey imaging system at 800nm (secondary antibody) and 700nm (molecular weight marker).

*Animal experiments:* Murine experiments were performed at the Wistar Institute according to protocols approved by the Wistar Institute Institutional Animal Care and Use Committee. Balb/c mice (Charles River Laboratories) were injected with 20ug of mAb i.p. and then infected i.n. with 10,000 TCID<sub>50</sub> of WT or K166Q-HA pH1N1 virus 12 hours later. As controls, some mice received an i.p. injection of PBS prior to infection. Weight loss and survival was recorded for 11 days. Ferret experiments were performed at the Vaccine and Gene Therapy Institute of Florida in accordance with the NRC Guide for the Care and Use of Laboratory Animals, the Animal Welfare

Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories handbook. Fitch ferrets (Marshall Farms, Sayre, PA) were infected with  $1 \times 10^6$  PFU of sH1N1 virus and bled 14 and 84 days later. These ferrets were then infected with the A/California/7/2009 pH1N1 strain and bled 14 days later. Some ferrets were sequentially infected with A/California/7/2009 (84 days between infections) and other ferrets were infected with only A/California/7/2009 and bled 14 days later.

HAI assays: Sera samples were pre-treated with receptor-destroying enzyme (Key Scientific Products Inc or Sigma-Aldrich) and HAI titrations were performed in 96 well round bottom plates (BD). Sera were serially diluted twofold and added to 4 agglutinating doses of virus in a total volume of 100  $\mu$ l. Turkey erythrocytes (Lampire) were added (12.5  $\mu$ l of a 2% vol/vol solution). The erythrocytes were gently mixed with sera and virus and agglutination was read out after incubating for 60 min at room temperature. HAI titers were expressed as the inverse of the highest dilution that inhibited 4 agglutinating doses of turkey erythrocytes. Each HAI assay was performed independently on three different dates. Sera that had at least 2-fold reduced HAI titers using K166Q HA mutant viruses in 3 independent HAI assays were labeled as 'K166 HA-specific'.

ELISA assays: Viruses for ELISAs were concentrated by centrifugation at 20,000 RPM for 1 hour. Concentrated viruses were then inactivated by B-Propiolactone (BPL; Sigma Aldrich) treatment. Viruses were incubated with 0.1% BPL and 0.1M HEPES (Cellgro) overnight at 4C followed by a 90 min incubation at 37C. 96 well Immulon 4HBX flat bottom microtiter plates (Fisher Scientific) were coated with 20 HAU/well BPL-treated virus overnight at 4C. Each human mAb was serially diluted in PBS and added to the ELISA plates and allowed to incubate for 2 hr at room temperature. As a control, we added the 70-1C04 stalk-specific mAb to verify equal coating of WT and K166Q HA virus. Next, peroxidase conjugated goat anti-human IgG (Jackson Immunoresearch) was incubated for 1hr at room temperature. Finally, Sureblue TMB Peroxidase Substrate (KPL) was added to each well and the reaction was stopped with addition of 250mM HCl solution. Plates were extensively washed with water between each ELISA step. Affinities

were determined by nonlinear regression analysis of curves of 6 mAb dilutions (18ug/ml to 74 ng/ml) using Graphpad Prism. mAbs were designated as K166-specific if they had a Kd at least 4 times greater for the K166Q mutant than for the WT virus.

*In vitro neutralization assays:* Sera were serially diluted and then added to 100 TCID<sub>50</sub> units of virus and incubated at room temperature for 30 mins. The virus-sera mixtures were then incubated with MDCK cells for 1 hr at 37C. Cells were washed and then serum-free media with TPCK-treated trypsin was added. Endpoints were determined visually 3 days later. Data are expressed as the inverse of the highest dilution that caused neutralization. All samples were repeated in quadruplicate and geometric mean titer is reported.

*Structural modeling of HA glycosylation sites:* Glycans were modeled onto positions 129 and 131 in the A/Solomon Islands/03/2006 HA crystal structure (PDB ID code 3SM5) using the GLYCAM-Web Glycoprotein Builder ([www.glycam.org](http://www.glycam.org)). The particular glycan used for modeling was an N-linked glycan with a trimannosyl core (DManpa1-6[DGlcNAcb1-2DManpa1-3]DManpb1-4DGlcNAcb1-4DGlcNAcb1-OH in Glycam notation), and default rotamer settings were used for modeling. To model the 131 glycosylation site, a T131N substitution was introduced using the PyMol structure viewer before the structure was uploaded to the GLYCAM-Web server.

*Computational and phylogenetic analyses of HA sequences:* The occurrence of different amino acid identities at HA residues 166, 156, 157, and 158 (H3 numbering) was analyzed by downloading all full-length human pandemic H1N1 sequences present in the Influenza Virus Resource (1) as of February 23, 2014. After purging sequences that were less than full length, contained ambiguous nucleotide identities, lacked full (year, month, day) isolation dates, or were otherwise anomalous, the sequences were aligned. Each calendar year was broken into four equal partitions beginning with January 1, and the frequencies of different amino acids at each residue of interest for each partition was calculated and plotted. Only amino acids that reached a frequency of at least 1% in at least one of the year partitions are labeled in the

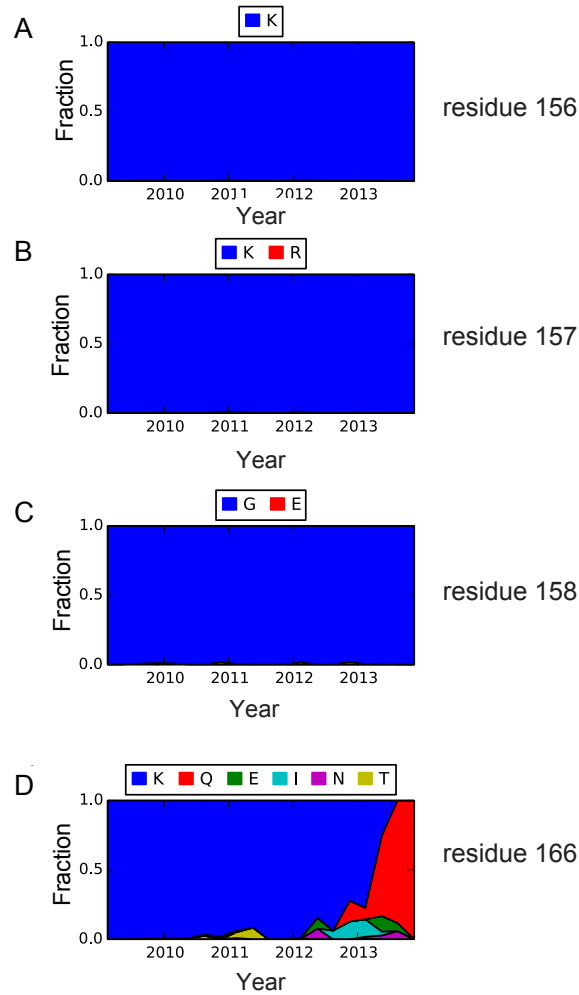


legend to the plot. For construction of phylogenetic trees, the sequence set was randomly subsampled to 10 sequences per quarter-year partition. BEAST (2) was then used to sample from the posterior distribution of phylogenetic trees with re-constructed sequences at the nodes, after date stamping the sequences, using a JTT (3) with a single rate category with an exponential prior, a strict molecular clock, and relatively uninformative coalescent-based prior over the tree. Figure 2-1C shows a maximum clade credibility summary of the posterior distribution with branches colored according to the reconstructed amino acid identity at site 166 with the highest posterior probability at their descendent nodes. The tree was visually rendered using FigTree. The input data and computer code used for this analysis can be found on GitHub at [github.com/jbloom/pdmH1N1\\_HA\\_K166\\_mutations](https://github.com/jbloom/pdmH1N1_HA_K166_mutations)

*Statistical analyses:* For all sera experiments, we excluded samples that did not have positive pH1N1 HAI titers. All samples that were pH1N1 HA-WT HAI negative were also pH1N1 HA-K166Q HAI negative. Samples were allocated to specific groups based on age of donor. The year of birth of each sample was available during the experiment, but this information was not assessed until each experiment was completed. Variance of raw HAI titers was similar between different age groups. Fisher's exact tests were completed using SAS version 9.3 software.

## Figures and Tables

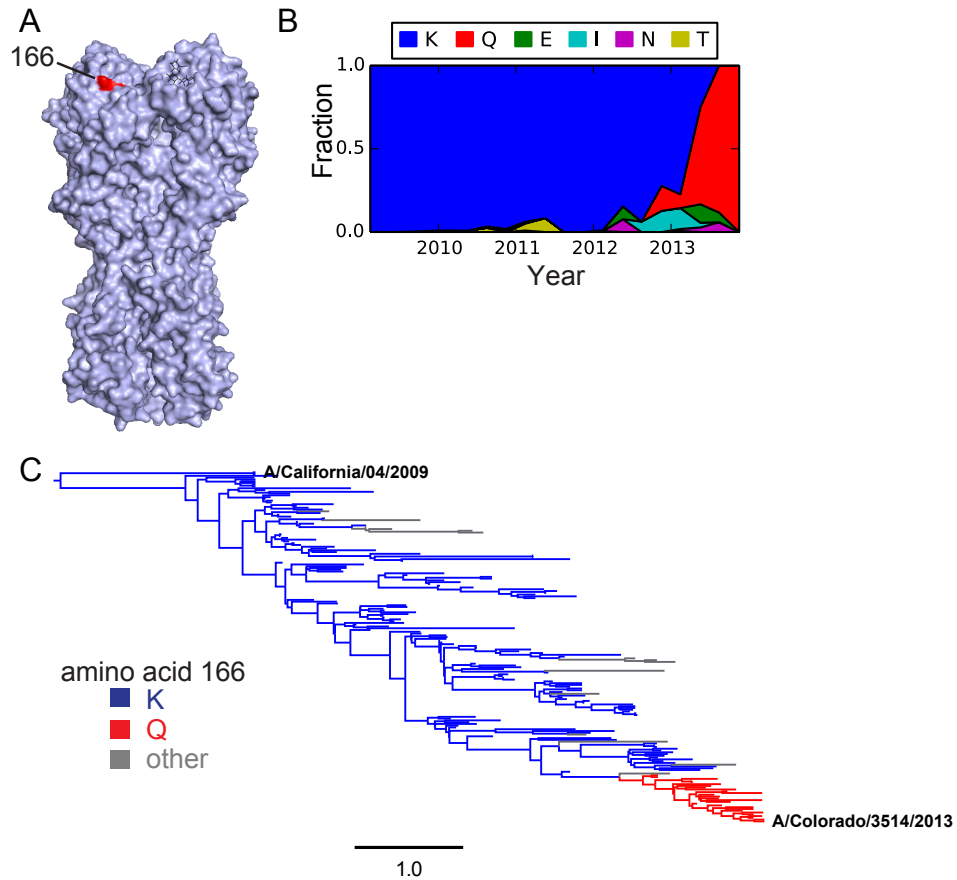
Figure 2-1



**Figure 2-1: Sequence variation of pH1N1 HA**

The residues in the dominant antigenic site recognized by primary ferret anti-sera (residues 156, 157, and 158 of the Sa antigenic site) are highly conserved in pH1N1 (**A-C**). No variation greater than 1% occurred at residue 156 and very little variation occurred at residues 157 and 158. For comparison, residue 166 (**D**; also shown in Fig 2-2B of manuscript) has undergone a complete change in the last year.

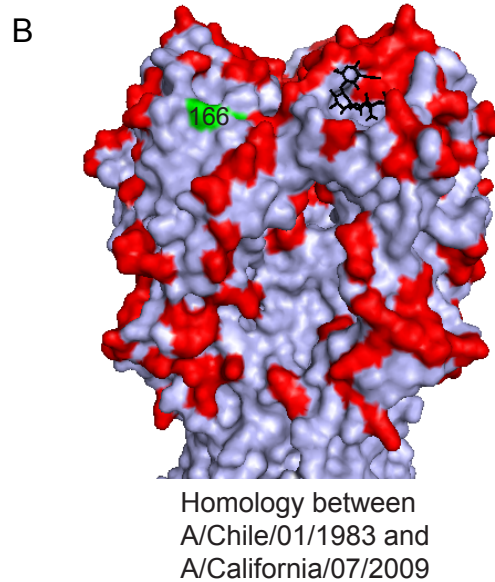
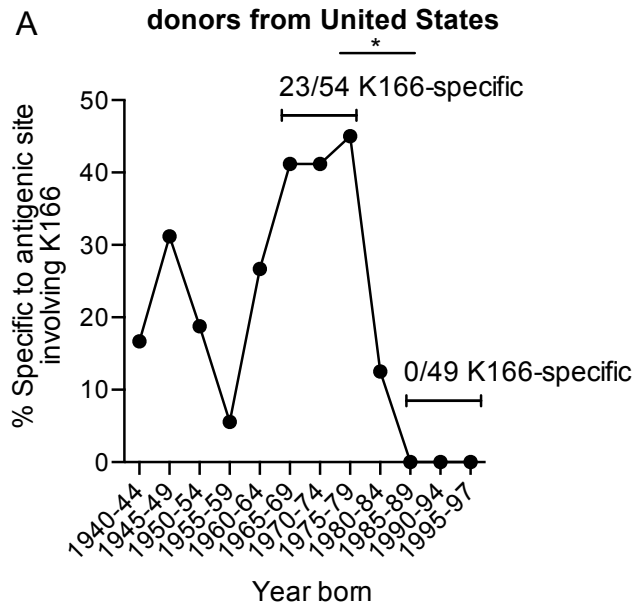
**Figure 2-2**



**Figure 2-2: pH1N1 viruses rapidly acquired HA substitution K166Q during the 2013-2014 influenza season**

(A) Residue K166 (red) is shown on the A/California/04/2009 HA trimer (PDB: 3UBN(Xu et al., 2010)). (B) Plotted is the frequency of different amino-acid identities at HA residue 166 in pH1N1 HA sequences as a function of time. Nearly all pH1N1 possessed K166 from 2009 to mid 2012, but most isolates possessed Q166 by the 2013-2014 season. (C) A phylogenetic tree of pH1N1 viruses with branches colored according to amino acid identity at site 166 illustrates the rapid fixation of K166Q in recent pH1N1 isolates.

Figure 2-3

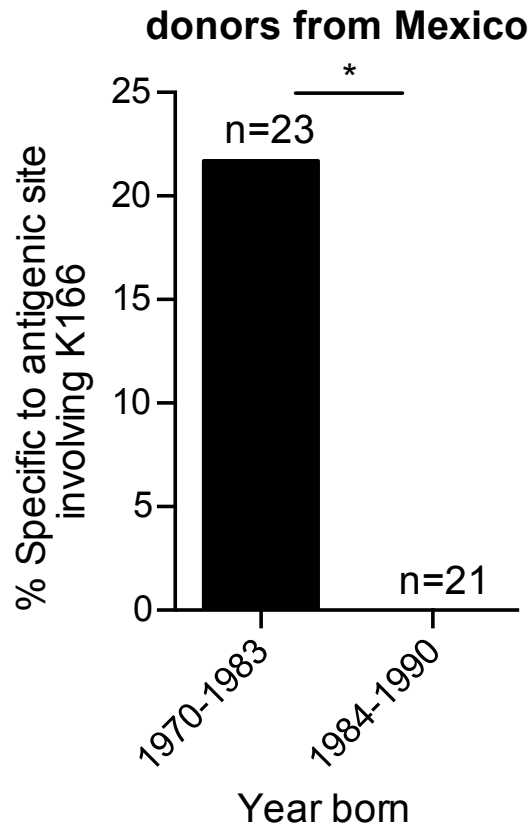


**Figure 2-3: Adult humans possess Abs that bind to a region of HA that was recently mutated in pH1N1**

(A) Sera were isolated from healthy donors (n=195) from the state of New York during the 2013-2014 influenza season. HAI assays were performed using viruses with either wild-type A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA substitution. For each sera

sample, we completed 3 independent HAI assays. Raw HAI data are reported in Table 2-1. Percentages of samples that had at least a 2-fold reduction in HAI titer using the mutant virus in 3 independent experiments are shown. K166-specificity of sera from individuals born between 1965-1979 is statistically significant compared to K166-specificity of sera from individuals born after 1985 (Fisher's exact test;  $p < 0.0001$ ). **(B)** Homology between the A/Chile/01/1983 sH1 and the A/California/04/2009 pH1 are shown using the crystal structure of the A/California/04/2009 HA (PDB: 3UBN (Xu et al., 2010)). Residue K166 is colored green. Amino acids that differ between A/Chile/01/1983 and A/California/04/2009 are shown in red. The glycan receptor is shown in black.

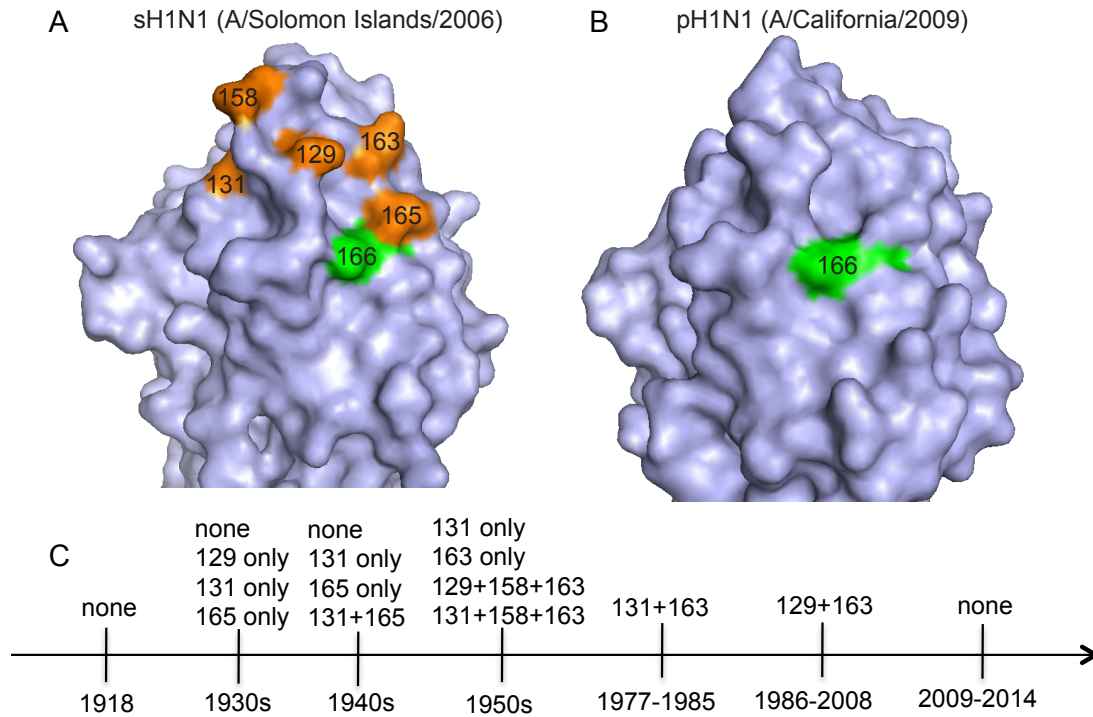
Figure 2-4



**Figure 2-4: Mexican donors born prior to 1984 possess Abs that bind to region of HA that was recently mutated in pH1N1**

Sera were isolated from healthy donors (n=45) at the Center for Research in Infectious Diseases at the National Institute of Respiratory Diseases in Mexico during the 2013-2014 influenza season. HAI assays were performed using viruses with either wild-type A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA substitution. For each sera sample, we completed 3 independent HAI assays. Raw HAI data are reported in Table 2-2. Percentages of samples that had at least a 2-fold reduction in HAI titer using the mutant virus in 3 independent experiments are shown. K166-specificity of sera from individuals born between 1970-1983 is statistically significant compared to K166-specificity of sera from individuals born after 1984 (Fisher's exact test;  $p < 0.0001$ ).

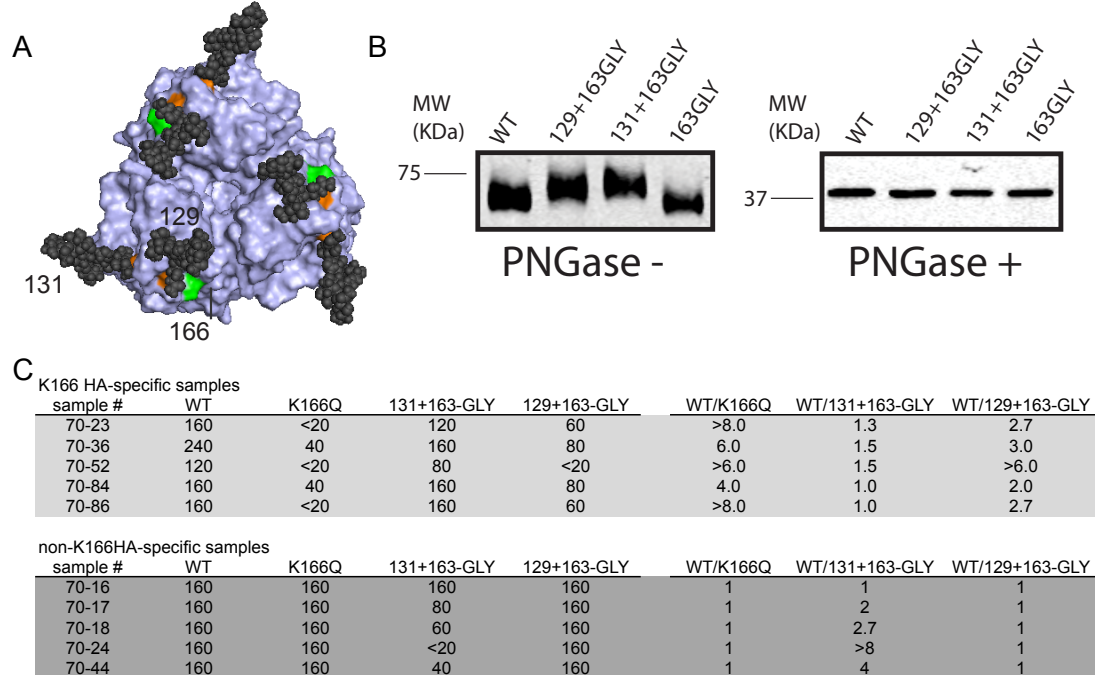
**Figure 2-5**



**Figure 2-5: Glycosylation status of historical H1N1 viruses**

The crystal structures of sH1N1 (**A**) and pH1N1 (**B**) HAs are shown (PDB #: 3SM5 and 3UBN). Glycosylation sites that have appeared from 1918-2008 in sH1N1 viruses are highlighted in orange and residue 166 is shown in green. (**C**) Glycosylation status of H1N1 viruses circulating during different time periods is shown as reported in Wei et al. *Science Translational Medicine* 2, 24ra21 (2010). Very few viral sequences are available for 1930-1950 viruses and variability of glycosylation sites in these viruses likely relates to egg adaptations. The majority of sH1N1 viruses circulating between 1977-1985 have the 131 and 163 glycosylation sites and the majority of sH1N1 viruses circulating between 1986-2008 have the 129 and 163 glycosylation sites. Of note, although residues 129 and 131 are very close in the linear sequence, they are located on opposite sides of the Sa/Sb ridge. The 131 glycosylation site is not expected to cover the K166 epitope, whereas the 129 glycosylation site potentially shields the K166 epitope.

**Figure 2-6**

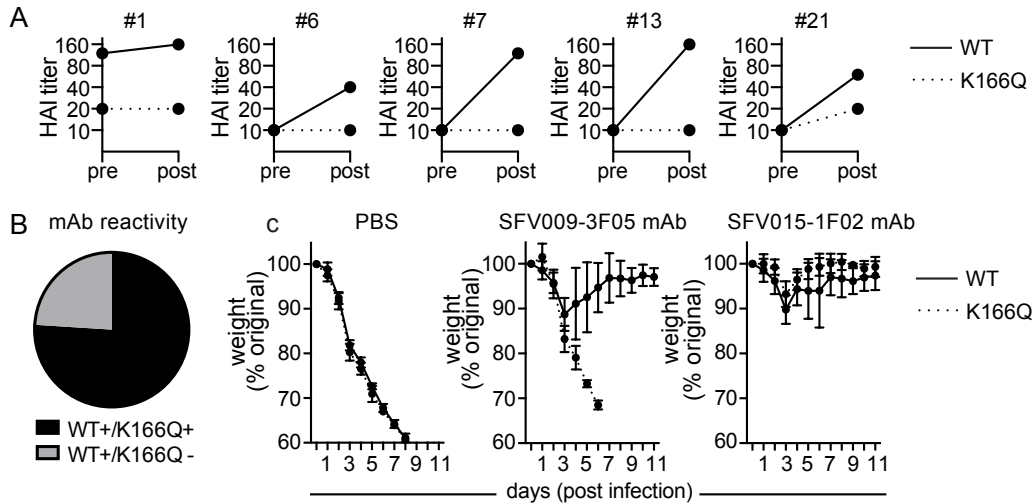


**Figure 2-6: Structural modeling of 129 and 131 glycosylation sites**

**(A)** Modeling of the putative glycosylation sites at residues 129 and 131 on the HA of A/Solomon Islands/6/2006 (PDB # 3SM5) was completed using Glycam software (see methods). The 129 glycosylation site present in most sH1N1 isolates circulating after 1985 is predicted to shield the antigenic site involving residue 166. The 131 glycosylation site is not predicted to shield the antigenic site involving residue 166. **(B)** Viruses possessing A/California/07/2009 HA with different putative glycosylation sites were created by reverse-genetics. HA from viruses with putative glycosylation sites introduced at residues 129+163 and 131+163 migrated slower compared to unmodified HA and HA from viruses with a putative glycosylation site at only residue 163. This indicates that residues 129 and 131, but not residue 163 was glycosylated in the reverse-genetics derived viruses. HA from all viruses migrates similarly following PNGase treatment. PNGase treatment was completed under reducing conditions, so HA migrated faster compared to no PNGase treatment. **(C)** HAI assays were completed using the reverse-genetics derived viruses possessing A/California/07/2009 with different glycosylation sites. Five K166 HA-specific samples and five non-K166 HA-specific human samples were tested.



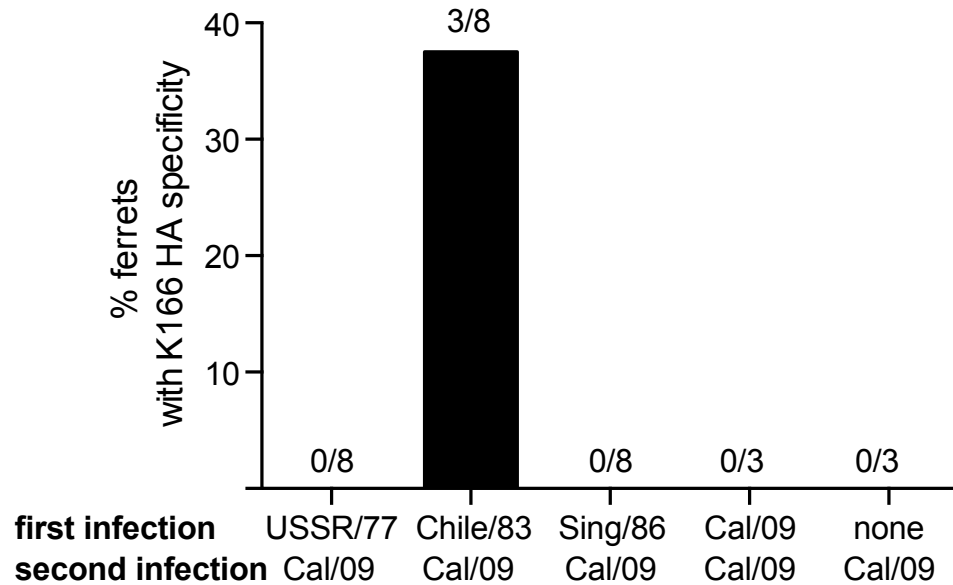
**Figure 2-7**



**Figure 2-7: Vaccination of middle-aged adults with the current pH1N1 vaccine strain elicits Abs that bind to a region of HA that is now mutated in most pH1N1 isolates**

**(A)** Healthy adult volunteers were vaccinated with a monovalent pH1N1 vaccine in 2009. Sera were isolated pre-vaccination and 30 days post-vaccination and HAI assays were performed using viruses with either wild-type A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA substitution. Shown are HAI titers for donors that possessed K166 HA-specific Abs following vaccination. Data are representative of 3 independent experiments. Raw HAI titers for all donors are shown in Table 4. **(B)** ELISA assays were completed using mAbs isolated from healthy adult volunteers that were vaccinated with a monovalent pH1N1 vaccine in 2009. ELISAs were coated either with A/California/07/2009 (WT) or A/California/07/2009 with a K166Q HA substitution. Shown are percentage of mAbs that bound to both viruses and percentage of mAbs that bound to the WT virus but not the mutant virus (n= 42 mAbs). Data are representative of 2 independent experiments. **(C)** A K166 HA-specific mAb (SFV009-3F05) or a mAb that recognizes both WT and K166Q-HA pH1N1 (SFV015-1F02) were injected into Balb/c mice (n=4 per group). 12 hours later, mice were then infected with 10,000 TCID<sub>50</sub> of WT or K166Q-HA virus and weight loss and survival were recorded for 11 days. Data are representative of 2 independent experiments.

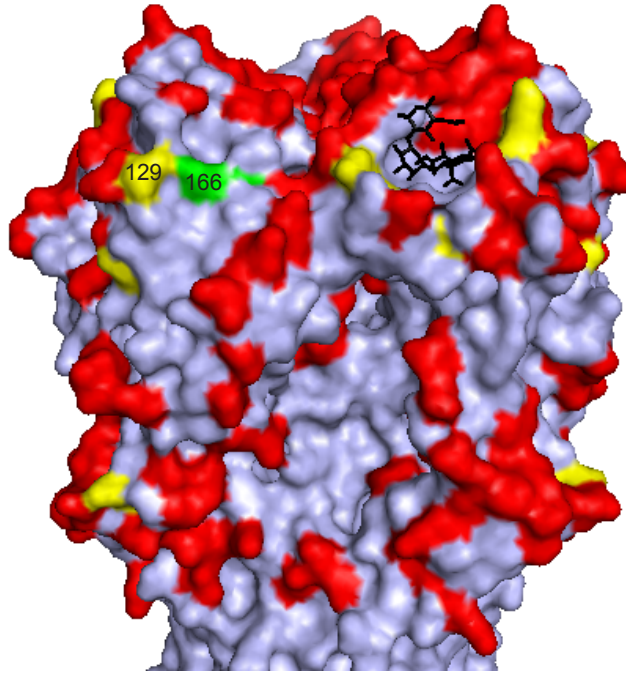
**Figure 2-8**



**Figure 2-8: Ferrets sequentially infected with A/Chile/1/1983 and A/California/7/2009 develop K166 HA-specific Abs**

Ferrets were infected with a sH1N1 virus and then re-infected 84 days later with the A/California/7/2009 pH1N1 virus. Sera were collected 14 days after the 2<sup>nd</sup> infection and HAI assays were completed using WT and K166Q-HA pH1N1 viruses. Shown are % of samples that had at least a 2-fold reduction in HAI titer using the K166Q HA mutant virus in 3 independent experiments. Raw HAI titers are shown in Table 2-5. The difference in K166 HA-specificity is statistically significant comparing the A/Chile/1/1983-A/California/7/2009 group with the rest of the groups (3 of 8 vs 0 of 22; Fisher's exact test  $p < 0.05$ ).

**Figure 2-9**



**Figure 2-9: Homology between A/USSR/90/1977, A/Chile/1/1983, and A/California/7/2009**

HA residues that differ between A/Chile/01/1983 and the A/California/7/2009 are shown in red. A few additional HA residues differ between the HAs of A/USSR/90/1977 and A/California/7/2009, and these are colored yellow. Of note, A/Chile/01/1983 and A/California/7/2009 both possess S125 whereas A/USSR/90/1977 possess R125. Residues 129 and 166 are next to each other in the structure. PDB file 3UBN (A/California/04/2009 HA) was used to make this figure.

**Table 2-1**

sample ID	YOB	experiment #1			experiment #2			experiment #3		
		HAI titers		ratio	HAI titers		ratio	HAI titers		ratio
		WT	K166Q	WT/K166Q	WT	K166Q	WT/K166Q	WT	K166Q	WT/K166Q
40-60	1940	160	80	2.00	160	80	2.00	160	60	2.67
40-49	1943	80	<20	>4.00	60	<20	>3.00	80	<20	>4.00
40-05	1948	160	80	2.00	160	80	2.00	160	80	2.00
40-02	1948	120	60	2.00	60	30	2.00	80	30	2.67
40-44	1948	320	80	4.00	320	80	4.00	320	80	4.00
40-14	1949	240	80	3.00	240	80	3.00	240	80	3.00
40-16	1949	240	<20	>12.00	160	<20	>8.00	160	<20	>8.00
50-55	1952	160	80	2.00	160	60	2.67	160	80	2.00
50-11	1952	120	40	3.00	80	30	2.67	80	30	2.67
50-43	1953	80	40	2.00	80	40	2.00	80	40	2.00
50-06	1959	120	60	2.00	80	40	2.00	80	30	2.67
60-04	1963	80	40	2.00	80	40	2.00	80	40	2.00
60-10	1963	160	80	2.00	240	80	3.00	160	60	2.67
60-13	1964	240	120	2.00	320	120	2.67	240	120	2.00
60-25	1964	60	20	3.00	80	30	2.67	60	20	3.00
60-14	1967	160	80	2.00	120	60	2.00	120	60	2.00
60-23	1967	320	120	2.67	320	160	2.00	320	160	2.00
60-44	1968	160	80	2.00	160	80	2.00	160	80	2.00
60-28	1969	320	160	2.00	320	160	2.00	320	160	2.00
60-41	1969	120	60	2.00	160	80	2.00	160	80	2.00
60-63	1969	120	60	2.00	120	60	2.00	120	60	2.00
60-35	1969	80	40	2.00	80	30	2.67	80	30	2.67
70-56	1971	80	40	2.00	80	30	2.67	80	40	2.00
70-52	1971	160	<20	>8.00	120	<20	>6.00	80	<20	>4.00
70-60	1972	160	80	2.00	160	80	2.00	160	80	2.00
70-74	1972	160	80	2.00	240	80	3.00	240	80	3.00
70-36	1972	240	60	4.00	240	30	8.00	320	30	10.67
70-84	1973	120	40	3.00	160	30	5.33	160	40	4.00
70-86	1973	160	40	4.00	160	30	5.33	160	40	4.00
70-13	1975	640	320	2.00	640	320	2.00	640	320	2.00
70-54	1975	160	80	2.00	80	30	2.67	120	60	2.00
70-25	1975	160	80	2.00	80	30	2.67	120	40	3.00
70-10	1975	160	30	5.33	80	30	2.67	80	30	2.67
70-23	1975	160	<20	>8.00	120	<20	>6.00	160	<20	>8.00
70-82	1976	160	80	2.00	160	60	2.67	160	80	2.00
70-50	1976	240	80	3.00	160	60	2.67	120	60	2.00
70-80	1978	320	160	2.00	320	160	2.00	320	160	2.00
70-19	1978	80	<20	>4.00	80	<20	>4.00	80	<20	>4.00
80-16	1980	80	30	2.67	60	20	3.00	80	40	2.00
80-48	1983	640	120	5.33	640	80	8.00	640	80	8.00

**Table 2-1: HAI titers using sera from healthy donors from the United States**

Sera were isolated from 195 healthy donors from the state of New York during the 2013-2014 influenza season. HAI assays were performed using viruses with either wild-type A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA substitution. For each sera sample, we completed 3 independent HAI assays. Shown are samples that had at least a 2-fold reduction in HAI titer using the mutant virus in 3 independent experiments.

**Table 2-2**

sample ID	YOB	experiment #1			experiment #2			experiment #3		
		WT	K166Q	ratio	WT	K166Q	ratio	WT	K166Q	ratio
N-33	1973	160	60	2.67	120	40	3.00	80	30	2.67
N-42	1975	40	<10	>4.00	30	<10	>3.00	30	<10	>3.00
N-93	1977	120	30	4.00	60	30	2.00	60	20	3.00
N-72	1979	30	<10	>3.00	30	<10	>3.00	20	<10	>2.00
N-70	1980	160	80	2.00	120	60	2.00	120	40	3.00

**Table 2-2: HAI titers using sera from healthy donors from Mexico**

Sera were isolated from 45 healthy donors from Mexico during the 2013-2014 influenza season.

HAI assays were performed using viruses with either wild-type A/California/07/2009 HA or

A/California/07/2009 HA with a K166Q HA substitution. For each sera sample, we completed 3

independent HAI assays. Shown are samples that had at least a 2-fold reduction in HAI titer using

the mutant virus in 3 independent experiments.

**Table 2-3**

sample #	YOB	HAI titers			<i>in vitro</i> neutralization titers	
		A/CAL/07/09- WT HA	A/CAL/07/09- K166Q HA	A/CHOP/1/2013	A/CAL/07/09- WT HA	A/CAL/07/09- K166Q HA
40-49	1943	20	<10	<10	24	12
40-44	1948	160	60	30	453	95
40-16	1949	80	<10	<10	160	20
70-52	1971	40	<10	<10	190	28
70-36	1972	120	40	10	381	95
70-84	1973	40	20	<10	160	57
70-86	1973	60	20	20	190	67
70-23	1975	80	<10	<10	226	20
70-19	1978	30	<10	<10	95	10
80-48	1983	320	60	30	1076	80
80-10	1986	160	320	160	761	1810
80-26	1986	80	160	80	113	160
80-74	1986	40	60	40	135	113
80-30	1987	20	40	10	67	113
80-34	1987	160	240	160	381	538
80-56	1988	20	20	<10	17	34
90-01	1990	40	40	10	48	80
90-24	1991	60	80	20	34	95
90-12	1992	160	320	60	190	226
90-13	1992	80	160	80	190	320

**Table 2-3: Characterization of K166 HA-specific sera**

We further characterized samples that had immunodominant K166 HA-specific Abs (light grey = K166 HA-specific sera from individuals born prior to 1985; darker grey = sera from individuals born after 1985). HAI assays were completed using viruses with the A/California/07/2009 HA engineered to have the K166Q HA substitution, as well as a primary pH1N1 virus isolated from the Children's Hospital of Philadelphia (CHOP) in 2013. Additional *in vitro* neutralization assays were completed using the reverse-genetics derived viruses. Data are representative of 2 independent experiments. For each neutralization assay, each sample was titered 4 times, and geometric mean of these quadruplicate samples is reported.

**Table 2-4**

<b>K166-specific donors</b>										
<b>visit 1 (pre-vaccine)</b>									<b>visit 2 (post-vaccine)</b>	
I.D.	YOB	USSR/77	Chile/83	Texas/91	NC/99	SI/06	CAL/09	CAL/09-K166Q	CAL/09-WT	CAL/09-K166Q
1	1979	240	160	320	60	80	120	20	160	20
6	1966	160	120	240	120	240	<10	<10	40	10
7	1966	40	60	30	10	60	<10	<10	120	<10
13	1982	80	40	80	40	60	10	<10	160	10
21	1958	60	30	80	40	80	<10	<10	60	20

<b>other donors</b>										
<b>visit 1 (pre-vaccine)</b>									<b>visit 2 (post-vaccine)</b>	
I.D.	YOB	USSR/77	Chile/83	Texas/91	NC/99	SI/06	CAL/09	CAL/09-K166Q	CAL/09-WT	CAL/09-K166Q
2	1976	80	80	120	<10	<10	60	60	80	60
5	1949	10	<10	<10	10	40	10	<10	40	40
8	1961	40	<10	30	80	120	<10	<10	80	80
9	1977	80	80	80	<10	<10	<10	<10	120	80
10	1983	80	<10	320	240	320	<10	<10	240	320
15	1961	<10	<10	<10	<10	<10	<10	<10	80	80
17	1956	40	<10	30	80	120	10	<10	20	20
19	1961	80	40	160	80	80	<10	<10	20	20
20	1945	40	<10	<10	<10	<10	<10	<10	240	320
22	1956	30	30	60	<10	<10	<10	<10	240	240
23	1975	40	<10	240	80	120	<10	<10	160	160
24	1968	<10	<10	30	<10	<10	<10	<10	60	60

**Table 2-4: Vaccination elicits K166 HA-specific responses**

HAI assays were completed using sera isolated from healthy donors before and after vaccination with the monovalent pH1N1 virus in 2009. Post- vaccination sera were collected 30 days after vaccination. HAI assays using post-vaccine sera were completed 3 independent times and assays using pre-vaccine sera were completed two independent times.

**Table 2-5**

first infection	second infection	group	animal #	day 14		day 84		day 98 (14 days post 2nd infection)	
				WT	K166Q	WT	K166Q	WT	K166Q
USSR/90/77	A/California/07/09	Group 1	7982	<10	<10	<10	<10	800	800
		Group 1	7983	<10	<10	<10	<10	800	800
		Group 1	7984	<10	<10	<10	<10	300	300
		Group 1	7985	<10	<10	<10	<10	800	600
		Group 1	7991	<10	<10	<10	<10	2400	2400
		Group 1	7993	<10	<10	<10	<10	800	1200
		Group 1	7994	<10	<10	<10	<10	600	600
A/Chile/1/1983	A/California/07/09	Group 1	7995	<10	<10	<10	<10	400	600
		Group 2	7987	<10	<10	<10	<10	1200	800
		Group 2	7988	<10	<10	<10	<10	800	400
		Group 2	7989	<10	<10	<10	<10	800	400
		Group 2	7990	<10	<10	<10	<10	1600	1600
		Group 2	7996	<10	<10	<10	<10	1200	400
		Group 2	7997	<10	<10	<10	<10	240	160
A/Singapore/06/86	A/California/07/09	Group 2	8000	<10	<10	<10	<10	600	800
		Group 2	8001	<10	<10	<10	<10	800	800
		Group 3	1487	<10	<10	80	120	6400	6400
		Group 3	1488	<10	<10	<10	<10	3200	3200
		Group 3	1481	<10	<10	<10	<10	2400	3200
		Group 3	1504	<10	<10	60	80	4800	6400
		Group 3	8002	<10	<10	<10	<10	1600	2400
A/California/07/09	A/California/07/09	Group 3	8009	<10	<10	<10	<10	1600	2400
		Group 3	8010	<10	<10	<10	<10	4800	6400
		Group 3	8012	<10	<10	<10	<10	1600	2400
		Group 4	8013	400	400	200	200	160	160
none	A/California/07/09	Group 4	8014	800	800	400	400	2400	2400
		Group 4	8015	800	800	300	200	2400	1600
		Group 5	1486	1600	2400	n.d.	n.d.	n.d.	n.d.
none	A/California/07/09	Group 5	1497	1600	1600	n.d.	n.d.	n.d.	n.d.
		Group 5	1492	1200	1600	n.d.	n.d.	n.d.	n.d.

**Table 2-5: Sera from ferrets sequentially infected with sH1N1 viruses and pH1N1**

Ferrets were infected with a sH1N1 virus and bled 14 and 84 days later. Animals were then infected with a pH1N1 strain and bled 14 days later (98 days post-first infection). Sera were isolated and HAI assays were completed using WT and K166Q-HA pH1N1 viruses. Data are representative of 3 independent HAI assays. The 3 ferrets with K166 HA- specific Ab responses had > 2 fold changes in HAI titer using the K166Q virus in 3 independent experiments.



## CHAPTER 3: CONTEMPORARY H3N2 VIRUS HAVE A GLYCOSYLATION SITE IN ANTIGENIC SITE B THAT ALTERS THE BINDING OF ANTIBODIES ELICITED BY EGG-ADAPTED VACCINE STRAINS

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Contributions: I generated mutant viruses by reverse genetics and completed all antigenic assays. Co-authors Kaela Parkhouse and Megan Gumina helped complete neutralization assays with human sera samples. Sebastian Diaz Perez completed mAb ELISAs. Kangchon Kim and Sarah Cobey performed statistical analyses on collected data. John Treanor and Andrea Sant designed the human vaccination studies (Figure 3-4). Patrick Wilson provided human mAbs. I wrote the manuscript with Scott Hensley and Sarah Cobey.

### Summary

H3N2 viruses continuously acquire substitutions in the hemagglutinin (HA) glycoprotein that abrogate binding of human antibodies. During the 2014-2015 influenza season, clade 3C.2a H3N2 viruses possessing a new predicted glycosylation site in antigenic site B of HA emerged, and these viruses remain prevalent today. The 2016-2017 seasonal influenza vaccine was updated to include a clade 3C.2a H3N2 strain; however, the egg-adapted version of this viral strain lacks the new putative glycosylation site. Here, we biochemically demonstrate that the HA antigenic site B of circulating clade 3C.2a viruses is glycosylated. We show that antibodies elicited in ferrets and humans exposed to the egg-adapted 2016-2017 H3N2 vaccine strain poorly neutralize a glycosylated clade 3C.2a H3N2 virus. Importantly, antibodies elicited in ferrets

infected with the current circulating H3N2 viral strain (that possesses the glycosylation site) and humans vaccinated with baculovirus-expressed H3 antigens (that possesses the glycosylation site motif) were able to efficiently recognize a glycosylated clade 3C.2a H3N2 virus. We propose that differences in glycosylation between H3N2 egg-adapted vaccines and circulating strains likely contributed to reduced vaccine effectiveness during the 2016-2017 influenza season. Further, our data suggest that influenza virus antigens prepared via systems not reliant on egg-adaptations are more likely to elicit protective antibody responses that are not affected by glycosylation of antigenic site B of H3N2 HA.

## **Introduction**

Influenza viruses continuously acquire substitutions in exposed epitopes of the hemagglutinin (HA) and neuraminidase (NA) proteins through a process called 'antigenic drift' (Yewdell, 2011). Influenza vaccine effectiveness can be low when there is a mismatch between vaccine strains and circulating strains (Belongia et al., 2016). Viral antigens included in influenza vaccines are routinely updated in an attempt to avoid antigenic mismatches (Carrat and Flahault, 2007; Schultz-Cherry and Jones, 2010). Current seasonal influenza vaccines possess antigens from 1 H1N1 strain, 1 H3N2 strain, and 1 or 2 influenza B strains.

H3N2 viruses began circulating in humans in 1968. Most neutralizing antibodies (Abs) recognize antigenic sites on the globular head of H3 (designated sites A-E), while rare Abs bind to the more conserved HA stalk or sialic acid binding domains (Krammer et al., 2015). Most H3N2 vaccine mismatches from 1968-2013 have been attributed to substitutions in antigenic site B of HA (Koel et al., 2013). None of the site B substitutions that emerged during this time period have led to new glycosylation sites on HA (Koel et al., 2013; Smith et al., 2004). This is surprising, given that the addition of glycans on HA can dramatically affect Ab binding (Abe et al., 2004; Das et al., 2011; Medina et al., 2013; Skehel et al., 1984; Wei et al., 2010; Wrigley et al., 1983). Instead, new glycosylation sites have repeatedly emerged and fixed in other antigenically important regions of H3 (Cherry et al., 2009).

Vaccine effectiveness was extremely low during the 2014-2015 influenza season (Zimmerman et al., 2016). During that season, influenza vaccines possessed antigens from a 2012 H3N2 virus that belonged to the 3C.1 HA genetic clade, while the majority of circulating H3N2 strains belonged to the 3C.2a and 3C.3a genetic clades (D'Mello et al., 2015). Both the 3C.2a and 3C.3a viruses differed at residues in HA antigenic site B compared to the 2014-2015 H3N2 vaccine strain (Chambers et al., 2015). Notably, 3C.2a viruses that circulated during the 2014-2015 season possessed a new predicted glycosylation site in HA antigenic site B (Chambers et al., 2015), and the 2014-2015 influenza vaccine exhibited especially low effectiveness against this clade (Flannery et al., 2016). In an effort to avoid an antigenic mismatch, the 2016-2017 influenza vaccine was updated to contain antigens from a 3C.2a H3N2 virus isolated in 2014 (2016). The majority of H3N2 viruses that circulated in the Northern Hemisphere during the 2016-2017 influenza season were 3C.2a viruses, however an interim estimate of vaccine effectiveness was only 43% against medically attended H3N2 infections (Flannery et al., 2017). Vaccine effectiveness was especially low in individuals that were 18-49 years old (Flannery et al., 2017). It is unclear why there was variable vaccine effectiveness during the 2016-2017 influenza season, given that the vaccine strain appeared to be well-matched to most circulating strains.

The majority of antigens for influenza vaccines are prepared in fertilized chicken eggs, and the 2016-2017 egg-adapted 3C.2a vaccine strain lacks the site B glycosylation site that is present on circulating 3C.2a H3N2 strains (Lin et al., 2017). Here, we completed a series of studies to determine if the difference in glycosylation of HA antigenic site B of H3N2 vaccine strains and circulating strains contributed to a previously unrecognized vaccine mismatch during the 2016-2017 influenza season.

## Results

### Recent H3N2 viruses possess a glycosylation site in antigenic site B of HA

A K160T HA substitution rapidly rose to fixation during the 2014-2015 influenza season, and nearly all currently circulating H3N2 viruses possess threonine (T) at HA residue 160 (Figure 3-1A). The K160T HA substitution is predicted to introduce an N-linked glycosylation site in antigenic site B of HA (Figure 3-1B, Table 3-1). H3N2 viruses with the K160T HA substitution grow poorly in chicken eggs (Lin et al., 2017), and the 2016-2017 egg-adapted H3N2 vaccine strain possesses a T160K HA reversion substitution (reference: GISAID isolate ID EPI\_ISL\_189811). We used reverse-genetics to create H3N2 viruses possessing HAs with T160 and K160, and we completed Western blot analyses to determine if the T160 and K160 HAs migrate differently in SDS-PAGE gels. HAs with T160 migrated with a higher molecular weight compared to HAs with K160 (Figure 3-1C, left). The HAs migrated similarly after PNGase treatment (Figure 3-1C, right), indicating that the difference in HA mobility in the absence of PNGase is due to differences in N-linked glycosylation.

To determine if the addition of this new antigenic site B glycan in HA affects antigenicity, we tested the binding of a panel of 26 human monoclonal antibodies that were elicited against an H3 virus from 2009, which lacks the new glycosylation site in antigenic site B of HA. All of these monoclonal antibodies bound to the HA of a 2009 virus (Figure 3-2). The majority of these monoclonal antibodies (77%) bound to a 2014 virus with the egg-adaptive K160 HA (which lacks the glycosylation site), while only a few (23%) bound to a 2014 virus with T160 (which possesses the glycosylation site) (Figure 3-2A). Raw binding data of a monoclonal antibody (024-10128-3C04) that binds efficiently to virus with K160 HA but not T160 HA is shown in Figure 3-2B. As a control, we also measured binding of an HA stalk-reactive monoclonal antibody that efficiently recognizes virus with either T160 HA or K160 HA (Figure 3-2C). Together, these data demonstrate that current circulating H3N2 influenza viruses possess a new glycosylation site in

antigenic site B of HA that affects antigenicity, and that this glycosylation site is not present in current egg-adapted H3N2 vaccine strains.

**Antibodies elicited in ferrets exposed to the 2016-2017 egg-adapted H3N2 vaccine strain poorly neutralize a circulating H3N2 viral strain**

We next completed a series of experiments to determine if the current egg-adapted H3N2 vaccine strain (that possesses K160) elicits different types of antibodies compared to the current circulating H3N2 viral strain (that possesses T160). We infected ferrets with viruses possessing either K160 HA or T160 HA, and we completed foci reduction neutralization tests (FRNTs) using sera collected from these animals 28 days after infection. We completed FRNT assays rather than conventional hemagglutination-inhibition assays since 3C2.a H3N2 viruses inefficiently agglutinate red blood cells. Antibodies elicited by the egg-adapted vaccine strain possessing K160 HA recognized the egg-adapted vaccine strain 4-8 fold more efficiently than the currently circulating H3N2 strain that possesses T160 HA (Figure 3-3A). This indicates that a large proportion of ferret antibodies elicited by the current egg-adapted H3N2 vaccine strain recognize the unglycosylated antigenic site B of HA. Interestingly, antibodies elicited by infection with the current circulating H3N2 viral strain possessing T160 HA recognized viruses with T160 HA and K160 HA equally (Figure 3-3B). This is consistent with the hypothesis that the new HA glycosylation site effectively 'shields' antigenic site B of HA, and that antibodies elicited by the viral strain possessing the new HA glycosylation site recognize epitopes not involving antigenic site B of HA.

**Antibodies elicited in humans vaccinated with the egg-adapted H3N2 strain poorly neutralize a circulating H3N2 viral strain**

We completed additional antigenic tests using sera isolated from humans (18-49 years old) before and after vaccination during the 2016-2017 influenza season. Although the majority of influenza vaccine antigens are prepared in fertilized chicken eggs, a small fraction of vaccine antigens are produced in insect cells or canine kidney cells. To account for this, we measured human antibody responses elicited by vaccine antigens prepared in eggs (Fluzone; n=22 donors),

MDCK cells (Flucelvax; n=26 donors), and insect cells (Flublok; n=22 donors). Importantly, the H3 antigens in the Fluzone and Flucelvax vaccines possess the egg-adapted K160 HA, while the recombinant H3 antigen in the Flublok vaccine possesses T160 HA.

Vaccine effectiveness was lower in younger adults compared to older adults during the 2016-2017 season (Flannery et al., 2017). Interestingly, we found that younger adults had higher pre-vaccination titers to the egg-adapted K160 HA compared to older individuals (Spearman's rho = 0.3,  $p < 0.01$ ; raw data from 3 independent experiments are shown in Table 3-2). Following vaccination, K160 HA titers were also higher in younger adults compared to older individuals (Spearman's rho=0.4), even after adjusting for higher pre-vaccination titers to K160 HA (Table 3-3). We speculate that the observed age-related differences in antibody titers to K160 HA might be due to birth year-related differences in H3N2 exposure history. Antibody titers to T160 HA were lower than antibody titers to K160 HA both before and after vaccination ( $p < 10^{-10}$  for each, Wilcoxon rank-sum test), but there were no birth year-related effects on antibody titers to T160 HA ( $p = 0.23$  pre-vaccination,  $p = 0.33$  post-vaccination; Table 3-3).

Some individuals in our study mounted strong antibody responses against T160 HA following vaccination (Table 3-2). Importantly, the majority of these individuals received the Flublok vaccine that possesses T160 HA (Figure 3-4A). Antibody titers against T160 HA increased ~4-fold following vaccination with Flublok, whereas there were only minimal increases in T160 HA antibody reactivity in most individuals following vaccination with Fluzone and Flucelvax (Figure 3-4A). Conversely, antibody increases against the K160 HA were similar following vaccination with the 3 different vaccine antigens (Figure 3-4B). These differential effects of the vaccines remained statistically significant after adjusting for age, pre-vaccination titer, and vaccination history (Table 3-3). These data indicate that H3N2 vaccine antigens produced during the 2016-2017 influenza season in eggs and MDCK cells (that possess K160 HA) elicit antibody responses that react poorly to current circulating H3N2 virus strains, whereas H3N2 vaccine

antigens produced in insect cells (that possess T160 HA) elicit antibody responses that react efficiently to current circulating H3N2 viral strains.

### **The effect of repeat vaccination on anti-H3 antibody responses during the 2016-2017 influenza season**

Many individuals receive influenza vaccines every year, however recent data suggest that repetitive vaccinations may be associated with reduced antibody responses (Huang et al., 2017; Leung et al., 2017; Thompson et al., 2016) and reduced vaccine effectiveness (McLean et al., 2014; Ohmit et al., 2013; Skowronski et al., 2016) during some influenza seasons. To determine if prior vaccinations impacted the development of H3 antibodies following vaccination of donors in our study, we examined neutralizing antibody data in relation to vaccine history. Donors in our study self-reported vaccination history in the previous two seasons (2014-2015 and 2015-2016). We excluded individuals who reported having received the live attenuated vaccine in previous seasons (n=1) and individuals who did not remember their vaccination status in either season (n=7), and then we grouped individuals into three categories: unvaccinated both seasons, vaccinated in only one season, and vaccinated in both seasons. Interestingly, vaccination history was uncorrelated with pre-vaccine titers to T160 HA and K160 HA, but it was the strongest independent predictor of final antibody titers and fold changes to both viral strains following vaccination (Table 3-3). Individuals that had been vaccinated in the previous two years exhibited overall lower antibody boosts (Figure 3-5), after adjusting for age and pre-vaccination antibody titers (Table 3-3).

## **Discussion**

It is unclear why there was only moderate vaccine effectiveness during the 2016-2017 influenza season, given that the 2016-2017 vaccine strains appeared to be well-matched to most circulating viral strains (Flannery et al., 2017). Our data suggest that a mismatch in antigenic site B of H3N2 viruses, caused by the propagation of the vaccine strain in eggs, likely contributed to this low vaccine effectiveness. When influenza viruses are passaged in new hosts, antigenic

properties of hemagglutinin can change since many residues of this protein are involved with both receptor binding and antibody recognition (Gambaryan et al., 1998; Gambaryan et al., 1999; Hensley et al., 2009; Li et al., 2013a). This is not the first example of antigenic properties of influenza viruses being altered by egg-adaptations. In 1978, Kilbourne showed that there were two antigenically distinct viruses in the 1976 X-53 swine influenza vaccine, and that these viruses had different growth properties in chicken embryos and canine kidney cells (Kilbourne, 1978). In 1983, Webster and colleagues demonstrated that influenza B viruses acquire substitutions that alter antigenicity when propagated in chicken eggs (Schild et al., 1983). Egg-adaptations resulting in antigenic changes have also been reported for H3N2 viruses (Chen et al., 2010b; Katz and Webster, 1989; Kilbourne et al., 1993; Meyer et al., 1993). It has been proposed that H3N2 egg-adaptations contributed to low vaccine effectiveness during the 2012-2013 influenza season (Skowronski et al., 2014), although this remains controversial (Cobey, 2017).

Nonetheless, the majority of influenza vaccine antigens continue to be prepared in eggs (Schultz-Cherry and Jones, 2010). One solution to the problem of egg-adaptations is to simply produce influenza antigens via a baculovirus system or in cell culture. The baculovirus system seems particularly well suited to avoid adaptive substitutions, however there are also potential problems with this approach. HA antigens prepared in insect cells are glycosylated with less complex sugars compared to mammalian cells (An et al., 2013), and it is possible that this affects antigenicity. In our study, some individuals vaccinated with baculovirus-prepared Flublok (that possessed T160 HA) mounted strong antibody responses that effectively neutralized a virus possessing an HA with a glycosylated antigenic site B; however, some Flublok-vaccinated individuals mounted antibody responses that reacted poorly to viruses with T160 HA.

It is important to note that there is more HA antigen in Flublok vaccine formulations compared to conventional egg-based vaccine formulations. It is possible that the increased amount of antigen in Flublok contributed to higher antibody responses against viruses that possess T160 HA, however, this is likely not the case since compared to the other vaccines



tested, Flublok did not induce a significantly greater response to K160 HA. Therefore, Flublok did not generate an overall higher antibody response in our study, but rather an antibody response that was better able to recognize viruses possessing T160 HA. This is interesting in light of a recent study demonstrating that the Flublok vaccine elicited more protective antibody responses in older adults compared to egg-based vaccines during the 2014-2015 season (Dunkle et al., 2017). This warrants further investigation because there was a large H3 antigenic mismatch during this season (Chambers et al., 2015). It is possible that the Flublok vaccine elicited antibodies of different specificities compared to egg-based vaccines during the 2014-2015 influenza season, perhaps to epitopes involving conserved residues of the HA receptor binding pocket.

Cell culture-expressed HA antigen might also avoid problems associated with egg-adaptations. The 2017-2018 recommended H3N2 component is the same as the 2016-2017 recommended H3N2 component, but starting this year, vaccine manufacturers that prepare antigens via cell culture systems will be allowed to use viral strains that have not been previously adapted to grow in eggs (<https://www.cdc.gov/flu/protect/vaccine/cell-based.htm>). H3N2 vaccine strains grown in this new system may possess HAs with a glycosylated antigenic site B, although substitutions that abrogate this glycosylation site have been previously reported upon serial passage of clade 3C.2a H3N2 viruses in MDCK cells (Lin et al., 2017).

Interim reports indicate that the 2016-2017 vaccine protected younger individuals from H3N2 infection less effectively compared to older individuals (Flannery et al., 2017). In our study, we found that younger adults were more likely to produce antibodies that efficiently neutralized K160 HA but weakly neutralized T160 HA after vaccination. These age-related differences may be due to differences in prior exposure histories. It is clear that prior influenza exposures can affect how an individual responds to new antigenically distinct viral strains (Cobey and Hensley, 2017). For example, individuals exposed to the 2009 pandemic H1N1 strain produce antibodies that target epitopes that are conserved in previously circulating seasonal H1N1 viral strains (Li et

al., 2012; Li et al., 2013b; Linderman et al., 2014; Wrarmert et al., 2011), particularly viral strains that they were likely exposed to in childhood. Early childhood infections also likely impact susceptibility to pandemic influenza virus strains. Gostic and colleagues recently demonstrated that early childhood infections with either H1N1 or H3N2 influenza viruses are associated with protection from H5N1 and H7N9 viruses later in life, presumably through induction of cross-reactive antibodies against epitopes that are conserved between these different viruses (Gostic et al., 2016). Further studies should explore if early childhood antigenic 'imprinting' with different H3N2 viral strains affects the specificity of antibodies elicited by seasonal influenza vaccination.

Vaccine effectiveness against H3N2 is often low (Belongia et al., 2016), especially among repeat vaccinees (McLean et al., 2014; Ohmit et al., 2013; Skowronski et al., 2016). Antigenic mismatch due to incorrect strain selection is an established cause of low vaccine effectiveness (Belongia et al., 2016). Future studies should address if egg-adapted substitutions constitute another form of antigenic mismatch that alters vaccine effectiveness in other influenza virus seasons. Antigens with egg-adapted mismatches might recall preexisting immune responses, including responses to previous vaccines. A major effort should be made to develop and utilize new systems that produce influenza antigens that are not dependent on egg or cell culture-adaptive substitutions. Antigens that do not possess adaptive substitutions will likely offer better protection against influenza virus strains that circulate in the human population.

## **Materials and Methods**

Viruses: The HA gene of a representative clade 3C.2a H3N2 virus (A/Colorado/15/2014) was cloned into the vector pHW2000 and the T160K substitution was introduced to remove the predicted glycosylation motif. Viruses containing H3 and N2 genes with A/Puerto Rico/8/1934 internal genes were rescued using the influenza reverse genetics system by transfecting a co-culture of 293T and MDCK-SIAT1 cells. Transfection supernatants were collected 3 d after transfection and stored at -80°C for use in neutralization assays. HA and NA genes of virus

expansions grown on MDCK-SIAT1 cells were sequenced to confirm that no additional substitutions arose during transfection.

*Glycosylation Western Blotting and Glycan Modeling:* Viruses possessing T160 and K160 HA were expanded on MDCK-SIAT1 cells and then concentrated at 20,000 rpm for 1 hr at 4°C using a SW-28 rotor. The amount of HA in each sample was normalized by ELISA with a human anti-HA monoclonal antibody for subsequent SDS-PAGE and Western blotting. To cleave N-linked glycans, samples were treated with PNGase-F (New England Biolabs) under reducing conditions. PNGase-treated and untreated samples were run on SDS-PAGE gels (Thermo Fisher Scientific) and transferred to a nitrocellulose membrane (Thermo Fisher Scientific). Blots were probed using anti-HA tag primary antibody clone HA-7 (Sigma-Aldrich, product number 59658) and an anti-mouse secondary antibody (Licor, product number 926-32212). In order to structurally model the N158 glycosylation introduced by T160, a K160T substitution was introduced into the crystal structure (PDB ID code 4O5I) using the program PyMol. Basic N-linked glycans were added to predicted N-linked glycosylation sites in the crystal structure using the GlyProt web server: <http://www.glycosciences.de/modeling/glyprot/php/main.php>.

*Human monoclonal antibody binding:* Human monoclonal antibodies were previously isolated from donor peripheral blood mononuclear cells following vaccination with the 2010-2011 influenza vaccine (Henry Dunand et al., 2015). ELISA plates were coated overnight at 4°C with a 2009 virus (A/Victoria/210/2009; the H3N2 component of the 2010-2011 influenza vaccine), a glycosylated 2014 clade 3C2.a virus with T160 HA, or an unglycosylated 2014 clade 3C2.a virus with K160 HA. ELISA plates were blocked with a 3% (w/vol) bovine serum albumin (BSA) solution in phosphate-buffered saline (PBS) for 2 hrs. Plates were washed 3 times with PBS containing 0.1% Tween20 and serial dilutions of each monoclonal antibody in ELISA buffer (1% BSA w/vol in PBS) were added to plates in 1% BSA in PBS. After 2 hrs of incubation, plates were again washed and a peroxidase-conjugated anti-human secondary antibody (Jackson ImmunoResearch, product number 109-036-098) diluted in ELISA buffer was added. After a 1 hr

incubation, plates were washed and a 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Seracare, product number 50-00-03) was added. The TMB reaction was stopped by adding HCl, and absorbance was measured using a plate reader. One-site specific binding curves were fitted in Prism software and the maximal binding against the 2009 vaccine strain was calculated for each monoclonal antibody. After background subtraction (from ELISA plates that did not have coated virus), binding of each monoclonal antibody relative to the vaccine strain was determined using the absorbance values at the lowest concentration of antibody that gave greater than 90% of maximal binding against the vaccine strain. Equivalent coating of each virus was checked using the stalk-reactive DY-2F04 monoclonal antibody.

Ferret sera: Ferret antisera were prepared at the AAALAC-accredited company Noble Life Sciences using protocols approved by the Nobel Life Sciences Institutional Animal Care and Use Committee. Naïve Fitch ferrets were intranasally infected with  $2 \times 10^5$  PFU of virus expressing either K160 HA or T160 HA. Serum samples were collected prior to infection and 28 days following infection. Sera used in antigenic assays were treated with receptor-destroying enzyme (Denka Seiken) for 2 hrs at 37°C, and the enzyme was then heat-inactivated at 55°C for 30 min.

Human Sera: Experiments using human sera were conducted with the approval of the University of Rochester and University of Pennsylvania Institutional Review Boards. Informed consent was obtained for all individuals enrolled. Serum samples were collected at the University of Rochester prior to and 28 days following vaccination. Serological experiments were completed at the University of Pennsylvania using de-identified samples. Prior to assays, sera samples were treated with receptor-destroying enzyme (Denka Seiken) for 2 hrs at 37°C, and the enzyme was then heat-inactivated at 55°C for 30 min.

Foci Reduction Neutralization Test (FRNT) Assays: Serum samples were serially diluted in 96-well round-bottom plates containing serum-free media. Approximately 200 focus-forming units (FFU) of reverse-genetics transfection supernatant of each virus were added to each diluted sera sample and the virus-sera mixtures were incubated at room temperature for 1 hr. The virus-sera

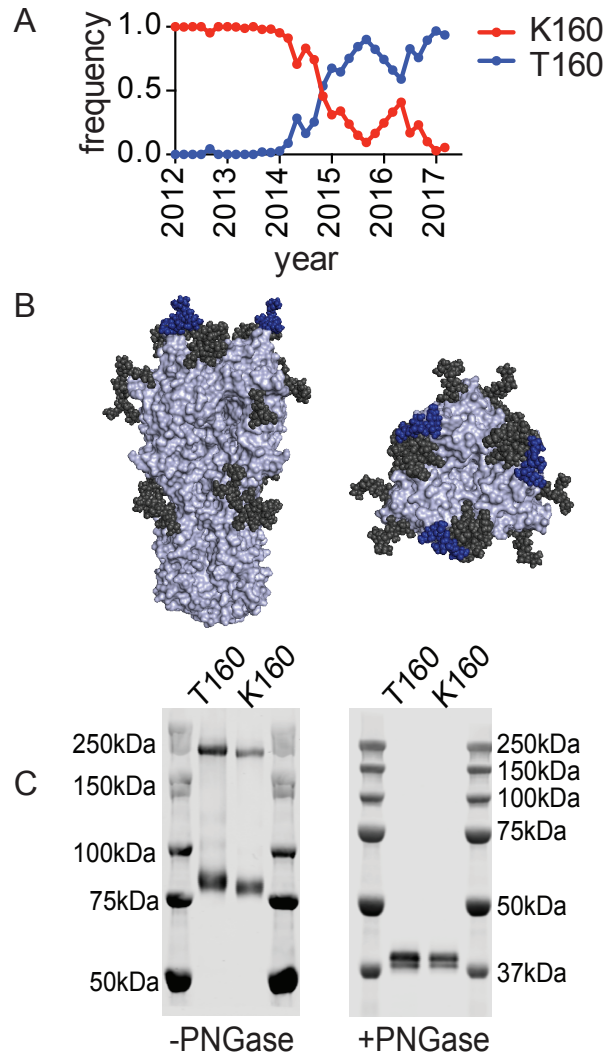
mixtures were then added to confluent monolayers of MDCK-SIAT1 cells and incubated at 37°C for 1 hr. After incubation, cells were washed with serum-free media and an overlay medium of serum-free media containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Thermo Fisher Scientific), gentamycin (Thermo Fisher Scientific), and 0.5% methylcellulose (Sigma Aldrich) (w/vol), was added to cells. Infected monolayers were incubated for 18 hours, after which the overlay medium was aspirated and the cells were fixed and permeabilized with ice-cold methanol-acetone (1:1 vol/vol). Infected monolayers were stained with anti-NP monoclonal antibody IC5-1B7 (BEI Reagent Resources, product number NR-43899) and an anti-mouse peroxidase-conjugated secondary antibody (MP Biomedicals, product number 855563). A 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Seracare, product number 5510-0030) was added to visualize foci. Following staining, plates were imaged and foci were quantified using an ELISPOT reader (Cellular Technologies Limited). FRNT<sub>90</sub> titers were reported as the reciprocal of the highest dilution of sera that reduced the number of foci by at least 90%, relative to control wells that had no serum or monoclonal antibody added. Undetectable titers were assigned a value of 10. All FRNT assays were repeated three times on separate days.

*Models of initial and final antibody titers and fold responses:* Three replicate measurements of pre- and post-vaccination antibody titers from each serum sample (see Table 3-2) were geometrically averaged, and the geometric mean titers were used for model fitting. Linear models that included age and vaccination history (receipt of trivalent inactivated vaccine in neither, only one, or both of the last two years) were fitted to log<sub>2</sub> pre-vaccination titers as continuous and factor variables, respectively. Models were similarly fitted to log<sub>2</sub> post-vaccination antibody titers, also including log<sub>2</sub> pre-vaccination titers and vaccine group (Flublok, Flucelvax, and Fluzone) as continuous and factor variables, respectively. Likelihood maximization was performed with the `lm` package in R v. 3.3.1. Code for the analysis is available at:

[https://cobeylab.github.io/H3N2\\_glycosylation/](https://cobeylab.github.io/H3N2_glycosylation/)

## Figures and Tables

Figure 3-1



**Figure 3-1: Contemporary H3N2 viruses possess a new substitution that introduces a glycosylation site in antigenic site B of HA**

**(A)** The K160T HA substitution rapidly rose to fixation during the 2014-2015 influenza season.

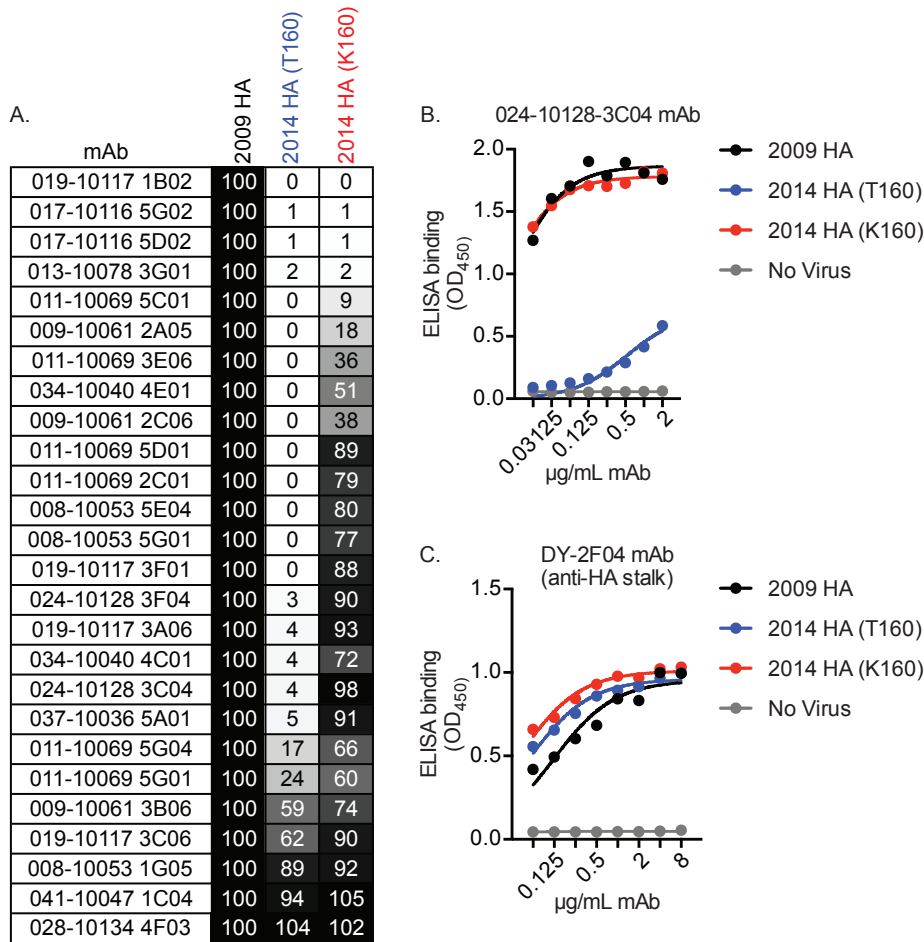
Shown are frequencies estimated from viral samples (from GISAID database) collected from

December 2011 to March 2017 and divided into two-month windows. **(B)** Putative glycosylated

sites on contemporary HAs are shown on the A/Victoria/361/2011 HA trimer (PDB ID code 4O5I).

The new putative site introduced by the K160T substitution is shown in blue, while the other putative sites are shown in black. **(C)** H3 viruses possessing either K160 HA or T160 HA were created by reverse-genetics. The molecular weights of the HAs of these viruses were determined using Western blots with an anti-HA antibody, either with or without prior PNGase treatment. PNGase treatment was completed under reducing conditions. On the –PNGase gel, the upper bands correspond to HA trimers and the lower bands correspond to HA monomers.

**Figure 3-2**

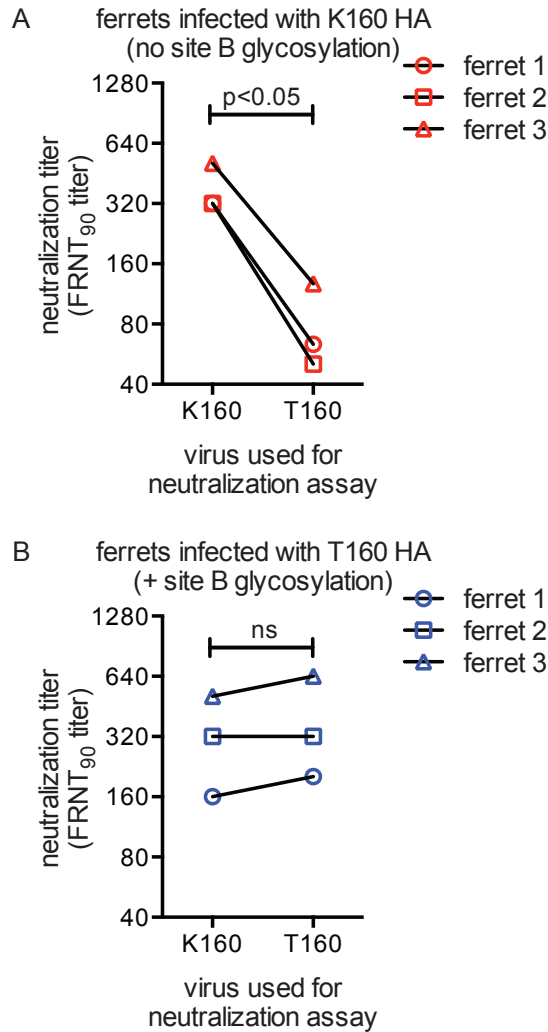


**Figure 3-2: Contemporary H3N2 viruses with T160 HA are antigenically distinct compared to H3N2 viruses with K160 HA**

**(A)** ELISA assays were completed to test the binding of 26 anti-H3 human monoclonal antibodies (mAbs) to a 2009 HA, a 2014 HA with T160, and a 2014 HA with K160. All antibodies in this panel were elicited by a 2009 HA following vaccination prior to the 2010-2011 season. Shown is % binding of antibodies to the 2014 viruses relative to binding to the 2009 virus. **(B)** ELISA binding data for an antibody that binds efficiently to virus with K160 HA but not T160 HA is shown. **(C)** ELISA binding data for an antibody that recognizes a conserved epitope on the HA stalk is shown to verify that ELISA plates were coated with similar amounts of HA antigen.



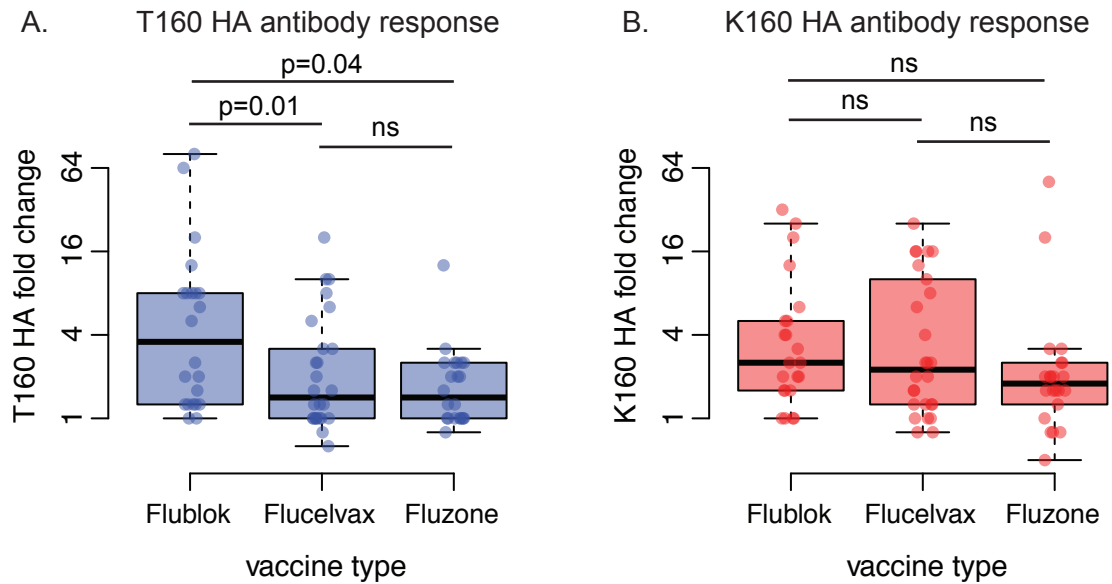
**Figure 3-3**



**Figure 3-3: Ferrets elicit different types of antibody responses when exposed to H3 viruses with K160 HA and T160 HA**

Ferrets (n=3 animals per group) were infected with viruses possessing **(A)** K160 HA or **(B)** T160 HA and sera were collected 28 days later. FRNTs were completed using viruses that possessed K160 HA or T160 HA. Neutralization titers are expressed as inverse dilution of sera that reduced foci by 90%. We completed 3 independent experiments with each sera. Shown are geometric means from the 3 independent experiments. Statistical significance was determined using a paired Student's t-test.

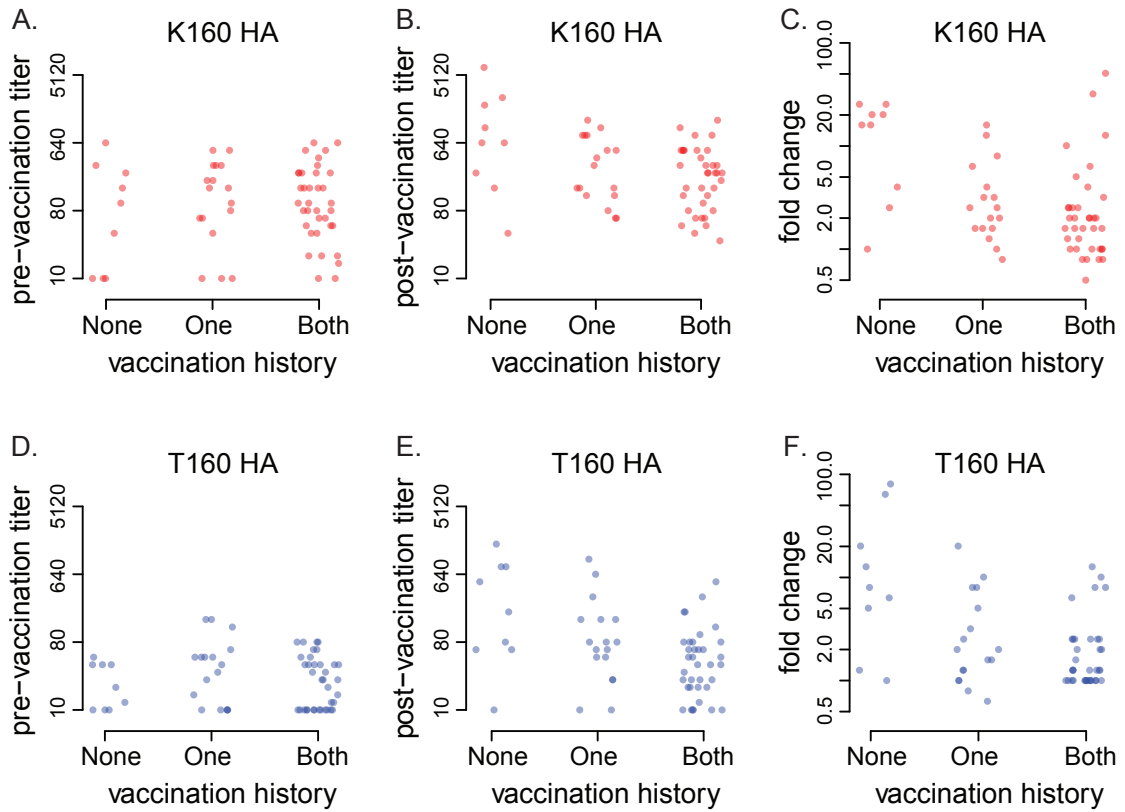
**Figure 3-4**



**Figure 3-4: Vaccine antigens possessing K160 HA and T160 HA elicit different responses in humans**

Donors were vaccinated with seasonal influenza vaccines, and sera were collected prior to and 28 days after vaccination. FRNTs were completed using viruses that possessed T160 HA or K160 HA. **(A)** Flublok induced higher fold changes to T160 HA than did Flucelvax and Fluzone ( $p=0.01$  and  $p=0.04$  in adjusted analysis, respectively; Table 3-3; ns, non-significant). **(B)** The vaccine types did not differ in their ability to induce responses to K160 HA ( $p>0.1$  in adjusted analysis; Table 3-3). Thick horizontal lines show the median fold changes of the geometric mean titers. Colored rectangles indicate the interquartile range and whiskers the 150% interquartile ranges. Individual data points are superimposed. See Table 3-2 for raw titer data.

**Figure 3-5**



**Figure 3-5: The effect of prior vaccine history on neutralizing antibody titers**

Vaccination history did not affect initial pre-vaccine titers to K160 HA or T160 HA (A,D), but previous vaccinations progressively reduced titers after vaccination (B,E). Fold change is shown in panels C and F. Shown are the geometric means from the 3 independent experiments. Each circle represents the geometric mean titer of an individual sample. Darker intensity indicates overlapping data points.

**Table 3-1**

**A predicted glycosylation site at residue 158 arose in 2014**

H3 residues

year	<u>158</u>	<u>159</u>	<u>160</u>
1968	G	S	T
1977	E	S	T
1979	E	S	K
1986	E	Y	K
1993	K	Y	K
2004	K	F	K
2009	N	F	K
2014-present	N	Y	T
predicted gly site	N	X (not P)	T

**Table 3-1: Historical status of the 158 glycosylation sequence motif**

**Table 3-2**

Donor #	Group	Age	experiment 1						experiment 2						experiment 3						vaccine history	
			pre-vaccination			post-vaccination			pre-vaccination			post-vaccination			pre-vaccination			post-vaccination			2014	2015
			T160	HA	K160	HA	T160	HA	K160	HA	T160	HA	K160	HA	T160	HA	K160	HA	T160	HA	K160	HA
1	FB	49	10	20	20	20	20	10	20	20	40	10	20	20	80	Inactivated	Inactivated					
2	FCV	38	10	10	20	40	10	10	20	80	10	10	40	80	Inactivated	Inactivated						
3	FZ	26	80	320	160	320	80	320	160	640	80	320	320	640	Inactivated	Inactivated						
4	FCV	37	160	320	160	640	160	640	160	640	160	640	160	1280	None	Inactivated						
5	FCV	30	40	160	40	160	40	160	40	160	40	160	40	160	40	160	Inactivated	Inactivated				
6	FB	26	10	80	80	640	10	160	80	640	20	160	160	640	Inactivated	Unknown						
7	FCV	32	80	80	320	640	40	80	640	80	160	1280	1280	None	Inactivated	Inactivated						
8	FCV	19	40	80	320	640	40	160	320	1280	20	80	320	1280	Inactivated	Inactivated						
9	FCV	48	10	10	10	10	10	10	10	80	10	10	10	80	None	None						
10	FB	29	10	320	40	640	10	320	80	1280	10	320	160	1280	None	Inactivated						
11	FCV	31	40	10	10	80	10	10	10	160	10	10	10	160	None	Inactivated						
12	FCV	30	10	40	10	80	10	80	10	80	10	80	10	80	Inactivated	Inactivated						
13	FZ	48	10	20	10	40	10	20	10	40	10	20	10	80	Inactivated	Inactivated						
14	FZ	49	10	160	10	80	10	160	10	160	10	160	10	160	Inactivated	Inactivated						
15	FB	37	80	80	80	80	40	80	40	80	80	80	80	80	Inactivated	Inactivated						
16	FZ	19	40	640	40	320	40	640	40	640	40	640	80	640	Inactivated	Inactivated						
17	FCV	18	10	20	40	320	10	20	40	320	20	20	40	320	Unknown	None						
18	FB	33	10	40	10	80	10	160	40	160	10	80	40	160	Inactivated	Inactivated						
19	FCV	27	10	160	40	320	20	320	20	320	20	320	40	320	Unknown	Inactivated						
20	FCV	22	40	160	40	320	20	160	80	640	80	320	80	640	Inactivated	None						
21	FB	32	40	80	40	160	40	80	40	160	40	160	80	320	Inactivated	Inactivated						
22	FZ	44	10	20	20	40	10	20	40	80	20	20	40	80	Inactivated	Inactivated						
23	FZ	20	10	40	10	40	10	40	10	40	10	80	10	80	Inactivated	Inactivated						
24	FZ	22	40	320	80	640	40	640	80	1280	40	640	80	2560	Inactivated	Inactivated						
25	FCV	24	10	40	10	80	10	80	10	80	10	80	10	80	None	Inactivated						
26	FCV	21	10	320	10	320	10	320	10	640	20	320	10	320	Unknown	Inactivated						
27	FCV	20	10	40	10	160	10	80	10	160	10	80	20	160	Inactivated	Inactivated						
28	FCV	35	10	80	20	160	10	80	20	320	20	160	20	320	Inactivated	Inactivated						
29	FZ	25	40	160	40	80	20	160	20	160	40	160	20	160	None	Inactivated						
30	FZ	32	10	40	10	40	10	40	10	80	10	80	10	80	Inactivated	Inactivated						
31	FB	33	40	160	40	320	20	160	40	320	20	320	80	640	None	Inactivated						
32	FB	19	20	80	160	320	20	80	160	320	40	80	320	320	Inactivated	Inactivated						
33	FCV	27	10	80	20	160	20	80	20	160	20	80	20	160	Inactivated	Inactivated						
34	FCV	21	10	10	80	160	10	10	80	160	10	10	80	160	None	None						
35	FCV	26	10	10	40	160	10	10	80	320	10	10	80	320	None	None						
36	FB	24	40	640	40	640	40	640	80	640	80	640	80	640	None	None						
37	FZ	47	20	40	20	80	20	40	20	80	40	80	40	160	Inactivated	Inactivated						
38	FZ	46	10	10	10	20	10	10	10	20	10	20	10	40	Live	Inactivated						
39	FB	22	10	320	1280	5120	20	320	1280	5120	40	320	2560	10240	None	None						
40	FZ	19	10	320	10	640	20	640	80	640	40	640	40	1280	Unknown	Unknown						
41	FB	42	40	40	320	320	40	40	640	640	40	40	640	640	Inactivated	Inactivated						
42	FZ	21	20	160	40	320	20	320	80	320	40	320	80	640	Inactivated	Inactivated						
43	FCV	31	10	10	80	160	10	10	40	160	10	10	40	160	Inactivated	None						
44	FZ	26	20	160	20	320	20	160	20	320	20	160	20	320	Inactivated	Inactivated						
45	FZ	30	40	640	40	80	40	640	80	640	80	640	40	640	Inactivated	Inactivated						
46	FZ	26	40	320	80	640	80	640	160	1280	80	640	160	640	Inactivated	Inactivated						
47	FB	45	10	10	80	40	10	10	80	80	10	10	80	80	None	Inactivated						
48	FB	23	80	40	80	80	40	80	80	320	40	80	80	160	None	Inactivated						
49	FCV	24	10	40	10	40	10	40	10	20	10	40	10	40	Inactivated	Inactivated						
50	FZ	20	40	160	160	320	40	160	160	640	80	160	160	640	Inactivated	None						
51	FB	20	40	160	40	160	40	320	40	320	40	320	40	320	Inactivated	Inactivated						
52	FB	19	40	160	40	160	40	320	80	320	80	320	80	320	Inactivated	Inactivated						
53	FCV	34	80	160	80	160	80	320	80	320	80	320	80	320	Inactivated	Inactivated						
54	FZ	21	80	160	80	320	80	320	160	640	80	320	80	640	Inactivated	Inactivated						
55	FCV	21	40	160	640	2560	40	160	640	2560	40	160	1280	2560	None	None						
56	FZ	42	160	320	320	640	160	640	320	1280	160	640	320	640	None	Inactivated						
57	FCV	24	80	320	80	320	80	320	80	320	80	640	80	320	Inactivated	Inactivated						
58	FB	31	80	40	40	40	40	40	80	80	40	40	80	80	None	Inactivated						
59	FB	19	40	320	1280	1280	80	320	640	1280	40	320	1280	1280	Inactivated	None						
60	FB	20	40	160	160	640	40	320	320	640	40	320	160	640	None	None						
61	FZ	21	20	160	80	320	40	160	80	320	40	160	80	320	Inactivated	Unknown						
62	FCV	37	160	320	160	320	160	320	160	320	80	320	160	320	None	Inactivated						
63	FZ	20	10	10	20	320	10	10	40	640	10	10	20	640	Inactivated	Inactivated						
64	FCV	20	10	80	40	160	10	80	20	160	10	80	20	160	Inactivated	None						
65	FB	40	20	40	640	640	10	40	1280	1280	10	40	640	1280	None	None						
66	FB	30	10	20	80	320	10	20	80	640	10	10	80	640	Inactivated	Inactivated						
67	FB	20	20	160	40	160	40	160	40	320	40	160	40	320	Inactivated	Inactivated						
68	FB	22	10	160	40	640	10	160	80	640	10	160	80	1280	Inactivated	Inactivated						
69	FCV	19	40	160	80	320	40	320	160	320	40	320	160	640	Unknown	None						
70	FZ	26	40	80	320	1280	40	80	640	2560	40	160	640	2560	None	None						

**Table 3-2: Summary of neutralization titers**

Shown are the prevaccination and postvaccination neutralization titers and prior vaccine history for individuals in Figure 3-4 and Figure 3-5. FB: Flublok, FCV: Flucelvax, FZ: Fluzone.

**Table 3-3**

	<b>Estimate</b>	<b>Std. err.</b>	<b>Sig.</b>	<b>Adj. R<sup>2</sup></b>	<b>Log lik.</b>
<b>Prevaccination titers to K160</b>				0.10	-119.74
Age	-0.07	0.02	**		
Vaccinated once	0.70	0.71			
Vaccinated twice	0.66	0.64			
<b>Prevaccination titers to T160</b>				0.02	-97.20
Age	-0.02	0.02			
Vaccinated once	0.69	0.50			
Vaccinated twice	0.17	0.45			
<b>Postvaccination titers to K160</b>				0.48	-94.13
Age	-0.04	0.02	*		
Vaccinated once	-1.38	0.49	**		
Vaccinated twice	-1.85	0.45	***		
Flucelvax receipt	-0.37	0.36			
Fluzone receipt	-0.09	0.38			
Prevacc. titer	0.46	0.09	***		
<b>Postvaccination titers to T160</b>				0.51	-101.40
Age	-0.02	0.02			
Vaccinated once	-1.67	0.55	**		
Vaccinated twice	-2.21	0.51	***		
Flucelvax receipt	-1.04	0.40	*		
Fluzone receipt	-0.89	0.43	*		
Prevacc. titer	0.84	0.15	***		

\* p<0.05, \*\* p<0.01, \*\*\* p<0.001

**Table 3-3: Summary of model fits**

# **CHAPTER 4: ANTIBODIES TARGETING THE H3 HEMAGGLUTININ RECEPTOR-BINDING SITE PLAY AN UNEXPECTED ROLE IN NEUTRALIZATION OF RECENT H3N2 VIRUSES**

Contributions: Megan Gumina and Kaela Parkhouse helped complete neutralization assays, and Megan Gumina helped complete absorption-neutralization and absorption-ELISA experiments shown in Figures 4-6 and 4-8. Patrick Wilson contributed mAbs, and Andrea Sant and John Treanor designed the human vaccination study (data shown in Figure 4-8).

## **Summary**

Influenza viruses escape neutralizing antibodies by acquiring substitutions in antibody epitopes in hemagglutinin (HA), a process known as antigenic drift. We mapped the binding footprints of a panel of H3-specific monoclonal antibodies isolated from humans vaccinated with the 2010-2011 seasonal influenza vaccine. Over a third of neutralizing antibodies were sensitive to substitutions at highly conserved positions in the HA receptor-binding site (RBS), and some neutralized contemporary H3N2 viruses with major antigenic changes. We measured the levels of a subset of RBS antibodies in serum of humans before and after vaccination. We found these RBS antibodies in frequencies ranging from 10-20% of individuals, with some neutralizing antibody responses focused against epitopes involving HA RBS residues. This suggests that in some individuals RBS-directed antibodies are present at levels that would likely provide protection, highlighting the need to better understand how these antibodies are elicited.

## **Introduction**

Influenza A viruses pose a serious public health threat, in large part due to their ability to rapidly escape human immunity (Yewdell, 2011). The viral attachment and entry factor hemagglutinin (HA) is the major target of neutralizing antibody responses against influenza viruses, and the presence of antibodies that bind the immunodominant globular head domain of HA and block attachment to cells is an established correlate of protection from infection (Coudeville et al., 2010). However, circulating influenza viruses continuously accumulate substitutions in antibody epitopes in HA, which may abrogate the binding of pre-existing antibody

responses. This process of immune evasion, known as antigenic drift, allows influenza viruses to escape population immunity. As a result, seasonal infection or vaccination provides limited protection from re-infection with antigenically drifted strains. Antigenic drift also undermines vaccine effectiveness because vaccines that are mismatched with circulating strains provide poor protection from infection. In order to combat antigenic drift, developing a “universal” influenza vaccine is a major priority. A universal influenza vaccine would protect individuals from antigenically drifted strains through longer-lasting and more broadly cross-reactive antibody responses (Erbelding et al., 2018). While specific approaches vary, a common strategy of universal influenza vaccine efforts is to elicit antibody responses that recognize functionally conserved sites on HA in order to limit the potential for viral escape. The highly conserved stalk domain of HA is a promising vaccine target, and several strategies have been pursued to elicit stalk-reactive immunity (Impagliazzo et al., 2015; Krammer et al., 2013; Yassine et al., 2015). However, as stalk antibodies generally neutralize poorly *in vitro* and require Fc-mediated effector functions in order to protect *in vivo* (DiLillo et al., 2014), it is important to also consider targeting functionally conserved sites in the HA head domain, such as the receptor-binding site (RBS).

In the 1980s, the crystal structure of H3 HA was used in combination with sequence data to define antigenic sites on the H3 head domain (Wiley et al., 1981; Wilson et al., 1981). Human antibodies that bind each site have been described (Okada et al., 2011), but there is evidence that sites A and B, which are adjacent to the RBS, are immunodominant in human antibody responses. As a result, substitutions in these sites play an especially important role in H3N2 antigenic drift. In a ferret model of influenza virus antigenic evolution, substitutions at a handful of positions in H3 antigenic sites A and B have been associated with large antigenic changes (Koel et al., 2013; Popova et al., 2012). In addition, antigenic site B has been a major site of antigenic escape in H3N2 viruses. Substitutions in site B that arose in 2014-2015 had a dramatic effect on the ability of pre-existing human antibodies to bind and neutralize H3N2 viruses (Beer et al., 2018; Chambers et al., 2015; Zost et al., 2017), and the acquisition of these substitutions was



also associated with drastically reduced vaccine effectiveness. In a recent example, the fixation of a new glycosylation site in site B abrogated pre-existing neutralizing antibody titers in a large number of individuals (Zost et al., 2017), illustrating the dramatic consequences of antigenic substitutions near the RBS.

Given that the surface area of the HA RBS is much smaller than the paratope of a single antibody, substitutions on the rim of the RBS have the potential to greatly impact antibody binding. However, despite the antigenic variation surrounding the RBS, some highly cross-reactive antibodies make contacts in the HA RBS. These RBS antibodies achieve this cross-reactivity through mimicry of the same contacts made by sialic acid, in some cases using strikingly similar mechanisms of recognition. Some antibodies make contacts with conserved RBS residues through a shared dipeptide motif (Krause et al., 2011a; Schmidt et al., 2015b; Whittle et al., 2011), while other antibodies insert a hydrophobic residue into the RBS (Xu et al., 2013). Broadly reactive RBS antibodies also generally make minimal contacts or tolerate substitutions at RBS-adjacent residues, in some cases due to atypically long HCDRs. However, unlike HIV RBS antibodies such as VRC01, HA RBS antibodies do not originate only from a handful of germline precursors or require extensive framework substitutions or germline polymorphisms. Rather, antibodies that recognize the H1 RBS have been shown to arise from a number of  $V_H$  gene segments (Schmidt et al., 2015b). While there is some evidence that antibodies targeting the RBS are more common than previously thought (McCarthy et al., 2018), the RBS antibodies that have been described structurally come from a comparatively small number of individuals. Despite the potential of RBS antibodies to contribute to more cross-reactive and antigenic drift-resistant neutralizing antibody responses, the frequency and relative abundance of these antibodies in individual repertoires and the human population remains unknown.

In order to better understand recent antigenic drift in H3N2 viruses, we have mapped the binding sites of a large panel of H3 mAbs isolated from donors vaccinated in 2010-2011. Substitutions in site B abrogate the binding of most neutralizing mAbs, consistent with recent

major changes in antigenic site B. However, some mAbs were able to tolerate large antigenic changes due to contacts in the RBS, and substitutions in the RBS abrogated the binding of a third of neutralizing antibodies. Using our antigenic mutant panel, we identified a subset of RBS antibodies that appear to make contact with Y98, an RBS residue that binds sialic acid and is completely conserved across HA subtypes. In serum from one individual, antibody contacts with Y98 mediated cross-reactivity with an antigenically drifted H3N2 strain, suggesting this individual mounted a convergent antibody response targeting the RBS. In order to answer the question of whether these RBS antibodies contribute to neutralization of currently circulating H3N2 strains, we designed an absorption-based approach to quantify the prevalence and abundance of RBS antibodies in bulk sera from humans before and after vaccination with recent seasonal influenza A vaccines. We found that in the case of an antigenically mismatched vaccine, some vaccine-induced neutralizing antibody responses were focused against epitopes involving Y98. Additionally, we discovered that RBS antibody titers were boosted by vaccination with vaccines that lack egg-adaptive substitutions in the RBS. We suggest that, in some individuals, RBS antibodies play an unappreciated role in protection from infection with antigenically drifted strains, and that vaccine regimens or immunogens designed to selectively recall these RBS antibodies would be a valuable component of universal vaccine strategies.

## **Results**

### **Characterization of mAbs**

We began by characterizing a panel of 33 anti-H3 human mAbs that were elicited by vaccination with the 2010/2011 trivalent influenza vaccine. (Henry Dunand et al., 2015). These antibodies originated from 13 different individuals. We first completed hemagglutination-inhibition (HAI) and micro-neutralization (MN) assays with the H3 component of the 2010/2011 vaccine, A/Victoria/210/2009 (Vic/09) to assess the ability of mAbs to prevent receptor binding and block virus infection respectively. Of the mAbs in the panel, 26 out of 33 inhibited agglutination of the vaccine strain (Figure 4-1A), indicating that they bound close enough to the HA globular head

domain to block sialic acid binding. Two mAbs that did not inhibit agglutination neutralized infection (Figure 4-1B), indicating that these HAI- mAbs likely neutralized by a different mechanism than preventing attachment. 5 mAbs did not give HAI or MN titers. In order to test the breadth of binding for these mAbs, we measured binding to a panel of different H3 viruses by ELISA (Figure 4-2). While most HAI+ mAbs bound to a relatively narrow range of viruses from 2005-2012, the HAI- mAbs bound a much wider range of viruses, with some mAbs binding strains from 1968-present. Surprisingly, two of these non-neutralizing mAbs cross-reacted with an H1 HA, indicating they bound an epitope conserved between group 1 and group 2 HAs. Taken together, these data demonstrated that most of the mAbs bound to the globular head domain and neutralized by blocking attachment to sialic acid, while a subset of more cross-reactive mAbs bound lower on the HA.

### **Fine-mapping of mAb Footprints**

In order to better understand the effects of individual substitutions on antigenic drift, we next sought to better define the epitopes of each mAb. To map the binding footprints of the mAbs, we introduced substitutions that had recently become fixed into the Vic/09 vaccine strain HA, as these were likely to have an antigenic effect. In parallel, we grew the Vic/09 strain in the presence of a subset of mAbs to select escape substitutions that abrogated mAb binding. We also used seasonal strain binding data (Figure 4-2) in combination with sequence comparisons to select substitutions that were likely to be responsible for the loss of binding to HA. The substitutions in our panel were predominantly located in antigenic sites A and B near the RBS, as these antigenic sites are immunodominant in human antibody responses. Our panel included substitutions at all the amino acid positions previously shown to be essential for antigenic escape from ferret sera (Koel et al., 2013). To map the epitopes of potential RBS antibodies in our panel, we also introduced substitutions at conserved positions in the RBS. In order to express HA substitutions that would likely have dramatic functional consequences, we generated virus-like particles (VLPs) bearing point-mutant HAs and tested binding of each mAb to VLPs in our panel using ELISA. We

then generated antigenic maps with mAb binding to each mutant normalized to the WT vaccine strain (Figure 4-3A). The majority of mAbs mapped to residues in antigenic site B. In particular, substitutions at positions 159 and 160 abrogated the binding of many mAbs, consistent with these substitutions having large antigenic effects in recent H3N2 evolution (Beer et al., 2018; Chambers et al., 2015; Zost et al., 2017). Some mAbs also mapped to sites lower on HA, away from the RBS. The two mAbs that neutralized but did not block attachment in HAI competed with a mouse H3 stalk mAb for binding in ELISA (Figure 4-4). Taken together, these data confirm that antigenic site B is the major target of neutralizing antibodies in human repertoires and demonstrate that single substitutions near the RBS can abrogate the binding of most human mAbs.

### **Identification of RBS Antibodies**

Surprisingly, substitutions at conserved positions in the RBS affected the binding of approximately a third of HAI+ mAbs (Figure 4-3A). Some mAbs that were sensitive to RBS substitutions were also abrogated by site B changes, likely because they make contacts with both the conserved RBS as well as highly variable positions in site B. Other RBS mAbs, however, were more tolerant of site B substitutions (Figure 4-3A). We chose to focus on a subset of these RBS mAbs that were sensitive to a Y98F substitution. Y98 is located at the base of the RBS, interacts with sialic acid, and is completely conserved in circulating strains from both group 1 and group 2 HAs. The Y98F substitution has been reported to dramatically decrease receptor binding (Martín et al., 1998) and impair viral replication *in vivo* (Bradley et al., 2011). We introduced the essentially isosteric tyrosine to phenylalanine substitution in order to produce a limited effect on the overall structure of the HA yet disrupt the binding of RBS antibodies.

### **A Subset of RBS Antibodies Reveals Convergent Targeting of the RBS**

While RBS that contact Y98 have been described (McCarthy et al., 2018) these antibodies have been thought to be rare. The five Y98-specific RBS mAbs were isolated from two different donors, and interestingly, all five mAbs arose from distinct heavy-chain rearrangements, indicating that multiple germline rearrangements converged on the RBS in each donor. Four of

the five Y98-specific mAbs utilized a J<sub>H</sub>6 gene segment, which has been previously reported as a common feature of mAbs recognizing the H1 RBS (Schmidt et al., 2015b). To test whether some of these Y98F-specific mAbs were able to tolerate site B substitutions due to their RBS contacts, we measured the binding of these mAbs against VLPs bearing WT and Y98F HAs. We also tested binding to HAs with a glycan introduced by a K160T substitution, and the double mutant Y98F K160T. As expected, while most Y98-specific mAbs were able to bind to the glycosylated HA, binding was greatly reduced against the double mutant (Figure 4-5A). This indicated that some of the RBS antibodies in our panel were able to use their contact with Y98 to maintain binding in the presence of large antigenic changes on the rim of the RBS. One of these Y98-specific mAbs, 019-10117 3C06, was able to neutralize an antigenically drifted strain, A/Hong Kong/4801/2014 (HK/14), which contains this site B glycosylation. This indicated that despite several years of antigenic drift, RBS contacts enabled continued binding and neutralization, and that antibodies like 019-10117 3C06 are capable of contributing to protection against strains circulating today.

### **Cross-Reactivity of RBS Antibodies in Donor Serum**

While the isolation of these RBS mAbs from plasmablasts indicated that they were recalled by vaccination, little is known about the levels of RBS antibodies in human sera. Some studies have demonstrated competition for binding between RBS antibodies and sera from autologous donors, however these experiments do not directly measure RBS antibody levels, as other antibodies that bind near the RBS could also compete with RBS antibodies for binding (Schmidt et al., 2015b). To test whether RBS antibodies targeting Y98 were present at detectable levels in donor sera, we completed ELISAs testing the binding of prevaccination and postvaccination donor sera against HAs from the Vic/09 vaccine strain, the antigenically drifted HK/14 strain, and Y98F mutants of Vic/09 and HK/14. We tested sera from 28 vaccinated individuals, representing 10 of the 13 donors in our panel. While most individuals showed no reduction in binding against the Y98F mutants, one individual, 019-10117, showed reduced

binding to both Vic/09 Y98F and HK/14 Y98F HAs (Figure 4-5B). This result indicated that antibodies targeting Y98 were prevalent in the sera of this individual, and that some of these serum antibodies were able to cross-react with an antigenically drifted strain that arose five years later. Interestingly, both the prevaccination (D0) and postvaccination (D21) antibody responses from 019-10117 were focused on Y98, indicating that while vaccination recalled RBS antibodies as part of the plasmablast response, the vaccine itself was not responsible for establishment of the Y98-specific repertoire.

### **RBS Antibodies Contribute to Neutralizing Titers Against an Antigenically-Mismatched Strain**

The presence of RBS specificity prior to vaccination and the distinct antibody lineages targeting Y98 suggested that the prior exposure history of 019-10117 resulted in an RBS-dominated antibody repertoire. This finding raised the question of how frequent similar RBS-directed antibody responses are in the human population and whether they can contribute to neutralization of contemporary circulating strains. We hypothesized that in some individuals, a fraction of neutralizing antibodies present in serum might be composed of RBS-directed antibodies. In order to dissect the contribution of RBS antibodies to neutralization in the sera of human donors, we developed an absorption-based approach to fractionate human serum samples. By incubating sera with 293F cells expressing either WT or Y98F HA, we could deplete antibodies that bound in the presence of Y98, while enriching for antibodies that failed to bind in the context of Y98F. Using mAbs of known specificity (019-10117 3C06 and 041-10047 1C04), we demonstrated that depletion of antibodies not specific for Y98 was robust and that Y98-specific antibodies would be pulled down following absorption with WT HA but left in solution following absorption with Y98F HA (Figure 4-6A). We first examined the antibody responses of 21 donors who were vaccinated during the 2015-2016 season. The H3 component of this vaccine, A/Switzerland/9715293/2013 (Switz/13), differs by 11 substitutions in a number of different epitopes compared with strains that circulated that season (Figure 4-6B). Given these potential

antigenic differences, we expected the vaccine to be antigenically mismatched with HK/14, a member of the clade that dominated circulation. As expected, we observed lower neutralization titers to HK/14 than Switz/13 after vaccination (Figure 4-6C), indicating that the vaccine was not antigenically matched with circulating strains. We used our absorption assay to fractionate prevaccination and postvaccination serum samples from each individual and measured the ability of the absorbed sera to neutralize HK/14 WT virus. In all cases, we were able to completely deplete the neutralization titer by absorbing with the HK/14 WT HA. While the neutralization titer of most individuals was completely depleted by HK/14 Y98F HA absorption, four individuals had neutralization titers following HK/14 Y98F HA absorption (Figure 4-6D). This indicated that after vaccination, these individuals possessed Y98-specific antibodies that cross-reacted with the HK/14 strain. We further confirmed that sera from these individuals contained antibodies specific for Y98 by absorbing with HK/14 WT and HK/14 Y98F HAs and measuring the binding of the absorbed sera in ELISA against HK/14 WT and HK/14 Y98F HAs (Figure 4E). These absorption-ELISA experiments revealed the presence of Y98-specific antibodies in the prevaccination sera of subjects 557 and 591. In contrast, subjects 574 and 595 had no detectable Y98-specific antibodies prior to vaccination. Taken together, these data demonstrate that antigenically mismatched vaccines are capable of recalling RBS antibodies and that in some individuals these RBS antibodies contribute to the neutralization titer against antigenically mismatched strains.

### **Are RBS Antibodies Better Boosted by a Vaccine Lacking Egg-adaptive RBS Substitutions?**

It should be noted that while some RBS residues are completely conserved in circulating influenza A strains, there is considerable variation near the RBS in vaccine strains as a result of culture-adaptive substitutions, and these substitutions can vary from year to year depending on the vaccine strain. There is increasing evidence that these changes in the RBS can affect vaccine responses. In the case of H1N1 viruses, an egg-adaptive RBS substitution has been shown to be the target of vaccine-induced neutralizing antibodies that fail to cross-react with circulating strains

(Garretson et al., 2018; Raymond et al., 2016). In the case of H3N2 viruses, we have previously demonstrated that an egg-adaptive H3 substitution, L194P, dramatically affects antibody binding and alters the conformational flexibility of antigenic sites surrounding the RBS (Wu et al., 2017). Consistent with this, the L194P substitution had a large antigenic effect on many of the antigenic site B and RBS mAbs. (Figure 4-7) This L194P substitution has been present in the H3N2 component of the vaccine since 2016 and will be present in the 2018-2019 Northern Hemisphere vaccine. However, vaccines produced in mammalian or insect cells can lack egg-adaptive substitutions. As a result, these vaccines might be able to more efficiently recall antibodies targeting the RBS. We previously reported that Flublok, a recombinant HA vaccine produced in a baculovirus expression system and lacking egg-adaptive substitutions, was able to better induce neutralizing antibody responses against circulating H3N2 strains compared with egg-adapted vaccine strains (Chapter 3). We hypothesized that due to a better-matched RBS, Flublok might more effectively recall RBS-directed antibodies. To test this hypothesis, we completed absorption-neutralization and absorption-ELISA assays using HK/14 WT and Y98F antigens with prevaccination and postvaccination serum samples from individuals vaccinated with either the baculovirus-produced vaccine Flublok or an egg-adapted vaccine strain (Fluzone) (Figure 4-8). Three individuals who were vaccinated with Flublok had neutralizing titers of RBS antibodies following vaccination. In comparison, one individual who was vaccinated with an egg-based vaccine had RBS antibodies contributing to neutralization following vaccination. We detected prevaccination RBS antibodies by absorption-ELISA in one Flublok subject and one Fluzone subject who went on to develop neutralization responses. Surprisingly, one of the individuals vaccinated with Flublok had nearly half of their neutralizing titer directed against Y98, indicating an extremely focused response. Due to the low frequency of Y98 specificity in both groups our study was not sufficiently powered to reject the null hypothesis that Flublok and Fluzone are equivalent in their ability to boost antibody responses targeting Y98. However, it is worth noting that the magnitude of the RBS antibody titers induced by Flublok is higher than that of Fluzone, and the role of culture-adaptive substitutions in vaccine responses warrants further investigation.



## Discussion

Here we have identified and characterized antibodies targeting the H3 RBS that were isolated from several individuals following vaccination. In one of these individuals, these RBS arose from at least three distinct lineages and were sensitive to a substitution at a single RBS residue, suggesting that the exposure history of that individual resulted in convergent targeting of the RBS by distinct germline rearrangements. Using an absorption-based approach we extended our findings to the polyclonal response against contemporary H3N2 strains by identifying antibodies specific for a single RBS residue in the sera of several individuals. In two separate cohorts, we found RBS-focused antibodies contributing to neutralization titers in ~10% of postvaccination samples. Importantly, in the case of a mismatched vaccine some individuals mounted an RBS response that cross-reacted with the circulating strain, indicating that these RBS antibodies were recalled by vaccination and would likely contribute to protection from infection.

Our work likely underestimates the prevalence of antibodies targeting the RBS in the human population for several reasons. Given that we only quantified Y98-specific antibodies in bulk sera, any RBS antibodies making critical contacts with other RBS residues were not measured by our absorption experiments. Additionally, RBS antibodies that maintained some level of binding in the presence of Y98 would have likely been at least partially depleted by absorption. It is worth noting that the Y98F substitution is used to ablate sialic acid binding in HA probes used to identify HA-specific B-cells by flow cytometry, in large part because the substitution does not have a major structural effect (Whittle et al., 2014). As a result, studies using these probes to select B-cells for characterization may underestimate the prevalence of Y98-specific antibodies.

Our absorption experiments reveal that some individuals mount a neutralizing response that is highly focused on RBS residues, in some cases constituting half of the total neutralizing antibody titer. However, how easy is it for the virus to escape this type of immunity? Deep

mutational scanning experiments have demonstrated that even a broadly-reactive RBS antibody can readily select substitutions that confer escape (Doud et al., 2018). On the other hand, other studies have demonstrated that RBS antibodies approaching the RBS from different angles select different escape substitutions, and as a result, some have suggested that complementary targeting of the RBS by different classes of RBS antibody could restrict the ability of the virus to completely escape immunity (Schmidt et al., 2015b). In addition, recent work has demonstrated that mutational reversion at an position in the RBS involves a complex network of epistatic substitutions within both RBS residues and residues in antigenic sites adjacent to the RBS (Wu et al., 2018). Together, this raises the important question whether some individuals with an RBS-dominated response might be more resistant to antigenic drift. There is emerging evidence that the neutralizing response of some individuals is functionally monoclonal. For example, the recent fixation of a glycosylation site in site B of H3N2 viruses had major antigenic consequences, resulting in the complete abrogation of pre-existing neutralizing antibody titers in a large number of individuals (Zost et al., 2017). In light of this glycosylation's dramatic antigenic effect, it is interesting that individual 019-10117 had detectable levels of RBS-directed antibodies present in serum both before and after vaccination in 2010. These serum RBS antibodies were able to cross-react with contemporary strains that contained this site B glycosylation and still circulate nearly eight years later. We suggest that individuals with RBS-focused responses might be less susceptible to antigenically drifted variants. While it is possible that escape from RBS immunity could occur through acquisition of multiple substitutions in or adjacent to the RBS, it is possible that this sequential escape might occur more slowly, and as a result, RBS-directed immunity might provide longer-lasting protection. An alternative possibility is that single substitutions on the rim of the RBS would be sufficient to confer escape from the RBS-directed antibody responses that we measure in human sera. Further studies will need to examine the ability of influenza virus to escape from RBS-directed immunity present in polyclonal sera.

While our study demonstrates that vaccination can sometimes recall antibodies targeting the RBS, do particular exposure histories favor the elicitation of RBS antibodies? While our

measurement of Y98-specific antibody frequency suggests ~10% of individuals have detectable levels of RBS antibodies contributing to neutralization in polyclonal sera, it has been speculated that germline rearrangements encoding antibodies that recognize the RBS should be common in naïve human repertoires (Schmidt et al., 2015b). While some studies have generated unmutated common ancestors and inferred the immunogenic stimuli for broadly-reactive antibody lineages targeting the RBS (McCarthy et al., 2018; Schmidt et al., 2015a), we know little about how prior immune history and repeated exposures influence the development of RBS antibodies. In addition, it has been speculated that the RBS of some HAs, such as H2, might be more intrinsically immunogenic (Krause and Crowe, 2014). In the field of HIV, major efforts have been made to study antibody-virus co-evolution and the development of broadly neutralizing antibody specificities in chronically infected individuals, with the goal of identifying Envs that favor the development of neutralizing breadth (Bonsignori et al., 2017; Landais et al., 2017; Rantalainen et al., 2018). Longitudinal studies in human cohorts could address similar questions for influenza, with the potential to fill in gaps in our understanding of how antibody responses are elicited, recalled, and altered by infection and vaccination (Erbelding et al., 2018). Studying the development of antibody responses targeting the HA RBS in these cohorts would provide valuable insights into the development of RBS-directed immunity, and could suggest possible immunogens or sequential vaccination regimens that would more effectively boost RBS antibodies compared with our current vaccines. In addition, it might be possible to alter immunogen structure in a way that favors the development of antibodies that bind the HA RBS. Glycan masking of irrelevant epitopes has recently been employed to focus the antibody response against the RBS of an Env immunogen (Duan et al., 2018), and a similar strategy could be employed in an influenza virus immunogen to selectively stimulate naïve B-cells capable of generating RBS-targeted antibodies.

Despite the complete conservation of some RBS residues in circulating strains, our existing process of vaccine production often results in changes at RBS residues or in RBS-proximal antigenic sites due to adaptive substitutions during vaccine strain propagation.

Importantly, these substitutions vary from year to year depending on the strain included in the vaccine. As an ironic result of adaptation to culture systems, many HA RBS residues are less conserved in our vaccines than they are in nature. Our work demonstrates that many antibodies elicited by vaccination make contacts in antigenic sites also contact RBS residues. In order to design next-generation vaccines that effectively elicit and boost RBS-targeted responses, our vaccines will need to be produced in systems where we can precisely control the structure of the RBS and adjacent residues.

## **Materials and Methods**

*Monoclonal Antibody Isolation and Purification:* mAbs were isolated from human donors as previously described (Smith et al., 2009b). Briefly, plasmablasts were single-cell sorted from peripheral blood mononuclear cells collected from donors seven days after vaccination with the 2010-2011 vaccine containing the H3N2 vaccine strain A/Victoria/210/2009. Single-cell RT-PCR was used to amplify  $V_H$  and  $V_L$  chains, which were cloned into human IgG expression vectors. mAbs were produced by transfecting 293T cells with plasmids encoding heavy and light chains and mAbs were purified using protein A/G affinity purification.

*Hemagglutination-inhibition (HAI) Assays:* mAbs were serially diluted twofold in a 96-well round-bottom plate in 50 $\mu$ L total volume of phosphate-buffered saline (PBS). After serial dilution, four agglutinating doses of virus in a total volume of 50 $\mu$ L PBS were added to each well. Turkey erythrocytes (12.5 $\mu$ L of a 2.5% [vol/vol] solution) were added and the sera, virus, and erythrocytes were gently mixed. After 1 hr at room temperature, plates were scanned and titers were determined as the lowest concentration of monoclonal antibody that fully inhibited agglutination. HAI assays were performed in duplicate on separate days.

*Microneutralization (MN) Assays:* mAbs were serially diluted twofold in a round-bottom 96-well plate in 50 $\mu$ L serum-free Minimal Essential Medium (MEM). 50 $\mu$ L of MEM containing 100 TCID<sub>50</sub>

of virus was added to serially diluted mAbs and the mAb-virus mixtures were incubated for 30 min at room temperature. Following incubation, the mAb-virus mixtures were added to confluent monolayers of Madin-Darby canine kidney (MDCK) cells in 96-well plates and incubated for 1 hr at 37°C. After incubation, the virus-antibody mixture was removed and cells were washed with 180µL MEM. After washing, serial dilutions of each mAb were added back to cell monolayers in infection media (MEM containing HEPES buffer, gentamycin, and TPCK-treated trypsin). The cells were incubated for 3 days and neutralization titers were determined as the lowest concentration of mAb that prevented cell death. MN assays were completed in duplicate on separate days.

Seasonal H3N2 strain ELISAs: A panel of seasonal H3N2 viruses spanning 1968 to 2013 was expanded by propagation in embryonated chicken eggs. Allantoic fluid was clarified centrifugation and concentrated by ultracentrifugation at 20,000 rpm in an SW-28 rotor. Viral pellets were resuspended overnight in PBS and chemically inactivated using B-Propiolactone (BPL; Sigma Aldrich). To measure mAb binding to the seasonal panel, ELISA plates were coated overnight at 4°C with concentrated virus in PBS or just PBS as a background control. The following day, plates were blocked with a 3% w/vol solution of bovine-serum albumin (BSA) in PBS for 2 hrs. After blocking, plates were washed five times with distilled water and twofold serial dilutions of each mAb were added to plates in a 1% w/vol solution of BSA in PBS. After 2 hrs of incubation, plates were washed and a peroxidase-conjugated goat anti-human secondary antibody was added in a 1% w/vol solution of BSA in PBS. After incubation for 1 hr, plates were washed and 50µL of TMB substrate was added to each well. The TMB reaction was quenched by addition of 25µL 250mM HCl and absorbance at 450nm was measured using a plate reader. In order to generate antigenic maps, one-site specific binding curves were fit to the data in GraphPad Prism software and the maximal binding ( $B_{max}$ ) was determined for each mAb. To generate antigenic maps from the ELISA data, we first selected the lowest mAb concentration that still gave at least 90% of the  $B_{max}$  signal. At this dilution, background signal was subtracted and signal for each point mutant was normalized to the Vic/09 WT HA VLP signal. Antigenic mapping ELISAs were

conducted for each mAb in duplicate on separate days, and the resulting values were averaged and represented as a heatmap.

VLP Antigenic Mapping ELISAs: Point mutants of A/Victoria/210/2009 HA were generated in a codon-optimized HA gene by site directed mutagenesis. Virus-like particles (VLPs) were generated by transfecting 293T cells with each point mutant along with plasmids encoding HIV gag, the NA from A/Puerto Rico/8/1934, and a human-airway trypsin-like protease (HAT). Supernatants from transfected 293T cells were collected 3 days following transfection and were concentrated by centrifugation at 19,000 rpm in an SW-28 rotor using a 20% sucrose cushion. VLP pellets were resuspended in PBS and stored at 4°C. ELISA plates were coated with HA-normalized point-mutant VLPs diluted in PBS or just PBS as a background control and stored overnight at 4°C. ELISAs and antigenic map generation were conducted as described for the H3N2 seasonal panel above.

Competition ELISAs: Plates were coated overnight at 4°C with the strain A/Hong Kong/1/1968 or with PBS. The following day, plates were blocked with 3% BSA in PBS (w/vol). Following 2 hrs of blocking, plates were washed three times with distilled water and serial dilutions of the mouse H3-stalk mAb F49 (Takara) were added to plates at a starting concentration of 32 µg/mL in 1% BSA in PBS. After 2 hrs, a fixed concentration of each human mAb was added. For each human mAb, a competition control was done using the H1-stalk mAb C179 at a starting concentration of 32 µg/mL to verify that competition was specific to F49. After 1 hr of incubation, plates were washed five times with distilled water and a peroxidase-conjugated goat anti-human IgG secondary was added in 1% BSA in PBS. After 1 hr, plates were washed five times with distilled water and developed with TMB as described previously. The binding of each human mAb in the presence of F49 was normalized to the binding of C179 at the same dilution.

Recombinant HA Production: Codon optimized HA genes for A/Hong Kong/4801/2014 WT and Y98F were cloned into expression vectors and the transmembrane domain was removed and replaced with the FoldOn trimerization domain from T4 fibritin, an AviTag site-specific biotinylation

sequence, and a His tag, as previously described (Whittle et al., 2014). Recombinant HAs were produced by transfecting 293F suspension cells with plasmids encoding the recombinant HA and the NA from A/Puerto Rico/8/1934. After four days, the supernatant was clarified by centrifugation and the HA proteins were purified by Ni-NTA affinity chromatography.

*Human Subjects and Serum Collection:* Experiments using deidentified human sera were conducted at the University of Pennsylvania with the approval of the institutional review boards of the University of Pennsylvania, the University of Rochester, and the University of Chicago. Informed consent was obtained for all individuals. In the case of individuals from whom mAbs were isolated, serum was collected at the time of vaccination and 21 days postvaccination. For individuals from the other vaccination cohorts, serum samples were collected at the time of vaccination and four weeks postvaccination. For assays using foci-reduction neutralization tests, serum were treated with receptor-destroying enzyme (RDE) for 2 hrs at 37°C. Following treatment, the enzyme was heat-inactivated by incubation at 55°C.

*Human Sera ELISAs:* ELISA plates were coated the day prior with 0.5µg/mL recombinant HAs (HK/14 WT, HK/14 Y98F, or a PBS background control) and blocked for 2 hrs on the day of the experiment with a solution addition of 3% BSA in PBS. After washing the plates three times with wash buffer containing 0.5% Tween20 (vol/vol) in PBS (PBS-T), serially diluted serum samples were added to the ELISA plates and incubated for 2 hrs. After incubation, plates were washed three times with PBS-T and a peroxidase-conjugated goat anti-human secondary antibody diluted in 1% BSA in PBS was added. After 1 hr of incubation with the secondary antibody, plates were washed three times with PBS-T and 50µL of a TMB substrate was added to each well. 25µL of 250mM HCl was used to quench the reaction and the absorbance at 450nm was measured using a plate reader. Background signal at each dilution was subtracted for each serum sample and one-site specific binding curves were fit to the data using GraphPad Prism. Human sera ELISAs were performed in triplicate on separate days.

*Foci-Reduction Neutralization Tests:* RDE-treated serum samples were serially diluted in 96-well plates in a total volume of 50 $\mu$ L. Approximately 200 focus-forming units of HK/14 WT virus in 50 $\mu$ L were added to each well and the virus-absorbed sera mixture was incubated for 1 hr at room temperature. After incubation, the virus-sera mixture was added to confluent monolayers of MDCK-SIAT1 cells and incubated for 1 hr at 37°C. After incubation, cell monolayers were washed with 180 $\mu$ L serum-free MEM and an overlay medium containing HEPES, gentamycin, and 0.5% methylcellulose was added. The cell monolayers were incubated for 18 hrs, after which the overlay was removed and the cells were fixed at 4°C for 2 hrs using an aqueous solution of 4% paraformaldehyde (vol/vol). After fixation, cell monolayers were permeabilized using 0.5% Triton-X100 in PBS (vol/vol). After fixation and permeabilization, monolayers were blocked with a solution of 5% fat-free milk in PBS for 1 hr. After blocking, a mouse anti-nucleoprotein antibody was added in 5% milk/PBS for 1 hr. After the primary incubation, a peroxidase-conjugated goat anti-mouse secondary antibody in 5% milk/PBS was added for 1 hr. After incubation with the secondary antibody, monolayers were stained using a TMB substrate and foci were imaged and quantified using an ELISpot reader. For staining, plates were washed with distilled water between each step. Percentage of infection was determined relative to wells that did receive any serum or antibody. Values reported are the concentration of serum or mAb that reduced the numbers of foci by at least 90%.

*Absorption-Neutralization and Absorption-ELISA Assays:* Two days prior to experiments, 293F suspension cells were transfected using 293fectin with plasmids expressing HK/14 WT HA, HK 14 Y98F HA, or a mock transfection control containing no plasmid or transfection reagent. On the day of the experiment, transfected cells were pelleted by centrifugation, washed twice with 293F media, and resuspended at the desired volume.

In the case of absorption-neutralization assays, RDE-treated serum samples were diluted in 293F media at an initial dilution of 1:10 and split into three fractions for the three absorption conditions. An equivalent volume of 293F media containing approximately  $8 \times 10^6$  transfected



cells/absorption reaction were added to each diluted serum sample and the samples were mixed by shaking for 1 hr at room temperature. After incubation, the cells were pelleted by centrifugation and the supernatant was transferred and re-centrifuged to clarify. Absorbed supernatant containing the sera was then serially diluted in 96-well round-bottom plates in serum-free MEM and FRNT assays were conducted as described. Absorption-neutralization experiments were completed in triplicate on separate days.

In the case of absorption-ELISA assays, serum samples were diluted in 293F media at an initial dilution of 1:50 and split into three fractions for the three absorption conditions. Transfected cells were added and absorption of serum antibodies was carried out as described above. Following absorption, absorbed serum samples were serially diluted at a starting dilution of 1:500 (factoring in absorption volume) in 1% BSA w/vol in PBS and human sera ELISAs were performed as described. Background binding for each sample at each dilution was subtracted and one-site specific binding curves were fit to the data using GraphPad Prism software. The area under the curve (AUC) was calculated for each curve. Absorption-ELISA experiments were done in triplicate on separate days.

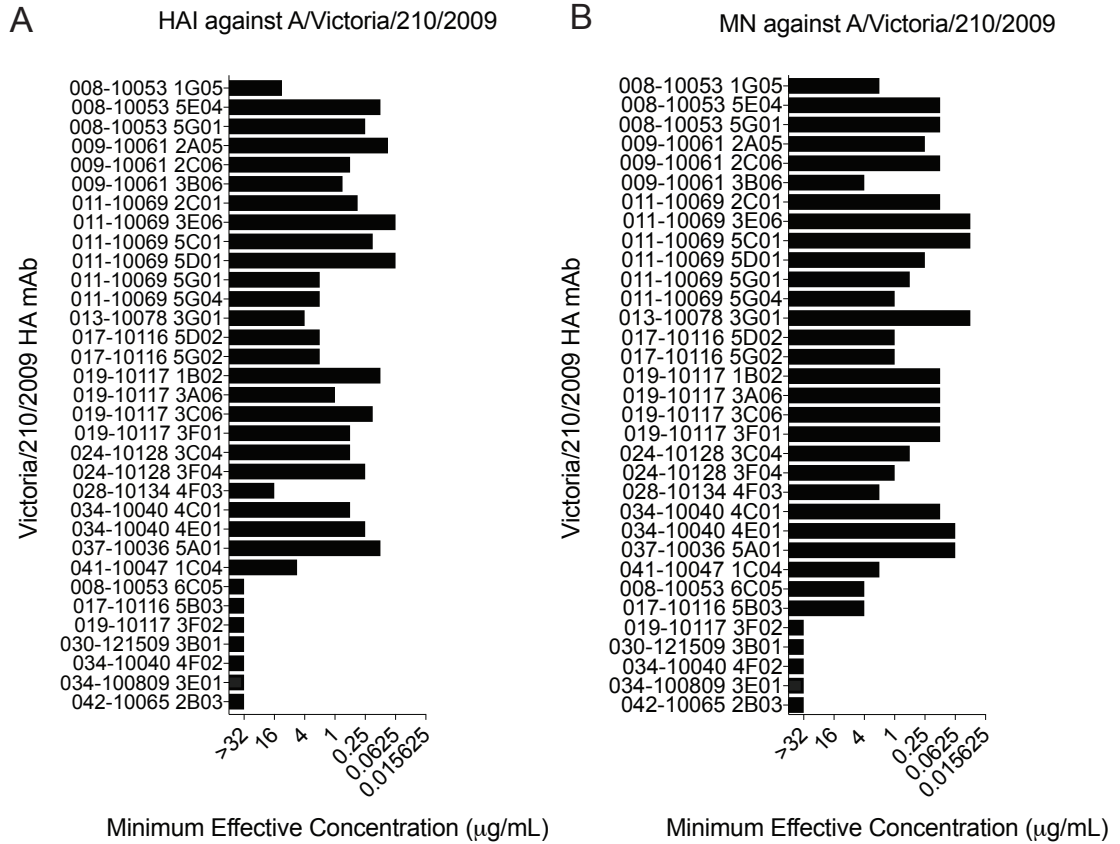
For both absorption-neutralization and absorption-ELISA experiments, RBS mAb 019-10117 3C06 and lower HA head mAb 041-10047 1C04 were initially diluted to a concentration of 32 $\mu$ g/mL in 293F media prior to the addition of cells. For absorption-neutralization experiments, the starting concentration for each mAb absorption condition in the FRNT was 16 $\mu$ g/mL (assuming no absorption). For absorption-ELISA experiments, the starting concentration for each mAb absorption condition in the ELISA was 3.2 $\mu$ g/mL (assuming no absorption).

Quantification and Statistical Analysis: Titer values for neutralization and ELISA experiments are reported as geometric mean  $\pm$  SD or geometric mean  $\pm$  95% CI as noted. Comparisons between groups for the 2015-2016 vaccinees were performed using either the nonparametric Krushal-Wallis test. Comparisons of ELISA titers against WT or Y98F HA following Y98F HA

absorption were performed using an unpaired Student's t-test. Statistical significance is indicated for the following thresholds: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns = non-significant.

## Figures and Tables

Figure 4-1



**Figure 4-1: Hemagglutination-Inhibition and micro-neutralization activities of mAbs**

**(A)** Hemagglutination inhibition titers of mAbs against the vaccine strain A/Victoria/210/2009. **(B)** Micro-neutralization titers against the vaccine strain A/Victoria/210/2009. Two mAbs, 008-10053 6C05 and 017-10116 5B03, neutralized infection but did not block viral attachment in HAI. Titers are representative of two independent experiments.

**Figure 4-2**

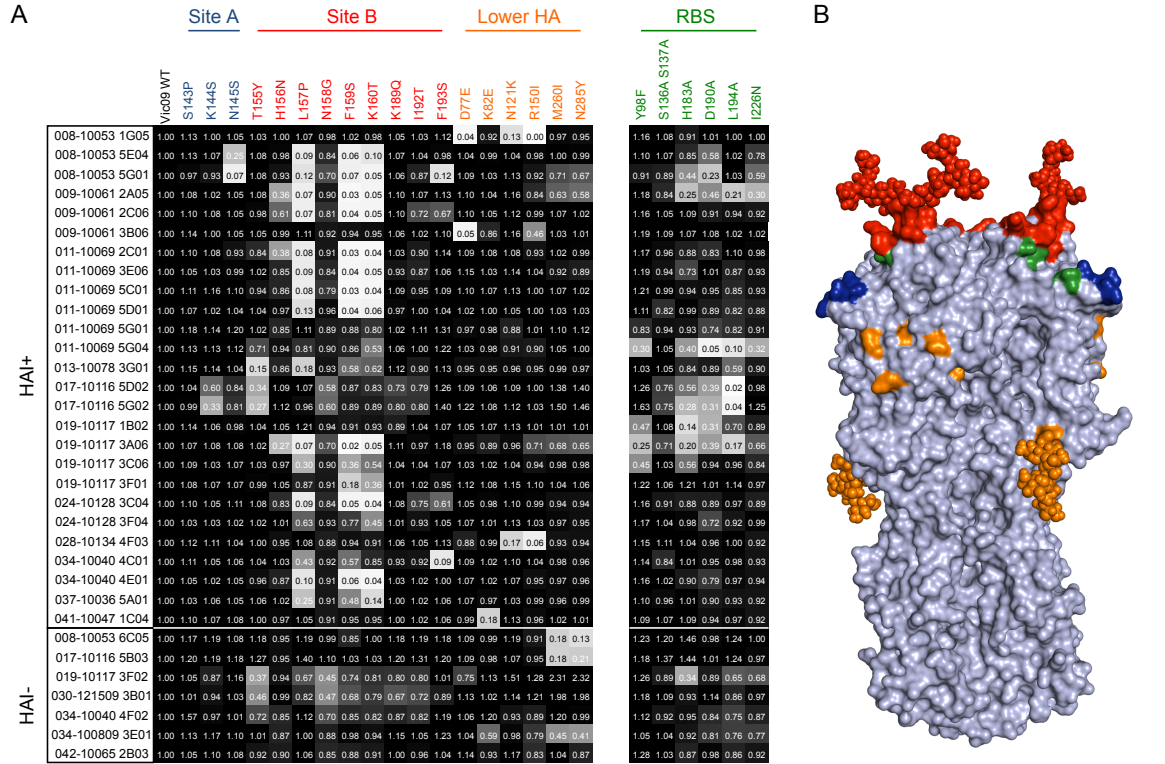
	1968	1974	1985	1994	2005	2009	2012	H1
008-10053 1G05	0.09	0.02	1.32	0.75	0.72	1.00	1.02	0.00
008-10053 5E04	0.01	0.00	1.10	0.58	0.72	1.00	0.97	0.00
008-10053 5G01	0.03	0.02	1.14	0.64	0.78	1.00	0.94	0.01
009-10061 2A05	0.02	0.01	0.02	0.41	0.53	1.00	0.96	0.00
009-10061 2C06	0.01	0.00	0.02	0.00	0.77	1.00	0.95	0.00
009-10061 3B06	0.02	0.00	0.15	0.23	0.70	1.00	0.89	0.01
011-10069 2C01	0.02	0.01	0.03	0.00	0.72	1.00	0.94	0.01
011-10069 3E06	0.02	0.00	0.03	0.00	0.73	1.00	0.95	0.00
011-10069 5C01	0.01	0.00	0.02	0.00	0.79	1.00	0.98	0.00
011-10069 5D01	0.03	0.01	0.04	0.01	0.82	1.00	1.01	0.01
011-10069 5G01	0.00	0.03	0.01	0.00	0.82	1.00	0.87	-0.01
011-10069 5G04	0.03	0.02	0.03	0.01	0.82	1.00	0.94	0.00
013-10078 3G01	0.07	0.04	0.08	0.02	0.86	1.00	0.93	0.04
017-10116 5D02	1.04	0.01	0.03	0.01	0.41	1.00	0.36	0.01
017-10116 5G02	0.73	0.00	0.03	0.00	0.08	1.00	0.22	0.01
019-10117 1B02	0.03	0.01	0.02	0.29	0.72	1.00	0.96	0.00
019-10117 3A06	0.01	0.00	0.02	0.11	0.76	1.00	0.95	0.00
019-10117 3C06	0.25	0.29	1.18	0.69	0.78	1.00	0.95	0.01
019-10117 3F01	0.03	0.02	0.05	0.22	0.78	1.00	0.98	0.00
024-10128 3C04	0.02	0.00	0.03	0.00	0.77	1.00	0.95	0.01
024-10128 3F04	0.02	0.01	0.04	0.39	0.74	1.00	1.00	0.00
028-10134 4F03	0.85	0.71	1.44	0.69	0.73	1.00	1.06	0.02
034-10040 4C01	0.02	0.01	0.03	0.01	0.83	1.00	0.98	0.01
034-10040 4E01	0.02	0.01	0.02	0.00	0.77	1.00	0.96	0.00
037-10036 5A01	0.02	0.01	0.04	0.00	0.87	1.00	1.00	0.01
041-10047 1C04	0.02	0.01	0.75	0.70	0.75	1.00	0.99	0.01
008-10053 6C05	0.55	0.64	1.60	0.24	0.27	1.00	0.90	0.00
017-10116 5B03	0.74	0.71	1.38	0.44	0.42	1.00	0.92	0.00
019-10117 3F02	0.96	0.87	0.93	0.70	0.92	1.00	1.13	0.61
030-121509 3B01	0.88	0.87	1.31	0.91	1.20	1.00	1.02	1.14
034-10040 4F02	1.13	1.11	1.47	0.69	0.88	1.00	0.02	0.01
034-100809 3E01	0.85	0.89	1.47	0.77	0.95	1.00	0.51	0.00
042-10065 2B03	0.98	0.90	1.33	0.64	0.77	1.00	0.96	0.00

**Figure 4-2: mAb binding to seasonal viruses**

mAb binding to a panel of seasonal H3N2 viruses spanning a wide range of H3N2 circulation (1968-2012). Numbers in squares are fraction of binding relative to Vic/09 WT. Colors of each square range from white (0% of WT binding) to black (100% of WT binding). H1:

A/California/07/2009 H1N1 strain

**Figure 4-3**



**Figure 4-3: Antigenic fine-mapping of mAb binding footprints**

**(A)** An antigenic map of residues affecting the binding of mAbs as measured by ELISAs using VLPs bearing WT and point mutant HAs. Numbers in squares are fraction of binding relative to Vic/09 WT. Colors of each square range from white (0% of WT binding) to black (100% of WT binding). Binding values are the average of two independent experiments. **(B)** Locations of substitutions in HA point mutant panel shown on the H3 structure. K160T results in the addition of a glycan at N158 (shown in red), while N285Y results in the loss of a glycan (shown in orange).

Figure 4-4

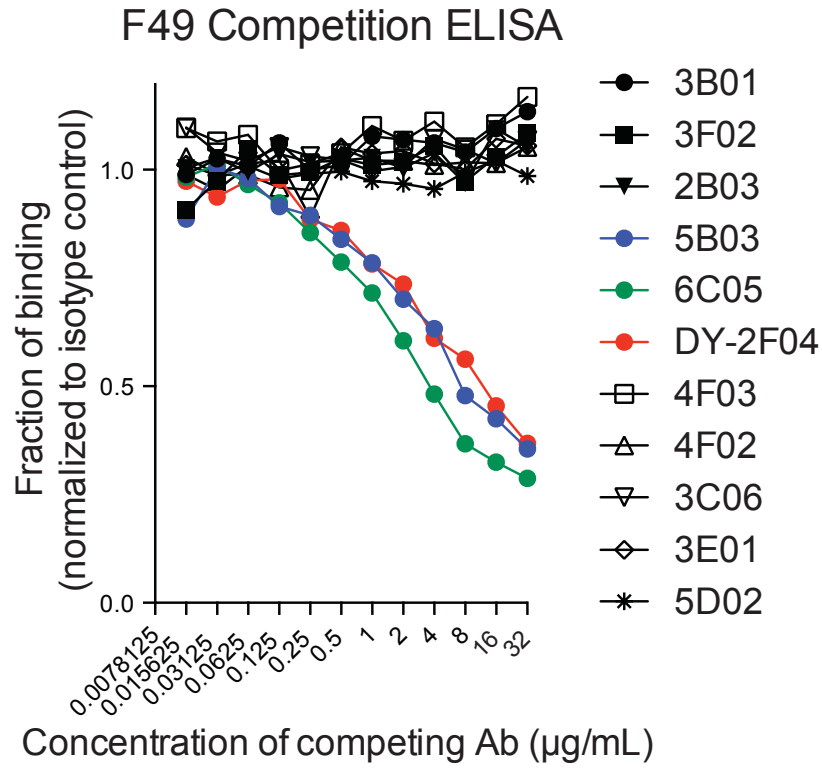
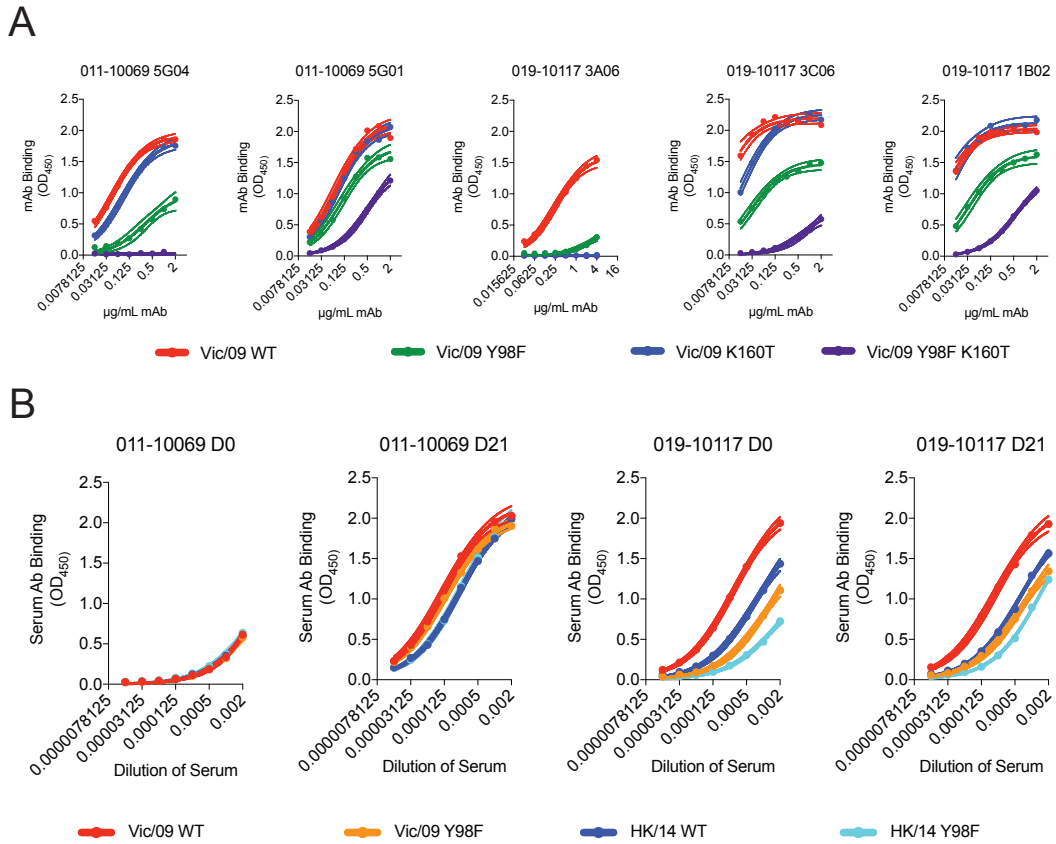


Figure 4-4: Some mAbs compete with H3-stalk mAb for binding

Competition ELISA measuring binding of human mAbs in the presence of increasing concentrations of F49, a mouse mAb that recognizes the H3 stalk. Binding is normalized to an isotype control (C179, an H1-specific mouse mAb that binds the H1 stalk but does not bind the H3 stalk). Antibodies that compete with F49 are 008-10053 6C05 (green) and 017-10116 5B03 (blue). F49, a previously described H3-stalk mAb, serves as a positive control (red).

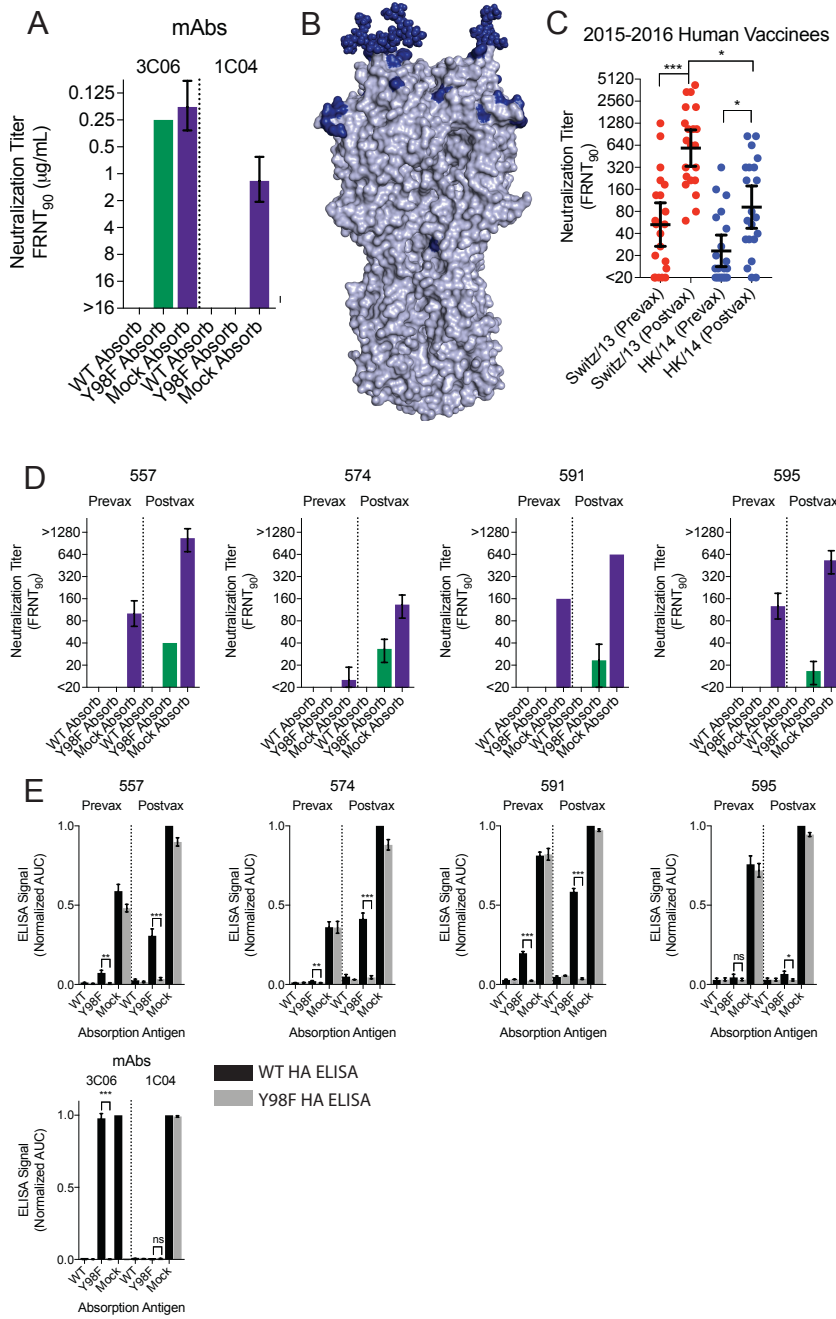
**Figure 4-5**



**Figure 4-5: RBS mAbs reveal convergent targeting of the RBS in two Individuals**

**(A)** RBS mAb reactivity to Vic/09 HA VLPs with Y98F, K160T, or the double mutant Y98F K160T. ELISA binding curves from experimental triplicates are shown. Dashed lines represent the 95% confidence interval for each curve. mAbs were derived from two different donors, 011-10069 and 019-10117. **(B)** Serum antibody binding from donors 011-10069 and 019-10117 at day 0 or day 21 following vaccination. Serum antibodies from 019-10117 exhibit reduced binding to Vic/09 Y98F HA and HK/14Y98F HA both before and after vaccination.

**Figure 4-6**



**Figure 4-6: RBS antibodies contribute to neutralizing titers against an antigenically-mismatched strain**

**(A)** Absorption with HK/14 WT HA depletes both RBS mAb 019-10117 3C06 and lower HA head



mAb 041-10047 1C04, while absorption with HK/14 Y98F HA does not deplete 019-10117 3C06. Titers shown are the geometric mean +/- geometric SD of three independent experiments. **(B)** Residue differences between 2015-2016 vaccine strain Switz/13 and circulating strain HK/14 shown in blue on the H3 structure. **(C)** Prevacination and postvaccination serum neutralization titers against Switz/13 and HK/14 from individuals vaccinated with the 2015-2016 vaccine. Titers shown are the geometric mean of three independent experiments. Black lines indicate geometric mean +/- 95% CI for each group. **(D)** Neutralization titers against HK/14 WT and **(E)** ELISA titers against HK/14 WT and HK/14 Y98F of serum samples from individuals after absorption with HK/14 WT, HK/14 Y98F, or cells only (mock). Titers shown in each instance are the geometric mean +/- the geometric SD from three independent experiments. ELISA data is area under the curve (AUC) for each condition normalized to postvaccination binding to HK/14 WT HA. Comparisons between WT and Y98F binding for the Y98F absorption condition were done using an unpaired Student's t-test (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns = non-significant).

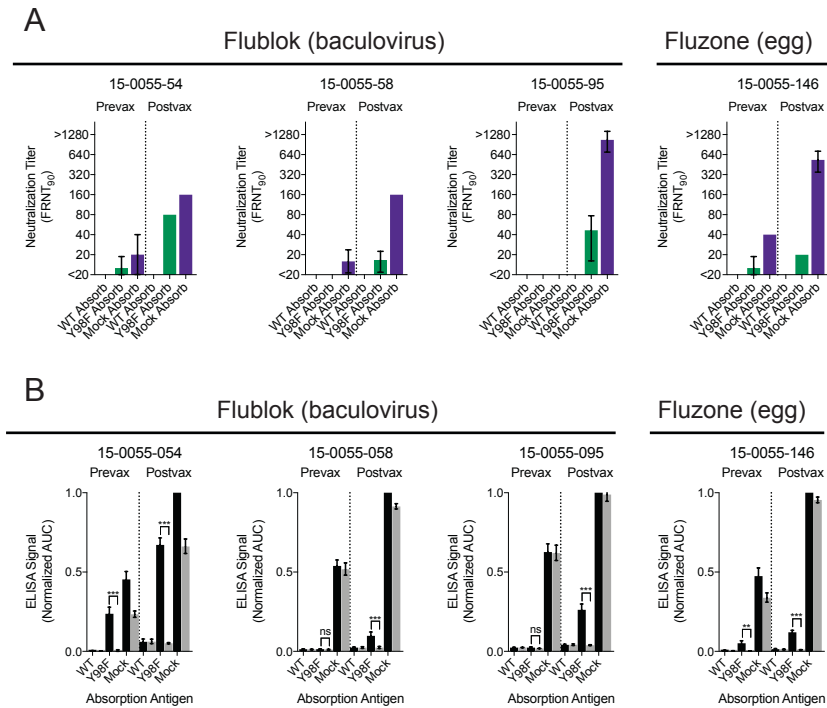
**Figure 4-7**



**Figure 4-7: The egg-adaptive H3 RBS substitution L194P affects the binding of a large number of mAbs**

mAb binding to Vic/09 WT or L194P VLPs. Binding is normalized to Vic/09 WT, and the value in each square denotes fraction of binding relative to Vic/09 WT. Colors of each square range from white (0% of WT binding) to black (100% of WT binding).

**Figure 4-8**



**Figure 4-8: RBS antibody levels in individuals who received different types of vaccines**

**(A)** Neutralization titers against HK/14 WT and **(B)** ELISA titers against HK/14 WT and HK/14 Y98F of prevaccination and postvaccination serum samples from three Flublok vaccinees and one Fluzone vaccinee following absorption with HK/14 WT, HK/14 Y98F, or cells only (mock).

Titers shown in each instance are the geometric mean +/- the geometric SD from three independent experiments. ELISA data is area under the curve (AUC) for each condition

normalized to postvaccination binding to HK/14 WT HA. Comparisons between WT and Y98F

binding for the Y98F absorption condition were done using an unpaired Student's t-test (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns = non-significant).

## CHAPTER 5: FUTURE DIRECTIONS AND CONCLUDING REMARKS

Parts of this chapter have been submitted for publication:

**Seth J. Zost\***, Nicholas C. Wu\*, Scott E. Hensley#, and Ian A. Wilson#. Immunodominance and antigenic variation of influenza virus hemagglutinin: implications for design of universal vaccine immunogens

(\* = equal contribution)

(# = co-corresponding author)

Improving influenza vaccine strain selection and viral surveillance requires a better understanding of how human antibody responses are shaped by prior exposure, how these antibody responses exert a selective pressure on influenza viruses, and the process by which influenza viruses escape human immunity. In the work presented here, I have explored the epitopes targeted in antibody responses of human individuals to influenza viruses and have defined the antigenic consequences of recent influenza HA substitutions that facilitate viral immune evasion. In this section, I will describe immediate implications of our work for improving viral surveillance and vaccine production as well as outline unresolved questions in the field that present a challenge to our efforts to understand the complex interplay of human immunity and influenza virus evolution. Addressing these questions will be an essential component of efforts to develop more effective universal influenza vaccines.

### **Practical ramifications**

In Chapter 2, we identified and characterized neutralizing antibody responses against a pandemic H1N1 HA epitope that mutated during the 2013-2014 influenza season. Importantly, we showed that many middle-aged individuals had an antibody response focused against this epitope, and that the K166Q substitution that arose during the 2013-2014 influenza season dramatically affected the ability of antibodies in these individuals to bind and neutralize

antigenically drifted viruses containing this substitution. In subsequent work, our lab has demonstrated that individuals with this K166 antibody specificity were more susceptible to infection during the 2013-2014 influenza season (Petrie et al., 2016), indicating that the K166Q substitution rendered some individuals susceptible to infection during this season.

Our findings in this study raise issues with our current “one size fits all” approach to influenza vaccination. For some individuals during the 2013-2014 influenza season, the vaccine strain elicited protective antibody responses against circulating H1N1 strains. However, for individuals with an antibody response focused against K166Q, the antibodies recalled by vaccination targeted this epitope that was mutated in circulating strains. As a result, the consequences of the antigenic mismatch between the H1N1 vaccine strain and circulating H1N1 strains depended on prior exposure history. Viral surveillance using primary ferret antisera missed this antigenic change. In addition, pooling human serum samples for the purposes of standardizing reference serum would also have likely missed this antigenic change. Fortunately, in 2016 the WHO updated the H1N1 strain in the vaccine, citing the reduced ability of sera from some middle-aged adults to inhibit agglutination of circulating strains. Our work and the work of others suggest public health agencies conducting viral surveillance should rely less heavily on ferret reference antisera and incorporate more human serological studies to identify escape from population immunity. In addition, identifying the epitopes targeted by individuals in the human population may allow us to identify sites of immune pressure that might be the site of a future antigenic substitution before substitutions in those sites begin to circulate.

In Chapter 3, we characterized the antigenic consequences of egg-adaptive substitutions in H3N2 HA that occurred during the process of vaccine strain propagation. One of these substitutions resulted in the loss of a glycosylation site that arose in circulating H3N2 strains during the 2014-2015 influenza season. We showed that while this glycosylation abrogated the binding of pre-existing antibodies in a large number of individuals, the HA in our egg-based vaccines lacked this glycosylation due to culture-adaptive substitutions. A vaccine produced in a

baculovirus system that lacked egg-adaptive substitutions and had this glycosylation motif more effectively induced neutralizing antibody responses against glycosylated circulating strains.

In addition, we demonstrated with collaborators that a L194P egg-adaptive substitution present in many of our vaccine strains has a large antigenic effect on the H3 RBS and antigenic site B. This is due to this substitution altering the conformational flexibility of sites near the RBS (Wu et al., 2017). These findings highlight shortcomings with our current process of vaccine production, and add to a growing body of evidence that egg-adaptive substitutions alter the antigenicity of our vaccines in key epitopes that mediate protection. Antigenic mismatches due to egg-adaptation likely compromise the effectiveness of our vaccines. Importantly, we now have licensed vaccine platforms that use MDCK cell culture and baculovirus recombinant protein expression. These platforms can produce HAs that are better matched with circulating influenza strains. Importantly, genetic vaccines that deliver DNA or RNA into cells are able to generate HA proteins that would more faithfully recapitulate circulating strains by avoiding egg-adaptive substitutions (Pardi et al., 2018). While the development of more effective universal vaccine immunogens is an important goal, existing vaccines that lack culture-adaptive substitutions could boost vaccine effectiveness if they were more widely implemented.

In Chapter 4, we identified and characterized antibody responses targeting conserved residues of the H3 RBS, and demonstrated that in some individuals these antibodies constitute a large fraction of the neutralizing antibody response against circulating strains. Importantly, RBS antibodies elicited by vaccination with an antigenically mismatched vaccine strain were capable of neutralizing strains with major antigenic differences. Importantly, these RBS antibody responses were relatively rare, occurring in approximately 10% of individuals. However, the ability of RBS-directed responses to cross-neutralize antigenically drifted strains suggests that for some individuals, RBS antibodies make a valuable contribution to protection.

## The role of functional monoclonality of antibody responses in influenza virus evolution

A common theme across our work presented here is that the neutralizing antibody responses of some individuals are highly focused against single epitopes, and that single antigenic substitutions can almost completely abrogate the neutralizing antibody titers of a large number of individuals. In essence, the neutralizing antibody responses of some individuals are not that different from a monoclonal antibody. In the case of responses against the 2009 pandemic H1N1, we have demonstrated that prior immunity shapes the response to infection and can lead to antibody responses that are highly monoclonal. We have also show that this functional monoclonality has consequences, as individuals who made a focused antibody response against H1N1 HA were more likely to be infected when an antigenic drift variant dominated circulation (Linderman et al., 2014; Petrie et al., 2016). In the case of responses against H3N2 viruses, we have demonstrated that the neutralizing antibody response of most humans is directed against antigenic site B. As a result, substitutions near this antigenic site render many individuals susceptible to infection with antigenically drifted strains. However, we have also identified individuals who make a more cross-reactive antibody response to H3N2 viruses that is primarily directed against a single residue in the H3 RBS.

The degree to which human antibody repertoires are functionally monoclonal has important ramifications for how antigenic drift variants might be selected during evolution of circulating viruses. We have known since the 1970s that while it is fairly easy for influenza viruses to escape a single monoclonal antibody *in vitro*, it is much harder for the virus to escape mixtures of monoclonal antibodies targeting different antigenic sites, as this would require viral antigenic variants to have simultaneous substitutions in more than one antigenic site, which is highly improbable (Yewdell et al., 1979). Based on this observation, polyclonal neutralizing antibody responses that contain high levels of antibodies specific for different antigenic sites should pose a daunting challenge for viral escape. it has been demonstrated in a mouse model that sub-saturating polyclonal neutralizing antibody responses present in sera select for HA substitutions

that increase receptor-binding avidity, allowing the virus to escape a neutralizing antibody response (Hensley et al., 2009). This also supports previous observations that influenza escape from antibody pressure is context dependent – while single antibodies can readily select for antigenic changes *in vitro* (Yewdell et al., 1979), mixtures of monoclonal antibodies select for absorptive substitutions that increase receptor-binding avidity, permitting viral infection in the presence of subsaturating concentrations of neutralizing antibodies (Yewdell et al., 1986). While this modulation of receptor avidity may be a mechanism that drives the acquisition of antigenic drift substitutions, it is clear that polyclonal sera from many individuals is sufficiently monoclonal to select antigenic drift variants *in vitro* (Davis et al., 2018; DeDiego et al., 2016; Li et al., 2016). Given that single antigenic substitutions can have large antigenic effects for many individuals, functional monoclonality may considerably simplify the problem of evading polyclonal antibody responses, as acquisition of a single antigenic substitution may render a large number of individuals susceptible to reinfection with circulating strains. In addition, these individuals with highly monoclonal responses may serve to select antigenic drift variants. It is possible to imagine these individuals playing a major role in facilitating escape from population immunity as a whole through sequential selection of antigenic substitutions.

What are possible explanations for this observed functional monoclonality? Our work with antibody responses against 2009 H1N1 demonstrates that prior immune history can focus antibody responses against epitopes conserved between strains seen in early childhood. On the other hand, primary antibody responses in both animal models and humans also exhibit functional monoclonality (Koel et al., 2013; Koel et al., 2015). In addition, antigenic site B of H3N2 HA seems to be the major target of neutralizing antibodies across a wider age range. One reason for observed monoclonality in H3N2 antibody responses may be the glycosylation status of the HA. Unlike the recently introduced HA of pandemic H1N1, H3N2 HA has progressively acquired multiple glycosylation sites since entering human circulation in 1968 (Tate et al., 2014). As a result, less surface area is available for antibody recognition on the H3 head domain, which may focus antibody responses to sites adjacent to the RBS. This may in part explain why the antigenic



site B glycosylation had such a dramatic effect on the binding and neutralization of pre-existing antibodies. Changes in glycosylation status may also shift immunodominance to other epitopes, and the effects of the N158 glycosylation on the immunodominance of antigenic site B in recent H3N2 viruses is under further investigation by members of our lab. Another potential factor may be the location of epitopes on the HA – if an antibody binds the top of HA, it might more potently inhibit attachment than an antibody that recognizes a site lower on HA. As a result, antibodies that bind residues near the RBS may play an outsize role in preventing viral attachment, and this may help explain the dramatic impact that substitutions near the RBS have on pre-existing neutralizing antibody titers.

### **The role of non-neutralizing antibody specificities in protection**

Our work has demonstrated that many individuals mount functionally monoclonal antibody responses against influenza virus HA at the level of neutralization. However, not all anti-HA antibodies are neutralizing *in vitro*. Although non-neutralizing antibody specificities are not explored in great detail in this work, in Chapter 4 we do describe monoclonal antibodies that have no neutralizing activity *in vitro*, yet are highly cross-reactive. For the most part, the role of non-neutralizing antibodies in protection from influenza virus infection remains unexplored. Some groups have described non-neutralizing antibodies that cross-react between group 1 and group 2 HAs. Cryo-electron microscopy reveals that some of these antibodies bind an epitope that requires an HA monomer to be displaced from the other monomers, suggesting that these antibodies recognize an alternative conformation of HA and bind to a cryptic epitope that may only be transiently exposed (Raymond et al., 2016). These antibodies appear to be common in human antibody repertoires. Although antibodies protected mice by passive transfer, the mechanism of protection is not immediately clear, and could be Fc-mediated. It is worth noting that despite their prevalence, these non-neutralizing antibody specificities are not measured by any of our existing assays used to select vaccine strains.

It is especially important to define the role of prior exposure in the development of non-neutralizing antibody specificities. Our work here has defined particular exposure histories that lead to antibody repertoires directed against sites conserved between strains. While the epitopes we describe are neutralizing, it is also possible to imagine prior exposure shifting the response to non-neutralizing epitopes lower on the HA. If these non-neutralizing antibodies are not protective or are less protective than antibodies that block viral attachment and neutralize *in vitro*, it is possible to imagine that some individuals may have a considerable fraction of their antibody response directed against non-neutralizing epitopes. If antibody responses become skewed towards non-neutralizing epitopes with repeated exposure, this could undermine protection in older individuals who have been repeatedly exposed to influenza virus, and could complicate efforts to use constructs such as chimeric HAs to refocus immunity towards protective epitopes in the HA stalk. Addressing the levels of non-neutralizing antibodies in human cohorts will answer important questions about the role, if any, that these antibodies play in protection.

### **The role of compensatory substitutions in influenza virus evolution**

Our work identifies HA substitutions that have major antigenic consequences. The impact of these substitutions on viral fitness, however, is less clear. Given that fitness costs associated with fixation of antigenic substitutions is a major constraint on influenza A viral evolution, it is likely that other HA substitutions play critical roles in viral evolution by offsetting these fitness costs. Although an antigenic change may allow the virus to escape antibody responses, HA antigenic changes are known to often alter avidity for sialic acid, which can adversely affect viral replication and the mutant virus's ability to spread. For example, the new glycosylation site in H3N2 HA results in the loss of the ability to agglutinate red blood cells, indicating that the introduction of this glycosylation motif close to the RBS impacts receptor binding avidity (Chambers et al., 2015). This alteration in receptor binding likely affects viral fitness because introducing this glycosylation into a previous vaccine strain resulted in a virus that could not be rescued, indicating that other substitutions likely played a compensatory role in the fixation of this

substitution in circulating strains (Chambers et al., 2015). These compensatory substitutions can facilitate antigenic drift by rescuing fitness defects (Hensley et al., 2009). In a striking example, compensatory substitutions offset the fitness cost of a substitution that confers resistance to oseltamivir, a commonly used antiviral (Bloom et al., 2010). This phenomenon of combinations of substitutions having a greater effect on fitness than each individual substitution is known as epistasis. In recent work, substitutions near the H3 RBS were demonstrated to be involved in complex epistatic networks, limiting the possible substitutions at key positions in the RBS (Wu et al., 2018).

Epistatic substitutions can also be antigenic substitutions themselves – work from others in our group using an H1N1 strain demonstrated that an HA substitution abrogated antibody binding but drastically reduced receptor avidity and viral fitness, while substitutions in a different antigenic site rescued the fitness defect and also prevented the binding of antibodies specific for the antigenic site in which they were located (Myers et al., 2013). In another example, Kryazhimskiy and colleagues used historical sequence data from viral surveillance to determine which substitutions were tightly associated, because epistatic substitutions undergoing positive selection are likely to be chronologically linked (Kryazhimskiy et al., 2011). The authors identified many pairs of possible epistatic substitutions in HA. many of these epistatic pairs were located in different antigenic sites, supporting the hypothesis that compensatory substitutions may also alter antigenic properties of influenza viruses. It is possible that many of these putative compensatory substitutions also abrogate the binding of antibodies.

While computational analysis is a powerful tool to identify possible compensatory substitutions, there are few experimentally characterized examples of epistasis in influenza virus evolution in the literature. Part of the difficulty in defining the role of compensatory substitutions in influenza virus evolution is the staggering scale of potential epistatic partners. For example, if there are ten substitutions that become fixed in HA, one of them might have an antigenic effect, but which of the other nine may play some compensatory role? Experimentally validating all

potential pairs of epistatic interactions by mutagenesis would be costly and time-consuming. In addition, which functional assays would be able to detect and quantify a change in viral fitness? In addition, NA substitutions can compensate for functional changes in HA, and vice versa (Hensley et al., 2011). While information on epistatic interactions would be valuable for predicting viral evolution, the practical challenge of conducting systematic and comprehensive experiments has proven daunting.

Fortunately, advances in molecular biology techniques and the advent of deep sequencing have enabled the study of the effects of individual substitutions en masse. These experiments, known as deep mutational scans, can measure the effect of essentially every possible HA amino acid substitution on viral replication, and deep mutational scanning of HA has shed light on the mutational tolerance of different sites on HA (Doud and Bloom, 2016; Thyagarajan and Bloom, 2014; Wu et al., 2014). Comparing these deep mutational scanning datasets can provide valuable insights into epistatic relationships; recent work has demonstrated different degrees of mutational tolerance at different sites in H1 and H3 HAs, including differences in the highly conserved stalk domains of these HAs (Lee et al., 2018). Interestingly, some residues had strong differences in the preference of amino acids at particular sites, suggesting that the mutational constraints differ between H1 and H3 HAs. This finding demonstrates that the evolutionary divergence between H1 and H3 HAs has resulted in different constraints on the evolution of these two HA subtypes. This information can be used to help predict the evolutionary fates of circulating strains (Lee et al., 2018). In parallel, other researchers are developing the theoretical frameworks and computational tools to infer epistatic interactions from the burgeoning sets of deep mutational scanning data (Otwinowski, 2018; Otwinowski et al., 2018). While in this work we have helped define selective pressures the human antibody response imposes on H3N2 viruses and have identified several antigenic substitutions that facilitate viral escape, further work on epistasis in influenza virus antigenic drift using the new approaches discussed here will better illuminate the sequence of mutational steps that influenza viruses need to undergo in order to successfully evade human immunity.

## **Towards a deeper understanding of human immune responses and antibody breadth**

A central challenge of universal vaccine efforts is to steer human antibody responses away from immunodominant, variable epitopes and towards subdominant, functionally conserved sites. Overcoming this challenge will require further understanding of the structural basis of broadly neutralizing HA and NA antibody binding epitopes and factors that influence immunodominance hierarchies of human antibody responses. Our work here and the work of others has shown that there are many similarities and many differences in the antibody responses to influenza virus from individual to individual, but we are by no means close to a comprehensive understanding of the complexities of human antibody responses against influenza viruses, and we will need fundamentally different approaches, study cohorts, and immunological tools to fill in the gaps in our knowledge.

In the case of HIV, a great deal has been learned about the development of broadly neutralizing antibody responses by studying antibody-virus co-evolution from the time of infection (Bonsignori et al., 2017; Landais et al., 2017; Rantalainen et al., 2018). The analogous situation in influenza is more challenging, as it requires following individuals from birth in longitudinal studies and defining how immunodominance changes over the course of a response and from response to response. These longitudinal cohort studies have the potential to answer fundamental questions about what antibody specificities dominate the plasmablast response versus B-cell memory and which lineages are recalled in the response to an antigenically drifted strain. Recent work in mice with a model antigen suggests that the responses of some individuals may skew towards a particular memory B-cell population as a result of high-avidity interactions of naïve B-cells with antigen (Pape et al., 2018). Studying the development of RBS-targeted antibody responses in these cohorts could give insight into strategies to selectively elicit these types of antibody responses in a larger number of individuals.

Importantly, these cohort studies will also allow us to explore differences in responses elicited by infection and vaccination. Some studies suggest that B-cells recalled in response to vaccination have a reduced ability to undergo somatic mutation relative to those recalled by an infection (Ellebedy et al., 2016). While titers elicited by vaccination in adults exhibit modest waning (Petrie et al., 2015), we know very little about the longevity of responses elicited in children. Cohorts that enroll individuals at birth will allow us to document the first exposures to influenza viruses in unprecedented detail. These studies will provide invaluable insight into immune imprinting, and can address important questions regarding the differences in the responses to infection and vaccination. Although the focus of the work presented here has been antibodies targeting HA, there is increasing evidence that NA antibodies can also play roles in protection from infection (Memoli et al., 2016). In some individuals, NA antibodies outnumber HA antibodies in the response to influenza virus infection, but our existing vaccines poorly elicit NA antibodies (Chen et al., 2018). Studying responses against multiple viral antigens in these cohorts will allow us to assemble a more holistic picture of influenza virus immunity, and could point the way towards using multiple immunogens to elicit protective titers against more than one viral antigen.

### **Defining new correlates of protection from infection**

Another major challenge will be to develop standardized assays to detect antibodies against different HA and NA epitopes. The standard assays used by global public health and viral surveillance agencies to select vaccine strains, HAI and microneutralization, almost exclusively detect antibodies that bind the HA head and block viral attachment. While these antibodies undoubtedly contribute to protection, antibodies against the stalk domain or other epitopes will not be detected using these assays. As a result, new assays to measure antibody functions such as NA inhibition and Fc-mediated effector engagement need to be developed and standardized. Dissecting the contribution of different epitopes to protection in universal vaccine trials will allow us to precisely determine which epitopes are targeted in different individuals and whether viral

escape is occurring at particular epitopes. It is worth noting that defining the role of immune correlates in protection from infection will require large cohorts, as teasing out the role of different antibody specificities targeting different sites will require considerable statistical power.

In addition, we should expand our understanding of protective correlates beyond simply studying serum antibody titers. Analyzing the antibody repertoire present at the anatomic sites relevant to influenza virus infection is challenging. While antibody titers present at mucosal surfaces in the nose, throat, and lung likely determine protection, these sites are difficult to study due to the need for invasive procedures to collect samples. As a result, the concentration and functionality of serum antibodies is the only antibody-mediated correlate of protection that has been rigorously investigated, and in particular, the IgG isotype has been the focus of most studies. IgG does play an important role in protection from infection and serum IgG likely reflects the levels of IgG present at mucosal surfaces in the lung, but it is highly likely that there are understudied aspects of antibody-mediated mucosal immunity and nuances of isotype specificity that remain to be explored (Chiu and Openshaw, 2015). For example, antibody isotypes such as multimeric IgA are predominantly localized to mucosal surfaces in the nose and lung, and anti-stalk IgA antibodies have been shown to be significantly more potent neutralizing antibodies *in vitro* (He et al., 2015). In addition, secondary lymphoid structures referred to as bronchus-associated lymphoid tissue can contain B-cells and other immune cells, and have been shown to protect mice from infection in the absence of peripheral lymphoid organs (Moyron-Quiroz et al., 2004). It is likely that our existing vaccines, particularly our live-attenuated versus inactivated vaccines, differ in their ability to induce mucosal immunity (Chiu and Openshaw, 2015). It is also important to note that mucosal immunity may be greatly influenced by immune imprinting, as the first infection likely influences the establishing of long-lived tissue-resident memory responses. Successful universal vaccine candidates may need to induce potent mucosal immune responses for protective responses. Recent work in herpes virus immunity used chemokines to establish protective tissue-resident T-cell memory, highlighting that manipulation of mucosal-resident immunity can pave the way to more protective vaccines (Shin and Iwasaki, 2012). Due to the

challenges of studying these mucosal responses in humans, some of these questions will be best addressed using animal models such as mice or ferrets.

## **Concluding remarks**

In the one hundred years since the 1918 pandemic, we have made great strides in our understanding of influenza virus biology and antibody-mediated immunity, and we have built a robust public health system to monitor influenza virus circulation and update the strains included in our vaccines. Despite these efforts, eliciting more protective antibody responses in humans with diverse prior exposure histories is still a daunting challenge. As highlighted in this work, this challenge is compounded by shortcomings with our current system of vaccine strain selection and vaccine production. Our current universal vaccine candidates are the product of decades of work across multiple disciplines, and represent the first attempt to control influenza virus immunodominance in order to elicit long-lived, protective responses against conserved sites. Although our current HA stalk-based universal vaccine candidates are promising, the development of a universal vaccine will likely be an iterative process, and a better understanding of the dynamics of immunodominance in humans will be essential for improving our vaccines. Understanding and manipulating the epitopes targeted by human antibody responses will be the crux of continued progress towards universal influenza immunity.



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