

Influenza vaccines and antivirals that target the conserved hemagglutinin stem

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Abstract

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Influenza is a major public health threat, and pandemics, such as the 2009 H1N1 outbreak, are inevitable. Due to low efficacy of seasonal flu vaccines and the increase in drug-resistant strains of influenza viruses, there is a crucial need to develop new antivirals and vaccines to protect from seasonal and pandemic influenza. Recently, several broadly neutralizing antibodies have been characterized that bind to a highly conserved site on the viral hemagglutinin (HA) stem region. These antibodies are protective against a wide range of diverse influenza viruses indicating that the HA stem may be an excellent target for vaccines and antivirals. This thesis describes the development of a novel antiviral that effectively targets the HA stem, examines the ability of a conventional HA-based vaccine to elicit stem-specific antibodies, and explores the potential of stem-based vaccines to induce broadly neutralizing antibodies. Here I show that a small engineered protein computationally designed to bind to the same region of the HA stem as broadly neutralizing antibodies mediated protection against diverse strains of influenza in mice by a distinct mechanism that is independent of a host immune response. Since an antiviral targeting the conserved stem results in broad protection, I next investigated immunogenicity and protective efficacy of an *E. coli* heat-labile enterotoxin (LT) adjuvanted multigenic (LT-MA) universal influenza DNA vaccine consisting of four HA

antigens, nucleoprotein (NP), and the ectodomain of the matrix protein (M2e) in nonhuman primates. Though the LT-MA DNA vaccine induced robust serum and mucosal antibody responses, it failed to induce broadly neutralizing antibodies. These results demonstrate that vaccination with full-length HA immunogen does not elicit stem-specific broadly neutralizing antibodies and further advancements in immunogen design are needed. In order to overcome this hurdle, I investigated a computationally designed DNA vaccine, based on the conserved HA stem, for the ability to induce antibody and T cell responses that provide protection from lethal influenza infection. Although these computationally designed headless HA immunogens were immunogenic and provided protection *in vivo*, they failed to elicit neutralizing antibodies. My results highlight the need for a vaccine immunogen that limits the immune response to the immunodominant head while directing the immune response to the stem and demonstrate that future vaccines will need to maintain the structural integrity of the fusion region in order to be successful. Together, these results have significant implications for the use of computational modeling to design new antivirals and vaccines against influenza and other viral diseases.

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DEDICATION

To my parents, for letting me be different and for always believing in me. To Mike, for being my person and for always showing up. To my grandparents, who taught me to do what you do well and do well whatever you do.

Chapter 1

Introduction

Influenza Virus

Influenza is an enveloped virus, with a segmented negative sense RNA genome. There are three types of influenza viruses, designated A, B, and C, with types A and B playing the major role in human infection [1]. Influenza A viruses infect birds, pigs, and horses, whereas types B and C are found primarily in humans [2, 3]. Human influenza A and B viruses cause seasonal epidemics, whereas influenza C viruses cause a mild respiratory illness and are not thought to cause epidemics. Influenza A viruses are continuously evolving due to mutations in the viral genome, resulting in HA variants that have distinct antigenic properties. These mutations result in antigenic drift, which is responsible for seasonal epidemics that occur with both influenza A and B viruses [1]. Additionally, the segmented genome allows reassortment to occur, resulting in novel viruses that are unrelated to pre-existing strains [4]. These major antigenic shifts result in the introduction of novel antigenic subtypes of the HA in to the population, which can spread rapidly, causing global disease pandemics, as was the case in the 2009 H1N1 “Swine Flu” pandemic [5].

Currently, the H1N1 and H3N2 viruses are the predominant subtypes of influenza A viruses that infect humans. Their relative abundance and importance change year to year, resulting in influenza seasons predominated by one subtype over another, or a mix of both. In the current 2015-2016 influenza season, H3N2 viruses predominated from October to mid-December, whereas H1N1 was the predominate subtype from mid-December to February [6]. Influenza B viruses do not have subtypes based on HA or NA, but are instead broken down into

lineages and strains. Currently circulating influenza B viruses belong to one of two lineages; B/Yamagata and B/Victoria [7].

The envelope of the influenza A virus contains two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which each play essential roles in viral infection (**Fig 1.1a**) [8]. There are eighteen subtypes of HA that share between 40% and 60% amino acid sequence identity and have been clustered in 2 phylogenetic groups: Group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and Group 2 (H3, H4, H7, H10, H14, and H15) (**Fig 1.1b**) [9]. HA is trimeric and responsible for attachment of the virus to sialic acid receptors on the host cell surface (**Fig 1.1c**). Initially, HA is produced as a precursor polypeptide, HA0, which is assembled in to a trimer and transported to the cell surface. HA0 is then cleaved by proteases to form two disulfide-linked subunits, HA1 and HA2. HA1 is the immunodominant highly variable head region responsible for receptor binding, whereas HA2 is subdominant more conserved stem region that undergoes a pH induced conformational change facilitating membrane fusion.

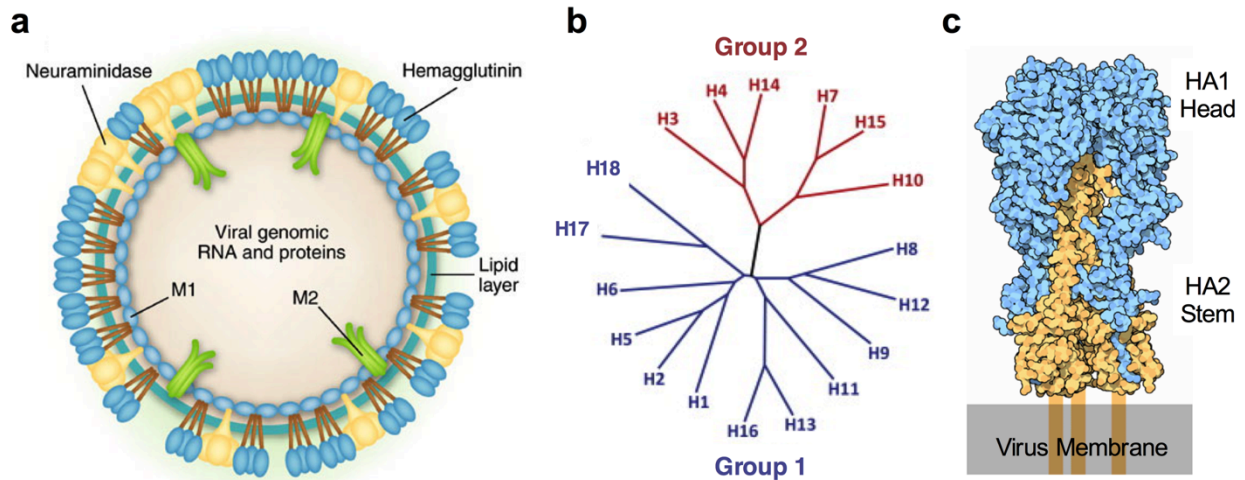


Figure 1.1: Influenza A virus and the main surface glycoprotein hemagglutinin. (a)

Influenza A is an enveloped virus that contains two surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA). The M2 protein forms an ion channel pore in the enveloped membrane. The M1 is a matrix protein that forms a coat inside the viral envelope, encapsulating the viral genome, which consists of 8 segmented negative-sense RNA pieces. (b) Phylogenetic tree of the hemagglutinin genes for all 18 known Influenza A virus subtypes. The HA subtypes are clustered into two phylogenetic groups, Group 1 and 2. (c) The hemagglutinin is a trimeric glycoprotein on the viral envelope. Each hemagglutinin is made up of two segments, the variable immunodominant HA1 Head (blue) and the highly conserved HA2 Stem (Yellow). The HA1 Head contains the Receptor Binding Site where influenza attaches to cells by HA binding to sialic acid. The HA1 region elicits the majority of neutralizing antibodies that block sialic acid binding. The HA2 stem contains the fusion peptide responsible for the low pH induced conformational change. (Figure adapted with permission from Wang et al. and Wu et al. [9, 10])

Infection is initiated by the virus attaching to the host cell via binding of HA with the host cell surface receptor, sialic acid (**Fig 1.2**). The sialic acid linkage to the sugar galactose determines host specificity; either alpha 2,3 in birds or alpha 2,6 in humans [11]. Endocytosis of the virus, followed by acidification of the endocytic vesicles, induces a conformational change in HA which facilitates fusion of the viral envelope with the endosomal membrane and release of the viral genome into the cytoplasm (**Fig 1.3**) [12]. During the low pH induced conformational change, the fusion peptide on the HA stem inserts in to the endosomal membrane (**Fig 1.3b**). Several low pH stems working in concert bring the viral and endosomal membrane in close contact facilitating fusion (**Fig 1.3c**) and the release of the viral genome in to the cytoplasm. The viral RNA is then translocated to the nucleus, where it is transcribed and replicated. The resulting mRNA is transported to the cytoplasm where viral proteins are generated at the ribosome. The viral proteins are then transported to the cell membrane where they aggregate and condense to produce the viral particle. The virus buds from the membrane and is released by neuraminidase activity (**Fig 1.2**).

HA- specific antibodies can neutralize infection by blocking the initial binding of HA to sialic acid or by interfering with the subsequent step of virus-host membrane fusion [13-15]. Antibodies specific to NA can prevent the release of virions from the infected cell surface. During influenza infection or after vaccination, neutralizing antibodies typically develop against epitopes on the HA head (HA1) and do not cross-react with other HA subtypes. Regions of HA1 are easily mutated without loss of function, whereas the conserved epitopes on the HA2 stem region are expected to be less tolerant of mutations [16-19].

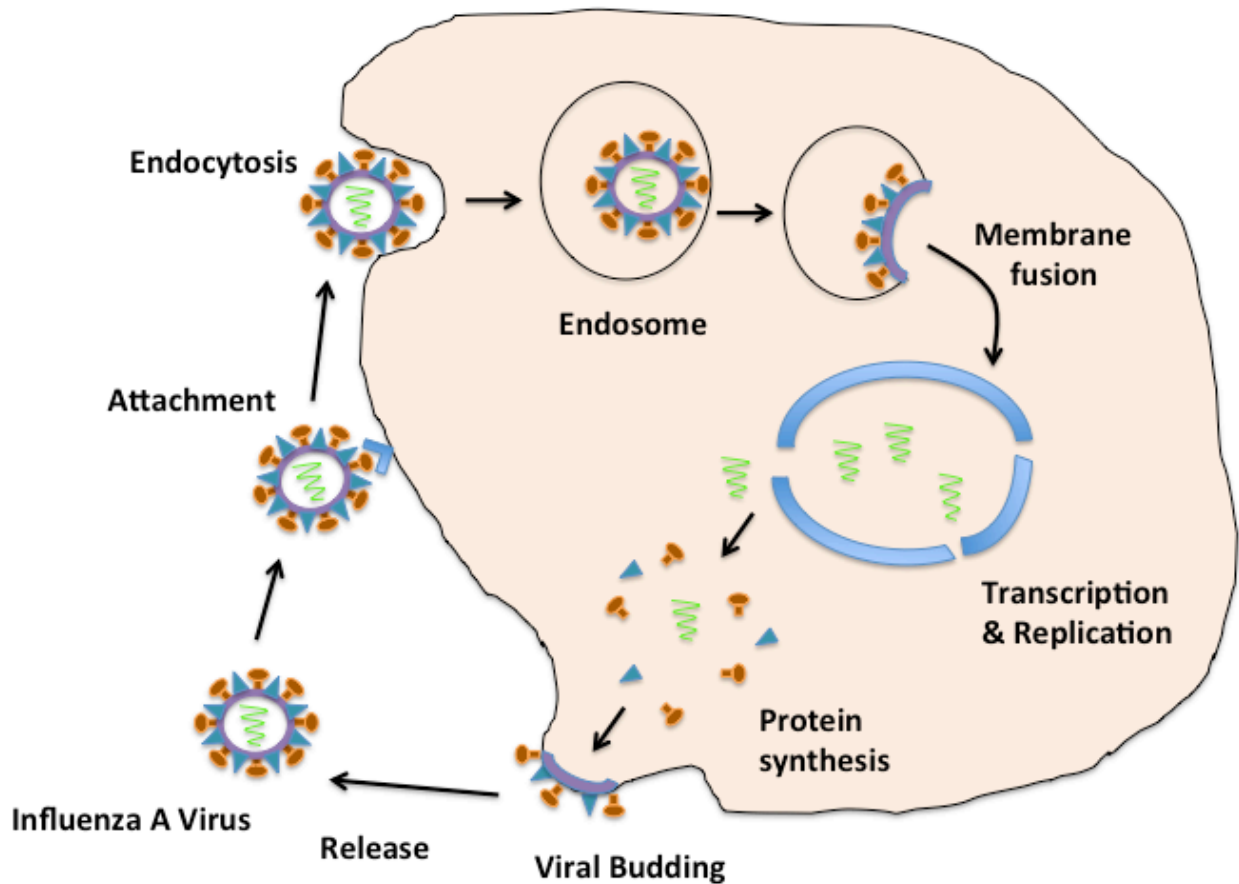


Figure 1.2: Influenza A virus life cycle. Influenza A virus infects epithelial cells by binding of HA to sialic acid on the host cell membrane. Entry is then mediated by endocytosis. In the host cell, acidification occurs in the endosome causing fusion of the viral and endosomal membranes. This fusion enables the release of the segmented viral genome into the cytoplasm. The viral genome is subsequently translocated to the nucleus, where it is transcribed and replicated. Protein synthesis occurs in the cytoplasm, and the resulting viral proteins are assembled at the cell membrane. The newly generated viral particles bud from the cells and are released by NA cleaving the sialic acid receptors to free the virus.

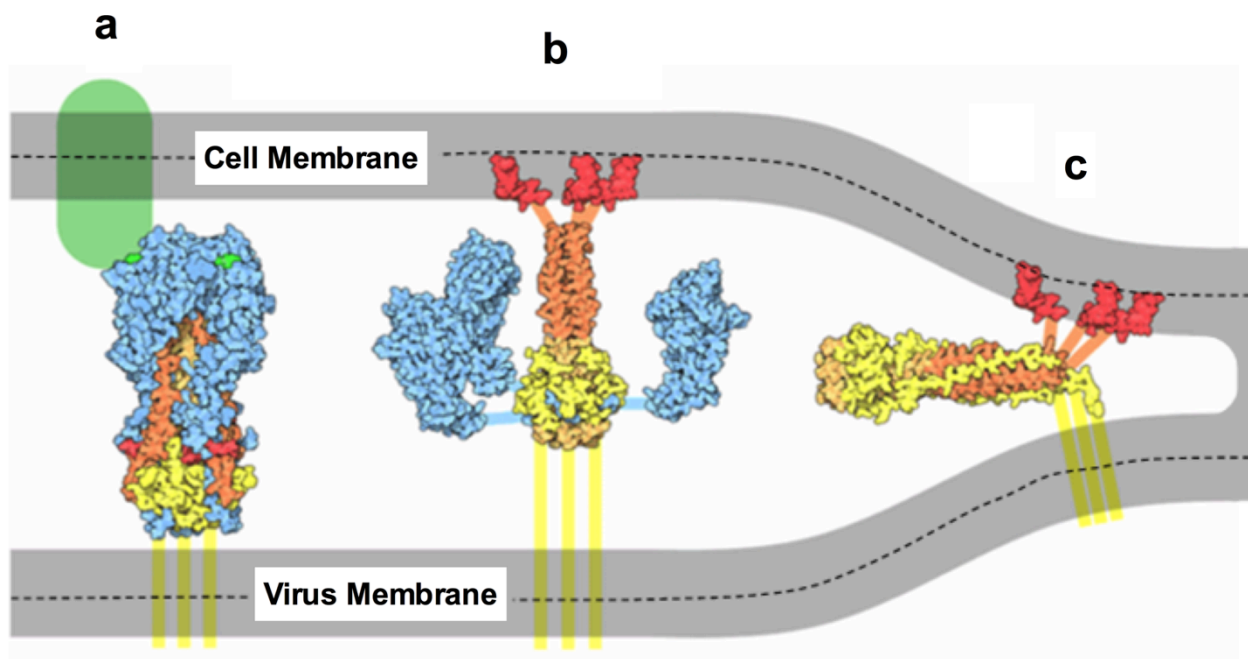


Figure 1.3: HA mediates binding and membrane fusion. (a) Influenza infects cells by first binding the hemagglutinin HA1 (blue) to sialic acid (green) on the cell membrane. (b) The virus is then endocytosed. The low pH in the endosome causes HA2 to undergo a conformational change that drives the fusion peptides (red) into the cell membrane; (c) The HA continues to change conformation and brings the cell and virus membrane together. Multiple HAs working in concert cause the two membranes to fuse together and the viral genome is released in to the cytoplasm. (Figure adapted with permission from Hamilton et al. [20]).

Influenza Virus Transmission and clinical symptoms

Influenza infects between 10-20% of the world's population each year, accounting for up to 200,000 hospitalizations and 36,000 deaths annually in the US, and 3–5 million cases of severe illness and 250,000–500,000 deaths worldwide [21, 22]. Influenza is a respiratory virus that spreads from person to person through large-particle respiratory droplet transmission, often by coughing, sneezing or direct contact of contaminated surfaces [23]. The incubation period for influenza is 1-4 days, with an average of 2 days, and virus can be shed from the day before symptoms start continuing for 5-10 days after symptom onset [24, 25]. Uncomplicated influenza illness is characterized by the abrupt onset of respiratory symptoms including fever, myalgia, headache, malaise, nonproductive cough, sore throat, and rhinitis. The majority of uncomplicated influenza illness typically resolves after 3-7 days, however cough and malaise can persist for >2 weeks [26]. Complicated influenza illness can include primary influenza viral pneumonia that is then exacerbated by an underlying medical condition, sinusitis, or co-infections with other viral or bacterial pathogens [26, 27]. Similarly, influenza can exacerbate pre-existing conditions like asthma and chronic obstructive pulmonary disease (COPD).

Influenza Vaccines

Vaccines are the gold standard for the control and prevention of infectious diseases but a “universal” influenza vaccine that confers broad-spectrum long-term protection remains elusive. Currently the following vaccines are licensed and approved for use in the US; live-attenuated, inactivated, and recombinant. These vaccines are either trivalent or quadrivalent and contain representative influenza strains from H1N1, H3N2, and influenza B virus subtypes that are predicted to circulate during that season. The quadrivalent vaccine contains two influenza A

viruses (H1N1 and H3N2) and two influenza B viruses. These vaccines offer limited protection, with no heterosubtypic breadth in neutralizing antibodies and only the live-attenuated vaccine induces T-cell responses. The seasonal vaccines induce strong HA-specific antibody responses and afford significant protection against matched circulating influenza strains but provide little or no protection against strains that are drifted from the vaccine. Annual reformulations of the vaccine are needed to keep pace with antigenic drift in HA, and a completely new vaccine is needed in the event of antigenic shift [1, 28]. Since the manufacture of these vaccines requires 6-9 months from identification of a new strain to distribution, current vaccines cannot be produced rapidly enough to protect against wide-scale mortality and morbidity that generally occurs within the first 3 months after the emergence of a new pandemic strain.

Immune response to Influenza

Exposure to influenza virus by either natural infection or vaccination initiates a cascade of humoral and cellular immune responses. Initial influenza infection induces a potent innate immune response, which serves to limit the extent of viral replication and virus spread. However, efficient viral clearance requires the activation, recruitment, proliferation, and effector functions by the adaptive immune system. The primary mediator of protection against infection is neutralizing antibodies targeting HA. These antibodies predominantly target the head region, specifically the receptor-binding sites, are strain-specific, and prevent attachment of the virus to host cells. These HA head-specific antibodies are the primary correlate of protection used to assess the efficacy of influenza vaccines [29]. In contrast to the strain-specific head antibodies, antibodies targeting the highly conserved HA stem have been shown to mediate broad protection [30-34]. These stem-directed antibodies have been shown to neutralize the virus by preventing

membrane fusion, but are dependent on Fc and Fc-receptor interactions [35, 36]. In addition to HA, NA specific antibodies also mediate protection by binding to NA and preventing the cleavage of sialic acid receptors and subsequent release of the virus [37, 38]. Furthermore, not all antibodies generated against influenza are neutralizing. Non-neutralizing antibodies can mediate protective functions such as complement-mediated lysis [39], phagocytosis [40], and antibody-dependent cellular cytotoxicity (ADCC) [41, 42].

T cells have also been shown to play an important role in mediating protection against influenza [43, 44]. Influenza specific CD4+ and CD8+ T cells have been shown to reduce viral shedding, limit disease severity, and accelerate viral clearance [45-51]. Importantly, T cells are capable of recognizing epitopes on internal proteins that are highly conserved across different influenza viruses. In humans, prior exposure to seasonal influenza results in memory T cells that cross-react with pandemic strains and correlate with reduced viral shedding and a lower incidence of clinical disease [52-59]. CD4+ T cells play a role in maintaining CD8+ T cell memory response, are capable of direct cytotoxic killing of virus-infected cells, and provide help to B cells for rapid antibody production [60-63]. Furthermore, CD8+ T cells limit pathogenesis by playing a critical role in rapid clearance of influenza virus by direct killing of virus-infected cells [64-67]. As a result, the goal is to design vaccines that induce strong antibody and T cell responses that are effective against a broad range of influenza subtypes and strains.

Influenza Antivirals

There are several antivirals for influenza that have been approved for both prophylaxis and treatment. These antivirals included the use of matrix protein (M2) inhibitors (amantadine and rimantadine), and neuraminidase-inhibitors (oseltamivir, zanamivir, and peramivir). The

FDA first approved the M2-inhibitors in 1994 for treatment and prophylaxis in healthy and immunocompromised patients [68, 69]. Unfortunately, the M2-inhibitors had serious side effects including anxiety, hallucinations, nightmares, confusion, and frequent central nervous system side-effects [70]. Furthermore, their use resulted in rapid emergence of resistance; the CDC determined that adamantane-resistance increased from 0.4% during 1994-95 to 92% during the 2005-06 influenza season [71]. During the 2009 pandemic, the CDC determined that 100% of influenza A viruses isolated from patients were resistant to rimantadine and adamantane. A single amino acid change at position 31 in the M2 gene confers resistance [72]. As a result, M2-inhibitors are no longer prescribed for the treatment of influenza infections.

The small-molecule neuraminidase inhibitors (NIs), oseltamivir (Tamiflu®, Roche) and zanamivir (Relenza®, GlaxoSmithKline) were first licensed in 1999. These NIs are effective against all Influenza A and B viruses. Despite large government stockpiling and a global sales market of almost \$500 million, the clinical effectiveness of NIs is still hotly debated. Recently, two systematic reviews of the data collected from clinical trials run by Roche and GSK suggest that NIs only reduce symptom duration by less than one day. The Cochrane review re-examined data from 20 clinical trials totaling more than 24,000 people, and showed that oseltamivir and zanamivir did not prevent person-to-person transmission or reduce the risk of hospitalization, and only reduced influenza symptoms by half a day [73, 74]. A different review reported that patients treated with oseltamivir had statistically significant reductions in the likelihood of requiring antibiotics (44%) and hospitalizations (63%) [75]. A further meta-analysis of observational data from the 2009 influenza A (H1N1) pandemic suggested that in hospitalized adult patients, NIs significantly reduced mortality by 25% overall, and by 65% if started within the first 48 hours of symptom onset [76]. However, the use of NIs increase the risk of adverse

effects such as nausea, vomiting, renal events, and psychiatric effects in adults and children. As a result, NIs are not usually prescribed as a prophylactic due to the major side effects and have only a limited therapeutic window (<48 hours from symptom onset). As a result, NIs have been reserved for patients with influenza who are at high-risk of complications, co-infections, or are rapidly deteriorating.

In addition to the controversy over efficacy, NIs have also been hampered by the emergence of resistance. During an uncomplicated influenza infection, all possible single nucleotide mutations and a large proportion of double mutations are generated [77]. Whereas most of these mutations sustain a fitness cost, some mutations result in reduced inhibition by NIs and are further selected for during drug therapy. The World Health Organization (WHO) reported that in 2008, 25% of the H1N1 isolates tested showed resistance to oseltamivir [78]. During the 2009 pandemic, oseltamivir resistant variants have emerged within several days of initiation of therapy in severe influenza [79, 80]. Development of resistance to NIs was more common between seasonal H1N1 viruses (27%) compared to seasonal H3N2 (3%) or influenza B viruses (0%) [81]. However, most influenza A and B virus strains are still susceptible to oseltamivir and zanamivir. During the 2013- 2014 season, the 2009 H1N1 viruses tested for surveillance were 98.2% and 100% susceptible to oseltamivir and zanamivir respectively [82]. The emergence of resistance will continue to be a source of concern and ongoing surveillance for NI resistance among influenza viruses is essential for public health and government stockpiling for future pandemics.

Broadly Neutralizing Antibodies against Influenza

Broadly neutralizing antibodies (nAbs) against a number of highly variable viruses have been reported, including hepatitis C, dengue, RSV, HIV, and influenza [10, 83, 84]. In contrast to the relatively large number of broadly neutralizing antibodies known for HIV, until recently only one such cross-protective antibody against influenza had been identified. Antibody C179 was isolated from a mouse that had been immunized with H2N2 virus, but was later found to cross-neutralize H1, H2, H5, H6, and H9 subtypes [19, 85]. C179 also bound to the HA stem suggesting that the epitope is fully conserved in some group 1 viruses. Recently, more novel broadly nAbs have been discovered that also bind to the conserved stem region of group 1 viruses [30-34]. Broadly nAbs CR6261 and F10 are the best studied among the antibodies from this family. These antibodies were isolated by phage display, are remarkably similar to one another at the sequence level and have very similar patterns of reactivity and neutralization when tested against a large number of influenza A viruses [86-89]. Like C179, CR6261 and F10 exhibit broad activity against only the group 1 HAs, including the H1, H2, H5, and H9 subtypes [90-92]. Importantly, CR6261 and F10 are protective in mouse models [87, 89, 91], suggesting that these antibodies may have therapeutic potential and that immunogens that elicit similar antibodies may result in broader immunity than current vaccines.

In contrast to the relatively large number of group 1 broadly nAbs, few antibodies have been reported with broad activity against group 2 HAs. It is unclear why broadly nAbs are not easily generated to group 2 subtypes since they are no more antigenically diverse than the group 1 subtypes. It is possible that the different glycosylation sites on group 2 HAs could be occluding antigenic sites on the stem that are readily available on group 1 HAs [18, 93, 94]. However, one human antibody with broad activity against group 2, CR8020, was recently isolated [92, 95]. Unlike most of the group 1 antibodies, CR8020 was isolated by screening immortalized antibody

secreting cells and was found to bind HAs from all six group 2 subtypes[95]. CR8020 also neutralized H3, H7, and H10 viruses *in vitro* and protected mice from lethal challenge with H3 and H7 viruses [95]. The crystal structure of CR8020 bound to HA revealed that CR8020 binds an epitope on the HA stem that is distinct from the site recognized by CR6261/F10 [84, 95, 96]. Thus, the structure of the CR8020 complex has identified a second site that may be targeted by antiviral therapies or vaccination.

Recently, the broadly neutralizing antibody FI6 has been characterized that binds to both group 1 and 2 HAs [32]. FI6 has been shown to neutralize the virus by inhibiting both cleavage of HA0 by trypsin and the pH induced conformation change required for membrane fusion [32]. The broadly neutralizing capacity of FI6 is due to its ability to accommodate the group-specific arrangements of residues near Trp21, which activate the low pH fusion mechanism [32]. FI6 appears to avoid the glycan found on group 2 HAs by nudging the sugar moiety out of the way, while still binding the conserved stem region. FI6 has been shown to be protective in mice and ferrets as both a prophylactic and therapeutic when delivered intravenously or intranasally [97]. Importantly, engagement with the host's FcγR and recruitment of natural killer (NK) cells for ADCC was crucial for optimum protection by FI6 and other stem binding broadly nAbs *in vivo* [35, 98]. While FcγR engagement by stem nAbs is required for optimal anti-viral efficacy, head directed nAbs do not require immune activation for protection. These findings are critical and has implications for the protection in the immune-compromised or elderly, who lack a fully functioning immune system and who comprise the majority of deaths from seasonal influenza each year [99].

Overall, broadly neutralizing antibodies have been discovered that bind to the conserved stem region of either group 1 or group 2 or both group 1 and 2 HAs, and have been shown to be

protective in several influenza challenge models. To date, no antibodies binding the highly conserved stem region has full anti-viral efficacy independent of immune activation.

HA Targeted Antivirals

The discovery of the highly conserved stem region of HA and identification of broadly neutralizing antibodies has spurred the development of anti-HA antivirals. Most are antibodies that are designed to target complicated hospitalized influenza cases. Crucell advanced CR8020 and CR6261 into clinical trials, completing Phase I and Phase IIa safety and efficacy studies [100]. However, a joint CR8020/CR6261 Phase II trial to test efficacy of a 2-hour infusion of 30 mg/kg of each antibody was withdrawn prior to enrollment [101]. The NIH reports that withdrawal was due to unfavorable results from an influenza challenge and efficacy trial. CellTrion is developing another anti-HA antibody called CT-P27. In April 2013, CellTrion received an Investigational New Drug (IND) approval to commence Phase 1 clinical studies [102]. Initial reports indicate 10 mg/kg and 20 mg/kg were safe and well tolerated. In November 2013 the company reported that Phase II trials will commence early in 2014 [103]. Another company, Visterra, is pursuing VIS-410, a monoclonal antibody. Preclinical studies have demonstrated efficacy against a range of influenza viruses including H1N1, H5N1, and H3N2 – used alone or in combination with existing antiviral drugs [104]. Currently, a Phase I trial is being recruited to evaluate safety of a single infusion of VIS-410 [100]. Finally, a 16-mer peptide, Flufirvitide-3 is being taken through clinical trials by Autoimmune Technologies [105]. This peptide is the only therapeutic targeting the stem region of HA currently in clinical trials. An initial nasal spray safety study has been completed, and the drug is being moved forward into a broad safety, tolerability, and PK trial [100]. While mAb therapies are in development, these

technologies are costly, time consuming, and complicated to produce due to the need for production in eukaryotic cells [106]. Furthermore, mAb and other therapies delivered intravenously are restricted to critical patients in hospitals.

Limitations of current vaccines and antivirals in preventing influenza

The clinical need for improved vaccines and antivirals against influenza is immediate and urgent. Seasonal influenza alone costs our economy nearly \$15 billion annually in direct and indirect health care costs. During pandemics, such as the 2009 H1N1, nearly 20% of the U.S. population was infected, and a subsequent pandemic could cost the US \$166 billion [107, 108]. A significant driver of the influenza market is population demographics and population growth. In particular, the elderly population (> 65 years old) is at an increased risk of influenza infections and is more likely to experience severe illness and co-infections due to limited immune defenses. Since the elderly do not readily develop robust immune responses, they are also less likely to be protected by vaccines. The aging population represents approximately 15% of the US population [109]. A key secondary consideration is that life expectancy nationally and globally has risen significantly [110]. The increase in life expectancy compounded with a growing older population places an even greater demand on healthcare services to provide efficient access for care and innovate efficacious drug therapies.

Objectives of this thesis

Over the past decades, the emergence of drug-resistant strains has rendered earlier antivirals such as the adamantane derivatives obsolete and increasing reports suggest that neuraminidase inhibitors may suffer the same fate, especially in circulating H1N1 strains. Due to

the high level of antigenic drift and shift that occur, there is a need for an influenza vaccine that confers broad spectrum, long-term protection not only from the seasonal circulating strains but from pandemics as well. Therefore, a cost effective, durable, broad acting influenza vaccine and/or antiviral that can be administered to reduce susceptibility in the population is a clear and unmet need. Targeting the conserved hemagglutinin stem could be the solution to this problem. The purpose of this thesis is three fold; 1) determine if a stem-based protein, designed to mimic broadly neutralizing antibodies, can provide protection against influenza in the absence of an Fc or immune activation; 2) determine if a DNA vaccine expressing the full-length HA can induce broadly neutralizing antibodies against the stem that contribute to protection; and 3) investigate novel stem-based DNA vaccines for the ability to induce broadly neutralizing antibodies against the stem.

Chapter 2

A Computationally Designed Hemagglutinin Stem-binding Protein Provides *in vivo* Protection from Influenza Independent of a Host Immune Response

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M.T.K. contribution: Working with D.H.F., conceived of study and wrote paper. Carried out all *in vivo* mouse influenza challenge studies with the exception of the oseltamivir comparison, prepared, analyzed all influenza viral load and cytokine experiments, designed and analyzed the *in vivo* ferret studies.

Abstract

Recently, several broadly neutralizing antibodies have been characterized that bind to a highly conserved site on the viral hemagglutinin (HA) stem region. These antibodies are protective against a wide range of diverse influenza viruses, but their efficacy depends on a host immune effector response through the antibody Fc region (ADCC). Here, I investigate the protective efficacy of a protein (HB36.6) computationally designed to bind with high affinity to the same region in the HA stem. I show that intranasal delivery of HB36.6 affords protection in mice lethally challenged with diverse strains of influenza independent of Fc-mediated effector functions or a host antiviral immune response. This designed protein prevents infection when

given as a single dose of 6.0 mg/kg up to 48 hours before viral challenge and significantly reduces disease when administered as a daily therapeutic after challenge. A single dose of 10.0 mg/kg HB36.6 administered 1-day post-challenge resulted in substantially better protection than 10 doses of oseltamivir administered twice daily for 5 days. Thus, binding of HB36.6 to the influenza HA stem region alone, independent of a host response, is sufficient to reduce viral infection and replication *in vivo*. Thus, through computational protein engineering, we have designed a new antiviral with strong biopotency *in vivo* that targets a neutralizing epitope on the hemagglutinin of influenza virus and inhibits its fusion activity. These results have significant implications for the use of computational modeling to design new antivirals against influenza and other viral diseases.

Introduction

New antivirals that broadly protect against a wide range of influenza variants are urgently needed to supplement the protective effects of vaccines and improve treatment options against seasonal influenza and future pandemics. Broadly neutralizing monoclonal antibodies (bnAbs) that bind the conserved HA stem can neutralize diverse influenza strains *in vitro*, suggesting that antivirals targeting the HA stem could provide similar widespread protection. BnAbs bind to the fusogenic region of the HA stem and inhibit the conformational rearrangements in HA required for membrane fusion [32, 84, 111]. Recent studies show that protection by HA-stem binding bnAbs is greatly enhanced through FcγR engagement *in vivo* [35, 98]. While antibody binding to the fusogenic region is sufficient for *in vitro* neutralization of the virus, Fc-FcγR interaction and activation of antibody-dependent cellular cytotoxicity (ADCC) are critical for *in vivo* efficacy of stem-binding bnAbs [35, 112].

The Baker lab had previously described two computationally designed small proteins that bind the HA stem region of multiple Group 1 influenza virus HA subtypes with equal or higher affinity than most bnAbs [113, 114]. These results demonstrated the feasibility of using computational modeling to design a protein that mimics the stem binding of bnAbs *in vitro*, but since the designed proteins lacked an Fc, it was unclear if they would be able to afford protection against a rigorous influenza challenge *in vivo*. Here, they optimized one of these HA stem binding protein for tighter binding using deep mutational scanning [115], and I investigated its ability to afford protection against influenza infection *in vivo*. I showed that intranasal administration of an HA stem binding protein reduces viral replication and provides strong protection against diverse influenza strains when administered as a prophylactic or therapeutic *in vivo*. I further showed that protection is independent of the host immune response, demonstrating that an antiviral can disrupt influenza infection *in vivo* via direct binding to the HA stem.

Results

HA stem-binding protein affords prophylactic and therapeutic protection against influenza *in vivo*.

The Baker lab optimized a broadly cross-reactive HA binding protein, HB36.5, which is a stable, 97-residue designed protein, by increasing its affinity against multiple HA subtypes [114]. They demonstrated by negative-stain electron microscopy and biolayer interferometry that HB36.6 binds to the designed target location on the HA (**Fig 2.1a, 2.1b, and 2.1c**) and had higher affinity than HB36.5 against a wide range of Group 1 influenza subtypes, with greater than 40-fold and 10-fold affinity increases against H2 and H5 HAs, respectively (**Fig 2.1d and**

Table 1). However binding affinity does not always translate in to neutralization. Therefore I examined the ability of HB36.6 to neutralize influenza *in vitro*. HB36.6 potently neutralized a panel of genetically distinct human (H1N1) and avian (H5N1) influenza viruses (range of genetic diversity between HA amino-acid sequences is 50-89%) with a 50% effective concentration (EC_{50}) range of 0.18-12.0 $\mu\text{g/ml}$ (**Fig 2.1e**). This level is comparable to the EC_{50} range of 0.27-0.34 $\mu\text{g/ml}$ observed for the monoclonal antibody, FI6v3, which has been shown to broadly neutralize Group 1 and 2 influenza strains [32]. In addition, HB36.6 was more potent than ribavirin, a broad-spectrum antiviral that neutralizes influenza *in vitro* [116-118] and protects against influenza in mice [117, 119] but had a higher EC_{50} of 15-18 $\mu\text{g/ml}$ against a representative subset of the same influenza strains (**Fig 2.1e**). However, HB36.6 did not neutralize either of the Group 2 strains tested or a Group 1 A/Hong Kong/2009 H9N2 strain, results that are consistent with computationally designed stem binders not binding Group 2 viruses [113] and FI6v3 weakly neutralizing the same H9N2 virus with an EC_{50} of 210 $\mu\text{g/ml}$.

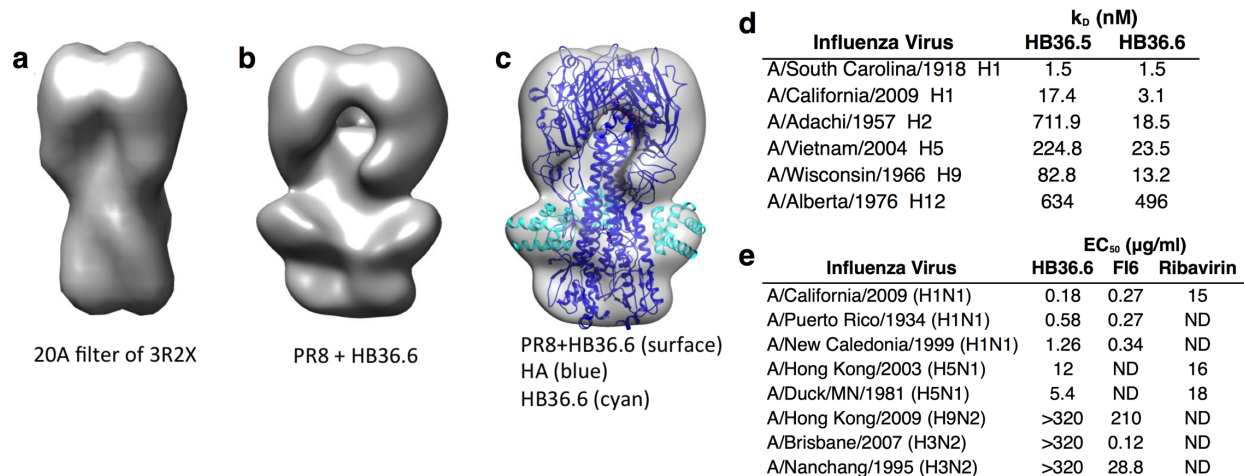


Figure 2.1: Characterization of HB36.6. (a) Crystal structure of A/South Carolina/1/1918 (H1) HA (derived from PDB 3R2X) filtered to 20 Å resolution. (b) Negative-stain EM reconstruction of PR8 HA bound to HB36.6. (c) X-ray structure of A/South Carolina/1/1918 (H1) HA (PDB 3R2X) (blue) in complex with HB36.6 (cyan) docked into PR8 EM reconstruction in b. HB36.6 fits well into the extra density in the stem region. (d) Equilibrium binding constants determined by bilayer interferometry for HB36.5 and HB36.6 against six HAs demonstrate broad improvements against a variety of Group 1 subtypes. (e) EC_{50} ($\mu\text{g/ml}$), compound concentration that reduces viral replication by 50%, of HB36.6, the monoclonal antibody FI6v3, and ribavirin against five representative strains from H1, H9, and H5 Group 1 viruses and two H3 Group 2 viruses. ND: not determined.

Influenza Virus	k_D (nM)
A/Puerto Rico/8/1934 (H1N1)	16
A/New Caledonia/20/1999 (H1N1)	3
A/Japan/305/1957 (H2N2)	52
A/turkey/Massachusetts/3740/1965 (H6N2)	16
A/duck/England/1/1956 (H11N6)	60
A/gull/Maryland/704/1977 (H13N6)	161

Table 2.1. HB36.6 broadly binds Group 1 HAs. Equilibrium binding constants determined by biolayer interferometry for HB36.6 against six HAs demonstrate broad binding affinity against a variety of Group 1 subtypes.

I next investigated the ability of HB36.6 to protect against influenza in mice. I administered a single intranasal (IN) dose of HB36.6 (6.0 mg/kg) to BALB/c mice at 2, 24, or 48 hours prior to challenge with a lethal dose (10 times the 50% mouse lethal dose or 10 MLD₅₀) of H1N1 A/California/04/2009 (CA09) virus. CA09 is a highly virulent Group 1 pandemic influenza strain that leads to rapid weight loss and death in mice within 5-8 days post-infection (d.p.i.) [120]. When administered up to 48 hours before challenge, a single pre-exposure dose of HB36.6 afforded complete protection with 100% survival and little (<10%) to no weight loss, whereas all untreated controls (Ctr) exhibited >30% weight loss, with none surviving (**Fig 2.2a**). Protection was specific to HB36.6 since a protein control (lysozyme, 6.0 mg/kg), administered either 48 or 2 hours before CA09 challenge provided no protection and resulted in weight loss and mortality comparable to the controls (**Fig 2.2a**). Protection was dependent on the IN route of delivery because the same dose of HB36.6 delivered intravenously (IV) provided no protection (**Fig 2.3**). When administered intranasally, HB36.6 was readily detected throughout the lung within 6 hours after administration indicating penetration into the lower respiratory tract (**Fig 2.4**). The observed prophylactic protection between 48-72 hours before challenge suggests a bioavailability range within this timeframe, although additional studies to determine the bioavailability and pharmacokinetics of HB36.6 will be needed.

Lower doses of 1.0, 0.1, and 0.01 mg/kg administered IN two hours prior to lethal challenge with CA09 also resulted in 100% survival with little (0.1 mg/kg) or no (1.0 mg/kg) weight loss whereas controls exhibited rapid weight loss and succumbed to the infection within 7 d.p.i. (**Fig 2.2b**). Mice that received the lowest IN dose tested (0.01 mg/kg) exhibited weight loss, yet survived 2-3 days longer than controls and 20% of mice completely recovered.

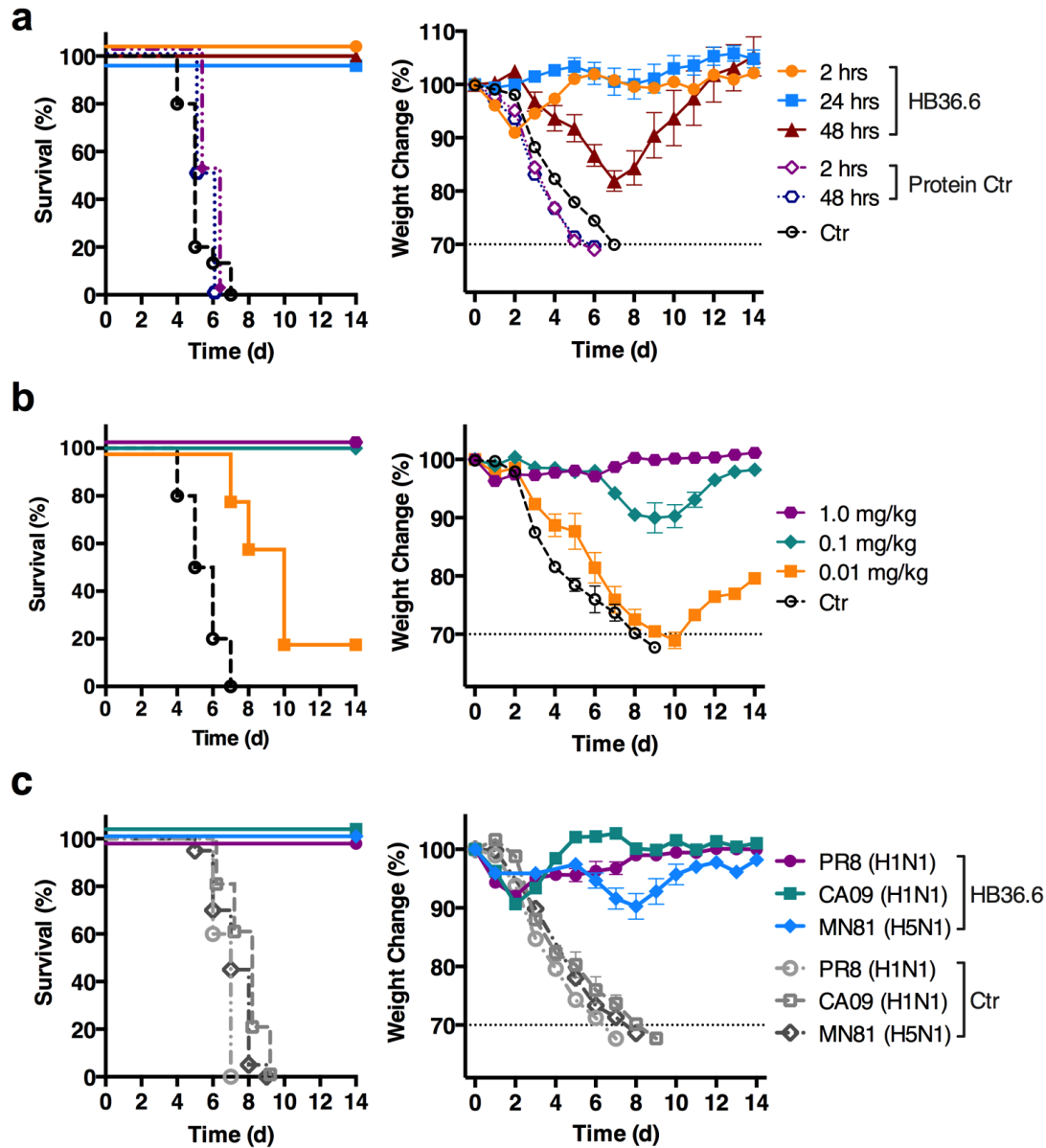


Figure 2.2: Intranasal delivery of HB36.6 affords prophylactic protection against lethal challenge by influenza virus. (a) Survival and weight change in BALB/c mice (n=10 per group) that received 6.0 mg/kg of HB36.6 administered intranasally (IN) at 2, 24, or 48 hours before challenge with 10 MLD₅₀ CA09 virus. The Protein Control (Ctr) group received 6.0 mg/kg of lysozyme at 2 or 48 hours before challenge with 10 MLD₅₀ CA09 virus. (b) Survival and weight change in BALB/c mice (n=5 per group) that received 0.01–1 mg/kg IN doses of

HB36.6 2 hours before challenge with 10 MLD₅₀ of CA09 virus. (c) Survival and weight change in BALB/c mice (n=10 per group) that received 3.0 mg/kg of HB36.6 IN 2 hours before IN infection with 10 MLD₅₀ of H1N1 CA09 virus, 6 MLD₅₀ H1N1 A/PR/8/34 (PR8), or 3 MLD₅₀ of H5N1 A/Duck/MN/1525/81 (MN81) virus. Mean and SEM are shown.

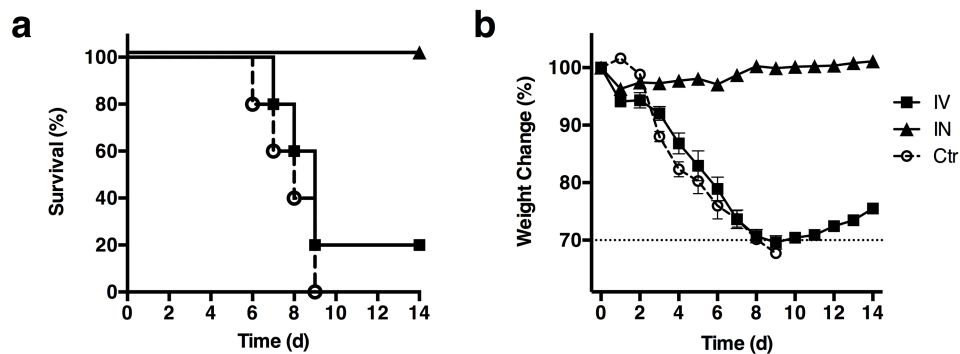


Figure 2.3: Intranasal but not intravenous delivery of HB36.6 protects mice against lethal Influenza virus challenge. (a) Survival and (b) weight change in mice that received 6 mg/kg body weight of HB36.6 intravenously (IV) or intranasally (IN) 2 hours before intranasal challenge with 10 MLD₅₀ of CA09 virus. Mean and SEM from n=5 Balb/c mice per group are shown.

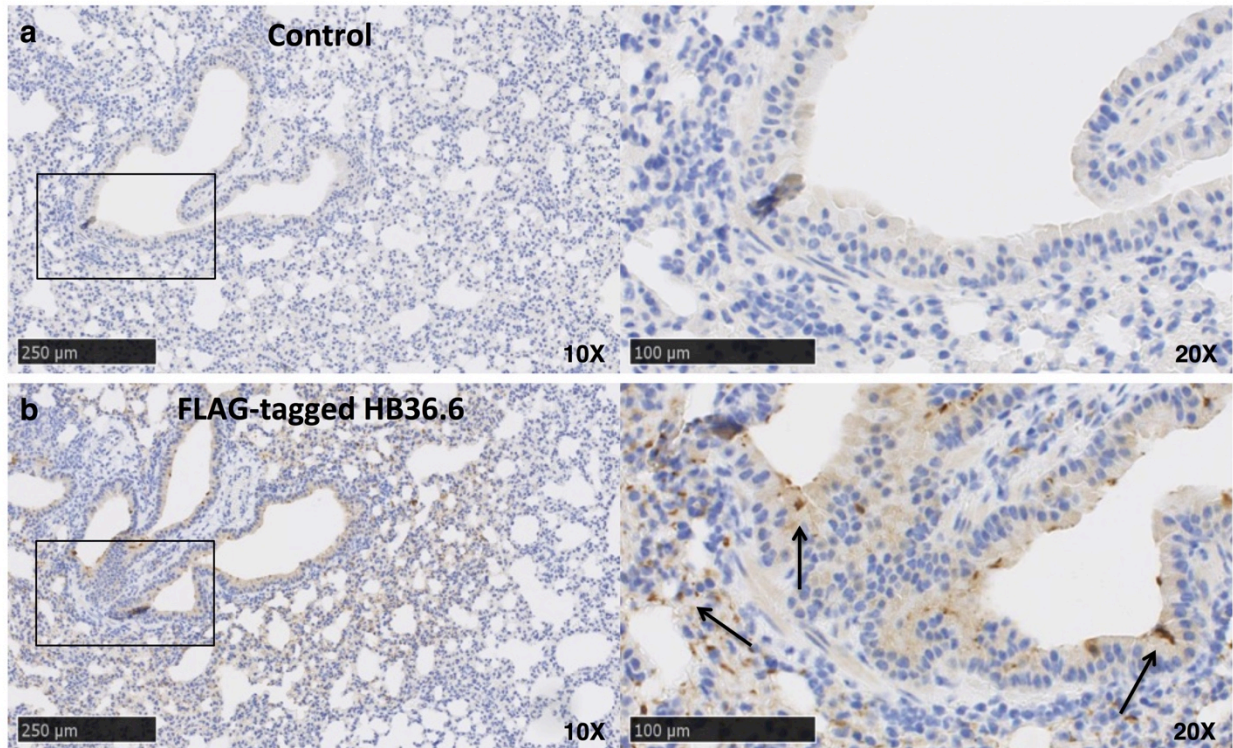


Figure 2.4: Intranasal administration of HB36.6 penetrates into the lung. To determine if IN delivery of HB36.6 penetrates into the lower respiratory tract, mice received 6.0 mg/kg of HB36.6 and 6 hours later, lung tissue (blue) was sectioned and stained using anti-FLAG antibodies (brown). Representative images, 10X and 20X (boxed area), from the right lung lobe of the lower respiratory tract from (a) untreated control, (b) HB36.6-treated. Arrows indicate areas of anti-FLAG staining.

To determine if HB36.6 can protect against genetically distinct strains *in vivo*, I investigated protection against H1 and H5 viruses that are the most virulent Group 1 subtypes that infect mice and cause the majority or most severe Group 1 influenza infections in humans. I inoculated mice IN with HB36.6 (3.0 mg/kg) two hours before challenge with either CA09, A/PR8/34 (PR8), or another highly virulent H1N1 mouse-adapted virus (PR8). In collaboration with Dr. Don Smee at Utah State University, we also examined protection against the H5N1 avian strain A/Duck/MN/1525/81 (MN81). The HA protein sequence of CA09 is 18% and 36% divergent from PR8 and MN81, respectively. HB36.6 provided robust protection against these two genetically distinct H1N1 viruses and the highly pathogenic H5N1 virus, with 100% of the mice surviving and no weight loss (**Fig 2.2c**). This result is consistent with the *in vitro* results showing that HB36.6 broadly binds and neutralizes H1 and H5 HAs (**Fig 2.1a and 2.1b**).

I next investigated HB36.6 for the ability to protect post-exposure. I challenged mice with CA09 and then treated with either a single IN dose of 3.0 mg/kg HB36.6 on day 0 (2 hours p.i.), +1, +2 or +3 p.i. or four daily IN doses on days +1-4 p.i. HB36.6 reduced weight loss and afforded complete recovery and protection from lethality in 100% of mice when administered daily for 4 days or as a single inoculation 2 hours p.i. and 60% protection from lethality when administered as a single inoculation + 1 day p.i. (**Fig 2.5a**). There was no significant difference in weight loss between mice receiving daily doses on days +1-4 p.i. or a single dose at 2 hours or day +1 p.i., suggesting that a single dose within 1 day post-exposure is sufficient to protect from disease. Although mice that received HB36.6 at day +2 or +3 p.i. succumbed to infection, disease onset was delayed. The majority of these mice died at 8-9 d.p.i., whereas 100% of the controls succumbed within 4-7 d.p.i. (2 d.p.i., $p = 0.0006$; 3 d.p.i, $p = 0.0031$ compared to controls) (**Fig 2.5a**). The protection was specific for HB36.6 binding to the HA since daily administration

of the scaffold protein (PDB ID 1u84) that HB36.6 was modeled on provided no protection (**Fig 2.5a**).

In collaboration with Dr. Don Smee at Utah State University, we next compared a single dose of HB36.6 to oseltamivir [74], an antiviral that targets influenza neuraminidase and is currently used to treat influenza in humans. Dr. Smee challenged mice with CA09 virus and then treated with either a single IN dose of HB36.6 (1.0-10 mg/kg) on day +1 p.i., or the recommended schedule of ten doses of oseltamivir administered by oral gavage, twice daily for 5 days starting on day +1 p.i.(5 mg/kg/day) [119, 121, 122]. Oseltamivir afforded a modest delay in time to death, but provided no protection (0%) from lethality. In contrast, escalating doses of HB36.6 protected mice from lethality with the highest dose (10 mg/kg) affording 70% efficacy (**Fig 2.5b**). Thus, a single dose of HB36.6 provided superior protection against a highly virulent influenza challenge when compared to ten doses (2 times per day for 5 days) of a leading influenza antiviral. Furthermore, post-infection treatment with a combination of a sub-optimal single dose of HB36.6 (1-10 mg/kg) and twice-daily doses of Oseltamivir resulted in 100% survival, indicating a synergistic effect when the two antivirals are combined (**Fig 2.5c**).

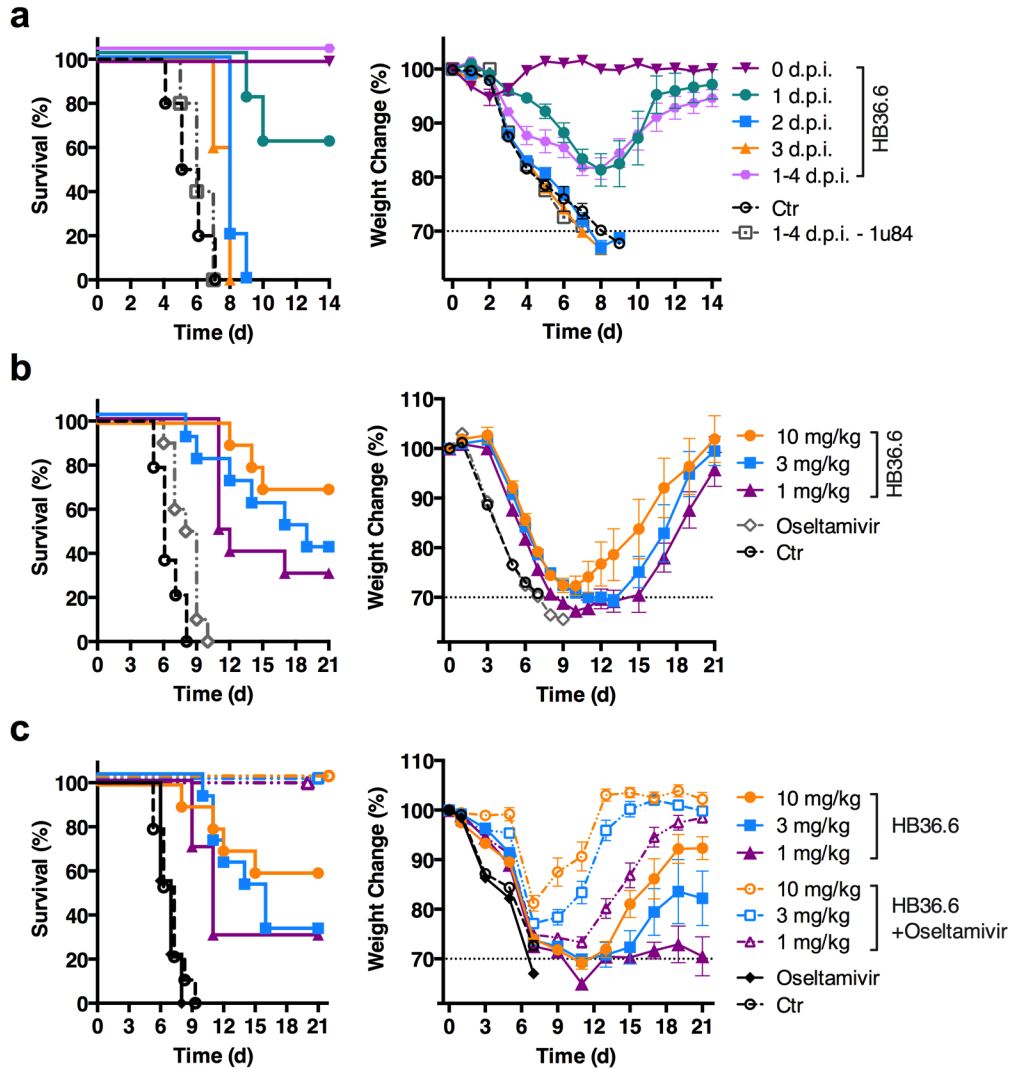


Figure 2.5: Intranasal delivery of HB36.6 affords therapeutic protection against lethal challenge by influenza virus. (a) Survival and weight change in BALB/c mice (n=10 per group) that received 3.0 mg/kg of HB36.6 IN on day 0 (2 hours post-infection) or +1, +2, or +3 days post-infection (d.p.i.) or once daily on days +1-4 post-infection with 10 MLD₅₀ CA09 virus. As a control, the scaffold protein 1u84 (3.0 mg/kg) was IN administered once daily on days +1-4 post-infection. (b) Survival and weight change in BALB/c mice (n=10 per group) that were challenged with 3 MLD₅₀ of CA09 virus and then received either a single dose of HB36.6 (0.1-10 mg/kg) IN on day +1 p.i., or oseltamivir (2.5 mg/kg/dose) by oral gavage twice a day on days

+1-5 p.i. (10 doses total). Mean and SEM are shown. (c) Survival and weight change in BALB/c mice (n=10 per group) that were challenged with 3 MLD₅₀ of CA09 virus and then received either a single dose of HB36.6 (1-10 mg/kg) IN on day +1 p.i., Oseltamivir (2.5 mg/kg/dose) by oral gavage twice a day on days +1-5 p.i. (10 doses total), or a combination of HB36.6 (1-10 mg/kg) by IN on day +1 plus oseltamivir (2.5 mg/kg/dose) by oral gavage twice a day on days +1-5 p.i. (10 doses total). Mean and SEM are shown.

HB36.6 reduces viral load and inflammation

To determine the effects of HB36.6 at the respiratory sites of virus exposure, I analyzed viral replication in nasal and lung compartments in mice that received a single IN dose of HB36.6 (6.0 mg/kg) either 24 hours before (Prophylactic- Pro) or after (Therapeutic- Ther) challenge with CA09. I collected nasal washes on days 2, 4, and 6 post-challenge and viral titers were measured by an end-point dilution assay (TCID₅₀). At each time-point p.i., mice that were treated with HB36.6 before (Pro) or after (Ther) challenge exhibited a substantial 1-3 log-fold reduction in mean viral titer when compared to untreated controls, with the lowest viral load observed in the prophylactic group (**Fig 2.6a**).

I next investigated the effects of HB36.6 on viral replication in the lung. I treated mice IN with HB36.6 (6.0 mg/kg) 1 day before or post-infection with CA09, collected lung tissue on days 2 and 4, and then stained for intracellular expressed influenza nucleoprotein (NP) to identify infected cells. Lung tissues from mice that received prophylactic or therapeutic administration of HB36.6 showed less viral replication in the lungs when compared to the untreated controls at day 4 p.i. (**Fig 2.6b**) and *in situ* enumeration of NP positive cells in the lung tissue confirmed significantly lower numbers of infected cells in the lung at day 4 in mice that received HB36.6 as a therapeutic compared to controls ($P \leq 0.0263$, **Fig 2.6c**). The lower nasal wash viral loads in the prophylactic group (**Fig 2.6a**) but comparable lung viral loads in the prophylactic and therapeutic groups (**Figs 2.6b and 2.6c**) suggest that prophylaxis with HB36.6 likely affords protection by binding and blocking the virus at the nasal site of exposure, whereas post-exposure therapy with HB36.6 affords protection by containing the burst of viral replication and progeny release in the lung resulting in reduction of viral load in the lung and blunting of the inflammatory response that typically initiates within 24 hours after challenge [120].

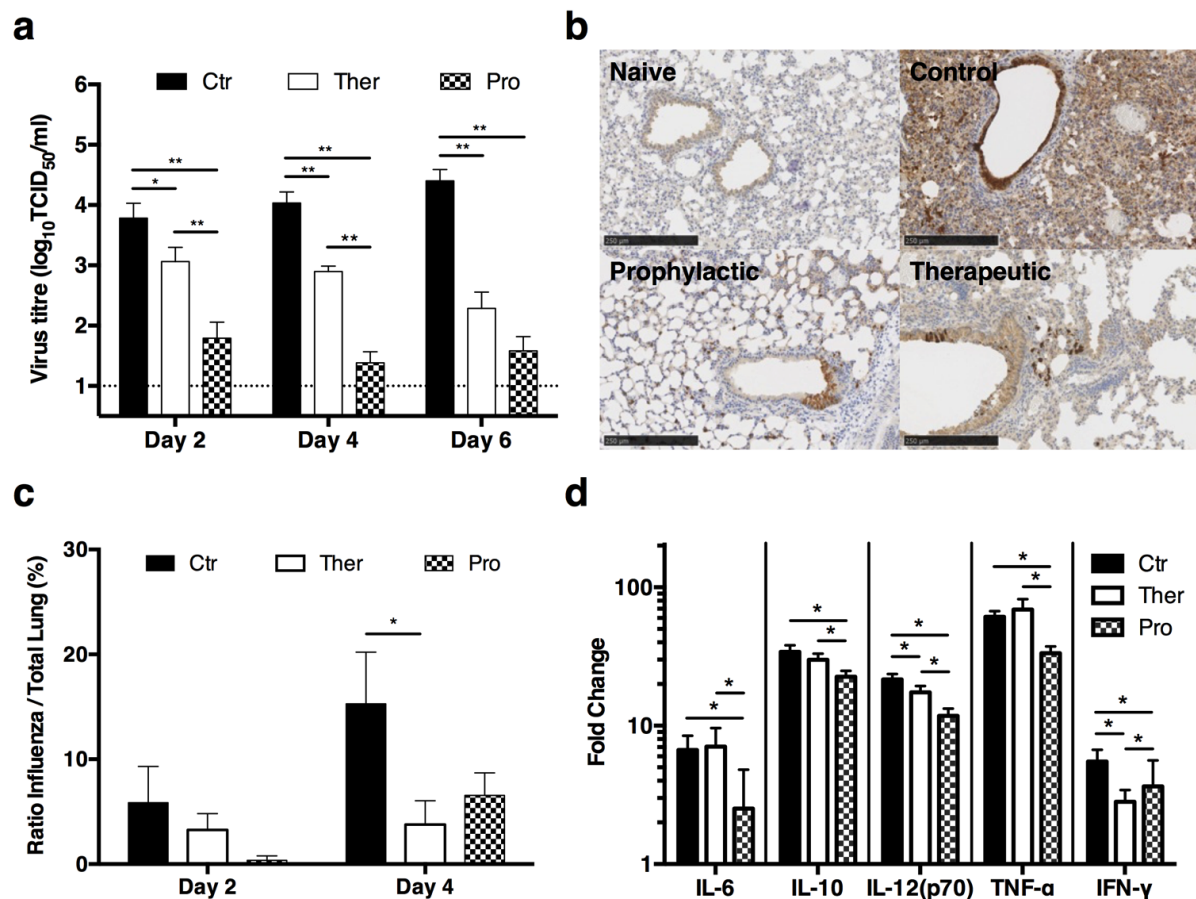


Figure 2.6: HB36.6 suppresses viral replication and inflammation in the lung. (a) Viral titers in nasal washes of untreated infected controls (Ctr) and mice that received 6.0 mg/kg HB36.6 either 1 day before (Prophylaxis, Pro) or 1 day after (Therapeutic, Ther) infection with 10 MLD₅₀ CA09 virus. Nasal washes collected on days 2, 4 and 6 post-infection were measured by determining the 50% tissue culture infectious dose (TCID₅₀) (bars indicate mean viral titer ±SD, n=18 mice per group, three replicate experiments). (b) IHC staining of intracellular influenza NP (H1N1) of representative lung sections from uninfected (Naïve) and untreated infected controls (Control) and HB36.6-treated mice (Prophylactic and Therapeutic) at 4 days post-infection with 10 MLD₅₀ CA09 virus. Mouse lungs were not inflated with formalin and consequently resulted in lung collapse and a more hypercellular appearance in the uninfected control. Images selected

show representative staining of influenza (NP) positive cells for each group. (c) Quantification of influenza positive cells in lung tissues was performed by measuring the area of positive staining compared to the total tissue on the slide (uniform random sampling of 50% lung tissue). (d) Inflammatory cytokines were assayed by Bio-Plex using supernatants from lung homogenates obtained from BALB/c mice on day 2 following infection with 10 MLD₅₀ CA09 virus (n=8 mice per group). The fold change over naïve-uninfected mice is shown. For a, c and d, significant differences between the Pro and Ther groups to the Ctr group are shown: *P < 0.05, **P < 0.001.

Influenza infection results in the expression of cytokines that induce inflammation and recruit activated immune cells to clear the infection. However, this inflammatory response damages the pulmonary epithelium and increases susceptibility to secondary infections by ~100 fold [123, 124]. To determine if HB36.6 protects from influenza-induced inflammation, mice were administered a single IN dose of HB36.6 (6.0 mg/kg) either 24 hours before (Pro) or 24 hours after (Ther) lethal challenge with CA09. Lungs were collected on day 2 p.i. and supernatants from lung homogenates were analyzed for the expression of inflammatory cytokines (IL-6, IL-10, IL-12(p70), TNF- α , IFN- γ). HB36.6 delivered as a prophylactic significantly lowered several cytokines, including the inflammatory cytokines IL-6 and TNF- α , when compared to controls ($P \leq 0.0012$, **Fig 2.6d**). HB36.6 delivered as a therapeutic also significantly lowered the amount of IL-12(p70) and IFN- γ when compared to controls ($P \leq 0.0007$, **Fig 2.6d**). These results suggest that reduction in viral load by HB36.6 provided an additional benefit of decreasing the cytokine responses that typically lead to increased inflammation and tissue damage. Together, these results show that HB36.6 blocks and interferes with viral spread, resulting in a lower viral replication, suppression of the cytokine response, and decreased lung inflammation. Furthermore, since HB36.6 lacks an Fc domain, these results show that engagement of the host Fc γ R is not required for protection in vivo.

HB36.6 does not induce a protective host antiviral response

Small proteins, such as HB36.6, may stimulate an immune response that could interfere with the effectiveness of a second administration or alternatively, stimulate antiviral responses that can contribute to protection [125]. Therefore, I administered four doses of HB36.6, 2 weeks apart, and observed that administration induced very low levels of antibody; however, 100% of

mice were still completely protected when challenged with a lethal dose of CA09 1 day after the 4th dose (**Fig 2.7**). These results indicate that HB36.6 is poorly immunogenic and repeat administration does not interfere with the antiviral activity of subsequent doses. However, induction of even a modest antibody response after multiple doses suggested HB36.6 likely stimulated a host innate response. To determine if HB36.6 administration induces antiviral cytokine responses that could contribute to protection, cytokines were measured at different time-points post-HB36.6 administration. Mice either received a single IN dose of HB36.6 (6.0 mg/kg) or the scaffold protein (PDB 1u84, 6.0 mg/kg) and lungs were collected at 2, 24 or 48 hrs post-administration. Supernatants from lung homogenates were analyzed for the expression of inflammatory cytokines (IL-6, IL-10, IL-12(p70), TNF- α , IFN- γ). Both HB36.6 and scaffold protein induced low levels of cytokines that peaked between 2-24 hrs post-administration and, by 48 hrs, the levels had dropped to pre-administration levels (**Fig 2.8a**). Importantly, cytokine levels after HB36.6 administration were significantly lower than levels induced by scaffold protein that afforded no protection from challenge. These data suggest that, although administration with HB36.6 induced a low cytokine response, the levels were too transient and/or low to contribute to protection.

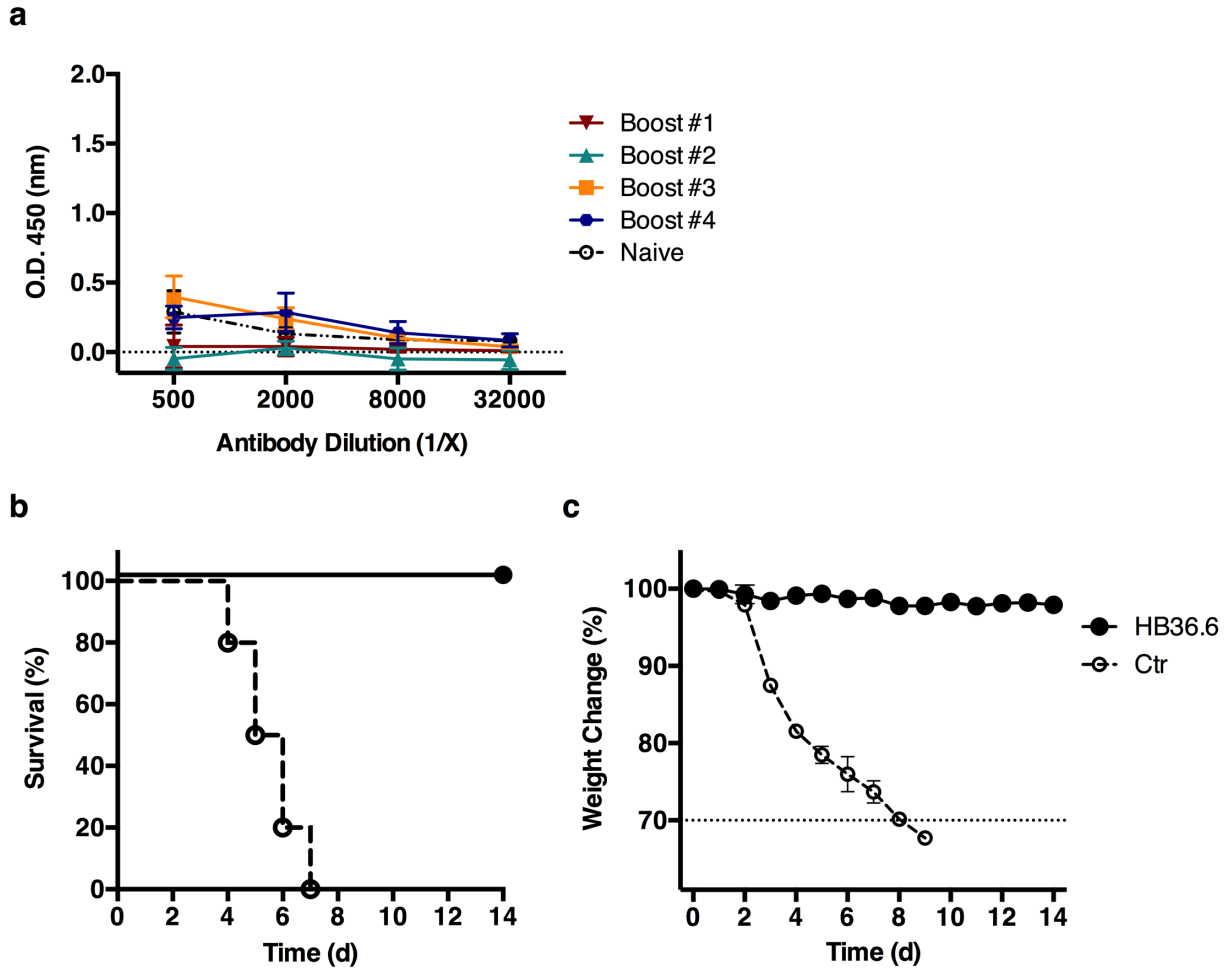


Figure 2.7: Repeat administration of HB36.6 induces low level antibody does not reduce protection from lethal challenge with CA09. Balb/c mice received 3 intranasal doses of HB36.6 (3.0 mg/kg) spaced two weeks apart and then received a 4th intranasal dose 2 weeks after the 3rd dose and 24 hours prior to lethal challenge with 10 MLD₅₀ of CA09 virus. (a) Antibody responses specific for HB36.6 were measured by ELISA in serum collected 2 weeks after each dose of HB36.6 (Doses #1-4), and determined in each mouse as the O.D. of HB36.6-FLAG minus the O.D. of FLAG-only. (b) Survival and (c) weight change in Balb/c mice following a 4th intranasal dose of HB36.6 and lethal challenge with CA09. Mean and SEM of n=10 mice per experimental condition are shown.

HB36.6 does not require a fully functioning immune response for protection

To investigate the possibility that HB36.6 may induce other host responses that could contribute to protection, I tested HB36.6 for protection against influenza in two severe immune-deficient mouse models: NOD SCID gamma (SCID) and MyD88^{-/-} mice. SCID mice lack mature T, B, and NK cells and are unable to develop an adaptive immune response [126, 127]. MyD88^{-/-} mice lack TLR signaling and are deficient in cytokine signaling, resulting in a severely dampened innate and adaptive immune response [128-130]. HB36.6 (6.0 mg/kg), scaffold protein (6.0 mg/kg), and another protein control (lysozyme, 6.0 mg/kg) were IN administered 2 hours before challenge with CA09. HB36.6 protected 100% of the SCID mice and 90% of the MyD88^{-/-} mice with only minimal weight loss (**Fig 2.8b**), whereas the control SCID and MyD88^{-/-} mice (1u84, Protein, and Naïve) exhibited significant weight loss and 0% survival. These results provide further evidence that the antiviral effect of HB36.6 is likely due to direct binding to the HA stem and is independent of an antiviral host response.

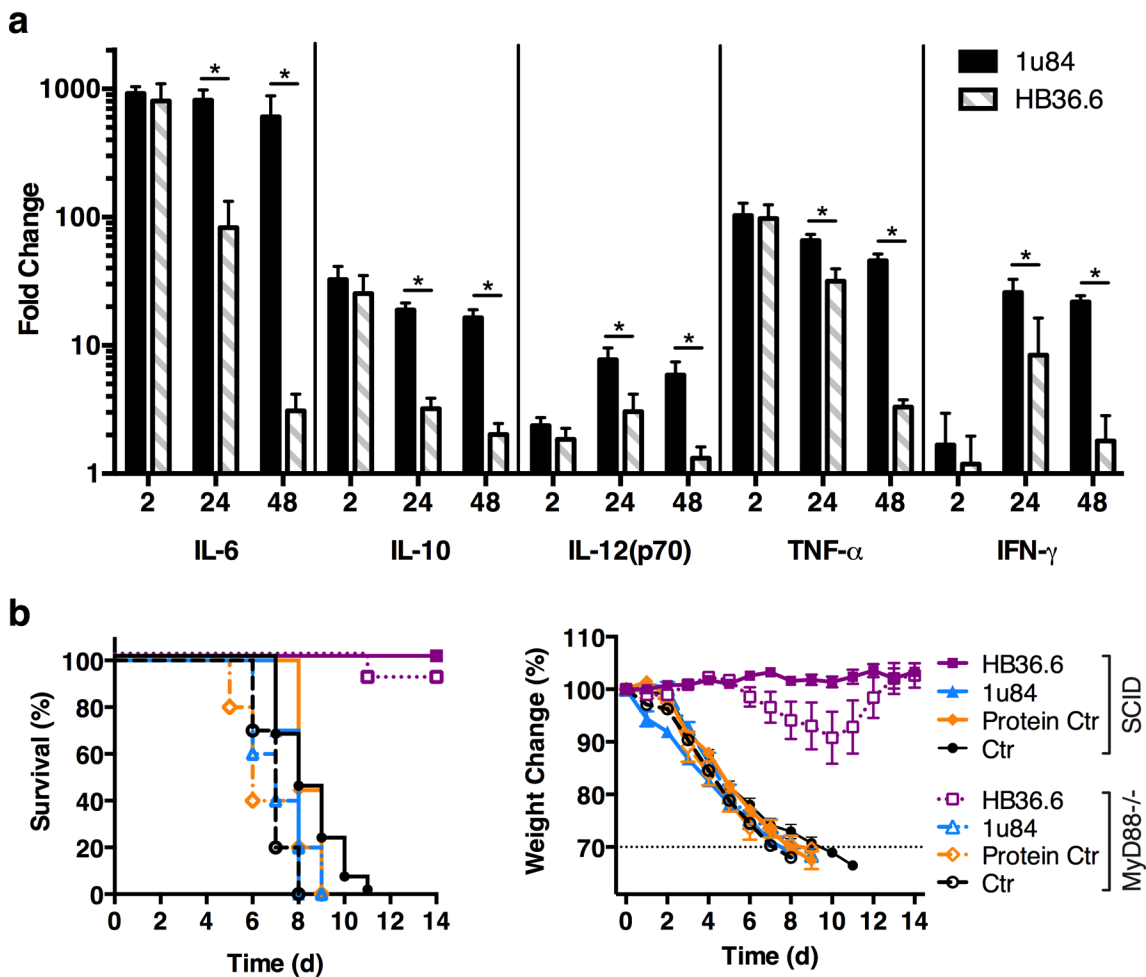


Figure 2.8: HB36.6 induces a transient cytokine response that is not required for protection. (a) Inflammatory cytokines were assayed by Bio-Plex using supernatants from lung homogenates obtained from BALB/c mice 2, 24 and 48 hours following administration with HB36.6 (6.0 mg/kg) or the scaffold protein (PDB ID 1u84) (6.0 mg/kg) (n=10 mice per group). Fold change over naïve mice is shown. *P < 0.05. (b) Survival and weight change in SCID and MyD88^{-/-} mice (n=10 per group) that received 6.0 mg/kg of HB36.6, scaffold protein, or Protein Ctr (lysozyme) IN 2 hours before IN infection with 10 MLD₅₀ CA9 virus.

HB36.6 reduces disease symptoms and viral shedding in ferrets.

Ferrets are a more reliable preclinical model for influenza since they are susceptible to human influenza viruses and develop similar clinical signs and disease [131]. To determine if HB36.6 could afford protection in ferrets, a single dose of either 2.5 or 10 mg/kg HB36.6, or sterile PBS (controls) was IN administered to ferrets 2 hrs prior to aerosol challenge with CA09 virus. The aerosol challenge results in droplet exposure to influenza and more closely resembles natural, airborne influenza virus exposure [132-135], which is the primary mechanism of influenza spread and transmission in humans. Similar to humans, ferrets challenged by aerosol with CA09 exhibit significant morbidity and a low rate of mortality [136]. This study was performed in collaboration with Dr. Kelly Stefano-Cole at the University of Pittsburgh. Ferrets were monitored daily for 3 days and scored for clinical signs of disease (**Fig 2.9**) and then necropsied on day 3. Temperature change and weight change was recorded for the groups over the course of the challenge. No significant difference between the control and HB36.6 treated groups was observed for both temperature (**Fig 2.10a**) and weight change (**Fig 2.10b**). Control (PBS) ferrets manifested significant clinical signs within 3 days p.i., whereas HB36.6-administered ferrets exhibited minimal or no disease (**Fig 2.10c**). These results demonstrate HB36.6 provided a significant benefit in reducing disease symptoms in ferrets. In addition, HB36.6 lowered viral loads in nasal washes at days 1 and 2 p.i, with the lowest viral loads observed in the 10 mg/kg treated HB36.6 group ($p=0.0026$, **Fig. 2.10d**). However there was no significant difference between the control and HB36.6 treated groups for viral loads in the lung lobes (**Fig 2.10e**), BAL, trachea, or nasal turbinates (**Fig 2.10f**). It's possible that higher doses of HB36.6 are required to see more of a difference in the lungs. Furthermore, the intranasal delivery method used in the ferrets may not be delivering the binder to the required locations to be

effective in reducing viral loads in the lungs. Aerosolized delivery in to the lungs may be necessary for better protection. However the reduction in viral shedding in the nasal washes does suggest potential for reducing transmission [137].

Sample: Clinical Scoring Observations

Animal ID # _____ 4-003 _____

Parameter	Degree of Parameter	Date:	9/13/14	9/14/14	9/15/14	9/16/14	9/17/14	9/18/14	9/19/14
Initials			BR	GB	AT/JL	JL/AT	JL/AT	AT/JL	JL
Time of Observation			9:00	9:00	8-12	8-11	8-12	8-12	9-11
Weight (Kg)			0.76	0.78	0.78	0.78	0.78	0.75	0.75
Temperature (C)			39.0	38.6	38.9	38.7	38.6	40.1	38.7
		Score							
Appearance	Posture	Normal	0			0	0		
		Hunched or abnormal posture	5					5	5
		Prostrate	10						
	Activity	Alert and playful	0			0	0		
		Alert but playful only when stimulated	2						
		Alert but not playful when stimulated	5					5	5
		Neither alert nor playful when stimulated	10						
	Skin/Eyes	Normal	0			0	0	0	0
	Piloerection	2							
	Ocular Discharge	5							
Movement	Activity	Normal	0			0	0		
		Lethargy	5					5	5
		Moribund	10						
		Neurological	Normal	0			0	0	0
		Tremors	10						
Hind limb weakness /paralysis		10						10	
Pulmonary Function	Respiration	Normal	0			0			
		Sneezing	2					2	2
		Shallow or Rapid	2						
		Open mouth breathing	5						
		Labored/coughing	10						10
Consumption/ Elimination	Food/water	Normal	0			0	0		
		Dehydrated	5					5	5
		Decreased	2						2
		Inappetent	5						
		Anorexic	10						
	Feces/Urine	Normal	0			0			
		No stool	2						
		Mucoid/Soft stool	2					2	2
		Diarrhea	5						
		Bloody feces	10						
	Vomiting	No	0			0	0	0	0
	Yes	10							
Total Score						0	4	24	46

Figure 2.9: Sample Clinical Scoring Observations for ferret study. Ferrets were evaluated daily for clinical signs of influenza infection. Researchers were blinded to ferret identity while collecting clinical scoring data. (Observational data collected by Dr. Anita Trichel and Dr. Kelly Stefano-Cole).

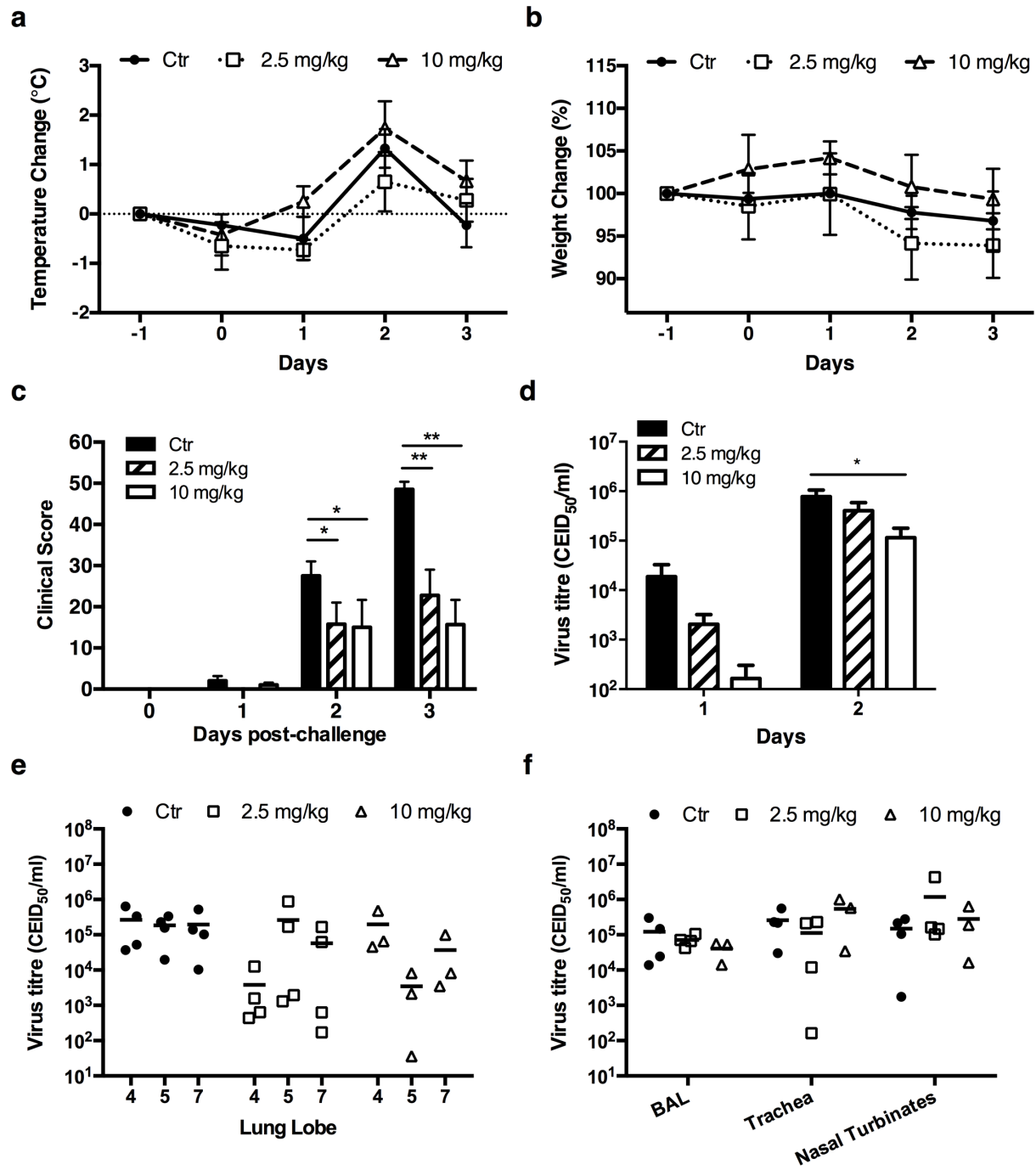


Figure 2.10: HB36.6 reduces clinical disease and viral loads in ferrets. HB36.6 (2.5 or 10 mg/kg in PBS, n=4 per group) was administered IN 2 hrs before aerosol challenge with CA09 virus. Controls received sham inoculations (PBS only). Sham controls (Ctr) and HB36.6-treated ferrets were monitored (blinded) twice daily following challenge for clinical symptoms. (a)

Change in temperature relative to the baseline temperatures obtained for the same animals prior to infection was determined at the indicated times after infection. **(b)** Change in weight of ferrets relative to baseline weights obtained before infection. **(c)** Clinical scores in Ctr and HB36.6-treated ferrets based on appearance, activity, movement, pulmonary function, consumption and elimination, see **Fig 2.10**. **(d)** Viral titers in controls (Ctr) and HB36.6 treated ferrets were measured on days 1 and 2 p.i. by semi-quantitative RT-PCR on RNA extracted from nasal washes and are expressed as relative CEID₅₀ compared to a standard curve of known titer. **(e)** Viral titers in lung lobes, **(f)** bronchoalveolar lavage (BAL), trachea, and nasal turbinates collected from Ctr and HB36.6 treated ferrets on day 3 post-infection. *P < 0.05, **P < 0.001. (Observational data and viral load analysis performed by Dr. Amy Hartman and Dr. Kelly Stefano-Cole).

Discussion

We showed previously that computationally designed proteins optimized for high affinity binding to a viral protein can neutralize viruses *in vitro* [113, 114]. However, prior to this study, it was not known if such proteins would have sufficient stability and potency to afford protection *in vivo*. Here, I provide the first proof-of-concept that a novel small protein that was computationally designed to mimic bnAbs and bind the highly conserved HA stem could be developed into a highly effective antiviral capable of neutralizing and affording robust protection against diverse strains of influenza *in vivo*. I show that HB36.6 neutralizes a panel of Group 1 H1 and H5 viruses *in vitro* and a single intranasal dose afforded significant protection against three highly divergent H1 and H5 influenza strains *in vivo*. This suggests that the range of neutralizing specificity of HB36.6 observed *in vitro* translated to protection against these strains *in vivo*.

My studies also show that HB36.6 mediates protection independent of a host response. This contrasts to previous studies employing intravenous injection of a bnAb (FI6v3) that HB36.6 binding was designed to mimic. These studies showed that engagement with the host's FcγR and recruitment of ADCC was crucial for optimum protection by bnAb *in vivo* [35, 98]. Here, I show that HB36.6, which lacks an Fc, still affords robust protection against different strains of influenza *in vivo*. This outcome may be due to intranasal delivery of HB36.6, which localizes the antiviral at the respiratory site of viral exposure and/or the ability of HB36.6 to bind the stem with high affinity [97]. Consistent with this possibility, Leyva-Grado et.al [97] showed that intranasal delivery of the fragment antigen-binding (Fab) region from the broadly neutralizing antibody, FI6v3, afforded a similar degree of protection as I report here for HB36.6.

A recent study showed that a host receptor binding peptide provided prophylactic protection against lethal influenza challenge that depended on the induction of an inflammatory

antiviral response [125]. The peptide did not work as a therapeutic, since antiviral cytokines are less effective after a viral infection is already established. In contrast, I found that HB36.6 induced only weak cytokine responses that were lower than the non-protective scaffold protein control and provided protection in two severe immune-deficient mouse models indicating a mechanism that is independent of a host antiviral cytokine immune response. Together, these results indicate that binding to the HA stem alone was sufficient for *in vivo* protection against influenza. These findings have implications for development of HB36.6 as a safe and effective alternative for protection from influenza. Here, I found that pre-exposure treatment with HB36.6 prevented infection without inducing an inflammatory response, hence it could be used pre-exposure to increase resistance to infection without the risk of inducing adverse inflammatory responses. Furthermore, since post-exposure inflammation mediates enhanced influenza disease and increased susceptibility to secondary infections [123], this also suggests HB36.6 could be used to treat influenza without the risk of exacerbating disease due to immune effector-mediated inflammation. Finally, the ability of HB36.6 to mediate protection independent of the host response has further implications for protection in the immune-compromised or elderly, who comprise the majority of deaths from seasonal influenza each year [99].

The CA09 strain used in our therapeutic challenge studies is highly virulent, rapidly disseminating into the lower lung of mice within hours after challenge and causing death in control mice within 8 days [120]. Although weight loss is not seen until later time-points, the robust inflammatory response responsible for these symptoms is initiated within hours after challenge [138]. The level of protection afforded by HB36.6 against this strain when used as a therapeutic suggests significant potential to provide post-exposure benefit and improve treatment of influenza infection when compared to current treatments. Consistent with this possibility, we

show that a single dose of HB36.6 administered to mice challenged with a highly virulent influenza strain outperformed a five-day, ten-dose regimen of oseltamivir, the lead antiviral approved for treatment of influenza in humans. This result is consistent with previously reported results showing that oseltamivir delayed, but did not protect from mortality in mice [139-141]. Furthermore combining sub-optimal doses of HB36.6 and oseltamivir resulted in synergistic protection, a result that suggests potential for use of HB36.6 as an approach to augment the effectiveness of existing marketed antivirals. Indeed, several studies have shown that therapeutic use of influenza antiviral combinations could increase antiviral potency, clinical effectiveness, and reduce resistance emergence [142, 143]. HB36.6 also reduced the viral burden and clinical signs in ferrets challenged by aerosol with influenza, a model that is more reflective of ‘natural’ virus infection in humans with respect to influenza pathogenicity and transmission [144]. Although this HA stem epitope is highly conserved the potential for emergence of resistance to HB36.6 will require further investigation.

Previous studies with bnAbs, small molecule inhibitors, and proteins designed to bind the HA stem demonstrate that targeting the HA stem affords protection by inhibiting the low pH-induced fusion of the viral membrane with the endosomal membrane [35, 98, 113, 114, 145]. The direct binding of HB36.6 to the highly conserved fusion region similarly inhibits key conformational rearrangements in the HA that drive the fusion of the viral and endosomal membranes, blocking entry of the viral RNA into the cell via the endosome [113, 114]. Intravenous delivery of bnAbs has been shown to be highly effective in mice and ferrets and is being developed for the hospital setting to treat severe and complicated influenza [32, 35, 97, 146]. However, due to the route of delivery and the high cost of monoclonal antibodies, this strategy is not viable for treatment of uncomplicated influenza in the general population. An

antiviral, such as HB36.6, that is effective intranasally could be more widely self-administered in the general population pre- or post-exposure to prevent infection or shorten recovery from the infection. HB36.6 could be produced in *E. coli* or by chemical synthesis, which would significantly decrease the cost of manufacturing and purification to around \$250/gram compared to the high cost of antibodies, which can range from \$2,000/gram to \$25,000/gram [147].

Seasonal drifted strains reduce vaccine efficacy and drug-resistant strains hinder the use of current antivirals in the prevention and treatment of influenza. These problems highlight the need for effective new antiviral drugs [148, 149]. Overall, my results show that computationally designed proteins have potent anti-viral efficacy *in vivo* and suggests promise for development of a new class of HA stem-targeted antivirals for both therapeutic and prophylactic protection against seasonal and emerging strains of influenza.

Materials and Methods

In vitro antiviral neutralization

MDCK (Madin Darby canine kidney) from American Type Culture Collection (ATCC, Manassas, VA) were grown in Growth medium comprising minimum essential medium (MEM) with non-essential amino acids, 5% FBS and 0.22% NaHCO₃. Influenza A/California/07/2009 (H1N1), A/Puerto Rico/08/1934 (H1N1), A/New Caledonia/20/1999 (H1N1), A/Hong Kong/213/2003 (H5N1), A/Nanchang/933/1995 (H3N2), A/Brisbane/10/2007 (H3N2), and A/Hong Kong/33982/2009 (H9N2), were obtained from the Center for Disease Control (Atlanta, GA). Influenza A/Duck/MN/1525/81 was kindly provided by Robert Webster (St. Jude Children's Research Hospital, Memphis, TN). The viruses were prepared in Madin Darby canine

kidney (MDCK) cells, placed in ampules and frozen at -80°C. Cells are seeded to 96-well flat-bottomed tissue culture plates at the proper cell concentration to establish confluent cell monolayers and incubated overnight at 37°C. Various dilutions of test compound were added to each well. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), FI6v3, and HB36.6 were tested in half-log increments from 320 µg/ml and below. Virus was added to test compound wells and to virus control wells at about 50-100 cell culture infectious dose per ml. The virus titer was determined by a prior titration, where the most diluted virus stock causes 100% cytopathic effect (CPE) in all wells at the particular virus dilution. Test medium without virus was added to all toxicity control wells and to cell control wells. The plates were incubated at 37°C for 72 hours. Sterile neutral red (0.034% in saline solution) was then added to each well. After two hours at 37°C, all medium was removed and the cells washed with PBS and inverted to drain. Neutral red was extracted from the cells by adding an equal volume mixture of absolute ethanol and Sørensen's citrate buffer, pH 4.2. The contents of each well are mixed gently and the optical density (O.D.) values of each well are obtained by reading the plates at 540 nm with a microplate reader.

HB36.6 and oseltamivir administration and influenza challenge

Animal studies approved by the University of Washington and Utah State University Institutional Animal Care and Use Committee. Female, 6-8 week-old BALB/c mice were randomly separated into groups, anesthetized and intranasally administered protein binder (HB36.6) at concentrations varying from 0.01 to 6.0 mg/kg. Two to forty-eight hours later, the mice were anesthetized with 2.5% isoflurane and challenged IN with 3-10 MLD₅₀ (fifty percent mouse lethal dose) of A/California/04/09 (H1N1) (CA09), A/PR/8/34 (H1N1) (PR8) or

A/Duck/MN/1525/81 (H5N1) (MN81). In a therapeutic setting, mice received the protein binder 0 (2 hours post-infection), +1, +2, +3, or +4 days post infection. The mice were monitored daily for weight loss and survival until 14 days post-infection. Animals that lost more than 30% of their initial body weight were euthanized by carbon dioxide in accordance with our animal protocols. Oseltamivir-treated mice received 2.5 mg/kg of oseltamivir (Roche, Palo Alto, CA) twice daily for 5 days (total of 10 doses) by oral gavage. Oseltamivir was dissolved in water prior to administration. The SCID (Non-Obese Diabetic (NOD), Severe Combined Immunodeficiency (SCID) gamma, strain NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice and the MyD88^{-/-} (strain B6.129P2(SJL)-Myd88tm1.1Defr/J) mice were purchased from Jackson Laboratory. At least five mice per group were used for each experiment. All mice used for the experiments are included for analyses. For mouse experiments, researchers were not blinded to animal identity.

Nasal and lung viral titers in mice

Nasal wash samples were collected by making an incision in the trachea and washing the nasal passages with 0.2 ml sterile PBS (pH 7.2). Supernatants from lung homogenates were collected by mincing whole lungs in 500µl MEM media, freeze thawing twice on dry ice, and then centrifuging at 13,000rpm for 10m. The viral titers in the nasal washes and supernatants from lung homogenates were determined using the TCID₅₀, as described previously [150]. In brief, monolayers of MDCK cells were inoculated with tenfold serial dilutions of mouse nasal washes in quadruplicate (three total replicates per sample). One hour after inoculation, the supernatants were removed and replaced with MEM media plus antibiotics and 1 µg/ml TPCK-trypsin (Sigma, St. Louis, MO). The viral cytopathic effect was observed for 3 days before viral

infectivity in MDCK cells was measured using a hemagglutination assay with 0.33% turkey erythrocytes. The tissue viral titers were calculated using the Reed and Muench method [151] and expressed as \log_{10} TCID₅₀/g of tissue.

Enzyme-linked immunosorbent assay (ELISA)

HB36.6-specific IgG antibody levels in mouse serum were assessed by ELISA. Maxisorp (Thermo Scientific-Nunc) were coated with 100 ng/well of HB36.6 in PBS overnight at 4°C. Plates were blocked with 5% nonfat milk powder in PBS for 1h at room temperature, and then washed three times with wash buffer (PBS-T; phosphate-buffered saline containing 0.05% Tween 20). Two-fold serial dilutions of samples were added to the wells and plates were incubated for 1hr at room temperature. Following three washes with PBS-T, plates were incubated with horseradish-peroxidase conjugated goat anti-mouse IgG (1/3,000 dilution) secondary antibodies (Thermo Scientific Pierce) for 1h at room temperature. After five washes with PBS-T, TMB substrate (KPL) was added to the wells for 30 min at room temperature. Color development was stopped by the addition of TMB Stop solution (KPL), and the plates were read at 450nm to measure relative optical densities (O.D.).

Bio-Plex analysis of cytokine production in lung homogenates

The concentrations of cytokines in lung tissue were measured. On days 2 and 4 post-infection, 8 mice per group were sacrificed and whole lung tissue was collected and immediately frozen. Lungs were thawed, weighed and lysed using the Bio-Plex Cell Lysis Kit (Bio-Rad, Hercules, CA). The levels of interleukin (IL)-6, IL-10, IL-12(p70), interferon (IFN)- γ , and tumor necrosis factor (TNF)- α in the lysate were measured using a Bio-Plex multiplex bead array kit

(Bio-Rad, Hercules, CA). The Bio-Plex assay was performed in accordance with the manufacturer's instructions.

Histology and immunohistochemistry

During in vivo challenge experiments, lungs were removed from mice and immersed in 10% neutral buffered formalin. IHC staining was performed by the UW Histology core. Following fixation, tissues were removed from formalin and placed in paraffin. Immunohistochemical staining was performed on the Leica Bond Automated Immunostainer. Sections were deparaffinized in Leica Bond Dewax Solution (Leica Cat No. AR922) and rehydrated through 100% EtOH. After antigen retrieval with EDTA buffer pH 9.0 (Leica Bond Epitope Retrieval Solution 2, Cat No AR9640) at 100°C for 20m, blocking endogenous peroxidase activity with 3.0% H₂O₂ for 5m, and blocking with 10% normal donkey serum in TBS for 20m, the sections were incubated with goat anti-influenza A virus, (Meridian Life Science Inc. Cat No. B65141G) at 1:2000 or normal goat IgG, isotype control, (Invitrogen Cat No. 02-6202) at (1:5000 dilution) both in Bond Primary Antibody Diluent (Leica Cat No. AR9352) for 30m at room temperature. Sections were then incubated with rabbit anti-goat IgG (Jackson ImmunoResearch Cat. No. 305-005-045) 1:1500 + 5% normal donkey serum for 8 minutes at RT followed by incubation with goat anti-rabbit poly-HRP polymer secondary detection (Leica Cat No DS9800) for 8m at room temperature. Sections were then incubated with Leica Bond Mixed Refine DAB substrate detection for 10 minutes at room temperature. (Leica Cat No DS9800). After washing with DIH₂O, the sections were counter stained with Hematoxylin solution (Leica Bond Refine Kit) dehydrated through 100% EtOH, cleared in Xylene and mounted with synthetic resin mounting medium and 1.5 coverslip.

Statistical and power analyses

All of the analyses were performed using Graphpad Prism version 5.01. A Student's t test (to compare two samples) and analysis of variance (ANOVA) (to compare multiple samples) were used for statistical analysis. Survival analyses were performed by using the Kaplan-Meier log-rank test. A P value of <0.05 was considered to be significant. For mice, the minimum group size was determined using weight loss data with 100% of control mice becoming infected with CA09. Based on a standard deviation of 2% in weight loss, a group size of $n=5$ yields $>80\%$ power to detect a minimum of a 10% difference between groups in weight loss using a two-sided t-test with an alpha value of 0.05.

Chapter 3

Multigenic DNA vaccine induces protective cross-reactive T cell responses against heterologous influenza virus in nonhuman primates

M.T.K. contribution: Working with D.H.F. and J.A.L., conceived of study and wrote paper. Carried out all the nonhuman primate antibody analysis and in vivo passive transfer mouse influenza challenge studies. Helped to collect and process samples for antibody, T cell, and viral load analysis. Collaborators contributing to this work; Jolie A. Leonard, Amy Hartman, Kelly Stefano-Cole, Todd Reinhart, and Deborah H. Fuller.

Abstract

Recent avian and swine-origin influenza virus outbreaks illustrate the ongoing threat of influenza pandemics. We investigated immunogenicity and protective efficacy of an *E. coli* heat-labile enterotoxin (LT) adjuvanted multigenic (LT-MA) universal influenza DNA vaccine consisting of four hemagglutinin (HA) antigens, nucleoprotein (NP) and the ectodomain of the matrix protein (M2e) in nonhuman primates. Though the LT-MA DNA vaccine induced robust serum and mucosal antibody responses, it failed to induce broadly neutralizing antibodies. In contrast, the LT-MA DNA vaccine induced strong cross-reactive NP and HA-specific T cell responses that resulted in reduced viral loads, rapid clearance of virus and lower levels of inflammation upon challenge with influenza, when compared to controls. These results demonstrate that the LT-MA DNA vaccine can induce strong cross-reactive T cell responses that can, independent of neutralizing antibody, mediate significant cross-protection in a nonhuman

primate model. These results support further development of multigenic influenza DNA vaccines for protection against circulating and emerging influenza strains with pandemic potential.

Introduction

There is a crucial need for an influenza vaccine that could provide broad spectrum, “universal” protection against a wider range of influenza variants including strains with pandemic potential. Due to the high level of antigenic drift and shift that occur, there is a need for an influenza vaccine that confers broad spectrum, long-term protection not only from the seasonal circulating strain but from pandemics as well. Attempts to bring universal influenza vaccines to market have been hampered by several obstacles including: overly simplistic immunogen design, limits in the number of antigens that can be included, poor delivery and immunogenicity, and anti-vector immunity. Furthermore, a successful universal influenza vaccine will likely need to induce antibody and T-cell responses to multiple conserved antigens due to the ability of the virus to rapidly mutate to evade host immunity.

DNA vaccines possess a number of characteristics that make them particularly well suited for a universal influenza vaccine [86, 152-154]. In the event of a pandemic threat, DNA vaccines offer an important advantage of accelerated vaccine development and production since the DNA vaccine sequences can be obtained directly from the clinical isolate and rapidly constructed and propagated using well-established molecular techniques without the need for cell culture or eggs [1, 28, 86]. DNA vaccines induce both antibody and T cell responses, and both arms of immunity contribute to cross-protection against different influenza variants [92, 155]. In addition, DNA vaccines have been shown to prime for B cells that produce broadly neutralizing antibody (bNAbs) against multiple HA variants within the same subtype, an effect that may be

due, in part, to cell surface expression of antigens in their natural conformation and induction of antibodies against conserved influenza sequences in the HA stalk region [16, 156-159]. Furthermore, many studies have shown that CD8⁺ T cells induced by vaccination play a critical role in rapid clearance of influenza virus, thus limiting pathogenesis [64-67]. CD4⁺ T cells are also induced following vaccination and play a role in maintaining CD8⁺ T cell memory response and providing help for B cells for rapid antibody production [60, 61].

Early studies showed DNA vaccines were poorly immunogenic in humans [160], but recent advances show that this poor performance can be overcome, in part, by improvement in vaccine delivery and co-delivery of adjuvants [161-163]. One such improvement is particle-mediated epidermal delivery (PMED), otherwise known as the gene gun, which involves the use of a needle-free device to deliver vaccines into the epidermis of the skin. PMED is needle-free and painless, making it attractive for widespread, rapid vaccination campaigns that could employ self-administration and direct distribution through local pharmacies [86]. The device has been designed to administer multiple doses for research purposes and as a disposable, single dose unit for clinical purposes. PMED DNA vaccines have induced protective levels of antibody and CD8⁺ T cell responses against a wide variety of diseases and in a wide range of small and large species including humans [90, 92, 154]. PMED also induces mucosal immune responses in the gut and lung that correlate with protection against mucosal exposure, by blocking the infection at the site of exposure [93, 94].

PMED has been tested in human clinical trials and shown to be a painless, safe, and well-tolerated method for delivery that produces only a mild, transient reaction at the vaccination site [92, 152, 164]. Earlier clinical studies employing injection of DNA with a needle resulted in poor immunogenicity [165]. In contrast, PMED has consistently induced significant humoral and

cellular immune responses in the majority of vaccine recipients in human clinical trials without adjuvants or viral vector boosting [152, 164, 166]. The success of this system is due to direct delivery of the DNA into the cell and the immune competence of the epidermis as a delivery site [16, 19, 86, 88]. These features are also believed to be responsible for the ability of PMED to induce robust immune responses in both small and large animals with very small doses of DNA [154]. In contrast to early DNA vaccines administered intramuscularly by needle, PMED DNA have been shown to be capable of inducing protective levels of immunity in most [167-169] or 100% [152, 170] of vaccinated humans without the need for an adjuvant or viral vector/protein boosting. However PMED-delivered DNA vaccines are still generally less immunogenic in humans as compared to currently licensed live attenuated or protein vaccines containing the same antigens [166, 171, 172]. Therefore additional strategies, such as co-delivery of adjuvants, are being developed to further increase DNA vaccine potency [154, 160, 173].

Genetic adjuvants are plasmids expressing immune-stimulatory genes that are co-administered with DNA vaccines to increase their immunogenicity [174]. The heat-labile enterotoxin from *E. coli* (LT) has been shown to be a powerful adjuvant that can be co-administered with DNA vaccines [174]. LT and a related adjuvant, cholera toxin (CT), are members of the AB5 class of bacterial toxins. Parenteral or mucosal administration of this adjuvant can be toxic. In contrast, LT can be safely delivered to the skin of animal and human subjects, even at high doses (i.e. 500mg) [175]. LT adjuvant activity is mediated in part by direct activation of dendritic cells (DC) and Langerhans cells (LC). LT also promotes migration of DC and LC to mucosal immune tissue such as Peyer's patches [94, 176-178]. The high concentration of DC, specifically LC, in the skin makes LT particularly attractive for enhancing the mucosal and systemic immunogenicity of vaccines administered via the skin and therefore well-suited to

PMED [94, 176, 178]. Consistent with this notion, we previously showed that co-delivery of plasmid expressing the A and B subunits of LT substantially increases DNA vaccine induction of antibody and T cell responses [174].

Here, we investigated immunogenicity and protective efficacy of an LT-adjuvanted multigenic (LT-MA) universal influenza DNA vaccine consisting of HA, M2, and NP antigens in the highly relevant preclinical nonhuman primate model. *Cynomolgus* macaques (*Macaca fascicularis*) have been used to study vaccine and antiviral efficacy against highly pathogenic human and avian influenza viruses [179-182]. Vaccinated cynomolgus macaques developed strong antigen-specific antibody responses and potent, cross-reactive T cell responses that resulted in lower viral loads and less inflammation upon challenge with influenza when compared to control macaques. The LT-MA DNA vaccine induced strong NP-specific T cell responses, which correlated with cross-protection. These results demonstrate the feasibility of using an adjuvanted universal influenza DNA vaccine for protection against drifted and shifted strains of influenza with pandemic potential.

Results

LT-MA DNA vaccine induces strong serum and mucosal antibodies in nonhuman primates.

A successful universal influenza vaccine will likely need to induce antibody and T-cell responses against multiple conserved antigens. To this end, we designed an LT-MA DNA vaccine consisting of four HA genes, the ectodomain of M2 (M2e) and the consensus sequence from the highly conserved influenza A nucleoprotein (NP) [183]. The M2e gene is a 23 amino acid conserved B cell epitope that is poorly immunogenic [184]. To address this, the M2e gene

was fused to a gene encoding the highly immunogenic hepatitis core antigen (HBcAg-M2e). This design results in the expression of HBcAg virus like particles carrying M2e epitopes on their surface [185] and is based on studies employing recombinant protein vaccines which have shown that conjugating M2e to HBcAg virus-like particles enhances M2e immunogenicity [186-188]. Similarly, we previously showed that immunizing with hybrid DNA vaccines expressing HBcAg virus like particles carrying HIV and SIV B or T cell epitopes induced stronger responses than DNA vaccines expressing the whole antigen or the epitopes alone [189, 190].

Each vaccine antigen was expressed and co-delivered on separate plasmids to reduce competition between vectors and maximize epitope expression [155]. The four HA genes consisted of 3 previously circulating seasonal H1, H3, and B strains (H1N1-A/New Caledonia/20/99, H3N2-A/Panama/2007/99, B/Jiangsu/10/03) and one avian H5N1 influenza virus that has recently infected humans (H5N1-A/Vietnam/1203/04) [191]. These immunogens were selected because M2e can induce cross-protective antibodies [192-194] and NP induces cross-reactive cellular responses that, in the absence of effective antibody, can provide some protection against drifted variants [52-59, 195]. The HA immunogens included in the LT-MA DNA vaccine mimic the composition of a seasonal influenza vaccine. Similar to currently marketed vaccines, HA DNA vaccines induce strong antibody against the highly variable HA head that do not contribute to cross-protection. However, HA DNA vaccines have been shown to also induce cross-protective antibody responses against the more conserved stem region of HA [16, 196].

Studies in mice have shown that influenza DNA vaccines induce strong responses and cross-protection against heterologous challenges [197-201]. However, vaccines that stimulate strong responses in mice are often less effective in humans. The nonhuman primate more closely

resembles humans in size and immune response. We therefore investigated the ability of the LT-MA DNA vaccine to induce antibody and T cell responses in cynomolgus macaques. To reduce competition between the vectors, each DNA vaccine vector was coated onto separate gold particles with the LT adjuvant at a 10:1 DNA vaccine to adjuvant ratio and then the gold beads were mixed prior to immunization into the skin with PMED. We previously showed this formulation results in expression of each vaccine plasmid in separate cells and induction of immune responses that are comparable to levels induced when immunizing with each plasmid separately [155]. Macaques received 3 immunizations with the LT-MA DNA vaccine spaced 6 weeks apart. Two weeks after each dose, blood was collected and I measured serum IgG responses by ELISA. After only a single immunization, IgG responses against each component of the vaccine (HA, NP, and M2e) were detected. Antibody responses against each antigen were boosted after the second dose but titers remained unchanged following the third dose in all 8 influenza-naïve animals (**Fig 3.1a**).

I then measured the mucosal IgG responses in the bronchioalveolar lavage (BAL) against M2e, NP and a representative HA antigen (NC99) by ELISA. Mucosal IgG were detectable two weeks after the first vaccine dose increased significantly following the second dose and, in contrast to the serum IgG antibodies, were further boosted following the third dose in 6 of 8 vaccinated macaques (**Fig 3.1b**). Therefore, the PMED delivered LT-MA DNA vaccine induced HA, M2e and NP-specific mucosal antibody in addition to systemic antibody responses, a result that is consistent with our previous findings showing that PMED induces both mucosal and systemic immune responses in nonhuman primates [155, 202, 203].

HA-specific neutralizing antibody responses were measured by hemagglutinin inhibition (HI) assay which measures neutralizing antibodies against the HA head region. The LT-MA

DNA vaccine induced HI antibody titers that greatly exceeded the minimum required for protection in humans (HI titer of 40). HI titers exceeded protective levels in 5/8 (62.5%) macaques after a single dose and in all 8 (100%) macaques after a third dose (**Fig 3.1c**). Significantly, 6 of these 8 animals developed robust HI titers that exceeded 1,000. These results confirm previous findings in nonhuman primates showing PMED DNA vaccination alone, without the need for boosting with recombinant protein or viral vectors, can induce antibody titers that greatly exceed protective levels of immunity against homologous strains of influenza [155].

To determine if these strong antibody responses induced broadly neutralizing antibodies, I analyzed sera from vaccinated macaques for the ability to neutralize homologous and heterologous H1 influenza strains *in vitro*. Consistent with the HI analysis, the sera neutralized the H1N1 virus that is homologous to the H1N1 HA immunogen that was included in the vaccine (NC99) (**Fig 3.1d**). However the serum failed to neutralize two heterologous H1N1 viruses A/PR/8/34 (PR8) or A/California/07/09 (CA09), which respectively share 89% and 80% sequence identity to the H1N1 vaccine strain NC99 (**Fig 3.1d**). These results indicate that the LT-MA DNA vaccine was able to elicit significant levels of neutralizing HA-specific antibodies in the serum of immunized macaques against a matching strain of influenza but not against more genetically distinct or shifted strains of influenza.

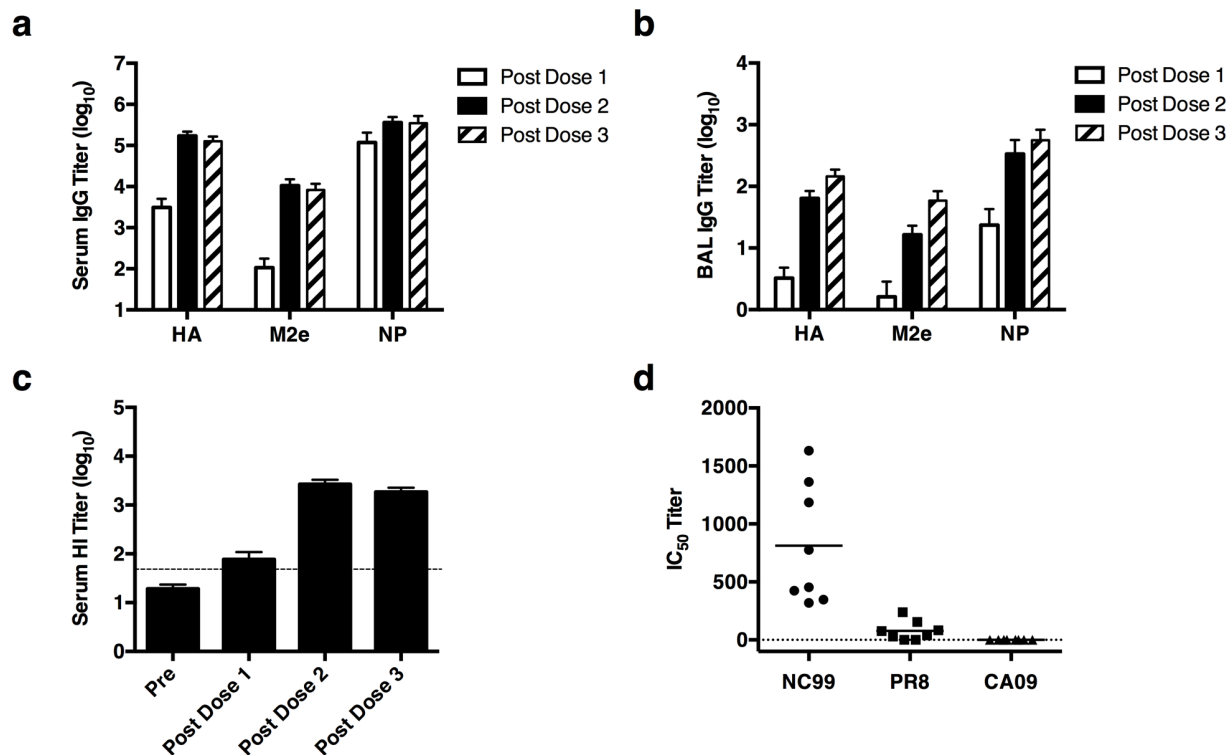


Figure 3.1: LT-MA DNA vaccine induces systemic and mucosal antibodies in NHP against HA, M2e, and NP. Cynomolgus macaques received 3 immunizations with the LT-MA DNA vaccine at 0, 6, and 12 weeks. Sera were collected from vaccinated macaques at various time-points and analyzed for the presence of IgG by ELISA and hemagglutinin inhibition (HI) assay. a) IgG antibody responses against the vaccine components M2e, NP and the representative HA NC99 in the serum. b) IgG antibody responses in the Bronchioalveolar lavage (BAL) collected at various time-points against the vaccine components M2e, NP and HA (NC99). c) Serum HI titers against NC99 virus. d) IC₅₀ titers of vaccinated macaque serum against the matching vaccine strain NC99 and the heterologous unmatched strains PR8 and CA09. Neutralization assays were performed on serum samples taken from vaccinated macaques two weeks after the final immunization.

DNA vaccine induces strong cross-reactive T cell responses in nonhuman primates.

To measure cellular immune responses induced in the blood by the vaccine, a postdoctoral fellow in the Fuller Lab, Dr. Jolie Leonard, performed IFN- γ ELISpot assays using pools of overlapping peptides comprising the entire amino acid sequence of HA, NP and M2e of the heterologous pandemic swine origin challenge strain, CA09 (H1N1). Modest cross-reactive IFN- γ responses were observed 2 weeks after the first vaccine dose, with a robust boost in magnitude after the second vaccine dose. The third vaccine dose did not increase mean IFN- γ responses, but responses were clearly boosted in those animals that exhibited low responses after the second dose. All 8 animals (100%) exhibited strong cross-reactive IFN- γ responses against CA09 peptides following the third dose (**Fig 3.2a**). The cellular responses observed were largely directed against HA and NP antigens, with only 4 of 8 animals generating M2e-reactive T cells (**Fig 3.2a-b**). However, all 8 vaccinated animals generated broadly reactive cellular responses to multiple HA and NP peptide pools (**Fig 3.2b**), and IFN- γ responses were significantly higher in the vaccinated animals when compared to responses in the unvaccinated, control animals, $p = 0.0008$ (**Fig 3.2c**).

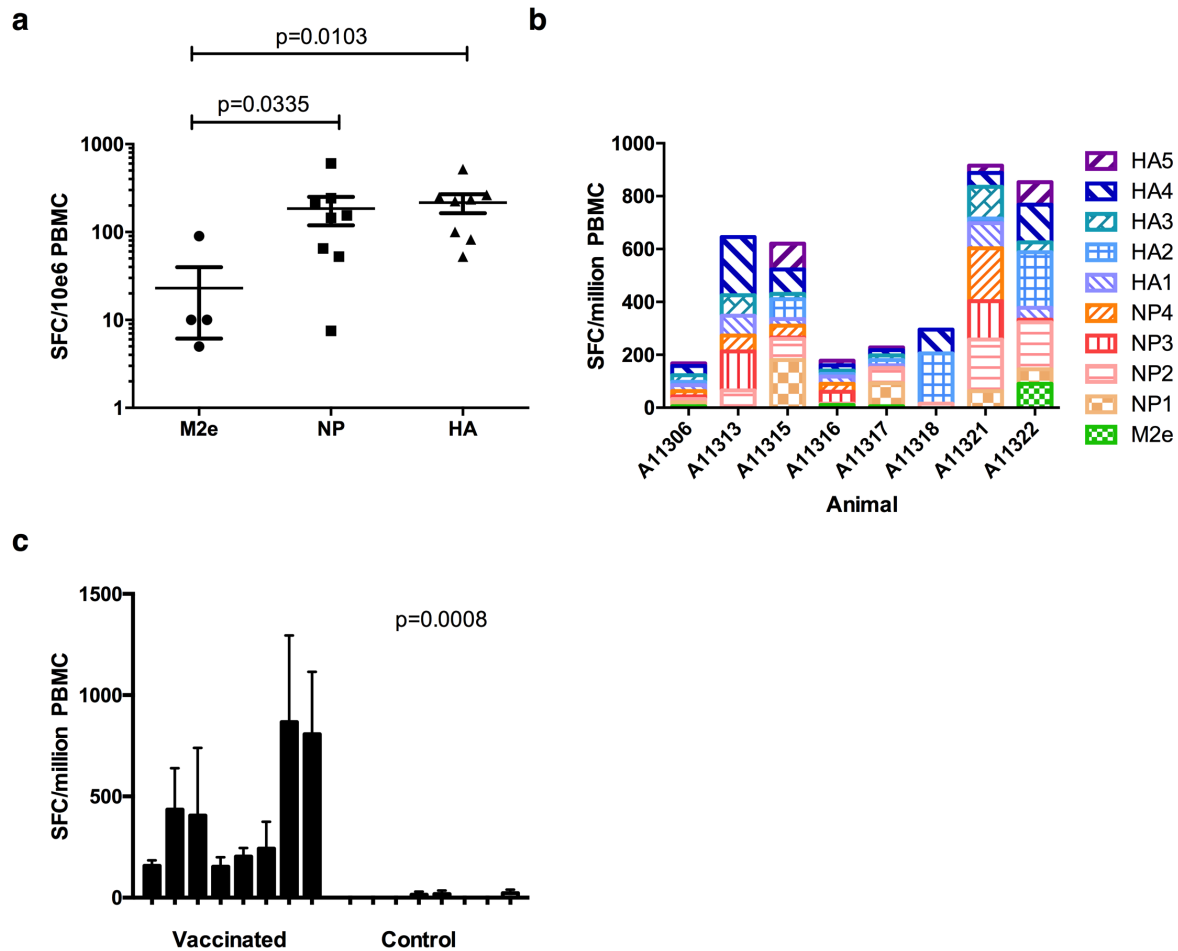


Figure 3.2: DNA immunization induces influenza-specific T cell responses. a) IFN- γ analysis was performed on peripheral blood mononuclear cells. Vaccine-induced responses to specific influenza antigens 2 weeks post third vaccination (week 14). Mean response for each animal is plotted. Error bars represent SEM. b) Responses of vaccinated animals to influenza peptide pools corresponding to subsets of HA, NP, and M2e at 2 weeks post third vaccination (week 14). c) Total cumulative IFN- γ responses against HA, NP, and M2e in vaccinated and control animals at 2 weeks post third vaccination (week 14). Mean of duplicate samples is plotted; error bars represent SEM. Responses after third immunization are significantly elevated over baseline; $p=0.0008$ as calculated by Mann-Whitney test. (Unpublished data courtesy of Dr. Jolie Leonard).

Protection from heterologous influenza challenge.

Two weeks post final vaccination (week 15) control and vaccinated macaques (n=8 macaques per group) were challenged with $10^{7.4}$ PFU of the heterologous H1N1 A/California/04/2009 (CA09) virus through a combination of intratracheal, intranasal, ocular and oral routes [179]. CA09 is a highly virulent Group 1 pandemic influenza strain that shares 80% amino acid sequence identity to the closest related immunogen used in the vaccine (H1N1 NC99) so the heterologous challenge with CA09 mimics vaccination against a seasonal strain and subsequent exposure to an unmatched pandemic strain. Infection of cynomolgus macaques with CA09 results in efficient replication of the virus in the lungs causing severe lung lesions and an influx of inflammatory infiltrates [179].

To assess the effects of the vaccine on protection, bronchioalveolar lavage (BAL) samples were taken at various time points post-challenge. Our collaborators Dr. Kelly Stefano-Cole and Dr. Amy Hartman at the University of Pittsburgh analyzed the BAL samples for viral load by semi-quantitative Taqman RT-PCR. The viral RNA titers in the vaccinated group were significantly lower than the control group at both days 3 and 7 post-challenge ($p < 0.0079$) (**Fig 3.3a**). At day 3, three macaques per group were necropsied and the right lung was collected and fixed in formalin to investigate the effects of vaccination on viral replication, tissue damage and inflammation in lung tissues. Dr. Todd Reinhart at the University of Pittsburgh performed in situ PCR against challenge virus on the right accessory lung (**Fig 3.3b**), and the right lower lung (**Fig 3.3c**). We observed lower viral RNA in the vaccinated lungs compared to the controls for both the right accessory and right lower lung. These results show that the LT-MA DNA vaccine reduced acute viremia and accelerated viral clearance in the lung demonstrating significant protection from the heterologous challenge.

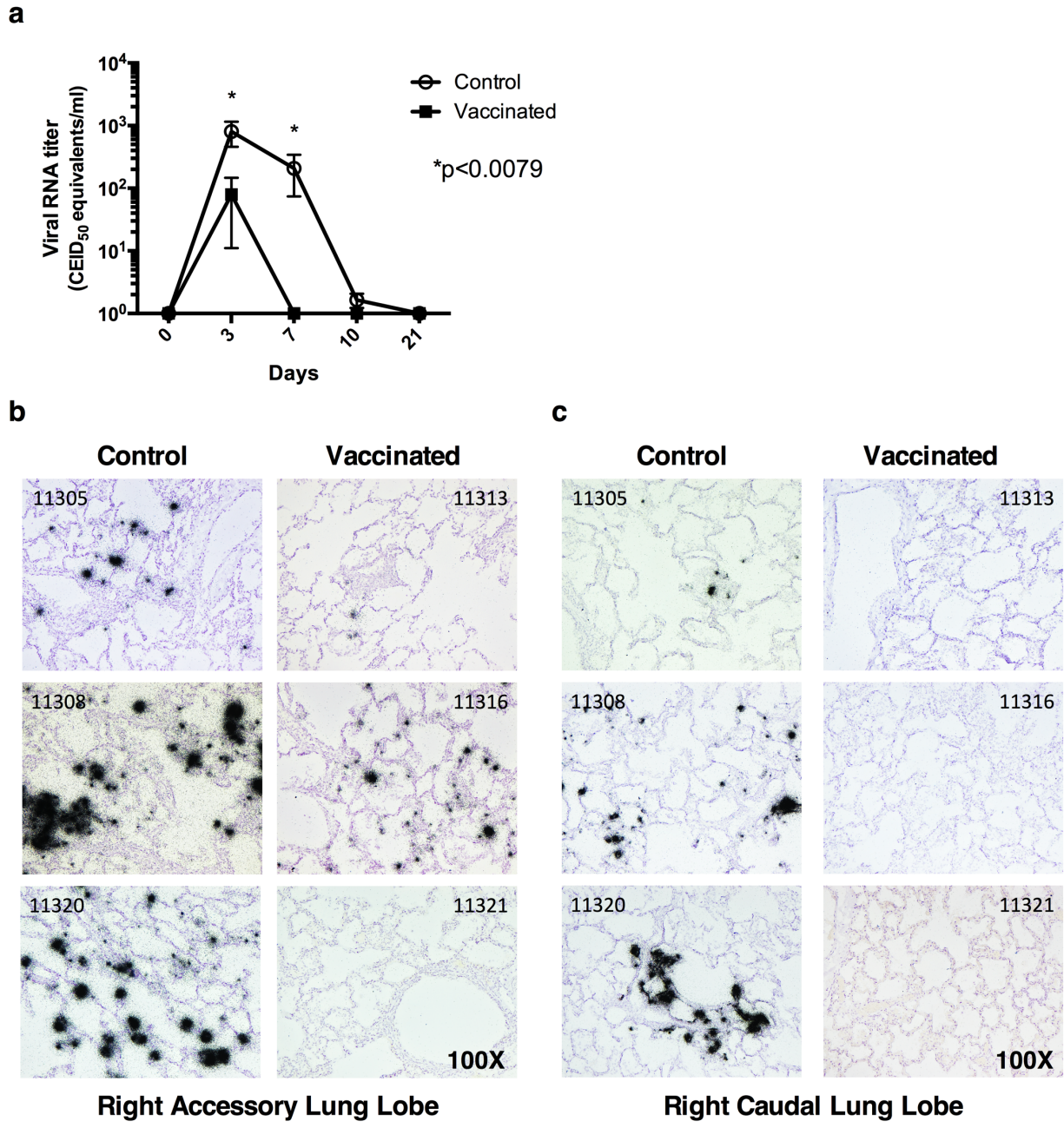


Figure 3.3: Vaccinated macaques have reduced viral RNA loads and viral replication in nasal washes and lungs. Three weeks post final vaccination (week 15), control and vaccinated macaques are challenged with $10^{7.4}$ PFU of A/California/04/2009 virus. a) Bronchioalveolar lavage (BAL) viral RNA titers in control and vaccinated macaques ($p < 0.0079$). In situ hybridization with influenza virus specific, ^{35}S -labeled riboprobes was used to localize challenge virus RNAs in the b) right accessory and c) right caudal lung lobes of control and vaccinated

macaques at day 3 post infection. Viral RNA signals are evident as collections of black silver grains over cells. Animal numbers are noted in the upper portion of each micrograph. (Unpublished data courtesy of Dr. Kelly Stefano-Cole, Dr. Amy Hartman and Dr. Todd Reinhart).

Vaccinated macaques displayed more rapid recall response and less inflammation post-challenge.

Influenza infection results in the induction of inflammatory responses that lead to tissue damage and disease. To determine if the LT-MA DNA vaccine protected from induction of inflammatory responses post-challenge, cytokine analysis was performed on BAL samples to assess soluble factors present in the lungs of the macaques at 3, 7, 11, and 21 days after heterologous challenge. As expected, all cytokines influenced by the infection peaked at days 3 or 7 post-infection (**Fig 3.4a**). Importantly, T-cell activating cytokine IL-2 was significantly elevated in the vaccinated animals during acute infection when compared to the controls ($p=0.0264$), as was leukocyte chemoattractant RANTES ($p=0.0430$) (**Fig 3.4a**), which is produced by epithelial cells in response to influenza infection in order to recruit leukocytes to the site of infection [204]. On the other hand, Eotaxin, MIG, IL-6 and TNF α , which are pro-inflammatory and chemoattractant responses that are induced by influenza infection, peaked early in the vaccinated group at 3 days post infection but then declined by 7 days post infection. This is in contrast to the control group, whose responses continued to increase or stay elevated between 3-7 days post infection (**Fig 3.4a**). Anti-influenza inflammatory responses were more rapidly resolved in the vaccinated animals than in the controls, an outcome that is consistent with the vaccine mediating more rapid viral clearance and reduced lung inflammation.

To confirm these findings, three animals from each treatment group were sacrificed at 3 days post infection in order to assess lung pathology and immune cell infiltrates. Dr. Jolie Leonard used flow cytometry to analyze the phenotypic profile of leukocytes isolated from lung tissue. Her analysis revealed significantly more CD8⁺ T lymphocytes present in the lungs of vaccinated macaques as compared to controls ($p = 0.018$, **Fig 3.4b**). Statistical significance was

difficult to achieve with an n-value of only three animals per group necropsied at this time point. However, the vaccinated animals appeared to have similar levels of CD4⁺ T lymphocyte and CD20⁺ B lymphocyte infiltration, and decreased CD14⁺/CD11b⁺ macrophage recruitment as compared to unvaccinated control animals (**Fig 3.4b-d**). These data support the hypothesis that the LT-MA DNA vaccine induced a rapid, local T lymphocyte recall response and decreased lung inflammation.

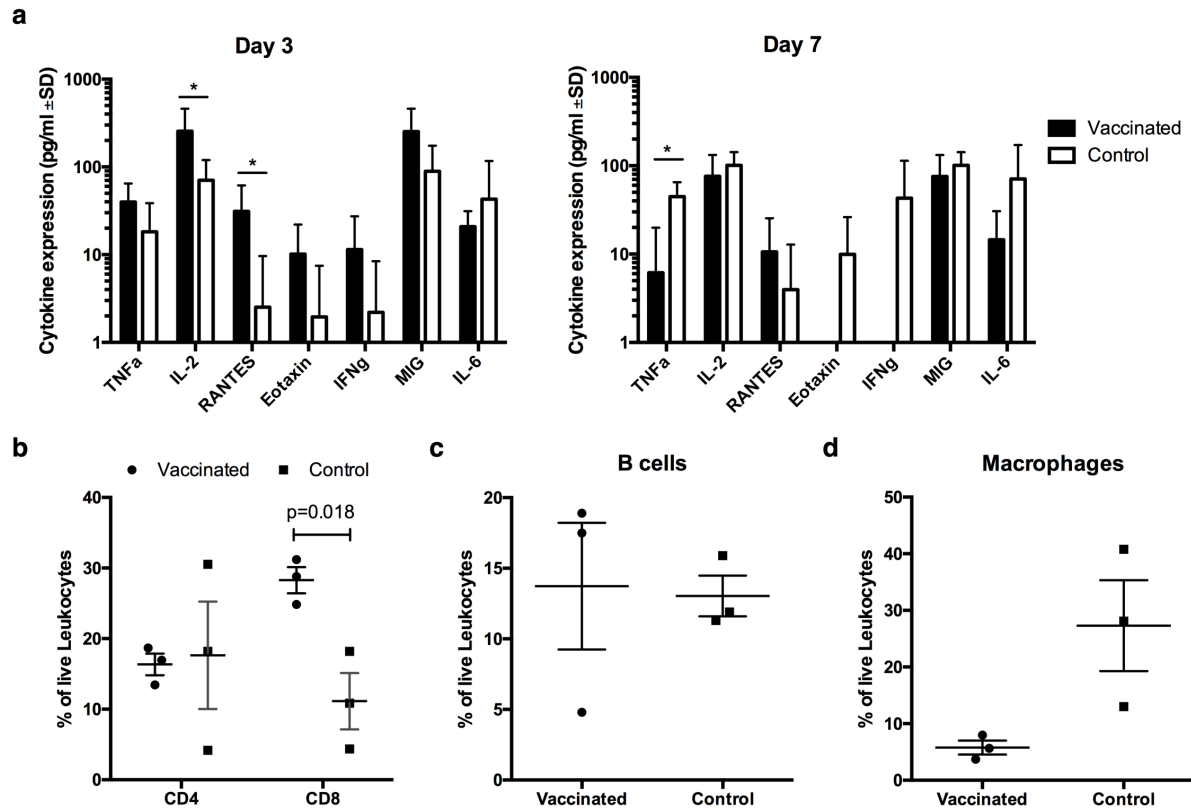


Figure 3.4: Vaccinated macaques displayed more rapid response and less inflammation. a)

Inflammatory cytokines were assayed by Bio-Plex using BAL obtained from vaccinated and control macaques on day 3 and day 7 following CA09 challenge (day 3 n=8 per group, day 7 n=5 per group) *P < 0.05. Percentage of b) CD4+ and CD8+ T cells (P=0.018), c) B cells, and d) macrophages in vaccinated and control macaques at day 3 post-challenge. (Unpublished phenotype data courtesy of Dr. Jolie Leonard).

Protection from influenza is mediated by T cell responses.

I next investigated the possible immune correlates of protection. The DNA vaccine induced strong cross-reactive T cell responses against the challenge strain, but we were unable to detect broadly neutralizing antibody responses. However, antibody-mediated protection can be based on effector mechanisms other than *in vitro* neutralization, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent complement-mediated lysis [41, 42]. To investigate the contribution of the antibodies to protection, I passively transferred purified IgG from vaccinated and control macaques to naive mice (25 mg/animal) by intraperitoneal (IP) injection 24 hours before lethal challenge with the mouse-adapted CA09 virus. The mouse-adapted CA09 virus leads to rapid weight loss and death in mice within 5-8 days post-infection [120]. IgG was purified from sera collected from vaccinated and control macaques before the first vaccine dose (Baseline, week 0), 3 weeks after final vaccine dose but before challenge (Vaccination, week 15) and 3 weeks after the challenge (Control, week 18) and (Vaccinated, week 18). Naive mice (PBS) received PBS by IP injection 24 hours before CA09 challenge and as expected, lost greater than 25% of their weight and were euthanized within 6-8 days. Similarly, mice that received IgG purified from sera collected from either control or vaccinated animals before the first vaccine dose (week 0) or after the final dose (week 15) succumbed within 7-8 days post-challenge (**Fig 3.5a**). As expected, IgG purified from sera collected post-challenge (week 18) after strong HI titers arose in response to the challenge virus, protected mice from lethal CA09 challenge (**Fig 3.5a**). These results indicate that although the LT-MA DNA vaccine induced robust antibody responses against the vaccine immunogens, these responses provided no protection against the heterologous virus challenge.

To investigate the contribution of T cell responses to protection, Dr. Jolie Leonard analyzed the magnitude, function and specificity of T cell responses that developed in response to CA09 three weeks post-challenge in vaccinated and control macaques. Analysis of the magnitude of the IFN- γ T cell responses by ELISpot post-challenge against CA-09-specific HA, NP and M2e peptide pools revealed significantly elevated cumulative IFN- γ responses in the vaccinated animals when compared to the controls (total responses, $p < 0.05$) with the greatest proportion of cross-reactive IFN- γ responses directed against NP peptide pools ($p < 0.02$) (**Fig 3.5b**). These results indicate that the DNA vaccine primed for robust recall T cell responses against the heterologous CA09 challenge strain.

To determine the effects of the DNA vaccine on T cell function, Dr. Jolie Leonard used intracellular cytokine staining to analyze individual and multifunctional T cell responses in response to stimulation with CA09 peptides. Vaccination increased the breadth and polyfunctionality of both CD4⁺ and CD8⁺ T cells when compared to the controls (**Fig 3.5c**). Specifically, vaccination significantly increased NP-specific CD4⁺ cells expressing TNF α ⁺/IL-2⁺/IFN γ ⁺ ($p=0.008$), TNF α ⁺/IL-2⁺ ($p = 0.048$), and IL-2⁺ ($p = 0.008$) at week 14. Following challenge, the vaccinated animals demonstrated significantly more multifunction effector responses than control animals at 11 days post infection (**Fig 3.5c**). Interestingly, both CD8⁺ and CD4⁺ HA-specific cells expressing TNF α ⁺/IL-2⁺ ($p = 0.048$, $p = 0.016$) were increased in the vaccinated animals compared to controls, as well as NP-specific CD4⁺ cells expressing TNF α ⁺/IL-2⁺ ($p = 0.032$). Protection provided by memory CD4⁺ and CD8⁺ T cells correlates with the capacity to produce multiple cytokines, including IFN- γ , TNF, and IL-2, rather than any one cytokine alone [205].

To determine if the magnitude of pre- or post-challenge T cell responses correlate with protection, I compared NP, HA and M2e-specific T cell responses measured by IFN γ ELISpot after the final DNA vaccine dose at week 3 weeks post-challenge to viral loads measured 3 days post-infection. The results in **Figure 3.6a** show a significant inverse correlation between the viral loads and only the NP-specific IFN- γ T cell responses measured after the final DNA vaccination ($p=0.0161$, $r=-0.8501$) but not 3 weeks post-challenge. In contrast there was no statistically significant correlation between the viral load and the magnitude of HA-specific or M2e-specific IFN- γ T cell responses measured post-vaccination or 3 weeks post-challenge (**Fig 3.6a-c**). I also compared antibody responses, monofunctional and polyfunctional T cell responses, cytokine responses measured at week 15 by Bio-Plex, and overall frequency of CD4+, CD8+, B cell and macrophage responses measured at week 15 by immunophenotyping and observed no statistically significant correlations. Taken together, these results indicate that vaccine-induced NP-specific IFN- γ T cell responses provided the primary mechanism of protection in this study.

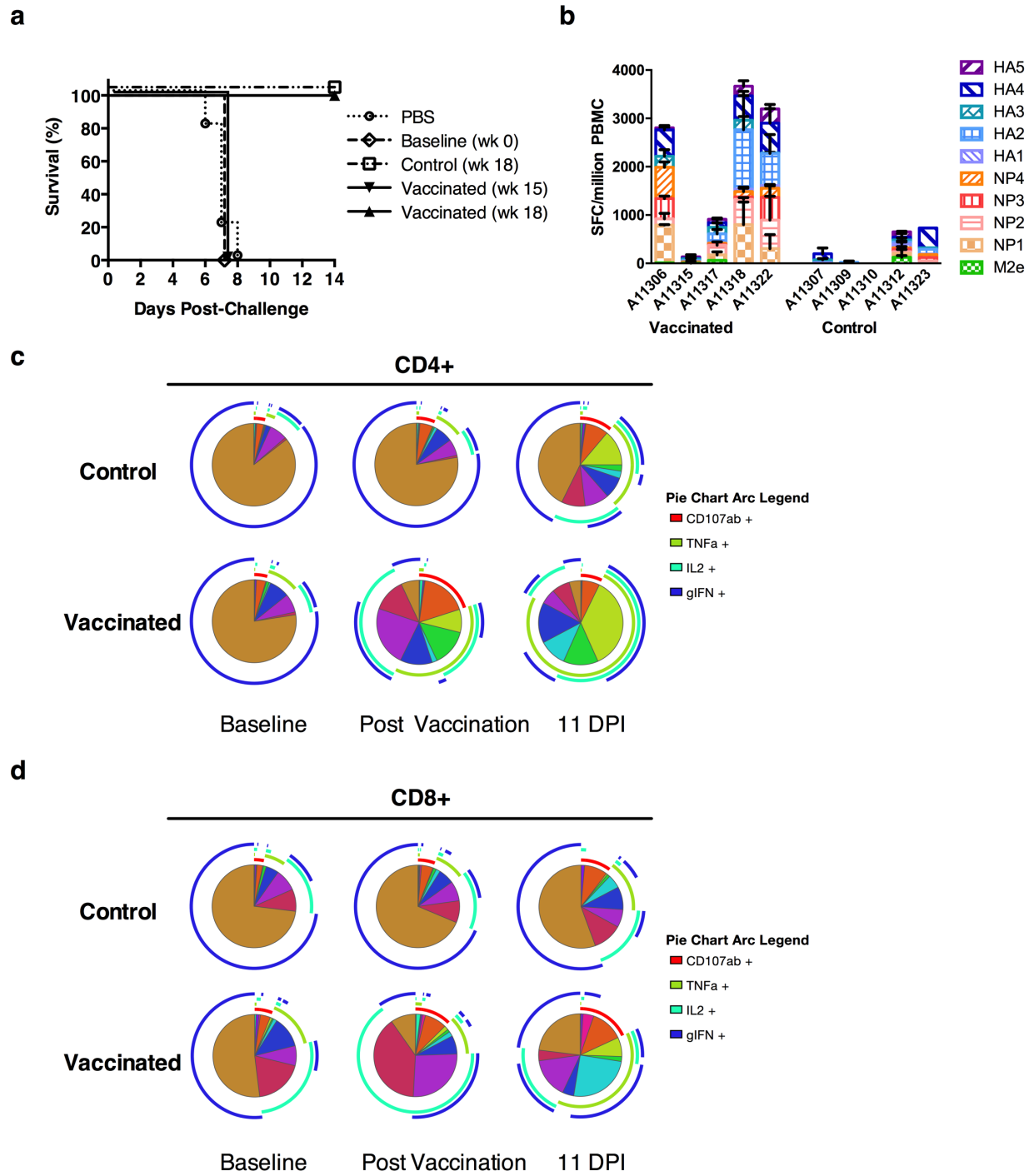


Figure 3.5: Protection from influenza is mediated by T cell responses and not antibody responses. (a) Passive transfer in mice of purified IgG from vaccinated and control macaques followed by influenza challenge with 10 MLD₅₀ CA09 virus. Survival of BALB/c mice (n = 10

per group) passively immunized (intraperitoneally) with 25 mg IgG 24 hrs before challenge with a lethal dose of CA09 influenza virus. (b) Sum of responses of vaccinated animals to influenza peptide pools corresponding to subsets of HA, NP and M2e at 3 weeks post challenge (week 18) $p < 0.05$. SPICE graphs displaying cumulative polyfunctional CD4+ (c) and CD8+ (d) T cell responses following stimulation with HA, NP, and M2e peptide pools at baseline (week 0), post vaccination (week 14) and 11 days post infection (DPI) (week 16+4 days). The pie charts summarize the cumulative CD4+ and CD8+ T cell responses to the various effector functions as either mono-or poly-functional responses. The colored sections of the pie correspond to the different cytokines expressed by the T cell responses (CD107ab, TNF- α , IL-2, IFN- γ) in mono-or poly-functional combinations. (Unpublished data courtesy of Dr. Jolie Leonard).

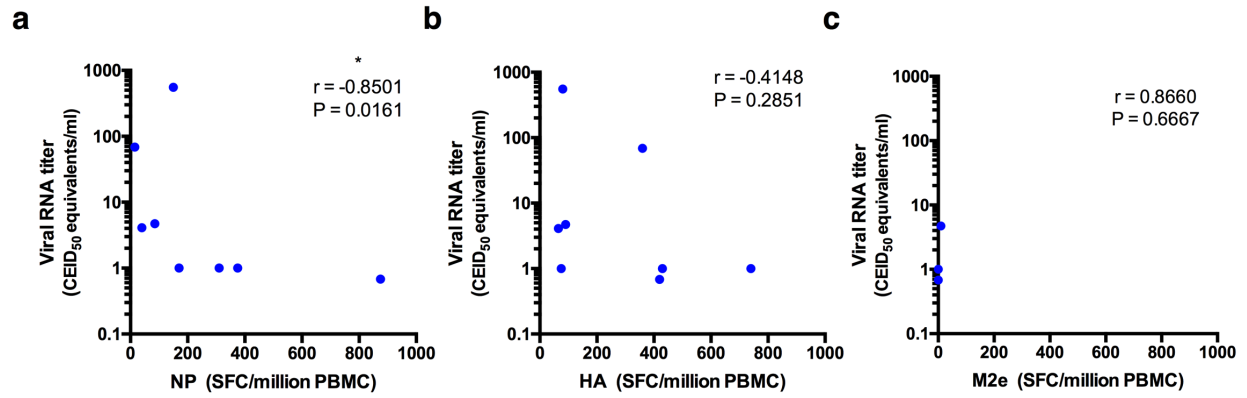


Figure 3.6: Protection from influenza correlates with NP-specific T cell responses.

Correlation between viral RNA titer (CEID₅₀ equivalents/ml) on day 3 post-infection and (a) NP-specific, (b) HA-specific, (c) M2e-specific IFN- γ T cell responses prior to challenge at 2 weeks post third vaccination (week 14).

Discussion

A successful universal influenza vaccine would need to induce a strong broadly neutralizing antibody response as well as generate robust cross-reactive T cell responses. Here we show that the LT-MA DNA vaccine induced strong binding antibody, protective levels of HA-specific antibody, and robust T cell responses in the highly relevant preclinical nonhuman primate model. This LT-MA DNA vaccine generated strong mucosal responses, eliciting both a humoral and cellular response, and provided a frontline defense against influenza infection [206, 207]. Several studies have shown that DNA vaccines expressing one or more influenza antigens can protect against influenza in multiple species including mice, swine, ferrets, and nonhuman primates, and protection has been correlated to both antibody and T cell responses [155, 208-211]. Heterosubtypic T cell responses reduce viral shedding, limit disease severity, and accelerate viral clearance [45-50]. In humans, prior exposure to seasonal influenza results in memory T cells that cross-react with pandemic strains and correlate with reduced viral shedding and a lower incidence of clinical disease [52-59].

This LT-MA DNA vaccine was effective in inducing strong NP-specific IFN- γ T cell responses that mediated enhanced viral clearance and significant cross-protection against a drifted strain of influenza [46, 195, 212]. In the absence of an effective antibody response, the LT-MA DNA vaccine induced CD4⁺ and CD8⁺ responses that still provided significant protection. Vaccinated animals also generated cross-reactive T cell responses. These results demonstrate that a PMED delivery of a universal influenza DNA vaccine formulated with a potent genetic adjuvant is alone, without requiring a viral vector or protein boost, sufficient to induce potent levels of antibody and T cell responses in a preclinical nonhuman primate model. Furthermore, we show these responses could mediate broad protection against a drifted

pandemic strain of influenza that shares a maximum 80% identity with the HA immunogens included in the vaccine.

DNA vaccines have been combined with other types of vaccines, such as live viral vectors or recombinant proteins in a prime-boost regimen that results in a synergistic immune response that is stronger than either vaccine alone [16, 196, 213]. The DNA vaccine priming appears to expand the antibody epitope repertoire and increase affinity maturation, and to direct development of T cells with effector memory phenotype [214-217]. The generation of broadly neutralizing antibodies was dependent on the DNA priming, which can increase the number and diversity of the CD4+ clones that stimulate B cells to secrete antibodies of greater magnitude and diversity [16, 218]. I observed a significant induction of HA-specific antibody responses following DNA prime/boost. In fact, my results show that a DNA vaccine alone can induce a robust HI titer that significantly exceeds protective levels in humans (1:40) and is comparable to levels induced by DNA priming followed by heterologous boosting with a viral vectored vaccine [16, 211]. However, DNA vaccination alone did not induce broadly neutralizing antibodies. This is in contrast to previous work demonstrating that a DNA prime-Ad5 boost induces broadly neutralizing antibodies in mice, ferrets and non-human primates [16]. Specifically, my results show that DNA vaccination, even if able to induce high antibody titers, was alone, not capable of generating broadly neutralizing antibodies. This suggests that the induction of broadly neutralizing antibodies by heterologous prime-boost regimens involving DNA, viral vectored vaccines or recombinant protein vaccines is not due to simply expanding responses primed by the DNA vaccine. Rather my results suggest that with an efficient delivery and potent adjuvants, DNA vaccines alone can induce robust antibody responses, however a viral vector or protein boost may be required to increase the diversity of the antibody repertoire [219, 220].

On the other hand, the PMED LT-MA DNA vaccine induced T cells that were broad in specificity, with the ability to cross-react to a variety of sequences and antigens in a heterologous influenza strain. This result is consistent with our previous studies showing an adjuvanted HA DNA vaccine administered by PMED induced broadly specific T cell responses against multiple sequences in HA and administration of a therapeutic PMED DNA vaccine induced broadly specific T cell responses in SIV-infected macaques that correlated with improved viral control [174, 221]. Furthermore, LT has been shown to stimulate robust antigen-specific CD8⁺ T cell responses, which aid in viral clearance [222]. In the absence of effective antibody-mediated protection, T cell responses can reduce viral shedding and mediate rapid clearance from infection and reduce inflammation [43]. We observed rapid recall responses and early induction of the pro-inflammatory cytokines in vaccinated macaques compared to controls. These results demonstrate that vaccination results in the induction of a robust antiviral response early in infection, while limiting the recruitment of inflammatory leukocytes, viral replication, and tissue damage. Our results show that vaccine stimulation of T cell responses against a highly conserved immunogen is sufficient to mediate robust heterologous protection from a highly divergent strain of influenza [43].

These results also suggest that a universal influenza vaccine should include multiple antigens, specifically HA and NP. Previous studies have shown that vaccination with a DNA vaccine expressing a consensus sequence for an H5N1 NP-induced T cell responses that resulted in reduced viral replication and increased protection [162]. Furthermore, another study demonstrated that even without the presence of neutralizing antibodies, macaques “primed” with a sublethal infection of influenza virus generated cross-reactive T cell responses to both HA and NP that mediated protection against heterologous challenge [43]. These results make a strong

case for generating a universal influenza vaccine that is capable of inducing cross-reactive T cell responses to enhance existing antibody-based modalities. Taken together, our results demonstrate that a LT-MA DNA vaccine generates robust cross-reactive T cell responses that even in the complete absence of antibody-mediated protection could still provide protection against influenza. A universal influenza DNA vaccine that induces robust cross-reactive T cell responses against influenza antigens, in addition to strong antibody responses against homologous strains could provide an important alternative to existing influenza vaccines by providing antibody-mediated protection against known circulating seasonal strains of influenza and at the same time inducing T cell responses that could provide added protection against emerging or unexpected pandemics.

Materials and Methods

Research animals and ethics statement

All animal experiments used in this study were approved by the University of Washington Institutional Animal Care and Use Committee, and in accordance with the guidelines of the United States National Research Council and the Weatherall Report. This study used male cynomolgus macaques (*macaca fascicularis*) between the ages of 4 and 6 (average 5). All procedures were performed under ketamine sedation (10 mg/kg) and all efforts were made to minimize suffering.

Plasmid construction and vaccine formulation

Codon optimized coding sequences for the hemagglutinin antigens, and a NP consensus sequence determined by alignment of the NP protein sequences of H1N1-A/New Caledonia/20/99, H3N2-A/Panama/2007/99, and H5N1-A/Vietnam/1203/04 were designed and constructed by GeneArt. The coding sequences were inserted into the Nhe1 and Bgl2 sites of PJV7563 [155], an empty DNA vaccine expression plasmid. HBcAg-M2e was constructed by inserting oligonucleotides that code for M2e into the Bsp120I site of plasmid PJV7063 [190], resulting in a plasmid that expresses a fusion of M2e in the immunodominant loop of HBcAg. The construction of the LT plasmid is described elsewhere [223]. Plasmid DNA was precipitated onto 1-3mm gold particles as previously described [166] at a rate of 1.8ug of each antigen plasmid and 0.2 µg LT adjuvant plasmid (10:1 ratio of DNA vaccine to adjuvant) per 1.0mg of gold. Animals were sedated, the leg and abdominal fur was clipped, and DNA-coated fold particles were accelerated into the skin of the abdominal and inguinal regions using a gene gun PMED device. DNA-coated gold particles were delivered at a helium pressure of 45 bar psi. Each actuation resulted in the delivery of 2 mg of DNA into the epidermis. A single dose consisted of 16 actuations for a sum of 32 mg. Each dose was administered 6 weeks apart.

Production of H1N1 Influenza A Virus

Sub-confluent MDCK cell monolayers in T75 flasks were washed and inoculated with 2ml serum-free MEM containing 0.5 multiplicity of infection (MOI) A/California/07/2009 (CA09) H1N1 Influenza A Virus obtained from J.T. Weinfurter (Wisconsin Primate Center, UW Madison) for 1 hour at 37°C/5% CO₂ [43]. The virus and cell inoculum was rocked every 15 minutes to avoid drying of the monolayer and then 18 ml additional medium was added to each

flask and the culture medium was harvested at 36 hours post-infection. Cell debris was pelleted at 200rpm for 10min at 4°C, and then filter-sterilized through a 0.22micron filter. Virus stock was frozen at -80°C in 1ml aliquots, and titered by plaque assay on MDCK cells.

Experimental challenge of cynomolgus macaques with influenza virus

Eight macaques per group (Vaccinated and Control) were intramuscularly anaesthetized with ketamine (10 mg/kg) and inoculated with a suspension containing $10^{6.5}$ p.f.u. ml⁻¹ of CA09 virus through a combination of intratracheal (4.5 ml), intranasal (0.5 ml per nostril), ocular (0.1 ml per eye) and oral (1 ml) routes (resulting in a total infectious dose of $10^{7.4}$ PFU). Macaques were monitored daily for clinical signs of disease including, sneezing, nasal discharge, weight loss and activity level. On days 0, 3, 7, 10 and 21 after infection, nasal and tracheal swabs and bronchial brush samples were collected. On day 3 after infection, 3 macaques per group were necropsied for virological and pathological examinations.

Enzyme-linked immunosorbent assay (ELISA)

HA-specific IgG antibody levels in mouse and macaque serum and bronchioalveolar lavage (BAL) were assessed by enzyme-linked immunosorbent assay (ELISA). Maxisorp plates (Thermo Scientific-Nunc) were coated with 100 ng/well of recombinant A/New Caledonia/20/99 (Protein Sciences), M2e, or NP in PBS overnight at 4°C. Plates were blocked with 5% nonfat milk powder in PBS for 1 h at room temperature, and then washed three times with wash buffer (PBS-T; phosphate-buffered saline containing 0.05% Tween 20). Three-fold serial dilutions of samples were added to the wells and plates were incubated for 1 hr at room temperature. Following three washes with PBS-T, plates were incubated with horseradish-peroxidase

conjugated goat anti-macaque IgG (1/5,000 dilution) secondary antibodies (Nordic Immunological Laboratories) for 1 hr at room temperature. After five washes with PBS-T, TMB substrate (KPL) was added to the wells for 30 min at room temperature. Color development was stopped by the addition of TMB Stop solution (KPL), and the plates were read at 450 nm. Antibody endpoint titers were calculated by nonlinear regression analysis of curves using GraphPad Prism and were determined to be two standard deviations above the baseline.

Hemagglutinin inhibition assay

RDE (Accurate Chemical & Scientific Corp.) treated macaque sera were analyzed for the presence of influenza A/New Caledonia/20/99 specific antibody using a hemagglutination inhibition (HI) assay as described [153]. Briefly, two-fold serial dilutions of RDE-treated sera were incubated with 4 hemagglutination units of influenza A/New Caledonia/20/99 virus. After 30 min at room temperature, 50 μ l of 0.5% turkey RBCs (Lampire Biological Laboratories) suspended in PBS was added to each well and incubated for an additional 30 minutes. Serum HAI titers are reported from the average of duplicate tests as the reciprocal dilution of serum found to inhibit hemagglutination.

Neutralization assay

Neutralization assays were performed using the PB1flank-eGFP viruses as previously described [224]. Briefly, macaque serum was heat inactivated at 56°C for 1 hr, then five-fold diluted in a 96-well plate, and virus was added at an MOI that ranged from 0.1 to 0.8 for the different viruses. Plates were incubated at 37°C for 1 hr to allow antibody binding, and then 4×10^4 MDCK-SIAT1-CMV-PB1 cells were added per well. After an 18-hr incubation, GFP

fluorescence intensity was measured using an excitation wavelength of 485 nm and an emission wavelength of 515 nm. Values are reported as percent infectivity remaining averaged over triplicate measurements.

Passive transfer studies

Sera from vaccinated and control macaques were collected at week 0 (Baseline, vaccinated and control macaques before vaccination and challenge, n=16), week 15 (Pre-Vaccinated, sera from vaccinated macaques pre-challenge only, n=8), week 18 (Post-Vaccinated, sera from vaccinated macaques post-challenge, n=5) and week 18 (Post-Controls, sera from control macaques post-challenge, n=5). IgG from immune sera was purified with protein G (Life Technologies) using the manufacturer's protocol. Female BALB/c mice (n = 10/group) received saline (Ctr) or 25 mg/animal of either Baseline, Pre-Vaccinated, Post-Vaccinated or Post-Control IgG via an intraperitoneal route. 24 hrs post passive transfer mice were challenge with a lethal dose (10 times the 50% mouse lethal dose or 10 MLD₅₀) of the mouse adapted H1N1 A/California/04/2009 (CA09) virus [120]. The mice were monitored daily for weight loss and survival until 14 days post-infection. Animals that lost more than 30% of their initial body weight were euthanized by carbon dioxide in accordance with our animal protocols.

Isolation of mononuclear cells from blood

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation. Erythrocytes were removed using ACK lysis buffer (BioWhittaker) and remaining cells washed with RPMI-1640 supplemented with 10% fetal bovine serum.

ELISPOT

PBMC's were stimulated with 10 individual peptide pools (1mg/mL each peptide) spanning the full amino acid sequences of HA, NP, and M2e. HA and NP peptides were 15-mers overlapping by 11 amino acids (BEI Resources), M2e peptides were 10-mers overlapping by 9 (Mimotopes). Each pool contained 14-27 individual peptides. Concanavalin A (Sigma) was used as a positive stimulation control (5mg/mL). DMSO served as a negative solvent control. Antigen-specific T cells secreting IFN- γ were detected using paired anti-macaque IFN- γ monoclonal antibodies (U-cytech-BV) as previously described. Spot forming cells (SFC) were enumerated using an Immunospot Analyzer with CTL Immunospot Profession Software (Cellular Technology Ltd.). Results are expressed as the mean number of SFC in replicate wells containing antigenic peptide, subtracting the number of spots from DMSO control wells from the same animal.

Viral titers by real-time RT-PCR

Viral loads were measured bronchoalveolar lavage samples by semi-quantitative real-time RT-PCR using the CDC protocol for swine influenza A (H1N1) (kit obtained from BEI Repository NR-15577) [225]. Briefly, RNA was extracted from BAL samples using a modified version of the Invitrogen PureLink Viral RNA/DNA kit (cat. # 12280-050). Semi-quantitative RT-PCR was performed using Invitrogen SuperScript III Platinum One-Step Quantitative Kit and Swine Influenza H1 (swH1) primers and probe from the BEI kit. A standard curve was generated by extracting RNA from 10-fold dilutions of CA09 virus of known chicken egg infectious dose 50 (CEID₅₀). The relative CEID₅₀ of unknown NHP samples were back calculated by comparison with CT values from this standard curve and are expressed as CEID₅₀

equivalents/ml. No template and positive (a sample from a prior experiment) template controls were included in each PCR run.

In Situ Hybridization for Influenza Virus RNAs. In situ hybridization (ISH) was performed using ³⁵S-labeled riboprobes to detect influenza virus RNAs in sections of formalin-fixed, paraffin-embedded lung tissues as described [226, 227]. Following stringent hybridization and washing, autoradiographic exposure times were 14 days. The following primers were used to obtain influenza virus partial cDNAs from viral segments 4 and 5: H1N1 Cal 2009 seg4 HA forward, 5'-TAA GAA GGC AAT ACT AGT AGT TCT GC-3'; H1N1 Cal 2009 seg4 HA reverse, 5'-TGC TAT TTC CGG CTT GAA CT-3'; H1N1 Cal 2009 seg5 NP forward, 5'-TAA GGC GTC TCA AGG CAC CAA ACG A-3'; and H1N1 Cal 2009 seg5 NP reverse, 5'-CAT TTT CAC CCC TCC AGA AA-3'. RT-PCR was performed using total RNA prepared from infected cells as template for cDNA synthesis followed by PCR. The resulting PCR products were agarose gel purified, ligated to pGEM-T vector (Promega) and DNA sequenced. The resulting cDNA-containing plasmids were linearized by restriction digestion and used as template for in vitro transcription to generate sense and anti-sense riboprobes.

Bio-Plex analysis of cytokine production in BAL

The concentrations of cytokines in the lung were measured by analyzing BAL on days 0, 3, 7, 11 and 21 days post-infection. The levels of interleukin IL-2, IL-6, IFN- γ , and TNF- α , RANTES, Eotaxin, and MIG in the lysate were measured using a Bio-Plex multiplex bead array kit (Bio-Rad, Hercules, CA). The Bio-Plex assay was performed in accordance with the manufacturer's instructions.

Statistical analyses

All of the analyses were performed using Graphpad Prism version 5.01. A Student's t test (to compare two samples) and analysis of variance (ANOVA) (to compare multiple samples) were used for statistical analysis. A P value of <0.05 was considered to be significant.

Chapter 4

Universal Influenza DNA vaccine based on the conserved stem region of hemagglutinin

M.T.K. contribution: Conceived of study and working with P.H. designed headless HA vaccine immunogens. Carried out all in vivo mouse vaccination and challenge studies, and analyzed all antibody and T cell responses. Collaborators contributing to this work; Possu Huang.

Abstract

Recent avian and swine-origin influenza virus outbreaks illustrate the ongoing threat of influenza pandemic. New vaccines that offer accelerated production and broader, more universal protection against drifted and shifted strains are needed. We have previously shown that a particle-mediated epidermal delivered (PMED) DNA vaccine expressing four HA antigens, nucleoprotein and the ectodomain of M2 (M2e) induced robust antibody and T cell responses in mice and non-human primates. However the antibodies generated were specific for the HA head and as a result were strain specific. We, and others, have shown that antibodies or small proteins that bind to the stem of HA can provide complete protection in mice, ferrets, non-human primates and humans. Therefore, a vaccine that elicits antibodies against the conserved stem region could provide universal protection against influenza. Here, I investigated a DNA vaccine for the ability to induce antibody and T cell responses against the conserved HA stem and provide protection from lethal influenza infection.

Introduction

A successful universal influenza vaccine will likely need to induce antibodies against antigens that don't mutate frequently but rather, are highly conserved between genetically distinct strains. The stem region of the influenza HA antigen is highly conserved and could, therefore, be an excellent target for a universal vaccine. HA stem-specific vaccines would shift the antibody response away from the immunodominant head domain to the more conserved stem domain. Interestingly, stem-specific antibodies occur naturally in humans, although at low frequencies [18, 95, 228]. Approaches to developing stem-based universal vaccines have included headless HA [229-232], recombinant soluble HA [158, 233-236], synthetic polypeptides [237], prime-boost regimens [16, 196], nanoparticles [238], and recombinant influenza viruses expressing chimeric HA (cHA) [158, 234]. Due to sequence conservation, a universal HA stem vaccine would likely require three components; an influenza A group 1 and group 2 HA, as well as an influenza B virus HA.

Since the stem is more conserved than the immunodominant HA head, antibodies elicited against the stem would likely confer better immunity against antigenic influenza variants without requiring annual reformulations including antigenic shift variants with pandemic potential. However, in its natural conformation, the stem is shrouded from immune recognition [239, 240]. This has complicated efforts to design and purify a stem antigen that retains conformationally dependent B cell epitopes and also elicits strong and broadly neutralizing antibody [240]. Here we combine a novel computational protein folding prediction program called RosettaRemodel with the numerous advantages of DNA vaccination to try and identify an optimal universal influenza stem-based vaccine that induces strong broadly neutralizing antibodies. We hypothesize that the computationally optimized DNA vaccine derived by this approach will elicit

broadly neutralizing antibodies against the stem and afford protection against shifted and drifted strains of influenza. If successful, this will generate a novel, more potent HA stem immunogen than has been possible by traditional vaccine design approaches and advance a new paradigm in vaccine discovery that translates computational modeling of immunogens to successful vaccines *in vivo*.

HA stem as a universal influenza vaccine immunogen

During influenza infection or after vaccination, neutralizing antibodies typically develop against epitopes on the HA head (HA1), and do not cross-react with other HA subtypes. Regions of HA1 are easily mutated without loss of viral fitness whereas the conserved epitopes on the HA stem (HA2) region are expected to be less tolerant of mutations [16, 87, 231, 239, 241-246] (**Fig. 1**). The HA2 subunit of the influenza virus hemagglutinin is relatively well conserved compared to the HA1 globular head domains. The HA2 domain has up to 85% sequence homology among different subtypes and 95% homology within strains of the same subtype [16, 31, 247-250]. Antibodies with broad activity against the HA stem can neutralize multiple influenza virus strains or subtypes, suggesting that a vaccine based on this region could elicit a broadly protective immune response [91, 239, 251, 252]. Current vaccines and natural infection do not generate significant stem-directed antibodies since the HA stem is poorly immunogenic in the presence of the immunodominant head [16, 242]. To address this, two groups have independently developed HA immunogens that contain the stem region without the globular head domain [242, 244, 247, 248, 253-255]. The vaccines induced nAb against a conserved long α -helix region (LAH) in the stem [244, 245]. However, antibody responses against the most genetically distinct variants within a phylogenetic group were relatively weak, possibly due to

failure of these antigens to fully expose shrouded conserved B cell epitopes within the stem region or loss of conformationally dependent epitopes during production and purification of the recombinant protein vaccines [231, 243, 244, 256]. These results demonstrate feasibility of developing the HA stem as a universal influenza vaccine immunogen but also show that further improvements in immunogen design are still needed to maximize immunogenicity.

Computationally designed immunogens.

Novel immunogens can be computationally designed using a variety of modeling methods [257]. The modeling program RosettaRemodel can facilitate the process by allowing systematic alterations to the structure of the protein, while accounting for epitope polarity, energy minimization and structural integrity [258]. Building novel immunogens in this fashion can quickly eliminate structures that fail on theoretical grounds and plausible structures can be further tested using experimental models. In certain cases, modeling tools can also re-enforce structural elements, such as introducing disulfides to achieve greater stability, which are aspects that have not been widely explored in previously reported stem-based HA immunogens. Computational modeling can identify numerous theoretical HA stem immunogen designs with the potential for improved immunogenicity, but to translate these novel immunogens to vaccine discovery, the immunogens must be tested *in vivo*. *In vivo* testing often employs recombinant protein formulations for the immunizations but this vaccine modality requires time-consuming and costly purification steps to produce correctly folded protein and the process of producing these proteins can alter the tertiary structure and limit the number or design of the immunogens tested. Viral vectored vaccine formulations can also be employed for immunogen screening but immune responses against the viral vector carrier prevent repeat immunizations and compete

with B cell responses against the test immunogen. A reliable and rapid method for producing and testing correctly folded candidate immunogens *in vivo* is therefore needed before the full potential of computational modeling as a tool for immunogen discovery can be realized.

DNA vaccines for immunogen discovery

DNA vaccines are ideal for immunogen discovery. They can be rapidly constructed, produced, and administered into animals within 1-2 weeks after computational modeling without requiring cell culture or extensive purification procedures. Importantly, in contrast to recombinant protein vaccines, DNA vaccines express and display antigens in their correctly folded and native conformation as secreted protein or as transmembrane proteins on cell surfaces [259]. This feature avoids the need for purification steps and speeds the immunogen screening process by eliminating the need to produce purified proteins and may also result in better exposure of putative epitopes when compared to protein-based vaccines. This may be one reason why HA DNA vaccines induce comparable titers but better protection against influenza when compared to conventional protein vaccine [16, 83, 255, 260, 261] a result that has been correlated to DNA vaccine induction of higher avidity or stem-specific antibody [261]. DNA vaccines also induce broader nAbs against the conserved HA stem than recombinant protein or conventional killed vaccines and immunization with HA DNA followed by protein, viral vectored, or traditional seasonal vaccination will increase stem-specific antibody responses [16, 83, 255]. DNA vaccines can therefore provide a dual advantage with respect to the speed they can be produced to support rapid comparison of multiple computationally predicted immunogens and in their ability to present the immunogen in its natural, correctly folded conformation *in vivo*.

DNA vaccines expressing whole HA antigen can induce cross-neutralizing antibodies against highly conserved influenza sequences, likely due to their ability to present antigen *in situ* to B cells in its native conformation [16, 83, 255]. A DNA vaccine designed to express only the HA stem in its native conformation will be more effective for inducing broadly neutralizing antibody and cross-protection than a DNA vaccine expressing the full length HA, since the responses induced will be focused only against the conserved stem. I hypothesized that a DNA vaccine computationally designed to express the conserved HA stem will induce antibodies that neutralize and protect against heterologous viruses. To explore this concept, I computationally designed HA stem immunogens to have optimal predicted stability and conformation, constructed DNA vaccines expressing these immunogens and then examined the immunogenicity and protective efficacy of these vaccines in mice. Combining computational modeling with the advantages of DNA vaccination will hopefully reveal an optimum stem immunogen that induces broadly nAbs and superior cross protection against influenza when compared to other HA stem-based vaccines.

Results

Group 2 Headless HA computational design

To test the feasibility of this approach, Dr. Possu Huang and I employed RosettaRemodel to computationally design two different headless HA vaccines; Trim 1 and Trim 2 (**Fig 1a**) using sequences from the Influenza A virus strain A/Hong Kong/1/68 (H3N2) (HK68) HA (Group 2 virus). These two vaccines were designed to minimize the immunodominant HA1 head while still maintaining the integrity of the stem region and trimeric structure. The entire

immunogen consists of the complete HA2 polypeptide and the regions of HA1 contributing to the stem region but lacks the immunodominant globular head domain of HA1 (**Fig 4.1a**). In addition, the HA stem immunogens were designed to have maximum stability and structure, while still exposing known B cell epitopes. Based on the structure prediction done by RosettaRemodel, disulfide bonds were placed in optimal positions in order to stabilize the structure (**Fig 4.1b**). Therefore, the two trims were combined with a single disulfide bond to create 7 possible combinations (Trim 1: T1-D1, T1-D2, T1-D3, T1-D4, Trim 2: T2-D1, T2-D2, T2-D3) (**Table 4.1**). The full-length HA HK68 was included as a positive control. Since the full-length HA has been shown to provide little or no protection against heterologous influenza viruses, I hypothesized that mice immunized with the full-length HA vaccines will not be protected from the heterologous challenge. In contrast, I expected the computationally optimized HA stem DNA vaccines will induce broader neutralizing and binding Ab and afford significant cross-protection against both the homologous and heterologous challenges that will correlate with induction of broadly binding Abs and nAbs. As an additional control, the headless HA vaccine construct from Steel et al. was included and disulfides were added at either position 3 or 4 to create two control headless HA combinations (HS-D3 and HS-D4) (**Table 4.1**) [231].

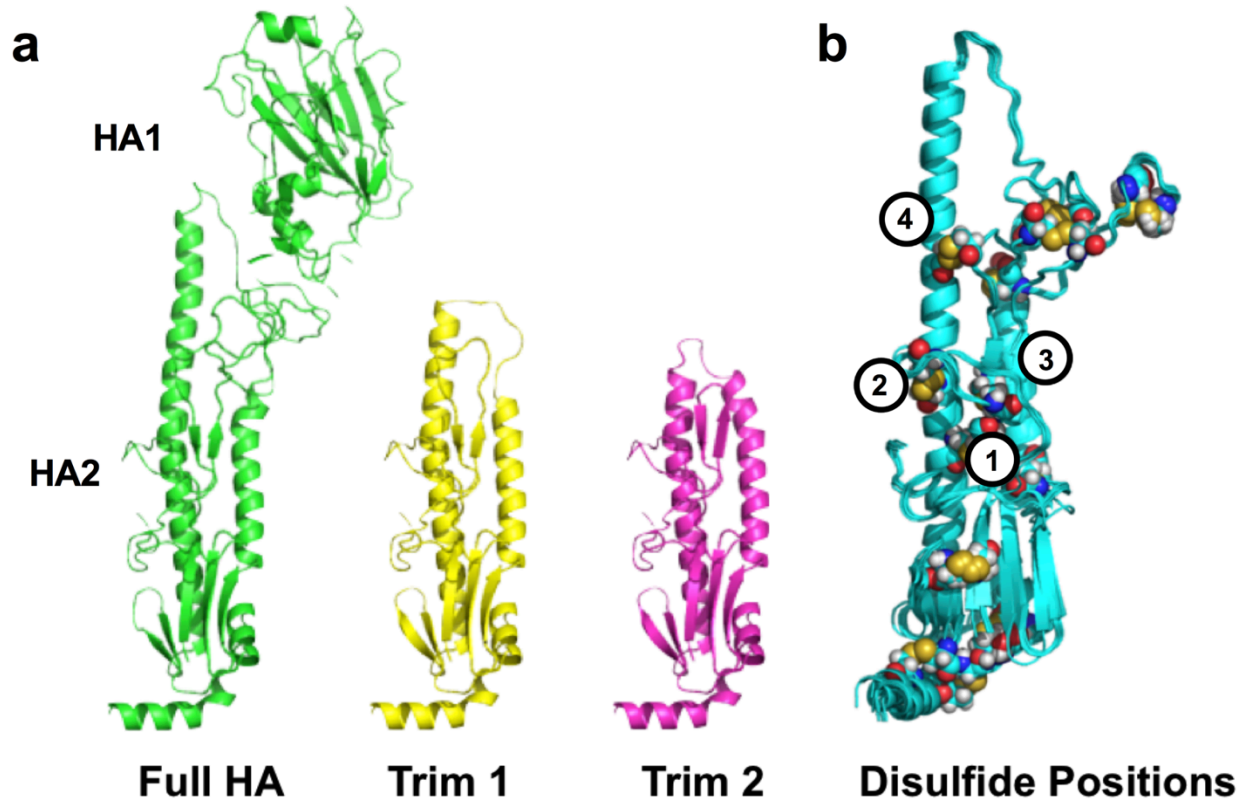


Figure 4.1: Headless HA trim vaccine constructs. (a) Crystal structure of the full-length HA which consists of the immunodominant head domain (HA1) and the conserved stem region (HA2), Trim 1, and Trim 2. (b) Headless HA structure from Steel et al. and the various possible disulfide positions available [231].

Vaccine construction, immunization and immunogenicity

Next, I constructed DNA vaccines expressing each headless HA trim combination in the vaccine vector JTF7563. Mice received a total of 3 immunizations, spaced 6 weeks apart, with 2 doses of the specific vaccine construct (**Table 4.1**). In addition to the two headless HA controls, a full-length HA that expresses both the head and stem (HA) was included. Two weeks following the final immunization, sera were collected and I measured antibody responses by ELISA against representative HA subtypes. The vaccines induced binding antibody against the homologous HK68 HA sequences, indicating computationally designed headless HA trims were immunogenic and conformationally correct (**Fig 4.2**). Importantly, both headless HA trim vaccines induced significantly stronger antibodies against heterologous viral variants including A/Canada/rv444/04 (H7N3, Group 2, 49% amino acid sequence identity to vaccine), A/California/07/09 (H1N1, Group 1, 44% amino acid sequence identity to vaccine), and A/Indonesia/5/05 (H5N1, Group 1, 42% amino acid sequence identity to vaccine), when compared to responses induced by the full-length HA (**Fig 4.2b**). This result demonstrates that the computationally designed stem vaccines (Trim 1 and 2) induce strong stem-specific antibody responses against influenza variants that are genetically distinct from the vaccine strain. Furthermore, there appears to be some difference between the positions of the disulfides on the trims and the antibody response. For example, construct T2-D1 had no antibody response so it's likely that the disulfide in position 1 from Trim 1 results in a non-functional or misshapen protein such that the resulting antibodies no longer recognize the original HA (**Fig 4.2c**). Furthermore, as expected, the full-length HA induces strong homologous antibody responses and weak heterologous antibody responses. These antibodies are likely directed against the immunodominant HA1 head and are strain specific. Interestingly, Trim 2 induced stronger

heterologous Group 2 antibody responses than the other vaccine constructs, whereas Trim 1 induced stronger Group 1 antibody responses (**Fig 4.3**).

Vaccine Construct		Description
Full-length HA	HA	Full-length HA (HA1 and HA2)
Headless HA	HS-D3	Headless HA stem from Steel et al. with disulfide in position 3
	HS-D4	Headless HA stem from Steel et al. with disulfide in position 4
Trim 1	T1-D1	Trim 1 and Disulfide 1
	T1-D2	Trim 1 and Disulfide 2
	T1-D3	Trim 1 and Disulfide 3
	T1-D4	Trim 1 and Disulfide 4
Trim 2	T2-D1	Trim 2 and Disulfide 1
	T2-D2	Trim 2 and Disulfide 2
	T2-D3	Trim 2 and Disulfide 3

Table 4.1: Group 2 Headless HA Vaccine Constructs. Vaccine constructs and descriptions for the various Headless HA vaccines. The headless HA constructs were adapted from Steel et al. [231]. The vaccine sequence is from the Influenza A virus strain A/Hong Kong/1/68 (H3N2) (HK68) HA (Group 2 virus).

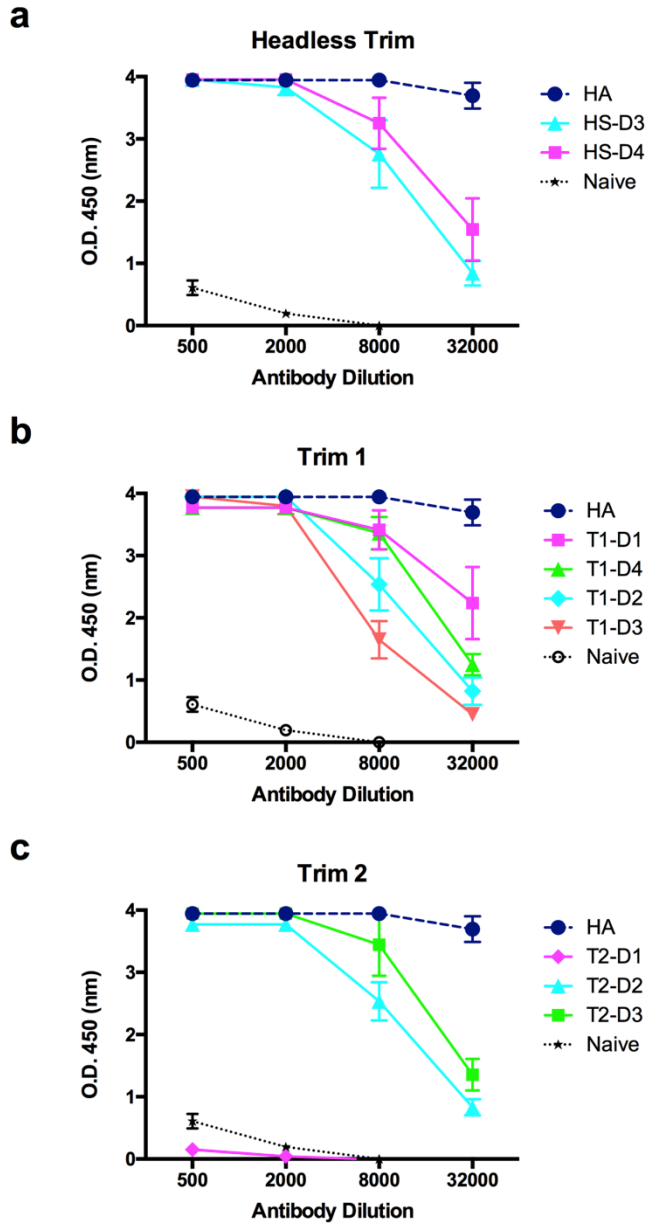


Figure 4.2: Headless HA vaccines induce antibodies against vaccine strain HA. Antibody titers against the homologous vaccine strain HA A/Hong Kong/1/68 (H3N2) for the (a) Headless Trim, (b) Trim 1, and (c) Trim 2 constructs. The full-length HA (HA) was included as a positive control, and serum from naïve unvaccinated mice (naïve) as a negative control. Shown is mean and SEM for three replicate experiments.

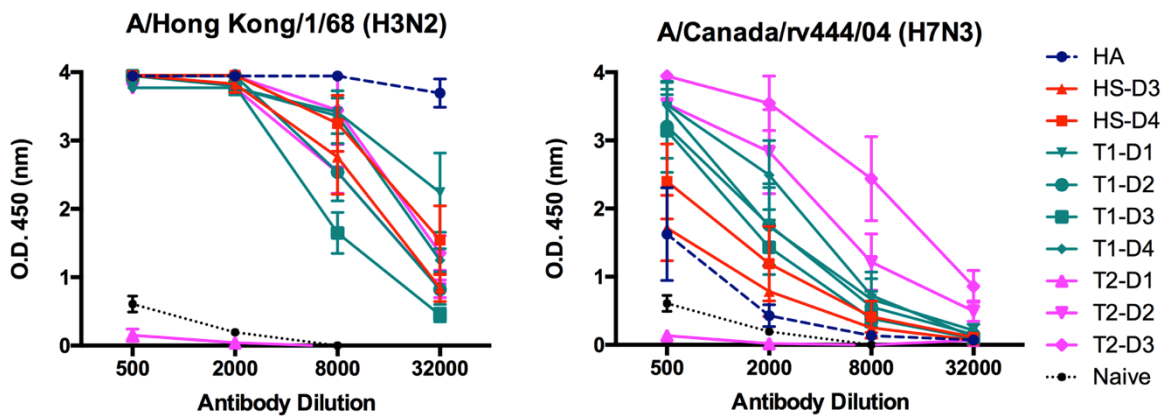
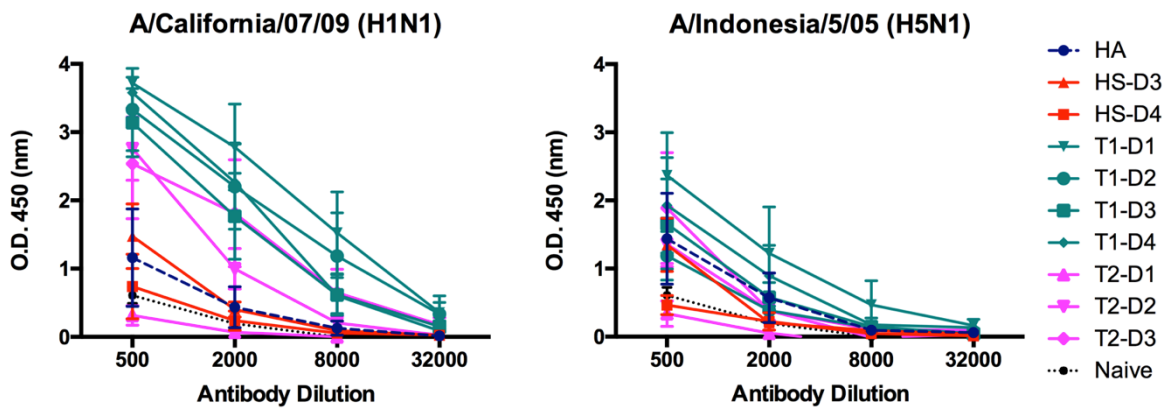
a**Group 2****b****Group 1**

Figure 4.3: Group 2 headless HA vaccines induce cross-reacting binding antibody titers against Group 1 and 2 HAs. Antibody titers against (a) the Group 2 HAs A/Hong Kong/1/68 (H3N2) and A/Canada/rv444/04 (H7N3), and (b) the Group 1 HAs A/California/07/09 (H1N1) and A/Indonesia/5/05 (H5N1). The full-length HA (HA) was included as a positive control, and serum from naïve unvaccinated mice (naïve) as a negative control. Shown is mean and SEM for three replicate experiments.

To determine if the headless HA vaccines induced broadly neutralizing antibodies, I analyzed sera using a GFP-based influenza virus neutralization assay developed by Dr. Jesse Bloom (FHCRC) [262]. Sera was collected from mice two weeks after the third vaccination and analyzed for ability to neutralize a panel of representative H1 (Group 1) and H3 (Group 2) human influenza viruses ranging from 42% - 97% amino acid sequence identity to the vaccine strain. This assay detects antibodies that recognize HA in its native trimeric confirmation and are functionally neutralizing. The full-length HA induced broadly neutralizing antibodies against the homologous H3 virus, X31, (**Fig 4.4**) however it failed to neutralize any other H3 or H1 virus. This result is consistent with previous studies showing that the full-length HA induces neutralizing antibodies directed at the immunodominant head domain that are strain specific [16, 242]. The broadly neutralizing antibody FI6 was included as a positive control since it binds to the stem of both group 1 and 2 HAs [32]. FI6 has been shown to neutralize the virus by inhibiting the pH induced conformation change required for membrane fusion [32]. FI6 has a strong affinity for the stem and neutralizes at a low IC_{50} indicating that a stem antibody is capable of completely neutralizing the viruses used in this assay (**Fig 4.4**). Unfortunately, none of the Headless HA (**Fig 4.4a**), Trim 1 (**Fig 4.4b**), or Trim 2 (**Fig 4.4c**) vaccine constructs induced neutralizing antibodies against the homologous virus X31 or any other heterologous H1 or H3 virus tested. Despite these results, all vaccine constructs were tested for protective efficacy, regardless of neutralization, because non-neutralizing binding antibodies could protect by other mechanisms (i.e. ADCC), and vaccine specific T cells may also play a role in protection.

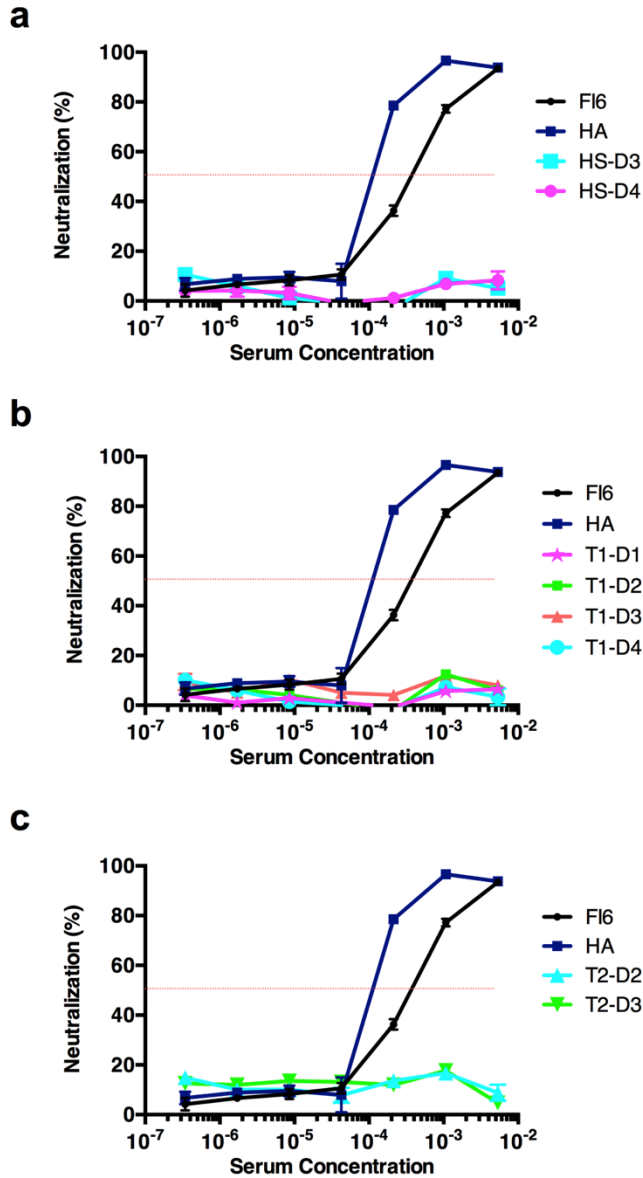


Figure 4.4: Headless HA vaccines do not induce neutralizing antibodies against vaccine strain HA. Percent neutralization of serum antibodies against the homologous vaccine strain HA A/Hong Kong/1/68 (H3N2) for the (a) Headless Trim, (b) Trim 1, and (c) Trim 2 constructs. The broadly nAb FI6 was included as a positive control. The dotted red line indicates 50% neutralization. Shown is mean and SEM for three replicate experiments.

Headless HA Vaccines provide protection in vivo

To determine if the binding antibody responses induced by these stem vaccines could provide protection against a lethal Influenza virus challenge, I challenged mice with a lethal dose of homologous virus, X31 (A/Hong Kong/1/68, H3N2). Two weeks after the third vaccination mice were intranasally (IN) challenged with 10 MLD₅₀ doses of X31. The full-length HA vaccine matched the challenge virus and afforded complete protection in mice against the homologous virus, as expected (**Fig 4.5**). The headless HA Trim 1 and Trim 2 vaccinated mice had a 75% and 65% survival respectively, indicating that the headless HA vaccines induced protective immune responses that recognized a wild-type (WT) influenza virus (**Fig 4.5b and c**). These results show that, even in the complete absence of neutralizing antibody against the immunodominant head, a vaccine consisting of only the stem region of HA can induce strong antibody responses and possibly T cell responses that can protect against a lethal virus challenge.

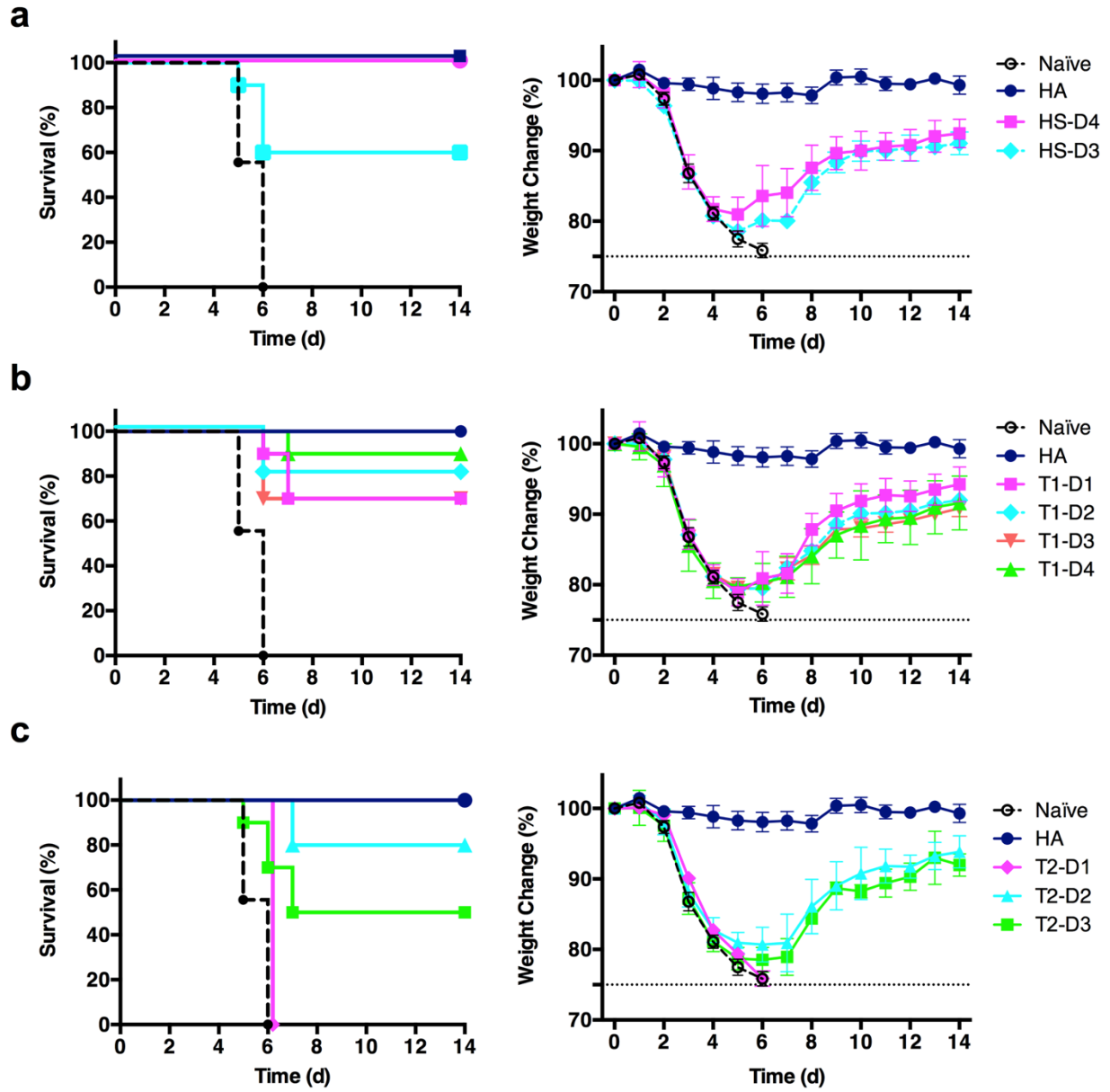


Figure 4.5: Group 2 Headless HAs provide protection against lethal homologous influenza challenge. Survival and weight change of HK68 Headless HA immunized mice after homologous virus challenge. Mice were PMED immunized 3 times with a DNA vaccine (n = 10 mice per group) expressing the respective HA construct; (a) Headless HA, (b) Trim 1, or (c) Trim 2. Two weeks after the final immunization, mice were challenged with 10 MLD₅₀ doses of X31 influenza virus. Mean and SEM are shown.

Depletion of T cell enhance protection

Since protection was observed despite the lack of neutralizing antibodies, I examined the potential contribution of the vaccine induced T cell responses. Mice were PMED vaccinated with 2 doses of the DNA vaccine expressing either the full-length HA (HA), HS-D4, T1-D4 or T2-D2 on weeks 0, 6 and 12. These vaccines were selected from each headless HA group that had the overall best survival and weight loss for their respective groups. Three days prior to challenge, mice were injected with monoclonal antibodies daily for 3 days to deplete either 1) CD4 T cells, 2) CD8 T cells or 3) CD4 and CD8 T cells. Two weeks after the final vaccination, mice were challenged IN with 10 MLD₅₀ of X31 virus. Reduced protection in any one of these groups will indicate that the depleted subset(s) contributed to protection. No change in protection will indicate antibody primarily mediates protection. In contrast, we observed enhanced protection when the CD4, CD8 or CD4/CD8 T cell subsets were depleted for all vaccine constructs, including the full-length HA (**Fig 4.6**). This is likely due to the cytokine storm observed during influenza infections, in which immune cells overproduce proinflammatory cytokines leading to increased tissue damage and high mortality rates in otherwise healthy patients [263, 264]. Consistent with this possibility, depletion of the CD4 T cells enhanced protection for all vaccines whereas depletion of the CD8 T cells resulted in no change or a slight decrease in weight loss. Based on these results it appears that the CD8 T cells are contributing to protection by clearing the virus and their depletion results in more weight loss. In contrast, the vaccine induces CD4 T cell responses that contribute to the proinflammatory cytokine response. When the CD4 T cells are depleted, there is a reduction in the proinflammatory cytokine response, which may enhance protection by limiting the effect of the cytokine storm during the influenza challenge.

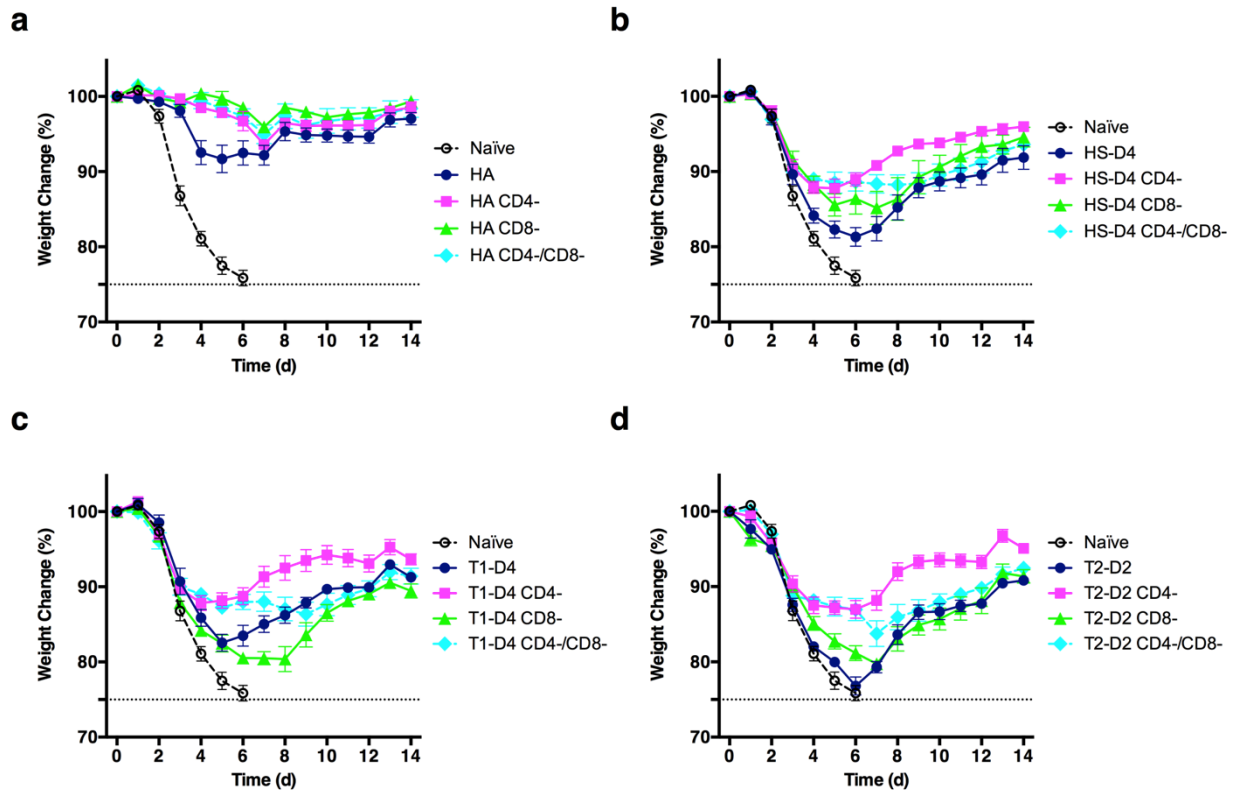


Figure 4.6: Depletion of CD4⁺ and CD8⁺ T cells enhance protection against lethal homologous influenza challenge in vaccinated mice. Weight change of immunized mice after T cell depletion and homologous virus challenge. Mice were PMED immunized 3 times with a DNA vaccine (n = 7 mice per group) expressing the respective HA construct; (a) Full-length HA, (b) Headless HA (HS-D4), (c) Trim 1 (T1-D4), or (d) Trim 2 (T2-D2). Two weeks after the final immunization, mice were challenged with 10 MLD₅₀ doses of X31 influenza virus. Mean and SEM are shown.

Headless HA Vaccines do not provide protection against heterologous viral challenge

To determine if the headless HA vaccines could provide protection against a lethal heterologous Influenza virus challenge, I challenged mice with a lethal dose of CA09 (A/California/07/2009, H1N1). Two weeks after the third vaccination mice were intranasally (IN) challenged with 10 MLD₅₀ doses of CA09. None of the vaccine constructs, including the full-length HA vaccine afforded protection in mice against the heterologous virus (**Fig 4.7**). These results clearly demonstrate that the binding abs observed in **Figure 4.3** do not translate in to cross-protection in vivo. Furthermore, it appears that any T cell responses due to vaccination did not provide any protection against the heterologous virus. Therefore, the overall design and composition of the vaccine needs to be reexamined in order to design a better headless HA vaccine that does induce broadly neutralizing antibodies and broad T cell responses that contribute to protection.

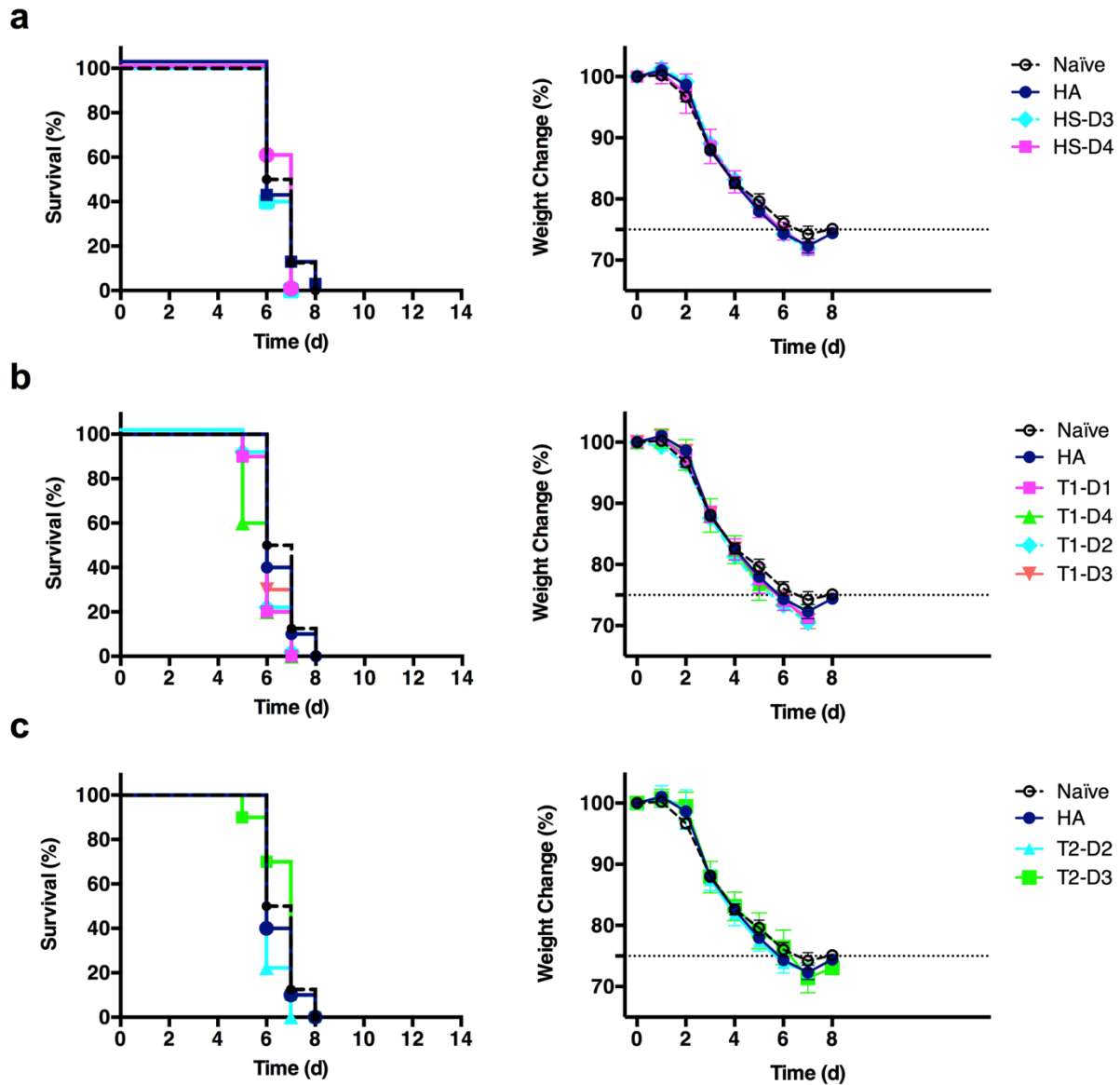


Figure 4.7: Group 2 Headless HA vaccines do not protect against lethal heterologous Group 1 influenza challenge. Survival and weight change of immunized mice after heterologous virus challenge with the Group 1 H1N1 virus A/California/07/2009 (CA09). Mice were immunized by PMED 3 times with a DNA vaccine (n=10 mice per group) expressing the respective HA construct; (a) Headless HA, (b) Trim 1, or (c) Trim 2. Two weeks after the final immunization, mice were challenged with 10 MLD₅₀ doses of CA09 influenza virus. Mean and SEM are shown.

Group 1 Headless HA computational modeling and vaccine design

To further expand on these results, Dr. Possu Huang and I used RosettaRemodel to computationally design headless HA vaccines based on A/California/07/2009 (H1N1) (CA09), a representative Group 1 strain from the swine flu pandemic [249, 250, 265]. The two trims, Trim 1 and Trim 2 were employed again for the CA09 strain (**Fig 4.1a**). Based on the structure prediction done by RosettaRemodel, disulfide bonds were placed in optimal positions in order to stabilize the structure as before, however some vaccines constructs were designed that had multiple disulfides in one structure (**Table 4.2**). The full-length HA CA09 was included as a positive control. As an additional control, the headless HA vaccine construct from Steel et al. was included and a second vaccine version with a disulfide at position 4 to create two control headless HA combinations (HS and HS-D4) (**Table 4.2**) [231].

Vaccine Construct		Description
Full-length HA	CA HA	A/California/07/09 (CA) Full-length HA (HA1 and HA2)
Headless HA	HS	Headless HA stem from Steel et al.
	HS-D4	Headless HA stem from Steel et al. with disulfide in position 4
Trim 1	T1-D1	Trim 1 and Disulfide 1
	T1-D2	Trim 1 and Disulfide 2
	T1-D3	Trim 1 and Disulfide 3
	T1-D4	Trim 1 and Disulfide 4
	T1-D1D2D3	Trim 1 and Disulfide 1, 2 and 3
Trim 2	T2-D1	Trim 2 and Disulfide 1
	T2-D2	Trim 2 and Disulfide 2
	T2-D3	Trim 2 and Disulfide 3
	T2-D1D3	Trim 2 and Disulfide 1 and 3

Table 4.2: Group 1 Headless HA Vaccine Constructs. Vaccine constructs and descriptions for the various Headless HA vaccines. The headless HA constructs were adapted from Steel et al. [231]. The vaccine sequence is from the Influenza A virus strain A/California/07/09 (H1N1) (CA09) HA (Group 1 virus).

Group 1 Vaccine construction, immunization and immunogenicity

Next, I constructed DNA vaccines expressing each headless HA trim combination into the vaccine vector JTF7563. Mice received a total of 3 immunizations, on weeks 0, 6, and 12, with 2 doses of the specific vaccine construct (**Table 4.2**). Two weeks following the final immunization, sera were collected and antibody responses were measured by ELISA against representative HA subtypes. The vaccines induced binding antibody against the homologous CA09 HA sequences, indicating computationally designed headless HA trims were immunogenic and conformationally correct (**Fig 4.8a**). In addition, the headless HA vaccines induced cross-binding antibodies to the drifted PR8 strain, which shares 80% amino acid sequence homology (**Fig 4.8a**). However, the headless HA trim vaccines did not induce binding antibodies against heterologous viral variants including A/Indonesia/5/05 (H5N1, Group 1, 63% amino acid sequence identity to vaccine), A/Hong Kong/1/68 (H3N2, Group 2, 44% amino acid sequence identity to vaccine), and A/Canada/rv444/04 (H7N3, Group 2, 40% amino acid sequence identity to vaccine) (**Fig 4.8**). Since the vaccines induced antibodies that recognized the full-length homologous HA, the headless HA vaccines resulted in functional protein that was not misshapen or malformed. However the headless HA vaccines did not induce cross-binding antibodies beyond the H1 subtype in contrast to what was observed for the H3 headless HA vaccines (**Fig 4.3**). This may be due to the location of a glycan on the Group 2 binding site that disrupts the binding pocket. Its possible that using the H3 vaccine as the backbone strain induces antibodies that can successfully avoid this glycan that then in turn can bind the Group 1 strains that lack the glycan.

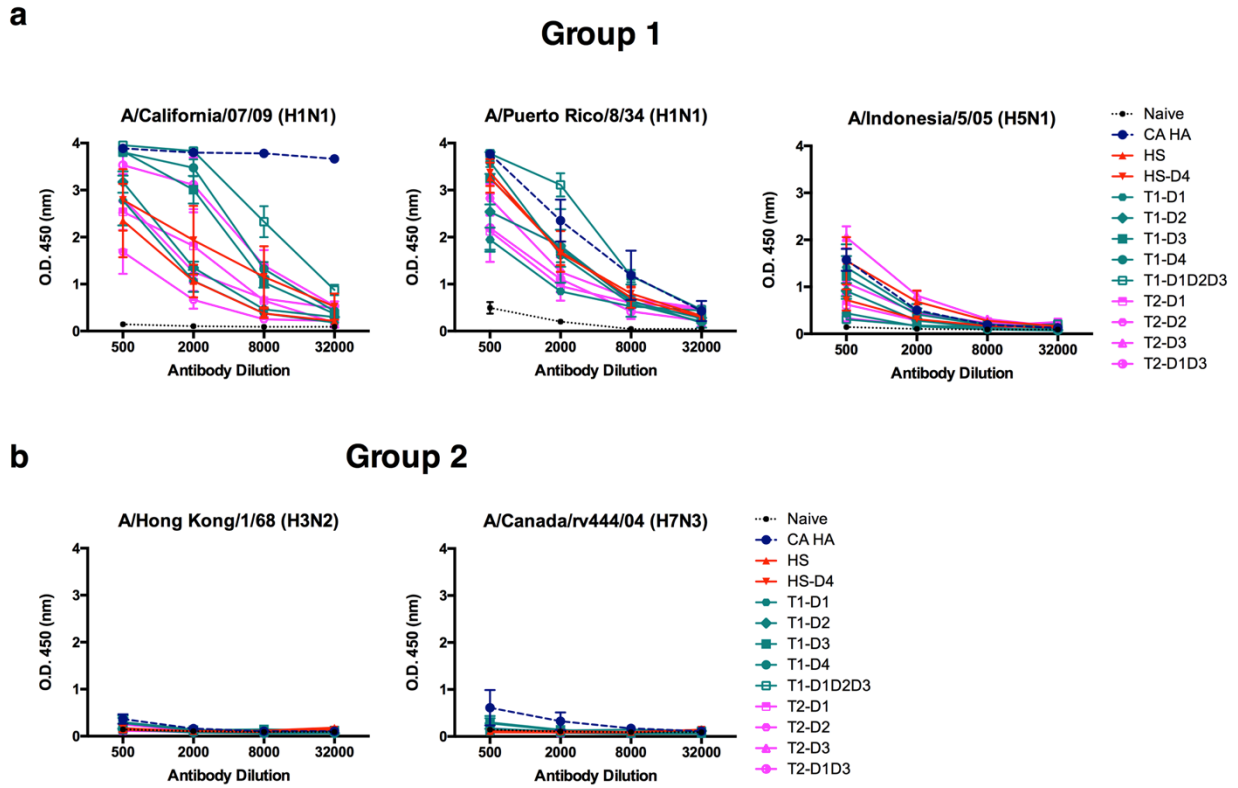


Figure 4.8: Group 1 Vaccines do not induce cross-reacting binding antibody titers against Group 1 and 2 HAs. Antibody titers against (a) the Group 1 HAs A/California/07/09 (H1N1) and A/Indonesia/5/05 (H5N1), and (b) the Group 2 HAs A/Hong Kong/1/68 (H3N2) and A/Canada/rv444/04 (H7N3). The full-length CA09 HA (CA HA) was included as a positive control, and serum from naïve unvaccinated mice (naïve) as a negative control. Shown is mean and SEM for three replicate experiments.

To determine if the headless HA vaccines induced broadly nAbs, I analyzed sera using the GFP-based influenza virus neutralization assay [262]. Sera was collected from mice two weeks after the third vaccination and analyzed for ability to neutralize a panel of representative H1 (Group 1) and H3 (Group 2) human influenza viruses ranging from 42% - 97% amino acid sequence identity to the vaccine strain. As previously seen with the H3 headless vaccines, none of the H1 Headless HA vaccine constructs induced neutralizing antibodies against the homologous virus CA09 or any other heterologous H1 or H3 virus tested.

Headless HA Vaccines provide protection in vivo

To determine if the stem-based vaccines could provide protection against a lethal homologous and heterologous influenza virus challenge, mice were challenged with a lethal dose of either the homologous H1N1 virus, CA09 or the heterologous H1N1 virus A/Puerto Rico/08/1934 (PR8). Two weeks after the third vaccination, I intranasally challenged mice with either 10 MLD₅₀ doses of CA09 or PR8. These viruses have been mouse adapted and replicate in lungs of mice but differ in pathology [266]. PR8 and CA09 share 82% amino acid sequence identity, which represents a heterologous virus challenge that is more closely related than a Group 1 to Group 2 heterologous challenge. The full-length CA HA vaccine matched the challenge virus and afforded complete protection in mice against the homologous virus, as expected (**Fig 4.9**). However against the heterologous PR8 virus, the mice had significant weight loss, losing over 20% of their total weight but had a survival rate of 80% (**Fig 4.10**). The full-length HA induces strong antibody responses against the head, which accounts for the complete homologous protection, however it's possible that it induces some heterologous T cell responses, which may account for the heterologous protection since weight loss was observed but 80% of

the mice survive. If the heterologous protection was antibody-mediated there would not be significant weight loss observed.

The two headless HA vaccines, HS and HS-D4, that were designed based on the Steel et al publication, resulted in 60% and 50% survival respectively against the homologous viral challenge [231]. Interestingly, the headless HA vaccine containing the added disulfide (HS-D4) had an 80% survival against the heterologous PR8 virus, in comparison to only 20% survival for the HS vaccine. These results indicate that adding a disulfide in position 4 enhanced protection observed, possibly by stabilizing the stem and the fusion peptide.

Further evidence of the importance of the disulfide bond can be seen in the Trim 1 vaccinated mice. The various disulfide positions in the same trim 1 result in a wide range of survival (0-80%) and weight loss (10-30%) (**Fig 4.9b**), indicating that the headless HA vaccines induced protective immune responses that recognized a wild-type (WT) influenza virus however the stability was enhanced by the position of the disulfide bond. Interestingly, as seen before with the H3 headless HA vaccines, these results show that even in the complete absence of neutralizing antibody, the vaccines conferred protection against a lethal virus challenge. These results demonstrate the feasibility of using computational modeling to design a novel immunogen to stimulate broadly reactive and protective immune responses.

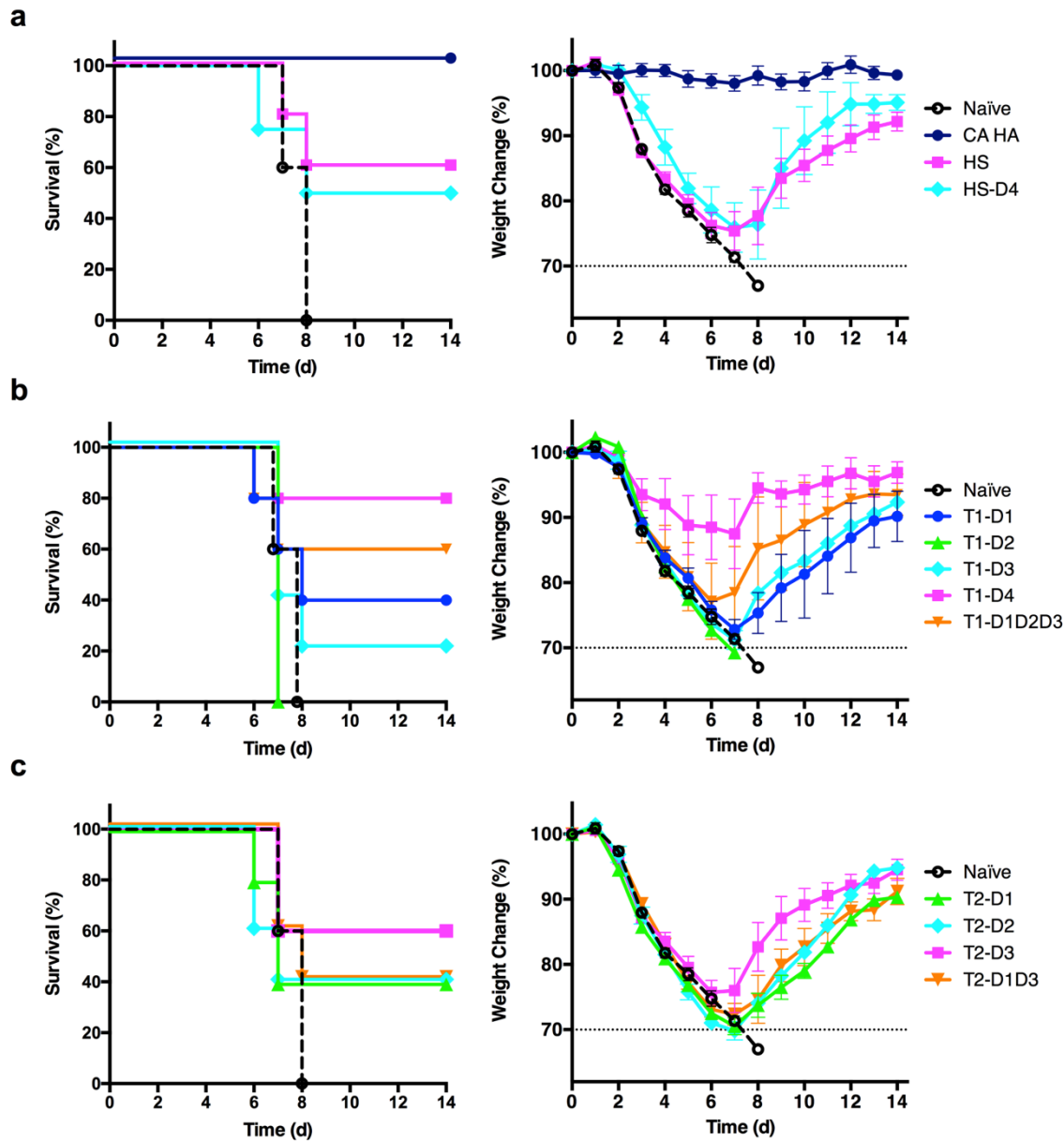


Figure 4.9: Group 1 Headless HAs provide protection against lethal homologous influenza challenge. Survival and weight change of CA09 Headless HA immunized mice after homologous virus challenge with the Group 1 CA09 H1N1 virus. Mice were PMED immunized 3 times with a DNA vaccine ($n = 10$ mice per group) expressing the respective H1 HA construct; (a) Headless HA, (b) Trim 1, or (c) Trim 2. Two weeks after the final immunization, mice were challenged with 10 MLD₅₀ doses of CA09 influenza virus. Mean and SEM are shown.

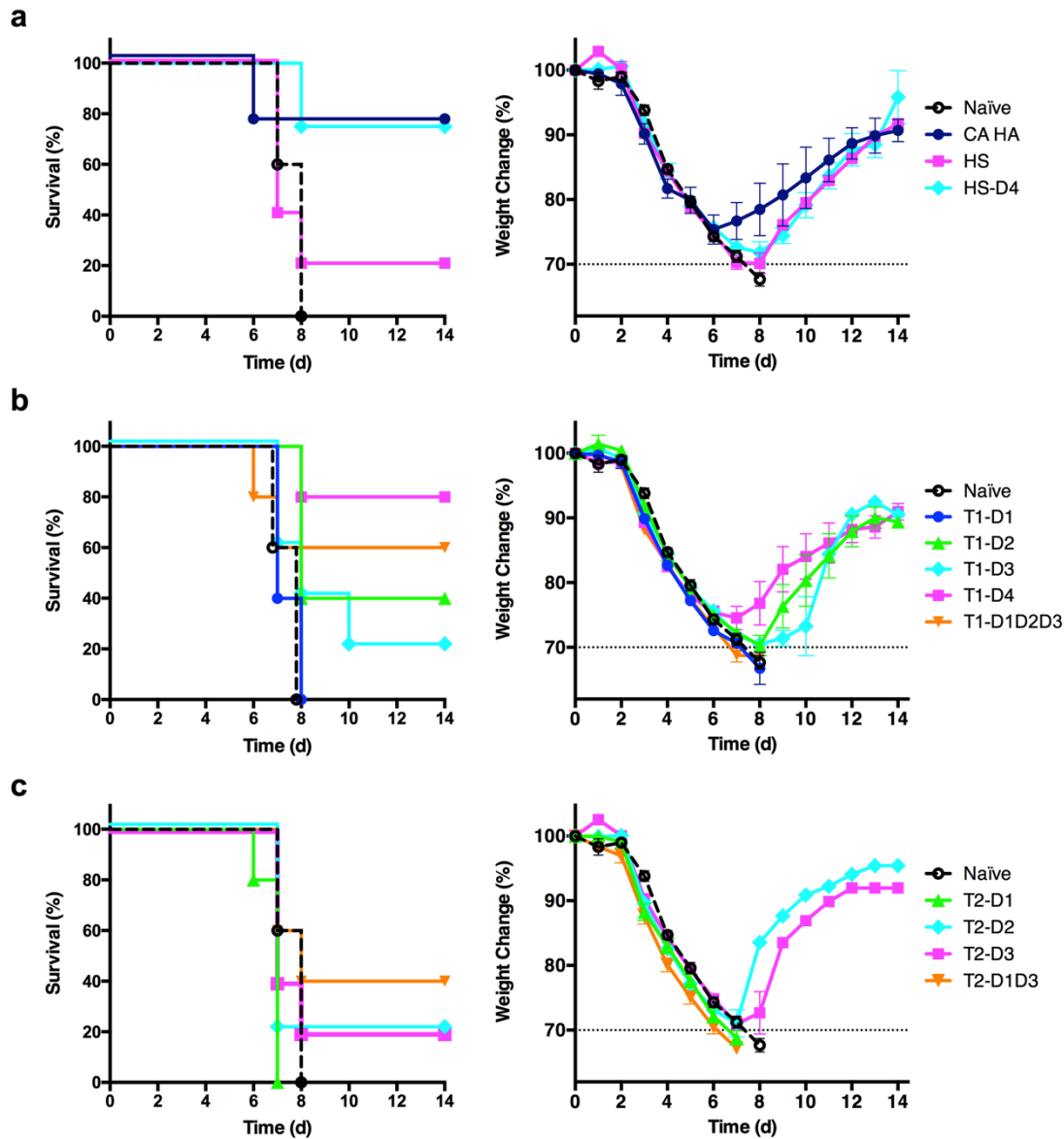


Figure 4.10: Group 1 Headless HAs provide protection against lethal heterologous Group 1 influenza challenge. Survival and weight change of immunized mice after heterologous virus challenge with the Group 1 A/Puerto Rico/08/1934 (PR8) virus which shares 82% amino acid sequence homology to the vaccine strain. Mice were PMED immunized 3 times with a DNA vaccine ($n = 10$ mice per group) expressing the respective HA construct; (a) Headless HA, (b) Trim 1, or (c) Trim 2. Two weeks after the final immunization, mice were challenged with 10 MLD_{50} doses of X31 influenza virus. Mean and SEM are shown.

Discussion

The goal of this project was to design novel HA stem immunogens that could be developed as a universal influenza vaccine. Such a vaccine could provide broader protection against a wider range of influenza variants than currently possible with existing vaccines. DNA vaccination is a fast method to screen multiple immunogens at one time and offers *in situ* presentation of putative B cell epitopes in their natural conformation. For clinical testing, the optimal immunogens could be formulated as protein or virus-like particle vaccines but could also be developed as a DNA vaccine. For administration of DNA vaccines, our laboratory employs particle-mediated epidermal delivery (PMED) [92] which results in highly efficient delivery of the DNA vaccines directly into cells of the skin [259]. The success of this system is due to direct delivery of the DNA into the cell and the *in vivo* expression of the antigen [88, 92, 267]. Thus, DNA vaccines are an ideal platform to rapidly design and screen various immunogens. Due to the vast capabilities of Rosetta computational design, hundreds and thousands of potential immunogens can be designed. Producing these immunogens as proteins for screening is not feasible given the number but formulating as a DNA vaccine allows for rapid generation of the vaccines for analysis of immunogenicity and protective efficacy in a matter of weeks. This allows for rapid results and an intelligent design feedback loop to influence new generations of designs.

Here I show that novel immunogens can be computationally designed, formulated in to a PMED DNA vaccine and that these vaccines are not only immunogenic but protect mice from lethal influenza challenge. The antibodies generated by these headless HA vaccines recognize the WT HA however they did not neutralize influenza viruses. Despite the lack of neutralizing antibodies, the vaccines still protected mice from lethal influenza challenge from homologous

and heterologous (no more than 20% amino acid sequence divergence) viruses. These results suggest that antibody-mediated protection was based on effector mechanisms other than *in vitro* neutralization. Possibilities include antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent complement-mediated lysis [41, 42]. My results suggest that HA stem-based vaccines do not necessarily need to induce neutralizing antibodies in order to provide broad protection. T cells may also be playing a significant role in the protection observed. However, previous studies have shown that CD8⁺ T cells do not significantly contribute to stem-based vaccine protection against a homologous challenge but their role may be more important in a heterologous challenge [158]. Further research on the vaccine induced T cell responses and their function are needed.

A recent study showed that humans vaccinated with the avian influenza H5N1 vaccine results in stem-specific HA antibody responses that cross-react with other influenza strains [268, 269]. This is likely due to recognition and boosting of the more conserved HA stem-region antibody responses since the recipients were naïve to the HA head. In addition, several studies have shown that chimeric HA vaccines, expressing the stem from one HA subtype and the head from a different subtype induced strong stem-specific antibody responses. The prime with one chimeric vaccine and boost with a second chimeric vaccine that both share the same stem portion but different head resulted in strong stem-specific antibody responses that were broadly neutralizing and protective [158, 234, 270]. These results demonstrate the importance of maximizing the immune response to the stem while limiting the impact of the immunodominant head domain.

The success of these chimeric HA vaccines illustrates the importance of using a vaccine immunogen that expresses both the HA1 and HA2 and not just the HA2 as I tried to do here. Due

to the structure of the HA antigen, both the HA1 and HA2 seem to be required to induce functioning antibodies. It is likely that the headless HA vaccines described here formed immunogens that resembled the HA2, hence the binding antibodies, however without the HA1 portion the fusion epitope might not have been displayed correctly which explains the lack of neutralizing antibodies.

The data from these experiments indicate that a stem-based strategy could be an effective universal vaccine strategy. I believe that there is great potential in combining the power of Rosetta computational design with the numerous advantages of DNA vaccines as a novel immunogen discovery platform.

Materials and Methods

Plasmid construction and vaccine formulation

All DNA constructs were synthesized using human/mouse codon optimized sequences (Genewiz) from the influenza strains A/Hong Kong/1/1968 (H3N2) or A/California/07/2009 (H1N1). The coding sequences were inserted into the Nhe1 and Bgl2 sites of PJV7563 [155], an empty DNA vaccine expression plasmid. Plasmid DNA was precipitated onto 1-3mm gold particles as previously described [166] at a rate of 1.8ug of each antigen plasmid per 1.0mg of gold.

PMED Vaccination

Animal studies approved by the University of Washington Institutional Animal Care and Use Committee. Female, 6-8 week-old BALB/c mice were randomly separated in to groups,

sedated, the abdominal fur was clipped, and DNA-coated gold particles were accelerated into the skin of the abdominal region using a gene gun PMED device. DNA-coated gold particles were delivered at a helium pressure of 45 bar psi. Each actuation resulted in the delivery of 2 mg of DNA into the epidermis. A single dose consisted of 2 actuations for a sum of 4 mg.

Influenza challenge

Two weeks after the final immunization, the mice were anesthetized with 2.5% isoflurane and challenged intranasally with 10 MLD₅₀ (fifty percent mouse lethal dose) of A/Hong Kong/1/68 (H3N2) (X31), A/California/04/09 (H1N1) (CA09), or A/PR/8/34 (H1N1) (PR8). The mice were monitored daily for weight loss and survival until 14 days post-infection. Animals that lost more than 25% of their initial body weight were euthanized by carbon dioxide in accordance with our animal protocols. At least five mice per group were used for each experiment. All mice used for the experiments are included for analyses. For mouse experiments, researchers were not blinded to animal identity.

Enzyme-linked immunosorbent assay (ELISA)

HA-specific IgG antibody levels in mouse serum were assessed by ELISA. Maxisorp (Thermo Scientific-Nunc) were coated with 100 ng/well of recombinant HA in PBS overnight at 4°C. Plates were blocked with 5% nonfat milk powder in PBS for 1h at room temperature, and then washed three times with wash buffer (PBS-T; phosphate-buffered saline containing 0.05% Tween 20). Two-fold serial dilutions of samples were added to the wells and plates were incubated for 1hr at room temperature. Following three washes with PBS-T, plates were incubated with horseradish-peroxidase conjugated goat anti-mouse IgG (1/3,000 dilution)

secondary antibodies (Thermo Scientific Pierce) for 1h at room temperature. After five washes with PBS-T, TMB substrate (KPL) was added to the wells for 30 min at room temperature. Color development was stopped by the addition of TMB Stop solution (KPL), and the plates were read at 450nm to measure relative optical densities (O.D.).

Neutralization assay

Neutralization assays were performed using the PB1flank-eGFP viruses as previously described [224]. Briefly, mouse serum was heat inactivated at 56°C for 1 h, then five-fold diluted in a 96-well plate, and virus was added at an MOI that ranged from 0.1 to 0.8 for the different viruses. Plates were incubated at 37°C for 1 h to allow antibody binding, and then 4×10^4 MDCK-SIAT1-CMV-PB1 cells were added per well. After an 18-h incubation, GFP fluorescence intensity was measured using an excitation wavelength of 485 nm and an emission wavelength of 515 nm. Values are reported as percent infectivity remaining averaged over triplicate measurements. The panel of viruses consisted of A/California/07/2009 (H1N1, CA09), A/Puerto Rico/08/1934 (H1N1, PR8), A/New Caledonia/20/1999 (H1N1, NC99), A/Hong Kong/1/1968 (H3N2, X31), A/Brisbane/10/2007 (H3N2, BNE07), and A/Wisconsin/67/2005 (H3N2, WI05).

T Cell Depletions and Challenge

Balb/c mice (n = 7 per group) were PMED vaccinated with 2 doses of the DNA vaccine expressing either the full-length HA (HA), HS-D4, T1-D4 or T2-D2 on weeks 0, 6 and 12. Three days prior to challenge, mice were injected with monoclonal antibodies daily for 3 days to deplete either 1) CD4 T cells, 2) CD8 T cells or 3) CD4 and CD8 T cells. Two weeks after the

final vaccination, mice were challenged intranasally with 10 MLD₅₀ of X31 virus. The mice were monitored daily for weight loss and survival until 14 days post-infection. Animals that lost more than 30% of their initial body weight were euthanized by carbon dioxide in accordance with our animal protocols.

Statistical and power analyses

All of the analyses were performed using Graphpad Prism version 5.01. A Student's t test (to compare two samples) and analysis of variance (ANOVA) (to compare multiple samples) were used for statistical analysis. Survival analyses were performed by using the Kaplan-Meier log-rank test. A P value of <0.05 was considered to be significant. For mice, the minimum group size was determined using weight loss data with 100% of control mice becoming infected with X31 or CA09. Based on a standard deviation of 2% in weight loss, a group size of n=5 yields >80% power to detect a minimum of a 10% difference between groups in weight loss using a two-sided t-test with an alpha value of 0.05. Statistics are performed using Graphpad Prism.

Chapter 5

Conclusions and future directions

Recent avian and swine-origin influenza virus outbreaks illustrate the ongoing threat of influenza pandemic. New vaccines and antivirals that offer complete protection or accelerated clearance would significantly help in limiting the threat of influenza. The global market for infectious disease diagnostics, vaccines, and therapeutics reached over \$100 billion in 2015 and is expected to grow at a rate of 7.5% over the next five years [271].

Overall, I have demonstrated that the HA stem is a good target for vaccines and antivirals. I have shown that a novel computationally designed protein can bind to the HA stem, prevent the pH induced conformational change, neutralize the virus and protect mice *in vivo* from lethal influenza challenge [272]. This is the first time a theoretical target, designed *in silico* has been translated in to an effective drug *in vivo*. This approach holds great promise for generating new effective drug candidates against influenza and other infectious diseases. We are currently working on designing a new Group 2 protein binder that when combined with HB36.6 would protect against all influenza subtypes. In addition to expanding the specificity, improvements to the overall design of the proteins are being made. The addition of disulfides will add stability to the proteins making them more stable at room temperature and have a longer shelf life. Furthermore, decreasing the size of the protein binders, to generate new “mini-binders,” will allow for chemical synthesis, which opens up new production, manufacturing and formulation opportunities.

Moreover, non-Ig protein scaffolds have great potential as new therapeutics for a variety of target indications. For example, respiratory syncytial virus (RSV) infects most children

leading to bronchitis and requiring hospitalization in ~2% of cases, with fatality rates as high as 30% [273]. The annual cost of disease is estimated at \$1.5 billion [274]. The only approved RSV antiviral is a monoclonal antibody called Synagis, which is only approved for premature births and is very expensive [275]. Since a monoclonal antibody exists and the mechanism of protection is known, RSV would be a prime target for our computationally designed mini-binders. In addition, RSV is a respiratory virus, which is ideal given the method of delivery for protein therapeutics. Therefore, de novo designed mini-binders could extend beyond influenza and treat various other respiratory infectious diseases.

I have also demonstrated that the power of computational design using RosettaRemodel can also be applied to designing novel vaccine immunogens. The success of HB36.6 as an antiviral supports the concept that the stem is an ideal target for both antivirals and vaccines. The goal now is to generate a complementary vaccine that generates antibodies that target the stem in the same way that HB36.6 does. I have shown here that the full-length HA, expressed as a DNA vaccine, does not induce broadly neutralizing antibodies against heterologous virus strains. However we have seen some success in computationally designing various headless HA vaccines that are immunogenic in mice. My results show that HA stem-only vaccine can provide protection against lethal influenza virus and should be considered in strategies to develop universal influenza vaccines. Overall I demonstrated that DNA vaccines are an optimal vaccine-discovering platform that when combined with RosettaRemodel can generate novel vaccines that are immunogenic and provide protection against lethal influenza infection.

Recently, several papers have been published that show success in generating stem-directed antibodies by vaccination. It appears that the success is in part due to minimizing the

immune impact of the head while directing the immune response to the stem. One study attached a headless HA immunogen to a ferritin cage that forms virus-like nanoparticles. They observed heterosubtypic protection in mice and ferrets against a highly pathogenic H5N1 virus and were able to demonstrate that the mechanism of protection was non-neutralizing antibodies directed against the HA stem [276]. Other groups have moved away from a “headless” HA and instead have used chimeric HAs that express the stem from one strain and the head from another [158, 277]. These vaccines have induced heterologous and heterosubtypic immunity in mice against the HA stem and show promise as a novel vaccine strategy.

What would be interesting is to combine the success of these chimeric HA vaccines, with the nanoparticle approach in a DNA platform background. Others and myself have shown that the immunogenicity of HA is substantially increased by multivalent presentation on a self-assembling nanoparticle or cage-like structure [238, 276]. The formulation as a DNA vaccine is key since the cage is made *in situ* and assembles *in vivo* rather than producing the vaccine as a purified protein, which may compromise the integrity of the cage vaccine. The combination of the chimeric HA with a nanoparticle cage and formulated as a DNA vaccine would combine the best, most promising approaches for a universal influenza vaccine. In summary, targeting the HA stem with novel protein binder therapeutics and innovative vaccine approaches will enhance current efforts to fight seasonal and pandemic influenza and reduce the disease burden experienced worldwide.

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EDUCATION

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Thesis: Development of Computationally designed vaccines and
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Hemagglutinin.

08/2006-05/2010 Bachelor of Science in Chemical and Biochemical Engineering
Colorado School of Mines, Golden, CO
Research: Cyanobacterial construction of hot spring siliceous
stromatolites in Yellowstone National Park.

HONORS

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06/11-06/15 NSF Graduate Research Fellowship Recipient
05/09-08/09 ASM Minority Undergraduate Research Fellowship,
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2008 Colorado School of Mines Employee of the Year Award
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TEACHING AND STEWARDSHIP

Courses and Lectures:

Graduate:		
Fall Quarter 2011	MICRO 302: General Microbiology Laboratory	Teaching Assistant
Winter Quarter 2012	MICRO 431: Prokaryotic Recombinant DNA Techniques	Teaching Assistant
Oct. 31-Dec 5, 2014 Assistant	CONJ539,	Guest Lecturer and Teaching
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Undergraduate Trainees:

2012-present	Hannah Kalinoski (Microbiology)
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Department Activities:

2011-2012	Recruitment Organizer
2012-2013	Faculty Meeting Representative
2013-2014	Admissions Committee Representative
Oct. 11-14, 2012	SACNAS National Conference, Seattle, WA – UW Microbiology Department Representative and Recruiter
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American Society for Microbiology
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RESEARCH

PEER-REVIEWED ARTICLES:

1. **Koday MT**, Nelson J, Chevalier A, Koday M, Kalinoski H, Stewart L, et al. (2016) A Computationally Designed Hemagglutinin Stem-Binding Protein Provides In Vivo Protection from Influenza Independent of a Host Immune Response. PLoS Pathog 12(2): e1005409. doi:10.1371/journal.ppat.1005409
2. Pepe-Rannek, C.C., Berelson, W.M., Corsetti, F.A., **Treants, M.E.**, Spear, J.R.,

“Cyanobacterial construction of hot spring siliceous stromatolites in Yellowstone National Park.,” *Environ Microbiol*, vol. 14, no. 5, pp. 1182–1197, May 2012.

MANUSCRIPTS SUBMITTED OR IN PREPARATION:

1. **Koday, MT**, Leonard, J, Forero, A, Koday, M, Bratt, DL, Fuller, JT, Murnane, R, Reinhart, RA, Duus, K, Hartman, AL, Stefano-Cole, K, Morrison, J, Katze, MG, Fuller, DH. (2016). Multigenic DNA vaccine induces protective cross-reactive T cell responses against heterologous influenza virus in nonhuman primates. (*In preparation*).

PATENTS

1. **Treants M**, Chevalier A, Baker D, Fuller D, March 21, 2014; Polypeptides for Treating and/or Limiting Influenza Infection and Related Methods of Use. US Provisional Patent Application 61/968,874
2. Baker D, Chevalier A, Nelson J, **Treants M**, Fuller D, July 23, 2014; Enhanced Influenza Hemagglutinin Binders. US Provisional Patent Application 62/028,139.

ABSTRACTS AND CONFERENCE PRESENTATIONS:

1. **Treants, M**, Nelson, J., Chevalier, A., Koday, M.A., Kalinoski, Baker, D., Fuller, D.H. Complete Prophylactic and Therapeutic Protection Against Influenza by a Computationally Designed Protein. *Novel Antiviral Therapies for Influenza and Other Respiratory Viruses: Bench to Bedside, Austin, TX. June 2014*. Oral presentation.
2. **Treants, M**, Leonard, J.A., Fuller, J.T., Bratt, D.L., Koday, M.A., Forero, A., Morrison, J., Palermo, R., Murnane, R.D., Agricola, B., Walker, R., Katze, M., Fuller, D.H. A universal influenza DNA vaccine induces robust systemic and mucosal immune responses and protection against a heterologous pandemic strain in Non-Human Primates. *Systems Biology of Infectious Diseases Conference, Seattle, WA. August 2014*. Poster presentation.
3. **Treants, M**, Nelson, J., Chevalier, A., Koday, M.A., Kalinoski, Baker, D., Fuller, D.H. Complete Prophylactic and Therapeutic Protection Against Influenza by a Computationally Designed Protein. *3rd Influenza Research and Development Conference, Boston, MA. July 2014*. Oral presentation.
4. **Treants, M**, Leonard, J.A., Bratt, D.L., Fuller, J.T., Murnane, R.D., Fuller, D.H. Multivalent Particle-mediated DNA vaccine against seasonal and pandemic influenza viruses elicit strong antibody responses in Non-Human Primates. *DNA Vaccine Conference, San Diego, CA. December 2012*. Oral presentation.
5. **Treants, M**, Khan, M., Brutkiewicz, R.R., Role of co-stimulatory molecules on the status and activation of NKT cells. *SRQP Conference, Indianapolis, IN. July 2009*. Poster presentation.
6. **Treants, M**, Khan, M., Brutkiewicz, R.R., Role of co-stimulatory molecules on the status and activation of NKT cells. *CIC SRQP Conference, Ann Arbor, MI July 2009*. Poster presentation.

presentation.

RESEARCH SUPPORT:

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Koday Treants/Fuller (MPI)

2/15/16 – 01/30/17

NIH via Virvio, Inc.

Optimization of Computationally Designed Antiviral for Influenza

The goal of this project is to investigate the efficacy of high-affinity, broad cross-reactive designed proteins in vivo against Group I Influenza A subtypes. Dr. Fuller is a Co-Investigator on this proposal, which is a subcontract from Virvio, Inc.

Role: Principal Investigator