Effect of a Codon Optimized DNA Prime on Induction of Anti-Influenza Protective Antibodies

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Christopher Parker

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APPROVED:

Shan Lu, M.D., Ph.D.	David Adams, Ph.D.	Samuel Politz Ph.D.
Department of Medicine	Dept of Biology and Biotech	Dept of Biology and Biotech
UMass Medical School	WPI	WPI
Major Advisor	Committee Member	Committee Member

ABSTRACT

An effective antibody response is essential for immunity against influenza virus infection and is the primary goal for vaccine development. In this study, codon optimized and wild type DNA vaccines expressing hemagglutinin (HA) antigens of human flu viruses A/H1N1/NewCal/20/99 (H1 serotype) were compared to test the antigenic differences of the constructs in mammalian systems. Furthermore, to determine if a prime-boost immunization strategy was more effective in eliciting a greater immune response, a codon optimized HA vaccine was administered as a prime in conjunction with the trivalent inactivated vaccine (TIV), Fluzone, as a boost and immune responses were measured. We found that protein expression and antibody response levels of HA antigens were increased with the codon optimized construct when compared to the wild type HA gene construct. Prime-boost vaccination of NZW rabbits was able to elicit a greater immune response when compared to TIV alone as measured by enzyme-linked immunosorbent assay (ELISA), hemagglutinin inhibition (HI) and neutralizing antibody (NAb) assays. Together, these studies indicate that optimal HA DNA vaccine formulations should be codon optimized and can be used as part of a prime-boost vaccination strategy.

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BACKGROUND

Influenza related sicknesses have become a global commonality during the winter months. The influenza virus is highly contagious, attacks the upper respiratory tract, and has plagued the world since nearly the beginning of written history. The "flu," as it is commonly known, primarily causes achy muscles, fever and digestive system symptoms, and can cause death in people with weakened immune systems (Langley and Faughnan, 2004). Some strains, however, cause global pandemics of catastrophic proportions such as the Spanish influenza of 1918-1919 during which up to 50 million people were killed worldwide, regardless of the status of their immune system (Palese and Garcia-Sastre, 2002). According to the World Health Organization (WHO), we are due for another pandemic with a human adapted form of the avian flu as the leading potential strain. Global pandemics of the past have reached all continents within 6-9 months but with easier travel, the next pandemic could infect all continents in less than 3 months (WHO, 2005).

Virological Features of Influenza Virus

Types of Influenza Virus

The influenza virus belongs to the family *Orthomyxoviridae* which are enveloped viruses with a segmented single-stranded RNA genome (Palese and Garcia-Sastre, 2002). There are three types of influenza currently in existence, influenza A, B and C. Influenza A and B are morphologically indistinguishable, while influenza C is differentiated by its glycoprotein spikes. Influenza A can spread among both humans and animals, influenza

B affects only humans, and while influenza C affects predominantly humans it recently has infected swine in China (Lamb and Krug, 1996). The influenza A virion is characterized by the 16 subtypes of hemagglutinin (HA protein) and by the 9 subtypes of neuraminidase (NA protein) both of which are protein spikes on the surface of the virus (Lamb and Krug, 1996; Murphy and Webster, 1996). Strains are named based on these two proteins. For example, the current avian flu strain designated H5N1, which currently has not established human-to-human transmission, is an influenza A virus with a subtype 5 HA protein and a subtype 1 NA protein. New influenza virus strains result primarily from antigenic changes of the hemagglutinin and neuraminidase proteins. Antigenic drift results from point mutations that occur during viral replication within a major serotype, while antigenic shift occurs when genes from animal influenza viruses are captured by the human virus via reassortment usually resulting in high mortality (Palese and Garcia-Sastrem, 2002).

Morphology of Influenza Viruses

The important features of the influenza virion are the mushroom-shaped neuraminidase proteins and rod-shaped hemagglutinin proteins (Figure 1). The NA and HA proteins extend inward through the lipid bilayer and into the matrix protein (M_1). Influenza A, B and C also encode transmembrane proteins M_2, NB and CM2, respectively. These proteins are used as ion channels. The outermost layer of the virus is a lipid bilayer under which M_1 is located. The matrix protein is thought to interact with the ribonucleoproteins (RNPs) in the nucleus of the virus (Lamb and Krug, 1996).

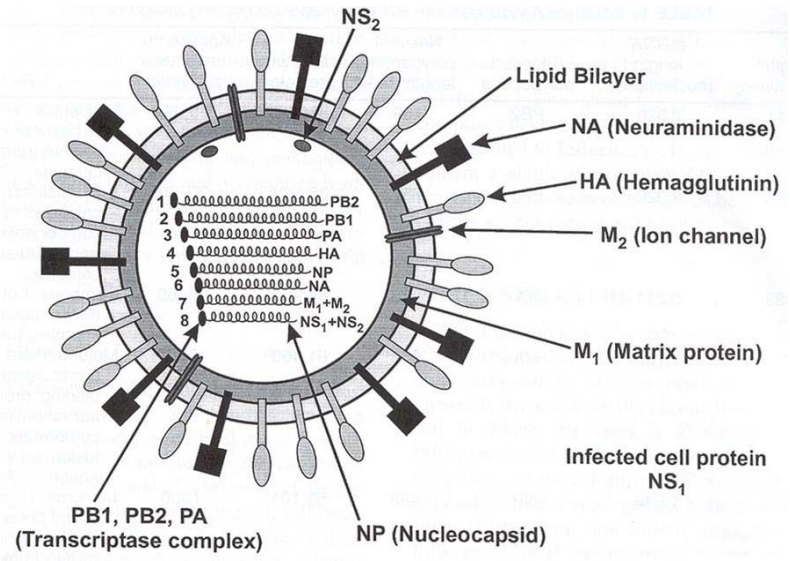


Figure 1: Morphology of the influenza virus. Note the Neuraminidase (black) and rod-like Hemagglutinin (gray) proteins, the primary antigenic components of the virus. (Lamb and Krug, 1996)

The number of RNA segments in the types of virions differ; influenza A and B have eight strands, while influenza C has only seven strands. Influenza C lacks an RNA segment for NA, although the glycoprotein HEF mimics some of the missing NA function in this type. Each of the eight strands has an associated transcriptase complex containing proteins PB1, PB2 and PA. Strand number one is the longest, while strand number eight is the shortest in length. The RNP is formed from the interaction of the nucleocapsid protein and an RNA strand (Lamb and Krug, 1996).

Protective Antigens

The hemagglutinin protein appears to be the most important surface protein. Its rod-shape (in trimeric form) makes it ideal for penetration of the virus into the cytoplasm of the cell it is trying to infect (Figure 2).

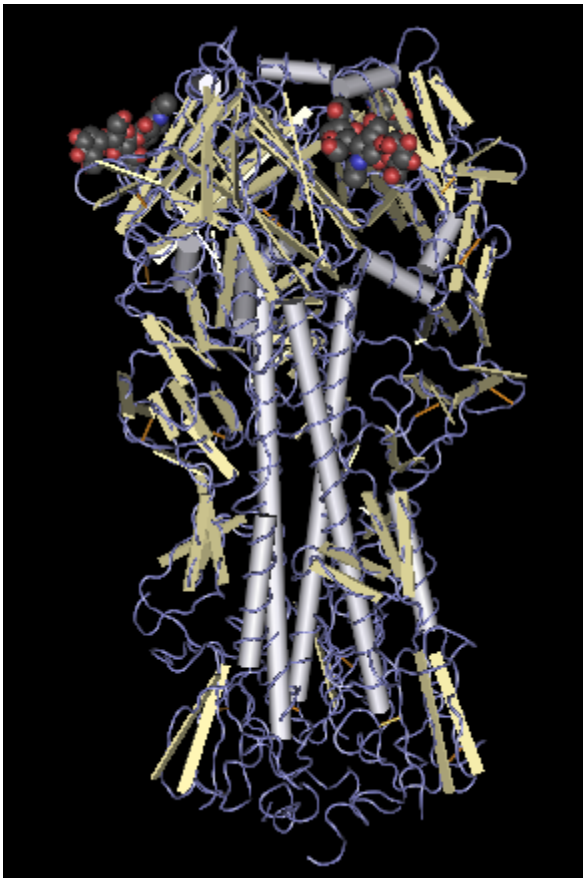


Figure 2: Trimeric HA structure
Three HA monomers converge to form a trimeric structure with hydrophobic fusion peptides hidden within the protein. Once the HA1 portion of the protein has bound to sialic acid of the host cell, it is taken up into an endosome in which the pH causes the protein to become unstable and fusion peptides are exposed to cleavage enzymes breaking the protein into HA1 and HA2 subunits still attached via disulfide bonds. (Protein Database)

The penetration allows for the eventual release of the viral nucleocapsids into the cell (Lamb and Krug, 1996). Hemagglutinin is also the primary protein against which antibody responses are mounted. The HA protein is expressed in the whole unit form, HA0, which is proteolytically cleaved into two subunits, HA1 and HA2, held together by a single disulfide bond. The HA1 subunit forms the orbicular head of the molecule, comprised of the receptor binding site and conformationally dependent epitopes against which neutralizing antibodies are primarily mounted (Dowdle et al., 1974; Webster et al., 1975; Koopmans et al., 2004). The HA2 subunit anchors the molecule into the lipid

membrane and is highly conserved among various influenza virus strains. Cleavage into HA1 and HA2 is required for the virus to become infectious making it a critical determinant in the ability of the virus to spread (Lamb and Krug, 1996).

Neuraminidase is a mushroom-shaped surface protein and is dispersed throughout the membrane (Hay, 1974). This protein permits the transport of the virus through the mucus membrane of the respiratory tract allowing the virus to find its target epithelial cells. It is known that the NA polypeptide is not present in influenza type C viruses. Some less common avian neuraminidases have a receptor binding site that causes hemagglutination (Murphy and Webster, 1996).

Replication

Entry of the virion into the target host cell is facilitated by the binding of the hemagglutinin spikes to the sialic acid of the target cell (Skehel and Wiley, 2000) (Figure 3). This binding can be reversed by polysaccharide cleavage by neuraminidase spikes. It is advantageous for the virus to do this because it prevents virus from being concealed by inappropriate cell types or in mucus. Sialic acid is common on cell surfaces in mucus and would neutralize the virus, rendering it useless. After binding, the cell engulfs the virus by endocytosis via endosomes. The vesicle is then acidified by the cell which allows for trypsin-like enzymes to cleave the HA monomers. This process activates the membrane-fusion function, and since the virus envelope and the endosome's membrane are so close to each other, they fuse together to form one membrane (Lamb and Krug, 1996). It has been shown that the H5 subtype of the influenza virus is not cleaved by trypsin which is available in the upper respiratory tract, but rather by furin which is

available in the lower respiratory tract (Walker et al., 1994). This may provide a possible reason for the current relatively low level of H5 viral spreading human-to-human via coughing or sneezing from the upper tract, and the current lack of an avian influenza pandemic.

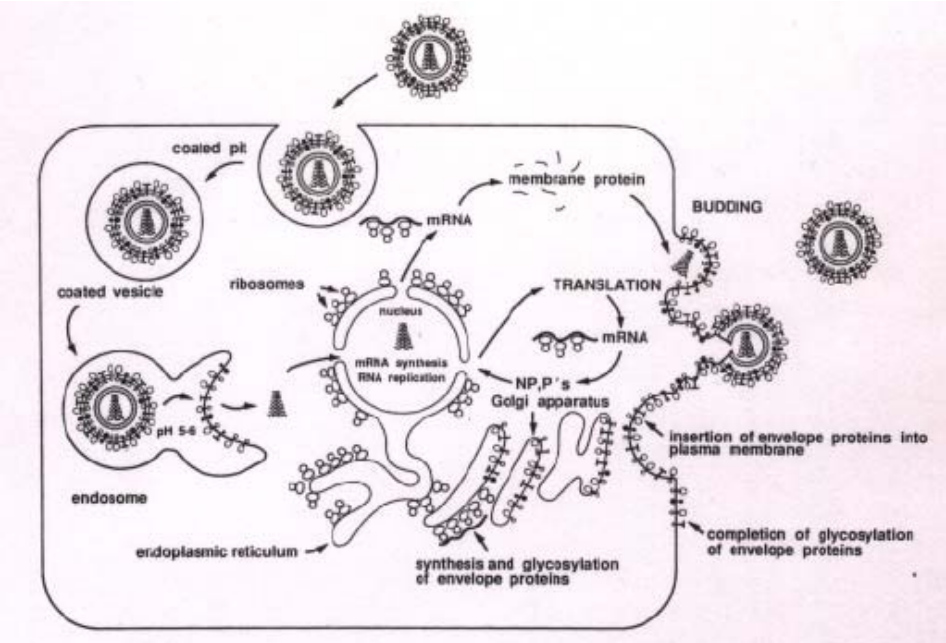


Figure 3: Influenza virus replication. The virus attaches itself to the outside of a host cell (upper part of diagram) and it enters the cell as a vesicle. The viral genes are transcribed and translated by the cell's enzymes and ribosomes. Instead of producing only new cellular material, the cell produces many new virus particles. The new virus particles are eventually released via budding (diagram right side), and infect their own host cells. (Lamb and Krug, 1996)

Next, a target sequence is cleaved in the nucleocapsid protein that results in passage of the nucleocapsid into the nucleus. When the virus first infects the cell, replication cannot occur because active host cell DNA synthesis is required, and virus replication is blocked by antibiotics like mitomycin C. Later, a protein is cleaved off of the RNA strand and a cap is exposed that acts as a primer. PB1 and PA complete the synthesis of the (+) sense strand. There are two types of (+) sense RNA formed, incomplete which serve as mRNA, and cRNA which serve as templates for the synthesis

of daughter (-) sense vRNAs. Most of the proteins created stay in the cytoplasm but the nucleoprotein drifts back to the nucleus where it interacts with the new vRNAs to form new nucleocapsids. Finally, the HA and NA proteins in the cytoplasm attach to a newly formed cell membrane which is exported from the host cell via budding (Murphy and Bang, 1952; Murphy and Webster, 1996).

Transmission and Prevention

Influenza virus is easily spread through the air when an infected person coughs or sneezes. The virus enters the body through the mucosal cells of the mouth or nose. It generally takes one to four days for a person to exhibit symptoms and the person is infectious for up to seven days following the first signs of symptoms. The virus survives most easily in cold and dry weather, which is also the greatest time of indoor person-to-person contact, making the population most susceptible during the winter months (Center for Disease Control, 2006).

Prevention techniques which include frequent hand washing, limited contact with eyes, nose and mouth, and coughing and sneezing into a tissue that is promptly thrown away do not always prevent the spread of the flu virus among the population. Therefore, antiviral drugs and flu vaccines must be developed. There are currently three antiviral drugs on the market (amantadine, rimantadine, and oseltamivir) that are approved for flu treatment and prevention. However, resistance to some antivirals has been found among circulating seasonal flu strains (Englund et al., 1998) underscoring the need for efficacious vaccines.

The foremost prevention measure against influenza infection is vaccination. Currently, there are two different forms of the flu vaccine which have different routes of administration. The most common form is made of a combination of three types of inactive flu viruses, and is administered via intramuscular injection. The newest vaccine is composed of a mixture of three live attenuated influenza viruses (LAIV) and is administered through a nasal spray. Each year, a new vaccine is produced that contains a mixture of three virus strains, two A strains and one B strain. It is these strains that are predicted to reach the United States during the next winter's flu season. Since the live attenuated version of the vaccine is still capable of replication and, therefore, possible infection, it is only recommended for healthy people ages five to forty-nine, while the inactivated virus vaccine can be given to anyone over the age of six months (CDC, 2006).

Current Influenza Vaccines

The flu vaccines used in the United States are trivalent, i.e., consisting of three different strains of influenza that are forecasted, by the World Health Organization, to arrive in the U.S. during any given flu season. The strains present in the United States for the 2006-2007 flu season are two type A viruses H3N2 and H1N1, and a type B virus (CDC, 2006). The inactivated vaccines are grown in eggs, purified and inactivated with chemicals to make them non-infectious. The effectiveness of the vaccine depends on the levels of similarity between the virus strains present in the population, the strains present in the vaccine, the strength of a person's immune system and their age (Palese and Garcia-Sastre, 2002). The live attenuated vaccines are created from the same three strains predicted by the WHO that are included in the inactive vaccine. This type of live

vaccine is based upon a master strain which is grown in eggs at low temperature and over many growth cycles as a way to increase the antigen variability. The vaccine is administered via a nasal spray instead of a needle injection, and is only recommended for healthy people between the ages of 5-49 years old. The age restrictions are in place because the vaccine consists of a weakened form of the actual virus and people with compromised immune systems due to age and/or disease can be susceptible to influenza viral replication and sickness (CDC, 2006). Currently, there are more vaccines in development including a universal flu vaccine based upon the antigenicity of the M_2 protein (Fan et al., 2004; Neirynck et al., 1999) and reverse genetics of the flu genome (Palese and Garcia-Sastre, 2002).

DNA Vaccines

Recently, DNA vaccines have been developed as another method to combat the flu virus. DNA vaccines are based upon the premise that only a portion of a virus' antigens delivered in the form of DNA is needed to elicit a sufficient immune response to combat infection. For DNA vaccines, naked DNA is transfected into target cells and the cells produce the encoded proteins. DNA vaccines are an effective way to create immunity because the antigens are produced within host cells and therefore, are presented via both Major Histocompatibility Complex I and II (MHC-I and MHC-II) pathways, so they can elicit strong T cell immune responses. A strong CD4+ T cell response is critical for the induction of therapeutically useful antibody responses. Another critical advantage of DNA vaccines is that the antigenic proteins are produced endogenously creating a native, tertiary structure compared to the chemically treated virions that are used in current flu vaccines which can incur changes in folding subsequently reducing the

immunogenicity of the vaccine. In addition, DNA vaccines elicit a response in both the humoral (antibodies) and cellular (cell-mediated) arms of the immune system (Lu et al., 1996; Lu et al., 1998; Robinson et al., 1999). Inactivated vaccines are not effective in generating strong immune responses, in particular, they produce poor cell-mediated immune responses (Johnson et al., 1999). Live attenuated vaccines are more effective in eliciting immune responses, however, as previously mentioned, there are significant safety concerns for people with a compromised immune system.

The amount of DNA required to mount a sufficient immune response varies between inoculation methods; however, in general very small amounts of DNA are needed. There are three common methods of inoculation: particle bombardment (gene gun), intramuscular injection (i.m.) and intradermal injection (i.d.). There are advantages and disadvantages to all inoculation methods. Less DNA is required for gene gun inoculation than is needed for i.m. or i.d. injections, and i.m and i.d. may require more immunizations (Bohm et al., 1996; Hartikka et al., 1996). The transfection efficiency of target cells is higher when using the gene gun, since the DNA is bombarded into the cells. The DNA dose required to elicit a good immune response in mice via i.m. injection ranges from 50 to 100 μg, while gene gun inoculation requires only approximately 6 μg at each immunization. The biggest drawback to using a gene gun for immunization is that it has not been approved for human use yet, whereas intramuscular injections are commonly used.

DNA vaccine plasmids are easy to engineer allowing for specific sequences to be designed as a way to increase immunogen variability. The addition of different leader sequences, promoters and other subunits and codon optimization can be utilized to

maximize the translational process. For example, the addition of a human tissue plasminogen activator (tPA) leader sequence can accelerate the humoral antibody responses in rabbits against spike protein which is the protective antigen against the SARS associated coronavirus (Wang et al., 2005a). Since only portions of viral DNA are used, there is no chance for subsequent infection after vaccination as there is when using live attenuated vaccines. Vaccines based on retroviral vectors always present the risk of becoming pathogenic or integrating into the host cell's genome with the possibility of carcinogenesis. However, adenoviral vectors are not pathogenic and are not integrative, therefore, they cause a strong immune response against the vector, hindering its effectiveness.

Influenza DNA Vaccines

Past DNA vaccines against the flu have yielded both positive and negative results. In a Phase I clinical trial, scientists at Merck developed a DNA flu vaccine and assayed for cell-mediated immunity. Researchers hoped that cell-mediated immunity (CMI) would be the most effective way to combat influenza (Donnelly et al., 1995) but the Merck trial failed to yield a detectable target result for possibly two reasons: either their assays were not effective enough at measuring CMI levels, or their DNA vaccine was not fully optimized (i.e. through codon optimization). On the other hand, in another study it was demonstrated that there were excellent humoral responses, and complete protection from a lethal dose of influenza virus from as little as 1 μg DNA given in two doses in a mouse model (Deck et al., 1997).

Prime-Boost Vaccine Strategy

A DNA vaccine can be paired with other combinations of flu vaccines to create an increased immune response. It has been shown that a prime-boost method of immunization elicits a greater immune response against HIV antigens as opposed to using one or the other modality alone (Richmond et al., 1998). Following these results, for this thesis it was reasoned that a DNA flu vaccine used as an immune primer followed by inoculation with an inactivated flu vaccine could boost immunity. If the flu DNA vaccine is administered first then boosted with the inactivated vaccine, a smaller dose of the inactivated vaccine will be needed, which in a future outbreak could help prevent dose shortages, as was seen in the 2004 flu season.

Codon Optimization

Codon optimization is performed as a way to increase protein expression in a particular cell line or organism by changing specific codons to those preferred by that species. In the case of DNA vaccines expressing viral proteins, the codons are switched to those preferred by the vaccinated species, especially to those genes that are highly expressed. The amino acid sequence of the viral vaccine protein remains the same, while the triplet of base pairs (codon) is altered to match the codon preferences in the vaccinated organism. Codons of low frequency in the original DNA are altered to codons of high frequency in the organism the DNA is being introduced to. It has been determined that there is a direct correlation between translational efficiency, which leads to high expression levels, and immunogenicity in host species. Nagata et al. (1999)

showed that the amount of codon optimization of a gene directly correlated with the translational efficiency in mammalian cells.

The influenza A genome is codon-biased meaning that some codons for specific amino acids are more common than others and/or that natural selection can distinguish between synonymous codons (Plotkin and Doshoff, 2003). The most commonly accepted explanation of codon bias is that codon appearance is optimized to match the amount of isoaccepting tRNAs available, allowing for an increased translational efficiency (Zuckerkandl and Pauling, 1965; Ikemura, 1981). Other theories include the ideas that codon bias is a result of selection for regulatory function mediated by ribosome pausing (Lawrence and Hartl, 1991) or selection against stop codons that lead to early termination (Fitch, 1980; Modiano et al., 1981). Whichever the explanation may be, the determination of which codons occur most frequently can be used to increase the efficiently of mRNA translation, and thus boost the overall immunogenicity of a specific viral protein in a vaccine. The codon usage varies in different viral strains because of mutations at the epitopic regions of the HA1 antigen which has more frequent mutations than others (Plotkin and Doshoff, 2003).

Codon optimized DNA vaccines have been developed for other infectious diseases, including HIV. André et al. (1998) created a HIV gp120 codon optimized DNA vaccine, syngp120, and performed cellular transfections and BALB/c mice studies. All wild type codons were replaced by codons found in human genes that are most highly expressed. The group found that codon optimized expression levels were significantly higher than WT levels among various assays. In 293T cells, expression levels increased 10-50 fold using the optimized sequence, in BALB/c mice, the optimized synthetic

sequence produced greatly higher antibody titers as measured by ELISA showing an increased humoral response.

Project Purpose

The purpose of this project was two fold. The first was to determine the immunogenic and expression differences of wild type and codon optimized H1 influenza DNA vaccines using enzyme-linked immunosorbent assays (ELISA) and Western blots. Second, we wanted to compare the effect of a prime-boost immunization schedule on protective antibody responses against the influenza virus compared to DNA vaccination alone. Mice, rabbits and 293T cells were used as model systems to more fully understand the ability of this type of immunization strategy to improve immunogenicity under a variety of conditions. This research will provide useful information and lay groundwork for future experiments involving other influenza DNA vaccines.

Materials and Methods

DNA Sequences

The influenza H1 strain A/H1N1/NewCal/20/99, GenBank accession number AJ 344014, and the influenza H3 strain A/H3N2/Panama/2007/1999 accession number DQ865956 were used as backbones for construct design for each strain of influenza. Codon optimized constructs were created by Geneart (Regensburg, Germany) from the wt sequence into a G + C rich content found in humans. The gene inserts were ligated into vector pSW3891.

Recombinant DNA Techniques

Agarose Gel Electrophoresis

Agarose was dissolved in 1x TAE buffer to a 1% (w/v) concentration. The mixture was then heated in a microwave oven until the agarose was completely dissolved. A Bio-Rad agarose gel casting system was used to pour the gel. After the agarose cooled, samples were loaded with 6X loading buffer. The gel was run in a Bio-Rad gel box at approximately 50-70 Volts for 1 1/2 to 2 hours. The gel was then stained in an Ethidium Bromide solution (25 ng/ml in TAE buffer) for 20-30 min. The stained gel was then photographed using an Olympus COMEDIA Master System 4.1^{TM} digital camera under UV light.

Restriction Endonuclease Digestion

The final volume for restriction enzyme digestions was 15 µl for single digestions

and 20 µl for double digestions. The appropriate enzyme concentration and buffer, bovine serum albumin (BSA), sterile H_20, and DNA concentrations were estimated according to the New England BioLabs catalog. DNA was digested at 37˚C for 90 min and analyzed on an agarose gel.

In vitro Expression of Influenza H1.HA0 Antigens

The expression of H1 DNA vaccine constructs was examined by transient transfection of 293T cells (Pear et al., 1993). Transfection was performed when cells were at approximately 50% confluence on 100mm dishes by calcium phosphate coprecipitation, using 20 µg of plasmid DNA per dish. The supernatants and cell-lysates were harvested 72 h after transfection. The H1 protein expression was confirmed by Western blot (see below). A quantitative enzyme-linked immunosorbent assay (ELISA) (see below) was used to measure the amounts of H1 produced from each DNA construct. A known amount of recombinant H1 protein was used to establish the standard curve.

DNA Vaccination Techniques

DNA Tubing Preparation

For all the procedures in this section, a Bio-Rad tubing prep station was used. Rislan tubing was dried for 1–2 hr by forcing N_2 at ~ 0.2 L/min into the tubing. The ends of the tubing were capped after drying to keep moisture out. Water was added to 1 µm gold beads (adjusted for each experiment) to achieve a final concentration of 100 mg/ml. The gold beads were centrifuged to the bottom of a microcentrifuge tube for ~10 sec and the water was removed with a pipette, leaving a small amount of water (~50

µl) in the tube. A 200 µl aliquot of spermidine (100 mg/ml in water) was added to the gold bead pellet and the mixture was vortexed thoroughly at high speed until the gold was completely re-suspended. Plasmid DNA was added to the beads/spermidine suspension. The mixture was vortexed on high, only briefly, as the DNA can shear easily. 400 µl $CaCl_2$ (2.5 M in water) was added drop-by-drop into the mixture while vortexing at medium speed. The mixture sat at room temperature for 3-5 min to allow precipitation and was then centrifuged for 8 sec, decanted and the supernatant was discarded. The coated gold beads were washed 5 times with 1ml dehydrated absolute ethanol. Each time, the ethanol-resuspended beads were centrifuged for 8-12 sec and the supernatant removed. The beads were resuspended in the total volume of absolute ethanol needed for each preparation in a 20 ml glass scintillation vial and capped tightly. A piece of tubing, 2 –3 inches beyond the right end of the tubing prep station, was cut. A 10 ml syringe was attached to the right end of the cut tubing. The glass vial of gold beads was sonicated briefly to completely suspend the beads and the suspension was drawn into the tubing with a syringe. The gold was allowed to settle out of the suspension in the tubing for 10 min. Then, the ethanol was slowly drawn from the tubing with the syringe at a rate of about 2 inches per second. The tubing was connected to the nitrogen port and the prep station was turned on to rotate the tubing and spread the gold over the inside of the tubing for 1 min. The nitrogen flow was turned on to 0.4 L/min to dry the remaining ethanol from the tubing for 5 min as it continued to rotate. The tubing was cut into one-half inch cartridges using the tubing cutter, sealed in a scintillation vial with parafilm and stored at –20˚C.

Anesthesia and Bleeding

Rabbits

A portion of skin was cleaned for the injection of anesthetic solution into the right hind leg quadriceps or lumbar muscle of each animal. The anesthetic solution consisted of Ketamine (100 mg/ml)/Xylazine (100 mg/ml)/saline (5:1). The total volume of the anesthetic solution each animal received was calculated based on the animal's weight (see Table I).

Dosage Per Animal (mg)			
Rabbit Weight (kg)	**Dose of Mixed Ket/Xyl (ml)**	**Ketamine (ml)**	**Xylazine (ml)**
2.0-2.5	*0.6*	*50.0*	*10.0*
2.5-3.0	*0.65*	*54.2*	*10.8*
3.0-3.5	*0.7*	*58.3*	*11.7*
3.5-4.0	*0.75*	*62.5*	*12.5*
4.0-4.5	*0.8*	*66.7*	*13.3*
4.5-5.0	*0.9*	*75.0*	*15.0*
5.0 & up	*1.00*	*83.3*	*16.7*

Table I: Dose and composition of the anesthetic solution injected to the rabbits

Rabbits were then bled from the central ear vein. The backside of the ear was rubbed briskly with an alcohol prep. Then, using a 23-gauge butterfly needle, blood was removed with a syringe until the desired amount was collected. The blood was then transferred to a vacuum tube.

Mice

The abdominal fascia was rubbed with an alcohol prep and 0.5-0.7 µl of anesthetic solution (Ketamine (100 mg/ml)/Xylazine (100 mg/ml)/Saline 4:1:10) was injected intraperitoneally using a tuberculin syringe. Once the animals were sedated, a

glass capillary tube was used to draw blood from the periorbital cavity. The abdominal area was then shaved and DNA-coated gold beads were inoculated using the gene gun. Each animal received 5 doses of 6 shots each according to the schedule in Table III.

DNA Immunization

Mice

Female Balb/C mice, 6–8 weeks old, were purchased from Taconic Farms (Germantown, NY) and housed in the animal facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School in accordance with IACUC approved protocol. Fifty mice were divided into five groups of ten mice each, Table II. Each group received five DNA immunizations by a Bio-Rad Helios gene gun (Bio-Rad, Hercules, CA) and were bled according to the schedule in Table III. The original sequence (wt) or codon optimized H1-expressing DNA pSW-3891 plasmids were coated onto the 1.0 μm gold beads at 2 μg of DNA/mg of gold. Each shot delivered 1 μg of DNA and a total of six non-overlapping shots were delivered to each anesthetized mouse at the shaved abdominal skin. The serum samples were collected immediately before and 2 weeks after each immunization.

Groups	# of Mice	DNA Vaccine
A	10	Saline I.M. (control)
B	10	wt I.M.
C	10	opt I.M.
D	10	wt G.G.
E	10	opt G.G.

Table II: Animal groups and immunizations administered for the mouse experiment. The table shows the composition of the DNA vaccine used to inoculate the mice in each group. Each mouse was given 100 μg of DNA per immunization for I.M. injection while the gene gun mice received 6 μg of DNA per immunization. I.M., intramuscular injection immunization; G.G., gene gun immunization; wt, original DNA sequence; opt, codon optimized DNA sequence

Immunization	I	II	III	IV			V	
Bleed	I	II	III	IV	V	VI	VII	VIII
Week #	0	2	4	8	10	11	13	15

Table III: Immunization and bleeding schedule for the mouse experiment
Bleedings were taken before immunizations when scheduled on the same day.

Rabbits

Female New Zealand White rabbits weighing 2 kg were purchased from Millbrook Breeding Lab (Amherst, MA). Animals were housed in the facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School following IACUC-approved protocols. Rabbits received DNA immunizations by a Helios gene gun (Bio-Rad, Hercules, CA). A H1 or H3 DNA vaccine plasmid was coated onto the 1.0-μm gold beads at 2 μg of DNA/mg of gold so that each shot delivered 1 μg of DNA. At each immunization, the rabbits were anesthetized, and a total of 36 non-overlapping shots were delivered to the shaved abdomen.

Codon Optimization Study

Two groups of two rabbits each were used in a pilot study. Each group was immunized with either wild type or codon optimized H1 HA DNA vaccines (Table IV). The immunization and bleeding schedules are shown in Table V.

Group	Rabbit #	DNA Vaccine
1	316-317	codon optimized H1 HA
2	381-382	wt A/H1N1/NewCal/20/99

Table IV: Animal groups and immunizations administered for the rabbit experiment. The table shows the composition of the DNA vaccine used to inoculate the rabbits in each group.

Immunization	I	II	III		IV		
Bleed	I	II	III	IV	V	VI	VII
Week #	0	2	4	6	8	10	12

Table V: Immunization and bleeding schedule for the codon optimization rabbit experiments

Prime-Boost Study

Four groups of five rabbits each were immunized with different priming and boosting vaccinations using various immunization schedules. Rabbits were given either the TIV Fluzone alone, or a combination of DNA and Fluzone in a prime boost schedule (Figure 4).

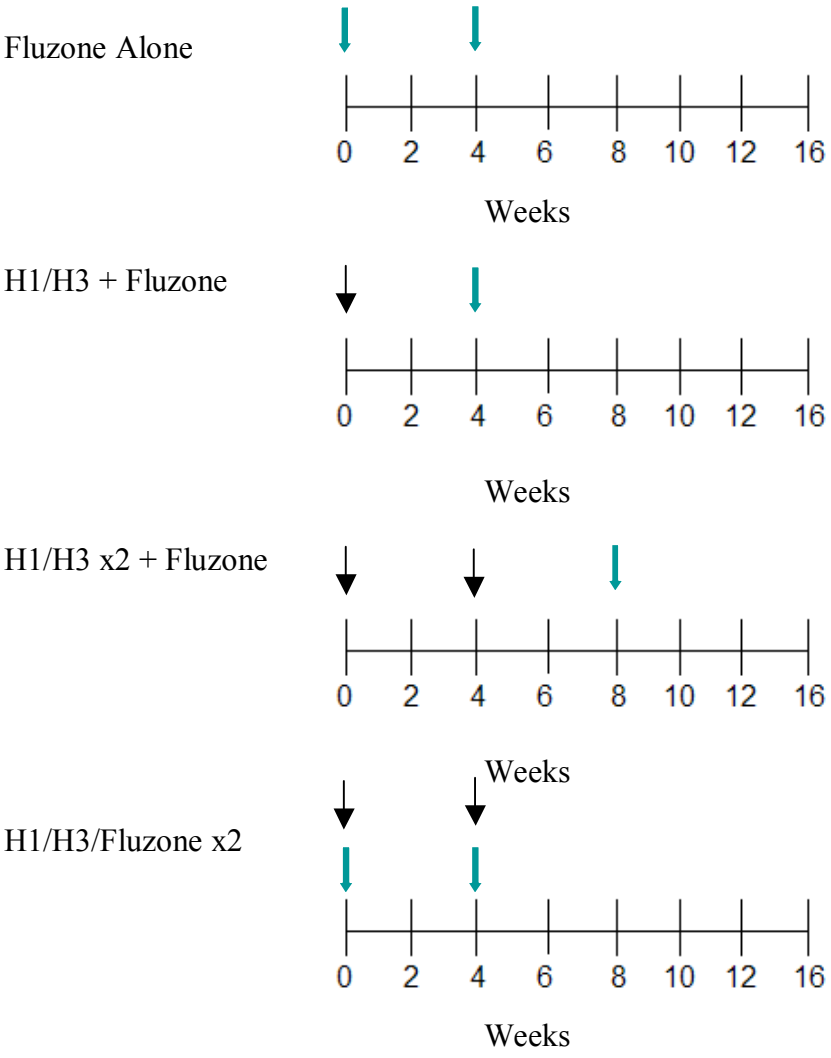


Figure 4: Immunization schedule for prime-boost rabbit experiment. Bleedings were taken at weeks 0,2,4,6,8,10,12,16. Black arrows indicate immunization with H1/H3 DNA vaccine and blue arrows indicate a boosting immunization with Fluzone TIV.

Immunological Techniques

ELISA

Concanavalin A (ConA) Antigen Capture ELISA for Detection of Antibody

96-well flat bottom plates were coated with 100 µl/well of Concanavalin A (ConA) at a concentration of 50 µg/ml in PBS, pH 7.4 and incubated for 1 hr at room temperature (RT). For all washes, the plates were rinsed 5 times with wash buffer (1x PBS plus tween-20) 3 times for 1 min, one time for 15 min, and one time quickly. 100 µl/well of PBS diluted H1.HA0.dTM, H3.HA0.dTM or Fluzone antigen at a 1:10 or 1:5 dilution were added to the plates and allowed to incubate for 1 hr at RT. The plates were washed, free ConA binding sites were blocked with 200 µl/well of blocking buffer (5% non-fat dry milk in whey dilution buffer), and plates were incubated overnight at 4˚C. After a series of washes, the plates were incubated in either 100 µl of anti- H1 rabbit sera at 1:5000 dilution or mouse sera at 1:1000 dilution in whey dilution buffer for 1 hr at RT. After a series of washes, plates were incubated in either 100 µl of biotinylated anti-rabbit IgG at 1:5000 or anti-mouse at 1:1000 in whey dilution buffer for 1 hour at RT. Plates were washed and incubated in 100 µl of streptavidin-conjugated horseradish peroxidase (HRPstreptavidin) at 1:10000 in whey dilution buffer for 1 hr at RT. The assay was developed using 3, 3', 5, 5'-tetramethylbenzidine (TMB) (1 TMB tablet/10 mL 0.05 M phosphate/citrate buffer/0.006% H_2O_2). 100 µl of TMB was added to each well allowing 3.5 min incubation time at RT. The reaction was stopped by adding 25 µl of 2N H_2SO_4 to each well and the OD was read at 450 nm using a Dynex Opsys MR™ plate reader.

SDS PAGE and Western Blot

A 10 % acrylamide/bis-acrylamide minigel in a tris-glycine buffer system was run with the supernatant and/or slysate obtained from harvesting the transfected 293T cells, for approximately 1.5 hr at 50 mA. Before loading into the minigel, samples were mixed with loading buffer and boiled for 3-5 min. Using a semi-dry blotting apparatus, the protein was transferred to a PVDF membrane for 1.5 to 2 hr at approximately 80 mA per gel. The cut piece(s) of membrane were pre-treated by submerging them into methanol for 2 min, then transferred to ddH_2O and washed. Finally, the membrane(s) were soaked in transfer buffer. A cut piece of 3MM Whatman™ paper (soaked in transfer buffer) was placed on the anode of the blotting apparatus followed by the membrane, the gel, and finally another piece of Whatman™ paper. After the transfer was done, the membrane was incubated overnight in 10 ml of blocking solution (PBS, Tween-20 0.1-0.2% v/v, I-Block™ 0.1% w/v) at 4ºC.

Antibody stocks were diluted to the desired concentration (1:200 for rabbit sera) with blocking solution. 5 ml of the diluted antibody were added to each membrane and were allowed to incubate on the rotator at low speed for 1 hr. The membrane was then washed 4 X 20 min, with 20 ml of blocking solution, on the rotator at high speed. The membrane was then incubated on the rotator at low speed with the secondary antibody solution (diluted 1:5000 in blocking solution) for 1 hr. The membrane was then washed with 20 ml of blocking solution four times, for a total of 1.5 hr, on the rotator at high speed.

Chemiluminescence was performed using the Tropix Western Light™ protein detection kit. Two pieces of thin, transparent plastic, big enough to cover the membrane

were cut. 1.5 ml of substrate was added per membrane. The "sandwich" was allowed to incubate for 5 min. The membrane was dried on a paper towel and transferred to a new dry sandwich. This was exposed to x-ray film in the darkroom for variable times.

RESULTS

Codon Optimization

Codon Usage

Two different hemagglutinin based DNA vaccines were created to test variability *in vitro* in 293T cells and *in vivo* in rabbit and mouse animal models. Both wild type and codon optimized sequences were used. Gene inserts were ligated into vector plasmid pSW3891 at BamHI and PstI restriction enzyme sites. Table VI shows the codon optimization for Leu, Lys, and Pro amino acids. Each amino acid can be encoded by multiple codons. The wild type HA codons (second column from the right) are shown in their wt frequencies. The optimized codons are shown in the right column with their frequencies. In this study, the wild type sequence was codon optimized to increase the level of preferred codon usage in mammalian cells, as indicated by the G + C content. The G + C content of the wild type gene was 41.24% while the codon optimized sequence was 60.13%.

Amino acid	Codon	HA coding seq.	
		wt	opt
Leu	TTA	14	0
	TTG	24	0
	CTT	10	2
	CTC	2	0
	CTA	21	0
	CTG	**29**	**98**
Lys	AAA	**69**	19
	AAG	31	**81**
Pro	CCT	24	5
	CCC	14	**95**
	CCA	**57**	0
	CCG	5	0

Table VI: Example Codon Usage of H1 HA Wild Type and Codon Optimized Genes. Numbers indicate percentage of codon usage for each codon of various amino acids in the influenza virus genome

A MacVector computer program was used to analyze the influenza HA gene (Figure 5A). A codon preference value of 1 indicates the same frequency of human preferred codons was used in this particular gene as would be expected from a human gene sequence. A value below 1 indicates a lower frequency usage of human preferred, G + C rich, codons. When a value greater than 1 is observed, it indicates a higher frequency of human preferred codons usage. As seen in Figure 5A, the WT gene sequence (left plot) contains a sequence at or below a value of 1 for the full length of the protein, whereas the codon optimized sequence (right plot) is well maintained over a value of 1 throughout the entire length of the protein making it more suited for expression in a human cellular environment.

Protein Expression

Hypothesis-1 of this thesis is that codon optimization of influenza H1 HA protein will increase its expression *in vitro*. In order to test expression levels of the optimized and wild type constructs, transient transfections and subsequent Western blots were performed (Figure 5B and C). 293T cells, a human embryonic kidney cell line, were inoculated with one of the wild type or codon optimized DNA and cell lysates and supernatants were collected. The cell lysates were used for Western blot analysis. Antibody detection was performed using rabbit sera inoculated with DNA encoding H1-HA0.tPA.dTM. The nomenclature for the rabbits inoculated with this optimized construct stands for H1 subtype, whole HA subunit HA0 with the construct containing a tPA leader sequence and the subsequent protein with the deletion of the transmembrane domain. As seen in the Western blot in Figure 4B, the codon optimized DNA sequence was expressed at a higher level than the wild type sequence. The protein bands on the

blots were then quantitatively measured using a densitometric approach (Figure 5C). Thus, this data support hypothesis-1 of this thesis that codon optimization of influenza H1 HA increases its expression *in vitro*.

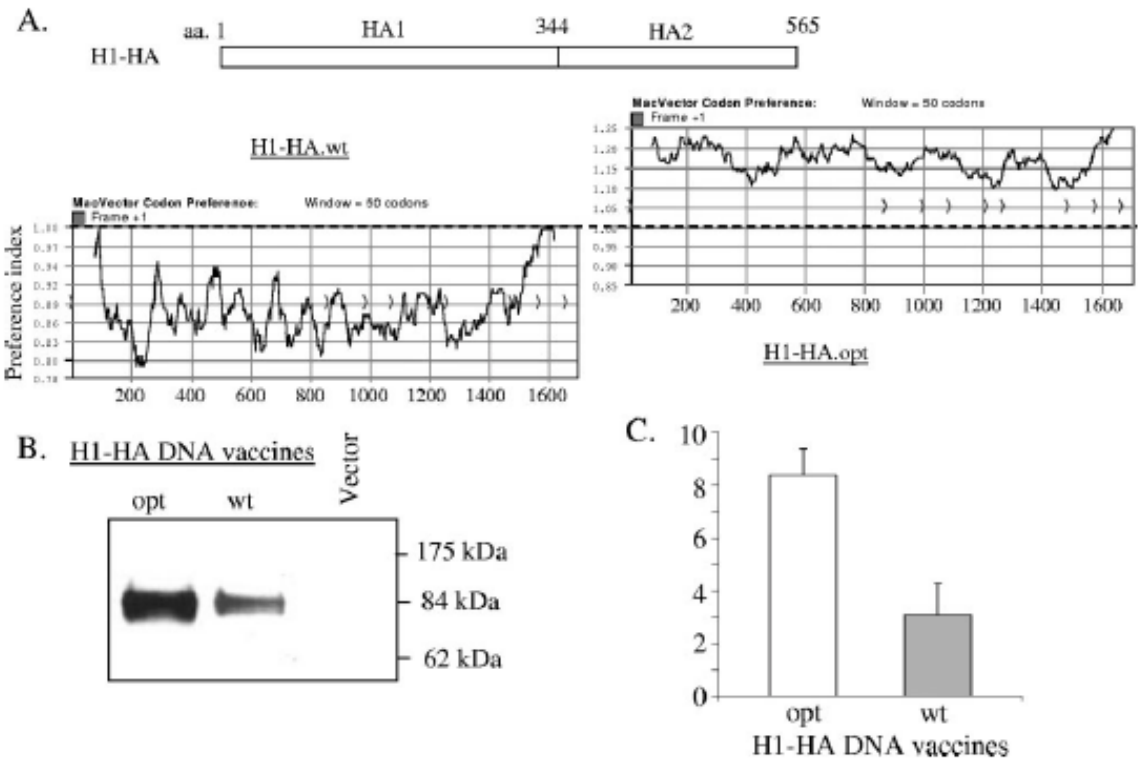


Figure 5: Influenza A H1 hemagglutinin (HA) gene codon preference and expression.

(A) Schematic diagram of influenza type A H1 HA.HA0 gene with HA1 and HA2 domains. The coding preferences of wild type (H1-HA.wt) and codon optimized (H1-HA.opt) H1 HA genes were analyzed using the computer software MacVector. Plots with values above 1.0 indicate bias toward codons more frequently used in mammalian systems, whereas plots below 1.0 indicate less preferred codons in mammalian systems.
(B) Western blot analysis of HA expression from either wild type (wt) or codon optimized (opt) H1-HA DNA vaccines in lysates of transiently transfected 293T cells. Lysates from cells transfected with empty DNA vector pSW3891 without HA antigen insert were included as a negative control.
(C) Quantification of HA expression from either wild type (wt) or codon optimized (opt) H1-HA DNA vaccines by densitometry. The relative amounts of HA antigens were measured by scanning HA-specific bands on Western blots by using an image processing system (Fujix Pictrography 3000, Science Lab 2003, Image Gauge 4.22). The measured AU showed the geometric means of four independent assays. Error bars indicate standard deviations.

Immunogenicity

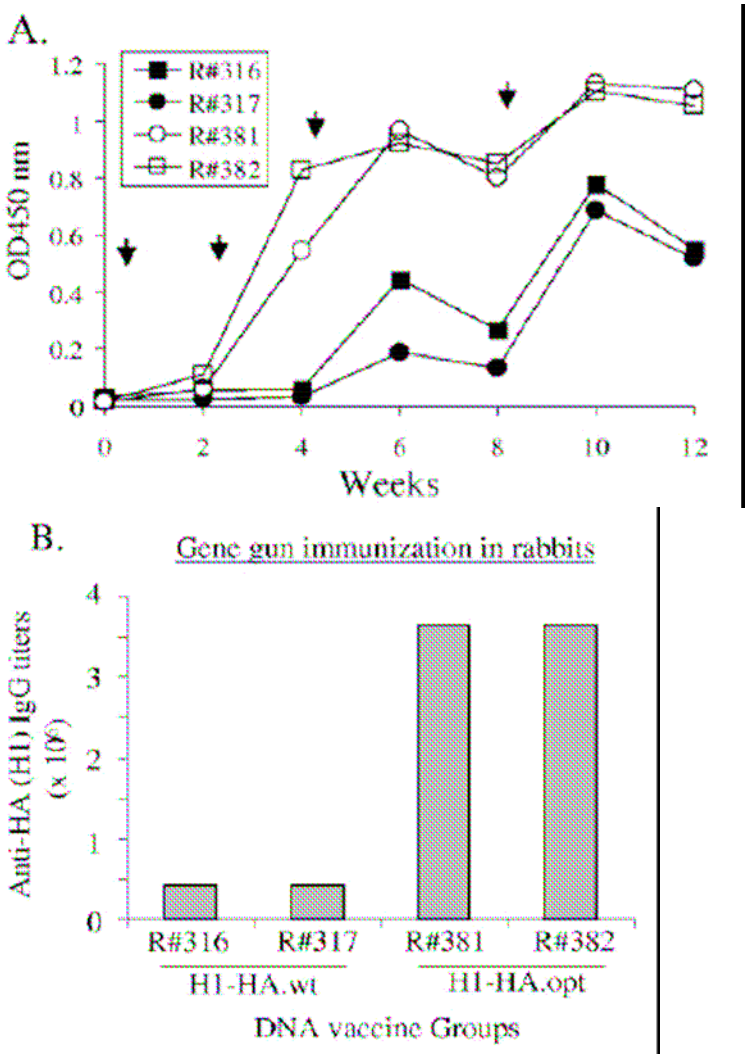
Animal Studies

Hypothesis-2 of this thesis is that codon optimized H1 HA DNA vaccines will produce a stronger immune response than non-codon optimized vaccines. Two animal models (mice and rabbits) were chosen to test the immune responses. The mouse model was used because it is more standardized and mice genetically homogenous unlike rabbits. Rabbits, on the other hand, allow for a greater volume of sera to be harvested and their immune response more closely parallels a human response. These two specific animal models, Balb/c mice and White New Zealand rabbits, are commonly used in vaccine studies and are highly documented in literature.

The relative immunogenicity between the wild type and codon optimized H1 HA DNA vaccines was studied in rabbit and mouse models. In a pilot rabbit experiment, the codon optimized H1 HA DNA vaccine was able to elicit high level anti-HA antibody responses after only two DNA immunizations whereas three to four immunizations were required to elicit good but still lower levels of anti-HA antibody response for the wild type H1 HA DNA vaccine (Fig. 6A). For the wild type HA DNA vaccine, even after multiple DNA-boosting immunizations, the peak level anti-HA antibody response expressed as antibody titer was still lower than that observed for the codon optimized HA DNA vaccine (Fig. 6B).

A similar study was conducted in mice, but with an increased group size (10 animals per group). The geometric mean end titer for the codon optimized HA DNA vaccine group was significantly higher than that of the wild type HA DNA vaccine group

($P< 0.001$) (Fig. 6C). Thus this data supports hypothesis-2, and has shown that codon optimization is effective for improving the immunogenicity of the flu H1 HA DNA vaccine.



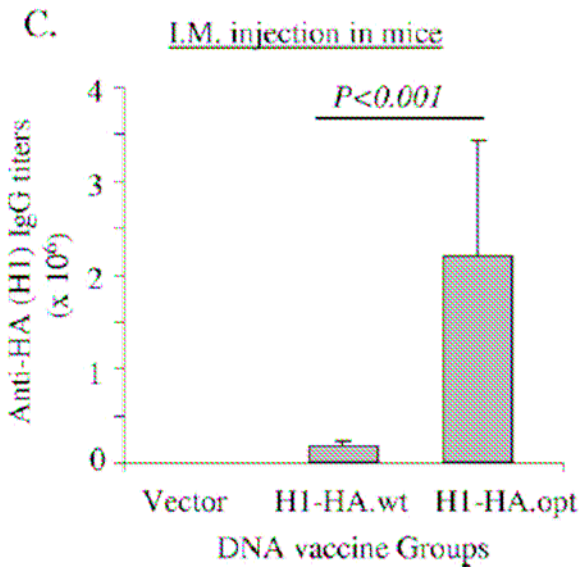


Figure 6: Relative immunogenicity of H1 HA wild type and codon optimized vaccines in rabbit and mouse models
Serum anti-HA IgG antibody responses induced with the vaccinations with either H1-HA.wt or H1-HA.opt DNA vaccines in NZW rabbits (A and B) and BALB/c mice (C) as measured by ELISA.
(A) Temporal anti-HA IgG responses in rabbits measured at a 1:5,000 serum dilution. The arrows indicate the time of gene gun-mediated DNA immunizations. Animals R#316 and R#317 received H1-HA.wt DNA vaccine, while R#381 and R#382 received H1-HA.opt DNA vaccine. OD450, optical density at 450 nm.
(B) End titers of serum anti-HA IgG responses at 2 weeks after the fourth DNA immunization from the same rabbits as shown in panel A. End titers were defined as the dilution at which the absorbance reading was 2X negative control background values.
(C) End titers of serum anti-HA IgG responses in BALB/c mice at 2 weeks after the fourth DNA immunization. Animals received either H1-HA.wt or H1-HA.opt DNA vaccines by intramuscular inoculation. Data shown are the geometric mean titers of 10 mice in each group with standard deviations (error bars). P values calculated using a student t-test.

Prime-Boost

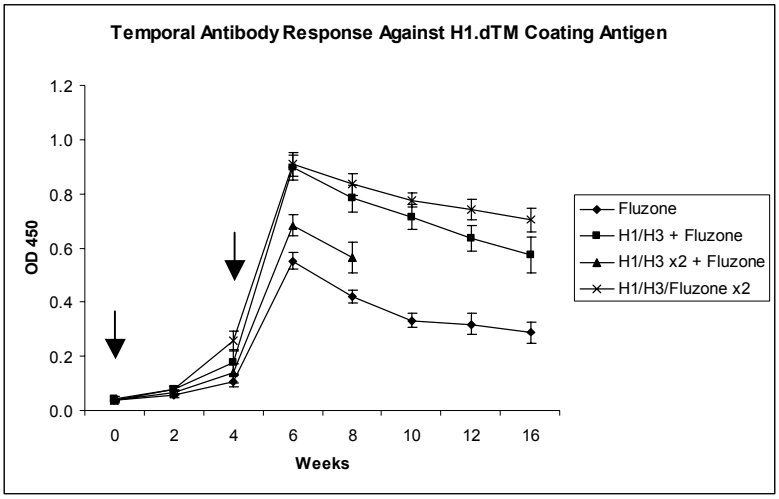
One of the previous challenges seen in the science of DNA vaccines was the inability of immunization with DNA alone to elicit effective antibody responses in humans. As a way to resolve this issue, the prime-boost immunization schedule was developed as described in the background. The concept and effect of priming with a DNA vaccine and boosting with protein immunization was shown in a HIV system by our lab previously (Wang et al., 2005a). Based on that data, hypothesis-3 of this thesis is that a prime-boost strategy will produce a stronger HA immune response than DNA

vaccination alone. We set out to determine the effect of prime-boost immunization on influenza HA DNA vaccines. Rabbits were immunized as previously described (Wang et al., 2006) with combinations of subtype H1/H3 DNA vaccines, and boosted with the commercial trivalent inactivated vaccine (TIV) Fluzone. Four different immunization schedules were tested in four groups of five rabbits each as shown in Materials and Methods (Figure 4): 1. Fluzone prime immunization with Fluzone Boost, 2. H1/H3 DNA prime with Fluzone boost, 3. two H1/H3 DNA primes with one Fluzone boost, and 4. H1/H3 DNA and Fluzone given at the same time twice. Temporal ELISAs were performed to look for trends in the anti-HA antibody response over time. Plates were coated with either H1.HA, H3.HA or Fluzone to look for antigen specific IgG responses (Figure 7). Similar trends were seen against H1 and H3 HA coating antigens in which any combination of DNA alone or prime-boost method elicited stronger antibody responses than the TIV alone (Figure 7A and B). When referring to immunization with DNA alone, we truncated the graph of the group receiving two DNA immunization plus a TIV boost before it received its TIV boost. The data indicates that immunization with TIV alone was significantly less effective at eliciting optimal immune responses as compared to immunization with either DNA alone ($p<0.05$) or multiple prime-boost strategies ($p<0.05$) (Figure 7).

The TIV Fluzone is chemically deactivated virions, meaning that unlike our DNA vaccines, it contains numerous proteins such as NA, M and M_2 to which immune responses can also be mounted in addition to HA. Since this is the case, when coating ELISA plates with Fluzone, we see a different temporal pattern because all of the extra antibodies induced by the boosting immunization are also being captured (Figure 7C).

It's because of this non-HA specific antibody response that only this Fluzone coating ELISA is being shown since it's not within the breath of this project's anti-HA focus.

A.



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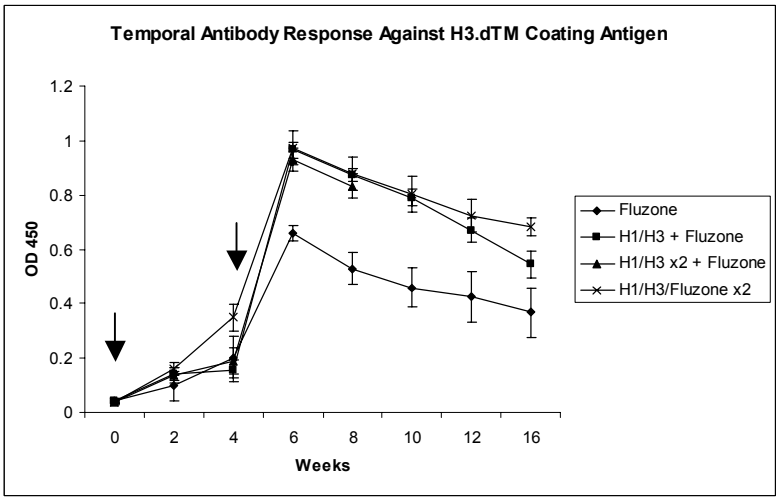

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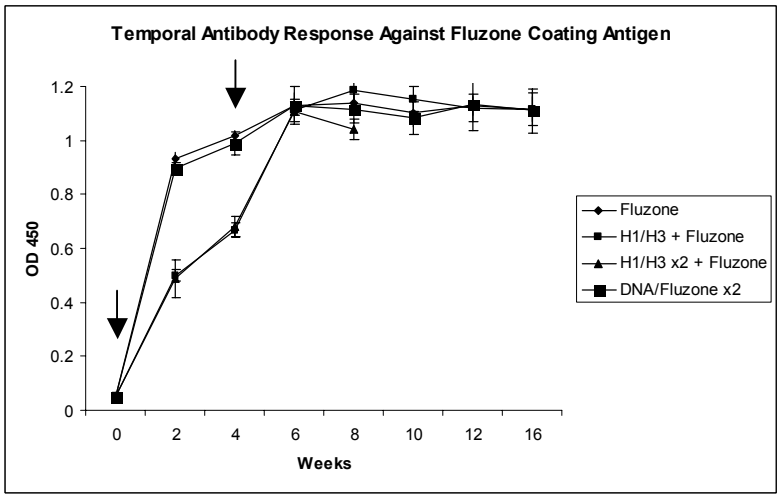
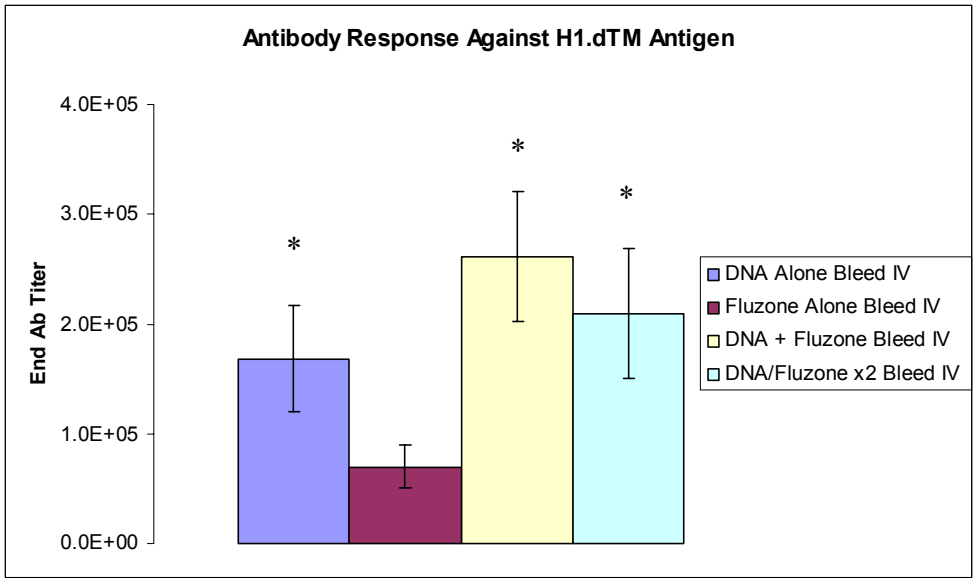


Figure 7: Time course of specific antibody responses measured by ELISA
Temporal anti-HA IgG antibody responses were measure by ELISA and captured by H1.dTM (A), H3.dTM (B), and Fluzone (C). Arrows indicate time of DNA or Fluzone immunization (see methods for specific schedule). The H1/H3 x2 + Fluzone group curve was truncated before its boost at week 8 to show the effect of immunization with DNA alone. Serum dilutions of 1:5000 were used and measure by OD 450nm and curves are an average of 5 rabbits. Error bars indicate standard error of the mean.

Peak IgG responses were reached at week 6, as shown by the temporal curve (Figure 7). In order to more accurately quantify the effect of prime-boost, end titers at peak levels of the anti-HA antibody response were determined (Figure 8). End titers were determined as the highest dilution at which antibody levels were 2x the negative control. All combinations of DNA prime and TIV boost elicited greater immune response as compared to TIV alone when tested against H1 HA dTM or H3 HAdTM. This data supports thesis hypothesis-3, and this assay alone can begin to show how effective our DNA vaccine is and how poor the current TIV given seasonally is.

A.



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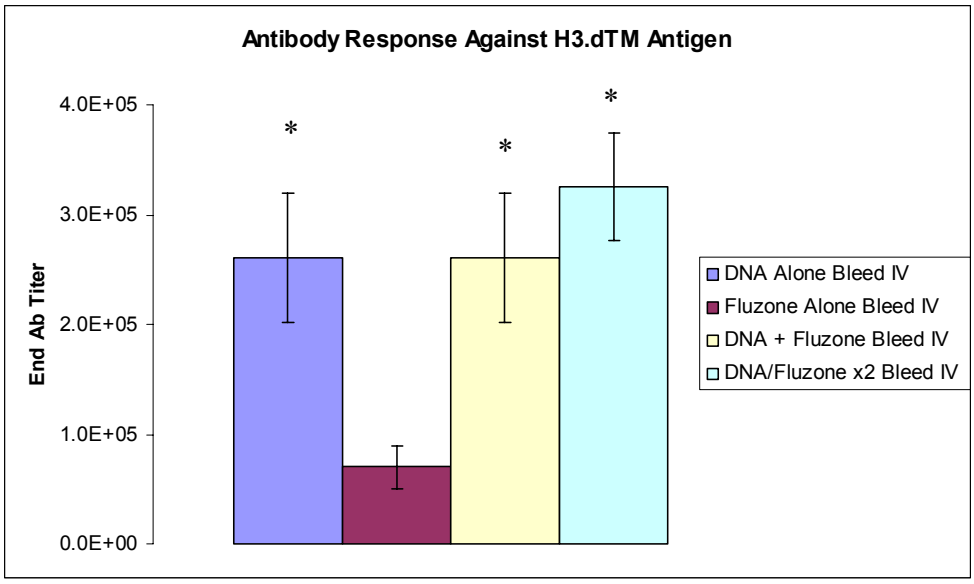


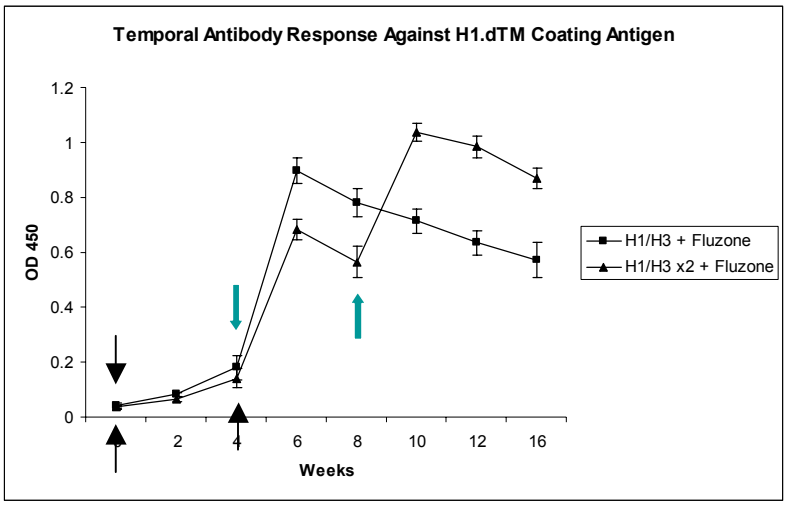
Figure 8: Peak anti-HA IgG antibody response in prime-boost model
Peak-level serum anti-HA IgG antibody responses in NZW rabbits at two weeks after second immunization induced by different H1/H3 HA DNA and Fluzone TIV vaccines as measured by ELISA. Antibodies were captured by H1.dTM (A) and H3.dTM (B). Data shown are the geometric mean titers of each group (five rabbits per group), with standard error (error bars). * $p<0.05$ compared to Fluzone alone as determined by the student t-test.

One Versus Two DNA Primes

In a separate part of the study, we wanted to look at the effect of priming the immune system with either one or two DNA immunizations on the immune response. An ELISA was performed to measure both the anti-HA IgG antibody response over time as well as peak antibody titers. Against H1.HA antigen, the DNA prime group, which received the boost on the same day that the second DNA was given to the 2x DNA group, was able to reach peak levels quicker than the two DNA alone groups (Figure 9A). However, once the 2x DNA prime group received the TIV boost, the peak antibody level in this group was significantly higher than the peak level of the 1x DNA prime group ($p<0.05$) (Figure 9B). However, at week 6, against the H3.HA antigen, the 2x DNA prime group after receiving only two DNA immunizations (i.e. this group had not yet

received the boost) had similar titers to the prime-boosted 1x DNA group (Figure 9C). After boosting, the 2x DNA prime group reached a peak titer which was significantly greater than the 1x DNA group ($p<0.05$) (Figure 9D).

A.



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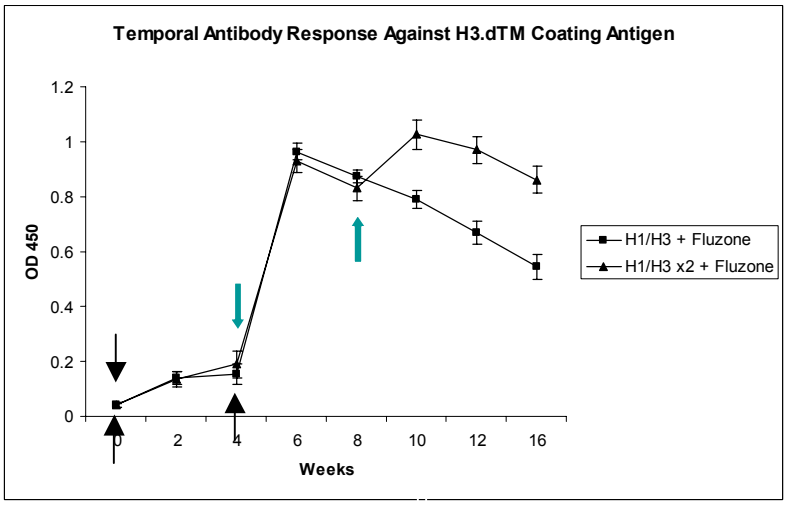

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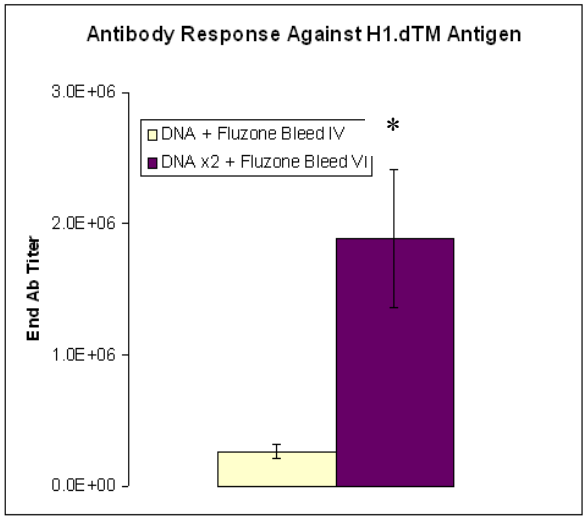

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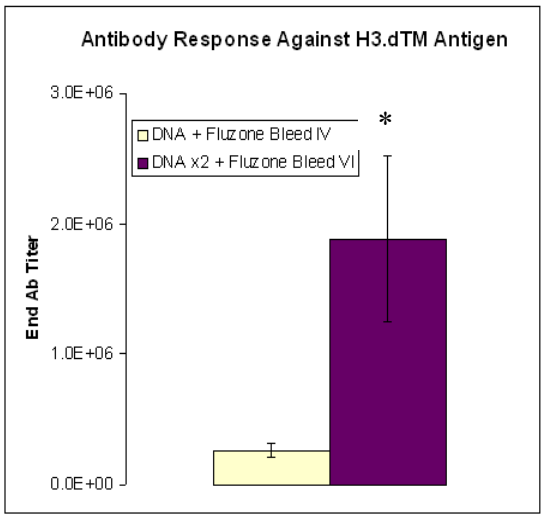


Figure 9: Prime-boost time course specific and peak anti-HA IgG antibody response
NZW rabbits were immunized with either one or two DNA vaccines and boosted once with Fluzone TIV. (A) and (D): H1.dTM (B) and (D): H3.dTM coated ELISA following temporal HA antibody response with peak responses measured as end titers. Peak titer bleeds taken at weeks 6 and 10 for one and two DNA prime groups respectively. Black arrows indicate DNA prime, blue arrows TIV boost. Arrows above the curves in parts A and B indicate immunization points for the one DNA prime group and arrows below the curves are for the two DNA prime group Data points are geometric mean of 5 rabbits with standard error (error bars). * indicates $p<0.05$, compared to one DNA + Fluzone.

Hemagglutinin Inhibition (HI) Assay

The functional activities of the anti-HA rabbit immune sera were investigated by determining the hemagglutinin inhibition (HI) antibody titers against H1 or H3 influenza viruses. The HI assay measures the ability of a particular antibody in a serum to bind virus and thus alter red blood cell (rbc) agglutination. The assay is performed by combining influenza virus and antibody containing serum in a V-bottomed 96-well plate. After 30 minutes of incubation, chicken rbc's are added to the mixture and then kept at 4˚C to allow potential cell agglutination. Antibodies that bind with high affinity to the virion hemagglutinin may prevent the virus from attaching to rbc's. Without the virus attached to rbc's the cells do not form a lattice matrix evenly coating the bottom of the well, but rather the non-viral bound rbc's simply settle to the bottom of a conical well (Figure 10). In an HI titration experiment, the relative affinities of different antisera for virus can be compared.

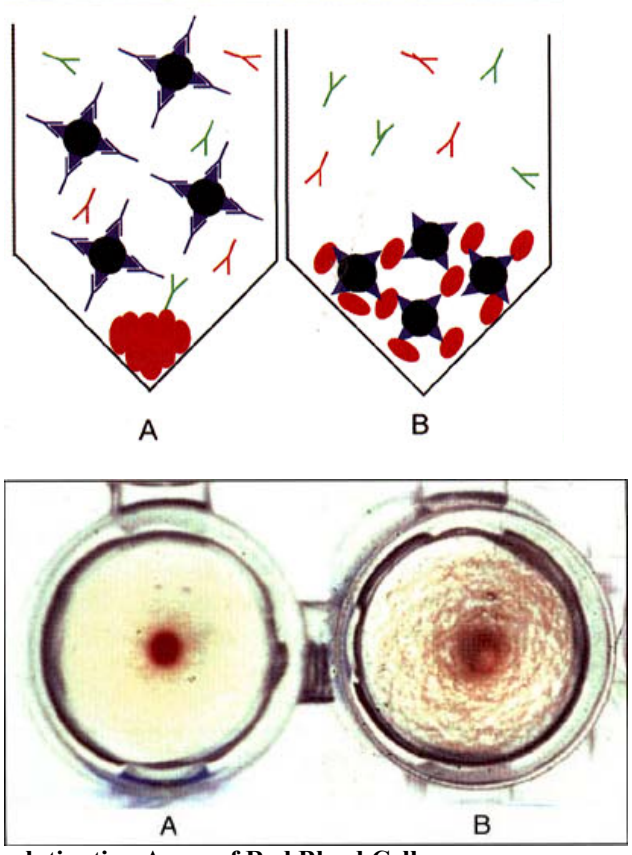
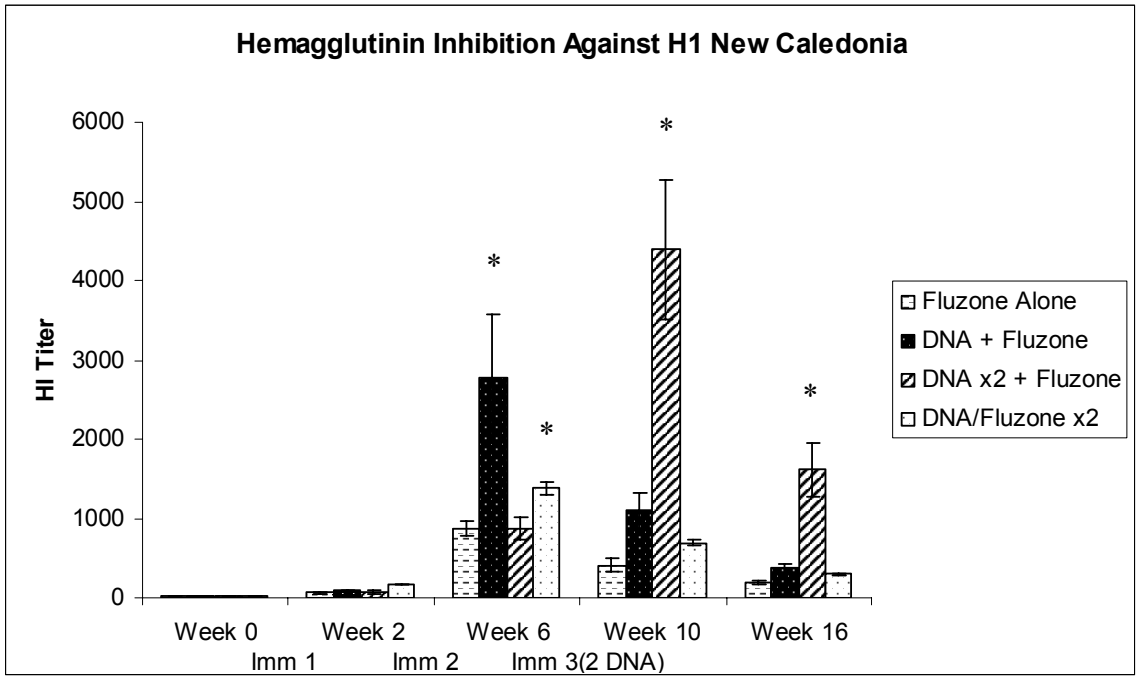


Figure 10: Example Hemagglutination Assay of Red Blood Cells
(A) HI antibodies against influenza HA are able to bind the influenza virus preventing the virus from attaching to rbc's. The non-viral bound rbc's then simply settle to the center of a conical well. (B) A negative control lacking HI antibodies. The influenza virus binds rbc's causing the rbc's to agglutinate and form a lattice matrix that more evenly coats the entire bottom of the well. (Kendall et. al, 1999)

After only one immunization, no vaccine combination was able to elicit a significant HI titer. However, after receiving a boost, HI titers for the 1x DNA plus TIV (boost) and the DNA/TIV together groups were significantly higher than DNA (2x DNA plus TIV group pre boost) ($p<0.05$) or TIV alone ($p<0.05$). However, after receiving the boost, the HI titer was increased in the 2x DNA plus TIV group to a level much higher than any of the other three groups, especially when compared to the group which had received the TIV alone. This observed increase in the HI titer in the 2x DNA plus TIV group remained high throughout the remainder of the study (i.e., through Week 16) (Figure 11).

A.



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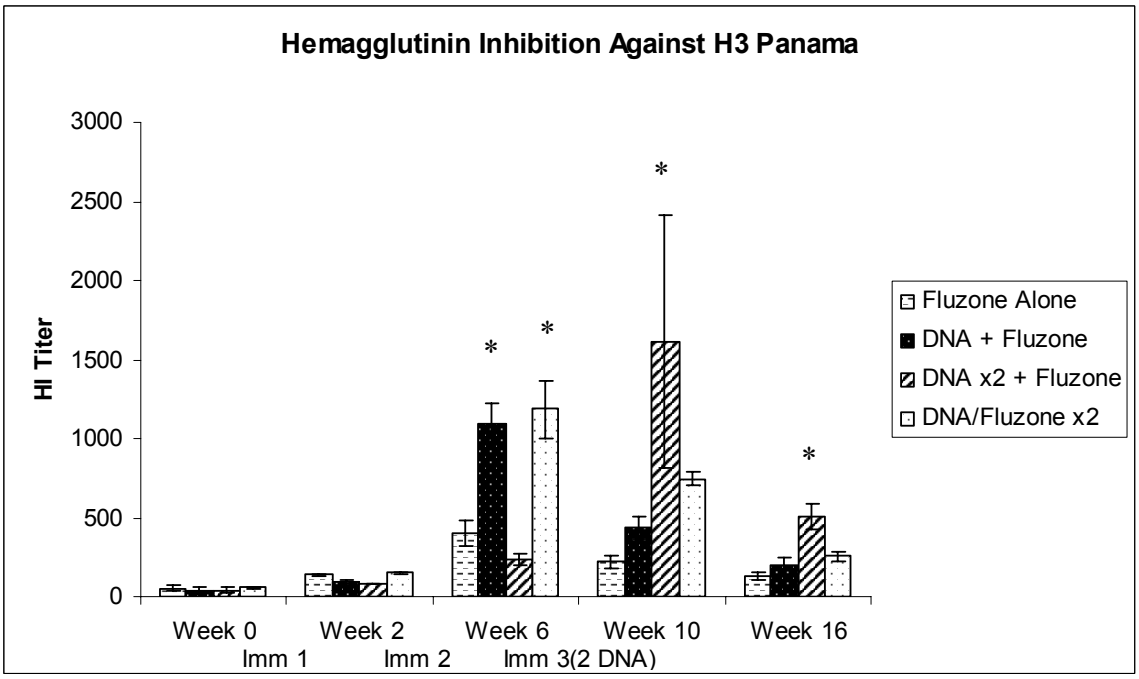


Figure 11: The HI antibody response in NZW rabbit sera
The HI antibody titers are shown as the geometric means for each group (three rabbits per group), with standard error (error bars), against (A) H1N1(A/New Caledonia/20/99) and (B) H3N2 (A/Panama/2007/99) viruses. * $p<0.05$ compared to Fluzone alone calculated by the student t-test

Neutralizing Antibody (NAb) Assay

Although HI titers are an effective way to measure a protective response, the assay itself only measures the ability of anti-HA antibodies in sera to bind influenza virus. The virus neutralization assay was created as a more effective way to relate to what takes place *in vivo* by measuring the blockage of viral entrance into host cells. When vaccinated, the mammalian immune system creates polyclonal antibodies against many epitopes on an antigenic protein and the HI assay measures the binding of all of these antibodies. The neutralizing antibody assay has the ability to measure the subset of these antibodies which more directly affect viral spreading, therefore allowing a more specific look at protective antibodies present. An effective antibody prevents the spreading of the virion into MDCK cells causing no plaques to be formed (Figure 12)

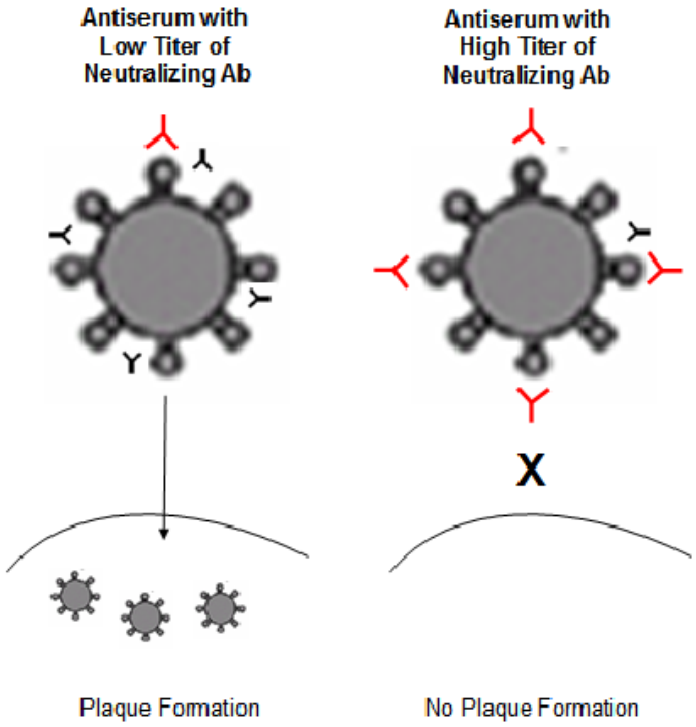
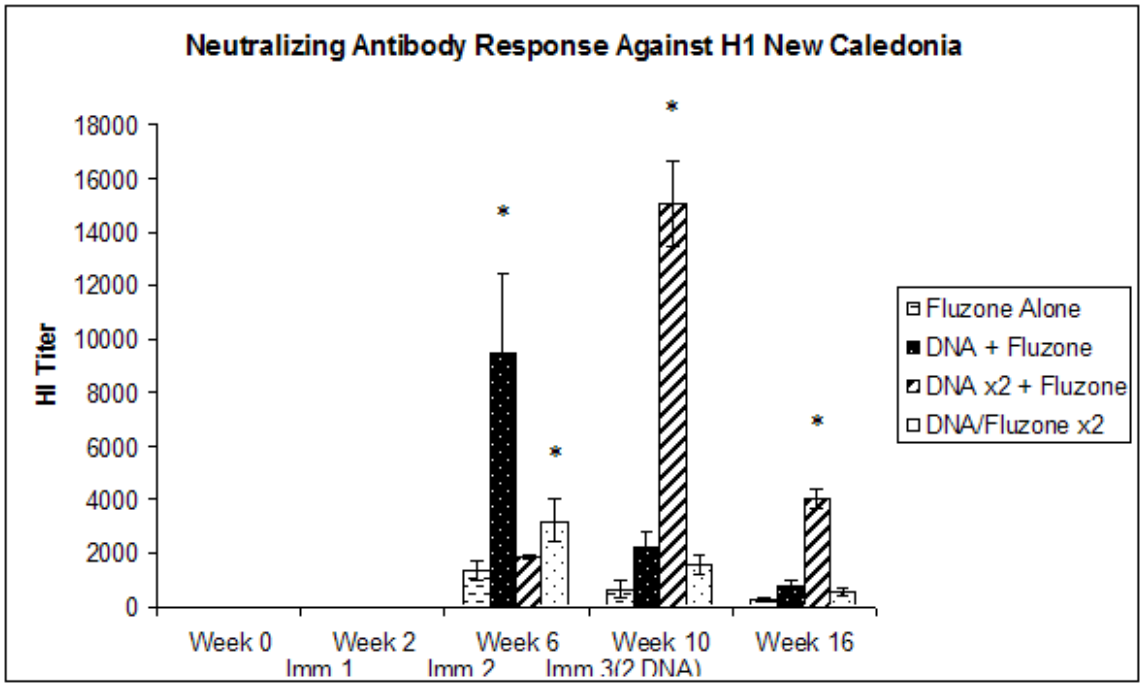


Figure 12: Virus Neutralization Assay

The assay is performed by adding influenza virus mixed with antibody containing serum to a 96-well plate containing a MDCK cell monolayer. After 24 hrs, the virus/serum sample is removed and replaced by normal growth medium. The neutralizing titer is defined as the highest dilution of serum that is able to neutralize 100 PFU of virus in MDCK cells and prevent plaque formation. On the right, neutralizing antibodies are able to bind the virus in a way that prevents the entrance and spreading of virions into the MDCK cell monolayer preventing the formation of plaques. A polyclonal rabbit antiserum will contain both neutralizing and non-neutralizing antibodies, and the neutralization assay will measure only the neutralizing antibodies. Red colored Y's represent neutralizing antibodies and black colored Y's represent non-neutralizing antibodies.

Neutralizing antibody (NAb) titers were found against H1 and H3 influenza viruses (Figure 13). The patterns of neutralizing antibody activities for different prime-boost schedules of HA DNA vaccines and TIV were similar to the patterns observed in the HI assay. With both assays, peak antibody levels against the H1 virus of the DNA/TIV administered together vaccine were less effective than the one DNA prime plus subsequent TIV. At week 6, the 1x DNA followed by TIV and the DNA/TIV together groups reached significantly higher NAb titers as compared to DNA alone ($p<0.05$) or TIV alone ($p<0.05$). At weeks 10 and 16, after receiving the TIV boost, the 2x DNA prime group reached NAb titers greater than any other prime-boost combination; this high level was against both H1 New Caledonia and H3 Panama virus subtypes through 16 weeks (Figure 13). Thus this data shows that for influenza HA antibodies, the DNA prime-boost vaccination strategy is superior to DNA vaccination alone or the TIV vaccine alone.

A.



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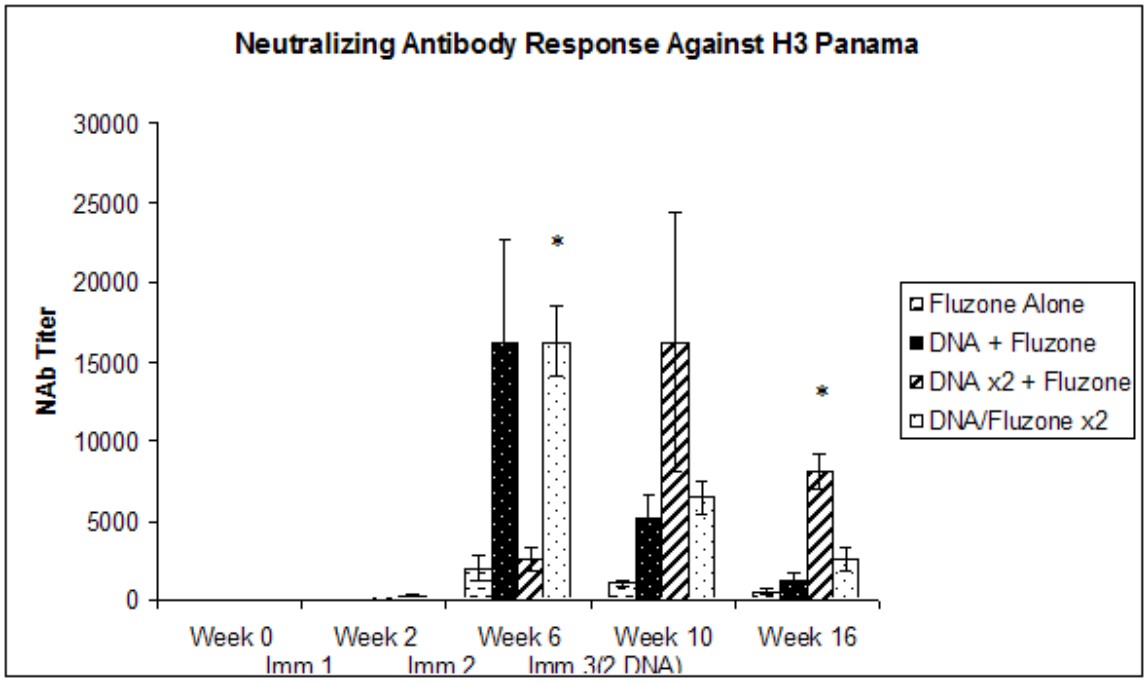

Figure 13: NAb response in NZW rabbit sera
The NAb antibody titers are shown as the geometric means for each group (three rabbits per group), with standard error (error bars), against (A) H1N1(A/New Caledonia/20/99) and (B) H3N2 (A/Panama/2007/99) viruses. * $p<0.05$ compared to Fluzone alone.

DISCUSSION

The data presented in the first part of this thesis provide evidence that codon optimization is effective for improving the antigen expression and immunogenicity of influenza HA DNA vaccines. In early flu DNA vaccine studies, only low or undetectable levels of HA-specific antibody responses were found after immunization with DNA. Recently, the use of codon optimization has been demonstrated to be an effective method for enhancing the immunogenicity of DNA vaccines which express HIV antigens (Wang, 2005a). In addition, codon optimized DNA vaccines have been used successfully against SARS (Wang, 2005b) and other pathogens.

In the current codon optimization study, we used a H1 subtype HA antigen to test the effect of codon optimization on the immunogenicity of an antigen. We compared the levels of HA expression and immunogenicity between wild type and codon optimized influenza HA DNA vaccines in rabbit and mouse animal models with two DNA vaccine delivery methods (gene gun and intramuscular injection). The data clearly show that the codon optimized HA DNA vaccines were able to induce significantly higher levels of HA protein levels *in vitro*, and enhanced HA-specific antibody responses when compared to the the wild type construct. We believe that this is, in part, due to the ability of codon optimized HA DNA vaccine to stimulate a quicker immune response and eventually increase peak-level anti-HA IgG antibody responses.

The ability of our H1 HA DNA vaccine to elicit high-titer, anti-HA antibodies, prior to exposure to the viral antigenic protein, paved the way for us to expand our study to determine the immune response against codon optimized, bivalent H1 and H3 subtype DNA vaccines. When matched with a protein boost, the effect of DNA vaccines are able

to be further enhanced, and this has been shown by many groups including our lab for other viruses. With a commonly used flu TIV already on the market, we have the unique fortune in the influenza DNA vaccine field to already have our boost readily produced. The rationale to any vaccine study is to either create a prevention tool for a pathogen to which a vaccine has yet to be created or to improve upon an existing one. We have chosen the latter, and have done so quite effectively and efficiently.

In the current studies, initial immunizations with DNA or TIV failed to elicit any significant level of antibody response. However, significant antibody responses were observed after the administration of a viral protein boost. As expected, the peak anti-HA IgG levels, at weeks 6 or 10 depending on immunization schedule, were similar against both H1 and H3 coated ELISA plates. We found that any combination of DNA priming and protein boosting was able to bind antibody (Figures 7 and 8) and protect against viral infection in protection assays (Figures 11 and 13) at higher levels when compared to TIV alone. These results show the increased effectiveness of our DNA vaccine when paired with the current TIV. We have been able to show that not only is immunization with DNA, at minimum, equal to the current seasonal vaccine but also that boosting with TIV after two DNA immunizations elicits about a 14 fold increase in protection against H1 and H3 viruses in the protective antibody assays (Figures 11 and 13).

Although not unexpected, we found that priming the immune system with DNA twice as compared to only once elicited a much stronger anti-HA IgG response after the boost both temporally and at peak titers (Figure 9). It is believed that the two DNA immunizations allow for a greater build up of B cell memory, so when the organism is

subsequently boosted with whole virus particles, the virion inoculation can more effectively induce both the cellular and humoral arms of the immune system.

When designing a vaccine trial study, the types as well as the amount of immunizations are very important. We wanted to examine if giving DNA and TIV together made any difference when compared to immunizing with DNA first and then boosting with TIV. In general, no significant differences can be seen in antibody response between the two groups, leading to an interesting conclusion. If the vaccine were to move into widespread use, using the DNA and TIV separately approach would be more practical from both a logistical and economic standpoint. This is because giving DNA and TIV at the same time, twice comprises twice as many injections, four shots total, as one DNA and one TIV given at different times, two shots total.

Current flu vaccines are effective in preventing infection, and prophylactic drugs like Amantadine can actively fight viral replication, however, there are issues with each treatment. Viral strains are being discovered in humans which are resistant to many inhibitors, and in 2004, there was a severe vaccine shortage around the world. Our H1/H3 DNA vaccine can not only be created faster than the traditional vaccine once the circulating viral strains have been identified, but may be able to fight the virus effectively enough that drugs like Amantadine would not be needed as often although that remains to be seen.

Future work remains on our flu vaccine system including the effects of adding the avian influenza H5 HA into making a trivalent H1,H3,H5 vaccine formulation. The ultimate goal is to see our vaccine tested in a human trial hoping that the increased

effectiveness of our codon optimized prime-boost DNA vaccine over TIV alone translates well into a human system.

REFERENCES

Andre S., Seed B., Eberle J., Schraut W., Bultmann A., Haas J. 1998 Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol*. **72**(2):1497-1503.

Bohm W., Kuhrober A., Paier T., Mertens T., Reimann J., Schirmbeck R. 1996 DNA
vector constructs that prime hepatitis B surface antigen-specific cytotoxic T lymphocyte and antibody responses in mice after intramuscular injection. *J Immunol Methods*. **193**(1):29-40.

Center for Disease Control. Questions & Answers: 2006-07 Influenza (Flu) Season http://www.cdc.gov/flu/about/qa/0607season.htm. Updated December 20, 2006.

Deck, R.R., C.M. DeWitt, J.J. Donnelly, M.A. Liu and J.B. Ulmer. 1997 Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* **15**:71-78.

Donnelly, J. J., A. Friedman, D. Martinez, D. L. Montgomery, J. W. Shiver, S. L. Motzel, J. B. Ulmer, and M. A. Liu. 1995. Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. *Nat Med* **1:**583-7.

Dowdle, W. R., J. C. Downie, and W. G. Laver. 1974. Inhibition of virus release by antibodies to surface antigens of influenza viruses. *J Virol* **13:**269-275.

Englund, J. A., R. E. Champlin, P. R. Wyde, H. Kantarjian, R. L. Atmar, J. Tarrand, H. Yousuf, H. Regnery, A. I. Klimov, N. J. Cox, and E. Whimbey. 1998. Common emergence of amantadine- and rimantadine-resistant influenza A viruses in symptomatic immunocompromised adults. *Clin. Infect. Dis.* **26:**1418-1424.

Fan, J., X. Liang, M. S. Horton, H. C. Perry, M. P. Citron, G. J. Heidecker, T. M. Fu, J. Joyce, C. T. Przysiecki, P. M. Keller, V. M. Garsky, R. Ionescu, Y. Rippeon, L. Shi, M. A. Chastain, J. H. Condra, M. E. Davies, J. Liao, E. A. Emini, and J. W. Shiver. 2004. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. *Vaccine* **22:**2993-3003.

Fitch, W. M. 1980. Estimating the total number of nucleotide substitutions since the common ancestor of a pair of homologous genes: Comparison of several methods and three beta hemoglobin messenger RNA's. *J. Mol. Evol.* **16:**153–209.

Hartikka J, Sawdey M, Cornefert-Jensen F, Margalith M, Barnhart K, Nolasco M, Vahlsing HL, Meek J, Marquet M, Hobart P, Norman J, Manthorpe M. (1996). An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum Gene Ther*. Jun 20; **7**(10):1205-1217.

Hay, A. J. 1974. Studies on the formation of influenza virus envelope. *Virology* **60**:398-418.

Ikemura, T. 1981. Correlation between the abundance of Escherichia coli transfer RNAs and the occurrence of the respective codons in its protein genes *J. Mol. Biol.* **146**:1–21.

Johnson R.P., Lifson J.D., Czajak S.C., Cole K.S., Manson K.H., Glickman R., Yang J., Montefiori D.C., Montelaro R., Wyand M.S., Desrosiers R.C. 1999. Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. *J Virol*. **73**:4952-4961.

Kendall L.V., Steffen E.K., Riley L.K. 1999. Hemagglutination Inhibition (HAI) Assay. *Contemporary Topics AALAS*. **58**:54.

Koopmans, M., B. Wilbrink, M. Conyn, G. Natrop, H. van der Nat, H. Vennema, A. Meijer, J. van Steenbergen, R. Fouchier, A. Osterhaus, and A. Bosman. 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* **363:**587-593.

Lamb, R.A. and Krug, R.M. Orthomyxoviridae: The Viruses and Their Replication. Fields Virology, Lippincott-Raven Publishers. (1996) p1353-1387.

Langley, J.M. and Faughnan, M.E., 2004**.** Prevention of influenza in the general population: recommendation statement from the Canadian Task Force on Preventive Health Care. *CMAJ* **171**: 1169-1170.

Lawrence, J. G. and Hartl, D. L. 1991. Unusual codon bias occurring within insertion sequences in *Escherichia coli*. *Genetica* **84:**23–29.

Lu, S., J. Arthos, D. C. Montefiori, Y. Yasutomi, K. Manson, F. Mustafa, E. Johnson, J. C. Santoro, J. Wissink, J. I. Mullins, J. R. Haynes, N. L. Letvin, M. Wyand, and H. L. Robinson. 1996. Simian immunodeficiency virus DNA vaccine trial in macaques. *J Virol* **70:**3978-3991.

Lu, S., R. Wyatt, J. F. Richmond, F. Mustafa, S. Wang, J. Weng, D. C. Montefiori, J. Sodroski, and H. L. Robinson. 1998. Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. *AIDS Res Hum Retroviruses* **14:**151-155.

Modiano, G., Battistuzzi, G. and Motulsky, A. G. 1981. Nonrandom patterns of codon usage and of nucleotide substitutions in human alpha- and beta-globin genes: an evolutionary strategy reducing the rate of mutations with drastic effects? *Proc. Natl. Acad. Sci. USA* **78:**1110–1114.

Murphy, B.R., and Webster, R.G. Orthomyxoviruses. Fields Virology, Lippincott-Raven Publishers. (1996) p1397-1433.

Murphy, J. S., and F. B. Bang. 1952. Observations with the electron microscope on cells of the chick chorio-allantoic membrane infected with influenza virus. *J. Exp. Med*. **95**:259-267.

Nagata, T., Uchijima, M., Yoshida, A., Kawashima, M., Koide, Y. 1999. Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: Analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem. and Biophys. Res. Comm*. **261:**445-451.

Neirynck, S., T. Deroo, X. Saelens, P. Vanlandschoot, W. M. Jou, and W. Fiers. 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat Med* **5:**1157-1163.

Palese, P., and A. Garcia-Sastre. 2002. Influenza vaccines: present and future. *J Clin Invest* **110:**9-13.

Pear, W.S., Nolan, G.P, Scott, M,.., Baltimore D. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* **18**:8392–6.

Plotkin, J.B., Doshoff, J. 2003. Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. *Proc Natl Acad Sci USA* **100:**7152-7157.

Richmond, J. F., S. Lu, J. C. Santoro, J. Weng, S. L. Hu, D. C. Montefiori, and H. L. Robinson. 1998. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. *J Virol* **72:**9092-9100.

Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, D. L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat Med* **5:**526-534.

Skehel, J. J., and D. C. Wiley. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69:**531-569.

Walker, J. A., S. S. Molloy, G. Thomas, T. Sakaguchi, T. Yoshida, T. M. Chambers, and Y. Kawaoka. 1994. Sequence specificity of furin, a proprotein-processing endoprotease, for the hemagglutinin of a virulent avian influenza virus. J. Virol. **68:**1213-1218.

Wang, S., Farfan-Arribas, D.J., Shen, S., T.H. Chou, Hirsch, A., Feng, H., Lu, S. 2005A. Relative contributions of codon usage, promoter efficiency and leader sequence

to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. Vaccine **24:**4531–4540.

Wang, S., T. H. Chou, P. V. Sakhatskyy, S. Huang, J. M. Lawrence, H. Cao, X. Huang, and S. Lu. 2005B. Identification of two neutralizing regions on the severe acute respiratory syndrome coronavirus spike glycoprotein produced from the Mammalian expression system. *J Virol* **79:**1906-1910.

Wang S, **Taaffe J**, **Parker C**, **Solorzano A**, **Cao H**, **Garcia-Sastre A**, **Lu S**. 2006. Hemagglutinin (HA) proteins from H1 and H3 serotypes of influenza A viruses require different antigen designs for the induction of optimal protective antibody responses as studied by codon optimized HA DNA vaccines. *J Virol* **23**:11628-37

Webster, R. G., W. G. Laver, and B. Tumova. 1975. Studies on the origin of pandemic influenza viruses V. Persistence of Asian influenza virus hemagglutinin (H2) antigen in nature. *Virology* **67:**534-543.

World Health Orgainzation. Ten things you need to know about pandemic influenza. http://www.who.int/csr/disease/influenza/pandemic10things/en/ Updated October 14, 2005.

Zuckerkandl, E. & Pauling, L. 1965. Molecules as documents of evolutionary history *J. Theor. Biol.* **8:**357–366.