Spring 2019

Evaluation of Self-Adjuvanting M2e Vaccine Efficacy in Response to Influenza A Virus Challenge

Dustin Heiden
University of South Dakota

EVALUATION OF SELF-ADJUVANTING M2E VACCINE EFFICACY IN RESPONSE TO INFLUENZA A VIRUS CHALLENGE

By
Dustin Heiden

A Thesis Submitted in Partial Fulfillment
Of the Requirements for the
University Honor's Program

Department of Biology
The University of South Dakota
May 2019

The members of the Honor's Thesis Committee appointed
to examine the thesis of Dustin Heiden
find it satisfactory and recommend that it be accepted.

Dr. Victor Huber
Associate Professor of Basic Biomedical Sciences
Director of the Thesis Committee

Dr. Bernie Wone
Assistant Professor of Biology

Dr. Michael Chaussee
Associate Professor of Basic Biomedical Sciences

Beate Wone
Instructor of Biology

ABSTRACT

Evaluation of Self-Adjuvanting M2e Vaccine Efficacy in Response to Influenza A Virus Challenge

Dustin Heiden

Thesis Director: Victor Huber, Ph.D.

Influenza is a negative-sense, ssRNA virus composed of 8 segments, and it is responsible for numerous cases of morbidity and mortality around the globe. As of today, vaccines are the best method to limit the pathogenesis of influenza viruses. Typically, influenza vaccines tend to incorporate hemagglutinin (HA) and neuraminidase (NA) surface proteins, and some can be complemented with a form of an adjuvant. However, HA and NA are problematic vaccine candidates due to their high susceptibility to mutate using antigenic drift and to change their genetic makeup using antigenic shift. The frequent alterations in HA and NA require a substantial amount of resources being utilized in the surveillance and subsequent bi-annual adjustment of the influenza vaccine administered to the public. To improve vaccine consistency, researchers are exploring the concept of a universal vaccine based on conserved components of the influenza virus, such as the hemagglutinin stalk and the ectodomain of the M2 (M2e) ion channel. Thus far, it has been shown that 9 out of 24 amino acids that constitute M2e are highly preserved and rarely exhibit mutations, making it a prominent candidate for vaccines. Investigating the immunogenic effects of a self-adjuvanting, liposome-based M2e vaccine in ferrets will shed light on whether M2e would be an effective vaccine candidate.

Keywords: Influenza, Universal Vaccine, Ferrets, M2e.

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CHAPTER ONE

Introduction:

The History and Importance of the Influenza Virus:

The Influenza A virus (IAV) is a respiratory pathogen that is easily transmitted from diseased patients to other vulnerable hosts via contact with and uptake of respiratory droplets (Deng, et al. 2015). The virus manipulates its host into producing more of its progeny, which in turn can lead to severe states of morbidity or even mortality (Dou, et al. 2018). The greatest of influenza pandemic dates back to the years 1918-1920, when a deadly outbreak of an H1N1 virus, more commonly known as the "Spanish Flu", resulted in the estimated deaths of 40-50 million people worldwide (Johnson and Mueller 2002). Since the 1918 pandemic, the human population has endured many other pandemics that were characterized by the emergence of new viral subtypes, including H2N2 in 1957, H3N2 in 1968, and new strains of H1N1 in 1977 and 2009 (Zimmer and Burke 2009). These strains are more commonly known as the Asian Flu (1957), Hong Kong Flu (1968), Russian Flu (1977), and the Swine Flu (2009) (McCormick 2015). According to the World Health Organization, there are approximately 5 million severe cases of influenza contributing to the death of about 650,000 individuals yearly ("Influenza (Seasonal), 2019). Many of the mortalities are not restricted to only primary viral infections but are the result of secondary bacterial superinfections (Huber, et al. 2011).

Influenza Replication Cycle:

The IAV is an enveloped, negative-sense, single-stranded RNA (ssRNA) virus that belongs to the *Orthomyxoviradae* family of viruses (Samji 2009). This pathogen comes in four types that are differentiated based on three main factors: their structure, proteins expressed, and the hosts that it affects (McCormick 2015). These four types are termed influenza A, influenza B, influenza C, and influenza D (Zimmer and Burke 2009). Currently, the most prominent virus type of concern is the IAV because it is the only viral type that is known to infect between species and generate pandemics in humans (Dou 2018). IAV is composed of eight segments: RNA Basic Polymerase 1 (PB1), RNA Basic Polymerase 2 (PB2), matrix proteins (M), nucleoprotein (NP), neuraminidase (NA), RNA Acidic Polymerase (PA), non-structural proteins (NS), and hemagglutinin (HA) as shown below in **Figure 1**(Klonoski 2018). Using these 8 genetic segments, the influenza virus produces approximately twelve proteins (Bouvier and Palese 2008). Upon infecting hosts, the influenza virus employs an acute infection cycle in which the pathogen will enter a host cell and manipulate its cellular machinery into producing replicates of itself (Zheng, Zhang, Guo, and Tao 2016). This process is characterized by a series of major events as follows: entry into the host cell; entry of viral ribonucleoproteins into the nucleus; transcription and replication of the pathogen; nuclear export; and assembly and budding at the cellular plasma membrane (Matsuoka 2013).

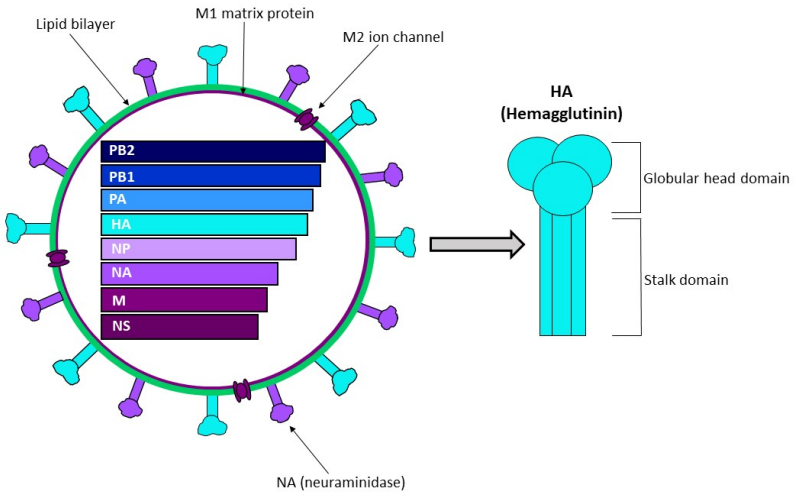

Figure 1: An Image showing the eight segments present in IAV and the major surface proteins, HA, NA, and M2 ion channel proteins (Wilson 2018).

For an IAV to commandeer cells, it utilizes one part of HA surface glycoprotein, known as the globular head region (HA1), to bind to the sialic acid residues (N-acetylneuraminic acid) coating the plasma membrane of the host cell (Vigerust 2011). Once bound to the surface of the cell, the influenza virus is taken up via receptor-mediated endocytosis (Matsuoka, et al. 2013). During the process of endocytosis, the pathogen is packaged into an endosome. Due to the endosome being a more acidic environment (pH between 5 and 6), the influenza virus undergoes a conformational change in the HA protein causing the exposure of the second subunit known as the stalk region or HA2 (Samji 2009). The HA stalk domain fundamentally functions to combine the viral envelope with the interior of the endosome. Following the fusion of the endosome with virus's envelope, the M2 ion channels are opened allowing protons to enter the virion, thus leading to the acidification of the of the pathogen's core (Pinto and Lamb 2005). In

response to the pH change, the viral ribonucleoproteins (vRNP) are ejected into the cytoplasm of the cell (Zheng, et al. 2016).

Upon entry into the cytoplasm, the viral contents follow a series of nuclear localization signals that lead it to the host cell's nucleus (Boulo, et al. 2007). Once in the nucleus, many key events must occur for successful viral replication. First, the negative-sense ssRNA IAV is coded in a positive direction by the pre-packaged RNA-dependent RNA polymerase enzyme, so the influenza virus can utilize the host's cellular machinery (Li, et al. 2001). During the positive coding process, the virus, through a method termed "cap-snatching," in an act of thievery will take the 5' methylated cap of pre-mRNA and use it to prime viral RNA for transcription (Dou, et al. 2018). Concurrently, other NS proteins actively work to prevent the exportation of host mRNA while viral RNA are free to exit the nucleus (Matsuoka, et al. 2013). With the cell has been successfully commandeered, the focus shifts to the translation of viral proteins from viral mRNA (Bui, et al. 2000). After the synthesis of viral proteins, IAV-associated proteins are transported to the endoplasmic reticulum and the golgi apparatus for final post-translational modifications (Vigerust 2011). Then, IAV constituents converge on the plasma membrane. The HA, NA, and M2 proteins integrate themselves into the host plasma membrane through associations with lipid rafts, therefore marking the site of budding (Boulo, et al. 2007). The M2 proteins, using its cytoplasmic tail, works in tandem with M1 proteins, contributing through lipid interactions, to package the vRNP into the completed virion (Iwatsuki-Horimoto, et al. 2006). Finally, through M2 mediated membrane interactions, the virion exits the host cell by budding (Samji 2009). During this process, NA proteins cleave sialic acid residues, so the IAV avoids immediate agglutination and can successfully leave the host cell (Nayak, et

al. 2009). It is important to note that during the infection cycle, all three proteins expressed on the surface of the virus, HA, NA, and the 23 amino acids of M2 (M2e) are expressed on the surface of infected cells. The viral replication process is shown in **Figure 2** below.

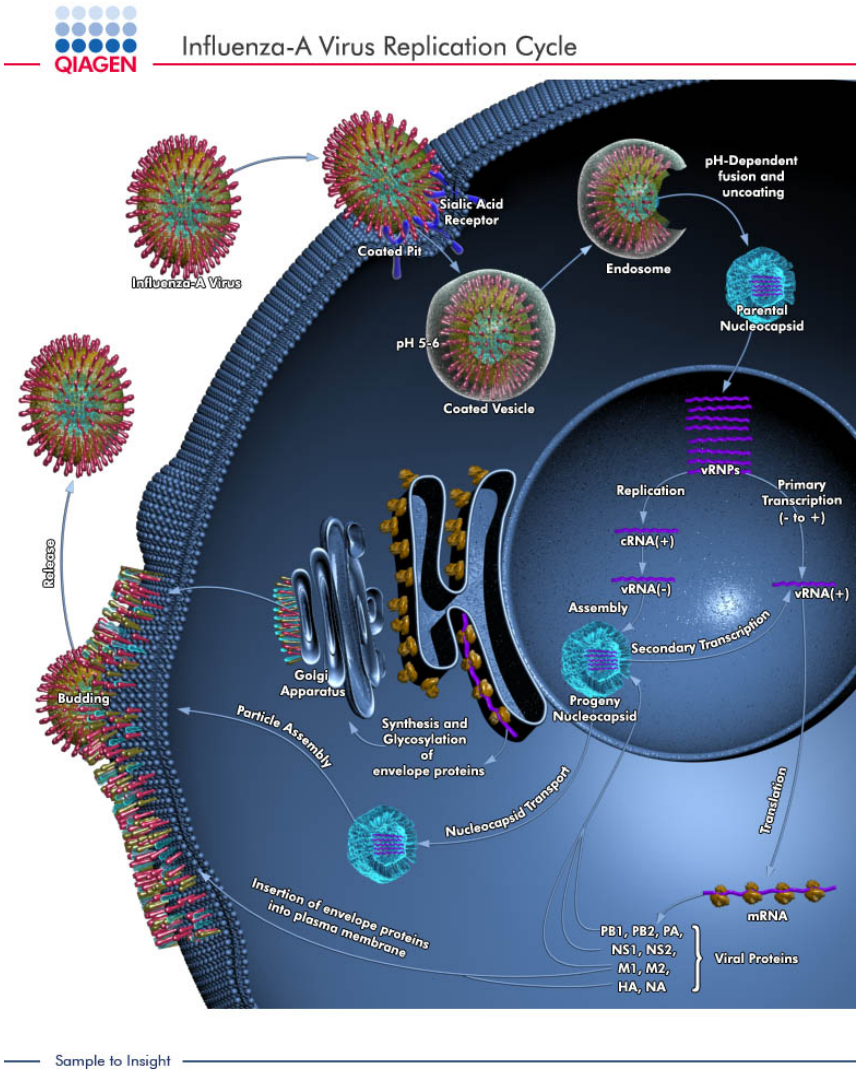

Figure 2: Qiagen© diagram depicting the influenza infection cycle (Qiagen, *Influenza A Virus Replication Cycle* 2009).

An Overview of the History of Influenza Vaccines:

Influenza Pandemics occur because of the emergence of a novel influenza virus and a lack of immunity among the host population (Lambert and Fauci 2010). In the early 1930's, scientists were able to successfully isolate the influenza virus from humans (McCormick 2015). This event was the first major step in the development of the influenza vaccine movement, and by the 1940's, the first inactive bivalent vaccine containing the of influenza A and B was developed, and these were grown in embryonated chicken eggs (Hannoun 2013). Around the same time, live-attenuated vaccines first entered clinical trials (I. Barberis, et al. 2016). The viruses used for live-attenuated vaccines were primarily grown in chicken eggs as is standard today as well, and by the end of the decade, a protocol for growing influenza viruses using in-vitro methods had been developed (Hannoun 2013). In 1952, the influenza first surveillance program was established with the purpose of watching for antigenic drift in the virus, particularly HA, and the emergence of new viral strains ("Influenza (seasonal)" 2019). Due to the reactogenicity of whole virus vaccines, there was a push for bettering the technique using the reassortment of influenza viruses onto a less-virulent laboratory strain (Parkman, et al. 1977) and creation of split and subunit vaccines.

In the next decade, the development of two new types of vaccines, the split vaccine and the subunit vaccine, were initiated (Krammer and Palese 2015). Initially, split vaccines were utilized leading into the seventies, but it was found that this style of vaccination was less immunogenic than its whole virus complement when it came to individuals who had never been inoculated. As a result, individuals receiving the vaccine for the first time had

to receive additional vaccine boosters (Parkman, Hopps, Rastogi, and Meyer 1977). By the late seventies the first inactive, trivalent subunit vaccines were developed. These vaccines contained only the HA and NA surface antigens of the H1N1, H3N2, and influenza B viruses and were advantageous over their split vaccine predecessor due to an increased level of immunogenicity (Kendal, Maassab, Alexandrova, and Ghendon 1987). However, two administrations of the vaccine were still required on first vaccination to ensure protection during epidemics (Gianchecchi, Trombetta, Piccirella, and Montomoli 2016).

In the last twenty years, vaccine technology has rapidly improved. FluMist®, a live-attenuated vaccine, was introduced as a means of intranasal inoculation in the early 2000's (Barberis, et al. 2016). By 2009, the FDA approved the usage of the MF-59 adjuvant-enhanced vaccines for young children because they are still building immunity and geriatric individuals due immunosenescence (Soema, Kompier, and Amorij 2015). An additional two years of vaccine research resulted in the approval of intradermal vaccine delivery. This new method of vaccinating proved to be better than previous intramuscular injections because it elicited a stronger innate and adaptive immune response through the enhancement of antigen presenting cell activity (Duarando, et al. 2011). In the next year, the first quadrivalent vaccine, Fluarix®, was approved for public use. Fluarix® had two influenza A strains and two influenza B antigens, providing the benefit of a reduction in viral mismatch and vaccine production while maintaining the standard set by trivalent vaccines (Belshe 2010). By 2013, FluBlock®, a recombinant influenza vaccine, was approved by the FDA for use in people between the ages of 18 and 49 years of age (Baxter, et al. 2011). Each of these vaccines work through the induction of immunological memory against HA in the form of memory T cells and antibodies (Pica and Palese 2013).

According to the Center for Disease Control (CDC), influenza vaccine administration in 2017-2018 prevented 7.1 million cases of morbidity and at least 8000 deaths, making vaccines the best method of controlling influenza outbreaks and offsetting the potential devastating effects of the virus ("Influenza (flu)", 2019).

Universal Vaccine Initiative:

Influenza has the tendency to change through antigenic drift and antigenic shift, causing epidemics and pandemics, respectively (McCormick 2015). When these new viruses emerge, humoral and cell mediated immune responses may not recognize them, and new vaccines have to be developed both annually and in the face of a pandemic (Staneková and Varečková 2010). However, certain proteins, such as the ectodomain of the M2 (M2e), are well preserved from an evolutionary standpoint (Pica and Palese 2013). It is the conserved protein epitopes of influenza viruses that have caught the attention of immunologists today, as they could be potential constituents for the development of universal influenza vaccines (Schotsaert, et al. 2009). The overarching goal of the universal vaccine initiative is the production of an influenza vaccine that generates lifelong protection against all strains of the influenza pathogen, with influenza A virus being the most important target due to its contribution to pandemics (Fiers, et al. 2009). By vaccinating using conserved proteins, like M2e, broad immunity in the form of M2e specific antibodies, specifically IgA and IgG, and cell mediated responses, can be induced against most influenza strains using a single vaccine (Lambert and Fauci 2010). However, M2e by itself does not elicit a strong immune response during natural infection (Pica and Palese 2013), and antibodies against M2e do not neutralize the virus. In order to increase the

immunogenicity of M2e vaccines, and allow antibodies induced to kill infected cells through antibody-dependent cellular cytotoxicity, or ADCC (Van den Hoecke 2017), an adjuvant needs to be incorporated into the vaccine (Filette, et al. 2006). It is hypothesized that by inoculating ferrets (*Mustela putorius furo*) using a liposome-based vaccine containing diptheria toxoid (DT), IAV peptide antigen derived from M2e, and the immunostimulatory synthetic glycolipid, Monophosphoryl 3-Deacyl Lipid A (3D-PHAD®), that immunity will be induced against the influenza A virus, A/California/04/2009 (Ca09), illustrating the potential for a self-adjuvanting, universal liposomal flu vaccines.

CHAPTER TWO

Methods and Materials:

M2e-Lipo-DT+PHAD Vaccine Design:

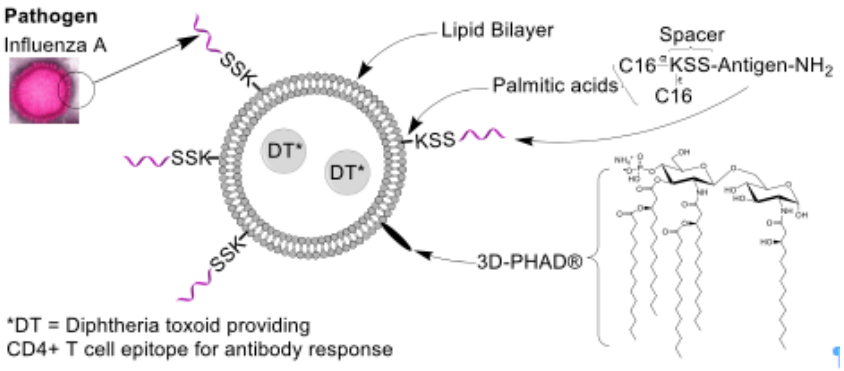

Figure 3: A theoretical image depicting the structure of the M2e-Lipo-DT+PHAD vaccine, with M2e being the component of influenza A expressed on the liposome.

The liposomal influenza vaccine platform (M2e-Lipo-DT+PHAD) utilized in this study was designed and provided by collaborators, Mehfuz Zaman, et al., at Griffith University, Australia (Zaman, et al. 2016). This vaccine is a spherically-shaped liposome made of neutrally charged, biocompatible phospholipid bilayers that were prepared as described (Zaman, et al. 2016). Various charged peptides are embedded in the liposome's surface. Protein spacers, termed SSK, act as antigen carriers and are covalently bonded to two palmitic acid molecules that have been integrated into the liposomal membrane. The

IAV M2e peptide was attached to the SSK spacers via the N-terminus. Each inoculant of liposomes contained approximately 16 μg of antigenic peptides per mL. 3D-PHAD® has been inserted into the membrane of the liposome using the lipid moiety extant in the glycolipid. The total concentration of glycolipid per mL was 13 μg. Inside the particle, resides the adjuvant diptheria toxoid (DT). DT serves two functions. The first is that lipidated peptides, such as DT, are targets for B cell antigen presentation, which in turn activates $CD4^+$ cells. The second purpose of DT is the formation of modular epitopic constructs that activate IgA producing B cells. Each vaccine particle housed approximately 16 μg of DT content per mL. Overall phospholipid content was calculated to be around 1 mg/mL (Zaman, et al. 2016). This was assessed by collaborator, Mehfuz Zaman (Griffith University, Australia). A visualization of the vaccine construct is illustrated above in **Figure 3.**

Ferrets:

Six young adult, male ferrets from an influenza-free ferret colony at Marshall Farms were brought into the University of South Dakota (USD) Sanford School of Medicine's animal facilities. The ferrets were allowed to acclimate for fourteen days. Following the acclimation period, all ferrets were tested to confirm that antibody levels were below a titer of 10 against A/California/04/2009 (Ca09), indicating that they were seronegative against the challenge virus at the time of vaccination. All procedures and experiments carried out were done so within biosafety level 2 facilities and in accordance to parameters set individually by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of USD.

Vaccine Preparation and Administration:

The M2e-Lipo-DT+PHAD M2e vaccine was received from collaborators in a freeze-dried condition as previously described (Zaman, et al. 2016). Initially, the vaccine contents and Dulbecco's Phosphate Buffered Saline (DPBS) were heated at 37°C for 5 minutes prior to adding 3.2 mL of DBPS to the dehydrated vaccine. Once added, the solution was mixed until a homogenous, cloudy mixture absent of bubbles was formed. This was repeated 3 times for the M2e vaccine and an additional 3 times for the blank liposome vials. Next, the ferrets were anesthetized using an isoflurane (4.5%) vaporizer as previously described (Huber, Kleimeyer, and McCullers 2008). Three of the ferrets were inoculated intranasally with 0.5mL per nostril of M2e-Lipo-DT+PHAD vaccine and held upright for 10-15 seconds to ensure the vaccine made it to the lungs. The inoculation process was repeated for the other three ferrets, using blank liposome solution. Ferrets were observed for 34 days post-vaccination and received booster inoculations on days 14 and 21 that were delivered as described for initial vaccine delivery. During this 34-day time period, sera were collected from ferrets on days 7, 14, 21, 28, and 34 and tested using HAI in a manner previously described (Huber and McCullers 2006), and Enzyme-linked Immunosorbent Assays (ELISA) as described below.

Serum Treatment:

Prior to, during, and after vaccination and subsequent viral challenge, the serostatus of the six ferrets were tested. Each ferret was subjected to isoflurane (4.5%) vaporization (Thermo Fisher Scientific) anesthesia individually. While anesthetized, approximately 1mL of serum was collected from the mammary vein of each ferret and allowed to clot for 3 hours at room temperature. Following serum collection, ferrets were observed for 10

minutes to ensure their recovery. The clotted blood samples were then centrifuged using an accuSpin Micro 17R tabletop centrifuge (Fisher Scientific) for 10 minutes at 6,000 RPMs to further separate the sera from the clotted cells. Then, 100uL of sera from each ferret was pipetted into separate 1.5 mL microcentrifuge tubes (Fisher Brand) and 300 µL of Receptor Destroying Enzyme (RDE) solution (Denka Seiken Co.) was added to each tube, as previously described (Cwach, Sandbulte, Klonoski, and Huber 2011). The RDE-treated samples were incubated overnight at 37°C. The next day, 300 µL of a 2.5% sodium citrate solution was added to each RDE-treated sample and incubated at 56°C to further reduce the interference of innate inhibitors naturally present in ferret serum. After incubation, 300 uL of 1x Phosphate Buffer Solution (PBS; pH = 7.43) was added to the ferret sera solutions prior to adding 50 uL of packed chicken red blood cells (CRBCs) to pre clear the sera of inhibitors that interact with CRBCs. The samples were placed at 4°C for one hour, with intermittent mixing occurring every 15 minutes. Sera were removed from CRBCs through centrifugation at 1,000 RPM for 10 minutes, and the treated ferret sera were then used for HAI assays, microneutralization assays, and ELISAs as described below.

HAI Assay:

The HAI assay was utilized to test the reactivity of the serum collected from ferrets. This technique was performed using a Ca09 virus stock that had been diluted to a 1:8 ratio based on HA content pre-determined by a Hemagglutinin assay, as previously described (Cwach, Sandbulte, Klonoski, and Huber 2011). After achieving the desired HA content, treated sera from vaccinated and unvaccinated ferrets were added to wells in column 1, A through F, of a non-sterile 96-well round bottom plate. 25 µL of 1x PBS (pH=7.43) were

added to each respective well in columns 2 through 12. The sera present in column 1 was serially diluted out across the wells 2- through 12. Next, 25 μL of diluted Ca09 virus was added to each well and was left to incubate at 4°C for 1 hour. The final volume in each well after the addition of the virus was 50 μL. Following the incubation period, 50 μL of 0.5% CRBC solution was added to each well and the plates were left to incubate at room temperature for 30 minutes. If a pellet is observed, then the hemagglutinin lattice formation by viral interactions with sialic acid residues present on CRBC has been inhibited by the presence of antibodies against Ca09 HA, denoting seroreactivity. If a pellet does not form and agglutination occurs, the virus was able to bind to CRBC illustrating that antibodies against Ca09 were not present at that dilution of sera.

Microneutralization Assay:

The microneutralization assay was performed as previously described (Cwach, Sandbulte, Klonoski, and Huber 2011). As the first step, a 96 well, flat bottom plate (Falcon) was seeded with 100 μL of Madin-Darby Canine Kidney (MDCK) cells in growth media at a concentration of 3.00 X 10^5 per mL and allowed to incubate overnight at 37° C with 5% CO_2. The next day, 60 μL of MDCK infection media was added to the whole plate except for column 1. Row H and column 12 were reserved for a cell control and Ca09 virus back titration respectively. To complete the cell controls, an additional 60 μL of MDCK infection media was added to row H. 120 μL of RDE-treated sera from each ferret was added to A1 through G1. Using the sera added to column 1, a serial, 1:2 dilution scheme was established across the plate with the exception of the column 12. Next, the viral back titration was prepared by adding 438 μL of Ca09 influenza virus, with a

previously determined $TCID_{50}$ value of 100, to a sterile Eppendorf tube. Then 138 µL from the previously mentioned tubes was serially diluted out through 6 other tubes. The total volume of each Eppendorf tube following the serial dilution of the Ca09 virus should be 300 µL. 60 µL of diluted virus from each tube was added, in order, to column 12 with the highest concentration in A12 and the lowest in G12. The total volume present in each well following the addition of the previous solutions was 120 µL. The 96 well plate was incubated at 37° C in a 5% CO_2 atmosphere for 2 hours to allow the virus and sera to interact.

Prior to inoculation with the virus:serum dilutions, created as described above, the supernatant was aspirated off of the 96 well plate containing MDCK cells that was prepared the previous day. The MDCK monolayers were washed with 200 µL of 1x, sterile PBS (pH = 7.43) two times. Next, supernatant containing Ca09 and RDE treated sera was transferred from its original plate to the 96 well plate containing MDCK cells. The cells antibody:virus mixtures were incubated at 37° C in a 5% CO_2 environment for two hours. Afterward, the plate with cells was rinsed with 200 µL of MDCK infection media. Finally, 100 µL of MDCK infection media supplemented with TPCK trypsin at a concentration of 2 µg /mL was added to the cells, and they were incubated overnight in a 37° C, 5% CO_2 environment.

The next day, the media was removed from the cells and replaced with 125 µL of sterile, 1x PBS twice as a method of washing the cells. Cells were then fixed to the plates using 100 µL of ice-cold fixative solution (80% acetone in 1x PBS) and left at room temperature for 10 minutes. After incubation, the fixative solution was removed, and the plate was allowed to air dry for 20 minutes. Plates were subject to a series of three washes

using wash solution (1x PBS + 0.05% Tween-20). Post-cleanse, cells were incubated in an antibody diluent buffer containing primary, mouse anti-NP monoclonal antibody (Millipore) in a 1:2000 ratio with 1x PBS, Bovine Serum Albumin (BSA), and tween-20 for 1 hour at room temperature. Following incubation, cells were washed with wash buffer four times. 100 µL of a secondary, polyclonal goat anti-mouse IgG-HRP antibody solution (1:2000, antibody: diluent solution; Sigma Aldrich) was added to the plate, which was incubated at room temperature for 1 hour. The plate was washed in a manner previously described six times. 100 µL of substrate buffer containing *o*-Phenylenediamine dihydrochloride (OPD) substrate, and hydrogen peroxide was utilized to activate the HRP attached to the secondary antibody. After 5 minutes of development, a color change from clear to yellow was denoted in samples that were positive, and 100 µL of a stop solution containing 1 N sulfuric acid was added to the plates. Plates were read using a Bio Tek Synergy HT plate reader at an absorbance of 490 nm. Absorbances collected were used to calculate the total amount of viral neutralization occurring.

M2e ELISA:

The ELISA was another method utilized for the detection of IgG/IgA antibody levels present in ferret sera pre-vaccination through post-vaccination and pre-challenge through post-challenge with Ca09, using methods similar to those previously described (Huber, Thomas, and McCullers 2009). Due to the nature of the ELISA, each test occurred over the course of three days. On day one, M2e peptide antigen in the form of the vaccine described above (**Figure 3**) was thawed on ice and diluted to a concentration of 10 µg /uL in 1x sterile PBS (pH=7.43). This process was repeated for the blank

liposomes. 50 µL of the InA antigen solution and blank liposome solution were added to their own 96 well flat bottom Maxisorp® ELISA plates. The plates were covered using para-film and stored at 4°C and incubated overnight to allow for the coating of the plates with their respective antigens.

On day 2 of the ELISAs, plates were removed from the 4°C and warmed to ambient temperature. The current solutions in the wells of the plates were removed and the plates were washed six times using a 200 µL of a 0.05% wash buffer (1x PBS in Tween-20, PBS-T). Following the washes, 100 µL of blocking buffer (10% FBS + PBS-T) was added to each of the wells to increase the sensitivity of the assay. The plates were para-filmed to prevent them from drying out and incubated at room temperature for 2 hours. Blocking buffer was removed and 50 µL of blocking buffer was added to all wells except those in the first column. 80 µL of blocking buffer along with 20 µL of the respective ferret sera sample was added to the first well. This 1:5 dilution of ferret sera that had been already diluted 1:10 through the RDE-treatment steps described above, made the initial serum dilution used in the ELISA a 1:50. The solution present in column 1 was serially diluted across columns 2 through 12 by transferring 50 µL of solution from the previous well to the next well, with the exception of H12 which was reserved as a blank. The plates were para-filmed once more and stored overnight at 4°C.

Day 3 of ELISA started with six washes using PBS-T as previously described. Next, primary, monoclonal antibody containing goat anti-ferret IgG (Thermo Scientific) was diluted in the 10% FBS in PBS-T blocking buffer at a 1:1,000 dilution. 50 µL of primary antibody solution was then applied to the plates prior to incubation for 1-hour incubation at room temperature. Plates were then washed with PBS-T to clear excess antibodies out

of the wells. Next, 50 μL of secondary, polyclonal rabbit anti-goat IgG-AP antibody (Cappel) solution was added to the wells at a 1:1,000 dilution and the plates were incubated for 1 hour at ambient temperature. Plates were washed in a manner previously described, and substrate buffer (1M Diethanolamine, pH = 9.8) was mixed with *p*-nitrophenyl phosphate tablets (1 tablet per 5 mL of substrate buffer, 1 mg/mL). The tablets were allowed to dissolve in a dark area to prevent premature photo reactivity. Once prepared, 50 μL of the substrate solution was added to each well. The plates were left at room temperature in the light to allow for development. Finally, the plates were read using at 405 nm using the EL808 plate reader (Bio-Tek). This process was repeated for ferret sera collected at days 0, 7, 14, 21, and 34 to characterize IgG and IgA response to the M2e-Lipo-DT+PHAD influenza A vaccine. This same process was also utilized to measure the IgA and IgG humoral response using sera collected days 1, 3, 5, and 7 post Ca09 challenge.

Ferret A/California/04/2009 Challenge and Observations:

Following the vaccination time period, the ferrets were challenged with the Ca09 virus at a dosage of $10^{6.25}$ infectious particles/mL. The virus was diluted to the inoculation dose 2using in 1x sterile PBS (pH=7.43), and 1 mL was administered intranasally to each anesthetized ferret (0.5 mL per nostril). After the intranasal inoculation with Ca09, ferrets were held upright for 10-15 seconds to ensure proper delivery of the pathogen. Over the 8-day course of the study, ferret symptoms related to influenza morbidity, specifically activity levels and sneezes, were observed as previously described (Huber and McCullers 2006). In relation to activity level, a score of 0 was given to ferrets that were alert and active without stimuli, a 1 was ascribed to ferrets who were alert, but required stimulus to be

playful, a 2 was given to ferrets that were alert, but not active despite the application of stimuli, and a 3 was given to ferrets were neither alert or playful. As for sneezes, a score of 0 was denoted for ferrets who did not sneeze. Ferrets who sneezed 1 to 10 times were given a score of 1. A score of 2 was assigned to ferrets that sneezed more than 10 time. A score of 3 was given to ferrets who display signs of respiratory distress. These scores were ascertained over a 10-minute observation period and served as a clinical score for ferrets, and the maximum value for this clinical score was 6. The daily temperature and body weight of each ferret was measured daily as additional ways to gauge the severity of infection.

Assessment of Viral Load Present in Ferrets:

To assess the viral load present in a ferret, their nasal washes were collected daily following the Ca09 challenge, as previously described (Huber and McCullers 2006). Prior to collecting nasal washes, ferrets were anesthetized using isoflurane (4.5%) vaporization and subsequent application of ketamine (Hospira) at a concentration of 30 mg/kg. Besides anesthesia, the administration of ketamine serves an accessory function of keeping the sneeze reaction intact. After anesthetizing the ferrets, 0.5 mL of PBS per nostrils was intranasally instilled. The response in the form of a sneeze was collected in 50-mL Tubes (Corning). To test the viral load present in the nasal fluid, MDCK cells grown at a concentration of $3.0x10^5$ per mL were plated on 48-well plates (Falcon). These plates containing confluent monolayers of MDCK cells were washed two times with 500uL of 1x sterile PBS. Infected with the nasal washes. Next, 50 µL of serially diluted (1:10) nasal wash was pipetted onto the cellular monolayers and left to rock gently on a Rocker 35® (Labnet) for 1 hour with intermittent plate taps every 15 minutes. Following the rocking

period, the inoculum was removed and supplanted by MDCK infection media supplemented with 1 μg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (TPCK-Trypsin; Worthington Biomedical). The cells were incubated at 37°C in 5% CO_2. Viral titers were reported as $TCID_{50}$ per milliliter of nasal wash fluid. While cytopathic effects (CPE), characterized by cellular morphology and destruction of the monolayer, can be utilized to qualitatively signify the presence of a virus in vitro, to accurately quantify the virus concentration present in the ferret nasal wash samples, a confirmation of the virus present in supernatant is done. This step involves taking 50 μL of supernatant from the 48-well plate (Falcon) that has been inoculated and incubate for 72 hours and transferring it to a 96-well round bottom plate (Fisherbrand). Next, 50 μL of 0.5% chicken red blood cell (CRBC) solution was added to the wells containing supernatant. The plate was then incubated at room temperature for 30 minutes. After the incubation period, the presence or absence of the influenza virus is identified. The results of a HA assay are read in opposition to the manner in which HAI assays are read. A negative result is characterized by a pellet forming at the bottom of the well. A positive result is marked by the lattice formation known as hemagglutination of CRBC due to HA interactions with the sialic acid residues present on the CRBC. The number of positive wells can then be used to calculate a $TCID_{50}$ value of the virus.

CHAPTER THREE

Results:

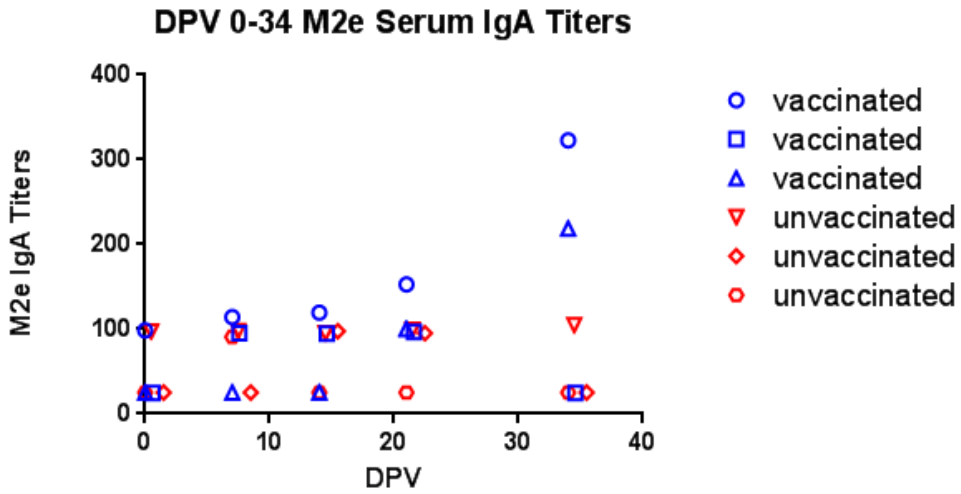

Figure 4: M2e-based ELISAs illustrate the differences between vaccinated and unvaccinated (blank liposome) ferrets regarding anti-M2e antibody levels produced.

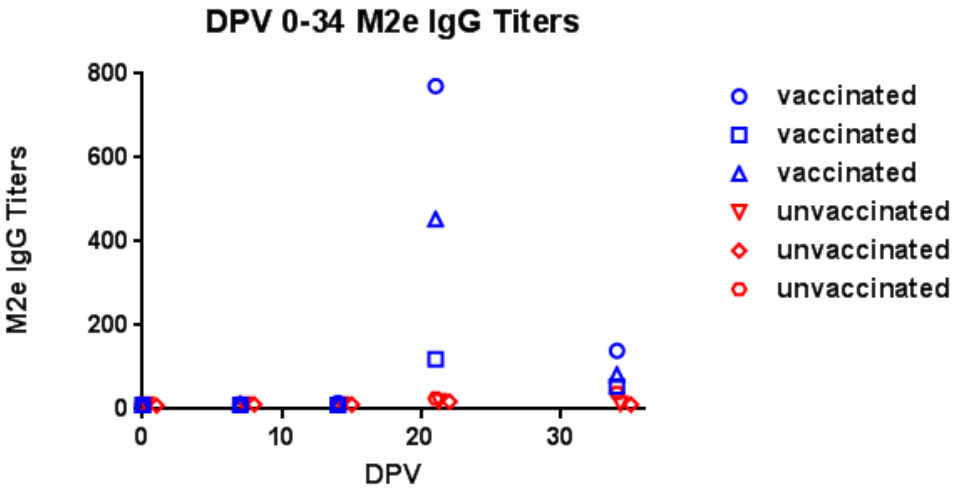

Figure 5: Results from M2e-based ELISAs exhibit the differences in IgG antibody production in ferrets who were vaccinated using the M2e-Lipo-DT+PHAD vaccine and ferrets who received blank liposome (unvaccinated).

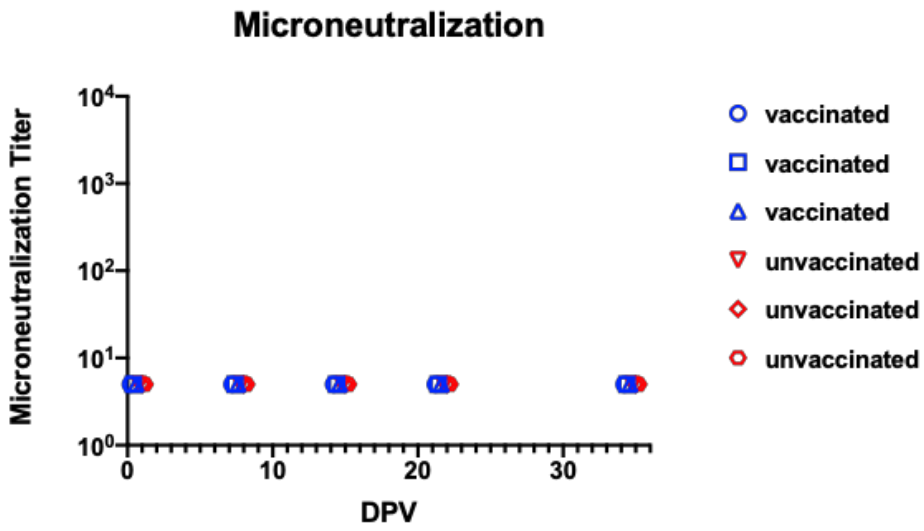

Figure 6: Microneutralization assay assessment shows that neither ferret sera contained antibodies capable of neutralizing Ca09 viruses in vitro prior to the viral challenge. Note that the unvaccinated group and vaccinated group had the same titers. As a result, one group is covering the other.

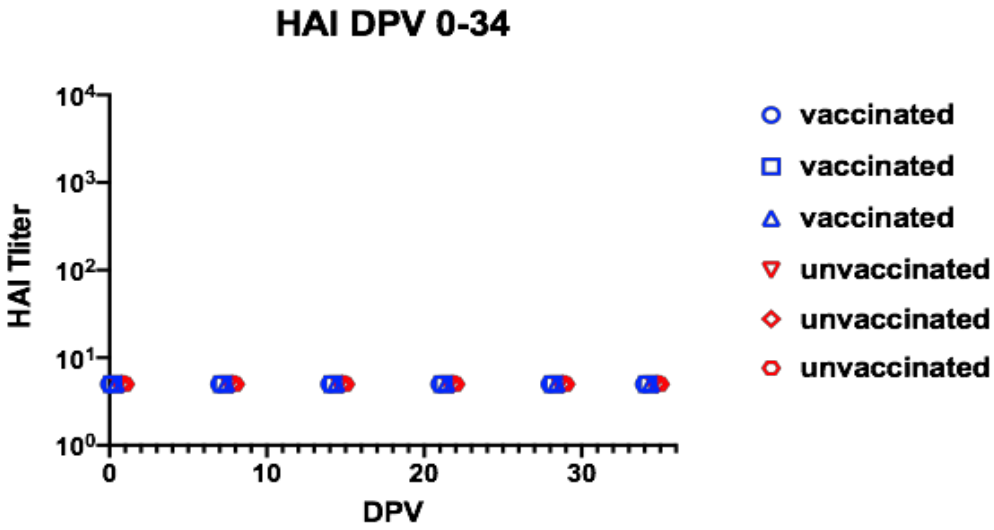

Figure 7: HAI assay data illustrates that none of the ferrets produced antibodies capable of interacting with the HA surface protein of IAV.

Days Post Vaccination (DPV) Results:

One of the goals of the testing the efficacy of the M2e-Lipo-DT+PHAD vaccine was to assess its ability to generate an IgA and IgG humoral immune response against M2e. To achieve this aim, a group of ferrets were inoculated intranasally with the liposome-based vaccine. Sera were collected on day 0 prior to vaccination and treated with RDE and sodium citrate solution to improve the sensitivity of the M2e-based ELISA. Days 7, 14, 21, and 34 days post vaccination, sera were collected from the ferrets and treated in a manner previously described. Following the collection of sera on days 14 and 21, the vaccine group of ferrets received an additional dosage of M2e-Lipo-DT+PHAD vaccine and the unvaccinated group was given a supplementary volume of blank liposome solution. The RDE and sodium citrate treated-ferret sera were subjected to analysis via M2e-based ELISAs and microneutralization assays.

The M2e-based ELISA was utilized to assess the titers of anti-M2e IgA and anti-M2e IgG levels present in the ferrets' sera. A 1:2 dilution scheme of treated sera was applied to 96 well, flat bottom plates and the absorbances, created from of each plate (one for each day) was ascertained. Absorbance results obtained from EL808 plate reader were used to calculate the anti-M2e IgG and M2e IgA concentrations from each day before and after vaccine or blank liposome administration. The results yielded from these assays on day 0 reveal that, initially, the ferrets had not seroconverted and thus were not producing IgA or IgG antibodies against the introduced InA M2e antigen. This is shown on **Figures 4** and **Figure *5***, respectively. These figures continue to illustrate the differences between vaccinated ferrets and blank liposome recipients. Days 7 and 14 of **Figure 4** show a consistent level of IgA following vaccination. After delivering the vaccine boosters on day

14 and 21, the IgA titers had a minimum 4-fold increase in comparison to the unvaccinated group (25 to 95). By day 34, anti-M2e IgA titers spiked. The ferrets inoculated with blank liposomes, in comparison, did not express high levels anti-M2e IgA. The M2e-based ELISA assessment, shown in **Figure 5**, showed that anti-M2e IgG was not present in the sera of any of the ferrets. However, day 21, following the application of a vaccine booster, exhibited a minimum significant 10-fold increase in the concentration of anti-M2e IgG antibody in the vaccinated group, with IgG titers reaching as high as 771, compared to those inoculated with blank liposomes only. By day 34, the anti-M2e IgG concentrations of the vaccinated group had tapered off, but still remained higher than the blank liposome group. Overall, the group of ferrets that were vaccinated and received subsequent boosters showed an increase in their antibody response against the M2e antigen when compared to the complementary group who were given blank liposomes. When blank liposomes were used as the coating antigen, antibodies were not detected for either the vaccinated or unvaccinated group, indicating that the blank liposomes alone were not immunogenic. The lack of immunogenicity against the blank liposomes was expected, because it is in line with previous findings (Zaman, et al. 2016)

To supplement the M2e-based ELISA results, the microneutralization assay was employed to assess the ability of the antibodies present in the treated-ferret serum to neutralize the virus that would be used in the next portion of the project to challenge the ferrets. From the absorbances collected via the Bio Tek Synergy HT plate reader, the amount of Ca09 neutralized was calculated. This assay revealed that despite the increase in production of anti-M2e IgG and IgA present in the ferret group that received the M2e-Lipo-DT+PHAD vaccine, the virus was still able to replicate successfully. The inability to

neutralize the Ca09 virus was illustrated in the unvaccinated group as well. The relationship between neutralization and the days post vaccination is shown in **Figure 6**.

HAI assays were utilized post-vaccination to investigate whether ferrets were producing antibodies in serum that were capable of binding to HA surface proteins on the Ca09 virus. After introducing CRBCs to plates containing virus and ferret sera from days 0, 7, 14, 21, and 34 post-vaccination, agglutination was observed, which illustrates that the virus was still able to interact with the sialic acids present on CRBCs. These interactions indicate, that despite producing higher levels of anti-M2e IgA and IgG, that neither the vaccinated or unvaccinated ferret groups were mounting an anti-HA humoral immune response. The inability of the antibodies produced by either ferret group to interact with Ca09 HA is shown above in **Figure 7**.

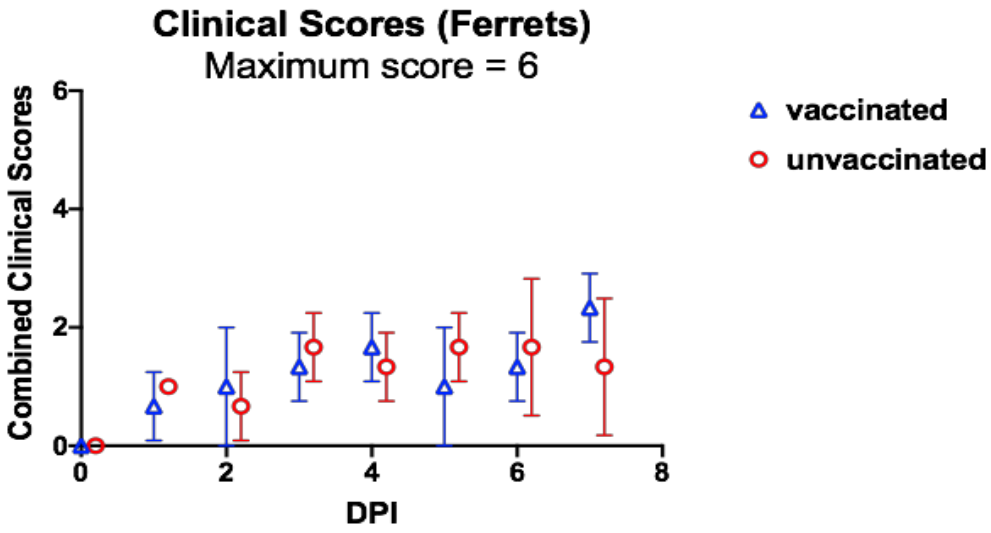

Figure 8: Clinical scores are the summation of daily sneeze scores and activity scores post-challenge with the influenza virus, Ca09. Activity is scored on a scale of 0-3, with 0 being the most active and 3 being

unresponsive. Sneezes are scored on a scale of 0-3, with 0 being no sneezes and a score of 3 is characterized by acute respiratory distress.

Days Post Infection (DPI) Results:

Following the 34-day post vaccination period, all ferrets were challenged with the influenza virus, Ca09 and observed for eight days. During the 8-day post infection period, all ferrets were observed for 10 minutes. During this observational period, the number of sneezes and activity level of each ferret was scored and summated into a daily clinical value shown in **Figure 8**. The number of sneezes is used as a method to qualitatively assess that the ferrets are infected with the Ca09 virus and that they are successfully shedding the virus. The activity level of the ferrets is used a qualitative, auxiliary method used to gauge how the ferret is handling the infection. A ferret who is infected with influenza tends to display more lethargic behaviors in direct correlation with the severity of the infection. Based on the **Figure 8**, initially, the vaccinated ferrets were not as symptomatic as the unvaccinated ferrets, suggesting a resistance to infection. By days 4 and 5 post infection, both ferret groups displayed consistent symptoms with one another. In terms of symptoms, by day 7 post-infection, ferrets from the unvaccinated group appeared to have resolved the infection sooner than their vaccinated counterparts. Overall, all ferrets manifested symptoms of an influenza A virus challenge.

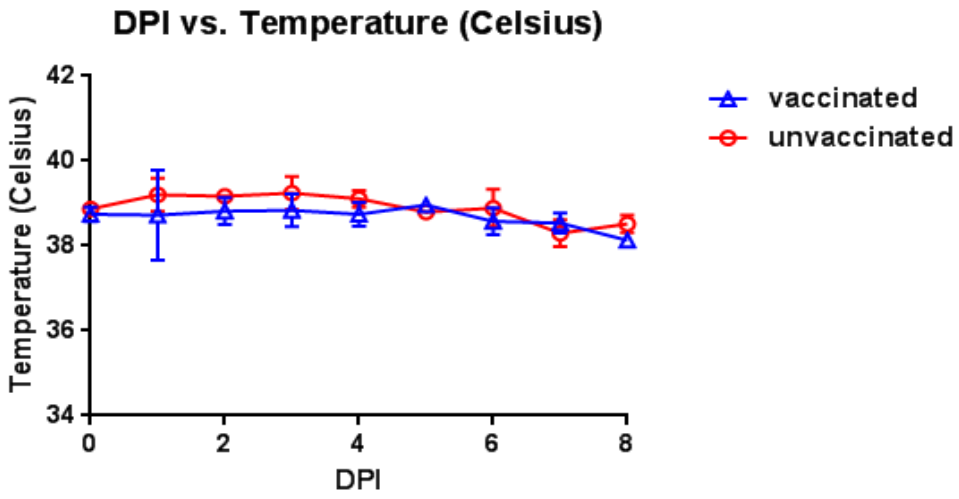

Figure 9: This model shows fluctuations in ferret temperatures, one of the common signs exhibited by the presence of an influenza infection.

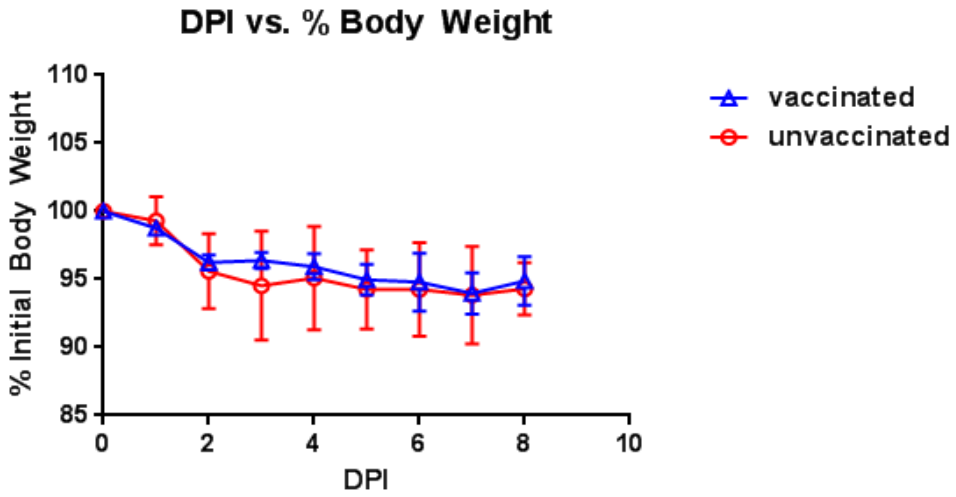

Figure 10: Influenza infections are often characterized by weight loss. This model shows the differences in the amount of weight lost between ferrets who were treated with M2e-Lipo-DT+PHAD vaccine and ferrets who received only blank liposome.

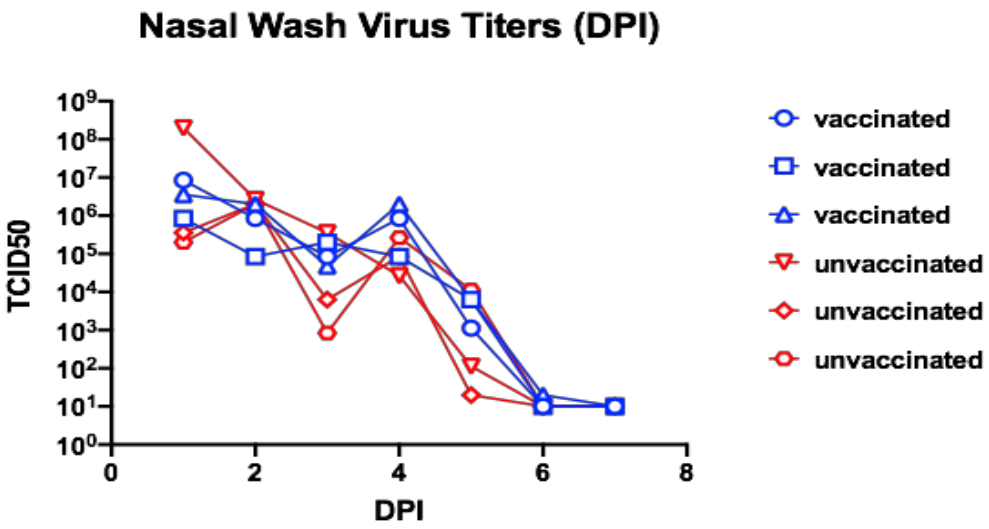

Figure 11: $TCID_{50}$ values, as determined via HA assays, show approximate viral titers across a 7-day infection period.

After the 10 minutes observations, ferrets were anesthetized using isoflurane and ketamine. While under the effects of anesthesia, ferrets from both groups were weighed and their temperatures were measured daily. Typically, when ferrets are challenged with an influenza infection, the ferret responds with signs, such as weight loss and a fever. The relationship between the infection time period and morbidity signs are illustrated in **Figure 9** and **Figure 10**. Initially, both ferret groups were running about the same temperature at approximately 39° C. After Ca09 challenge, both groups had temperatures between 38° C and 40°C. On average ferrets have a body temperature of approximately 38.83° C. Following intranasal inoculation with Ca09, most of the ferrets, regardless of vaccination, exhibited a low-grade fever. As shown in **Figure 9**, ferrets that received vaccination prior to challenge had lower temperatures, as a whole, in comparison to the blank liposome recipients. After infection, ferrets from both groups showed signs of weight loss, especially by day 2 post infection. Following day 2 post infection, the ferrets from the vaccinated group experienced a less drastic reduction in weight. In the unvaccinated group, the weight

loss shown was more drastic, dropping to just under 95% of their original body weight as denoted by **Figure 10**. By day 6 and 7 post challenge, the groups weighed about the same in terms of percent-initial body weight.

After taking the weight and temperatures of each ferret, both the vaccinated and unvaccinated groups were subjected to nasal washes using 1x PBS (pH =7.43). The nasal washes collected were serially diluted and plated over a 48 well plate containing MDCK cells as previously described. The plates were allowed incubate for 4 days at 37° C before measuring the viral content using the HA assay. Results from the HA assay were determined on the basis of the formation of a pellet, signifying a negative result, or hemagglutination, proving a positive result. Based on the data gathered, the vaccinated ferrets have reduced initial viral titers (approximately $10^{6.5}$) compared to the unvaccinated group which exhibited viral titers as high as 10^{8}. This trend continued throughout the course of infection as shown in **Figure 11**. Day 3 showed that 2 out of the 3 ferrets in the unvaccinated group had a lower viral titer than the vaccine recipients, but by the next day the groups had similar titers again. This trend renewed again by day 5. Finally, vaccine and blank liposome recipients cleared the infection by 7.

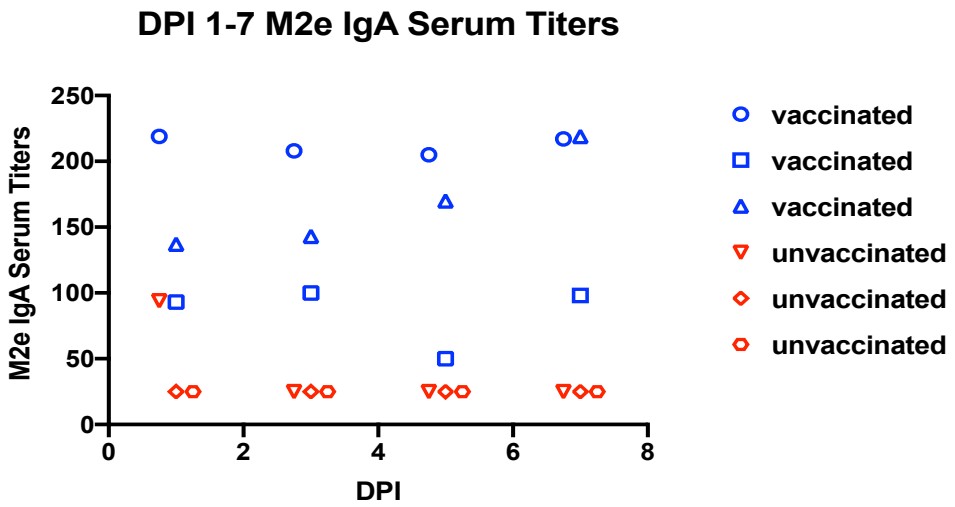

Figure 12: M2e-based ELISA results depict how prior vaccination and subsequent antigen exposure, or the lack thereof, affects the generation of an anti-M2e IgA response.

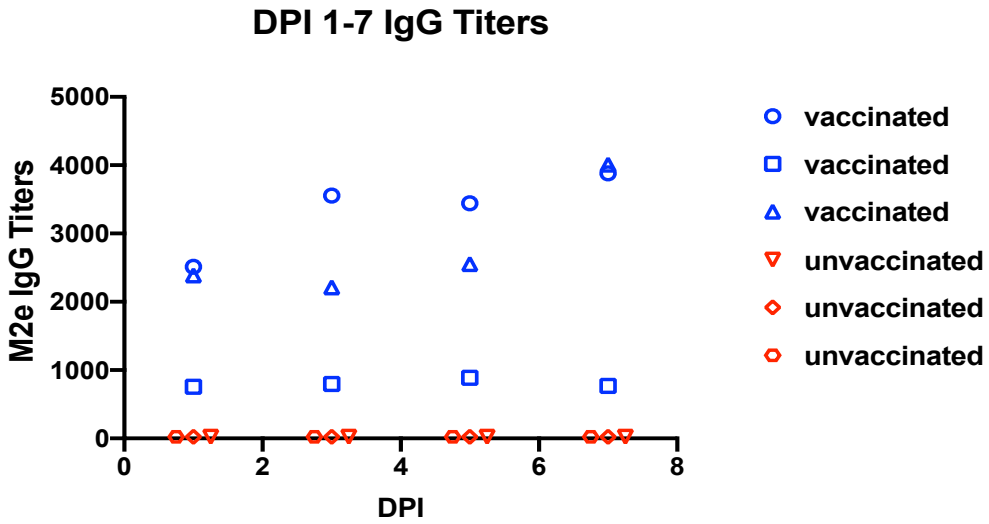

Figure 13: M2e-based ELISA results demonstrate the efficacy of the M2e vaccine in ferrets in relation to anti-M2e IgG generation during a 7-day post infection period versus the IgG humoral response of blank liposome recipients.

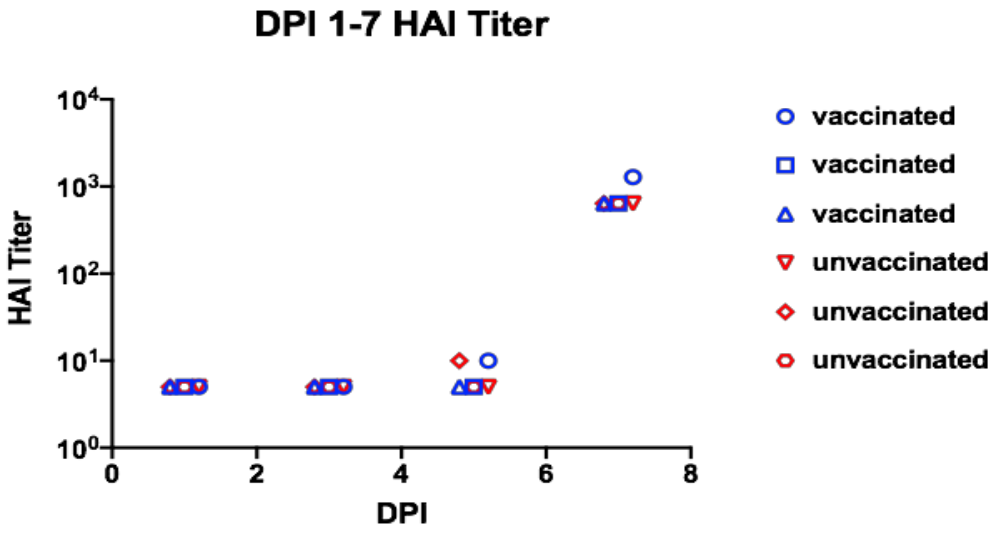

Figure 14: HAI assays demonstrate the ability of each ferret, vaccinated or not, to neutralize via sera antibody-viral HA interactions. The unvaccinated group exhibited the same HAI titers as the vaccinated group.

Once anesthetized on days 1, 3, 5, and 7 following infection with the Ca09 virus, sera from the vaccinated and unvaccinated groups was collected and treated in a manner previously described. The treated sera were used in a variety of assays, such as M2e-based ELISAs and HAI assays. To assess the formation of anti-M2e IgG and IgA in response to Ca09 challenge, M2e-based ELISAs were employed in a manner previously described. Analysis of the absorbances produced via Bio Tek EL808 revealed that anti-M2e IgA titers in the vaccinated group, immediately after infection, were exponentially higher than the ferrets who were inoculated with blank liposomes (**Figure 12**). Furthermore, one of the ferrets in the vaccinated group exhibits a reduction in the anti-M2e IgA titers on day 7, correlating with the clearance of the pathogen.

M2e-based ELISAs that were utilized to determine the amount of anti-M2e IgG in sera revealed that the ferrets who were vaccinated had a multiple fold increase in anti-InA IgG in response to Ca09 challenge compared to their unvaccinated counterparts.

Vaccinated ferrets maintain higher titers of anti-M2e IgG throughout the course of infection ranging from 76 to 412. In comparison, the blank liposome group did not mount a great IgG mediated humoral response against the M2e protein following the induction of morbidity displaying a constant anti-M2e IgG titer of approximately 10. Even as the Ca09 infection persisted, M2e-based ELISAs did not detect the significant production of anti-M2e IgG antibodies in ferrets who were inoculated previously with blank liposomes. **Figure 13** shows the relationship between anti-M2e IgG titers in the vaccinated and unvaccinated ferrets over the 7-day infection period.

Ferret sera collected on days 1, 3, 5, and 7 post-infection with the Ca09 influenza A virus was also used in HAI assays to assess the ability of antibodies present in the sera to interact with the HA surface protein of the virus. Initially, neither the vaccine or blank liposome recipients produced anti-HA antibodies against the Ca09 influenza virus. As the infection proceeded, the concentration of anti-HA antibodies present in the sera of both groups increased exponentially to 640 with one ferret in the vaccinated group reaching titers of 1280. The most notable increases in the ability of antibodies present in the ferret sera to interact with Ca09 HA was on days 5 and 7 as shown by **Figure 14**. Thus, the vaccinated group produced higher levels of antibodies against M2e, indicating that the vaccine induced immunity against this protein. However, both vaccinated and unvaccinated animals produced the same level of anti-HA against the Ca09 pathogen, and responded similarly to challenge. The impact of these findings on the use of M2e as a universal vaccine are discussed below.

CHAPTER FOUR

Discussion:

Why Ferrets?

The ferret is a reputable model organism when it comes to researching influenza viruses. Unlike murine-based models, ferrets are a particularly useful model when it comes to studying the pathogenicity and transmissibility of influenza, because this mammalian species can be infected, without viral adaptation, by both human and avian variants of the influenza virus (Matsuoka, Lamirande, and Subbarao 2009). This is further complemented by the ferret's analogous respiratory structure to humans. As such, the viral interactions between host and pathogen are very similar (Belser, Katz, and Tumpey 2011). The ferret demonstrates usefulness when it comes to modeling viral transmission due to the induction of sneezing following infection (Matsuoka, Lamirande, and Subbarao 2009). Sneezing results in the expelling of virus in the form of respiratory droplets, a sign commonly seen in human patients with influenza. Another sign exhibited by ferrets that is common in humans diseased with influenza is the induction of a fever (Belser, Katz, and Tumpey 2011). In tandem with demonstrating signs similar to those found in humans, is the ability to vaccinate ferrets and reduce viral spread.

Why the M2e-Lipo-DT+PHAD Vaccine?

The M2e-Lipo-DT-PHAD vaccine was selected for this study for many reasons. The first motive for using this vaccine was due to the vaccine's ability to display multiple antigenic molecules, such as the exhibition of antigenic peptides attached to a lipid tail that is embedded into the liposome itself (Zaman, et al. 2016). Conjunction of lipid tails to protein spacers and antigen proteins leads to better recognition by B cell epitopes generating a more immunogenic response (Ingale, et al. 2007). This lipid-protein-M2e antigen conjugation is a favorable design, because antigen peptides by themselves are poorly immunogenic. However, through the covalent attachment of a peptide, such as M2e antigen, to a carrier protein, a stronger cell-mediated response is induced (Yussef, Pierobon, Reverstat, and Lennon-Duménil 2013). In addition, the liposome and associated peptides are potent activators of toll-like receptors (TLR), such as TLR-2, and other aspects of innate immunity (Zaman and Toth 2013). Complementary to TLR-2 responses, is the affinity that antigen presenting cells (APCs) have for liposomes (Watson, Endsley, and Huang 2012). This allows for a more "natural" immune response in which the APC phagocytizes antigen and delivers it to peripheral immune sites. Next, is the ability of the M2e-Lipo-DT-PHAD vaccine to generate an IgA mucosal immune response (Lee and Nguyen 2015). This begins with the intranasal delivery of the vaccine. The nasal route mimics the natural way that influenza is transmitted from one host to another. Intranasally delivery of the M2e-Lipo-DT-PHAD vaccine allows for the potential to generate defensive immunity against pathogens, such as influenza, that spread through mucous portals. Furthermore, the liposome and encapsulated DT has been shown to generate a strong IgA response through the formation of modulating epitopic constructs that stimulate IgA

secreting B cells. The M2e-Lipo-DT-PHAD vaccine platform demonstrates the capability to stimulate a cell-mediated response, such as the activation of CD4+ cells, which aid in the process of the humoral response (Mozdzanowska, et al. 2005). In previous human vaccinations, it has been determined that liposome-based vaccines are safe for human use (Watson, Endsley, and Huang 2012).

Post-M2eA-Lipo-DT+PHAD Vaccination

Current vaccines are designed with the sole intent of targeting the subunits of the HA and NA surface proteins of the influenza virus, thus neutralizing the pathogen through the production of anti-HA or anti-NA antibodies (Kendal, Maassab, Alexandrova, and Ghendon 1987). This directive is problematic due to the high mutation rate of HA and NA in conjunction with the concurrent circulation of additional viral strains (Hannoun 2013). As a result of influenza's tendency to antigenically shift and drift, the production of vaccines is dampened (Schotsaert, Filette, Fiers, and Saelens 2009). However, this hindrance could be averted through the addition of a more conserved peptide, such as the ectodomain of the M2 protein. 9 out of the 24 amino acids that compose M2e are highly conserved across all influenza strains (Wu, et al. 2007). It is due to the conserved peptide sequence that makes M2e a promising vaccine candidate. This is further supported by previous studies that suggest that M2e-based vaccines convey cross-protection against multiple influenza strains in mice, rhesus monkeys, and ferrets (Fan, et al. 2004).

In line with the hypothesis, the results gathered from the post vaccination of ferrets suggest that a liposome-based vaccine incorporating multiple peptide antigens, such as M2e peptides and DT, and a self-adjuvanting glycolipid (PHAD) in ferrets has been identified.

It has been demonstrated that not only is this vaccine capable of eliciting a significant IgG humoral response to the M2e antigen, M2e, but due to the intranasal delivery, in tandem with the vaccine design, was able to generate an IgA response as well. The unvaccinated group did not develop a humoral response against Ca09 M2e. However, despite the exceptionally high anti-M2e humoral response in vaccinated ferrets, the antibodies produced during the vaccination period were shown to be non-neutralizing in function and, as was expected, did not have an affinity for Ca09 HA. This was illustrated through the microneutralization assay and HAI assay, respectively. These assays introduced the sera of ferrets that had been vaccinated, and thus contained anti-M2e antibodies, to the Ca09 influenza A virus.

There are multiple explanations concerning why the anti-M2e humoral response does not neutralize the infectious agent. The first is that the expression of M2e on a virion is low in comparison to HA and NA whereas an infected cell abundantly expresses M2e proteins on the surface of the cell (Bakkouri, et al. 2011). Due to the low concentration of M2e present on a virion, antibodies are less likely to target the protein. Furthermore, if antibodies bind to the ectodomain of M2e, it doesn't necessarily block the M2 channel, so a pH change can still be induced allowing for viral replication to continue. However, it is worth using M2e *complemented* vaccines to employ other antibody-mediated effector functions, such as ADCC, to destroy infected cells (Fiers, et al. 2009). In short, when anti-M2e antibodies bind to the M2e that is expressed by infected cells, the Fc portion of the antibody is exposed to other effector cells, such as natural killer cells and macrophages. The effector cells will bind to Fc portion of the anti-M2e antibody via the FcγrIII and trigger the effector function of the bound cell (Bakkouri, et al. 2011).

Post-A/California/04/2009 Challenge/Relevancy to Superinfections

After the 34-day post vaccination period, both vaccinated and unvaccinated ferrets were challenged with Ca09 influenza A virus. The ferrets who were vaccinated against M2e displayed an immediate IgA and IgG response compared to the ferret group that received blank liposomes suggesting immunological memory was generated against M2e. Despite the vaccinated group having higher antibody titers, they displayed viral titers equivalent to the unvaccinated group as shown by **Figure11**. This shows that the M2e vaccine was not strong enough to protect the ferrets against Ca09 infection, which suggests that the vaccine would need to be complemented with an HA or NA component. The unvaccinated ferrets did not elicit a natural, humoral response against M2e, which suggests that M2e does not natural elicit an immune response.

However, previous microneutralization data suggests that the antibodies were non-neutralizing in function. Extrapolation of microneutralization results coupled with the presence of reduced viral titers and an elevated anti-M2e humoral response suggests that these antibodies operating in an alternative manner. The data produced from M2e-based ELISAs showed that following the challenge with Ca09 that vaccinated ferrets produced a substantial amount of anti-M2e IgA antibodies. IgA is composed of two monomers linked by a J chain and a secretory unit (Abbas 2019). Due to the secretory unit, IgA is able to be taken up by the basolateral side of mucosal cells without being degraded inside the cell and is transferred to the apical side where the infection is taken place (Renegar, Small, Boykins, and Wright 2004). Given that IgA is the antibody primarily associated with mucosal immunity, it is partially responsible for reducing the viral load present in the respiratory

system vaccinated ferrets as seen by days 1-3 post infection. Again, the unvaccinated ferrets did not elicit an anti-M2e IgA response showing M2e is not innately immunogenic.

The results derived from post vaccination and post infection show that the anti-M2e IgG being produced is not performing a direct neutralization function, but it is binding to M2e that is expressed on the surface of influenza-riddled cells. The mechanism triggered through the binding of anti-M2e IgG to M2e on the surface of infected cells has yet to be defined, but it has been shown in previous research that major contrivances of immunity are marking for opsonophagocytosis, the activation of the complement cascade, resulting in the formation of the membrane attack complex, and functional FcγRIII interactions that yield ADCC (Jegerlehner, Schmitz, Storni, and Bachmann 2004). Bakkouri et. al, produced results that demonstrated the necessity of FcγRIII interactions of effector cells with anti-M2e antibodies bound to influenza-diseased cells in clearing the influenza related morbidity (Bakkouria, et al. 2011). As a result, it is thought that effector cells in conjunction with anti-M2e antibodies, are reducing the spread of the disease through ADCC. Again, these antibodies have been shown to be non-neutralizing in nature and lack specificity for HA, as determined by HAI assays, suggesting an alternative mode of operation which requires further exploration.

The M2e-Lipo-DT-PHAD vaccine induced antibodies against M2e, but the antibodies were not neutralizing and did not initially reduce viral load, in comparison to the ferrets who received blank liposomes during the first three days following Ca09 challenge. This illustrates that while M2e is a potential vaccine candidate due to its conserved nature between influenza A strains, it is an infection permissive vaccine that may not be protective if delivered alone. It was not until days 5-7 that a significant decrease, and eventual

clearance of the Ca09 pathogen was shown. This correlates with an elevation in the production of anti-HA antibodies as shown by the day 5-7 of **Figure 14**. Given that none of the ferrets produced anti-HA antibodies following vaccination and in turn did not have immunological memory against Ca09 HA, the reduction of virus in response to an anti-HA humoral response illustrates the importance of an immune response against HA is when it comes to reducing the pathogenicity of influenza. The lack of production of anti-M2e antibodies versus the generation of an anti-HA humoral response in unvaccinated ferrets demonstrates that naturally the host generates an immune response based on antigen availability. Since the HA concentration on IAV is exponentially higher than the M2e content present, the natural immune response will be towards HA. Future vaccines that evaluate M2e should consider including HA and NA components in the vaccine design. This vaccine could be tested against influenza viruses that have undergone antigenic drift where the anti-HA or anti-NA response may not be 100% protective. However, in these situations the immunity induced against the HA, NA, and M2e may complement each other and provide complete protection against this drift variant.

CHAPTER FIVE

Conclusion:

Contextualizing for the Future

What do these results suggest for continuing the development of M2e-based vaccines? It illustrates that individuals who have been vaccinated with an M2e-based vaccine will potentially contract influenza because the humoral response induced against M2e does not protect against immediate morbidity. According to previous studies, the mortality rate associated with influenza infections are primarily due to complications inflicted by secondary bacterial infections, such as those caused by *S. pneumoniae* and *S. pyogenes* (Chaussee, et al. 2011). The data of this study illuminates the fact that since anti-M2e antibodies do not neutralize the influenza virus results illustrate that the M2e-Lipo-DT-PHAD vaccine is an infection permissive vaccine. While it is imperative to explore the utility of M2e peptides for universal vaccine candidacy, it is equally as important to realize the limitations of using M2e as the sole vaccine peptide despite its obvious advantages, such as its conserved nature and inexpensive production. The permissibility to influenza morbidity, despite vaccination, leaves patients susceptible to subsequent infection, and potentially mortality (Peltola and McCullers 2004). Instead, M2e should be included in conjunction with HA and NA content that is utilized in contemporary trivalent and quadrivalent vaccines. This claim is supported by the data produced by this experiment, which recognizes the importance of humoral immunity against HA in clearing influenza infections.

If M2e is to be incorporated in vaccines, it is essential to understand the immune response being induced. It has been shown that a humoral response against M2e is not based on neutralization, but rather other mechanisms, such as opsonophagocytosis, complement activation, and ADCC (Fiers, et al. 2009). The future of this project aims to characterize these responses, specifically those related to ADCC. In order to assess the contribution of anti-M2e antibodies to activation of the ADCC pathway, the Huber lab seeks to carry out similar studies as the ones conducted in this project. However, in tandem with ELISAs, microneutralization assays, and HAI assays, an ADCC assay will be utilized to define the interactions anti-M2e IgG and IgA play in the activation of effector cells, such as natural killer cells and respiratory resident macrophages (Jegaskanda, et al. 2013). A similar research design can be utilized to assess the activation of the complement pathway via complement dependent cytotoxicity assays (Co, et al. 2014). It is through characterizing these responses that a better understanding of the immunity induced by M2e based vaccines can be achieved. This is crucial in generating the best immunonological memory possible to confront potential influenza infections that a patient may encounter. If an immunization can be developed that inhibits primary infections, subsequent secondary infections can be prevented, drastically reducing the mortality rate associated with influenza infections.

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