Plant-Expressed Recombinant Universal Influenza A Vaccine Candidates

by

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ABSTRACT

Influenza is a deadly disease that poses a major threat to global health. The surface proteins of influenza A, the type most often associated with epidemics and pandemics, mutate at a very high frequency from season to season, reducing the efficacy of seasonal influenza vaccines. However, certain regions of these proteins are conserved between strains of influenza A, making them attractive targets for the development of a 'universal' influenza vaccine. One of these highly conserved regions is the ectodomain of the influenza matrix 2 protein (M2e). Studies have shown that M2e is poorly immunogenic on its own, but when properly adjuvanted it can be used to induce protective immune responses against many strains of influenza A. In this thesis, M2e was fused to a pair experimental 'vaccine platforms': an antibody fusion protein designed to assemble into a recombinant immune complex (RIC) and the hepatitis B core antigen (HBc) that can assemble into virus-like particles (VLP). The two antigens were produced in *Nicotiana benthamiana* plants through the use of geminiviral vectors and were subsequently evaluated in mouse trials. Mice were administered three doses of either the VLP alone or a 1:1 combination of the VLP and the RIC, and recipients of both the VLP and RIC exhibited endpoint anti-M2e antibody titers that were 2 to 3 times higher than mice that received the VLP alone. While IgG2a:IgG1 ratios, which can suggest the type of immune response (T_H1 vs T_H2) an antigen will elicit, were higher in mice vaccinated solely with the VLP, the higher overall titers are encouraging and demonstrate a degree of interaction between the RIC and VLP vaccines. Further research is necessary to determine the optimal balance of VLP and RIC to maximize IgG2a:IGg1 ratios as well as whether such interaction would be observed through the use of a variety of diverse antigens, though the results of other studies conducted in this lab suggests that this is indeed the case. The results of this study demonstrate not only the successful development of a promising new universal influenza A vaccine, but also that co-delivering different types of recombinant vaccines could reduce the total number of vaccine doses needed to achieve a protective immune response.

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INTRODUCTION

1.1 The Impact of Influenza

Influenza is a historically and socially significant disease that is characterized by the sudden onset of a high fever, runny nose, fatigue, muscle pains, sneezing, coughing, and general malaise (World Health Organization, 2018). In a meta-analysis of six influenza seasons in the United States (from the 2010-2011 season to the 2015-2016 season), it was estimated that the number of influenza-related illnesses ranged from 9.2 million to 35.6 million per season, including 140,000 to 710,000 influenza-related hospitalizations (Rolfes et al., 2018). Further, the disease is also estimated to cost the U.S. economy anywhere from $6.3 to $25.3 billion annually (Putri et al., 2018). On a global scale, influenza-associated mortality typically ranges from 291,243 to 645,832 influenza-associated respiratory deaths per year (Iuliano et al., 2018), with most (>99%) deaths of children below the age of 5 years old occurring in developing countries. However, should a pandemic strain of influenza similar in severity to the 1918 Spanish Flu pandemic occur, the cost to human life could be catastrophic and the damage to the world' economy would be staggering ;the World Bank estimates that such a pandemic would cost the global economy approximately 5% of its GDP in the pandemic's first year alone (Burns et al., 2006).

Due to the disease's impact around the world, there have been calls for substantial improvement in the methods used to estimate the global burden of influenza (e.g. including data on nonrespiratory complications of influenza infection, severe influenza case data, etc.) (Bresse et al., 2018), indicating that not only a potentially substantial number of cases are currently left unreported and unaccounted for within the literature, but that influenza may be a larger problem than is currently believed. While influenza vaccines lessen the annual impact of influenza on the world, it is of the utmost importance that vaccines with high rates of efficacy are developed in a timely manner.

1.2 Influenza Vaccines Need Improvement

Over six influenza seasons in the U.S., from 2010 to 2016, it was determined that overall vaccination rates ranged from 42%-47% of the population, preventing anywhere from 1.6 million

to 6.7 million illnesses, 790,000-3.1 million outpatient medical visits, 39,000-87,000 hospitalizations, and 3,000-10,000 influenza-related deaths (Rolfes et al., 2018). However, seasonal influenza vaccines are routinely associated with low rates of vaccine efficacy (VE); the U.S. Centers for Disease Control and Prevention (CDC) reported VEs of 56% for the 2012-2013 (Jackson et al., 2013), 61% for the 2013-2014 season (Flannery et al., 2014), 23% for the 2014-2015 season (Flannery et al., 2015), 48% for the 2015-2016 season (Jackson et al., 2017), and 48% for the 2016-2017 season (Flannery et al., 2017). Furthermore, during the 2017/2018 influenza season, VE against the circulating strain of influenza A (H3N2) was estimated to be as low as 25% in the United States (Centers for Disease Control and Prevention, 2018), 17% in Canada (Skowronski et al., 2018) and 10% in Australia (Sullivan et al., 2017) despite the fact that the 2017/2018 influenza vaccine contained influenza of the same subtype and clade. This was due, in part, to three mutations in hemagglutinin (HA), a protein on the influenza virus' surface.

The vulnerability of influenza vaccines to small mutations like those observed in the 2017/2018 strain's HA protein is due primarily to the vaccines' composition, which involve including three to four strains of inactivated or attenuated influenza virus. Predictions, and subsequent recommendations, are made on an annual basis as to which three to four strains will most likely be in circulation during that year's influenza season based on influenza surveillance data collected by the CDC and the World Health Organization. Then, vaccines composed of the predicted strains are mass-produced, traditionally by growing and passaging the virus in chicken eggs, and shipped before the influenza season starts. However, due to the structure and behavior of the influenza virus as a multipartite RNA virus with high rates of mutation, reassortment, and recombination, the annual predictions often miss the mark. Therefore, it comes as no surprise that the globally circulating influenza virus, in practice, often varies significantly from those predicted ahead of the season, substantially reducing the VE of a given season's influenza vaccine.

1.3 The Four Types of Influenza Viruses

There are four types influenza viruses, A, B, C, and D, though influenza C is not commonly associated with disease in most populations, being observed only in isolated, sporadic

outbreaks in children younger than the age of six years old and elderly populations (Matsuzaki et al., 2006; Smith et al., 2016). Influenza D has been shown to only infect swine and cattle (Hause et al., 2014) (Smith et al., 2016). Influenza A and B, meanwhile, regularly circulate and cause disease in humans, with rates of influenza-related hospitalizations being higher in seasons where influenza A viruses dominate (Thompson et al., 2004). Influenza A is also more routinely the cause of epidemics and pandemics (Hay et al., 2001), as influenza A viruses have a higher mutation rate than influenza B viruses (Nobusawa & Sato, 2006) and consequently have a higher propensity to evade the protective immune response that influenza vaccines or infections confer. The higher mutation rate of influenza A viruses, when compared to influenza B viruses, is partially due to influenza B viruses generally being limited to infecting humans (Hay et al., 2001). Meanwhile, influenza A viruses are able to infect a range of mammalian and avian hosts, including, but not limited to, pigs, wild and domesticated water fowl, and humans (Hay et al., 2001), increasing the likelihood of antigenic shift between human and zoonotic strains. Influenza A's association with high levels of hospitalization, seasonal epidemics, and global pandemics makes the need for a 'universal' influenza A vaccine that maintains its efficacy and protection from season to season, despite the virus' high rate of mutation, absolutely essential to preventing the influenza pandemics of the future.

1.4 The Structure of Influenza A

Influenza is a member of the *Orthomyxoviridae* family of viruses, a group of single-stranded negative sense RNA viruses, and its genome is comprised of eight segments each containing a single gene (McGeoch et al., 1976). The influenza A genome encodes at least ten proteins and up to 14 proteins through strain-dependent alternative splicing (Eisfeld et al., 2015; Suarez et al., 2016). The ten common influenza A proteins can be grouped into (a) the surface proteins hemagglutinin (HA), neuraminidase (NA), and the matrix 2 protein (M2); (b) the internal proteins nucleoprotein (NP), the matrix 1 protein (M1), polymerase basic protein 1 and 2 (PB) and polymerase acidic protein (PA); and (c) the two non-structural proteins (NS) (Suarez et al., 2016). Segment 1 encodes PB2, segment 2 encodes PB1, segment 3 encodes PA, segment 4 encodes HA, segment 5 encodes NP, segment 6 encodes NA, segment 7 encodes both M1 and M2, and

segment 8 encodes NS1 and NS2 (Inglis et al., 1976). HA and NA, in particular, are common targets of recombinant influenza vaccines, as HA's primary role is to facilitate viral entry into target cells through binding to sialic acid-containing receptors on the host cell (Skehel & Wiley, 2000) and NA promotes the release of newly-formed influenza virions through the removal of sialic acid residues on both the host cell and the nascent virion (Mitnaul et al., 2000). However, while the neutralization of either could provide protection against influenza A infection, HA and NA mutate frequently from season to season (Webster et al., 1982). This has led to the search for conserved, protective epitopes in not only HA (Kramer & Palese, 2019) and NA (Kosik et al., 2019), but also other influenza A proteins, so that a 'universal' influenza vaccine that is effective from season to season can be developed. One epitope that has received much attention in recent years has come not from HA or NA, but the M2 protein.

1.5 M2e as a Target for Universal Influenza A Vaccines

M2 is a tetrameric integral membrane protein that, despite being found at low levels on influenza A virion, facilitates viral uncoating in its role as a proton channel (Lamb et al., 1985). M2e is the ectodomain of M2, and it has not changed significantly since it was first identified in 1933 (Fiers et al., 2004). This highly-conserved region of M2 is poorly immunogenic on its own, but when conjugated or fused to potent adjuvants or carriers, it becomes a potent protective determinant against influenza A (Mardanova & Ravin, 2018). M2 is expressed on the surface of infected cells at nearly the same rate as NA, but is incorporated into virions much less than NA, with only 14 to 68 molecules of M2 per virion versus 198-211 molecules of NA, suggesting that M2 is selectively excluded from budding virions (Lamb et al., 1985; Zebedee et al., 1988). Despite this, vaccines targeting M2e have demonstrated protection in several studies, with this protection having been determined to be due not to the prevention of infection, but instead through Fc-receptor dependent antibody-dependent cell cytotoxicity (ADCC) and alveolar macrophage antibody-dependent cell-mediated phagocytosis (ADCP) of infected cells (El Bakkouri et al., 2011). Additionally, it has been discovered that lung-resident T_H17 CD4 T cells specific for M2e tetramers are broadly effective against influenza infection, indicating that the anti-M2e response is not limited only to antibody-dependent responses (Eliasson et al., 2018).

In clinical trials, vaccines targeting M2e have been well-tolerated, with studies investigating M2e expressed recombinantly on hepatitis B core antigen (HBc) (Fiers et al., 2009), even in the presence of anti-HBc antibodies, and fused to flagellin (Turley et al., 2011) demonstrating safety and efficacy. Several other clinical trials have been conducted around the world investigating M2e's potential as a vaccine antigen (Scorza et al., 2016) to varying degrees of protective value. However, to the best of our knowledge, no studies have attempted to express M2e recombinantly in a recombinant immune complex (RIC), a promising 'universal vaccine platform' that could boost the immunogenicity of M2e substantially and whose modularity could allow for the addition of other prominent and conserved influenza targets and adjuvants to adjust to whatever potential hurdles the influenza epidemics and pandemics of the future may have to offer.

1.6 Recombinant Immune Complex Vaccines

RICs, fundamentally, are composed of immunoglobulin molecules specific for an antigen that are fused to said antigen (Chargelegue et al., 2005). This allows for the binding region of one antibody to bind to the antigen recombinantly fused to another antibody, resulting in the formation of large, highly immunogenic antibody-antigen complexes (Chargelegue et al., 2005). RICs can be engineered into 'universal vaccine platforms' through the use of antibodies specific for an epitope tag, which allows for the same antibody to be used regardless of the antigen so long as the antibody's corresponding epitope tag is expressed on the antigen (Mason et al., 2016) (**Figure 1)**.

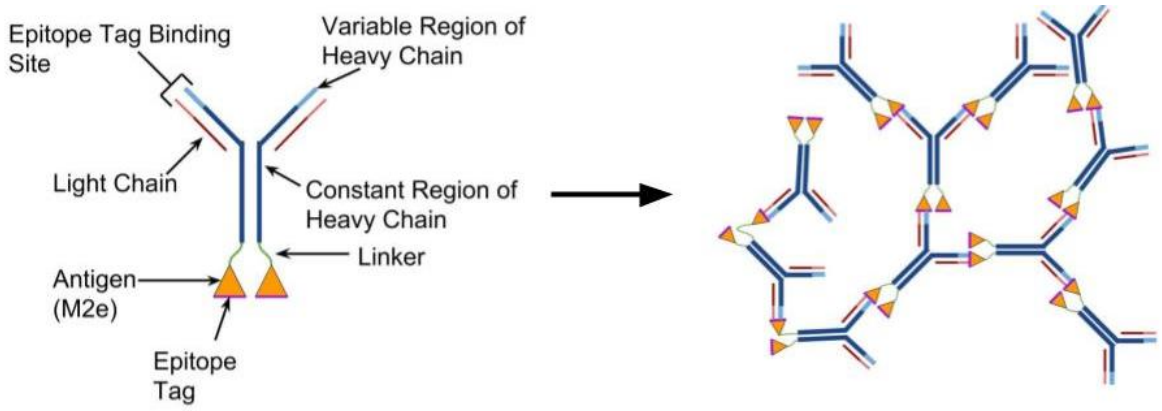

Figure 1: Diagram of universal recombinant immune complex. The epitope tag of one immunoglobulin is bound to the binding site of other immunoglobulins, forming complexes that contain the target antigen.

RICs take advantage of existing immunological mechanisms by utilizing antibodies' natural interaction with Fcγ receptors (Fridman, 1991; Van den Hoecke et al., 2017), which results in the phagocytosis and processing of the RICs and the target antigens contained within. Additionally, the increased concentration of antigens within the RIC can allow for increased B cell-receptor cross-linking, increasing B cell stimulation and activation (Avalos & Ploegh, 2014). RICs have been evaluated as treatment platforms for diseases like Ebola fever (Phoolcharoen et al., 2011) and tuberculosis (Pepponi et al., 2014) as well as vaccine platforms for HIV (Hioe et al., 2009), Dengue fever (Kim et al., 2015), and HPV (Diamos et al., 2019). Because of the platform's ability to potentiate the immunogenicity of a given antigen, the development of an M2e-containing RIC (M2e-RIC) could act as a first step toward developing a truly universal influenza vaccine.

1.7 Virus-Like Particle Vaccines

Virus-like particles (VLPs) are non-infectious, protein-based nanoparticles derived from virus capsids that self-assemble into virus-like structures and can be modified to recombinantly express vaccine antigens, making them an adaptable vaccine platform for combatting a myriad of diseases (Rohovie et al., 2017). One of the VLP vaccines against human papillomavirus have been commercially available in the United States since the 2006 approval of Merck & Co.'s Gardasil, and more recently, potent, VLP-based vaccines have been shown significant efficacy against diseases like Zika virus (Yang et al., 2017), norovirus (Diamos & Mason, 2018), and even

influenza (Pushko et al., 2017; Ramirez et al., 2018), each of which make use of the hepatitis B core antigen (HBcAg) as the platform for their VLPs.

HBcAg offers several advantages as a vaccine platform: it is both a T-cell dependent and T-cell independent antigen (Milich et al., 1986), it preferentially evokes a T_H1 response instead of a T_h2 response like other hepatitis B antigens (Milich et al., 1995), is a potent activator of macrophages (Cooper et al., 2005), and can be used effectively to present heterologous antigens without compromising the assembly or integrity of the HBc VLP (Schödel et al., 1992). Delivering HBcAg VLPs with non-HBc antigens at the c/e1 loop of HBc α-helical spike, a region also known as the 'major insertion region' (MIR), has been shown to evoke immune responses to both the HBcAg and the inserted antigen (Whitacre et al., 2009). To form VLPs, HBcAg monomers first assemble into dimers, which in turn form full VLPs when expressed in eukaryotes (Pumpens et al., 2001; Mechtcheriakova et al., 2006). This technique has been refined to allow for larger proteins to be expressed through the use of the 'tandem core' approach, which involves fusing two HBcAg reading frames together, which enables the expression of foreign antigens on both, neither, or only one of the MIRs of the HBc tandem core (Peyret et al., 2015) (**Figure 2**). Opting to express foreign antigens on only one of the two spike regions of the HBc dimer reduces the steric hindrance between foreign antigens, which consequently increases the maximum size of the potential foreign antigens that can be included in the VLP (Peyret et al., 2015).

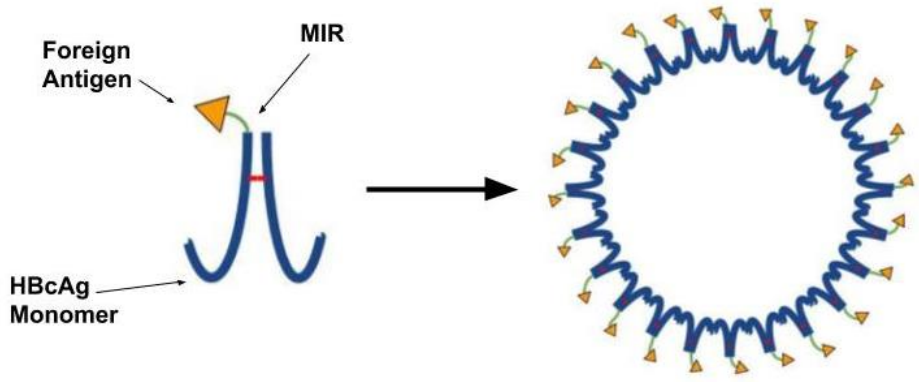

Figure 2: Assembly of HBcAg VLPs using the tandem core approach.

VLPs offer several advantages over more traditional vaccination approaches. To start, they can self-assemble to resemble the structure of their native virus, providing the immune

system with a more authentic target and consequently improving VLPs' immunogenicity (Chackerian, 2014). Further, because they lack genomic information, they are unable to replicate, improving the safety of any VLP delivered as a vaccine. Additionally, owing to their fundamental nature of being solely a recombinant protein, they are able to be produced at much faster rates than live-attenuated and inactivated viruses, as there is no need to use production systems, like eggs, that would support virus replication. This simultaneously lowers the cost and opens the doors to a wide variety of different production methods that can be chosen based on the needs for glycosylation, folding, speed, etc. desired for any given VLP. Furthermore, it has been demonstrated that by co-delivering VLPs with RICs a greater immune response can be obtained than through delivering either alone (Diamos et al., 2019). The immunogenicity of these vaccines can be enhanced through the use of glycoengineered plants to glycosylate the vaccines in favorable patterns (Shields et al., 2002; Maverakis et al., 2015)).

1.8 Production of Biopharmaceuticals in Plants

As described previously (Favre, 2018), the use of plants as a production vector for recombinant vaccines has many advantages. The production of valuable and viable biopharmaceuticals and vaccine antigens in plants is well-documented as being a cost-effective alternative to other means of biopharmaceutical production (Streatfield et al., 2001; Fischer & Emans, 2000; Tiwari et al., 2009; Rybicki, 2010). It has been shown that M2e and M2e-containing vaccines has been able to be expressed effectively in plants (Nemchinov & Natilla, 2007). Further, the use of geminiviral vectors has been demonstrated to significantly increase the yield of proteins expressed in plants systems.

Geminiviral vectors allow for the insertion of desired genes into a self-replicating plant virus vector (Davies & Stanley, 1989; Stanley, 1993), which facilitates the production of vaccine antigens in plants (Chen et al., 2011). Geminiviral replication proteins amplify gene expression through the use of cellular DNA replication machinery in the nucleus, where the DNA uses soluble histones to form a 'viral minichromosome' (separate of the host genome) (Paprotka et al., 2015). This amplification of genes of interest is achieved through the inclusion of geminiviral replicon elements in the expression cassette. Specifically, the inclusion of the genes Rep and

Rep A, as well as geminiviral short and long intergenic regions, *in cis* allows for the genes of interest to be amplified once delivered into the plant (Lazarowitz & Shepherd, 2008; Hefferon, 2014). Delivery of the expression cassette containing both the genes of interest and geminiviral replicon elements is enhanced through the use of the hypervirulent EHA105 strain of *Agrobacterium* tumefaciens, which can be used to transfer an expression cassette flanked by the left and right border sequences of the *A. tumefaciens* Ti plasmid into plants.

Plants are prime candidates for producing recombinant vaccines, as their glycosylation patterns can be modified to improve vaccine efficacy. For instance, some biopharmaceutical production methods inadvertently fucosylate their products, which can be counterintuitive as fucose inhibits binding of various targets by Fc gamma RIII receptors, which decreases the efficacy of antibody-based therapeutics (Shields et al., 2002). However, engineering plants to feature knocked out fucosylation pathways, as well as upregulated GnGn glycosylation (which increases binding to Fcγ RIIIA receptors (Maverakis et al., 2015), can increase the efficacy of plant-expressed biopharmaceuticals. Specifically, GnGn *N. benthamiana* plants have been engineered to produce human N-glycosylation by downregulating the endogenous β1,2-xylosylation (XylT) and α1,3-fucosyltransferase (FucT) genes (Strasser et al., 2008). This is key, as fucosylation inhibits FcγR recognition which reduces the efficacy of immunoglobulin-based treatments (Niwa et al., 2005), and β1,2-xylosylation and core α1,3-fucose are absent from humans entirely, which could provoke unwanted immune responses against non-GnGn plant-produced therapeutics. The lack of α1,6-fucose, which is normally present in humans but not in plants, may actually be beneficial, as the lack of fucose improves antibody-dependent cellular cytotoxicity (Shields et al., 2002), making GnGn plants an ideal production system for plant-produced biopharmaceuticals and a viable production vector for a universal influenza A vaccine.

2. METHODS

2.1 Vector Construction:

A codon-optimized sequence for the expression of a dimeric human consensus M2e protein (SLLTEVETPIRNEWGCRCNDSSD**GGSGG**SLLTEVETPIRNEWGCRCNDSSD) in *N. benthamiana* was designed (Neirynck et al., 1999; Blokhina et al., 2013; Mardanova et al., 2015; Krishnavajhala et al., 2018), and restriction sites for the restriction enzymes BamHI (New England Biolabs) and SpeI (New England Biolabs) and binding sites for the M13F and M13R primers flanking the BamHI and SpeI sites were added to the ends of the sequence to maximize compatibility with existing vectors. M2e monomers were linked by a glycine-serine linker to minimize interference of the linker with the protein and RIC as a whole. Tandem repeats of M2e increases the chance of cross-linking occurring in B cell receptors on B cells and also increases the chances of M2e being degraded and displayed by proteosomal digestion and MHC presentation. This increases the immunogenicity of the vaccine. 250 ng of the sequence was ordered from IDT (Integrated DNA Technologies, Coralville, IA) as a gBlock, which was promptly resuspended by centrifuging the geneblock for 5 seconds at 3,000 xg and resuspending the resulting pellet in TE buffer to a final concentration of 10 ng/µl. The geneblock was then amplified by high-fidelity PCR using M13F and M13R primers and run on a 1% agarose gel, with the resulting band being excised and the DNA contained within being isolated by dissolving the gel fragment in sodium iodide (a chaotropic salt that dissolves agarose) and heating for approximately 10 minutes. The DNA was precipitated out of the solution by mixing the solution with a small amount (7 µl) of silicon dioxide suspension, which binds DNA under high salt conditions. The solution was pelleted, washed with 50% ethanol/50mM NaCl to remove NaI, and the DNA was eluted from the silicon dioxide with sterile water. Following this, the amplified M2e sequence was then digested with BamHI and SpeI. The vector backbone plasmid, pBYR11eMa-h6D8-L2 (Diamos et al., 2019), was digested with SbfI and SpeI for the vector fragment, and separately with SbfI and BamHI for the 2264 bp fragment. The three fragments were ligated using T4 DNA ligase (New England Biolabs) overnight in a 16^{o}C water bath.

The ligated plasmid was then precipitated using ammonium acetate and 2-propanol to increase purity and decrease the volume of the plasmid in solution (the precipitation allowed for a volume reduction from 20 µl to 3 µl, vastly increasing the concentration of the plasmid in solution). Following precipitation, 2 µl of the plasmid was electroporated into competent DH5α *E. coli*, which was allowed to grow in a 2 mL Eppendorf tube containing 500 µl YENB broth for one hour to allow the *E. coli* to recover. This 500 µl of broth was subsequently plated onto an LB+kanamycin plate and allowed to grow overnight in a 37^{o}C incubator. Ten colonies were selected and screened via PCR using the 6D8H-F and Ext3-R primers (Table 1) with the two colonies producing the brightest bands on the agarose gel being selected for plasmid preparation via lysis of the cells (using an EDTA-containing buffer to prevent DNAse activity) and precipitation of the plasmid through mixing with ammonium acetate and ethanol to precipitate the DNA. pBYR11eMa-h6D8M2e was isolated via centrifugation and resuspension in TE buffer. Isolated pBYR11eMa-h6D8M2e from the *E. coli* were Sanger sequenced in the region of the plasmid containing M2e to ensure sequence integrity.

To generate the plasmid encoding the VLP, pBYR2eK2M-HBcheM2e was constructed. pET-28b (Novalgen) and the M2e geneblock were digested with NcoI and XhoI. These fragments were ligated r to generate the plasmid pET28b-M2e. Then, the plasmid pBY037P3-HbcheL2ic (Diamos et al., 2019) and the M2e gBlock were digested with BamHI and SpeI, with the fragments being ligated to form pBY037P3-HbcheM2e, which contained M2e fused to the MIR of an HBc monomer. pBY037P3-HbcheM2e was digested with NcoI and SpeI to obtain the 926 bp fragment. pBYR2eK2M-HbcheZE3 (Pardhe et al. manuscript submitted as “M18-193L_Pardhe-Zika”) was digested with SbfI and SpeI for the vector fragment, and separately with SbfI and NcoI to obtain the 821 bp fragment with promoter and 5’UTR. These fragments were ligated together to form pBYR2eK2M-HbcheM2e, which contained the HBc dimer with M2e inserted into the second HBc monomer’s MIR region.

2.2 Agroinfiltration

After verifying the presence of M2e in pBYR11eMa-h6D8M2e and pBYR2eK2M-HBcheM2e, the plasmids were electroporated into *Agrobacterium tumefaciens* EHA 105 cells,

which were allowed to recover in 500 µl YENB broth for one hour. The cells were then plated on LB+kan plates and incubated at 28^{o}C for two days. Following this, cultures of the transformed *A. tumefaciens* were grown overnight at 28^{o}C on a shaker in YENB, rifampicin (2.775 µg/ml) and kanamycin (50 µg/ml). These cultures were PCR screened after which cultures identified to contain pBYR11eMa-h6D8M2e and pBYR2eK2M-HBcheM2e were spun down and resuspended in 1x infiltration buffer to an OD of 0.260. Three GnGn *N. benthamiana* plants (Strasser et al., 2008) ranging from five to six weeks old were infiltrated (specifically in the leaves) with the *A. tumefaciens* suspensions (Huang and Mason, 2004) and allowed to grow at room temperature for five days. The plants were watered daily.

2.3 Extraction and Purification of Recombinant Influenza A Vaccines

Five days after agroinfiltration, plant leaves were homogenized with an electric blender in ice cold buffer (100 mM tris, 50 mM NaCl, 10 mM EDTA, 2 mM PMSF, 0.1 Triton, pH=8.0). No significant necrosis was observed in any of the infiltrated or uninfiltrated leaves. The blended plant leaves were then stirred for 20 minutes at 4^{o}C, after which the solution was filtered through four-ply miracloth to remove plant fibers. 1 ml of this solution was taken as a sample of 'crude extract' and frozen at -80^{o}C for later analysis. 1M phosphoric acid was then added to lower the pH of the extraction to 4.6 for one minute to precipitate plant proteins like RuBisCo, with 2M Tris Base being added to raise the pH of the sample back up to 7.6. The extraction was then centrifuged at 16,000 xg for 20 minutes at 4^{o}C. The supernatant was isolated and centrifuged for another 30 minutes at 16,000 xg at 4^{o}C. Then, the supernatant was again isolated and centrifuged for a final 10 minutes at 16,000 xg at 4^{o}C to remove as much precipitated plant protein and other insoluble matter in the extraction as possible (as RICs are present in the soluble fraction). The supernatant was then run through vacuum filter sterilizers (pore size=0.45 micron) to remove any remnant bacteria that might have remained after centrifugation.

For the M2e-RIC, a protein G resin column (containing protein G conjugated to agarose beads) (Thermo Fisher Scientific, Waltham, MA, USA) was prepared by running the RIC extraction buffer through the column. The extraction was then run through the prepared column, after which the column was washed again with the RIC extraction buffer again to remove any final

contaminants from the column. The RICs were then eluted from the column using a glycine solution (100 mM glycine, pH=2.5), with five 1.5 ml elutions being taken from the column and pH-neutralized using Tris base (100 µl of 1M Tris pH=8.0 in each elution). 50 µl aliquots from each elution were immediately frozen in a -80^{o}C freezer for later analysis to prevent the larger elutions from degrading due to frequent freezing and thawing. Additionally, small volumes from each elution were used in spectrophotometry to determine the concentration of RICs present in each elution.

For the M2e-VLP, a sucrose gradient and subsequent ultracentrifugation was used to purify the VLPs out of the plant extract as described previously by members of this laboratory (Diamos et al., 2019). Briefly, 6 mL of extract was centrifuged at 148,000 xg for 2.5 hours at 4^{o}C through a 13 ml sucrose cushion gradient composed of layered 25% and 70% sucrose in phosphate-buffered saline (PBS). VLPs, which have a density between the density 25%-and 70% sucrose, was extracted ere extracted out of the cushion and dialyzed against PBS for further purification and to remove residual sucrose. The use of a sucrose cushion allows for more gentle purification of the VLPs and can increase the yield of the purification (Peyret et al., 2015). The purified M2e-VLPs were then analyzed via spectrophotometry and frozen at -80^{o}C.

An Eppendorf BioPhotometer^{tm} RS232c was used to determine the concentration of RICs within each elution. Using the Beer-Lambert Law ($A=\varepsilon*b*c$, where A=absorbance, ε=extinction coefficient, b=length of the path of the light in centimeters, and c=concentration), concentration can of the RIC can be determined by rearranging the equation to solve for ‘c’. The extinction coefficient of human IgG is approximately 1.4 (Eisenberg, 1976), and with the length of the path of the light being 1 cm, A_{280} values can be used to determine concentration.

2.4 Western Blotting and Coomassie Staining

For the RIC, samples of crude extract, the extract post-filtration, the extract post-acid precipitation, the wash buffer, and five elutions, and a protein ladder standard (GOLDBIO BLUEstain) were run on two 10% SDS-PAGE gels (Bio-Rad) simultaneously under non-reducing conditions. Fresh SDS was added to the running buffer to maximize resolution. One gel was

stained using Coomassie Brilliant Blue dye for an hour, after which the gel was destained overnight using deionized water.

The other gel was used to transfer proteins to a PVDF membrane for 20 minutes at 110V. The membrane was blocked in 5% PBSTM (1x phosphate-buffered saline (PBS) containing tween and 5% skim milk) overnight at 4^{o}C, after which the membrane was rinsed in deionized water three times. Then, the membrane was rotated in a 37^{o}C incubator in a 1% PBSTM (1x PBS and tween and 1% skim milk) solution containing mouse anti-6D8 antibody (Wilson et al., 2000) at a 1:2000 dilution to detect the 6D8 epitope tag on the RIC (Phoolcharoen et al., 2011). Following this, the membrane was washed again in deionized water and incubated and rotated at 37^{o}C in a 1% PBSTM solution containing goat anti-mouse antibody conjugated to horseradish peroxidase (Sigma) at a 1:500 dilution for one hour. After this, the membrane was washed in deionized water and exposed to a mixture of developing reagents. The membrane was used to develop photosensitive film at an exposure time of 1 minute in the dark.

Additional SDS-PAGE gels were run under similar conditions and using an anti-M2e antibody, MAb 65 (Kolpe et al., 2018) as the primary antibody for western blotting and mouse-anti-kappa chain antibody as the secondary antibody. MAb 65 was expressed in plants and purified in-house. Both RICs and VLPs were probed for the presence of M2e.

2.5 Electron Microscopy

Purified samples of the M2e VLP were incubated on 75/300 mesh grids coated with formvar and washed twice with deionized water. The VLPs were then negatively stained with 2% aqueous uranyl acetate and analyzed using transmission electron microscopy (TEM). TEM was performed with a Phillips CM-12 microscope, and images were acquired with a Gatan model 791 CCD camera.

2.6 Immunization of Mice

All mice were handled in compliance with ASU IACUC regulations and in accordance with the Animal Welfare Act. Groups of 6 female Balb/c mice, 6-8 weeks old, were immunized subcutaneously with three doses of antigen, each containing an equal mass of 5 µg of M2e presented on either VLPs or a 1:1 ratio of the M2e-RIC and M2e-VLP. Doses were administered

in a1:1 ratio with the alum adjuvant Imject Alum (Thermo Fisher Scientific, Waltham, MA). Doses were administered on day 0, 28, and56, and serum collection was done as described (Santi et al., 2008) by submandibular bleed on days 0, 28, 56, and 86.

2.7 Antibody Quantification

Mouse sera were analyzed via enzyme-linked immunosorbent assay (ELISA). 100 µl of a stock solution of 1 mg/ml synthetic monomeric human consensus M2e peptide (GenScript Biotech Corp., NJ) was diluted into 5.8 ml of 50 mM carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH=9.6) (Ebrahimi et al., 2012) to generate a 17 µg/ml solution of M2e peptide in carbonate buffer. 50 µl of this solution was used to coat each well of 96-well plates (850 ng/well) overnight at 4^{o}C. Following this, plates were allowed to warm to room temperature for 20 minutes, after which the plates were rinsed thrice with PBST and blocked with 100 µl of 5% PBSTM per well at room temperature for 15 minutes. Mouse sera were diluted in 1% PBSTM to dilutions ranging from 1:50 to 1:6250 for dose one, 1:8,000 to 1:1,000,000 for dose two, and 1:40,000 to 1:5,000,000 for dose three. After blocking with 5% PBSTM, 50 µl of diluted mouse sera were added to each well and the plate was incubated overnight at 4^{o}C. The following day, the plates were incubated at 37^{o}C for 20 minutes and then rinsed thrice with PBST. Following this, a mixture of goat anti-mouse IgG2a antibodies, goat anti-mouse IgG1 antibodies, goat anti-mouse kappa chain antibodies, and goat anti-mouse IgG antibodies, all conjugated to HRP (Santa Cruz Biotechnology Inc., TX) in 1% PBSTM was prepared. Each antibody was present in the solution at a 1:5700 dilution. This solution was used to detect the total antibody titers within each sera sample by adding 50 µl of the mixture to each well and subsequently incubating the plates at 37^{o}C for 1 hour. Furthermore, additional plates used to determine the titers of IgG2a and IgG1 within each sample. Plates were rinsed five times with PBST and incubated for 45 minutes with 50 µl of TMB (3,3',5,5'-Tetramethylbenzidine) being added to each well. After this, the TMB-HRP reaction was stopped through the addition of HCl and the absorbance of the plates were read using a Molecular Devices SpectraMax 340PC Microplate Reader at 450 nm. Endpoint titers were calculated using GraphPad Prism (GraphPad Sofware, Inc.) to calculate the geometric mean of the ELISA results to determine geometric mean titers.

2.8 Analysis of Cytokine Production in Mice

Mouse splenocytes were removed and homogenized via mashing in a 70 µm nylon strainer with the plunger of a 3 ml syringe. The strainer and plunger were then washed using 13 ml of RPMI complete (RPMI, 10% heat-inactivated PBS, 1% P/S/G) into a microcentrifuge tube to collect cells. Cells were then centrifuged for 5 mins at 1200 RPM at 4^{o}, with the supernatant being removed thereafter and the cells being resuspended in 2 ml red cell lysis buffer (ACK) and incubated for 2 minutes at room temperature. The cells were then quenched in 8 ml RPMI complete and centrifuged again for 5 minutes at 1200 RPM at 4^{o}C. The cell pellet was then washed twice with 10 ml RPMI complete and subsequently resuspended in 2 ml RPMI.

Splenocytes were then plated in 96-well round bottom plates at a concentration of 10^{6} cells per well. The plate was then centrifuged and the splenocytes were resuspended in 180 µl of assay media (RPMI complete, 1.11 ng/ml human IL-2, 5.5 µl/ml GolgiPlug (BD Biosciences-US)). Splenocytes were then exposed to either 20 µl of 10µg/ml synthetic M2e peptide (GenScript Biotech Corp., NJ), 20 µl of RPMI complete, or 20 µl PMI/ionomycin) and incubated at 37^{o}C for 5 hours. Following incubation, cells were pelleted at 1300 rpm for 3 minutes, the supernatant was removed, and cells were washed with 1x fluorescence-activated cell sorting (FACS) buffer. Cells were stained with anti-CD8 (1:100) and anti-CD4 (1:100) in 100 µl FACS buffer and incubated for 30 minutes at 4^{o}C, after which cells were washed twice with FACS buffer to remove excess unbound stain. The cells were then fixed and permeabilized through resuspension in 100 µl of Fixation/Permeabilization solution (BD Biosciences, USA). Cells were then stained for intracellular cytokines using 50 µl of staining solution; CD4 responses were assayed via staining in a solution containing 1:100 dilutions of anti-IL-4, anti-IL-21, and anti-IFN-γ in Permeabilization/Wash buffer (BD Biosciences, USA). while CD8 responses were assayed via staining with a solution containing 1:100 dilutions of anti-IL-2, anti-TNFα, and anti-IFN-γ in Permeabilization/Wash buffer. Cells were then washed twice with Permeabilization/Wash buffer, resuspended in 200 µl FACS buffer, and analyzed via FACS using an LSR Fortessa (**Fig 9**).

3. RESULTS

3.1 Construction of Recombinant Influenza Vaccines

Two recombinant universal influenza A vaccine candidates were designed and expressed using *Agrobacterium tumefaciens*-mediated transfer of geminiviral vectors into glycoengineered *Nicotiana benthamiana* plants (Strasser et al., 2008). The vaccines, using both the recombinant immune complex (RIC) hepatitis B core antigen (HbcAg) virus-like particles (VLP) as vaccine platforms to boost immunogenicity, presented a consensus sequence of the ectodomain of the matrix 2 protein of human influenza A, M2e (SLLTEVETPIRNEWGCRCNDSSD). The antigen was constructed as a dimer, with a 2xGGS linker linking the two copies of M2e together to minimize steric hindrance and other unwanted interactions between the two copies, codon-optimized for expression in *Nicotiana benthamiana*, and inserted into the C-terminal end of the human IgG 6D8 heavy chain gene encoded in pBYR11eMa-h6D8M2e (**Figure 3**) and into the MIR of the C-terminal copy of the tandem dimer HBcAg encoded in pBYR2eK2M-HBcheM2e (**Figure 4),** both geminiviral vector plasmids containing several elements to enhance transcription and protein expression.

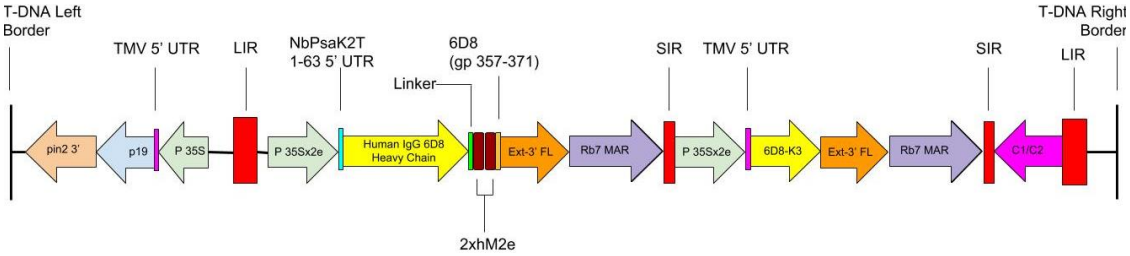

Figure 3: The vector encoding the M2e-RIC, pBYR11eMa-h6D8M2e. Pin2 3' is the 3' end of the Pin2 gene's promoter. The p19 gene encodes the p19 protein of the tomato bushy stunt virus, a suppressor of post-translational gene silencing (Chen et al., 2011). The TMV 5' UTR is a viral translational enhancer that is spurred on by the binding of HSP101, which recruits the translational initiation factors eIF4G and eIF3 (Gaille 2002, Diamos et al., 2016). P35s is a viral promoter sequence. NbPsaK2T (Nb=*Nicotiana benthamiana,* PsaK=photosystem I reaction center subunit, T=truncated) 1-63 5' UTR is used as a leader sequence and is directly upstream of the initiation codon; previous work in this laboratory found that this was the optimal 5' UTR for expressing vaccine antigens in plants in a comparison of 23 5' UTRs (Diamos et al., 2016). The Human IgG 6D8 Heavy Chain gene encodes the heavy chain of the humanized anti-ebola antibody specific for ebola glycoprotein epitope 6D8, while the 6D8-K3 gene encodes the antibody's light chain. This is linked by a glycine-serine linker to dimeric 2x M2e, with each copy of M2e being linked to the other by a glycine-serine linker. 6D8 (gp 357-371) is the ebola glycoprotein epitope 6D8, which serves as the epitope binding tag for this RIC. Ext-3' FL is the extensin gene's 3' flanking region, which was used as a terminator and was previously found through research in this laboratory to enhance protein production relative to other more common terminators (Diamos et al., 2016). The Rb7 MAR (matrix attachment region) increases the likelihood and magnitude of transgene expression in plants (Halweg et al., 2005). C1/C2 encodes the geminiviral bean yellow dwarf virus (BeYDV) Rep/RepA proteins, which require the BeYDV LIR and SIR to function properly, as the LIR contains a bi-directional promoter and stem-loop structure essential for the initiation of rolling-circle replication and the SIR is the origin of C-strand synthesis and supplies termination and polyadenylation signals (Chen et al., 2011). The Rep/RepA proteins facilitate the formation of a transgene-containing 'viral minichromosome' within the plant cells' nuclei and subsequently enhances protein expression (Chen et al., 2011). Finally,

the T-DNA left and right borders are the borders of the expression cassette that is transferred to the cells of the plant by *Agrobacterium tumefaciens*.

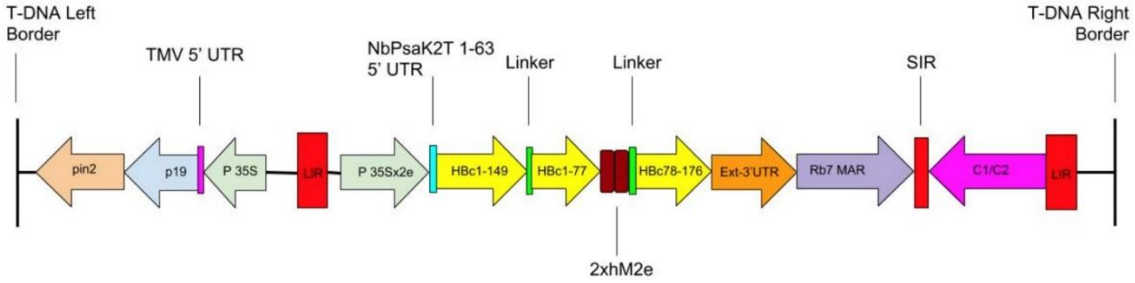

Figure 4: The vector encoding the M2e-VLP, pBYR2eK2M-HBcheM2e. The vector uses many of the same elements as the M2e-RIC vector, pBYR11eMa-h6D8M2e (Fig. 3), but features HBc1-149, HBc1-77, and HBc78-176 genes encoding the heptatis B core antigen assembled in a 'tandem core' fashion, in the place of the human IgG heavy and light chain sequences.

3.2 Production of the M2e-RIC and M2e-VLP in Plants

After the expression and purification of the recombinant vaccines via protein G chromatography for the RIC and sucrose gradient purification and dialysis for the VLP, samples were characterized via SDS-PAGE and subsequent Coomassie Brilliant Blue staining and western blotting (**Figures 5 and 6**). The RIC was probed using anti-6D8 antibody and anti-M2e antibody to while the VLP was probed solely with anti-M2e antibody. Samples were compared to a standard protein ladder, with the RIC being further compared to an IgG standard to elucidate the suspected differences between the heavy chains and the light chains of the RIC and the standard. Samples probed with the anti-M2e probe demonstrated a clear signal, indicating that both the RIC and the VLP contained the M2e antigen. Further, RICs probed with the anti-6D8 epitope tag demonstrated the presence of the epitope tag. Signal above the expected 164 kDa could be interpreted as suggesting the presence of complex formation, though additional studies to characterize the structure of the RIC binding to other RICs would be necessary to determine whether these bands are indicative of complex formation of aggregation driven by other, unexpected factors. Regardless, the results of these characterization studies confirmed the presence of the target antigen, M2e, and other characteristics of the vaccines. Furthermore, M2e-VLPs were analyzed using TEM, with the images generated confirming the structure of VLP (**Figure 7**).

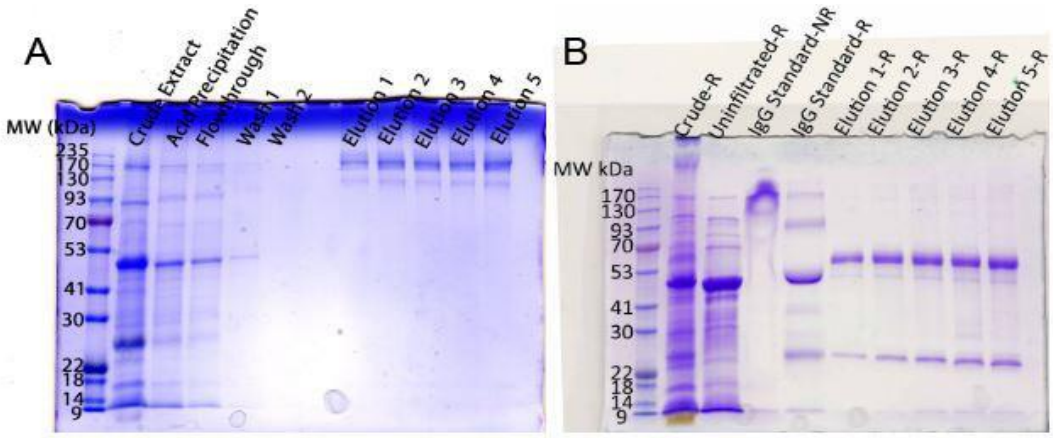

Figure 5: Coomassie staining of RIC plant extract. The approximate size of the M2e RIC is 164 kDa, with the heavy chain-2xM2e-h6D8 fusion being roughly 57 kDa in size and the anti-6D8 light chain being roughly 25 kDa in size. **(A)** Samples were run under non-reducing conditions, with the bands of the size of the RIC being clearly present in the elutions from the protein G resin and the removal of other contaminants post-acid precipitation and post-chromatography being evident. Bands of a larger size than the expected 164 kDa could indicate immune complex formation. **(B)** All samples were run under reducing conditions (indicated by a '**-R**', with the exception of an IgG standard, which was run under both reducing and non-reducing conditions. The heavy chain of the RIC, when reduced, has a greater size than that of the IgG standard's, which is due to the heavy chain of the RIC being fused to the M2e dimer and 6D8 epitope tag, which adds an additional 7 kDa to its size. Additionally, the light chains of the IgG standard and the RIC groups are of the same size, indicating that the 2xM2e and epitope tag were not expressed on the light chain of the RIC. These data suggests the successful expression of M2e-RIC.

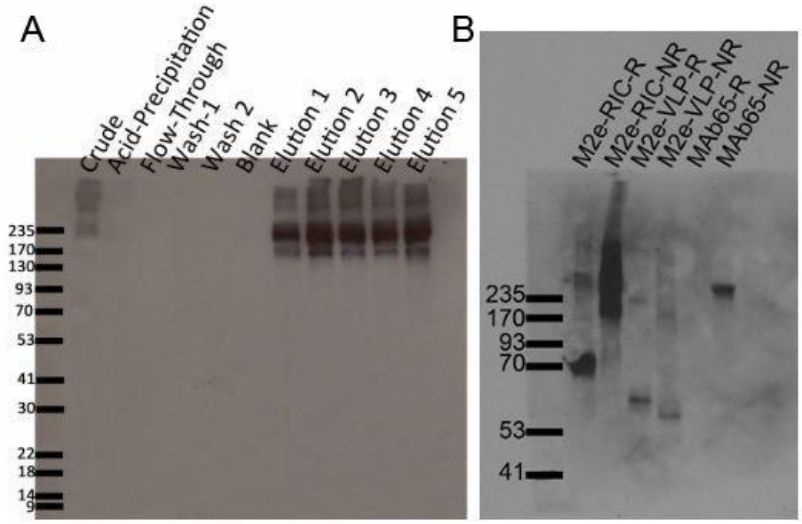

Figure 6: Western blotting of RIC and VLP samples. **(A)** Gel was run concurrently with gel in **Figure XX A** and samples were run under non-reducing conditions. The samples were probed with anti-h6D8 antibody, anad a clear signal is seen in both the crude and the elution fractions, demonstrating the presence of the epitope tag. Bands are present at the expected 164 kDa range, and larger bands may suggest immune complex formation.(**B)** Samples of the M2e-RIC, M2e-VLP, and an anti-M2e antibody, MAb65, were run under both reducing and non-reducing conditions. The samples were probed with MAb65 to confirm the presence of M2e, and the detection antibody was anti-human light chain. A single band is present in the M2e-RIC reducing conditions group that is around the expected size of the M2e-RIC's heavy chain-2xM2e-epitope tag fusion. Because the samples were run under reducing conditions, no band of the heavy chain's size would have appeared if it did not contain M2e, as the anti-human light chain probe would not have bound to the heavy chain on its own. The probe, however, did bind to the light chains of the M2e RIC and MAb65 further down the gel (not pictured). Additionally, bands are present in both samples of the VLP, confirming the presence of M2e in the VLP.

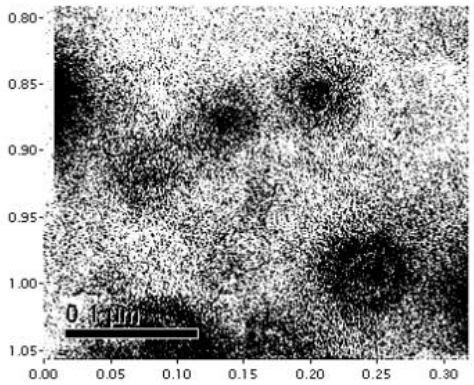

Figure 7: Transmission Electron Microscopy image of M2e-VLP. The formation of VLPs indicated by this image, with the particle in the upper-right quartile being the clearest indication of proper virus-like particle formation.

3.3 Analysis of Mouse Sera and Splenocytes

Two groups of five BALB/c mice were immunized with either the M2e-VLP alone or a combination of the M2e-RIC and the M2e-VLP at days 0, 28, and 56, with bleeds at 0, 28, 56, and 86. Mouse sera was analyzed via ELISA, with total antibody titers being measured after each bleed, and IgG1, and IgG2a titers being measured at the conclusion of day 63. Total antibody titers were consistently 2-3 times higher at all time points in the M2e-RIC/M2e-VLP combination group, though the ratio of IgG2a to IgG1, was lower in the combination group relative to the group that received the M2e-VLP alone (**Figure 8).**

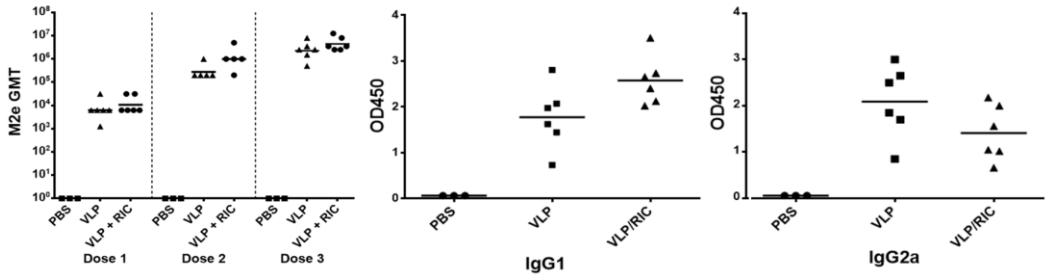

Figure 8: Mouse antibody titers post-vaccination. Total anti-M2e antibody titers were higher, on average, in mice that received both the M2e-RIC and the M2e VLP vaccines than in mice that received only the M2e-VLP vaccine (Dose 1: $1.17*10^4$ vs $6.25*10^3$; Dose 2: $1*10^6$ vs $3.8*10^5$; Dose 3: $4.43*10^6$ vs $2.25*10^6$, respectively. IgG2a to IgG1 ratios, however, were higher in the group receiving only the M2e-VLP vaccine compared to the group receiving both vaccines. Although dilutions were different for IgG1 and IgG2a titer ELISAs, preventing direct comparison, the VLP/RIC group demonstrated IgG2a:IgG1 ratios that were 64% lower relative to those of the VLP-alone group when the ratio of IgG2a to IgG1 is is arbitrarily set at 1.0, as has been done similarly in previous studies (Diamos et al., 2019)..

Splenocyte analysis revealed that mice vaccinated with the M2e-VLP had higher levels of IFN-γ and IL-4 positive splenocytes when stimulated with synthetic M2e peptide than mice vaccinated with both vaccines. Interestingly, mice receiving the combination vaccine had, on average, lower proportions of IFN-γ and IL-4 positive splenocytes post-stimulation than both pre-stimulation cells and cells from unvaccinated mice (**Figure 9**).

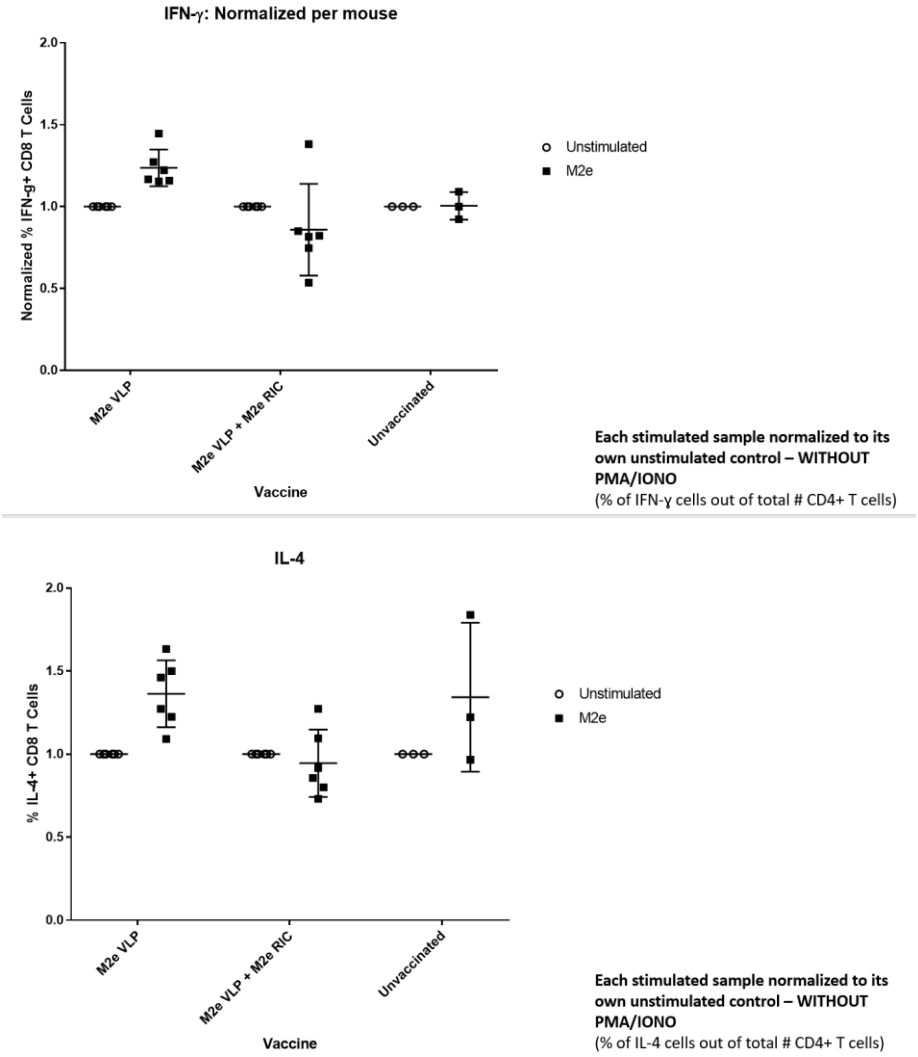

Figure 9: Analysis of cytokine production in mouse splenocytes. Splenocyte analysis revealed that the combination VLP/RIC vaccine induced lower levels of both IL-4 and IFN-γ when compared to both unvaccinated and the VLP alone group.

4. DISCUSSION

In this thesis, two universal influenza A vaccine candidates were developed and expressed in plants. A degree of interaction was noted between the two, as mice that were vaccinated with both the M2e-VLP and M2e-RIC exhibited 2-3 fold higher total anti-M2e antibody titers after each dose. These results are support the findings of research conducted previously by members of this laboratory wherein it was found that RICs and VLPs containing the HPV antigen L2 induced higher responses when the vaccine candidates were co-delivered (Diamos et al., 2019). Given the immense differences in size and other properties between M2e and L2, combining RICs and VLPs may potentiate their efficacy beyond the potential of either vaccine individually when used to vaccinate against many diseases. Higher antibody titers after each combinatorial dose could result in fewer total doses needed to achieve a protective response, which could improve vaccination rates due to a lesser reliance on patient compliance to return for booster doses. Further, fewer doses of a given vaccine could lessen the economic cost of vaccinating the population, as fewer total vaccines will be needed to achieve a sufficient level of protection. The production of the vaccines in plants further compounds the reduction of the economic cost of the vaccine. Most promising, however, is that previous studies have shown that M2e-HBcAg VLPs inducing anti-M2e antibody titers of only 10^4 provided substantial protection against lethal doses of influenza (Neirynck et al., 1999), indicating that the combinatorial vaccine candidate could be protective after only one dose. In the case of influenza, a potent universal influenza vaccine requires only one dose to confer protection could have serious implications in staving off potential epidemics and pandemics before they begin while simultaneously providing a bulwark against ongoing epidemics. Even if the vaccines developed in this thesis are unable to fully protect recipients after a single dose, the M2e-RIC/M2e-VLP combinatorial vaccine could be used to adjuvant existing flu vaccines to increase their efficacy from season to season. However, there are some key hurdles that must be overcome before the M2e-RIC/M2e-VLP vaccine can be seriously evaluated as a candidate in clinical trials.

Both IgG1 and IgG2a play important roles in viral immunity against influenza; in one study, mice with high expression of IgG1 had lower lung viral titers and high influenza virus

neutralization, but lower survival rate when challenged with significantly high doses of influenza virus (Huber et al., 2006). Mice that had both isotypes fared the best, though it was noted that mice that had low IgG1 expression and high IgG2a had the same survival rates as those that had equivalent expression of both IgG1 and IgG2a (Huber et al., 2006). This could be due to IgG2a antibodies' propensity to stimulate complement activation much more effectively than IgG1 in mice (Neuberger & Rajewsky, 1981). The data presented in this thesis suggest that, if mice were challenged with high doses of influenza, those that received the VLP only would have better outcomes than those receiving both groups. Substantially higher IgG2a responses in mice would be desirable if the combinatorial vaccine were to be evaluated as a candidate in clinical trials.

Additionally, as T_H1 cytokines, like IFN-γ, induce isotype switching to IgG2a and T_H2 cytokines, like IL-4, a cytokine associated with deleterious effects on viral clearance due to its mediation of down-regulation of antiviral cytokine expression (Sharma et al., 1996), induce isotype switching to IgG1 (Mossman & Coffman, 1989), the fact that IL-4 production increased to a level higher than that of IFN-γ after stimulation with M2e peptide suggests that the mice receiving the VLP alone may have been undergoing an isotype switch to a T_H2-biased phenotype. This is not desirable, as T_H1 responses are the predominant antiviral response, and a shift to a T_H2 response may not produce as strong of an antiviral response. However, it should be noted that data from the splenocyte assay is not consistent with the results of previous studies; M2e-VLPs have been documented to induce robust T_H1-biased responses and have been associated with high levels of IFN-γ production when challenged with influenza virus post-vaccination (Kim et al., 2018). Further analyses are necessary to confirm the patterns of cytokine expression for the vaccines in this thesis.

It is important to note that the majority of the most promising universal influenza vaccines target more than one site on the influenza virion, with many recent vaccines pursuing some combination of some or all of the following HA, M1, NP, and M2e (Atsmon et al., 2012; Ellebedy et al., 2014; Ingle et al., 2017). Targeting multiple conserved regions of the influenza virion would aid in preventing influenza from escaping via a single mutation in one of its proteins; the fewer avenues of escape the better, especially when any of the virus' proteins could mutate. Indeed,

despite its high conservation, even M2e has the potential to mutate, with the M2e of avian and swine influenza A featuring mutations at different points in the protein (Liu et al., 2005; Zhou et al., 2012). While consensus sequences are useful to a point, including multiple conserved, immunogenic antigens in the design of universal influenza vaccines would likely make the vaccine more effective for a longer period of time. Because of the flexibility of both the VLP and the RIC platforms, several antigens could be packaged into each platform while reaping the benefits of the interactions between the platforms. This makes the combinatorial vaccine a potent tool in the fight against infectious diseases, from influenza to HPV and beyond.

FUTURE DIRECTIONS

To further improve upon the universal influenza vaccines presented in this thesis, other influenza targets should be added to the RIC and VLP platforms. The stalk region of HA2-2, from residue 76 to 130, has shown promise as a vaccine antigen (Stepanova et al., 2018), as have several regions of NP, M1 protein (Atsmon et al., 2012), and NA (Eichelberger et al., 2018). However, most importantly, influenza B antigens should be added to future recombinant universal influenza vaccines to make the vaccines truly 'universal' against the two major epidemic types of influenza. The vast majority of universal influenza vaccines in development focus exclusively on influenza A, despite influenza B also posing a serious threat to public health (Mosnier et al., 2017; Caini et al., 2017). With schools having been closed in Hong Kong to mitigate the impact of a deadly influenza B epidemic (Ali et al., 2018) and consecutive influenza A and influenza B infections substantially worsening influenza epidemics (Möst & Weiss, 2016), the need for an effective universal influenza B vaccine has never been clearer. Conserved protective influenza B epitopes within the influenza B HA protein have been identified (Bianchi et al., 2005; Dreyfus et al., 2012), and some recent studies have investigated the use of influenza B NP in vaccine candidates (Lee et al., 2019), though not many vaccines have been developed focusing on influenza B. Because of the ease with which new antigens can be added to the VLP and RIC platforms, future studies investigating the combinatorial vaccine approach should include influenza B antigens as a part of the vaccine to mitigate the risk that influenza B poses. Further, while influenza D does not currently represent a threat to human health, and influenza C does not generally cause disease in humans, attention should be given to developing prototype vaccines against these diseases as influenza's mutations and changes can be difficult to predict (Smith et al., 2016; Su et al., 2017).

Table 1. Summary of Nucleotide Sequences Used	
M2e gBlock	**5'-** GTAAAACGACGGCCAGTGGATCCTCT TTGCTTACCGAGGTTGAGACCCCTATT AGAAACGAGTGGGGTTGCAGATGTAA CGATTCTTCCGACGGaGGtTCTGGaggt TCCCTTTTGACTGAAGTgGAGACTCCA ATcAGgAACGAATGGGGATGcAGATGC AACGACTCCTCTGACGGAGGTGGAact agtCATGGTCATAGCTGTTTCC-**3'**
M2e-Nco-F Primer	**5'-**tagccatgGGATCCTCTTTGCTTACCG- **3'**
M2e-Xho-R Primer:	**5'-**tcgctcgagactagtTCCACCTCCGTC-**3'**
6D8H-F Primer:	**5'**-TGAGGCTCTTCACAATCA-**3'**
Ext3-R Primer:	**5'**-CTTCTTCTTCTTCTTTTCTCATTGTC- **3'**
Ext3i-R Primer:	**5'**-CAATTTGCTTTGCATTCTTGAC-**3'**
M13-F Primer:	**5'-**GTAAAACGACGGCCAGT-**3'**
M13-R Primer:	**5'**-GGAAACAGCTATGACCATG-**3'**

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