BINDING SPECIFICITY OF M2E-SPECIFIC MONOCLONAL ANTIBODIES TO DIVERSE INFLUENZA A VIRUSES

By

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(Under the Direction of Stephen M. Tompkins)

ABSTRACT

Influenza A virus (IAV) causes contagious respiratory illness and significant morbidity and mortality annually. Disease incidence is high because vaccination nor infection elicit protective immunity due to antigenic drift or shift of surface proteins hemagglutinin and neuraminidase. Also found on the surface of IAV is the matrix protein 2 (M2) proton channel, which has a highly conserved, 23- amino acid ectodomain (M2e). A panel of M2e-specific murine monoclonal antibodies (mAbs) were generated and assayed for breadth of reactivity and efficacy in a murine model with species specific IAVs having distinct M2e sequences. We show mAbs binding to all viruses tested but with distinct binding patterns. Interestingly, relative affinity for a given virus did not directly correlate with efficacy *in vivo*, suggesting a complex interaction of protection. Identification of precise epitopes for each mAb will inform use of therapeutic Mab cocktails as well as potential for universal protection with diverse IAVs.

INDEX WORDS: Influenza A virus; IAV, matrix protein 2; M2, M2e, mAbs

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CHAPTER 1

INTRODUCTION

Influenza continues to be a top health concern worldwide despite the development of a vaccine in the 1940s (1). This is due to the high mutation rate of the immune-dominant surface proteins Hemagglutinin (HA) and Neuraminidase (NA) (2). This process, also known as antigenic drift, is the reason we are subject to seasonal vaccinations (3, 4). Influenza A virus (IAV) can undergo another process known as antigenic shift, or a rapid change of genetic material that occurs when two different serotypes of IAV coinfect the same cell and exchange RNA segments (3). Antigenic shift of IAV has caused pandemics dating back to the middle ages and can cause the seasonal vaccine to be completely ineffective (1). Both antigenic drift and antigenic shift in HA and NA reduce the efficacy of seasonal influenza vaccines. This necessitates not only the frequent updating of the vaccine to current circulating strains, but also a correct prediction of seasonal epidemic strains due to production of vaccines ahead of exposure. This leaves the human population in need of an effective universal prophylactic or therapeutic to contain seasonal epidemics and potential pandemics. While therapeutics have been developed and FDA approved as recently as October 2018, there is not one treatment to which viral escape mutants have not been isolated (5, 6). It would therefore be advantageous to expand therapeutic protection to less variable viral targets. A potential viral target that would achieve this is the M2 ectodomain (M2e) and induction of M2e-specific antibodies (Abs) (7-11).

M2 is a 97-amino-acid transmembrane protein found on the surface of IAV (12-14). The M2 protein contains three distinct regions: a 24 amino acid N terminal ectodomain, a 19 amino acid transmembrane region, and a 57 amino acid C terminal cytoplasmic tail (14-16). After a splicing event, the mature protein of M2 forms homotetramers (17-19) that have pH-inducible proton channel activity to aid in cell entry and IAV maturation (18, 19). The sequence of the 23-amino-acid N terminus ectodomain of M2 has remained highly conserved among IAV strains isolated since 1918 (15, 20). M2e remains highly conserved due in part to its genetic relation to M1, which is the most conserved protein of IAV (21). M2 is encoded by a spliced RNA of the 7^{th} viral gene segment that also codes for M1 (12, 13). The splicing removes most of the nucleotides that encode M1 except for 26 nucleotides which essentially encode the entire M2e (12, 13). The 26 nucleotides encoding M2e are bicistronic, or in the same reading frame as M1. Therefore, this genetic relation of M2e to M1 can be expected to lower the degree of variability in M2e.

The low degree of change in the M2e sequence could also be explained to the absence of M2e-specific Abs mounted in response to IAV infection and therefore there is no pressure for the virus to change (11, 22). While M2e is 99% conserved across IAV strains and has a low mutation rate (22, 23), the M2e-specific immune response is currently lacking, where less than 20% of infected individuals produce M2e antibodies in response to IAV infection (24). This perhaps is due to M2e's small size and low frequency on the membrane of mature virus particles (25, 26). Therefore, it would be advantageous to develop a universal prophylactic or therapeutic agent to prevent or treat IAV by

incorporating M2e-specific monoclonal antibodies (mAbs) to generate a protective immune response that is currently lacking in humans.

mAbs are laboratory-produced proteins that act as substitute antibodies to restore, enhance, or mimic the natural human immune system through various antigen dependent mechanisms of action. Generally, mAbs are produced through a well-established protocol known as hybridoma technology developed in 1975 (27). This protocol has followed the same structure for over 40 years in which a specific antigen is injected into a mammal, harvest the antigen-specific plasma cells from the spleen, and fuse these antigen-specific plasma cells with a cancerous immune cell known as a myeloma cell. This fusion with myeloma cells results in hybrid myeloma or hybridoma cells that are long-lived and produce clonal, antigen-specific antibodies (27). M2e-specific mAbs have been developed and studied since the early 1990's and have been shown to be protective against IAV through multiple mechanisms of action including mAb dependent cytotoxicity, phagocytosis, and complement activation (28, 29). Of these studies, two M2e-specific mAbs, 14C2 and human TCN-032, have gone through extensive experimentation on their protective potential against recognized strains of IAV (24, 29-31).

Extensive research has been conducted to determine the protectiveness of M2e as a vaccine platform given its poor immunogenicity. Previous studies in various animal models have overcome this poor immunogenicity by attaching M2e to larger carrier molecules. Some of these carrier molecules include bacteria (7, 32), flagellin (33), liposomes (34), and hepatitis B virus core (7, 10, 35). While this does produce varying protection and increased survival against lethal IAV challenge in various animal models (9, 10, 35-40), it does not determine whether these vaccine candidates protect humans. In

addition, only half of children and adults get vaccinated against influenza each year leaving a universal vaccine to IAV using M2e less likely due to herd immunity not being established. To combat these problems, researchers have moved to generating high affinity mAbs to M2e to use as a potential prophylactic.

Our collaborator, Dr. Silke Paust, generated a panel of murine M2e-specific mAbs representing multiple murine IgG subclasses (**Table 1.1**) from B cells isolated from the spleen of an AuNP-M2e+CpG vaccinated BALB/c mouse. Seven clones (391, 472, 522, 602, 770, 934, 1191) were identified to bind to M2-consensus peptide (41, 42). These 7 M2e Mabs produced by the AuNP-M2e+CpG vaccine were then tested for binding and protection against IAV infection. At UGA, I specifically focused on the potential of these 7 mAbs to bind to cells infected with 8 disparate IAVs (H1N1 A/PR/8/1934, pH1N1 A/CA/04/2009, H1N1 A/FM/1/1947, H1N1 A/sw/NE/A01444614/2013, H3N2 A/sw/TX/A01049914/2011, H1N2 A/sw/MO/A01444664/2013, H5N1 A/Vietnam/1203/2004, and H7N9 A/Anhui/1/2013) by ELISA. These viruses were selected for having diverse M2e sequences, as well as representing zoonotic and pandemic threats (**Table 1.2**). We saw strong binding of 391, 472, 522, 602, and 1191 to all IAV infected cells which suggests these antibodies bind to conserved epitopes of the N terminal M2e. Mabs 770 and 934 show comparatively lower binding to all infected cells (**Figure 1.1**). An NP-specific mAb was used as a control in ELISAs to demonstrate cells were intact and that our M2e-specific mAbs were binding epitopes on the surface (N terminal M2e). While M2 is more highly expressed on infected cells than influenza virions (43, 44), some M2e-specific antibodies have been shown to bind to influenza virions and induce protection by blocking ion channel activity (44-46). Therefore, we tested to see if our

antibodies were capable of binding to influenza virions from the eight different influenza strains tested in infected cell ELISAs. Again, 391, 472, 522, 601, and 1191 bound to all influenza virions at a high level. 770 showed moderate binding to all strains with the exceptions of pH1N1 A/CA/07/2009 and H1N1 A/sw/NE/A01444614/2013. It was observed that weak binding still occurred in virion ELISAs for 934 (**Figure 1.2**). In conclusion, we found that all 7 M2e-specific mAbs were able to bind both infected cells and virions to varying degrees. The variety of M2e sequences tested confirms that these antibodies bind to highly conserved epitopes and have potential to be candidates to provide universal protection against IAV. In comparison, 14C2 is not capable of binding M2e on the virion itself but does show recognition of M2e on the surface of influenza-infected cells and M2 expressing cell lines (30).

While these 7 M2e-specific mAbs were able to bind both infected cells and virions, we wanted to test the protection provided to determine the universality of that protection by challenging mice with either pH1N1 A/CA/07/09, H5N1 A/Vietnam/1203/2004, or H7N9 A/Anhui/1/2013. pH1N1 A/CA/07/09 was chosen as it represents the pandemic "swine flu" strain from 2009 and is comparable to the currently circulating H1N1 strain. Both H5N1 A/Vietnam/1203/2004 and H7N9 A/Anhui/1/2013 are avian IAVs with high pandemic potential and have infected 100's of individuals over the past two decades. The mice were prophylactically treated with a low dose (25µg) or a high dose (100µg) of our 6 M2e-specific mAbs and then challenged with a $10XLD_{50}$ dose of each virus. All mAbs, except for 770, were about 40-50% protective against pH1N1 at the low dose of 25 µg and ≥80% protective at a high dose of 100µg. 770 was the least protective antibody when challenged with pH1N1, reflecting the weak binding we observed against infected cells

and virions (**Figure 1.1B and 1.2B**). Almost all mice in both low and high dose groups succumbed to disease before the end of the study (**Figure 1.3A-B**). For mice challenged with highly virulent H5N1, we found that in both the low and high dose groups all antibodies were at least partially protective (**Figure 1.3C-D**). 391, 472, 522, 602, and 934 were each partially protective at one or both doses, with 100 μg of 934 protecting 90% of mice. For H7N9, all antibodies tested were highly protective at both low and high doses (**Figure 1.3E-F**). Comparing the *in vivo* protection studies to the ELISA binding pattern assays, we can see that in some cases, relative specificity and affinity of a mAb for a given virus did not directly correlate with efficacy *in vivo*, suggesting a more complex interaction mediating protection. Identification of epitopes of each M2e-specific mAb will inform use of therapeutic mAb cocktails as well as potential for universal protection from infection with diverse IAVs.

My thesis will specifically focus on the binding specificity and relative epitope mapping of the mAbs towards 8 disparate IAV strains to further understand and develop a protective mAb treatment for the prevention of IAV infection. As seen in Table 1.2, M2e amino acids 1-10 are universally conserved among all IAV strains regardless of host species, whereas the rest of the M2e sequence has greater diversity across hosts. It is hypothesized that the 7 M2e mAbs produced from a vaccine that appears to have universal protection in mice, will potentially bind universal epitopes, suggesting similar binding patterns and relative affinities.

Aim 1: To assess binding patterns and relative epitopes of each mAb *in vitro* by competition ELISA against 8 disparate IAV strains.

Table 1.1. Generated murine M2e-specific mAbs and their represented murine IgG subclass.

Clone Name	**Isotype**
391	**IgG1**
472	**IgG2a**
522	**IgG1**
602	**IgG2a**
770	**IgG2a**
934	**IgG3**
1191	**IgG2b**

Table 1.2. N terminal M2e sequences of human, avian, and swine IAV strains compared to the consensus sequence.

Virus	Subtype	Amino Acid Sequence																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Consensus sequence		M	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D
A/PR/8/1934	H1N1	M	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	G	S	S	D
A/CA/07/2009	pH1N1	M	S	L	L	T	E	V	E	T	P	T	R	S	E	W	E	C	R	C	S	D	S	S	D
A/FM/1/1947 (WT & MA)	H1N1	M	S	L	L	T	E	V	E	T	P	T	K	N	E	W	E	C	R	C	S	D	S	S	D
A/Vietnam/1203 /2004	H5N1	M	S	L	L	T	E	V	E	T	P	T	R	N	E	W	E	C	R	C	S	D	S	S	D
A/Anhui/1/2013	H7N9	M	S	L	L	T	E	V	E	T	P	T	R	T	G	W	E	C	N	C	S	G	S	S	E
A/sw/NE/A01444614/2013	H1N1	M	S	L	L	T	E	V	E	T	P	T	R	N	G	W	E	C	K	C	N	D	S	S	D
A/sw/TX/A01049914/2011	H3N2	M	S	L	L	T	E	V	E	T	P	T	R	S	E	W	E	C	R	C	S	D	S	S	D
A/sw/MO/A01444664/2013	H1N2	M	S	L	L	T	E	V	E	T	P	T	R	N	G	W	E	C	K	C	N	D	S	S	D

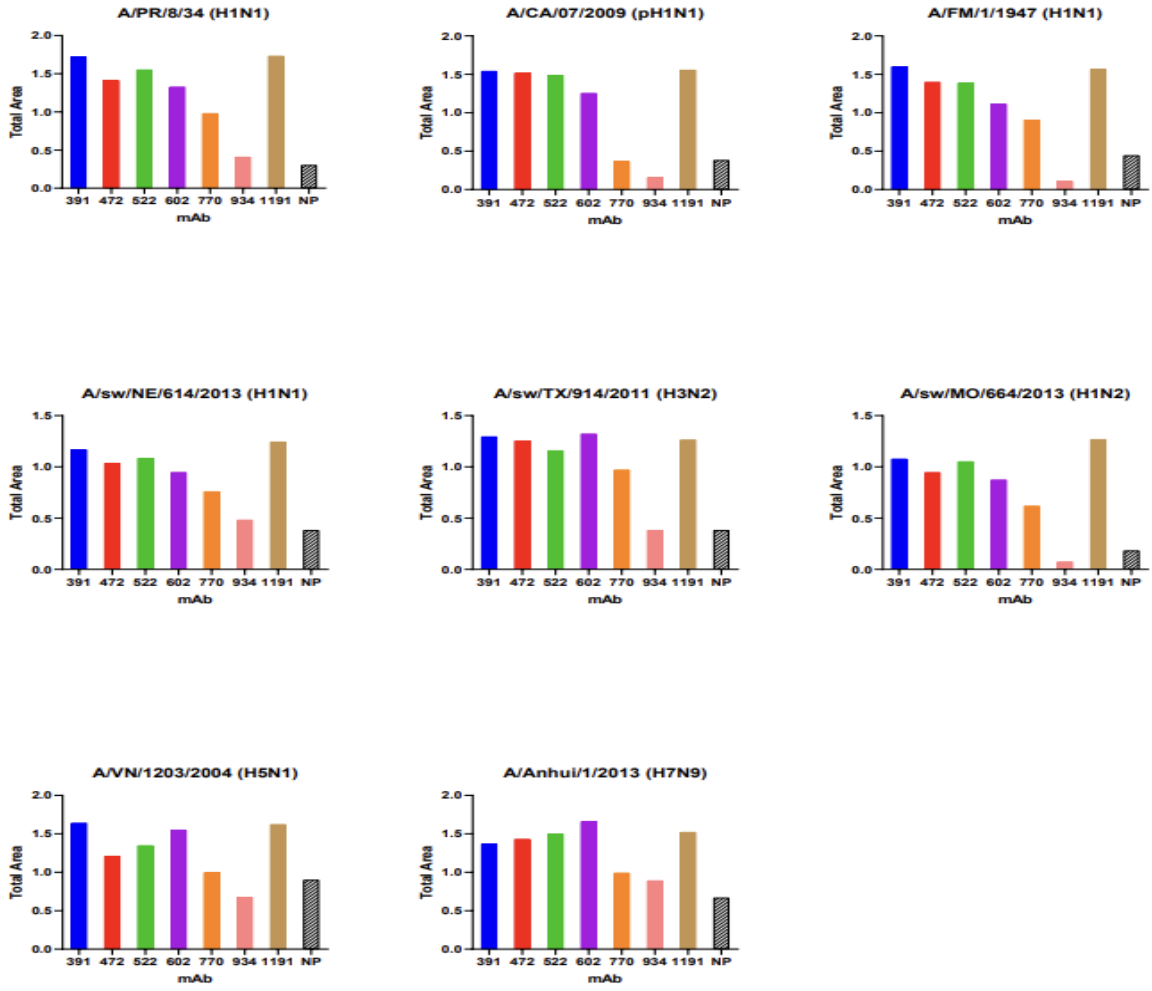

Figure 1.1. M2e-mAbs bind to influenza A infected cells. (A-H) MDCK cells, infected with the specified virus at a MOI = 0.1 for 12 hours, were used as the coating antigen for ELISAs, and the indicated mAb clone tested for reactivity. Nuclear Protein (NP) and a NP-specific MAb were used as a positive control. Background staining of uninfected MDCK cells was subtracted. Area Under Curve analysis performed using GraphPad Prism 7 and presented as total relative binding response of each mAb clone.

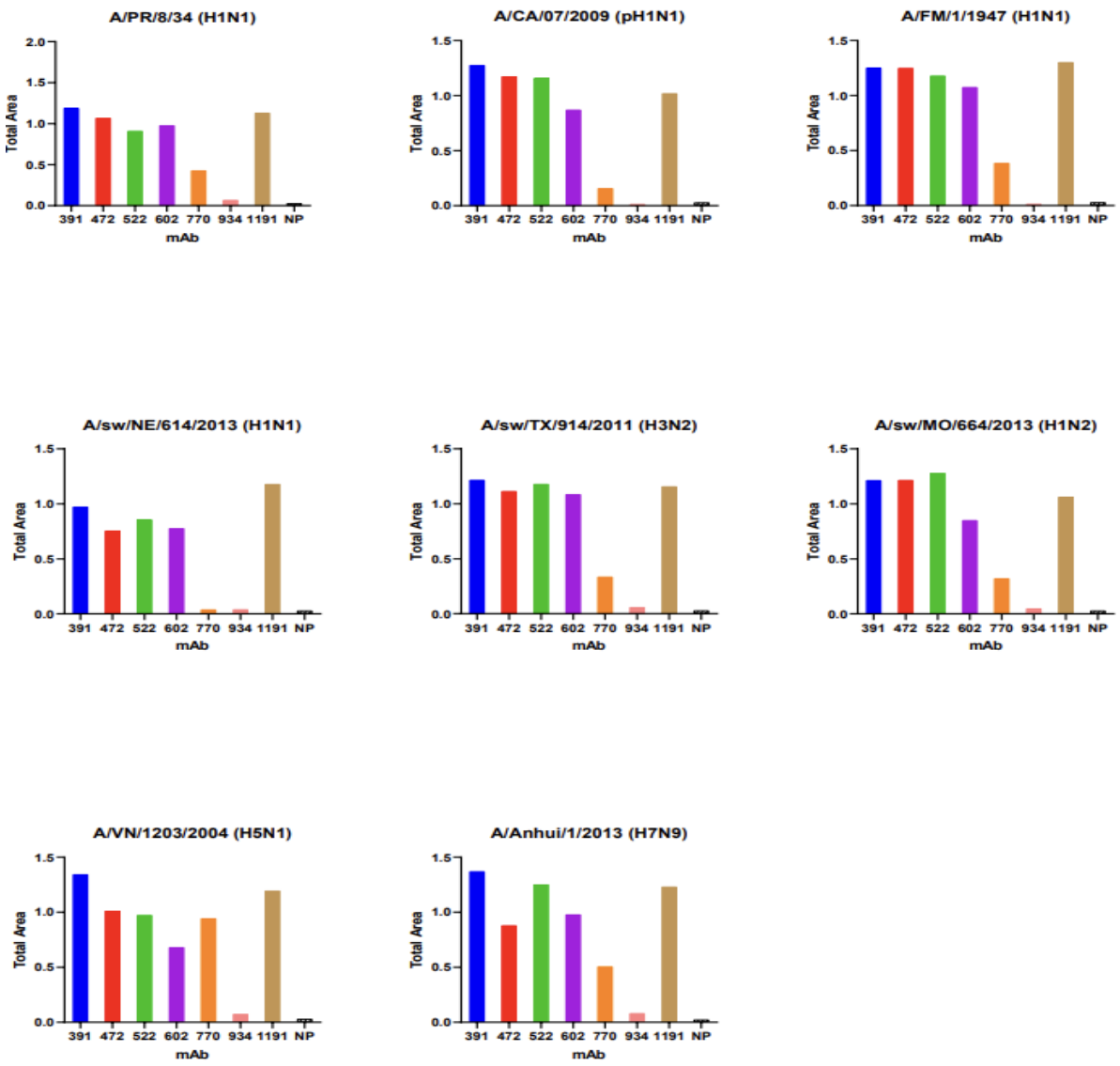

Figure 1.2. M2e-mAbs bind to influenza virions. (A-H) Purified influenza A virions were used as the coating antigen for ELISAs, and the indicated MAb clone tested for virion reactivity. Background was subtracted. Area Under Curve analysis performed using GraphPad Prism 7 and presented as total relative binding response of each mAb clone.

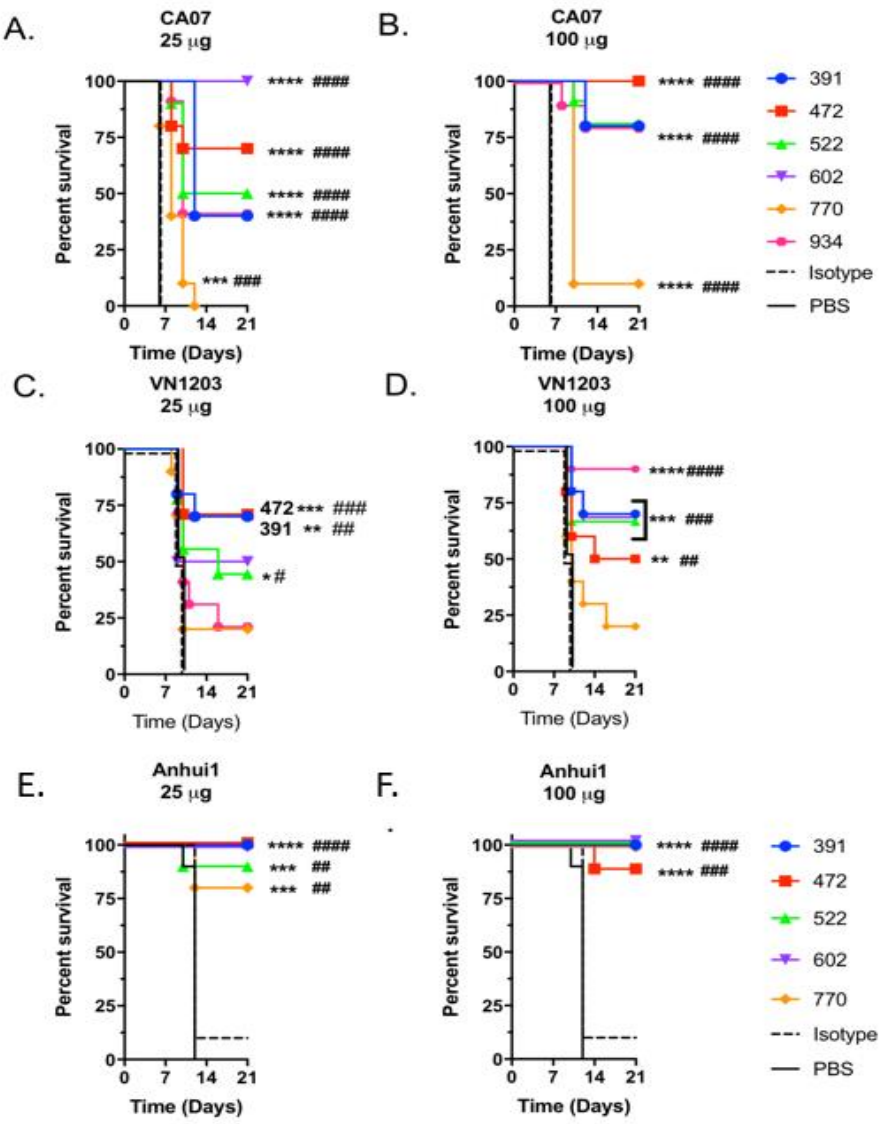

Figure 1.3. M2e-mAbs protect from lethal influenza A infection. (A-F) BALB/c mice were passively immunized with indicated dose of M2e-MAb clone one day prior to infection with (A-B) pH1N1 A/CA/04/2009, (C-D) H5N1 A/Vietnam/1203/2004, or (E-F) H7N9 A/Anhui/1/2013. Experiments had an endpoint based on a comprehensive point system evaluating symptoms and disease severity. (A-F) n=10. ** $p<0.005$, * $p<0.05$, log-rank analysis. * indicates significance compared to PBS control, # indicates significance compared to isotype control.

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CHAPTER 2

LITERATURE REVIEW

INFLUENZA A VIRUS OVERVIEW

Influenza viruses are characterized by segmented, negative strand RNA genomes and belong to the *Orthomyxoviridae* family of viruses. This family of viruses contains seven genera, and four of these genera contain influenza viruses (1). Influenza species in the *Alphainfluenzavirus* can infect a broad range of hosts including humans, swine, horses, a wide array of avian species, and in some instances-marine mammals(2-4). *Betainfluenzavirus* typically only infect humans but have been found to infect other mammals with limited disease pathology(5) (6). *Gammainfluenzavirus* are capable of infecting humans, swine, and dogs but typically cause limited disease pathology as well (7). *Deltainfluenzavirus* were recently discovered in 2016 and categorized as a new genus of *Orthomyxoviridae*. These species of viruses infect swine and cattle, with no human infections being observed(8-10). Species within the *Alphainfluenzavirus* and *Betainfluenzavirus* genera pose the greatest threat to the human population, causing seasonal epidemics that facilitate the need to vaccinate annually (6). This review will focus primarily on species within the *Alphainfluenzavirus* genera. The genome of influenza A viruses (IAV) is composed of 8 gene segments that code for 10 proteins: a multiunit polymerase complex with two basic proteins (PB1, PB2) and an acidic protein known as PA, hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein 1 and 2 (M1, M2), and two non-structural proteins (NS1, NS2). IAVs are divided into subtypes based on two proteins found on the surface of the virion: HA and NA (i.e.

H5N1, H3N2). There are currently 18 HA and 11 NA subtypes characterized however, only 3 HA (H1, H2, H3) and 2 NA (N1, N2) subtypes have been shown to circulate in humans.

HA and NA are critical to the life cycle and transmission of IAV. Cleavage of the HA glycoprotein (HA_0), into disulphide-linked HA_1 and HA_2 subunits is critical for IAV infectivity of host cells (11), whereas NA is crucial for virus release from infected cells (12, 13). The IAV HA glycoprotein binds to sialic acid receptors on the surface of the host cell membrane and binding is dependent on the linkage of the sialic acid to the penultimate galactose within carbohydrate side chains (14, 15) - avian influenza viruses bind α2,3-linked sialic acid moieties, whereas mammalian (human) influenza viruses preferentially bind to α2,6-linked sialic acid moieties (16). Upon HA binding to sialic acid receptors, the virion is internalized by receptor-mediated endocytosis, and exposure to the low pH in the endosome results in insertion into the host cell membrane (17-19). The eight viral ribonucleotides (vRNPs) are trafficked to the nucleus, where transcription and replication of the IAV genome occurs (20, 21). Newly synthesized proteins are transported to the surface of the host cell membrane for viral budding (22). Following bud formation and closure, NA cleaves the virus from sialic acid receptors present at budding sites of infected cells. While HA and NA are highly expressed on the surface of IAV and are crucial for viral replication and infectivity, another protein also plays a key part in the replication life cycle of IAV.

Each year in the United States an average of 20% of the population is infected with IAV and this results in an estimated 200-500,000 deaths each year. These rates are typically greater in high risk groups such as the young and elderly, pregnant women, and

the immunocompromised (23). IAV also poses the threat of generating pandemics by a new subtype able to infect and transmit within the human population. This process is known as antigenic shift and results from multiple IAV strains that infect a single host cell and exchange gene segments to create a novel virus. Four influenza pandemics have occurred since the 1900's. The first and worst pandemic was the "Spanish" flu in 1918, which was caused by the introduction of an H1N1 virus to the naïve human population and resulted in over 50 million deaths worldwide. This H1N1 virus continued to circulate within the human population until it was replaced by the emergence of the H2N2 "Asian" influenza virus in 1957. In 1968 the H3N2 "Hong Kong" influenza virus replaced the H2N2 virus and circulated alone until 1977 when the H1N1 "Russian" influenza strain re-entered the human population. H3N2 and H1N1 have co-circulated since (23-26). In early 2009 a novel H1N1 of swine origin emerged and resulted in the first influenza pandemic of the 21st century (27, 28).

INNATE IMMUNE RESPONSE TO INFLUENZA INFECTION

A huge challenge for humans in developing an effective immune response to IAV is the acute nature of the infection. Meta-analyses of human challenge studies with seasonal IAV has shown clinical symptoms peaking on day 2 post-infection and resolving in 10 days, while viral shedding lasts an average of 4 days (29). Because of such a short infection period, the innate immune response is critical for restricting and clearing IAV. This is accomplished through several mechanisms. Within infected cells, the viral RNA is recognized by pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (TLR-3 and TLR-7), retinoic acid-inducible gene 1 (RIG-1), and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR). Activation through any of

these immune pathways typically leads to induction of Type I interferon (IFN)-mediated antiviral responses and secretion of proinflammatory cytokines, which helps to restrict virus replication. Type-I IFNs, which include IFN-α and IFN-β, are the primary cytokines produced in response to IAV infection and result in the transcriptional up regulation of multiple genes which inhibit viral replication as well as up-regulate MHC class-I expression and modulate the T cell responses towards a TH1 bias (30). Another important cytokine of note expressed following IAV infection is interleukin-1β (IL-1β) which functions to mediate local inflammation (30). IL-1β expression has been shown to be dependent on the activation of the innate immune complex, termed the inflammasome, which can be activated through the stimulation of NLRs (31). Of note, researchers show that during IAV infection, the proton channel activity of the M2 protein can stimulate IL-1β production through inflammasome activation mediated through the NOD-like receptor NLRP3 (32). Additionally, alveolar macrophages, natural killer (NK) cells, and dendritic cells (DCs) all play a role in innate immunity to IAV by clearing virus-infected cells through phagocytosis and induction of apoptosis (33-35).

The humoral arm of the innate immune response primarily mediates IAV infection by generation of antibodies that target the immune dominant surface-exposed viral proteins HA and NA. The HA glycoprotein is a lectin (carbohydrate binding protein), and is present on the surface of the virion as a trimer of protomers (36). Each HA protomer is composed of two structural domains – the membrane-proximal stem domain, which upon cleavage by host cell serine proteases, liberates the fusogenic peptide, and the membrane-distal globular head, which contains the receptor binding site (RBS) (37). Antigenic drift in circulating strains of IAV is caused by changes to the

antigenic sites in the globular head. In contrast, the stem-domain region is remarkably conserved, with only 3 amino acid changes noted for both H1 and H3 in the same time period (38). The stem-domain region is also immunogenic and antibodies that target this region have been found in humans after infection and vaccination, although at markedly reduced levels compared to those targeting the globular head (39, 40). Extensive research has shown that HA-specific antibodies are inherently protective and function to neutralize the virus and prevent infection (41). NA-specific antibodies are non-neutralizing in nature and have been shown to provide protection through neuraminidase activity inhibition preventing the release of new virions and slowing progression of infection (42, 43). While these antibodies produced during IAV infection are protective, the high variability in HA and NA proteins means the antibodies are typically strain-specific and this can render antibody protection ineffective through several mechanisms discussed later. Due to this variability in protection, antibodies targeting conserved regions of the virus are appealing because they could perhaps provide protection against diverse strains of influenza.

CURRENT IAV TREATMENT AND PREVENTION

There are two primary control measures for IAV: vaccination and antiviral drug treatments. Antivirals for IAV have been developed and even FDA approved as recently as October 2018 (44, 45). There are three classes of drugs on the market for controlling IAV infection: three NA inhibitors, two M2 channel blockers, and one endonuclease inhibitor (44). Two NA inhibitors for prophylactic and therapeutic treatments are oseltamivir (Tamiflu™) and zanamivir (Relenza™), which function to inhibit neuraminidase activity by inhibiting the ability of new virion release from infected cells

(46-49). Resistance to NA inhibitors has been a growing concern for many years. Prior to the novel swine H1N1 pandemic, nearly 100% of seasonal H1N1 strains were resistant to oseltamivir and 30% zanamivir resistance(47, 48, 50, 51). There have been cases of resistance to NA inhibitors with the novel swine H1N1 (46-48) but this subtype has maintained a high level of susceptibility to NA inhibitors to date. While not in use currently, the FDA approved the emergency-use authorization of peramivir in response to the swine H1N1. Peramivir (Rapivab^{TM}) was an experimental NA inhibitor with limited Phase II/III data at the time of approval, it was only used for patients with severe influenza infection (52). Finally, in clinical trials with the endonuclease inhibitor baloxavir marboxil (Xofluza^{TM}), the virus did develop resistance to in some patients (53).

To date, vaccination is the primary and most efficient method for preventing IAV infection. The development of seasonal influenza vaccines occurs in February each year in the United States for vaccine use in the following season (reviewed in (54)). The seasonal vaccine requires the identification of circulating strains (one H1N1, H3N2, and influenza B virus) through a global surveillance network with strain selection for the United States and the northern hemisphere. There are two primary types of vaccines for influenza: quadrivalent inactivated influenza vaccines (TIV) and live attenuated influenza vaccines (LAIV). Both types contain the same chosen strains for that year and are licensed for use in the United States. The inactivated vaccines come in three major formulations: inactivated whole-virus, “detergent”-split, or subunit vaccines (reviewed in (54)). All three major formulations are expanded in embryonated chicken eggs or cell culture, inactivated using formalin or β-propiolactone, and purified for use. The split-virus vaccine has an additional treatment step with detergent to dissociate the viral lipid

envelope, exposing all viral proteins and subviral elements (55, 56). In subunit vaccines, additional purification steps are added to enrich the HA protein (56-58). Both split-virus and subunit vaccines have high immunogenicity in the vaccinated populations and reduced reactogenicity when compared to the whole-virus vaccine, therefore most contemporary vaccines since the 1970s have been split-virus or subunit formulations (reviewed in (54)). Standard doses for the TIV contains 15 µg of HA per strain (45 µg total) and is administered intramuscularly as a single dose for the human population over the age of 9 years. In children 6 months-8 years old, split-virus and subunit vaccines have been proven to be less immunogenic, requiring two doses 4 weeks apart to achieve the 1:40 titer (59, 60). TIVs provide immunity primarily by inducing antibodies that target the protective epitopes on HA. Some formulations may also induce NA-specific antibodies that do not protect from infection but may modulate the resulting disease. TIV remains the most popular vaccine, primarily because of its widespread availability and relatively low cost.

Antiviral drugs targeting M2, known as amantadine and its derivative rimantadine, have been previously approved for use in controlling IAV infection. These antivirals inhibit the proton channel activity of M2 by two different routes: binding to and blocking the M2 proton channel in the "open" state (61) or by binding to M2 in the "closed" state and preventing conformational change (62). Amantadine functions in binding to the amphiphilic, transmembrane α-helices of the M2 protein. This binding locks the M2 protein in its closed conformation (63). Once M2 is locked in the closed conformation, the proton channel is inhibited. When the proton channel is inhibited, this halts subsequent infection and replication of IAV as the protons are not able to flux into

the membrane, disrupting the capsid formation (64-67). Widespread use of Amantadine presented drawbacks which are evidenced by the rapid emergence of amantadine-resistant mutations within the transmembrane-domain of the M2 protein. These amantadine-resistant mutations are now dominant in circulating H3N2 strains of human IAV. These strains are resistant to amantadine binding but do not inhibit the transmission and replication of IAV by single amino acid substitutions at positions 26, 27, 30, 31, or 34 (68-70). It was reported that in the 2001-2002 IAV season in the United States, about 1.8% of the circulating IAV isolates tested positive for amantadine/rimantadine resistance, 2 years later that frequency had jumped to over 12% (71). According to the Centers for Disease Control and Prevention (CDC), high levels of susceptibility to the adamantanes exist in the 2009 IAV (H1N1) and human IAV (H3N2) circulating worldwide at that time. This led the CDC to halt recommendation of the use of amantadine and rimantadine for the treatment of IAV, although the drugs remain approved in case of a need for use against pandemic IAVs. This illustrates the need for more effective prevention through vaccination.

LAIVs were created to mimic the natural infection that occurs with influenza and in doing so, elicit cellular and humoral immunity while restricting virus replication to the nasal passages. LAIVs were first developed in the 1960s by serial passage of IAV in eggs under suboptimal conditions (72, 73). Serial culture under lower and lower temperatures resulted in temperature sensitive phenotypes of IAV. These viruses were adapted to grow at 25 °C (cold-adapted) to mimic the normal temperature of the nasal passage in humans. These viruses were also adapted not to grow at temperatures higher than 35 °C because this is the temperature of the respiratory tract. These attenuated viruses developed into

"master donor" strains as they were stable, immunogenic, and nontransmissible (72, 73). Master donor strains contribute their internal genes (all genes except the HA and NA genes) either by classic reassortment in eggs or by reverse genetics to generate vaccine strains with the desired HA and NA of the circulating strains for that given year. In contrast to TIV, LAIV is delivered intranasally, induces a longer-lasting antibody titer, and is immunogenic in children 2 to 7 years old (74). However, because of the high risk of vaccines using live viruses, the LAIV is not recommended for immunocompromised individuals or individuals in close contact with these vulnerable populations. Also, LAIV would not be an optimal candidate for use against avian zoonotic strains since these typically do not replicate in the human upper respiratory tract (75, 76). This vaccine platform, despite its drawbacks, continues to develop different strategies for manipulating the viruses internal genes.

Both current vaccine strategies do present certain drawbacks due to reduced vaccine efficacy enhanced by antigenic drift within the circulating viruses. It is thought that two phenomena play a role in antigenic drift. The first is immune pressure primarily through neutralizing antibodies exerted on the virus, resulting in the generation of escape mutants. The second is due to the viral RNA dependent RNA polymerase involved in viral replication which lacks proofreading capabilities often leading to the accumulation of nucleotide errors which can alter the antigenic surface of the virus (77). Because of this, much focus has been put on the development of new vaccine candidates and therapeutics targeting conserved viral antigens which could provide protection against multiple subtypes and would be most effective if potential pandemic viruses occur in the human population. Numerous vaccine platforms within the past couple of years have

been developed to elicit robust protective antibody and T cell responses primarily against highly conserved antigens including the NP and M2 proteins (45, 78-82). While much research into IAV and vaccines is currently focusing on a universal, highly conserved epitope, these candidates are not meant to replace traditional strain-matched HA based vaccines but rather slow the progression of infection from a divergent IAV strain.

M2 PROTEIN: STRUCTURE AND FUNCTION

As stated before, M2 is a 97 amino acid type III transmembrane protein (83). M2 exists as a homotetramer comprised of disulfide linked M2 dimers that are held in their tetrameric form through non-covalent, hydrostatic interactions (84-86). The M2 protein primarily functions in IAV replication through the acidification of the internal viral compartment that results in the dissociation of RNP complexes from M1 (87). The M2 protein contains three distinct regions: a 24 amino acid N terminal ectodomain, a 19 amino acid transmembrane region, and a 57 amino acid C terminal cytoplasmic tail (86, 88, 89). The transmembrane domain of M2 contains a cholesterol-binding region that has been shown to localize surface M2 to the lipid raft region where HA ad NA are densely aggregated (90). However, only some of these M2 molecules actually associate with the lipid rafts and are incorporated into newly budded virions, a majority of M2 does not associate with the lipid rafts and is ubiquitously expressed across the cell surface (91). Studies have shown that surface expressed M2 can play a role in virion formation as well as viral budding (92, 93). The M2 cytoplasmic tail has been shown to aid in viral assembly by coordination with the HA, NA, and M1 proteins to have successful viral packaging (83). M2 also plays an important role in the regulation of pH late in IAV infection. The ubiquitous expression of M2 throughout the trans-Golgi network (TGN)

increases the pH of the TGN to equal that of the host cell's cytosol (62, 64, 94). The neutralization of the TGN's low pH by M2 is vital because it prevents the HA protein from converting to its low pH conformation, which would render HA incapable of infection (64). Therefore, antibody targeting of M2 can disrupt its assistance in regulating HA in the TGN.

M2E: A PRIME TARGET FOR VACCINE DEVELOPMENT

Despite the poor immune response generated against M2e during IAV infection it is possible for M2e to induce antibodies that can influence viral replication. This was first described by the generation and characterization of the M2e-specific monoclonal antibody 14C2 (95). mAb 14C2 was generated in 1988 by injecting the foot pads of BALB/c mice with polyacrylamide-purified M2 from A/WSN/33-infected cell lysates and was shown to be partially protective against recognized IAV strains (96). 14C2 binding is dependent on the less conserved positions 11 and 14 of the extracellular N terminus of M2 and mutations in these positions among human, avian, and swine IAV can alter binding and protection of the mAb (89, 96). Furthermore, 14C2 is not capable of binding M2e on the virion itself but does show recognition of M2e on the surface of influenza-infected cells and M2 expressing cell lines (96). It is possible that the low levels of M2 incorporated on the packaged IAV as well as steric hindrance could prevent 14C2 from directly accessing the amino acids used for binding on free virus (96). 14C2 has been shown to be partially protective against IAV through two distinct mechanisms of action. The mAb is able to inhibit viral replication/cell-to-cell transfer by a non-neutralizing mechanism with recognized IAV strains (89). Furthermore, ablation of viral inhibition was linked to mutations in M2 protein regions where 14C2 does not interact or

bind with in the cytoplasmic tail of M2, as well as mutations in M1 (89). This finding indicated that 14C2 viral inhibition is dependent on disruption of the cytoplasmic tail of M2 that associates with the M1 protein which is necessary for viral assembly and successful budding (89, 96). However, an *in vivo* study suggested that passive protection of mice by 14C2 is not restricted to direct viral inhibition of susceptible strains, indicating multiple protective mechanisms of the M2e-specific mAb (97).

The human mAb TCN-032 preferentially binds a more universal region (proximal region) of the extracellular N terminus of M2 (and M1) found in 99.8% of IAV strains. The proximal leader region of M2e is 100% conserved among avian, swine, and human IAV isolates and is identified as amino acids 1 through 10 (98). While TCN-032 binds a more conserved region than 14C2, 14C2 binds M2e in a linear specific manner at amino acids 8-15 and TCN-032 is conformation dependent. This means that TCN-032 can only bind to full length, tetrameric M2 proteins, and cannot bind M2 peptides of any form like 14C2 (89, 99). Furthermore, TCN-032 showed very limited protection when tested in clinical trials (99, 100). Ramos, Mitcham (100) shows in clinical trials using TCN-032 as a therapeutic treatment that there was an overall 35% reduction in symptoms and a reduction in viral shedding. However, the length of illness and reduction in severe symptoms was not significantly decreased. This is concerning when thinking of the limitations TCN-032 could present in a clinical setting where patients may not present symptoms or only present symptoms 1- or 2-days post infection (100, 101).

M2e-mAbs have been found to induce protection through a variety of non-neutralizing mechanisms including antibody mediated phagocytosis by alveolar macrophages, NK cell mediated antibody dependent cellular cytotoxicity (ADCC), and

complement induction (102-106). In a recent study conducted by Eliasson et al. (107), researchers showed that B cell-deficient mice are very poorly protected by a mucosal M2e-based vaccine even though these mice mount a considerable CD4 T-cell response against M2e. This study involved the use of serum antibodies following vaccination and this does not accurately reflect the mechanisms of protection involved following treatment with M2-specific monoclonal antibodies. In another study that utilizes actual M2-specific monoclonal antibodies they demonstrated both ADCC mechanisms and complement activation were involved in protection (104). In contrast to both of these studies, many researchers believe that protection depends on the epitope of the M2e-specific monoclonal antibody. It has been shown that antibodies recognizing the N-terminus of M2 (AA 2–12) have virus-neutralizing activity against both influenza A and B *in vitro* (108). The authors suggest that this part of M2 may contain one epitope that can induce antibodies with inhibitory activities against both viruses, but there is no additional evidence to confirm that anti-M2e immunity can suppress influenza B virus replication. In 2011, El Bakkouri *et al.* (105) demonstrated the crucial role of Fc gamma receptors (FcγR) in the *in vivo* protection by M2e-specific IgG isotypes. In this study, wild-type and FcR $\gamma^{-/-}$ BALB/c mice were passively immunized with anti-M2e immune serum, followed by lethal challenge with a mouse-adapted influenza virus. Despite showing similar distribution of anti-M2e IgG titers and antibody isotype in both mouse strains, the FcR $\gamma^{-/-}$ mice were significantly less protected than wild-type animals further concluding the non-neutralizing effects of M2e-specific monoclonal antibodies. Further experiments demonstrated that the activating receptor FcγRIII associated with the common γ-chain is required for anti-M2e IgG1 isotype-mediated protection (109).

Similar results were yielded in a study using wild-type mice and FcR $\gamma^{-/-}$ genotype mice where results showed similar levels of antibodies (IgG1 and IgG2a) after immunization with M2e5x virus-like particles (VLP), the vaccine was significantly less protective in mice without an FcR γ-chain (110). In addition, Van den Hoecke *et al.* (111) showed that protection against IAV with different IgG antibody isotypes requires different FcR subtypes. This study suggests that protection with IgG1 requires FcγRIII, while IgG2a requires all three activating FcγRs. Altogether, there are a wide variety of mechanisms by which M2e-monoclonal antibodies could be effective including the blocking of ion channel activity, prevent membrane scission during viral budding, and decrease the expression of M2 on the surface of infected cells (112-115). Licensing of a new M2e-based therapeutic is challenging due to the lack of clearly defined mechanisms of protection by M2e-specific monoclonal antibodies. In addition, the M2e-specific protection is lower compared to HA- neutralizing immunity, therefore the problem of proving non-inferiority over existing influenza vaccines remains an issue. Nevertheless, the conserved M2 ectodomain can be used as a supplement to overcome strain specificity and improve long-term cross-protection of currently approved seasonal influenza vaccines.

ANTIBODY THERAPIES

Antibodies as a treatment for infection is not a novel idea as this type of therapy can be traced back to the 20th century when sera from infected humans who had recovered was used to treat other infected individuals. However, antibodies as a treatment for infection has been revolutionized by the discovery of hybridoma technology in 1975 (116) which led to the rapid generation of monoclonal antibodies (mAbs) for human use.

This protocol has followed the same structure for over 40 years in which you inject a specific antigen of choice into a mammal, harvest the antigen-specific plasma cells from the spleen, and fuse these antigen-specific plasma cells with a cancerous immune cell known as a myeloma cell. This fusion with myeloma cells causes long-lived antigen-specific antibodies (116) to be used in experimentation. However, the early excitement in this new technology was rapidly replaced by disappointment when it became clear that these molecules were facing serious problems when used as therapeutics. The first mAbs were murine molecules and were recognized as foreign objects when injected into patients, leading to elimination by the patient's immune system. Moreover, in order to be effective, antibodies often need to interact with receptors displayed on effector cells or the complement cascade. Because of their murine nature, these early antibodies did not interact properly with components of the human immune system and their biological efficacy was severely restricted. This led to the development of chimeric and humanized mAbs in efforts to limit the immunogenicity of the nonhuman constructs (117, 118). There are several methods existing today to engineer antibodies for human use. One method uses an antigen to antibody libraries from immunoglobulin V_H and V_L variable regions of genes of non-immune, vaccinated, or naturally infected individuals. In this method, antibody libraries are presented to antigens by phages (119, 120), bacteria (121), or yeast (122). In addition, other methods make use of antibodies that are cloned from a single-memory B cell (123, 124) or plasma B cells (125, 126) isolated from vaccinated or naturally infected animals and human donors. More recently, heavy and light chain paired mAbs have been generated by deep sequencing of the B-cell IgG repertoire (127).

A major development in antibody engineering was the possibility to create chimeric antibodies. The binding of IgG antibodies is dependent on the variable regions of the heavy and light chains. As antibodies are often well conserved through evolution, it was now possible to create chimeras through fusing murine variable domains that are responsible for the binding activity, with human constant domains (128) for a new generation of therapeutic candidates (129). These chimeric antibodies are 70% human and possess a fully human Fc portion, which allows them to be less immunogenic in humans and to interact with human effector cells and the complement cascade. To decrease even further the murine portion of mAbs, researchers replaced the hypervariable loops of a fully human antibody with the hypervariable loops of the murine antibody of interest, this approach is called complementarity-determining region grafting (130). These antibodies, termed 'humanized', are 85–90% human and are even less immunogenic than chimeric antibodies. However, complementarity-determining region grafting is more demanding than a mere fusion and often time direct mutagenesis is used to restore the original affinity present in the murine antibody. Most of the approved mAbs in current use as treatments are either chimeric or humanized. Another major improvement in antibody technology came with the development of *in vitro* selection methods, the most successful being phage display. This *in vitro* technique relies on the ability to establish a physical link between a protein fused to a filamentous phage capsid protein (p3 or p8) displayed at the surface of phage M13 and its corresponding gene contained in the encapsulated DNA. If the molecule binds to the antigen of interest, its gene is immediately available, allowing sequencing and further multiplication of the specific clone. Because of these *in vitro* selection methods, it is now possible to rapidly

and efficiently select fully human antibody fragments against virtually any antigen by using ‘universal’, large, non-immunized libraries (131). Phage display has been able to obtain ligands with sub-picomolar affinities for their relevant antigen, outperforming the affinities of most conventional mAbs. During the same decade, a complementary approach was developed to create fully human antibodies within a murine model. Transgenic ‘humanized’ mice were created by replacing the entire mouse IgG repertoire with a human repertoire (132). Upon immunization, these humanized mice produce human IgGs and conventional hybridoma techniques can be used to clone human antibodies with the required properties. Moreover, they directly lead to full-length IgG, which is often the format for therapy. However, humanized mice cannot be used effectively when the immunogen is toxic or when the targeted antigen shares a high degree of homology with its murine ortholog which is a major drawback to using these humanized mice.

The creation of chimeric, humanized, or fully human antibodies was a major breakthrough and led to a wave of FDA-approved antibodies. Many antibodies are commercialized as therapeutics, mainly for cancer and immune disorders. While impressive results have been achieved in cancer and immune disorder therapy, mAb-based treatments face several limitations that limit their widespread use as therapeutics, especially for viral infections. Some drawbacks include high production costs, feasibility of large-scale production, and *in vivo* pharmacokinetics of generated antibodies (133). Monoclonal antibodies are large (~150 kDa) proteins containing numerous disulphide bonds and post-translational modifications necessitating a sophisticated eukaryotic machinery to be produced in their active form. Moreover, most studies have shown that

these molecules have to be injected in large amounts to even achieve clinical efficacy. This results in the production of therapeutic antibodies with very large cultures of mammalian cells followed by extensive purification steps, leading to extremely high production costs and limiting the wide use of these drugs. Additionally, antibody uptake in humans depends on a balance between favorable pharmacokinetics and efficient penetration and retention in the targeted tissues. Various characteristics of mAbs such as molecular size, shape, affinity and valency control these properties. In one study performed in murine xenograft models, mAbs directed against tumor-specific antigens largely remained in the blood and no more than 20% of the administered dose of mAbs actually interacted with the tumor (134). This represents probably one of the major limitations faced by mAbs.

The only mAb that has been FDA approved for use as a prophylactic for viral infections in the United States is Palivizumab, a mAb specific to respiratory syncytial virus (RSV). RSV is a major cause of virus-induced respiratory disease and hospitalization in infants and young children. Palivizumab is a neutralizing mAb that is used clinically to prevent serious RSV-related respiratory disease in high-risk infants. Motavizumab, an affinity-optimized version of palivizumab, was developed to improve protection against RSV, but was not FDA approved because of concerns of non- specific tissue binding (135). This highlights one of the risks of affinity-optimization of mAbs. Palivizumab binds RSV F-protein, which plays a role in virus attachment and mediates fusion, acting at a point after F-protein initiates interaction with the cell membrane and before virus transcription indicating that the mAb may prevent conformational changes in the F-protein required for the fusion process (135). Monthly administration of

palivizumab is safe and effective for prevention of serious RSV illness in premature children. Of note, while this mAb does not have FDA approval, an urgent global quest for effective therapies to prevent and treat COVID-19 disease is ongoing. REGN-COV2 is a cocktail of two potent human neutralizing antibodies that target different, non-overlapping epitopes on the SARS-CoV-2 spike protein. Studies in rhesus macaques and hamsters demonstrate that REGN-COV2 can greatly reduce virus load in the lower and upper airway when administered prophylactically or therapeutically. This mAb cocktail could lead to the usual standard of care for both non-hospitalized and hospitalized patients with COVID-19.

The delivery of antibodies through passive immunization for protection against influenza infection is especially appealing because it would allow administration of protective antibodies to high risk groups that are generally poor responders to traditional vaccination such as the very old, very young, or immunocompromised (23). Given the conserved nature of M2e, an antibody therapy based on M2 would prove beneficial in the event of a pandemic strain of influenza. The administration of M2 specific antibodies able to recognize a novel influenza virus could slow the progression and spread of a pandemic strain which would allow time for the development of conventional strain-matched vaccines.

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CHAPTER 3

BINDING SPECIFICITY OF M2E-SPECIFIC MONOCLONAL ANTIBODIES TO DIVERSE INFLUENZA A VIRUSES[1]

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ABSTRACT

Influenza virus infection causes significant morbidity and mortality worldwide as humans fail to make a universally protective memory immune response to influenza A. This is due to the immune dominant surface proteins Hemagglutinin and Neuraminidase that undergo antigenic drift and shift, resulting in new influenza A strains to which humans can not recognize. Additionally, escape mutants have been reported for all treatments for influenza A. Influenza A will remain a significant threat to human health in the absence of a universal vaccine or treatment. The extracellular domain of the M2-proton channel (M2e) is an appealing antigenic target for a universal treatment as it is highly conserved across influenza A serotypes, has a low mutation rate, and is essential for viral infection and replication. Previously, we generated seven M2e-mAbs and utilized *in vitro* and *in vivo* assays to validate the binding and protection of our novel M2e-mAbs. Based on these results, we wanted to further validate the specificity and affinity of our M2e-mabs. Our data shows our M2e-mAbs interfere with one another for binding across multiple serotypes, suggesting similar epitopes. Our antibody cocktail significantly protects highly susceptible Balb/c mice from lethal challenge with pH1N1 A/CA/07/2009, H5N1 A/Vietnam/1203/2004, and H7N9 A/Anhui/1/2013 by improving survival rates and weight loss. Based on these results, our M2e-mAb cocktail shows strong potential as a universal influenza A treatment.

INTRODUCTION

Influenza is a negative sense, single-stranded RNA virus that is composed of eight gene segments that make up its genome. Two of these gene segments encode the surface proteins Hemagglutinin (HA) and Neuraminidase (NA); the immune dominant targets of influenza A virus (IAV). Due to the high mutation rate of HA and NA, seasonal vaccine efficacy is often highly variable and difficult to predict. This necessitates not only the frequent updating of the vaccine to current circulating strains, but also a correct prediction of seasonal epidemic strains due to production of vaccines ahead of exposure. Treatments for influenza have been developed and FDA approved as recently as October 2018 (1, 2). However, there is no current influenza treatment to which viral escape mutants have not been isolated (3, 4). Considering the variability in the efficacy of the influenza vaccine and antiviral treatments for influenza disease, it is critical to develop a treatment for IAV which is universal and avoids viral escape mutants (5).

The sequence of the 23-amino-acid N terminus ectodomain of M2 has remained highly conserved among IAV strains isolated since 1918 (6, 7). M2e remains highly conserved due in part to its genetic relation to M1, which is the most conserved protein of IAV (8). The low degree of change in the M2e sequence could also be related to the limited M2e-specific antibody response mounted during IAV infection, resulting in little pressure for the virus to change (9, 10). While M2e is 99% conserved across IAV strains and has a low mutation rate (9, 11), the M2e-specific immune response is currently lacking, where less than 20% of infected individuals produce M2e antibodies in response to IAV infection (12). This perhaps is due to M2e's small size and low frequency on the membrane of mature virus particles (13, 14). Therefore, it would be advantageous to

develop a universal prophylactic or therapeutic agent to prevent or treat IAV by incorporating M2e-specific monoclonal antibodies (mAbs) to generate a protective immune response that is currently lacking in humans.

Considering that antibody mediated responses are protective against IAV infection and the basis behind the seasonal influenza vaccines (15), we were determined to produce antibody therapeutics targeting the highly conserved influenza protein M2e and using the AuNP-M2e-CpG vaccine in mice for the production of our M2e-specific antibodies (16, 17). M2e-mAbs are protective against IAV (16, 18). However, many M2e-mAbs are limited in their binding to different strains and develop escape mutants (19, 20). The M2e-specific antibodies we produced bind to M2e expressed on virions and infected cells expressing a range of M2e sequences which represent the M2e variations seen in M2 across human, swine, and avian influenza A viruses. This cross reactivity in binding and/or protection suggests that 5 of our M2e-mAbs (391, 472, 522, 602, 934, and 1191) bind to epitopes in the highly conserved N-terminal region (AA 1-10) of M2e, similar to TCN-032. mAb 770 on the other hand, likely binds to an epitope that is more strain specific at AAs between position 11 and 19 due to its distinct decrease in binding to influenza A viruses with mutations in this region, similar to 14C2.

Upon considering this high binding to virions as well as infected cells and the cross-reactivity of our M2e-mAbs, we further investigated the epitope binding specificity of each M2e-mAb with IAV strains having considerable diversity within the M2e sequence as well as representing zoonotic and pandemic threats. We hypothesize that most of our M2e-mAbs bind universal-highly conserved regions of the M2e epitope and therefore will interfere with each other for binding *in vitro*. Additionally, we believe that

a cocktail of our M2e-mAbs will increase protection against lethal IAV challenge at low doses. We analyzed the potential of our M2e-mAbs to bind specific epitopes using purified IAV virions and competing the antibodies to each other. Then we analyzed the potential of our antibody cocktail as a universal treatment for IAV using BALB/c mice, which are highly susceptible to and an established model of influenza A infection (21).

MATERIALS AND METHODS

Viruses

Human influenza A viruses (IAVs) A/PR/8/1934 (H1N1), A/CA/07/2009 (pH1N1), and A/FM/1/1947-MA (H1N1) were propagated in the allantoic cavity of embryonated chicken eggs at 37°C for 48h. Avian influenza viruses A/Anhui/1/2013 (H7N9) and A/Vietnam/1203/2004 (H5N1) were propagated in the allantoic cavity of embryonated chicken eggs for or 37°C for 18-24h. Swine influenza viruses A/sw/NB/A01444614/2013 (H1N1), A/sw/TX/A01049914/2011 (H3N2), and A/sw/MO/A01444664/2013 (H1N2) were provided via the National Swine Surveillance repository (National Veterinary Services Laboratories, Ames, IA) and propagated in MDCK-ATL cells in MEM containing 0.05% TPCK-Trypsin (Worthington) at 37°C for 48-72h. MDCK-ATL cells were obtained from the International Reagent Resource (Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA). All experiments using H7N9 or highly pathogenic H5N1 avian influenza virus were reviewed and approved by the institutional biosafety program at the University of Georgia and were conducted in biosafety level 3 enhanced

containment. Work with highly pathogenic avian influenza virus followed guidelines for use of Select Agents approved by the CDC.

Virus Purification

IAVs were treated with 1:1000 Betapropiolactone (BPL; final concentration of 2μM) and inactivated for 72h at 4°C. Inactivation of human and swine IAVs was verified by plaque assay. Inactivated virus was spun in tabletop centrifuge at 2000g for 10 minutes at 4°C and then 36 ml of clarified virus was added to a 50 ml ultrafuge tube and spun in ultracentrifuge (ThermoFisher Scientific, Sorvall™ WX+ Ultracentrifuge Series, Rotor AH-629) at 12,280 x g for 30 minutes at 4°C. 30 ml of clarified supernatant was transferred to a new 50ml ultrafuge tube and 5ml of chilled 30% sucrose in NTE buffer (NaCl-Tris-EDTA buffer) was added to the very bottom of the ultrafuge tube. Virus was then pelleted through sucrose cushion at 77, 000 x g for 2 hours at 4°C. Sucrose/media supernatant was aspirated without disturbing pellet and the pellet resuspended in 500μl PBS. The virus was dialyzed (10kd MWCO; ThermoFisher Scientific) for 12 hours in PBS. Samples were removed from dialysis cassettes, aliquoted, and stored at -80°C.

M2e Monoclonal antibodies

Murine M2e monoclonal antibodies (mAbs) 391, 472, 522, 602, 770, 934, and 1191 were produced through Hybridoma technology and provided via Baylor College of Medicine (Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX).

Antibody Biotinylation

All mAbs were reacted with 25µl EZ-Link Hydrazide Biotin (ThermoFisher Scientific) dissolved in DMSO. The reaction was carried out at room temperature for 2 hours, and the biotinylated mAbs were purified by dialysis (10kd MWCO; ThermoFisher Scientific) for 12 hours in PBS. Biotinylated mAbs were removed from dialysis cassettes, aliquoted, and stored at -80°C.

ELISA Analysis of Biotinylated mAbs

Nunc Maxisorp Flat-Bottom plates (ThermoFisher Scientific) were coated overnight at 4°C with 100 µl/well of purified A/PR/8/1934 (H1N1) at 0.5 µg/mL in bicarbonate buffer (pH 9.6). After washing 3 times with PBS containing 0.05% Tween 20 (PBS/T) plates were blocked with 100 µl/well of 1% Bovine serum Albumin (BSA) in PBS for 2 hours. After washing again, 50 µl of the indicated biotinylated mAb was added in 4-fold dilutions and incubated at 4°C overnight. After washing with PBS/T, 50µl/well of a 1:10,000 dilution of HRP-Streptavidin IgG (Vector Laboratories) was added to plates and incubated for 1 hour at room temperature. Plates were washed with PBS/T and 50µl/well of TMB substrate (Vector Laboratories) was added for 10-15 minutes for color change; reaction was stopped with the addition of 50 µl/well H_2SO_4. Absorbance was measured at OD_{450} nm. The optical densities of the assay wells containing no mAbs were subtracted from the optical densities of the antigen-stimulated mAb samples to calculate the results. Data not shown.

Competition ELISAs

Nunc Maxisorp Flat-Bottom plates (ThermoFisher Scientific) were coated overnight at 4°C with 100µl/well of purified influenza virions at 0.5 µg/mL in bicarbonate buffer (pH 9.6). After washing 3 times with PBS containing 0.05% Tween 20 (PBS/T) plates were blocked with 100 µl/well of 1% Bovine serum Albumin (BSA) in PBS for 2 hours. After washing again, 50 µl of the indicated biotinylated mAb was added at a fixed dilution and incubated at RT for 2 hours. The fixed dilution was calculated to be the 50% absorbance rate of each indicated mAb (~2 µg/ml for each indicated mAb). 50 µl of the indicated unlabeled mAb was then added in 4-fold dilutions and incubated for 1 hour at room temperature. After washing with PBS/T, 50 µl/well of a 1:10,000 dilution of HRP-Streptavidin IgG (Vector Laboratories) was added to plates and incubated for 1 hour at room temperature. Plates were washed with PBS/T and 50 µl/well of TMB substrate (Vector Laboratories) was added for 10-15 minutes for color change; reaction was stopped with the addition of 50µl/well H_2SO_4. Absorbance was measured at OD_{450} nm. The optical densities of the assay wells containing no competitor mAbs were subtracted from the optical densities of the antigen-stimulated mAb samples to calculate the results. A table was generated to understand the degree of inhibition more readily. The table represents the lowest concentration a competitor mAb was able to bind to each IAV strain and still cause interference with the biotinylated mAb. Concentrations were color coded with dark blue representing binding even at the lowest concentration and orange as no binding (or interference).

Mice

Female BALB/c mice were purchased from Envigo RMS, Inc., Indianapolis, Indiana, USA. The institutions' Animal Care and Use Committees approved all protocols for animal experiments.

Cocktail Protection Studies

Mice were given an IP injection of the specified mAb cocktail at specified dose 24 hours before virus challenge with 10XLD50 of the specified virus. H1N1 challenge virus was administered in 20 µl of PBS intranasally to mice anesthetized with isoflurane. The pH1N1 and H5N1 challenge viruses were administered in 30 µl of PBS intranasally to mice anesthetized with Ketamine/xylazine. The H7N9 virus was administered intranasally to mice anesthetized with 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co). Each challenge with pH1N1, H7N9, and H5N1 viral inoculum was back-tittered on MDCK cells to confirm dose. If specified, subsets of mice were humanely euthanized, and tissues collected for virus titer 3 days post-infection (dpi). All animals were monitored for body weight and humane endpoints for euthanization. Survival and weight loss were monitored for up to 21 dpi or until all animals recovered to at least 90% starting body weight.

Viral Lung Quantification

Lungs were homogenized in 1 mL of sterile PBS using a Tissuelyser homogenizer (Qiagen), clarified by centrifugation, and titrated for infectious virus by 50% tissue culture infectious dose ($TCID_{50}$) assay. The 50% infectious dose was calculated using the method of Spearman-Karber (22).

Statistical Analysis

All statistics were performed using Graphpad Prism 7. Survival analysis utilized a Mantel-Cox log rank test. All statistics for a particular dataset are indicated in the figure legends.

RESULTS

Competition ELISAs

To test the binding specificity of the M2e-mAbs we competed them by competition ELISAs where we tested eight influenza A purified virions: H1N1 A/PR/8/1934, pH1N1 A/CA/04/2009, H1N1 A/FM/1/1947,H1N1 A/sw/NE/A01444614/2013, H3N2 A/sw/TX/A01049914/2011, H1N2 A/sw/MO/A01444664/2013, H5N1 A/Vietnam/1203/2004, and H7N9 A/Anhui/1/2013 with 5 of our M2e-mAbs. We saw mAbs 472, 522, 602, and 1191 consistently able to bind IAV strains and inhibit the binding of the labeled antibody even at low concentrations (**Figure 3.1-Figure 3.6**). The interference of antibody 770 was comparatively lower against all strains and specifically against pH1N1 A/CA/04/2009 where interference was completely absent (**Figure 3.5**). Interestingly, the interference of all antibodies with the exception of 770 appeared to occur even at very low concentrations (**Table 3.1**). Interestingly, 602 inhibition of biotinylated mAbs 472 and 1191 was decreased in viruses H7N9 A/Anhui/1/2013, H1N1 A/sw/NE/614/2013, and H1N2 A/sw/MO/664/2013 (**Figure 3.2 and Figure 3.6**). Additionally, mAb 770 only shows interference with mAb 602 and 391 in these same viruses (**Figure 3.1 and Figure 3.4**). H7N9 A/Anhui/1/2013, H1N1 A/sw/NE/614/2013, and H1N2 A/sw/MO/664/2013 are the only viruses that carry the same mutation at position

14 (**Table 1.2**). This suggests mAbs 391 and 602 may bind an epitope that is overlapping the conserved region of M2e and the variable region of M2e.

Cocktail Protection Studies

To determine if our M2e-specific antibodies' high binding efficiency and high interference with one another translated to protection, we challenged mice with pH1N1 A/CA/04/2009, H5N1 A/Vietnam/1203/2004, or H7N9 A/Anhui/1/2013. Both low and high concentrations of the cocktail M2e-mAbs were more than partially protective against pH1N1 and increased protection from lethal challenge even at a low dose (**Figure 3.7A**). When comparing the 20 μg cocktail dose to a 25 μg single dose of 472 or 522 M2e Mab (**Figure 1.3A)** in pH1N1 infection, we see increased protection with the cocktail of antibodies even at a lower dosage (**Figure 3.7A**). However, 602 single dose at 25 μg protected 100% of mice from IAV challenge (**Figure 1.3A**), which was higher than the antibody cocktail (**Figure 3.7A**). These are only potential comparisons to the single dose mAb data as concentrations do not match. The cocktail dose increased from 60% protection at 10 μg to 75-80% protection with the 20 μg dose. The M2e-mAb cocktails also significantly decreased weight loss, especially the 20 μg dose (data not shown). However, neither dose of the antibody cocktails induced a decrease in viral titer at day 3 (**Figure 3.7D**). In challenges with a $10XLD_{50}$ of highly virulent H5N1 A/Vietnam/1203/2004, we found that the 10 μg cocktail dose administered prophylactically was partially protective against lethal challenge but protection increases in the 20 μg cocktail dose to 90% (**Figure 3.7B**). Furthermore, the 10 μg and 20 μg cocktail dose induced a small decrease in viral titer in the lung at day 3 (**Figure 3.7E**). Finally, for H7N9 A/Anhui/1/2013 challenges,

both low dose and high dose of the cocktail was extremely protective against H7N9 A/Anhui/1/2013 (**Figure 3.7C**). Additionally, we did see a reduction in viral titer in mice treated with both 10 μg and 20 μg of cocktail (**Figure 3.7F**) and significantly lower weight loss in treated mice (data not shown).

DISCUSSION

The primary goal of this study was to begin understanding and characterizing the specific epitopes on M2e of diverse IAVs that our M2e-mAbs bind to. The annual influenza vaccine effectiveness is highly variable and difficult to predict due to antigenic drift and shift of the immune dominant surface proteins HA and NA (5, 23). The six FDA approved antiviral drugs for treatment of influenza infection are unable to avoid the development of viral resistance leading to the isolation of additional mutations which increase the transmission of these viruses (3, 24-27). Given the high dependence of vaccine efforts on correct predictions of influenza strains in circulation, the emergence of viral resistance in all current influenza A therapies, and the high mortality rate due to influenza infections, it is necessary to explore other options for treating influenza that would potentially be universally protective. To that end, we consider the potential for a monoclonal antibody therapy. In our previous study, we found that these M2e-specific mAbs bind to M2e expressed on both purified virions and infected cells expressing a range of M2e sequences which represent zoonotic and pandemic M2e variations across human, swine, and avian IAVs. This data suggested that 5 of the murine M2e-mAbs (391, 472, 522, 602, 1191) potentially bind to epitopes in the highly conserved N-terminal region and 770 and 934 potentially bind a different region of the M2e epitope between AA positions 11 through 23 where an increased number of mutations have been found in this region on different strains

of IAV. In the present study, the interference patterns between the antibodies to all strains confirms our previous thoughts as mentioned above on the epitopes we believe these antibodies are binding to. mAbs 472, 522, 602, and 1191 had strong interference patterns across all IAV strains suggesting they are binding similar epitopes in the conserved region of M2e. In contrast, 770 had very low interference with all antibodies tested suggesting that 770 binds an alternative region of the M2e epitope. Furthermore, these results demonstrate that our M2e-specific antibody cocktail is highly protective against avian and swine flu strains representing zoonotic and pandemic potential even at low doses. mAb 1191 was included in the competition data due to its high binding in ELISAs but does not protect due to its IgG2b isotype, therefore it was not included when formulating the cocktail of antibodies for protection studies (**Table 1.1**). Due to the low binding in virion and infected cell ELISAs, 934 was not included in the competition data. Additionally, 391 was excluded from initial experiments due to production and purification issues encountered at the time. Competition ELISAs for 391 were run at a later date and therefore, there is no data in the chart to display for 391 as a competitive antibody against the other mAbs in the panel.

Considering the overlapping epitopes suggested in interference assays, it is interesting that the three mAbs (472, 522, and 602) were more effective as a cocktail at a lower dose than the individual mAbs at a high does. Assuming the mAbs bind to a similar epitope, it suggests that competition does not affect efficacy *in vivo*. This could be for several reasons: this difference is isotype dependent where the IgG1 or IgG2a antibody has higher affinity to the M2e region than the other antibody isotype. This difference could be due in part by the finite amount of antigen available for antibody binding in an *in vitro*

setting, it is not indicative of actual IAV infection and this could be reason for such high competition. Furthermore, it is possible that the epitopes these antibodies bind to overlap but are not identical therefore causing enhanced protection in an *in vivo* model.

These results strongly suggest that this antibody cocktail is protective against the additional strains tested in our competition ELISA studies. The protection provided to all these strains would imply that this antibody cocktail has potential as a universal IAV treatment.

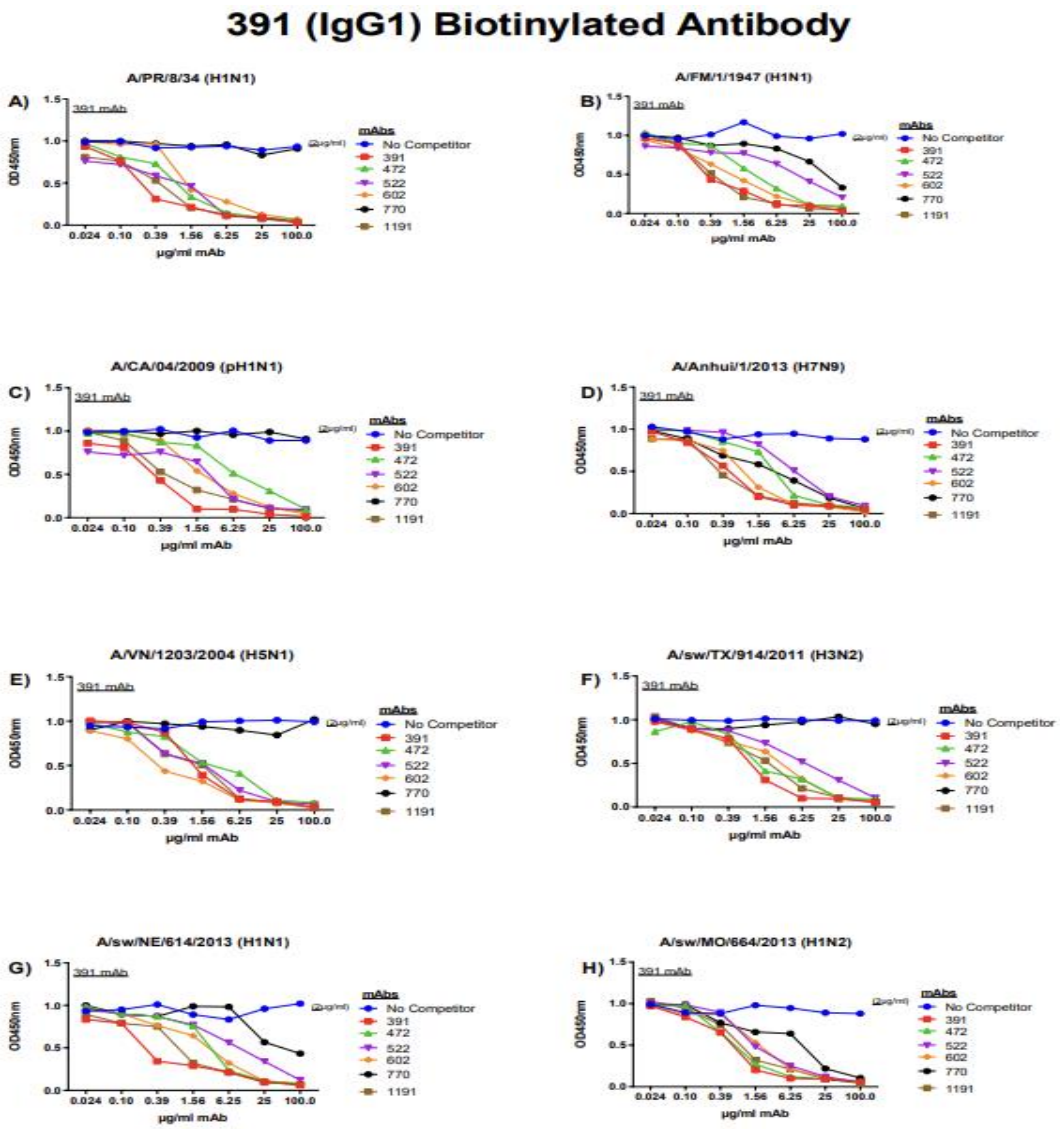


Figure 3.1. M2e-specific mAbs compete at varying degrees with biotinylated 391 M2e-specific mAb against different influenza A virions. (A-H) Purified influenza A virions were used as the coating antigen for competition ELISAs. The 50% absorbance rate of biotinylated 391 was added to the antigen coated wells and the indicated un-labeled mAb clone was added in 4-fold dilutions to test for inhibition activity. Background was subtracted. OD_{450} = Optical Density 450 nm.

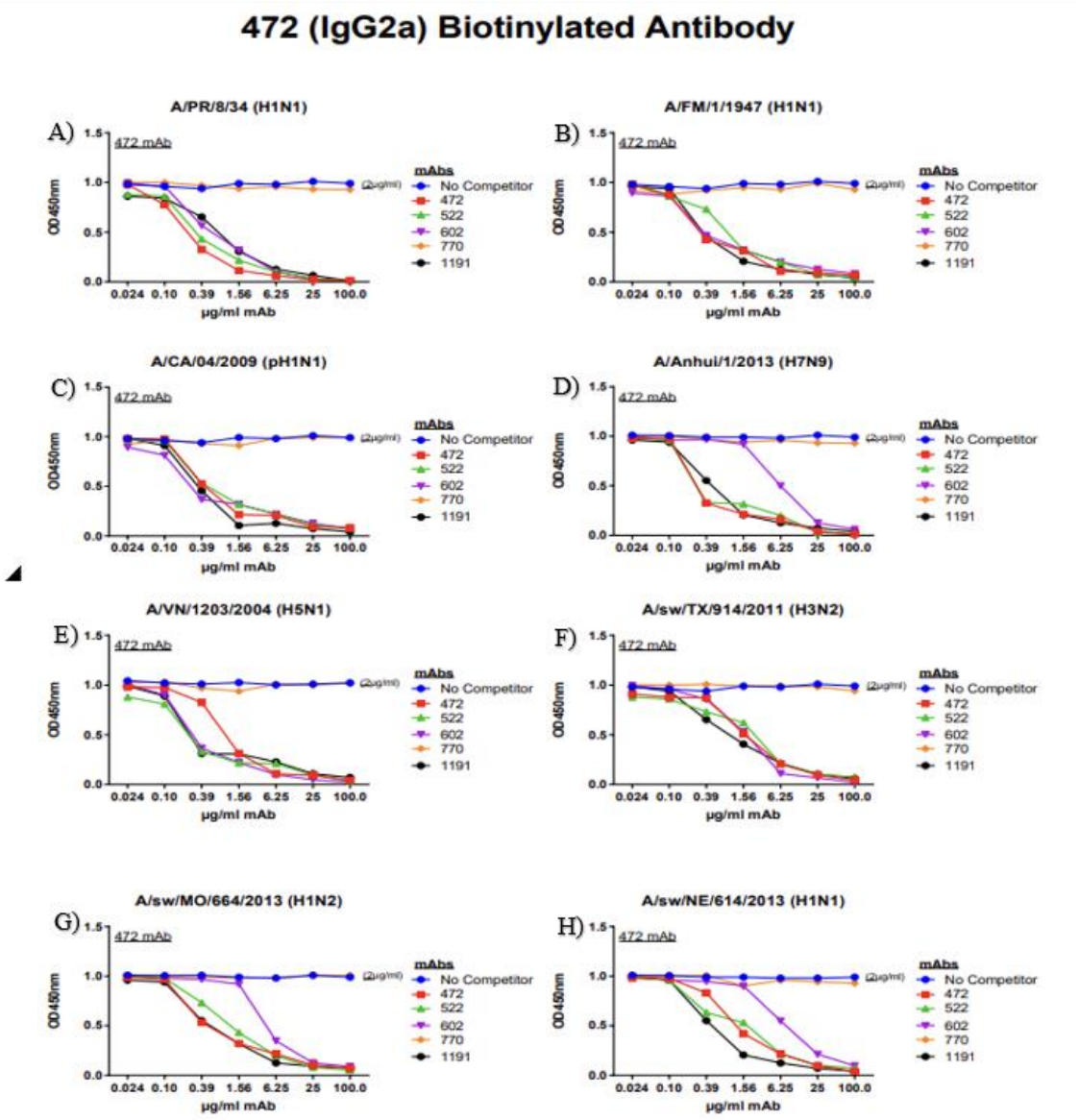


Figure 3.2. M2e-specific mAbs compete at varying degrees with biotinylated 472 M2e-specific mAb against different influenza A virions. (A-H) Purified influenza A virions were used as the coating antigen for competition ELISAs. The 50% absorbance rate of biotinylated 472 was added to the antigen coated wells and the indicated un-labeled mAb clone was added in 4-fold dilutions to test for inhibition activity. Background was subtracted. OD_{450} = Optical Density 450 nm.

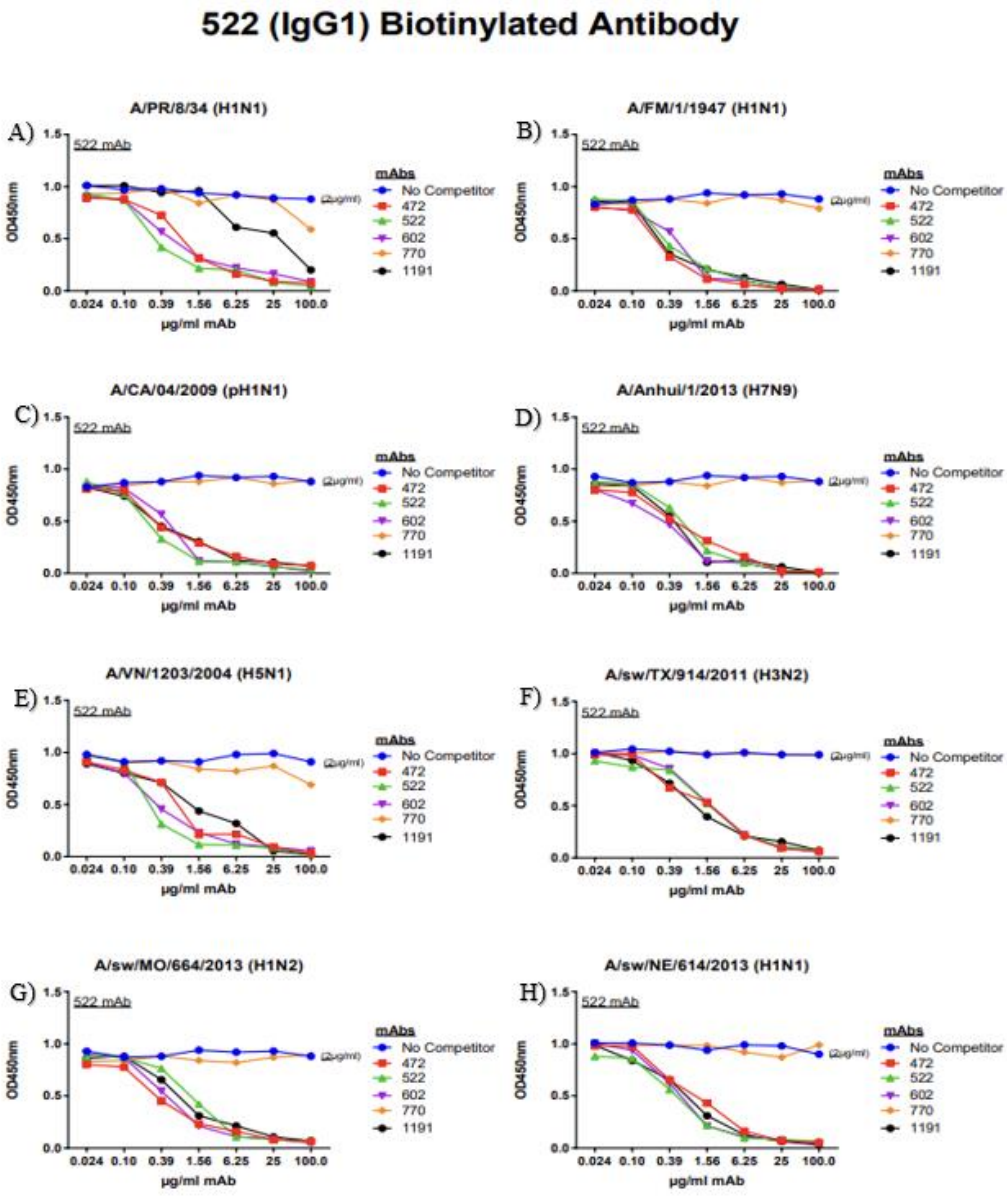


Figure 3.3. M2e-specific mAbs compete at varying degrees with biotinylated 522 M2e-specific mAb against different influenza A virions. (A-H) Purified influenza A virions were used as the coating antigen for competition ELISAs. The 50% absorbance rate of biotinylated 522 was added to the antigen coated wells and the indicated un-labeled mAb clone was added in 4-fold dilutions to test for inhibition activity. Background was subtracted. OD_{450} = Optical Density 450 nm.

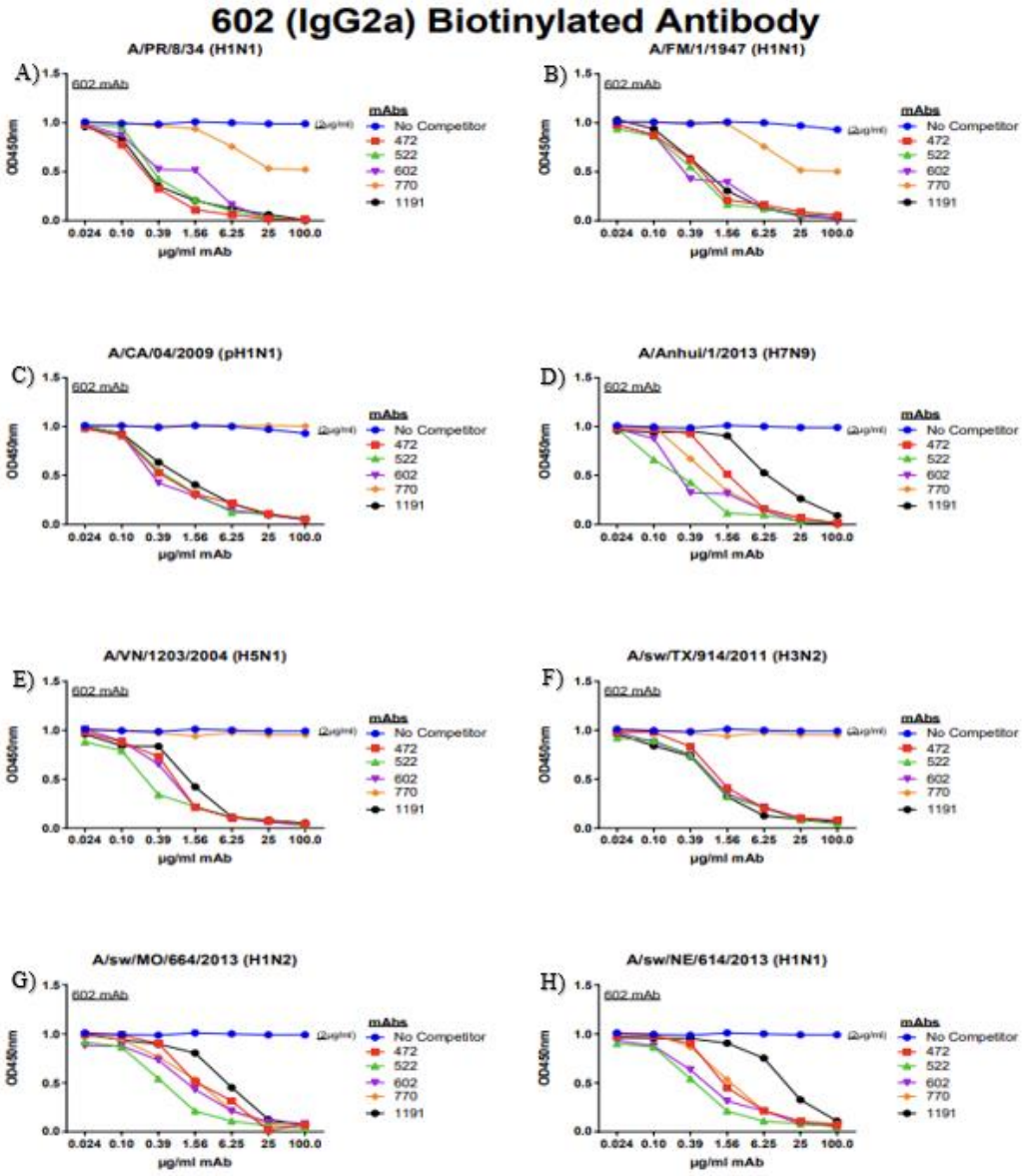


Figure 3.4. M2e-specific mAbs compete at varying degrees with biotinylated 602 M2e-specific mAb against different influenza A virions. (A-H) Purified influenza A virions were used as the coating antigen for competition ELISAs. The 50% absorbance rate of biotinylated 602 was added to the antigen coated wells and the indicated un-labeled mAb clone was added in 4-fold dilutions to test for inhibition activity. Background was subtracted. OD_{450} = Optical Density 450 nm.

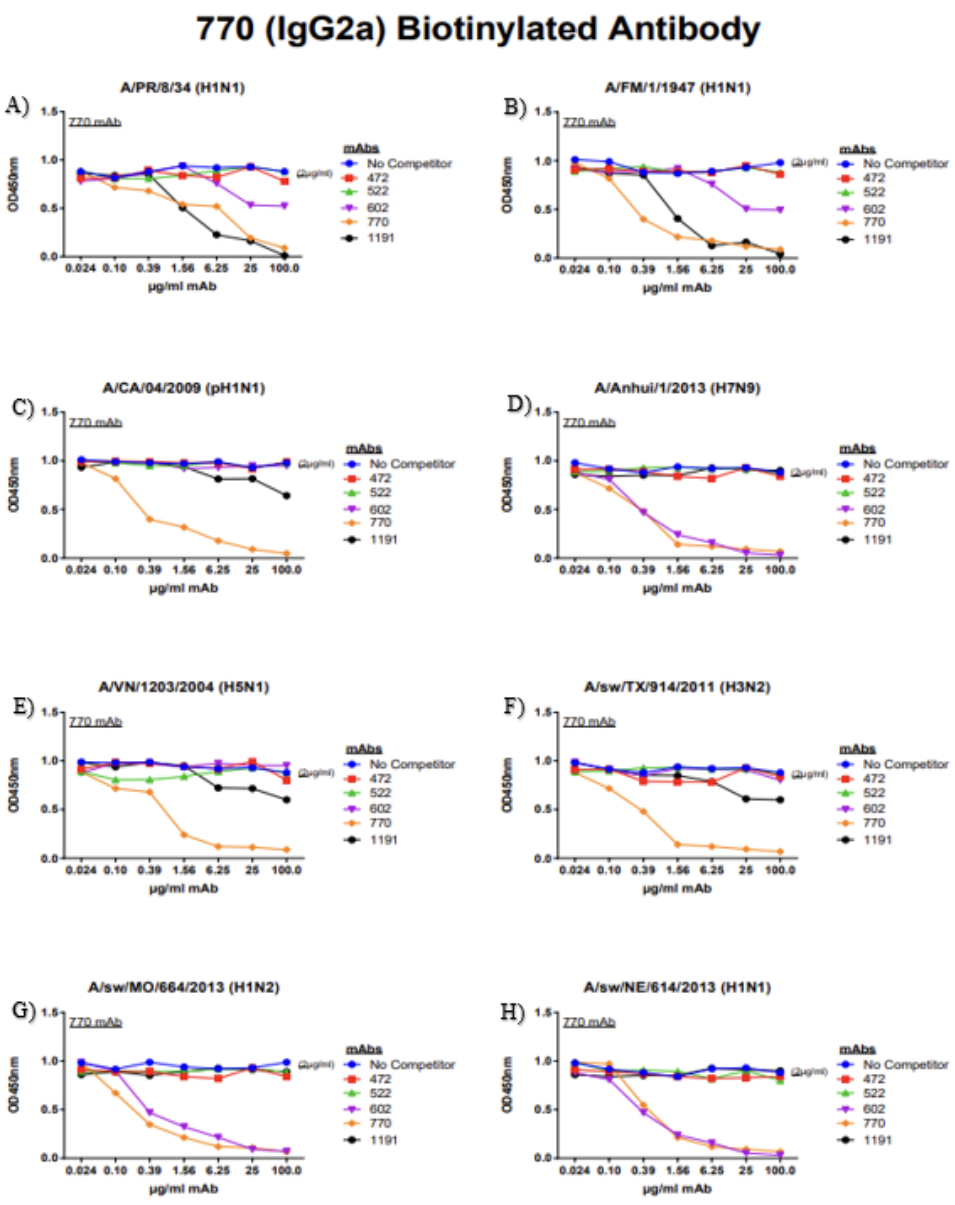


Figure 3.5. M2e-specific mAbs compete at varying degrees with biotinylated 770 M2e-specific mAb against different influenza A virions. (A-H) Purified influenza A virions were used as the coating antigen for competition ELISAs. The 50% absorbance rate of biotinylated 770 was added to the antigen coated wells and the indicated un-labeled mAb clone was added in 4-fold dilutions to test for inhibition activity. Background was subtracted. OD_{450} = Optical Density 450 nm.

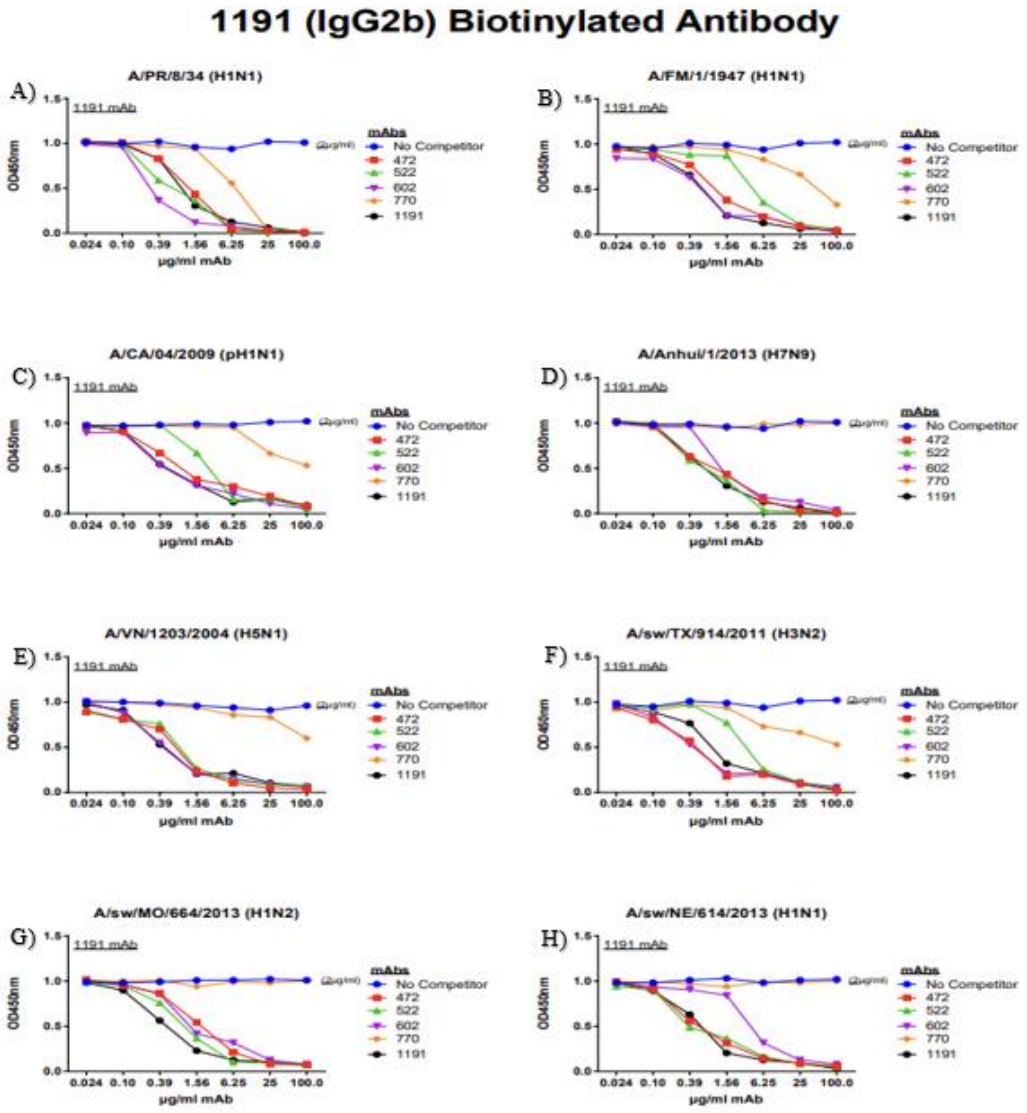
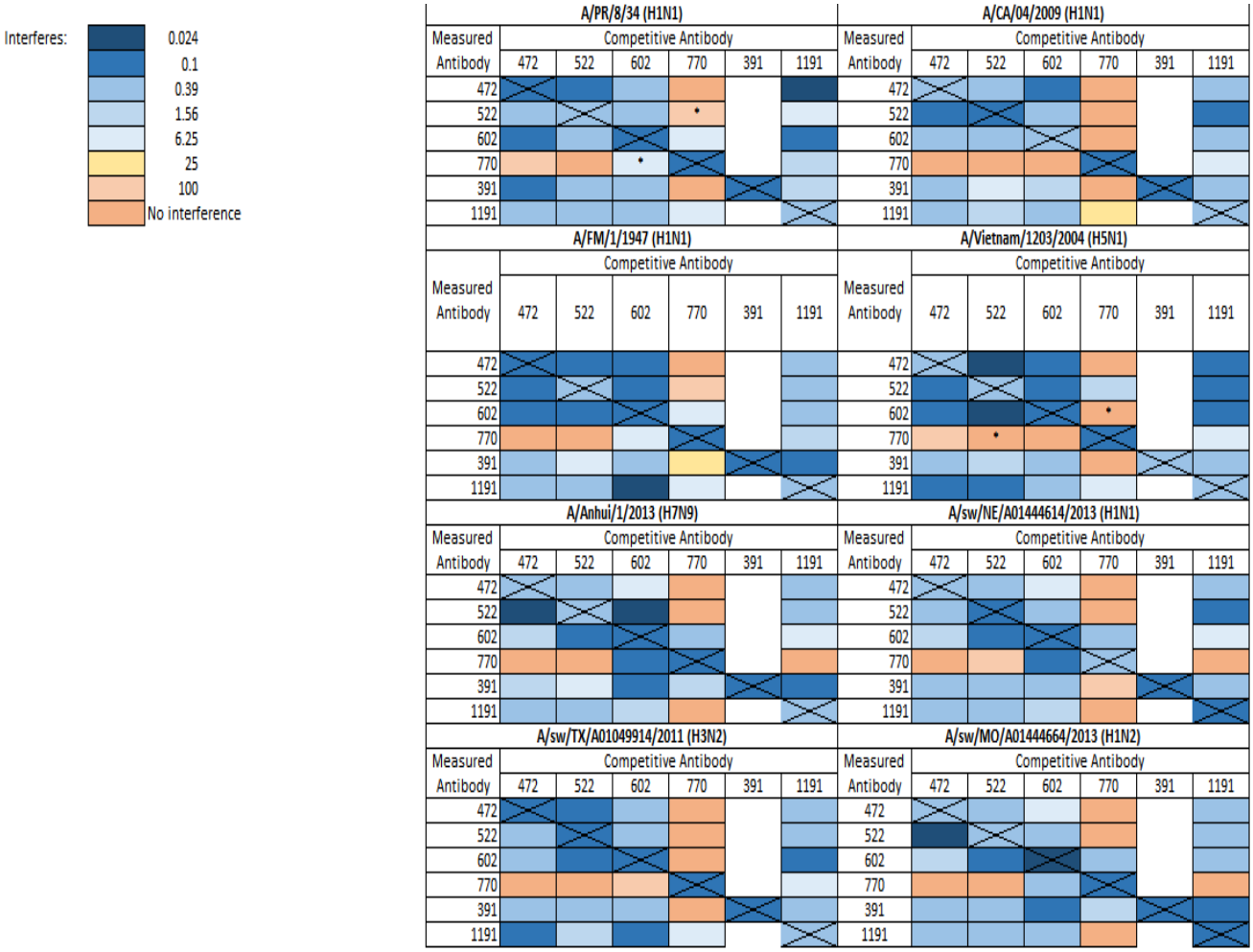


Figure 3.6. M2e-specific mAbs compete at varying degrees with biotinylated 1191 M2e-specific mAb against different influenza A virions. (A-H) Purified influenza A virions were used as the coating antigen for competition ELISAs. The 50% absorbance rate of biotinylated 1191 was added to the antigen coated wells and the indicated un-labeled mAb clone was added in 4-fold dilutions to test for inhibition activity. Background was subtracted. OD_{450} = Optical Density 450 nm.

Table 3.1. Competing M2e mAbs



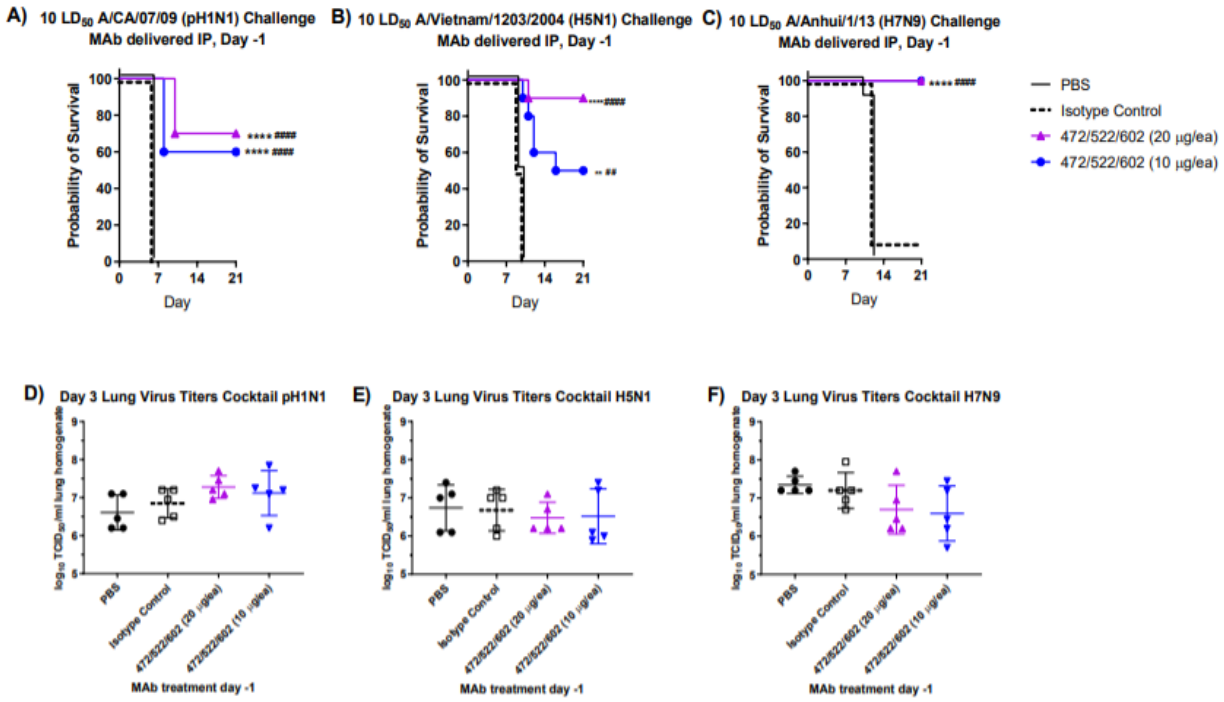


Figure 3.7.M2e-specific antibodies are more protective against lethal challenge as a cocktail. (A-C) BALB/c mice were passively immunized with indicated dose of M2e-MAb cocktail one day prior to infection with (A) pH1N1 A/CA/04/2009, (B) H5N1 A/Vietnam/1203/2004, or (C) H7N9 A/Anhui/1/2013. (D-F) Lungs for viral titers removed on day 3 post infection. Viral titers measured via plaque assay. Experiments performed followed endpoints based on comprehensive point system evaluating symptoms and disease severity. (A-C) n=10 (D-F) n=5. ** $p<0.005$, * $p<0.05$, log-rank analysis. * indicates significance compared to PBS control, # indicates significance compared to isotype control.

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CHAPTER 4

DISCUSSION

Influenza remains a public health concern as vaccines are mainly effective against matching vaccine strains and fail to induce a cross-protective immune response against constantly emerging antigenic variants of IAV (1, 2). The six FDA approved antiviral drugs for treatment of influenza infection include three NA inhibitors, one endonuclease inhibitor, and two M2 channel blockers. Unfortunately, all six treatments for influenza infection are unable to avoid the development of viral resistance, leading to the isolation of additional mutations which increase the transmission of these viruses (3-7). Given the high dependence of vaccine efforts on the correct prediction of influenza strains in circulation, the emergence of viral resistance in all current influenza A therapies, and the high mortality rate due to influenza infections, it is necessary to explore other options for treating influenza that would potentially be universally protective. To that end, we consider the potential for a monoclonal antibody therapy.

Monoclonal antibodies provide a very appealing strategy for preventing or treating viral infections because of their specificity, relatively long half-life, and limited toxicity as just a few examples. It is well established that antibody protection is effective against IAV. Each year the efficacy of seasonal vaccines is partially determined by the titers of neutralizing antibodies (antibodies which prevent infection through binding to the virus) produced (8). However, antibodies also provide protection through non-

neutralizing mechanisms as well and have been shown to be effective against IAV (9-14). The protection provided by antibodies can be sufficient to prevent lethality as seen when transferred immunized serum and IAV specific monoclonal antibodies are protective in animal models (9, 15, 16). Antibodies to the hemagglutinin (HA) can neutralize the virus and readily prevent infection, but these antibodies are mostly subtype or strain specific and do not have use as antibody therapies (17). Neuraminidase (NA) antibodies have been proven to protect against IAV infection through an undefined non-neutralizing mechanism (18). Unexpectedly, antibodies against the influenza nucleoprotein (NP) have also been shown to be protective in mice (19). Finally, the M2 protein has been widely explored as a target for both vaccines and drug therapies through the use of mAbs.

M2 is an alternative target as it is expressed on virus-infected cells, it is more highly conserved compared to HA and NA, the other surface viral antigens, and antibody responses to M2 proteins have been demonstrated to protect against human and avian influenza virus infections (20-22). M2e is conserved at least in part because it is generated as a spliced transcript and the first 9 amino acids are shared by M1 capsid and M2 pore proteins. M2e-mAbs specifically have been found to induce protection through a variety of Fc dependent mechanisms, including antibody mediated phagocytosis by alveolar macrophages, NK cell mediated antibody dependent cellular cytotoxicity (ADCC), and complement induction (22-26). Effective mechanisms employed by M2e-specific monoclonal antibodies is dependent on the M2e epitope it recognizes. Some of these mechanisms include preventing membrane scission during viral budding, decreasing the expression of M2 on the surface of infected cells, and the blocking of ion channel activity (27-30).

Our seven murine M2e-mAbs were developed at Baylor School of medicine in mice from the AuNP-M2e-CpG vaccine that appears to confer universal protection (31, 32). This approach can be compared to the monoclonal antibody TCN-032, that was one of 17 M2e-specific Ab producing B cells isolated from 23 seropositive patients from a cohort of 140 adults (33). By using mice to produce the mAbs we are able to enrich antigen-specific B cells and the affinity of the mAbs produced through repeated and directed vaccination. Our 7 M2e- specific mAbs have potential for being a universal IAV treatment. We previously found that these antibodies bind to M2e expressed on both purified virions and infected cells expressing a range of M2e sequences which represent zoonotic and pandemic M2e variations across human, swine, and avian IAVs. This cross reactivity seen in binding suggests that 5 of the murine M2e-mAbs (391, 472, 522, 602, 1191) potentially bind to epitopes in the highly conserved N-terminal region at AA positions 1-10 of M2e, which can be compared to the mAb TCN-032 that binds AA 1-5 of M2e. mAbs 770 and 934 potentially bind a different region of the M2e epitope between AA positions 11 through 23 where an increased number of mutations have been found in this region on different strains of IAV. This is similar to the mAb 14C2 that binds to AA 6-15. This epitope region is more strain specific, resulting in a decrease in binding to IAV strains with an increased number of mutations in this region of M2e.

To further test the binding specificity of our M2e-mAbs we competed our highly cross-reactive antibodies (with the exception of 391) and 770 against all 8 IAV strains mentioned previously. We saw strong interference with 472, 522, 602, and 1191 against each other when tested with all eight strains. This high interference even at low concentrations for each antibody can confirm that these antibodies are likely binding to

epitopes in the highly conserved N-terminal region and that these antibodies share the same-or very similar epitopes. The interference of antibody 770 was comparatively lower against all strains and specifically against pH1N1 A/CA/07/2009 where interference was completely abolished. This low interference with 770 likely means that this antibody is binding an epitope in the less conserved N-terminal region and is not competing for binding with the other M2e-specific antibodies. Due to the low binding in virion and infected cell ELISAs, 934 was not included in the competition data. 391 was also excluded due to production and purification issues encountered at the time.

Confirming the potential for these antibodies to be universally protective, almost all of our M2e-specific antibodies protected against pH1N1 A/CA/077/2009, H5N1 A/Vietnam/1203/2004, and H7N9 A/Anhui/1/2013. Highly effective M2e-specific antibodies were able to be titrated down to low doses and still remain effective. A dose of 25 μg of 391, 472, 522, and 602 is also partially, if not completely, protective against pH1N1 A/CA/04/2009, H5N1 A/Vietnam/1203/2004, and H7N9 A/Anhui/1/2013. mAb 472 followed by 602 seem to be the most highly protective against all strains. mAbs 391 and 522 are strongly protective against pH1N1 A/CA/07/2009, H5N1 A/Vietnam/1203/2004, and H7N9 A/Anhui/1/2013. Because these four antibodies appear to bind strongly to all these strains via ELISA, it is possible that this difference in protection is isotype dependent, as 472 and 602 are IgG2a antibodies and 391 and 522 are IgG1 antibodies. This is consistent with literature comparing the Fc mediated protection of M2e-specific IgG1 and IgG2a antibodies (34).

The cocktail antibody protection study demonstrated that 472, 522, and 602 when combined together are highly effective against pH1N1 A/CA/07/2009, H5N1

A/Vietnam/1203/2004, and H7N9 A/Anhui/1/2013 at a very low dose of 10 μg. This shows that these antibodies are highly effective against avian and swine strains of influenza that are large public health concerns (35). mAb 1191 was included in the competition data due to its high binding in ELISAs but does not protect due to its IgG2b isotype, therefore it was not included when formulating the cocktail of antibodies for protection studies. It is interesting that the cocktail of antibodies was able to protect mice against IAV infection at such a low dosage, as the competition data demonstrates a high interference with each other in the same epitope region across all strains. It is possible as before that this difference is isotype dependent where the IgG1 or IgG2a antibody has higher affinity to the M2e than the other. This can also be explained in part by the finite amount of antigen available for antibody binding in an *in vitro* setting, it is not indicative of actual IAV infection and this could be reason for such high competition. Furthermore, it is possible that the epitopes these antibodies bind to overlap but are not identical therefore causing enhanced protection in an *in vivo* model.

In future experiments, it would be beneficial to isotype switch the M2e-specific antibodies to the same IgG1 isotype so as we do not see as stark of a change in binding and protection with different isotypes. Once the antibodies are able to be isotype switched, it would be beneficial to repeat virion and M2e infected cell ELISAs to see if this alters binding of any sort for each antibody. It would especially be interesting for 934 as we see very little binding in both virion and infected cell ELISAs but over 50% protectiveness in *in vivo* studies due in part to its IgG3 isotype. It would also be worthwhile to repeat protection studies with all mAbs *in vivo* to examine if antibody protectiveness increases with isotype switch. Furthermore, determining the exact Fc mediated effector function of

each antibody could give insight as to how these antibodies protect against lethal challenge of IAV infection. Additionally, measuring the interactions of our mAbs in real time would provide insight on their true binding affinity and rates of association and dissociation with the M2e.

Our M2e-specific mAbs possess unique characteristics which support further studies in efforts of developing a universal therapeutic for humans. Passive immunization using M2e mAbs could allow for the protection of certain immunocompromised groups which often are unable to receive or respond poorly to traditional active vaccination. This could also be used for rapid protective immunity to first responders on the frontlines of an IAV pandemic. While we hypothesize that our M2e-mAbs could be a universal treatment based on their recognition of and protection against a wide breadth of M2e variants, we are aware that other M2e-specific antibody treatments in the past have produced escape mutants when tested *in vivo* (27, 36). Further examination of these antibodies and their ability to avoid escape mutants will be required to determine their true potential as a universal IAV treatment. Overall, IAV continues to be a costly health problem for the human population. Continuing studies on the interaction of this virus with the immune system will help expand our knowledge of this disease and aide in the development of new methodologies for its prevention and treatment.

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