

EXPLOITING THE MATRIX PROTEIN 2 FOR PROTECTION AGAINST INFLUENZA VIRUS INFECTION

by

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(Under the Direction of S. Mark Tompkins)

ABSTRACT

Influenza virus infection continues to impose significant global health and economic consequences each year despite large-scale vaccination efforts. Constant antigenic changes along with the threat of pandemic strains necessitate periodic reformulation of traditional influenza vaccines. One approach which could help reduce the disease burden associated with the loss of vaccine efficacy due to antigenic drift and shift is to generate vaccine strategies induce protective immunity against conserved viral proteins. The matrix protein 2 (M2) with its small ectodomain (M2e) has remained remarkably conserved across influenza A virus strains and possess as an ideal target for antibody-mediated protection. The purpose of the research presented here focuses on the development of two novel vaccine approaches with the common goal of exploiting the M2 protein for protection against influenza virus infection. The first approach utilizes novel M2e-specific single-chain minibodies. Passive immunization with M2 minibodies conferred protection against a lethal influenza virus challenge. In addition, M2 minibodies were shown to facilitate activation of Fc-mediated immune functions proposing mechanism(s) of protection involved in immunity. The second

approach involves the use of recombinant adenovirus (rAd) vectors used for vaccination with conserved influenza virus proteins able to induce rapid immunity protective against challenge with a pandemic influenza virus strain. rAd vectors encoding the M2 protein from influenza were capable of inducing serum antibody titers able to recognize the M2e sequence of the vaccine antigen as well as the divergent M2e sequence of the pandemic challenge virus. Taken together, the novel vaccine approaches presented here represent potential prophylactic and therapeutic interventions to combat both seasonal and pandemic influenza virus infection.

INDEX WORDS: influenza virus, pandemic, matrix protein 2, monoclonal antibodies, passive immunization, recombinant adenovirus

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DEDICATION

To my wife and best friend, Shan. Without your amazing love and support none of this would have been possible.

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CHAPTER 1

INTRODUCTION

Influenza virus infection continues to cause significant morbidity and mortality across the globe and remains an important public health and socioeconomic concern. In the U.S. alone, seasonal influenza infection affects 5-20% of the total population with an estimated overall case mortality of 36,000 annually (1, 2). This significant disease burden persists even in the face of influenza-specific antiviral drugs and large-scale vaccination efforts. Current vaccination strategies primarily focus on the induction of neutralizing antibodies recognizing the highly variable surface glycoprotein hemagglutinin (HA). However, due to its variable nature, antigenic mutations often occur within HA resulting in the ability of circulating strains to avoid pre-existing immunity and lead to the loss of vaccine efficacy, a term known as 'antigenic drift'. Also, the introduction of a novel HA subtype not previously encountered by the population (antigenic shift) can contribute to not only the loss of vaccine protection but result in the spread of a global influenza pandemic. This phenomenon was exemplified by the emergence of the 2009 H1N1 pandemic virus. The increased disease prevalence due to antigenic drift and shift warrants the exploration of vaccination efforts able to protect the population against influenza virus infection regardless of the circulation HA subtype.

A promising alternative strategy under investigation is the generation of protective immunity against a conserved antigenic target. One appealing candidate is

the matrix protein 2 (M2) which contains a small ectodomain sequence (M2e) that has been found to be remarkably conserved across influenza A virus strains (3). Due to its surface availability, M2 presents itself as a potential candidate for antibody-based therapies. Indeed, early work utilizing M2 as an antigenic target discerned that M2-specific antibodies not only have the capability of suppressing influenza virus growth in tissue culture but also in the lungs of infected mice (4, 5). In light of this, our research efforts have focused on the induction of protective immunity utilizing M2 as an antigenic target. The overall goal of the research project presented here is the development of two novel vaccination platforms focused on M2-specific antibodies. One strategy involves the development of novel single-chain minibodies specific for the M2e of influenza while the second employs the use of a novel recombinant viral vector encoding the M2 for vaccination. Both approaches were developed around the central hypothesis that novel vaccination strategies targeting the M2 protein will protect against influenza virus infection including pandemic influenza. Three main specific aims were developed to address this hypothesis:

1. The generation and *in vitro* characterization of a humanized single-chain minibody specific for the M2e of influenza virus.
2. Determination of the *in vivo* capability of M2 minibodies to protect against influenza virus challenge as well as addressing the immune mechanism(s) of protection involved.
3. Demonstrating the effectiveness of vaccination with recombinant adenovirus vectors encoding conserved viral antigens for protection against challenge with a pandemic influenza virus.

The construction of M2 minibodies would provide a unique therapeutic tool for protection against influenza virus infection through passive immunization. This approach is especially appealing for certain groups including the very young and old as well as other immunocompromised populations which often benefit less from traditional influenza vaccines. Along with this, the development of a novel adenovirus vaccine capable of inducing cross-protective immunity would allow for protection against divergent influenza strains. The two vaccine approaches discussed here are by no means meant to replace traditional strain-matched HA-based vaccines; however, they represent vaccines which could be stockpiled and rapidly distributed following the emergence of a pandemic virus thereby slowing the progression of infection allowing for production of strain-matched vaccines and ultimately reducing the overall health and economic burden associated with influenza pandemics.

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CHAPTER 2

LITERATURE REVIEW

Overview of Influenza A Viruses

Influenza viruses are members of the family *Orthomyxoviridae* and are characterized by segmented, negative-strand RNA genomes. Influenza viruses are further classified into three distinct genera: influenza A, B, and C viruses. Influenza A viruses infect a broad range of host species including humans, swine, horses, certain marine mammals, and a wide array of avian species (1-4). Influenza B viruses typically only infect humans but have occasionally been isolated from other mammalian species including seals (2, 5, 6). Influenza C viruses also infect humans, swine and dogs, but cause limited disease (2). This review will focus primarily on influenza A viruses (IAV). The negative-sense genome of IAV is composed of 8 gene segments that code for up to 11 proteins: hemagglutinin (HA), neuraminidase (NA), matrix 1 and 2 protein (M1, M2), nucleoprotein (NP), two non-structural proteins (NS1, NS2), and a multiunit polymerase complex composed of two basic proteins (PB1, PB2) and one acidic protein (PA). A protein (PB1-F1) generated from a +1 shift in the reading frame of the PB1 protein has also been described (7).

Influenza viruses are subtyped according to the expression of the surface glycoproteins HA and NA (i.e. H1N1, H5N1) with 16 HA subtypes and 9 NA subtypes characterized; however, only 3 HA subtypes (H1, H2, H3) and 2 NA subtypes (N1, N2)

have been found to circulate within the human population. HA and NA play crucial roles in the replication of influenza virus in host cells. HA functions in the viral attachment to cell surface sialic acid receptors and also mediates viral-host membrane fusion following endocytosis (2). NA allows the release of budding progeny virus through the cleavage of sialic acid residues (2). It has also been shown that NA prevents virus inactivation by mucins in the respiratory tract (8). HA and NA are highly expressed on the virion surface with a HA to NA ratio of 4 to 1. Another protein expressed at lower levels on the virion surface which also plays a key role in virus replication is the M2 protein (9, 10). M2 is a homotetrameric transmembrane protein that forms an ion channel which functions to facilitate the release of the viral ribonucleoprotein (RNP) complexes following membrane fusion. M2 will be discussed in greater detail later in this review.

Each year in the United States an average of 5-20% of the population become infected resulting in an estimated 36,000 deaths with rates typically greater in high risk groups such as the young and elderly, pregnant women, and the immunocompromised (11, 12). Influenza also poses the threat of generating pandemics through the introduction of a new antigenic subtype able to infect and transmit within the human population. This process is termed “antigenic shift” and results from the reassortment of gene segments from multiple IAV strains into a novel virus. Since the early 1900s four influenza pandemics have been described. The first and worst was the “Spanish” influenza pandemic in 1918, which was caused by an H1N1 virus 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 re-entered the human population and the two subtypes have co-circulated since (2). In early 2009 a swine-origin “novel H1N1” virus emerged and resulted in the first influenza pandemic of the 21st century (13-15).

Immune Response to IAV Infection

Following influenza virus infection, both innate and adaptive immune responses are required to control virus replication. Much of our understanding of the influenza-specific immune response has been highlighted using the mouse-model of influenza virus infection. While responses observed in the murine model may not always be directly translatable to the human population (16), the vast amount of knowledge and resources available within this model provide excellent insight into human immunology.

The first responses to IAV infection are those of the innate immune system initiated through virus activation of pattern recognition receptors (PRRs) resulting in interferon/cytokine production and inflammation. Several PRRs have been shown to play a role in controlling virus infection including Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I), and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Due to both the immediate *and* downstream effects that the innate immune responses can have, the quality of these initial responses can impact the subsequent adaptive responses after infection.

TLR7 and TLR3 are PRRs which recognize single and double-stranded RNA respectively, resulting in the production of type-I interferons (17, 18). Type-I interferons

(IFNs), which include IFN- α and IFN- β , are the primary cytokines produced in response to virus 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 ensuing T cell responses towards a T_H1 bias (19, 20). Another PRR involved in antiviral responses following infection is RIG-I. It has been demonstrated that IAV infection can activate RIG-I which also results in type-I IFN production (21, 22). Another important cytokine expressed following virus infection is interleukin-1 β (IL-1 β) which functions to mediate local inflammation (19). IL-1 β expression has been shown to be dependent on the activation of another innate immune complex, termed the inflammasome, which can be activated through the stimulation of NLRs (23). Recently it has been shown that during IAV infection, the ion channel activity of the M2 protein can stimulate IL-1 β production through inflammasome activation mediated through the NOD-like receptor NLRP3 (24).

Also critical following IAV infection are the responses of the adaptive arm of the immune system which include both humoral and cell-mediated responses directed against specific viral epitopes. Antibodies specific for the HA, NA, NP, M1 and M2 proteins can be detected during and after IAV infection (25). Perhaps the most important antibody responses are those which recognize the HA surface protein. Extensive research has shown that HA-specific antibodies are protective and primarily function to neutralize the virus thus preventing infection (26-28). NA-specific antibodies, while non-neutralizing in nature, have been shown to provide protection through neuraminidase activity inhibition preventing the release of new virions thereby slowing the progression of infection (29, 30). Although protective, antibody responses targeting

the highly variable HA and NA proteins are typically found to be strain-specific and can be rendered ineffective through several mechanisms discussed later. Antibodies targeting conserved regions of the virus are therefore appealing because they would perhaps provide protection in the face of virus variability. Recently, antibodies specific for more conserved regions within the HA protein, including the stalk region and receptor-binding pocket, have been described exhibiting broadly-reacting neutralizing activity (31-33). Antibody responses targeting the conserved M2 protein will be discussed later in this review.

Antibodies also play a role in mediating responses at the interphase of the innate and adaptive immune system. These responses are primarily initiated through the recognition of antibody Fc regions by various components of the innate immune system. One such mechanism is through the 'classical' activation of the complement pathway resulting in the lysis of virus-infected cells. Several studies have highlighted the role of complement activation by influenza-specific antibodies in controlling influenza virus infection (34-37). Antibodies can also function to activate innate cellular functions mediated through Fc receptor recognition. The opsonization of virus particles can result in Fc receptor-mediated phagocytosis by macrophages (38). Also, influenza-specific antibodies can lead to the clearance of virus-infected cells by macrophages and natural killer cells (39, 40).

Also critical in controlling IAV infection are the cell-mediated responses of the adaptive immune system which are primarily orchestrated by CD4⁺ and CD8⁺ T cell populations. This crucial involvement was first highlighted in studies utilizing mice deficient in one or both cell populations (41-43). Perhaps the best characterized

responses are those of CD8⁺ cytotoxic T lymphocytes (CTLs). Influenza-specific CTLs recognize viral antigens presented via MHC class I molecules and are able to mediate the killing of infected cells through the release of cytolytic proteins such as perforin and granzymes as well as through the induction of apoptosis through Fas/FasL interactions. The development of MHC class I tetramer technology (44) has exponentially increased the ability to analyze CTL responses and has lead to the characterization of multiple influenza-specific epitopes recognized in both mice and humans (45-49).

Though not as well characterized as CD8⁺ T cell responses, due primarily to the difficulty in developing MHC class II tetramers, several studies have highlighted the function of virus-specific CD4⁺ T cells in the clearance of influenza virus infection (43, 50). While some evidence has suggested direct cytolytic activity against virus-infected cells (51), CD4⁺ T cells primarily promote viral clearance through the enhancement of B cell responses (42, 52) as well as the maintenance and recall of memory CD8⁺ T cell populations (53, 54).

IAV Prevention and Treatment

Currently, two primary control measures are used to combat influenza illness: vaccination and antiviral drugs. Two classes of antiviral drugs have been associated with the prevention and control of influenza virus; M2 ion channel inhibitors and neuraminidase inhibitors. M2 inhibitors will be discussed in detail later. The two currently approved neuraminidase inhibitors for prophylactic and therapeutic use are oseltamivir (Tamiflu™) and zanamivir (Relenza™), which function to inhibit neuraminidase activity, resulting in the inability of new virion release from infected cells

(55). Resistance to neuraminidase inhibitors has been a growing concern in recent years. Prior to the novel H1N1 pandemic, nearly 100% of seasonal H1N1 strains were resistant to oseltamivir with zanamivir resistance less prevalent (56, 57). While the novel 2009 H1N1 has maintained a high level of susceptibility to neuraminidase inhibitors cases of resistance have been reported (58-60). Recently in response to the spread of the novel 2009 H1N1 influenza the FDA approved the emergency-use authorization of peramivir, an experimental neuraminidase inhibitor with limited Phase II/III data, for patients with severe influenza infection (61).

Vaccination is currently the primary method for preventing influenza virus infection. Development of seasonal influenza vaccines 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 occurring in February for vaccine use in the following influenza season (62). Two primary types of seasonal influenza vaccination methods, both containing the chosen strains for that year, are licensed for use in the United States; inactivated influenza vaccines and live attenuated influenza vaccines (LAIV). The inactivated vaccines are expanded in embryonated hen eggs, purified, and inactivate using formalin or β -propiolactone. The inactivated viruses are either presented as whole virus vaccines or, more commonly, as split or subunit vaccines. Currently, whole virus inactivated vaccines are not licensed for use in the United States. Each vaccine dose contains 15 μ g (7.5 μ g for children <3 years) of quantified HA from each of the three strains and is administered intramuscularly, with one advantage of inactivated influenza vaccines being that they can be given to the majority of the population greater than 6 months in

age. The focus of inactivated vaccines is the induction of neutralizing serum antibodies against HA with a serum antibody titer or ≥ 40 (as determined by hemagglutination inhibition (HAI) assay) associated with protection (63).

For generation of LAIVs the selected strains are reassorted with a master donor virus and the resulting vaccine strains express three phenotypic characteristics; cold adapted (*ca*), temperature sensitive (*ts*), and attenuated (*att*). The *ca* phenotype is a virus with efficient replication at 25°C, the *ts* phenotype results in a virus that is unable to replicate at 37 to 39°C, and the *att* phenotype is a virus that does not result in influenza illness. LAIVs are administered intranasally with virus replication restricted to the nasopharynx region. Unlike the inactivated vaccine, LAIVs are only recommended for healthy individuals 2-49 years old. LAIVs are also associated with not only inducing neutralizing serum antibody titers against HA but also generating mucosal antibody and cell-mediated responses (64).

The current vaccine strategies are associated with a number of drawbacks. The vaccine strains for both platforms are propagated in embryonated hen eggs and as a result anyone with egg-related allergies cannot receive either vaccine. Propagation in eggs also results in production issues. Of the greatest importance however, is the need to antigenically match the current circulating strains for high vaccine efficacy. As seen with the emergence of the novel 2009 H1N1 (65), it takes time from the report of the first cases until the generation, production and distribution of a matched-vaccine. Even with a well matched vaccine, the vaccine efficacy in the population can still be as low as 70% (66). This reduced vaccine efficacy with the current vaccines can also be enhanced by antigenic drift within the circulating viruses. Antigenic drift is the antigenic variability

associated with certain proteins, primarily HA and NA, resulting in immune evasion of the virus. It is believed that two phenomena play a role in antigenic drift. The first is immune pressure exerted on the virus, primarily through neutralizing antibodies, often resulting in the generation of escape mutants (2). The second is attributed to the viral RNA dependent RNA polymerase (RdRp) involved in viral replication which lacks proofreading capabilities often resulting in the accumulation of nucleotide errors which can alter the antigenic surface of the virus (67). As with vaccine efficacy, the antigenically variable NA and HA has lead to escape mutants and resistance to certain neuraminidase inhibitors (reviewed in (68)).

Recently, there has been much focus on the development of new vaccine candidates and therapeutics targeting conserved viral antigens which could provide protection against multiple influenza subtypes and would be most effective in the face of emergent antigenically distinct viruses possessing pandemic potential. While the current LAIV vaccine has been shown to induce limited cross-protective responses (69, 70), numerous vaccine platforms have been employed to elicit robust protective antibody and T cell responses, primarily against highly conserved antigens including the NP and M2 proteins (71-77). One promising candidate is vaccination with recombinant adenovirus vectors encoding conserved influenza proteins. Recombinant adenovirus vectors (rAd) encoding influenza genes of interest were originally used to boost responses in mice which had been previously primed through naked DNA vaccination (71, 72, 78). Recently, the use of rAd delivery has been explored as a standalone vaccination strategy (73, 75). Such vaccination has been found to induce protective antibody and T-cell responses against homologous and heterologous influenza virus challenge. One

drawback to these protective responses is that they do not provide sterilizing immunity as with neutralizing antibodies. In light of this, vaccines inducing immunity to cross-protective antigens NP and M2 are not meant to replace traditional strain-matched HA-based vaccines but rather slow the progression of infection from a divergent influenza strain allowing for the generation and distribution of strain-matched vaccines.

Influenza M2 Protein

As previously mentioned, a means of overcoming the loss of influenza vaccine efficacy attributed to antigenic drift would be to target antigenic proteins which are more conserved within the viral genome and there by more guarded against nucleotide variability (79). One potential target is the influenza M2 protein. As stated above, the M2 protein forms a transmembrane ion channel on the virion surface and functions in viral replication through acidification of the internal viral compartment resulting in the dissociation of the RNP complexes from M1 (80). Under the low pH conditions in the endosome (~pH 5) key amino acids in the transmembrane domain undergo a conformation change which allows the influx of ions into the internal space resulting in the dissociation of the RNP complexes from the M1 protein allowing for release of the viral genome into the cytoplasm. M2 expression in the host cell Golgi complex suggests that M2 also plays a role in viral packaging through preventing pH induced changes in HA (81, 82). Although M2 expression on the virion surface is much lower compared to HA (1-2% of HA) (83), it is highly expressed on the surface of infected cells (9, 10, 84). Recent studies have shown that surface-expressed M2 plays a role in mediating filamentous virion formation as well as virus budding (85, 86). The structure of M2 is

that of a homotetramer with each subunit composed of a 54 amino acid (aa) internal domain, a 19 aa transmembrane domain, and a 23 aa extracellular (M2e) domain (80). It is this M2e region that has maintained high homology across human IAV strains. Several mechanisms have been proposed by which M2e has maintained high rate of conservation. The first deals with the M2e gene sequence itself. M2 is generated from a splice variant of the M1 protein in which the first 9 aa are identical between M1 and M2 and residues 10-24 are the result of a +1 shift in the M1 sequence. As a result the entire M2e sequence overlaps with M1. Because M1 is also a highly conserved protein it is suspected that any mutations within the M2e coding region are deselected (10). A second mechanism proposed by which M2e has remained relatively unchanged is the lack of a substantial sustained immune response directed against M2e following influenza virus infection, therefore exerting less immune pressure on M2 (87-89).

As mentioned previously, antiviral drugs targeting M2 (amantadine and rimantadine) have been used to control influenza virus infection, through the inhibition of the ion channel activity of M2. Recent evidence suggests this is accomplished by either by binding to and blocking the M2 ion channel in the “open” state (90) or by binding to M2 in the “closed” state and preventing conformational change (91). Because the transmembrane domain exhibits greater antigenic variability compared to the M2e domain, a high rate of resistance to amantadine and rimantadine has been seen in human IAV strains. This resistance is associated with amino acid changes within the transmembrane domain that either prevent the binding of inhibitors or changes that retain inhibitor binding ability but do not affect ion channel activity (92).

Despite the poor immune response generated against M2e during influenza virus infection it is possible for M2e to induce antibodies that influence viral replication. This was first described by the generation and characterization of the M2e-specific monoclonal antibody 14C2 (83). This monoclonal antibody has been shown to restrict influenza virus replication *in vitro* as well as decrease the amount of cellular expressed M2 protein (83, 93, 94). Passively transferred 14C2 mAbs have also been associated with the reduction of influenza virus replication *in vivo* in addition to conferring protection against viral challenge (95). The fact that it is possible to generate an M2-specific protective immune response has led to the exploitation of M2e as a vaccine antigen utilizing various vaccine platforms and, depending on the vaccine strategy used, been shown to generate antibody and cell-mediated immune responses (71, 96-104). Advancements in molecular biology techniques along with the generation of transgenic mouse models have also led to the generation of fully human monoclonal antibodies targeting M2 that are able to confer protection against influenza virus challenge when used in either a prophylactic or therapeutic setting (105-107). Because of the protection mediated by M2-specific immune mechanisms and the high rate of conservation exhibited by M2e, M2e has been proposed as an immunological target with the potential to confer heterosubtypic immunity against varying IAV strains.

The mechanisms of protection utilized by M2-specific antibodies are not well understood. One study suggests that M2-specific antibodies generated in response to vaccination with M2 protein provide protection through antibody dependent cellular cytotoxicity (ADCC) activity against influenza virus infected cells mediated by natural killer (NK) cells. This protection was also afforded when serum from vaccinated mice

was transferred into mice deficient in the complement protein C3 suggesting that complement activation does not play an important role in protection (108). It should be noted that this study involved the use of serum antibodies following vaccination and more than likely does not accurately reflect the mechanisms of protection involved following treatment with M2-specific monoclonal antibodies. This is highlighted in a recent study utilizing M2-specific monoclonal antibodies which demonstrated both ADCC mechanisms and complement activation involved in protection (107). In contrast to this, it has been suggested that M2-specific antibody protection is independent of ADCC mechanism but instead related to the biochemical nature of the specific epitopes (109). This could account for the *in vitro* activity exhibited by M2-specific antibodies on viral replication in the absence of any immune effector mechanisms. Regardless of the mechanisms involved, as mentioned above, one drawback to the protection provided by M2-specific antibodies is that M2-specific antibodies are non-neutralizing antibodies and do not prevent influenza infection. Rather, they reduce virus load and the time to clear infection, similar to the benefits of NA inhibitor treatment and prophylaxis.

Monoclonal Antibody Therapies

The development of antibody based therapies was revolutionized by the discovery of hybridoma technology (110) and rapidly lead to the generation of monoclonal antibodies (mAbs) for human use (111). However, it was quickly found that the use of murine monoclonal antibodies in humans was associated with certain effects including a high rate of immunogenicity against the murine constructs as well as decreases in serum half-life and effector functions. This led to the development of

chimeric and humanized antibodies in efforts to limit the immunogenicity of the non-human constructs (112, 113). Chimeric antibodies contain murine variable regions associated with human constant regions whereas humanized antibodies only retain the murine complementary determining regions (CDRs) on a human antibody scaffold. The latest technological advance in engineering antibodies is the generation of fully human monoclonal antibodies through either the creation of phage display libraries or the use of transgenic mice containing human antibody gene sequences (114). While these constructs are still associated with minor immunogenicity the effects are generally well tolerated and several humanized or fully human antibodies have been developed for use in humans (115).

The use of monoclonal antibody-based therapies is not without associated drawbacks including feasibility of large scale production, high production costs, and even the *in vivo* pharmacokinetics of ex vivo generated antibodies (116, 117). Due to the relatively short serum half-life of administered mAbs which often results in the need for multiple administrations to maintain therapeutic levels, a number of recent studies have explored the potential of therapeutic antibody gene delivery for the sustained *in vivo* expression of mAbs (118-125). Multiple delivery methods have been implored including the use of viral-vectors encoding the gene of interest as well as delivery of naked DNA through intramuscular or intravenous routes. Recently, several studies have shown the effectiveness of intramuscular DNA delivery followed by electroporation resulting in efficient DNA uptake in skeletal muscle cells resulting in the sustained mAb expression (124-127). One particular study of interest demonstrated the intramuscular delivery followed by electroporation of DNA encoding a mAb specific for the HA protein

of the A/PR/8/34 strain of influenza resulted in therapeutic levels of expression of HA-specific mAbs able to protect against a lethal viral challenge (125).

One method for identifying and generating novel monoclonal antibodies is through the screening of antibody gene libraries using phage or yeast display technology resulting in the isolation of the heavy and light chain variable gene segments associated with recognition of the target of interest. Traditionally, generation of full-length monoclonal antibodies requires insertion of these identified gene segments into separate plasmids for the expression of each antibody chain. One major drawback associated with this method of mAb production is the difficult task of intracellular incorporation of both plasmids along with the need for equal expression of both heavy and light chains. As an alternative approach, several studies have explored inserting the identified variable gene segments into novel vectors resulting in expression of single-chain antibody fragments (scFv) associated with antibody constant regions (scFv-Fc) (123, 128-130). In one particular study of interest Di Niro et al (123) described the generation of novel single-chain antibody fragments, which the authors termed 'miniantibodies', capable of mimicking certain full-length monoclonal antibody properties including dimeric structuring, target-specific recognition, and the capacity to mediate Fc-related immune functions such as complement fixation.

The delivery of antibodies through passive immunization for protection against influenza infection is appealing because it would allow administration of protective antibodies to groups that are generally poor responders to traditional vaccination such as the very old, very young, or immunocompromised. These are also groups traditionally at high risk of complications from influenza virus infection (12). It could also

serve as an option for people who cannot receive the traditional vaccine due to egg-based allergies or other contraindications. Given the conserved nature of M2e, an antibody therapy based on M2 would prove beneficial in the event of the emergence of a pandemic strain of influenza. The administration of M2 specific antibodies able to recognize a novel influenza strain could potentially slow the progression and spread of a pandemic strain allowing time for the development of conventional strain-matched vaccines. Also, having a tool that can be administered on a prophylactic or therapeutic basis would be critical for rapid, post exposure control or first-responder protection in the case of a pandemic.

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CHAPTER 3

A HUMANIZED ANTI-M2 SCFV SHOWS PROTECTIVE IN VITRO ACTIVITY AGAINST INFLUENZA ¹

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Abstract

M2 is one of the most conserved influenza proteins, and has been widely prospected as a potential universal vaccine target, with protection predominantly mediated by antibodies. In this paper we describe the humanization of 14C2, a potent monoclonal antibody against M2. We show that the humanized antibody demonstrates similar activity to the parental mAb: it is able to recognize M2 in its native context on cell surfaces and is able to show protective in vitro activity against influenza, and so represents a potential lead antibody candidate for universal prophylactic or therapeutic intervention in influenza.

Introduction

Each year in the United States five to twenty percent of the population contract influenza virus, more than 200,000 people are hospitalized from complications, and approximately 36,000 people die (1-3). Vaccines are the mainstay of prophylaxis, but there are technical and safety issues that must be overcome. These include difficulties in predicting which viral strains may emerge, preparing sufficient quantities of vaccine, poor immunogenicity in the elderly and very young, and difficulty in storage and administration. Influenza anti-viral drugs are an important adjunct to vaccination; however, substantial drug resistance has developed to two of the four currently approved anti-viral drugs (4). Furthermore, only two anti-viral drugs (rimantadine, and oseltamivir) are approved for chemoprophylaxis of influenza virus infection (5). The evidence for viral resistance to anti-viral agents indicates that more than one drug is necessary to combat influenza.

This has led to the search for new therapeutic and vaccine approaches for influenza; in particular, the concept of a “universal vaccine”. The M gene of influenza A encodes two proteins in overlapping frames: M1, the capsid protein and M2, an ion channel protein. Both M1 and M2 are highly conserved, with M2 encoding a small ectodomain (M2e) (6), making it a potential target for antibody-based immunity. The ability of a monoclonal anti-M2e antibody to reduce viral replication (7) suggested M2e as a potential vaccine target. Slepushkin et al. demonstrated protection following M2e vaccination using baculovirus-expressed protein, with serum antibody responses detected against both amino- and carboxy-terminal M2e peptides, and presumed to be responsible for the protection against lethal challenge with a matched (H2N2) influenza

A virus (8). In 1999, Neirynck et al. described a “universal vaccine” based upon the 23 a.a. ectodomain of M2 and demonstrated protection against H3N2 challenge virus with an M2e sequence identical to or differing by one amino acid from the vaccine constructs (9). Multiple antigenic peptide (MAP) vaccines have also been shown to be protective (10). While most human H1 and H3 influenza viruses share complete homology with the M2e consensus sequence (called M2con here), M2e-specific antibodies have not been shown to bind to M2e peptides with considerable sequence divergence. In a study of M2e-carrier conjugate vaccines, serum antibodies specific for M2con or the M2e sequence of A/PR/8/34 (A/PR8, H1N1) did not cross-react with M2e peptides from H5 and H7 avian viruses having 3 or 4 mismatches out of 24 a.a. (11). In another study, immunization with plasmid containing the entire M gene from A/PR8 was shown to protect against matched (H1N1) challenge, however there was limited evidence for M2-specific immune responses (12, 13). Importantly, while a recent study used matched M2e peptide-liposome vaccines of various subtypes (14), none of the previously published work has documented protection against challenge with influenza viruses across substantial M2e sequence differences from the immunizing antigen and specifically against potential pandemic H5N1 influenza challenge. In contrast, we have obtained evidence that M2-specific antibody responses can potentially be broadly cross-reactive and protect against divergent influenza virus challenge (15). While vaccines based on M2 may become very useful, it is also possible that human or humanized antibodies recognizing M2 may be effective as passive vaccines or therapeutics.

The sequences of six murine anti-M2e mAbs, generated either by consecutive pulmonary infection (10) or immunization with purified M2 (7), have recently been

published (16). These all have very similar recognition properties, with the recognized epitope located between amino acids 4-16 of the external portion of M2 (16). Interestingly, these mAbs all use the same VH, DH and JH genes, with minor differences (less than 7%) between them, and only two different kappa light chains. One of these antibodies, 14C2, recognizes M2e when expressed on the cell membrane after infection (7), and reduces viral plaque size (7) and viral production levels (17) in vitro. Both 14C2 and another antibody, M2-80, have also been shown to have significant protective effects in mice (18-20).

The demonstrated anti-viral activity of 14C2 suggests that it would be a good candidate for humanization (21-30), a term describing a series of techniques in which the sequence of a murine antibody is changed so that it more closely resembles a human antibody sequence. Conceptually, this involves taking the binding loops of the murine antibody and grafting them onto a human variable region framework in such a way that they are still able to recognize the antigen of interest. This often involves the retention of some critical murine framework amino acids required to maintain the correct orientation of the binding site loops, as well as subsequent mutation and selection to maintain affinity. Humanization has been widely used, and nine approved drugs are humanized antibodies. Although a number of different methods to carry out humanization have been developed (21-30), none of them has been demonstrated to be significantly superior to any other. Here we describe the humanization of the M2e-specific murine mAb, 14C2, demonstrate specificity for the native M2 protein, and confirm anti-viral activity of the humanized single-chain minibodies.

Materials and Methods

Viruses, cell lines, and monoclonal antibodies

The influenza virus used in these studies was A/Udorn/307/72 (A/Udorn, kindly provided by Dr. Suzanne Epstein, FDA/CBER, Bethesda, MD, USA). A/Udorn was cultured in the allantoic cavity of 9 day-old embryonated chicken eggs for 3 days at 35°C. Allantoic fluid was collected, cleared by centrifugation, aliquoted, and stored at -80°C. Virus titers were confirmed by TCID₅₀, plaque, and/or hemagglutination assays as described. Madin-Darby canine kidney (MDCK) cells (ATCC) were cultured in DMEM containing 5-10% FBS. FreeStyle™ 293-F cells (293-F; Invitrogen, Carlsbad, CA, USA) were cultured according to the manufacturer's instructions. The hybridoma for the 14C2-S1-4 mAb (IgG2a; gift of Dr. Walter Gerhard, The Wistar Institute, Philadelphia, PA, USA) was cultured in DMEM+10% FBS. The hybridoma for the the purified mouse control mAb, anti-influenza NP (IgG2a, gift of Dr. Jon Yewdell, NIAID, Bethesda, MD, USA) was cultured in DMEM+10% FBS.

scFv and minibody cloning

The h14C2 VH and VL genes were ordered from Blue Heron Biotechnology. They were cloned individually into our phage display vector pDAN5 (31) using BssHII and BspEI for VL and KpnI and NheI for VH. The characteristics, and the cloning procedures, of the mouse and the human version of the constructs encoding for miniantibodies are reported in detail in (32), specifically, the cloning of the h14C2 scFv, as well as of the control D1.3 (33) was performed by excising the scFv genes from the pDAN5 phage display vector using BssHII and NheI and cloning directly into the

minibody constructs using the same enzymes, and so replacing the resident 2.8 scFv gene.

scFv expression

The pDAN5 plasmids containing scFvs (h14C2 or D1.3, the control scFv) were transformed into DH5 α F' and cultured on 2XTY agar plates (containing 3% glucose and carbenicillin 50 μ g/ml). The following day a small streak of bacteria was added to 10 ml of carbenicillin/glucose 2XTY media and grown to 0.5 OD₆₀₀. The bacteria were centrifuged and re-suspended in 10 ml of carbenicillin/IPTG (250 mM) 2XTY media and protein production was allowed to proceed at 30°C overnight. The following day, bacteria were centrifuged and the culture supernatant used in ELISA.

Peptide synthesis

The M2con peptide was synthesized on a CEM microwave synthesizer using standard 0.1 mmol Fmoc chemistry. All reagents were Biochem or HPLC grade obtained through Sigma Aldrich, Novabiochem or Fisher Scientific, respectively. The peptides were deprotected with TFA/Water/TIS (95:2.5:2.5) and DTT added (2 mg/mL). Crude peptide purities were in excess of 70% and the peptides were purified using a linear gradient from 92:8 to 60:40 Water/ACN with 0.1% TFA. Analyses of the purified peptides were obtained on a Thermo Electron LCQ Deca in ESI + mode with M²⁺ of the peptides seen as dominant signal. The peptide was obtained in over 50% of theoretical yield in > 98% chemical purities.

ELISA testing of scFv on M2 peptides

Unlabeled purified M2 peptide was conjugated to BSA and KLH using Inject maleimide activated supercarrier immune modulator (BSA) and mCKLH (Pierce Inc.) kits according to the manufacturer's instructions. The success of conjugation was evaluated by 14C2 IgG based ELISA. Antigens were added to Nunc Maxisorp 96 well plate at 1 µg/100 µl concentration and incubated at 4°C overnight. Excess protein was washed with PBSLT (1XPBS with 0.01% tween) and 200 µl of wonder block (0.3% BSA, 0.3% milk, 0.3% fish gelatin) and incubated for 1 hour at RT. After washing with PBSLT, 70 µl of scFv culture supernatant and 30 µl blocking agent were added per well and incubated for 1 hour. Unbound reagents were washed using PBST (1XPBS with 0.1% tween) and PBSLT. The bound scFv was detected using anti-SV5 antibody (1 mg/ml) followed by anti-mouse HRP (Dako Inc. 0.5 mg/ml). The HRP activity was detected using TMB (Sigma, Inc.) and quenched with 1M H₂SO₄. ELISA values are given as absorbance at 450 nm. The mouse 14C2 IgG was purchased from Affinity BioReagents Inc. and used at 1 mg/ml concentration. Its binding was also detected as described.

Expression of scFv in 293 cells

The various minibody constructs were expressed using the FreeStyle™ 293 Expression System (Invitrogen), following the manufacturer's instructions. In brief, 293-F cells were cultured in suspension in FreeStyle™ 293 Expression Medium, transfected with 300µg of plasmid per 250ml of cells and cultured for 72. Culture supernatants were collected, filtered and assayed for minibody expression by M2-specific assay, V5 epitope or Fc in western blot and ELISA assays. In some cases, scFvs were purified

using HiTrap protein G columns (GE Healthcare) or ProPur Protein A spin A columns (Nunc, Rochester, NY, USA) following standard procedures. To assess concentrations of minibodies in culture supernatants, purified minibodies and culture supernatants were titrated and assayed by western blot. Proteins were detected by V5 tag and concentration in supernatants determined as compared to the purified minibodies using densitometry.

Creation of HEK 293 M2 cell lines

The M2 cDNA from influenza A/PR/8/34 (PR8-M2, Genbank accession # AF389121.1) was synthesized and cloned into the pJ5 vector (Integrated DNA Technologies, Coralville, IA, USA) with HindIII and EcoRV restriction sites at the 5' and 3' end, respectively. PR8-M2 was cloned by restriction digest and ligation into pcDNA5/FRT/TO (Invitrogen). Cloning was confirmed by sequence analysis. To generate the M2 consensus construct (M2con) the pcDNA5/FRT/TO-M2-PR8 plasmid was mutated using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. In brief, nucleotides 61-63 of the cDNA were altered from GGT to GAT, changing Glycine 21 to Aspartic Acid, converting the PR8 M2e sequence to the consensus M2e sequence. The mutation was confirmed by sequence analysis. The new construct, pcDNA5/FRT/TO-M2con has the consensus M2e sequence and PR8 transmembrane and cytoplasmic sequences.

The inducible 293-M2 cell lines were generated using the Flp-In™ T-REx™ 293 Cell Line system (Invitrogen) following the manufacturer's instructions. This system enables insertion of a cDNA in to a unique insertion site in the genome using site-

specific DNA recombination. Gene expression is regulated by a Tet repressor and is induced by the addition of tetracycline. 293-M2 cells were cultured in DMEM containing 10% FBS containing blasticidin and hygromycin. The M2 expression was induced by adding tetracycline to the media (1 µg/ml final). Cells were incubated for 72 hours, fixed, and M2 expression measured by flow cytometry using the M2-specific mAb, 14C2-S1-4. M2 positive clones of 293-PR8M2 and 293-M2con were selected and used throughout the studies.

Cell ELISA

The cell-based ELISA for M2-specific antibody binding was done as previously described (34) with modifications. In brief, 239 control, 293-PR8M2, or 293-M2con cells were plated in 96-well tissue culture plates in DMEM containing 10% FBS and tetracycline (1 µg/ml final; to induce M2 expression), and incubated at 37°C, 5% CO₂. In some experiments control wells included 293-PR8M2 or 293-M2con without tetracycline induction. Seventy-two hours later, plates were gently washed with PBS, fixed with 4% formaldehyde, washed, and then blocked with PBS containing 5% non-fat dry milk and 0.5% BSA. Antibodies or minibodies were serially diluted in PBS containing 1% BSA and incubated with the cells for 2 hours at 37°C. Plates were washed and incubated with secondary antibody (goat anti-mouse IgG (H+L) or goat anti-human IgG (H+L) phosphatase labeled; Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD, USA). Plates were developed with pNPP Phosphatase Substrate (Kirkegaard & Perry Laboratories) following the manufacturer's instructions. Absorbance was measured at 405 nm on a 96-well plate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometry

Control 293, 293-PR8M2, and 293-M2con cells were cultured in DMEM containing 10% FBS with or without tetracycline (1 µg/ml final) to induce M2 expression. Seventy-two hours later, cells were harvested by washing with PBS (no Mg⁺⁺ or Ca⁺⁺) + 2.5 mM EDTA, fixed with 4% formaldehyde, washed, and then blocked with PBS containing, 2% FBS, 0.02% azide, and FcBlock (BDPharmingen). Antibodies or minibodies were diluted in PBS containing 2% FBS + 0.02% azide and incubated with the cells for 20 minutes at on ice. Cells were washed and incubated with secondary antibody (goat anti-mouse IgG-Alexa 488 or goat anti-human IgG-Alexa 488, Invitrogen) in PBS containing 2% FBS + 0.02% azide for 30 minutes on ice. Fluorescence was measured on a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blotting

Control 293, 293-PR8M2, and 293-M2con cells were cultured, induced, and harvested as in the flow cytometry protocol. Cells were then either fixed with 4% formaldehyde or left untreated. All samples were then lysed using PBS containing 0.5% Triton X-100. The lysates were briefly sonicated and separated in non-reducing 12.5% Tris-HCl, SDS-PAGE gels following standard protocols. The separated proteins were transferred to PVDF membranes (Millipore) and blocked using TBS-0.05% tween 20 containing 5% non-fat dry milk. Blots were incubated with antibodies or minibodies (diluted in TBS-0.05% tween 20 + 2% non-fat dry milk, washed and then incubated with detection antibody (goat anti-mouse IgG (H+L) or goat anti-human IgG (H+L) phosphatase labeled; Kirkegaard & Perry Laboratories, Inc). Blots were developed

using ECF reagent (GE Healthcare, Piscataway, NJ, USA) and imaged using a Typhoon Imager (GE Healthcare).

In vitro plaque inhibition assay

MDCK cells were plated into 12 well plates and allowed to grow to near-confluence. Approximately 30 pfu of A/Udorn virus was diluted into MEM containing 1 µg/ml TPCK-treated trypsin (Worthington Biochemical, Lakewood, NJ, USA) and antibody or minibody as indicated. Virus with minibody was incubated for 30 minutes at 4°C. The plates were washed with MEM to remove serum-containing medium and the virus was added to each well in 0.1 ml. Cells were infected for 2 hours at 37°C and then overlaid with MEM + 1.2% Avicel (35), 1 µg/ml TPCK-treated trypsin, and antibody or minibody at the indicated concentrations. Plates were incubated at 37°C for 2 days, the overlay gently washed off using PBS, fixed with ice-cold methanol/acetone (40%:60%), air-dried, and the plaques visualized by crystal violet counter-stain. Plates were imaged on a Typhoon Imager and plaques counted manually.

Results

M2e conservation

The sequences of M2e proteins from all influenza A infecting humans were downloaded from the Los Alamos influenza database (www.flu.lanl.gov) (36). This was pared down from 1476 to 1353 sequences after all partial sequences were removed. Of these 937 (69.2%) are represented by the proposed consensus vaccine sequence (MSLLTEVETP IRNEWGCRCN DSSD) (9), and a further 294 (21.7%) differed by only

one amino acid, indicating that 90.9% of the N terminal portion of M2e of all sequenced influenza A virus infecting humans are identical to the proposed vaccine strain, or differ from it by only a single amino acid. Of those sequences that differ by more than one amino acid, 78 (5.8%) are represented in the database by less than ten independent isolates. All the H5N1 M2e sequences from viruses that have infected humans differ from the vaccine strain by at least 3 amino acids, with two changes (I11T and N20S) being found in all and one change (G16E) found in most. These changes, however, are not restricted to H5N1: 55 (4.1%) of the non H5N1 strains also show the I11T change, and 34 (2.5%) the N20S change. Table 3.1 shows a comparison of the different M2e sequences, including the two most common H5N1 sequences.

Antibody humanization

The VH and VK sequences of 14C2 (16) were compared to the human VH and VK gene families using IgBLAST (NCBI). As the first seven amino acids of 14C2 were not reported, and a number of other antibodies with the same specificity use the same VH gene, the first seven amino acids of those sequences were used (in italics in table 2). The closest human V genes were VH1-2 and B3 (VK3). Figure 3.1 shows the homology between the 14C2 sequences and the closest human sequences. These human sequences were used as templates for humanization by assigning a risk score to each amino acid on the basis of an analysis of 6 papers that examined the effects of changing framework residues that might have an effect on affinity (21-26). At those amino acid positions in which there was a risk of affecting affinity the murine residues were retained, whereas the human residues of VH1-2 and B3 were used at those

positions that appeared to have no effect on affinity or where no information on the effect of affinity was available. This approach has been successfully used in the humanization of a botulinum specific antibody without the need for additional mutation (37). In total, seven murine amino acids were retained (4 in VH and 3 in VK, underlined in Figure 3.1). The CDRs were transplanted completely, while the J regions used were consensus J sequences. The genes encoding these proteins were synthesized as separate VH and VL genes (Blue Heron Biotechnology), and cloned into pDAN5, our standard phage display vector (31).

h14C2 recognition of M2

In a first test of reactivity the scFv was shown to bind to the M2con peptide (Figure 3.2). For further testing, the scFv was recloned into two minibody constructs, in which the scFv was directly fused to the CH2 of an IgG Fc domain, as previously described (38). This provides additional stability, dimerization and potential effector functions. Dimerization is particularly important, as it has been shown that monomeric 14C2 Fab does not inhibit viral assembly (17). The humanized scFv was named h14C2, and the humanized minibody with the h14C2 scFv fused to human CH2 and CH3 constant domains, was named h14C2 human minibody. The murine equivalent, with the h14C2 scFv fused to murine CH2 and CH3 constant regions was named h14C2 murine minibody.

Although recognition of peptide is promising, M2 on the cell surface is present as a tetramer, and it is recognition of cell surface M2, and its subsequent aggregation into coated pits that is responsible for the reduction of virus production observed by some

anti-M2 antibodies, such as 14C2 (17). Furthermore, over 75% of antibodies developed against M2 during virus infection recognize cell expressed M2, but not the synthetic peptide (34). For this reason it was important to show that h14C2 recognizes native M2 as expressed on the cell surface. This was addressed using the h14C2 minibodies in three ways: flow cytometry, western blotting and ELISA.

The targets for these experiments were HEK 293 cells transfected with the full length cDNA encoding the M2 gene of A/PR/8/34 or the same construct with a point mutation (G21D), which reflects the human consensus M2e sequence. To avoid M2-mediated toxicity, expression of the M2 genes was regulated by a tetracycline-inducible promoter. Figure 3.3 shows that cell-expressed M2 proteins were recognized by the original 14C2 murine monoclonal as well as the h14C2 human and murine minibodies in flow cytometry. Signals for the 14C2 mAb and the h14C2 murine minibody are almost comparable as both are recognized by an anti-mouse secondary antibody. However, the murine minibody lacks the CH1 and light chain constant domains, which is likely to reduce the signal somewhat. The h14C2 human minibody is recognized by an anti-human secondary antibody making direct comparison difficult. Notwithstanding these caveats, incubation with equivalent amounts of the mAb and minibodies detected similar levels of the M2 protein on the surface of induced transfectants and showed no reactivity to untransfected or uninduced controls (Figure 3.3). Moreover, this confirms that the h14C2 murine minibody is able to recognize M2 within its native context on the cell surface.

During infection, M2 is expressed as a 97 amino acid viroporin present on the cell surface as a tetramer. The M2 proteins form a dimer of homo-dimers (6). Western

blots of fixed and non fixed 293-M2con and control 293 cell lysates were used to test whether the M2-specific minibodies could recognize M2 in the monomeric, dimeric, and/or tetrameric forms. Under standard polyacrylamide gel electrophoresis M2 migrates as a monomer, while fixation prior to reduction maintains M2 in tetrameric, dimeric, and monomeric forms (34). Western blotting with the 14C2 mAb or the human or murine h14C2 minibodies shows (Figure 3.4 A-E) that all forms (monomer, dimer and tetramer) of M2 are recognized by the humanized minibody, with recognition indistinguishable from that of the parental 14C2 mAb.

Finally, results similar to the flow cytometric analysis were obtained using a cell based ELISA (Figure 3.5), in which clear, titratable binding by all M2-specific constructs is shown to the induced and transfected cells, but not the untransfected cells. Fixed cells were incubated with titrated concentrations of 14C2 mAb, h14C2 human minibody, or h14C2 mouse minibody. Purified h14C2 human minibody detected native M2 on the fixed 293-con-M2 cells at least as well as the 14C2 mAb with the h14C2 human minibody titering at approximately 10 ng/ml and 14C2 mAb titering at about 40 ng/ml (Figure 3.5 A), although absolute comparisons are difficult for the reasons given above. Comparison titrations of supernatants from 14C2 mAb and h14C2 mouse minibody cultures, normalized to equivalent protein concentration by SDS-PAGE densitometry, show that the murine mAb and minibody have similar sensitivities, titering out at a 1:25,600 dilution (Figure 3.5 B).

h14C2 in vitro neutralization data

The 14C2 mAb has previously been demonstrated to inhibit influenza virus replication in an in vitro plaque-reduction assay (7, 17). As the h14C2 minibodies recognized native M2 at least as well as the mAb, the ability of the h14C2 murine minibody to inhibit influenza infection was tested in a similar assay. A/Udorn was incubated with 14C2 mAb, h14C2 murine minibody, or mAb control. In each case, the virus was incubated with M2-specific or control constructs at the time of infection and then during the 48 virus culture. Figure 3.6 shows the reduction of both plaque size and plaque number after incubation with 14C2 mAb alone or the h14C2 mouse minibody. Culture with 100 µg/ml of h14C2 mouse minibody resulted in reductions in plaque size and number, although culture with 25 µg/ml of the 14C2 mAb was sufficient to reduce plaque size and number to a similar extent. Culture with 20 µg/ml of purified 14C2 minibody was not sufficient to reduce virus replication (data not shown). Culture with the control mAb did not affect virus replication.

Discussion

While annual vaccination is a primary means to reduce seasonal influenza-related illness, antiviral therapies can be used to prevent illness or reduce the length or severity of disease (1). There are four antiviral drugs currently licensed in the US for use against influenza: the neuraminidase inhibitors, zanamivir and oseltamivir, and the M2 inhibitors, amantadine and rimantadine. Zanamivir and oseltamivir act by blocking the active site of neuraminidase, preventing cleavage of sialic acid during virus egress from infected cells and causing virus aggregation at the cell surface, while amantadine and

rimantadine act by blocking the ion channel of the M2 pore protein, preventing ion influx and release of the viral nucleic acids into the cellular cytoplasm (39). Oseltamivir and zanamivir are approved for chemoprophylaxis for seasonal or avian influenza virus, and both have been shown to be similarly effective against H5N1 virus (5). Moreover, the neuraminidase inhibitors have fewer side effects than the M2 inhibitors rimantadine and amantadine, and drug resistance seems to develop less frequently (40). Oseltamivir is currently the drug of choice for the treatment of human seasonal and H5N1 influenza and zanamivir is also suggested as a second choice (1, 5, 41). In contrast, the licensed M2 inhibitors are no longer recommended for use as the incidence of drug resistant viruses has steadily increased (42-44). More recently, the frequency of oseltamivir resistant viruses has also increased (45) and NA inhibitor-resistant avian influenza viruses have been identified as well (46). As such, the development of novel antiviral drugs for the prevention or treatment of influenza virus infection is of high priority.

Antibodies provide an appealing strategy for the prevention or treatment of viral infections. Their specificity, relatively long half life and limited toxicity are just a few of the strengths of this therapeutic modality. Although polyclonal antibodies are FDA approved for eight pathogens (hepatitis B, CMV, botulism, RSV, rabies, tetanus, VZV and vaccinia) (47), there is a clear preference for therapeutics that are better defined. There were initial hopes that rodent mAbs could be used in therapy, but their immunogenicity has led to efforts to create mAbs which are more human in their sequences. With the advent of modern molecular biology, three main classes of mAbs with lower immunogenicity have been developed. These include chimeric antibodies, in which murine V regions are fused to human constant regions (48), humanized

antibodies, in which murine antigen binding loops are grafted onto human variable region framework sequences (49), and fully human antibodies, the latter being made by phage display (reviewed in (50)), or by applying traditional hybridoma technology to mice transgenic for the human immunoglobulin loci (reviewed in (51)). As chimeric antibodies retain some residual immunogenicity, humanized and human antibodies are most frequently used and are equally represented in clinical trials and approved drugs (52).

Recombinant antibodies offer many advantages for the treatment of diseases (52), including those caused by infectious agents (53), and viruses in particular (54). Compared to antibodies produced in animals, they have greater potency, defined activity, lack infectious agents, avoid the development of serum sickness caused by immune responses to non-human antibodies, and with a half life of up to 4 weeks, provide long periods of protection with relatively infrequent dosage schedules. For all indications, eighteen mAbs have received regulatory approval and over 150 are now in clinical development (52, 53). One mAb against respiratory syncytial virus, providing significant reduction in morbidity, has been approved for treatment of high risk pediatric cases and mAbs against over twenty other infectious agents, including SARS, rabies, West Nile virus, HIV, Dengue, Ebola, Hepatitis A, B and C, anthrax, *E. coli* and *Staphylococcus*, are under development (53, 54). In addition to their therapeutic value, antibodies also have potential as passive vaccines, which can translate into months of protection following prophylactic administration: long enough to cover a flu season, or the community duration of a pandemic (55). As the means of production of human mAbs are well known, the process of manufacturing, as well as the necessary

toxicology and clinical safety testing requirements are well understood. This results in a rapid development timeline, once suitable candidates have been identified. This is especially true for antibodies recognizing infectious epitopes, rather than human proteins, in which inadvertent unexpected reactions may occur (56).

Influenza provides a number of viral targets for antibody therapies (57). Antibodies to the hemagglutinin can neutralize the virus and readily prevent infection, however these antibodies are subtype and in many cases strain or clade specific and so have limited use as antibody therapies (58), even though the efficacy of the annual vaccines is related to their ability to induce HA antibodies. Neuraminidase antibodies, while not neutralizing may also protect against infection (59). Unexpectedly, antibodies against the influenza nucleoprotein (NP), which coats the viral RNA have also been shown to be protective in mice (60), although previous studies suggest NP immunization protects via T and not B cell responses (61, 62). Finally, the M2 protein has been widely explored as a target for both vaccines and drug therapies. M2 is an appealing target as it is expressed at high levels on virus-infected cells, it is relatively conserved compared to other surface viral antigens, and antibody responses to M2 proteins have been demonstrated to protect against human and avian influenza virus infections (15, 63-65). M2e, the M2 ectodomain 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 (6).

In this paper we describe the humanization of 14C2 (7), a well characterized anti-M2e monoclonal antibody. Once recognition of the M2 peptide was established for the humanized h14C2 scFv it was converted into the minibody format (38), in which the

scFv is fused directly to either human or murine CH2-CH3 domains. Minibodies are similar to full length antibodies in their activity, but have the advantage that only one gene is required, making cloning and expression considerably easier. We were able to show that the minibody derivatives had equivalent specificity to the original mAb and recognized native M2 proteins as shown by a variety of assays including flow cytometry, western blot and ELISA. They also show in vitro activity against influenza, by reducing the size and number of plaques. However, the in vitro activity of the humanized minibody was dependent upon high concentrations of minibody. It has been previously shown that the anti-viral activity of the 14C2 mAb is dependent upon the bivalent structure of the Ab, since Fab fragments do not restrict virus replication (17). While the minibody scFv binding sites are dimerized in the minibody construct, the lack of the CH1 domain reduces the distance that the two scFvs can span, and this may account for the lower activity, that can be overcome by higher concentrations.

As with all antiviral drugs, development of antiviral resistance to M2e-specific antibodies is a concern. While drug resistance to M2 ion channel inhibitors has readily developed, the mutations enabling drug resistance are focused in the ion channel (66, 67), a region that does not overlap with M1 and may be more amenable to mutation. This is in contrast to M2e that overlaps with M1, and is more conserved as a result. Viruses resistant to M2-specific antibody therapy have been selected by extended infection of immune compromised mice in the presence of the 14C2 mAb. However, analysis of the mutant viruses showed that changes were limited to two mutants, suggesting there is a limitation to changes within the M2 ectodomain. Moreover, M2-resistant viruses were not always generated after 14C2 treatment and the infection was

controlled, suggesting that multiple mutations in multiple gene segments may be required to allow changes in M2e (20). Moreover, the mechanism of action of M2-specific antibody treatment is not fully understood. In vitro data suggest that the 14C2 mAb acts by aggregating the M2 protein on the surface of cell and preventing incorporation into budding virions, or hindering a role for the M2 cytoplasmic domain in virus assembly (17). In contrast, there is no evidence that 14C2 causes released virus to aggregate on the surface of cells or interferes with ion channel activity (17). In vivo, M2-specific antibodies may reduce virus replication by the same mechanisms described in vitro, however, there is also evidence they may function via antibody-dependent cell-mediated cytotoxicity (ADCC) (64, 68) or complement dependent cytotoxicity (CDC) (64) to clear virus-infected cells.

The availability of this humanized anti-M2 antibody will allow further investigation of these issues, as well as the possibility of specificity broadening to include avian influenza M2's in a manner similar to that which has been described for some anti-botulinum antibodies (69) using yeast display.

Table 3.1. Sequences of M2e from all viruses infecting humans in the LANL flu database represented 10 times or more.

M2e sequence	Frequency	Notes
MSLLTEVETP <u>I</u> RNEW <u>G</u> CRCN <u>D</u> SSD	937	Consensus vaccine M2con
..... ..N.	163	
..... T.....	45	
..... T....E...S	30	H5N1
..... ..E.....	26	
..... ..S.....	18	
..... ..Y.....	18	
..... ..G...	15	PR8
..... ..G.....	11	
.....L T..G.....S	10	H5N1

VH

```
<-----FWR1-----> <CDR1><-----FWR2---> <-----CDR2----->
14C2 QVQLQQSGPEVVRPGVSVKISCKGSGYIFT DYAMH WVKQSHAKSLEWIG VISTYTGKTNYSQKFKG
62.6 VH1-2 QVQLVQS.A..KK..A...V...A...T.. ..R.APGQG...M.
humanized QVQLVQSGAEVKKPGASVKVSCASGYTFT DYAMH WVRQAPGQGLEWIG VISTYTGKTNYSQKFKG

<-----FWR3-----> <--CDR3--> <---FW4--->
14C2 KATMTVDKSSSTAYMELARLTSEDSSVYYCAR RGDYDAWFAY WGQGLTVTSA
62.6 VH1-2 RV...R.T.I.....S..R.D.TA.....
humanized RATMTVDKSSISTAYMELSRSDDTAVYYCAR RGDYDAWFAY WGQGLTVTSS
```

VL

```
<-----FWR1-----> <-----CDR1-----> <-----FWR2---> <-CDR2>
14C2 DIVMSQSPSSLAVSVGEKVSMTC KSSQRLLYSSDQKNYLA WYQKPGQSPKVLIIY WASTRVS
78.2 B3 VK3 ....T...D.....L..RATIN. ....P..L... ....E.
humanized DIVMTQSPDSLAVSLGERATMNC KSSQRLLYSSDQKNYLA WYQKPGQPPKVLIIY WASTRVS

<-----FWR3-----> <--CDR3--> <---FW4--->
14C2 GVPDRFTGSESGTDFTLTISVKAEDLAVYYC QQYYTYPLT FGAGTKLELK
78.2 B3 VK3 .....S..G.....LQ...V.....
humanized GVPDRFSGSESGTDFTLTISSLQAEDVAVYYC QQYYTYPLT FGQGTKVEIK
```

Figure 3.1. Sequence of 14C2 and humanized antibody genes. The VH and VL sequences of 14C2, the closest human V genes and the humanized sequence. The different parts of the variable region (FWR: framework; CDR: complementarity determining region) are shown. Dots represent homology. Retained murine amino acids are underlined. When the 14C2 VH gene was sequenced (16), the first seven amino acids, in italics, were not determined and the sequence used here is derived from the M2-80 sequenced VH gene (16).

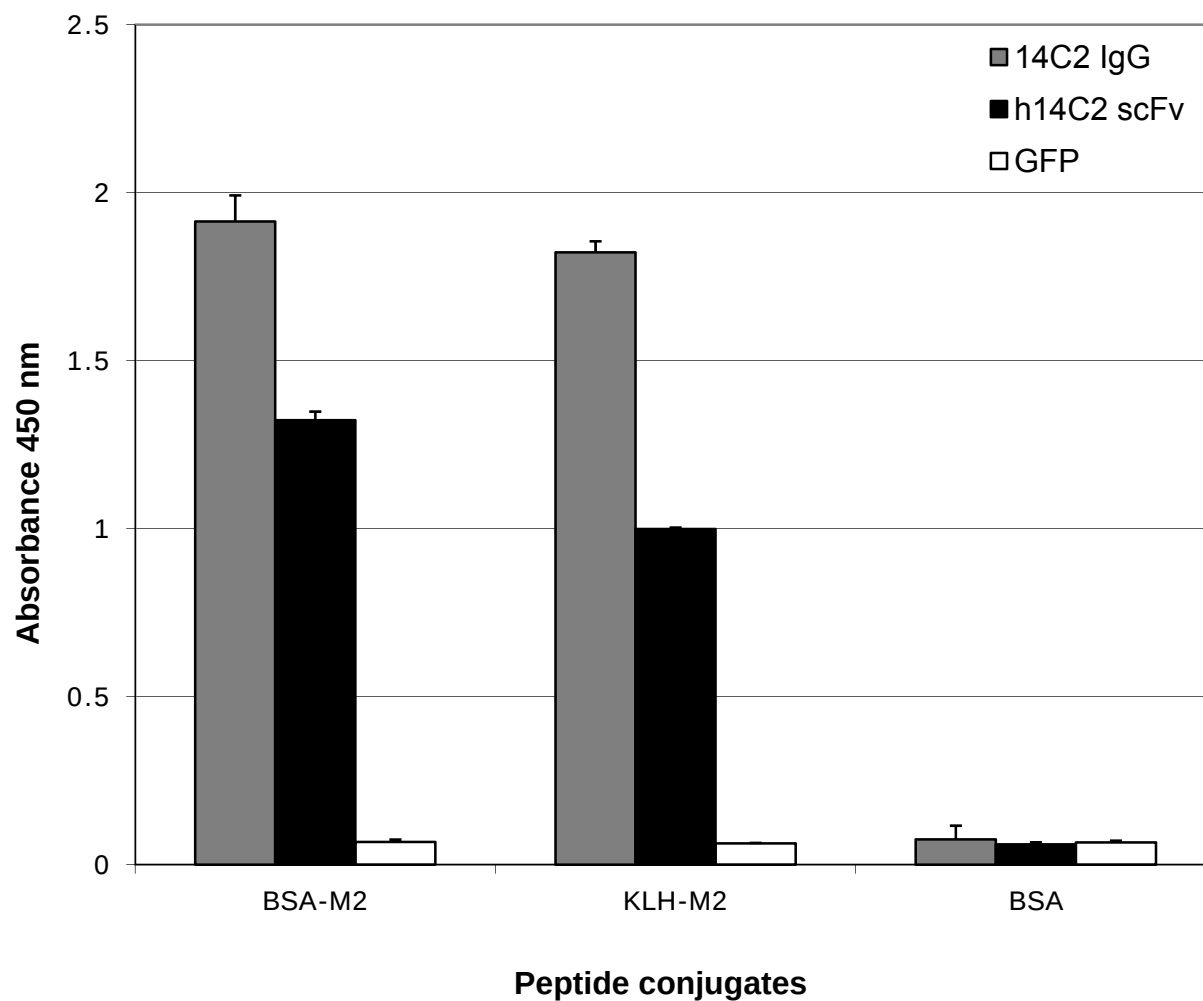


Figure 3.2. Peptide ELISA for the detection of M2. Recognition of the M2con peptide by h14C2 scFv and the parental 14C2 mAb.

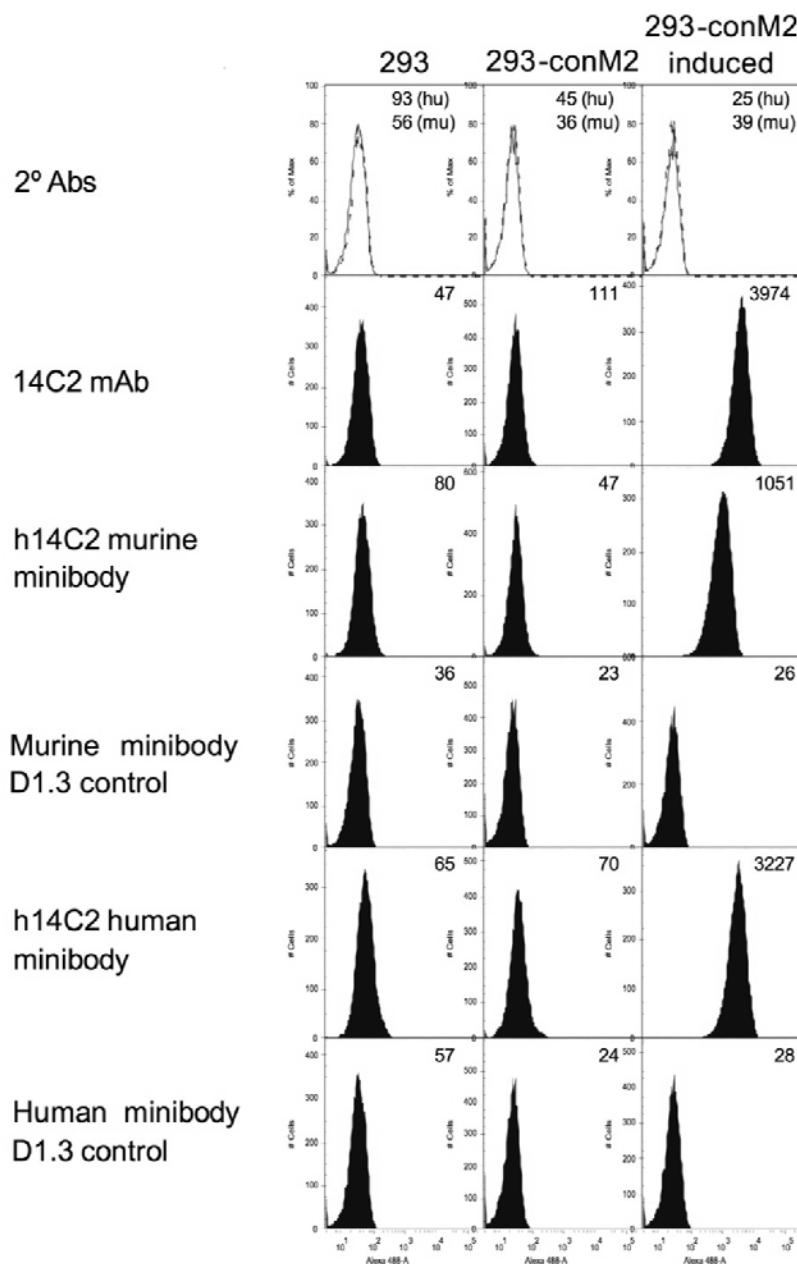


Figure 3.3. Surface recognition of M2 using flow cytometry. Detection of control 293 cells or 293 cells transfected with M2con gene (induced or not induced) by 14C2, h14C2 murine minibody, and h14C2 human minibody by. Murine secondary antibody controls are represented with dashed lines and human secondary antibody controls are represented with solid lines. Values in the upper right corners of each histogram indicate the mean fluorescent intensity of the peak.

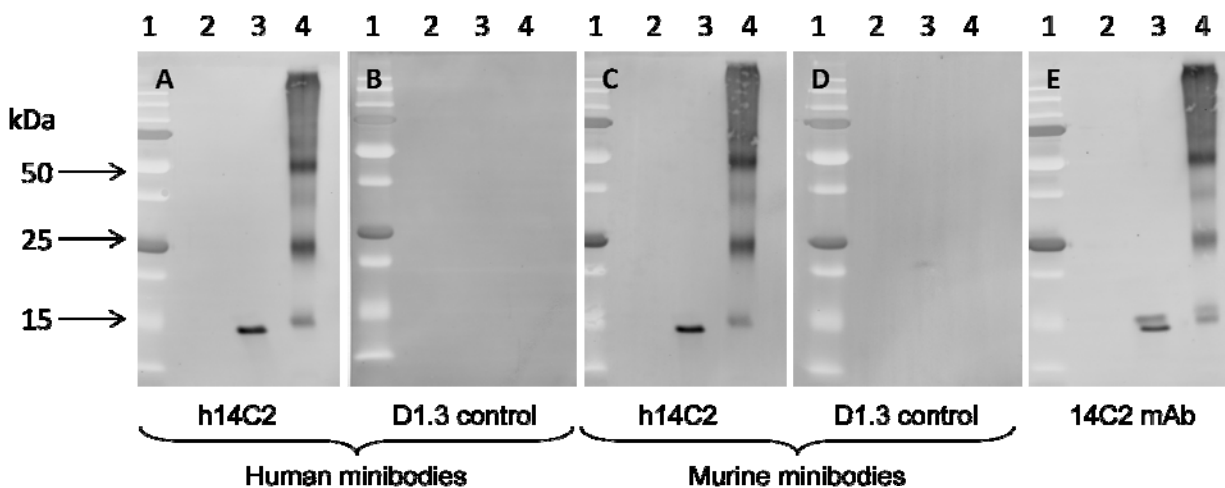


Figure 3.4. Western blot recognition of control 293 cells or 293 cells transfected with M2con gene. Western blot lanes: 1) Marker; 2) 293 cells; 3) 293-M2con; 4) 293-M2con, Fixed.

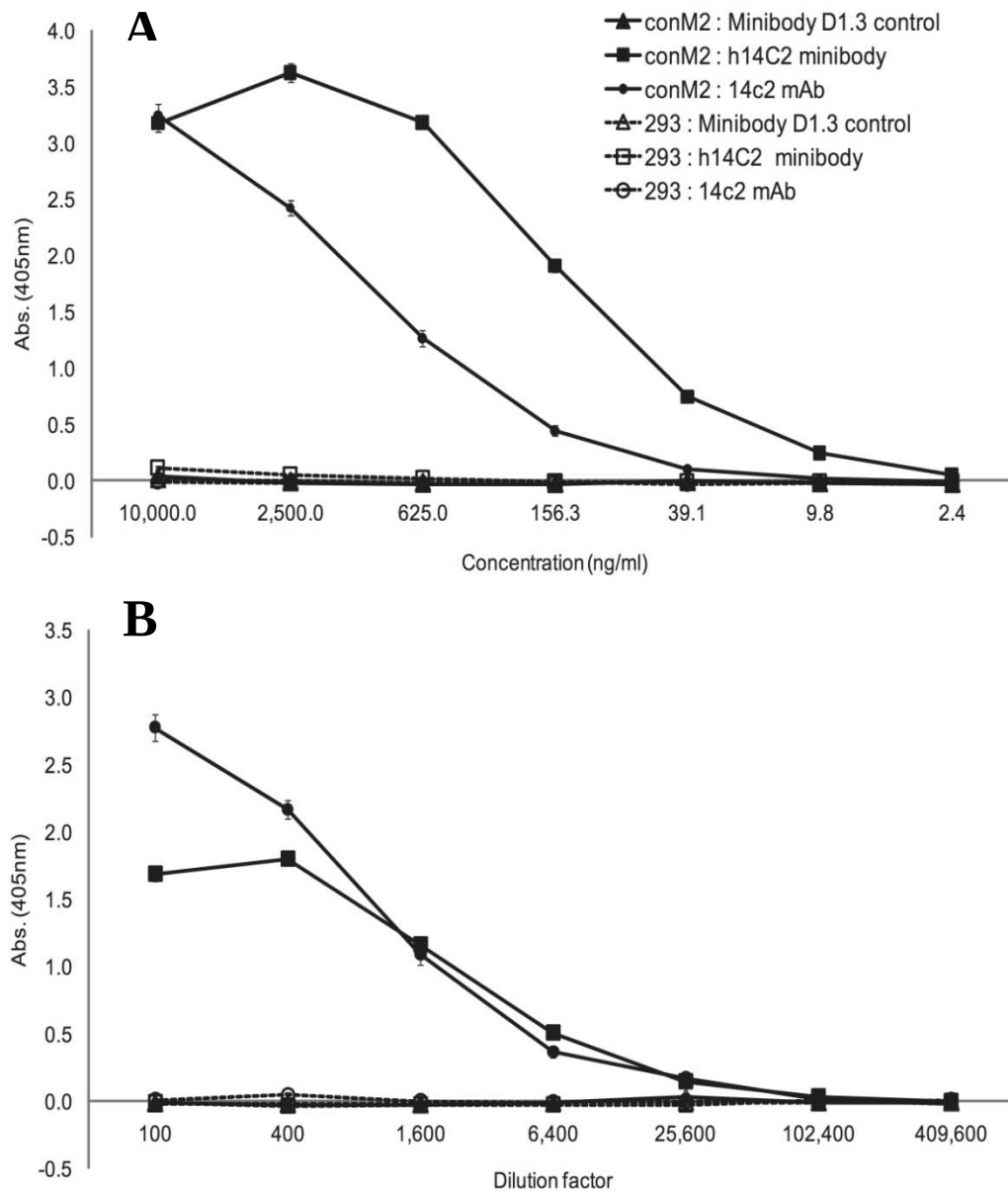


Figure 3.5. Recognition of control 293 cells (293) or 293 cells transfected with M2con gene by cell-based ELISA. A) human minibody constructs; B) murine minibody constructs.

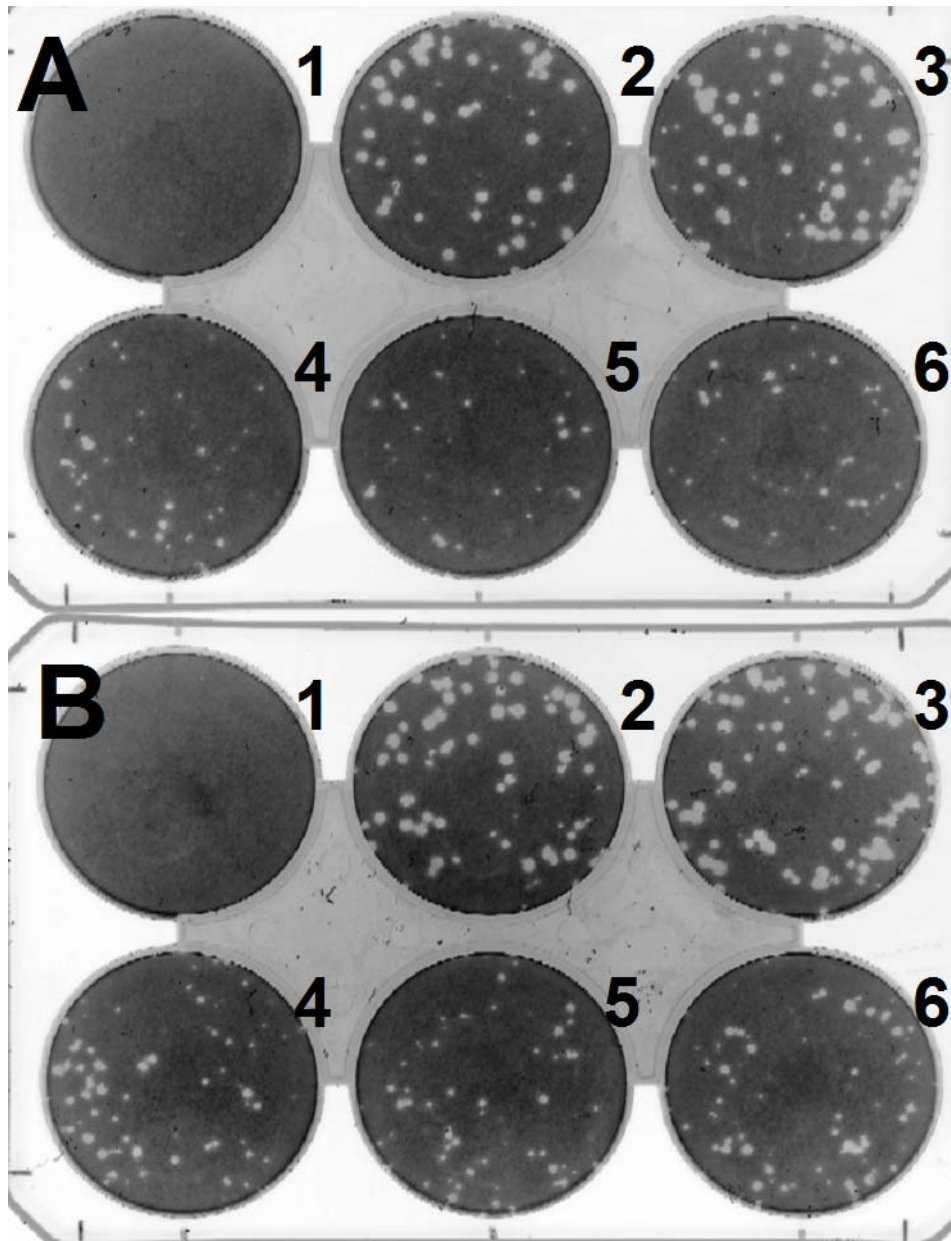


Figure 3.6. In vitro plaque reduction of viral infection. A) A/Udorn virus was cultured into MDCK cells alone (2), in the presence of anti-NP mAb (negative control, 3), or with 25µg/ml 14C2 mAb (4-6). B) A/Udorn virus was cultured into MDCK cells alone (2), in the presence of anti-NP mAb (negative control, 3), or with 100µg/ml m14C2 minibody (4-6). Well 1 shows uninfected MDCK cells on each plate.

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CHAPTER 4

PASSIVE IMMUNIZATION WITH A NOVEL MINIBODY SPECIFIC FOR INFLUENZA

M2 PROTEIN PROTECTS MICE AGAINST LETHAL VIRUS CHALLENGE ¹

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Abstract

The highly conserved M2 protein of influenza has been investigated as the potential target for cross-protective vaccine strategies mediated by protective antibodies. Passive immunization with M2-specific antibodies would allow for rapid protection, including immunocompromised populations, following the emergence of a divergent strain of influenza. We have previously described the *in vitro* activity of a novel single-chain minibody specific for the M2 protein of influenza. In this study we demonstrate the *in vivo* protective efficacy of our M2 minibody following passive immunization in mice. We also show the activation of Fc-mediated immune functions providing potential mechanisms of protection. The results portrayed here suggest the potential use of M2 minibodies as a protective intervention against influenza virus infection.

Introduction

Influenza A virus infection is still a major cause of disease in the United States affecting an estimated 5-20% of the population annually resulting in roughly 36,000 deaths (1). The greatest risk for influenza infection and subsequent complications is found among the very young and old, pregnant women and many other immunocompromised populations (2). Current vaccine strategies focus on the induction of neutralizing antibodies targeting the hemagglutinin (HA) protein of influenza strains; however, the highly variable nature of HA frequently requires the reformulation of seasonal influenza vaccines and often results in the decrease of vaccine efficacy due to antigenic drift (3). This loss of protection can be further amplified with the emergence of a pandemic strain of influenza exemplified by the recent introduction of the novel pandemic H1N1 in 2009 (4).

Passive immunotherapy using influenza-specific antibodies offers a distinct alternative to traditional vaccination, including the treatment of immunocompromised groups, such as the very young and old, who typically benefit less from active vaccination. The transfer of antibodies recognizing a conserved viral epitope would also give the added advantage of protecting against newly emergent viral strains. Advanced stockpiling and rapid distribution could be effective to reduce morbidity and mortality and slow the progression of a pandemic while strain-matched vaccines are generated (5, 6).

The matrix protein 2 ectodomain (M2e) offers an appealing target for antibody-based therapies due to a high rate of conservation among human influenza A virus isolates (7). M2e-specific antibodies have been shown to restrict *in vitro* virus replication

(8) as well as protect against virus challenge following passive transfer into naïve mice (9). Numerous studies have characterized the protective efficacy of M2-specific antibodies through both passive immunotherapy (10-13) and active vaccination (14-18). Recently, M2e antibodies specific for the consensus M2e sequence were shown to cross-react with the M2e from the recent pH1N1 and aid in protection against a lethal virus challenge despite multiple amino acid differences (19).

Several recent studies have proposed several mechanisms through which M2-specific antibodies mediate protection. Very little M2 is found on the virus surface; however, M2 is highly expressed on the surface of influenza infected cells (8, 20). Although one recent study suggests neutralizing activity of M2-specific antibodies (21), it is generally thought the presence of M2 antibodies does not result in virus neutralization but rather the elimination of virus infected cells. The immune mechanisms previously shown to be utilized by M2-specific antibodies include; complement-dependent cytotoxicity (CDC), antibody-mediated cellular cytotoxicity (ADCC), as well as potential involvement of alveolar macrophages (10-13, 16, 22).

We have previously demonstrated the *in vitro* activity of a novel single-chain 'minibody' specific for the M2e of influenza virus (23). This M2 minibody contains the humanized variable regions of the well characterized 14C2 mAb (8) and displayed the ability to recognize M2 expressed on the surface of virally infected cells as well as restrict virus growth in a plaque reduction assay. Here, we describe the *in vivo* efficacy of these M2 minibodies, showing they are capable of protecting mice against a lethal influenza virus challenge following a single passive immunization. We further show functional activation of both CDC and ADCC activities by the M2 minibodies, providing

potential mechanisms of protection. These novel M2 minibodies represent a potential immunotherapeutic tool which could be used against influenza virus infection.

Materials and Methods

Viruses, cell lines, and monoclonal antibody

The influenza virus used in this study was A/PR/8/34 (PR8). Viral stocks were generated by culture in the allantoic cavity 9-day embryonated chicken eggs for 72 hours at 35°C. Allantoic fluid containing virus was clarified by centrifugation, aliquoted, and stored at -80°C. Madin-Darby Canine Kidney (MDCK) cells (ATCC) were cultured in DMEM + 5% FBS. HEK 293H cells were cultured in DMEM containing 10% FBS. HEK 293 cells expressing the M2 protein from PR8 mutated to reflect the consensus M2e (293-M2con) sequence generated previously (23) were maintained in DMEM + 10% FBS containing blasticidin and hygromycin B. The hybridoma for expression of the 14C2-S1-4 (14C2) mAb (IgG2a; gift of Dr. Walter Gerhard, The Wistar Institute, Philadelphia, PA) was maintained in DMEM + 10% FBS.

M2 minibody expression and purification

The construction of the expression plasmid for the humanized M2e-specific minibody containing the human IgG1 Fc region (h14C2) and control minibody (D1.3) is described elsewhere (23). For minibody expression, plasmid DNA was transfected into 293H cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacture's protocol. Stable transfectants were selected for and maintained in growth media containing hygromycin B. For minibody expression, transfected cells were grown

to near confluence, after which growth media was removed and replaced with Hyclone SFM4MAb media (Thermo Fisher Scientific, Waltham, MA). Minibodies and 14C2 mAb were purified using Nunc ProPur Protein G spin columns (Thermo Fisher Scientific) following the manufacture's protocol. Purified minibodies/mAb were dialyzed against PBS, filtered through 0.22 μ m syringe filters and protein concentrations determined using a Pierce BCA Protein Assay (Thermo Fisher Scientific) as directed.

Mice and passive immunization

Four to six week old female BALB/c mice were purchased from NCI and housed under specific pathogen-free conditions. All animal protocols and procedures were approved by the University of Georgia Institutional Animal Care and use Committee. For passive immunization, mice received a single intraperitoneal administration of minibodies or mAb at a dose of 400 μ g/mouse (~20 mg/kg).

Presence of M2e-minibodies in serum

To determine the bioavailability of our minibodies after immunization, two cohorts of three mice received either 14C2 mAb or h14C2 minibody as described above. Alternating groups of mice were warmed and tail bled via the ventral artery at: 8 hr, 24 hr, 48 hr, Day 4, 7, 14, and 21 post immunizations. A baseline sample was taken prior to immunizations. The presence of minibody/mAb was determined by ELISA using the M2e consensus peptide following standard procedures. Briefly, 1 μ g/well of M2e peptide was absorbed to the surface of Nunc Immulon 2HB microtiter plates (Thermo Fisher Scientific). After blocking, serum samples were diluted 100-fold in Staining Wash Buffer

(SWB, PBS + 1% BSA) and allowed to incubate for 1 hr at room temperature. Standard curves for sample quantification were generated using either purified 14C2 mAb or h14C2 minibody at an initial concentration of 10 µg/ml followed by 3-fold serial dilutions. Standards were made in SWB containing naïve mouse serum at the diluted 100-fold to account for any serum-specific background signal. M2e-specific minibody/mAb was detected using either anti-mouse or anti-human IgG secondary antibodies labeled with alkaline phosphatase (KPL, Gaithersburg, MD) followed by the addition of pNPP substrate (KPL). Optical densities (OD) were measured at 405 nm using a Safire 2 microplate reader (Tecan, Männedorf, Switzerland).

Virus challenge

Groups of n=10 BALB/c mice were passively immunized as described above with; 14C2 mAb, h14C2 minibody, D1.3 control minibody, or left unvaccinated. Eight to ten hours after immunization, mice were anesthetized using Avertin (2-2-2-tribromoethanol) and challenged intranasally with 40 x LD₅₀ of PR8 in 50 µl. Animals were monitored daily for weight loss and survival. Mice which exhibited $\geq 35\%$ loss of original body weight were humanely euthanized.

Complement-dependent cytotoxicity assay

The ability of M2e-specific minibodies to mediate CDC was evaluated against 293-M2con target cells and 293 controls. Cells were induced for M2 expression as previously described (23) and added to 96-well round bottom plates at a concentration of 10⁴ cells/well. Target cells were incubated with the indicated concentration of

minibody/mAb in the presence of 8% (v/v) rabbit sera as a source of complement at 37°C and 5% CO₂ for 5 hrs. Rabbit serum which was heat-inactivated at 56°C for 45 min. was included as a control. All samples were run in quadruplicate. Cytotoxicity was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) following the manufacture's protocol. Cytotoxic values were calculated using the following formula: % Specific Lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) x 100. Data is presented as '% M2-specific lysis' by correcting for non-specific lysis through subtraction of control 293 only lysis.

Antibody-dependent cellular cytotoxicity assay

The ability of M2e-specific minibodies to mediate ADCC was evaluated using 293-M2con target cells and human natural killer (NK) cells as immune effector cells. Target cells were prepared as described above. Human peripheral blood mononuclear cells (PBMCs) were isolated using whole blood from a healthy donor through density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). NK cells were isolated from purified PBMCs through negative selection using a MACS human NK Cell Isolation Kit and an autoMACS cell separator (Miltenyi Biotec, Auburn, CA). Target cells were added to a 96-well round bottom plate (10⁴ cells/well) and incubated with minibody/mAb at a final concentration of 10 µg/ml for 1 hr at 4°C. After incubating, human NK cells were added at the indicated effector:target (E:T) ratio. Culture plates were then briefly centrifuged at 250 x g for 4 minutes allowed to incubate at 37°C and 5% CO₂ for 5 hrs. All samples were run in quadruplicate. Cytotoxicity was measured

using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) as mentioned above.

Graphs and statistics

All graphs and statistics were generated using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Availability after passive immunization

Single-chain Fv antibodies are known to be rapidly cleared from the bloodstream in mice (24). In efforts to increase the bioavailability, the humanized variable regions of the M2-specific 14C2 mAb were expressed as minibodies as previously described (23, 25). To determine the presence and persistence of our M2e-specific minibodies after passive immunization, BALB/c mice received a single intraperitoneal bolus of M2e minibody or 14C2 mAb at a dose equivalent to 20 mg/kg. As shown in Figure 4.1, stable levels of M2e minibodies were detected in the serum out to Day 4 post immunization with detectable levels still present at Day 7. As expected, high levels of 14C2 mAb were present in the serum following passive immunization and maintained longer than the minibody.

Protection against influenza virus challenge

Passive immunizations with monoclonal antibodies specific for M2 have been shown previously to protect mice against a lethal virus challenge (10, 11, 13). Specifically, a chimeric derivative of the 14C2 mAb has previously demonstrated the ability to protect mice from lethal challenge to varying degrees (12). We set out to determine the protective efficacy of our M2 minibodies as compared to the native 14C2 mAb. Groups of 10 mice received a single passive immunization and challenged as described in the Material and Methods. Passive immunization with M2e-specific minibodies resulted in 80% survival following a lethal PR8 virus challenge (Figure 4.2A). This protection also correlated with significant differences in weight loss compared to control mice (Figure 4.2B). All animals that received the control minibody or which were left untreated succumbed to infection or were euthanized by day 10 post challenge. Mice which received the full length 14C2 mAb exhibited 100% survival.

M2e minibodies mediate complement activation

One mechanism by which M2-specific antibodies have been shown to mediate protection is through the classical activation of complement resulting in the lysis of influenza infected cells (11, 12). We have previously shown that our M2e minibodies are able to recognize cellular express M2 protein (23) and that similar minibody constructs containing different variable regions are able to fix the C1q component of the complement cascade (25). To determine the capability of our M2 minibodies to mediate CDC we utilized target cells expressing the M2 protein from PR8 containing the consensus ectodomain sequence (293-M2con) and included rabbit serum as a source

of complement. M2 minibodies, in the presence of complement, were able to mediate the specific-lysis of target cells expressing the influenza M2 protein (Figure 4.3). This level of cytotoxicity was comparable to the 14C2 mAb. When heat-inactivated rabbit serum was used all cytotoxic activity was lost (data not shown) which attributes the cytotoxic effect to functional complement activity. Control minibodies were unable to mediate the lysis of target cells in the presence of complement.

NK cell recognition of M2e minibodies

Another mechanism by which M2-specific antibodies have been shown to mediate protection against influenza infection is through NK cells (11, 12, 16). In this model, antibodies bound to M2 expressed on the surface of infected cells would activate NK cells via Fc receptors resulting cell lysis. Given that our M2 minibodies contain the human IgG1 Fc domain we determined the ability of our M2 minibodies to mediate ADCC by using human NK cells. As shown in Figure 4.4, purified human NK cells incubated with target cells in the presence of M2 minibodies resulted in the significantly enhanced lysis of M2-expressing target cells compared to control minibodies. This level of cytotoxicity was also much greater than that observed for the 14C2 mAb most likely due to a diminished interaction of the murine Fc domain with human Fc receptors.

Discussion

The constant antigenic drift of seasonal human influenza A viruses along with the ever present threat of a future pandemic warrants the need for protective therapies targeting a conserved viral antigen. The development of protective antibodies focused

on such antigens would allow for rapid distribution following the emergence of a potential pandemic virus as well as the protection of high-risk groups who might not respond efficiently to traditional vaccination methods. In this study we examine the protective efficacy of a novel single-chain minibody which recognizes the M2 protein expressing on the surface of infected cells.

The development of single-chain fragment variable (scFv) antibodies has previously been explored for use in other disease models including Rabies virus, West Nile virus and Hantavirus (26-28). One drawback of scFv therapies is the rapid clearance of the single-chain antibodies from the circulation (24). Fusion of scFvs to an antibody Fc domain, similar to our minibodies, has been shown to increase the serum half-life after *in vivo* delivery (29). Following a single i.p. administration, M2 minibodies containing human Fc domains were detected in the serum of mice up to Day 7 post vaccination. The use of minibodies with murine Fc regions would most likely increase serum concentrations and period of circulation to levels similar to those observed for the 14C2 mAb. Minibody constructs containing human IgG1 Fc domains were used in this study over murine IgG1 due to the increased affinity for Fc receptors and propensity for complement activation (30-34).

Following a single passive immunization of M2 minibodies, BALB/c mice were significantly protected against a lethal challenge of PR8 influenza virus. Surviving mice also exhibited significantly less weight loss compared to control groups. Interestingly, this survival was not associated with significant differences in lung virus titers analyzed at various timepoints post challenge (data not shown). We offer multiple suggestions which could possibly explain these results. First, recent analysis of cytokines present in

the airways of mice immunized and challenged in a similar manner showed significantly higher levels of IL-1 α and IL-1 β present at Day 3 post challenge in mice which received the control minibody or were left naïve compared to mice which receive the M2-specific mAb or minibody (data not shown). IL-1 is a potent proinflammatory cytokine which has been shown to be associated with increased immunopathology in the lungs of mice following influenza virus infection (35). Perhaps the ability of the M2 minibodies to partially control the initial burden of virus infection results in a lowered proinflammatory environment and contributes to the observed increased survival.

Secondly, the lack of significantly decreased lung virus titers could be an artifact of the specific antibody and challenge virus combination used in our study as others have noted inconsistencies in reproducing previously published data using the 14C2 mAb. Fu et al. were unable to successfully protect mice using purified 14C2 IgG and only achieved protection using whole ascities fluid. Also, the mAbs described in that study were capable of conferring protection against influenza virus challenge; however, no effect on viral growth kinetics was observed (10). A recent study which generated a chimeric version of the 14C2 mAb reported only a 60% survival rate after challenge with a lower lethal dose of PR8 followed by 3 i.p. immunizations (12). It should be noted however, that those authors followed a more stringent humane endpoint (20% weight loss) which could account for overall survival differences. The therapeutic potential of our M2 minibodies when delivered after virus infection has yet to be determined.

The protection provided by M2-specific antibodies has previously been shown to be mediated through complement and NK cell activation resulting in the lysis of virus infected cells (11, 12, 16). We were able to demonstrate the ability of our M2 minibodies

to both activate CDC and ADCC resulting in the specific lysis of cells expressing influenza M2 protein. Not shown here, M2-specific minibodies were also able to mediate CDC against cell targets expressing the M2 protein from the A/Vietnam/1203/04 strain of influenza virus suggesting their potential heterosubtypic protective capability. It has previously been shown that certain modifications within the antibody Fc domain can enhance the ability to activate complement or increase affinity for Fc receptors (34, 36-38). One advantage of our minibody constructs over traditional monoclonal antibodies is the ability to readily manipulate the constructs through molecular techniques. This could allow for generation of minibodies exhibiting enhanced protection through more efficient activation of immune mechanisms associated with protection.

Another advantage of our minibody constructs is the potential for genetic passive immunization for the expression of minibodies *in vivo*. We have previously demonstrated this capability using a similar minibody construct through naked DNA delivery (25). Genetic delivery could also be achieved through the use of a recombinant viral vector such as adeno-associated virus (39). Both delivery methods have been shown to result in prolonged expression of the gene of interest.

In conclusion, we have demonstrated the *in vivo* protective efficacy through passive immunization of a novel single-chain minibody recognizing the highly-conserved M2 protein of influenza. Our M2 minibodies represent a unique therapeutic tool which could not only be stockpiled and rapidly distributed following the emergence of novel influenza strains with pandemic potential but also offer a protective alternative to active vaccine for high-risk groups such as the elderly and immune-compromised. Such applications would hopefully slow the progression of a potential pandemic allowing for

the generation and distribution of strain-matched vaccines as well as reduce the overall health and economic impact often associated with pandemic influenza.

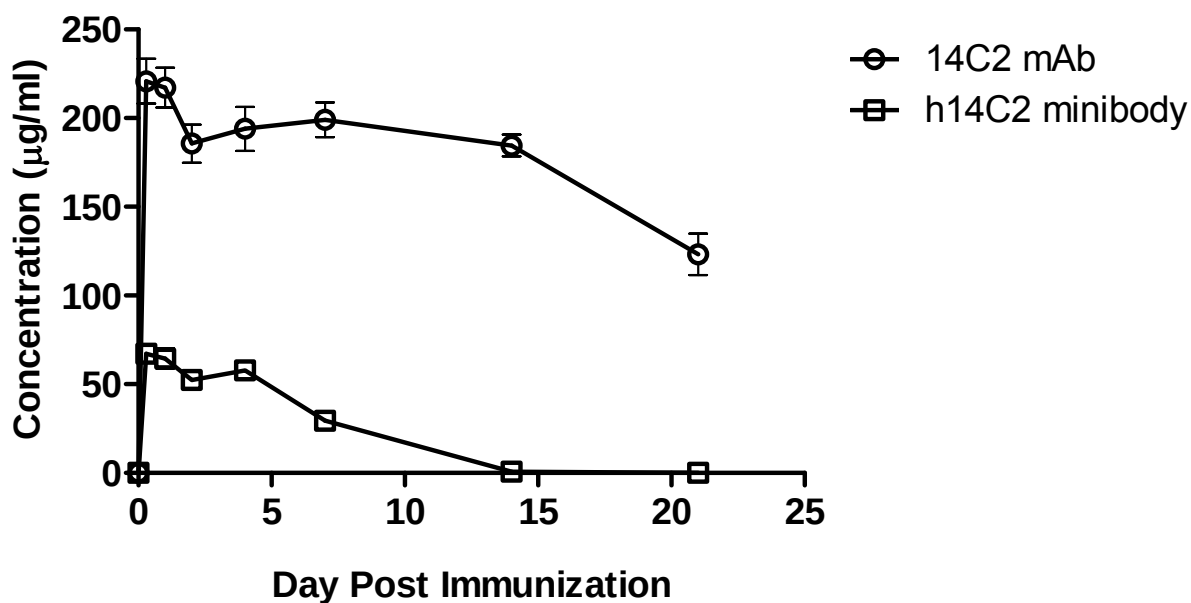


Figure 4.1. Serum M2 minibody concentrations. M2 minibody and 14C2 mAb concentrations in the serum of mice following a single intraperitoneal administration. Alternating cohorts of mice for each group were bled at the indicated timepoint. Each data point represents the average value for three mice.

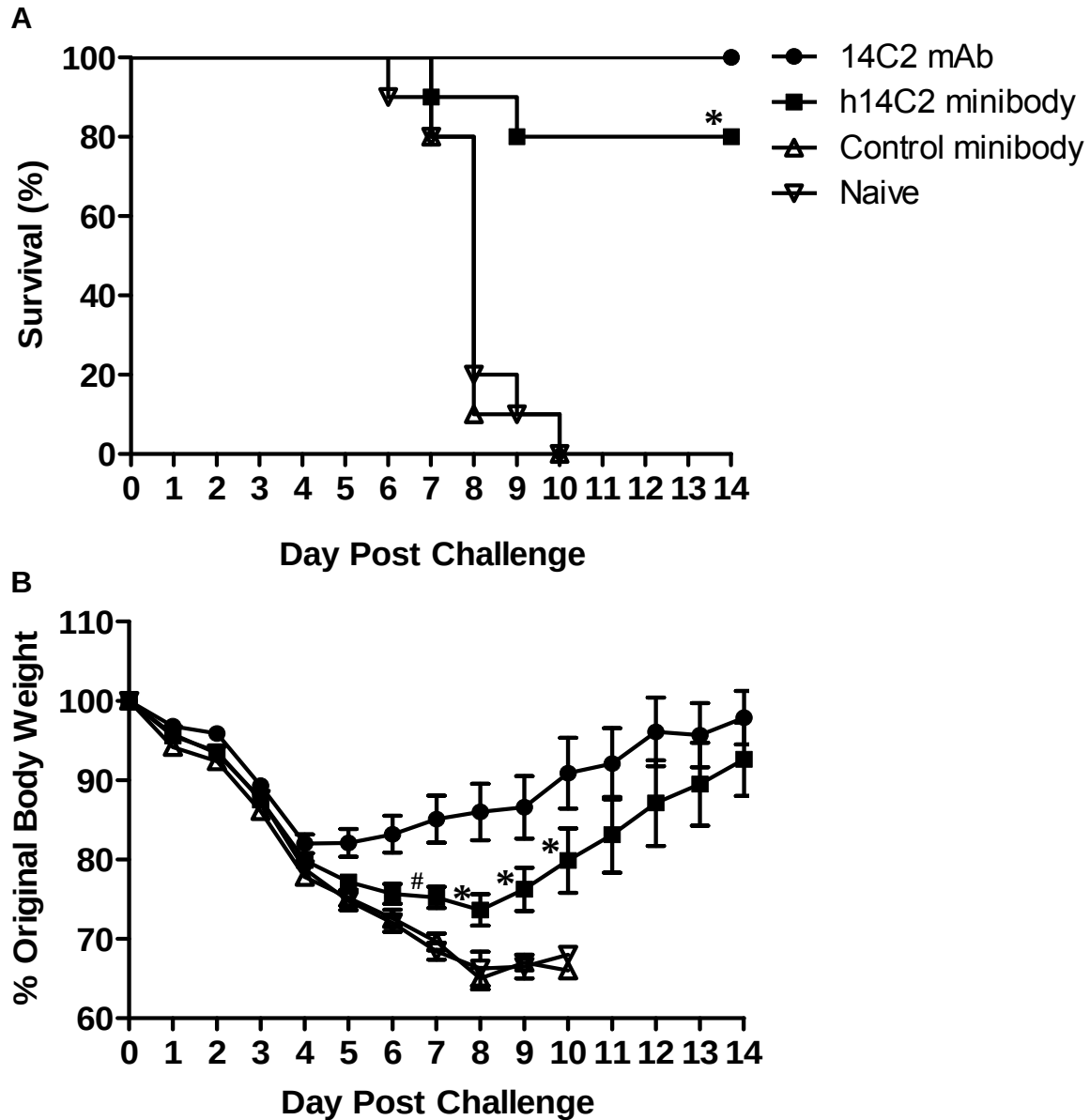


Figure 4.2. Protective efficacy of M2 minibodies. Passive immunization of M2 minibodies protects against influenza virus challenge. Mice were immunized as described in Materials and Methods and challenged intranasally with 40 x LD₅₀ of PR8. Mice were monitored for survival (A) and weight loss (B). * p < 0.0005 versus Naïve; Log-rank test (A); or # p < 0.01, * p < 0.05 versus Naïve; Two-way repeated measures ANOVA (B).

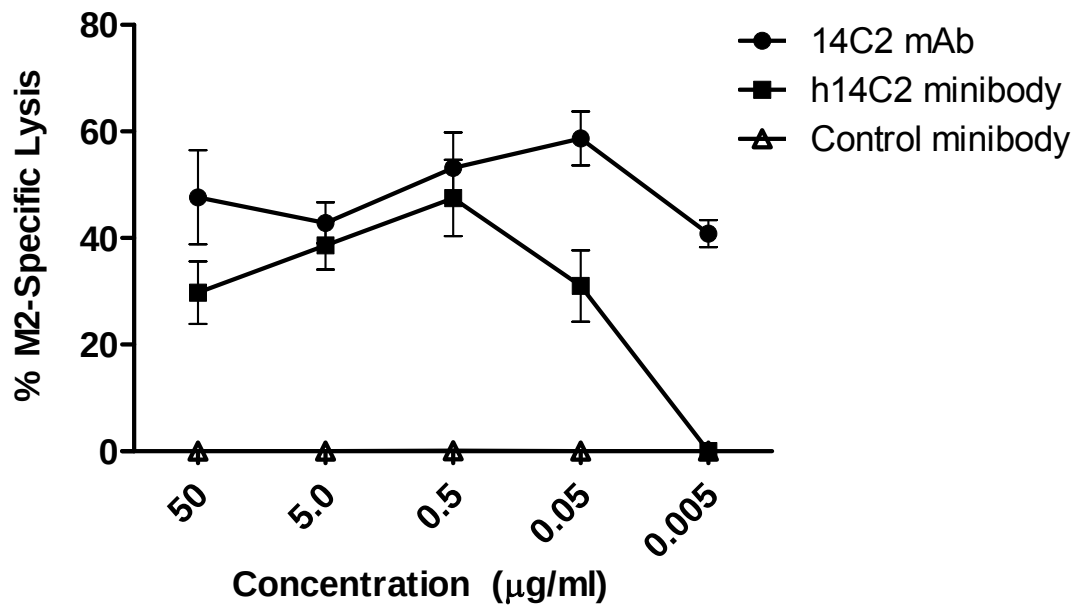


Figure 4.3. M2 minibody activation of complement. Target cells expressing influenza M2 protein and control cells were treated with the indicated concentration of minibody/mAb in the presence of rabbit complement. The release of lactate dehydrogenase was quantified as a measure of cytotoxicity. The data are representative of multiple experiments.

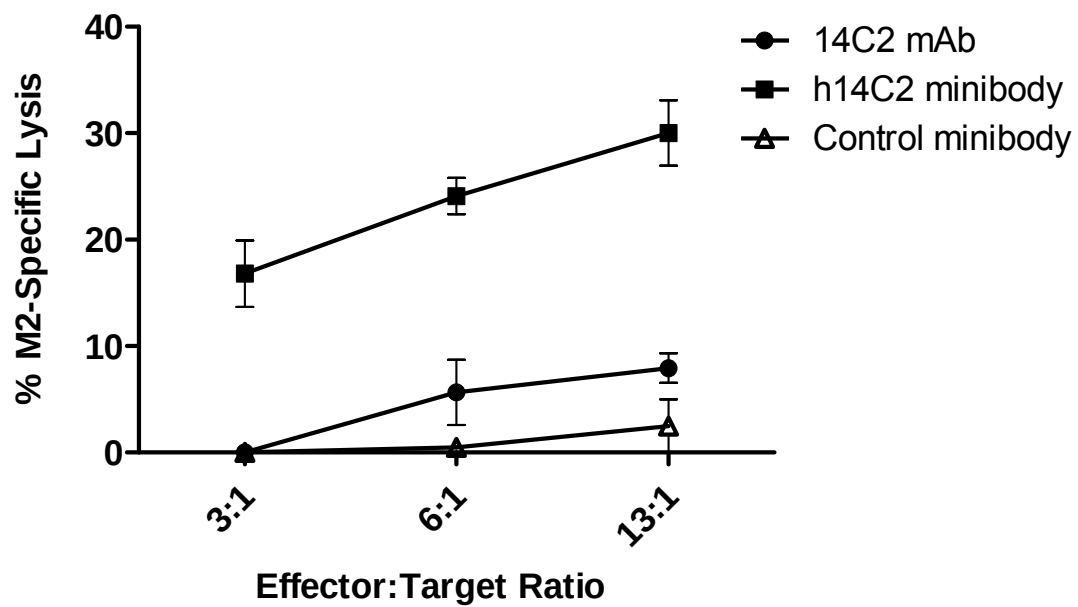


Figure 4.4. M2 minibodies facilitate NK cell-mediated ADCC. Target cells expressing influenza M2 protein and control cells were treated with minibody/mAb and incubated with human NK cells at the indicated E:T ratios. The release of lactate dehydrogenase was quantified as a measure of cytotoxicity. The results are representative of two experiments.

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CHAPTER 5

A RECOMBINANT ADENOVIRUS VACCINE INDUCES CROSS-PROTECTIVE IMMUNITY AGAINST pH1N1 CHALLENGE IN MICE

Abstract

The rapid spread of the 2009 H1N1 pandemic influenza virus (pH1N1) highlighted problems associated with relying on strain-matched vaccines. The lengthy process of strain identification, manufacture, and testing are required for current strain-matched vaccines and delay vaccine availability. Vaccines inducing immunity to conserved viral proteins could be manufactured and tested in advance and provide cross-protection against novel influenza viruses until strain-matched vaccines become available. Here we test a novel vaccine for cross-protection against pH1N1 infection. BALB/c and C57BL/6 mice were intranasally immunized with a single dose of recombinant adenoviruses expressing nucleoprotein (NP) and matrix 2 (M2) (NP+M2 rAd). Antibodies cross-reactive with the pH1N1 M2 ectodomain (M2e) were seen in NP+M2 rAd immunized BALB/c, but not C57BL/6 mice. Lung T cells from immunized mice cross-reacted with pH1N1 peptides despite some divergence between the vaccine and challenge virus sequences. NP+M2 rAd immunization protected BALB/c and C57BL/6 mice against challenge with a mouse-adapted pH1N1 virus. Cross-protective vaccines such as NP+M2 rAd can protect mice against pH1N1 challenge within 3 weeks

of immunization. Protection was not dependent on recognition of the highly variable external viral proteins and could be achieved with a single vaccine dose. Despite sequence differences between vaccine and challenge virus epitopes, extensive cross-reactivity of lung T cells was detected following immunization. This study highlights the potential for cross-protective vaccines to defend against novel influenza virus strains early in a pandemic.

Introduction

Current approved influenza vaccine strategies focus primarily on the induction of strong antibody responses against the hemagglutinin (HA) protein due to their ability to potentially neutralize virus infection. It is estimated; however, despite the use of strain-matched vaccines, seasonal influenza results in the hospitalization of millions and the death of thousands in the U. S. annually (1). This is attributed to the highly mutagenic nature of the HA glycoprotein resulting in antigenic drift which subverts preexisting vaccine-induced immunity. The emergence of a distinctively unique influenza virus through antigenic shift can result in the majority of the population being unprotected and, due to the time required to identify, develop, and distribute strain-matched vaccines can lead to vastly increased health and economic burden worldwide.

The recent 2009 H1N1 pandemic (pH1N1) is a testament to the unpredictable nature of influenza. The pH1N1 virus is thought to have originally emerged in Mexico in February of 2009 with the first reported cases identified in the U.S. in April (2). The virus quickly spread across the globe with the WHO officially declaring a pandemic in June 2009. In spite of rapid strain identification, the logistics associated with vaccine

preparation and distribution prevented the first pH1N1 vaccines from being available in the U.S. until October 2009. By this time infection rates, estimated at 11%-21%, were at their highest (3).

This situation highlights the need for vaccination approaches not reliant upon the highly-variable HA protein. Through the targeting of highly conserved proteins, protective heterosubtypic immunity could be generated against a wide variety of influenza strains and subtypes. While this protective immunity would not prevent virus infection, such vaccines could be prepared in advance and rapidly distributed should a potentially pandemic strain emerge thus slowing the spread of the virus while strain-specific vaccines are generated.

Two such antigenic targets which have been explored for the induction of heterosubtypic immunity are the highly conserved nucleoprotein (NP) (4-6) and matrix 2 (M2) protein (7-10). Characterization of immune responses generated against NP and M2 have been shown to not only mediate protection through antibody-specific responses but through cell-mediated responses as well (11). Previous work has shown that priming vaccination using plasmid DNA encoding NP and M2 followed by boosting with recombinant adenovirus (rAd) vectors encoding the same antigens was able to induce immune responses able to confer protection against a lethal heterosubtypic virus challenge, including the highly-pathogenic H5N1 (12, 13). It has also been demonstrated that a single intranasal immunization using NP and M2 rAd was able to induce rapid, long lasting protective immunity in as little as two weeks after vaccination (14). Although the use of rAd virus vaccine approaches are still in pre-clinical development, recent Phase I clinical trials focused on the induction of M2-specific immunity in healthy

volunteers have been completed and in both studies were found to be immunogenic, safe, and well-tolerated (15).

We report here the capability of a single intranasal vaccination of rAd vectors expressing NP and M2 (NP+M2 rAd) able to induce rapid, cross-protective immune responses against the 2009 H1N1 pandemic virus. NP+M2 rAd vaccination induced both humoral and cell-mediated responses reactive against antigenically distinct pH1N1 epitopes which were ultimately able to provide protection against a lethal challenge. This study highlights the effectiveness of heterosubtypic vaccination against potential future pandemic viruses.

Material and Methods

Ethics statement

All animal protocols and procedures were approved by Institutional Animal Care and Use Committees at the Center for Biologics Evaluation and Research (protocol #1991-06) the University of Georgia (protocol #A3437-01) in animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experiments were performed according to institutional guidelines.

Vaccines, viruses and peptides

Recombinant adenovirus vectors (Ad5- Δ E1 Δ E3) expressing influenza A or B nucleoprotein (kindly provided by Gary Nabel, NIH Vaccine Research Center) and consensus M2 have been described previously (7, 16) and bulk stocks were produced by ViraQuest, Inc. (North Liberty, IA). The 2009 pandemic H1N1 challenge virus is a

mouse-adapted strain of A/California/04/09 (ma-CA/04) (17). Challenge stocks were grown in MDCK cells for 48 hours at 37°C and 5% CO₂ with 1 µg/ml TPCK-treated trypsin (Worthington Biochemical Inc.; Lakewood, NJ). HA protein from influenza virus A/California/04/09 (H1N1) was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH as a recombinant protein in baculovirus, NR-15258. Recombinant NP was purchased from Imgenex (San Diego, CA) and has the A/PR/8/1934 (PR8) sequence. HIV p24 gag₂₈₅₋₃₀₇ and PR8 M2₂₋₂₄ (M2e) peptides were synthesized at the CBER core facility. All other PR8 and ma-CA/04 peptides were purchased from GenScript (Piscataway, NJ).

Mice and immunizations

Six to eight week-old female BALB/cANcr (BALB/c) or C57BL/6Ncr (B6) mice were purchased from NCI and maintained under specific pathogen-free conditions. Animals were anesthetized with isoflurane and immunized intranasally with 50 µl of vaccine dropwise to the nares. Animals received a dose of 5×10^9 virus particles (vp) each of NP rAd and M2 rAd, or 1×10^{10} vp of B/NP rAd.

Challenge infections

Mice were anesthetized with 250 µl of Avertin (2-2-2-tribromoethanol, Aldrich Chemical Co.; Milwaukee, WI) administered via i.p. injection. Lethal doses (LD₅₀) of ma-CA/04 were determined for each mouse strain. Challenge experiments used 5 LD₅₀ of ma-CA/04 administered in 50 µl. Animals which lost ≥25% of their weight were humanely euthanized. Animals for analysis of lung virus titers were chosen before the

start of the experiment. All challenge experiments were performed in ABSL-3 facilities at the University of Georgia.

Antibody analysis

96-well flat bottom immunoplates (Nalge Nunc International; Rochester, NY) were coated at 4°C overnight with 1 µg/ml of recombinant NP (Imgenex; San Diego, CA) or 15 µg/ml of M2e peptide in 0.125 M saline, 0.007 M borate buffer. For HA ELISAs, recombinant H1 HA was diluted in PBS to 1µg/ml for plate coating. ELISA assays were performed as previously described (18). Optical densities (OD) were determined at 405 nm using a Multiskan EX spectrophotometer (Thermo Fisher Scientific Inc.; Waltham, MA) and Ascent analysis software. Data are expressed as endpoint titers, defined as the highest dilution of sample giving an OD>3 standard deviations (SD) above the mean of the starting dilution of naïve sera.

ELISPOT analysis

Lung tissue was harvested and processed on interferon- γ ELISPOT was performed as previously described (7, 13) by stimulation with indicated peptides.

Determination of lung viral titers

For viral titer analyses, lungs were removed and placed into 2 ml Safe-Lock Tubes (Eppendorf AG; Hamburg, Germany) containing 1 ml of PBS and a 5 mm diameter steel ball-bearing. Lung tissue was homogenized using a TissueLyser (Qiagen; Valencia, CA) for 2 minutes at 30 oscillations/second. Lung homogenates

were centrifuged at 2376 G for 5 minutes at 4°C and the clarified supernatant aliquoted and stored at -80°C until used. MDCK cells were plated in 96-well tissue culture plates at a density of 3×10^4 cells per well (100 μ l/well) the day before infection. Lung homogenate supernatants were diluted in a 10-fold series in quadruplicate using 'Infection Media' (MEM + 2mM L-glutamine + penicillin [100 IU/ml], streptomycin [100 μ g/ml], amphotericin B [0.25 μ g/ml] + TPCK-treated trypsin [1 μ g/ml]). The cell monolayers were rinsed twice using PBS to remove all serum-containing medium, and then 200 μ l of diluted test sample was added. Culture plates were then incubated at 37°C and 5% CO₂ for 72 hours. After incubation, 50 μ l of supernatant was removed for virus testing by hemagglutination. Fifty microliters of 0.5% chicken erythrocytes were added to supernatants in a 96-well round bottom plate and incubated for 30-60 minutes. Wells were observed for the presence of hemagglutination and TCID₅₀ titers calculated using the Reed and Muench method (19).

Graphs and statistics

All graphs were created using GraphPad Prism version 5 (GraphPad Software; La Jolla, CA). Statistics were performed using SigmaStat version 3.1 (Systat Software, Point Richmond, CA) or GraphPad Prism. Antibody and viral titer data were log-transformed prior to statistical analysis.

Results

Vaccine and challenge virus sequence differences

It has long been known that T cell involvement is crucial in mediating cross-protective immunity against influenza virus infection (20-22). This protection is dependent on T cell recognition of viral epitopes expressed in the context of MHC molecules. The dominant MHC class I NP epitopes recognized by the two strains of mice used in this study are NP₁₄₇₋₁₅₅ (BALB/c) and NP₃₆₆₋₃₇₄ (B6) (23-25). While the NP and M2 proteins have typically been found to be highly conserved among influenza A viruses, several amino acid changes exist between the sequences used for vaccination and the ma-CA/04 challenge virus. Table 5.1 lists the peptides used for stimulation in this study and highlights the sequence differences found between the two. These peptides were chosen because they are known or suspected to contain key MHC class I and II epitopes recognized by BALB/c and B6 mice.

Vaccination induces robust serum antibody responses

Following a single intranasal immunization of rAd virus, mice were bled at 3 and 5 weeks post vaccination to determine serum antibody responses. BALB/c mice immunized with NP+M2 rAd exhibited robust anti-NP antibody titers at both time points following vaccination while all control mice failed to induce a detectable response (Figure 5.1A). Although the titers were lower compared to BALB/c titers, NP+M2 rAd vaccination also induced significant anti-NP antibody responses in B6 mice (Figure 5.1B).

BALB/c mice immunized with NP+M2 rAd virus demonstrated significant M2e-specific antibody titers at both 3 weeks (Figure 5.2A) and 5 weeks (Figure 5.2B) post vaccination. The antibodies present were not only able recognize the PR8 M2e peptide but were also cross-reactive against the ma-CA/04 M2e peptide despite several amino acid differences. B6 mice failed to develop detectable M2e-specific serum antibody titers following rAd virus vaccination (data not shown).

Lung T cell responses

To determine the capability of NP+M2 rAd vaccination at inducing cross-reactive T cell responses, IFN- γ ELISPOT assays were conducted using NP and M2 peptides representing both vaccination and challenge sequences containing previously reported dominant and subdominant T cell epitopes (Figure 5.3). Lung T cells were harvest from a subset of identically vaccinated mice at 3 weeks post vaccination which coincided with virus challenge in protection experiments.

Within the BALB/c strain, mice immunized with NP+M2 rAd virus developed robust responses against the NP₁₄₇₋₁₅₅ epitope (Figure 5.3A), which, as seen in Table 5.1, is 100% identical between the vaccination and ma-CA/04 challenge sequence. Moderate responses were detected against the PR8 M2₂₋₂₄ peptide; however, vaccine-induced M2-specific T cells failed to cross-react with the ma-CA/04 M2₂₋₂₄ peptide. Reactions to the MHC class II NP₅₅₋₆₉ peptide were generally quite low; however, T cells exhibited a slightly higher response against the ma-CA/04 sequence.

As expected, B6 mice immunized with NP+M2 rAd virus developed robust T cell responses against the dominant NP₃₆₆₋₃₇₄ epitope (Figure 5.3B). Responses against the

corresponding ma-CA/04 peptide were similar, though slightly lower, in spite of a single amino acid difference at position 371 (M371V). Similarly moderate responses were detected against the NP₂₆₀₋₂₈₃ epitope which differ by only one amino acid at the C-terminal end while negligible responses were observed against M2₂₋₂₄.

NP+M2 rAd vaccination protects against lethal challenge

Next we tested the ability of NP+M2 rAd vaccination to protect against challenge with a divergent pandemic virus. Three weeks after receiving a single intranasal immunization of rAd virus, mice were challenge with a lethal dose of mouse-adapted 2009 H1N1 pandemic influenza and monitored for morbidity and mortality (Figure 5.4). All BALB/c mice vaccinated with NP+M2 rAd survived challenge (Figure 5.4A) and experienced significantly reduced weight loss compared to control mice (Figure 5.4B). While B6 mice were not completely protected, 10 out of 12 NP+M2 rAd vaccinated mice survived challenge (Figure 5.4C) and exhibited minimal overall weight loss (~15%) compared to controls (Figure 5.4D).

Lung viral titers

In order to determine the role of vaccine-induced immune responses in controlling influenza virus replication following challenge, lung virus titers were determined in a subset of identically vaccinated mice at days 3 and 6 post challenge (Figure 5.5). BALB/c mice receiving a single dose vaccination of NP+M2 rAd had significantly reduce (~100-fold) lung viral titers at day 6 post challenge compared to control groups (Figure 5.5A). In B6 mice, NP+M2 rAd vaccinated mice also exhibited

significantly lower titers at day 6 compared to controls (Figure 5.5B). No statistically significant difference in viral titers were observed in NP+M2 rAd vaccinated mice at day 3 in either mouse strain or between rAd control vaccinated and naïve mice at either timepoint.

Discussion

In this study we demonstrated the capability of vaccination with recombinant adenovirus encoding the conserved influenza A proteins NP and M2 at inducing cross-reactive immune responses. These responses developed rapidly (within 3 weeks) after a single mucosal administration and were able to protect mice of different genetic backgrounds against a lethal challenge with a mouse-adapted strain of the recently emerged 2009 pandemic H1N1 virus.

Concerning NP-mediated immunity, robust anti-NP serum antibody titers were induced after NP+M2 rAd vaccination along with strong T cell responses against known dominant NP epitopes. While the exact mechanisms of protection contributed by NP-specific antibodies are not fully understood, they have been shown to provide protection against viral challenge (26-28). NP-specific T cells recognizing dominant MHC class I epitopes have also been shown to contribute to protection in the absence of antibodies (29). While the dominant BALB/c MHC class I epitope (NP₁₄₇₋₁₅₅) is 100% conserved between the vaccination and challenge sequence, a single amino acid mutation (M371V) exists in the corresponding B6 epitope (NP₃₆₆₋₃₇₄). This mutation is found at position 6 within the epitope and corresponds to a residue which makes contact with the T cell receptor (30, 31). Despite this critical difference, T cells primed against the PR8

NP used in vaccination were cross-reactive in IFN- γ ELISPOT assays when stimulated with the divergent ma-CA/04 epitope. This cross-reactivity has also been shown in studies employing tetramer staining (32).

The primary contribution of M2-mediated immunity is through antibody recognition of the M2e. While M2e-specific antibodies are non-neutralizing, they have been shown to restrict virus growth *in vitro* as well as protect against influenza virus challenge (7, 10, 33-35). This protection has been attributed to the activation of Fc-mediated immune responses including complement fixation and antibody-dependent cellular cytotoxicity (35, 36). Recently, alveolar macrophages have been shown to play a crucial role in mediating M2-specific immunity (37).

Although immune responses against M2 after vaccination differed depending on the genetic background of the mice, both strains were protected against ma-CA/04 challenge following NP+M2 rAd vaccination. BALB/c mice developed a strong cross-reactive M2e-specific antibody response able to recognize the sequence of both the vaccinating and challenge antigen. BALB/c mice also exhibited a moderate M2e-specific T cell response following NP+M2 rAd vaccination. This is in accordance to previous work that suggests the presence of a BALB/c-specific MHC class II epitope (38). While the ma-CA/04 M2e varies by 5 amino acids and appears to be sufficient enough to eliminate any T cell cross-reactivity, the presence of the MHC class II epitope during vaccination most likely contributes to the strong cross-reactive antibody response.

B6 mice failed to mount an M2e-specific antibody response following NP+M2 rAd vaccination which is in accordance to previous work (38). In spite of this, the M2 rAd component was still included because our previous work has shown that co-

immunization with NP+M2 rAd provided enhanced protection compared to vaccination with either construct individually (38). Interestingly, others have reported the induction of M2e-specific antibodies in B6 mice following vaccination with a chimpanzee rAd vector expressing a fusion protein composed of 3 M2e sequences from diverse influenza strains linked to NP (8). This, along with our results here, suggests that the induction of M2e-specific antibody responses in B6 mice is dependent upon not only the immunizing vector but also the context of the antigen delivered.

In humans, one caveat against the use of a recombinant adenoviral vector for vaccine delivery is the presence of pre-existing immunity which could potentially affect vaccine efficacy. Several alternative techniques could be employed to avert this pre-existing immunity including the use of a non-human adenovirus (8) or a human adenovirus of rare serotype (39).

In this study we investigated the rapid induction of protective immunity following a single immunization with a rAd virus vaccine able to protect against a lethal challenge with a pandemic virus. Vaccination with the NP+M2 rAd virus induced robust T cell responses as evident by IFN- γ production as well as high levels of serum antibodies. We have shown previously in other studies that this protection can persist for at least 10 months and is able to withstand challenge with highly virulent viruses. The recent emergence of the 2009 H1N1 pandemic influenza highlights the need for a cross-protective influenza vaccine available should another pandemic arise. This vaccine could be stockpiled in advance and rapidly distributed in the face of an impending pandemic. Such preparation would not only slow the progression of a future pandemic

allowing for the generation and distribution of strain-matched vaccines but would also lessen the global health and economic burden attributed to influenza pandemics.

Table 5.1. Peptides used in this study.

Virus ^a	Peptide ^b	Sequence	Analyses ^c
HIV	p24 gag ₂₈₅₋₃₀₇	Q G P K E P F R D Y V D R F Y K T L R A E Q A	B6+BALB/c
A/PR/8/34	NP ₁₄₇₋₁₅₅ ^d	T Y Q R T R A L V	B6+BALB/c
A/PR/8/34	NP ₅₅₋₆₉	R L I Q N S L T I E R M V L S	BALB/c
ma-CA/04	NP ₅₅₋₆₉	R L I Q N S <u>I</u> T I E R M V L S	BALB/c
A/PR/8/34	NP ₂₆₀₋₂₈₃	A R S A L I L R G S V A H K S C L P A C V Y G P	B6
ma-CA/04	NP ₂₆₀₋₂₈₃	A R S A L I L R G S V A H K S C L P A C V Y G <u>L</u>	B6
A/PR/8/34	NP ₃₆₆₋₃₇₄	A S N E N M E T M	B6
ma-CA/04	NP ₃₆₆₋₃₇₄	A S N E N <u>V</u> E T M	B6
A/PR/8/34	M2 ₂₋₂₄	S L L T E V E T P I R N E W G C R C N G S S D	B6+BALB/c
ma-CA/04	M2 ₂₋₂₄	S L L T E V E T P <u>I</u> R <u>S</u> E W <u>E</u> C R C <u>S</u> <u>D</u> S S D	B6+BALB/c

NP and M2 peptides used in this study for analysis of immune responses. Amino acids in the challenge virus sequence which differ from the A/PR/834 sequence are underlined.

^aSource virus of specified peptide.

^bProtein and sequence range of the peptide.

^cMice tested with peptide.

^dSequence is 100% identical in vaccine and challenge viruses.

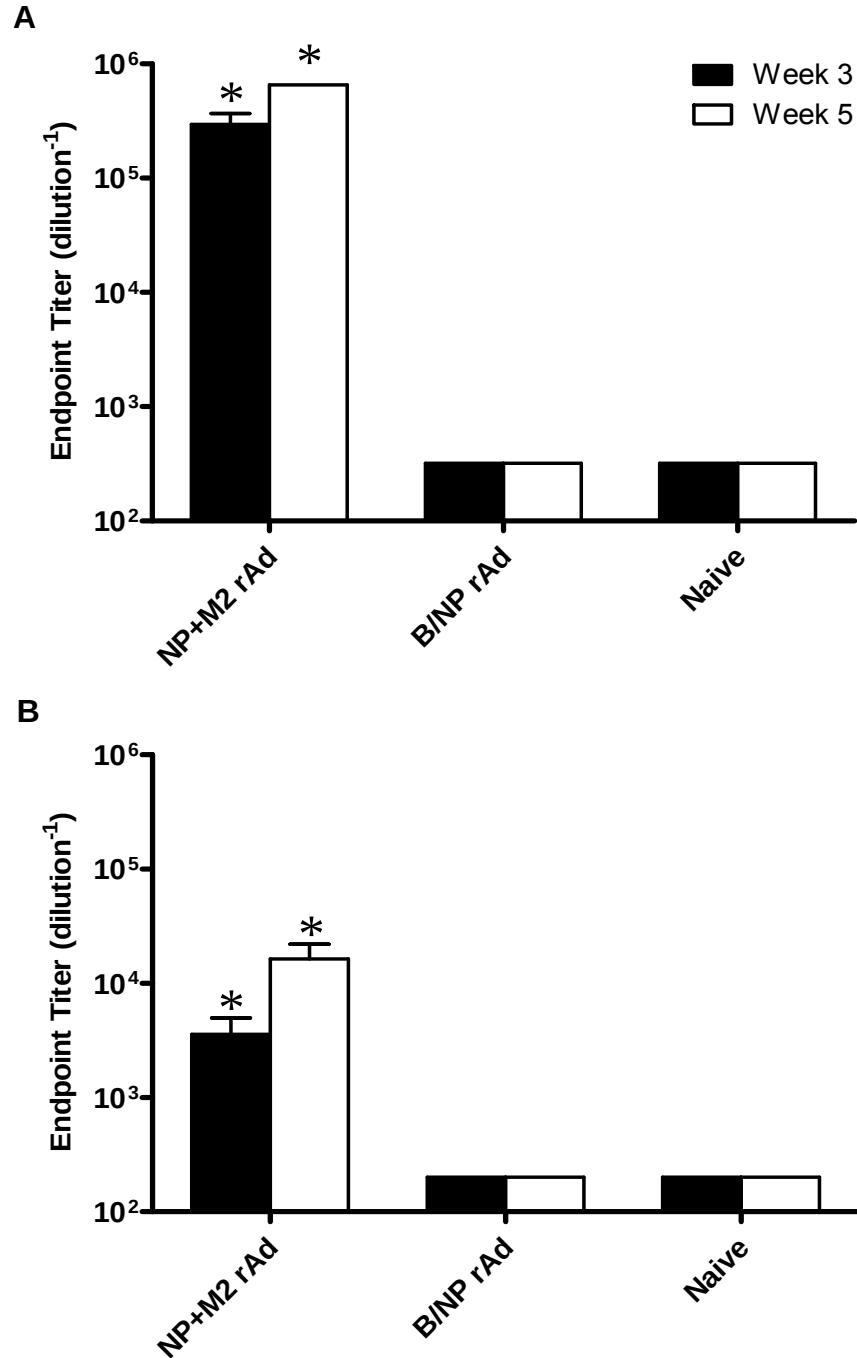


Figure 5.1. Anti-NP serum antibody titers. Anti-NP IgG levels in the serum of immunized mice at various timepoints following immunization. BALB/c (A) or B6 (B) mice were immunized with 1×10^{10} vp of NP+M2 rAd, B/NP rAd, or left unvaccinated. Serum was obtained at 3 weeks (black bars) or 5 weeks (open bars) post-vaccination and tested for anti-NP IgG as described in Materials and Methods. Shown are the mean endpoint titers $\pm$ SD; $n=5$ per group. * $p \leq 0.0001$ versus naïve; One Way ANOVA with Student-Newman-Keuls post test.

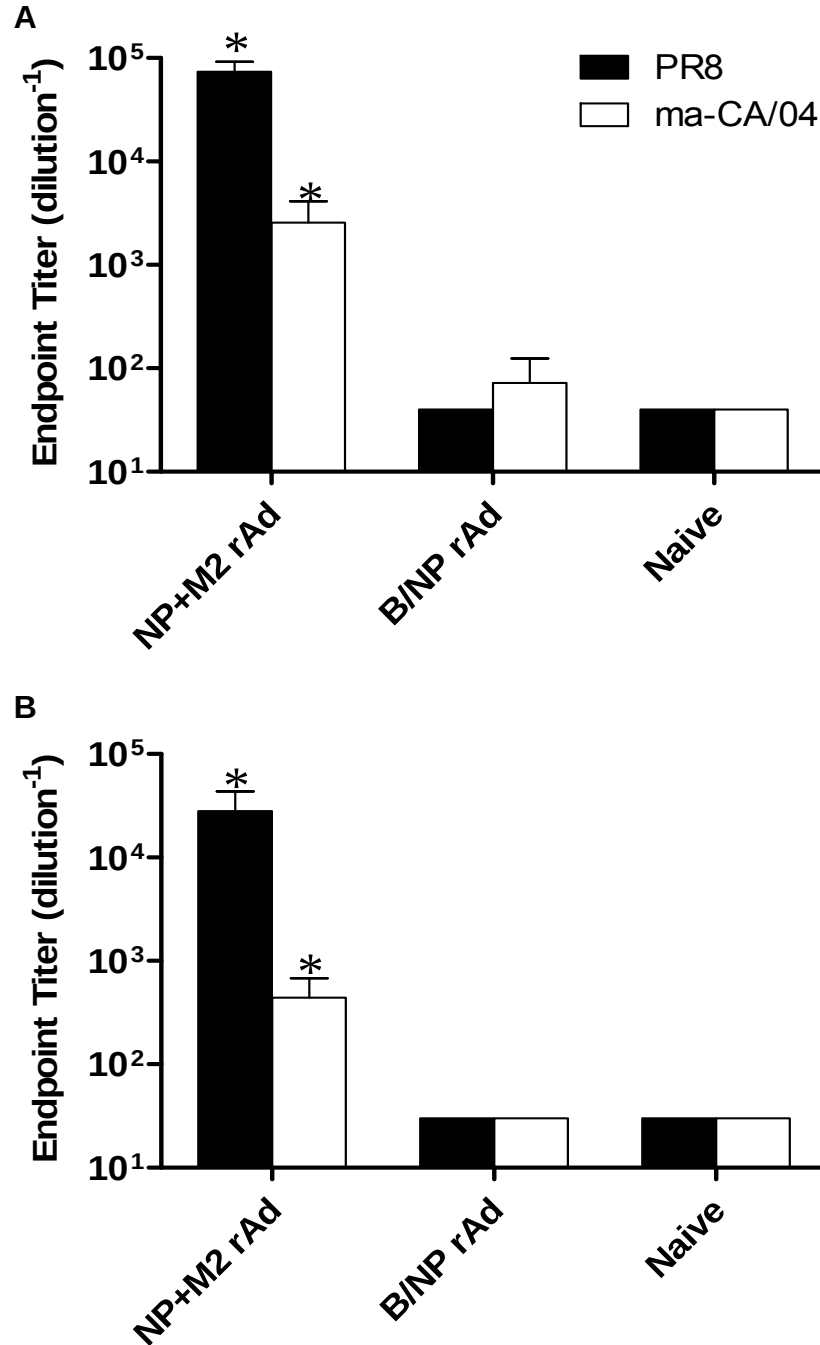


Figure 5.2. Anti-M2e serum antibody titers. Anti-M2e IgG levels in the serum of immunized mice at various timepoints following immunization. BALB/c (A and B) mice were immunized as in Figure 1 or left unvaccinated. Serum was obtained at 3 weeks (A) or 5 weeks (B) post-vaccination and tested for anti-M2e IgG antibody. Anti-M2e titers were determined using the PR8 peptide sequence (black bars) and the ma-CA/04 peptide sequence (open bars). Shown are the mean endpoint titers $\pm$ SD; n=5 per group. * $p \leq 0.0001$ versus naïve; One Way ANOVA with Student-Newman-Keuls post test.

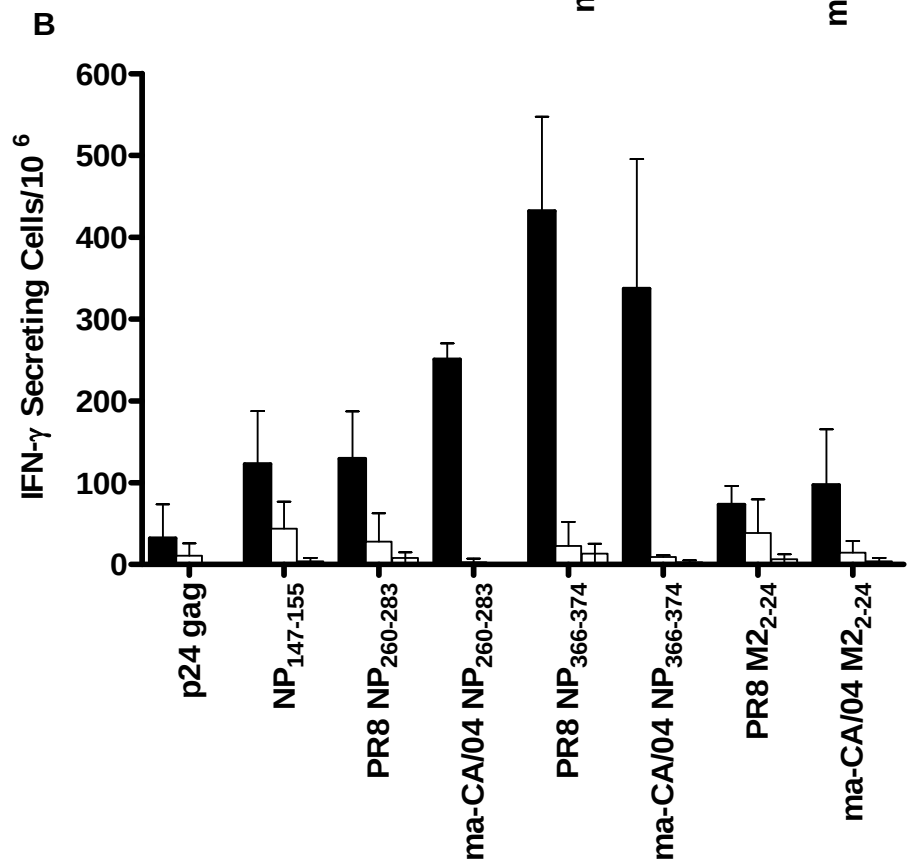
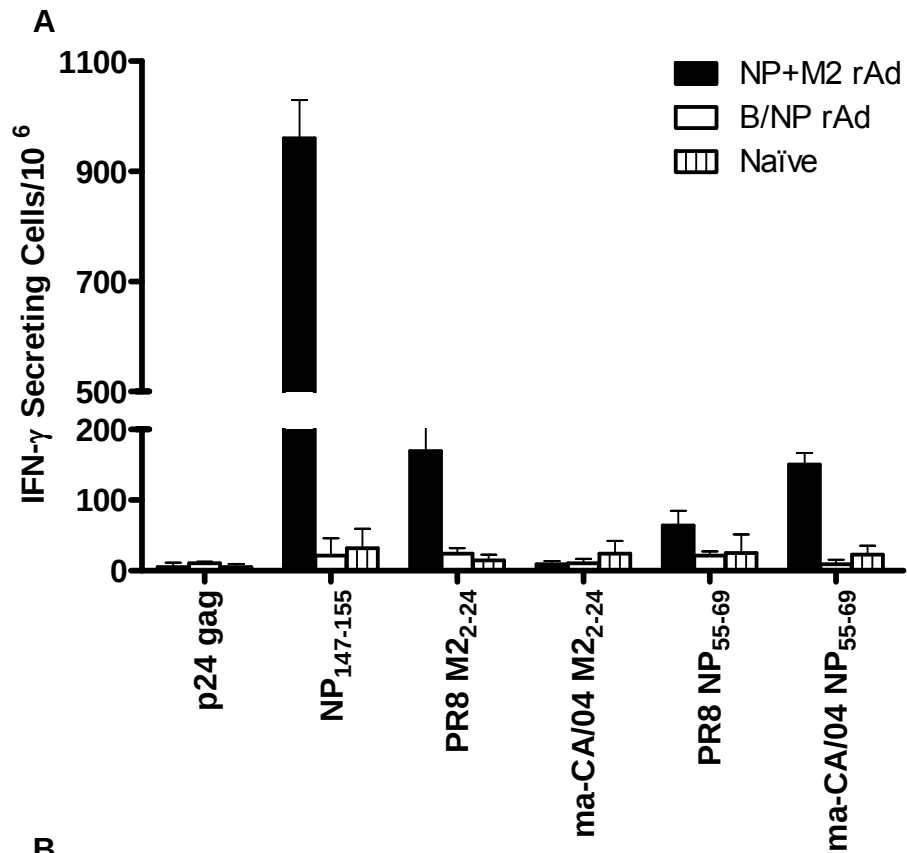
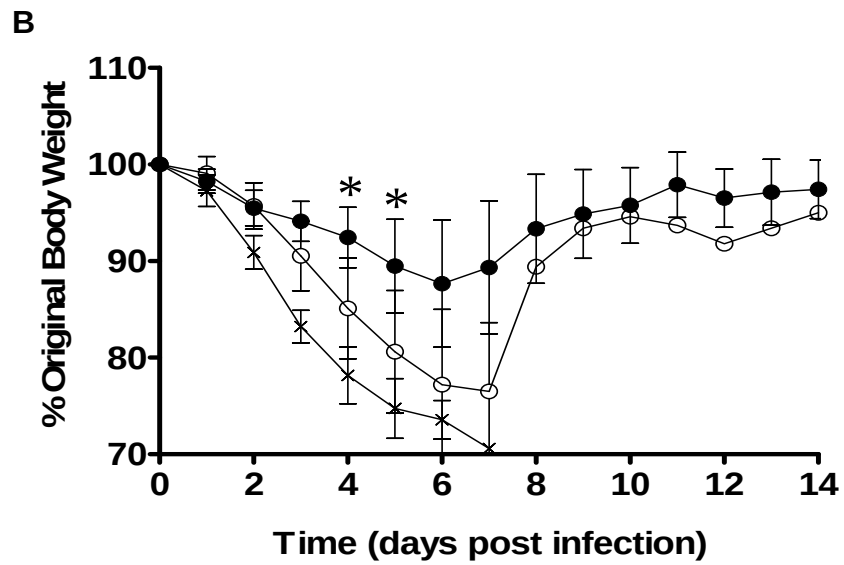
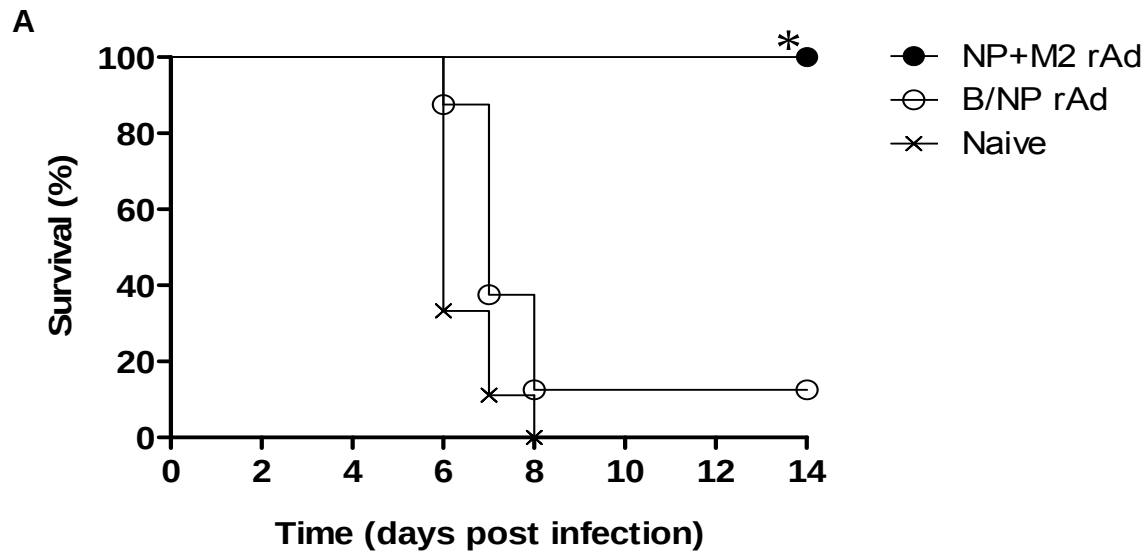


Figure 5.3. Lung T cell responses. IFN- γ responses of lung T cells following stimulation with peptides of vaccine sequences or challenge virus sequences. BALB/c (A) or B6 (B) mice were immunized as in Figure 1, or left unvaccinated. Three weeks post-vaccination, T cell responses were determined by IFN- γ ELISPOT as described in Materials and Methods. Pooled lung cells were tested using cells from 5 (BALB/c) or 7 (B6) mice per group. Shown are the mean of triplicate measurements of IFN- γ secreting cells per million $\pm$ SD.



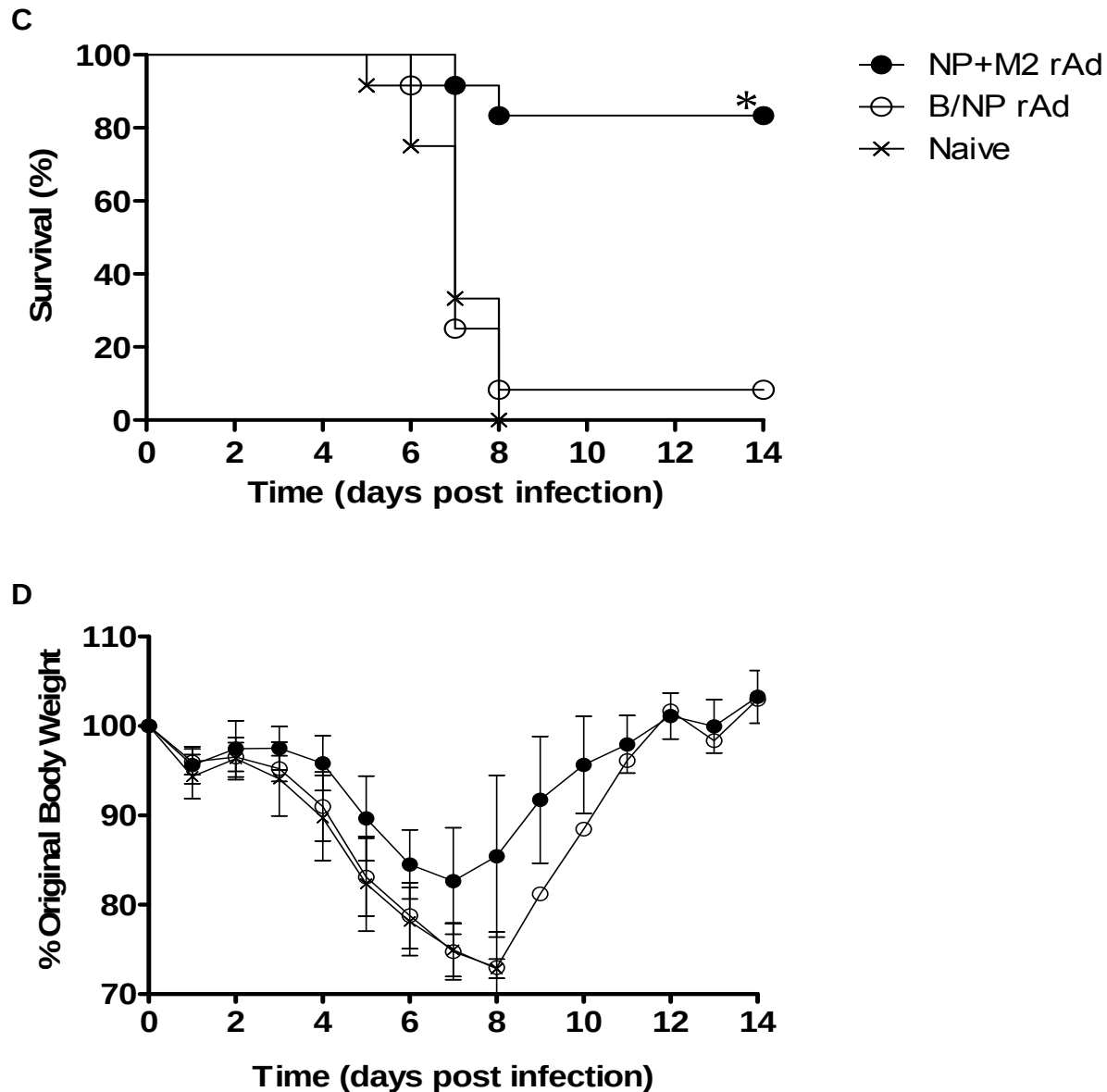


Figure 5.4. Weight loss and survival. Vaccine effectiveness in protection against challenge with ma-CA/04. Groups of BALB/c (A and B) or B6 (C and D) mice were immunized as in Figure 1, or left unvaccinated. Three weeks after immunization, animals were challenged with 5 MLD₅₀ of ma-CA/04 intranasally and monitored for survival (A and C) and weight loss (B and D). Weight loss graph shows average of n=9 (BALB/c) or n=12 (B6) mice $\pm$ SD. * $p \leq 0.0001$ versus naïve; log rank test (A and C); or $p \leq 0.01$ two-way repeated measures ANOVA with Holm-Sidak post test (B and D).

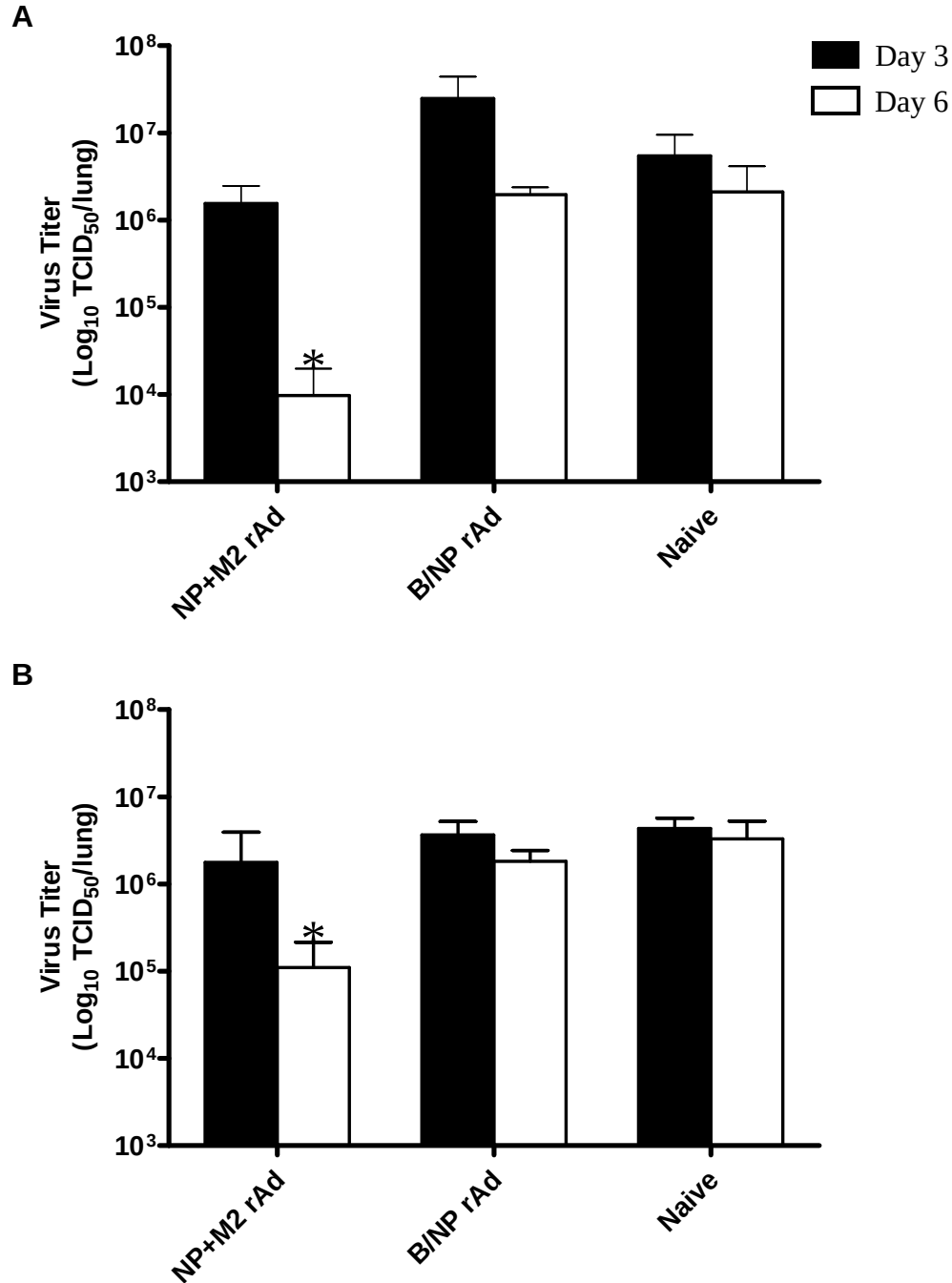


Figure 5.5. Lung virus titers. Viral replication in the lungs following ma-CA/04 challenge. BALB/c (A) or B6 (B) mice were immunized as in Figure 1, or left unvaccinated. Three weeks after immunization, animals were challenged with 5 MLD₅₀ of ma-CA/04 intranasally. Lung viral titers were determined on day 3 (solid bars) and day 6 (open bars) by TCID₅₀ assay as described in Material and Methods. Shown are the mean $\pm$ SD; n=4 per group. * $p \leq 0.05$ versus naïve; Kruskal-Wallis test with Student-Newman-Keuls post test.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

The constant antigenic drift of seasonal influenza A viruses combined with the ever present threat of pandemic influenza necessitates the need to explore vaccination strategies focused on conserved viral antigens. The M2 protein fits the bill as a potential candidate due to the high conservation rate observed for the M2e sequence among influenza virus strains combined with its surface expression on virions and infected cells making it an ideal target for antibody-based therapies. The overall goal of the work presented here was to exploit these characteristics of M2 in efforts to protect against influenza virus infection.

One approach taken was through the development of novel M2-specific single-chain minibodies. The generation of plasmid-derived minibodies containing the humanized variable regions of the well characterized M2-specific 14C2 mAb allowed for the ‘proof of concept’ investigation of minibodies specific for the M2 protein of influenza. Our M2 minibodies exhibited the capability of recognizing not only M2e peptide but also native M2 protein expressed on the surface of transfect or virus infected cells (Chapter 3). The ability of our M2 minibodies to reduce the plaque sizes of influenza virus in an *in vitro* assay suggested their anti-viral activity. This activity was subsequently confirmed in a series of *in vivo* and *in vitro* assays (Chapter 4). Ultimately, the passive immunization of mice with a single administration of M2 minibodies conferred protection

against a lethal influenza virus challenge. This protection also correlated with a reduction in weight-loss following virus challenge. Also of importance, M2 minibodies were shown to facilitate cytotoxic Fc-mediated immune functions against cells expressing native M2 protein. M2 minibodies were capable of facilitating the classical pathway activation of complement resulting in the lysis of M2-specific target cells. In a translation approach M2 minibodies containing the human IgG1 Fc-domain, when bound to surface M2, were able to activate human NK cells through Fc-receptor recognition resulting in target cell lysis. This activity suggests the potential of our M2 minibodies to function as a human therapeutic intervention against influenza virus infection.

Our M2 minibodies possess several unique characteristics which support further studies in efforts of developing a novel therapeutic tool for human use. First, passive immunization using M2 minibodies would allow for the protection of certain immunocompromised groups which often are unable to receive or respond poorly to traditional active vaccination. Passive immunization could also be used confer rapid protective immunity to first responders on the frontlines of an influenza pandemic. Second, because minibodies are encoded and expressed via DNA plasmids, certain alterations could be made using simple molecular techniques. For instance, exchange of the IgG Fc-domain for IgA might allow for increased delivery of minibodies to mucosal tissues enhancing protective immunity at the site of infection. Also, certain modifications to amino acid residues within the minibody Fc region could increase their affinity for complement activation or Fc receptor recognition resulting in improved cytotoxic effects. Lastly, M2 minibodies could be delivered through genetic passive immunization using

their encoding DNA plasmid. This may perhaps be achieved through the direct administration of naked DNA via gene gun or delivery using a viral vector such as adeno-associated virus, both of which have been shown to induce expression over a prolonged period of time.

The second technique used in this study to utilize the M2 protein for protective immunity against influenza virus infection was a more traditional, active vaccination approach (Chapter 5). A single vaccination with recombinant adenovirus vectors encoding the M2 (and NP) protein of influenza was sufficient to generate rapid protective immunity against a lethal challenge with the recently emerged pandemic H1N1 virus. M2-specific antibodies were detected post vaccination that were reactive against the vaccinating M2e sequence as well as the divergent M2e sequence of the pandemic challenge virus. One disadvantage of using a human adenovirus as a vaccine vector is the high prevalence of preexisting adenovirus immunity present in the human population. This could be subverted by the use of either a rare serotype human adenovirus or a nonhuman adenovirus. Another possible method would be the use of a different viral vector which has been shown to have little prior immunity found in humans. One potential candidate is parainfluenza virus 5 which in our hands has been shown to induce the generation of M2-specific antibodies in mice (unpublished observations).

In conclusion, the novel vaccination strategies presented in this study represent unique approaches for protection against influenza virus infection through antibody-mediated immunity targeting the conserved M2 protein. These vaccination methods however are in no way intended to replace current strain-matched HA-based vaccines.

Advanced stockpiling and rapid distribution of the vaccines presented here could provide a first line of defense in the face of a pandemic caused by the emergence of a divergent influenza virus, thus slowing the progression of infection allowing for the generation of strain-matched vaccines. This could ultimately reduce the overall global health and economic consequences associated with influenza pandemics.