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Author(s):	Gabbard, J., Velappan, N., Di Niro, R., Schmidt, J., Jones, C.A., Tompkins, S.M. and Bradbury, A.R.M.
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A humanized anti-M2 scFv shows protective *in vitro* activity against influenza

[1]Gabbard, J., [2]Velappan, N., [3]Di Niro, R., [2]Schmidt, J., [1]Jones, C.A., [1]Tompkins, S.M.* and [2]Bradbury, A.R.M.*

***corresponding authors: SMT: smt@uga.edu; ARMB: amb@lanl.gov**

[1] Influenza Pathogenesis & Immunology Research Center
University of Georgia
College of Veterinary Medicine
Department of Infectious Diseases
Animal Health Research Center
111 Carlton Street, building 1077
Athens, GA 30602-1563

[2] B division,
Los Alamos National Laboratory
TA-43, HRL-1,
Los Alamos,
NM 87545

[3] Centre for Immune Regulation
Institute of Immunology
Rikshospitalet
University of Oslo
Sognsvannsveien 20,
N-0027 Oslo,
Norway

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 (Fiore *et al.*, 2008, Hoyert *et al.*, 2005, Podewils *et al.*, 2005). 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 (Hayden and Hay, 1992). Furthermore, only two anti-viral drugs (rimantadine, and oseltamivir) are approved for chemoprophylaxis of influenza virus infection (Govorkova *et al.*, 2001). 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) (Palese, 2006), making it a potential target for antibody-based immunity. The ability of a monoclonal anti-M2e antibody to reduce viral replication (Zebedee and Lamb, 1988) 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 (Slepushkin *et al.*, 1995). 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 (Neirynck *et al.*, 1999). Multiple antigenic peptide (MAP) vaccines have also been shown to be protective (Mozdzanowska *et al.*, 2003). While most human H1 and H3 influenza viruses share complete homology with the M2e consensus sequence (called conM2 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 conM2 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. (Fan *et al.*, 2004). 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 (Okuda *et al.*, 2001, Watabe *et al.*, 2001). Importantly, while a recent study used matched M2e peptide-liposome vaccines of various subtypes (Ernst *et al.*), 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 (Tompkins *et al.*, 2007). 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 (Mozdzanowska, et al., 2003) or immunization with purified M2 (Zebedee and Lamb, 1988), have recently been published (Zhang *et al.*, 2006). These all have very similar recognition properties, with the recognized epitope located between amino acids 4-16 of the external portion of M2 (Zhang, et al., 2006). 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 (Zebedee and Lamb, 1988), and reduces viral plaque size (Zebedee and Lamb, 1988) and viral production levels (Hughey *et al.*, 1995) in vitro. Both 14C2 and another antibody, M2-80, have also been shown to have significant protective effects in mice (Mozdzanowska *et al.*, 1999, Treanor *et al.*, 1990, Zharikova *et al.*, 2005).

The demonstrated anti-viral activity of 14C2 suggests that it would be a good candidate for humanization (Carter *et al.*, 1992, Chothia *et al.*, 1985, Hwang *et al.*, 2005, Kettleborough *et al.*, 1991, Pedersen *et al.*, 1994, Roguska *et al.*, 1994, Routledge *et al.*, 1991, Studnicka *et al.*, 1994, Tsurushita *et al.*, 2005, Vargas-Madrazo and Paz-Garcia, 2003), 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 (Carter, et al., 1992, Chothia, et al., 1985, Hwang, et al., 2005, Kettleborough, et al., 1991, Pedersen, et al., 1994, Roguska, et al., 1994, Routledge, et al., 1991, Studnicka, et al., 1994, Tsurushita, et al., 2005, Vargas-Madrazo and Paz-Garcia, 2003), 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.

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) (Macken *et al.*, 2001). 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) (Neirynck, et al., 1999), 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 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 (Zhang, et al., 2006) 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). Table 2 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 (Chothia, et al., 1985, Hwang, et al., 2005, Pedersen, et al., 1994, Roguska, et al., 1994, Studnicka, et al., 1994, Vargas-Madrazo and Paz-Garcia, 2003). 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 (Razai *et al.*, 2005). In total, seven murine amino acids were retained (4 in VH and 3 in VK, underlined in Table 2). 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 (Sblattero and Bradbury, 2000).

h14C2 recognition of M2

In a first test of reactivity the scFv was shown to bind to the conM2 peptide (figure 1). 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 (Di Niro *et al.*, 2007). 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 (Hughey, et al., 1995). 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 (Hughey, et al., 1995). Furthermore, over 75% of antibodies developed against M2 during virus infection recognize cell expressed M2, but not the synthetic peptide (Feng *et al.*, 2006). 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), expressing the consensus M2e. To avoid M2-mediated toxicity, expression of the M2 genes was regulated by a tetracycline-inducible promoter. Figure 2 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 2). 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 (Palese, 2006). Western blots of fixed and non fixed 293-conM2 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 (Feng, et al., 2006). Western blotting with the 14C2 mAb or the human or murine h14C2 minibodies shows (figure 3a-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 4), 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 4a), 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 4b).

m14C2 in vitro neutralization data

The 14C2 mAb has previously been demonstrated to inhibit influenza virus replication in an in vitro plaque-reduction assay (Hughey, et al., 1995, Zebedee and Lamb, 1988). 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 5 shows the reduction of both plaque size and plaque number after incubation with 14C2 mAb alone or the h14C2 mouse minibody. Culture with 100μg/ml of h14C2 mouse minibody re-

sulted in reductions in plaque size and number, although culture with 25μg/ml of the 14C2 mAb was sufficient to reduce plaque size and number to a similar extent. Culture with 20μg/ml of purified 14C2 minibodies 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 (Fiore, et al., 2008). 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 (Wright, 2006). Oseltamivir and zanamivir are approved for chemoprohylaxis for seasonal or avian influenza virus, and both have been shown to be similarly effective against H5N1 virus (Govorkova, et al., 2001). Moreover, the neuraminidase inhibitors have fewer side effects than the M2 inhibitors rimantadine and amantadine, and drug resistance seems to develop less frequently (Moscona, 2005). 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 (Fiore, et al., 2008, Govorkova, et al., 2001, Gubareva *et al.*, 2001). In contrast, the licensed M2 inhibitors are no longer recommended for use as the incidence of drug resistant viruses has steadily increased (Barr *et al.*, 2007, Bright *et al.*, 2005, Bright *et al.*, 2006). More recently, the frequency of oseltamivir resistant viruses has also increased (Lackenby *et al.*, 2008) and NA inhibitor-resistant avian influenza viruses have been identified as well (de Jong *et al.*, 2005). 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. Al-

though polyclonal antibodies are FDA approved for eight pathogens (hepatitis B, CMV, botulism, RSV, rabies, tetanus, VZV and vaccinia) (Zeitlin *et al.*, 2000), 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 (Morrison *et al.*, 1984), humanized antibodies, in which murine antigen binding loops are grafted onto human variable region framework sequences (Jones *et al.*, 1986), and fully human antibodies, the latter being made by phage display (reviewed in (Winter *et al.*, 1994)), or by applying traditional hybridoma technology to mice transgenic for the human immunoglobulin loci (reviewed in (Lonberg, 2005)). 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 (Reichert *et al.*, 2005).

Recombinant antibodies offer many advantages for the treatment of diseases (Reichert, et al., 2005), including those caused by infectious agents (Reichert and Dewitz, 2006), and viruses in particular (Marasco and Sui, 2007). 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 (Reichert and Dewitz, 2006, Reichert, et al., 2005). 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 (Marasco and Sui, 2007, Reichert and Dewitz, 2006). 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 (Bartlett, 2006). 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 (Feldman *et al.*, 2000).

Influenza provides a number of viral targets for antibody therapies (Beigel and Bray, 2008). 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 (Simmons *et al.*, 2007), 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 (Gillim-Ross and Subbarao, 2007). Unexpectedly, antibodies against the influenza nucleoprotein (NP), which coats the viral RNA have also been shown to be protective in mice (Carragher *et al.*, 2008), although previous studies suggest NP immunization protects via T and not B cell responses (Epstein, 2003, Ulmer *et al.*, 1993). Finally, the M2 protein has been widely explored as an target for both vaccines and drug therapies. M2 is an appealing target as it is expressed to 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 (Fiers *et al.*, 2004, Tompkins, et al., 2007, Tripp and Tompkins, 2008, Wang *et al.*, 2008). 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 (Palese, 2006).

In this paper we describe the humanization of 14C2 (Zebedee and Lamb, 1988), 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 (Di Niro, et al., 2007), 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 (Hughey, et al., 1995). 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 (Schnell and Chou, 2008, Stouffer *et al.*, 2008), 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 (Zharikova, et al., 2005). 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 (Hughey, et al., 1995). In contrast, there is no evidence that 14C2 causes released virus to aggregate on the surface of cells or interferes with ion channel activity (Hughey, et al., 1995). 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) (Jegerlehner *et al.*, 2004, Wang, et al., 2008) or complement dependent cytoxicity (CDC) (Wang, et al., 2008) 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 (Garcia-Rodriguez *et al.*, 2007) using yeast display.

Figure legends

Table 1: sequences of M2e from all viruses infecting human in the LANL flu database represented 10 times or more. The number each sequence was found in the database is indicated. Dots represent homology.

Table 2: 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 (Zhang, et al., 2006), the first seven amino acids, in italics, were not determined and the sequence used here is derived from the M2-80 sequenced VH gene (Zhang, et al., 2006).

Figure 1: Recognition of the M2con peptide by h14C2 scFv and the parental mAb, 14C2.

Figure 2: Recognition of control 293 cells or 293 cells transfected with conM2 gene (induced or not induced) by 14C2, h14C2 murine minibody, and h14C2 human minibody by flow cytometry. 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: Western blot recognition of control 293 cells or 293 cells transfected with conM2 gene (induced). Western blot lanes: 1) Marker; 2) 293 cells; 3) 293-conM2; 4) 293-conM2, Fixed.

Figure 4: Recognition of control 293 cells (293) or 293 cells transfected with conM2 gene and induced (conM2) by cell-based ELISA. a) human minibody constructs; b) murine minibody constructs.

Figure 5: In vitro plaque inhibition 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.

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 $TCID_{50}$, 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 Yewdwll, 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 (Sblattero and Bradbury, 2000) 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 (Di Niro *et al.*, 2008), specifically, the cloning of the h14C2 scFv, as well as of the control D1.3 (Mariuzza *et al.*, 1983) was performed by excising the scFv genes from the pDAN5 phage display vector using BssHII and NheI and cloning directly into the minibody contructs 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 DH5αF', grown on 2XTY agar plates (containing 3% glucose and carbenicillin 50μg/ml). The following day a small streak of bacteria was added to 10ml of carbenicillin/glucose 2XTY media and grown to 0.5 OD_{600}. The bacteria were centrifuged and re-suspended in 10ml of carbenicillin/IPTG (250mM) 2XTY media and protein produc-

tion 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 M^{2+} 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 Imject 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 1µg/ 100µl concentration and incubated at 4°C overnight. Excess protein was washed with PBSLT (1XPBS with 0.01% tween) and 200µl of wonder block (0.3% BSA, 0.3% milk, 0.3% fish gelatin) and incubated for 1 hour at RT. After washing with PBSLT, 70µl of scFv culture supernatant and 30ml 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 (1mg/ml) followed by anti-mouse HRP (Dako Inc. 0.5mg/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 1mg/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 titered 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, Genebank 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 (conM2) 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-conM2 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 (1μg/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-conM2 were selected and used throughout the studies.

Cell ELISA

The cell-based ELISA for M2-specific antibody binding was done as previously described (Feng, et al., 2006) with modifications. In brief, 239 control, 293-PR8M2, or 293-conM2 cells were plated in 96-well tissue culture plates in DMEM containing 10% FBS and tetracycline (1μg/ml final; to induce M2 expression), and incubated at 37°C, 5% CO_2. In some experiments control wells included 293-PR8M2 or 293-conM2 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 405nm on a 96-well plate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometry

Control 293, 293-PR8M2, and 293-conM2 cells were cultured in DMEM containing 10% FBS with or without tetracycline (1μg/ml final) to induce M2 expression. Seventy-two hours later, cells were harvested by washing with PBS (no Mg^{++} or Ca^{++}) + 2.5mM 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-conM2 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, UDA) 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 were 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 0.1ml. Cells were infected for 2 hours at 37°C and then overlaid with MEM + 1.2% Avicel (Matrosovich *et al.*, 2006), 1 µg/ml TPCK-treated trypsin, and antibody or minibody at the indicated concentrations. Plates were incubates 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.

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Table 1: conservation of M2e

M2e sequence	frequency	Notes
MSLLTEVETP IRNEWGCRCN DSSD	937	Consensus vaccine conM2
....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

Table 2: 14C2 and humanized sequences

VH

```
                <------------FWR1------------> <CDR1> <-----FWR2---> <------CDR2----->
                113131-333-333---31-1-1--------------11-1-333-1-111------------------
14c2            QVQLQQSGPEVVRPGVSVKISCKGSGYIFT DYAMH WVKQSHAKSLEWIG VISTYTGKTNYSQKFKG
62.6 VH1-2      QVQLVQS.A..KK..A...V...A...T..        ..R.APGQG...M.
humanized       QVQLVQSGAEVKKPGASVKVSCKASGYTFT DYAMH WVRQAPGQGLEWIG VISTYTGKTNYSQKFKG

                <--------------FWR3------------>    <CDR3>   <---FW4--->
                -1-1-1-1331-1-1-----333-3-111111-------------11-1-------
14c2            KATMTVDKSSSTAYMELARLTSEDSSVYYCAR  RGDYDAWFAY WGQGTLVTVSA
62.6 VH1-2      RV...R.T.I.......S..R.D.TA......
humanized       RATMTVDKSISTAYMELSRLRSDDTAVYYCAR  RGDYDAWFAY WGQGTLVTVSS
```

VL

```
                <---------FWR1--------> <------CDR1-----> <------FWR2---> <-CDR2>
                313131-3------3--3--1-1-------------------11-1-33--131111--------
14c2            DIVMSQSPSSLAVSVGEKVSMTC KSSQRLLYSSDQKNYLA WYQQKPGQSPKVLIY WASTRVS
78.2 B3 VK3     ....T...D.....L..RATIN.                    ........P..L...
humanized       DIVMTQSPDSLAVSLGERATMNC KSSQRLLYSSDQKNYLA WYQQKPGQPPKVLIY WASTRVS

                <-------------FWR3-------------> <--CDR3-> <--FWR4-->
                ---3---1-131131-1------33----111-----------11-13---3
14c2            GVPDRFTGSESGTDFTLTISSVKAEDLAVYYC QQYYTYPLT LTFGAGTKLELK
78.2 B3 VK3     ......S..G...........LQ...V.....
humanized       GVPDRFSGSESGTDFTLTISSLQAEDVAVYYC QQYYTYPLT FGQGTKVEIK
```

Figure 1: recognition of M2e peptide

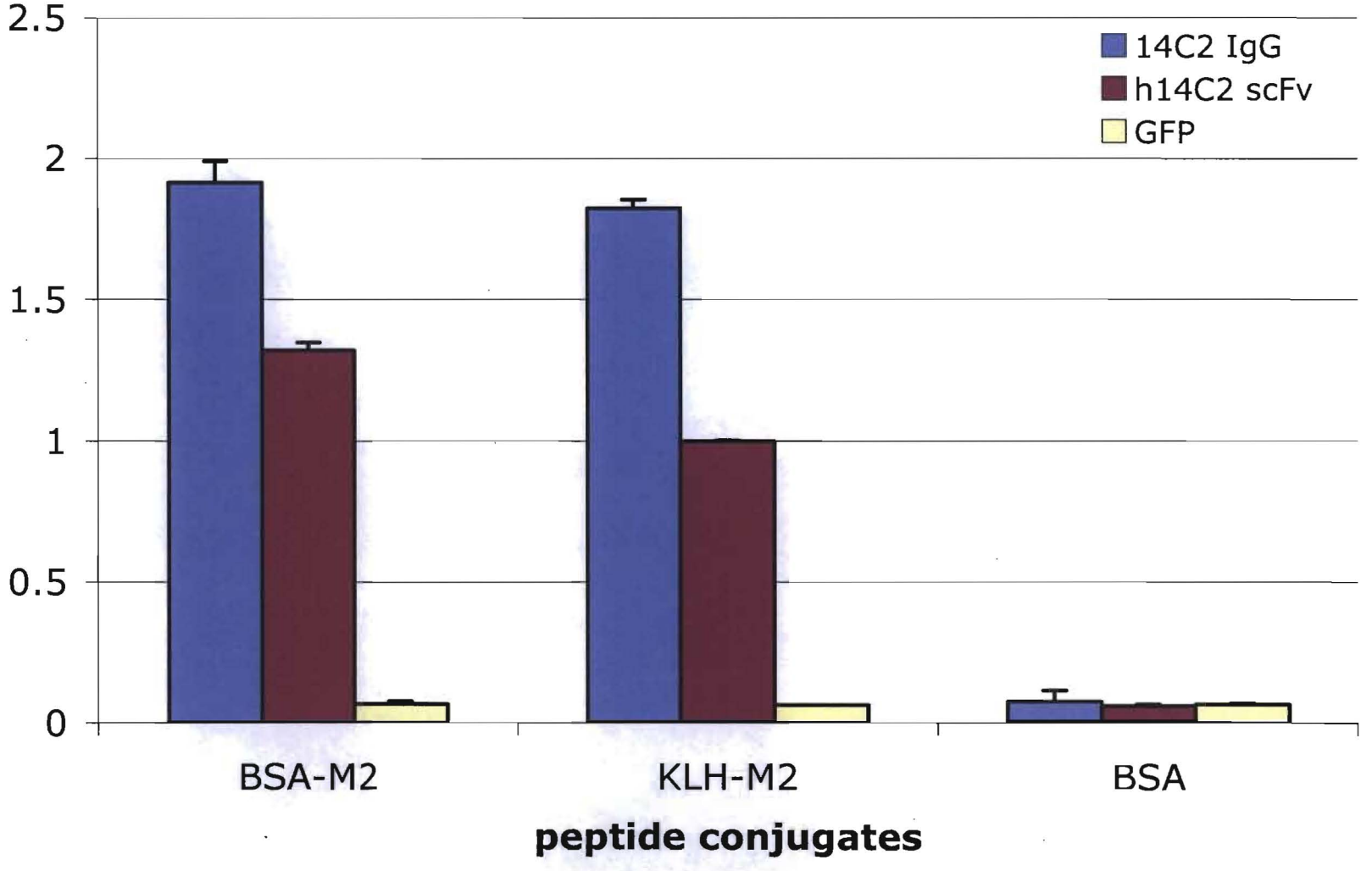

Figure 1: recognition of M2e peptide

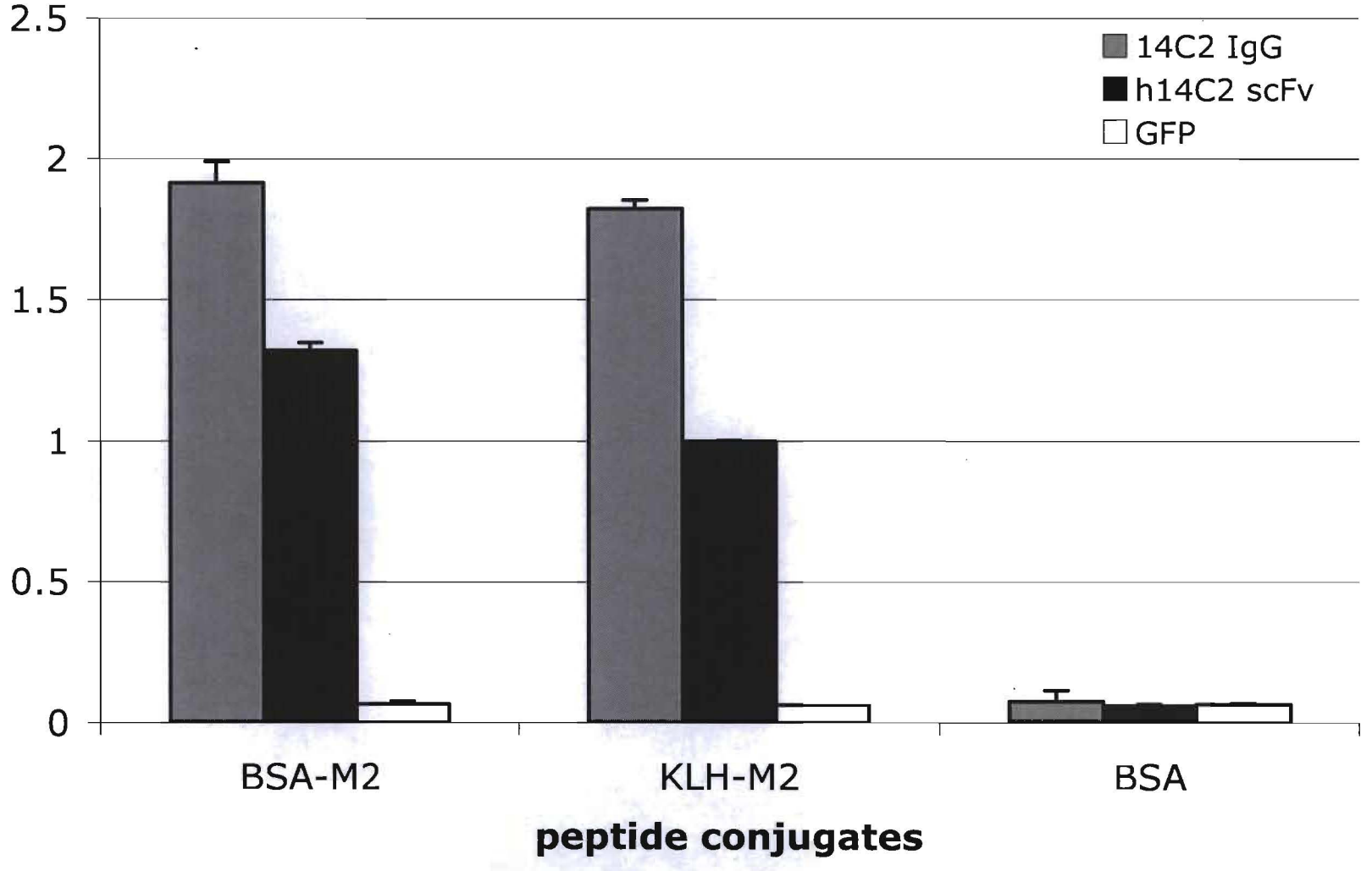

Figure 2: recognition of surface M2 by flow cytometry

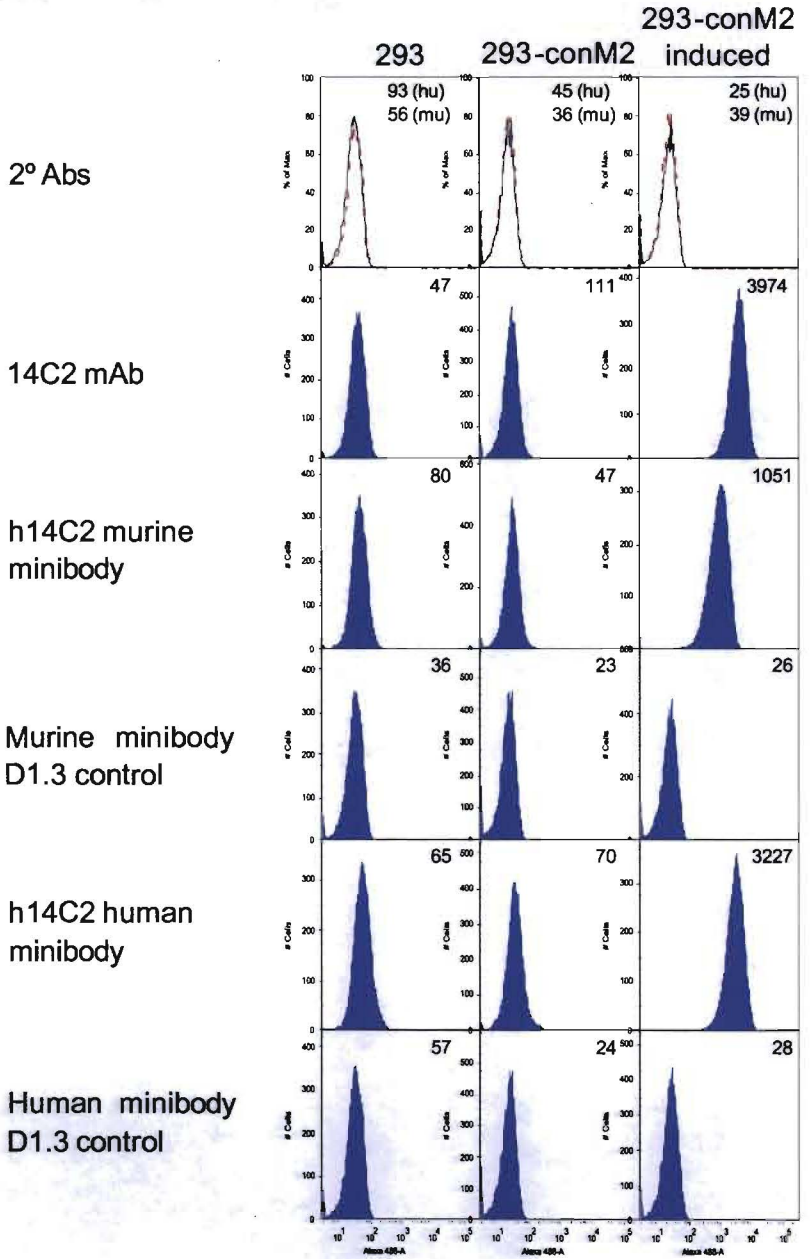

Figure 3: western blotting of M2

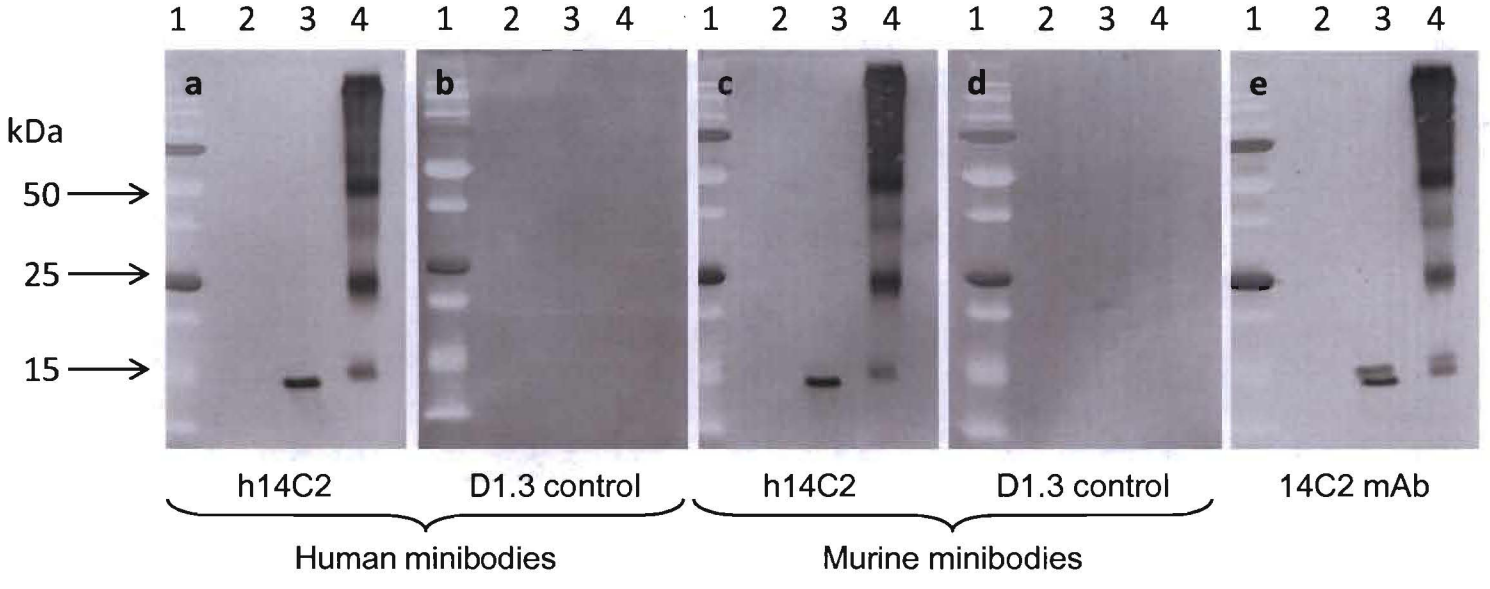

Figure 4: ELISA based recognition of M2 on cell surfaces

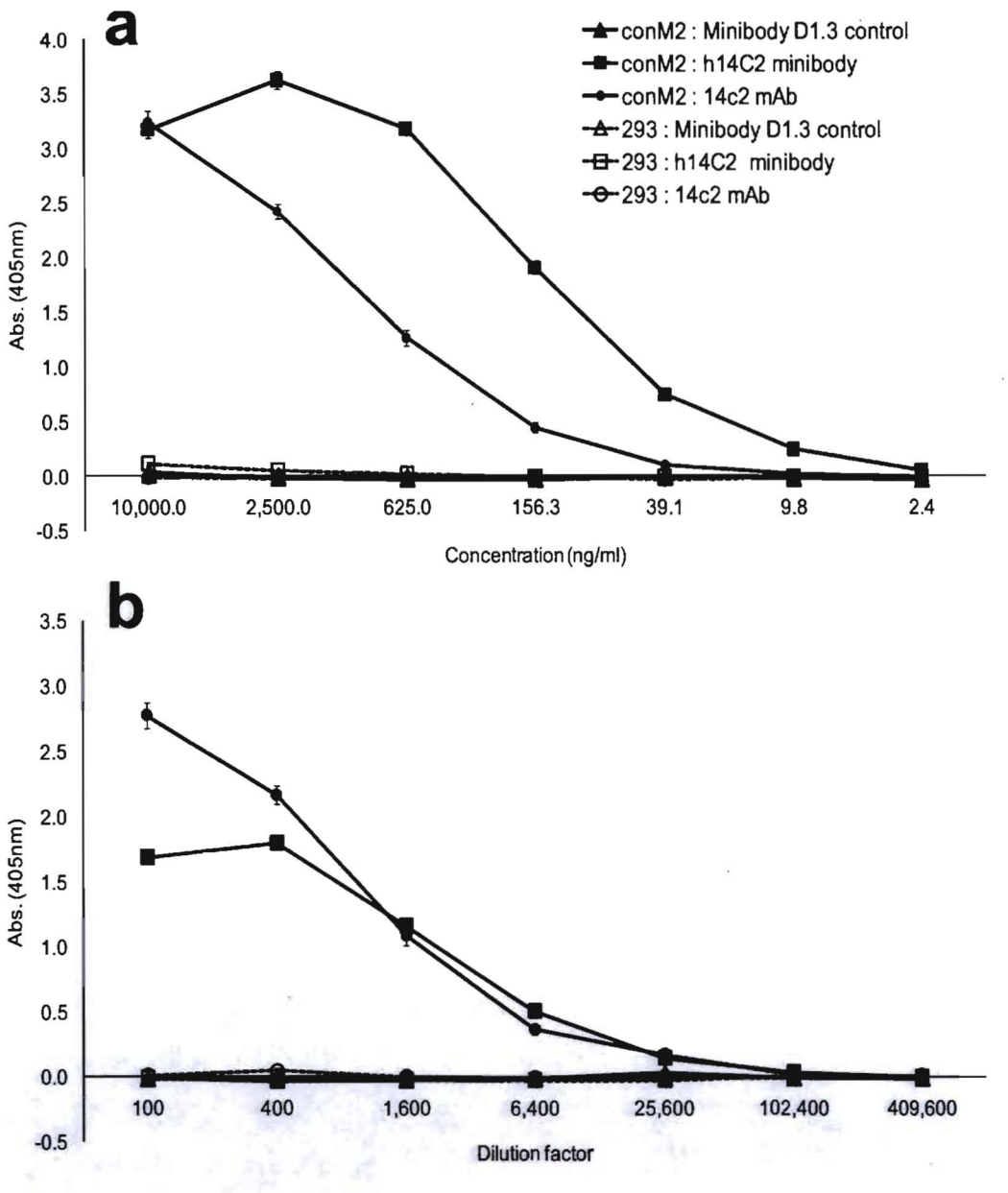

Figure 5: in vitro inhibition of viral infection