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Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein

Rongfang Wang[a,1], Aihua Song[a,1], James Levin[a], Dawna Dennis[a], Noelle J. Zhang[a], Hitoshi Yoshida[a,b], Lilia Koriazova[a], Lydia Madura[a], Laura Shapiro[a], Atsushi Matsumoto[b], Hideaki Yoshida[b], Toshifumi Mikayama[b], Ralph T. Kubo[a,d], Sally Sarawar[c], Hilde Cheroutre[d,2], Shinichiro Kato[a,b,*,2]

[a] *Kirin Pharma USA, Inc., La Jolla, CA, USA*
[b] *Kirin Pharma Co. Ltd., Tokyo, Japan*
[c] *Torrey Pines Institute for Molecular Studies, San Diego, CA, USA*
[d] *La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA*

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ABSTRACT

Influenza is one of the most prevalent viral diseases in humans. For some high-risk human populations, including the infant, the elderly, and the immunocompromised, who may not benefit from active immunization, passive immunotherapy with antibodies reactive with all influenza A strains may be an alternative. In this study, we characterized several fully human monoclonal antibodies (MAb) reactive with M2e, which were generated from transchromosomic mice engineered to produce fully human antibodies following immunization with a consensus-sequence M2e peptide. The MAbs showed strong binding to M2e peptide and to virus infected MDCK cells. One MAb recognizing the highly conserved N-terminal portion of consensus M2e displayed high binding to the majority of M2e variants from natural viral isolates, including highly pathogenic avian strains, which were recently reported to infect humans. Passive immunotherapy with this MAb in mice resulted in significant reduction in virus replication in the lung and protection from lethal infection when administered either prophylactically or therapeutically. These results suggest the potential of the anti-M2e human MAb with broad binding spectrum as a universal passive immunotherapeutic agent to infection by influenza A virus.

1. Introduction

Influenza A remains a major viral disease of humans, and typically affects 5–10% of adults during annual influenza epidemics. This results in approximately 20,000–40,000 infection related deaths and more than 100,000 hospitalizations annually in the USA alone. Infection rates are greater in high-risk populations, which include infants, young children, the elderly, and immunosuppressed persons (Simonsen et al., 2000; Thompson et al., 2003). Influenza viral infection is a significant and growing health concern for people at risk, as well as for healthy young adults due to increasing incidence of transmission of avian viral strains (H5N1, H7N2 and H9N2) to humans (Fouchier et al., 2004; Hayden, 2004).

Influenza infection elicits both T and B cell (antibody) responses, both of which play important roles in viral clearance (Couch, 2003; Jegerlehner et al., 2004; Mozdzanowska et al., 1999; Palladino et al., 1995). The annual influenza vaccine aims to stimulate the generation of anti-hemagglutinin (HA) neutralizing antibodies, which confer protection from homologous strains (Jegerlehner et al., 2004; Palladino et al., 1995; Virelizier, 1975). Vaccine efficacy in healthy young adults ranges from 70% to 90%, if the strains included in the vaccine match the circulating ones (Regoes and Bonhoeffer, 2006). However, the major shortcoming of this strategy is that it targets the highly variable HA determinant, and predicting the major HA types that pose the greatest annual epidemic threat is a significant limitation to the current vaccine strategy.

Abbreviations: M2, matrix protein 2; M2e, ectodomain of M2; MAb, monoclonal antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; HA, hemagglutinin; NA, neuraminidase.

* Corresponding author at: Kirin Pharma USA, Inc., 9420 Athena Circle, La Jolla, CA, USA. Tel.: +1 858 952 7040; fax: +1 858 952 7001.
E-mail address: skato@kirinpharma-usa.com (S. Kato).

[1] Co-first author of this article.
[2] Co-last author of this article.

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Furthermore, the conventional influenza vaccine has to be adapted every year to follow the antigenic drift and shift of the virus, and large-scale vaccine production takes months to prepare, which may limit the ability to provide immediate protection when a pandemic occurs (Kaiser, 2006). Recently it was demonstrated that neutralizing anti-H5N1 human MAbs protected mice from infection with highly pathogenic avian strains (Simmons et al., 2007), but again, this passive immunotherapy still targets the highly variable HA molecule.

Efforts have been made to develop a vaccine targeting another transmembrane protein of influenza A, matrix protein 2 (M2) (Mozdzanowska et al., 2003; Neirynck et al., 1999). M2 is expressed on the influenza virus surface as tetramers (Holsinger and Lamb, 1991; Sugrue and Hay, 1991) and functions as a proton channel required for the release of ribonucleoprotein complexes from the viral membrane after the viral entry (Helenius, 1992; Pinto et al., 1992; Zhirnov, 1990). M2 is expressed at low levels on the virus particle (1/50 of the HA density) but is abundant on infected cells (50% of HA level) (Feng et al., 2006). Unlike HA and NA, the extracellular domain of M2 (M2e), a 23 amino acid peptide, has remained highly conserved among all human influenza viruses, due in part, to being co-transcribed with another essential but non-surface exposed protein, matrix protein 1 (Lamb and Choppin, 1981). In particular, the first eight amino acids of M2e are rarely mutated in human and avian viruses (Helenius, 1992). This distinguishing characteristic makes M2e an attractive target for a 'universal' vaccine (Fan et al., 2004; Fiers et al., 2004; Mozdzanowska et al., 2003; Neirynck et al., 1999). The strategy to target M2e was initially inspired by the observation that passive immunotherapy with mouse anti-M2e MAb (14C2) reduced human influenza A virus replication in the lungs of mice (Treanor et al., 1990). However, M2e is weakly immunogenic (Fiers et al., 2004). M2e specific antibody responses are poorly induced in humans during natural influenza infection (Black et al., 1993; Feng et al., 2006; Liu et al., 2003), and the initial attempt to vaccinate mice and ferrets with M2-expressing recombinant vaccinia virus showed no evidence of protection (Epstein et al., 1993; Jakeman et al., 1989). Several groups examined various types of vaccine constructs engineered by conjugating multiple copies of M2e peptide with a carrier protein, such as hepatitis B virus core (HBc) protein and KLH (Fan et al., 2004; Jegerlehner et al., 2004; Mozdzanowska et al., 2003; Neirynck et al., 1999). Multiple immunizations of conjugated M2e derived from a human influenza strain have successfully protected more than 90% of animals against infection from not only homologous but also heterologous human strains (Fan et al., 2004; Neirynck et al., 1999). Although this vaccine approach displays substantial protection in several species, it requires multiple injections of high dose immunogens with one or two adjuvants to achieve high level of anti-M2 antibody titer. This encumbers the translation of the current M2 vaccine strategy to humans, especially to young children, the elderly and immunocompromised populations, who in general benefit less from active immunization.

Here we report the generation and characterization of fully human MAbs targeting M2e, and the use of one of these MAbs (Z3G1) in passive transfer experiments. Our data demonstrated that Z3G1 provided significant protection from influenza A infection in mice through the mechanisms of ADCC and CDC, reducing the lung viral titer at the peak stage of the viral infection without diminishing the endogenous anti-HA antibody response. Furthermore, this MAb recognizes a broad spectrum of M2e variants, including those with distinctive amino acids in the middle region of M2e (Liu et al., 2005; Zebedee and Lamb, 1988; Zharikova et al., 2005), which are typically observed in the M2e from avian strains (Liu et al., 2005). These characteristics of the MAb support its potential as "universal" passive immunotherapeutic agent.

2. Materials and methods

2.1. Peptide synthesis and generation of peptide-BSA conjugates

All peptides were synthesized by either A & A laboratories, LLC or Abgent (San Diego, CA). The purity of each peptide was >95% determined by HPLC. The peptide conjugates (M2e-BSA) were prepared using the EDC conjugation kit (PIERCE Biotechnology, Inc., Rockford, IL) according to the manufacturer's instruction.

2.2. Mice

Human transchromosomic mice harboring a human chromosomal fragment containing the immunoglobulin heavy chain locus and transgenic human kappa light chain genes (KM Mice™), or an artificial human chromosome containing the heavy and lambda light chain loci (λHAC Mice) were obtained from Kirin Pharma Co. Ltd., Tokyo, Japan. The mouse heavy and kappa chain loci have also been deleted in these mice. C57BL/6, FcγRIII knockout (B6.129P2-Fcgr3tm1Sjv/J) and C3 knockout (B6.129S4-C3tm1Crr/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the animal facility at the La Jolla Institute for Allergy and Immunology under specific pathogen-free conditions. All animal studies were conducted according to the guidelines of the La Jolla Institute for Allergy and Immunology IACUC committee.

2.3. Generation of anti-M2e monoclonal antibodies

KM or λHAC mice were immunized subcutaneously with 20 μg of M2e-BSA conjugates in Complete Freund's adjuvant (CFA, Sigma, St. Louis, MO). The conjugate concentration was determined by Lowry using BSA as a standard. Mice were boosted subcutaneously with 20 μg of the same material as used in the primary immunization in Incomplete Freund's adjuvant (IFA, Sigma, St. Louis, MO), and then with the material in RIBI (Corixa, Hamilton, MT) intraperitoneally with an interval of 21 days. The mice were boosted again with 10 μg of M2e peptide intravenously 3 days prior to the isolation of spleen cells.

Hybridomas were generated by the fusion of splenocytes to myeloma cells (SP2/O-Ag14, ATCC, Rockville, MD) in the presence of 50% polyethylene glycol (Boehringer Mannheim, Indianapolis, IN) followed by the selection with HAT. Approximately 2000 hybridoma growth-positive wells of each fusion were screened by peptide based ELISA. Cells positive for binding to M2e peptide were cloned by four rounds of limiting dilution. The specificity of M2e MAbs was further confirmed by the binding to native M2 molecules on the surface of MDCK cells (Madin-Darby Canine Kidney epithelial cells) (ATCC, Rockville, MD) infected with either A/PR/8/34 or A/HK/1/68 in a cell-based ELISA.

The mouse monoclonal anti-M2e MAb, 14C2, was purchased from Abcam (Abcam, Cambridge MA).

2.4. Influenza A virus-infected cell-based ELISA

Briefly, MDCK cells (4.5×10^4/well) were cultured in minimal essential media (MEM) containing 10% FCS, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 1 mM sodium pyruvate (InVitrogen, Carlsbad, CA) (cMEM) in 96-well flat bottom plates for approximately 24 h to form confluent cell monolayers. They were then infected with 20× $TCID_{50}$ of influenza A virus (A/PR/8/34 from ATCC, Rockville, MD; A/HK/1/68 kindly provided by Dr. J. Katz, CDC, Atlanta, GA) in serum free MEM at room temperature for 30 min. The cells were then washed and cultured in cMEM containing 1 μg/ml trypsin (Worthington Biochem Corp, Lakewood, NJ) for 20–27 h. The M2 proteins

expressed on the surface of MDCK cells were detected by M2e mAbs using a series of 4-fold dilutions, and developed by HRP-conjugated goat anti-human IgG (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA) and TMB substrate (DAKO, Carpinteria, CA). The plates were read at 450 nm. The binding activity (EC50) was calculated using Prism4 software.

2.5. Peptide based ELISA

M2e-BSA, M2e peptide or M2e variant peptides diluted in carbonate–bicarbonate buffer (pH 9.3–9.5) were coated on Nunc MaxiSorp 96-well flat bottom plates (Fisher, CA) at 5 μg/ml overnight at 4 °C. After washing twice with wash buffer (0.05% Tween 20 in PBS), plates were blocked with blocking buffer (1% BSA and 5% FCS in PBS) for 1 h at room temperature, followed by the incubation with 50 μl of 2-fold serial dilutions of M2e mAbs for 1 h at room temperature. The plates were then washed 3 times with wash buffer, and incubated with HRP-conjugated goat anti-human IgG (1:20,000) for 1 h at room temperature, followed by development with TMB substrate. The absorbance was read at 450 nm within 30 min. The binding activity (EC50) was calculated using Prism4 software. For the M2e variants listed in Fig. 1, the sequences were found in Genbank from at least one viral isolate. M2: A/Hong Kong/1/68 (H3N2), M2EG: A/New York/687/1995 (H3N2), M2K: A/Wisconsin/3523/88 (H1N1), M2G: A/Puerto Rico/8/34 (H1N1), M2FG: A/X-31 (H3N2), M2DLTGS: A/Hong Kong/485/97 (H5N1), M2LTKGS: A/Hong Kong/542/97 (H5N1), M2LTGS: A/Hong Kong/156/97 (H5N1), M2LTGEKS: A/Hong Kong/1074/99 (H9N2), M2TGEKS: A/Canada/rv504/2004 (H7N3), M2TES: A/Viet Nam/1203/2004 (H5N1), M2TGS: A/chicken/Shanghai/F/98 (H9N2), M2TGEK: A/Netherlands/33/03 (H7N7), M2KTGEKS: A/Quail/Arkansas/16309-7/94 (H7N3NSA), M2TDGEKS: A/chicken/Pennsylvania/13552-1/98 (H7N2NSB), M2HTGEKS: A/chicken/California/1002a/00 (H6N2), M2P: A/swine/Quebec/192/81 (H1N1), M2SG: A/swine/Tennessee/25/77 (H1N1), M2TGE: A/DK/ST/5048/2001 (H3N8), M2KGENS: A/Turkey/VA/158512/02 (H7N2), M2GHTGKS: A/chicken/HongKong/SF1/03 (H9N2), M2PHTGS: A/chicken/HongKong/YU427/03 (H9N2).

2.6. Surface plasmon resonance measurements

The affinities of the anti-M2 antibodies for the M2e consensus sequence and its variants were determined by surface plasmon resonance (SPR) technology using a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). Anti-M2e MAbs were directly immobilized on three sample flow cells of a CM5 sensor chip by amine ligation according to the manufacturer's instructions. Non-relevant human anti-HSA MAb or mouse polyclonal IgG were immobilized on a fourth reference flow cell. MAb capture levels of 6000 response units (RU) were optimized to yield a maximum antigen binding of approximately 200 RU, which is within the range recommended for kinetic analysis (50–250 RU). M2e peptides were injected over the four flow cells in the appropriate range of concentrations in HBS-EP-DMSO buffer (Hepes-buffered saline, pH 7.4, 3 mM EDTA, 0.05% Biacore surfactant, 1.25% DMSO) at a flow rate of 50 ml/min for 120 s. Dissociation was carried out in running buffer (HBS-EP-DMSO) for 1200 s. Regeneration of the sensor chip was performed by using 60 μl of 10 mM Glycine–HCl, pH 1.5.

2.7. SPR data analysis

Association and dissociation rates (k_a and k_d, respectively) and overall affinity ($K_D = k_d \times k_a^{-1}$) were calculated using BIAevaluation 3.2 software (Biacore AB) using a 1:1 Langmuir binding model with mass transfer and the global analysis method (simultaneous

Human	Avian	Variant	Amino Acid Sequence	Z3G1	C40G1	14C2
+	+	M2e	SLLTEVETPIRNEWGCRCNDSSD	■	■	■
+	+	M2EG	----------**E**----**G**---	■	■	■
+	+	M2K	----------**K**------	■	■	■
+		M2G	----------**G**---	■	■	■
+		M2FG	-**F**----------**G**---	■	■	■
+	+	**M2DLTGS**	------**D**-**LT**--**G**-----**S**----	■	■	■
+	+	**M2LTKGS**	--------**LTK**-**G**-----**S**----	■	□	□
+	+	**M2LTGS**	--------**LT**--**G**-----**S**----	■	□	□
+	+	**M2LTGEKS**	--------**LT**--**G**-**E**-**K**-**S**----	■	□	□
+	+	**M2TGEKS**	---------**T**--**G**-**E**-**K**-**S**----	■	□	■
+	+	**M2TES**	---------**T**----**E**---**S**----	■	■	■
	+	M2TGS	---------**T**--**G**-----**S**----	■	□	■
+	+	M2TGEK	---------**T**--**G**-**E**-**K**------	■	□	□
	+	M2KTGEKS	------**K**--**T**--**G**-**E**-**K**-**S**----	■	□	□
	+	M2TDGEKS	---------**T**-**DG**-**E**-**K**-**S**----	■	□	□
	+	M2HTGEKS	--------**HT**--**G**-**E**-**K**-**S**----	■	□	□
	+	M2P	--**P**----------	■	■	■
	+	M2SG	----------**S**---------**G**-	■	■	■
	+	M2TGE	---------**T**--**G**-**E**--------	■	□	■
	+	M2KGENS	----------**KG**-**E**-**N**-**S**----	■	□	■
	+	M2GHTGKS	----**G**---**HT**--**G**---**K**-**S**----	□	□	□
	+	M2PHTGS	---**P**----**HT**--**G**-----**S**----	□	□	□

Fig. 1. Binding spectrum of anti-M2e antibodies to M2e variants. Binding of serially 4-fold diluted human and mouse anti-M2e MAbs to M2e variant peptide and BSA conjugates (M2e-BSA) was measured by the peptide-based ELISA. The binding activity was presented as EC50, calculated with Prism4 software. (■, black): EC50 < 1×10^9 M; (■, grey): EC50 > 1×, but <10×10^9 M; (□, white): EC50 > 10×10^9 M. Bolded amino acids in the amino acid sequence are the portions where consensus mutations are frequently seen in avian virus strains. Six M2e variants (bold) were derived from highly pathogenic avian virus strains, which were reported to be transmitted to humans. Data are representative of at least three independent experiments.

processing of the data corresponding to seven concentrations of M2e peptides used for each set of experiments). Each dataset was double referenced by subtracting both reference and buffer signals from binding responses.

2.8. Plaque assay

MDCK cells (9×10^5) were cultured in cMEM for 16–20 h in 6-well flat bottom plates, followed by infection with a series of 10-fold dilutions of virus in serum free MEM at room temperature for 30 min. The monolayers were washed with PBS and overlayed with 0.8% carboxymethyl–cellulose sodium salt (CMC) (medium viscosity) (Sigma, St. Louis, MO) in MEM containing 1% nonessential amino acids, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate, 1 mM sodium pyruvate and 1 μg/ml of trypsin. Cells were incubated in a 5% CO_2 incubator at 37 °C for 3 days. The cells were then fixed with 10% formaldehyde solution and plaques were counted after staining with 0.1% crystal violet (LabChem, Inc., Pittsburg, PA).

2.9. Hemagglutination inhibition (HI) assay

The HI assay was performed following the standard protocol (Coligan et al., 2005), using four hemagglutinating units of A/HK/1/68 (H3N2) and 0.8% chicken erythrocytes (Colorado Serum Company, Denver, CO) for hemagglutination.

2.10. In vivo efficacy of anti-M2 MAbs

Female C57BL/6, FcγRIII−/− or C3−/− mice (7–8 week old) were anesthetized by intraperitoneal injection of 15 μl/g body weight of Avertin (1:1 w/v of 2,2,2-tribromoethanol: *tert*-amylalcohol, Sigma, St. Louis, MO), and then infected intranasally with 30 μl of a lethal (3× MLD50, 1 MLD50 = 550 pfu/ml) or sub-lethal (1/3× MLD50) dose of A/PR/8/34 or A/HK/1/68. Various doses of the antibodies were given 1, 3 or 6 days before infection or 5 h to 5 days post-infection. A control group received isotype matched irrelevant human anti-HSA monoclonal antibody (Kirin Pharma Co., Ltd., Tokyo, Japan). In some experiments, C57BL/6 mice were injected intraperitoneally with 30 μg of Cobra Venom Factor (CoVF, Quidel Corporation, San Diego, CA) 1 day before and 2 days after the infection to deplete complement. Mice were observed daily for 22 days for survival. For measurement of the lung viral titer, the infected lungs were removed at various time points after infection, snap frozen in liquid nitrogen and homogenized in PBS. Virus titers were measured by plaque assay, as described above.

2.11. Statistics

Numeric data, including the anti-HA titers, were compared by Mann–Whitney tests, *p* values were two-tailed and significance was set at 5%. Mouse survival was compared by Logrank test (life table). All statistical analysis was performed using Prism4 software.

3. Results

3.1. The generation of fully human anti-M2 MAbs with high binding activity

To generate fully human antibodies, we used λHAC or KM[TM] mice (Kakeda et al., 2005; Larin and Mejia, 2002). Mice were immunized with a consensus 23 amino acid M2e peptide (SLLTEVETPIRNEWGCRCNDSSD) conjugated to BSA (M2e-BSA) as described in Section 2.

Table 1
Antigen binding activity of anti-M2e human MAbs

Antibody	Ig subclass	Binding activity ($\times 10^{-9}$) M		
		Peptide ELISA	Cell-based ELISA	
		M2-BSA	A/PR/8/34	A/HK/1/68
C40	G4κ	1.1	0.61 ± 0.16	n.d.
C40G1[a]	G1κ	0.5	0.52 ± 0.03	0.54 ± 0.06
L66	G1λ	0.9	0.56 ± 0.10	0.53 ± 0.05
N547	G1λ	0.9	1.05 ± 0.17	1.22 ± 0.06
Z3	G3λ	0.5	0.48 ± 0.08	n.d.
Z3G1[a]	G1λ	0.3	0.50 ± 0.03	0.49 ± 0.07

Values shown in the above table are either EC50 from a single experiment (peptide ELISA) or EC50 ± S.D. from three separate experiments (cell-based ELISA). All experiments were repeated at least three times. n.d.: not done.

[a] C40G1 and Z3G1 are the recombinantly produced MAbs with different IgG isotype (IgG1) from the original isolated mAbs, C40 (IgG4) and Z3 (IgG3).

A panel of IgG anti-M2e human MAbs was generated (data not shown), and four antibodies (C40, L66, N547 and Z3) were selected for further investigation based on their strong binding activities. Among the four antibodies, L66, N547 and Z3 were generated from λHAC mice, each containing a human γ chain paired with a human λ chain, while C40 was derived from a KM mouse, carrying a human γ chain paired with a human κ chain. Interestingly, the majority of the anti-M2 human MAbs generated in this study were human λ chain bearing MAbs derived from λHAC mice.

The original IgG isotypes of the four MAbs were IgG1 (L66 and N547), IgG3 (Z3) and IgG4 (C40). As demonstrated in Table 1, these four MAbs exhibited specific binding to the M2e peptide with binding activities (EC50, equilibrium concentration at 50%) in the range of 10^{-9} M by peptide-based ELISA (Table 1). Due to the poor efficacy of C40 observed in the in vivo study (see Fig. 4) and the known shorter half life of IgG3 in vivo, C40 and Z3 were class switched to IgG1 (designated C40G1 and Z3G1), and these IgG1 type recombinant antibodies were produced by transfected Chinese Hamster Ovary (CHO) cells. The binding activity of C40G1 and Z3G1 to M2e remained comparable with C40 and Z3, respectively, when measured by the peptide based ELISA (Table 1), and therefore, further studies were performed using these IgG1 MAbs instead of the original C40 and Z3. Importantly, all four IgG1 MAbs displayed strong binding activity to native M2 molecules expressed on virus infected MDCK cells determined by the cell-based ELISA, and the EC50 of these MAbs were in the 10^{-9} M range (Table 1). The binding activity to the native M2 molecule from A/PR/8/34 with a single amino acid mutation at position 21 (G21D) within the M2e portion was comparable with the binding activity to the native M2 from A/HK/1/68 with the consensus sequence of M2e (Table 1), indicating that the binding epitopes of these MAbs were likely located at the N-terminal or central region rather than C-terminal region of M2e. An irrelevant human IgG1 antibody, specific to human serum albumin (HSA), was used as a negative control, and showed undetectable binding to either M2e peptide or virus infected MDCK cells (data not shown). None of these MAbs were found to bind BSA (data not shown). Moreover, the binding could be specifically inhibited by M2e peptide or M2e-BSA (data not shown).

Next, an attempt was made to map the epitopes of these four anti-M2e MAbs using a series of N-terminal and C-terminal sequentially truncated synthetic peptides (data not shown). The results indicated that L66, N547 and Z3G1 recognized distinct but overlapping epitopes within the N-terminal portion of M2e, while C40G1 bound to a six amino acid epitope positioned in the center of M2e (Table 2). The epitope of the mouse MAb, 14C2 (purchased from Abcam, Cambridge MA), also resided relatively close to the middle region of M2e (Table 2), similar to the epitope of C40G1. Taken together, these results show that we have generated several fully

Table 2
Antibody epitopes

	1 5 10 15 20 24
Consensus	MSLLTEVETPIRNEWGCRCNDSSD
Antibody	
L66	SLLTEVETPIRNEWG
N547	LLTEVETPIRNEWG
Z3G1	LLTEVETPIR
C40G1	TPIRNE
14C2*	EVETPIRNEW

Epitopes were mapped by the ELISA using a series of N-terminal and C-terminal sequentially truncated M2e peptides. *14C2 is mouse anti-M2e MAb.

human IgG1 MAbs recognizing different portions of M2e with high binding activity.

3.2. A fully human anti-M2 MAb shows broad spectrum binding activity

We then investigated whether these MAbs were able to recognize a broad spectrum of M2e variants. We analyzed several hundreds of M2e sequences derived from human and avian virus strains listed in Genbank and the Los Alamos National Laboratory's flu database or kindly provided by Dr. J. Katz, CDC, Atlanta (data not shown). Although, M2e is highly conserved when compared with the other two influenza surface molecules, HA and NA, which constantly undergo antigenic drift (Kilbourne, 1999), several mutations were found in the 23 amino acids of M2e. However, amino acids at positions 2, 7, 9, 15 and 22, remained constant among isolates. We measured the binding activity of two of our MAbs (Z3G1 and C40G1) to a panel of M2e variant peptides from naturally occurring human and avian influenza strains. These two antibodies represented the human anti-M2 antibodies recognizing the epitope located either in the N-terminal or center portion of the M2e. Notably, the results demonstrated that Z3G1 could successfully bind to the majority of M2e variants, including various M2e peptides from avian strains, with the EC50 at the level of 10^{-9} M. Importantly, it bound to M2e peptides from highly pathogenic avian strains that were recently reported to be transmitted to humans, such as A/Hong Kong/156/97 (M2LTGS, H5N1) (Suarez et al., 1998), A/Vietnam/3046/2004 (M2TES, H5N1) (Li et al., 2004), A/Hong Kong/485/97 (M2DLTGS, H5N1) (Hiromoto et al., 2000; Lin et al., 2000), A/Hong Kong/542/97 (M2LTKGS, H5N1) (Shaw et al., 2002), A/Canada/504/2004 (M2TGEKS, H7N3) (Obenauer et al., 2006; Tweed et al., 2004), and A/Hong Kong/1074/99 (M2LTGEKS, H9N2) (Shaw et al., 2002). In contrast, C40G1 failed to bind a number of M2e variants that have amino acid mutations in the center portion of M2e (Fig. 1). The mouse MAb, 14C2, was used for comparison. Since 14C2 also recognizes an epitope located in the center region of M2e similar to C40G1 (Zebedee and Lamb, 1989; Zhang et al., 2006) (Table 2), it shared a similar binding pattern with C40G1 with poor binding activity to many avian M2e variants with mutations in the amino acid 10–20 positions of M2e.

The binding affinity of two human MAbs, Z3G1 and C40G1, and one mouse MAb to the M2e peptide and two M2e variant peptides was determined by Biacore analysis (Table 3). In the analysis, anti-M2e MAbs were directly immobilized on three sample flow cells of the sensor chip, and M2e peptides were injected over the flow cells. As for the dissociation constants (K_D) of these MAbs to the M2e peptide, C40G1 and Z3G1 showed comparable binding affinity to the mouse anti-M2e MAb, 14C2, with K_D of the same order of magnitude, 10^{-10} M. The two human MAbs showed much higher binding affinity to the M2TES peptide than the mouse MAb, 14C2. Consistently with the peptide based ELISA data, only Z3G1 showed strong binding affinity to the M2LTGS peptide, while C40G1 as well as 14C2 did not bind even at 100 μM under these experimental conditions. Overall, there was a good correlation between Biacore and peptide-based ELISA data. All the results strongly suggest that Z3G1 has a better binding profile than C40G1 or mouse MAb, 14C2.

3.3. Prophylactic treatment with the anti-M2 MAb protects mice from the viral challenge

To explore the therapeutic potential of these fully human anti-M2e MAbs, we examined the in vivo protective efficacy of Z3G1 in a mouse model of influenza. We first examined the protective effect of Z3G1 in a prophylactic setting, administering the MAb prior to challenge by virus. Mice were administered Z3G1 at various doses (10, 30 or 100 μg/mouse, i.p.), followed by intranasal challenge with a lethal dose of A/HK/1/68 virus (3× MLD50) after 24 h. Mice in a control group were injected with an isotype matched irrelevant human anti-HSA MAb (100 μg/mouse, i.p.). Mouse survival was monitored over 22 days. Notably, mice treated with Z3G1 were significantly protected from a lethal dose viral challenge, even at 10 μg/mouse (~0.5 mg/kg) (Fig. 2A). This indicated that antibody treatment inhibited viral growth, thus preventing viral pneumonia and eventual death. The body weight of individual mice was measured, and mice in all groups lost weight in a comparable rate before day 10. Surviving mice, in both Z3G1 treated and anti-HSA treated groups, began to gain weight and recovered between day 12 and 14. This suggested that the antibody treatment did not block the initial infection, but prevented the infection from progression and facilitated the virus elimination.

To test whether Z3G1 did in fact reduce viral replication in vivo, lung viral titers were determined at various time points during the infection by the plaque assay. The mice were treated with either Z3G1 or the control anti-HSA MAb (30 μg/mouse, i.p.) 1 day prior to the challenge of a sub-lethal dose of A/HK/1/68 (0.3x MLD50, i.n.). Lungs of infected mice were harvested at day 2, 5, and 8, for measurement of viral titers. The lung weight was measured, and no significant difference was detected between Z3G1 treated and anti-HSA treated groups. Nevertheless, the antibody treatment significantly reduced the viral titer at day 5 and 8 (Fig. 2B), which are the peak and late stages of the infection, respectively, with the sub-lethal dose. This suggested that the antibody treatment conferred significant protection during the first few days of infection, before adaptive immune responses came into play. Viral titers declined at

Table 3
Dissociation constants of anti-M2 antibodies towards the consensus sequence and its variants as determined by surface plasmon resonance

MAb	M2e		M2TES		M2LTGS	
	K_D (nM)	RA	K_D (nM)	RA	K_D (nM)	RA
Z3G1	0.19 ± 0.02	100	0.35 ± 0.04	54	1.04 ± 0.15	18
C40G1	0.49 ± 0.06	39	0.89 ± 0.22	21	NB	NA
14C2 (mouse mAb)	0.51 ± 0.09	37	361.67 ± 14.53	0.05	NB	NA

RA stands for relative affinity calculated according to the following equation: RA (%) = (K_D of Z3G1 − M2e)/(K_D of Mab_x − $peptide_y$) × 100. K_D data are presented as average of three sensograms with standard deviation. NB means no specific binding was observed up to 100 μM of M2 peptides concentration. NA means not applicable.

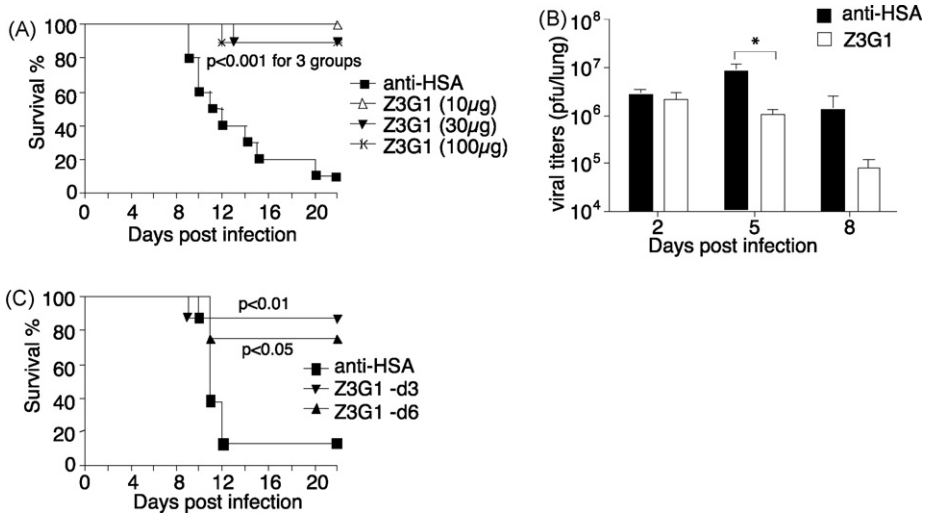

Fig. 2. Protective activity of the anti-M2e human MAb from the virus infection in the prophylactic setting. C57BL/6 mice were infected intranasally with either lethal or sub-lethal dose of A/HK/1/68 virus (3-fold and 0.3-fold of MLD50 respectively). (A) Mice received the human anti-M2e MAb (Z3G1) at 100 μg (n = 10), 30 μg (n = 10) or 10 μg (n = 7), or an isotype control human MAb (anti-HSA) at 100 μg (n = 10) 1 day before the lethal dose of virus infection. Mouse survival was observed daily for 22 days. P value, anti-HSA group vs. Z3G1 groups. (B) Mice (n = 8) received the anti-M2e MAb (Z3G1) or the isotype control MAb (anti-HSA) at 100 μg 1 day before the sub-lethal dose of virus infection. Infected lungs were harvested at various time points, and the virus titer of individual mice was measured by plaque assay. Data are shown as means + S.E.M., and means are the means of all animals in two experiments. *, $P < 0.05$, and ***, $P < 0.001$, anti-HSA group vs. Z3G1 group. (C) Mice (n = 8) were treated with the anti-M2e MAb (Z3G1) or the control antibody (anti-HSA) at 100 μg 3 or 6 days prior to the lethal dose of virus infection. Mouse survival was monitored daily for 22 days. P value, anti-HSA group vs. Z3G1 groups. Data are representative of at least two independent experiments.

day 8 in both groups, as the virus was probably cleared by the adaptive immune response, such as CD8 T cell and humoral responses (Allan et al., 1990; Doherty et al., 1997; Eichelberger et al., 1991; Mozdzanowska et al., 1997, 1999; Palladino et al., 1995; Scherle et al., 1992; Topham and Doherty, 1998). Taken together, our data clearly indicated that passive immunotherapy with Z3G1 in the prophylactic setting substantially inhibited viral replication in the lung and the development of severe pneumonia and also reduced mortality.

Next we investigated the approximate length of time a single administration of Z3G1 would protect mice from lethal challenge when used prophylactically. Mice were treated with Z3G1 (100 μg/mouse, i.p.) 3 or 6 days prior to infection with a lethal dose of A/HK/1/68, and survival was monitored over 22 days. The result demonstrated that Z3G1 was able to protect mice even when given 6 days prior to challenge (Fig. 2C). The serum concentration of Z3G1 was approximately 15–20 μg/ml at day 6 at the time of infection (data not shown). The minimum protective dose was defined as the serum concentration at the time of infection that could significantly protect mice from the lethal challenge. The minimum protective serum concentration of Z3G1 was approximately 10–20 μg/ml (data not shown). The ability to maintain an effective serum concentration is an important characteristic for a therapeutic when it is used prophylactically. These results suggest the feasibility of prophylactic use of anti-M2e MAb-based passive immunotherapy for durable protection from infection with influenza A virus.

3.4. Therapeutic treatment with the anti-M2 MAb protects mice from the viral infection

Based upon the in vivo efficacy of the anti-M2e MAb, Z3G1, observed in the prophylactic administration, we further asked whether the MAb was capable of controlling established viral infections. Preliminary studies in the therapeutic setting using some of the early candidate anti-M2 antibodies demonstrated that early treatment, e.g. within 24 h post-infection, and multiple doses were critical for the efficacy (data not shown). Similar therapeutic regimens were applied using Z3G1. Mice were infected with a lethal dose of A/HK/1/68, followed by the treatment with Z3G1 with different administration regimens at various time points after infection. Mortality was monitored over 22 days. Mice treated with Z3G1 5 h post-viral challenge followed by two additional half-dose treatments at days 3 and 5 were all protected from a lethal dose of influenza A virus (Fig. 3A). Mice receiving the first treatment at 24 h post-infection and further treatments with two consecutive doses during the next 2 days were also protected, although the results were statistically significant only at the high dose (100 μg/mouse/dose). These results suggested that Z3G1 was efficacious against an established and ongoing virus infection. The reduced protective efficacy at the lower dose (30 μg/mouse) suggested the demand of not only early intervention but also higher antibody dose to sufficiently control an established viral infection. To examine the effect of Z3G1 administered after infection on viral replication, lung viral titers were measured on day 5 after infection with a sub-lethal dose of A/HK/1/68 infection. All three regimens significantly reduced viral titers in the lung by 3–5-fold compared to the control group (Fig. 3B), although the effect was less dramatic than in the prophylactic setting. Similar observation was made in the study of the neutralizing MAb against respiratory syncytial virus, which exhibited higher efficacy when the MAb was given 24 h before the infection than 48 h after the infection (Mejias et al., 2004; Wyde et al., 1995). It appeared that moderate but significant viral reduction at the early stage of infection, before the adaptive immune response is effective, significantly contributed to viral clearance and eventually affected mortality. These results indicate the potential use of Z3G1 for post-exposure prophylaxis at the early stage of infection in both epidemic and pandemic settings. The inhibitory effect of late Z3G1 treatment, e.g. treatment at 48 or 72 h post-infection, was also examined, and the result demonstrated that Z3G1 treatment after 48 h could still significantly suppress the viral replication, while the treatment after 72 h showed a trend towards protection but did not reach significance (Fig. 3C). More studies using a range of viral infection doses will be carried out to evaluate the effect of Z3G1 at late times of infection on not only viral titers but also the symptom improvement as well as survival.

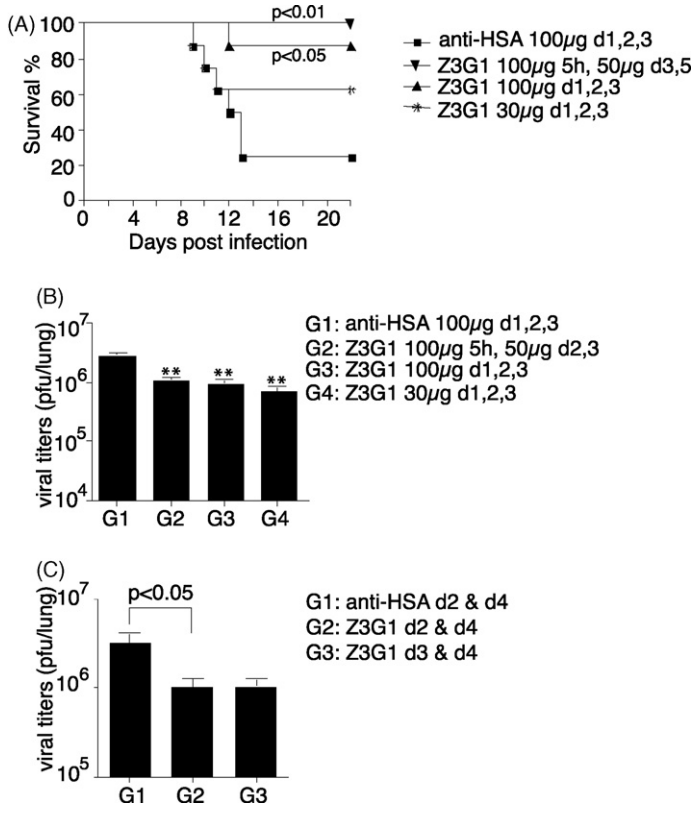

Fig. 3. Protective activity of the anti-M2e MAb from virus infection in the therapeutic setting. C57BL/6 mice ($n = 8$) were infected intranasally with either a lethal dose or a sub-lethal dose of A/HK/1/68 virus. (A) Mice were treated with anti-M2e MAb (Z3G1) or the isotype control MAb (anti-HSA) at the specified doses and times after infection with a lethal dose of virus. Survival was monitored daily for 22 days. *P* value, anti-HSA group vs. Z3G1 groups. (B) Mice ($n = 8$) were treated with anti-M2e MAb (Z3G1) or the control MAb (anti-HSA) at various doses during the first 3 days after a sub-lethal dose of virus and lungs were harvested at day 5 post-infection for plaque assay. **, $P < 0.01$, anti-HSA group vs. Z3G1 groups. Data are representative of two independent experiments. (C) Mice ($n = 14$–17) were treated with anti-M2e MAb (Z3G1) or the control MAb (anti-HSA) at day 2 (50–100 μg/mouse) and day 4 (50 μg/mouse) post a sub-lethal dose infection and lungs were harvested at day 5 post-infection for plaque assay. Data are representative of three independent experiments. Data are shown as means + S.E.M., and means are the means of all animals in three experiments.

3.5. ADCC and complement are required for the anti-M2 MAb mediated protection in mice

Similar to previously reported mouse anti-M2e antibodies (Jegerlehner et al., 2004; Mozdzanowska et al., 1999; Treanor et al., 1990), none of our MAbs showed direct virus neutralizing activity in vitro (data not shown). As M2 is expressed at extremely low density on the viral particle compared to HA and NA (Black et al., 1993; Mozdzanowska et al., 2003) and at high level (Lamb et al., 1985; Zebedee and Lamb, 1988) on the surface of infected cells, it is likely that the in vivo protection of the anti-M2e MAb we have demonstrated is mediated through antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-mediated cytotoxicity (CDC) (Jegerlehner et al., 2004; Mozdzanowska et al., 1999). This is initially supported by our observation that C40G1 (IgG1) but not C40 (IgG4) protected mice in vivo (Fig. 4A), which was consistent with the results of the in vitro studies demonstrating that C40G1 but not C40 showed ADCC (Suplementary Fig. A) and CDC activities (Suplementary Fig. B). To directly demonstrate the role of ADCC and CDC in passive immunity of the anti-M2 antibody, we assessed the ability of Z3G1 to confer the protection from influenza virus infection in FcγRIII−/− and C3−/− mice, respectively. The FcγRIII−/− mice do not have NK cell mediated antibody-dependent cytotoxicity function, while the C3−/− mice cannot activate the complement pathway. Wild type, FcγRIII−/− and C3−/− mice were administered Z3G1 (100 μg/mouse) 1 day prior to infection with a sub-lethal dose of A/HK/1/68 (0.3× MLD50). The lung viral titer from all mice was measured at day 5 of post-infection. Clearly, Z3G1 failed to reduce the lung viral titers in mice lacking either ADCC or complement, while it significantly decreased the lung viral titers in wild type mice (Fig. 4B). A different approach was taken to analyze the role of complement in anti-M2 MAb mediated protection, where wild type C57BL/6 mice were treated with CoVF (30 μg/mouse) 1 day before and 2 days after the infection to deplete the complement. Again, Z3G1 was not able to reduce the lung viral titer in complement-depleted mice in comparison to untreated control animals (data not shown). Taken together, these data clearly indicated that both ADCC and complement were required for non-neutralizing anti-M2 antibody mediated protection in vivo.

3.6. Anti-M2 MAb treatment does not influence the endogenous antibody response

As Z3G1 treatment reduced the lung viral titer, a question was raised on whether a Z3G1 therapeutic might influence the endogenous immune response, in particular, the generation of anti-HA neutralizing antibodies, critical for the protection from the secondary infection. To address this question, anti-HA titers in Z3G1 or anti-HSA treated mice were measured using the hemagglutination inhibition assay. Notably, comparable levels of the anti-HA titer in Z3G1 treated mice were observed after the viral infection (Fig. 5, $P > 0.05$, Z3G1 group vs. anti-HSA group at day 9, 14 and 21). The

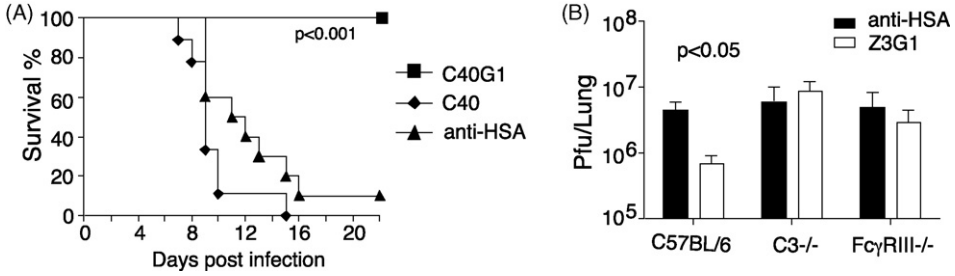

Fig. 4. ADCC and complement were both required for anti-M2e MAb mediated protection in mice. (A) C57BL/6 mice ($n = 8$) were infected intranasally with the lethal dose of A/PR/8/34 (3.5× MLD50). Mice were treated with C40G1 (IgG1), or C40 (IgG4) or the control MAb (anti-HSA, IgG1) (200 μg/mouse, intraperitoneally) at day 1, 2 and 3 post-infection, and the mouse survival was monitored daily for 22 days. *P* value, anti-HSA group vs. C40G1 group. Data are representative of two independent experiments. (B) C57BL/6, FcγRIII−/− and C3−/− mice ($n = 5$) were treated with the anti-M2e MAb (Z3G1) or the control antibody (anti-HSA), followed by the infection with the sub-lethal dose of A/HK/1/68 (1/3× MLD50). Lungs were harvested at day 5 post-infection to measure the viral titer via the plaque assay. Data are representative of two independent experiments.

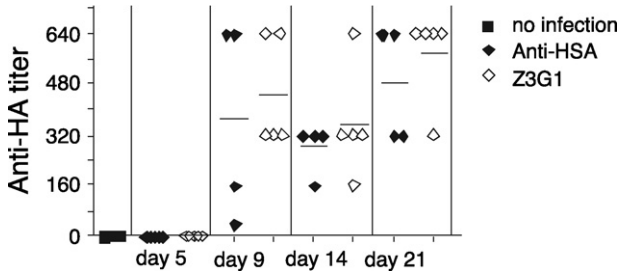

Fig. 5. Z3G1 treated mice generated comparable levels of anti-HA antibodies. C57BL/6 mice were treated with the anti-M2e MAb (Z3G1) or the control antibody (anti-HSA), followed by the infection with the sub-lethal dose of A/HK/1/68 (1/3× MLD50). Tail blood was collected at day 0 (pre-infection), 5, 9, 14 and 21. The anti-HA titer was measured via the standard HI assay.

result suggested that Z3G1 treatment did not influence the endogenous antibody response, despite the reduction of viral titer in the lung.

4. Discussion

To circumvent the limitations of the current influenza vaccine strategies that are mainly associated with the high variability of HA and NA, researchers have turned to the other surface antigen, M2. M2e seems to be a good candidate antigen for a vaccine because of its high degree of conservation with little variation since the emergence of the highly virulent pandemic strain of 1918 (Fiers et al., 2004). This high conservation of M2e is due, at least in part, to the special characteristics of the co-transcription of the two different proteins, M1 and M2, from the same genome fragment (Mozdzanowska et al., 2003; Neirynck et al., 1999). However, the inadequate antibody titer induced by the M2e vaccine is a particular challenge resulting from the low immunogenicity of M2e.

Here we describe the generation of several fully human IgG1 MAbs targeting M2e. These MAbs recognized not only the synthetic M2e peptide but also the native form of the M2 protein expressed on virus infected cells with high efficiency, and this translated into significant protection in mice from lethal dose infection of influenza A virus, even at a dose of 10 μg of MAb given 1 day prior to the infection. It is known that plasma half life of human IgG1 is about 20 days in the human body. As M2 is a virus-derived protein, the anti-M2e MAb may remain in the circulation longer than other MAbs targeting endogenous antigens. In fact, mice were protected from a lethal dose of the virus with 100 μg/mouse of Z3G1, even when they were given Z3G1 6 days prior to the infection. Taken together, one might expect protection from virus infection for a substantial period of time if the antibody is administered in sufficient quantity. This is an important aspect when the antibody is used prophylactically, and also indicates the advantage of antibody-based prophylaxis as compared to the conventional anti-viral therapy.

The potential disadvantage of anti-M2e antibody is that the anti-M2e antibody targets infected cells and do not kill or neutralize the virus. As the in vitro neutralization assay might not entirely reflect the in vivo situation, the ability of anti-M2e antibody to neutralize the virus in vivo cannot be completely excluded. The direct comparison of anti-M2e antibody, anti-HA neutralizing antibody and anti-viral drugs in controlling influenza infection should be analyzed in a future study. The fact that Z3G1 was capable of protecting mice when given 5 or 24 h post-infection, indicates the potential use of this MAb for post-infection prophylaxis and its therapeutic potential in the early stages of infection. Given that the anti-M2 antibody is not directly targeting the virus, high dose or multiple doses might be necessary to significantly inhibit the viral spreading. We have begun to evaluate the Z3G1 efficacy at late infection stages, e.g. 48 or 72 h post-infection, which are more relevant to the clinical situation. Our early study demonstrated that Z3G1 treatment at 48 h post-infection could significantly reduce the viral titer, while the treatment at 72 h only displayed a trend towards protection. As in the mouse infection model with a sub-lethal dose, virus can be eliminated rapidly after the establishment of endogenous adaptive immune response, which might obscure the antibody effect. Therefore, the effect of Z3G1 treatment probably needs to be investigated further with a range of viral infection doses in a kinetic analysis. Alternatively, this may indicate that the non-neutralizing anti-M2 antibody may have only moderate effects in the late stages of infection, and combinational therapy of anti-M2 and other anti-viral drugs may enhance the efficiency. Currently, combination treatment with anti-viral drugs targeting different steps of viral replication proved to have increased success in controlling influenza virus and decreased possibility in the emergence of escape mutants (Ilyushina et al., 2007; Masihi et al., 2007).

In this study, we evaluated the therapeutic efficacy by assessing lung viral titer and survival in a mouse model, of which the latter could serve as the indication of symptom improvement. Kinetic analysis of histopathology might also be useful, but the study of anti-RSV MAb (palivizumab) showed no or minor difference on the histopathologic scores at the peak (day 5) and late (day 70) time points in both prophylactic and therapeutic regimens (Mejias et al., 2005). This suggested that there might be no correlation of the lung histopathology change and MAb mediated lung viral titer reduction and overall symptom improvement in the murine pneumonia model. However, histopathology analysis in the animal model that closely resembles the human situation may help evaluate the therapeutic efficacy.

As anti-M2e antibodies do not neutralize the virus in vitro (Jegerlehner et al., 2004; Mozdzanowska et al., 1999; Treanor et al., 1990) and M2 is known to be expressed at high levels on the surface of virus infected cells (Lamb et al., 1985; Zebedee and Lamb, 1988), ADCC and CDC seem to be plausible mechanisms of action of our anti-M2e MAbs. Our observation of in vivo efficacy of C40G1 (IgG1) but not C40 (IgG4) indirectly supports this proposed mechanism because, in contrast to IgG1, IgG4 is known to have only weak ADCC and CDC activities. Our data showing that Z3G1 was not capable of protecting FcγRIII−/− and C3−/− mice from the influenza infection provided solid evidence that both ADCC and complement were of paramount importance for passive immunotherapy. ADCC mediated by NK cells through FcγRIII was required for the in vivo efficacy, which cannot be compensated solely by the CDC. The reduction of anti-M2 efficacy without the complement might not only result from the lack of CDC but also from the absence of some complement proteins, such as C3a and C5a, which can augment the recruitment of phagocytic cells to the infection site, thus initiating a local inflammatory response. This explains why Z3G1 could not confer the protection in C3−/− mice with intact ADCC. Jegerlehner et al. indicated that the protection conferred by the M2e vaccine is mediated mostly via ADCC rather than CDC (Jegerlehner et al., 2004; Mozdzanowska et al., 1999; Treanor et al., 1990). They demonstrated that passive immunotherapy with serum from mice previously vaccinated with M2e could protect C3−/− mice as well as wild type mice (Jegerlehner et al., 2004). The discrepancy between our data and theirs could be due to the specific experimental settings, in particular, the fact that we used monoclonal antibody while they used mouse serum. We showed that our MAb Z3G1 was able to recognize a variety of M2e peptides derived from human and avian isolates, including highly pathogenic H5N1 strains. The result indicates the potential of this MAbs as a "universal" passive immunotherapeutic for pandemic avian virus infection. One of the expected advantages of the M2e vaccine over the conven-

tional vaccine is that induced anti-M2e antibodies may recognize M2e derived from a variety of viral isolates. However, Fan et al. recently showed that sera from M2e vaccinated mice failed to bind synthetic M2e peptides corresponding to a pathogenic H5N1 strain, (A/Hong Kong/156/97, M2e corresponding to M2LTGS of Fig. 1), and another avian strain (A/Chicken/New York/95, M2e corresponding to M2TGEKS of Fig. 1) (Fan et al., 2004). These results indicate that the immunodominant epitope of anti-M2e antibodies induced by the M2e vaccine exists in the central region of M2e, which is highly variable and represents the host restriction specificities (Liu et al., 2005). Thus, the potential drawback of the M2e vaccine is its possible induction of a suboptimal antibody response that may not necessarily recognize M2e variants derived from various avian viruses.

Mouse anti-M2e MAbs, 14C2, 8C6 and 1B12 also recognize the middle portion of M2e, and similar to the antibody response after M2e vaccination, these MAbs showed weak or no binding to avian strains (Fig. 1) (Liu et al., 2004, 2005; Zebedee and Lamb, 1988). Our data also showed that the human anti-M2e MAb, C40G1, exhibited limited binding to a panel of M2e variant peptides (Fig. 1). The binding spectrum of C40G1 was similar to 14C2, and this observation is consistent with the fact that these two antibodies share the epitopes localized in the middle portion of the M2e.

In contrast to C40G1 or 14C2, Z3G1 recognized the N-terminal region of M2e. The epitope recognized by this antibody spans the region between amino acids 3 and 12 (LLTEVETPIR), in which the region, LLTEVET, is known to be highly conserved among all influenza A virus strains. It is known that M1 and M2 are co-transcribed from the same gene in the seventh genome fragment of influenza A virus (Lamb and Choppin, 1981). Particularly, the first 26 nucleotides from the start codon coding MSLLTEVET, were fully shared between M1 and M2. Therefore, the very low mutation frequency in this region might be attributable to the unique co-transcriptional character of the two genes in addition to the poor antibody response to this region, as evidenced in M2 vaccination studies (Fan et al., 2004). Furthermore, there is a tendency towards several common amino acid changes in the majority of avian virus strains as compared to human strains, which are represented by I11T, E14G, G16E, R18K and N20S and are mostly localized in the middle region of M2e (Liu et al., 2005). Z3G1 bound to the majority of M2e variant peptides from avian virus strains that have those common amino acid changes, which indicates the therapeutic potential of this MAbs to pandemic flu caused by the cross-species transmission of avian viruses to humans. Further studies of binding on avian viral infected cells as well as in vivo efficacy using H5N1 infected mouse and/or ferret models will help assess the therapeutic potential of Z3G1 during the pandemics.

It is known that the treatment of influenza infection with amantadine causes amino acid mutations in the transmembrane region of the M2 protein with high frequency (Hayden, 2004). The frequency of escape mutant generation under the Z3G1 treatment of virus infected mice remains to be analyzed. Zharikova et al. recently reported the low frequency and highly restricted single amino acid mutation in the ectodomain of the M2 protein after the treatment of virus infected SCID mice with 14C2 (Zharikova et al., 2005). These results suggest the possibility of low mutation rate of amino acids in this region even in the presence of an anti-M2e MAb.

In conclusion, our results suggest a prospective 'universal' passive immunotherapy for not only epidemic human virus infection but also pandemic avian virus infection. Our results also suggest that human anti-M2e MAb based therapy has a better profile as compared with the vaccine and might be a promising therapeutic particularly in certain high-risk groups including healthcare workers, newborns, the elderly, patients with chronic diseases and the immunocompromised.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2008.06.002.

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