A Pan-H1 Anti-Hemagglutinin Monoclonal Antibody with Potent Broad-Spectrum Efficacy *In Vivo*

Gene S. Tan, Florian Krammer, Dirk Eggink, Alita Kongchanagul, Thomas M. Moran, and Peter Palese

Department of Microbiology, Mount Sinai School of Medicine, New York, New York, USA

Seasonal epidemics caused by antigenic variations in influenza A virus remain a public health concern and an economic burden. The isolation and characterization of broadly neutralizing anti-hemagglutinin monoclonal antibodies (MAb) have highlighted the presence of highly conserved epitopes in divergent influenza A viruses. Here, we describe the generation and characterization of a mouse monoclonal antibody designed to target the conserved regions of the hemagglutinin of influenza A H1 viruses, a subtype that has caused pandemics in the human population in both the 20th and 21st centuries. By sequentially immunizing mice with plasmid DNA encoding the hemagglutinin of antigenically different H1 influenza A viruses (A/South Carolina/1/1918, A/USSR/92/1977, and A/California/4/2009), we isolated and identified MAb 6F12. Similar to other broadly neutralizing MAb previously described, MAb 6F12 has no hemagglutination inhibition activity against influenza A viruses and targets the stalk region of hemagglutinins. As designed, it has neutralizing activity against a divergent panel of H1 viruses *in vitro*, representing 79 years of antigenic drift. Most notably, MAb 6F12 prevented gross weight loss against divergent H1 viruses in passive transfer experiments in mice, both in pre- and postexposure prophylaxis regimens. The broad but specific activity of MAb 6F12 highlights the potent efficacy of monoclonal antibodies directed against a single subtype of influenza A virus.

Antigenic drift caused by periodic amino acid changes on the globular head of hemagglutinin (HA) is one of the hallmarks of influenza A virus immune evasion (28). It is also this phenomenon that requires the current influenza vaccine to be reformulated annually to match the upcoming circulating strain in the human population. Yet, seasonal influenza A virus epidemics, usually targeting the young and the old, still kill about 250,000 people worldwide each year (34). There are other countermeasures in addition to vaccines, such as the neuraminidase inhibitors oseltamivir (Tamiflu) and zanamivir (Relenza), which prevent viral egress, or the M2 ion channel inhibitor amantadine, to combat influenza A virus infections (1). However, frequent usage can select for escape mutants and ultimately give rise to drug-resistant influenza virus strains (12). Thus, there is always need for better vaccines and additional antiviral therapeutics.

The HA glycoprotein is an excellent target for an antiviral therapeutic, simply because it is the chief target of the immune response that elicits the most robust neutralizing antibodies during vaccination or natural infection. Composed of the HA subunit 1 (HA1) and HA subunit 2 (HA2) moieties, this homotrimeric molecule (i) is involved in the initial binding and internalization of the viral particle into the endosomal pathway, (ii) mediates the release of the viral ribonucleoprotein (RNP) from the endosome to the cytosol, and (iii) is involved in the budding of viral particles from the cell plasma membrane (23). The initial step requires the receptor-binding site found on the apex of the globular head (HA1) to attach to sialic acid, the host cell receptor. Upon acidification of the endosome containing the virus-receptor complex, the HA1:HA2 trimer undergoes drastic conformational change (2, 3), whereby HA1 disengages from HA2 and subsequently allows the fusion peptide located on the amino-terminal end of the HA2 subunit to mediate fusion of the viral and endosomal membranes (8). It is not surprising that antibodies target these two steps in viral entry. In fact, the typical anti-HA neutralizing antibody sterically blocks viral attachment to its cellular ligand by binding in or around the receptor-binding site of the globular head. Although

highly effective, they are strain specific and have little or no broad-spectrum activity (28).

Currently, there are 17 known influenza A virus subtypes, which are divided into two distinct phylogenetic groups, 1 and 2; subtype H1 from the former and H3 from the latter are presently cocirculating in the human population. Lately, there have been several reports of the isolation and characterization of human monoclonal antibodies (MAb) capable of recognizing and neutralizing a diverse number of influenza A virus subtypes. For several of these human monoclonal antibodies, heterosubtypic binding and neutralizing activity have been demonstrated against group 1 viruses (31, 32), group 2 viruses (6), and most recently, between the two divergent groups (4). Crystal structure analysis of MAb CR6261 was shown to bind to both H1 and H5 HA (5) on the short α -helix of the HA2 subunit, while MAb CR8020 bound to the membrane-proximal region of HA2 on both H3 and H7 HAs (6). Interestingly, FI6, a monoclonal antibody found to bind to 16 influenza A virus subtypes demonstrated binding also to the short α -helix of H1 and H3 (4). Conversely, a more recent study reported the isolation of a human MAb that recognizes a conserved region of the globular head of pandemic-related strains of H1 viruses (11). All these studies reveal the presence of amino acid conservation among divergent HAs. There are, however, limitations in the breadth of efficacy for monoclonal antibodies such as FI6, in that it lacks the ability to prevent transient weight loss against H1 or H3 challenges even at the highest concentrations in mice.

Here, we sought to design an immunization regimen to hone

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Address correspondence to Peter Palese, peter.palese@mssm.edu.

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the immune response and generate a monoclonal antibody that is broadly neutralizing and specific to only one subtype. It is interesting that when Okuno and colleagues previously generated the first reported heterosubtypic MAb, C179, which neutralized H1, H2, H5, H6, and H9 influenza A viruses, by solely immunizing mice with A/Okudo/1957 (H2) virus (20), they initially had to sort through hundreds of strain-specific MAb-producing hybridomas (19). We know from our own studies that the more-conserved HA stalk can elicit a broadly reactive response (7, 30) and that sequential immunization with diverse HAs can increase our chances of generating cross-neutralizing monoclonal antibodies (33). By immunizing successively with antigenically different HAs, the idea is to limit the B-cell immune response against the more variable globular domain to a primary response while allowing the immune response against the more conserved stalk region to be boosted. In the present study, we applied a similar immunization strategy in generating a pan-H1 monoclonal antibody that has broad and potent neutralizing activity against a diverse panel of H1 viruses *in vitro* but also provides considerable protection *in vivo*.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby canine kidney (MDCK) and 293T cells were maintained in Dulbecco's minimum essential medium (DMEM; Mediatech, Inc.) supplemented with 10% HyClone fetal bovine serum (Thermo Fisher Scientific, Inc.) and 100 units/ml of penicillin–100 µg/ml of streptomycin. The following viruses were grown in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Charles River Laboratories, Inc.): A/swine/Iowa/15/1930 (sw30) (H1), A/Puerto Rico/8/1934 (PR8) (H1), A/USSR/92/1977 (USSR77) (H1), A/Texas/36/1991 (TX91) (H1), A/New Caledonia/20/1999 (NC99) (H1), A/Solomon Islands/3/2006 (SI06) (H1), A/Brisbane/59/2007 (Bris07) (H1), A/Hong Kong/1/1968 (HK68) (H3), and a reassortant expressing the HA and the neuraminidase (NA) of A/Vietnam/1203/2004 (rVN04) (H5) with the internal genes of PR8 (H1). A reassortant expressing the HA and NA of A/California/4/2009 (rCal09) (H1) with the internal genes of PR8 (H1) was grown in MDCK cells.

Generation of MAb and screening. Six to 8-week-old female BALB/c mice (Jackson Laboratories, Inc.) were sequentially immunized intramuscularly with a DNA pCAGGs plasmid (17) carrying H1 virus HA, followed immediately with an electrical stimulation at the same site of immunization (Trigrid Systems; Ichor Medical Systems) (13, 14). The plasmids carrying the HA of A/South Carolina/1/1918 (SC18) (H1), USSR77 (H1)m and A/California/4/2009 (Cal09) (H1) were used to immunize mice at 2-week intervals. Three to 4 weeks after the last immunization, mice were boosted with 50 µg of a UV-inactivated purified whole-virus preparation of Bris07 (H1) virus intravenously. Mice were sacrificed and their spleens sterilely removed. The spleen was dissociated with a 10-ml 20-gauge needle into a single-cell suspension in serum-free 1× DMEM. Splenocyte and SP2/0 myeloma cells (in log phase) were combined in a 5:1 ratio and fusion mediated by using polyethylene glycol (molecular weight, 4,000) (33). The splenocyte and SP2/0 mixture was resuspended in 1× DMEM supplemented with hypoxanthine and thymidine (Life Technologies Corp.), and hybridomas selected by addition of azaserine (Sigma-Aldrich) 24 h after fusion. Hybridomas were grown for 10 to 14 days until screening.

MDCK cells in a 96-well format were infected at a multiplicity of infection (MOI) of 0.5 with rCal09 (H1) virus and grown in the absence of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. Twelve to 16 h postinfection (hpi), cells were fixed with 0.5% paraformaldehyde (PFA)–1× phosphate-buffered saline (PBS) and blocked with 5% nonfat (NF) milk–1× PBS. Supernatants from the hybridoma culture were added and incubated for 1 h at room temperature (RT). The monolayer was washed thrice with 1× PBS and then incubated with a goat anti-

mouse IgG γ -chain-specific antibody conjugated to horseradish peroxidase (HRP; Millipore) for 1 h at RT. Cells were washed thrice, and immunostaining using 3-amino-9-ethylcarbazole (AEC) substrate (BD Pharmingen) was used to visualize positive reactivity to rCal09 (H1) virus.

Expansion and purification of MAb. Hybridoma cultures were expanded in hybridoma serum-free medium (Life Technologies Corp.) to a final volume of 500 to 700 ml. Cultures were harvested by low-speed centrifugation (30 min, 5,500 × g) when viability of the culture dropped (visual examination under a microscope), and culture supernatants were passed through a 0.22-µm sterile filtration unit. The filtered supernatant was then passed through a gravity flow column containing protein G-Sepharose 4 Fast Flow beads (GE Healthcare) (9). The Sepharose beads were washed with 150 ml of sterile PBS (pH 7.4). Finally, MAb 6F12 or 7B2 was eluted with 45 ml of 0.1 M glycine-HCl buffer (pH 2.7). The eluate was immediately neutralized with 2 M Tris-HCl buffer (pH 10). The antibody was then concentrated and buffer exchanged against PBS (pH 7.4) using Amicon Ultra centrifugal filter units (10-kDa cutoff; Millipore). The protein concentration was determined by measuring the absorbance at 280 nm with a Nanodrop spectrophotometer.

Preparation of purified whole virus. Purified viral particles were prepared by harvesting allantoic fluid or tissue culture medium and spun at 82,705 × g for 2 h at 4°C over a 20% sucrose cushion (33). Pelleted viruses were then washed once with 1× PBS and spun at 82,705 × g for an hour at 4°C, reconstituted with 1× PBS, and stored at –80°C until further use.

Immunofluorescence. MDCK cells were infected at an MOI of 5 with USSR77 (H1), TX91 (H1), NC99 (H1), Bris07 (H1), rCal09 (H1), HK68 (H3), or rVN04 (H5) for 12 to 16 h in the absence of TPCK-treated trypsin. Cells were then fixed with 0.5% PFA–1× PBS for 30 min at RT and blocked with 5% NF milk–1× PBS for 30 min at RT. MAb were diluted in 5% NF milk–1× PBS and incubated at RT for 1 h at a final concentration of 5 µg/ml. The cell monolayer was washed three times with 1× PBS and then incubated with an Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody (Invitrogen) at a dilution of 1:1,000 for 1 h at RT. Fluorescence reactivity was visualized using an Olympus IX70 inverted fluorescence microscope. A chimeric HA (cH9/1) construct with the stalk domain of an H1 (PR8) HA and the globular head domain of an H9 (A/guinea fowl/Hong Kong/WF10/99) HA was constructed as described before (24). Wild-type PR8 HA (H1), A/guinea fowl/HK/WF10/99 HA (H9), cH9/1 HA, and HK68 HA (H3) were expressed in High Five insect cells by using a recombinant baculovirus vector (10) or in 293T cells by plasmid transfection. Cells were stained as described above with MAb 6F12 or anti-H3 stalk MAb 12D1 (33).

Enzyme-linked immunosorbent assay (ELISA). Fifty microliters of purified preparations of hemagglutinins (at 2.5 µg/ml) or whole viruses (at 5.0 µg/ml) were used to coat Costar 96-well enzyme immunoassay/radioimmunoassay (EIA/RIA) high-binding plates (Corning Inc.) overnight at 4°C. The next day, plates were washed twice with 0.1% Tween 20–1× PBS (TPBS) and blocked with 5% NF milk–1× PBS for 30 min at RT. Starting dilutions of select MAb were either 100 or 30 µg/ml and incubated at RT for 2 h. After the incubation, plates were washed thrice with TPBS, then incubated with a 1:5,000 dilution of a goat anti-mouse IgG γ -chain-specific antibody conjugated to HRP (Millipore), and incubated at 37°C for 1 h. Plates were then washed thrice with TPBS and developed with 200 µl of Sigmafast OPD peroxidase substrate (Sigma-Aldrich) for 15 to 30 min in the dark. The signal was read at an absorbance of 405 nm or 490 nm when stopped with 50 µl of 3 M sulfuric acid. For positive controls, sera from infected Cal09, JP57, and B/Yamagata/1988 mice were used as controls, as well as the following MAb: PY102 (26), XY102 (18), 8 (BEI NR-2731), and G1-26 (BEI NR-9691). All MAb and secondary antibodies were diluted in 1% bovine serum albumin (BSA)–1× PBS. A nonlinear regression curve was generated using GraphPad Prism 4.0, and the 50% effective dose (EC₅₀) was calculated.

Competitive ELISA. MAb 6F12 was first biotinylated using the ChromaLink One-Shot antibody biotinylation kit (Solulink). Plates were coated with purified baculovirus-expressed Cal09 HA (NR-15749; ob-

tained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH) as described above and incubated overnight at 4°C. Plates were washed twice with TPBS and then blocked with 5% NF milk–1× PBS for 30 min at RT. After the block, competition was done by preincubating Cal09 HA with 10 µg of human MAb CR6261 or mouse MAb C179 (TaKaRa Bio Inc.) for 1 h at RT. Plates were then washed three times with TPBS, and MAb 6F12 was incubated at a starting dilution of 100 µg/ml. The standard ELISA protocol as described above was followed. Of note, biotinylated MAb 6F12 was used with the mouse MAb C179, and a streptavidin antibody conjugated to HRP (Millipore) was used as a secondary antibody.

pH-induced conformational change ELISA. EIA/RIA plates were coated with purified baculovirus-expressed Cal09 HA (NR-15749; BEI) as described above and then blocked with 5% NF milk–1× PBS for 30 min at RT. Plates were washed with TPBS twice, then incubated with appropriate pH-buffered solution (15 mM citric acid and 150 mM NaCl) for 30 min at RT, and then washed again with TPBS. To remove the globular HA1 subunit, 0.1 M dithiothreitol (DTT) was used to reduce the disulfide bond that connects HA1 to HA2 after treatment with pH-buffered solutions (6). Thereafter, a standard ELISA protocol was followed as described above.

To test whether MAb 6F12 can prevent conformational change, a purified preparation of rCal09 virus was preincubated with 10 µg of 6F12 (IgG2b) before exposure to acidic solution at pH 4.4. The purified rCal09 virus was further reduced with 0.1 M DTT. An anti-head MAb, 7B2 (IgG2a), was then used as a primary MAb to detect intact globular head after reduction. The secondary antibody used was an anti-mouse IgG2a-specific antibody conjugated to HRP (Southern Biotech) at a dilution of 1:5,000. A standard ELISA protocol was followed as described above.

Plaque reduction neutralization assay (PRNA). Dilutions of MAB were first preincubated with 60 to 80 PFU of virus for 1 h at RT on a shaker. The virus and MAB mixture was then used to infect a monolayer of MDCK cells in duplicate in a 6-well format and incubated at 37°C for 1 h with intermittent rocking every 10 min. The agar overlay was supplemented with corresponding MAB dilutions. At 2 days postinfection (dpi), the monolayer was fixed with 4% PFA–1× PBS for 30 min and then permeabilized with 0.5% Triton X-100 for 20 min. Cells were blocked with 5% NF milk–1× PBS for 30 min at RT and were incubated accordingly with either infected sera (1:500) or PR8 nucleoprotein-specific MAB HT103 (5 µg/ml) (21) for 1 h at RT. An anti-mouse secondary antibody conjugated to HRP was used at a 1:1,000 dilution. Plaques were visualized using TrueBlue peroxidase substrate (KPL Inc.), and the reaction was stopped with tap water. Plaques were counted for each antibody, and the percent inhibition was calculated versus the no-MAB group value. A nonlinear regression curve was generated using GraphPad Prism 4.0, and the 50% inhibitory concentration (IC₅₀) was calculated using the regression curve. An anti-glutathione S-transferase (GST) MAB, 22A6 (Mount Sinai School of Medicine), with an isotype of IgG2b, was employed as an isotype MAB control and used in parallel to all the neutralization assays for each virus with no observed dose-dependent inhibition.

Microneutralization and hemagglutination inhibition (HI) assays. Test viruses were diluted to 100 50% tissue culture infectious doses (TCID₅₀) per 50 µl with 1× minimum essential medium (MEM) and then incubated with a series of dilutions of MAB 6F12, starting at a concentration of 100 µg/ml for 1 h at 37°C, 5% CO₂. MDCK cells in a 96-well plate format were then washed with 1× PBS and infected with 100 µl of the virus and MAB mixture for 1 h at 37°C, 5% CO₂. Cells were washed once with 1× MEM and then refed 1× MEM supplemented with TPCK-treated trypsin with or without MAB 6F12. At 18 to 22 hpi, cells were fixed and permeabilized with ice-cold 80% acetone and air dried. Cells were blocked with hydrogen peroxide for 30 min, followed by 5% NF milk–1× PBS for another 30 min, both at RT. A mouse anti-nucleoprotein antibody conjugated to biotin (Millipore) was used as a primary antibody, and then streptavidin antibody conjugated to HRP (Millipore) was used as a

secondary antibody to detect reactivity. Sigmafast OPD tablets were used as a substrate, and absorbance was read at 405 nm.

Twenty-five microliters of 8 chicken erythrocyte hemagglutination units (4 wells) of rCal09 virus was preincubated with 25 µl of different dilutions of MAB 6F12 or 7B2 for 1 h on ice. Fifty microliters of 0.5% of chicken erythrocyte suspension was added to the virus and MAB mixture, gently shaken, and incubated on ice for an additional hour. PBS with virus and no MAB was used as a negative control, while PBS with no virus and no MAB served as a background control.

In vitro selection of antibody escape mutants. rCal09 virus was passaged on MDCK cells in 1× MEM supplemented with 2 µg/ml TPCK-treated trypsin and 1% BSA and grown at 37°C in 5% CO₂. Cultures were started with an MOI of 0.05 and an antibody concentration of 1 µg/ml (corresponding to the approximate IC₅₀). Virus was passaged 1:10 or 1:100 every 2 or 3 days when the presence of cytopathic effect was observed. The antibody concentration was doubled after every passage to increase selective pressure. After the 10th passage, viral RNA was isolated and sequenced. Escape cultures were performed in quadruplicates, and two cultures were grown and passaged in parallel in the absence of antibody to control for possible cell adaptive mutations. Sequencing analysis of the generated escape variants revealed one mutation that was not present in the parental virus strain. This mutation was introduced in the wild-type HA background in the pCAGGS plasmid and used for transfection and immune fluorescence microscopy to investigate MAB 6F12 binding.

Animal pre- and postexposure prophylaxis experiments. Six- to 8-week-old female BALB/c mice (Jackson Laboratories, Inc.) were treated intraperitoneally with 30, 15, 7.5, 3.0, 1.0, or 0.5 mg/kg of body weight of MAB 6F12 or 30 mg/kg of isotype MAB control 22A6 for 2 h before an intranasal infection with 5 50% mouse lethal doses (mLD₅₀) of the following viruses: PR8, sw30, or A/Netherlands/602/2009 (NL09). Similarly, 6- to 8-week-old female DBA.2 mice (Jackson Laboratories, Inc.) were treated with MAB 6F12 as described above and challenged with 5 mLD₅₀ of SI06. All mice were monitored daily, and their weights were recorded until the end of the 2-week experiment. Death was determined by a 25% body weight loss threshold used in challenges against PR8, sw30, and SI06, while a 31.5% body weight loss cutoff was used for NL09. The latter cutoff weight was used with permission from the Institutional Animal Care and Use Committee (IACUC).

To determine viral lung titers in BALB/c mice treated with MAB, mice were administered 15 mg/kg of MAB 6F12 or 22A6 prior to intranasal infection with 5 mLD₅₀ of PR8 or NL09. At 3 and 6 dpi, three mice from each MAB-treated group were sacrificed and their lungs harvested. Lungs were homogenized (Fastprep-24; MP Biomedical) in 1 ml of 1× PBS and spun at 16,000 × g for 15 min to pellet tissue debris, and supernatants were collected. Supernatant samples were stored at –80°C until titers were determined by plaque assay as described previously (33).

For postexposure prophylaxis, BALB/c mice were first intranasally infected with 5 mLD₅₀ of NL09 and then administered 30 mg/kg of MAB 6F12 intraperitoneally at 24, 48, 72, 96, 120, or 144 hpi. Mice were monitored daily for signs of illness, and their weights were recorded.

RESULTS

Generation of a pan-H1 monoclonal antibody. Following our success in generating and characterizing a monoclonal antibody that had broadly neutralizing activity against H3 influenza viruses (33), we sought to generate an antibody that would focus on the other major influenza A virus subtype (H1) circulating in humans with a similar strategy. Here, we sequentially immunized BALB/c mice with DNA plasmids carrying antigenically distinct HAs representing H1 viruses encompassing the initial “1918 influenza pandemic” to the most recent one in 2009, with the intent of boosting the B-cell responses to the conserved regions of HA (28). Thus, mice were immunized with the HAs of the following viruses: (i) SC18 (H1), (ii) USSR77 (H1), and lastly, (iii) Cal09 (H1).

FIG 1 MAb 6F12 recognizes a panel of H1 influenza A viruses. (A) MDCK cells were infected at an MOI of 5 with USSR77 (H1), TX91 (H1), NC99 (H1), Bris07 (H1), rCal09 (H1), HK68 (H3), or rVN04 (H5) viruses and at 12 hpi fixed with 0.5% paraformaldehyde. Reactivity was detected using immunofluorescence with MAb 6F12, 7B2 (Cal09 specific), or E10 at 5 $\mu\text{g/mL}$. MAb E10 is an M2-specific MAb that is used as an infection control. EIA/RIA plates were coated with baculovirus-expressed HA proteins of PR8 (B), Cal09 (C), JP57 (D), VN04 (E), HK99 (F), HK68 (G), or Yam88 (H) or purified preparation of whole virus of rCal09, Bris07, and USSR77 (I) in duplicate at a starting concentration of 100 or 30 $\mu\text{g/mL}$ for MAb 6F12. Sera from infected mice or MAb PY102, 8, G1-26, or XY102 were used as positive controls.

Three days before fusion of the splenocytes with its partner myeloma cells, a mouse was boosted intravenously with a purified preparation of Bris07 (H1) virus. Hybridoma supernatants were screened for their ability to react to rCal09 by immunostaining, and positive hits were subcloned until a monoclonal population of hybridoma cells reactive to Cal09 was obtained.

Two thousand hybridoma clones were screened, and two had particularly strong signals against rCal09 virus and were chosen for further characterization. Of the two, monoclonal antibody 6F12 was found to be reactive based on immunofluorescence at 5 $\mu\text{g/mL}$ to USSR77-, TX91-, NC99-, Bris07-, and rCal09-infected MDCK cells (Fig. 1A). As expected, MAb 6F12 bound to purified H1 HAs (Fig. 1B and C). Notably, unlike other reported broadly reactive MAb against group 1 viruses (31, 32), MAb 6F12 did not recognize baculovirus-expressed H2 (A/Japan/1957) (Fig. 1D), H5 (A/Vietnam/1203/04) (Fig. 1E), or H9 (A/guinea fowl/Hong

Kong/WF10/1999) (Fig. 1F) HAs, being strictly a pan-H1 MAb, and as expected did not bind to a group 2 H3 (A/Hong Kong/1/1968) (Fig. 1G) HA or an influenza B virus (Fig. 1H) HA. Monoclonal antibody 7B2, on the other hand, strictly recognized rCal09 virus and is therefore strain specific (Fig. 1A). A MAb against a highly conserved region of the M2 ectodomain (E10) was used as an infection control for all the viruses. Monoclonal antibody 6F12 also bound to purified preparations of rCal09, Bris07, and USSR77 viruses (Fig. 1I).

MAb 6F12 targets the stalk region of H1 HA. In light of its binding profiles to several H1 viruses based on immunofluorescence and past published results, we predicted that MAb 6F12 would also be stalk specific. In order to test our hypothesis, we competed MAb 6F12 with two previously described group 1 MAb, CR6261 (32) and C179 (20), in an ELISA. In both cases, MAb CR6261 and C179 clearly competed with MAb 6F12 and greatly

FIG 2 MAb 6F12 binds to the stalk domain of HA. (A) EIA/RIA plates were coated with baculovirus-expressed Cal09 HA and then incubated with MAb CR6261 (human) or C179 (mouse) at a concentration of 100 μg/ml. An ELISA using biotinylated MAb 6F12 at a starting concentration of 100 μg/ml was performed with a streptavidin antibody conjugated to HRP, used as a secondary antibody. Positive competition was detected by an increase in the EC_{50} of samples preincubated with MAb CR6261 or C179 over samples that did not show increases. (B) Wild-type HA (PR8, HK68, and HK99) or chimeric HA (ch9/1) was expressed in High Five insect cells using a recombinant baculovirus vector and fixed with 0.5% PFA at 48 hpi. Reactivity was detected by immunofluorescence with MAb 6F12 or pan-H3 12D1 at 1 μg/ml. (C) Eight chicken hemagglutination units (4 wells) of rCal09 virus was preincubated with MAb 6F12 or 7B2 with a starting concentration of 50 μg/ml before addition of 50 μl of 0.5% chicken red blood cells. PBS with virus and no MAb was used as a negative control, while PBS with no virus and MAb served as a background control.

increased its EC_{50} by approximately 7- and 10-fold, respectively (Fig. 2A). This was not so surprising, as the spatial arrangements for a group 1 antibody to bind to the stalk region are large and may sterically hinder and easily outcompete a similar anti-stalk antibody irrespective of specific binding residues (4, 31, 36). Moreover, our own data demonstrated that MAb C179 and CR6261 also competed with each other in the same assay (data not shown).

To further elucidate the predominant binding site of MAb 6F12, we constructed a chimeric HA composed of the globular domain of an H9 (A/guinea fowl/Hong Kong/WF10/1999) HA with the stalk region of an H1 (A/Puerto Rico/8/1934) HA, ch9/1 HA. As anticipated, MAb 6F12 bound only to ch9/1 HA and did not recognize wild-type H9 HA, indicating that the binding site of MAb 6F12 is located on the stalk region of H1 HAs (Fig. 2B).

Typical influenza A virus-neutralizing antibodies or sera will only have potent activities against closely related strains within a specific time period (28) due to mutational drift on the surface of the globular head of HA. For example, since MAb 7B2 was found to react only with rCal09 virus, it is not surprising that we observed HI activity against rCal09 virus, with an endpoint titer of 1.6 μg/ml. Conversely, because the chief binding site of MAb 6F12 is located on the stalk region, MAb 6F12 did not show any HI activity against rCal09 virus (Fig. 2C). Taken together, our immunofluorescence and HI data demonstrate that MAb 6F12 does not bind in or around the receptor-binding site on the globular head but rather binds to a conserved region of the stalk of H1 HA.

MAb 6F12 has a potent neutralizing ability against numerous H1 influenza viruses. To assess whether the cross-reactivity of MAb 6F12 correlates to the broadly neutralizing activity, we performed a series of neutralizing assays against a panel of H1 viruses. In plaque reduction neutralization assays, in which antibody is incorporated into the agar overlay in addition to preincubation

with virus, MAb 6F12 had neutralizing activity against all of the pre-pandemic seasonal H1s (USSR77, TX91, and NC99), the pandemic rCal09 virus, a classical swine sw30 virus, and the mouse-adapted PR8 virus. Of note, MAb 6F12 was most potent against USSR77, with an IC_{50} of 1.8 μg/ml, and had the highest IC_{50} of 17.5 μg/ml against sw30. As expected, rVN04 was not neutralized by MAb 6F12, even at a concentration of 100 μg/ml (Fig. 3). In addition, MAb 6F12 demonstrated robust neutralizing activity against rCal09 virus in a microneutralization assay, with endpoint titers of 0.8 μg/ml and 1.6 μg/ml with or without MAb added in the liquid medium, respectively (data not shown).

MAb 6F12 binds to the prefusion conformation of HA. Since MAb 6F12 did not have HI activity but did neutralize, we assumed it mediates its antiviral activity in the second stage of entry. To examine this, we exposed purified egg-grown preparations of rCal09 virus to different pH-buffered solutions for 30 min prior to doing an ELISA. As shown in Fig. 4A, an example of an HI-positive MAb, 7B2, retained its ability to bind under all acidic (pH 5.6 to 4.4) and neutral conditions. Only after the globular head is removed with exposure to a reducing agent such as DTT is 7B2 binding abrogated (6). Monoclonal antibody 6F12, on the other hand, had optimal binding only at neutral pH, but it had markedly lowered binding kinetics as the pH was lowered from pH 7 to pH 4.4 (Fig. 4B), similar to what was seen with another anti-stalk MAb, C179 (Fig. 4C). Furthermore, when MAb 6F12 was added prior to pH-induced conformation change of HA, MAb 6F12 prevented dissociation of HA1 from HA2 by locking both into the prefusion conformation, as shown in Fig. 4D. Our data indicate that the stalk-based epitope of MAb 6F12 is present at neutral pH but is gradually abrogated as the pH is decreased. We also found that MAb 6F12 prevented the pH-induced conformational change of HA.

FIG 3 MAb 6F12 neutralizes H1 viruses, but not an H5 virus, in a plaque reduction neutralization assay. Sixty to 80 PFU of PR8 (H1), sw30 (H1), USSR77 (H1), TX91 (H1), NC99 (H1), rCal09 (H1), or rVN04 (H5) virus was preincubated with dilutions of MAb 6F12 at a starting concentration of 100 $\mu\text{g/mL}$ at room temperature prior to infection of a monolayer of MDCK cells. The agar overlay was also supplemented with the proper dilutions of MAb 6F12. At 48 hpi, the monolayers were fixed with 4% PFA and permeabilized with 0.5% Triton X-100. Plaques were visualized either through immunostaining using MAb HT103 (anti-PR8 nucleoprotein), sera (anti-USSR77), or crystal violet staining. The IC_{50} was calculated by fitting data with a nonlinear regression curve using GraphPad Prism. An isotype IgG2b (22A6) control was also tested in parallel, with no dose-dependent inhibition observed (data not shown).

Selection and characterization of a MAb 6F12 escape mutant.
To investigate the possibility of influenza A virus escape from MAb 6F12 inhibition and to further define the region that is bound by MAb 6F12, rCal09 virus was grown in MDCK cells

under selective pressure of antibody, starting at a concentration of 1 $\mu\text{g/mL}$. At each passage the antibody concentration was doubled, and after passage 10, viral RNA was isolated and amplified for sequencing. Only one mutation was observed at residue 44 of the HA2 subunit, while no mutations were observed in the control cultures. The A44₂V substitution was cloned into a Cal09 HA expression vector, and the mutant HA was tested for MAb 6F12 binding by immunofluorescence (Fig. 5A). The mutant Cal09 A44₂V HA clearly lost all binding to MAb 6F12, while binding of MAb 7B2 (an anti-head antibody) or serum from mice infected with Cal09 was not affected.

When modeling the A44₂V mutation on the crystal structure of the SC18 HA (Protein Database 1RD8), it was found that this residue (represented as a red region) is positioned in the short α -helix at the interior interface of the stalk (Fig. 5B and C), confirming the data that MAb 6F12 targets the stalk domain of HA. However, this position at the short α -helix seems inaccessible in the native prefusion conformation of HA, suggesting that either MAb 6F12 binds after conformational changes during the fusion process or that this escape mutation induces small conformational changes in nearby residues, thereby indirectly disrupting the epitope of MAb 6F12.

Broad and potent prophylactic efficacy of MAb 6F12 *in vivo*.
We also wanted to investigate whether the *in vitro* neutralization activity translates to *in vivo* activity in a mouse model. We tested the prophylactic neutralizing activity of MAb 6F12 against several strains of H1 viruses: the mouse-adapted laboratory strain PR8, the classical swine influenza sw30, which resembles the 1918 pandemic H1N1 strain (29), the prepandemic seasonal strain SI06, and finally, a mouse-adapted 2009 pandemic strain, namely, NL09 (15).

FIG 4 MAb 6F12 binds to the prefusion conformation of rCal09 HA. EIA/RIA plates were coated with purified preparations of whole rCal09 virus at 5 $\mu\text{g/mL}$ and exposed to buffered solutions of pH 7.0, 5.6, 5.0, and 4.4 for 30 min before performing an ELISA with MAb 7B2 (A), 6F12 (B), or C179 (C) at a starting concentration of 100 $\mu\text{g/mL}$. To remove the globular head (HA1), the whole-virus preparation was exposed to 0.1 M DTT after exposure to pH-buffered solutions prior to the ELISA. Purified preparations of whole rCal09 virus were preincubated with MAb 6F12 (IgG2b) at a concentration of 100 $\mu\text{g/mL}$ before exposure to acidic buffer (pH 4.4) and then reduced with 0.1 M DTT. (D) An ELISA with MAb 7B2 (IgG2a) was then performed using an isotype-specific secondary antibody.

FIG 5 An alanine-to-valine mutation at position 44 in the HA2 subunit of Cal09 HA abrogates MAb 6F12 binding. (A) 293T cells were transfected with wild-type Cal09 (H1), Cal09 A44₂V (H1), or wild-type HK68 (H3) HA, and at 24 h posttransfection cells were fixed with 0.5% paraformaldehyde. Reactivity was detected by immunofluorescence using Cal09-infected sera or MAb 6F12 or 7B2 at 5 μ g/ml. (B and C) PyMOL was used to model the location of residue 44 on the HA2 subunit of A/South Carolina/1/1918 (H1) HA (1RD8). Residue 44 of HA2 is indicated in red in the model of the homotrimeric molecule of HA (B) or a monomer of HA2 (C). The HA1 is shown in light gray, while the HA2 is shown in light blue. HA molecules are not drawn to scale.

Groups of five mice were intraperitoneally injected with different concentrations of MAb 6F12, PBS, or a matched mouse IgG2b isotype control antibody. Two hours later, animals were challenged with 5 mL₅₀ of one of the four divergent H1N1 influenza virus strains. Mice were weighed daily and sacrificed when they reached 75% of their starting weight. Mice pretreated with 15 mg/kg of antibody all survived the challenge with PR8 virus without showing any signs of clinical illness or weight loss (Fig. 6A). Animals treated with 7.5 or 3 mg/kg lost significant amounts of weight (10 to 15% and 15 to 20%, respectively) but were still

partially (80 and 60%) protected from death. The group treated with 1 mg/kg lost weight in a similar manner as animals treated with PBS or the isotype control and succumbed on day 9 or before.

Classical swine influenza virus isolates have been shown to be particularly pathogenic in mice and in ferrets (16, 29). Here, we used the sw30 virus, which is highly lethal in mice. Animals pretreated with 30 or 7.5 mg/kg of MAb 6F12 as described above did not show any clinical signs or weight loss upon challenge with this virus strain and had a survival rate of 100% (Fig. 6B).

Having demonstrated protective neutralizing activity against

FIG 6 MAb 6F12 provides preexposure protection against several H1 virus strains. Six- to 8-week-old BALB/c or DBA.2 mice were intraperitoneally administered 30, 15, 7.5, 3.0, 1.0, or 0.5 mg/kg of MAb 6F12 2 h prior to challenge with 5 mL₅₀ of PR8 (A), sw30 (B), SI06 (C), or NL09 (D) virus. PBS and an isotype IgG2b (22A6) at a concentration of 30 mg/kg served as negative controls. Mice were monitored daily for signs of illness and weight change. The ratios beside the legends indicate the number of survivors over the total number of animals in each group. Mice were administered 15 mg/kg of MAb 6F12 or 22A6 2 h before infection with 5 mL₅₀ of PR8 or NL09 virus. At 3 and 6 days postinfection, three mice from each group were randomly sacrificed and lungs were harvested. Lungs were then homogenized, and viral lung titers were determined by plaque assay (E). Undetectable viral lung titers are indicated by an asterisk.

historical isolates of H1N1 viruses, we sought to evaluate protection against recent virus isolates. We started with the prepandemic seasonal influenza virus strain SI06, which shares only 80% HA protein identity with the currently circulating pandemic H1N1 strains and is separated from PR8 and sw30 viruses by almost 60 years of antigenic drift. For this particular experiment, we chose the DBA.2 mouse model, as these mice, in contrast to BALB/c mice, have been shown to be susceptible to a lethal challenge with prepandemic seasonal influenza isolates (25). When treated with 15 or 7.5 mg/kg, these mice lost 10 to 15% of their initial body weight, but they started to regain weight on day 6 and had a survival rate of 100% (Fig. 6C). Mice treated with PBS or the isotype control showed severe weight loss and had survival rates of 0 and 20%, respectively.

Next, we wanted to demonstrate the efficacy of MAb 6F12 against the pandemic 2009 H1N1 virus that is currently circulating in the human population, which replaced prepandemic seasonal H1 strains (22). Mice were pretreated with 30, 15, 7.5, 3, 1, or 0.5 mg/kg and then challenged with the mouse-adapted isolate, NL09. Animals that received 30, 15, 7.5, or 3 mg/kg showed no signs of distress or clinical illness (Fig. 6D). Groups that were treated with 30 or 15 mg/kg showed no weight loss at all; groups treated with 7.5 or 3 mg/kg lost approximately 5% of their initial body weight but regained weight quickly. Animals treated with the isotype control, PBS, or 1 or 0.5 mg/kg suffered from severe weight loss and succumbed to infection.

Finally, we assessed the virus lung titers of animals that were inoculated with 5 mL_D₅₀ of PR8 or NL09 when pretreated with 15 mg/kg of MAb 6F12 or isotype control antibody. Animals ($n = 3$) were sacrificed at 3 or 6 days postinoculation, lungs were harvested, and virus titers were measured by plaque assay. Lung titers were in accordance with the survival and weight loss data presented above; pretreated animals showed an approximately 1-log reduction in lung titer on day 3 in both PR8- and NL09-infected groups compared to the isotype control (Fig. 6E). A comparable reduction in viral lung titer was observed in the 6F12-treated group, of approximately 1 to 1.5 logs lower in PR8- and NL09-infected mice, respectively. Of note, one mouse from each of the 6F12-treated group infected with NL09 or PR8 had an undetectable viral titer with the assay used.

Therapeutic efficacy of MAb 6F12 *in vivo*. After demonstrating prophylactic protection against four divergent H1N1 strains, we set out to test the efficacy of MAb 6F12 in a therapeutic post-exposure setting. In order to study the postexposure efficacy of MAb 6F12 in a more sensitive manner, permission was given by the IACUC to set the end point for the postexposure challenge studies to 68.5% of the animals' initial weights. Thus, mice were challenged with 5 mL_D₅₀ of NL09 and were given 30 mg/kg 6F12 intraperitoneally at 24, 48, 72, 96, 120, or 144 hpi. The group treated at 24 hpi did not show any signs of clinical illness or weight loss but gradually gained weight (Fig. 7). Animals treated at 48 and 72 hpi lost approximately 10% of their initial weight but showed no other symptoms of disease and regained weight quickly (Fig. 7). The group treated 96 hpi lost approximately 20% of its initial weight but recovered fast and had a survival rate of 100%. At 120 hpi (5 days), treatment was still able to partially protect from mortality (80% survival), although these animals suffered from severe weight loss. Animals treated at 144 hpi and the control groups all succumbed by day 11 and were sacrificed (Fig. 7).

FIG 7 MAb 6F12 provides postexposure protection against NL09 virus. Six- to 8-week-old BALB/c mice were infected with 5 mL_D₅₀ of NL09 virus and treated intraperitoneally with 30 mg/kg of MAb 6F12 at 24, 48, 72, 96, 120, or 144 hpi. Mice were monitored daily for clinical signs of illness and weight change. The ratios beside the legend indicate the number of survivors over the total number of animals in each group.

DISCUSSION

With a strategy similar to one we previously used in making a pan-H3 antibody (33), we generated an antibody that has broad and potent activity against a single subtype of influenza A virus both *in vitro* and *in vivo*. By sequentially immunizing mice with DNA encoding H1 HAs representing 91 years of antigenic drift, we produced and isolated a monoclonal antibody that strictly recognizes H1 HAs and additionally neutralizes a divergent panel of H1 viruses *in vitro*. Specifically, MAb 6F12 was shown to bind to the most recent pandemic H1 (Cal09) virus, in addition to three prepandemic H1 viruses, by immunofluorescence and by ELISA. Of note, MAb 6F12 did not detect H1 HA in a Western blot analysis under reducing and denaturing conditions (data not shown), which suggests that the epitope recognized by MAb 6F12 is not linear but is probably conformational.

Plaque reduction neutralization experiments correlated well with binding assays in that MAb 6F12 demonstrated pan-neutralizing activity against a broad range of H1 virus strains, including the mouse-adapted PR8 virus and a classical swine sw30 virus. The calculated IC₅₀ of MAb 6F12 against the different H1s varied, with TX91, NC99, PR8, and USSR77 viruses having robust to modest sensitivity while rCal09 and sw30 had the highest IC₅₀s. Monoclonal antibody 6F12 also had strong activity against Cal09 in the more traditional microtiter neutralization assay format, indicating an endpoint titer of 0.8 µg/ml. Conventional neutralization assays, whether in a plaque or liquid assay (microtiter), require only preincubation of MAb or sera with virus, without antibody added to the agar overlay or the liquid medium during the multicycle replication assay. However, since anti-stalk antibodies do not prevent endocytosis of the viral particle but rather the fusion of the viral and endosomal membranes (20), we previously found that a continuous presence of antibody is required to optimally measure the efficacy of anti-stalk antibodies (33). The continuous presence of anti-stalk antibodies enhances the neutralization assay during a multicycle replication cycle. This could be due to the mechanism by which anti-stalk antibodies mediate neutralization. Instead of interfering with ligand and receptor binding, our data suggest that anti-stalk MAB are internalized along with the viral particle bound to the HA at a prefusion conformation and,

thus, inhibit the fusion peptide from engaging the endosomal membrane. However, we found that MAb 6F12 still neutralized without being added to the semisolid agar, albeit at a higher IC_{50} of 11 μ g/ml.

The generation of an escape mutant to MAb 6F12 revealed that an alanine residue at position 44 of the HA2 of rCal09 is crucial for MAb 6F12 binding. Interestingly, this also maps to the short α -helix, similar to the binding regions of MAb CR6261, F10, and FI6. What is interesting is that residue 44 is on the interior face of the short α -helix and does not seem accessible for antibody interaction at native pH. We speculate that perhaps the change from an alanine to a valine may affect adjacent residues that ultimately alter the epitope of MAb 6F12. In the absence of a crystal structure, we can only speculate on how MAb 6F12 interacts with this particular region of HA. Although MAb CR6261 and F10 are group 1-specific monoclonal antibodies, the spatial orientation by which they bind to the hydrophobic groove adjacent to the short α -helix of HA extensively overlaps with another heterosubtypic MAb, FI6, which recognizes all influenza A virus HA subtypes. Not surprisingly, we demonstrated that MAb 6F12 does compete with MAb CR6261 or C179, another group 1-specific MAb, and thus should occupy this space. With regard to the specific antibody and HA interface, we know that although MAb CR6261 and FI6 both bind to the hydrophobic groove of HA2, the former uses all three of its heavy chain complementarity-determining regions (HCDRs), while the latter uses only HCDR3.

A majority of the published reports of broadly neutralizing monoclonal antibodies against influenza A viruses have indicated heterosubtypic activity within group 1 (20, 31, 32) or group 2 (6) viruses and have shown pan-neutralizing activity *in vitro*. However, few studies have reported extensive data demonstrating protective efficacy *in vivo*. Here, we show that not only does 6F12 have a pan-neutralizing ability *in vitro*, but also that it protects against a diverse number of H1 viruses in the mouse model. MAb 6F12 demonstrates protection against a mouse-adapted PR8 virus and robustly against a classical swine virus, sw30, with little or no weight loss. Moreover, MAb 6F12 also protects against two representative human H1 viruses, SI06 and the more recent pandemic NL09 virus. Notably, MAb 6F12 still gave full protection at 3 mg/kg (60 μ g/mouse), with only as a 5% weight loss. This was reflected in the significant decrease in viral lung titers at 3 and 6 days postinfection in MAb 6F12-treated mice compared to the control group. To further examine the efficacy of MAb 6F12 *in vivo*, we treated mice after infection. Our data demonstrated a clear time-dependent effect of MAb 6F12 (at 30 mg/kg) treatment postexposure to NL09 virus, with little or minimal weight loss of mice treated 1 to 4 days postinfection. At 5 days posttreatment, survival dropped slightly, to 80%.

We hypothesize that this robust protection is chiefly due to MAb 6F12 being strictly a pan-H1 monoclonal antibody. Likewise, we believe that a heterosubtypic MAb such as MAb C179, although protective against an H1 or H5 challenge, does so by sacrificing efficacy. For example, even at 1,000 μ g, roughly 50 mg/kg (20 g/mouse), MAb C179 led to only 80% survival when treatment was 2 days after lethal challenge with H1 (19) or H5 (27) virus. Similarly, the recently reported pan-group 1 and 2 MAb FI6 demonstrated binding to all influenza A virus subtypes but failed to prevent initial weight loss in FI6-treated mice, even at its highest concentration against a sublethal challenge of H3 and a lethal challenge of H1 (4). As much as survival is the final readout for

mouse experiments, morbidity, here reflected by weight change, is as important in measuring *in vivo* efficacy.

Based on recent findings that cross-reactive antibodies that target conserved epitopes in the HA stalk naturally occur in humans upon exposure to the proper immunogen (24, 35), it is intriguing to speculate on a vaccination regimen that would mimic the neutralizing activity of MAb 6F12 and induce pan-H1 immunity.

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