A Broadly Reactive Human Anti-hemagglutinin Stem Monoclonal Antibody That Inhibits Influenza A Virus Particle Release

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ABSTRACT

Many broadly reactive human monoclonal antibodies against the hemagglutinin (HA) stem of influenza A virus have been developed for therapeutic applications. These antibodies typically inhibit viral entry steps, especially the HA conformational change that is required for membrane fusion. To better understand the mechanisms by which such antibodies inhibit viral replication, we established broadly reactive human anti-HA stem antibodies and determined the properties of these antibodies by examining their reactivity with 18 subtypes of HA, evaluating their in vivo protective efficacy, identifying their epitopes, and characterizing their inhibitory mechanisms. Among the eight human monoclonal antibodies we generated, which recognized at least 3 subtypes of the soluble HA antigens tested, clone S9-1-10/5-1 reacted with 18 subtypes of HA and protected mice from lethal infection with H1N1pdm09, H3N2, H5N1, and H7N9 viruses. This antibody recognized the HA2 helix A in the HA stem, and inhibited virus particle release from infected cells but did not block viral entry completely. These results show that broadly reactive human anti-HA stem antibodies can exhibit protective efficacy by inhibiting virus particle release. These findings expand our knowledge of the mechanisms by which broadly reactive stem-targeting antibodies inhibit viral replication and provide valuable information for universal vaccine development.

Keywords: Influenza A virus, Human monoclonal antibody, HA stem, Broadly reactive

1. Introduction

Influenza A virus possesses eight segmented, negative-sense viral RNAs (vRNAs) as its genome. Two of these vRNAs encode hemagglutinin (HA) and neuraminidase (NA), which are major viral antigenic proteins on the virus particle. The trimeric type I transmembrane glycoprotein HA is classified into 18 subtypes (H1 to H18) that can be combined into two separate phylogenetic groups: group 1 encompasses H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18, whereas group 2 includes H3, H4, H7, H10, H14, and H15 (Gamblin and Skehel, 2010; Tong et al., 2013; Webster et al., 1992). HA is produced as HA0, which is then cleaved into HA1 and HA2. The HA1-HA2 monomer assembles as trimers consisting of an apical globular head, which is derived from the central region of HA1, and a stem region, which consists of HA2 and the N- and C-terminals of HA1 (Wilson et al., 1981). The globular head and stem regions are involved in receptor binding and membrane fusion, respectively. Antibodies against the highly antigenic region around the receptor-binding site on the globular head ordinarily inhibit receptor binding steps, and therefore virus infectivity is neutralized (Caton et al., 1982). Because of the high immunological pressure imposed by these antibodies, the antigenicity of the globular head varies by accumulating mutations that allow escape from recognition by these antibodies. In contrast, a limited number of antibodies against the HA stem are present in ordinary human sera because the HA stem is not highly immunogenic under normal circumstances (Sui et al., 2011), and is, in fact, highly conserved among heterotypic HAs. The antibodies against the HA stem typically neutralize virus by inhibiting membrane fusion steps (Brandenburg et al., 2013). The vast majority of anti-HA globular head antibodies are strain or subtype-specific, whereas many anti-HA stem antibodies recognize several subtypes of HA. Therefore, antibodies against the HA stem are highly desired as a novel antiviral therapy and a target for a universal vaccine.

Known human monoclonal antibodies against the heterotypic HA stem are classified into 3 types based on their reactivity. The first type recognizes several subtypes of HA that belong to group 1; CR6261 (Ekiert et al., 2009; Throsby et al., 2008), F10 (Sui et al., 2009), and is, in fact, highly conserved among heterotypic HAs. The antibodies against the HA stem typically neutralize virus by inhibiting membrane fusion steps (Brandenburg et al., 2013). The vast majority of anti-HA globular head antibodies are strain or subtype-specific, whereas many anti-HA stem antibodies recognize several subtypes of HA. Therefore, antibodies against the HA stem are highly desired as a novel antiviral therapy and a target for a universal vaccine.

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Marco et al., 2012), and A06 (Kashyap et al., 2010) are member of this type. The second type of antibodies includes CR8020 (Ekiert et al., 2011), and CR8043 (Friesen et al., 2014), which react with several subtypes of HA belonging to group 2. The third and final type binds to many subtypes of HA belonging to both groups 1 and 2; CR9114 (Dreyfus et al., 2012), F6v3 (Corti et al., 2011), 39.29, 81.39 (Nakamura et al., 2013), CT149 (Wu et al., 2015), VIS410 (Tharakaraman et al., 2015), 1,12 (Wyzynzuki et al., 2015), ICA, 3C4 (Hu et al., 2013), 05-2G02 (Li et al., 2012), 045-05310-2B06, S6-B01 (Henry Dunand et al., 2015), PNS6A28 (Clementi et al., 2011), MEDI8852 (Kallewaard et al., 2016), 15a.09, 31.b.09, 16.a.26, and 31.a.83 (Joyce et al., 2016) belong to this type. CR9114 also reacts with the HA stem of influenza B virus (Dreyfus et al., 2012). Many of these antibodies inhibit viral growth in vitro by predominantly interfering with viral membrane fusion during viral entry. Some of the anti-HA stem antibodies require Fcγ receptor-mediated antibody-dependent cellular cytotoxicity (ADCC) to afford efficient protection in vivo to reduce the number of infected cells (DiLillo et al., 2014; DiLillo et al., 2016; Jegaskanda et al., 2014). Thus, several antibody-dependent inhibitory mechanisms serve to protect against influenza A virus infection in vivo. Therefore, the characterization of inhibitory mechanisms utilized by human antibodies should help in the development of a universal vaccine.

Anti-influenza agents are under development, because antiviral therapy helps reduce the burden of seasonal influenza and provides the first line of defense against pandemic influenza before vaccines are available. Concerns regarding widespread resistance to M2 inhibitors and the emergence of NA inhibitor-resistant viruses emphasize the need to develop novel anti-influenza agents. The broadly reactive neutralizing anti-HA stem antibodies could fill the need for novel broad spectrum anti-influenza virus agents. Such antibodies should have the potential to prevent infection with seasonal influenza H1N1pdm09 and H3N2 viruses, as well as protect against influenza viruses that cause interspecies infections between humans and animals, including H5N1 and H7N9 viruses. Here, we established a broadly reactive anti-HA stem antibody that protected mice from lethal infection with H1N1pdm09, H3N2, H5N1, or H7N9 viruses. This antibody mainly inhibited virus particle release from infected cells in vitro. These results expand our knowledge of the mechanisms by which broadly reactive stem-targeting antibodies inhibit viral replication.

2. Materials and Methods

2.1. Ethics and Biosafety Statements

Human blood was collected by following protocols approved by the Research Ethics Review Committee of the Institute of Medical Science, the University of Tokyo. Signed informed consent was obtained from all participants.

All experiments with mice were performed in accordance with the University of Tokyo’s Regulations for Animal Care and Use and were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo.

All experiments with H5N1 and H7N9 viruses were performed in biosafety level 3 (BSL3) laboratories at the University of Tokyo, which were approved for such use by the Ministry of Agriculture, Forestry, and Fisheries, Japan.

2.2. Cells

Madin-Darby canine kidney (MDCK) cells were maintained in Eagle’s minimal essential medium (MEM) containing 5% newborn calf serum (NCS). Hela cells (kindly provided by T. Odagiri, National Institute of Infectious Diseases) were maintained in Eagle’s minimal essential medium (MEM) containing 10% fetal calf serum (FCS). Human embryonic kidney 293T cells and Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS. These cells were incubated at 37 °C under 5% CO2.

2.3. Viruses

A/California/04/2009 (CA04; H1N1pdm09), its mouse-adapted strain (MA-CA04) (Sakabe et al., 2011), mouse-adapted A/California/2/68 (MA-Aichi; H3N2), A/Perth/16/2009 (H3N2), A/Vietnam/1203/2004 (VN1203; H5N1), A/geese/Egypt/0929-NLQP/2009 (H5N1), and A/Anhui/1/2013 (Anhui/1; H7N9), were propagated in MDCK cells or eggs, and tittered in MDCK cells.

2.4. Recombinant HAs

Recombinant soluble HAs of subtype H1 (A/California/07/2009), H3 (A/Wyoming/03/2003, and A/Perth/16/2009), H5 (A/Egypt/N05056/2009, and A/Indonesia/5/2005), and H7 (A/Netherland/219/2003) were purchased from Sino Biological.

2.5. Construction of Plasmids

Open reading frames of the HA gene derived from CA04 (H1N1pdm09), A/Singapore/1/57 (H2), A/Tokyo/UT-IMS6-1/2013 (H3), A/duck/Czechoslovakia/56 (H4), VN1203 (H5), A/turkey/Massachusetts/3740/65 (H6), Anhui/1 (H7), A/turkey/Ontario/6118/68 (H8), A/turkey/Wisconsin/66 (H9), A/chicken/Germany/N/49 (H10), A/duck/Memphis/546/74 (H11), A/duck/Alberta/60/76 (H12), A/gull/Maryland/704/77 (H13), A/mallard/Astakhov/263/82 (H14), A/duck/Australia/341/83 (H15), A/black-headed gull/Sweden/5/99 (H16), A/little yellow-shouldered bat/Guatemala/164/2010 (H17), and A/flat-faced bat/Peru/033/2010 (H18), were cloned into pCAGGS.

Mutations in the HA gene of CA04 were generated by polymerase chain reaction (PCR) amplification with primers possessing the desired mutations (primer sequences available upon request). All constructs were sequenced to confirm the absence of unwanted mutations.

2.6. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Cell Fusion

Blood (10 ml) was obtained from healthy volunteers (n = 37) who received one or two influenza H5N1 vaccines, an inactivated, adjuvanted whole-virus vaccine to A/Egypt/N03072/2010 (clade 2.2.1) or A/Indonesia/505/2005 (clade 2.1.3.2), one week after vaccination. The PBMCs were prepared by centrifugation through Ficoll-Paque Plus (GE Healthcare). The isolated PBMCs were fused with SPYMECs cells (MBL), which is a fusion partner cell line, as previously described (Kubota-Koketsu et al., 2009). Fused cells were cultured in DMEM supplemented with 15% FCS in 96-well plates for 10–14 days in the presence of hypoxanthine-aminopterin-thymidine (HAT). The first screening of the culture medium for antibodies against HA was performed by using an enzyme-linked immunosorbent assay (ELISA), as described below. Specific antibody-positive wells were then subjected to cell expansion and cloning by dilution. The subsequent screenings were also performed by using the ELISA.

2.7. ELISA

Ninety-six-well microplates coated with recombinant HA proteins derived from A/California/07/2009 (H1N1pdm09), A/Wyoming/03/2003 (H3N2), A/Perth/16/2009 (H3N2), A/Egypt/N05056/2009 (H5N1), A/Indonesia/5/2005 (H5N1), and A/Netherlands/219/2003 (H7N7), or purified H5N1 virus (A/geese/Egypt/0929-NLQP/2009) were reacted with the culture medium of the hybridomas, followed by a peroxidase-conjugated goat anti-human IgG, Fcγ Fragment specific antibody (Jackson Immuno-Research).
2.8. Construction and Expression of Monoclonal Human IgG

Total RNA was extracted from the hybridomas by using an RNeasy Mini Kit (Qiagen) or ISOGEN (Nippon gene). The V\(_4\) and V\(_\lambda\) sequences of the antibodies were determined by using a GeneRacer Kit (Invitrogen) and were cloned into the expression vector, Mammalian PowerExpress System (TOYOBO), together with the constant gamma heavy (IgG\(_\gamma\)) and kappa light chain coding sequences. Determined nucleotide sequences were analyzed and compared to sequences in the National Center for Biotechnology Information (NCBI) database by using the IgBlast software (http://www.ncbi.nlm.nih.gov/igblast/). Stable antibody-expressing cells were established by transfecting the plasmid into CHO cells and selecting with 20 μg/ml puromycin, and then adapting them to serum-free media CDM4CHO (GE Healthcare). The human antibody in 1 l of 1-week-cultured serum-free media was purified by using a HisScreen MabSelect SuRe LX column (GE Healthcare) and the automated chromatography system AKTA pure 25 (GE Healthcare). Fab fragment of human IgG clone S9-1-10/5-1 was prepared by using a Pierce Fab preparation kit (Thermo Scientific).

2.9. Evaluation of the In Vivo Protective Efficacy of the mAbs in Mice

Baseline body weights of 6-week-old female BALB/c mice (Japan SLC) were measured. Four mice (randomly selected) per group were intraperitoneally injected with PBS or the indicated antibodies at 0.2, 0.6, 1.7, 5, or 15 mg/kg. One day later, the mice were anesthetized and inoculated with 10 mouse lethal dose 50 (MLD\(_{50}\)) (50 μl) of the indicated viruses. Body weight and survival were monitored daily for 14 days. Mice with body weight loss of ~25% of their pre-infection values were humanely euthanized.

2.10. Virus Neutralization Assay

Virus neutralization was performed in accordance with the World Health Organization (WHO) manual on animal influenza diagnosis and surveillance released in 2002 with some modifications. Briefly, purified antibody (50 μg/ml) in quadruplicate was serially two-fold diluted with MEM containing 0.3% bovine serum albumin (BSA-MEM) prior to being mixed with 100 TCID\(_{50}\) (50% tissue culture infectious doses) of the indicated viruses at 37 °C for 30 min. The mixtures were inoculated into MDCK cells and incubated for 1 h at 37 °C. After the cells were washed twice with BSA-MEM, the cells were incubated with BSA-MEM containing 1% agarose, 1 μg/ml TPCK-treated trypsin, and the serially two-fold diluted indicated antibody for 2 days at 37 °C. The plaques were visualized by removing the overlays and staining with crystal violet. The data were subjected to nonlinear regression analysis to derive EC\(_{50}\) values (GraphPad Prism).

2.11. Antibody Treatment After Virus Infection

MDCK cells in quadruplicate were infected with 100 TCID\(_{50}\) of the indicated virus at 37 °C for 1 h. After being washed twice with BSA-MEM, the cells were incubated with BSA-MEM containing 1 μg/ml TPCK-treated trypsin and the cytopathic effect (CPE) was examined. Antibody titres required to reduce virus replication by 50% (IC\(_{50}\)) were determined by using the Spearman-Kärber formula.

2.12. Plaque Reduction Assay

For experiments with virus that was pre-treated with antibody prior to infection, the indicated antibody (50 μg/ml in triplicate) was serially two-fold diluted with BSA-MEM prior to being incubated with 100 PFU of CA04 at 37 °C for 30 min. The antibody-virus mixtures were inoculated into MDCK cells and incubated for 1 h at 37 °C. After the cells were washed twice with BSA-MEM, the cells were incubated with BSA-MEM containing 1 μg/ml TPCK-treated trypsin and 1% agar for 2 days at 37 °C. For experiments with virus that was incubated with the antibody post-infection, MDCK cells in quadruplicate were infected with 75 plaque-forming unit (PFU)/well of CA04 at 37 °C for 1 h. After being washed twice with BSA-MEM, the cells were incubated with BSA-MEM containing 1% agarose, 1 μg/ml TPCK-treated trypsin, and the serially two-fold diluted indicated antibody for 2 days at 37 °C. The plaques were visualized by removing the overlays and staining with crystal violet. The data were subjected to nonlinear regression analysis to derive EC\(_{50}\) values (GraphPad Prism).

2.13. Virus Release Inhibition Assay

MDCK cells were infected with CA04 at a multiplicity of infection (MOI) of 1. After incubation at 37 °C for 1 h, the cells were washed twice, and then incubated with BSA-MEM containing 1 μg/ml TPCK-treated trypsin, and a human IgG (clone S9-1-10/5-1, 4-6-19/6, which recognizes the HA head, or 1429C6/3-3, which recognizes influenza B virus HA, at 0.08, 0.4, 2, or 10 μg/ml or 1, 4, 16, or 64 nM) or a Fab fragment (1, 4, 16, or 64 nM) for 18 h at 37 °C. In some experiments, 100 μM oseltamivir carboxylate (Roche) and/or 20 μM of bacterial sulfaide from Clostridium perfringens (Roche) were also included in the medium. The cells were then analyzed by western blotting and transmission electron microscopy (TEM). For western blotting, total cell lysates and culture media samples prepared in Sample buffer (Life Technologies) were loaded onto Any kD Mini-Protean TGX precast gels (Bio-Rad). Separated proteins were transferred to Immobilon-P (Millipore) and probed with a mouse monoclonal anti-M1 antibody, clone C111 (Takara bio), and a mouse monoclonal anti-ACTB antibody, clone AC-74 (SIGMA). For TEM, the cells were pre-fixed with 2.5% GLA in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4 °C. They were then washed with the same buffer and post-fixed with 2% OsO\(_4\) in the same buffer for 1 h at 4 °C. After dehydration through a series of ethanol gradients followed by propylene oxide, the samples were embedded in Epon 812 resin mixture (TAAB Laboratories) and polymerized at 70 °C for 2 days. Ultrathin sections (50 nm) were stained with 2% uranyl acetate in 70% ethanol and Reynolds’s lead solution, and examined with a Tecnai F20 electron microscope (FEI) at 200 kV.

2.14. Reactivity of Human mAbs

293T cells were transfected with each HA-expressing plasmid by use of Trans-IT 293 (Takara bio). At 24 h post-transfection, the cells were fixed with 4% paraformaldehyde, and then permeabilized with 0.2% Triton X-100. Antigens were probed with S9-1-10/5-1, CR9114 (Dreyfus et al., 2012), or 4-6-19/6, followed by Alexa Fluor 488-conjugated donkey anti-human IgG (H + L) (Jackson Immuno-Research) or peroxidase-conjugated donkey anti-human IgG (H + L) (Jackson Immuno-Research) and SIGMAFAST 3,3’-Diaminobenzidine tablets (SIGMA).

2.15. K\(_{d}\) Determination

K\(_d\) was determined by bio-layer interferometry (BLI) using an Octet Red 96 instrument (Fortebio). Reombinant HAs [A/California/07/2009 (H1N1pdm09)], A/Perth/16/2009 (H3N2), A/Egypt/N05056/2009 (H5N1), and A/Netherlands/219/2003 (H7N7)] were used for these measurements. HAs at 10 μg/ml in 1x kinetics buffer (1x PBS, pH 7.4, 1% BSA, and 0.002% Tween 20) were loaded onto Ni-NTA biosensors (Fortebio) and incubated with various concentrations of S9-1-10/5-1 or CR9114. All binding data were collected at 30 °C. The experiments comprised 4 steps: (1) HA loading onto the biosensor until the shift reached 0.5 nm; (2) baseline acquisition (60 s); (3) association of S9-1-10/5-1 or CR9114 for the measurement of k\(_{\text{on}}\) (300 s); and (4) dissociation of S9-1-10/5-1 or CR9114 for the measurement of k\(_{\text{off}}\) (900 s). Three to five concentrations of S9-1-10/5-1 or CR9114 were used, with the highest concentration varying, depending on the affinity, from 37 to 70 μM.
333 nM. Baseline and dissociation steps were carried out in buffer only. The ratio of $k_{on}$ to $k_{off}$ determined the $K_d$ reported here.

2.16. Selection of Escape Mutants

Escape mutants were selected by culturing MA-CA04 in the presence of S9-1-10/5-1, S9-1-10/5-1 (250 μg/ml), serially five-fold diluted, was incubated with 10- or 100-fold diluted virus for 30 min at 37 °C. Then, the mixtures were inoculated into MDCK cells. After 1 h incubation at 37 °C, BSA-MEM containing 1 μg/ml TPCK-treated trypsin was added. The cells were then cultured for 3 days, after which the supernatants in each well were collected and used for the next selection. The selections (n = 4) were performed for 4-6 cycles to obtain escape mutants. The open reading frame for HA was directly sequenced from the supernatant population in the supernatants of virus samples that showed CPE at the highest antibody concentration.

2.17. Binding Competition of S9-1-10/5-1 With CR9114 and 4-6-19/6

Binding competition was performed by using an Octet Red 96 instrument. After binding of recombinant HA (A/California/07/2009; H1N1pdm09) to the Ni-NTA biosensors at 30 °C for 600 s, the sensors were incubated with S9-1-10/5-1, CR9114, or 4-6-19/6 (10 μg/ml in 1X kinetic buffer) for 600 s at 30 °C, and then incubated with a secondary antibody (S9-1-10/5-1, CR9114, or 4-6-19/6; 10 μg/ml in 1X kinetic buffer) for 600 s at 30 °C.

2.18. Fusion Inhibition Assay

HeLa cells were transfected with a plasmid encoding HA derived from CA04 by using TransIT-LT1 (Takara Bio). At 24 h post-transfection, the cells were incubated for 15 min at 37 °C with BSA-MEM containing 5 μg/ml acetylated trypsin to cleave the HA0 into the HA1 and HA2 subunits, and then treated with a human mAb, S9-1-10/5-1, anti-HA stem CR9114, anti-HA head 4-6-19/6, or anti-B-HA 1429C6/3-3, at 20, 50, or 200 μg/ml for 1 h at 37 °C. Polykaryon formation was induced by exposing the cells to low-pH buffer (145 mM NaCl, 20 mM sodium citrate pH 5.5) for 5 min at room temperature. After the exposure, the low-pH buffer was replaced with MEM containing 10% FCS. The cells were incubated for 15 min at 37 °C with BSA-MEM containing 10% FCS. After incubation, the cells were fixed with 10% neutral buffered formalin, and then stained with Giemsa's solution.

2.19. Antibody Binding to Virus Particles

We used BLI using an Octet Red 96 instrument to estimate the relative $k_{on}$, which is the observed rate constant. Human monoclonal antibodies (clones S9-1-10/5-1, CR9114, 4-6-19/6, or 1429C6/3-3) at 2 μg/ml in binding buffer (PBS pH 7.4 including 0.1% BSA) were loaded onto anti-human IgG Fc capture biosensors (Fortebio) and incubated with 3 concentrations of the purified CA04/PB2-KO virus, which is a replication incompetent PB2-knockout PR8 virus possessing HA and NA segments derived from CA04 (Ozawa et al., 2011). All binding data were collected at 30 °C. The experiments comprised 3 steps: (1) antibody loading onto the biosensor (300 s); (2) baseline acquisition (60 s); and (3) association of CA04/PB2-KO virus for the measurement of $k_{obs}$ (600–3600 s).

2.20. Statistical Analysis

Data are expressed as mean ± SD. The two-tailed unpaired t-test was used for two-group comparison. P<0.01 was considered statistically significant.

3. Results

3.1. Establishment of Broadly Reactive Human Anti-HA Monoclonal Antibodies (mAbs)

One week after one or two vaccinations with influenza H5N1 vaccine, PBMCs were isolated and fused with fusion partner SPYMEG cells (Kubota-Koketsu et al., 2009) to obtain hybridoma clones that expressed a human antibody. After screening by ELISA using recombinant H1-, H3-, H5-, and H7-HAs and a purified H5N1 virus, positive hybridomas were cloned by dilution. Ultimately, 8 hybridoma clones (S9-1-10/5-1, 3352E69, 10-4-7/1, 4-6-8-4, 3381E12, 3381A11, 3352D13, and 3352E71) producing a human mAb, which recognized at least 3 subtypes of the HAs tested, were established (Table 1). By ELISA, S9-1-10/5-1 and 3352E69 recognized H1-, H5-, and H7-HA, and H1-, H3-, and H7-HA, respectively. The other 6 clones recognized H1-, H3-, and H7-HA. None of the clones reacted with HA derived from influenza B virus. The nucleotide sequence of the VH and VL regions for the 8 human mAbs was determined, and their complementarity determining region (CDR) 3 sequences were analyzed to determine the closest germline sequences using the IgBLat software in the NCBI database. Among the 8 human mAbs, 10-4-7/1, 3381E12, and 3391A11 used the IGHV3-33 gene, whereas 4-6-8-4, and 3352D13 used the IGKV1-24 gene (Table 1). S9-1-10/5-1, 3352E69, and 3352E71 used the IGKV4-59, IGKIV1-18, and IGKV3-11 genes, respectively (Table 1).

3.2. In Vivo Protective Efficacy of Broadly Reactive Human mAbs in Mice

To evaluate the in vivo efficacy of the 8 human mAbs we obtained, each human mAb or 1429C6/3-3, which recognizes influenza B virus HA, at 15 mg/kg, or phosphate buffered saline (PBS), was intraperitoneally administrated to 4 mice per group, which were then challenged 1 day later via intranasal infection with 10 MLD₅₀ of A/Vietnam/1203/2004 (VN1203; H5N1). All mice that received S9-1-10/5-1 survived, whereas 75% (3/4) of mice that were administered the other 7 human mAbs died (Fig. 1a). All mice that received PBS or 1429C6/3-3 died within 10 days of challenge. These results show that S9-1-10/5-1 possesses in vivo protective efficacy against lethal infection with a highly pathogenic H5N1 virus.

Next, we examined the dose-dependent protective efficacy of S9-1-10/5-1 against lethal challenges with H1, H3, H5, and H7 viruses. S9-1-10/5-1 at 15, 5, 1.7, 0.6, and 0.2 mg/kg, or PBS, was intraperitoneally injected into 4 mice per group. One day later, the mice were intranasally infected with 10 MLD₅₀ of mouse-adapted A/California/04/2009 (MA-CA04; H1N1pdm09), mouse-adapted A/Anhui/2/68 (MA-Aichi; H3N2), VN1203, or A/Anhui/1/2013 (Anhui1/1; H7N9). After challenge with MA-CA04 or VN1203, most of the mice that received the higher doses of S9-1-10/5-1 survived the 14-day observation period with little to no body weight loss (Fig. 1b). At the lower doses, however, severe body weight loss was observed. Nevertheless, even at the lowest dose, S9-1-10/5-1 partially protected mice from lethal infection with H1N1pdm09 or H5N1 virus. In the cases of MA-Aichi and Anhui1/1, most of the mice that received the higher doses of S9-1-10/5-1 survived the 14-day observation period but showed severe body weight loss (Fig. 1b). At the lower doses, almost all of the mice had died by 8 days post-challenge. These data indicate that S9-1-10/5-1 has in vivo protective efficacy against lethal infection with H1N1pdm09, H3N2, H5N1, and H7N9 viruses.

3.3. Inhibition of Virus Propagation by S9-1-10/5-1

Broadly reactive human monoclonal antibodies against the HA-stem region that protect mice from lethal infection generally inhibit virus entry, especially the membrane fusion step (Brandenburg et al., 2013; Laursen and Wilson, 2013). S9-1-10/5-1 protected mice from lethal infection with H1, H3, H5, and H7 viruses. We therefore hypothesized that
S9-1-10/5-1 would neutralize these viruses at the entry step in cultured cells. To test this hypothesis, we examine the neutralization activity of S9-1-10/5-1 in virus neutralization assays. Briefly, 100 TCID$_{50}$ of A/Cali-
ifornia/04/2009 (CA04; H1N1pdm09), A/Perth/16/2009 (Perth/16; H3N2), MA-Aichi, A/geese/Egypt/0929-NLQP/2009 (Egypt; H5N1), and Anhui/1 viruses were incubated with serially two-fold-diluted S9-1-
10/5-1. MDCK cells were incubated with the mixtures, washed twice to remove the antibody and uninfected viruses, and then the presence or absence of cytopathic effect (CPE) was examined at 3 days post-infec-
tion. Contrary to our expectation, S9-1-10/5-1 did not neutralize any of the viruses tested even at the highest concentration (50 μg/ml) under the conditions tested (Table 2). Under identical conditions, CR9114 neu-
tralized CA04 and Perth/16 viruses; the IC$_{50}$ values were 7.4 and 25 μg/ml (Table 2). In our virus neutralization assay, S9-1-10/5-1 was re-
moved after virus infection, which means that the virus was not ex-
posed to S9-1-10/5-1 during multiple cycles of replication. Therefore, we examined virus propagation in the presence of S9-1-10/5-1. Briefly, MDCK cells were infected with 100 TCID$_{50}$ of CA04, Perth/16, MA-Aichi, Egypt, and Anhui/1 viruses, washed twice, and then incubated in the presence of a serially two-fold-diluted S9-1-10/5-1 for 3 days at 37 °C before CPE was examined. S9-1-10/5-1 inhibited the propagation of CA04, Egypt, and Anhui/1 viruses; the IC$_{50}$ values ranged from 0.55–
8.8 μg/ml (Table 2). S9-1-10/5-1 failed to suppress the replication of Perth/16 and MA-Aichi viruses in vitro (Table 2). Similarly, CR9114
inhibited replication of CA04 and Perth/16 under the same conditions.

To further confirm these results, we performed a plaque reduction

| mAb         | Heavy chain  | Light chain  |
|-------------|--------------|--------------|
|             | VH           | VL           |
|             | CDR3         | CDR3         |
| S9-1-10/5-1 | IGHV4-59*01  | IGKV3-15*01  |
|             | ARMSTCQHHHHYFGIV| HQQYNWLSFT   |
| 3352E69     | IGHV1-18*01  | IGKV1-39*01  |
|             | AGTPIGYCQWSGYDCGLDP | GQHYTTPT    |
| 10-4-7/1    | IGHV1-33*01  | IGKV1-39*01  |
|             | ARAPGVCTGCGRFPYAMIDV | GQHYTTPT    |
| 4-8-6/4     | IGHV1-24*01  | IGKV2-28*01  |
|             | ATGFCATCNYGMDV | MQNNQTPPT    |
| 3381E12     | IGHV3-33*01  | IGKV3-11*01  |
|             | AGLGTVGGCGICRNYQDMDV | QCQCGWPLT   |
| 3381A11     | IGHV3-33*01  | IGKV3-11*01  |
|             | VRGLGVCSCYLYTRQMDV | QCQCGWPLT   |
| 3352D13     | IGHV1-24*01  | IGKV2-28*01  |
|             | ATGCSCSTTYCSEF | MQNNQTPPT    |
| 3352E71     | IGHV3-11*05  | IGKV3-39*01  |
|             | ARAPQENHHCGDLYFID | HQQYTTPT    |

Table 1: Genetic hallmarks of the human mAbs that recognized at least 3 subtypes of HA.

Fig. 1. In vivo protective efficacy of S9-1-10/5-1 in mice. (a) In vivo protective efficacy of 8 broadly reactive human mAbs in mice. Four mice per group were intraperitoneally injected with PBS or the indicated antibodies at 15 mg/kg. One day later, the mice were challenged with 10 MLD$_{50}$ of VN1203 virus. Body weight and survival were monitored daily for 14 days. 1429C6/3-3, which recognizes influenza B virus HA, and PBS were used as negative controls. (b) In vivo protective efficacy of S9-1-10/5-1 against H1, H3, H5, and H7 viruses. Four mice per group were intraperitoneally injected with PBS or S9-1-10/5-1 at 15, 5, 1.7, 0.6, or 0.2 mg/kg. One day later, the mice were challenged with 10 MLD$_{50}$ of MA-CA04 (H1N1pdm09), MA-Aichi (H3N2), VN1203 (H5N1), or Anhui/1 (H7N9) virus. Body weight and survival were monitored daily for 14 days. PBS was used as a negative control. Mouse body weights are expressed as mean ± SD.
Inhibition of virus replication by S9-1–10/5-1.

| Subtype | Virus strain | S9-1–10/5-1 Pre | Post | CR9114 Pre | Post |
|---------|--------------|----------------|------|------------|------|
| H1N1    | A/California/04/2009 | >50 ▲ | 1.1 | 7.4 | 1.1 |
| H3N2    | A/Perth/16/2000     | >50 >50 25 | 1.3 |
| H5N1    | A/geese/Egypt/0929-NLQP/2009 | >50 >50 25 | 1.3 |
| H7N9    | A/Anhui/1/2013     | >50 >50 25 | 1.3 |

▲ Virus was incubated with antibody. MDCK cells were incubated with the virus-antibody mixture, washed twice to remove the antibody and uninfected virus, and then the presence or absence of CPE was examined at 3 days post-infection.

Table 2

Inhibition of virus replication by S9-1–10/5-1.

To test this theory, we performed a virus release inhibition assay. Briefly, MDCK cells were infected with CA04 virus at an MOI of 1, and then incubated with 0.08, 0.4, 2, or 10 μg/ml of S9-1-10/5-1, 4-6-19/6, which is one of the broadest reactive human anti-HA stem antibody (Dreyfus et al., 2012). Among the HA subtypes belonging to group 1, H1-, H2-, H5-, H6-, H8-, H9-, H11-, H13-, H15-, and H16-HAs were clearly recognized by S9-1-10/5-1 whereas H12-HA was weakly detected (Fig. 3). Among the HA subtypes belonging to group 2, H4-, H7-, H10-, H14-, and H15-HAs were recognized by S9-1-10/5-1. H3-HA was not detected by S9-1-10/5-1 in this assay. CR9114 recognized all but H2 HA. These results show that S9-1-10/5-1 reacts with divergent HA subtypes belonging to both groups.

We next determined the binding kinetics of S9-1-10/5-1 with H1-, H3-, H5-, and H7-HAs by bio-layer interferometry (BLI) using an Octet Red 96 instrument and compared them with those for CR9114. Binding of S9-1-10/5-1 to H1-HA was 8.5 × 10⁻¹² M, whereas S9-1-10/5-1 bound with lower affinity (3.4 × 10⁻⁸ M) to H3-HA (Table 3). S9-1-10/5-1 showed similar binding affinities to H5-HA (1.4 × 10⁻¹⁰ M) and H7-HA (9.0 × 10⁻¹⁰ M), CR9114 exhibited higher affinities to all test HAs than did S9-1-10/5-1. These data show that S9-1-10/5-1
recognizes H1-, H3-, H5-, and H7-HAs to different extents. Taken together, S9-1-10/5-1 reacts with all 18 subtypes of HA.

3.5. Epitope Analysis of S9-1-10/5-1

Since broadly reactive antibodies usually bind to the HA-stem, we asked whether S9-1-10/5-1 recognizes the HA-stem. First, to map the epitope targeted by S9-1-10/5-1, we tried to generate escape mutants of CA04 virus under antibody pressure. By virus passages under antibody selection, we obtained 4 escape mutants, which were not neutralized by 250 μg/ml of S9-1-10/5-1. We identified the mutations in the HA gene by direct sequencing, and found that two escape mutants possessed the T378I (H3-numbering) substitution and one mutant possessed both the E110V and the A373T substitutions (Fig. 4a). To verify that these amino acid substitutions contributed to the escape from S9-1-10/5-1, we expressed wild-type or these mutant HA s by plasmid transfection and tested their reactivity with S9-1-10/5-1 and CR9114. HA-E110V, and HA-S383P substitution, and one mutant possessed the T378I (H3-numbering throughout) substitution, one mutant possessed the S383P substitution, and one mutant possessed both the E110V and the A373T substitutions (Fig. 4a). To verify that these amino acid substitutions contributed to the escape from S9-1-10/5-1, we expressed wild-type or these mutant HA s by plasmid transfection and tested their reactivity with S9-1-10/5-1 or 4-6-19/6, which recognizes the HA head. All of the HA s tested were recognized by 4-6-19/6 (Fig. 4b). Wild-type HA, HA-E110V, and HA-A373T were recognized by S9-1-10/5-1, HA-E110V/373T and HA-S383P were weakly recognized by S9-1-10/5-1, whereas HA-T378I was barely recognized by S9-1-10/5-1. These results demonstrate that an amino acid mutation in the HA stem plays an important role in escape from S9-1-10/5-1 and suggest that S9-1-10/5-1 targets the HA stem.

Further, to secondly examine whether S9-1-10/5-1 targets the HA stem or head, we performed an antibody binding competition assay. Briefly, H1-HA immobilized to a biosensor was saturated with S9-1-10/5-1 or 4-6-19/6, which recognizes the HA head. All of the HA s tested were recognized by 4-6-19/6 (Fig. 4b). Wild-type HA, HA-E110V, and HA-A373T were recognized by S9-1-10/5-1, HA-E110V/373T and HA-S383P were weakly recognized by S9-1-10/5-1, whereas HA-T378I was barely recognized by S9-1-10/5-1. These results demonstrate that an amino acid mutation in the HA stem plays an important role in escape from S9-1-10/5-1 and suggest that S9-1-10/5-1 targets the HA stem.

3.6. Actions of S9-1-10/5-1 on Virus Entry and Release

To determine why S9-1-10/5-1 did not block virus entry, we tested whether S9-1-10/5-1 inhibited the membrane fusion step by examining HA-mediated polykaryon formation induced by low pH exposure. Prior to low pH exposure, HA-expressing cells were treated with 5 μg/ml of trypsin and 20, 50, or 200 μg/ml of S9-1-10/5-1, the anti-stem CR9114, the anti-head 4-6-19/6, or the anti-B-HA 1429C6/3-3. We found that S9-1-10/5-1 and CR9114 suppressed HA-mediated polykaryon formation at a similar level, although polykaryons formed even at the highest concentration (Fig. 5a). In contrast, 4-6-19/6 completely inhibited HA-mediated polykaryon formation at all tested concentrations, whereas 1429C6/3-3 failed to suppress it (Fig. 5a). These data suggest that S9-1-10/5-1 serves as a fusion inhibitor because it behaves similarly to CR9114, which is a known fusion inhibitor (Dreyfus et al., 2012). Next, we examined whether S9-1-10/5-1 binds to the HA on the virion surface. A biosensor immobilized with S9-1-10/5-1, CR9114, 4-6-19/6, or 1429C6/3-3 was exposed to purified virus possessing CA04-HA and -NA. Binding of S9-1-10/5-1 to virus particles was slightly lower than that of CR9114 (Fig. 5b); kobs, which is the observed rate constant, of CR9114 to virus particles was 11.6 times higher than that of S9-1-10/5-1 (Fig. 5b). Virus particles efficiently bound to 4-6-19/6 but not to anti-B HA 1429C6/3-3 as expected (Fig. 5b). Although S9-1-10/5-1 bound to soluble H1-HA with a kobs rate similar to that of CR9114 (see Table 3) and bound to the HA on the cell surface (see Figs. 3 and 5a), S9-1-10/5-1 showed a lower ability to access the HA on the virion surface compared with CR9114 (Fig. 5b and c). Taken together, these data suggest that the low potency of S9-1-10/5-1 for binding to the HA on the virion surface plays a central role in the inability of S9-1-10/5-1 to block virus entry completely.

To investigate the mechanism by which S9-1-10/5-1 inhibited virus particle release, we examined whether S9-1-10/5-1 indirectly inhibited viral neuraminidase activity. MDCK cells infected with CA04 at an MOI of 1 were mock-incubated or incubated with oseltamivir carboxylate (OC), S9-1-10/5-1 (S9), or 4-6-19/6 (4-6) with or without bacterial sialidase. At 18 h post-infection, total cell lysates and culture supernatants including virus particles were analyzed by western blotting.

### Table 3

| Virus (subtype) | S9-1-10/5-1 | CR9114 |
|----------------|------------|---------|
|               | kon (1/Ms) | koff (1/s) | KD (M) | kon (1/Ms) | koff (1/s) | KD (M) |
| A/California/07/2009 (H1N1pdm09) | 7.9E + 05 | 6.7E − 06 | 8.5E − 12 | 7.8E + 05 | <1.0E − 07 | <1.0E − 12 |
| A/Perth/16/2009 (H3N2) | 5.9E + 04 | 2.0E − 03 | 3.4E − 08 | 1.0E + 05 | 3.3E − 04 | 3.3E − 09 |
| A/Egypt/200/2009 (H5N1) | 6.8E + 05 | 9.4E − 05 | 1.4E − 10 | 3.6E + 05 | <1.0E − 07 | <1.0E − 12 |
| A/Netherlands/219/2003 (H7N7) | 4.1E + 05 | 3.7E − 04 | 9.0E − 10 | 3.6E + 05 | 2.4E − 05 | 6.7E − 11 |
using an anti-M1 antibody. Sialidase treatment did not affect the virus particle release inhibited by S9-1-10/5-1 or 4-6-19/6, but did increase virus particle release in the presence of OC (Fig. 5d). M1 levels were similar in all cell lysates tested. These results indicate that S9-1-10/5-1 does not affect viral neuraminidase activity. Next, we investigated whether bivalency of S9-1-10/5-1 is required for its inhibition of virus particle release. To this end, we tested an Fab fragment of S9-1-10/5-1 for this activity. MDCK cells infected with CA04 at an MOI of 1 were incubated with 1, 4, 16, or 64 nM of IgG or Fab fragment of S9-1-10/5-1. Viral particle release was analyzed by western blotting with the anti-M1 antibody. M1 was not detected in the supernatant samples that contained 16 or 64 nM of IgG fragments of S9-1-10/5-1, whereas it was detected in all supernatant samples containing Fab fragments of S9-1-10/5-1 (Fig. 5e). M1 was similarly detected in the cell lysates from these samples. These data demonstrate that bivalent S9-1-10/5-1 acts as an inhibitor of virus particle release, suggesting that the IgG fragment of S9-1-10/5-1 tethers virions via crosslinking HA molecules between neighboring virions.

**Fig. 4.** Epitope analysis of S9-1-10/5-1 on H1-HA. (a) Mutations found in H1-HA after passages of MA-CA04 in the presence of S9-1-10/5-1. MA-CA04 was passaged in the presence of various concentrations of S9-1-10/5-1 in MDCK cells. After 4–6 passages, the HA sequence was analyzed and the identified mutations were mapped on the 3D structure of the H1-HA trimer by using the molecular graphics system PyMOL. Four independent experiments were performed. Mutations (positions are presented as H3-numbering) shown in the same color were found in the same sample. (b) Escape mutations from S9-1-10/5-1. Wild-type HA and mutant HAs expressed in 293T cells were stained with S9-1-10/5-1 or 4-6-19/6 (anti-HA head). Scale bars, 100 μm. (c) S9-1-10/5-1 competed with the human anti-stem antibody CR9114. Additional binding of the indicated human mAbs to the immobilized HA of A/California/07/2009 (H1N1pdm09) saturated with 10 μg/ml S9-1-10/5-1 (left), CR9114 (middle), or 4-6-19/6 (right), was measured by bio-layer interferometry.

**Fig. 5.** Actions of S9-1-10/5-1 on virus entry and release. (a) Inhibition of viral HA-mediated membrane fusion by S9-1-10/5-1. Hela cells were transfected with a plasmid expressing wild-type CA04-HA. After trypsin treatment, the cells were incubated with the indicated human monoclonal antibody. Polykaryon formation was induced by low pH exposure. Scale bars, 100 μm. (b) Binding of S9-1-10/5-1 to virus particles. Binding of the purified CA04/PB2-KO virus through a sucrose cushion to the immobilized human mAbs, S9-1-10/5-1, CR9114, 4-6-19/6, and 1429C6/3-3, was measured by bio-layer interferometry. (c) Relative $k_{on}$ to virus particles. Relative $k_{on}$, which is the observed rate constant, was calculated from the binding kinetics of human mAbs to three concentrations of CA04/PB2-KO virus. The $k_{on}$ of S9-1-10/5-1 was set to 1. Data are expressed as mean ± SD. ** indicates $P < 0.01$, according to the t-test. (d) Viral neuraminidase activity was not inhibited by S9-1-10/5-1. MDCK cells infected with CA04 at an MOI of 1 were mock-incubated or incubated with 100 μM oseltamivir carboxylate (OC), 0.4 or 2 μg/ml S9-1-10/5-1 (S9), or 0.4 or 2 μg/ml 4-6-19/6 (4-6) with or without 20 μM of bacterial sialidase derived from Clostridium perfringens. (e) Fab fragments of S9-1-10/5-1 failed to inhibit virus particle release. MDCK cells infected with CA04 at an MOI of 1 were incubated with 1, 4, 16, and 64 nM of IgG or Fab fragment of S9-1-10/5-1. (D–E) Cell lysates and supernatants were analyzed by western blotting with the anti-M1 antibody. ACTB was detected as a loading control.
4. Discussion

Here we established 8 human mAbs that reacted with at least 3 subtypes of HA among H1-, H3-, H5-, and H7-HA. Typically, antibodies that are positive in an initial screen are re-screened in an in vitro neutralization assay prior to in vivo assessment. The in vitro neutralization assay examines whether the antibody inhibits virus entry when it is conducted without incubating the virus-infected cells with the antibody. Therefore, the broadly reactive human monoclonal antibodies against the HA stem of influenza A virus that have been reported to date predominantly interfere with virus entry by abolishing the membrane fusion step (Cho and Wrammert, 2016). Here, we first evaluated the in vivo protective efficacy of our 8 established mAbs in a mouse infection model and found one antibody that was protective; we took this approach because non-neutralizing antibodies sometimes can suppress viral replication in vivo (Tharakaraman et al., 2015). This approach ensured that we would not exclude protective non-neutralizing antibodies. We were able to pick up a human protective monoclonal antibody, S9-1-10/5-1, which inhibited virus particle release from the cell surface but did not block virus entry completely. Our strategy allowed us to find a broadly reactive antibody that mainly inhibited virus particle release. The S9-1-10/5-1 protected mice from lethal infection with seasonal and enzootic influenza A viruses including H1N1pdm09, H3N2, H5N1, and H7N9 viruses. S9-1-10/5-1, therefore, has the potential to be an antiviral drug to treat severe influenza virus infection and to prevent influenza virus infection under epidemic and pandemic conditions.

S9-1-10/5-1 competed for binding to HA with CR9114, which targets the HA stem. Escape mutations from S9-1-10/5-1 were mostly found in helix A of HA2, which forms part of the HA stem. Although we have not yet determined the precise epitope of S9-1-10/5-1, the helix A of HA2 is likely the major target of S9-1-10/5-1. The helix A of HA2 is also reported to be a binding site for other broadly reactive human monoclonal antibodies, including CR9114, CT149, 39.29, MED8852, and FI6v2 (Corti et al., 2011; Dreyfus et al., 2012; Kallewaard et al., 2016; Nakamura et al., 2013; Wu et al., 2015). Although these antibodies may recognize the same or a similar region as S9-1-10/5-1, they all inhibit virus entry steps, whereas S9-1-10/5-1 does not. S9-1-10/5-1 binds to the HA on the cell surface. However, it showed a lower ability to access to the HA on the virion surface; structural analysis of the binding mode of S9-1-10/5-1 may reveal the difference in the inhibitory mechanisms between S9-1-10/5-1 and other entry-inhibiting anti-stem antibodies. An understanding of the precise mechanism by which S9-1-10/5-1 inhibits virus particle release will contribute to our understanding of broadly reactive human antibodies.

S9-1-10/5-1 recognized all 18 subtypes of HA derived from both groups 1 and 2. Several human monoclonal antibodies against the HA stem have been reported to react with both groups of HA. These antibodies usually use the IGHV1-69, IGHV3-30, IGHV1-18, IGHV3-23, IGHV4-4, or IGHV6-1, which are thought to provide a framework for broadly reactive antibodies (Corti and Lanzavecchia, 2013). This is the primary report to show that the IGHV4-59 gene is used by a broadly protective human monoclonal antibody. Usage of the IGHV4-59 gene by S9-1-10/5-1 may contribute to its unique inhibitory mechanism. The establishments of additional human monoclonal antibodies against the HA stem that mainly inhibit virus particle release would shed light on this possibility.

S9-1-10/5-1 inhibited the in vitro and in vivo replication of H1N1pdm09 (group 1), H5N1 (group 1), and H7N9 (group 2) viruses, whereas it suppressed the replication of H3N2 virus in vivo but not in vitro. These results indicate the possible involvement of cellular factors that are lacking in vitro for protection especially against H3N2 virus in vivo. Among the many cellular factors in vivo, we presume that Fcγ receptors, which are required for the protection afforded by anti-HA stem antibodies against influenza A virus in vivo (DiLillo et al., 2014; DiLillo et al., 2016), play an important role in this protection. The interaction between the anti-HA stem antibody and FcγRs triggers ADCC, leading to a reduction in the number of infected cells (Jegaskanda et al., 2014). This mechanism may also contribute to the protection afforded by S9-1-10/5-1 against H1N1pdm09, H5N1, and H7N9 viruses in vivo.

In summary, here we showed that the broadly reactive human monoclonal antibody S9-1-10/5-1 suppresses the replication of heterotypic influenza A viruses both in vitro and in vivo by mainly inhibiting virus particle release from infected cells. These data provide experimental evidence of the antiviral potential of S9-1-10/5-1 and provide the basis for further clinical testing. The finding of a broadly reactive antibody that mainly inhibits virus particle release provides valuable information toward the development of a universal vaccine.

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**Conflicts of Interest Statement**

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**Author Contributions**

S.Y. and Y.K. designed the study. S.Y., R.U., M.I., M.K., S.N., A.Y., and K.O. performed the experiments. S.Y., R.U., M.I., and K.Y. analyzed the data. T.S. and K.I. assisted with the experiments. S.Y. and Y.K. wrote the manuscript. All authors reviewed and approved the manuscript.

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**References**

Brandenburg, B., Koudstaal, W., Goudsmit, J., Klaren, V., Tang, C., Bujny, M.V., Korse, H.J., Kwaks, T., Ottersen, J.J., Jurasek, J., et al., 2013. Mechanisms of hemagglutinin targeted influenza virus neutralization. PLoS One 8, e80034.

Caton, A.J., Brownlee, G.G., Yewdell, J.W., Gerhard, W., 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31, 417–427.

Cho, A., Wrammert, J., 2016. Implications of broadly neutralizing antibodies in the development of a universal influenza virus vaccine. Curr. Opin. Virol. 17, 110–115.

Clementi, N., De Marco, D., Mannini, N., Solforosi, L., Moreno, G.J., Guibareva, L.V., Mishin, V., Di Pietro, A., Vicenzì, E., Siccardi, A.G., et al., 2011. A human monoclonal antibody with neutralizing activity against highly divergent influenza subtypes. PLoS One 6, e28001.

Corti, D., Lanzavecchia, A., 2013. Broadly neutralizing antiviral antibodies. Annu. Rev. Immunol. 31, 705–742.

Corti, D., Sugasaitan Jr., A.L., Pinna, D., Silacci, C., Fernandez-Rodriguez, B.M., Vanzetta, F., Santos, C., Luke, C.J., Torres-Velez, F.J., Temperton, N.J., et al., 2010. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest. 120, 1663–1673.
Corti, D., Voss, J., Gamblin, S.J., Codoni, G., Macagno, A., Jarrossay, D., Vachieri, S.G., Pinna, D., Minola, A., Vanzetta, F., et al., 2011. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science 333, 850–856.

De Marco, D., Clementi, N., Mancini, N., Solfors, L., Moreno, C.J., Sun, X., Tumpey, T.M., Gubareva, L.V., Mishin, V., Clementi, M., et al., 2012. A non-VH1-69 heterosubtypic neutralizing monoclonal antibody protects mice against H1N1 and H5N1 viruses. PLoS One 7, e34415.

Dilillo, D.J., Tan, G.S., Palese, P., Ravetch, J.V., 2014. Broadly neutralizing hemagglutinin stalk-specific antibodies require Fab/Fc interactions for protection against influenza virus in vivo. Nat. Med. 20, 143–151.

Dilillo, D.J., Palese, P., Wilson, P.C., Ravetch, J.V., 2016. Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. J. Clin. Invest. 126, 605–610.

Dreyfus, C., Laursen, N.S., Kwaks, T., Zuijdgeest, D., Khayat, R., Ekiert, D.C., Lee, J.H., Metlagel, Z., Bufny, M.V., Jongeneelen, M., et al., 2012. Highly conserved protective epitopes on influenza B viruses. Science 337, 1343–1348.

Ekiert, D.C., Bhatia, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., Wilson, I.A., 2009. Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.

Ekiert, D.C., Friesen, R.H., Bhatia, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., et al., 2011. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.

Friesen, R.H., Lee, P.S., Stoop, E.J., Hoffman, R.M., Ekiert, D.C., Bhatia, G., Yu, W., Jurasek, J., Koudstaal, W., Jongeneelen, M., et al., 2014. A common solution to group 2 influenza virus neutralization. Proc. Natl. Acad. Sci. U. S. A. 111, 445–450.

Gamblin, S.J., Skehel, J.J., 2010. Influenza hemagglutinin and neuraminidase membrane glycoproteins. J. Biol. Chem. 285, 28403–28409.

Henry Dunand, C.J., Leon, P.E., Kaur, K., Tan, G.S., Zheng, N.Y., Andrews, S., Huang, M., Qu, X., Huang, Y., Salgado-Ferrer, M., et al., 2013. Preserving human antibodies neutralize recently emerged H7N9 influenza strains. J. Clin. Invest. 125, 1255–1268.

Hu, W., Chen, A., Xiao, Y., Xia, S., Ling, Z., Xu, K., Wang, T., Xu, Y., Cui, J., Wu, H., et al., 2013. Fully human broadly neutralizing monoclonal antibodies against influenza A viruses generated from the memory B cells of a 2009 pandemic H1N1 influenza virus vaccine recipient. Virology 435, 320–328.

Jegaska, S., Reading, P.C., Kent, S.J., 2014. Influenza-specific antibody-dependent cellular cytotoxicity: toward a universal influenza vaccine. J. Immunol. 193, 469–475.

Joyce, M.G., Wheatley, A.K., Thomas, P.V., Chuang, G.Y., Soto, C., Baider, R.T., Druz, A., Georgiev, I.S., Gillespie, R.A., Kanekiyo, M., et al., 2016. Vaccine-induced antibodies that neutralize group 1 and group 2 influenza A viruses. Cell 166, 609–623.

Kaliewaard, N.L., Corti, D., Collins, P.J., Neu, U., McAuliffe, J.M., Benjamin, E., Wachter-Rosati, L., Palmer-Hill, F.J., Yuan, A.Q., Walker, P.A., et al., 2016. Structure and function analysis of an antibody recognizing all influenza A subtypes. Cell 166, 596–608.

Kashyap, A.K., Steel, J., Rubrum, A., Estelles, A., Briante, R., Ilyushina, N.A., Xu, L., Swale, R.E., Faynboym, A.M., Foreman, P.K., et al., 2010. Protection from the 2009 H1N1 pandemic influenza by an antibody from a combinatorial survivor-based libraries. PLoS Pathog. 6, e1000990.

Kubota-Koletsu, R., Mizuta, H., Oshita, M., Ideno, S., Yunoki, M., Kuhara, M., Yamamoto, N., Okuno, Y., Ikuta, K., 2009. Broad neutralizing human mononal antibodies against influenza virus from vaccinated healthy donors. Biochem. Biophys. Res. Commun. 387, 180–185.

Lauren, N.S., Wilson, I.A., 2013. Broadly neutralizing antibodies against influenza viruses. Antivir. Res. 98, 476–483.

Li, G.M., Chiu, C., Wrammert, J., McCausland, M., Andrews, S.F., Zheng, N.Y., Lee, J.H., Huang, M., Qu, X., Edupuganti, S., et al., 2012. Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. Proc. Natl. Acad. Sci. U. S. A. 109, 9047–9052.

Nakamura, G., Chai, N., Park, S., Chiang, N., Lin, Z., Chiu, H., Fong, R., Yan, D., Kim, J., Zhang, J., et al., 2013. An in vivo human-plasmablast enrichment technique allows rapid identification of therapeutic influenza A antibodies. Cell Host Microbe 14, 93–103.

Ozawa, M., Victor, S.T., Taft, A.S., Yamada, S., Li, C., Hatta, M., Das, S.C., Takashita, E., Kakugawa, S., Maher, E.A., et al., 2011. Replication-incompetent influenza A viruses that stably express a foreign gene. J. Gen. Virol. 92, 2879–2888.

Sakabe, S., Ozawa, M., Takano, I., Iwastuki-Horimoto, K., Kawaoi, Y., 2011. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. Virus Res. 158, 124–129.

Sui, J., Hwang, W.C., Perez, S., Wei, G., Aird, D., Chen, L.M., Santelli, E., Stec, B., Cadwell, G., Ali, M., et al., 2009. Structural and functional bases for broad–spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol. 16, 265–273.

Sui, J., Sheehan, J., Hwang, W.C., Bankston, L.A., Burchett, S.K., Huang, C.Y., Liddington, R.C., Beigel, J.H., Marasco, W.A., 2011. Wide prevalence of heterosubtypic broadly neutralizing human anti-influenza A antibodies. Clin. Infect. Dis. 52, 1003–1009.

Tharakaraman, K., Subramanian, V., Viswanathan, K., Sloan, S., Yen, H.L., Barnard, D.L., Leung, Y.H., Soretter, K.J., Koch, T.J., Delaney, J.C., et al., 2015. A broadly neutralizing human monoclonal antibody is effective against H7N9. Proc. Natl. Acad. Sci. U. S. A. 112, 10890–10895.

Throsby, M., van den Brink, E., Jongeneelen, M., Poon, L.L., Alard, P., Cornelissen, L., Bakker, A., Cox, F., van Deventer, E., Guan, Y., et al., 2008. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One 3, e3942.

Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J., et al., 2013. New world bats harbor diverse influenza A viruses. PLoS Pathog. 9, e1003657.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoi, Y., 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56, 152–179.

Wilson, I.A., Skehel, J.J., Wiley, D.C., 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature 289, 366–371.

Wu, Y., Cho, M., Shore, D., Song, M., Choi, J., Jiang, T., Deng, Y.Q., Bourgeois, M., Almir, L., Yang, H., et al., 2015. A potent broad–spectrum protective human monoclonal antibody crosslinking two haemagglutinin monomers of influenza A virus. Nat. Commun. 6, 7708.

Wyrzucki, A., Dreyfus, C., Kohler, I., Steck, M., Wilson, I.A., Hangartner, L., 2014. Alternative recognition of the conserved stem epitope in influenza A virus hemagglutinin by a VH3-30-encoded heterosubtypic antibody. J. Virol. 88, 7083–7082.

Wyrzucki, A., Bianchi, M., Kohler, I., Steck, M., Hangartner, L., 2015. Heterosubtypic antibodies to influenza A virus have limited activity against cell-bound virus but are not impaired by strain-specific serum antibodies. J. Virol. 89, 3136–3144.