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Research Paper

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.

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*-terminus 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), 3.1 (Wyrzucki et al., 2014), FE43, FE17 (Corti et al., 2010), PN-SIA49 (De

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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), FI6v3 (Corti et al., 2011), 39.29, 81.39 (Nakamura et al., 2013), CT149 (Wu et al., 2015), VIS410 (Tharakaraman et al., 2015), 1.12 (Wyrzucki et al., 2015), 1C4, 3C4 (Hu et al., 2013), 05-2G02 (Li et al., 2012), 045-05310-2B06, S6-B01 (Henry Dunand et al., 2015), PN-SIA28 (Clementi et al., 2011), MEDI8852 (Kallewaard et al., 2016), 56.a.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% CO_2.

2.3. Viruses

A/California/04/2009 (CA04; H1N1pdm09), its mouse-adapted strain (MA-CA04) (Sakabe et al., 2011), mouse-adapted A/Aichi/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 titrated 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/Astrakhan/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/05/2005 (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 SPYMEG 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/Netherland/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_H and V_L 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_1) 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 HiScreen MabSelect SuRe LX column (GE Healthcare) and the automated chromatography system ÄKTA 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 μg/ml TPCK-treated trypsin for 3 days at 37 °C before 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.

2.11. 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 *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin and the serially two-fold diluted indicated antibody for 3 days at 37 °C before the 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 mU of bacterial sialidase 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 Reynold'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 by 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). Recombinant HAs [A/California/07/2009 (H1N1pdm09), A/Perth/16/2009 (H3N2), A/Egypt/N05056/2009 (H5N1), and A/Netherland/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_{on} (300 s); and (4) dissociation of S9-1-10/5-1 or CR9114 for the measurement of k_{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

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 mixed 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 second 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. After incubation for 90 min at 37 °C, 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_{obs}, 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 vaccines, 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, 4-8-6/4, 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-, H5-, 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 IgBlast software in the NCBI database. Among the 8 human mAbs, 10-4-7/1, 3381E12, and 3391A11 used the *IGHV3-33* gene, whereas 4-8-6/4, and 3352D13 used the *IGHV1-24* gene (Table 1). S9-1-10/5-1, 3352E69, and 3352E71 used the *IGHV4-59*, *IGHV1-18*, and *IGHV3-11* 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 MLD_{50} 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 MLD_{50} of mouse-adapted A/California/04/2009 (MA-CA04; H1N1pdm09), mouse-adapted A/Aichi/2/68 (MA-Aichi; H3N2), VN1203, or A/Anhui/1/2013 (Anhui/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 Anhui/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

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

	Heavy chain		Light chain	
mAb	VH	CDR3	VL	CDR3
S9-1-10/5-1	IGHV4-59*01	ARMSRGVFSSSRFFGLDV	IGKV3-15*01	QQYNNWLSFT
3352E69	IGHV1-18*01	AGTNPWEQNYGEGLDP	IGKV1-39*01, or IGKV1D-39*01	QQSYTTPRT
10-4-7/1	IGHV3-33*01	ARAPGYCTGGSCRTRYFYYMDV	IGKV1-39*01, or IGKV1D-39*01	QQSYSTPLS
4-8-6/4	IGHV1-24*01	ATGTFCSATNCYGMDV	IGKV2-28*01, or IGKV2D-28*01	MQNQQTPPT
3381E12	IGHV3-33*01	ARGLGYCGGGICRNRYDYQMDV	IGKV3-11*01	QQCYGWPLT
3381A11	IGHV3-33*01	VRGLGYCSGYLCRTRYDQQMDV	IGKV3-11*01	QQCRDWPLT
3352D13	IGHV1-24*01	ATGSYCSTTYCSFNF	IGKV2-28*01, or IGKV2D-28*01	MQAQQTPLT
3352E71	IGHV3-11*05	ARAFQENLHGYGDLLVFDI	IGKV1-39*01, or IGKV1D-39*01	HQTWT

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/California/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-infection. 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 neutralized 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 removed after virus infection, which means that the virus was not exposed 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

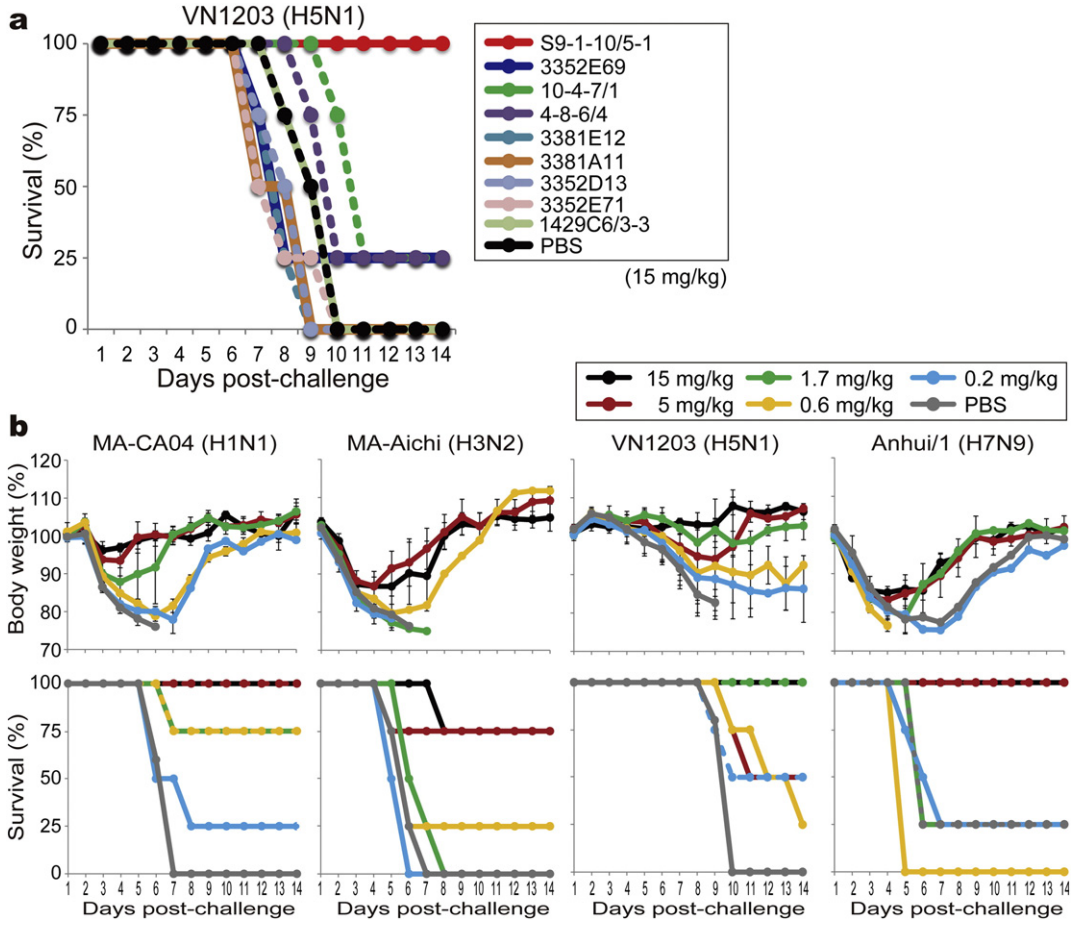

Fig. 1. *In vivo* protective efficacy of S9-1-10/5-1 in mice. (a) *In vivo* protective efficacy of 8 broadly reactive human mAbs in mice. Four mice per group were intraperitoneally injected with PBS or the indicated antibodies at 15 mg/kg. One day later, the mice were challenged with 10 MLD_{50} of VN1203 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.

Table 2
Inhibition of virus replication by S9-1-10/5-1.

Subtype	Virus strain	S9-1-10/5-1		CR9114	
		Pre[a]	Post[b]	Pre	Post
H1N1	A/California/04/2009	>50[c]	1.1	7.4	1.1
H3N2	A/Perth/16/2009	>50	>50	25	1.3
	A/Aichi/2/68 (mouse-adapted)	>50	>50	–[d]	–
H5N1	A/geese/Egypt/0929-NLQP/2009	>50	0.55	–	–
H7N9	A/Anhui/1/2013	>50	8.8	–	–

[a] 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.
[b] MDCK cells were infected with virus, washed twice, and then incubated in the presence of antibody for 3 days before CPE was examined.
[c] IC_{50} value (μg/ml).
[d] Not done.

assay in which CA04 was treated with S9-1-10/5-1, CR9114, or 1429C6/3-3 prior to, or after infection. S9-1-10/5-1 efficiently inhibited virus spread (red dashed line; $EC_{50} = 0.145$), whereas it poorly inhibited virus entry (red solid line; $EC_{50} = 7.61$) (Fig. 2a). In contrast, CR9114 inhibited both virus entry and virus spread with similar efficiency (blue dashed and solid lines; $EC_{50} = 0.144$ and 0.301) (Fig. 2a). 1429C6/3-3 failed to inhibit plaque formation under either test condition (black solid and dashed lines). From these results, we theorized that S9-1-10/5-1 would mainly inhibit virus particle release from infected cells, unlike the anti-head antibodies which functions at both steps. 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 recognizes the HA head of CA04, or 1429C6/3-3, which recognizes influenza B viral HA. At 18 h post-infection, total cell lysates and culture supernatants including virus particles were analyzed by western blotting using an anti-M1 antibody; M1 is one of the major components of the virus particle. In the absence of antibody, M1 was detected in the supernatant and in the cell lysate (Fig. 2b). M1 was detected in all supernatant samples containing 1429C6/3-3, whereas it was not detected in the supernatant samples that contained 2 or 10 μg/ml S9-1-10/5-1 or 4-6-19/6. M1 was similarly detected in the cell lysates from these samples. To further confirm these results, we observed the virus particles being released from the cell surface by using electron microscopy. We saw that virus budding at the surface of cells treated with 1429C6/3-3 was similar to that for cells that were not treated with antibody (Fig. 2c). However, at the surface of cells treated with S9-1-10/5-1 or 4-6-19/6, the released virions aggregated, indicating that virus release was inhibited. These data show that S9-1-10/5-1 suppresses virus propagation in cell culture by mainly inhibiting virus release from the cell surface.

3.4. Reactivity of S9-1-10/5-1 to All 18 HA Subtypes Belonging to Groups 1 and 2

S9-1-10/5-1 recognized H1-, H5-, and H7-HAs by ELISA, inhibited *in vitro* propagation of H1, H5, and H7 viruses, and protected mice from lethal infection with H1, H3, H5, and H7 viruses. These results suggest that S9-1-10/5-1 recognizes divergent HA subtypes belonging to groups 1 and 2. To examine whether S9-1-10/5-1 reacts with H1- through H18-HA, 293T cells were transfected with individual plasmids encoding H1- through H18-HA, and then stained with S9-1-10/5-1 or CR9114, 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-, H16-, H17-, and H18-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 HAs. 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

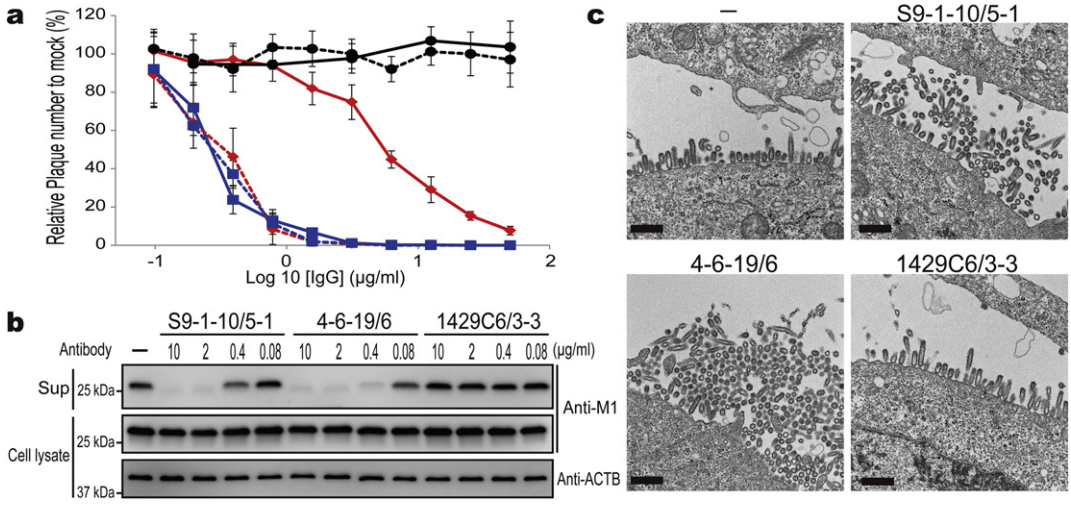

Fig. 2. Inhibition of virus particle release by S9-1-10/5-1. (a) Plaque reduction assay in MDCK cells infected with CA04 virus pre- or post-treated with S9-1-10/5-1. For the pre-treatment assay, CA04 was incubated with S9-1-10/5-1 (red solid line), CR9114 (blue solid line), or 1429C6/3-3 (black solid line). MDCK cells (3 wells per condition) were incubated with the virus-antibody mixture, washed twice to remove the antibody and uninfected virus, and then plaques were counted at 2 days post-infection. For the post-treatment assay, MDCK cells (4 wells per condition) were infected with CA04, washed twice, and then overlaid with agar in the presence of S9-1-10/5-1 (red dashed line), CR9114 (blue dashed line), or 1429C6/3-3 (black dashed line) for 2 days before the plaques were counted. The average plaque number in the mock treatment wells was set to 100%. Data are expressed as mean ± SD. (b) Detection of M1 in the cell lysates and supernatants of MDCK cells infected with CA04. MDCK cells were infected with CA04 at an MOI of 1 and then incubated with 10, 2, 0.4, or 0.08 μg/ml of S9-1-10/5-1, 4-6-19/6 (anti-HA head), or 1429C6/3-3 (anti-B-HA) for 18 h. Cell lysates and supernatants were analyzed by western blotting. ACTB was detected as a loading control. Each experiment was performed twice, and representative data are shown. (c) TEM images of MDCK cells infected with CA04. MDCK cells were infected with CA04 at an MOI of 1 and then incubated with 2 μg/ml of S9-1-10/5-1, 4-6-19/6, or 1429C6/3-3 for 18 h. Ultrathin sections were examined by TEM. Scale bars, 500 nm.

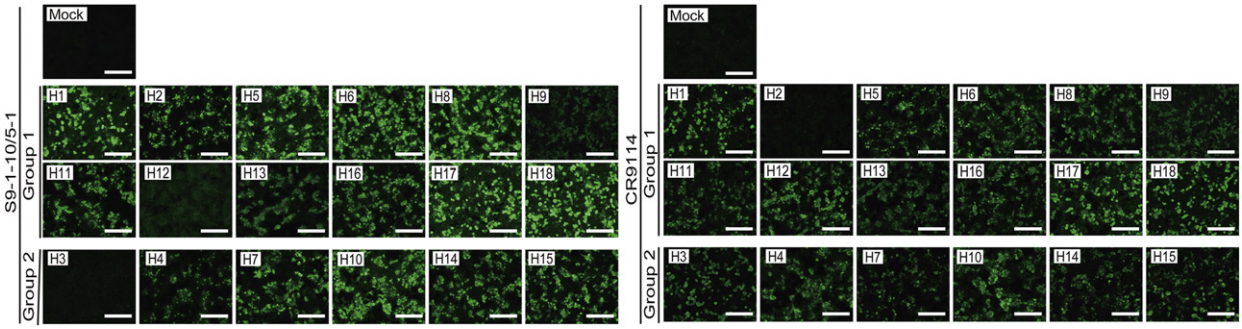

Fig. 3. Binding of S9-1-10/5-1 to H1- through H18-HA subtypes. 293T cells were mock transfected or transfected with plasmids encoding H1- to H18-HA subtypes. Expressed HAs were detected by S9-1-10/5-1 or CR9114, followed by an anti-human IgG conjugated with Alexa Fluor 488. Scale bars, 100 μm.

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 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 HAs 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 HAs 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/A373T 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.

Second, to further 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 (Fig. 4c, left), CR9114, which recognizes the HA stem (Fig. 4c, middle), or 4-6-19/6, which recognizes the HA head (Fig. 4c, right). Each biosensor was additionally exposed to S9-1-10/5-1, CR9114, or 4-6-19/6. S9-1-10/5-1 competed with CR9114 but not with 4-6-19/6 for binding to H1-HA (Fig. 4c, left), and *vice versa* (Fig. 4c, middle). 4-6-19/6 did not compete with S9-1-10/5-1 or CR9114 (Fig. 4c, right). Taken together, these results indicate that S9-1-10/5-1 recognizes 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); k_{obs}, 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. 5c). 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 k_{on} 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
Binding kinetics of S9-1-10/5-1 with recombinant HAs.

	S9-1-10/5-1			CR9114		
Virus (subtype)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (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/N05056/2009 (H5N1)	6.6E + 05	9.4E − 05	1.4E − 10	3.5E + 05	<1.0E − 07	<1.0E − 12
A/Netherland/219/2003 (H7N7)	4.1E + 05	3.7E − 04	9.0E − 10	3.6E + 05	2.4E − 05	6.7E − 11

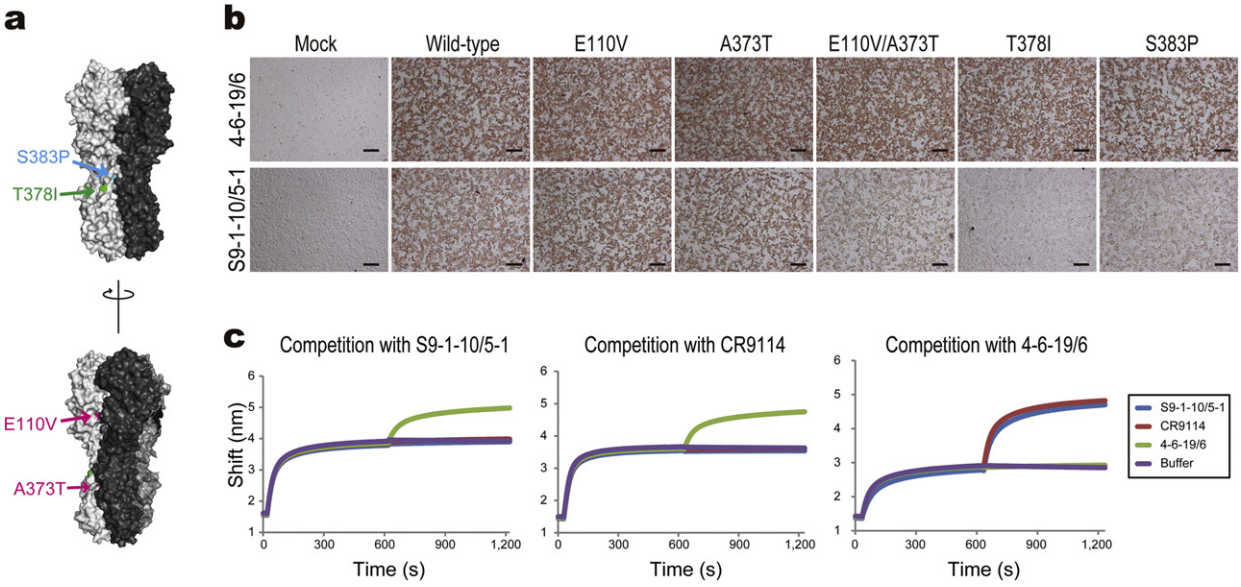

Fig. 4. Epitope analysis of S9-1-10/5-1 on H1-HA. (a) Mutations found in H1-HA after passages of MA-CA04 in the presence of S9-1-10/5-1. MA-CA04 was passaged in the presence of various concentrations of S9-1-10/5-1 in MDCK cells. After 4–6 passages, the HA sequence was analyzed and the identified mutations 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.

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.

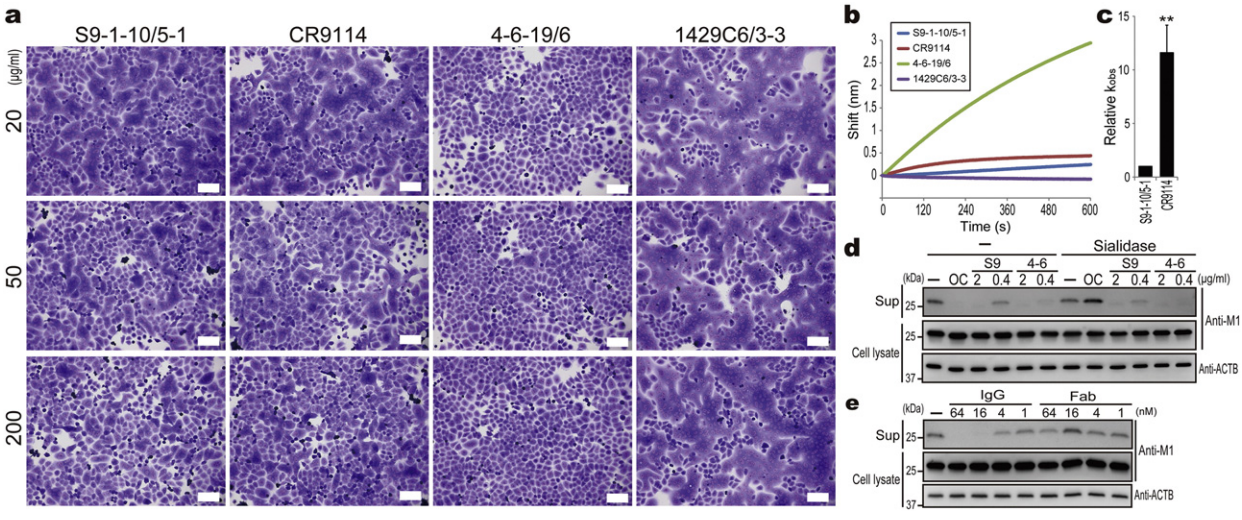

Fig. 5. Actions of S9-1-10/5-1 on virus entry and release. (a) Inhibition of viral HA-mediated membrane fusion by S9-1-10/5-1. Hela cells were transfected with a plasmid expressing wild-type CA04-HA. After trypsin treatment, the cells were incubated with the indicated human monoclonal antibody. Polykaryon 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_{obs} to virus particles. Relative k_{obs}, 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_{obs} 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 mU 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, MEDI8852, and FI6v3 (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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