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Human protective monoclonal antibodies against the HA stem of group 2 HAs derived from an H3N2 virus-infected human

Seiya Yamayoshi¹, Mutsumi Ito¹, Ryuta Uraki¹, Tadahiro Sasaki², Kazuyoshi Ikuta², Yoshihiro Kawaoka^{1,3,4,5,#}

¹Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Japan

²Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan

³Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, USA

⁴Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Japan

⁵ERATO Infection-Induced Host Responses Project, Japan Science and Technology Agency, Japan

Summary

Objectives: Broadly reactive human monoclonal antibodies against the HA stem of influenza A virus are being developed as therapeutic agents as well as to understand the epitopes that are essential for a universal influenza virus vaccine.

Methods: We isolated and characterized two hetero-reactive human monoclonal antibodies from an H3N2 virus-infected human.

Results: These antibodies, which predominantly bound to the HA stem of group 2 HAs, used IGHV3-66 and IGHV4-38-2 germline genes, respectively. They possessed in vitro neutralizing ability, and in vivo protective efficacy against lethal infection with H3N2 or H7N9 virus. Escape mutations revealed that one of the protective antibodies recognized the α -helix A of HA2, and the other recognized the C-terminal portion of the fusion peptide and the β -sheet that precedes the α -helix A of HA2.

#Correspondence and requests should be addressed to: Yoshihiro Kawaoka, DVM, PhD; yoshihiro.kawaoka@wisc.edu, Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan.

Author contributions

S.Y. and Y.K. designed the study. S.Y., M.I., and R.U. performed the experiments. S.Y., M.I., R.U., 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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Conflicts of interest

Y.K. has received speaker's honoraria from Toyama Chemical and Astellas Inc., has received grant support from Chugai Pharmaceuticals, Daiichi Sankyo Pharmaceutical, Toyama Chemical, Taun's Laboratories, Inc., Otsuka Pharmaceutical Co., Ltd., and Denka Seiken Co., Ltd., and is a co-founder of FluGen.

Conclusions: Of many human protective monoclonal antibodies against the HA stem, two human protective monoclonal antibodies were isolated in this study that predominantly recognize epitopes on the HA stem of group 2 and use unique IGHV3-66 and IGHV4-38-2 germline genes.

Keywords

Influenza A virus; human monoclonal antibody; HA stem; group 2

Introduction

The hemagglutinin (HA) protein of influenza A virus is the major antigenic protein on the virion. HA is phylogenetically divided into 18 subtypes (H1 to H18) that can be genetically separated to two groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15) (1, 2). 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 region and a stem region (3). Some anti-HA stem antibodies recognize several subtypes of HA because stem epitopes are highly conserved. However, very few anti-HA stem antibodies are normally present in human sera because the HA stem is sub-immunodominant.

Hetero-reactive human monoclonal antibodies against the HA stem are roughly classified into 2 types based on the reported epitopes. The first type of antibody includes CR6261 (4), F10 (5), and 3.1 (6), which recognize the group 1 HAs, and CR9114 (7), CT149 (8), 39.29 (9), FI6v3 (10), S9-1-10/5-1 (11), and MEDI8852 (12), which recognize the HAs of both group 1 and 2 and mainly target the α -helix A of HA2. The second type of antibodies includes CR8020 (13), CR8043 (14), 042-100809-2F04 (15), and 41-5E04 (16), which recognize the group 2 HAs and target the C-terminal portion of the fusion peptide and the β -sheet that precedes α -helix A of HA2. Most of these antibodies inhibit viral growth in vitro by inhibiting the conformational change of HA that is required for viral membrane fusion (17). Some of them, including S9-1-10/5-1, suppress virus growth in vitro by inhibiting virus particle release (11). Moreover, the anti-HA stem antibodies trigger antibody-dependent cellular cytotoxicity (ADCC), which affords effective protection in vivo (18–20).

Novel types of antivirals are needed because of concerns about the emergence of NA inhibitor-resistant viruses, including the highly pathogenic avian influenza H7N9 virus that was recently isolated from humans (21–23). One of the most promising approaches is the use hetero-reactive anti-HA stem antibodies. Such antibodies could suppress seasonal influenza H1N1pdm09 and H3N2 viruses, as well as zoonotic H5N1 and H7N9 viruses. Here, we attempted to obtain hetero-reactive human monoclonal anti-HA antibodies from an H3N2 virus-infected human.

Materials and methods

Ethics and biosafety statements.

Human blood was collected according to protocols that were approved by the Research Ethics Review Committee of the Institute of Medical Science, the University of Tokyo. Written informed consent was obtained from all participants.

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

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.

Cells.

Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium (MEM) containing 5% newborn calf serum (NCS). Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. These cells were incubated at 37 °C under 5% CO₂. Expi293F cells (Thermo Fisher Scientific), maintained in Expi293 expression medium (Thermo Fisher Scientific), were incubated on an orbital shaker platform rotating at 125 rpm at 37 °C under 8% CO₂.

Viruses.

A/California/04/2009 (CA04; H1N1pdm09), its mouse-adapted strain (MA-CA04) (24), mouse-adapted A/Aichi/2/68 (MA-Aichi; H3N2), A/Perth/16/2009 (Perth/16; H3N2), A/Vietnam/1203/2004 (VN1203; H5N1), A/geese/Egypt/0929-NLQP/2009 (H5N1), A/Anhui/1/2013 (Anhui/1; H7N9), and its mutant virus possessing 11 amino acid mutations in PA (PA-11Mut; H7N9) (25), were propagated in MDCK cells or eggs, and titrated by use of a plaque assay and/or TCID₅₀ (50% tissue culture infectious doses) values in MDCK cells.

Cell fusion and cloning.

Blood (5 ml) was obtained from a volunteer one week after the onset of natural H3N2 influenza virus infection. Peripheral blood mononuclear cells (PBMCs), prepared by centrifugation through Ficoll-Paque Plus (GE Healthcare), were fused with SPYMEG cells (MBL), which is a fusion partner cell line, and selected as previously described (11, 26). The first screening and cell cloning were performed by using an enzyme-linked immunosorbent assay (ELISA) with recombinant HAs [from A/Christchurch/16/2011 (H1N1pdm09), A/Victoria/361/2011 (H3N2), A/Egypt/N05056/2009 (H5N1), and A/Netherlands/219/2003 (H7N7)], which were purchased from Sino Biological, or with purified H3N2 virus (A/Tokyo/UT-IMS6-1/2013), as previously described (11).

Construction and expression of monoclonal human IgG.

Plasmids expressing human IgG consisted of each VH and VL sequence of the hybridoma clone and the constant gamma heavy (IgG₁) and lambda light chain coding sequences were

constructed using a Mammalian PowerExpress System (TOYOBO), as previously described (11). Determined nucleotide sequences of the variable region were analyzed and compared to sequences in the NCBI database by using the IgBlast software (<http://www.ncbi.nlm.nih.gov/igblast/>). Each plasmid encoding heavy and light chains was transfected into Expi293F cells by using ExpiFectamine 293 (Thermo Fisher Scientific) according to the manufacturer's protocol. At 4–6 days post-transfection, the human antibody in the culture media was purified by using a HiScreen MabSelect SuRe LX column (GE Healthcare) and the automated chromatography system ÄKTA pure 25 (GE Healthcare). The concentration of the purified antibodies was measured by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Reactivity of human mAbs.

293T cells were transfected with each plasmid expressing the H1- to H18-HA (11) by use of Trans-IT 293 (Takara bio). At 48 h post-transfection, the cells were fixed with 4% paraformaldehyde and then incubated with 1417infE21, 1417infC10, or FI6v3, which recognizes all HA subtypes (10), at 1 µg/ml, followed by a peroxidase-conjugated goat anti-human IgG, Fcγ Fragment-specific antibody (Jackson Immuno-Research). TMB (3,3',5,5'-Tetramethylbenzidine) solution was added to each well and incubated for 5 min at room temperature. The reaction was stopped by the addition of H₂SO₄. The optical density at 450 nm (OD₄₅₀) was measured by using a VersaMax plate reader (Molecular Devices). The OD₄₅₀ values of the mock-transfected wells incubated with each mAb was subtracted as background.

K_D determination.

K_D was determined by bio-layer interferometry (BLI) using an Octet Red 96 instrument (ForteBio), as reported previously (11). Briefly, recombinant HAs [from A/California/07/2009 (H1N1pdm09), Perth/16 (H3N2), A/Egypt/N05056/2009 (H5N1), and A/Netherlands/219/2003 (H7N7)], which were purchased from Sino Biological, were loaded onto Ni-NTA biosensors (ForteBio) and incubated with various concentrations of 1417infE21 or 1417infC10. Three to six concentrations of each antibody were used. The ratio of k_{on} to k_{off} determined the K_D reported here.

Virus neutralization assay.

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 TCID₅₀ 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. BSA-MEM containing TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin was added to each well and the cells were incubated for 3 days at 37 °C. The cytopathic effect (CPE) was examined, and antibody titers required to reduce virus replication by 50% (IC₅₀) were determined by using the Spearman-Kärber formula.

Selection of escape mutants.

Escape mutants were selected by culturing Perth/16 in the presence of 1417infE21 or 1417infC10, as described previously (11). Selections (n=2) were performed for 6–16 cycles to obtain escape mutants. The open reading frame of the HA of the escape mutants was directly sequenced.

Construction of plasmids.

The HA and NA genes of Perth/16 virus were cloned into the RNA polymerase I plasmid (27) by using a standard technique. Mutations in the HA gene were generated by PCR amplification of the respective RNA polymerase I plasmid with primers possessing the desired mutations (primer sequences available upon request). All constructs were sequenced to confirm the absence of unwanted mutations.

Virus rescue.

Plasmid-based reverse genetics for virus generation was performed as previously described (27). Two RNA polymerase I plasmids encoding the HA or NA gene of Perth/16 and six RNA polymerase I plasmids encoding the other six segments of A/Puerto Rico/8/34 (H1N1) were used. Each rescued virus was propagated in MDCK cells and stored as a stock virus. The titers of the stock viruses were determined in MDCK cells. Each HA gene of all rescued viruses was sequenced to confirm the absence of unwanted mutations.

Competitive binding assay.

Competitive binding was assessed by using an Octet Red 96 instrument, as reported previously (11). After binding of recombinant HA (A/Perth/16/2009; H3N2) to the Ni-NTA biosensors, the sensors were incubated with 1417infE21, 1417infC10, CR8020 (13), or F008–090 (28) (10 µg/ml) for 600 seconds, and were then incubated with a second antibody (1417infE21, 1417infC10, CR8020, or F008–090; 10 µg/ml) for 600 seconds.

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 per group were intraperitoneally injected with PBS or the indicated antibodies at a concentration of 30 mg/kg. One day later, the mice were anaesthetized and inoculated with 10 mouse lethal dose 50 (MLD₅₀) (50 µl) of the indicated viruses. Body weight and survival were monitored daily for 14 days. Mice with body weight loss of more than 25% of their pre-infection values were humanely euthanized.

Results

Acquisition of broadly reactive human anti-HA mAbs.

One week after the onset of H3N2 influenza virus infection, PBMCs were isolated and fused with fusion partner SPYMEG cells to obtain hybridomas that expressed human antibodies. On the basis of the results of the ELISA, positive hybridomas were cloned by limiting dilution. We established 2 hybridoma clones (1417infE21 and 1417infC10) that produced antibodies that reacted with at least 2 subtypes of the HAs tested (Table 1). By ELISA,

1417infE21 recognized H1-, H3-, and H7-HAs, whereas 1417infC10 recognized H3- and H7-HAs. Based on the nucleotide sequence of the VH and VL regions for these three human monoclonal antibodies (mAbs), the closest germline gene and the CDR3 sequence were determined by using the IgBlast software. Clones 1417infE21 and 1417infC10 used the IGHV3-66 and IGHV4-38-2 germline genes, respectively (Table 1). This result indicates that the two clones are derived from different B cell ancestors.

Reactivity of the three human monoclonal antibodies to the 18 HA subtypes.

We next attempted to determine the breadth of reactivity of the two mAbs by immunostaining using 293T cells transiently expressing the HA of different subtypes, that is, H1- through H18-HA. Both mAbs recognized all 6 HA subtypes belonging to group 2 (Table 2). 1417infE21 reacted with some group 1 HAs including H1-, H5-, H6-, and H8-HA, whereas 1417infC10 did not recognize any of the group 1 HAs (Table 2). These results indicate that the both mAbs primarily recognize HA subtypes that belong to group 2.

We next determined the binding kinetics of the two mAbs to recombinant HAs by bio-layer interferometry (BLI) using an Octet Red 96 instrument. Binding of 1417infE21 to H3-HA was 1.27×10^{-10} M (Table 3). 1417infE21 bound with approximately 10 times lower affinity (1.28 or 1.01×10^{-9} M) to H1- or H7-HA and approximately 100 times lower affinity (9.49×10^{-9} M) to H5-HA (Table 3). Binding of 1417infC10 to H3-HA was less than 1.0×10^{-12} M. 1417infC10 bound with lower affinity (6.48×10^{-8} M) to H7-HA. These data show that 1417infE21 preferentially recognizes group 2 HAs over group 1 HAs.

Neutralization activity of 1417infE21 and 1417infC10.

We next examined whether the two mAbs possess neutralization activity against H1N1pdm09, H3N2, H5N1, and H7N9 viruses in vitro. Briefly, 100 TCID₅₀ of A/California/04/2009 (H1N1pdm09), A/Perth/16/2009 (H3N2), A/geese/Egypt/0929-NLQP/2009 (H5N1), and A/Anhui/1/2013 (H7N9) viruses were incubated with serially two-fold-diluted antibodies and then inoculated to MDCK cells. The presence or absence of cytopathic effect (CPE) was examined at 3 days post-infection. 1417infE21 inhibited the propagation of H1N1pdm09, H3N2, and H7N9 viruses with 50% inhibitory concentration (IC₅₀) values of 50, 1.3, and 12.5 µg/ml, respectively, but failed to suppress the replication of H5N1 virus at the highest concentration tested (50 µg/ml) (Table 4). 1417infC10 neutralized H3N2 virus with an IC₅₀ value of 2.2 µg/ml, but failed to suppress the other three viruses tested.

Epitope analysis of 1417infE21 and 1417infC10.

Since 1417infE21 and 1417infC10 possessed neutralizing ability, we tried to map the epitopes of these mAbs by generating escape mutant H3N2 viruses. We obtained two escape mutants for each mAb by virus passages under antibody selection and analyzed the escape mutations by direct sequencing of their HA gene. We found that the mutant viruses that partially escaped from 1417infE21 had acquired V186I (H3 numbering throughout) and I374F substitutions, or G78D, I374S, and I522T substitutions. The mutant viruses that fully escaped from 1417infC10 possessed K62E, Q156H, V186G, E344K, V347M, and D489E substitutions, or I226N and G362D substitutions. To determine which substitutions mainly

contributed to the escape, we prepared mutant viruses possessing the various amino acid substitutions (listed in Table 5) by reverse genetics, and examined whether these mutant viruses could escape from the mAbs. The analysis revealed that the I374F and I374S substitutions, and the E344K and G362D substitutions, were important for escape from 1417infE21 and 1417infC10, respectively (Table 5 and Fig. 1A). Since CR8020 binds to the HA stem of group 2, the sensitivity of each virus to CR8020 was also examined. CR8020 failed to neutralize the mutant viruses that escaped from 1417infC10 but did suppress other viruses tested (Table 5). These results indicate that 1417infE21 and 1417infC10 likely recognize the HA stem. Moreover, the epitopes of 1417infE21 and 1417infC10 are not completely identical, and the epitopes of 1417infC10 and CR8020 are similar to each other.

To further examine whether these mAbs target the HA stem or head, we performed a competitive binding assay using recombinant H3-HA. 1417infE21 competed with 1417infC10 and CR8020, which recognizes the H3-HA stem, but not with F008–090, which recognizes the H3-HA head, for binding to H3-HA (Fig. 1B). 1417infC10 also competed with CR8020 and 1417infE21 but not with F008–090. CR8020 and F008–090 did not affect each other's binding to H3-HA. These results demonstrate that 1417infE21 and 1417infC10 recognize the HA stem.

In vivo protective efficacy of 1417infE21 and 1417infC10 in mice.

To evaluate the in vivo efficacy of 1417infE21 and 1417infC10, each mAb (at 30 mg/kg) or a control (i.e., the anti-B-HA 1429C6/3–3 or PBS), was intraperitoneally administrated to 4 mice per group, which were then challenged 1 day later via intranasal infection with 10 MLD₅₀ of mouse-adapted A/California/04/2009 (MA-CA04; H1N1pdm09), mouse-adapted A/Aichi/2/68 (MA-Aichi; H3N2), A/Vietnam/1203/2004 (VN1203; H5N1), or A/Anhui/1/2013 possessing 11 amino acid mutations in its PA (PA-11Mut; H7N9). The last virus was generated previously by using reverse genetics and showed higher mouse virulence than its wild-type counterpart (25). After challenge with MA-CA04, MA-Aichi, or PA-11Mut, most of the mice that received 1417infE21 survived for the 14-day observation period with no or transient body weight loss (Fig. 2). However, 1417infE21 protected only one of four mice from lethal infection with VN1203. In the case of 1417infC10, most of the mice that were challenged with MA-Aichi or PA-11Mut survived with various degrees of body weight loss, whereas all mice challenged with MA-CA04 or VN1203 died. All mice that received PBS or 1429C6/3–3 died within 11 days of challenge. These data demonstrate that 1417infE21 and 1417infC10 have in vivo protective efficacy against lethal infection with H3N2 and H7N9 viruses.

Discussion

Here we report the characterization of two novel hetero-reactive human monoclonal antibodies (mAbs) that mainly reacted with group 2 HAs. 1417infE21 and 1417infC10 possessed both in vitro neutralization activity against H3N2 virus and in vivo protective efficacy against H3N2 and H7N9 viruses in a mouse infection model. Furthermore, 1417infE21 protected mice from lethal infection with H1N1pdm09 virus, which belongs to group 1. CR8020, CR8043, 042-100809-2F04, and 41-5E04 have been reported as human

protective mAbs against the HA stem of group 2 (13–16). Recently, many human cross-reactive mAbs against the HA stem of group 2 have been reported but their precise epitopes have not been determined (29). Since this class of human mAbs is understudied relative to human mAbs against the HA stem of group 1 or both groups, our findings contribute to our understanding of cross-reactive mAbs against the HA stem. CR8020, CR8043, 042-100809-2F04, and 41-5E04 are derived from the IGHV1-18, IGHV1-3, IGHV3-23, and IGHV3-53 genes, respectively (13–15), whereas 1417infE21 and 1417infC10 used the IGHV3-66 and IGHV4-38-2 genes, respectively. IGHV1-18 and IGHV3-53, together with IGHV6-1 and IGVH1-2, are major germline genes for broadly reactive mAbs against the HA stem after H7N9 vaccination (29). IGHV3-23, which is used by PN-SIA49 (30), FB110 (31), 31.a.83 (32), 1E1, and 1F2 (33), is among the most frequently identified genes from broadly protective human antibodies. IGHV1-3 is unique for CR8042 among the human anti-HA stem mAbs. This is thus the first report to demonstrate that the unique IGHV3-66 and IGHV4-38-2 genes are responsible for the hetero-protective human monoclonal antibodies against the HA stem.

Escape mutations (E344K and G362D) for 1417infC10 were mapped to the C-terminal portion of the fusion peptide and the β -sheet that precedes α -helix A of HA2, which are the epitopes of the protective human mAbs against the group 2 HAs, CR8020 (13), CR8043 (14), and 042-100809-2F04 (15). In addition, CR8020 failed to neutralize the mutant virus that escaped from 1417infC10, indicating that both mAbs recognize an overlapping epitope. The broadly neutralizing mouse mAb 9H10, which targets the group 2 HA stem, also recognizes a similar region (34). Therefore, the C-terminal portion of the fusion peptide and the β -sheet that precedes the α -helix A of HA2 form the major common epitope among the group 2 HAs. In contrast, the escape mutations (I374F and I374S) for 1417infE21 mapped to α -helix A of HA2, which is usually targeted by the broadly protective human mAbs against the group 1 HA stem, CR6261 (4), F10 (5), and 3.1 (6), or against the group 1 and 2 HA stem, CR9114 (7), CT149 (8), 39.29 (9), FI6v3 (10), S9-1-10/5-1 (11), and MEDI8852 (12). Since the epitope of 1417infE21 is similar to that preferred by human mAbs that bind to group 1 or to both groups, 1417infE21 may recognize several group 1 HAs as well as all group 2 HAs. Collectively, based on their epitopes, 1417infC10 is an authentic mAb against the group 2 HA stem, and 1417infE21 is a novel type of mAb targeting the group 2 HA stem. An understanding of the precise epitope of both mAbs through co-crystallization will further our understanding of these broadly reactive human antibodies.

Several broadly protective human anti-HA stem antibodies were isolated from volunteers who were vaccinated with H5N1 pre-pandemic or H1N1pdm09 pandemic vaccine (11, 32, 35), or who infected with H1N1pdm09 or H5N1 virus (8, 10, 36). Since these viruses possessed distinct antigenicity from seasonal H1N1 and H3N2 viruses, antibodies against the HA stem were efficiently elicited (35, 37, 38). However, memory B cells against the HA stem generally appear with low frequency after seasonal vaccination or infection (35, 39). Here we isolated 1417infE21 and 1417infC10 from a human volunteer who was naturally infected with H3N2 virus, indicating that natural infection with H3N2 virus can elicit hetero-reactive antibodies against the HA stem. In our study, the antibody responses against the HA stem were likely enhanced by antigenic drift since the epitopes in the HA stem remained unchanged after antigenic drift, as previously suggested (40). Further analysis of

the relationship between antigenic drift and anti-stem antibody response may be useful in the development of a vaccine that targets the HA stem.

1417infE21 and 1417infC10 protected mice from lethal infection with H1N1pdm09 and H7N9 viruses, respectively, even though these mAbs did not have high in vitro neutralization activity against these viruses. These results indicate that Fc γ receptor-mediated antibody-dependent cellular cytotoxicity (ADCC) may play a role in this protection because an interaction between the antibody and the Fc γ receptor is sometime required for the in vivo protection against influenza A virus afforded by some anti-HA stem antibodies (18, 20, 41). Elucidation of other cellular factors involved in the antibody-mediated protection against influenza A virus infection would contribute to the development of an effective vaccine.

In summary, we showed that hetero-reactive human mAbs, which use a novel IGVH gene, suppress influenza A viruses that possess group 2 HA in vitro and in vivo. These data provide experimental evidence of the antiviral potential of these mAbs and provide the basis for clinical testing.

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References

1. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev.* 1992 3;56(1):152–79. [PubMed: 1579108]
2. Wu Y, Wu Y, Tefsen B, Shi Y, Gao GF. Bat-derived influenza-like viruses H17N10 and H18N11. *Trends in microbiology.* 2014 4;22(4):183–91. [PubMed: 24582528]
3. Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature.* 1981 1 29;289(5796):366–73. [PubMed: 7464906]
4. Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, et al. Antibody recognition of a highly conserved influenza virus epitope. *Science.* 2009 4 10;324(5924):246–51. [PubMed: 19251591]
5. Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol.* 2009 3;16(3):265–73. [PubMed: 19234466]
6. Wyrzucki A, Dreyfus C, Kohler I, Steck M, Wilson IA, Hangartner L. Alternative recognition of the conserved stem epitope in influenza A virus hemagglutinin by a VH3–30-encoded heterosubtypic antibody. *Journal of virology.* 2014 6;88(12):7083–92. [PubMed: 24719426]
7. Dreyfus C, Laursen NS, Kwaks T, Zuijgeest D, Khayat R, Ekiert DC, et al. Highly conserved protective epitopes on influenza B viruses. *Science.* 2012 9 14;337(6100):1343–8. [PubMed: 22878502]

8. Wu Y, Cho M, Shore D, Song M, Choi J, Jiang T, et al. A potent broad-spectrum protective human monoclonal antibody crosslinking two haemagglutinin monomers of influenza A virus. *Nat Commun.* 2015;6:7708. [PubMed: 26196962]
9. Nakamura G, Chai N, Park S, Chiang N, Lin Z, Chiu H, et al. An in vivo human-plasmablast enrichment technique allows rapid identification of therapeutic influenza A antibodies. *Cell Host Microbe.* 2013 7 17;14(1):93–103. [PubMed: 23870317]
10. Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science.* 2011 8 12;333(6044):850–6. [PubMed: 21798894]
11. Yamayoshi S, Uraki R, Ito M, Kiso M, Nakatsu S, Yasuhara A, et al. A Broadly Reactive Human Anti-hemagglutinin Stem Monoclonal Antibody That Inhibits Influenza A Virus Particle Release. *EBioMedicine.* 2017 3;17:182–91. [PubMed: 28286060]
12. Kallewaard NL, Corti D, Collins PJ, Neu U, McAuliffe JM, Benjamin E, et al. Structure and Function Analysis of an Antibody Recognizing All Influenza A Subtypes. *Cell.* 2016 7 28;166(3):596–608. [PubMed: 27453466]
13. Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, et al. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science.* 2011 8 12;333(6044):843–50. [PubMed: 21737702]
14. Friesen RH, Lee PS, Stoop EJ, Hoffman RM, Ekiert DC, Bhabha G, et al. A common solution to group 2 influenza virus neutralization. *Proceedings of the National Academy of Sciences of the United States of America.* 2014 1 7;111(1):445–50. [PubMed: 24335589]
15. Henry Dunand CJ, Leon PE, Kaur K, Tan GS, Zheng NY, Andrews S, et al. Preexisting human antibodies neutralize recently emerged H7N9 influenza strains. *The Journal of clinical investigation.* 2015 3 2;125(3):1255–68. [PubMed: 25689254]
16. Henry Dunand CJ, Leon PE, Huang M, Choi A, Chromikova V, Ho IY, et al. Both Neutralizing and Non-Neutralizing Human H7N9 Influenza Vaccine-Induced Monoclonal Antibodies Confer Protection. *Cell Host Microbe.* 2016 6 08;19(6):800–13. [PubMed: 27281570]
17. Brandenburg B, Koudstaal W, Goudsmit J, Klaren V, Tang C, Bujny MV, et al. Mechanisms of hemagglutinin targeted influenza virus neutralization. *PLoS One.* 2013;8(12):e80034. [PubMed: 24348996]
18. DiLillo DJ, Tan GS, Palese P, Ravetch JV. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. *Nat Med.* 2014 2;20(2):143–51. [PubMed: 24412922]
19. Jegaskanda S, Reading PC, Kent SJ. Influenza-specific antibody-dependent cellular cytotoxicity: toward a universal influenza vaccine. *J Immunol.* 2014 7 15;193(2):469–75. [PubMed: 24994909]
20. He W, Tan GS, Mullarkey CE, Lee AJ, Lam MM, Krammer F, et al. Epitope specificity plays a critical role in regulating antibody-dependent cell-mediated cytotoxicity against influenza A virus. *Proceedings of the National Academy of Sciences of the United States of America.* 2016 10 18;113(42):11931–6. [PubMed: 27698132]
21. Zhu W, Zhou J, Li Z, Yang L, Li X, Huang W, et al. Biological characterisation of the emerged highly pathogenic avian influenza (HPAI) A(H7N9) viruses in humans, in mainland China, 2016 to 2017. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin.* 2017 5 11;22(19).
22. Ke C, Mok CKP, Zhu W, Zhou H, He J, Guan W, et al. Human Infection with Highly Pathogenic Avian Influenza A(H7N9) Virus, China. *Emerging infectious diseases.* 2017 7;23(8):1332–40. [PubMed: 28580899]
23. Zhang F, Bi Y, Wang J, Wong G, Shi W, Hu F, et al. Human infections with recently-emerging highly pathogenic H7N9 avian influenza virus in China. *The Journal of infection.* 2017 7;75(1):71–5. [PubMed: 28390707]
24. Sakabe S, Ozawa M, Takano R, Iwastuki-Horimoto K, Kawaoka Y. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. *Virus Res.* 2011 6;158(1–2):124–9. [PubMed: 21458512]

25. Yamayoshi S, Yamada S, Fukuyama S, Murakami S, Zhao D, Uraki R, et al. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses. *Journal of virology*. 2014 3;88(6):3127–34. [PubMed: 24371069]
26. Kubota-Koketsu R, Mizuta H, Oshita M, Ideno S, Yunoki M, Kuhara M, et al. Broad neutralizing human monoclonal antibodies against influenza virus from vaccinated healthy donors. *Biochem Biophys Res Commun*. 2009 9 11;387(1):180–5. [PubMed: 19580789]
27. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. *Proceedings of the National Academy of Sciences of the United States of America*. 1999 8 03;96(16):9345–50. [PubMed: 10430945]
28. Okada J, Ohshima N, Kubota-Koketsu R, Ota S, Takase W, Azuma M, et al. Monoclonal antibodies in man that neutralized H3N2 influenza viruses were classified into three groups with distinct strain specificity: 1968–1973, 1977–1993 and 1997–2003. *Virology*. 2010 2 20;397(2):322–30. [PubMed: 19969317]
29. Andrews SF, Joyce MG, Chambers MJ, Gillespie RA, Kanekiyo M, Leung K, et al. Preferential induction of cross-group influenza A hemagglutinin stem-specific memory B cells after H7N9 immunization in humans. *Science immunology*. 2017 7 14;2(13).
30. Burioni R, Canducci F, Mancini N, Clementi N, Sassi M, De Marco D, et al. Monoclonal antibodies isolated from human B cells neutralize a broad range of H1 subtype influenza A viruses including swine-origin Influenza virus (S-OIV). *Virology*. 2010 3 30;399(1):144–52. [PubMed: 20096915]
31. Corti D, Suguitan AL Jr., Pinna D, Silacci C, Fernandez-Rodriguez BM, Vanzetta F, et al. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *The Journal of clinical investigation*. 2010 5;120(5):1663–73. [PubMed: 20389023]
32. Joyce MG, Wheatley AK, Thomas PV, Chuang GY, Soto C, Bailer RT, et al. Vaccine-Induced Antibodies that Neutralize Group 1 and Group 2 Influenza A Viruses. *Cell*. 2016 7 28;166(3):609–23. [PubMed: 27453470]
33. Hu W, Chen A, Miao Y, Xia S, Ling Z, Xu K, et al. Fully human broadly neutralizing monoclonal antibodies against influenza A viruses generated from the memory B cells of a 2009 pandemic H1N1 influenza vaccine recipient. *Virology*. 2013 1 20;435(2):320–8. [PubMed: 23084424]
34. Tan GS, Lee PS, Hoffman RM, Mazel-Sanchez B, Krammer F, Leon PE, et al. Characterization of a broadly neutralizing monoclonal antibody that targets the fusion domain of group 2 influenza A virus hemagglutinin. *Journal of virology*. 2014 12;88(23):13580–92. [PubMed: 25210195]
35. Li GM, Chiu C, Wrammert J, McCausland M, Andrews SF, Zheng NY, et al. Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2012 6 5;109(23):9047–52. [PubMed: 22615367]
36. Kashyap AK, Steel J, Oner AF, Dillon MA, Swale RE, Wall KM, et al. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proceedings of the National Academy of Sciences of the United States of America*. 2008 4 22;105(16):5986–91. [PubMed: 18413603]
37. Ellebedy AH, Krammer F, Li GM, Miller MS, Chiu C, Wrammert J, et al. Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. *Proceedings of the National Academy of Sciences of the United States of America*. 2014 9 09;111(36):13133–8. [PubMed: 25157133]
38. Wrammert J, Koutsouanos D, Li GM, Edupuganti S, Sui J, Morrissey M, et al. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *The Journal of experimental medicine*. 2011 1 17;208(1):181–93. [PubMed: 21220454]
39. Andrews SF, Huang Y, Kaur K, Popova LI, Ho IY, Pauli NT, et al. Immune history profoundly affects broadly protective B cell responses to influenza. *Science translational medicine*. 2015 12 02;7(316):316ra192.
40. Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, et al. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. *Journal of virology*. 2013 4;87(8):4728–37. [PubMed: 23408625]

41. DiLillo DJ, Palese P, Wilson PC, Ravetch JV. Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. *The Journal of clinical investigation*. 2016 2;126(2):605–10. Epub 2016/01/06. eng. [PubMed: 26731473]

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Highlights

- We isolated two hetero-protective anti-HA stem antibodies.
- These antibodies suppress group 2 influenza A viruses in vitro and in vivo.
- They recognize epitopes on the HA stem.

Figure 1. Epitope analysis on H3-HA.

(A) Mutations found in H3-HA after passages of Perth/16 in the presence of 1417infE21 (cyan) or 1417infC10 (green). Perth/16 was passaged in the presence of various concentrations of 1417infE21 or 1417infC10 in MDCK cells. After 6–16 passages, the HA sequence of the escape mutant viruses was analyzed and the identified escape mutations were mapped onto the 3D structure of the H3-HA trimer by using the molecular graphics system PyMOL. Two independent experiments were performed. Mutations are shown with H3 numbering. (B) Competitive binding of 1417infE21 or 1417infC10 to H3-HA. Additional binding of the indicated human mAbs to the immobilized H3-HA saturated with 1417infE21, 1417infC10, CR8020, or F008-090, was measured by means of bio-layer interferometry.

Figure 2. In vivo protective efficacy in mice.

Four mice per group were intraperitoneally injected with PBS or the indicated antibodies at 30 mg/kg. One day later, the mice were challenged with 10 MLD₅₀ of MA-CA04 (H1N1pdm09), MA-Aichi (H3N2), VN1203 (H5N1), or PA-11Mut (H7N9) virus. Body weight and survival were monitored daily for 14 days. 1429C6/3-3, which recognizes influenza B virus HA, and PBS served as negative controls.

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Table 1.

Genetic hallmarks of the human mAbs.

mAb	Heavy chain		Light chain	
	VH	CDR3	VL	CDR3
1417infE21	IGHV3-66*01	ARVPQLRFFDWPNYAMDV	IGLV2-8*01	SSHSGSSNLWV
1417infC10	IGHV4-38-2*02	AGTNPWEQNYGEGLDP	IGLV1-51*01	GTWDRSLSVGL

Table 2.

Reactivity of the human mAbs with HA expressed in cells

mAb	Group 1					Group 2													
	H1	H2	H5	H6	H8	H9	H11	H12	H13	H16	H17	H18	H3	H4	H7	H10	H14	H15	
1417infE21	++ ^a	-	+	+	+	-	-	-	-	-	-	-	++	+	+	+	+	+	
1417infC10	-	-	-	-	-	-	-	-	-	-	-	-	++	+	+	++	+	+	
Fl6v3	++	++	++	++	++	++	+	++	+	+	++	++	++	++	++	+	+	++	

^aReactivity of each mAb (1 µg/ml) was stratified according to optical density at 450 nm, ++ (>0.5), + (0.1–0.5), and – (<0.1).

Table 3.

Dissociation constants of the human mAbs with recombinant HAs.

Subtype	Virus strain	Kd value (M)	
		1417infE21	1417infC10
H1N1pdm09	A/California/07/2009	1.28×10^{-9}	NT ^a
H3N2	A/Perth/16/2009	1.27×10^{-10}	$<1.0 \times 10^{-12}$
H5N1	A/Egypt/N05056/2009	9.49×10^{-9}	NT
H7N9	A/Netherland/219/2003	1.01×10^{-9}	6.48×10^{-8}

^aNT indicates not tested.

Table 4.

IC50 values (µg/ml) of the human mAbs

Subtype	Virus strain	1417infE21	1417infC10
H1N1pdm09	A/California/04/2009	50	>50
H3N2	A/Perth/16/2009	1.3	2.2
H5N1	A/geese/Egypt/0929-NLQP/2009	>50	>50
H7N9	A/Anhui/1/2013	12.5	>50

Table 5.IC₅₀ values (µg/ml) of the human mAbs against mutant viruses

Virus strain tested 1	1417infE21	1417infC10	CR8020
Wild-type	0.8	0.9	0.8
HA-VI86I/1374F	17.7	2.2	0.6
HA-V186I	1.9	4.4	1.3
HA-I374F	12.5	2.2	0.8
HA-G78D/I374S/I522T	14.9	1.3	0.9
HA-G78D	1.6	1.6	0.7
HA-I374S	35.4	1.9	1.1
HA-I522T	1.9	3.1	1.1
HA-K62E/Q156H/H183L/V186G/E344K/V347M/D489E	0.6	>50	>50
HA-K62E/Q156H/H183L/V186G	0.7	0.9	0.66
HA-E344K	0.9	14.9	>50
HA-V347M	1.6	2.2	1.6
HA-E344K/V347M	0.6	>50	>50
HA-D489E	2.2	3.1	1.1
HA-I226N/G362D	0.6	>50	>50
HA-I226N	1.6	3.1	0.9
HA-G362D	0.5	10.5	>50