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A novel neutralizing antibody against diverse clades of H5N1 influenza virus and its mutants capable of airborne transmission

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ABSTRACT

Highly pathogenic avian influenza A virus H5N1 continues to spread among poultry and has frequently broken the species barrier to humans. Recent studies have shown that a laboratory-mutated or reassortant H5N1 virus bearing hemagglutinin (HA) with as few as four or five mutations was capable of transmitting more efficiently via respiratory droplets between ferrets, posing a serious threat to public health and underscoring the priority of effective vaccines and therapeutics. In this study, we identified a novel monoclonal antibody (mAb) named HAb21, that has a broadly neutralizing activity against all tested strains of H5N1 covering clades 0, 1, 2.2, 2.3.4, and 2.3.2.1. Importantly, HAb21 efficiently neutralized diverse H5N1 variants with single or combination forms of mutations capable of airborne transmission. We demonstrated that HAb21 blocked viral entry during the receptor-binding step by targeting a previously uncharacterized epitope at the tip of the HA head. This novel epitope closely neighbors the receptor-binding site (RBS) and the interface of HA trimer and is highly conserved among divergent H5N1 strains. Our studies provide a new tool for use either for therapeutic purposes or as a basis of vaccine development.

1. Introduction

Infection of the highly pathogenic avian influenza (HPAI) H5N1 virus continues to be a serious threat to public health worldwide. As of 24 January 2014, there have been 650 confirmed human H5N1 cases, resulting in 386 deaths (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Although most the human cases have likely been transmitted from avian species, the high frequency of naturally-occurring mutations and reassortment may adapt new H5N1 strains capable of human to human transmission (Russell et al., 2012; Stevens et al., 2006; Yamada et al., 2006). This possibility has been underlined by recent studies that few mutations could confer H5N1 virus capable of airborne transmission (Herfst et al., 2012; Imai et al., 2012; Russell et al., 2012). As reported, the A/Indonesia/5/2005 avian A/H5N1 influenza virus required as few as five amino acid substitutions (Herfst et al., 2012) and the A/Vietnam/1203/2004 A/H5N1 influenza virus required four substitutions and reassortment (Imai et al., 2012), to become transmissible via respiratory droplets between ferrets, the best animal model for humans. Although these studies triggered a keen debate over global biosecurity and biosafety (Berns et al., 2012a,b; Fouchier et al., 2012a,b,c; Le Duc and Franz, 2012), the results did reveal specific HA mutations that drive receptor conversion from the more avian-like α2-3-linked sialic acid to the more human-like α2-6-linked sialic acid thus alarming a potential risk for human H5N1 pandemic (Herfst et al., 2012; Imai et al., 2012; Russell et al., 2012). Therefore, there is a strong need to develop effective countermeasures especially antiviral drugs and vaccines in preparedness, and to this end it is highly important to further explore the immunological properties of H5N1 virus and its mutants.

The hemagglutinin (HA) glycoprotein of influenza A virus (IAV) plays an essential role for viral infection by mediating viral binding to target cells and subsequent fusion between viral and cellular membranes (Caton et al., 1982; Stevens et al., 2006; Wilson et al., 1981). It is initially synthesized as a precursor polypeptide (HA0) and then cleaved into disulfide-linked HA1 and HA2 subunits. The HA1 subunit forms a globular head that contains the

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receptor-binding site (RBS), while the HA2 subunit forms a helix-rich stem that anchors the cell membrane. It is now known that both HA1 and HA2 of IAVs are major antigens to induce neutralizing antibodies during viral infection or immunization thus serving as the most important targets for developing antiviral vaccines and therapeutics (Li and Poon, 2009; Yen and Peiris, 2009). Among 16 subtypes of HA, only a few have been finely characterized with respect to their antigenic structures, and this significantly hampers the development for HA-based antiviral approaches. By selecting escape mutants against a panel of monoclonal antibodies (mAbs), three antigenic sites of H5N1 HA were initially characterized and they overlapped exclusively with the antigenic sites A and B of H3 HA or the site Sa of H1 HA (Ha et al., 2001, 2002; Kaverin et al., 2007, 2002). Several novel epitopes in HA1 or HA2 of H5N1 have recently been identified by new mAbs derived from H5N1-infected individuals or immunized animals (Du et al., 2013a; Ekiert et al., 2009; Hu et al., 2012; Oh et al., 2010; Qian et al., 2013; Simmons et al., 2007; Sui et al., 2009; Sun et al., 2009). Notably, a number of newly-isolated mAbs were mapped to the conserved epitopes that overlap the RBS in HA1 head (Cao et al., 2012; Du et al., 2013a; Hu et al., 2012; Khurana et al., 2009; Oh et al., 2010; Simmons et al., 2007) or around the fusion peptide region in HA2 stem (Corti et al., 2011; Ekiert et al., 2009; Sui et al., 2009).

Besides the high values for dissecting the antigenic structure, mAbs with potent inhibitory activity are generally considered to be more effective and specific (safer) as candidate therapeutics and prophylactics (Corti et al., 2011; Ekiert et al., 2009, 2011; Hu et al., 2012; Simmons et al., 2007; Sui et al., 2009; Yoshida et al., 2009; Yu et al., 2008). The successful treatment with convalescent plasma for H5N1-infected patients did emphasize an antibody-based treatment approach (Luke et al., 2006; Zhou et al., 2007). It is agreed that passive immunotherapy with neutralizing mAbs can provide immediate protection against H5N1 infection thereby saving lives of severely ill patients. In this study, we identified a broadly H5N1-neutralizing mAb, named HAb21. The present data showed that it targets a novel conserved conformation-dependent epitope in the head region of HA protein and can efficiently block the receptor-binding step. Promisingly, HAb21 potently neutralized diverse clades and subclades of H5N1 viruses and those H5N1 variants carrying single or combination forms of mutations responsible for the above-noted airborne transmission. We believe that this novel mAb can serve as a vital tool for developing anti-H5N1 vaccines and immunotherapeutics.

2. Materials and methods

2.1. Recombinant HA proteins and peptides

A baculovirus transfer vector (pAcGP67-A) that expresses the ectodomain of HA from A/Vietnam/1203/04 avian A/H5N1 virus was kindly provided by Dr. James Stevens in the Department of Molecular Biology, the Scripps Research Institute (La Jolla, CA). A foldon trimerizing sequence from T4 bacteriophage and a hexa His-tag were fused to the C-terminus of HA so that the engineered protein was produced at a largely trimeric form, designated as Tri-HA (Stevens et al., 2006). Transfection and virus amplification were carried out according to the baculovirus expression system manual (BD Biosciences, San Jose, CA). Soluble Tri-HA proteins were purified from cell culture supernatants by metal affinity chromatography using Ni–NTA resin (Qiagen Inc., Valencia, CA).

A panel of plasmids expressing truncated HA proteins of the A/Vietnam/1203/04 H5N1 virus (according to mature H5 HA numbering), including HA1, HA2, HA1–130 (a.a. 1–130), HA100–250 (a.a. 100–250), HA230–346 (a.a. 230–346) fused with the Fc portion of human IgG1 were constructed. The fusion proteins were expressed in 293T cells by transfection and purified by Protein A Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ). A set of recombinant HA proteins derived from the A/Vietnam/1194/04 (VN04-HA), A/Common magpie/HongKong/2256/06 (HK06-HA), A/Hongkong/213/03 (HK03-HA), A/Anhui/1/05(AH05-HA), A/Qinghai/14/08 (QH08-HA) were purchased from the Sino Biological Inc., Beijing. 77 overlapping peptides that span the ectodomain of HA protein of the A/H5N1/Vietnam/1203/2004, range from 15–20 amino acids in length, were synthesized as described previously (Cao et al., 2012).

2.2. Generation of HA-specific mAbs

Mouse immunization and mAb production were performed according our protocols described previously (He et al., 2006). In brief, four BALB/c mice (6 weeks old) were subcutaneously vaccinated with 20 μg of Tri-HA proteins (resuspended in phosphate-buffered saline, PBS) in the presence of MLP + TDM adjuvant (Sigma, Saint Louis, MO) and boosted three times with 10 μg of the same antigen and adjuvant at 3-week intervals. After a high titer of anti-HA antibodies were developed, the splenocytes from immunized mice were harvested and fused with SP2/0 myeloma cells. Cell culture supernatants from the wells containing hybridoma colonies were screened by an enzyme-linked immunosorbent assay (ELISA) using the Tri-HA as a coating antigen. Positive cells were expanded, retested and subcloned to generate stable hybridoma cell lines. MAbs were purified from culture supernatants using Potein A Sepharose 4 Fast Flow (GE Healthcare). The isotypes of mAbs were determined with a mouse monoclonal antibody isotyping kit (SouthernBiotech, Birmingham, AL).

Human anti-HA1 mAbs, AVFluIgG01 and AVFluIgG03, and mouse anti-HA2 mAb 9G1G9 (provided by the Sino Biological Inc., Beijing) were previously described (Cao et al., 2012).

2.3. Live H5N1-based neutralization assay

A live-virus-based neutralization assay was performed according to the protocol described previously (Du et al., 2013a). Briefly, Madin Darby canine kidney (MDCK) cells were seeded in 96-well cell culture plate at 2×10^4 per well in 100 μl of MEM growth medium and the plate was then incubated at 37 °C overnight. Next day, serially twofold diluted mAb was incubated with 100 50% tissue culture infective doses ($TCID_{50}$) of H5N1 strains at 37 °C for 1 h. The tested virus strains included A/Vietnam/1194/04, A/HongKong/483/97 and A/Shenzhen/406H/06. 100 μl of neutralized samples were then transferred into a monolayer of MDCK cells and the cell culture plates were incubated at 37 °C for 48 h. Virus infectivity was recorded by the presence of cell cytopathic effect (CPE) and the titer was calculated by the Reed–Muench method (Reed and Muench, 1938).

2.4. Hemagglutination inhibition (HI) assay

HI assay was performed to verify the inhibitory ability of mAbs. Briefly, mAb was twofold serially diluted in triplicates in 96-well U-bottom plates (Greiner bio-one, Germany). The H5N1 virus, A/HongKong/483/97 or A/Vietnam/1194/04, was adjusted to 8 HAU/50 μl and added into each well. The plates were incubated at 37 °C for 1 h followed by the addition 50 μl per well of 0.5% turkey erythrocytes. The plates were then incubated at room temperature for 1 h. The HI titer was determined as the highest dilution that completely inhibited hemagglutination. Negative control was included in each plate.

2.5. Construction of plasmids expressing diverse IAV HAs

A panel of 13 plasmids that express diverse full-length HAs of IAVs were constructed by using pcDNA3.1 vector (Invitrogen, Carlsbad, CA), including the HAs of H5N1 (A/Hong Kong/156/1997, A/Vietnam/1203/2004, A/Xinjiang/1/2006, A/Qinghai/59/2005, A/Anhui/1/2005, A/Shenzhen/406H/2006 (clade 2.3.4), A/Hubei/1/2010; the HAs of H1N1 (A/South Carolina/1/1918, A/Brisbane/59/2007, A/California/06/2009), and the HAs of newly emerging H7N9 (A/Shanghai/1/2013, A/Anhui/1/2013). A panel of 13 plasmids expressing HA mutants of the A/Qinghai/59/05, including single (N154D, N220K, Q222L, T315I), or combination forms (N154D/N220K, N154D/Q222L, N154D/T315I, N220K/Q222L, N220K/T315I, N154D/N220K/Q222L, N154D/N220K/T315I, N154D/Q222L/T315I, N154D/N220K/Q222L/T315I) of mutations were described previously (Du et al., 2013b). A control plasmid expressing the G protein of vesicular stomatitis G protein (VSV-G) was previously described (Qian et al., 2013).

2.6. Pseudovirus-based neutralization assay

Lentivirus-based pseudoviruses were generated as our previously described protocols (Cao et al., 2012). Briefly, 293T cells were co-transfected with three plasmids, including a backbone plasmid encoding an Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE), a HA-expressing plasmid, and a H5N1 neuraminidase (NA)-expressing plasmid. H5N1 pseudovirus-containing supernatants were harvested 48 h posttransfection and used for single-cycle infection of MDCK cells. Briefly, MDCK cells were plated at 10^5 cells/well in 96-well tissue culture plates and grown overnight. The pseudoviruses were preincubated with serially diluted mAbs at 37 °C for 1 h before being added to cells. The culture was refed with fresh medium 24 h later and incubated for an additional 48 h. Cells were washed with PBS and lysed using cell lysis buffer included in a luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well luminometer plates (Corning Inc., Corning, NY), followed by addition of luciferase substrate (Promega). Relative luciferase activity was determined immediately and the percent neutralization and 50% inhibitory concentration (IC_{50}) were calculated.

2.7. Virus binding assay

A virus binding assay was performed by using the A/Vietnam/1203/04 H5N1 pseudovirus as previously described (Cao et al., 2012). Briefly, virus was first incubated with serially diluted mAbs in DMEM containing 1% BSA at 4 °C overnight. MDCK cells were seeded in 24-well plates and grew 24 h at a monolayer. The mixture of pseudovirus and mAb was inoculated onto MDCK cells and incubated at 4 °C for 2 h, followed by rinsing the cells four times with PBS containing 1% BSA to remove unbound viruses. Then, the cells were lysed in 250 μl of DMEM using a freeze–thaw method. The amount of cell-bound viruses in the presence or absence of mAbs was quantified by an HIV-1 p24 ELISA kit (Vironostika HIV-1 antigen microelisa system, Biomerieux). Percent inhibition and IC_{50} were calculated.

2.8. Postattachment assay

A postattachment assay was performed as previously described (Cao et al., 2012). Briefly, the A/Vietnam/1203/04 pseudovirus was added to MDCK monolayer in a 96-well plate and incubated at 4 °C for 6 h. The unbound viruses were removed by washing cells four times with cold PBS. A tested mAb was serially diluted and added to the monolayer at 4 °C for 2 h, followed by washing two times. After addition of fresh DMEM, the cells were incubated at 37 °C for 72 h. The luciferase activity of the cells was measured by using the luciferase assay system (Promega). The percent inhibition and IC_{50} were calculated.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Reactivity of mAbs with various HA antigens was determined by a standard ELISA. Briefly, a recombinant protein (Tri-HA, HA1, HA2 or truncated HA1 fragments) was diluted at 1 μg/ml in 0.1 M carbonate buffer (pH 9.6) and coated onto 96-well microtiter plates at 4 °C overnight. The plates were blocked with 3% BSA for 1 h at room temperature and then washed with PBS–0.05% Tween 20 (PBS–T). A tested mAb (diluted at 10 μg/ml) was added into wells and incubated at 37 °C for 1 h, followed by three washes with PBS–T. Bound antibodies were detected with HRP-conjugated goat anti-mouse or human IgG (Sigma) at 37 °C for 1 h, followed by three washes. The reaction was visualized by addition of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma), and stopped with 2 M H_2SO_4. The absorbance at 450 nm was measured by an ELISA plate reader (Bio-Rad, Hercules, CA).

To detect the reactivity of mAbs with denatured HA proteins, the ELISA plates were coated with Tri-HA proteins at a concentration of 1 μg/ml and then treated with dithiothreitol (DTT) at a concentration of 10 mM for 1 h at 37 °C. The plate wells were then treated with 50 mM iodoacetamide for 1 h at 37 °C. After three washes, a regular ELISA was performed as described above.

2.10. Peptide library and biopanning

A random phage display library (Ph.D.-12), wherein the displayed peptides (12-mer) are fused to the N terminus of gIII protein, was purchased from New England Biolabs (Beverly, MA). Affinity selection of the phage clones from the library was performed according to the manufacturer's instructions. Briefly, the ELISA plates were coated with 0.1 μg/ml mAb (HAb21) in 0.1 M $NaHCO_3$ buffer (pH 8.6) at 4 °C overnight and blocked with 0.5% BSA in $NaHCO_3$ buffer for 1 h at 37 °C. After six washes with TBS–T, the phage library (1×10^{11}) was added into the coated wells and incubated at room temperature for 1 h. The wells were extensively washed with TBS–T for 10 times and the bound phages were eluted with 0.2 M Glycine–HCl (pH 2.2) containing 1 mg/ml BSA and neutralized with 1 M Tris–HCl (pH 9.1). The eluted phages were amplified by infecting log-phage *Escherichia coli* ER2738, and then concentrated by polyethylene glycol (PEG) precipitation. The phages were tittered and submitted to the second round of selection. After 3–4 rounds of selection, the selected phage clones were identified by single-strand DNA sequencing and the encoded peptide sequences (mimotopes) were deduced.

2.11. Phage ELISA

To verify the positive phage clones, an indirect phage ELISA was performed as previously described (Cao et al., 2012). Briefly, the 96-well plates were coated with HAb21 at 1 μg/ml and blocked by 0.5% BSA. Affinity-selected phages were first amplified in 2 ml ER2738 culture and then added 100 μl diluted culture supernatant to the coated wells. The plate was incubated for 1 h at room temperature and then washed three times with TBS–T. HRP-conjugated murine anti-M13 antibodies (Sigma) were added to the wells and incubated at 37 °Cfor 1 h. The wells were extensively washed with TBS–T and reacted with TMB substrate (Sigma). Absorbance at 450 nm was measured with an ELISA reader.

2.12. Prediction of HAb21 binding residues by Mapitope

The potential binding residues of mAb (HAb21) were computationally predicted by the Mapitope algorithm (Bublil et al., 2007; Mayrose et al., 2007), which has been validated for efficient mapping of the conformation-dependent binding sites or epitopes based on the affinity-selected mimotopes by a number of studies (Bublil et al., 2006; Cao et al., 2012; Tarnovitski et al., 2006; Wan et al., 2013). In principle, the algorithm of Mapitope is based on the assumption that the epitope is separated into several amino acid pairs (AAP) contributing for the binding to the antibodies and the entire set of peptides are enriched with AAP which mimic a genuine epitope.

2.13. Site-directed mutagenesis

A panel of HA mutants were generated by site-directed mutagenesis using the QuikChange XL kit (Stratagene, La Jolla, CA). The plasmid encoding the HA of A/Vietnam/1203/2004 was used as a template. The primers used for construction of HA mutants were designed and synthesized according to the manufacturer's instructions. The introduced mutations were verified by DNA sequencing and the protein expression was confirmed by Western-blot.

2.14. Immuofluorescence assay (IFA)

The reactivity of mAbs with wild-type (WT) HA or HA containing a specific mutation was measured by IFA as described previously (Cao et al., 2012). In brief, the plasmids encoding HA were transiently expressed in 293T cells by transfection. After 24 h, the cells were fixed by 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature, followed by three washes with PBS. The cells were then incubated with 1% BSA in PBS for 30 min to block unspecific binding of the antibodies. HAb21 or a control mAb was added to the cells at a final concentration of 10 μg/ml and incubated at room temperature for 1 h in a humidified chamber. After washes, bound antibodies were detected by using FITC-conjugated anti-mouse antibodies and observed under an immunofluorescence microscope.

3. Results

3.1. Isolation of a broadly H5N1-neutralizing mAb

By using a standard hybridoma approach, two anti-HA mAbs, designated HAb02 and HAb21, were successfully isolated from immunized mice, both belong to the IgG1 isotypes. In the first step, we determined their antiviral activity with live-virus based neutralization assay. As shown in Fig. 1A and Table 1, HAb21 exhibited potent neutralizing activity against three index H5N1 strains, including A/HongKong/483/97 (clade 0), A/Vietnam/1194/04 (clade 1), and A/Shenzhen/406H/06 (clade 2.3.4). Consistently, HAb21 could efficiently inhibit HA-mediated hemagglutination of A/HongKong/483/97 and A/Vietnam/1194/04 H5N1 viruses (Fig. 1B). In contrast, HAb02 failed to inhibit any of tested viruses in both assay systems. Further, we expanded our characterization by using a panel of HA pseudoviruses. As shown in Fig. 2 and Table 1, HAb21 but not HAb02 had potent activity to inhibit the cell entry of divergent H5N1 strains, including clades 0, 1, 2.2, 2.3.2.2, and 2.3.4. However, both mAbs had no neutralizing activity against the HAs of influenza H1N1 viruses and newly emerging H7N9 viruses. Therefore, there results suggested that HAb21 is a broadly neutralizing mAb against diverse H5N1 strains.

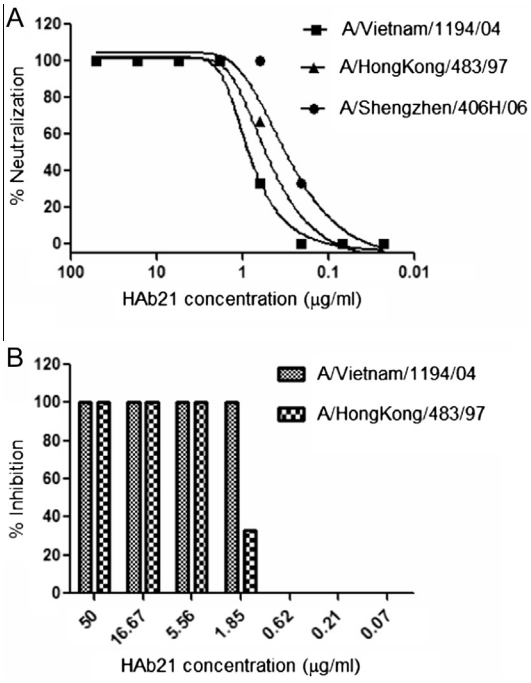

Fig. 1. Inhibitory activity of HAb21 against multiple influenza H5N1 viruses. (A) Neutralization of H5N1 viruses A/HongKong/483/97, A/Vietnam/1194/04, and A/Shenzhen/406H/06) by HAb21. (B) Inhibition of A/HongKong/483/97 and A/Vietnam/1194/04 H5N1 viruses-mediated hemagglutination by HAb21.

Table 1
Neutralizing activity of HAb21 against diverse IAVs.*

Viruses used	Clades	IC_{50} (μg/ml)	
		HAb21	HAb02
Wild-type H5N1 virus			
A/HongKong/483/97	0	0.44	>50
A/Vietnam/1194/04	1	0.64	>50
A/Shenzhen/406H/06	2.3.4	0.21	>50
H5N1 pseudovirus			
A/HongKong/156/97	0	0.42	>50
A/Vietnam/1203/04	1	0.55	>50
A/Xinjiang/1/06	2.2	0.58	>50
A/Qinghai/59/05	2.2	1.83	>50
A/Anhui/1/05	2.3.4	0.54	>50
A/Shenzhen/406H/06	2.3.4	0.7	>50
A/Hubei/1/10	2.3.2.1	1.36	>50
H1N1 pseudovirus			
A/South Carolina/1/1918	NA	>50	>50
A/Brisbane/59/2007	NA	>50	>50
A/California/06/2009	NA	>50	>50
H7N9 pseudovirus			
A/Shanghai/1/2013	NA	>50	>50
A/Anhui/1/2013	NA	>50	>50

* Representative data from three independent repeat experiments. NA, not applicable.

3.2. Potent neutralization of H5N1 mutants transmissible by air

It was intriguing to know whether HAb21 was able to neutralize H5N1 mutants capable of airborne transmission as recently shown by Imai and others (Herfst et al., 2012; Imai et al., 2012; Russell et al., 2012). We thus constructed a panel of H5N1 pseudoviruses carrying single (N154D, N220K, Q222L, and T315I) and combination forms of the mutations and used in the microneutralization assays. Compared to the wild-type QH-HA pseudovirus, all the mutant H5N1 viruses showed comparable sensitivity

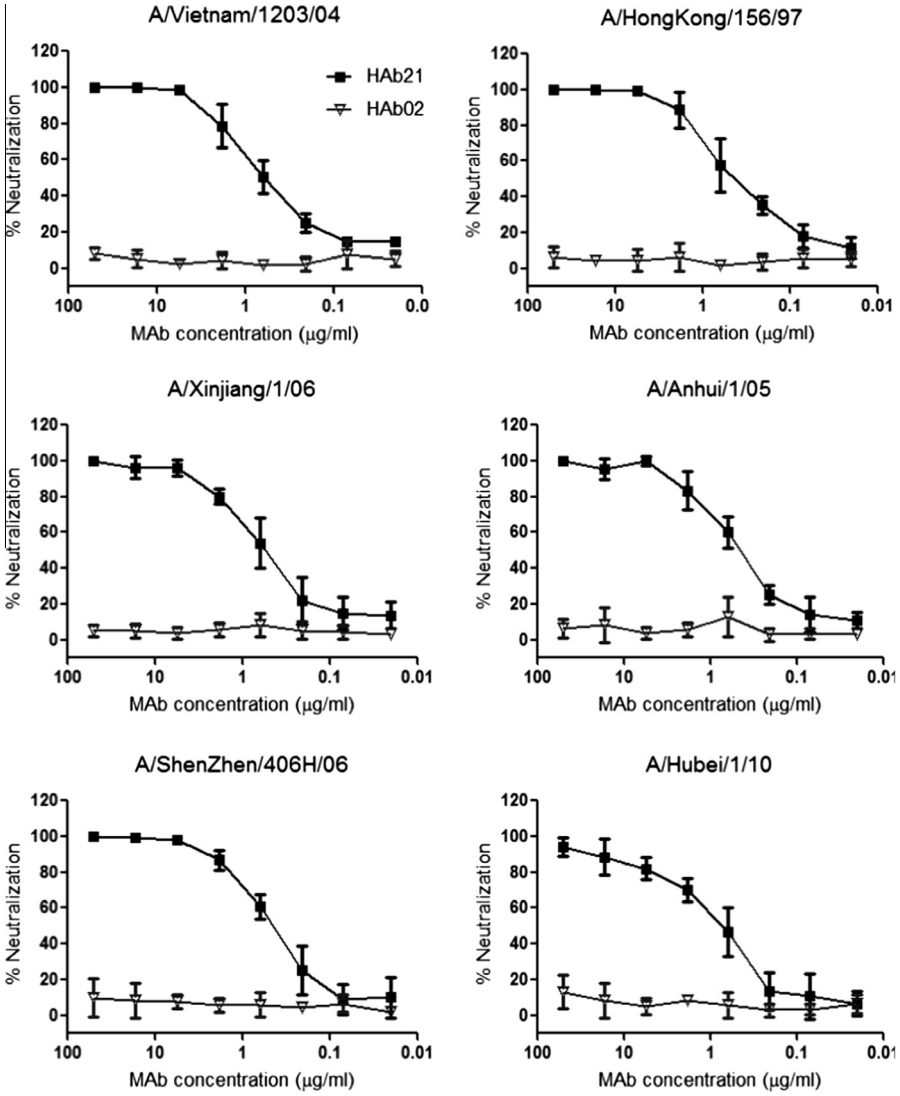

Fig. 2. Cross-clade neutralizing activity of anti-HA mAbs against a panel of H5N1 pseudoviruses. Infection of MDCK cells by each H5N1 pseudovirus was assessed in the presence of HAb21 or HAb02 at a series of threefold dilutions. Data were derived from the results of 3 independent experiments and are expressed as means ± SD.

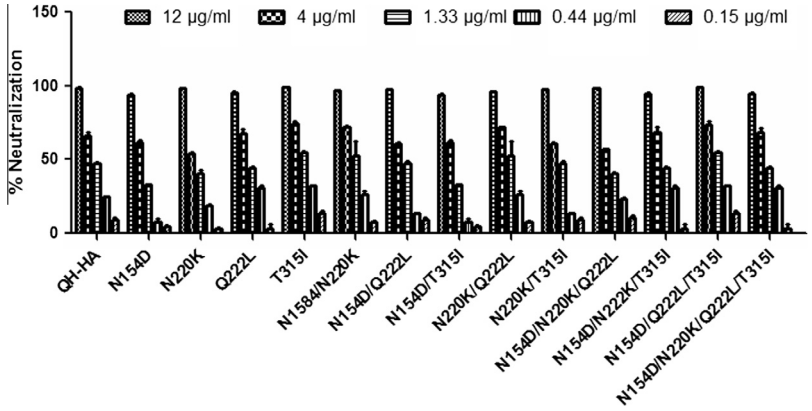

Fig. 3. Neutralizing activity of HAb21 against a panel of H5N1 mutants. Total of 13 H5N1 pseudoviruses bearing single or combination forms of amino acid substitutions that mediate viral airborne transmission between ferrets were constructed. The QH-HA represents the pseudovirus of A/Qinghai/59/2005. HAb21 was tested at five different concentrations and the percent neutralization was calculated. Data were derived from the results of 3 independent experiments and are expressed as means ± SD.

to the neutralization by HAb21 (Fig. 3). Promisingly, this mAb could maintain its high activity to inhibit those pseudoviruses with multiple substitutions, such as N154D/N220K/Q222L, N154D/N220K/T315I, N154D/Q222L/T315I, and N154D/N220K/Q222L/T315I. This result suggested that HAb21 targets a conserved epitope not involving in the above mutations.

3.3. HAb21 efficiently block the virus–receptor binding

The entry of influenza A virus into target cells is mediated by its HA protein through two steps: binding to the receptor on the cell surface which results in the endocytosis of virus, and then the viral membrane fuses with the endosomal membrane to release the viral genetic materials into the host (Skehel and Wiley, 2000). The HA-specific neutralizing antibodies can block either the receptor binding or membrane fusion, or both steps. To explore the mechanism of HAb21-mediated inhibition on the H5N1 virus, we performed both virus binding and postattachment experiments as described. As shown in Fig. 4A and B, HAb21 efficiently blocked HA-mediated virus binding onto the MDCK cells at a dose-dependent manner, similar to that of the control mAb, AVFluIgG01. The postattachment assay indicated that HAb21 had no inhibitory function after the H5N1 virus bound to the cells. Taken together with its potent inhibition on the HA-mediated hemagglutinin, we concluded that HAb21 exerts its neutralization only through interfering the viral binding step.

3.4. HAb21 recognizes a HA conformation-dependent epitope

We measured the reactivity of HAb21 and HAb02 with various HA-derived antigens by ELISA. Unexpected but interestingly, HAb21 did not react with a group of other recombinant HA proteins except the immunogen Tri-HA (Fig. 5A). Furthermore, HAb21 could not react with both HA1 and HA2 subunits and those truncated HA fragments (a.a. 1–130, 100–250, 230–346). However, HAb02 reacted strongly with all the tested antigens and its binding site could be clearly localized onto the HA1 subunit (Fig. 5B). No reaction was observed for both mAbs with a panel of 77 synthetic peptides that cover the full-length HA protein (data not shown).

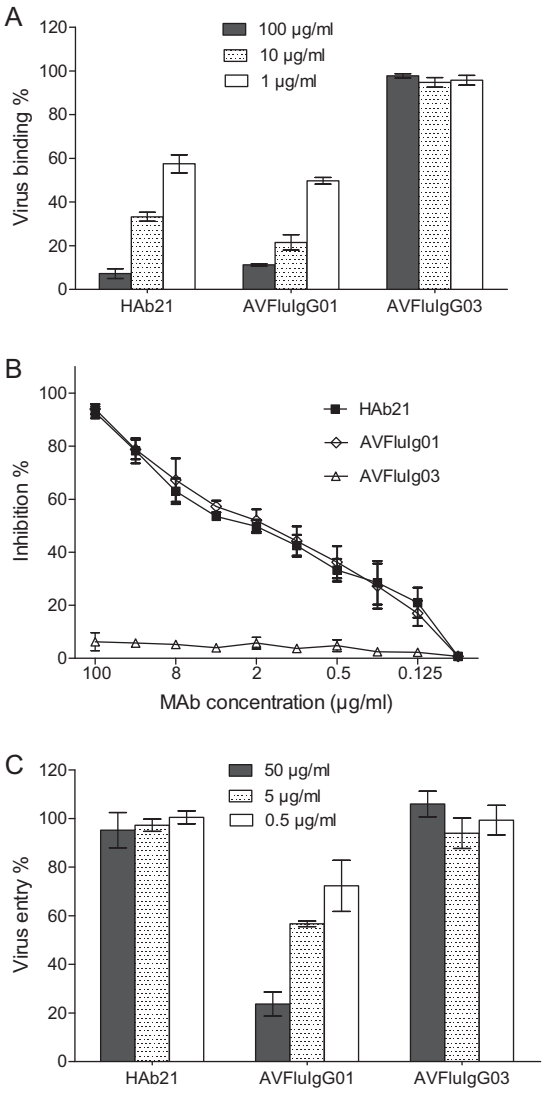

Fig. 4. Inhibitory activity of mAbs on two steps of H5N1 entry. (A) Inhibition of mAbs against HA-mediated virus-receptor binding. Binding of the A/Vietnam/1203/04 H5N1 pseudovirus to MDCK cells was expressed as a percentage of the reading obtained in the absence of antibody, which was set at 100%. (B) HAb21 inhibited the pseudovirus binding at a dose-dependent manner, similar to the control mAb AVFluIgG01. (C) Postattachment neutralization of mAbs against the A/Vietnam/1203/04 H5N1 pseudovirus. Unlike AVFluIgG01, HAb21 failed to block the virus entry after binding to the MDCK cells. Data were derived from the results of 3 independent experiments and are expressed as means ± SD.

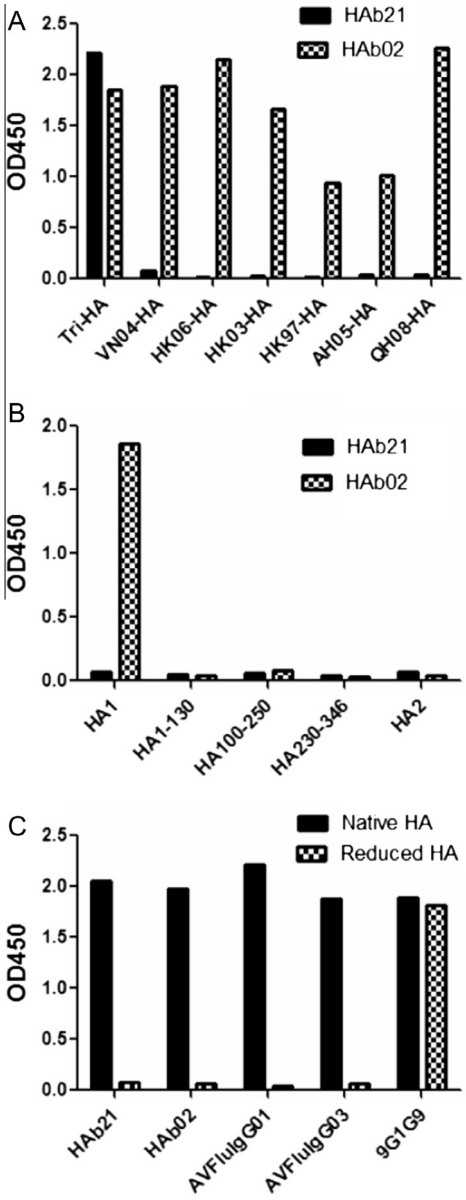

Fig. 5. Reactivity of mAbs with different HA-derived antigens in ELISA. (A) Reactivity of HAb21 and HAb02 with the recombinant HA proteins from diverse clades of H5N1. (B) Reactivity of HAb21 and HAb02 with the truncated HA proteins. (C) Reactivity of mAbs with the native and DTT-reduced HA proteins. HA proteins were used at 1 μg/ml and mAbs were tested at a final concentration of 10 μg/ml.

To reveal the epitopes recognized by HAb21 and HAb02, we compared the reactivity of mAbs with the native HA and DTT-treated HA proteins. It is well known that DTT treatment of a protein would disrupt the disulfide bonds thus impairing the conformation-dependent epitopes recognized by antibodies. As shown in Fig. 5C, the reduction of HA protein completely abolished the binding by HAb21 and HAb02, and by two conformation-dependent control mAbs (AVFluIgG01 and AVFluIgG03). Severing as a control antibody recognizing a linear epitope within HA2 subunit, 9G1G9 reacted equally with the native and reduced HA proteins. Collectively, these results suggested that HAb21 and HAb02 target the disulfide bond-dependent conformational epitopes presented on the HA protein.

3.5. Computational prediction of HAb21 epitope

We sought to explore the binding sites of HAb21 by selecting mimotopes from a random peptide phage display library, as previously used to map conformation-dependent mAbs (Cao et al., 2012; Irving et al., 2010; Khurana et al., 2009; Wan et al., 2013). Based on the positive phage clones isolated from the library, 10 different peptide minotopes were identified by sequencing their DNA inserts (Fig. 6A). The binding specificity of these peptides to HAb21 was re-tested by phage-based ELISA as described. From the selected mimotopes, we could not find apparent consensus sequences or significantly homologous motifs on the HA protein that can be used to deduce the HAb21 epitope. Therefore, we predicted the potential binding residues of HAb21 by applying the Mapitope, a powerful computational algorithm for mapping antibody epitopes based on the mimotopes (Bublil et al., 2007; Huang et al., 2008). As shown in Fig. 6B, the Mapitope predicted a total of 22 residues, which are exclusively located in the global head region of HA1 subunit. Notably, several patches of residues (a.a. 122–127, a.a. 154–156, a.a. 182–186, a.a. 192–194, a.a. 211–214) were predicted by the program, implying their possible involvement for HAb21 binding.

3.6. Identification of the epitope residues by mutagenesis

To identify the epitope residues critical for HAb21 binding, we generated a panel of HA mutants carrying single amino acid substitutions corresponding to the above-noted residues. All residues were mutated to alanine except Ala188 and Ala189, which were changed to glycine. The wild-type (WT) and mutated HA proteins were transiently expressed on the surface of 293T cells by transfection and the reactivity of HAb21 and a control mAb with each of HA mutants was examined by IFA assay. As shown in Fig. 7, substitutions of His125, Asp183, Ala184, Ala185, Glu186, Asn193, Pro194, Pro211, Asn244 could completely abolish or dramatically decrease the binding of HAb21 to the HA expressed on the cell surface, suggesting their potential involvement in the antibody epitope. Mutations of other 13 residues had no or minor effects on the HAb21 binding thus they could be excluded for the epitope composition. As a control for HA protein expression, no mutations affected the binding ability of a human mAb directing against the HA2 subunit (data not shown).

A

Affinity-selected mimotopes

WHWSWWNPNQLT	YTDLTSDTLVRG
NSWVAIQTQDEY	NHWREPTPDTIN
VEAHNQWTGLVA	TLRVPPNPNMNV
LPGRAHDPWKVP	GHWLWWNPNHQH
KQHNTKYSVVLR	SLITKHHERAIK

B

Predicted residues by Mapitope

Trp122	Ser123	Ser124	His125	Glu126
Ala127	Asn154	Ser155	Thr156	Asn182
Asp183	Ala184	Ala185	Glu186	Gln192
Asn193	Pro194	Pro211	Arg212	Ile213
Ala214	Asn244			

Fig. 6. Computational prediction of HAb21 epitope by minotopes. (A) Sequences of affinity-selected minotopes from a random peptide phage display library. (B) Predicted amino acid residues by Mapitope. Numbering is relative to mature H5 HA as described in text.

3.7. Mapping the HAb21 epitope on the tip of HA1 head

Interestingly, all the characterized HAb21-binding residues are not involved in the previously reported antigenic sites, suggesting that HAb21 targets a novel conserved epitope on the H5N1 HA protein. We labeled the potential epitope residues on the 3-dimensional structure (3D) of a H5N1 HA protein (PDB: 2IBX) with the PyMol program. Apparently, 8 residues (residues184–186, 93–94, 211, 244) were closely located on the tip region of HA1 head (Fig. 8A), neighboring the interface of HA trimer (Fig. 8B) and overlapping the receptor-binding site (RBS) with one residue (Glu186) (Fig. 8C). But the residue His125 was localized on the opposite face of the HA1 tip thus cannot be physically contacted by HAb21, suggesting that its mutation may indirectly affect the antibody binding. As compared to the known antigenic sites mapped by a number of mouse and human mAbs (Fig. 9), HAb21 recognizes a novel conformation-dependent epitope in the HA1 head. By analyzing a large panel of H5N1 HA sequences (Table S1), all the characterized residues are highly conserved among diverse H5N1 clades and subclades, especially no mutations were identified for Asp183, Glu186, Asn193, Pro194, Pro211, and Asn244 among 98 H5N1 isolates, explaining the broad-spectrum neutralizing activity of HAb21.

4. Discussion

Similar to the HA protein of other influenza A viruses, the HA of H5N1 virus is a major antigen to induce neutralizing antibodies thus serving as an important target for developing influenza vaccines and immunotherapeutics. Here, we generated a novel mAb (HAb21) which has a broad-spectrum neutralizing activity against diverse clades of H5N1 strain and especially those H5N1 mutants capable of airborne transmission. In mechanism, HAb21 targets a novel conserved epitope at the tip of the globular HA head and blocks the receptor-binding step of H5N1 virus.

Since 1997, highly pathogenic H5N1 virus has spread to numerous countries in Asia, Europe, and Africa, infecting not only large numbers of poultry but also an increasing number of humans. Based on the sequences of HA, 10 clades of H5N1 virus have emerged in various species. So far the H5N1 strains isolated from human cases predominantly fall into clades 0, 1 and 2 (Smith et al., 2006), with that the clade 2 can be further divided into multiple subclades and sub-subclades. It is thought that continuous reassortment and adaptation may evolve new H5N1 strains capable of human-to-human transmission. Recent studies that the laboratory-mutated or reassortant H5N1 viruses could be more efficiently transmitted among ferrets through respiratory droplets did pose a series threat to public health (Herfst et al., 2012; Imai et al., 2012). Herfst et al. (2012) identified a minimal set of substitutions containing two receptor-binding amino acids (Q222L and G224S) in HA1 head region, which may change the virus from

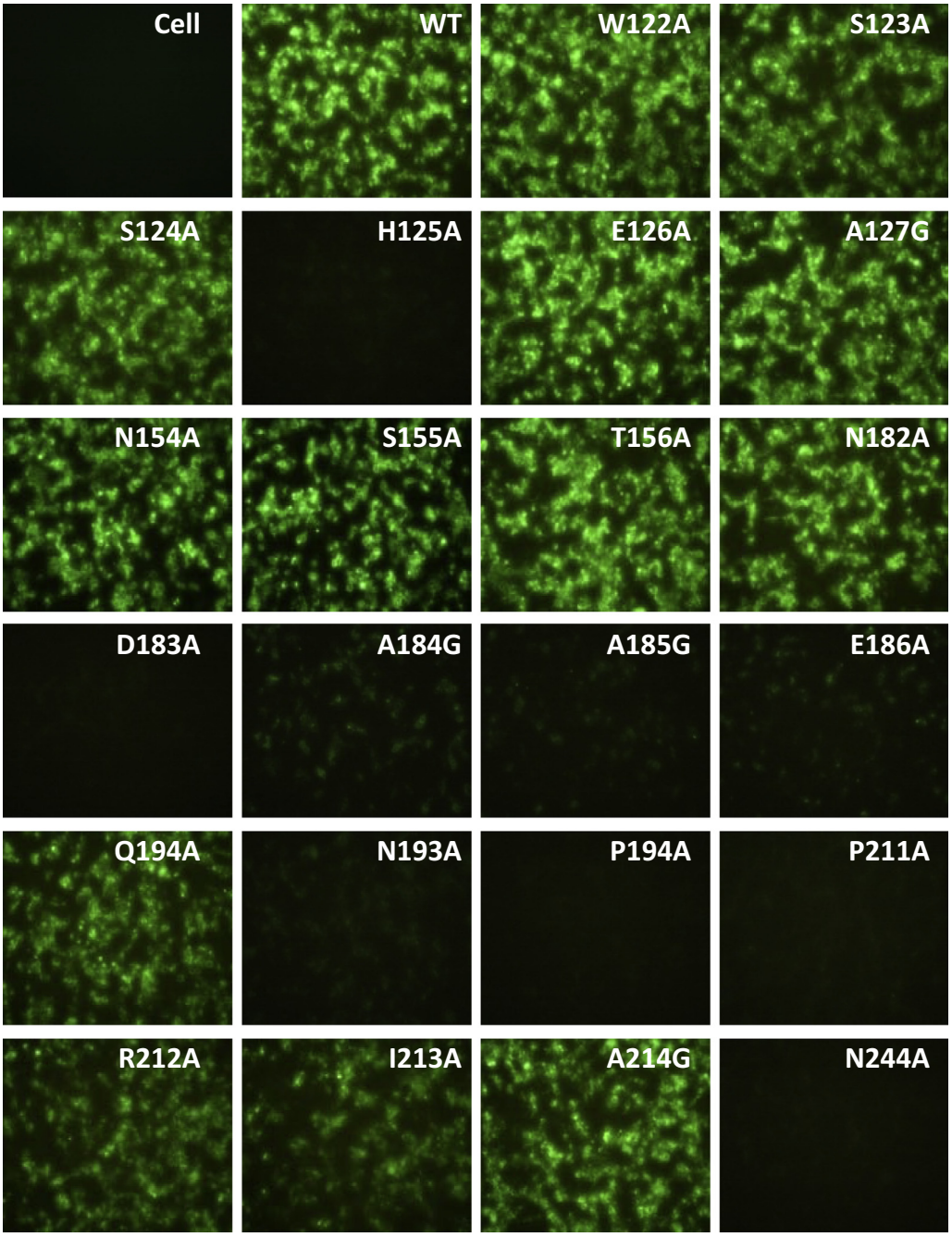

Fig. 7. Mutagenesis analyses of the predicted epitope residues. The reactivity of mAbs with wild-type (WT) HA or HA containing site-directed mutations analyzed by immunofluorescence assay (IFA). The WT HA or mutants were transiently expressed on 293T cells by transfection and then immunostained by HAb21 or a control mAb at a final concentration of 10 μg/ml.

α2-3 to α2-6 specificity. Three additional mutations in the set were T156A in HA, which disrupts the N-linked glycosylation sequon, H103Y in the HA trimer-interface, and E627K in PB2, which is a common mammalian polymerase adaptation. Independently, Imai et al. (2012) identified two receptor-binding amino acid mutations, N220K and Q222L, which together are known to change the sialic acid linkage preference to the more human-like. In addition, N154D, which disrupts the same N-linked glycosylation sequon as the T156A substitution, and T315I in the stalk region, were also identified. Therefore, a robust global surveillance should be in place for timely detection of newly arising H5N1 strains in animals; however, such a coordinated surveillance and control effort has not always been successful. To fight against a future influenza pandemic by emerging H5N1 virus, various antiviral strategies should be prepared. As an alternative to vaccines, neutralizing mAbs represent a vital strategy to provide immediate protection against infection by those highly pathogenic IAVs. Encouragingly, several mAbs specific for the HA2 subunit were reported to possess broad neutralization against multiple strains of IAVs (Corti et al., 2011; Ekiert et al., 2011; Sui et al., 2009). For example, human mAbs F10 and CR6261 were effective against all tested group 1 IAVs (Ekiert et al., 2011; Sui et al., 2009), while CR8020 could neutralize most of group 2 viruses including H3N2 and H7N7 subtypes (Ekiert et al., 2011). Impressively, mAb F16 was reported to recognize the HAs of all 16 subtypes and neutralize both group 1 and 2 IAVs (Corti et al., 2011). These HA2-specific mAbs may be

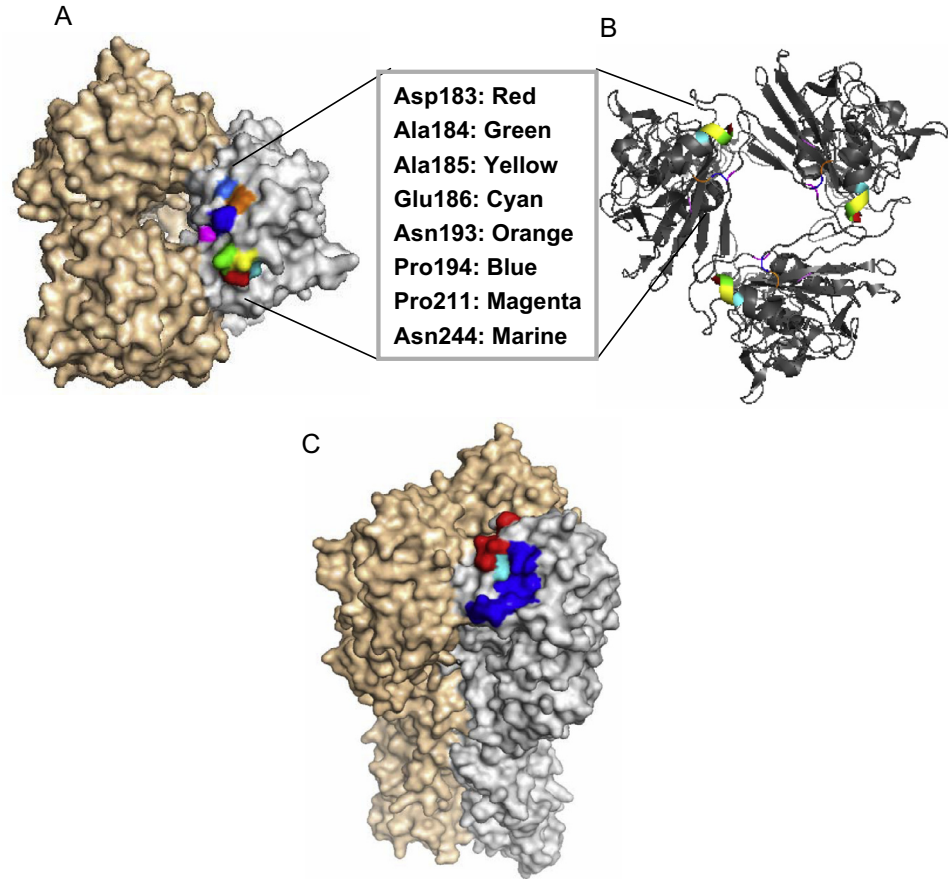

Fig. 8. Surface representation of HAb21 epitope on the HA trimer model. (A) Top view of the trimeric HA structure. The amino acids critical for HAb21 binding were respectively colored. (B) The amino acids critical for HAb21 binding were shown in ribbon representation. (C) Side view of HAb21 epitope (red) relative to the receptor binding site (blue). The overlapping residue Glu186 was colored in cyan. The H5 HA structure (PDB ID: 2IBX) was used to generated the diagram was generated by PyMOL. Amino acid positions are designated in mature H5 numbering as described in text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

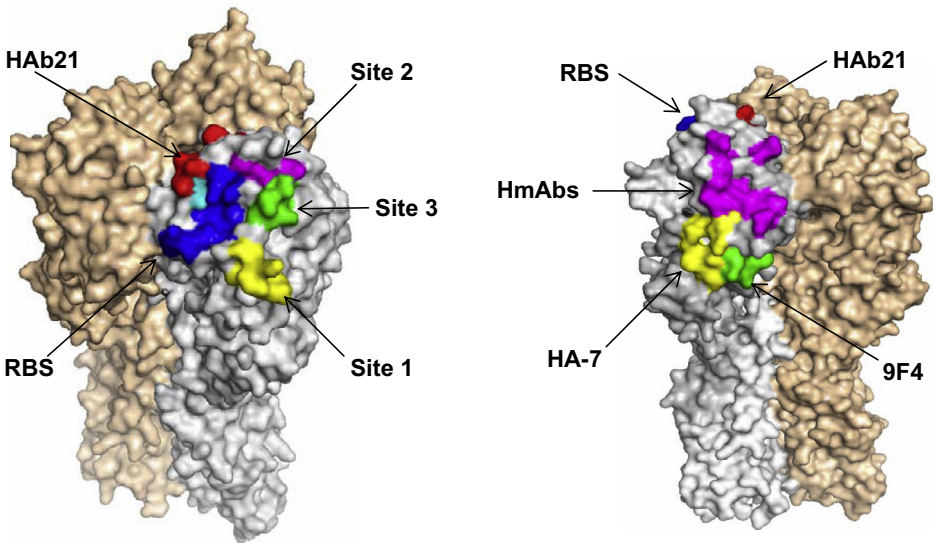

Fig. 9. Analysis of the antigenic sites on the H5N1 HA. Left: HAb21 epitope (red) in context of the receptor-binding site (blue), and the antigenic site 1 (yellow), site 2 (pink), site 3 (green). Right: Surface show of newly-characterized mAb epitopes. The epitopes targeted by a panel of human mAbs were colored in pink, the HA-7 epitope was colored in yellow, and the 9F4 epitope was colored in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

used as effective passive immunotherapeutics against a broad of IAVs during influenza pandemics or epidemics because of their ability to neutralize a variety of IAVs in addition to H5N1 virus. Meanwhile, a number of mAbs specific for the HA1 subunit were also identified from H5N1-infected individuals or HA-immunized animals, such as human mAbs FLA5.10 (Simmons et al., 2007),

FLD21.140 (Simmons et al., 2007), AVFluIgG01 (Sun et al., 2009), AVFluIgG03 (Sun et al., 2009), 65C6 (Hu et al., 2012), 100F4 (Qian et al., 2013) and mouse mAbs 9F4 (Oh et al., 2010) and HA-7 (Du et al., 2013a). Compared to HA2-specific mAbs, these HA1-specific mAbs usually possess a limited breadth to neutralize IAVs, but they are important reagents to structurally and functionally characterize the highly pathogenic H5N1 viruses. Unlike HA2-sepicific neutralizing mAbs which mainly block the viral membrane fusion step, HA1 mAbs can neutralize H5N1virus with multiple mechanisms, including inhibition of the receptor-binding, membrane fusion or both steps. It is possible that H5N1-specific mAbs may provide an effective passive immunotherapeutic effect in treatment of H5N1-infected patients. Our neutralization data indicated that HAb21 was able to inhibit all the tested H5N1 isolates, including 1 of clade 0, 2 of clade 1 and 5 of clade 2 viruses, and more importantly, those H5N1 mutants bearing the above substitutions individually or in different combinations, highlighting its potential application as a therapeutic or prophylactic. We are not concerning an human anti-mouse antibody response (HAMA) for use of a murine anti-H5N1 mAb such as HAb21, as only limited injections may be needed to save lives of severe patients infected by a deadly H5N1 virus. Also, a mouse mAb could be successfully humanized if a long term use is required.

Previous studies identified three antigenic sites in the HA1 subunit of H5N1 HA as shown in Fig. 9 (Kaverin et al., 2007; Stevens et al., 2006). The site 1, an exposed loop comprising HA1 residues136–141 (140–145 in H3 numbering), overlaps with the antigenic site A of H3 and Ca2 of H1; the site 2, HA1 residues 152–153 (156–157 in H3 numbering), corresponds to the antigenic site B in H3 serotype; and the site 3, HA1 residues 125–129 (129–133 in H3 numbering), is restricted to the Sa site of H1 and H9 HAs. Typically, these epitopes are mainly clustered around the receptor-binding site (RBS) in the HA1 head (Kaverin et al., 2007; Stevens et al., 2006). Recently, several novel and conserved antigenic sites of H5N1 HA have been identified by mAbs. First, a group of human neutralizing mAbs (FLA5.10, FLD21.140, AVFluIgG01, 65C6) were independently isolated from H5N1-infected patients and their epitopes were exclusively mapped to overlap three key sites (^{115}QIIP^{118}, ^{121}SWS^{123}, ^{164}YNNT^{167}) (Cao et al., 2012; Hu et al., 2012; Khurana et al., 2009; Simmons et al., 2007; Sun et al., 2009). Notably, the epitope of FLA5.10 was also overlapping with the previously characterized antigenic site 3 with residues ^{126}EASL^{129} (Khurana et al., 2009), whereas the 65C6 epitope comprises a residue involving in the Sa site of H1 at position 161 (Lys161) (Hu et al., 2012). Interestingly, the epitopes for two mouse mAbs (9F4 and HA-7) were located to the previously uncharacterized sites below the globular head of the HA1 subunit, far away from the RBS (Du et al., 2013a; Oh et al., 2010). On the surface of HA, the 9F4 epitope was mapped to the residues 256–259 (Oh et al., 2010), while the HA-7 epitope was found to contain two discontinuous fragments (the residues 72–75 and 110–115) (Du et al., 2013a). In comparison, our mAb HAb21 targets a novel conformational epitope not overlapping any known epitopes in the HA1 head mapped by mouse and human mAbs. Specifically, HAb21 epitope contains the resides at positions 183–186, 193–194, 211 and 244. On the 3D structure, these epitope residues are adjacent to each other at the tip of the HA1 head, in close proximity to the RBS and to the inside interface of HA trimer. Therefore, a HA trimer-dependent conformation might be required for the integration of the HAb21 epitope. In other words, a HA monomer might lose ability to present this unique epitope, partially explaining why HAb21 reacted well with a trimeric immunogen (Tri-HA) but not other recombinant HA proteins (Fig. 5A). Indeed, a crystal structure of HAb21 in complex with HA would be helpful to further characterize the HAb21 epitope and the HAb21/HA interactions in detail.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.03.005.

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