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Human 4F5 single-chain Fv antibody recognizing a conserved HA1 epitope has broad neutralizing potency against H5N1 influenza A viruses of different clades

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ABSTRACT

Influenza A viruses present a significant threat to public health worldwide. High-affinity human scFv antibodies against a conserved epitope can potentially provide immunity to diverse viruses and protect against future pandemic viruses. A library of phage-displayed human scFv containing 6.0×10^8 members was generated from lymphocytes of H5N1 virus vaccinated individuals. Using the recombinant H5N1 virus hemagglutinin ectodomain (HA1), 4F5 scFv was identified with neutralizing activity against both clade 2 and 9 H5N1 viruses. In embryonated chicken eggs, the antiviral activity of 4F5 scFv conferred a 100% survival rate and at least a 62.5% survival rate against different clades of H5N1 viruses by pre-treatment and post-treatment, respectively. 4F5 scFv belongs to the VH-3-43 family according to the IMGT database, and a peptide (76)WLLGNP(81) containing half of an α-helix in HA1 was identified as the binding pocket. The conserved binding epitope of this novel broadly neutralizing scFv may become key in the design and implementation of vaccines or RNA interference against H5N1 viruses.

1. Introduction

Influenza A viruses have caused four human pandemics in the last century and still pose threats to public health (Gregg et al., 1978; Kilbourne, 2006; Oxford, 2000; L'vov et al., 2004). Infection and transmission in humans by the highly pathogenic H5N1 avian flu in the past few years (Andrade et al., 2009; Olsen et al., 2005; Ungchusak et al., 2005; Wang et al., 2008), particularly in Southeast Asian countries (Kandun et al., 2006; Chotpitayasunondh et al., 2005), with either high mortality or morbidity have heightened fear that the next influenza pandemic is due (Doerr et al., 2006; Ebrahim, 2004). Mutation of the viral genome due to antigenic drift and shift (Russell and Webster, 2005; Shih et al., 2007; Matrosovich et al., 2000), especially in the hemagglutinin (HA) and neuraminidase (NA) genes, necessitates the development of prophylactic and therapeutic interventions that can provide broad protection.

Vaccination undoubtedly is the principal strategy for prevention and control of influenza (Subbarao et al., 2006; Chen et al., 2008; Nakaya et al., 2011). However, the current vaccines have limitations in that they can only protect those at risk from the viruses circulating at the time of development (Sasaki et al., 2011; Tang et al., 2008). Another possible effective countermeasure against influenza is antibody-based therapy (Lu et al., 2006; Nguyen et al., 2010). The resurgence of this type of therapy has been fueled by the reports of individuals surviving severe influenza infection after transfusion with convalescent plasma (Kong and Zhou, 2006). Of the 3 major surface glycoproteins on the virion, HA is the primary target for neutralizing antibodies. HA is a glycoprotein, composed of HA1 and HA2 subunits, and three HA monomers form a homo-trimer to function in receptor binding by HA1 (Chiu et al., 2009) and membrane fusion by HA2. HA has been classified into 16 antigenically distinct subtypes, and it is generally believed that neutralizing antibodies are not cross-reactive among HA subtypes. However, the identification of monoclonal antibodies with broad, heterosubtypic neutralizing activity (Sakabe et al., 2010; Ohshima et al., 2011; Ekiert et al., 2009; Hultberg et al., 2011; Oh et al., 2010; Wrammert et al., 2011; Clementi et al., 2011) suggests that such conserved epitopes in HA do exist.

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Recently, a binding pocket was characterized on HA for the fusion inhibitor tert-butyl hydroquinone (Sui et al., 2009), which shows great promise as a new target for therapy. The HA2 domain is rich with α-helices, which can form hydrophobic pockets and facilitate binding with antibodies. Antibodies binding to these concave pockets have been reported to neutralize different clades or even subtypes of influenza A viruses. There are also three α-helices in the HA1 domain, but antibodies against these sites with cross-reactivity to other viruses have not been previously reported.

In this study, we isolated and characterized a human single-chain Fv (scFv) antibody, designated 4F5, with neutralizing activity against different clades of H5N1 influenza A virus. It bound to a conserved peptide (76)WLLGNP(81) in the HA1 domain and showed satisfactory antiviral effects against challenge with H5N1 viruses in embryonated chicken eggs. Altogether, our findings indicate that the scFv may be developed for use as prophylaxis or treatment of influenza A virus infections.

2. Materials and methods

2.1. Viruses

The viruses used in this study were: A/chicken/Hongkong/369/2003 (H5N1) (GenBank: KC784945.1), A/goose/Jilin/514/2005 (H5N1) (GenBank: KC784947.1), A/Jiangsu/4/2007 (H5N1) (GenBank: KC784948.1), A/goose/Guangdong/08/2005 (H5N1) (GenBank: KC784944.1), A/Jiangsu/6/2008 (H5N1) (GenBank: KC784946.1) and A/Jiangsu/1/2007 (H5N1) (GenBank: EU434686.1). The whole-virion H5N1 vaccine was manufactured in embryonated hens' eggs using the reassortant strain NIBRG-14 (A/Vietnam/1194/2004-A/PR/8/34) as vaccine virus. The A/Vietnam/1194/2004 virus belongs to clade 1, and the inactivated vaccine was developed by the Beijing Sinovac Biotech Co. Ltd. and China Center for Disease (Qiu and Yin, 2008).

2.2. Expression of H5N1 recombinant HA1 (rHA1) protein in a Bac-to-Bac baculovirus expression system

Total RNA was isolated from A/Jiangsu/1/2007 (H5N1), and HA1 was amplified with H5-specific primers. The amplified products were subcloned into pFastBac and transformed into *Escherichia coli* DH10Bac. The positive recombinant bacmid was isolated and transfected into Sf9 cells, as described previously (Li et al., 2010). Recombinant HA1 proteins were detected with a horseradish peroxidase (HRP)-conjugated anti-His antibody. An anti-H5N1 virus mouse monoclonal antibody (mAb) affinity chromatography column was used to purify the rHA1 protein.

2.3. Construction of scFv antibody library

Lymphocytes were isolated from healthy donors from the Chinese Han population who were immunized with whole-virion H5N1 vaccines. Written informed consent was obtained from each volunteer. V_H and V_L genes were amplified by RT-PCR using a group of specific constant region primers (Barbas et al., 2004). ScFv fragments were spliced by overlapping PCR and ligated into pComb3XSS phagemid. A scFv phage display library was constructed after the ligations were introduced into competent *E. coli* XL1-Blue by electroporation. The library was rescued and amplified using VCSM13 helper phage.

2.4. Selection of rHA1-specific clones from the scFv library

For phage bio-panning, rHA1 was coated onto ELISA plates. Amplified phage mixtures were then incubated with the coated wells, and the unbound phage were removed by washing with PBST. Bound phage were eluted with glycine–HCl, pH 2.2. Eluted phage were neutralized to pH 7.0, followed by infection of *E. coli* XL1-Blue. After three rounds of selection, the phage were amplified, plated on agar plates and incubated overnight. Single bacterial colonies were picked and tested by phage-ELISA.

The scFv coding regions of the positive colonies were amplified and sequenced. Their framework regions (FRs) and complementary determining regions (CDRs) were determined by the Kabat system (Kabat et al., 1979). Amino acid sequence alignments of the V_H regions and V_L regions of the scFv antibodies were performed to compare with the human immunoglobulin sequences (IMGT Database) (Lefranc, 2011; Ehrenmann et al., 2010).

The positive scFv-phagemids were extracted from *E. coli* XL1-Blue colonies and transformed into non-suppressor *E. coli* Top10F'. These clones were cultured for large-scale soluble scFv production, and HisTrap™ chromatography was used to purify the scFv proteins.

2.5. Microneutralization assay

Neutralizing antibody titers were determined by microneutralization assays performed on Madin–Darby canine kidney (MDCK) cells, following a previously described procedure (Hu et al., 2008). The 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Reed and Muench method (Krah, 1991; Reed and Muench, 1938). Briefly, serially diluted scFv antibodies were incubated with 100 $TCID_{50}$ of virus before adding to the MDCK cells. Plates were incubated for 40 h before fixation of the cells with acetone. The presence of viral protein was detected with an anti-NP antibody. The concentration for 50% of maximal effect (EC_{50}) of the antibody was expressed as the lowest concentration of scFv antibodies giving 50% neutralization of 100 $TCID_{50}$ of virus in MDCK cells. Anti-CTGF mAb and PBS were used as controls.

2.6. Hemagglutination inhibition (HI) assay

In a microtiter plate, a series of 2-fold dilutions of the H5N1 virus (50 μl per well) were incubated at 37 °C for 1 h with an equal volume of 0.5% chicken erythrocytes. Hemagglutination (HA) titers were calculated.

In another microtiter plate, a series of 2-fold dilutions were made with antibody (25 μl per well), and an equal volume (25 μl) of virus suspension (diluted to contain 4-HA units per 25 μl) was added to each well. After incubation for 1 h, 50 μl of 0.5% erythrocyte was added and incubated at 37 °C for 1 h. The HI titers were expressed as the lowest concentration of scFv antibodies that completely inhibited hemagglutination. Anti-CTGF mAb and PBS were also used as controls.

2.7. Western blotting and immunofluorescence assay

Inactivated viruses were separated by SDS–PAGE and detected with the scFv by Western blotting. Immunofluorescence assays were used for analysis of scFv binding to native HA proteins in MDCK cells infected with H5N1 viruses. MDCK cells were infected with H5N1 viruses separately until the cytopathic effect reached 60–70%.The cells were then fixed and incubated with the scFv antibody. The mouse anti-His antibody and Alexa Fluor555-labeled goat anti-mouse antibody were added to the cells in succession. After DAPI staining, the cells was observed by using a laser scanning confocal microscope.

2.8. Epitope mapping of HA

The Peptide Library Kit Ph.D.-12 was used for HA epitope selection. The scFv was incubated with phage from the library, and the next process of panning was the same as that described for scFv antibody panning. After the third cycle, the phage clones were sequenced.

HA1 protein mutants were produced with primers introducing mutations in the scFv-HA1 protein binding site. The amplified mutant products were transfected into Sf9 cells using the Bac-to-Bac baculovirus expression system, as above. The infected Sf9 cells were detected with HRP-conjugated anti-His antibody and HRP-conjugated scFv, respectively.

The putative binding site of H5N1 virus with scFv was also evaluated by using three dimensional (3D) models. 3D structures of the HA protein and scFv were modeled and analyzed using UCSF Chimera (Yang et al., 2012), and the putative binding site was predicted.

2.9. Treatment with 4F5 scFv pre- and post-challenge with H5N1 influenza A virus confers survival to embryonated chicken eggs

Groups of embryonated chicken eggs were used in all experiments (Sauerbrei et al., 2006). The 50% egg infectious dose (EID50) of each virus was determined by the Reed and Muench method. In the pre-treatment groups, the eggs were given several treatment doses (25, 50, 75, 100 and 200 μg/kg) of the scFv 0.5 h prior to viral challenge (10 EID_{50}), and the control was injected with PBS only. In the post-treatment groups, the scFv was employed 1 h after the eggs were infected by viruses (10 EID_{50}), and several doses of the antibody (100, 150, 200, 250 and 500 μg/kg) were tested. The survival rates of the embryos were recorded until day 8 post-inoculation.

3. Results

3.1. Production of rHA1 proteins

The full-length gene sequence (~1000 bp) encoding HA1, was amplified and successfully subcloned into pFastBac. Sf9 cells were infected with the recombinant bacmid at 10 multiplicities of infection (MOI). Western blotting showed that the recombinant protein was present in both the cell culture supernatant and cell pellets. Coomassie Brilliant Blue staining of the purified rHA1 protein on an SDS–PAGE gel revealed a 43 kD band corresponding to the expected molecular weight of the recombinant protein (Fig. 1).

3.2. Production of rHA1-specific scFv antibodies

There were 36 individuals among the 40 volunteers who showed positive responses to the H5N1 vaccine. After RNA extraction, RT-PCR and overlapping PCR, scFv gene fragments of about 750 bp were generated, ligated into pComb3XSS and transformed into competent cells. A phage display library containing 6.0×10^8 clones was constructed successfully. Following three rounds of panning, 564 clones were selected from the overnight culture plates. Among them, 25 clones showed high positivity against rHA1 (Fig. 2A), which were all confirmed to contain the 750 bp scFv sequence.

All positive clones were sequenced, and nine unique sequences were obtained. The FRs and CDRs of each clone were analyzed according to the Kabat numbering system. As indicated in the alignment, most of these clones are closely associated with the VH-3 family compare with IMGT Database (Fig. 2B). After expression and purification using HisTrap FF crude columns, each of the

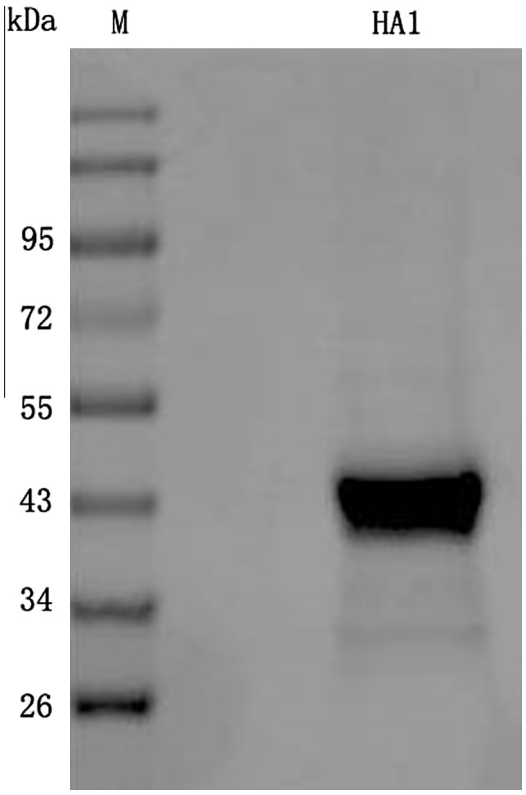
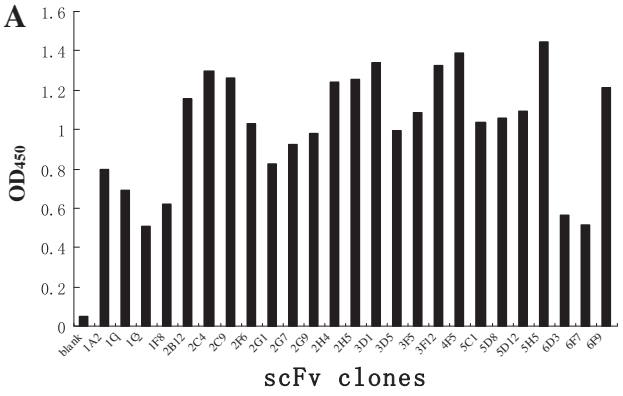


Fig. 1. SDS–PAGE analysis of the purified rHA1 protein. A 43 kD band was observed, corresponding to the predicted molecular weight of HA1.



B

NO.	Clones	IGHV	IGLV
1A2	6	IGHV3-23*04	IGKV3-20*01
1C1	5	IGHV3-74*02	IGKV4-1*01
1F8	1	IGHV3-7*02	IGKV1D-13*01
2C9	1	IGHV5-a*04	IGLV1-51*01
2G1	6	IGHV4-34*12	IGLV1-44*01
3D1	2	IGHV3-43*02	IGKV1-5*03
3F5	1	IGHV3-43*02	IGKV3-15*01
4F5	2	IGHV3-43*02	IGLV1-51*01
6F9	1	IGHV3-43*02	IGLV2-14*01

Fig. 2. Specificity and sequence analysis of anti-H5N1 influenza A virus rHA1 scFv genes selected from the library. (A) ELISA binding of scFv antibodies to rHA1. The 25 clones shown in the figure were selected based on high positive binding to recombinant HA1 by phage-ELISA. (B) Sequence analysis of V_H and V_L genes selected from the phage display library. These clones belong to the VH-3, VH-4 and VH-5 families, with the majority related to the VH-3 family.

scFv showed a specific band of about 26–34 kD, while no band was found in the negative control TOP10F'cultured pellet.

3.3. Microneutralization assays

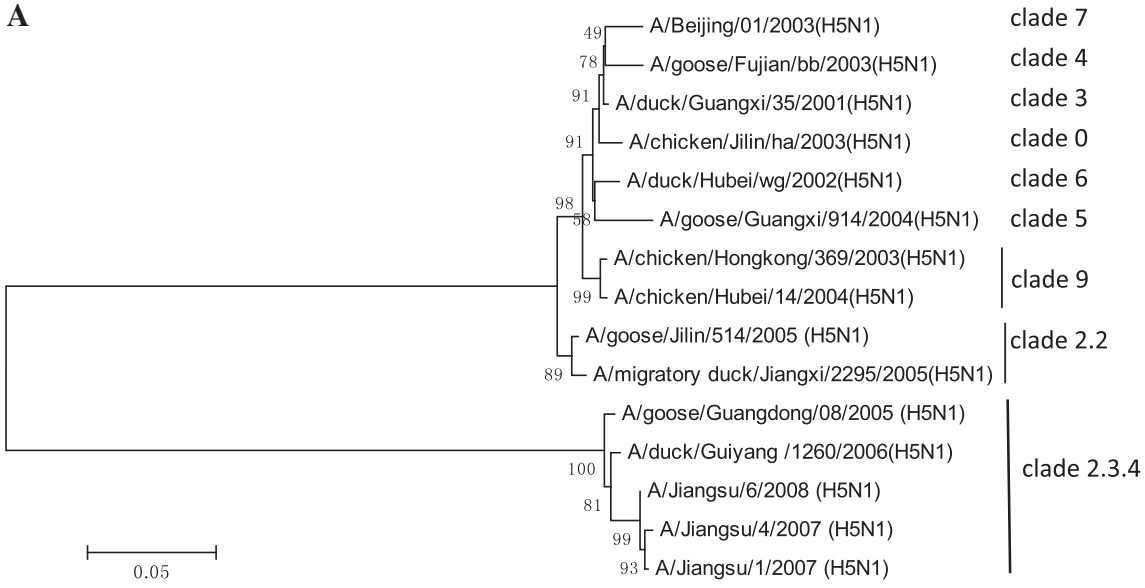
Subclades of the the six H5N1 viruses used in this study were first determined by RT-PCR amplification of the HA gene from extracted virus RNA samples and compared with reference sequences downloaded from the WHO sequence database (http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html). Distance calculation, tree construction methods and bootstrap analysis were implemented using MEGA version 5.0 (Tamura et al., 2011). The phylogenetic analysis indicated that the A/goose/Guangdong/08/2005 (H5N1), A/Jiangsu/4/2007 (H5N1), A/Jiangsu/1/2007 (H5N1) and A/Jiangsu/6/2008 (H5N1) strains clustered closely to viruses from clade 2.3.4; the A/goose/Jilin/514/2005 (H5N1) strain clustered closely to viruses from clade 2.2; and the A/chicken/Hongkong/369/2003 (H5N1) strain clustered closely to viruses from clade 9 (Fig. 3A).

All nine purified scFv antibodies were tested for efficacy in the neutralization assay. Four of the scFvs could neutralize all six H5N1 viruses, with 4F5 showing the best effect, while others had neutralization activity against only the A/Jiangsu/1/2007 (H5N1) virus or none of them. No neutralization activity was detected with the anti-CTGF mAb or PBS controls to any of the six viruses.

As shown in Fig. 3B, the EC_{50} values of 4F5 scFv against A/goose/Guangdong/08/2005 (H5N1), A/goose/Jilin/514/2005 (H5N1) and A/chicken/Hongkong/369/2003 (H5N1) were less than 0.1 μg/ml, while those for A/Jiangsu/6/2008 (H5N1) and A/Jiangsu/4/2007 (H5N1) were high, up to 6.25 μg/ml. The EC_{50} of 4F5 scFv against A/chicken/Hongkong/369/2003 (H5N1), A/goose/Guangdong/08/2005 (H5N1) and A/goose/Jilin/514/2005 (H5N1) ranged from 0.1 to 0.39 μg/ml, while those for A/Jiangsu/4/2007 (H5N1), A/Jiangsu/6/2008 (H5N1) and A/Jiangsu/1/2007 (H5N1) ranged from 0.39 to 6.25 μg/ml, nearly 1–62 times higher than the values above. The EC_{50} for A/goose/Guangdong/08/2005 (H5N1) was significantly different from the ones for A/Jiangsu/4/2007 (H5N1), A/Jiangsu/6/2008 (H5N1) and A/Jiangsu/1/2007 (H5N1), although they all belong to clade 2.3.4. Meanwhile, the EC_{50} for A/goose/Guangdong/08/2005 (H5N1) was comparable to those for A/goose/Jilin/514/2005 (H5N1) and A/chicken/Hongkong/369/2003 (H5N1), which are avian in origin, as well as A/goose/Guangdong/08/2005 (H5N1). In general, the EC_{50} values for the H5N1 influenza A viruses of human origin exceeded those for H5N1 viruses of avian origin.

3.4. HI assays

HI tests were used to detect whether the 4F5 scFv could neutralize viruses by preventing the binding of virions to target cells.



B

Influenza A virus	4F5 (μg/ml)	
	EC_{50} activity	HI activity
A/chicken/Hongkong/369/2003	<0.1	0.54
A/goose/Jilin/514/2005	<0.1	1.08
A/Jiangsu/4/2007	6.25	4.30
A/goose/Guangdong/08/2005	<0.39	1.08
A/Jiangsu/6/2008	6.25	4.30
A/Jiangsu/1/2007	0.39	2.15

Fig. 3. Analyses of the six H5N1 influenza A viruses used in the study and the neutralizing activity and HI activity of the purified 4F5 scFv against these viruses. (A) Clades of different H5N1 influenza A viruses. Phylogenetic tree of H5N1 influenza A viruses, based on full-length HA sequences. The nucleotide sequences were analyzed by using the neighbor-joining method. (B) Neutralizing activity (EC_{50}) and HI activity of purified 4F5 scFv against H5N1 influenza A viruses.

In HI assays, binding of the scFv to, or nearby, the receptor binding site (RBS), would cause steric hindrance that prevents hemagglutination. As shown in Fig. 3B, 4F5 scFv displayed HI activity against the six viruses, but at different concentrations. The minimum concentrations of 4F5 scFv required to inhibit hemagglutination of the H5N1 viruses A/chicken/Hongkong/369/2003 (H5N1), A/goose/Jilin/514/2005 (H5N1) and A/goose/Guangdong/08/2005 (H5N1) ranged from 0.54 to 1.08 μg/ml; while the HI concentrations for A/Jiangsu/4/2007 (H5N1), A/Jiangsu/6/2008 (H5N1) and A/Jiangsu/1/2007 (H5N1) were higher, from 2.15 to 4.30 μg/ml. The anti-CTGF mAb and PBS did not show HI activity to any of the six viruses.

3.5. Western blotting and immunofluorescence assays

As shown in Fig. 4, when detected with 4F5 scFv, all six viruses showed a ~70 kD protein band, which is the predicted molecular weight of the full-length HA protein (HA0). Another protein band around 43 kD was also detected for each virus corresponding to HA1. This result also confirmed that the binding site of 4F5 scFv is located on the HA1 domain. The 43 kD band also appeared with the positive control recombinant HA1 protein.

To investigate the binding activity of the scFv to the native form of the HA protein, immunofluorescence assays were performed. When cells were observed, binding of 4F5 scFv to MDCK cells infected by the six viruses was evident by the purple colored nuclei, with red signals around the nucleus. Meanwhile, no red signals were observed with normal MDCK cells. Thus, 4F5 scFv was able to bind to the native as well as the denatured forms of the HA protein.

3.6. Epitope mapping of the HA protein with scFv antibodies

HA epitopes were identified by the Ph.D-12 random peptide phage display approach. After three rounds of selection, 20 clones were randomly isolated, and their DNA sequences were determined. Some of them were frequently found in several clones. The sequences were compared with the HA1 sequences. As shown in Fig. 5A, the isolated 12-mer mimotopes share a common sequence, WLLP, homologous to (76)WLLGNP(81) in the amino acid sequence of HA1 (Fig. 5B).

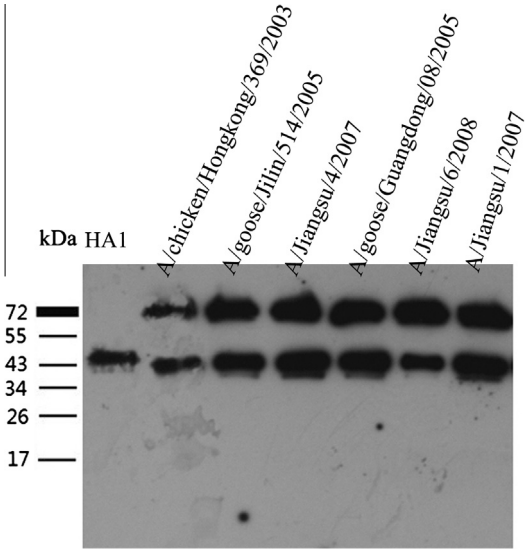


Fig. 4. Western blotting analysis of 4F5 scFv binding to denatured HA in reducing conditions. Note that the 4F5 scFv clone binds with two protein species with molecular masses of about 70 kD and 43 kD, the H5N1 influenza A HA protein and its major subunit HA1, respectively.

In further analyses, an HA1 mutant was designed by introducing two mutations, T > G at 230 bp and 233 bp, resulting in two amino acids changes (from WLLGNP to WRRGNP). As shown in Fig. 6B,when the mutant HA1 protein was probed with an HRP-conjugated anti-His antibody, one 43 kD band could be observed, while no specific band emerged when the HRP-conjugated 4F5 scFv was used (HRP was conjugated to 4F5 by the $NaIO_4$ method (Tsang et al., 1995)). This result further confirmed the WLLGNP epitope as the binding site for 4F5 scFv.

Antigen/antibody reactions occur when the epitope and paratope fit tightly, and hydrophobic amino acids play an important role in formation of the epitope. We confirmed that the WLLGNP sequence with its conserved hydrophobic residues is the binding epitope for 4F5 scFv. To determine whether these hydrophobic amino acids can form a concave pocket to interact with the scFv, 3D structures of the HA protein was modeled by using UCSF Chimera Viewer, and the putative binding site was observed.

For influenza A viruses, there are currently five antibody binding epitopes identified (Matrosovich et al., 2000; Duvvuri et al., 2009). According to these reports, amino acid sequences and their positions of the five epitopes in HA1 (A/Jiangsu/ 1/ 200 7 (H5N1)) were shown in Fig. 5C. The target binding site for 4F5 scFv is located in the E epitope region which contains the (76)WLLGNP(81) peptide (Fig. 6A and C). From the 3D models, an α-helix binding pocket formed by those residues can be clearly observed (Fig. 6C and D). Thus, this specific peptide can mimic the epitope on the HA protein and bind with the scFv.

3.7. Antiviral effects of scFv treatment pre- and post-challenge with H5N1 viruses in embryonated chicken eggs

4F5 scFv was used to treat eggs before or after challenge with two viruses of different origins, A/chicken/Hongkong/369/2003 (H5N1) and A/Jiangsu/6/2008 (H5N1). In the group pre-treated with 4F5 scFv at 100 μg/kg, the survival rate of eggs after challenge with A/chicken/Hongkong/369/2003 (H5N1) was 100% on day 8; meanwhile, with the A/Jiangsu/6/2008 (H5N1) challenge virus, pre-treatment with 4F5 scFv at 200 μg/kg was required to obtain 100% *antiviral effect*. As expected, the PBS control treatment did not protect the eggs from lethal viral challenge (Fig. 7A and B).

In groups post-treated with 4F5 scFv at 500 μg/kg, the survival rates were 100% and 62.5% after challenge with A/chicken/Hongkong/369/2003 (H5N1) and A/Jiangsu/6/2008 (H5N1), respectively. Furthermore, higher concentrations of 4F5 scFv did not improve the survival of A/Jiangsu/6/2008 (H5N1) challenged eggs, while all embryos died in the PBS treated group (Fig. 7C and D).

4. Discussion

H5N1 viruses that have spread from wild birds to domestic poultry can potentially infect humans. Thus, it is essential to develop effective therapies for the worldwide population at risk for zoonotic infections as well those subsequently infected by human-to-human transmissions.

Although there are other membrane-bound proteins on the virion, such as NA and M2, HA is the primary target for neutralizing antibodies (Daniels et al., 1987). HA is a homotrimer, with each monomer consisting of 2 subunits, HA1 and HA2. In this study, only the HA1 region was expressed. Since HA1 cannot form trimers without HA2, the HA1 monomer may have exposed epitopes, which are normally buried in the native protein, for rare functional antibody binding.

A

12-mer peptide	Spot intensity
APWNAPT**WLLP**P	5
TSWTAPL**WLLP**A	4
NKWSPPA**WLLP**P	4
YTSRI**WLLP**LAK	1
LQPWSSS**WLLP**N	3
TAS**WLLP**RTRPV	1
STTLPTPTNRTS	1
IPSFTMSARTGA	1

B

Viruses	Clade	--------70S----------80S-------
A/chicken/Hongkong/369/2003 (H5N1)	9	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/goose/Jilin/514/2005(H5N1)	2.2	CSVAG**WLL**GN**P**MCDEF*L*NVP
A/Jiangsu/4/2007(H5N1)	2.3.4	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/goose/Guangdong/08/2005(H5N1)	2.3.4	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/Jiangsu/6/2008 (H5N1)	2.3.4	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/Jiangsu/1/2007(H5N1)	2.3.4	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/Beijing/01/2003(H5N1)	7	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/goose/Fujian/bb/2003 (H5N1)	4	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/duck/Guangxi/35/2001 (H5N1)	3	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/chicken/Jilin/ha/2003 (H5N1)	0	CSVAG**WLL**GN**P**MCDEF*I*NVP
A/duck/Hubei/wg/2002(H5N1)	6	CSVAG**WLL**GN**P**MCDEF*T*NVP
A/goose/Guangxi/914/2004(H5N1)	5	CSVAG**WLL**GN**P**MCDEF*I*NVP

C

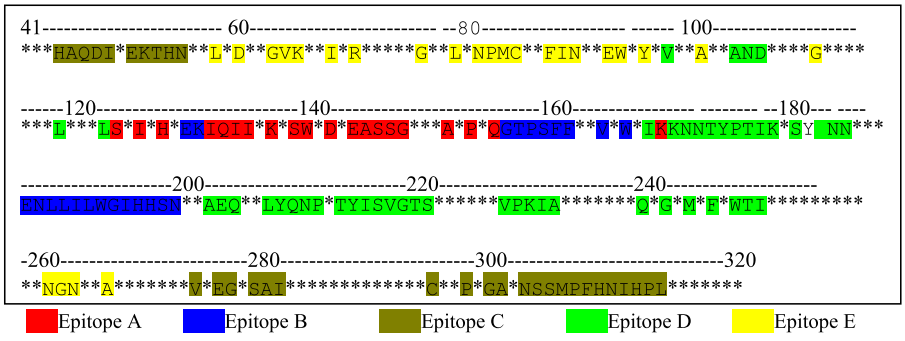


Fig. 5. Epitope mapping of the HA protein binding site with the 4F5 scFv antibody. (A) 12-mer peptides obtained from the Ph.D. 12 library. The isolated 12-mer mimotopes share a common sequence, WLLP, homologous to (76)WLLGNP(81) within the HA amino acid sequence. (B) Protein sequence alignment for residues 70 to 90 in HA of the H5N1 influenza A viruses belong to different clades. The differences in amino acid residues between the different strains are highlighted in italic type. (C) Amino acid sequences and their positions of 5 antibody-binding sites (or epitopes) in HA1 (A/Jiangsu/1/2007 (H5N1)). Different epitope amino acids marked with different colors.

By panning the library, we obtained four scFvs which could neutralize all six H5N1 viruses. Previous reports showed that antibody neutralizing potency is dependent on the H5N1 virus clade to which it binds. Interestingly, the EC_{50} and HI values of 4F5 scFv were related to the origin of the H5N1 viruses in this study. Although 4F5 scFv was screened with the HA1 of A/Jiangsu/1/2007 (H5N1), which belongs to clade 2.3.4, its EC_{50} for clade 9 A/chicken/Hongkong/369/2003 (H5N1) virus was lower than those for other human origin viruses in clade 2.3.4. This observation could be explained by the highly pathogenic H5N1 influenza A viruses possessing the polybasic amino acid sequence at the HA-cleavage site, which is easily cleavable from HA0 into HA1 and HA2 during replication in cells. It is also possible that 4F5 scFv is less accessible to the H5N1 viruses of human origin than to those of avian origin.

As expected, 4F5 scFv was able to bind to the native as well as the denatured forms of the HA protein of viruses from different clades. Detection of the 70 kD HA0 and 43 kD HA1 established that the 4F5 could block virus binding to the host cell, consistent with results of the HI assay. Based on these results and analysis of the 3D models, we concluded that 4F5 scFv binds at a distance from the RBS, which may cause steric hindrance during engagement of the receptor with the virus, thereby impeding viral entry. The 4F5 scFv yielded a 100% survival rate and at least a 62.5% survival rate by pre-treatment and post-treatment, respectively, relative to viral challenge of embryonated eggs. The results indicated that the scFv could be used against avian influenza, but it demonstrated a relatively low efficacy against human strains. For the treatment of human origin strains, changing the mode of antibody delivery or use of the scFv in combination treatment with a vaccine will considered in further studies.

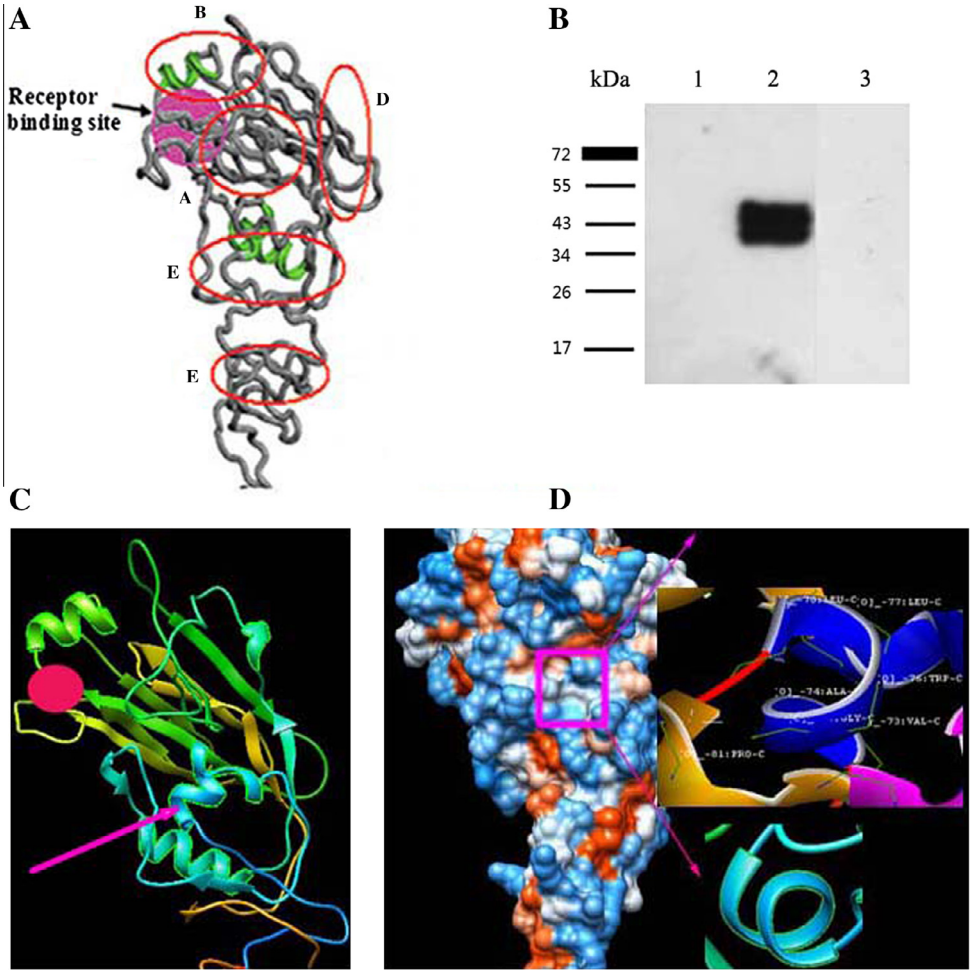


Fig. 6. Mutant analysis and 3D modeling used to map the binding epitope in the HA1 subunit. (A) HA1 monomer subunit (Protein Data Bank ID code 1hgf) showing five known antibody-binding sites (or epitopes) and the RBS. (B) Western analysis of the mutant HA1 protein. Lane 1 was normal Sf9 cells, the mutant HA1 protein detected with an HRP-conjugated anti-His antibody (lane2), one 43 kD band could be observed, while no specific band emerged when the HRP-conjugated 4F5 scFv was used (lane3). (C) The target binding site for 4F5 scFv is located in the E epitope region, which contains the (76)WLLGNP(81) peptide, the arrow indicates the binding pocket. (D) A surface representation of A/Jiangsu/1/2007(H5N1) HA1 protein, the conserved pocket on hemagglutinin shown in the pink rectangle. The WLL residues, at position 76 and 78 are labeled, can form half of an α-helix (upper right corner), and the whole a-helix was shown in the lower right corne.

In further analyses, the Ph.D.-12 library, mutant assay and 3D model were used to map the binding epitope of 4F5 scFv to the WLLGNP peptide in the HA1 subunit. The WLL residues can form half of an α-helix, one of the three α-helixes in the HA1 domain (Fig. 6C and D). An amino acid sequence alignment showed that this epitope is well conserved among all viruses in this study. According to the influenza database, this epitope is conserved in over 95% of sequenced H5N1 viruses strains sequenced (http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html).

Sui et al. screened nine antibodies employing the germline gene VH1-69 which effectively neutralized all group 1 influenza viruses tested (Sui et al., 2009). The crystal structure of one such antibody binding to H5 was shown to block infection by inserting its heavy chain into a conserved pocket in the stem region, thus preventing membrane fusion. (Ekiert et al., 2009) obtained the Fab CR6261 recognizing a highly conserved helical region in the membrane-proximal stem of HA1 and HA2, and the antibody was found to neutralize the virus by blocking conformational rearrangements associated with membrane fusion. (Oh et al., 2010) developed the mAb 9F4 recognizing HA proteins of three heterologous strains of H5N1 influenza A viruses of clades 1, 2.1 and 2.2, and epitope mapping revealed that the mAb binds to a previously undescribed epitope below the globular head of the HA1 subunit 2010. Recent reports of these broad neutralizing mAbs against influenza viruses that are they could not induce chicken erythrocytes to generate hemagglutination inhibition, although they could bind different amino acids in both HA1 and HA2. Compared to these mAbs, 4F5 scFv in our study showed hemagglutination inhibition. The WLLGNP peptide is a novel conserved epitope of HA1 not reported in other papers, This provide groundwork for the design of novel vaccine constructs aimed at providing broad-spectrum immunity to influenza viruses. Furthermore, the IGHV family of 4F5 scFv was analysed. According to the early studies, some of virus-neutralizing human antibodies belong to the IGHV1 family. In this family, the IGHV1-69 is the common one with typical characteristics of antibody (Ekiert et al., 2009; Sui et al., 2009; Zhou et al., 2007). However, antibodies belong to IGHV3 family have also been reported in recently researches (De Marco et al., 2012; Kuwata et al., 2013), and the 4F5 scFv was one member of the family. Further studies on 4F5 scFv will helpful to understand IGHV3 family antibodies' biological features.

In conclusion, we used a human immune antibody phage-display library to select a neutralizing scFv antibody that was shown to be effective against clade 2 and clade 9 H5N1 viruses. Binding of 4F5 scFv likely impedes viral entry by steric hindrance, and it

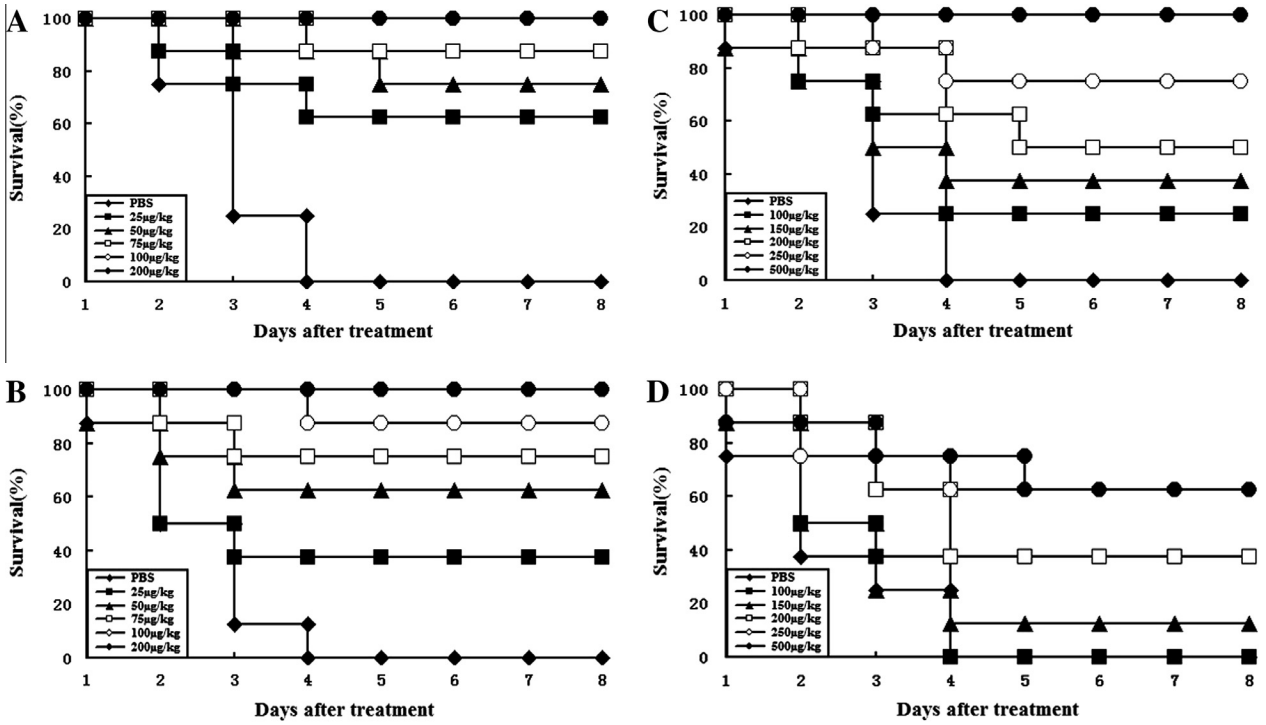


Fig. 7. Survival of embryonated chicken eggs conferred by 4F5 scFv antibody treatment against A/chicken/Hongkong/369/2003 (H5N1) (clade 9) and A/Jiangsu/6/2008 (H5N1) viruses (clade 2.3.4). In the pre-treatment groups, 8 embryonated chicken eggs were injected with the 4F5 scFv antibody at different doses and then challenged with A/chicken/Hongkong/369/2003 (H5N1) (clade 9) or A/Jiangsu/6/2008 (H5N1) (clade 2.3.4) (10 EID_{50}) 0.5 h later. Survival of the eggs was observed daily until day 8 after the challenge (A, B). In the post-treated groups, 4F5 scFv was administered 1 h after the viral challenge, and the survival of the eggs was monitored as described above (C, D).

showed satisfactory protection against H5N1 influenza A viruses in pre- and post-challenge studies in an embryonated chicken eggs model. The unique conserved WLLGNP binding epitope for 4F5 scFv located at the HA1 domain can be utilized as a potential candidate for vaccine preparation or RNA interference in the event of an H5N1 influenza A virus pandemic.

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