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Chimerization and characterization of a monoclonal antibody with potent neutralizing activity across multiple influenza A H5N1 clades

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ABSTRACT

The persistent evolution and circulation of highly pathogenic avian influenza H5N1 viruses pose a serious threat to global heath and hamper pandemic preparedness through conventional vaccine strategies. Combination passive immunotherapy using non-competing neutralizing antibodies has been proposed as a viable alternative to provide broad protection against drift variants. This necessitates the pre-pandemic production and characterization of potently neutralizing monoclonal antibodies (MAbs). One such antibody, MAb 9F4 was shown to provide heterologous protection against multiple H5N1 clade viruses, including one of the recently designated subclades, namely 2.3.4, through binding to a novel epitope, warranting its further development and characterization as a therapeutic candidate. In this study, the conversion of MAb 9F4 from mouse IgG_{2b} to mouse-human chimeric (xi) IgG_1 and IgA_1 was achieved. These chimeric MAb versions were found to retain high degrees of binding and neutralizing activity against H5N1. The demonstration that xi-IgA_1-9F4 retains a fairly high level of neutralizing activity, which is ~10-fold lower than the corresponding xi-IgG_1 isotype, suggests that this MAb could be further developed and engineered for intranasal administration.

1. Introduction

Highly pathogenic avian influenza A (HPAI) virus H5N1 remains a serious threat to global health due to its unabated and widespread geographical circulation (Chen et al., 2006; Wang et al., 2008a). Although human cases remain sporadic, the absence of human herd immunity, the high lethality (WHO, 2014) and potential ability of HPAI H5N1 to gain efficient human transmissibility (Herfst et al., 2012; Imai et al., 2012), all point towards a potentially catastrophic pandemic.

The establishment and continual antigenic drift of H5N1 viruses in poultry and wild bird populations has led to the evolution of diverse lineages with distinct geographical distribution (Guan and Smith, 2013; Pfeiffer et al., 2011). This ongoing evolution of H5N1 viruses hampers vaccine development and enables emerging resistance to both adamantanes and neuraminidase inhibitors (Chao et al., 2012; Le et al., 2005). The increased usage of antiviral drugs may also contribute to the development of resistance (Tang et al., 2008). As such, there is a strong urgency for alternative strategies to be developed. Since antibodies are crucial in the protection against infection, passive immunotherapy is increasingly being explored as a viable option [reviewed in (Ye et al., 2012)]. The effectiveness of this approach has been documented for treatment of severe influenza illness during the 1918 pandemic influenza (Luke et al., 2006) and, more encouragingly, H5N1 virus infected patients (Wang et al., 2008b; Zhou et al., 2007). Furthermore, several preclinical studies demonstrate the protective ability of neutralizing monoclonal antibodies (MAbs) against lethal H5N1 challenge (Du et al., 2013; Meng et al., 2013; Ye et al., 2010). The use of MAb is advantageous over traditional convalescent blood products in terms of availability and inter-batch consistency.

Due to its abundance and role in virus entry, the surface hemagglutinin (HA) glycoprotein elicits the production of neutralizing antibodies and this forms the basis of conventional vaccination and most passive immunotherapeutic strategies. These antibodies confer protection against infection as they block viral entry into host cells by interfering with virus attachment or by preventing HA-mediated membrane fusion during virus uncoating. However,

Abbreviations: HA, hemagglutinin; HApp, lentiviral pseudotyped particles; HPAI, highly pathogenic avian influenza; MAb, monoclonal antibodies; xi, chimeric.

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because influenza replication is error prone, selection of escape mutants may occur if strategies are based on single MAb formulations (O'Donnell et al., 2012). To overcome this problem, a combination of non-competing MAbs can be used synergistically to confer broad protection while preventing emergence of escape variants. Proof of this concept has been shown for several respiratory viruses, including HPAI H5N1 (Prabakaran et al., 2009; ter Meulen et al., 2006). Thus, a library of well characterized neutralizing MAbs could facilitate rapid accessibility to and selection of the appropriate combinations for passive immunotherapy during an outbreak.

Although fully human anti-H5N1 HA neutralizing MAbs have been described, the generation of such antibodies typically require H5N1 convalescent donors as cross-protective antibodies obtained from patients previously immunized with other subtypes of influenza are rare (Corti et al., 2011). Consequently, the mouse hybridoma technology continues to be a popular method for the *in vitro* generation of anti-H5N1 HA MAbs. However, murine antibodies will elicit a non-self immune response in humans, rendering them useless or even harmful if used directly for immunotherapy. A common solution is to make mouse-human chimeric constructs, consisting of the original mouse variable antibody domains fused to human constant domains. The resultant chimeric (xi-) MAb should retain the binding properties of the original mouse MAb, but with reduced immunotoxicity.

MAb 9F4 is a mouse IgG_{2b} antibody with neutralizing activity against multiple H5N1 viruses and recognizes a novel epitope (^{260}I/LVKK^{263}, according to H3 numbering) (Oh et al., 2010) that is situated away from previously characterized antigenic sites on HA globular head (Underwood, 1982; Wiley et al., 1981). This suggests that MAb 9F4 may be used in synergy with other characterized MAbs for combination immunotherapy. In this study, we tested the ability of MAb 9F4 to bind HA of one of the recently designated subclades, namely 2.3.4, of H5N1 and extended the antigenic characterization of the MAb. Because of its potent neutralizing activity across multiple H5N1 clades and subclades, two chimeric (xi-) versions of the MAb, xi-IgG_1-9F4 and xi-IgA_1-9F4, were generated and tested to assess their therapeutic potential.

2. Materials and methods

2.1. Cell lines and transient transfection

293FT cells were from Invitrogen. MDCK and HeLa cells were from American Type Cell Collection (Manassas, VA, USA). All cell lines were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Growth media for 293FT and HeLa cells were further supplemented with non-essential amino acids and antibiotics.

Transient transfection experiments were performed using Lipofectamine™ 2000 reagent (Invitrogen), according to manufacturer's instruction. Where needed, transfected cells were used directly for immunofluorescence experiments or lysed with a lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% NP-40, 0.5% deoxycholic acid (sodium), 0.025% SDS, and 1 mM phenylmethylsulfonyl fluoride for downstream ELISA and Western blot analysis.

2.2. HA expressing plasmids and HA recombinant proteins

The HA expressing plasmids used in this study contained full length HA coding sequences from Hatay04 [clade 1 virus: A/chicken/Hatay/2004(H5N1)], VN04 [clade 1 virus: A/Vietnam/1203/2004(H5N1)], Indo05 [clade 2.1 virus: A/Indonesia/5/2005(H5N1)], India06 [clade 2.2 virus: A/chicken/India/NIV33487/2006(H5N1)] and DL06 [clade 2.3.4 virus: A/duck/Laos/3295/2006(H5N1)] (Genbank accession numbers AJ867074, EF541403,EU146622, EF362418 and DQ845348, respectively).

2.3. Mouse MAbs

Mouse MAb 9F4 and mouse MAb 8F8 were generated using previously established protocol (Oh et al., 2010). For all assays, MAb 8F8, specific for M1 of Hatay04, was used as a negative control antibody.

2.4. Cloning and expression of xi-IgG_1-9F4 and xi-IgA_1-9F4

Total RNA was extracted from MAb 9F4 hybridoma by using RNeasy kit (Qiagen) and used for first strand cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen). Variable heavy (VH) and variable light (VL) genes were amplified in subsequent PCR using Expand High Fidelity PCR (Roche). The Ig-primer set (Novagen) was used for these reactions, according to manufacturer's instruction. PCR products were cloned into pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen) and sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Variable regions were then defined using the IMGT database (Ehrenmann et al., 2010).

Variable region specific primers were designed to introduce *Mfe1* and *Xho1*; and *ApaL1* and *Pst1* restriction sites to respectively flank MAb 9F4 VH and VL coding sequences by PCR. This enabled the ligation of MAb 9F4 VH to human IgG1 heavy chain constant (CH) domain and MAb 9F4 VL to light chain kappa constant domain (CL) in a single IgG1 constant region expression vector, as previously described (Hanson et al., 2006).

Variable region specific primers were designed to introduce *EcoRI* and *NheI*; and *EcoRI* and *BsiWI* restriction sites to respectively flank MAb 9F4 VH and VL coding sequences by PCR. This enabled the ligation of MAb 9F4 VH to the human IgA_1 CH domain within pFUSEss-CHIg-hA1 cloning plasmid and the MAb 9F4 VL to the human CL kappa domain within pFUSE2ss-CLIg-hK cloning plasmid. Both pFUSEss-CHIg-hA1 and pFUSE2ss-CLIg-hK cloning plasmids were purchased from InvivoGen. After successful incorporation of MAb 9F4 sequences, the plasmids were co-transfected into 293FT cells as described.

The chimeric constructs were transiently transfected into 293FT cells as described. Expression of xi-IgG_1-9F4 was checked by immunofluorescence analysis while expression of xi-IgA_1-9F4 was checked by Western blot. Cell culture supernatants containing the respective chimeric MAb were collected at 24 and 72 h post transfection. xi-IgG_1-9F4 and xi-IgA_1-9F4 MAbs were extracted from the pooled supernatants using a HiTrap protein G and HiTrap protein A columns (GE Healthcare) respectively, according to manufacturer's instructions. Purity of chimeric MAb was confirmed using SDS–PAGE analyses.

2.5. Immunofluorescence analysis

293FT or MDCK cells were seeded on coverslips 24 h prior to transient transfection with appropriate expression vectors. 24 h post transfection, the coverslips were washed twice with 1× phosphate-buffered saline (PBS) and cells were fixed with 4% paraformaldehyde (PFA) for 10 min. The coverslips were washed and cells were permeabilized with 0.1% Triton-X for 10 min, where necessary. The coverslips were washed and blocked with 1% BSA in 1XPBS for 30 min and incubated with primary MAbs diluted in 1% BSA in 1XPBS for 2 h. After washing to remove unbound MAbs, the cells were incubated with Alexa Fluor® 488-conjugated goat anti-human IgG or Alexa Fluor® 488 conjugated goat anti-mouse

IgG (Molecular Probes®) for 1 h. Unbound secondary antibodies were removed by washing and the coverslips were mounted onto microscope slides using Fluorosave mounting medium (Calbiochem, Merck Chemicals Ltd). Images were obtained using an epi-fluorescence microscope (Olympus BX60).

2.6. Pseudotyped lentiviral particle neutralization assay

Lentiviral pseudotyped particles (HApp) harboring the H5N1 HA glycoprotein were generated by co-transfection of 293FT cells with an H5N1 HA expression plasmid and the envelope-defective pNL4.3.Luc.R^- E^- lentiviral vector. HA sequences corresponding to the aforementioned viruses were used to generate HApp as previously described (Oh et al., 2010). The neuraminidase gene from Hatay04 was also co-transfected to facilitate the release of pseudotyped particles from the 293FT cells. The culture supernatants were collected 24 h post transfection, and stored at −80 °C until use.

The HApp neutralization assay was performed as previously described (Oh et al., 2010). Briefly, MAbs were serially diluted in DMEM and mixed with an equal volume of HApp for 1 h. The mixture was used to infect MDCK cells, which were seeded in 12-well plates 24 h prior to infection. The infected MDCK cells were incubated at 37 °C for 72 h and were lysed with 250 μl of 1× luciferase cell lysis buffer (Promega) per well. 50 μl of the lysate was tested for luciferase activity by the addition of 50 μl of luciferase substrate (Promega) and luminescence was measured with a luminometer (Infinite M200, Tecan). Viral entry, as reflected by the relative light units (RLU), was expressed as a percentage relative to the absence of antibody. Each experiment was performed in duplicate.

2.7. ELISA

The total binding affinity of MAbs for specific test antigen was determined by direct ELISA. 96 well ELISA plates were coated with recombinant proteins or transfected cell lysates overnight at 4 °C and blocked with 5% milk for 1 h. Serially diluted MAbs in 2% milk were added to the plates and incubated for 1 h at 37 °C. The plates were washed six times with PBS containing 0.05% Tween-20 (PBST) and incubated with horseradish-peroxidase-conjugated secondary antibodies (ThermoScientific) for 1 h at 37 °C. The plates were washed six times with PBST before the reaction was visualized using the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (ThermoScientific) and stopped with 2 M H_2SO_4. The absorbance at 450 nm (A450) was measured using a plate reader.

2.8. Syncytial inhibition assay

HeLa cells seeded on glass coverslips were transiently transfected with Hatay04-HA as described. The cells were then treated with two test concentrations of each MAb for 1 h at 37 °C in 5% CO_2, 48 h post transfection. Unbound MAbs were removed by washing the cells with 1XPBS prior to treatment with low pH buffer for 15 min at 37 °C in 5% CO_2. Excess low pH buffer was removed by washing and the cells were allowed to recover in growth media for 3 h at 37 °C in 5% CO_2. Cells were stained with CellMask Orange (Invitrogen) at 1:5000 dilution and fixed with 4% PFA. Finally, the cells were mounted onto glass slides using VectorShield mounting media with DAPI (Vector Laboratories). Images were obtained using an epi-fluorescence microscope (Olympus BX60).

2.9. Statistics

Unpaired *t*-test was used to evaluate whether mouse and xi-MAbs differed in their binding or neutralizing activity from at least 3 sets of values for each ELISA and HApp neutralization assays.

3. Results

3.1. MAb 9F4 binds and prevents viral entry into MDCK cells mediated by HA of clade 2.3.4 H5N1

In 2007, a shift from clade 1 to clade 2.3.4 was reported for human H5N1 infections in Vietnam (Le et al., 2008). Clade 2.3.4 viruses have since disseminated to Myanmar, Laos, China, Hong Kong and Bangladesh, where they have been isolated from humans and domestic birds (WHO, 2009, 2012). As clade 2.3.4 viruses retain the previously identified MAb 9F4 epitope site (Fig. 1A), we tested the ability of MAb 9F4 to bind to HA from a clade 2.3.4 H5N1 virus by immunofluorescence analysis on non-permeabilized cells. As shown in Fig. 1B, MAb 9F4 binds to native DL06-HA transiently expressed on the surface of MDCK cells.

The neutralizing ability of MAb 9F4 against HApp harboring DL06-HA was also examined. HApp contain the firefly luciferase reporter gene and permits the sensitive quantification of pseudovirus entry into host cells, which have been shown to display similar entry characteristics and neutralization titers as live virus (Garcia and Lai, 2011). MAb 9F4 inhibited the entry of DL06-HApp in a dose dependent manner, similar to clade 1 VN04-HApp ($p > 0.05$ at all concentrations tested). Whereas, the negative control antibody was unable to inhibit HApp entry into MDCK cells even when used at 10,000 ng/ml, which is 10 times higher than the highest concentration of MAb 9F4 used (Fig. 1C). The half-maximal inhibitory concentration (IC_{50}) for DL06-HApp was about 10 ng/ml, similar to clade 1 VN04-HApp as previously reported (Oh et al., 2010) and included in this experiment as a positive control.

3.2. Production of xi-IgG_1-9F4 and xi-IgA_1-9F4

The ability of MAb 9F4 to potently neutralize clade 1 and multiple clade 2 viruses from subclades 2.1, 2.2 (Oh et al., 2010) and 2.3.4 (Fig. 1C) makes it an attractive lead antibody for passive immunotherapy as viruses from these clades and subclades have caused human infection (Abdel-Ghafar et al., 2008; WHO, 2012). To minimize potential rejection of MAb 9F4 for use in humans, a mouse-human chimeric form of MAb 9F4, named as xi-IgG_1-9F4, was generated. Firstly, the VH and VL chains of MAb 9F4 were obtained from the messenger RNA of the hybridoma by using PCR method (Supplementary Fig. S1). To generate xi-IgG_1-9F4, specific gene fragments of VH and VL were then fused to the coding regions for CH chain of human IgG1 and CL of the kappa chain, respectively. The expression of xi-IgG_1-9F4 in 293FT cells was then checked by immunofluorescence staining. Positive immunofluorescence only in the presence of Alexa Fluor® 488-conjugated goat anti-human IgG confirmed the chimerization of MAb 9F4. No immunofluorescence was detected in the presence of Alexa Fluor® 488-conjugated goat anti-mouse IgG, indicating successful replacement of heavy and light chains to human forms (Supplementary Fig. S2A).

Similarly, a chimeric IgA_1 form of MAb 9F4 was generated by fusing 9F4 VH and VL to the coding regions for CH chain of human IgA_1 and CL of the kappa chain, respectively. 293FT cells were used as producer cells and expression of xi-IgA_1-9F4 was detected using anti-human-IgA-HRP conjugate antibody in Western blot analysis (Supplementary Fig. S2B).

3.3. xi-IgG_1-9F4 retains binding and neutralization ability

xi-IgG_1-9F4 binding to native H5 HA from multiple H5N1 clades was detected by fluorophore-conjugated-anti-human IgG (Fig. 2A) but not in the presence of fluorophore-conjugated-anti-mouse IgG (data not shown). This indicates that conversion to xi-IgG_1 was successful and does not impede cross-clade binding.

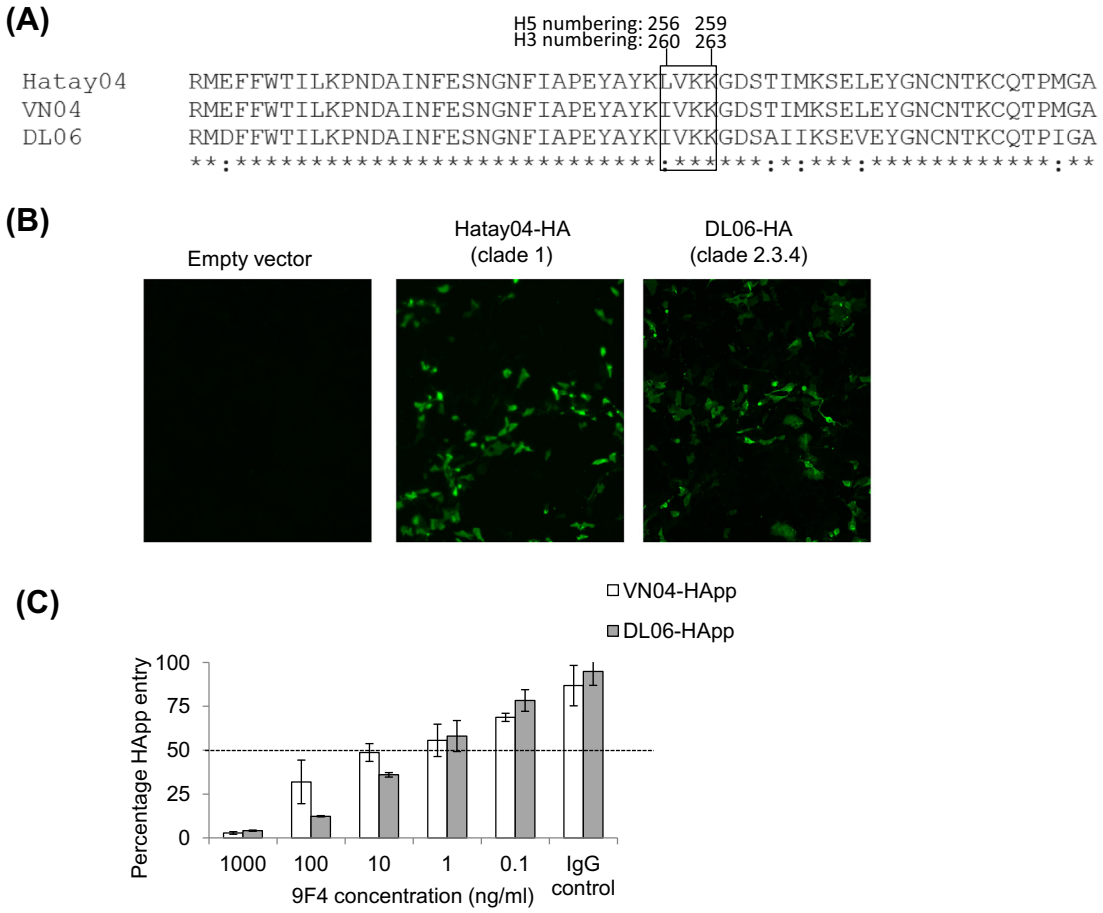

Fig. 1. MAb 9F4 binds and prevents viral entry into MDCK cells mediated by HA of clade 2.3.4 H5N1. (A) Alignment of residues 229 to 288 in the HA protein of a clade 2.3.4 H5N1 virus (DL06) with the corresponding domain in two clade 1 viruses (Hatay04 and VN04). An epitope within the HA1 subunit previously found to be essential for the interaction with MAb 9F4, which was generated using Hatay04-HA, is boxed. The H3 numbering is used throughout this paper. For comparison, the mature H5 numbering is included in this diagram. (B) MDCK cells were transfected with empty vector or DL06-HA or Hatay04-HA expressing plasmids. Binding of 9F4 to surface expressed recombinant HAs was detected via immunofluorescence assay performed on non-permeabilized cells. Cells were stained with MAb 9F4 followed by Alexa Fluor® 488-conjugated goat anti-mouse IgG antibody. Hatay04-HA (clade 1) transfected cells were included as a positive control. Original magnification ×10. (C) Pseudotyped lentiviral particles harboring the HA proteins from H5N1 influenza viruses of clade 1 (VN04-HApp) and clade 2.3.4 (DL06-HApp) were incubated with different concentrations of MAb 9F4 for 1 h before inoculation onto MDCK cells. Luciferase activity in the cell lysates was determined 72 h post-infection. Viral entry, as indicated by the luciferase activity measured in relative light units (RLU), was expressed as a percentage of the reading obtained in the absence of antibody, which was set at 100%. A control MAb 8F8 of the same isotype was used at 10,000 ng/ml. The experiments were repeated three times, and representative data are shown. Each histogram shows the mean of the values from duplicate wells. Error bars, standard deviations. Inset dotted lines demarcate approximate IC_{50}.

Next, the pseudotyped lentivirus particle neutralization assay was used as a quantitative measure of xi-IgG_1-9F4 activity compared to mouse 9F4. As shown in Fig. 2B–E, both mouse and xi-IgG_1-9F4 inhibited the entry of HApp containing the HA of various H5 clades in a dose dependent manner. As expected, the negative control antibody was consistently unable to inhibit HApp entry even when used at 10,000 ng/ml. Neutralization of Indo05-HApp and India06-HApp mediated by mouse and xi-IgG_1-9F4 was similar at all MAb concentrations tested. Neutralization of VN04-HApp and DL06-HApp mediated by xi-IgG_1-9F4 differed from mouse 9F4 only at the highest concentration tested, where xi-IgG_1-9F4 reduces HApp entry by approximately 90% compared to complete neutralization by mouse 9F4. Nevertheless, xi-IgG_1-9F4 retains high neutralizing potency similar to mouse 9F4, with an approximate IC_{50} of 10 ng/ml for all HApp tested.

3.4. Neutralization ability of xi-IgA_1-9F4 is decreased due to reduction in binding affinity

Unlike xi-IgG_1-9F4, the ability of xi-IgA_1-9F4 to neutralize VN04-HApp was significantly reduced at all MAb concentrations tested. xi-IgA_1-9F4 only inhibited 75% of VN04-HApp entry at 1000 ng/ml and has an IC_{50} of 100 ng/ml (Fig. 3A).

To account for the reduction in neutralization, we performed a comparative ELISA using total cell lysates from 293FT cells transiently expressing VN04-HA, Hatay04-HA and DL06-HA. These cell lysates contain all expressed forms of HA (precursor HA0 and mature disulfide-linked HA1–HA2 on cell surface) and were therefore suitable for assessing total binding affinity. The negative IgG control was used to determine the cut-off and the endpoint titer, defined as the MAb concentration that produces an A450 reading that is equivalent or lower than the cut-off (Frey et al., 1998).

While xi-IgG_1-9F4 and mouse 9F4 bound comparably to all H5 HA and at all MAb concentrations tested, binding by xi-IgA_1-9F4 was significantly decreased (Fig. 3B–D). The endpoint titer for xi-IgA_1-9F4 was 1250 ng/ml for all H5 HA tested, whereas xi-IgG_1-9F4 and mouse 9F4 still exhibited strong binding at this concentration.

3.5. Mouse and mouse-human chimeric form of MAb 9F4 comparably inhibit HA mediated fusion at low pH

It was previously suggested that MAb 9F4 inhibits fusion of viral and host endosomal membranes as MAb 9F4 did not show hemagglutination inhibition activity and was able to prevent low pH mediated HA conformational change (Oh et al., 2010). As xi-IgG_1-9F4

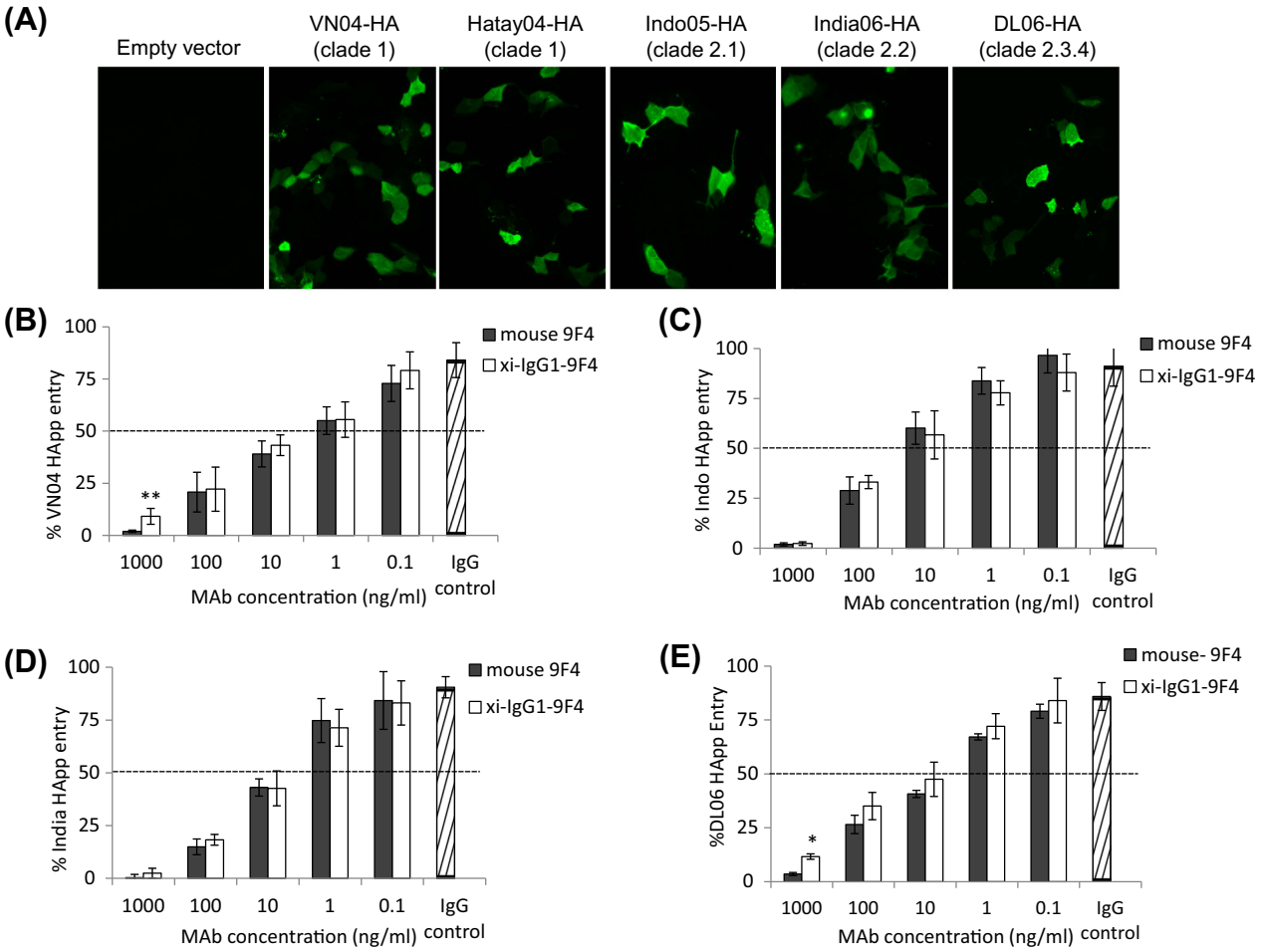

Fig. 2. xi-IgG$_1$-9F4 retains binding and neutralization ability against multiple H5N1 clades. (A) MDCK cells were transfected with empty vector or the various H5-HA expressing plasmids. Binding of xi-IgG1-9F4 to surface expressed recombinant HAs was detected via immunofluorescence assay performed on non-permeabilized cells. Cells were stained with xi-IgG1-9F4 followed by Alexa Fluor® 488-conjugated goat anti-human IgG antibody. Original magnification ×40. (B–E) Pseudotyped lentiviral particles harboring the HA proteins from the various representative clade H5N1 influenza viruses were incubated with different concentrations of MAb 9F4 or xi-IgG$_1$-9F4 for 1 h before inoculation onto MDCK cells. Luciferase activity in the cell lysates was determined 72 h post-infection. Viral entry, as indicated by the luciferase activity measured RLU, was expressed as a percentage of the reading obtained in the absence of antibody, which was set at 100%. A control MAb 8F8 of the same isotype was used at 10,000 ng/ml. The experiments were repeated three times, each in duplicates. Each histogram shows the mean of the values from all data. Error bars, standard deviations. Differences in binding by mouse and xi-IgG$_1$-9F4 were evaluated by unpaired *t*-test ($^*p < 0.05$, $^{**}p < 0.01$). Inset dotted lines demarcate approximate IC$_{50}$.

showed comparable binding and neutralizing activity as mouse-9F4, the ability of xi-IgG$_1$-9F4 to inhibit fusion was determined by means of a syncytial inhibition assay. Briefly, HeLa cells expressing HA were subjected to low pH treatment to allow HA-mediated cell membrane fusion. The resultant syncytia formation was analyzed by means of immunofluorescence staining. No syncytial formation was observed for untransfected cells (Fig. 4, first column), while large multinucleated syncytia bodies were observed for HA expressing HeLa cells in the absence of antibodies (Fig. 4, second column). It was observed that the pre-incubation of HA expressing HeLa cells with either mouse-9F4 and xi-IgG$_1$-9F4 reduced the amount and size of syncytia formation at a MAb concentration of 10 μg/ml and this reduction was more pronounced at 50 μg/ml (Fig. 4, fourth and fifth column). In contrast, the pre-incubation of HA expressing HeLa cells with an irrelevant mouse MAb 8F8 prior to low pH treatment did not prevent syncytia formation (Fig. 4, third column).

4. Discussion

Anti-H5N1 HA neutralizing antibodies can be classified according to their binding sites [reviewed in (Velkov et al., 2013)]. The majority of HA neutralizing MAbs targets the membrane distal receptor binding site (RBS) located on the globular head of HA1. Consequently, the selective antibody pressure drives antigenic drift and antibody escape. HA2 selective antibodies target the highly conserved fusion peptide region and therefore display broad cross-clade and varying degrees of heterosubtypic protection (Smirnov et al., 1999). However, a small number of neutralizing MAbs targeting non-RBS regions in HA1 have also been described. These MAbs are less well understood with some of them inhibiting the viral attachment step and others inhibiting post-attachment events. Some of these MAbs have been reported to provide homosubtypic cross-clade protection by binding conformation dependent epitopes (Cao et al., 2012; Hu et al., 2012). The novelty of these epitopes suggests that these MAb could be suitable in combination approaches with RBS selective or HA2 selective MAb in a polyclonal passive immunotherapeutic fashion and further discovery and evaluation of MAb within this obscure class is thus warranted.

MAb 9F4 is an example of neutralizing MAb targeting a non-RBS domain in HA1. MAb 9F4 protected mice against lethal H5N1 challenge and neutralizes clade 1, 2.1, 2.2 (Oh et al., 2010) and 2.3.4 HA-lentiviral pseudotyped particles (Fig. 1C). MAb 9F4 was found to be potently neutralizing, with IC$_{50}$ and IC$_{95}$ values approximating 10 and 100 ng/ml, respectively for the various H5N1 clades.

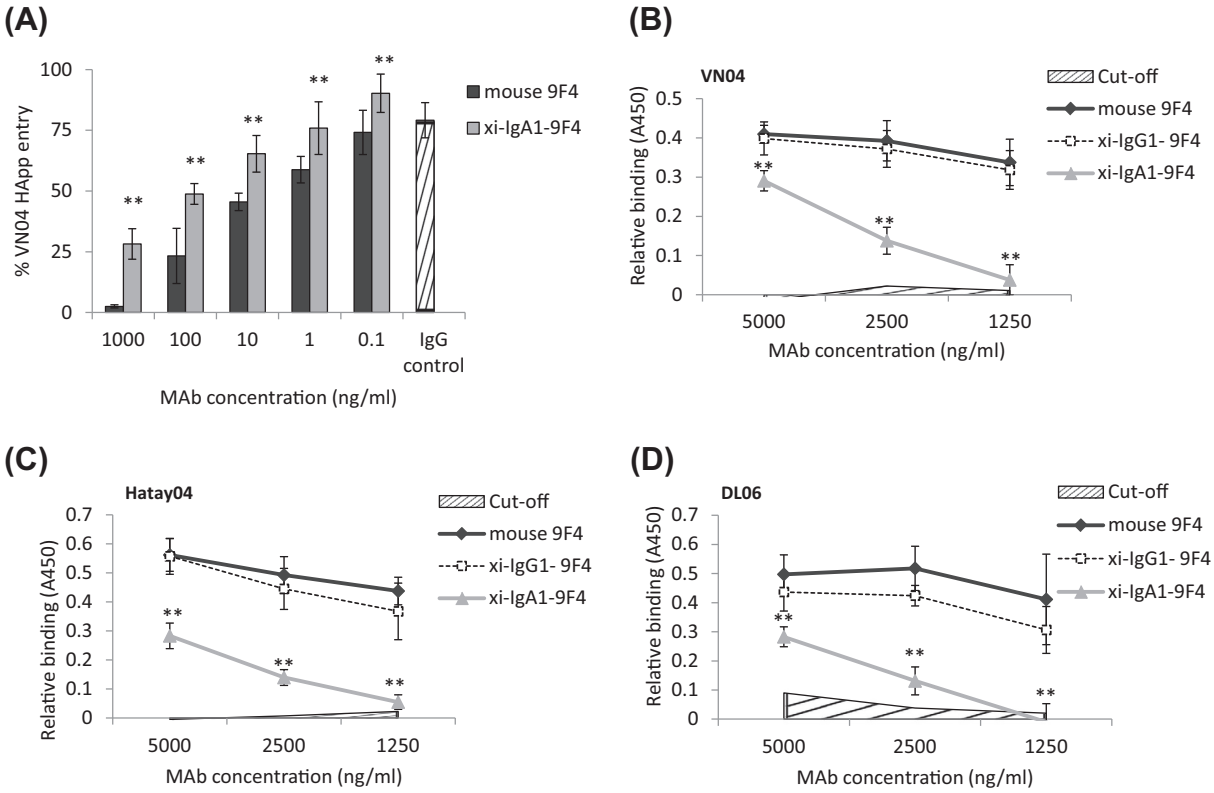

Fig. 3. Neutralization ability of xi-IgA$_1$-9F4 is reduced due to decreased binding affinity. (A) Pseudotyped lentiviral particles harboring the HA proteins from VN04 were incubated with different concentrations of MAb 9F4 or xi-IgA$_1$-9F4 for 1 h before inoculation onto MDCK cells. Luciferase activity in the cell lysates was determined 72 h post-infection. Viral entry, as indicated by the luciferase activity measured RLU, was expressed as a percentage of the reading obtained in the absence of antibody, which was set at 100%. MAb 8F8 was used at 10,000 ng/ml as a negative control MAb. The experiments were repeated three times, each in duplicates. Each histogram shows the mean of the values from all data. Error bars, standard deviations. Differences in binding by mouse and xi-IgA$_1$-9F4 were evaluated by unpaired *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$). Inset dotted lines demarcate approximate IC$_{50}$. (B–D) Comparative ELISA as performed to measure the binding of different forms of MAb 9F4 to fixed amount of cell lysates obtained from cells transfected with a cDNA construct expressing various H5 HA. All readings are normalized against cell lysates from 293FT cells transfected with empty vector alone. The experiments were repeated three times. Each point shows the mean of the values from all data. Error bars, standard deviations. The cut-off level was determined using a control mouse MAb. Differences in binding by mouse, xi-IgG$_1$-9F4 and xi-IgA$_1$-9F4 were evaluated by unpaired *t*-test ($^{**}p < 0.05$).

This is comparable to the anti-HA activity of other potently neutralizing MAbs (Cao et al., 2012; Corti et al., 2011; Du et al., 2013). To reduce immune rejection in humans, two chimeric forms of MAb 9F4 were created using recombinant molecular techniques. While xi-IgG$_1$-9F4 retained total binding affinity and neutralizing potency of mouse-9F4, xi-IgA$_1$-9F4 showed reduced binding and a 10-fold increase in the IC$_{50}$ value in the HApp neutralization assay. Since all three forms of the MAb 9F4 contain the same variable regions, the differences in binding affinity and neutralizing potency could be attributed to the differences in constant region domains. Although the variable antibody regions are usually expected to be sufficient for binding, constant regions have also been shown to participate through steric hindrances and inducing conformational changes in the targeted antigen (Nason et al., 2001).

As outcome of passive immunotherapy could be dependent on the efficacy by which passively transferred MAbs reach the sites of viral replication, 9F4 was reformatted into two chimeric isotypes in this study.

The xi-IgG$_1$ isotype was engineered as most characterized anti-H5N1 HA MAbs are IgG (Burioni et al., 2010; Corti et al., 2011; Hanson et al., 2006; Hu et al., 2012; Sun et al., 2009). However, the degree of protection observed by parentally administered IgG MAbs in mice could be due in part to the disseminated nature of viral replication in murine models. Although H5N1 has been reported to cause disseminated infection in humans, the lungs remain the main site of viral replication (Sirinonthanawech et al., 2011; Uiprasertkul et al., 2005). While IgG transudates from plasma to the lungs to mediate protection after intravenous administration, very high dosages are required to effectively eliminate nasal viral shedding (Renegar et al., 2004). To improve recovery of IgG at the lungs, vectored delivery directly at the nasopharyngeal mucosa has been suggested as a practical strategy. This approach has yielded encouraging results in mouse and ferret models of H5N1 infection, with the added advantage of antibody expression lasting up to 100 days (Limberis et al., 2013).

In this study, xi-IgA$_1$-9F4 was generated as this isotype is predominant in the nasal mucosa during influenza infection (Burlington et al., 1983) and the presence of specific secretory IgA in the upper respiratory tract is associated with resistance to severe respiratory disease (Weltzin and Monath, 1999). Thus far, only one IgA, generated using mouse hybridoma (Ye et al., 2010), has been reported. IgA potentially offers a few advantages over IgG. Firstly, IgA does not fix complement via the classical pathway (Woof and Russell, 2011) and is therefore believed to be less pro-inflammatory than IgG MAbs. This feature could be particularly important for H5N1 infection, where disease severity correlates with exacerbated inflammation. Secondly, IgA permits intranasal administration (Ye et al., 2010), allowing IgA to neutralize influenza A virus at the primary site of infection, thereby preventing colonization and invasion of host cells. Alternatively, dimeric IgA can be generated for systemic administration, allowing IgA to bind to polymeric IgG receptors (pIgR) located at the basal membrane of epithelial cells for transepithelial transport to the mucus layer (Tamura et al., 2005). Both routes of administration enable IgA access to the upper respiratory tract, where inhibition of viral

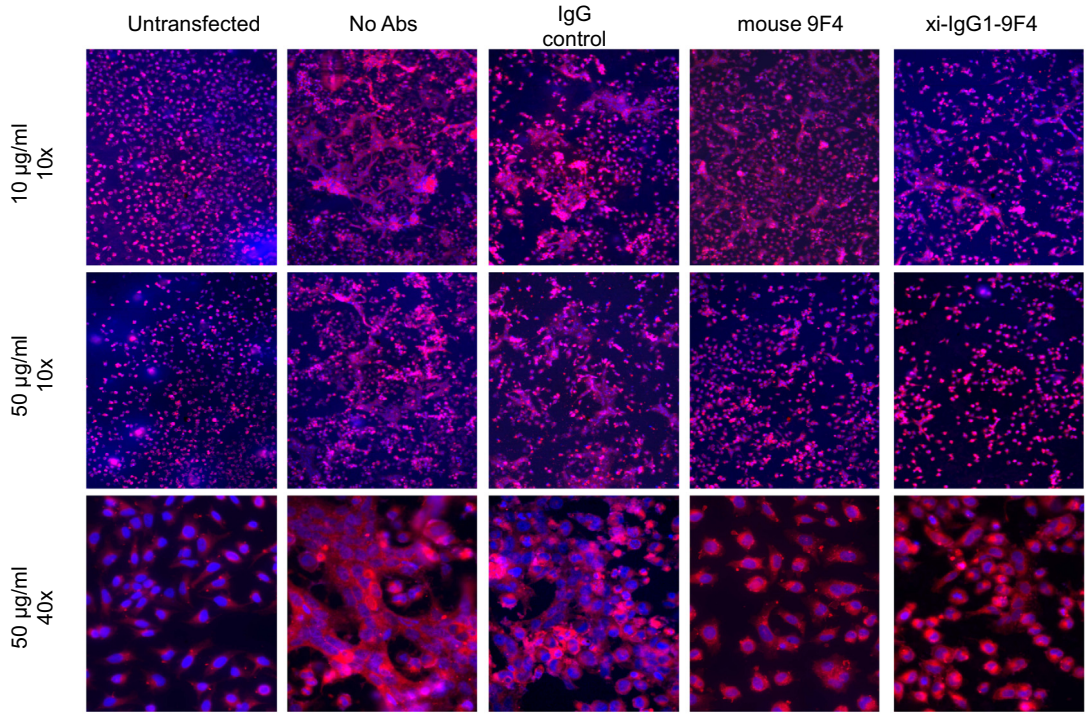

Fig. 4. Mouse and mouse-human chimeric forms of MAb 9F4 comparably inhibit HA mediated fusion at low pH. HeLa cells were transiently transfected with a cDNA construct expressing Hatay04-HA and then incubated with mouse-9F4 or xi-IgG$_1$-9F4 at two different concentrations. Control cells were not treated or incubated with control mouse-8F8 antibody. Subsequently, the unbound MAbs were removed by washing the cells with 1XPBS prior to treatment with low pH buffer and followed by recovery, fixation and staining. Plasma membrane is stained orange (CellMask Orange) and nucleus is stained blue (DAPI). Pictures shown are representative of 20 fields and 3 independent experiments. The top two panels were taken at original magnification ×10 while the bottom panel was taken at original magnification ×40.

replication can occur. In contrast, IgG MAb activity is localized in the lung. Interestingly, it was recently shown that IgA, but not IgG, prevents transmission of influenza viruses in guinea pig model (Seibert et al., 2013).

Dimeric IgA will also encounter intracellular virus present within endosomes during transepithelial transport (Tamura et al., 2005). MAbs that prevent the fusion process can therefore bind to virus present within the endosomes and interfere with virus uncoating during entry. The polymerization of IgA also enhances its antiviral immune responses due to the increased ability for antigen agglutination (Tanimoto et al., 2012). Polymeric IgA variants of originally IgG antibodies have been shown to improve antibody reactivity to specific antigen for other diseases affecting mucosal tissues (Liu et al., 2003), thus, the generation of polymeric xi-IgA1-9F4 is a possible future direction in improving its neutralizing potency.

In summary, two chimeric forms of MAb 9F4 have been constructed and were found to retain varying degrees of binding and neutralizing activity against multiple clades of HPAI H5N1. These chimeric MAbs may be suitable candidates for the development of passive immunotherapeutic agents.

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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.2014.04.011.

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