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Monovalent H5 vaccine based on epitope-chimeric HA provides broad cross-clade protection against variant H5N1 viruses in mice

Fang He [a], Mookkan Prabakaran [a], Subaschandrabose Rajesh kumar [a], Yunrui Tan [a], Jimmy Kwang [a,b,*]

[a] Animal Health Biotechnology, Temasek Life Sciences Laboratory, Singapore, Singapore
[b] Department of Microbiology Faculty of Medicine, National University of Singapore, Singapore, Singapore

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ABSTRACT

H5N1 HPAI virus continues to be a severe threat for public health, as well as for the poultry industry, due to its high mortality and antigenic drift rate. There is no monovalent vaccine available which provides broad protection against those major circulating strains. In the present study, a monovalent H5 vaccine strain was developed with antigenic sequence analysis and epitope mutations. H5 from Indonesia strain (A/Indonesia/CDC669/2006) was used as backbone sequence. Three amino acids were mutated to express immunogenic epitopes from other circulating H5N1s in the backbone. RG influenza virus expressing the epitope-chimeric H5 can react in HI with multiple H5 monoclonal antibodies which fail to neutralize wild type CDC669. High titers in HI and virus neutralization against different clades H5N1s (clade 1, 2, 4 and 7) were detected using sera from mice immunized with the epitope-chimeric H5N1. The monovalent vaccine with RG-epitope-chimeric H5N1 protected mice from lethal challenge with H5N1s of different clades, including clade 1.0, 2.1, 2.2 and 2.3. This study indicates that the broad immune response elicited by this single H5N1 virus allows it to be a promising candidate for a monovalent H5 universal vaccine.

1. Introduction

Recurrence of highly pathogenic avian influenza (HPAI) virus subtype H5N1 in humans and poultry continues to be a serious concern to public health. Since their emergence in Asia over a decade ago, highly pathogenic avian influenza H5N1 viruses have spread to over sixty countries on three continents and are endemic among poultry in South East Asia and Africa (Peiris, de Jong, and Guan, 2007). It has caused disease in several mammals, including humans, often with lethal consequence. Up to date, H5N1 has resulted in 641 human cases worldwide, including 380 deaths (http://www.who.int/influenza/human_animal_interface/EN_GIP_20131008CumulativeNumberH5N1cases.pdf, 2013). Although so far no sustained human to human transmission of the virus has been observed, the concern remains that, if human transmissibility was acquired, a severe pandemic could occur (Guan et al., 2004; Imai et al., 2012).

Vaccination remains the most effective and economically prudent strategy to combat the threat posed by avian influenza viruses with pandemic potential (Baz et al., 2013). However, it will be a challenge to produce an effective vaccine if a pandemic comes up suddenly and spreads rapidly. Therefore, efforts are being undertaken to develop pandemic vaccines that use less antigen and induce cross-protective responses. The highly conserved ion channel protein (M2) (Wu et al., 2007) and the nucleoprotein (NP) of influenza virus have been evaluated for the induction of cross-protective cellular immunity and viral clearance (Chen and Subbarao, 2009). However, these proteins are poorly immunogenic and antibodies elicited are infection permissive. Thus, the development of a vaccine based on influenza virus hemagglutinin (HA), the principal determinant of immunity to influenza virus, remains the most favorable option to prevent infections by HPAI influenza viruses (Gambotto et al., 2008). H5N1 viruses are phylogenetically characterized in different lineages or clades of H5N1 (2005, 2008). Differences in hemagglutinin sequences further result in distinguishable antigenicity (Russell et al., 2008). Due to variations in the HA sequences, particularly within the neutralizing epitope regions, conventional HA based H5N1 vaccines appear not to be protective against heterologous strains or phylogenetically variant clades of H5N1 (Shore et al., 2013). Hence, the strategy to exploit a cocktail of antigenically different triple or more virus strains was developed to elicit broad protection (Prabakaran et al., 2010). However, either the propagation of individual vaccine seed viruses or the development of co-expression recombinant vaccines is time-consuming, technique-demanding and expensive (Pica and Palese, 2013).

* Corresponding author at: Animal Health Biotechnology, Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, Singapore 117604, Singapore. Tel.: +65 68727473; fax: +65 68727007.
E-mail address: kwang@tll.org.sg (J. Kwang).

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Therefore, it is important to develop a prepandemic monovalent vaccine that induces cross-clade protection against antigenically distinct H5N1 strains. Efforts have previously made by several research groups to generate a monovalent universal H5N1 vaccine based on the different technologies. Ancestral protein sequences were selected at several nodes of HA and NA gene phylogenies that represent ancestors to diverse H5N1 virus clades, in order to enhance the cross reactivity and availability of H5N1 influenza vaccines (Ducatez et al., 2011). Based on a new method, computationally optimized broadly reactive antigen (COBRA), VLPs presenting a synthetic HA elicited broad humoral immunity in nonhuman primates against multiple H5N1 isolates from different clades (Giles and Ross, 2011). Further studies indicate that the single COBRA HA antigen elicited a broader antibody response and reduced morbidity and viral titers more effectively than a polyvalent mixture of primary H5N1 HA antigens (Giles et al., 2012).

In the present study, a broadly immunogenic H5 was developed based on epitope engineering in order to present representative epitopes from different major H5N1 clades. The feasibility of a monovalent vaccine expressing the epitope-chimeric H5 against multiple clades of H5N1s was evaluated in a mouse model challenged with phylogenetically variant H5N1 strains.

2. Materials and methods

2.1. Ethics statement

All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of the National Institute of Infectious Diseases (NIID). Experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee of the Temasek Life Sciences Laboratory, National University of Singapore, Singapore. (IACUC approval number TLL-12-014).

All experiments involving human H5N1 strains were performed in a biosafety level 3 (BSL-3) containment laboratory in compliance with CDC/NIH and WHO recommendations and were approved by the Agri Veterinary Authority (AVA) of Singapore.

2.2. Viruses and cell lines

H5N1 human influenza viruses A/Indonesia/CDC669/2006 was obtained from the Ministry of Health (MOH), Republic of Indonesia. The H5N1 viruses from different phylogenetic clades or subclades were rescued by reverse genetics. Briefly, the hemagglutinin (HA) and neuraminidase (NA) genes of H5N1 viruses from clade 1.0 (A/Vietnam1203/04), clade 2.2.1.1 (A/Egypt/3300-NAMRU3/2008), clade 2.2 (A/barheaded goose/Qinghai/12/05), 2.3.2.1 (A/Akita/1/08), 2.3 (A/Anhui/1/05 and A/Jiangsu/2/07), 4 (A/goose/Guiyang/337/06), 7.0 (A/chicken/Shanxi/2/06) and 7.1 (A/Vietnam/NCVD-03/08) were synthesized (GenScript) based on the sequences in the NCBI influenza virus database. The synthetic HA and NA genes were cloned into a dual promoter plasmid for influenza A virus reverse genetics (Prabakaran et al., 2009a). The dual-promoter plasmids were obtained from the Centers for Disease Control and Prevention, Atlanta, GA. Reassortant viruses were rescued by transfecting plasmids containing HA and NA along with the remaining six influenza virus genes derived from high-growth master strain A/Puerto Rico/8/34 (H1N1) into cocultured 293T and MDCK cells by using Lipofectamine 2000 (Invitrogen Corp.). At 72 h posttransfection the culture medium was inoculated into embryonated eggs or MDCK cells. The HA and NA genes of reassortant viruses from the second passage were sequenced to confirm the presence of the introduced HA and NA genes and the absence of mutations. Stock viruses were propagated in the allantoic cavity of 10-day-old embryonated eggs, and virus containing allantoic fluid was harvested and stored in aliquots at 80 °C. Virus content was determined by a standard hemagglutination assay as described previously (He et al., 2008).

MDCK cells were maintained in Dulbeccos Modified Eagle Medium (DMEM; Life Technologies, USA) containing 10% Fetal Bovine Serum (FBS; Life Technologies, USA). 293T were maintained in Opti-MEMI (Life Technologies, USA) containing 5% FBS. After 48 h the transfected supernatants were collected and virus titers were determined by standard hemagglutination assays. The tissue culture infectious dose 50 ($TCID_{50}$) of reassortant virus was then calculated by the Muench–Reed method (1938).

2.3. Production and characterization of Mab

A panel of different H5 neutralizing MAbs was used. The MAbs were produced as described previously. Briefly, BALB/c mice were immunized twice 2 weeks apart by the subcutaneous injection of individual BEI-inactivated H5N1 virus (A/Indonesia/CDC669/2006 A/Vietnam/1203/2004 or A/Anhui/1/05) mixed with Montanide ISA563 adjuvant (Seppic, France). Mice received an additional intravenous injection of the same viral antigen 3 days before the fusion of splenocytes with SP2/0 cells. Hybridoma culture supernatants were screened by immunofluorescence assays. Hybridomas that produced specific MAbs were cloned by limiting dilution, expanded, and further subcultured. The hybridoma culture supernatant was clarified and tested for the hemagglutination inhibition activity as described below.

2.4. Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assays were performed as described previously (Prabhu et al., 2009). Briefly, Mabs or receptor-destroying enzyme (RDE)-treated sera were serially diluted (2-fold) in V-bottom 96-well plates and mixed with 4 HA units of H5 virus. Plates were incubated for 30 min at room temperature, and 1% chicken RBCs were added to each well. The hemagglutination inhibition endpoint was the highest antibody dilution in which agglutination was not observed.

2.5. Microneutralization assay

Neutralization activity of Mab or serum against H5 strains was analyzed by microneutralization assay as previously described (He and Kwang, 2013). Briefly, antibody samples were serially two-fold diluted and incubated with 100 TCID50 of different clades of H5 strains for 1 h at room temperature and plated in duplicate onto MDCK cells grown in a 96-well plate. The neutralizing titer was assessed as the highest antibody dilution in which no cytopathic effect was observed by light microscopy.

2.6. Immunization and challenge

Specific-pathogen-free female BALB/c mice (6 weeks old) were obtained from the Laboratory Animals Centre, National University of Singapore. 10 mice per each experimental group were vaccinated subcutaneously two times at a regular interval of 14 days with 100 ul (HA titer, 128) of inactivated RG H5N1 virus carrying the epitope-chimeric HA or other inactivated H5N1 viruses (A/Indonesia/CDC669/2006 and A/Vietnam/1203/2004), with the adjuvant Montanide ISA563 (water-in-oil emulsion; Seppic, France). PBS with the same adjuvant was used as a reference vaccine control. The serum was collected from 10 mice per experimental group on day 28. Levels of hemagglutination inhibition activity and serum cross-clade neutralizing antibody were measured.

Three weeks after the final vaccination, mice were transferred into an animal BSL-3 containment facility. 8 mice per group were challenged intranasally with 5 50% mouse lethal doses (MLD50) of clade 1.0 (A/Vietnam/1203/2004, VN1203), clade 2.1 (A/Indonesia/CDC669/06, CDC669), clade 2.3 (A/Anhui/1/05, Anhui) and clade 2.2.1.1 (A/Egypt/3300-NAMRU3/2008) HPAI H5N1 virus strains. The MLD50 of influenza virus required for intranasal challenge experiments was predetermined. Mice were observed daily to monitor body weight and mortality. Monitoring continued until all animals died or until day 14 after challenge.

2.7. Histopathological analysis

Mice were necropsied, and the lungs were stored in 10% (wt/vol) neutral buffered formalin, embedded in paraffin, and sectioned. The sections were deparaffinized using Hist-choice (Amersco) and rehydrated in sequentially graduated ethanol baths. The slides were stained with hematoxylin and eosin and pathological evaluation was performed by light microscope (Olympus, UK). The images were captured by digital imaging system (Nikon, USA).

2.8. Statistical analysis

The data are expressed as arithmetic means and standard deviations (SD). An unpaired two-tailed Student's *t* test was performed to determine the level of significance in the difference between the means of two groups. One-way analysis of variance (ANOVA) was also used to test for differences between groups.

3. Results

3.1. Combination of major H5 epitopes from different clades on one single H5

Isolated from a human lethal case in 2006, A/Indonesia/CDC669/06 was one of the representative strains from the clade 2, the largest clade in H5 phylogenetic trees (Shore et al., 2013). In this study, H5 of CDC669 was selected as the backbone for the epitope chimeric H5 (EC H5). The protein sequences of the major antigenic sites equivalent to those in H1 structure (Caton et al., 1982) were aligned among H5N1s from different clades (Fig. 1). Amino acid positions 155, 156 and 189 were found to be some of the most variable positions among all the antigenic regions on H5. The three amino acids were mutated to other predominant forms from other clades on the same sites. The three mutations were indicated as S155N, T156A and R189K individually. The rest of amino acids involved in epitopes remained unchanged to sustain the antigenic features of clade 2.1 CDC669. Hence, the representative epitopes of different clades were displayed together on a single H5 in order to elicit cross-clade immunity.

3.2. Characterization of the epitope-chimeric H5

The epitope-chimeric H5 was expressed in a RG H5N1 virus with PR8 background. Expression of H5 was determined with Mab 4C2, a Mab with broad recognition of H5 (Prabakaran et al., 2009b), in infected MDCK cells. Hemagglutination activity was detected in HA

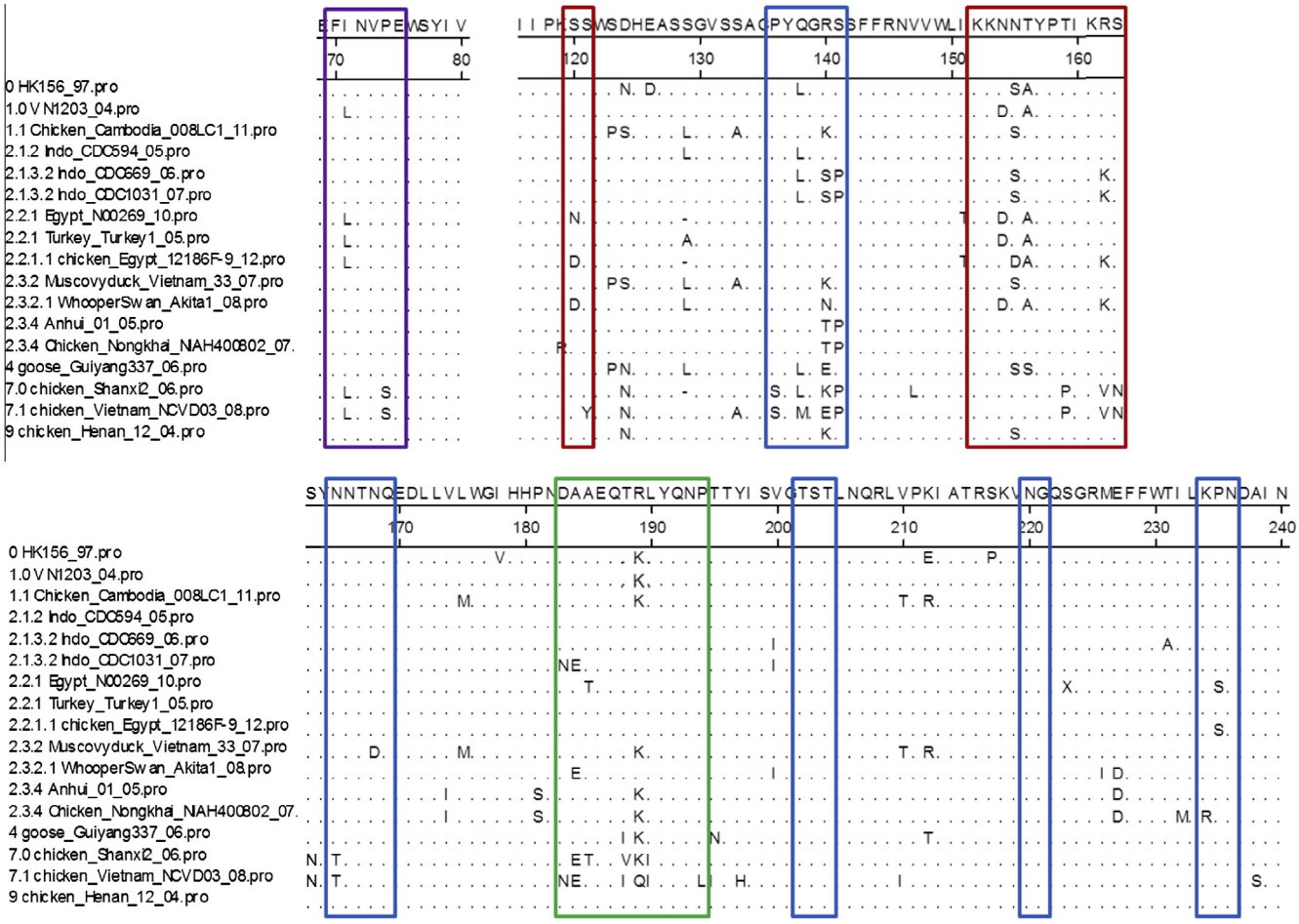

Fig. 1. Identification of variable amino acids in antigenic epitopes among different clades. H5 protein sequences of different major clades were aligned. Amino acid consensus sequences of H5N1 HA clades were highlighted at positions equivalent to the H1 antigenic sites, Ca (in blue boxes), Cb (in a purple box), Sa (in red boxes) and Sb (in green boxes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

test with the EC H5. A panel of neutralizing Mabs was produced previously in mice immunized with clade 1.0 VN1203, clade 2.1 CDC669 or clade 2.3 Anhui individually. HI tests with these Mabs were performed to compare the antigenicity between EC H5 and wild type CDC669. As shown in Table 1, the EC H5 can be recognized and inhibited by all the H5 Mabs tested in HI, except 11G12, a Mab specific to clade 1 (He and Kwang, 2013). CDC669 fails to react with many Mabs which were generated from other H5N1s. Some Mabs which are able to neutralize CDC669 showed a higher titer against the EC H5 in HI test. The observations indicate that the EC H5 presents different antigenic properties from CDC669. The mutations in the epitopes allow the EC H5 to be more accessible to neutralization with variant monoclonal antibodies.

3.3. Cross-clade neutralizing antibody titers in sera

Hemagglutination inhibition (HI) titers measure the efficacy of the antibody response to inhibit HA function. As shown in Fig. 2A, the sera from mice immunized with RG-EC monovalent vaccine had efficiently induced HI titer against heterologous viruses of different clades on day 28. Higher titers were observed with sera from the RG-EC group against all the tested strains of clade 1, clade 2, clade 4 and clade 7 as compared to sera from other single H5s against heterologous strains. HI titers of sera from RG-EC H5 vaccinated mice were lower than the groups with homologous vaccinations against either clade 1.0 VN1203 or clade 2.1 CDC669. Virus neutralization was performed to determined functional antibodies responsible for the protective immunity against influenza (Fig. 2B). The similar results regarding the comparison between EC H5 and other single strains were observed in both HI and virus neutralization test, confirming that the epitope-chimeric H5 exhibits improved cross-clade immunogenicity and suggesting that EC H5 is able to elicit broader protection against multiple clades of H5N1 than other monovalent strains.

3.4. Challenge studies after vaccination

Three weeks after the final immunization, all groups of mice were challenged intranasally with 5 MLD50 of HPAI H5N1 virus strains from clade 1.0 VN1203, clade 2.1 CDC669, clade 2.3 Anhui and clade 2.2.1.1 (A/Egypt/3300-NAMRU3/2008) respectively. Groups of mice immunized with RG-EC H5 obtained 100% protection against clade 2.1, clade 2.2 and clade 2.3 viruses (Fig. 3C, E and G) and 87.5% survival rate against clade 1.0 (Fig. 3A). However, mice immunized with other monovalent H5N1 vaccines showed only 50–75% survival rate against any heterologous challenge respectively.

The progression of infection was indicated by various trends of a decrease in body weight for the different groups. In groups of mice immunized with a RG-EC H5 strain, no significant decreases in body weight ($\leqslant$13.3%) were observed for mice upon the challenges from different heterologous H5N1s respectively (Fig. 3B, D, F and G)). However, mice vaccinated with other single H5N1 strains showed at least a 17% loss in body weight against any heterologous challenge, although the body weight of survived mice was gradually regained after 6 days post-challenge. The group of RG-EC H5 presented similar body weight loss (<5.1%) as the group of CDC669 (<1.9%) upon the homologous challenge of CDC669, while significant improvements were showed in preventing weight loss upon other heterologous challenges as compared to CDC669 vaccinated groups

Histopathology studies were performed for the mice vaccinated with different monovalent H5N1 strains and challenged with clade 2.3 Anhui or clade 2.2.1.1 (A/Egypt/3300-NAMRU3/2008) HPAI H5N1 viruses. On day 5 postinfection, lungs of mice vaccinated with clade 1.0 VN1203, clade 2.1 CDC669 or PBS, had pulmonary lesions consisting of moderate to severe necrotizing bronchitis and histiocytic alveolitis with associated pulmonary edema upon heterologous challenges (Fig. 4). The mice immunized RG-EC H5 lacked lesions in the lungs upon the challenge from clade 2.3 Anhui and had minimal bronchitis against clade 2.2.1.1 (A/Egypt/3300-NAMRU3/2008) H5N1 viral challenges.

Moreover, viral load in lungs was evaluated in H5 immunized mice upon viral challenges by measuring the viral titers in the mouse lungs (Fig. 5). The lungs were collected on day 5 after viral challenge when the high virus titers of more than 10^6 were detected in the infected but untreated mice which died within the following day. The mice immunized with RG-EC H5 showed lowest viral load upon challenge with clade 2.3 Anhui or clade 2.2.1.1 (A/Egypt/3300-NAMRU3/2008) as compared to other monovalent vaccinations. Virus titers in lungs from RG-EC H5 vaccinated mice were at least 10 times lower than the group immunized with clade 2.1 CDC669 upon the challenge with clade 1.0 VN1203, though the group vaccinated with homologous strain presented even lower titers.

All these results indicated that immunization with RG-EC H5 provided more efficacious protection than other monovalent vaccines against variant heterologous challenge.

4. Discussion

The rapid evolution of new sublineages of influenza A/H5N1 virus is considered to be the most likely culprit for the next pandemic (Watanabe et al., 2011). Newer vaccine approaches for pandemic preparedness against these viruses are needed, given the limitations of vaccines currently approved for H5N1 viruses in terms of their production timelines and the ability to induce cross-clade protective immune responses (Prieto-Lara and Llanos-Mendez, 2010). Efforts have been made by several research groups to produce influenza vaccines with broad immunogenicity

Table 1
Comparison of HI activity with Mabs between RG-EC H5 and CDC669.

Mab[a]	Parental virus clade	Parental virus	HI titer against parental virus	HI titer against RG-EC H5	HI titer against CDC669
4F8	2.1	A/Indonesia/CDC669/06	64	128	64
3B1	2.1	A/Indonesia/CDC669/06	32	128	32
5C5	2.1	A/Indonesia/CDC669/06	32	64	32
3H11	2.1	A/Indonesia/CDC669/06	64	64	64
C	2.3.4	A/Anhui/1/05	64	64	32
F	2.3.4	A/Anhui/1/05	64	64	32
H	2.3.4	A/Anhui/1/05	32	32	<4
G	2.3.4	A/Anhui/1/05	32	16	<4
6B8	1.0	A/Vietnam/1203/2004	64	16	<4
2F11	1.0	A/Vietnam/1203/2004	64	32	16
11G12	1.0	A/Vietnam/1203/2004	32	<4	<4

[a] Antibody concentration of each Mab is 100 ug/ml.

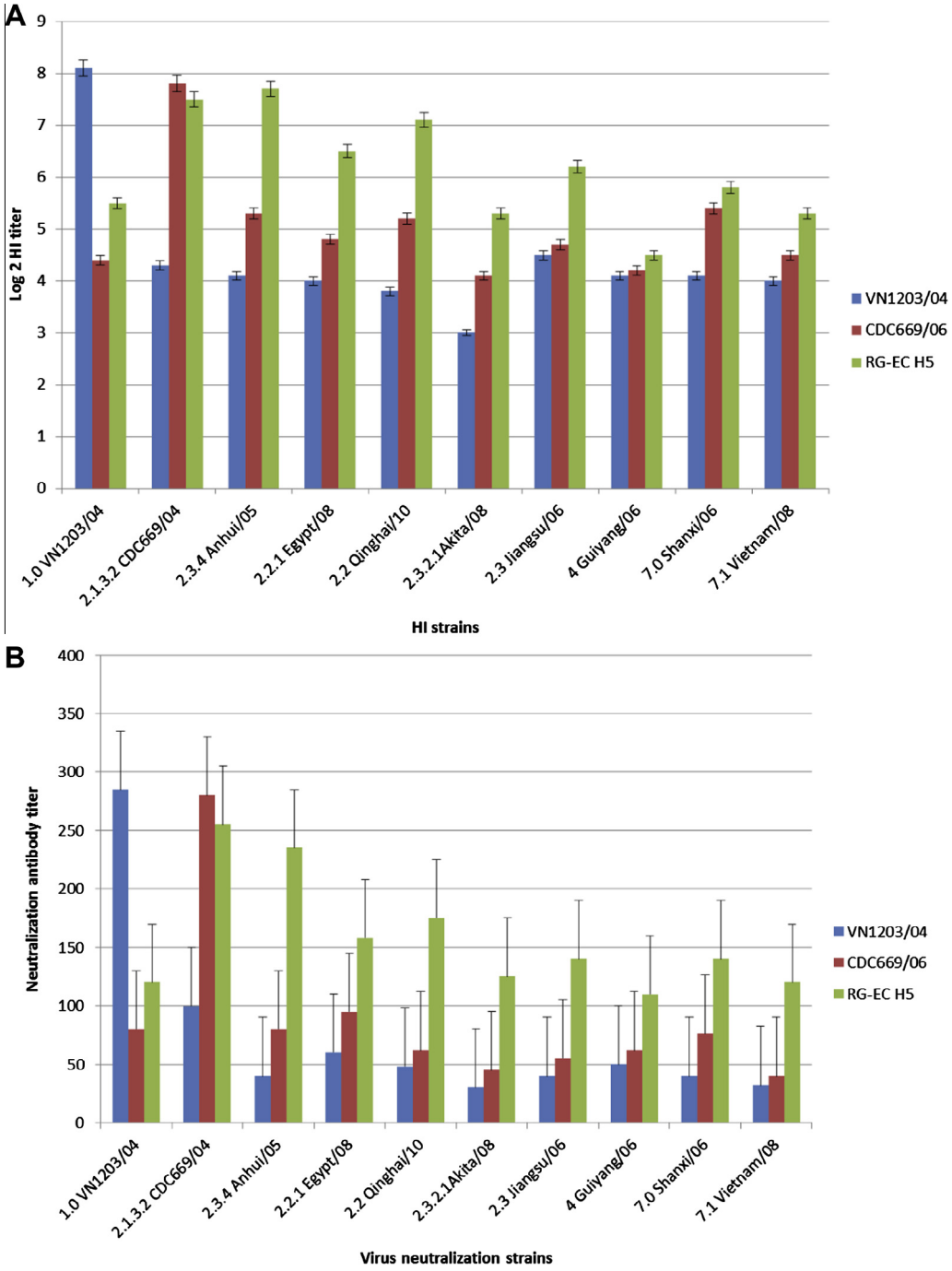

Fig. 2. Cross-clade tests with immunized mouse sera. Serum samples were collected on Day 28 (14 days after the final immunization) from mice immunized with RG-EC H5, A/Vietnam/1203/2004, A/Indonesia/CDC669/2006 or PBS respectively. (A) HI titer against different clades of H5N1s. (B) Virus neutralization titer against different clades of H5N1s. Clade number was listed before each virus name accordingly. Each point represents the arithmetic mean value (n = 10) ± SD.

by engineering HA sequences (Giles et al., 2012; Dukhovlinov et al., 2013). Some vaccine candidates by these approaches have been shown to elicit broader, more cross-reactive antibody responses than those conventional vaccines derived from a single strain. However, the approach with an "ancestral" H5 was found to present a biased immunogenicity to strains of clade 1, which are not currently predominant (Chen et al., 2008). Besides, consensus H5s to centralize the immunogenicity of the HA were mainly delivered as DNA vaccines (Laddy et al., 2007). Some synthesized sequences may not sustain all the major antigenic epitopes and not yield a structurally and functionally intact protein (Chen et al., 2008). Computationally optimization on HA sequence shows its merits (Giles and Ross, 2011), but it is technique-demanding and may not be widely used against sudden outbreaks. An epitope-chimeric H5 presented in this study meets both needs as a monovalent broad vaccine against variable circulating H5N1s. Having broad reactivity to multiple monoclonal antibodies and immunogenicity to different H5N1 clades, the developed single H5N1 elicited effective cross-clade protection against lethal H5N1 challenges in mice.

Considerable amino acid variations within antigenic sites of HA lead to the emergence of antigenically distinct influenza H5N1

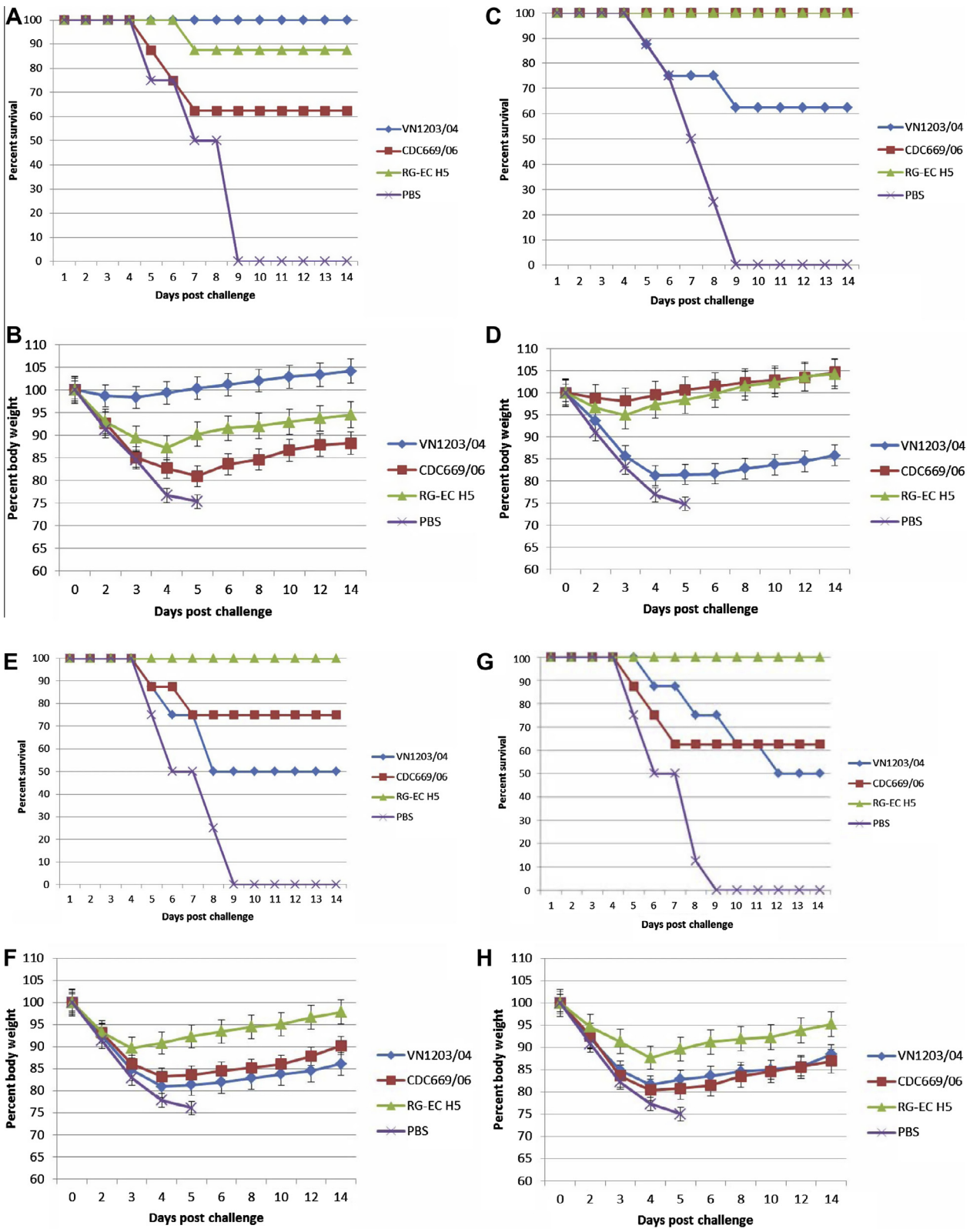

Fig. 3. Protection of mice against lethal challenge with clade 1.0, clade 2.1, clade 2.2 or clade 2.3 H5N1 virus. The results are expressed in terms of percent survival (A, C, E and G) and percent body weight (B, D, F and H) (at the beginning of the trial), respectively. Groups of mice ($n = 8$) were subcutaneously immunized with RG-EC H5, A/Vietnam/1203/2004, A/Indonesia/CDC669/2006 or PBS respectively. Three weeks after the final vaccination, mice were intranasally infected with 5 MLD50 of clade 1.0 (A/Vietnam/1203/2004) (A and B), clade 2.1 A/Indonesia/CDC669/2006 (C and D), clade 2.3.4 A/Anhui/1/05 (E and F) or clade 2.2.1.1 A/Egypt/3300-NAMRU3/2008 (G and H) HPAI H5N1 virus strains. Mice were monitored throughout a 14-day observation period.

viruses (Plotkin, Dushoff, and Levin, 2002). The continuous evolution of H5N1 within each clade has generated multiple second, third and fourth order clades defined by their phylogenetic clustering and genetic distance, especially in clade 2 (Shore et al., 2013; Sun et al., 2013; Younan et al., 2013). Currently, the majority of viruses circulating worldwide and causing human infections

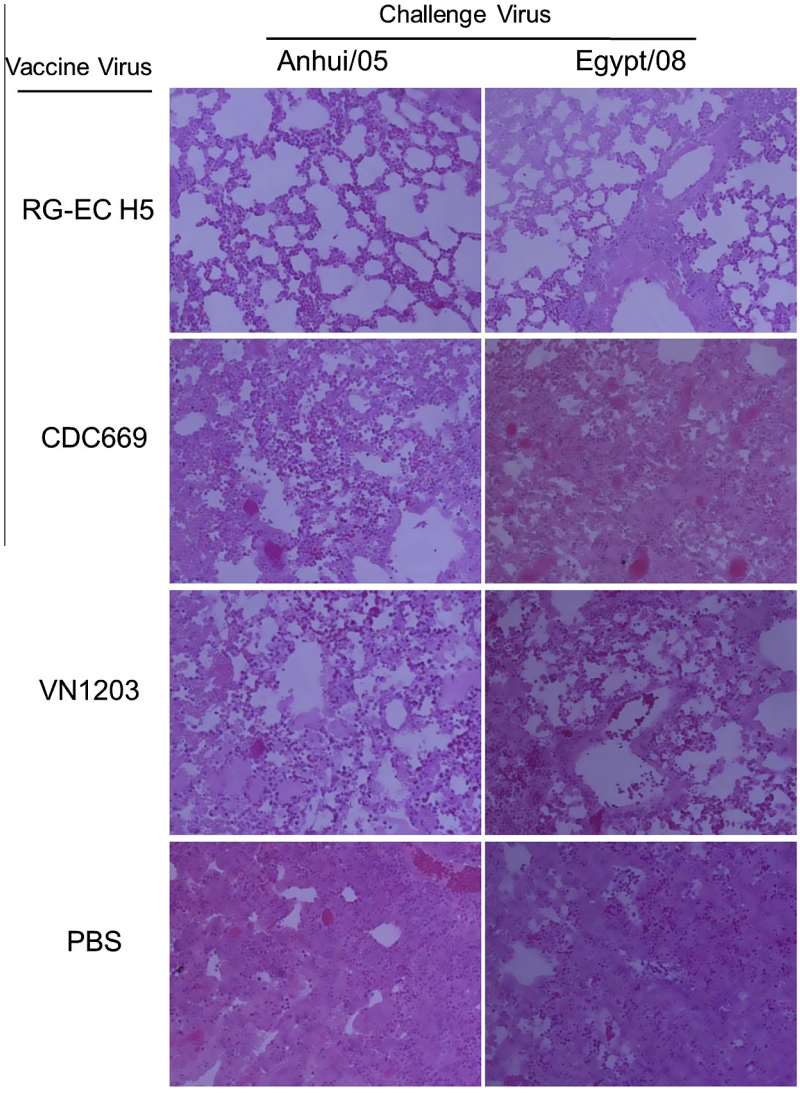

Fig. 4. Histopathology of lung tissue in vaccinated mice. Shown are photomicrographs of hematoxylin- and eosin-stained lung sections of mice 5 days after challenge with clade 2.3.4 A/Anhui/1/05 or clade 2.2.1.1 A/Egypt/3300-NAMRU3/2008 H5N1 strains. Mice were immunized with RG-EC H5, A/Vietnam/1203/2004, A/Indonesia/CDC669/2006 or PBS respectively. The immunization strain and challenge strain were shown accordingly.

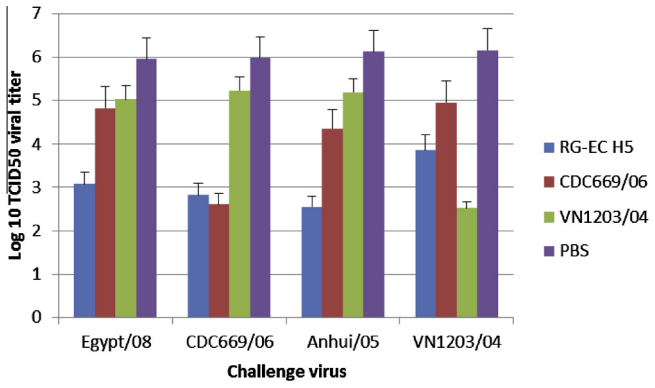

Fig. 5. Measurement of viral infectivity titers in the lungs of vaccinated mice. Mice were subcutaneously immunized with RG-EC H5, A/Vietnam/1203/2004, A/Indonesia/CDC669/2006 or PBS respectively. Lung samples were collected from mice 5 days after challenge with 5 MLD50 of clade 1.0 (A/Vietnam/1203/2004), clade 2.1 A/Indonesia/CDC669/2006, clade 2.3.4 A/Anhui/1/05 or clade 2.2.1.1 A/Egypt/3300-NAMRU3/2008. The results of virus load in lungs were expressed in terms of mean value of $\log_{10}$ $TCID_{50}$. Each point represents the arithmetic mean value ($n = 2$) ± SD.

were identified in clades 1 and 2, such as clades 1.1, 2.1.3.2, 2.2.1 and 2.3.4 (Forrest et al., 2009; Yang et al., 2009). Therefore, different vaccine combinations from these clades were selected by different research groups and regulatory agencies in order to produce a universal H5N1 vaccine. Recently, clade 2.2.1 A/Egypt/N03072/2010, clade 2.3.2.1 A/Hubei/1/2010 and clade 2.3.4 A/Anhui/1/2005 were selected by the WHO as vaccine candidates based on their history related to human fatal cases. In the previous study in the lab, a vaccine formulation containing H5s of clade 1.0 VN1203, clade 2.1.3.2 CDC669 and clade 2.3.4 Anhui was confirmed to be effective against multiple clades of H5N1s (Prabakaran et al., 2010). Considering all these recommendations, in order to generate a monovalent H5 which is able to elicit cross protection against all these major H5N1s responsible to human infection, a H5 from a representative circulating strain of clade 2 is preferred. Many H5 Mabs were produced in the lab with different H5N1 antigens. It was noticed that more H5 Mabs with broad spectrum to all clades of H5N1 were generated from CDC669 than any other H5N1 strains (He et al., 2010; Ho et al., 2009; Prabakaran et al., 2009a,b), suggesting the cross-clade immunogenicity of CDC669. Taken together, in the study, H5 from A/Indonesia/CDC669/2006

of clade 2.1.3.2 was selected as the backbone for the monovalent universal H5. As shown in the results, after further mutations in the epitopes, the EC H5 from CDC669 gained reactivity to those previously un-reactive Mabs and sustained the original reactivity to CDC669 Mabs. This finding paves way for this single EC H5 to a potential universal H5N1 vaccine.

Among all the amino acid variations in antigenic sites, three significant ones were found in Sa (155, 156aa), Sb (189aa) and Ca (138–141) sites (Kaverin et al., 2002). Three amino acids in Sa and Sb were mutated to other predominant forms in order to generate the epitope-chimeric H5. The variable region in Ca was kept same as wild type CDC669, contributing to the antigenic features of clade 2.1. As a part of receptor binding site, amino acids in the 130 loop is highly variable among different clades (Hensley et al., 2009; Stevens et al., 2006). For example, there are more than 7 possible amino acids identified on the 140th aa. Together with other variations in the neighboring positions, the number of possible combinations of amino acids in the 130 loop is very high. A screening may be required to determine the most suitable form for a universal H5 vaccine. In the future study, mutations could be induced to this site to improve and expand the immunogenic spectrum of the EC H5 vaccine.

The EC-H5 fails to react to Mab 11G12 (He and Kwang, 2013), a monoclonal antibody specific to H5N1 of clade 1, implying the relatively weaker immunogenicity to clade 1. With the mutation on the 189th aa from R(Arginine) to K(Lysine), the predominant amino acid in the position among viruses of clade 1.0, more mice survived with the RG-EC H5 immunization upon the challenge with VN1203 of clade 1.0. Though 100% protection was not achieved, the performance of RG-EC H5 was much better as compared to wild type CDC669 with a survival rate at 50% against VN1203. This could be further improved by the induction of other mutations in Sa region, such as 152aa, the epitope targeted by Mab 11G12. Tests with various monoclonal antibodies targeting different epitopes can serve as a rapid method to predict the antigenicity of H5s before animal tests and also provide clues for improvement.

Antigenic differences in epitopes may render current vaccines unqualified for the prevention of influenza globally (Fouchier and Smith, 2010). Exploit of multivalent vaccines significantly increases vaccine cost and delays vaccine developing progress against a sudden influenza outbreak. The availability of a simple and broadly protective vaccine for influenza H5N1 is a high priority in preparedness for a future influenza pandemic. The study provides not only a monovalent vaccine candidate with broad spectrum but also contributes a new method to flu vaccine development. Instead of using the strains from major outbreaks or severe cases, epitope engineering on a single HA to produce an epitope chimeric H5 overcomes difficulties in virus strain adaption and multiple antigen co-expression. A monovalent H5 vaccine with broad spectrum to H5N1s worldwide will greatly reduce cost in both vaccine development and production. New combinations of epitopes in a single HA could be easily designed, evaluated and identified to provide protection against infections mediated by newly emerged H5N1s.

Authors' contributions

Conceived and designed the experiments: FH, MP, JK. Performed the experiments: MP, SRK, FH, YT. Analyzed the data: FH MP JK. Wrote the paper: FH, MP.

Competing interests

Authors claim no conflict of interests.

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