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Development of a universal influenza vaccine using hemagglutinin stem protein produced from *Pichia pastoris*

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ABSTRACT

The development of a universal influenza vaccine has become a major effort to combat the high mutation rate of influenza. To explore the use of the highly conserved stem region of hemagglutinin (HA) as a universal vaccine, we produced HA-stem-based protein using yeast expression systems. The glycosylation effects on the immunogenicity and protection activities were investigated. The yield of the A/Brisbane/59/2007 HA stem produced from *Pichia pastoris* reached 100 mg/l. The immunogenicity of HA stem proteins in various glycoforms was further investigated and compared. All glycoforms of the HA stem protein can induce cross-reactive antibody responses, antibody-dependent cellular cytotoxicity (ADCC)-mediated protection as well as T-cell responses, with broad protection in mice. The monoglycosylated form of the A/Brisbane/59/2007 HA stem produced in yeast, together with the glycolipid C34 as the adjuvant, can elicit greater ADCC responses, better neutralizing activities against heterologous strains, and broader protection in mice.

1. Introduction

The spread of influenza A virus results in seasonal pandemics of human respiratory disease, which causes serious public health issues and economic burdens. Several influenza pandemics have occurred in the past century, including the Spanish flu in 1918, the seasonal H3N2 outbreaks in 2015, and the recent 2009 H1N1 pandemic. The antigenic variants of the influenza virus, such as the variants through antigenic drift and shift, may cause epidemics of influenza. Antigenic drifts are antigenicity alterations by small changes in the genes of influenza viruses while the virus replicates. Antigenic shifts are major changes, which can eventually result in a new influenza subtype. In addition, avian influenza viruses, such as H5N1, H7N7, H7N9, and H10N8, have the potential transmissibility from domestic poultry or wild birds to humans (Chen et al., 2014a; Chong et al., 2016; Cui et al., 2016; Kudo et al., 2012).

Since 1945, vaccination has been the most effective method to protect humans against influenza virus infection. However, due to the high mutation rate leading to changes in antigenicity, the influenza vaccine needs to be reformulated every year to provide efficient protection in the influenza season (Osterholm, 2005). The most common technology used for the production of influenza vaccine is to amplify influenza virus in the allantoic cavity of specific pathogen free (SPF) embryonated hens' eggs followed by virus inactivation. However, there are several limitations of current influenza vaccines, including expensive and time-consuming processes, potential infection of active virus due to incomplete inactivation, lack of cross-reactivity and efficacy in populations with weaker immune protection, such as children, elderly and unprimed populations. Therefore, to control the spread of influenza virus in case of a global infectious-disease threat, a faster and more efficient approach for influenza vaccine production is in demand. Next-generation vaccines such as molecular vaccines using recombinant proteins have been developed to overcome the limitations of traditional techniques (Krammer and Palese, 2015; Sedova et al., 2012; Soema et al., 2015). The recombinant influenza vaccines can be produced faster with no concerns for potential viral infections during the production process and avoid incomplete viral inactivation as they are independent of egg supplies.

The surface of influenza A virus is composed of a lipid bilayer with two viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and one tetrameric transmembrane matrix protein 2 (M2) (Nogales and Martinez-Sobrido, 2016). These components of influenza are considered as suitable targets for influenza vaccine development. HA is a good antigen candidate for vaccine development because it is the major protein on the influenza virus surface. HA is expressed as a trimer, which is composed of monomers that contain a highly variable immunogenic globular head domain (HA1)

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and a conserved HA stem domain (HA2) (Cho and Wrammert, 2016; Impagliazzo et al., 2015). HA is post-translationally modified by glycosylation which can accelerate the folding process and enhance protein stability (Hanson et al., 2009; Mitra et al., 2006; Wormald and Dwek, 1999). Recent studies suggested that the glycan masking of influenza is a strategy to avoid antibody response and neutralization (Das et al., 2011). We also showed that the receptor-binding ability and immunogenicity of HA can be increased by shortening the *N*-glycans of HA (Wang et al., 2009a). HA with shortened N-glycans may expose more epitopes and induce more antibodies. To overcome the rapid antigenic drift, recent studies toward the development of broadly protective influenza vaccine have focused on the HA stem region. Human antibody 3E1, for example, has been reported to target the HA stem region and neutralize H1 and H5 subtype viruses (Wang et al., 2016b). Several recent reports demonstrated the use of HA stem domain to induce broad protection against various influenza virus strains. The HA stem region has been produced using various expression systems. For example, Lu et al. (2014) used *Escherichia coli*-based cell-free protein synthesis followed by refolding to produce the trimeric HA stem protein. Using a rational design and library approach, HA stem antigens based on a H1 subtype sequence elicited cross-protective antibodies in mice and non-human primates (Impagliazzo et al., 2015), indicating that the influenza vaccine based on the HA stem region could facilitate the development of an universal influenza vaccine.

Various strategies for the development of recombinant proteins as vaccines have been investigated. The vaccine can be prepared from mammalian cells (Madin Darby canine kidney cells, Vero cells and Per.C6 cells), insect cells (sf9 cells), plant cells, bacterial cells, and yeast cells (Krammer and Palese, 2015; Lei et al., 2016; Wang et al., 2016a). Since 1980, yeast cells have been used for the large-scale productions of intracellular and extracellular proteins of the human or animal origins (Romanos et al., 1992). The vaccines produced from yeast cells have proven to be safe and effective for human uses, such as RECOMBIVAX HB (Hepatitis B vaccine) and Gardasil (Human papillomavirus) (Bill, 2015; Poland and Jacobson, 2004). The yeast systems are more economical and easier to scale up than other eukaryotic expression systems and can be used to produce glycoproteins (Bollok et al., 2009). Several different yeast strains, such as *Pichia pastoris*, *Kluyveromyces lactis, Saccharomyces cerevisiae*, and *Yarrowia lipolytica*, have been used for protein expression (Buckholz and Gleeson, 1991; Muller et al., 1998). In general, the production yields of *Saccharomyces cerevisiae* are lower than other yeast expression systems (Buckholz and Gleeson, 1991; Muller et al., 1998). On the other hand, *P. pastoris* has been developed as a successful expression system that can be cultured in high-density fermentors (Cereghino and Cregg, 1999). Various studies revealed that the *Pichia* expression system may provide a simple tool to produce approximately 15–200 mg/l HA protein (Athmaram et al., 2011; Kopera et al., 2014; Lin et al., 2016; Pietrzak et al., 2016). Nonetheless, the production of HA stem region using *P. pastoris* has not been reported so far.

In order to develop a universal influenza vaccine, we exploited the *P. pastoris* expression system to produce the A/Brisbane/59/2007 HA stem as the antigen. The *N*-glycans of the A/Brisbane/59/2007 HA stem proteins were manipulated and investigated regarding their effects on immune response and protection profiles. We also compared the yield and immunogenicity of the HA stem protein produced in yeast and mammalian (HEK 293T) cells. The results showed that immunization of mice with the mono-glycosylated form of the H1 stem protein expressed in yeast can elicit greater antibody responses associated with strong neutralization activity, as well as ADCC-mediated cross-protection against H5N1 viruses.

2. Materials and methods

2.1. Cells and viruses

Human embryonic kidney 293T (HEK 293T) cells and Madin-Darby canine kidney (MDCK) cells were purchased from ATCC and grown in Dulbecco's Modified Eagle medium plus 10% fetal bovine serum. *P. pastoris* Mut^S strain KM71H was purchased from Invitrogen. The attenuated reassortant H5N1 influenza virus A/Vietnam/1194/2004 (RG14), and the H1N1 influenza virus strains A/Brisbane/59/2007 and A/WSN/1933 were procured from the reference collection of the National Institute for Biological Standards and Control (Potters Bar, United Kingdom). All viruses were cultivated in the allantoic cavity of SPF-embryonated eggs and titered in MDCK cells to determine the values of 50% tissue culture infective dose ($TCID_{50}$).

2.2. Preparation of HA stem proteins from P. pastoris by shake flask culture

The recombinant HA stem gene was synthesized (Genscript) using the sequence of mini-HA#4900 (Impagliazzo et al., 2015), which was based on H1N1 A/Brisbane/59/2007, and then constructed into pPicZA vector (Invitrogen). The plasmid was linearized and then transformed into the *P. pastoris* Mut^S strain KM71H (Invitrogen) by electroporation according to the manufacturer's protocols (Invitrogen). The transformants were selected on YPD (1% yeast extract, 2% bactopeptone, and 2% glucose) agar plates containing 100 μg/ml zeocin. The transformants were confirmed for gene insertions by screening using polymerase chain reaction with 5′-AOXI (GACTGGTTCCAATTGACAAGC) and 3′-AOXI (GCAAATGGCATTCTGACATCC) as the primers. *P. pastoris* clones were inoculated into 400 ml BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base (YNB), 1% glycerol, and 100 mM potassium phosphate; pH 6.0) using shake-flask (Corning) cultures at 28 °C for 24 h. The expression of HA stem protein was induced with 1% methanol in BMMY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.5% methanol, and 100 mM potassium phosphate; pH 6.0). After 96 h, the HA stem proteins (y-stem) were purified from the culture media by using Ni-NTA resin (GE Healthcare). To create the monoglycosylated HA stem region (HA y-stem$_{mg}$) with a single GlcNAc at the glycosylation sites, the purified HA stem (y-stem) protein was treated with EndoH (NEB) at 37 °C for 16 h. The purified y-stem and y-stem$_{mg}$ were further analyzed by immunoblotting and gel filtration chromatography using a Superdex 200 increase 10/300 GL column (GE Healthcare) as described previously (Stevens et al., 2004).

2.3. Preparation of HA stem proteins from P. pastoris by fed-batch cultivation

The KM71H-stem strain was pre-cultured in a 3 l shake flask with 400 ml BMGY medium for 16–18 h at 30 °C. After the OD_{600} of the culture reached 12–16, the culture was inoculated for fed-batch cultivation in a 5-l bioreactor (Sartorius Biostat A) with 2 l FM22 medium (42.9 g/l of KH_2PO_4, 5 g/l of $(NH_4)_2SO_4$, 1 g/l of $CaSO_4{\cdot}2H_2O$, 14.3 g/l of K_2SO_4, 11.7 g/l of $MgSO_4{\cdot}7H_2O$, and 40 g/l of glycerol) supplemented with 8.7 ml of *Pichia* trace minerals 4 (PTM4, 2 g/l of $CuSO_4{\cdot}5H_2O$, 0.08 g/l of NaI, 3 g/l of $MnSO_4{\cdot}H_2O$, 0.2 g/l of $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/l of H_3BO_3, 0.5 g/l of $CaSO_4{\cdot}2H_2O$, 0.5 g/l of $CoCl_2$, 7 g/l of $ZnCl_2$, 22 g/l of $FeSO_4{\cdot}7H_2O$, 0.2 g/l of biotin, and 1 ml/l of H_2SO_4) (Liu et al., 2016). The cells were cultivated at 28 °C, pH 5.6 at 40–80% dissolved oxygen (DO) (controlled by agitation/air flow cascade at 300–950 rpm). A glycerol-fed batch phase was initiated upon the consumption of glycerol by feeding the cells with 10× YP medium containing glycerol (20 g yeast extract, 40 g peptone, 23.8 g yeast nitrogen base, and 80 g glycerol in 200 ml ddH_2O with 24 ml PTM4 trace salt) at a rate of 20 ml l^{-1} h^{-1}. After 20–30 h of growth, the methanol-fed batch phase was initiated by feeding methanol (containing 12 ml/l PTM trace salt) at a rate of 3.5 ml l^{-1} h^{-1} for 72 h. The HA stem proteins (y-stem) were purified and processed to obtain y-stem$_{mg}$ as described above.

2.4. Preparation of stem protein from human embryonic kidney 293 cells

The sequence encoding the HA stem gene (Impagliazzo et al., 2015) was synthesized (Genscript) and constructed into pCMV vector (Invitrogen). Human epithelial kidney (HEK) 293T cells were routinely

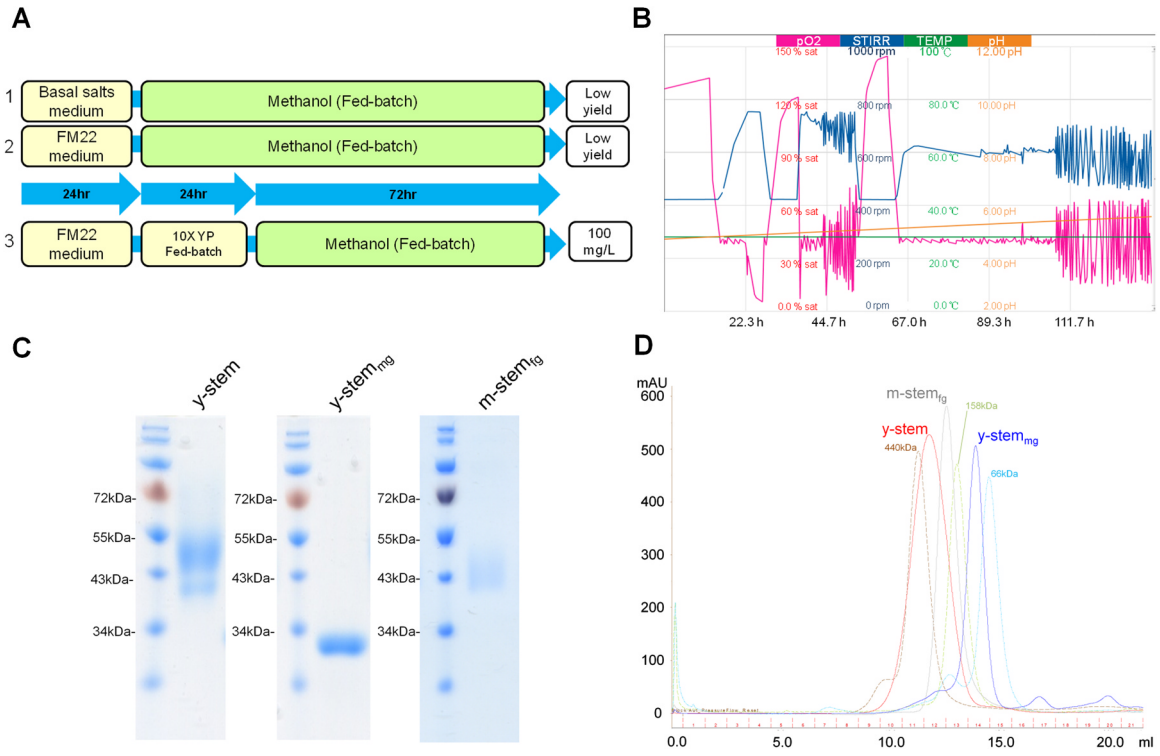

Fig. 1. Production of HA stem proteins using fermentation fed-batch process. (A) The HA stem gene of mini-HA#4900 was synthesized and constructed, and then transformed to *P. pastoris* for expression. Three fermentation processes were evaluated for production of HA stem protein (y-stem). (B) A representative result of the third process was shown in Fig. 1A. The yield using this process reached 100 mg/l culture. (C) The purified y-stem protein was processed for glycan removal to generate y-stem$_{mg}$. In addition, the HA stem protein was produced from HEK293T cells (m-stem$_{fg}$). The purified proteins were analyzed by SDS-PAGE with coomassie blue staining method. The purified proteins were used for the following mice studies. (D) Molecular weight and the oligomeric status of m-stem$_{fg}$ (gray line), y-stem (red line) and y-stem$_{mg}$ (blue line) were confirmed by size exclusion chromatography.

maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco). The plasmid containing the gene was transfected into HEK293T cells by TransIT-LT1 (Mirus Bio LLC) according to the manufacturer's protocols. The HA stem protein (m-stem$_{fg}$) was purified from the culture media by Ni-NTA resin (GE Healthcare) and confirmed by immunoblotting and gel filtration chromatography.

2.5. Identification of N-linked glycosylation on HA stem proteins

The purified y-stem, m-stem$_{fg}$, or y-stem$_{mg}$ (5 μg) was dissolved in 10 μl of 50 mM ammonium bicarbonate (ABC), pH 8.0. The protein samples were further denatured using 50% tetrafluoroethylene and the disulfide bonds were reduced via addition of 1 μl of 40 mM dithiothreitol (DTT). After incubation at 65 °C for 1 h, the protein samples were added 4 μl of 40 mM iodoacetamide and incubated at room temperature for 1 h in the dark for alkylation. Protein samples were diluted with 80 μl of 25 mM ammonium bicarbonate and then treated with 0.2 μg trypsin (Sigma) at 37 °C overnight. The digested protein samples were dried using a Speedvac evaporator and re-dissolved in 20 μl of distilled water. The samples were subjected to LC-MS/MS analysis for determination of the glycosylation sites and the types of the N-glycans (Tsai et al., 2013).

2.6. Vaccination studies in mice

Six to eight-week-old female BALB/c mice (n = 10 per group) were intramuscularly immunized with three doses of 30 μg of purified y-stem, m-stem$_{fg}$, or y-stem$_{mg}$ protein in 100 μl of PBS, pH 7.4 containing 50 μg of adjuvant Al(OH)$_3$ (Sigma) or 2 μg of the glycolipid C34 (Lin et al., 2010). The control groups of mice were immunized intramuscularly with 100 μl phosphate buffer saline (PBS). The vaccinations were given at 2-week intervals. Fourteen days after the second and the third immunizations, the blood was drawn from immunized mice and the sera were collected for ELISA and neutralization analysis.

2.7. Determination of HA-specific antibodies by ELISA

HA-specific antibody titers were determined by direct ELISA using y-stem$_{mg}$, y-stem, the H1N1 A/Brisbane/59/2007 HA, and the H5N1 Vietnam/1194/2004 HA proteins as the coating antigen. The HA protein from H1N1 A/Brisbane/59/2007, or H5N1 A/Vietnam/1194/2004 was expressed as secreted protein using HEK 293T cells and the purified proteins were used for ELISA (Chen et al., 2014b). The 96-well ELISA plate was coated with 100 μl of purified protein diluted in 100 mM sodium bicarbonate (pH 8.8) at a concentration of 5 μg/ml per well, and incubated at 4 °C for overnight. Alternatively, the MDCK cells (1×10^4 cells/well of a 96-well plate) were infected with the A/Brisbane/59/2007 or A/Vietnam/1194/2004 viruses (MOI = 1) for 48 h, and fixed with methanol:acetone (1:1) at room temperature for 10 min. The solution in each well was removed and subsequently added with 200 μl of blocking buffer (1% BSA, 137 mM NaCl, 20 mM Tris-base, and 0.05% Tween 20; pH 7.4) at 37 °C followed with incubation for 1 h. The blocking buffer was removed and the coated plates were washed three times with wash buffer TBST (137 mM NaCl, 20 mM Tris-base, and 0.05% Tween 20; pH 7.4). The antisera (150 μl) collected from HA-stem immunized mice on day 42 in two-fold serial dilution were added to the coated plates and incubated at 37 °C for 1 h. After removal of antisera followed by washing, 200 μl of the horseradish peroxidase (HRP)-conjugated anti-mouse IgG (PerkinElmer) were added to each well and incubated at 37 °C for 1 h. The solution was removed, and the coated plates were washed six times with wash buffer. The plates were developed with 100 μl of the Super Aquablue ELISA substrate (eBioscience) for 1 min. The reaction was stopped by adding 100 μl of 0.625 M oxalic acid. The absorbance of each well was measured at

Table 1
The glycan structures of HA stem proteins analyzed using LC–MS/MS.

Glycan name	Glycan structure	N28			N40			N150			N233		
		m-stem$_{fg}$	y-stem	y-stem$_{mg}$	m-stem$_{fg}$	y-stem	y-stem$_{mg}$	m-stem$_{fg}$	y-stem	y-stem$_{mg}$	m-stem$_{fg}$	y-stem	y-stem$_{mg}$
None		0%	0%	0%	0%	0%	0%	97%	97%	48%	1%	8%	15%
Deamidated		0%	0%	0%	0%	0%	0%	0%	2%	0%	0%	46%	0%
N		0%	4%	100%	0%	0%	100%	0%	1%	53%	0%	0%	85%
Man5		0%	0%	0%	11%	0%	0%	0%	0%	0%	0%	0%	0%
N-N3H5S0F0		0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%
NNH3NNF1-G0		5%	0%	0%	0%	0%	0%	0%	0%	0%	12%	0%	0%
Man6N1		0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%
BiF1-H		0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%
Bi		0%	0%	0%	15%	0%	0%	0%	0%	0%	0%	0%	0%
N-N5H4S0F0		0%	0%	0%	21%	0%	0%	0%	0%	0%	0%	0%	0%
N-N5H3S0F1		2%	0%	0%	0%	0%	0%	0%	0%	0%	15%	0%	0%
Man8		6%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%
BiF1		0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	0%
Man9		0%	7%	0%	0%	60%	0%	0%	0%	0%	0%	18%	0%
BiH1		0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%
BiN1		0%	0%	0%	8%	0%	0%	0%	0%	0%	0%	0%	0%
BiN1F1-H		0%	0%	0%	0%	0%	0%	0%	0%	0%	20%	0%	0%
N-N6H4S0F0		0%	0%	0%	2%	0%	0%	0%	0%	0%	0%	0%	0%
N-N6H3S0F1		0%	0%	0%	2%	0%	0%	0%	0%	0%	2%	0%	0%
BiN1F2-H		0%	0%	0%	3%	0%	0%	0%	0%	0%	2%	0%	0%
BiN1F1		0%	0%	0%	0%	0%	0%	1%	0%	0%	9%	0%	0%

(*continued on next page*)

Table 1 (*continued*)

Man10		0%	59%	0%	0%	29%	0%	0%	0%	0%	0%	19%	0%
N-N6H4S0F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%
BiS1F1		4%	0%	0%	0%	0%	0%	0%	0%	0%	4%	0%	0%
N-N5H4S1F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	5%	5%	0%
Man11		0%	29%	0%	0%	7%	0%	0%	0%	0%	0%	0%	0%
BiN1S1F1		4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
N-N6H5S0F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%
N-N5H4S1F2		0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%
BiN1S1F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	0%	0%
Man12		5%	0%	0%	0%	2%	0%	0%	0%	0%	0%	2%	0%
Man13		0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	2%	0%
N-N5H4S2F1		1%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	0%
TriS1F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	0%
N-N6H5S1F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	0%
TriS1N1F1		0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%
TriS2N1F1		3%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%
BiF1N1S2		1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TriS2F1		3%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TriS3F1		60%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TetraS4F1		6%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
TetraH2S3		0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%
PentaS3F1		0%	0%	0%	29%	0%	0%	0%	0%	0%	0%	0%	0%
Others		1%	0%	0%	3%	0%	0%	1%	0%	0%	4%	0%	0%

405 nm using SpectraMax M5 (Molecular Devices). The endpoint antibody titer was defined as the last dilution fold of antisera to give an absorbance 2.5-fold higher than the absorbance of the pre-immune serum (50× dilution). Optionally, for detection of isotypes and subtypes of HA specific antibodies, a step of the addition of 50 µl of anti-IgA, IgM, IgG1, IgG2a, IgG2b and IgG3 (Merck) antibodies for 1 h

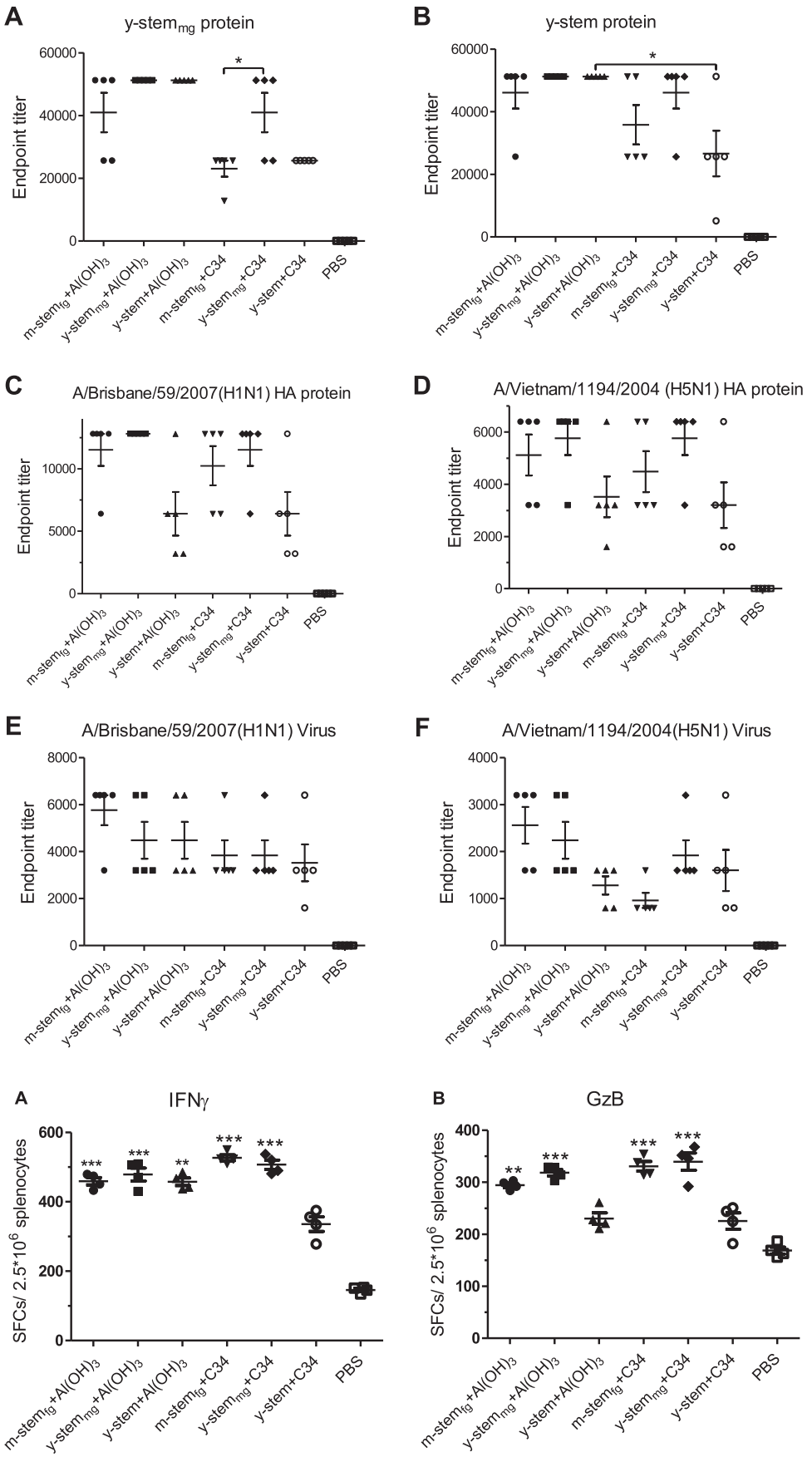

Fig. 2. The broadly cross-reactive antibodies assessed by ELISA. BALB/c mice were intramuscularly immunized with three doses of the recombinant proteins y-stem, m-stem$_{fg}$, or y-stem$_{mg}$ adjuvanted with Al(OH)$_3$ or C34. The mice were immunized at 2-week intervals. Fourteen days after the third immunizations, the antisera were drawn from immunized mice and analyzed using ELISA with the y-stem$_{mg}$ (A), the y-stem (B), and the A/Brisbane/59/2007 H1N1 HA protein (C) and the A/Vietnam/1194/2004 H5N1 HA protein (D) as the coating antigens. Alternatively, the MDCK cells infected with A/Brisbane/59/2007 H1N1 (E) or A/Vietnam/1194/2004 H5N1 (F) were used for ELISA studies. The endpoint antibody titer was defined as the last dilution fold of antisera to reach the absorbance 2.5 times higher than the negative control (pre-immune serum). Data represents the mean ± standard deviation. Results were analyzed using one-way ANOVAs; differences considered statistically significant were marked as "*" ($p < 0.05$).

Fig. 3. T cell response in splenocytes evaluated by ELISpot assay. BALB/c mice were immunized with HA stem proteins, the splenocytes of immunized mice were obtained after three immunizations and the IFN-γ (A) and GzB (B)-secreting cells were assessed using ELISpot assay. The number of spot-forming cells (SFCs) is presented as mean ± standard deviation. Results were analyzed using one-way ANOVAs. Differences considered statistically significant (individual group compared to the group of the y-stem + C34) were marked as "*" for $p < 0.05$, "**" for $p < 0.01$, and "***" for $p < 0.005$.

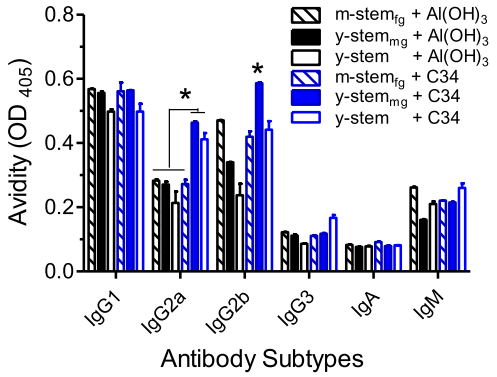

Fig. 4. Subtypes of Elicited antibodies. The antisera from immunized mice with HA stem proteins were characterized for IgA, IgM, IgG1, IgG2a, IgG2b and IgG3 by direct ELISA using the y-stem$_{mg}$ as the coating antigen. Data represent the mean ± standard deviation. Results were analyzed using one-way ANOVAs; differences considered statistically significant were marked as "*" ($p < 0.05$).

incubation followed by washing were added prior to the addition of 200 µl of anti-rabbit IgG-HRP (Merck) for 1 h incubation. The solution was removed, and the plates were washed six times with wash buffer and developed with 100 µl of the TMB substrate (Merck) for 1 min. The reaction was stopped by adding 100 µl of 1 M phosphoric acid. The absorbance of each well was measured at 450 nm using SpectraMax M5 (Molecular Devices).

2.8. Bioassay for antibody dependent cell mediated cytotoxicity

MDCK cells were seeded on 96 well flat-bottom plates at a concentration of 1×10^4 cells per well. After incubation for 24 h, MDCK cells were infected with influenza viruses at multiplicity of infection (MOI) of 1 for 24 h. The medium was then replaced with RPMI 1640 medium containing 4% low IgG FBS followed by the addition of serial dilutions of antisera from HA stem protein-vaccinated mice for a 30 min incubation at 37 °C. Jurkat effector cells expressing mouse FcγRIII and FcγRIV (Promega) were resuspended in RPMI 1640 medium containing 4% low IgG FBS and the cells at a target-effector ratio of 1–4.5 were added to assay plates. After incubation at 37 °C for 6 h, Bio-Glo Luciferase Assay Buffer (Promega) was added and luminescence was quantified using a plate reader (CLARIOstar).

2.9. Enzyme-Linked Immunospot (ELISpot) assay

ELISpot plates were pre-coated with anti-mouse IFN-γ (Mabtech AB) or anti-mouse granzyme B (GzB) (R&D Systems Inc) according to the manufacturer's instructions. The plates were washed four times and incubated for 30 min with RPMI-1640 supplemented with 10% fetal bovine serum. For the detection of IFNγ and GzB-secreting cells, the splenocytes of immunized mice were obtained on day 42 and cultured in ELISpot plates at 5×10^5 cells per well at 37 °C in 5% CO_2. The peptides derived from A/Brisbane/59/2007 HA (DTVDTVLEK, HDSNVKNLY, EIGNGCFEF, and MESVKNGTY) were equally mixed and added at a total of 2 µg per well for re-stimulation. After 24–48 h, the cells were removed and incubated with biotinylated anti-mouse IFNγ or GzB-specific antibody. The plates were washed five times before the addition of streptavidin conjugated with alkaline phosphatase (ALP) and the BCIP/NPT substrate for development. After drying, the number of resulting spots was analyzed with an Immune Spot Reader (Cellular Technology Ltd.).

2.10. Neutralization assay

Culture supernatant containing 100-fold $TCID_{50}$ of virus was mixed with equal volume of two-fold serially diluted serum and incubated at room temperature for 1 h. The mixture was added onto MDCK monolayer cells in 96-well plates followed by incubation at 37 °C overnight. The mixture was removed, and the conditioned medium (MEM with 0.3% BSA and 1 µg/ml TPCK-treated trypsin) was added. After incubation at 37 °C for 72 h, the cells were added to 30 µl CellTiter-Glo (Promega) to determine the number of viable cells based on quantification of the ATP concentrations. The neutralizing activity of serum was determined as the maximal dilution fold that significantly protected the cells from virus-induced death. The neutralization titer, the antisera dilution resulted in 50% virus induced cell death, was calculated by using GraphPad Prism software.

2.11. Virus challenge experiments

After three vaccinations at two week intervals, the immunized mice were challenged intranasally with 30× LD_{50} (the virus doses leading to 50% of the death of mice) of H1N1 A/WSN/1933 viruses and 5× LD_{50} of H5N1 A/Vietnam/1194/2004 viruses. After infection, the survival rate and body weight of mice were recorded for 14 days. The percentage of body weight was calculated for each individual animal per group by comparing the daily weight to the pre-challenge weight, and the mice losing more than 25% of their initial weight were sacrificed and scored as dead. Mice studies were approved by the Institutional Animal Care and Use Committee of Academia Sinica.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism software. The experiments were performed in triplicates and the data were presented as mean ± standard deviation. All results were examined by using one-way ANOVA with Tukey's tests; differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Expression, purification, and characterization of the A/Brisbane/59/2007 HA stem proteins

To evaluate the immunogenicity of the A/Brisbane/59/2007 HA stem with different glycans, we used *Pichia pastoris* and human cells (HEK 293T) to produce the HA stem-based protein. The HA stem gene was synthesized using the sequence of mini-HA#4900 (Impagliazzo et al., 2015), which was based on H1N1 A/Brisbane/59/2007 and constructed into pCMV and pPicZA vector, and then transformed to *P. pastoris* KM71H strain and human cells (HEK 293 T), respectively. For protein expression in *P. pastoris*, the transformed strain was inoculated into 400 ml BMGY in 3 l shake flask at 28 °C for 24 h. The expression of the A/Brisbane/59/2007 HA stem protein was subsequently induced with 1% methanol in BMMY medium. After 96 h, the final wet cell weight was about 70 g per liter culture. The A/Brisbane/59/2007 HA stem protein (y-stem) were purified from the culture media by using Ni-NTA resin with the yield of 10 mg/l. We also tested the production of A/Brisbane/59/2007 HA stem protein from the *Pichia* expression system using the fermentation process in order to increase the economic value as well as the production yield (Fig. 1A). Initially, basal salts medium (process 1 in Fig. 1A) or FM22 medium (process 2 in Fig. 1A) was used in the first 24 h. When the dissolved O_2 decreased (pO_2), methanol was introduced in the fed-batch phase to start the protein production process. Unfortunately, both processes resulted in low cell growth and low protein yield. To increase the biomass production, glycerol (10×) was added in the fed-batch phase before the methanol induction (process 3 in Fig. 1A). When the pO_2 dropped, methanol was then added in the fed-based phase to induce protein expression. After 72–96 h in the methanol fed-batch phase, the culture medium was collected (Fig. 1B) and processed for purification by Ni-NTA resin to give the HA stem

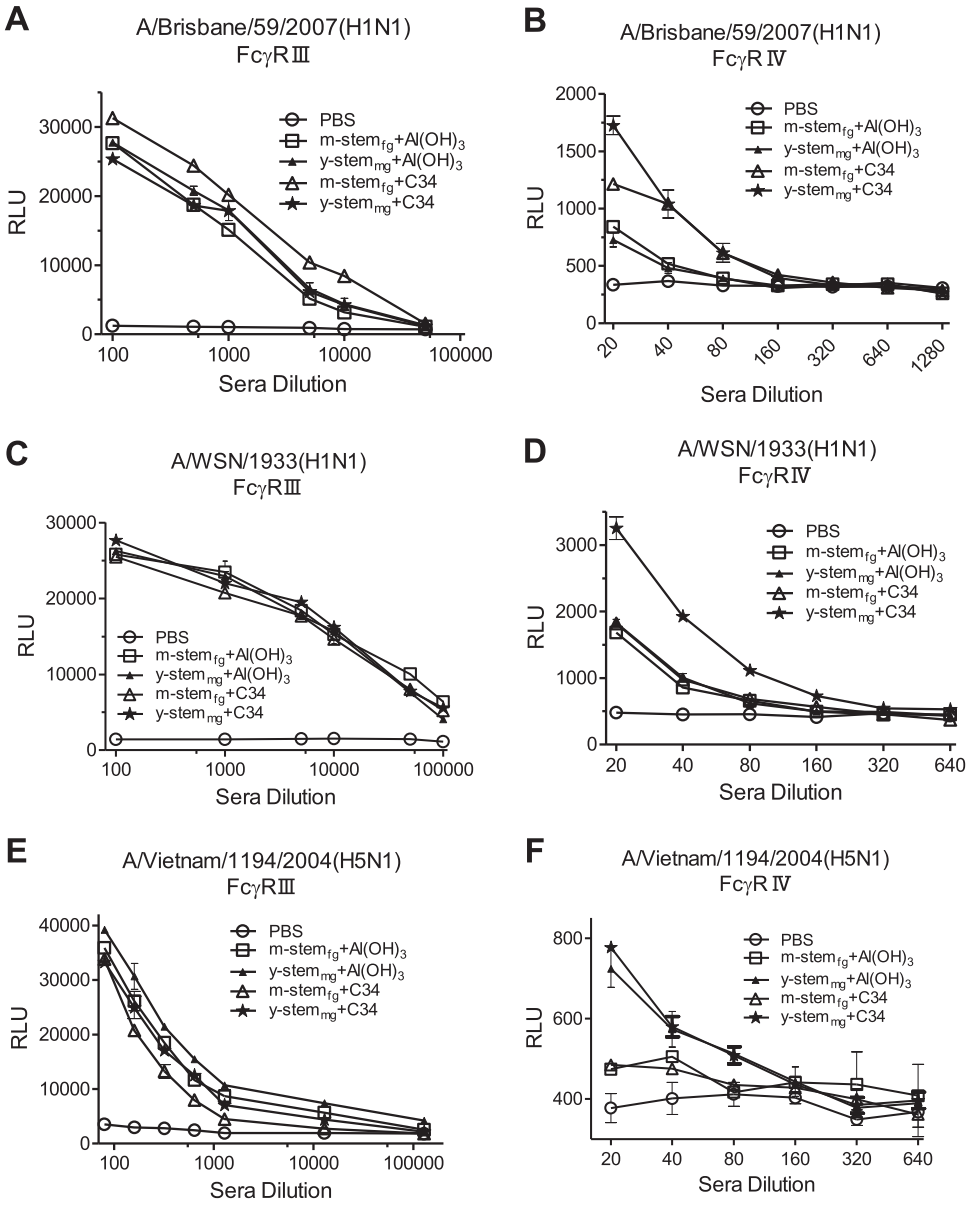

Fig. 5. ADCC responses of antisera from HA stem protein vaccinated mice. The antisera collected from mice immunized with adjuvanted m-stem$_{fg}$ and y-stem$_{mg}$ were incubated with MDCK cells which were infected with A/Brisbane/59/2007 (H1N1) (A and B), A/WSN/1933 (H1N1) (C and D), or A/Vietnam/1194/2004 (H5N1) (E and F) for 30 min. Subsequently, the ADCC bioassay was performed using Jurkat effector cells expressing mouse FcγRIII (A, C, and E) and FcγRIV (B, D, and F) and the relative luminescence unit (RLU) was measured and presented as mean ± standard deviation.

protein (y-stem). The yield was up to 100 mg/l, a 10-fold increase compared to that from the shake-flask culture.

The y-stem prepared from *P. pastoris* is highly glycosylated as it appeared as a wide protein band with apparent molecular weight much higher than the calculated molecular weight of 31 kDa (Fig. 1C). The monoglycosylated form of y-stem was further prepared by treating y-stem$_{fg}$ with EndoH to remove the *N*-glycan followed by purification with size exclusion chromatography. The apparent molecular weight of y-stem$_{mg}$ greatly decreased from the apparent molecular weight of y-stem and now is close to 31 kDa, indicating that most of *N*-glycans has been removed by EndoH treatment (Fig. 1C). Moreover, the molecular weight and the oligomeric status of the y-stem and the m-stem$_{fg}$ were confirmed by size exclusion chromatography (Fig. 1D). The results showed that the y-stem showed a broader peak than the m-stem$_{fg}$, indicating that the y-stem might have more *N*-glycans or longer *N*-glycans or both. Furthermore, the y-stem$_{mg}$ showed a sharp peak between 66 kDa and 158 kDa, consistent with the molecular weight of a trimeric form (93 kDa). We also prepared the A/Brisbane/59/2007 HA stem protein from human cells (HEK293T) for comparison. We obtained approximately 1 mg of the A/Brisbane/59/2007 HA stem protein (m-stem$_{fg}$) from 1 l cell culture. As shown in Fig. 1C, the apparent molecular weight of m-stem$_{fg}$ is higher than the expected molecular weight of 31 kDa calculated from the sequences, indicating that the protein prepared from 293T cells is glycosylated.

The glycosylation sites and the glycan structures of the purified y-stem, y-stem$_{mg}$, and m-stem$_{fg}$ were analyzed by LC–MS/MS. There were four *N*-glycosylation sites (N28, N40, N150, and N233) predicted on the stem region. Consistent with the prediction, all four sites were confirmed to be glycosylated. The *N*-glycans of m-stem$_{fg}$ were diverse while the *N*-glycans of y-stem are composed of high-mannose glycans (Table 1). It is also confirmed that the y-stem$_{mg}$ contained 85–100% one GlcNAc at its *N*-glycosylation sites in N28, N40, and N233. The other glycan site N150 was partially glycosylated.

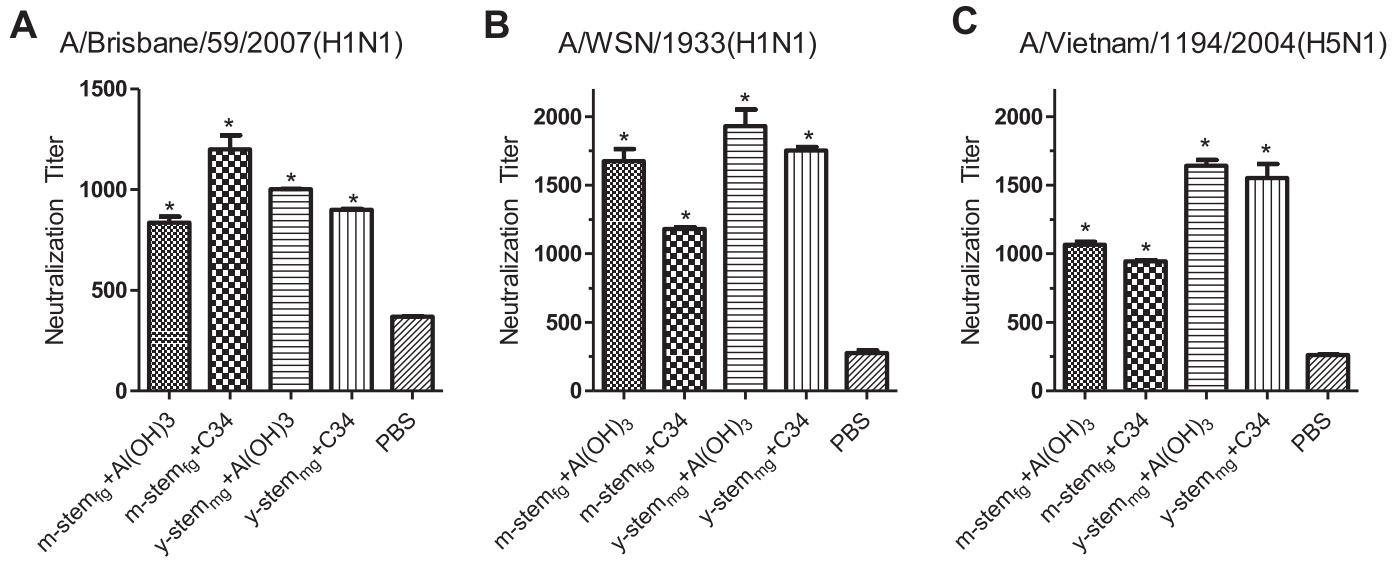

Fig. 6. Induction of neutralizing antibodies against H1N1 and H5N1 virus. The neutralization activities of antisera from m-stem$_{fg}$ and y-stem$_{mg}$ immunized mice were monitored by using (A) H1N1 A/Brisbane/59/2007, (B) H1N1 A/WSN/1933 viruses, and (C) H5N1 A/Vietnam/1194/2004. The neutralizing titer of serum was determined as the dilution fold that significantly protected 50% of the cells from virus-induced death and was presented as mean ± standard deviation. Results were analyzed using one-way ANOVAs; differences considered statistically significant were marked as "*" ($p < 0.05$).

3.2. Induction of broadly cross-reactive antibodies by the HA stem protein

The binding profiles of the antibodies elicited by the A/Brisbane/59/2007 HA stem proteins were further evaluated. BALB/c mice were intramuscularly immunized with three doses of the recombinant proteins y-stem, m-stem$_{fg}$, or y-stem$_{mg}$ adjuvanted with Al(OH)$_3$ or C34. The glycolipid C34 is an analogue of α-galactosylceramide, which was isolated from marine sponges and is shown to bind CD1d on dendritic cells to modulate the immune response (Carreno et al., 2014). Among all the α-galactosylceramide analogues tested, C34 was shown to have superior effects on several carbohydrate-based vaccines (Huang et al., 2013). We therefore use aluminum, a commonly-used agent for immunization, and C34 glycolipid as adjuvants to evaluate the immunogenicity of the HA stem proteins in various glycoforms. The mice were immunized on day 0, 14, and 28, and the antisera were collected on day 42 and analyzed using direct ELISA. The binding to the original immunogen (the y-stem$_{mg}$ or the y-stem) was first monitored (Figs. 2A and B). It was surprisingly found that the antiserum induced by C34-adjuvanted fully glycosylated m-stem$_{fg}$ or y-stem showed less binding to the y-stem$_{mg}$ or the y-stem protein. On the other hand, the C34-adjuvanted y-stem$_{mg}$ induced comparable binding antibodies to the aluminum-adjuvanted proteins. In order to elucidate whether the antiserum elicited by the A/Brisbane/59/2007 HA stem proteins can recognize the HA proteins, another ELISA was performed with the A/Brisbane/59/2007 HA protein and the A/Vietnam/1194/2004 HA protein as antigens. The data showed that the HA stem proteins could elicit antibodies which recognized the HA from H1N1 as well as H5N1 strains (Figs. 2C and D). Furthermore, the antibody titers elicited by the y-stem$_{mg}$ are higher than the y-stem, but is comparable to the titers induced by the m-stem$_{fg}$. No significant differences were observed between the Al(OH)$_3$- and C34-adjuvanted proteins. Alternatively, we used the MDCK cells infected with A/Brisbane/59/2007 H1N1 virus (Fig. 2E) or with A/Vietnam/1194/2004 H5N1 virus (Fig. 2F) to analyze the binding profiles of the antiserum. Although the binding to the A/Brisbane/59/2007-infected cells with all the antisera showed no significant differences, the binding of y-stem$_{mg}$ antiserum exhibited higher binding capacity than the binding of y-stem antiserum to MDCK cells infected with a heterologous strain A/Vietnam/1194/2004 H5N1 virus.

3.3. Cell-mediated immunity elicited by the HA stem protein

To determine the cytokine-secreting T cells in HA stem protein-immunized mice, the splenocytes of immunized mice were obtained after three immunizations and the IFN-γ and GzB-secreting cells were assessed upon stimulation of HA proteins. As shown in Fig. 3, the m-stem$_{fg}$ and y-stem$_{mg}$ adjuvanted with Al(OH)$_3$ or C34 elicited more IFN-γ and GzB-secreting T cells than the y-stem adjuvanted with C34 did. These results confirmed that the HA stem protein could stimulate T cell responses against viral antigens.

3.4. Subtypes of elicited antibodies and induction of ADCC activities

To further characterize the subtypes of antibodies induced by different glycoforms of HA stem proteins, the levels of IgG1, IgG2a, IgG2b and IgG3, IgA, and IgM in antisera from immunized mice were determined using ELISA. High levels of HA-specific IgG1 antibody responses were elicited by all HA stem proteins. Interestingly, the levels of IgG2a in the antisera from mice immunized with the y-stem$_{mg}$ and the y-stem adjuvanted with C34 were significantly higher than other antisera (Fig. 4).

IgG plays an important role in antibody-dependent cellular cytotoxicity (ADCC) with Fcγ receptors (FcγR) against influenza infection (Nimmerjahn et al., 2015; Vanderven et al., 2017). Therefore, we investigated whether the IgG antibodies stimulated Fc-mediated effector mechanisms, the ADCC reporter bioassay was performed using Jurkat effector cells expressing mouse FcγRIII and FcγRIV to test the ADCC activities of antisera from HA stem protein-immunized mice (Fig. 5). As expected, the y-stem$_{mg}$ and the m-stem$_{fg}$ induced similar levels of ADCC activities through FcγRIII against the homologous virus A/Brisbane/59/2007 (Fig. 5A). Surprisingly, stronger ADCC activities through FcγRIV were elicited when C34 was used as the vaccine adjuvant (Fig. 5B). The

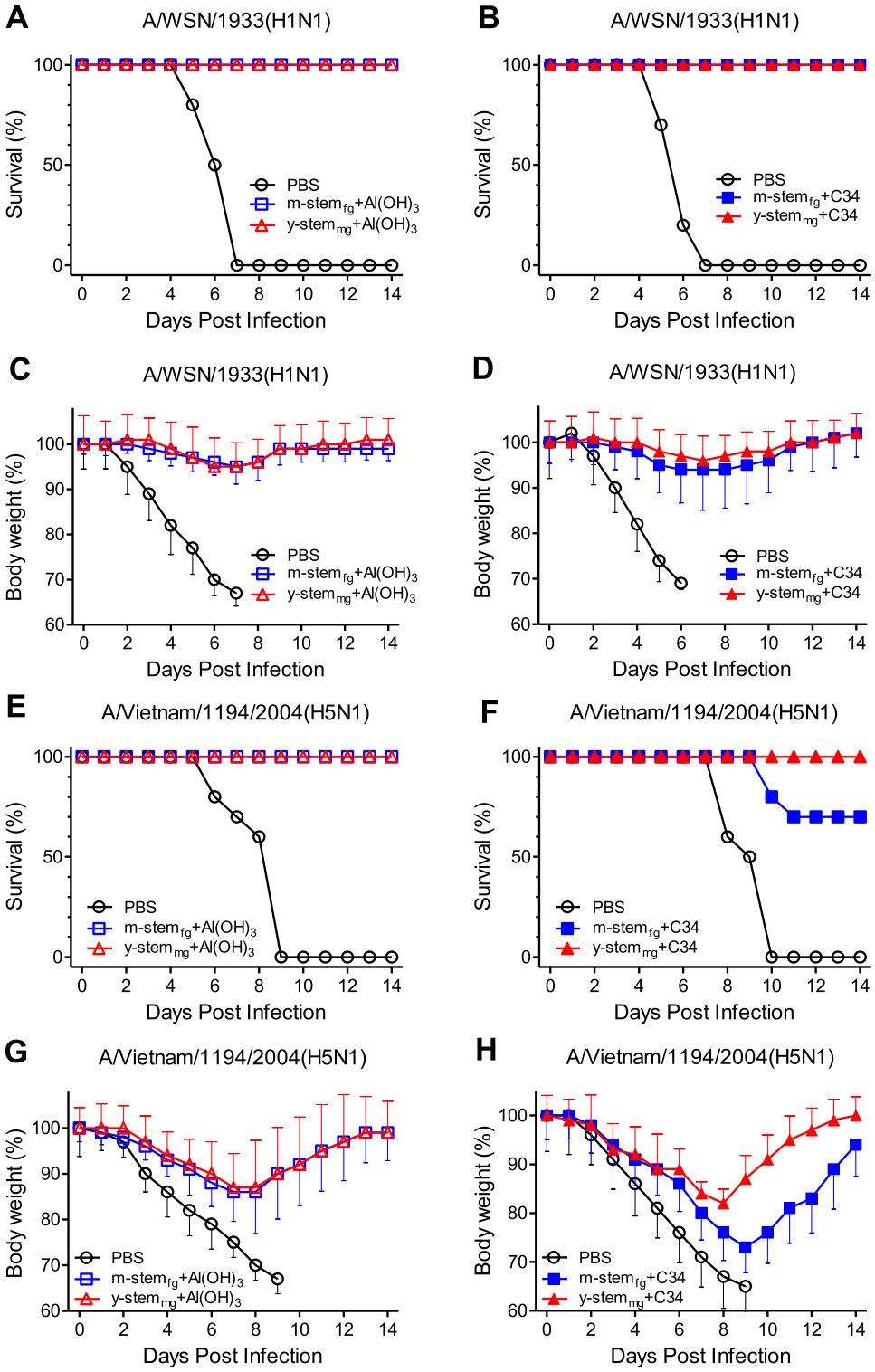

Fig. 7. Cross-protective immunity in mice challenged with viruses. BALB/c mice were immunized intramuscularly with three doses of m-stem$_{fg}$ and y-stem$_{mg}$ with adjuvant Al(OH)$_3$ (A, C, E, G) or C34 (B, D, F, H) over a 2-week interval (n = 10 per group). After resting for 1 week, the immunized mice were challenged intranasally with H1N1 A/WSN/1933 viruses (A–D) and H5N1 A/Vietnam/1194/2004 viruses (E–H). Survival rates (A, B, E and F) and body weights (C, D, G and H) were recorded for 14 days. Body-weight loss is presented as mean ± standard deviation. Shown are representatives from three independent experiments.

same phenomenon were observed on the induced ADCC levels against the A/WSN/1933 (H1N1) virus (Figs. 5C and 5D). It was also noticed that the y-stem$_{mg}$ protein can elicit higher ADCC activities against the heterologous H5N1 virus A/Vietnam/1194/2004 (Figs. 5E and F).

3.5. Neutralizing antibodies against H1N1 and H5N1 viruses

Our previous data showed that the immunogenicity of m-stem$_{fg}$ and y-stem$_{mg}$ were greater than the y-stem; therefore, the neutralization

activities of the antisera from immunized mice were further investigated. The results exhibited that the antisera from the m-stem$_{fg}$- as well as the y-stem$_{mg}$- immunized mice could neutralize the H1N1 and H5N1 viruses and protect cells from virus-induced death (Fig. 6). The neutralization activities of m-stem$_{fg}$ vs. y-stem$_{mg}$ were found comparable against the homologous strain A/Brisbane/59/2007 (Fig. 6A). Nonetheless, compared to the antisera from m-stem$_{fg}$, the antisera from y-stem$_{mg}$ provided significantly higher neutralizing activity against the heterologous viruses H1N1 A/WSN/1933 and H5N1 A/Vietnam/1194/2004 (Figs. 6B and 6C). The results demonstrated that m-stem$_{fg}$ and y-stem$_{mg}$ could induce broadly neutralizing antibodies against H1N1 and H5N1 viruses, and the neutralizing activity of y-stem$_{mg}$ was greater than m-stem$_{fg}$, specifically against the heterologous strains.

3.6. Evaluation of the cross-protection of the HA stem protein against H1N1 and H5N1 viruses

The benchmark of an influenza vaccine is the protection against a lethal virus challenge. Challenge experiments were performed by infecting vaccinated mice with H1N1 and H5N1 viruses to evaluate the immunogenicity of purified A/Brisbane/59/2007 HA stem proteins. BALB/c mice were immunized intramuscularly with three doses of m-stem$_{fg}$ and y-stem$_{mg}$ with adjuvant Al(OH)$_3$ or C34 (n = 10 per group). After resting for 1 week after the third immunization, the immunized mice were challenged intranasally with 30× LD$_{50}$ of H1N1 A/WSN/1933 viruses and 5× LD$_{50}$ of H5N1 A/Vietnam/1194/2004 viruses. As shown in Fig. 7, the mice vaccinated with m-stem$_{fg}$ and y-stem$_{mg}$ survived well upon the challenge of H1N1 A/WSN/1933 viruses (Figs. 7A and B). The bodyweight of the m-stem$_{fg}$ and y-stem$_{mg}$-immunized mice gradually decreased upon the virus challenge, but recovered at the 8th day post infection (Figs. 7C and D). For the mice challenged with H5N1 A/Vietnam/1194/2004 virus at 5× LD$_{50}$, all mice immunized with aluminum-adjuvanted mice survived, however, three out of 10 mice immunized with C34-adjuvanted m-stem$_{fg}$ died at the 6th day post infection (Figs. 7E and F). Similar to the pattern observed upon the challenge with the H1N1 A/WSN/1933 virus, the bodyweight of the m-stem$_{fg}$ and y-stem$_{mg}$-immunized mice gradually decreased upon the H5N1 A/Vietnam/1194/2004 virus challenge, but recovered at the 8th day post infection (Figs. 7G and H). In conclusion, the challenge data indicated that the HA stem vaccine could provide cross-protection against H1N1 and H5N1 viruses. In addition, the monoglycosylated form of the stem protein y-stem$_{mg}$, compared to the glycosylated m-stem$_{fg}$, provides broader protection activities.

4. Discussions

Development of a universal vaccine is the ultimate goal to prevent pandemic influenza. Recent studies show that the influenza nucleoprotein (NP) (Yewdell et al., 1981; Zheng et al., 2014), neuraminidase (NA) (Sandbulte et al., 2007), M2 (Fu et al., 2009; Huleatt et al., 2008; Tompkins et al., 2007; Wang et al., 2008, 2009b), and HA (Ekiert et al., 2009; Impagliazzo et al., 2015; Mallajosyula et al., 2014; Prabhu et al., 2009; Throsby et al., 2008) can elicit cross-protective antibodies. Therefore, the viral proteins NP, NA, M2 and HA might be used as universal vaccine candidates. The production of the extracellular domain of matrix protein 2 (M2e), which was highly conserved among human influenza viruses, could be developed as a potential universal influenza vaccine (Lee et al., 2015). HA2 is also highly conserved in H1 and H5 (Ekiert et al., 2009; Impagliazzo et al., 2015). Among all the influenza proteins, HA is the most abundant on the surface of influenza virus; therefore, HA is a better candidate for vaccine development. Previous studies reported that mini-HA#4900, prepared from mammalian (293T) cells, was found to elicit cross-protection in mice and in non-human primates (Impagliazzo et al., 2015).

Yeast cells have been used for the large-scale productions of recombinant protein; it is more economical, safer, and easier to scale up than other expression systems (Bollok et al., 2009). Recent studies demonstrated that the *P. pastoris* expression system may provide a simple tool to produce recombinant HA proteins for influenza vaccine. When the *P. pastoris* system was used to express influenza HA, approximately 15–40 mg/l of HA proteins were secreted into the culture medium and the *N*-glycosylation sites of HA were demonstrated to be the same as those of the native HA, albeit with different *N*-glycan forms (Kopera et al., 2014; Wang et al., 2007; Xu et al., 2006). Recently, the expression of the HA1 gene of influenza virus H5N1 by *P. pastoris* resulted in a yield of 120 mg/l through the use of fed-batch fermentation (Lin et al., 2016). The yields up to 200 mg/l were obtained for the expression of the extracellular domain of H5N1 HA by *P. pastoris* (Pietrzak et al., 2016). The full length HA of the epidemic H1N1 strain (A/California/04/2009) was expressed in *P. pastoris*, and the secreted soluble HA protein was able to elicit neutralizing antibodies both in mice and rabbits (Athmaram et al., 2011). These data indicated that the *P. pastoris* expression system can provide a safe, effective, and economical platform to speed up the large-scale production of recombinant influenza HA protein. Nonetheless, the production of HA stem region using *P. pastoris* expression system has not been reported so far.

The production of the HA stem proteins in yeast has not been reported. In this report, we explored the production of the HA stem protein from the *Pichia* expression system with fermentation process and selected the KM71H Mut^s strain for the production of the HA stem from the *Pichia* expression system as universal vaccine candidate. *P. pastoris* KM71H Mut^s strain has a deletion of the AOX1 gene and only expresses the AOX2 gene. This strain grows slower than the wild-type strain but has the advantage of better yield (Cregg et al., 1989; Chiruvolu et al., 1997). The yields of mini-HA#4900 were 10–21 mg/l (Impagliazzo et al., 2015). Our results showed that y-stem can be produced in a trimeric form in the yeast system. The HA stem protein was secreted into the culture medium with a yield of approximately 10 mg/l in the culture flask. The yield was further improved to 100 mg/l by using the fermentation process.

The HA stem protein produced using *Pichia* was demonstrated to successfully elicit cross-reactive antibody responses to purified HA proteins or virus-infected cells (Fig. 2). ELISpot assays demonstrated that the HA stem protein can stimulate T cell responses. Compared with the y-stem, higher amounts of IFN-γ- and GzB-secreting T cells were stimulated by m-stem$_{fg}$ and y-stem$_{mg}$ (Fig. 3). In addition, the major subtype of HA specific antibodies was IgG1, and a significant increase in IgG2a and IgG2b was observed in the antisera immunized with the y-stem$_{mg}$ and C34 compared to other antisera. Fc receptors are important mediators of opsonophagocytosis and ADCC. Mouse FcγRIIb and FcγRIII have high affinity with IgG1. Mouse IgG2a and IgG2b can also bind FcγRIIb, FcγRIII and FcγRIV (Nimmerjahn et al., 2015). Therefore, through FcγRIV, higher IgG2a and IgG2b titer might elicit higher ADCC activity. It was also observed that y-stem$_{mg}$ adjuvanted with C34 induced higher ADCC activities through FcγRIV than others (Fig. 5). ADCC-mediated antibodies were able to crosslink FcγRs and thereby promote killing of infected cells. Thus, immunization with the HA stem protein may induce IgG responses to provide protective immunity through Fc-mediated function, and the generation of ADCC-mediated protection by the HA stem protein would be a good concept in the development of a universal influenza vaccine (DiLillo et al., 2014; Jegaskanda et al., 2014).

The *N*-glycans of HA can stabilize the protein structure (Wormald and Dwek, 1999), accelerate the folding (Jitsuhara et al., 2002), promote secondary structure formation (Imperiali and O'Connor, 1999), reduce aggregation, and increase folding cooperativity (Hanson et al., 2009; Mitra et al., 2006). The glycosylation of HA also influenced the immunogenicity of the HA protein. It was reported that the monoglycosylated form of HA can induce higher neutralizing antibodies and increase HA-specific B-cell repertoires (Chen et al., 2014b). On the other hand, Eggink et al. (2014) masked the immunodominant head domain of HA by introducing more glycosylation. The resulted

hyperglycosylated HA was able to enhance stalk-directed seroreactivity in immunized mice (Eggink et al., 2014). Various studies also indicate that the immunogenicity of envelope proteins is reduced with glycan shield (Coss et al., 2016; Helle et al., 2011). In our results, the A/Brisbane/59/2007 stem protein produced in yeast (y-stem) was glycosylated with high-mannose type, such as Man9, Man10, and Man11 (Table 1) and indeed induced lower antibody titer against the homologous and the heterologous strains. The glycosylation of the m-stem$_{fg}$ protein was less extensive than that of y-stem (Fig. 1D and Table 1). The antigenicity of m-stem$_{fg}$ is better than y-stem whether Al(OH)$_3$ or C34 was used as the adjuvant. The binding activities of the antiserum from the m-stem$_{fg}$ and the y-stem$_{mg}$-immunized mice were comparable. However, the neutralization activities of the y-stem$_{mg}$ antiserum were greater than the m-stem$_{fg}$ antiserum, especially against the heterologous H1N1 A/WSN/1933 and H5N1 A/Vietnam/1194/2004 strain (Fig. 6B and C). Even though the HA would be glycosylated naturally in the infected system, it is proposed that the glycans on protein surface are flexible and the specific antibodies can still recognize the specific peptide epitopes. Our results showed that the antiserum induced by y-stem$_{mg}$ can recognize the glycosylated HA proteins as well as the virus-infected MDCK cells (HA would be glycosylated using the machinery inside the MDCK cells) (Fig. 2). The higher seraactivities of the y-stem$_{mg}$ antiserum were also supported by the stronger ADCC responses through FcγRIV than other antisera.

Adjuvants are substances used to improve the immunogenicity of antigens. There are few adjuvants approved for human uses, such as MF59 and aluminum salts. To increase the immune responses of a given antigen, it was proposed and proven that α-galactosylceramides can be presented by the CD1d surface protein on dendritic cells to activate invariant NKT cells and thus can augment a wide variety of immune responses, especially the production of cytokines such as IL-4 for the class-switch activity and IFN-γ for the adjuvant effects (Carreno et al., 2014). Through extensive structure-activity-relationship studies (Li et al., 2010; Lin et al., 2010), it was reported that C34 has higher avidity and stability than the original α-galactosylceramide to drive the Th1 polarization (Wu et al., 2011). We also found that C34 has superior antibacterial and antiviral activities in a murine model (Lin et al., 2010). The use of C34 as vaccine adjuvants to improve the immune response has also been demonstrated in the studies using oligosaccharide-based vaccine (Huang et al., 2013) as well as using DNA-based vaccine (Hung et al., 2014). When mice were administered with C34-adjuvanted H5N1 HA-based DNA vaccine (pCHA5), a high antibody titer against H5 can be induced and a higher survival rate was increased upon virus challenges than the vaccine alone (Hung et al., 2014). In our results, the y-stem$_{mg}$ with C34 stimulated higher ADCC activities compared to the y-stem$_{mg}$ with Al(OH)$_3$. Therefore, C34 could also be used as a good adjuvant with protein vaccine.

In conclusion, we have successfully established a manufacturing technology for an efficient and productive method to produce HA stem proteins that can elicit high seraactivities and immune responses. Moreover, the mono-glycosylated form of the HA stem protein can offer heterologous protection against H1N1 and H5N1 viruses. The production of monoglycosylated HA stem protein from yeast is efficient with low operating cost and could be an alternative for the development of a universal vaccine to combat with the influenza pandemics.

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