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Recombinant influenza H1, H5 and H9 hemagglutinins containing replaced H3 hemagglutinin transmembrane domain showed enhanced heterosubtypic protection in mice

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ABSTRACT

Influenza A viruses cause annual epidemics and irregular pandemics. A vaccine with heterosubtypic protection (hetero-protection) has been needed. In the present study, various influenza H1, H3, H5, and H9 hemagglutinin (HA) proteins were expressed in insect cells, and then mice were subcutaneously immunized with the expressed HA proteins, and challenged by influenza A viruses (A/Puerto Rico/8/1934 (H1N1) or A/chicken/Guangdong/96 (H9N2)). The results first showed that wild-type H3 hemagglutinin (HA) (H3-WT), but not a transmembrane domain (TM) mutant, had hetero-protection against both H1N1 and H9N2 with survival rates of 17% and 33% respectively, and that wild-type H1 (H1-WT), H5 (H5-WT) and H9 (H9-WT) had no hetero-protection against H1N1 or H9N2 except for H5-WT against H1N1 with a survival rate of 17%. Then the H3-WT TM replaced the TMs of H1-WT, H5-WT and H9-WT to generate recombinant H1-TM, H5-TM and H9-TM respectively, and whether the H3-WT TM-dependent hetero-protection could be transferred to these TM mutants was investigated. The results showed that the H3-WT TM-dependent hetero-protection was transferable. H1-TM against H9N2 and H9-TM against H1N1 were with survival rates of 33% and 17% respectively, and H5-TM against both H1N1 and H9N2 with survival rates of 50% and 17% respectively. Furthermore, higher dosage H5-TM scored 100% hetero-protection against H1N1. These results demonstrated that replacement of the TMs of non-H3 HAs with H3-WT TM could enhance their hetero-protection. These findings would help the development of future influenza vaccines against pandemics such as the recently appeared H7N9 infection.

1. Introduction

Influenza A viruses are members of the family Orthomyxoviridae with a segmented genome consisting of eight segments encoding at least 11 proteins [6,7]. They are classified into subtypes based on the hemagglutinin (HA) and neuraminidase (NA) expressed on viral surfaces [2]. The recent discoveries of H17N10 and H18N11 subtypes in bat have expanded the influenza A viruses into 18 HA subtypes [8,9]. HA is the main surface antigen and responsible for virus entry and fusion activity [6,7]. The accumulation of point mutations within the antibody-binding sites of HA is called antigenic drift causing annual epidemics, and the introduction of a HA naïve to humans by a reassortant virus is referred as antigenic shift associated with irregular pandemics [2]. The recent emergence of avian influenza H7N9 in China is a vivid reminder that the danger of influenza pandemics is still imminent [1,5].

The main defense against influenza epidemics and pandemics is vaccine including seasonal trivalent/quadrivalent inactivated virus (TIV/QIV) vaccine and live attenuated virus vaccine [2]. The inactivated virus vaccine was first introduced more than 60 years ago. Current QIV vaccine consists of a H1N1 strain, a H3N2 strain, B Yamagata, and B Victoria strains are now becoming available [34]. However, the TIV/QIV vaccine has been in need of at least two imperative improvements; one is to overcome its dependence upon chicken eggs for viral propagation, and other to increase its cross-protective immunity (hetero-protection) because the TIV/QIV vaccine provides little protection when there is a

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gross antigenic mismatch between the vaccine strains and circulating strains [4]. The commercially available trivalent HA subunit (THS) vaccine ('FluBlok') consisting of three insect-cell-derived full length HAs same as the seasonal TIV vaccine represents the success of overcoming chicken-egg dependence [3,10,11]. As for hetero-protection, both TIV and THS vaccines have been largely overlooked that universal vaccines have been developed in other platforms; for example, the hemagglutinin HA2 stalk domain-based vaccine, NP-based DNA vaccine, M2e-based subunit vaccine and cross-reactive monoclonal antibodies [2,6,12–15,35].

The studies by some groups have demonstrated a plausible correlation between HA stability and immunity [16]; for example, when the ectodomain of HA proteins is fused to the foldon domain of fibritin from bacterial phage T4 [17], GCN4pII trimerization repeat [18], or ferritin [19], the fusion proteins showed increased stability and consequently improved potency and breadth of cross-reactive immunity.

Our previous study showed that wild-type H3 (H3-WT) had higher thermal stability than a mutant (H3-SL) with mutation of two cysteines present only in the transmembrane domain (TM) [20]. Since the two TM cysteines in H3 HA are unique, this study extended to investigate whether H1, H5 and H9 HAs without these two TM cysteines had less hetero-protection and if so whether replacement of their TMs with H3-WT TM could increase hetero-protection. Our data showed that the recombinant H1, H5 and H9 HA proteins with replaced H3-WT TM exhibited enhanced hetero-protection.

2. Materials and methods

2.1. Cell line and viruses

Spodoptera frugiperda cells (Sf9 cells) were maintained in serum free SF900II medium (Gibco). The influenza A/Puerto Rico/8/1934 (H1N1) was obtained from Wuhan Institute of Virology, CAS, and the influenza A/chicken/Guangdong/96 (H9N2) [23] from South China Agricultural University. These two viruses were adapted in BALB/c mice for virus challenge in our laboratory, and their 50% mouse lethal doses (MLD50) were determined. The reassortant H5N1 virus A/Harbin/Re-1/2003 (Re-1) derives its HA and NA genes from A/goose/Guangdong/1/96(H5N1) virus, and six internal genes from the high-growth A/Puerto Rico/8/34 (PR8) virus. Re-1 was used as inactivated virus particles obtained from Harbin Veterinary Research Institute (Harbin, China) [24]. The A/swine/Guangdong/01/1998(H3N2) was isolated by and maintained in our laboratory. All live viruses were first propagated in embryonated chicken eggs, and then inactivated and purified for being used for immunological studies.

2.2. H1, H5 and H9 gene cloning and mutagenesis; recombinant baculovirus generation, and baculovirus infection

Site-specific mutagenesis, cloning, and recombinant baculovirus generation and infection were performed as previously described [20]. Briefly, all DNA fragments were first amplified by PCR and cloned directly into pMD-18T vector (Takara) and then amplified from pMD-18T by PCR and cloned into the pFastBac1 vector to generate recombinant baculoviruses (Invitogen, Carlsbad, CA, USA). All clones were verified by sequencing.

2.3. Western blotting

Cell lysates were separated on a 10% polyacrylamide gel, and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked and subsequently probed with murine monoclonal antibodies (MAbs) respectively specific for influenza H1, H3, H5 or H9 HA (Novus Biologicals) and commercial ECL kit (Pierce).

2.4. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [25]. Briefly, Sf9 cells were infected with the recombinant baculovirus expressing HA proteins at a multiplicity of infection (MOI) of 3–5. After 72 h infection, Sf9 cells were washed, fixed, and then probed with primary murine mAbs specific for influenza H1, H5 and H9 HA (Novus Biologicals), followed by species-specific, Cy3-conjugated secondary antibody (PTG, 00009-1). Finally, cells were stained with 4′-6′-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) as counterstain, and photographed under a scanning confocal microscopy (Leica TCS-SP5).

2.5. Flow cytometry

Flow cytometry was performed as previously described [26]. Briefly, Sf9 cells infected with rBVs were suspended and incubated with primary murine mAbs specific for influenza H1, H5 and H9 HA (Novus Biologicals) for 30 min on ice; followed by species-specific, FITC-conjugated secondary antibody (PTG, 00009-1). The cells were washed, fixed in 4% paraformaldehyde and analyzed by a FACS Calibur instrument (Becton Dickinson, San Jose, CA).

2.6. Hemagglutination activity assay

The hemagglutination activity assays for Sf9 cell-expressed HA proteins were performed as previously described [20]. Briefly, the infected Sf9 cells expressing WT and mutant HAs were ultrasonicated to prepare HA suspensions for assaying HA titers of HA proteins. For assaying the thermal effects, the HA suspensions were treated at indicated temperatures (37, 44, 46, 48, 50, 52 or 54 °C) for 10 min before the erythrocytes were added. The relative ratio was calculated as the percentage of the residual HA titer after incubation over the initial HA titer for each batch; then the mean relative ratios were calculated based on three batches. Inactivated influenza viruses were used for assaying immunized sera.

2.7. Hemagglutination inhibition assay

The hemagglutination inhibition assay was performed as previously described [27]. Briefly, 25 μl of each influenza virus (PR8, H3N2, Re-1 or H9N2) with four HA units was used for HI assay. The HI titer was defined as the highest dilution of the serum able to inhibit hemagglutination.

2.8. HA preparation for vaccines

Sf9 cells were infected with recombinant or wild-type baculoviruses as above described. 3 days after infection, the cells were collected, ultrasonicated, and then centrifuged at 2000 × *g* for 30 min at 4 °C. The supernatants were centrifuged at 120,000 × *g* for 3 h at 4 °C. The resulting precipitates were resuspended in PBS and loaded onto a stepped 30–60% sucrose gradient for HA protein enrichment, and centrifuged at 65,000 × *g* for 16 h at 4 °C. Fractions were collected from the gradient interphases [28,29], and their HA contenst were analyzed.

2.9. Animals, immunization, and viral challenge

Six-week-old female BALB/c mice were purchased from experimental animal center of Sun Yat-sen University and housed in microisolator units. WT and TM-HAs with oil emulsion were given

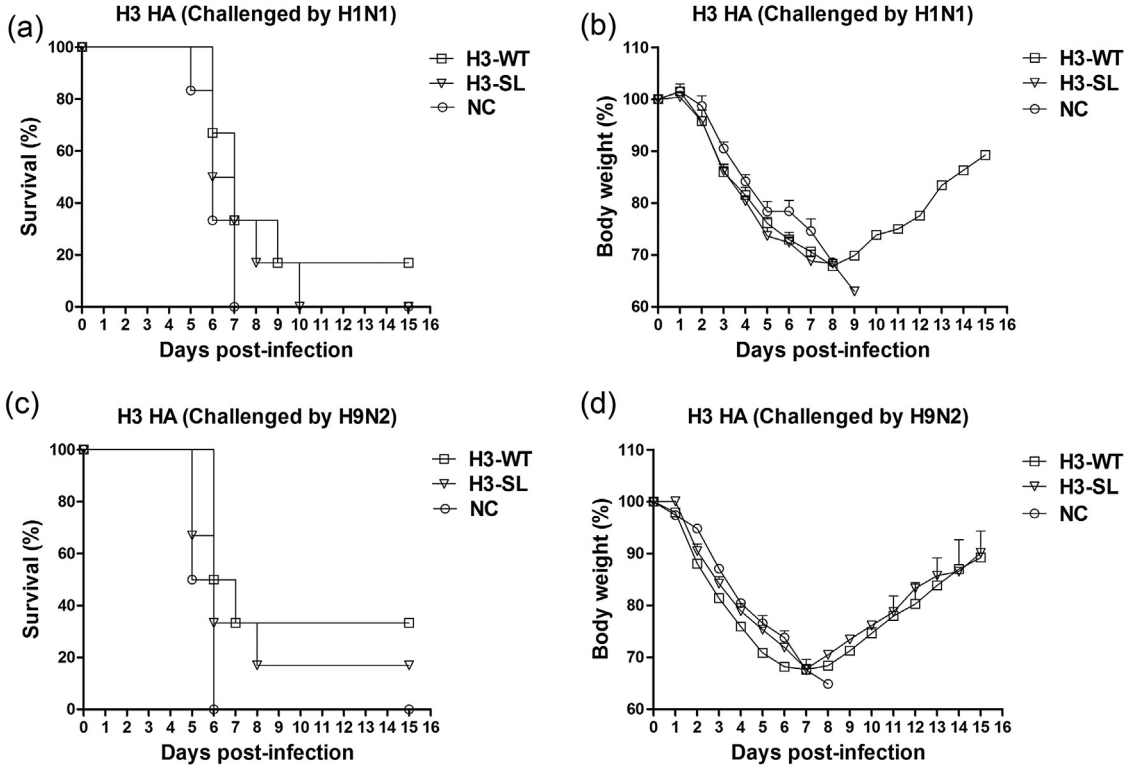

Fig. 1. Survival rates and relative body weight graphs of mice vaccinated with H3-WT or H3-SL HAs infected with H1N1 or H9N2 viruses.
(a) Survival rates of H3-WT and H3-SL groups against H1N1 infection. Three groups of six-week-old female BALB/c mice ($n = 6$) were respectively vaccinated subcutaneously with 3 μg of H3-WT, H3-SL or Sf9 cell lysate as negative control (NC) and boosted once 2 weeks later. On day 28 after the initial vaccination, the mice were intranasally challenged with 3× MLD_{50} of H1N1 virus. Survival rates and body weight losses were monitored for 15 days.
(b) Relative body weight graphs of H3-WT or H3-SL vaccinated mice when challenged by H1N1 virus.
(c) Survival rates of H3-WT and H3-SL groups against H9N2 infection. Same numbers of mice were vaccinated and processed the same as in (a) except for being intranasally challenged with 3× MLD50 of H9N2 virus.
(d) Relative body weight graphs of H3-WT or H3-SL vaccinated mice when challenged by H9N2 virus.

by subcutaneous injection at 0 week and 2 weeks. Sf9 cell lysate (infected by wild-type baculoviruses) was included as negative control. Blood samples were collected 2 weeks after each immunization. For challenge, vaccinated mice were first anesthetized by ether and then challenged intranasally with a lethal dose (3× MLD50) of H1N1 or H9N2 viruses in a 40 μl inoculum volume [30] at 3 weeks after final boost immunization. The mice were monitored daily for 15 days after the challenge for survival and weight loss. For some experiments, the specific lethal doses for challenges were denoted.

2.10. ELISA for anti-HA IgG antibodies

HA-specific immunoglobulin G (IgG) or IgG isotype antibody titers in mouse sera were analyzed by enzyme-linked immunosorbent assay (ELISA) as previously described [31,32]. Briefly, purified inactivated virus particles of H1N1, H3N2, H5N1 or H9N2 at a concentration of 3 μg/ml were coated, incubated with serial dilutions of each serum sample (37 °C for 1 h) and detected by HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 polyclonal antibodies. Optical densities were read at 450 nm using

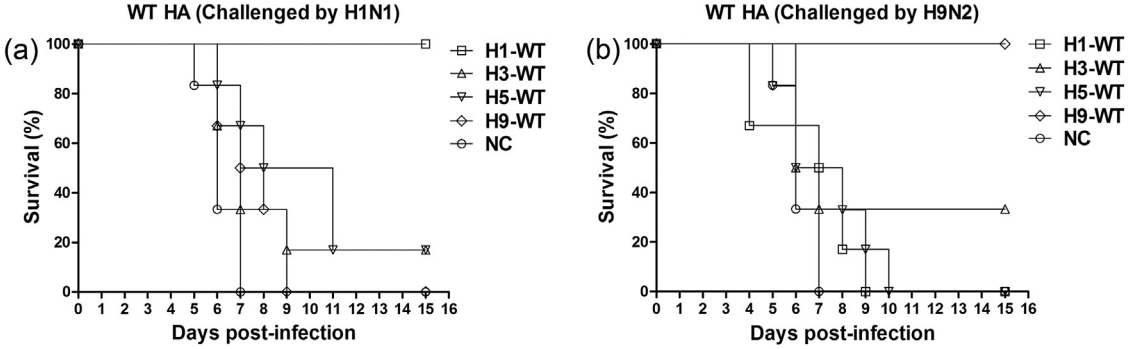

Fig. 2. Survival rates of mice vaccinated with H1-WT, H3-WT, H5-WT and H9-WT HAs infected with H1N1 or H9N2 viruses.
(a) Survival rates of H1-WT, H3-WT, H5-WT and H9-WT groups against H1N1 infection. Five groups of six-week-old female BALB/c mice ($n = 6$) were respectively vaccinated subcutaneously with 3 μg of H1-WT, H3-WT, H5-WT, H9-WT or Sf9 cell lysate (NC) and boosted once 2 weeks later. All mice were intranasally challenged with 3× MLD50 of H1N1 virus and monitored for 15 days.
(b) Survival rates of H1-WT, H3-WT, H5-WT and H9-WT groups against H9N2 infection. Same numbers of mice were vaccinated and processed the same as in (a) except for being intranasally challenged with 3× MLD50 of H9N2 virus.

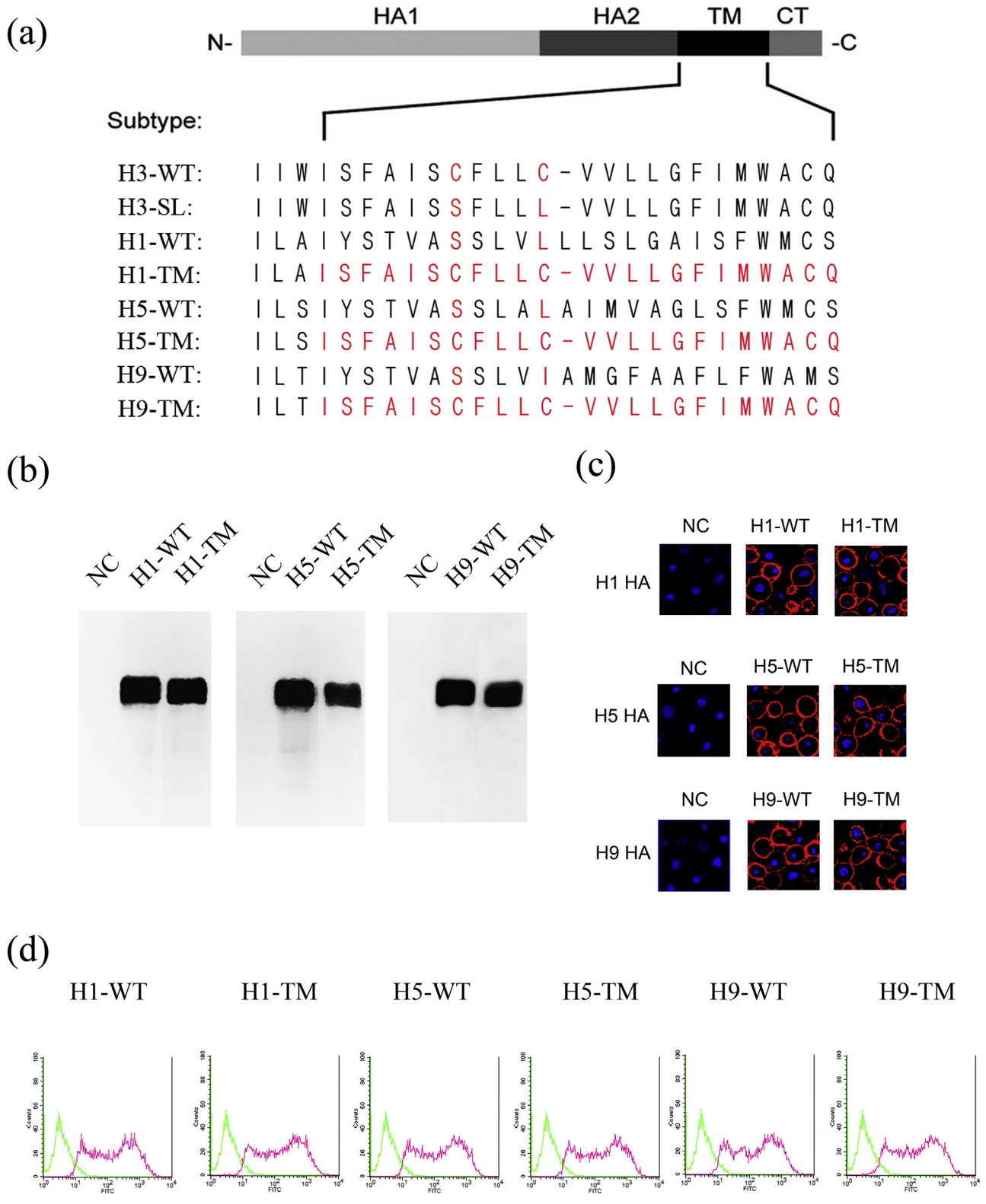

Fig. 3. Nomenclature and characterization of HAs with engineered transmembrane domains (TMDs).
(a) Nomenclature of H1, H3, H5 and H9 HAs with engineered TMDs. Amino acid sequences of H3-WT, H3-SL, H1-WT, H1-TM, H5-WT, H5-TM, H9-WT and H9TM were aligned and designated.
(b) Western blots of H1-WT, H1-TM, H5-WT, H5-TM, H9-WT and H9-TM protein expressions. Sf9 cells were infected by rBVs expressing each of the HA proteins respectively and the resultant cell lysates were separated on SDS–PAGE gels; and then HA proteins were detected by Western blot. Sf9 cells infected wt-BV were included as a negative control (NC).
(c) Indirect immunofluorescence assay of H1-WT, H1-TM, H5-WT, H5-TM, H9-WT, and H9-TM protein expression. Sf9 cells were infected as in (b), and fixed and stained for the expression of HA proteins. Sf9 cells infected wt-BV were included as a negative control (NC).
(d) Flow cytometric analysis of H1-WT, H1-TM, H5-WT, H5-TM, H9-WT, and H9-TM protein expression. Sf9 cells were infected as in (b), and fixed and stained using murine monoclonal antibody (mAb) specific for H1, H5 and H9 HA. Surface HA expression was shown in pink line, and Sf9 cells infected wt-BV were included as a negative control (green line).(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a spectrophotometer (Bio-Tek ELx800UV, USA). The end-point titer was determined as the reciprocal of the final dilution giving a three-fold optical absorbance of negative control [33].

2.11. ELISPOT assay

The number of splenocytes secreting IFN-γ or IL-4 upon antigen stimulation was determined using the ELISPOT assay (EZ-Sep, DAKEWE, China) according to the manufacturer's instructions. Briefly, pre-coated anti-mIFN-γ or -IL-4 96-well plates were seeded with splenocytes (5×10^5/well) and stimulated with purified inactivated influenza viruses (H1N1, H3N2, H5N1 or H9N2 respectively) at a concentration of 10 μg/ml. After processing, the spots were counted using an ImmunoSpot ELISPOT reader (Bioreader4000, BIO-Sys, Germany).

2.12. Statistics analysis

Statistical analyses were performed using Student's two-tailed test with equal variance. A P values (P) less than 0.05 were considered statistically significant. The correspondence between the average cross-reactive anti-HA IgG titer and survival rate was analyzed by linear regression using SPSS 13.0 software.

3. Results

3.1. Wild-type H3 hemagglutinin (HA) (H3-WT), but not a transmembrane domain (TM) mutant (H3-SL), showed hetero-protection

Our previous study showed that wild-type H3 (H3-WT) had higher thermal stability than a mutant (H3-SL) with mutation of two cysteines present only in the transmembrane domain (TM) [20]. This study first compared the hetero-protection of H3-WT and H3-SL against H1N1 or H9N2 infections. Groups of mice ($n = 6$) were subcutaneously immunized twice with 3 μg H3-WT or H3-SL respectively; sf9 cells infected with wild-type recombinant baculovirus (WT-rBV) was included as negative control (NC). When infected intranasally by 3× MLD_{50} H1N1 (PR8) or H9N2 (A/Chicken/Guangdong/96), H3-WT group showed survival rates of 17% and 33% against H1N1 and H9N2 respectively (Fig. 1a and c); in contrast, H3-SL group showed a survival rate of 17% against H9N2, but no hetero-protection against H1N1 (Fig. 1c). ELISA and hemagglutinin inhibition (HI) assays revealed that H3-WT group elicited apparent higher, but not significant, H3N2-reactive IgG antibody titers, H3N2-specific HI titers and H1N1-, H5N1- and H9N2-cross-reactive antibody titers than H3-SL group (data not shown). All survived mice showed weight losses before recovery (Fig. 1b and

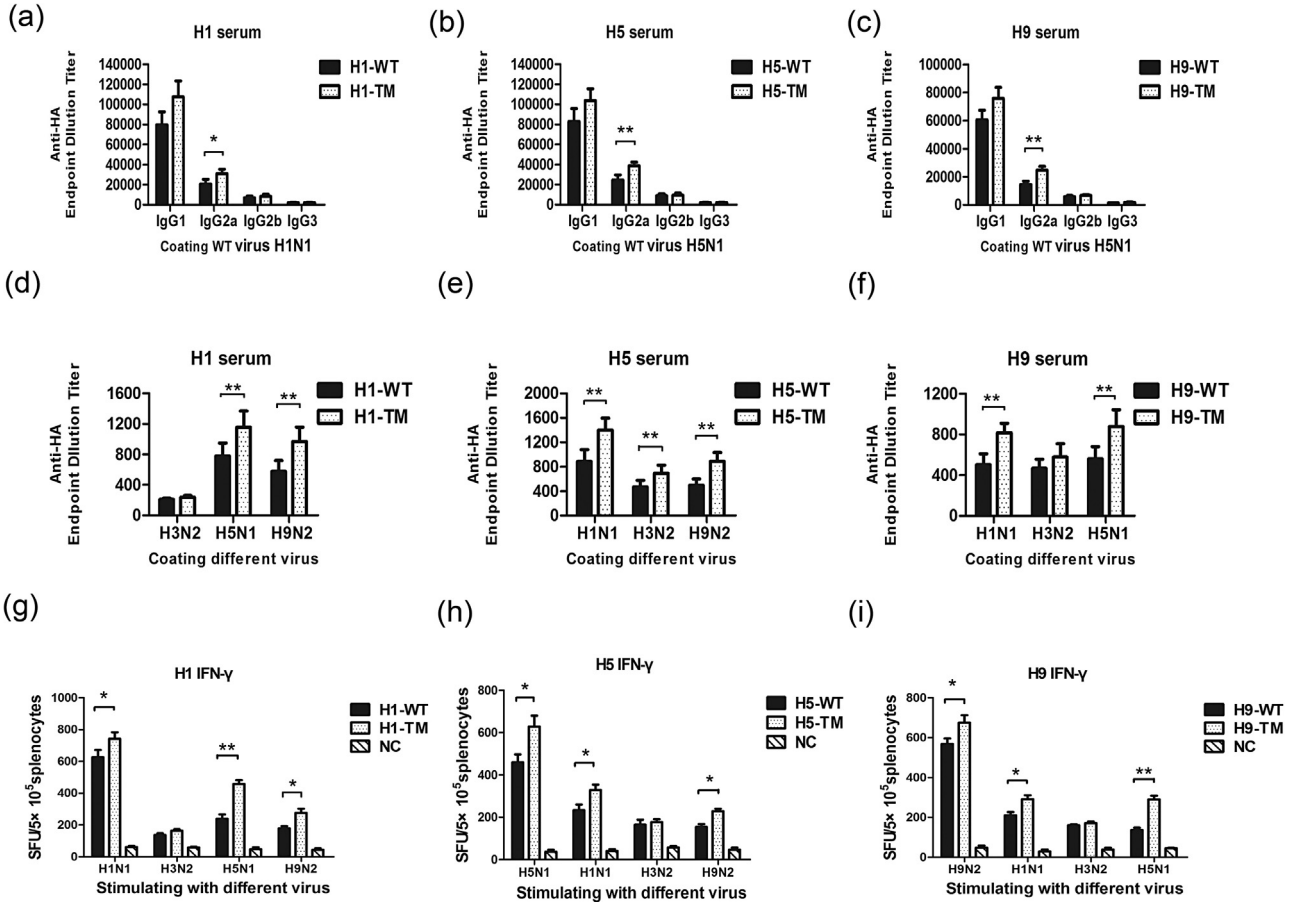

Fig. 4. Characterization of humoral and cellular immune responses.
(a–c) IgG isotype antibody titers against cognate inactivated viruses. Immunized sera from vaccinated mice were collected and measured for their IgG isotype antibody titers against cognate inactivated viruses by ELISA as described in the Section 2.
(d–f) Cross-reactive IgG titers against heterosubtypic inactivated viruses. Immunized sera from vaccinated mice were collected and measured for their cross-reactive IgG titers against heterosubtypic inactivated viruses by ELISA as described in the Section 2.
(g–i) Number of IFN-γ- or IL-4-secreting splenocytes. Splenocytes from mice immunized with H1-WT, H1-TM, H5-WT, H5-TM, H9-WT or H9-TM proteins were isolated 3 weeks after the final immunization and IFN-γ- or IL-4-secreting cells were determined by ELISPOT assays. Cognate or heterosubtypic inactivated viruses were used as stimulants. The results are expressed as the number of spots in 5 × 106 splenocytes.
Statistical significance between the values for HA-TM and HA-WT is indicated; * denotes $P < 0.05$; ** denotes $P < 0.01$.

d). These results were the first to demonstrate that H3-WT had the capability of hetero-protection, and that H3 TM is critical for the hetero-protection of H3-WT.

3.2. Wild-type H1, H5 and H9 HAs showed less hetero-protection than H3-WT

Since the two TM cysteines mutated in H3-SL are uniquely present in H3 HA [20], it was hypothesized that other HAs should have less hetero-protection than H3-WT. Wild-type H1 (H1-WT), H5 (H5-WT) and H9 (H9-WT) HAs (collectively, Hx-WT) were selected because H1N1 is included in TIV vaccine, H9N2 circulating in chickens, and H5N1 has the potential of causing pandemics. As expected, when the mice immunized with H1-WT, H5-WT or H9-WT were infected with H1N1 or H9N2 viruses, there was no hetero-protection except that H5-WT group had a survival rate of 17% against H1N1 (Fig. 2a and b).

3.3. Replaced transmembrane domain had no apparent impact on in vitro expression of H1, H5 and H9 HA proteins in Sf9 cells

Whether the hetero-protection of H3-WT could be transferred into H1, H5 and H9 HAs was investigated by replacing the TMDs of H1, H5, and H9 HAs with H3 HA TM (designated as H1-TM, H5-TM and H9-TM (collectively, Hx-TM)) (Fig. 3a). The expression of Hx-TM in Sf9 cells was not affected by TM replacement as demonstrated by Western blot (Fig. 3b), immunofluorescence staining (Fig. 3c) and flow cytometry (Fig. 3d). Furthermore, Hx-TM showed

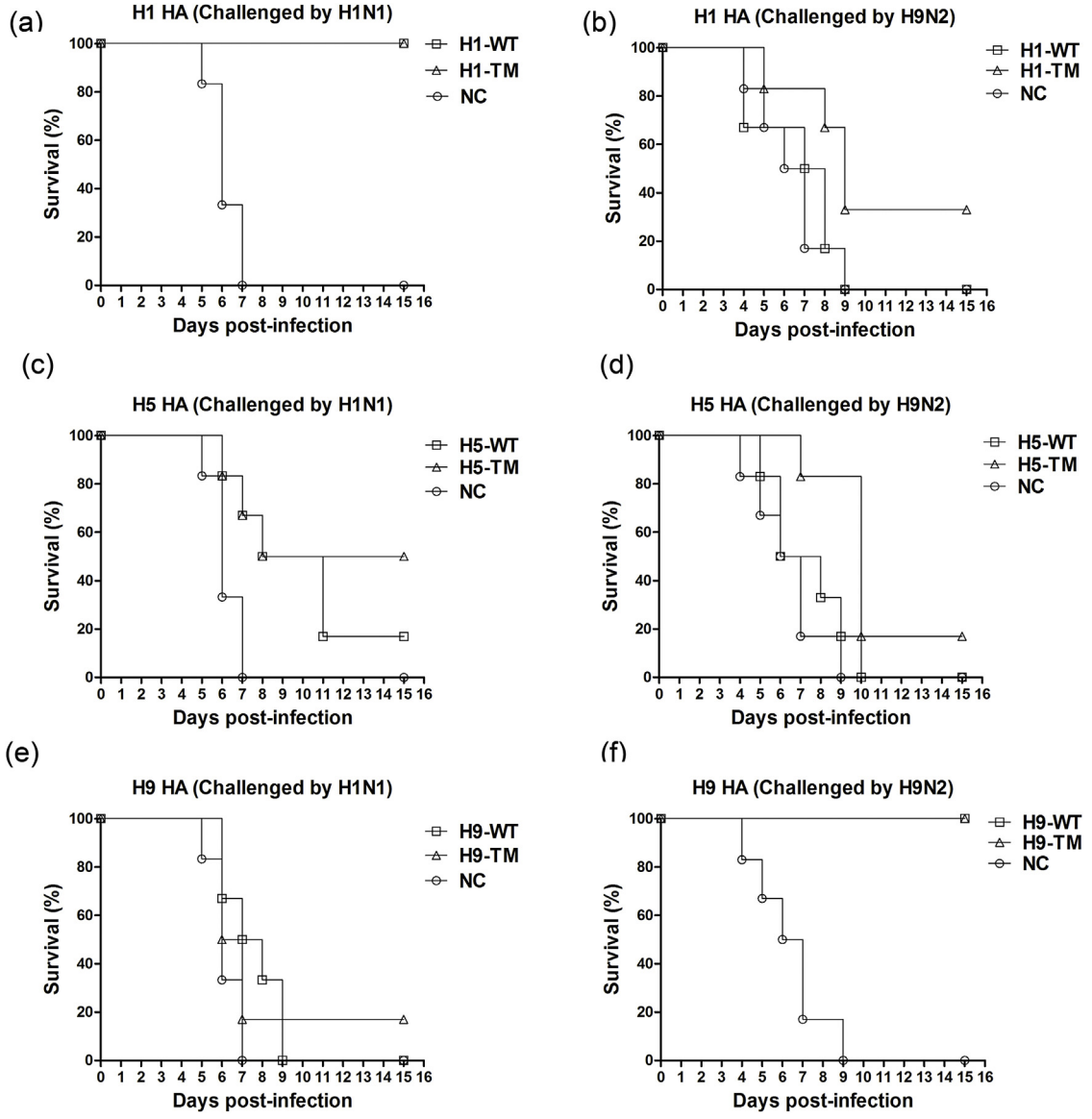

Fig. 5. Homologous or heterosubtypic protection against H1N1 or H9N2 infections.
Groups of mice were immunized subcutaneously twice with 3 μg of H1-WT, H1-TM, H5-WT, H5-TM, H9-WT or H9-TM proteins (except for (g)) and intranasally infected by 3× MLD50 H1N1 or H9N2 viruses; the mice were monitored for their survival rates and body weight for 15 days.
(a) Survival rates of H1-WT and H1-TM groups against H1N1 virus.
(b) Survival rates of H1-WT and H1-TM groups against H9N2 virus.
(c) Survival rates of H5-WT and H5-TM groups against H1N1 virus.
(d) Survival rates of H5-WT and H5-TM groups against H9N2 virus.
(e) Survival rates of H9-WT and H9-TM groups against H1N1 virus.
(f) Survival rates of H9-WT and H9-TM groups against H9N2 virus.

significant increase in their thermal resistance over corresponding Hx-WT (data not shown).

3.4. Hx-TM proteins showed increased antibody and cytokine responses in mice

The effects of TM replacement on antibody and cytokine responses were analyzed. Hx-WT and Hx-TM were expressed in Sf9 cells and enriched by stepped centrifugation for immunization as described in Section 2. The sera from immunized mice were analyzed by ELISA using purified inactivated viruses as antigens, hemagglutinin inhibition assay using cognate inactivated viruses, and ELISPOT assay using purified inactivated viruses as stimulants. Hx-TM elicited higher but not significant homologous IgG titers than corresponding Hx-WT (data not shown). However, Hx-TM elicited a significant higher homologous IgG2a response than corresponding Hx-WT ($P = 0.02–0.032$) (Fig. 4a–c). In addition, even though the cross-reactive IgG titers were low, Hx-TM elicited significant higher cross-reactive IgG titers than corresponding Hx-WT (Fig. 4d–f). With regard to HI titers, TM replacement increased apparent but not significant homologous HI titers (data not shown). For cytokine production, Hx-TM elicited significant higher IFN-γ

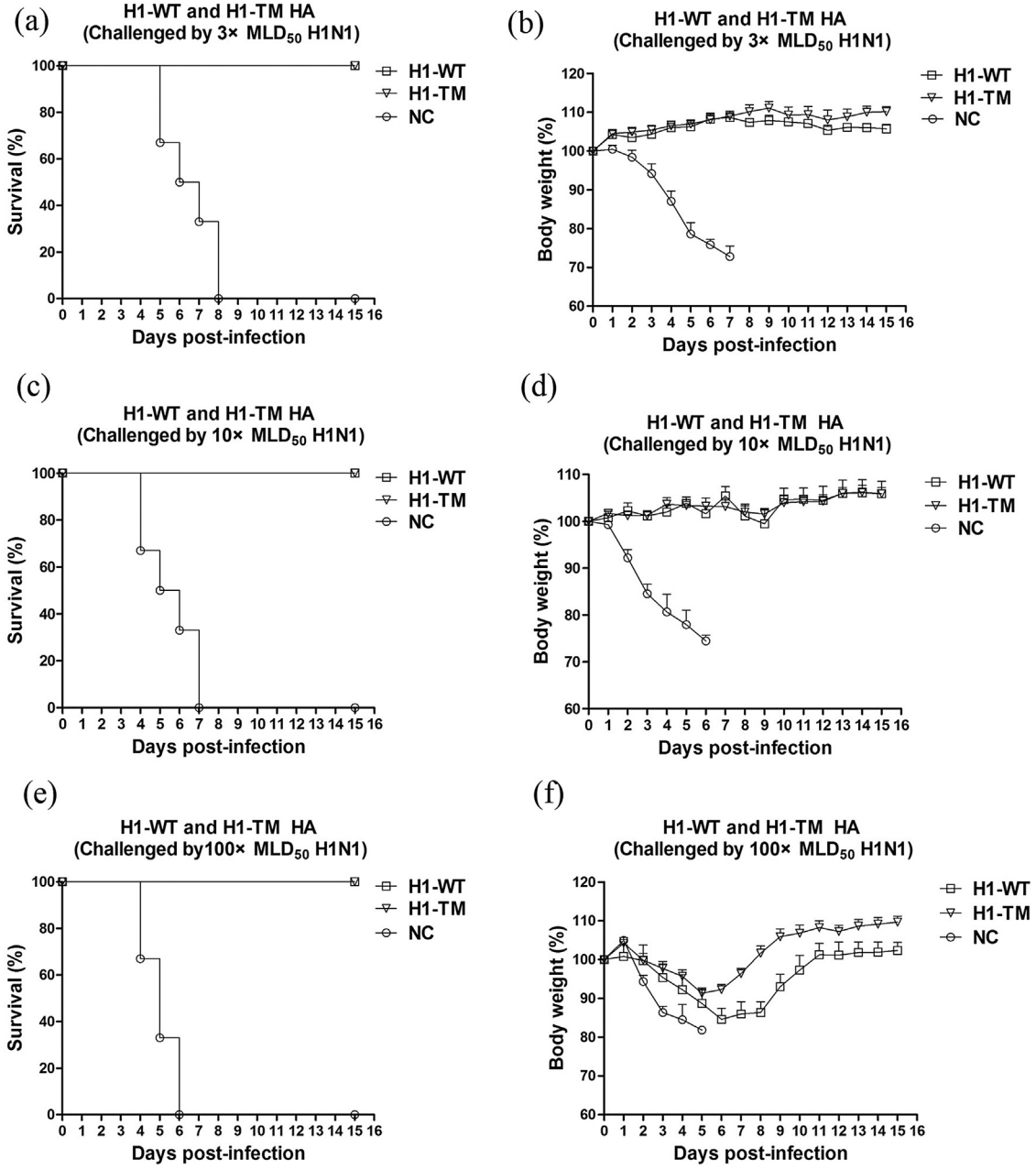

Fig. 6. Homologous protection of H1-WT and H1-TM HA vaccinated mice against different infection dosages of H1N1 virus.
Groups of six female BALB/c mice were vaccinated subcutaneously twice with 3 μg H1-WT, H1-TM or Sf9 cell lysate (infected by wild-type baculoviruses) as negative control. Two weeks after boost, the mice were intranasally challenged with 3× MLD_{50}, 10× MLD_{50} or 100× MLD_{50} H1N1 viruses. During infection, the body weight of each immunized and infected mouse was measured for 15 consecutive days; the body weight results are expressed in terms of percent body weight compared to the beginning of the viral challenge.
(a, c, e) Survival rates of immunized mice challenged with 3× MLD_{50}, 10× MLD_{50} or 100× MLD_{50} H1N1 viruses respectively.
(b, d, f) Relative body weight graphs of immunized mice challenged with 3× MLD_{50}, 10× MLD_{50} or 100× MLD_{50} H1N1 viruses respectively.

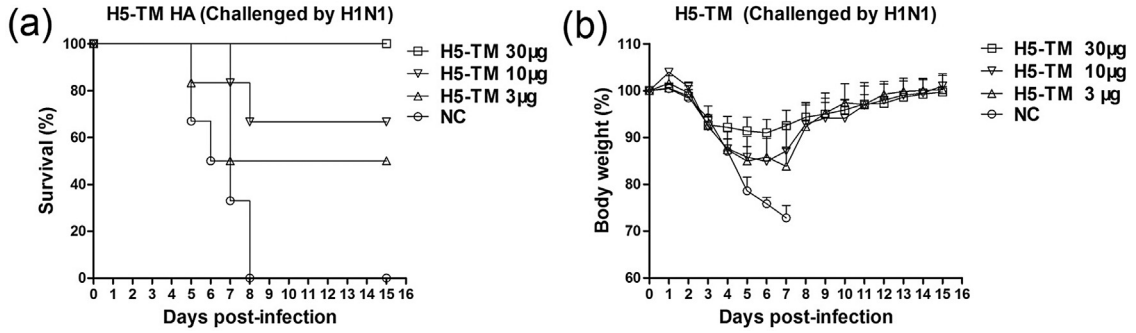

Fig. 7. Heterosubtypic protection and relative body weight graphs of mice vaccinated with three different dosages of H5-TM HAs infected with 3× MLD50 H1N1.
Groups of mice were immunized in the manner described above with three different dosages (i.e., 3 μg, 10 μg or 30 μg) of H5-TM respectively. Two weeks after boost, the groups immunized with H5-TM were infected with 3× MLD50 H1N1 and monitored for 15 consecutive days.
(a) Survival rates of H5-TM groups immunized with 3 μg, 10 μg, or 30 μg HA proteins against H1N1 virus.
(b) Relative body weight graphs of mice immunized with three different dosages of H5-TM (3 μg, 10 μg or 30 μg) and infected with 3× MLD50 H1N1 virus.

production than corresponding Hx-WT (Fig. 4g–i) but not IL-4 production (data not shown).

3.5. Hx-TM proteins exhibited enhanced heterosubtypic protections

Whether the increased cross-reactive antibodies and IFN-γ production could be translated into hetero-protection was investigated. Groups of mice were subcutaneously immunized twice with 3 μg Hx-WT and Hx-TM respectively and then infected intranasally with 3× MLD_{50} H1N1 or H9N2 viruses. H1-WT/H1-TM and H9-WT/H9-TM groups showed survival rates of 100% against homologous H1N1 or H9N2 respectively (Fig. 5a and f). Hx-TM all showed higher hetero-protection than corresponding Hx-WT (Fig. 5b–e); H1-TM group against H9N2 and H9-TM group against H1N1 with survival rates of 33% and 17% respectively, and H5-TM group against both H1N1 and H9N2 with survival rates of 50% and 17% respectively. Homologous infection resulted in no weight loss, while heterosubtypic infection caused severe weight loss (data not shown).

3.6. H1-TM protein showed less body weight loss against higher dosage homologous infection than H1-WT HA protein

Whether the same amount of H1-WT and H1-TM proteins had differential capability of protections against homologous infection in mice was investigated. Different groups of mice were vaccinated with 3 μg H1-WT or H1-TM HA proteins, and then infected with 3× MLD50, 10× MLD50 or 100× MLD50 of H1N1. The results showed that the weight loss for the H1-TM HA vaccinated group was significantly less than that for the H1-WT HA vaccinated group when infected with 100× MLD50 of H1N1 while the survival rates were the same (Fig. 6a–f).

3.7. Higher dosage of H5-TM proteins showed improved heterosubtypic immunity

To test the dosage effects of HA proteins, different groups of mice were vaccinated with 3 μg, 10 μg or 30 μg H5-WT or H5-TM HA proteins. The increase of HA protein dosages resulted in an increase of serum HA-specific IgG titers against homologous H5N1 virus and heterosubtypic H1N1 virus (data not shown). The protection against heterosubtypic H1N1 infection increased along with the increase of HA protein dosages; strikingly, 100% protection was achieved with vaccination of 30 μg H5-TM HA proteins with mild weight loss while 10 μg H5-TM HA proteins provided 67% protection with severe weight loss (Fig. 7a and b).

4. Discussion

This study demonstrated that H3-WT TM is critical for H3 HA-induced hetero-protection, and further that H3-WT TM-dependent hetero-protection could be transferred to H1, H5 and H9 HAs by replacing their TMs with H3-WT TM.

Previous studies have suggested a plausible correlation of HA stability and heterosubtypic immunity. Du et al. showed that Hemagglutinin 1 (HA1) fragment of A/Anhui/1/2005(H5N1) was fused to either Fc of human IgG (HA1-Fc) or foldon plus Fc (HA1-Fdc) and the fusion proteins expressed in 293T cells provided cross-clade protection [17]. Weldon et al. showed that a soluble HA (sHA), derived from the H3N2 virus A/Aichi/2/68, was modified at the C-terminus with a GCN4pII trimerization repeat to stabilize the native trimeric structure of HA, and the fusion protein expressed in insect cells elicited significantly higher IgG and HAI titers than unmodified sHA [18]. More recently, Kanekiyo et al. showed that the ectodomain of HA protein was fused to ferritin to form nanoparticles with eight trimeric viral spikes on its surface, and the nanoparticle vaccine improved the potency and breadth of influenza virus immunity [19]. In addition, Li et al. showed that the full-length ectodomain of the spike (S) protein of severe acute respiratory syndrome (SARS) was fused to the foldon domain derived from T4 bacteriophage, and the fusion protein induced a significantly higher titer of neutralizing antibody [21]. The results that H3-TM with higher thermal stability had better hetero-protection than H3-SL provide one more evidence supporting such a plausible correlation [16].

Our results demonstrated that H1, H5 and H9 HAs containing replaced H3-WT TM showed increased thermal stability and hetero-protection, indicating that H3-WT TM-based hetero-protection could be transferred with two important features. One is that the recipient is not restricted to one particular HA subtypes, and the other is that the transferred hetero-protection is restricted to one heterosubtypic infection. It strongly suggested the general applicability of TM replacement for HAs, a requisite for a universal vaccine.

This study has undoubtedly raised many questions that need answers. For example, it is imperative to know whether the effect of TM replacement is H3 TM-specific, and whether H3 TM could be further optimized for eliciting even higher degrees of hetero-protection. Furthermore, the increased IFN-γ production provides one explanation for the increased IgG2a titers in replacement Hx-TM mutants, but how the replacement of TM affects IFN-γ production is not clear. In addition, all immunized sera showed no detectable HI titers against heterosubtypic viruses (data not shown); thus it would be interesting to know whether the

cross-reactive antibodies provided hetero-protection by antibody-dependent cellular toxicity [22].

In summary, our results demonstrated that hetero-protection of H1, H5 and H9 HA proteins could be enhanced by replacing their TMs with H3-WT TM. This strategy of replacing TMs of HA proteins would be conceivably applicable to other HA proteins, especially the HA proteins from emerging pandemic influenza viruses. This study helps to increase the hetero-protection of TIV and THS vaccines and to develop effective vaccines against future influenza pandemics and other viral pathogens.

Conflict of interest: We declare that we hve no finacial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in this manuscript.

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