



Published in final edited form as:

Virology. 2017 September ; 509: 82–89. doi:10.1016/j.virol.2017.06.001.

Protein nanoparticle vaccine based on flagellin carrier fused to influenza conserved epitopes confers full protection against influenza A virus challenge

Lei Deng¹, Jong R. Kim², Timothy Z. Chang³, Han Zhang², Teena Mohan¹, Julie A. Champion³, and Bao-Zhong Wang^{1,*}

¹Center for Inflammation, Immunity & Infection, Georgia State University Institute for Biomedical Sciences, Atlanta, GA, USA

²Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA

³Georgia Institute of Technology, School of Chemical & Biomolecular Engineering, Atlanta, GA, USA

Abstract

Currently marketed influenza vaccines only confer protection against matching influenza virus strains. The influenza A composition of these vaccines needs to be annually updated. Vaccines that target conserved epitopes of influenza viruses would in principle offer broad cross-protection against influenza A viruses. In our study, we investigated the specific immune responses and protective efficacy of protein nanoparticles based on fusion proteins of flagellin carrier linked to conserved influenza epitopes. We first designed the fusion protein by replacing the hyperimmunogenic region of flagellin (FliC) with four tandem copies of the ectodomain of matrix protein 2 (f4M2e), H1 HA2 domain (fHApr8) or H3 HA2 domain (fHAaichi). Protein nanoparticles fabricated from these fusion proteins by using DTSSP crosslinking retained Toll-like receptor 5 agonist activity of FliC. Intranasal immunization with f4M2e, f4M2e/fHApr8 or f4M2e/fHAaichi nanoparticles induced vaccine antigen-specific humoral immune responses. It was also found that the incorporation of the H1 HA2 domain into f4M2e/fHApr8 nanoparticles boosted M2e specific antibody responses. Immunized mice were fully protected against lethal doses of virus challenge.

Keywords

influenza; nanoparticle; vaccine; Matrix protein 2 ectodomain; Hemagglutinin; flagellin

*Corresponding author. Dr. Bao-Zhong Wang, bwang23@gsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Competing Interests

There are no conflicting interests.

Introduction

Influenza is a preventable disease and vaccination is the most effective way to prevent influenza infection. Due to the low efficacy of protection of the seasonal vaccines against mismatching influenza A virus strains, seasonal influenza epidemics still cause an estimated 250,000 deaths worldwide each year (Prevention, 2010). New emerging viruses escape from prevailing herd immunity by antigenic drift and shift in the head domains of the major glycoproteins HA and NA, which necessitates incorporating conserved epitopes in the development of a universal influenza vaccine.

Hemagglutinin (HA) is the major influenza surface glycoprotein. The pre-fusion state HA0 is cleaved by furin-like proteases into HA1 and HA2 subunits (Skehel and Wiley, 2000). The majority of HA1 forms the globular head, which contains a receptor binding site and accumulates point-mutations on its surface. The membrane-proximal stalk region is predominantly comprised of HA2 and is structurally conserved across HA subtypes (de Vries et al., 2015). Based on the sequence conservation, HA sequences from 18 subtypes have been organized into two phylogenetic groups (groups 1 and 2) (Zhang et al., 2014). The conserved stalk domain contains a greater proportion of broadly neutralizing antibody binding sites than the variable head domain. In recent years, many efforts have been made to develop universal vaccines consisting of HA2 and portions of HA1. The adjuvanted stalk domain vaccines were highly immunogenic in animal models, with the specific humoral immunity playing the main role in protecting against lethal doses of homo- and heterosubtypic influenza A virus challenges (Bommakanti et al., 2010; Impagliazzo et al., 2015; Steel et al., 2010; Valkenburg et al., 2016; Yassine et al., 2015). The induced antibodies targeting the stalk region presumably prevent the conformational change of HA at low pH, thereby blocking the fusion of viral and host endosomal membranes. It is speculated that the conserved HA stalk domain could elicit protection against viral infection.

The extracellular domain of matrix protein 2 (M2e) is 23-amino acid in length and is highly conserved among influenza A viruses (Deng et al., 2015a). M2e is a promising conserved antigen for developing universal influenza A vaccines. The M2e-specific immune responses elicited by seasonal vaccines or natural infection are weak due to its low immunogenicity (Feng et al., 2006; Zhong et al., 2014). This low immunogenicity results from its relatively low epitope density and smaller size (Hutchinson et al., 2014). Nevertheless, many M2e based vaccines coupling the M2e epitope to carriers were able to induce robust M2e specific humoral immunity and were able to provide full protection against lethal doses of homosubtypic and heterosubtypic influenza A virus challenges (Deng et al., 2015b; El Bakkouri et al., 2011; Neiryneck et al., 1999; Wang et al., 2014b). Clinical trials demonstrated that the proper range of doses of recombinant M2e-flagellin influenza vaccines (STF2.4xM2e) was safe and induced high antibody responses to M2e in healthy adults (Turley et al., 2011). It was reported previously that the supplemental use of M2e based vaccine with inactivated PR8 virus could induce enhanced anti-influenza virus IgG responses and Th1-biased immune responses (Song et al., 2011).

Bacterial flagellins are the natural ligands of Toll-like receptor (TLR) 5 and are promising potential adjuvants (Huleatt et al., 2008; Smith et al., 2003; Wang et al., 2014a; Wang et al.,

2010). The replacement of the variable region of FliC with M2e does not result in the impairment of innate TLR signaling (Smith et al., 2003). Previous experiments have shown that the replacement of M2e with the central variable region of FliC largely improved M2e immunogenicity (Wang et al., 2014a; Wang et al., 2010). In this study, we incorporated four tandem copies of M2e or HA2 domain in the hyperimmunogenic region of FliC. Due to the self-adjuvanting property of this fusion protein, it was presumed that a cocktail of these recombinant proteins would provide effective protection against influenza A virus challenge.

Nanoparticles are a promising antigen delivery system. Previously, we designed self-assembling protein nanoclusters consisting of M2e epitopes stabilized with a GCN4 tetramerization motif. This nanoparticle vaccine was composed of antigen proteins and trace amounts of stabilizing cross-linker. Due to the high antigen load and nanoparticle morphology, these nanoclusters were very immunogenic *in vivo* (Wang et al., 2014b). The combined use of conserved epitopes in a vaccine to confer broad protection is a desired goal in the development of a universal influenza vaccine. In this study, we engineered FliC fusion proteins by replacing the central hyperimmunogenic region of FliC with four tandem copies of the ectodomain of matrix protein 2 (f4M2e), H1 HA2 domain (fHApr8) or H3 HA2 domain (fHAaichi). To test whether incorporation of the HA2 domain can boost the M2e specific antibody responses, we evaluated the *in vivo* immunogenicity and protectivity of the crosslinked nanovaccines generated from f4M2e alone and a mix of f4M2e with the HA2 domain fusion protein.

Materials and Methods

Immunogen design and expression

The flagellin (FliC) fusion proteins were generated by replacing the hyperimmunogenic region of FliC with four tandem copies of M2e in which two point-mutations, C17S and C19S, were made. Within the f4M2e construct, the order of modified M2e sequences from N- to C- terminal was: human H3N2 consensus M2e, SLLTEVETPIRNEWGSRSSDSSD; A/California/04/2009 H1N1 M2e, SLLTEVETPTRSEWESRSDSSD; A/Viet Nam/1194/2004 H5N1 M2e, SLLTEVETPTRNEWESRSDSSD; A/Shanghai/02/2013 H7N9 M2e, SLLTEVETPTRTGWESNSSGSSE. The H1 HA2 domains from A/Puerto Rico/08/1934 or H3 HA2 domain from A/Aichi/02/1968 were used to generate fHApr8 and fHAaichi, respectively. The method to generate the gene encoding the fusion proteins was described previously (Wang et al., 2012). Briefly, a DNA fragment encoding the variable region (aa 177 to 401 in FliC) was deleted from the *S. Typhimurium* FliC gene and replaced with the sequences of interest (Fig 1). The coding sequences of interest were PCR amplified and ligated into the desired position in the pET22b F+S plasmid. A sequence encoding a 6xHistidine tag was added to the 3'-terminus in frame to generate the full-length gene encoding the secreted fusion protein. The integrity of constructs was confirmed by DNA sequencing analysis. Histidine-tagged recombinant FliC fusion proteins were purified from an *Escherichia coli* protein expression system as described previously (Skountzou et al., 2010). Purified proteins were dialyzed against phosphate-buffered saline (PBS) and stored at -80 °C.

Nanoparticle fabrication

The nanoparticles (Nps) were formed by DTSSP (3,3'-Dithiobis(sulfosuccinimidylpropionate), Sigma, US) crosslinking. Five hundred microliters (μl) of f4M2e (2.2 mg/ml), a mix of f4M2e and fHApr8 at a 1:1 weight ratio, or a mix of f4M2e and fHAaichi at a 1:1 weight ratio was stirred at a speed of 600 rpm with a final concentration of 0.197 mM DTSSP at 4 °C for 1 hour. Soluble protein was then removed by buffer exchange against fresh PBS using a 300K size diafiltration tube (Pall Corporation, US). The samples were centrifuged at speed 5,500 \times g for 20 min at 4 °C. The centrifugation was repeated twice. Dynamic light scattering (DLS) was performed in PBS with a Malvern Zetasizer Nano ZS to assess the size distribution of the collected f4M2e, f4M2e/fHApr8 and f4M2e/fHAaichi Nps.

Toll-like receptor 5 specific bioactivity assay

The Toll-like receptor (TLR) 5 activating abilities of the FliC fusion protein Nps were evaluated using a human TLR5 and NF- κ B/luciferase transiently transfected HEK293T cell based assay. Briefly, 6 \times 10⁶ HEK293T cells were transfected with 10 μg pUNO-hTLR5 and 3 μg pGL4.32 (Invivogen, CA) with Lipofectamine 2000 per the manufacturer's instructions (Invitrogen, NY). After a 24-hour transfection period, cells were seeded at a concentration of 5 \times 10⁴ cells/well in a 96-well plate and incubated overnight. Cells were then stimulated for 5 hours by 1:5 serially diluted Nps of f4M2e, f4M2e/fHAaichi, f4M2e/fHApr8, a mix of f4M2e/fHAaichi and f4M2e/fHApr8, or positive control of soluble FliC protein in serum-free media. Afterwards, the media was removed, and 50 μl of serum-free media with 50 μl of Bright-Glo luciferase assay reagent (Promega, WI) was added to each well. Luminescence was measured using GloMax[®] Explorer (Promega, WI).

In vitro assays for inflammation

JAWS II murine dendritic cells (DCs, passages 6-15) were plated at 10⁵ cells/ml in 24-well plates in complete culture medium consisting of α -Minimal Essential Media (α -MEM) with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA), 5 ng/mL murine GM-CSF (Peprotech, Rocky Hill, NJ), 4mM L- glutamine, 10 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. These plates were incubated in an CO₂ incubator at 37 °C and 5% CO₂ for *in vitro* measurement of secreted IL-1 β levels. After 24 hours of incubation, cells were stimulated with 10 $\mu\text{g}/\text{ml}$ of f4M2e, f4M2e/fHAaichi or f4M2e/fHApr8 in fresh complete medium. An equal volume of DPBS (Thermo Fisher Scientific, MA) was used as a negative control treatment. Interleukin IL-1 β was assessed in supernatants after 24 hours of stimulation by ELISA (R&D Systems, Minneapolis, MN).

Serum antigen-specific antibody titration using ELISA

M2e Peptides (M2e sequence from H1N1: SLLTEVETPTRSEWESRSSDSSDP; H3N2: SLLTEVETPIRNEWGSRSDSSDP; H5N1: SLLTEVETPTRNEWESRSSDSSDP; H7N9: SLLTEVETPTRTGWESNSSGSSEP) and HA proteins were immobilized (100 ng of antigen per well) and probed with serially diluted immune serum samples. After washing, HRP-conjugated anti-mouse Ab was added. After 2 hours of incubation, HRP substrate

TMB solution (Prod# 34029, Thermo Scientific, US) was added and the absorbance at 450 nm was measured.

Enzyme-Linked ImmunoSpot (ELISPOT) assay

Spleens of three mice per group were isolated three weeks post the boosting immunization. To evaluate the Interleukin (IL)-2 secreting splenocytes, multiscreen 96-well filtration plates (Millipore, Bedford, MA, US) were coated with anti-mouse IL-2 antibody (JES6-1A12, Biolegend) at 2 µg/ml in PBS and incubated overnight at 4 °C. Coated plates were washed with 0.05% Tween 20 in PBS (PBST) before 200 µl of RPMI 1640 with 10% FBS was added to each well for 2 hours at 37 °C to block non-specific binding. Freshly prepared splenocytes were suspended at a concentration of 2×10^5 cells/ml in complete RPMI medium and 100 µl was added to each well. After overnight stimulation with M2e peptide from human consensus H3N2 M2e (SLLTEVETPIRNEWGSRSDSSDP) at 37 °C, plates were overlaid with 50 µl (2 µg/ml) of Biotinylated anti-mouse IL-2 antibody (JES6-5H4, Biolegend) and incubated for 1 hour. After washing three times with PBST, 100 µl HRP-conjugated streptavidin (1:1,000 in PBST) was added and incubated at room temperature for 1 hour. After washing with PBST, 3-3'-diaminobenzidine tetrahydrochloride (DAB; Research Genetics Inc., Huntsville, AL, USA) was added to develop spots in the plates. The plates were rinsed with water and air dried before counting using an ImmunoSpot ELISPOT reader (BIOSYS, Karben).

Immunization and challenge studies

Six to eight-week-old female BALB/c mice (Jackson Laboratory) were intranasally immunized with either 10 µg f4M2e Nps, 10 µg f4M2e+fHAaichi Nps, or PBS as negative control. Ten mice per group were immunized twice at a 4-week interval. To compare antibody responses, sera were collected 2 weeks after each immunization. For challenge infections, all mice were lightly anesthetized with isoflurane and then were intranasally infected with mouse adapted H3N2 (A/Aichi/2/68) ($4 \times 50\%$ lethal dose, LD₅₀ in 30µl PBS) four weeks after the boost vaccination. Mice were observed daily to monitor changes in survival rate and body weight.

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mouse studies were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) under protocol number 2003060. Female BALB/c mice (six to eight-week old) were purchased from the Jackson Laboratory and housed in the animal facility at Emory University. Bleeding, infecting and sampling were performed under anesthesia that was induced by inhalation of isoflurane to reduce mouse suffering.

Statistical analysis

To determine the statistical significance, a two-tailed Student's *t*-test was used to compare two different conditions. Two-way ANOVA was used to analyze the significant differences among vaccinated groups. A *P*-value less than 0.05 was considered to be significant. The

analysis was done by using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA) for Windows.

Results

Characterization of fusion protein nanoparticles

The flagellin (FliC) fusion proteins were constructed by replacement of the variable region (aa 177 - 401) of FliC with the H1 HA2 or H3 HA2 domain (Fig 1 A). The expression and sizes of recombinant fusion proteins were determined by using Coomassie blue stained SDS-PAGE gel and Western blot analysis using an anti-FliC antibody. The results indicated that the expressed soluble proteins fHApr8 and fHAaichi both are at 48 kDa, the same as expected based on their amino acid composition (Fig 1 B-E). The expression and characterization of f4M2e were described previously (Wang et al., 2014a). These data demonstrated that all three fusion proteins can be successfully expressed and purified from an *E. coli* prokaryotic expression system.

Next, protein nanoparticles (Nps) from the three fusion proteins were fabricated by DTSSP crosslinking. DTSSP crosslinked Nps were collected by centrifugation using a 300K diafiltration tube. Dynamic light scattering (DLS) analysis of the f4M2e, f4M2e/fHApr8 and f4M2e/fHAaichi Nps indicated the presence of a single, reproducible particle population distribution with hydrodynamic diameters of $54 \text{ nm} \pm 13.5 \text{ nm}$, $46.7 \text{ nm} \pm 24.1 \text{ nm}$ and $81.7 \text{ nm} \pm 18.9 \text{ nm}$, respectively (Table 1).

Flagellin is a principal component of bacterial flagella and can be recognized by mammalian TLR5. The activation of TLR5 mobilizes the nuclear factor NF- κ B and stimulates tumor necrosis factor- α production (Hayashi et al., 2001). To assess whether FliC fusion protein Nps still retain TLR5 ligand function of FliC, human TLR5 and NF- κ B/luciferase reporter genes were transiently transfected into HEK293T for a cell- based assay. As shown in Fig 2 A, nanoparticles showed TLR5 specific bioactivity, activating the NF- κ B/luciferase reporter gene. The caspase-1 dependent cytokine IL-1 β exerts enhancement effects on the initiation of the protective Th1 and Th17 responses (van de Veerdonk et al., 2011). An *in vitro* stimulation assay showed that all types of nanoparticles could induce DCs to secrete significant levels of the IL-1 β in cell culture supernatants, compared to the negative control PBS group (Fig 2 B).

Protein nanoparticles induced strong IgG responses and broadly cross-reactive M2e specific antibodies

To determine if intranasal immunization with f4M2e, f4M2e/fHApr8 and f4M2e/fHAaichi Nps can induce strong M2e-specific humoral responses, immune sera were collected 2 weeks post each immunization and evaluated for antigen specific IgG titers using ELISA. Four tandem copies of M2e protein or various M2e peptides from H1N1, H3N2, H5N1 or H7N9 were used as coating antigens. As shown in Fig 3 A, two weeks post the priming immunization, all immunization groups showed detectable levels of 4M2e- specific IgG titers. Of note, 4M2e-specific IgG titers in sera from mice receiving f4M2e/fHAaichi Nps were significantly higher than other immunization groups ($p < 0.05$). Two weeks post-

boosting, all Nps induced strong 4M2e specific IgG titers. The f4M2e/fHApr8 Np immunization group induced the highest 4M2e-specific humoral immune responses, with an antibody titer around two times greater than that of the f4M2e Np and f4M2e/fHAAichi Np immunization groups (Fig 3 B, $p < 0.05$). The PBS control group had undetectable M2e specific antibody levels after two immunizations.

Next, to evaluate the cross-reactivity of M2e-specific antibodies induced by Nps, sera collected 2 weeks post the boosting immunization were assayed with ELISA by using M2e variant peptides as coating antigens. The induced serum antibodies from all the 4M2e immunized mice groups had broad spectrum binding activity to M2e variant peptides (Fig 3 C).

The induction of antibodies against the HA stalk domain can potentially increase the protection efficacy and breadth. The incorporation of fHApr8 or fHAAichi into f4M2e Nps was also able to induce HA2 domain specific antibody responses (Fig 3 D). In conclusion, the generated protein Nps not only induced strong and cross-reactive M2e specific antibodies, but also induced antibodies against the HA stalk domain.

M2e nanoparticles activated M2e-specific T cell immunity

IgG2a monoclonal antibody (mAb) is associated with more effective viral clearance *in vivo* than IgG1 mAb (Van den Hoecke et al., 2017). To assess effector T cell differentiation associated cytokine responses, M2e specific IL-2 secreting splenocytes from all immunization groups were evaluated by ELISPOT. As shown in Fig 4, all M2e-vaccinated mice groups showed significantly higher numbers of IL-2 secreting splenocyte populations after M2e peptide re-stimulation compared to mock-immunized mice ($p < 0.05$). However, there is no significant differences among f4M2e, f4M2e/fHApr8 and f4M2e/fHAAichi vaccination groups. All M2e immunized mice generated M2e specific IL-2 secreting T cell responses.

M2e nanoparticle protects mice from lethal H3N2 virus challenge

To evaluate the protective efficacy of nanoparticles, mice were intranasally immunized twice with f4M2e Nps, f4M2e/fHAAichi Nps or PBS at 4-week intervals and intranasally infected with $4 \times LD_{50}$ of mouse-adapted H3N2 (A/Aichi/02/1968) virus 4 weeks after the boost. The morbidity and mortality of infected mice was monitored for 14 days. As shown in Fig 5, all mice that received M2e vaccines were fully protected from lethal H3N2 virus challenge. In comparison, mice mock-immunized with PBS were not protected from lethal challenge and all died at or before day 8 post infection (Fig 5 A). Both f4M2e and f4M2e/fHAAichi Nps vaccinated mice experienced slight body weight loss but started recovering at day 8 post infection (Fig 5 B). M2e or blended Nps immunizations conferred full protection against lethal dose H3N2 virus challenge, with no significance in weight loss difference between these two groups.

Discussion

Tandem copies of M2e fused to TLR5 ligand flagellin were highly immunogenic *in vivo*, induced robust and long-lasting M2e-specific antibody responses and provided full

protection against influenza A virus challenges (Huleatt et al., 2008; Mardanova et al., 2016; Stepanova et al., 2015; Wang et al., 2014a). In our study, the variable region of FliC was replaced with three types of weak antigens: four tandem copies of M2e, H1 HA2 domain and H3 HA2 domain (Fig 1). It is presumed that the FliC fusion could inhibit the formation of the low-pH conformation of the HA2 domain. Protein nanoparticles were generated from these proteins to further increase their immunogenicity. The TLR5 bioactivity assay showed that all DTSSP-crosslinked protein nanoparticles retained the function of TLR5 activation (Fig 2 A). Possibly because of the steric effect of Nps, the luciferase activity strength of f4M2e was lower than the f4M2e/fHApr8 and f4M2e/fHAaichi groups. This may negatively impact the TLR5 signaling mediated immune responses induction by f4M2e.

The high sequence conservation of M2e among all known human influenza A viruses is critical for its development as a universal human influenza A vaccine candidate. Though M2e residues 10 to 24 are relatively more variable compared the almost absolutely conserved N-terminal 9 amino acids (Deng et al., 2015a), the M2e sequence variation in the membrane proximal part is not comparable to the amino acid changes in HA and NA. M2e has low immunogenicity possibly because of its smaller size and lower abundance in virions compared to HA and NA. Therefore, M2e is often fused to a carrier to enhance its immunogenicity in animal experiments. The HA2 domain is considerably more conserved than the HA1 domain (approximately 90% and 67% for influenza A H1 and H3 subtypes in extracellular parts) and is a promising candidate immunogen for development as a universal influenza vaccines (Bommakanti et al., 2010). It has been reported that the HA stalk domain based vaccines comprising HA2 and portions of HA1 were immunogenic *in vivo* and able to provide protection against heterologous and heterosubtypic influenza A virus strains (Impagliazzo et al., 2015; Yassine et al., 2015). However, the subviral particles in which the HA1 was chemically removed did not confer protection to immunized mice and rabbits (Graves et al., 1983). In this study, we compared the immunogenicity of f4M2e, f4M2e/fHApr8 and f4M2e/fHAaichi Nps in a mouse model. Our results showed that all three constructs induced strong M2e-specific IgG responses (Fig 3 B). Of note, Np containing 5 µg fHAaichi and 5 µg f4M2e induced higher levels of M2e specific antibody titers compared with 10 µg f4M2e Np (Fig 3 B). Possibly, the introduction of T-helper epitopes from HA2 resulted in the increase of M2e specific antibody responses. The H3 HA2 domain in the vaccine also induced HA2 domain specific antibodies (Fig 3 D).

The degree of M2e density is critical for the level of induced M2e specific humoral immune responses (De Filette et al., 2005; Neiryneck et al., 1999; Zhou et al., 2012). To increase the M2e epitope density in the vaccine and the cross-reactivity, four tandem copies of M2e epitopes from H1N1, H3N2, H5N1 and H7N9 were incorporated into the FliC carrier protein. The induced M2e specific serum antibodies could bind to the 4 types of M2e peptides with similar binding activity (Fig 3 C). The comparable bindings to various M2e variants demonstrated that the tandem positions of these M2e copies in fusion proteins do not affect the immunogenicity of these M2e variants.

Cellular immunity plays an important role in protection against viral infection (Salerno-Goncalves and Sztejn, 2006). The M2e-specific CD4⁺ cellular immunity contributes to virus clearance (Eliasson et al., 2008; Eliasson et al., 2017). T cell derived Interleukin (IL) -2 is

associated with the differentiation of effector T cells after antigen mediated activation (Boyman and Sprent, 2012). Our results showed significantly higher numbers of IL-2-secreting splenocytes from the M2e vaccine-boosted groups after overnight human consensus M2e peptide re-stimulation in ELISPOT experiment, compared with the PBS group (Fig 4).

The incorporation of several types of conserved influenza A virus antigens provides a promising way to develop universal influenza vaccines. In conclusion, immunization with the combination of M2e and HA2 domain-based Nps can induce both M2e- and HA2-specific humoral immune responses and provide full protection against a lethal dose of H3N2 virus challenge. The M2e-specific antibody titers were significantly enhanced by incorporation of HA2 domain in the same Np vaccines.

We presumed that the solely HA2 domain elicited specific immunity was less protective than the acquired immunity against the entire stalk domain comprising HA2 and portions of HA1. The results highlight the necessity to generate constructs expressing HA stalk domains simulating their structures in their native protein context. The study of which antigens to include, which adjuvant to use and how the protein nanoparticles can best exhibit the native-like structures of antigens presents a promising opportunity to develop a strongly and broadly protective influenza vaccine.

Acknowledgments

This research was supported by National Institutes of Health (NIH)/ National Institute of Allergy and Infectious Diseases (NIAID) Grant R01 AI101047.

References

- Bommakanti G, Citron MP, Hepler RW, Callahan C, Heidecker GJ, Najar TA, Lu X, Joyce JG, Shiver JW, Casimiro DR, ter Meulen J, Liang X, Varadarajan R. Design of an HA2-based *Escherichia coli* expressed influenza immunogen that protects mice from pathogenic challenge. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:13701–13706. [PubMed: 20615991]
- Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nature reviews. Immunology*. 2012; 12:180–190.
- De Filette M, Min Jou W, Birkett A, Lyons K, Schultz B, Tonkyro A, Resch S, Fiers W. Universal influenza A vaccine: optimization of M2-based constructs. *Virology*. 2005; 337:149–161. [PubMed: 15914228]
- de Vries RD, Altenburg AF, Rimmelzwaan GF. Universal influenza vaccines, science fiction or soon reality? *Expert review of vaccines*. 2015; 14:1299–1301. [PubMed: 26104835]
- Deng L, Cho KJ, Fiers W, Saelens X. M2e-Based Universal Influenza A Vaccines. *Vaccines*. 2015a; 3:105–136. [PubMed: 26344949]
- Deng L, Ibanez LI, Van den Bossche V, Roose K, Youssef SA, de Bruin A, Fiers W, Saelens X. Protection against Influenza A Virus Challenge with M2e-Displaying Filamentous *Escherichia coli* Phages. *PloS one*. 2015b; 10:e0126650. [PubMed: 25973787]
- El Bakkouri K, Descamps F, De Filette M, Smet A, Festjens E, Birkett A, Van Rooijen N, Verbeek S, Fiers W, Saelens X. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. *J Immunol*. 2011; 186:1022–1031. [PubMed: 21169548]

- Eliasson DG, El Bakkouri K, Schon K, Ramne A, Festjens E, Lowenadler B, Fiers W, Saelens X, Lycke N. CTA1-M2e-DD: a novel mucosal adjuvant targeted influenza vaccine. *Vaccine*. 2008; 26:1243–1252. [PubMed: 18243429]
- Eliasson DG, Omokanye A, Schon K, Wenzel UA, Bernasconi V, Bemark M, Kolpe A, El Bakkouri K, Ysenbaert T, Deng L, Fiers W, Saelens X, Lycke N. M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection. *Mucosal immunology*. 2017
- Feng J, Zhang M, Mozdzanowska K, Zharikova D, Hoff H, Wunner W, Couch RB, Gerhard W. Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. *Virology journal*. 2006; 3:102. [PubMed: 17150104]
- Graves PN, Schulman JL, Young JF, Palese P. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. *Virology*. 1983; 126:106–116. [PubMed: 6189287]
- Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 2001; 410:1099–1103. [PubMed: 11323673]
- Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, Tang J, McDonald W, Song L, Evans RK, Umlauf S, Tussey L, Powell TJ. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine*. 2008; 26:201–214. [PubMed: 18063235]
- Hutchinson EC, Charles PD, Hester SS, Thomas B, Trudgian D, Martinez-Alonso M, Fodor E. Conserved and host-specific features of influenza virion architecture. *Nature communications*. 2014; 5:4816.
- Impagliazzo A, Milder F, Kuipers H, Wagner MV, Zhu X, Hoffman RM, van Meersbergen R, Huizingh J, Wannigen P, Verspuij J, de Man M, Ding Z, Apetri A, Kukrer B, Sneekes-Vriese E, Tomkiewicz D, Laursen NS, Lee PS, Zakrzewska A, Dekking L, Tolboom J, Tettero L, van Meerten S, Yu W, Koudstaal W, Goudsmit J, Ward AB, Meijberg W, Wilson IA, Radosevic K. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science*. 2015; 349:1301–1306. [PubMed: 26303961]
- Mardanov ES, Kotlyarov RY, Kuprianov VV, Stepanova LA, Tsybalova LM, Lomonosoff GP, Ravin NV. High immunogenicity of plant-produced candidate influenza vaccine based on the M2e peptide fused to flagellin. *Bioengineered*. 2016; 7:28–32. [PubMed: 26710263]
- Neirynek S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nature medicine*. 1999; 5:1157–1163.
- Prevention, C.f.D.C.a. Estimates of deaths associated with seasonal influenza --- United States, 1976–2007. *MMWR. Morbidity and mortality weekly report*. 2010; 59:1057–1062. [PubMed: 20798667]
- Salerno-Goncalves R, Szein MB. Cell-mediated immunity and the challenges for vaccine development. *Trends in microbiology*. 2006; 14:536–542. [PubMed: 17055276]
- Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual review of biochemistry*. 2000; 69:531–569.
- Skountzou I, Martin Mdel P, Wang B, Ye L, Koutsonanos D, Weldon W, Jacob J, Compans RW. Salmonella flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. *Vaccine*. 2010; 28:4103–4112. [PubMed: 19654062]
- Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, Cookson BT, Aderem A. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nature immunology*. 2003; 4:1247–1253. [PubMed: 14625549]
- Song JM, Van Rooijen N, Bozja J, Compans RW, Kang SM. Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:757–761. [PubMed: 21187388]
- Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, Haye K, Garcia-Sastre A, Palese P. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *mBio*. 2010:1.
- Stepanova LA, Kotlyarov RY, Kovaleva AA, Potapchuk MV, Korotkov AV, Sergeeva MV, Kasianenko MA, Kuprianov VV, Ravin NV, Tsybalova LM, Skryabin KG, Kiselev OI. Protection against

- multiple influenza A virus strains induced by candidate recombinant vaccine based on heterologous M2e peptides linked to flagellin. *PLoS one*. 2015; 10:e0119520. [PubMed: 25799221]
- Turley CB, Rupp RE, Johnson C, Taylor DN, Wolfson J, Tussey L, Kavita U, Stanberry L, Shaw A. Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults. *Vaccine*. 2011; 29:5145–5152. [PubMed: 21624416]
- Valkenburg SA, Mallajosyula VV, Li OT, Chin AW, Carnell G, Temperton N, Varadarajan R, Poon LL. Stalking influenza by vaccination with pre-fusion headless HA mini-stem. *Scientific reports*. 2016; 6:22666. [PubMed: 26947245]
- van de Veerdonk FL, Joosten LA, Shaw PJ, Smeekens SP, Malireddi RK, van der Meer JW, Kullberg BJ, Netea MG, Kanneganti TD. The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. *European journal of immunology*. 2011; 41:2260–2268. [PubMed: 21681738]
- Van den Hoecke S, Ehrhardt K, Kolpe A, El Bakkouri K, Deng L, Grootaert H, Schoonooghe S, Smet A, Bentahir M, Roose K, Schotsaert M, Schepens B, Callewaert N, Nimmerjahn F, Staeheli P, Hengel H, Saelens X. Hierarchical and Redundant Roles of Activating FcγR3s in Protection against Influenza Disease by M2e-Specific IgG1 and IgG2a Antibodies. *Journal of virology*. 2017:91.
- Wang BZ, Gill HS, He C, Ou C, Wang L, Wang YC, Feng H, Zhang H, Prausnitz MR, Compans RW. Microneedle delivery of an M2e-TLR5 ligand fusion protein to skin confers broadly cross-protective influenza immunity. *Journal of controlled release : official journal of the Controlled Release Society*. 2014a; 178:1–7. [PubMed: 24417966]
- Wang BZ, Gill HS, Kang SM, Wang L, Wang YC, Vassilieva EV, Compans RW. Enhanced influenza virus-like particle vaccines containing the extracellular domain of matrix protein 2 and a Toll-like receptor ligand. *Clinical and vaccine immunology : CVI*. 2012; 19:1119–1125. [PubMed: 22647270]
- Wang BZ, Xu R, Quan FS, Kang SM, Wang L, Compans RW. Intranasal immunization with influenza VLPs incorporating membrane-anchored flagellin induces strong heterosubtypic protection. *PLoS one*. 2010; 5:e13972. [PubMed: 21124769]
- Wang L, Hess A, Chang TZ, Wang YC, Champion JA, Compans RW, Wang BZ. Nanoclusters self-assembled from conformation-stabilized influenza M2e as broadly cross-protective influenza vaccines. *Nanomedicine : nanotechnology, biology, and medicine*. 2014b; 10:473–482.
- Yassine HM, Boyington JC, McTamney PM, Wei CJ, Kanekiyo M, Kong WP, Gallagher JR, Wang L, Zhang Y, Joyce MG, Lingwood D, Moin SM, Andersen H, Okuno Y, Rao SS, Harris AK, Kwong PD, Mascola JR, Nabel GJ, Graham BS. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nature medicine*. 2015; 21:1065–1070.
- Zhang H, Wang L, Compans RW, Wang BZ. Universal influenza vaccines, a dream to be realized soon. *Viruses*. 2014; 6:1974–1991. [PubMed: 24784572]
- Zhong W, Reed C, Blair PJ, Katz JM, Hancock K. Serum antibody response to matrix protein 2 following natural infection with 2009 pandemic influenza A(H1N1) virus in humans. *The Journal of infectious diseases*. 2014; 209:986–994. [PubMed: 24325965]
- Zhou C, Zhou L, Chen YH. Immunization with high epitope density of M2e derived from 2009 pandemic H1N1 elicits protective immunity in mice. *Vaccine*. 2012; 30:3463–3469. [PubMed: 22446634]

Highlights

- Stable protein nanoparticles can be fabricated using DTSSP crosslinker.
- Crosslinked protein nanoparticles are highly immunogenic in mouse models and induce strong antibody responses against M2e and HA stalk domains.
- Mice receiving nanoparticle vaccinations are fully protected against lethal dose influenza A virus challenges.

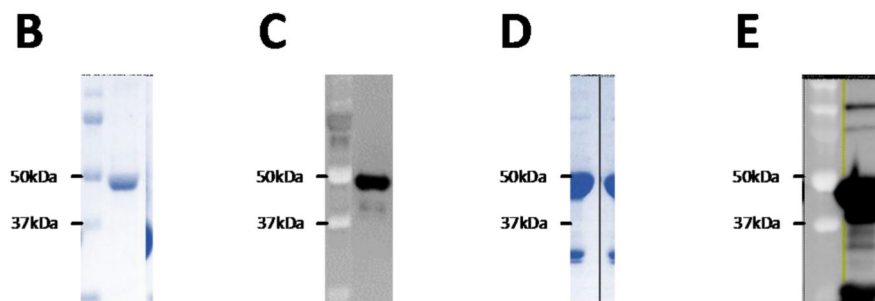
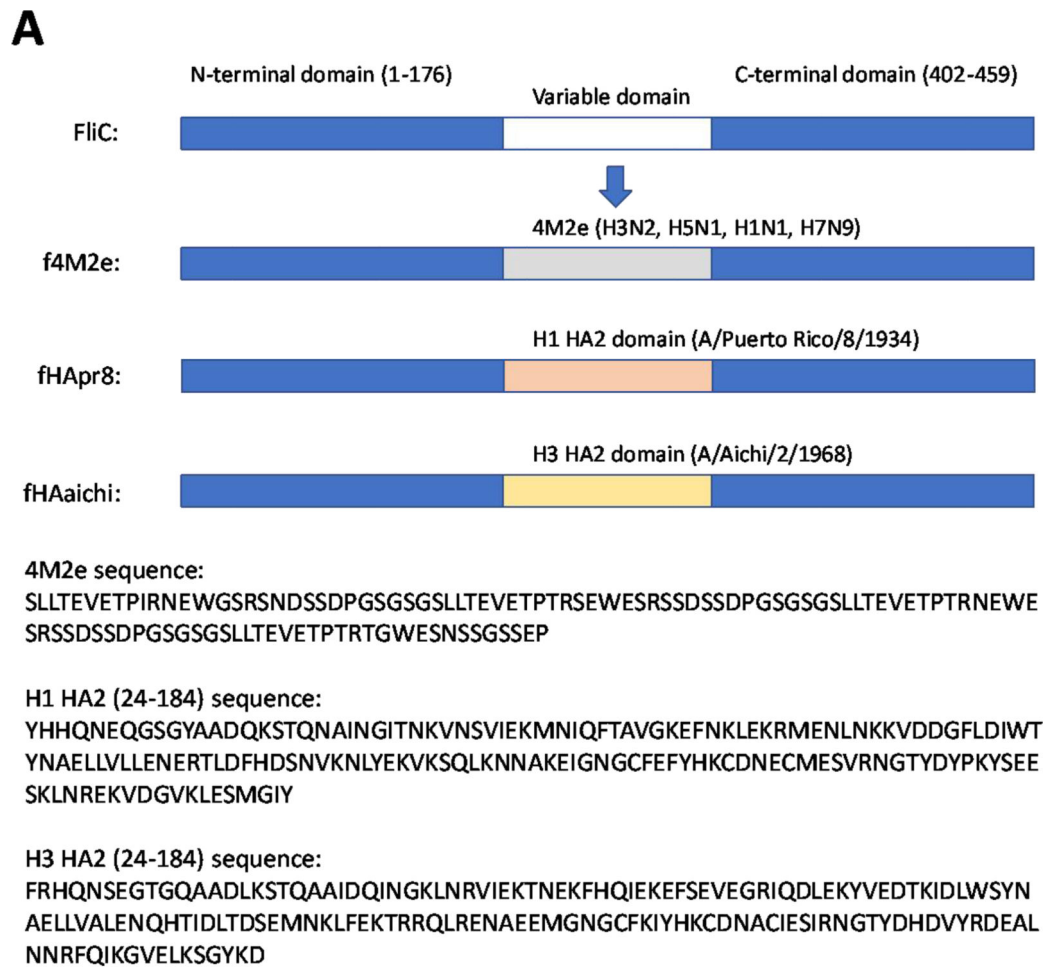


Fig 1. Construction, purification, and characterization of fusion proteins. (A) The variable domain of FliC (177-401) was replaced with 4M2e (f4M2e), H1 HA2 domain (fHApr8) and H3 HA2 domain (fHAaichi), respectively. Four tandem copies of M2e sequence contains M2e peptides from human H3N2 consensus M2e (SLLTEVETPIRNEWGSRSDSDSD), A/California/7/2009 H1N1 M2e (SLLTEVETPTRSEWESRSDSDSD), A/Viet Nam/1194/2004 H5N1 M2e (SLLTEVETPTRNEWESRSDSDSD) and A/Shanghai/02/2013 H7N9 M2e (SLLTEVETPTRTGWESNSSGSSE). The H1 (A/Puerto Rico/8/1934) HA2 domain

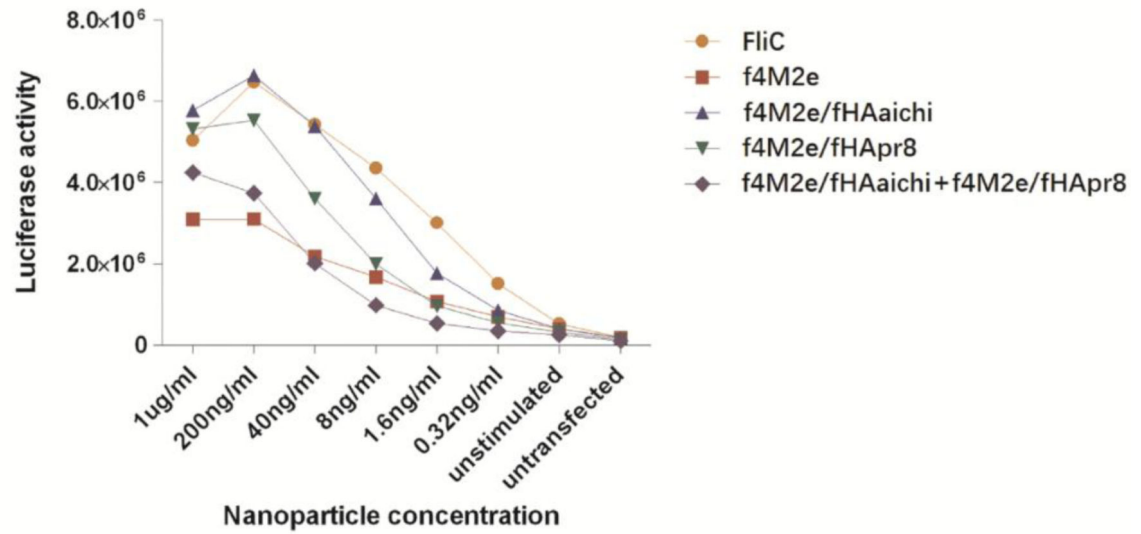
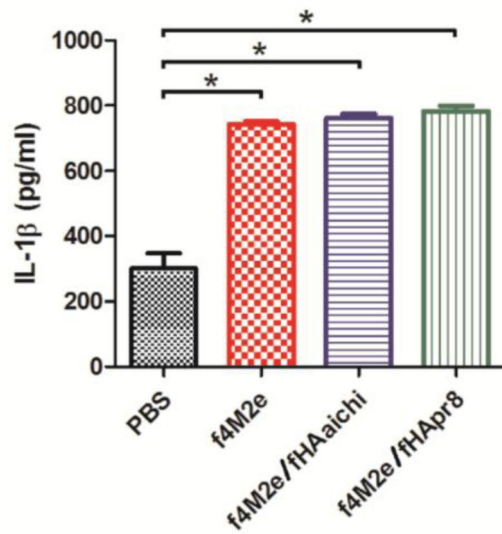
(24-184) sequence and H3 (A/Aichi/2/1968) HA2 domain (24-184) sequence are shown below. Commassie blue stained SDS-PAGE gel and Western blot analysis of purified fHApr8 (B, C) and fHAaichi (D, E).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

A**B****Fig 2.**

Characterization of FliC fusion protein nanoparticles. (A) TLR5 agonist bioactivity of FliC fusion protein nanoparticles. The TLR5 and NF- κ B/luciferase transiently transfected HEK293T cells were used to determine the bioactivity of serially diluted FliC fusion protein nanoparticles. Soluble FliC protein was used as a positive control. (B) IL-1 β secretion by nanoparticle-stimulated murine JAWS II DC cells. PBS mock-stimulation group was used as negative control. * indicates $p < 0.05$ (t -test).

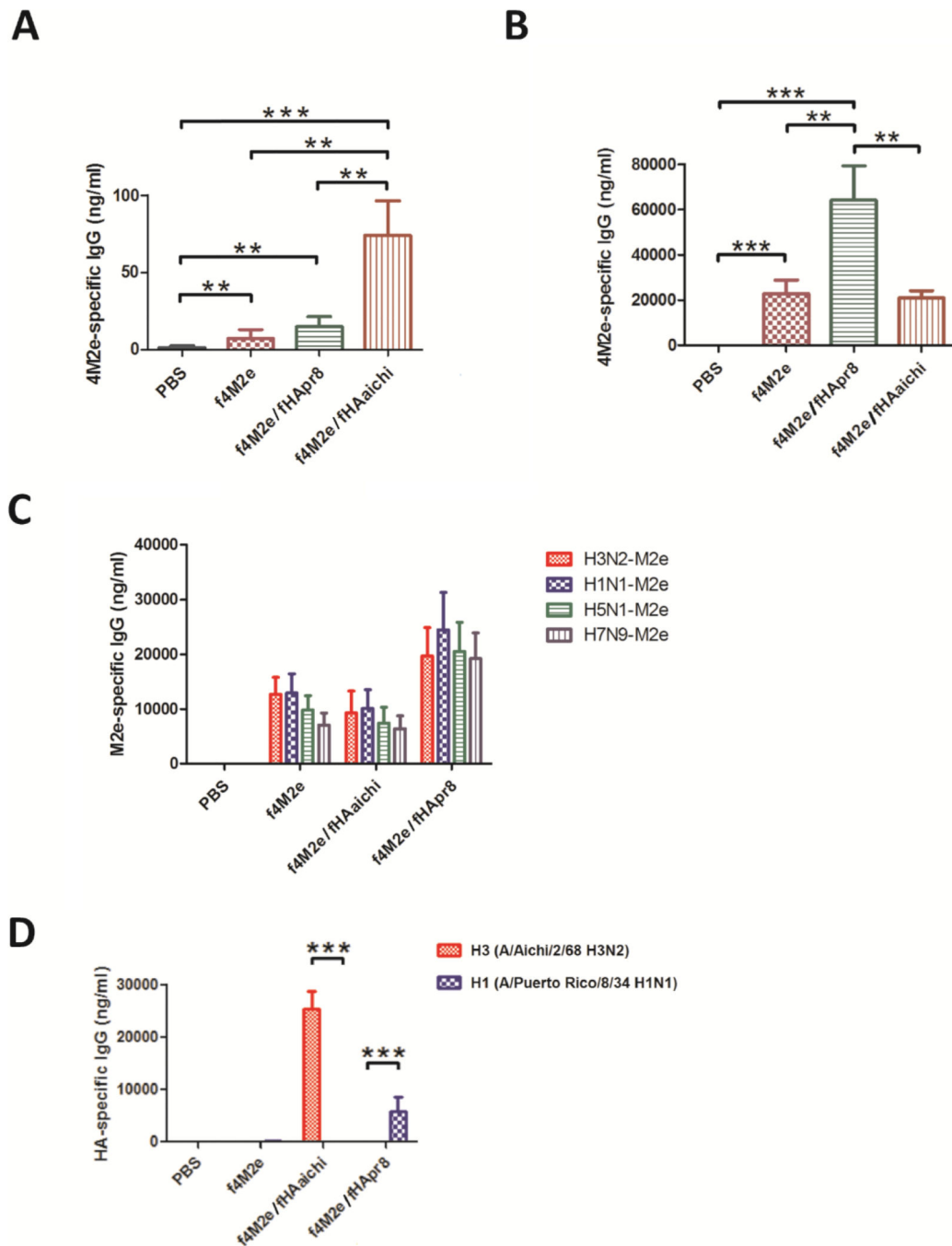


Fig 3. Serum antibody responses induced by crosslinked protein nanoparticles. Mice were intranasally immunized twice, immune sera collected 2 weeks post each immunization were compared in ELISA analysis. Data are depicted as the mean \pm SD (n=10). (A) Serum 4M2e-specific IgG levels after the priming immunization. (B) Serum total 4M2e-specific IgG levels after the boosting immunization. (C) Cross-reactivity of post boosting immune sera to different M2e peptides from H3N2, H1N1, H5N1 and H7N9. (D) Serum H3- and H1-stalk

domain specific IgG levels after the boosting immunization. ** indicate $p < 0.01$; *** indicate $p < 0.005$ (*t*-test).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

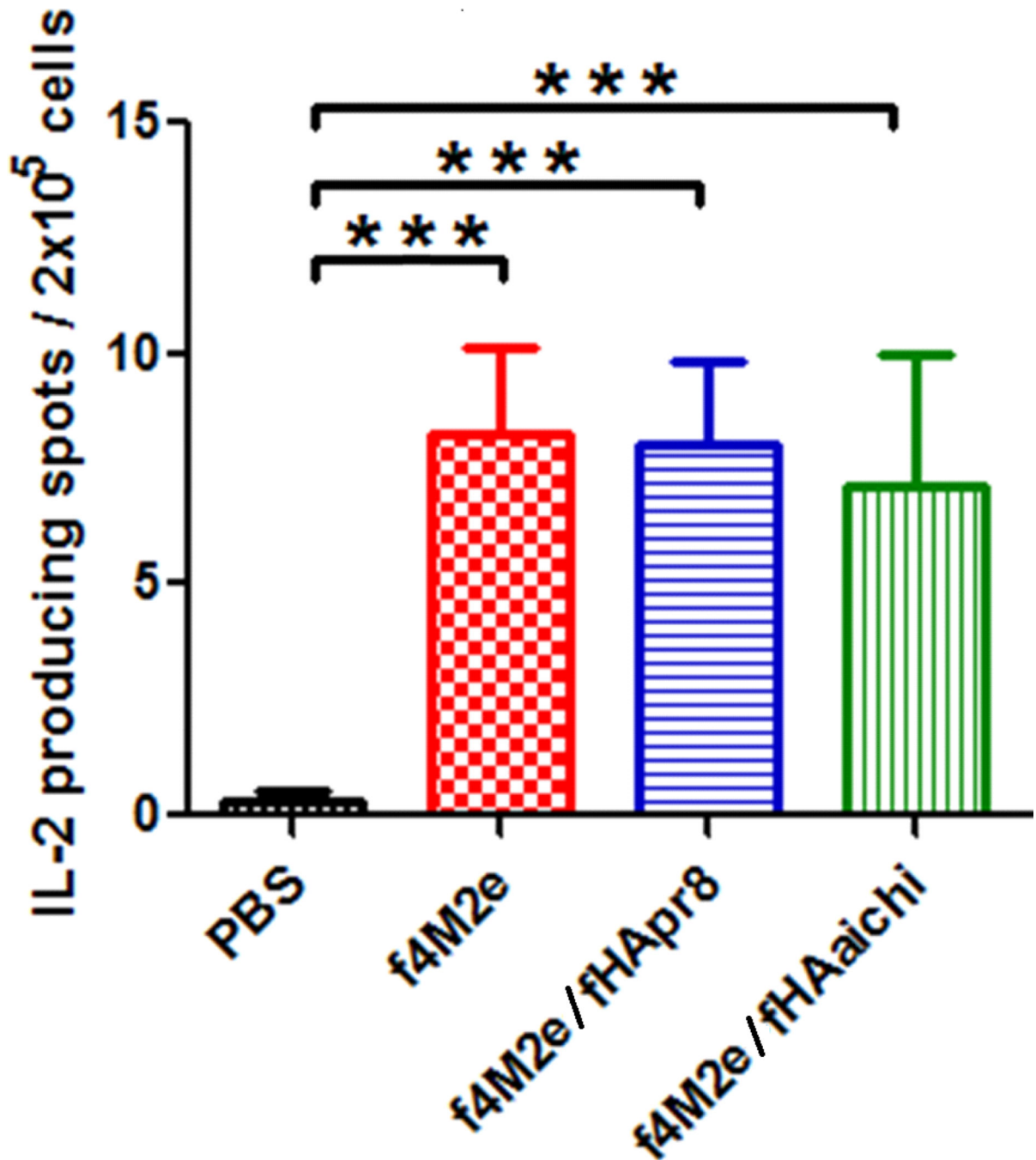
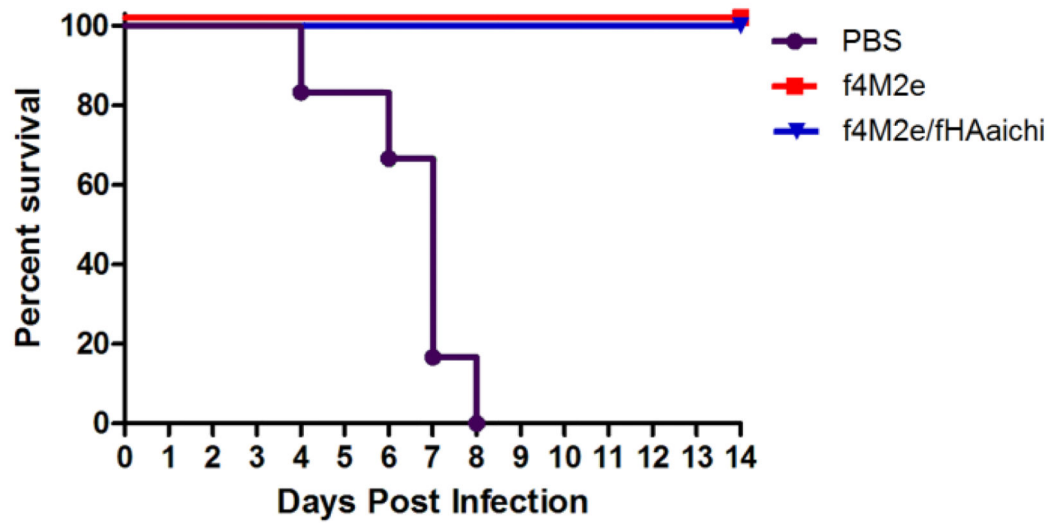
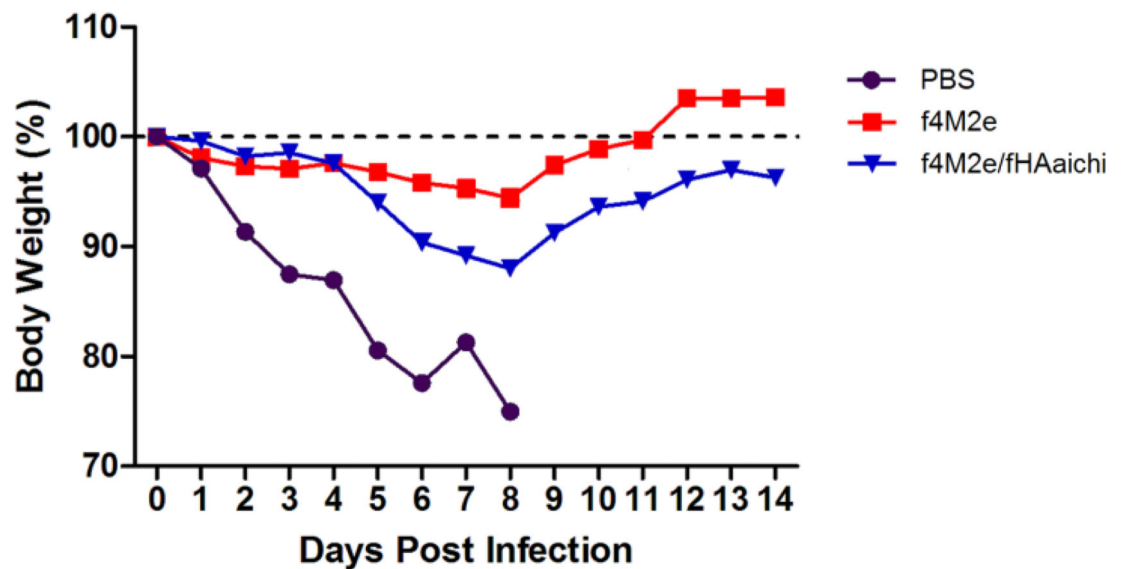


Fig 4. M2e-specific cellular immune responses. Splenocytes were collected 3 weeks after the boosting immunization and were re-stimulated overnight with M2e peptide. The IL-2 cytokine secreting cell colonies were determined by using ELISPOT assay. *** indicate significant difference ($p < 0.005$) compared to PBS group.

A**B****Fig 5.**

Protective efficacy against infection with H3N2 strain. Immunized BALB/c mice were intranasally challenged with 4×50% lethal dose of A/Aichi/2/68 H3N2 virus in a volume of 30 μ l PBS. (A) Mouse survival rate and (B) body weight changes were monitored daily for 14 days post infection.

Table 1

Size distribution and Zeta potential parameters of crosslinked protein nanoparticles measured by Malvern Zetasizer.

Nanoparticle Type	Size (nm)	Stdev(nm)	Zeta Pot.	Stdev
f4M2e	54	13.5	-7.1	0.26
f4M2e/fHApr8 (1:1)	46.7	24.1	-10.9	1.43
f4M2e/fHAaichi (1:1)	81.7	18.9	-7.94	0.83

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript