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Double-Layered M2e-NA Protein Nanoparticle Immunization Induces Broad Cross-Protection against Different Influenza Viruses in Mice

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The development of a universal influenza vaccine is an ideal strategy to eliminate public health threats from influenza epidemics and pandemics. This ultimate goal is restricted by the low immunogenicity of conserved influenza epitopes. Layered protein nanoparticles composed of well-designed conserved influenza structures have shown improved immunogenicity with new physical and biochemical features. Herein, structure-stabilized influenza matrix protein 2 ectodomain (M2e) and M2e-neuraminidase fusion (M2e-NA) recombinant proteins are generated and M2e protein nanoparticles and double-layered M2e-NA protein nanoparticles are produced by ethanol desolvation and chemical crosslinking. Immunizations with these protein nanoparticles induce immune protection against different viruses of homologous and heterosubtypic NA in mice. Double-layered M2e-NA protein nanoparticles induce higher levels of humoral and cellular responses compared with their comprising protein mixture or M2e nanoparticles. Strong cytotoxic T cell responses are induced in the layered M2e-NA protein nanoparticle groups. Antibody responses contribute to the heterosubtypic NA immune protection. The protective immunity is long lasting. These results demonstrate that double-layered protein nanoparticles containing structure-stabilized M2e and NA can be developed into a universal influenza vaccine or a synergistic component of such vaccines. Layered protein nanoparticles can be a general vaccine platform for different pathogens.

1. Introduction

Influenza virus infection can cause severe respiratory disease during yearly influenza seasons by evading prevailing immunity via frequent mutations on the globular head of the hemagglutinin protein (HA). Scientists have tried to develop a new generation of influenza vaccines to induce broadly reactive influenza immunity and avoid strain-specific immune responses by including conserved influenza structures while excluding highly strain-specific components in new vaccine immunogens. Conserved antigens such as the HA stalk domain, the M2 ectodomain (M2e), and T cell epitopes from the internal nucleoprotein have been incorporated into vaccine formulations to generate cross-protection against various viruses of different subtypes.[1–3]

Influenza neuraminidase (NA) is another important membrane glycoprotein containing four identical polypeptides. This tetrameric protein facilitates the cleavage between HA and sialic acid during viral budding. For a long time, NA was underestimated by the vaccinology community due to its relatively poor immunogenicity and the immune dominance of HA.[4] However, recent studies have shown NA to be a promising antigen for vaccine development: (1) Compared to HA, NA experiences relatively slower antigenic drift.[5] (2) A universally conserved NA epitope between 222 and 230 induced NA-inhibiting antibodies against all influenza types.[6,7] (3) Therapeutic NA-specific monoclonal antibodies protected mice from lethal doses of homologous and heterologous influenza infection.[8] These discoveries demonstrated that NA has the potential to be developed into a universal influenza vaccine if it is presented in an immunogenic form, such as protein nanoparticles.

The small tetrameric influenza M2 protein (the third viral membrane protein) comprises 97 amino acids and contains a highly conserved ectodomain. The eight N-terminal amino acid residues (SLLTEVET) from the ectodomain are over 99% conserved among all subtype A influenza viruses.[9] Although the M2 protein does not induce a strong immune response in nature due to its small size and a low count on the virion surface, researchers have increased the immunogenicity of M2e by designing polymeric peptides of M2e or incorporating M2e into nanoparticle systems to induce M2e-specific immunity.[2,10]

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adhm.201901176.

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DOI: 10.1002/adhm.201901176

A double-layered protein nanoparticle vaccine platform has demonstrated a superior immune response for less immunogenic antigens.[3,10,11] The induction of immune responses to conserved epitopes located inside influenza antigenic proteins with natural antigenic influenza proteins or current inactivated vaccines is an almost impossible task because of the immunodominance of strain-specific epitopes in the proteins. Designing recombinant fusion proteins containing conserved influenza epitopes with natural-like structures is the optimal avenue to avoid the immunodominance of strain-specific epitopes and emphasize immune responses to these conserved epitopes. By employing fine-turned protein nanoparticles, our previous studies have demonstrated that these multi-epitope subunit vaccines are highly potent and could be developed into a universal influenza vaccine.[3,11] The clinical trials of a multi-epitope universal influenza vaccine (MiondVax's M-001) have also demonstrated the advantage of this approach in inducing broadly reactive influenza immunity.[12]

Nanoparticle designs have been applied to vaccine development to improve cellular and humoral immune responses.[13,14] For example, the interbilayer-crosslinked multi-lamellar vesicle-loaded antigens elicited a strong CD8 T cell response;[15] self-assembling ferritin-based HA2 nanoparticles displayed a stable structure and elicited broadly neutralizing antibodies;[16] poly(lactic-co-glycolic acid) (PLGA) polymer nanoparticles carrying murine melanoma antigenic peptides primed significant antigen-specific CD8 T cells.[17] While these different nanoparticles have various biochemical features that increase the immunogenicity of the comprising antigens, they also have some weaknesses because of their unique structures and compositions. For example, polymer nanoparticles have a relative low antigen load because they need the polymer to form the nanoparticle scaffold to trap antigens inside the particles or to bind antigens on their surfaces. Polymer selection is a limitation as well because of their weak biocompatibility.[18] Inorganic nanoparticles have a weakness similar to polymer nanoparticles. Self-assembling peptide-derived protein nanoparticles may trigger off-target immune responses.[16]

Our desolvation protein nanoparticles are composed almost entirely of the immunogenic proteins with a tiny amount of crosslinkers for fixation, and thus have the highest possible loads of target antigen. Because of their almost pure protein composition, the layered protein nanoparticles are highly biocompatible. Using a reducible crosslinker like a disulfide bond between two cytidines in a protein means the layered protein nanoparticles will release proteins in a physiological redox condition. The extended antigen-presentation observed in previous studies demonstrated the slow antigen release from the protein nanoparticles.[3] These features are expected to contribute to the high immunogenicity of the layered protein nanoparticles.[3,10,11,14,19,20]

In this study, we generated structure-stabilized M2e-NA fusion protein and combined structure-stabilized M2e-NA and M2e into self-disassembling layered protein nanoparticles.[14,20] We used N1 from A/Vietnam/1203/2004 (Viet, H5N1) and N2 from A/Aichi/1968 (Aichi, H3N2) to construct representative NA vaccine antigens. We compared the immunogenicity of these protein nanoparticles, evaluated cross-protection against influenza challenges, and explored the immunological mechanisms of the protection.

2. Results

2.1. Fabrication and Characterization of Double-Layered M2e-NA Protein Nanoparticles

We constructed genes encoding targeted conserved antigens (M2e, M2e-N1 fusion protein, and M2e-N2 fusion protein) (**Figure 1**A), generated recombinant baculoviruses (rBVs) with these genes, and expressed and purified the recombinant proteins in insect cells. Results from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining and Western blotting analysis demonstrated that the proteins were purified to high purity, appearing as a major band in gel staining (Figure 1B), and the protein identities were confirmed by antigen-specific antibody detection in the Western blots (Figure S1, Supporting Information). After bis(sulfosuccinimidyl) suberate (BS3) cross-linking to fix the polymeric states of the purified proteins, we observed four-fold higher molecular weight in the Western blots compared with samples without crosslinking treatment—demonstrating that the recombinant M2e-N1 and M2e-N2 were in tetrameric forms (Figure S1B,C, Supporting Information). After the BS3 crosslinking, we observed that the dominant M2e is the dimeric form, with some monomeric and tetrameric forms (Figure S1A, Supporting Information).

We generated M2e protein nanoparticles by ethanol desolvation as previously described.[3,11] Double-layered M2e-N1 or M2e-N2 nanoparticles were generated by coating M2e-N1 or M2e-N2 fusion proteins onto the surfaces of M2e nanoparticles with 3′-dithiol bis (sulfosuccinimidylpropionate) (DTSSP) crosslinking (diagramed Figure 1C).[3] The size ranges of the resulting double-layered protein nanoparticles were 211.0 +/− 60.14 nm and 190.4 +/− 53.52 nm for M2e-N1 and M2e-N2 nanoparticles, respectively (Figure 1D, M2e-N1 Nano and M2e-N2 Nano). In comparison, the M2e core nanoparticle size was 176.4 +/− 48.3 nm (Figure 1D, M2e Nano). The layered M2e-N1 and M2e-N2 protein nanoparticles showed negative ζ-potentials of −27.77 +/−1.16 mV and −21.03 +/− 0.93 mV (Figure S1D, Supporting Information), and the loading ratios of M2e-N1 and M2e-N2 to the total protein in their layered protein nanoparticles were 62.1% +/− 2.5% and 59.3% +/− 1.8%, respectively. Neuraminidase activity assays showed the retention of NA activity in the resulting layered M2e-NA nanoparticles (Figure 1F). The Coomassie blue staining and Western blotting analysis demonstrated the M2e and NA composition of the layered protein nanoparticles (Figure 1E and Figure S2, Supporting Information). Transmission electron microscopy (TEM) observation further revealed the roughly spherical shape of the layered protein nanoparticles with irregular surfaces (Figure 1G).

2.2. Double-Layered M2e-NA Protein Nanoparticle Immunization Induced Immune Protection against Homologous and Heterologous NA Viral Challenge

Unlike the NA protein in live influenza viruses or inactivated vaccines, the NA fusion proteins on the surfaces of the layered protein nanoparticles were at high density and were not shielded by other larger immunogenic proteins, such as HA.

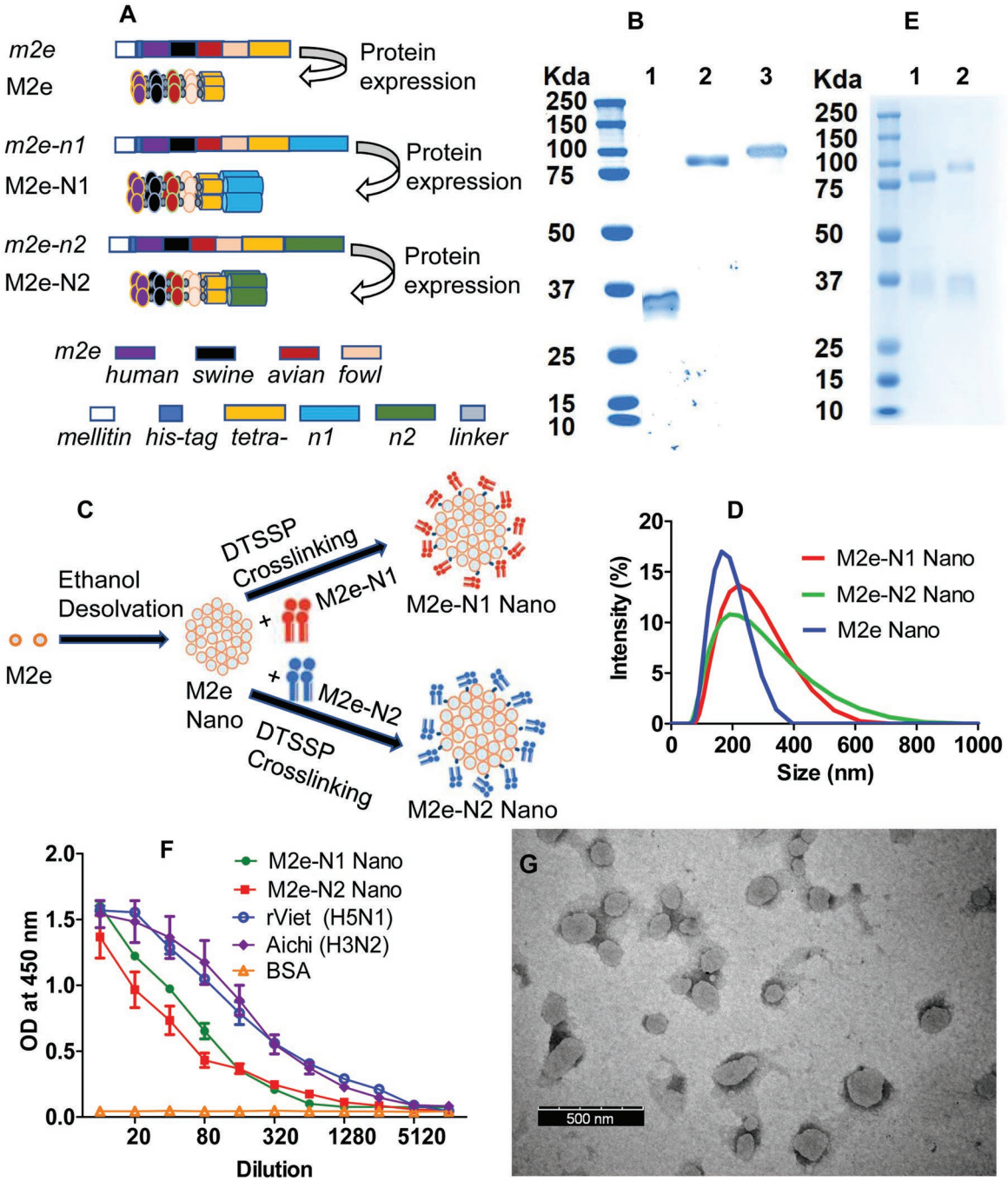


Figure 1. The fabrication of double-layered M2e-NA protein nanoparticles. A) Diagrams of M2e and M2e-NA fusion proteins and their coding sequence compositions. GS linker: glycine and serine GGSGGG; M2e: a recombination of four tandem M2e sequences from human, swine, avian, and domestic fowl influenza viruses stabilized by the tetrabrachion tetramerization sequence; M2e-N1 fusion protein: sequence of NA ectodomain (36H to 449K from Viet (H5N1) NA) fused with the four tandem M2e repeats and stabilized by the tetrabrachion sequence; M2e-N2 fusion protein: as M2e-N1 but the sequence of NA ectodomain is from Aichi (H3N2) NA(38K to 469I). B) Characterization of purified recombinant proteins with Coomassie blue staining. Line 1, recombinant M2e; Line 2, M2e-N1; Line 3, M2e-N2. C) Diagrams of layered protein nanoparticle generation. M2e protein nanoparticles (M2e Nano) were generated by ethanol desolvation. Double-layered M2e-N1 and M2e-N2 protein nanoparticles (M2e-N1 Nano and M2e-N2 Nano) were formed by crosslinking M2e-N1 and M2e-N2 fusion proteins onto the surfaces M2e Nano as coatings, respectively. D) The size range of protein nanoparticles. Red, M2e-N1 Nano; Green, M2e-N2 Nano; Blue, M2e Nano. E) Coomassie blue staining of protein nanoparticles in an SDS-PAGE gel. Line 1, double-layered M2e-N1 protein nanoparticles; Line 2, double-layered M2e-N2 protein nanoparticles. F) Neuraminidase activity. NA activity was acquired by using ELLA as described in the Experimental Section. The concentration of M2e-N1 nanoparticle, M2e-N2 nanoparticle, Vietnam virus, or Aichi virus was 1 mg mL^{-1} (total protein). G) TEM image of double-layered M2e-NA nanoparticles. All types of protein nanoparticles have a similar morphology. The image was acquired using double-layered M2e-N2 protein nanoparticles as an example. Bar scale, 500 nm.

We hypothesized that these traits made the NA fusion proteins highly immunogenic in the nanoparticles. Immune serum antibody titrations showed that the double-layered nanoparticle immunizations significantly increased NA and M2e-specific IgG antibody levels (the total serum IgG, as well as the IgG1 and IgG2a subtypes) compared with the soluble protein mixture immunization groups (sM2e+NA) (**Figure 2**A,C). Mice in the layered M2e-NA nanoparticle groups produced significantly higher M2e and NA-specific IFN-γ secreting splenocytes after the boost immunization (Figure 2B,D).

Because the neuraminidase inhibition (NAI) of immune sera is a correlate of NA mediated immune protection, we performed an NAI test to show the neutralizing ability of immune sera from every group. Sera from the layered M2e-N1 nanoparticle and M2e-N2 nanoparticle groups showed potent neutralization to both homologous and heterologous NA influenza viruses. The M2e-N1 nanoparticle immune sera showed a significant decrease in OD_{450nm} (indicating an elevated NAI) compared with the sM2e+N1 (soluble protein mixture) immune sera up to a 20 480-fold dilution. The layered M2e-N2 nanoparticle sera showed a significant decrease up to a 32 000-fold dilution in OD_{450nm} compared with the corresponding soluble protein mixture or untreated N2 virus (Aichi, or A/Hong Kong/99 (Hong Kong, H9N2)) NA activity (Figure 2E,F). However, immune sera from layered M2e-N1 or M2e-N2 nanoparticle groups showed lower levels of NAI activity against the heterosubtypic NA influenza viruses. Layered M2e-N1 nanoparticle immune sera showed a significant decrease in OD_{450nm} compared with the untreated Aichi/68 virus NA activity down to a 640-fold dilution while M2e-N2 nanoparticle sera showed a significant decrease in OD_{450nm} compared with the untreated A/California/2009 (Cal, H1N1, the 2009 pandemic strain) virus NA activity down to only a 500-fold dilution (Figure 2E,F).

We challenged mice in the layered M2e-N1 nanoparticle group with doses of $3 \times LD_{50}$ of the reassortant A/Vietnam/1203/2004 (rViet, H5N1, PR8 backbone with HA and NA from Viet), or $5 \times LD_{50}$ Cal and mice in the M2e-N2 nanoparticle group with doses of $5 \times LD_{50}$ of Aichi (H3N2) or Hong Kong (H9N2) viruses to verify immune protection against homologous or heterologous viral infection (**Figure 3**).[11] Mice receiving the M2e-N1 or M2e-N2 nanoparticle immunizations completely survived their respective NA virus challenges (Figure 3A,B). Mice receiving the soluble sM2e+N1 immunization had a 100% survival rate against the rViet challenge, but the sM2e+N2 immunization demonstrated partial protection against the homologous viral challenge (Figure 3A,B). The nanoparticle immunization groups tended to lose less body weight than the soluble protein immunization groups, with the Hong Kong (H9N2) viral challenge being an exception (Figure 3). Furthermore, the layered protein nanoparticle immunization groups had significantly reduced viral titers in the lungs compared with the soluble protein groups 5 d after the homologous viral challenges (**Figure 4**A). Histological examinations of the lung tissue from the challenged mice indicated that layered protein nanoparticle groups showed a lower degree of immune cell infiltration and inflammation near the alveolar walls compared to the sM2e-NA soluble protein mixture groups, but the naïve control mice presented a heavier inflammatory state and severe tissue damage (Figure 4B).

2.3. Double-Layered M2e-NA Protein Nanoparticles Induced Strong T Cell Responses

Next, we compared different T cell responses induced by double-layered M2e-NA nanoparticles and sM2e+NA protein mixtures. CD4 T cells facilitate B cell differentiation and maintenance of CD8 cytotoxic T cell response, while CD8 T cells have a role in viral clearance from the lungs.[21] The layered M2e-N1 nanoparticle immunization significantly increased the number of IFN-γ secreting CD4 and CD8 T cells in the lungs 5 d after the rViet (H5N1) infection (**Figure 5**A,B,E,F). The layered M2e-N2 nanoparticle immunization induced even stronger T cell responses, demonstrated by higher numbers of IFN-γ secreting CD4 and CD8 T cell population in the lung 5 d after the Aichi (H3N2) challenge infection (Figure 5C,D,E,F). In contrast, the soluble protein mixture immunization did not show significant T cell population activation upon the challenge infection of homologous NA influenza viruses (Figure 5A,B, sM2e+N1 vs the rViet challenge; Figure 5C,D, sM2e+N2 verse the Aichi challenge).

To investigate whether the CD8 T cell responses are correlates of the immune protection, we depleted CD8 T cells from M2e-NA nanoparticle immunized mice by injecting 300 µL anti-CD8 antibody clone 2.43 (Cat. No. BE0061; BioXCell) 24 h before and after the challenge infection. CD8 T cell depletion groups showed a greater body weight loss and a decreased survival rate over immunized groups without the depletion (Figure 5G,H). These results indicated that layered M2e-NA protein nanoparticles conferred immune protection at least partially through CD8 T cell responses against influenza infection.

2.4. Antibody Responses Induced by M2e-NA Nanoparticles Contributed to Cross-Protection against Heterosubtypic Influenza Viruses

We tested the cross-protection of the nanoparticles against heterosubtypic virus challenges. Because NA fusion proteins in this study are derived from N1 in Viet (H5N1) and N2 in Aichi (H3N2), $3 \times LD_{50}$ of A/Philippines/2/82 (Philippines, H3N2) was applied to the M2e-N1 nanoparticle immunization set while $5 \times LD_{50}$ of Cal (H1N1) was applied to the M2e-N2 nanoparticle immunization set to test the heterosubtypic protection. Immunized mice in the M2e-N1 nanoparticle group showed a 100% survival against the Philippines virus challenge while the sM2e+N1 soluble protein mixture induced partial protection (**Figure 6**A). M2e-N2 nanoparticle immunized mice showed a 60% survival against the Cal virus challenge while mice from the corresponding sM2e+N2 soluble protein group, as well as naïve mice, reached their endpoints at day 7 post the challenge infection (Figure 6B).

Passive transmission of immune sera can show the immunological role of the antibodies without T cell responses. To verify whether the sera from M2e-NA nanoparticle immunization confer cross-protection against heterologous influenza viruses, we intraperitoneally (I.P.) injected naïve mice with 300 µL sera from double-layered protein nanoparticle-immunized mice 24 h before the challenge infection. Sera from

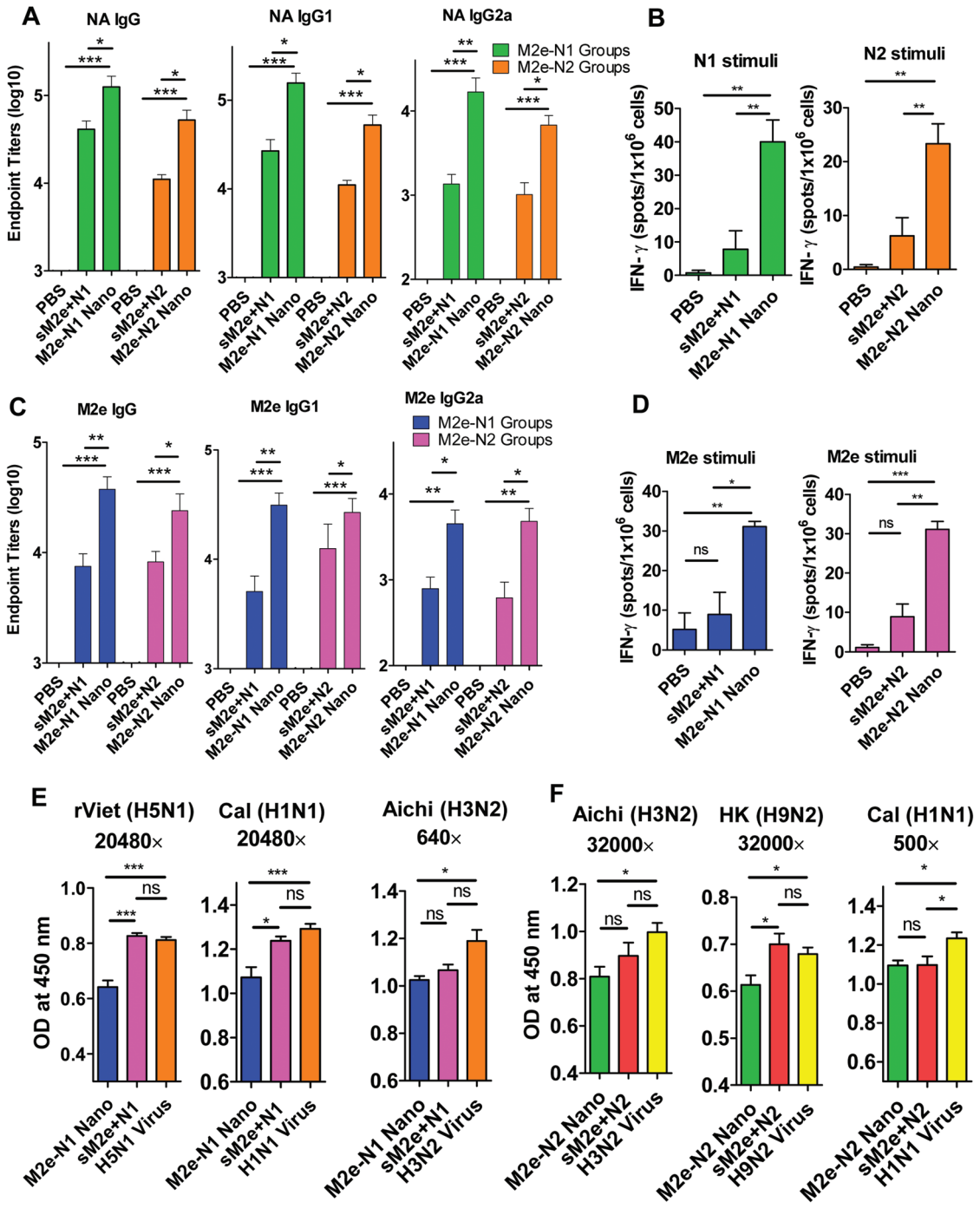


Figure 2. Humoral and cellular immune responses of vaccinated mice. A) Detection of NA-specific antibody responses by ELISA. Neuraminidase 1 (NA1) from Viet and Neuraminidase 2 (NA2) from Aichi were coated on the 96-well plates and sera from different groups were tested. B) Enumeration of NA-specific IFN-γ secreted splenocytes by ELISpot. The spleens of boost immunized mice were collected before the influenza challenge and N1 or N2 peptide pools were added to the homogenized splenocytes for stimulation. C) M2e-specific antibody responses. M2e peptide was coated on the 96-well plates and sera from different groups were tested for anti-M2e titers. D) Enumeration of M2e activated IFN-γ secreted splenocytes by ELISpot. The spleens of boost immunized mice were collected before the influenza challenge and M2e peptide pools were added to the homogenized splenocytes for activation. E,F) NAI test against different strains of influenza viruses. Sera from M2e-N1 group set (E) were mixed with 8000 $TCID_{50}$ rViet (H5N1), Cal (H1N1), and Aichi (H3N2). The OD450 value was measured by using ELLA. The OD values have been compared with the virus only and naive serum reads. The end dilution titers of sera for the maximum significant inhibition are as follows: rViet (H5N1), 20 480; Cal (H1N1), 20 480; Aichi (H3N2), 640. Sera from M2e-N2 group set (F) were mixed with 8000 $TCID_{50}$ Aichi (H3N2), Hong Kong (HK, H9N2), and Cal (H1N1). The end dilution titer of sera for the maximum significant inhibition is as follows. Aichi (H3N2): 32 000, Hong Kong (H9N2): 32 000, Cal (H1N1): 500. Data represent mean ± SEM. The statistical significance was analyzed with one-way ANOVA followed by Tukey's test ($n = 5$; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, $p > 0.05$).

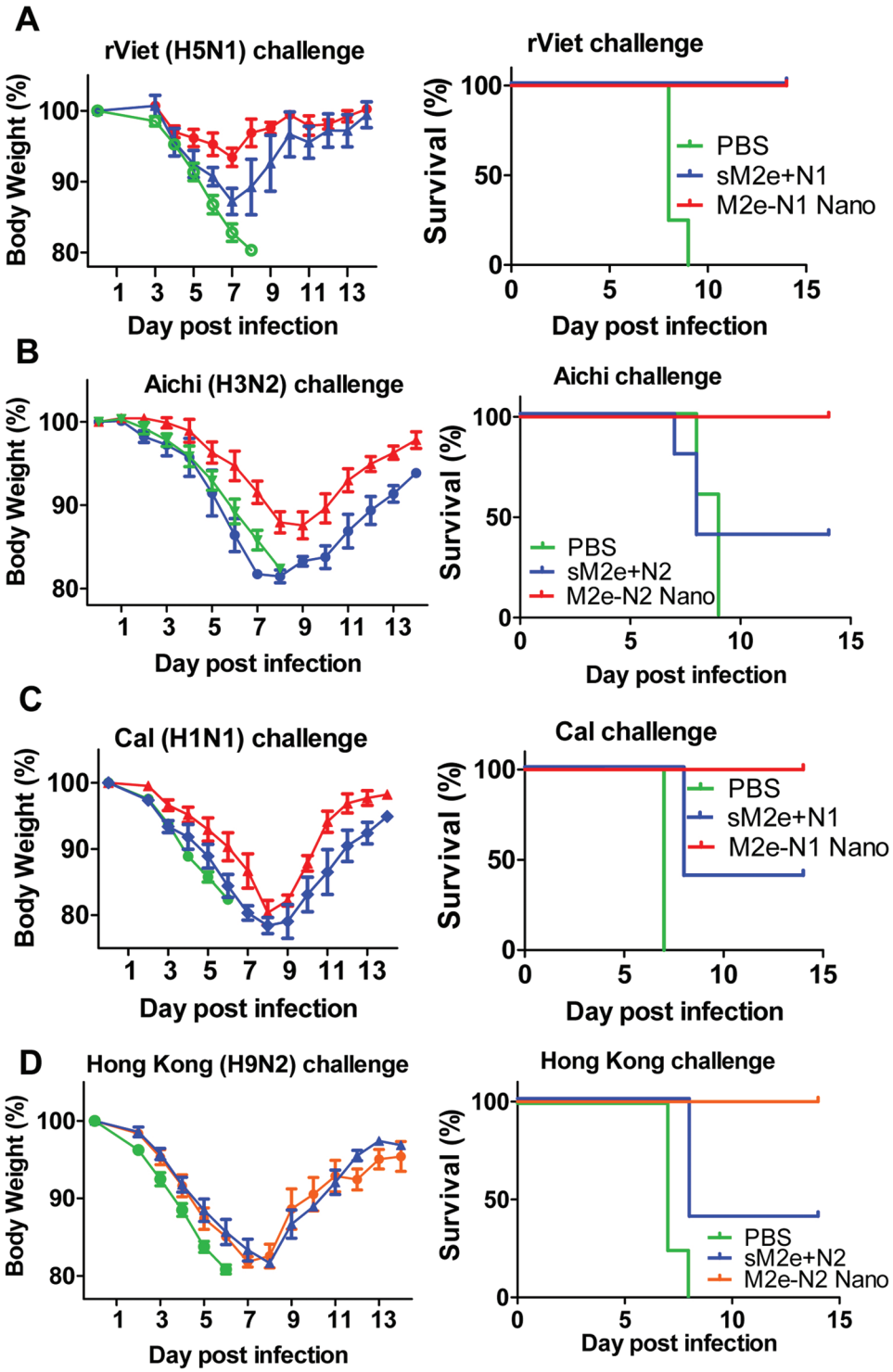


Figure 3. Immune protection against homologous and heterologous NA virus challenge. A) Body weight monitoring and survival rate of M2e-N1 group set against the challenge of 3 × LD_{50} rViet (H5N1). B) Body weight monitoring and survival rate of M2e-N2 group set versus 5 × LD_{50} Aichi (H3N2) challenge. C) Body weight monitoring and survival rate of M2e-N1 group set versus 5 × LD_{50} Cal (H1N1) challenge. D) Body weight monitoring and survival rate of M2e-N2 group set versus 5 × LD_{50} Hong Kong (H9N2) challenge. Data represent mean ± SEM. The difference of survival rate was analyzed by using the log-rank (Mantel–Cox) test ($n = 5$; $^{*}p < 0.05$; $^{**}p < 0.01$).

M2e-N1 nanoparticle and M2e-N2 nanoparticle immunized mice conferred significant protection against rH5N1 and H3N2 influenza virus infection as shown by 100% survivals and preventing severe body weight loss, respectively (Figure 6C, M2e-N1 Nano; Figure 6D, M2e-N2 Nano). Meanwhile, immune sera from sM2e+N1 and sM2e+N2 soluble protein-immunized

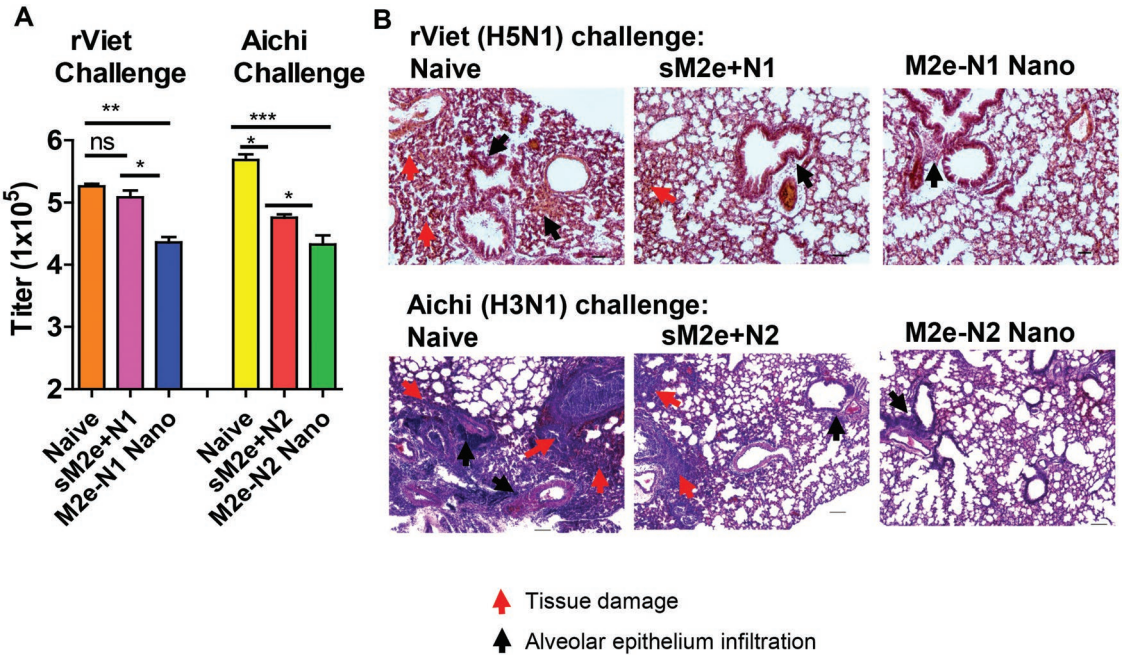


Figure 4. Lung viral titers and histology of M2e-N1 and M2e-N2 group sets. A) Viral titers of lungs from different immunization groups at 5 d after the infection. B) Histology examination by using H&E staining. Arrows show tissue damage and alveolar epithelium infiltration. Data represent mean ± SEM. The statistical significance was analyzed with one-way ANOVA followed by Tukey's test for comparison of groups. ($n = 5$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, $p > 0.05$.)

mice showed weaker protection than their corresponding layered protein nanoparticles, demonstrated by significantly more severe morbidity in the soluble protein mixture groups upon challenge (Figure 6C, sM2e+N1; Figure 6D, sM2e+N2). These results demonstrated that serum antibody responses contributed the heterosubtypic protection in the layered protein nanoparticle immunization.

2.5. Double-Layered M2e-NA Protein Nanoparticles Induced Durable Antibody Responses and Immune Protection

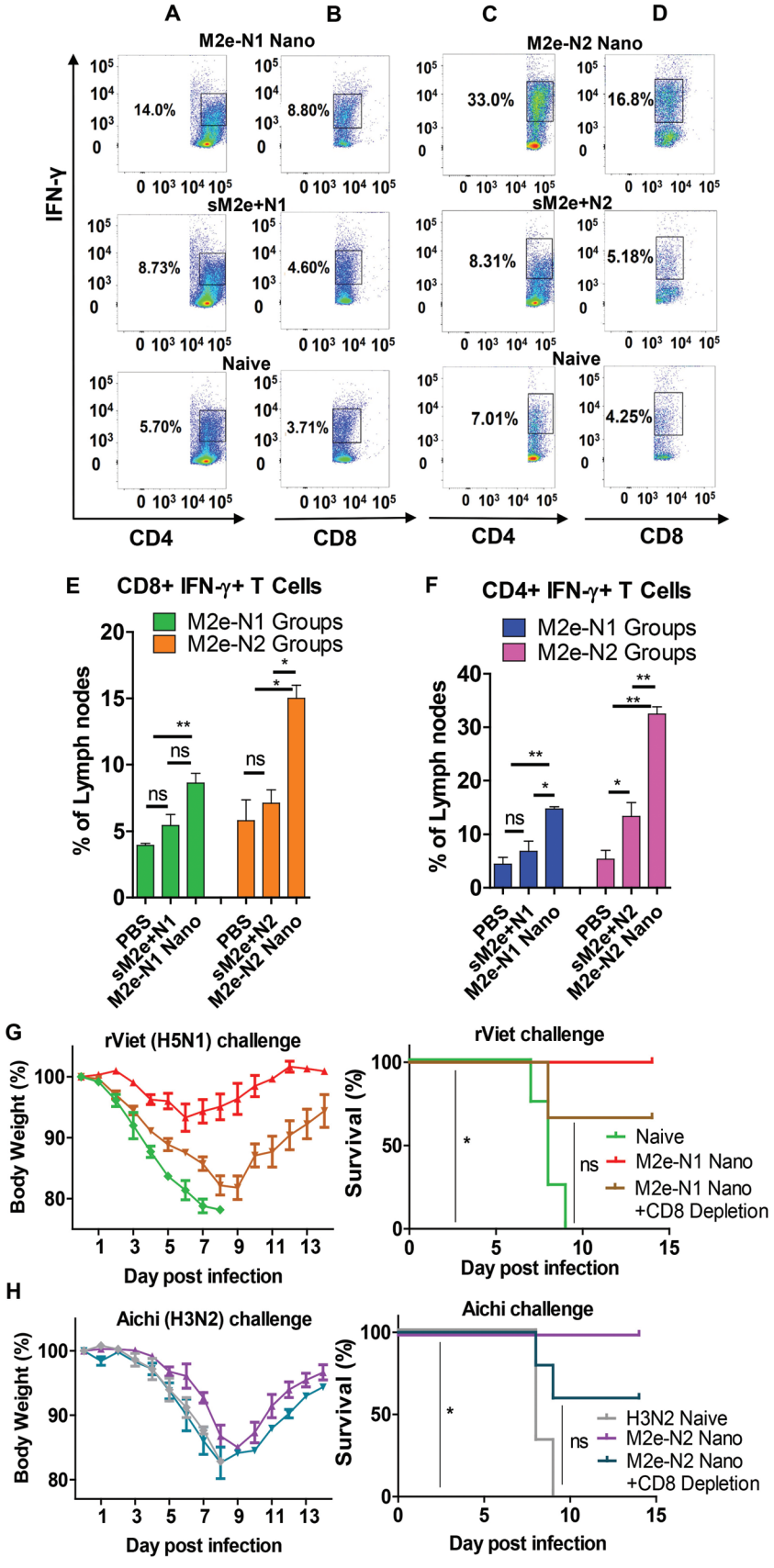
A long-lasting immune response is a necessary feature for an ideal vaccine. Here, we evaluated the longevity of the immunity induced by the double-layered protein nanoparticle formulations. We immunized mice with M2e-N1 nanoparticles, M2e-N2 nanoparticles, or soluble M2e and M2e-NA proteins twice with a 4 week interval. After 4 months, we collected the immune sera from the mice. Immunized mice in both double-layered M2e-NA protein nanoparticle groups maintained significantly higher levels of M2e and NA-specific antibodies than the soluble protein mixture groups (**Figure 7**A,B).

We then challenged the layered M2e-N1 protein nanoparticle-immunized mice with rViet (H5N1) and the layered M2e-N2 protein nanoparticle-immunized mice with Aichi (H3N2). The immune protection was unchanged against the viral challenges up to 4 months after the immunizations. The double-layered protein nanoparticle immunizations limited morbidity to a greater degree than the soluble protein immunizations (Figure 7C,D).

3. Discussion

Influenza is a leading cause of death by infection. Circulating viruses can rapidly accumulate mutations that enable influenza epidemics to occur worldwide. An occasional gene reassortment may cause influenza A pandemics. The outbreak of another influenza pandemic continues to be "not a question of if, but when."[22] The current seasonal influenza vaccine strategy is insufficient to prevent these outbreaks.[6,23] A universal influenza vaccine will negate the need for vaccinations each season and serve as a countermeasure against the emergence of novel pandemics by offering universal protection.[24] The realization of this ambitious goal needs a deep integration of recent advances in vaccinology, including structure-based immunogen designs, recombinant protein engineering, nanotechnology-based drug formulation, delivery, and controlled releases.[14]

Recombinant antigenic proteins as immunogens for a new generation of influenza vaccines have several advantages over traditional seasonal influenza vaccines.[14] Current seasonal influenza vaccines are typically produced in eggs.[25] This production method requires a long lead time and precludes egg-allergic patients from receiving this type of vaccines.[26] Besides, the egg supply could be highly limited in an influenza pandemic. In this study, we produced the M2e or M2e-NA fusion proteins in insect cells using the rBV-based protein-expressing system, removing the need for long incubation times and the possibility of egg-allergen contamination in the vaccine. Meanwhile, due to the small size of the M2e peptide, it was easy to construct M2e-NA fusion protein without disrupting the NA protein structure or NA activity. These intact M2e-NA fusion



proteins increased the M2e antigen load and enhanced the M2e immunogenicity. The retention of the polymeric state of antigenic proteins is a potent trigger of immune responses to not only linear epitopes but also conformational structures in the immunogens.

Multi-layered protein nanoparticles have the potential to integrate different antigenic proteins and adjuvants as vaccines.[11,14,20] We generated M2e nanoparticle cores by ethanol desolvation and then crosslinked M2e-NA onto the core particle surfaces as the coatings using DTSSP to produce double-layered protein nanoparticles. Several exceptional physical and biochemical features of these nanoparticles make them high immunogenic. The protein nanoparticles have very high antigen loads because of their almost pure protein composition and the lack of self-assembling structures or nanocarriers (such as polymers). Avoiding the use of self-assembling structures or nanocarriers also avoids off-target immune responses.[1] The disulfide bond in DTSSP that fixes the nanoparticles is reducible in the intracellular redox environment, allowing the protein nanoparticles to slowly disassemble and release free antigenic proteins after uptake by antigen-presenting cells (Figure S3, Supporting Information), creating a longer period of antigen processing and presentation, which consequently results in strong immune responses. The antigen depot effect of these nanoparticles in secondary lymphoid organs has been demonstrated previously.[11]

Figure 5. T cell responses in M2e-NA nanoparticle immunized mice. Characterization of T cell response by selected cell markers with FACS. After stimulation by N1 or N2 peptides, the homogenized lung cells were stained by antibodies against CD3, CD45, CD4, CD8, and intracellular cytokines IFN-γ. Lymphocytes were marked by selecting CD3, CD45+ gated cells. A) CD4+, IFN-γ+ T cells; B) CD8+, IFN-γ+ T cells. Cells were stimulated with N1 peptide pool. C) CD4+, IFN-γ + T cells; D) CD8+, IFN-γ+ T cells. Cells were stimulated with N2 peptide pool. Percentages of E) INF-γ-secreting CD8 T cells and F) CD4 T cells were acquired from flow cytometry data in (A)–(D). G) T cell depletion assay of M2e-N1 nanoparticle immunized mice versus 3 × LD_{50} H5N1. H) T cell depletion assay of M2e-N2 nanoparticle immunized mice versus 5 × LD_{50} H3N2. Data represent mean ± SEM. The statistical significance was analyzed with one-way ANOVA followed by Tukey's test for comparison of groups, and the survival rate was analyzed by using the log-rank test ($n = 5$; *$p < 0.05$; **$p < 0.01$; ns, $p > 0.05$).

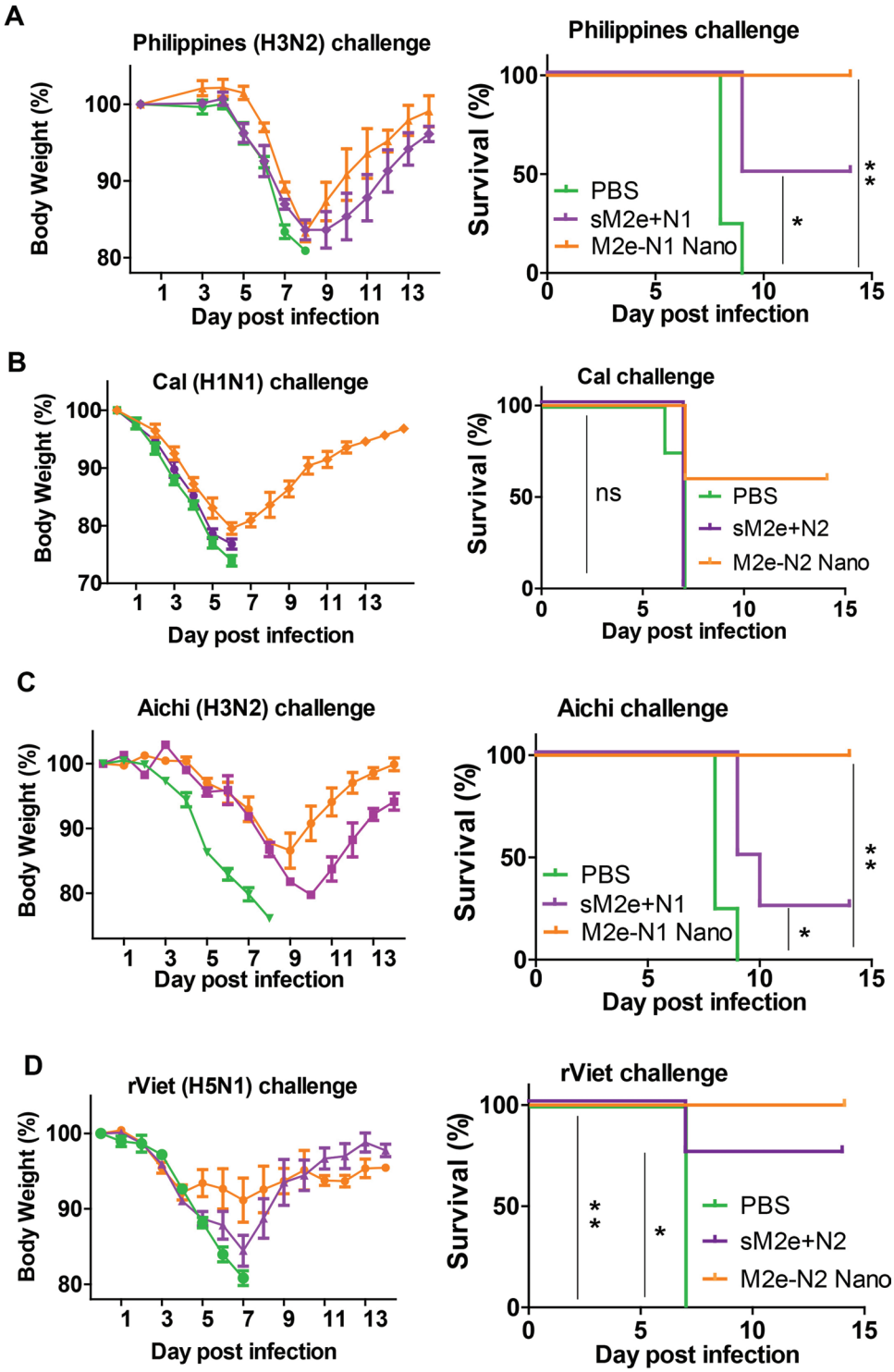


Figure 6. Immune protection against heterosubtypic NA virus challenge. A) M2e-N1 group set versus the challenge of 5 × LD_{50} Philippines (H3N2). B) M2e-N1group set versus the challenge of 5 × LD_{50} Cal (H1N1). C,D) Body weight changes of mice upon passive immune serum transfers. Immune sera (300 µL) from layered M2e-N1 protein nanoparticle or sM2e+N1 immunized mice were I.P. injected to mice 24 h before the challenge infection with 3 × LD50 Aichi (C). Immune sera (300 µL) from layered M2e-N2 protein nanoparticle or sM2e+N2 immunized mice were I.P. administered to mice 24 h before the 3 × LD50 rViet challenge infection (D). Body weight changes were monitored for 14 d post the challenge. Data represent mean ± SEM. The statistical significance was analyzed with one-way ANOVA followed by Tukey's test for comparison of groups, and the survival rate was analyzed by using the log-rank test ($n = 5$; $^{*}p < 0.05$; $^{**}p < 0.01$; ns, $p > 0.05$).

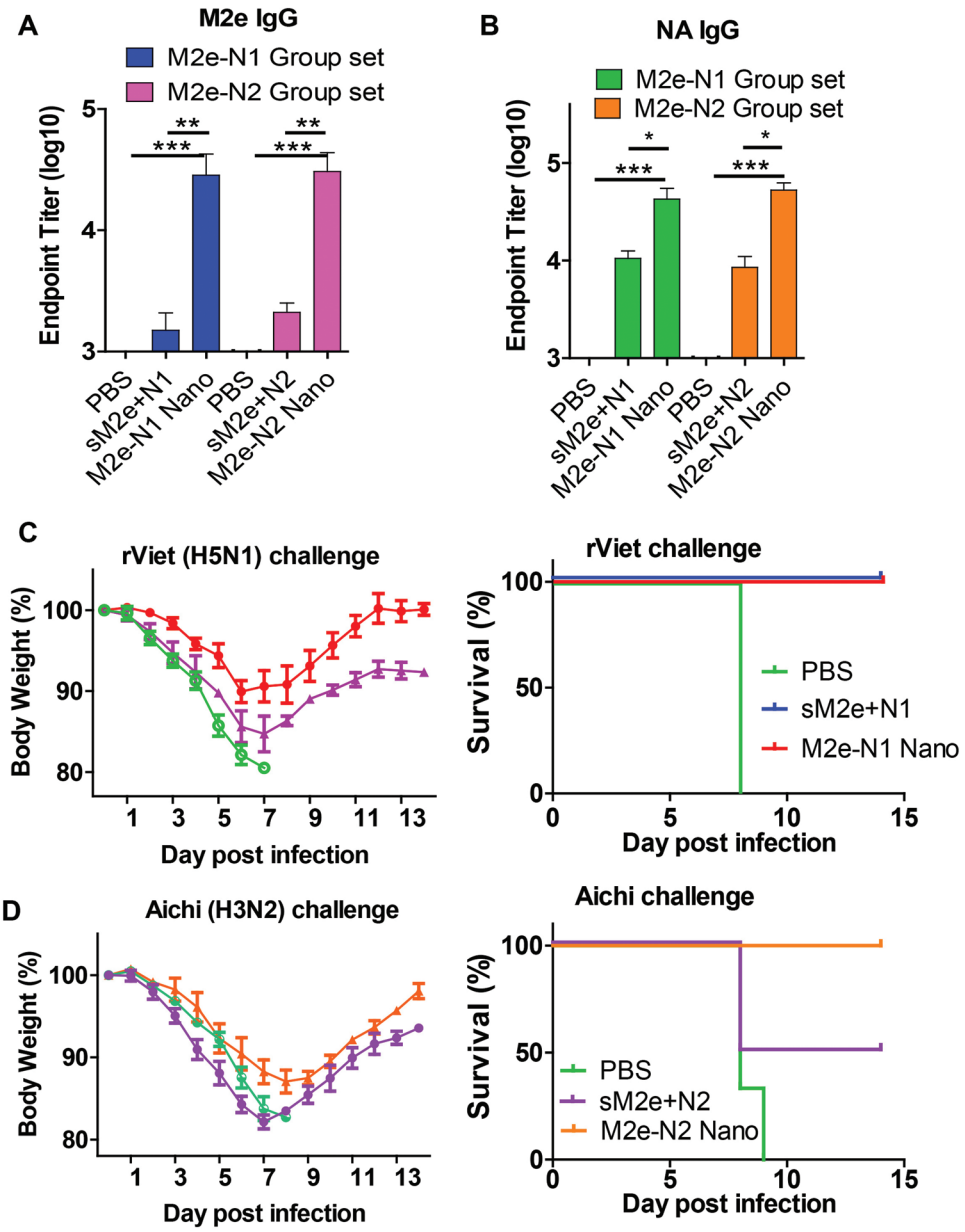


Figure 7. Long-term immune protection. A,B) Long-term antigen-specific antibody binding titers. M2e-specific (A) and NA-specific (B) antibody titers in immune sera from mice 4 months after boost immunizations were measured by ELISA. C,D) Long-term immune protection. M2e-N1 group set was challenged with 3 × LD_{50} rViet 4 months after the boost immunization (C). M2e-N2 group set was challenged with 3 × LD50 Aichi 4 months after the boost immunization (D). Data represent mean ± SEM. The statistical significance was analyzed with one-way ANOVA followed by Tukey's test for comparison of groups, and surviving curve was analyzed using the Log-rank test with GraphPad Prism ($n = 5$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, $p > 0.05$).

The outer layer of the double-layered protein nanoparticles is an ideal platform to display viral surface antigens to the immune system in a multivalent conformation.[10] The layered protein nanoparticle platform is very adaptable because it allows for the generation of nanoparticles with any combination of immunogens and protein adjuvants via changing the core or coating proteins. These protein nanoparticles can be further coated with other immunogenic proteins or protein adjuvants to create a layered nanostructure.[27] Even without an adjuvant, the double-layered M2e-NA protein nanoparticles significantly increased the anti-NA antibody responses compared with the soluble M2e-NA proteins.

The stability of nanoparticles is one key issue for vaccines development. Prior research results have shown that the desolvation-driven and DTSSP-fixed influenza hemagglutinin nanoparticles retained the intact structures (demonstrated by its hemagglutination activity retention) and immunogenicity for a storage period of 3 months at 25 °C.[19] In the current study, we examined nanoparticles after a storage period of 6 months at 4 °C. The stored nanoparticles showed similar

ζ-potential and size distribution (Figure S2D,E, Supporting Information), indicating that the NA-M2e nanoparticles are stable during long-term storage. Meanwhile, under the nonreducing environment (SDS-PAGE without β-mercaptoethanol), the nanoparticles stayed on the top of the gel (Figure S1B, Supporting Information), while nanoparticles only disassembled to corresponding M2e-NA and M2e proteins under the reducing environment (Figure 1F). We also observed an overtly extended antigen-presentation period by the layered protein nanoparticle immunization over soluble proteins, demonstrating the slow antigen release and antigenic protein availability for antigen-processing and presentation.[3,19]

In recent years, researchers have paid more attention to influenza NA immunity.[7,23] Current seasonal influenza vaccines do not induce strong anti-NA immune responses due to the immunodominance of HA. The increased attention to NA immunity is warranted by findings showing that anti-NA antibodies persist in adults and elderly people for years;[28] that anti-NA antibodies from the individuals naturally infected by the influenza virus showed broadly cross-reactive activity;[7] and that H1N1 NA immunized mice showed cross-protection to lethal challenges by reassortant H5N1 viruses.[29] These findings have indicated the importance of anti-NA antibody responses in a broadly protective influenza vaccine. From the phylogenic organization and crystal structures, NA can be divided into two groups: group 1 contains N1, N4, N5, and N8, while group 2 contains N2, N3, N6, N7, and N9.[30] NA from group 1 and group 2 display substantial sequence and structural differences near the active site, mainly because the group1 NA has a cavity on the "150-loop" (residues 147–152).[31] NA immune sera inhibited homologous and heterologous influenza NA activities and thus protected mice against these viruses within the same NA phylogenies group.[32–34] However, such NA immunity did not exhibit heterosubtypic protection.[35] In this study, we observed a similar result where M2e-N1 nanoparticles induced robust NA inhibition titers to homologous H5N1 and heterologous H1N1 NA, but a low inhibition titer to the heterosubtypic H3N2 NA. We saw a similar NAI pattern against homologous, heterologous, or heterosubtypic NA in the M2e-N2 nanoparticle immunization groups as well. These weaknesses could be overcome by further optimization of the protein nanoparticle designs, the use of adjuvants, or a cocktail combination of different nanoparticles in the vaccine formulation. We constructed M2e-NA fusion protein as the particle shell but not NA alone or the mixture of M2e and NA because one tetramerization sequence can stabilize both M2e and NA in the fusion protein. This particle design was foreseen to trigger both strong T cell responses to the core M2e and antibody responses to the coating NA/M2e, as we have found that double-layered nanoparticles elicited strong T cell responses to the core antigens and antibody responses to the coating antigens.[3,11] As seen in the present study, although all immunized mice in the M2e-N1 group set survived the $3 \times LD_{50}$ rViet (H5N1) challenge (Figure S4B, Supporting Information), the M2e-N1 nanoparticle group lost the least body weight (less than 10%) in all groups before the recovery, indicating a better immune protection (Figure S4A, Supporting Information). While groups of M2e-core + soluble M2e-N1 and soluble M2e + soluble M2e-N1 lost 10% to 15% of their body weights, the M2e-core only group lost the most (up to 20%) body weight prior to the recovery. These results indicated that the double-layered M2e-NA fusion protein nanoparticles induced better protective immunity when compared with M2e core only or M2e core with soluble M2e-NA fusion protein (Figure S4, Supporting Information).

Previous studies have indicated the importance of M2e-specific monoclonal antibody or M2e vaccine-derived immune sera in protecting mice against various types of influenza viruses.[36] We included the M2e antigen in our nanoparticle designs to compensate for the expected lower heterosubtypic NA immunity. The M2e-containing nanoparticles induced high antibody titers against M2e, as demonstrated previously.[11] Despite the low NAI against heterosubtypic influenza strains, we observed that immunized mice were protected against heterosubtypic virus challenge, indicating the synergistic role of M2e immunity in the protection induced by the double-layered M2e-NA protein nanoparticles.

NA has been known to induce both CD4 and CD8 T cell responses.[37] Upon influenza infection, both $CD8^+$ CTL and $CD4^+$ helper T cells differentiate and proliferate into effector cells with the increasing IFN-γ secretion.[38] We observed that double-layered M2e-NA protein nanoparticles increased the populations of effector T cells secreting IFN-γ and that the protection of M2e-NA nanoparticle-immunized mice was impaired after CD8 T cell depletion—demonstrating M2e and NA-specific CTL responses contribute to the protection observed in the study. In our previous studies, we have found protein nanoparticles with M2e inner cores triggered strong T cell immune responses contributing to the overall cross-protective immunity.[11] Thus, we further measured T cell responses to NA. We concluded that NA-specific T cell responses, in addition to M2e T cell responses, played an important role in the protein nanoparticle-induced cross-protection.

Adjuvants are critical to the efficacy of a vaccine. Adjuvants can program an immune response induced by an immunogen for speed, magnitude, breadth, longitude, and memory.[39] However, only one adjuvant, the aluminum salts, is approved for human use in the USA.[40] Many other adjuvants in preclinical or clinical stages are subjected to further studies because of their potentially harmful impact on humans. Particulate vaccines themselves have demonstrated adjuvant functions. The repetitive antigenic surfaces of nanoparticle vaccines mimic the pathogen structures to which the host immune system has been evolved to fight against. Speedy uptake of nanoparticles by phagocytes (macrophages and dendritic cells) facilitates antigen absorbance, processing, and presentation necessary to trigger a robust immune response.[41] Nanoparticles have also demonstrated to generate cellular stress, similar to other crystalline structures, leading to activation of the DAMP signaling pathway and generation of proinflammatory cytokines.[42] Our protein nanoparticles include features of the high levels of antigen loads, enhancement of DC uptake, elevated antigen depot effect, antigen processing, and antigen presentation in draining lymph nodes.[3,11,14,19] These complementary features are hypothesized to collectively contribute to the elevated immune responses seen.

We have previously found that protein nanoparticles of conserved influenza antigens preferentially induced non-neutralizing antibody responses.[3,11] The protection seen upon

the passive serum transfer indicated that the M2e-specific antibody responses might confer protection via antibody-dependent cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADPC), as previously observed.[33] Although we observed neutralizing NA-specific antibody responses in this study, NA-specific non-neutralizing antibodies may still be involved in the immune protection through antibody-dependent, Fc-mediated immune effector function, like the ADCC and ADPC activity of HA-stalk and M2e antibody-mediated protection.[8] These results and the CD8 T cell depletion results demonstrate that the double-layered protein nanoparticle format induced not just protective humoral immune responses but also protective cellular immune responses.

Current inactivated virus vaccines emphasize HA dominant immunity with strain specificity.[43] Layered M2e-NA protein nanoparticles can effectively induce immune responses to the relatively conserved NA. We have previously demonstrated the enhanced immunogenicity of double-layered protein nanoparticles containing HA-stalk antigens.[11] As a reasonable continuation, we will investigate if a combination of both HA-stalk antigen layered protein nanoparticles and NA layered protein nanoparticles will induce more complementary broadly protective responses without strain specificity and immunodominance.

Highly effective vaccines are expected to induce durable protective immunity. We found that the double-layered M2e-NA nanoparticles efficiently elicited long-lasting protective immune responses. The durable immunity could result from two important features that are integrated into the protein nanoparticles: the high load of antigens in the almost pure protein nanoparticles and the controlled release of antigenic proteins in the intracellular redox environment after uptake by antigen-presenting cells. These features grant the immune system a relatively long period of antigen retention, processing, and presentation, which is necessary for the differentiation of lymphocytes for higher affinity and long-term memory.[44]

4. Conclusion

In conclusion, the double-layered M2e-NA protein nanoparticles were highly immunogenic, inducing broadly reactive immune responses against both M2e and different NA antigens. The induced immunity conferred protection against viruses of different NA subtypes. Both antibody and T cell immune responses contributed to cross-protection. Our results show that double-layered M2e-NA protein nanoparticles have the potential to be developed into influenza universal vaccines, either alone or as a synergistic component in more complicated influenza universal vaccine formulations. The approach of combining stabilized antigenic proteins into layered protein nanoparticles could be a general vaccine strategy for different pathogens.

5. Experimental Section

Ethics Statement: This study was carried out by strictly following the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were approved by the Georgia State University Institutional Animal Care and Use Committee (IACUC) under protocol No. A19030.

Design, Expression, and Characterization of Recombinant Proteins and Fabrication of Protein Nanoparticles: For the construction of the tetrameric M2e protein-encoding gene (*m2e*), encoding sequences of a honeybee melittin signal peptide (*melittin*), a hexahistidine (His)-tag (*his-tag*), a tetrameric motif tetrabrachion (*tetra-*), and four tandem copies of different *M2e* were fused in frame and subcloned into the transferring vector pFastBac for rBV generation.[45] The full-length coding DNA and peptide sequences of the recombinant M2e are listed in Note S1 of the Supporting Information.

For the construction of the M2e-NA fusion protein-encoding genes (*m2e-n1* and *m2e-n2*), encoding sequences of neuraminidase 1 (*n1*) and 2 ectodomains (*n2*) (N1 from A/Vietnam/1203/2004 (Viet, H5N1, Genbank Accession EF541467, 36H to 449K); N2 from A/Aichi/2/1968 (Aichi, H3N2, Genbank Accession AB295606, 38K to 469I)) were fused to the above tetrameric M2e encoding gene in frame (M2e-N1 and M2e-N2 peptide sequences and their encoding genes, *m2e-n1* and *m2e-n2*, are listed in Notes S2 and S3 of the Supporting Information).[46] The resulting tetrameric M2e-NA encoding genes were cloned into pFastBac and used to generate rBVs expressing M2e-NA fusion proteins.

Ni-NTA resins (Thermo Fisher, Cat. No. 88221) were used for the purification of the above recombinant proteins from insect cells infected with resulting rBVs, as described previously.[3]

Double-layered M2e-N1 nanoparticles or M2e-N2 nanoparticles were fabricated and characterized, as described previously.[11] In brief, the M2e core particles were formed by slowly adding a four-fold volume of ethanol to a volume of M2e solution in phosphate buffered solution (PBS) while stirring. The coating layer of M2e-NA fusion proteins was crosslinked onto the M2e cores by using DTSSP (Cat. No. 21578, Thermo Scientific, Waltham, MA). After one and a half hour incubation for the crosslinking reaction, the nanoparticle-containing supernatant was centrifuged at 15 000 rpm for 30 min. The nanoparticle pellet was collected, the pellet was resuspended in PBS, and the nanoparticle suspension was stabilized by sonication at a 40% amplitude on an ice bath. The size and surface potential of the layered M2e-NA protein nanoparticles were measured by dynamic light scattering analysis with a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA). BS3 crosslinking followed by Western blots was performed to determine the polymeric state of the purified recombinant M2e and M2e-NA fusion proteins. A 10% SDS-PAGE followed with Coomassie blue staining was applied for the characterization of the M2e-NA nanoparticle compositions. The ratio of M2e-NA to M2e protein was measured using GelQuantNET software after Coomassie blue staining. To detect the M2e epitope in the M2e-NA nanoparticles, monoclonal antibody 14C2 (Thermo Fisher Scientific, Cat. No. MA1-082) was used in the Western blotting analysis.

Protein Nanoparticle TEM Observation: Protein nanoparticles were suspended in Millipore H_2O. Fine forceps were used to hold a formvar/carbon-coated TEM grid while nanoparticle solution (5 µL) was applied. A drop (5 µL) of 1% phosphotungstic acid (PTA, pH 7.4) was immediately applied to the nanoparticle solution on the TEM grid. The nanoparticle/PTA solution was incubated on the TEM grid for 1 min. After the incubation, the nanoparticle/PTA solution was wicked from the side of the TEM grid with blot paper. The grid was air dried at room temperature and stored for TEM imaging on a Jeol JEM-100CX II at 100 kV. Digital images were acquired with an Apogee Imaging Systems CCD camera system and software.

Immunization and Challenges in Mice: Six to eight week old BALB/c mice from Jackson Laboratory were used for the immunization and challenge experiments (n = 5). There were three mouse groups for each M2e-NA nanoparticle group set: (1) PBS; (2) M2e-NA layered nanoparticle (10 µg total protein with ≈6 µg of M2e-NA in outer layer and ≈4 µg of M2e in the core); (3) soluble M2e and soluble M2e-NA protein mixture (a dose of 10 µg total protein with 6 µg M2e-NA and 4 µg M2e); Mice received primary and boost immunizations at a 4 week interval by an intramuscular route. Immune sera were collected 21 d after the boost immunization.

For challenge experiments, $3 \times LD_{50}$ reassortant Viet (rViet, H5N1), $5 \times LD_{50}$ A/California/2009 (Cal, H1N1), or A/Philippines/1982 (Philippines, H3N2) in 30 µL PBS was intranasally applied to the M2e-N1 group set; while $5 \times LD_{50}$ of Aichi (H3N2), A/Hong Kong/1073/99 (Hong Kong, H9N2), Cal (H1N1) was applied to the M2e-N2 group set.[10,47] Mice were challenged 28 d after the boost immunization. Body weight changes and survival rates of the infected mice were monitored for 14 d after the challenge infections.

Humoral and Cellular Immune Response Assays: Enzyme-linked immunosorbent assay (ELISA) was performed for IgG antibody levels specific to NA1, NA2, or M2e proteins. The 96-well plates were coated with NA1 (Viet, Cat. No. 11676-VNAHC, Sinobiological), NA2 (Aichi NA, Cat. No. 40199-VNAHC, Sinobiological), or M2e peptide (Synpeptide, Seq: SLLTEVETPT; Lot No.: JT-61134), respectively.

The number of antigen-specific IFN-γ secreting cells after the boost immunization was evaluated using an ELISpot method as described previously.[10] Three weeks after the boost, splenocytes were collected as single-cell suspensions. Each well of 96-well filtration plates (Cat. No. MSIPS4W10, Fisher Scientific) was loaded with 5×10^5 splenocytes. A final concentration of 2 µg mL^{-1} of M2e, NA1(BEI Resources, NR-19258), or NA2 (BEI Resources, NR-2608) peptide pools was used for corresponding lymphocyte stimulation.

Flow Cytometry: Immunized mice were challenged with $5 \times LD_{50}$ of rViet (H5N1) or Aichi (H3N2) viruses. Five days after the infection, lungs from different groups were collected and homogenized into single-cell suspensions by using a Percoll gradient (44% and 67%) centrifugation method.[48] The resuspended lung cell cultures were stimulated with NA1 or NA2 peptide mixture (4 µg mL^{-1}) for 5 h in RPMI 1640 medium with Golgi stopper (2 µg mL^{-1}). Before the fixation, the stimulated lung cells were stained by surface marker CD3-APC, CD4-PE-Cy7, CD8-APC-Cy7, and CD45-FLTC (BD Biosciences). Then intracellular cytokine IFN-γ-BV711(BioLegend) staining was applied to the cells above after the fixation and permeabilization by using a Cytofix/Cytoperm kit (BD Biosciences). Stained cells were acquired on a BD LSRFortessa and analyzed using FlowJo software.[48]

Lung Viral Titration and Histological Analysis: Mice were sacrificed at day 5 post the challenge infection with $1 \times LD_{50}$ rViet or Aichi viruses. For the histological analysis, lung samples were treated as described previously with minor modifications.[44] Briefly, the dehydrated and fixed lung samples were embedded in paraffin for sectioning. Lung sample sections were then stained with hematoxylin and eosin. The pictures were taken using a Keyence BZ-X710 microscope. For lung virus titration, lung samples were homogenized by using Percoll gradient centrifugation as described previously.[48] The lung supernatants containing influenza viruses were serially ten-fold diluted. Then diluted lung supernatants (100 µL) were added to 96-well plates in which Madin-Darby canine kidney (MDCK) cells (100 µL, 1×10^5) per well were coated. Lung supernatants and the MDCK mixture were cultivated for 120 h at 37 °C, 5% CO_2. After the incubation, MDCK supernatants (50 µL) from each well were transferred to 96-well assay plates and mixed with chicken red blood cells (50 µL 0.5%). Lung viral titers were determined by measuring hemagglutination activity and calculated by the Reed–München method.[49]

Neuraminidase Activity Assay: NA activity was evaluated by using enzyme-linked lectin assays (ELLAs) with virus substrates as described previously.[35,50] Fetuin proteins (Sigma, Cat. No. F3385) were coated on a 96-well plate. For the ELLA of double-layered M2e-NA nanoparticles, 50 µL (1 mg mL^{-1}) nanoparticles were added to the fetuin-coated plates in the first well, then serially (2x) diluted. The whole plates were incubated at 37 °C for 2 h. For the NAI test, serially two-fold diluted, heat-inactivated sera (50 µL) were mixed with different influenza viruses (8000 $TCID_{50}$, 50 µL) at 37 °C for 30 min. Then the serum–virus mixtures were inoculated to the fetuin-coated plates and incubated at 37 °C for 2 h. An horseradish peroxidase (HRP)-conjugated lectin from Arachis hypogaea peanut (Sigma, Cat. No. L6135-1MG) was added and then incubated for 2 h. The color development and plates were finally read in ELISA reader at 450 nm as described previously.[34]

T Cell Depletion and Immune Serum Passive Transmission: Groups of mice ($n = 5$) were treated with anti-CD8 antibody (clone 2.43, Cat. No. BE0061; BioXCell) for CD8 cell depletion.[3] Briefly, the clone 2.43 antibody (200 µg) was I.P. injected into mice twice with a 24 h interval prior and post the challenge infection. For serum passive transmission, sera (300 µL) from M2e-N1 or M2e-N2 nanoparticle groups were injected to naïve mice by the I.P. route 24 h before infection. The survival and body weight changes of infected mice were monitored for 14 d after the infection.

Statistical Analysis: Data represent mean ± SEM ($n = 5$). Statistical significance was analyzed by using one-way ANOVA followed by Tukey's multiple comparison post-test. P values of less than 0.05 ($p < 0.05$) were considered to be significant.[34] A comparison of the survival rate was performed using the log-rank (Mantel–Cox) test. The analysis was performed with Graphpad Prism (GraphPad Sofware; San Diego, CA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the US National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases (NIAID) under grants R01AI101047, R01AI116835, and R01AI143844 to B.-Z.W. The electron microscopy study was performed in part at Georgia Institute of Technology for Electronics and Nanotechnology, a member of the National Nanotechnology Coordinated Infrastructure (NNCI), which is supported by the National Science Foundation (Grant ECCS-1542174). The content in this study is solely the responsibility of the authors and does not necessarily represent the official views of the funders.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

broadly reactive immunity, immune cross-protection, layered protein nanoparticles, universal influenza vaccines

Received: August 23, 2019
Revised: November 18, 2019
Published online:

[1] H. M. Yassine, J. C. Boyington, P. M. McTamney, C. J. Wei, M. Kanekiyo, W. P. Kong, J. R. Gallagher, L. Wang, Y. Zhang, M. G. Joyce, D. Lingwood, S. M. Moin, H. Andersen, Y. Okuno, S. S. Rao, A. K. Harris, P. D. Kwong, J. R. Mascola, G. J. Nabel, B. S. Graham, *Nat. Med.* **2015**, *21*, 1065.

[2] M. C. Kim, J. M. Song, E. O, Y. M. Kwon, Y. J. Lee, R. W. Compans, S. M. Kang, *Mol. Ther.* **2013**, *21*, 485.

[3] L. Deng, T. Z. Chang, Y. Wang, S. Li, S. Wang, S. Matsuyama, G. Yu, R. W. Compans, J. D. Li, M. R. Prausnitz, J. A. Champion, B. Z. Wang, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E7758.

[4] a) B. E. Johansson, T. M. Moran, C. A. Bona, S. W. Popple, E. D. Kilbourne, *J. Immunol.* **1987**, *139*, 2010; b) B. E. Johansson, T. M. Moran, E. D. Kilbourne, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 6869.

[5] K. B. Westgeest, C. A. Russell, X. Lin, M. I. Spronken, T. M. Bestebroer, J. Bahl, R. van Beek, E. Skepner, R. A. Halpin, J. C. de Jong, G. F. Rimmelzwaan, A. D. Osterhaus, D. J. Smith, D. E. Wentworth, R. A. Fouchier, M. de Graaf, *J. Virol.* **2014**, *88*, 2844.
[6] T. M. Doyle, A. M. Hashem, C. Li, G. Van Domselaar, L. Larocque, J. Wang, D. Smith, T. Cyr, A. Farnsworth, R. He, A. C. Hurt, E. G. Brown, X. Li, *Antiviral Res.* **2013**, *100*, 567.
[7] Y. Q. Chen, T. J. Wohlbold, N. Y. Zheng, M. Huang, Y. Huang, K. E. Neu, J. Lee, H. Wan, K. T. Rojas, E. Kirkpatrick, C. Henry, A. E. Palm, C. T. Stamper, L. Y. Lan, D. J. Topham, J. Treanor, J. Wrammert, R. Ahmed, M. C. Eichelberger, G. Georgiou, F. Krammer, P. C. Wilson, *Cell* **2018**, *173*, 417.
[8] H. Wan, H. Yang, D. A. Shore, R. J. Garten, L. Couzens, J. Gao, L. Jiang, P. J. Carney, J. Villanueva, J. Stevens, M. C. Eichelberger, *Nat. Commun.* **2015**, *6*, 6114.
[9] W. Liu, P. Zou, J. Ding, Y. Lu, Y. H. Chen, *Microbes Infect.* **2005**, *7*, 171.
[10] L. Wang, A. Hess, T. Z. Chang, Y. C. Wang, J. A. Champion, R. W. Compans, B. Z. Wang, *Nanomedicine* **2014**, *10*, 473.
[11] L. Deng, T. Mohan, T. Z. Chang, G. X. Gonzalez, Y. Wang, Y. M. Kwon, S. M. Kang, R. W. Compans, J. A. Champion, B. Z. Wang, *Nat. Commun.* **2018**, *9*, 359.
[12] E. van Doorn, H. Liu, T. Ben-Yedidia, S. Hassin, I. Visontai, S. Norley, H. W. Frijlink, E. Hak, *Medicine* **2017**, *96*, e6339.
[13] a) R. Pati, M. Shevtsov, A. Sonawane, *Front. Immunol.* **2018**, *9*, 2224; b) L. Powles, S. D. Xiang, C. Selomulya, M. Plebanski, *Vaccines* **2015**, *3*, 894.
[14] Y. Wang, L. Deng, S. M. Kang, B. Z. Wang, *Expert Rev. Vaccines* **2018**, *17*, 967.
[15] J. J. Moon, H. Suh, A. Bershteyn, M. T. Stephan, H. Liu, B. Huang, M. Sohail, S. Luo, S. H. Um, H. Khant, J. T. Goodwin, J. Ramos, W. Chiu, D. J. Irvine, *Nat. Mater.* **2011**, *10*, 243.
[16] K. S. Corbett, S. M. Moin, H. M. Yassine, A. Cagigi, M. Kanekiyo, S. Boyoglu-Barnum, S. I. Myers, Y. Tsybovsky, A. K. Wheatley, C. A. Schramm, R. A. Gillespie, W. Shi, L. Wang, Y. Zhang, S. F. Andrews, M. G. Joyce, M. C. Crank, D. C. Douek, A. B. McDermott, J. R. Mascola, B. S. Graham, J. C. Boyington, *mBio* **2019**, *10*, e02810.
[17] Z. Zhang, S. Tongchusak, Y. Mizukami, Y. J. Kang, T. Ioji, M. Touma, B. Reinhold, D. B. Keskin, E. L. Reinherz, T. Sasada, *Biomaterials* **2011**, *32*, 3666.
[18] P. Sahdev, L. J. Ochyl, J. J. Moon, *Pharm. Res.* **2014**, *31*, 2563.
[19] T. Z. Chang, L. Deng, B. Z. Wang, J. A. Champion, *PLoS One* **2018**, *13*, e0202300.
[20] L. Deng, B. Z. Wang, *ACS Infect. Dis.* **2018**, *4*, 1656.
[21] a) D. M. Brown, E. Roman, S. L. Swain, *Semin. Immunol.* **2004**, *16*, 171; b) K. J. Flynn, G. T. Belz, J. D. Altman, R. Ahmed, D. L. Woodland, P. C. Doherty, *Immunity* **1998**, *8*, 683.
[22] a) S. Chandra, *Emerging Infect. Dis.* **2013**, *19*, 616; b) P. J. Allen, *Pediatr. Nurs.* **2006**, *32*, 76.
[23] F. Krammer, R. A. M. Fouchier, M. C. Eichelberger, R. J. Webby, K. Shaw-Saliba, H. Wan, P. C. Wilson, R. W. Compans, I. Skountzou, A. S. Monto, *mBio* **2018**, *9*, e02332.
[24] H. Zhang, L. Wang, R. W. Compans, B. Z. Wang, *Viruses* **2014**, *6*, 1974.
[25] F. C. Zhu, H. Wang, H. H. Fang, J. G. Yang, X. J. Lin, X. F. Liang, X. F. Zhang, H. X. Pan, F. Y. Meng, Y. M. Hu, W. D. Liu, C. G. Li, W. Li, X. Zhang, J. M. Hu, W. B. Peng, B. P. Yang, P. Xi, H. Q. Wang, J. S. Zheng, *N. Engl. J. Med.* **2009**, *361*, 2414.
[26] J. R. Miller, H. A. Orgel, E. O. Meltzer, *J. Allergy Clin. Immunol.* **1983**, *71*, 568.
[27] D. Verma, N. Gulati, S. Kaul, S. Mukherjee, U. Nagaich, *J. Pharm.* **2018**, *2018*, 1.
[28] M. Rajendran, R. Nachbagauer, M. E. Ermler, P. Bunduc, F. Amanat, R. Izikson, M. Cox, P. Palese, M. Eichelberger, F. Krammer, *mBio* **2017**, *8*, e02281.
[29] M. R. Sandbulte, G. S. Jimenez, A. C. Boon, L. R. Smith, J. J. Treanor, R. J. Webby, *PLoS Med.* **2007**, *4*, e59.
[30] S. J. Gamblin, J. J. Skehel, *J. Biol. Chem.* **2010**, *285*, 28403.
[31] R. J. Russell, L. F. Haire, D. J. Stevens, P. J. Collins, Y. P. Lin, G. M. Blackburn, A. J. Hay, S. J. Gamblin, J. J. Skehel, *Nature* **2006**, *443*, 45.
[32] C. Y. Wu, Y. C. Yeh, J. T. Chan, Y. C. Yang, J. R. Yang, M. T. Liu, H. S. Wu, P. W. Hsiao, *PLoS One* **2012**, *7*, e42363.
[33] K. El Bakkouri, F. Descamps, M. De Filette, A. Smet, E. Festjens, A. Birkett, N. Van Rooijen, S. Verbeek, W. Fiers, X. Saelens, *J. Immunol.* **2011**, *186*, 1022.
[34] Y. Wang, Y. J. Jung, K. H. Kim, Y. Kwon, Y. J. Kim, Z. Zhang, H. S. Kang, B. Z. Wang, F. S. Quan, S. M. Kang, *Viruses* **2018**, *10*, E471.
[35] T. J. Wohlbold, R. Nachbagauer, H. Xu, G. S. Tan, A. Hirsh, K. A. Brokstad, R. J. Cox, P. Palese, F. Krammer, *mBio* **2015**, *6*, e02556.
[36] L. Deng, K. J. Cho, W. Fiers, X. Saelens, *Vaccines* **2015**, *3*, 105.
[37] E. Assarsson, H. H. Bui, J. Sidney, Q. Zhang, J. Glenn, C. Oseroff, I. N. Mbawuike, J. Alexander, M. J. Newman, H. Grey, A. Sette, *J. Virol.* **2008**, *82*, 12241.
[38] a) E. Roman, E. Miller, A. Harmsen, J. Wiley, U. H. Von Andrian, G. Huston, S. L. Swain, *J. Exp. Med.* **2002**, *196*, 957; b) M. M. Hufford, T. S. Kim, J. Sun, T. J. Braciale, *Curr. Top. Microbiol. Immunol.* **2015**, *386*, 423.
[39] S. Awate, L. A. Babiuk, G. Mutwiri, *Front. Immunol.* **2013**, *4*, 114.
[40] H. HogenEsch, *Vaccine* **2002**, *20* (Suppl 3), S34.
[41] M. Zaric, O. Lyubomska, O. Touzelet, C. Poux, S. Al-Zahrani, F. Fay, L. Wallace, D. Terhorst, B. Malissen, S. Henri, U. F. Power, C. J. Scott, R. F. Donnelly, A. Kissenpfennig, *ACS Nano* **2013**, *7*, 2042.
[42] V. Hornung, F. Bauernfeind, A. Halle, E. O. Samstad, H. Kono, K. L. Rock, K. A. Fitzgerald, E. Latz, *Nat. Immunol.* **2008**, *9*, 847.
[43] L. A. Grohskopf, E. Alyanak, K. R. Broder, E. B. Walter, A. M. Fry, D. B. Jernigan, *Mmwr Recomm. Rep.* **2019**, *68*, 1.
[44] Y. Wang, G. Huang, J. Wang, H. Molina, D. D. Chaplin, Y. X. Fu, *Eur. J. Immunol.* **2000**, *30*, 2226.
[45] J. Stetefeld, M. Jenny, T. Schulthess, R. Landwehr, J. Engel, R. A. Kammerer, *Nat. Struct. Biol.* **2000**, *7*, 772.
[46] P. M. Schmidt, R. M. Attwood, P. G. Mohr, S. A. Barrett, J. L. McKimm-Breschkin, *PLoS One* **2011**, *6*, e16284.
[47] J. M. Song, Y. C. Kim, A. S. Lipatov, M. Pearton, C. T. Davis, D. G. Yoo, K. M. Park, L. M. Chen, F. S. Quan, J. C. Birchall, R. O. Donis, M. R. Prausnitz, R. W. Compans, S. M. Kang, *Clin. Vaccine Immunol.* **2010**, *17*, 1381.
[48] K. H. Kim, Y. T. Lee, H. S. Hwang, Y. M. Kwon, M. C. Kim, E. J. Ko, J. S. Lee, Y. Lee, S. M. Kang, *J. Virol.* **2015**, *89*, 11692.
[49] L. J. R. H. Muench, *Am. J. Epidemiol.* **1938**, *27*, 493.
[50] T. M. Doyle, B. Jaentschke, G. Van Domselaar, A. M. Hashem, A. Farnsworth, N. E. Forbes, C. Li, J. Wang, R. He, E. G. Brown, X. Li, *J. Biol. Chem.* **2013**, *288*, 18283.