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Microneedle delivery of an M2e-TLR5 ligand fusion protein to skin confers broadly cross-protective influenza immunity

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Abstract

Influenza vaccines with broad cross-protection are urgently needed to prevent an emerging influenza pandemic. A fusion protein of the TLR5-agonist domains from flagellin and multiple repeats of the conserved extracellular domain of the influenza matrix protein 2 (M2e) was constructed, purified and evaluated as such a vaccine. A painless vaccination method suitable for possible self-administration using coated microneedle arrays was investigated for skin-targeted delivery of the fusion protein in a mouse model. The results demonstrate that microneedle immunization induced strong humoral as well as mucosal antibody responses and conferred complete protection against homo- and heterosubtypic lethal virus challenges. Protective efficacy with microneedles was found to be significantly better than that seen with conventional intramuscular injection, and comparable to that observed with intranasal immunization. Because of its advantages for administration, safety and storage, microneedle delivery of M2e-flagellin fusion protein is a promising approach for an easy-to-administer universal influenza vaccine.

Keywords

Cross-protection; Influenza M2e; Microneedle array; TLR5 ligand; Universal influenza vaccine

Introduction

Influenza is one of the most serious vaccine-preventable viral diseases of humans [1, 2]. The major limitations of current influenza vaccines include the strain specificity, resulting in the need to produce new vaccines every influenza season, uncertainty in the choice of the correct strains, a slow production process requiring embryonated eggs, as well as the

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inability to prevent an influenza pandemic which may emerge. Rapid and effective vaccination with a broadly cross-protective vaccine, or universal vaccine, is a promising approach for prevention of both seasonal and pandemic influenza [3, 4].

Skin is an attractive site for the administration of vaccines and immunomodulators because it contains various immune cells including keratinocytes and Langerhans cells (specialized dendritic cells) in the epidermis, dendritic and mast cells in the dermis, and T and B cells in the skin-draining lymph nodes [5]. Microneedle arrays (MNs) are designed to penetrate the stratum corneum, the outer layer of the skin, and deposit a vaccine or drug into the epidermis and dermis. Using this approach, vaccine is applied as coatings to the surfaces of metal MNs or encapsulated in a polymer [6]. In another approach, hollow microneedles have been used to inject influenza vaccines into the skin demonstrating efficacy and dose sparing [7, 8]. Furthermore, this immunization method generated an antigen-specific antibody response that was superior to those induced by subcutaneous (SC) or IM routes [9-12]. In addition to enhanced immunogenicity, MN administration has been shown to be painless, simple to administer, and well accepted by patients and healthcare providers [13, 14].

The extracellular domain of the membrane-bound matrix protein 2 (M2e) in human influenza A viruses is completely conserved in its N-terminal 9 amino acids (aas), and has minor changes in the membrane-proximal region [15]. However, because of its low incorporation level and relatively small size, M2 is not effectively sensed by host immune cells during virus infection or conventional vaccination [16, 17]. Nevertheless, some M2e-based vaccine candidates protected immunized mice from low-dose lethal virus challenge [16, 18-22]. Improved protection was also observed when an M2-based virus-like particle (VLP) antigen was used as a supplement to inactivated viral vaccines [23]. Thus, M2e is considered to be a promising antigen for the development of broadly protective influenza vaccines.

Bacterial flagellins are the natural ligands of Toll-like receptor (TLR) 5 and can be used as adjuvants [21, 24, 25]. Previously we have found that a membrane-anchored form of the *Salmonella typhimurium* phase I flagellin (FliC) can be co-incorporated into influenza VLPs as an adjuvant molecule [26-28]. The central variable region of FliC is unnecessary for its TLR5 binding activity, and has been found to be hyperimmunogenic because of the self-adjuvant property of FliC [24]. We previously found that a variable region-deleted FliC in VLPs enhanced mucosal antibody responses [26, 28]. In the present study, we designed a recombinant fusion protein comprised of FliC with a repetitive M2e replacement of the central variable region. Due to the self-adjuvanting property of this fusion protein, we hypothesized that this replacement would improve M2e immunogenicity. By using coated MN arrays to deliver the M2e fusion protein to the skin and comparing this approach to conventional intramuscular and intranasal routes, we assessed whether this new vaccination approach induced broadly protective immunity in mice, as a proof-of-concept for its potential use as a simple-to-administer universal influenza vaccine for further development.

Material and Methods

Cell lines and viruses

Spodoptera frugiperda sf9 insect cells (ATCC: CRL-1711), Madin-Darby canine kidney (MDCK, ATCC: PTA-6500) and RAW264.7 (ATCC: TIB-71) cells were maintained as described previously [27]. Mouse-adapted influenza A/PR/8/34 (H1N1) and A/Philippines/2/82 (H3N2) viruses were prepared as described previously [29]. The LD₅₀ (lethal dose inducing 50% mortality) of these strains was determined by infection of mice with serial viral dilutions and calculated by the method of Reed and Muench [30].

Generation of constructs expressing tandem 4 repeats of M2e (4.M2e) and a 4.M2e-flagellin fusion protein (4.M2e-tFliC)

The DNA sequence encoding four individual repeats of a human viral consensus M2e (SLLTEVETPIRNEWGSRSDSSDP) and flexible linker sequences were produced and cloned into the expression vector pET-22b (Novagen, EMD Biosciences, Madison, WI) under the T7 promoter and lac operator with a 6-histidine tag sequence in frame to facilitate the purification of the recombinant 4.M2e. To generate a gene encoding a fusion protein in which the variable region of FliC is replaced by 4.M2e, the DNA fragment encoding the variable region (aa 177-401 in FliC) in *S. typhimurium* FliC gene was replaced by the 4.M2e coding sequence described above [31]. The resulting sequence was cloned into pET-22b with a 6-histidine tag sequence in frame as described above for the 4.M2e construct. The integrity of the constructs was confirmed by DNA sequencing analysis.

Protein purification

Histidine-tagged recombinant 4.M2e and 4.M2e-tFliC were purified from an *E. coli* protein expression system as described previously [32]. Recombinant FliC and tFliC were purified for comparison. Purified proteins migrated as one band by Coomassie blue staining and Western blotting analysis, and were dialyzed against phosphate buffered saline (PBS) and stored at -80°C .

TLR-5-specific bioactivity assay

The TLR5-agonist activity of the purified 4.M2e-tFliC was evaluated as described previously [27], and compared to soluble recombinant flagellin and 4.M2e. After a 24 h treatment, levels of TNF- α production in TLR5-positive cell cultures stimulated by the recombinant proteins were determined by ELISA using a TNF- α assay kit (eBioscience, San Diego, CA).

Fabrication of microneedle arrays

A linear array containing five microneedles at an intramicroneedle spacing of 1.5 mm was fabricated from 75 μm -thick stainless steel (304) sheets using an infrared laser (Resonetics Maestro, Nashua, NH, USA) as an etching tool [33, 34]. After electropolishing, the thickness of the microneedles reduced to 50 μm , and each microneedle in the array measured 700 μm in length and 160 μm in width at the base, tapering to a sharp tip.

Coating MNs with 4.M2e-tFliC

To develop a uniform coating of the recombinant 4.M2-tFliC on MNs, a microprecision dip-coating process was used as described previously [34, 35]. The coating solution was composed of excipients including 1% (w/v) carboxymethylcellulose sodium salt (low viscosity, USP grade, CarboMer, San Diego, CA, USA), 0.5% (w/v) Lutrol F-68 NF (BASF, Mt. Olive, NJ, USA), and recombinant 4.M2-tFliC (4 mg/ml). The carboxymethylcellulose and Lutrol F-68 NF are not believed to have direct effects on immunogenicity, as shown previously [36]. MNs were repeatedly dipped into the coating solution to build up the desired coating level on microneedle surfaces.

To determine the mass of antigen coated on MNs, individual rows of coated MNs were thoroughly vortexed in 160 μl DI water to fully dissolve the antigen [34]. The antigen content in the resulting solution was determined using a micro bicinchoninic acid (BCA) assay kit according to the manufacturer's instructions (Pierce BCA protein assay kit, Thermo Fisher Scientific, IL, USA).

Immunization and challenge

A uniform coating of the 4.M2e-tFliC protein was formed on MNs without contaminating the base. An amount of $1.4 \pm 0.1 \mu\text{g}$ of antigen was coated on a single row of five MNs assessed as described above. Five arrays were used to deliver approximately $7 \mu\text{g}$ of 4.M2e-tFliC per mouse. Because the $700 \mu\text{m}$ -long microneedles were longer than the thickness of mouse skin, the antigen coating dissolved off the microneedles all along the microneedle insertion track, which delivered antigen to the epidermis, dermis and subcutaneous space. Mice were immunized three times at 4-week intervals by MNs. For MN immunization, skin on the back of mice was first treated with a depilatory (Nair, Church & Dwight Company, Princeton, NJ, USA) to remove hair, and then microneedle devices were inserted into the skin and held in place for approximately three minutes to achieve delivery of the coated 4.M2e-tFliC. Previous studies have shown that most of the coating dissolves off the microneedles within 3 min [33, 36]. Mouse groups were also simultaneously immunized with $10 \mu\text{g}$ of recombinant 4.M2e or 4.M2e-tFliC by intramuscular (IM) injection using a conventional 28-gauge needle or by the intranasal (IN) route (nasal drops) for comparison.

Three months after the last immunization, mouse groups were challenged with mouse-adapted A/PR8 (H1N1) or A/Philippines (H3N2) viruses to confirm the protection level. For this response, mice were lightly anesthetized by inhalation of isoflurane, and $10 \times \text{LD}_{50}$ of virus in $30 \mu\text{l}$ PBS was administered into the mouse nostrils. Mouse body weight and survival were monitored daily for 15 days.

Determination of antibody levels and lung virus loads

M2e-specific antibody levels in immune sera were measured by ELISA using chemically-synthesized M2e peptide as capture antigen. To determine the antibody levels recognizing native M2, the M2-expressing MDCK cells were used for binding immune sera by cell surface ELISA as described previously [31, 37]. To measure the lung virus titers, mouse lungs were collected and pooled in each group 4 days post-challenge. Lung virus titers were determined by a MDCK cell-based plaque assay as described previously [28, 29].

Statistical analysis

An unpaired student *t*-test was performed to compare the difference between two groups in Figures 3 and 4. A two-way ANOVA with Bonferroni's post test was performed to compare the body weight change among groups post-challenge in Figures 5 and 6. Data depict mean $\pm$ standard deviation (SD). The analysis was done by using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). P values of less than 0.05 ($P < 0.05$) were considered to be statistically significant. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P > 0.05$ (n.s.).

Results

Construction, purification and characterization of recombinant 4.M2e-tFliC and MN array coating

Constructs (Fig.1) expressing recombinant 4.M2e and 4.M2e-tFliC fusion protein were generated as described in Materials and Methods. Recombinant proteins were purified as previously described, and the full length and variable region-truncated flagellin proteins were also purified for comparison [32]. The purified proteins showed a single main band after SDS-PAGE followed by Coomassie blue staining (Figure 2A). Both FliC and the variable region-truncated form (tFliC) were detected by anti-flagellin antibodies (lane 1 and 2 in Figure 2B) but not anti-M2e antibodies (lane 1 and 2 in Figure 2C). In contrast, recombinant 4.M2e could be probed by anti-M2e antibodies (lane 3 in Figure 2C) but not

anti-flagellin antibodies (lane 3 in Figure 2B). Recombinant 4.M2e-tFliC fusion protein was recognized by both anti-flagellin (lane 4 in Figure 2B) and anti-M2e (lane 4 in Figure 2C) antibodies in Western blotting analysis, confirming its structure as a fusion protein.

We determined if 4.M2e-tFliC fusion protein retains the innate signaling activity of flagellin as the natural ligand of TLR5. As shown in Figure 2D, the 4.M2e-tFliC stimulated TLR 5 (+) mouse macrophage cell cultures to produce TNF- α , with the highest TNF- α production at a stimulating concentration of 100 ng/ml, which is comparable to the bioactivity of recombinant soluble FliC. In contrast, recombinant 4.M2e did not show any TLR 5 agonist activity. These results demonstrate that the resulting fusion protein retained the activity of flagellin as a ligand of TLR 5. As shown in Figure 2E, 4.M2e-tFliC could be selectively coated as uniform coatings on the surface of MN arrays without contaminating the handling part of the device.

Skin-targeted delivery using MNs elicited systemic as well as mucosal antibody responses

To determine if MN delivery of 4.M2e-tFliC could be developed into an easily administered universal influenza vaccine, we compared the M2e-specific antibody titers induced by the MN route to IM or IN immunization. As shown in Figure 3A, MN immunization elicited comparable serum IgG endpoint titers to the IM route (IM route showed a higher titer but the difference was not significant, $P>0.05$) and induced significant higher IgG titers than IN immunization ($P<0.05$). We also compared the humoral IgG isotype profiles of the above immunized mice. As shown in Figure 3B, conventional IM injection of 4.M2e-tFliC induced IgG1-dominant humoral antibody responses (IgG1/IgG2a around 2.0, $P<0.05$), while IN immunization induced a comparatively balanced Th1/Th2 response (IgG1/IgG2a around 0.91; $P>0.05$, n.s.). In contrast, MN delivery of 4.M2e-tFliC to skin induced an IgG2a-dominant antibody response (IgG1/IgG2a is 0.5; $P<0.05$). Recombinant 4.M2e induced extremely low IgG titers by either the IM or IN immunization route. Immune sera from different groups recognizing the native M2 protein expressed on MDCK cells were measured by cell surface ELISA (Figure 3C). The serum IgG showed a similar binding pattern to M2e in ELISA compared to its binding to native M2 by cell surface ELISA, demonstrating internal consistency of the two assays. Our previous studies showed that IN immunization with M2e VLPs induced better protection than IM immunization, although the IM route induced much higher serum IgG end-point titers [31], demonstrating that mucosal antibody responses are more important correlates for M2e-induced immune protection.

Because of the specialized properties of the skin as an immune organ and the TLR5 ligand activity in the fusion protein, we assessed whether MN delivery of 4.M2e-tFliC to skin induced enhanced mucosal responses compared to conventional IM or IN immunization. As shown in Figure 4, MN immunization induced a moderate mucosal antibody response compared to that found by IM or IN immunization in mice. Both IgA and IgG end-point titers in lung lavages from the MN immunized mice were 5-fold higher than those from mice in an IM group ($P<0.05$). As expected, IN immunization induced the highest mucosal antibody levels: lung lavage IgA and IgG titers were respectively 2 ($P<0.05$) and 1.7-fold ($P<0.05$) higher compared to those in the MN group, indicating that mucosal immunization is the best route for inducing mucosal antibody responses although IN immunization induced much lower humoral antibody response as shown in Figure 3.

Skin-targeted delivery of 4.M2e-tFliC fusion protein using MNs induced complete protection from lethal viral challenge infection

To test if the above immune responses protect mice from lethal viral infection, immunized mice were challenged with mouse-adapted A/Philippines virus (H3N2, $10\times$ LD₅₀), in which the M2e shares the same sequence as the human viral consensus M2e in our construct. As

shown in Figure 5A, mice in both MN and IN groups immunized with 4.M2e-tFliC completely survived the viral challenge. In comparison, 83% (5 of 6 mice in the group) of the conventional IM immunized mice survived the viral challenge, although higher serum IgG levels had been induced. Although both MN and IN immunizations also elicited full protection upon Philippines virus challenge, mice lost less body weight in the IN group (12%) compared to the MN group (18%, Figure 5B) ($P=0.0276$), revealing the effectiveness of IN immunization in providing protection against influenza infection in mice.

Although M2e is highly conserved, some sequence variation occurs among influenza A viruses. To test if MN delivery of 4.M2e-tFliC induced immune protection against viruses with an aa variation in M2e, immunized mice were also challenged with mouse-adapted A/PR8 virus, which differs from the M2e sequence of the construct by one aa. As shown in Figure 5C, mice in both MN and IN groups were completely protected from A/PR8 challenge infection. In contrast, only partial protection (4 of 6 mice survived) was seen in IM immunized mice. However, 4.M2e-tFliC induced weaker protection to A/PR8 virus challenge, as shown by the increased body weight loss in both MN and IN immunized mice when compared to A/Philippines viral challenge infection (for MN, $P=0.0114$; for IN, $P=0.0062$; Figure 5D). These results demonstrate that MN immunization induced better protection in mice compared to conventional IM injection.

Post-challenge lung virus loads of immunized mice

For an effective influenza vaccine, the best protection is reflected by its ability to prevent virus infection and limit the virus titers in lungs post-challenge. Our results demonstrated that mice in 4.M2e immunized groups showed very high virus loads in lungs on day 4 post-challenge, more than 5×10^6 pfu/lung after either IM or by IN immunization, indicating the low immunogenicity of this antigen (Figure 6). Immunization with the fusion protein 4.M2etFliC greatly increased immune protection, as shown by the lower lung virus loads in MN, IM or IN immunized mice, consistent with the increased protective efficacy and mucosal antibody levels observed above. MN delivery showed greater effectiveness in reducing virus titers from immunized mouse lungs post-challenge when compared to that of IM immunized mice with 6.7×10^4 pfu/lung versus 1.0×10^5 pfu/lung after Philippines virus challenge, and 1.2×10^4 pfu/lung versus 2.1×10^4 pfu/lung after PR8 virus challenge, respectively. Mice immunized by the IN route showed the lowest lung virus titers after either A/Philippines or A/PR8 virus challenge, demonstrating that the best protective efficacy occurred by IN immunization.

Discussion

Since non-human influenza viruses may acquire the capacity for transmission in humans, emergence of new influenza pandemics is an important concern. With the frequent infection by highly pathogenic avian influenza A (HPAI) H5N1 in humans in recent years, and the recent human infection by a novel avian influenza virus (H7N9) in China [38, 39], this concern has become more urgent. The motivation for the present study is that a simple-to-administer universal influenza vaccine would greatly reduce the morbidity and mortality of a newly emerged influenza pandemic when general resources such as vaccine production, storage, transportation and healthcare service facilities are limited. Since conserved epitopes generally have low immunogenicity, we integrated multiple approaches in this study to increase M2e immunogenicity including employing antigen repeats to increase epitope density, fusing an immune stimulator (TLR5-recognizing domains from flagellin) to initiate appropriate innate signaling, and using MN delivery of antigen to skin to enhance antigen-specific immune responses [10, 11, 40]. We demonstrated that combining these approaches provides a potent strategy to control possible emerging influenza pandemics.

As demonstrated previously, M2e-specific antibodies are the main players in M2e vaccine-induced immune protection [37, 41]. Overall, high IgG titers were observed by all three immunization routes in this study, indicating the effectiveness of fusing the TLR5 ligand to M2e for increasing antibody responses. The IgG isotype profiles may reflect which arm of CD4⁺ T helper (Th) cells is activated in the early stage of an immunization or infection, and can suggest which mechanism of antibody-mediated effector functions may be employed, such as the Th1-associated complement fixation, antibody dependent cellular cytotoxicity (ADCC), or Th2-associated pathogen neutralization [27, 42]. We observed higher humoral antibody titers by IM immunization with 4.M2e-tFliC which resulted in a high IgG1/IgG2a ratio in mice, indicating a Th2-associated antibody response. In contrast, IN immunization induced a balanced Th1/Th2 response, while MN delivery of 4.M2e-tFliC to skin induced a Th1-biased immune response with an IgG2a-dominant IgG subtype profile. M2e-specific antibody is not neutralizing [43]. It is believed that IgG2a allows for more efficient complement activation, promoting ADCC as well as opsonization compared to IgG1 [42]. The M2e-specific IgG2a isotype has been recognized as the main player in M2e induced antibody protection [44]. This may explain why the IgG2a-dominant IgG isotype profile associated with MN delivery of 4.M2e-tFliC to the skin conferred better protection against lethal challenge infections than conventional IM immunization and was comparable to IN immunization, which elicited the highest mucosal antibody responses.

The potency of IN immunization has been recognized for inducing protective immunity against respiratory infections such as influenza [31, 45]. Although administration of 4.M2e-tFliC as intranasal drops in this study showed better effectiveness in reducing disease symptoms in immunized mice upon lethal viral challenge, a concern is the potential toxicity of nasal adjuvants (such as flagellin in this study) to the central nervous system (CNS) [46]. MN delivery may be a simpler approach for worldwide use, in particular when an influenza pandemic is emerging. This is because MNs contain vaccine in a dry state without need for reconstitution, and have demonstrated increased thermal stability [47, 48], MNs have a small package size and thereby can be more easily stockpiled and rapidly distributed; and MNs are simple to administer, thereby enabling rapid delivery by minimally trained healthcare personnel or possible self-administration by patients themselves [6, 13, 49, 50].

In the present study, four repetitive copies of the human influenza viral consensus M2e sequence were used to replace the variable central region of flagellin in the 4.M2e-tFliC fusion protein. The advantage of this replacement includes a decreased immune response against flagellin and increased mucosal adjuvant efficacy [51]. Previously, a membrane-anchored form of the fusion protein was incorporated into influenza VLPs and was found to elicit broad cross-protection by IN immunization [31]. It is known that dendritic cells (DCs) are the targets of flagellin in initiating the TLR5-associated innate signaling pathway [52]. MN delivery of 4.M2e-tFliC to the skin may efficiently utilize the innate-signaling function of flagellin because the skin contains large numbers of Langerhans cells (epithelia and mucosa-associated DCs) and dermal DCs. The activation of these APCs promotes antigen-presentation and cytokine production, which drive antigen-specific adaptive responses [11, 53].

Another benefit of MN delivery of vaccines to the skin is its potential dose sparing efficacy. In a number of studies, a much lower dose is needed for MN delivery to generate the same immune responses as delivery using higher vaccine doses via the IM or IN routes [40, 54]. Although we did not specifically investigate dose sparing effects, the present results indicate that a lower dose (7 μ g) of 4.M2e-tFliC administered to skin by MN delivery generated a better protection than by IM immunization and was comparable to IN immunization, both of which were given at a 10 μ g dose of 4.M2e-tFliC. Whether the dose may be reduced further using MN delivery to achieve comparable protective efficacy to the other routes is to be

determined. However, the known dose-sparing efficacy of MN delivery provides a great benefit for preventing an emerging influenza pandemic because the available vaccine production capacity can yield more vaccine doses.

In conclusion, protective efficacy with microneedle delivery of M2e-flagellin fusion to skin is significantly better than that seen with conventional intramuscular injection, and comparable to that observed with intranasal immunization. The immunity confers influenza cross-protection. With the advantage for administration, safety and storage, skin vaccination using microneedle-based delivery of M2e-flagellin fusion protein is a promising approach for an easy-to-administer universal influenza vaccine.

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M2e sequence: (M) SLLTEVETPIRNEWGC RC NDSSDP

Used sequence: SLLTEVETPIRNEWG S RS NDSSDP

4.M2e:

(AAA)SLLTEVETPIRNEWGSRSDSSDPAAAGTSAASLLTEVET
PIRNEWGSRSDSSDPAAALQAAASLLTEVETPIRNEWGSRSD
SSDPAAAACAAASLLTEVETPIRNEWGSRSDSSDPA(AAA)

Figure 1. Schematic diagrams of constructs.

In 4.M2e, four M2e repeats were bridged by short linker sequences to form the tandem repetitive 4.M2e. In the 4.M2e-tFliC fusion protein, three alanine residues were inserted as flexible linkers prior to and after the tandem 4.M2e sequence.

Figure 2. Purification and characterization of recombinant proteins.

4.M2e and 4.M2e-tFliC were purified from *E.coli* protein expression by affinity chromatography as described in Materials and Methods. A, Coomassie blue staining; B, Western blot probed with anti-flagellin polyclonal antibodies and developed with HRP color substrata DAB (3,3'-Diaminobenzidine); C, Western blot probed with anti-M2e monoclonal antibody (14C2). Recombinant flagellin (FliC) and the central variable region-truncated flagellin (tFliC) were purified for comparison. Lanes 1 to 4, FliC, tFliC, 4.M2e, and 4.M24-tFliC, respectively. D, TLR5 agonist bioactivity. The bioactivities of recombinant proteins were measured by comparison of their ability in stimulating TNF- α production using the mouse macrophage cell line RAW 264.7; E, A MN device containing a row of five MNs coated with 4.M2e-tFliC. The bright coatings were formed on the microneedle surfaces as described in Materials and Methods.

Figure 3. Serum IgG endpoint titers and M2-specific IgG-binding.

ELISA assay plates were coated with 100 μ l/well of M2e peptide (5 μ g/ml). Immune serum samples were diluted 2 $\times$ stepwise, and 100 μ l of diluted samples were applied to plates for antibody-binding. Bound antibodies were detected by binding HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2 antibody and color was developed with TMB substrate. The greatest dilution which presented an OD₄₅₀ 2 $\times$ higher than that of the negative control (naïve group) was designated as the endpoint titer (mean $\pm$ SD, n=6). Serum IgG binding to native M2 protein expressed on cell surfaces was determined using cell surface ELISA. MDCK cells were infected with PR8 at a multiplicity of infection (MOI) of 1. Cells were washed with PBS and fixed with 10% formalin 12 h post-infection. Samples diluted 80-fold were applied to determine antibody binding. Data depict the OD₄₅₀ (mean $\pm$ SD, n=3 replicates) of infected cells subtracting the background of uninfected cells. A, Serum IgG; B, IgG isotypes; C, Serum IgG recognizing M2.

Figure 4. Mucosal antibody endpoint titers.

Immunized mouse lungs were collected and lavaged with 1 ml PBS containing 0.05% Tween 20 per lung twice. IgG and IgA endpoint titers were determined as described above for serum IgG but the secondary antibody was HRP-conjugated goat anti-mouse IgA antibody for IgA endpoint measurement. A, IgA endpoint titer; B, IgG endpoint titer. (mean +SD, n=6).

Figure 5. Virus challenge.

Three months after the last immunization, mice were IN infected with $10 \times LD_{50}$ A/Philippines virus (A and B) or A/PR8 (C and D). Mouseadapted virus in $30 \mu\text{l}$ PBS was slowly applied to the nares of mice. Mouse survival and body weight changes were monitored for 15 days. A and C, Mouse survival; B, Body weight change. (n=6).

Figure 6. Lung viral loads on day 4 postchallenge.

Six mice in each group were infected IN with $10 \times LD_{50}$ of A/PR8 (H1N1) or A/Philippines (H3N2) viruses. Mouse lungs were collected on day 4 post-challenge. Each lung was ground and cleared in 1 ml of DMEM. Virus titers of lung extracts were titrated using a standard plaque assay with MDCK cells. Lung viral titer was expressed as pfu/lung.