Enhanced Influenza Virus-Like Particle Vaccines Containing the Extracellular Domain of Matrix Protein 2 and a Toll-Like Receptor Ligand

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The extracellular domain of matrix protein 2 (M2e) is conserved among influenza A viruses. The goal of this project is to develop enhanced influenza vaccines with broad protective efficacy using the M2e antigen. We designed a membrane-anchored fusion protein by replacing the hyperimmunogenic region of Salmonella enterica serovar Typhimurium flagellin (FliC) with four repeats of M2e (4.M2e-tFliC) and fusing it to a membrane anchor from influenza virus hemagglutinin (HA). The fusion protein was incorporated into influenza virus M1-based virus-like particles (VLPs). These VLPs retained Toll-like receptor 5 (TLR5) agonist activity comparable to that of soluble FliC. Mice immunized with the VLPs by either intramuscular or intranasal immunization showed high levels of systemic M2-specific antibody responses compared to the responses to soluble 4.M2e protein. High mucosal antibody titers were also induced in intranasally immunized mice. All intranasally immunized mice survived lethal challenges with live virus, while intramuscularly immunized mice showed only partial protection, revealing better protection by the intranasal route. These results indicate that a combination of M2e antigens and TLR ligand adjuvants in VLPs has potential for development of a broadly protective influenza A virus vaccine.

Influenza is one of the most important viral diseases in humans, with significant medical and economic burdens (23, 31). Vaccination is the most effective approach for prevention of influenza virus infection. However, the major limitations of the current influenza vaccines include the need to produce new vaccines every season and uncertainty in choice of the correct strains, as well as the fact that the vaccines are produced by a slow process requiring embryonated eggs. Because of these limitations, a broadly protective vaccine that is based on relatively conserved protein domains and egg-independent production would be a promising approach (6, 12, 14, 32).

Matrix protein 2 (M2) of influenza A viruses is a highly conserved transmembrane protein exhibiting pH-dependent proton transport activity (1). In human isolates of different subtypes, the extracellular domain (M2e) of M2 is completely conserved in its N-terminal 9 amino acids (aa) and has minor changes in the membrane-proximal region (25). However, because of its low copy number and small size compared to the hemagglutinin (HA) and neuraminidase spikes, M2e is poorly immunogenic (4, 42). Nevertheless, some M2e-based vaccine candidates protected immunized mice from low-dose lethal virus challenge (8, 10, 15, 16, 39, 42). Improved protection was also observed when an M2-based virus-like particle (VLP) antigen was used as a supplement to inactivated viral vaccines (36). Thus, M2e is considered a promising antigen for the development of a universal influenza vaccine.

The bacterial flagellins are the natural ligands of Toll-like receptor 5 (TLR5) (35) and can be used as adjuvant (16, 18). In most isolates of Salmonella, two genes encode flagellar antigens. fliC encodes the phase I flagellin FlIC, and fljB encodes the phase II flagellin FljB (43), and they are coordinately expressed by a phase-variation mechanism (33). Both FlIC and FljB share conserved N and C termini which form the flagellar filament backbone (22) and contain motifs recognized by TLR5. Previously, we have found that a membrane-anchored form of the Salmonella enterica serovar Typhimurium phase I flagellin (FlIC) can be coincorporated into influenza VLPs as an adjuvant molecule (40). The variable central region of FlIC has been found to be hyperimmunogenic and unnecessary for its TLR5 binding activity (35). In the present study, we designed a membrane-anchored fusion protein comprised of FlIC with a repetitive M2e replacement of its central variable region and incorporated this into influenza virus M1-based VLPs. We further determined whether these VLPs induce broadly protective immunity in a mouse model.

MATERIALS AND METHODS

Ethics statement. Mice were sterile housed and treated according to Emory University guidelines, and all animal studies were approved by the Emory University Institutional Animal Care and Use Committee.

Cell lines and viruses. Spodoptera frugiperda Sf9, Madin–Darby canine kidney (MDCK; ATCC PTA-6500), and RAW264.7 (ATCC TIB-71) cells were maintained as described previously (40). Mouse-adapted influenza A/PR/8/34 (A/PR8; H1N1) and A/Philippines/2/82 (A/Philippines; H3N2) viruses were prepared as described previously (28). The lethal dose inducing 50% mortality (LD50) of these strains was determined by infection of mice with serial virus dilutions and calculated by the method of Reed and Muench (29).

Construction of repetitive M2e (4.M2e) and a membrane-anchored 4.M2e-flagellin fusion protein (4.M2e-tFlIC). Four DNA fragments encoding individual repeats of a consensus M2e sequence (SLLTEVETPIR

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doi:10.1128/CVI.00153-12

Received 12 March 2012 Returned for modification 13 April 2012 Accepted 7 May 2012 Published ahead of print 30 May 2012

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doi:10.1128/CVI.00153-12

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M2e sequence: (M) SLTVEVTPRNWGRSNNDSSDP  
Sequence used: SLTVEVTPRNWGRSNNDSSDP

4.M2e:  
AAASLTVEVTPRNWGRSNNDSSDDAGTSAI4ALSLLTEETIPR
NEWGRSNNDSSDP44AQL44ALSLLTEETIPRNWGRSNNDSD
DDAAACCA1ALLTVEVTPRNWGRSNNDSSDPAAACCKL

4 M2e
Flagellin (FlIC)
Membrane-anchored 4.M2e-tFlIC

M2e Histag Melittin SP HA TM HA CT
ND1-2 (1-176) D3 CD2-1 (402-459)

FIG 1 Sequences and schematic diagram of constructs. The M2e sequence shown is the consensus of the human influenza A virus M2e sequence. In 4.M2e, four repetitive M2e regions (underlined) are located in a tandem. Sequences in italics are flexible linkers. A 6-histidine tag-encoding DNA was fused in frame. In the membrane-anchored 4.M2e-tFlIC, the central immunogenic region of FlIC (domain 3 [D3], aa 177 to 401) was replaced by the 4.M2e sequence. The melittin signal peptide (SP) was fused to the N terminus of 4.M2e-tFlIC (N-terminal domains 1 and 2 [ND1-2] in FlIC) to enable its ectodomain to reach the exocytic pathway and is removed in the mature protein by insect cell signal peptidase (44). The transmembrane and cytoplasmic domains (TM/CT) of A/PR8 virus HA were attached to the C terminus of the 4.M2e-tFlIC (C-terminal domains 2 and 1 [CD2-1] in FlIC).

NEWGRSNNDSSDP (30) and flexible linker sequences (Fig. 1) were produced by primer-extension PCR and ligated to generate the encoding gene sequence 4.M2e. Two cysteines at sites 17 and 19 of M2e were replaced by serine residues (9), and a 6-histidine tag sequence was added in frame to facilitate the purification of the 4.M2e protein. 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 to 401 in FlIC) was deleted from the S. Typhimurium FlIC gene (40) and replaced by the 4.M2e-coding sequence. The sequences encoding an N-terminal signal peptide (SP) from honeybee melittin and a C-terminal membrane anchor from the A/PR8 influenza virus HA were added in frame (40) to generate the full-length gene encoding the membrane-anchored fusion protein (Fig. 1). The integrity of constructs was confirmed by DNA sequencing.

Protein and VLP production. Histidine-tagged recombinant 4.M2e was purified from an Escherichia coli protein expression system as described previously (34). Purified proteins migrated as one band by Coomassie blue staining and Western blotting and were dialyzed against phosphate-buffered saline (PBS) and stored at −80°C. A recombinant baculovirus (rBV) expressing the membrane-anchored 4.M2e-tFlIC was generated by using a Bac-to-Bac kit (Invitrogen). Recombinant BVs expressing membrane-anchored FlIC and M1 were described previously (40). The 4.M2e-tFlIC/M1 VLPs were produced by coinfection of S9 cells with rBVs expressing the membrane-anchored 4.M2e-tFlIC and M1 at multiplicities of infection (MOIs) of 6 and 3, respectively. The M2e content in 4.M2e-tFlIC/M1 VLPs was 1.5% when normalized by Western blotting using purified 4.M2e as a standard. VLPs were concentrated from the supernatants of S9 cell culture and characterized by protein assay, Western blotting, sterility assay, and electron microscopy (EM), as described previously (40).

TLR5-specific bioactivity assay. The TLR5-activating activities of the recombinant protein and VLPs were evaluated using a RAW264.7 cell-based assay (40). After a 24-h treatment, levels of tumor necrosis factor alpha (TNF-α) production stimulated by proteins or VLPs in both TLR5-positive and -negative cell cultures in supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using a TNF-α assay kit (eBioscience, San Diego, CA). TLR5 bioactivity was presented as the level of TNF-α production in TLR5-positive cells subtracted from that in TLR5-negative cells.

Immunoassay and challenge. Groups of six inbred female BALB/c mice (from Charles River Laboratories) were immunized three times with 10 μg 4.M2e or 50 μg 4.M2e-tFlIC/M1 (VLPs equal to 0.75 μg M2e) by either the intramuscular (i.m.) or intranasal (i.n.) route at 4-week intervals. Preimmune sera were collected at 1 week before prime immunization. Immune sera and lung lavage samples were collected at 4 weeks after the last immunization. Blood samples were collected by retro-orbital plexus puncture. For virus challenge, mice were lightly anesthetized by inhalation of isoflurane, and 5 LD₅₀ of mouse-adapted A/PR8 or A/Philippines virus (in 25 μl PBS) were administered into the mouse nostrils. Mouse body weight and survival were monitored daily for 15 days. Mice were sacrificed at day 4 postinfection to determine lung virus loads. Lung virus titers were determined as previously described (40).

Antibody responses. M2e-specific serum IgG endpoint titers were determined by ELISA as described previously (28) using synthesized M2e peptide-coated 96-well ELISA plates (Nunc Life Technologies). Serum M2e-specific antibody levels were also determined using cell surface ELISA (16). MDCK cells in 96-well culture plates (90% confluence) were infected with A/PR8 virus at an MOI of 1 (5 × 10⁴ PFU). After 12 h of growth, the plates were washed and the cells were fixed with 0.5% glutaraldehyde at 4°C for 30 min. After three washes with PBS, test sera were added to the wells in 80-fold dilutions and incubated for 2 h at room temperature. Antibody binding to uninfected MDCK cells was subtracted as background. M2e-specific antibody levels were presented as the value of the optical density at 450 nm (OD₄₅₀).

At 4 weeks postimmunization, mouse lungs were collected and lavaged twice with 1 ml PBS containing 0.05% Tween 20 per lung. The mucosal responses, expressed as endpoint titers of M2e-specific IgA and IgG, were determined in lung lavage fluid of immunized mice by ELISA, as previously described (28), using horseradish peroxidase (HRP)-conjugated anti-mouse IgA or IgG as developing antibodies.

Statistical analysis. All experiments were repeated at least twice in this study. Comparisons of vaccinated groups were performed using a non-matching two-way analysis of variance with Bonferroni’s posttest. Comparison of survival curves was performed using a log-rank (Mantel-Cox) test. The analyses were done by using GraphPad Prism (version 5.00) software for Windows (GraphPad Software, San Diego, CA). P values of less than 0.05 were considered statistically significant.

RESULTS
Characterization of VLPs. To improve the immunogenicity of M2e, the membrane-anchored 4.M2e-tFlIC protein was incorporated into influenza virus M1 VLPs (Fig. 2A), and the incorporation was confirmed by Western blotting assays using either anti-FlIC antibody (Fig. 2C, lane 1) or anti-M2e antibody (14C2; Fig. 2D, lane 1). The integrity of VLPs was confirmed by electron microscopy (Fig. 2B). We also produced VLPs containing M1 only (Fig. 2E, lane 2) as controls.

Because TLR5 is the known extracellular sensor of flagellin, we determined the ability of the membrane-anchored 4.M2e-tFlIC in VLPs to function as a TLR5 ligand. As shown in Fig. 2F, 4.M2e-tFlIC showed TLR5-specific bioactivity, inducing mouse macrophage cell line RAW264.7 to produce TNF-α over a concentration range of 0.01 ng to 1,000 ng/ml. The 50% effective concentration (the concentration which produces 50% of maximal activity [EC₅₀]) of the 4.M2e-tFlIC/M1 VLPs was 60 ng/ml, while that of the soluble flagellin was 0.8 ng/ml. Considering that the soluble FlIC has a much lower molecular weight than the VLPs, their relative EC₅₀ titers are within the expected ranges.

4.M2e-tFlIC/M1 VLPs induce high IgG responses. To evaluate the potential of 4.M2e-tFlIC/M1 VLPs to be universal influenza vaccines, the immune responses induced by either i.m. or i.n. immunization were compared. The result in Fig. 3A shows that...
Enhanced Influenza VLP Vaccines

FIG 2 Characterization of 4.M2e-tFlIC VLPs. (A) Diagram of influenza virus 4.M2e-tFlIC VLPs. The membrane-anchored form of the 4.M2e-tFlIC is inserted into the lipid bilayer of the envelope. (B) Negative-stain EM of VLPs. (C to E) Characterization of VLPs. VLP samples were applied to SDS-polyacrylamide gels, followed by Western blotting. Lanes 1, 4.M2e-tFlIC VLPs; lanes 2, M1-only VLPs. Protein bands were detected by mouse anti-FlIC antibody (FlIC-1) (C), mouse anti-M2e antibody (14C2) (D), and anti-M1 antibody (GA2B; Abcam) (E). (F) TLR5 agonist activity of flagellin. The mouse macrophage cell line RAW264.7, which expresses TLR2 and TLR4 without TLR5, was used to determine the bioactivity of 4.M2e-tFlIC VLPs. Soluble recombinant FlIC (sFlIC) was used as a positive control; M1-only VLPs and recombinant 4.M2e were used as negative controls. TLR5-expressing RAW264.7 cells were prepared by transfection with the vector pUNO-hTLR5 expressing the human TLR5, as described previously (40). Data represent means ± standard deviations (SDs) from triplicate repeats.

The 4.M2e-tFlIC/M1 VLPs trigger robust M2e-specific humoral responses in mice immunized i.m. or i.n. compared to those triggered by recombinant 4.M2e (P < 0.001), which induced only low titers of anti-M2e IgG. The serum IgG titers in i.m. and i.n. immunizations were 6.5 × 10^5 and 4.0 × 10^5, respectively. Although the 4.M2e-tFlIC/M1 VLPs induced high levels of M2e-specific antibody responses, only antibodies capable of targeting native tetrameric M2 may confer protection (42). Influenza virus-infected MDCK cells express high levels of M2 on the cell membrane and can be used to determine M2-specific antibody binding (36). Therefore, we further evaluated antibody binding to native M2 protein by an MDCK cell-based ELISA. As shown in Fig. 3B, antibodies in immune sera recognized and bound M2 expressed on MDCK cell surfaces, as determined by ELISA. Sera from the 4.M2e peptide-immunized mice showed very low binding close to background. In contrast, 4.M2e-tFlIC/M1 VLPs induced significantly higher M2-specific antibody responses (P < 0.01) by either i.m. or i.n. immunization. Comparatively, the i.m. route showed a trend to induce higher systemic immune responses than the i.n. route, although the difference between the i.m. and i.n. routes was not statistically significant (Fig. 3A and B). The levels of antibody binding to M2 on cell surfaces in all groups showed a pattern similar to the M2e-specific IgG ELISA titers, demonstrating that the M2e-specific antibodies confer M2 recognition and are correlated with binding to the native M2.

4.M2e-tFlIC/M1 VLPs induce strong mucosal immune responses. The respiratory tract is the dominant site of influenza virus infection and replication, and effective mucosal antibody responses can prevent the initiation of viral infection in the respiratory tract (5). The results shown in Fig. 3 indicate that 4.M2e-tFlIC/M1 VLPs induced higher titers of M2e-specific IgA (Fig. 3C) and IgG (Fig. 3D) in mouse lungs after i.n. immunization than 4.M2e did (P < 0.001) and the advantage of the i.n. immunization route in inducing mucosal immune responses (P < 0.05). These data demonstrate that flagellin is effective as a mucosal adjuvant when fused with M2e antigen in VLPs.

Breadth of protective efficacy. To determine whether the above-described vaccine candidates confer enhanced protection against virus challenge, immunized mice were challenged i.n. with 5 LD50s (250 PFU) of mouse-adapted A/Philippines (H3N2) virus in which M2e has the same sequence as the consensus sequence used. As shown in Fig. 4, mice in both the i.m. and i.n. groups immunized with 4.M2e-tFlIC/M1 VLPs completely survived the H3N2 virus challenges (Fig. 4A and B). However, mice in the i.n. group lost less body weight (11%; Fig. 4C) than mice in the i.m. group (18%, Fig. 4D). Mice immunized with 4.M2e protein, as well as the naive mice, reached their endpoints (25% body weight loss; Fig. 4) at day 7 to 10.

We further evaluated the protection against A/PR8 (H1N1) virus challenge induced by the above-described vaccine candidates. The M2e sequence of A/PR8 virus M2 has 1 aa different from the consensus sequence used, and no other human influenza virus shares this M2e sequence. We found that mice immunized i.m. with 4.M2e-tFlIC/M1 VLPs partially survived A/PR8 virus challenge at a dose of 5 LD50s (5 of 6 mice; Fig. 5A) with a 19% body weight loss (Fig. 5C). In contrast, mice in the i.n. group completely survived the challenge (Fig. 5B) but lost 13% body weight (Fig. 5D). Mice in the 4.M2e protein group, as well as the naive mice, reached 25% weight loss endpoints. These results demonstrate that the M2e vaccines induced better protection against viruses having less of an amino acid difference in M2, consistent with our previous observations (36, 37).

A common feature of the above-described challenge results is that immunity induced by the i.n. route is more effective in providing protection against influenza virus infection. i.m. immunizations induced higher titers of serum M2-specific IgG responses than i.n. immunizations, as shown in Fig. 3, but the i.n. vaccination induced better protection, demonstrating the advantage of mucosal immunity in M2e-induced protection. These data demonstrate that the M2e-specific antibodies, in particular, the mucosal antibodies, correlate with protective efficacy.

Because the M2e-specific antibodies do not neutralize the infectivity of influenza viruses but instead may exert their protective effect at the level of the infected cell (15, 17, 39, 42), we further

August 2012 Volume 19 Number 8 cvi.asm.org 1121
evaluated the effect of 4.M2e-tFlIC/M1 VLPs in decreasing viral replication in the lung. As shown in Fig. 6, all surviving groups have lung virus loads lower than $1 \times 10^4$ for the A/PR8 challenge virus or $1 \times 10^5$ for A/Philippines challenge virus. In the 4.M2e-tFlIC VLP group, mice receiving i.n. immunization showed a 10-fold lower lung viral load than those receiving i.m. immunization with A/PR8 virus challenge and a 1.25-fold lower lung viral load with A/Philippines virus challenge. These results further demon-

FIG 3 Antibody responses. (A) Serum IgG recognizing M2e in immunized mice. To determine serum M2e-specific IgG titers, ELISA plates were coated with 100 μl/well of synthesized M2e peptide (5 μg/ml; at the Emory University Biochemical Core Facility). (B) Endpoint titers of IgG recognizing native M2 protein. MDCK cells were infected with A/PR8 viruses at an MOI of 1. Uninfected cells were used as a control. At 12 h postinfection, cells were washed with PBS and fixed with 0.5% glutaraldehyde. Samples diluted 80-fold were applied to detect antibody binding, as described in Materials and Methods. Data depict the OD$_{450}$ (mean ± SD) with infected cells, with the background of uninfected cells subtracted. Groups 1 and 2 represent mouse groups immunized with 4.M2e and 4.M2e-tFlIC/M1 VLPs, respectively. (C) Lung lavage IgA. IgA endpoint titers were determined as described for the serum IgG endpoint titer in panel A, but the secondary antibody used was HRP-conjugated goat anti-mouse IgA antibody. (D) Lung lavage IgG. Representative data are the geometric mean (GM) ± 95% confidence interval (CI) of six mice in each group at 4 weeks after the last immunization. Asterisks on the top of a bar present the statistical difference of the group to its counterpart in the M2e group. The statistical difference between other groups is labeled with a connecting line. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 4 A/Philippines (H3N2) live virus challenge. Four weeks after the last immunization, 6 mice of each group were infected i.n. with 5 LD$_{50}$s of mouse-adapted A/Philippines viruses (250 PFU). Survival of mouse groups immunized i.m. (A) or i.n. (B) and body weight changes of i.m. (C) and i.n. (D) groups were monitored and recorded for 15 days to indicate the protective efficacy of the vaccines. In panels C and D, the data represent means of the body weight change (percentage of prechallenge body weight) of 6 mice in each group.
strate the advantage of mucosal immunization for protection against influenza.

**DISCUSSION**

Because it is highly conserved in all A-type influenza viruses, M2e has been studied as a universal influenza vaccine (8, 10, 16, 26). Here we used a combined approach to target M2e for the development of such a vaccine. First, multiple copies of M2e (4.M2e) in our vaccine candidates increased the density of epitopes. Second, replacement of the highly immunogenic variable region of FliC with 4.M2e may endow M2e with high immunogenicity because the resulting 4.M2e-tFliC fusion protein retains the TLR5 ligand property of FliC. VLPs are advantageous as immunogens in inducing strong immune responses since they mimic the structures of viruses which the immune system has evolved to fight. The HA cytoplasmic domain contains signals guiding the envelope glycoprotein to assemble into VLPs (7). Previously, we have found that a fusion protein with an HA membrane anchor can be incorporated into M1 VLPs (40). The VLP approach enables M2e to be delivered in a particulate form and to be presented in its native external membrane microenvironment. Particulate repetitive epitopes are more effective antigens because of their increased epitope density (13, 20, 21). Several groups have also reported enhanced immune responses induced by physical repeats of M2e (11, 16). The potential of this approach has been demonstrated by inducing a high level of M2-specific immunity and complete protection against lethal live virus challenge, as shown in the present study.

As the natural ligand of TLR5, flagellin has been found to be an effective adjuvant. It has been used to enhance the immunogenicity of antigens in mixtures with antigens and the physical association as fusion proteins, and it has been coincorporated into the same particles with antigens (16, 24, 40). The replacement of the variable region of FliC with 4.M2e should not impair its innate TLR signaling because flagellins from different bacterial strains show significant differences in their variable regions (2, 35). A fusion protein composed of 4 repeating M2e peptides attached to the C terminus of the phase II flagellin (FljB) was reported to induce partial protection in mice against a lethal influenza virus infection with reduced clinical symptoms (16). Our results demonstrate that the membrane-anchored 4.M2e-tFliC in VLPs retains innate TLR5 signaling activity. As expected, these VLPs in-

**FIG 5** A/PR8 (H1N1) virus challenge. Four weeks after the last immunization, mice were infected i.n. with 5 LD₅₀ of mouse-adapted A/PR8 virus (125 PFU). Survival of mouse groups immunized i.m. (A) or i.n. (B) and body weight changes of groups immunized i.m. (C) or i.n. (D) were monitored and recorded as described in the legend to Fig. 4.

**FIG 6** Lung viral load on day 4 postchallenge. Four weeks after the last immunization, three mice in each group were infected i.n. with 5 LD₅₀ of mouse-adapted A/PR8 (H1N1) or A/Philippines (Phi; H3N2) virus. Mouse lungs were collected on day 4 postchallenge. Each lung was ground and cleared in 1 ml of Dulbecco modified Eagle medium. Virus titers of lung extracts were titrated using a standard plaque assay with MDCK cells. Lung viral titer was expressed as the number of PFU/lung. Data depict means ± SDs of three mice from each group. Groups 1 and 2 represent mouse groups immunized i.m. or i.n. with 4.M2e and 4.M2e-tFliC/M1 VLPs, respectively. Statistically significant differences between immunized groups and the naive group are indicated: *, P < 0.05; **, P < 0.01.
duced high levels of M2e-specific immune responses, in particular when they were given by i.n. immunization, in which complete protection against either A/PR8 or A/Philippines virus with little body weight loss was induced. These promising results are probably derived from the advantages of our approach: the physical association of the FlIC TLR5 binding domains with 4.M2e and the replacement of the FlIC hyperimmunogenic central region with 4.M2e. Also, these results are consistent with results reported in other studies which indicate that the TLR5 recognition domains of flagellin are not associated with the central variable region (2, 35) and that a variable region-truncated FlIC is more effective as a mucosal adjuvant (27). Because the variable region of FlIC is hyperimmunogenic, another advantage of the approach is that replacement of the variable region of FlIC with M2e sequences minimizes antibody responses to FlIC (27), although other work showed that induction of anti-FlIC antibody is not a limitation for its adjuvant properties (3, 40).

Several different immunization routes have been used to deliver M2e-derived antigens for inducing protective immunity. i.n. immunization was reported to be an advantageous approach in inducing protective immunity compared to other routes (9). Our results also demonstrated that i.n. immunization with 4.M2e-tFlIC/M1 VLPs induces high systemic immune responses as well as strong mucosal immunity and confers more effective protection against live virus challenge, as demonstrated by the complete protection against challenge with H1N1- or H3N2-subtype viruses with minimal body weight loss. Also, virus replication in the lungs of immunized mice is restricted, as revealed by lower lung virus titers, although they are still higher than those in the lungs of mice immunized with HA-containing VLPs (40, 41). i.n. immunization stimulates the nasopharynx-associated lymphoreticular tissue and induces local mucosal immunity (19). Furthermore, the variable region-truncated flagellin has been reported to be more efficient as a mucosal adjuvant, as discussed above. These observations provide a basis for the finding that the M2e-tFlIC/M1 VLPs induce enhanced mucosal immunity.

Because the M2-specific antibodies are not neutralizing and recent work demonstrated that M2 VLP antigens used in combination with the trivalent influenza vaccines or inactivated viral vaccines showed enhanced subtypic protection (36, 38), the approaches described here provide new alternatives for such combinations. Thus, a fusion protein containing a replacement of the variable region of FlIC with repetitive M2e in VLPs is promising for further development of universal influenza A virus vaccines.

ACKNOWLEDGMENTS
This study was supported by grants AI068003, AI093772, and AI087782 from the National Institutes of Health.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Erin-Joi Collins for her valuable assistance in the preparation of the manuscript.

REFERENCES


