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

Bao-Zhong Wang [a,*], Harvinder S. Gill [a,b], Cheng He [c], Changbo Ou [c], Li Wang [a], Ying-Chun Wang [a], Hao Feng [a], Han Zhang [a], Mark R. Prausnitz [d], Richard W. Compans [a,**]

[a] Department of Microbiology and Immunology, and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA
[b] Department of Chemical Engineering, Texas Tech University, Lubbock, TX 79409, USA
[c] Key Laboratory of Animal Epidemiology and Zoonosis, Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing 100094, China
[d] School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, 30332, USA

ARTICLE INFO

ABSTRACT

Influenza vaccines with broad cross-protection are urgently needed to prevent an emerging influenza pandemic. A fusion protein of the Toll-like receptor (TLR) 5-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.

1. 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 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 (MN) arrays 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 intramuscular (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 variation 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

* Correspondence to: B.Z. Wang, Department of Microbiology and Immunology, and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA. Tel.: +1 404 712 1735; fax: +1 404 727 3295.
** Correspondence to: R.W. Compans, Department of Microbiology and Immunology, and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA. Tel.: +1 404 727 2015; fax: +1 404 727 8250.
E-mail addresses: bwang3@emory.edu (B.-Z. Wang), rcompan@emory.edu (R.W. Compans).

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 hyper-immunogenic 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 IM and intranasal (IN) 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.

2. Material and methods

2.1. 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 LD_{50} (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].

2.2. 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 (SLLTEVETPIRNEWGSRSNDSSDP) and flexible linker sequences were produced and cloned into the expression vector pET-22b (Novagen, EMDBiosciences, 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 (aas 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.

2.3. Protein purification

Histidine-tagged recombinant proteins 4.M2e and 4.M2e-tFliC were purified from an *Escherichia coli* protein expression system as described previously [32]. Recombinant proteins 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.

2.4. 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 FliC 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).

2.5. Fabrication of microneedle arrays

A linear array containing five microneedles at an intra-microneedle 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.

2.6. Coating MNs with 4.M2e-tFliC

To develop a uniform coating of the recombinant 4.M2e-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.M2e-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).

2.7. 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 ± 0.1 μg of antigen was coated on a single row of five MNs assessed as described above. Five arrays were used to deliver approximately 7 μg of 4.M2e-tFliC per mouse. Because the 700 μm-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 3 min 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 μg of recombinant 4.M2e or 4.M2e-tFliC by IM injection using a conventional 28-gauge needle or by the 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 $10 \times LD_{50}$ of virus in 30 μl PBS was administered into the mouse nostrils. Mouse body weight and survival were monitored daily for 15 days.

2.8. 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].

2.9. Statistical analysis

An unpaired Student t-test was performed to compare the difference between two groups in Figs. 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 Figs. 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 (***), and P > 0.05 (n.s.).

3. Results

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

Constructs (Fig. 1) expressing recombinant 4.M2e protein and 4.M2e-tFliC fusion protein were generated as described in Material and methods. Recombinant proteins were purified as previously described, and the full length and variable region-truncated flagellin proteins, FliC and tFliC, were also purified for comparison [32]. The purified proteins showed a single main band after SDS-PAGE followed by Coomassie blue staining (Fig. 2A). Both FliC and tFliC were detected by anti-flagellin antibodies (lanes 1 and 2 in Fig. 2B) but not anti-M2e antibodies (lanes 1 and 2 in Fig. 2C). In contrast, recombinant 4.M2e could be probed by anti-M2e antibodies (lane 3 in Fig. 2C) but not anti-flagellin antibodies (lane 3 in Fig. 2B). Recombinant 4.M2e-tFliC fusion protein was recognized by both anti-flagellin (lane 4 in Fig. 2B) and anti-M2e (lane 4 in Fig. 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 Fig. 2D, the 4.M2e-tFliC stimulated TLR5 (+) 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 TLR5 agonist activity. These results demonstrate that the resulting fusion protein retained the activity of flagellin as a ligand of TLR5. As shown in Fig. 2E, 4.M2e-tFliC could be selectively coated as

M2e sequence: (M) SLLTEVETPIRNEWG*C* R*C* NDSSDP

Used sequence: SLLTEVETPIRNEWG *S* R*S* NDSSDP

4.M2e:

(*AAA*)SLLTEVETPIRNEWGSRSNDSSDP*AAGTSAAA*SLLTEVET PIRNEWGSRSNDSSDP*AAALQAAA*SLLTEVETPIRNEWGSRSND SSDP*AAAACAAA*SLLTEVETPIRNEWGSRSNDSSDPA(*AAA*)
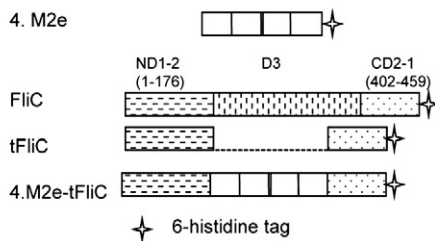

Fig. 1. Schematic diagrams of constructs. In 4.M2e, four M2e repeats were bridged by short linker sequences to form the tandem repetitive 4.M2e. In the 4.M2e-tFliC fusion protein, three alanine residues were inserted as flexible linkers prior to and after the tandem 4.M2e sequence.
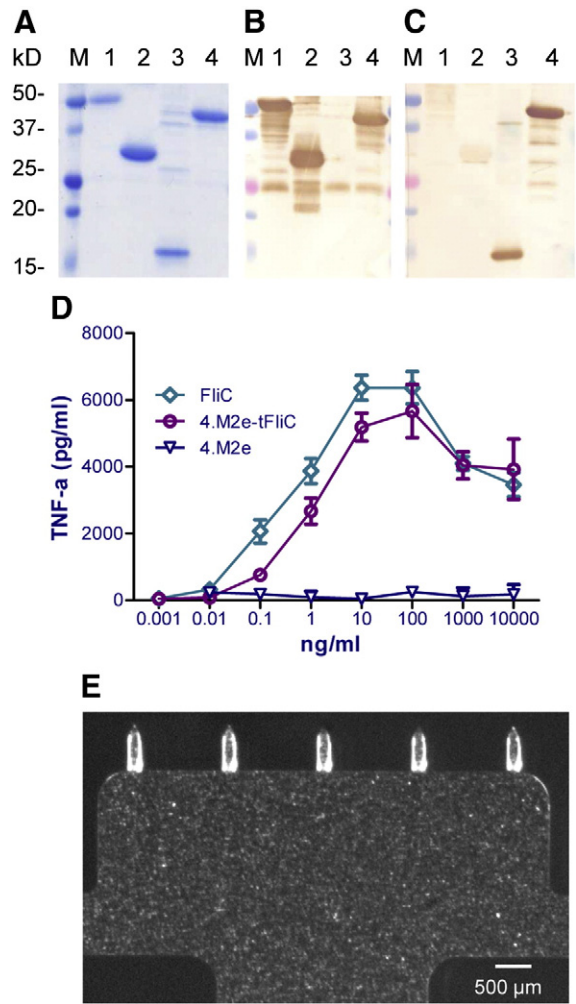

Fig. 2. Purification and characterization of recombinant proteins. 4.M2e and 4.M2e-tFliC were purified from E. coli protein expression by affinity chromatography as described in Material and methods. A, Coomassie blue staining; B, Western blot probed with anti-flagellin polyclonal antibodies and developed 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.M2e-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 Material and methods.

uniform coatings on the surface of MN arrays without contaminating the handling part of the device.

3.2. 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 Fig. 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 Fig. 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,
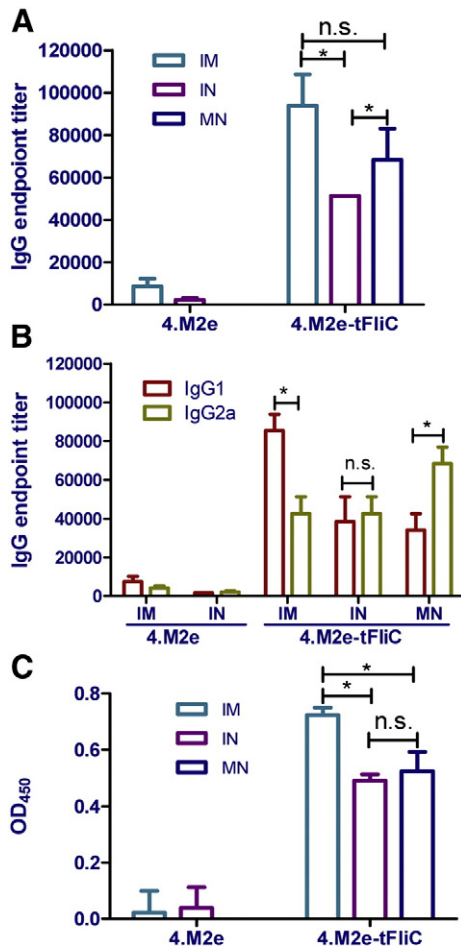
Fig. 3. Serum IgG endpoint titers and M2-specific IgG-binding. ELISA assay plates were coated with 100 μl/well of M2e peptide (5 μg/ml). Immune serum samples were diluted 2× stepwise, and 100 μl of diluted samples was applied to plates for antibody-binding. Bound antibodies were detected by binding HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2 antibody and color was developed with TMB substrate. The greatest dilution which presented an OD450 2× higher than that of the negative control (naïve group) was designated as the endpoint titer (mean + 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 ± SD, n = 3 replicates) of infected cells subtracting the background of uninfected cells. A, serum IgG; B, IgG isotypes; C, serum IgG recognizing M2.

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 (Fig. 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 immunizations. As shown in Fig. 4, MN immunization induced a moderate mucosal antibody response compared to that found by IM or IN immunizations in mice. Both IgA and IgG end point titers in lung lavages from the MN
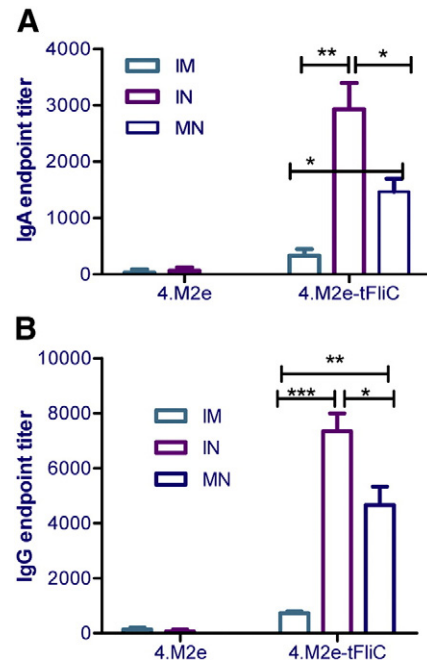

Fig. 4. Mucosal antibody endpoint titers. Immunized mouse lungs were collected and lavaged with 1 ml PBS containing 0.05% Tween 20 per lung twice. IgG and IgA endpoint titers were determined as described above for serum IgG but the secondary antibody was HRP-conjugated goat anti-mouse IgA antibody for IgA endpoint measurement. A, IgA endpoint titer; B, IgG endpoint titer (mean ± SD, n = 6).

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-fold (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 Fig. 3.

3.3. 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× LD$_{50}$), in which the M2e shares the same sequence as the human viral consensus M2e in our construct. As shown in Fig. 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%, Fig. 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 Fig. 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; Fig. 5D). These results
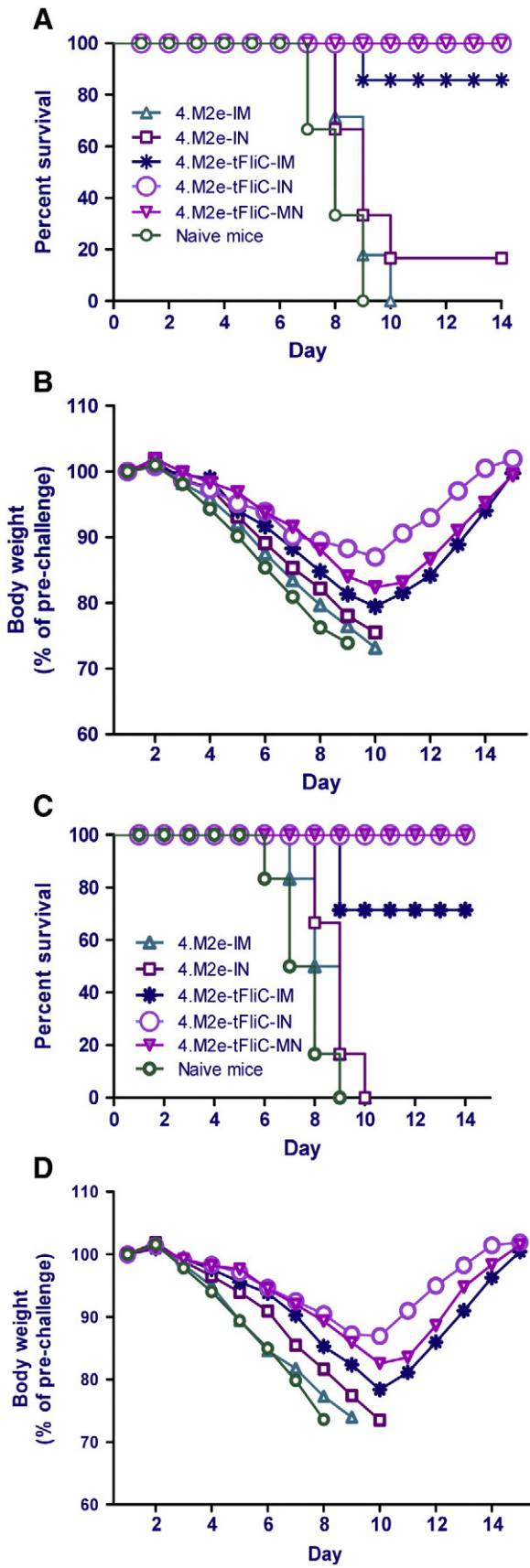
Fig. 5. Virus challenge. Three months after the last immunization, mice were IN infected with $10\times LD_{50}$ A/Philippines virus (A and B) or A/PR8 (C and D). Mouse-adapted virus in 30 μl PBS was slowly applied to the nares of mice. Mouse survival and body weight changes were monitored for 15 days. A and C, mouse survival; B, body weight change (n = 6).

demonstrate that MN immunization induced better protection in mice compared to conventional IM injection.

3.4. 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 demonstrate 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 by IM immunization or by IN immunization, indicating the low immunogenicity of this antigen (Fig. 6). Immunization with the fusion protein 4.M2e-tFliC 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.

4. 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.
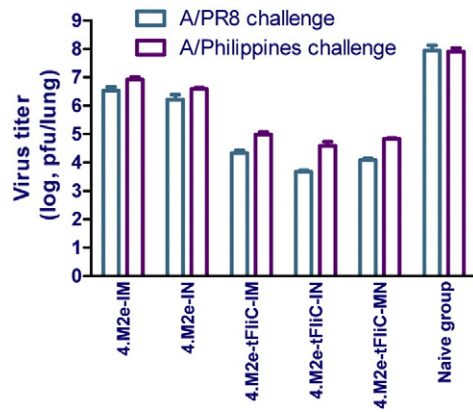

Fig. 6. Lung viral loads on day 4 postchallenge. Six mice in each group were infected IN with $10\times LD_{50}$ of A/PR8 (H1N1) or A/Philippines (H3N2) viruses. Mouse lungs were collected on day 4 post-challenge. Each lung was ground and cleared in 1 ml of DMEM. Virus titers of lung extracts were titrated using a standard plaque assay with MDCK cells. Lung viral titer was expressed as pfu/lung.

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

References

[1] M.I. Meltzer, C.B. Bridges, Economic analysis of influenza vaccination and treatment, Ann. Intern. Med. 138 (2003) 608–609.

[2] S. Schultz-Cherry, J.K. Dybing, N.L. Davis, C. Williamson, D.L. Suarez, R. Johnston, M.L. Perdue, Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses, Virology 278 (2000) 55–59.

[3] J. Fan, X. Liang, M.S. Horton, H.C. Perry, M.P. Citron, G.J. Heidecker, T.M. Fu, J. Joyce, C.T. Przysiecki, P.M. Keller, V.M. Garsky, R. Ionescu, Y. Rippeon, L. Shi, M.A. Chastain, J.H. Condra, M.E. Davies, J. Liao, E.A. Emini, J.W. Shiver, Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys, Vaccine 22 (2004) 2993–3003.

[4] W. Gerhard, K. Mozdzanowska, D. Zharikova, Prospects for universal influenza virus vaccine, Emerg. Infect. Dis. 12 (2006) 569–574.

[5] M. del Pilar Martin, W.C. Weldon, V.G. Zarnitsyn, D.G. Koutsonanos, H. Akbari, I. Skountzou, J. Jacob, M.R. Prausnitz, R.W. Compans, Local response to microneedle-based influenza immunization in the skin, mBio 3 (2012) e00012–e.

[6] Y.C. Kim, J.H. Park, M.R. Prausnitz, Microneedles for drug and vaccine delivery, Adv. Drug Deliv. Rev. 64 (2012) 1547–1568.

[7] J.B. Alarcon, A.W. Hartley, N.G. Harvey, J.A. Mikszta, Preclinical evaluation of microneedle technology for intradermal delivery of influenza vaccines, Clin. Vaccine Immunol. 14 (2007) 375–381.

[8] F. Ansaldi, P. Canepa, A. Ceravolo, L. Valle, D. de Florentiis, R. Oomen, F.R. Vogel, M. Denis, S.I. Samson, G. Icardi, Intanza(®) 15 mcg intradermal influenza vaccine elicits cross-reactive antibody responses against heterologous A (H3N2) influenza viruses, Vaccine 30 (2012) 2908–2913.

[9] G.J. Fernando, X. Chen, T.W. Prow, M.L. Crichton, E.J. Fairmaid, M.S. Roberts, I.H. Frazer, L.E. Brown, M.A. Kendall, Potent immunity to low doses of influenza vaccine by probabilistic guided micro-targeted skin delivery in a mouse model, PLoS One 5 (2010) e10266.

[10] S.P. Sullivan, D.G. Koutsonanos, M. Del Pilar Martin, J.W. Lee, V. Zarnitsyn, S.O. Choi, N. Murthy, R.W. Compans, I. Skountzou, M.R. Prausnitz, Dissolving polymer microneedle patches for influenza vaccination, Nat. Med. 16 (2010) 915–920.

[11] W.C. Weldon, M.P. Martin, V. Zarnitsyn, B. Wang, D. Koutsonanos, I. Skountzou, M.R. Prausnitz, R.W. Compans, Microneedle vaccination with stabilized recombinant influenza virus hemagglutinin induces improved protective immunity, Clin. Vaccine Immunol. 18 (2011) 647–654.

[12] A. Kumar, P. Wonganan, M.A. Sandoval, X. Li, S. Zhu, Z. Cui, Microneedle-mediated transcutaneous immunization with plasmid DNA coated on cationic PLGA nanoparticles, J. Control Release 163 (2012) 230–239.

[13] J.C. Birchall, R. Clemo, A. Anstey, D.N. John, Microneedles in clinical practice—an exploratory study into the opinions of healthcare professionals and the public, Pharm. Res. 28 (2011) 95–106.

[14] H.S. Gill, D.D. Denson, B.A. Burris, M.R. Prausnitz, Effect of microneedle design on pain in human volunteers, Clin. J. Pain 24 (2008) 585–594.

[15] K. Mozdzanowska, J. Feng, M. Eid, G. Kragol, M. Cudic, L Otvos Jr., W. Gerhard, Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2, Vaccine 21 (2003) 2616–2626.

[16] S.L. Zebedee, R.A. Lamb, Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions, J. Virol. 62 (1988) 2762–2772.

[17] R.A. Black, P.A. Rota, N. Gorodkova, H.D. Klenk, A.P. Kendal, Antibody response to the M2 protein of influenza A virus expressed in insect cells, J. Gen. Virol. 74 (Pt 1) (1993) 143–146.

[18] P.G. Hughey, P.C. Roberts, L.J. Holsinger, S.L. Zebedee, R.A. Lamb, R.W. Compans, Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly, Virology 212 (1995) 411–421.

[19] J.J. Treanor, E.L. Tierney, S.L. Zebedee, R.A. Lamb, B.R. Murphy, Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice, J. Virol. 64 (1990) 1375–1377.

[20] M. De Filette, W. Martens, K. Roose, T. Deroo, F. Vervalle, M. Bentahir, J. Vandekerckhove, W. Fiers, X. Saelens, An influenza A vaccine based on tetrameric ectodomain of matrix protein 2, J. Biol. Chem. 283 (2008) 11382–11387.

[21] J.W. Huleatt, V. Nakaar, P. Desai, Y. Huang, D. Hewitt, A. Jacobs, J. Tang, W. McDonald, L. Song, R.K. Evans, S. Umlauf, L. Tussey, T.J. Powell, Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin, Vaccine 26 (2008) 201–214.

[22] J. Denis, E. Acosta-Ramirez, Y. Zhao, M.E. Hamelin, I. Koukavica, M. Baz, Y. Abed, C. Savard, C. Pare, C. Lopez Macias, G. Boivin, D. Leclerc, Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform, Vaccine 26 (2008) 3395–3403.

[23] J.M. Song, N. Van Rooijen, J. Bozja, R.W. Compans, S.M. Kang, Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 757–761.

[24] K.D. Smith, E. Andersen-Nissen, F. Hayashi, K. Strobe, M.A. Bergman, S.L. Barrett, B.T. Cookson, A. Aderem, Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility, Nat. Immunol. 4 (2003) 1247–1253.

[25] V. Le Moigne, G. Robreau, W. Mahana, Flagellin as a good carrier and potent adjuvant for Th1 response: study of mice immune response to the p27 (Rv2108) Mycobacterium tuberculosis antigen, Mol. Immunol. 45 (2008) 2499–2507.

[26] E.V. Vassilieva, B.Z. Wang, A.N. Vzorov, L. Wang, Y.C. Wang, J. Bozja, R. Xu, R.W. Compans, Enhanced mucosal immune responses to HIV virus-like particles containing a membrane-anchored adjuvant, mBio 2 (2011) e00328-e.

[27] B.Z. Wang, F.S. Quan, S.M. Kang, J. Bozja, I. Skountzou, R.W. Compans, Incorporation of membrane-anchored flagellin into influenza virus-like particles enhances the breadth of immune responses, J. Virol. 82 (2008) 11813–11823.

[28] B.Z. Wang, R. Xu, F.S. Quan, S.M. Kang, L. Wang, R.W. Compans, Intranasal immunization with influenza VLPs incorporating membrane-anchored flagellin induces strong heterosubtypic protection, PLoS One 5 (2010) e13972.

[29] F.S. Quan, C. Huang, R.W. Compans, S.M. Kang, Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus, J. Virol. 81 (2007) 3514–3524.

[30] L.J. Reed, H. Muench, A simple method of estimating fifty percent endpoint, Am. J. Hyg. 27 (1938) 5.

[31] B.Z. Wang, H.S. Gill, S.M. Kang, L. Wang, Y.C. Wang, E.V. Vassilieva, R.W. Compans, Enhanced influenza virus-like particle vaccines containing the extracellular domain of matrix protein 2 and a Toll-like receptor ligand, Clin. Vaccine Immunol. 19 (2012) 1119–1125.

[32] I. Skountzou, P. Martin Mdel, B. Wang, L. Ye, D. Koutsonanos, W. Weldon, J. Jacob, R.W. Compans, Salmonella flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine, Vaccine 28 (2010) 4103–4112.

[33] H.S. Gill, M.R. Prausnitz, Coating formulations for microneedles, Pharm. Res. 24 (2007) 1369–1380.

[34] H.S. Gill, M.R. Prausnitz, Coated microneedles for transdermal delivery, J. Control Release 117 (2007) 227–237.

[35] H.S. Gill, M.R. Prausnitz, Pocketed microneedles for drug delivery to the skin, J. Phys. Chem Solids 69 (2008) 1537–1541.

[36] Q. Zhu, V.G. Zarnitsyn, L. Ye, Z. Wen, Y. Gao, L. Pan, I. Skountzou, H.S. Gill, M.R. Prausnitz, C. Yang, R.W. Compans, Immunization by vaccine-coated microneedle arrays protects against lethal influenza virus challenge, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 7968–7973.

[37] J.M. Song, B.Z. Wang, K.M. Park, N. Van Rooijen, F.S. Quan, M.C. Kim, H.T. Jin, A. Pekosz, R.W. Compans, S.M. Kang, Influenza virus-like particles containing M2 induce broadly cross protective immunity, PLoS One 6 (2011) e14538.

[38] L. Mei, P.P. Song, Q. Tang, K. Shan, R.G. Tobe, L. Selotlegeng, A.H. Ali, Y.Y. Cheng, L.Z. Xu, Changes in and shortcomings of control strategies, drug stockpiles, and vaccine development during outbreaks of avian influenza A H5N1, H1N1, and H7N9 among humans, Biosci. Trends 7 (2013) 64–76.

[39] R. Gao, B. Cao, Y. Hu, Z. Feng, D. Wang, W. Hu, J. Chen, Z. Jie, H. Qiu, K. Xu, X. Xu, H. Lu, W. Zhu, Z. Gao, N. Xiang, Y. Shen, Z. He, Y. Gu, Z. Zhang, Y. Yang, X. Zhao, L. Zhou, X. Li, S. Zou, Y. Zhang, X. Li, L. Yang, J. Guo, J. Dong, Q. Li, L. Dong, Y. Zhu, T. Bai, S. Wang, P. Hao, W. Yang, Y. Zhang, J. Han, H. Yu, D. Li, G.F. Gao, G. Wu, Y. Wang, Z. Yuan, Y. Shu, Human infection with a novel avian-origin influenza A (H7N9) virus, N. Engl. J. Med. 368 (20) (2013) 1888–1897.

[40] F.S. Quan, Y.C. Kim, R.W. Compans, M.R. Prausnitz, S.M. Kang, Dose sparing enabled by skin immunization with influenza virus-like particle vaccine using microneedles, J. Control Release 147 (2010) 326–332.

[41] R.B. Belshe, F.K. Newman, J. Cannon, C. Duane, J. Treanor, C. Van Hoecke, B.J. Howe, G. Dubin, Serum antibody responses after intradermal vaccination against influenza, N. Engl. J. Med. 351 (2004) 2286–2294.

[42] T.F. Gajewski, M. Pinnas, T. Wong, F.W. Fitch, Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations, J. Immunol. 146 (1991) 1750–1758.

[43] M. Schotsaert, M. De Filette, W. Fiers, X. Saelens, Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments, Expert Rev. Vaccines 8 (2009) 499–508.

[44] K. El Bakkouri, F. Descamps, M. De Filette, A. Smet, E. Festjens, A. Birkett, N. Van Rooijen, S. Verbeek, W. Fiers, X. Saelens, Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection, J. Immunol. 186 (2011) 1022–1031.

[45] J. Mestecky, S.M. Michalek, Z. Moldoveanu, M.W. Russell, Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans, Behring Inst. Mitt. (1997) 33–43.

[46] F.W. van Ginkel, R.J. Jackson, Y. Yuki, J.R. McGhee, Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues, J. Immunol. 165 (2000) 4778–4782.

[47] X. Chen, G.J. Fernando, M.L. Crichton, C. Flaim, S.R. Yukiko, E.J. Fairmaid, H.J. Corbett, C.A. Primiero, A.B. Ansaldo, I.H. Frazer, L.E. Brown, M.A. Kendall, Improving the reach of vaccines to low-resource regions, with a needle-free vaccine delivery device and long-term thermostabilization, J. Control Release 152 (2011) 349–355.

[48] Y.C. Kim, F.S. Quan, R.W. Compans, S.M. Kang, M.R. Prausnitz, Stability kinetics of influenza vaccine coated onto microneedles during drying and storage, Pharm. Res. 28 (2011) 135–144.

[49] R.J. Pettis, A.J. Harvey, Microneedle delivery: clinical studies and emerging medical applications, Ther. Deliv. 3 (2012) 357–371.

[50] K. van der Maaden, W. Jiskoot, J. Bouwstra, Microneedle technologies for (trans) dermal drug and vaccine delivery, J. Control Release 161 (2012) 645–655.

[51] C. Nempont, D. Cayet, M. Rumbo, C. Bompard, V. Villeret, J.C. Sirard, Deletion of flagellin's hypervariable region abrogates antibody-mediated neutralization and systemic activation of TLR5-dependent immunity, J. Immunol. 181 (2008) 2036–2043.

[52] A.N. Honko, S.B. Mizel, Effects of flagellin on innate and adaptive immunity, Immunol. Res. 33 (2005) 83–101.

[53] D.G. Koutsonanos, M. del Pilar Martin, V.G. Zarnitsyn, S.P. Sullivan, R.W. Compans, M.R. Prausnitz, I. Skountzou, Transdermal influenza immunization with vaccine-coated microneedle arrays, PLoS One 4 (2009) e4773.

[54] P. Van Damme, F. Oosterhuis-Kafeja, M. Van der Wielen, Y. Almagor, O. Sharon, Y. Levin, Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults, Vaccine 27 (2009) 454–459.