Enhanced Memory Responses to Seasonal H1N1 Influenza Vaccination of the Skin with the Use of Vaccine-Coated Microneedles

Yeu-Chun Kim,1,a Fu-Shi Quan,2,a Dae-Goon Yoo,2 Richard W. Compans,2 Sang-Moo Kang,2 and Mark R. Prausnitz1

1School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, and 2Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia

Background. Morbidity and mortality due to influenza could be reduced by improved vaccination.

Methods. To develop a novel skin delivery method that is simple and allows for easy self-administration, we prepared microneedle patches with stabilized influenza vaccine and investigated their protective immune responses.

Results. Mice vaccinated with a single microneedle dose of trehalose-stabilized influenza vaccine developed strong antibody responses that were long-lived. Compared with traditional intramuscular vaccination, stabilized microneedle vaccination was superior in inducing protective immunity, as was evidenced by efficient clearance of virus from the lung and enhanced humoral and antibody-secreting cell immune responses after 100% survival from lethal challenge. Vaccine stabilization was found to be important, because mice vaccinated with an unstabilized microneedle vaccine elicited a weaker immunoglobulin G 2a antibody response, compared with the stabilized microneedle vaccine, and were only partially protected against viral challenge. Improved trafficking of dendritic cells to regional lymph nodes as a result of microneedle delivery to the skin might play a role in contributing to improved protective immunity.

Conclusions. These findings suggest that vaccination of the skin using a microneedle patch can improve protective efficacy and induce long-term sustained immunogenicity and may also provide a simple method of administration to improve influenza vaccination coverage.

Influenza remains a critical respiratory disease globally and is caused by viruses that are continuously under-going antigenic change [1, 2]. Vaccination is the most cost-effective public health measure for the prevention of disease caused by these pathogens [3]. Most vaccines are administered to humans by hypodermic needle injections, including intramuscular (IM), subcutaneous, and, in some cases, intradermal vaccinations. Vaccination using hypodermic needles requires trained medical personnel; thus, there are limitations for mass vaccination. Also, generation of biohazardous needle waste and needle-associated injuries and diseases are additional problems associated with lower rates of vaccination coverage. Simpler vaccination methods that are less painful and are easier for possible self-administration could significantly reduce morbidity and mortality due to such vaccine-preventable diseases as influenza [4–6].

Intradermal or transcutaneous vaccination of the skin has been suggested as an attractive method and has been performed to improve vaccine efficacies [7–9]. In particular, improved vaccine immunogenicity may be enabled by targeting the antigen-presenting Langerhans cells and dermal dendritic cells (DCs) in
the skin via intradermal delivery [9]. However, transdermal delivery is blocked by the outermost barrier layer of the skin (the stratum corneum) [8], and intradermal injection is time consuming, painful, and unreliable, and requires trained medical personnel [10].

Recently, we and others fabricated micron-scale needles that pierce the stratum corneum, to administer drugs, proteins, and DNA into skin [11–13]. Microneedles can be assembled into patches suitable for self-administration by means of low-cost manufacturing [12], and they have been reported to be painless and well-tolerated by human subjects [14, 15]. Some research has addressed vaccine delivery via the intradermal route using single hollow microneedles involving delivery of a liquid vaccine formulation by clinical personnel [16]. More-recent studies have examined deliveries of influenza vaccine to mice by use of coated microneedle patches with high-dose vaccines [17, 18]. Additional studies have assessed intradermal vaccination with influenza vaccines by use of hypodermic needles [7, 9]. However, the limitations of performing detailed immunologic studies in humans, especially to assess memory responses after viral challenge, and the difficulty of making intradermal injections in thin mouse skin have resulted in limited study of memory responses to influenza vaccination of the skin.

In the present study, we used microneedles to target vaccine delivery to the skin of mice with the use of a microneedle patch designed for simple administration with minimal training, and we studied the resulting immune responses before and after challenge. Our study also examined the immunogenic effect of influenza antigen stabilization using trehalose during microneedle vaccination formulation.

**MATERIALS AND METHODS**

**Preparation of inactivated influenza virus.** Formalin-inactivated influenza H1N1 A/PR/8/34 virus was prepared as described elsewhere [19]. For imaging experiments, inactivated whole virus was labeled by mixing 200 μL of inactivated virus (3 mg/mL) with 10 μL of octadecyl rhodamine B chloride (R18; Invitrogen) and incubating it at 25°C for 1 h. Unbound R18 molecules were removed by ultracentrifugation (at 28,000 g for 1 h).

**Fabrication and coating of microneedles and measurement of hemagglutination activity.** Stainless-steel microneedles were fabricated using laser cutting and electropolishing [20]. To apply a vaccine coating, microneedles were dipped 6 times for virus challenge, lightly anesthetized mice were intra-nasally infected with the mouse-adapted A/PR8 virus (50 μL of 20 × 10^6) 5 weeks after vaccination [19]. Mice were observed daily to monitor body weight changes and mortality rates. Animals with >25% loss in body weight were euthanized to minimize their suffering. All animal studies were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

**Antibody responses and antibody-secreting cells.** Influenza virus–specific antibodies of different isotypes (immunoglobulin G [IgG], IgG1, IgG2a, and IgG2b) were determined by following the standard enzyme-linked immunosorbent assay (ELISA) protocol, as described elsewhere [19].

To determine recall immune responses, bone marrow and Microneedles were imaged by bright-field microscopy (Olympus) with a CCD camera (Leica Microsystems) and fluorescence microscopy (Olympus) with a CCD camera (Diagnostic Instruments). To image delivery of vaccine into the skin, microneedles coated with R18-labeled virus were inserted into human cadaver skin for 10 min and were fixed by freezing in histologic mounting compound (Tissue-Tek) for 10 min, after which time microneedles were removed and skin was sectioned using a cryostat (Microm). This use of human skin was approved by the institutional review board of the Georgia Institute of Technology.

To measure hemagglutination (HA) activity, vaccine-coated microneedles were decoated in PBS at 4°C for 12 h. To determine HA titers, 50 μL of dissolved vaccines in PBS was serially diluted in 50 μL of PBS mixed with an equal volume of a fresh 0.5% suspension of chicken red blood cells (Lampire) and was incubated at 25°C for 1 h. The titers were determined as the end-point dilutions inhibiting the precipitation of red blood cells [21].

**Vaccination and infection with challenge virus.** Eight- to 10-week-old female BALB/c mice (10 mice/group) were anesthetized with 110 mg/kg ketamine (Abbott Laboratories) mixed with 11 mg/kg xylazine (Phoenix Scientific) and injected IM. The skin on the back of the mouse was exposed by removing the hair with depilatory cream (Nair), washed with 70% ethanol, and dried. An in-plane 5-needle array of microneedles coated with 0.4 μg of inactivated influenza virus was manually inserted into the skin and left in place for 10 min. For an IM control, 0.4 μg of inactivated influenza virus in 100 μL of PBS was injected IM into the upper quadriceps muscles of mice (50 μL per leg). The mock control mice were treated using similar microneedles prepared using coating solution without influenza vaccine. To determine the amount of inactivated virus vaccine coated on a microneedle, vaccine-coated microneedles were incubated in PBS at 4°C for 12 h, and the amount of dissolved protein was measured using a BCA Protein Assay Kit (Pierce Biotechnology).

For virus challenge, lightly anesthetized mice were intranasally infected with the mouse-adapted A/PR8 virus (50 μL of 20 × 10^6) 5 weeks after vaccination [19]. Mice were observed daily to monitor body weight changes and mortality rates. Animals with >25% loss in body weight were euthanized to minimize their suffering. All animal studies were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

To determine recall immune responses, bone marrow and
cells were harvested at day 4 after challenge were cultured in 96-well plates at $5 \times 10^5$ cells/well. Supernatants (4 × dilutions) were used to determine virus-specific antibody levels at days 1, 3, and 6 after culture [22].

**Analysis of lung samples.** Lung viral titers at day 4 after challenge were determined by counting the number of plaques that formed after incubation with serially diluted lung extracts on the Madin-Darby canine kidney cells [19]. Inflammatory cytokines (interleukin [IL]–6 and interferon [IFN]–γ) in lung samples collected at day 4 after challenge were analyzed by Ready-Set-Go cytokine kits (eBioscience), in accordance with the manufacturer’s procedure [23]. Lung antibodies were similarly determined by ELISA performed using serially diluted lung extracts [23].

**DC labeling and analysis.** We applied an approach similar to one previously used to track skin DCs draining to regional lymph nodes [24, 25]. Fluorescein isothiocyanate (FITC) (Sigma) was reconstituted in the coating buffer to a final concentration of 1 mg/mL. Microneedles were coated with FITC and inserted into mouse skin as described above. Equal amounts of FITC-coated microneedles dissolved into 100 µL of PBS were injected IM. After 24 h, mice were killed, lymph nodes were collected, and DCs were prepared using collagenase and DNase I [26]. Suspensions containing $5 \times 10^7$ single cells were then stained with DC phenotypic markers and analyzed using FACSscan flow cytometry (Becton Dickinson). Granular cells were gated and analyzed for CD11c+ and CD11c+FITC+ cell populations.

**Statistical analysis.** Every assay was measured using $\geq$3 samples for which the arithmetic mean and standard error of the mean were calculated and presented in the figures in this article. A 2-tailed paired Student’s $t$ test was performed when 2 time points were compared for the same set of animals (Table 1). When $\geq$3 conditions were compared, one-way analysis of variance (ANOVA; $\alpha = .05$) was performed. In some cases, nonparametric methods that expressed median values were compared to validate the results. In all cases, $P < .05$ was considered to denote statistical significance.

**RESULTS**

**Fabrication of microneedle patches and delivery into skin.** Microneedles fabricated by laser cutting stainless-steel sheets (Figure 1A) were designed to be long enough to penetrate through the stratum corneum and viable epidermis and into the superficial dermis by gentle manual insertion but short enough to avoid pain [15]. Our delivery strategy involved dip-coating solid microneedles with formulations of influenza vaccine (A/PR/8/34) that dry onto the microneedles and then rapidly dissolve in the skin. Dip-coating produced thick, uniform coatings localized to microneedle shafts (Figure 1B and 1C). Insertion of microneedles into the skin led to dissolution (Figure 1D and 1E) and deposition (Figure 1F and 1G) in the skin within minutes.

Addition of trehalose disaccharide to the microneedle coating formulation significantly improved the retention of HA activity of influenza vaccine antigens after drying on microneedles (Table 1). The optimized formulation retained 64% of HA activity after coating. This finding indicated an increase in antigen stability associated with trehalose, at least over the 24-h time frame of the stabilization study.

**Antibody responses after delivery of stabilized microneedle influenza vaccine.** We next evaluated immune responses induced by stabilized microneedle vaccine delivery and compared them with responses to IM injection in mice. Groups of BALB/c mice ($n = 10$) received single-dose vaccination via skin delivery using a microneedle array coated with 0.4 µg of inactivated whole virus with trehalose (MN+Tre) or without trehalose.

### Table 1. Hemagglutination (HA) Activity, Virus-Specific Serum Antibody Levels Expressed in Terms of End-Point Dilution Titers ($\times 10^4$) before and after Challenge, and Ratios of Virus-Specific Immunoglobulin G (IgG) 2a and IgG1 Antibodies before and after Challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>HA activity*</th>
<th>Before challengeb</th>
<th>After challengec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgG1</td>
<td>IgG2a</td>
</tr>
</tbody>
</table>
| Mock  | ... | 0.2 ± 0.5 | 0.2 ± 0.03 | 0.2 ± 0.03 | 0.1 | ... | 0.2 ± 0.05 | 0.2 ± 0.03 | 0.2 ± 0.03 | 0.2 ± 0.03 | 0.1 ± 0.02 | ...
| MN    | 1.6 ± 0.3 | 19.2 ± 2.4 | 25.6 ± 1.6 | 3.2 ± 0.7 | 0.8 ± 0.07 | 0.125 | 25.6 ± 3.9 | 51.2 ± 6.7 | 12.8 ± 0.7 | 0.4 ± 0.02 | 0.25 |
| MN+Tre| 63.7 ± 7.9 | 51.2 ± 2.9 | 128 ± 2.1 | 102.4 ± 5.8 | 96 ± 2.1 | 8 | 102.4 ± 5.6 | 38.4 ± 8.9 | 153.6 ± 16 | 9.6 ± 0.9 | 4 |
| IM    | 100 | 51.2 ± 6.1 | 64 ± 0.5 | 102.4 ± 4.3 | 64 ± 1.7 | 16 | 19.2 ± 2.1 | 3.2 ± 0.2 | 25.6 ± 2.8 | 1.6 ± 0.2 | 8 |

**NOTE.** HA activity was measured as an indicator of the functional integrity of hemagglutinin on the inactivated viral vaccine and is expressed as a percentage of activity relative to the same mass of unprocessed antigen. Titers are expressed as the highest dilution having a mean optical density value (measured at 450 nm) that was greater than the mean value plus 3 standard deviations of naive serum samples. Total IgG titers did not equal the sum of IgG isotype titers, probably because of differences in experimental measurement sensitivities. Such discrepancies have also been observed previously [27]. The same animals were used to generate the data in Figure 3C and Table 1. IM, intramuscular vaccination with intact influenza vaccine; MN, microneedle vaccination without trehalose formulation; MN+Tre, microneedle vaccination with trehalose formulation; mock, microneedle vaccination without influenza vaccine.

* Data denote the percentage of HA activity or the percentage ± standard error of HA activity.

b Serum samples collected at week 4 after vaccination ($n = 10$).

c Serum samples collected at day 4 after challenge ($n = 4$).

d IgG2a/IgG1 ratios were determined based on antibody titers.
Memory Responses to H1N1 Vaccination

Figure 1. Microneedle coated with influenza vaccine. A, Image of a 5-microneedle array (scale bar, 500 μm). Bright-field (B, D) and fluorescence (C, E) micrographs of a microneedle coated with red-fluorescent inactivated influenza virus before (B, C) and 10 min after (D, E) insertion into human cadaver skin (scale bar, 200 μm). Histologic section of human cadaver skin fixed after insertion of a vaccine-coated microneedle imaged by (F) bright-field microscopy showing skin deformation and the needle track across the epidermis and into the superficial dermis and by (G) fluorescence microscopy showing deposition of red-fluorescent vaccine coating in skin (scale bar, 200 μm).

In contrast, microneedle delivery without trehalose (MN delivery) showed lower levels of antibodies, as well as a shift in the isotype profile, such that IgG1 was the dominant antibody isotype (IgG2a/IgG1 ratio for MN delivery, 0.125) with significantly lower IgG2a and IgG2b antibody responses (Table 1).

Overall, these results suggest that the stabilized microneedle (MN+Tre) vaccine can induce antibody responses higher than (in the case of IgG1) or comparable to those achieved by IM vaccination. Moreover, vaccine stabilization with trehalose during microneedle coating was critical for maintaining the isotype (MN). Considering that the thickness of mouse skin is ~500–600 μm [24] and that the length of the microneedles is 700 μm, most vaccine was probably deposited within the mouse skin. The IM control group was vaccinated IM with 0.4 μg of unprocessed inactivated virus vaccine. An additional group was mock-treated using microneedles without vaccine.

Total IgG and IgG2a levels after stabilized microneedle vaccination were similar to those noted after IM vaccination (P > .05) (Table 1). Interestingly, both types of microneedle delivery (MN and MN+Tre) induced higher levels of IgG1 antibodies than did IM delivery (P < .01). For stabilized MN and IM deliveries, IgG2a was the dominant isotype (IgG2a/IgG1 ratio: for MN+Tre delivery, 8; for IM delivery, 16) (Table 1).

Figure 2. Protection against lethal challenge infection. Vaccinated mice were challenged with a lethal dose (20 × LD₅₀) of a highly pathogenic A/PR8 influenza virus 5 weeks after a single vaccination (n = 10). A, Body weight change. B, Monitoring of survival rates daily for 14 days (n = 6). Similar survival rates were obtained in 2 independent experiments indicating reproducible results. Dead animals were removed, and only live animals were counted for the body weight analysis, reflecting the rebound in body weight as a result of recovery. For the analysis performed on day 4 after challenge, 4 of 10 mice were killed, and the remaining 6 mice were monitored. IM, intramuscular vaccination with unprocessed influenza vaccine; MN, microneedle vaccination with influenza vaccine formulated in the absence of trehalose; MN+Tre, microneedle vaccination with influenza vaccine formulated in the presence of trehalose (15%); mock, microneedle vaccination without vaccine.
Figure 3. Protective efficacy of microneedle vaccination. A, Lung virus titers. Lungs from individual mice were extracted (1 mL of media/mouse lung [n = 4]). The detection limit for lung viral titers was 50 pfu per milliliter of lung extracts of individual mice. B, Lung inflammatory interleukin-6 and interferon-γ cytokines (n = 4). C, Virus-specific antibodies in lungs (n = 4). Lungs were collected from individual mice at day 4 after challenge, and antibody levels determined by enzyme-linked immunosorbent assay are expressed as end-point dilution titers. Groups of mice are as described in the Figure 2 legend. IM, intramuscular vaccination with unprocessed influenza vaccine; MN, microneedle vaccination with influenza vaccine formulated in the absence of trehalose; MN+Tre, microneedle vaccination with influenza vaccine formulated in the presence of trehalose (15%); mock, microneedle vaccination without vaccine. *MN+Tre vs mock, MN, and IM (P < .05); **MN+Tre vs mock and MN (P < .05); +MN+Tre vs mock (P < .05).

Protection against lethal infection with challenge virus. Vaccinated mice were challenged with a lethal dose of influenza A/PR8 virus (20 × LD₅₀) at 5 weeks after single-dose vaccination (Figure 2). Mice immunized with a stabilized microneedle vaccine (MN+Tre) or by IM delivery were completely protected without experiencing body weight loss. However, the MN group without trehalose experienced body weight losses ranging from 15% to >25%, which is statistically significant (P < .05). The survival rate of mice in the MN group was decreased to 67%. All mice in the mock control group died or had to be killed by day 6. Thus, MN+Tre vaccination provided protection equal to that associated with IM vaccination, and the formulation of microneedles to maintain influenza vaccine stability was critically important in providing protective immunity against lethal infection with challenge virus.

Protective efficacy of microneedle vaccination. Determination of lung viral titers could be informative to indicate the strength of the protective immune capacity of the host to control the challenge virus replication. We found that, at day 4 after challenge, the IM and MN groups had significantly reduced lung viral titers compared with mock control mice (Figure 3A) (P < .01). Of interest, the viral titers of mice in the MN+Tre group were below the limit of detection, indicating drastically improved clearance of challenge virus by day 4 compared with that noted in the MN and IM groups (P < .05).

We also measured production of lung proinflammatory cytokines known to cause tissue damage and increased mortality [25]. Challenged mice in the mock group exhibited high levels of inflammatory cytokines, whereas no IFN-γ and significantly lower levels of IL-6 were detected in the lungs of mice in the MN+Tre group, compared with those of mice in the MN group (Figure 3B) (P < .05). Of note, mice in the MN+Tre group had lower levels of IL-6 than did mice in the IM group (P < .05). Overall, these results indicate that microneedle delivery of sta-
Memory Responses to H1N1 Vaccination

**Figure 4.** Rapid recall and long-term immune responses. Bone marrow and spleen cells were harvested at day 4 after challenge \( (n = 4) \), and the kinetics of virus-specific immunoglobulin G (IgG) antibody production were determined. Antibody levels in in vitro culture supernatants diluted 4-fold were determined by enzyme-linked immunosorbent assay (ELISA) and expressed as the optical density value (measured at 450 nm) after 1–6 days of incubation and are expressed in concentrations (nanograms per milliliter) using standard mouse antibodies. **A,** Bone marrow cell cultures \( (5 \times 10^5 \text{ cells/well}) \) in the absence of influenza virus antigen stimulation \( (n = 4) \) (**P** < .05). **B,** Spleen cell cultures \( (5 \times 10^5 \text{ cells/well}) \) in the plate coated with inactivated influenza viral antigen \( (n = 4) \) (**P** > .005). Groups of mice \( (n = 4) \) (**P** > .005) are as described in the legend of Figure 2. **C,** Long-term maintenance of antibody levels by microneedle vaccination. In an independent experiment to assess long-term antibody responses, virus-specific antibody responses were determined over a 9-month period in mice \( (n = 6) \) vaccinated in the skin with trehalose-formulated microneedle vaccine \( (0.7 \text{ mg of inactivated influenza virus}) \). Time 0 is the IgG value from the serum samples obtained before vaccination of mice with microneedle vaccine. Serial diluted serum samples were used for ELISA, and antibody levels are expressed in concentrations (micrograms per milliliter) from a mouse antibody standard curve. IM, intramuscular vaccination with unprocessed influenza vaccine; MN+Tre, microneedle vaccination with influenza vaccine formulated in the presence of trehalose (15%); mock, microneedle vaccination without vaccine. **MN+Tre vs mock and IM.**

Rapid-recall humoral immune responses induced by microneedle delivery. To better understand the improved protection observed in association with microneedle vaccination, we compared influenza virus–specific antibodies in serum and lungs at day 4 after challenge between the MN+Tre and IM groups. Both microneedle vaccine groups (MN and MN+Tre) showed significantly higher levels of virus-specific total IgG and isotype antibodies, including IgG1 and IgG2a isotypes, at day 4 after challenge, compared with levels noted before challenge (Table 1) (**P** < .05). In contrast, at day 4 after challenge, antibody levels in mice in the IM group were lower than the levels noted before challenge (Table 1) (**P** < .05). After challenge, IgG2a was still the dominant isotype in both the MN+Tre and IM groups, which exhibited IgG2a/IgG1 ratios ranging from 4 to 8. The MN group showed IgG1 as a dominant isotype.

Of importance, levels of virus-specific antibodies, including IgG, IgG1, and IgG2a, in the lungs were considerably higher in the MN+Tre group than in the IM and MN groups (Figure 3C). It is interesting to note that total IgG from the IM group was higher in the lung but slightly lower in serum samples, compared with corresponding samples from the MN group.
Figure 5. Dendritic cell (DC) migration to the draining lymph nodes. After 1 day of treatment of 5 mice with microneedle or intramuscular (IM) delivery of fluorescein isothiocyanate (FITC), the inguinal lymph nodes were harvested, and CD11c+ and CD11c+FITC+ DC populations were analyzed by flow cytometry. Percentages of gated populations in the upper quadrants of each dot plot are shown. The plots are representative of 2 independent experiments.

 Trafficking of DCs to the lymph nodes. These improved anamnestic responses could be explained by vaccine targeting to both epidermal Langerhans cells and dermal DCs via delivery to the skin [26]. To assess this possibility, we compared the levels of CD11c+ DCs in lymph nodes (Figure 5) [28]. Mice receiving microneedle delivery showed higher populations of DCs trafficked to the lymph nodes than did mice receiving IM delivery. Of importance, at 1 day after delivery, microneedle delivery induced in inguinal lymph nodes CD11c+FITC+ DC populations approximately twice as large as those noted after IM delivery. These results suggest that microneedle delivery to the skin led to more-effective migration of DCs capturing antigens to the lymph nodes, compared with IM vaccination. Because DCs are known to be professional antigen-presenting cells capable of stimulating naive T and B cells [29], effectively targeting an influenza antigen to dermal DCs might explain efficient induction of recall immune responses after delivery to the skin using microneedles.

DISCUSSION

The findings reported in the present study show that MN+Tre vaccination of the skin induced better protective immunity, as measured by lung virus titer, recall B cell responses, and long-lived plasma cells, than did MN or IM vaccination. In addition, virus-specific postchallenge serum total IgG and isotype antibody (IgG2a and IgG2b) levels were significantly higher in the MN+Tre group than in the MN and IM groups.

The IgG2a isotype antibody is known to promote a cascade of complement activation and to be more efficient in clearing viral and bacterial infections [30–32]. A high level of IgG2a induced by the MN+Tre vaccination might have contributed to effective clearing of the challenge virus, compared with IM vaccination. The total levels of binding antibodies and protective efficacies were much lower in the MN group than in the MN+Tre group.
Our mechanistic analysis suggests that improved immunogenicity starts with more-efficient antigen delivery to DCs in the skin, which migrated to the regional lymph node after vaccination using microneedles. This, in turn, enables more-efficient activation of naïve B cells to generate long-lived plasma cells and memory B cells, which rapidly increase lung and serum antibody levels after challenge. As evidence for long-lived immunity, we observed the long-term maintenance of virus-specific antibodies for >9 months after a single vaccine dose using microneedles. In contrast, a previous study demonstrated that IM vaccination with split influenza vaccines showed a decreasing trend of virus-specific antibody levels 21 days after vaccination [27]. The combination of these increased humoral and cellular anamnestic responses can explain the effective control of viral replication in the lungs resulting in lower lung inflammatory cytokines and the associated excellent protection against viral challenge.

Whole inactivated influenza vaccines contain a lipid-bilayer membrane that contains hemagglutinin as a major glycoprotein. Hemagglutinin is the most important antigenic target for inducing protective immunity. Recent studies demonstrated that solid microneedle vaccination with high doses of inactivated influenza virus induced protection similar to that provided by IM vaccination, although the stability of microneedle vaccines was not investigated [17, 18]. The stability of vaccine is assumed to be important for its immunogenic efficacy. We believe it is notable that a relatively simple formulation of trehalose was able to maintain immunogenicity of whole-virus vaccine after drying onto microneedles and rehydration in the skin.

The immune responses induced by MN delivery were different from those induced by MN+Tre delivery in terms of quantity and quality. It is interesting to note that the pattern of antibody isotypes was strikingly opposite between the MN and MN+Tre groups of mice. We speculate that the functional integrity of hemagglutinin in the influenza vaccine might be important for effective interactions with receptors to recognize pathogen-associated molecular patterns, such as Toll-like receptors that are expressed on antigen-presenting cells, including Langerhans cells and dermal DCs [33] or B cells [34]. Interactions between the hemagglutinin of influenza vaccines and receptors on antigen-presenting cells are likely to induce Th1 helper type 1 (Th1) cytokines, which would significantly influence the pattern of antibody isotypes. In contrast, macrophages engulfing particulate antigens without engaging receptor interactions were shown to induce Th2 type immune responses associated with IgG1 isotype antibodies [35].

Apart from immunologic merits, microneedles have potential logistic advantages too. Vaccination using microneedles has a reduced probability of bloodborne pathogen transmission resulting from inaccessibility to the bloodstream. In future work, microneedles can be developed as a patch suitable for self-administration with a design engineered to prevent re-use by using dissolvable or retractable microneedles. In addition, microneedles are expected to be cost efficient for mass vaccination, at a manufacturing price similar to a needle and syringe [12, 36]. These characteristics of microneedles could increase coverage of seasonal and pandemic influenza vaccination by facilitating school-based vaccination of children and easy access to vaccination in elder-care facilities to minimize risks of cross-contamination and long delays associated with injection-based vaccination at centralized clinics [4]. Self-administration should be carefully monitored by medical personnel for potential concerns regarding any side effects.

In conclusion, we found that microneedle vaccination of the skin generated better control of viral replication and reduced inflammatory responses in the lungs, probably as a result of skin-derived DC activation and antibody-secreting plasma cell responses in bone marrow, compared with such responses noted after IM injection. These immunologic advantages, combined with logistic benefits, indicate that microneedle delivery to the skin may offer a strategy for improved influenza vaccination, which might also be applicable to delivery of other vaccines.

Acknowledgments

This study was performed at the Emory University School of Medicine and the Georgia Institute of Technology Center for Drug Design, Development, and Delivery and the Institute for Bioengineering and Biosciences. We thank Thuc-Vy Le for assistance in preparing lymph nodes, Huan Nguyen for the mouse-adapted influenza virus A/PR8/34 strain, and Mark Allen for use of his laser microfabrication facilities.

References