

MATERIALS AND METHODS

Microneedle Fabrication and Coating

As described elsewhere [14], metal microneedles were fabricated by laser-etching stainless steel sheets (McMaster-Carr). Microneedles were prepared in rows of 5 microneedles. Each microneedle measured 700 μm long, with a cross sectional area of 170 μm by 55 μm at the base and tapering to a sharp tip. Microneedles were dip-coated using a coating solution formulated with 1% (w/v) carboxymethylcellulose (Carbo-Mer), 0.5% (w/v) Lutrol F – 68NF (BASF), 15% (w/v) D-(+)-trehalose dihydrate (Sigma), and 5 $\mu\text{g}/\text{mL}$ inactivated H1N1 2009 virus vaccine [21].

Cells and Viruses

Madin-Darby canine kidney cells (ATCC CCL 34, American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (Mediatech) containing 10% fetal bovine serum (Hyclone; ThermoFisher Scientific). Influenza virus stocks (A/California/04/09, H1N1) were prepared, purified, and inactivated, as described elsewhere [22]. Hemagglutination (HA) activity was determined using turkey red blood cells (LAMPIRE Biological Laboratories) [23]. The mouse-adapted virus was obtained using 5 serial passages in lungs of BALB/c mice. The LD_{50} was calculated using the Reed-Muench formula, and viral titer was determined by plaque assay [14].

Vaccinations and Characterization of Immune Responses

Female BALB/c mice (Charles River Laboratory; 30 mice per group; age, 6–8 weeks) received 1 dose (5 μg) of the vaccine administered with microneedles or subcutaneously, which is the route most closely related to skin vaccination. For microneedle delivery, the mice were treated by manual insertion of the microneedles into skin on the dorsal surface for 5 min [14, 16]. A placebo group was treated in the same way with uncoated metal microneedles. Unimmunized mice were used as an additional negative control. Animals were bled retro-orbitally 2, 4, and 24 weeks after vaccination under systemic anesthesia [14]. Six weeks and 24 weeks after vaccination, mice ($n = 5$) were challenged with $10 \times \text{LD}_{50}$ dose of live mouse-adapted H1N1 virus and were monitored for 14 days for signs of morbidity (body weight changes, fever, and hunched posture) and mortality. A weight loss of $>25\%$ was used as the experimental endpoint, at which mice were euthanized according to the Institutional Animal Care and Use Committee guidelines. Four days after challenge of an independent cohort, blood samples were collected to determine humoral immune responses and lung samples were collected to determine virus and antibody titers, cytokine expression levels, and histopathological changes. All serum samples and lung homogenates were individually processed to determine humoral immune responses (total IgA, IgG, IgG isotypes, and hemagglutination inhibition [HAI] titers) [14]. Cellular immune responses were estimated using cytokine enzyme-linked immunosorbent assay (ELISA) [14, 22]. Finally, a cohort of immunized unchallenged mice ($n = 5$ per group) was euthanized 12 weeks after vaccination, and spleen and bone marrow were collected for the measurement of influenza-specific IgA and IgG antibody-secreting cells (ASC) and plasma cells by enzyme linked immunospot assay (ELISPOT) [24–26]. All animal studies had approval of the Emory University's Institutional Animal Care and Use Committee.

Histopathological Examination

Lung, liver, and spleen tissue samples were collected and fixed in 100% formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin [27]. The stained samples were examined for signs of pathological changes under light microscopy.

Statistics

The statistical significance of the differences was calculated by 2-tailed unpaired Student's *t* test and 1-way analysis of variance (ANOVA; including Bonferroni's multiple comparison test) or 2-way ANOVA. Values of $P \leq .05$ were considered to be statistically significant. Unless otherwise stated, independent experiments were run at least in triplicates.

References

14. Koutsonanos DG, del Pilar Martin M, Zarnitsyn VG, et al. Transdermal influenza immunization with vaccine-coated microneedle arrays. *PloS One* 2009; 4:e4773.
16. Sullivan SP, Koutsonanos DG, Del Pilar Martin M, et al. Dissolving polymer microneedle patches for influenza vaccination. *Nat Med* 2010;16:915–20.
21. Kim YC, Quan FS, Compans RW, Kang SM, Prausnitz MR. Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity. *J Control Release* 2010; 142:187–95.
22. Skountzou I, Quan FS, Jacob J, Compans RW, Kang SM. Transcutaneous immunization with inactivated influenza virus induces protective immune responses. *Vaccine* 2006; 24:6110–9.
23. Compans RW. Hemagglutination-inhibition: rapid assay for neuraminic acid-containing viruses. *J Virol* 1974; 14:1307–9.
24. Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods* 1983; 65:109–21.
25. Slifka MK, Ahmed R. Long-lived plasma cells: a mechanism for maintaining persistent antibody production. *Curr Opin Immunol* 1998; 10:252–8.
26. Crotty S, Aubert RD, Glidewell J, Ahmed R. Tracking human antigenspecific memory B cells: a sensitive and generalized ELISPOT system. *J Immunol Methods* 2004; 286:111–22.
27. Itoh Y, Shinya K, Kiso M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* 2009; 460:1021–5.

User Defined ID Format

General format:

- Study ID_species_treatment_challenge_sample type_Experiment

Abbreviations:

Abbreviation	Description
DK	Dimitrios G. Koutsonanos
M	Mouse
MN	Microneedles
SC	Subcutaneous
UC	Uncoated
L	Lung Sample
S	Serum
D	Day
Vac	Vaccination
Inf	Infected
Histo	Histopathology
BM	Bone Marrow
SP	Spleen