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A protective role of murine langerin+ cells in immune responses to cutaneous vaccination with microneedle patches

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Cutaneous vaccination with microneedle patches offers several advantages over more frequently used approaches for vaccine delivery, including improved protective immunity. However, the involvement of specific APC subsets and their contribution to the induction of immunity following cutaneous vaccine delivery is not well understood. A better understanding of the functions of individual APC subsets in the skin will allow us to target specific skin cell populations in order to further enhance vaccine efficacy. Here we use a Langerin-EGFP-DTR knock-in mouse model to determine the contribution of langerin+ subsets of skin APCs in the induction of adaptive immune responses following cutaneous microneedle delivery of influenza vaccine. Depletion of langerin+ cells prior to vaccination resulted in substantial impairment of both Th1 and Th2 responses, and decreased post-challenge survival rates, in mice vaccinated cutaneously but not in those vaccinated via the intramuscular route or in non-depleted control mice. Our results indicate that langerin+ cells contribute significantly to the induction of protective immune responses following cutaneous vaccination with a subunit influenza vaccine.

Cutaneous immunization has gained increasing interest due to its ability to induce robust host immune responses1. Although the cornerstone of influenza prevention is vaccination, the current conventional method of annual influenza vaccination is intramuscular injection of inactivated trivalent subunit or split vaccine which can only provide moderate protection against influenza2. Microneedle technology platform takes the advantage of the immunological potential of skin and relies on controlled and rapid delivery of the antigen to epidermal and dermal layers3. The length of the microneedles is 600–700 µM which is appropriate for both mouse and human skin despite their difference in thickness4. In the process of skin insertion the needles span both the epidermis and the dermis delivering the vaccine to both layers5.

Skin is the largest immunological organ in the body. In addition to harboring a large number of T lymphocytes, it is densely populated by antigen presenting cells (APC) which are important sentinels against pathogens5,6. The epidermis is populated by Langerhans cells (LCs), which are specialized APCs characterized by the expression of langerin (CD207), a type II transmembrane C-type lectin7,8, and MHCII molecules15,17. Although langerin expression was initially thought to be unique for LCs, it is also expressed in subpopulations of DCs and migrating LCs in the dermis and within skin-draining lymph nodes (LN)5,7–15. Several subsets of dermal DCs (dDC) are observed in both human and mouse dermis. In mice, the dermis contains at least five different DC subsets which can be differentiated based on their expression of langerin, CD11b, CD103, and CD8α markers. Most antigens delivered to the skin are captured by APCs which migrate to skin-draining lymph nodes, although some can move to draining lymph nodes via a cell-independent mechanism9. Among lymph node-resident DCs, langerin+ /
CD8+ cells constitute about 20%15 and are reportedly superior to other dermal DC in promoting T-helper type 1 (Th1) cell differentiation.

A few studies have investigated the induction of adaptive immune responses in mice following gene gun delivery of OVA or β-galactosidase21–24 or microneedle delivery of recombinant human adenovirus encoding HIV-1 gag4. The contribution though of individual APC subsets in protective immunity to microneedle immunization with influenza subunit vaccines has not been completely elucidated. In previous studies we have shown that following microneedle vaccination with Alexa 488 labeled influenza virus the majority of the influenza antigen-positive cell emigrating from auricular explants in the medium were CD11c+ whereas the numbers of CD11c-negative cells were approximately 3-fold lower. FACS analysis showed that more than 50% were activated and mature25. The findings were in agreement with earlier reports26. Based on our preliminary observations and on other reported studies that dermal langerin+ DCs are once again detectable within the dermis and 2–3 weeks for vaccination, we coupled the Langerin-EGFP-DTR mouse model with influenza-A induction IgG2c antibody titers follow microneedle vaccination (Supplementary Figure 1C). There was no statistical significance between levels of IgG2c in the PBS-treated Langerin-EGFP-DTR group (Figure 3C) and the DT-treated C57BL/6 group (Supplementary Figure 2C), p = 0.4629 by Student unpaired 2-tailed t-test. There was no difference between PBS and DT treated C57BL/6 mice in influenza-specific binding antibody titers, ensuring that diphertheria toxin treatment targets specifically DT receptors and does not cause a systemic toxic effect (data not shown)27.

We next determined how the loss of langerin+ cells impacts the generation of antibodies against influenza hemagglutinin using the hemagglutination inhibition (HAI) assay or the microneutralization assay (NT) both of which are considered correlates of vaccine-induced protective immunity28. The DT+ group had significantly lower serum HAI titers than the DT− group on both days 14 (p = 0.009) and 28 (p = 0.016) (Figure 4A). A two-fold decrease in the neutralizing antibody titers (NT) was also observed in LC-depleted mice (Figure 4B). These results indicate that functional antibody responses to cutaneous vaccination are impaired in LC-depleted mice.

Virus-specific antibody secreting cells (ASC) in draining lymph nodes and spleens are decreased following langerin+ depletion. Since we observed reduced humoral immune responses in the DT-treated mice, cutaneous lymph nodes and spleens were analyzed for IgM and IgG-secreting cells at 0, 7 and 14 days after immunization. We chose these time points because we have observed that between days 7 and 14 peak influenza-specific IgM and IgG ASC peak responses occur. At least a twelve-fold higher frequency of IgM-secreting cells was observed in lymph nodes of control mice (DT−) at day 7 compared to the DT+ mice (Figure 4C). At day 14, despite a decline of IgM-secreting cells in the DT− group, the numbers remained two-fold higher than those in the DT+ mice (p ≤ 0.001). Mice treated with DT had also lower numbers of IgG-secreting cells at both days 7 and 14 post-vaccination (Figure 4D).

Overall influenza specific IgM and IgG secreting cells in spleens were significantly lower than those observed in lymph nodes. Thus, the numbers of IgM- secreting cells in spleens were already reduced by 50% in DT+ mice 7 days after immunization when compared to the controls (p = 0.005). Despite lower numbers of IgG-secreting cells in spleens of DT+ mice as compared to the controls the differences were not as significant as in lymph nodes, indicating that the effect of LC depletion is more pronounced at sites proximal to antigen delivery.

Langerin+ cell depletion reduces IFN-γ and IL-4 secreting cells in lymph nodes. Naïve T cell differentiation into Th1 or Th2 cells following antigen presentation depends on the cytokine environment. Th2 cells produce IL-4 and mediate humoral immune responses, while Th1 cells secrete IFN-γ and mediate cellular immune responses32. We found that the numbers of IL-4 secreting cells collected on day 7 and 14 from the lymph nodes of DT-treated Langerin-EGFP-DTR mice were reduced by at least 90% (Figure 5A). Since IL-4 is involved in isotype class switch33, this result is consistent with the delayed kinetics of IgM and IgG secreting plasma cells observed in lymph nodes of DT-treated mice at day 14. Similarly, significantly lower numbers of IFN-γ secreting cells were detected in
lymph nodes of the DT+ group as compared to control mice (Figure 5B), consistent with the decreased levels of IgG2c. Although influenza specific IL-4 and IFN-γ secreting cells were detected in spleens of the DT+ and DT− mice, no difference was observed between groups in these cell numbers (Figure 5A and 5B). Thus depletion of langerin+ cells alters the generation of cells able to produce cytokines relevant to class switching and cellular immune responses in lymph nodes proximal to the site of immunization.

Protective immunity is compromised in langerin−-depleted mice following cutaneous vaccination with microneedles. To investigate the effect of langerin− depletion prior to cutaneous immunization on protective immunity, DT-treated mice were initially challenged with 5 × LD₅₀ of mouse-adapted A/California/07/09 (H1N1) virus five weeks after vaccination. Greater weight losses (7–8%) were observed in langerin−-depleted mice when compared to control (DT−) mice (3–4%) at 6 to 8 days post-infection (Figure 6A). Both DT+ and DT− groups survived this challenge, whereas the unvaccinated ( naïve) mice died by day 8 (Figure 6B). When the challenge infection was repeated with a four-fold higher dose of virus, the DT+ group incurred 12–15% weight losses while the DT− group lost only about 8–10% of the initial body weight at 6–8 days (Figure 6C). Although there was no statistical significance of differences between survival curves likely due to relatively small numbers of mice per group (10 per group), it is worth noting that the DT+ group showed a 25% higher mortality than the DT− cohort.
Consistent with these results, analysis of virus load in lungs of mice infected with the $20 \times \text{LD}_{50}$ dose showed about 1.5-log lower titers in control mice (DT$^2$) than the naive uninfected control. In contrast the DT$^1$ mice exhibited only about 0.5-log lower virus lung titers than the naive mice (Figure 6E). The average reduction of virus load correlated the average serum HAI titers measured on day 28 in each vaccinated group and the unvaccinated control. Thus, the virus load (expressed in PFU/g tissue) of the DT$^2$ group with an average serum HAI titer of 24 was eight times lower than the virus load of DT$^1$ mice with an HAI titer of 16, and twenty one times lower than the lung titers of naive mice with HAI titer at the detection limit.

In contrast to cutaneous immunization with vaccine coated microneedles, similar levels of influenza-specific binding and functional antibodies (Figure 7A–E) were observed in DT$^2$ or DT$^1$ mice vaccinated via the intramuscular route which, when challenged with $20 \times \text{LD}_{50}$ homologous virus, demonstrated about 1.5 log reduction of viral lung titers when compared to naive mice (Figure 7F). These results support the conclusion that langerin$^+$ cells play an important role in protective immunity elicited by cutaneous vaccination.

**Discussion**

Microneedle technology has become one of the most promising novel vaccine delivery platforms. From the immunological point of view, epidermal and dermal layers of skin are densely populated in antigen presenting cells, hence skin is a very attractive site for vaccine delivery. In this study metal microneedles coated with subunit influenza vaccine were used to deliver subunit influenza vaccine antigen to mouse epidermis and dermis skin.

We observed that 95% of the vaccine is eliminated from the intact skin within 24 hours, whereas only 65% of the vaccine is eliminated from langerin$^+$ cell-depleted skin. The depletion of langerin$^+$ cells prior to cutaneous vaccination resulted in impaired humoral and cellular immune responses. Influenza-specific functional antibody titers were reduced and the Th1 and Th2 profiles in the draining lymph nodes were altered. Absence of langerin$^+$ cells affected isotype switching and resulted in a major reduction of IgG2c antibodies. This reduction is particularly significant because these antibodies have high affinity for complement proteins and Fc receptors, mediating opsonization and presentation of antigens to cells. IgG2a antibodies from BALB/c mice (corresponding to IgG2c in C57BL6) have been associated with increased efficacy of influenza vaccination, virus neutralization, protection against infection, and immunity to heterologous viruses. Consistent with the decrease of IgG2c titers, the frequency of IFN-$\gamma$ secreting lymphocytes in DT-treated mice was markedly reduced, further supporting the role of langerin$^+$ cells.

Figure 2 | Langerin-EGFP DTR mice were injected with DT (DT$^+$) or PBS (DT$^-$) two days prior to immunization with 5 $\mu$g of A/CA/07/09 vaccine. Spleens (SP) and lymph nodes (LNs) were collected on days 7 and 14; sera on days 14 and 28 post-immunization. Mice were challenged 35 days after immunization.

Figure 3 | Humoral immune responses are reduced in mice depleted of langerin$^+$ cells prior to skin vaccination. Anti-influenza binding antibodies were determined by ELISA in sera collected from DT$^+$ and DT$^-$ mice 14 and 28 days after immunization. (A) IgG, (B) IgG1 and (C) IgG2c antibody titers. (D) IgG1/IgG2c antibody ratios. Values are expressed as mean $\pm$ SEM ($n$ = 15).
Significant decrease and altered kinetics of induction of influenza-specific IgM and IgG antibody secreting cells were mainly observed in lymph nodes proximal to site of vaccination and to a lesser degree in the spleens of DT+ and DT− groups. The mechanism for the slow switch is not yet known but we will follow up in subsequent studies. Interestingly the significantly reduced numbers of IgM and IgG ASC correlated well with the neutralizing antibody titers in the DT+ group. It is likely that the intensity of ASC responses are less pronounced in the spleen because systemic passage of antigens through the vascular supply of the skin, hair follicles and lymphatics is limited, although in humans antigens may have preferential interaction with local cell subsets because the numbers of hair follicles are greatly reduced when compared to mice. However, the qualitative and quantitative differences in antibody secreting cell numbers in local lymph nodes and significant decrease in IgG2c titers in sera suggest that lack of langerin+ cells in mice vaccinated using microneedles results in impaired clonal expansion of B cells to ASCs.

Although it was reported that the bulk of langerin+ (including CD8+) DCs are present in splenic marginal zones around white pulp nodules, and hence ideally placed to take up antigens delivered by the bloodstream, the langerin+ CD8+ DCs in spleen inefficiently take up antigens from blood44. Consistently with these observations, we found that depletion of langerin+ cells did not alter host immunity following intramuscular immunization. These findings provided further evidence that skin langerin+ cells are more important in the induction of immune responses to cutaneous vaccination as they are in close proximity to antigen delivery site.

The role of epidermal LCs in immunomodulation has been reassessed in recent studies, indicating that langerin+ cells may play an important role in the induction of adaptive immune responses21,24,45.

Figure 4 | Depletion of langerin+ cells prior to skin vaccination results in decreased functional antibody titers and fewer antigen-specific IgM and IgG secreting cells in LNs. (A) Hemagglutination inhibition (HAI) and (B) neutralizing antibody (NT) titers in sera collected 14 and 28 days after immunization. Values are expressed as geometric mean with a ±95% confidence interval (n = 15). ELISPOT assay of (C) IgM and (D) IgG secreting cells from spleens and inguinal lymph nodes on day 7 and 14 after vaccination. Values are expressed as mean ± SEM (n = 5). Plasma cell numbers of vaccinated mice were considered positive if the numbers of spots were higher than the sum of naive infected group spots + 3 × SDev.

Figure 5 | Depletion of langerin+ cells prior to skin immunization results in reduced T-cell responses in LNs. Cutaneous lymph nodes (LN) and spleens (S) were collected on day 7 and 14 after immunization of Langerin-EGFP DTR mice. (A) IL-4 and (B) IFN-γ secreting cells were quantified by ELISPOT. Values are expressed as mean ± SEM (n = 5).
In one study the authors suggested that langerin$^+$ cells are not required for CD8 T-cell priming following delivery of live adenovirus HIV-1 Gag vaccine via dissolvable microneedles. Although our results also showed that langerin$^+$ cells were dispensable for the induction of immune responses in spleen, they are important for the induction of immune responses in skin draining lymph nodes following vaccination with microneedle patches coated with influenza vaccine. There are several possible reasons for these differences between their study and ours. First, influenza immunization relies on robust humoral immune responses, whereas live adenovirus vaccines induce robust CD8 T-cell responses hence the role of APC subsets may be vaccine-dependent. Secondly, the delivery technology was different as we used metal microneedle arrays vs. polymer needles. Thirdly, the site of immunization was different as we applied microneedles to dorsal flank skin and determined the frequencies of APCs in inguinal lymph nodes as opposed to dorsal surface of the foot to analyze popliteal lymph nodes. Differences in the contribution of langerin$^+$ cells to the induction of immune responses were also observed when the antigen was applied to the ear versus flank skin, suggesting that use of different sites of immunization will impact the results obtained.

We did not distinguish the role of LCs versus langerin$^+$ dDCs in the induction of immune responses to cutaneous vaccination because with the microneedle platform vaccine is delivered to both the epidermal and dermal skin layers. Previous studies using gene gun vaccination for protein or DNA vaccines reached differing conclusions on the role of LCs and langerin$^+$ dDCs. It has been postulated that LCs are not required for the induction of humoral or cellular immune responses or that they may facilitate IgG1 production, a Th2-dependent process or may play an immunosuppressive role in unperturbed skin. In the case of microneedle insertion, an event that perturbs the skin architecture and initiates a sequence of inflammatory events due to chemokine and cytokine secretion, the involvement of LCs may upregulate the immune responses instead. As for the involvement of langerin$^+$ dDCs in immune responses it...
has been suggested that they may only mediate differentiation of CD8+ T cells or that they may participate in the initiation of humoral immune responses and optimal production of IgG2a/c and IgG2b antibodies early in the response thus promoting Th1 cell differentiation as compared to other DCs. Our findings are in agreement with reports on the importance of both langerin+ dDCs and LCs in the induction of optimal CD8+ T cell responses following skin vaccination since we showed that depletion of langerin+ cells led to a dramatic reduction of IgG2c antibodies and decrease of IFN-γ secreting T cells.

While we found that langerin+ cells contribute significantly to the induction of immune responses in regional lymph nodes following cutaneous immunization, the survival rates of langerin+ cell depleted animals in the challenge study indicated that they only play a partial role in the protective efficacy of this vaccination approach. The reduction of protection against a lethal dose of influenza virus demonstrated a correlation with the functional antibody titers as well as the percent of vaccine retention in murine skin treated with DT.

It will be of interest to further dissect the role of other APC populations using additional transgenic mouse models following cutaneous delivery. Also, the approach used for antigen delivery (metal versus polymer microneedles) may affect the recruitment of various cell subsets and antigen-APC interaction due to different physicochemical properties affecting vaccine diffusion rate. More detailed

Figure 7 | Depletion of langerin+ cells prior to intramuscular immunization does not affect immune responses. (A) IgG, (B) IgG1, (C) IgG2c, (D) HAI and (E) NT antibody titers were measured in sera collected on days 14 and 28 after intramuscular immunization of Langerin-EGFP-DTR mice. (F) Mice were challenged with 20 × LD50 of homologous virus and titers of virus in lungs 4 days after challenge were assessed by plaque assay (4–5 mice/group). IgG titers and PFU/g lung tissue are expressed as the mean ± 95% confidence interval.

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understanding of the immunological mechanisms involved in cutaneous immunization and the identification of specific subsets of APCs that are important for capturing antigens and for induction of immune responses could enable further improvements in vaccine efficacy by including adjuvants that target the skin APCs or by targeting antigens to selected DC subtypes.

**Methods**

**Cells and virus stocks.** Madin-Darby canine kidney (MDCK) cells (CCL 34, ATCC, Manassas, VA) were maintained in Dulbecco’s Modified Eagle’s Medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum (HyClone, Thermo Scientific, Rockford, IL). Influenza virus stocks (A/California/07/2009 (H1N1)) were propagated in MDCK cells. The hemagglutination (HA) activity was determined using turkey blood cells (LAMPIRE, Piperville, PA). Mouse-adapted virus was obtained by serial passage in lungs of C57BL/6 mice, and titers were determined by plaque assay. The LD₅₀ was determined using Reed-Munch formula.

**Animals.** Female Langerin-EGFP-DTR mice were kindly provided by Dr. Bernard Malissen (Centre d’Immunologie de Marseille-Luminy, France). Six- to eight-week-old female wild type C57BL/6 mice were purchased from Harlan Laboratories (Tampa, FL). All mice were bred and housed at Emory University. All experiments were performed in accordance to Emory University’s Institutional Animal Care and Use Committee guidelines.

**Depletion of Langerin⁺ cells.** For systemic depletion, Langerin-EGFP-DTR and C57BL/6 mice (6–8 weeks old) were injected intraperitoneally with 1 mg of DT (Sigma, St. Louis, MO) two days prior and on the day of vaccination. Depletion was confirmed by either direct fluorescence staining with anti-CD11c PE Alexa 488 antibody (Proteins, Inc.), an all-trans retinal taste for transgenic animals, or by flow cytometry of isolated LN subsets.

**Immunization.** Mice were immunized with 10⁻⁵ mg of influenza subunit vaccine were inserted into the skin for 5 min. For comparison, mice from both strains were immunized with the same dose of vaccine.

**Immunizations, challenge and sample collection.** Langerin-EGFP-DTR mice (25–35 g) were immunized with influenza vaccine as described previously. H1N1 A/California/07/2009 subunit vaccine (261 μg HA/ml stock solution) was provided by Novartis Vaccines and Diagnostics (Rockford, IL). Influenza virus stocks (A/California/07/2009 (H1N1)) were propagated in MDCK cells. The hemagglutination (HA) activity was determined using turkey blood cells (LAMPIRE, Piperville, PA). Mouse-adapted virus was obtained by serial passage in lungs of C57BL/6 mice, and titers were determined by plaque assay. The LD₅₀ was determined using Reed-Munch formula.

**Post-challenge lung titers.** Lung homogenates were prepared in DMEM and viral titers were assessed per gram of tissue by plaque assay.

**Statistics.** The statistical significance of differences was calculated by two-tailed unpaired Student’s t-test and one-way ANOVA including Bonferroni’s multiple comparison test. The statistical significance of differences between survival curves was calculated with Mantel-Cox Test and Gehan-Breslow-Wilcoxon Test. A p value less than 0.05 was considered statistically significant. Means and standard deviations were calculated from quadruplicate runs and at least two independent animal experiments.

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Author contributions

J.P.F. designed the majority of the study, E.V. designed part of the study, L.S. designed part of the study, E.S.E. and J.P.F. maintained the animal colony and carried out all the animal work, J.P.F., E.S.E., E.V. and M.T.T. performed experiments, analyzed data and prepared the figures. J.W.L. prepared the vaccine coated microneedles. B.P.P. consulted in the in vivo skin experiments and contributed in manuscript editing and relevant citations. J.P.F., M.R.P., R.W.C. and L.S. wrote the manuscript. All authors discussed the results and commented on the manuscript. All experiments were performed in accordance to Emory University’s Institutional Animal Care and Use Committee guidelines.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: M.R.P. is an inventor on patents licensed to companies developing microneedle-based products, is a paid advisor to companies developing microneedle-based products, and is a founder/shareholder of such companies. This possible conflict of interest is being managed by Georgia Institute of Technology and Emory University.

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