

ARTICLE

DNA vaccination strategy targets epidermal dendritic cells, initiating their migration and induction of a host immune response

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The immunocompetence and clinical accessibility of dermal tissue offers an appropriate and attractive target for vaccination. We previously demonstrated that pDNA injection into the skin in combination with surface electroporation (SEP), results in rapid and robust expression of the encoded antigen in the epidermis. Here, we demonstrate that intradermally EP-enhanced pDNA vaccination results in the rapid induction of a host humoral immune response. In the dermally relevant guinea pig model, we used high-resolution laser scanning confocal microscopy to observe direct dendritic cell (DC) transfections in the epidermis, to determine the migration kinetics of these cells from the epidermal layer into the dermis, and to follow them sequentially to the immediate draining lymph nodes. Furthermore, we delineate the relationship between the migration of directly transfected epidermal DCs and the generation of the host immune response. In summary, these data indicate that direct presentation of antigen to the immune system by DCs through SEP-based *in vivo* transfection in the epidermis, is related to the generation of a humoral immune response.

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INTRODUCTION

The skin is an attractive vaccination target for a number of reasons: it is accessible, and due to the presence of resident professional antigen presenting cell (APC) populations, is a highly immunocompetent tissue. Professional APCs are adept at antigen capture, and upon appropriate activation, efficiently migrate to regional lymph nodes and mediate the induction of the immune response.¹ Multiple preclinical experiments and clinical trials have demonstrated the effectiveness of vaccinating in the skin to drive robust immune responses.^{2–8}

pDNA vaccination strategies offer significant advantages over the conventional attenuated or inactivated vaccines. DNA vaccines can be manufactured to a large scale quickly, are easy to formulate and most importantly, are able to generate both humoral and T cell responses to single or multiple target antigens. However, initial DNA vaccines were of low immune potency especially in larger mammals.⁹ The inability to efficiently deliver pDNA to cells *in vivo* was cited as the major reason for the lack of efficacy of naked DNA vaccines in larger animals and humans. As such, considerable effort has been attached to the development of enhanced delivery technologies to improve the uptake and expression of pDNA *in vivo*. Delivery techniques, including electroporation (EP),^{3,7,10,11} gene gun,^{12,13} tattooing,^{2,14} and microneedles,¹⁵ have been developed to reliably enhance gene expression in the skin tissue. EP is a physical technique based on applying brief electrical pulses to the tissue of choice to open cell membranes in a transient and reversible manner. This facilitates the direct transport of pDNA into the cell. Upon

comparison to naked DNA vaccination, a 10- to 100-fold enhancement of the immunological response was observed when EP was employed as an enabling delivery technology.^{16–18} While historically intramuscular (IM) EP has been the target tissue of choice, recently, considerable effort has been employed to develop intradermal (ID) EP techniques toward clinical applications.^{3,5,19,20} A minimally-invasive surface ID EP platform (SEP) was recently shown to significantly enhance the expression of reporter gene plasmid in the skin and induce robust immunity.^{3,21–23} Histological analysis revealed rapid reporter gene expression exclusively in the epidermis following SEP treatment.²⁴ Furthermore, in specific animal vaccine models, we have demonstrated enhanced immunity using EP-based pDNA protocols that target the skin over those targeting the muscle.²⁵ As such, the accessibility of the skin combined with the shallow penetration depths required for drug delivery, result in a less invasive and therefore potentially more tolerable clinical procedure.^{19,26} However, a greater understanding of the cellular mechanisms involved in the generation of immunity following pDNA delivery to the skin enhanced by EP is needed. Previous studies have investigated the nature of EP-enhanced DNA transfection at the skin, demonstrating efficient reporter gene expression and the generation of robust immune responses.^{3,10,11,21,23,27} The mechanisms that operate following EP-enhanced direct cell transfection and the generation of immunity have yet to be delineated. Here, we identify a migratory cell population that are directly transfected following SEP-enhanced delivery, and elucidate mechanisms involved in the generation of immunity following antigen encoding plasmid

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delivery. Specifically, we have identified the direct targeting of an epidermal dendritic cell (DC) population and delineate a mechanism involved in the priming of a host humoral immune response.

RESULTS

Induction of rapid humoral immunity after ID delivery of a DNA vaccine

The ability of a vaccine to induce rapid immunity is a highly desirable trait, especially in halting the spread of infectious disease. However, DNA vaccination by IM administration is associated with a time lag before productive immunity is achieved.²⁸ This may be due to the small numbers of professional APCs residing in this tissue available for *in vivo* transfection, suggesting that the plasmid-encoded antigen is indirectly presented to the immune system.²⁹ Since the skin contains a significant population of resident DCs as illustrated in Figure 1a, their direct transfection with antigen-encoding DNA may facilitate a rapid response.^{30,31} We hypothesized that skin DNA vaccination would directly target this DC population, potentially resulting in a detectable immune response in a shorter period of time than muscle immunization. To test this, we used the guinea pig model. Guinea pig skin has a similar thickness and structure to that of human skin, and is thus considered an optimal surrogate model.^{32,33}

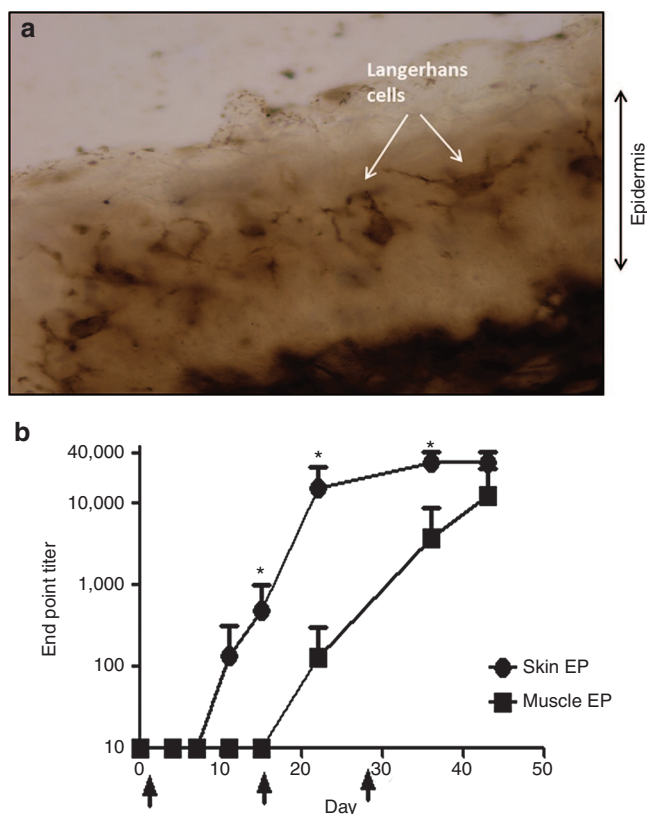


Figure 1 Accelerated immunity after DNA vaccination targeting the dendritic cell-rich epidermis. (a) Image of guinea pig epidermis highlighting Langerhans cell populations after ATPase staining. Magnification 40x. (b) The kinetics of the generation of humoral immunity was detected by enzyme-linked immunosorbent assay after skin or muscle pH5HA immunization of Hartley guinea pigs. Peripheral blood IgG against the A/Vietnam/2004 H5HA antigen end point titers are plotted (mean + standard error of the mean). **P* < 0.05 = end point titer between skin and muscle immunization groups (five guinea pigs per group). ↑ = immunization day.

To determine whether there was differential in kinetics in the generation of humoral immune responses between ID/SEP- and IM/EP-enhanced pDNA delivery, we immunized two groups of guinea pigs using each modality. Group 1 was vaccinated IM (quad muscle (100 µg pH5HA (A/Vietnam/2004) influenza DNA + IM EP), and group 2 ID (abdominal skin (100 µg pH5HA (A/Vietnam/2004) influenza DNA + SEP)), on days 1, 15, and 29. Figure 1b shows the kinetics of the generated humoral immunity by ELISA-determined end point titers. Binding antibody titers were generated significantly faster in the skin epidermis-targeted group over the muscle-immunized group (*P* < 0.05 day 15–36). Anti-H5HA IgG antibody titers (1 in 150) were first detected at day 11 in the ID group 2 and at day 22 in the IM group 1. By day 42 (14 days after third immunization), the difference in anti-H5HA binding titers between the IM and ID-treated groups were no longer significant, indicating the magnitude of the IM response reaches that of the ID response after a time lag. In summary, EP-based pDNA immunization induces a rapid immune response when the treatment is targeted to the epidermal layer of the skin.

pDNA delivery to the epidermis results in direct transfection of DCs which migrate into the dermal tissue

To investigate the mechanism mediating the host immune response observed after skin vaccination, we focused on identifying the cell population directly transfected in the epidermis. We previously reported skin delivery of a reporter gene plasmid followed by SEP resulted in gene expression only in the epidermis.^{3,24} Here, we confirmed these findings by collecting serial optical sections of a skin biopsy using laser scanning confocal microscopy, where a reporter gene plasmid had been delivered using SEP. Imaging reveals distinct red fluorescent protein expression located specifically in the epidermal region (Figure 2). The majority of the red fluorescent protein-positive cells stained with a K10 keratinocyte-specific fluorescein isothiocyanate (FITC)-conjugated antibody, indicating the nonmigratory keratinocyte to be the predominant cell type transfected by this delivery modality (Figure 2). As would be expected, the outer stratum corneum barrier layer—a layer of dead keratinocytes that have sloughed off the epidermis, also stained green.

While the predominant cell type transfected was the keratinocyte, there were clearly reporter gene-positive cells in the epidermis that had distinctly different morphologies to their keratinocyte counterparts. To investigate further, we analyzed reporter gene-positive skin biopsies at higher magnifications. Analysis revealed a distinct population of green fluorescent protein (GFP)-positive interdigitating cells between the keratinocytes. These cells exhibited a distinct morphology, suggestive of DCs. Analysis confirmed reporter gene-positive transfected DC-like cells in the epidermal region as early as 1–2 hours after DNA injection and SEP-based EP. Figure 3a (panels 1 and 2) shows maximum projected stacks of confocal images of GFP-expressing cells with a typical dendrite morphology in the epidermis. Imaging revealed GFP⁺ DC-like cells at the top of the epidermis (Figure 3a—panel 1) and deeper within this layer (Figure 3a—panel 2), located close to the basement membrane of the epidermis—the structural border between the epidermis and dermis. The rapidity of the GFP expression and diffusion throughout the cytoplasm of these cells supported these cells directly taking up plasmid and expressing the gene product endogenously, rather than captured exogenous protein.

To determine whether these dendritic-like cells possessed the potential to transport antigen out of the epidermis and into the dermis, where they could enter the lymphatic vessels, we investigated

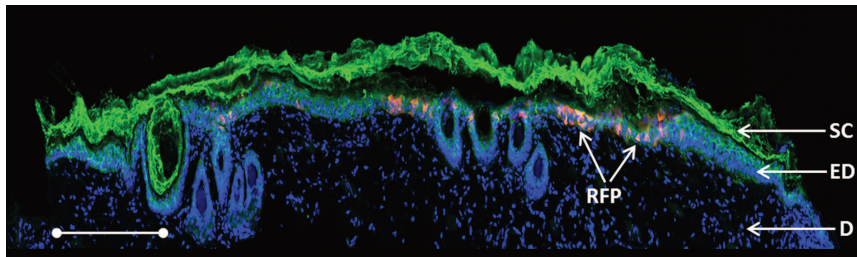


Figure 2 Panoramic image of the dermal tissue after pDNA delivery in the presence of surface electroporation (SEP). Guinea pigs were treated intradermally with 50 μ g pRFP and the injection site immediately electroporated with the SEP device. The mapped and stitched multipanel and multisection confocal image reconstruction revealed reporter gene (red fluorescent protein (RFP)) expression in the epidermal layer of guinea pig skin aligned to the site where plasmid injection and electroporation with SEP was performed. Image acquired 6 hours after treatment. Magnification 10 \times . D, dermis; ED, epidermis; SC, stratum corneum. RFP (red), K10 stain (green), Hoechst (blue). Scale bar represents 1 mm.

their movement in the skin tissue. To monitor the movement of transfected DC-like cells, we acquired optical stacks of confocal images in a time course (1–24 hours) study. We focused on the epidermal and dermal regions of the skin, looking for motile GFP-positive cells. Maximum projections of confocal images of DC-like cells in the dermal tissue 6 hours after treatment are shown in Figure 3a, panels 3 and 4. Panel 4 shows a reporter gene-positive dendritic-like cell at the edge of what appears to be a vessel, as revealed by differential interference contrast. We believe that these DC-like cells are migrating out of the dermis via lymphatic vessels to the draining lymph nodes.

We proceeded to quantify the movement and kinetics of motile GFP⁺ cells in the dermis, multiple skin biopsy sections were analyzed and cell counts taken at defined time points (1, 2, 4, 6, 8, and 24 hours post-treatment). Figure 3b shows that the percentage of GFP⁺ cells in the dermis peaked 6 hours after treatment (0.17% of the cells GFP⁺ area⁻¹). The percentage of GFP⁺ cells in the dermis decreased after 6 hours to 0.075% at 8 hours and 0.055% at 24 hours. Although there was a significant decrease in percentage of GFP⁺ cells after 6 hours, the raw numbers counted in the dermis only slightly decreased (Figure 3c). This phenomenon was due to an increased influx of cells into the dermal tissue after 6 hours, thus decreasing the GFP⁺ cells as a percentage of total dermal cells. As a further confirmation of these findings we analyzed the trafficking of GFP-transfected cells by flow cytometry. Epidermal sheets were mechanically separated from dermal sheets, the tissues digested, and cell suspensions were analyzed by flow cytometry. Results confirmed peak numbers of epidermal-transfected cells had migrated into the dermis within 6 hours of treatment (Figure 3d). Together, this data suggests that migratory DCs are initially transfected in the epidermis and migrate into the dermis within several hours of SEP treatment.

To further confirm that these cells were professional APCs, we proceeded to demonstrate their interaction with T cells. To accomplish this, guinea pigs harboring immunity to influenza H5HA were treated with a cocktail formulation of H5HA and GFP plasmids administered to the skin. This experimental protocol rests on the assumption that some reporter gene-positive cells also presented H5HA-derived antigens. After skin administration of a cocktail formulation containing pGFP and pHIV-EnvC followed by SEP we had previously confirmed coexpression of GFP and EnvC—using an anti-EnvC antibody, in epidermal cells (unpublished data). Here, we demonstrated that reporter gene-positive cells with DC morphology interacted with CD4⁺ and CD8⁺ T cells in the dermis (Figure 4a). When we administered GFP plasmid without the H5HA plasmid into the opposite flank of the guinea pig, no colocalization of GFP⁺ cells with lymphocytes was observed. These observations indicated

indirectly, that these migratory cells were major histocompatibility class I and II positive. Expression of major histocompatibility class II is associated with professional APCs. Furthermore, we detected GFP⁺ migratory cells stained with an antibody (MsGp2) that strongly reacts with Langerhans cells³⁴ (Figure 4b).

Reporter gene expression is detected in the skin draining lymph nodes 12 hours after SEP treatment

After capturing antigen, activated DCs leave the dermis by entering the lymphatic vessels, and migrate to the draining lymph nodes (DLN).³⁵ Lymphocyte priming occurs primarily in the lymph nodes, thus early arrival of APCs carrying skin delivered antigen could initiate an immune response. We sought to determine the migration kinetics between transfection in the epidermis and the transfected cells reaching the draining lymph nodes.

In an attempt to identify reporter gene-positive cells in DLNs following plasmid delivery in the skin, pGFP was administered with SEP to guinea pig abdominal skin. Inguinal lymph nodes were harvested at 0, 6, 12, 24, 48, and 72 hours. Flow cytometric analysis revealed a population of GFP⁺ cells (84–152 cells per lymph node) in the draining inguinal lymph nodes as early as 12 hours after treatment (Figure 5a). The intrinsic autofluorescence associated with analyzing guinea pig cells by fluorescence-activated cell sorting (FACS) caused a number of between 10 and 20 cells to leak into the GFP channel, and this accounts for the background cell numbers measured at 0 and 6 hours. The number of GFP⁺ cells detected in the inguinal lymph node peaked at 24 hours (average 168 per lymph node). The numbers of GFP⁺ cells detected in the lymph node declined slightly at later time points. Previous groups have reported similar numbers of reporter gene-positive cells in murine lymph nodes after skin immunization.^{36–38} Furthermore, histological analysis of intact lymph node sections detected reporter gene-positive cells in the T cell zone of the cortex (the 24-hour time point is depicted in Figure 5b). We performed control experiments in which we delivered pGFP into the skin without SEP, and we observed only background numbers of GFP⁺ cells in the DLNs after analysis by FACS.

Data presented in the above sections delineates the pathway via which *in vivo* transfected APCs migrate out of the epidermis to the dermis and enter the lymphatics, reaching the DLN's within 12 hours of plasmid delivery and SEP treatment. In contrast to the kinetics we demonstrate after skin administration of reporter plasmid, after the EP-assisted delivery of pGFP into rat muscle, Gronevik *et al.* analyzed GFP mRNA expression in the draining lymph node between 0 and 96 hours after treatment, and failed to detect its expression.³⁹ Furthermore, we could not detect GFP⁺ cells in the draining lymph nodes 24 hours (peak GFP⁺ cell numbers were detected 24 hours after skin pGFP + SEP delivery) after quad muscle

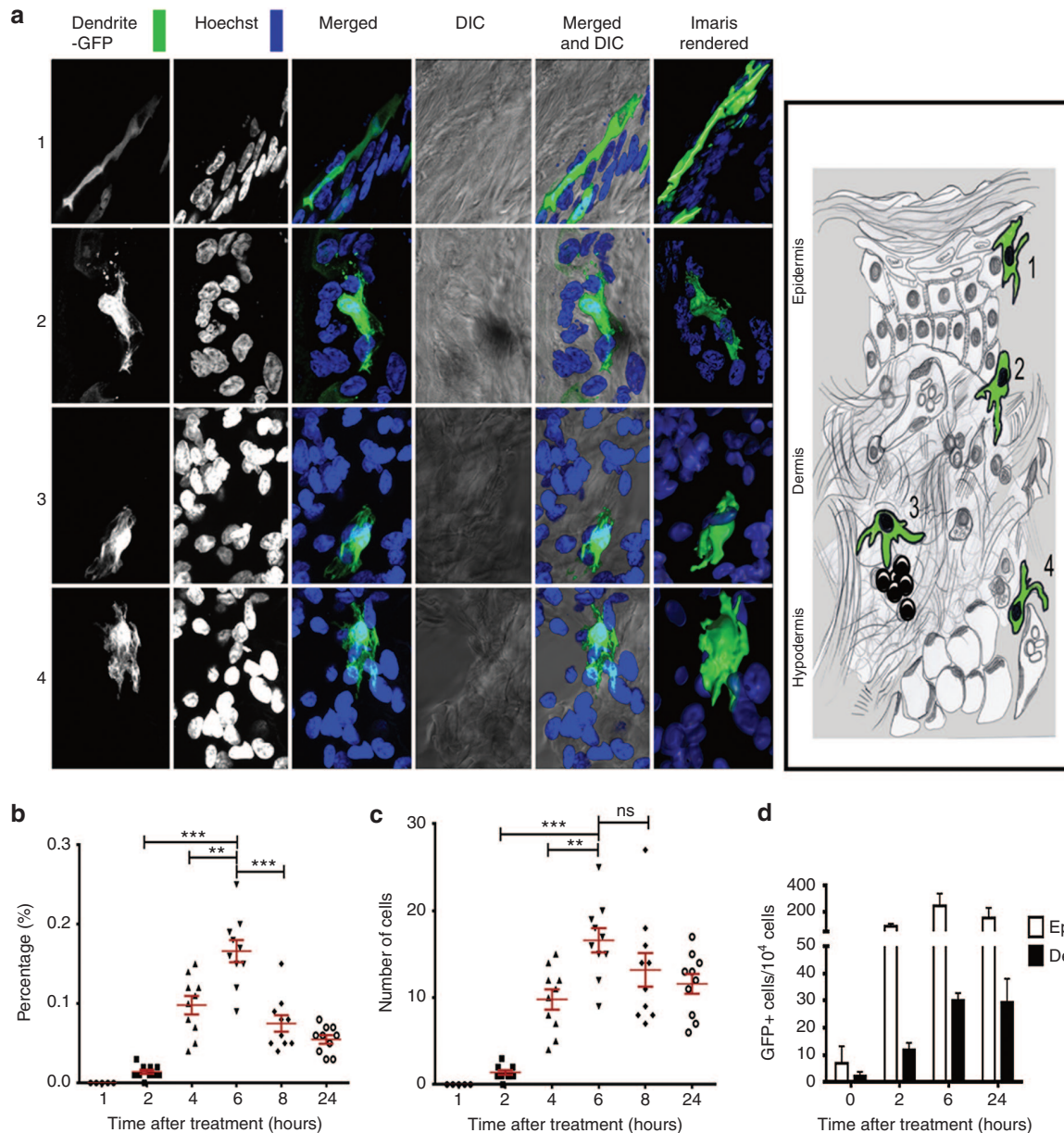


Figure 3 Imaging and quantification of *in vivo* transfected cells in the dermal tissues after pDNA delivery in the presence of surface electroporation (SEP). Guinea pigs were treated ID with 50 μ g pGFP and the injection site immediately electroporated with the SEP device. **(a)** Depiction of migration of reporter gene (GFP) transfected cells from the epidermis to the dermis. Showing GFP⁺ cells of dendritic cell-like morphology in the epidermis (panels 1 and 2), and the dermis (panels 3 and 4). Images were acquired with a Zeiss LSM 780 laser scanning confocal microscope, and further processed in 3D using Imaris software. Differential interference contrast images compliment the fluorescent images revealing dermal matrix, and the cartoon image identifies the dermal location of the cells in each panel. Reporter gene-positive cells counted in the dermis between 1 and 24 hours after treatment as a percentage **(b)** or total number **(c)** of cells in the analyzed section. **(d)** Single cell suspensions were made from epidermal and dermal sheets, between 1 and 24 hours after treatment. Number of GFP⁺ cells were determined by FACS. Graphs **b–d**: mean \pm standard error of the mean plotted. *** $P < 0.001$, ** $P < 0.01$.

pGFP + Twin-injector EP delivery (data not shown). We believe the rapid arrival of *in vivo* transfected DCs from the epidermis contributes to the enhanced immune response kinetics observed following skin versus muscle vaccination.

Migration of DC cells out of the dermis correlates to the generation of immunity

The data presented above suggest that a population of directly transfected APCs have emigrated from the skin by 8 hours (Figure 3b–d), and have reached the draining lymph node within 12 hours of treatment (Figure 5a). Next we aimed to determine whether these early

epidermal migratory cells could prime an immune response. To do this, we excised the skin at the site of injection at defined time points postimmunization. Figure 6 demonstrates that excising the site 2 hours after pNP influenza SEP treatment completely abrogated the generation of an immune response. However, when excision was delayed for 8 hours after treatment a response (1 in 850 anti-NP titer) was detected, this response was ~60% of the intact treatment site associated humoral response.

This data suggests that SEP-based pDNA vaccination can successfully deliver pDNA directly to a population of epidermal APCs in a manner that results in their activation and migration to the draining lymph nodes. We believe this may be the mechanism mediating the

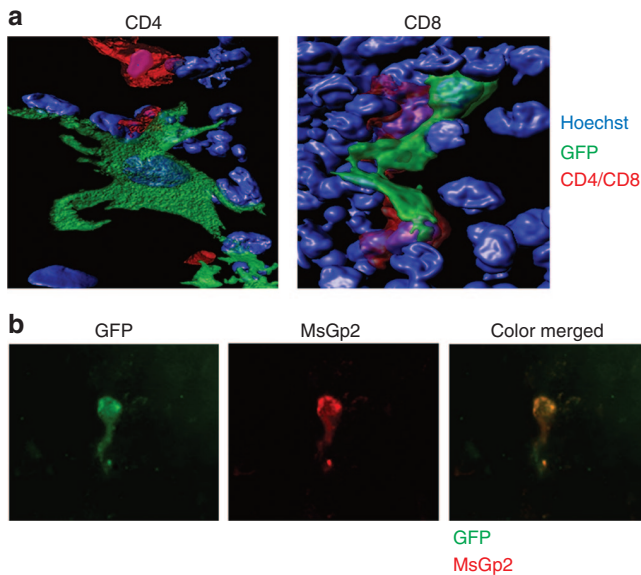


Figure 4 Identification and interaction with T cells of dendritic cells expressing GFP reporter gene after surface electroporation (SEP) treatment. **(a)** Guinea pigs harboring immunity to the A/Vietnam/2004 H5HA strain of influenza were treated intradermally with a pDNA cocktail containing 50 μ g pGFP and pH5HA in the presence of SEP. Dermis tissue was harvested 24 hours after treatment and stained with anti-CD4 or anti-CD8 PE-conjugated antibodies. Confocal images were modeled using IMARIS software. **(b)** Six hours after pGFP administration in the presence of SEP, guinea pig skin sections were stained with the Langerhans cells reactive antibody, MsGp2. Images depicts a GFP⁺ cell staining positive with MsGp2.

accelerated immune response observed following skin compared to muscle vaccination.

DISCUSSION

DNA vaccines are a promising solution to many unmet medical needs, and combining them with enhanced delivery via EP may be the key to clinical success. Dermal EP may offer a tolerable, easy to use platform that would be applicable for mass vaccination while maintaining vaccine efficacy. While the benefits of ID vaccine delivery have been proven in the clinic,^{6,8,40} ID delivery of DNA vaccines is at an earlier developmental stage, and mechanisms of antigen presentation are less understood. The findings presented here delineate a mechanism mediating the induction of host immune response following EP-enhanced epidermal pDNA immunization. Using a surface EP device that permits transfection of the epidermis, but not lower dermal layers, we demonstrate the direct transfection of a migratory DC population in the epidermis, and the kinetics of their migration to the dermis and into the lymphatics. Once in the draining lymph node, we surmise that they present the encoded antigen to the adaptive immune system. Our results suggest the epidermal layer of skin may be an optimal target for immunization protocols aimed at generating rapid immunity.

One aim of this study was to identify a mechanism that could mediate the accelerated immunity observed after epidermal, as compared to IM DNA vaccination (Figure 1b). To our knowledge, this is the first observation of such a phenomenon in DNA vaccine models using EP as a delivery aide. Other studies have reported higher or equivalent antibody responses being generated in DNA vaccine studies after skin EP compared to muscle EP^{3,25,41}, while others have reported lower levels.^{42,43} The model antigen used in each of these studies may account for these differences. Studies describing

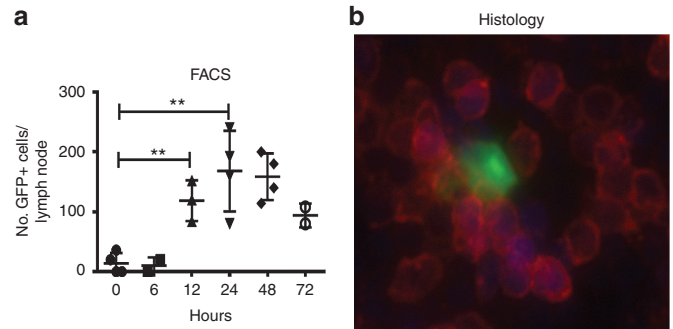


Figure 5 Expression of reporter gene in cells in draining lymph node. Guinea pigs were treated intradermally with 250 μ g pGFP in the presence of surface electroporation on the abdominal flank. Inguinal lymph nodes were excised at between 0 and 72 hours **(a)** and at 24 hours **(b)** after treatment. **(a)** Flow cytometric quantification of number of GFP-positive per draining lymph nodes at the indicated time after treatment (mean + standard error of the mean plotted). ** $P < 0.01$. **(b)** Fluorescent microscopy of the paracortex region of the inguinal lymph node. GFP (green), CD4 (red), and dapi (blue).

higher humoral responses after IM delivery used the weakly immunogenic Luciferase antigen whose expression level in the skin is 100th of that detected in the muscle.⁴³ Such a vast difference in expression levels of a weakly immunogenic antigen could account for the discrepancies in immune responses generated between delivery sites. The studies observing higher or equivalent antibody responses after ID delivery used strongly immunogenic Influenza antigens, so antigen expression levels per se may not have so much an effect on host immunity generated by this antigen. Surprisingly, in another study, we found no significant difference in the kinetics of the generation of humoral responses upon pDNA + SEP delivery into guinea pig skin or muscle.²² Lower EP voltage (15V) was used in the Lin *et al.*²³ study compared to 25V in this study. Further investigation has suggested that the EP electrical parameters affect the immune kinetics. Higher EP voltages were associated with stronger immune responses after the prime stage immunization. We have yet to determine the mechanism behind this phenomenon, but it is likely to be associated with the increase in innate cell activation at the treatment site that is observed with higher voltages.

In this study, we aimed to delineate the mechanism involved in the presentation of antigen to the immune system after epidermal delivery enhanced by EP. We hypothesized that the direct *in vivo* transfection and migration of a professional APC population from the skin as a potential mechanism. To investigate this, we utilized histological methods to determine the cell phenotypes transfected in the guinea pig epidermis. The majority of cells (upwards of 90%) residing in the epidermis are keratinocytes. This dictates, as shown in Figure 2, that keratinocytes would be the major cell population transfected after SEP treatment. However, keratinocytes are non-migratory cells and display low immunostimulatory capacity, they are not considered to be APCs capable of directly priming an adaptive immune response.⁴⁴

To determine if cell types, other than keratinocytes, were being transfected with pDNA in the epidermis we utilized high resolution laser scanning confocal microscopy to obtain serial sections and/or stitched sections of the entire epidermis and revealed a population of cells with a distinct dendritic morphology to be expressing the reporter gene (Figure 3). To demonstrate that these were a professional APC population, we observed their ability to interact with CD4⁺ and CD8⁺ T cells in the dermis (Figure 4a), and stain with the Langerhans cell reactive antibody, MsGp2 (Figure 4b).

Kinetic analysis of transfected DCs revealed a distinct migration pattern. Epidermal migratory cells were detected in the draining lymph nodes as early as 12 hours after treatment (Figure 5). Other groups have demonstrated similar migration kinetics in murine models after skin immunization with pDNA.^{37,38} These protocols in rodent models have also reported low numbers of skin migratory reporter gene-positive cells in the lymph nodes 24 hours after treatment.^{37,38} DCs are extremely efficient in their ability to process antigen and present it to the immune system in a stimulatory manner. They are considered the most potent APC.¹ The capacity of the Langerhans cell to prime an immune response has been shown to be 1,000-fold greater than that of a keratinocyte.⁴⁵ Thus due to the potent stimulatory ability of DCs, such low numbers of reporter gene-positive migratory cells in the DLN is not so surprising (Figure 5). This is supported by data in Figure 6 that indicates the population of pDNA+ skin migratory cells that have left the skin within 8 hours of treatment to be capable of priming an immune response. Data presented in Figures 5 and 6 predicted that numbers as low as 100 epidermal APCs capable of priming systemic immunity.

To our knowledge, this is the first report describing the cellular migration kinetics involved in the priming of an immune response after epidermal vaccination in a guinea pig model. Similar studies assessing the impact of genetic vaccination directed to the skin have been performed in murine models.^{2,36–38,46} These vaccine studies have established that skin-resident APC populations can take up pDNA, express the encoded antigens, and deliver them in a stimulatory manner to the adaptive immune system. However, the exact subsets of skin epidermal or dermal APC and antigen presentation mechanism involved in the priming of the immune system are debated. With some studies suggesting the epidermal directly transfected Langerhans population priming the immune response,^{37,38,46} and other studies suggesting a dermal DC population to be involved in an indirect priming mechanism.^{36,47} It is due to distinct differences between the skin of mice to that of higher mammals, and the pDNA transfection modalities used, that these referenced studies were unable to only directly target the epidermal layers, instead, cells residing in the dermis and even deeper tissue were likely to be transfected in these studies.⁴⁸ As such, this dictates that some of the migratory cells detected in the draining lymph nodes may be of dermal origin, and not epidermal derived APCs. This is an important consideration as dermally derived DCs and epidermal DCs have been reported to be functionally distinct, in respect to the generation of humoral

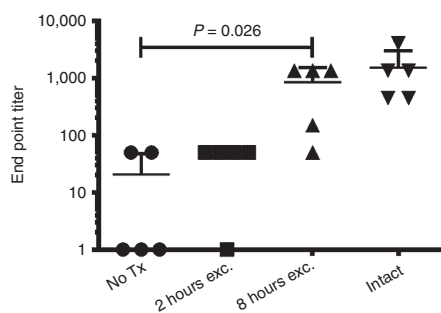


Figure 6 Correlation of the migration of dendritic cells out of the dermis to the generation of an immune response. Naive guinea pigs (five per group) were treated intradermally with 100 μ g pNP and the site electroporated with surface electroporation. The treatment site was either left intact, or excised 2 or 8 hours after injection. Guinea pigs were bled at day 28 after treatment and anti-nucleoprotein IgG antibody titers detected by enzyme-linked immunosorbent assay (mean + standard error of the mean plotted). Two independent experiments were performed, and one representative data set is shown.

versus cellular immunity, and tolerance versus productive immunity.⁴⁹ Currently, there is no consensus of opinion on the differential roles played by dermal and epidermal DCs in mediating immune responses, as the idiosyncrasies of each study dictates the conclusion drawn. For example, Allan *et al.*³⁶ compared the lymph node migration kinetics of epidermal CD205^{high} and dermal CD205^{low} DCs in mice. They reported after flank skin painting of mice with FITC in acetone and dibutyl phthalate that lymph node dermal-derived FITC+DC numbers peaked at 24 hours, while epidermal-derived FITC DCs peaked later at 72 hours. Figure 5 demonstrates in our study that the number of epidermal-derived migratory DC-like cells peaked at 24 hours. There are multiple differences between our models to explain this discrepancy. Most importantly may be the delivery technology that we used. In addition to stimulating pore formation within the cell membrane to allow the direct passage of large molecules into the cell, EP has recently been shown to directly activate immature Langerhans cells.⁵⁰ In addition to maturation and the development of a highly immunogenic phenotype, activation triggers the migration of DCs out of resident tissue toward a lymphoid organ, with the aim of priming an immune response.¹ EP may have not only aided in the direct delivery of pDNA into the DC, but also initiated DC migration. Thus, accounting for the difference in DC migration kinetics observed between pDNA delivery modalities.

Our study does not rule out a role for indirect cross-presentation of antigen to prime an immune response after delivery of pDNA vaccines to the epidermis. Data shown in Figure 6 imply that the directly transfected APC population that rapidly migrates from the skin may not fully prime the immune response. The observation that an immune response generated after 8-hour skin excision is 60% of that when the treatment site is left intact, suggests either a significant number of directly transfected epidermal DCs have not left the skin, and/or indirect capture of antigen manufactured in nonmigratory cells augments the immune response. The former argument is supported by the observation of GFP-positive cells in the dermis with the diffuse cytoplasmic pattern of GFP expression (characteristic of direct transfection) at later than 8 hours after treatment (Figure 3). The latter argument is supported by the identification at the 24-hour time point of low numbers of cells with speckled GFP cytoplasmic expression in the DLNs, which we believe to be GFP⁺ vesicles, indicative of uptake of exogenous GFP protein (data not shown). Future studies are being planned to further characterize the functional differences between cells expressing a diffuse versus speckled GFP pattern, along with the use of chemical small molecule inhibitors that can block the capture of exogenous protein, and thus inhibit the cross-presentation pathway. In conclusion, we believe both the direct transfection and indirect antigen capture mechanisms play a role in the overall immunity generated.

Unfortunately, due to the lack of reagents (antibodies to detect cytokines in intracellular staining or ELISpot assays), we were unable to analyze T cell responses in the guinea pig model. In Figure 4a, we successfully captured DC-like cells interacting with T cells in the dermis of guinea pigs harboring immunity to the administered plasmid immunogen, suggesting a role for the transfected DC in T cell activation. To investigate the ability of SEP-mediated transfection of epidermal DCs to prime T cells, we have begun studies in the Wistar rat. This rat strain possesses a skin structure similar to the guinea pig, and SEP-mediated transfection is limited to the epidermis. Preliminary studies are revealing robust T cells responses after pDNA vaccination with SEP (unpublished data).

Studies by other groups have demonstrated that skin DNA vaccination enhanced by EP resulted in increased gene expression in the epidermis and dermis, followed by robust immune responses

in both mice and guinea pigs.^{7,11,21,43} However, these studies did not directly connect pDNA transfection and cell migration with the generation of an immune response. Here, we have specifically focused on the epidermis, extending our knowledge by identifying the cells transfected, their migration pattern and delivery of antigen to the immune system. We believe this to be the first study that identifies the essential role for epidermal-derived DCs in the priming of a host immune response after EP-based pDNA vaccination. We believe the immunization strategy outlined here has the potential to be applied to medical situations where there is a need for rapid protective immune response. The generation of rapid immunity would be crucial in a pandemic or bioterrorism situation. An EP device which targeted the epidermal region and was easily deployable, combined with an efficacious DNA vaccine could be an effective strategy for the generation of protective immunity in the general population.

MATERIALS AND METHODS

EP devices

The epidermal targeting surface EP device (SEP) was an electrode array consisting of 4×4 array of gold-plated trocar needle of 0.43 mm diameter at a 1.5 mm spacing (Inovio Pharmaceuticals, San Diego, CA). The SEP array is pressed down on the skin bleb made by Mantoux delivery of 50 μ l plasmid formulation, so that all the electrodes across the array contact with the skin. The electrodes do not penetrate the live skin layers. The muscle targeting twin-injector device was a 2 27-gauge needle array with 4 mm spacing between electrodes (Inovio Pharmaceuticals). These electrodes are first inserted into the quad muscle, and using an insulin syringe, 125 μ l of plasmid formulation is injected into the muscle between the electrodes, and the electrodes are then pulsed. Both devices were used in conjunction with the ELGEN1000 (Inovio Pharmaceuticals). Electrical parameters delivered using the ELGEN1000 were three 25 V pulses, each of 100 ms duration for skin, and two 125 V cm^{-1} pulses lasting 60 ms each were for muscle.

Plasmids

gWiz-GFP or gWiz-RFP reporter gene plasmids were purchased from Aldevron (Fargo, ND). NP plasmid encodes the full-length nucleoprotein derived from the A/Puerto Rico/8 (H1N1) strain of influenza. pH5HA is a SynCon vaccine construct that encodes a consensus sequence of hemagglutinin of A/Vietnam/2004 (H5HA) strain of influenza virus. Briefly, consensus sequence for pH5HA vaccine was generated by aligning multiple primary sequences (obtained from the Los Alamos National Laboratory influenza sequence database) and choosing the most common amino acid at each position to generate a sequence not necessarily found in nature (synthetic) but which retained characteristics of the component sequences chosen.⁵¹ Sequences were then optimized for codon usage, RNA structure, and GC content and were synthesized. Synthetic genes were then subcloned into a pVax expression vector with a CMV promoter (Invitrogen, Carlsbad, CA). The size of the plasmid was 4.733 kb. All plasmids were diluted in 1× phosphate-buffered saline (PBS) before injection.

Animals

Female Hartley guinea pigs (6 months old and weighing ~350–400 g) were purchased from Charles River Laboratories (Wilmington, MA). The guinea pigs were group housed (four per cage) with *ad libitum* access to food and water. Animals were acclimated for 2 weeks prior to experimentation. All animals were housed and handled at BioTox (San Diego, CA) according to the standards of the Institutional Animal Care and Use Committee.

Treatments and EP

All animals were sedated with isoflurane prior to treatment. Before treatment, the injection site was shaved. Skin pDNA injections (50 μ l in guinea pigs) were performed on the abdominal flank by the Mantoux method (needle parallel to skin) with a 29-gauge insulin needle. Immediately following pDNA injection, EP was performed by applying the 4×4 electrode array at the injection site on the skin. Animal IM pDNA injections were into the quadriceps (150 μ l in guinea pigs). Immediately following injection, EP was performed by inserting 2-needle electrode array at the site of injected muscle. Electrotransfer was achieved through pulse generation from the ELGEN1000.

Histology

Animals were sacrificed under standard institutional protocol. 8-mm skin biopsies were removed from the treatment area postmortem and transported on ice to the laboratory for processing. Biopsies were fixed in 4% paraformaldehyde at 4 °C for ~12 hours. After washing in 1× PBS (three times for 5 minutes at room temperature (RT)), biopsies were incubated in 30% sucrose at 4 °C for 12 hours. After washing in 1× PBS, skin biopsies were embedded in OCT compound and sectioned at a thickness of 15 μ m for basic fluorescent, or 30 μ m for confocal microscopy, using an OTF Bright Cryostat (Cambridge, UK). Keratinocytes were detected in the epidermis using rabbit anti-keratin 10 antibody (Assay Biotech, Sunnyvale, CA). T cells were detected in the dermis using mouse anti-guinea pig CD4 (clone CT7) and CD8 (clone CT6) both AbD Serotec, Oxford, UK). To detect pDNA transfected Langerhans cells in the epidermis, we first separated the epidermal sheet using a previously described method.⁵² 6-mm skin biopsies were incubated in Thermolysin-solution for ~12 hours at 4 °C. Thermolysin-solution was a cocktail of 25 mg Thermolysin from *Geobacillus stearothermophilus* (Sigma, Saint Louis, MO) and 50 ml Thermolysin-buffer (10 mmol/l HEPES, 142 mmol/l NaCl, 6.7 mmol/l MgCl_2 , 1 mmol/l CaCl_2 in D.I. water pH 7.4) After incubation, biopsies were washed with 1× PBS (three times for 5 minutes at RT). Epidermal sheets were peeled off. Sheets were fixed for 10 minutes in acetone at -20 °C. After washing, epidermal sheets were stained for Langerhans cells with MsGp2-antibody (AbD Serotec).

Secondary antibodies used were anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 555 (Life-Technologies, Grand Island, NY) (MsGp2). Hoechst 33342 (Life Technologies), was used to visualize nuclei. Slides were then mounted with Fluoromount (Ebioscience, San Diego, CA) and viewed using widefield or confocal microscopy.

To visualize guinea pig epidermal Langerhans cells, we adapted Robins and Brandon's protocol.⁵³ Briefly, we fixed 30 μ m guinea pig skin sections in cacodylate formalin (0.2 mol/l cacodylate buffer, 20% paraformaldehyde, 6.85 g sucrose in 40 ml D.I. water) at 4 °C for 60 minutes. After washing with 1× PBS, we incubated the skin sections in prewarmed ATP-substrate-solution (0.25 mol/l Tris-maleate-buffer, 0.1 mol/l magnesium sulfate, 2 g glucose, 30 mg adenosin-5-triphosphate, and 2% lead nitrate in 40 ml D.I. water, pH 7) at 37 °C for 90 minutes. After rinsing, ATPase stain was developed in 1% ammonium sulfide for 2–5 minutes at RT.

Imaging

Widefield fluorescence microscopy was performed using an Olympus BX51 with a MagnaFire combo camera for photo acquisition (Olympus, New York City, NY). Magnafire software was used to acquire the images. Confocal images were obtained with a Zeiss LSM 780 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) and processed with Zen 2012 software (Zeiss) and further processed as 3D images in IMARIS software (Bitplane, Belfast, UK). Z stacks of images (obtained at 0.3 μ m intervals) were collected sequentially using 63× objective and then maximum projected into single flattened stacks for figures.

Separation of epidermal from dermal skin layers, and digestion

To analyze the frequency of GFP-transfected cells in epidermis and dermis, the skin-layers from 8-mm skin-biopsies taken at predetermined intervals after pGFP/SEP treatment were separated with Thermolysin as described above.⁵² Ten epidermal sheets were digested in 20 ml keratinocyte-digestion-medium (0.5 mmol/l calcium chloride, 0.05% Trypsin in Dulbecco's modified Eagle's medium) in a shaker-incubator (37 °C, 185 rpm, 1 hour). Ten dermal samples were digested in 30 ml collagenase medium (2.5% FBS, 100 U/ml collagenase type IV in RPMI) in a shaker-incubator (37 °C, 185 rpm, 1 hour). After vigorous vortex of the epidermal samples, 4.5 ml FBS was added immediately, followed by 20 ml Keratinocyte-medium (0.5 ml calcium chloride in Dulbecco's modified Eagle's medium). To the dermal samples, 20 ml wash medium (1% FCS, 1× HEPES in Dulbecco's modified Eagle's medium) was added. All samples were filtered through 70 μ m cell strainers. Cells were washed and resuspended in FACS buffer (2% FBS, 1 mmol/l EDTA in 1× PBS). GFP expression in skin cells was analyzed using the Accuri C60 flow cytometer (BD Biosciences, Franklin Lakes, NJ).

ELISA

Antibody responses against influenza NP and H5HA were performed as previously described.²² Optical densities were read at 450 nm, and determined to be a positive titer if optical density was two times that of

background control. The bottom positive titer on the plate was plotted as the end-point titer.

Statistical analysis

Data are expressed as mean + standard error of mean for each group. Statistical differences between groups were evaluated using a Student's *t*-test using GraphPad Prism 5.0b software. *P* < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

T.R.F.S., K.S., D.H.A., J.M.M., J.C.S., J.R.M., N.Y.S., and K.E.B. are employees of Inovio Pharmaceuticals and receive salary, benefits, and own stock/stock options in the company. The authors have no other relevant affiliations or financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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