Skin Transfection Patterns and Expression Kinetics of Electroporation-Enhanced Plasmid Delivery Using the CELLECTRA-3P, a Portable Next-Generation Dermal Electroporation Device

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The CELLECTRA-3P dermal electroporation device (Inovio Pharmaceuticals, Plymouth Meeting, PA) has been evaluated in the clinic and shown to enhance the delivery of an influenza DNA vaccine. To understand the mechanism by which this device aids in enhancing the host immune response to DNA vaccines we investigated the expression kinetics and localization of a reporter plasmid (pGFP) delivered via the CELLECTRA-3P. Histological analysis revealed green fluorescent protein (GFP) expression as early as 1 hr posttreatment in the epidermal and dermal layers, and as early as 2 hr posttreatment in the subdermal layers. Immunofluorescence techniques identified keratinocytes, fibrocytes, dendritic-like cells, adipocytes, and myocytes as the principal cell populations transfected. We proceeded to demonstrate elicitation of robust host immune responses after plasmid DNA (pDNA) vaccination. In guinea pigs equivalent humoral (antibody binding titers) immune responses were observed between protocols using either CELLECTRA-3P or intramuscular electroporation to deliver the DNA vaccine. In nonhuman primates, robust interferon- γ enzyme-linked immunospot and protective levels of hemagglutination inhibition titers after pDNA vaccination were observed in groups treated with the CELLECTRA-3P. In conclusion, these findings may assist in the future to design efficient, tolerable DNA vaccination strategies for the clinic.

INTRODUCTION

AN ATTRACTIVE CLINICAL STRATEGY for vaccine delivery is to directly target the skin (intradermal, ID). This is due to a number of features specific to the dermal regions, which make it an excellent immunization target. Skin is the most accessible organ of the human body and is easily monitored, but most importantly, it is a highly immunocompetent target.^{1,2} Indeed, the skin contains a resident population of professional antigen-presenting cells (APCs), specifically a large number of Langerhans cells and dermal dendritic cells.

The skin has three broad tissue types: the epidermis, dermis, and subdermis. The epidermis is a continually keratinizing stratified epithelium. Making up approximately 80–90% of the cellular population of the epidermis, the predominant cell type is the keratinocyte. These cells play both

structural and immunological roles.³ The epidermis is also home to the Langerhans cells, a professional APC population.^{1,2}

The dermis is the thickest of the skin layers and is composed primarily of connective tissue containing a dense collagen matrix, elastic fibers, and extracellular matrix interspersed with fibroblast cells and as such provides a scaffold for the skin.¹ The subdermis (or hypodermis) is the layer of tissue directly underneath the dermis and is composed mainly of areolar (loose) connective tissue and adipose tissue.

DNA vaccines are an example of a next-generation approach that offers major benefits over their conventional vaccine counterparts.^{4–7} DNA vaccines are gene-based expression plasmids that encode specific antigens and can mimic the immunological effects of infection because they are

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directly transfected into the host cell. Gene expression occurs via the host's own cellular machinery. This allows for antigen presentation through both the MHC class I and II pathways. DNA vaccines are able to generate both robust antibody and T cell responses.⁶⁻⁹ This ability means that DNA vaccination has the potential to offer a therapeutic solution against many complex diseases such as HIV/AIDS and cancers. Gene-based vaccines also offer the ability to develop, optimize, and manufacture large doses of vaccine in a cost-effective, rapid manner and to deliver various combinations of antigens avoiding immune responses to the vector itself. Because of the inherent stability of DNA vaccines, they do not require cold-chain storage, which is a major logistical issue with some current conventional vaccines and biologics. This has obvious major implications for their distribution and use in developing countries.

A major obstacle to effective vaccination via gene-based methods is the low efficiency of intracellular delivery. Outside of mouse models, the delivery of naked plasmid DNA (pDNA) by standard intramuscular (IM) injection is notoriously inefficient. In past studies, this has led to an inability to achieve strong immune responses in large mammals, such as monkeys, and humans immunized with naked DNA.⁴⁻⁷ One physical method to temporarily increase cell permeability is *in vivo* electroporation (EP), and this method has become the vanguard when considering delivery modalities for DNA vaccination.

EP involves the use of brief electrical pulses whose application results in the creation of transient hydrophilic pathways within the lipid bilayer membranes of mammalian cells. This allows the transport of DNA and other macromolecules through a cell membrane that was previously impermeable to these molecules. Therefore, EP has the ability to increase both the uptake and the extent to which drugs and DNA are delivered to the target tissue of interest.¹⁰⁻¹⁴ Historically, EP used in combination with drugs such as bleomycin has been shown to increase cell cytotoxicity and thus has been used for local treatment of cancer (electrochemotherapy).^{15,16} DNA vaccination with EP has been primarily targeted to muscle tissue and, at present, multiple clinical trials are being conducted using this route of delivery.¹⁷⁻²¹

In an attempt to improve the vaccination experience from the patients' perspective, a significant move toward developing EP devices that target the highly accessible dermal region has been made. Because skin as a target tissue is considerably shallower from a depth perspective than skeletal

muscle, dermal EP devices can be designed to be much less invasive and even completely noninvasive. This has the important implication from a patient tolerability standpoint of not activating deep nerves and muscles. To be a clinically relevant platform, it is vital that ID-EP still maintains equivalent efficacy in comparison with IM-EP procedures. Historically, it had been proposed that IM-EP generated robust cellular responses and ID-EP humoral responses. However, the current understanding of the platform implies that ID-EP can generate both antibody and cellular responses equally well.²²

Skin electroporation devices have been assessed both in experimental animals and in the clinic.²³⁻²⁵ The CELLECTRA-3P (Inovio Pharmaceuticals, Plymouth Meeting, PA) is a minimally invasive electroporation device that targets dermal and subcutaneous layers of the skin²⁶⁻²⁸ with mild EP conditions and minimal tissue damage. This device has entered the clinic in two studies (ClinicalTrials.gov identifiers: NCT01403155 and NCT01405885) sponsored by Inovio Pharmaceuticals addressing the delivery of a multistrain influenza DNA vaccine. The reduced depth of the minimally invasive electrodes has been shown to significantly increase the tolerability of the procedure compared with IM-EP.²⁹ Because this approach is considered more tolerable, the ability to deliver prophylactic immunizations with minimal invasiveness has become a reality using this device platform.

In a previous publication, the CELLECTRA-3P device platform was shown to improve plasmid vaccine delivery and potency in pigs and rhesus macaques.²⁶ This previous publication detailed proof-of-concept immunogenicity studies with this delivery modality in two animal models; however, we wished to gain a deeper understanding of the mechanism of action of the device with respect to plasmid delivery, identifying the cellular populations transfected, where they reside within the skin, and the impact these events have on immunogenicity. We previously published mechanistic studies evaluating the mode of action of our surface electroporation (SEP) device.^{22,30,31} Whereas the SEP and CELLECTRA-3P devices both target the intradermal space and rely on the Mantoux injection for plasmid delivery, each is distinct in its design and electrical parameters and as such the resulting immune response can differ. We were therefore also keen to understand the mechanism of action behind the CELLECTRA-3P and use this information to better understand the immune responses generated when vaccine was delivered by this modality. To achieve this knowledge, a time

course assessment of reporter gene expression was performed, using histological methods of analysis. We were able to identify the reporter gene-transfected cells as well as assess the kinetics of expression in defined layers of the skin. In the guinea pig and macaque models, we show equivalency of immune response in both magnitude and breadth when comparing this delivery platform with IM electroporation.

The results from this study provide insights into the mode of action after EP-enhanced DNA delivery targeting the dermal tissue. These findings may have implications for future understanding and application of this electroporation technology in clinical studies.

MATERIALS AND METHODS

Electroporation devices

ID-EP was performed with the 3P array, a microarray consisting of three needle electrodes, each 3 mm in length, forming an isosceles triangle with 5-, 5-, and 3-mm spacing between the needles, connected to the CELLECTRA-3P adaptive constant current device that delivers two sets of two 52-msec pulses with 198 msec between the end of the first pulse and beginning of the second pulse at 0.2-A constant current, a maximum of 200 V, and with approximately 3 sec between the first set of pulses and second set of pulses, delivering a total energy of 8.3 J. The disposable 3P array, which inserts into the skin, was redesigned to include a needle stick guard adding extra protection for the clinical staff. The new, handheld unit uses a single built-in control and signal generating printed circuit board (PCB) with a rechargeable lithium ion battery power source. Communication and data downloading capabilities were also redesigned for this prototype device through the addition of a wireless Bluetooth communication link. The Windows-based user interface program can be uploaded on any Windows-based laptop, tablet, and/or pocket PC. In nonhuman primates (NHPs), IM-EPs were performed with the 5P array, an array consisting of five needle electrodes, each 19 mm in length, forming a pentagon with 5.84-mm spacing between each needle, connected to the CELLECTRA-5P adaptive constant current device delivering three pulses at 0.5-A constant current, a maximum of 200 V, 52-msec pulse length with 1 sec between pulses, delivering a total of 15.6 J. Because each pulse briefly changes the impedance of the skin, the actual parameters vary slightly from pulse to pulse and it is thus estimated that the actual energy delivered by CELLECTRA-3P and CELLECTRA-5P

varies within a certain range and is determined to be approximately 6.2 and 11.7 J, respectively. In guinea pigs, IM-EPs were performed with the ELGEN twin injector, an applicator carrying two standard 1-ml syringes with needles serving as electrodes, 16 mm long, 4 mm apart, connected to the ELGEN-1000 pulse generator, delivering two 60-msec pulses at an applied voltage of 60 V and current of 200 mA (total of 1.44 J).

Plasmids

gWiz-GFP reporter gene plasmid was purchased from Aldevron (Fargo, ND). pH1HA is a SynCon vaccine construct that encodes a synthetic consensus sequence of hemagglutinin (HA) from H1N1 influenza viruses. EnvA, EnvB, and EnvC are SynCon plasmids based on Env antigen sequences derived from clade A, clade B, and clade C HIV, respectively.³² Gag and Pol are SynCon plasmids based on Gag and Pol antigen sequences derived from sequences that encompass most global HIV viruses.³³ All plasmid were diluted in 1× phosphate-buffered saline (PBS) before injection.

Animals

Female Hartley guinea pigs (6 months old) weighing ~350–400 g were used in this study. The guinea pigs were group housed (four per cage) with *ad libitum* access to food and water. Animals were acclimated for 2 weeks before experimentation. All animals were housed and handled according to the standards of the Institutional Animal Care and Use Committee (IACUC) at BioTox Sciences (San Diego, CA). Rhesus macaques (*Macaca mulatta*) of Chinese origin weighing 2.11–4.57 kg were individually housed at BIOQUAL (Rockville, MD) and acclimated for 2 weeks before experimentation under standard conditions. All animal treatment protocols were approved by the BIOQUAL IACUC.

Treatment and tissue processing

Guinea pigs were shaved and depilated 1 day before EP treatment. Each treatment at each time point consisted of a single injection of 50 µg of gWiz-GFP (Aldevron) in 50 µl of 1× PBS delivered intradermally by the Mantoux injection method and immediately followed by electroporation of the site, using the CELLECTRA-3P device.²⁶ The Mantoux intradermal injection is a standard clinical technique involving a small-gauge needle (usually 29G) inserted parallel to the skin bevel up. At the chosen time points, 8-mm biopsy punches of the treatment site were made postmortem, and biopsies were fixed in 4% paraformaldehyde (Sigma-Aldrich, St.

Louis, MO) at 4°C overnight. The next day, skin biopsies were buffered in a 25% sucrose solution (Sigma-Aldrich) and stored at 4°C. For sectioning biopsies were embedded in O.C.T. compound (Sakura, Tokyo, Japan) and sectioned at a thickness of 15 μ m, using an OTF cryostat (Bright Instruments, Cambridge, UK). Sections from all time points were stained with hematoxylin and eosin (BBC Biochemicals, Mount Vernon, WA) and viewed by bright-light microscopy. The fifth treatment of each time point for each animal was collected and stored at -20°C for gross imaging.

Immunohistochemistry

Skin sections from 1, 2, 4, 6, 24 and 48 hr post-treatment were stained with unconjugated primary antibodies, including anti-heat shock protein 47 (Hsp47) (clone M16.10A1; Novus Biologicals, Littleton, CO) and anti-keratin 10 (Assay Biotech, San Francisco, CA). Sections were then stained with an anti-mouse Alexa Fluor 555 (Life Technologies, Carlsbad, CA)-conjugated secondary antibody. An additional stain, Hoechst 33342 (Life Technologies), was used to visualize nuclei. The slides were then mounted with Fluoromount (eBioscience, San Diego, CA) and viewed by fluorescence microscopy.

Imaging

Fluorescence microscopy was carried out with an Olympus BX51 with a U-TV1X-2/U-CMAD 3 combo camera for photo acquisition (Olympus, Melville, NY). MagnaFire software was used to acquire the images. Confocal images were obtained with a Zeiss LSM 780 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and processed with Zen 2012 software (Carl Zeiss). Z stacks of images (obtained at 0.3- μ m intervals) were collected sequentially, using a $\times 63$ objective and then maximally projected into single flattened stacks for figures.

Animal immunizations

Guinea pigs. Female Hartley guinea pigs were divided into two groups of five. Animals were treated on days 0, 21, and 42, and therefore each animal received a total of three immunizations. Treatment was as follows: group 1 received two 100- μ l injections of 100 μ g of pH1HA and each injection site was immediately electroporated, using the CELLECTRA-3P device; group 2 received one 100- μ l injection, in the quadriceps muscle, of 50 μ g of pH1HA immediately followed by EP, using the ELGEN-1000 twin injector.

Nonhuman primates. Rhesus macaques were vaccinated intradermally or intramuscularly via

the quadriceps muscle followed by EP with the CELLECTRA-3P or CELLECTRA-5P, respectively. Each monkey received two immunizations, 5 weeks apart, at a dose of 1 mg for each of the following plasmids: pEnvA, pEnvB, pEnvC, pGag, and pPol for a total of 5 mg of cocktail per animal per treatment. The injection volume was 0.1 ml in five separate sites for a total of 0.5 ml for the group undergoing EP with the CELLECTRA-3P array. The injection volume was 0.5 ml in a single site for the group undergoing EP with the CELLECTRA-5P array. Each monkey also received two immunizations, 5 weeks apart, at a dose of 1 mg of pH1HA per immunization and the injection volume was 0.1 ml for the group undergoing EP with the CELLECTRA-3P array and 1 ml for the group undergoing EP with the CELLECTRA-5P array.

Sample collection

Peripheral blood. Blood samples were collected from the guinea pigs' cranial vena cava before treatment and 1 week after each immunization. Blood was centrifuged and serum was stored at -20°C until antibody titer testing by ELISA.

Blood samples were collected from rhesus macaques 2 weeks after each immunization. Animals were anesthetized with ketamine (10 mg/kg) mixed with acepromazine (0.1 mg/kg). Blood was collected in EDTA tubes and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by standard Ficoll-Hypaque density gradient centrifugation and resuspended in complete culture medium (RPMI 1640 with 2 mM L-glutamine supplemented with 10% heat-inactivated fetal bovine serum [FBS], penicillin [100 IU/ml], streptomycin [100 μ g/ml], and 55 μ M 2-mercaptoethanol).

ELISA

Guinea pig. Antibody responses against influenza H1 antigen [HA(Δ TM)(H1N1/A/California/2009); Immune Tech, New York, NY] were detected by ELISA as previously described. Optical densities (ODs) were read at 450 nm with a Molecular Devices SpectraMax PLUS 384 microplate reader, and determined to be a positive titer if the OD was two times that of the background control. The bottom positive titer on the plate was plotted as the end-point titer.³⁴

Enzyme-linked immunospot

Monkey interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) assays were performed as previously described.²⁶ Antigen-specific responses were determined by subtracting the number of spots in the negative control wells from the wells

containing peptides. Results are shown as the mean value (spot-forming units [SFU]/ 10^6 PBMCs) obtained for triplicate wells. After subtracting the negative control, the mean value in the wells with the PBMCs collected postvaccination had to exceed 50 SFU/ 10^6 PBMCs and be at least four times higher than prevaccination reactivity to be considered a positive response.

Hemagglutination inhibition testing

Rhesus macaque sera were treated with receptor-destroying enzyme (RDE) by diluting 1 part serum with 3 parts enzyme and incubated overnight in a 37°C water bath. The enzyme was inactivated by a 30-min incubation at 56°C followed by addition of 6 parts PBS for a final dilution of 1:10. Hemagglutination inhibition (HAI) assays were performed in V-bottom 96-well microtiter plates, using four hemagglutinating units (HAU) of virus and 1% horse red blood cells. Virus stock (H1N1/Mexico/2009 strain) used for the assays was obtained from the influenza branch of the Centers for Disease Control (CDC, Atlanta, GA).

Statistical analysis

Data are presented as means $\pm$ SD calculated from triplicate wells of pooled lymphocytes from each experimental group. Where appropriate, the statistical difference between immunization groups was assessed using a two-tailed, paired Student *t* test that yielded a specific *p* value for each experimental group. Comparisons between samples with *p* < 0.05 were considered to be statistically different and therefore significant.

RESULTS

Development of a hand-held, compact, easy-to-use dermal electroporation device

In a bid to build on an electroporation platform in current clinical and research use, a hand-held, nontethered electroporation device was designed and built for animal testing to reduce the size and improve the usability of the current CELLECTRA-3P device, originally connected to a pulse generator.²⁶ The new, handheld unit (Fig. 1a) sits in an inductive charging station (Fig. 1b). This single unified control board allows for reduced complexity for the end user, and reduced overall costs. The use of lithium ion battery technology for the power source provides greater energy density, longer charge-holding times, and faster recharging times. The electrode arrays are captured in individual sockets within the array head (Fig. 1c). The three-needle electrodes form a microarray to cover the

DNA injection site. This depth of penetration treats the entire skin thickness and as such targets the dermal cells in the epidermis, dermis, and subdermis. A schematic describes the firing pattern of the device (Fig. 1d).

Gene delivery enhanced by dermal electroporation induces sustained expression on the surface and underside of the skin

To assess the expression pattern resulting from gene delivery with the CELLECTRA-3P dermal EP device, a reporter plasmid (pGFP) was injected into guinea pig skin and the site immediately electroporated with the CELLECTRA-3P device. Animals were killed 72 hr posttreatment and the skin was excised and visualized under a fluorescence microscope (Fig. 2). Qualitative analysis of the gross localization pattern of GFP expression after delivery with EP revealed speckled expression on the surface of the skin, primarily in the region between the three electrodes, and distinct striated expression patterns on the underside of the skin. The insertion position of the three electrodes was visible on the surface of the skin for approximately 3 days postprocedure. The biopsies shown in Fig. 2 are representative examples of those seen in multiple treatments on multiple animals.

Histological analysis reveals GFP expression appearing in the epidermis, dermis, and subdermis

While the skin surface and underside localization patterns delineate the global expression trends, skin is a squamous epithelial tissue so it was possible that cells between the observable upper and lower surfaces were also being transfected. To investigate this further, biopsies were taken at defined time points and fixed sections were prepared. Under a fluorescence microscope, full skin sections (epidermal, dermal, and subdermal layers) from five treatment sites harvested from three guinea pigs were observed and scored for GFP-positive cells by two blinded technicians (Fig. 3 and summarized in Table 1). One hour posttreatment, positive cells were identified in both the epidermal (Fig. 4a) and dermal (Fig. 4b) sections of skin. The GFP-positive cells in the epidermis appeared to be closer to the basement membrane and were likely to be located in the stratum basale level of the epidermis and the mid to upper epidermis (stratum granulosum). Over the period of 24 hr, these GFP-positive cells moved toward the upper surface of the skin, flattening as they traversed the epidermal layer. Although GFP-positive cells in the dermis could be observed 1 hr posttreatment, they became

Figure 1. Development of a hand-held, compact, easy-to-use version of the CELLECTRA-3P dermal electroporation (EP) device. **(a)** Battery-operated, hand-held, nontethered EP applicator docked on **(b)** a charging station alongside a handheld tablet, which can be used for parameter programming and data collection. **(c)** Disposable array head with 3-mm-long electrodes encompassing sharps protection and fluid protection. **(d)** Schematic of electrode firing pattern of array.

Figure 2. Gene delivery enhanced by dermal electroporation induces sustained expression on the surface and underside of the skin. Shown is gross imaging of a guinea pig skin treatment site, surface and underside, injected with a reporter plasmid (pGFP) and immediately electroporated (*right*) with the CELLECTRA-3P intradermal device. The skin shown was collected 72 hr posttreatment and is representative of multiple treatments on multiple different guinea pigs. Skin was imaged by fluorescence microscopy. Red arrows indicate the insertion points of the three electrodes.

Figure 3. Histological analysis reveals universal green fluorescent protein (GFP) expression throughout the skin. Guinea pig skin was injected with a reporter plasmid (pGFP, green) and immediately electroporated. Skin was biopsied 2, 4, and 24 hr posttreatment, sectioned, and stained with an anti-keratinocyte antibody (Alexa Fluor 555 secondary, red) and with Hoechst (blue) counterstain. Skin sections were imaged on a fluorescence microscope. Images are representative of multiple treatments at each time point.

visibly more abundant at about 6 hr and were highly enriched by 24 hr (Fig. 4b). GFP could also be detected in the subdermal adipocyte (Fig. 4c) and connective muscle layers (Fig. 4d), although the GFP signal here appeared later than in the epidermis and dermis. Magnified Hoechst-counterstained

images from the 24-hr time point biopsies (Fig. 5) demonstrate the morphology of the transfected cells in the epidermis, dermis, and subdermis of skin. The large, circular morphology of the cells in the fat layer is indicative of adipocytes (Fig. 5e) and the smaller, dense, oval morphology of the cells in the connective tissue layer is indicative of myocyte cross-sections (Fig. 5f). Full identification of the specific cell type was confirmed through double staining with a cell-specific marker antibody for the keratinocytes and the fibroblasts. As the predominant cell type in the dermal layer, the majority of cells transfected in the dermis were fibroblasts (Fig. 5c). We have previously demonstrated the direct transfection of both keratinocytes³⁰ and dendritic cells (DCs)³¹ in the epidermis using another dermal EP platform, the SEP-EP. The reporter gene cellular expression pattern in the epidermis after CELLECTRA-3P treatment appears similar to that observed using the SEP (Fig. 5a and b), suggesting that the same populations of cells in the epidermis (keratinocytes and DCs) are targeted by both device platforms. Transfected dendritic-like cells were

Table 1. Proportion of Green Fluorescent Protein-Expressing Cells at Various Time Points in Each Skin Layer Relative to the Total Number of Resident Cells

Skin layer	Time after treatment (hr)						
	0	1	2	4	6	24	48
Epidermis	—	Δ	ΔΔΔ	ΔΔΔΔ	ΔΔΔΔ	ΔΔΔ	ΔΔΔ
Dermis	—	Δ	ΔΔΔ	ΔΔΔ	ΔΔΔΔ	ΔΔΔΔ	ΔΔΔ
Subdermis							
Adipose layer	—	—	Δ	Δ	ΔΔ	ΔΔΔΔ	ΔΔΔΔ
Muscle layer	—	—	Δ	Δ	Δ	ΔΔΔΔ	ΔΔΔΔ

Symbols: —, no expression; Δ, lowest ratio of positive reporter gene cells to resident population cells; ΔΔΔΔ, highest ratio of positive reporter gene cells to resident population cells.
Note: GFP-expressing cells were counted by fluorescence microscopy in skin sections from a total of five treatment sites harvested from three guinea pigs.

Figure 4. Histological analysis reveals GFP expression appearing in the epidermis, dermis, and subdermis. Shown are sections of guinea pig skin injected with reporter plasmid (pGFP) and immediately electroporated. Skin was collected and processed before treatment (NT) and at various time points post-treatment, sectioned, and stained with Hoechst counterstain (blue). Skin sections were imaged on a fluorescence microscope. **(a)** Magnified image of the epidermal layer. **(b)** Magnified image of the dermal layer. **(c)** Magnified image of adipose tissue in the subdermal layer. **(d)** Magnified image of connective muscle tissue in the subdermal layer. Images are representative of multiple experiments performed at each time point.

Figure 5. Histological analysis reveals GFP expression appearing in multiple skin-resident cell types. Guinea pig skin was injected with pGFP (green) and immediately electroporated. **(a and b)** Skin sections stained with an anti-keratinocyte antibody (Alexa Fluor 555 secondary, red) and with Hoechst (blue) counterstain. **(c and d)** Skin sections stained with an anti-Hsp47 antibody (Alexa Fluor 555 secondary, red) and with Hoechst counterstain (blue). Sections are from skin collected **(a)** 1 hr, **(b–d and f)** 24 hr, or **(e)** 48 hr posttreatment. Images are representative of multiple experiments performed at each time point.

Figure 6. Enhanced delivery of antigenic plasmid by intradermal EP generates immune responses equivalent to intramuscular EP. **(a)** ELISA end-point titers measured in the sera of guinea pigs administered a plasmid expressing flu antigens immediately followed by either intradermal (CELLECTRA-3P, $n=5$) or intramuscular (ELGEN-1000 twin injector, $n=5$) electroporation. Treatments were performed at weeks 0, 3, and 6, and sera were collected and analyzed at weeks 3, 6, and 12. **(b)** IFN- γ enzyme immunospot assays (ELISPOTs) were performed on peripheral blood mononuclear cell (PBMC) populations of nonhuman primates (NHPs) administered a cocktail of plasmids expressing HIV antigens immediately followed by either intradermal (CELLECTRA-3P, $n=5$) or intramuscular (CELLECTRA-5P, $n=5$) electroporation and **(c)** hemagglutination inhibition assays (HAIs) on sera isolated from rhesus macaques 7 days after their second immunization with a plasmid expressing HIV **(b)** or influenza H1HA **(c)** hemagglutination inhibition assays (HAIs) on sera isolated from rhesus macaques 2 weeks after their second immunization with a plasmid expressing influenza H1HA strain MX/4108/2009 followed by either intradermal (CELLECTRA-3P, $n=5$) or intramuscular (CELLECTRA-5P, $n=5$) electroporation.

detected in the dermis at 24 hr posttreatment (Fig. 5d). It should be noted that at the treatment sites of guinea pigs that received Mantoux injections of pGFP without electroporation, a small number of transfected cells can be observed; this number is insignificant compared with the number of transfected cells observed in the skin of guinea pigs that received the treatment and EP.

Enhanced delivery of encoding antigen plasmid by dermal EP generates immune responses equivalent to intramuscular electroporation

The majority of DNA-based vaccine clinical trials involving electroporation target the muscle. However, if equivalent efficacy could be produced with an intradermal delivery device, this may offer a less invasive and more tolerable alternative for the patient. To establish the impact of the delivery modality and target tissue, we investigated humoral responses in guinea pigs after delivery of a DNA vaccine encoding an influenza antigen enhanced either by IM-EP or ID-EP with the CELLECTRA-3P. The candidate vaccine was pH1HA (pGX2005 Syn-Con vaccine construct that encodes a consensus sequence of hemagglutinin [HA] from H1N1 viruses). Animals (five guinea pigs per group) were immunized three times, 3 weeks apart, and blood samples were taken at regular intervals to obtain serum for use in an ELISA. At all evaluated time points, the end-point antibody titers detected in the sera of animals electroporated with the ID device were equivalent to those of the IM-EP group (Fig. 6a).

To evaluate the technology in another clinically relevant animal model, we proceeded to immunize rhesus macaques with DNA vaccines against HIV antigens (EnvA, EnvB, EnvC, Gag, Pol) and influenza antigen (H1HA). Animals were immunized twice, 5 weeks apart, and blood taken to perform either IFN- γ ELISPOT (HIV antigens) or hemagglutination inhibition assay (HAI) for the influenza antigen. Two weeks after the second immunization, although the breadth of the responses varied between the two delivery modalities, the overall IFN- γ spot numbers in the ELISPOT readout were similar (2349 and 2383 SFU/million PBMCs for IM-EP and ID-EP, respectively) (Fig. 6b). Importantly, strong cellular responses were observed against all five vaccine antigens, using both delivery devices. To investigate functional antibody levels, sera from the immunized animals 2 weeks after the second immunization were assayed for HAI activity against influenza H1HA strain MX/4108/2009. Whereas control samples registered a mean HAI titer of 1:20 or below, the average responses from both the IM-

EP and ID-EP groups were over those required for protection (greater than 1:40), with the IM-EP group reaching 1:190 and the ID-EP group reaching 1:280 (Fig. 6c). These data with the influenza pH1HA vaccine support our previous observation with a pH5HA vaccine. We demonstrated induction of comparable cellular and humoral responses and protection from H5N1 virus challenge in NHPs after IM-EP or ID-EP delivery of pH5HA.²⁷

CONCLUSIONS

Intradermal electroporation is a platform technology that offers tolerable delivery of DNA vaccines in the clinic for prophylactic immunization. Many groups have shown the usefulness of this technology in a range of animal models in preclinical studies.^{22,26,35,36} Furthermore, skin EP delivery of pDNA has been used in clinical trials²⁵ (ClinicalTrials.gov identifiers: NCT01587131 and NCT01405885). Multiple modalities of ID-EP devices exist, ranging from contactless to fully penetrating electrodes. Because the devices have various modes of action, each will likely target different compartments in the skin. This may result in the transfection of different resident cell populations and as such, have the capacity to elicit various immune responses. Whereas a wealth of published literature demonstrates the ability of the ID platform to elicit robust immune responses in a spectrum of animal models and in the clinic,^{23–25} less is understood about the mechanism of action of dermal EP, especially related to the resulting expression patterns, transfected cell populations, and immune cell infiltration.

The tethered CELLECTRA-3P has now been evaluated in the clinic and has proven to be a generally safe, well-tolerated, and effective modality to deliver a DNA-based vaccine against influenza to human skin (ClinicalTrials.gov identifiers: NCT01587131 and NCT01405885). Although this was an excellent proof of concept of the technology, we sought to further advance the platform by generating a next-generation device that was more compact and easier to use in a widespread clinical setting.

The firing pattern of the CELLECTRA-3P device (Fig. 1d) is proprietary and was developed through extensive evaluations of pulse lengths, delays, and voltage and current inputs. The order of the pulsing allows for optimal electric field coverage of the area of skin between the electrodes. We believe that it is the simultaneous and coincident presence of the electric field and of the GFP plasmid that results in the expression patterns observed in Fig. 2. We observed speckled transfection of the upper

surface of the skin that was localized almost entirely within the pulsing area between the electrodes. Because the electrodes for this device are 3 mm long, it was not surprising that transfection occurred through the entire thickness of the skin and reached the subdermal region. Interestingly, the kinetics of this expression varied from layer to layer, with the majority of expression in the epidermis appearing between 2 and 4 hr, the majority of dermal expression appearing between 6 and 24 hr, and the majority of subdermal GFP expression appearing at the 24-hr time point (Figs. 3 and 4). This may be a function of transfection efficiency and expression driven by EP related to cell size and natural turnover, because the keratinocyte is relatively small with a high turnover rate, to the larger fibroblastic cells, and myocytes and adipocytes that are larger still with a much slower turnover rate.¹ It may also be a function of the electric field strength, which diminishes with increasing distance away from the electrodes and varies between different layers of the skin. The wide variety of cell population transfected by this intradermal device differs from the exclusive transfection of keratinocytes and DCs in the epidermal layer by another intradermal EP device, the SEP^{22,30,31} (Fig. 5a–f). This is likely a function of both the EP parameters used and the device design, but may additionally explain the differences observed between the resulting immune responses when an antigen plasmid is delivered by each modality. The persistence of antigenic expression may be extended in the skin with the use of the CELLECTRA-3P because the turnover of myocytes and adipocytes is significantly lower than that of keratinocytes.

Cellular infiltration at the treatment site is not observed until 4 days posttreatment after EP in a muscle.³⁷ After ID-EP with the CELLECTRA-3P, we observed significant monocyte/granulocyte trafficking to the treatment site at the 4- to 6-hr time point and continued to increase to 24 hr (data not shown). Clearly there are significant differences between skin and muscle as target tissues, but this finding seeks to highlight the benefits of intradermal vaccinations from the perspective of rapid dynamics. In a previous publication,³⁸ we demonstrated local tissue damage after EP with the CELLECTRA-3P. This damage, however, was extremely localized and was observed only in the direct vicinity of the penetrating electrodes. Optimizing electroporation conditions is a balance between expression to drive immunogenicity and inflammation, which can impact tolerability. Studies suggest that immunogenicity likely depends on improving the level and

context in which the antigen is expressed and recruiting APCs to present antigen.¹⁰ The influx of immune cells to the epidermis and dermis of the treatment site has been noted previously. However, previous analyses have not expanded to the subdermal region. In one study, we noted significant cell infiltration into the adipose and connective muscle layers resulting in, at the 24-hr time point (data not shown), an inflammatory response leading to localized tissue disruption. Electroporation as a procedure is proinflammatory and the local inflammation can act as an adjuvant to recruit APCs and expand the immune response.^{10,23,30} We are currently investigating the effect EP has on recruitment of inflammatory cells to the target tissue in relation to inflammation and cell death, and how these mechanisms relate to the ensuing immune response.

The data generated here describe a highly efficient mechanism to deliver DNA to skin that generates optimal expression in a variety of cell types. To evaluate the efficacy of DNA delivery, we compared the CELLECTRA-3P with IM-EP using a standard clinically tested device in both guinea pig and nonhuman primate models. It was previously shown that ID-EP significantly increases immune responses in animals that receive the EP immediately after vaccination compared with animals that receive only the vaccine injection.^{7,26} In the guinea pigs, because of the lack of available immunological tools at the time we were only able to evaluate humoral responses, we noted that at the week 6 time point, the titers from the ID-EP group were higher in magnitude (1:24,300 vs. 1:4410) (Fig. 6a). However, at the other time points analyzed, the responses between groups were equivalent.

Guinea pigs are an excellent animal model for dermatological research because their skin physiology is extremely similar to that of human skin. However, with respect to general physiology and the immune system, a more clinically relevant surrogate model is the nonhuman primate. In this experimental model, we were able to investigate both cellular responses and functional antibody responses. The cellular responses generated between the two devices were similar in magnitude although there was some variability in the breadth of responses (Fig. 6b). The HAI titer responses in the NHPs were comparable in magnitude (1:190 [IM-EP] vs. 1:280 [ID-EP]), both surpassing defined protective titers (Fig. 6c).

When designing clinical protocols involving plasmid transfer, a solid understanding of the mechanism of action of the vaccine delivery platform is crucial. The information gained from this study might allow us to better understand the immune

responses generated by this delivery modality and as such design optimal prime–boost regimens from a timing perspective, taking into account expression kinetics and trafficking of immune sensing cells to the treatment site. From the results of this study, we can conclude that the CELLECTRA-3P is a new and promising pDNA vaccine delivery device.

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AUTHOR DISCLOSURE

Authors D.H.A., J.M.M., A.S.K., K.S., J.B.M., T.R.F.S., N.Y.S., and K.E.B. are employees of Inovio Pharmaceuticals and as such receive compensation in the form of salary, stock options, and bonuses.

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