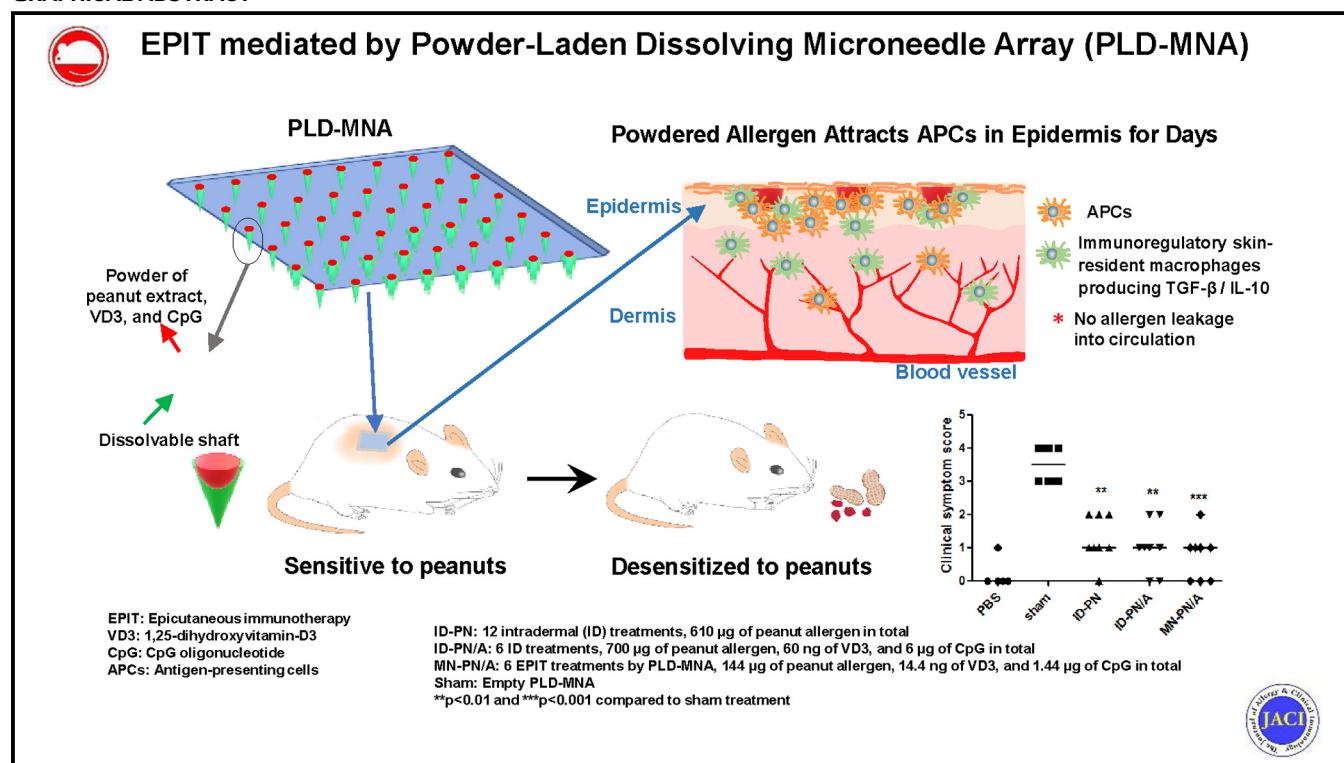


Delivery of allergen powder for safe and effective epicutaneous immunotherapy



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GRAPHICAL ABSTRACT



Background: More effective and safer immunotherapies to manage peanut allergy are in great demand despite extensive investigation of sublingual/oral immunotherapy and epicutaneous immunotherapy (EPIT) currently in the clinics. **Objective:** We sought to develop a powder-laden, dissolvable microneedle array (PLD-MNA) for epidermal delivery of powdered allergens and to evaluate the efficacy of this novel EPIT in peanut-sensitized mice.

Methods: PLD-MNA was packaged with a mixture of powdered peanut allergen (PNA), 1,25-dihydroxyvitamin D₃ (VD3), and CpG. Its epidermal delivery and therapeutic efficacy were

evaluated alongside PNA-specific forkhead box P3-positive regulatory T cells and IL-10⁺ and TGF- β 1⁺ skin-resident macrophages.

Results: PLD-MNA was successfully laden with PNA/VD3/CpG powder and capable of epidermal delivery of most of its content 1 hour after application onto intact mouse skin concomitant with no significant leakage into the circulation or skin irritation. PLD-MNA-mediated EPIT substantially reduced clinical allergy scores to 1 from 3.5 in sham control mice ($P < .001$) after 6 treatments accompanied by lower levels of PNA-specific IgE and intestinal mucosal mast cells and

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eosinophils over sham treatments. Moreover, in comparison with allergens administered intradermally, powdered allergens delivered by means of PLD-MNA preferentially attracted immunoregulatory macrophages and stimulated the cells to produce IL-10, TGF- β , or both at the immunization site, which might account for increased numbers of regulatory T-like cells in lymph tissues in association with systemic tolerance. PNA/VD3/CpG-laden PLD-MNA was safe and required only 6 treatments and one fifth of the PNA adjuvant dose, with improved outcomes when compared with 12 conventional intradermal immunotherapies.

Conclusions: PLD-MNA holds great promise as a novel, safe, effective, and self-applicable modality to manage IgE-mediated allergies. (*J Allergy Clin Immunol* 2020;145:597-609.)

Key words: *Epicutaneous immunotherapy, tolerogenic adjuvant, powdered peanut allergens, immunoregulatory macrophages, dissolvable microneedle arrays, regulatory T cells*

IgE-mediated food allergy is a growing health concern worldwide, affecting 6% to 8% of children and up to 10% of the general population in the United States.^{1,2} Although food allergies to milk and egg are common, peanut allergy dominates, with peanut causing frequent allergic reactions in more than 2% of children in the United States.³ These children are at a daily risk of peanut-induced anaphylaxis, which can be extremely severe and sometimes life-threatening, yet few treatment options are available to them beside strict dietary avoidance and carrying EpiPens to briefly interrupt the life-threatening anaphylaxis. Allergen-specific immunotherapy, including subcutaneous immunotherapy (SCIT), oral immunotherapy, sublingual immunotherapy, and epicutaneous immunotherapy (EPIT), has the prospect of clinical benefits, but none of them have been approved by the US Food and Drug Administration for managing peanut allergy in part because of the high risk of anaphylaxis in association with early clinical trials of SCIT¹ and adverse events in the mouth and gastrointestinal tract in highly sensitized subjects after oral and sublingual immunotherapy.⁴⁻⁶

The epidermis is a nonvascularized tissue that can greatly limit entrance of allergens into the bloodstream, minimizing anaphylactic risk. Also, it is enriched in antigen-presenting cells (APCs), especially immunoregulatory macrophages.⁷ In accordance with this, delivery of allergens into intact skin, but not damaged skin (eg, tape-stripped skin),⁸ has been shown to activate APCs in the epidermis, promote allergen-specific regulatory T (Treg) cells, and significantly inhibit allergic responses in preclinical and clinical studies.^{3,9,10} Moreover, EPIT could induce Treg cells with long-lasting suppressive function at levels greater than other routes of immunotherapy.¹¹ In addition, the epidermis is one of the more self-administrable and convenient sites for patients.

These advantages have attracted increasing attention to exploring various forms of EPIT in the management of peanut allergy, and some of them have been advanced to clinical studies in the past 2 decades.^{3,12-17} However, how to sufficiently deliver allergens into the epidermis without breaching skin integrity or provoking T_H2 immune responses remains a significant challenge because most allergens are macromolecules and impermeable to the skin.^{8,12}

To date, the most advanced EPIT is Viaskin, an epicutaneous delivery system (DBV Technologies, Bagneux, France) that has completed phase III clinical trials showing a high rate (95%) of

Abbreviations used

APC:	Antigen-presenting cell
DC:	Dendritic cell
dLN:	Draining lymph node
EGFP:	Enhanced green fluorescent protein
EPIT:	Epicutaneous immunotherapy
FoxP3:	Forkhead box P3
ID-PN:	Intradermal immunization with PNA alone
ID-PN/A:	Intradermal immunization with PNA/CpG/VD3
mLN:	Mesenteric lymph node
mMCP-1:	Mouse mast cell protease 1
MNA:	Microneedle array
MN-PN/A:	PLD-MNA loaded with PNA/VD3/CpG
OVA:	Ovalbumin
OVA-555:	OVA powder labeled with Alexa Fluor 555
PLD-MNA:	Powder-laden, dissolvable microneedle array
PNA:	Peanut allergen
SCIT:	Subcutaneous immunotherapy
sIg:	Specific immunoglobulin
SRB:	Sulforhodamine B
Treg:	Regulatory T
VD3:	1,25-Dihydroxyvitamin D ₃

patient compliance and beneficial effects for patients with peanut allergy from the age of 4 to 11 years.³ Importantly, there was no report of anaphylaxis in the clinical study, reaffirming good safety of the EPIT.³ Viaskin creates an occlusive chamber on the skin in which moisture is rapidly generated, and powdered allergens sprayed on the supporting membrane are dissolved and released from a supporting membrane entering the epidermis. However, only less than 10% of allergens could be delivered into the epidermis after 48 hours of application,^{18,19} which limits its efficacy because the allergen dose delivered into the skin is critical.²⁰⁻²³

Microneedle arrays (MNAs) superficially coated with birch pollen on each microneedle were also developed to deliver the allergens into the skin in human subjects, demonstrating improved allergen delivery,⁸ but this minimally invasive epidermal delivery has not been investigated in the treatment of peanut allergy.⁸ We used an ablative fractional laser to generate an array of self-renewable microchannels in the skin onto which a patch decorated with an identical pattern of antigen powder dots was aligned, resulting in approximately 80% powder delivery in approximately 1 hour in mouse, human, and pig skin concurrent with little skin irritation.^{17,24-26} The powder was retained within the epidermis for an extended period of time, constantly stimulating the immune system for days.²⁵⁻²⁷ In addition, we identified CpG, an agonist of Toll-like receptor 9 for T_H1 immune responses, and 1,25-dihydroxyvitamin D₃ (VD3), an immunosuppressant, to be a potent tolerogenic adjuvant when combined.¹⁷

To tailor this powder delivery technology for clinical uses, we recently engineered powder-laden, dissolvable microneedle arrays (PLD-MNAs) that could be directly applied onto intact skin to deliver powdered antigens into the epidermis²⁶ without the need for a laser to perforate the skin.^{17,25} In the current investigation, we showed that PLD-MNAs could sufficiently deposit a mixture of powdered peanut allergen (PNA), VD3, and CpG into the epidermis, giving rise to safer and more effective immunotherapy with a reduced number of treatments and much less

PNA in each treatment than conventional intradermal immunotherapy in a peanut-sensitized murine model.

METHODS

Animals

Female BALB/c mice at the age of 4 to 5 weeks and C57 BL/6J mice at the age of 6 to 8 weeks were purchased from the Jackson Laboratory (Bar Harbor, Me). MHC class II-enhanced green fluorescent protein (EGFP) mice expressing EGFP fused with the MHC class II molecule were a generous gift of Drs M. Boes and H. Ploegh, Harvard Medical School.²⁸ Mice were maintained under pathogen-free conditions at room temperature ($21^{\circ}\text{C} \pm 1^{\circ}\text{C}$), relative humidity ($45\% \pm 5\%$), and a 12-hour light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee, Massachusetts General Hospital, Boston, Mass.

PLD-MNA fabrication

A female MNA mold was made of polydimethylsiloxane (Sylgard 184; Dow Corning, Midland, Mich) through a soft lithographic process and comprised a total of 6×9 microneedles each at a height of $200\text{ }\mu\text{m}$ and a base diameter of $100\text{ }\mu\text{m}$, as previously described.^{26,29} The mold was cast with 8% sodium carboxymethyl cellulose (molecular weight, 90,000 Da; Sigma, St Louis, Mo) in distilled water, generating an MNA with a cave at approximately 25 to $30\text{ }\mu\text{m}$ in depth in the upper part and a sharp tip in the lower part of each microneedle.²⁶ PNA was prepared from defatted peanut powder (Byrd Mills, Ashland, Va) by using acetone extraction 3 times to remove any residual fat, followed by complete air-drying. Peanut extract was then dissolved in PBS at 4°C and precipitated with 80% ammonium sulfate for 2 hours at 0°C . The precipitate was collected after centrifugation at 15,000 rpm/min for 30 minutes, dissolved in PBS, and dialyzed in deionized water at 4°C 3 times, followed by passing through a $100\text{-}\mu\text{m}$ cell strainer. The resultant PNA was frozen at -70°C , lyophilized, and checked for protein concentrations by using the Bradford method. The defatted PNA was mixed with VD3 (Akron, Ill) and CpG (CpG-ODN 1826; TriLink BioTechnologies, San Diego, Calif) at a ratio of PNA/VD3/CpG equivalent to 100:0.01:1 and freeze-dried. The lyophilized mixture was loaded into the caved PLD-MNA through multiple rounds of centrifugation at 3000 rpm/min at 4°C , as previously described.²⁶

A standard curve was established by mixing sulforhodamine B (SRB) powder with a mixture of PNA/VD3/CpG prepared above at a 1:4 ratio by weight in dH_2O , followed by quantification of SRB fluorescent intensity on SpectraMax (Molecular devices, Sunnyvale, Calif), to quantify the amount of PNA proteins loaded to each PLD-MNA. The amount of PNA was correlated linearly with SRB absorbance at 560 nm and estimated to be about 12 μg of peanut protein per array.²⁶ Powdered ovalbumin (OVA)–Alexa Fluor 488 (Life Technologies, Eugene, Ore) at 10 μg per PLD-MNA was loaded similarly for the antigen uptake study.

Preparation of single-cell suspensions from the skin

A PLD-MNA loaded with powdered OVA–Alexa Fluor 488 (10 μg) was applied onto the shaved dorsal skin of individual mice to analyze antigen uptake after PLD-MNA application. Intradermal injection of a similar amount of OVA–Alexa Fluor 488 in a volume of 10 μL was also carried out for comparisons. After application for 1, 6, 24, and 48 hours, mice were killed. Skin samples of $0.5 \times 1\text{ cm}^2$ in size beneath the patch or at the intradermal injection site was excised, cut into small pieces of 1 to 2 mm^3 , and incubated for 30 minutes in RPMI containing 10% FBS, 400 U/mL Collagenase-D, and 0.1% DNase (both from Roche Applied Sciences, Mannheim, Germany). After digestion, skin fragments were gently pressed against a $70\text{-}\mu\text{m}$ nylon filter and then a $40\text{-}\mu\text{m}$ nylon filter to obtain single-cell suspensions for flow cytometry.

Flow cytometry

Single-cell suspensions prepared from the skin, spleens, inguinal lymph nodes, and mesenteric lymph nodes (mLNs) were stained with the following

antibodies after blocking with anti-CD16/32 antibody (Fc Block; clone 93; eBioscience, San Diego, Calif). Surface biomarkers were identified by using antibodies directed at CD4 (GK 1.5), CD11b (M1/70), CD11c (N418), CD25 (PC 61), CD44 (IM7), CD62L (MEL-14), CD169 (3D6.112), CD127 (IL-7 receptor α), and F4/80 (BM8). Intracellular biomarkers were recognized by antibodies specific for TGF- β 1 (LAP; TW7-16B4), forkhead box P3 (FoxP3; MF14), and IL-10 (catalog no. 554467), all from BioLegend (San Diego, Calif), except for anti-IL-10 antibody, which was from BD Biosciences (San Jose, Calif). For intracellular staining, cells were fixed after surface staining, permeabilized with an IC Fixation & Permeabilization Buffer Set from eBioscience, and then stained with indicated antibodies. 4'-6-Diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) was used to determine cell viability. Stained cells were enumerated on a FACS Aria (BD Biosciences) and analyzed with FlowJo software (version 7.6.5; TreeStar, Ashland, Ore).

Intravital confocal imaging

The ears of MHC class II–EGFP mice were inserted with an OVA powder labeled with Alexa Fluor 555 (OVA-555) powder-laden MNA and imaged by using intravital 2-photon confocal microscopy (Olympus FV-1000; Olympus, Center Valley, Pa) to monitor antigen uptake over time, as previously described.^{29,30} The OVA-555 powder-loaded MNA was also imaged by using confocal microscopy before and after skin insertion to show microneedle shafts melting away. In addition, 3-dimensional reconstruction was made by using ImageJ software (National Institutes of Health, Bethesda, Md) to visualize OVA powder delivered into the skin.

Measurement of levels of circulating OVA

Two PLD-MNAs, each loaded with 10 μg of OVA–Alexa Fluor 647 powder, were applied to the upper and lower dorsal skin of a mouse after hair removal to examine any allergen leakage into the circulation after application of PLD-MNAs. In parallel, the same amount of OVA–Alexa Fluor 647 in 10 μL of PBS was administered by using either intradermal or subcutaneous injection. Blood samples were taken at the indicated times after OVA delivery, and fluorescent intensity of plasma OVA over plasma control was quantified by using SpectraMax (Molecular devices), as previously described.⁵

Analysis of immunoregulatory macrophages in the skin and Treg cells in the spleen

Mice were sensitized to OVA (50 μg ; Sigma-Aldrich) emulsified with alum (1 mg; Thermo Fischer Scientific, Waltham, Mass) by using intraperitoneal administration on days 1 and 14. Sensitized mice were treated on day 25 with PLD-MNAs loaded with powdered OVA, CpG, and VD3 at the same ratio as above. The trio were also intradermally injected in 10 μL of PBS, and the empty PLD-MNAs (no powder) served as controls. Skins involved were excised on day 27, and spleens were collected on day 40 for preparation of single-cell suspensions and analysis of skin immunoregulatory macrophages and spleen Treg cell-like cells by using flow cytometry, respectively.

Therapeutic potentials of PLD-MNAs encapsulated with PNA/VD3/CpG

Mice were sensitized to peanut proteins (Greer Laboratories, Lenoir, NC) by using intraperitoneal administration of 25 $\mu\text{g}/\text{d}$ peanut proteins emulsified with 1 mg of alum on days 1, 14, 21, and 75. Sensitized mice were treated on day 25 for 1 hour with 2 PLD-MNAs containing a total of 24 μg of PNA, 2.4 ng of VD3, and 0.24 μg of CpG per mouse: one on the upper and the other on the lower dorsal skin. The same treatments were conducted on days 33 and 41 in phase I and days 67, 73, and 80 in phase II in a total of 6 treatments. A separate group of sensitized mice were treated on a similar schedule with intradermal injection of 10 μL of PBS containing increasing doses of 50, 100, or 200 μg of PNA plus VD3 (10 ng) and CpG (1 μg) in phase I and the same doses in phase

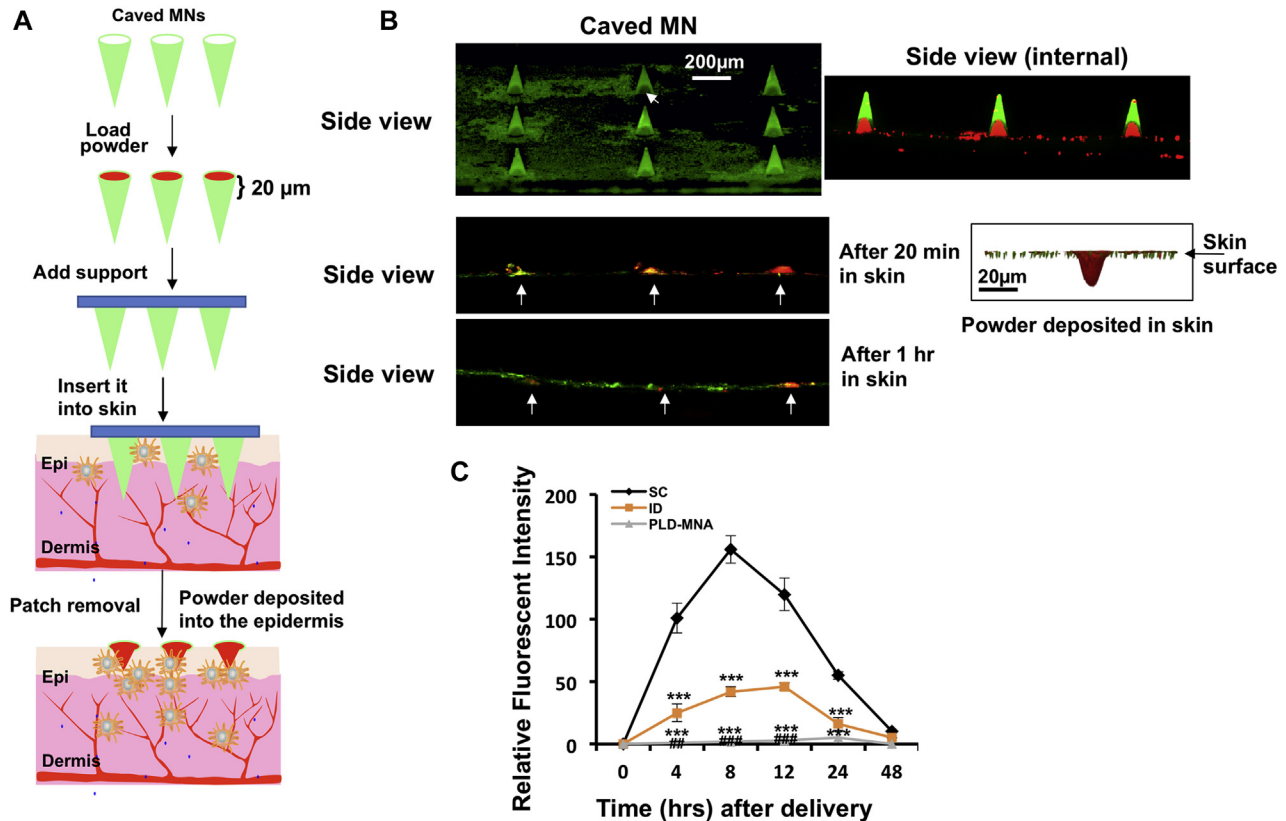


FIG 1. OVA powder is laden into a PLD-MNA and delivered into the epidermis. **A**, Illustration of a PLD-MNA loaded with OVA powder to deliver it into the epidermis: green, caved microneedles; red, OVA powder; blue, a support. **Epi**, Epidermis. Cells with dendritic morphology are APCs. **B**, Proportion of a PLD-MNA before and after application into mouse skin. PLD-MNA was made by using carboxymethyl cellulose mixed with a trace amount of fluorescein isothiocyanate (green; upper left) and laden with OVA-555 powder (red; upper right), followed by confocal microscopic scanning. Shown is a portion of the PLD-MNA before powder loading and a cave pointed by an arrow in the upper left, 3 representative microneedles encapsulated with OVA-555 powder in the upper right, OVA-laden PLD-MNA after insertion into the skin for 20 minutes (middle left) or 1 hour (bottom left), and a 3-dimensional image of a compressed powder deposited in the skin in the middle left. Note: The shafts dissolved in 20 minutes, and most of the powder disappeared in the caves within 1 hour after skin application (middle and bottom panels on left). MN, PLD-MNA. **C**, Insignificant leakage of OVA administered through PLD-MNAs compared with intradermal or subcutaneous injection. OVA-555 was measured in the blood at indicated times after delivery. Data are shown as means $\pm$ SDs ($n = 6$). *** $P < .001$ compared with subcutaneous injection or ### $P < .01$ and #### $P < .001$ compared with intradermal injection.

II at an interval of 1 week. In addition, intradermal immunization with a dose-escalation pattern mimicking human SCIT was carried out in parallel, in which the sensitized mice were treated with 5, 10, 20, 40, 80, or 150 μ g of PNA alone in phase I and similar doses in phase II in a total of 12 treatments. Between the phase I treatment (days 28-64) and after the phase II treatment (days 84-105), the mice were challenged intragastrically with 15 mg of peanut proteins at an interval of 4 days for 10 times or 6 times, respectively. On the final day (day 105), peanut-challenged mice were killed after 2 hours of the final challenge. Blood samples, jejunum, spleens, and mesenteric and inguinal lymph nodes were collected for analyses.

Clinical symptom scores

During each peanut challenge, all mice were scored for clinical symptoms up to an hour as follows³¹: 0, no symptoms; 1, intense scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, a profuse liquid stool recorded as diarrhea, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and tail; 4, no activity after prodding or tremor and convulsion; and 5, death.

ELISAs

Fifty microliters of blood was obtained from the mouse tail vein at different time points and assayed for peanut-specific antibodies by using ELISA.^{17,32} In brief, plates (Corning Life Sciences, Corning, NY) were incubated overnight at 4°C with 50 μ g/mL peanut proteins (Greer Laboratories), blocked, and then reacted with serum at different dilutions for 2 hours at room temperature. PNA-specific IgE, IgG₁, and IgG_{2a} were detected by using horseradish peroxidase-labeled isotype-specific secondary antibodies (all from Southern-Biotech, Birmingham, Ala). Mouse mast cell protease 1 (mMCP-1) protein levels were measured by using an ELISA kit according to the manufacturer's instructions (eBioscience).

Statistical analysis

The significance of differences between 2 groups or among multiple groups was analyzed by using the Mann-Whitney *U* test or 1-way ANOVA, followed by Tukey multiple comparison tests, respectively, with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered significant.

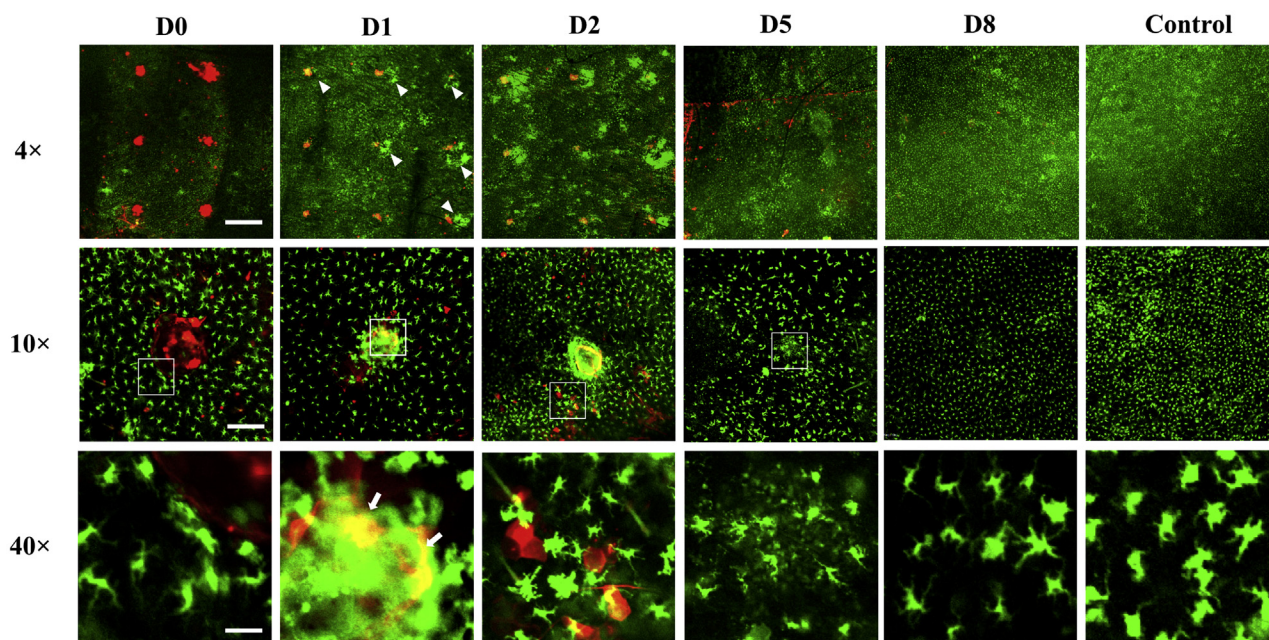


FIG 2. Powdered allergen deposited by a PLD-MNA actively attracts APCs and promotes antigen uptake in the epidermis. OVA-PLD MNA was applied into the ears of MHC class II-EGFP mice for 15 minutes and removed. The skin involved was imaged by using 2-photon confocal microscopy to track OVA-555 in the epidermis from day 0 to day 8. The untreated ears of the same mice served as controls. OVA powder deposited in the epidermis had an identical pattern of a PLD-MNA, and APCs were heavily accumulated around each powder dot (white triangles; top view, upper panels). The area highlighted in each middle panel was enlarged in corresponding bottom panels. White arrows indicate OVA-555 taken up by MHC class II-positive APCs, as suggested by a merged yellow color of green and red. Scale bars represent 500, 200, and 50 μm in the upper, middle, and bottom panels, respectively. Green, Green fluorescent protein-positive APCs, mainly macrophages and DCs; red, OVA-555. Magnifications are shown at left.

RESULTS

Fabrication and characterization of PLD-MNAs

How a PLD-MNA can deliver allergen powder precisely into the epidermis is shown in Fig 1, A. A cave was formed in the basal part of each microneedle as a result of condensation during polymerization.²⁶ The caves each were approximately 25 to 30 μm in depth, which was similar to the depth of the mouse epidermis, and could be filled directly with powdered allergens through multiple rounds of centrifugation.²⁶ After package of the powder, the caves were sealed by a supporting layer, and the PLD-MNA was peeled off and inserted into the shaved skin. Microneedle shafts melted away in approximately 15 to 20 minutes by interstitial fluid after skin application of the PLD-MNA, exposing the powder in the epidermis (Fig 1, A, fourth and fifth panels), despite the fact that the tip of the PLD-MNA penetrated into the dermis (fourth panel) because the powder was encapsulated in the basal part of the microneedles only (fifth panel).

We fabricated 6×9 microneedles per array with a natural polymer, carboxymethyl cellulose, a typical biocompatible and dissolvable polymer that has been widely used in biomedical and pharmaceutical fields.^{33,34} The polymer was mixed with a trace amount of fluorescein isothiocyanate before polymerization for imaging the microneedles with confocal microscopy, a portion of which was shown with caves in the basal part of the microneedles (Fig 1, B, upper left, arrow). To better visualize the caves and powder package, finely meshed OVA-555 was laden into a PLD-MNA, and the resultant PLD-MNA was scanned by using 2-photon confocal microscopy showing OVA packaged within the cave of each microneedle (Fig 1, B, upper right, red).

After application of the PLD-MNA into the skin for 20 minutes, PLD-MNAs were removed, and their shafts were dissolved completely, but some powder remained in some of the caves while completely disappearing in others (Fig 1, B, middle left). Within the skin, the powder could be seen mirroring the cave in the epidermis (Fig 1, B, middle right). If the PLD-MNA was kept in the skin for 1 hour, all microneedles in the array were devoid of powder (Fig 1, B, bottom left). Unlike allergens in a liquid form, powdered allergen could be drained into the epidermis by sucking interstitial fluid from the skin tissue and diffusing against the interstitial fluid influx similarly to the powder draining into laser-generated microchannels previously demonstrated.²⁷ Therefore, even if the powder was encircled within the stratum corneum at the outermost layer or above the skin because of incomplete insertion, the powder could still enter the skin.

Although efficiently delivering OVA into the epidermis, PLD-MNA application for 1 hour in the skin did not result in OVA leakage into the circulation. The amount of OVA in the circulation did not differ significantly from the basal level during the 48-hour experimental period after 2 PLD-MNAs, each loaded with 12 μg of OVA-Alexa Fluor 647, were applied to the dorsal skin of a mouse (Fig 1, C). In parallel, OVA levels were readily measurable in the blood after the same amount of OVA-Alexa Fluor 647 in a volume of 10 μL was administered either intradermally or subcutaneously, peaking at 8 or 12 hours, respectively. The negligible entry of allergens into the circulation warrants the safety of PLD-MNAs because allergen entrance into the circulation is a prerequisite for triggering anaphylaxis.

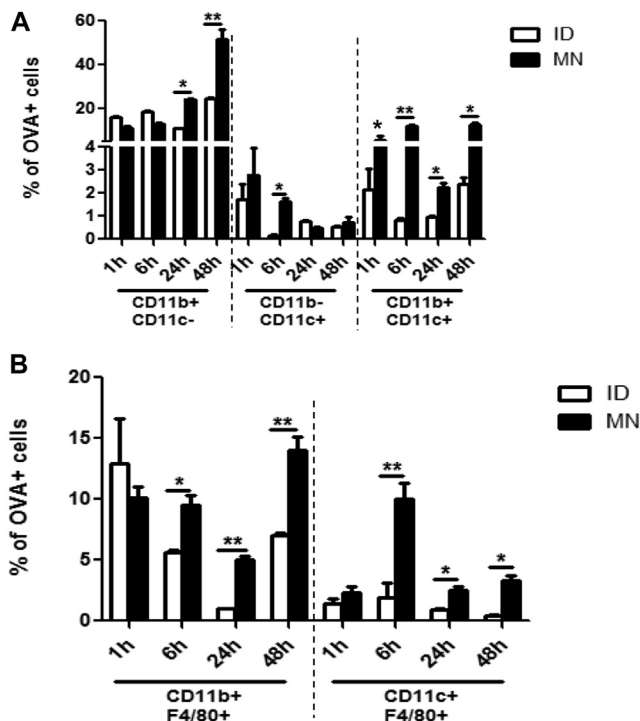


FIG 3. Powdered antigen attracts more macrophages than liquid antigen at the site of immunization. Antigen uptake was analyzed for cells expressing CD11b⁺ or CD11c⁺ (A) and F4/80⁺ (B) at indicated times by using flow cytometry after administering powdered or liquid OVA into the skin. Data are expressed as means $\pm$ SEMs (n = 6). *P < .05 and **P < .001 compared with intradermal immunization. ID, Intradermal; MN, PLD-MNA.

Dynamic accumulation of APCs around individual allergen-deposited microdots and their uptake of the powdered allergen were investigated by using intravital confocal microscopy of MHC class II-EGFP mice.^{25,28,35} When an OVA-555-laden PLD-MNA was applied into mouse ears for 15 minutes and removed, OVA-555 powder was clearly deposited within the skin and readily recognized on a top view (Fig 2, day 0). The OVA attracted APCs that were heavily concentrated around each powder dot, with a peak on days 1 and 2 (Fig 2, white triangles, upper panels), which could be readily seen at high magnification (Fig 2, middle panels). These APCs took up OVA, as suggested by emerged yellow colors of green and red on days 1 and 2 (Fig 2, bottom panels, arrows), and migrated away, resulting in fewer and fewer of these congregated APCs over the next 2 days. The gradual reduction in the number of APCs concurred with corresponding decreases in powder quantity or density in the epidermis from day 1 to day 5. Normal numbers and patterns of APCs were restored in the skin indistinguishable to the control skin after 8 days of PLD-MNA application (Fig 2).

Powdered allergen enhances accumulation of macrophage-like cells in the skin

APCs that accumulated around and captured the powdered antigen were next analyzed in the skin after PLD-MNA application. To this end, a PLD-MNA loaded with OVA-Alexa Fluor 488 was applied onto the shaved dorsal skin, and intradermal injection of the same amount of OVA-Alexa Fluor 488 in 10 μ L of PBS served as a control. The skin beneath the patch or intradermal

injection site was excised within 1, 6, 24, or 48 hours of application, and the skin cells were subject to flow cytometric analysis. Overall, OVA-Alexa Fluor 488 was preferably captured by macrophages identified as CD11b⁺CD11c⁻F4/80⁺ cells or macrophage-like cells marked as CD11b⁺CD11c⁺F4/80⁺ cells over CD11b⁻CD11c⁺ dendritic cells (DCs), with significantly greater efficiencies of antigen capture after PLD-MNA application than intradermal injection (Fig 3). Differences in OVA capture after the 2 approaches of skin administration became more significant at later time points (24 or 48 hours) than at earlier time points (1 hour) for macrophages (Fig 3), but it was without a significant difference for DCs with the exception when OVA capture was examined at 6 hours after immunization. Moreover, proportions of OVA⁺CD11b⁺CD11c⁺ cells or macrophage-like cells were significantly greater at all time points at the skin receiving powdered OVA than that receiving liquid OVA (Fig 3, A). Likewise, CD11b⁺CD11c⁻F4/80⁺ and CD11b⁻CD11c⁺F4/80⁺ cells, both displaying a macrophage-like phenotype, were more likely to capture OVA at the administration site after 1 hour of powdered over liquid OVA administration (Fig 3, B). Enhanced antigen uptake after administering OVA powder over liquid was presumably ascribed to gradual dissolving of the powder by interstitial fluid *in situ* in coordination with APC migration toward the powder-deposited site for an extended period of time, intrinsically creating antigen “depot” effects like alum adjuvant. On the contrary, OVA delivered intradermally was diffused out quickly, as suggested by increasing circulation of OVA in a few hours after intradermal injection (Fig 1, C).

PLD-MNAs containing VD3/CpG enhances tolerogenic macrophages in the skin and Treg-like cells systemically

We screened various prominent experimental adjuvants for their ability to induce anti-inflammatory cytokines at the site of intradermal immunization and found that a combination of VD3 and CpG, but not either alone, vigorously bolstered IL-10 and TGF- β production.¹⁷ Thus we packaged the adjuvant plus OVA into a PLD-MNA. When the PLD-MNA was applied to sensitized mice (Fig 4, A), macrophage-like cells producing TGF- β and IL-10 were augmented at levels much greater than those seen with intradermal or sham immunization (Fig 4, C and D). The significant increases were demonstrated both by the total numbers and percentages of IL-10⁺ and/or TGF- β ⁺ cells in mice immunized with PLD-MNA over intradermal delivery of the same amount of OVA/VD3/CpG (Fig 4, C and D). The phenotypes of the skin macrophages producing IL-10 (Fig 4, E and F) or TGF- β (Fig 4, G and H) were mainly CD11b⁺CD11c⁻CD169⁺F4/80⁺ cells, among which distinct double-positive CD11b⁺CD169⁺, CD11b⁺F4/80⁺, and CD169⁺F4/80⁺ cells producing IL-10 were increased by 2.5-, 3-, or 5-fold in percentages (Fig 4, F) or by 6-, 7-, or 7-fold in numbers (Fig 4, E), respectively, when compared between PLD-MNA EPIT and intradermal EPIT. These tissue-resident CD169⁺ macrophages are well documented to be immunoregulatory.⁷

Moreover, PLD-MNA enhanced TGF- β expression in CD11b⁺CD169⁺, CD11b⁺F4/80⁺, and CD169⁺F4/80⁺ cells by 1.5-, 2-, and 3-fold, respectively, compared with intradermal injection (Fig 4, G), although the percentages of these double-positive cells were either increased similarly or not affected by

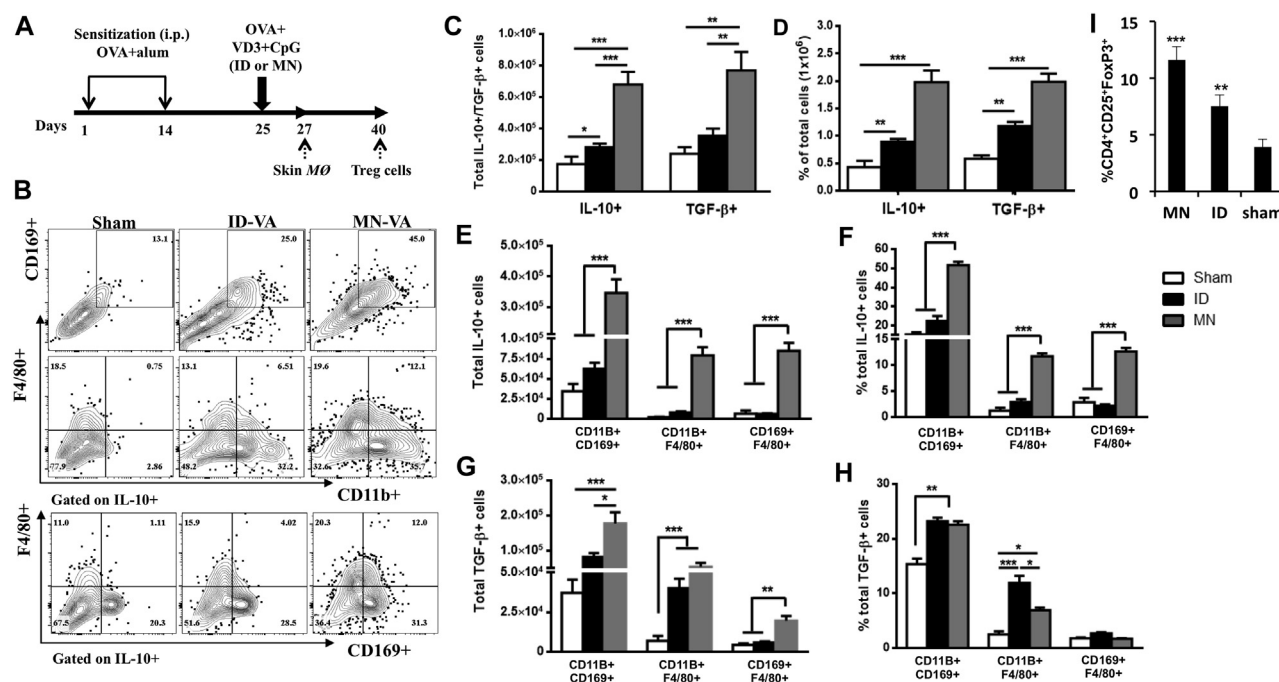


FIG 4. Accumulation of skin-resident macrophages with immunoregulatory function determined by using PLD-MNA. **A**, Depiction of experimental timelines for OVA sensitization, EPIT, and analyses of skin macrophages (*MΦ*) and spleen Treg cells. *i. p.*, Intraperitoneal. **B**, Skin cells were prepared 48 hours after an indicated EPIT and stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) and antibodies against CD11b, CD11c, and CD169, followed by intracellular staining with anti-IL-10 or anti-TGF- β 1 antibody. **C** and **D**, Using sequential gating strategies as shown in Fig 4, **B**, DAPI-negative live cells were first analyzed for IL-10⁺ and TGF- β ⁺ cells. **E-H**, Phenotypes of IL-10⁺ cells (Fig 4, **E** and **F**) and TGF- β ⁺ cells (Fig 4, **G** and **H**) were subdivided into CD11b⁺CD169⁺, CD11b⁺F4/80^{hi}, and CD169⁺F4/80^{hi} cells. Percentages (Fig 4, **F** and **H**) and total numbers (Fig 4, **E** and **G**) of these subsets were analyzed. Flow cytometric profiles of TGF- β ⁺ cells were similarly obtained for those IL-10⁺ cells. **I**, Percentages of CD4⁺CD25⁺FoxP3⁺ T cells relative to total CD4⁺ T cells were determined by using flow cytometry in the spleens of sensitized mice 2 weeks after sham or EPIT with OVA, VD3, and CpG administered either intradermally or by means of PLD-MNA (*n* = 6). **P* < .05, ***P* < .01, and ****P* < .001 compared with sham treatment or between indicated groups. ID, Intradermal; MN, PLD-MNA.

the 2 EPIT approaches, except for CD11b⁺F4/80⁺ cells, numbers of which were lower in mice receiving OVA/VD3/CpG through the PLD-MNA than those receiving the trio through intradermal immunization (Fig 4, **H**). Representative flow cytometric profiles on gating of IL-10⁺ cells were presented in Fig 4, **B**, and a similar gating strategy was used to analyze TGF- β ⁺ cells (data not shown). CD11b⁺CD11c⁺ DCs were not significantly different in numbers or percentages between the 2 immunotherapies, although they both increased IL-10 and/or TGF- β production similarly over sham treatment (data not shown). In accordance with a high level of IL-10 and TGF- β in the skin, CD4⁺CD25⁺Treg-like cells in the spleens of OVA-sensitized mice were significantly greater in the mice treated with PLD-MNA EPIT over those receiving intradermal EPIT (Fig 4, **I**). Collectively, powdered allergen presents advantages over liquid allergen in attraction of immunoregulatory macrophages and induction of immune tolerance in sensitive animals.

PLD-MNA induces immune tolerance and attenuates allergic responses

Mice were first sensitized by using PNA emulsified with alum once a week for 3 consecutive weeks, as well as on day 75 after the first injection (Fig 5, **A** and **B**). To treat these sensitized mice, the

amount of PNA powder encapsulated in a PLD-MNA must be determined, which was estimated to be 12 μ g per patch on average from 3 independent experiments each with 6 PLD-MNAs analyzed similarly to the previous study.²⁶ In the pilot study we found that 2 patches were required to sufficiently suppress IgE in the mice. Therefore 2 patches containing a total of 24 μ g of PNA, 2.4 ng of VD3, and 0.24 μ g of CpG were applied onto each sensitized mouse once every 8 days 3 times in phase I and repeated the 3 treatments in phase II, as depicted in Fig 5, **B** (PLD-MNA loaded with PNA/VD3/CpG [MN-PN/A]). Intradermal immunization with the same amount of PNA/VD3/CpG did not induce significant tolerance in phase I therapy and thus was not continued (Fig 6, **H**). Instead, an escalating PNA dose (50, 100, or 200 μ g), along with 10 ng of VD3 and 1 μ g of CpG in each treatment, was intradermally administered on a similar schedule (intradermal immunization with PNA/CpG/VD3 [ID-PN/A]; Fig 5, **B**). The PNA and adjuvant used in intradermal EPIT were at the minimum amounts required to significantly suppress allergic responses, which were 5 times more than those used for PLD-MNA EPIT. In addition, a separate group of mice were intradermally immunized with an escalating dose of 5, 10, 20, 40, 80, or 150 μ g of PNA alone every 2 or 3 days for a duration of 15 days in a total of 6 treatments in phase I, followed by an interval of 4 weeks and then repetition of phase I immunotherapy

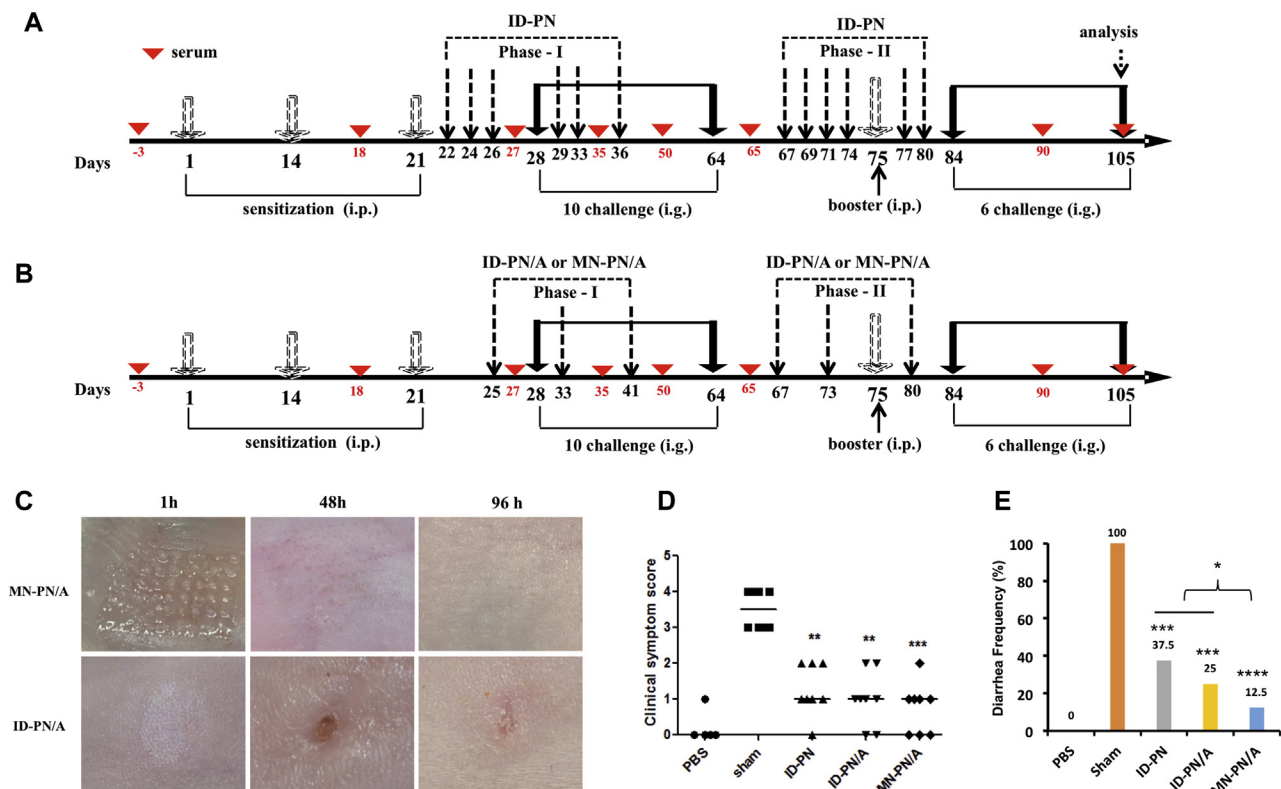


FIG 5. Amelioration of peanut-induced allergic symptoms by EPIT in sensitized mice. **A** and **B**, Experimental schedules of peanut sensitization, immunotherapy, and challenges. *i.g.*, Intragastric; *i.p.*, intraperitoneal. **C**, Representative photographs of skin captured at indicated times after application of a MN-PN/A or intradermal injection of the trio (*ID-PN/A*). **D** and **E**, EPIT sufficiently suppressed clinical symptoms and diarrhea frequency compared with sham treatment. *ID-PN*, Intradermal immunization with PNA alone (6 treatments with a total of 305 μ g of PNA in phase I); *ID-PN/A*, intradermal immunization with PNA/CpG/VD3 (3 treatments with a total of 350 μ g of PNA, 30 ng of VD3, and 3 μ g of CpG in phase I); *MN-PN/A*, EPIT by PLD-MNAs containing PNA/VD3/CpG (3 treatments with a total of 72 μ g of PNA, 7.2 ng of VD3, and 0.72 μ g of CpG in phase I). Data are shown as means $\pm$ SEMs ($n = 5$ in PBS group and $n = 8$ in the sham and treatment groups). * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with sham treatment or between indicated groups. *ID*, Intradermal; *MN*, PLD-MNA.

with a total of 12 treatments, mimicking a dose escalation study in clinics, as depicted in Fig 5, A (Intradermal immunization with PNA alone [*ID-PN*]). The sham group was treated with empty PLD-MNA free of PNA or adjuvant. As shown in Fig 5, C, PLD-MNA completely dissolved within 1 hour after its application into the shaved dorsal skin of a mouse. The application site exhibited normal skin appearance after 48 hours (Fig 5, C, middle). On the contrary, significant skin inflammation occurred at 48 hours and did not resolve within 96 hours after intradermal immunization of PNA, regardless of whether the duo adjuvant was included (Fig 5, C). The adverse skin irritation was observed consistently in each treatment, irrespective of whether it was in phase I or phase II therapy. A delay in skin healing or a skin lesion could shift the immune response to T_H2 and diminish the efficacy of EPIT.³⁶

To see how these treated mice could tolerate peanut ingestion (intragastric), we challenged the mice repeatedly with intragastric peanut extract once every 4 days, with a total of 10 challenges from days 28 to 64 between the 2 immunotherapies and then 6 challenges from 84 to 105 days after discontinuation of treatment. Clinical symptoms of each mouse were recorded up to 1 hour after each challenge, and the final clinical symptoms and diarrhea

frequency recorded on day 105 were presented in Fig 5, D and E, respectively. As expected, clinical symptoms were scored the highest in the sham treatment group among all treatments (Fig 5, D and E). All mice in the sham group had diarrhea, and all mice in the nonsensitized group did not have diarrhea (PBS). All 3 EPITs suppressed the clinical symptoms and diarrhea substantially in sensitized mice with MN-PN/A significantly better than in mice with *ID-PN/A* or *ID-PN* in diarrhea frequency (Fig 5, D and E). The results suggest that 6 PLD-MNA immunizations are highly sufficient to reduce clinical symptoms, and the efficacy is comparable with or better than that of 12 *ID-PN*-mediated EPITs. Moreover, despite a similar efficacy between intradermal EPIT and PLD-MNA EPIT in the presence of the duo adjuvant, intradermal EPIT not only provoked significant skin irritation (Fig 5, C) but also required 5 times more PNA to achieve similar therapeutic benefits compared with PLD-MNA EPIT.

Allergen-specific humoral and local allergic responses after EPIT

PNA-specific immunoglobulins (sIgE, sIgG₁, and sIgG_{2a}) were quantified at the indicated days in sensitized mice treated with an

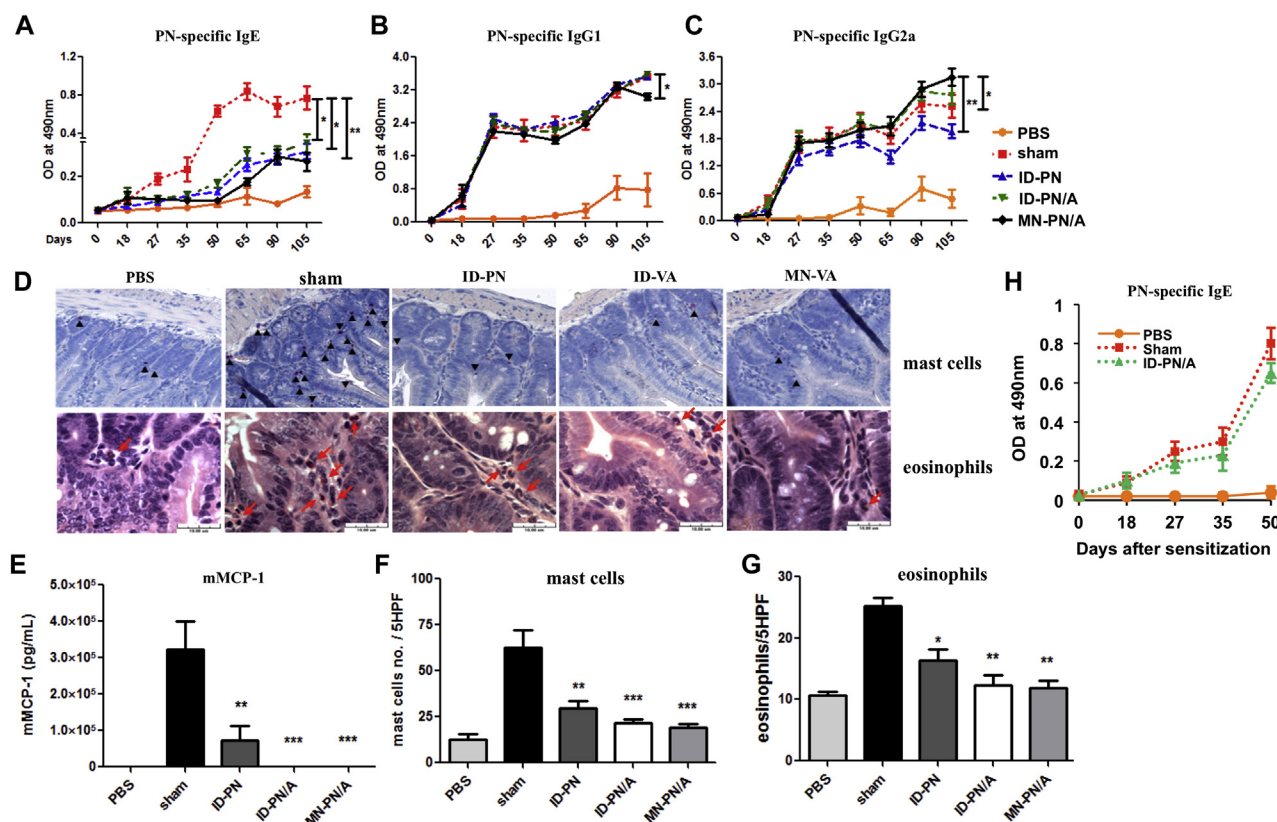


FIG 6. EPIT reduces PNA-specific IgE and allergic responses. **A–C** and **H**, Peanut-specific IgE (Fig 6, **A** and **H**), IgG₁ (Fig 6, **B**), and IgG_{2a} (Fig 6, **C**) levels were measured in the blood of mice treated with the indicated EPIT or sham treatment at indicated days by using ELISA. **D**, Representative histology shows mast cell and eosinophil infiltration into the jejunum of the small intestine. **E–G**, Reduction of mMCP-1 titers (Fig 6, **E**), mast cell counts (Fig 6, **F**), and eosinophil counts (Fig 6, **G**) caused by EPIT were either measured by using ELISA or counted manually. Mast cells and eosinophils were counted from 5 high-power fields (HPF) in each group by using NanoZoomer software. **H**, A low dose of PNA/VD3/CpG (24 μ g of PNA, 2.4 ng of VD3, and 0.24 μ g of CpG similar to that used in PLD-MNA EPIT) or sham PBS was intradermally administered in a similar schedule in phase I (Fig 5, **B**). Data are shown as means $\pm$ SEMs ($n = 5$ in the PBS group and $n = 8$ in the sham and treatment groups). * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with sham treatment or between indicated groups.

indicated EPIT in the presence or absence of the duo adjuvant (Fig 6, A–C). Peanut sensitizations increased sIgE production that began to be detectable on day 18 (Fig 6, A). sIgE production was intensified during repeated challenges, peaked on day 65, and sustained at peak levels after another round of repeated challenges from days 84 to 105 (Fig 6, A). All EPITs significantly reduced sIgE production in sensitized mice to levels comparable with those in nonsensitized mice up to day 50, regardless of EPIT procedures and in the presence or absence of the duo adjuvant (Fig 6, A). In spite of increasing sIgE production by repeated challenge on days 28 to 64 in sham control mice, sIgE production was consistently inhibited in all treated mice, even after EPIT discontinuation. The comparable effects of these EPITs on suppression of sIgE production clearly suggested that the duo adjuvant could reduce the number of EPITs to 3 from 6 treatments in phase I with a similar therapeutic efficacy, irrespective of whether the trio was administered by means of PLD-MNA or intradermal injection. Likewise, in phase II the same PNA dosage and the number of EPITs were used to treat the sensitized mice after 10 repeated challenges, which continuously controlled sIgE production in the mice. This proved again that 3 EPITs in the presence of the duo adjuvant could be as efficient as 6 intradermal EPITs with PNA

alone. The sIgE level remained significantly lower in treated compared with sham mice throughout the experimental period in spite of increased levels of sIgE production toward the end of the experiment after repetitive challenges in sham mice. These data demonstrated that the presence of the duo adjuvant in EPITs could reduce the number of EPITs by half and sufficiently control sIgE production in peanut-sensitized mice (Fig 6, A).

Similar increases in sIgG₁ and sIgG_{2a} levels were observed in the sensitized mice, irrespective of the form of EPIT, which became substantial on day 27 and peaked after the second round of repetitive challenges (Fig 6, B and C). Notably, mice receiving PLD-MNAs appeared to respond to the second round of multiple challenges slightly better among the 3 EPITs, with lower sIgG₁ levels on the final day (Fig 6, B). Twelve intradermal immunotherapies with PNA alone were inferior to 6 doses of intradermal EPIT or PLD-MNA EPIT in terms of sIgG_{2a} production in the presence of the duo adjuvant, especially in the second round of peanut challenges, although there was no difference between intradermal EPIT and PLD-MNA EPIT (Fig 6, C).

We next analyzed mMCP-1, mast cells, and eosinophils in the jejunum of the intestine (Fig 6, D–G). All 3 EPITs strongly suppressed the infiltration of mast cells and eosinophils into the

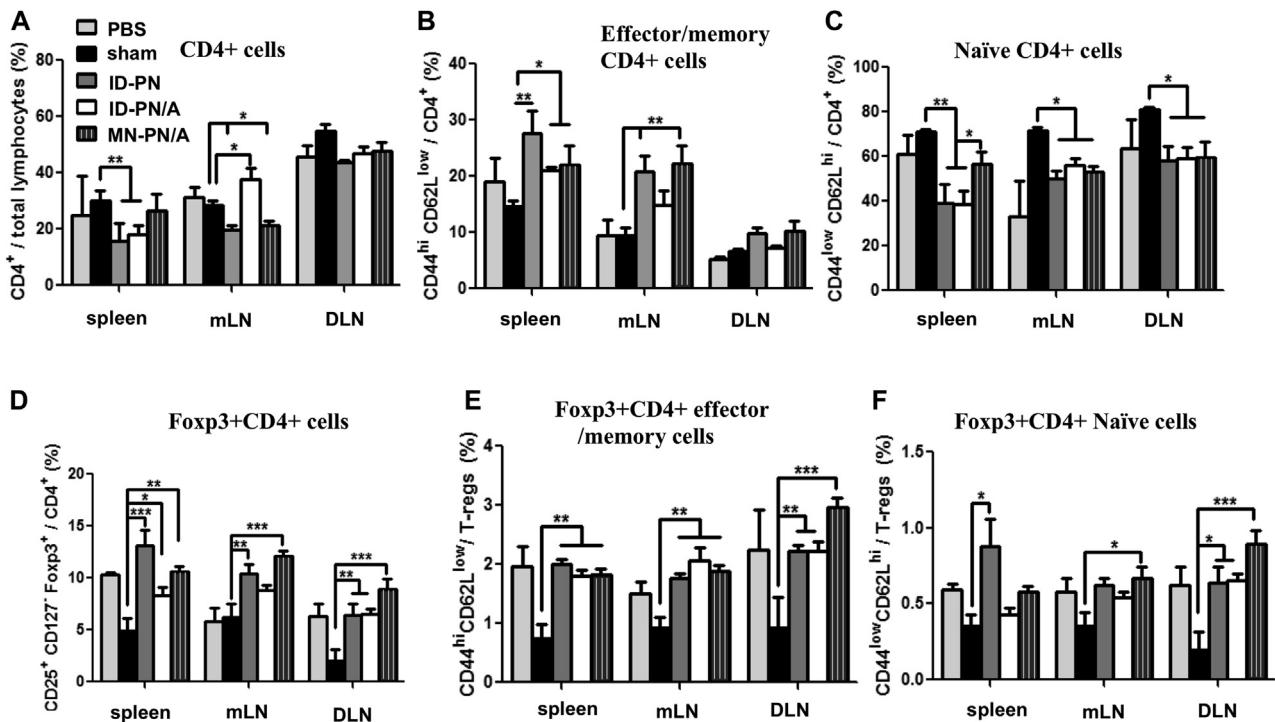


FIG 7. EPIT induces PNA-specific CD4⁺ Treg cells. CD4⁺ and CD4⁺ Treg cells were analyzed in spleens, dLNs, and mLNs by using flow cytometry. Cells were identified as CD4⁺ (A), effector/memory (CD4⁺CD44^{hi}CD62L^{low}; B), naïve (CD4⁺CD44^{low}CD62L^{hi}; C), total Treg (FoxP3⁺CD25⁺CD4⁺; D), FoxP3⁺CD4⁺ effector/memory (E), and FoxP3⁺CD4⁺ naïve T (F) cells. Data are shown as means $\pm$ SEMs ($n = 5$ in the PBS group and $n = 8$ in the sham and treatment groups). * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with sham treatment or between indicated groups.

jejunum of the intestine (Fig 6, D, F, and G). Suppression of mast cells was further ascertained by a corresponding decrease in mMCP-1 levels mainly produced by mast cells (Fig 6, E). The presence of the duo adjuvant was slightly more effective than its absence. The result suggests that EPIT can alter humoral and local immune responses toward immune tolerance and that immune tolerance responses can be further strengthened by the duo adjuvant.

EPIT strongly induces FoxP3⁺CD4⁺ T cells

Phenotypes of CD4⁺ T cells were characterized in spleens, mLNs, and draining lymph nodes (dLNs) at the end of the experiments by using flow cytometry. The sensitization did not alter CD4⁺ T-cell composition significantly in all lymph tissues examined (Fig 7, A-C) but diminished FoxP3⁺CD4⁺ T-cell counts substantially when compared with those in nonsensitized mice, regardless of which subsets of the cells were analyzed in all 3 lymph tissues, with the exception of mLNs, in which total FoxP3⁺CD4⁺ T-cell counts were unaltered (Fig 7, D-F). These CD25⁺FoxP3⁺CD127⁺CD4⁺ T cells are commonly referred to as Treg CD4⁺ T cells,³⁷⁻⁴¹ although their inhibitory function needs to be verified in the future. In general, all 3 EPITs increased the level of effector/memory CD4⁺ T cells concurrent with a reciprocal decrease in numbers of naïve CD4⁺ T cells in the spleens and mLNs when compared with those in sham-treated mice (Fig 7, B and C). However, these EPITs did not alter the percentages of CD4⁺ cells or effector/memory CD4⁺ T cells significantly in dLNs, except for naïve CD4⁺ T cells, which were

diminished by the 3 EPITs compared with sham treatment. In addition to increases in numbers of effector/memory CD4⁺ T cells, Treg cells were significantly increased in these 3 lymph tissues by all 3 EPITs compared with sham treatment irrespective of whether Treg cells were analyzed as percentages of total Treg cells (CD4⁺CD25⁺FoxP3⁺; Fig 7, D), effector/memory Treg cells (CD4⁺CD44^{hi}CD62L^{low}FoxP3⁺; Fig 7, E), and, to a lesser extent, FoxP3⁺ naïve CD4⁺ T cells (Fig 7, F). Although intradermal immunization with PNA alone was stronger in stimulation of total and naïve Treg cells in the spleen, MN-PN/A showed more effective in augmentation of these Treg cells in mLNs and dLNs (Fig 7, D and F).

The increased numbers of Treg cells by the 3 EPITs are in good agreement with their effectiveness in control of clinical symptoms and sIgE in these allergic mice. In addition to FoxP3⁺ Treg cells, previous studies have shown a significant contribution of gut-homing LAP⁺FoxP3⁺ Treg cells¹⁵ and CD62L⁺ Treg cells with a hypomethylated FoxP3 locus⁴² to immune tolerance induced by Viaskin. Whether these FoxP3⁺ Treg cells were induced and contributed to the immune tolerance stimulated by PLD-MNA EPIT remains unknown and would be subject to future investigation.

DISCUSSION

In the current study we engineered PLD-MNAs for safe and effective EPIT against IgE-mediated peanut allergy. This treatment is needle free and potentially self-applicable and can sufficiently deposit powdered allergens into the epidermis in a

minimally invasive manner and in a relatively short period of time.

Second, PLD-MNA EPIT did not provoke any overt skin inflammation at the site of patch application in marked contrast to intradermal EPIT, which caused overt skin irritation. Microneedle-generated pores were so small that they could be completely sealed in 48 hours by rapidly growing epithelial cells surrounding each pore.^{29,43} The fast restoration of skin integrity has been shown to associate with T_H1 -dominated immune responses, whereas slow healing favors T_H2 immune responses.⁴⁴

Third, the powder deposited in the epidermis was gradually hydrated and spread over the epidermis, and some entered the upper dermis, but not the bloodstream, which was a key to anaphylaxis prevention.¹⁶ Moreover, the slow process of hydration and diffusion of powdered allergens resulted in the allergens sustaining within the skin for an extended period of time when compared with liquid allergens injected intradermally. Thus allergens deposited by using PLD-MNA can constantly stimulate the immune system for days, simulating multiple small doses of immunotherapy, which promote immune tolerance.^{17,29,43}

Finally, recent clinical studies of MNAs showed good patient compliance and a high feasibility of self-applicability, highlighting the clinical relevance of the current study.^{45,46}

In the past decade, various forms of EPIT have been investigated in an attempt to develop safer therapeutic alternatives to conventional SCIT in the management of IgE-mediated food allergies and airway hyperresponsiveness.^{3,12-17} However, the outermost layer of the skin, the stratum corneum, forms a tight permeability barrier to macromolecules, which makes epidermal delivery of allergens into intact skin extremely challenging. Although gentle physical disruption of the skin by means of tape stripping, followed by allergen patch application, increased allergen penetration of the epidermis in human subjects,⁸ this physical skin aberration provoked release of proinflammatory cytokines, such as thymic stromal lymphopoietin, and T_H2 immune responses as a consequence of skin damage.¹² In contrast, Viaskin delivers allergens through intact skin and evokes a minimal T_H2 immune response.³⁶

Another innovative approach to facilitate sufficient skin delivery are the various MNAs, such as dissolvable, coating, or hollow MNAs, which have been extensively explored in the past 2 decades.^{8,45} However, none of them can preserve allergenicity by 100% as seen with the PLD-MNA. For instance, dissolvable MNAs are fabricated by mixing the allergens with monopolymer before polymerization to embed allergens into microneedle shafts. However, the polymerization process can substantially diminish this allergenicity, thus requiring optimization of the excipients and stabilizers to adequately preserve the allergenicity in an allergen-specific manner. Optimization of the excipients and stabilizers is time-consuming and varies from one allergen to another. Similarly, coating allergens on the surfaces of microneedles in the array also depends on a specific buffer, stabilizers, and excipients for a given allergen. Because some of the allergens are not well defined, the optimization of these undefined allergens might be difficult or impossible. In marked contrast, PLD-MNAs can be laden with any allergens as long as a powder form of the allergens is available. In theory, this universal powder-loadable PLD-MNA can be easily stored, transported, and ready to use for specific immunotherapy of various allergies. Currently, lyophilized powdered

extracts of various allergens are available for skin prick testing or specific immunotherapy and can be directly loaded into the MNAs for EPIT without the need for additives, stabilizers, excipients, or specific allergens to be identified. Apart from allergen preservation, direct delivery of powdered allergens offers additional advantages over liquid allergens because a powdered form can better avoid chemical modification and degradation, even after long-term storage, and no reconstitution is required before administration.

It is worthwhile to point out that complete insertion of the PLD-MNA is not always necessary for complete delivery of the encapsulated powder because the powder can be drained into the skin by the interstitial fluid influx. In this regard we recently showed that powder above the skin could sufficiently enter the skin through a microchannel beneath the powder.^{26,27} The unique property of powder being drained into the skin by the interstitial fluid warrants consistency of the therapy, even if the PLD-MNA is inserted imperfectly, which can happen when the PLD-MNA is self-applied. Moreover, a modified version of this PLD-MNA would allow delivery of a high amount of allergen into the skin in hours by raising the height of the basal cave above the skin, for example, as we recently described.³² The loading capacity can also be raised by enlarging and prolonging the microneedles for human uses because of the much greater thickness of human skin than mouse skin and the increased the density of the microneedles.

Recently, we showed that intradermal immunization of VD3 plus CpG bolstered IL-10 transcription 1000-fold and TGF- $\beta 1$ 40-fold at the immunization site in the skin.¹⁷ The duo adjuvant vigorously suppressed sIgE production and clinical symptoms induced by the model allergen OVA.¹⁷ CpG as an adjuvant suppresses allergic responses by inducing a specific T_H1 immune response,^{14,17,47-49} whereas the immunomodulatory nature of VD3 (calcipotriol) has been shown to directly polarize tolerogenic macrophages and Treg cells.^{50,51} Moreover, VD3 suppressed IgE production in B cells, as well as antigen-specific T cells.^{51,52} The current investigation extends the immunoregulatory roles of VD3 and CpG to peanut allergy. VD3/CpG adjuvant reduced treatment dosage by half with a similar therapeutic efficacy, irrespective of whether the trio were administered intradermally or by using PLD-MNA. Adjuvant has been extensively investigated for boosting various vaccines, but no tolerogenic adjuvant has been licensed to induce immune tolerance in the clinic to date. Our current and previous studies suggest that the VD3/CpG duo adjuvant might be a potential candidate to induce immune tolerance in human subjects.¹⁷

In summary, we showed that 6 EPITs in the presence of CpG and VD3 applied in 2 phases, either intradermally or by using PLD-MNA, could control peanut allergy with comparable efficacy as 12 intradermal EPITs with PNA alone, as evidenced by reduction of clinical symptoms, allergen-specific IgE levels, and intestinal mMCP-1 levels and mast cell and eosinophil counts in allergic mice. Moreover, PLD-MNA requires a much lower amount of PNA, CpG, and VD3 and exhibits safer profiles, as suggested by negligible leakage of allergens into the circulation and a lack of overt skin irritation. Immune tolerance induced by EPIT was associated with significant increases in memory/effector Treg cells marked with FoxP3⁺CD4⁺CD44^{hi}CD62L^{low} and total Treg cells compared with sham treatment. Therefore PLD-MNA could potentially confer safer and more effective treatments of peanut allergy and be readily extended to other IgE-mediated allergies,

although its efficacy and safety remain to be determined in human subjects.

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Key messages

- PLD-MNA-mediated EPIT might be safe for managing peanut allergy because it does not evoke significant side effects locally or result in allergen leakage into the circulation in a mouse model.
- PNA/VD3/CpG-laden PLD-MNA is effective in a mouse model and can potentially reduce the number of treatments by half, with a significant shift of the immune response toward Treg cell responses.
- PLD-MNA might be self-applicable because of its safety and consistence of delivery.
- The powder of PNA, VD3, and CpG deposited in the epidermis strongly attracts immunoregulatory skin-resident macrophages marked by CD11b⁺CD169⁺ and stimulates them to secrete IL-10, TGF- β 1, or both in favor of differentiation and expansion of FoxP3⁺ Treg-like cells, conferring long-lasting benefits.

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