



# Successful transdermal allergen delivery and allergen-specific immunotherapy using biodegradable microneedle patches

Ji Hye Kim <sup>a,1</sup>, Jung U. Shin <sup>a,1</sup>, Seo Hyeong Kim <sup>a,b</sup>, Ji Yeon Noh <sup>a</sup>, Hye Ran Kim <sup>a</sup>, Jungsoo Lee <sup>a</sup>, Howard Chu <sup>a</sup>, Kyoung Yong Jeong <sup>c</sup>, Kyung Hee Park <sup>c</sup>, Jung Dong Kim <sup>d</sup>, Hong Kee Kim <sup>d</sup>, Do Hyeon Jeong <sup>d</sup>, Tai-Soon Yong <sup>e</sup>, Jung-Won Park <sup>c</sup>, Kwang Hoon Lee <sup>a,b,\*</sup>

<sup>a</sup> Department of Dermatology & Cutaneous Biology Research Institute, Yonsei University College of Medicine, Seoul, South Korea

<sup>b</sup> Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea

<sup>c</sup> Department of Internal Medicine, Institute of Allergy, Yonsei University College of Medicine, Seoul, South Korea

<sup>d</sup> Raphas, South Korea

<sup>e</sup> Department of Environmental Medical Biology, Institute of Tropical Medicine, Arthropods of Medical Importance Resource Bank, Yonsei University College of Medicine, Seoul, South Korea

## ARTICLE INFO

### Article history:

Received 9 August 2017

Received in revised form

1 October 2017

Accepted 3 October 2017

Available online 4 October 2017

### Keywords:

*Dermatophagoides farinae*

Atopic dermatitis

Allergen-specific immunotherapy

Microneedle

NC/Nga mouse

## ABSTRACT

Allergen-specific immunotherapy (SIT) is an effective treatment modality for allergic diseases such as atopic dermatitis (AD). However, frequent visits over a 3-year period as well as looming adverse events tend to discourage patient compliance. Therefore, a more convenient, effective, and safe method of SIT is needed.

For several decades, use of microneedles has been promoted as an efficient and precise transdermal drug delivery method. In this study, we developed *Dermatophagoides farinae* (*D. farinae*) extract (DfE)-loaded microneedle patches, and evaluated their safety and efficacy as a novel SIT method. After 4 weeks of patch application, efficient allergen delivery and successful induction of immune response to DfE were demonstrated in mice, with no apparent adverse events. AD-induced NC/Nga mice received microneedle immunotherapy (MNIT) (10 µg), subcutaneous immunotherapy (SCIT) (10 µg), SCIT (100 µg), or placebo. Both MNIT (10 µg) and SCIT (100 µg) treatments improved clinical and histologic manifestations of AD skin lesions, altered immunoglobulin production, dampened Th2 cellular response, and boosted Treg infiltrates, without significant side effects; whereas SCIT (10 µg) or placebo subsets failed to show any effects. Based on the favorable safety and efficacy profiles demonstrated in mice by MNIT in the current study, we believe that MNIT may serve as a new SIT modality.

© 2017 Elsevier Ltd. All rights reserved.

## 1. Introduction

Despite numerous treatment modalities that are available for allergic diseases, allergen-specific immunotherapy (SIT) is advantageous in that it is a disease-modifying, causal treatment that induces long-lasting tolerance to allergens [1].

Atopic dermatitis (AD) is a chronic, relapsing, inflammatory skin

\* Corresponding author. Department of Dermatology and Cutaneous Biology Research Institute, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul, 120-752, South Korea.

E-mail address: [kwanglee@yuhs.ac](mailto:kwanglee@yuhs.ac) (K.H. Lee).

<sup>1</sup> The two authors contributed equally to this work and should be considered as first authors.

disease that affects 20% of children and 1–3% of adults [2,3]. Recently, SIT has received support in treating AD, backed by randomized controlled trial [4] as well as meta-analyses [5], and especially by its success in patients sensitized with house dust-mite allergen, the most commonly sensitized allergen in atopic dermatitis patients [6–8]. There are several routes for SIT. Subcutaneous immunotherapy (SCIT) is the most commonly used and generally effective route for allergen injection. However, the need for frequent visits over a long period has undermined patient adherence. To overcome this limitation, sublingual immunotherapy (SLIT), which delivers allergen via mucosal layer, has been developed. However, effectiveness of SLIT is less certain than that of SCIT [9,10]; therefore, a more effective, safe, and convenient method for allergen delivery is needed.

Microneedle patch is a transdermal drug delivery method consisting of micrometer-sized needles in arrays, on a backing material [11,12]. It allows patients to safely self-administer treatment without pain, while presenting minimal risk of bleeding, infection, or injury, compared with conventional hypodermic needles. Accordingly, microneedle patch has been actively investigated as a potent drug delivery strategy, especially for vaccination [13–15]. Recently, however, epicutaneous [16] and allergen-coated microneedle [17,18] methods of allergen delivery have emerged as alternative means of inducing immune reactions, and as novel vehicles for SIT.

In this study, we developed *Dermatophagoides farinae* (*D. farinae*) extract (*DfE*)-loaded sodium hyaluronate-based microneedle patches, and assessed their ability to deliver allergen into the skin of mice. We then evaluated safety and efficacy of this strategy to determine its potential use in SIT.

## 2. Methods

### 2.1. Preparation of microneedle patches

Droplet-born air blowing (DAB) method was used to prepare *DfE*-loaded microneedle patches [19]. First, *DfE* was passed through an Acrodisc Syringe Filter with 0.45  $\mu$ m Supor membrane (Pall Corp, Port Washington, NY, USA), and admixed with HA powder in phosphate-buffered saline (PBS) (pH 7.4) to load microneedle patches (4 °C) at various concentrations (0, 0.2, 2, and 10  $\mu$ g/patch). The specified concentrations of *DfE* and sodium hyaluronate (intrinsic viscosity: 0.14 m<sup>3</sup>/kg, HA-EP1; Bloomage Freda Biopharm Co Ltd, Jinan, China) were placed in solution (HA concentration: 16.8% w/w), we fabricated corresponding *DfE*-loaded microneedle patches through precision control of solution droplets, with each droplet delivered separately and air-blown to form a microneedle. Each patch was designed as 76-microneedle array (needle pitch, 1.4 mm; needle length, 0.25 mm). *DfE* was provided by the Institute of Allergy of Yonsei University (Seoul, Korea) [20].

### 2.2. Measurement of *DfE* allergenicity

Allergenicity of *DfE* was determined by inhibition ELISA [21]. A 96-well plate was coated with 100  $\mu$ l of *DfE* (10  $\mu$ g/ml) and incubated overnight (4 °C). Then, sera from four subjects who were highly sensitized to *D. farinae* were pooled, and diluted 1:3 with 1% bovine serum albumin in PBS containing 0.05% Tween20 (PBST). The 1:3 diluted sera and serially six-fold diluted test solutions were pre-incubated with *DfE* (4 °C), then incubated in a coated plate (2 h, 37 °C). Finally, goat biotinylated anti-human IgE (Vector Laboratories Inc., Burlingame, CA, USA) was added (1 h, 37 °C), followed by horseradish peroxidase-streptavidin (HRP; Vector Laboratories) incubation (1 h, room temperature [RT]). Color was developed by adding 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Kirkegaard & Perry Laboratories [KPL] Inc., Gaithersburg, MD, USA) to each well, and then interrupted using TMB stop solution (KPL). Absorbance at 450 nm was measured via microplate reader, with percent inhibition calculated as follows:  $(1 - A_t/A_0) \times 100$ , where  $A_t$  stands for absorbance of test solution at 450 nm, and  $A_0$  for absorbance of non-test solution. Allergenicity was determined by calculating the concentration of allergen required to produce 50% inhibition of a standard extract.

### 2.3. Evaluation of *DfE*-loaded microneedle patches

#### 2.3.1. Safety test

Before applying microneedle patches, mice were anesthetized with 3–5% isoflurane using isoflurane vaporizer (VetEquip Inc.,

Livermore, CA, USA). Hair on the back of mice ( $n = 3$ ) was shaved, and microneedle patches were applied. Self-adherent bandages (3 M Coban, St Paul, MN, USA) were used to wrap around the torsos, covering the patches to prevent detachment. After 2 h, bandages and patches were gently removed. Patch applications were performed twice per week for 4 weeks. Transepidermal water loss (TEWL) was measured (Tewameter TM210; Courage and Khazaka Electronic, Cologne, Germany) before and immediately after patch application, and again after 2 h. For this purpose, mice were anesthetized in an atmosphere-controlled room ( $24 \pm 2$  °C,  $55 \pm 15\%$  relative humidity).

#### 2.3.2. Allergen delivery capacity

To evaluate dendritic cell migration, 6-week-old female BALB/c mice were treated with 10  $\mu$ g of *DfE*-loaded microneedle patch or subcutaneously injected with 10  $\mu$ g or 100  $\mu$ g of *DfE* twice a week for 4 weeks. Next, cells were isolated from skin draining lymph nodes and stained with anti-CD11c Alexa 488 (N418) and anti-Der f1 (MA-6A8) antibodies. Secondary PerCP (sc-358939; Santa Cruz Biotechnology, Dallas, TX, USA) for anti-Der f1 was stained and read sample using FACSVerse flow cytometer (BD Biosciences).

### 2.4. Allergen-specific immunotherapy in AD mice

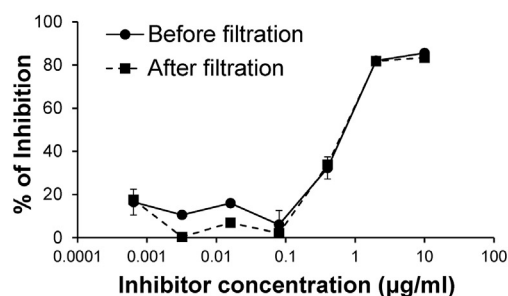
Induction of AD in a mouse model was achieved using previously described methods [6]. In brief, 100 mg of *DfE* ointment (Biostir-AD; Biostir Inc., Kobe, Japan) was applied to shaved dorsal skin and each ear surface of pathogen-free, female NC/Nga mice twice per week, for 8 weeks. After 3 weeks of induction, mice were divided into four groups as follows: 1) AD controls; 2) microneedle immunotherapy (MNIT), 10  $\mu$ g; 3) SCIT, 100  $\mu$ g; and 4) SCIT, 10  $\mu$ g. Immunotherapy was performed twice per week for 5 weeks, along with AD induction. In MNIT (10  $\mu$ g) group, *DfE*-loaded microneedle patches (10  $\mu$ g) were applied, while again wrapping the patched areas in self-adherent bandages (3 M) to prevent detachment. After 2 h, bandages and patches were gently removed. Mice in SCIT groups were subcutaneously injected with either 10  $\mu$ g or 100  $\mu$ g of *DfE*, respectively, in 100  $\mu$ l of PBS. Control animals received the same volume of PBS, without *DfE* (Supplementary Fig. 1). All experiments were performed under protocols approved by the Animal Research Ethics Board of Yonsei University (Seoul, Korea).

### 2.5. Scoring of dermatitis severity

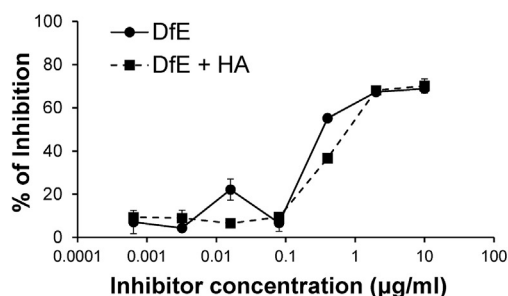
Scoring AD (SCORAD) index for mice, grading erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion were used to gauge severity of dermatitis. Each symptom was rated as 0 (none), 1 (mild), 2 (moderate), or 3 (severe), combining individual marks for a total score [22,23]. Each animal was scored once each week, by two independent researchers. Photographs were also obtained every week, under the same camera settings and lighting conditions.

### 2.6. Histopathological assessment of dermatitis

Dorsal skin tissues, taken from same sites in each group, were fixed in 4% paraformaldehyde, processed routinely, and serially sectioned (4- $\mu$ m thick) for hematoxylin-eosin (H&E) staining. Epidermal thickness was determined by image analysis software (MetaMorph; Molecular Devices Corp, Sunnyvale, CA, USA), and inflammatory infiltrates (eosinophils, other leukocytes) were assessed microscopically (BX53; Olympus Corp, Tokyo, Japan). Three different images were taken of each slide. Results were expressed as mean  $\pm$  standard error of mean (SEM), based on eight different digital images.

**A**

|                   | 50% inhibition concentration (μg/ml) |
|-------------------|--------------------------------------|
| Before filtration | $1.020 \pm 0.118$                    |
| After filtration  | $1.112 \pm 0.011$                    |

**B**

|                 | 50% inhibition concentration (μg/ml) |
|-----------------|--------------------------------------|
| <i>DfE</i>      | $1.112 \pm 0.018$                    |
| <i>DfE</i> + HA | $1.256 \pm 0.017$                    |

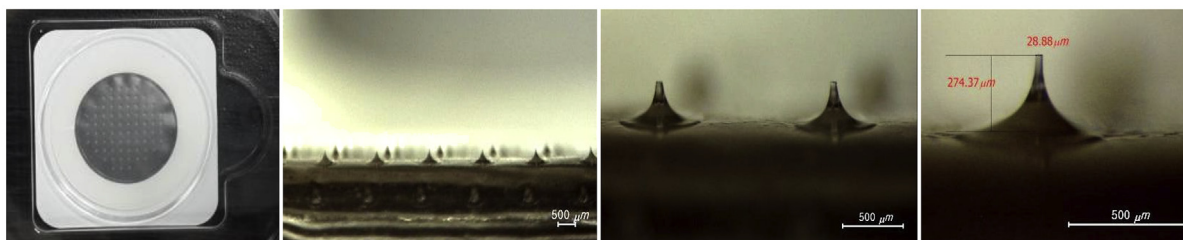
**C**

**Fig. 1. Characteristics of *DfE*-loaded microneedle patches.** Allergenicity of *DfE*: (A) before ( $1.020 \pm 0.118$  μg/ml) and after ( $1.112 \pm 0.011$  μg/ml) filtration through 0.45-μm membrane, and (B) *DfE* alone ( $1.112 \pm 0.018$  μg/ml) or in combination with HA ( $1.256 \pm 0.017$  μg/ml), measured by inhibition ELISA (50% inhibitory concentration); (C) Microscopic images of *DfE*-loaded microneedle patches at 8X, 30X, and 50X magnifications (Bar = 50 μm). *DfE*, *D. farinae* extract; HA, hyaluronate; ELISA, enzyme-linked immunosorbent assay; TEWL, transepidermal water loss.

### 2.7. Enzyme-linked immunosorbent assay (ELISA) of immunoglobulins and der f1 allergen

Using commercially available kits, total IgE (Mouse IgE ELISA MAX Delux; BioLegend Inc., San Diego, CA, USA), total IgG4 (Mouse Immunoglobulin G4 ELISA Kit; MyBioSource, San Diego, CA, USA), and Der f1 dust-mite allergen (Der f1 ELISA kit; INDOOR Biotechnologies Inc., Charlottesville, VA, USA) were assayed according to the manufacturers' instructions. To determine the concentration of Der f1 in *DfE*-loaded microneedle patch for long-term storage, we used patches manufactured at the same time with same allergen. Assay was performed at each time point as time passed and three different patches were used to determine the concentration.

### 2.8. RNA purification and quantitative RT-PCR

Mouse skin samples were homogenized (Precellys 24 Homogenizer; Bertin Technologies, Toulouse, France), and total RNA were extracted (RNeasy Plus Mini Kit; Qiagen, Hilden, Germany) by using

a commercial kit. Following that, RNA reverse transcription was performed (AccuPower RT PreMix; Bioneer, Daejeon, Korea). cDNA and Taqman gene expression master mix (Applied Biosystems, Waltham, MA, USA) were combined with mouse Taqman primers IL-4 (Mm00445259\_m1), IL-10 (Mm00439614\_m1), IL-13 (Mm00434204\_m1), Foxp3 (Mm00475162\_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915\_g1) in 20 μl reactions, and relative quantification was done (StepOnePlus PCR System/7500 Software v2.0.1; Applied Biosystems).

### 2.9. Flow cytometry

Lymph node cells were incubated (6 h) in phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma-Aldrich Corp, St Louis, MO, USA), ionomycin (1 μg/ml; Sigma-Aldrich), and Golgistop (2 μg/ml; BD Biosciences, Franklin Lakes, NJ, USA). Thereafter, cells were stained by a fixable viability dye (eBioscience Inc., San Diego, CA, USA), and then either anti-CD4 FITC (GK1.5) or anti-CD4 PE-cyanine5.5 (RM4-5) antibodies (eBioscience) were applied. For intracellular staining,

cells were immersed in fixation/permeabilization buffer (eBioscience), and anti-IL-4 APC (11B11), anti-IL-10 PE (JESS-16EB), or anti-Foxp3 APC (FJK-16s) antibodies (eBioscience) were applied. FACSVerse flow cytometer (BD Biosciences) was used for sample analysis, coupled with FlowJo Software (v7.6.2; TreeStar, San Carlos, CA, USA) for data handling.

## 2.10. Immunofluorescence staining

Paraffin-embedded mouse skin samples were deparaffinized and rehydrated. Antigen retrieval was carried out in citrate buffer (pH 6.0) (Sigma, MO, USA). Frozen sections were fixed in acetone, and primary antibodies were applied for overnight incubation (4 °C). After washing with PBST, secondary antibodies were applied (1 h, RT). Slides were mounted in VECTASHIELD, a medium containing 4',6-diamidino-2-phenylindole (H-1200; Vector Laboratories). All sections were ultimately examined by confocal microscopy (LSM 780; Carl Zeiss, Oberkochen, Germany). The following mouse-specific antibodies were utilized: anti-CD4 (ab200594; Abcam, Cambridge, UK), anti-Der f1 (MA-6A8; INDOOR Biotechnologies Inc.), anti-MHC II Alexa Fluor 488 (Biolegend), anti-IL-4 FITC (BVD6-24G2; eBioscience), anti-mouse Foxp3 (236A/E7; Abcam), anti-rat IgG Alexa Fluor 555 (ab150158; Abcam), and anti-mouse Alexa Fluor 633 (A21126; Life Technologies, Carlsbad, CA, USA).

## 2.11. Statistical analyses

If not described, all assays were repeated at least three times, expressing data as mean  $\pm$  SEM and Gaussian distribution was checked by Komogorov-Smirnov and Shapiro Wilk normality test. Standard software (GraphPad Prism 5 v4.03; GraphPad Software Inc., San Diego, CA, USA) was used for all computations. Differences between two groups were assessed by two-tailed Student's *t*-test. For comparisons of more than two groups, one-way ANOVA and Tukey-Kramer tests were utilized. In analysis of SCORAD, two-way ANOVA and Bonferroni post-test were applied. Statistical significance was set at  $P < 0.05$ , while denoting levels of significance in figures as follows: \* $P < 0.05$ , \*\* $P < 0.01$ , or \*\*\* $P < 0.001$ .

## 3. Results

### 3.1. Stability of DfE admixed with sodium hyaluronate (HA)

In order to manufacture DfE-loaded microneedle patches, DfE constituents dissolved in sodium HA needed to be  $< 0.45 \mu\text{m}$ . Therefore, we filtered DfE and checked its allergenicity thereafter, to find that the levels had not been significantly altered (50% inhibitory concentration [mean  $\pm$  SD]: before filtration,  $1.020 \pm 0.118 \mu\text{g/ml}$ ; after filtration,  $1.112 \pm 0.011 \mu\text{g/ml}$ ) (Fig. 1A). We then examined the effect of HA on DfE allergenicity, and determined that allergenicity of DfE and HA in solution was

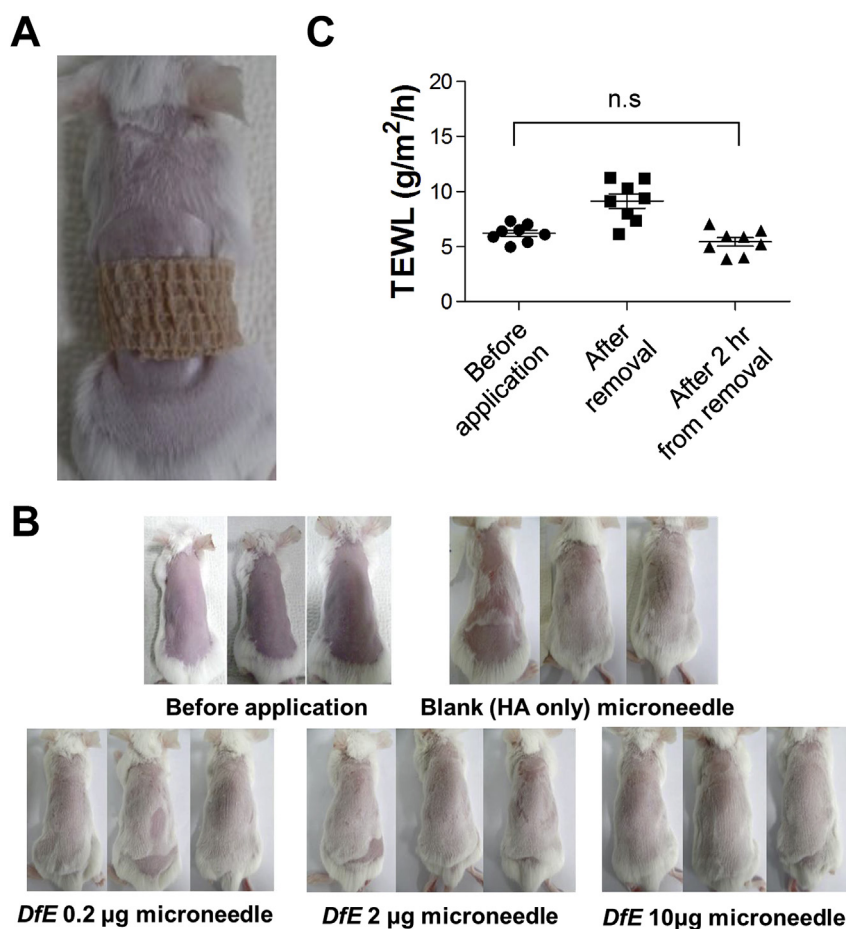


**Fig. 2. Clinical manifestations of mouse model.** (A) Photo of DfE-loaded microneedle patch applied to BALB/c mouse; (B) BALB/c mice before and after 4-week application of microneedle patches (no signs of adverse effects during experiment); (C) TEWL slightly increased upon patch removal, but was restored within 2 h ( $P = \text{ns}$ ). DfE, *D. farinae* extract; TEWL, transepidermal water loss.

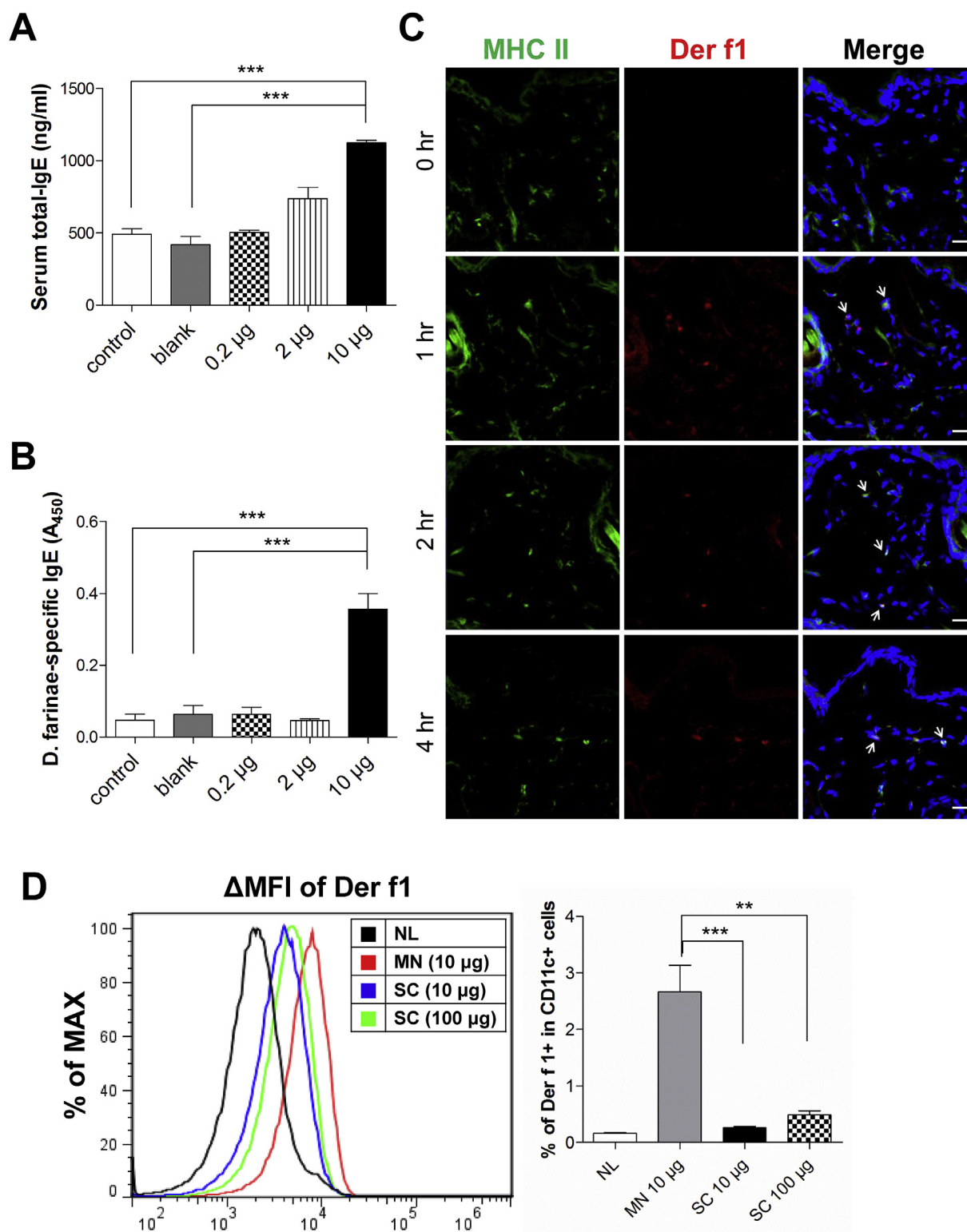


**Fig. 3. Immune responses in serum and DfE delivery into skin.** Female BALB/c mice with blank (no DfE-load) or DfE-loaded microneedle patches at 0.2 µg, 2 µg, or 10 µg applied for 4 weeks: (A) Total serum IgE concentration; (B) DfE-specific IgE level (absorbance at 450 nm). Only in 10 µg DfE-loaded microneedle patch, both IgE determinations were increased ( $***P < 0.001$ ); (C) 10 µg DfE-loaded microneedle patches applied for 1, 2, and 4 h. MHC II (green) served as dendritic cell marker. Notable co-localizations of MHC II and Der f1 (red) were detected in dermis (white arrows). (D) Der f1 populations of CD11c + dendritic cells in skin draining lymph nodes. ΔMFI is most increased in 10 µg DfE-loaded microneedle patch group. The percentage of Der f1+ in CD11c + cells was also increased in 10 µg DfE-loaded microneedle patch group ( $**P < 0.01$ ,  $***P < 0.001$ ). DfE, *D. farinae* extract; MHC, major histocompatibility complex.

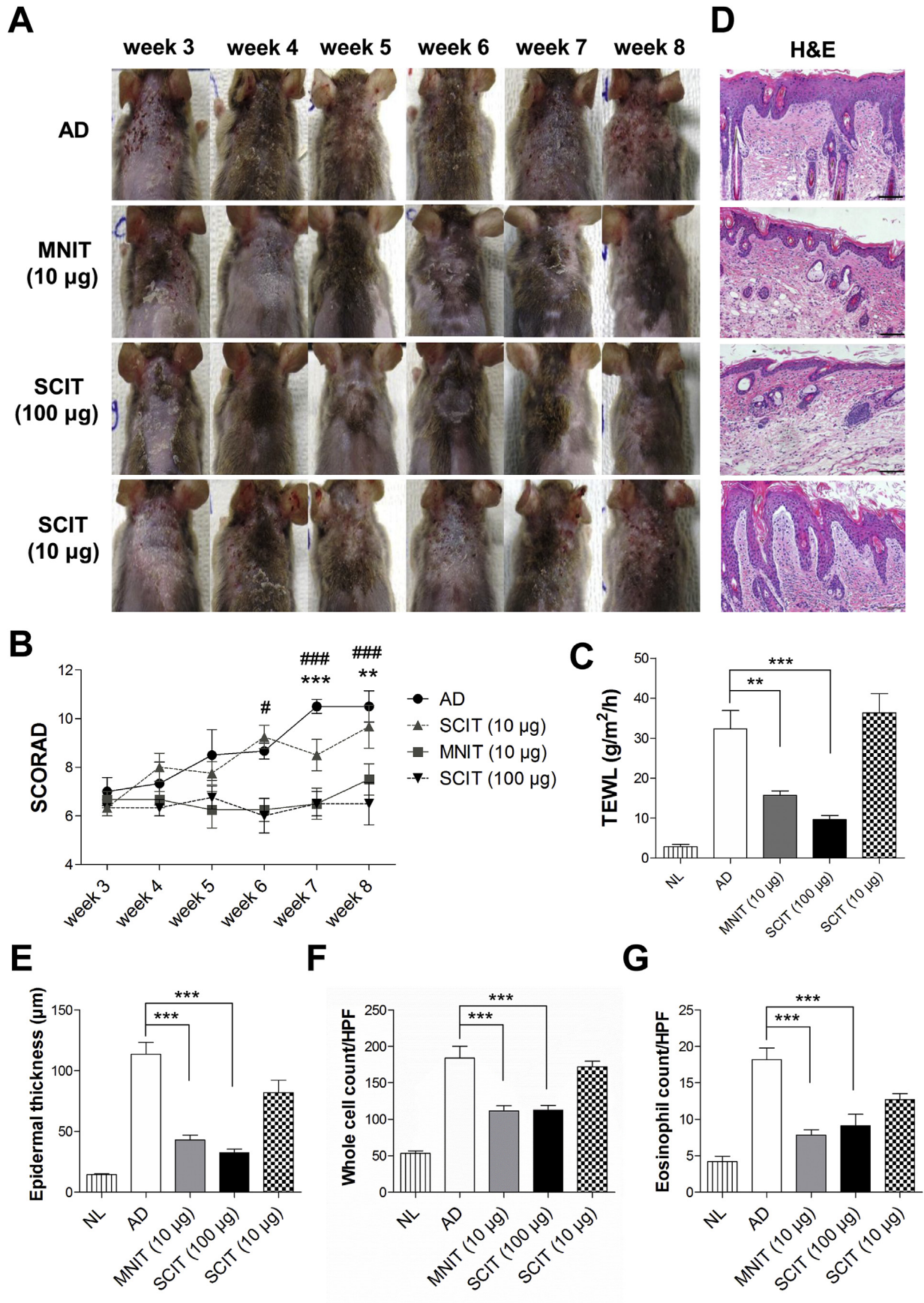


**Fig. 4.** Clinical and histologic features of AD-induced mouse model of SIT. (A) Changes in clinical appearance of skin, Weeks 3–8 (dermatitis noted since Week 3); (B) SCORAD, Weeks 3–8 (\*AD vs MNIT 10 µg; #AD vs SCIT 100 µg); (C) TEWL measured at Week 8 (\* $P < 0.05$ , \*\* $P < 0.01$ ). MNIT (10 µg) and SCIT (100 µg) subsets showed clinical improvement, compared to AD and SCIT (10 µg) subsets; (D) H&E-stained mouse skin (Bar = 100 µm); (E) Epidermal thickness; and (F) Total and (G) eosinophil cell counts (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). AD, atopic dermatitis; SIT, allergen-specific immunotherapy; SCORAD, Scoring AD; MNIT, microneedle immunotherapy; SCIT, subcutaneous immunotherapy; TEWL, transepidermal water loss.

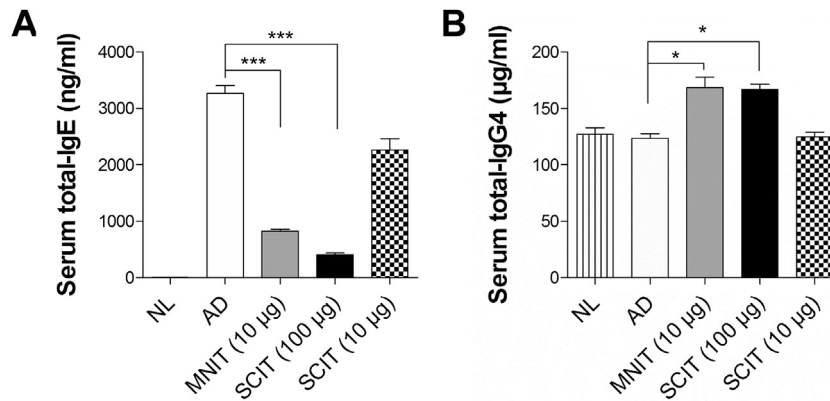


**Fig. 5.** Enzyme-linked immunosorbent assay of mouse serum immunoglobulins. Serum levels of (A) total IgE and (B) IgG4 in mice. At Week 8, total IgE levels were markedly lower, IgG4 levels were significantly increased in MNIT (10 μg) and SCIT (100 μg) subsets, compared to AD control and SCIT (10 μg) subsets (\* $P < 0.05$ , \*\*\* $P < 0.001$ ). MNIT, microneedle immunotherapy; SCIT, subcutaneous immunotherapy.

comparable to that of *DfE* alone (50% inhibitory concentration [mean  $\pm$  SD]: *DfE* + HA,  $1.256 \pm 0.017$  μg/ml; *DfE*,  $1.112 \pm 0.018$  μg/ml) (Fig. 1B).

### 3.2. Manufacturing of *DfE*-loaded microneedle patches

*DfE*-loaded microneedle patches were manufactured at various concentrations (0, 0.2, 2, and 10 μg/patch) by DAB method. A microneedle patch has two parts; an adhesive border and a microneedle array in mid-patch. Images of microneedles at 8X, 30X, and 50X magnifications were examined with a zoom microscope (JSZ-7XT; Samwon Scientific, Seoul, Korea) (Fig. 1C).

### 3.3. Stability of *DfE* in microneedle patches after irradiation and long-term storage

Before applying to SCIT mouse model, *DfE*-loaded microneedle patches were exposed to gamma radiation (5 kGy), and concentration of Der f1 (a major allergen of *DfE* [24]) was assayed (ELISA). At various doses of irradiation, we found no change in Der f1 concentration (Supplementary Fig. 2A). Furthermore, as *DfE* contains its own proteases that degrade rapidly at RT, we evaluated the stability of *DfE* microneedle-loading as a function of temperature and time. *DfE*-loaded microneedle patches and *DfE* in PBS were stored at RT or at 4 °C for 12 weeks. After 12 weeks at RT, Der f1 concentration declined significantly in both microneedle patches and in PBS (34% and 40%, respectively); whereas Der f1 concentration was fairly stable at 4 °C, and fell to 81% and 86% in microneedle patches and in PBS, respectively (Supplementary Fig. 2B).

### 3.4. Safety of *DfE*-loaded microneedle patches

*DfE*-loaded microneedle patches were applied twice per week for 4 weeks in female BALB/c mice, while checking for related side effects (Fig. 2A) such as infection, bleeding, and erythema. However, nothing of significance was found (Fig. 2B). Likewise, to assess whether *DfE*-loaded microneedle patches disrupt skin barrier, TEWL was measured before and after patch application. Although a slight rise in TEWL was detected just after patch removal, baseline levels were restored after 2 h (Fig. 2C).

### 3.5. Delivery of *DfE* into skin by loading of microneedle patches

*DfE*-loaded microneedle patches were applied to female BALB/c

mice twice weekly for 4 weeks to investigate the effects of serial dosing on total IgE (Fig. 3A) and *D. farinae*-specific IgE (Fig. 3B) levels, both of which showed elevation at the end of 4 weeks. Such increases were most apparent with the use of 10 μg of *DfE*-loaded microneedle patch. To confirm dermal allergen delivery, identification of Der f1 in skin after applying 10 μg of *DfE*-loaded microneedle patches was pursued, using an immunofluorescence stain. After 1, 2, and 4 h-application of *DfE*-loaded microneedle patches, presence Der f1 in dermis was confirmed. Remarkably, Der f1 was co-localized with major histocompatibility complex (MHC) II, suggesting that dermal dendritic cells (DCs) had captured this allergen (Fig. 3C). In addition, we treated BALB/c mice with 10 μg of *DfE*-loaded microneedle patches or *DfE* (10 μg or 100 μg) subcutaneously twice a week for 4 weeks. In skin draining lymph nodes, Der f1 + CD11c + dendritic cells were most increased in 10 μg of *DfE*-loaded microneedle applied group compared to any other subcutaneous injection groups (Fig. 3D). From these results, we concluded that delivered *DfE* by microneedle captured by dendritic cells and migrated to lymph nodes and this induces more effective immune responses compared to subcutaneous injection.

### 3.6. *DfE*-loaded microneedle patches vs. conventional subcutaneous *DfE* injection

#### 3.6.1. Clinical and histologic improvement

To assess the potential for whether *DfE*-loaded microneedle patches can serve as a novel SIT strategy, we compared them to subcutaneous injection of *DfE* in AD-induced NC/Nga mice. As shown in Fig. 4A and B, MNIT (10 μg) and SCIT (100 μg) halted further exacerbation of clinical symptoms, whereas AD controls and mice in SCIT (10 μg) subset showed continued aggravation, progressing to severe dermatitis. At Week 8, TEWL of dorsal skin was measured to evaluate skin barrier function. In both MNIT (10 μg) and SCIT (100 μg) subsets, TEWL levels were significantly lower, compared to those in AD controls and SCIT (10 μg) recipient mice (Fig. 4C). Also, H&E staining (Fig. 4D) revealed significantly thinner epidermis (Fig. 4E) and fewer numbers of infiltrating cells (Fig. 4F), including eosinophils (Fig. 4G).

#### 3.6.2. Serum immunoglobulin alteration

Since SIT is known to modulate antibody responses, we measured total IgE and IgG4 in serum. At Week 8, total IgE levels in MNIT (10 μg) and SCIT (100 μg) subsets were markedly lower than those of AD controls or SCIT (10 μg) recipient mice (Fig. 5A).

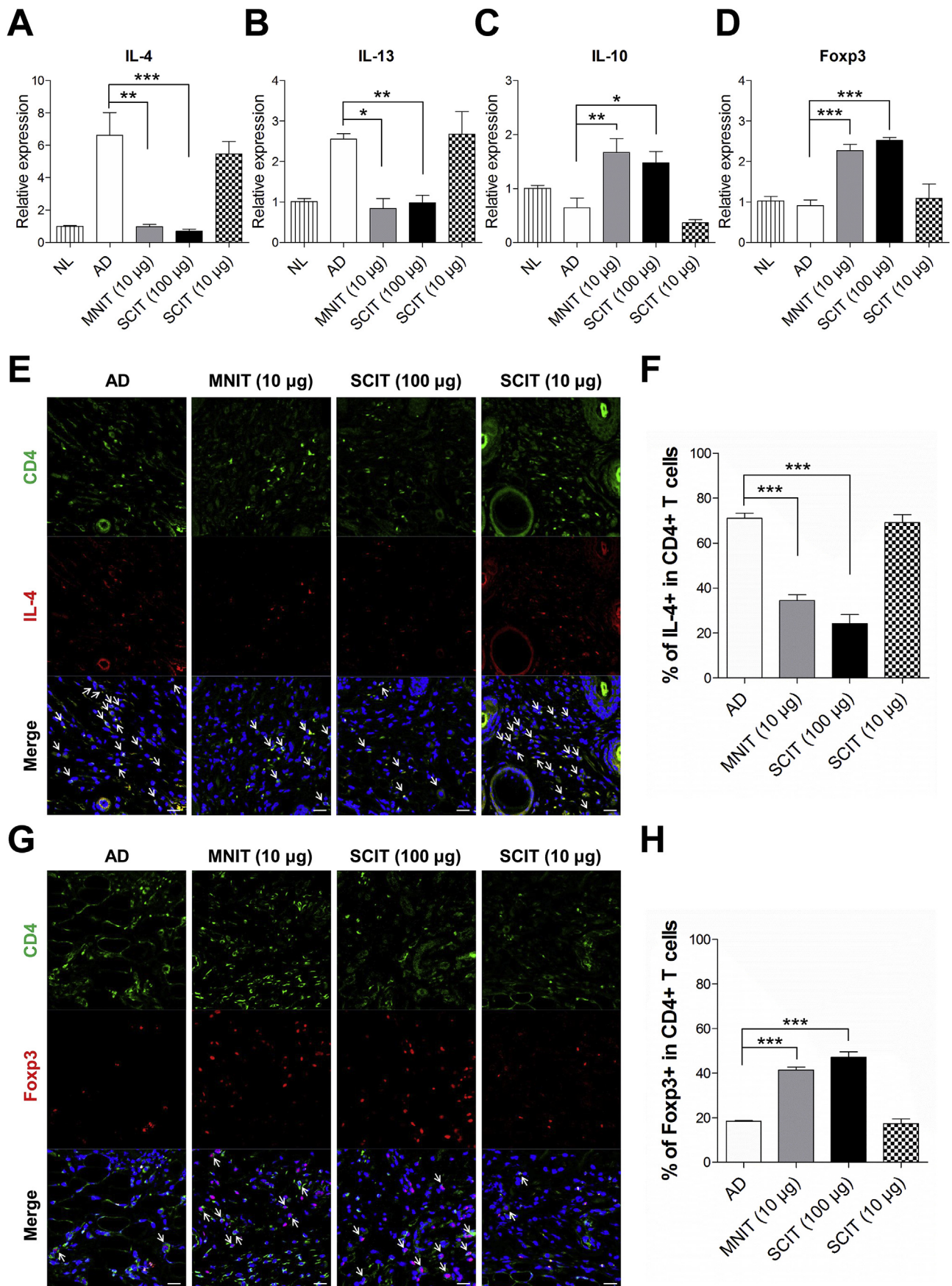


**Fig. 6. Th2 and Treg-related cytokine gene expression in mouse skin.** Expression levels of IL-4, IL-13, IL-10, and Foxp3 mRNA in mouse skin, determined by quantitative real-time PCR: (A) IL-4 and (B) IL-13 expression levels were low in MNIT (10 µg) and SCIT (100 µg) subsets, compared to in AD and SCIT (10 µg) subsets; (C) IL-10 and (D) Foxp3 expression levels were much higher in MNIT (10 µg) and SCIT (100 µg) subsets than in AD and SCIT (10 µg) subsets. Fluorescence images of mouse skin (all 400X): (E) CD4 (red) and IL-4 (green); (G) CD4 (red) and Foxp3 (green). IL-4 and Foxp3 in CD4+ T cells quantified: (F) Percent IL-4+CD4+ T cells were significantly lower in MNIT (10 µg) and SCIT (100 µg) subsets, compared to in AD and SCIT (10 µg) subsets; and (H) Percent Foxp3+CD4+ T cells were significantly higher in MNIT (10 µg) and SCIT (100 µg) subsets, compared to in AD and SCIT (10 µg) subsets (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). AD, atopic dermatitis; MNIT, microneedle immunotherapy; SCIT, subcutaneous immunotherapy.

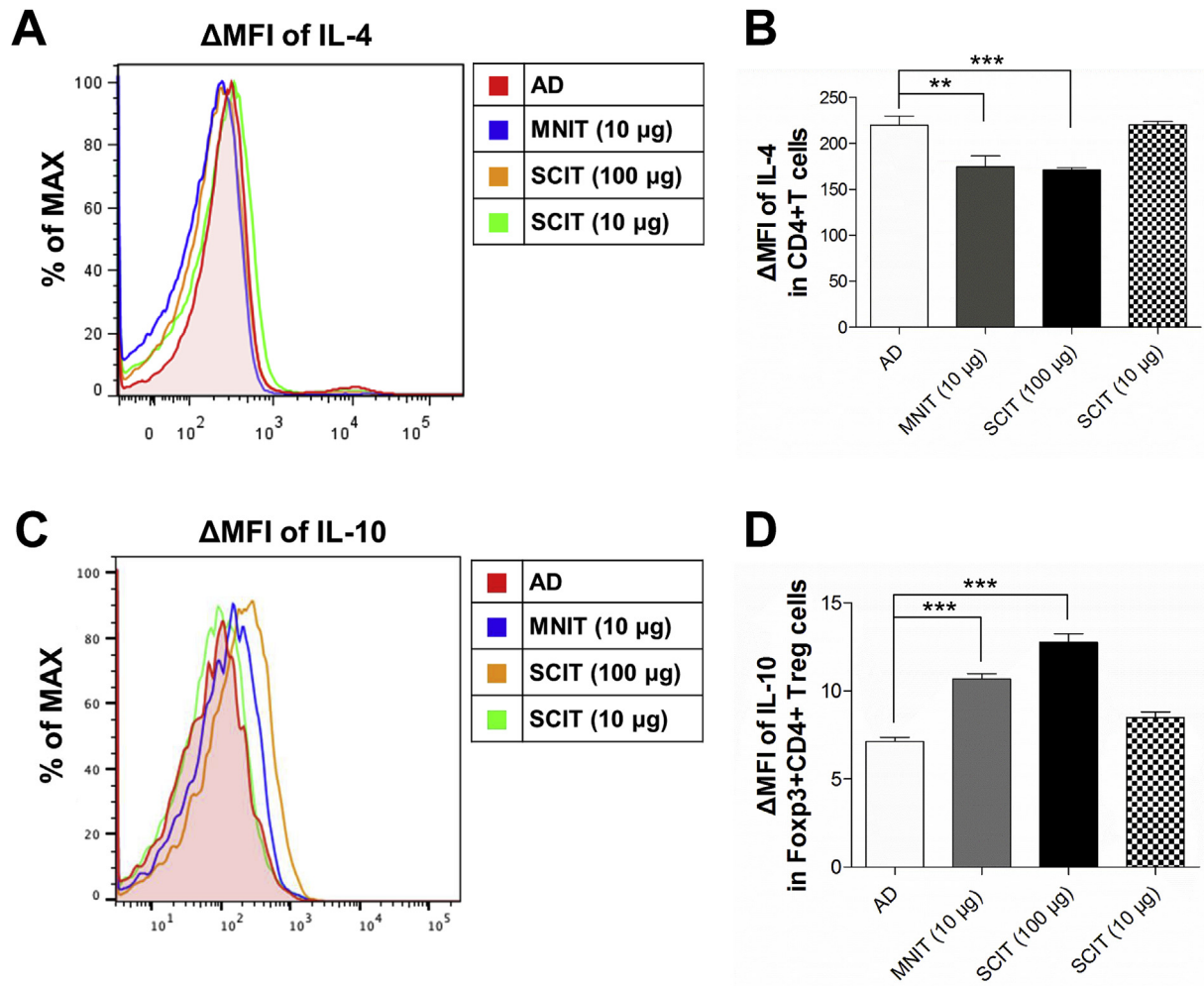


**Fig. 7.** Cytokines in Th2 and Treg cells of skin draining lymph nodes. T-cell populations in skin draining lymph nodes in mouse: (A, B) IL-4 levels in CD4+T cells ( $\Delta$ MFI each group): AD, 219.8  $\pm$  4.53; MNIT (10  $\mu$ g), 174.5  $\pm$  11.92; SCIT (100  $\mu$ g), 171.0  $\pm$  2.57; and SCIT (10  $\mu$ g), 220.0  $\pm$  3.84; (C, D) IL-10 levels in Foxp3+CD4+ T cells ( $\Delta$ MFI each group): AD, 7.130  $\pm$  0.23; MNIT (10  $\mu$ g), 10.69  $\pm$  0.30; SCIT (100  $\mu$ g), 12.76  $\pm$  0.49; and SCIT (10  $\mu$ g), 8.490  $\pm$  0.33 (\* $P$  < 0.05, \*\* $P$  < 0.01). AD, atopic dermatitis; MNIT, microneedle immunotherapy; SCIT, subcutaneous immunotherapy.

Conversely, IgG4 levels were significantly increased in MNIT (10  $\mu$ g) and SCIT (100  $\mu$ g) subsets, compared to others (Fig. 5B).

### 3.6.3. Suppression of Th2 cells and induction of regulatory T (Treg) cells in skin

In skin, mRNA levels of Th2 cytokines, IL-4, and IL-13 were significantly lower, while level of regulatory cytokine, IL-10, was higher in both MNIT (10  $\mu$ g) and SCIT (100  $\mu$ g) subsets (Fig. 6A–C). Also, mRNA expression of master transcription factor of Treg cells, Foxp3, was comparatively higher in MNIT (10  $\mu$ g) and SCIT (100  $\mu$ g) subsets (Fig. 6D). In correlation with RNA expression, immunofluorescence staining revealed fewer IL-4+CD4+T cells (Fig. 6E and F) and more Foxp3+CD4+ Treg cells (Fig. 6G and H) in infiltrates of MNIT (10  $\mu$ g) and SCIT (100  $\mu$ g) subsets, compared to those in AD controls and SCIT (10  $\mu$ g) recipient mice.

### 3.6.4. Changing cytokine expression in lymph nodes during SIT

Cytokine expression levels in lymph node cells were evaluated by flow cytometry. Expression of IL-4 in CD4+T cells was significantly lower in MNIT (10  $\mu$ g) and SCIT (100  $\mu$ g) subsets by comparison (Fig. 7A and B), whereas IL-10 expression level in Foxp3+CD4+T cells was significantly higher in the same subsets (MNIT [10  $\mu$ g] and SCIT [100  $\mu$ g]) (Fig. 7C and D).

## 4. Discussion

SIT is considered a causative treatment for allergic disease that not only controls clinical symptoms, but also modifies the natural course of disease to prevent further sensitization to other allergens [25,26]. However, factors such as the need for frequent hospital visits, long treatment periods, and other issues (i.e., aversion to conventional needles, possible anaphylaxis) have limited the efficacy of such therapy. Indeed, many patients undergoing SLIT (55–82%) or SCIT (13–89%) were proven to be noncompliant [27,28]. Therefore, there is a clear need for a novel method of SIT that is convenient, highly effective, and safe. In this study, we developed DfE-loaded microneedle patch as a potentially new method of SIT, while testing its safety and efficacy of allergen delivery in AD-induced mouse model.

Recently, the advantages of transdermal drug systems, including avoidance of liver metabolism, controlled delivery, compliance favorability, and low systemic side effects of microneedles, have gained considerable attention [16]. Skin-associated lymphoid tissue (SALT) that consists of keratinocytes, Langerhans cells (LCs), dermal DCs, mast cells, macrophages, basophils, and T cells attest to the fact that skin is a potent immunologic organ [29]. In skin, there are 1000 LCs per mm<sup>2</sup> [30], and adult human skin contains 20 billion T

cells [31]. Dermal DCs are comprised of various subsets but are generally involved in B-cell responses, whereas LCs are more inclined to participate in cellular immunity [32,33]. However, recent evidence has suggested that LCs are also involved in peripheral tolerance induction. LCs that migrate to nearby lymph nodes mediate deletional tolerance of CD4<sup>+</sup> and CD8<sup>+</sup> T cells [34,35], stimulate development of Treg cells [36], and promote production of IL-10 [35]. Skin-derived CD103<sup>+</sup> DCs are also presumed to be tolerogenic, able to trigger Treg-cell generation [37]. Given the apparent importance of both LCs and dermal DCs in induction of peripheral tolerance via SIT, precise allergen delivery that targets these particular cells seems imperative.

In the present study, we anticipated the use of microneedles in allergen delivery to be a viable and compelling alternate method of immunotherapy. Thus, we formulated a *DfE*-loaded microneedle patch that can be compared with conventional subcutaneous injection. We found that microneedle patch successfully delivered *DfE* into the skin, bringing significant improvement in clinical and histologic manifestations of AD. In addition, antibody production and T-cell responses were altered, while successfully suppressing serum IgE, inducing IgG4, reducing Th2 influx, and increasing cutaneous Treg cells. In addition, we demonstrated similar efficacies for 10 µg *DfE*-loaded microneedle patch and 100 µg *DfE*-SCIT, with 10 µg *DfE*-SCIT showing no effect. These findings are in line with those of other studies, which also reported that microneedles enable dose-sparing of vaccinations (1/100) by activating dendritic cells compared to intramuscular injection [38,39]. Low dose required for *DfE* immunization by microneedle as well as capability of delivering allergen directly to epidermis and dermis (instead of highly vascular subcutis) diminish the risk of systemic allergic reaction (i.e., anaphylaxis).

Since it is important to maintain allergenicity of *DfE* when loading microneedles, we decided to use DAB method, in which each droplet is air-blown to solidify and form a microneedle. DAB fabrication conditions are also less extreme, devoid of heat or UV irradiation imposed by conventional cast molding in biodegradable microneedle fabrication [40–43]. By design, it is possible to load each microneedle without loss, providing precise concentration of allergen by controlling droplet volume. DAB-fabricated biodegradable microneedles can be produced within 10 min, which is beneficial in terms of sustained allergen activity. Herein, we have also shown that allergenicity of *DfE* is not altered by admixture of HA, culminating in a successfully induced immune reaction.

Another potential route for SIT is epicutaneous immunotherapy (EPIT), which has been recently highlighted, especially in addressing food allergy [16,44–47]. Epicutaneous delivery of antigen has proven to be effective in treating allergy to grass pollen [46,47] and milk [44]. A recently reported phase III study of peanut EPIT has established its safety and efficacy after a 52-week treatment period [45], although immunoglobulin production was only modestly altered. In case of airway allergy, vaccination by microneedle was as effective as SCIT [48]. Since both LCs and dermal DCs are pivotal in inducing peripheral tolerance, such modest effect may indicate that epicutaneous allergen delivery will only target epidermal LCs (not dermal DCs). Therefore, allergen delivery to both epidermis and dermis by microneedle may enhance efficacy of SIT.

To our knowledge, this is the first documented use of allergen-loaded microneedles for SIT of AD. Our *DfE*-loaded microneedle patches showed stable allergenicity, which at 10 µg of *DfE* was sufficient to induce immunologic responses rivaling 100 µg of subcutaneous injection, without side effects. Based on the success of this mouse model, we contend that our *DfE*-loaded microneedle patch may serve as convenient and effective new means of immunotherapy, which would help encourage patient compliance

and reduce side effects. It may similarly be applied to other allergic diseases, such as allergic rhinitis, asthma, and food allergies.

## Conflicts of interest

The authors declare no conflict of interest in this study.

## Financial support

This study was supported by a grant from the Ministry of Health & Welfare, Republic of Korea (grant number: HI14C1324).

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.biomaterials.2017.10.013>.

## References

- [1] S.R. Durham, D.Y. Leung, One hundred years of allergen immunotherapy: time to ring the changes, *J. Allergy Clin. Immunol.* 127 (2011) 3–7.
- [2] T. Bieber, Atopic dermatitis, *N. Engl. J. Med.* 358 (2008) 1483–1494.
- [3] M. Boguniewicz, D.Y. Leung, Atopic dermatitis: a disease of altered skin barrier and immune dysregulation, *Immunol. Rev.* 242 (2011) 233–246.
- [4] N. Novak, T. Bieber, M. Hoffmann, R. Folster-Holst, B. Homey, T. Werfel, et al., Efficacy and safety of subcutaneous allergen-specific immunotherapy with depigmented polymerized mite extract in atopic dermatitis, *J. Allergy Clin. Immunol.* 130 (2012) 925–931 e924.
- [5] J.M. Bae, Y.Y. Choi, C.O. Park, K.Y. Chung, K.H. Lee, Efficacy of allergen-specific immunotherapy for atopic dermatitis: a systematic review and meta-analysis of randomized controlled trials, *J. Allergy Clin. Immunol.* 132 (2013) 110–117.
- [6] S. Jin, J.U. Shin, J.Y. Noh, H. Kim, J.Y. Kim, S.H. Kim, et al., DOCK8: regulator of Treg in response to corticotropin-releasing hormone, *Allergy* 71 (2016) 811–819.
- [7] J. Lee, C.O. Park, K.H. Lee, Specific immunotherapy in atopic dermatitis, *Allergy Asthma Immunol. Res.* 7 (2015) 221–229.
- [8] H. Zhong, X. Deng, Z. Song, U. Darso, W. Chen, S. Chen, et al., Immunological changes after ASIT in AD allergen-specific immunotherapy and their potential correlation with clinical response in patients with atopic dermatitis patients sensitized to house dust mite, *J. Eur. Acad. Dermatol. Venereol.* 29 (2015) 1318–1324.
- [9] D.R. Wilson, M.T. Lima, S.R. Durham, Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis, *Allergy* 60 (2005) 4–12.
- [10] L.S. Cox, D. Larenas Linnemann, H. Nolte, D. Weldon, I. Finegold, H.S. Nelson, Sublingual immunotherapy: a comprehensive review, *J. Allergy Clin. Immunol.* 117 (2006) 1021–1035.
- [11] K. Ita, Transdermal delivery of drugs with microneedles-potential and challenges, *Pharmaceutics* 7 (2015) 90–105.
- [12] T.M. Tuan-Mahmood, M.T. McCrudden, B.M. Torrisi, E. McAlister, M.J. Garland, T.R. Singh, et al., Microneedles for intradermal and transdermal drug delivery, *Eur. J. Pharm. Sci.* 50 (2013) 623–637.
- [13] Y. Hiraishi, T. Nakagawa, Y.S. Quan, F. Kamiyama, S. Hirobe, N. Okada, et al., Performance and characteristics evaluation of a sodium hyaluronate-based microneedle patch for a transcutaneous drug delivery system, *Int. J. Pharm.* 441 (2013) 570–579.
- [14] E. Jacoby, C. Jarrahian, H.F. Hull, D. Zehrung, Opportunities and challenges in delivering influenza vaccine by microneedle patch, *Vaccine* 33 (2015) 4699–4704.
- [15] Y.C. Kim, F.S. Quan, D.G. Yoo, R.W. Compans, S.M. Kang, M.R. Prausnitz, Improved influenza vaccination in the skin using vaccine coated microneedles, *Vaccine* 27 (2009) 6932–6938.
- [16] G. Senti, S. von Moos, T.M. Kundig, Epicutaneous allergen administration: is this the future of allergen-specific immunotherapy? *Allergy* 66 (2011) 798–809.
- [17] A.K. Shakya, H.S. Gill, A comparative study of microneedle-based cutaneous immunization with other conventional routes to assess feasibility of microneedles for allergy immunotherapy, *Vaccine* 33 (2015) 4060–4064.
- [18] L. Spina, M. Weisskopf, S. von Moos, N. Graf, T.M. Kundig, G. Senti, Comparison of microneedles and adhesive-tape stripping in skin preparation for epicutaneous allergen delivery, *Int. Arch. Allergy Immunol.* 167 (2015) 103–109.
- [19] J.D. Kim, M. Kim, H. Yang, K. Lee, H. Jung, Droplet-born air blowing: novel dissolving microneedle fabrication, *J. Control Release* 170 (2013) 430–436.
- [20] K.Y. Jeong, S.Y. Choi, J.H. Lee, I.Y. Lee, T.S. Yong, J.S. Lee, et al., Standardization of house dust mite extracts in Korea, *Allergy Asthma Immunol. Res.* 4 (2012) 346–350.
- [21] K.Y. Jeong, S.Y. Choi, I.S. Han, J.H. Lee, J.S. Lee, C.S. Hong, et al., The effects of storage conditions on the stability of house dust mite extracts, *Allergy Asthma Immunol. Res.* 5 (2013) 397–401.
- [22] M. Yamamoto, T. Haruna, K. Yasui, H. Takahashi, M. Iduhara, S. Takaki, et al.,

- A novel atopic dermatitis model induced by topical application with dermatophagoides farinae extract in NC/Nga mice, *Allergol. Int.* 56 (2007) 139–148.
- [23] N. Lee, J.U. Shin, S. Jin, K.N. Yun, J.Y. Kim, C.O. Park, et al., Upregulation of CD47 in regulatory T cells in atopic dermatitis, *Yonsei Med. J.* 57 (2016) 1435–1445.
  - [24] M. Pernas, I. Sanchez-Ramos, R. Sanchez-Monge, M. Lombardero, C. Arteaga, P. Castanera, et al., Der p 1 and Der f 1, the highly related and major allergens from house dust mites, are differentially affected by a plant cystatin, *Clin. Exp. Allergy* 30 (2000) 972–978.
  - [25] S.R. Durham, S.M. Walker, E.M. Varga, M.R. Jacobson, F. O'Brien, W. Noble, et al., Long-term clinical efficacy of grass-pollen immunotherapy, *N. Engl. J. Med.* 341 (1999) 468–475.
  - [26] D.B. Golden, A. Kagey-Sobotka, P.S. Norman, R.G. Hamilton, L.M. Lichtenstein, Outcomes of allergy to insect stings in children, with and without venom immunotherapy, *N. Engl. J. Med.* 351 (2004) 668–674.
  - [27] B.G. Bender, J. Oppenheimer, The special challenge of nonadherence with sublingual immunotherapy, *J. Allergy Clin. Immunol. Pract.* 2 (2014) 152–155.
  - [28] W.R. Reisacher, J.M. Visaya, Patient adherence to allergy immunotherapy, *Curr. Opin. Otolaryngol. Head. Neck Surg.* 21 (2013) 256–262.
  - [29] J.W. Streilein, Skin-associated lymphoid tissues (SALT): origins and functions, *J. Invest. Dermatol.* 80 (Suppl) (1983) 12s–16s.
  - [30] V. Flacher, C.H. Tripp, P. Stoitzner, B. Haid, S. Ebner, B. Del Frari, et al., Epidermal Langerhans cells rapidly capture and present antigens from C-type lectin-targeting antibodies deposited in the dermis, *J. Invest. Dermatol.* 130 (2010) 755–762.
  - [31] R.A. Clark, B. Chong, N. Mirchandani, N.K. Brinster, K. Yamanaka, R.K. Dowgiert, et al., The vast majority of CLA+ T cells are resident in normal skin, *J. Immunol.* 176 (2006) 4431–4439.
  - [32] E. Klechevsky, R. Morita, M. Liu, Y. Cao, S. Coquery, L. Thompson-Snipes, et al., Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells, *Immunity* 29 (2008) 497–510.
  - [33] H. Ueno, N. Schmitt, E. Klechevsky, A. Pedroza-Gonzalez, T. Matsui, G. Zurawski, et al., Harnessing human dendritic cell subsets for medicine, *Immunol. Rev.* 234 (2010) 199–212.
  - [34] V. Flacher, C.H. Tripp, D.G. Mairhofer, R.M. Steinman, P. Stoitzner, J. Idoyaga, et al., Murine Langerin+ dermal dendritic cells prime CD8+ T cells while Langerhans cells induce cross-tolerance, *EMBO Mol. Med.* 6 (2014) 1191–1204.
  - [35] E. Shklovskaya, B.J. O'Sullivan, L.G. Ng, B. Roediger, R. Thomas, W. Weninger, et al., Langerhans cells are precommitted to immune tolerance induction, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 18049–18054.
  - [36] M. Gomez de Agüero, M. Vocanson, F. Hacini-Rachinel, M. Taillardet, T. Sparwasser, A. Kissenpfennig, et al., Langerhans cells protect from allergic contact dermatitis in mice by tolerizing CD8(+) T cells and activating Foxp3(+) regulatory T cells, *J. Clin. Invest.* 122 (2012) 1700–1711.
  - [37] M. Williams, K. Crozat, S. Henri, S. Tamoutounour, P. Grenot, E. Devilard, et al., Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells, *Blood* 115 (2010) 1958–1968.
  - [38] G.J. Fernando, X. Chen, T.W. Prow, M.L. Crichton, E.J. Fairmaid, M.S. Roberts, et al., Potent immunity to low doses of influenza vaccine by probabilistic guided micro-targeted skin delivery in a mouse model, *PLoS One* 5 (2010), e10266.
  - [39] F.S. Quan, Y.C. Kim, R.W. Compans, M.R. Prausnitz, S.M. Kang, Dose sparing enabled by skin immunization with influenza virus-like particle vaccine using microneedles, *J. Control Release* 147 (2010) 326–332.
  - [40] Y.C. Kim, J.H. Park, M.R. Prausnitz, Microneedles for drug and vaccine delivery, *Adv. Drug Deliv. Rev.* 64 (2012) 1547–1568.
  - [41] J.H. Park, M.G. Allen, M.R. Prausnitz, Biodegradable polymer microneedles: fabrication, mechanics and transdermal drug delivery, *J. Control Release* 104 (2005) 51–66.
  - [42] C.M. Schoellhammer, D. Blankschtein, R. Langer, Skin permeabilization for transdermal drug delivery: recent advances and future prospects, *Expert Opin. Drug Deliv.* 11 (2014) 393–407.
  - [43] Q.L. Wang, D.D. Zhu, Y. Chen, X.D. Guo, A fabrication method of microneedle molds with controlled microstructures, *Mater. Sci. Eng. C Mater. Biol. Appl.* 65 (2016) 135–142.
  - [44] C. Dupont, N. Kalach, P. Soulaïnes, S. Legoue-Morillon, H. Piloquet, P.H. Benhamou, Cow's milk epicutaneous immunotherapy in children: a pilot trial of safety, acceptability, and impact on allergic reactivity, *J. Allergy Clin. Immunol.* 125 (2010) 1165–1167.
  - [45] S.M. Jones, S.H. Sicherer, A.W. Burks, D.Y. Leung, R.W. Lindblad, P. Dawson, et al., Epicutaneous immunotherapy for the treatment of peanut allergy in children and young adults, *J. Allergy Clin. Immunol.* 139 (2017) 1242–1252 e1249.
  - [46] G. Senti, N. Graf, S. Haug, N. Ruedi, S. von Moos, T. Sonderegger, et al., Epicutaneous allergen administration as a novel method of allergen-specific immunotherapy, *J. Allergy Clin. Immunol.* 124 (2009) 997–1002.
  - [47] G. Senti, S. von Moos, F. Tay, N. Graf, T. Sonderegger, P. Johansen, et al., Epicutaneous allergen-specific immunotherapy ameliorates grass pollen-induced rhinoconjunctivitis: a double-blind, placebo-controlled dose escalation study, *J. Allergy Clin. Immunol.* 129 (2012) 128–135.
  - [48] A.K. Shakyia, C.H. Lee, H.S. Gill, Cutaneous vaccination with coated microneedles prevents development of airway allergy, *J. Control Release* (2017), <https://doi.org/10.1016/j.jconrel.2017.08.012> [Epub ahead of print], pii: S0168-3659(17)30779-4.

AD induction with D1

Immunotherapy with MNIT (10  $\mu$ g)/SCIT (100  $\mu$ g)/SCIT (10

---

