Development of *Dermatophagoides farinae*-loaded microneedle patches for allergen specific immunotherapy in allergic diseases

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Development of *Dermatophagoides farinae*-loaded microneedle patches for allergen specific immunotherapy in allergic diseases

Directed by Professor Kwang Hoon Lee

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Ji Hye Kim

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ABSTRACT

Development of *Dermatophagoides farinae*-loaded microneedle patches for allergen specific immunotherapy in allergic diseases

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(Directed by Professor Kwang Hoon Lee)

Allergen specific immunotherapy (ASIT) which induces allergen-specific tolerance is an effective treatment modality in atopic patients. However, frequent visits over a 3-yr period results in a low patient compliance. To overcome this limitation, sublingual immunotherapy (SLIT) has been developed, however, the effects on atopic dermatitis (AD) are controversial. Therefore, a more convenient and effective allergen delivery is necessary.

Microneedle patch is an efficient transdermal drug delivery method across the skin which consists of hundreds of micrometer sized needles. When it applied to the skin, it creates micron sized pores and deliver drug into the skin.

The aim of this study is to develop allergen-loaded microneedle patches and confirm the stability and allergenicity of the allergen which is loaded into the microneedles. Also, ASIT with these allergen-loaded microneedle patches was performed in the AD mouse model.

Dermatophagoides farinae (*D. farinae*) extract was loaded in hyaluronic acid (HA) based microneedle patches and the stability was validated. There was no decrease of allergenicity after loading the *D. farinae* into the HA microneedle patches. The *D. farinae*-loaded HA microneedle patches were applied to female

BALB/c mice. The 4-wk application of the *D. farinae*-loaded microneedle patches did not show any adverse effects. In addition, the 4-wk application of 10 μg of *D. farinae*-loaded microneedle patches increased the serum total IgE and *D. farinae*-specific IgE levels. In the NC/Nga AD mouse model, there was a decrease of T_H2 cytokines such as IL-4, IL-13 and an increase of Foxp3 and IL-10 in the microneedle patch immunotherapy (MNIT) group compared to the AD group. In addition, MNIT was effective as conventional subcutaneous immunotherapy (SCIT).

From these results, we confirmed the *D. farinae*-loaded HA microneedle patches successfully deliver allergen into the skin and elicit immune responses. In human, allergen-loaded microneedle patches might be useful in ASIT for allergic diseases.

Key words : *Dermatophagoides farinae*, atopic dermatitis, allergen specific immunotherapy, microneedle, NC/Nga

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I. INTRODUCTION

Allergen specific immunotherapy (ASIT) has been used as an effective treatment for IgE-mediated diseases like allergic rhinitis, asthma, and atopic dermatitis (AD).[1-4] The principle of ASIT is to induce allergen specific peripheral tolerance and reduce allergic reaction by repeated injection of the sensitized allergen. In atopic patients, especially sensitized with house dust mite allergen, the effectiveness of ASIT has been proven in clinical research.[5,6] The main mechanism of ASIT is the change of T cell subtypes and immunoglobulin (Ig) responses.[2,7,8] By ASIT, allergen-specific regulatory T cells (Treg) are induced and subsequently secrete IL-10. IL-10 has complex activities on the immune response like a decrease of T cells[9] and an activation of IgG4.[10] IgG4, one of the IgG subclasses, is induced and blocks the binding of IgE to the high-affinity receptor for IgE (FcεRI) on macrophages and neutrophils.[2,8]

There are various routes for ASIT. Subcutaneous injection is the most common and effective route, through an allergen injection. However, it requires trained medical staff and repetitive injections of the allergen over a 3 to 5-yr period, which makes for a low compliance. To overcome this limitation, sublingual immunotherapy (SLIT), which delivers allergen via the mucosal

layer, has been developed. However, the precise mechanism of SLIT is unclear and the effectiveness is lower than that of subcutaneous immunotherapy (SCIT).[11,12] Also, the effectiveness in AD is controversial.[13,14] Epicutaneous immunotherapy (EPIT) is a method of applying an allergen on the skin surface. However, in this method, a physical barrier must be disrupted or the allergen applied for a long period to penetrate the skin barrier.[15,16] Therefore, a more convenient and safe method for allergen delivery is required.

Microneedles are a new transdermal drug delivery method which consists of hundreds of micrometer sized needles in arrays on a backing.[17,18] It creates micron sized pores into the skin, enhancing drug delivery across the skin. A microneedle patch is a microneedle array with a bandage, allowing for simplified application to the skin. Microneedles can be categorized into 4 types according to drug loading and delivery methods: solid, coated, hollow, and dissolving.[19] Among them, the dissolving type of microneedle is made of biodegradable components like hyaluronic acid (HA), so when it is applied to the skin, it can dissolve and release the drug into the skin.[20] Microneedle drug delivery has many advantages. Patients can safely administer it by themselves. There are no risks of bleeding, infection, and/or injury by a regular hypodermic needle. Also, there is no pain as the microneedles do not stimulate pain nerves. For these reasons, microneedles are highlighted as a replacement for hypodermic needles and there is considerable research about vaccine and drug delivery in this area.[20-22] Further, microneedles have a potential use for ASIT by improving allergic responses induced by allergen delivery.[23,24]

In this study, *Dermatophagoides farinae* (*D. farinae*) extract was used as an allergen and the *D. farinae*-loaded HA based microneedle patches were developed. House dust mite allergen has a characteristic of degrading rapidly at room temperature,[25] so the allergenicity of the allergen which was loaded in the microneedles was established.

For vaccination, microneedle delivery is more effective than conventional intramuscular delivery. In many related studies, microneedle delivery results in the same vaccination effect with a much lower dose than intramuscular vaccination.[26-28] Because the vaccine loaded in microneedles is delivered into the epidermis and dermis, it can directly activate dendritic cells which act as an antigen presenting cell.[29] Therefore, it is assumed that microneedle immunotherapy (MNIT) could result in effective therapy with a lower dose of allergen when compared to the conventional SCIT. For this reason, dose-sparing effect of the MNIT was tested in the AD mouse model and compared its therapeutic effect with that of the SCIT.

II. MATERIALS AND METHODS

1. Enzyme-linked immunosorbent assay (ELISA)

A. Inhibition ELISA

The allergenicity of *D. farinae* extract was determined by inhibition ELISA.[25] The *D. farinae* extract was diluted in a 50 mM sodium carbonate buffer (pH=9.6) (Sigma, MO, USA). A 96-well plate was coated with 100 μl of 10 μg/ml of *D. farinae* extract overnight at 4℃. The sera from 4 subjects were pooled and diluted 1:3 with 1% BSA in PBS-T. The 1:3 diluted sera with 6-fold serial diluted inhibitor solutions were pre-incubated at 4℃. Next, these sera were treated in a pre-coated plate for 1-2 hr at 37℃. Then, goat biotinylated anti-human IgE (Vector, Burlingame, CA, USA) was treated for 1 hr at 37℃. After washing, streptavidin-peroxidase was treated for 30 min at room temperature. Color development was performed by TMB (SureBlue^{TM} TMB Microwell Peroxidase Substrate (1-Componet), KPL) and stopped with stop solution. The percentage of inhibition was calculated as $(1-A_i/A_0)*100$, which A_i stands for the absorbance at 450 nm with an inhibitor, and A_0 for the absorbance without an inhibitor. The allergenicity was measured by calculating the concentration of *D. farinae* allergen required to produce 50% inhibition of a standard extract.

B. Der f 1 ELISA

Der f 1 concentrations were measured using a Der f 1 ELISA kit (Indoor Biotechnologies Inc., Charlottesville, VA, USA) following the manufacturer's instructions.

C. Serum Immunoglobulin ELISA

Mouse serum Immunoglobulin concentration was determined using a mouse IgE ELISA kit (BioLegend, San Diego, CA, USA) and a mouse IgG4 ELISA kit (MyBioSource, San Diego, CA, USA). All experiments were performed following the manufacturer's instructions. In addition, *D. farinae*-specific IgE was measured. 20 μg/ml of *D. farinae* extract in 50 mM carbonate buffer (pH 9.6) (Sigma) was incubated in a 96-well plate overnight at 4°C. The plate was blocked with 3% skim milk in PBS containing 0.05% Tween 20 for 1 hr at 37°C. Thereafter, the plate was incubated with diluted mouse serum samples overnight at 4°C. Subsequently, bionylated rat anti-mouse IgE was treated on the plate for 2 hr, and then peroxidase-streptavidin was incubated for 1 hr at room temperature. The color development was performed by TMB and stop solution (KPL). The absorbance was measured at 450 nm by microplate reader.

2. HA based microneedle patches

The *D. farinae* extract was provided by the Department of Internal Medicine and Institute of Allergy, Yonsei University (Seoul, Korea).[30] The *D. farinae* extract was mixed with a HA with the dose of 0.2 μg, 2 μg, 10 μg/patch. These mixtures were made into microneedles using the droplet-born air blowing method specialized by Rapas.[31] Briefly, *D. farinae* mixed HA solution was dispensed onto two plates. The plates were placed each other and elongated. Next, solidify the mixtures with an air blower. Finally, the solidified mixtures were separated from each surface and made into two microneedle arrays.

3. Safety test in BALB/c mice

6-wk-old female BALB/c mice were raised in an air-conditioned room maintained at 24 ± 2℃ and 55 ± 15% humidity. The mice were maintained for 1 wk before experiments began. The hair on the back of the mice was shaved, and the microneedle patches were applied (n=3/group). A 3M-coban was banded around the body to avoid it detaching from the skin. After 2 hr, the 3M-coban and patches were gently removed. Patch application was performed twice a wk, for 4 wk.

4. Immunotherapy in AD mouse model

6-wk-old female NC/Nga mice were purchased from SLC Japan (Shizuoka, Japan) to make the AD mouse model.[32] The hair on the back of the mice was shaved. For AD induction, 200 μl of 4% sodium dodecyl sulfate (SDS) solution (Sigma-Aldrich, Saint Louis, MO, USA) was applied to disrupt the skin barrier. After 2 hr, 100 mg of *D. farinae* body extract (*Df*E) ointment (Biostir-AD; Biostir Inc., Kobe, Japan) was topically applied twice a wk for 8 wk. At week 4, ASIT was performed by subcutaneous injection or *D. farinae*-loaded microneedle patch application. For SCIT, *D. farinae* extract dissolved in PBS was injected with a dose of 100 μg or 10 μg. For MNIT, a 10 μg of *D. farinae*-loaded microneedle patches were applied, twice a wk, for 5 wk.

5. Measurement of transepidermal water loss (TEWL)

The TEWL was measured on the skin of the shaved mice back with a Tewameter TM210 (Courage and Khazaka, Cologne, Germany) to evaluate skin barrier function. For measurement, the mice were anesthetized in a room maintained at 24 ± 2℃ and 55 ± 15% humidity.

6. Immunofluorescence staining

For the frozen section, the mice's skin was fixed with 4% paraformaldehyde for 2 hr and frozen in OCT at -80℃. The tissue was fixed with acetone and blocked with 5% BSA for 1 hr and treated with a primary antibody overnight at 4℃. After washing with 1% TBST, the tissue was treated with a fluorescent labelled secondary antibody. Finally, the tissue was mounted with a VECTASHIELD™ Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and investigated with a fluorescent microscope. The primary antibodies were the anti-Der f 1 antibody (Indoor Biotechnologies Inc.) and the anti-mouse MHC II-Alexa-488 antibody (Biolegend), and the secondary antibody was the anti-mouse Alexa-633 (Life Technologies, Carlsbad, CA, USA). For the paraffin section staining, Deparaffinization and antigen retrieval were done. Next, the primary CD4 antibody (Abcam, Cambridge, MA, USA) and the secondary Alexa-555 (Abcam) were stained, and then, IL-4-FITC (eBioscience, San Diego, CA, USA) and Foxp3 (Abcam) were stained respectively. In Foxp3 staining, the secondary Alexa-633 (Life technologies) was also stained.

7. Flow cytometry

Cells were isolated from mouse lymph nodes and cultured in the anti-CD3, -CD28 (eBioscience) coated plates. The cells were treated with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience) and then the cells were stained with a fixable viability dye for 30 min at 4℃. After washing, the cells were labeled with anti-CD4 antibody conjugated with fluorescent dye (eBioscience). For intracellular labeling, cells were fixed and permeabilized with a cytofix/cytoperm buffer (eBioscience) and labeled with anti-mouse IL-4, IL-10 and Foxp3 conjugated with fluorescent dye. Labeled cells were quantified

using a BD LSR Fortessa^{TM} flow cytometer, and then the data was analyzed using FlowJo Software (BD Bioscience, San Jose, CA, USA).

8. Quantitative Real-Time PCR (qRT-PCR)

Mouse skin samples were homogenized using a Precellys 24 Homogenizer (Bertin Technologies, France). Next, the total RNA was extracted using RNeasy Plus Mini kit (Qiagen, Hilden, Germany) per the manufacturer's instructions and then subjected to a reverse transcription. A quantitative PCR was performed at least two times for each sample using 2 μl of cDNA supplemented with the appropriate primers (Applied Biosystems, Foster City, CA, USA). Primers specific for mice; IL-4 (Mm00445259_m1), IL-13 (Mm00434204_m1), Foxp3 (Mm00475162_m1), IL-10 (Mm01288386_m1), and GAPDH (Mm99999915 _g1) were mixed with cDNA and qPCR master mix (Applied Biosystems) in a 20 μl reaction. qRT-PCR was performed using a StepOnePlus PCR system (Applied Biosystems). The expression levels of mRNA were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method.

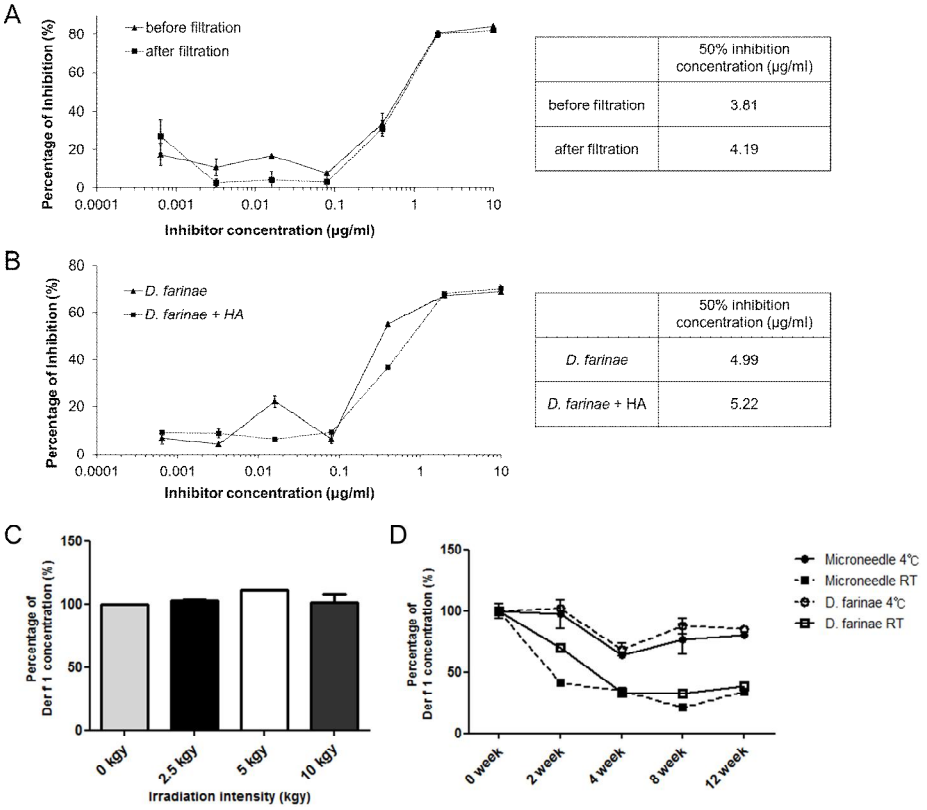
9. Statistical analysis

All the statistical analyses were conducted using Graph Pad Prism, version 5 (GraphPad Software, San Diego, CA, USA). All data represented means ± SEMs. The significance of differences among multiple groups was determined using one-way ANOVA with a Tukey's post hoc test. A p value <0.05 was considered statistically significant. Significance is indicated in the figures as follows: * p<0.05; ** p<0.01; *** p<0.001.

III. RESULTS

1. Stability of the *D. farinae* extract

Prior to manufacturing allergen-loaded microneedle patches, the allergenicity of *D. farinae* extract was examined. To load the microneedles, particle size needed to be below 0.45 μm. So, a filtration of dissolved *D. farinae* extract was done and the allergenicity was examined by inhibition ELISA. The samples were *D. farinae* extract dissolved in PBS (before filtration) and *D. farinae* extract filtering with 0.4 μm filter (after filtration). The protein concentrations of both samples were measured with a BCA assay and similar protein concentration samples were used. The allergenicity was calculated by the concentration which produces a 50% inhibition of the standard *D. farinae* extract. Before filtration, the 50% inhibition concentration was 3.81 μg. After filtration, the concentration of 50% inhibition was 4.19 μg (Fig. 1A). The allergenicity of filtrated *D. farinae* extract was slightly decreased, about 10%. Next, we confirmed the allergenicity of *D. farinae* extract in HA, the base polymer of the microneedles. Filtrated *D. farinae* extract was mixed with HA (*D. farinae* + HA) and dried completely. For the test, this dried mixture was dissolved in PBS and the protein concentration was also measured. Next, the allergenicity was compared to *D. farinae* extract. The 50% inhibition concentration of *D. farinae* extract was 4.99 μg and the *D. farinae* + HA was 5.22 μg (Fig. 1B). The difference is 0.05%. From these results, it was confirmed that the allergenicity of *D. farinae* extract is maintained after filtration and being loaded into the HA.



	50% inhibition concentration (μg/ml)
before filtration	3.81
after filtration	4.19

	50% inhibition concentration (μg/ml)
D. farinae	4.99
D. farinae + HA	5.22

Figure 1. Stability of the *D. farinae*-loaded microneedle patches by inhibition ELISA and Der f 1 ELISA. (A) The allergenicity of *D. farinae* extract (before filtration) and *D. farinae* extract with 0.4 μm filtration (after filtration) were measured by inhibition ELISA. Before filtration, 50% inhibition concentration was 3.81 μg/ml. After filtration, the concentration was 4.19 μg/ml. (B) The 50% inhibition concentration was 4.99 μg/ml and 5.22 μg/ml for *D. farinae* extract and *D. farinae* extract with HA (*D. farinae* + HA) respectively. (C) Der f 1 concentration of the conditionally irradiated *D. farinae*-loaded microneedle patches. There were no changes caused by irradiation (p=ns). (D) The changes of Der f 1 concentration in the *D. farinae*-loaded microneedle patches stored at room temperature and 4℃ for 12 wk.

2. Manufacturing of the *D. farinae*-loaded microneedle patches

We manufactured various concentrations (0 μg, 0.2 μg, 2 μg, and 10 μg/patch) of *D. farinae* extract-loaded microneedle patches using a droplet born air blowing method by Rapas.[31] A microneedle patch consists of two parts; a sticky patch lesion along the border and a microneedle array in the middle. The microneedle length is 272.9 ± 6.45 μm. Microscopic images of microneedles with the magnification of X8, X30, and X50 were shown (Fig. 2).

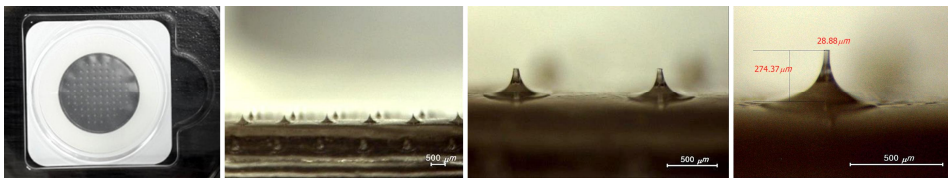


Figure 2. Microscopic images of the *D. farinae*-loaded microneedle patches. The photograph of a manufactured *D. farinae*-loaded microneedle patch and microscopic images in the magnification of X8, X30, and X50 each (Bar = 500 μm).

3. Stability of the *D. farinae*-loaded microneedle patches

For clinical use, we irradiated *D. farinae*-loaded microneedle patches and examined the concentration change of the loaded allergen with the method of Der f 1 ELISA. Der f 1 is the major allergen of *D. farinae*.[33] With several intensities of irradiation, there was no change of the Der f 1 concentration (Fig. 1C). From this result, it was decided to use 10 kgy of irradiation intensity. The *D. farinae* extract contains its own proteases, so it degrades rapidly at room temperature. We examined the storage stability of the *D. farinae*-loaded microneedle patches per storage temperature and term. The *D. farinae*-loaded microneedle patches and *D. farinae* extract were stored at room temperature (RT) and 4℃ for 12 wk. The initial Der f 1 concentrations of microneedle

patches and *D. farinae* extract were determined to be 19.57 ng/ml and 19.32 ng/ml, respectively. After 12 wk, the concentration of *D. farinae* stored at RT decreased more than 70%, but less than 20% when stored at 4℃ (Fig. 1D). At 4℃, the Der f 1 concentration remained 81% in the microneedle patches, and 86% in *D. farinae* extract. However, at RT, the Der f 1 concentration in the microneedle patches and *D. farinae* extract remained 34% and 40%, respectively. From these results, storage temperature is important and *D. farinae* extract loaded in a microneedle patch remains for at least 12 wk at 4℃.

4. Safety of the *D. farinae*-loaded microneedle patches

When removing the attached microneedle patches from the skin, there is a slight irritation. To estimate the irritation on the skin, we measured the TEWL, an indicator of skin barrier functionality. First, the basal TEWL on the backs of shaved BALB/c mice was measured. Next, microneedle patches were applied for 2 hr. After 2 hr, the patches were gently removed from mice's skin and the TEWL where the needle and patch lesion were attached was measured. After waiting for another 2 hr for the skin barrier to recover, the TEWL was measured again at the same lesion. There was an increase of the TEWL right after removal of the microneedle patches at both lesions, but this subsided to a basal level within 2 hr (Fig. 3A-B). Also, manufactured microneedle patches were applied twice a wk, for 4 wk in female BALB/c mice. There were no adverse effects such as infection, bleeding, and/or erythema caused by microneedles (Fig. 3C).

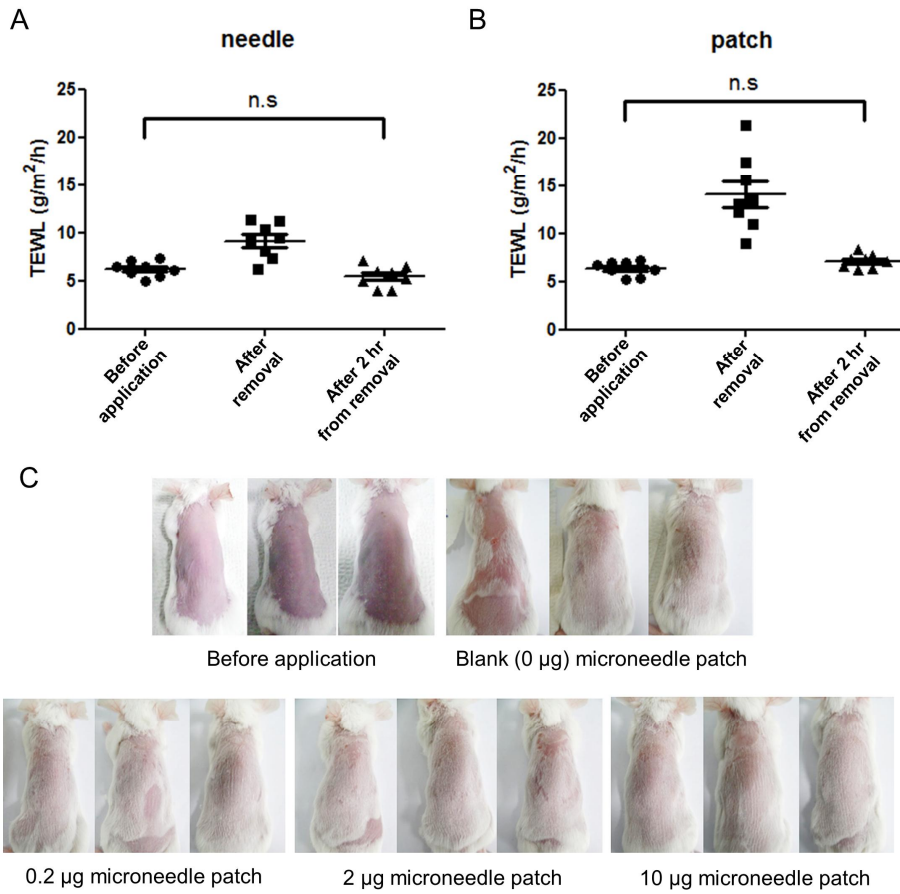


Figure 3. Characteristic of the *D. farinae*-loaded microneedle patches in BALB/c mice. The TEWL of the skin where a microneedle patch was applied. There was a slight increase of the TEWL when removing from the skin both (A) needle and (B) patch lesions, but recovered within 2 hr (p=ns). (C) Images of BALB/c mice before and after a 4-wk application of microneedle patches. During the experiment, there were no signs of adverse effects.

5. Allergen delivery of the *D. farinae*-loaded microneedle patches

Microneedle patches with a serial dose of *D. farinae* were applied to female BALB/c mice twice a wk for 4 wk to evaluate immune responses by *D. farinae* (n=3). In week 4, serum was harvested and the total IgE and *D. farinae*-specific IgE levels measured. Total IgE level had increased in the group of 2 μg and 10 μg (Fig. 4A), and *D. farinae*-specific IgE had increased only in the group of 10 μg of *D. farinae*-loaded microneedle patches (Fig. 4B). So, we determined that 10 μg of *D. farinae* is enough to elicit immune reactions. When the *D. farinae*-loaded microneedle patches were applied onto the skin, the microneedles which were made of HA dissolved and released the loaded molecules into the skin, especially in the epidermis and dermis.[20] In normal mouse skin, the epidermal thickness is about 10 μm and the dermis is about 250 μm. The microneedle length is 272.9 ± 6.45 μm. So, microneedles could penetrate the skin into the dermis. To confirm the allergen delivery into the dermis, 10 μg of *D. farinae*-loaded microneedle patches were applied to BALB/c mice and harvested the skin lesion where the patch was applied at the point of 1, 2 and 4 hr. The harvested mice skin was made into a frozen section and stained with MHC II, a marker of dendritic cells, and Der f 1 antibodies. In fluorescent images, there were co-localizations of MHC II and Der f 1 in the dermis (Fig. 4C). From this result, it was confirmed that *D. farinae* extract loaded in microneedles was successfully delivered into the dermis and could activate dermal dendritic cells.

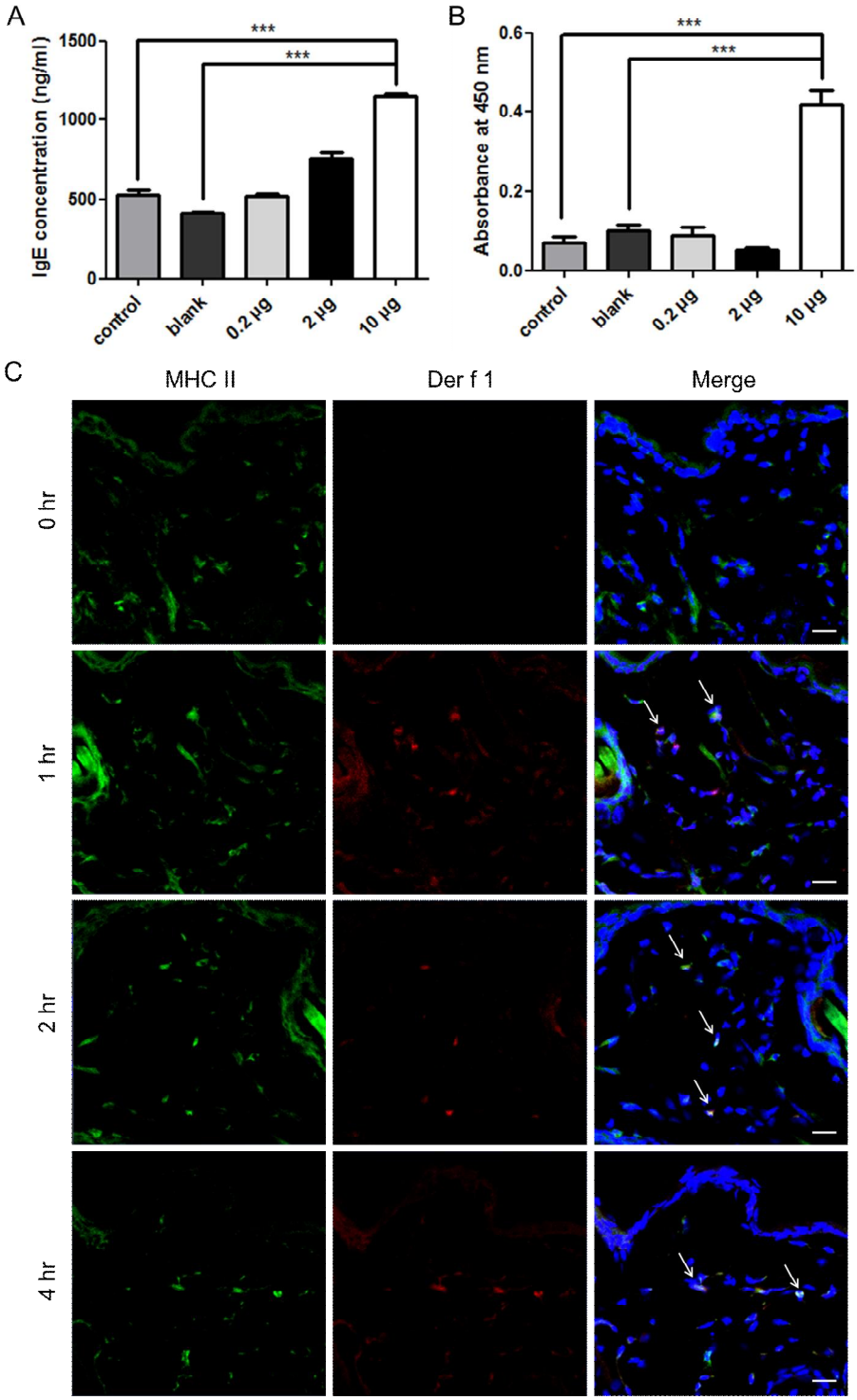


Figure 4. Immune responses in serum and *D. farinae* delivery into the skin. Female BALB/c mice were applied blank (0 μg), 0.2 μg, 2 μg, and 10 μg of *D. farinae*-loaded microneedle patches for 4 wk. (A) Serum total IgE concentration. (B) *D. farinae*-specific IgE level represented by absorbance at 450 nm. Only in applying 10 μg of *D. farinae*-loaded microneedle patches, both IgE levels were increased (***p<0.001). (C) 10 μg of *D. farinae*-loaded microneedle patches were applied for 1, 2, and 4 hr. MHC II (green) was used as dendritic cell marker. Co-localizations of MHC II and Der f 1 (red) were detected in the dermis (white arrows, Bar = 20 μm).

6. Immunotherapy with the *D. farinae*-loaded microneedle patches

6-wk-old female NC/Nga mice were used to induce AD and mice were divided into 5 groups (n=4/group); normal (NL, non-treated), AD (control), MNIT (10 μg), SCIT (100 μg), and SCIT (10 μg). For AD induction, 100 μg of *D. farinae* extract ointment was applied twice a wk, for 8 wk. Barrier disruption with 4% SDS was performed 2 hr before applying the *D. farinae* ointment. At week 3, the eczematous lesion on the neck and back skin of the mice could be examined.

For ASIT, starting at week 4, *D. farinae* extract was injected subcutaneously or *D. farinae*-loaded microneedle patches were applied twice a wk for 5 wk. In the MNIT group, a 10 μg of *D. farinae* which is 1/10 of the dose used in the SCIT group was loaded in the microneedles. In the SCIT group, a 100 μg of *D. farinae* extract dissolved in PBS was injected subcutaneously. In addition, a 10 μg of *D. farinae* extract injection group was added which is the same dose of the MNIT group. In the AD group, only PBS was injected (Fig. 5A).

The mice were photographed weekly to observe the changes of individual mice (Fig. 5B). At week 8, the TEWL was measured to evaluate the skin barrier function. In the MNIT (10 μg) and SCIT (100 μg) groups, the TEWLs at week 8 were lower than the AD and SCIT (10 μg) groups (Fig. 5C). In addition, mouse

serum was harvested and total IgE and IgG4 levels were measured. ASIT accompanied by an increased IgG4 isotype results in blockage of IgE activities.[34] Total IgE levels of the MNIT (10 μg) and SCIT (100 μg) groups were much lower than the AD and SCIT (10 μg) groups (Fig. 6E). Conversely, IgG4 levels were increased in the MNIT (10 μg) and SCIT (100 μg) groups compare to the other groups (Fig. 6F).

Paraffinized mice skin was stained with hematoxylin and eosin for histological evaluation. The skin of the AD and SCIT (10 μg) groups had increased epidermal thickness. Also, whole cell and eosinophil count were increased. In contrast, the MNIT (10 μg) and SCIT (100 μg) groups showed decreased epidermal thickness and eosinophil count compared to the AD and SCIT (10 μg) groups (Fig. 6A-D).

To confirm the therapeutic effect of ASIT, we measured the changes of T_H2 and Treg cells using various methods; flow cytometry in draining lymph nodes, fluorescence staining and qRT-PCR in skin.

First, for flow cytometry, lymphocytes from skin draining lymph nodes were stained with CD4, IL-4 (T_H2), and IL-10 (Treg) antibodies. In CD4+ T cells, the level of IL-4+ cells was lower in the MNIT (10 μg) and SCIT (100 μg) groups than the AD and SCIT (10 μg) groups. The ΔMFI of each groups were measured; 223.5 ± 11.5 in the AD, 149 ± 11.0 in the MNIT (10 μg), 168.5 ± 5.5 in the SCIT (100 μg), 214.5 ± 8.5 in the SCIT (10 μg) group (Fig. 7A). In contrast, the level of IL-10+ cells in CD4+ T cells was increased in the MNIT (10 μg) and SCIT (100 μg) groups compared to the AD and SCIT (10 μg) groups. The ΔMFI of each groups were measured; 7.285 ± 0.36 in the AD, 11.35 ± 0.15 in the MNIT (10 μg), 13.25 ± 0.65 in the SCIT (100 μg), 10.10 ± 0.40 in the SCIT (10 μg) group (Fig. 7B).

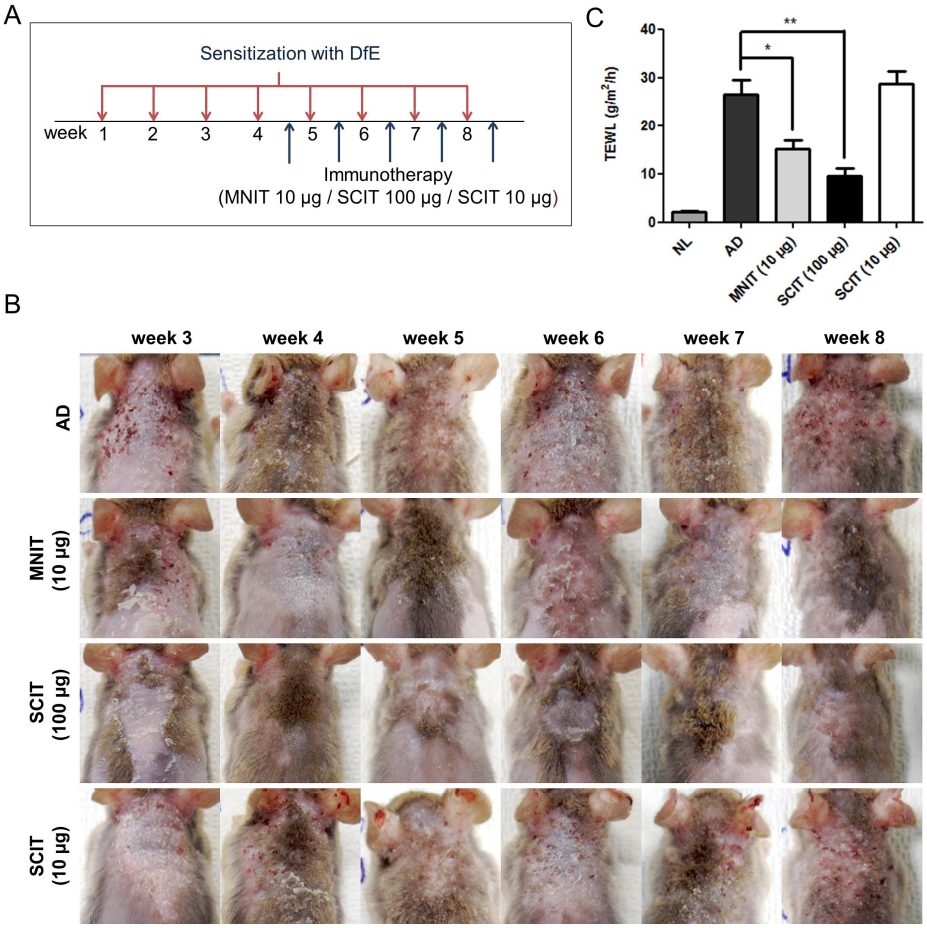


Figure 5. Experimental procedure and clinical features of AD mouse model. (A) Scheme of experimental procedures. Female NC/Nga mice were divided into 5 groups (n=4/group): NL (untreated), AD, MNIT (ASIT with 10 μg of *D. farinae*-loaded microneedle patch), SCIT (ASIT with 100 μg of *D. farinae*), and SCIT (ASIT with 10 μg of *D. farinae*). (B) Changes of clinical feature from week 3 to week 8. From week 3, dermatitis features were detected. (C) The TEWL measured at week 8 (*p<0.05, **p<0.01). The MNIT (10 μg) and SCIT (100 μg) groups showed relieved clinical features when compared to the AD and SCIT (10 μg) groups.

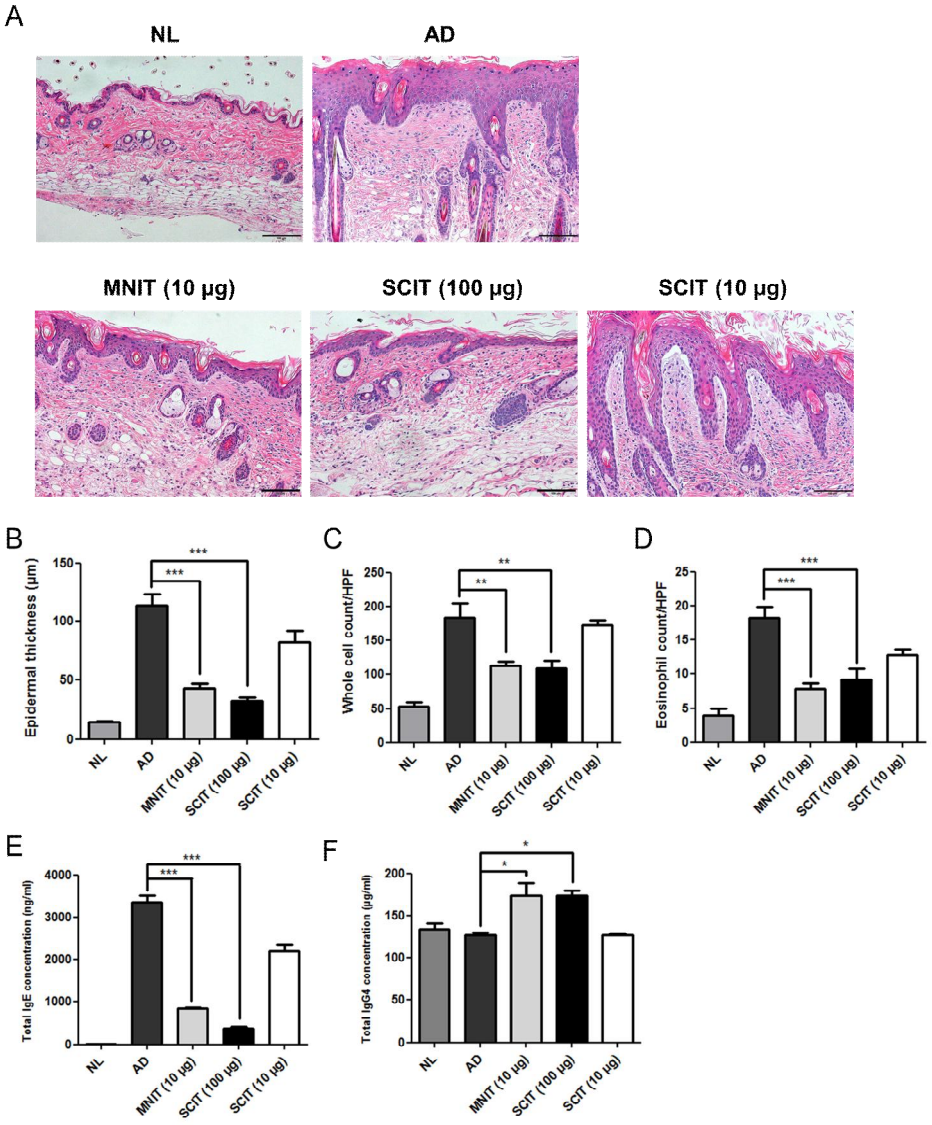


Figure 6. Histological features of mice skin and serum Ig concentrations.

(A) Hematoxylin and eosinophil staining of mice skin (Bar = 100 μm). (B) Epidermal thickness, (C) whole cell count, and (D) eosinophil count were measured. (E) Total IgE level and (F) IgG4 level were measured in mice serum (*p<0.05, **p<0.01, ***p<0.001).

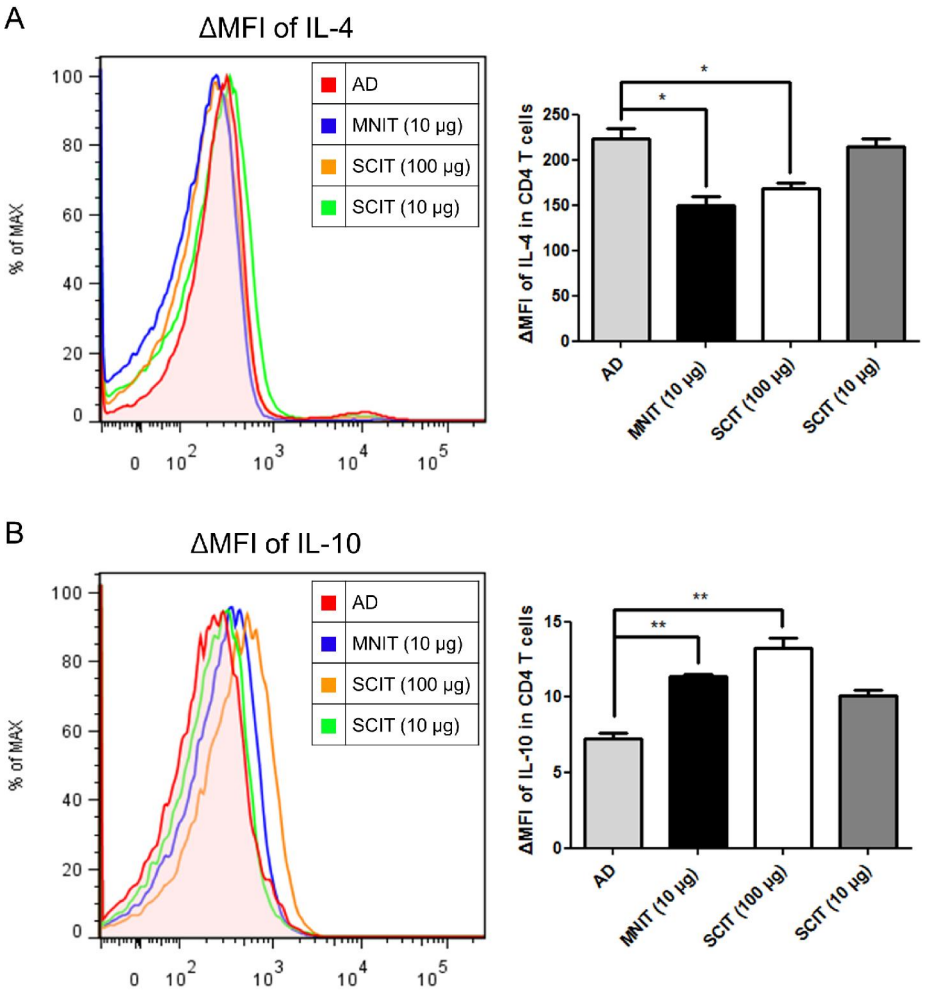
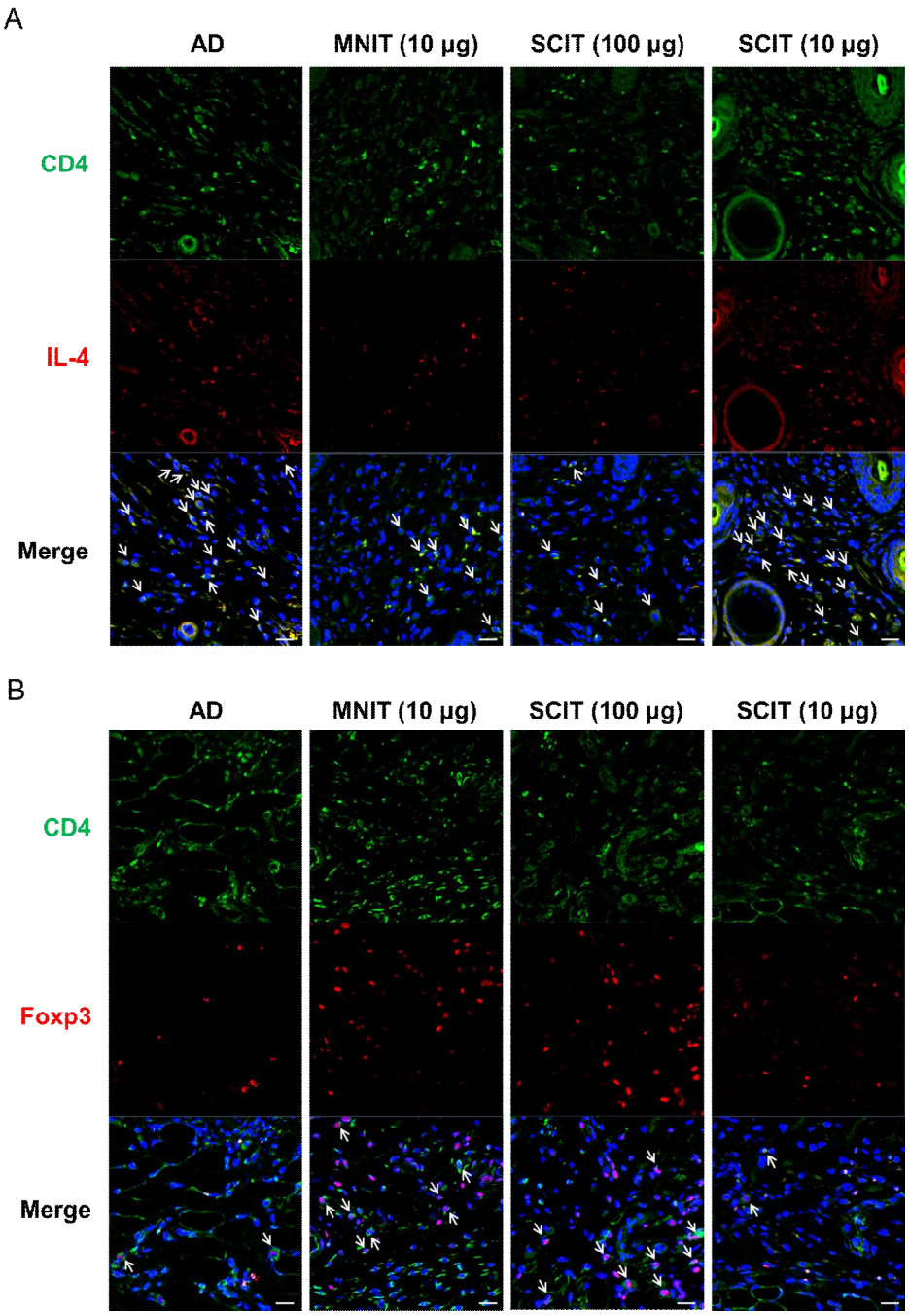


Figure 7. T_H2 and Treg cytokines in draining lymph nodes. T cell subtypes of lymphocytes in mice skin draining lymph nodes. (A) The level of CD4+ IL-4+ cells. The ΔMFI of each groups: 223.5 ± 11.5 in the AD, 149 ± 11.0 in the MNIT (10 μg), 168.5 ± 5.5 in the SCIT (100 μg), 214.5 ± 8.5 in the SCIT (10 μg) group. (B) The level of CD4+ IL-10+ cells. The ΔMFI of each groups: 7.285 ± 0.36 in the AD, 11.35 ± 0.15 in the MNIT (10 μg), 13.25 ± 0.65 in the SCIT (100 μg), 10.10 ± 0.40 in the SCIT (10 μg) group (*p<0.05, **p<0.01).

Second, with mice skin tissue, fluorescence staining was performed. Skin tissue was stained with CD4 and IL-4 antibodies for T_H2 cells, or CD4 and Foxp3 antibodies for Treg cells (Fig. 8A-B). From the result of calculating the numbers of CD4+ and CD4+ IL-4+/Foxp3+ cells, the MNIT (10 μg) and SCIT (100 μg) groups have a lower proportion of CD4+ IL-4+ cells in CD4+ T cells (Fig. 8C) and more CD4+ foxp3+ Treg cells in CD4+ T cells (Fig. 8D), and this result is reversed in the AD and SCIT (10 μg) groups. The AD and SCIT (10 μg) groups showed increased T_H2 cells and low numbers of Treg cells.

Last, to quantify cytokine gene expression levels in mice skin, total RNAs were extracted from skin tissue and synthesis cDNA. Then, qRT-PCR was performed. Both the MNIT (10 μg) and SCIT (100 μg) groups showed similar patterns of gene expression level of cytokines. IL-4 and IL-13 were decreased (Fig. 9A-B). In contrast, IL-10 and Foxp3 were increased compared to the AD and SCIT (10 μg) groups (Fig. 9C-D). From these results, reduction of T_H2 cytokines and induction of Treg cells, it was confirmed that the MNIT (10 μg) group has therapeutic effect and it is similar to the SCIT (100 μg) group. However, the SCIT (10 μg) has no therapeutic effect, just similar state to the AD group in all experiments.



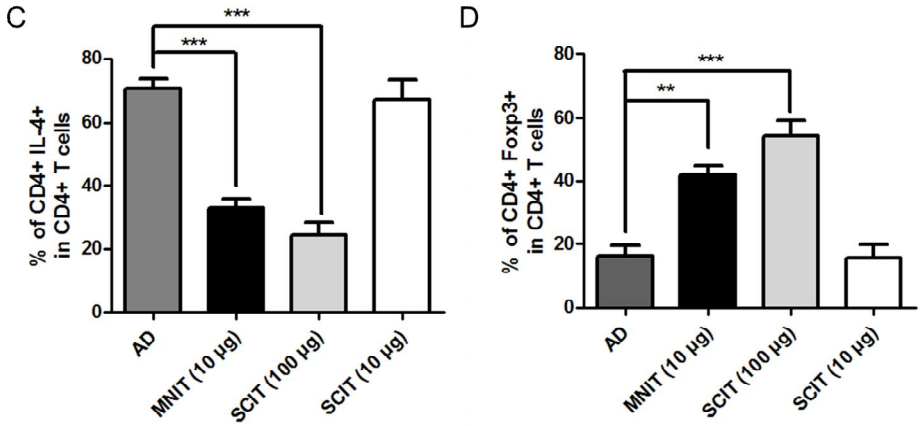


Figure 8. T_H2 and Treg cells in fluorescence staining. Fluorescence images of the paraffinized mice skin. All the magnification is X400 (Bar = 20 μm). (A) CD4 (red) and IL-4 (green), (B) CD4 (red) and Foxp3 (green) were stained. IL-4+ or Foxp3+ cells in CD4+ cells were counted and quantified. (C) The percentages of CD4+ IL-4+ cells were decreased in the MNIT (10 μg) and SCIT (100 μg) groups when compared to the AD and SCIT (10 μg) groups. (D) The percentages of CD4+ Foxp3+ cells were increased in the MNIT (10 μg) and SCIT (100 μg) groups when compared to the AD and SCIT (10 μg) groups (***p<0.001).

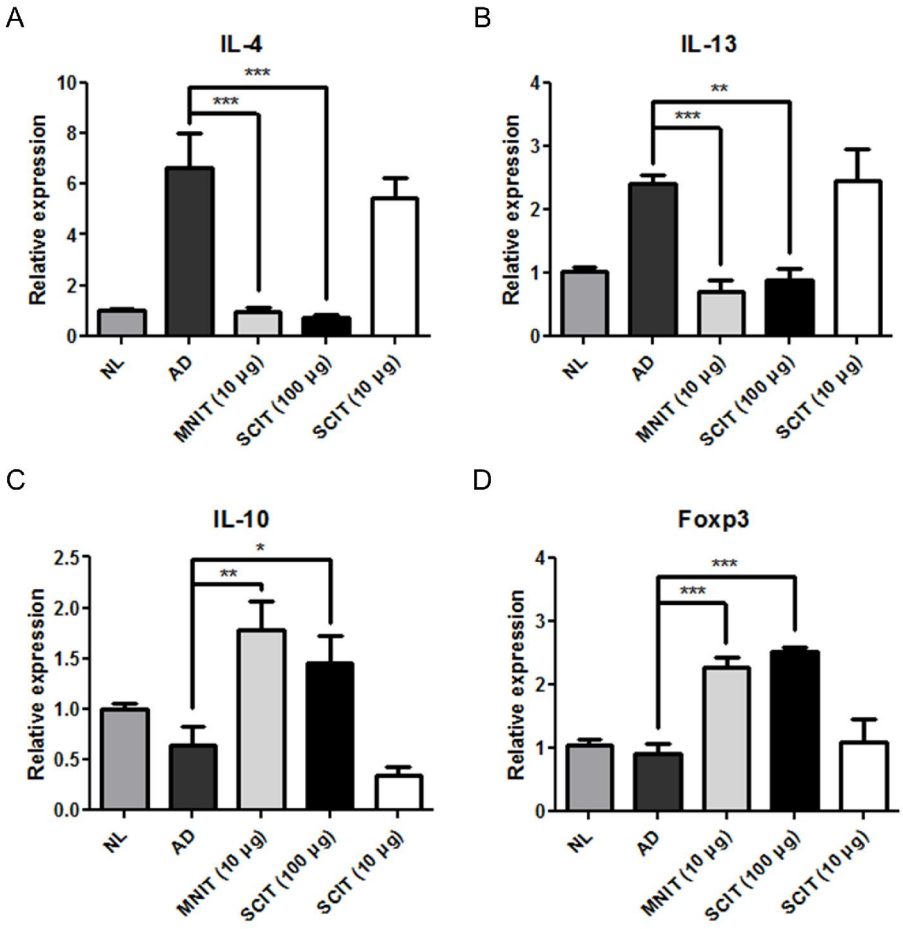


Figure 9. Cytokine gene expression levels in mice skin. Expression levels of IL-4, IL-13, IL-10, and Foxp3 mRNA were determined by qRT-PCR in mice skin. (A-B) IL-4 and IL-13 expression was much low in the MNIT (10 μg) and SCIT (100 μg) groups than the AD and SCIT (10 μg) groups. (C-D) IL-10 and Foxp3 expressions were much higher in the MNIT (10 μg) and SCIT (100 μg) groups than the AD and SCIT (10 μg) groups (*p<0.05, **p<0.01, ***p<0.001).

IV. DISCUSSION

ASIT is an important and unique treatment modality because of its potential of modifying the disease progression by addressing the underlying cause of allergic diseases.[35,36] Conventionally, subcutaneous injection is performed for allergen administration. However, frequent visits over a 3 to 5-yr period results in a low patient compliance rate. For this reason, SLIT which holding a formulation in the mouth has been developed. This method is more safe but less efficient than SCIT.[12,37] Moreover, there is a report that the compliance of SLIT is lower than that of SCIT.[38]

Recently, EPIT is suggested as an effective treatment because it targets dendritic cells in the epidermis (Langerhans cells) and dermis (dermal dendritic cells).[15,39] The dendritic cells uptake antigens and migrate into draining lymph nodes. They represent the antigens to the naïve lymphocytes, effectively inducing subsequent allergic reactions.[8,39-41] However, the physiological skin barrier must be disrupted to deliver allergen across the epidermis. Various methods of skin barrier disruption such as tape stripping, electroporation, alcohols, and terpenes could elicit adverse effects.[15]

Recently, there has been a rise in transdermal delivery with microneedles.[15,24] Conventional hypodermic needles have many disadvantages such as a possibility of hypersensitivity like bleeding, bruising, and/or discomfort. There is a risk of contamination and/or an accidental hypodermic needle injury. Moreover, trained medical staff is necessary for proper administration. In contrast, microneedles have many advantages; there is no need for trained medical staff, it is non-invasive, with painless administration, and there are no risks of hypodermic needle accidents.[17,42] Therefore, it is assumed that microneedles could be an alternative route for ASIT by conveniently delivering the allergen into the skin.

In this study, the *D. farinae*-loaded HA based microneedle patches for ASIT were developed. HA is a biodegradable polysaccharide and can be found in human tissue, body fluids, and mainly in the skin. Because HA is also used as a cosmetic component, it is a useful and safe matrix for microneedles and it also has sufficient mechanical force.[20,42,43]

For the first, the allergenicity and stability of the *D. farinae*-loaded microneedle patches was confirmed by inhibition ELISA analysis. However, house dust mite allergen contains various proteases.[33] For this reason, the storage condition is the most important for long-term use. Especially in ASIT, to overcome the low patient compliance, allergen-loaded microneedle patches should be stable. In this experiment, when stored at 4℃, Der f 1 concentration of the *D. farinae*-loaded microneedle patches was maintained about 81% after 12 wk. However, the concentration decreased to 34% when stored at room temperature (Fig. 1). Thus, storage temperature is the most important factor for long-term storage. For clinical use, irradiation was performed to the *D. farinae*-loaded microneedle patches and there was no decrease of Der f 1 concentration. This research found that the *D. farinae*-loaded microneedle patches are stable enough to apply to mouse models.

Allergen delivery was confirmed as fluorescence staining of skin tissue which was *D. farinae*-loaded microneedle patches were applied. There was co-localization of MHC II+ cells and Der f 1 in the dermal lesion, meaning *D. farinae* extract was captured by dermal dendritic cells. Also, by repeated application of 10 μg of *D. farinae* using microneedle patches, an increased serum total IgE and *D. farinae*-specific IgE were detected (Fig. 4). So, it was determined that 10 μg of *D. farinae*-loaded microneedle patches were enough to elicit immune responses in mouse.

One of the challenges of microneedle patches is the limitation of loading dose.[17] For a large amount of loading, the microneedle array size or length should be increased which could induce pain. In many other studies, especially

on vaccinations, microneedles enable dose-sparing about 1/100 compared to an intramuscular injection.[26,27] This is because the microneedles mainly delivered vaccine into the epidermis and dermis. In the same way of EPIT, by activating dendritic cells, more efficient delivery is possible. Also, in allergen delivery, microneedles can be applied.[23] So, for ASIT study in AD mouse model, 10 μg of *D. farinae* was loaded into the microneedle patches. It is 10% of the dose used in the SCIT group (100 μg).

In allergic diseases, T_H2 cell and associated cytokines (IL-4, IL-5, and IL-13) are generally elevated.[44,45] These cytokines induce class switching to IgE, which sensitizes mast cell and basophil by interacting the high-affinity receptor, FcεRI. The crosslinking of the IgE-FcεRI results in degranulation of mast cell and basophil and this induces the allergic reactions.[8] The mechanism of ASIT is closely related to the tolerance to the sensitized allergen. By ASIT, the IgE level decreases and IgG4 level increases because of activated Treg cells. Treg cells produce IL-10 and TGF-β which have the function of class switching to IgA, IgG, and IgG4.[46] Especially, elevated IgG4 contributes to blocking IgE activity.[2] Also, Treg cells have immune-suppressive effects which induces the decrease of T_H1 (IFN-γ, IL-2) and T_H2 cytokines.[9]

So, the therapeutic effect of ASIT was confirmed by observing the change of T_H2 and Treg cells.[47] At both clinical and histological analysis level, the MNIT (10 μg) and SCIT (100 μg) groups showed relieved aspects compared to the AD group (Fig. 5, 6). In skin and skin draining lymph nodes, T_H2 cytokines were decreased. Also, Treg cells and IL-10 were significantly increased after the MNIT as SCIT (100 μg). However, SCIT (10 μg) group showed no therapeutic effect from the treatment, it is similar to an AD condition (Fig. 7, 8, 9).

In the future, microneedle patches could be developed for patients with allergic diseases as an ASIT. However, for human use, optimal dose and schedule should be tested. Also, the stability of various allergens in microneedle patches should be confirmed. In this study, the storage stability for only 12 wk

was measured, but, further storage of *D. farinae* concentration and its allergenicity, at least for 6 months, should be confirmed for stable long-term use.

In summary, ASIT in the AD mouse model with the *D. farinae*-loaded microneedle patches successfully induced a therapeutic effect with a lower dose (1/10) of allergen compared to the SCIT. That is, transepidermal injection by microneedle is more efficient than subcutaneous injection by activating an antigen presenting cells network and this also results in a dose-saving effect. Also, these results suggest a potential use in humans. Allergen-loaded microneedle patches can be applied to other allergic diseases such as allergic asthma or rhinitis as an ASIT.

V. CONCLUSION

In our study, the *D. farinae*-loaded microneedle patches were developed for ASIT and applied to the *D. farinae* sensitized AD mouse model. *D. farinae* extract in a HA based microneedle has a stable allergenicity and can be maintained at least for 12 wk when stored at 4℃. The *D. farinae*-loaded microneedle patches induced no severe adverse effects and successfully induce allergic responses. In the AD mouse model, ASIT with 10 μg of *D. farinae*-loaded microneedle patches successfully induced Treg responses and serum IgG4.

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ABSTRACT (IN KOREAN)

알레르기 질환의 알러젠 특이 면역치료를 위한 *Dermatophagoides farinae* 탑재 마이크로니들 패치의 개발

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김 지 혜

알러젠 특이 면역치료란 알레르기 비염, 천식, 아토피피부염과 같은 IgE 매개 질환에서 매우 효과적인 치료법으로 감작된 알러젠의 반복적인 투여를 통해 알러젠 특이 peripheral tolerance를 유도하여 알레르기 반응을 완화시키는 치료 방법이다. 그러나, 3년 이상의 장기간 동안 피하주사를 통해 알러젠을 반복적으로 투여해야 하기 때문에 환자의 순응도가 낮은 단점이 있다. 이러한 한계를 극복하기 위해 설하 (sublingual) 면역 치료법이 고안되었지만 피하 주사법에 비하여 효과가 떨어진다는 단점이 있다. 따라서, 더 편리하고 효과적인 알러젠 전달법이 필요하다. 마이크로니들 패치는 수백 마이크로미터 길이의 바늘에 약물을 탑재하여 패치 형태로 만든 것으로 피부에 마이크로 단위의 구멍을 만들어 약물을 전달한다. 기존의 피하주사 방식에 비해 고통이 없고 출혈이나 바늘에 의한 사고 위험이 없으며, 환자 스스로도 사용 가능하다는 장점이 있다.

따라서, 본 연구에서는 아토피피부염을 유발하는 집먼지 진드기인 *Dermatophagoides farinae* (*D. farinae*) 알러젠을 탑재한 마이크로니들 패치를 개발하였고, 이를 아토피피부염 마우스 모델에 적용하여 면역치료 효과를 확인하고자 하였다. 먼저, *D. farinae* 추출물을 탑재한 히알루론 산 기반의 마이크로니들 패치를 제작하여 이의 안정성 및

항원성을 실험을 통해 확인하였다. 암컷 BALB/c 마우스에 *D. farinae* 탑재 마이크로니들 패치를 일주일에 2번씩, 4주간 부착하였고 실험 기간 동안 부작용은 관찰되지 않았다. 또한, 10 μg의 *D. farinae* 탑재 마이크로니들 패치를 부착한 마우스에서 혈청의 total IgE 및 *D. farinae* 특이 IgE 수치가 증가함을 통해 알러젠 전달에 의한 면역 반응을 확인하였다. 마지막으로, NC/Nga 아토피피부염 마우스 모델 실험에서 마이크로니들로 면역치료를 시행한 군에서 IL-4, IL-13과 같은 T_H2 연관 사이토카인의 감소와 Foxp3와 IL-10의 증가를 림프절과 피부 조직에서 관찰하였으며, 기존의 피하 면역치료와 유사한 수준의 면역치료 효과를 확인하였다.

이러한 결과를 통해 마이크로니들 패치를 통한 알러젠 전달이 기존의 방식을 대체할 편리하고 효과적인 알러젠을 전달 방법임을 검증하였고, 향후 여러 알레르기 질환의 알러젠 특이 면역치료에 적용 가능성을 제시하고자 한다.

핵심되는 말 : *Dermatophagoides farinae*, 아토피피부염, 알러젠 특이 면역치료, 마이크로니들, NC/Nga