

Time-dependent progression from the acute to chronic phases in atopic dermatitis induced by epicutaneous allergen stimulation in NC/Nga mice

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Abstract: Atopic dermatitis (AD) is a complicated skin condition influenced by genetic background and environmental factors. In this study, we applied *Dermatophagoides farinae* body extract (DfE) to the barrier-disrupted skin of NC/Nga mice twice a week for 8 weeks to identify the clinical and immunological factors in AD progression. Repeated application of the DfE to the skin of NC/Nga mice showed the similar consequences for the natural course of progression in human AD, histologically and immunologically. We confirmed that the AD-like skin lesions in NC/Nga mice did not last for the whole period of our experiment in spite of repeated topical applications of DfE twice a week. Topical DfE stimulation increased the skin mRNA expressions of Th1-, Th2- and Th17-related cytokines in the acute phase. The expression patterns of IL-4 and IL-13 in splenic T cells and skin lesions were consistent with the time course alterations of clinical

features of AD-like skin symptoms. We also showed that there was a remission phase either just before or right after the chronic phase in this experimental model. Interestingly, splenic T-cell-derived IL-5 expression began to increase in the chronic phase, while skin-derived IL-5 mRNA expression increased in the acute phase. In conclusion, our results suggest that we should pay attention to the characteristics of each stage of AD progression and choose a suitable corresponding stage of animal model not only to elucidate the pathogenesis of AD but also to develop and evaluate therapeutic drugs for AD.

Key words: atopic dermatitis – cytokines – *Dermatophagoides farinae* – NC/Nga mouse

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Introduction

Atopic dermatitis (AD) is not a simple inflammatory skin disease but a chronic, relapsing, multifactorial skin disorder. It is considered to be the first step of the atopic march, which is characterized by the progression of atopic disorders from AD in infants to allergic rhinitis and finally to asthma in children and adults (1). The pathogenesis of AD has been attributed to a complex interaction between the host's susceptibility genes, resulting in disrupted epidermal barrier function and the abnormal immune regulation to allergens as well as microbial antigens (2,3).

In general, to understand the pathogenesis of human AD, mice are commonly used as AD experimental models because of their ease of genetic manipulation (4,5). Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) is the first AD-like murine model that spontaneously develops human AD-like symptoms when it is raised under air-uncontrolled conventional conditions (4). However, NC/Nga mice with spontaneous AD symptoms are not widely available to study human AD. In most countries, exposure to only conventional environments is not enough to induce human AD-like clinical symptoms in NC/Nga mice. Therefore, epicutaneous stimulation with certain environmental allergens may be an important factor to induce human AD-like symptoms in NC/Nga mice.

Sensitization to house dust mites (HDMs) through a disturbed skin barrier is a well-known risk factor for induction of AD. The higher prevalence of HDMs on the skin of AD patients compared with healthy normal controls may affect the exacerbation of AD (6). In particular, *Dermatophagoides* species, such as *Dermato-*

phagoides pteronyssinus and *Dermatophagoides farinae*, are the most prevalent HDMs and the source of the most potent allergens on the skin of AD patients (6–8).

Although various animal models have been used to analyse the pathogenesis of AD and to examine therapeutic drugs for the disease, there are few reports to evaluate each stage of AD-like symptoms that occur in the natural course of human AD.

It has also been controversial how similar AD-like manifestations at each stage of AD animal model are to the various representative symptoms indicating in human AD progression courses from acute to chronic phase. In our experimental setting, we presented the similarities and differences between human and murine AD in the each stage of progression from acute to chronic phase for 8 weeks.

Materials and methods

Animals

Female 3-week-old specific pathogen free (SPF) NC/Nga mice were purchased from SLC Japan (Shizuoka, Japan) and were maintained for 2 weeks before starting the experiment. These mice were all housed under SPF conditions. All experiments with mice were performed in accordance with the regulations and the approval of the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University (Approval No. 10-1012).

Preparation of *Dermatophagoides farinae* extract

Dermatophagoides farinae body extract (DfE) ointment was purchased from Biostir Inc. (Kobe, Japan), and lyophilized *D. farinae* crude extract was purchased from Yon-Sei University (Seoul,

South Korea). The protein concentration of *D. farinae* crude extract was measured using a Bradford assay kit (Bio-Rad Laboratory, Hercules, CA, USA). Bovine serum albumin was used as a standard for protein concentration.

Induction of AD-like skin lesion

At 5 weeks of age, mice were anesthetized and then the hair on their dorsal area was shaved once a week. Barrier disruption was induced with 150 μ l of 4% sodium dodecyl sulphate (SDS) treatment before the application of *DfE* ointment. One hundred milligram *DfE* ointment (Biostir Inc.) was swabbed to both ears and the dorsal skin of each mouse twice a week for 8 weeks.

Evaluation of AD-like skin lesions

Scoring of dermatitis was assessed macroscopically in a blinded fashion using the following scoring procedure. The development of (i) dryness, (ii) erythema, (iii) oedema and (iv) excoriation was scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The total symptom severity score was defined as the sum of the individual scores.

Histological evaluation of the skin

Mice were sacrificed at each week of the 8 week experiment. The skin removed from a lesion site was fixed in 4% neutral phosphate-buffered formalin (pH 7.2) for 24 h at room temperature, embedded in paraffin and serially sectioned at 4 μ m thickness. It was then stained with haematoxylin and eosin (H&E) to assess epidermal hyperplasia and with toluidine blue (TB) to assess for mast cell infiltration.

Measurement of total and *Dermatophagoides farinae*-specific IgE, G1 and G2a in plasma

Three days after the final application of *DfE* ointment, mice were sacrificed and blood was taken to obtain the plasma by centrifuging at 16 000 g for 15 min. Total and *D. farinae*-specific plasma IgE, G1 and G2a was measured using the mouse OptEIA™ set (BD Biosciences, San Diego, CA, USA) and the mouse ELISA Quantification set (Bethyl Lab. Inc., Montgomery, TX, USA). The concentration was estimated using a standard sample from the manufacturer's set.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from the ear skin with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA); reverse transcription was performed with RevertAid™ first-strand cDNA synthesis kit (Fermentas, Hanover, MD, USA), and then, cDNAs were amplified with specific primers for genes using a PCR master mix (Fermentas) according to the manufacturer's instructions. The Bio-Rad CFX96™ (Bio-Rad Laboratory) Real-Time PCR System was used for the relative quantification of the target genes with the SsoFast™ EvaGreen® (Bio-Rad Laboratory) Supermix.

Analysis of Th1, Th2 and Th17 cytokines in splenocytes

To evaluate the production levels of T-cell-derived cytokines, spleens of an individual group were obtained, and the isolated splenocytes were resuspended in RPMI 1640 medium containing 10% fetal bovine serum (Gibco®; Life Technologies, Grand Island, NY, USA). Splenocyte suspensions were seeded 3×10^6 cells per well in 24-well-flat-bottom microplates and then incubated in the absence or presence of concanavalin A (Con A) (Sigma-Aldrich Co., St. Louis, MO, USA) at 2.5 μ g/ml for 48 h. Following the incubation, the culture supernatants were collected to determine the production levels of cytokines, including IL-4, IL-5, IL-13, IL-17A and IFN- γ , using mouse OptEIA™ set (BD Biosciences).

Statistical analysis

Experimental values represent the mean $\pm$ SEM of seven mice per group. Statistical significance was determined by using a paired *t*-test (a parametric test). Values of $P < 0.05$ were considered significant. All analyses were conducted using SPSS version 12.0 KO software package (SPSS, Chicago, IL, USA).

Results

Time course changes of clinical skin features and severities in a human AD-like murine model

In this study, we induced human AD-like symptoms in NC/Nga mice by repeated topical applications of *D. farinae* whole body extracts (*DfE*) to barrier-disrupted skin for 8 weeks. The skin barriers of mice were disrupted by topical treatment of 4% SDS to shaved dorsal skin twice a week. However, the skin barrier disruption that resulted from intermittent applications of 4% SDS was not enough to induce AD-like skin lesions in our experimental animal model.

Figure 1a shows the representative clinical features of AD-like skin symptoms, which were induced by repeated epicutaneous *DfE* stimulations, at each week of our 8-week experiment.

One week after *DfE* stimulation, the skin surface became dry and rough. At week 2, skin dryness in particular led to skin scratching and persisted for the entire period of our experiment. At the third week, increased scratching caused fluids extravasation from tiny blisters, which resulted in the formation of thin dry crusts on the skin surface. Interestingly, at the 4th week, the mice seemed to recover. After that, AD-like skin lesions around both ears and upper dorsal areas of the mice were extremely aggravated with excessive scratching behaviours and persisted until the 6th week. We also weekly scored the four major clinical skin symptoms of AD, including dryness, erythema, oedema and excoriation (Fig. 1b). The total skin severity score, the sum of each individual score for four major clinical symptoms, peaked at the 5th week. Interestingly, clinically observed AD-like skin symptoms in NC/Nga mice seemed to begin to improve between the 7th and the

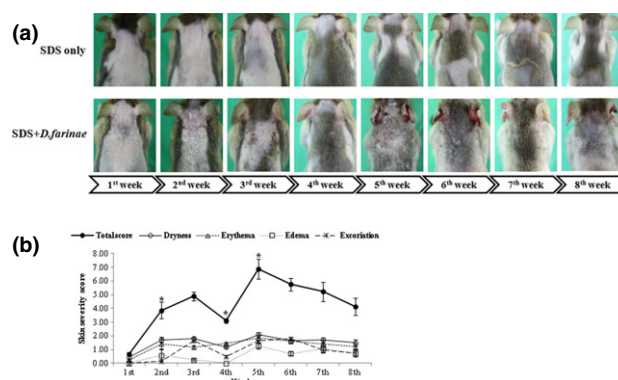


Figure 1. Time course changes of clinical skin features and skin severity scores in a human atopic dermatitis (AD)-like murine model. (a) Representative clinical features of AD-like skin lesions in NC/Nga mice. After shaving, 4% sodium dodecyl sulphate (SDS) solution and/or 100 mg *DfE* ointment was topically applied onto the dorsal skin and both surfaces of each ear twice a week for 8 weeks. (b) Clinical severity score of AD-like skin lesions in NC/Nga mice. Total scoring of dermatitis severity was expressed as the sum of individual scores judged by the symptoms of dryness/scaling, erythema/haemorrhage, oedema, excoriation/erosion. Each data represent the mean $\pm$ SEM of seven mice. *Statistically significant at $P < 0.05$ using a paired *t*-test compared with all other groups. *DfE*, *Dermatophagoides farinae* body extract.

8th week, even though *DfE* was continuously applied to the skin surface with SDS-derived barrier disruption.

Time course changes of skin histology in a human AD-like murine model

Every week, the skin lesions removed from the *DfE*-applied upper dorsal areas were stained with haematoxylin and eosin (H&E) for the analysis of eosinophil infiltration and with TB for the analysis of mast cell degranulation.

Repeated epicutaneous *DfE* stimulations to the barrier-disrupted skin of NC/Nga mice induced the representative histological changes of AD progression from acute to chronic skin lesions, including epidermal spongiosis and hyperplasia (Fig. 2a). In particular, at the 2nd week, H&E-stained upper dorsal skin of mice showed typical histological features of acute AD lesions, such as spongiosis in the spinous layer of the epidermis (asterisk in Fig. 2a) and significant infiltration of eosinophils in the dermis (arrows in Fig. 2a). At the 5th and 6th weeks, the H&E-stained skin sections demonstrated all of the characteristic changes observed in chronic lesions of AD patients, including markedly diffuse epidermal hyperplasia with elongation of rete ridges (arrow head in Fig. 2a) and increased infiltration of inflammatory cells in the dermis.

We also demonstrated the time course alteration of mast cell degranulation in AD-like skin lesions by TB staining (Fig. 2b). Repeated epicutaneous *D. farinae* stimulation resulted in an increase in the total number of mast cells. The increased numbers of degranulated mast cells in the dermis started to increase at the 2nd week and peaked at the 6th week. At the 5th and 6th week, extrusion of basophilic TB-stained granules indicated that most of the mast cells were partially or completely degranulated.

Time course changes of the expression levels of total and *Dermatophagoides farinae*-specific plasma immunoglobulin subclasses in a human AD-like murine model

The expression levels of immunoglobulin (Ig) antibodies have been reported to be closely correlated with clinical severity of dermatitis. We measured the alteration in the expression levels of total and *D. farinae*-specific IgE, G1 and G2a in plasma extracted from each group of mice at every week. As indicated in Fig. 3a, time course changes of total plasma IgE expression levels showed a close correlation with the alteration pattern of clinical skin severity in NC/Nga mice (Fig. 1). Total IgE levels in plasma also

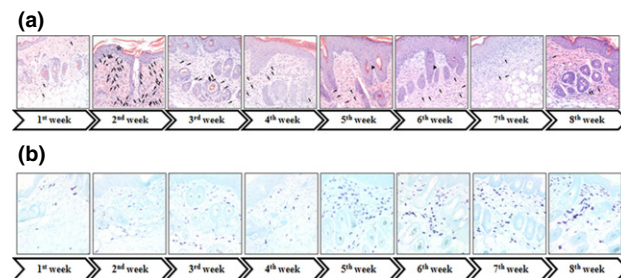


Figure 2. Time course changes of histological aspects in human atopic dermatitis (AD)-like murine model. Paraformaldehyde-fixed and paraffin-embedded upper dorsal skin samples from the mice were sectioned and then stained with haematoxylin and eosin (H&E) and toluidine blue (TB) to count the eosinophils and mast cells, respectively. Representative (a) H&E- and (b) TB-stained skin sections from each group of mice are shown (original magnification, 400 $\times$). The asterisk (*) indicates a spongiotic area within the epidermis. The arrow head (▶) indicates an elongated and expanded rete ridge, and the arrow (→) indicates an eosinophil in the dermis.

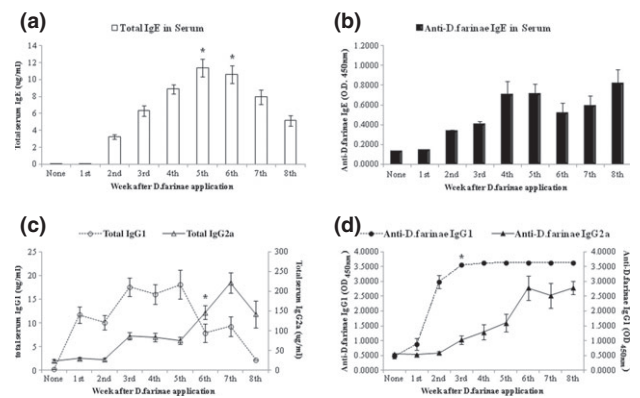


Figure 3. Time course changes of the expression levels of total and *Dermatophagoides farinae*-specific immunoglobulin subclasses in a human atopic dermatitis (AD)-like murine model. Total and *D. farinae*-specific IgE, IgG1 and IgG2a expression levels in plasma were measured by ELISA. Each graph represents the time course changes of expression patterns of (a) total IgE, (b) *D. farinae*-specific IgE, (c) total IgG1 and IgG2a, and (d) *D. farinae*-specific IgG1 and IgG2a in plasma. Each data point represents the mean $\pm$ SEM of seven mice. *Statistically significant at $P < 0.05$ using a paired *t*-test compared with all other groups.

peaked at the 5th week in the same manner as the total skin severity score did in Fig. 1b. Interestingly, repeated epicutaneous *DfE* applications induced a gradual increase in the plasma levels of *D. farinae*-specific IgE expression (Fig. 3b), which was slightly different from the time course changes of total plasma IgE levels.

We also examined the time course changes of total and *D. farinae*-specific IgG1 and IgG2a levels in plasma. While total IgG1 levels were significantly downregulated from the 6th week to the 8th week, total plasma levels of IgG2a were incrementally upregulated until the 7th week and then downregulated at the 8th week (Fig. 3c). At the 6th week, the expression levels of total and *D. farinae*-specific IgG2a in plasma started to increase (Fig. 3c,d), while the total and *D. farinae*-specific IgE showed a slight decrease (Fig. 3a,b). Repeated *DfE* application to barrier-disrupted skin resulted in greater induction of the *D. farinae*-specific IgG1 subclass compared with the *D. farinae*-specific IgG2a in plasma from the 2nd week (Fig. 3d).

Time course changes of splenic T-cell- and lesional-skin-derived cytokine production in a human AD-like murine model

It has been reported that various cytokines derived from T helper (Th) cells, such as Th1, Th2 and Th17, are involved in the development of AD-like skin inflammation. In this study, we analysed the effects of repeated *DfE* stimulations on the changes of cytokine expression patterns in concanavalin A (Con A)-stimulated splenic T cells and lesional skin of NC/Nga mice.

In splenocytes, IL-4 and IL-13 were significantly elevated twice during the whole period of our experiment at the 2nd and the 6th weeks. We also showed that, at the 4th week, the production levels of IL-4 and IL-13 in activated splenic T cells were significantly downregulated (Fig. 4a,c). On the other hand, expression of IL-5 dramatically increased at the 5th and 6th week, but then significantly decreased at the 7th week (Fig. 4b). And IL-17A expression was dominant only at the 2nd week (Fig. 4d).

As shown in Fig. 4f–j, topical *DfE* stimulation increased skin mRNA expressions of Th1-, Th2- and Th17-related cytokines at

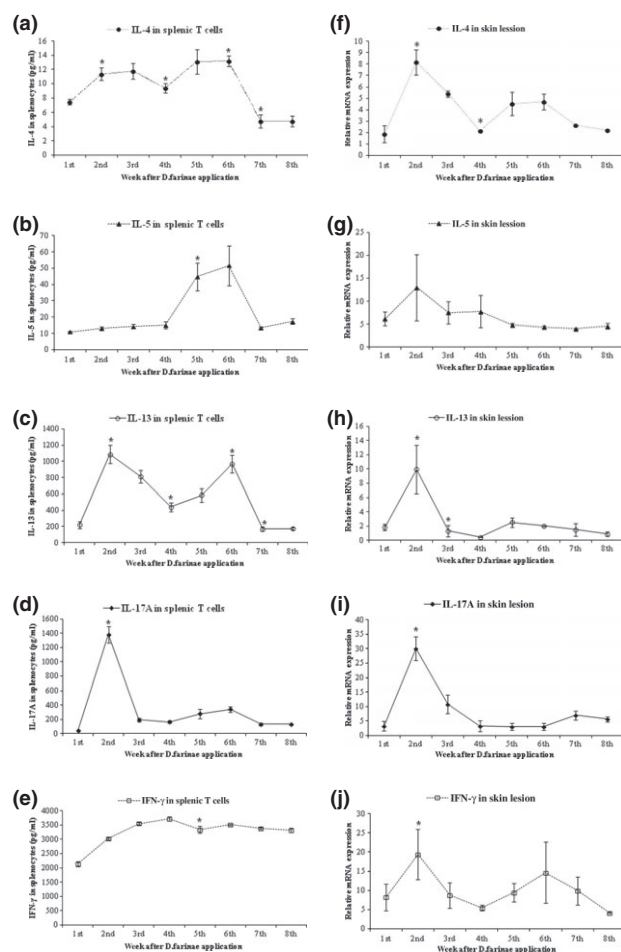


Figure 4. Time course changes of splenic T-cell- and lesional-skin-derived cytokine production in a human atopic dermatitis (AD)-like murine model. Splenic T-cell- and lesional-skin-derived cytokine production was evaluated in the spleen and the dorsal skin of NC/Nga mice by ELISA and real-time quantitative PCR, respectively. Each graph represents the time course changes of splenic T-cell-derived (a) IL-4, (b) IL-5, (c) IL-13, (d) IL-17A and (e) IFN- γ protein production and also demonstrates the time course changes of lesional-skin-derived (f) IL-4, (g) IL-5, (h) IL-13, (i) IL-17A and (j) IFN- γ mRNA expression. Each data point represents the mean $\pm$ SEM of seven mice. *Statistically significant at $P < 0.05$ using a paired t-test compared with all other groups.

the 2nd week. Interestingly, skin-derived IL-5 mRNA expression increased in the acute phase (Fig. 4g), while splenic T-cell-derived IL-5 expression began to increase in the chronic phase (Fig. 4b).

We also found that the production of splenic T-cell-derived IFN- γ , one of representative Th1 cytokines, was increased from the 1st week to the 8th week, although the expression level was slightly decreased at the 5th week (Fig. 4e). However, the time course pattern of skin-derived IFN- γ mRNA expression (Fig. 4j) was consistent with the expression pattern of IL-4 and IL-13 in spleen and skin.

Discussion

Atopic dermatitis has various clinical features like chronic relapsing courses of skin inflammation. It has been reported that AD starts within the first 2 years of life, and a high proportion of the patients go into remission during childhood. In severe cases, AD persists with chronic relapsing symptoms until adulthood (9–11). However,

there are some difficulties in defining the objective indicators of the diagnosis or prognosis for human AD due to the complicated pathogenesis of AD onset or aggravation, which is affected by genetic and environmental factors. Therefore, it is important to establish a suitable AD experimental model and to evaluate the similarities to and differences from human AD. To date, however, there have been few studies analysing the time course changes of the clinical and immunological aspects of the progression or aggravation of AD. Controversy exists regarding how similar AD-like manifestations at each stage of AD animal models are to the various representative symptoms in human AD from the acute to chronic phases.

In this study, we induced human AD-like skin inflammation and AD-related immune responses in genetic atopic prone mice by repeated epicutaneous applications of an environmental allergen to barrier-disrupted skin. We also evaluated the time course changes of the clinical and immunological factors, which are characteristic of AD.

In AD patients, skin dryness is known to appear at all stages of the disease. Clinically unaffected AD skin, which is not normal, has increased dryness and has a greater irritant skin response compared with normal healthy skin. Dryness leads to itching, scaling, redness and tiny blisters in the skin (2,3). Our AD-like animal model also demonstrated the same features seen in the natural course of AD progression in humans. In contrast with the results from previous studies, however, neither skin barrier disruption nor skin dryness induced by topical detergent application was enough to induce and aggravate AD-like symptoms in this study. Interestingly, we also found an interphase between the acute and chronic phases of AD-like symptoms in our animal model. At the interphase, clinical skin features of mice seemed to be recovered, even though the stained skin sections showed typical histological features of AD, including epidermal thickening and increased infiltration of inflammatory cells. Furthermore, this interphase was repeated at the end of the chronic phase.

Most of the studies on the mechanisms of AD have been focused on the imbalance in Th1 and Th2 in immune regulation. Increased scratching behaviours in pruritic skin lesions may exacerbate symptoms by damaging the skin barrier and induce switching from the Th2-predominant acute phase to the Th1-dominant chronic phase of AD (12). Compared with normal healthy skin, acute lesions of AD showed increased infiltration of Th2 cells expressing IL-4 and IL-13 mRNA, but not IFN- γ . Chronic AD skin lesions have significantly higher IL-5 and IFN- γ expression, as compared with acute AD lesions (2,13), whereas IL-13 still remains at high levels (13–15). It was also recently reported that IL-17A may be implicated in the pathogenesis of AD. Increased IL-17A expression was found only in the acute skin lesions of patients with AD, but not in chronic (16).

Activated splenic T cells extracted from the acute stage of mice also showed predominant Th2- and Th17-derived cytokine expression, such as IL-4, -13 and -17A. Activated splenic T-cell-derived IL-13 expression was remarkably increased in both acute and chronic stages of the AD-like animal model, similar to the skin lesions of human AD. However, activated splenic T cells maintained a high level of IFN- γ expression during most of our experiment, which was different from the changes in human AD progression.

The skin mRNA expression pattern of Th1-, Th2-, and Th17-related cytokines in our AD-like animal model showed a slight

difference in comparison with the pattern of human AD skin lesions. In the acute stage, the AD-like skin lesions showed significantly increased mRNA expressions of Th2 cytokines such as IL-4, IL-5 and IL-13. Interestingly, a representative Th1 cytokine, IFN- γ , significantly increased in AD-like skin lesion of the acute stage as well as the chronic stage. We showed that splenic T-cell-derived IL-5 production dramatically increased in the chronic stage. On the other hand, the skin mRNA expression of IL-5 significantly increased in the acute stage. IL-5 has been also reported as the major factors of maturation and differentiation for eosinophils in mice and human (17). We showed that the increased skin mRNA expression of IL-5 in the acute stage was consistent with the significantly high infiltration of eosinophils in the dermal skin from the acute stage of mice. We also identified that both splenic T-cell-derived protein production and skin-derived mRNA expression of IL-17A were significantly enhanced in the acute phase of our AD-like animal model. We thought that epicutaneous immunization of NC/Nga mice with the *D. farinae* allergen may drive local or systemic Th17 responses associated with significantly enhanced skin inflammation.

It has been also reported that T-cell activation patterns are closely associated with IgE production and that Th2-IgE-dominated immune responses result in aggravation of skin barrier dysfunction (18). In the acute phase of AD, the majority of allergen-specific T cells, which are derived from skin lesions, show a Th2-cytokine-dominated pattern. Activated Th2 cell-derived cytokines, such as IL-4 and IL-13, play important roles in leading to immunoglobulin (Ig) isotypes switching to IgE synthesis by B cells. In particular, IL-13 was reported as the major cytokine in the activation of B cells and IgE production in AD (13,19). Our results also showed that repeated epicutaneous *D. farinae* allergen stimulation first induced the production of Th2 type antibodies, such as IgE and IgG1, and then was followed by upregulation of the IgG2a subclass as a Th1-type antibody.

Previously, we have reported the effect of repeated applications of *D. farinae* on the aggravation of AD-like symptoms in NC/Nga mice (20). In this study, we demonstrated that time course changes of AD-like clinical and histological manifestations, which were induced in NC/Nga mice by repeated *D. farinae* stimulation to barrier-disrupted skin for 8 weeks, showed considerable similarities to the changes observed in the progression from the acute to chronic stages of patients with AD. Repeated topical allergen stimulation to the barrier-disrupted skin of AD-prone mice was able to convert acute skin lesions to chronic lesions through the different expression patterns of cytokines at each phase of AD

progression. Our experimental setting in this study offers the advantage of allowing evaluation of the time course changes of clinical and immunological aspects in AD-like murine model, NC/Nga mice, affected by topically applied *D. farinae* allergen. To date, there have been few studies analysing the time course changes of clinical and immunological aspects according to the progression or aggravation of AD. It has also been controversial how similar AD-like manifestations at each stage of AD animal model are to the various representative symptoms indicating in human AD progression courses from acute to chronic phase. In our experimental setting, we presented the similarities and differences between human and murine AD in the each stage of progression from acute to chronic phase for 8 weeks.

We also hypothesized that there may be a clinical remission phase either just before or right after the chronic phase in the animal model. In both interphases, clinical AD-like skin symptoms seemed to be relieved, and Th2-type cytokines (IL-4 and IL-13) were downregulated in the same manner, correlating to clinical symptoms. However, the remission phase did not significantly affect the levels of increasing total and *D. farinae*-specific IgE expression in plasma, an indicator with close correlation with clinical severity of AD.

According to our results, each stage of AD progression depends on the schedule of repeated allergen exposure in the genetic atopic prone murine model. Therefore, to evaluate the effects of newly developed anti-AD drugs in animal models, it may be very important to choose the optimal stage of AD progression.

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Author contributions

Ji-Yun Kim performed the research, designed the research study, analysed the data and wrote the paper; Mi Sook Jeong performed the research and analysed the data; Mi Kyung Park and Mi-Kyung Lee analysed the data; Seong Jun Seo designed the research study analysed the data and wrote the paper.

Ethics approval for animal care

All experiments with mice were performed in accordance with the regulations and the approval of the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University (Approval No. 10-1012).

Conflict of interests

All authors have no potential conflicts of interests. There are no conflicts of interest to declare.

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