의학박사 학위논문

A Study on Group 3 Innate Lymphoid Cells in Atopic Dermatitis-like Skin Inflammation

아토피 피부염 유사 피부염증에서 그룹 3 선천성 림프구 세포에 관한 연구

2022년 8월

서울대학교 대학원

의과학과 의과학전공

김 민 호

A thesis of the Degree of Doctor of Philosophy

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ABSTRACT

A Study on Group 3 Innate Lymphoid Cells in Atopic Dermatitis-like Skin Inflammation

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Atopic dermatitis (AD) is a chronic allergic disease associated with itching and eczema. Patients with AD have a poor quality of life due to itching and a high treatment burden. Recent advances in the treatment for AD have focused on regulating the immune response. Previous studies have reported that a number of patients with AD present a type 2 immune response related to IL-4 and IL-13. Treatments that regulate these cytokines are currently being investigated. However, it is necessary to identify other mechanism associated with AD because the proportion of patients with improvements over 90% is low. Unlike T cells, innate lymphoid cells (ILCs) are emerging as important cells in innate immunity because

they can immediately produce effector cytokines corresponding to T cells by alarmin cytokines that stimulate ILCs. Studies have shown that group 2 innate lymphoid cells (ILC2s) are activated in the skin of patients with AD; however, in-depth research is required, and it is unknown whether ILCs other than ILC2s are involved in AD. This study aimed to identify previously unknown mechanisms of AD immunopathogenesis and the role of group 3 innate lymphoid cells (ILC3s). To this end, mouse models for AD and samples from healthy subjects and AD patients were used. After applying house dust mites to the dorsal skin of mice, flow cytometry was performed to analyze the distribution of ILCs. Both ILC2s and ILC3s were found to be increased in the skin. To investigate the effect of ILC3s, sorted ILC3s were injected into the dorsal skin, where they accelerated the development of AD in response to house dust mites. Conversely, administration of an IL-17A neutralizing antibody delayed the induction of AD. Furthermore, expression of IL-33 was decreased in the skin of AD mice injected with IL-17A antibody. The expression of IL-33 was increased in human keratinocytes and fibroblasts expressing IL-17A receptors during coculture with human ILC3s. Finally, the levels of ILC3s and ILC2s were increased in peripheral blood mononuclear cells from patients with AD, and approximately 40% of ILCs expressed cutaneous lymphocyte-associated antigens. A positive correlation between ILC3s and neutrophils was also observed in the blood of patients with AD. Taken together, the results of this study suggest that ILC3s are involved in type 2 immune responses in AD by increasing the expression of IL-33, and reveal a role for IL-17A, which has not been previously reported. Thus, inhibiting IL-17A may provide a complementary treatment for patients with AD that remain uncontrolled on immunotherapy.

Keywords: Innate lymphoid cell, Atopic dermatitis, IL-17A, IL-33, keratinocyte, fibroblast

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ABBREVIATIONS

AD: atopic dermatitis

CLA: cutaneous lymphocyte-associated antigen

EASI: eczema area and severity index

FLG: filaggrin

HDM: house dust mites

ILCs: innate lymphoid cells

ILC1s: group 1 innate lymphoid cells

ILC2s: group 2 innate lymphoid cells

ILC3s: group 3 innate lymphoid cells

NCR: natural cytotoxicity receptors

Oxa: oxazolone

PBMCs: peripheral blood mononuclear cells

SdLN: skin-draining lymph node

SDS: Sodium dodecyl sulfate

INTRODUCTION

1. Background

1.1 Features of atopic dermatitis

Atopic dermatitis (AD) is one of the most widespread inflammatory skin diseases, affecting both males and females [1, 2]. AD commonly starts in childhood can also occur in adulthood [3, 4]. The prevalence of AD has increased over the last few decades, and varies among countries and races [2]. For example, the prevalence of AD is lower among White children than among Black children [5, 6].

AD is characterized by intense itching (pruritus) and eczematous lesions, and can be acute, intermediate, or chronic. The clinical characteristics of AD vary depending on age, race, and country [7]. In children, eczema is widespread across the body, and affects the skin underneath the diaper. From the age of 1 year, lesions tend to be more confined to the flexural folds. Adults more often present with chronic eczema on their hands and feet compared with children. Pruritus is the term used to describe the intensity of itching with different severities of AD. Pruritus is exacerbated by humidity, stress, and heat [8]. Scratching induces excoriations. If AD persists into adulthood, erythema, redness, and eczema tend to appear on the face. In addition to chronic eczema, acute lesions also occur repeatedly over a period of time [9].

AD is often the initial step and a risk factor for the ‘atopic march’, which can result in the development of asthma, food allergy, and allergic rhinitis [10, 11, 12]. Approximately 70% of patients with severe AD develop asthma; on the other hand,

only approximately 20% of patients with mild AD and 8% of general population develop asthma. The severity of AD is correlated with the probability of developing allergy rhinitis and with elevated levels of immunoglobulin E antibodies. In a cohort study, 15% of children with AD were also reported to develop food allergy. The mechanisms underlying the atopic march remain largely unknown; however, potential mechanisms include a primary defect in the epithelial barrier and lack of dermal integrity [10, 11, 12].

One question is what mechanisms underlie the symptoms of AD. In this study, I will investigate the causes of AD and identify mechanisms to regulate these causes.

1.2 Pathogenesis of atopic dermatitis

The cause of AD is not fully understood, although evidence indicates that genetic and environmental factors, skin barrier dysfunction, and immune dysregulation are involved [13, 14, 15, 16].

Approximately 30–50% of white patients carry mutations in the gene encoding filaggrin (FLG), which promote skin barrier dysfunction and increase the risk for the early onset of AD [15, 17]. FLG promotes the production of moisturizing factors, and mutations in this protein increase transepidermal water loss, resulting in dry skin. Disruption of the skin barrier enables the penetration of allergens and subsequent sensitization. These skin barrier abnormalities lead to transepidermal water loss. The skin of patients with AD is also deficient in ceramides and antimicrobial peptides [18]. *Staphylococcus aureus* is involved in the development of AD, and colonizes the skin in about 90% of patients [18].

Environmental factors include sensitization to foods, allergens, and air pollution exposure, in addition to the hygiene hypothesis. Eggs, milk, soy, peanut, and wheat are responsible for 90% of allergic reactions in children [19]. House dust mites (HDM) are also an important cause of AD. House dust mites produce a wide variety of allergens (cysteine protease) based on recognition by IgE [20]. Airborne proteins produced by HDM have the ability to penetrate in the skin and increase the severity of AD [21].

AD lesions are characterized by the infiltration of Th2 cells and increased expression of type 2 cytokines, including IL-4 and IL-13 [22, 23, 24]. In addition,

alarmins, including IL-33, TSLP and IL-1β, are released from keratinocytes and induce the activation of innate immune cells, such as eosinophils and mast cells, which secrete type 2 cytokines in AD lesions **(Figure 1)** [25, 26, 27, 28, 29, 30]. IL-4 induces the differentiation of naïve T cells into Th2 cells. Both IL-4 and IL-13 are crucial for the long-term maintenance of Th2 cells and the synthesis of Th2 cytokines. Th2 cells produce inflammatory cytokines, including IL-4, IL-5, and IL-13, which are crucial for recruiting eosinophils and mast cells. Basophils and mast cells are activated by IL-4 and IL-13 to release histamine, serotonin, and mediators. Both IL-4 and IL-13 are involved in isotype switching from IgM to IgE synthesis, which is a feature of allergic AD. Recently, these cytokines were found to promote neurogenic itch by activating pruritogenic sensory neurons [31]. IL-4 and IL-13 may contribute to barrier impairment in AD and downregulate FLG and tight junctions in the skin barrier [32]. Hyperplasia and attenuated differentiation are caused by the action of IL-4 and IL-13 on keratinocytes. Decreased antimicrobial peptide production has been demonstrated in response to IL-4 and IL-13, which makes the skin more prone to infiltration from foreign infectious agents [33]. IL-13 affects dermal fibroblasts, increasing the production of collagen leading to fibrosis and remodeling through the downregulation of MMP-13. This leads to a thickening of the dermis, which is a feature of AD. Therefore, IL-4 and IL-13 play critical roles in the Th2 immune response in AD, contributing to the activated immune response and skin barrier dysfunction.

One question is how effective the suppression of IL-4 and IL-13 is in relation to type 2 immune responses in human. Recent clinical trials focused on blocking these cytokines have shown that a high proportion of patients has improvements in

symptoms exceeding 50%; however, less than half of patients presented improvements of more than 90% [34]. A further question is whether another mechanism is associated with AD.

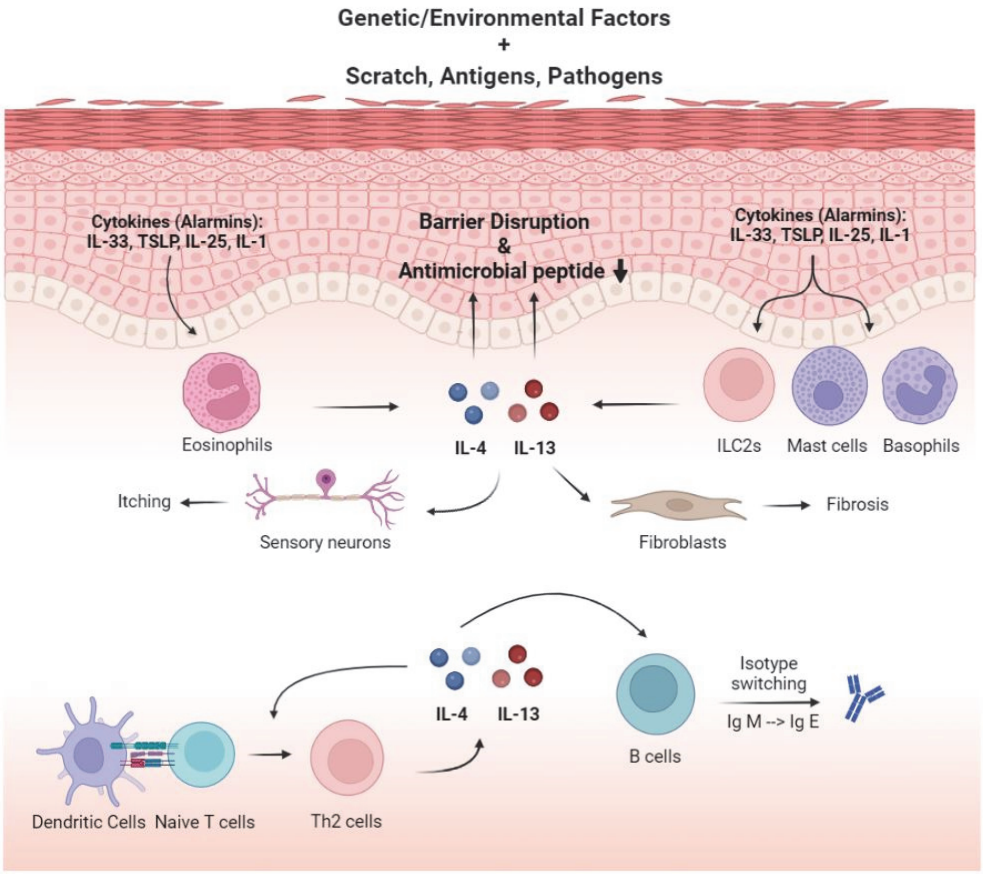

Figure 1. Immunopathogenesis of atopic dermatitis

Infiltration of Th2 cells and increased expression of type 2 cytokines, including IL-4 and IL-13, are observed in AD. In addition, alarmins, including IL-33, TSLP, and IL-1β, are released from keratinocytes and activate innate immune cells such as eosinophils and mast cells, which secrete type 2 cytokines in AD lesions.

1.3 Heterogeneity of atopic dermatitis.

AD is characterized by a heterogenous and complex pathophysiology. Stratification of AD is based on age, disease severity (acute versus chronic AD), age at onset, serum immunoglobulin E levels (intrinsic versus extrinsic AD), and the ethnicity origin of patients [35]. Regarding immunology, the heterogeneity of AD is associated with the region and ethnicity of the patients. Although Th2 skewing is common in AD subtype, recent studies have reported systemic immune involvement, such as increased Th17 skewing, in several AD subtypes. Substantial differences in cytokine profiles have been reported between white and Asian patients [36]. Patients from Japan and Korea have been reported to possess a Th2 profile and present high Th17 expression [37]. Patients with intrinsic AD present similar Th2, but higher Th17 expression compared to patients with extrinsic AD [38]. AD lesions of pediatric patients with AD contain higher levels of Th17-related cytokines compared with those of adult patients with AD [39, 40]. The results of those studies indicated that patients with AD present a type 2 immune response; however, intrinsic, Asian, pediatric patients with AD presented different cytokine profile associated with Th2- and Th17-related cytokines. These findings highlight a different understanding of AD and treatment approach for intrinsic AD, in Asian, infants and children. Therefore, Th17-related cytokines as well as type 2 immune response may be involved in the pathogenesis of AD.

The role of cytokines associated with Th17 cells, and whether these cytokines are related to the pathogenesis of AD remain unclear. Although non-hematopoietic cells are predominant, ILCs also reside in the skin. ILCs will be investigated since

these cells are close to T cells in AD lesions.

1.4 Innate lymphoid cells and atopic dermatitis

Innate lymphoid cells (ILCs) are a recently identified type of innate immune cells. ILCs are known as counterparts of T cells in innate immunity. ILCs possess the functional characteristics of T cells, lack antigen-specific receptors, and do not undergo clonal selection and expansion. For this reason, ILCs can be promptly activated prior to the induction of T cells, and participate in immune responses. ILCs comprise three groups of cells that possess lymphoid morphology, lack the expression of lineage markers, express CD90 (mouse) and CD127 (IL-7Rα, mouse and human), and produce cytokines such as T helper cells [41, 42]. ILCs consist of three subsets based on the expression of surface markers, key transcription factors, and cytokines. ILCs are stimulated and regulated by various factors, including cytokines, alarmins, hormones, and cell-cell signaling.

Group 1 ILCs (ILC1s) are activated in response to stimulating cytokines, such as IL-12, IL-15, and IL-18 [43]. ILC1s express T-bet and produce IFN-γ [44]. ILC1s respond to tumor cells, viruses, and intracellular microbes, including bacteria and parasites. ILC1s are less known than other subsets, and it is difficult to clearly distinguish them from NK cells.

Group 2 ILCs (ILC2s) respond to epithelium-derived cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). ILC2s are characterized by the expression of ST2 (mouse) and CRTH2 (human). They express GATA3 and produce type 2 cytokines, such as IL-4, IL-5, IL-13, and amphiregulin [45, 46, 47]. ILC2s respond following exposure to *Nippostrongylus brasiliensis* and produce IL-13 [48]. Among the three ILC subsets, ILC2s have been the most actively researched.

ILC2 levels are higher in patients with asthma compared to healthy volunteer [49, 50]. Furthermore, ILC2 levels are increased in nasal polyps of patients with chronic rhinosinusitis [51]. The percentage of blood ILC2s is increased in patients with allergic rhinitis when these cells are sensitized to allergens [52]. Amphiregulin promotes airway epithelial integrity and lung tissue homeostasis and is secreted by ILC2s [53].

Group 3 ILCs (ILC3s) are activated in response to extracellular microbes, such as commensal microbiota and fungi, and produce IL-17, IL-22 and GM-CSF following activation by IL-1β and IL-23A [54, 55, 56, 57]. ILC3s constitutively express RORγt and CD117. Patients with psoriasis have been reported to possess high levels of $NKp44^{+}$ ILC3s in PBMCs and AD lesions [58, 59]. The numbers of IL-17–producing ILCs are increased in the ileum and colon of patients with Crohn's disease [60]. Obesity-induced airway inflammation is mediated by NLRP3-dependent production of IL-1β and induced IL-17A production by ILC3s [61].

Type 2cytokines are overexpressed in patients with AD. Activation of ILC2 is the central part of type 2 immune responses. Skin keratinocytes are the first line of defense against allergens. ILC2s have been identified in the dermis [62]. Following exposure to allergens, keratinocytes release alarmins, IL-25, IL-33, and TSLP. These cytokines have been reported to be increased in the skin of patients with AD. ILC2s activated by alarmins produce type 2 cytokines [62, 63]. Basophils and ILC2s colocalize lesions in the skin of patients with AD. Depletion of basophils and blockade of IL-4 reduce the levels of ILC2s and skin inflammation [64].

A role for ILC1s or ILC3s in the pathogenesis of AD has not been explored. If

ILC1s or ILC3s are revealed to play a role in the immunopathogenesis of AD, this may represent a novel treatment approach to relieve the symptoms of AD.

Group	Group 1 ILCs	Group 2 ILCs	Group 3 ILCs
Stimulating cytokines	IL-12 IL-15 IL-18	IL-25 IL-33 TSLP	IL-1β IL-23
ILCs			
Transcription factors	T-bet	GATA3	RORγt
Cytokine production	IFN-γ	IL-4 IL-5 IL-13 Amphiregulin	IL-17A/F IL-22 GM-CSF
Related disease	Tumor Virus Intracellular microbes	Atopic dermatitis Asthma Allergic rhinitis Chronic rhinosinusitis with nasal polyps	Psoriasis IBD Obesity-associated airway inflammation

Figure 2. Classification of innate lymphoid cells

ILCs comprise three subsets based on the expression of surface markers, key transcription factors, and cytokines.

2. Purpose of the study

AD is a chronic allergic disease associated with itching and eczematous lesions. Patients experience damage to their skin due to the effects of itching, and their quality of life is poor as a result of treatment costs and depression [65]. AD is the initial step and a risk factor for the 'atopic march', which results in development of asthma, food allergies, and allergic rhinitis in a majority of patients [10, 11, 12]. For these reasons, patients require non-steroidal treatment for AD, since steroids are associated with side effects.

Protection of the skin barrier and regulation of the immune response are major factors underlying the causes of AD. Protecting the skin barrier is important for the prevention of AD; however, the skin barrier in patients with AD is damaged. Treatments that regulate the immune response are effective for patients with AD. Many studies have shown that a number of patients with AD present a type 2 immune response associated with IL-4 and IL-13 [16, 66]. The treatment to blocking these cytokines for patients with AD is ongoing. Since only a small proportion of patients experience symptomatic improvements of more than 90%, research on currently the unknown mechanism of AD is needed.

ILC2s have been characterized, starting with a report that non-T, non-B cells produce IL-13 protein via IL-25 [67, 68]. ILCs are emerging as important cells in innate immunity, because they immediately produce effector cytokines parallel to T cells. Previous studies have demonstrated that ILCs are present in the skin and that ILC2s are activated in patients with AD [62, 63, 69]; however, in-depth studies are needed. In addition, it is not known whether ILCs other than ILC2s are associated

with the immunopathogenesis of AD, not related to IL-4 and IL-13.

The objectives of this study were to identify unknown mechanisms and the role of ILCs, other than ILC2s, in the pathogenesis of AD, through the use of mouse models of AD and samples from healthy subjects and patients with AD.

MATERIALS AND METHODS

Mice

Female NC/Nga mice, aged 4 weeks, from Japan SLC, Inc. were purchased and housed under specific-pathogen-free conditions. C57BL/6 and Rag1$^{-/-}$ mice were purchased from KOATECH (Pyeongtaek, Republic of Korea) and Jackson Laboratory (Bar harbor, USA), respectively. All experiments described in this manuscript were approved by the Institutional Animal Care and Use Committee in Seoul National University Hospital (SNUH-IACUC, #14-0124-C2A0(1)). Animals were maintained in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) international-accredited facility (#001169) and were cared for according to the 8th Edition (2010) of the Guide for the Care and Use of Laboratory Animals of the National Resource Council (NRC).

Atopic dermatitis model

House dust mite ointment (D. farina, HDM) was purchased from Biostir Inc. NC/Nga mice were anesthetized, and 200 μl of 4% sodium dodecyl sulfate (SDS) was applied to the shaved dorsal skin and ear using a cotton swab. (Japan). HDM (130mg) was applied to the same area in 3h. This process was conducted twice a week for 2 weeks. Skin lesions were assessed as a symptom score comprised of (i) erythema/hemorrhage, (ii) scarring and dryness, (iii) edema, and (iv) excoriation and erosion; each symptom was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe) by an experienced dermatologist. The total score was taken as the symptom scores [70].

For MC903-induced skin inflammation, 100 μl of MC903 (45 μM , Santa Cruz Biotechnology, Dallas, TX) or ethanol was applied to the shaved dorsal skin of C57BL/6 and $Rag1^{-/-}$ mice. (See Figure 13 for treatment regimen.)

For oxazolone-induced skin inflammation, C57BL/6 and $Rag1^{-/-}$ mice were sensitized by topical application of 3% oxazolone (Sigma, Burlington, MA) on the shaved abdomen (30 μl). After 5 days, mice were treated topically with 100 of 0.6% oxazolone on the shaved dorsal skin every other day for 10 days. (See Figure 16 for treatment regimen.)

Murine cell isolation for flow cytometry analysis

For isolating skin cells, dorsal skin was obtained and chopped using blade and scissors after removal of subcutaneous fat. Dissected skin samples were incubated with collagenase IV (1 mg/ml, Worthington, Lakewood, USA) and DNase I (0.1 mg/ml, Sigma, St Louis, USA) at 37℃ for 90 min with vigorous shaking. Skin samples were filtered through a 40 μm strainer and red blood cells were lysed with RBC lysis buffer (Sigma). Skin draining lymph nodes was obtained and ground in 40 μm strainer for single cell isolation.

Flow cytometry analysis

For Live/Dead staining, Live/Dead dye (BioLegend, Califonia, USA) was used. To block non-specific binding isolated cells were incubated with CD16/32 antibody (BD Biosciences) for 15min. For surface staining, the cells were labeled by he following antibodies: FITC anti-FcεRIα, anti-F4/80, anti-Ly6C, anti-TCRγδ; PE anti-CD11b, anti- CD3ε; APC anti- CD117, anti-F4/80, anti- TCRγδ; PE- Cy7 anti-CD127, anti-CD90.2, anti-Ly6G; BV-421 anti-CD25; BV-650 anti-CD127,

streptavidin; BV-785 anti-CD25; purified anti-CLA; biotin anti-rat light chain κ (all purchased from BioLegend). From BD Biosciences: FITC anti-CD11b, anti-CD11c, anti- CD19, anti-CD3ε, anti-CD49b; BV-421 anti-Siglec-F. From eBioscience (ThermoFisher Scientific, Massachusetts, USA): PerCP- Cy5.5 anti-CD45; PE-streptavidin; biotin anti-ST2. For intracellular cytokine staining, isolated cells were stimulated for 4 hours at 37 ℃ with phorbol 12-myristate 13-acetate (50 ng/ml, Miltenyi Biotec, Bergisch Gladbach, Germany), ionomycin (0.5 μg/ml, Sigma) and Golgi stop (0.7 μl/ml, BD Biosciences, New Jersey, USA). FITC anti-IL-17A; APC anti-IFN-γ, anti-Pro-IL-1β, anti-IL-22; BV-421 anti-IL-17A, (all purchased from BioLegend); FITC anti-IL-4 (BD Biosciences); PE anti-IL-13; PE-Cy7 anti-IL-13 (eBioscience).

To stain human ILCs, PBMCs were isolated from healthy volunteers and AD patients by centrifugation using a Ficoll-Paque PLUS density gradient (GE Healthcare, State of llinois, USA). Single cells from blood were blocked with human BD Fc Block™ (BD Biosciences, NJ, USA). Antibodies used for flow cytometric analysis were as follows: PerCP-Cy5.5 anti-CD45; FITC anti-CD3ε, anti-CD19, anti-CD11b, anti-CD11c, anti-CD14, anti-CD49b, anti-FcεRIα; PE-Cy7 anti-CD127; BV-421 anti-CD117; PE anti-CRTH2 (BD Biosciences); Purified anti-CLA; Biotin anti-rat Ig light chain κ; BV-650 streptavidin. The rest of antibodies were purchased from Biolegend.

The samples were acquired using LSRII and LSR Fortessa (BD Biosciences). Data were analyzed with FlowJo 10 software.

Adoptive transfer of ILC3s

Cells were isolated from skin-draining lymph node and spleen of HDM-induced AD mice. ILC3s were sorted by Aria III (BD Biosciences) after depletion of lineage positive cells using micro beads (Lineage depletion kit, Miltenyi Biotec). 5,000-7,000 cells were intradermally injected into 2 x 2 cm-area of the dorsal skin 3 hours after shaving or 4% SDS treatment.

Neutralization of IL-17A

Mice were intraperitoneally injected with anti-mouse IL-17A (100, clone TC11-18H10.1, Biolegend) or isotype IgG1 (clone RTK2071, Biolegend) on the day in the schedule before application of HDM ointment or oxazolone.

Histological examination

The samples were fixed with 4% paraformaldehyde at 4℃ overnight before processing into paraffin wax. Serial sections (4 μm) were mounted onto silane-coated slides (Dako, Japan) and ither subjected to hematoxylin and eosin (H&E) staining for morphological analysis, or to toluidine blue staining for mast cell examination. Images were analyzed using Image J software.

Quantitative real-time PCR

Total RNA was extracted from mice skin samples or cultured primary human skin cells (keratinocytes and fibroblasts) using RNAiso Plus (Takara Bio Inc. Japan). cDNA was synthesized using a RevertAid First Strand cDNA synthesis Kit (Thermo Scientific). Quantitative RT-PCR was performed on an ABI 7500

(Applied Biosystems, California, USA) The data were normalized to RPLP0, and relative expressions were depicted using ΔCt or ΔΔCt methods.

Table 1. Primers used in real-time PCR

Genes	Forward (5'-3')	Reverse (5'-3')
Il17a	GCAATGAAGACCCTGATAGATATCC	TTCATGTGGTGGTCCAGCTTT
IL13	TGAGGAGCTGAGCAACATCACACA	TGCGGTTACAGAGGCCATGCAATA
Tslp	CGGATGGGGCTAACTTACA	TCCTCGATTTGCTCGAACTT
Il33	GATGGGAAGAAGCTGATGGTG	TTGTGAAGGACGAAGAAGGC
Il23a	CCAGCAGCTCTCTCGGAATC	TCATATGTCCCGCTGGTGC
Il1b	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
Cxcl1	GCTGGGATTCACCTCAAGAA	TCTCCGTTACTTGGGGACAC
Rplp0	TGCCACACTCCATCATCAAT	CGAAGAGACCGAATCCCATA
TSLP	GCTATCTGGTGCCCAGGCTAT	CGACGCCACAATCCTTGTAAT
IL33	TCAGGTGACGGTGTTGATGG	ACAAAGAAGGCCTGGTCTGG
CXCL1	CCCCAGCAATCCCCGGCTC	TGGGGTCCGGGGGACTTCAC
RPLP0	TGGGCTCCAAGCAGATGC	GGCTTCGCTGGCTCCCAC

Cell culture

Primary human dermal fibroblasts and epidermal keratinocytes were obtained from foreskin and cultured in DMEM (Welgene, Korea) with 10% FBS, and keratinocytes growth medium with supplements (Clonetics, Sweden) respectively. All human samples (foreskin and blood) were obtained under written informed consent, in accordance with the approvals by the Institutional Review Board of Seoul National University Hospital (#1503-018-654).

To investigate the effect of IL-17A on the cells, cells were starved without supplements overnight and then treated with recombinant human IL-17A (100 ng/ml, R&D systems, Minnesota, USA) for 12 hours.

Human ILCs sorting

Human PBMCs were isolated from peripheral blood of healthy volunteers by centrifugation on a Ficoll-Paque PLUS density gradient (GE Healthcare). After red blood cells were lysed with RBC lysis buffer (sigma), PBMCs were stained by CD16/32 antibody (BD Biosciences) for blockade of nonspecific binding and then FITC anti-CD3ε, anti-CD19, anti-CD11b, anti-CD235α (Biolegend) for 30min. Anti-FITC microbeads were used to deplete $FITC^+$ cells by LS MACS column (Miltenty Biotec). To enhance the purity of sorted ILCs, the $FITC^-$ cells were stained by PerCP-Cy5.5 anti-CD45; FITC anti-CD3ε, anti-CD19, anti-CD11b, anti-CD11c, anti-CD14, anti-CD49b, anti-FcεRIα; PE-Cy7 anti-CD127; BV-421 anti-CD117 (Biolegend); PE anti-CRTH2 (BD Biosciences) for 20min. ILC3s ($CD45^+$ Lin^- $CD127^+$ $CRTH2^-$ $CD117^+$) and other ILCs (not ILC3s) were sorted by AriaIII (BD Biosciences). Sorted cells were washed by the keratinocyte basal medium and

HBSS 1:1 mixed medium.

Coculture of keratinocytes and fibroblasts with ILCs

Primary human dermal fibroblasts were seeded at a density of 150,000 cells in a 12-mm diameter polycarbonate membrane with a pore size of 3 μm (Milicell, Merck Millipore Ltd., Massacusetts, USA) in DMEM medium and incubated for 2 days in 24-well plate. The next day, after washing with PBS for twice, 1.5 x 10^5 of primary human epidermal keratinocytes were seeded onto the same culture inserts in KGM medium. The medium was changed into the keratinocyte basal medium without supplements (KBM, Lonza) and then incubated overnight. 10^5 of isolated ILC3s or ILC others (ILCs other than ILC3s) were cocultured in the lower compartment of the insert with the established fibroblasts and keratinocytes in the KBM and HBSS (Hank's Balanced Salt Solution) 1:1 mixed medium for 48h. The medium was provided only to the lower compartment, being supplemented with human recombinant IL-1β (40 ng/ml, R&D systems) and IL-7 (5 ng/ml, Miltenyi Biotec). Polycarbonate membranes removed and washed three times with PBS. The membranes were harvested for further analysis.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (GraphPad software, California, USA). Comparisons between two groups were performed using Mann-Whitney U test. Comparisons of multiple groups were performed using one-way ANOVA with Kruskal-Wallis with Dunn's post hoc test. Sperman rank correlation was used for the relationship between two variables. Data were represented as

mean with standard error of the mean (SEM) as stated. P-values less than 0.05 were considered significant.

RESULTS

1. Increased type 2 and type 3 immune responses in HDM-induced NC/Nga mice model of atopic dermatitis

To evaluate the immune responses involved in development of AD, I used an HDM-induced NC/Nga murine AD model, which has a similar phenotype to human AD [71]. Mice treated with an ointment containing HDM (Dermatophagoides farina) antigens twice a week for 2 weeks showed prominent eczematous lesions in the dorsal skin, and AD symptom scores increased (**Figure 4**). Besides, epidermal thickness and the number of infiltrating eosinophils and mast cells were increased in HDM-induced mice than in control mice (**Figure 5**). Because type 2 cytokines are important for development of AD, I measured production of several cytokines by cells in skin-draining lymph nodes (SdLN) by flow cytometry. As expected, HDM treatment increased the number of IL-13–producing lymphocytes. It also increased the number of IL-17–producing lymphocytes. However, IFNγ–producing lymphocytes didn't change (**Figure 6**). Moreover, expression of *Il17*a and *Il13* mRNA increased markedly in the dorsal skin of HDM-induced AD mice (**Figure 7**). HDM-derived cysteine proteases disrupt the epithelial barrier, resulting in production of innate cytokines from epithelial cells [72]. The expression of innate cytokines such as *Il33* and *Tslp*, which induce type 2 immune responses, was greater in AD skin than in control skin. In addition, expression level of *Il1b* and *Il23*, which induce type 3 immune responses, were increased (**Figure 8A, B**). The increase in the expression level of *Il1b* and *Il17a* in the skin of HDM-induced mice led me to focus on ILC3s because these cells produce IL-17A in response to IL-1β

[61]. The greater numbers of IL-17A–producing ILC3s and IL-13–producing ILC2s in the skin of HDM-induced mice than in that of control mice were analyzed by flow cytometry (**Figure 9A, B**). Secretion of IL-22–producing ILC3s from skin, on the other hand, was not observed. IL-17A secretion by ILC3s increased to the greatest extent among IL-17A–producing cells in the skin after exposure to HDM, although the percentage of T cells was greater than that of ILC3s (**Figure 10A, B**).

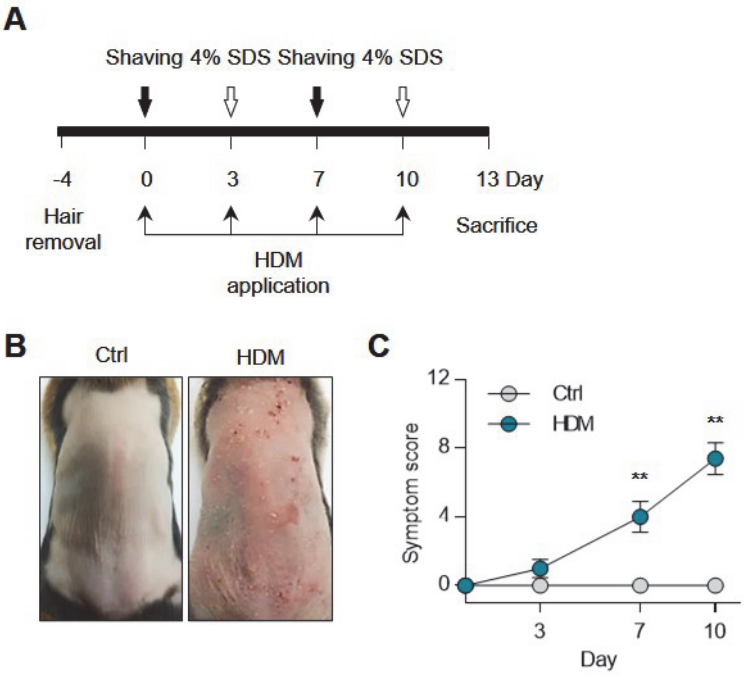

Figure 3. HDM-treated NC/Nga murine AD

A, The scheme of HDM-induced AD model. **B,** Representative skin images of the control and mice with HDM-induced AD. **C,** Symptom scoring of control versus mice with HDM-induced AD (n = 5 per group). Data was depicted as mean± SEM. Mann-Whitney U test was performed. $^{**}p<0.01$.

*Figure 4 C was kindly provided by Seon-Pil Jin.

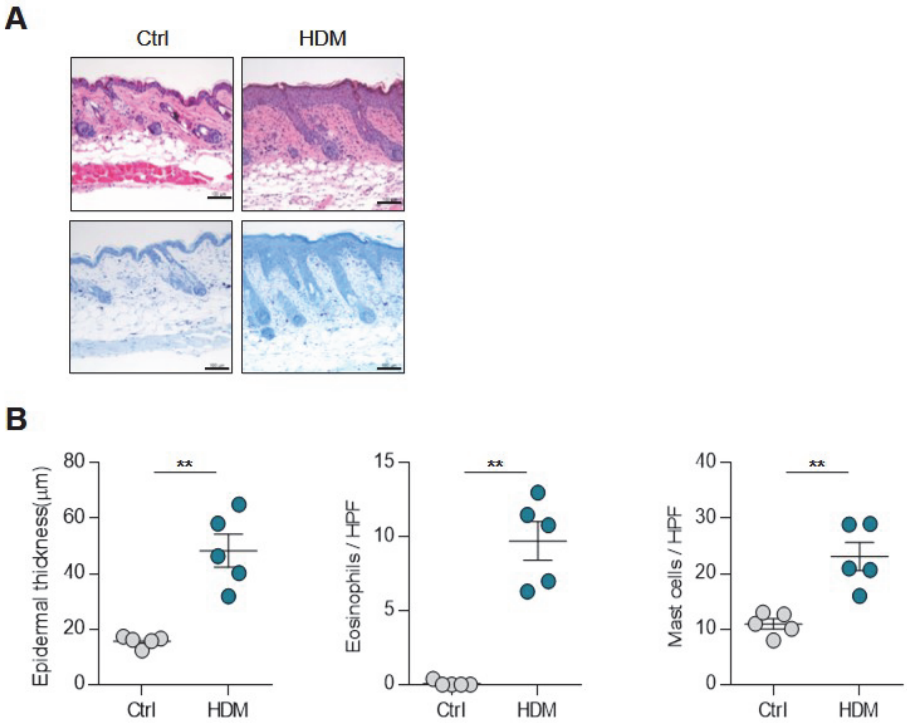

Figure 4. Phenotypes of HDM-induced AD mice model.

A, Representative H&E and toluidine blue-stained biopsy specimen from dorsal skins. Scale bar = 100 μm. **B,** Epidermal thickness, eosinophils and mast cells counts per high-power field (HPF). Data was depicted as mean ± SEM. Data was obtained from two to three independent experiments. Mann-Whitney U test was performed. $^{**}p<0.01$.

Figure 5 was kindly provided by Seon-Pil Jin.

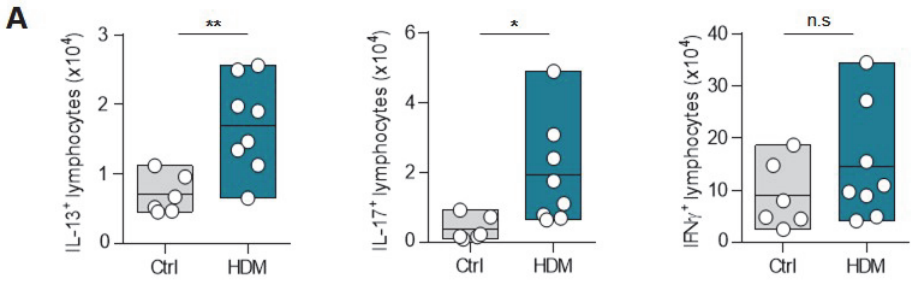

Figure 5. The number of IL-13– or IL-17A–producing lymphocytes was increased in skin-draining lymph nodes of AD mice

A, Absolute numbers of cytokine–producing lymphocytes in inguinal lymph nodes. Box lines express min, mean, and max. Data was obtained from two to three independent experiments (n= 6 - 8 per group). Mann-Whitney U test was performed. $^{*}p<0.05$, $^{**}p<0.01$.

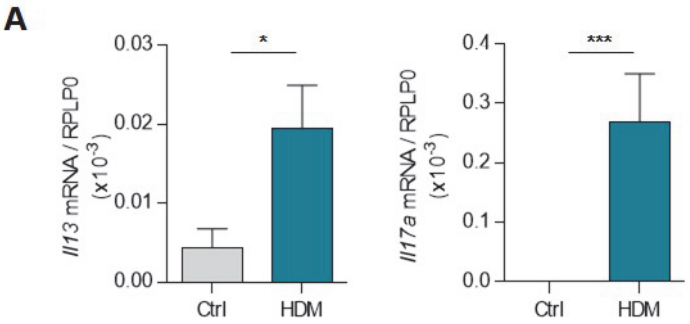

Figure 6. mRNA expression of *Il13* and *Il17a* was increased in the dorsal skin of HDM-induced AD mice

A, mRNA expression of *Il13* and *Il17a* in the dorsal skin. (n = 7 - 9 per group). Mann-Whitney U test was performed. Data are depicted as mean + SEM. $^{*}p<0.05$, $^{***}p<0.001$.

*Figure 7 was kindly provided by Seon-Pil Jin.

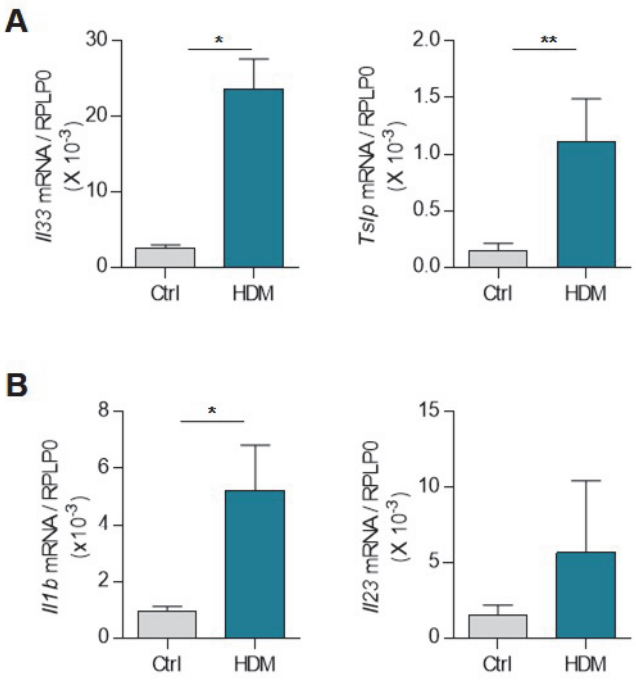

Figure 7. The expression level of innate cytokines was higher in AD mice than control

A, mRNA expression of *Il33* and *Tslp* in the dorsal skin. **B,** mRNA expression of *Il1b* and *Il23* in the dorsal skin. Data are obtained from two independent experiments (n = 4 - 5 per group). Mann-Whitney U test was performed. Data are depicted as mean + SEM. $^{*}p<0.05$, $^{**}p<0.01$.

*Figure 8 was kindly provided by Seon-Pil Jin.

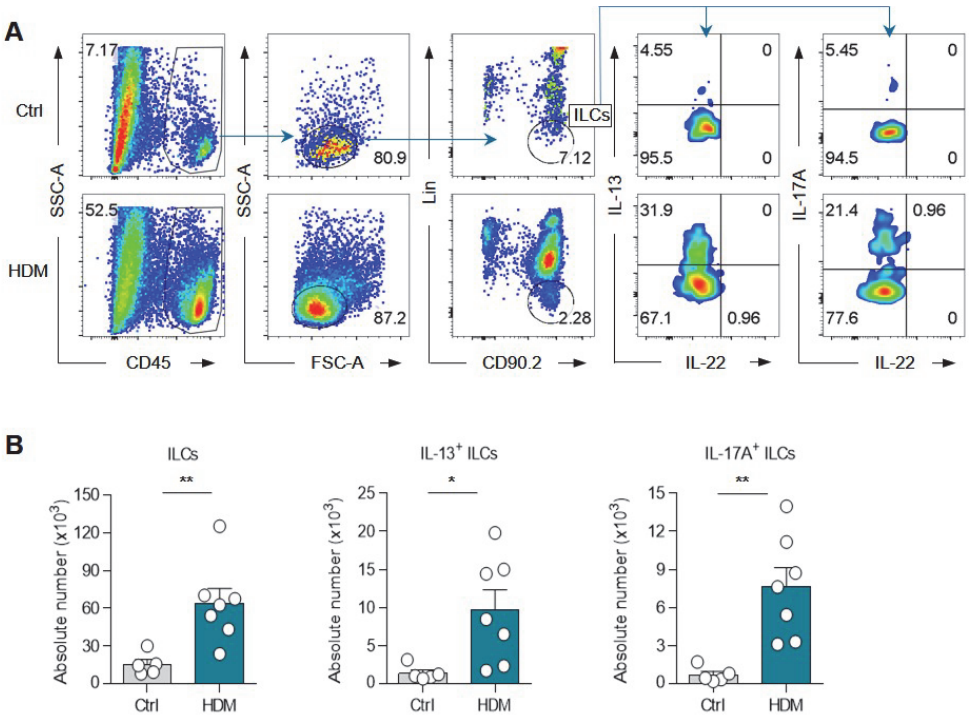

Figure 8. ILC3s as well as ILC2s are present at high numbers in the dorsal skin of an HDM-induced AD model

A, Gating strategy of cytokine-production from ILCs ($CD45^+$ $Lympho^+$ Lin^- $CD90.2^+$). **B,** Absolute numbers of $IL\text{-}13^+$ ILCs, $IL\text{-}17A^+$ ILCs in the skin. Data are representative of two independent experiments. (n = 5 - 7 per group). Mann-Whitney U test was performed (b-e). Data are depicted as mean + SEM. $^*p<0.05$, $^{**}p<0.01$.

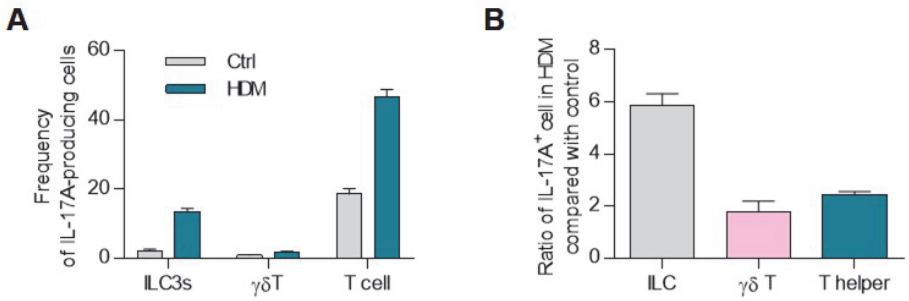

Figure 9. IL-17A–producing ILC3s increased to the largest extent in HDM-induced AD model

A, Frequency of IL-17A–producing cells in the skin of control and AD mice. (n = 2 - 3 per group). **B,** Ratio of IL-17A positive in each cell of HDM-induced NC/Nga compared with control. Data are representative of two independent experiments (n = 3). Data are depicted as mean + SEM.

2. ILCs contributed to AD-like skin inflammation regardless of a strain, allergen or adaptive immunity

To ascertain whether the increase in ILC3s is a strain or allergen-specific, I induced AD with HDM, MC903, and oxazolone in syngenic C57BL/6 mice and $Rag^{-/-}$ mice, which do not contain mature B and T lymphocytes. Topical application of vitamin D3 analog MC903 induces skin inflammation resembling immune perturbations observed in acute lesions of patients with AD [73]. Multiple challenges with a synthetic hapten, oxazolone, causes skin inflammation involving a shift from a typical T helper type 1 dominated delayed-type hypersensitivity response to a chronic T helper type 2 dominated inflammatory response that observed in human AD [74]. Similar to previous results, I discovered an increase in IL-13– or IL-17A–producing lymphocytes from Skin-draining lymph nodes in HDM induced C57BL/6 (**Figure 11**). Also, the absolute number of ILC2s and ILC3s was increased in HDM-induced C57BL/6 mice (**Figure 12**). However, MC903-treated C57BL/6 mice showed a predominantly type 2 response (**Figure 13**) and ILCs wasn't changed in the skin (**Figure 14**). The number of IL-13–producing ILCs was only increased in MC903-treated $Rag1^{-/-}$ mice (**Figure 15**). In the case of mice with oxazolone-induced AD, I noted an increase in the production of all cytokines secreted by lymphocytes (**Figure 16**) or ILCs (**Figure 17**). Furthermore, IL-13– or IL-17A– producing ILC did in oxazolone-induced $Rag1^{-/-}$ mice (**Figure 18**).

Therefore, these data suggest that not only ILC2s but also ILC3s may be

associated with the pathogenesis of AD.

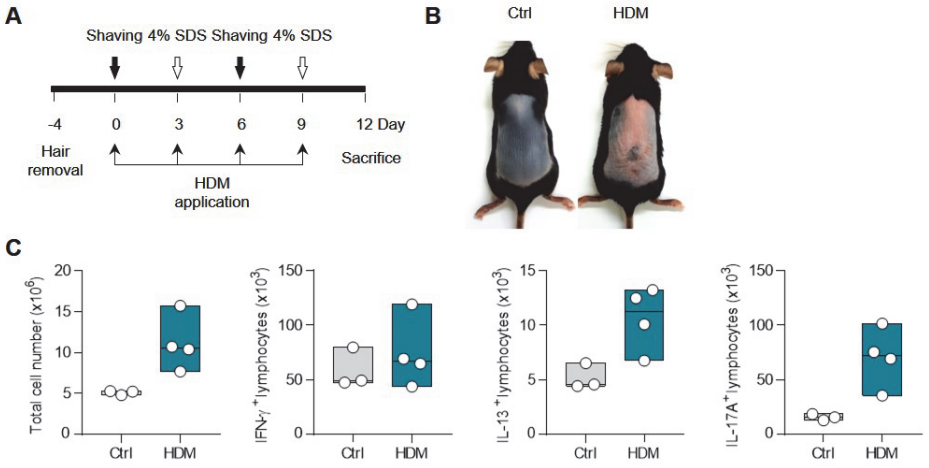

Figure 10. HDM enhanced both IL-13 and IL-17A production in the skin-draining lymph nodes from C57BL/6 mice

A, The scheme of HDM-induced AD C57BL/6 mice. **B,** Representative images of the control and HDM-induced mice. C, Total cell count and cytokine–producing lymphocytes in SdLN from control and HDM-induced C57BL/6 mice. (n= 3 - 4 per group) Box lines express min, mean, and max. Mann-Whitney U test was performed. $^{*}p<0.05$.

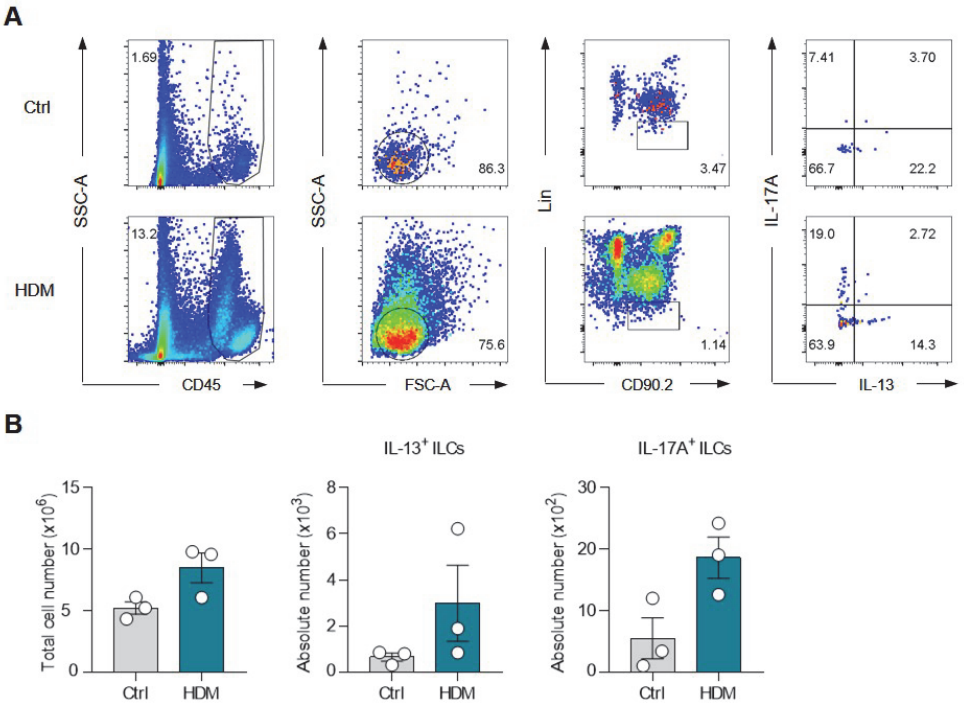

Figure 11. ILC2s as well as ILC3s were enhanced in HDM-induced C57BL/6

A, Representative dot plots of cytokine–producing ILCs in the dorsal skin. **B,** Total cell count and IL-13$^+$ or IL-17A$^+$ ILCs in the dorsal skin. (n= 3 per group). Data are depicted as mean ± SEM.

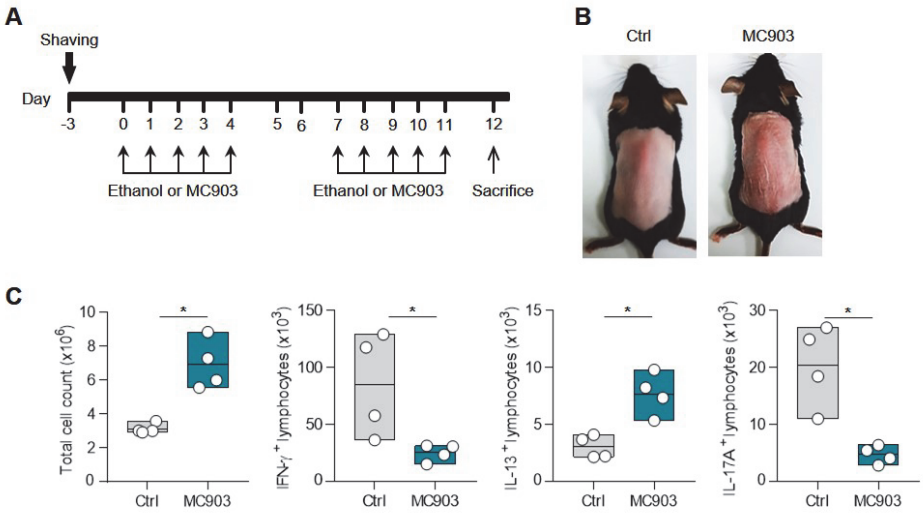

Figure 12. Predominant type 2 immune response in MC903-treated C57BL/6 mice

A, The scheme of MC903-treated AD-like model. **B,** Representative images of the EtOH and MC903-treated C57BL/6 mice. **C,** Total cell count and cytokine–producing lymphocytes in the SdLN. (n = 4 per group). Box lines express min, mean, and max. Mann-Whitney U test was performed. $^{*}p<0.05$.

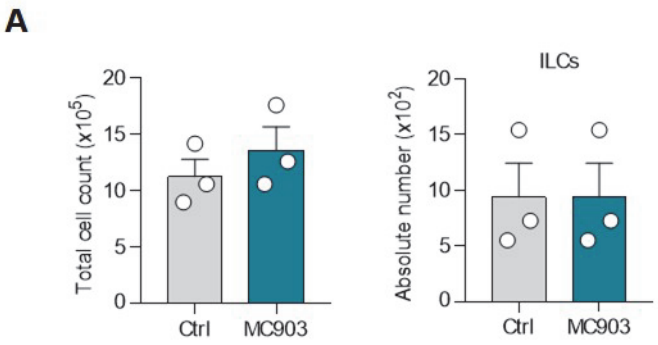

Figure 13. ILCs was not changed in MC903-treated AD mice

A, Total cell count and absolute number of ILCs in the dorsal skin. (n = 3 per group). Mann-Whitney U test was performed. Data are depicted as mean + SEM.

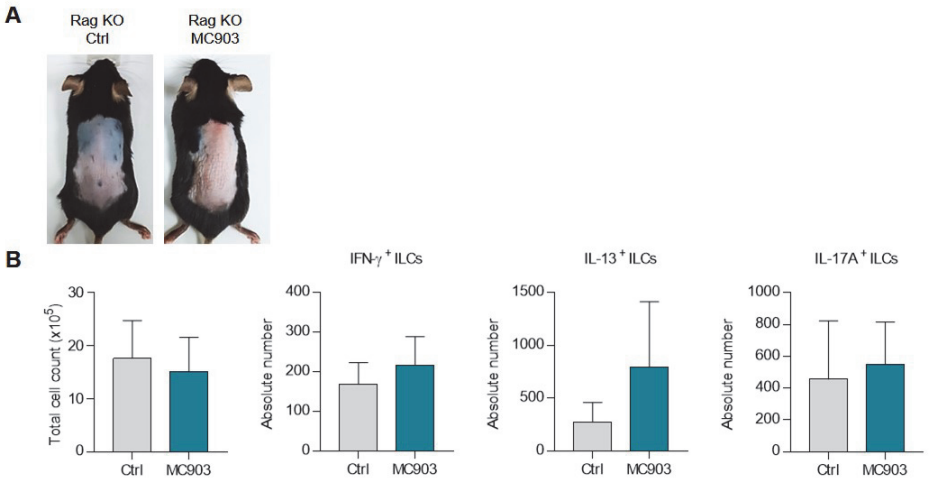

Figure 14. ILC2s in MC903-treated Rag KO mice were higher than those in control mice

A, Representative images of the EtOH and MC903-treated Rag KO mice. **B,** Total cell count and absolute number of ILCs in the dorsal skin from MC903-induced Rag KO mice. (n= 3 - 4 per group) Data are depicted as mean + SEM.

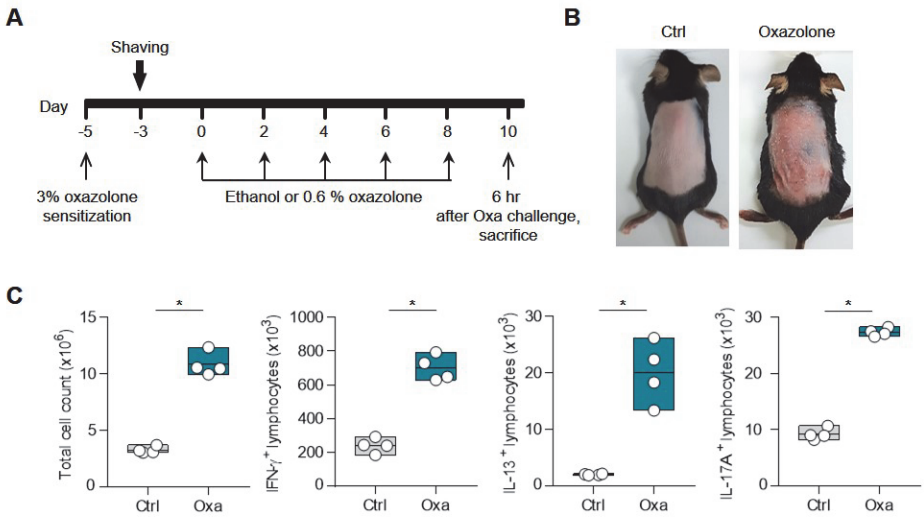

Figure 15. All cytokines were enhanced in oxazolone-induced skin inflammation

A, Experiment design of oxazolone-induced skin inflammation. **B,** Representative images of control and oxazolone-induced mice. **C,** Total cell count and cytokine–producing lymphocytes in the SdLN. Data are representative of two independent experiments (n = 4 per group). Box lines express min, mean, and max. Mann-Whitney U test was performed. $^{*}p<0.05$.

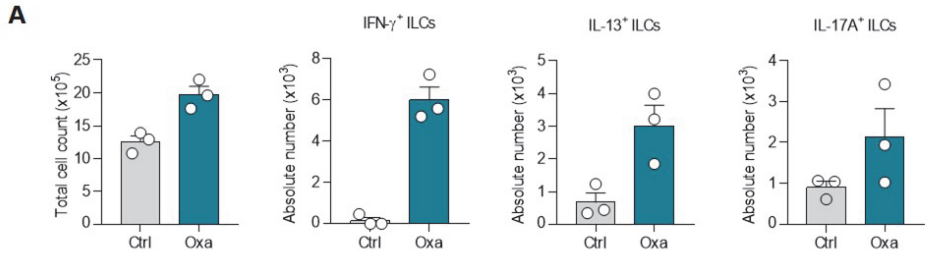

Figure 16. Both ILC2s and ILC3s are higher in oxazolone-induced skin inflammation

A, Total cell count and absolute number of cytokine–producing ILCs in the dorsal skin. Data are representative of two independent experiments. Data are depicted as mean + SEM.

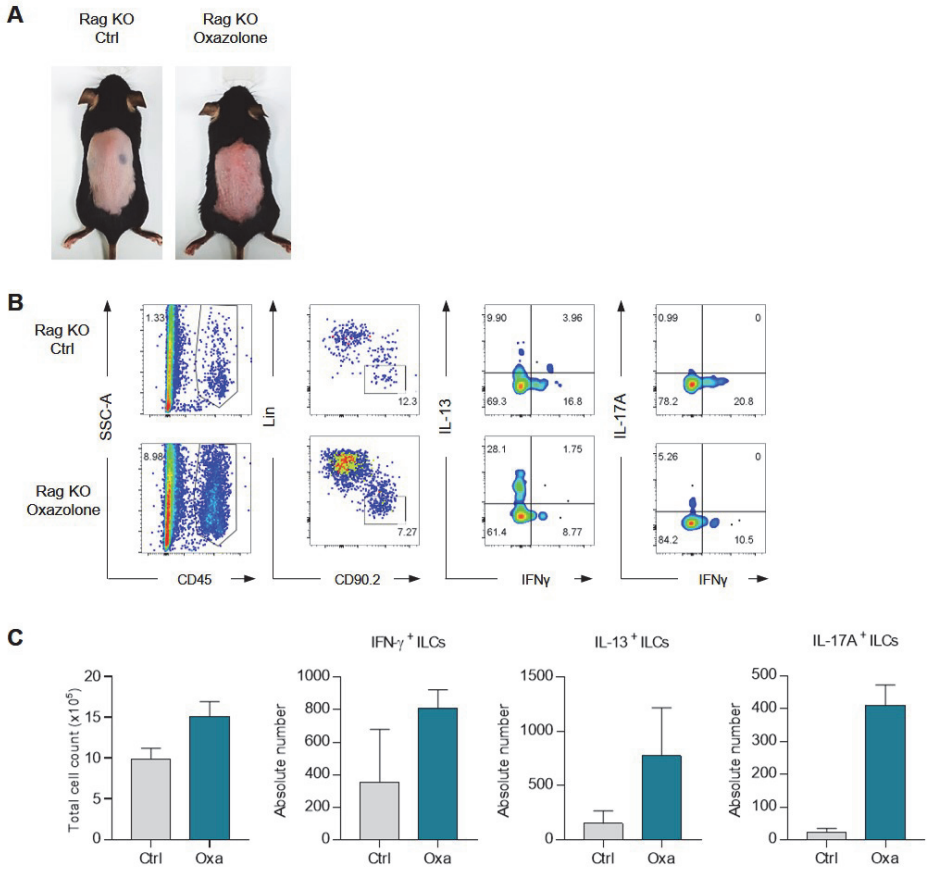

Figure 17. ILCs contributed to AD-like skin inflammation without adaptive immunity

A, Representative images of the EtOH and oxazolone-induced Rag deficient mice. **B,** Representative dot plots of cytokine-producing ILCs in the dorsal skin from oxazolone-induced Rag deficient mice. C, Total cell count and absolute number of cytokine positive ILCs in the dorsal skin. (n= 3 per group). Data are depicted as mean + SEM.

3. Neutrophils are the major cells producing IL-1β among immune cells in the AD environment

Next, I tried to identify the cells that produce IL-1β, which is essential to inducing IL-17A production by ILC3s [61]. Intracellular cytokine staining revealed that both $CD45^+$ cells and $CD45^-$ cells were a source of IL-1β in the dorsal skin of HDM-induced NC/Nga mice. However, the IL-1β expression from $CD45^+$ cells was higher than that from $CD45^-$ cells (**Figure 19A**). It is well known that IL-1β has to be cleaved by cytoplasmic caspase 1 to attain biological activity [75]. The increased expression of mature IL-1β was analyzed in the dorsal skin of HDM-induced mice by western blot (**Figure 19B**). Neutrophil expressed the highest levels of IL-1β in lesional skin among IL-1β–producing $CD45^+$ cells (**Figure 20**).

Therefore, increased IL-1β levels in an atopic environment may contribute to the heterogeneity of disease by inducing type 3 cytokines in addition to type 2 cytokines.

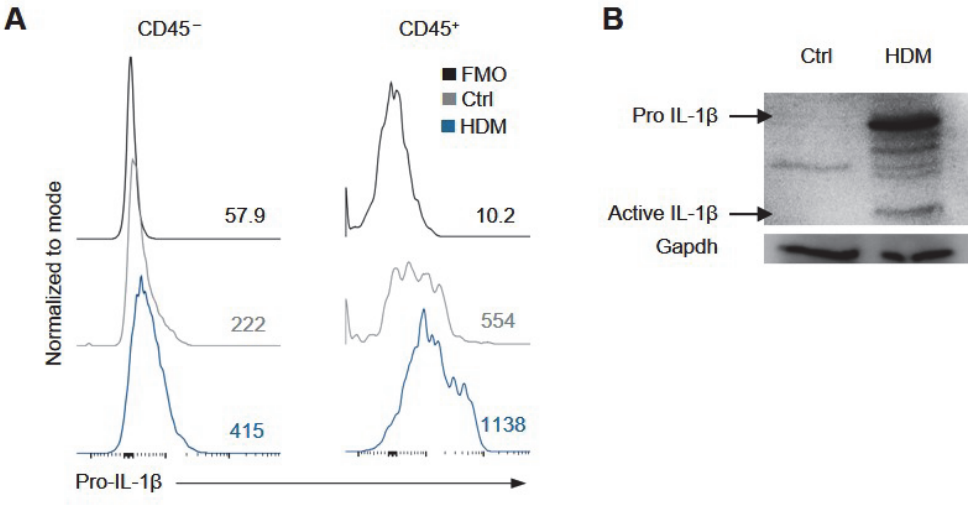

Figure 18. IL-1β was more expressed in CD45^{+} cells compared with CD45^{-} cells

A, Pro-IL-1β expression among CD45^{-} and CD45^{+} cells from the dorsal skin. FMO, fluorescence minus one. **B,** IL-1β protein was detected by western blot in the dorsal skin from control and HDM-induced NC/Nga mice.

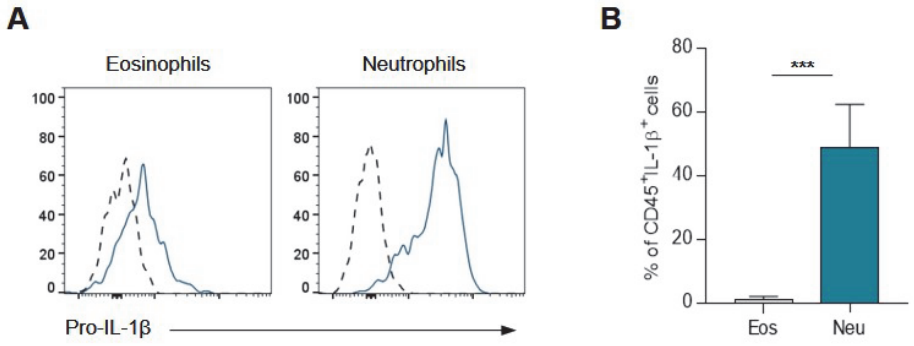

Figure 19. Neutrophils expressed IL-1β in the dorsal skin of AD murine model

A, Pro-IL-1β expression of eosinophils and neutrophils. Data are representative of two independent experiments (n = 3). **B,** Percentage of IL-1β producing cells in AD skin. Eosinophils ($CD45^+$ $CD11b^+$ Siglec F^+), Neutrophils ($CD45^+$ $CD11b^+$ Siglec F^- $Ly6G^+$). Data were obtained from two independent experiments (n = 7 per group). Mann-Whitney U test was performed. Data are depicted as mean + SEM. $^{***}p<0.001$.

4. ILC3s are involved in the development of AD-like skin inflammation

Since the previous results suggest that ILC2s and ILC3s produce IL-13 or IL-17A, respectively, I needed to confirm the effect of ILC3s alone under atopic conditions. To examine this, I sorted ILC3s in SdLN and spleen from mice with application of HDM and injected them intradermally into recipient mice during induction of AD. I sorted ILC3s from SdLN and spleen to reduce the isolation time and to obtain a sufficient number of cells, although the number of ILCs producing IL-17A was higher in the skin than others (**Figure 21A)** The purity of sorted ILC3s and cytokine production was confirmed by intracellular cytokine staining (**Figure 22B**). Administration of ILC3s to recipient mice accelerated development of AD (**Figure 22**) and increased epidermal thickness and infiltration by mast cells, eosinophils and neutrophils (**Figure 23**). Furthermore, the number of IL-4–, IL-13–, IL-17A–producing cells in SdLN was increased by the transfer of ILC3s (**Figure 24**). mRNA expression of *Il13* and *Il1b* also was increased in the dorsal skin from AD mice injected with ILC3s (**Figure 25**).

These results suggest that ILC3s themselves are sufficient to exacerbate the symptoms of AD.

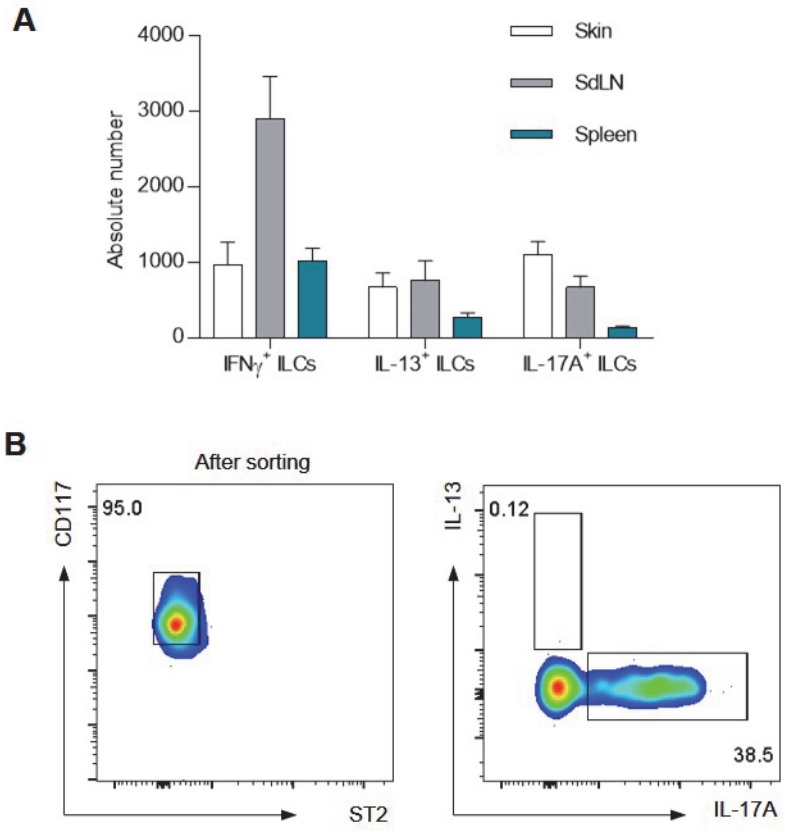

Figure 20. The confirmation of sorted ILC3s in HDM-induced mice

A, Absolute numbers of IFNγ$^+$, IL-13$^+$ and IL-17A$^+$ ILCs. (n= 4). **B,** The purity of sorted ILC3s (CD45$^+$ Lympho$^+$ Lin$^-$CD127$^+$ CD25$^+$) analyzed with AriaIII. Lin: CD3ε, CD11b, CD11c, CD19, CD49b, F4/80, FcεRIα, TCRγδ. Cytokine expression of ILC3s (CD45$^+$ Lympho$^+$ Lin$^-$ CD127$^+$ CD25$^+$ CD117$^+$ ST2$^-$).

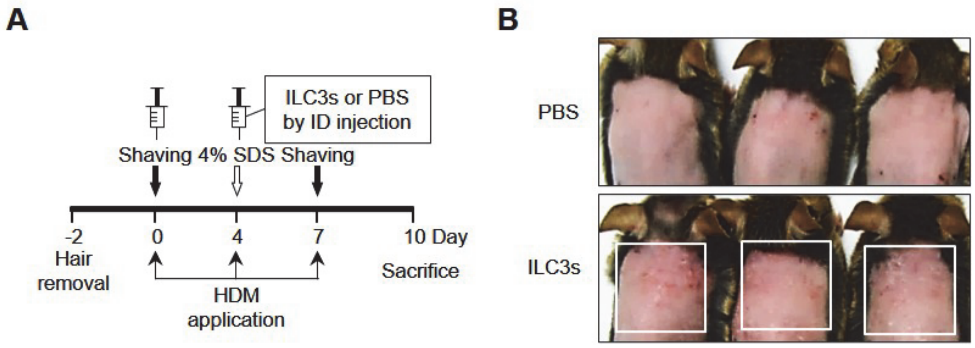

Figure 21. ILC3s accelerate the HDM-induced AD.

A, The scheme of HDM-induced AD model and adoptive transfer of ILC3s. **B,** Representative images of HDM-induced AD mice transferred with PBS or ILC3s.

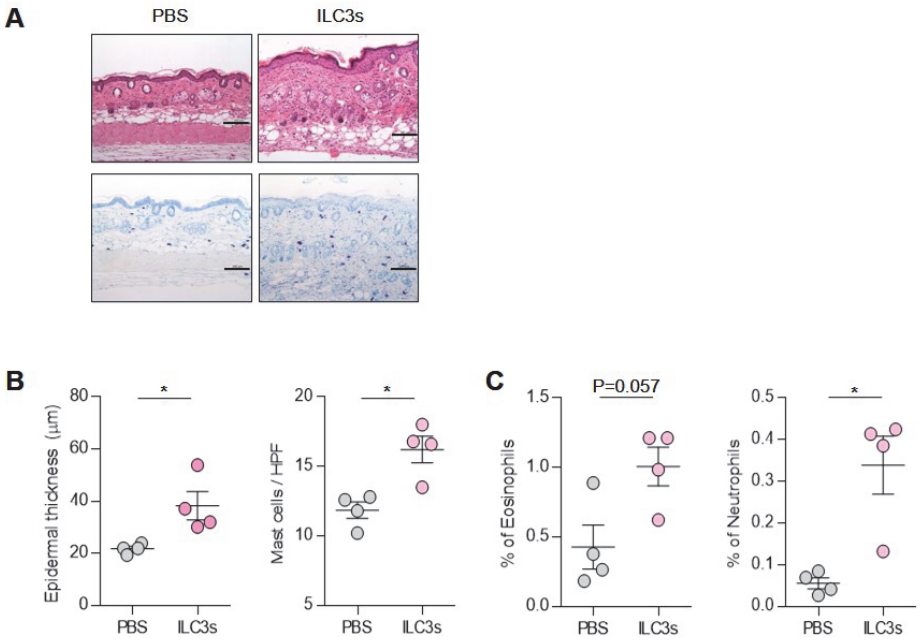

Figure 22. Phenotypes of HDM-induced AD mice injected with ILC3s

A, Representative H&E and toluidine blue-stain of each group. Scale bar = 100 μm. **B,** Epidermal thickness, mast cell counts per HPF. **C,** The frequency of eosinophils and neutrophils among $CD45^+$ cells in the dorsal skin of PBS treated or ILC3s transferred AD mice were analyzed by flow cytometry. Data are depicted as mean ± SEM. $^*p<0.05$.

*Figure 23A and B was kindly provided by Seon-Pil Jin.

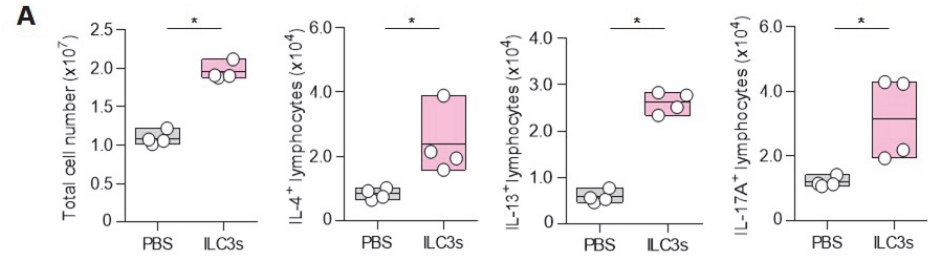

Figure 23. Increased type 2 and type 3 immune response in AD mice injected with ILC3s compared with PBS

A, Absolute numbers of IL-4$^+$, IL-13$^+$, and IL-17A$^+$ lymphocytes in skin-draining lymph nodes. Data are representative of two independent experiments (n = 4 per group). Mann-Whitney U test was performed. Box lines express min, mean, and max. $^*p<0.05$.

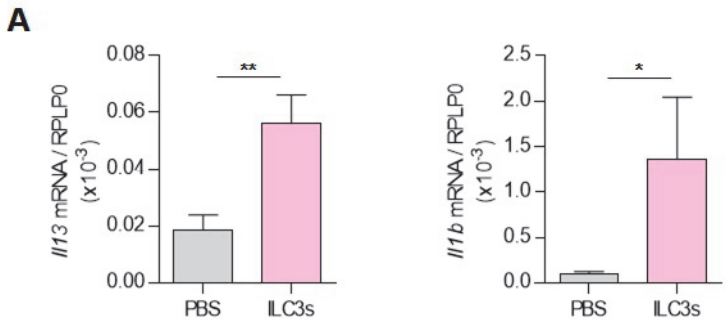

Figure 24. ILC3s enhanced inflammatory cytokines in AD mice injected with ILC3s

A, mRNA expression of *Il13* and *Il1b* in the dorsal skin from control or ILC3s transferred mice. Data from two independent experiments (n= 6 - 7 per group). Data are depicted as mean + SEM. Mann-Whitney U test was performed. $^{*}p<0.05$, $^{**}p<0.01$.

*Figure 25 was kindly provided by Seon-Pil Jin.

5. Blockade of IL-17A attenuates development of HDM-induced AD in NC/Nga mice

Because the genetic background of NC/Nga mice is not fully understood, IL-17A–neutralizing antibody was used to block IL-17A secreted by ILC3s (**Figure 26A**). The efficacy of anti-IL-17A was validated by downregulation of *Cxcl1* and *Il6* mRNA, the representative chemokines induced by IL-17A [76] (**Figure 26B**). Both ear and epidermal thickness of HDM-induced AD mice treated with the anti-IL-17A antibody was less than those of isotype control mice (**Figure 27A, C**). Moreover, Skin inflammation in mice treated with an IL-17A blocking antibody was less severe than that in mice treated with an isotype control antibody (**Figure 27**). I also analyzed the expression of several innate cytokines to confirm the effects of IL-17A blockade on HDM-induced AD. Remarkably, the expression of *Il33*, an alarmin cytokine that plays important roles in atopic inflammation, was reduced by IL-17A blockade, although that of *Tslp* was not in mice with HDM-induced AD (**Figure 28**).

In addition to HDM-induced NC/Nga mice, I further examined oxazolone-induced C57BL/6 mice to confirm whether IL-17A downregulated the expression level of IL-33 (**Figure 29A**). Eosinophils in dorsal skin of mice treated IL-17A antibody were less infiltration than those in dorsal skin of mice treated isotype (**Figure 29B**). As HDM-induced NC/Nga mice, mRNA expression of *Il33* was decreased in oxazolone-induced C57BL/6 mice treated with IL-17A blocking (**Figure 29C**).

These results indicate that there may be an unknown pathway for IL-17A–

mediated IL-33 modulation of skin inflammation.

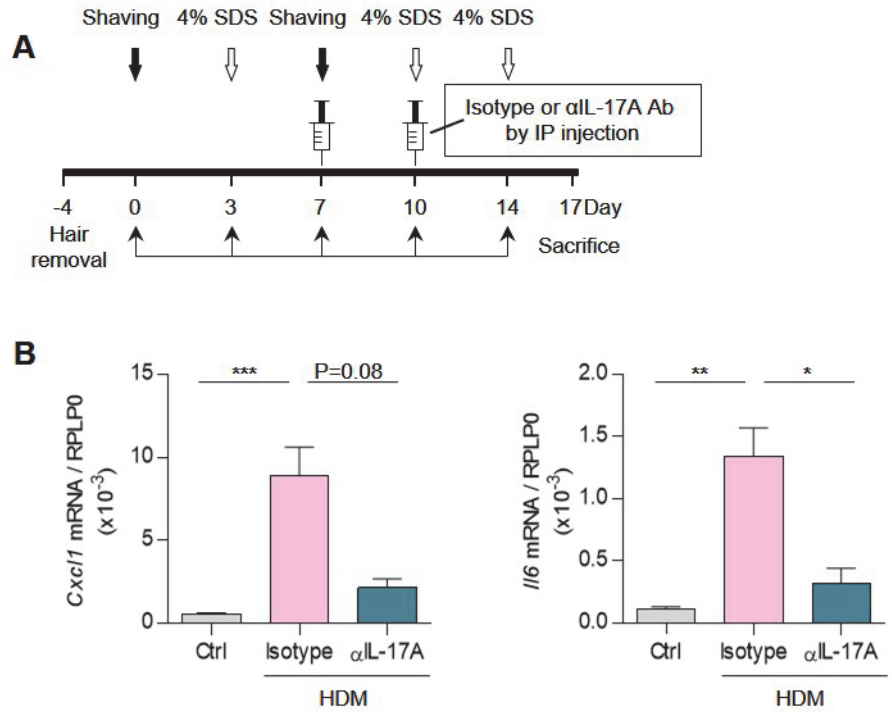

Figure 25. The expression of IL-17A targeted genes

A, The scheme of IL-17A neutralization in HDM-induced AD mice. **B,** *Cxcl1* and *Il6* mRNA expression in the dorsal skin were tested as positive control of IL-17A neutralization. Data were obtained from two independent experiments (n= 4 - 6 per group). Data were depicted as mean + SEM. Kruskal-Wallis with Dunn's post hoc test was performed. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

*Figure 26 B was kindly provided by Seon-Pil Jin.

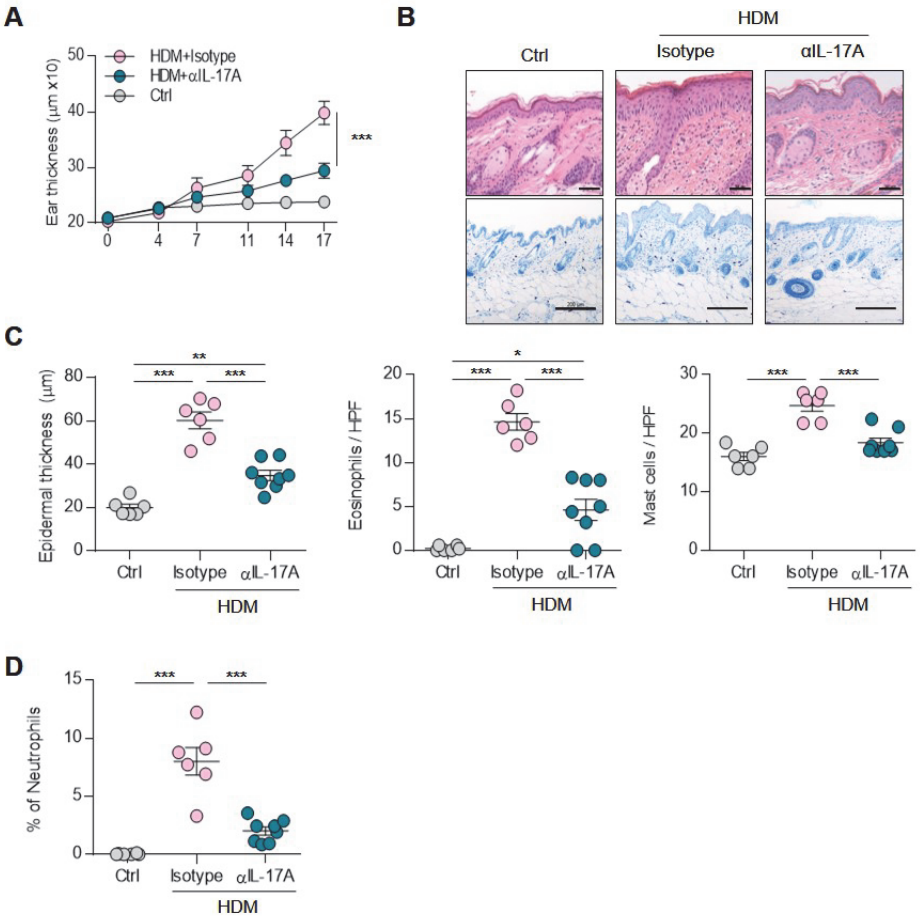

Figure 26. IL-17A–neutralizing impedes the induction of AD

A, Time-dependent change in ear thickness. (n = 7 - 11 per group). Isotype control Ab versus anti-IL-17A Ab on the day 17. Mann-Whitney U test was performed. **B,** H&E and toluidine blue-stained dorsal skin from control and HDM-induced AD mice treated with isotype or anti-IL-17A Ab. Scale bar = 50 μm (H&E), and 200 μm (toluidine blue). **C,** Epidermal thickness, Eosinophils and mast cells counts per high-power field (HPF). **D,** frequency of neutrophils among $CD45^+$ cells. (n = 6 - 8 per group). Data are depicted as mean ± SEM. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

*Figure 27 B and C were kindly provided by Seon-Pil Jin.

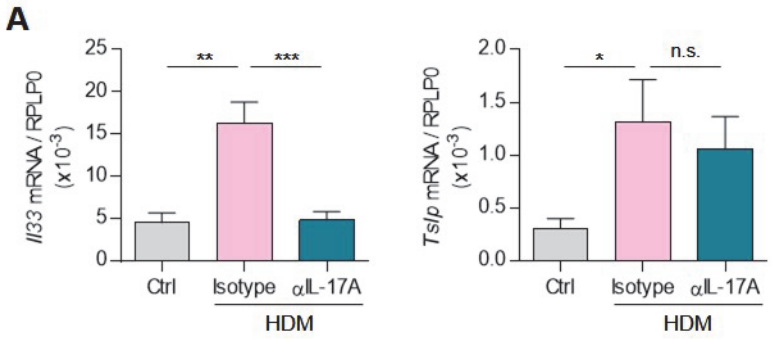

Figure 27. mRNA expression of *Il33* was downregulated in HDM-induced Nc/Nga mice treated with IL-17A antibody

A, mRNA expression of *Il33* and *Tslp* in the dorsal skin. Data were obtained from two independent experiments (n = 4 - 6 per group). Kruskal-Wallis with Dunn's post hoc test was performed. Data are depicted as mean + SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ns, not significant.

*Figure 28 was kindly provided by Seon-Pil Jin.

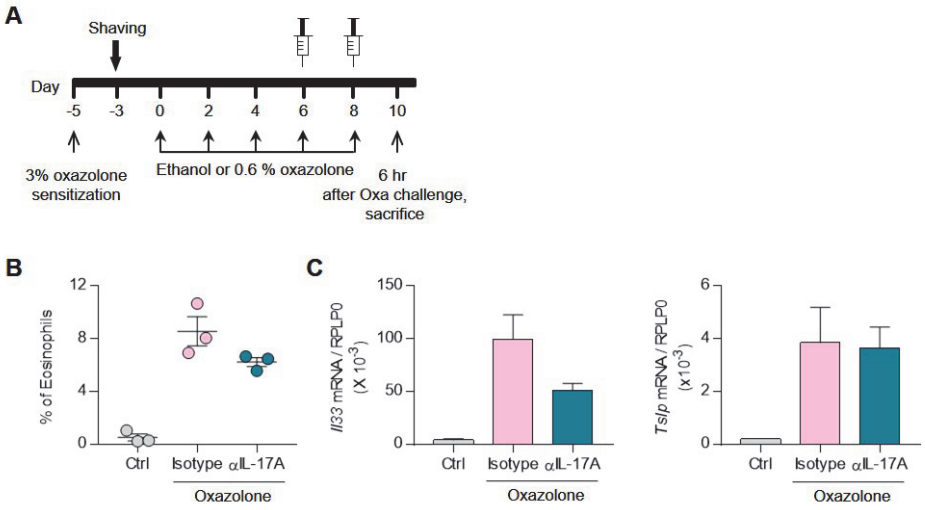

Figure 28. mRNA expression of *Il33* was downregulated in oxazolone-induced C57BL/6 mice treated with IL-17A antibody

A, mRNA expression of *Il33* and *Tslp* in the dorsal skin. Data were obtained from two independent experiments (n = 4 - 6 per group). Kruskal-Wallis with Dunn's post hoc test was performed. Data are depicted as mean + SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ns, not significant.

6. ILC3s induce IL-33 secretion by human keratinocytes and fibroblasts

Because blockade of IL-17A reduced expression of *Il33* and infiltration by immune cells in the lesional skin, I hypothesized that IL-17A regulates secretion of innate cytokines by skin cells such as keratinocytes and fibroblasts that have IL-17A receptor (IL-17RA). To address this hypothesis, I treated recombinant human IL-17A protein (rhIL-17A) directly to the culture medium of human primary keratinocytes or fibroblasts. mRNA expression of *CXCL1*, which is induced by IL-17A signaling, was increased. Moreover, treatment with rhIL-17A led to marked induction of *IL33* in both human primary keratinocytes and fibroblasts, which represent skin cells (**Figure 30A, B**).

Based on that adoptive transfer of murine ILC3s worsened the symptoms of AD and that rhIL-17A induced expression of *IL33*, I next examined whether ILC3s induce expression of IL33 from skin cells and whether this could enhance type 2 immune response. I sorted ILC3s from human peripheral blood mononuclear cells (PBMCs) and then ILC3s were cocultured with human primary keratinocytes and fibroblasts using a transwell system with rhIL-7 and rhIL-1β which are cytokines for development and stimulating ILC3s, respectively (**Figure 31A**). Coculture of stimulated ILC3s induced expression of *IL33* and *CXCL1* by keratinocytes and fibroblasts. Coculture with other types of ILCs, however, did not (**Figure 31B**). In addition, when keratinocytes and fibroblasts were cultured separately, expression of *IL33* was lower than when these cells were cultured together (**Figure 32**).

Therefore, these results suggest that IL-17A from ILC3s might be responsible for

increased expression of *IL33*, which induces type 2 immune responses.

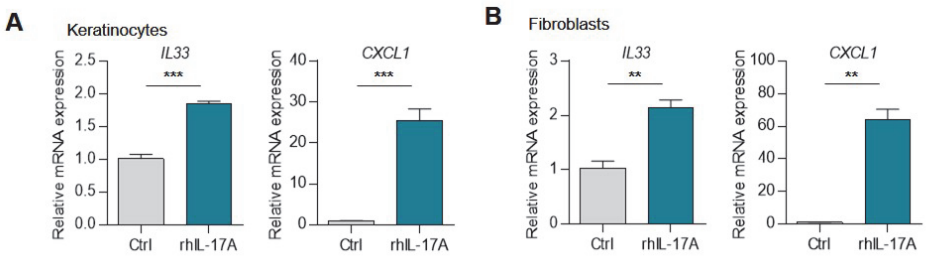

Figure 29. rhIL-17A upregulated mRNA expression of *IL33* in human keratinocytes or fibroblasts

A, B, Relative mRNA expression of *IL33* and *CXCL1* by rhIL-17A in human keratinocytes and fibroblasts, respectively. Each gene expression was normalized to the expression level of the control group. Data were obtained from three independent experiments. (n = 7 per group). Mann-Whitney U test was performed.
*Figure 30 was kindly provided by Seon-Pil jin.

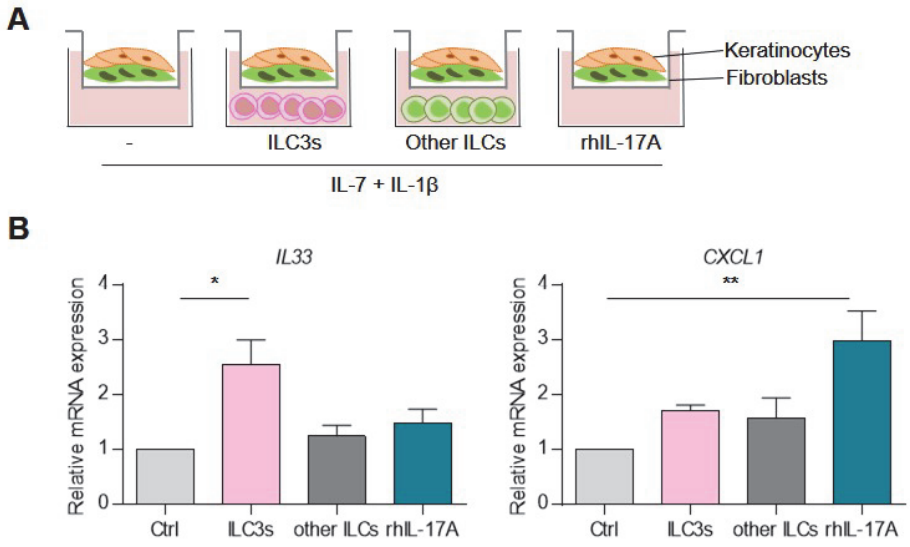

Figure 30. ILC3s stimulate the expression of *IL33* from the human keratinocytes and fibroblasts

A, Experimental design of coculture of human ILC3s with keratinocytes and fibroblasts. ILC3s ($CD45^+$ $Lympho^+$ Lin^- $CD127^+$ $CRTH2^-$ $CD117^+$), other ILCs ($CD45^+$ $Lympho^+$ Lin^- $CD127^+$ $CD117^-$). Lin: CD3ε, CD11b, CD11c, CD14, CD19, CD49b, FcεRIα. **B,** Relative mRNA expression of *IL33* and *CXCL1* from human keratinocytes and fibroblasts in transwell inserts. Data were obtained from three independent experiments. Data are depicted as mean + SEM (n = 3). Kruskal-Wallis with Dunn's post hoc test was performed. $^*p<0.05$, $^{**}p<0.01$

*Figure 31B was kindly provided by Seon-Pil jin and Sunhyae Jang.

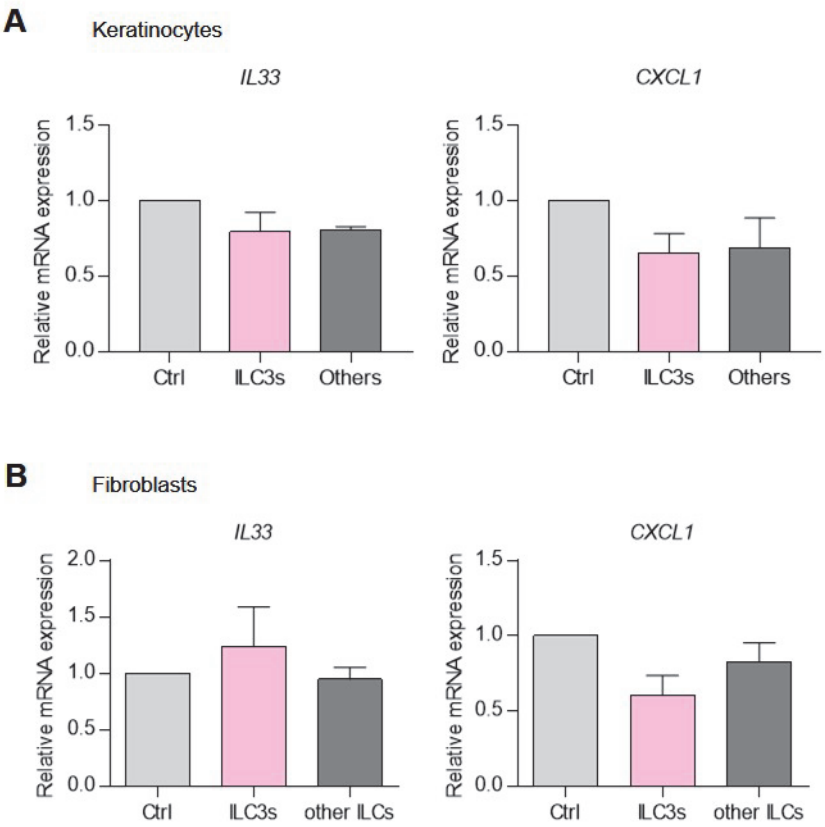

Figure 31. The coculture of human ILC3s with monolayer of keratinocytes or fibroblasts

Relative mRNA expression of *IL33* and *CXCL1* from human keratinocytes **(A)** or fibroblasts **(B)** in transwell inserts. Data were obtained from two independent experiments. Data are depicted as mean + SEM (n = 2).

*Figure 32 was kindly provided by Sunhyae Jang.

7. Patients with AD show an altered ILC composition in peripheral blood

To test whether ILC3s were also elevated in patients with AD like AD mice model, I examined population of ILCs in the PBMCs by flow cytometry. Human ILC populations (lineage$^-$ CD127$^+$) can be classified into three groups according to the expression of CD117 and CRTH2 [42]. Freshly isolated PBMCs from healthy control and patients with AD consisted of a distinct population of ILCs (**Figure 33A**). Patients with AD had a higher percentage of ILCs in the PBMCs. I used two analytical tools for staining, surface marker or transcription factor about ILCs subset. The percentage of ILC2s (CRTH2$^+$ or GATA3$^+$) and ILC3s (c-Kit$^+$ or RORγt$^+$) in the PBMCs from patients with AD were larger than in healthy controls. ILC1s wasn't, however, changed (**Figure 33 and 34**). It was observed that ILC3s and ILC1s changed in ILCs **(Figure 35)**. ILC3s showed a strong positive correlation with neutrophils in the blood from patients with AD, but correlation between ILC2s and eosinophils didn't. There was a positive correlation between ILC3s and neutrophils, but no change in neutrophils was observed in the PBMCs from patients with AD (**Figure 36**). To assess whether ILCs within the PBMC population have the potential to home in to the skin, I investigated the expression of cutaneous lymphocyte-associated antigen (CLA) by ILCs. About 40% of ILCs expressed CLA within the PBMCs from both healthy control and AD patients (**Figure 37**). Increased ILC2s and ILC3s in the PBMCs from patients with AD could affect the skin lesions in atopic dermatitis. As a previous study, the phenotype of Asian AD patients presented a higher Th17 activation, along with an increase in ILC3s.

Although ILC3s are not considered critical for the pathogenesis of AD, these experiments indicate that the interaction between ILCs and nonimmune cells such as keratinocytes and fibroblasts induces IL-33 production, which contributes to the development of AD.

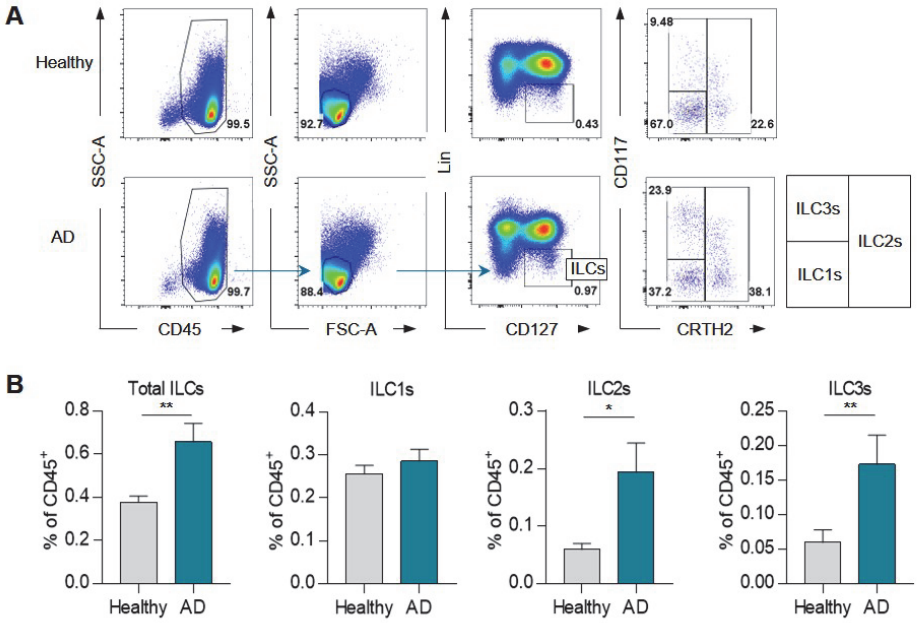

Figure 32. ILC3s account for a large proportion among ILCs in PBMCs from patient with AD

A, Representative dot plots of ILCs ($CD45^+$ $Lympho^+$ Lin^- $CD127^+$) in PBMCs from healthy control or patients with AD. Mean percentage of each cell in plot. Lin: CD3ε, CD11b, CD11c, CD14, CD19, CD49b, FcεRIα. **B,** Frequency of ILCs and the distribution of each subset within the entire ILCs among $CD45^+$ cells (n = 9 per group). Mann-Whitney U test was performed. Data are depicted as mean + SEM. $^*p<0.05$, $^{**}p<0.01$.

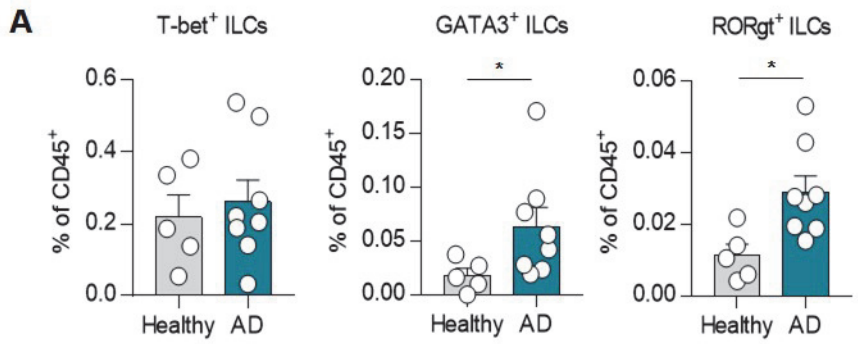

Figure 33. ILC3s as well as ILC2s were increased in PBMCs from AD patients

A, Frequency of ILCs expressing T-bet, GATA3 or RORγt among $CD45^+$ cells (n = 5 – 8 per group). Mann-Whitney U test was performed. Data are depicted as mean + SEM. $^*p<0.05$.

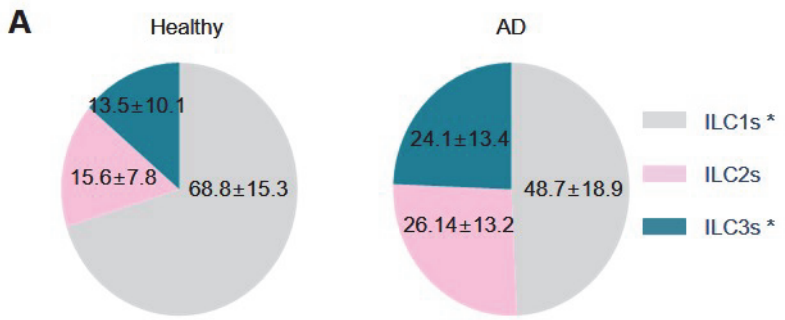

Figure 34. ILC3s and ILC1s in ILCs were changed in PBMCs from AD patients

A, Pie chart showing the mean ± SD frequency of each ILC subset among ILCs. ILC1s and ILC3s were significantly changed in ILCs.

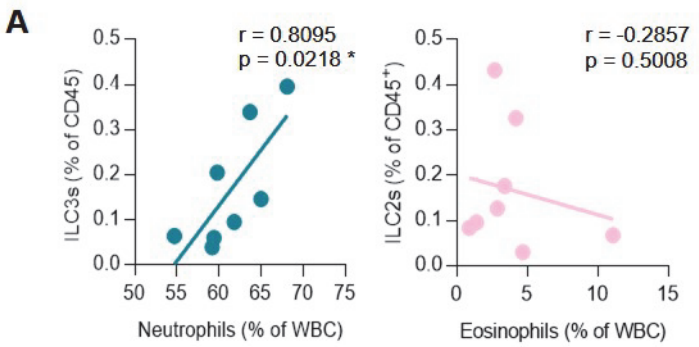

Figure 35. Positive correlation between ILCs and neutrophils in patients with AD

A, Correlation between ILC3s and neutrophils (left) and ILC2s and eosinophils (right) in peripheral blood from AD patients. The correlation was assessed by using Spearman rank correlation.

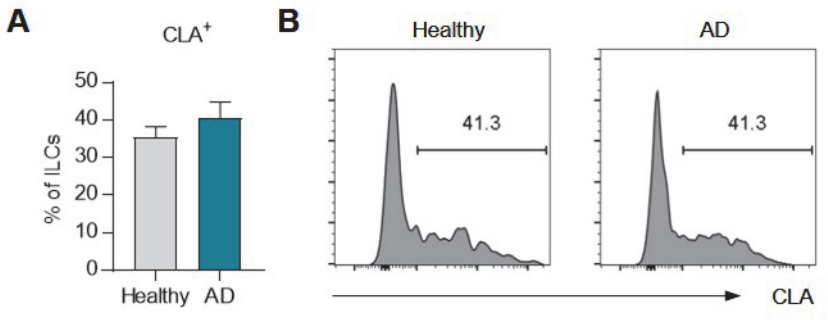

Figure 36. Cutaneous lymphocyte antigen was expressed on innate lymphocyte cells in human PBMCs

A, Frequency of CLA^+ ILCs in PBMCs from healthy volunteer and AD patients. **B,** CLA expression of ILCs from healthy control and patients with AD. (n= 6 - 7 per group). Data were depicted as mean + SEM.

DISCUSSION

The aim of this study was to investigate the immunopathogenesis of AD and the role of ILC3s in AD. Here, I identified a previously unreported role for ILC3s and IL-17A in the pathogenesis of AD. This study demonstrated that the levels of ILC3s, as well as ILC2s, were increased in the skin of mice and PBMCs from patients with AD. ILC3s accelerated the pathogenesis of AD; however, blocking IL-17A signaling impeded the severity of AD by downregulating the expression of IL-33. IL-17A–producing ILC3s increased the production of IL-33 from human keratinocytes and fibroblasts. Therefore, early secretion of IL-17A could strengthen type 2 immune responses by inducing alarmin signals from keratinocytes and fibroblasts. Consequently, ILC3s contribute to the pathogenesis of AD.

Several studies have reported the contribution of ILC2s to the pathogenesis of AD [62, 63, 77, 78, 79]. However, the role of ILC3s in the immunopathogenesis of AD remains unknown. This study first found that ILC3s contribute to the pathogenesis of AD. ILC3s are associated with Th17 cytokine-related conditions, such as psoriasis, Crohn's disease, and autoimmune diseases [80, 81, 82]. Although type 2 cytokines are important in AD, AD is a complex disease with a high degree of heterogeneity with respect to clinal phenotype. Previous studies have reported changes in IL-17A levels in patients with AD [36, 38, 40]. The present study utilized a mouse model of AD to delineate the role of ILC3 in the pathogenesis of AD. The results of several studies in mice support the present findings, and the pathogenic role of IL-17A in AD. For example, filaggrin-deficient mice develop spontaneous eczematous inflammation as they get older, and appear to be predominant by IL-

17A [83]. A mouse model of ovalbumin-induced asthma and AD revealed that IL-4– and IL-13– null mice administered epicutaneous ovalbumin develop significant eczematous inflammation and express high levels of IL-17A. Blocking IL-17A reverses the development of airway hyperresponsiveness and skin inflammation [84, 85]. IL-17A deficiency attenuated the development of AD by decreasing the number of IL-4–producing $CD4^+$ T cells in flaky-tail mice [86]. As noted above, recent studies have not revealed a role for IL-17A in the development of AD. Thus, the results of the present study suggest that IL-17A–producing cells, in addition to ILC3s, interact with keratinocytes and fibroblasts to promote the release of IL-33.

The NC/Nga model of AD is most similar to human lesions, although no knockout strain is available. To address the effect of ILC3s, ILC3s from the spleen and lymph nodes were isolated and injected into the dorsal skin of NC/Nga mice. Only a small number of ILC3 (5,000–7,000) accelerated the development of AD. Unlike T cells, low levels of ILCs are present in the tissue and contributes an immediate immune response by stimulating cytokines without antigen-specific responses. IL-17A secreted by ILC3s induced the expression of IL-33 in keratinocytes and fibroblasts. When keratinocytes and fibroblasts were cultured with ILC3s separately, the increase in IL-33 expression was lower than that observed in the combined culture. The expression of cytokines and growth factors was modulated following individual culture with keratinocytes and fibroblasts [87, 88]. This suggested that crosstalk between keratinocytes and fibroblasts may be important for the secretion of innate cytokine. The combination of IL-17A and IL-33 exacerbated neutrophilic inflammation and airway hyperreactivity in a murine model of asthma. This was associated with increased levels of CXC chemokines

and with infiltration by neutrophils [89]. Taken together, data suggest that IL-17A–mediated type 2 immune responses might amplify and sustain chronic inflammation in AD. Here, the proportion of ILC3s in PBMCs from patients with AD was higher than that in healthy volunteers. Bruggen *et al.* used in situ mapping to detect prominent aryl hydrocarbon receptor-positive ILC3s in the skin of mice and patients with AD. Notably, most ILCs present in healthy human skin are ILC1s and ILC3s, not ILC2s [69]. Consequently, skin-resident ILC3s may rapidly sense environmental changes by interacting with keratinocytes and fibroblasts under atopic conditions.

ILC3s have also been reported in patients with psoriasis, which is chronic inflammatory skin disease characterized by inflamed scaly lesions [90]. Compared to AD, psoriasis is highly involved in the IL-23/IL-17/IL22 axis of innate and adaptive immunity [80]. Therefore, the results obtained in mouse model could be mistaken as an immune response to psoriasis. However, the histology of skin in model mice differs from that in patients with psoriasis. In addition, IL-4 and IL-13 are absent in skin from patients with psoriasis. Secukinumab is used in the treatments of patients with psoriasis, and neutralizes IL-17A. This treatment resulted in a Psoriasis Area Severity Index 90/100 response of 78/53%, respectively, at Week 52 [91]. Conversely, AD treatments are focused on the inhibition of IL-4 and IL-13. Eczema Area and Severity Index (EASI) 90 was found to average 30% at Week 16, which is low compared to that reported for psoriasis. Thus, AD may be driven by a complex immune mechanism. The results of the present study suggest that ILC3s play a critical role in the pathogenesis of AD and psoriasis. Villanova *et al.* identified ILC3s in the epidermis and dermis of skin from healthy volunteers [59].

They also reported the presence of distinct populations of ILC3s in PBMCs from patients with psoriasis and AD. ILC3s, which carry natural cytotoxicity receptors (NCR), are increased in PBMCs from patients with psoriasis; however, these cells do not show increased numbers in PBMCs from patients with AD. Consistent with their findings, ILC3s in PBMCs from patients with AD were mostly NCR^- ILC3s (data not shown). Furthermore, the expression of CLA in ILCs does not differ between healthy volunteers and patients with AD. Thus, increased ILC3s and ILC2s in PBMCs, which migrate to the skin from patients with AD, contribute to the pathogenesis of AD.

There is potential plasticity of ILC1s-ILC3s in PBMCs (Figure 34). The percentage of ILC1s and ILC3s among ILCs changed significantly. $CD127^+$ ILC1s can differentiate toward ILC3s in response to IL-1β and IL-23 [92]. Differentiated ILC3s upregulate the expression of IL-22 and RORγt in the small and large intestine following the injection of ex-$ROR\gamma t^+$ ILC3s. (ILC3s-derived ILC1s). While further study is needed to confirm the plasticity of ILCs, the transdifferentiation of ILC3s to ILC1s may represent a potential treatment target for the alleviation of AD symptoms.

Interestingly, the results obtained from the neutrophils of mice and humans in the present study were conflicting. The observation that neutrophil levels do not increase in patients with AD was consistent with observations in previous studies. However, neutrophils from AD mice were found to have an important role in the development of AD. The infiltration of neutrophils in AD lesions was the main source of IL-1β that stimulated ILCs to produce IL-17A, although this was an inactive precursor, pro-IL-1β. IL-1β is a potent activator of ILC2s, which enhances

the responsiveness of ILC2s to epithelial-cell-derived cytokines such as IL-25, IL-33, and TSLP [93]. Neutrophils stimulate both ILC2s and ILC3s in an AD mice model. Patients with AD experience chronic itching in eczema. Walsh *et al.* reported that neutrophils promote chronic itching during the development of AD via activation of sensory neurons [94]. The present results revealed a positive correlation between ILC3s and neutrophils (Figure 35). Collectively, these data indicate that AD patients who have high levels of ILC3s in PBMCs may suffer severe itching because of neutrophils.

IL-33 is a member of the IL-1 superfamily of cytokines, and is expressed by epithelial and endothelial cells. IL-33 is considered to be an alarmin and is released during necrosis and apoptosis [95]. IL-33 is well known as a stimulator or activator of type 2-related immune responses, and induces eosinophils, mast cells, and basophils to promote survival and the production of inflammatory cytokines in these cells [96, 97]. IL-33 can also activate dendritic cells to drive polarization of naïve T cells to Th2 cells, and Th2 cells to produce IL-4, IL-5, and IL-13. Serum IL-33 is positively correlated with EASI scores in patients with AD [98]. Thus, IL-33 plays a pivotal role in the pathogenesis of AD. Considering the IL-17A–mediated IL-33 expression reported in the present study, IL-33 might have potent roles in the pathogenesis of psoriasis, which mainly involves Th17-related cytokines. However, it is unclear whether IL-33 is involved in the pathogenesis of psoriasis. Tamagawa-Mineoka *et al.* reported no significant difference in the levels of serum IL-33 between patients with psoriasis and healthy subjects [98]. However, another group reported that serum IL-33 levels are increased in patients with psoriasis [99]. Studies in an imiquimod-induced mice model have also reported controversial results.

Clinical and histological evaluation of imiquimod-induced psoriasis revealed no difference between wild-type and IL-33-knock out mice [100]. Zeng *et al.* reported that mice intradermally injected with IL-33 present a psoriasis-like phenotype and that IL-33 deficiency ameliorates imiquimod-induced psoriasis in mice [101]. Skin thickness is increased in imiquimod-induced mice injected with IL-33, and IL-33 promotes proliferation and autophagy-related protein expression in keratinocytes [102]. Therefore, these data indicate that IL-33 is associated with skin inflammation. In addition, IL-17A–mediated IL33 expression, as identified in this study, is likely to be more important in the pathogenesis of AD than in that of psoriasis.

In this study, the NC/Nga mouse model was used to determine whether ILC3s are associated with the pathogenesis of AD. However, there were several differences in the ILC population between mouse models. ILC distribution in AD lesions differed depending on the materials used to induce AD, such as HDM, oxazolone, and MC903. Because the protein expression of IL-1α/β was not increased in a mouse model of MC903-induced AD, there was no change in the levels of ILC3s [103, 104]. A Th2-dominant cytokine profile was observed in the MC903-induced model of AD; however, Li *et al.* reported a decrease in IL-13 when using IL-1 α/β knockout mice [104]. Those authors suggested that IL-1 α/β may play a minor role in MC903-induced AD. To confirm that this was consistent with the present study, further research should be performed using an IL-1 α/β blocking antibody.

There are limitations to this study to identify the sole effect of ILC3s. Genetic engineering in NC/Nga mice is not permitted. Because the genetic background of NC/Nga mice is not fully understood [105], an IL-17A blocking antibody was

alternatively used in this study. This approach inhibits the functions of cells that produce IL-17A, as well as ILC3s. Since the results were similar to those obtained in NC/Nga mice in the HDM-induced AD mice model using C57BL/6 mice, further experiments using C57BL/6 mice should be performed. Use of an inducible conditional knock out system for loss-of-function, for example, $Id2^{CreER}$ x $Rorc(\gamma t)^{flox}$, would be of interest [106]. ILC populations were investigated using PBMCs from patients. Unlike mice experiments, I was unable to obtain human tissue; therefore, the population of ILCs in human skin could not be determined. Instead, expression of cutaneous lymphocyte antigen, skin-homing molecules, was determined in ILCs. Approximately 40% of ILCs expressed cutaneous lymphocyte antigen and could migrate from the blood to the skin. I found that Asian patients with AD presented high levels of ILC3s as well as Th17 cells [36].

AD is a chronic and relapsing skin condition characterized by a type 2 immune response [107]. Several monoclonal antibodies are currently used for the treatment of AD. For example, dupilumab, which blocks the IL-4 receptor α and inhibits IL-4 and IL-13 signaling, has recently been approved by the U.S. Food and Drug Administration for the treatment of adult patients with moderate-to-severe AD [108, 109]. The therapeutic effect of dupilumab was found to exceed 85% in a phase III trial [110]. Although dupilumab demonstrated high effectiveness for adolescent AD, it did not lead to a complete recovery of the condition [110]. The EASI 50, EASI 75, and EASI 90 results indicate the percentage of patients with symptomatic improvements over 50, 75, and 90%. EASI 50 was at least 85%, while EASI 90 was between 30 and 50% in cohorts of patients from Korea, China, The Netherlands, and Italy [34, 111]. It is possible that the treatment effects differ due to the

heterogenous phenotype of AD, which involves an immune response unrelated to IL-4 and IL-13. This study identified a pathway that strengthens the type 2 immune response via ILC3-induced IL-33 expression. These findings suggest that ILC3s, IL-17A, and IL-33 may represent potential therapeutic targets. Although no agent is available that directly inhibits ILC3s, a small-molecule FimH antagonist, Sibofimloc, is currently being investigated in a phase II clinical trial in patients with Crohn's disease. This drug reduces the pathogenicity of ILC3s by manipulating the microbiome [112]. Another target is IL-33 secreted by keratinocytes and fibroblasts. Etokimab and itepekimab, monoclonal antibodies against IL-33, have been studied in a phase II clinical trial in patients with AD. Although they are less effective than dupilumab, they remain candidates for the treatment of AD [113, 114]. Finally, IL-17A is a treatment target for AD. Secukinumab, which binds to IL-17A, does not exert its effects due to activation of type 2 immune response [115]. Ungar *et al.* reported an improvement in AD patients receiving secukinumab compared to those receiving a placebo; however, the improvement was not statistically significant. One phenotype of AD is characterized by a combination of increased Th2- and Th17-related cytokines. In this study, IL-17A was found to enhance the type 2 immune response in AD. Therefore, the combination of dupilumab with secukinumab may provide a treatment option for patients with AD who are not controlled on dupilumab **(Figure 37)**.

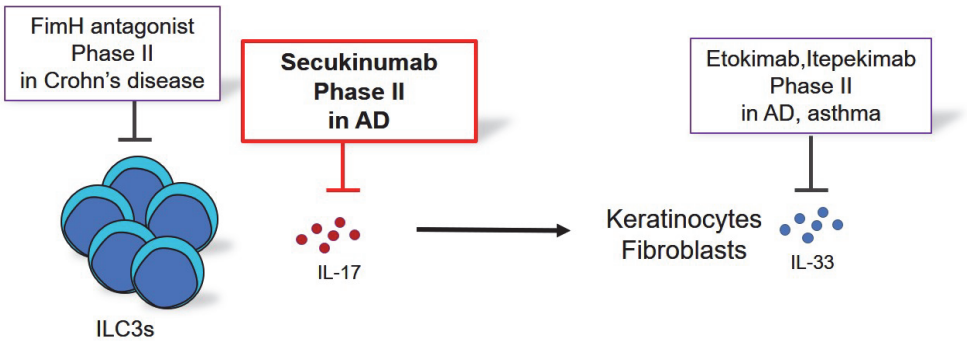

Figure 37. Therapeutic target discovered from this study

A small-molecule FimH antagonist, Sibofimloc, is being investigated in a phase II clinical trial in patients with Crohn's disease. FimH reduces the pathogenicity of ILC3s by manipulating the microbiome. Etokimab and itepekimab have been investigated in a phase II clinical trial in patients with AD. Although they are less effective than dupilumab, it remains a candidate for the treatment of AD. Secukinumab, which binds to IL-17A, has been investigated in a phase II clinical trial in patients with AD; however, alternatives or personalized treatments are needed.

CONCLUSION

In conclusion, this study revealed that ILC3s play a novel role in the immunopathogenesis of AD by increasing IL-33 production from non-immune cells. In AD lesions from mice, high levels of ILC3s were found to secrete IL-17A. This cytokine promoted keratinocytes and fibroblasts to express IL-33, which is an alarmin that exacerbates the type 2 immune responses. Furthermore, the levels of both ILC2s and ILC3s were increased in peripheral blood from Asian patients with AD, suggesting a contribution of human ILC3s to the development of AD. In this study, if type 2 immune responses are not considered, the effect of ILC3s and IL-17A could be mistaken as the sole immune response to AD. However, the findings of this indicate that another axis induces type 2 immune responses, which is representative of the immune response in AD. These results indicate that the objectives of the study were met, which aimed to investigate whether ILC3s and IL-17A are associated with AD. In addition, a role of ILC3s in AD was identified. Thus, these findings may support research on alternative therapeutic strategies for patients with AD that are uncontrolled on immunotherapy (**Figure 38**).

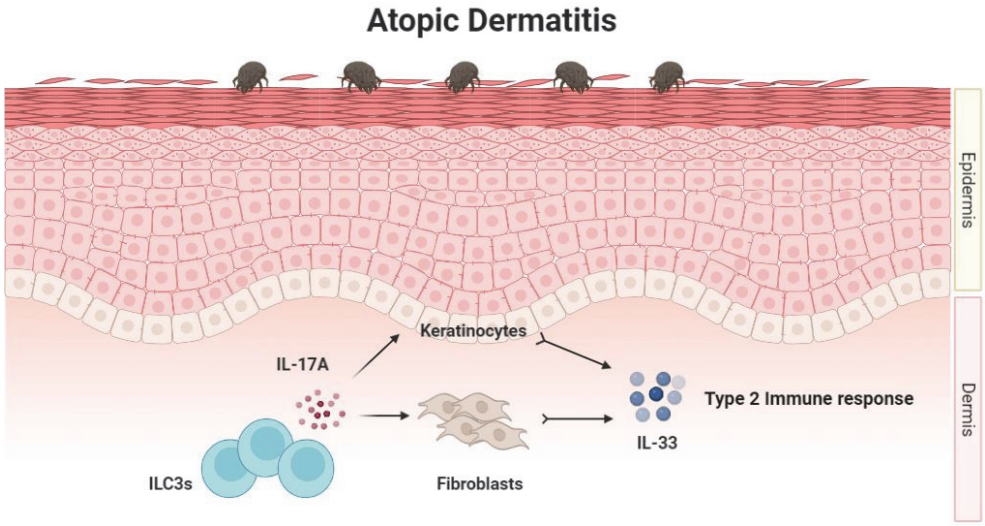

Figure 38. Graphical summary of this study

ILC3s play a role in the immunopathogenesis of AD in a mouse model. In AD lesions, increased ILC3s secrete IL-17A, which induces IL-33 expression by keratinocytes and fibroblasts. IL-33 is one of the alarmins that exacerbates type 2 immune responses.

REFERENCE

1. Weidinger, S. & Novak, N. Atopic dermatitis. *Lancet* **387**, 1109-1122 (2016).

2. McKenzie, C. & Silverberg, J.I. The prevalence and persistence of atopic dermatitis in urban United States children. *Ann Allergy Asthma Immunol* **123**, 173-178 e171 (2019).

3. Silverberg, J.I. *et al.* Atopic dermatitis in the pediatric population: A cross-sectional, international epidemiologic study. *Ann Allergy Asthma Immunol* **126**, 417-428 e412 (2021).

4. Barbarot, S. *et al.* Epidemiology of atopic dermatitis in adults: Results from an international survey. *Allergy* **73**, 1284-1293 (2018).

5. Brunner, P.M. & Guttman-Yassky, E. Racial differences in atopic dermatitis. *Ann Allergy Asthma Immunol* **122**, 449-455 (2019).

6. Kim, Y. *et al.* Racial/Ethnic Differences in Incidence and Persistence of Childhood Atopic Dermatitis. *J Invest Dermatol* **139**, 827-834 (2019).

7. Stander, S. Atopic Dermatitis. *N Engl J Med* **384**, 1136-1143 (2021).

8. Huet, F. *et al.* Characteristics of Pruritus in Relation to Self-assessed

Severity of Atopic Dermatitis. *Acta Derm Venereol* **99**, 279-283 (2019).

9. Yew, Y.W., Thyssen, J.P. & Silverberg, J.I. A systematic review and meta-analysis of the regional and age-related differences in atopic dermatitis clinical characteristics. *J Am Acad Dermatol* **80**, 390-401 (2019).

10. Spergel, J.M. & Paller, A.S. Atopic dermatitis and the atopic march. *J Allergy Clin Immunol* **112**, S118-127 (2003).

11. Tsakok, T. *et al.* Does atopic dermatitis cause food allergy? A systematic review. *J Allergy Clin Immunol* **137**, 1071-1078 (2016).

12. Gustafsson, D., Sjoberg, O. & Foucard, T. Development of allergies and asthma in infants and young children with atopic dermatitis--a prospective follow-up to 7 years of age. *Allergy* **55**, 240-245 (2000).

13. Grey, K. & Maguiness, S. Atopic Dermatitis: Update for Pediatricians. *Pediatr Ann* **45**, e280-286 (2016).

14. Narla, S. & Silverberg, J.I. The Role of Environmental Exposures in Atopic Dermatitis. *Curr Allergy Asthma Rep* **20**, 74 (2020).

15. Patrick, G.J., Archer, N.K. & Miller, L.S. Which Way Do We Go? Complex Interactions in Atopic Dermatitis Pathogenesis. *J Invest Dermatol* **141**, 274-284 (2021).

16. Langan, S.M., Irvine, A.D. & Weidinger, S. Atopic dermatitis. *Lancet* **396**, 345-360 (2020).

17. Irvine, A.D., McLean, W.H. & Leung, D.Y. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med* **365**, 1315-1327 (2011).

18. Egawa, G. & Kabashima, K. Multifactorial skin barrier deficiency and atopic dermatitis: Essential topics to prevent the atopic march. *J Allergy Clin Immunol* **138**, 350-358 e351 (2016).

19. Bjorksten, B. Genetic and environmental risk factors for the development of food allergy. *Curr Opin Allergy Clin Immunol* **5**, 249-253 (2005).

20. Bessot, J.C. & Pauli, G. [House dust mites allergens]. *Rev Mal Respir* **28**, 475-495 (2011).

21. Fuiano, N. & Incorvaia, C. Dissecting the causes of atopic dermatitis in children: less foods, more mites. *Allergol Int* **61**, 231-243 (2012).

22. Czarnowicki, T., He, H., Krueger, J.G. & Guttman-Yassky, E. Atopic dermatitis endotypes and implications for targeted therapeutics. *J Allergy Clin Immunol* **143**, 1-11 (2019).

23. Bao, K. & Reinhardt, R.L. The differential expression of IL-4 and IL-13

and its impact on type-2 immunity. *Cytokine* **75**, 25-37 (2015).

24. Honda, T. & Kabashima, K. Reconciling innate and acquired immunity in atopic dermatitis. *J Allergy Clin Immunol* **145**, 1136-1137 (2020).

25. Endo, Y. *et al.* The interleukin-33-p38 kinase axis confers memory T helper 2 cell pathogenicity in the airway. *Immunity* **42**, 294-308 (2015).

26. Hammad, H. & Lambrecht, B.N. Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity* **43**, 29-40 (2015).

27. Howell, M.D. *et al.* Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* **120**, 150-155 (2007).

28. Nomura, I. *et al.* Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* **171**, 3262-3269 (2003).

29. Mashiko, S., Mehta, H., Bissonnette, R. & Sarfati, M. Increased frequencies of basophils, type 2 innate lymphoid cells and Th2 cells in skin of patients with atopic dermatitis but not psoriasis. *J Dermatol Sci* **88**, 167-174 (2017).

30. Barata, L.T. *et al.* IL-4- and IL-5-positive T lymphocytes, eosinophils, and mast cells in allergen-induced late-phase cutaneous reactions in atopic

subjects. *J Allergy Clin Immunol* **101**, 222-230 (1998).

31. Oetjen, L.K. *et al.* Sensory Neurons Co-opt Classical Immune Signaling Pathways to Mediate Chronic Itch. *Cell* **171**, 217-228 e213 (2017).

32. Bao, L. *et al.* A molecular mechanism for IL-4 suppression of loricrin transcription in epidermal keratinocytes: implication for atopic dermatitis pathogenesis. *Innate Immun* **23**, 641-647 (2017).

33. Bao, L., Shi, V.Y. & Chan, L.S. IL-4 up-regulates epidermal chemotactic, angiogenic, and pro-inflammatory genes and down-regulates antimicrobial genes in vivo and in vitro: relevant in the pathogenesis of atopic dermatitis. *Cytokine* **61**, 419-425 (2013).

34. Zhou, B. *et al.* Efficacy and Safety of Dupilumab in Chinese Patients With Atopic Dermatitis: A Real-World Study. *Front Med (Lausanne)* **9**, 838030 (2022).

35. Bieber, T. *et al.* Clinical phenotypes and endophenotypes of atopic dermatitis: Where are we, and where should we go? *J Allergy Clin Immunol* **139**, S58-S64 (2017).

36. Noda, S. *et al.* The Asian atopic dermatitis phenotype combines features of atopic dermatitis and psoriasis with increased TH17 polarization. *J Allergy Clin Immunol* **136**, 1254-1264 (2015).

37. Torrelo, A. Atopic dermatitis in different skin types. What is to know? *J Eur Acad Dermatol Venereol* **28 Suppl 3**, 2-4 (2014).

38. Suarez-Farinas, M. *et al.* Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis. *J Allergy Clin Immunol* **132**, 361-370 (2013).

39. Czarnowicki, T. *et al.* Early pediatric atopic dermatitis shows only a cutaneous lymphocyte antigen (CLA)(+) TH2/TH1 cell imbalance, whereas adults acquire CLA(+) TH22/TC22 cell subsets. *J Allergy Clin Immunol* **136**, 941-951 e943 (2015).

40. Esaki, H. *et al.* Early-onset pediatric atopic dermatitis is TH2 but also TH17 polarized in skin. *J Allergy Clin Immunol* **138**, 1639-1651 (2016).

41. Spits, H. & Cupedo, T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol* **30**, 647-675 (2012).

42. Vivier, E. *et al.* Innate Lymphoid Cells: 10 Years On. *Cell* **174**, 1054-1066 (2018).

43. Fuchs, A. *et al.* Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells.

Immunity **38**, 769-781 (2013).

44. Gordon, S.M. *et al.* The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* **36**, 55-67 (2012).

45. Moro, K. *et al.* Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* **463**, 540-544 (2010).

46. Halim, T.Y., Krauss, R.H., Sun, A.C. & Takei, F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* **36**, 451-463 (2012).

47. Liang, H.E. *et al.* Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat Immunol* **13**, 58-66 (2011).

48. Price, A.E. *et al.* Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* **107**, 11489-11494 (2010).

49. Bartemes, K.R., Kephart, G.M., Fox, S.J. & Kita, H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol* **134**, 671-678 e674 (2014).

50. Smith, S.G. *et al.* Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol* **137**, 75-86 e78 (2016).

51. Mjosberg, J.M. *et al.* Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* **12**, 1055-1062 (2011).

52. Lao-Araya, M., Steveling, E., Scadding, G.W., Durham, S.R. & Shamji, M.H. Seasonal increases in peripheral innate lymphoid type 2 cells are inhibited by subcutaneous grass pollen immunotherapy. *J Allergy Clin Immunol* **134**, 1193-1195 e1194 (2014).

53. Monticelli, L.A. *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* **12**, 1045-1054 (2011).

54. Cella, M. *et al.* A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* **457**, 722-725 (2009).

55. Luci, C. *et al.* Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat Immunol* **10**, 75-82 (2009).

56. Sonnenberg, G.F., Monticelli, L.A., Elloso, M.M., Fouser, L.A. & Artis, D. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity* **34**, 122-134 (2011).

57. Hoorweg, K. *et al.* Functional Differences between Human NKp44(-) and NKp44(+) RORC(+) Innate Lymphoid Cells. *Front Immunol* **3**, 72 (2012).

58. Teunissen, M.B.M. *et al.* Composition of innate lymphoid cell subsets in the human skin: enrichment of NCR(+) ILC3 in lesional skin and blood of psoriasis patients. *J Invest Dermatol* **134**, 2351-2360 (2014).

59. Villanova, F. *et al.* Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44+ ILC3 in psoriasis. *J Invest Dermatol* **134**, 984-991 (2014).

60. Geremia, A. *et al.* IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med* **208**, 1127-1133 (2011).

61. Kim, H.Y. *et al.* Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med* **20**, 54-61 (2014).

62. Roediger, B. *et al.* Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol* **14**, 564-573 (2013).

63. Kim, B.S. *et al.* TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med* **5**, 170ra116 (2013).

64. Kim, B.S. *et al.* Basophils promote innate lymphoid cell responses in inflamed skin. *J Immunol* **193**, 3717-3725 (2014).

65. Manjelievskaia, J. *et al.* The direct and indirect costs of adult atopic dermatitis. *J Manag Care Spec Pharm* **27**, 1416-1425 (2021).

66. Werfel, T. *et al.* Cellular and molecular immunologic mechanisms in patients with atopic dermatitis. *J Allergy Clin Immunol* **138**, 336-349 (2016).

67. Fort, M.M. *et al.* IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* **15**, 985-995 (2001).

68. Bjorkstrom, N.K., Kekalainen, E. & Mjosberg, J. Tissue-specific effector functions of innate lymphoid cells. *Immunology* **139**, 416-427 (2013).

69. Bruggen, M.C. *et al.* In Situ Mapping of Innate Lymphoid Cells in Human Skin: Evidence for Remarkable Differences between Normal and Inflamed Skin. *J Invest Dermatol* **136**, 2396-2405 (2016).

70. Yamamoto, M. *et al.* A novel atopic dermatitis model induced by topical application with dermatophagoides farinae extract in NC/Nga mice. *Allergol Int* **56**, 139-148 (2007).

71. Vestergaard, C., Yoneyama, H. & Matsushima, K. The NC/Nga mouse: a model for atopic dermatitis. *Mol Med Today* **6**, 209-210 (2000).

72. Nakamura, T. *et al.* Reduction of skin barrier function by proteolytic activity of a recombinant house dust mite major allergen Der f 1. *J Invest Dermatol* **126**, 2719-2723 (2006).

73. Moosbrugger-Martinz, V., Schmuth, M. & Dubrac, S. A Mouse Model for Atopic Dermatitis Using Topical Application of Vitamin D3 or of Its Analog MC903. *Methods Mol Biol* **1559**, 91-106 (2017).

74. Kim, D., Kobayashi, T. & Nagao, K. Research Techniques Made Simple: Mouse Models of Atopic Dermatitis. *J Invest Dermatol* **139**, 984-990 e981 (2019).

75. Franchi, L., Eigenbrod, T., Munoz-Planillo, R. & Nunez, G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* **10**, 241-247 (2009).

76. Datta, S. *et al.* IL-17 regulates CXCL1 mRNA stability via an AUUUA/tristetraprolin-independent sequence. *J Immunol* **184**, 1484-1491 (2010).

77. Salimi, M. *et al.* A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med* **210**, 2939-2950 (2013).

78. Schwartz, C. *et al.* Spontaneous atopic dermatitis in mice with a defective skin barrier is independent of ILC2 and mediated by IL-1beta. *Allergy* **74**, 1920-1933 (2019).

79. Imai, Y. *et al.* IL-33-Induced Atopic Dermatitis-Like Inflammation in Mice Is Mediated by Group 2 Innate Lymphoid Cells in Concert with Basophils. *J Invest Dermatol* **139**, 2185-2194 e2183 (2019).

80. Di Cesare, A., Di Meglio, P. & Nestle, F.O. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *J Invest Dermatol* **129**, 1339-1350 (2009).

81. Monteleone, I., Sarra, M., Pallone, F. & Monteleone, G. Th17-related cytokines in inflammatory bowel diseases: friends or foes? *Curr Mol Med* **12**, 592-597 (2012).

82. Harrington, L.E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* **6**, 1123-1132 (2005).

83. Oyoshi, M.K., Murphy, G.F. & Geha, R.S. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *J Allergy Clin Immunol* **124**, 485-493, 493 e481 (2009).

84. He, R., Oyoshi, M.K., Jin, H. & Geha, R.S. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc Natl Acad Sci U S A* **104**, 15817-15822 (2007).

85. He, R. *et al.* Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13. *J Allergy Clin Immunol* **124**, 761-770 e761 (2009).

86. Nakajima, S. *et al.* IL-17A as an inducer for Th2 immune responses in murine atopic dermatitis models. *J Invest Dermatol* **134**, 2122-2130 (2014).

87. Werner, S., Krieg, T. & Smola, H. Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol* **127**, 998-1008 (2007).

88. Wojtowicz, A.M. *et al.* The importance of both fibroblasts and keratinocytes in a bilayered living cellular construct used in wound healing. *Wound Repair Regen* **22**, 246-255 (2014).

89. Mizutani, N., Nabe, T. & Yoshino, S. IL-17A promotes the exacerbation of IL-33-induced airway hyperresponsiveness by enhancing neutrophilic inflammation via CXCR2 signaling in mice. *J Immunol* **192**, 1372-1384 (2014).

90. Griffiths, C.E. & Barker, J.N. Pathogenesis and clinical features of

psoriasis. *Lancet* **370**, 263-271 (2007).

91. Magnolo, N. *et al.* Efficacy of Secukinumab Across Subgroups and Overall Safety in Pediatric Patients with Moderate to Severe Plaque Psoriasis: Week 52 Results from a Phase III Randomized Study. *Paediatr Drugs* **24**, 377-387 (2022).

92. Bernink, J.H. *et al.* Interleukin-12 and -23 Control Plasticity of CD127(+) Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria. *Immunity* **43**, 146-160 (2015).

93. Ohne, Y. *et al.* IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat Immunol* **17**, 646-655 (2016).

94. Walsh, C.M. *et al.* Neutrophils promote CXCR3-dependent itch in the development of atopic dermatitis. *Elife* **8** (2019).

95. Moussion, C., Ortega, N. & Girard, J.P. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* **3**, e3331 (2008).

96. Iikura, M. *et al.* IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Lab Invest* **87**, 971-978 (2007).

97. Cayrol, C. & Girard, J.P. Interleukin-33 (IL-33): A nuclear cytokine from

the IL-1 family. *Immunol Rev* **281**, 154-168 (2018).

98. Tamagawa-Mineoka, R., Okuzawa, Y., Masuda, K. & Katoh, N. Increased serum levels of interleukin 33 in patients with atopic dermatitis. *J Am Acad Dermatol* **70**, 882-888 (2014).

99. Mitsui, A. *et al.* Serum IL-33 levels are increased in patients with psoriasis. *Clin Exp Dermatol* **41**, 183-189 (2016).

100. Athari, S.K. *et al.* Collagen-induced arthritis and imiquimod-induced psoriasis develop independently of interleukin-33. *Arthritis Res Ther* **18**, 143 (2016).

101. Zeng, F. *et al.* An Autocrine Circuit of IL-33 in Keratinocytes Is Involved in the Progression of Psoriasis. *J Invest Dermatol* **141**, 596-606 e597 (2021).

102. Duan, Y. *et al.* IL-33 contributes to disease severity in Psoriasis-like models of mouse. *Cytokine* **119**, 159-167 (2019).

103. Li, C. *et al.* Experimental atopic dermatitis depends on IL-33R signaling via MyD88 in dendritic cells. *Cell Death Dis* **8**, e2735 (2017).

104. Zheng, J. *et al.* A novel function of NLRP3 independent of inflammasome as a key transcription factor of IL-33 in epithelial cells of atopic dermatitis.

Cell Death Dis **12**, 871 (2021).

105. Suto, H. *et al.* NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol* **120 Suppl 1**, 70-75 (1999).

106. Cording, S. *et al.* Mouse models for the study of fate and function of innate lymphoid cells. *Eur J Immunol* **48**, 1271-1280 (2018).

107. Boguniewicz, M. Biologic Therapy for Atopic Dermatitis: Moving Beyond the Practice Parameter and Guidelines. *J Allergy Clin Immunol Pract* **5**, 1477-1487 (2017).

108. Thaci, D. *et al.* Efficacy and safety of dupilumab monotherapy in adults with moderate-to-severe atopic dermatitis: a pooled analysis of two phase 3 randomized trials (LIBERTY AD SOLO 1 and LIBERTY AD SOLO 2). *J Dermatol Sci* **94**, 266-275 (2019).

109. Snast, I. *et al.* Are Biologics Efficacious in Atopic Dermatitis? A Systematic Review and Meta-Analysis. *Am J Clin Dermatol* **19**, 145-165 (2018).

110. Stingeni, L. *et al.* Moderate to severe atopic dermatitis in adolescents treated with dupilumab: a multicenter Italian real-world experience. *J Eur Acad Dermatol Venereol* (2022).

111. Zhao, Y. *et al.* The efficacy and safety of dupilumab in Chinese patients with moderate-to-severe atopic dermatitis: a randomized, double-blind, placebo-controlled study. *Br J Dermatol* **186**, 633-641 (2022).

112. Gury-BenAri, M. *et al.* The Spectrum and Regulatory Landscape of Intestinal Innate Lymphoid Cells Are Shaped by the Microbiome. *Cell* **166**, 1231-1246 e1213 (2016).

113. Chen, Y.L. *et al.* Proof-of-concept clinical trial of etokimab shows a key role for IL-33 in atopic dermatitis pathogenesis. *Sci Transl Med* **11** (2019).

114. Wechsler, M.E. *et al.* Efficacy and Safety of Itepekimab in Patients with Moderate-to-Severe Asthma. *N Engl J Med* **385**, 1656-1668 (2021).

115. Ungar, B. *et al.* Phase 2 randomized, double-blind study of IL-17 targeting with secukinumab in atopic dermatitis. *J Allergy Clin Immunol* **147**, 394-397 (2021).

국문초록

아토피 피부염 유사 피부염증에서 그룹 3 선천성 림프구 세포에 관한 연구

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김민호

아토피 피부염은 가려움증과 습진성 피부염을 동반한 만성 알레르기성 질환이다. 아토피 피부염 환자는 가려움증으로 인한 삶의 질 저하와 치료비 부담을 겪고 있다. 최근 치료 방향은 아토피 피부염의 면역 반응을 조절하는 것에 집중되어 있다. 다수의 환자에서 IL-4와 IL-13과 연관된 제2형 면역 반응을 보이는 선행 연구들이 보고되어 있다. 이것을 조절하는 치료가 진행 중이지만 90% 이상 호전된 환자의 비율이 낮기 때문에 아토피 피부염의 밝혀지지 않은 기전에 관한 연구가 필요하다. 선천성 림프구 세포

(ILCs)는 T 세포와 달리 ILCs를 자극할 수 있는 사이토카인에 의해서 즉시 T 세포에 상응하는 effector cytokine을 생성할 수 있기 때문에 선천 면역에서 중요한 세포로 떠오르고 있다. 그룹 2 선천성 림프구 세포(ILC2s)가 아토피 피부염 환자의 피부에서 활성화되어 있다는 선행 연구가 보고되었지만 자세한 연구가 필요하며 ILC2s 외에 다른 ILCs가 발병 기전에 관여하는지 알려져 있지 않다. 본 연구는 아토피 피부염 발병 시 다른 면역 발병 기전과 그룹 3 선천성 림프구 세포(ILC3s)에 대한 역할을 규명할 목적으로 진행하였다. 이를 위해 본 연구에서는 아토피 피부염 마우스 모델과 아토피 피부염 환자, 건강한 사람의 시료를 사용하였다. 본 연구자는 집먼지진드기를 마우스의 피부에 도포한 후, 피부 면역 세포 분석을 시행하였다. 그 결과 ILC2s뿐만 아니라 ILC3s도 증가하여 있는 것을 확인하였다. 집먼지진드기로 아토피 피부염을 유도하는 동안 ILC3s만 분류한 것을 마우스 피부에 주입한 결과 아토피 피부염의 발병이 가속되었다. 반대로 IL-17A 중화항체로 신호 경로를 차단하는 것은 아토피 피부염의 유발을 지연시켰다. 또한, IL-17A 중화항체를 사용한 아토피 피부염 마우스의 피부에서 IL-33의 mRNA 발현이 감소하는 것을 확인하였다. IL-17A 수용체를 발현하고 있는 사람의 각질형성세포와 섬유아세포 층과 ILC3s를 같이 배양할 때, 각질형성세포와 섬유아세포에서 IL-33의 발현이 증가하는 것을 확인하였다. 마지막으로 아토피 피부염 환자의 혈액 내에 ILC2s뿐만 아니라 ILC3s도 증가하였음을 확인하였고 ILCs의 대략 40% 정도가 피부 림프구 항원을 발현하였다. 아토피 피부염 환자의 혈액에서 ILC3s와 호중구 사이의 양의 상관 관계도 관찰되었다. 모두 종합해 보면 본 연구의 결과는 아토

피 피부염 발병에 ILC3s가 IL-33를 증가시켜 제2형 면역 반응에 관여하는 새로운 기전을 제시하였고 기존에 증명하지 못한 IL-17A의 역할을 밝혔다. 이 연구에 따르면 IL-17A을 억제하는 것은 기존 면역 치료의 효과가 미비한 환자들에게 보완 치료법이 될 수 있다.

주요어: 선천성 림프구 세포, 아토피 피부염, IL-17A, IL-33, 각질형성세포, 섬유아세포

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