



IL-17A–Producing Innate Lymphoid Cells Promote Skin Inflammation by Inducing IL-33–Driven Type 2 Immune Responses

Min Ho Kim^{1,10}, Seon-Pil Jin^{2,3,10}, Sunhyae Jang⁴, Ji-Yeob Choi⁵, Doo Hyun Chung^{6,7}, Dong Hun Lee^{2,3,8}, Kyu Han Kim^{2,3,8} and Hye Young Kim^{1,9}

Atopic dermatitis (AD) is a chronic, pruritic, inflammatory skin disease characterized by type 2 cytokines secreted by T helper type 2 cells and group 2 innate lymphoid cells. Despite a high degree of heterogeneity, AD is still explained by type 2 immunity, and the role of IL-17A, which is increased in acute, pediatric, or Asian patients with AD, remains poorly understood. Here, we aimed to investigate the role of IL-17A–producing group 3 innate lymphoid cells (ILC3s), which are unexplored immune cells, in the pathogenesis of AD. We found that the numbers of ILC3s in the skin of AD-induced mice were increased, and that neutralizing IL-17A delayed development of AD. Moreover, adoptive transfer of ILC3s accelerated the symptoms of AD. Mechanically, ILC3s induced IL-33 production by nonimmune skin cells, keratinocytes, and fibroblasts, which promoted type 2 immune responses. Because AD has a complex pathophysiology and a broad spectrum of clinical phenotypes, the presence of ILC3s in the skin and their interaction with nonimmune skin cells could explain the pathogenesis of cutaneous AD.

Journal of Investigative Dermatology (2020) **140**, 827–837; doi:10.1016/j.jid.2019.08.447

INTRODUCTION

Atopic dermatitis (AD) is an allergic disorder characterized by chronic dermatitis (itching, redness, and cracked skin). The prevalence of AD in developed countries is 10–20% (Weidinger and Novak, 2016); however, the pathogenesis of AD is not well defined because of the diverse phenotypes and endotypes of disease (Bieber et al., 2017). Although impaired

skin barrier integrity is a critical defect in AD, patients with AD also show aberrant immune activation characterized by increased expression of epithelial cell–derived cytokines such as IL-33, IL-25, and thymic stromal lymphopoietin in the skin (Kim et al., 2013; Salimi et al., 2013; Savinko et al., 2012; Tatsuno et al., 2015); these cytokines trigger production of type 2 cytokines (such as IL-4 and IL-13), which are major drivers of AD. Dupilumab, a monoclonal antibody (Ab) targeting the IL-4 receptor and blocking the IL-4 and IL-13 signaling pathways, has been used to treat AD. However, the therapeutic effect (75% of symptom relief) of dupilumab is incomplete because of the heterogeneity of AD (Simpson et al., 2016). Therefore, other factors might play essential roles in AD. From this perspective, recent studies suggested that IL-17A contributes to the pathogenesis of AD (Esaki et al., 2016; He et al., 2007; Koga et al., 2008; Nakajima et al., 2014; Noda et al., 2015; Oyoshi et al., 2009; Suárez-Fariñas et al., 2013; Toda et al., 2003). Nevertheless, the role of IL-17A in the pathogenesis of AD remains unclear.

Innate lymphoid cells (ILCs) are immune cells that lack lineage markers associated with T cells, B cells, dendritic cells, macrophages, and granulocytes, but express CD90 (THY1 antigen), CD25 (IL-2 receptor α), and CD127 (IL-7 receptor α) (Wojno and Artis, 2012). Although ILCs lack antigen-specific receptors, they produce effector cytokines in response to environmental stimuli. The ILC family can be subdivided into three groups based on expression of transcription factors and production of effector cytokines: group 1 ILCs, which include classical natural killer cells and T-bet–dependent IFN- γ –producing cells; group 2 ILCs (ILC2s), ROR α – and GATA3–dependent, IL-5– and IL-13–producing cells; and group 3 ILCs (ILC3s), ROR γ t–dependent, IL-17A– and/or IL-22–producing cells (Vivier et al., 2018; Woo et al., 2014).

¹Laboratory of Mucosal Immunology, Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea; ²Department of Dermatology, Seoul National University College of Medicine, Seoul, Korea; ³Laboratory of Cutaneous Aging Research, Biomedical Research Institute, Seoul National University Hospital, Seoul, Korea; ⁴Laboratory of Cutaneous Aging and Hair Research, Seoul National University Clinical Research Institute, Seoul National University Hospital, Seoul, Korea; ⁵Laboratory of Behavioral Systems Epidemiology, Department of Biomedical Sciences, Seoul National University Graduate School, Seoul National University College of Medicine, Seoul, Korea; ⁶Department of Pathology, Seoul National University College of Medicine, Seoul, Korea; ⁷Laboratory of Immune Regulation in Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea; ⁸Institute of Human-Environment Interface Biology, Medical Research Center, Seoul National University, Seoul, Korea; and ⁹Institute of Allergy and Clinical Immunology, Seoul National University Medical Research Center, Seoul, Korea

¹⁰These authors contributed equally to this work.

Correspondence: Kyuhan Kim, Department of Dermatology, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea. E-mail: kyuhkim@snu.ac.kr or Hye Young Kim, Laboratory of Mucosal Immunology, Department of Biomedical Sciences, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea. E-mail: hykim11@snu.ac.kr

Abbreviations: Ab, antibody; AD, atopic dermatitis; HDM, house dust mite; ILC, innate lymphoid cell; ILC2, group 2 innate lymphoid cell; ILC3, group 3 innate lymphoid cell; PBMC, peripheral blood mononuclear cell; rh, recombinant human

Received 7 February 2019; revised 6 August 2019; accepted 16 August 2019; accepted manuscript published online 16 October 2019; corrected proof published online 19 December 2019

All subsets of ILCs are present in both the epidermis and dermis of healthy human skin (Brüggen et al., 2016; Dyring-Andersen et al., 2014). Dermal ILC2s are essential cells for AD as they produce IL-5 and IL-13 (Kim et al., 2013; Roediger et al., 2013). However, in situ mapping of human ILCs reveals that substantial numbers of aryl hydrocarbon receptor-expressing ILC3s reside in the skin of patients with AD (Brüggen et al., 2016). In addition, the T helper type 17-related immune axis is active both in some murine models of AD (He et al., 2007; Nakajima et al., 2014; Oyoshi et al., 2009) and in the skin and peripheral blood mononuclear cells (PBMCs) from patients with AD (Esaki et al., 2016; Koga et al., 2008; Noda et al., 2015; Suárez-Fariñas et al., 2013; Toda et al., 2003). Although the role of ILC3s and IL-17A in AD remains poorly understood, these results suggest that ILC3s, as well as ILC2s, have the potential to contribute to the pathogenesis of AD.

Here, we analyzed the ILC populations in human PBMCs and a murine model of AD to evaluate the immune and epidermal factors that contribute to the development of AD. Increased numbers of IL-17A-producing ILC3s were found in the lesional skin from house dust mite (HDM)-treated mice, oxazolone-treated mice, and in PBMCs from patients with AD. Furthermore, we demonstrated that blockade of IL-17A attenuated disease progress and adoptive transfer of ILC3s accelerated disease development. Also, ILC3s increased IL-33 production from keratinocytes and fibroblasts via IL-17A production. Taken together, these results suggest that not only type 2 immune cells but also IL-17A-secreting ILC3s play a pathogenic role in the development of AD.

RESULTS

Increased type 2 and type 3 immune responses in various models of AD

First, we used an HDM-induced NC/Nga murine AD model, which has a similar phenotype to human AD (Vestergaard et al., 2000), to evaluate the immune responses involved in development of AD. Mice treated with an ointment containing HDM (*Dermatophagoides farinae*) antigens showed prominent eczematous lesions in the dorsal skin, and AD symptom scores increased (Supplementary Figure S1a–c). Also, epidermal thickness and the number of infiltrating eosinophils and mast cells were greater in HDM-treated mice than in control mice (Figure 1a and Supplementary Figure S1d). Because type 2 cytokines are essential for development of AD, we measured production of several cytokines by cells in skin-draining lymph nodes by flow cytometry. As expected, HDM treatment increased the number of IL-13-producing lymphocytes, however, it also increased the number of IL-17A-producing lymphocytes (Supplementary Figure S1e). Moreover, expression of *Il17a* and *Il13* mRNA increased markedly in the dorsal skin of HDM-induced AD mice (Figure 1b). HDM-derived cysteine proteases disrupt the epithelial barrier, resulting in production of innate cytokines from epithelial cells (Nakamura et al., 2006). The expression level of innate cytokines such as *Il33* and *Tslp*, which induce type 2 immune responses, was higher in AD skin than in control skin; in addition, expression of *Il1b* and *Il23* increased, as did the number of IL-17A-producing cells (Figure 1c).

The marked increase in the amount of *Il1b* and *Il17a* in the skin of HDM-treated mice led us to focus on ILC3s because these cells produce IL-17A rapidly in response to IL-1 β (Kim et al., 2014). Flow cytometry analysis revealed greater numbers of IL-17A-producing ILC3s and IL-13-producing ILC2s in the skin of HDM-treated mice than in that of control mice (Figure 1d and e). However, secretion of IL-22 from skin ILC3s was not observed. Among IL-17A-producing cells in the skin, IL-17A secretion by ILC3s increased to the greatest extent after exposure to HDM, although the percentage of T cells was greater than that of ILC3s (Figure 1f and g).

To ascertain whether the increase in ILC3s is a strain or allergen-specific, we induced AD with HDM, MC903, and oxazolone in syngenic C57BL/6 mice and Rag^{-/-} mice, which do not contain mature B and T lymphocytes. (Supplementary Figures S2 and S3 and Figure 2). Topical application of vitamin D3 analog MC903 induces skin inflammation resembling immune perturbations observed in acute lesions of patients with AD (Moosbrugger-Martinez et al., 2017), and 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 such as that observed in human AD (Kim et al., 2019). Similar to previous results, we found an increase in the percentage of ILC2s and ILC3s in HDM-induced C57BL/6 mice (Supplementary Figure S2). However, the number of ILCs in MC903-treated C57BL/6 mice did not increase (Figure 2c); in addition, they showed a predominantly type 2 response (Supplementary Figure S3a and b). In the case of mice with oxazolone-induced AD, we noted an increase in the ILC population and an increase in the production of all cytokines (including IL-17A) secreted by ILCs (Figure 2). Therefore, these data suggest that not only ILC2s but also ILC3s may be associated with the pathogenesis of AD.

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

Next, we tried to identify the cells that produce IL-1 β , which is important for inducing IL-17A production by ILC3s (Kim et al., 2014). Intracellular cytokine staining revealed that both CD45⁺ cells and CD45⁻ cells were a source of IL-1 β in the skin of HDM-treated mice; however, IL-1 β expression in CD45⁺ cells was higher (Supplementary Figure S4a). It is well known that IL-1 β has to be cleaved by cytoplasmic caspase 1 to attain biological activity (Franchi et al., 2009). Western blot analysis confirmed increased expression of mature IL-1 β in the dorsal skin of HDM-treated mice (Supplementary Figure S4b). Among IL-1 β -producing CD45⁺ cells, neutrophils expressed the highest levels of IL-1 β in lesional skin (Supplementary Figure S4c and d). Therefore, increased IL-1 β levels in an atopic environment may contribute to the heterogeneity of disease by inducing type 3 cytokines in addition to type 2 cytokines.

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

Because the previous results suggest that ILC2s and ILC3s produce mixed effects, we needed to confirm the effect of ILC3s alone under atopic conditions. To test this, we sorted

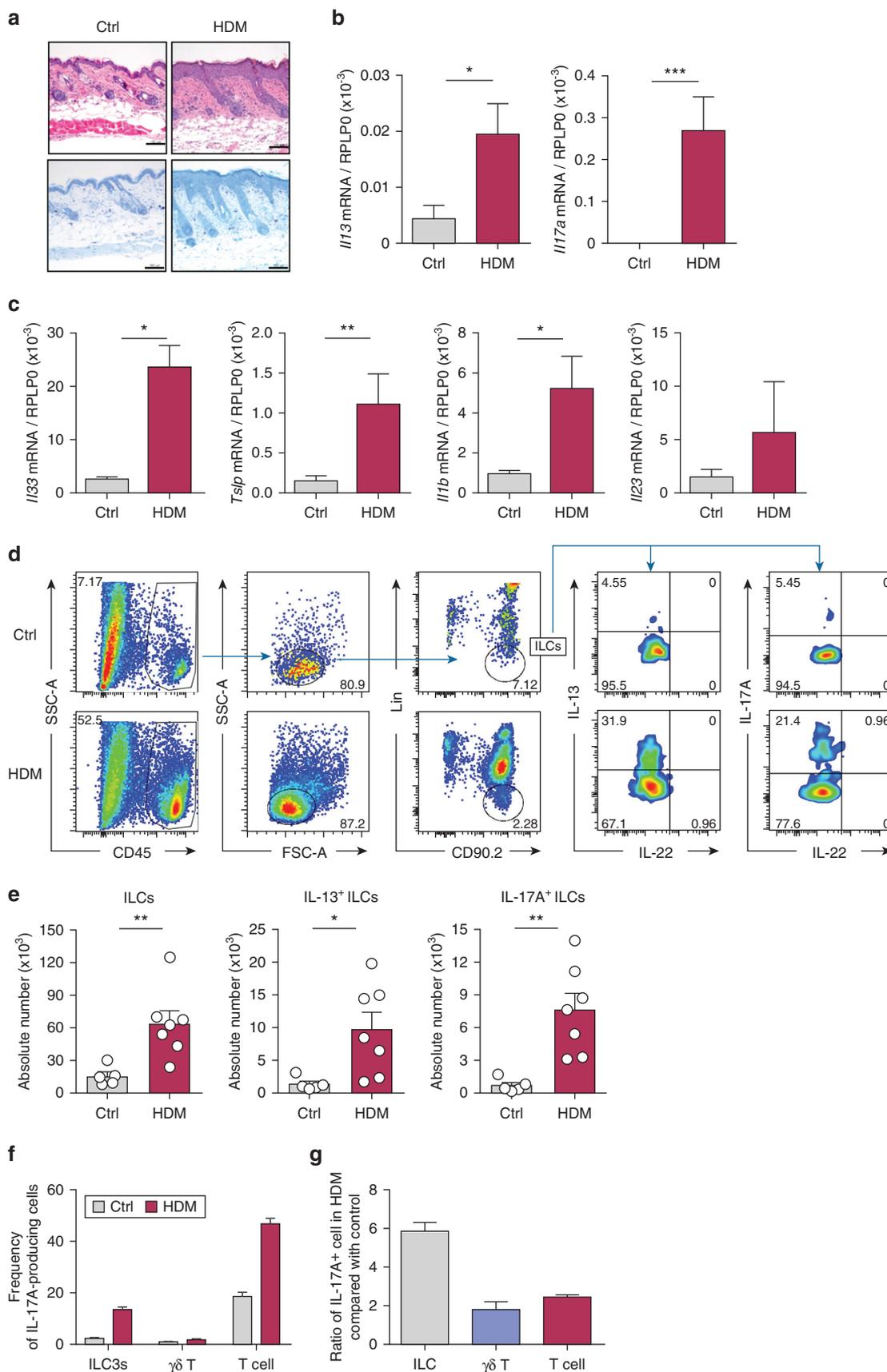


Figure 1. ILC3s as well as ILC2s are present at high numbers in the dorsal skin of an HDM-induced AD model. (a) Representative H&E- and toluidine blue-stained biopsy specimen from dorsal skins. Bar = 100 μ m. (b) mRNA expression of *Il13* and *Il17a* in the dorsal skin (n = 7–9 per group). (c) mRNA expression of innate cytokines in the dorsal skin. Data are obtained from two to three independent experiments (n = 4–5 per group). (d) Gating strategy of cytokine production from ILCs (CD45⁺ Lympho⁺ Lin⁻ CD90.2⁺). (e) Absolute numbers of IL-13⁺ ILCs and IL-17A⁺ ILCs in the skin. Data are representative of

ILC3s from mice with HDM-induced AD and injected them intradermally into recipient mice during induction of AD (Figure 3a). Although the percentage of ILCs producing IL-17A was higher in the skin than in skin-draining lymph nodes and the spleen, we sorted ILC3s from skin-draining lymph nodes and the spleen to reduce the isolation time and to obtain a sufficient number of cells (Supplementary Figure S5a and b). The purity of sorted ILC3s, along with cytokine production, was confirmed by intracellular cytokine staining (Supplementary Figure S5c). Administration of ILC3s to recipient mice accelerated development of AD and increased epidermal thickness and infiltration by inflammatory granulocytes such as mast cells, eosinophils, and neutrophils (Figure 3b–e). Moreover, transfer of ILC3s increased the number of IL-4⁺, IL-13⁺, and IL-17A⁺-producing cells in the skin-draining lymph nodes as well as inflammatory cytokines in the dorsal skin (Figure 3f and Supplementary Figure S5d). These results suggest that ILC3s themselves are sufficient to exacerbate the symptoms of AD.

Blockade of IL-17A attenuates development of HDM-induced AD-like skin inflammation in NC/Nga mice

Because the genetic background of NC/Nga mice is not fully understood, IL-17A-neutralizing Ab secreted by ILC3s (Figure 4a). The efficacy of anti-IL-17A Ab was validated by downregulation of *Cxcl1* and *Il6* mRNA, the representative chemokines induced by IL-17A (Datta et al., 2010) (Supplementary Figure S6). Both ear and epidermal thickness of AD-induced mice treated with the anti-IL-17A Ab was less than that of isotype control mice (Figure 4b–d). In addition, skin inflammation in mice treated with an IL-17A-blocking Ab was less severe than that in mice treated with an isotype control Ab (Figure 4c). Absence of IL-17A signaling resulted in reduced infiltration of eosinophils, mast cells, and neutrophils (Figure 4c–e). Blocking IL-17A in mice with oxazolone-induced AD led to a reduction in infiltration by inflammatory cells (Figure 4g and h).

We also analyzed the expression of several innate cytokines to address the effects of IL-17A blockade on HDM-induced skin inflammation. Notably, expression of *Il33*, an alarmin cytokine that plays crucial roles in atopic inflammation, was reduced by IL-17A blockade, but that of *Tslp* was not in mice with both HDM-induced and oxazolone-induced AD (Figure 4f and i). These results indicate that there may be an unknown pathway for IL-17A-mediated IL-33 modulation of skin inflammation.

ILC3s induce IL-33 secretion by human keratinocytes and fibroblasts

Because blockade of IL-17A reduced expression of *Il33* and infiltration by immune cells (Figure 4e and f), we hypothesized that IL-17A regulates secretion of innate cytokines by skin cells such as keratinocytes and fibroblasts, thereby increasing type 2 immune responses. To address this hypothesis, we added recombinant human (rh) IL-17A

protein (rhIL-17A) directly to the culture medium of human primary keratinocytes or fibroblasts, which express IL-17 receptor A constitutively. Expression of *CXCL1* mRNA, which is induced by IL-17A signaling, increased. Moreover, treatment with rhIL-17A led to marked induction of *IL33* in both human primary keratinocytes and fibroblasts, which represent skin cells (Figure 5a and b).

Because adoptive transfer of murine ILC3s worsened the symptoms of AD, and because rhIL-17A induces *IL33* mRNA expression, we next examined whether ILC3s induce *IL33* expression directly from skin cells and whether this could enhance type 2 immune responses. ILC3s from human PBMCs were cocultured with human primary keratinocytes and fibroblasts using a Transwell system, and rhIL-7 and rhIL-1 β were added to stimulate ILC3s (Figure 5c). Coculture of stimulated ILC3s induced expression of *IL33* and *CXCL1* by keratinocytes and fibroblasts; however, coculture with other types of ILCs (including ILC2s and group 1 ILCs) did not (Figure 5d). In addition, when keratinocytes and fibroblasts were cultured separately, expression of IL33 was lower than when these cells were cultured together (Supplementary Figure S7). It has been reported that cytokine and growth factor expression was modulated when keratinocytes and fibroblasts were cultured separately (Werner et al., 2007; Wojtowicz et al., 2014). Therefore, these results suggest that IL-17A from ILC3s might be responsible for increased expression of IL33, which induces type 2 immune responses.

Patients with AD show an altered ILC composition in peripheral blood

To test whether ILC3s were also elevated in patients with AD, we examined ILCs in the PBMC population by flow cytometry. Human ILC populations can be classified into three groups according to the expression of CD117 and CRTH2 (Vivier et al., 2018). Freshly isolated PBMCs from healthy individuals and patients with AD contained a distinct population of ILCs (Figure 6a). Patients with AD had a higher percentage of ILCs, and the percentage of ILC2s (CRTH2⁺ and GATA3⁺) and ILC3s (c-Kit⁺ and ROR γ t⁺) in the PBMC population from patients with AD were higher than in healthy controls (Figure 6b and c). We noted that not only ILC2s, but also ILC3s, were increased in patients with AD (Figure 6d). Although the neutrophil population in patients with AD was not greater than that in healthy controls (data not shown), ILC3s showed a strong positive correlation with neutrophils in the blood from patients with AD (Figure 6e). To assess whether ILCs within the PBMC population have the potential to home in to the skin, we investigated the expression of cutaneous lymphocyte antigen by ILCs. Approximately 40% of ILCs within the PBMC population expressed cutaneous lymphocyte antigen (Supplementary Figure S8). Although ILC3s are not considered essential for the pathogenesis of AD, these experiments indicate that the

two independent experiments (n = 5–7 per group). Mann-Whitney U test was performed (b–e). (f) Frequency of IL-17A-producing cells in the skin of control mice and mice with AD (n = 2–3 per group). (g) Ratio of IL-17A-positive cells of HDM-induced NC/Nga mice compared with control mice. Data are representative of two independent experiments (n = 3). Data are depicted as mean + SEM. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant. AD, atopic dermatitis; Ctrl, control; FSC-A, forward scatter area; H&E, hematoxylin and eosin; HDM, house dust mite; ILC, innate lymphoid cell; ILC2, group 2 innate lymphoid cell; ILC3, group 3 innate lymphoid cell; SEM, standard error of the mean; SSC-A, side scatter area.

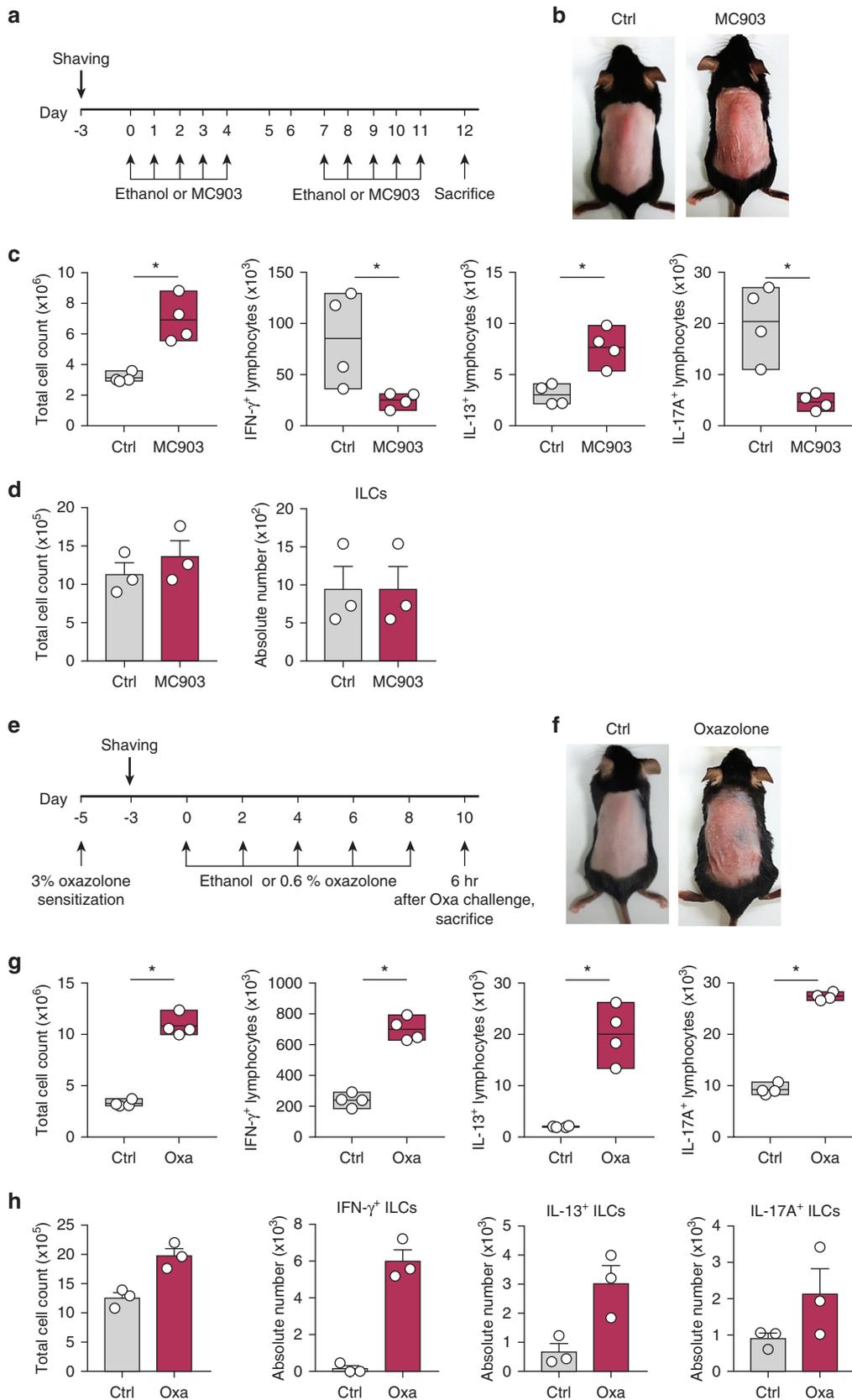


Figure 2. Both ILC2s and ILC3s are higher in oxazolone-treated skin inflammation. (a) The scheme of MC903-treated AD-like model. (b) Representative images of the ethanol and MC903-treated C57BL/6 mice. (c) Total cell count and cytokine-producing lymphocytes in the SdLN (n = 4 per group). Mann-Whitney U test was performed. (d) Total cell count and absolute number of ILCs in the dorsal skin (n = 3 per group). (e) Experiment design of oxazolone-induced skin inflammation. (f) Representative images of control and oxazolone-induced mice. (g) Total cell count and cytokine-producing lymphocytes in the SdLN. Data are representative of two independent experiments (n = 4 per group). Box lines express minimum, mean, and maximum. Mann-Whitney U test was performed. (h) 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. *P < 0.05. AD, atopic dermatitis; Ctrl, control; ILC, innate lymphoid cell; ILC2, group 2 innate lymphoid cell; ILC3, group 3 innate lymphoid cell; Oxa, oxazolone; SdLN, skin-draining lymph node; SEM, standard error of the mean.

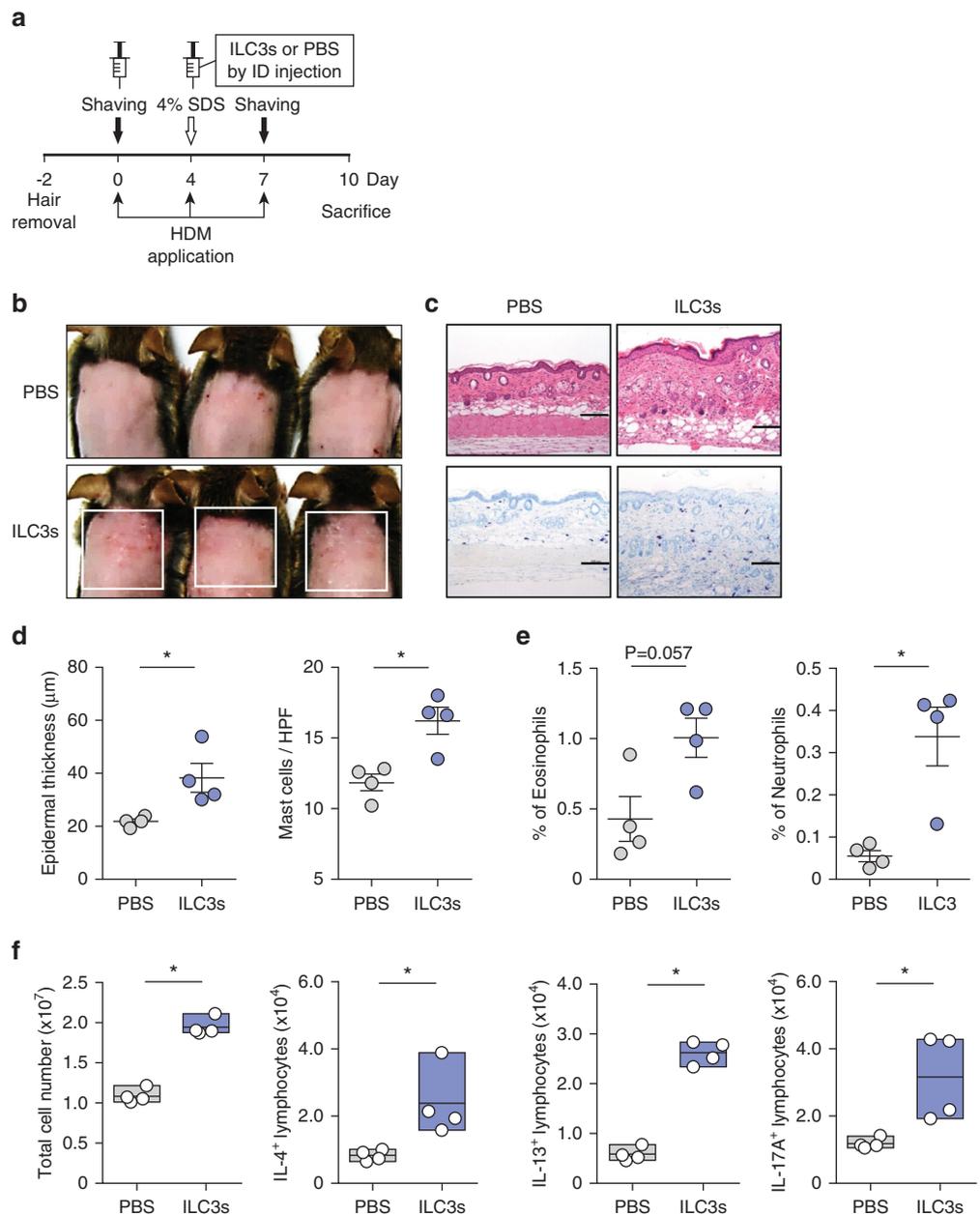
interaction between ILC3s secreting IL-17A and nonimmune cells in the skin induces IL-33 production, which contributes to the development of AD.

DISCUSSION

Several studies report the contribution of ILC2s to the pathogenesis of AD (Kim et al., 2013; Roediger et al.,

Figure 3. 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. (c) Representative H&E- and toluidine blue-staining of each group. Bar = 100 μ m. (d) Epidermal thickness and mast cell counts per HPF. (e) The frequency of eosinophils and neutrophils among CD45⁺ cells in the dorsal skin of PBS-treated or ILC3-transferred AD mice were analyzed by flow cytometry. Data are depicted as mean \pm SEM. (f) 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 minimum, mean, and maximum. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. AD, atopic dermatitis; H&E, hematoxylin and eosin; HDM, house dust mite; HPF, high-power field; ID, intradermal; ILC3, group 3 innate lymphoid cell; PBS, phosphate buffered saline; SEM, standard error of the mean.



2013; Salimi et al., 2013). However, the role of ILC3s in the immunopathogenesis of AD is poorly understood. Here, we identified a previously unreported role for ILC3s in development of AD by using several AD model. We demonstrated that the levels of IL-17A in the skin of mice with HDM-, and MC903-, and oxazolone-induced AD, in addition to type 2 cytokines, increased markedly. Furthermore, adoptive transfer of ILC3s to recipient mice accelerated the development of AD, and blockade of IL-17A reduced the severity of AD. IL-17A-secreting ILC3s increased production of IL-33 by human keratinocytes and fibroblasts. Therefore, early secretion of IL-17A during development of AD might strengthen type 2 immune responses by inducing alarmin signals from skin cells. Likewise, both the ILC2 and ILC3 populations increased in peripheral blood from patients with AD, suggesting a contribution of ILC3s to the development of AD.

Although type 2 cytokines are considered key players in AD, AD is a complex disease with a high degree of heterogeneity with respect to clinical phenotype, which is based on age-of-onset (Esaki et al., 2016), race (Noda et al., 2015), acute versus chronic course (Gittler et al., 2012; Koga et al., 2008), therapeutic response, and infectious or allergic triggers. Recently, the PRACTALL document proposed diverse phenotypes of AD, including type 2 immune response and non-type 2 immune response AD with a combination of T helper type 1-, T helper type 17-, and T helper type 22-driven inflammation, as well as epithelial dysfunction (Muraro et al., 2017). Moreover, the human transcriptomic profile suggested substantial differences in the cytokines driving chronic inflammation between patients from Western and Asian populations; patients from Japan and Korea show strong IL-17A expression in skin lesions in addition to the expected type 2 cytokines. Also, another study reports greater

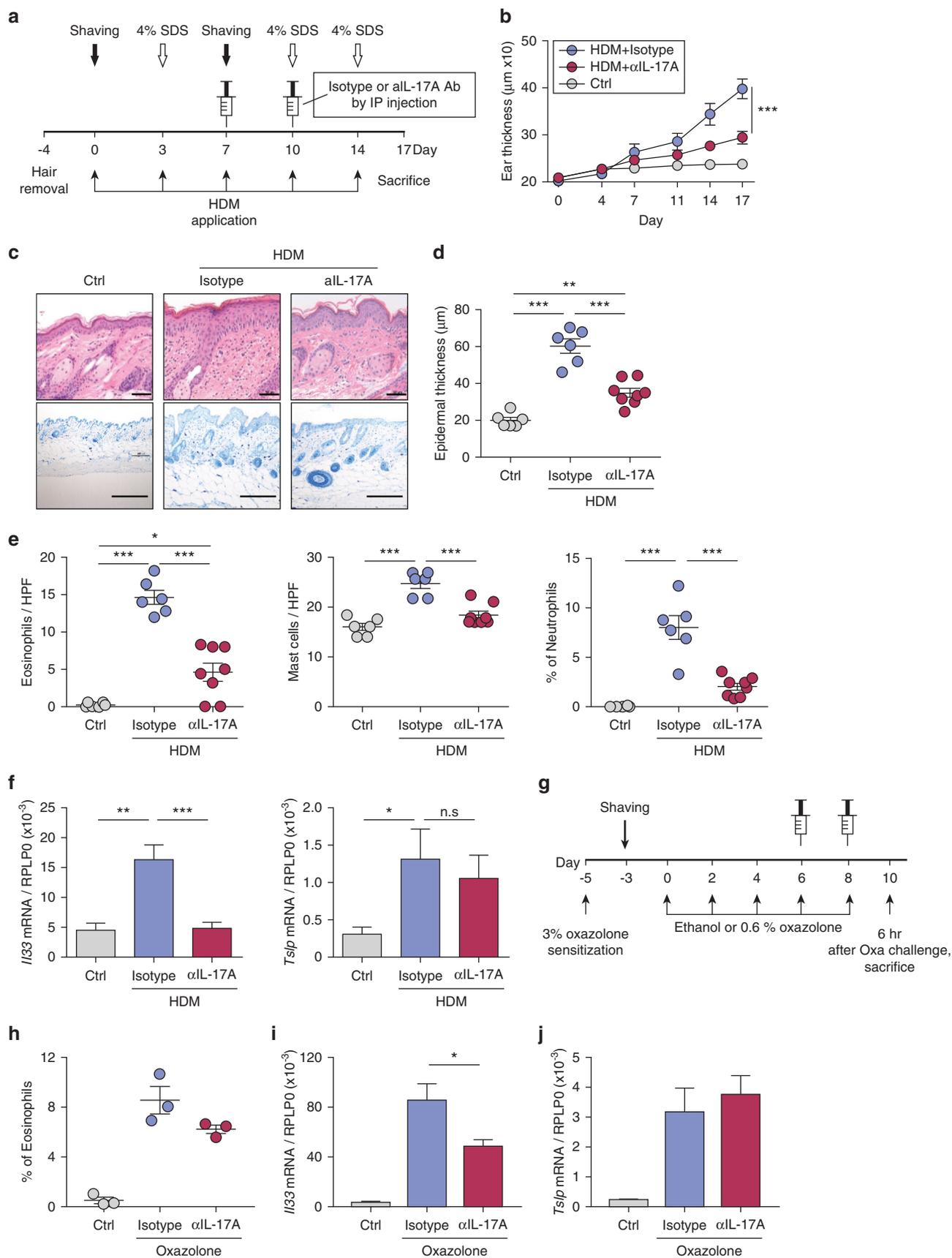
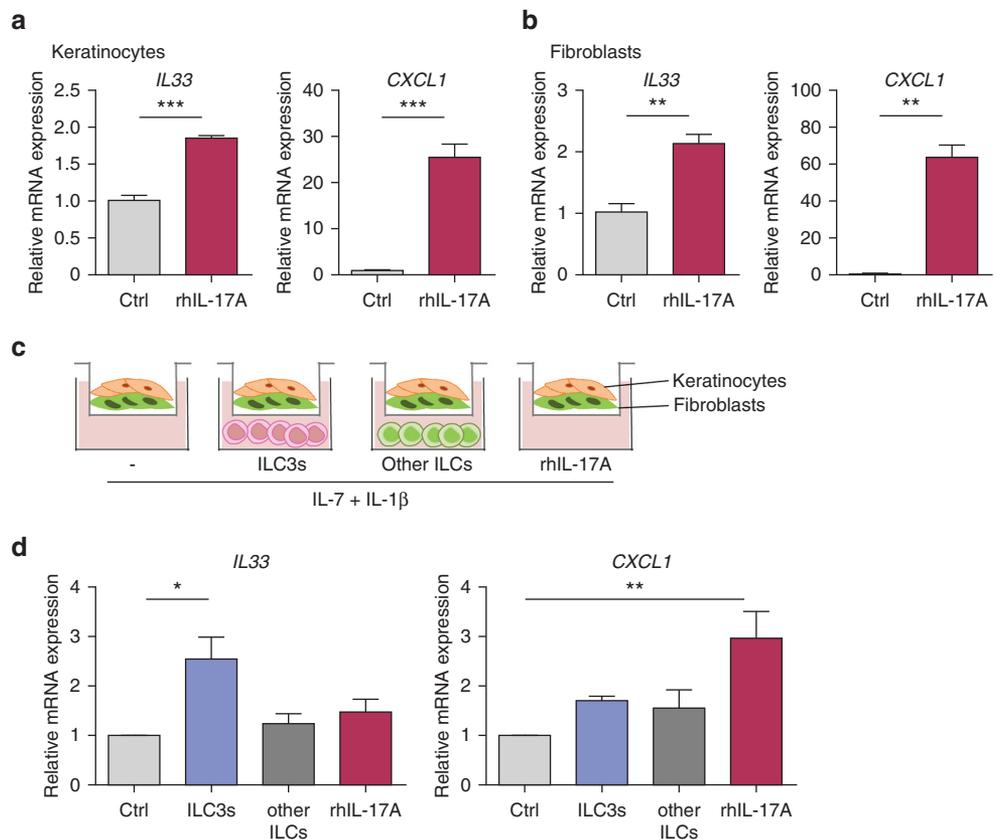


Figure 4. IL-17A-neutralizing impedes the induction of AD. (a) The scheme of IL-17A neutralization in mice with HDM-induced AD. (b) Time-dependent change in ear thickness ($n = 7-11$ per group). Isotype control Ab versus anti-IL-17A Ab on day 17. Mann-Whitney U test was performed. (c) H&E- and toluidine blue-stained dorsal skin from control and mice with HDM-induced AD treated with isotype or anti-IL-17A Ab. Bar = 50 μm (H&E), and 200 μm

Figure 5. ILC3s stimulate the expression of IL-33 from the human keratinocytes and fibroblasts. (a, b)

Relative mRNA expression of *IL33* and *CXCL1* by rhIL-17A from human (a) keratinocytes and (b) fibroblasts. 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. (c) 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: CD3_e, CD11b, CD11c, CD14, CD19, CD49b, and FcεRI α . (d) 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, ***P < 0.001. ILC3, group 3 innate lymphoid cell; ns, not significant; rh, recombinant human; SEM, standard error of the mean.



epidermal hyperplasia and cellular infiltration, and higher levels of T helper type 17–related cytokines and antimicrobials (IL-17A, IL-19, CCL20, LL37, and peptidase inhibitor 3/elafin), in lesional skin from children with AD (Esaki et al., 2016). Despite the heterogeneity of the disease, most research has taken a T helper type 2–biased approach and neglected other pathways. Therefore, it is critical to ascertain whether and how IL-17A is involved in the pathogenesis of AD because IL-17A could be a part of a multiphenotypic inflammatory response.

Several mice studies support the pathogenic role of IL-17A in atopic diseases. For example, filaggrin-deficient mice develop spontaneous eczematous inflammation as they get older, and this inflammation appears to be driven predominantly by IL-17A (Oyoshi et al., 2009). Also, a mouse model of ovalbumin-induced asthma and AD revealed that IL-4/IL-13 null mice sensitized epicutaneously with ovalbumin develop significant eczematous inflammation and increased IL-17A expression. Blockade of IL-17A reverses development of airway hyperresponsiveness and skin inflammation in this model (He et al., 2009). Nakajima et al. reported that IL-17A deficiency attenuated development of AD using flaky-tail

(*Flg^{fl/fl} ma/ma*) mice; they found that V γ 5⁻ dermal $\gamma\delta$ T cells were the major source of IL-17A (Nakajima et al., 2014). However, involvement of ILC3s as one of the principal sources of IL-17A, and the mechanistic impact of this cytokine on AD, remains unclear.

Here, we observed an increase in the number of ILC3s in various murine models of AD. We also used an IL-17A–blocking Ab to demonstrate the potential therapeutic effects of IL-17A, which is secreted by ILC3s. It would be better to examine the difference between ILC3s from wild-type and IL-17^{-/-} mice, but it is not technically possible because the genetic background of NC/Nga mice is not fully understood (Suto et al., 1999). Instead, we identified the role of ILC3s in the following experiments. In an oxazolone-induced AD model, skin inflammation was induced in Rag1^{-/-} mice as well as wild-type C57BL/6 mice. To demonstrate the sole effect of ILC3s, we performed adoptive transfer and coculture experiments. Adoptive transfer of ILC3s into recipient mice accelerated the symptoms of AD. Coculture of sorted ILC3s with human keratinocytes and fibroblasts showed that IL-17A–producing ILC3s induced expression of IL-33 by skin cells. When keratinocytes and

(toluidine blue). (d) Epidermal thickness and (e) eosinophil and mast cell counts per HPF, and frequency of neutrophils among CD45⁺ cells (n = 6–8 per group). Data are depicted as mean \pm SEM. (f) 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. (g) The scheme of IL-17A blockade in oxazolone-induced C57BL/6 mice. (h) Frequency of eosinophils in dorsal skin. Data are representative of two independent experiments (n = 3 per group). (i) mRNA expression of *Il33* and *Tslp* in the dorsal skin of oxazolone-induced C57BL/6 mice treated with isotype or anti-IL-17A Ab. Mann-Whitney U test was performed. Data were obtained from two independent experiments (n = 7 per group). Data are depicted as mean + SEM. *P < 0.05, **P < 0.01, ***P < 0.001. Ab, antibody; AD, atopic dermatitis; Ctrl, control; H&E, hematoxylin and eosin; HDM, house dust mite; HPF, high-power field; ns, not significant; IP, intraperitoneal; SEM, standard error of the mean.

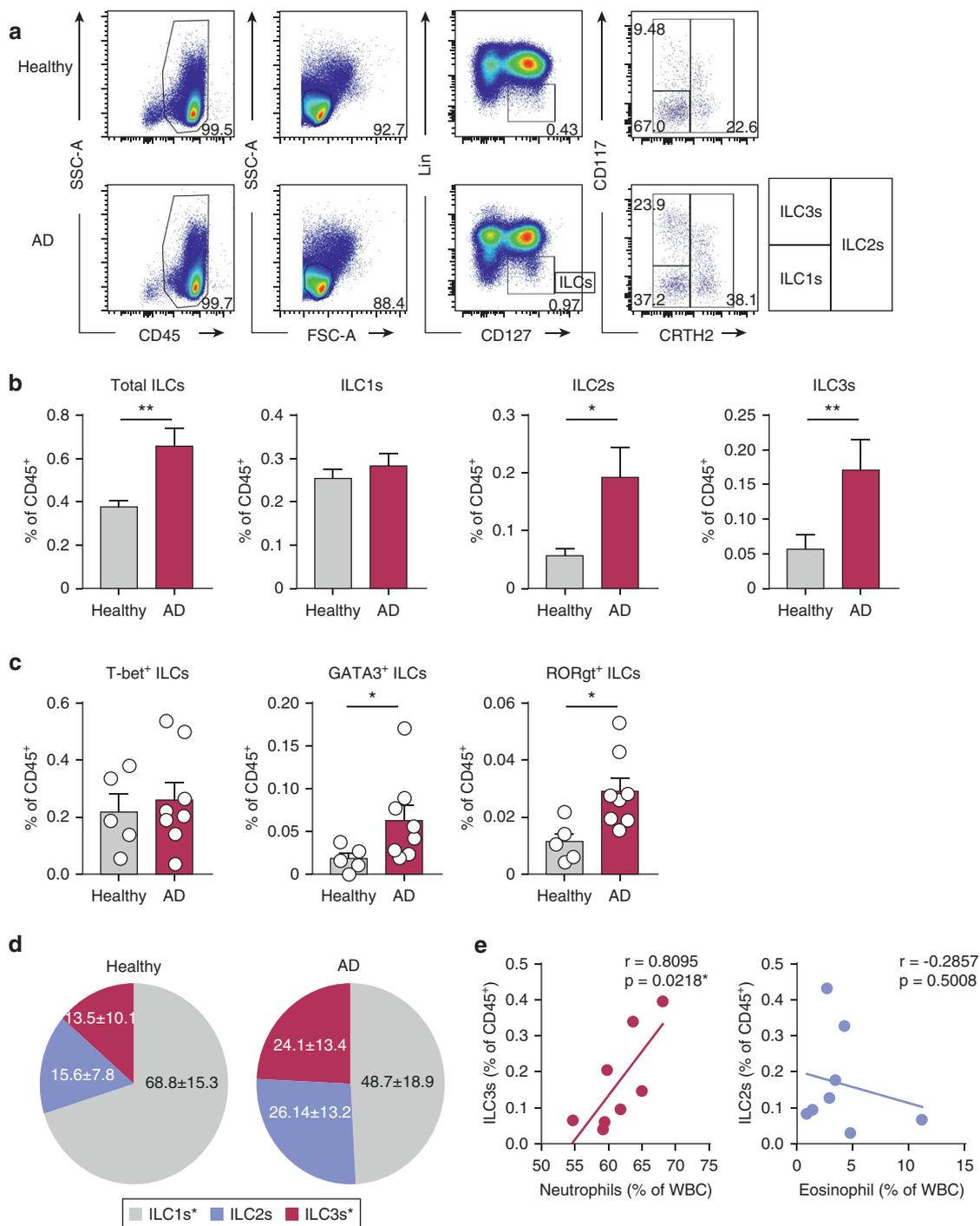


Figure 6. ILC3s account for a large proportion among ILCs in PBMCs from patients with AD. (a) Representative dot plots of ILCs (CD45⁺ Lympho⁺ Lin⁻ CD127⁺) in PBMCs from healthy control or patients with AD. Mean percentage of each cell in plot. Lin: CD3ε, CD11b, CD11c, CD14, CD19, CD49b, and FcεRIα. (b) Frequency of ILCs and the distribution of each subset within the entire ILCs among CD45⁺ cells (n = 9 per group). (c) 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. (d) Pie chart showing the mean ± SD frequency of each ILC subset among ILCs. (e) Correlation between ILC3s and neutrophils (left) and ILC2s and eosinophils (right) in peripheral blood from patients with AD. The correlation was assessed by using Spearman rank correlation. *P < 0.05, **P < 0.01. FSC-A, forward scatter area; SSC-A, side scatter area; AD, atopic dermatitis; ILC, innate lymphoid cell; ILC2, group 2 innate lymphoid cell; ILC3, group 3 innate lymphoid cell; PBMC, peripheral blood mononuclear cell; SD, standard deviation; SEM, standard error of the mean.

fibroblasts were cultured separately, the increase of IL-33 was less than the combined culture. It has been reported that cytokine and growth factor expression was modulated when keratinocytes and fibroblasts were cultured separately (Werner et al., 2007; Wojtowicz et al., 2014). It suggests that

crosstalk between keratinocytes and fibroblasts may be important for innate cytokine secretion, although further studies are required. Similarly, Xu et al. (2018) demonstrated IL-17A-regulated IL-25 expression by keratinocytes. Stimulation with IL-17A, but not IL-17C, IL-1, or tumor necrosis

factor- α , induced robust expression of *Il25* in primary murine keratinocytes. Moreover, *IL-17^{-/-}* mice showed reduced expression of IL-25 in lesional skin in the imiquimod-induced psoriasis model. IL-17A/IL-25 signaling in keratinocytes induces cell proliferation and production of various cytokines and chemokines in a STAT3-dependent manner. Although the models are different, Mizutani et al. (2014) found that the combination of IL-17A and IL-33 exacerbated neutrophilic inflammation and airway hyperreactivity in a murine model of asthma. This phenomenon is associated with increased levels of CXC chemokines, including *Cxcl1*, *Cxcl2*, and *Cxcl5*, and with infiltration by neutrophils (Mizutani et al., 2014). Taken together, the IL-17A/IL-33 circuit might regulate keratinocytes and fibroblasts, which amplifies and sustains chronic inflammation in AD.

Finally, we found that the percentage of ILC3s within the PBMC population from patients with AD was higher than that in healthy volunteers. Similarly, Bruggen et al. (2016) used *in situ* mapping to detect prominent aryl hydrocarbon receptor-positive ILC3 populations in the skin of mice with AD and in patients with AD. It should be noted that most ILCs present in healthy human skin are group 1 ILCs and aryl hydrocarbon receptor-positive ILC3s, not ILC2s (Bruggen et al., 2016). Therefore, skin-resident ILC3s might sense environmental changes rapidly by interacting with nonimmune cells such as skin fibroblasts and keratinocytes under atopic conditions.

It is still unclear whether IL-17A-producing ILC3s and ILC2s act in concert with their T helper cell counterparts, nor is it clear which type of microenvironment contributes to the pathogenic process. Nevertheless, this study provides functional evidence of ILC3s in the skin of patients with AD; the increase in ILC3 numbers drives development of AD by coordinating immune responses in the skin. Because AD is triggered by multicytokine responses in the skin, ILCs may be the primary cell type that senses these initial stimuli, thereby acting as modulators of immune responses by interacting in concert with various immune cells and nonimmune skin cells (keratinocytes and fibroblasts). Taken together, these findings will increase our understanding of the mechanisms underlying onset of AD. They also indicate a need for personalized or precision medicine appropriate for heterogeneous subtypes of AD.

MATERIALS AND METHODS

Detailed materials and methods are provided in the [Supplementary Materials and Methods](#).

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (SNUH-IACUC, #14-0124-C2A0(1)), and animals were maintained in the facility accredited by AAALAC International (#001169) by Guide for the Care and Use of Laboratory Animals, 8th edition, NRC (2017).

Atopic dermatitis (AD) model

NC/Nga mice were anesthetized, and 200 μ l of 4% SDS was applied to the shaved dorsal skin and ear using a cotton swab. After 3 hours, house dust mite (HDM) ointment (130 mg) was applied to the same area. This process was conducted twice a week for 2 weeks. SDS treatment was used for inducing skin barrier disruption.

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, NJ) and DNase I (0.1 mg/ml, Sigma) at 37 °C for 90 minutes with vigorous shaking. Skin samples were filtered through a 40- μ m strainer and red blood cells were lysed with Red Blood Cell Lysis Buffer (Sigma).

Flow cytometry analysis

For live/dead staining, Live/Dead dye (BioLegend, San Diego, CA) was used. To block nonspecific binding, isolated cells were incubated with CD16/CD32 antibody (BD Biosciences, Franklin Lakes, NJ) for 15 minutes.

Cell culture

Primary human dermal fibroblasts and epidermal keratinocytes were obtained from foreskin and cultured in DMEM (WelGENE, Daegu, Korea) with 10% fetal bovine serum and keratinocyte growth medium with supplements (Lonza, Basel, Switzerland) 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. 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, Minneapolis, MN) for 12 hours.

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 μ m (Millicell, Merck Millipore Ltd., Burlington, MA) in DMEM and incubated for 2 days in a 24-well plate. The next day, after washing with phosphate buffered saline twice, 1.5×10^5 primary human epidermal keratinocytes were seeded onto the same culture inserts in keratinocyte growth medium. The medium was changed into the keratinocyte basal medium without supplements (KBM, Lonza, Basel, Switzerland) and then incubated overnight. Isolated ILC3s or ILC others (ILCs other than ILC3s; 1×10^5 cells) were cocultured in the lower compartment of the insert with the established fibroblasts and keratinocytes in KBM and Hank's Balanced Salt Solution 1:1 mixed medium for 48 hours. The medium was provided only to the lower compartment, being supplemented with human recombinant IL-1 β (40 ng/ml, R&D Systems) and IL-7 (5 ng/ml, Miltenyi Biotec). Polycarbonate membranes were removed and washed three times with PBS. The membranes were harvested for further analysis.

Data availability statement

No data sets were generated or analyzed during this study.

ORCIDs

Min Ho Kim: <https://orcid.org/0000-0001-6782-8468>
Seon-Pil Jin: <https://orcid.org/0000-0002-8290-4278>
Sunhyae Jang: <https://orcid.org/0000-0001-7745-8130>
Ji-Yeob Choi: <https://orcid.org/0000-0001-5365-8189>
Doo Hyun Chung: <https://orcid.org/0000-0002-9948-8485>
Dong Hun Lee: <https://orcid.org/0000-0002-2925-3074>
Kyu Han Kim: <https://orcid.org/0000-0001-8376-9090>
Hye Young Kim: <https://orcid.org/0000-0001-5978-512X>

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health & Welfare Affairs, Republic of Korea (HI15C3083 and HI15C1736), the National Research Foundation of Korea (SRC 2017R1A5A1014560), and the Cooperative Research Program of Basic Medical Science and Clinical Science from Seoul National University College of Medicine (800-20140168). Min Ho Kim received a scholarship from the BK21-plus education program, provided by the National Research Foundation of Korea.

AUTHOR CONTRIBUTIONS

Conceptualization: MHK, SPJ, DHL, HYK; Formal Analysis: MHK, SPJ, JYC; Funding Acquisition: KHK, HYK; Investigation: MHK, SPJ, SHJ; Methodology: MHK, SPJ; Project Administration: DHL, KHK, HYK; Resources: DHC, DHL, KHK, HYK; Supervision: KHK, HYK; Visualization: MHK, HYK; Writing - Original Draft Preparation: MHK, SPJ, HYK; Writing - Review and Editing: MHK, SPJ, DHC, DHL, KHK, HYK.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2019.08.447>.

REFERENCES

- Bieber T, D'Erme AM, Akdis CA, Traidl-Hoffmann C, Lauener R, Schäppi G, et al. Clinical phenotypes and endophenotypes of atopic dermatitis: where are we, and where should we go? *J Allergy Clin Immunol* 2017;139: S58–64.
- Brüggen MC, Bauer WM, Reininger B, Clim E, Captarencu C, Steiner GE, et al. In situ mapping of innate lymphoid cells in human skin: evidence for remarkable differences between normal and inflamed skin. *J Invest Dermatol* 2016;136:2396–405.
- Datta S, Novotny M, Pavicic PG, Jr., Zhao C, Herjan T, Hartupce J, et al. IL-17 regulates CXCL1 mRNA stability via an AUUUA/tristetraprolin-independent sequence. *J Immunol* 2010;184:1484–91.
- Dyring-Andersen B, Geisler C, Agerbeck C, Lauritsen JP, Gúdjonsdóttir SD, Skov L, et al. Increased number and frequency of group 3 innate lymphoid cells in nonlesional psoriatic skin. *Br J Dermatol* 2014;170:609–16.
- Esaki H, Brunner PM, Renert-Yuval Y, Czarnowicki T, Huynh T, Tran G, et al. Early-onset pediatric atopic dermatitis is TH2 but also TH17 polarized in skin. *J Allergy Clin Immunol* 2016;138:1639–51.
- Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 2009;10:241–7.
- Gittler JK, Shemer A, Suárez-Fariñas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQ, et al. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J Allergy Clin Immunol* 2012;130:1344–54.
- He R, Kim HY, Yoon J, Oyoshi MK, MacGinnitie A, Goya S, 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* 2009;124: 761–70.e1.
- He R, Oyoshi MK, Jin H, Geha RS. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc Natl Acad Sci USA* 2007;104:15817–22.
- Kim BS, Siracusa MC, Saenz SA, Noti M, Monticelli LA, Sonnenberg GF, et al. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med* 2013;5:170ra16.
- Kim D, Kobayashi T, Nagao K. Research techniques made simple: mouse models of atopic dermatitis. *J Invest Dermatol* 2019;139:984–90.e1.
- Kim HY, Lee HJ, Chang YJ, Pichavant M, Shore SA, Fitzgerald KA, et al. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med* 2014;20:54–61.
- Koga C, Kabashima K, Shiraishi N, Kobayashi M, Tokura Y. Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol* 2008;128: 2625–30.
- 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* 2014;192:1372–84.
- Moosbrugger-Martinez 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* 2017;1559:91–106.
- Muraro A, Lemanske RF Jr, Castells M, Torres MJ, Khan D, Simon HU, et al. Precision medicine in allergic disease—food allergy, drug allergy, and anaphylaxis—PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma and Immunology. *Allergy* 2017;72:1006–21.
- Nakajima S, Kitoh A, Egawa G, Natsuaki Y, Nakamizo S, Moniaga CS, et al. IL-17A as an inducer for Th2 immune responses in murine atopic dermatitis models. *J Invest Dermatol* 2014;134:2122–30.
- Nakamura T, Hirasawa Y, Takai T, Mitsuishi K, Okuda M, Kato 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* 2006;126: 2719–23.
- Noda S, Suárez-Fariñas M, Ungar B, Kim SJ, de Guzman Strong C, Xu H, et al. The Asian atopic dermatitis phenotype combines features of atopic dermatitis and psoriasis with increased TH17 polarization. *J Allergy Clin Immunol* 2015;136:1254–64.
- Oyoshi MK, Murphy GF, Geha RS. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *J Allergy Clin Immunol* 2009;124:485–93, 493.e1.
- Roediger B, Kyle R, Yip KH, Sumaria N, Guy TV, Kim BS, et al. Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol* 2013;14:564–73.
- Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med* 2013;210:2939–50.
- Savinko T, Matikainen S, Saarialho-Kere U, Lehto M, Wang G, Lehtimäki S, et al. IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. *J Invest Dermatol* 2012;132:1392–400.
- Simpson EL, Bieber T, Guttman-Yassky E, Beck LA, Blauvelt A, Cork MJ, et al. Two phase 3 Trials of Dupilumab versus Placebo in Atopic Dermatitis. *N Engl J Med* 2016;375:2335–48.
- Suárez-Fariñas M, Dhingra N, Gittler J, Shemer A, Cardinale I, de Guzman Strong C, et al. Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis. *J Allergy Clin Immunol* 2013;132:361–70.
- Suto H, Matsuda H, Mitsuishi K, Hira K, Uchida T, Unno T, et al. NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol* 1999;120(Suppl. 1):70–5.
- Tait Wojno ED, Artis D. Innate lymphoid cells: balancing immunity, inflammation, and tissue repair in the intestine. *Cell Host Microbe* 2012;12: 445–57.
- Tatsuno K, Fujiyama T, Yamaguchi H, Waki M, Tokura Y. TSLP directly interacts with skin-homing Th2 cells highly expressing its receptor to enhance IL-4 production in atopic dermatitis. *J Invest Dermatol* 2015;135: 3017–24.
- Toda M, Leung DY, Molet S, Boguniewicz M, Taha R, Christodoulopoulos P, et al. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J Allergy Clin Immunol* 2003;111:875–81.
- Vestergaard C, Yoneyama H, Matsushima K. The NC/Nga mouse: a model for atopic dermatitis. *Mol Med Today* 2000;6:209–10.
- Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell* 2018;174:1054–66.
- Weidinger S, Novak N. Atopic dermatitis. *Lancet* 2016;387:1109–22.
- Werner S, Krieg T, Smola H. Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol* 2007;127:998–1008.
- Wojtowicz AM, Oliveira S, Carlson MW, Zawadzka A, Rousseau CF, Baksh D. The importance of both fibroblasts and keratinocytes in a bilayered living cellular construct used in wound healing. *Wound Repair Regen* 2014;22: 246–55.
- Woo Y, Jeong D, Chung DH, Kim HY. The roles of innate lymphoid cells in the development of asthma. *Immune Netw* 2014;14:171–81.
- Xu M, Lu H, Lee YH, Wu Y, Liu K, Shi Y, et al. An interleukin-25-mediated autoregulatory circuit in keratinocytes plays a pivotal role in psoriatic skin inflammation. *Immunity* 2018;48:787–98.e4.

SUPPLEMENTARY MATERIALS AND METHODS

Animals

Female 4-week-old NC/Nga mice were purchased from Japan SLC, Inc. and housed under specific pathogen-free conditions. C57BL/6 and Rag1^{-/-} mice were purchased from the KOA-TECH (Pyeongtaek, Korea) and the Jackson Laboratory (Bar Harbor, ME), respectively. All experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (SNUH-IACUC, #14-0124-C2A0(1)), and animals were maintained in the facility accredited by AAALAC International (#001169) by Guide for the Care and Use of Laboratory Animals, 8th edition, NRC (2017).

Atopic dermatitis (AD) model

HDM ointment (*Dermatophagoides farinae*) was purchased from Biostir Inc. (Osaka, Japan). The manufacturing process was to (i) breed *D. farinae* for 2 months, (ii) collect mite bodies and remove debris, (iii) extract and purify mite allergens (Der f 1) partially, and (iv) mix the mite allergens with hydrophilic petrolatum. Skin lesions were evaluated as a symptom score comprised of the following: (i) erythema and 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 sum of the scores was taken as the symptom scores (Yamamoto et al., 2007).

For MC903-induced skin inflammation, 100 μ l of MC903 (45 μ M, Santa Cruz Biotechnology, Dallas, TX) or ethanol was daily applied to the shaved dorsal skin of C57BL/6 and Rag1^{-/-} mice. For oxazolone-treated skin inflammation, C57BL/6 and Rag1^{-/-} mice were sensitized by topical application of 3% oxazolone (Sigma, Burlington, MA) on the shaved abdomen (30 μ l). After 5 days, mice were treated topically with 100 μ l of 0.6% oxazolone on the shaved dorsal skin every other day for 10 days.

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, NJ) and DNase I (0.1 mg/ml, Sigma) at 37 °C for 90 minutes with vigorous shaking. Skin samples were filtered through a 40- μ m strainer and red blood cells were lysed with Red Blood Cell Lysis Buffer (Sigma). Skin draining lymph nodes were obtained and ground in a 40- μ m strainer for single cell isolation.

Flow cytometry analysis

For surface staining, the cells were labeled by the following antibodies: FITC anti-Fc ϵ R1 α , anti-F4/80, anti-Ly6C, and anti-TCR γ δ ; PE anti-CD11b and anti-CD3 ϵ ; APC anti-CD117, anti-F4/80, and anti-TCR γ δ ; PE-Cy7 anti-CD127, anti-CD90.2, and anti-Ly6G; BV-421 anti-CD25; BV-650 anti-CD127 and streptavidin; BV-785 anti-CD25; purified anti-CLA; and biotin anti-rat light chain κ (all purchased from BioLegend). From BD Biosciences the following were purchased: FITC anti-CD11b, anti-CD11c, anti-CD19, anti-CD3 ϵ , and anti-CD49b; and BV-421 anti-Siglec-F. From eBioscience (ThermoFisher Scientific, Waltham, MA), the following were purchased: PerCP-Cy5.5 anti-CD45; PE-streptavidin; and biotin anti-ST2. For intracellular cytokine staining, isolated cells were stimulated for 4 hours at 37 °C

with phorbol 12-myristate 13-acetate (50 ng/ml, Miltenyi Biotec, Bergisch Gladbach, Germany), ionomycin (0.5 μ g/ml, Sigma), and Golgi stop (0.7 μ l/ml, BD Biosciences). After stimulation, cells were fixed and permeabilized using the fixation/permeabilization solution kit (BD Biosciences). FITC anti-IL-17A; APC anti-IFN- γ , anti-Pro-IL-1 β , anti-IL-22; BV-421 anti-IL-17A, (all purchased from BioLegend); FITC anti-IL-4 (BD Biosciences); PE anti-IL-13; and PE-Cy7 anti-IL-13 (eBioscience) were used at the concentrations recommended by the manufacturers. The samples were acquired using LSRII and LSRFortessa (BD Biosciences). Data were analyzed with FlowJo 10 software.

To stain human innate lymphoid cells (ILCs), peripheral blood mononuclear cells were isolated from healthy volunteers and patients with AD by centrifugation using a Ficoll-Paque PLUS density gradient (GE Healthcare, Chicago, Illinois). Single cells from blood were blocked with human BD Fc Block (BD Biosciences). Antibodies used for flow cytometric analysis were as follows: PerCP-Cy5.5 anti-CD45; FITC anti-CD3 ϵ , anti-CD19, anti-CD11b, anti-CD11c, anti-CD14, anti-CD49b, and anti-Fc ϵ R1 α ; PE-Cy7 anti-CD127; BV-421 anti-CD117; PE anti-CRTH2 (BD Biosciences); purified anti-CLA; biotin anti-rat Ig light chain κ ; and BV-650 streptavidin. The rest of antibodies were purchased from BioLegend. The samples were acquired using LSRII and LSRFortessa (BD Biosciences). Data were analyzed with FlowJo 10 software.

Adoptive transfer of group 3 ILCs (ILC3s)

Cells were isolated from skin-draining lymph nodes and spleens of mice with HDM-induced AD. ILC3s were sorted by AriaIII (BD Biosciences) after depletion of lineage positive cells using micro beads (lineage depletion kit, Miltenyi Biotec). Mice were injected intradermally with 5,000–7,000 cells in a 2 \times 2 cm area of the dorsal skin 3 hours after shaving or 4% SDS treatment.

Neutralization of IL-17A

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

Histologic examination

The samples were fixed with 4% paraformaldehyde at 4 °C overnight before processing into paraffin wax. Serial sections (4 μ m) were mounted onto silane-coated slides (Dako, Japan) and either subjected to hematoxylin and eosin 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 (fibroblasts and keratinocytes) using RNAiso Plus (Takara Bio, Kyoto, Japan). cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed on an ABI 7500 (Applied Biosystems, Waltham, MA). The data were normalized to *RPLP0*, and relative expressions were depicted using Δ Ct or $\Delta\Delta$ Ct methods.

Human ILCs sorting

Human peripheral blood mononuclear cells 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 Red Blood Cell Lysis Buffer (Sigma), peripheral blood mononuclear cells were stained by CD16/32 antibody (BD Biosciences) for blockade of nonspecific binding, FITC anti-CD3 ϵ , anti-CD19, anti-CD11b, and anti-CD235 α (Biolegend). Anti-FITC microbeads were used to deplete FITC⁺ cells by LS MACS column (Miltenyi Biotec). The FITC⁻ cells were stained by PerCP-Cy5.5 anti-CD45; FITC anti-CD3 ϵ , anti-CD19, anti-CD11b, anti-CD11c, anti-CD14, anti-CD49b, and anti-Fc ϵ RI α ; PE-Cy7

anti-CD127; BV-421 anti-CD117 (Biolegend); and PE anti-CRTH2 (BD Biosciences). ILC3s (CD45⁺ Lin⁺ CD127⁺ CRTH2⁻ CD117⁺) and other ILCs (not ILC3s) were sorted by ArialIII (BD Biosciences).

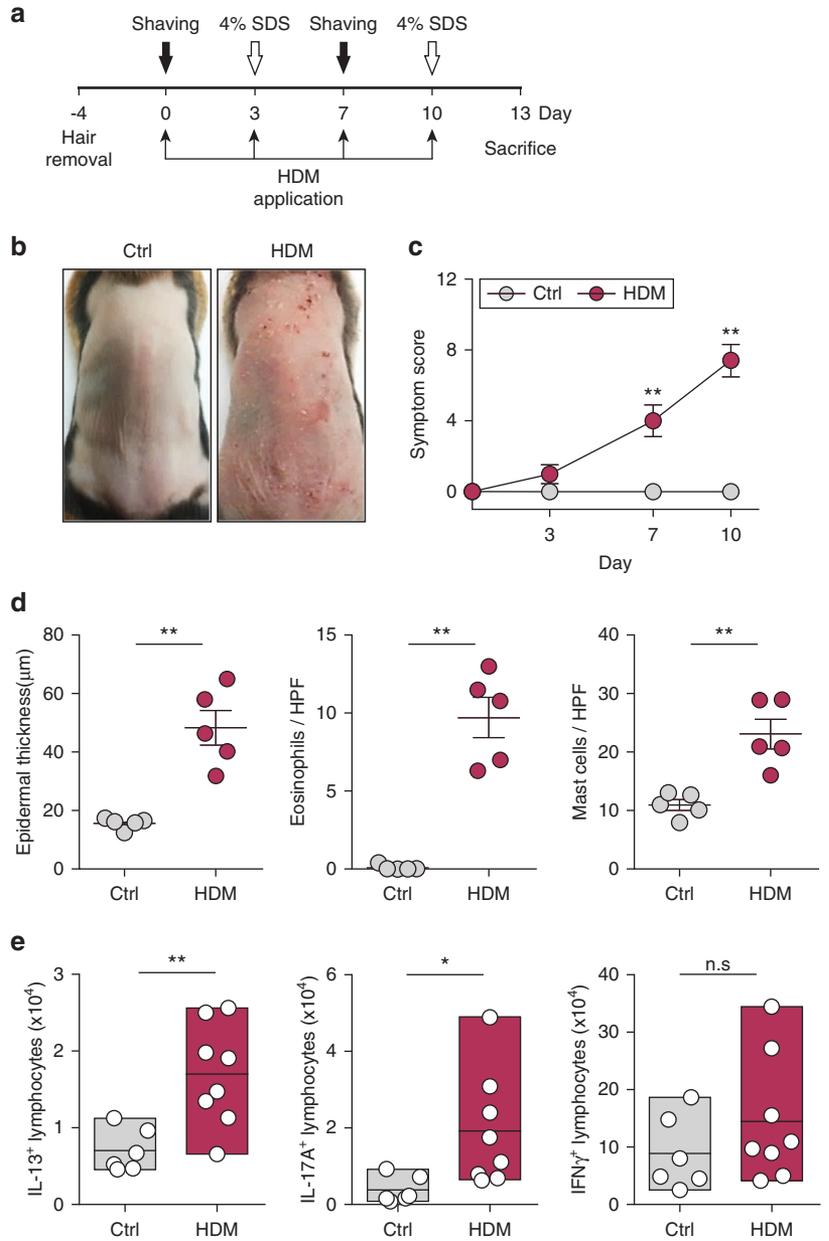
Statistical analysis

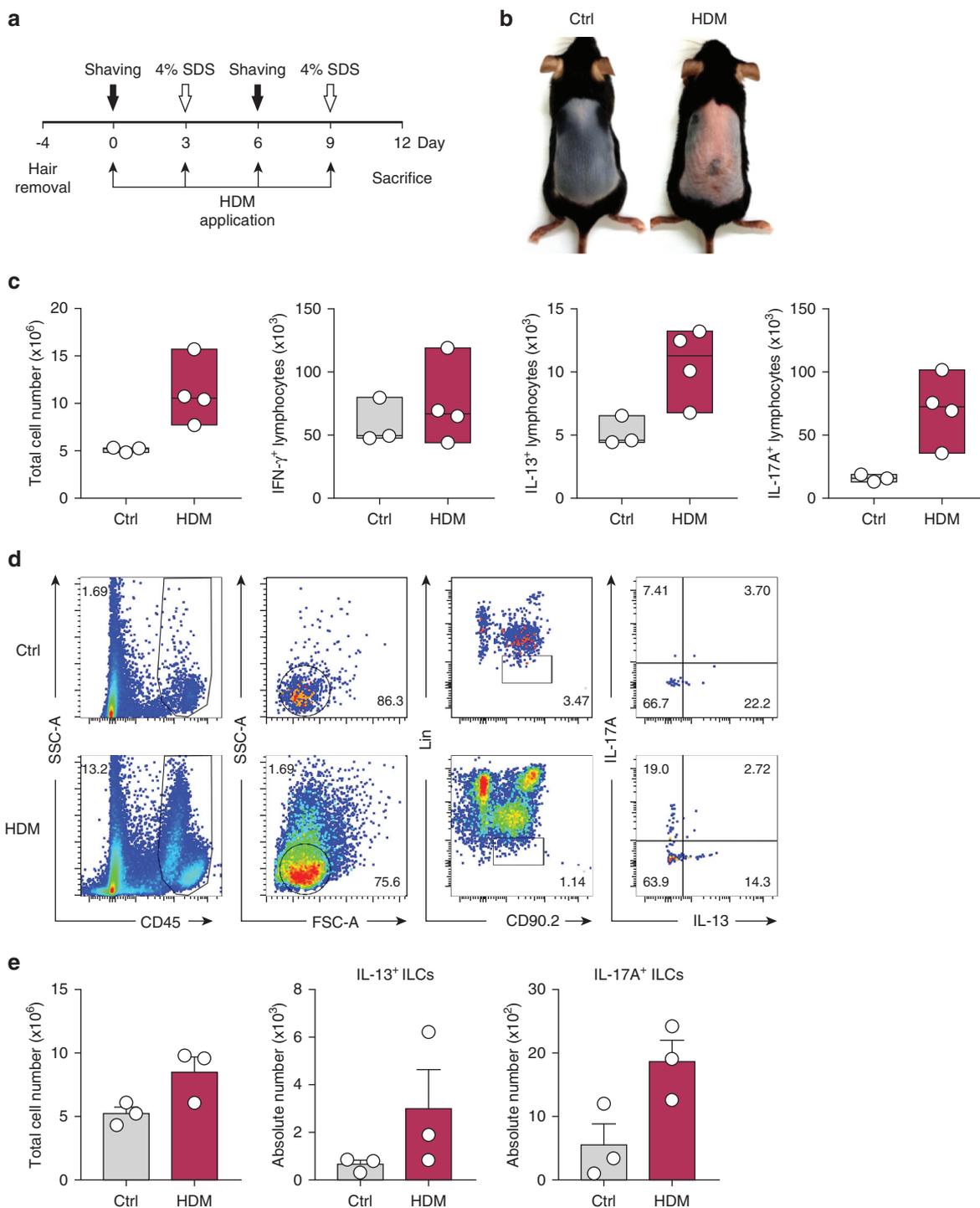
Data were analyzed using Prism 7 (GraphPad software, La Jolla, CA). Comparisons between two groups were performed using Mann-Whitney U test. Comparisons of multiple groups were performed using one-way analysis of variance with Kruskal-Wallis with Dunn's post hoc test. Spearman rank correlation was used for the relationship between two variables.

Supplementary Figure S1.

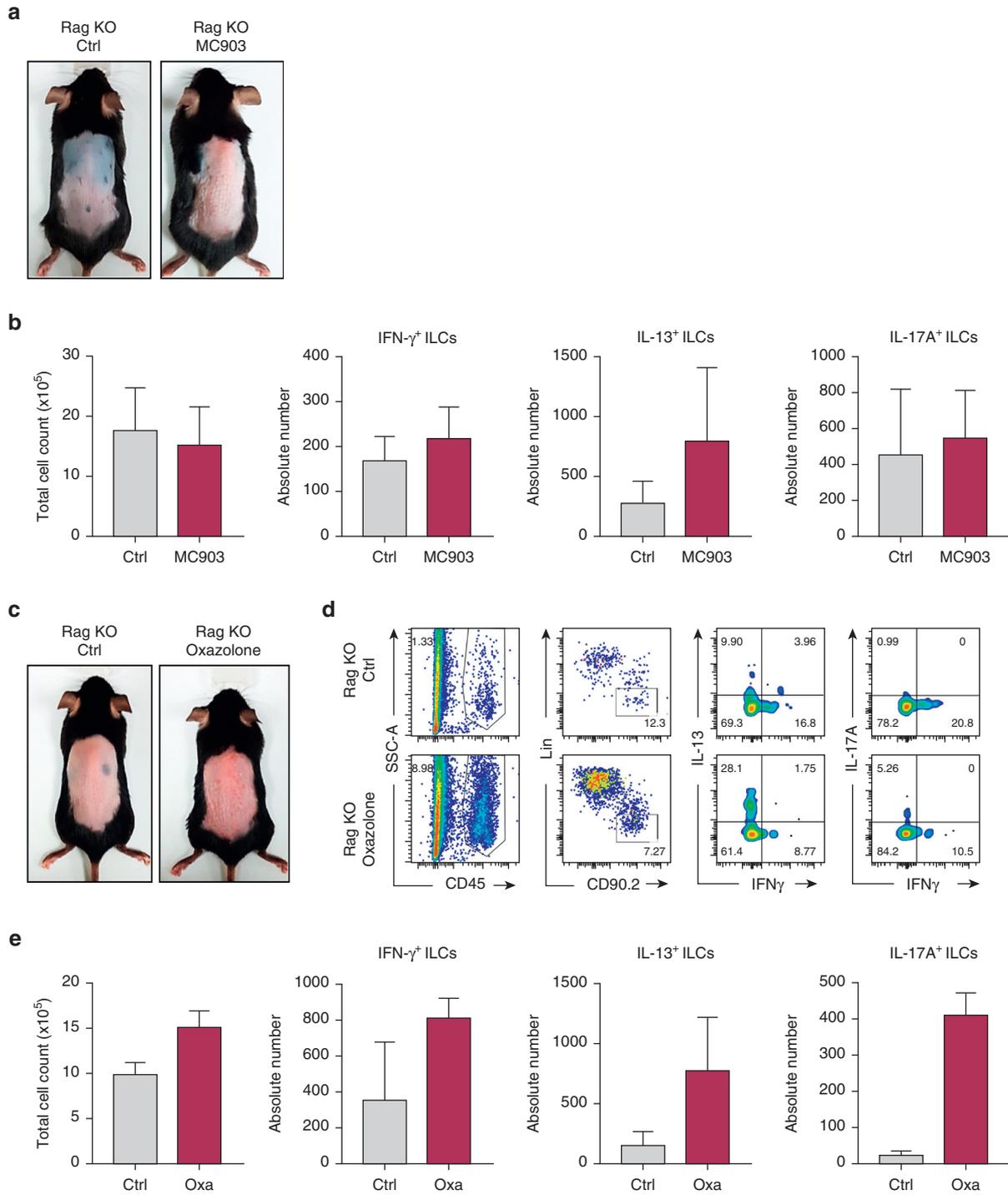
Phenotypes of mice with HDM-induced AD model.

(a) The scheme of HDM-induced AD model. (b) Representative 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. (d) Epidermal thickness, eosinophils, and mast cells counts per HPF. Data was depicted as mean ± SEM. (e) Absolute numbers of cytokine-producing lymphocytes in inguinal lymph nodes. Box lines express minimum, mean, and maximum. 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, ***P < 0.001. AD, atopic dermatitis; Ctrl, control; HDM, house dust mite; HPF, high-power field; SEM, standard error of the mean.





Supplementary Figure S2. HDM enhanced both IL-13 and IL-17A production in the skin regardless of mouse strain. (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/7 mice ($n = 3-4$ per group). (d) Representative dot plots of cytokine-producing ILCs in the dorsal skin. (e) Total cell count and IL-13 $^+$ or IL-17A $^+$ ILCs in the dorsal skin ($n = 3$ per group). Data are depicted as mean + SEM. AD, atopic dermatitis; Ctrl, control; FSC-A, forward scatter area; HDM, house dust mite; ILC, innate lymphoid cell; SdLN, skin-draining lymph node; SEM, standard error of the mean; SSC-A, side scatter area.

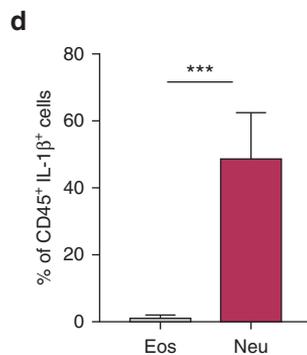
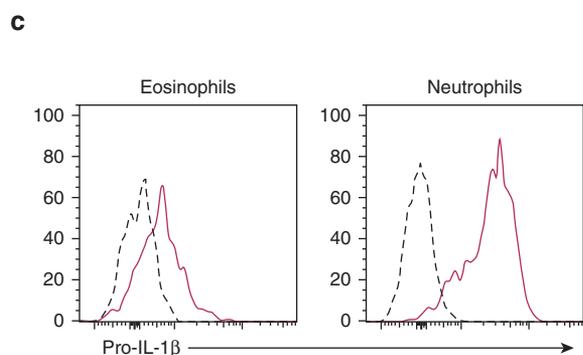
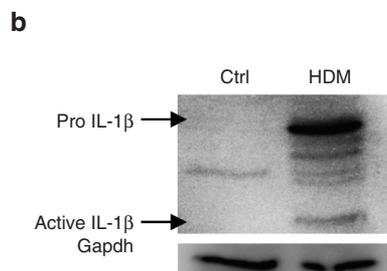
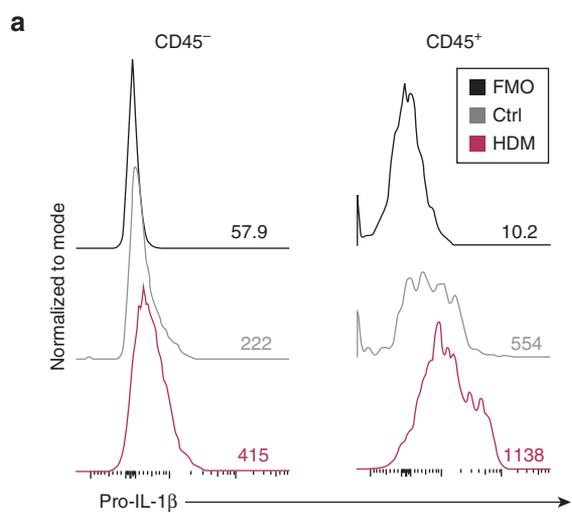


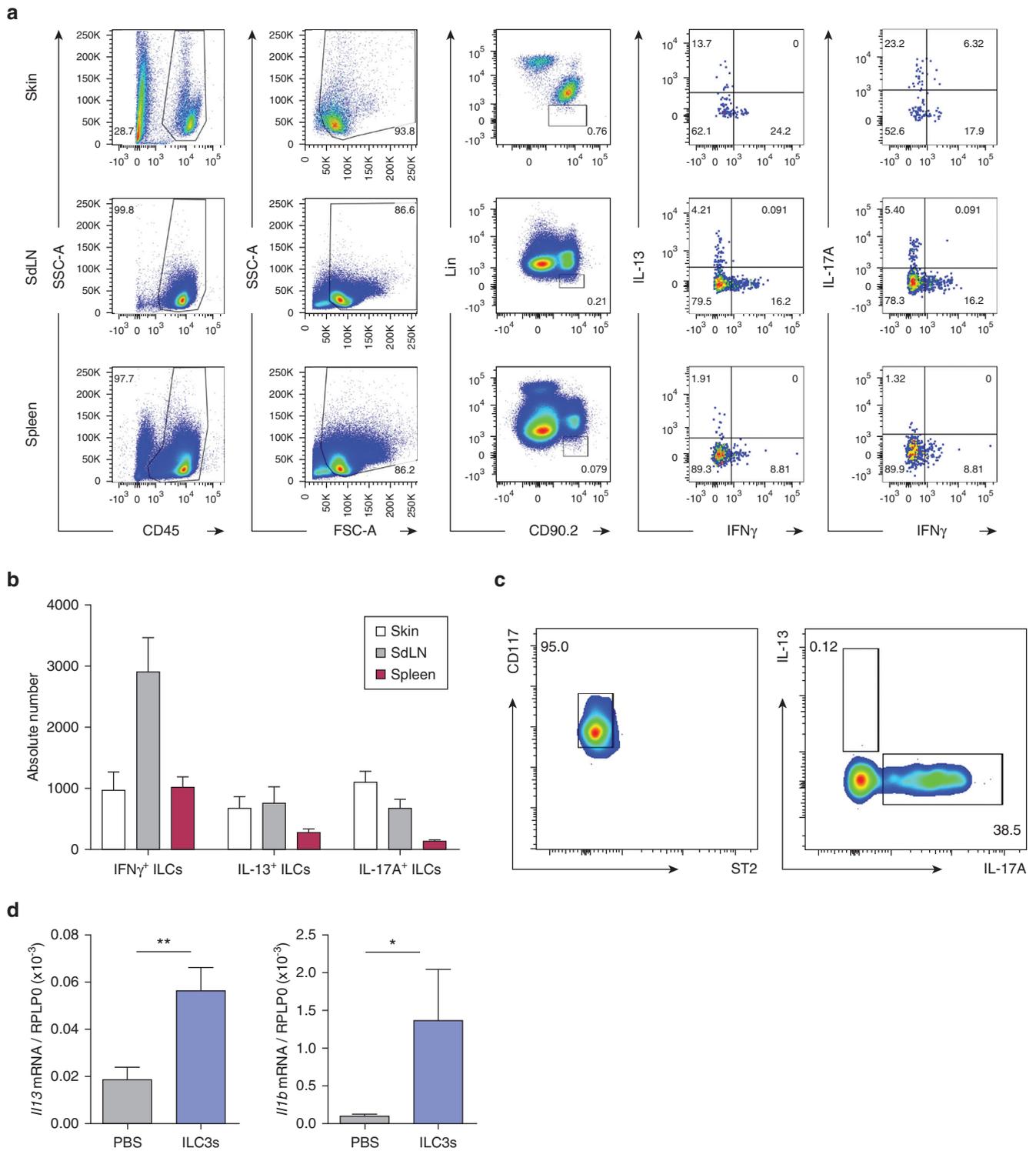
Supplementary Figure S3. ILCs contributed to skin inflammation regardless of adaptive immunity. (a) Representative images of the ethanol and MC903-treated Rag KO mice. (b) Total cell count and absolute number of ILCs in the dorsal skin from MC903-induced Rag KO mice ($n = 3-4$ per group) (c) Representative images of the ethanol and oxazolone-induced Rag deficient mice. (d) Representative dot plots of cytokine-producing ILCs in the dorsal skin from oxazolone-induced Rag deficient mice. (e) Total cell counts and absolute number of cytokine-positive ILCs in the dorsal skin ($n = 3$ per group). Data are depicted as mean + SEM. Ctrl, control; ILC, innate lymphoid cell; KO, knockout; Oxa, oxazolone; SEM, standard error of the mean.

Supplementary Figure S4.

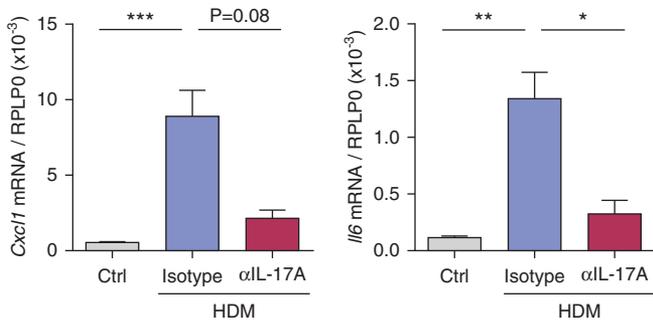
Neutrophils are dominant immune cells to produce IL-1 β in the skin of mice with HDM-induced AD.

(a) Pro-IL-1 β expression among CD45 $^{-}$ and CD45 $^{+}$ cells from the dorsal skin. (b) IL-1 β protein was detected by western blot in the dorsal skin from control and HDM-induced NC/Nga mice. (c) Pro-IL-1 β expression of eosinophils and neutrophils. Data are representative of two independent experiments (n = 3). (d) Percentage of IL-1 β -producing cells in AD skin. Eosinophils (CD45 $^{+}$ CD11b $^{+}$ Siglec F $^{+}$) and 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. AD, atopic dermatitis; Ctrl, control; Eos, eosinophil; FMO, fluorescence minus one; HDM, house dust mite; Neu, neutrophil; SEM, standard error of the mean.

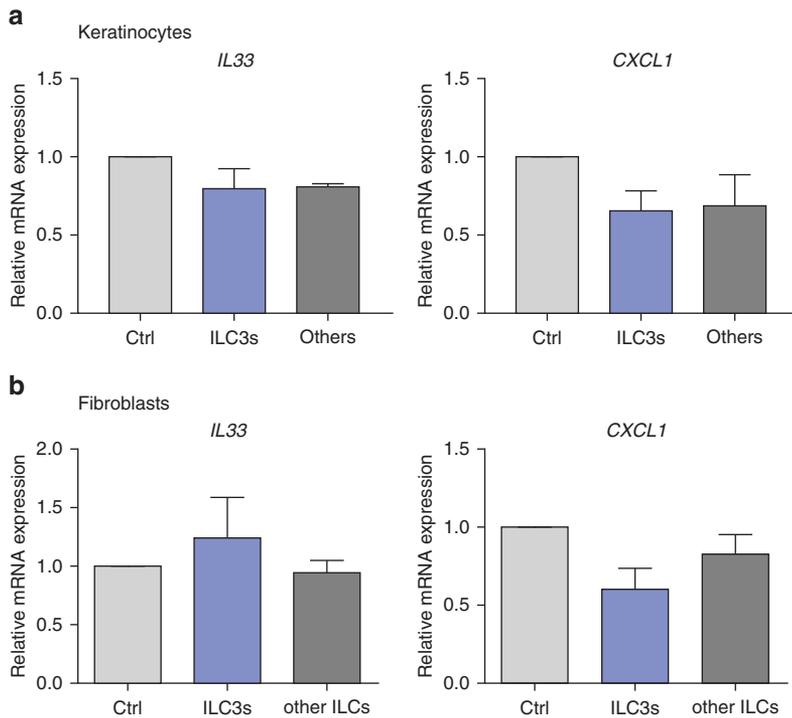




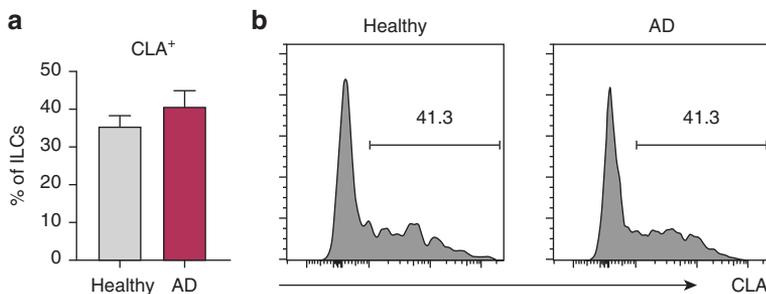
Supplementary Figure S5. The effect of ILC3s on AD. (a) Representative dot plots of cytokine-producing ILCs in the skin, SdLN, and spleen. (b) Absolute numbers of IFN γ ⁺, IL-13⁺, and IL-17A⁺ ILCs (n = 4). (c) The purity of sorted ILC3s (CD45⁺ Lympho⁺ Lin⁻ CD127⁺ CD25⁺) analyzed with AriaIII. Lin: CD3 ϵ , CD11b, CD11c, CD19, CD49b, F4/80, Fc ϵ RI α , and TCR γ δ . Cytokine expression of ILC3s (CD45⁺ Lympho⁺ Lin⁻ CD127⁺ CD25⁺ CD117⁺ ST2⁻). (d) mRNA expression of *Il13* and *Il1b* in the dorsal skin from control- or ILC3-transferred mice. Data from two independent experiments (n = 6–7 per group). Data are depicted as mean + SEM. Mann-Whitney U test was performed. * $P < 0.05$, ** $P < 0.01$. AD, atopic dermatitis; ILC3, group 3 innate lymphoid cell; PBS, phosphate buffered saline; SdLN, skin-draining lymph node; SEM, standard error of the mean.



Supplementary Figure S6. The expression of IL-17A–targeted genes. *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. Ctrl, control; HDM, house dust mite; SEM, standard error of the mean.



Supplementary Figure S7. The coculture of human ILC3s with monolayer of keratinocyte or fibroblasts. Relative mRNA expression of *IL33* and *CXCL1* from human (a) keratinocytes or (b) fibroblasts in Transwell inserts. Data were obtained from two independent experiments. Data are depicted as mean + SEM (n = 2). Ctrl, control; ILC3, group 3 innate lymphoid cell; SEM, standard error of the mean.



Supplementary Figure S8. CLA was expressed on innate lymphocyte cells in human PBMCs. (a) Frequency of CLA⁺ ILCs in PBMCs from healthy volunteers and patients with AD. (b) CLA expression of ILCs from healthy controls and patients with AD (n = 6–7 per group). Data were depicted as mean + SEM. AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; ILC, innate lymphoid cell; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

Supplementary Table S1. Primers Used in Real-Time PCR

Genes	Forward(5'-3')	Reverse(5'-3')
<i>Il17a</i>	GCAATGAAGACCCTGATAGATATCC	TTCATGTGGTGGTCCAGCTTT
<i>Il13</i>	TGAGGAGCTGAGCAACATCACACA	TGCGGTTACAGAGGCCATGCAATA
<i>Tslp</i>	CGGATGGGGCTAACTTACA	TCCTCGATTGCTCGAACTT
<i>Il33</i>	GATGGGAAGAAGCTGATGGTG	TTGTGAAGGACGAAGAAGGC
<i>Il23a</i>	CCAGCAGCTCTCTCGGAATC	TCATATGTCCCGCTGGTGC
<i>Il1b</i>	TGTAATGAAAGACGGCACACC	TCTTCTTGGGTATTGCTTGG
<i>Cxcl1</i>	GCTGGGATTCACCTCAAGAA	TCTCCGTTACTGGGGACAC
<i>Rplp0</i>	TGCCACACTCCATCATCAAT	CGAAGAGACCGAATCCATA
<i>TSLP</i>	GCTATCTGGTGGCCAGGCTAT	CGACGCCACAATCCTTGTAAT
<i>IL33</i>	TCAGGTGACGGTGTGATGG	ACAAGAAGGCCTGGTCTGG
<i>CXCL1</i>	CCCCAGCAATCCCCGGCTC	TGGGGTCCGGGGGACTTCAC
<i>RPLP0</i>	TGGGCTCCAAGCAGATGC	GGCTTCGCTGGCTCCAC