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Type 2 Innate Lymphoid Cells and Skin Fibrosis in a Murine Model of Atopic Dermatitis-Like Skin Inflammation

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ABSTRACT

Background: Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease. Although murine studies have demonstrated that type 2 innate lymphoid cells (ILCs) mediate type 2 skin inflammation, their role in skin fibrosis in AD remains unclear. This study investigated whether type 2 ILCs are involved in skin fibrosis using an AD-like murine model.
Methods: C57BL/6 mice were treated epicutaneously with *Aspergillus fumigatus* (*Af*) for 5 consecutive days per week for 5 weeks to induce skin fibrosis. Mature lymphocyte deficient *Rag1*$^{-/-}$ mice were also used to investigate the role of type 2 ILCs in skin fibrosis.
Results: The clinical score and transepidermal water loss (TEWL) were significantly higher in the AD group than in the control group. The AD group also showed significantly increased epidermal and dermal thicknesses and significantly higher numbers of eosinophils, neutrophils, mast cells, and lymphocytes in the lesional skin than the control group. The lesional skin of the AD group showed increased stain of collagen and significantly higher levels of collagen than the control group (10.4 ± 2.2 μg/mg vs. 1.6 ± 0.1 μg/mg, $P < 0.05$). The AD group showed significantly higher populations of type 2 ILCs in the lesional skin compared to the control group (0.08 ± 0.01% vs. 0.03 ± 0.01%, $P < 0.05$). These findings were also similar with the AD group of *Rag1*$^{-/-}$ mice compared to their control group. Depletion of type 2 ILCs with anti-CD90.2 monoclonal antibodies significantly improved clinical symptom score, TEWL, and infiltration of inflammatory cells, and significantly decreased levels of collagen were observed in the AD group of *Rag1*$^{-/-}$ mice (1.6 ± 0.0 μg/mg vs. 4.5 ± 0.3 μg/mg, $P < 0.001$).
Conclusion: In the *Af*-induced AD-like murine model, type 2 ILCs were elevated, with increased levels of collagen. Additionally, removal of type 2 ILCs resulted in decreased collagen levels and improved AD-like pathological findings. These findings suggest that type 2 ILCs play a role in the mechanism of skin fibrosis in AD.

Keywords: Type 2 Innate Lymphoid Cell; Skin Fibrosis; Atopic Dermatitis, Mouse Model

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Disclosure

The authors have no potential conflicts of interest to disclose.

Author Contributions

Conceptualization: Yoon J[1], Moon GJ, Yu J. Data curation: Lee J. Formal analysis: Lee J, Yoon J.[2] Investigation: Yoon J,[1] Lee J, Park A, Yoon J.[2] Methodology: Lee J, Park A, Yoon J.[2] Software: Lee J. Supervision: Yoon J,[2] Yu J. Validation: Park A, Kim JR, Moon GJ. Visualization: Kim JR, Moon GJ. Writing - original draft: Yoon J,[1] Lee J. Writing - review & editing: Yoon J,[1] Moon GJ, Yu J.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease characterized by intense itch and recurrent eczematous lesion.[1] The disease is highly prevalent in children and inflicts a substantial socioeconomic burden on patients.[2-5] The two major pathogeneses of AD are skin barrier defect and cutaneous inflammation typically defined by increase in both T helper (Th) 2-mediated and Th1-mediated immune responses.[6-8] The chronic cutaneous inflammation induces skin fibrosis characterized by lichenification and fibrotic papules, involving various inflammatory responses including interleukin (IL)-33, thymic stromal lymphopoietin (TSLP), and IL-13, and epidermal defects such as loss of Notch1 in keratinocytes.[9-12]

Studies on the innate cell sources of type 2 cytokines, known to be related to allergic diseases, have identified group 2 innate lymphoid cells (ILCs) as critical sources of innate effector cells in type 2 immunity.[13,14] Type 2 ILCs produce type 2 cytokines such as IL-5, IL-9, and IL-13 in response to IL-25 and IL-33, leading to allergic inflammation.[14,15] We previously reported the involvement of type 2 ILCs in skin inflammation in a murine model of *Aspergillus fumigatus* (*Af*)-induced AD-like skin lesions.[16]

Recently, type 2 ILCs were reported to be involved in fibrosis in a murine model of pulmonary fibrosis, extending beyond their role in type 2 inflammation.[17,18] However, no studies have shown that type 2 ILCs also play a role in fibrosis induced by chronic cutaneous inflammation. Therefore, we investigated whether skin fibrosis is associated with type 2 ILCs in a murine model chronically induced by *Af*.

METHODS

Murine model of AD

Female 8-week-old C57BL/6 mice were purchased from Orient Bio INC (Seongnam, Korea). *Af* antigen was purchased from Greer Laboratories (Lenoir, NC, USA). All animal experiments were performed at a specific pathogen-free (SPF) facility. Skin fibrosis was induced by chronic AD-like cutaneous inflammation through epicutaneous application of *Af* extract as previously described.[19] Briefly, animals were anesthetized with intraperitoneal injections of tiletamine-zolazepam (60 mg/kg, Zoletile50®; Vibac Laboratories, Carros, France) and xylazine (10 mg/kg, Rumpun®; Bayer Korea, Ansan, Korea). The back of the mice was shaved and 40 μg of *Af* extract was applied epicutaneously once a day to a 1 cm^2 area on the dorsal skin surface for 5 consecutive days per week for 5 weeks. The control mice were treated with normal saline solution. *Rag1*$^{-/-}$ (Jackson Laboratory, Bar Harbor, ME, USA) mice which lack mature lymphocytes were used to elucidate the role of group 2 ILCs. Skin tissue, lymph nodes, and blood were collected on day 33 after sacrifice.

Antibody treatment

Isotype control (LTF-2) and anti-CD90.2 monoclonal antibodies (mAbs) (30H12) were purchased from Bio X Cell (West Lebanon, NH, USA). To remove type 2 ILCs, 30 μg/mouse anti-CD 90.2 mAb was administrated intraperitoneally (i.p.) every 2 days from 2 days before *Af* application until day 30 in *Rag1*$^{-/-}$ mice.

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Clinical parameters

The clinical scores of the skin lesions were assessed by a single investigator on days 0, 18, 25, and 32. Dryness, scaling, erosion, excoriation, and hemorrhage were scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), with the sum of these items defined as the clinical score (maximum score, 15). Epidermal permeability barrier function was evaluated by measuring transepidermal water loss (TEWL) using a Vapometer® SWL-3 (DelfinTechnologies Ltd., Kuopio, Finland) on day 0, 18, 25, and 32.

Histological examination of the skin

Skin samples were cut into 1 mm × 1 mm pieces and fixed at room temperature for 18 hours in 10% formalin solution (vender). After embedding the tissue in paraffin, 5 μm thick sections were cut and stained with hematoxylin and eosin (H&E) for measuring skin thickness and counting inflammatory cells. Toluidine blue (TB) was used to identify mast cells and Masson's trichrome (MT) was used to identify collagen fibers. The stained samples were mounted with mounting medium and analyzed using a bright-field microscopy under high power (400× magnification; Olympus, Tokyo, Japan).

Measurement of serum total immunoglobulin (Ig) E

To collect blood samples, the mice were anesthetized, and laparotomy was performed. After laparotomy, blood was collected from the portal vein using a 1 mL syringe. Using BD Vacutainer® SST™ II Advance (BD Biosciences, San Jose, CA, USA), centrifugation was performed at 2,000 rpm, 20°C, for 10 minutes to separate serum. The remaining samples were stored at −70°C. Total IgE levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BD OptEIA ELISA; BD Biosciences) according to the manufacturer's protocol, and absorbance was measured at a wavelength of 450 nm using a VICTOR X3 microplate reader (PerkinElmer, Waltham, MA, USA).

Flow cytometry

Skin tissues were chopped into small pieces and incubated in RPMI-1640 media (Gibco™; Thermo Fisher Scientific, Waltham, MA, USA) containing 2.5 mg/mL collagenase IV (Worthington Biochemical, Lakewood, NJ, USA) and 0.2 mg/mL DNase 1 (Roche Molecular Systems, Somerville, NJ, USA) at 37°C for 60 minutes. ILC2 labeled antibody was used for fluorescence analysis, and the cells were analyzed using Canto II (BD Biosciences). The data were analyzed and graphically presented using FlowJo software (BD Biosciences). PI staining (APC-cy7) (Invitrogen™; Thermo Fisher Scientific) was performed to determine whether the cells were alive or dead. The markers CD45, CD3, Ly-6G, CD11b, Ter-119m, IgG2b, CD25, CD127, and IL-17A were used. IL-13 and IL-33R (BioLegend, San Diego, CA, USA) were used, along with APC, Amcyan, FITC, PE, PE-CY7, Pacific blue, and PerCP-CY5.5 for each fluorescence. ILCs were defined as lineage-negative (Lin^-) $CD127^+$ cells and type 2 ILCs were defined as $CD25^+IL\text{-}33R^+$ cells.

Collagen assay

A Sircol-soluble collagen assay kit (Biocolor, Belfast, UK) was used to measure the amount of collagen in the skin tissue. Equal weights of minced skin tissue (30 mg) were ground and incubated with pepsin and 0.5 M acetic acid (in a 1:10 ratio of pepsin to tissue wet weight) overnight at 4°C. Following this, 100 μL of Sircol dye reagent was added and shaken for 30 minutes. The mixture was then centrifuged at 12,000 rpm for 10 minutes, and the supernatant was discarded after adding 750 μL of acid-salt wash for rinsing. The resulting pellet was mixed with an alkaline reagent and gently agitated before measuring the absorbance at 555 nm using a microplate reader.

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Total ribonucleic acid (RNA) preparation and quantitative real-time polymerase chain reaction (RT-PCR) analysis

After placing the skin tissue in Qiazol (Qiagen, Hilden, Germany) and homogenizing it, RNA was quantified at 1,000 ng/μL following the miRNeasy mini kit protocol (Qiagen). Subsequently, complementary deoxyribonucleic acid (cDNA) synthesis was carried out using the WizScript™ cDNA Synthesis kit (Wizbio, Sungnam, Korea), following the manufacturer's instructions, with cDNA synthesized from 2 μg RNA. RT-PCR was performed on a Quant Studio 6 Flex (Applied Biosystems, Waltham, MA, USA) using the synthesized cDNA, and primers for IL-4 (Mm00445259_m1), interferon (IFN)-γ (Mm01168134_m1), and TSLP (Mm00498739_m1) were used, based on 18s (Mm00415898_m1), with the TaqMan® Gene Expression Assay (Thermo Fisher Scientific).

Statistical analysis

Data normality was assessed using the Shapiro–Wilk test, and subsequent testing was conducted using the Student's unpaired *t*-test or Mann–Whitney *U* test. Statistical significance was established at $P < 0.05$. Statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, Inc., La Jolla, CA, USA).

Ethics statement

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Asan Medical Center and Ulsan University College of Medicine. The IACUC abides by the Institute of Laboratory Animal Resources guide (permit number: 2014-12-064).

RESULTS

AD-like skin inflammation and fibrosis induced by epicutaneous application of Af

Skin fibrosis was induced by treatment with 40 μg *Af* for 5 days per week for 5 weeks (**Fig. 1A**). Erythematous and edematous skin changes with excoriation and increased dryness were observed in the saline-treated control group. Clinical symptom scores (mean ± standard deviation) were also significantly higher in the *Af*-treated group than those in the control group at day 18 (*Af*-treated group, 6.6 ± 0.4 vs. control group, 0.6 ± 0.2), day 25 (7.7 ± 0.6 vs. 0.0 ± 0.0), and day 32 (10.0 ± 1.0 vs. 0.0 ± 0.0), and degree of TEWL was significantly increased in the *Af*-treated group compared to the control group at day 18 (15.0 ± 0.6 g/m^{2}h vs. 5.3 ± 0.5 g/m^{2}h), day 25 (20.5 ± 1.1 g/m^{2}h vs. 2.1 ± 0.2 g/m^{2}h), and day 32 (22.0 ± 1.9 g/m^{2}h vs. 2.8 ± 0.3 g/m^{2}h) (all $P < 0.001$). Serum concentrations of total IgE were significantly higher in the *Af*-treated group than in the control group ($P < 0.001$) (**Fig. 1B**).

To examine whether long-term treatment with *Af* leads to pathological changes, such as chronic inflammation and fibrosis, in the mouse skin, skin tissues were histopathologically examined using H&E, TB, and MT staining. Histopathological examination of the lesional skin of the *Af*-treated mice revealed marked hyperplasia of the epidermis (thickness of the *Af*-treated group, 24.5 ± 4.5 μm vs. thickness of the control group, 12.1 ± 0.8 μm, $P = 0.026$) and dermis (154.7 ± 12.3 μm vs. 98.0 ± 14.2 μm, $P = 0.014$), more infiltration into the dermis of inflammatory cells including eosinophils (*Af*-treated group, 5.4 ± 0.3/mm^2 vs. control group, 0.6 ± 0.1/mm^2), neutrophils (6.8 ± 0.5/mm^2 vs. 1.2 ± 0.4/mm^2), lymphocytes (4.5 ± 0.1/mm^2 vs. 1.8 ± 0.3/mm^2), and mast cells (5.0 ± 0.2/mm^2 vs. 1.6 ± 0.2/mm^2) (all $P < 0.001$; **Fig. 1C**).

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Moreover, IFN-γ (1.00 vs. 3.28 ± 1.51, $P = 0.163$), TSLP (1.00 vs. 2.34 ± 0.54, $P = 0.038$), and IL-4 (1.00 vs. 115.47 ± 63.29, $P = 0.101$) increased in the *Af*-treated group than in the control group (**Fig. 1D**). The AD group (10.4 ± 2.2 μg/mg) showed a significantly higher collagen level than the control group (1.6 ± 0.1 μg/mg) ($P < 0.001$; **Fig. 1E**).

The populations of Lin$^-$CD127$^+$25$^+$IL-33R$^+$ cells (type 2 ILCs) were significantly higher in the skin of the *Af*-treated group (0.08 ± 0.01%) compared to the control group (0.03 ± 0.01%), when CD45$^+$ was measured as the denominator ($P = 0.022$) (**Fig. 1F**).

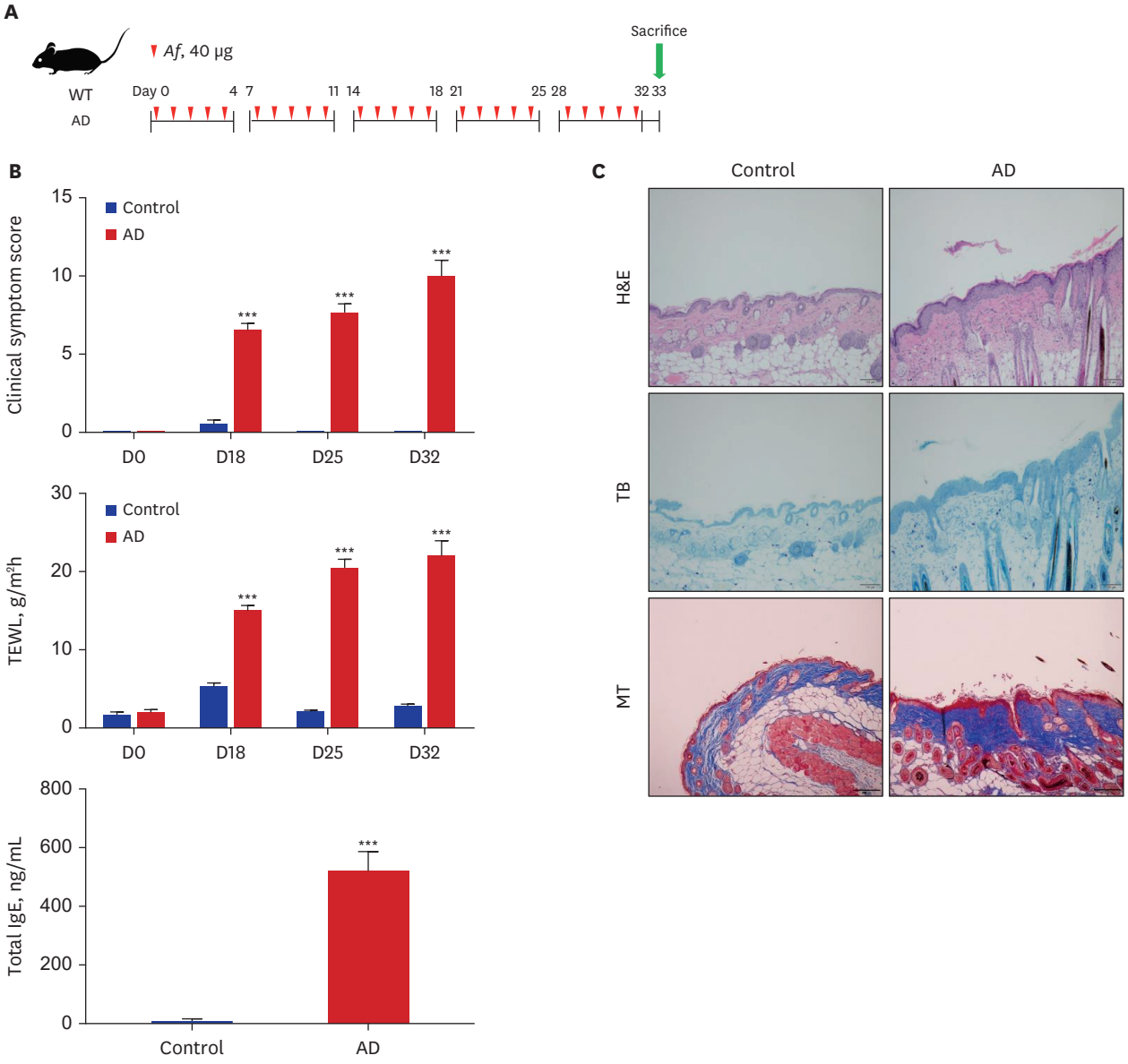

Fig. 1. Skin fibrosis induced by *Af* in wild type mice. (**A**) Schematic protocol of this study. For 5 weeks, *Af* was epicutaneosly applied to the dorsal skin of mice for 5 consecutive days per week. (**B**) Clinical symptom scores, TEWL, and serum total IgE levels in *Af*-induced atopic dermatitis murine model. (**C**) H&E (top), TB (middle), and MT (bottom) staining were performed on skin tissue sections (×400 magnification, scale bar = 200 μm). (**D**) mRNA expression of IFN-γ, TSLP, and IL-4 in the lesional skin of atopic dermatitis murine model. (**E**) Collagen levels in the lesional skin as measured by collagen assay. (**F**) Flow cytometry was used to measure the proportion of type 2 ILCs. ILCs were defined as lineage negative (Lin$^-$) CD127$^+$ cells, and type 2 ILCs were defined as CD25$^+$IL-33R$^+$ cells.
Af = *Aspergillus fumigatus*, TEWL = transepidermal water loss, Ig = immunoglobulin, H&E = hematoxylin and eosin, TB = toluidine blue, MT = Masson's trichrome, IFN = interferon, TSLP = thymic stromal lymphopoietin, IL = interleukin, ILC = innate lymphoid cell.
$^*P < 0.05$, $^{***}P < 0.001$.

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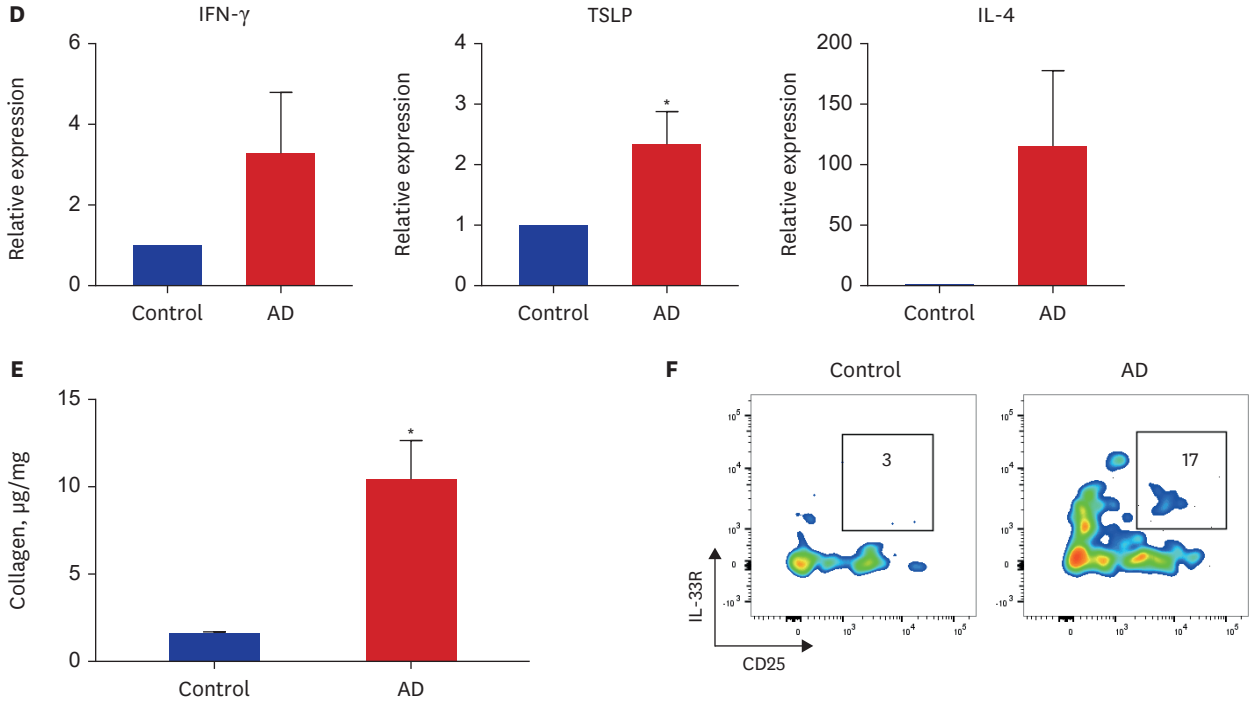

Fig. 1. (Continued) Skin fibrosis induced by *Af* in wild type mice. (**A**) Schematic protocol of this study. For 5 weeks, *Af* was epicutaneosly applied to the dorsal skin of mice for 5 consecutive days per week. (**B**) Clinical symptom scores, TEWL, and serum total IgE levels in *Af*-induced atopic dermatitis murine model. (**C**) H&E (top), TB (middle), and MT (bottom) staining were performed on skin tissue sections (×400 magnification, scale bar = 200 μm). (**D**) mRNA expression of IFN-γ, TSLP, and IL-4 in the lesional skin of atopic dermatitis murine model. (**E**) Collagen levels in the lesional skin as measured by collagen assay. (**F**) Flow cytometry was used to measure the proportion of type 2 ILCs. ILCs were defined as lineage negative (Lin^-) $CD127^+$ cells, and type 2 ILCs were defined as $CD25^+IL\text{-}33R^+$ cells.
Af = *Aspergillus fumigatus*, TEWL = transepidermal water loss, Ig = immunoglobulin, H&E = hematoxylin and eosin, TB = toluidine blue, MT = Masson's trichrome, IFN = interferon, TSLP = thymic stromal lymphopoietin, IL = interleukin, ILC = innate lymphoid cell.
$^*P < 0.05$, $^{***}P < 0.001$.

Type 2 ILCs in skin fibrosis of *Af*-treated *Rag1*$^{-/-}$ mice

To elucidate the role of type 2 ILCs in skin fibrosis induced by *Af*, *Rag1*$^{-/-}$ mice lacking in mature lymphocytes were used. It was similarly treated with 40 μg *Af* for 5 days, resting for 2 days, for 5 weeks (**Fig. 2A**). The lesional skin of *Af*-treated *Rag1*$^{-/-}$ mice showed gross erythematous and edematous changes with excoriation and greater dryness than that of saline-treated *Rag1*$^{-/-}$ mice. Similar to the AD group of wild type mice, both clinical symptom score and TEWL were significantly increased in the *Af*-treated *Rag1*$^{-/-}$ mice at day 18 (6.2 ± 0.4 g/m^{2}h and 16.2 ± 1.4 g/m^{2}h vs. 0.4 ± 0.2 g/m^{2}h and 4.2 ± 0.6 g/m^{2}h, respectively), day 25 (8.2 ± 0.7 g/m^{2}h and 19.6 ± 1.4 g/m^{2}h vs. 0.0 ± 0.0 g/m^{2}h, 4.1 ± 0.4 g/m^{2}h, respectively), and day 32 (8.6 ± 1.0 g/m^{2}h and 16.6 ± 3.1 g/m^{2}h vs. 0.0 ± 0.0 g/m^{2}h and 2.7 ± 0.2 g/m^{2}h, respectively) compared to the control group (all $P < 0.001$; **Fig. 2B**).

Histopathological examination of the lesional skin of the *Af*-treated *Rag1*$^{-/-}$ mice showed increased epidermal (*Af*-treated group, 28.5 ± 2.1 μm vs. control group, 16.7 ± 1.3 μm, $P = 0.001$) and dermal thickness (189.4 ± 11.3 μm vs. 100.3 ± 5.8 μm, $P = 0.048$), and increased mast cell (7.7 ± 1.2/mm^2 vs. 2.6 ± 0.6/mm^2, $P = 0.004$), eosinophil (6.8 ± 1.0/mm^2 vs. 1.9 ± 0.3/mm^2, $P = 0.002$), neutrophil (9.6 ± 0.9/mm^2 vs. 2.9 ± 0.6/mm^2, $P < 0.001$), and lymphocyte (5.8 ± 0.6/mm^2 vs. 2.4 ± 0.3/mm^2, $P < 0.001$) infiltration, and increased collagen stain in H&E, TB and MT staining compared to the control group (**Fig. 2C**). In addition, the collagen was measured to determine the degree of fibrosis in the lesional skin of the *Af*-treated *Rag1*$^{-/-}$ mice model (**Fig. 2D**). The AD group (3.9 ± 1.2 μg/mg) showed a higher collagen level tendency than the control group (1.6 ± 0.0 μg/mg) ($P = 0.174$).

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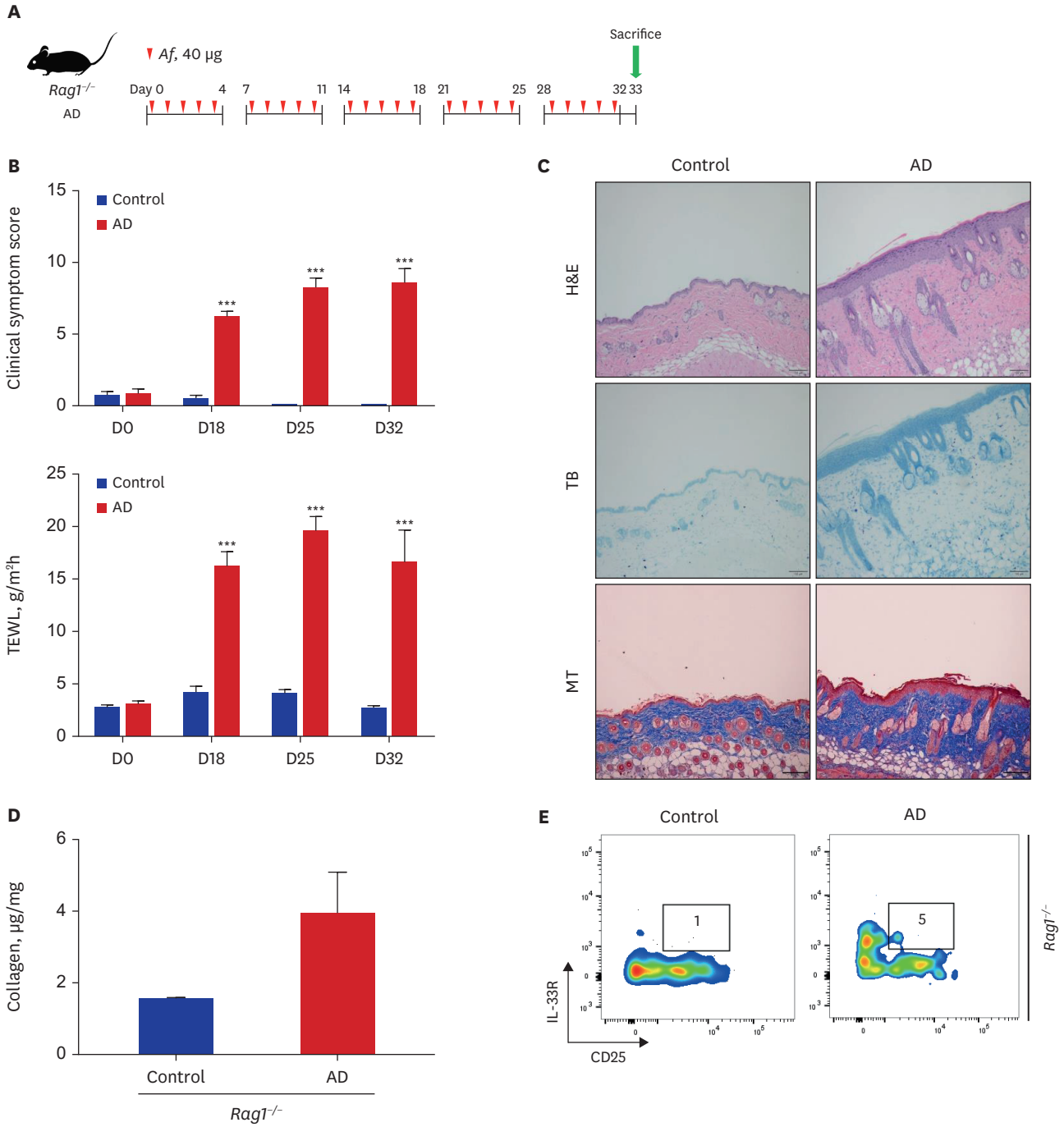

Fig. 2. Skin fibrosis induced by *Af* in *Rag1*$^{-/-}$ mice. (**A**) Schematic protocol of this study. For 5 weeks, *Af* was epicutaneosly applied to the dorsal skin of mice for 5 consecutive days per week. (**B**) Clinical symptom scores and TEWL in *Af*-induced atopic dermatitis murine model. (**C**) H&E (top), TB (middle), and MT (bottom) staining were performed on skin tissue sections (×400 magnification, scale bar = 200 μm). (**D**) Collagen levels in the lesional skin as measured by collagen assay. (**E**) Flow cytometry was used to measure the proportion of type 2 ILCs. ILCs were defined as lineage negative (Lin^{-}) CD127^{+} cells, and type 2 ILCs were defined as CD25^{+}IL-33R^{+} cells.
Af = *Aspergillus fumigatus*, TEWL = transepidermal water loss, Ig = immunoglobulin, H&E = hematoxylin and eosin, TB = toluidine blue, MT = Masson's trichrome, ILC = innate lymphoid cell, IL = interleukin.
$^{***}P < 0.001$.

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The populations of Lin$^-$CD127$^+$CD25$^+$IL-33R$^+$ cells were higher in the skin of the *Af*-treated *Rag1*$^{-/-}$ mice (0.1 ± 0.04%) compared to the control *Rag1*$^{-/-}$ mice (0.03 ± 0.01%), when CD45$^+$ was measured as the denominator; however, the difference was not significant (P = 0.240; **Fig. 2E**).

Elimination of type 2 ILCs and skin fibrosis in *Af*-treated *Rag1*$^{-/-}$ mice

For further investigating the role of type 2 ILCs in skin fibrosis, an anti-CD90.2 mAb was used to deplete type 2 ILC2 in *Rag1*$^{-/-}$ mice.[20] Anti-CD90.2 and isotype of rat IgG2b were intraperitoneally injected in the control group from 2 days before *Af* application to day 30 at an interval of 2 days (**Fig. 3A**). Both clinical symptom score and TEWL at day 18 (2.6 ± 0.5 g/m^{2}h and 8.4 ± 1.1 g/m^{2}h, respectively), day 25 (2.5 ± 0.3 g/m^{2}h and 6.6 ± 0.8 g/m^{2}h, respectively), and day 32 (2.4 ± 0.5 g/m^{2}h and 7.7 ± 0.6 g/m^{2}h, respectively) were significantly decreased in anti-CD90.2-treated *Rag1*$^{-/-}$ mice compared to those at day 18 (6.4 ± 0.5 g/m^{2}h and 18.1 ± 2.0 g/m^{2}h, respectively), day 25 (5.3 ± 0.9 g/m^{2}h and 16.0 ± 2.0 g/m^{2}h, respectively), and day 32 (6.5 ± 0.3 g/m^{2}h and 18.9 ± 2.1 g/m^{2}h, respectively) in isotype-treated control mice (all P < 0.001; **Fig. 3B**). Both epidermal (22.0 ± 4.7 μm vs. 29.1 ± 1.8 μm) and dermal (130.9 ± 10.1 μm vs. 202.0 ± 30.4 μm) thickness in anti-CD90.2-treated *Rag1*$^{-/-}$ mice were not significantly different from those in isotype-treated control mice (P = 0.188 and P = 0.057, respectively; **Fig. 3C**). The depletion of type 2 ILCs in the anti-CD90.2-treated *Rag1*$^{-/-}$ mice was accompanied by significantly reduced immune cells including mast cell (control group, 9.4 ± 1.0/mm^2 vs. anti-CD90.2-treated group, 3.5 ± 0.6/mm^2, P < 0.001), eosinophil (8.3 ± 0.8/mm^2 vs. 2.1 ± 0.6/mm^2, P < 0.001), neutrophil (12.6 ± 1.3/mm^2, 5.0 ± 0.9/mm^2, P = 0.001), and lymphocyte (7.8 ± 0.9 mm^2 vs. 2.4 ± 0.6/mm^2, P < 0.001). Collagen levels were significantly decreased in the anti-CD90.2-treated *Rag1*$^{-/-}$ mice (1.6 ± 0.0 μg/mg) than in the isotype-treated group (4.5 ± 0.3 μg/mg) (P < 0.001; **Fig. 3D**).

The populations of Lin$^-$CD127$^+$CD25$^+$IL-33R$^+$ cells were lower in the skin of anti-CD90.2-treated *Rag1*$^{-/-}$ mice (0.008 ± 0.01%) compared to isotype-treated *Rag1*$^{-/-}$ mice (0.03 ± 0.01%), when CD45$^+$ was measured as the denominator; however, the difference was not significant (P = 0.29; **Fig. 3E**).

DISCUSSION

This study investigated the role of type 2 ILCs in skin fibrosis using a murine model of AD-like skin inflammation. Epicutaneous exposure to *Af* extract for 5 weeks induced skin fibrosis, as demonstrated by increased collagen levels and AD-like skin inflammation. We found a trend of increased type 2 ILCs in the lesional skin of *Af*-treated mice, although the difference was not statistically significant. Similar to the results in wild-type mice, collagen levels significantly increased with an increase in type 2 ILCs in lymphocyte-depleted *Rag1*$^{-/-}$ mice treated with *Af* compared to control *Rag1*$^{-/-}$ mice. Depletion of type 2 ILCs using anti-CD90.2 significantly decreased both staining and collagen levels.

According to previous mouse model studies, type 2 ILCs play a role in the development of AD even in the absence of adaptive immunity.[21-23] As expected from conventional immunobiology of type 2 inflammation, type 2 ILC-derived type 2 cytokines, including IL-4, IL-5, and IL-13, are responsible for skin inflammation and barrier dysfunction in mice and human studies.[24-26] TSLP, IL-33, and IL-25 have the capacity to activate type 2 ILCs, and these cytokines can be amplified when exposed to allergens such as house dust mite extracts,[26,27] viruses such as respiratory syncytial virus, rhinovirus, and influenza,[28-30] and fungus.[31]

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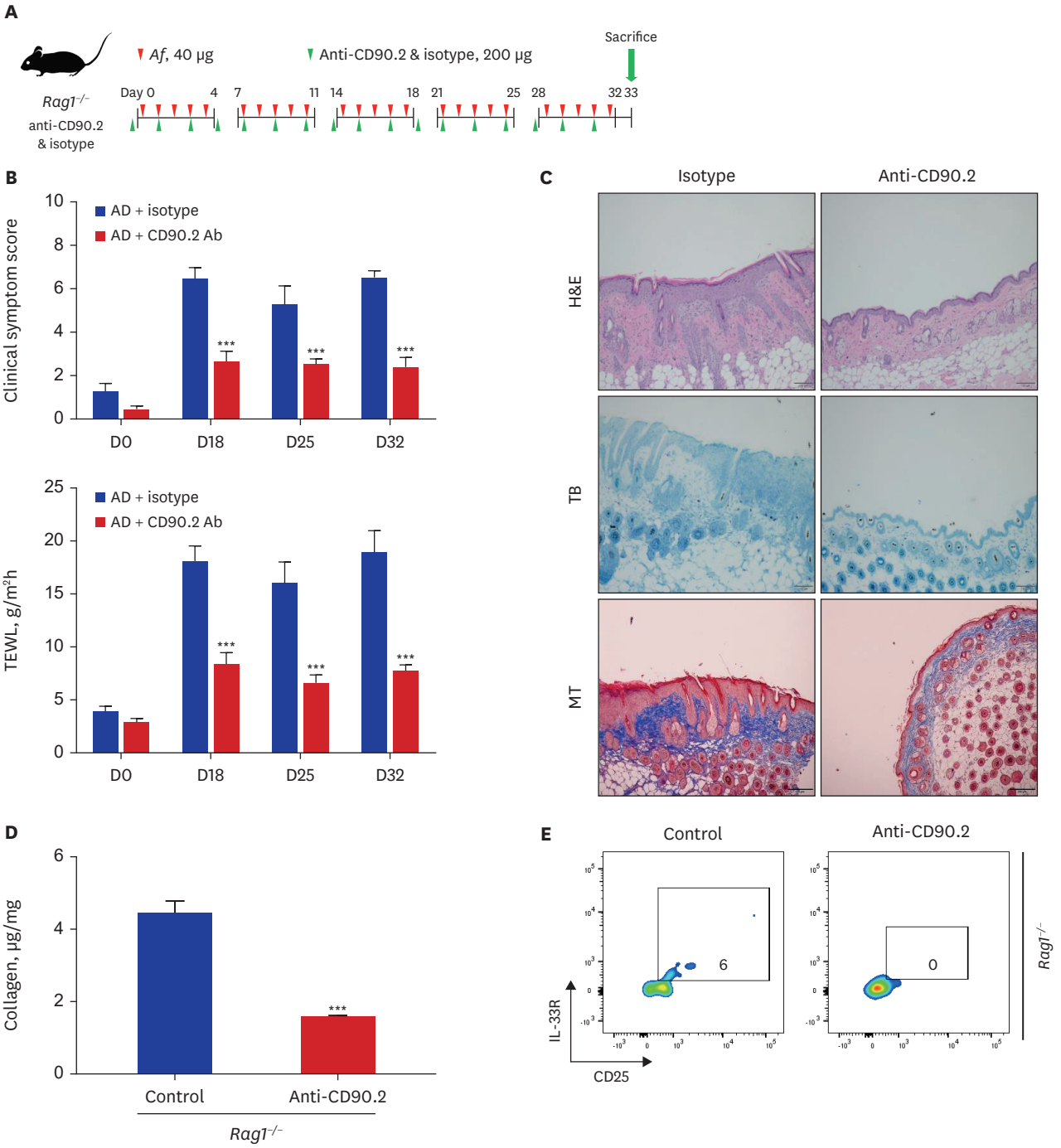

Fig. 3. Depletion of type 2 ILCsimproved skin fibrosis in *Rag1*$^{-/-}$ mice. (**A**) Schematic protocol of this study. For 5 weeks, *Af* was epicutaneosly applied to the dorsal skin of mice for 5 consecutive days per week. (**B**) Clinical symptom scores and TEWL in *Af*-induced atopic dermatitis murine model. (**C**) H&E (top), TB (middle), and MT (bottom) staining were performed on skin tissue sections (×400 magnification, scale bar = 200 μm). (**D**) Collagen levels in the lesional skin as measured by collagen assay. (**E**) Flow cytometry was used to measure the proportion of ILCs. ILCs were defined as lineage negative (Lin^{-}) CD127^{+} cells, and type 2 ILCs were defined as CD25^{+}IL-33R^{+} cells.
ILC = innate lymphoid cell, *Af* = *Aspergillus fumigatus*, TEWL = transepidermal water loss, Ig = immunoglobulin, H&E = hematoxylin and eosin, TB = toluidine blue, MT = Masson's trichrome, IL = interleukin.
$^{***}P < 0.001$.

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However, the mechanisms underlying AD fibrosis are not fully understood. Similar to other diseases, it is known to be associated with Th2 and Th17 immune responses.[32,33] It was demonstrated that IL-13 was associated with the development of skin fibrosis in AD.[34] Under the influence of Th2 immune response, the secretion of transforming growth factor (TGF)-β leads to fibroblasts differentiating into myofibroblasts, and secreting a significant amount of collagen.[35,36] Using *Rag1*$^{-/-}$ mice with depleted lymphocytes, such as Th2 and Th17 cells, this study elucidated that type 2 ILCs in fibrosis is independent of adaptive immunity, highlighting a potential role of type 2 ILCs in the development of skin fibrosis. Studies investigating the association between type 2 ILCs and skin fibrosis in AD are scarce. Type 2 ILCs are implicated in the induction of fibrosis in other organs. Type 2 ILCs are involved in fibrosis in a murine model of pulmonary fibrosis and have been found in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis.[18,37] Other studies have explained the role of type 2 ILCs in organ fibrosis in mucosal immune response.[38] The elucidated mechanism involves the production of Th2 cytokines by type 2 ILCs in response to IL-25, IL-33, and TSLP. Given that Th2 cytokines such as IL-13 and IL-5 also mediate tissue fibrosis, type 2 ILCs can play a role in modulating remodeling and fibrosis, particularly in mucosal tissues. Additionally, Th2 cytokines recruit and stimulate eosinophils to release the profibrotic cytokines such as TGF-β, platelet-derived growth factor, and IL-13. This promotes the differentiation of fibroblasts to myofibroblasts,[39] which could potentially induce intestinal[40] and hepatic fibrosis.[41] However, whether fibrosis is a consequence of chronic inflammation caused by type 2 ILCs or whether type 2 ILCs are directly involved in the development of fibrosis in our study remains unclear. Therefore, more in-depth mechanistic research is required to determine the interrelationship between skin fibrosis caused by type 2 ILCs.

We used *Af*-induced AD model with histological findings and immune responses similar to those in humans. Epicutaneous application of *Af* for 5 weeks induced skin fibrosis concomitant with chronic skin inflammation, including both neutrophil and eosinophil infiltration, involving both type 2 and type 1 immune responses. Therefore, this model may be well suited for investigating fibrosis in human AD, which manifests as a combination of both immune response types.

In conclusion, our study implicates the role of type 2 ILCs in the development of skin fibrosis in a murine model of AD-like skin inflammation. Further investigations are warranted to elucidate the underlying mechanisms and to evaluate the therapeutic potential of targeting type 2 ILCs in the management of AD-associated fibrosis.

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