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of May 16, 2011*J Immunol*; Prepublished online 16 May 2011;

doi:10.4049/jimmunol.1100504

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IL-13 Induces Skin Fibrosis in Atopic Dermatitis by Thymic Stromal Lymphopoietin

Min-Hee Oh,* Sun Young Oh,* Jinho Yu,* Allen C. Myers,* Warren J. Leonard,[†] Yong Jun Liu,[‡] Zhou Zhu,* and Tao Zheng*

Skin fibrotic remodeling is a major feature in human atopic dermatitis (AD). Inflammation and tissue fibrosis are common consequences of Th2 responses. Elevated IL-13 and thymic stromal lymphopoietin (TSLP) have been found in the AD skin lesions. Fibrocytes can be recruited to inflamed tissues to promote wound healing and fibrosis. Dermal transgenic expression of IL-13 causes an AD-like phenotype with fibrosis and increased TSLP. However, the role of TSLP in fibrotic remodeling is unknown. In this study, we investigated the role of TSLP and fibrocytes in the generation of IL-13–induced skin fibrosis. In AD lesion, cessation of IL-13 transgene expression resulted in reduced skin inflammation but with no effect on further progression of fibrosis. This was accompanied by markedly increased CD34⁺/procollagen 1⁺ fibrocytes. Furthermore, fibrocytes express TSLP receptor (TSLPR), and TSLP directly promotes PBMC-derived fibrocytes to produce collagen. Neutralization of TSLP or genetic deletion of TSLPR in IL-13 transgenic mice resulted in a significant reduction in fibrocytes and in skin fibrosis. Furthermore, reduction of fibrosis by depletion of TSLP was independent of IL-13. Interestingly, the number of fibrocytes was highly increased in the skin samples of AD patients. These data indicate that the progression of skin fibrosis in IL-13–induced AD occurs via TSLP/TSLPR-dependent but IL-13–independent novel mechanisms by promoting fibrocyte functions. *The Journal of Immunology*, 2011, 186: 000–000.

Atopic dermatitis (AD), one of the most common inflammatory skin diseases, is characterized by epidermal and dermal infiltration of inflammatory cells and skin fibrotic and vascular remodeling. IL-13 is known as a major stimulator of inflammation and tissue remodeling at sites of Th2 inflammation, such as in asthma. We reported previously that selective expression of IL-13 in the skin in a transgenic (Tg) murine model of AD causes a skin inflammatory phenotype characterized by increased eosinophils, mast cells, activated Langerhans cells, CD4⁺ T cells, and significant skin remodeling. The skin remodeling includes markedly increased collagen deposition in the skin associated with significantly increased expression of thymic stromal lymphopoietin (TSLP) by keratinocytes (1).

TSLP is a critical cytokine that promotes Th2 immunity in AD (2). TSLP exerts its biological function by binding to its functional heterodimeric receptor complex composed of the TSLP receptor (TSLPR) and the IL-7R α -chain (3, 4) and signals through the STAT5 pathway. TSLP has been shown to be highly increased in the skin lesions of patients with AD, but not in skin lesions of nickel-induced dermatitis or systemic lupus erythematosus (5, 6). TSLP can induce phenotypes of asthma or AD when selectively overexpressed in Tg mice or when driven by topical application of vitamin D3 (7–9). Intradermal-administered exogenous TSLP can induce skin subcuticular fibrosis in wild-type (WT) mice (10). However, the role of TSLP and TSLPR signaling in the generation of skin fibrosis in AD is unknown.

Circulating fibrocytes, a newly characterized bone marrow-derived cell type in the blood, account for 0.1–0.5% of circulating mononuclear cells. These cells express both hematopoietic markers (CD34, CD45) and collagen markers (procollagen 1 [ProCol 1], collagen 1), and can differentiate into myofibroblasts and fibroblasts after recruitment by chemokines produced at the inflamed site (11, 12). Fibrocytes are associated with skin fibrosis, pulmonary fibrosis, and tumors, and functionally, these cells contribute to the remodeling response by secreting matrix metalloproteinases and connective tissue proteins such as vimentin and collagens I and III (13–15). Recent studies indicate that the increase of circulating fibrocytes correlates with persistent airway flow obstruction in patients with asthma, and fibrocyte localization in the airway smooth muscle presents a prominent feature of airway remodeling in severe asthmatic patients (16, 17). Furthermore, IL-13 has recently been shown to promote the differentiation of CD14⁺ mononuclear cells into fibrocytes, whereas Th1 cytokines, IL-12, and IFN- γ inhibit this process (18). However, the role of fibrocytes in AD and the roles of IL-13 and TSLP in regulating these cells are unknown.

In the current study, using an inducible Tg IL-13 model of AD, we showed that after AD developed, inactivation of the IL-13 transgene resulted in a dramatic reduction of inflammatory

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Received for publication March 3, 2011. Accepted for publication April 18, 2011.

This work was supported by National Institutes of Health Grants AI075025 (to T.Z.) and HL079349 (to Z.Z.) and by the Intramural Research Program of the National Heart, Lung, and Blood Institute/National Institutes of Health (to W.J.L.).

M.-H.O. and S.Y.O. performed the experiments and collected and analyzed the data; J.Y. assisted in experiments; A.C.M. provided reagents and assisted in the immunohistochemistry experiments and in the analysis of the results; W.J.L. generated the TSLPR knockout mice, assisted with design of the study, and edited the manuscript; Y.J.L. provided conceptual advice on the study in general and on the biology of TSLP in particular, collected and provided human skin samples and reagents, and edited the manuscript; Z.Z. assisted in the conception and design of the study, provided the TRE-IL-13 transgenic mice, advised on the experiments and analysis of data, and helped in writing and editing the manuscript; and T.Z. conceived and designed the study, supervised and performed the experiments, analyzed the data, and wrote the manuscript.

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Abbreviations used in this article: AD, atopic dermatitis; Ct, cycle threshold; Dox, doxycycline; ET-1, endothelin-1; HPF, high-power field; IHC, immunohistochemistry; PDGF, platelet-derived growth factor; ProCol 1, procollagen 1; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor; WT, wild-type.

responses in the skin; however, the skin fibrosis continuously worsened, suggesting that, unlike skin inflammation, the maintenance and progression of skin fibrosis in IL-13-induced AD is through an IL-13-independent mechanism. To understand the underlying cellular and molecular mechanisms of skin fibrotic remodeling in AD, we studied the role of TSLP in skin fibrosis, the accumulation, differentiation, and function of fibrocytes, and the expression of profibrogenic mediators in IL-13-induced AD.

Our studies revealed that the number of CD34 and ProCol 1 double-positive fibrocytes in the dermal and hypodermal areas of IL-13 transgene(+) skin lesions was significantly increased compared with the Tg(−) mice, and these fibrocytes express TSLPR. The mRNA levels of CXCL12 and CXCR4, which form a critical biologic axis for the migration of peripheral fibrocytes to inflammatory and injured tissues, were highly increased in Tg(+) AD skin samples. Blockage of TSLP with a monoclonal neutralizing Ab or genetic deletion of TSLPR in IL-13 Tg(+) mice significantly reduced the magnitude of skin pathogenic fibrosis. This is the first reported study, to our knowledge, demonstrating that neutralization of TSLP or genetic deletion of TSLPR in mice significantly inhibits IL-13-induced skin fibrosis, whereas the levels of IL-13 in the skin of these mice were not changed, suggesting that the profibrotic effect of TSLP in AD is independent of the effect of IL-13. Furthermore, blocking TSLP with monoclonal neutralizing Ab after the transgene IL-13 was inactivated and the level of IL-13 in the skin of Tg(+) mice was comparable to that of Tg(−) mice, whereas the level of TSLP in the lesional skin remained elevated, resulted in significant attenuation of collagen accumulation, further supporting that the effects of TSLP on skin fibrotic remodeling are independent of IL-13. Importantly, we found a novel role of TSLP on skin fibrogenesis in AD by directly exerting its fibrogenic-promoting effects on fibrocytes, including enhancing the differentiation of fibrocytes and production of collagen by fibrocytes. We found that the number of CD34/ProCol 1 double-positive fibrocytes was highly increased in the skin biopsy samples from patients with AD. These findings provided mechanistic insights into the role of TSLP and TSLPR signaling in the pathogenic fibrosis of skin remodeling in AD induced by IL-13.

Materials and Methods

Animals

The inducible skin-specific IL-13 Tg mice [K5-tTA-IL-13(+) or simply Tg(+)] on a C57BL/6 genetic background were generated by our laboratory as previously described (1). To control the expression of IL-13 specifically and inducibly in the skin, doxycycline (Dox) was added to the drinking water (1 mg/ml) to suppress tTA binding to its target and to keep the IL-13 transgene off until the K5-tTA-IL-13 mice were 6 wk old. The experiments were initiated by withdrawing Dox from the drinking water. In all experiments, Tg(−) littermate controls received the same amount of Dox or no Dox for the same length of time. For the reversibility experiments, after the development of AD, the Tg(+) mice were randomly assigned to receive Dox water to turn off the IL-13 transgene *in vivo* (the transgene On-Off group) or to receive normal drinking water to keep the IL-13 transgene on (the transgene On-On group). The TSLPR gene null mice were generated as described previously (19). These mice on C57BL/6 genetic background were crossbred with the K5-tTA mice and TRE-Tight-IL-13 mice, both of which were on the C57BL/6 background. The genotype of the K5-tTA-IL-13 mice was determined as described previously (1). The genotype of the TSLPR-null mice was determined using three PCR primers: primer A, 5'-AACCTCTCCC-ACAAGAAGTCCAGAAGT-3'; Neo primer, 5'-ATCGCCTTCTATCG-CCTTCTT-3'; and primer B, 5'-AGACTTTACCTGATTCCTGCCTTG-3'. Primers A and B amplify a 250-bp segment of the endogenous TSLPR gene. The Neo primer and primer B identify the targeted gene and give a 650-bp product (19). The offspring with genotypes K5-tTA-IL-13/TSLPR^{+/+} [or simply Tg(+)/TSLPR^{+/+}] and K5-tTA-IL-13/TSLPR^{−/−} [or simply Tg(+)/TSLPR^{−/−}] were compared in the experiments. All procedures involving animals in this study were approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

Clinical disease scores

Tg(+) and Tg(−) mice were examined for skin lesions three times per week, and the clinical scores for disease severity were recorded as described with slight modifications (1, 20, 21).

Preparation of skin protein extracts and measurement of cytokines and collagens

Protein samples were prepared as described previously (1). Briefly, frozen skin tissues were placed in liquid nitrogen, crushed with a mortar and pestle, and weighed. Triton X-100 0.25% (w/v) in PBS was added to the skin powder. The homogenate was stirred at 4°C overnight and then centrifuged at 3000 × g for 15 min to remove debris. Supernatants were stored in small aliquots at −80°C until assayed. All samples were normalized to the weight of the skin samples. Cytokines or collagen in the skin samples were determined using ELISA kits or Sircol assay (Biocolor, Belfast, U.K.) per the manufacturer's instructions.

Quantitative real-time PCR

Total RNA from the skin samples was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA was reverse-transcribed using the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using Power SYBR Green Master Mix and the ABI PRISM 7700 Detection system (Applied Biosystems, Foster City, CA) to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Target cDNA levels were normalized to GAPDH, an internal reference, using the equation $2^{-[\Delta Ct]}$, where ΔCt is defined as $Ct_{\text{target}} - Ct_{\text{internal}}$. Specific gene expression was presented in arbitrary units relative to the lowest detectable expression. Values shown were the average of two independent experiments.

Fluorescent immunohistochemistry and Abs

To detect skin fibrocytes, skin tissues were fixed with Shandon Glyo-Fixx (Thermo Fisher Scientific, Waltham, MA), and then immunofluorescence was performed on deparaffinized mouse skin tissue slides. The slides were blocked with donkey blocking solution (10% donkey serum [Sigma-Aldrich, St. Louis, MO], 1% BSA [Sigma-Aldrich], and 0.5% Tween 20) in PBS for 1 h. After washing, tissue sections were incubated at 4°C overnight with a goat anti-mouse ProCol 1A1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and a rat anti-mouse CD34 Ab (eBioscience, San Diego, CA). After rinsing, tissue sections were incubated with Alexa Fluor 488-labeled donkey anti-goat IgG (A11055; Invitrogen), Alexa Fluor 594 donkey anti-rat IgG (Invitrogen), and DAPI (Roche Diagnostics, Mannheim, Germany) at room temperature for 2 h. After washing, tissue sections were mounted using PermaFluor (Thermo Fisher Scientific). The skin sections were examined using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) at 488 nm to assess ProCol 1, 594 nm to assess CD34, and 405 nm to assess cell nuclei.

The expression of TSLPR on skin fibrocytes was assessed on deparaffinized mouse skin slides. Briefly, after blocking and washing, tissue sections were incubated at 4°C overnight with a rabbit anti-ProCol 1A1, a rat anti-CD34, and a goat anti-mouse TSLPR Ab (Santa Cruz Biotechnology). After washing, the tissue sections were incubated with Alexa Fluor 350-labeled donkey anti-rabbit IgG (Invitrogen), Alexa Fluor 594-labeled donkey anti-rat IgG (Invitrogen), and Alexa Fluor 488-labeled donkey anti-goat IgG (Invitrogen) at room temperature for 2 h. The skin sections were washed and mounted using PermaFluor (Thermo Fisher Scientific). The skin sections were examined under a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) at 350 nm to assess ProCol 1, 594 nm to assess CD34, and 488 nm to assess TSLPR.

To detect fibrocytes in cryosectioned human skin samples, the sections were analyzed as described previously with minor modifications (22). Briefly, the slides were fixed with 4% paraformaldehyde and then blocked with donkey blocking solution for 1 h. After washing, tissue sections were incubated with a goat anti-human ProCol 1A2 and a mouse anti-human CD34 (Santa Cruz Biotechnology) at 4°C overnight followed by Alexa Fluor 488-conjugated donkey anti-goat IgG (Invitrogen) and with Alexa Fluor 594-conjugated donkey anti-mouse IgG (Invitrogen) at 4°C overnight. Cell nuclei were detected by incubating the tissue sections with DAPI for 10 min. After washing, tissue sections were mounted using PermaFluor (Thermo Fisher Scientific) and examined using confocal microscopy as above.

Cultured mouse fibrocytes

Purification of mouse fibrocytes was performed as described by Abe et al. (23) with slight modification. Briefly, fibrocytes from WT (C57BL/6)

mouse blood through cardiac puncture were isolated by Histopaque 1083 (Sigma-Aldrich) density gradient centrifugation following the manufacturer's protocol. These cells were cultured (2×10^6 cells/well) in DMEM supplemented with 10% FBS (Invitrogen), 4 mM L-glutamine, penicillin, streptomycin, 50 μ M 2-ME, 20 mM HEPES, 2 $\times$ nonessential amino acids, and 2 mM sodium pyruvate. After 72 h incubation, non-adherent cells were washed out, and attached cells were cultured in the presence of recombinant TSLP (50 ng/ml) (R&D Systems, Minneapolis, MN) and rIL-13 (10 ng/ml). The supernatants and cells were then collected after a 10 d-culture for collagen assay.

Flow cytometry

Skin tissues were chopped into small pieces and incubated in DMEM containing 10% fetal serum, 20 μ g/ml DNase I (Sigma-Aldrich) for 1 h at 37°C. Mononuclear cells from skin single-cell suspensions were isolated by Histopaque 1083 (Sigma-Aldrich) density gradient centrifugation following the manufacturer's protocol. The cells were stained with allophycocyanin-conjugated anti-CD45 (BD Biosciences) and PE-conjugated anti-TSLPR (R&D Systems) or respective isotype control Ab. To stain for collagen type I, cells were incubated with anti-collagen type I (Millipore) or isotype control and subsequently stained with FITC-conjugated secondary Ab. Samples were acquired on an FACSCalibur instrument (BD Biosciences), and the outputs were analyzed using FlowJo (Tree Star).

Quantification of fibrocytes

For quantification of positive cells of fluorescent-labeled fibrocytes in the skin and fibrocytes purified from PBMC, slides were coded, and positive cells were counted blindly under $\times 200$ original magnification with an eyepiece graticule. Five fields per section were counted in mouse skin samples, and five fields per section of cultured fibrocytes (four slides per condition) were counted per high-power field (HPF; 0.02 cm²) (24).

Skin fibrosis score

As described (24), the severity of skin fibrosis was scored on a 1–4 scale according to the extent of fibrosis within the dermis, with a score of 4 representing severe fibrosis that extended to the whole dermis. The within-observer coefficient of variation for repeated measures was <5%. Data were expressed in the text as mean $\pm$ SEM.

Neutralization of TSLP in vivo

Neutralizing TSLP at the time of activation of the transgene IL-13: IL-13 Tg(+) mice ($n = 4$ each group) were given a specific neutralizing monoclonal TSLP Ab (R&D Systems) at 10 mg/kg i.p. weekly for four injections at the time of withdrawal of Dox water to turn on the IL-13 transgene in the skin, followed by three biweekly i.p. injections. For the control group, Tg(+) mice were administered an IgG2a isotype control Ab (R&D Systems). The mice were sacrificed 3 d after the last dose to obtain the skin samples for evaluation.

Neutralizing TSLP 4 wk after inactivation of the transgene IL-13: IL-13 Tg(+) mice ($n = 4$ each group) were given a specific neutralizing monoclonal TSLP Ab (see above) or a control Ab IgG2a at 10 mg/kg i.p. every 5 d for seven injections. The mice were sacrificed 3 d after the last dose.

Quantitative measurement of skin collagen content

To determine the collagen content in the skin samples, the Sircol collagen assay (Biocolor) was performed following the manufacturer's instructions. Briefly, Sirius red reagent (50 μ l) was added to each skin homogenate (50 μ l) and mixed for 30 min. The collagen dye complex was precipitated by centrifugation at $16,000 \times g$ for 5 min, washed with ethanol, and dissolved in 0.5 M NaOH. Finally, the samples were introduced into a microplate reader and the absorbance determined at 540 nm. All samples were normalized to the weight of the skin samples.

Histochemistry

Masson's Trichrome staining was used to detect the collagen fibers and collagen deposition in the mouse skin. This assay was performed in mouse skin samples following the manufacturer's instructions. The collagen fibers were stained blue, the nuclei were stained black, and the background was stained red. The extent of the positive staining was scored as described (24). Gomori's Trichrome staining was performed for detection of collagen in human skin samples using Trichrome Stain AB Solution following the manufacturer's instructions (Sigma-Aldrich). The collagen fibers were stained blue, the nuclei were stained purple/black, and normal muscle myofibrils were stained green-blue, whereas intermyofibrillar muscle membranes, cytoplasm, and muscle were stained red.

Skin biopsy samples

Skin biopsy samples from AD patients and normal volunteers were described previously (25). After explanation of the nature of research and obtaining informed consent from patients, 3–6-mm punch biopsies were taken from either lesional or nonlesional skin from AD ($n = 3$) or normal healthy individuals ($n = 4$). Skin samples were immediately frozen in liquid nitrogen and stored at -80°C . The study was approved by the local ethics committee.

Statistical analysis

The Student *t* test was used to determine the significance of difference between two groups, and one-way ANOVA was used for comparison among multiple groups. All data were expressed as mean $\pm$ SEM. Difference with $p < 0.05$ was considered statistically significant.

Results

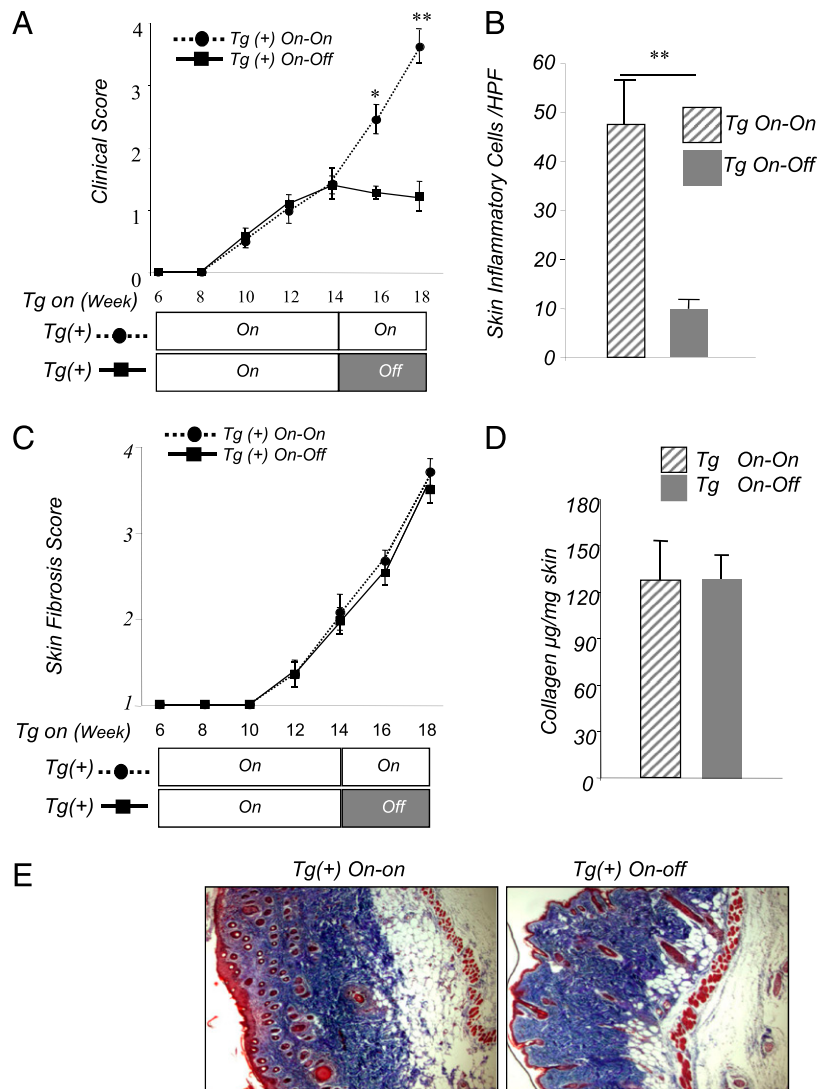
Progression of skin fibrosis despite attenuation of skin inflammation

We previously showed that overexpression of IL-13 in the skin caused fibrotic changes in the lesional skin of AD in IL-13 Tg(+) mice, which was characterized by increased collagen accumulation and increased profibrogenic mediators including TGF- β 1 and MCP-1 in the AD skin (1). In the current study, we first evaluated whether IL-13–induced skin fibrosis could be reversed or halted after the IL-13 transgene was turned off in vivo. After withdrawal of Dox in the drinking water (the transgene was turned on) for 8–10 wk, Tg(+) mice developed AD including pruritus, hair loss, and erythema (clinical score <2). These mice were then randomly assigned to receive Dox water or normal water. The mice that continued to receive normal water (the transgene was kept on: the transgene On-On group) developed more severe and extensive dermatitis. In contrast, the Tg(+) mice given Dox water to turn off the IL-13 transgene (the transgene On-Off group) showed no worsening of their AD (Fig. 1A). This change became apparent after the mice were given Dox for 2 wk, although the skin lesions did not completely resolve at the end of the experiment. When the skin lesions were histologically examined, there were significantly less inflammatory cells in the AD skin of the transgene On-Off group compared with the AD skin of the transgene On-On group (Fig. 1B). However, despite the marked attenuation in skin inflammation, the magnitude of extensive collagen deposition in the dermal and hypodermal areas and the amount of collagen accumulation in the AD skin in the transgene On-On group and in the transgene On-Off group were comparable (Fig. 1C, 1D), correlating with the fibrosis scores (Fig. 1E). These data showed that the pathogenic fibrotic changes in AD progressed despite attenuated inflammation, suggesting that the progression of the skin fibrogenesis is independent of IL-13.

Increased fibrocytes and profibrogenic mediators in the AD skin

Studies in various animal models of wound healing or fibrotic diseases have suggested a causal link between fibrocyte accumulation and ongoing tissue fibrogenesis or vascular remodeling in response to tissue inflammation (13). To understand the mechanisms of the skin fibrotic remodeling in AD, we investigated whether IL-13 could play a role in recruiting fibrocytes to the site of skin inflammation in AD. These newly identified circulating cells carry both markers for hematopoietic cells (CD34 and CD45) and markers for myofibroblasts (ProCol 1a, collagen 1, and fibronectin) (18, 26). When lesional skin samples of IL-13 Tg(+) mice were stained for cells coexpressing fluorescent-labeled CD34 and ProCol 1, increased numbers of fibrocytes were visualized, and they were primarily located in the dermal and hypodermal areas adjacent to microvessels in IL-13 Tg(+) skin. In contrast,

FIGURE 1. Progression of skin fibrosis in AD despite the attenuation of skin inflammation. After withdrawal of Dox in the drinking water (the transgene was turned on) for 8–10 wk, IL-13 Tg(+) mice began to develop AD (clinical score <2). These mice were then randomly assigned to receive Dox to inactivate the IL-13 transgene [IL-13 Tg(+) On-Off group] or normal water to keep the transgene on [IL-13 Tg(+) On-On group]. Mice were sacrificed, and skin samples were obtained at the age of 18 wk. **A**, Clinical score of AD: amelioration of the AD after the IL-13 transgene was turned off in the Tg(+) On-Off mice (solid line) and On-On group (dotted line). **B**, Quantification of skin inflammatory cells (HPF) in the skin stained by H&E. **C**, Quantification of the skin fibrosis (fibrosis score): progression of skin fibrosis in AD in the Tg(+) On-On mice (dotted line) and in the Tg(+) On-Off mice (solid line). **D**, Quantification of collagen contents (by Sircol assay; Biocolor) in the skin. **E**, No significant difference in the skin collagen deposition between the two groups of mice by Masson's Trichrome staining (original magnification $\times 10$). Shown are representatives of six individual samples for each group. For A–D, $n = 6$ for each group. * $p < 0.05$, ** $p < 0.005$.



these cells were rarely seen in Tg(–) skin (Fig. 2A). Quantification of CD34/ProCol 1 dual-positive fibrocytes by immunohistochemistry (IHC) with fluorescent microscopy and the percentage of CD45/collagen dual-positive fibrocytes by FACS showed a significantly higher number of cells in the lesional AD skin than those in the skin of Tg(–) mice (Fig. 2B, 2C). These data indicate that increased fibrocytes in the AD skin in Tg(+) animals may contribute to IL-13-induced skin fibrosis.

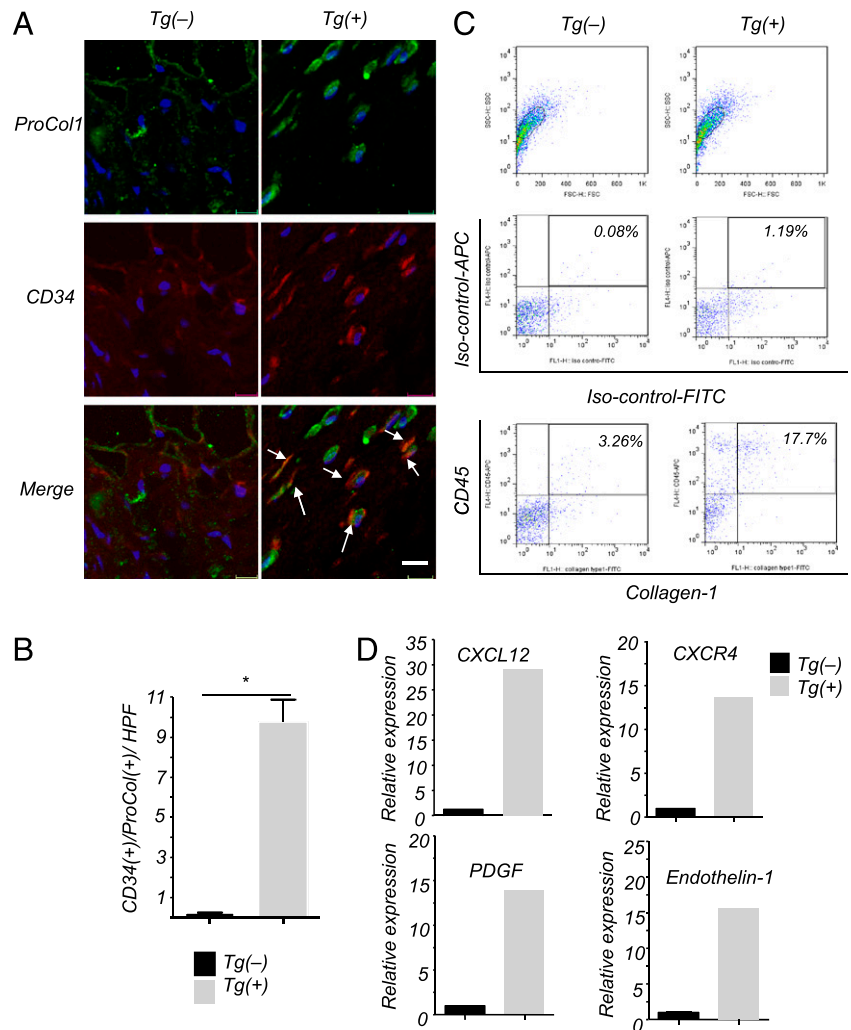
Because the migration of fibrocytes to inflamed tissues is critically dependent on the CXCL12/CXCR4 pathway in both idiopathic pulmonary fibrosis in humans and in bleomycin-induced lung fibrosis in mice (27), we next examined the mechanisms of IL-13-induced accumulation of fibrocytes in the skin of AD. It is known that CXCR4 is a predominant chemokine receptor on both human and mouse fibrocytes. Its expression on fibrocytes is enhanced by platelet-derived growth factor (PDGF) (27), and the differentiation of fibrocytes to mature fibroblasts and myofibroblasts is promoted by stimulation of TGF- β 1 and endothelin-1 (ET-1) (23, 26). Studies were undertaken to examine the factors critical in regulating tissue fibrosis and migration and maturation of fibrocytes, including CXCL12/stromal cell-derived factor-1, CXCR4, PDGF, ET-1, and TGF- β 1. Real-time PCR was used to compare the expression of these mediators between the lesional skin samples of Tg(+) mice and those of Tg(–) mice. Expression of genes encoding CXCL12, CXCR4, PDGF, and ET-1 was sig-

nificantly enhanced in the AD skin of IL-13 Tg(+) mice compared with that of IL-13 Tg(–) mice (Fig. 2D). These data suggest that the CXCL12–CXCR4 biological axis may play an important role in recruiting circulating fibrocyte precursors to the AD skin in Tg(+) mice, and upregulation of PDGF, ET-1, and TGF- β 1 may enhance the migration and maturation of fibrocytes precursors in the process of fibrotic remodeling in AD skin.

Ab-mediated neutralization of TSLP resulted in amelioration of skin fibrosis

Our previous study showed that the level of TSLP is robustly elevated in both acute and chronic lesional skin of IL-13 Tg(+) mice and that TSLP is highly expressed in keratinocytes in the Tg(+) skin. Thus, we examined whether a specific neutralizing Ab against TSLP administered at the time of activation of the IL-13 transgene in vivo could reduce IL-13-mediated skin fibrosis. Tg(+) mice were treated with a monoclonal anti-TSLP Ab or an IgG2a isotype control via i.p. injection four times weekly, then three times biweekly. Masson's Trichrome stain for detection of collagen deposition and Sircol assay (Biocolor) for quantification of collagen in the skin were performed to compare the levels of collagen in chronic lesional AD skin samples from the two groups of Tg(+) mice. The lesional skin samples from IL-13 Tg(+) mice that were treated with control IgG2a Ab showed intense and extensive Masson's Trichrome staining and higher skin fibrosis

FIGURE 2. Increased CD34⁺/ProCol 1⁺ fibrocytes and mediators critical in the biology of fibrocytes in the AD skin of Tg(+) mice. **A**, CD34⁺/ProCol 1⁺ fibrocytes (white arrows) in the skin of Tg(+) mice were evaluated by confocal microscopy. Scale bar, 10 μ m. **B**, Quantification of fibrocytes in the skin by fluorescent IHC. Double-positive cells were counted in five HPF per section, five sections per mouse ($n = 4$ for each group, Mean $\pm$ SEM. **C**, Percentage of CD45⁺/collagen-1⁺ fibrocytes in the skin tissue by flow cytometry. **D**, Quantitative comparison by real-time PCR of mRNA encoding CXCL12, CXCR4, PDGF, and ET-1. The results were normalized to GAPDH (reference gene) and expressed in arbitrary units relative to the lowest detectable expression. The data were representative of two or three independent experiments. * $p < 0.05$.



scores (Fig. 3A, 3B). Conversely, mice treated with anti-TSLP had markedly reduced collagen deposition and significantly lower skin fibrosis scores than those mice that received IgG2a control Ab. The level of skin collagen by Sircol assay (Biocolor) and the levels of both total and activated TGF- β 1 by ELISA in the AD skin samples showed that blockage of TSLP using anti-TSLP significantly reduced the amount of the skin collagen and TGF- β 1 in the AD skin of IL-13 Tg(+) mice compared with those in the AD skin of IL-13(+) mice that received control IgG2a (Fig. 3C, 3D). Furthermore, skin inflammation and clinical disease of AD were significantly attenuated in the Tg(+) mice that received anti-TSLP compared with the Tg(+) mice given control Ab only (Fig. 3E, 3F).

Neutralization of TSLP resulted in a reduction in skin fibrocytes and downregulation of critical profibrotic mediators

We next examined the role of TSLP in IL-13-induced accumulation of fibrocytes in the skin fibrotic remodeling in AD. Using IHC with fluorescent-labeled Abs to CD34 and ProCol 1, we compared the numbers of the double-positive fibrocytes in the lesional skin samples from Tg(+) mice treated with either anti-TSLP or IgG2a isotype control. The numbers of fibrocytes were significantly reduced in the lesional skin samples of anti-TSLP-treated Tg(+) mice compared with those in the skin of the Tg(+) mice given isotype control (Fig. 4A, 4B), suggesting that TSLP is important in regulating accumulation of fibrocytes in the AD skin.

We next examined mediators that are critical in migration and differentiation of fibrocytes in skin wound healing and fibrosis.

Real-time PCR analysis showed that the expression of genes encoding CXCL12, CXCR4, PDGF, and ET-1 was significantly downregulated in the AD skin samples from the mice treated with anti-TSLP compared with those treated with control Ab (Fig. 4C). Skin inflammatory responses were remarkably attenuated in Tg(+) mice that were given anti-TSLP. Conversely, numerous inflammatory cells were seen in the skin of Tg(+) animals that received control Ab IgG2a (Fig. 3E). When viewed together, these studies demonstrated that neutralizing TSLP in Tg(+) mice led to downregulation of the expression of profibrogenic mediators, which in turn led to reduced migration and differentiation of fibrocytes and collagen accumulation. These results suggest that TSLP critically contributes to skin fibrosis of AD, possibly through regulating fibrogenic mediators and exacerbating inflammatory responses. The levels of IL-13 in the AD skin samples from both groups of the mice were comparable, indicating that the reduction in skin fibrosis by the treatment of specific neutralizing TSLP was not mediated by inhibiting the production of Tg IL-13, which indicates that blocking TSLP-mediated attenuation of magnitude of skin fibrogenesis is not dependent on the tissue effect of IL-13 (Fig. 4D).

Role of TSLPR in skin collagen deposition in IL-13-induced AD

The role of TSLP and TSLPR signaling in promoting Th2 inflammation in asthma and AD has been recognized (2, 5, 7, 9). However, their role in skin fibrosis in AD is unknown. We

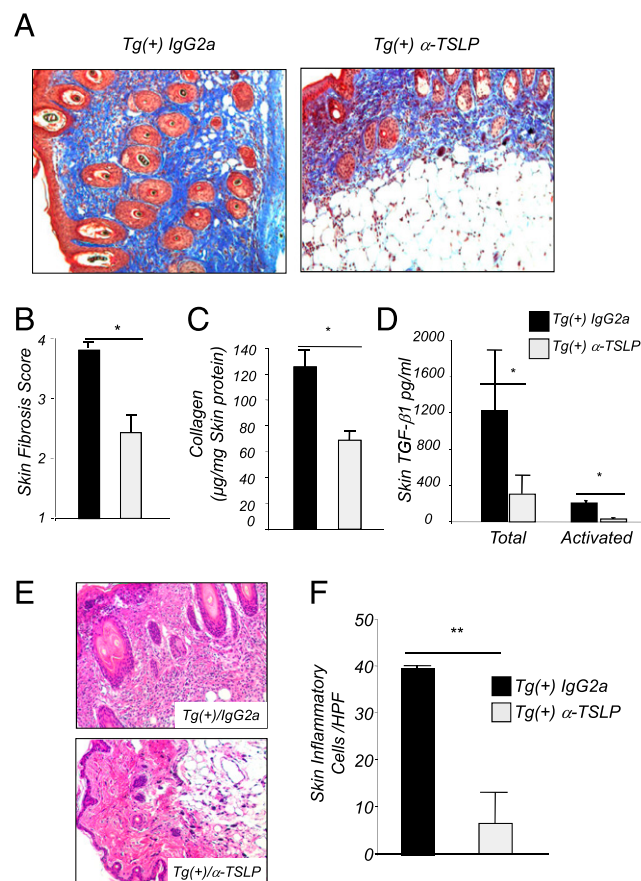


FIGURE 3. Specific mAb-mediated neutralization of TSLP resulted in amelioration of IL-13-induced skin fibrosis and inflammation. Six-week-old Tg(+) mice received the first dose of anti-TSLP mAb or control IgG2a at the time of the transgene being activated. Anti-TSLP was given i.p. at 10 mg/kg weekly for four doses and biweekly for three doses. The mice were examined for scores of dermatitis and skin fibrosis ($n = 4$ for anti-TSLP group and $n = 5$ for IgG2a group). **A**, Using Masson's Trichrome staining, increased collagen deposition in the dermal and s.c. areas of the skin from IgG2a-treated Tg(+) mice compared with markedly reduced collagen deposition in anti-TSLP-treated Tg(+) mice. Original magnification $\times 10$. **B**, Quantitative evaluation of skin fibrosis score. **C**, Quantification of collagen content in the skin using the Sircol collagen assay (Biocolor). **D**, The levels of total and active forms of TGF- $\beta 1$ assessed by ELISA. Histological evaluation of skin samples from Tg(+) mice given IgG2a and Tg(+) mice given anti-TSLP (**E**; H&E, original magnification $\times 10$) and quantification of skin inflammatory cells per HPF (**F**). For **B–D**, results are shown as mean $\pm$ SEM. $*p < 0.05$, $**p < 0.001$.

investigated the importance of the TSLP–TSLPR signaling pathway in skin fibrosis in AD induced by IL-13. K5-tTA–IL-13 mice with the WT TSLPR gene Tg(+)/TSLPR^{+/+} and null mutation of TSLPR gene Tg(+)/TSLPR^{-/-} were compared for the magnitude of collagen deposition and the level of collagen content in the AD skin samples. As shown by Masson's Trichrome staining, extensive and more dense collagen was seen in the dermal and hypodermal areas in the skin of Tg(+)/TSLPR^{+/+} animals. In contrast, the deposition and distribution of collagen were remarkably reduced in the skin of Tg(+)/TSLPR^{-/-} mice (Fig. 5A). The skin inflammation was attenuated in Tg(+)/TSLPR^{-/-} mice compared with that in Tg(+)/TSLPR^{+/+} mice (Fig. 5B). In accord with these findings (Fig. 5A), the amount of collagen in the AD skin by quantitative Sircol collagen assay (Biocolor) was significantly lower in Tg(+)/TSLPR^{-/-} mice than that in the AD skin of Tg(+)/TSLPR^{+/+} mice (Fig. 5C). In addition, the levels of total and ac-

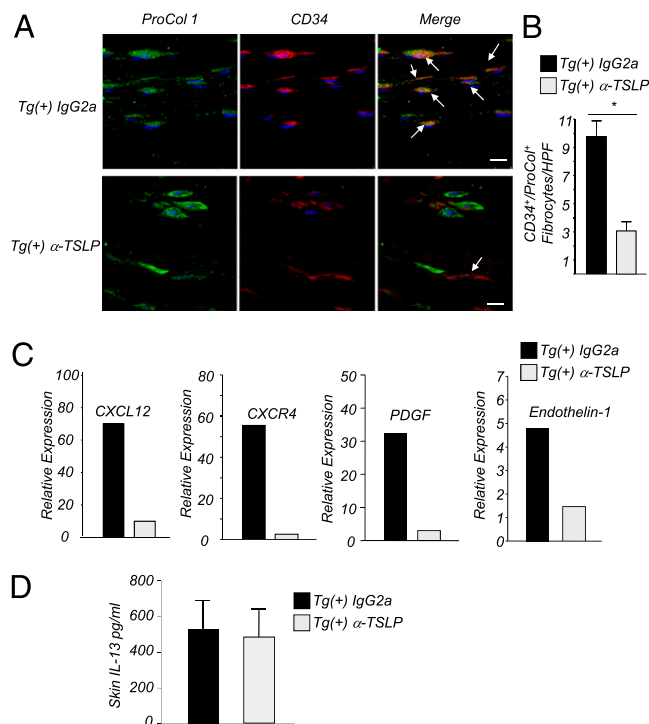


FIGURE 4. Neutralization of TSLP in Tg(+) mice reduced accumulation of skin fibrocytes. **A**, Accumulation of skin fibrocytes in Tg(+) mice treated with anti-TSLP or IgG2a isotype control by confocal microscopy (white arrows: double-positive cells). Scale bars, 10 μ m. **B**, Quantitative evaluation of fibrocytes in the skin by fluorescent IHC with double labeling of CD34 and ProCol 1 Abs. Double-positive cells were quantified (five HPF per section). The data are expressed as mean $\pm$ SEM ($n = 6$ for each group). **C**, Quantitative real-time PCR analysis of mRNA encoding CXCL12, CXCR4, PDGF, and ET-1. The results were normalized to GAPDH (reference gene) and expressed in arbitrary units relative to the lowest detectable expression. The data were representative of two or three independent experiments. **D**, The levels of IL-13 in the skin by ELISA in the anti-TSLP or IgG2a isotype control groups ($n = 4$ to 5 each group). $*p < 0.05$.

tivated forms of TGF- $\beta 1$ in the AD skin samples from Tg(+)/TSLPR^{-/-} mice were significantly decreased compared to those in the AD skin of Tg(+)/TSLPR^{+/+} mice (Fig. 5D).

Role of TSLPR in accumulation of fibrocytes in the AD skin

To evaluate the contribution of the TSLPR in the accumulation of fibrocytes in the AD skin, using fluorescent IHC, we compared the number of double-positive CD34/ProCol 1 cells in the lesional skin of AD between IL-13 Tg(+)/TSLPR^{+/+} and IL-13 Tg(+)/TSLPR^{-/-} animals. The number of fibrocytes in the chronic AD skin was remarkably lower in the Tg(+) mice carrying the null mutation of TSLPR than those Tg(+) mice carrying the WT TSLPR alleles, although still higher than those in the skin from Tg(-) mice with either the WT or null TSLPR gene (Fig. 5E). A comparison of expression of genes encoding CXCL12, CXCR4, PDGF, and ET-1 in the skin was made between Tg(+)/TSLPR^{+/+} and Tg(+)/TSLPR^{-/-} mice. In the absence of TSLPR in the Tg(+) mice, the levels of expression of these genes were reduced compared with those in Tg(+) carrying the WT TSLPR gene (Fig. 5F). Taken together, these studies indicate that TSLP signaling is critically involved in promoting skin fibrogenesis in AD induced by IL-13 by upregulating the expression of profibrotic mediators and enhancing the accumulation of fibrocytes and that TSLP signaling also contributes to the generation of inflammatory responses in IL-13-induced AD. Incomplete inhibition in IL-13-mediated tissue

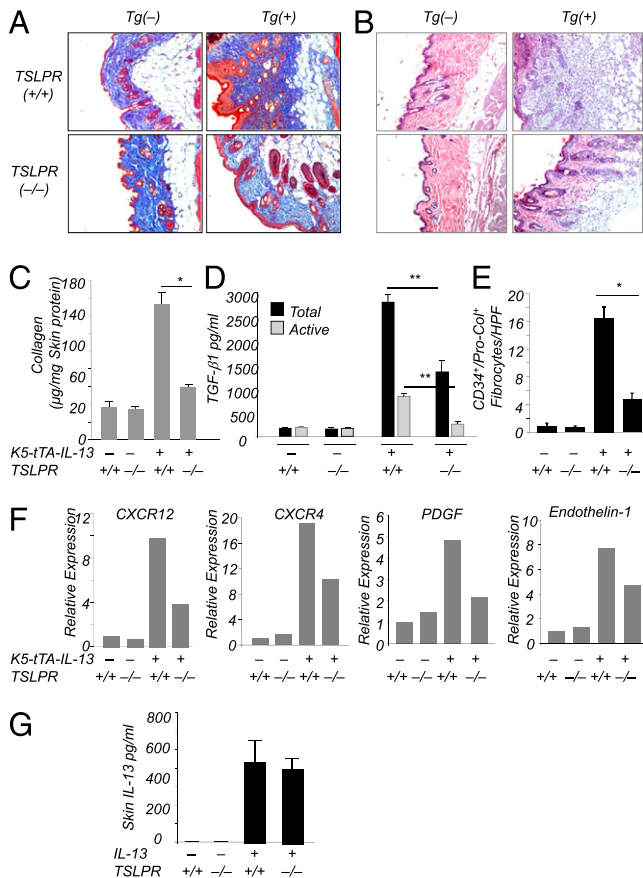


FIGURE 5. Role of TSLPR in IL-13-induced skin fibrosis in AD in IL-13 Tg(+) mice with WT(+/+) and null(-/-) TSLPR loci. Reduced collagen deposition by Masson's Trichrome staining (A) and reduced skin inflammatory response by H&E staining (B) were seen in the skin of Tg(+) TSLPR^{-/-} mice compared with that in the skin of Tg(+) TSLPR^{+/+} mice. A and B are representative of five individual samples from each group. Original magnification $\times 10$. Total collagen content of the skin by Sircol assay (C) and the levels of total and active forms of TGF- β 1 in the skin by ELISA (D). E, IHC with fluorescent anti-CD34/anti-ProCol 1 was used to assess double-positive fibrocytes in the skin. F, Real-time RT-PCR was used to evaluate the levels of mRNA encoding CXCR12, CXCR4, PDGF and ET-1 in the skin samples from Tg(-) and IL-13 Tg(+) mice with WT(+/+) and null(-/-) TSLPR loci ($n = 7$ for each group). G, The level of IL-13 in the skin by ELISA. The values in C–G are the mean $\pm$ SEM of three separate evaluations of at least eight animals for each group. * $p < 0.05$, ** $p < 0.005$.

effects in the skin of Tg(+) mice with null mutation of TSLPR suggests that part of the IL-13-mediated effects are TSLPR independent. The levels of IL-13 in the AD skin samples from both groups of mice were not altered, a finding in accord with that in the TSLP neutralization study (Fig. 4D), further supporting that the reduction in skin fibrosis in the absence of TSLPR in Tg(+) mice was not mediated by inhibiting the production of Tg IL-13, but rather by a tissue effect downstream of IL-13 (Fig. 5G).

Fibrocytes express TSLPR, and TSLP stimulates fibrocyte proliferation, differentiation, and collagen production

TSLP exerts its biological functions by binding to its receptor complex IL-7R α and TSLPR and signaling through STAT5. Serial studies were undertaken to explore the mechanisms by which TSLP enhances skin fibrosis. TSLPR has been shown to be expressed on mouse skin keratinocytes, airway epithelial cells, mast cells, dendritic cells, and mouse T cells (28), but whether TSLPR is expressed on fibrocytes is unknown. We first determined whether fibrocytes were the target cells of TSLP. Fluorescent IHC with

anti-CD45, anti-ProCol 1, and anti-TSLPR and FACS with anti-CD45, anti-Col 1, and anti-TSLPR were used to evaluate the expression of TSLPR on fibrocytes. Many CD34/ProCol 1/TSLPR triple-positive cells were readily seen in the dermal and hypodermal regions of AD skin (Fig. 6A). CD34 and ProCol 1 double-positive cells in the skin from Tg(-) mice were rarely seen, and TSLPR, CD34 and ProCol 1 triple-positive cells were absent in the skin samples from Tg(-) mice. A high percentage (97%) of CD45/Col-1 dual-positive fibrocytes was seen to express TSLPR (Fig. 6B). We then explored whether TSLP could directly exert its effect on fibrocytes. Fibrocytes purified from peripheral blood of WT mice were stimulated with recombinant TSLP (50 ng/ml) or IL-13 (10 ng/ml) for 10 d, and the cell morphology was examined. Cells stimulated with TSLP exhibited the typical fibrocyte morphology of elongated body with multiple projections, whereas those stimulated with IL-13 and unstimulated cells were seen with not well-differentiated elongated morphology or as undifferentiated small round cells (Fig. 6C). Further, measured by the Sircol assay (Biocolor), fibrocytes stimulated with TSLP produced significantly higher levels of collagen than those cells stimulated with IL-13 and unstimulated cells. The cells stimulated by both TSLP and IL-13 produced even higher levels of collagen than by TSLP alone, although IL-13 alone was not able to stimulate fibrocytes to produce collagen (Fig. 6D). These results demonstrated that fibrocytes express TSLPR, and TSLP is a potent stim-

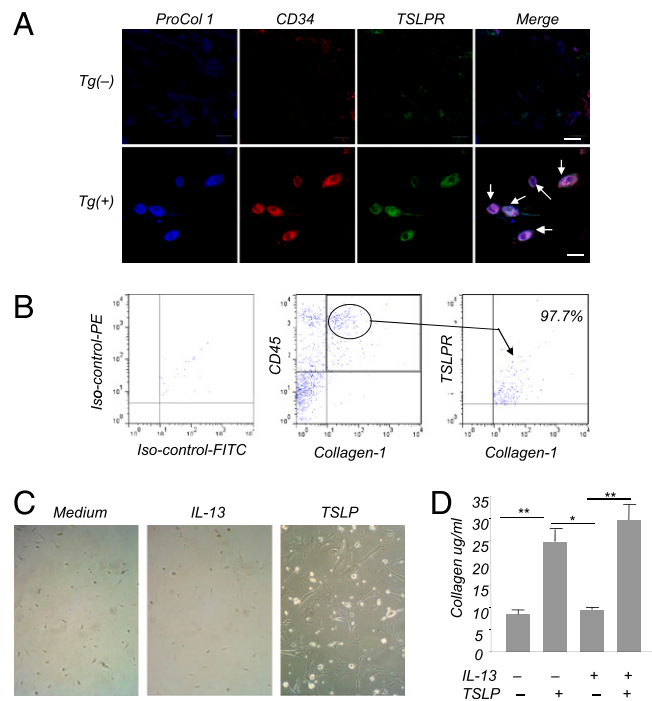


FIGURE 6. TSLPR expression and TSLP effects on skin fibrocytes. A, Expression of TSLPR on fibrocytes in the AD skin samples of K5-tTA-IL-13 mice. Skin samples from Tg(-) and Tg(+) mice were stained for TSLPR, CD34, and ProCol 1 by immunofluorescence using specific Abs and examined by confocal microscopy. White arrows indicate triple-positive cells. Slides are representatives of five different samples from each group. Scale bars, 10 μ m. B, CD45 and Col 1 double-positive fibrocytes purified from the skin of Tg(+) mice were stained and analyzed for TSLPR. C, Morphology of fibrocytes purified from PBMC of WT mice that were cultured in the presence of TSLP (50 ng/ml) or IL-13 (10 ng/ml) for 3 d (original magnification $\times 100$), and using Sircol assay (D), soluble collagen content from supernatants of cultured fibrocytes stimulated with TSLP (50 ng/ml) and IL-13 (10 ng/ml) for 10 d were measured. Data are representative of three independent experiments. $n = 5$ per group. * $p < 0.05$, ** $p < 0.01$.

ulator of fibrocytes to produce collagen. IL-13 does not directly act on fibrocytes but seems to potentiate the profibrogenic effect of TSLP on fibrocytes.

Taken together, these data indicated that fibrocytes are the target cells for TSLP and that the novel roles of TSLP on fibrocytes are critical in promoting the skin fibrotic remodeling through directly enhancing fibrocyte differentiation and collagen production. The fibrogenic effect of TSLP in AD in Tg(+) mice was significantly dampened by neutralizing TSLP (Fig. 3) and genetic deletion of TSLPR (Fig. 5), whereas the levels of IL-13 (the Tg IL-13 and perhaps endogenous IL-13) in the skin in these mice were unaltered (Figs. 4D, 5G), strongly suggesting that the effects of TSLP on skin fibrosis in AD are independent of the effect of IL-13.

Attenuation of skin pathological fibrogenesis in AD by blocking TSLP is mediated through an IL-13-independent pathway

Studies were undertaken to further evaluate whether the inhibitory effect of skin fibrogenesis by blocking TSLP is independent of the effect of IL-13. To eliminate the effect of Tg IL-13 in the skin of Tg(+) mice, the IL-13 transgene was turned off and kept off throughout the duration of the experiments. Four weeks after the IL-13 transgene was turned off in Tg(+) mice with AD, the level of IL-13 in the skin of these mice was reduced to the level comparable to that of Tg(−) mice (i.e., below the ELISA detection limit). However, the level of TSLP in the skin of Tg(+) mice was not altered by the inactivation of the IL-13 transgene (Fig. 7A). The Tg(+) mice were randomly assigned to receive either anti-TSLP mAb or IgG2a isotype control Ab i.p. every 5 d for seven doses. The level of collagen deposition in the skin was evaluated by Trichrome staining and compared between the two groups. Strong and extensive collagen deposition was seen in the dermal and subdermal areas of the AD skin of the Tg(+) On-Off group mice treated with control IgG2a. Conversely, remarkably reduced collagen and collagen scores were seen in the skin of the Tg(+) On-Off mice that received anti-TSLP (Fig. 7B, 7C). In addition, numbers of CD34 and ProCol 1 double-positive cells evaluated by IHC were significantly lower in the skin of the Tg(+) On-Off mice that received anti-TSLP than those in the skin of control mice (Fig. 7D). Inflammatory responses in the skin samples from both groups of mice were milder and comparable after the IL-13 gene was inactivated (Fig. 7E, 7F). When viewed together, these studies further support the notion that the inhibitory effect of blocking TSLP on the pathogenic fibrogenesis in the AD skin of Tg(+) mice that had diminished level of IL-13 in the skin was clearly unassociated with IL-13.

Increased collagen deposition and fibrocytes in human AD skin

Skin fibrotic changes, including increased collagen deposition and the levels of mRNA expression of IL-11 and IL-17, are noted in the chronic lesions of human AD (24). The resident fibroblast has been traditionally viewed as the primary cell type involved in promoting tissue fibrosis. Accumulating evidence supports that fibrocytes, capable of differentiating into fibroblasts and myofibroblasts, as well as adipocytes, contribute to pulmonary fibrosis (29). However, the role of fibrocytes in the skin of AD patients is unknown. Using Gomori's Trichrome staining, we compared the collagen deposition in the skin samples from normal subjects and biopsy samples of chronic lesional skin from AD patients. Almost the entire dermal and hypodermal regions of the skin from AD patients were strongly stained by Gomori's Trichrome (Fig. 8A). In contrast, only a small amount of Gomori's Trichrome positive collagen was visualized in the dermal layer of the skin from normal subjects. We then used dual-color immunofluorescence

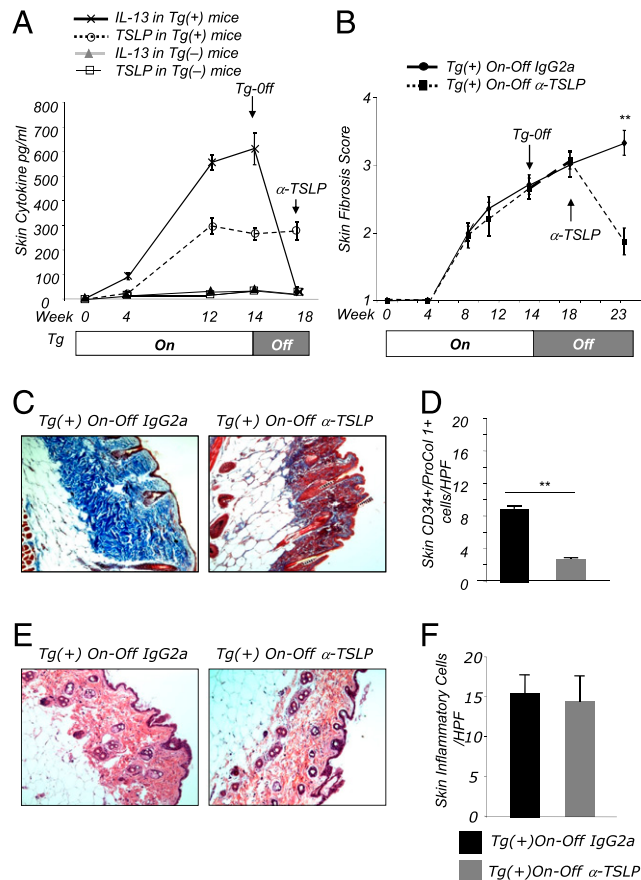


FIGURE 7. TSLP blockade induced attenuation of skin fibrogenesis in AD was independent of IL-13. Tg(+) mice, when the IL-13 level in the skin reached the level comparable to that in Tg(−) skin, after inactivation of the transgene IL-13, were randomly assigned to receive neutralizing TSLP mAb [Tg(+) On-Off-αTSLP group] and control Ab IgG2a [Tg(+) On-Off-IgG group]. The mice received the treatments i.p. every 5 d for seven injections. A, Kinetics of the levels of IL-13 and TSLP in the skin samples of Tg(+) and Tg(−) mice. Collagen deposition in the dermal and subdermal areas by Masson's Trichrome staining (blue color) was evaluated, and the kinetics of quantification of the collagen deposition are shown (B, C). Quantification (HPF) of CD34⁺/ProCol 1⁺ fibrocytes by IHC in the skin (D). The skin inflammatory response by H&E staining and numbers of inflammatory cells (E, F). Original magnification (C, E) ×10. ***p* < 0.01.

IHC analysis with anti-CD34 and anti-ProCol 1 to examine the presence of fibrocytes in the skin from normal subjects and AD patients. The number of CD34/ProCol 1 double-positive fibrocytes was highly increased in the dermal and hypodermal regions of the lesional skin from AD patients, whereas double-positive cells were rarely seen in the skin samples from normal subjects (Fig. 8B). These studies demonstrated that along with fibrosis, increased accumulation of fibrocytes is a feature in the human AD skin, and these cells may contribute to the pathogenesis of skin fibrosis in human AD.

Discussion

Tissue remodeling has been well documented in asthmatic airways, and airway remodeling is a consequence of chronic inflammatory episodes, which are associated predominantly with Th2 cytokines IL-4, IL-13, and IL-5 (30). In human AD, chronic lichenified skin lesions have undergone tissue remodeling due to chronic inflammation and are characterized by thickened plaques with increased markings (lichenification) and dry, fibrotic papules (24, 31). Dermal fibrosis corresponding to lichenified lesions is a char-

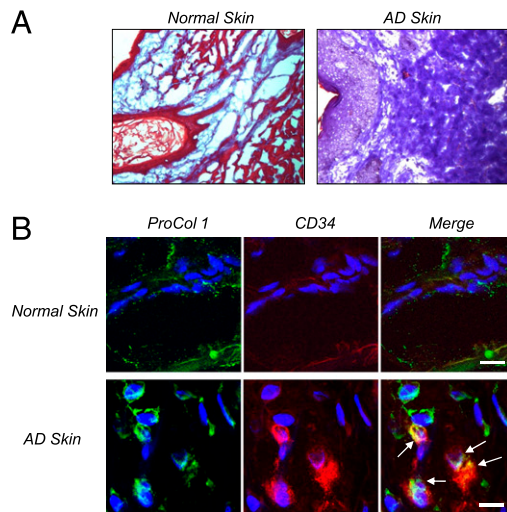


FIGURE 8. Increased collagen and fibrocytes in human AD skin. In skin biopsy specimens from normal subjects and patients with AD, Gomori's Trichrome staining (original magnification $\times 10$) was used to evaluate collagen deposition (A) and IHC (B) with fluorescent anti-CD34/anti-ProCol 1 was used to assess expression of double-positive fibrocytes (white arrows) in the skin. Shown are representatives of three individual samples from each group. Scale bars, 10 μ m.

acteristic feature of chronic AD (31). Many factors—cytokines (IL-13, TGF- β 1), chemokines (MCP-1, MIP-1), angiogenic factors (vascular endothelial growth factor), growth factors (PDGF), peroxisome proliferator-activated receptors, acute phase proteins (SAP), and caspases—have been identified as important regulators of fibrosis (32). However, the pathogenesis of fibrotic remodeling in chronic AD is far from being comprehensively understood. We previously reported that IL-13 is a potent stimulator of the skin fibrotic changes in AD, which is characterized by greatly increased collagen deposition and elevated levels of TSLP, MCP-1, MIP-1 α , MIP-1 β , and TGF- β 1 in the AD skin (1).

We initiated this study to understand the mechanisms that are involved in IL-13-induced skin fibrosis in AD. We observed that inactivation of the IL-13 transgene in vivo in K5-tTA-IL-13 animals after they developed AD resulted in a significant improvement in the clinical manifestation of AD. However, the reduced inflammatory responses in the AD skin did not correlate with amelioration of the skin fibrotic changes. Rather, the magnitude of skin fibrosis was heightened, indicating that the chronic inflammation induced by IL-13 contributes to initiation of skin fibrotic remodeling, but persistency and progression of skin fibrosis in AD does not require IL-13 or IL-13-induced inflammation. Furthermore, the number of CD34⁺/ProCol 1⁺ fibrocytes in the dermal and hypodermal areas in the AD skin of K5-tTA-IL-13 mice was highly increased. This suggests that the chronic inflammation induced by IL-13 contributes to the pathogenic skin fibrotic process by recruiting fibrocytes and potentiating profibrotic tissue effect of TSLP on fibrocytes, which in turn leads to deposition of more connective tissue elements that progressively remodel and destroy the normal skin architecture, leading to a progressive and irreversible process.

Fibrocytes constitutively release profibrotic and angiogenic factors and can modulate ongoing inflammatory reactions by releasing a number of chemokines, cytokines, and growth factors (15, 33). In tissues, fibrocytes can differentiate into other mesenchymal cells, such as myofibroblasts and adipocytes (11, 12). Fibrocytes synthesize new collagen or acquire myofibroblast markers, which have been detected in extensive remodeling of the bronchial wall

or pulmonary fibrosis, in the skin of patients with nephrogenic systemic fibrosis, in human hypertrophic scars, and in atherosclerotic lesions (34–36). In this study, we investigated whether fibrocytes were present and increased in the AD skin. We found that CD34/ProCol 1 double-positive fibrocytes were virtually absent in the skin of Tg(–) mice, whereas a marked increase in the number of fibrocytes was seen in the chronic AD skin of IL-13 Tg(+) mice. In addition, the expression level of CXCL12, the only known ligand for CXCR4 (37), and the level of CXCR4, a dominant receptor expressed by both human and mouse fibrocytes, were both significantly enhanced in the AD skin of Tg(+) mice. This differential expression of CXCL12 in the AD skin tissue creates a chemotactic gradient required for the trafficking of CXCR4-expressing fibrocytes, as the migration of these cells to the inflamed tissue is critically dependent on the CXCL12–CXCR4 biological axis (29). Furthermore, the elevated levels of activated TGF- β 1 and the upregulation of genes encoding ET-1 and PDGF in the AD skin may cooperate with one another to contribute to the propagation of the pathogenic fibrotic responses in IL-13-induced AD.

Several studies with TSLP-neutralizing mAbs and TSLPR-deficient mice demonstrated that the TSLP signaling is required for the development of allergen-driven inflammatory responses in the lung and skin (2, 9, 38). Indeed, several lines of evidence support a critical role of TSLP signaling in the generation of Th2 responses and in the development of helminth-induced Th2 responses (9, 31, 39, 40). Nevertheless, the role of TSLP/TSLPR signaling in skin fibrosis in AD remains unclear. Our initial studies showed that the level of TSLP was significantly elevated in the early stage of AD lesions of Tg(+) animals (1). We showed in this study that specific mAb-mediated neutralization of TSLP starting before the mice developed AD led to a significant reduction in skin fibrosis and skin inflammation. Furthermore, blockage of TSLP in vivo significantly reduced the number of fibrocytes in the lesional AD skin, which may be mediated by: 1) inhibiting the expression of CXCL12/CXCR4 to block the trafficking of CXCR4⁺ fibrocytes in response to CXCL12 in AD skin, which is an especially important chemokine signaling during skin fibrosis in AD; and 2) reducing the expression of TGF- β 1, PDGF, and ET-1 to inhibit the maturation of fibrocytes from their precursors and differentiation of fibrocytes to fibroblasts and myofibroblasts in the local tissues.

In the current study, we further demonstrated that in the absence of TSLPR, the magnitude of skin pathological fibrosis in chronic AD skin was markedly reduced in IL-13 Tg(+) mice. These findings are in accord with the results of TSLP neutralization and indicate that the TSLP signaling is important in regulating the skin fibrotic remodeling in AD. Our results showed that TSLPR is required in generating and maintaining skin fibrosis in IL-13-induced AD. The significantly reduced magnitude of skin pathogenic fibrosis is correlated with reduced accumulation of fibrocytes and profibrogenic mediators critical for fibrocyte migration and differentiation. Blocking the accumulation of fibrocytes in the skin pathological fibrogenesis in AD is important, which can lead to reduced production of cytokines that induce collagen deposition (15, 23, 26, 41), reduced production of proangiogenic mediators that promote angiogenesis (42), and decreased ability of fibrocytes as potent APCs to recruit and activate T cells (43).

TSLPR was shown to be expressed by many cell types in lymphoid and nonlymphoid tissues, including keratinocytes, airway epithelial cells, dendritic cells, mast cells, B cells, CD34⁺ progenitor cells, and CD4⁺ and CD8⁺ T cells (3, 4, 25, 44–51). For the first time to our knowledge, we showed that fibrocytes express TSLPR, the fibrogenic-promoting role of TSLP is mediated by

directly stimulating fibrocytes to differentiate and produce collagen, and IL-13 potentiated the effect of TSLP on collagen production in vitro.

Together, these data suggest that TSLP may be able to stimulate the production of cytokines/chemokines critical in the recruitment of fibrocytes into the AD skin, and TSLP may promote fibrocyte differentiation and production of collagen. However, whether TSLP also has an effect on fibrocyte apoptosis remains to be investigated.

Our studies of the effects of IL-13 on skin inflammation and skin fibrosis showed that the two processes did not change in parallel when the expression of the IL-13 transgene in the skin was discontinued after the mice developed AD. This indicates that once the skin fibrotic remodeling was initiated by IL-13, the progression of skin fibrosis did not require IL-13. In contrast, there was a significant attenuation in skin inflammation in mice with AD when TSLP was neutralized, and there was a reduction, though to a lesser extent, in skin inflammation in AD in Tg(+) mice carrying a null mutation of TSLPR. Nevertheless, both neutralization of TSLP and genetic deletion of TSLPR in Tg(+) mice demonstrated significant inhibition of skin fibrotic remodeling, suggesting that TSLP and its signaling through TSLPR are critically involved in skin inflammation and in the maintenance and progression of IL-13-initiated skin fibrosis in AD. As marked attenuation of skin pathogenic fibrogenesis by blocking TSLP and disrupting TSLP signaling pathway was achieved, the levels of IL-13 in the skin were not altered between Tg(+)/TSLPR^{+/+} and Tg(+)/TSLPR^{-/-} groups and anti-TSLP-treated and control Ab-treated groups, which indicates that the deficiency of TSLPR and blocking TSLP did not inhibit the expression of the IL-13 transgene and that the progression of skin pathogenic fibrosis in AD was mediated largely by the TSLP pathway. Importantly, after inactivation of the IL-13 transgene, when the level of IL-13 in the skin was reduced to the level comparable to that of WT skin and the level of TSLP in the skin remained high, blocking TSLP significantly diminished collagen accumulation and the number of fibrocytes, further demonstrating that suppression of skin fibrosis by interrupting the TSLP pathway is independent of the IL-13 effects (Fig. 7).

The interaction between TSLP with its receptor TSLPR seems to have different functions depending on the disease models studied. A recent study showed that TSLPR has no functional impact on the development of protective Th2 immune responses postinfection with helminth pathogens *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*; however, it is necessary for the development of protective Th2 responses upon infection with the helminth *Trichuris muris* (52). Interestingly, our findings in the current study are somewhat different from those in a recent report by Ramalingam et al. (40) in which TSLPR signaling was found to play a limited role in the development of helminth *Schistosoma mansoni*-induced tissue fibrosis. One possible reason for the discrepancy between that study and ours is that in our system, the skin inflammation and fibrosis is initiated by IL-13, whereas the Ramalingam study (40) was performed in a *Schistosoma* infection model.

To determine if the finding of fibrocytes in the skin fibrogenesis in our mouse model of AD was also important in the pathogenic fibrosis in human AD, we examined the number of CD34 and ProCol 1 double-positive fibrocytes in the skin biopsies from AD patients with chronic lesions and compared with those from normal subjects. Increased fibrocytes, which were associated with a robust and extensive increase in collagen deposition, were readily detected in the AD skin compared with those in the skin of normal subjects. However, whether the TSLP-fibrocyte axis is operative in human AD requires further investigation.

Collectively, our studies using a mouse model of IL-13-induced AD demonstrated that IL-13 is a potent mediator in generating pathologic fibrosis in AD, and, for the first time to our knowledge, these studies provided evidence that the TSLP and TSLPR signaling pathway is critical in the pathogenesis of the detrimental tissue remodeling and skin fibrosis in AD. Our findings in human AD identified a remarkable accumulation of fibrocytes in the skin biopsies of AD patients, suggesting that fibrocytes may play an important role in the skin fibrotic remodeling. Additional studies are needed ultimately to translate these experimental and clinical findings to the development of novel therapeutic approaches that will allow selective manipulation of these cells to attenuate pathologic fibrosis in AD.

Acknowledgments

We thank Janet Dorer for proofreading the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

- Zheng, T., M. H. Oh, S. Y. Oh, J. T. Schroeder, A. B. Glick, and Z. Zhu. 2009. Transgenic expression of interleukin-13 in the skin induces a pruritic dermatitis and skin remodeling. *J. Invest. Dermatol.* 129: 742–751.
- Al-Shami, A., R. Spolski, J. Kelly, A. Keane-Myers, and W. J. Leonard. 2005. A role for TSLP in the development of inflammation in an asthma model. *J. Exp. Med.* 202: 829–839.
- Pandey, A., K. Ozaki, H. Baumann, S. D. Levin, A. Puel, A. G. Farr, S. F. Ziegler, W. J. Leonard, and H. F. Lodish. 2000. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nat. Immunol.* 1: 59–64.
- Park, L. S., U. Martin, K. Garka, B. Gliniak, J. P. Di Santo, W. Muller, D. A. Largaespada, N. G. Copeland, N. A. Jenkins, A. G. Farr, et al. 2000. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. *J. Exp. Med.* 192: 659–670.
- Liu, Y. J. 2006. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J. Exp. Med.* 203: 269–273.
- Liu, Y. J. 2007. Thymic stromal lymphopoietin and OX40 ligand pathway in the initiation of dendritic cell-mediated allergic inflammation. *J. Allergy Clin. Immunol.* 120: 238–244; quiz 245–256.
- Yoo, J., M. Omori, D. Gyarmati, B. Zhou, T. Aye, A. Brewer, M. R. Comeau, D. J. Campbell, and S. F. Ziegler. 2005. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J. Exp. Med.* 202: 541–549.
- Li, M., P. Hener, Z. Zhang, S. Kato, D. Metzger, and P. Chambon. 2006. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. *Proc. Natl. Acad. Sci. USA* 103: 11736–11741.
- Zhou, B., M. R. Comeau, T. De Smedt, H. D. Liggitt, M. E. Dahl, D. B. Lewis, D. Gyarmati, T. Aye, D. J. Campbell, and S. F. Ziegler. 2005. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat. Immunol.* 6: 1047–1053.
- Jessup, H. K., A. W. Brewer, M. Omori, E. A. Rickel, A. L. Budelsky, B. R. Yoon, S. F. Ziegler, and M. R. Comeau. 2008. Intradermal administration of thymic stromal lymphopoietin induces a T cell- and eosinophil-dependent systemic Th2 inflammatory response. *J. Immunol.* 181: 4311–4319.
- Hong, K. M., M. D. Burdick, R. J. Phillips, D. Heber, and R. M. Strieter. 2005. Characterization of human fibrocytes as circulating adipocyte progenitors and the formation of human adipose tissue in SCID mice. *FASEB J.* 19: 2029–2031.
- Hong, K. M., J. A. Belperio, M. P. Keane, M. D. Burdick, and R. M. Strieter. 2007. Differentiation of human circulating fibrocytes as mediated by transforming growth factor-beta and peroxisome proliferator-activated receptor gamma. *J. Biol. Chem.* 282: 22910–22920.
- Bellini, A., and S. Mattoli. 2007. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibrosis. *Lab. Invest.* 87: 858–870.
- Quan, T. E., S. E. Cowper, and R. Bucala. 2006. The role of circulating fibrocytes in fibrosis. *Curr. Rheumatol. Rep.* 8: 145–150.
- Quan, T. E., S. Cowper, S. P. Wu, L. K. Bockenstedt, and R. Bucala. 2004. Circulating fibrocytes: collagen-secreting cells of the peripheral blood. *Int. J. Biochem. Cell Biol.* 36: 598–606.
- Wang, C. H., C. D. Huang, H. C. Lin, K. Y. Lee, S. M. Lin, C. Y. Liu, K. H. Huang, Y. S. Ko, K. F. Chung, and H. P. Kuo. 2008. Increased circulating fibrocytes in asthma with chronic airflow obstruction. *Am. J. Respir. Crit. Care Med.* 178: 583–591.
- Saunders, R., S. Siddiqui, D. Kaur, C. Doe, A. Sutcliffe, F. Hollins, P. Bradding, A. Wardlaw, and C. E. Brightling. 2009. Fibrocyte localization to the airway smooth muscle is a feature of asthma. *J. Allergy Clin. Immunol.* 123: 376–384.

18. Shao, D. D., R. Suresh, V. Vakil, R. H. Gomer, and D. Pilling. 2008. Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation. *J. Leukoc. Biol.* 83: 1323–1333.
19. Al-Shami, A., R. Spolski, J. Kelly, T. Fry, P. L. Schwartzberg, A. Pandey, C. L. Mackall, and W. J. Leonard. 2004. A role for thymic stromal lymphopoietin in CD4(+) T cell development. *J. Exp. Med.* 200: 159–168.
20. Leung, D. Y., R. L. Hirsch, L. Schneider, C. Moody, R. Takaoka, S. H. Li, L. A. Meyerson, S. G. Mariam, G. Goldstein, and J. M. Hanifin. 1990. Thymopentin therapy reduces the clinical severity of atopic dermatitis. *J. Allergy Clin. Immunol.* 85: 927–933.
21. Akei, H. S., E. B. Brandt, A. Mishra, R. T. Strait, F. D. Finkelman, M. R. Warrier, G. K. Hershey, C. Blanchard, and M. E. Rothenberg. 2006. Epicutaneous aeroallergen exposure induces systemic TH2 immunity that predisposes to allergic nasal responses. *J. Allergy Clin. Immunol.* 118: 62–69.
22. Mori, L., A. Bellini, M. A. Stacey, M. Schmidt, and S. Mattoli. 2005. Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. *Exp. Cell Res.* 304: 81–90.
23. Abe, R., S. C. Donnelly, T. Peng, R. Bucala, and C. N. Metz. 2001. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J. Immunol.* 166: 7556–7562.
24. Toda, M., D. Y. Leung, S. Molet, M. Boguniewicz, R. Taha, P. Christodouloupoloulos, T. Fukuda, J. A. Elias, and Q. A. Hamid. 2003. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J. Allergy Clin. Immunol.* 111: 875–881.
25. Soumelis, V., P. A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, et al. 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* 3: 673–680.
26. Schmidt, M., G. Sun, M. A. Stacey, L. Mori, and S. Mattoli. 2003. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J. Immunol.* 171: 380–389.
27. Mehrad, B., M. D. Burdick, and R. M. Strieter. 2009. Fibrocyte CXCR4 regulation as a therapeutic target in pulmonary fibrosis. *Int. J. Biochem. Cell Biol.* 41: 1708–1718.
28. Lu, N., Y. H. Wang, Y. H. Wang, K. Arima, S. Hanabuchi, and Y. J. Liu. 2009. TSLP and IL-7 use two different mechanisms to regulate human CD4+ T cell homeostasis. *J. Exp. Med.* 206: 2111–2119.
29. Strieter, R. M., E. C. Keeley, M. D. Burdick, and B. Mehrad. 2009. The role of circulating mesenchymal progenitor cells, fibrocytes, in promoting pulmonary fibrosis. *Trans. Am. Clin. Climatol. Assoc.* 120: 49–59.
30. Elias, J. A., Z. Zhu, G. Chupp, and R. J. Homer. 1999. Airway remodeling in asthma. *J. Clin. Invest.* 104: 1001–1006.
31. Leung, D. Y., M. Boguniewicz, M. D. Howell, I. Nomura, and Q. A. Hamid. 2004. New insights into atopic dermatitis. *J. Clin. Invest.* 113: 651–657.
32. Wynn, T. A. 2008. Cellular and molecular mechanisms of fibrosis. *J. Pathol.* 214: 199–210.
33. Chesney, J., C. Metz, A. B. Stavitsky, M. Bacher, and R. Bucala. 1998. Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. *J. Immunol.* 160: 419–425.
34. Ishida, Y., A. Kimura, T. Kondo, T. Hayashi, M. Ueno, N. Takakura, K. Matsushima, and N. Mukaida. 2007. Essential roles of the CC chemokine ligand 3-CC chemokine receptor 5 axis in bleomycin-induced pulmonary fibrosis through regulation of macrophage and fibrocyte infiltration. *Am. J. Pathol.* 170: 843–854.
35. Sakai, N., T. Wada, H. Yokoyama, M. Lipp, S. Ueha, K. Matsushima, and S. Kaneko. 2006. Secondary lymphoid tissue chemokine (SLC/CCL21)/CCR7 signaling regulates fibrocytes in renal fibrosis. *Proc. Natl. Acad. Sci. USA* 103: 14098–14103.
36. Varcoe, R. L., M. Mikhail, A. K. Guiffre, G. Pennings, M. Vicaretti, W. J. Hawthorne, J. P. Fletcher, and H. J. Medbury. 2006. The role of the fibrocyte in intimal hyperplasia. *J. Thromb. Haemost.* 4: 1125–1133.
37. Ma, Q., D. Jones, P. R. Borghesani, R. A. Segal, T. Nagasawa, T. Kishimoto, R. T. Bronson, and T. A. Springer. 1998. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. USA* 95: 9448–9453.
38. He, R., M. K. Oyoshi, L. Garibyan, L. Kumar, S. F. Ziegler, and R. S. Geha. 2008. TSLP acts on infiltrating effector T cells to drive allergic skin inflammation. *Proc. Natl. Acad. Sci. USA* 105: 11875–11880.
39. Zhou, B., M. B. Headley, T. Aye, J. Tocker, M. R. Comeau, and S. F. Ziegler. 2008. Reversal of thymic stromal lymphopoietin-induced airway inflammation through inhibition of Th2 responses. *J. Immunol.* 181: 6557–6562.
40. Ramalingam, T. R., J. T. Pesce, M. M. Mentink-Kane, S. Madala, A. W. Cheever, M. R. Comeau, S. F. Ziegler, and T. A. Wynn. 2009. Regulation of helminth-induced Th2 responses by thymic stromal lymphopoietin. *J. Immunol.* 182: 6452–6459.
41. Metz, C. N. 2003. Fibrocytes: a unique cell population implicated in wound healing. *Cell. Mol. Life Sci.* 60: 1342–1350.
42. Hartlapp, I., R. Abe, R. W. Saeed, T. Peng, W. Voelter, R. Bucala, and C. N. Metz. 2001. Fibrocytes induce an angiogenic phenotype in cultured endothelial cells and promote angiogenesis in vivo. *FASEB J.* 15: 2215–2224.
43. Chesney, J., M. Bacher, A. Bender, and R. Bucala. 1997. The peripheral blood fibrocyte is a potent antigen-presenting cell capable of priming naive T cells in situ. *Proc. Natl. Acad. Sci. USA* 94: 6307–6312.
44. Friend, S. L., S. Hosier, A. Nelson, D. Foxworthe, D. E. Williams, and A. Farr. 1994. A thymic stromal cell line supports in vitro development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells. *Exp. Hematol.* 22: 321–328.
45. Sims, J. E., D. E. Williams, P. J. Morrissey, K. Garka, D. Foxworthe, V. Price, S. L. Friend, A. Farr, M. A. Bedell, N. A. Jenkins, et al. 2000. Molecular cloning and biological characterization of a novel murine lymphoid growth factor. *J. Exp. Med.* 192: 671–680.
46. Reche, P. A., V. Soumelis, D. M. Gorman, T. Clifford, M. Liu Mr, S. M. Travis, J. Zurawski, Y. J. Johnston, H. Liu, Spits, et al. 2001. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J. Immunol.* 167: 336–343.
47. Quentmeier, H., H. G. Drexler, D. Fleckenstein, M. Zaborski, A. Armstrong, J. E. Sims, and S. D. Lyman. 2001. Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation. *Leukemia* 15: 1286–1292.
48. Omori, M., and S. Ziegler. 2007. Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *J. Immunol.* 178: 1396–1404.
49. Rochman, I., N. Watanabe, K. Arima, Y. J. Liu, and W. J. Leonard. 2007. Cutting edge: direct action of thymic stromal lymphopoietin on activated human CD4+ T cells. *J. Immunol.* 178: 6720–6724.
50. Allakhverdi, Z., M. R. Comeau, D. E. Smith, D. Toy, L. M. Endam, M. Desrosiers, Y. J. Liu, K. J. Howie, J. A. Denburg, G. M. Gauvreau, and G. Delespesse. 2009. CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation. *J. Allergy Clin. Immunol.* 123: 472–478.
51. Rochman, Y., and W. J. Leonard. 2008. The role of thymic stromal lymphopoietin in CD8+ T cell homeostasis. *J. Immunol.* 181: 7699–7705.
52. Massacand, J. C., R. C. Stettler, R. Meier, N. E. Humphreys, R. K. Grencis, B. J. Marsland, and N. L. Harris. 2009. Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. *Proc. Natl. Acad. Sci. USA* 106: 13968–13973.