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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.

A

topic 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-7Rα-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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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; HIF, 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 I 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 TSLP.

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 TSLP 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 TSLP 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, resulting 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 I 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 TSLP 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-Ta-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-Ta-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 TSLP gene null mice were generated as previously described (19). These mice on C57Bl/6 genetic background were compared in the experiments. All procedures in-
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 μM 2-ME, 20 mM HEPES, 2× 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 IL-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 μg/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 ×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 (HPP; 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 ± 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 μl) was added to each skin homogenate (50 μl) and mixed for 30 min. The collagen dye complex was precipitated at centrifugation of 16,000 × 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 myofibers 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 ± 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-β1 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-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,
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-β1 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-β1. 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 significantly 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-β1 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.
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-β1 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-β1 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
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, 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 ×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-β1 in the chronic AD skin were assessed by ELISA. Histological evaluation of skin samples from Tg(+) mice given IgG2a and Tg(+) mice given anti-TSLP (E; H&E, original magnification ×10) and quantification of skin inflammatory cells per HPF (F). For B–D, results are shown as mean ± SEM. *p < 0.05, **p < 0.001.

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 ×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-β1 assessed by ELISA. Histological evaluation of skin samples from Tg(+) mice given IgG2a and Tg(+) mice given anti-TSLP (E; H&E, original magnification ×10) and quantification of skin inflammatory cells per HPF (F). For B–D, results are shown as mean ± SEM. *p < 0.05, **p < 0.001.

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 μm. 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 ± 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.

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...
ELISA. The values in at least eight animals for each group.

TSLPR loci (the TSLP neutralization study (Fig. 4C)) groups of mice were not altered, a finding in accord with that in 
dependent. The levels of IL-13 in the AD skin samples from both 
suggests that part of the IL-13–mediated effects are TSLPR in-
effects in the skin of Tg(+) mice with null mutation of TSLPR
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 in-
flammatory 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 ×10. Total collagen content of the skin by Sircol assay (C) and the levels of total and active forms of TGF-β1 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 ± 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 in-
dependent. 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-7Rα 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 hypo-
dermal 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 mor-
phology 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 undiffer-
entiated 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 demon-
strated that fibrocytes express TSLPR, and TSLP is a potent stim-

FIGURE 6. TSLP 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 with 
immunofluorescence using specific Abs and examined by confocal microscopy. White arrows indicate triple-pos-
itive cells. Slides are representatives of five different samples from each 
group. Scale bars, 100 μm. B, CD45 and Col 1 double-positive fibrocytes 
were stained for TSLPR and FACS with anti-
CD45, anti-ProCol 1, and anti-TSLPR. 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 mor-
phology 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 undiffer-
entiated 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 demon-
strated that fibrocytes express TSLPR, and TSLP is a potent stim-
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 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 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 un-associated 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 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 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.
acteristic of chronic AD (31). Many factors—cytokines (IL-13, TGF-β1), 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-1α, MIP-1β, and TGF-β1 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 inflammatory responses in the AD skin did not correlate with inactivation of the IL-13 transgene in vivo in K5-tTA–IL-13 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, blockade 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 important chemokine signaling during skin fibrosis in AD; and 2) reducing the expression of TGF-β1, 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, CD4+ 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 TSLP 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.

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Disclosures
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