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TSLP elicits IL-33–independent innate lymphoid cell responses to promote skin inflammation

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Abstract

Innate lymphoid cells (ILCs) are a recently identified family of heterogeneous immune cells that can be divided into three groups based on their differential developmental requirements and expression of effector cytokines. Among these, group 2 ILCs produce the type 2 cytokines IL-5 and IL-13 and promote type 2 inflammation in the lung and intestine. However, whether group 2 ILCs reside in the skin and contribute to skin inflammation has not been characterized. Here, we identify for the first time a population of skin-resident group 2 ILCs present in healthy human skin that are enriched in lesional human skin from atopic dermatitis (AD) patients. Group 2 ILCs were also found in normal murine skin and were critical for the development of inflammation in a murine model of AD-like disease. Remarkably, in contrast to group 2 ILC responses in the intestine and lung, which are critically regulated by IL-33 and IL-25, ILC responses in the skin and skin-draining lymph nodes were independent of these canonical cytokines but were critically dependent on thymic stromal lymphopoietin (TSLP). Collectively, these results demonstrate an essential role for IL-33– and IL-25–independent group 2 ILCs in promoting skin inflammation.

Introduction

Innate lymphoid cells (ILCs) are a family of immune cells that are heterogeneous in their tissue location, cytokine production and effector functions (1). ILCs are characterized by the

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Data and materials availability: *Il17rb*^{−/−}, *Tslpr*^{−/−}, and *Il33*^{−/−} mice, monoclonal anti-TSLP antibody, and TSLP cDNA plasmid were obtained through an MTA with Amgen. BAC-transgenic *Rorc*(*yt*)-Gfp^{TG} mice were obtained through an MTA with G. Eberl. *Id2*^{−/−} fetal liver chimeras were obtained through an MTA with A.W. Goldrath and C.Y. Yang.

lack of expression of cell lineage markers associated with T cells, B cells, dendritic cells (DCs), macrophages and granulocytes but express CD90 (Thy1 antigen), CD25 (IL-2R α) and CD127 (IL-7R α) (2). These cells derive from a common lymphoid progenitor, and their development is dependent on the common γ -chain (γ c or CD132) and the transcription factor Inhibitor of DNA binding 2 (Id2) (1). Although ILCs lack antigen-specific receptors, they demonstrate striking functional similarities to T helper (T_H) cell populations. Based on their developmental requirements for defined transcription factors and their expression of cell surface markers and effector cytokines, ILCs are currently categorized into three distinct populations: group 1 ILCs, which include classical NK cells and T-bet-dependent IFN- γ -producing ILCs (referred to as ILC1s); ROR α - and GATA3-dependent group 2 ILCs, which include IL-5- and IL-13-producing ILCs such as natural helper cells (NHCs), nuocytes and innate helper type 2 (Ih2) cells (termed ILC2s); and ROR γ t-dependent group 3 ILCs, which include IL-17A- and/or IL-22-producing ILCs (termed ILC3s) (3).

The functions of group 1 ILCs, such as classical NK cells, in antiviral immunity and tumor surveillance have been well documented {Bellucci, 2012 #375; Finton, 2012 #376}. Recent studies have identified that group 2 and group 3 ILCs exhibit specialized functions at different epithelial barrier surfaces (4, 5). For example, while group 3 ILCs are activated by IL-23, maintain intestinal epithelial barrier function, and regulate host responses to commensal and pathogenic bacteria in the intestine (6), group 2 ILCs are reported to be critically dependent on the epithelial cell-derived cytokines IL-33 and IL-25 and have been shown to promote anti-helminth immunity and regulate inflammation and/or epithelial repair in the lung (4, 7–10). Collectively, these findings indicate that the anatomical localization and functional specialization of group 2 and group 3 ILCs allow them to orchestrate immunity, inflammation and tissue homeostasis at barrier surfaces.

The composition and functional potential of ILCs in the skin remain poorly understood. Barrier disruption in atopic dermatitis (AD) can result from spontaneous inflammation and inappropriate immune responses to putative environmental allergens or microbial signals. AD is a chronic, relapsing inflammatory disease of the skin barrier that affects approximately 15–20% of children and 2–9% of adults, costing up to \$3.8 billion annually in the US alone {Mancini, 2008 #262}. AD in humans has been associated with enhanced expression of the predominantly epithelial cell-derived cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) in the skin, which elicit the T_H2 cell-associated cytokine responses that are known to promote disease {Brandt, 2011 #247}.

Although both IL-33 and IL-25 have been shown to elicit group 2 ILCs that express IL-5, IL-9, IL-13, and amphiregulin (Areg) in murine gut-associated lymphoid tissue (GALT), fat-associated lymphoid clusters (FALC), and lung parenchyma (11), the role of these cytokines and group 2 ILC responses in promoting inflammation in the skin remains to be defined. Analogous populations of group 2 ILCs have recently been identified in healthy human blood, intestine, and lung, as well as inflamed nasal polyps from patients suffering from allergic rhinosinusitis (12, 13). Previous studies have demonstrated that group 2 ILCs in mice are critical mediators of allergic inflammation in the lung (10, 14). Whether group 2 ILCs are also present at the skin barrier surface, what role these cells play in skin inflammation, and what factors regulate their homeostasis and function have not been examined.

In this study, we demonstrate that group 2 ILC-like cells are present in healthy skin of both humans and mice. Depletion of group 2 ILCs in mice was associated with a significant reduction in inflammation and AD-like disease. Strikingly, cutaneous group 2 ILC responses and AD-like inflammation were independent of IL-33 and IL-25 but critically dependent on

TSLP. TSLP-dependent cutaneous ILC responses may offer a new therapeutic target for the treatment of AD.

Results

Group 2 ILCs are enriched in human AD skin lesions

To interrogate whether group 2 ILCs are present in human skin, we performed flow cytometric analysis of cells isolated from the skin tissue of healthy control subjects. We identified a population of lineage-negative (Lin^-) cells that lacked expression of cell surface markers associated with T cells (CD3, $\text{TCR}\alpha\beta$), B cells (CD19), DCs (CD11c), macrophages/monocytes (CD16), NK cells (CD56), or mast cells and basophils ($\text{Fc}\epsilon\text{RI}\alpha$), but expressed CD25 (IL-2R α) and IL-33R (ST2) (Fig. 1A)—a surface phenotype consistent with group 2 ILCs described at other tissue sites (4, 10). To test whether group 2 ILC responses are associated with skin inflammation, we examined skin tissue from lesions of human AD patients. Flow cytometric analysis of the lesions revealed a significant increase in the frequency of group 2 ILCs in lesional AD skin compared to healthy control skin (Fig. 1, B and C).

Group 2 ILCs found in human peripheral blood can express CD25, CD127, CRTH2 and/or CD161 (12, 13). Consistent with these reports, we also identified Lin^- CD25 $^+$ CD127 $^+$ cells in the peripheral blood from healthy control subjects that expressed CRTH2 and CD161 (fig. S1A). However, Lin^- CD25 $^+$ IL-33R $^+$ ILC2s isolated from healthy human skin did not express CRTH2 or CD161 (fig. S1B). Further, ILCs isolated from healthy skin or AD lesions did not express group 3 ILC-associated markers such as CD4, NKp44 or ROR γ t (fig. S1, B to C). Strikingly, Lin^- CD25 $^+$ IL-33R $^+$ ILC2s isolated from lesional AD skin expressed CRTH2 and CD161 (fig. S1C), indicating that the ILC2s present in AD lesions are either a distinct population of ILCs or are in a different state of activation from those ILC2s found in the healthy skin.

Group 2 ILCs are enriched in the skin of a mouse model of AD

Similar to the results observed in human skin, flow cytometric analysis of cells isolated from the skin of naïve C57BL/6 wild-type (WT) mice revealed a population of Lin^- CD25 $^+$ IL-33R $^+$ ILCs (Fig. 2A). Phenotypic analysis revealed that these skin-resident ILCs expressed CD127, CD90.2 (Thy1.2), c-Kit, Sca-1, CD44, and ICOS (Fig. 2B), similar to group 2 ILCs previously identified in the intestine, lymphoid tissue, and lung of mice (11). Consistent with other group 2 ILC populations (4), analysis of Lin^- CD25 $^+$ CD90 $^+$ ILCs in murine skin and skin-draining lymph nodes (dLNs) of Id2-deficient bone marrow chimeras revealed the developmental dependence of ILCs in the skin on Id2 expression (fig. S2, A to B). Our analysis of healthy murine skin-resident ILCs reveals a significant population of Lin^- CD25 $^+$ IL-33R $^+$ that lack expression of the group 3 ILC-associated markers CD4,

Supplementary Materials

Methods

fig. S1. Phenotypic analysis of blood and skin-resident group 2 ILCs in humans.

fig. S2. Development of skin-associated ILCs requires Id2.

fig. S3. Topical MC903 treatment results in progressive eczematous (AD-like) dermatitis.

fig. S4. Group 3 ROR γ t $^+$ ILC responses do not influence the progression of MC903-induced AD-like disease.

fig. S5. Anti-CD90.2 mAb treatment depletes CD25 $^+$ ILCs and attenuates AD-like dermatitis.

fig. S6. IL-25-IL25R signaling is dispensable for the elicitation of skin-associated group 2 ILC responses.

fig. S7. Transient blockade of TSLP signaling abrogates skin-associated group 2 ILC responses and attenuates AD-like dermatitis in C57BL/6 WT mice.

fig. S8. Transient blockade of TSLP signaling abrogates skin-associated group 2 ILC responses and attenuates AD-like dermatitis in the absence of T and B cells in *Rag1* $^{-/-}$ mice.

fig. S9. Skin-associated group 2 ILCs respond directly to IL-33 and TSLP.

fig. S10. Skin-associated group 2 ILCs promote TH2 cell-associated cytokine responses and are sufficient to cause AD-like disease.

NKp46, and ROR γ t (Fig. 2C). Therefore, as in normal human skin, these data demonstrate the existence of a population of ROR γ t-negative, IL-33R-expressing group 2 ILCs in healthy murine skin.

Group 2 ILCs have recently been recognized as critical mediators of allergic airway inflammation in multiple murine models (10, 15). However, the potential role of group 2 ILCs in regulating inflammation in the skin is unknown. An experimental murine model of AD-like inflammation was previously reported in which topical treatment with the vitamin D analog calcipotriol (MC903) resulted in skin inflammation associated with T_H2 cell-associated cytokine production (16). In line with these findings, topical treatment of C57BL/6 WT mice with MC903 resulted in the development of a chronic eczematous dermatitis associated with dry scaly skin or xerosis on day 7 that became progressively worse by day 14 (fig. S3A) and elevated serum IgE (fig. S3B). Histopathologic changes resembling human AD were also observed, including thickening of the stratum corneum (orthokeratosis), epidermal hyperplasia (acanthosis), dilated blood vessels and a mixed dermal inflammatory infiltrate consisting of mononuclear leukocytes and granulocytes (fig. S3C).

Employing this murine model system, we tested whether group 2 ILC responses were associated with AD-like inflammation in the skin and found that C57BL/6 WT mice exhibited an increased frequency of CD25⁺ IL-33R⁺ group 2 ILCs in the skin (Fig. 3A) similar to that observed in human AD skin (Fig. 1). In addition, there was a significant increase in the frequency and absolute number of CD25⁺ IL-33R⁺ group 2 ILCs in the skin dLNs (Fig. 3B).

To test whether group 2 ILCs are activated in the context of AD-like inflammation, CD25⁺ CD90⁺ ILCs isolated from the skin dLNs were examined for expression of IL-5 and IL-13 by intracellular cytokine staining. In association with the development of AD-like inflammation following MC903 treatment, C57BL/6 WT mice exhibited an increase in the frequency of CD25⁺ CD90⁺ ILCs producing IL-5 and IL-13 in the skin dLNs (Fig. 3C). Further, the accumulation and activation of group 2 ILCs was associated with increased ear thickening (Fig. 3D).

Enrichment of group 2 ILCs in AD skin is independent of adaptive immunity

Although the contribution of the adaptive immune response in experimental AD is well-characterized (17–19), the role of innate immune cells in AD-like inflammation remains poorly understood. To test whether AD-like inflammation can occur independently of adaptive immunity, lymphocyte-deficient *Rag1*^{−/−} mice were treated topically with MC903. AD-like inflammation induced in *Rag1*^{−/−} mice was associated with the accumulation of CD25⁺ IL-33R⁺ group 2 ILCs in the ear skin (Fig. 4A). In addition, there was a significant increase in the frequency and absolute number of CD25⁺ IL-33R⁺ group 2 ILCs in the skin dLNs (Fig. 4B). As observed in C57BL/6 WT mice, ILCs in the skin dLNs of MC903-treated *Rag1*^{−/−} mice produced both IL-5 and IL-13, which were significantly enriched in the setting of AD-like inflammation (Fig. 4C). Associated with the group 2 ILC responses and their expression of type 2 cytokines, *Rag1*^{−/−} mice also exhibited increased ear thickening (Fig. 4D).

There was a population of Lin[−] CD25⁺ IL-33R[−] cells in the skin dLNs of *Rag1*^{−/−} mice that expressed ROR γ t, consistent with a group 3 ILC phenotype (fig. S4A). Therefore, to test whether ROR γ t-dependent ILCs contribute to AD-like inflammation, *Rorc*^{−/−} mice were treated with topical MC903 for 7 days. Both C57BL/6 WT and *Rorc*^{−/−} mice exhibited comparable levels of ear thickening (fig. S4B) and similar histopathologic changes in the skin (fig. S4C), indicating that ROR γ t-dependent group 3 ILCs do not[A1] contribute to the

pathogenesis of MC903-induced AD-like disease. Collectively, these data show that AD-like inflammation can be initiated independently of adaptive immunity and $\text{ROR}\gamma^+$ cells, and that the accumulation and activation of group 2 ILCs is associated with an innate form of AD-like inflammation. Further, these data suggest a potential role for skin-associated group 2 ILCs in regulating innate immunity and inflammation in the skin.

Depletion of ILCs attenuates AD-like dermatitis in mice

To test the functional role of group 2 ILCs in AD-like inflammation, we used an anti-CD25 monoclonal antibody (mAb) to deplete ILCs in mice lacking T and B cells (20). *Rag1*^{-/-} mice were given either isotype or anti-CD25 mAb, and ILC responses and AD-like inflammation were assessed at day 7. Administration of anti-CD25 mAb effectively depleted CD25⁺ IL-33R⁺ ILCs in the skin dLNs, demonstrating the effectiveness of this strategy (Fig. 5A). Depletion of CD25⁺ ILCs resulted in reduced IL-5 and IL-13 production in the ear skin (Fig. 5B). Further, this reduction in type 2 cytokine responses was associated with significantly diminished ear thickening (Fig. 5C). Finally, ILC depletion resulted in reduced orthokeratosis and acanthosis, as well as a reduction in dermal inflammation consisting of mononuclear leukocytes and granulocytes (Fig. 5D).

To confirm these findings employing an alternative depletion strategy, *Rag1*^{-/-} mice were treated with anti-CD90.2 mAb, and AD-like inflammation was assessed. This method also resulted in effective depletion of CD25⁺ ILCs (fig. S5A), attenuation of ear thickening (fig. S5B), reduced histopathological changes associated with AD-like inflammation (fig. S5C) and attenuated dermal infiltration of mononuclear leukocytes and granulocytes (fig. S5C). These observations employing two different depletion strategies indicate a critical role for CD25⁺ IL-33R⁺ ILCs in promoting AD-like inflammation.

IL-33 is dispensable for group 2 ILC responses in the skin

IL-33 has recently been reported to be a dominant cytokine that promotes group 2 ILC responses and inflammation in models of allergic airway disease and helminth infection (8–10). AD is primarily a type 2 inflammatory disease in which levels of IL-33 are elevated {Pushparaj, 2009 #170}. Therefore, to test whether IL-33–IL-33R signaling is required to promote group 2 ILC responses in the skin and AD-like inflammation, *Il33*^{-/-} mice were tested in the AD-like disease model. Unexpectedly, genetic deletion of IL-33 did not result in the reduction of CD25⁺ IL-33R⁺ group 2 ILC responses in the skin dLNs in the context of AD-like inflammation (Fig. 6A). Further, similar increases in ear thickness (Fig. 6B) and AD-like histopathological changes were evident in both C57BL/6 WT and *Il33*^{-/-} mice (Fig. 6C), indicating that group 2 ILC-dependent skin inflammation was independent of IL-33–IL-33R interactions.

In addition to IL-33, IL-25 has been shown to elicit group 2 ILC responses in both mouse and human tissues and is reported to be elevated in lesional human AD skin (8, 9, 12, 21). Therefore, employing mice deficient in IL-25R (*Il17rb*^{-/-}), we tested whether group 2 ILC responses associated with AD-like inflammation were dependent on IL-25–IL-25R signaling. Similar to the results observed in *Il33*^{-/-} mice (Fig. 6), genetic disruption of IL-25–IL-25R signaling did not affect CD25⁺ IL-33R⁺ group 2 ILC responses in the skin dLNs (fig. S6A). Ear thickness (fig. S6B) and histopathological changes observed in *Il17rb*^{-/-} mice were similar to those observed in C57BL/6 WT mice following treatment with MC903 (fig. S6C).

Group 2 ILC responses in the skin are dependent on TSLP–TSLPR interactions

Previous studies have demonstrated that keratinocyte-derived TSLP expression is associated with progression of AD-like disease (16). Recent *in vitro* studies have suggested that both

murine and human group 2 ILCs are responsive to TSLP stimulation (13, 20), but the potential influence of TSLP signaling on ILC responses *in vivo* remains unknown. We observed that the development of AD-like inflammation resulted in robust induction of TSLP from inflamed skin but not control skin (Fig. 7A). Further, group 2 ILCs isolated from C57BL/6 WT mice expressed the TSLP receptor (TSLPR), as well as its co-receptor subunit CD127, indicating potential direct responsiveness of these cells to TSLP (Fig. 7B). To directly test whether TSLP–TSLPR interactions are necessary for group 2 ILC responses in the skin, *Tslpr*^{−/−} mice were examined in the model of AD-like inflammation, and group 2 ILC responses were analyzed at day 7. Genetic deletion of TSLPR significantly impaired CD25⁺ IL-33R⁺ group 2 ILC responses, as measured by both frequency and absolute cell number (Fig. 7C). Ear thickness (Fig. 7D) and histopathologic changes of orthokeratosis, acanthosis, and mononuclear leukocyte and granulocyte infiltration were also markedly ameliorated following induction of AD-like inflammation in *Tslpr*^{−/−} mice (Fig. 7E).

Transient disruption of TSLP–TSLPR signaling also inhibited group 2 ILC responses in AD-like inflammation in both lymphocyte-sufficient and lymphocyte-deficient hosts. C57BL/6 WT and *Rag1*^{−/−} mice were treated with a neutralizing anti-TSLP mAb in the context of MC903-induced AD-like inflammation. mAb-mediated neutralization of TSLP resulted in a marked reduction in the frequency and absolute number of CD25⁺ IL-33R⁺ group 2 ILCs in the skin dLNs following induction of AD-like inflammation in both C57BL/6 WT (fig. S7A) and *Rag1*^{−/−} mice (fig. S8A). Decreased ILC responses were also associated with diminished ear thickness in both C57BL/6 WT (fig. S7B) and *Rag1*^{−/−} mice (fig. S8B) and less orthokeratosis, acanthosis, and dermal mononuclear and granulocytic infiltrates in the skin in both C57BL/6 WT mice (fig. S7C) and *Rag1*^{−/−} mice (fig. S8C).

To test whether TSLP can directly activate group 2 ILCs, Lin[−] CD25⁺ IL-33R⁺ cells were sort-purified from the skin dLNs of MC903-treated C57BL/6 WT mice and cultured *in vitro* (fig. S9A). Similar to group 2 ILCs isolated from the lung (20), IL-33 stimulation induced expression of effector cytokines IL-5 and IL-13 from purified group 2 ILCs isolated from the skin dLNs. Critically, culturing group 2 ILCs with rTSLP in addition to rIL-33 resulted in enhanced production of IL-5 and IL-13, indicating that TSLP can act directly on group 2 ILCs (fig. S9B). Taken together, these results indicate that the TSLP–TSLPR pathway is a direct and crucial regulator of IL-25- and IL-33-independent group 2 ILC responses and type 2 inflammation in the skin.

TSLP-elicited group 2 ILC responses are independent of IL-33 signaling

In vitro studies have shown that activation of murine and human group 2 ILCs by IL-33 in combination with TSLP can enhance IL-5 and IL-13 production, indicating that in some circumstances TSLP may act in synergy with IL-33 (fig. S9) (13, 20). To test whether TSLP alone is sufficient to elicit IL-33-independent group 2 ILC responses, both C57BL/6 WT and *Il33*^{−/−} mice were injected intravenously with a cDNA plasmid encoding TSLP (22). Delivery of TSLP cDNA resulted in a significant increase in the frequency and absolute cell number of CD25⁺ IL-33R⁺ group 2 ILCs in the skin dLNs of both C57BL/6 WT (Fig. 8A) and *Il33*^{−/−} (Fig. 8B) mice. Although the magnitude of group 2 ILC responses in *Il33*^{−/−} mice was lower than in C57BL/6 WT mice, *Il33*^{−/−} mice still exhibited a significant increase in the frequency and absolute number of group 2 ILCs in the skin dLNs following TSLP-cDNA injection (Fig. 8B).

TSLP-elicited group 2 ILCs promote AD-like disease in lymphocyte-sufficient mice

TSLP-elicited group 2 ILCs are critical to the pathogenesis of AD-like disease in lymphocyte-deficient mice, but whether they contribute to inflammation in the context of a functional adaptive immune system has not been tested. Employing a gain-of-function

approach, group 2 ILCs were sort-purified from MC903-treated C57BL/6 WT mice and adoptively transferred by intradermal injection into a naïve C57BL/6 WT recipient mice (fig. S10A). Following adoptive transfer, the recipient mouse demonstrated enhanced IL-4, IL-5, and IL-13 production from T cells in the skin dLNs (fig. S10B) and histological analysis revealed AD-like features in the skin (fig. S10C). Thus, group 2 ILCs can promote AD-like disease in healthy, lymphocyte-sufficient C57BL/6 WT mice in the absence of external stimuli and tissue damage.

Discussion

Recent studies have demonstrated that group 2 ILCs are present in the lung and intestine of humans and mice and can play major roles in promoting immunity, inflammation, and tissue repair at those barrier surfaces (4, 7–10, 13, 15, 23, 24). Group 2 ILCs require expression of Id2, ROR α , and GATA-3 for their development (1, 15, 23, 24), are classically activated by IL-33 and IL-25, and express the effector molecules IL-5, IL-13, and amphiregulin (25). However, whether group 2 ILCs are present in the skin and influence inflammation at cutaneous sites has not previously been assessed.

The present study provides three conceptual advances that broaden our understanding of the regulation and function of group 2 ILCs. First, we describe the presence of a constitutive population of Lin[−] CD25⁺ IL-33R⁺ group 2 ILCs in the healthy skin of both mice and humans. Second, we demonstrate that group 2 ILCs accumulate in lesional human skin of AD patients and that depletion of murine ILCs significantly ameliorates skin inflammation in a model of AD-like inflammation, indicating that group 2 ILCs play an essential role in promoting skin inflammation. Third, in contrast to the previously described roles for IL-33 and IL-25 in promoting group 2 ILC responses in airway hyperreactivity or intestinal anti-helminth immunity in the intestine, group 2 ILC responses in the skin were independent of these canonical pathways—but were critically dependent on TSLP–TSLPR interactions. The identification of a previously unrecognized dependence of skin group 2 ILCs on TSLP highlights a new pathway by which pathologic group 2 ILC responses can be elicited in the skin in the absence of IL-33–IL-33R or IL-25–IL-25R interactions. Collectively, these findings demonstrate that heterogeneous epithelial cell–derived, cytokine-dependent pathways can promote group 2 ILC responses and related type 2 cytokine–associated skin inflammation.

The role of TSLP in promoting T_H2 cytokine-associated inflammation in AD was first highlighted when lesional human AD skin was found to exhibit elevated expression of TSLP (26). In addition, genetic variants in TSLP were subsequently found to be significantly associated with the development of AD and its most severe complications (27), further supporting a role for TSLP in the pathogenesis of this disease. Consistent with these findings, previous studies have found that TSLP can influence dendritic cells, CD4⁺ T_H2 cells, and basophils to promote T_H2 cytokine responses and inflammation in models of AD and other allergic diseases (22, 28–30). Our findings that skin-resident group 2 ILC responses in a murine model of AD-like inflammation are TSLP-dependent, but independent of canonical IL-33–IL-33R or IL-25–IL-25R signaling, reveal a mechanism by which TSLP may promote early type 2 cytokine responses and inflammation in the context of AD.

A recent report indicated that TSLP–TSLPR signaling is not required for the development of group 2 ILCs in the periphery, nor is it necessary for bone marrow–derived progenitors to differentiate into mature, resting group 2 ILCs (23). Although TSLP–TSLPR pathway is not essential for the development of cutaneous group 2 ILCs, our data reveal a previously unappreciated role for TSLP–TSLPR signaling in promoting group 2 ILC responses in the context of skin inflammation. Mjösberg *et al.* recently showed that TSLP is upregulated in

nasal polyps of patients with chronic allergic rhinosinusitis and that TSLP can act synergistically with IL-33 *in vitro* to induce IL-5 and IL-13 cytokine production in human group 2 ILCs (13). In contrast to these *in vitro* studies, we show *in vivo* in mice that the TSLP-TSLPR pathway can promote pathologic group 2 ILC responses in the absence of IL-25-IL-25R or IL-33-IL-33R pathways to promote T_H2 cytokine-associated skin inflammation.

Although the IL-33-IL-33R pathway appears to be a critical signal for eliciting group 2 ILC responses during anti-helminth immunity in the intestine or airway hyperreactivity in the lung (8, 9, 14), the contribution of TSLP in regulating these responses, potentially through synergy with IL-33, remains unknown. In this context, these data provoke a fundamental question as to whether TSLP and IL-33 elicit functionally similar or distinct populations of group 2 ILCs. Future genome-wide transcriptional profiling and functional studies of TSLP-elicited versus IL-33-elicited group 2 ILCs will be essential to further characterize and compare these two potentially distinct populations of group 2 ILCs. The ability of TSLP to act in concert with IL-33 or drive IL-33-independent ILC responses may depend in part on the nature of the inflammatory stimulus or the tissue-specific microenvironment. Future studies are also needed to dissect the relative contributions of the IL-25, IL-33, and TSLP cytokine signaling pathways in the regulation of group 2 ILC responses at distinct barrier surfaces.

Although we have identified that TSLP-elicited group 2 ILCs can function independently of IL-33 and IL-25 to promote AD-like inflammation, we did not determine the effector mechanisms by which group 2 ILCs directly or indirectly promote AD-like inflammation. Previous studies have demonstrated that recombinant TSLP-induced skin inflammation is dependent on the recruitment of eosinophils (31). Whether group 2 ILC-derived IL-5 and IL-13 directly promote skin inflammation or do so indirectly by eliciting the activation and/or recruitment of other innate cell populations such as eosinophils, mast cells, or alternatively activated macrophages requires further exploration.

In this study, we employed the murine model of MC903-induced AD-like disease to recapitulate human AD. MC903-treated mice were previously reported to exhibit enhanced susceptibility to respiratory atopy in models of allergic airway disease in addition to developing a chronic eczematous dermatitis associated with xerosis (32). In conjunction with demonstrating elevated serum IgE, this model of AD meets at least 2 of the 4 major and 2 of the 23 minor Hanifin and Rajka criteria (3 major and 3 minor criteria required for positive diagnosis) used for the diagnosis of AD used in human patients. Extending our murine studies into human AD, we identified that Lin⁻ CD25⁺ IL-33R⁺ group 2 ILCs are enriched in lesional human AD skin. Although we did not identify other human group 2 ILC-associated markers such as CCR2 and CD161 in healthy human skin (12, 13), we cannot exclude the possibility that they constitute a rare population in healthy human skin that accumulates in human AD skin, as they were readily identified in the lesion. Additionally, future studies will be required to determine whether skin-resident group 2 ILCs in AD produce IL-5 and IL-13, and respond to TSLP as demonstrated in our mouse studies. Although our studies identify the enrichment of group 2 ILCs in human AD, whether or not other ILC subsets, such as group 1 and group 3 ILCs, are present in human skin remains to be determined. Along these lines, a recent study has shown that IL-17- and IL-22-producing ROR γ ⁺ group 3 ILCs promote psoriasiform inflammation in a murine model of psoriasis (33). Given that psoriasis is associated with T_H17 and T_H22 cytokine-associated responses, future studies exploring the presence of ROR γ ⁺ group 3 ILCs in both healthy human skin and lesional skin from psoriasis patients are warranted.

In summary, the results presented in this report identify the presence of group 2 ILCs in both healthy human and murine skin as well as their enrichment in both human AD and murine AD-like inflammation. Further, these data demonstrate a previously unrecognized a mechanism that is independent of IL-33–IL-33R and IL-25–IL-25R pathways, but is dependent on TSLP for group 2 ILC responses in promoting experimental AD-like inflammation in the skin. In addition to identifying the ability of group 2 ILCs to promote AD-like disease, we also demonstrate that group 2 ILCs can amplify the scope and intensity of the adaptive T_H2 cell response. Therefore selective targeting of TSLP and TSLP-dependent group 2 ILCs could prove to be a beneficial strategy in the treatment of AD and multiple allergic diseases.

Materials and Methods

Mice

C57BL/6 WT, *Rag1*^{−/−}, and *Rorc*^{−/−} mice were purchased from the Jackson Laboratory. *Tslpr*^{−/−}, *Il17rb*^{−/−}, and *Il33*^{−/−} mice were provided by Amgen. BAC-transgenic *Rorc*(γt)-Gfp^{TG} mice were provided by G. Eberl (Pasteur Institute). The generation of Id2-deficient bone marrow chimeras is described in Supplementary Methods. All mice were maintained and/or bred in specific pathogen-free facilities at the University of Pennsylvania. All protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC), and all experiments were performed according to the guidelines of the University of Pennsylvania IACUC.

Analysis of human skin

Healthy human control skin samples were obtained as residual healthy marginal skin from patients undergoing routine dermatologic surgery. Human AD skin samples were obtained directly from the lesional skin of patients with a clinical diagnosis of AD and meeting the U.K. Working Party's Criteria for Atopic Dermatitis (34). Use of human skin samples for research purposes was approved by the University of Pennsylvania IRB.

Skin tissues were obtained as 4-mm punch biopsy specimens and digested in 0.25 mg/mL Liberase TL (Roche) in DMEM media for 3 hours at 37°C. Samples were mashed through 70 μm cell strainers and washed with DMEM media [supplemented with 5% FBS, 1% L-glutamine (GIBCO), 1% Penicillin/Streptomycin (GIBCO)]. For analysis of ILC populations, single-cell suspensions of skin tissue underwent flow cytometric analysis (Supplementary Methods).

MC903 treatment, antibody treatments, and cDNA injections

For MC903 treatment, mice were treated once daily topically with 2 nmol of MC903 (calcipotriol, Tocris Bioscience) in 20 μL of ethanol (vehicle) on both ears. For monoclonal antibody treatments, anti-CD25 mAb (clone PC-61.5.3) and anti-CD90.2 mAb (30H12) were purchased from BioXCell. Anti-CD25 mAb and anti-CD90.2 mAb treatments in *Rag1*^{−/−} mice were administered intraperitoneally (i.p.) every 2 days at a dose of 300 μg/mouse starting two days prior to treatment with MC903. Anti-TSLP mAb treatments in mice were administered i.p. every 3 days at a dose of 200 μg/mouse starting on the day prior to treatment with MC903. For TSLP cDNA injections, mice were injected i.v. with 10 μg of control or TSLP-encoding cDNA plasmid (provided by Amgen).

ILC adoptive transfer

Lin[−] CD25⁺ IL-33R⁺ group 2 ILCs were sort-purified from the skin dLNs of MC903-treated (Day 7) C57BL/6 WT mice using a BD FACSAria cell sorter. ILCs (5,000–7,500) were then suspended in 50 μL of PBS and injected intradermally into naïve C57BL/6 mice.

Statistical analysis

Data are means $\pm$ SEM unless indicated otherwise. Statistical significance was determined by unpaired Student's t-test. Statistical analyses were performed using Prism GraphPad software v5.0.

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Fig. 1. Group 2 ILCs are present in healthy human skin and are enriched in human AD skin lesions. **(A)** Identification of skin-resident ILCs in healthy human control skin by flow cytometry as lineage (Lin)⁻negative FcεRIα⁻ CD25⁺ IL-33R⁺ cells. Data from human control skin tissue are representative of 9 healthy control subjects. **(B)** Identification of skin-resident ILCs in human AD skin by flow cytometry as Lin⁻ FcεRIα⁻ CD25⁺ IL-33R⁺ cells. Data from human lesional AD skin are representative of 5 AD patients. **(C)** Frequencies of Lin⁻ FcεRIα⁻ CD25⁺ IL-33R⁺ cells from normal skin of healthy controls and lesional skin from

AD patients. All cell frequencies are given as a percentage of total Lin⁺ cells. *** $P < 0.001$, Student's t test.

Fig. 2.

Skin-resident group 2 ILCs are present in murine skin. **(A)** Identification of skin-resident ILCs in C57BL/6 wild-type (WT) mice by flow cytometry as Lin⁻ FcεRIα⁻ CD25⁺ IL-33R⁺ cells. All cell frequencies are given as a percentage of total Lin⁻ cells. SSC, side scatter. **(B)** Expression of cell surface markers on Lin⁻ CD25⁺ IL-33R⁺ murine skin-resident ILCs (solid black line) compared to fluorescence minus one controls (FMO; gray-shaded). **(C)** Expression of cell surface markers and RORγt-GFP on Lin⁻ CD25⁺ IL-33R⁺ murine skin-resident ILCs from BAC-transgenic Rorc(γt)-Gfp^{TG} mice. Data from murine skin tissue are

representative of more than 5 independent experiments, $n = 3\text{--}4$ mice per group per experiment.

Fig. 3. Skin-resident group 2 ILCs are enriched in a mouse model of AD-like inflammation. C57BL/6 WT mice were treated with vehicle control (EtOH) or MC903. (A) Representative flow cytometry plots and frequencies of Lin⁻ CD25⁺ IL-33R⁺ ILCs from ear skin. (B) Representative flow cytometry plots, frequencies, and absolute cell numbers of Lin⁻ CD25⁺ IL-33R⁺ ILCs from the skin dLNs. (C) Representative flow cytometry plots and frequencies of IL-5⁺ IL-13⁺ ILCs from the skin dLNs of treated mice that were also stimulated with PMA/ionomycin. Flow cytometry plots are gated on Lin⁻ CD25⁺ CD90⁺ cells. (D) Ear thickness measurements. All data are from Day 7 of treatment and are representative of

more than three independent experiments, $n = 3-4$ mice per group per experiment. Cell frequencies in A and B are given as a percentage of total Lin⁻ cells. All statistical analyses of ear thickness measurements were performed on Day 7. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test.

Fig. 4.

Skin-resident group 2 ILCs are enriched in AD-like inflammation independent of adaptive immunity. *Rag1*^{-/-} mice were treated with vehicle control (EtOH) or MC903. (A) Representative flow cytometry plots and frequencies of Lin⁻ CD25⁺ IL-33R⁺ ILCs from *Rag1*^{-/-} ear skin. (B) Representative flow cytometry plots, frequencies, and absolute cell numbers of Lin⁻ CD25⁺ IL-33R⁺ ILCs from the skin dLNs. (C) Representative flow cytometry plots and frequencies of IL-5⁺ IL-13⁺ ILCs from the skin dLNs of treated mice also stimulated with PMA/ionomycin. Flow cytometry plots are gated on Lin⁻ CD25⁺ CD90⁺ cells. (D) Ear thickness measurements. All data are from Day 7 of treatment and are

representative of more than 3 experiments, $n = 3-4$ mice per group per experiment. Cell frequencies in A and B are given as a percentage of total Lin⁻ cells. All statistical analyses of ear thickness measurements were performed on Day 7. * $P < 0.05$, ** $P < 0.01$, Student's t test.

Fig. 5. Depletion of ILCs attenuates AD-like dermatitis and type 2 cytokine responses in the skin. *Rag1*^{-/-} mice were treated with vehicle control (EtOH) + isotype mAb, MC903 + isotype mAb, or MC903 + anti-CD25 mAb. **(A)** Representative flow cytometry plots, frequencies, and absolute cell numbers of Lin⁺ CD25⁺ IL-33R⁺ ILCs from the skin dLNs of *Rag1*^{-/-} mice. Cell frequencies are given as a percentage of total Lin⁺ cells. **(B)** IL-5 and IL-13 cytokine levels from ear skin homogenates, measured by ELISA. **(C)** Ear thickness measurements. **(D)** H&E staining of ear skin tissue. Closed black arrows indicate orthokeratosis, closed gray arrows indicate acanthosis, open green arrows indicate

mononuclear leukocytes, open black arrows indicate granulocytes. Scale bar, 100 μm for the upper panel; 25 μm for the lower panel. All data in (A, B and D) are from Day 7 of treatment and are representative of more than 4 experiments, $n = 3\text{--}4$ mice per group per experiment. All statistical analyses of ear thickness measurements were performed on Day 7. * $P < 0.05$, Student's t test.

Fig. 6. IL-33 is dispensable for the elicitation of skin-associated group 2 ILC responses in the skin. C57BL/6 WT or *Il33*^{-/-} mice were treated with vehicle control (EtOH) or MC903. (A) Representative flow cytometry plots, frequencies, and absolute cell numbers of Lin⁻ CD25⁺ IL-33R⁺ ILCs from the skin dLNs of treated mice. All cell frequencies are given as a percentage of total Lin⁻ cells. (B) Ear thickness measurements. (C) H&E staining of ear skin tissue. Closed black arrows indicate orthokeratosis, closed gray arrows indicate acanthosis, open green arrows indicate mononuclear leukocytes, open black arrows indicate granulocytes. Scale bar, 100 μ m for the upper panels; 25 μ m for lower panels. All data in A and C are from Day 7 of treatment and are representative of 2 or more experiments, $n = 3-4$ mice per group per experiment. All statistical analyses of ear thickness measurements were performed on Days 3 and 7.

Fig. 7. Skin-associated group 2 ILC responses and AD-like dermatitis are critically dependent on TSLP signaling. C57BL/6 WT or *Tslpr*^{-/-} mice were treated with control (EtOH) or MC903. **(A)** TSLP cytokine levels from ear skin explants following treatment, as measured by ELISA. N.D., not detected. **(B)** Representative flow cytometry plots demonstrating expression of TSLPR on Lin⁻ CD25⁺ IL-33R⁺ cells from skin dLNs of C57BL/6 WT mice (solid black line) compared to *Tslpr*^{-/-} mice (gray-shaded) and expression of CD127 (solid black line) on Lin⁻ CD25⁺ IL-33R⁺ cells compared to FMO controls (gray shaded). **(C)** Representative flow cytometry plots, frequencies, and absolute cell numbers of skin dLN

Lin⁻ CD25⁺ IL-33R⁺ ILCs in C57BL/6 WT and *Tslpr*^{-/-} mice. All cell frequencies are given as a percentage of total Lin⁻ cells. **(D)** Ear thickness measurements. **(E)** H&E staining of skin tissue. Closed black arrows indicate orthokeratosis, closed gray arrows indicate acanthosis, open green arrows indicate mononuclear leukocytes, open black arrows indicate granulocytes. Scale bars, 100 μ m for the upper panels; 25 μ m for the lower panels. All data in A, B, C and E are from Day 7 of treatment and are representative 3 or more experiments, $n = 3-4$ mice per group per experiment. All statistical analyses of ear thickness measurements were performed on Day 7. * $P < 0.05$, Student's t test.

Fig. 8. TSLP elicits group 2 ILC responses independently of IL-33 signaling. C57BL/6 WT or *Il33*^{-/-} mice were treated with control cDNA plasmid or TSLP cDNA plasmid. (A) Representative flow cytometry plots, frequencies, and absolute cell numbers of skin dLN Lin⁻ CD25⁺ IL-33R⁺ ILCs from WT mice taken 3 weeks after treatment. (B) Representative flow cytometry plots, frequencies, and absolute cell numbers of skin dLN Lin⁻ CD25⁺ IL-33R⁺ ILCs from *Il33*^{-/-} mice taken 3 weeks after treatment. All cell frequencies are given as a percentage of total Lin⁻ cells. All data are representative of 2 or more experiments, $n = 3-4$ mice per group per experiment. * $P < 0.05$, ** $P < 0.01$, Student's t test.