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Intradermal Administration of Thymic Stromal Lymphopoietin Induces a T Cell- and Eosinophil-Dependent Systemic Th2 Inflammatory Response

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The epithelial-derived cytokine thymic stromal lymphopoietin (TSLP) is sufficient to induce asthma or atopic dermatitis-like phenotypes when selectively overexpressed in transgenic mice, or when driven by topical application of vitamin D3 or low-calcemic analogues. Although T and B cells have been reported to be dispensable for the TSLP-induced inflammation in these models, little is known about the downstream pathways or additional cell types involved in the inflammatory response driven by TSLP. To characterize the downstream effects of TSLP in vivo, we examined the effects of exogenous administration of TSLP protein to wild-type and genetically deficient mice. TSLP induced a systemic Th2 inflammatory response characterized by increased circulating IgE and IgG1 as well as increased draining lymph node size and cellularity, Th2 cytokine production in draining lymph node cultures, inflammatory cell infiltrates, epithelial hyperplasia, subcuticular fibrosis, and up-regulated Th2 cytokine and chemokine messages in the skin. Responses to TSLP in various genetically deficient mice demonstrated T cells and eosinophils were required, whereas mast cells and TNF-α were dispensable. TSLP-induced responses were significantly, but not completely reduced in IL-4- and IL-13-deficient mice. These results shed light on the pathways and cell types involved in TSLP-induced inflammation. ***The Journal of Immunology,*** **2008, 181: 4311–4319.**

The biological consequences of cytokine overexpression in vivo have been demonstrated in numerous transgenic mouse models. These models provide insight into the underlying mechanisms of action, target cells, downstream pathways, and potential disease associations that result from aberrant production of these potent factors. In allergic disorders such as asthma and atopic dermatitis (AD),[2] the contributions of the prototypical Th2 cell-derived cytokines IL-4, IL-5, IL-9, and IL-13 have long been the focus of research. More recently, the emerging contributions of epithelial cell-derived cytokines such as thymic stromal lymphopoietin (TSLP) to the inflammatory response provide a greater appreciation for the active role of the epithelium rather than solely as a barrier to the environment (1). TSLP is a cytokine closely related to IL-7 and is primarily expressed by epithelial cells in response to specific TLR agonists, physical injury, and inflammatory cytokine stimulation (2–4). TSLP has been reported to play a role in the initiation and maintenance of the allergic inflammatory response (5, 6). Initially, TSLP was found to activate human $CD11c^+$ myeloid dendritic cells (DC), thus inducing the up-regulation of costimulatory molecules and the production of Th2 cell-attracting chemokines CCL17 and CCL22. Naive $CD4^+$ T cells exposed to TSLP activated DC differentiate into inflammatory Th2 cells defined by the production of Th2 cytokines, high levels of TNF-α, and a decrease in IL-10 and IFN-γ (7). Expanding the possible role of TSLP in the orchestration of allergic inflammatory responses, mast cells (MC) were identified as additional sentinel immune cells that are colocalized at the epithelial surface and are capable of responding to TSLP in proinflammatory settings. In the presence of IL-1, TSLP stimulates MC to produce significant levels of the proinflammatory cytokines IL-5, IL-6, IL-13, and GM-CSF, and chemokines such as CCL1 and CXCL8. The combination of TSLP and IL-1 did not induce MC degranulation or the release of preformed mediators. These data suggest a non-T cell-dependent mechanism by which TSLP may influence innate immune response and Th2 inflammation (3).

The involvement of TSLP in allergic inflammation has been demonstrated in vivo, in which it was found to be highly expressed in the lesional skin of AD patients (3, 7) and increased in asthmatic airways correlating with Th2-attracting chemokine expression and disease severity (8). In transgenic mouse models, overexpression of TSLP leads to systemic inflammation (9, 10), and when specifically expressed in airway epithelial cells or keratinocytes leads to the development of asthma and AD-like phenotypes, respectively (11–13). Similarly, in nontransgenic models, aberrant TSLP expression can be induced by keratinocyte-specific ablation of retinoid X receptors or topical application of vitamin D3 and low-calcemic analogues. This expression leads to the development of an AD-like phenotype comparable to that seen in TSLP transgenic mice (13, 14). Collectively, these findings provide further support for the role of TSLP as a critical factor capable of initiating allergic disease. Interestingly, the phenotype seen in both TSLP transgenic and vitamin D3-driven models was reported to be independent of T and B cells (12, 14).

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[2] Abbreviations used in this paper: AD, atopic dermatitis; C_T, cycle threshold; DC, dendritic cell; DLN, draining lymph node; KO, deficient; MC, mast cell; MSA, mouse serum albumin; TSLP, thymic stromal lymphopoietin; WT, wild type.

To further elucidate the pathways and cell types involved in the TSLP-induced inflammatory response in vivo, we characterized the effects of repeated administration of TSLP protein into the skin of wild-type (WT) mice and mice genetically deficient in key immune response pathways. Intradermal administration of TSLP protein over a 2-wk time frame consistently induced a systemic inflammatory response that was largely Th2 in nature and with some similarities to TSLP transgenic mice. Although a significant, diffuse inflammatory cell infiltrate containing eosinophils and mast cells was observed along with s.c. fibrosis, there was no development of skin lesions. Thus, this model differs from previously published mouse models of atopic dermatitis (12–14). Th2 cytokine production in restimulated draining lymph node (DLN) cultures was increased in a dose-dependent manner, as were circulating levels of IgE and IgG1, typical of a Th2 response. Additionally, Th2 cytokine and chemokine messages were up-regulated in skin mRNA samples following TSLP treatment. In contrast to results reported in TSLP transgenic mice (12, 14), T cells were a required component of the TSLP-induced response, whereas the presence of B cells was not sufficient to induce inflammation. TSLP-driven inflammation was independent of mast cells and signaling of TNF-α through the TNF-RI in this system, and displayed partial dependence on IL-4 and IL-13. We also show in this study for the first time that eosinophils are required not only for local TSLP-induced skin inflammation, but for many systemic effects of TSLP. Collectively, these data elucidate the dependence of TSLP on Th2 pathways and cell types in skin and systemic inflammation.

Materials and Methods

Mice and injections

All mice were housed in a specific pathogen-free facility at Amgen or Benaroya Research Institute, according to Institutional Animal Care and Use Committee standards. IL-7Rα (15), IL-4Rα (16), STAT6 (17), IL-4 (18), TNF-RI (19)-deficient (KO) mice, ΔdblGATA (20), and $Kit^{W\text{-}sh}$ mice (21), and WT controls were obtained from Jackson ImmunoResearch Laboratories. CCR4 (22) and RAG2 (23) KO mice, athymic nude mice (24), and WT controls were obtained from Taconic Farms. IL-13 KO mice (25) were a gift from A. McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). Female mice (n = 5/group) between 6 and 14 wk of age were used, with the exception of the ΔdblGATA experiment in which males and females, aged 20–22 wk of age, were used.

Intradermal injections were performed, as previously described (26), with minimal modifications to the dose volume and injection regimen. Injections were given under anesthesia using a sterile 26-gauge needle. Briefly, fur was stripped from the lower backs of mice, and 10 μg of TSLP (R&D Systems) or mouse serum albumin (MSA; Sigma-Aldrich) was injected intradermally in a 100 μl vol of sterile PBS by inserting the needle into the superficial dermis as close to the epithelium as possible. Bolus injections remained at the site for ~30–60 min and slowly diffused into the surrounding tissues. Injections were performed three times per week over a 2-wk period (see Fig. 1*A*). Seventy-two hours after the final injection, blood and tissues were collected for analysis. Experimental protocols were approved by the Institutional Animal Care and Use Committees of both Amgen and Benaroya Research Institute.

Histology

Skin samples from the injection site (~1 cm × 1 cm) were collected from each animal and fixed in 10% neutral buffered formalin for 24–48 h. After fixation, samples were processed and cut into 4-μm sections following routine procedures. Individual sections were stained with H&E, astra blue, modified Masson's trichrome, and/or Congo red. Samples were scored for the severity and character of the inflammatory response using a subjective grading scale in which 0 = no findings, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. All of the studies were initially read unblinded, because there was typically an obvious difference between the groups. In studies in which inflammation was mild or moderate, the slides were blinded, randomized, and reread to determine the histology score. All studies were read by the same pathologist using the same subjective grading scale. The total histology score is a sum of scores, including edema, epithelial hyperplasia, subcuticular fibrosis, and inflammation.

RNA isolation/cDNA/TaqMan analysis

One-quarter of the injection site was put into RNAlater (Qiagen) or flash frozen in liquid nitrogen and skin sections pooled by treatment group for subsequent RNA isolation and TaqMan analysis. RNA was isolated using an RNeasy Maxi kit (Qiagen). First-strand cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems) using random hexamers. Samples were distributed on plates at 20 ng/well and run in triplicate. 6-carboxy-fluorescein/minor groove binder-labeled gene expression assays or VIC/minor groove binder-labeled β-actin housekeeper assays were purchased from Applied Biosystems. Single-plex TaqMan PCR were set up using TaqMan Universal PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7900HT Sequence Detection System. Cycle threshold (C_T) values were determined using Sequence Detector software version 2.2.2 (Applied Biosystems) and transformed to $2^{-\Delta CT}$ for relative expression comparison of genes to β-actin.

Determination of serum concentrations of IgE, IgG1, and IgG2a

IgE serum concentrations were measured using an Opt EIA IgE kit (BD Biosciences). OD was read at 450 nm on a plate reader (Molecular Devices) using Deltasoft 3 software (BioMetallics). IgG1 and IgG2a serum concentrations were measured using a standard ELISA protocol. Briefly, Maxisorp microtiter plates (Nunc) were coated with goat anti-mouse IgG1 (Southern Biotechnology Associates) or IgG2a (Southern Biotechnology Associates) capture Abs in carbonate coating buffer (pH 9.5) at 1 μg/ml overnight at 4°C. Nonspecific binding was blocked with 10% goat serum (Life Technologies) diluted in PBS. Serially diluted serum samples were incubated in anti-mouse IgG1- or IgG2a-coated plates for 1 h at room temperature, and unbound material was removed by washing with PBS containing 0.05% Tween 20. Bound IgG1 or IgG2a was detected by incubation with a goat anti-mouse IgG1 or IgG2a Ab conjugated to HRP diluted in PBS containing 50% goat serum for 1 h at room temperature. Plates were developed using tetramethylbenzidine peroxidase substrate solution (Kirkegaard & Perry Laboratories) and serum Ab concentrations calculated using purified IgG1 and IgG2a standards (Southern Biotechnology Associates), as described for the IgE ELISA.

Cell purification

Inguinal lymph nodes were dissected from injected mice, and single-cell suspensions were prepared by pressing tissues through 70-μm nylon mesh in a 60-mm petri dish. Cells were washed with staining buffer (PBS (pH 7.4), Life Technologies; 5% FBS, JRH Biosciences) and collected by centrifugation. Cell pellets were resuspended in staining buffer containing 1 μg/ml Fc block (BD Biosciences) and viable cells enumerated by trypan blue (Life Technologies) exclusion.

Multiparameter flow cytometric analysis

FcRs were blocked before staining using 1 μg/ml Fc block (BD Biosciences) in PBS. Cells were incubated with the indicated fluorochrome-conjugated mAbs (BD Biosciences), as follows: I-A/I-E-FITC (2G9), CD80-PE (16-10A1), or CD86-PE (GL1); washed; and fixed in PBS containing 0.5% paraformaldehyde (Alfa Aesar). Samples were collected on a FACSCalibur (BD Biosciences), and multiparameter data analysis was performed using FCS Express version 3.0 (De Novo Software).

In vitro restimulation

Cells were cultured for 48 h in IMDM (Life Technologies) containing 10% heat-inactivated FBS (JRH Bioscience), 1 mM Na pyruvate (Life Technologies), 100 μM nonessential amino acids (Life Technologies), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies), and 0.1% 2-ME (Sigma-Aldrich). For in vitro restimulation, 10 μg/ml anti-CD3 (clone 500A2; Amgen) was precoated in PBS into wells of a 96-well U-bottom plate. A total of 2.5 μg/ml anti-CD28 (clone 37.51; BD Pharmingen) was added in soluble form to cell cultures, or PMA (Sigma-Aldrich) was added at a final concentration of 10 ng/ml. Supernatants were analyzed on mouse cytokine/chemokine multiplex plate (Linco Research).

Statistical analysis

All statistical calculations were performed using GraphPad Prism version 4.01. Student's unpaired t test and ANOVA (Tukey-Kramer multiple comparisons test) were used to determine the statistical significance of the data.

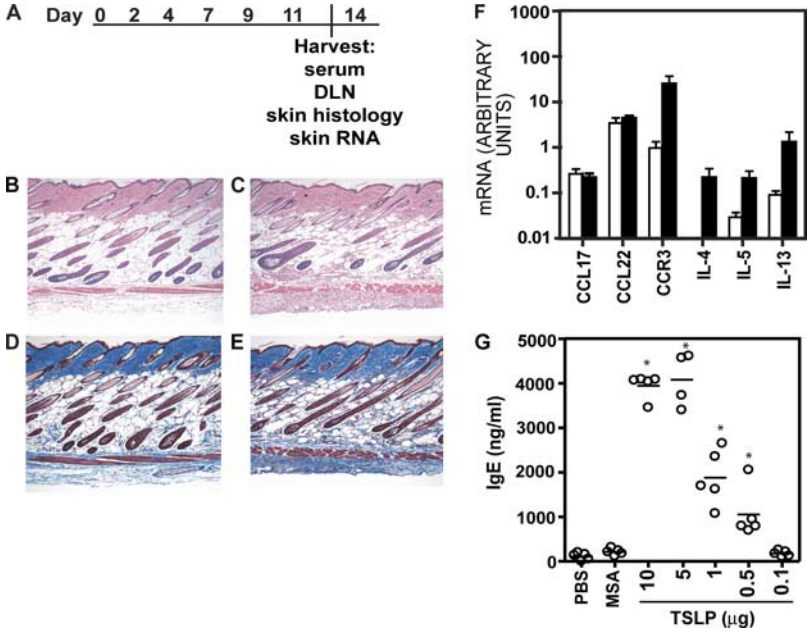

FIGURE 1. Injection of TSLP induces localized skin inflammation and fibrosis accompanied by increased Th2 cytokine and chemokine gene expression. *A*, Mice ($n = 5$/group) received injections of TSLP (10 μg) or MSA (10 μg) three times per week for 2 wk. *B* and *C*, H&E-stained sections of MSA (*B*)- and TSLP (*C*)-injected skin showing increased inflammatory infiltrate in TSLP-treated mice at $\times$10 magnification. *D* and *E*, Masson's trichrome-stained sections of MSA (*D*)- and TSLP (*E*)-injected skin showing increased fibrosis in TSLP-treated mice at $\times$10 magnification. *F*, TaqMan analysis of cytokine and chemokine gene expression in MSA (□)- or TSLP (■)-injected skin. Bars represent pools of skin from five individual mice. The mean mRNA levels from triplicate measurements are normalized for β-actin expression. *G*, Serum IgE concentrations are increased in mice receiving TSLP ($n = 5$/group) and correlate with TSLP dose. *, $p < 0.05$, relative to MSA-injected mice. Results shown are representative of >20 mice analyzed.

Results

Injection of TSLP results in a Th2 phenotype

To better understand the role of TSLP and its associated pathways in inflammation, we developed a model in which recombinant protein was introduced into the intradermal space. BALB/c or C57BL/6 mice were injected with recombinant, carrier-free TSLP or MSA as a protein control. Mice received a series of six injections on their backs every other day over a 2-wk period (Fig. 1*A*).

Mice receiving TSLP displayed a number of similarities to mice containing skin-specific K5 and K14-TSLP transgenes (12, 13). H&E-stained sections of skin displayed localized edema, mild epithelial hyperplasia, and a marked increase of inflammatory infiltrate in the subcutis (Fig. 1*C*) compared with mice receiving MSA (Fig. 1*B*). The cellular infiltrate was composed largely of eosinophils and mononuclear cells with smaller numbers of mast cells and neutrophils. Moderate to severe subcuticular fibrosis was also evident in skin sections of TSLP-injected mice as seen by Masson's trichrome staining (Fig. 1*E*), but was absent in mice injected with MSA (Fig. 1*D*). However, in contrast to what has been observed in K5-TSLP mice, mice receiving exogenous TSLP lacked

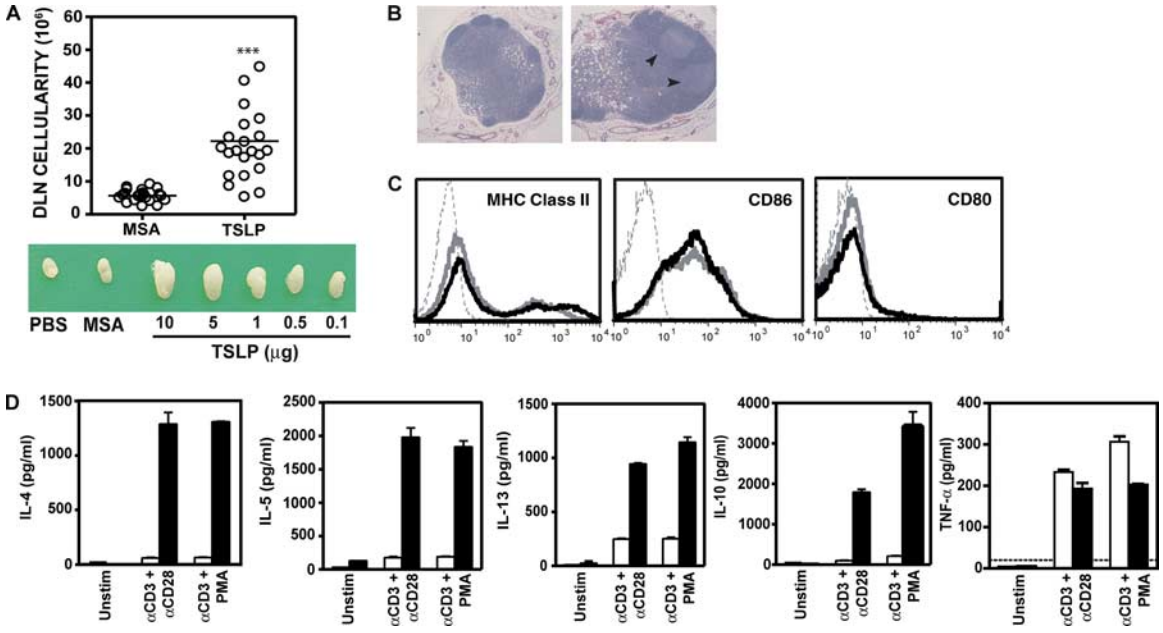

FIGURE 2. Injection of TSLP induces a Th2 cell phenotype, lymphadenopathy, and increased DLN cellularity along with germinal center formation. *A*, Skin DLN were harvested and counted. Mice receiving TSLP display increased DLN cellularity and lymphadenopathy, which correlated with the dose of TSLP received. *B*, H&E-stained section of DLN from mice treated with MSA (*left*) or TSLP (*right*) depicts pale sections indicating germinal center formation in mice treated with TSLP. *C*, Intradermal injection of TSLP (black) induces a slight up-regulation of MHC class II on DLN cells compared with MSA treatment (gray) or isotype control (dotted). CD80 and CD86 were unaffected by administration of TSLP. *D*, Restimulated DLN cells from mice injected with TSLP (■) produce greater concentrations of the Th2 cytokines IL-4, IL-5, IL-13, and IL-10, but equivalent or reduced TNF-α compared with cells injected with MSA (□). Error bars in *D* represent the mean of triplicate wells ± the SD. Results shown are representative of >20 mice analyzed.

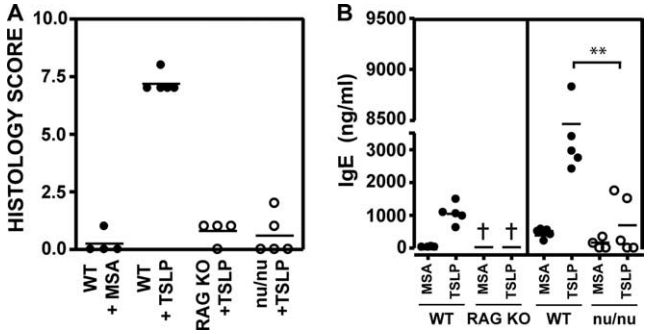

FIGURE 3. Skin inflammation resulting from injection of TSLP requires T cells, but is independent of B cells. RAG2 KO and nu/nu mice received injections of TSLP or MSA ($n = 5$/group). *A*, Skin sections were stained with H&E and scored as in *Materials and Methods*. *B*, Serum IgE concentrations were not increased in RAG2 KO or nu/nu mice (○) receiving injections of TSLP compared with WT controls (●) receiving TSLP ($n = 5$/group). IgE concentrations did not increase in MSA-treated mice, regardless of genotype. †, not detected. **, $p < 0.001$, compared with WT mice injected with TSLP.

dermatitis and xerosis. Thus, whereas intradermal injection of TSLP results in localized skin inflammation, it lacks these hallmark characteristics of atopic dermatitis.

Consistent with the increase in inflammatory infiltrate, TaqMan analysis of gene expression in TSLP-injected skin revealed significant increases in CCR3, IL-4, IL-5, and IL-13 levels compared with MSA-injected skin, and a modest increase in CCL22 expression (Fig. 1*F*). CCL17 expression, however, did not increase appreciably in TSLP-injected skin. This result was unexpected because TSLP induces CCL17 production from DC in humans and mice (7, 11), and CCL17 is known to be expressed by keratinocytes in the affected skin of patients with AD (27) and increased in the skin of K14-TSLP transgenic mice (13).

IgE production, a hallmark of an allergic response, was also evaluated in mice receiving exogenous TSLP. C57BL/6 mice were injected with a dose titration of TSLP ranging from 0.1 to 10 μg. Serum IgE concentrations increased 20-fold in mice receiving 10 or 5 μg of TSLP and correlated with TSLP dose thereafter (Fig. 1*G*). Additional analysis of serum Igs from TSLP-injected mice showed an increase in serum IgG1 concentration and no change in IgG2a concentration (data not shown), consistent with a Th2 response and previous observations in TSLP transgenic mice (10, 12, 13).

An increase in DLN cellularity and lymphadenopathy was evident in mice receiving TSLP (Fig. 2*A*), consistent with data from K5-TSLP mice. This increase in cellularity correlated with TSLP dose and affected only the local DLN. Histological analysis of the DLN from TSLP-injected mice revealed enlarged lymphoid follicles with large germinal centers (Fig. 2*B*). Concomitant with this, there was an increase in absolute $CD19^+B220^+$ B cell number in the DLN by FACS analysis (data not shown).

Treatment of human myeloid DC with TSLP has been shown to induce their maturation as measured by up-regulation of HLA-DR and the costimulatory molecules CD40, CD80, and CD86 (28). Although there was a slight, reproducible up-regulation of MHC class II expression on DLN cells of mice injected with TSLP, neither CD80 nor CD86 expression was significantly increased compared with MSA treatment (Fig. 2*C*). In humans, naive $CD4^+$ T cells exposed to TSLP-stimulated DC are reported to produce large amounts of IL-4, IL-5, IL-13, and TNF-α, but little or no IFN-γ or IL-10 (7). In contrast, restimulated DLN cells from mice injected with TSLP produced increased amounts of IL-4, IL-5, IL-13, and IL-10, but not TNF-α (Fig. 2*D*).

TSLP signals through a heterodimeric receptor consisting of the IL-7Rα chain and a common γ-chain-like receptor (TSLP-R) (29). To investigate the specificity of the TSLP-induced inflammatory response, TSLP was injected into IL-7Rα KO or TSLP-R KO

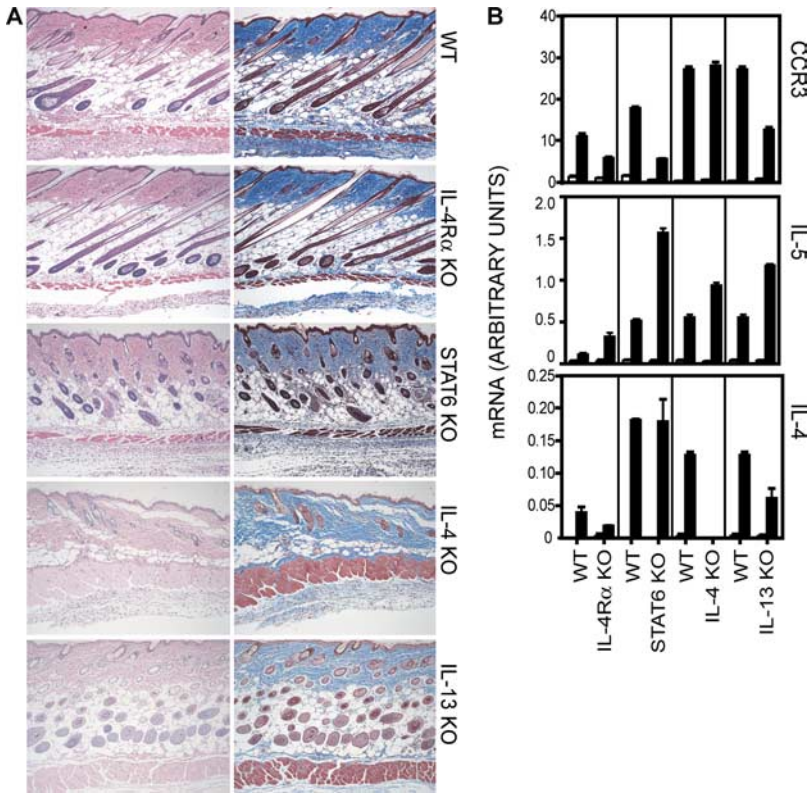

FIGURE 4. Skin inflammation resulting from injection of TSLP is partially dependent on IL-4 and IL-13. IL-4Rα-, STAT6-, IL-4-, or IL-13-deficient mice received injections of MSA or TSLP ($n = 5$/group). *A*, Skin sections were stained with H&E (*left column*) or Masson's trichrome blue (*right column*), and cellular infiltrate, edema, and fibrosis were assessed. TSLP elicited mild inflammation in all KO mice tested compared with the more moderate to severe inflammation seen in WT mice. Photographs are at ×10 magnification. *B*, TaqMan analysis of cytokine and chemokine gene expression in MSA (□)- or TSLP (■)-injected skin from WT or KO mice. Bars represent pools of skin from five individual mice. The mean mRNA levels from triplicate measurements are normalized for β-actin expression.

Table I. *Mean histological scores from IL-4Rα KO, STAT6 KO, and WT mice intradermally injected with TSLP*[a]

	WT	IL-4Rα KO	WT	STAT6 KO
Inflammation	3.0	2.0	4.0	3.0
Neutrophils	1.0	0.0	1.0	1.0
Mononuclear cells	2.0	1.0	2.0	2.0
Eosinophils	3.0	2.0	4.0	3.0
Edema	1.0	1.0	3.0	2.0
Epithelial hyperplasia	0.0	0.0	2.0	1.0
Fibrosis, subcuticular	2.0	0.0	4.0	3.0

[a] Skin sections from mice receiving intradermal TSLP were scored based on inflammatory infiltrate, edema, epithelial hyperplasia, and subcuticular fibrosis. 0 = No findings, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

mice. Neither IL-7Rα KO nor TSLP-R KO mice displayed appreciable increases in inflammatory infiltrate, serum IgE concentration, or DLN cellularity compared with MSA-injected mice (data not shown). These data demonstrate that the effects of TSLP injection are due to TSLP signaling through its receptor, rather than a nonspecific effect of injection of protein into skin.

TSLP-induced inflammation is T cell dependent

Similar to K5-TSLP transgenic mice, WT mice injected with TSLP display an overt Th2 phenotype that suggests a role for $CD4^+$ T cells in the initiation or protraction of skin inflammation. Because myeloid cells and mast cells are able to respond to TSLP (3, 30), we sought to determine whether T and B cells were required for the pathology that develops in response to TSLP administration.

Mice deficient in RAG2 and athymic nude (nu/nu) mice were injected with TSLP or MSA. Neither RAG2 KO nor nu/nu mice developed skin inflammation compared with TSLP-injected WT mice (Fig. 3*A*). Very few mononuclear cells or eosinophils were present in the skin of lymphocyte-deficient mice, and there was a lack of subcuticular fibrosis, epithelial hyperplasia, and edema. These results do not recapitulate those from the K5-TSLP transgenic mice, in which skin inflammation occurred in the absence of αβ T cells (12). Thus, whereas intermittent injection of TSLP protein is insufficient to induce an inflammatory response in the skin in the absence of T and B cells, chronic overexpression of TSLP in the skin may compensate for the lack of T and B lymphocytes, as demonstrated in TSLP transgenic mice. Additionally, native TSLP has been shown to be a more potent inducer of inflammation compared with rTSLP (3), and this increased potency may override the need for T cells in the K5-TSLP transgenic mice.

RAG2 KO mice lacked any production of serum IgE in response to either MSA or TSLP injection (Fig. 3*B*). The nu/nu mice injected with TSLP exhibited a small amount of IgE production, but this was one-third of what was produced in WT mice injected with TSLP (Fig. 3*B*). Because nu/nu mice develop largely normal B cell responses (31), this suggests that B lymphocytes contribute minimally to the phenotype induced by TSLP in the absence of T cells.

TSLP-induced inflammation is partially dependent on IL-4 and IL-13

To elucidate the role of IL-4 and IL-13 in the development of TSLP-induced inflammation, we injected TSLP or MSA into mice deficient in IL-4 or IL-13, or into mice deficient in IL-4Rα or STAT6, which are required for IL-4 and IL-13 signaling (32–36).

Injection of TSLP into IL-4Rα and STAT6 KO mice elicited a mild inflammatory response, which was higher than in MSA-injected WT or KO mice, but less than that seen in TSLP-injected WT mice (Fig. 4*A* and Table I). Interestingly, whereas IL-4Rα KO mice injected with TSLP lacked s.c. fibrosis, STAT6 KO mice exhibited some residual fibrosis compared with MSA-treated mice. Because STAT6 is required for IL-4Rα signaling, similar results were expected in both KO mice. Histology scores were greater in WT control mice in the STAT6 KO experiment compared with the IL-4Rα KO experiment, which may account for the difference in fibrosis. Alternatively, these results may suggest the existence of another pathway through which IL-4/IL-13 signals. Although IL-4 and IL-13 have many overlapping biological activities, their use of intracellular Jak kinases differs, and STAT1, 3, and 5 have been reported to be phosphorylated in response to IL-13 (37).

Similar to IL-4Rα and STAT6 KO mice, TSLP-injected IL-4 KO and IL-13 KO mice displayed a decrease in inflammatory cell infiltrate compared with WT mice injected with TSLP, but retained a mild amount of inflammation (Fig. 4*A*). IL-4 KO mice injected with TSLP displayed minimal subcuticular fibrosis, whereas IL-13 KO mice receiving TSLP lacked fibrosis in the skin, consistent with the role of IL-13 in fibrosis and remodeling (38).

IgE was undetectable in the serum of TSLP-injected IL-4Rα and STAT6 KO mice (data not shown). This was expected because IL-4 is required for class switching to IgE in mice (39). Serum IgE concentrations were not assessed in IL-4 and IL-13 KO mice.

TaqMan analysis of skin of TSLP-injected mice revealed that whereas CCR3 gene expression required IL-13 and its signaling components (Fig. 4*B*), IL-5 gene expression did not. IL-5 was not only independent of IL-4 and IL-13, but its expression was higher in IL-4Rα, STAT6, IL-4, and IL-13 KO mice compared with WT controls injected with TSLP (Fig. 4*B*). These results were confirmed by IL-5 protein quantification in skin lysates (data not shown).

IL-4 gene expression was generally lower in IL-4Rα KO and IL-13 KO mice compared with WT mice injected with TSLP, but

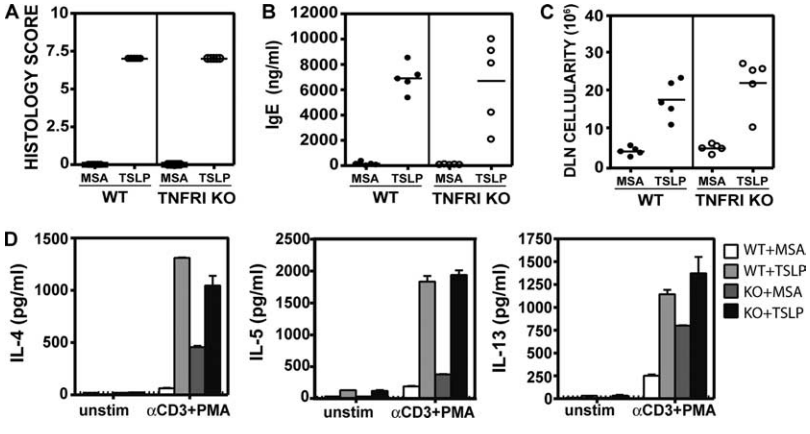

FIGURE 5. Inflammation resulting from injection of TSLP is independent of TNF-α signaling. *A*, Histology scores from WT and TNF-RI KO mice treated with TSLP (n = 5/group) are equivalent, with similar amounts and types of inflammatory infiltrate. *B*, Serum IgE concentrations from WT (●) or TNF-RI-deficient mice (○) injected with TSLP are comparable. *C*, DLN cellularities are equivalent in WT (●) and TNF-RI-deficient mice (○) receiving injections of TSLP. *D*, TNF-RI deficiency does not affect TSLP-induced Th2 cytokine production. DLN cells from TSLP-injected WT (light gray bars) or TNF-RI KO mice (■) produce similar levels of IL-4, IL-5, and IL-13 when restimulated in vitro. Error bars in *D* represent the mean of triplicate wells ± the SD.

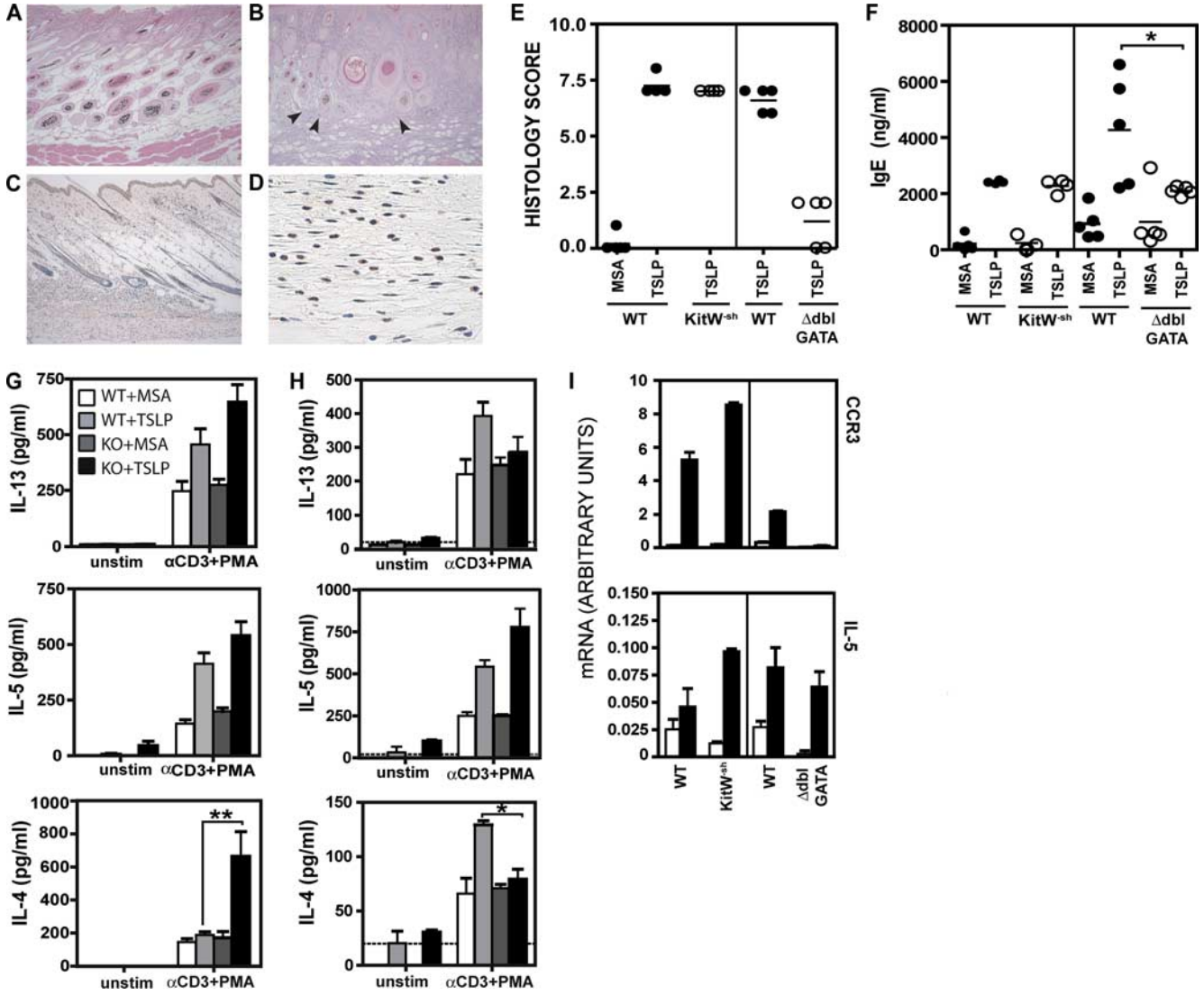

FIGURE 6. TSLP-induced inflammation is mast cell independent, but eosinophil dependent. Astra blue staining from MSA (*A*)- and TSLP (*B*)-injected mice reveals mast cell infiltrate in the dermis of TSLP-injected mice (×10 magnification). *C* and *D*, Congo red-stained TSLP-injected skin reveals eosinophil infiltrate at ×10 (*C*) and ×100 (*D*) magnification (*D* taken from the *bottom center* of Fig. 6*C*). *E*, Histology scores of H&E-stained sections of WT, Kit$^{W\text{-}sh}$ mutant ($n = 4$/group), and ΔdblGATA ($n = 5$/group) mice. *F*, Serum IgE concentrations in mast cell-deficient mice injected with TSLP were equivalent to WT mice, whereas ΔdblGATA mice display a decrease in serum IgE. *, $p < 0.05$, compared with WT injected with TSLP. *G* and *H*, Cytokine production was measured from restimulated DLN cells from Kit$^{W\text{-}sh}$ (*G*) or ΔdblGATA (*H*) mice injected with MSA (dark gray bars) or TSLP (■) and compared with WT mice injected with MSA (□) or TSLP (light gray bars). DLN cell cultures from mast cell-deficient mice injected with TSLP produce equivalent amounts of IL-13 and IL-5, but increased IL-4. **, $p < 0.001$, compared with WT. DLN cell cultures from eosinophil-deficient mice injected with TSLP produce equivalent amounts of IL-5 and IL-13, but decreased IL-4. *, $p < 0.05$, compared with WT. Error bars in *G* and *H* represent the mean ± the SD. *I*, CCR3 gene expression in TSLP-injected Kit$^{W\text{-}sh}$ mice is slightly increased compared with WT, but is decreased in ΔdblGATA mice. IL-5 gene expression is increased in Kit$^{W\text{-}sh}$, but not ΔdblGATA mice compared with WT. Bars represent pools of skin from four (Kit$^{W\text{-}sh}$) or five (ΔdblGATA) individual mice. The mean mRNA levels from triplicate measurements are normalized for β-actin expression.

was equivalent in STAT6 KO mice. As expected, IL-4 gene expression was absent in IL-4 KO mice (Fig. 4*B*).

TSLP-induced inflammation is independent of TNF-α

Stimulation of human DC with TSLP induces the development of Th2 cells that are unique in their production of high amounts of the proinflammatory cytokine TNF-α and absence of IL-10 production (7). Ito et al. (30) speculate that these TNF-α^{+}IL-10^{-} cells represent a subclass of Th2 cells that are pathogenic and are responsible for the induction of allergic inflammation.

To determine whether TNF-α was critical in development of a TSLP-induced inflammatory response, TSLP was injected into mice deficient in the TNF-I receptor (p55, TNF-RI). TNF-α signaling through TNF-RI was dispensable for generation of TSLP-induced skin inflammation, because skin sections from TSLP-injected TNF-RI KO mice received similar scores as WT controls (Fig. 5*A*). Likewise, TNF-α did not play a significant role in the development of a systemic response because TNF-RI KO and WT controls produced similar concentrations of serum IgE when injected with TSLP (Fig. 5*B*).

Although it has been suggested that the TNF-II receptor (p75, TNF-RII) is required for migration of Langerhans cells from the skin to the DLN (40), TNF-RI is not required for Langerhans cell migration. Consistent with this, the DLN of TSLP-injected TNF-RI KO mice exhibited DLN cellularities similar to WT controls (Fig. 5*C*).

Equivalent levels of IL-10 (data not shown), IL-4, IL-5, and IL-13 were produced by restimulated DLN cells from TNF-RI KO mice injected with TSLP compared with WT controls (Fig. 5*D*). TNF-α concentrations in the supernatants were also not significantly altered in TNF-RI KO mice compared with WT controls (data not shown).

TSLP-induced inflammation is independent of mast cells and dependent on eosinophils

The cellular infiltrate in skin receiving injections of TSLP is composed mainly of eosinophils and mononuclear cells, with smaller numbers of mast cells and neutrophils (Figs. 1*C* and 6*B*). Astra blue staining of skin sections revealed an increase in mast cells in TSLP-injected skin (Fig. 6*B*) compared with MSA-injected skin (Fig. 6*A*). Likewise, there is a striking increase of eosinophils in the dermis, as seen by Congo red staining (Fig. 6, *C* and *D*). To determine the contribution of MCs and eosinophils to this inflammatory phenotype, we injected TSLP intradermally into mice containing the $Kit^{W\text{-}sh}$ mutation or ΔdblGATA mice, respectively.

Skin sections from $Kit^{W\text{-}sh}$ mice injected with TSLP received similar scores for edema, epithelial hyperplasia, and fibrosis as WT mice (Fig. 6*E*). The inflammatory infiltrate differed slightly between $Kit^{W\text{-}sh}$ and WT mice, with fewer mononuclear cells present in skin sections of $Kit^{W\text{-}sh}$ mice injected with TSLP, but similar numbers of eosinophils.

Strikingly, skin sections from ΔdblGATA mice injected with TSLP displayed an appreciable decrease in mononuclear cell infiltrate, a complete loss of epithelial cell hyperplasia, and a slight decrease in subcuticular fibrosis compared with WT mice. The overall decrease in skin inflammation in ΔdblGATA mice compared with WT mice (Fig. 6*E*) suggests a requirement for eosinophils in TSLP-induced skin inflammation.

Likewise, the absence of eosinophils, but not mast cells, affected the systemic response to TSLP. Serum IgE concentrations in mast cell-deficient mice injected with TSLP were similar to WT mice, but ΔdblGATA mice lacked an increase in serum IgE concentration (Fig. 6*F*). This reduction in serum IgE when compared with WT controls may be explained by the extremely robust IgE production by three of the WT mice, which was greater than has been typically observed. Alternatively, these results may represent a role for eosinophils in B cell responses, which has been heretofore unappreciated.

Although there was a trend toward slight increases in IL-13, IL-5, and IL-10 production by restimulated DLN cells from $Kit^{W\text{-}sh}$ mice receiving TSLP compared with their WT counterparts, this increase was not significant (Fig. 6*G*, and data not shown). There was, however, a significant increase in IL-4 production by $Kit^{W\text{-}sh}$ mice injected with TSLP compared with WT (Fig. 6*G*). ΔdblGATA mice receiving TSLP produced less IL-13, IL-10, and IL-4, and a slight, but insignificant increase in IL-5 compared with WT mice (Fig. 6*H*, and data not shown).

CCR3 message was increased in the skin of $Kit^{W\text{-}sh}$ mice injected with TSLP, but was nearly absent in ΔdblGATA injected with TSLP compared with WT mice (Fig. 6*I*, *top*). This dramatic loss of CCR3 message in the ΔdblGATA mice most likely reflects the absence of eosinophils in the injection site. There was a trend toward increased IL-5 gene expression (Fig. 6*I*, *bottom*) in TSLP-injected $Kit^{W\text{-}sh}$ mice compared with WT mice, and similar IL-5 levels in ΔdblGATA mice injected with TSLP compared with WT mice.

Discussion

TSLP is produced from epithelial cells of the airway and skin in proinflammatory settings, and may have a profound influence on both the innate and adaptive arms of the immune response through activities on sentinel immune cells localized in these tissues (1–4). Recent data demonstrating abundant expression of TSLP in keratinocytes within the lesional skin of AD patients (7) increased expression in asthmatic airways correlating with disease severity (8), and the demonstration that TSLP is both necessary and sufficient to induce asthma and AD-like disease in animal models suggests a critical role for TSLP in the occurrence of inflammatory disease in the skin and airway (11, 12). Originally described as an epithelial cell-derived factor that activates and instructs human myeloid DC to induce the expansion of a proinflammatory Th2 T cell population from naive T cells (7), TSLP is well positioned to potentially influence multiple cells involved in the innate and adaptive immune response. Recently, it has been demonstrated that native TSLP released from airway epithelial cells in response to proinflammatory cytokines, mechanical injury, or select TLR ligands potently activates MC, inducing the release of multiple cytokines and chemokines (3), thus expanding the known cell types capable of responding to TSLP.

In this study, we present a TSLP-driven model of inflammation in which we inject TSLP into the skin of WT and genetically deficient mice to further elucidate downstream pathways, immune mediators, and mechanisms of action underlying TSLP-induced inflammation.

WT mice injected with TSLP displayed a marked increase of inflammatory infiltrate in the subcutis, composed largely of eosinophils and mononuclear cells. Commensurate with the role of TSLP in the activation of DC and differentiation of naive $CD4^+$ into Th2 cells, we observed a slight increase of $Langerin^+$ cells, $CD11c^+$ DC, and $CD4^+$ T lymphocytes, but not $CD8\alpha^+$ lymphocytes in the skin by immunohistochemistry (data not shown). Unlike K5-TSLP transgenic mice, no skin lesions developed as a result of TSLP injection, but there was a localized increase in edema and mild epithelial hyperplasia, as well as evidence of fibrosis by Masson's trichrome staining compared with control MSA-injected mice. Associated with the increase in fibrosis, there was an increase in IL-13 message in the skin of TSLP-injected mice, as well as an increase in IL-6 and MMP-9 protein levels (data not shown), all of which are suggested to play a role in remodeling and development of fibrosis (41, 42). TSLP-stimulated DC have been shown to induce production of the CCR4 ligands CCL17 and CCL22 (7, 11). Although a modest induction of CCL22 message was seen in the skin of mice receiving TSLP, little to no CCL17 message was detected. Injection of TSLP into CCR4-deficient mice resulted in a comparable or slightly exacerbated inflammation in the skin compared with WT mice, suggesting CCL17 and CCL22 are not critical in this model (data not shown). Injection of TSLP also induced class switching to produce prototypical Th2 Igs, IgE and IgG1, and resulted in many changes in the local DLN. DLN from TSLP-injected mice displayed lymphadenopathy and germinal center formation, increased expression of MHC class II, and increased production of the Th2 cytokines IL-4, IL-5, and IL-13. Contrary to what has been published for human cells, TSLP injection did not increase production of TNF-α, but rather elicited high amounts of IL-10 from restimulated DLN cells. This may reflect a difference in human TSLP biology compared with mouse. Alternatively, because we found that TNF-RI was not required for the development of TSLP-induced inflammation, TNF-α may not play a role in this model.

The dependence of TSLP-induced inflammation on T cells was somewhat surprising due to published data on the persistence of skin inflammation in K5-TSLP transgenic mice crossed to TCR-β KO mice (12). One possible explanation for this discrepancy is that sustained production of TSLP may potently activate myeloid cells

and granulocytes, thus overriding the need for T lymphocytes, whereas intermittent injection of TSLP is unable to achieve this level of activation. Additionally, we have observed that native TSLP is a more potent inducer of inflammation than recombinant protein (our unpublished observations) (3), and this may explain the disparity between the transgenic mice and our model. Finally, T cells bearing $\gamma\delta$ TCR are known to be present in the epidermis (43). Although it has been demonstrated that $\alpha\beta$, rather than $\gamma\delta$ T cells are required in an epicutaneous OVA model of AD (44), we cannot conclusively rule out the potential contribution of $\gamma\delta$ T cells in this TSLP-driven model of inflammation.

The requirement for T cells in this model suggests that downstream mediators of Th2 cells may be critical in the development of the TSLP-induced inflammation observed in this study. In support of this, IL-4 and IL-13 message levels are up-regulated in TSLP-injected skin, as is IL-4 protein concentration (data not shown). Additionally, there is an increase in IL-4 and IL-13 production from restimulated DLN cell cultures.

To determine the involvement of IL-4 and IL-13 signaling in the pathology induced by TSLP administration, we injected TSLP into mice deficient in IL-4, IL-13, IL-4Rα, or STAT6. Strikingly, in the absence of IL-4, IL-13, or their signaling components, IL-5 message is highly up-regulated in the skin of TSLP-injected mice compared with WT controls. The genes encoding IL-4 and IL-5 form a cluster along with IL-3 and GM-CSF on chromosome 11 in the mouse (45). Although IL-4 and IL-5 are often coexpressed in Th2 cells, it is likely that mechanisms exist to control their transcription independently of each other, and this increase in IL-5 message perhaps reflects compensatory production in the absence of IL-4 and IL-13.

A residual level of inflammation was also seen by histological analysis, suggesting some components of the TSLP-induced effects in this model are independent of IL-4- and IL-13-mediated activities. The fibrosis remaining in the STAT6 KO was surprising when compared with the results from the IL-4Rα KO. IL-13 has been suggested to have STAT6-independent activities, either through the recruitment of STAT3 to the intracellular domain of IL-13Rα1 (46) or through signaling via the IL-13Rα2. Long considered a decoy receptor, recent data suggest that IL-13 signaling through IL-13Rα2 may result in fibrosis via production of TGF-β (47).

Mast cells represented a small subset of the inflammatory infiltrate resulting from TSLP injection. Because TSLP can directly activate human mast cells (3) and recent data have suggested that mast cells play a primary rather than secondary role in the development of cutaneous disorders (48, 49), we injected TSLP into the skin of mast cell-deficient $Kit^{W\text{-}sh}$ mutant mice. We saw no amelioration of inflammation in the absence of mast cells, suggesting that they are not required in this model. Interestingly, there was a slight increase in eosinophil infiltrate in mast cell-deficient mice (data not shown) and slight increases in IL-5 message in skin as well as increased IL-5 production from restimulated DLN cells. These data, taken together with the data from the IL-4- and IL-13-deficient mice, strongly suggest a role for IL-5 and eosinophils in the pathology arising from TSLP administration. In agreement with this, increased circulating levels of IL-5 have been reported in transgenic mice systemically overexpressing TSLP (10). Injection of TSLP into eosinophil-deficient mice resulted in a striking loss of inflammatory infiltrate along with the loss of prototypical Th2 responses. This result was unanticipated based on the data from RAG2 KO and athymic nude mice, in which inflammation was also abrogated. IL-5, which is required to initiate development and release of eosinophils from the bone marrow (50), was reduced in the skin of T cell-deficient mice at both the mRNA and protein level. These data suggest that, in this model, both T cells and eosinophils must be present to generate TSLP-induced inflammation, and that the coordinated release of IL-5 by Th2 cells and cytotoxic granule proteins or other eosinophilic mediators accounts for the skin inflammation and systemic Th2 responses. Alternatively, eosinophils may be a primary cell type responsive to TSLP, similar to what has been shown for mast cells. In support of this, we have observed TSLP-R protein expression on tissue-infiltrating eosinophils in lung samples from mouse airway inflammation models and human asthma patient samples (our unpublished observations).

The active role of the epithelium in the initiation and regulation of mucosal inflammation has become increasingly clear. Emerging information about epithelial-derived cytokines such as IL-33, IL-25, and TSLP (51, 52) sheds further light on the immunological mechanisms underlying the contribution of the epithelium to the inflammatory process. TSLP has been shown to be involved in the initiation of the atopic phenotype, both in mouse models (12–14) and in skin sections from AD patients (7). In this study, we present a TSLP-driven mouse model of inflammation that exhibits many characteristics of human AD, including infiltrate of $CD4^+$ cells, eosinophils, and mast cells; production of Th2 cytokines; and class switching to produce IgE. Despite differences between this model and TSLP transgenic mice, intradermal injection of TSLP may provide useful insight toward elucidating the downstream mediators of TSLP activity and identification of target molecules for therapeutic intervention.

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Disclosures

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