

Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice

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The cytokine thymic stromal lymphopoietin (TSLP) has been linked to human allergic inflammatory diseases. We show here that TSLP expression was increased in the lungs of mice with antigen-induced asthma, whereas TSLP receptor-deficient mice had considerably attenuated disease. Lung-specific expression of a *Tslp* transgene induced airway inflammation and hyperreactivity characterized by T helper type 2 cytokines and increased immunoglobulin E. The lungs of *Tslp*-transgenic mice showed massive infiltration of leukocytes, goblet cell hyperplasia and subepithelial fibrosis. TSLP was capable of activating bone marrow-derived dendritic cells to upregulate costimulatory molecules and produce the T helper type 2 cell-attracting chemokine CCL17. These findings suggest that TSLP is an important factor necessary and sufficient for the initiation of allergic airway inflammation.

Asthma is a disease of dysregulated immunity characterized by chronic inflammation in the airways in response to a wide variety of stimuli, including airborne allergens, infectious agents, chemical irritants and others¹. The hallmarks of the disease include infiltration of the airways by leukocytes, varying degrees of subepithelial fibrosis, mucus hyperproduction, hypertrophy and hyperplasia of smooth muscle, goblet cell hyperplasia^{2,3} and, in most cases, increased serum immunoglobulin E (IgE)⁴. Studies have established a critical function for the T helper type 2 (T_H2) cytokines interleukin 4 (IL-4), IL-5, IL-9 and IL-13 in the asthmatic response. IL-4 is key in CD4⁺ T cell commitment to a T_H2 phenotype and the induction of IgE production⁵, whereas IL-5 regulates eosinophil development, activation and tissue recruitment⁶. IL-13 and IL-9 are important in mucus production and airway hyper-responsiveness^{7–9}.

Thymic stromal lymphopoietin (TSLP) was first identified as a growth-promoting factor in cultured supernatants of a thymic stromal cell line. As with the closely related cytokine IL-7, TSLP was initially recognized as a pre-B cell growth factor^{10,11}, differing from IL-7 in its ability to drive B cells to the B220⁺IgM⁺ immature B cell stage. However, other studies have suggested that TSLP can exert effects beyond the B cell lineage. Transgenic mice overexpressing TSLP under control of the proximal *Lck* promoter develop mixed cryoglobulins with renal involvement closely resembling cryoglobulinemic glomerulonephritis as well as systemic inflammatory diseases involving kidney, spleen, liver, lung and skin¹². *In vitro* studies with a human TSLP ortholog have shown that TSLP can stimulate the differentiation and activation of CD11c⁺ dendritic cells (DCs), inducing expression of the T_H2 cell-attracting chemokines CCL17 and CCL22 (ref. 13). In

addition, naive CD4⁺ T cells primed by TSLP-stimulated DCs produce the proallergic cytokines IL-4, IL-5, IL-13 and tumor necrosis factor while at the same time diminishing production of IL-10 and interferon- γ (IFN- γ) when restimulated¹³. Thus, TSLP seems to be capable of initiating an activation cascade that could lead to atopic inflammation and subsequent disease. In support of that model, TSLP expression is associated with acute and chronic atopic dermatitis in human patients¹³. In these patients, epidermal keratinocytes in inflamed skin express TSLP, whereas those in uninvolved skin do not. Expression of TSLP is also associated with the maturation and migration of local DCs.

Asthma is one of a triad of common allergic inflammatory diseases (along with atopic dermatitis and allergic rhinitis). These diseases have several features in common, including the presence of cellular infiltrates, increased serum IgE, T_H2 cytokines and the chemokine CCL17 (ref. 14). TSLP is normally expressed by epithelial cells in the lungs and skin^{15,16}. That finding along with the increased TSLP in the epithelial cells of atopic dermatitis patients¹³ suggest a general function for TSLP in allergic inflammatory disease. Here we show that TSLP was upregulated in the lungs of mice with antigen-induced airway inflammatory disease and that amounts of TSLP correlated with the extent of pulmonary leukocytic infiltration. TSLP receptor-deficient (*Tslpr*^{−/−}) mice had considerably reduced airway disease, as did, unexpectedly, *Tslpr*^{+/−} mice. To directly test the ability of TSLP to initiate airway inflammation, we generated mice that expressed TSLP specifically in lung epithelial cells under control of the promoter of the gene encoding human surfactant protein C. These mice developed an eosinophilic airway inflammatory disease with asthma-like characteristics such as

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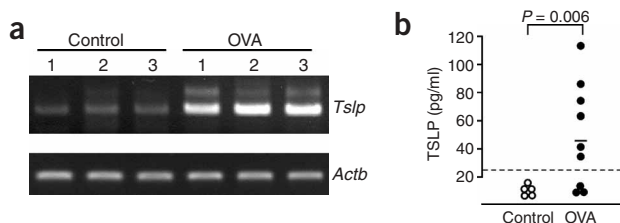


Figure 1 Upregulation of *Tslp* in the lungs of mice with OVA-induced airway inflammation. (a) RT-PCR detection of *Tslp* mRNA in the lung. Each lane represents an individual mouse. *Actb*, β -actin, 20 cycles (loading control). (b) ELISA detection of TSLP in BAL fluid. ELISA was done on the first 1-ml fraction of BAL fluid, with a detection limit of 25 pg/ml (dashed line). Small horizontal bar represents the average value of all nine mice. The three values below the detection limit were treated as 0. $P = 0.006$, two-tailed t-test.

T_H2 -biased $CD4^+$ T cell infiltration, eosinophilia, increased serum IgE, airway hyper-responsiveness and airway remodeling. These results indicate that TSLP is an important cytokine for the initiation of allergic inflammatory diseases such as asthma.

RESULTS

Tslp expression in a mouse asthma model

Ovalbumin (OVA)-induced murine models of allergic airway disease have helped to demonstrate the potential underlying mechanisms and have been important in identifying factors involved in the development of allergen-induced inflammation and airway hyper-responsiveness. To determine the function of TSLP in OVA-induced asthma, we assessed *Tslp* expression in the lungs of mice with OVA-induced airway inflammatory disease. Initially we isolated RNA from mice that we primed with OVA or PBS (control) and challenged with OVA. *Tslp* mRNA was upregulated only in the lungs of mice sensitized and intranasally challenged with OVA (Fig. 1a). We extended those studies by directly measuring TSLP in bronchoalveolar lavage (BAL) fluid from these mice by enzyme-linked immunosorbent assay (ELISA). In six of nine mice sensitized and intranasally challenged with OVA, we detected TSLP in the BAL

fluid, with a detection limit of 25 pg/ml (Fig. 1b). The amount of TSLP correlated well with the total cell counts in BAL fluid, such that mice with undetectable TSLP had the lowest cell counts and mice with high TSLP concentrations had high cell counts. None of the mice in the PBS-sensitized control group had detectable TSLP (Fig. 1b). These data indicate that TSLP expression is upregulated in a mouse model of asthma and suggest possible involvement of TSLP in disease development.

Involvement of TSLP signaling in a mouse asthma model

To investigate further the involvement of TSLP signaling in allergic inflammation, we sensitized and intranasally challenged groups of *Tslpr*^{-/-}, *Tslpr*^{+/-} and *Tslpr*^{+/+} mice with OVA. Total BAL fluid cell counts in *Tslpr*^{-/-} mice were reduced to about 15% of those in *Tslpr*^{+/+} mice (Fig. 2a). Although they demonstrated no significant difference in the percentage of lymphocytes in BAL fluid (data not shown), *Tslpr*^{-/-} mice sensitized and challenged with OVA had a significantly higher percentage of macrophages ($P < 0.05$) and lower proportion of eosinophils ($P < 0.05$) than did *Tslpr*^{+/+} mice (Fig. 2a). Furthermore, as in nonsensitized but OVA-challenged wild-type mice, alveolar macrophages from *Tslpr*^{-/-} mice were small and nonvacuolated, whereas those from *Tslpr*^{+/+} mice were large and highly vacuolated (Supplementary Fig. 1 online).

Lung histology showed that the larger airways and blood vessels of the *Tslpr*^{+/+} mice were lined with infiltrating leukocytes (Fig. 2b, arrowhead), whereas heterozygous *Tslpr*^{+/-} mice had an 'attenuated' phenotype with patches of leukocytes along the larger airways (Fig. 2b, arrowhead). Even more notably, there were only scattered leukocytes along the larger airways in the *Tslpr*^{-/-} mice sensitized and challenged with OVA. Similarly, goblet cell hyperplasia and, hence, mucus production were also reduced in *Tslpr*^{-/-} mice (Fig. 2b). We stained lung sections from mice of all genotypes with periodic acid-Schiff (PAS) stain. Whereas there was goblet cell hyperplasia in the large airways in all three sets of mice (data not shown), PAS⁺ cells in the medium-sized airways were very different in these mice. Medium-sized airways containing goblet cells were abundant in sections from *Tslpr*^{+/+} mice, rare in sections from *Tslpr*^{+/-} mice with reduced PAS-staining cells and absent from sections from *Tslpr*^{-/-} mice.

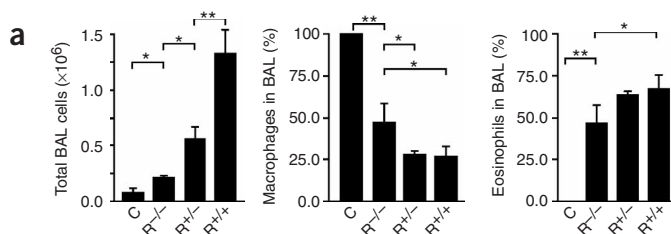
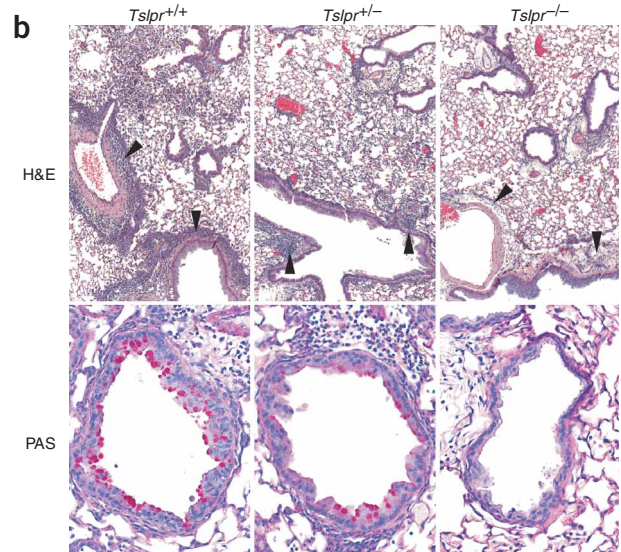


Figure 2 TSLP receptor knockout reduces airway inflammation and goblet cell hyperplasia in OVA-induced mouse asthma. (a) Total BAL fluid cells and percentage of macrophage and eosinophils in BAL fluid. C, nonsensitized but OVA-challenged wild type; *R*^{-/-}, *Tslpr*^{-/-}; *R*^{+/-}, *Tslpr*^{+/-}; *R*^{+/+}, *Tslpr*^{+/+}. Data represent mean $\pm$ s.d. ($n = 3-4$). $P = 0.00000489$ (left), $P = 0.031$ (middle), and $P = 0.038$ (right), one-way analysis of variance. *, $P < 0.05$, and **, $P < 0.01$, post-hoc Bonferroni's multiple comparison tests. (b) Lung histopathology of mice (genotypes, above images) sensitized and challenged with OVA. Lung sections stained with hematoxylin and eosin (H&E) show attenuated airway inflammation in *Tslpr*^{-/-} mice. Arrowheads indicate inflammatory cell infiltration along the larger airways and blood vessels. Lung sections stained with PAS show reduced mucus overproduction, so that there are no PAS⁺ cells in the medium-sized airways in *Tslpr*^{-/-} mice. Original magnification, $\times 5$ (hematoxylin and eosin) or $\times 20$ (PAS). Data represent one of two independent experiments.



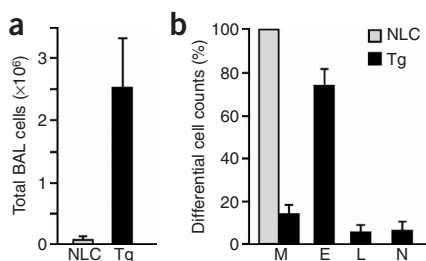


Figure 3 BAL fluid of *SPC-Tslp* mice has an eosinophilic infiltrate. Total counts (a) and differential counts (b) of inflammatory cells in BAL fluid from transgenic mice (Tg) and normal littermate control mice (NLC). Data represent mean $\pm$ s.d. ($n = 6$) and one of more than five independent experiments. M, macrophages; E, eosinophils; L, lymphocytes; N, neutrophils.

Characterization of mice transgenic for lung-specific *Tslp*

To directly test the function of TSLP in airway inflammation, we generated mice expressing a *Tslp* transgene under control of the promoter of the gene encoding surfactant protein C (called '*SPC-Tslp* mice' here). We selected one line and backcrossed it to the BALB/c strain for further analysis. This line had increased TSLP expression in the lung (10.48 ± 2.05 ng/ml of BAL fluid) but had no detectable TSLP in the serum (detection limit for this ELISA, 19.5 pg/ml). Consistent with this tissue-restricted expression, we did not find any of the systemic abnormalities associated with systemic overexpression of TSLP^{12,17}, such as altered B lymphopoiesis or myelopoiesis (Supplementary Fig. 2 online). In addition, whereas up to 80% of transgenic mice that systemically overexpressed TSLP¹⁷ died by 7 weeks of age and had substantial skin lesions, the *SPC-Tslp* mice were capable of living up to 7–8 months without any visible skin lesions before succumbing to lung failure.

Analysis of BAL fluid isolated from the lungs of *SPC-Tslp* mice demonstrated cellular infiltrates. The total number of cells collected from *SPC-Tslp* mice reached $2.5 \times 10^6 \pm 0.8 \times 10^6$ at 2 months of age, compared with $0.07 \times 10^6 \pm 0.04 \times 10^6$ in normal littermate control mice (Fig. 3a). Whereas leukocytes from the BAL fluid of normal littermate control mice were almost exclusively monocytes and/or macrophages (Fig. 3b), BAL fluid cells from *SPC-Tslp* mice were mainly eosinophils (75% of total BAL fluid cells). In addition, the macrophages from the *SPC-Tslp* mice were considerably enlarged and many were multinucleated (data not shown).

Further analysis of the cellular infiltrate in the *SPC-Tslp* mice by flow cytometry showed that most T lymphocytes were CD4⁺ (data not shown). We used intracellular staining to assess the cytokines produced by the CD4⁺ T cells from the lungs of *SPC-Tslp* mice. Although we consistently detected expression of IL-4 in over 30% CD4⁺ T cells from BAL fluid, IFN- γ -expressing cells seldom reached 10%

Figure 4 *SPC-Tslp*-transgenic mice have T_H2-biased inflammation. (a) IL-4, IL-5, IL-13 and IFN- γ production by CD4⁺ T cells isolated from BAL fluid and analyzed by intracellular cytokine staining. (b) IL-4 and IFN- γ production by CD4⁺ T cells isolated from BAL fluid, mediastinal (lung-draining) lymph nodes and peripheral (mixture of axillary and inguinal) lymph nodes and analyzed by intracellular cytokine staining. Numbers in top right corners indicate percentage of cells in each quadrant. Data in a,b represent more than five independent experiments. (c) Serum IgE concentrations in *SPC-Tslp*-transgenic mice (Tg) and normal littermate control mice, measured by ELISA with a detection limit of 30 ng/ml. Small horizontal bar represents the mean value of ten mice. The two values below the detection limit were treated as 0. $P = 0.0176$, two tailed t -test.

(Fig. 4a,b). CD4⁺ T cells from the BAL fluid of these mice also produced IL-5 and IL-13, although at lower frequency than IL-4, consistent with a T_H2 phenotype (Fig. 4a). The increased frequency of IL-4-expressing CD4⁺ T cells was limited mainly to the lung and lung-draining lymph nodes, as skin-draining lymph nodes had a staining pattern similar to that of normal controls (Fig. 4b).

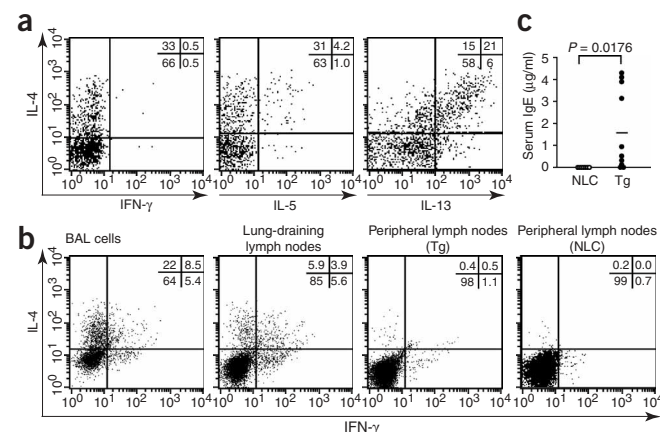
In addition to the mouse line described above, we generated several additional founder lines. Three of these founders had much more deleterious phenotypes; one founder mouse died at 7 weeks of age and two founder mice developed difficulty breathing, with heavy reliance on abdominal musculature, at 11 weeks of age. We killed the last two mice and did a preliminary analysis. BAL fluid from these mice had a substantial cellular infiltrate, with 1.8×10^7 to 4.0×10^7 total cells, compared with 0.1×10^5 to 1.0×10^5 total cells for the age-matched wild-type mice. Further examination of the BAL fluid showed that it contained increased IL-4 and IgE but no IFN- γ (data not shown), supporting the conclusion that expression of TSLP in the lungs of these mice resulted in a robust T_H2-type response.

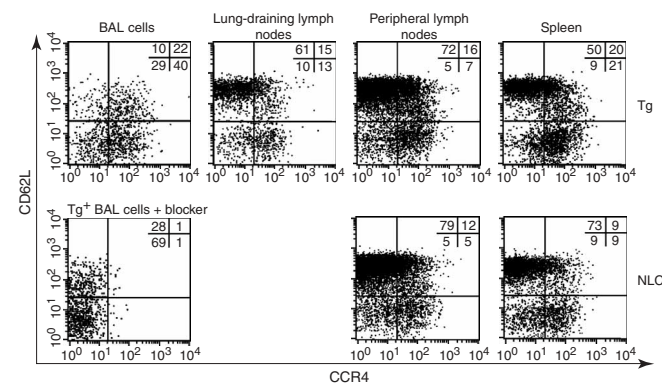
Increased serum IgE in *SPC-Tslp* mice

Allergic inflammation is typically accompanied by increased serum IgE, whose synthesis heavily relies on the combined effects of T_H2 cytokines¹⁸. Thus, we also measured total IgE in the sera of many transgenic mice. Whereas the IgE of control mice was below the limit of detection (30 ng/ml), *SPC-Tslp* mice had much more serum IgE, approaching 5 μ g/ml in some mice (Fig. 4c). Thus, expression of the lung-specific *Tslp* transgene led to a T_H2 environment similar to that in human patients with asthma.

Lung-infiltrating CD4⁺ T cells express CCR4

Chemokine receptor expression by CD4⁺ T cells from *SPC-Tslp* mice was also consistent with their T_H2 status. We used a CCL22-IgG3 fusion protein to probe expression of the CCL22 and CCL17 chemokine receptor CCR4, which is expressed by nearly all IL-4-producing CD4⁺ T cells in normal humans¹⁹ and is found on most BAL fluid CD4⁺ T cells isolated from human asthmatics²⁰. Accordingly, most (62%) CD4⁺ T cells from the BAL fluid of the *SPC-Tslp* mice were CCR4⁺ (Fig. 5). Additionally, compared that of normal littermate control mice, the frequency of CD4⁺CCR4⁺ T cells was increased in both the spleens and lung-draining mediastinal lymph nodes of the transgenic mice. In contrast, CCR4 expression by CD4⁺ T cells in skin-draining peripheral lymph nodes was similar to that in normal control mice, emphasizing the tissue specificity of the TSLP-induced T cell response. A large fraction of the CCR4⁺ CD4⁺ T cells in BAL fluid





were CD62L⁺, and the increase in CCR4⁺ expression was paralleled by a decrease in CD4⁺ T cell expression of CCR7 (Fig. 5 and Supplementary Fig. 3 online). These results indicate that the CD4⁺ T cells that infiltrated the lungs of *SPC-Tslp* transgenic mice had acquired a phenotype characteristic of fully differentiated T_H2 effector cells.

Lungs of *SPC-Tslp* mice show airway remodeling

To evaluate the histopathology of the lungs from *SPC-Tslp* mice, we collected lungs from normal littermate control and transgenic mice 3 month of age or older and fixed, sectioned and stained tissue samples. Histological analysis demonstrated substantial peribronchiolar and perivascular inflammatory infiltrates in the lungs of the transgenic mice that were not present in those of normal littermate control mice (Fig. 6a–d). These cells were mainly mononuclear in nature and included many plasma cells and lymphocytes, with fewer macrophages and rare multinucleated giant cells. In more severe cases, there were many eosinophilic crystal-laden macrophages (Fig. 6d). There were also mast cells in the vicinity of airways (Fig. 6e) and blood vessels (Fig. 6f) in lung tissues stained with metachromatic Giemsa stain.

Remodeling of the conducting and small airways of the lung is a common feature of patients with asthma^{2,3}. This remodeling takes the form of epithelial cell hyperplasia, subepithelial fibrosis, mucus metaplasia and increased smooth muscle mass. Examination of the hematoxylin and eosin-stained sections of severely affected lungs isolated from transgenic mice showed focal hyperplasia of bronchiolar epithelial cells (Fig. 6c,d). This hyperplastic change was often accompanied by goblet cell hyperplasia, as demonstrated in PAS-stained sections (Fig. 6g). Another prominent histological feature of more

Figure 5 Lung-infiltrating CD4⁺ T cells upregulate CCR4 while downregulating CD62L. Expression of CCR4 and CD62L by CD4⁺ T cells from the BAL fluid, mediastinal lymph nodes, peripheral lymph nodes and spleens of *SPC-Tslp* (Tg⁺) mice as well as from the peripheral lymph nodes and spleen of normal littermate control mice was analyzed by flow cytometry. Bottom left, specificity of CCL22-Fc fusion staining (cells of BAL fluid from transgenic mice blocked with monoclonal antibody to CCL22). An absence of T cells in BAL fluid and too few cells in lung-draining lymph nodes prevented a similar assessment of normal littermate control mice. Numbers in top right corners indicate percentage of cells in each quadrant. Data represent one of three independent experiments.

severely affected lungs was the presence of fibroplasia, which in sections stained with Masson's trichrome was shown to surround airways and vessels (Fig. 6h). All the abnormalities in the lungs of *SPC-Tslp*-transgenic mice, including infiltration of eosinophils, lymphocytes and mast cells, epithelial cell hyperplasia, subepithelial fibrosis and mucus metaplasia, were not present in the lungs of normal littermate control mice (Supplementary Fig. 4 online).

Airway hyper-responsiveness in *SPC-Tslp* mice

To determine if lung-specific expression of TSLP resulted in airway hyper-responsiveness, we obtained enhanced pause (P_{enh}) measurements both basally and in response to increasing doses of inhaled methacholine. The *SPC-Tslp* mice developed considerable airway hyper-responsiveness in response to even the lowest dose of methacholine (2.5 mg/ml). The maximum P_{enh} of *SPC-Tslp* mice was considerably higher than that of normal littermate control at all doses tested (Fig. 7). Additionally, in response to all doses of methacholine, but not during challenge with PBS alone, *SPC-Tslp* mice developed labored breathing with considerable reliance on abdominal musculature, which normal littermate control mice did not, even in response to higher doses of methacholine. Therefore, increased TSLP expression in the lung led to spontaneous airway hyper-responsiveness.

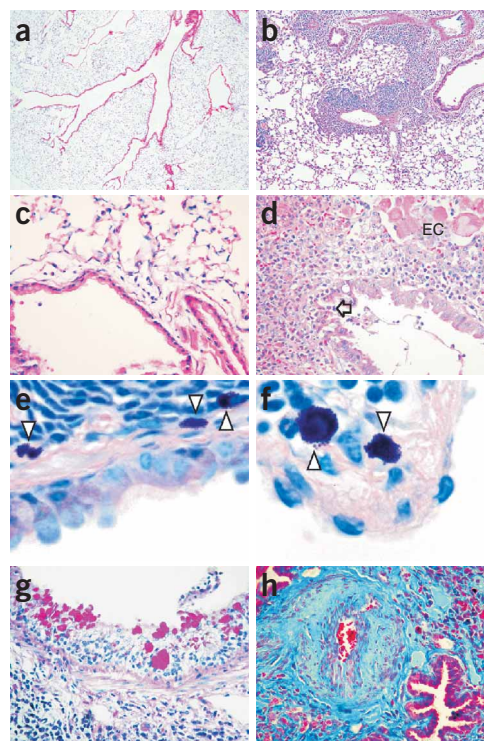


Figure 6 Lungs from *SPC-Tslp* mice have inflammatory infiltrates and show signs of airway remodeling. (a–d) Hematoxylin and eosin staining of lung sections from normal littermate control mice (a,c) and transgenic mice (b,d). (a,b) Low-power magnification shows perivascular and peribronchiolar accumulation of dark-staining inflammatory cells. (c,d) High-power magnification shows the accumulation of mixed inflammatory cells near a focal disruption of bronchiolar epithelium (open arrow). There is also a small cluster of eosinophilic crystal-laden macrophages (EC). (e,f) Giemsa staining of transgenic mouse lung shows mast cells (open arrowheads) in the vicinity of airways (e) and blood vessels (f). With higher magnification (f), dark purple-stained mast cell granules are visible. (g) PAS staining of transgenic mouse lung shows goblet cell hyperplasia and mucus overproduction. (h) Masson's trichrome staining shows considerable vascular and perivascular fibrosis accompanied by medial hypertrophy. The lumen of this thickened vessel is identified by the presence of red blood cells. Original magnification, $\times 10$ (a,b), $\times 100$ (f) or $\times 40$ (c–e,g,h). Data represent more than five independent experiments.

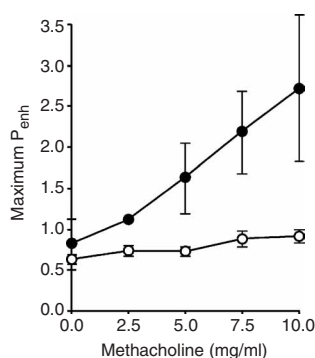


Figure 7 Airway responsiveness. Whole-body plethysmography. P_{enh} measurements of airway hyperreactivity in unrestrained mice were made basally and in response to increasing doses of methacholine. Data represent mean $\pm$ s.d. ($n = 6$) and one of two independent experiments. $P = 0.0269$, transgenic versus normal littermate controls mice, and $P = 0.0012$, comparison of methacholine doses (two-way repeated-measures analysis of variance).

TSLP activates bone marrow-derived DCs

In humans, TSLP can activate DCs and thus may be key in the induction of allergic dermatitis¹³. We found that mouse bone marrow-derived DCs (BMDCs) expressed both the TSLP receptor and IL-7 receptor- α (IL-7R α), the two receptor subunits that constitute the functional TSLP receptor complex (Fig. 8a). When cultured in the presence of TSLP for 48 h, BMDCs produced the T_H2 cell-attracting chemokine CCL17 in a dose-dependent way (Fig. 8b). TSLP-induced CCL17 production was IL-7R α dependent and was blocked by a TSLP-neutralizing antibody (data not shown). Additionally, the expression of major histocompatibility complex class II and costimulatory molecules such as CD40, CD80 and CD86 on BMDCs was increased in response to 20 ng/ml of TSLP (Fig. 8c). This suggests that TSLP is sufficient for mediating the qualitative alteration of BMDCs to a more mature phenotype. Similarly, expression of these costimulatory molecules on CD11c⁺ cells derived from lungs of SPC-Tslp mice was upregulated compared with that of normal littermate

control mice (data not shown). These data suggest that TSLP is capable of inducing DC maturation in mice as well as in humans.

DISCUSSION

In humans, asthma, atopic dermatitis and allergic rhinitis form the triad of common atopic IgE-dependent diseases²¹. Patients suffering from one member of the triad often show symptoms of one or both of the other members, suggesting a common genetic or initiating element in these diseases. Published work has shown that the cytokine TSLP may be critically involved in initiating the pathogenesis of atopic dermatitis¹³. Several lines of evidence from our work here have demonstrated involvement of TSLP in the pathogenesis of asthma. TSLP expression was upregulated in mice sensitized and challenged with OVA. Furthermore, both Tslpr^{+/-} and Tslpr^{-/-} mice showed a considerable reduction in disease severity when sensitized and challenged with OVA. Finally, mice expressing a Tslp transgene in lung epithelial cells developed a spontaneous airway inflammatory disease with the cardinal features of human asthma. Collectively these data indicate that TSLP is both necessary and sufficient for the development of allergic inflammatory disease in the lungs.

The data presented here are consistent with the initiation of an inflammatory cascade by TSLP that eventually leads to the pathology seen in asthmatics. We propose the following model as a way to explain the involvement of TSLP in the induction of the allergic phenotypes observed. Airway epithelial cells respond to an environmental stimulus (such as an allergen) by producing TSLP. Local CD11c⁺ DCs, responding to TSLP, differentiate, express CCL17 and CCL22 to attract T cells with a T_H2 bias, and migrate to the draining lymph node. CD4⁺ T cells that are subsequently primed by these TSLP-activated DCs differentiate into IL-4-producing T_H2 cells, traffic back to the lung and trigger allergic inflammation through the production of additional cytokines and chemokines. The presence of these mediators leads to the infiltration of additional inflammatory cells (neutrophils, eosinophils and mast cells) and the production of IgE by B cells, establishing a chronic inflammatory cycle that gives rise to disease pathology (such as airway obstruction through mucus overproduction and subepithelial fibrosis). Our data support the central tenants of this model: TSLP was upregulated in the lungs

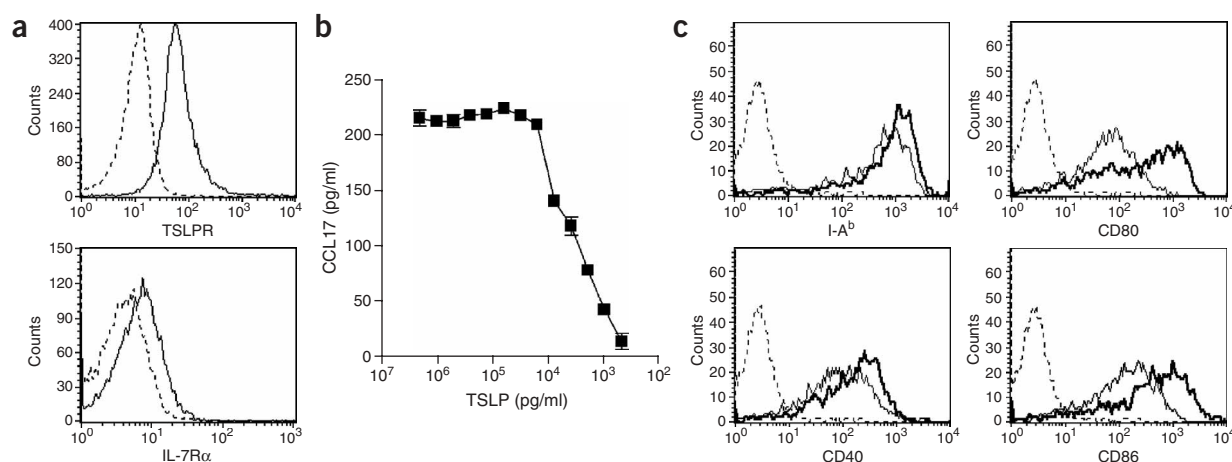


Figure 8 Activation of BMDCs by recombinant mouse TSLP. (a) Expression of a functional TSLP receptor complex, TSLP receptor (TSLPR) and IL-7R α , on BMDCs. Dotted lines, isotype control; solid lines, antibody stain. (b) Expression of CCL17 by BMDCs after stimulation *in vitro* with graded doses of TSLP, measured by ELISA of supernatants at 48 h. (c) Expression of major histocompatibility complex class II and costimulatory molecules on DCs is upregulated in response to TSLP. BMDCs were stimulated *in vitro* with 20 ng/ml of TSLP for 48 h and surface expression of I-A^b, CD40, CD80 and CD86 was assessed by flow cytometry. Dotted lines, isotype control; thin lines, DCs cultured without TSLP; thick lines, DCs cultured in TSLP. Data represent one of more than five independent experiments.

in an OVA-induced mouse allergic asthma model; *Tslpr*^{-/-} mice had considerably reduced antigen-induced airway inflammation; expression of TSLP by lung epithelial cells led to spontaneous airway inflammation; and BMDCs responded to TSLP treatment by producing CCL17 and upregulating costimulatory molecules.

T_H2 -dominated inflammation has been regarded as a cornerstone of the asthmatic diathesis, as more IL-4 and IL-5 have been detected in bronchial biopsies, BAL fluid and blood of asthmatic patients than in those of normal people²². Murine models of pulmonary inflammation and asthma have elegantly demonstrated that T_H2 cells and their cytokines are essential for the development of asthmatic features, including lung eosinophilia, mucus hypersecretion, mast cell hyperplasia and bronchial hyper-responsiveness²³. The increase in T_H2 CD4⁺ cells and their products in the lungs of the *SPC-Tslp* mice is consistent with involvement of this cytokine in driving T_H2 cell differentiation *in vivo*. In addition to its involvement in T_H2 cell differentiation and recruitment via DC activation, mouse TSLP preferentially stimulates the proliferation and survival of CD4⁺ single-positive thymocytes and peripheral T cells²⁴. We have found that TSLP can directly promote the T_H2 differentiation of purified CD4⁺ T cells cultured *in vitro* (M. Omori and S.F.Z., unpublished data). Therefore, TSLP may affect the development of T_H2 immune responses at multiple stages through its effects on DCs and CD4⁺ T cells.

Our analysis of homing receptors suggests that the pathogenic CD4⁺ T cells in *SPC-Tslp* mice express the T_H2 -associated chemokine receptor CCR4 and have lost the lymph node homing-receptors CD62L and CCR7. This phenotype was particularly prevalent in BAL fluid and resembled the expression pattern of CCR4 and CCR7 reported for CD4⁺ T cells in the BAL fluid of asthma patients^{20,25}. The CCR4 ligands CCL22 and CCL17 are both present in the BAL fluid of asthma sufferers²⁶, and we found substantial expression of CCL17 and CCL22 in the lungs of *SPC-Tslp* mice (data not shown). These data collectively suggest that CCR4 and its ligands are important in the recruitment of T_H2 cells to the lung in *SPC-Tslp*-transgenic mice.

Transgenic mouse models with expression of T_H2 cytokines have been used extensively to study the functions of cytokines in pulmonary pathophysiology^{9,27–30}. Because the expression of T_H2 cytokines is a consequence of T_H2 polarization, those transgenic mice probably cannot be used to address the crucial events that occur before T_H2 polarization in the pathogenesis of asthma. In contrast, TSLP is a factor that probably acts early in the pathogenesis of allergy because of its ability to activate DCs, a key event for the initiation of allergic sensitization and disease³¹. The generation of a complete asthma-like phenotype in *SPC-Tslp* mice, including airway inflammation, airway remodeling and airway hyper-responsiveness, serves as strong evidence for the involvement of TSLP in the development of allergic asthma.

METHODS

Animals. Wild-type BALB/c mice were purchased from Jackson Laboratories. *Tslpr* knockout mice³² were backcrossed to BALB/c for seven generations. All animals were housed in specific pathogen-free conditions in the Benaroya Research Institute animal facility, and all experiments were done as approved by the Benaroya Research Institute Institutional Animal Care Committee (Seattle, Washington).

OVA sensitization and airway challenge. Groups of mice 8–10 weeks of age were sensitized on days 1 and 14 by intraperitoneal injection of 50 µg OVA (grade V; Sigma) emulsified in 1.3 mg aluminum hydroxide gel (Sigma) in a total volume of 200 µl. For nonsensitized control mice, PBS plus aluminum hydroxide gel without OVA was injected. Anesthetized mice were challenged intranasally with 150 µg OVA in 40 µl PBS for 4 d starting from day 21. Mice were analyzed 24 h after the last challenge.

Generation of transgenic mice. Mouse *Tslp* cDNA was cloned into a construct containing the promoter of the gene encoding human surfactant protein C as well as SV40 small T intron plus a polyadenylation sequence (a gift from J. Whitsett, Children's Hospital Medical Center, Cincinnati, Ohio). The resulting *SPC-Tslp* plasmid was purified with a Plasmid Maxi kit (Qiagen). After digestion of the plasmid with *NdeI* and *NotI*, the *SPC-Tslp* transgene was separated by electrophoresis and was purified with a QIAquick Gel Extraction kit (Qiagen) before injection into [(C3H × C57BL/6 F₁) × C57BL/6] F₂ eggs. Transgene-positive founder mice were identified by PCR analysis of DNA from tail tissue, using primers for *Tslp* (5'-GACAGCATGGTCTCTCTCAG-3' and 5'-CTGGAGATTGCATGAAGG-3'). The established lines were backcrossed to BALB/c mice for at least six generations.

BAL, tissue fixation and staining. Mice were killed by intraperitoneal injection of an overdose of avertin. The lungs were subjected to BAL five times with 1 ml of PBS instilled via a tracheal polyethylene catheter. The first BAL fraction was centrifuged at 1,400g for 5 min and the supernatant was used to measure cytokine expression. The pellet was pooled with the subsequent four lavages. BAL fluid cells were resuspended in PBS plus 1% BSA and were counted with a hemocytometer. Differential cell counts were made using cytospin cell preparations stained with a modified Wright-Giemsa stain on a Hematek 2000 slide stainer (Bayer Corp, Diagnostics Division).

After lavage, lungs were excised completely from the chest, were inflated with 10% neutral buffered formalin (Fisher BioTech) and were fixed in the same solution overnight at 25 °C. Tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin, Giemsa stain, Masson's trichrome and PAS.

RNA isolation and RT-PCR. After excision, tissues were placed in liquid nitrogen and crushed with a mortar and pestle. Total RNA was then isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA was further purified with a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions.

Reverse transcription used 1 µg total RNA for first-strand cDNA synthesis with SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen) in a total volume of 20 µl. A portion of the resulting reverse-transcription product (1 µl) was used for PCR amplification. The primers used for *Tslp* were same as those used for genotyping. PCR conditions to amplify *Tslp* were 95 °C for 4 min for initial denaturing and followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

ELISA. IgE ELISA reagents were purchased from BD Biosciences Pharmingen. CCL17 ELISA reagents were purchased from R&D Systems. ELISA was done according to the manufacturer's instructions.

TSLP was quantified in BAL fluid and serum samples by TSLP-specific ELISA. The 96-well plates were coated in PBS overnight at 4 °C with 1 µg/ml of monoclonal antibody to mouse TSLP (2H7; Amgen). The coating solution was discarded and plates were blocked for 1 h at 25 °C in 0.05% Tween-20 in PBS (PBST) plus 10% nonfat dry milk. Sera samples were diluted in PBST plus 5% normal mouse sera (Bioreclamation). BAL samples were diluted in PBST. The blocking solution was discarded and plates were washed six times in PBST. Samples were added to plates, which were incubated for 1 h at 25 °C and then washed again. Biotinylated antibody to mouse TSLP (anti-mouse TSLP; BAF555; R&D Systems) was then added at a concentration of 100 ng/ml, followed by incubation for 1 h at 25 °C and then another wash. Poly80 HRP:Streptavidin (Research Diagnostics) was diluted 1:5,000 in PBST, then was added to plates, followed by incubation for 30 min at 25 °C. Plates were washed and a 1:1 mixture of TMB Peroxidase Substrate plus Peroxidase Solution B (Kirkegaard and Perry Laboratories) was added to the plates for 10 min at 25 °C. Color development was stopped with 2 N H₂SO₄ and plates were 'read' at 450 nm.

Flow cytometry. For the detection of cytokine production at the single-cell level, intracellular staining was done as described²⁷ with the minor modification that monensin was included throughout the stimulation. As a negative control, cells were also cultured in the presence of monensin but without stimulation with phorbol 12-myristate 13-acetate and ionomycin. All conjugated monoclonal antibodies used in this experiment were purchased from eBiosciences except biotinylated goat anti-mouse IL-13 (R&D systems).

Chemokine-IgG3 fusion proteins were used for flow cytometry of CCR4 and CCR7 expression. The cDNA encoding the mature form of mouse CCL22 (amino acids 25–91) or CCL19 (amino acids 26–108) was cloned in-frame downstream of the human CD5 signal sequence and upstream of the 'hinge' region of human IgG3 in the Peak13 expression vector. Protein was produced by transient transfection of these plasmids into COS7 cells by DEAE-dextran. Supernatants from transfected cells were used directly for lymphocyte staining, and fusion protein binding was detected with phycoerythrin-conjugated F(ab')₂ fragments of goat anti-human IgG (Jackson ImmunoResearch). As a specificity control, staining was blocked by the preincubation of protein with monoclonal blocking anti-CCL22 (clone 158132) or anti-CCL19 (clone 87102) (R&D Systems). CD62L expression was assessed with allophycocyanin-conjugated anti-CD62L (clone MEL-14; eBiosciences).

For BMDCs, cells were stained in flow cytometry buffer (PBS containing 2% FBS, 1% normal rat serum, 1% normal hamster serum, 1% normal mouse serum and 10 mg/ml of monoclonal antibody 2.4G2 (rat anti-mouse Fc receptor)). Monoclonal antibodies to CD11c (HL3), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), CD127 (IL-7Ra, SB/199) and I-Ab (AF6-120.1) were purchased from Caltag. Monoclonal antibody to the TSLP receptor (114704) was purchased from R&D Systems.

Physiological evaluation. P_{enh} measurements of airway hyperreactivity in unrestrained mice were made basally and in response to increasing doses of aerosolized methacholine in PBS by whole-body plethysmography (Buxco Electronics) as described³³. Each methacholine dose was given over a 2.5-minute period and the maximum P_{enh} was measured during the subsequent 10-minute period. The mice were measured for 3 consecutive days and results from three readings were averaged.

Mouse BMDC cultures. Mouse BMDCs were obtained as described³⁴. Bone marrow cells were cultured for 10 d in McCoy's medium supplemented with 200 ng recombinant human Flt3L (Amgen), essential and nonessential amino acids, 1 mmol/l of sodium pyruvate, 2.5 mmol/l of HEPES buffer, pH 7.4, vitamins, 55 nmol/l of 2-mercaptoethanol, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 0.3 mg/ml of L-glutamine and 10% FBS. *In vitro* activation of the DCs from Flt3L-supplemented cultures was achieved by the addition of various concentrations of recombinant mouse TSLP (R&D Systems). The supernatant was collected 48 h after culture inception and was analyzed by ELISA for CCL17 content, and the expression of surface molecules was assessed by flow cytometry.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Immunology website for details).

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