

Reversal of Thymic Stromal Lymphopoietin-Induced Airway Inflammation through Inhibition of Th2 Responses¹

Baohua Zhou,^{2,3*} Mark B. Headley,^{*†} Theingi Aye,^{*} Joel Tocker,[‡] Michael R. Comeau,[‡] and Steven F. Ziegler^{3*†}

Lung-specific thymic stromal lymphopoietin (TSLP) expression is sufficient for the development of an asthma-like chronic airway inflammatory disease. However, the nature of the downstream pathways that regulate disease development are not known. In this study, we used IL-4- and Stat6-deficient mice to establish the role of Th2-type responses downstream of TSLP. IL-4 deficiency greatly reduced, but did not eliminate, TSLP-induced airway hyperresponsiveness, airway inflammation, eosinophilia, and goblet cell metaplasia, while Stat6 deficiency eliminated these asthma-like symptoms. We further demonstrate, using the chronic model of TSLP-mediated airway inflammation, that blockade of both IL-4 and IL-13 responses, through administration of an anti-IL-4R α mAb, reversed asthma-like symptoms, when given to mice with established disease. Collectively these data provide insight into the pathways engaged in TSLP-driven airway inflammation and demonstrate that simultaneous blockade of IL-4 and IL-13 can reverse established airway disease, suggesting that this may be an effective approach for the therapy of Th2-mediated inflammatory respiratory disease. *The Journal of Immunology*, 2008, 181: 6557–6562.

Asthma is a multifactorial disease characterized by chronic inflammation of the lungs in response to aerosol allergens. The hallmarks of the disease include infiltration of the bronchial mucosa by leukocytes, varying degrees of subepithelial fibrosis, mucus hyperproduction, goblet cell metaplasia, and pronounced elevation in serum IgE (1). Studies over the past few years have established critical roles for CD4⁺ Th2 cells and Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 in the asthmatic response. IL-4 plays a key role in CD4⁺ T cell commitment to a Th2 phenotype and the induction of IgE production (2). IL-5 is known to promote the differentiation, maturation, and endothelial adherence (thus tissue recruitment) of eosinophils (3, 4). IL-13 and IL-9 control mucus production and airway hyperreactivity (AHR)⁴ (5–7).

Several lines of evidence demonstrate that thymic stromal lymphopoietin (TSLP) is a critical and essential factor for allergic inflammation (8, 9). In humans, TSLP treatment of dendritic cells (DCs) leads to their functional maturation, and naive CD4⁺ T cells that are primed by these DCs take on an inflammatory Th2 phe-

notype, producing IL-4, IL-5, IL-13, and TNF- α (10, 11). Furthermore TSLP-activated human CD11c⁺ DCs play important roles in the maintenance and further polarization of Th2 central memory cells in allergic diseases (8). Consistent with a role in allergic inflammation, TSLP expression levels are increased in the lesional skin of atopic dermatitis patients (10) and in the lungs of asthmatics (12).

In mice, TSLP is both necessary and sufficient to initiate allergic airway inflammation (13, 14). For example, TSLP is up-regulated in the lungs of mice in an Ag-driven model of airway inflammation, and mice that express a lung-specific TSLP transgene (SPC-TSLP mice) develop an airway disease similar to human asthma (13). Similarly, mice that express a skin-specific TSLP transgene develop a spontaneous inflammatory disease of the skin similar to human atopic dermatitis (15, 16). Concomitant with disease development in all these animals was a robust Th2 response. Additionally, mice lacking the TSLP receptor fail to develop airway inflammation in an Ag-induced mouse model of airway disease (13, 14).

Although it is becoming clear that TSLP is an important determinant in allergic inflammation, the downstream mediators that are triggered by TSLP remain to be determined. In this report, the role of Th2 responses in TSLP-induced airway inflammation was assessed using SPC-TSLP mice. IL-4-deficient SPC-TSLP mice displayed markedly attenuated disease development, including no significant AHR, while Stat6-deficient SPC-TSLP mice were devoid of airway inflammation and remodeling. Furthermore, treatment of mice with an Ab specific for IL-4R α and capable of blocking both IL-4 and IL-13 biologic activity was able to reverse the TSLP-induced airway hyperresponsiveness and greatly reduce airway inflammation and remodeling. Taken together, these results indicate that intact Th2 responses are an essential downstream element in the TSLP-induced pathogenesis of asthma-like airway inflammation and simultaneous blockade of IL-4 and IL-13 may be an effective approach for the therapy of Th2-mediated inflammatory respiratory disease.

Materials and Methods

Animals

BALB/c mice were purchased from The Jackson Laboratory. IL-4^{-/-} and Stat6^{-/-} mice were also purchased from The Jackson Laboratory and then

*Immunology Program, Benaroya Research Institute, Seattle, WA 98101; [†]Department of Immunology, University of Washington School of Medicine, Seattle, WA 98196; and [‡]Department of Inflammation, Amgen, Seattle, WA 98119

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² Current address: Department of Pediatrics, H. B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202.

³ Address correspondence and reprint requests to Dr. Baohua Zhou, Department of Pediatrics, H. B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202, or Dr. Steven F. Ziegler, Benaroya Research Institute, 1201 9th Avenue, Seattle, WA 98101. E-mail addresses: zhouba@iupui.edu or sziegler@benaroyaresearch.org

⁴ Abbreviations used in this paper: AHR, airway hyperreactivity; DC, dendritic cell; TSLP, thymic stromal lymphopoietin; BAL, bronchoalveolar lavage; PAS, periodic acid Schiff; MAP, MultiAnalyte profiling; P_{enh}, enhanced pause; RIgG, rat IgG.

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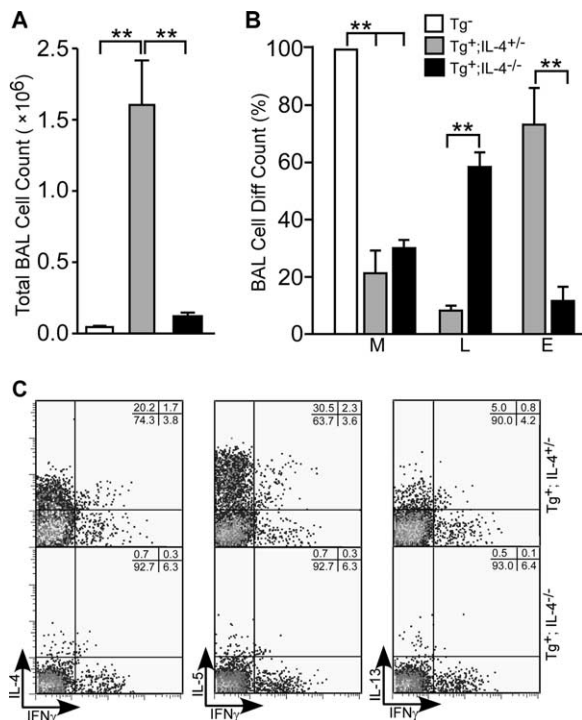


FIGURE 1. IL-4 deficiency greatly reduced TSLP-induced airway inflammation and Th2 polarization. **A**, Total cell counts in the BAL ($n = 4$). **B**, Differential cell counts in the BAL ($n = 4$). M, Macrophage; L, lymphocyte; E, eosinophil. **C**, Intracellular cytokine staining of CD4⁺ T cells in BAL from IL-4^{-/-}/SPC-TSLP mice showing that these cells failed to differentiate a Th2 phenotype with no detectable IL-4, IL-5, and IL-13 expression. Plots represent one of three independent experiments. **, Significant difference ($p < 0.01$, ANOVA test with Bonferroni posttests).

subsequently bred to SPC-TSLP transgenic mice (13) under specific pathogen-free conditions in the Benaroya Research Institute animal facility. All experiments were performed as approved by the Benaroya Research Institute Institutional Animal Care Committee.

Bronchoalveolar lavage (BAL), tissue fixation, and staining

Mice were euthanized by i.p. injection of a lethal dose of avertin. The lungs were subjected to BAL four times with 1 ml of PBS through a tracheal polyethylene catheter. The first BAL fraction was centrifuged at $1400 \times g$ for 5 min and the supernatant was used in MultiAnalyte profiling (MAP) cytokine analysis (see below). The pellet was pooled with the subsequent three lavages. BAL fluid cells were resuspended in PBS plus 1% BSA and counted. Differential cell counts were performed using cytospin cell preparations stained with a modified Wright-Giemsa stain on a Hematek 2000 slide stainer (Bayer).

After lavage, lungs were excised completely from the chest cavity, inflated with 10% neutral buffered formalin (Fisher BioTech) and fixed in the same solution overnight at room temperature. Tissues were embedded in paraffin, sectioned, and stained with H&E and periodic acid Schiff (PAS).

Cytokine profile of BAL fluid by MAP analysis

Samples of the first BAL fluid fraction (see above) were submitted for quantitative MAP analysis at Charles River Laboratories following the recommended procedure for BAL fluid.

Intracellular staining and FACS analysis

To examine Th2 cytokine expression by the CD4⁺ T cells in BAL fluid, intracellular staining was performed as described previously (13). After staining, cells were analyzed by FACS (BD Biosciences).

Evaluation of airway hyperresponsiveness

Enhanced pause (P_{enh}) measurements of AHR in unrestrained mice were made basally and in response to increasing doses of aerosolized methacholine (Sigma-Aldrich) in PBS using whole body plethysmograph (Buxco

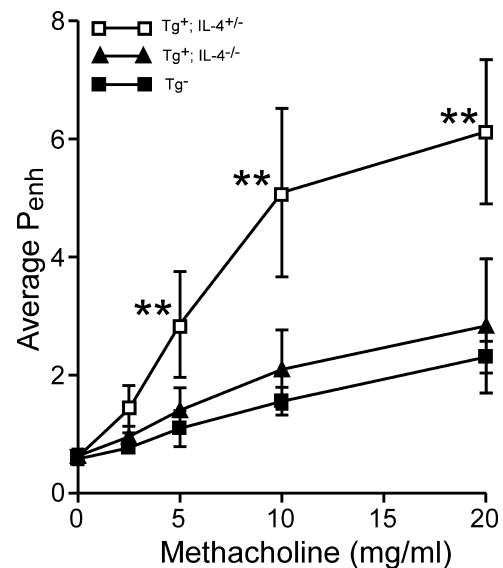


FIGURE 2. IL-4^{-/-}/SPC-TSLP mice do not develop significant AHR. Airway responsiveness of animals was assessed by P_{enh} reading in response to increasing dose of methacholine. Data represent mean $\pm$ SD ($n = 4-5$). **, Significant difference ($p < 0.01$, two-way repeated measures ANOVA with Bonferroni posttests) between IL-4^{+/-}/SPC-TSLP mice and either IL-4^{-/-}/SPC-TSLP mice or transgene negative littermate controls.

Electronics) as previously described with slight modification (13). Each methacholine dose was given over a 3-min period and the average P_{enh} value was measured during the following 5-min period.

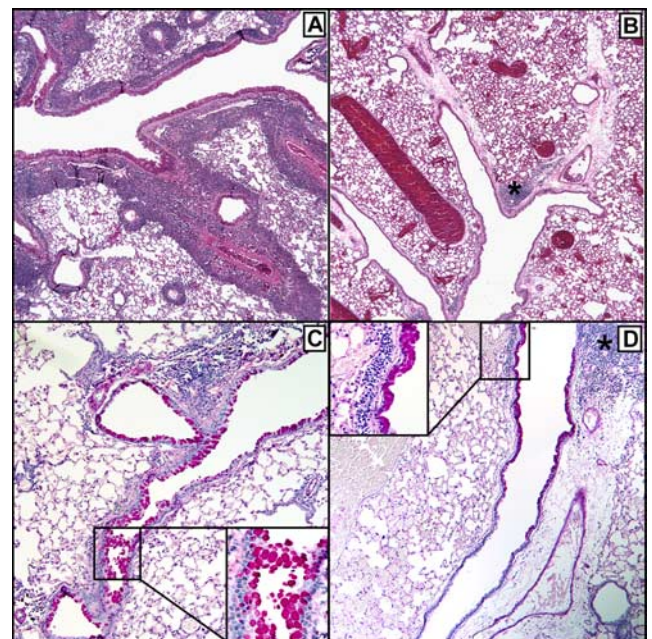


FIGURE 3. IL-4 deficiency greatly reduced TSLP-induced inflammatory infiltrates and goblet cell metaplasia. **A** and **B**, H&E staining of lung sections from IL-4^{+/-}/SPC-TSLP mice (**A**) and IL-4^{-/-}/SPC-TSLP mice (**B**). **C** and **D**, PAS staining of lung sections from IL-4^{+/-}/SPC-TSLP mice (**C**) and IL-4^{-/-}/SPC-TSLP mice (**D**). Although inflammation and goblet cell metaplasia in IL-4^{+/-}/SPC-TSLP mice could be seen at most of the airways, inflammation in IL-4^{-/-}/SPC-TSLP mice were rare (* in **B** and **D**) and goblet metaplasia was seen only around the regions with inflammation (**D**). Original magnification $\times 5$ for **A** and **B**, and $\times 10$ for **C** and **D**. Data represent one of three experiments.

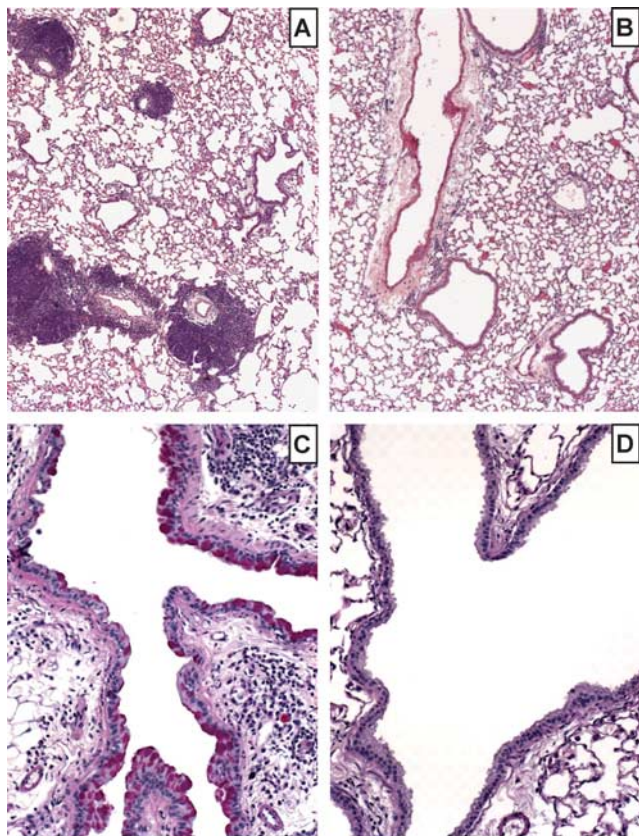


FIGURE 4. Stat6-deficient mice are not susceptible to TSLP-induced inflammatory infiltrates and goblet cell metaplasia. *A* and *B*, H&E staining of lung sections from Stat6^{+/}/SPC-TSLP mice (*A*) and Stat6^{-/-}/SPC-TSLP mice (*B*). *C* and *D*, PAS staining of lung sections from Stat6^{+/}/SPC-TSLP mice (*C*) and Stat6^{-/-}/SPC-TSLP mice (*D*). Original magnification $\times 5$ (*A* and *B*) or $\times 20$ (*C* and *D*). Data represent one of two independent experiments.

Anti-IL-4R α (M1) Ab treatment

A chimeric Ab against IL-4R α (referred to as M1) was used to block both IL-4 and IL-13 signaling pathways (17). M1 was derived from a rat anti-IL-4R mAb in which the rat Fc region has been replaced by muIgG1. M1 Ab was given two times a week via i.p. injection (1 mg/mouse). For control animals, an equivalent dose of normal rat IgG (RIG) (Sigma-Aldrich) was used.

Data and statistical analysis

ANOVA with Bonferroni posttests was performed with Prism version 4.00 (GraphPad). For analysis of physiologic data (P_{enh}), two-way ANOVA with repeated measures was used. Data were graphed using the same software and values for all measurements were expressed as mean $\pm$ SD.

Results

Reduced TSLP-mediated airway eosinophilia and hyperresponsiveness in IL-4-deficient mice

IL-4 has been shown to be important for mediating proinflammatory functions in asthma including differentiation of Th2 cells leading to Th2 cytokine release, induction of the IgE isotype switch, and promotion of eosinophil transmigration across endothelium (18). To assess the role of IL-4 in the accumulation of inflammatory cells and development of TSLP-mediated lung inflammation, SPC-TSLP transgenic mice were crossed to IL-4^{-/-} mice and analyzed for disease development at 2 mo of age. No differences were seen in disease progression and severity in IL-4^{+/+}/SPC-TSLP and IL-4^{-/-}/SPC-TSLP mice, and the lungs of IL-4-sufficient SPC-TSLP mice contained a significant inflammatory infil-

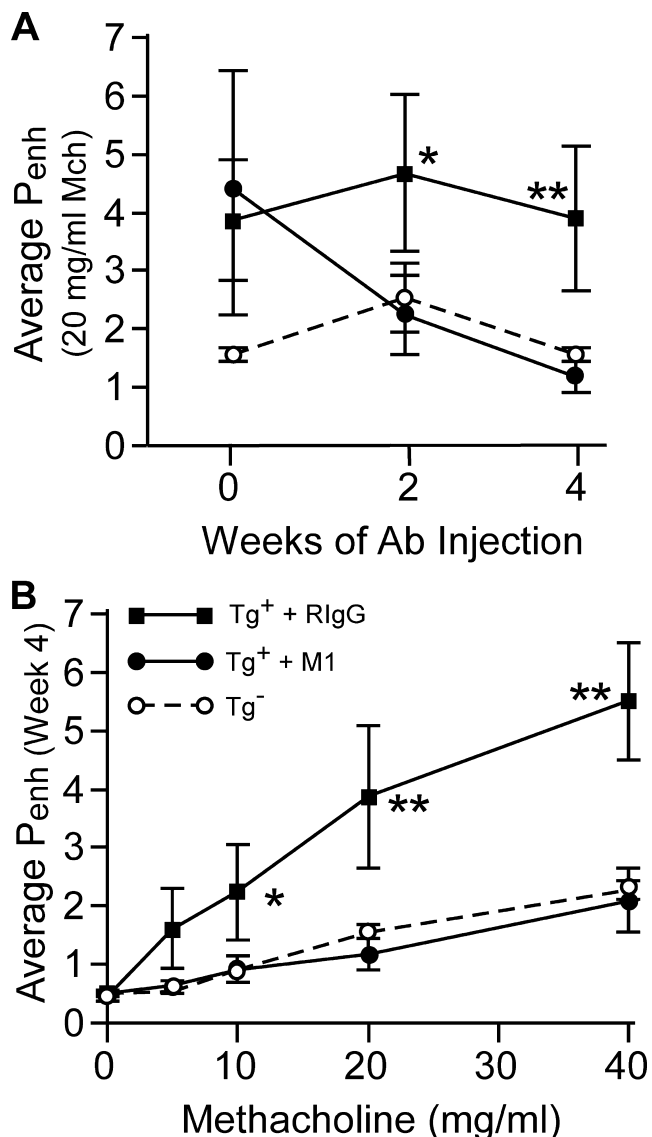


FIGURE 5. Anti-IL-4R α Ab (M1) efficiently reverses SPC-TSLP transgene-induced AHR. *A*, Change in P_{enh} in response to 20 mg/ml methacholine during the course of Ab treatment ($n = 4$). *B*, Airway responsiveness of animals after 4 wk of Ab treatment were assessed by P_{enh} measurements in response to increasing dose of methacholine ($n = 4$). Significant difference between M1-treated animals and normal RIG-treated animals are shown as *, $p < 0.05$; **, $p < 0.01$ (two-way repeated measures ANOVA with Bonferroni posttests).

trate consisting largely of eosinophils (Fig. 1*A*) (13). In contrast, the lungs of IL-4-deficient SPC-TSLP mice displayed dramatically reduced cellular infiltrates not significantly different from that seen in normal littermate controls (Tg⁻; Fig. 1*A*). Unlike IL-4^{+/+}/SPC-TSLP mice in which $\sim 70\%$ of BAL fluid cells were eosinophils, BAL fluid cells in IL-4^{-/-}/SPC-TSLP mice consisted mostly of lymphocytes ($\sim 60\%$) with less than 10% eosinophils (Fig. 1*B*). However, the absolute number of lymphocytes in the BAL fluid of IL-4-deficient mice was still decreased relative to IL-4-sufficient mice (1.2×10^5 vs 1.8×10^5 , respectively). In addition, CD4⁺ T cells in the BAL fluid of IL-4^{-/-}/SPC-TSLP mice were not only deficient for IL-4 but also lacked IL-5 and IL-13 expression (Fig. 1*C*).

To determine whether IL-4 deficiency also affects TSLP-induced AHR, we measured P_{enh} by unrestrained whole body plethysmograph. IL-4^{+/+}/SPC-TSLP mice showed significant AHR

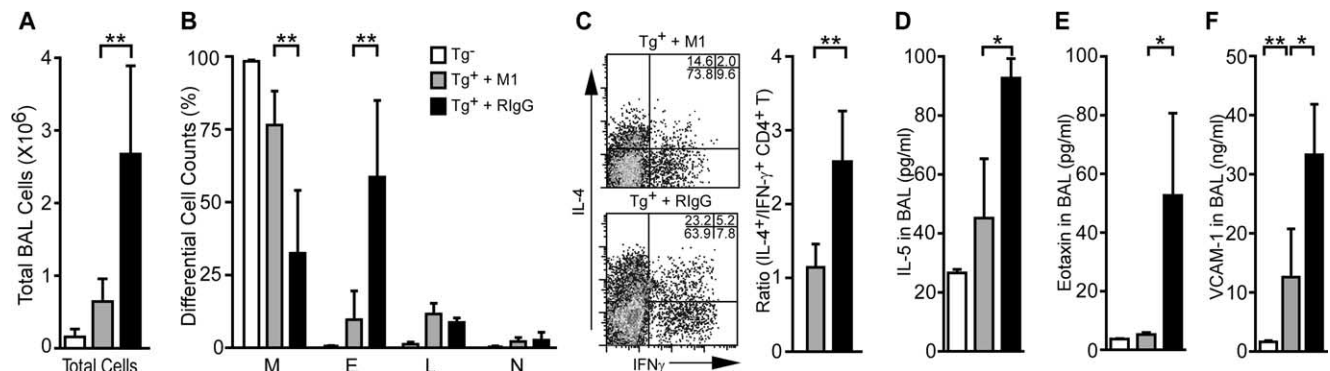


FIGURE 6. Anti-IL-4R α Ab (M1) efficiently reverses SPC-TSLP transgene-induced airway inflammation. **A**, Total BAL cell counts in M1-treated SPC-TSLP mice were significantly reduced. **B**, BAL cell differential counts showing significantly reduced airway eosinophilia by M1 treatment. **C**, M1 treatment significantly reduced the percentage of Th2-biased CD4⁺ T cells in BAL. **D–F**, MAP analysis of cytokines in BAL fluid showed that levels of IL-5 (**D**), eotaxin (**E**), and VCAM-1 (**F**) were significantly reduced in M1 treated SPC-TSLP mice. One-way ANOVA with Bonferroni posttests were performed to examine differences between multiple groups except in **C** where two groups were compared by unpaired *t* test. Data represent mean $\pm$ SD (*n* = 4). *, *p* < 0.05; **, *p* < 0.01; Tg: SPC-TSLP transgene; RIgG: normal RIgG control Ab.

even at low doses of methacholine (5 mg/ml), which continued to escalate, whereas the IL-4^{-/-}/SPC-TSLP mice did not display significant AHR over the littermate control mice at any of the doses of methacholine tested (Fig. 2).

Similar to our previous report (13), lungs from IL-4^{+/-}/SPC-TSLP mice had severe peribronchial and perivascular inflammatory infiltrates (Fig. 3A), consisting primarily of eosinophils, lymphocytes, and multinucleated giant cells. These mice also displayed goblet cell metaplasia and mucus overproduction, as shown by PAS staining (Fig. 3C). In contrast, these cardinal features of TSLP-induced airway inflammation were largely absent in the IL-4^{-/-}/SPC-TSLP mice, with only the occasional area of mild infiltration and PAS staining (Fig. 3, *B* and *D*).

Stat6 deficiency leads to a complete loss of TSLP-mediated asthma-like airway inflammation

Stat6 is an essential downstream mediator of IL-4 and IL-13 responses and for development of Th2 cells (19). Consistent with its function in Th2 responses, Stat6-deficient mice were protected from Ag-induced airway eosinophilia, AHR, and mucus production (20, 21). To determine whether Stat6 is required for TSLP-induced pathogenesis, we crossed SPC-TSLP transgenic mice with Stat6-deficient mice. The resulting Stat6^{-/-}/SPC-TSLP mice were completely devoid of any symptoms of TSLP-mediated airway inflammation (Fig. 4). Lungs from these mice lacked inflammatory infiltrates, as well as goblet cell metaplasia and mucus overproduction. These data are consistent with a crucial role of Th2-type responses in TSLP-induced airway inflammatory disease.

Therapeutic blockade of IL-4 and IL-13 reversed disease progression in SPC-TSLP mice

The results from the previous experiments demonstrated that an intact Th2 immune response was critical for the development of TSLP-mediated airway inflammation. However, these studies were performed in mice lacking the ability to mount complete Th2 responses and thus could not address whether these responses were required for continued inflammation in the SPC-TSLP mice. We next addressed whether blockade of Th2 cytokine responses after the initiation of airway inflammation, in a therapeutic-type model, could control or reverse TSLP-induced disease progression. We used a chimeric Ab (M1) specific for the IL-4R α -chain, a component of both the IL-4 and IL-13 receptors, which has been shown to be capable of blocking the biologic activity of both IL-4 and

IL-13 (17). For these studies SPC-TSLP mice exhibiting signs of airway inflammation (positive AHR) were treated with M1 Ab twice weekly and AHR was monitored by whole body plethysmograph. SPC-TSLP mice treated with M1, but not control Ab, showed a remarkable modulation of AHR, with *P*_{enh} reduced to the level of littermate control mice within 2 wk of treatment and sustained reductions over the course of Ab treatment (Fig. 5).

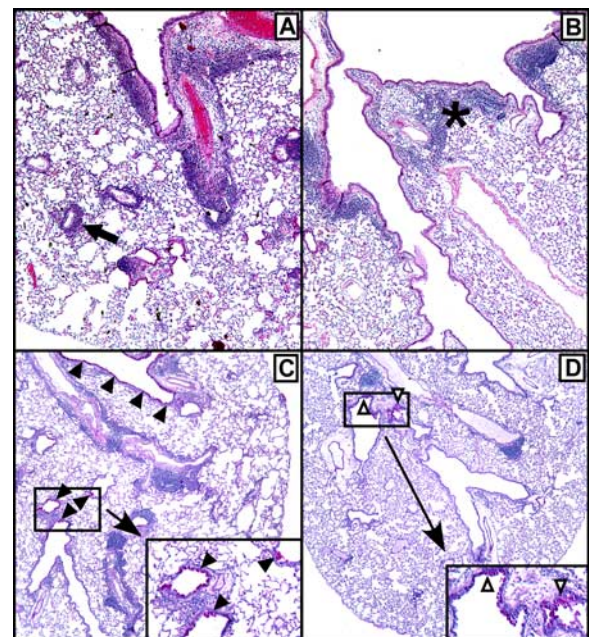


FIGURE 7. Anti-IL-4R α Ab (M1) treatment suppressed TSLP-induced pulmonary inflammatory infiltrates and goblet cell metaplasia. **A** and **B**, H&E staining of lung sections from normal RIgG-treated SPC-TSLP mice (**A**) and M1-treated SPC-TSLP mice (**B**). Although pulmonary inflammatory infiltrates in RIgG-treated animals spread to the distal smaller airways (arrow), they were only found at the very proximal large airways in M1-treated animals (star). **C** and **D**, PAS staining of lung sections from RIgG-treated SPC-TSLP mice (**C**) and M1-treated SPC-TSLP mice (**D**). Similar to the inflammatory infiltrates, purple-stained goblet cells in RIgG-treated animals were present in not only the proximal large airways but also the distal smaller airways (solid arrowhead) whereas they were only present in proximal larger airways (open arrowhead) in M1-treated animals. Original magnification $\times 5$.

After 4 wk of M1 treatment, the animals were sacrificed and analyzed for evidence of airway inflammation. M1-treated SPC-TSLP mice showed a marked reduction in BAL fluid cellularity, which was even lower than that before the Ab treatment ($0.64 \pm 0.3 \times 10^6$ vs $1.6 \pm 0.3 \times 10^6$, respectively). Differential cell counts showed M1-treated SPC-TSLP had a BAL cellular composition more similar to littermate control mice than SPC-TSLP mice treated with control Ab (Fig. 6, A and B).

We next examined cytokine production in the lungs of SPC-TSLP mice treated with M1 or control Ab. Although SPC-TSLP mice treated with control Ab displayed a typical Th2-bias in the lung, Th2-bias was shifted back in M1-treated SPC-TSLP animals displaying an equal proportion of Th1- and Th2-biased CD4⁺ T cells (Fig. 6C) with significant lower levels of IL-5 in BAL fluid (Fig. 6D). Furthermore, M1 treatment also suppressed expression of the chemokine eotaxin (Fig. 6E), a potent eosinophil attractant. Similarly, VCAM-1, which directs the migration of T lymphocytes, monocytes, basophils, and especially eosinophils to the site of allergic inflammation (22, 23), was significantly suppressed in M1-treated animals (Fig. 6F).

Consistent with the cytokine profile of the M1-treated SPC-TSLP mice, histopathological analysis also showed reduced airway inflammation and goblet cell metaplasia (Fig. 7). Although inflammatory infiltration in control Ab-treated animals was observed not only around the proximal airways but also around the distal small airways (Fig. 7A, arrow), infiltrates in M1-treated animals were mild and only seen around proximal major conducting airways (Fig. 7B). Similarly, goblet cell metaplasia could be found in virtually all airways in control Ab-treated animals (Fig. 7C, solid arrowhead), whereas it was only rarely observed in the proximal major conducting airways in M1-treated SPC-TSLP animals (Fig. 7D, open arrowhead).

Discussion

TSLP has been implicated in a variety of inflammatory diseases, including asthma and atopic dermatitis (9, 10). In fact, TSLP has been described as a master switch for the development of allergic inflammation (8, 9), based on its association with Th2-mediated inflammatory states. Consistent with these human studies, mice that express tissue-specific TSLP transgenes develop spontaneous inflammatory disease. For example, expression of a lung-specific TSLP transgene (the SPC-TSLP mice) (13) is sufficient to drive a Th2 inflammatory disease with all the cardinal features of asthma (13). In the current study, we have demonstrated that Th2 responses are a necessary downstream component in TSLP-initiated pathogenesis. In addition, the disease seen in this model mimics human asthma in that it is chronic in nature and involves responses to innocuous environmental Ags for disease development. SPC-TSLP mice lacking an adaptive immune system fail to develop full disease. More importantly, intranasal administration of TSLP and a foreign Ag, but not TSLP or Ag alone, on BALB/c mice results in rapid onset of asthma-like airway inflammation (M. B. Headley, B. Zhou, and S. F. Ziegler, manuscript submitted). These data suggest that the spontaneous asthma-like airway inflammation seen in SPC-TSLP mice is a result of allergic response to environment Ags present in their cages (M. B. Headley, B. Zhou, and S. F. Ziegler, manuscript submitted). Thus, the SPC-TSLP mice represent a robust model of chronic allergic airway inflammation and thus provide a useful tool to assess therapeutic efficacy of drug candidate on reversing chronic airway inflammation and airway hyperresponsiveness seen in allergic asthma.

Studies in both human patients and animal models of asthma have established a critical role for Th2 cells and their effector cytokines IL-4, IL-5, and IL-13. IL-4, playing a dominant role in the

differentiation of Th2 cells in vivo and in vitro (24, 25), is of particular interest in the pathogenesis of allergic inflammation. Mice deficient in IL-4 have impaired lung Th2 immune response and attenuated allergic airway inflammation in allergen-induced asthma model (2, 26). Consistent with these data, we have now shown that IL-4 is required for the full development of TSLP-induced chronic airway inflammation. Interestingly, two recent reports have also shown that OX40-OX40L interactions are involved in TSLP-mediated Th2 responses (11, 27). The data presented here would suggest that IL-4/IL-13 responses are also downstream of OX40-OX40L.

IL-13 is a pleiotropic cytokine with immunoregulatory activities that partially overlap with those of IL-4 (28). The redundancy in biologic responses to IL-4 and IL-13 may be explained by the fact that their receptors share the common IL-4R α -chain (29). Recent studies suggested that IL-4 is essential for the initiation of Th2-polarized immune responses to allergenic peptides, while IL-13 alone may mediate the main physiological consequences of disease, namely AHR, mucus hypersecretion, and subepithelial fibrosis (28). This may explain the residual airway inflammation and goblet cell metaplasia present in IL-4^{-/-}/SPC-TSLP mice (Fig. 3), a phenomenon similarly observed in Ag-induced asthma model with IL-4^{-/-} mice (30). Indeed, on the Stat6^{-/-} background, SPC-TSLP transgene induced airway inflammation and goblet cell metaplasia are almost completely abolished (Fig. 4). Since CD4⁺ cells in the BAL of IL-4^{-/-}/SPC-TSLP mice had no detectable IL-13 expression (Fig. 1), the residual inflammation and goblet cell metaplasia seen in these mice, but not in Stat6^{-/-}/SPC-TSLP mice, might suggest that other cell types are capable of IL-13 production following TSLP exposure. For example, Rag2^{-/-}/SPC-TSLP mice, which lack T and B cells, still exhibited a certain degree of inflammation and mucus overproduction. Both IL-4 and IL-13 could be detected in the lungs of these mice (data not shown). Recent evidence suggests that this could be mast cells as human mast cells stimulated by TSLP in the presence of IL-1 and TNF produced high levels of Th2 cytokines (31).

The data presented here is the first demonstration of therapeutic efficacy of IL-4R α blockade on disease progression of chronic allergic asthma. Previous studies have shown that the Ab was able to prevent airway responses in an acute asthma model (32). In this study, we used the SPC-TSLP mice with established disease to test the therapeutic efficacy of IL-4/IL-13 blockade, a more clinically relevant study. The M1 Ab efficiently reversed AHR and greatly reduced airway inflammation, eosinophilia, and goblet cell metaplasia (Figs. 5–7). Furthermore, M1 treatment reduced the number of Th2-polarized CD4⁺ T cells in the airways and suppressed levels of Th2 cytokines such as IL-5 in BAL fluid (Fig. 6). With added benefit, M1 treatment also suppressed expression of IL-4- and IL-13-induced proteins such as the chemokine eotaxin and VCAM-1 (Fig. 6), genes important for eosinophil recruitment to the diseased tissues. Recently it has been reported that an inhaled IL-4R α antisense oligonucleotide suppressed airway inflammation, inhibited production of airway Th2 cytokines and chemokines, and reduced goblet cell metaplasia and mucus overproduction in Ag-induced asthma model (33). Taken together, these data suggest that inhibition of IL-4R α , even locally in the lung, represents a promising therapeutic approach for allergy and asthma.

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Disclosures

The authors have no financial conflict of interest.

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