

Fms-Like Tyrosine Kinase 3 Ligand Decreases T Helper Type 17 Cells and Suppressors of Cytokine Signaling Proteins in the Lung of House Dust Mite–Sensitized and –Challenged Mice

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We previously reported that Fms-like tyrosine kinase 3 ligand (Flt3-L) reversed airway hyperresponsiveness (AHR) and airway inflammation, and increased the number of regulatory CD11c^{high}CD8 α ^{high}CD11b^{low} dendritic cells and CD4⁺CD25⁺ICOS⁺Foxp3⁺IL-10⁺ T-regulatory cells in the lung of allergen-sensitized and -challenged mice. In this study, we evaluated the effect of Flt3-L on Th17 cells and expression of suppressors of cytokine signaling (SOCS) proteins in the lungs of house dust mite (HDM)–sensitized and –challenged mice. BALB/c mice were sensitized and challenged with HDM, and AHR to methacholine was established. Mice were treated with Flt3-L (5 μ g, intraperitoneal) daily for 10 days. Levels of IL-4, -5, -6, -8, and -13, and transforming growth factor (TGF)- β in the bronchoalveolar lavage fluid (BALF) were examined by ELISA. Flt3-L treatment reversed existing AHR to methacholine and substantially decreased eosinophils, neutrophils, IL-5, -6, -8, and IL-13, and TGF- β levels in the BALF. HDM-sensitized and -challenged mice showed a significant increase in lung CD4⁺IL-17⁺IL-23R⁺CD25⁻ T cells with high expression of retinoic acid–related orphan receptor (ROR)- γ t transcripts. However, administration of Flt3-L substantially decreased the number of lung CD4⁺IL-17⁺IL-23R⁺CD25⁻ T cells, with significantly decreased expression of ROR- γ t mRNA in these cells. HDM sensitization caused a significant increase in the expression of SOCS-1, -3, and -5 in the lung. Flt3-L treatment abolished the increase in SOCS-1 and SOCS-3 proteins, whereas SOCS-5 expression was significantly reduced. These data suggest that the therapeutic effect of Flt3-L in reversing the hallmarks of allergic asthma in a mouse model is mediated by decreasing IL-6 and TGF- β levels in the BALF, which, in turn, decrease CD4⁺IL-17⁺IL-23R⁺ROR- γ t⁺CD25⁻ T cells and the expression of SOCS-1 and SOCS-3 in the lung of HDM-sensitized and -challenged mice.

Keywords: airway hyperresponsiveness; house dust mite; retinoic acid–related orphan receptor- γ t; suppressors of cytokine signaling; T helper cell type 17

Asthma is a chronic inflammatory lung disease of the airways, characterized by reversible airway obstruction (1), airway hyperresponsiveness (AHR) (2), and airway inflammation (3). CD4⁺ T cells have been found in the lungs of postmortem subjects who died of severe acute asthma attacks, suggesting that CD4⁺ T cells have a central role in the pathogenesis of asthma (4). An imbalanced ratio of T helper cell (Th) type 1 and Th2 cells leads to the development of a Th2 polarized response and pathophysiological changes seen in airways of subjects with asthma (5).

CLINICAL RELEVANCE

Fms-like tyrosine kinase 3 ligand (Flt3-L) is a potent immunomodulator in allergic airway inflammation and airway hyperresponsiveness in asthma. Here, we found that Flt3-L decreases lung T helper type 17 cells that are one of the major cells involved in allergic airway inflammation. These findings enhance our knowledge base of the disease process, and may be helpful in developing a better therapeutic approach.

The Th1 and Th2 model was first proposed over 2 decades ago (6, 7), and this paradigm has been the cornerstone of understanding cellular development of Th1 cells in response to viruses, intracellular bacteria, and cancer (8). On the other hand, the development of a Th2 polarized response is initiated by multicellular parasites and extracellular bacterial infections (9).

Th17 cells are the most recently identified member of CD4⁺ T cell subset, and it has been defined by its secreted product, IL-17 (10). There are several isoforms of IL-17 that include IL-17A to IL-17F, and they are homodimeric peptides between 35 and 52 kD (11). IL-17 is a proinflammatory cytokine (12) secreted by Th17 cells and consists of six isoforms—IL-17A through IL-17F (13)—but it can be produced by CD8⁺ T cells and NKT cells (14). Th17 cells develop in response to IL-6 and transforming growth factor (TGF)- β (15), and these differentiated cells express IL-23R, which is stabilized and/or maintained by IL-23 signaling (16). In general, Th17 cells mount a defense against extracellular bacteria (i.e., *Klebsiella pneumoniae*) (17) and fungi (i.e., *Candida albicans*) (18) by the secretion of IL-17 and the recruitment of neutrophils (19). Recent data have shown that Th17 cells may indirectly participate in virus-associated pathologies (20), such as human immunodeficiency virus (21). However, Th17 cells have been implicated in the development of autoimmune diseases, including multiple sclerosis (22), rheumatoid arthritis (23), and lupus (24), and accumulating data suggest a correlation with initiation and pathogenesis of allergic diseases, such as atopic dermatitis, allergic rhinitis, food allergy, and asthma (25).

There is a plethora of environmental antigens that trigger the development of proinflammatory T cells, and these lymphocytes play important roles in pathogenesis of asthma (26). House dust mite (HDM), a common indoor antigen consist of *Dermatophagoides farinae* (27) and *Dermatophagoides pteronyssinus* (28), is a potent inducer of atopic asthma (29), and mite allergy affects approximately 10–20% of the population (30). People who are allergic to dust mites respond to the proteins within the bodies and feces of the mites (31). Exposure to dust mite at high levels is an important factor in the development of asthma in children (32). The proteins of HDM are found mostly in pillows,

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mattresses, carpeting, and upholstered furniture (33); therefore, this household allergen is a high-risk factor for the initiation of allergic asthma.

Fms-like tyrosine kinase (Flt) 3 (or Flk2) is a member of the class III tyrosine kinase receptor family (34), and it was independently cloned by two groups of investigators (35). Flt3 was cloned from murine placenta based upon its similar sequence homology to c-fms (36). The c-fms (cellular) oncogene is a homolog of v-fms (viral) oncogenes, and it was originally encoded by the Susan McDonough strain of feline sarcoma virus (37). The mouse and human ligands (Flt3-L) for the Flt3/Flk2 receptor were cloned, and were found to share structural similarities with c-kit-L and M-CSF (38, 39). *In vivo* administration Flt3-L in mice leads to a significant increase of dendritic cells (DCs) in all primary and secondary lymphoid tissues (40), and in humans it induces both CD11c⁺ and CD11c⁻ DC subsets (41).

Recently, our laboratory reported that treatment of Flt3-L in ovalbumin-sensitized and -challenged mice reversed AHR to methacholine and airway inflammation (42). In addition, we found that ovalbumin-sensitized mice treated with Flt3-L increased the number of regulatory CD11c^{high}CD8 α ^{high}CD11b^{low} DCs in the lung (42). We also found that administration of Flt3-L significantly increased the number of CD4⁺CD25⁺Foxp3⁺IL-10⁺ T cells in the lungs of cockroach-sensitized and -challenged mice (43). Furthermore, adoptive transfer of T-regulatory cells (Tregs) reverses AHR and airway inflammation (45). The secretion of cytokines has been shown to play a role in the regulation of T cell response. The regulation of Th1 and Th2 development in steady-state conditions has implied that suppressors of cytokine signaling (SOCS) proteins play a significant role in the proper immune response, and the expression of these protein molecules is also regulated by cytokines (44). Therefore,

in this study, we examined the potential effect of Flt3-L on the expression of SOCS proteins and suppression of Th17 cells in the lungs of a clinically relevant model of HDM-induced allergic asthma.

MATERIALS AND METHODS

Animals

Female BALB/c mice (4–5 wk old) were purchased from Harlan Laboratories (Indianapolis, IN), and were housed in separate cages according to treatment protocol. Food and water were provided *ad libitum*. In accord with National Institutes of Health guidelines, the research protocol of this study was approved by the Institutional Animal Care and Use Committee of Creighton University (Omaha, NE).

Sensitization and Treatment Protocol

Allergic airway inflammation was induced by intranasal injections of 20 μ g allergenic extract of whole bodies of mites, *D. farinae* and *D. pteronyssines* (Hollister-Stier Laboratories LLC, Spokane, WA), on Days 0 and 14, followed by aerosol sensitization with 1% HDM for 20 minutes on Days 28–30 with an Aeroneb Pro nebulizer (Aerogen, Somerset, PA). Mice were again challenged on Day 32 with 5% HDM for 20 minutes followed by the measurement of pulmonary functions 24 hours later to establish AHR to methacholine (Figure 1A), by the established methods in our laboratory (42–45). Starting Day 33, HDM-sensitized- and -challenged mice with established AHR to methacholine were randomized into two groups: the first experimental group received 5 μ g Flt3-L (Peprotech, Inc Rocky Hill, NJ) intraperitoneal treatment for 10 days, and the second group received sterile PBS, the vehicle of Flt-3L. The nonsensitized control mice were sham treated with sterile PBS. On Day 45, mice in all groups were challenged with 5% aerosolized HDM, and nonsensitized control mice were administered only sterile PBS. On Day 46, AHR to methacholine was measured by whole-body plethysmography (Buxco Electronics, Troy,

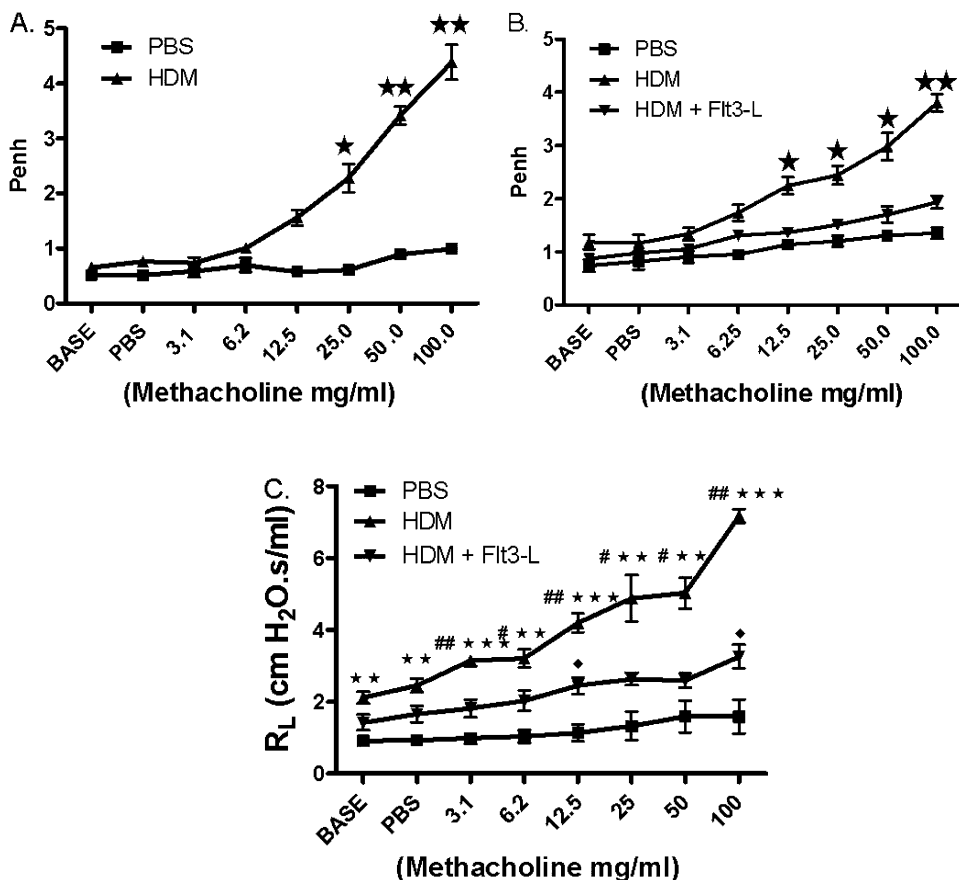


Figure 1. Airway hyperresponsiveness (AHR) to methacholine (Mch). (A) On Day 33, AHR to Mch was established, followed by 10-day treatment with Fms-like tyrosine kinase ligand (Flt3-L; 5 μ g/d, intraperitoneal). (B) On Day 45, AHR to Mch was measured in enhanced pause (Penh) values after Flt3-L treatment. (C) AHR was confirmed by the measurement of specific airway resistance (R_L). Data are shown as means (\pm SEM) from five mice in each experimental group. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ compared with PBS group; # $P < 0.05$, ## $P < 0.01$ compared with house dust mite (HDM); closed diamonds, $P < 0.05$ compared with PBS group.

NY), and the data reported in enhanced pause (Penh) values. On Day 47, AHR to methacholine was confirmed by the invasive tracheotomy method of measuring specific airway resistance, followed by collection of bronchoalveolar lavage fluid (BALF), blood, lungs, and spleen.

Tissue Preparation and Isolation of Th17 Cells

To isolate Th17 cells, mice were killed and lungs were harvested. These tissues were cut into fragments and digested with collagenase-D (1 mg/ml; Roche Laboratories, Burlington, NC) and 5 ml of RPMI 1,640 (Cambrex, East Rutherford, NJ). The samples were incubated at 37°C in a CO₂ incubator for 60 minutes. Tissues were disrupted with a 1-ml syringe, and red blood cells were removed from the suspension with Tris-buffered ammonium chloride solution and neutralized with PBS4 solution. The suspension was centrifuged at 350 × g for 15 minutes. Supernatant was discarded and the pellet washed in 10 ml Hanks' balanced buffered solution, centrifuged, and resuspended in AutoMACS running buffer. The cell suspension was labeled with the CD4⁺ microbead kit (Miltenyi Biotec, Auburn, CA) and sorted by AutoMACS (miltenyi Biotec, Auburn, CA). The sorted cells were labeled with conjugated peridinin chlorophyll protein (PerCP) CD4 antibody and allophycocyanin (APC) CD25 antibody, and further purified by FACSaria (BD Bioscience, San Diego, CA). The purified CD4⁺CD25⁻ T cells were processed for Th17 cells by a three-step process: (1) the purified CD4⁺CD25⁻ T cells were labeled with IL-17 catch reagent and incubated for 5 minutes at 37°C (warm 37°C serum-free medium was added to the CD4⁺CD25⁻ T cells and incubated for an additional 45 minutes on a slow, continuous rocker); (2) CD4⁺CD25⁻ T cells were washed, centrifuged, and resuspended in cold running buffer, and labeled with IL-17 detection antibody, incubated for 10 minutes on ice; and (3) cells were washed, centrifuged, and resuspended in buffer and label with anti-PE microbeads, and sorted for IL-17-secreting cells with AutoMACS. The CD4⁺CD25⁻ T cells were analyzed for IL-17 expression by FACS (BD Bioscience). Overall purity and viability of the CD4⁺CD25⁻ T cell population in the lung tissue ranged between 94 and 95% and 95 and 98%, respectively. The purity of the CD4⁺CD25⁻ T cells used to analyze the expression of IL-23R was 60–70%, as the cells in these experiments were isolated with AutoMACS, and not the FACSaria cell sorter.

Flow Cytometry and Antibodies

A FACScan (Becton Dickinson, San Diego, CA) was used for analytical flow cytometry, and data were processed with CellQuest Pro (Becton Dickinson) by standard protocol for cell preparation. Cells were stained with PerCP CD4 (L3T4) and APC CD25 (BC96), purchased from eBioscience (San Diego, CA). In addition, cells were stained with mouse PE IL-17 Secretion and Detection assay kit (Miltenyi Biotec).

BALF and Cytokine Measurements

After death, lungs were gently lavaged with 1 ml of warm saline (37°C) via a tracheal cannula. Total cell counts were performed with a Coulter counter (Beckman and Coulter, Brea, CA). All samples were centrifuged at 400 rpm for 10 minutes, and the supernatant was stored in a -80°C freezer until assays were performed for IL-4, -5, -6, -13, and TGF-β levels with an ELISA Detection Ready-Set-Go kit (eBioscience), according to the manufacturer's protocol. The IL-8 levels were examined with the Mouse GRO/KC Assay kit—IBL 96 well (Immuno-Biological Labs Co., Ltd., Minneapolis, MN), according to the manufacturer's protocol.

Immunohistochemistry

Paraffin sections (5 μm) were cut from the tissue-embedded blocks, placed onto a warm (37°C) water bath, and placed onto superfrost plus slides (VWR International, Lutterworth, Leicestershire, UK). The sections were deparaffinized and hydrated in xylene and solutions of ethanol gradients (100, 95, 80, and 70%). For immunostaining, antigen was exposed with target retrieval solution by boiling the sections in a steam cooker for 30 minutes. Sections were cooled to room temperature for 15 minutes and washed three times for 5 minutes each. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol for 30 minutes. All procedures for blocking of nonspecific binding sites, incubation with primary antibody (1–2 h), secondary antibodies, and

avidin-biotin complex) protocol were performed as recommended by the manufacturer (Vectastain ABC elite kit, PK-7800; Vector Laboratories, Burlingame, CA). The substrate used was 2,2-diaminobenzene in which positive staining was indicated by the presence of brown precipitate. The sections were counterstained with Gills no. 2 hematoxylin for 7 seconds. Negative controls were run without the primary antibody. Results were examined by light microscopy.

RT-PCR

To analyze the expression of retinoic acid-related orphan receptor (ROR)-γt transcripts, the mRNA was prepared from isolated CD4⁺IL-23R⁺IL-17⁺ T cells with Trizol (Sigma-Aldrich, St. Louis, MO) reagent protocol. Gene Amp PCR System 2,400 (Perkin-Elmer, Waltham, MA) was used at 31 cycles for: (1) ROR-γt, forward 5'-TGA GGA AAC CAG GCA TCC TGA ACT-3', reverse 5'-TGT GTG GTT GTT GGC ATT GTA GGC-3' (melting temperature, T_m, 55°C); and (2) ROR-γt, forward 5'-GTT TGG CCG AAT GTC CAA GAA GCA-3', reverse 5'-ATT GAT GAG AAC CAG GGG CCG TGT A (melting temperature, T_m, 55°C).

Statistical Analysis

Data were analyzed with GraphPad Prism statistical analysis and graphing software. Unpaired Student's *t* test was used to determine differences between two groups. Multiple group comparison was made with ANOVA. A *P* value of less than 0.05 was considered significant.

RESULTS

Effect of Flt3-L Treatment on AHR to Methacholine in HDM-Sensitized and -Challenged Mice

Before the treatment with Flt3-L or PBS, HDM-sensitized and -challenged mice showed elevated AHR to methacholine on Day 33, as measured by noninvasive whole-body plethysmography (Figure 1A). HDM-sensitized and -challenged mice treated with Flt3-L exhibited a significant reduction of AHR to methacholine compared with PBS control mice (Figure 1B). A more rigorous method with invasive tracheostomy in anesthetized mice to measure specific airway resistance confirmed the findings of AHR (Figure 1C).

Effect of Flt3-L Treatment on Leukocytes in the BALF from HDM-Sensitized and -Challenged Mice

HDM sensitization and challenge significantly increased the number of eosinophils and neutrophils in the BALF compared with PBS control mice. The administration of Flt3-L substantially decreased the density of BALF eosinophils and neutrophils to the PBS control level (Figure 2).

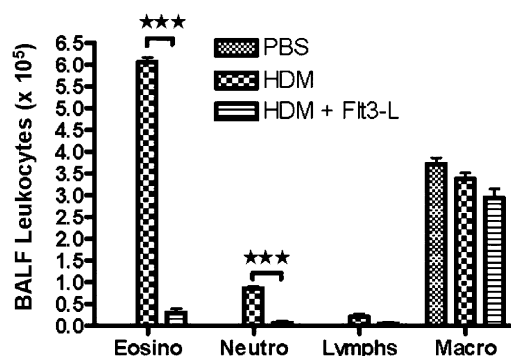


Figure 2. Bronchoalveolar lavage fluid (BALF) leukocytes after Flt3-L treatment. BALF was centrifuged, and pelleted cells were counted and differential analysis was performed with standard morphological criteria. A total of 300 cells was examined per cytospin slide, and absolute cell numbers were calculated per milliliter of the BALF based on the percentage of individual cell in a slide. Data are shown as means (±SEM) for five animals in each experimental group. ****P* < 0.001.

Effect of Flt3-L Treatment on Th2 cytokines in the BALF of HDM-Sensitized and -Challenged Mice

The levels of IL-5 and -13 were significantly increased in the BALF of HDM-sensitized and -challenged mice. However, treatment with Flt3-L in these mice substantially reduced the BALF levels of IL-5 and -13. There was no significant effect of Flt3-L on the level of BALF IL-4. (Figure 3).

Effect of Flt3-L Treatment on IL-8 in the BALF of HDM-Sensitized and -Challenged Mice

Increased neutrophils in the BALF could be due to increases in IL-8 levels. Therefore, we evaluated the levels of IL-8 in the BALF of each experimental group. The IL-8 levels were substantially higher in the BALF of HDM-sensitized and -challenged mice than in PBS control mice. Treatment with Flt3-L in HDM-sensitized and -challenged mice significantly reduced the BALF IL-8 levels (Figure 4).

Effect of Flt3-L Treatment on IL-6 and TGF-β in the BALF of HDM-Sensitized and -Challenged Mice

HDM sensitization and challenge significantly increased the levels of BALF IL-6 and TGF-β compared with PBS control mice. In contrast, administration of Flt3-L in HDM-sensitized and -challenged mice significantly lowered the BALF IL-6 and TGF-β levels, almost to the levels in PBS control mice (Figures 5A and 5B).

Effect of Flt3-L on CD4⁺CD25⁻ T Cells Expressing IL-17 Isolated from the Lungs of HDM-Sensitized and -Challenged Mice, and from PBS Control Mice

Because IL-6 and TGF-β promote the development of Th17 cells, we isolated CD4⁺CD25⁻ T cells from the lung and gated for expression of IL-17 to compare PBS, HDM, and HDM plus Flt3-L groups. The expression of IL-17 on lung CD4⁺CD25⁻ T cells is shown in representative contour plots (Figure 6A). The percentage of lung CD4⁺CD25⁻ T cells expressing IL-17 from PBS control mice was substantially lower (Figure 6B). However, HDM sensitization and challenge significantly increased the percentage of CD4⁺CD25⁻ T cells expressing IL-17 in the lungs. CD4⁺CD25⁻ T cells, isolated from the lungs of HDM-sensitized and -challenged mice treated with Flt3-L, exhibited a substantial decrease in IL-17 expression (Figure 6B).

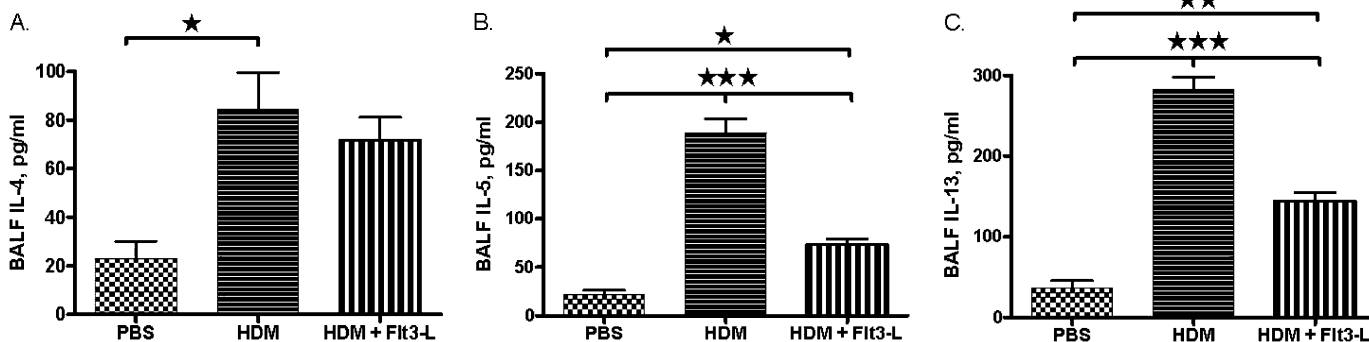


Figure 3. T helper (Th) 2 cytokines in the BALF of HDM-sensitized and -challenged mice. Th2 cytokine levels in the BALF of and HDM-sensitized and -challenged mice with and without Flt3-L treatment. (A) IL-4; (B) IL-5; and (C) IL-13. Data are shown as means (±SEM) for five animals in each experimental group. *P < 0.05, **P < 0.01, ***P < 0.001.

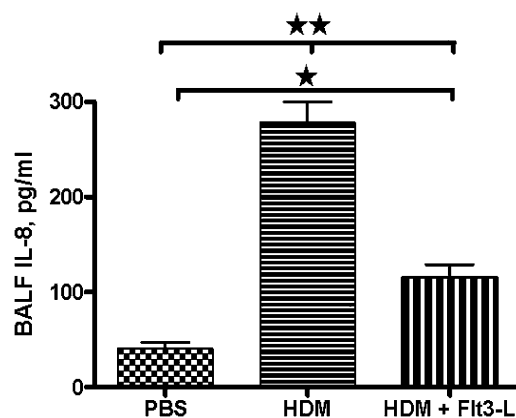


Figure 4. IL-8, a potent chemokine for neutrophil recruitment. Collected BALF samples were analyzed for levels of IL-8 expression in HDM-sensitized and -challenged mice with and without Flt3-L treatment, and PBS control mice. Data are shown as means (±SEM) for five animals in each experimental group. *P < 0.05, **P < 0.01.

Effect of Flt3-L on the Expression of IL-23R on CD4⁺CD25⁻ T Cells Isolated from the Lungs of HDM-Sensitized and -Challenged Mice

The expression of IL-23R on lung CD4⁺CD25⁻ T cells is shown in representative dot plots (Figure 7A). CD4⁺CD25⁻ T cells isolated from the lungs of PBS control mice had low to no expression of IL-23R (Figure 7B). HDM sensitization and challenge significantly increased the expression of IL-23R on lung CD4⁺CD25⁻. However, CD4⁺CD25⁻ T cells isolated from the lungs of HDM-sensitized and -challenged mice treated with Flt3-L showed a substantial decrease in IL-23R expression (Figure 7B).

Therapeutic Effect of Flt3-L on the mRNA Expression of ROR-γt in Lung CD4⁺IL-17⁺CD25⁻ T Cells

We analyzed the expression of ROR-γt transcripts in purified CD4⁺CD25⁻IL-17⁺ T cells from lung tissue of each experimental group. The expression of ROR-γt transcripts was substantially increased in the HDM-sensitized and -challenged mice without Flt3-L treatment. Treatment with Flt3-L significantly reduced the expression of ROR-γt mRNA to the PBS control level (Figure 8).

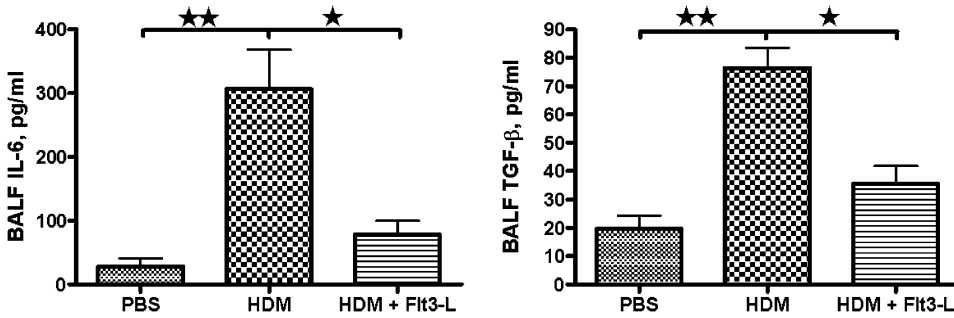


Figure 5. Transforming growth factor (TGF)-β and IL-6 levels in the BALF. BALF samples were immediately centrifuged and frozen. The cytokines, IL-6 (A) and TGF-β (B), in all samples were measured in the stored samples. Data are shown as means (±SEM) for five animals in each experimental group. *P < 0.05, **P < 0.01.

Effect of Flt3-L Treatment on mRNA Levels of ROR-γt in Lung CD4⁺IL23R⁺CD25⁻ T Cells

Lung CD4⁺IL-23R⁺CD25⁻ T cells isolated from PBS control mice exhibited no detectable levels of ROR-γt transcripts. However, sensitization and challenge with HDM antigen caused a significant increase in the levels of ROR-γt transcripts in the lung CD4⁺IL-23R⁺CD25⁻ T cells. The administration of Flt3-L in HDM-sensitized and -challenged mice abolished the ROR-γt mRNA expression in the lung CD4⁺IL-23R⁺CD25⁻ T cells (Figure 9).

Expression of SOCS-1, -3, and -5 after Flt3-L Treatment in the Lungs of HDM-Sensitized and -Challenged mice

SOCS proteins negatively regulate cytokine signaling. IL-6 is a proinflammatory cytokine, and is known to be regulated by SOCS proteins, specifically SOCS-1 and -3 (45, 46). Because

development of Th17 cells is regulated by the secretion and activity of IL-6 and TGF-β, we examined the expression of SOCS proteins in the lungs of HDM-sensitized and -challenged mice with and without Flt3-L treatment. PBS control mice expressed low to undetectable levels of SOCS-1, -3, and -5 in the lungs (Figures 10A–10C). However, the lungs of HDM-sensitized and -challenged mice exhibited substantially higher expression of SOCS-1, -3, and -5 compared with PBS control mice. Flt3-L treatment abolished the increase in SOCS-1 and -3 protein, whereas SOCS-5 expression was significantly reduced (Figures 10A–10C).

DISCUSSION

In this study, the clinically relevant aeroallergen, HDM, induced the classical hallmarks of allergic airway inflammation

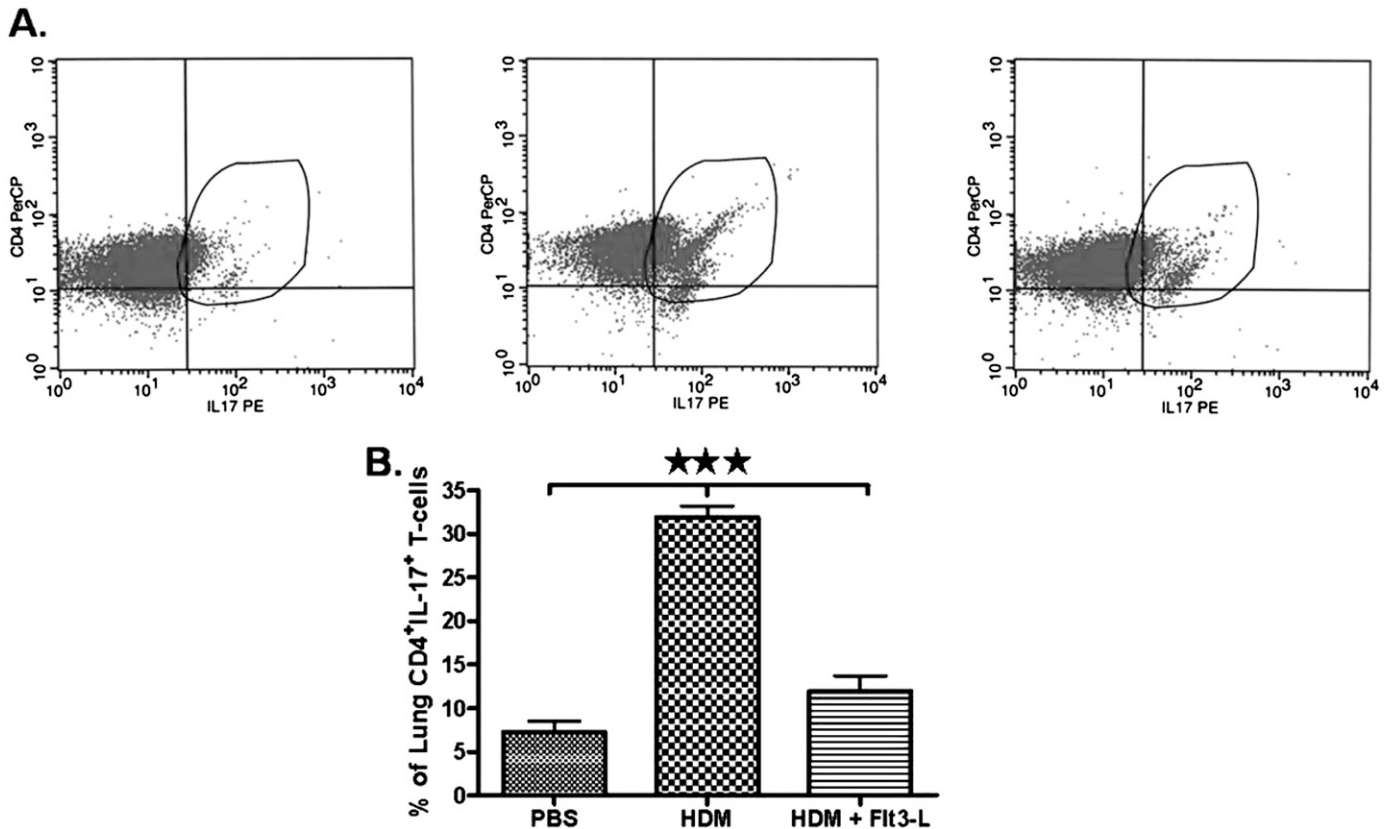


Figure 6. Expression of IL-17–producing lung CD4⁺CD25⁻ T cells. (A) Dot plots showing a comparative analysis of IL-17–producing CD4⁺CD25⁻ T cells isolated from lungs of PBS (left panel), HDM (middle panel), and HDM/Flt3-L (right panel). (B) Cumulative analysis of IL-17–producing CD4⁺CD25⁻ cells in the lungs of mice in each experimental group. Data are shown as means (±SEM) from five mice in each experimental group. ***P < 0.001.

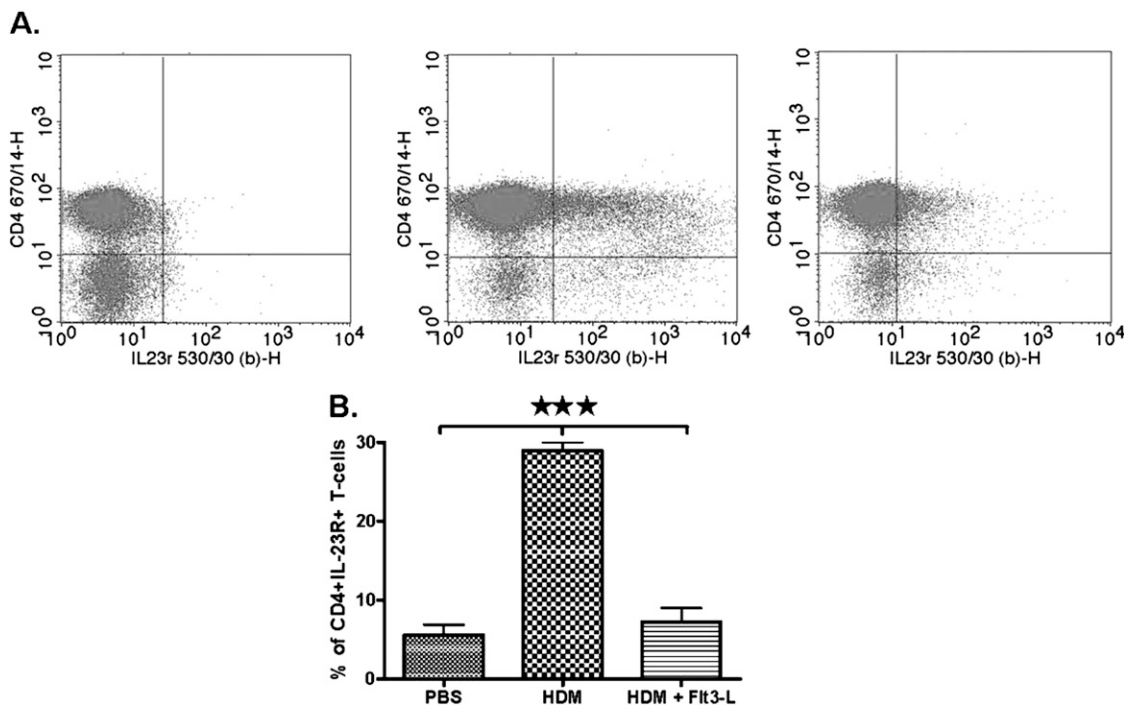


Figure 7. Expression of IL-23R on lung CD4⁺CD25⁻ T cells. (A) Dot plots showing a comparative analysis of IL-23R with CD4⁺CD25⁻ T cells isolated from lungs of PBS (left panel), HDM (middle panel), and HDM/Flt3-L (right panel). (B) Cumulative analysis of IL-23R expression in each experimental group. Data are shown as means (\pm SEM) from five mice in each experimental group. *** $P < 0.001$.

and asthma, including elevated AHR, and high levels of BALF IL-6 and TGF- β . In addition, we found that sensitization with HDM antigen caused a substantial increase in CD4⁺IL23R⁺IL-17⁺CD25⁻ T cells in the lungs of these mice, and these cells expressed significantly higher levels of ROR- γ t transcripts, the master transcriptional factor for Th17 cells. In addition, HDM sensitization caused a significant increase in the expression of SOCS-1, -3, and -5 in the lungs of challenged mice. Flt3-L treatment significantly decreased CD4⁺IL-23R⁺IL-17⁺CD25⁻ T cells in the lungs of HDM-sensitized and -challenged mice, and decreased expression of ROR- γ t transcripts. In addition, Flt3-L treatment reversed AHR, airway inflammation, and BALF IL-6 and TGF- β , and completely abolished the expression of SOCS-1 and -3 in the lung of HDM-sensitized and -challenged mice. These data suggest that the therapeutic effect of Flt3-L in allergic asthma could involve a reduction in the cytokines that increases Th17 cells.

In the HDM model, we found significantly higher BAL eosinophilia and neutrophilia, and Flt3-L treatment inhibited the density of these inflammatory cells in the BALF. The increased level of neutrophils in the BALF of HDM-sensitized mice may correlate with the increased number of CD4⁺IL-17⁺CD25⁻ T cells. IL-17 has been implicated in the recruitment of neutrophils in subjects with acute to severe asthma (47), and is expressed in BALF and sputum of patients with asthma (48, 49). Expression and activity of IL-23R on Th17 cells help in the maintenance of Th17 cells after their differentiation. In this study, HDM sensitization caused high expression of IL-23R on lung CD4⁺IL-17⁺CD25⁻ T cells, and Flt3-L treatment significantly decreased the expression of IL-23R on lung CD4⁺IL-17⁺CD25⁻ T cells to the PBS control mice.

The expression of ROR- γ t mRNA was substantially increased in the HDM-sensitized and -challenged mice, and treatment with Flt3-L significantly reduced the expression of ROR- γ t mRNA to control level. The low expression of ROR- γ t transcripts in the PBS control group could be due to the quiescent and/or an intermediate state of CD4⁺CD25⁻IL-17⁺

T cells, and these cells are activated during immune response. Recently, studies have shown that CD4⁺ T cells can coexpress ROR- γ t and Foxp3, and, under proper stimulation, these cells can differentiate into Tregs or Th17 cells (47). However, further investigation is warranted to elucidate these subsets and their functional role.

The specific mechanism(s) that mediates the mobilization and recruitment of neutrophils to the lung airways is ill defined. However, the secretion of IL-17 has been implicated in granulopoiesis and specific recruitment of neutrophils in various tissue types (50). Caldwell and colleagues (51) showed that the depletion of CD4⁺ T cells with antibody significantly reduced recruitment of neutrophils and injury after hepatic ischemia-reperfusion. However, little is known about the molecular mechanism(s) by which CD4⁺ T cells orchestrate neutrophil recruitment in the airways in response to stimuli other than allergens (52). To date, data suggest that Th17 cells are specific for recruiting neutrophils to the airways in the presence of exogenous antigen (52), and the Th17/neutrophil signaling axis could be mediated by airway epithelial cells (53). In this study, we found a significant increase in CD4⁺IL-23R⁺IL-17⁺CD25⁻ T cells in the lungs of HDM-sensitized and -challenged mice, and increased levels of neutrophils in the BALF of the same mice. However, the mechanism underlying the recruitment of neutrophils by Th17 cells remains to be elucidated. Recent evidence reported by Fielding and colleagues (54) suggests that IL-6 may regulate the mobilization and recruitment of neutrophils to the site of inflammation during acute-phase response. The BALF of HDM-sensitized and -challenged mice showed significantly higher levels of IL-6 and TGF- β , and this could synergistically drive the development of Th17 cells (55, 56), and may explain the increased density of neutrophils in the BALF of the HDM-sensitized and -challenged mice. Alternatively, neutrophil recruitment to the airways could be mediated directly by HDM allergen. HDMs produce enzymes called proteases that cleave peptide bonds of tight junction in the epithelial cell layer (57-59), and this action occurs by the

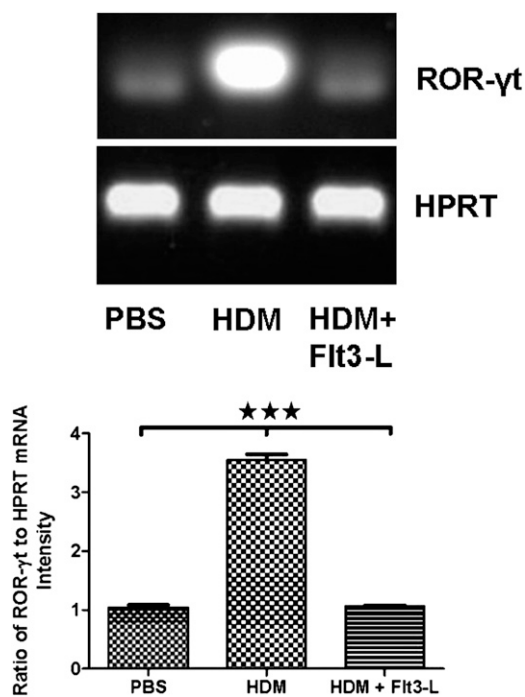


Figure 8. Effect of Flt3-L on retinoic acid-related orphan receptor (ROR)- γ t expression in CD4⁺CD25⁻IL-17⁺ T cells. Cells were isolated from the lungs HDM-sensitized and -challenged mice with and without Flt3-L for expression of ROR- γ t mRNA (970 bp). The purity of these CD4⁺CD25⁻IL-17⁺ T cells was greater than 98%. Densitometric analyses confirmed the PCR data, as shown by the ratio of mRNA intensity of ROR- γ t to intensity of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are shown as means (\pm SEM) from five mice in each experimental group. ****P* value < 0.001.

binding of proteases to protease activator receptors that are found on airway epithelial cells (60). The binding of proteases causes the production and secretion of IL-6 and -8 (61). In addition, protease activator receptor-2 receptors are found on fibroblasts, and activated fibroblasts secrete TGF- β (62). In addition, it is well established that IL-6 and TGF- β are responsible for development of Th17 cells, and the maintenance of Th17 cells is regulated by IL-23R on Th17 cells. Therefore, it is tempting to speculate that the increase in Th17 in the lungs of HDM-sensitized and -challenged mice is due to IL-6 and TGF- β , and the secretion of IL-17 from Th17 cells and IL-8 from epithelial cells lead to the recruitment of neutrophils. In this study, we found IL-8 levels to be substantially increased in the BALF of HDM-sensitized and -challenged mice. IL-8 is a potent chemokine to recruit neutrophils. However, IL-17 could be a potential stimulus for the release of IL-8 and -6 from the structural cells in the lungs, such as epithelial cells and macrophages (65). Indeed, IL-17 substantially increases IL-8 secretion from epithelial cells and induces migration of neutrophils. In the presence of IL-17 antibody, IL-8 secretion was substantially reduced together with the inhibition of neutrophil recruitment the lungs (66). Ye and colleagues (17) demonstrated in IL-17 knockout mice that IL-17 was essential for the up-regulation of granulocyte colony-stimulating factor (G-CSF) to induce granulopoiesis, macrophage-inflammatory protein-2 (MIP-2), MIP-2, and TNF- α , which promoted the recruitment of neutrophils (17). In a model of rheumatoid arthritis, IL-17 mediates IL-8 and -6 secretion via activation of phosphatidylinositol 3-kinase/Akt and NF- κ B (67). Thus, most of the data

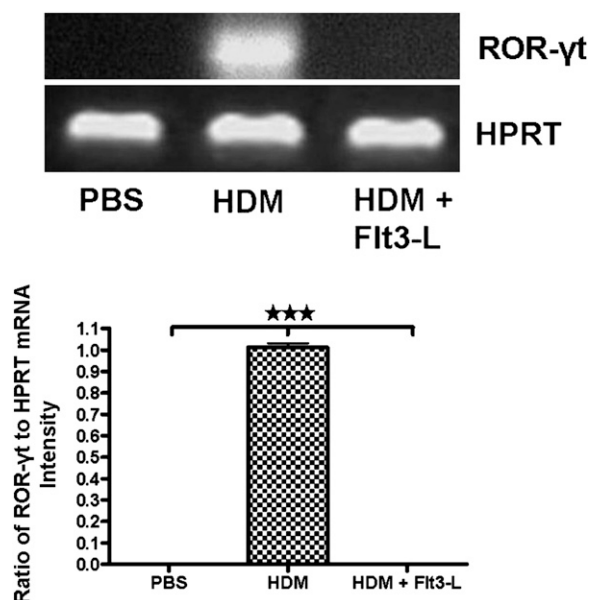


Figure 9. The effect of Flt3-L on lung CD4⁺ IL-23R⁺CD25⁻ T cells expressing ROR- γ t transcripts. CD4⁺IL-23⁺CD25⁻ T cell isolated from the lungs of PBS control mice showed very low but detectable levels ROR- γ t. However, HDM sensitization induced a significant increase in ROR- γ t transcripts. Flt3-L significantly decreased ROR- γ t expression compared with HDM and PBS control group. Densitometric analyses confirmed the PCR data, as shown by the ratio of mRNA intensity of ROR- γ t to the intensity of the housekeeping gene, HPRT. Data are shown as means (\pm SEM) from five mice in each experimental group. ****P* value < 0.001.

support the view that IL-17 causes neutrophil recruitment via IL-8 secretion in the lung. Nonetheless, the therapeutic effect of Flt3-L in the HDM-sensitized mice substantially decreased the levels of IL-6, -8, and TGF- β in the BALF; however, TGF- β and IL-8 were not lowered to the PBS control level. The remaining levels of TGF- β in the BALF may be a result of a skewed development of CD4⁺CD25⁺ Tregs, which also use TGF- β for development and function, or Flt3-L treatment may require a longer duration of therapy to completely remove TGF- β . Interestingly, we found an increase in Th2 cytokines, IL-4, -5, and -13, in HDM-sensitized and -challenged mice without Flt3-L treatment. Although not examined in the study, a recent new IL-17 family constituent, IL-25, has been shown to decrease significantly eosinophilia in the lung by increased production of IL-5 and -13, but not IL-4 (63). In addition, we found a significant decrease in eosinophilia in the lungs of HDM-sensitized and -challenged mice after treatment with Flt3-L.

IL-6 has also been implicated in the expression of SOCS proteins in several disease pathologies. Neuwirt and colleagues (65) demonstrated in prostate cancer cells that IL-6 induced a significant up-regulation of SOCS-1, which promoted the differentiation and survival of these cells. In addition, insulin resistance in adipocytes showed a substantial increase in SOCS-1 expression after treatment with IL-6 (64). SOCS-1 is highly expressed in committed Th17 cells, and it has been suggested that SOCS-1 could be a mechanism for IL-4 and IFN- γ resistance by committed Th17 cells (66). To this end, IL-6 and SOCS-1 expression appears to be pivotal in the development of Th17 cells, and Flt3-L was able to reverse or block their development by inhibiting IL-6, SOCS-1, and/or TGF- β .

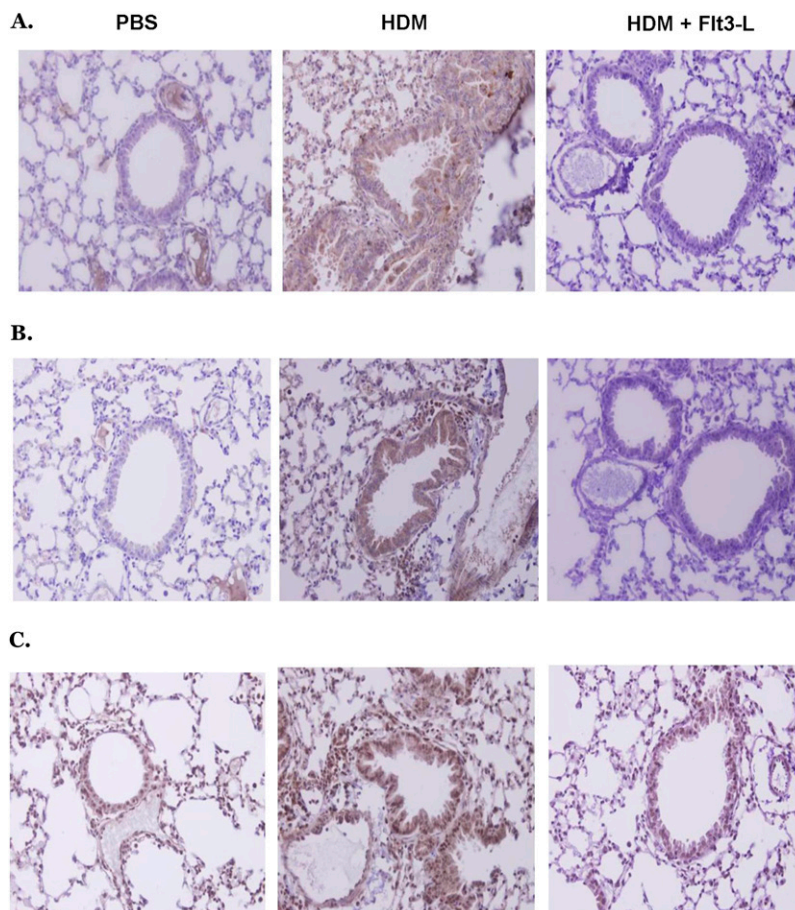


Figure 10. Effect of Flt3-L on the expression of (A) suppressors of cytokine signaling (SOCS)-1, (B) SOCS-3, and (C) SOCS-5 in lungs of HDM-sensitized and -challenged and PBS control mice. Flt3-L caused a decrease in SOCS-1, -3, and -5 expression compared with that in HDM-sensitized and -challenged mice without Flt3-L treatment. However, SOCS-5 was not completely removed after Flt3-L treatment. The histological slides are representative of five mice in each experimental group. *Left panels, PBS; middle panels, HDM; right panels, HDM plus Flt3-L.*

SOCS-3 has been reported to be a negative regulator of Th17 cells due to its ability to suppress IL-6 and -23 in a STAT3-dependent pathway (46). However, SOCS-3 has been shown to preferentially promote the development of Th2 cells (67). Kinjyo and colleagues (68) demonstrated that SOCS-3-deficient CD4⁺ T cells secreted high levels of TGF- β and moderate levels of IL-10, suggesting the development of Th3 Treg phenotype. In contrast, the presence of IL-6 with TGF- β blocks the development of Tregs and promotes development of Th17 cells (69). To this end, we have found that Flt3-L completely inhibited the expression of SOCS-3 induced by HDM sensitization, significantly decreased IL-6 and TGF- β , as well as CD4⁺CD25⁺IL-17⁺ T cells, and the effect Flt3-L may arise from the increased differentiation and development of Tregs. In addition, we found high expression of SOCS-5 in the lungs of HDM-sensitized mice; however, Flt3-L treatment did not abolish the expression of SOCS-5. The role of SOCS-5 in allergic asthma remains to be elucidated, but data suggest that SOCS-5 may have overlapping roles with other SOCS proteins. Alternatively, SOCS-5 might not be essential in the differentiation of either Th1 or Th2 cells (70).

In summary, we have delineated that HDM sensitization and challenge causes a substantial increase in CD4⁺IL-23R⁺IL-17⁺ROR- γ t⁺ T cells in the lungs. However, Flt3-L therapy significantly decreased CD4⁺IL-23R⁺IL-17⁺ROR- γ t⁺ T cells in the lung after HDM sensitization and challenge. The therapeutic effect of Flt3-L could be due to significant decreases in IL-6 and TGF- β levels in the lung, and by decreased expression of IL-23R on Th17 cells. This mechanism could be modulated by the expression of SOCS proteins, and possibly by the development

of regulatory DCs and/or Tregs in the lungs. Nonetheless, the interaction of Th17 cells and Tregs warrants further investigation.

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