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Pulmonary IL-17E (IL-25) Production and IL-17RB⁺ Myeloid Cell-Derived Th2 Cytokine Production Are Dependent upon Stem Cell Factor-Induced Responses during Chronic Allergic Pulmonary Disease

Vladislav Dolgachev,* Bryan C. Petersen,* Alison L. Budelsky,† Aaron A. Berlin,* and Nicholas W. Lukacs2*

In the present studies local neutralization of allergen-induced stem cell factor (SCF) leads to decreased production of Th2 cytokines, a reduction in inflammation, allergen-specific serum IgE/IgG1, and attenuation of severe asthma-like responses. The local blockade of pulmonary SCF also resulted in a significant reduction of IL-17E (IL-25). Sorted cell populations from the lung indicated that IL-25 was produced from c-kit⁺ cells, whereas Th2 cytokine production was primarily from c-kit⁻ cell populations. SCF stimulated c-kit⁺ eosinophils produced IL-25, whereas bone marrow-derived mast cells did not. Using 4get mice that contain a IL-4-IRES-eGFP that when transcribed coexpress GFP and IL-4, our studies identified cells that comprised a CD11b⁺, GR1⁺, Ly6C⁺/⁻, c-kit⁻, CD4⁻, CD11c⁻, MHC class II⁺ cell population as a source of IL-4 in the lung after chronic allergic challenge. In the bone marrow a similar cell was identified with approximately a third of the IL-4⁺ cells also expressing c-kit⁺. The pulmonary and bone marrow IL-4⁺ cell populations were significantly reduced upon local pulmonary anti-SCF treatment. Subsequently, when IL-25R was examined during the chronic allergic responses the expression was found on the IL-4⁺ myeloid cell population that expressed CD11b⁺GR1⁺. Interestingly, the IL-25R⁺ cells in the bone marrow were also all CD11b⁺GR1⁺, similar to the lung cells, but they were also all c-kit⁺, potentially suggesting a maturation of the bone marrow cell once it enters the lung and/or is stimulated by SCF. Overall, these studies suggest a complex relationship between SCF, bone marrow-derived IL-25-responsive myeloid cells, Th2 cytokines, and chronic allergic disease. The Journal of Immunology, 2009, 183: 5705–5715.

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Abbreviations used in this paper: SCF, stem cell factor; CRA, cockroach allergen; AHR, airway hyperresponsiveness.

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The expression of IL-25R corresponds to the IL-4 treatment predominantly alters a Th2 cytokine-producing myeloid IL-25 responsiveness in the lung and the bone marrow. The results. Additionally, blocking SCF also has a significant effect on neutralized in the lung there is a significant reduction of inflam-
molecules of naive mice by blocking the responses using an IL-25R blocking Ab (30). Thus, a non-T cell source of Th2 cytokines may also act to enhance allergic inflammation. This novel area may help explain how chronic asthmatics continue to have significant problems in the face of immune modulatory treatment protocols directed at T cell responsiveness.

The data presented in these studies indicate that when SCF is neutralized in the lung there is a significant reduction of inflammatory cells that is accompanied by a reduction of IL-25 production. Additionally, blocking SCF also has a significant effect on IL-25 responsiveness in the lung and the bone marrow. The results further indicate that the reduction in Th2 cytokines after anti-SCF treatment predominantly alters a Th2 cytokine-producing myeloid cell and has a significant impact on chronic disease phenotypes. The expression of IL-25R corresponds to the IL-4' myeloid cells. These data give further insight into the source of Th2 cytokines that is dependent upon SCF/c-kit and IL-25 for driving the severity of disease.

Materials and Methods

Animals and allergen model

BALB/c/J wild-type and 4get mice were purchased from The Jackson Laboratory and used at 6–8 wk of age. The allergen we used was a clinical grade skin test cockroach allergen (CRA) that has little endotoxin contamination (<20 ng/ml). Our chronic model has been set up as outlined previously and as depicted below (18). Briefly, animals are immunized syntropically by i.p. sensitization emulsified with IFA and after 14 days they are given four intranasal exposures of allergen (1.5 g/10 μl) 4 days apart followed by two intratracheal administrations (5 g/mouse) 4 days apart. The intratracheal challenge allowed the administration of the allergen directly into the airways to induce the most intense disease exacerbation. We analyzed the animals histologically and biochemically for changes in airway way responses as listed below. This methodology provides a chronic and consistent exacerbating airway model with severe mucus overproduction and peribronchial fibrotic response, a difficult aspect to initiate in mice.

Measurement of airway hyperreactivity

Airway hyperreactivity was measured using mouse plethysmography, which is specifically designed for the low tidal volumes (Buxco Research Systems), as previously described (3, 31). Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. The intubated mice were ventilated at a rate of 200 μl at a rate of 120 breaths/min. The airway resistance was measured in the closed plethysmograph by directly assessing tracheal pressure and comparing the level to corresponding box pressure changes. These values were monitored and immediately transformed into resistance measurements using computer-assisted calculations. Once baseline levels had stabilized and initial readings were taken, a methacholine challenge was given via the cannulated tail vein. After determining a dose-response curve (0.001–0.5 mg), an optimal dose was chosen (0.250 mg of methacholine). This dose was used throughout the rest of the experiments in this study. After the methacholine challenge, the response was monitored and the peak airway resistance was recorded as a measure of airway hyperreactivity.

Collagenase dispersed lungs and bone marrow cell extraction

Lungs and lung draining mediastinal lymph nodes were isolated and dispersed using 1 mg/ml collagenase A (Roche Applied Science) in RPMI 1640 (Invitrogen) with 5% FCS (Atlas Biologicals) and 2 U/ml DNase (Sigma-Aldrich) at 37°C for 45 min. Cells were dispersed to a single-cell population by flushing tissue through a 16-gauge cannula. After lysis of RBC, cells were counted and numbers used in the calculation of specific cell populations were based upon differential staining in flow cytometry.

In vivo neutralization of SCF

Neutralization of SCF was conducted using a polyclonal rabbit anti-murine SCF Ab developed in our laboratory (2). The protein A column purified anti-SCF or control Ab was administered intratracheally with CRA at the time of the final two intratracheal challenges along with intranasal treatment daily given between the final two allergen challenges (200 μg/mouse) for a total of five daily administrations of Ab.

Quantification of cytokines

RNA was isolated from the upper right lobes of lung, lymph nodes, and bone marrow using TRizol (Invitrogen). Levels of mRNA were assessed using quantitative PCR analysis (TaqMan) with predeveloped primers and probe sets from Applied Biosystems. Quantification of the genes of inter-
estrogens were normalized to GAPDH and expressed as fold increases over the negative control for each treatment at each time point. Protein levels of cytokines were quantified using a Bio-Plex bead-based (Luminex) cytokine assay purchased from Bio-Rad Laboratories.

Flow cytometry analysis

Analyses of pulmonary and bone marrow cell populations by flow were assessed using previously established standard techniques. Briefly, Dulbecco’s PBS (DPBS) with 1% PBS and 0.09% sodium azide were used at all time points in staining procedures. A total of 1 × 10⁶ cells was used with viability staining with the Live/Dead staining kit (Invitrogen) along with the Fc receptors blocked using mouse Fc block (1 g/100 μl; BD Phari-
gramin) for 20 min on ice. Diluted fluorescent Abs specific for the different surface markers or control Abs were incubated for 10 min at 4°C. Cells were washed and the pelleted cells were resuspended in DPBS containing 1% PBS and 0.09% sodium azide and analyzed by flow cytometry on the same day to avoid loss of fluorescence intensity. In some experiments we also performed intracellular staining of formalin-fixed cells. The following monoclonal mAbs were obtained from BD Biosciences: CD11b-PE-Cy7, CD8a-PE-Cy5, streptavidin-allophycocyanin-Cy7, and MHC II (I-A-PE); from BioLegend: Ly6C-PE, Gr-1-AF700, and streptavidin-allophycocya-
in-Cy7; and from eBioscience: CD8a-PE-Cy5, FceRI α-AF647, c-kit-allo-
ophycocyanin, c-kit-PE-Cy7, Gr-1-PE, and anti-I-A-PE. Data were col-
culated in an LSR II BD Biosciences flow cytometer and analyzed using FlowJo software (Tree Star). The mAb to IL-17Rβ was biotinylated and used for flow cytometry.

Ag-elicted peritoneal eosinophil purification

Eosinophils were elicted by injection of thioglycolate plus soluble egg Ag into the peritoneum of Schistosoma mansoni-infected mice. The injection of soluble egg Ag into infected mice induces a pool of circulating eosinophils. Eosinophils were purified by negative selection using the MACS system. The Abs used were anti-
Thy1 (for T cells), anti-B220 (for B cells), and anti-class II (for APCs). After the plate adherence and MACS separation, the population of cells contained >97% eosinophils contaminated with neutrophils (∼1–2%) and mononuclear cells (lymphocytes and macrophages). Adherent cell populations were removed by plastic adherence. The nonadherent cells were washed and resuspended in PBS/BSA and eosinophils were purified by negative selection using the MACS system. The Abs used were anti-
Thy1 (for T cells), anti-B220 (for B cells), and anti-class II (for APCs). After the plate adherence and MACS separation, the population of cells contained >97% eosinophils contaminated with neutrophils (∼1–2%) and mononuclear cells (1–2%).

Bone marrow-derived mast cells

The generation of mast cells was accomplished as previously described (1) by culturing bone marrow with rSCF (50 ng/ml) and IL-3 (30 ng/ml) for 8 days. The cells were evaluated for FceRI by flow cytometry to establish mast cell phenotype along with cytokisin analysis. The cells were then used for evaluation of SCF-induced IL-25 and IL-25R expression.

Analysis of IgG1 and IgE levels in plasma

Anesthetized animals were bled by cardiac puncture. The syringes were placed in ice and blood was allowed to coagulate. Samples were centrifuged for 18 min at 800 × g for 30 min. The resulting serum was used to determine IgG1 and IgE levels. IgG and IgE capturing and detection Abs were from R&D Systems. The specific level was determined by direct ELISA on CRA-
coated 96-well plates as previously described using appropriate goat.
anti-mouse secondary Abs and reported as the mean absorbance at OD_{492} for comparison (32).

**Statistical analysis**

Data were evaluated by one-way ANOVA and, where appropriate, further evaluated with the parametric Student-Newman-Keuls test for multiple comparisons or the nonparametric Mann-Whitney rank-sum test.

**Results**

*Inhibition of SCF regulates Th2 cytokines and IL-25 responsiveness in the lung*

In previous studies, blocking SCF in the airway demonstrated a significant attenuation of airway hyperresponsiveness (AHR), eosinophil accumulation, mucus overproduction, and collagen deposition (2–4, 15, 16, 18). To better define these responses we examined the expression of Th2 cytokines and found the anti-SCF treatment significantly reduced the mRNA and protein expression of IL-4, IL-5, and IL-13 in the lungs of allergic mice (Fig. 1A and B). Interestingly, in these same mice the lymph node response was also examined and no alteration of T cell-associated Th2 cytokines was observed in animals treated with anti-SCF (data not shown). We suggested that the anti-SCF treatment was altering locally recruited cell populations that could influence or directly produce Th2 cytokines. In this model of chronic allergic responses we found no significant allergen-induced increase in IL-17 or IFN, and no change was observed with the anti-SCF treatment (data not shown). The data in Fig. 1C illustrate that during chronic allergen-induced disease the leukocyte numbers increase within the lung, and when animals were treated with anti-SCF the number of specific leukocyte subsets in the lung were significantly reduced, including the c-kit^{+} populations. Surprisingly, the number of T cells was not significantly altered. In contrast, when we examined the myeloid lineage cells, CD11b^{+} mononuclear and granulocytic (primarily eosinophils) populations, there was a significant reduction in these subsets. Examination of the histology demonstrated a significant decrease in leukocyte accumulation in the lung (Fig. 1D), especially eosinophils, along with reduced intensity of mucus and goblet cell presence as previously described (18). Finally, corresponding to the reduction in severity and Th2 cytokines, when allergen-specific IgE and IgG1 was examined there was a significant reduction with allergic animals that were treated with anti-SCF (Fig. 1E).

A recent set of studies has demonstrated that IL-25 can influence both T and non-T cell populations to produce significant levels of Th2 cytokines (25) and therefore may not entirely depend upon Ag-driven responses. In the present studies the expression of IL-25 was investigated in lungs of chronic allergen-challenged animals. There was a significant up-regulation of IL-25 during allergic responses in the lungs of challenged mice (Fig. 2), but not in draining lymph nodes during our studies (data not shown). When we depleted SCF by local airway administration of neutralizing Ab we observed a significant reduction in IL-25 expression, suggesting that one of the consequences leading to attenuation of disease with anti-SCF may be reduced IL-25-mediated responses. However, these results were not clear on whether SCF directly or indirectly drove IL-25 production.

Given the above data suggesting that blocking SCF locally would reduce Th2 cytokines and IL-25 production, our studies sought to verify that this was a direct mechanism in the lung. To more definitively identify what cell population was producing the cytokines, we sorted c-kit^{+} and c-kit^{-} cells from allergen-challenged mice. The mRNA was immediately isolated from the sorted cells and it was determined whether they were the source of IL-25 and Th2 cytokines. The data clearly indicate that while the c-kit^{+} cell population was the source of IL-25, the c-kit- cell population was the predominant source of the Th2 cytokines (Fig. 2B). Two cell populations that primarily express c-kit and can play a central role in promoting chronic allergic disorders are mast cells and eosinophils. In particular, c-kit^{+} eosinophils have been shown to respond to SCF closely associated with the induction of Th2 cytokines (15, 33). To further assess the possibility that eosinophils are a source of IL-25, purified eosinophils (>97% pure) were stimulated with SCF and the expression of IL-25 was assessed by real-time PCR. Eosinophils significantly up-regulated IL-25 expression when exposed to as little as 1 ng/ml SCF. We also grew mast cells from bone marrow and assessed whether these cells expressed IL-25 after SCF stimulation (Fig. 2D). No increase in IL-25 expression was observed after SCF stimulation in the mast cell cultures. Additionally, when mast cells or eosinophils were incubated with IL-25, no increase in Th2 cytokines, IL-4, IL-5, or IL-13, was observed, and no surface expression of IL-17RB could be detected by flow cytometry or by quantitative PCR (data not shown). Together, these studies begin to outline the activation pathway required for SCF-mediated IL-25 generation in eosinophils and suggest that they may be the predominant source of IL-25, but do not respond to IL-25, during allergen-induced disease.

*IL-25-induced Th2 cytokines in lungs and bone marrow of allergic mice*

We next assessed whether there were increases in IL-25-responsive cells in the lungs of the chronic allergen-challenged mice vs cells from naive mice, as previous studies have suggested that IL-25 can directly induce Th2 cytokines in an Ag-independent manner (20, 25). To understand this aspect, our experiments used enzymatically dispersed lungs from naive or chronic allergen-challenged mice as above. The dispersed cells were then exposed to rIL-25 (10 ng/ml; R&D Systems) in the absence of allergen, and the expression of Th2 cytokines was assessed. Interestingly, when we examined IL-25-induced Th2 cytokines there was a significant increase in the expression of Th2 cytokines, IL-4, IL-5, and IL-13, in lung cells from chronic allergen-challenged mice compared with cells from lungs of naive animals (Fig. 3A). Isolated cells from lungs of allergic animals treated with anti-SCF demonstrated a significant reduction in the Th2 cytokines IL-5 and IL-13 compared with allergic animals treated with control Ab. However, the response was not completely diminished in the anti-SCF-treated animals. An important issue to address is whether the IL-25-responsive cells that are found in the lung during allergic responses are locally or peripherally derived. A previous publication described that IL-25-responsive cells can be identified in the bone marrow (25). To examine whether the bone marrow was also altered during the chronic allergic response in the lung, we isolated cells from the long bones of the hind legs of the mice and characterized their responsiveness to IL-25. The data in Fig. 3B illustrate that when isolated bone marrow cells were stimulated by IL-25 (10 ng/ml per 3 x 10^{6} cells), there was a significant up-regulation of IL-4 and IL-13 in the bone marrow from allergic mice but not from naive mice. Additionally, when bone marrow from allergic mice treated with anti-SCF as above was stimulated with IL-25, significantly less IL-4 and IL-13 production was observed. These data suggest that the allergic response in the lungs altered the generation of IL-25-responsive cells in the bone marrow and was dependent in part on the production of pulmonary SCF. When we examined the plasma level of SCF in chronic allergen-challenged mice, we observed a significant increase in SCF levels compared with naive mice that were significantly reduced when mice were treated with...
anti-SCF into the airway (34), further supporting a feedback mechanism. Overall, these data thus far illustrate several important points: (1) IL-25 is up-regulated in the lung after chronic allergen challenge in a SCF-dependent manner, (2) IL-25-responsive cells are increased in the lung and bone marrow during chronic allergic responses, (3) the IL-25-induced Th2 cytokine production could be mediated in an Ag-independent manner, (4) IL-25 and Th2 cytokines are derived from two different

FIGURE 1. Neutralization of SCF attenuates IL-4, IL-5, and IL-13 expression in the lungs (A) and significantly reduces inflammation (B) during chronic allergen-induced pulmonary disease. Chronic allergen-sensitized and -challenged mice were given control or anti-SCF purified IgG Abs into the airways during the final two allergen challenge periods on a daily basis. Twenty-four hours after the final allergen challenge the lungs were harvested from the mice. The lungs were processed for mRNA isolation and cytokines were assessed by real-time PCR analysis with fold increase calculated in comparison to levels in lungs from naive mice (A). Cytokine protein levels were measured in lung homogenates processed using a lysis solution consisting of protease inhibitors and subsequently subjected to Bioplex analysis (B). Twenty-four hours after the final chronic allergen challenge lungs from control Ab or anti-SCF-treated animals were dispersed by collagenase to a single-cell suspension. Total cells counted and individual cell populations were characterized by flow cytometry analysis using specific cell subset markers. Total numbers of individual subsets were calculated using the flow cytometry analysis and the total numbers counted from individual mice (C). Lungs of individual mice were processed for histology, stained with H&E, and representative airways were photographed at ×400 and ×1000 magnification (D). In correspondence to the reduction of Th2 cytokines allergen-specific serum IgE and IgG1 were significantly reduced in anti-SCF-treated animals (E). *, p < 0.05 comparing anti-SCF-treated to control Ab-treated animals with five to seven mice per group.
cell populations, and (5) eosinophils are potentially a significant SCF-induced source of IL-25.

IL-25 exacerbates asthmatic disease and reconstitutes anti-SCF attenuation of local responses

To further describe the relationship between SCF and IL-25, we have recapitulated the model previously published where rIL-25 instilled into the airway can enhance the disease response (22, 23). In these studies rIL-25 (100 ng/mouse) was instilled into the airways of allergic mice upon the final challenges of allergen along with anti-SCF. The data demonstrate that when we added IL-25 into allergic control mice, we observed the expected exacerbation of pathophysiologic disease. Additionally, IL-25 administration reconstituted the AHR response in mice that received anti-SCF, or an alternative view may be that the anti-SCF treatment reduced IL-25-induced effects (Fig. 4A).

Analysis of the cytokine expression levels during the allergen-induced responses demonstrated that administration of rIL-25 into the airways of the anti-SCF-treated animals reconstituted the Th2 cytokine responses compared with control Ab-treated allergic animals (Fig. 4B). Finally, when histology of the tissue was examined, IL-25 clearly enhanced the disease based upon

![Graph A](Image)

**Figure 2.** SCF regulates pulmonary IL-25 production in c-kit⁺ eosinophils. Assessment of IL-25 expression levels was assessed in lungs of naive, chronic allergen challenge, and anti-SCF-treated animals (A). To identify the source of SCF-induced IL-25 in the lung chronic allergen-challenged animals had dispersed lung cells separated by MACS cell sorting for c-kit 24 h postchallenge. Immediately after the sort, c-kit⁺ or c-kit⁻ populations were processed for mRNA analysis for designated cytokines (B). Eosinophils were isolated from *S. mansoni*-infected mice (see Materials and Methods) and stimulated with SCF and assessed for IL-25 expression by quantitative PCR (C). Bone marrow-derived mast cells were grown in vitro with rSCF (50 ng/ml) and rIL-3 (50 ng/ml) for 2 wk and stimulated with various doses of SCF (D). *, p < 0.05.
inflammation and mucus staining, while the coadministration of IL-25 with anti-SCF showed an altered exacerbating response (Fig. 4C). Taken together, these studies have further linked IL-25-induced disease with the local overexpression of SCF within the lung and together appear to play a significant role in the progression of the disease.

Identification of IL-4-producing cells in the lungs of allergic mice using 4get mice and expression of IL-25R

To further investigate the Th2 cytokine producing population in the lung, we utilized 4get mice that possess a bicistronic expression of GFP along with IL-4 (IL-4-IRES-eGFP; 4get). Upon flow cytometry characterization of GFP+ cells in lungs of allergic animals, distinct subsets were positive for GFP/IL-4 (Fig. 5). Representative flow cytometry histograms demonstrate the distribution of total lung cells (Fig. 5A, left panels) and IL-4+/GFP+ lung cells (Fig. 5A, right panels). The lower right panel clearly depicts IL-4+CD11b+ cells as the main source of IL-4 in lung of chronic allergen-challenged animals. In our initial studies we compared a

FIGURE 3. IL-25 induces Th2 cytokines directly from dispersed lung cells. To begin to identify the direct target of IL-25, we isolated lungs cells from naive or allergic animals treated them with rIL-25 in vitro (A). In initial studies we examined the IL-25-induced Th2 cytokine expression from the lung cells. The protein expression data were assessed using Bioplex proteome analysis. Data represents means ± SE from five to six mice per group. *, p < 0.05 comparing allergic to naive lung cells; #, p < 0.05 comparing anti-SCF-treated to control Ab-treated animals. To assess the global effect of SCF expression in lungs of allergic animals on IL-25-induced Th2 cytokine responses bone marrow was assessed (B). Femurs from naive, chronic allergen-challenged mice, or animals treated with anti-SCF were flushed with PBS and isolated cells (2 × 10^6 cells/ml) were stimulated in vitro with IL-25 (10 ng/ml). After 4 h mRNA expression was assessed by quantitative PCR. Data represent means ± SE from four to five mice per group. *, p < 0.05.

FIGURE 4. Exogenous administration of rIL-25 (100 ng/ml) reconstitutes AHR responses (A) and the Th2 cytokine expression (B) in anti-SCF-treated animals. Chronic allergen-challenged animals were given anti-SCF and/or IL-25 during the final two allergens. AHR was measured at 24 h after final challenges. In separate experiments, animals were assessed for Th2 cytokines by isolating whole lung mRNA and performing quantitative PCR. Histologic examination of H&E-stained lungs from allergen-treated mice indicates a significant alteration in the inflammation in anti-SCF-treated animals that was enhanced and reconstituted by exogenous rIL-25 treatment (C). Studies were performed with five to eight mice per group. *, p < 0.05 comparing anti-SCF treated animals to control Ab-treated group in the IL-25 expression data; #, p < 0.05 in rIL-25 plus anti-SCF compared with anti-SCF alone; **, p < 0.05 of IL-25 treatment compared with allergen only.
chronic allergen-challenged animal with naive 4get mice and surprisingly found that most of the cells that were IL-4+ were CD11b+ while only a minority of the cells were CD4+. Subsequent experiments, illustrated in Fig. 6, were used to further characterize the IL-4+, CD11b+ myeloid cell population and illustrate that none of the CD11b+ cells was c-kit+, while half were Ly6C+ and nearly half were GR1+ (~45%). Finally, when we gated on the CD11b+/IL-4+ population, ~25% of cells were GR1+/Ly6C+ (Fig. 6B). The chronic allergen-sensitized and-challenged animals displayed a significant increase in GFP/IL-4+ cells in the lungs compared with naive animals, and these were significantly reduced upon anti-SCF treatment (Fig. 6, A and C). As demonstrated by the quantitative analysis in Fig. 6C, anti-SCF significantly reduced the IL-4+ cell populations in the lung with every marker we examined. To assure ourselves that the GFP reporter expression driven by the IL-4 promoter correlates directly in this model with IL-4 protein production, we utilized intracellular staining for IL-4 of the cells from the lungs of chronic allergen-treated animals (Fig. 6, D and E). The protein expression data correlate extremely well with the IL-4/GFP reporter assessment.

Because earlier literature had implicated basophils and mast cells as a significant source of Th2 cytokines, our analysis included FcεRI cells that would comprise basophils and mast cells (data not shown). Only a small number of the IL-4+ cells were FcεRI+, and they were not altered in the anti-SCF-treated animals, and none of the IL-4+ cells were c-kit+. Additional markers that were examined included CD11c and MHC class II, but neither of these markers displayed any correlation to the IL-4/GFP+ population in the lungs of allergic animals (data not shown). Additionally, the IL-4+ CD4+ T cells were not reduced upon anti-SCF treatment in the animals (data not shown). Overall, there appear to be several CD11B+ IL-4+ populations in the lung based upon additional surface marker staining.

Since our previous data had suggested that chronic allergen challenges resulted in increased IL-25 responsiveness, we examined the expression of IL-17RB (IL-25R) using a recently described specific mAb (30). The data illustrate that all of the IL-4+ IL-17RB+ cells were CD11B+, with most expressing GR1 and approximately half expressing Ly6C (Fig. 6F). When animals were treated with anti-SCF, the total numbers of IL-17RB+ cells were significantly reduced (Fig. 6G), reflecting the apparent responsiveness to IL-25 restimulation above. These data further suggest a correlation between chronic Th2 cytokine production, SCF, and IL-25 responsiveness.

Because we also identified that there were IL-25-responsive Th2 cytokine-producing cells in the bone marrow of allergic animals, the expression patterns of these cells were also examined. The data in Fig. 7 indicate that compared with naive 4get animals there was an up-regulation of IL-4 production in cells from the bone marrow of allergic mice. Additionally, the animals treated with anti-SCF into the airway have a decreased expression of the IL-4+ cells, with the wild-type mice having ~5% and anti-SCF-treated mice having ~2% IL-4/GFP+ cells within the bone marrow (Fig. 7A). Analysis of the individual markers again demonstrated a similar phenotype as in the lung, with nearly all of the IL-4+ cells being CD11B+ (>95%), most GR1+ (>80%), some Ly6C+ (~25%), and, unlike the lung, many were c-kit+ (~60%), perhaps suggesting a maturing phenotype on its way to losing c-kit expression once it migrates into the lung (Fig. 7A). Additionally, when we examined FcεRI very little expression was observed in the IL-4+ population, similar to the lung cell data (data not shown). Thus, it appears that a similar cell population is also increased in the bone marrow and that neutralization of SCF in airways of allergen-challenged mice reduces the number of the cell population. We also examined the expression of IL-17RB+ on the bone marrow-derived cells and found that ~8% of the overall IL-4+ cells in the bone marrow expressed IL-17RB+, again at a lower percentage than those found in the lung (Fig. 7B). However, the IL-17RB+ cells were CD11B+, GR1+ cells similar to the IL-17RB+ cells in the lung, but were also c-kit+, suggesting that perhaps the cells mature before reaching the lung or once they migrate into the lung. These data support the findings in Fig. 3B demonstrating IL-25-induced Th2 cytokines in bone marrow of chronic allergen-challenged mice. Overall, these data are striking, and while the maturation of this cell population is unclear, it likely demarcates an important and potentially pathogenic cell that can contribute to the allergic inflammation.

**Discussion**

Previous studies have established a significant role for stem cell factor during the development of allergic airway inflammation that was associated with severity and disease progression (12, 16, 18, 35, 36). During allergic airway responses SCF appears to impact...
mast cell activation, eosinophil accumulation and activation, as well as having an effect on the airway remodeling and physiologic responses. In the present studies the focus was on the cellular mechanism of how SCF altered the ongoing responses in a therapeutic model. While the IL-25 expression by eosinophils in response to SCF may provide a pivotal disease-enhancing effect
locally in the lungs during an allergen challenge, the most interesting results pertain to the IL-4+ myeloid cells that respond to IL-25, resulting in Th2 cytokine production. Previous studies have identified eosinophils as a potential source of IL-25 (27). SCF-stimulated eosinophils have enhanced binding to VCAM-1 and fibronectin (33) and enhanced survival (17), and SCF also can induce profibrotic and chemotactic mediators in eosinophils (15) and now IL-25 production. In the present research a link was made establishing a specific role of SCF/c-Kit activation for the generation of IL-25, leading to the progression and severity of chronic allergic airway disease. While a number of studies have recently been published that demonstrate that IL-25 has a significant role in the production of Th2 cytokines and influences the severity of inflammatory Th2-associated disease models, the mechanism of activation and progression is not completely clear (21–23, 27, 37). Studies have demonstrated that IL-25 induces enhanced Th2 cytokine production through at least two different cell populations. An Ag-specific T cell response is enhanced by IL-25 and induces additional Th2 cytokines to be produced, as well as augments Th2 cell polarization and memory (23, 27). A second bone marrow-derived non-T cell population also appears to respond to IL-25 and produce cytokines (25). These latter data help to define the mechanism of how IL-25 directly induced Th2 cytokines when injected in vivo in the original studies (20) and in a recent study when IL-25 was directly injected into the airway of mice (30). The recognition that SCF influences the production of Th2 cytokines in the lung is reflected by previous studies using locally delivered anti-SCF for alteration of severe asthma-like responses (16, 18, 35). This latter aspect is also highlighted by our findings indicating that anti-SCF does not alter lymph node-associated Th2 cytokine production (data not shown), a finding in the lymph node that was also demonstrated when IL-25 blockade was used (21).
While we have not compared the relative level of Th2 cytokines that each IL-4+ cell population produces, most of the IL-4+ cells within the lung appear to be CD11B- myeloid cells of varying phenotype based upon surface protein expression assessed by flow cytometry. The use of an IL-25R-specific Ab (30) identified a subset of the myeloid cell population that can produce IL-4 with a discrete cell population that was CD11B+ GR1+ and c-kit+ in the lung after chronic allergen challenge. A striking result was the downstream effects found in the bone marrow during allergic responses where the generation of the IL-25-responsive cells was reduced when SCF was blocked in the lung. These cells were phenotypically similar to those found in the lung except that they also expressed c-kit. Thus, the effects of SCF appear to be global in nature, even though the protein appears to be made locally in response to allergen challenge. These results are consistent with the generation of IL-25-responsive cells in the bone marrow during helminth-induced Th2 responses that demonstrated a c-kit+ myeloid cell capable of producing IL-4 (25). We would suggest that in the previous publication, which utilized a very immunogenic, comparatively acute parasitic response, that the c-kit+ population from the bone marrow accumulated in tissue, whereas our chronic allergen challenge led to enhanced SCF production locally in the lung, leading to a more mature cell phenotype. It might be intriguing to examine whether longer treatment protocols might help to eliminate this population that persisted in the lung that is likely derived from the c-kit+ bone marrow population. A recent study has identified an IL-13-producing myeloid cell that is MHC class II+ during persistent viral infections (29), while another finding indicated that a c-kit+ (SCF receptor) dendritic cells promote Th2 and Th17 cytokine associated with allergic disease (38, 39). It is tempting to speculate that these are related populations to the one we have identified in a differentially activated immune environment. While bone marrow-derived cells express Th2 cytokines in response to IL-25, we would suggest that the cells depend upon SCF to allow maturation and/or skewing toward Th2 cytokine producing cells. It would follow that when SCF was blocked locally in the lung that would be reduced local Th2 cytokines due to a reduction in IL-25.

The intriguing finding that pulmonary generated SCF influences the development of an IL-25-responsive cell population in the bone marrow may demonstrate an important feedback mechanism. While we have not thoroughly examined other potential signals, it may be that the generation of the IL-25-responsive cell populations depend upon additional cytokines, such as IL-4 or IL-25 itself, that are also needed to establish the proper immune environment. Since SCF also influences the generation of chemokines during allergen-induced responses (16, 17, 40), the recruitment of the IL-25-responsive cell from the bone marrow to the lungs may also be altered upon local blockade of SCF. A previous study identified that an IL-25-responsive cell population that produced Th2 cytokines was CCR2- and CCR3-positive (25), corresponding to chemokines that are highly up-regulated during Th2-type responses, that is, CCL2 and CCL11 family chemokines. These data along with the finding that many of these cells have a CD11b+GR1+ (Ly6C+Ly6G+) “inflammatory” monocyte phenotype suggest that perhaps this cell population may be present in multiple inflammatory sites, including the gut and bladder (41, 42). This phenotypic designation would predict that they should respond to CCR2 ligands for migration into the lungs as originally defined (43). Whether additional maturation signals are needed for the cell to obtain the ability to respond to IL-25 and/or produce Th2 cytokines is an area for further investigation.

Most studies have examined T cells as a focus of IL-25 responsiveness. Studies have suggested that IL-25 augments the skewing of Th2 cells by not only direct effects on the T cell but also by indirect effects on the APC (21, 22, 23, 27). This response has been coordinated with other factors that appear to affect the nature of the response, such as TSLP and IL-13. Interestingly, the most profound effects of IL-25 are found in the presence of APC and specific Ag, further supporting both direct and indirect effects on T cells. In the present studies, our analysis of CD4 T cells indicated that a relatively small percentage of the lung CD4 cells were also IL-4+, which may represent differentiated Th2-type cells (27). Although the source of IL-25 has not been fully elucidated, it can be produced by a number of cell populations. While we did not detect IL-25 in bone marrow-derived mast cells stimulated with SCF, they have been described to produce IL-25 by other stimuli (44). A recent study has suggested that epithelial cells produce IL-25 in response to innate signals from allergens (22). This latter observation may be especially relevant during respiratory allergen and virus-induced exacerbations within a Th2 environment to activate IL-25 production, leading to subsequent exacerbation by Th2-producing myeloid cells. How these responses correlate to the findings in the present studies with blockade of SCF is unclear, but they suggest that there are likely multiple sources of IL-25 that could exacerbate the Th2 environment. Interestingly, the inhibition observed by SCF blockade could be overcome by the addition of IL-25 into the airway. While the numbers of IL-4+ T cells were not significantly altered with anti-SCF and the number of IL-4/GFP+ cells did not increase as significantly as did the myeloid cell population during chronic allergen challenge, the T cells likely produce much higher levels of Th2 cytokine on a per cell basis. It may be that during exacerbations the increased number of Th2 cytokine-producing myeloid cells adds a significant level of additional cytokine, resulting in the most severe disease.

Taken together, these studies demonstrate an important mechanistic role for SCF that correlates to observations in human asthma where an increased expression of SCF in the lung and in serum after allergen challenges was observed and correlated to the severity of disease (11–14). Thus, a complex network of activation may be emerging with SCF as an important initiating cytokine locally in the lung with its ability to activate IL-25 production during allergic responses as well as contribute to the maturation of the IL-17RB+ cells in the lung and/or bone in a Th2 immune environment. A key feature is production of IL-25 by SCF-stimulated eosinophils, which are only present during allergic disease. While there appear to be multiple populations of IL-4-producing cells in the lung, the IL-17RB+ cells appear to be CD11b+GR1+. This overall concept supports the role of SCF for local lung as well as bone marrow-associated effects influencing the overall responses. It is now reasonable to speculate that targeting SCF in the airway during allergic asthma could provide an effective treatment for reducing IL-25 and Th2 cytokines and therefore allergic asthmatic responses.

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Disclosures

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