

Thymic stromal lymphopoietin and IL-33 modulate migration of hematopoietic progenitor cells in patients with allergic asthma

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Background: Thymic stromal lymphopoietin (TSLP) and IL-33 are considered important initiators of type 2 immunity. In asthmatic patients allergic inflammatory responses are associated with increased lung homing of bone marrow–derived CD34⁺ hematopoietic progenitor cells (HPCs), which include eosinophil lineage–committed progenitor cells. In this study we investigated the role of TSLP and IL-33 in the recruitment of progenitor cells to the airways in asthmatic subjects.

Objectives: We sought (i) to examine the effect of allergen inhalation challenge on expression levels of receptors for TSLP (thymic stromal lymphopoietin receptor [TSLPR] and CD127) and IL-33 (ST2) and (ii) investigate the functional effects of these cytokines on HPCs.

Methods: Consenting patients with mild atopic asthma (n = 19) with an FEV₁ of 70% or greater and methacholine PC₂₀ of 16 mg/mL or less were recruited. Blood- and sputum-extracted progenitors were phenotyped by flow cytometry before and 24 hours after allergen challenge. Functional responses, including cytokine production and migration to TSLP and IL-33, were assessed *in vitro*.

Results: Significant increases in mature eosinophil, HPC, and eosinophil lineage–committed progenitor cell counts in sputum were observed 24 hours after allergen and were associated with a significant allergen-induced increase in HPCs expressing TSLPR, CD127, and ST2. Pre-exposure to TSLP and IL-33 primed the migration of HPCs to a potent progenitor cell chemoattractant, stromal cell–derived factor 1 α (CXCL12). Incubation with TSLP and IL-33 stimulated significant production of IL-5 and IL-13, but not IL-4, by HPCs. This priming effect was inhibited by blocking antibodies to TSLPR and ST2, respectively, and IL-13 receptor α 1 in both scenarios.

Conclusions: In allergic asthmatic responses increased lung homing of HPCs may be orchestrated by TSLP and IL-33 through an IL-13–dependent axis. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Hematopoietic progenitor cells, thymic stromal lymphopoietin, asthma

Asthma is characterized by airway inflammation, reversible airway obstruction, and airway hyperresponsiveness. Eosinophilic inflammation is believed to be a major contributor to the physiologic changes and remodeling seen in asthmatic patients.^{1,2} Eosinophils are present and activated within the airway lumen and tissue of patients with current asthma, are increased in number when asthma is uncontrolled³ or severe,⁴ and are decreased in number when asthma is controlled.⁵ Treatment strategies that aim to control airway eosinophilia are significantly more effective at decreasing the number of exacerbations than guidelines-based clinical strategies and are steroid sparing and thus less expensive for improving asthma control.⁶⁻⁸ Understanding physiologic processes that promote local airway eosinophilia in asthmatic patients are critical for the development of novel treatment modalities.

A considerable body of evidence supports an association between allergen-induced asthmatic responses and mobilization of progenitors from the bone marrow.^{9,10} We and others have shown that affected tissues support the local differentiation, proliferation, and maturation of hematopoietic progenitor cells (HPCs), which home to the site of allergen exposure in patients with allergic airways diseases.¹¹⁻¹³ Investigating factors that promote lung homing of bone marrow–derived progenitor cells will provide tools to investigate the role of progenitors in the pathogenesis of eosinophilic asthma and provide novel treatment strategies for improved asthma control.

An emerging hypothesis proposes that chronic respiratory diseases, including allergic asthma, are primarily driven by alterations of the mucosal interface compartment.¹⁴ In asthmatic patients airway epithelial cells activated by allergens, viruses, or environmental pollutants produce cytokines, including thymic stromal lymphopoietin (TSLP), IL-25, and IL-33.¹⁵⁻¹⁷ These cytokines are considered to be essential early initiators of type 2 immune responses and are proposed to contribute to the pathogenesis of asthma.^{18,19} In genome-wide association studies, single nucleotide polymorphisms of genes for TSLP, IL-33, and the IL-33 receptor ST2 have been identified as being associated with atopy²⁰ and increased susceptibility to bronchial asthma in general.²¹⁻²³ Studies have now shown that TSLP and IL-33 are detectable at higher levels in the airways of asthmatic patients

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Abbreviations used

EoP: Eosinophil lineage-committed progenitor
 HPC: Hematopoietic progenitor cell
 IL-4R: IL-4 receptor
 IL-13R: IL-13 receptor
 PE: Phycoerythrin
 TSLP: Thymic stromal lymphopoietin
 TSLPR: Thymic stromal lymphopoietin receptor

and correlate with disease severity.^{15,16,24} In addition, increased amounts of TSLP and IL-33 have been reported to be spontaneously released at picomolar concentrations from airway epithelial cells cultured from patients with severe asthma.^{15,16} Interestingly, TSLP and IL-33, which are known to activate various mature cells of the innate and adaptive immune pathways, have now been shown to act directly on CD34⁺ HPCs to stimulate T_H2 cytokine production,²⁵ basophilopoiesis in mice,²⁶ lineage commitment,²⁷ and outgrowth of eosinophil/basophil colony-forming units from human CD34⁺ HPCs.²⁸ We hypothesize that airway epithelium-derived cytokines act directly on HPCs to promote lung homing during allergic asthmatic responses.

The novel findings of this study are that after allergen inhalation challenge in patients with mild asthma, there is increased lung homing of HPCs that express receptors for TSLP and IL-33. *In vitro* experiments showed that TSLP and IL-33 were equipotent at priming the directional migration of HPCs and that this effect was mediated through autologous production of IL-13. These data suggest that TSLP and IL-33 might act to stimulate local eosinophilia by promoting the lung homing of HPCs to the airways in asthmatic patients.

METHODS**Subjects**

Nonsmoking subjects with mild stable asthma were recruited for this study. Subjects (aged 19–60 years) had positive skin prick test responses, FEV₁ of 70% of predicted value or greater, and baseline methacholine PC₂₀ of 16 mg/mL or less. Subjects who were healthy for 4 weeks before the study were steroid naive, infrequently used inhaled β_2 -agonists, and provided written informed consent. All experimental procedures were reviewed and approved by the institutional research ethics board.

Study design

Allergen/diluent challenges were randomized with a 2-week washout period. Subjects attended the laboratory. At visits 1 and 4, medical history, skin prick tests, spirometry, methacholine inhalation, and sputum induction were performed; at visits 2 and 5, allergen/diluent inhalation challenge were performed; and at visits 3 and 6 (24 hours after challenge), methacholine inhalation and sputum induction were performed.

Airway measurement and allergen inhalation challenge

Methacholine inhalation challenge was performed by using the tidal breathing method, as previously described.^{29,30} Subjects inhaled incremental doubling concentrations of methacholine phosphate with a Wright nebulizer. PC₂₀ was calculated as the provocative concentration of methacholine causing a 20% decrease in FEV₁.

Allergen inhalation challenge was performed as described in detail in the **Methods** section in this article's Online Repository at www.jacionline.org.³¹

TABLE I. Subjects' lung function and sputum details

	Allergen challenge	Diluent challenge
Early phase (% change in FEV ₁)	−32.24 (1.75)*	1.89 (0.58)
Late phase (% change in FEV ₁)	−13.38 (0.58)*	3.58 (0.83)
Methacholine PC ₂₀ (mg/mL)		
Before challenge	4.29 (0.87)	4.86 (1.44)
24 h After challenge	1.83 (0.31)*	6.70 (1.89)
Total sputum cells (×10 ⁶ cells/mL)		
Before challenge	2.41 (0.54)	3.27 (0.77)
24 h After challenge	4.66 (1.06)*	3.35 (0.60)
Sputum eosinophils (×10 ⁴ cells/mL)		
Before challenge	4.23 (4.98)	6.63 (4.98)
24 h After challenge	35.91 (11.99)*	5.96 (2.38)

Values are presented as geometric mean ($\pm$ SEM) in parentheses. There was a significant difference for both early- and late-phase methacholine PC₂₀, total sputum cell counts, and sputum eosinophil counts.

**P* < .05 compared with diluent.

and subjects' characteristics are summarized in **Tables E1** and **E2** in this article's Online Repository at www.jacionline.org.

Sputum induction and cell isolation

Sputum was induced by means of hypertonic saline inhalation, as previously described.³² The sputum samples were processed by selecting mucus plugs, mixing with 4 parts 0.1% dithiothreitol and Dulbecco PBS, and filtered through a 52- μ m nylon mesh.³³ The cells in the filtrate were pelleted and resuspended in Dulbecco PBS. Cytospin preparations were prepared on glass slides and stained with Diff-Quik (American Scientific Products, McGaw Park, Ill) for differential counts expressed as percentage means of duplicate slides (400 cells counted per slide). The remaining cells were placed in aliquots for flow cytometry.

Progenitor cell isolation from peripheral blood

Peripheral blood from venipuncture was drawn into heparin (1000 U/mL) and layered on Accu-Prep (Accurate Chemical & Scientific Corp, New York, NY) for density gradient sedimentation. Monocytes were depleted by means of adherence to plastic (for 2 hours in a 5% CO₂ atmosphere at 37°C), and CD34⁺ cells were enriched by means of positive selection with MACS immunomagnetic beads (Miltenyi Biotec, Auburn, Calif). Cell purity of CD34⁺ cells was greater than 94%, and viability was greater than 93%.

Immunofluorescence staining and flow cytometric gating strategy

Expression levels of the receptors for TSLP (thymic stromal lymphopoietin receptor [TSLPR] and CD127:IL-7R α) and IL-33 (ST2) were determined by means of flow cytometry. Peripheral blood- and sputum-extracted cells were immunostained with CD34-e450, CD45-AmCyan, CD125-fluorescein isothiocyanate, TSLPR-PerCP-eFluor710, CD127-biotin-streptavidin-phycoerythrin (PE)-Texas red, and ST2-PE or relevant isotype controls (BD Biosciences, Mississauga, Ontario, Canada; eBioscience, San Diego, Calif; and R&D Systems, Minneapolis, Minn). Cells were identified as HPCs (CD34^{high}CD45^{dull}; see **Fig E1, A**, in this article's Online Repository at www.jacionline.org) and eosinophil lineage-committed progenitor (EoP) cells (CD34^{high}CD45^{dull}CD125^{high}), as previously described, after acquisition of 300,000 events.³⁴ The gating strategy is described in **Fig E1, B**.

Progenitor cell transmigration assays

The migrational responses of progenitors were assessed in Transwell chambers (24-well cell clusters; 6.5-mm Transwell with 5- μ m pore polycarbonate membrane insert filters; Corning Costar, Corning, NY), as

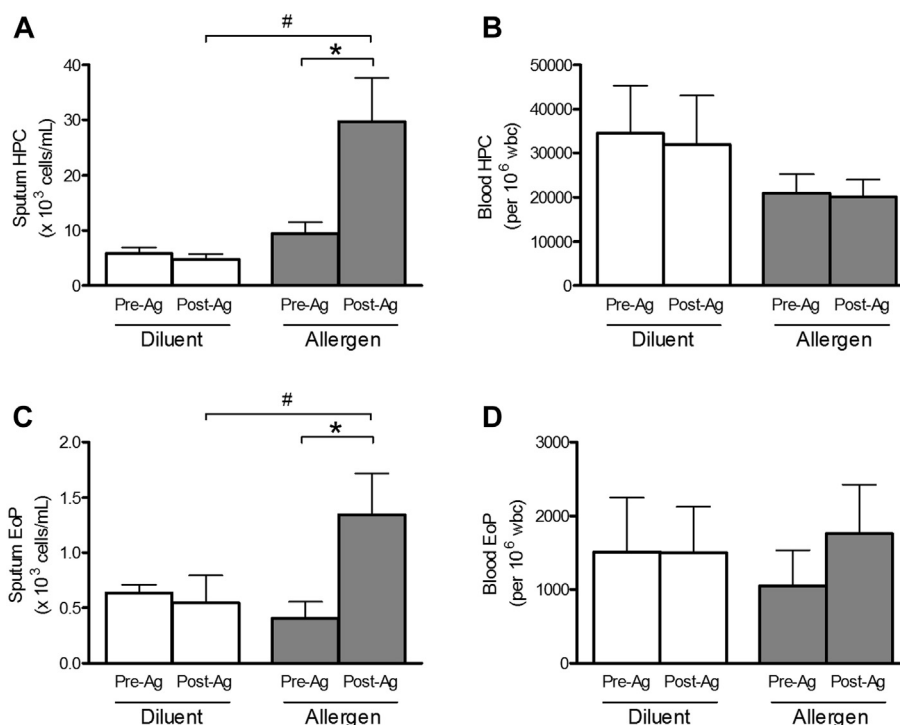


FIG 1. Fluctuations in sputum and blood HPC (A and B, respectively) and EoP cell (C and D, respectively) counts in patients with mild asthma after diluent (open bars) or allergen (Ag; solid bars) inhalation challenge. HPC and EoP cell counts significantly increased 24 hours after allergen compared with baseline values. Data are mean $\pm$ SEM (n = 15). * P < .05 compared with baseline. # P < .05 compared with diluent.

previously described^{34,35} and as described in detail in the [Methods](#) section in this article's Online Repository. Migrated cells were expressed as a percentage of the total cells added to the top Transwell (the gating strategy is shown in [Fig E2](#) in this article's Online Repository at www.jacionline.org). Cells were preincubated for 15 minutes with nystatin (25 μ g/mL) or amphotericin B (10 μ g/mL) before the priming and transmigration assay (Sigma, St Louis, Mo) to evaluate the influence of lipid raft formation on migration, as previously described.^{35,36}

Stimulated intracellular cytokine detection

Enriched CD34⁺ cells (1×10^6 cells/mL) in Iscove medium plus 10% FBS and monensin were stimulated with TSLP, IL-33, or both (for 6 hours in a 5% CO₂ atmosphere at 37°C) and fixed in 500 μ L of 1% paraformaldehyde. Cells were immunostained with CD34-PerCP and CD45-allophycocyanin-Cy7, IL-5-fluorescein isothiocyanate, IL-13-PE, and IL-4-PE (BD Biosciences) and enumerated by using flow cytometry.

Statistical analysis

Data are expressed as mean $\pm$ SEM. The allergen-induced changes from baseline were analyzed by using paired t tests on GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif). Priming of migration was analyzed by using ANOVA. For data that were not normally distributed, statistics were performed on log-transformed data. The α value was set at .05.

RESULTS

Airway physiology and inflammation

After allergen challenge, subjects had a significant maximal decrease in FEV₁ during the early and late responses (P < .05) compared with that seen after diluent challenge (P < .05, [Table I](#)). In sputum there was a significant increase in total cell

counts and mature eosinophil counts when preallergen values were compared with those 24 hours after postallergen ([Table I](#)).

Sputum progenitor cells: Allergen-induced TSLPR and IL-33 receptor expression

Progenitor cell assessments in sputum were performed using sequential multigating flow cytometric analyses (see [Fig E1](#)). As previously shown, we found that in response to allergen but not diluent challenge, there was a significant increase in HPC counts when prechallenge values were compared with 24-hour postchallenge values (P < .05; [Fig 1, A](#)).^{33,34} In addition, there were significantly more HPCs in the sputum when 24-hour postchallenge values were compared with 24-hour postdiluent values (P < .05). Similarly, we found a significant increase in the absolute number of EoP cells in induced sputum when comparing 24-hour postallergen values with prechallenge and 24-hour postdiluent values (P < .05; [Fig 1, C](#)). No significant changes in blood HPC and EoP cell counts were found ([Fig 1, B and D](#)).

Expression of TSLP receptor components (TSLPR and CD127) and IL-33 receptor (ST2) were detected at both the protein and mRNA levels on HPCs (see [Figs E1 and E3](#) in this article's Online Repository at www.jacionline.org). After allergen challenge, phenotypic analyses of progenitor cells in sputum showed a significant increase in absolute number of HPCs expressing TSLPR, CD127, and ST2 compared with prechallenge and 24-hour postdiluent values (P < .05, [Fig 2](#)).

In contrast, we found no significant allergen-induced increase in absolute numbers of EoP cells expressing TSLPR, CD127, or ST2 in sputum (see [Table E3](#) in this article's Online Repository at www.jacionline.org). In addition, no significant change in these

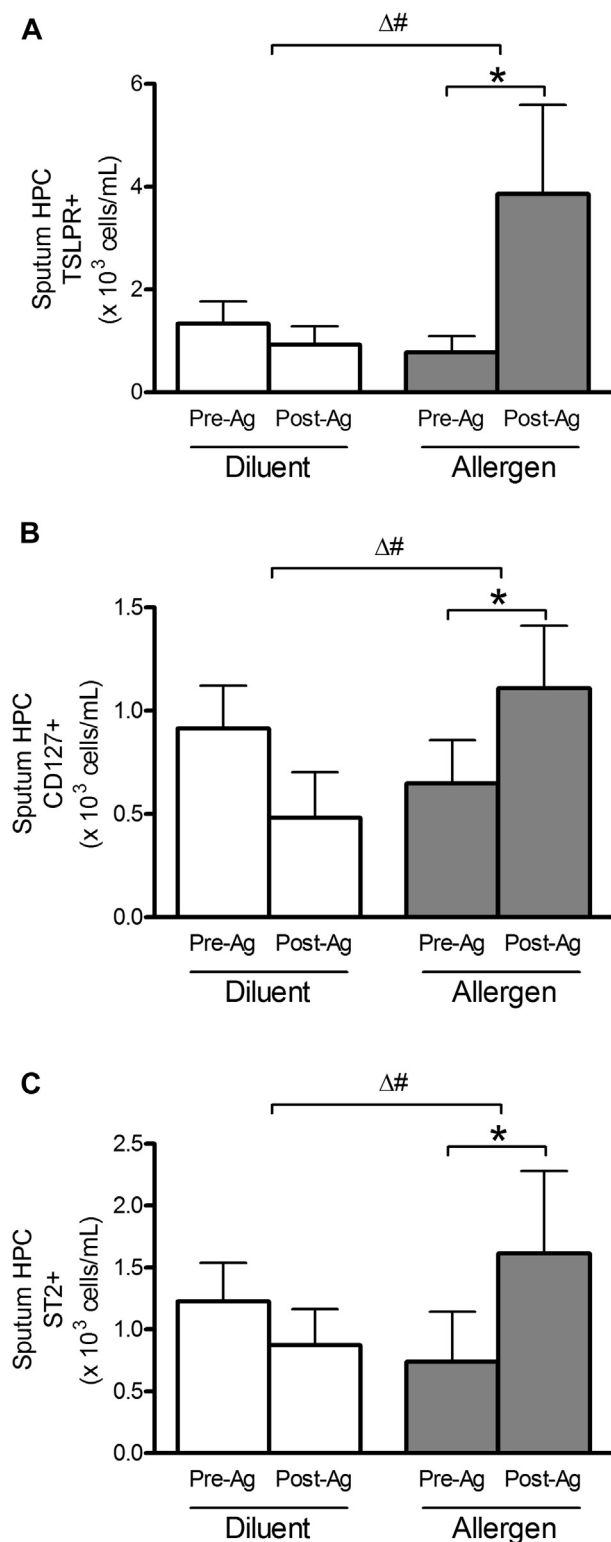


FIG 2. Changes in sputum HPC expression of TSLPR (A), CD127 (B), and ST2 (C) for diluent (open bars) and allergen (Ag; solid bars) inhalation challenge. Numbers of HPCs expressing TSLPR, ST2, and CD127 significantly increased after allergen. Data are mean $\pm$ SEM ($n = 10$). * $P < .05$ compared with baseline. # $P < .05$, Δ comparison with diluent.

receptors was detected on either HPCs or EoP cells in the blood when prechallenge values were compared with 24-hour postchallenge values (see Table E4 in this article's Online Repository at www.jacionline.org).

TSLP and IL-33 priming of HPC migration *in vitro*

Activation of CD34⁺ progenitor cells by TSLP and IL-33 and its effect on lineage commitment and promotion of eosinophilopoiesis/basophilopoiesis has been described.^{25,27} However, there is no information on the effect of these cytokines on the lung homing of progenitor cells. The role of TSLP and IL-33 in modulating the migrational responses of HPCs from peripheral blood was investigated *in vitro* by using Transwell chambers. Although TSLP and IL-33 did not stimulate migration of HPCs directly, overnight incubation with physiologically relevant doses of TSLP (optimal at 1 pg/mL for 18 hours at 37°C in a 5% CO₂ atmosphere) significantly enhanced the migration of HPCs to a suboptimal dose of CXCL12 (10 ng/mL), a potent progenitor cell chemoattractant ($P < .05$; Fig 3, A, and see Fig E4 in this article's Online Repository at www.jacionline.org for fluorescence-activated cell sorting analysis plots). Similarly, preincubation with IL-33 (optimal at 1 pg/mL for 18 hours at 37°C in a 5% CO₂ atmosphere) significantly primed the migrational response of HPCs to CXCL12 ($P < .05$; Fig 3, B). TSLP and IL-33 were equipotent at enhancing HPC migrational responses to CXCL12. We found no additive or synergistic effect of these cytokines (Fig 3, A and B). In addition, TSLP and IL-33 did not prime the migrational responses of HPCs to other chemoattractants, including eotaxin, RANTES, or monocyte chemoattractant protein 1 (see Fig E5 in this article's Online Repository at www.jacionline.org). Phenotypic analyses of the responding cells showed that neither TSLP nor IL-33 primed the migrational responses of blood-extracted EoP cells *in vitro* (Fig 3, C and D).

Inhibition of TSLP and IL-33 priming of HPC migration

An enriched population of CD34⁺ cells was incubated with a blocking TSLPR antibody (1–100 ng/mL) for 15 minutes prior to incubation with TSLP (1 pg/mL) to determine the specificity of the TSLP priming effect. TSLPR (10 ng/mL) treatment significantly inhibited the priming effect of TSLP on CXCL12-stimulated migration of HPCs to preprimed levels ($P < .05$; Fig 3, E). Similarly, CD34⁺ cells were incubated with ST2 antibody for 15 minutes before incubation with an optimal priming dose of IL-33 (1 pg/mL) to determine the specificity of the IL-33 priming effect. ST2 antibody (10 ng/mL) significantly decreased IL-33 priming of CXCL12-stimulated migration to preprimed levels ($P < .05$; Fig 3, F). The HPC priming effect of TSLP was not inhibited by ST2 antibody, and the priming effect of IL-33 was not inhibited in the presence of TSLPR (Fig 3, E and F).

TSLP and IL-33 stimulated T_H2 cytokine generation by HPCs

An enriched population of CD34⁺ cells from the blood of patients with mild asthma was incubated with concentrations of

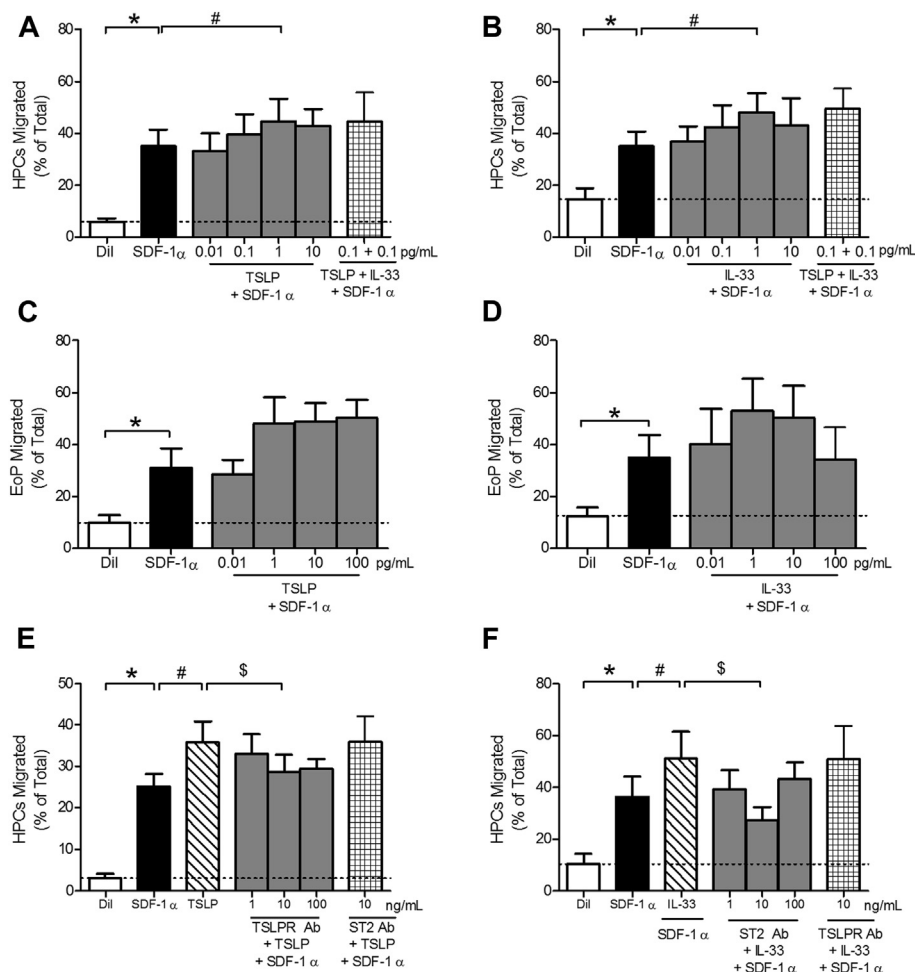


FIG 3. TSLP and IL-33 priming of HPC (A and B) and EoP cell (C and D) migration. Preincubation with TSLP or IL-33 primed migration of HPCs. TSLP and IL-33 primed migration of HPCs. TSLP and IL-33 receptor antibody blocked priming (E and F). Data are mean $\pm$ SEM (n = 8). * P < .05 compared with diluent. # P < .05 compared with CXCL12. \$ P < .05 compared with priming response. Ab, Antibody; Dil, diluent; SDF-1 α , stromal cell-derived factor 1 α .

TSLP or IL-33 that recapitulated the migration priming experiments (0.1-10 pg/mL) to determine whether the epithelium-derived cytokines can induce IL-5, IL-13, and IL-4 expression by HPCs. Compared with diluent, exposure to either TSLP (1 pg/mL) or IL-33 (1 pg/mL) significantly increased the proportion of HPCs expressing intracellular IL-5 (P < .05; Fig 4, A) and IL-13 (P < .05; Fig 4, B). No effect on IL-4 expression by HPCs was observed after incubation with either TSLP or IL-33 (Fig 4, C).

Inhibition of TSLP and IL-33 priming of HPCs by IL-13 receptor (IL-13R) α 1

We have previously shown that IL-13 and IL-4, but not IL-5, can prime the migrational responses of HPCs but not EoP cells.³⁵ In the current study we investigated whether the priming effects of TSLP and IL-33 are mediated indirectly through autologous production of IL-13 by HPCs. When CD34⁺ cells were incubated with a blocking IL-13R α 1 antibody (0.1-10 ng/mL) for 15 minutes before incubation with TSLP or IL-33 (1 pg/mL), there was a significant inhibition of the priming effect to CXCL12-stimulated migration (P < .05; Fig 5, A and B). In the presence of the IL-4 receptor (IL-4R) blocking antibody

(IL-4R α , 0.1-10 ng/mL), there was no effect on the priming effects of TSLP or IL-33 to CXCL12-stimulated migration of HPCs (Fig 5, C and D).

Effect of lipid raft disruption on TSLP and IL-33 priming effects on HPCs

To investigate the mechanism by which TSLP and IL-33 prime the migrational response of HPCs to CXCL12, we examined the level of cell-surface expression of CXCR4. Incubating an enriched population of HPCs with either TSLP (0.1-10 pg/mL) or IL-33 (0.1-10 pg/mL; for 18 hours in a 5% CO₂ atmosphere at 37°C) had no effect on either the proportion of HPCs expressing CXCR4 or the intensity of CXCR4 expression on HPCs (see Fig E6 in this article's Online Repository at www.jacionline.org).

To determine whether increased incorporation of CXCR4 into signaling lipid rafts within the plasma membrane is involved in the observed priming effect of TSLP and IL-33, we examined the effect of lipid raft disruption on CXCL12-stimulated migration of HPCs. Blood-derived CD34⁺ cells were incubated with previously optimized doses of polyene antibiotics, amphotericin B (10 μ g/mL), and nystatin (25 μ g/mL) for 15 minutes before

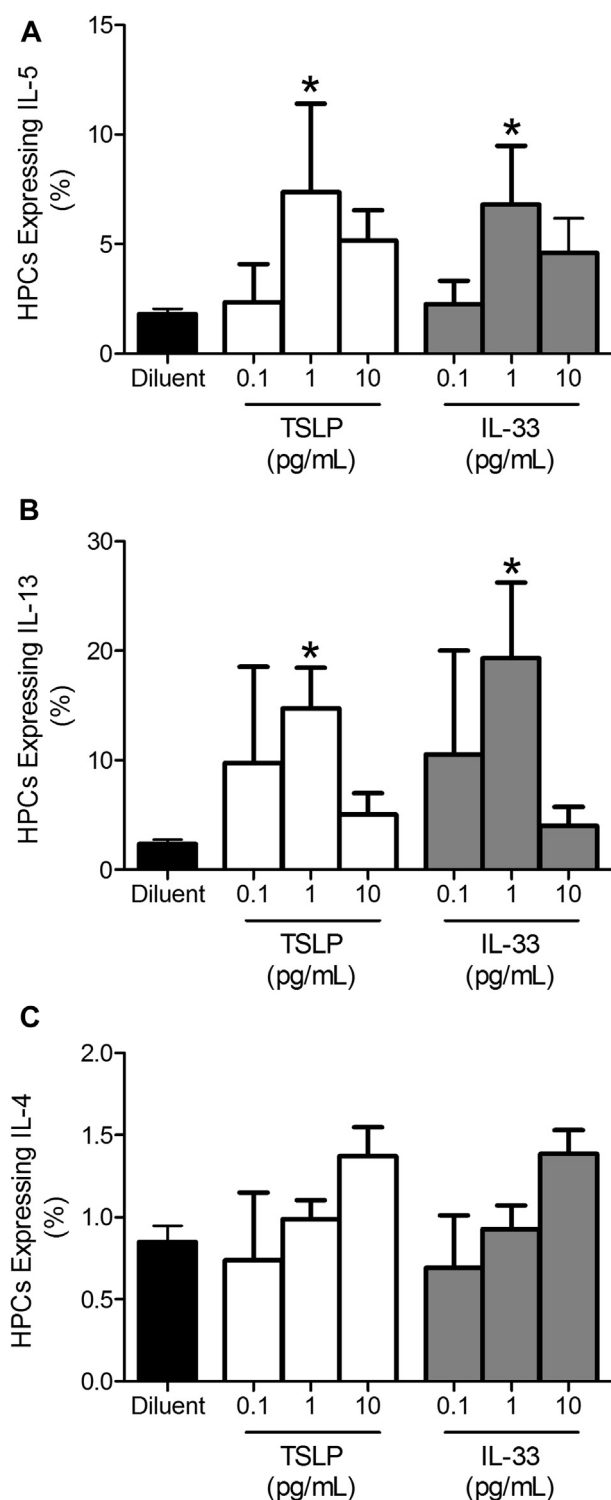


FIG 4. Effect of TSLP and IL-33 on intracellular expression of IL-5, IL-13, and IL-4 in HPCs. Incubation for 6 hours with TSLP (1 pg/mL) or IL-33 (1 pg/mL) increased the percentage of HPCs expressing IL-5 (**A**) or IL-13 (**B**) but not IL-4. Data are means $\pm$ SEMs ($n = 12$). * $P < .05$, comparison between response to diluent and various doses of cytokine.

priming with TSLP (1 pg/mL) or IL-33 (1 pg/mL).³⁵ Both compounds inhibited TSLP and IL-33 priming of CXCL12-stimulated migration of HPCs but not the unprimed migrational response of HPCs to CXCL12. This indicates that increased

incorporation of CXCR4 into lipid raft moieties might play a role in promoting enhanced signaling events, thus increasing the migrational responsiveness of HPCs (Fig 6).

DISCUSSION

Allergic asthmatic responses are associated with activation of the hematopoietic compartment in bone marrow and a contemporaneous increase in populations of HPCs within the airways with the capacity to differentiate into mature proinflammatory cells.^{13,37} Therapies that control the lung homing and local tissue differentiation of HPCs attenuate the development of airway eosinophilia and airway dysfunction, which are characteristic features of asthma.^{33,38} More recently, anti-TSLP therapy has been shown to be effective in reducing ambient and allergen-induced eosinophilia in blood and sputum in patients with mild atopic asthma, and this was associated with improvements in methacholine PC₂₀.³⁰ In light of the increasing evidence for a role of epithelium-derived cytokines in orchestrating type 2 immunity,¹⁴ the current study explored the role of TSLP and IL-33 in orchestrating the lung homing of progenitor cell populations to the airways. The novel findings of this study are as follows: (1) there was an allergen-induced increase in the numbers of sputum-derived HPCs that expressed receptors for TSLP and IL-33 in patients with mild asthma; (2) these cytokines primed the migrational responsiveness of HPCs at picogram levels *in vitro*; and (3) this priming effect was mediated by autocrine production of IL-13. These data suggest that TSLP and IL-33 can stimulate the lung homing of primitive non-lineage-committed HPCs to the airways through an IL-13-dependent axis in patients with allergic asthma.

Allakhverdi et al²⁵ first demonstrated the expression of receptors for TSLP (TSLPR and IL-7R α :CD127) and IL-33 (ST2) on cord blood-derived HPCs. We have replicated these findings at the mRNA and protein levels using blood and airway-derived progenitor cells (see Figs E1 and E2). In addition, we have shown that after allergen inhalation challenge, there was a selective increase in the numbers of HPCs expressing TSLPR, CD127, and ST2 within the airways. The relevance of these findings is highlighted by our *in vitro* findings that both TSLP and IL-33 can prime the migrational responsiveness of HPCs at physiologically relevant concentrations (1–10 pg/mL) and that this priming effect can be inhibited in the presence of blocking receptor antibodies for each respective cytokine. We found no additive or synergistic effect of these cytokines, suggesting that the priming effect is mediated through a common signaling pathway. In addition, the priming effect of these cytokines was selective for CXCL12, a potent progenitor cell chemoattractant that is downregulated in bone marrow and upregulated in the airways during allergic inflammatory responses, thus mediating the egress and lung homing of HPCs to airway allergic inflammatory responses.^{12,39} We have also shown that pretreatment with a CXCR4 antagonist attenuated allergen-induced lung homing of HPCs, airway eosinophilia, and airway dysfunction in a mouse model of asthma.^{40,41}

TH2-associated cytokines are produced by HPCs after direct stimulation with TSLP and IL-33 *in vitro* or after overnight incubation with nasal epithelial supernatants from patients with allergic rhinitis in whom the activity was inhibited by neutralizing anti-TSLP.²⁵ In agreement with this report, we found significant increases in the proportion of HPCs expressing intracellular IL-5 and IL-13 but not IL-4. However, we found negligible levels of these cytokines in the supernatants, with the likely explanation

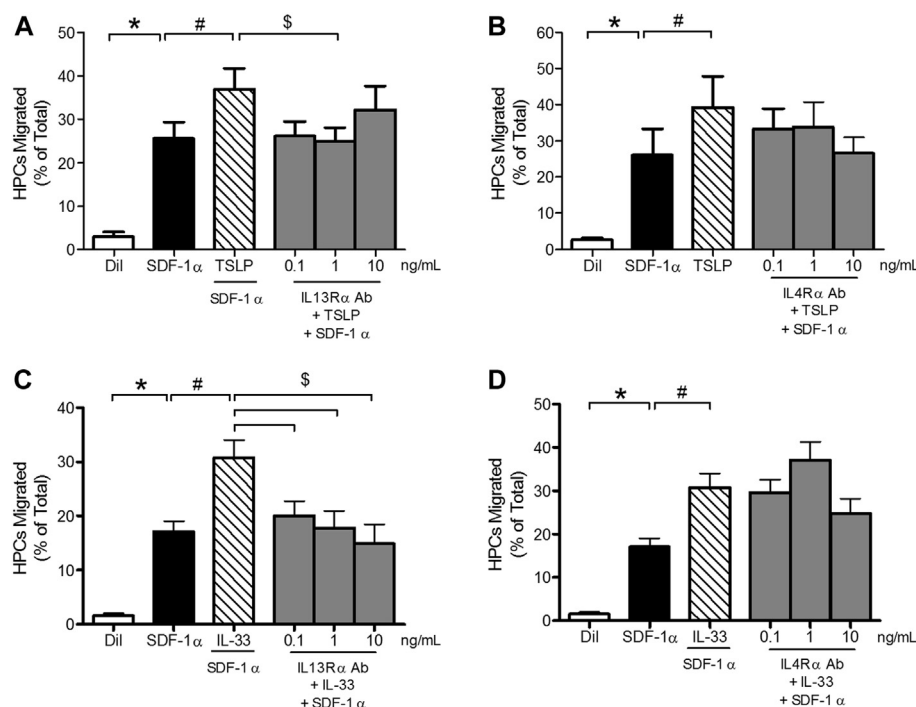


FIG 5. Effect of receptor blocking antibodies on TSLP- and IL-33–primed migration of HPCs. IL-13R α blocked priming in both cases (**A** and **C**), whereas blocking IL-4R α had no effect (**B** and **D**). Data are means $\pm$ SEMs ($n = 7$). * $P < .05$ compared with diluent. # $P < .05$ compared with CXCL12. \$ $P < .05$ compared with the priming response. Ab, Antibody; Dil, diluent; SDF-1 α , stromal cell–derived factor 1 α .

being that we used picogram compared to nanogram levels of these cytokines in the absence of costimulatory factors, such as stem cell factor, as previously reported.²⁵ We have shown that IL-13 can prime CXCL12-induced HPC migration³⁵ but not eosinophil/basophil colony formation, whereas IL-5 can induce eosinophil/basophil colony formation⁴² but not CXCL12-induced HPC migration.³⁹ To determine the role of autologously produced IL-13 in the TSLP- and IL-33–mediated effects, we used added blocking IL-13R α 1 and IL-4R α antibodies to the HPC migration assays. Blocking IL-13R α 1, but not IL-4R α , inhibited the TSLP- and IL-33–induced priming, suggesting an IL-13–dependent axis for the homing of HPCs to the airways in asthmatic patients. It is likely that within the T_H2 milieu of the lungs and autologous IL-5 production, the recruited HPCs can be stimulated to lineage-commit and differentiate into proinflammatory effector cells, such as eosinophils and basophils. This process of *in situ* differentiation has previously been shown in nasal explant tissues from patients with allergic rhinitis after *ex vivo* challenge with either IL-5 or ragweed allergen.¹¹

Our results show that the priming effects of TSLP and IL-33 are dependent on the cholesterol content in the cell membrane because this was disrupted by polyene antibiotics. We have previously shown that IL-13 priming of HPC responses to CXCL12 involves increased incorporation of CXCR4 into lipid rafts, where colocalization with signaling moieties critical for the dynamic remodeling of actin cytoskeleton and formation of cortical F-actin structures occurs.³⁵ Thus TSLP and IL-33 enhance HPC migration through an IL-13–dependent pathway.

In summary, our data show that after allergen challenge in patients with allergic asthma, there is a significant recruitment of

HPCs to the airways and that these cells express receptors for TSLP and IL-33. Through stimulation of autocrine IL-13 production, TSLP and IL-33 might orchestrate the lung-homing HPCs by enhancing their migrational responsiveness to progenitor cell chemoattractants. In light of the obvious biology of IL-13 in asthmatic patients⁴³ and the clinical efficacy of anti-IL-13 treatments in patients with poorly controlled eosinophilic asthma, particularly patients with high periostin levels⁴⁴ or dipeptidyl peptidase 4,⁴⁵ both of which are biomarkers of epithelial cell activation, it would be of interest to investigate the effect of modulating IL-13 activity on the lung homing of HPCs and its association with the development of airway eosinophilia and lung dysfunction.

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Key messages

- Increased numbers of HPCs expressing receptors for TSLP and IL-33 were detected in the airways of atopic asthmatic patients after allergen inhalation challenge.
- *In vitro* experiments show that TSLP and IL-33 can prime the migrational response of HPCs through autocrine production of IL-13.
- TSLP and IL-33, primarily airway epithelium–derived cytokines, promote lung-homing of precursors for proinflammatory effector cells through an IL-13 dependent axis in patients with allergic asthma.

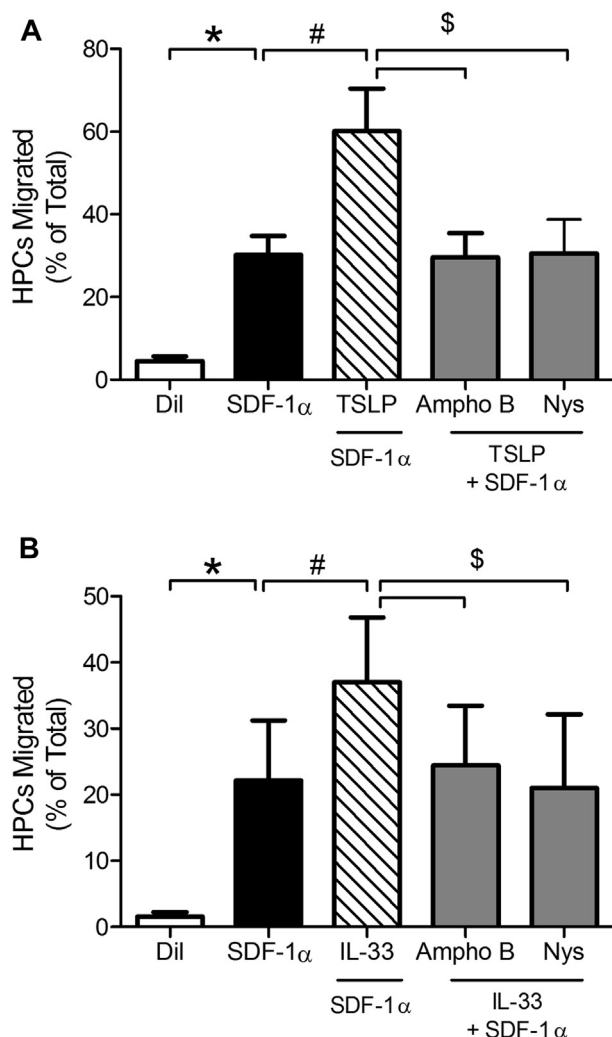


FIG 6. Priming of HPCs is dependent on incorporation of CXCR4 into GM1-enriched lipid rafts. TSLP (**A**) and IL-33 (**B**) priming is inhibited after lipid raft disruption ($n = 4$). * $P < .05$ compared with diluent. # $P < .05$ compared with CXCL12. \$ $P < .05$ compared with priming response. Ab, Antibody; Ampho B, amphotericin B; Dil, diluent; Nys, nystatin; SDF-1 α , stromal cell-derived factor 1 α .

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SUPPLEMENTAL METHODS

Allergen Challenge

A Wright nebulizer operated by oxygen at 50 psi and at a flow rate of 8 L/min producing an output of 0.13 mL/min was used to deliver allergen solutions. Subjects were instructed to wear nose-clips and to breathe normally from the mouthpiece during a 2-minute inhalation period. All subjects inhaled allergen to which they were sensitized, and allergen was selected using results from a skin prick test. The starting concentration of allergen was also determined from the skin prick test and from the methacholine PC20 according to the formula published by Cockcroft *et al.*^{E1} The skin prick test was performed using extracts of several allergens. Duplicate skin-prick tests were performed with doubling dilutions of allergen from 1:4 to 1:4096. The wheal diameter was measured in two perpendicular directions at 10 minutes and the mean wheal diameter is determined for each dilution. A positive response was defined as the threshold concentration that produces a 2-mm wheal over the diluent response. The extract that produces a 2-mm wheal in the skin prick test was the same extract used for the allergen inhalation. The starting concentration for the allergen challenge was two doubling concentrations lower than that predicted to cause a 20% fall in FEV₁ (allergen PC20).

Each concentration of allergen was inhaled for 2 minutes and the FEV₁ was measured 10 minutes after the inhalation. If the FEV₁ fell by 10% or more, the measurement was repeated at 10-minute intervals until no further fall was observed. The dose of inhaled allergen was increased in a doubling manner until the FEV₁ had decreased by at least 20%. After inhalation of the highest allergen concentration, the FEV₁ was measured at 10, 20, 30, 45, 60, 90, and 120 minutes, and then at hourly intervals until 7 hours after the inhalation. Subjects returned the next day for their 24 hour FEV₁ measurement.

The time course after allergen challenge has been well described in the literature.^{E2, E3} The observed early phase bronchoconstriction occurs within 10 minutes of allergen exposure and reaches a maximum after 30 minutes. The early response usually resolves within 1 to 3 hours. The late response occurs after 3 to 4 hours and reaches a maximum around 6 to 10 hours. The late response can last for 24 hours or more.

Protocol: mRNA expression in CD34+ HPCs from peripheral blood

cDNA was synthesized directly from CD34+ HPCs (extracted from peripheral blood) using μ MacSTM One-step cDNA kit (Milenyi Biotec, Auburn, CA). PCR amplification was performed on StepOne Plus Real-time ABI systems (Life Technologies, ON, Canada) with PrimePCRTM SYBR® Green primer assays for ST2 (Unique Assay ID: qHsaCID0010352), TSLPR (Unique Assay ID: qHsaCID0008607), CD127 (Unique Assay ID: qHsaCID0015056) and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-rad, Coralville, IA), as per manufacturer's instructions. Generated Ct (threshold cycle) values for all target genes were ratiometrically analysed relative to the endogenous control gene, HPRT1 (Unique Assay ID: qHsaCID0016375). Additionally, for visual confirmation, all amplified products were further subjected to electrophoresis (1% agarose gel using SYBR® Safe DNA Gel Stain, Life Technologies), with subsequent image acquisition on a Molecular Imager®Gel DocTM XR+ System with Image Lab software (Bio-rad). Positive control – lung fibroblast cDNA generously gifted by Dr. S Mukherjee (post-doctoral fellow, McMaster University, ON). No RT control

(refers to mRNA that has not been further processed with reverse transcriptase during cDNA synthesis) showed no bands, and had undetectable Ct values (data not shown above).

Progenitor Cell Transmigration Assays

The migrational responses of progenitors was assessed in transwell chambers (24-well cell clusters, 6.5 mm Transwell® with 5 µm pore polycarbonate membrane insert filters; Corning Costar, NY, USA) as previously described and in detail in the online supplement^{E4, E5}. The chemoattractant (600 µL) SDF-1 (CXCL12;10 ng/mL; R&D Systems) or diluent (IMDM plus 10% FBS) was loaded into the lower well and CD34+ cells (5×10^4) were added to the upper transwell inserts. Cells in the lower well (representing migrated responding cells) were immunostained and enumerated by flow cytometry. Migrated cells were expressed as a percentage of the total cells added to the top transwell (gating strategy shown in Supplemental Figure E2).

Cells were pre-incubated with either TSLP or IL-33 (R&D Systems) for 18 h (37°C, 5% CO₂) prior to the migration assay. In receptor blocking experiments, cells were incubated with TSLPR, ST2, IL-4Rα or IL-13Rα1 antibodies (R&D Systems) for 15 min prior to the priming with TSLP or IL-33 (gating strategy shown in Supplemental Figure E2). To evaluate the influence of lipid raft formation on migration, cells were pre-incubated for 15 min with nystatin (25 µg/mL) or amphotericin B (10 µg/mL) prior to the priming and transmigration assay, (Sigma, St Louis, MO, USA), as previously described^{E5, E6}.

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SUPPLEMENTAL FIGURES LEGENDS

Supplemental Figure E1. Sequential Multi-gating strategy: to identify HPC (CD34+45+ cells) in sputum using FlowJo software on a LSRII (A). Expression of TSLPR, CD127 and ST2 on sputum cells pre and post-Ag from allergic asthmatics. FlowJo plots of specific antibody and isotype control antibody for TSLPR, CD127 and ST2 (B).

Supplemental Figure E2: Sequential Multi-gating strategy: to identify HPC (CD34+45+ cells) using FlowJo software on a FACS Calibur. Scatter plots show (a) SSC and CD45 – region R1 identifies nucleated white blood cells (WBC). (b) Region R2 identifies CD34+ marker, which is present on HPCs, and which were CD34+45+. Figure C shows how true CD34+ were further isolated based upon low granularity and low CD45+ marker expression. Figure D shows the location of the HPCs on a graph of Side Scatter (SSC) vs Forward Scatter (FSC). HPCs are characterized by medium size (medium FSC) and low granularity (low SSC).

Supplemental Figure E3. Baseline expression of ST2, CD127 and TSLPR in unstimulated CD34+ HPCs extracted from peripheral blood (top) qRT-PCR analysis showing mRNA expression of all three target genes plotted relative to HPRT1 (endogenous control). Data represented as mean $\pm$ SD (n=6). (Bottom) Gene expression panel: samples analysed by qRT-PCR were run on 1% agarose gel to confirm baseline expression. Bands were detected as per their respective amplicon sizes (HPRT1 – 90 bp; ST2 – 114 bp; CD127 -120 bp; TSLPR -150 bp). Positive control – lung fibroblasts.

Supplemental Figure E4: Hemopoietic progenitor cells (within the region R4) represent responding cells in the lower transwell chamber that have migrated to (A) diluent, (B) SDF-1 α (10 ng/mL) (C) cells pre-incubated with TSLP (1 pg/mL) and migrated to SDF-1 α . (D) Demonstrates attenuation of TSLP priming of HPC migration in the presence of anti-TSLP receptor antibody (10 ng/mL). (E) Demonstrates attenuation of TSLP priming of HPC to SDF-1 in the presence of the anti-IL-13R α blocking antibody. (F) Demonstrates migration of HPCs to TSLP (1pg/mL).

Supplemental Figure E5: Effect of TSLP and IL-33 (1 pg/mL) on migration of HPC (CD34+45+ cells) and EoPs (CD34+45+125+) towards RANTES (A and B), Eotaxin (C and D) and MCP-1 (E and F). There was no significant difference observed in the % of migrated HPCs or EoPs towards RANTES, Eotaxin or MCP-1. Data are presented as mean $\pm$ SEM (n = 5).

Supplemental Figure E6: Effect of TSLP and IL-33 on expression of SDF-1 α receptor, CXCR4 on HPC (CD34+45+ cells). There was no significant difference observed in the proportion of HPC expressing CXCR4 after overnight incubation with (A) TSLP or (B) IL-33 compared to diluent control. Similarly, there was no change in the density of surface expression of CXCR4 on HPC following incubation with (C) TSLP or (D) IL-33. Data are presented as mean $\pm$ SEM (n = 7).

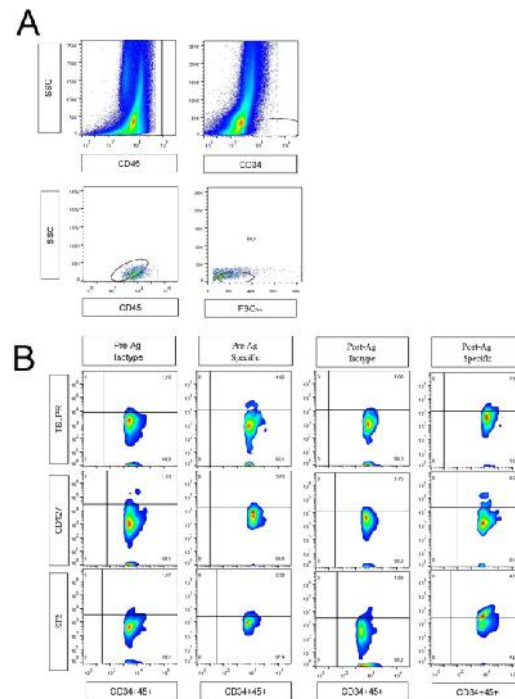


Fig E1

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Thymic stromal lymphopoietin and IL-33 modulate migration of hematopoietic progenitor cells in patients with allergic asthma

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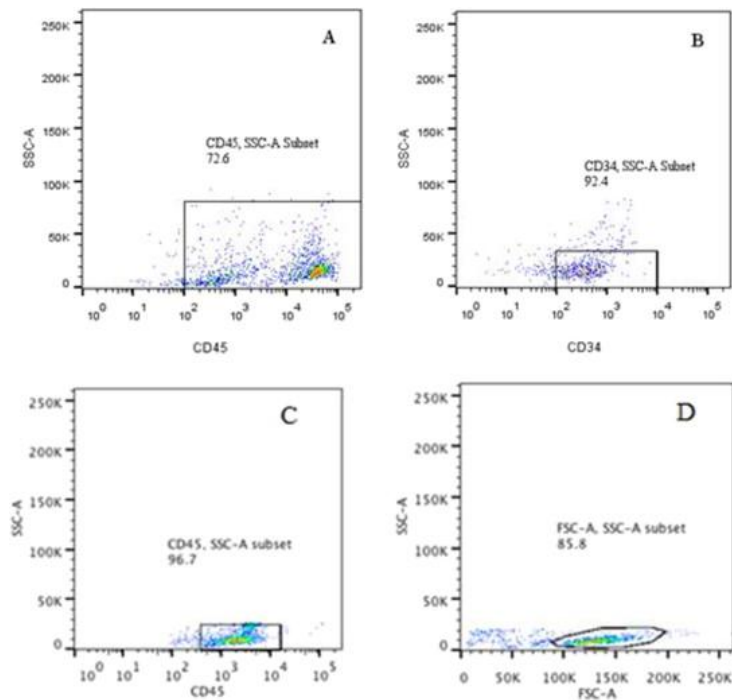


Fig E2

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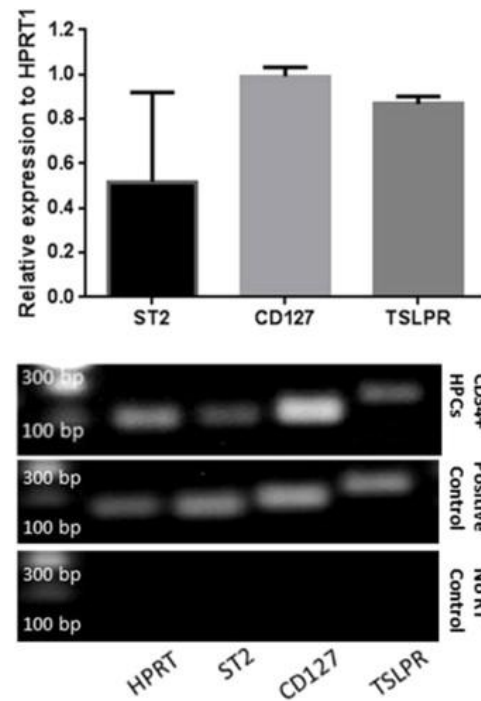


Fig E3

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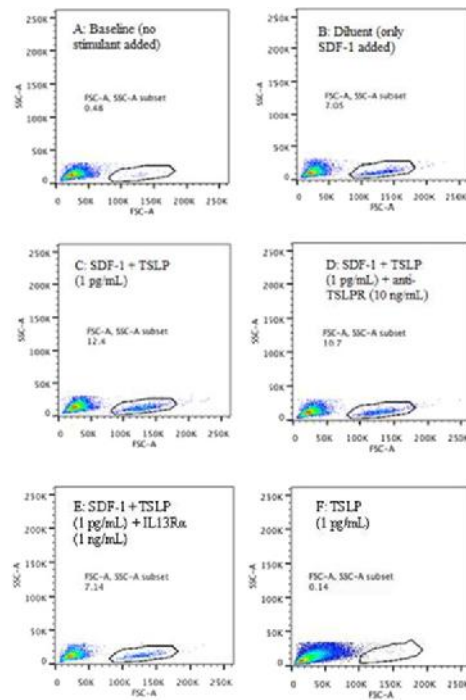


Fig E4

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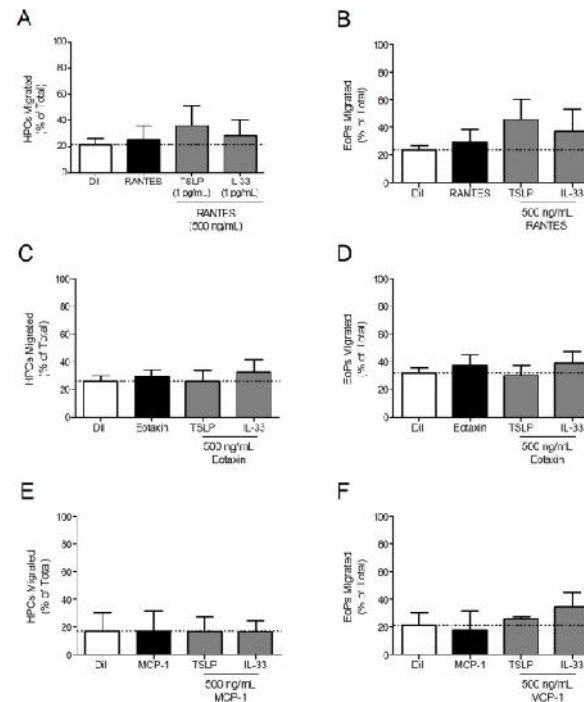


Fig E5

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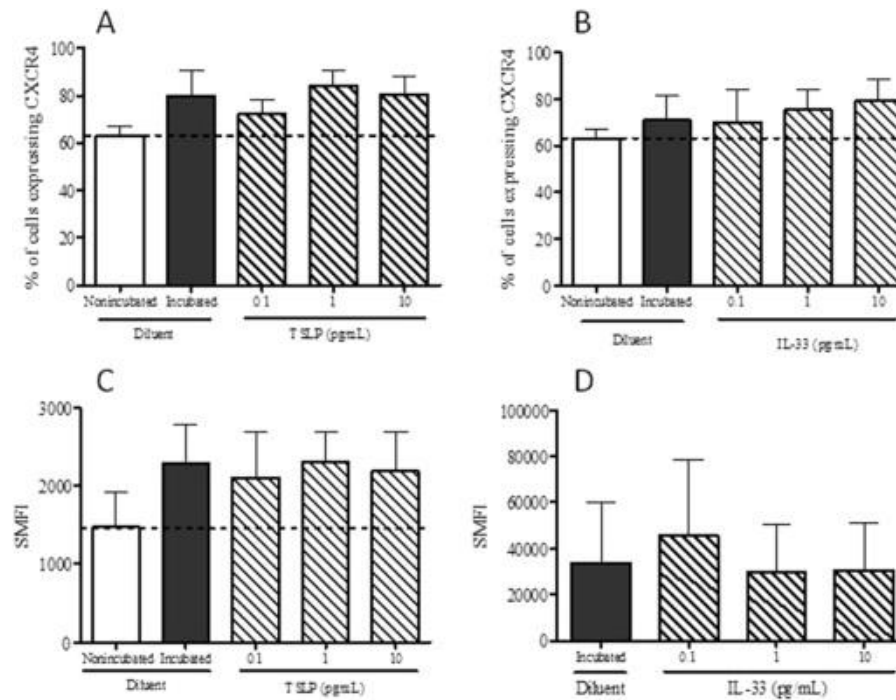


Fig E6

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Supplemental Table E1. Sensitization Profiles

Allergen	Total IgE	Specific IgE	Skin Test Positive (Histamine)	DOG	CAT	HORSE	FEATHER	DICTYOPTERA	HD MDF	HD MDP	ALTER-NARIA	HORMO-DENDRUM	ASPER-GILLUS	TREE	GRASS MIX	RAG-WEED	WEED MIX
Cat	NS	NS	2x2	2x2	10x10	2x2	2x2	0x0	0x0	0x0	1x1	1x1	0x0	0x0	0x0	0x0	0x0
Ragweed	742	0.17	3x3	2x2	4x4	2x2	2x2	0x0	9x6	6x6	2x2	2x2	3x4	2x2	13x9	13x9	8x7
Ragweed	NS	NS	4x4	6x4	5x4	3x3	2x2	0x0	12x5	5x7	2x2	0x0	0x0	5x4	15x8	11x6	4x3
CAT	598	19.8	3x3	4x4	7x5	0x0	0x0	0x0	4x4	4x4	4x4	4x4	2x2	0x0	0x0	0x0	0x0
Ragweed	102	5.4	4x4	5x4	5x5	3x3	2x2	0x0	10x3	5x4	2x2	0x0	0x0	4x4	12x9	13x8	4x4
Grass	73.3	0.05	3x3	2x2	0x0	0x0	0x0	0x0	3x3	3x4	0x0	0x0	0x0	7x8	25x8	2x2	0x0
HD MDP	14.7	4.78	3x3	0x0	0x0	0x0	0x0	0x0	7x7	11x7	2x2	0x0	0x0	0x0	0x0	0x0	0x0
Ragweed	702	10.2	2x2	3x3	5x4	0x0	0x0	0x0	4x4	5x4	6x5	4x4	0x0	2x2	7x5	8x5	0x0
HD MDP	NS	NS	3x3	2x2	9x4	1x1	2x2	2x2	7x7	12x10	4x5	2x2	2x2	5x5	7x6	5x5	2x2
Cat	116	10.3	5x5	4x4	6x6	3x3	3x2	2x2	2x2	0x0	2x2	3x3	6x6	4x4	3x3	3x3	3x3
Horse	NS	NS	3x3	2x2	2x2	10x6	3x3	0x0	4x4	5x3	3x3	3x3	3x3	2x2	0x0	0x0	2x2
Cat	218	12.3	4x4	3x3	5x5	4x4	2x2	2x2	10x3	3x3	3x3	3x3	2x2	3x3	7x5	8x4	2x2
Alternaria	40	7.45	5x5	0x0	3x4	3x3	2x2	0x0	4x4	3x3	6x5	0x0	3x3	4x4	0x0	3x3	0x0
Cat	NS	NS	2x2	3x3	5x5	6x5	2x2	0x0	0x0	0x0	10x10	2x2	0x0	2x2	2x2	2x2	2x2
Ragweed	126	1.48	3x3	0x0	2x2	0x0	0x0	0x0	0x0	0x0	0x0	0x0	0x0	0x0	0x0	3x3	2x2
Horse	44.7	2.41	2x2	3x3	3x3	10x8	2x2	0x0	2x2	1x1	0x0	0x0	0x0	4x4	0x0	0x0	0x0
HD MDP	398	41.9	3x3	3x3	2x2	0x0	0x0	0x0	10x6	10x7	2x2	0x0	2x2	0x0	2x2	3x3	4x4
Horse	39.9	0.58	3x3	4x3	5x	8x8	0x0	0x0	0x0	0x0	0x0	0x0	0x0	4x3	3x3	0x0	0x0
HD MDP	NS	NS	3x3	0x0	2x2	0x0	0x0	0x0	4x3	7x5	0x0	0x0	0x0	2x2	4x4	2x2	0x0

Definition of abbreviations: HD MDF - house dust mite *Dermatophagoides farinae*; HD MDP - house dust mite *Dermatophagoides pteronyssinus*; All subjects had a 0x0 response to diluent. NS - no sample.

Supplemental Table E2. Subject Characteristics.

Subject	Sex	Age (years)	Allergen	FEV₁ (% predicted)	PC20 (Baseline)
001	F	28	Cat	106.2	2.92
002	M	35	Ragweed	102.6	0.826
003	F	42	Ragweed	103.2	0.63
004	M	19	Cat	83.9	7.26
005	F	41	Ragweed	98.8	9.98
006	M	22	Grass	92.5	10.2
007	M	20	HDMDP	93.3	10.71
008	M	26	Ragweed	109.4	2.2
009	M	26	HDMDP	85.4	7
010	M	60	Cat	90.9	0.26
011	F	31	Horse	97	1.51
012	F	23	Cat	102.4	1
013	M	33	Alternaria	86.8	8
014	F	25	Cat	88	1.69
015	F	23	Ragweed	102.1	0.65
016	F	22	Horse	99.2	5.92
017	F	53	HDMDP	90.6	2.97
018	F	23	Horse	95.4	7.7
019	M	51	HDMDP	92.2	0.13

Definition of abbreviations: F - female; M - male; HDMDP - house dust mite *Dermatophagoides farinae*; HDMDP - house dust mite *Dermatophagoides pteronyssinus*; FEV₁ - forced expiratory volume in 1 second; PC20 - provocative concentration of methacholine causing a 20% drop in FEV₁.

Supplemental Table E3. Expression of TSLPR, ST2 and CD127 on sputum extracted EoP.

Sputum EoP	Diluent		Allergen	
	Pre	Post	Pre	Post
TSLPR+ (cells/mL)	29±26	38±30	40±34	40±38
ST2+ (cells/mL)	15±14	30±20	29±26	20±14
CD127+ (cells/mL)	38±25	34±27	15±9	10±8

Definition of abbreviations: EoP - eosinophil progenitor cell. Values are presented as mean $\pm$ SEM in parentheses. There were no significant differences in receptor expression levels on sputum EoPs when allergen was compared to diluent challenge.

Supplemental Table E4. Expression of TSLPR, ST2 and CD127 on blood extracted HPCs and EoPs.

Blood	HPC				EoP			
	Diluent		Allergen		Diluent		Allergen	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
TSLPR+ (per million wbc)	997±298	1032±476	798±395	1268±601	13±5	21±11	17±11	18±7
ST2+ (per million wbc)	1229±363	971±432	841±386	957±591	36±17	22±7	55±37	41±24
CD127+ (per million wbc)	720±316	1414±822	806±433	868±556	8±5	13±11	10±6	9±3

Definition of abbreviations: HPC - hematopoietic progenitor cells. Values are presented as means $\pm$ SEM in parentheses. There were no significant differences in receptor expression levels on blood HPCs or EoPs when allergen was compared to diluent challenge.