

Human Bronchial Epithelial Cell-derived Factors from Severe Asthmatic Subjects Stimulate Eosinophil Differentiation

Brittany M. A. Salter^{1*}, Steven G. Smith^{1*}, Manali Mukherjee¹, Sophie Plante², Sakktée Krishna¹, Graeme Nusca¹, John Paul Oliveria¹, Anam Irshad¹, Gail M. Gauvreau¹, Jamila Chakir², Parameswaran Nair¹, and Roma Sehmi¹

¹Division of Respiriology, Department of Medicine, McMaster University, Hamilton, Ontario, Canada; and ²Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval, Québec City, Québec, Canada

Abstract

Activated bronchial epithelial cells (BEC) release various alarmins, including thymic stromal lymphopoietin (TSLP), that drive type 2 inflammation. We hypothesize that BEC-derived factors promote *in situ* eosinophil differentiation and maturation, a process that is driven by an IL-5-rich microenvironment in asthmatic airways. To assess the eosinophilopoietic potential of epithelial-derived factors, eosinophil/basophil colony forming units (Eo/B-CFU) were enumerated in 14-day methylcellulose cultures of blood-derived nonadherent mononuclear cells incubated with BEC supernatants (BECN) from healthy nonatopic controls ($n = 8$), mild atopic asthmatics ($n = 9$), and severe asthmatics ($n = 5$). Receptor-blocking antibodies were used to evaluate the contribution of alarmins. Modulation of the mRNA expression of transcription factors that are crucial for eosinophil differentiation was evaluated. BECN stimulated the clonogenic expansion of eosinophil progenitors *in vitro*. In the presence of IL-5, Eo/B-CFU numbers were significantly greater in cocultures of BECN from severe asthmatics compared with other groups. This was attenuated in the presence of a TSLP (but not an IL-33) receptor-blocking antibody. Recombinant human TSLP (optimal at 100 pg/ml) stimulated Eo/B-CFU growth, which was significantly enhanced in the presence of IL-5 (1 ng/ml). Overnight culture of CD34⁺ cells with IL-5 and TSLP synergistically increased

GATA-binding factor 2 and CCAAT/enhancer-binding protein α mRNA expression. The eosinophilopoietic potential of factors derived from BEC is increased in severe asthma. Our data suggest that TSLP is a key alarmin that is produced by BECs and promotes *in situ* eosinophilopoiesis in a type 2-rich microenvironment.

Keywords: bronchial epithelial cells; eosinophilopoiesis; severe asthma; TSLP; TSLPR

Clinical Relevance

Eosinophilia is a persistent feature of difficult-to-control severe asthma, and controlling this inflammation is important to control asthma exacerbations and reduce dependency on high-dose steroid therapy. We show that bronchial epithelial cells from severe asthmatics release thymic stromal lymphopoietin, and that this cytokine has the potential to drive local eosinophil growth and differentiation. This is enhanced in a type 2-rich environment (e.g., in the presence of IL-5), thus driving local airway eosinophilia. Targeting thymic stromal lymphopoietin may attenuate local eosinophilia by inhibiting local eosinophilopoietic processes.

A considerable body of evidence supports an association between allergen-induced asthmatic responses and the mobilization of progenitors from bone marrow (BM) (1).

Once homed to the site of allergic inflammation, affected tissues have been shown to support the local differentiation, proliferation and maturation of

hemopoietic progenitor cells (2). Specifically, we have shown that airway structural cells (i.e., airway smooth muscle-derived IL-5 and

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Roma Sehmi, B.Sc., Ph.D., McMaster University, 1280 Main Street West, HSC 3U32, Hamilton, ON, L8S 4K1 Canada. E-mail: sehmir@mcmaster.ca.

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granulocyte-macrophage colony-stimulating factor [GM-CSF]) stimulate eosinophilopoiesis (3); however, we did not perform a comparison between normal and asthmatic tissue donors in that study. In a baseline cross-sectional study, we recently reported markedly increased numbers of eosinophil progenitors in the sputum of severe asthmatics (SA) compared with both mild atopic asthmatics (MA) and healthy nonatopic controls (NC), indicative of an exaggerated eosinophilopoietic environment locally within the airways in severe asthma (4). We propose that in chronic disease, *in situ* differentiation in the presence of a locally elaborated milieu of cytokines produced by structural cells provides an ongoing source of effector cells such as eosinophils.

Eosinophil progenitor cells home to the lungs after allergen challenge, a process that coincides with elevated local levels of IL-5 but precedes the development of airway eosinophilia (5, 6). Localized differentiation of BM-derived hemopoietic progenitors is inferred from findings that CD34⁺ cells extracted from human nasal polyp tissue have the capacity to undergo IL-5 stimulated proliferation and differentiation into eosinophil/basophil colony-forming units (Eo/B-CFU), *in vitro* (7). Experiments using nasal explant tissue from allergic rhinitis have shown increases in major basic protein-positive eosinophils in conjunction with a reduction in CD34⁺IL-5Rα⁺ mRNA cells after antigen challenge or exposure to IL-5 *ex vivo*, indicative of a local maturation process (8). Increased numbers of CD34⁺ cells and CD34⁺IL-5Rα⁺ mRNA⁺ cells were detected in bronchial biopsies from MA compared with NC, and CD34⁺IL-5Rα⁺ mRNA⁺ cell numbers correlated with asthma severity (9). This raised the possibility that controlling the local differentiation and expansion of CD34⁺IL-5Rα⁺ cells within lung tissue may be a prerequisite for reducing eosinophilic inflammation in the airway in asthma. In agreement with this, a study using intravenous anti-IL-5 treatment in mild asthmatics showed a significant, but not complete, attenuation of CD34⁺IL-5 mRNA⁺ cell numbers (eosinophilopoietic progenitor cells [EoP]) in the lung mucosa (but not in peripheral blood or BM), indicating that controlling local differentiative events may be important for attenuating lung eosinophilia in asthma (10).

Airway epithelial cells form a continuous, highly regulated physical

barrier that lines the airway lumen, creating an interface between the host and environment that acts as the first line of defense against inhaled microorganisms, particulate matter, and allergic antigens. Epithelial cells express pattern recognition receptors and Toll-like receptors that detect environmental stimuli, including viral and allergenic antigens. Subsequent activation of pattern recognition receptors and Toll-like receptors triggers epithelial cell release of soluble cytokines, chemokines, growth factors, and antimicrobial peptides, which stimulate the immune system in response to potential threats, thereby acting as alarmins. Among the alarmin cytokines that are released from the epithelium, thymic stromal lymphopoietin (TSLP) has been shown to be a key upstream promoter of type 2 cytokine (IL-4, IL-5, and IL-13)-driven inflammation, which is characteristic of allergic asthma (11, 12).

TSLP protein and mRNA, which are detectable at higher levels in asthmatic airways, have been shown to correlate with disease severity (13, 14), and cultured primary bronchial epithelial cells (BEC) from SAs spontaneously release detectable levels of TSLP (15, 16). Recent studies with myeloid progenitor cells have shown that TSLP can act directly on CD34⁺ cells, stimulating a proinflammatory phenotype (type 2 cytokine production [IL-5 and IL-13]), a proliferative phenotype (Eo/B lineage commitment) (17–19), and priming migrational responses *in vitro*, in an IL-13-dependent manner (20). In addition, supernatants from both activated primary airway epithelial cells and nasal-explant supernatants from chronic rhinitis stimulate CD34⁺ cells to produce IL-5, an activity that is inhibited by anti-TSLP antibodies (17). Considering the above findings and the increased presence of EoP in the airways of SA with persistent eosinophilia (4), we hypothesized that factors released from the activated bronchial epithelium would enhance the *in situ* eosinophil differentiation and maturation of the CD34⁺ progenitor population, driven by an IL-5-rich type 2 microenvironment as seen in severe eosinophilic asthma.

Our aim in this study was to investigate the effect of epithelium-derived factors released by primary cultures of BEC obtained from SA, MA, and NC on the growth and differentiation of myeloid progenitor cells *in vitro*.

Methods

Study Patients and Samples

Study subjects for bronchial biopsies were recruited from the Asthma Clinic at the Institut Universitaire de Cardiologie et de Pneumologie de Québec (Québec City, PQ, Canada). Airway epithelial cells were isolated from bronchial biopsy tissue from NC ($n = 8$), MA ($n = 9$), and SA ($n = 5$) (Table 1). Progenitor cell populations were isolated from the blood of healthy atopic subjects at McMaster University who were skin-prick-test positive for common aeroallergens but had no history of asthma. All subjects in this study provided informed consent, and the reported experimental procedures were reviewed and approved by the institutional research ethics boards of the Institut Universitaire de Cardiologie et de Pneumologie de Québec and McMaster University, respectively.

Primary Epithelial Cell Cultures

Cells were extracted by overnight collagenase digestion of bronchial biopsies and tissue-extracted airway epithelial cells, characterized, and cultured as previously described (21, 22) and detailed in the data supplement. Of note, we have previously shown that primary epithelial cells isolated from bronchial biopsies keep their phenotype and reproduce the *in vivo* phenotype. Specifically, we have shown that the primary cells are similar to the cultured cells in terms of proliferative responses (16), cytokine and chemokine production (16, 23), and microRNA profile (24).

Methylcellulose Culture to Enumerate Eo/B-CFU

Progenitor cells were isolated from the blood (100 ml) of atopic subjects based on previous data showing that CD34⁺ cells are increased in number and eosinophilopoietic potential compared with progenitors from normal healthy controls (25). Heparinized blood (1,000 units/ml) was diluted with McCoy's 5A media and subjected to density-gradient sedimentation on Accuprep (Axis-Shield). Monocytes were depleted by adherence to plastic (2 h, 5% CO₂, and 37°C) and nonadherent mononuclear cells (NAMNC; 1×10^6 cells/plate) containing an enriched population of CD34⁺ hemopoietic progenitors resuspended in Iscove's modified Dulbecco's medium (with 1% penicillin-streptomycin and

Table 1. Baseline Subject Characteristics of the Bronchial Epithelial Cell Supernatant Donors

Characteristic	Severe Asthmatics	Mild Atopic Asthmatics	Normal Nonatopic Controls
Number of patients	n = 5	n = 9	n = 8
Age (yr)	59.7 ± 1	22.5 ± 1.3	32.4 ± 5.3
Sex (M/F)	3/2	0/9	5/3
Atopy (yes/no)	4/5	9/0	0/8
FEV ₁ % predicted	69 ± 1.8	99 ± 0.9	102 ± 7.4
FEV ₁ (liters)	1.73 ± 0.1	3.3 ± 0.1	3.75 ± 0.2
Methacholine PC ₂₀ (mg/ml)	N/A	2.3 ± 0.9	48.7 ± 3.6
Medications	LTRA, ICS, OCS, LABA, SABA	Intermittent SABA	None

Definition of abbreviations: ICS = inhaled corticosteroid; LABA = long-acting β -adrenoceptor agonist; LTRA = leukotriene receptor antagonist; N/A = not available; OCS = oral corticosteroid; PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁; SABA = short-acting β -adrenoceptor agonist.

Normal controls were skin-prick-test-negative, nonsmokers with no history of respiratory or systemic diseases. Mild asthmatics were skin-prick-test-positive for common aeroallergens, with methacholine PC₂₀ of <8 mg/ml, FEV₁ > 70% predicted, and intermittent use of β_2 -agonists. Severe asthmatics had a FEV₁ < 70% predicted and used high-dose inhaled corticosteroids (>750 μ g of fluticasone propionate or equivalent in combination with long-acting β_2 -agonist daily) and daily-maintenance oral corticosteroids. None of the asthmatics had experienced an asthma exacerbation or respiratory tract infection 4 weeks before entry into the study. Data are presented as mean $\pm$ SD.

0.5 μ M of 2 mercaptoethanol), 20% FCS, and 0.9% methylcellulose. They were plated in duplicate with BECSNs with or without an optimal dose of IL-5 (1 ng/ml; R&D Systems, Minneapolis, MN) for 14 days (37°C, 5% CO₂). Cultures were also performed with neutralizing antibodies to TSLP (TSLP receptor [TSLPR]-blocking antibody [5 μ g/ml; R&D Systems], IL-33: IL-33 receptor-blocking antibody [ST2, 1 μ g/ml; R&D Systems], IL-5R α -blocking antibody [3 μ g/ml; R&D Systems]) or the relevant isotype controls. The outgrowth of progenitors into clonally derived aggregates of >40 daughter cells (defined as CFU) was identified by distinct morphological features under light microscopy as Eo/B-CFU (tight, compact, round refractile cell aggregates) (26, 27).

TSLP and IL-5 Mediated the Eosinophil Lineage Commitment of CD34⁺ Progenitor Cells

Blood-derived CD34⁺ progenitor cells were isolated using MACS CD34 Microbeads (Miltenyi Biotech) and cultured in the presence of IL-5 (1 ng/ml), TSLP (100 pg/ml), in the presence or absence of culture media (18 h, 5% CO₂, 37°C). Details regarding the RNA extraction and quantitative real-time PCR

of stimulated CD34⁺ cells for expression of eosinophil-lineage fate determine transcription factors such as GATA-binding factor 2 (GATA-2) and CCAAT/enhancer-binding protein α (CEBP- α) are provided in the data supplement.

Statistical Analyses

Data are expressed as mean $\pm$ SEM, and the numbers of Eo/B-CFU in 14 days of culture were analyzed using one-way repeated-measures ANOVA with a *post hoc* Bonferroni test on GraphPad Prism5 software (GraphPad Software Inc.). The fold increase in mRNA expression of transcription factors in CD34⁺ cells was analyzed using a two-way ANOVA with Tukey's multiple-comparison tests. A significant *P* value was set at less than 0.05.

Results

Clonogenic Potential of BEC Supernatants

NAMNC with an enriched population of CD34⁺ progenitor cells from atopic subjects were cultured in methylcellulose with BEC supernatants (BECSN; 1:10 and 1:100 dilution) from NC (*n* = 8), MA (*n* = 9), and SA (*n* = 5). BECSN at a 1:100 final

dilution from NC, MA, and SA increased the outgrowth of Eo/B-CFU compared with negative controls, and these responses were comparable to those achieved with an maximal stimulating dose of IL-5 (1 ng/ml) alone (Figure 1A). Higher concentrations of BECSN (1:10 dilution) from SA, but not from NCs or MAs, stimulated increased levels of Eo/B-CFU compared with negative controls, although in all cases the level of clonogenic stimulation was lower than the 1:100 dilution. This may be due to an inhibitory effect of greater concentrations of the relevant growth factor in the more concentrated culture supernatants, as seen for IL-5 at 10 versus 1 ng/ml (Figure 1A).

BEC can produce eosinophilopoietic cytokines, including IL-5 (28). To test whether the eosinophilopoietic activity in BECSN was a factor other than IL-5, we added an optimal concentration of IL-5 (1 ng/ml) to cultures of BECSN from all groups. Cocultures of BECSN (1:100 dilution) from MA and SA, but not NCs, with IL-5 (1 ng/ml) significantly increased the outgrowth of Eo/B-CFU compared with IL-5 alone. In addition, IL-5 cocultures with BECSN from SA stimulated significantly greater outgrowth of Eo/B-CFU compared with BECSN from MA (*P* < 0.05) (Figure 1B).

Effect of Blocking TSLPR, ST2, and IL-5R α on Eo/B-CFU Induced by Severe Asthma Supernatants

The increase in Eo/B-CFU stimulated by BECSN from SA (1:100 dilution) in combination with IL-5 (1 ng/ml) was significantly attenuated in the presence of TSLPR-blocking antibody (5 μ g/ml, a dose that was previously optimized in pilot experiments compared with the isotype control; *P* < 0.05; Figure 2A). To investigate the role of IL-33, another key alarmin released by activated bronchial epithelium upon extraneous insults, a neutralizing antibody targeting ST2 was used in the cultures. In contrast to TSLPR, ST2 blockade had no effect on attenuating colony growth stimulated by BECSN from SA (Figure 2A).

In separate experiments, we looked at the effect of IL-5R α blockade on SA BECSN-induced eosinophilopoiesis. In the presence of a blocking IL-5R α antibody (3 μ g/ml), there was no significant attenuation of SA BECSN (1:100 dilution)-induced Eo/B-CFU compared with the isotype control (Figure 2B).

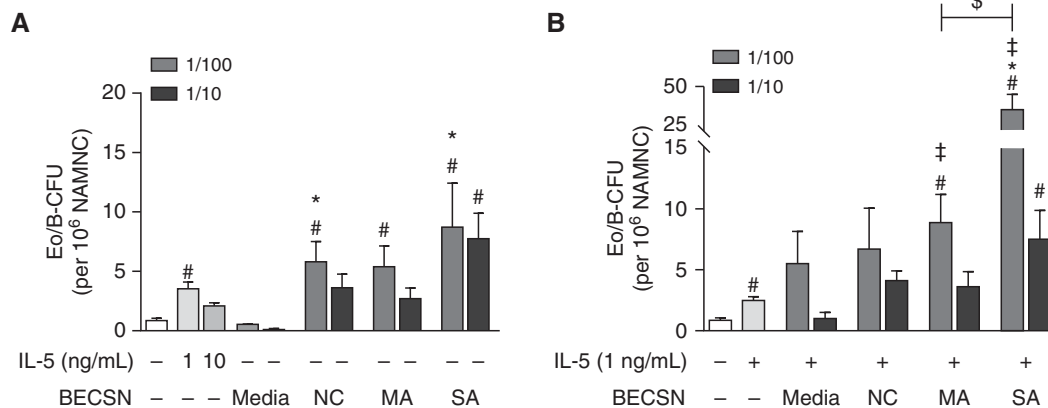


Figure 1. Clonogenic potential of bronchial epithelial cell supernatants (BECSN). Nonadherent mononuclear cells from atopics were cultured in (A) methylcellulose with BECSN (1:10 and 1:100 dilution) from healthy nonatopic controls (NC; $n = 8$), mild asthmatics (MA; $n = 9$) and severe asthmatics (SA; $n = 5$), and (B) in the presence of an optimal concentration of IL-5 (1 ng/ml). Data are represented as mean $\pm$ SEM. #, *, and ‡ indicate significant increase compared with negative control, BEC media control, and IL-5, respectively; \$ indicates significant difference in between-group comparisons; $P < 0.05$, ANOVA, Bonferroni correction. B-CFU = basophil colony forming units; BEC = bronchial epithelial cell; Eo = eosinophil; NAMNC = nonadherent mononuclear cell.

Clonogenic Capacity of Recombinant Human TSLP

Cultures of peripheral blood NAMNC from atopic subjects with recombinant human TSLP (rhTSLP; 1–100 pg/ml) stimulated significant Eo/B-CFU growth compared with the negative control ($P < 0.05$; Figure 3). In combination with IL-5 (1 ng/ml), rhTSLP at 1 and 100 pg/ml stimulated significantly greater Eo/B-CFU growth compared with TSLP alone ($P < 0.05$; Figure 3). Eo/B-CFU outgrowth stimulated by rhTSLP at all tested concentrations (1–100 pg/ml) was significantly attenuated by a neutralizing TSLPR antibody (5 μ g/ml), but not by the

isotype control ($P < 0.05$; Figure 4). Eo/B-CFU outgrowth stimulated by cultures in the presence of both rhTSLP (1–100 pg/ml) and IL-5 (1 ng/ml) was significantly attenuated to levels comparable to those observed with IL-5 alone (Figure 4).

Effect of TSLP on Eosinophil Lineage-Specific Transcription Factor Expression

To elucidate the molecular mechanisms involved in the synergistic clonogenic effect of TSLP and IL-5, we evaluated variations in the expression of two key eosinophilopoietic transcription factors, GATA-2 and CEBP- α , in an enriched population of CD34⁺ cells

(purity > 98%) using quantitative real-time PCR. Isolated CD34⁺ cells stimulated with IL-5 (1 ng/ml) and TSLP (100 pg/ml) alone did not show any significant change in expression compared with the untreated control. However, in combination, IL-5 and TSLP stimulated significant fold increases in both GATA-2 and CEBP- α mRNA levels with respect to untreated CD34⁺ cells and TSLP (alone) ($P < 0.05$; Figure 5). Whereas CEBP- α showed a significant fold increase in the presence of both IL-5 and TSLP compared with IL-5 alone, GATA-2 showed only a trend of increasing expression that was not significant (Figure 5).

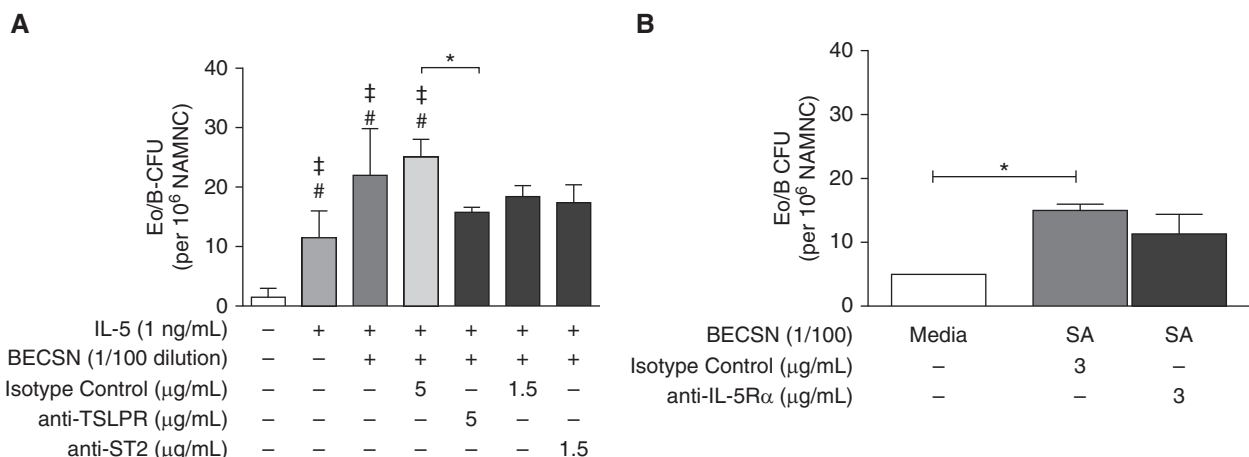
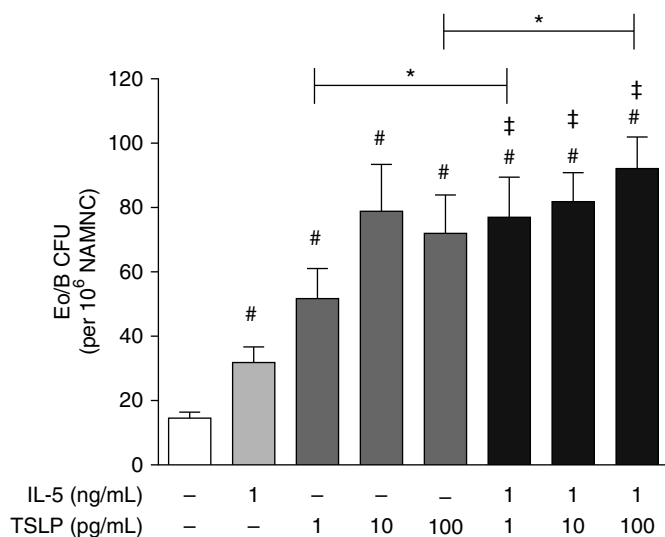


Figure 2. Effect of alarmin receptor blockade on BECSN-stimulated eosinophil differentiation. Outgrowth of Eo/B-CFU stimulated with (A) BECSN from SA ($n = 4$) $\pm$ IL-5 (1 ng/ml); in the presence of neutralizing TSLPR antibody (5 μ g/ml) or ST2 blocking antibody (1.5 μ g/ml) was assessed. (B) Effect of neutralizing IL-5R α (3 μ g/ml) and the respective isotype controls on clonogenic activity of BECSN from SA are plotted. #, ‡, and * indicate significant difference compared with negative control, IL-5, and the respective isotype control, respectively. Data are represented as mean $\pm$ SEM. $P < 0.05$, ANOVA, Bonferroni correction. ST2 = IL-33 receptor-blocking antibody; TSLPR = thymic stromal lymphopoietin receptor.

A



B

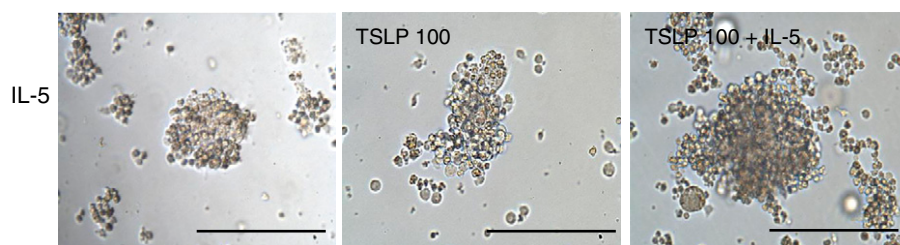


Figure 3. Effect of IL-5 and TSLP on eosinophil differentiation. (A) Enumeration of Eo/B-CFU from cocultures of nonadherent mononuclear cells from atopic subjects ($n = 7$) with TSLP (1–100 pg/ml) in the presence or absence of 1 ng/ml IL-5. Data are presented as mean CFU $\pm$ SEM; # $P < 0.05$ compared with diluent control, ‡ $P < 0.05$ compared with TSLP-only cultures, * $P < 0.05$ compared with IL-5 alone. ANOVA, Bonferroni correction. (B) Representative micrographs of Eo/B-CFU colonies. Colonies were increased in number and size in IL-5 and TSLP cocultures. Images were captured on an Eclipse TE2000-E (Nikon) with a Retiga 2000R camera (QImaging). Scale bars: 100 μ m.

Discussion

The findings of this study demonstrate that factors released by BEC can promote eosinophilopoiesis, the potential for which increases with asthma severity. In addition, we show that TSLP, an epithelial-derived alarmin, can promote eosinophil-lineage commitment and differentiation, an effect that is enhanced in the presence of IL-5 *in vitro*. These findings indicate that BECs release growth factors (in particular TSLP) that contribute to ongoing eosinophilic inflammation and can be considered to be a driving factor for local eosinophilopoietic processes within the airways in asthma.

In this study, we tested the hypothesis that in asthmatics, BEC-derived factors can stimulate the local tissue eosinophilopoietic

response of BM-derived hemopoietic progenitor cells. We have previously reported that human airway smooth muscles produce IL-5 and GM-CSF that can stimulate clonogenic expansion of Eo/B-CFU, *in vitro* (3). This demonstrated that a major structural cell of the lung has the capacity to direct eosinophil differentiation and maturation from progenitor cells, which in turn may perpetuate airway eosinophilia and consequently tissue remodeling in patients with chronic asthma.

Emerging evidence suggests that *in situ* eosinophilopoietic processes in the airways sustain tissue eosinophilia and perpetuate asthma severity (4, 29). For example, recent clinical trials of anti-IL-5 reported effective depletion of blood eosinophils in all patients, but only an ~50% reduction in exacerbation frequencies (30, 31).

Moreover, in a subgroup of SAs on low-dose anti-IL-5 therapy, although blood eosinophils were attenuated, sputum eosinophilia remained unaffected, suggestive of airway eosinophilopoietic processes maintained locally (4). In contrast, a high-dose anti-IL-5 biologic completely attenuated both blood and sputum levels of eosinophils (32).

To test the hypothesis that in asthmatics, structural cells such as the bronchial epithelium may play a role in driving the local tissue eosinophilopoietic response to recruited BM-derived hemopoietic progenitor cells, we first established that factors from BEC of SA have increased clonogenic potential. This was evident from the significant increase in Eo/B-CFU when cocultured with NAMNC (Figure 1) in comparison with NC and MA. This growth response of NAMNC was markedly upregulated in combination with IL-5, and attenuated in the presence of a neutralizing TSLPR antibody (Figure 2A). To further confirm our observations, we assessed the number of Eo/B-CFU after culture of rhTSLP and/or IL-5 for 14 days. Cultures of rhTSLP significantly stimulated the outgrowth of Eo/B-CFU compared with the negative control, which was further increased in the presence of IL-5 (Figure 3). This effect was attenuated in the presence of a TSLPR-blocking antibody (Figure 4), thereby confirming a key role for the TSLP/TSLPR axis in eosinophilopoietic activity in BECSN.

To confirm a role for TSLP within SA airways, we conducted additional experiments to determine whether a blockade of IL-5R α would result in inhibitory effects similar to those caused by TSLPR blockade on SA BECSN-induced eosinophilopoiesis. Our data show that the presence of an IL-5R α -neutralizing antibody did not attenuate the effects of SA BECSN on the number of Eo/B-CFU (Figure 2B). This suggests that in addition to IL-5, there may be other key growth factors, including TSLP, within SA airways that play a role in eosinophilopoiesis.

TSLP in combination with IL-3 was previously shown to stimulate the outgrowth of Eo/B-CFU *in vitro* (20). However, in contrast to our findings, the authors found that TSLP alone could not induce eosinophilopoiesis, and TSLP had no effect on IL-5- or GM-CSF-stimulated colony

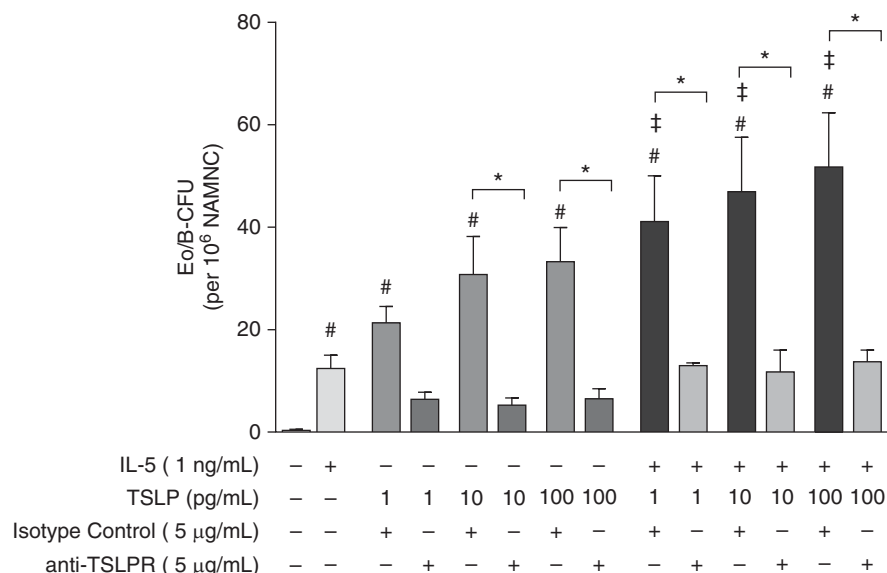


Figure 4. Effect of treatment with anti-TSLPR on IL-5 and TSLP-stimulated eosinophil/basophil differentiation. Enumeration of the Eo/B-CFU setup in methylcellulose semisolid cultures in the presence or absence of IL-5 (1 ng/ml) and TSLP (1–100 pg/ml) $\pm$ anti-TSLPR antibody ($n = 6$). Data are represented as mean $\pm$ SEM; #, †, and * indicate significant difference compared with negative control, IL-5, and the respective isotype controls, respectively. $P < 0.05$, ANOVA, Tukey's multiple comparison *post hoc* test.

outgrowth. This may be attributable to differences in the TSLP concentrations used. We have shown that TSLP can promote IL-5 and IL-13 production by hematopoietic progenitor cells, and prime migration to stromal cell derived factor-1 α in a concentration range of 1–100 pg/ml (20). Préfontaine and colleagues (15) and Semlali and colleagues (16) found 76.9 ± 3.4 pg/ml of TSLP in the BECSN from asthmatic patients, compared with 43.6 ± 2.9 pg/ml in NC. This is consistent with the concentration range we employed in this study to induce eosinophilopoiesis. In contrast, Hui and colleagues (19) used a concentration dose of 10 ng/ml of TSLP and, as mentioned previously, found no significant effect of TSLP alone on Eo/B-CFU growth. Collectively, these observations suggest that human progenitor cells are sensitive to picogram levels of TSLP, and that TSLP induces eosinophilopoiesis independently of other growth factors.

Expanding on our previous findings (20), we have shown that overnight stimulation with SA BECSNs in combination with exogenous TSLP significantly increased EoP intracellular IL-5 (Figure E1 in the data supplement). These findings suggest that in addition to

mediating effects through the TSLP/TSLPR axis, TSLP may, in part, exert its effect on EoP through the induction of autologous IL-5 production, which would then further stimulate EoP function. Furthermore, these results suggest that TSLP may be a more upstream mediator than IL-5 in promoting eosinophilopoiesis in severe asthma; however, this will need to be investigated further.

To confirm the role of TSLP in eosinophilopoiesis, we assessed the expression of key transcription factors. In the steady state, eosinophils differentiate and mature from CD34⁺IL5R α ⁺ EoP derived from common lymphoid progenitors. The lineage commitment of common lymphoid progenitors into eosinophils requires a threshold expression of fate-determining transcription factors, namely, GATA, XBP1, PU.1, CEBP- α , and low FOG-1 (33). In humans, GATA-2 is both necessary and sufficient to drive eosinophil development, and CEBP- α is required for eosinophil terminal differentiation (34). We quantified GATA-2 and CEBP- α mRNA levels in purified CD34⁺ cells at baseline and after stimulation with TSLP $\pm$ IL-5. It was previously established that IL-5 stimulates the proliferation and differentiation of CD34⁺ cells that express IL-5R α , but does

not play an instructive role in eosinophil lineage commitment (33). In agreement with this, we saw no change in GATA-2 or CEBP- α levels with IL-5. Interestingly, TSLP at the concentration that allowed significant outgrowth of Eo/B-CFU (100 pg/ml) in NAMNC cultures did not cause any change in the baseline mRNA expression of either GATA-2 or CEBP- α in CD34⁺ cells. This may be due to TSLP-stimulated IL-5 production by other immune cells in a heterogeneous cell population (NAMNCs) in the methylcellulose cultures. However, the ability of TSLP to stimulate IL-5 production from CD34⁺ cells (17) in this case was probably not adequate to induce significant changes in the cellular machinery in an autologous manner. The same concentration of TSLP and IL-5 stimulated CD34⁺ cells for significant fold increases in both transcription factors, which corroborates the observation of a synergistic effect of IL-5 + TSLP on Eo/B-CFU outgrowth in methylcellulose assays. Thus, it can be deduced that in an IL-5-rich microenvironment associated with allergic inflammation, TSLP promotes eosinophil lineage commitment and *in situ* differentiation of recruited progenitors. We acknowledge that there are multiple

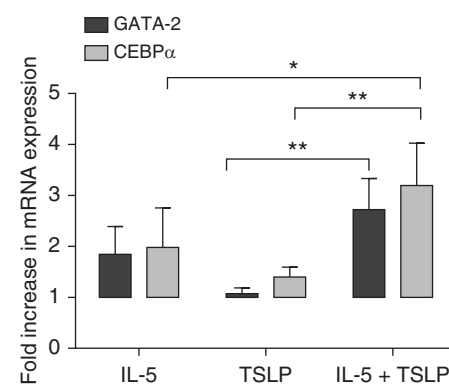


Figure 5. Regulation of eosinophil-lineage-specific transcription factors in CD34⁺ cells by IL-5 and TSLP. Fold increase in mRNA expression of CCAAT/enhancer binding protein α (CEBP- α) (gray bars) and GATA-binding factor 2 (GATA-2) (black bars) in CD34⁺ progenitor cells isolated from atopic donors ($n = 6$) in the presence of IL-5 (1 ng/ml) $\pm$ TSLP (100 pg/ml) compared with untreated cells, incubated for 18 hours at 37°C, 5% CO₂. Data are represented as mean fold increase $\pm$ SEM with respect to the housekeeping genes β -glucuronidase and hypoxanthine phosphoribosyltransferase 1. * indicates significant difference between groups, $P < 0.05$. ** $P < 0.01$. Two-way ANOVA, Tukey's multiple-comparison *post hoc* test.

transcription factors that regulate eosinophilopoiesis. However, given the limited number of CD34⁺ cells obtained through isolation, we chose GATA-2 and CEBP- α because of their roles in modulating initial and terminal differentiation, respectively. The relative contributions of additional transcription factors involved in TSLP-induced eosinophilopoiesis warrants further investigation.

It is important to note that several growth factors within the airways may have both positive and negative effects on local eosinophilopoietic activities. In addition to IL-5, other overlapping/redundant mediators can promote eosinophil differentiation, including IL-3, GM-CSF, IL-9, and cysteinyl leukotrienes. Conversely, various mediators have a negative influence on eosinophil differentiation, such as the type 1 cytokines IL-12 and IFN- γ . There appears to be a complex balance between both negative and positive mediators, which results in an overlapping proinflammatory environment that fosters *in situ* hematopoiesis and thus provides a continuous source of eosinophilia. As such, we cannot rule out the possibility that other growth factors present within the BECSN could have worked synergistically with TSLP to promote eosinophil differentiation. It would be interesting to determine the synergistic effect of TSLP with other growth factors present within the BECSN aside from IL-5, including GM-CSF and IL-3. However, for the purposes of this study, given the importance of IL-5, we examined the synergistic effect of IL-5 and TSLP acting as a primary regulator in eosinophilopoiesis.

There are a few notable limitations to this study. First, we acknowledge that there was a difference in the medication used across subject groups. As mentioned in METHODS, SA were on high-dose inhaled corticosteroids with daily oral corticosteroids, whereas MA used intermittent β_2 -agonists. It is possible that these medications could have

downregulated inflammatory activity within the epithelial supernatant, thus downplaying the effect that these supernatants had on our subsequent *in vitro* experiments. These medications could, in part, explain the difficulty we encountered in trying to obtain readable levels of TSLP via ELISA. On the other hand, given that the SA had a FEV₁ of <70% despite receiving high-dose steroid therapy, it is not surprising that the BECSN yielded significantly eosinophilopoietic effects in our experiments. It is clear that SA have greater local inflammation within the airways, which contributes to their poorly controlled asthma symptoms, as we previously confirmed (4). Second, there is a notable difference in the ages of the subject groups, with the NAs being markedly younger than the SAs. Unfortunately, it is not known whether age can affect the function of BECs. In the current study the lack of subjects in the same age group across asthma severity made it difficult to control for this variable. Third, we recognize that the levels of epithelial-derived cytokines (TSLP, IL-25, and IL-33) in the BECSN, were below the limit of detection by the Luminex bead assay (limit of detection for IL-25 and IL-33: 0.07 pg/ml and 0.58 pg/ml, respectively) although specific bioactive isoforms of each cytokines were not assessed. Given that we were specifically interested in the effect of epithelial-derived TSLP on eosinophilopoiesis, we treated colonies with neutralizing TSLPR antibody to determine the influence of this cytokine within the BECSN on progenitor cell differentiation. The inhibitory effect of anti-TSLPR on BECSN-induced eosinophilopoiesis suggests that TSLP was present at sufficient levels within the BECSN to promote differentiation. Fourth, it is important to note that there are other epithelial-derived cytokines that may affect eosinophilopoiesis, including IL-33 and IL-25 (35). However, in this study we found that the eosinophilopoietic potential of

the BECSN derived from asthmatic epithelium was not inhibited in the presence of a blocking ST2 antibody (Figure 2) that was previously shown to attenuate IL-33-mediated progenitor cell migrational responses (20). Therefore, IL-33 may sustain airway eosinophilia by promoting the migration of progenitor cells and mature eosinophils into the lungs, and stimulating the release of soluble mediators that are crucial for eosinophil biology, rather than by direct clonogenic expansion *per se*. We were unable to study the contribution of IL-25 due to the lack of availability of a reliable blocking antibody to the IL-25 receptor. However, we do not discount the possible contribution of IL-25 to the clonogenic potential demonstrated by the BECSN in the methylcellulose assays. Nevertheless, the importance of TSLP in maintaining eosinophilia in allergic asthma was recently confirmed by a clinical trial of anti-TSLP that allowed depletion of blood and sputum eosinophils in the treatment arm (36). Thus, taken together, these findings indicate that TSLP is an important driver of progenitor cell differentiation that may be released upstream of the main eosinophil growth factor, IL-5, thereby promoting eosinophilic inflammation within the airways.

In conclusion, for the first time in an *in vitro* experimental setup, we have demonstrated that factors derived from BEC from patients with severe asthma contain the ability to drive *in situ* eosinophilopoiesis, with TSLP being a key driver of this process. This study highlights the importance of understanding the alternative mechanisms of *in situ* processes that promote the persistence of airway eosinophilia. Controlling this activity, may improve the clinical management of severe eosinophilic asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Title: **Human Bronchial Epithelial Cell-Derived Factors from Severe Asthmatic
Subjects Stimulate Eosinophil Differentiation**

Authors: Brittany MA Salter, PhD, Steven G Smith, PhD, Manali Mukherjee, PhD,
Sophie Plante, MSc, Sakktee Krisna BSc, Graeme Nusca, BSc,
John Paul Oliveria, BSc, Anam Irshad, BSc, Gail M Gauvreau, PhD,
Jamila Chakir, PhD, Parameswaran Nair, MD, PhD and Roma Sehmi, PhD

ONLINE DATA SUPPLEMENT

ONLINE SUPPLEMENT

METHODS

Primary Epithelial Cell Cultures

Cells were extracted by overnight collagenase digestion of bronchial biopsies and tissue-extracted airway epithelial cells were characterized by immunofluorescence and flow cytometry using anti-cytokeratin antibody (Calbiochem, CA, US), as previously described (21, 22). Epithelial cells were cultured in DMEM-12 medium containing FBS (10%), insulin (5 µg/mL), hydrocortisone (0.4 µg/mL), EGF (10 ng/mL), transferrin (5 µg/mL), cholera toxin (10^{-10} M), T3 (2×10^{-9} M), Pen/Strep) in 12-well plates (3×10^5 cells/well) until they reached 80% confluence (16). Cells were then stimulated overnight with DMEM-12 supplemented with 1% FBS and basal epithelial cell supernatants (BECSN) were collected and stored at -80°C until functional studies were conducted.

We have previously shown that primary epithelial cells isolated from bronchial biopsies keep their phenotype and reproduce the in vivo phenotype. Specifically, we have shown that the primary cells are similar to the cultured cells in terms of proliferative responses (Semlali A et al. Allergy; 2010 Nov;65(11):1438-45), cytokines and chemokine production (Semlali A et al. JACI, 125(4):844-50) and Larose, M et al. JACI 2015 Oct;136(4):904-13) and miRNA profile (Haj-Salem I et al. Allergy. 2015 Feb;70(2):212-9).

TSLP and IL-5 Mediated Eosinophil-lineage Commitment of CD34⁺ Progenitor Cells

Blood CD34⁺ progenitor cells were isolated using MACS CD34 Microbeads (Miltenyi Biotech, CA, USA) and cultured in presence of IL-5 (1 ng/mL), TSLP (100 pg/mL), both or diluent control (18 h; 5% CO₂; 37°C). Cells were then washed, re-suspended in 100 µL RNAlater (Thermo Fischer Scientific, CA, US) and stored at -70°C. PCR amplification was performed using StepOne Plus Real-time ABI Systems (Life Technologies, ON, Canada) with PrimePCR™ SYBR® Green primer assays for CCAAT/enhancer-binding protein (CEBP/α, Unique Assay ID: qHsaCED0019045), GATA-binding factor-2 (GATA-2, Unique Assay IDL qHsaCID0010528) and soAdvanced™ Universal SYBR® Green Supermax (Bio-rad, IA, US), as per manufacturer's instructions. Generated Ct values (threshold cycles) were used to enumerate fold change in mRNA expression for target genes compared to untreated CD34⁺ cells, using the ΔΔCt method with respect to endogenous control genes, HPRT1 (Unique Assay ID: qHsaCID0016375) and GUSB (Unique Assay ID: qHsaCID0011706). Endogenous controls were selected using a pre-designed reference gene panel (Reference Genes H96, Bio-Rad, IA, US).

Effect of BECSN stimulation on activation status of eosinophil progenitors

NAMNCs (1x10⁶ cells/ plate) were cultured in 24-well plates in RPMI-Complete for 15 minutes with BECSN (1/1000) in the presence or absence of rhTSLP (100 pg/mL; R&D Systems, MN, US). Cells were then fixed and permeabilized with the BD Phosphoflow kit (BD Bioscience, CA, US) and stained with CD45 APCH7 (BD Bioscience, CA, US), CD34 PerCP Cy5.5 (Miltenyi Biotech, CA, US), IL-5Rα PE (Miltenyi Biotech, CA, US), and phosphor-STAT5 PE Cy7 (BD Bioscience, CA, US). Using similar conditions but

stimulating overnight and adding Monensin (1/1000; Ebioscience, CA, US) to the cultures 6 h prior to cell harvesting, cells were washed with PBS, and stained with CD45 APCH7, CD34 PerCP Cy5.5, IL-5R α APC and TSLPR Alexa Fluor 700 (R&D Systems, MN, US). Cells were further fixed and permeabilized, then stained for the following intracellular signaling moieties: Bcl-xL FITC (Abcam, ON, Canada) or cytokines IL-13 BV421 (Biolegend, CA, US), and IL-5 PE (Biolegend, CA, US). Events were acquired using a BD Biosciences LSRII Flow Cytometer (BD Bioscience, CA, US) and eosinophil progenitor cells identified as CD45^{dim}CD34⁺IL-5R α ⁺ gating, as previously described (1).

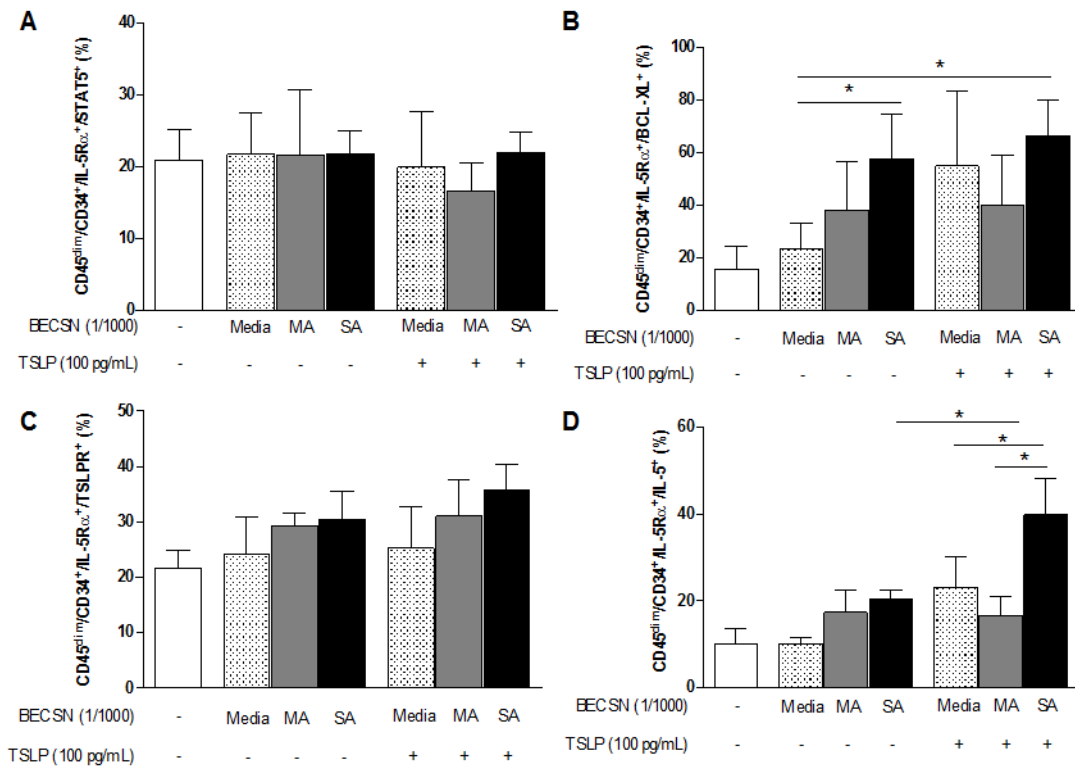
Results

Effect of BECSN on activation status of Eosinophil Progenitor Cells

Short-term cultures of peripheral blood-derived NAMNCs from atopic subjects with BECSN from either MA and SA BECSN (1/000) in the presence or absence of rhTSLP (100 pg/mL) did not stimulate significantly the TSLP phosphorylation of STAT5 in the eosinophil progenitor cell populations compared to media control (Figure E1, A).

Overnight cultures of peripheral blood NAMNCs from atopic subjects with SA BECSN (1/000) in the presence and absence of rhTSLP (100 pg/mL) significantly increased the proportion of eosinophil progenitor cells expressing intracellular levels of the anti-apoptotic pro-survival molecule Bcl-xL, compared to the media control ($P < 0.05$) (Figure E1, B). There was no significant increase in Bcl-xL expression with respect to culturing with MA BECSN (1/000) in the presence or absence of rhTSLP (100 pg/mL) (Figure E1, B). Although a trend for increased expression of TSLPR was observed on eosinophil progenitor cells cultured with BECSN (1/000) from MA and SA alone or in the presence of rhTSLP (100 pg/mL), this effect was not significant (Figure E1, C). Stimulation with

SA BECSN (1/000) in the presence of rhTSLP (100 pg/mL) significantly increased the proportion of eosinophil progenitor cells expressing intracellular levels of IL-5, compared to MA BECSN with TSLP and media control with TSLP ($P<0.05$) (Figure E1, D). In addition, SA BECSN with TSLP induced significantly higher percent IL-5 expression compared to SA BECSN alone ($P<0.05$) (Figure E1, D).



Effect of BECSN alone or in the presence of exogenous TSLP on Eosinophil Progenitors: Enumeration of the percentage of eosinophil progenitor (CD45^{dim}CD34⁺IL-5Rα⁺) cells expressing (A) phosphorylated STAT5; (B) Bcl-xL; (C) TSLPR; (D) intracellular IL-5 performed by flow cytometry. Following 15 min (STAT5) or 18 h (BCL-XL, TSLPR, IL-5) stimulation of non-adherent mononuclear cells from atopic subjects ($n = 3$) containing an enriched population of progenitor cells with BECSN (1/1000) from mild asthmatics (MA) or severe asthmatics (SA) alone or in the presence exogenously added TSLP (100 pg/mL). Data are presented as mean percent cell expression $\pm$ SEM of three separate experiments performed in duplicate where * indicates $P<0.05$ compared to media culture control; statistical analyses by ANOVA and tukey post-hoc analyses.

Supplementary References:

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