

Thymic stromal lymphopoietin activation of basophils in patients with allergic asthma is IL-3 dependent

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Background: Thymic stromal lymphopoietin (TSLP) released after antigenic stimulation of allergic asthmatic airways is a key initiator of type 2 inflammation. Basophils are important effectors of allergic inflammation in the airways. Murine basophils have been shown to respond to TSLP independently of IL-3 by increasing functional thymic stromal lymphopoietin receptor (TSLPR) expression.

Objective: The purpose of this study was to investigate the effect of TSLP stimulation on human basophil function.

Methods: Ten patients with mild allergic asthma underwent diluent and allergen inhalation challenges. Peripheral blood and sputum samples were collected at baseline and 7 and 24 hours after challenge, and bone marrow samples were collected at baseline and 24 hours after challenge to measure basophil TSLPR expression. *In vitro* experiments were conducted on purified human basophils to measure the effect of TSLP on degranulation, expression of activation markers and T_H2 cytokines, and eotaxin-induced shape change.

Results: Allergen inhalation increased basophil numbers in the airways and significantly upregulated the expression of activation markers, T_H2 intracellular cytokines, and receptors for TSLP, IL-3, and eotaxin in blood, bone marrow, and sputum basophils. *In vitro* stimulation with TSLP primed basophil migration to eotaxin and induced rapid and sustained basophil activation mediated directly through TSLPR and indirectly through an IL-3-mediated basophil autocrine loop. Basophils responded to TSLP at a similar magnitude and potency as the well-described basophil-activating stimuli IL-3 and anti-IgE. **Conclusion:** Our findings indicate that basophil activation during early- and late-phase responses to inhaled allergen might be driven at least in part by TSLP. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Basophils, allergic asthma, thymic stromal lymphopoietin, epithelium-derived cytokines, CD203c, T_H2 cytokines

Asthma is a chronic respiratory disease characterized by reversible airway obstruction, airway hyperresponsiveness, and heterogeneous inflammation of the airways of which eosinophils and basophils are prominent components. The cause of this

Abbreviations used

EAR: Early asthmatic response
GM-CSFR: GM-CSF receptor
IL-3R: IL-3 receptor
IL-5R: IL-5 receptor
LAR: Late asthmatic response
TSLP: Thymic stromal lymphopoietin
TSLPR: Thymic stromal lymphopoietin receptor

disorder is multifaceted, and recent evidence indicates an array of genetic and environmental mechanisms at play.

The epithelium-derived cytokine thymic stromal lymphopoietin (TSLP) has been identified as a master switch for allergic inflammation in several mouse models.¹ TSLP is produced in response to proinflammatory stimuli, which in turn promotes primarily type 2 inflammatory responses. In both animal and *in vitro* human models, this mediator has been shown to exert its effects on innate and adaptive immune cells, including dendritic cells, macrophages, lymphocytes, granulocytes, mast cells, innate lymphoid type 2 cells, and CD34⁺ progenitor cells.²

In studies of human asthma, TSLP mRNA expression in the airways was greater in asthmatic patients compared with that seen in healthy control subjects and correlated with asthma severity.³⁻⁵ Gauvreau et al⁶ demonstrated that treatment with an mAb to TSLP in patients with mild allergic asthma significantly attenuated allergen-induced early asthmatic responses (EARs) and late asthmatic responses (LARs) in parallel with a reduction in blood and sputum eosinophil numbers. Given that allergen-induced EARs and LARs are mediated by mast cell and basophil activation, the above findings suggest that TSLP can activate these cells in addition to other inflammatory cells, such as eosinophils.

Emerging evidence demonstrates that epithelium-derived factors can regulate basophil function. TSLP can promote murine and human bone marrow progenitor cells to differentiate into basophils.⁷ Human peripheral CD34⁺ cells preincubated with IL-3 and TNF- α have enhanced sensitivity to TSLP-mediated basophil lineage commitment,⁸ and mast cell-activated bone marrow mesenchymal stromal cells produce TSLP, which can enhance the differentiation of CD34⁺ progenitors into eosinophil/basophil colonies.⁹ Furthermore, in the presence or absence of IL-3, TSLP can induce murine basophil receptor changes, including increased CD69, CD62 ligand, CD11b, CD123, IL-33 receptor, and IL-18 receptor expression.⁷ Basophils are a significant source of TSLP, enabling the possibility of a positive feedback loop.¹⁰ It has recently been confirmed that human peripheral basophils express thymic stromal lymphopoietin receptor (TSLPR), which can be upregulated after incubation with IL-3.¹¹ Additionally, allergen stimulation leads to increased TSLPR expression on circulating basophils from patients with atopic asthma compared with nonatopic control subjects.¹² Lastly,

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Disclosure of potential conflict of interest: G. M. Gauvreau has received research support from Genentech, Sanofi, and CSL Pharma. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication November 12, 2014; revised March 19, 2015; accepted for publication March 25, 2015.

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<http://dx.doi.org/10.1016/j.jaci.2015.03.039>

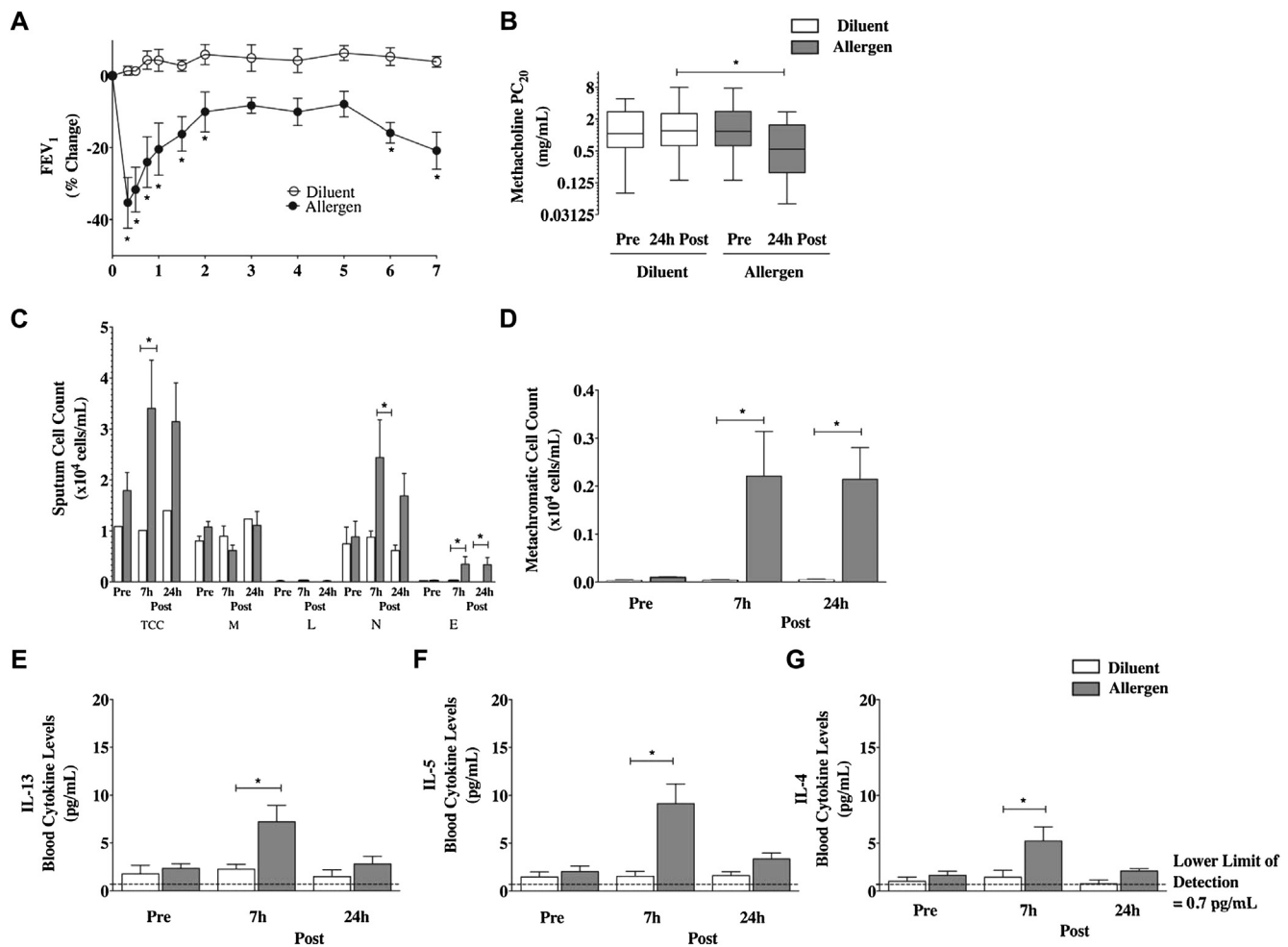


FIG 1. Allergen inhalation induces airway changes in patients with mild allergic asthma. **A** and **B**, Percent change in FEV₁ (Fig 1, **A**) and methacholine PC₂₀ (in milligrams per milliliter; Fig 1, **B**) for allergen (gray bars) versus diluent (open bars). **C** and **D**, Sputum cell counts after diluent (open bars) and allergen (gray bars). **E**, Eosinophils; **L**, lymphocytes; **M**, macrophages; **N**, neutrophils; **TCC**, total cell count. **E-G**, T_H2 cytokines in serum after allergen (gray bars) versus diluent (open bars). Data are presented as means $\pm$ SEMs, with the exception of methacholine PC₂₀, which was presented as geometric means $\pm$ geometric SEMs. *Significantly different from diluent control ($P < .05$).

a correlation has been found between increased airway TSLP expression and exaggerated basophil responses in patients with eosinophilic esophagitis, and TSLP neutralization, basophil depletion, or both ameliorates disease-like symptoms, suggesting that the interaction between TSLP and basophils might be an important component of airway inflammation.¹³

Although TSLP has been identified as an important initiator of type 2 inflammatory responses in both animal and human models of allergic asthma, the basophil-TSLP axis in human allergic inflammatory responses has not been well characterized. The purpose of this study was to further elucidate the relationship between basophils and TSLP in human allergic asthma.

METHODS

Subjects

Ten patients with mild allergic asthma were enrolled in a diluent-controlled allergen bronchoprovocation study (see Table E1 in this article's Online Repository at www.jacionline.org). Subjects were required to have a positive skin test response to common aeroallergens, a methacholine PC₂₀ value of

16 mg/mL or less, FEV₁ of 70% of predicted value or greater, and a dual-phase response to allergen ($\geq 20\%$ decrease in FEV₁ within 2 hours after allergen and $\geq 15\%$ decrease in FEV₁ between 3 and 7 hours after allergen).

Blood for *in vitro* experiments was collected from 8 donors with positive skin prick test responses to common aeroallergens. Approval was granted from the Hamilton Integrated Research Ethics Board, and all subjects provided signed informed consent.

Study design

Baseline measurements of methacholine PC₂₀, peripheral blood, bone marrow aspirate, and sputum were conducted on day 1. Subjects were randomized to inhale diluent or allergen on day 2. Blood and sputum samples were obtained 7 hours after challenge, and blood, bone marrow, and sputum samples were collected 24 hours after challenge on day 3. This triad was repeated after a 2-week recovery period. Allergen and diluent challenges were conducted, as previously described (see the Methods section in this article's Online Repository at www.jacionline.org).¹⁴

Blood, bone marrow, and sputum samples

Approximately 10 mL of blood was collected into sodium heparin vacutainers. The same volume of bone marrow was aspirated from the iliac

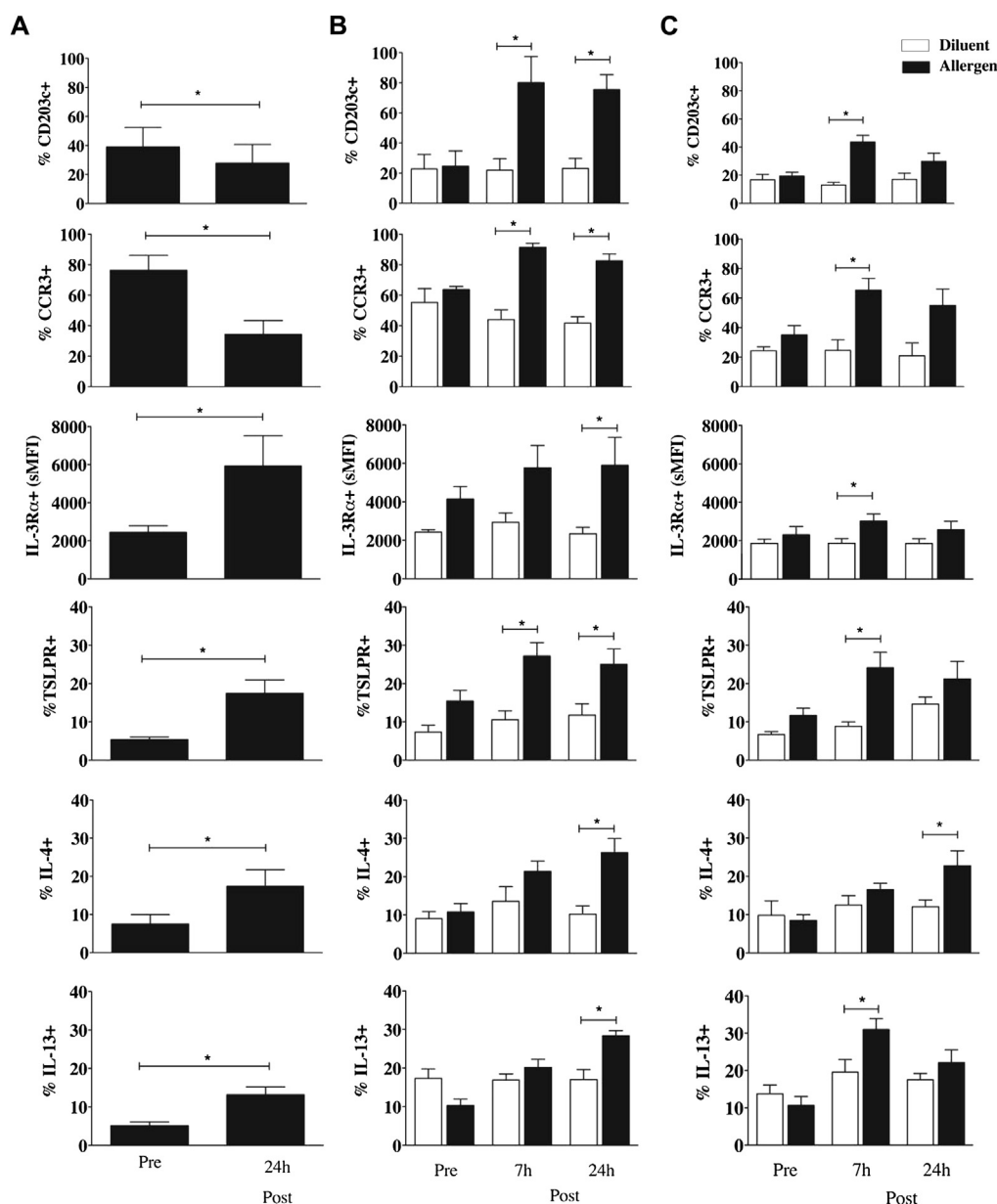


FIG 2. Effects of allergen inhalation on basophil function. Basophil expression of CD203c, CCR3, IL-13, IL-3Rα, and TSLPR in bone marrow before and 24 hours after allergen (A) and in peripheral blood (B) and sputum (C) before and 7 and 24 hours after allergen (black bars) compared with diluent (open bars). Data are presented as means $\pm$ SEMs ($n = 10$). *Significantly different from preallergen values for bone marrow and diluent control values for peripheral blood and sputum ($P < .05$). sMFI, Specific mean fluorescent intensity.

crest into heparin (1000 U/mL) and then diluted in McCoy's 5A (Gibco, Life Technologies, Grand Island, NY), layered on Lymphoprep (Axis Shield, Dundee, United Kingdom), and centrifuged at 2200 rpm for 20 minutes at room temperature. Sputum samples were induced by using hypertonic saline, and mucous plugs were selected from samples and then dispersed with Dulbecco PBS.¹⁵ Cytospin preparations were created from sputum cells and stained with Diff-Quick for differential cell counts and with toluidine blue for metachromatic cell counts. More than 95% of sputum metachromatic cells are basophils.¹⁶ TSLP ELISA and IL-4/IL-13/IL-5 Multiplex Immunoassay kits (Bio-Rad Laboratories, Ontario, Canada) with lower limits of detection of 32 and 0.7 pg/mL, respectively, were used to measure TSLP and T_H2 cytokine levels in bone marrow, peripheral blood, and sputum supernatant samples collected before and after challenges. Whole blood, cells collected from bone

marrow buffy coat, and sputum cells were immunostained to measure the expression of extracellular receptors CD203c, CCR3, IL-3 receptor (IL-3R) α , and TSLPR, as well as the intracellular cytokines IL-13 and IL-4 (see the [Methods](#) section in this article's Online Repository).

Purification of basophils for cell culture

One hundred milliliters of blood from allergic patients was diluted with McCoy's 5A and layered on Lymphoprep. Erythrocytes were lysed, and basophils were purified by using negative selection with magnetic beads (STEMCELL Technologies). Basophil cell suspensions were greater than 98% pure, as determined by using flow cytometry, and greater than 90% viable, as

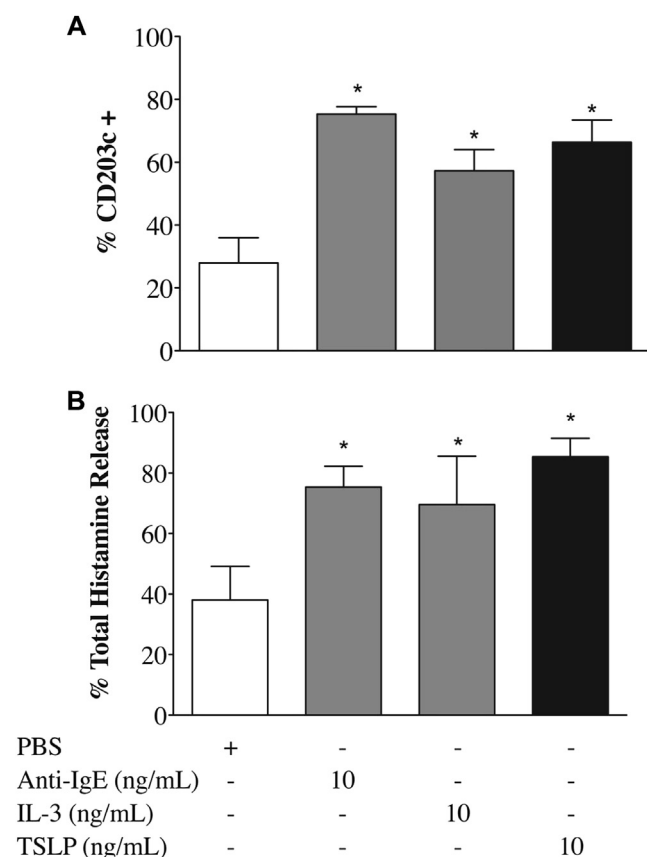


FIG 3. Effects of 1 hour of stimulation with TSLP on basophil activation. Effects of 1 hour of stimulation with PBS (open bars) or optimal concentrations of anti-IgE (10 ng/mL) and IL-3 (10 ng/mL; gray bars) or TSLP (10 ng/mL; black bars) on basophil CD203c expression measured by flow cytometry (A) and histamine degranulation measured by using an ELISA (B) are shown. Data are expressed as means $\pm$ SEMs. *Significantly different from PBS-negative control ($P < .05$).

determined by using trypan blue (see Fig E1 in this article's Online Repository at www.jacionline.org).

One-hour basophil cultures

Purified basophils were cultured for 1 hour at 37°C with or without an optimal concentration of TSLP (10 ng/mL; R&D Systems, Minneapolis, Minn) determined through dose response. IL-3 (R&D Systems) and anti-IgE (both 10 ng/mL; Sigma-Aldrich, St Louis, Mo) served as positive controls. Basophils were immunostained to assess CD203c expression by using flow cytometry, and culture media were collected for histamine measurements by using ELISA (IBL-America, Minneapolis, Minn). Basophils heated for 10 minutes at 90°C provided a measure of total histamine release.

Eighteen-hour basophil cultures

Purified basophils were incubated for 18 hours at 37°C in RPMI-C with monensin (eBioscience, San Diego, Calif), with or without optimal concentrations of IL-3, anti-IgE, and TSLP (all 10 ng/mL) and with or without neutralizing antibodies targeting TSLPR, IL-3R α , IL-5 receptor (IL-5R) α , GM-CSFR α , common β -chain (all 10 μ g/mL, R&D Systems). Basophils were immunostained to measure levels of the cell-surface receptors CD203c, CCR3, and TSLPR and intracellular cytokine expression of IL-3, IL-4, IL-5, IL-13, and GM-CSF by using flow cytometry (see Fig E2 in this article's Online Repository at www.jacionline.org).

Shape-change assay

Purified basophils were incubated with or without TSLP (0.1, 1, 10, and 100 ng/mL) at 37°C in RPMI-C for 0, 30, 45, 60, or 180 seconds to determine whether TSLP had a direct effect on basophil shape change. Purified basophils were incubated for 18 hours at 37°C in RPMI-C with either PBS or TSLP (10 ng/mL) to determine whether TSLP primed the basophil shape-change response to eotaxin. After incubation, cells were stimulated with either an optimal dose of the chemoattractant eotaxin (5 ng/mL, R&D Systems) or PBS for 0, 30, 45, 60, or 180 seconds. Basophils were fixed with ice-cold 1% paraformaldehyde for 10 minutes to stop the reaction, and cell shape change was measured by using the parameter front-angle light scatter with flow cytometry.

Statistical analysis

All data are presented as means $\pm$ SEMs. Flow cytometric results are expressed as the mean percentage of positive cells. In cases in which basophils constitutively express the marker of interest, results are expressed as specific mean fluorescent intensity. Histamine is expressed as a percentage of total histamine release. Statistical analysis for the inhalation challenges was performed by using 2-way ANOVA and the *post hoc* Bonferroni test. The *in vitro* experiments were analyzed by using 1-way ANOVA with a *post hoc* Tukey test. Significance was accepted at a P value of less than .05.

RESULTS

Allergen inhalation induces airway bronchoconstriction, hyperresponsiveness, and inflammation in patients with mild allergic asthma

Inhalation of allergen induced a 20.1% $\pm$ 5.5% decrease in FEV₁ during the EAR (10-120 minutes) and a 20.8% $\pm$ 5.2% decrease in FEV₁ during the LAR (3-7 hours) compared with no change after diluent inhalation (Fig 1, A). The methacholine PC₂₀ value decreased by 1 doubling dose after allergen challenge compared with no change after diluent (Fig 1, B).

Compared with diluent, allergen inhalation significantly increased total cell, eosinophil, neutrophil (Fig 1, C), and basophil (metachromatic cell; Fig 1, D) numbers in sputum and levels of the T_H2 cytokines IL-4, IL-5, and IL-13 in peripheral blood at 7 hours after allergen (Fig 1, E and F). IL-4, IL-5, and IL-13 could not be detected in bone marrow or sputum. TSLP could not be detected in bone marrow or sputum samples but could be detected in the blood of 1 subject.

Allergen inhalation activates basophils in bone marrow, peripheral blood, and sputum

Numbers of basophils in blood and sputum increased during the LAR after inhaled allergen challenge^{16,17} and provide a significant source of IL-4 and IL-13.¹⁸ We sought to evaluate basophil expression of T_H2-related cytokines and receptors in samples collected from bone marrow, blood, and airways after allergen inhalation. In the bone marrow there was a significant increase in IL-3R α , TSLPR, IL-4, and IL-13 expression at 24 hours after allergen compared with preallergen baseline values, whereas CD203c and CCR3 expression significantly decreased (Fig 2, A). Circulating basophils expressed peak levels of CD203c, CCR3, and TSLPR at 7 hours after allergen, which remained increased at 24 hours. IL-3R α , IL-4, and IL-13 expression increased in circulating basophils at 24 hours after allergen (Fig 2, B). In addition, sputum basophil CD203c, CCR3, TSLPR, IL-3R α , and intracellular IL-13 expression peaked at 7 hours after allergen, with IL-4 expression peaking

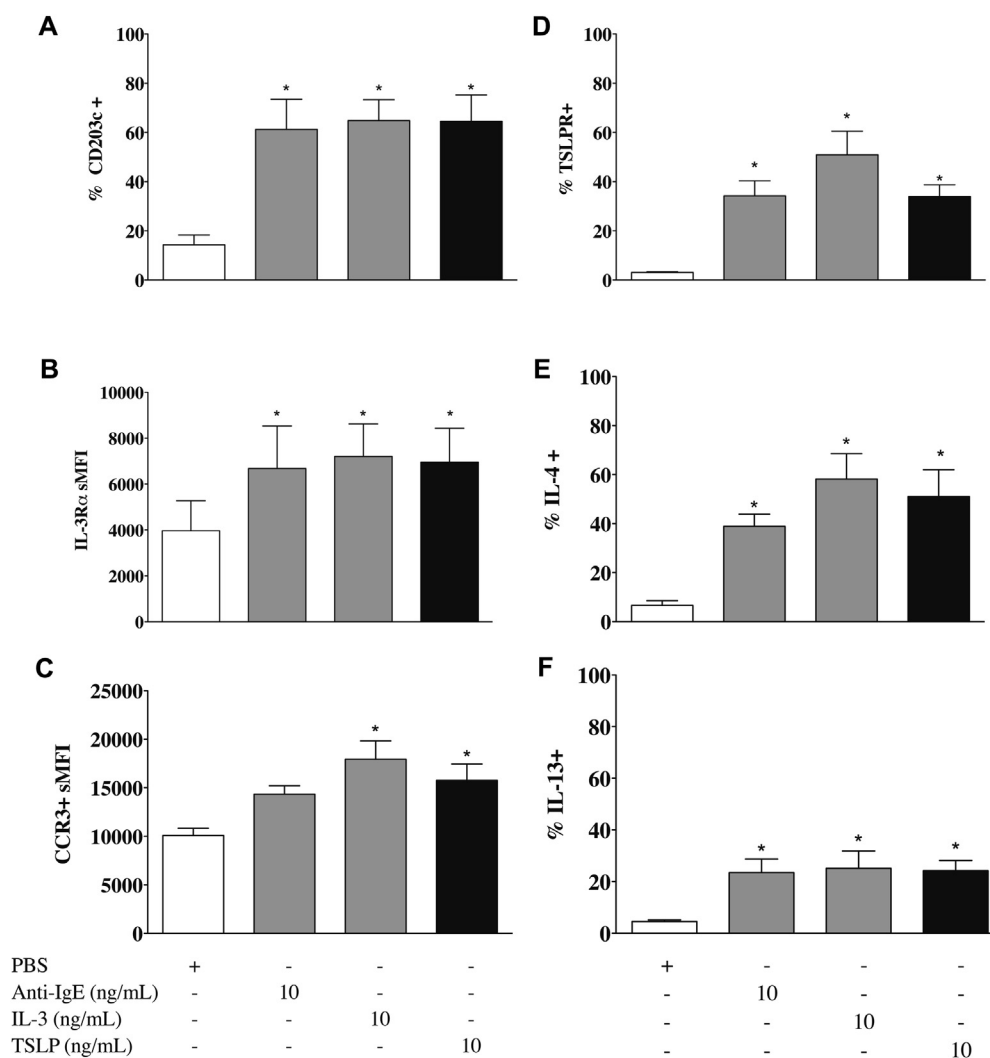


FIG 4. Effects of 18 hours of incubation with TSLP on basophil function. Data for basophils cultured for 18 hours with PBS as a negative control (open bars) or optimal concentrations of anti-IgE (10 ng/mL) and IL-3 (10 ng/mL; gray bars) or TSLP (10 ng/mL; black bars) and then measured for expression of CD203c (A), IL-3Rα (B), CCR3 (C), TSLPR (D), IL-4 (E), and IL-13 (F) are shown. Data are expressed as means $\pm$ SEMs. *Significantly different from PBS-negative control ($P < .05$). sMFI, Specific mean fluorescent intensity.

at 24 hours (Fig 2, C). There was a significant positive correlation between the magnitude of the airway response to allergen (increase in airway responsiveness to methacholine and the LAR) and peripheral blood basophil CD203c, TSLPR, and intracellular IL-13 and IL-4 expression (see Figs E3-E5 in this article's Online Repository at www.jacionline.org). In addition, the frequency of sputum basophils expressing TSLPR at 7 hours after allergen significantly correlated with a shift in methacholine PC₂₀ ($P < .05$, data not shown).

TSLP induces rapid and long-lasting effects on basophil function

To investigate mechanisms through which TSLP might promote allergen-induced EARs, we evaluated the effect of TSLP on basophil degranulation and activation *in vitro*. Purified human basophils stimulated with an optimal concentration of TSLP (see Fig E6 in this article's Online Repository at

www.jacionline.org) for 1 hour yielded marked increases in expression of CD203c and release of histamine comparable with those seen after stimulation with optimal concentrations of anti-IgE and IL-3 (Fig 3). Incubation of basophils for 18 hours with an optimal concentration of TSLP (see Fig E7 in this article's Online Repository at www.jacionline.org) induced significantly higher expression of intracellular IL-4 and IL-13 (Fig 4). We also observed significant increases in intracellular IL-3 and IL-5, but not GM-CSF, expression (see Fig E8 in this article's Online Repository at www.jacionline.org). Incubation of basophils with TSLP for 18 hours upregulated TSLPR and increased IL-3Rα and CCR3 expression to a similar magnitude as incubation of basophils with IL-3. Basophils incubated with anti-IgE for 18 hours had no change in CCR3 expression.

Treatment with neutralizing antibodies to TSLPR, IL-3Rα, and the common β -chain for IL-3/IL-5/GM-CSF significantly inhibited TSLP-induced increases in intracellular expression of IL-4 and IL-13, as well as CD203c and IL-3Rα (Fig 5). Blocking

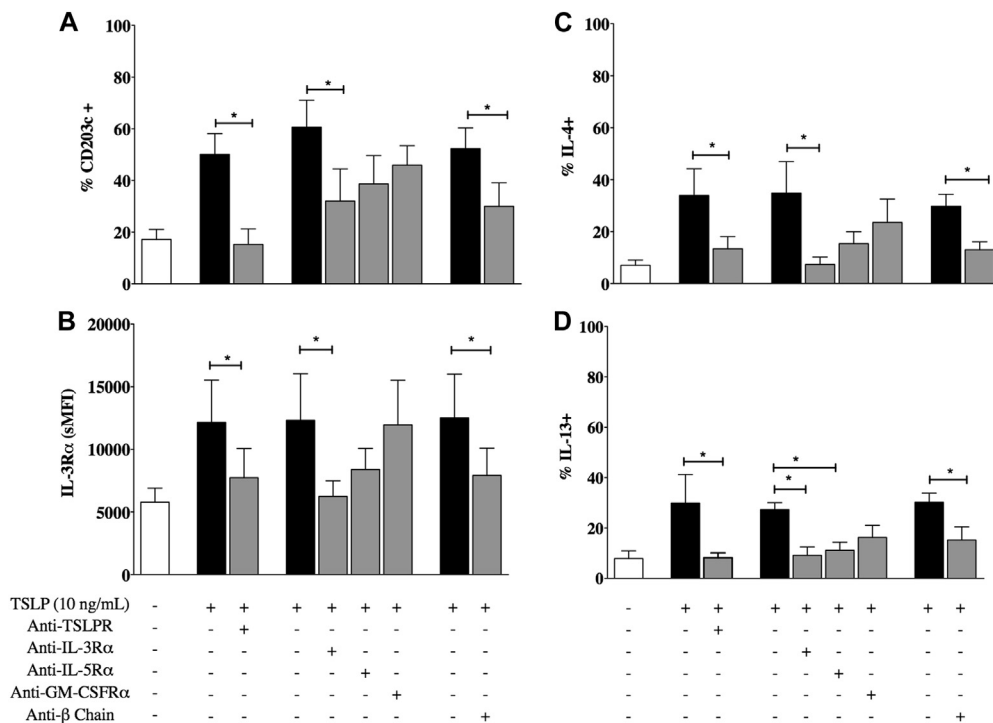


FIG 5. Blocking TSLPR, IL-3R α , and the common β -chain neutralizes the effects of TSLP on basophils. The effect of 18 hours of incubation with PBS-negative control (open bars) or TSLP (10 ng/mL; black bars) and neutralizing antibodies to TSLP, IL-3, IL-5, GM-CSF, and the common β -chain (10 μ g/mL; gray bars) on markers of basophil function measured based on expression of CD203c (A), IL-3R α (B), IL-4 (C), and IL-13 (D) is shown. Data are expressed as means $\pm$ SEMs. *Significantly different from isotype control ($P < .05$). sMFI, Specific mean fluorescent intensity.

IL-5R α inhibited only intracellular IL-13 expression, whereas blocking GM-CSF receptor (GM-CSFR) α had no effect on any measured TSLP-induced responses.

TSLP primes basophil shape change in response to eotaxin

The migrational responsiveness of basophils to the CCR3 cognate ligand eotaxin was evaluated by using shape change as a surrogate measure for chemotaxis.¹⁹ Compared with the PBS diluent control, TSLP did not directly stimulate basophil shape change (see Fig E9 in this article's Online Repository at www.jacionline.org), but overnight incubation of basophils with TSLP induced a significantly greater shape change in response to stimulation with eotaxin (Fig 6, A). This priming effect, which was observed by 45 seconds after eotaxin stimulation and was maintained for up to 3 minutes, demonstrates a functional consequence for TSLP-induced upregulation of CCR3 on basophils.

After incubation of basophils with TSLPR neutralizing, there was significant inhibition of the TSLP priming effects on basophil shape change in response to eotaxin (Fig 6, B). Furthermore, blocking IL-3R α and the common β -chain with neutralizing antibodies significantly reduced the priming effect of TSLP, whereas blocking IL-5R α and GM-CSFR α had no effect (Fig 6, B).

DISCUSSION

TSLP has been proposed to be a master switch for allergic inflammation by activating an array of inflammatory cells that

drive type 2 inflammatory responses.^{1,2} Gauvreau et al⁶ have recently shown that TSLP blockade significantly attenuated airway functional and inflammatory responses to inhaled allergen in allergic asthmatic patients. In the current study we have extended these findings to show that basophils directly respond to TSLP by producing mediators that can contribute to the development of allergen-induced EARs and LARs.

The EAR develops after IgE-mediated release of histamine and cysteinyl leukotrienes from airway mast cells and basophils; the reported inhibitory effects of TSLP blockade on the EAR⁶ suggests that TSLP is also involved with activation of these cells in the airways after allergen inhalation (Fig 7). Stimulation of human basophils for 1 hour with TSLP upregulated the activation marker CD203c, together with release of histamine at a level similar to that seen after stimulation with anti-IgE and IL-3,²⁰⁻²² indicating potent effects of TSLP on basophil degranulation. During the LAR, peripheral and airway basophils increase in number¹⁶ and activation¹⁷ and release IL-4 and IL-13 after antigen activation.^{18,23} We show that incubation of basophils overnight with TSLP increased intracellular expression of T_H2 cytokines and upregulated receptors for TSLP and IL-3R α . We found that upregulation of IL-3R α by TSLP was blocked by neutralizing antibodies to TSLPR, IL-3R α , and the common β -chain for IL-3/IL-5/GM-CSF but not through neutralization of IL-5R α or GM-CSFR α , thereby demonstrating that the effects of TSLP are dependent on the IL-3-specific pathway mediated through a functional IL-3R component. The IL-3-dependent autocrine activation loop in basophils has been previously described in human *in vitro* studies in which basophils produce

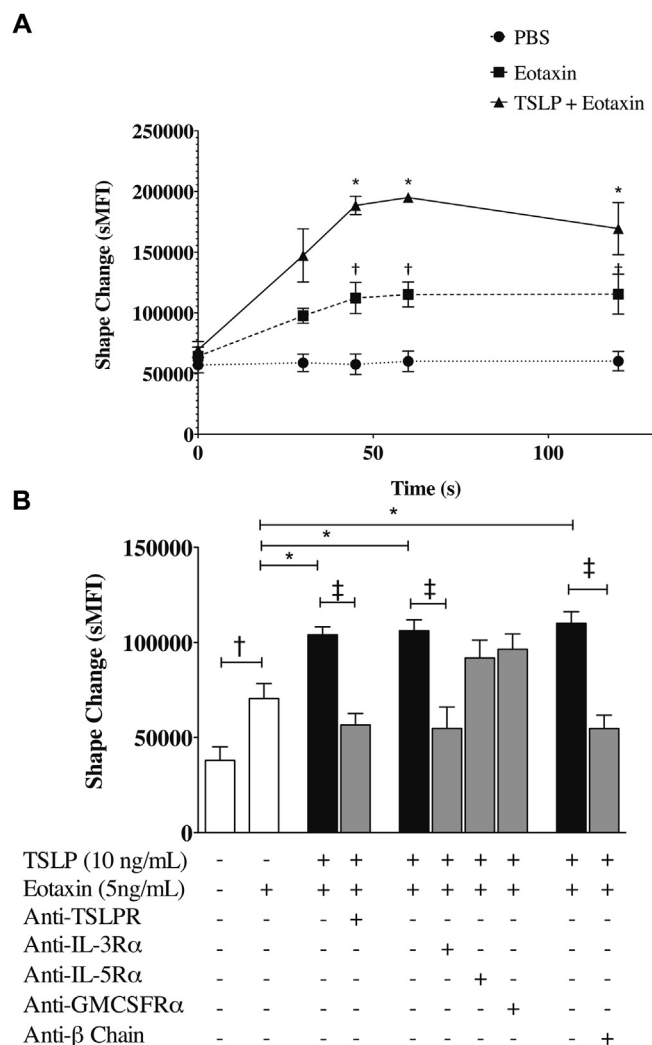


FIG 6. TSLP priming increases eotaxin-induced basophil shape change. **A**, Effect of 18 hours of incubation of basophils with or without TSLP (10 ng/mL) on shape change measured at 0, 30, 45, 60, and 180 seconds after eotaxin stimulation (5 ng/mL). Shape change was assessed by using flow cytometry and measuring the change in specific mean fluorescent intensity (sMFI) of front-angle light scatter. *Significantly different from eotaxin or †significantly different from PBS-negative control ($P < .05$). **B**, Effect of pretreatment with neutralizing antibodies (10 μ g/mL) on 18 hours of TSLP-induced increase of basophil shape change measured 45 seconds after stimulation with 5 ng/mL eotaxin (gray bars) compared with corresponding isotype controls (black bars). Data are expressed as means $\pm$ SEMs ($n = 8$). *Significantly different from eotaxin (open bars), †significantly different from PBS-negative control (open bars), and ‡significantly different from isotype control (black bars; $P < .05$).

IL-3 within 4 hours in response to low-level IgE/FcεRI cross-linking.²⁴ Furthermore, basophils were shown to rapidly bind the IL-3 they produced, as demonstrated by inhibition of their functional activity by treatment with neutralizing anti-IL-3R.²⁴ In addition, we demonstrated that TSLP could increase basophil production of IL-3, which is another mechanism through which TSLP can enhance basophil signaling through IL-3R. Elaboration of preformed mediators and enhanced expression of T_H2 cytokines and receptors in samples of purified human basophils demonstrates for the first time a primary effect of TSLP on the effector function of these cells.

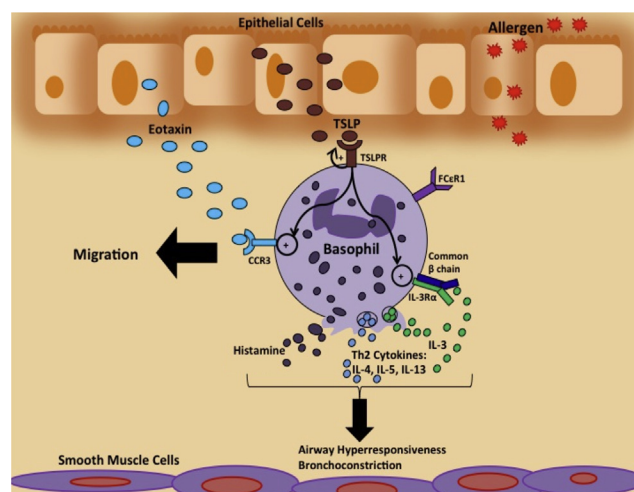


FIG 7. TSLP-basophil axis in the airways during allergic stimulation. TSLP released in airways binds to TSLPR on basophil surfaces, resulting in release of histamine; upregulation of TSLPR, IL-3, and CCR3 receptors; and increased intracellular expression of T_H2 cytokines. TSLP thereby induces basophil migration and effector responses known to drive EARS and LARs.

TSLP stimulated up-regulated basophil CCR3 expression, which was associated with increased responsiveness to eotaxin and also was dependent on signaling through IL-3R. CCR3 expression was further evaluated in blood, bone marrow, and airway basophils of patients with mild allergic asthma after allergen inhalation. In bone marrow basophils there was a decrease in CCR3 (and CD203c) expression at 24 hours after allergen, whereas CCR3 (and CD203c) expression was upregulated on both peripheral and sputum basophils. Taken together with the increase in sputum basophil numbers after allergen, this suggests that a subset of activated basophils that express CCR3 migrate in response to eotaxin from the bone marrow into the periphery and finally to the airways after allergen inhalation. This is consistent with mechanisms of eosinophil migration from bone marrow,²⁵ with reports of increased peripheral eotaxin levels by 5 hours after allergen challenge²⁶ and with the current data demonstrating that TSLP upregulates CCR3 basophil expression for up to 18 hours.

During the LAR from 7 to 24 hours after allergen, we found that intracellular IL-4 and IL-13 levels in peripheral and sputum basophils increased but appeared to lag behind the increased serum levels measured at 7 hours after allergen. Furthermore, we found peripheral IL-13 and IL-4 basophil expression at 24 hours after allergen correlated with disease severity. We also observed an increase in IL-3Rα expression for up to 24 hours after exposure to allergen on both peripheral and sputum basophils, which suggests that the level of cell activation and responsiveness to surrounding IL-3 within the periphery and airways becomes increased during the LAR. Given that our *in vitro* studies show TSLP to promote basophil IL-3Rα and CD203c expression, as well as intracellular T_H2 cytokine expression, it is possible that the increased expression seen after allergen inhalation might be at least in part due to interaction with TSLP (Fig 7). Lastly, one of the most important findings of this study was the upregulation of basophil TSLPR expression within the bone marrow, periphery, and airways after allergen, suggesting that basophils

might have increased responsiveness to TSLP after allergen inhalation in asthmatic patients. Furthermore, TSLPR basophil expression at 7 to 24 hours after allergen positively correlated with pulmonary function and airway reactivity, which further suggests that increased responsiveness of basophils to TSLP might be a contributing factor to LAR development.

Our study had a few limitations. First, given the invasiveness of bone marrow collection, we could only collect samples at 2 time points. No bone marrow samples were collected during the diluent challenge for statistical comparison; however, the bone marrow samples were both collected in the morning to control for diurnal variation.

Second, we were unable to detect secreted TSLP within the bone marrow or sputum either because of low levels or local degradation of the protein. The frequency of subjects with detectable TSLP expression in blood (10%) in the current study is lower than the frequency of children reported to have detectable levels of TSLP in blood (33%),²⁷ and this might be due to differences in the populations studied or performance of the assays used. Previous studies have shown that airway TSLP mRNA expression is increased in asthmatic patients and correlates with disease severity³⁻⁵ and that anti-TSLP antibody blocks allergen-induced airway responses.⁶ Thus we expect TSLP levels to be increased in the airways after allergen inhalation; however, future studies are needed to determine the relative protein levels of TSLP within the airways. Similarly, we could not detect secreted IL-13 or IL-4 from basophil cultures, possibly because of the low cell numbers available for culture. Instead, we measured intracellular cytokine expression by means of flow cytometry.

Lastly, because of limited basophil cell numbers, it was difficult to conduct migration experiments, and thus we assessed cell shape change as a surrogate measure of responsiveness to eotaxin.

In summary, we have demonstrated that the basophil-TSLP axis generates type 2 inflammatory responses with potency similar to anti-IgE and IL-3 (Fig 7). Allergen inhalation in patients with allergic asthma significantly increases basophil numbers within the airways, and this recruitment of basophils might be primed by TSLP. Furthermore, basophils express increased TH2 cytokine levels during the LAR, which are consistent with their response to TSLP stimulation *in vitro*. We conclude that initiation and promotion of basophil proinflammatory function after allergen inhalation in patients with allergic asthma is at least in part due to activation by TSLP in an IL-3-dependent manner.

Key messages

- TSLPR expression increases on basophils in blood, bone marrow, and airways after allergen inhalation in patients with allergic asthma.
- TSLP induces proinflammatory responses in human basophils.

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**Thymic Stromal Lymphopoietin Activation of Basophils in Allergic Asthma is IL-3
Dependent**

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Online Data Supplement

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ONLINE REPOSITORY MATERIALS AND METHODS

Subject Exclusion Criteria

Subjects were excluded if they were current or ex-smokers with more than 10 pack-years, developed lower respiratory infections or used inhaled or oral steroids 4 weeks prior to study. Anti-histamines, caffeine, and non-steroidal anti-inflammatory agents were prohibited 48 hours before study visits.

Methacholine Challenge

The methacholine inhalation challenge was carried out by tidal breathing from a Wright nebulizer.^{E1} Doubling concentrations of methacholine chloride (Methapharm, ON) were inhaled orally from a Hans Rudolph valve at 2 min intervals. The FEV₁ was measured following inhalation at 30 s and 90 s or until it stopped falling. The percent fall was calculated from the post-diluent FEV₁ value. The test was terminated when a fall in FEV₁ of at least 20% of the lowest post-saline value occurred. The methacholine PC₂₀ was then calculated using linear interpolation.

Allergen and Diluent Challenge

Allergen challenges were conducted by administering doubling inhaled concentrations of allergen extract, as previously described^{E2} and diluent challenges were conducted using 3 inhalations of 0.9% saline for 2 min each. The early asthmatic response was the largest percent fall in FEV₁ between 0 and 2 h, and the late asthmatic response was the largest percent fall in FEV₁ between 3 and 7 h post-challenge.

1-h Dose-Response Basophil Cultures

Purified basophils were cultured for 1 h at 37°C with increasing concentrations of TSLP (1, 10, 100 ng/mL) (R&D Systems). Following incubation, the cell pellet was collected, then

washed with PBS and centrifuged at 300 g for 10 min at 4°C. The cell pellet was then re-suspended in FACS buffer and immunostained to assess CD203c expression by flow cytometry. In addition, the supernatant was collected from culture to measure basophil histamine release via a commercially available ELISA (IBL-America). Basophils heated for 10 min at 90°C provided a measure of total histamine release.

18-h Basophil Cultures

Purified basophils were incubated for 18 h at 37°C in RPMI-C with the golgi-blocking agent, monensin (Ebioscience) with or without increasing concentrations of TSLP (0.1, 1, 10, 100 ng/mL). Basophils were immunostained to measure extracellular receptors CD203c, CCR3, and TSLPR, and intracellular cytokine expression of IL-3, IL-4, IL-5, IL-13, and GM-CSF by flow cytometry.

Flow cytometry staining and analysis

Cells for flow cytometry experiments were immunostained with isotype controls or antibodies to extracellular receptors CD45, HLA-DR, CCR3 (BD Biosciences), TSLPR, IL-3R α (Ebioscience), CD203c (MACS Miltenyi) for 30 min at 4°C. To measure intracellular cytokine expression, cells were washed, fixed and permeabilized, then stained with isotype controls or antibodies to IL-4, IL-13 (Ebioscience), GM-CSF (BD Biosciences), IL-3, IL-5 (R&D Systems). Cells were washed and acquired with a LSR II flow cytometer (BD Biosciences). Analyses were performed using Flow-Jo software (TreeStar). Basophils were defined as the CD45⁺/HLA-DR⁻/IL-3R α ⁺ population (Supplementary Figure 2). For whole staining of blood and bone marrow, at least 300, 000 CD45⁺ events were acquired, with the collection of the same cell number for each time-point pre/post-diluent/allergen challenge per subject. Similarly, at least 1, 000, 000 events were acquired for sputum samples, with the same cell number collected for each time-point

pre/post-diluent/allergen challenge per subject. Lastly, for the *in vitro* culture studies, at least 10,000 CD45⁺ events were acquired per culture condition.

Statistical Analysis

All data in the manuscript are presented as mean $\pm$ SEM. In this study the EAR and LAR were assessed as the maximum allergen-induced fall in FEV₁ %, based on analysis of the minimum FEV₁ % remaining over 0-3 h and 3 to 7 h, respectively, post-challenge.

Methacholine PC₂₀ values were log-transformed before analyses, and expressed as the geometric mean and range. The Methacholine PC₂₀ between Day 1/Day 3 of both diluent and allergen inhalation challenges were compared.

Flow cytometry results are expressed as mean percent positive cells. In cases where basophils constitutively express the marker of interest the results are expressed as specific Mean Fluorescent Intensity (sMFI). Histamine is expressed as percent of total histamine release. The cytokines measured by ELISA are shown as concentration (pg/mL).

Statistical analysis for the inhalation challenges was performed using 2-way ANOVA and post-hoc Bonferroni test. The *in vitro* experiments were analyzed using 1-way ANOVA with a post-hoc Tukey test. Significance was accepted at P<0.05.

83

84 **ONLINE REPOSITORY TABLE**

Sex	Age (y)	% Predicted FEV ₁	Methacholine PC ₂₀ , mg/mL	Ag Inhaled	Final [Ag] Inhaled
M	61	96.00	0.66	CAT	1:32
F	24	80.00	1.46	RGW	1:16.15
M	52	95.00	0.11	HDM	1:68
F	40	104.00	0.18	CAT	1:120
M	25	86.00	1.44	RGW	1:83
F	24	93.00	0.45	HDM	1:84
M	26	93.00	0.33	HDM	1:32
M	20	94.00	3.71	GRS	1:4
F	34	70.00	0.26	CAT	1:512
M	26	96.00	0.64	CAT	1:83
No. M/F 6:4	Mean 33.2±13.6	Mean 90.7±9.6	Geometric Mean= 0.55 Range= 0.11 to 3.71		

85

86 **Table I: Subject Characteristics**

87 *Ag*, Allergen; *EAR*, early asthmatic response; *F*, female; *HDM*, house dust mite; *LAR*, late
88 asthmatic response; *M*, male; *RGW*, ragweed; *GRS*, grass. Data presented as mean ± SEM for
89 Age and FEV₁ and geometric mean with range for methacholine PC₂₀.

90

ONLINE REPOSITORY FIGURE LEGNEDS

Figure E1. Basophil purity post-isolation. (A-D) Representative dot plots showing flow cytometric analysis of basophil purity through gating around CD45⁺/CD123⁺/HLA-DR⁻ population. The final purity after gating around the basophil population is 98.8%. (E) Representative toluidine blue-stained cells at 40x magnification (0.65mm diameter field).

Figure E2. Basophil gating strategy. Representative whole blood dot plots showing strategic basophil gating used for flow cytometry analysis. (A) SSC vs. CD45 Alexa Fluor 700 (B) SSC vs. HLA-DR APCH7 (C) SSC vs. CD123 PECy7. (D) Histogram demonstrating isotype vs specific antibody for assessment of receptor expression on basophils.

Figure E3. Correlation between CD203c basophil expression and magnitude of allergen-induced responses. Peripheral blood basophil expression of CD203c at 7 and 24 hours post-allergen is positively related to maximum % fall in FEV₁ during the late asthmatic response 3-7h post-allergen (A and B) but is not related to the doubling dose shift in methacholine PC₂₀ from pre- to 24 hours post-allergen (C and D). Data are presented as mean±SEM.

Figure E4. Correlation between TSLPR basophil expression and disease severity. Peripheral blood basophil expression of TSLPR at 7 and 24 hours post-allergen is positively related to maximum % fall in FEV₁ during the late asthmatic response 3-7h post-allergen (A and B) but is not related to the doubling dose shift in methacholine PC₂₀ from pre- to 24 hours post-allergen (C and D). Data are presented as mean±SEM.

Figure E5. Correlation between intracellular expression of IL-4 and IL-13 in peripheral blood basophils and magnitude of allergen-induced late asthmatic response. Peripheral blood basophil

expression of IL-4 (A) and IL-13 (B) is positively related to maximum % fall in FEV₁ during the late asthmatic response 3-7h post-allergen. Data are presented as mean±SEM.

Figure E6. The effects of 1 hour stimulation of purified peripheral blood basophils with PBS (open bars) or increasing concentrations of TSLP (0.1, 1, 10, 100 ng/mL) (black bars), on the expression of CD203c (A) and histamine release (B). Data expressed as mean±SEM. * P<0.05 compared to PBS control.

Figure E7. The effects of 18 hours incubation of purified peripheral blood basophils with PBS (open bars) or increasing concentrations of TSLP (0.1, 1, 10, 100 ng/mL) (black bars), on the expression of CD203c (A), IL-3R α (B), CCR3 (C), TSLPR (D), IL-4 (E), and IL-13 (F). Data expressed as mean±SEM. * P<0.05 compared to PBS control.

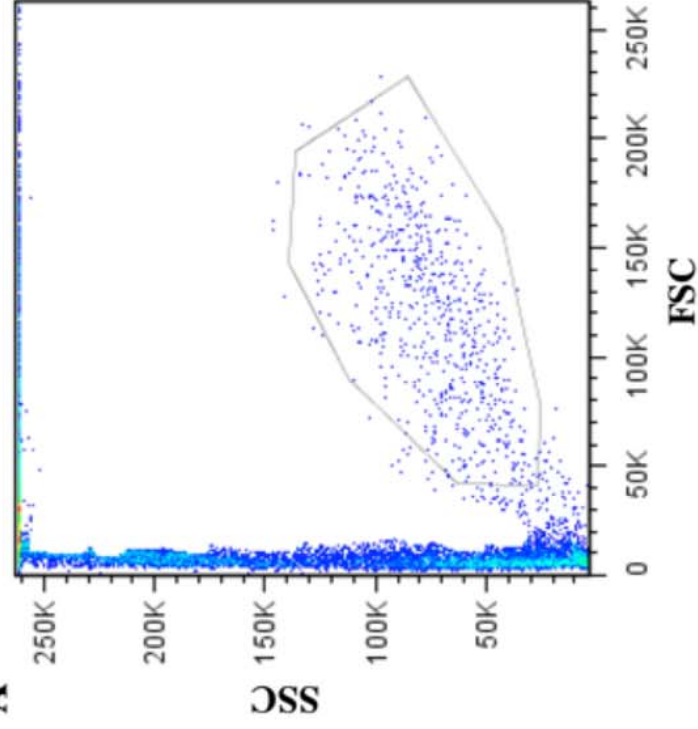
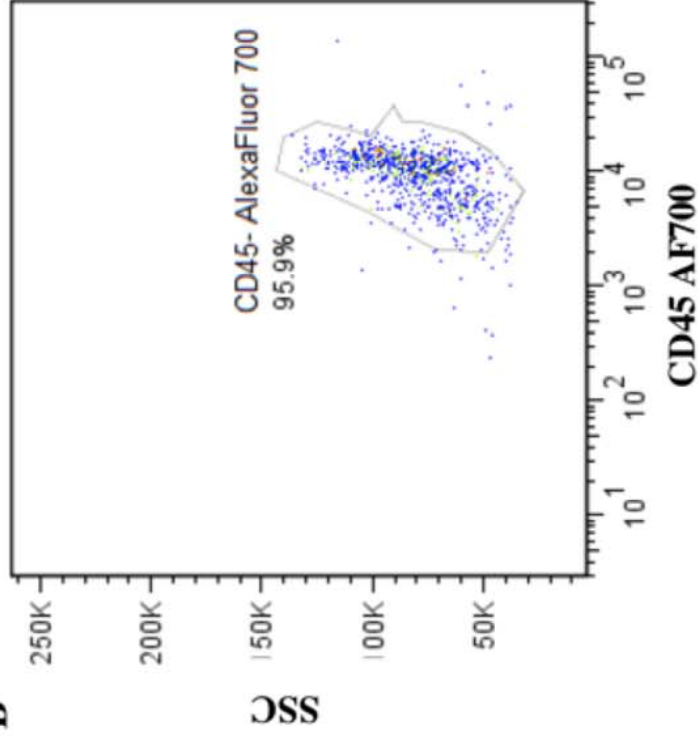
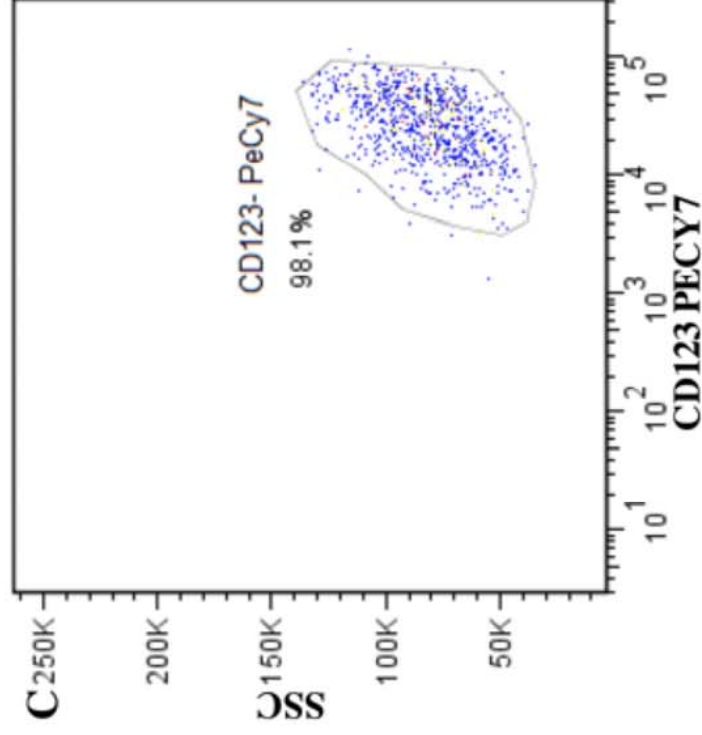
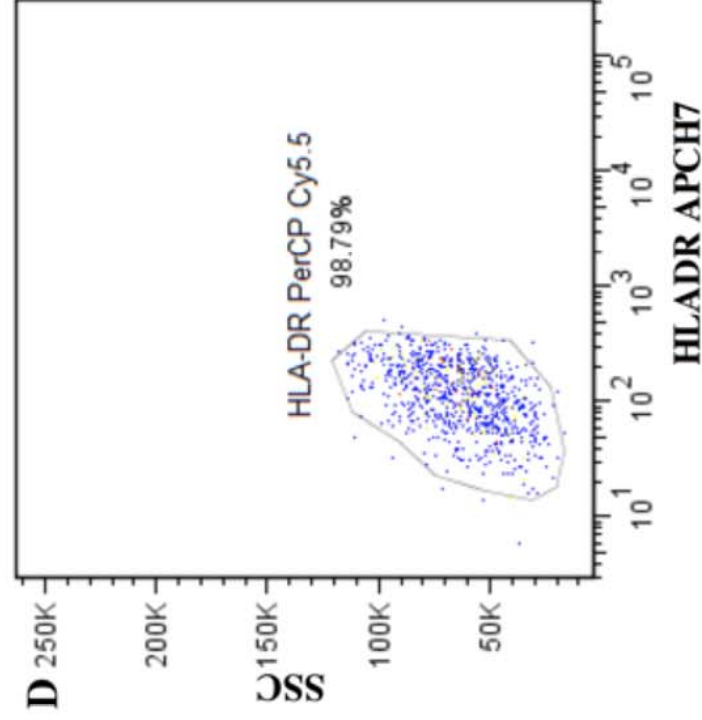
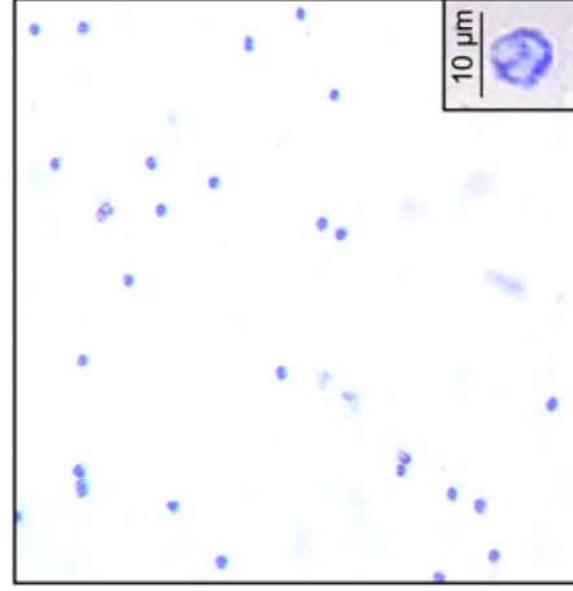
Figure E8. The effects of 18 hours incubation of purified peripheral blood basophils with PBS (open bars) or 10 ng/ml TSLP on the intracellular expression of eosinophilopoietic cytokines IL-3, IL-5, and GM-CSF. Data expressed as mean±SEM. * P<0.05 compared to PBS control.

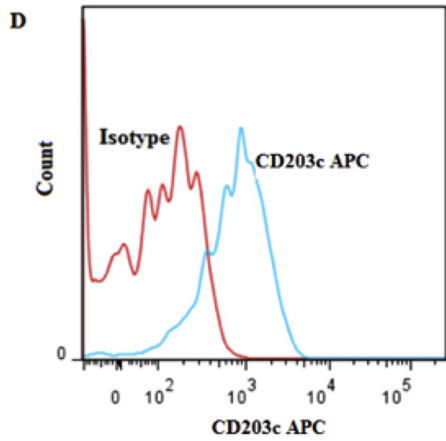
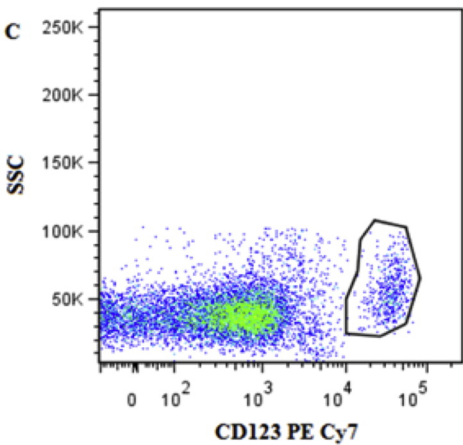
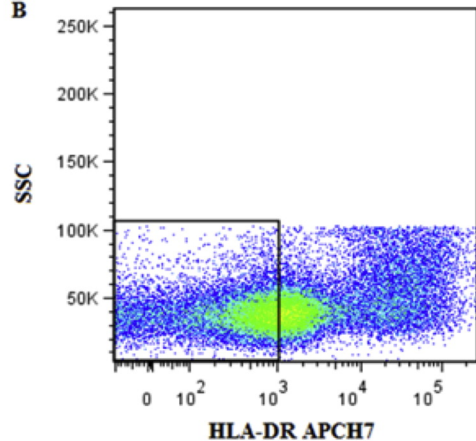
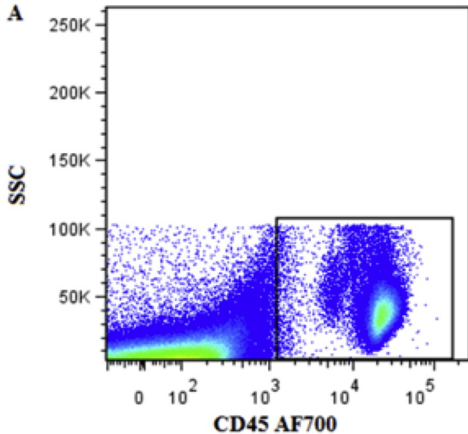
Figure E9. TSLP does not directly induce shape change in basophils measured at 0, 30, 45, 60, and 180 seconds after stimulation with 10 ng/ml TSLP. Data expressed as mean±SEM. * P<0.05 compared to PBS control.

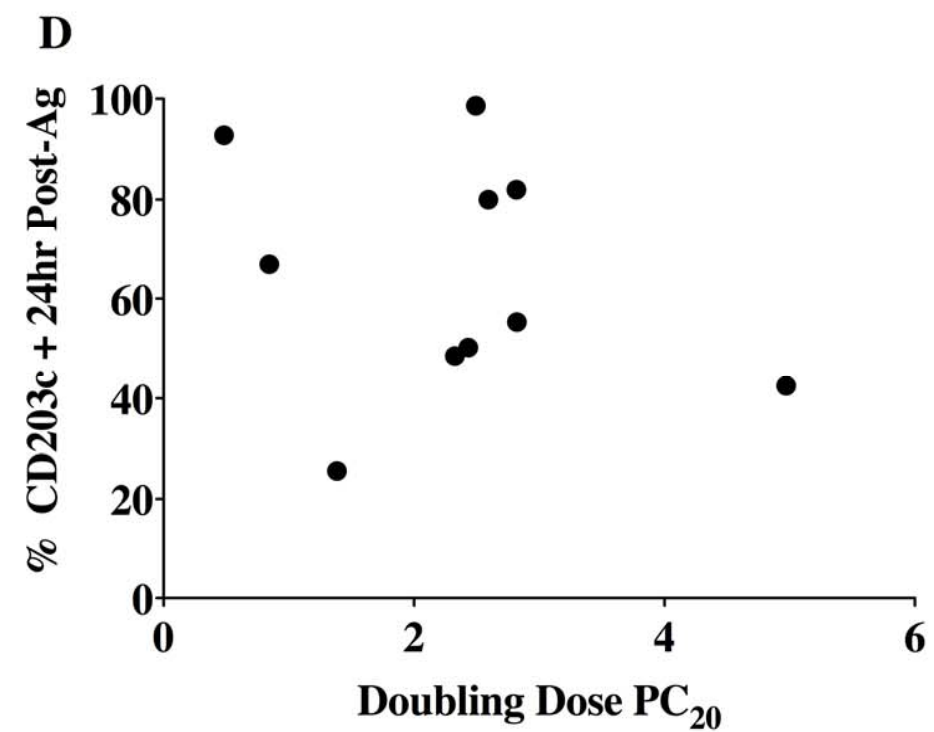
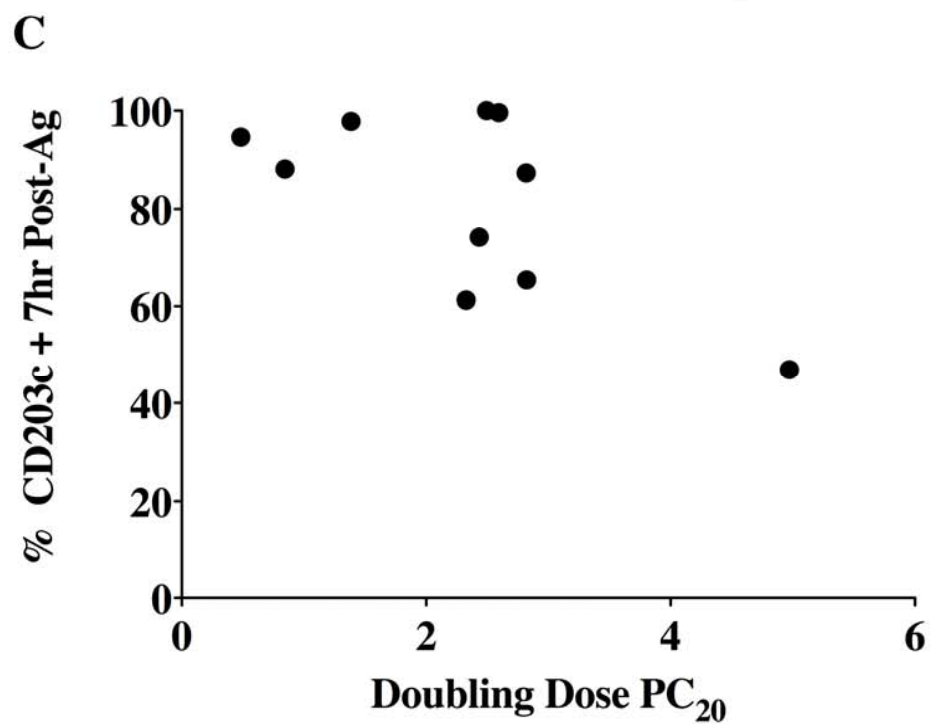
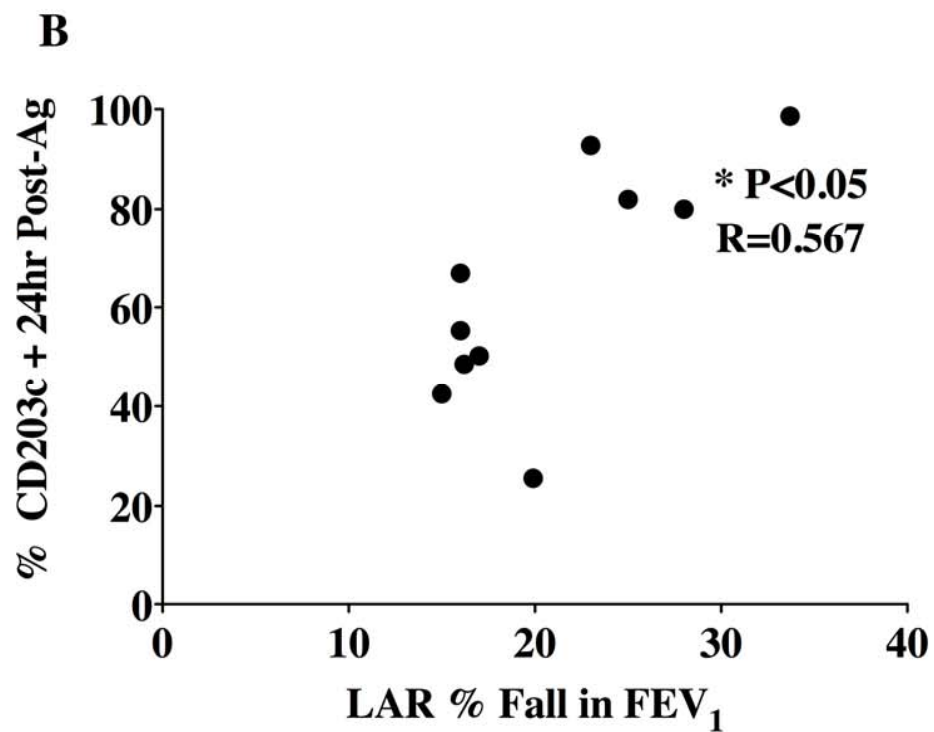
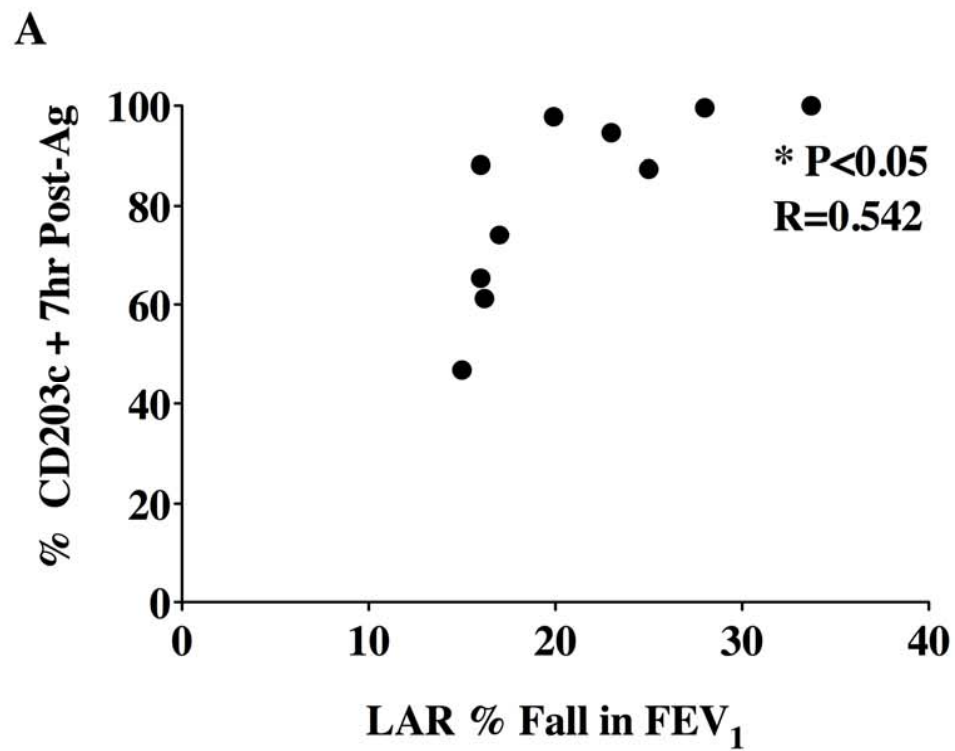
130 **ONLINE REPOSITORY REFERENCES**

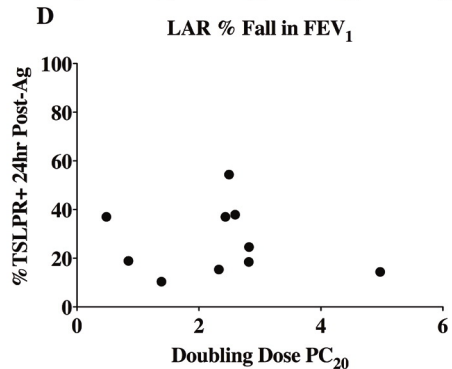
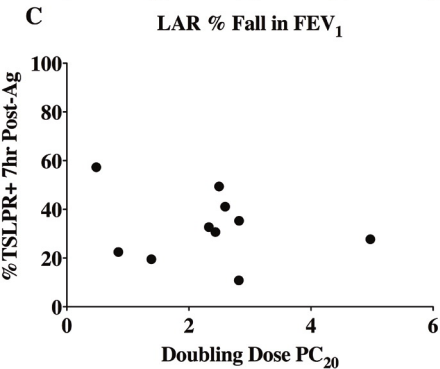
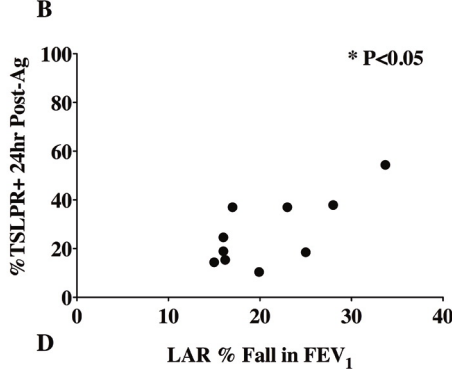
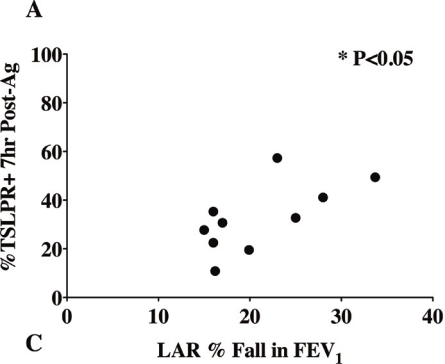
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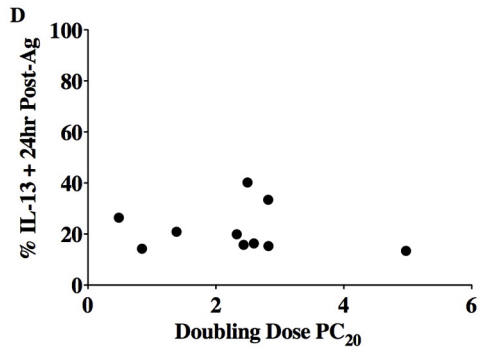
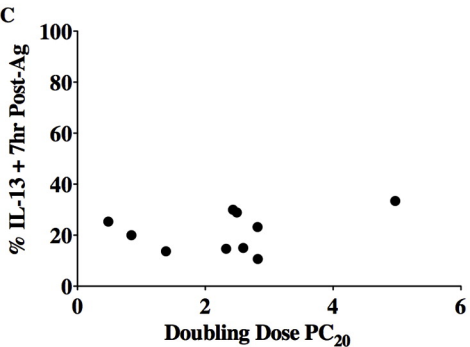
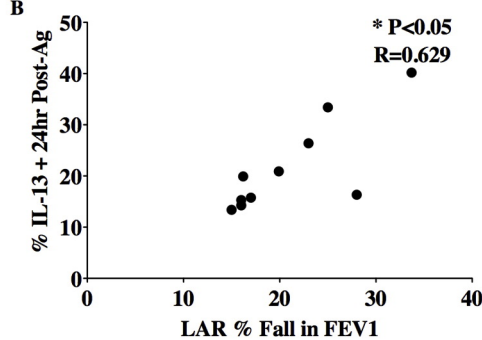
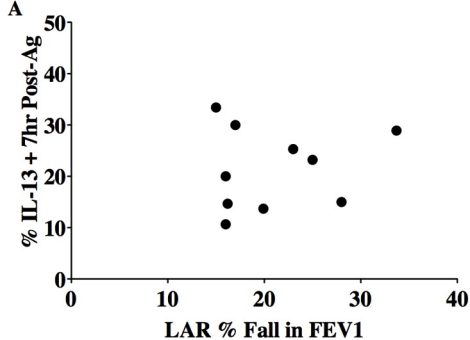
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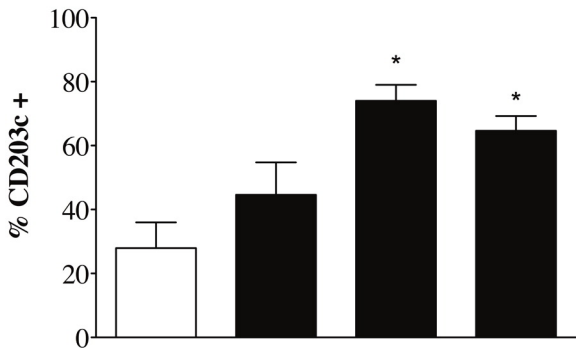
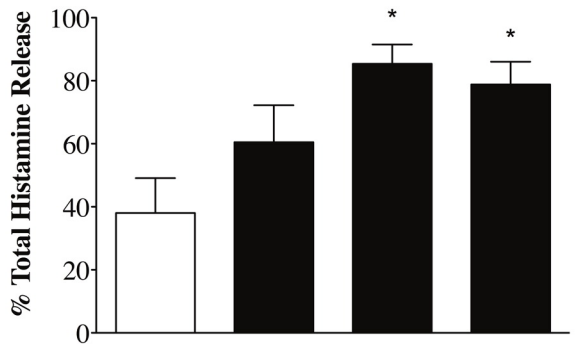
A**B****C****D****E**



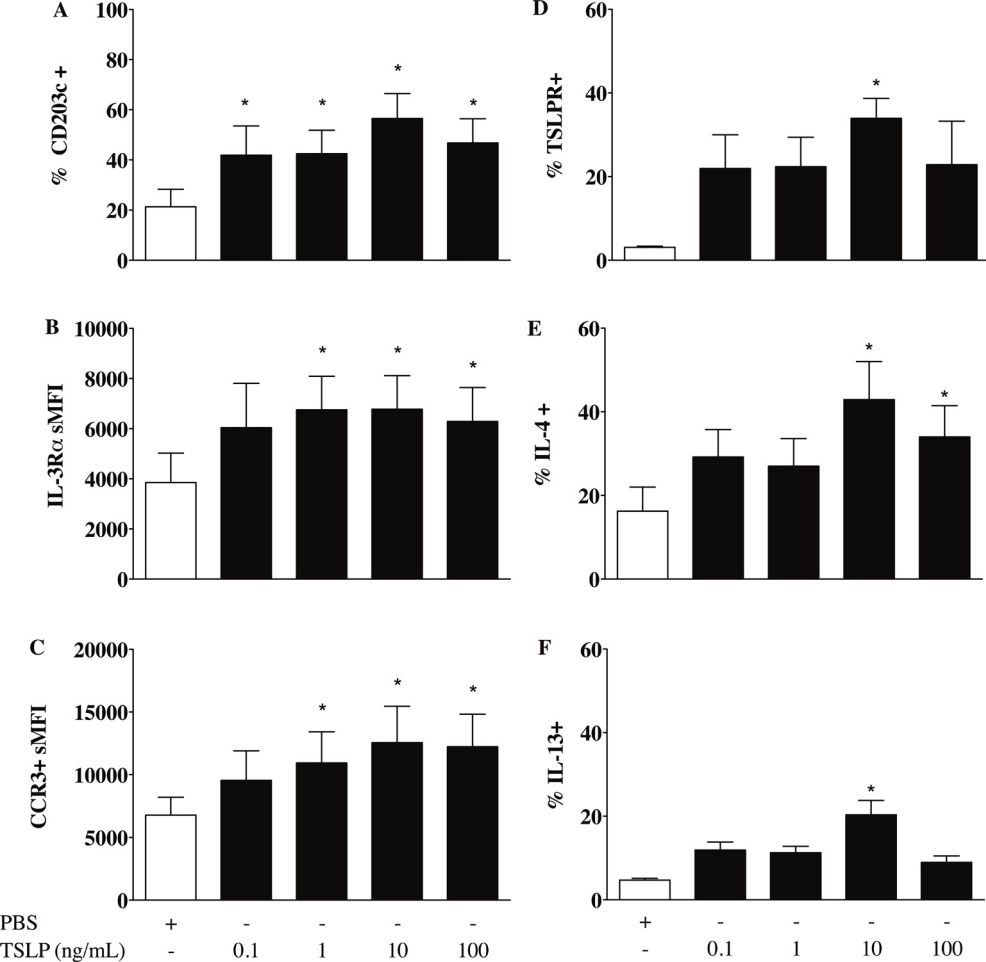


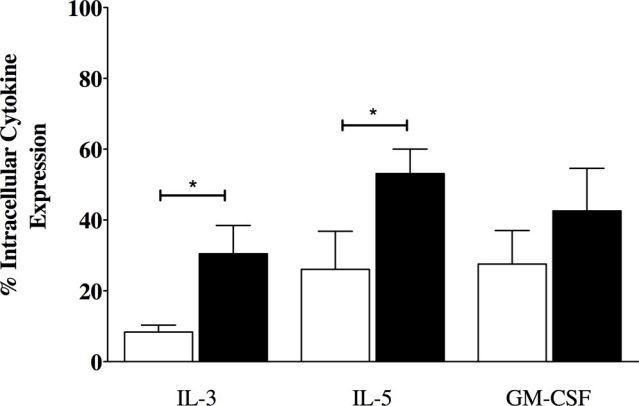


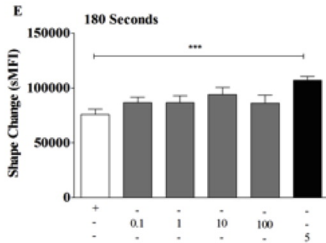
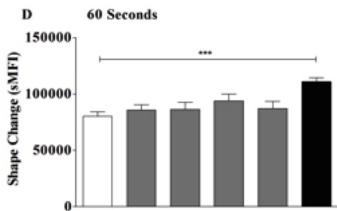
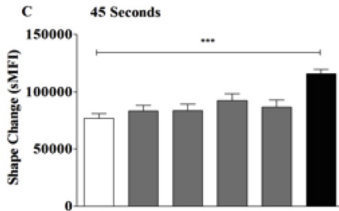
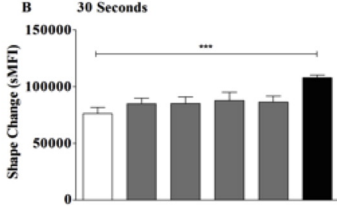
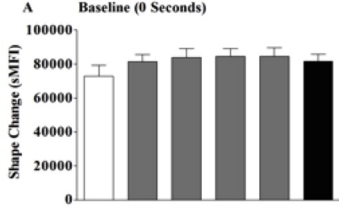


A**B**

PBS	+	-	-	-
TSLP (ng/mL)	-	1	10	100







	+	-	-	-	-	-
PBS	+	-	-	-	-	-
TSLP (ng/mL)	-	0.1	1	10	100	-
Eotaxin (ng/mL)	-	-	-	-	-	5