

IL-33 and Its Receptor ST2 after Inhaled Allergen Challenge in Allergic Asthmatics

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Keywords

Eosinophils · Neutrophils · Alarmins · Interleukin-33 · ST2 · Soluble ST2 · Interleukin-5

Abstract

Background: Previous murine models have demonstrated interleukin (IL)-33 to be an important mediator of type-2 inflammation and to promote airway hyperresponsiveness in allergic asthma. A number of inflammatory cells produce IL-33 and eosinophils express ST2 mRNA. The relationship between IL-33 and eosinophils in allergic asthma, however, remains unclear. **Objective:** The aim of this work was to evaluate in vitro the effect of allergen inhalation on IL-33 levels and expression of its receptor (ST2L) on eosinophils in allergic asthmatics, and the effect of IL-33 stimulation on eosinophil activity. **Methods:** Plasma and sputum IL-33, soluble ST2 (sST2) levels, and ST2L expression on eosinophils were measured in 10 healthy controls and 10 allergic asthmatics. Asthmatics underwent allergen and diluent inhalation challenges. Blood and sputum samples were collected to measure IL-33, sST2, and ST2L eosinophil expression before and 24 h after allergen inhalation. Purified blood eosinophils from allergic asthmatics were incubated overnight with IL-33 to as-

sess ST2 and intracellular IL-5 expression. **Results:** Baseline levels of IL-33 in sputum and sST2 in plasma and sputum were similar in allergic asthmatics compared to healthy controls. In addition, there was no difference in blood or sputum eosinophil ST2L expression in healthy controls versus allergic asthmatics. Eosinophil ST2L expression was significantly increased 24 h postallergen inhalation in allergic asthmatics. In vitro stimulation of human eosinophils with IL-33 and LPS significantly increased eosinophil ST2L expression and IL-33 stimulation increased intracellular IL-5 expression, which was attenuated by treatment with sST2 and ST2 blockade. **Conclusion and Clinical Relevance:** In mild asthmatics, there was a significant upregulation of ST2 surface expression on eosinophils from blood and sputum following allergen inhalation challenge. In vitro, IL-33 stimulation of eosinophils increases both ST2 membrane expression and IL-5 production. These results support a role for IL-33 in causing allergen-induced eosinophilia. Blockade of IL-33 and ST2 signaling may present a novel therapeutic avenue for asthma treatment.

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Introduction

Asthma is a chronic disease of the airways characterized by airway inflammation, reversible airway obstruction, and airway hyperresponsiveness (AHR). Dysfunctional cellular responses of both the adaptive and innate immune systems are important in the underlying pathogenesis of asthma. Epithelial-derived cytokines, such as interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin, have been suggested to be involved in the pathogenesis of allergic asthma [1].

IL-33 belongs to the IL-1 cytokine superfamily and is expressed by epithelial cells in the gut, skin, and lung, as well as immune cells, including dendritic cells, mast cells, and monocytes [2–4]. IL-33 exerts its activity through the IL-33 receptor complex (IL-33R), which consists of the IL-33-bound IL-1 receptor like-1 component (IL-1R1; ST2) and a ubiquitously expressed, shared coreceptor called IL-1 receptor accessory protein (IL-1RAcP) [5]. There are 2 isoforms of ST2, the transmembrane form (ST2L) and soluble ST2 (sST2), with the latter able to serve as a decoy receptor for IL-33 [5]. ST2L is expressed on eosinophils, mast cells, innate lymphoid cell type II (ILC2), basophils, dendritic cells, natural killer cells, Th2 lymphocytes, and airway smooth muscle cells [6–10]. A variety of stimuli, including bacteria, viruses, fungi, allergen, and pollutants, can trigger the release of IL-33 from airway epithelial cells and dendritic cells [11, 12].

In mouse models, transgenic overexpression [13] or administration of IL-33 [14] generates airway eosinophilia, upregulates Th2 cytokine expression, and elevates serum IgE, AHR, and mucus hypersecretion. Conversely, neutralization of IL-33 [15], or its receptor ST2L [16, 17], reduces airway inflammation, IgE levels, Th2 cytokine expression, goblet cell hyperplasia, and AHR. Furthermore, IL-33 mRNA expression has been found to be greater in epithelial cells of airway biopsies in asthmatics compared to healthy controls, with severe asthmatics expressing markedly greater levels as opposed to mild asthmatics [18–20]. Similarly, high levels of sST2 and IL-33 have been found in the plasma and sputum of adults and children with acute asthma in comparison to healthy controls [21–24].

Allergic asthma is associated with airway eosinophilic inflammation. Eosinophils are present in increased numbers within the airway lumen and tissue of allergic asthmatics when compared with healthy normal controls, and reducing airway eosinophilia reduces asthma exacerbation rates [25]. The important role of eosinophils in asthma supports the continued need to develop novel targets

to reduce the mediator function of these cells. Cherry et al. [26] identified ST2L mRNA and protein expression in eosinophils. They further showed that IL-33 induced eosinophil superoxide production and degranulation at similar levels to the positive control of IL-5. In addition, IL-33 was able to increase eosinophil survival and induce IL-8 production, which was inhibited by anti-ST2L [26]. Suzukawa et al. [27] additionally showed that mRNA expression for ST2L in eosinophils and *in vitro* stimulation with IL-33 increased eosinophil adhesiveness and CD11b expression as well as viability, which was inhibited with a neutralizing antibody to ST2. These findings demonstrate that eosinophils express mRNA for ST2L and that IL-33 can promote the proinflammatory function.

Given the important role of eosinophils in allergic asthma, the current study endeavored to expand on these findings to determine the relationship between eosinophils and IL-33 in the context of allergic asthma. Furthermore, there is a paucity of data with respect to the interaction between IL-33, ST2L, and sST2 in allergic asthmatics, and how this changes following allergen inhalation challenge. As such, this study examined the levels of IL-33 and sST2, as well as ST2L expression on eosinophils in both the peripheral blood and sputum of healthy controls and allergic asthmatics at baseline and following allergen inhalation in mild allergic asthmatics. The effects of stimulation with IL-33 *in vitro* on eosinophil ST2L and intracellular IL-5 expression were also evaluated.

Methods

Subjects

Ten nonsmoking subjects, aged 19–60 years, with mild allergic asthma were enrolled in this study. Subjects were required to have positive skin prick test responses to common aeroallergens, FEV₁ of $\geq 70\%$ of predicted, and baseline methacholine PC₂₀ of < 16 mg/mL, and a dual-phase response to allergen (fall in FEV₁ of $\geq 20\%$ by 2 h and $\geq 15\%$ 3–7 h postallergen). Ten healthy nonatopic, non-asthmatic controls (with both a negative skin prick test and methacholine PC₂₀ challenge > 16 mg/mL) were also enrolled. Subjects were excluded from the study if they were pregnant or nursing, current or ex-smokers with more than 10 pack-years, had developed lower respiratory infections/exacerbations, or used inhaled and/or oral steroids 4 weeks prior to the study. Antihistamines, caffeine, and nonsteroidal anti-inflammatory agents were prohibited 48 h before any study visit. All participants in the study provided informed written consent. The study was approved by the Hamilton Integrated Research Ethics Board.

Study Design

Ten healthy controls attended the laboratory for a single visit, which entailed a detailed medical history, skin prick test, spirometry, methacholine inhalation, sputum induction, and collection of

100 mL of peripheral blood via venipuncture. These baseline sputum and peripheral blood samples were used to compare the level of IL-33 and sST2 to that of 10 allergic asthmatics through an ELISA. In addition, peripheral blood was used for one set of in vitro experiments to determine the effect of LPS stimulation on ST2L eosinophil expression.

In addition, asthmatic subjects underwent either an allergen or diluent challenge, randomly allocated, with a 2-week washout period. At the first study visit on day 1, a detailed medical history, skin-prick test, spirometry, methacholine inhalation, sputum induction, and peripheral blood were obtained. Subjects then underwent either an allergen or diluent inhalation challenge on day 2, followed by sputum induction and peripheral blood collection 7 h postchallenge. On day 3, 24 h postchallenge, methacholine inhalation testing, spirometry, sputum induction, and peripheral blood collection were performed. Asthmatic subjects returned after the 2-week wash-out period to donate 100 mL of peripheral blood for subsequent in vitro experiments.

Methacholine inhalation challenges were performed using Wright nebulizer and the tidal breathing method, as previously described [28]. PC₂₀ was calculated as the provocative concentration of methacholine inducing a 20% decrease in FEV₁. Allergen and diluent inhalation challenges were performed as previously described [29].

Peripheral Blood and Induced-Sputum Sample Processing

Peripheral blood was collected into a sodium heparin for cell isolation and into containing EDTA vacutainers for differential cell counts. The eosinophil-rich fraction was collected from whole blood following dilution 1:1 with McCoys 5A and density gradient centrifugation on Accu-Prep (Accurate Chemical & Scientific Corp., Westbury, NY, USA) at 2,200 rpm for 20 min at room temperature. The granulocyte layer was then harvested and red blood cells were lysed and immunostained as described below.

Sputum samples were induced using hypertonic saline and mucous plugs were selected from samples followed by disbursement with DPBS, as previously described [30]. In brief, sputum samples were centrifuged at 790 g at 4 °C for 10 min, followed by the collection of supernatant, centrifugation at 1,500 g at 4 °C for 10 min, and aliquoting of supernatant into Eppendorf tubes that were frozen at -20 °C. Two cytopsins were prepared from sputum cells and stained with Diff-Quick for differential cell counts. Four hundred cells were counted per slide for cell differential counts. The results were expressed both as the percent of total cells and as cells per gram of sputum. The mean value of the 2 slides was used.

Next, 100 µL of the granulocyte-rich fraction from peripheral blood and sputum-extracted cells were immunostained with specific antibodies or corresponding isotype controls in the dark at 4 °C for 30 min, washed with FACS buffer at 500 g for 10 min at 4 °C, resuspended in PBS, and stored at 4 °C until flow cytometry acquisition. A BD LSRII flow cytometer was used to identify eosinophils and neutrophils, with the percent cell expression of ST2L and standard mean fluorescent intensity being measured.

Commercially available multiplex immunoassay kits were used to measure IL-33 (R&D Systems, Minneapolis, MN, USA; lower limit of detection 1.65 pg/mL) and sST2 (Elabscience, Houston, TX, USA; lower limit of detection: 18.75 pg/mL) in peripheral blood plasma and sputum samples collected before and after challenges.

Eosinophil Purification and in vitro Cultures with IL-33

Peripheral blood (100 mL) was drawn into heparin vacutainers, diluted in McCoys 5A, and layered on Accu-Prep (Accurate Chemical & Scientific Corp.) for density gradient sedimentation. The granulocyte layer was harvested, red blood cells were lysed, and eosinophils were then isolated using a MACS column CD16+ neutrophil depletion kit (Miltenyi Biotech, Auburn, CA, USA). Postisolation, the eosinophil population had 100% viability and >97% purity.

Initial experiments were performed to assess the effect of LPS, a known eosinophil activation mediator, on ST2L expression. Isolated and purified eosinophils from 10 healthy controls and 10 allergic asthmatics were cultured in a 24-well plate (1 × 10⁶/mL) in RPMI-Complete with media control, PBS, or LPS 0.1 ng/mL (R&D Systems) for 18 h (37 °C; 5% CO₂ atmosphere). Cells were harvested following incubation and immunostained to measure the percent eosinophil expression of ST2L via flow cytometry. Cell viability was assessed via Trypan blue staining. Two negative controls were employed in this experiment.

To assess the effect of IL-33 on eosinophil ST2L expression, isolated and purified eosinophils from 10 allergic asthmatics were cultured in a 24-well plate (1 × 10⁶/mL) in RPMI-Complete with media control or IL-33 at concentrations of 0.01, 0.1, 1, 10, or 100 ng/mL (R&D Systems) for 18 h (37 °C; 5% CO₂ atmosphere). These concentrations were chosen based on previous in vitro experiments conducted with IL-33 and basophils [31]. Cells were harvested following incubation and immunostained to measure the percent eosinophil expression of ST2L via flow cytometry. Cell viability was assessed via Trypan blue staining.

To measure the effect of IL-33 on eosinophil intracellular IL-5 expression, isolated and purified populations of eosinophils from 10 allergic asthmatics were cultured in a 24-well plate (1 × 10⁶/mL) in RPMI-Complete with media control or IL-33 (0.01, 0.1, 1, 10, 100 ng/mL; R&D Systems) for 18 h (37 °C; 5% CO₂ atmosphere). These concentrations were chosen based on previous in vitro experiments conducted with IL-33 and basophils [31]. Eosinophils were also pretreated with sST2 (R&D Systems; 0.01, 0.1, 0.5 µg/mL), neutralizing antibody to ST2L (0.01, 0.1, 0.5 µg/mL; R&D Systems), or corresponding isotype controls for 30 min prior to the addition of IL-33 for 18 h (37 °C; 5% CO₂ atmosphere). Monensin (1:1,000 dilution; Biolegend, San Diego, CA, USA) was added 6 h prior to the completion of cultures. Cell viability was assessed via Trypan blue staining. Cells were harvested postincubation and immunostained for the assessment of IL-5 intracellular expression via flow cytometry.

Immunofluorescence Staining and Flow Cytometric Gating Strategy

Samples were immunostained with monoclonal antibodies (mAb) including CD45-PerCPCy5.5 (BD Biosciences, Mississauga, ON, Canada), CD16-V450 (eBioscience, San Diego, CA, USA), CCR3-PE (Ebioscience), ST2L-FITC (MBL International, Woburn, MA, USA), and relevant isotype controls for 30 min at 4 °C. Eosinophils were defined as the CD45+/CD16-/CCR3+ population. We first gated around the CD45+ population in the SSC versus CD45 plot, followed by gating around the CD16- population in the SSC versus CD16 plot, and a final gate around the CCR3+ population in the SSC versus CCR3 plot (see online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000488015). Conversely, neutrophils were identified as the CD45+/CD16+/CCR3- population (see online suppl. Fig. 1).

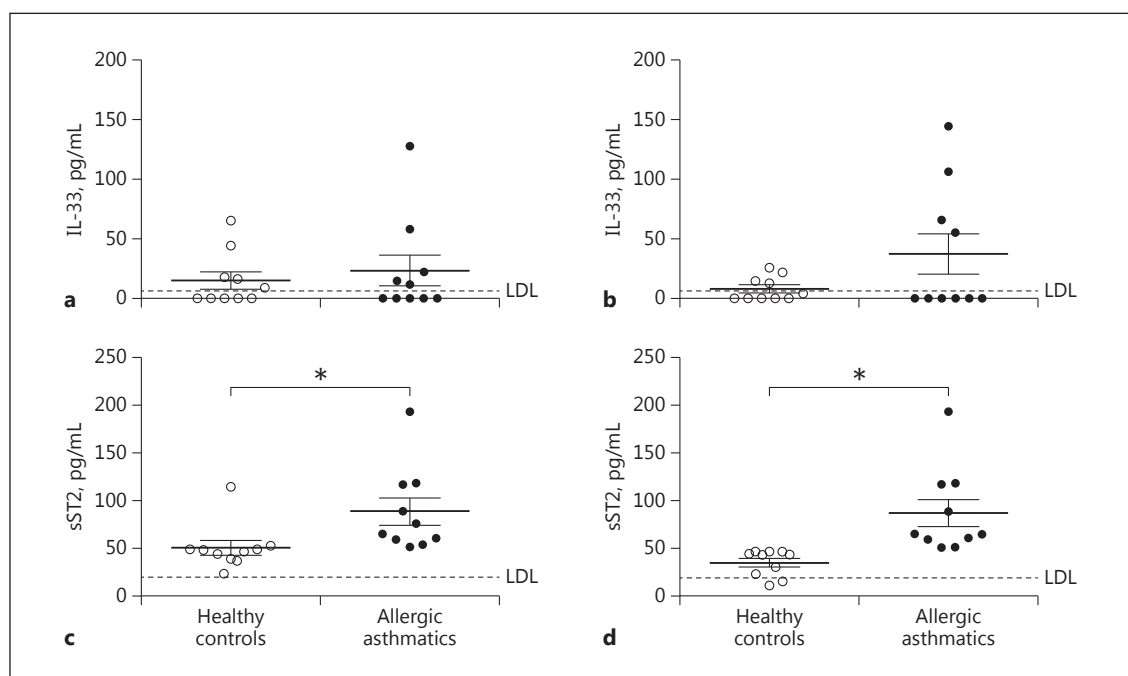


Fig. 1. Baseline IL-33 and sST2 levels in mild allergic asthmatics and healthy controls. Baseline samples from healthy controls (open circles; $n = 10$) and mild allergic asthmatics (black circles; $n = 10$) demonstrating measured plasma IL-33 (a), sputum supernatant IL-33 (b), plasma sST2 (c), and sputum supernatant sST2 (d). Data are presented as the mean $\pm$ SEM pg/mL. * $p < 0.05$, significant difference between the 2 subject groups. The lower detection limit (LDL) of was set to 1.65 pg/mL for IL-33 and 18.75 pg/mL for sST2.

To measure the intracellular cytokine expression, MACS-purified eosinophils were stained as above, washed, fixed, and permeabilized, then stained with isotype control or specific antibody for IL-5-APC (Biolegend). The cells were washed with FACS buffer and acquired with a LSR II flow cytometer (BD Biosciences). Analyses were performed using Flow-Jo software (Tree-Star, Ashland, OR, USA).

Statistics

Data are expressed as the standard error of the mean (SEM). The statistical analysis for changes to eosinophil/neutrophil ST2L expression following the inhalation challenges were performed using 2-way ANOVA and post hoc Bonferroni test on GraphPad Prism 5 software (GraphPad Software, CA, USA). In vitro experiments were analyzed using 1-way ANOVA with a post hoc Tukey test. Significance was accepted at $p < 0.05$.

Results

The healthy and allergic asthmatic subjects were similar in age, gender, and BMI (Table 1). Allergic asthmatics had a mean FEV₁ percent predicted of $87.7 \pm 2.6\%$ and methacholine PC₂₀ of 4.32 ± 1.5 mg/mL, whereas values

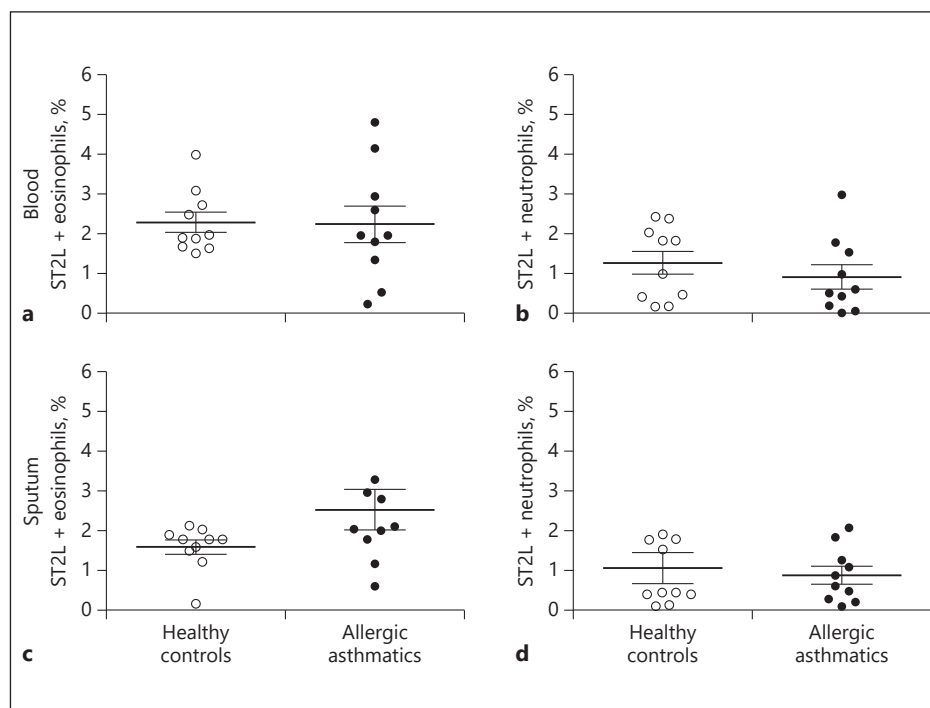
Table 1. Subject characteristics

	Allergic asthmatics ($n = 10$)	Normal controls ($n = 10$)
Age, years	31.2 $\pm$ 4.0	37.6 $\pm$ 3.5
Male, %	50	50
BMI	24.71 $\pm$ 0.9	25.21 $\pm$ 1.3
Pulmonary function		
FEV ₁ , % predicted	87.7 $\pm$ 2.6	95.9 $\pm$ 2.9
FVC, % predicted	92.5 $\pm$ 3.6	94.7 $\pm$ 3.44
FEV ₁ /FVC	95.1 $\pm$ 1.93	101.4 $\pm$ 1.86
Methacholine PC ₂₀ , mg/mL	4.32 $\pm$ 1.5	>16

for the healthy controls were $95.9 \pm 2.9\%$ and >16 mg/mL, respectively (Table 1).

For all the study subjects enrolled in this study, plasma IL-33 levels were below the limit of detection undetectable in 50% of both the healthy controls and allergic asthmatics. In the remaining 50% of subjects, plasma IL-33 levels were not significantly different between healthy controls and allergic asthmatics (Fig. 1a). In contrast,

Fig. 2. Baseline percent eosinophil ST2L expression in mild allergic asthmatics and healthy controls. Baseline samples from healthy controls (open circles; $n = 10$) and mild allergic asthmatics (black circles; $n = 10$) demonstrating the percentage of peripheral blood and sputum eosinophils and neutrophils expressing ST2L. **a** Peripheral blood eosinophils. **b** Peripheral blood neutrophils. **c** Sputum eosinophils. **d** Sputum neutrophils. Data are presented as the mean $\pm$ SEM. No significant difference was found at baseline between healthy controls and allergic asthmatics.



sputum IL-33 levels were significantly greater in allergic asthmatics compared to healthy controls ($p < 0.05$; Fig. 1b). Levels of sST2 were measurable in all subjects, and were significantly higher in both plasma and sputum supernatant of allergic asthmatics compared to healthy controls ($p < 0.05$; Fig. 1c, d). There was no significant difference in baseline levels of ST2L expression by either peripheral blood eosinophils (Fig. 2a) or neutrophils (Fig. 2b), or sputum eosinophils (Fig. 2c) or neutrophils (Fig. 2d) from asthmatics compared to healthy controls.

In the atopic asthmatic subjects, allergen inhalation challenge induced a maximum early drop in FEV₁ of $34.02 \pm 2.0\%$ and late drop in FEV₁ of $20.7 \pm 3.5\%$, compared to no change in FEV₁ following diluent inhalation (see online suppl. Fig. 2A). The methacholine PC₂₀ decreased 1 doubling dose following allergen challenge, compared to no change after the diluent (see online suppl. Fig. 2B).

Following allergen inhalation, there was no significant increase in plasma or sputum levels of IL-33 or sST2, as determined by ELISA. There was, however, a significant increase in the proportion of blood and sputum eosinophils expressing ST2L at 24 h postallergen inhalation compared to the diluent control and baseline ($p < 0.05$; Fig. 3). Conversely, there was no effect of allergen inhala-

tion on the percent of neutrophils expressing ST2L in peripheral blood or sputum (Fig. 3).

To determine whether eosinophil activation can result in upregulation of ST2L expression, we conducted in vitro experiments to assess the effect of overnight incubation with LPS on eosinophil ST2L expression on both healthy controls and allergic asthmatics. We found a significantly increased percentage of eosinophils from allergic asthmatics expressing ST2 following stimulation with 0.1 ng/mL of LPS compared to the media control ($p < 0.05$; Fig. 4a). Furthermore, the percentage of eosinophils expressing ST2L was markedly higher in allergic asthmatics compared to healthy controls following LPS stimulation, but not media control ($p < 0.05$; Fig. 4a).

We next investigated whether IL-33 stimulation increased the expression of its own receptor on eosinophils. Purified eosinophils from allergic asthmatics were incubated overnight with media control or IL-33 (0.01–100 ng/mL). IL-33 significantly increased the expression of ST2L when compared to the media control, which was optimal at 10 ng/mL ($p < 0.05$; Fig. 4b).

By flow cytometry, we found that incubation of purified eosinophils with IL-33 stimulated increased intracellular IL-5 expression (see online suppl. Fig. 3). Our results showed a significant increase in the percentage of eosinophils expressing intracellular IL-5, which was optimal at

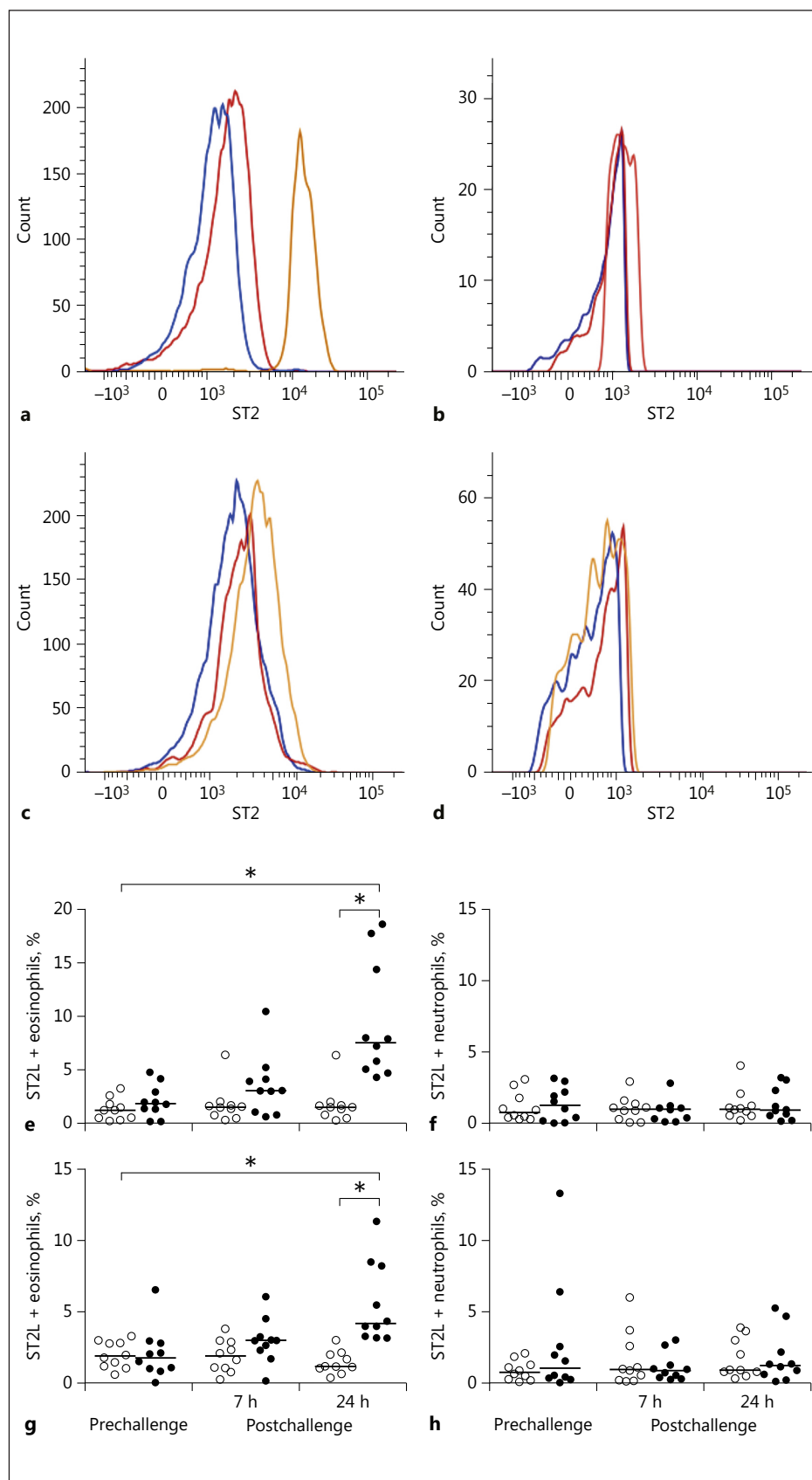


Fig. 3. Eosinophil and neutrophil expression of ST2 after allergen and diluent inhalation. Ten mild allergic asthmatics were randomized to undergo both allergen and diluent airway inhalation challenges. Sample plots show flow cytometric enumeration of ST2L expression on blood eosinophils (a); blood neutrophils (b); sputum eosinophils (c), and sputum neutrophils (d), where the blue histogram represents preallergen isotype control, red depicts the preallergen specific stain, and orange shows the postallergen specific stain. Individual data points from diluent (open) and allergen (closed) inhalation challenge showing percent expression of ST2L for blood eosinophils (e) and blood neutrophils (f), sputum eosinophils (g), and sputum neutrophils (h) are shown, where horizontal lines represent the median value of the data set ($n = 10$). * $p < 0.05$, significant difference between either prechallenge levels or diluent challenge at the same time point.

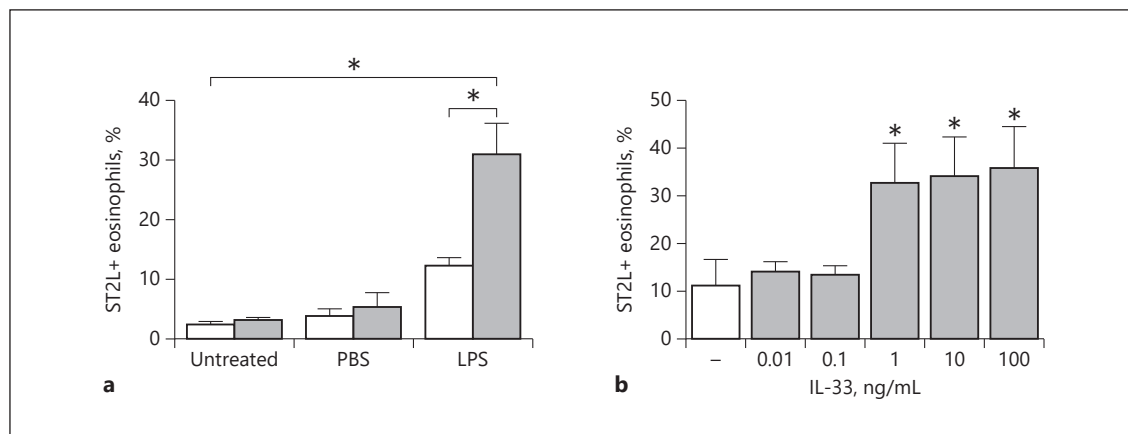


Fig. 4. ST2 expression on purified eosinophils from blood after IL-33 stimulation in vitro. **a** Purified eosinophils from the blood of 10 healthy controls (white bars) and 10 allergic asthmatics (black bars) were stimulated for 18 h with media control, PBS, or LPS (0.1 ng/mL). The percent of eosinophils expressing ST2L was then assessed. Stimulation with LPS significantly increased ST2L expression in allergic asthmatics compared to media control and

healthy controls. **b** Isolated peripheral blood eosinophils from 10 allergic asthmatics were stimulated for 18 h with media control or with IL-33 (0.01–100 ng/mL) to assess for the change in ST2L expression; 1–100 ng/mL of IL-33 induced a significant expression of ST2L on eosinophils compared to the media control. Data are presented as the mean $\pm$ SEM. * $p < 0.05$.

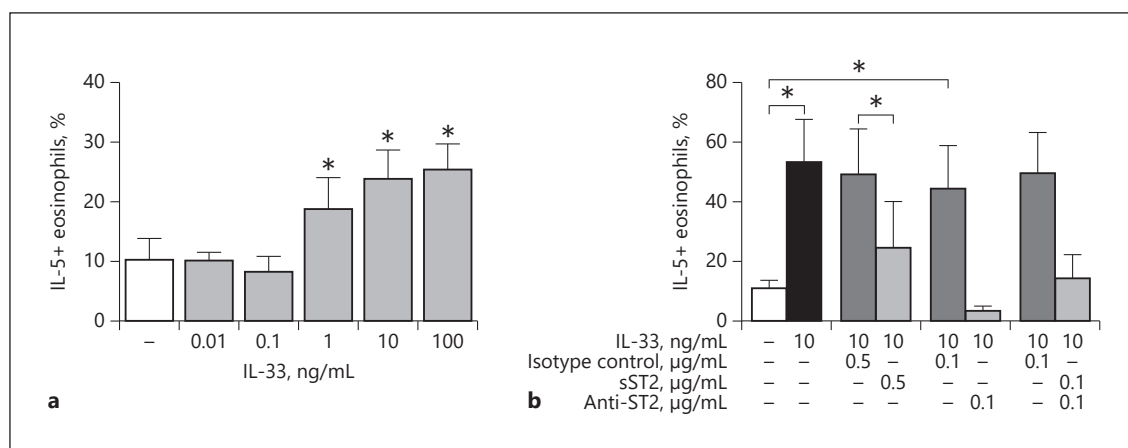


Fig. 5. Intracellular IL-5 expression in peripheral blood eosinophils after stimulation of with IL-33 and the addition of sST2, an anti-ST2 mAb, or both in vitro. **a** Purified blood eosinophils from 10 mild allergic asthmatics were stimulated for 18 h with media control or IL-33 (0.01–100 ng/mL; black bars) and then assessed for the percent eosinophil expression of intracellular IL-5 (the FACs gating is shown in online suppl. Fig. 3). **b** Isolated peripheral

blood eosinophils from 10 mild allergic asthmatics were stimulated for 18 h in vitro without (open bar) or with IL-33 (10 ng/mL; black bar) and treated with/without sST2 (0.5 μ g/mL), or anti-ST2 (0.1 μ g/mL; light grey bars) or corresponding isotype controls (dark grey bars), followed by measurement of the percent eosinophil expression of intracellular IL-5. Data are presented as the mean $\pm$ SEM. * $p < 0.05$, significant difference from comparison.

10 ng/mL IL-33 compared to the media control ($p < 0.05$; Fig. 5a). Lastly, the enhanced expression of eosinophil intracellular IL-5 induced by 10 ng/mL of IL-33 was significantly attenuated by treatment with either sST2 (0.5 μ g/mL) or an anti-ST2 mAb (0.1 μ g/mL), or both combined ($p < 0.05$; Fig. 5b).

Discussion

IL-33 has been suggested to be a mediator of allergic inflammatory responses through the promotion of maturation, activation, and chemotaxis of numerous leukocyte types, including Th2 cells, mast cells, CD34 cells, ILC2s,

basophils, natural killer cells, and eosinophils [7, 10, 18, 19]. In addition, treatment with anti-IL-33 or sST2 in an OVA-challenge murine model inhibited AHR and IL-5 levels in BALF, as well as eosinophil numbers [16]. Other studies have shown that the administration of IL-33 leads to the induction of eosinophilia and Th2 inflammation within murine airways [14, 15, 32]. IL-33^{-/-} mice have shown attenuated eosinophil influx into the BALF and Th2 inflammation [33]. Similarly, anti-IL-33 mAb inhibited eosinophil recruitment and Th2 cytokine production [16, 34].

IL-33 and sST2 has been previously shown to be increased in the peripheral blood and airways of asthmatic patients [21, 35, 36]. The principal source of IL-33 in allergic disease is not fully known; however, airway epithelial cells, smooth muscle cells, and mast cells have been identified as key sources [18, 19, 37, 38]. Unlike previous reports [21, 22], we found no difference in plasma IL-33 levels between allergic asthmatics and healthy controls. This may be attributed to having underpowered comparisons due to the lack of detectable IL-33 levels in 5 subjects from each group. Consistent with previous findings [21], however, the allergic asthmatic group showed a trend for higher sputum levels of IL-33 and higher levels of sST2 in plasma and sputum compared to healthy controls, although this was not significant. Elevated sST2 levels in allergic asthmatics suggest localized production of sST2 within the airways that may act as an inhibitor of IL-33-induced inflammation. This has been demonstrated in cases of sepsis and asthma exacerbation [36, 39, 40].

Interestingly, we found no difference in baseline levels of ST2L expression on eosinophils between healthy controls and mild asymptomatic allergic asthmatics. However, allergen inhalation in atopic asthmatics induced a significant increase in the percent of eosinophils expressing ST2L in both peripheral blood and sputum samples taken 24 h after the allergen but not the diluent challenge. Consistent with the findings of Cherry et al. [26], we found no significant expression of ST2L on neutrophils, and furthermore these levels did not significantly change following allergen inhalation challenge. These findings suggest that at baseline levels there is no difference between ST2L between healthy controls or allergic asthmatics; however, following exposure to an allergen there are inflammatory processes that drive the increase in ST2L on eosinophils, which facilitates the increased binding of IL-33 and thus further propagates asthma pathogenesis. This is, in part, supported by the fact that LPS induces ST2L expression on allergic asthmatics but not healthy controls *in vitro*.

Conversely, neither IL-33 nor sST2 plasma and serum levels changed at 7 and 24 h postallergen inhalation. It is interesting that no changes in these levels were found; however, we propose that the timelines chosen may have been too late to examine changes in IL-33. This is suggested by data from Fux et al. [40], who demonstrated that IL-33 increased in BALF at 10 min postallergen, but declined to baseline at 18 h postallergen. Cohen et al. [41] reported that rapid oxidation drives conformation change, involving the formation of 2 sulfide bridges in IL-33 that causes it to become inactive, thus limiting its potency *in vivo*. This is not thought to interfere with IL-33 detection via available ELISAs; however, it may limit the ability of the ELISA assay to differentiate between active IL-33 and inactive IL-33. In addition, it is possible that, similar to IL-33, sST2 becomes elevated early after allergen inhalation to regulate IL-33 responses.

We conducted additional *in vitro* studies to understand the relationship between IL-33 and eosinophils. We initially determined whether the common activator of eosinophils and neutrophils, LPS, can upregulate ST2L expression on eosinophils. Our *in vitro* study demonstrated that overnight incubation with LPS significantly increased the proportion of eosinophils expressing ST2L in allergic asthmatics; however, this response was not seen in healthy controls. These findings suggest that upstream inflammatory mediators, like LPS, can induce ST2L expression on eosinophils to promote increased responsiveness to IL-33. Furthermore, this mechanism seems to be specific to allergic asthmatics, which may be an underlying mechanism contributing to airway pathogenesis in this disease.

IL-33 has been previously shown to induce the production of superoxide and IL-8 directly, and indirectly enhance IL-3, IL-5, or GM-CSF-mediated IL-8 production by human eosinophils [26]. Moreover, IL-33 enhances eosinophil adhesion through upregulation of CD11b expression and survival independently of IL-5, IL-4, and GM-CSF [28]. We expanded on these findings to demonstrate that overnight stimulation with IL-33 can induce eosinophil intracellular IL-5 expression, which was blocked following treatment with anti-ST2L or sST2. These findings suggest that IL-33 stimulation of eosinophils can induce type-2 cytokine release, which can further perpetuate the inflammatory response inherent in asthma.

There were some notable limitations to our study. It would have been valuable to look at additional cell type expressions of ST2L before and after allergen inhalation to compare these levels to those of eosinophils. However, given the limited number sputum cells and the limited antibody colors available for ST2L, we focused on eosinophils

and used neutrophils as a negative control. We have previously observed increases in ST2L basophil expression following allergen inhalation, which may be used as a comparison [31]. We further acknowledge that measurement of mRNA expression of ST2L could have been valuable; however, this was not identified as an outcome measure in the initial study design. Lastly, it would have been beneficial to look at additional outcome measures aside from IL-5; however, the focus of this study was mediators that promote type 2 inflammatory responses such as IL-5.

The allergen-induced increases of ST2L expression on both blood and sputum eosinophils suggest that IL-33 may act both locally and systemically; however, we could not demonstrate an allergen-induced increase in plasma IL-33, which would have supported this hypothesis. Taken together, these results support an important role for IL-33 in allergen-induced airway inflammation. Further evaluation of this hypothesis aided by studies examining the effects of mAbs directed against IL-33 or its receptor ST2L in attenuating allergen-induced airway responses in asthmatic subjects is warranted.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

P.D.M. conducted all the diluent and allergen challenge sample collection, immunostaining, flow cytometry acquisition, statistical analysis, and drafted the manuscript. B.M.S. conducted in vitro experiments, immunostaining, flow cytometry acquisition, statistical analysis, and drafted the manuscript. P.O.B., G.M.G., and R.S. helped design the experiments and assisted in writing the manuscript. J.P.O., S.G.S., E.G.A., and D.T. assisted with technical support with experiments.

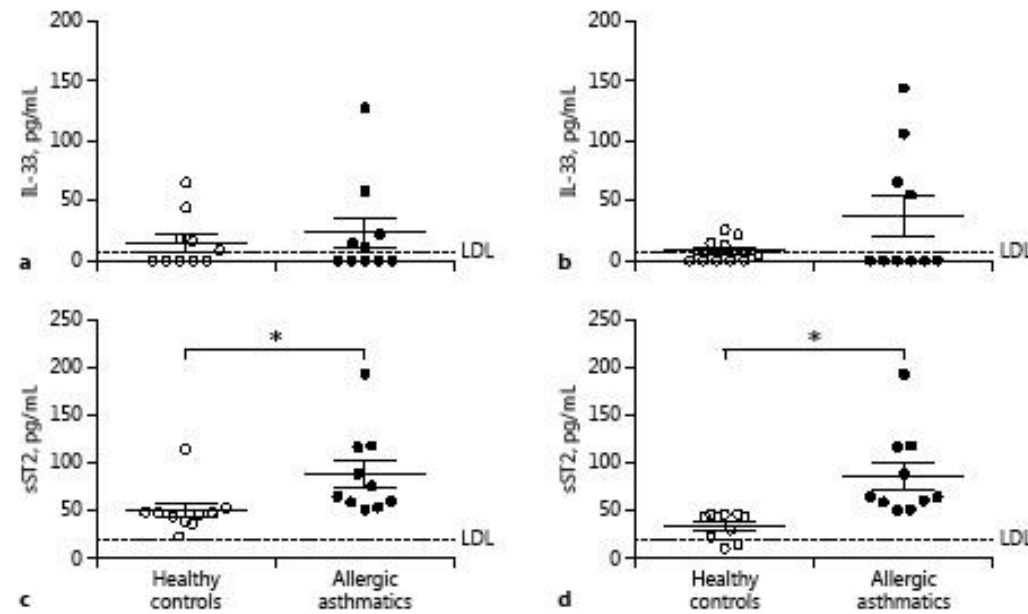
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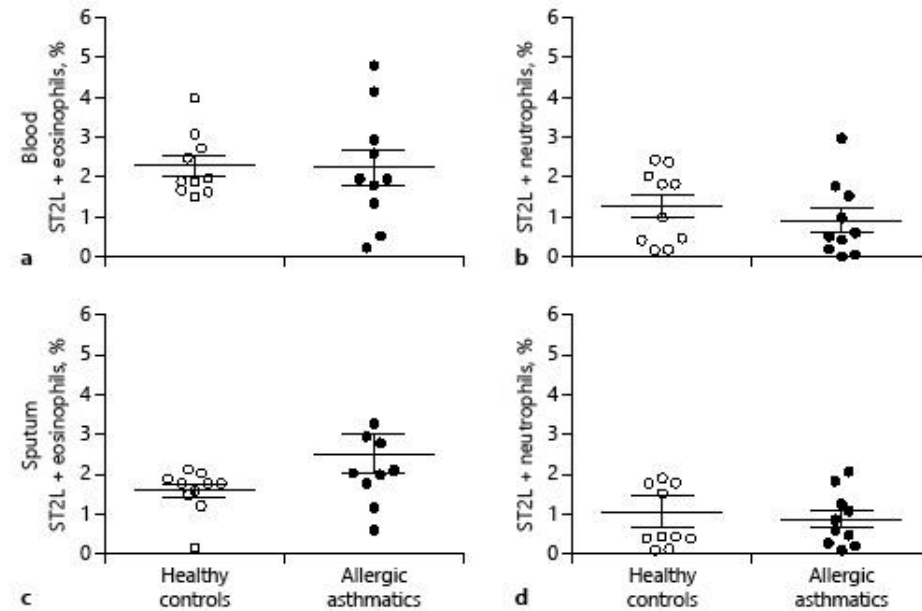
IL-33 and Its Receptor ST2 after Inhaled Allergen Challenge in Allergic Asthmatics

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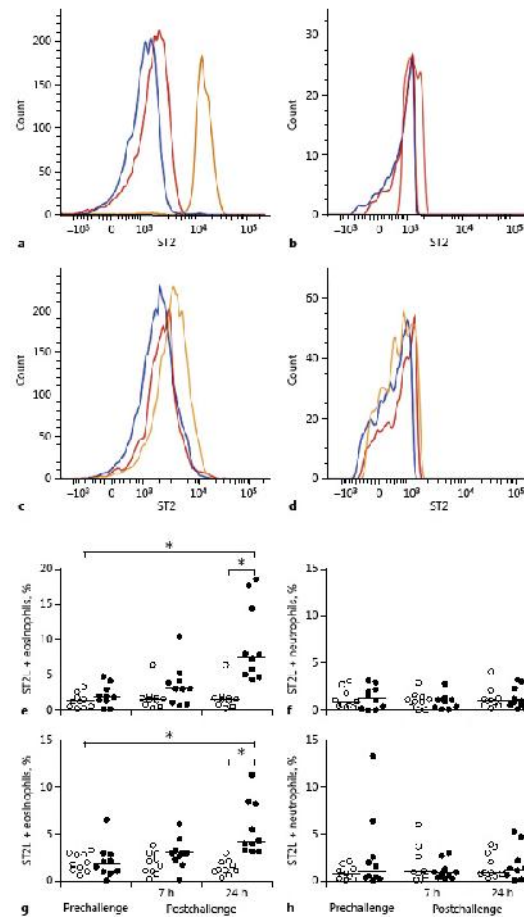
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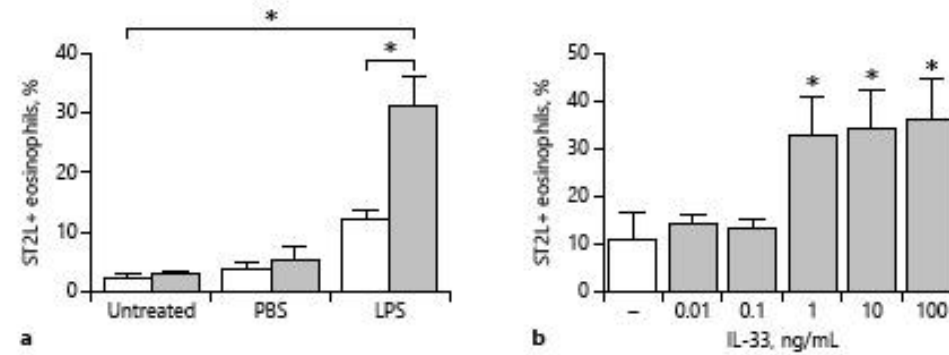
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