

# Increased Levels of Interleukin-33 and Thymic Stromal Lymphopoietin in Exhaled Breath Condensate in Chronic Bronchial Asthma

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## Key Words

Asthma · Epithelium · Exhaled breath condensate · Interleukin-33 · Thymic stromal lymphopoietin

## Abstract

**Background:** Epithelium-derived cytokines such as thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33 are important contributors to inflammation in asthma. Exhaled breath condensate (EBC) is a noninvasive method used to assess the inflammation of airways. Our aim was to assess the levels of TSLP, IL-25, IL-33, and its receptor ST2/IL-1 R4 in EBC in patients with asthma and to correlate these with serum levels and asthma control. **Methods:** EBC and serum levels of TSLP, IL-25, IL-33, and ST2/IL-1 R4 were measured in 44 patients with chronic bronchial asthma (14 in the uncontrolled phase) and 19 healthy control participants. **Results:** EBC levels of IL-33 and TSLP and serum levels of IL-33 were statistically higher in patients with asthma than in controls. IL-25 and ST2/IL-1 R4 were present in EBC at barely detectable levels and were not analyzed. The EBC and serum levels of all studied mediators did not differ between controlled and uncontrolled asthma patients, except for the serum level of ST2/IL-1 R4, which was higher in uncontrolled asthma. There were no correlations between serum and EBC

levels of TSLP and IL-33 or between either serum and EBC levels and the forced expiratory volume in 1 s or the total IgE level. **Conclusions:** Higher levels of IL-33 and TSLP in EBC provide evidence supporting a role for these mediators in asthma. Their levels do not discriminate between controlled and uncontrolled asthma. The local reaction within the epithelium is independent of the systemic reaction.

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## Introduction

Bronchial asthma is a chronic inflammatory disease caused by interactions between genetic and environmental factors. More and more data suggest that the pathomechanism of allergic diseases and asthma originates in the epithelium. The epithelium is immunologically active, and it is considered to be an important tissue in allergic reactions because it is the first line of protection against allergens, microbes, and noxious agents and it is a barrier between the environment and the parenchyma [1, 2]. Moreover, aberrant expression of various genes such as the Th2-induced genes *POSTN*, *SeRPINB2*, and *CLCA1* within the epithelium has been proposed to be an important contributor to allergic reactions [3]. It has also

**Table 1.** Patient characteristics

	Asthma patients (n = 44)	Controls (n = 19)
Age <sup>a</sup> , years	43 (33–54)	44 (38–56)
Male/female ratio	8/35	8/9
FEV <sub>1</sub> , % predicted	86 (72–96)	n.a.
FEV <sub>1</sub> /FVC	78 (72–82)	n.a.
Ratio of controlled/uncontrolled asthma patients	30/14	n.a.
Inhalant allergen sensitization	22 (50)	n.a.
Serum total IgE, IU/ml	82 (25–433)	n.a.
Smokers	0	0
<i>Use of asthma controllers</i>		n.a.
Inhaled corticosteroids		
<400 µg BUD	8 (18)	
400–800 µg BUD	17 (39)	
>800 µg BUD	19 (43)	
aLTR	16 (36)	
LABA	24 (54)	
Theophylline	6 (13)	

Values are shown as medians (IQR) or numbers (%). n.a. = Not applicable; BUD = budesonide; aLTR = antileukotrienes; LABA = long-acting  $\beta$ -mimetics. <sup>a</sup> Values are comparable in asthma patients and controls.

been suggested that epithelial function influences the degree of Th2 type inflammation [1].

Thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33 belong to a group of epithelium-derived cytokines that link the innate and adaptive immune responses related to Th2 cytokine-mediated reactions. The roles of TSLP, IL-25, and IL-33 have been investigated in inflammatory diseases such as allergy. TSLP may promote an innate allergic response to indoor allergens such as house dust mites and cause asthma, and it is thought to be a central regulator of allergic asthma [4, 5]. Genetic variants of TSLP have been found in children with asthma and allergic rhinitis [6]. ST2L/IL-1 R4 is an isoform of one of two receptors for IL-33. ST2 is expressed by natural killer cells, natural killer T cells, mast cells, monocytes, dendritic cells, granulocytes, and human Th2 cells, as well as nasal epithelium [7–9]. Both ST2L/IL-1 R4 and IL-33 serum levels are elevated in some allergic diseases, including acute bronchial asthma, in both children and adults [10–12]. IL-33 expression by epithelial cells and smooth muscle cells has also been observed in bronchial asthma [13, 14]. IL-25 epithelial expression is in-

creased in patients with asthma and it is associated with the Th2 response [15].

Exhaled-breath condensate (EBC) has been used to measure various markers of airway inflammation in patients with asthma. It is a simple, noninvasive, and safe method of airway sampling [16]. EBC is collected using special condensing devices that freeze the exhaled air. Nonvolatile particles from the epithelial lining fluid of the airway are thus suspended in aerosol form and can subsequently be detected [17, 18]. To the best of our knowledge, epithelium-derived cytokines have not yet been studied in EBC. Thus, we decided to examine whether the epithelium-derived cytokines IL-25, IL-33, and TSLP and a soluble form of the IL-33 receptor ST2L/IL-1 R4 are present in detectable amounts in the EBC of patients with asthma and if there is a relationship between serum and EBC levels of these cytokines. The secondary aim of this study was to analyze whether the EBC and serum levels of the above-mentioned cytokines could be used as markers for bronchial asthma control.

## Materials and Methods

We enrolled 44 patients with chronic bronchial asthma into this study. Asthma was diagnosed according to Global Initiative for Asthma (GINA) recommendations based on the clinical history, physical findings, and lung function test results [19]. Twenty-two patients (50%) had allergic bronchial asthma, confirmed based on their clinical history, skin prick test results, and allergen-specific IgE estimation. Fourteen of the patients assessed (32%) were in the uncontrolled phase of the disease. Partially controlled and uncontrolled asthma were diagnosed according to GINA recommendations based on the clinical picture, anamnestic data, and spirometry values [19]. The patients with bronchial asthma were allowed to use inhalant glucocorticosteroids, antileukotrienes, theophylline, and short- or long-acting  $\beta_2$ -mimetics (table 1).

As controls, we included 19 healthy volunteers (9 males, median age 44 years, range 37.5–55.5) with normal lung function and no signs of bronchial asthma or other systemic diseases. The precise characteristics of both groups are shown in table 1. Exclusion criteria included acute respiratory infections in the previous 4 weeks, any pathological changes in the mouth, and chronic inflammatory or malignant conditions that, in the researchers' opinion, could bias the results. EBC and blood samples were collected from all patients and controls. Skin prick tests and spirometry were performed for all patients with asthma. The Local Ethical Committee of the Medical University of Silesia approved this study. The participants provided written informed consent.

### *EBC Collection, Exhaled Nitric Oxide Assessment, and Spirometry*

EBC was collected using an EcoScreen II Turbo condenser (Medivac, Italy) according to American Thoracic Society/European Respiratory Society recommendations [20]. EBC was collected in the morning, 2 h before meals. The participants used nose

**Table 2.** Serum levels of IL-33, IL-25, TSLP, and ST2L/IL-1 R4 and EBC levels of IL-33 and TSLP in asthmatic patients, controlled and uncontrolled asthmatic patients, and controls

	Asthma group (n = 44)	Controlled asthma (n = 30)	Uncontrolled asthma (n = 14)	Controls (n = 19)	p <sup>a</sup>	p <sup>b</sup>
<i>Serum level, pg/ml</i>						
IL-33	58.8 (36–157)	55 (34–159)	70 (56–155)	31 (23–47)	0.004	0.61
IL-25	2.285 (1.21–17.45)	2.1 (1.2–17.2)	10.2 (1.3–19.2)	11.1 (23–47)	0.058	0.46
TSLP	77 (42–129.1)	66.7 (40–124.9)	100 (54–144)	45.9 (34.4–65)	0.054	0.31
ST2L/IL-1 R4	650 (454.5–830.9)	567 (413–782)	833.9 (500–976)	670 (469–835)	0.99	0.022
<i>EBC level, pg/ml</i>						
IL-33	5 (2.2–9)	5 (2.2–9)	6 (2.2–9.0)	1.9 (1.2–2.6)	0.0008	0.55
TSLP	39 (23–55)	41 (24.8–55)	31 (12.5–40)	13.1 (10.25–23.45)	0.0002	0.11

Values are presented as medians (IQR) unless otherwise stated. <sup>a</sup> For comparisons between the asthma group and controls. <sup>b</sup> For comparisons between the controlled and uncontrolled asthma groups.

clips and were allowed to periodically swallow saliva to maintain a dry mouth during collection. The procedure lasted 20 min and about 1.5 ml EBC was collected. The EBC and serum samples were stored at –80°C until analyzed. Spirometry was performed using a Medgraphics CPFS/D USB spirometer (MGC Diagnostics, USA) according to European Respiratory Society standards [21].

#### Skin Prick Tests

Skin prick tests were performed according to European Academy of Allergy and Clinical Immunology guidelines using Allergopharma (Germany) extracts [22].

#### Measurement of Serum Levels of ST2L/IL-1 R4, TSLP, IL-25, and IL-33 and Serum Levels of Total and Allergen-Specific IgE

Commercial enzyme-linked immunosorbent assays were used to measure EBC and serum levels of IL-25 (Wuhan EIAAB Science, China), IL-33, ST2L/IL-1 R4, and TSLP (R&D Systems, USA). The assays were performed using the protocols recommended by the manufacturers. The sensitivity of the assays was as follows: IL-25, 6.9 pg/ml; IL-33, 1.65 pg/ml; ST2L/IL-1 R4, 13.5 pg/ml, and TSLP, 9.87 pg/ml. In the case of values lower than the method sensitivity limit, the samples were quantified based on extrapolation of standard curves generated for each set of samples assayed. Only the proteins that recorded a level at least half of that of the detection limit were analyzed (table 2). The proteins with a value lower than half that of the detection limit were excluded from further analyses. Total and allergen-specific IgE levels were determined using an enzyme-linked immunosorbent assay (Allergopharma).

#### Statistical Analysis

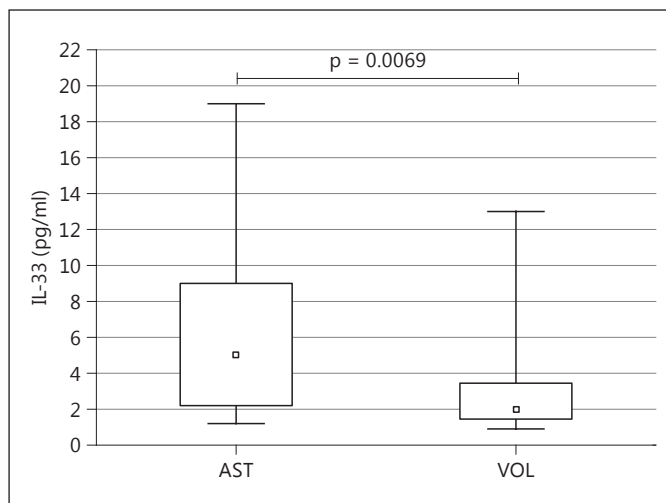
Results are expressed as median values with interquartile ranges (IQR). We used nonparametric tests (Mann-Whitney U rank sum test, Kruskal-Wallis test, and Spearman's correlation test) because of the nonnormal distribution of the data. All analyses were performed with the software package STATISTICA 10 (Statsoft Polska, Poland).  $p < 0.05$  was considered statistically significant.

## Results

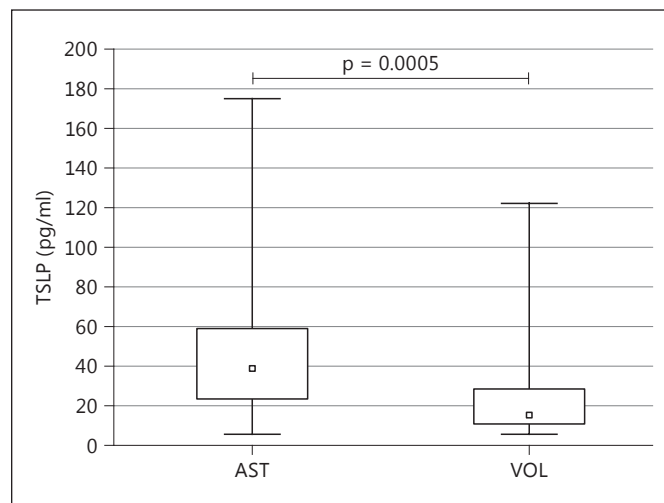
IL-25 and ST2L/IL-1R4 levels were under the limit of detection in almost all of the EBC samples. The values were quantified based on extrapolation of standard curves generated for each set of samples assayed; however, because of the very low values, and possibly poor reliability, these data were not analyzed further and comparisons were not performed (table 2). Values below half of the minimal detection limit were found in 2 patients for IL-33 (<0.825 pg/ml) and in 2 patients for TSLP (<4.935 pg/ml). These data were excluded from further analyses.

EBC levels of IL-33 and TSLP were statistically higher in patients with asthma than in controls (fig. 1, 2). The serum level of IL-33 was statistically higher in patients with asthma than in controls [58.8 pg/ml (IQR 36–157) vs. 31 pg/ml (IQR 23–47), respectively,  $p = 0.004$ ] (fig. 3). There was a trend for higher serum levels of TSLP [77 pg/ml (IQR 42–129.1) vs. 45.9 pg/ml (IQR 34.4–65), respectively,  $p = 0.054$ ] and IL-25 [2.285 pg/ml (IQR 1.21–17.45) vs. 11.1 pg/ml (IQR 23–47), respectively,  $p = 0.058$ ] in patients with asthma compared to controls, but this was not statistically significant (table 2). There were no significant correlations between serum and EBC levels of TSLP and IL-33 in asthma patients ( $r_s = 0.03$  and  $0.137$ , respectively) or controls ( $r_s = 0.104$  and  $0.23$ , respectively).

EBC levels of IL-33 and TSLP and serum levels of IL-25, IL-33, and TSLP did not significantly differ between the patients with controlled and uncontrolled asthma. Serum levels of ST2L/IL-1 R4 were statistically higher in patients with uncontrolled asthma [833.9 pg/ml (IQR



**Fig. 1.** IL-33 levels in EBC from patients with asthma and healthy controls. The square indicates the median value, the box indicates the IQR, and the whiskers indicate the total range. AST = Patients with asthma; VOL = controls.



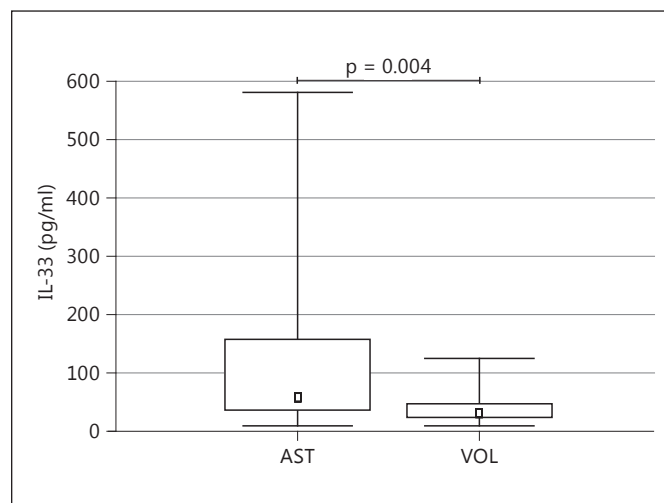
**Fig. 2.** TSLP levels in EBC from patients with asthma and healthy controls. The square indicates the median value, the box indicates the IQR, and the whiskers indicate the total range. AST = Patients with asthma; VOL = controls.

500–976) vs. 567 pg/ml (IQR 413–782), respectively]. The serum and EBC levels of TSLP and IL-33 did not correlate with the forced expiratory volume in 1 s [ $FEV_1$ ;  $r_s = 0.05$  (TSLP in serum), 0.31 (IL-33 in serum),  $-0.11$  (TSLP in EBC), and 0.15 (IL-33 in EBC)] or the total serum level of IgE [ $r_s = 0.26$  (TSLP in serum),  $-0.39$  (IL-33 in serum), 0.01 (TSLP in EBC), and  $-0.33$  (IL-33 in EBC); Spearman's correlation test].

The doses of inhaled glucocorticosteroids used did not interfere with serum IL-25, IL-33, ST2L/IL-1 R4, and TSLP levels or with EBC IL-33 and TSLP levels (in all comparisons  $p > 0.05$ , Kruskal-Wallis test).

## Discussion

In the present study, we explored the levels of 3 epithelium-derived cytokines (i.e. IL-25, IL-33, and TSLP) and a soluble form of the IL-33 receptor ST2L/IL-1 R4 in EBC and in the serum of patients with asthma and healthy controls. We additionally compared data between patients with controlled and uncontrolled disease. Only 2 of the examined mediators, i.e. IL-33 and TSLP, were detectable in EBC in amounts greater than half of the detection limit both in asthma patients and in healthy controls. We found significantly higher EBC levels of both IL-33 and TSLP, and a significantly higher serum level of IL-33, in patients with asthma compared to healthy controls. The



**Fig. 3.** IL-33 in serum from patients with asthma and healthy controls. The square indicates the median value, the box indicates the IQR, and the whiskers indicate the total range. AST = Patients with asthma; VOL = controls.

values of EBC and serum levels did not differ between controlled and uncontrolled asthma.

Both IL-33 and TSLP belong to a group of epithelium-derived cytokines. Our results indicate that these two cytokines are present in EBC from patients with bronchial asthma, and that this is a stable feature regardless of the

control level of asthma (controlled vs. uncontrolled). To the best of our knowledge, these cytokines have not previously been studied in the EBC of patients with asthma. Our results are of interest because both of the epithelium-derived cytokines, i.e. IL-33 and TSLP, are important activators of allergic inflammation and therapeutic targets for asthma. Some studies have been reported on animal models in which the therapeutic possibility of blocking IL-33 and TSLP was observed. Blockade of a receptor for TSLP by monoclonal antibodies in a cynomolgus monkey model of asthma and administration of anti-IL-33 antibodies in a murine model of asthma have both been shown to reduce allergic inflammation [23, 24]. Moreover, allergic inflammation was dramatically reduced in TSLP receptor-deficient mice, with less production of proinflammatory cytokines, such as IL-1 $\beta$ , or Th-2-related cytokines, such as IL-13 and IL-33 [25]. Neutralization of TSLP with anti-TSLP monoclonal antibodies reversed allergic inflammation and inhibited airway remodeling in chronic allergen-induced asthma in mice [26]. Galactooligosaccharides have been found to suppress IL-33 expression at the site of inflammation in house dust mite-induced asthma in mice [27]. In a human study, it was recently shown that anti-TSLP antibody reduced allergen-induced bronchoconstriction and indexes of airway inflammation before and after allergen challenge [28].

IL-33 is constitutively expressed in epithelial barrier tissues. Shortly after exposure to allergens or other noxious stimulators, such as pollutants or infections, IL-33 has been shown to induce Th2 cytokine production by group 2 innate lymphoid cells and to initiate allergic inflammation. Owing to this response, IL-33 is also called the 'alarmin' cytokine [29, 30]. We found no difference in the levels of analyzed cytokines in EBC between patients with controlled and uncontrolled asthma. These data are surprising given that during rhinovirus-induced asthma

exacerbation inflammation is dependent on IL-33 [31]. There are data suggesting that some parameters found in EBC can distinguish patients with different asthma control or severity. Tomasiak-Łozowska et al. [32] examined the acid-base equilibrium in EBC and found lower pH values and ammonia concentrations and significantly higher levels of nitrite/nitrate in patients with unstable asthma, and they concluded that these parameters may be useful as markers of asthma severity. The same authors found higher levels of eotaxin-1 in EBC from patients with unstable asthma than in patients with stable disease and healthy controls [33]. During asthma exacerbation, the pH of EBC decreases and H<sub>2</sub>O<sub>2</sub>, 8-isoprostane, and cysteinyl-leukotriene concentrations increase [17, 34].

In our study, we found statistically higher serum levels of ST2L/IL-1 R4 in uncontrolled asthma compared to controlled asthma. However, there were no differences between either subgroup of patients and controls. Although we observed statistical significance, the clinical relevance seems to be low given that the IQR were close to each other.

In conclusion, two epithelium-derived cytokines, i.e. IL-33 and TSLP, are found at higher levels in EBC from patients with asthma compared to healthy controls, supporting the important role of these mediators in bronchial asthma. The EBC levels of these two mediators do not correlate with their serum levels, which suggest that the local epithelial reaction is independent of the systemic reaction. The levels of IL-33 and TSLP in EBC do not discriminate between controlled and uncontrolled asthma.

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