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Expression of TSLP and IL-33 receptors on sputum macrophages of asthma patients and healthy subjects

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ABSTRACT

Objective: Local cytokine milieu (especially Th2 inflammatory type) secreted into the asthmatic airways affect the alternative activated macrophages polarization (M2). TSLP and IL-33 are important alarmins of allergic response associated with Th2 inflammation. The aim of the study was to investigate the expression of the receptors for epithelial derived cytokines: TSLP (TSLPR) and IL-33 (ST2) on induced sputum CD206 positive macrophages from asthma and healthy subjects and analyze the relationships between these receptors and clinical features of the disease. **Methods:** Immunofluorescence staining for CD206 and TSLPR or ST2 on sputum macrophages was performed in 20 adult patients with stable asthma – 75% with atopy (3 intermittent, 12 mild-to-moderate, 5 severe, of which 11 were on biological anti-IgE treatment) and 23 healthy adult controls – 48% with atopy. **Results:** Our study demonstrated an increased expression of TSLP and IL-33 receptors on bronchial CD206 positive macrophages in asthma group. TSLPR but not ST2 had also greater expression on CD206 negative macrophages in asthma patients. Increased expression of both investigated receptors was related to longer disease duration and impaired lung function. We observed increased count of CD206_{low}TSLP_{high} macrophages as well as positive correlation of these cells with total serum IgE in patients with atopy. **Conclusions:** The macrophage response during allergic reaction is likely to be connected with TSLP but rather not with IL-33 action. Our study indicates an important role of crosstalk between macrophages, TSLP and IL-33 in asthma pathophysiology.

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KEYWORDS

TSLP; IL-33;
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Pulmonary macrophages are important immune cells that are involved in the recognition, ingestion and killing of pathogens, clearance of debris, initiation and regulation of inflammatory responses and adaptive immunity [1]. As macrophages polarize into various phenotypes which are characterized by functional diversity and the presence of various surface markers, they represent a heterogeneous cell population. Two major subpopulations are labeled as M1 and M2 phenotypes. The differentiation of macrophages into M1 or M2 type is controlled by a local milieu including a panel of stimulating cytokines [2]. Surface markers of macrophages M2 (also called alternative activated) are much better defined than those for M1 macrophages. Macrophage differentiation into M2 cells mainly depends on stimulation by Th2 cytokines (IL-4 and IL-13) and this cell phenotype is characterized by low

expression of MHCII, CD86, and iNOS2 but high levels of arginase-1, family proteins chitinase-like Ym1/2 and Fizz1/RELM- α (found in inflammatory zone 1), and cell surface receptors such as CD163 and macrophage mannose receptor (MRC1), also called CD206 [3–5]. CD206 function is associated with increased efferocytosis (clearance of apoptotic cells) by M2 macrophages [6]. M2 macrophages promote Th2 response by producing CCL17, CCL22 and CCL24. Therefore, the predominance and the high activity of these cell types result in eosinophilic inflammation. Although the phenotypic identification of M1 and M2 macrophages is well established in mice, much less is known about human M1/M2 macrophages.

Some data suggest that dysfunction of macrophages may play a role in the pathogenesis of obstructive lung diseases [7]. Most studies on pulmonary macrophage

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used bronchoalveolar lavage fluid (BALf) containing mainly alveolar macrophages (AMs) or biopsies of lung tumors that were rich in tumor associated macrophages (TAMs). Much less is known about bronchial macrophages (BMs) and their subpopulations. Induced sputum (IS) is an easily accessible and valuable respiratory sample containing high numbers of macrophages, which mainly reflects the cellular profile of macrophages located in the trachea, large and medium-sized bronchi. It has been demonstrated that sputum/bronchial phagocytes from healthy individuals are more active than phagocytes from the alveolar region [8]. It was also shown that macrophages obtained from IS of asthma patients are phenotypically and functionally altered compared to healthy individuals [9]. This is due to a different pattern of gene expression [10].

Macrophages are capable of responding to important respiratory epithelium derived drivers of allergic inflammation, including thymic stromal lymphopoietin (TSLP) and IL-33 action [11,12]. It has been demonstrated that bronchial epithelial cells from asthmatics constitutively express more TSLP and IL-33 than those from healthy subjects [13,14]. The levels of TSLP and IL-33 in asthmatic airways correlate with disease severity [15,16] and with basal membrane thickness [17]. TSLP and IL-33 amplify the polarization of alternative activated macrophages *in vivo* possibly via downstream of IL-4 and IL-13 [18–20]. Macrophages respond to IL-33 and contribute to the induction of type 2 immunity by production of IL-5 and IL-13 [21]. AMs from an acute exacerbation in mice exhibited enhanced expression of markers of alternative activation, together with enhanced expression of both mRNA and immunoreactivity for IL-33 in the airways [22].

Th2 type of inflammation in asthma links TSLP and IL-33 (important alarmins of allergic response) with M2 macrophages (their polarization is dependent on Th2 associated cytokines). In the face of uncertain data regarding the associations between epithelial derived cytokines (TSLP, IL-33) and M2 macrophages in humans we undertook the study aimed at the evaluation of this issue. We tested the hypothesis that in asthmatic airways TSLP and IL-33 action is more associated with M2 macrophages than other subsets of macrophages. This study was designed to (1) investigate the expression of receptor for TSLP (TSLPR) and receptor for IL-33 (ST2) on sputum CD206 positive macrophages from asthma and healthy subjects; (2) analyze the relationships between TSLPR and ST2 expression on sputum CD206 positive macrophages and clinical features of the disease.

Material and methods

Study design

This was a prospective, cross-sectional study performed between 2014 and 2016 which involved 20 adult patients with stable asthma and 23 adult healthy controls. Asthma patients were recruited from the pulmonary outpatient department of the Medical University Hospital in Warsaw. Exclusion criteria for all subjects were disease exacerbation or symptoms of respiratory tract infection in the previous 3 months.

The diagnosis of asthma and its severity was made in accordance with the Global Initiative for Asthma (GINA) guidelines [23]. All patients with asthma underwent the following evaluations: medical history, physical examination, spirometry with flow-volume curve, airway obstruction reversibility test (when applicable), methacholine bronchial challenge, allergy skin prick tests (SPT, 15 allergens, [Supplementary material, Table S1](#)), serum C-reactive protein (CRP), total IgE level, and sputum induction. Airway hyper-responsiveness (minimum one day before sputum induction) and bronchial obstruction reversibility were tested according to the recommendations of the European Respiratory Society (ERS) and the American Thoracic Society (ATS) [24,25].

The control group comprised healthy volunteers with no history of obstructive lung disease. In this group spirometry (normal spirometry was mandatory for inclusion), SPT, serum CRP, total IgE level, and sputum induction were performed.

The study protocol was approved by the Institutional Review Board. Informed consent was obtained from all individual participants included in the study.

Sputum induction

The procedure of sputum induction was the same in control and asthma group and was preceded by inhalation of 400 µg of salbutamol and subsequent pulmonary function testing. After post-bronchodilator spirometry, patients inhaled sterile hypertonic saline (NaCl) at room temperature at increasing concentrations (3, 4 and 5% solutions) via an ultrasonic nebulizer (ULTRA-NEBTM 2000, DeVilbiss, Somerset, PA, USA) during which subjects were asked to cough and expectorate at 5 min intervals. The duration of each inhalation was 7 min. FEV₁ was measured after each inhalation and when the patient felt uncomfortable. Salbutamol was administered if FEV₁ fell ≤80% of the pre-induction value. The induction was terminated

when ≥ 5 ml of sputum with visible white “plugs” was expectorated or when the nebulization with all three NaCl concentrations was completed or when FEV₁ decreased by at least 20% from baseline (post bronchodilator) value.

Sputum processing

Induced sputum was processed immediately on receipt as described elsewhere [26]. The volume of the sputum was measured and the plugs were separated and weighed. A freshly prepared 0.1% solution of dithiothreitol (DTT, Sigma Aldrich, St. Louis, MO) in sterile phosphate-buffered saline (PBS) was added in a volume equal to fourth the weight of the sputum, and the mixture was shaken for 15 min. Subsequently, a double volume of sterile PBS was added and the mixture was vortexed briefly. After filtration through 70 μ m nylon cell strainer (Falcon, Corning Brand, NY, Columbia, USA) the sputum was centrifuged for 10 min at 1800 g. The cells were counted in Neubauer hemocytometer, percentage of dead cells and epithelial cells (squamous and ciliated) was assessed. Total number of immunological cells ($\times 10^6$) and total cell count (10^6 cells/g sputum) were calculated. Six smears from each patient were performed: four smears were stored at -80°C for further investigation, two smears from each subject were stained with May-Grünwald-Giemsa method to assess the percentage of immunological cells, based on the morphology of 300 cells from various fields. The criteria for appropriate IS quality were as follows: less than 50% epithelial cells, and more than 300 non-epithelial cells on one slide.

Biochemical serum measurements

Total IgE concentration was evaluated in serum using ELISA Biomerieux mini Vidas (Marcy-l'Étoile, France) according to the manufacturer's instruction (measurement range 0.5–1000 kIU/L). Serum CRP was measured by immunoturbidimetric assay on Roche/Hitachi Cobas c systems (range 0.3–350 mg/L).

Immunofluorescent staining for CD206 and TSLPR or ST2 in sputum smears

An indirect method of immunostaining that involved a two-step process was applied to detect the expression of TSLPR and ST2. Based on the literature data, CD206 was selected as a marker of M2 macrophage population [27–29]. IS slides (one slide double staining: CD206 expression and one cytokine receptor)

were incubated in acetone at -20°C for 20 min and dried at room temperature (RT). Nonspecific binding was blocked in 10% normal goat serum blocking solution (Thermo Fisher Scientific, Waltham, MA) for 30 min. The blocking solution was aspirated, and rat anti-CD 206 monoclonal antibody (1:100, R&D Systems, Minneapolis, MN) was added (overnight at 4°C) on each slide. The slides were rinsed three times with PBS without Ca^{2+} and Mg^{2+} . Then rabbit anti-TSLPR (1:50, Thermo Fisher Scientific, Waltham, MA), or rabbit anti-ST2 (1:50, Thermo Fisher Scientific, Waltham, MA) primary antibodies were added (overnight at 4°C). The slides were rinsed three times with PBS without Ca^{2+} and Mg^{2+} and incubated with secondary polyclonal antibody: anti-rat Alexa Flour 488 and anti-rabbit Alexa Fluor 555 (1:200, Thermo Fisher Scientific, Waltham, MA) for 1 h at RT. The slides were mounted using Vectashield with DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA). Immunofluorescence was analyzed under an Eclipse 80i microscope (Nikon, Tokyo, Japan).

TSLPR and ST2 expression was evaluated with semi-quantitative method based on the color of immunostainings. To evaluate the immunopositivity of studied receptors we considered as positive those macrophages which exhibited green, red or orange fluorescence in cytoplasmic region. The level of infiltration (low vs. high) was set arbitrarily based on the development of immunostaining color. Estimation was performed by three experienced researchers in a blinded manner. Three hundred (3×100 cells) consecutive macrophages were counted in different areas of slides. Macrophages were evaluated in light microscope on the basis of the size of the cell and the ratio of the nucleus of the cell to the cytoplasm. Macrophages were assigned to one of subgroups (Figure 1):

- CD206_{high} TSLPR_{high} or CD206_{high} ST2_{high} (CD206_{high} TSLPR/ST2_{high}) macrophages (orange)
- CD206_{low} TSLPR_{high} or CD206_{low} ST2_{high} (CD206_{low} TSLPR/ST2_{high}) macrophages (red)
- CD206_{high} TSLPR_{low} or CD206_{high} ST2_{low} (CD206_{high} TSLPR/ST2_{low}) macrophages (green)

For each slide the percentage of particular subgroup of macrophages was assessed.

Statistical analysis

Statistical analysis was performed with the use of Statistica 12.0 software package (StatSoft Inc., Tulsa, OK). Differences between categorical and continuous

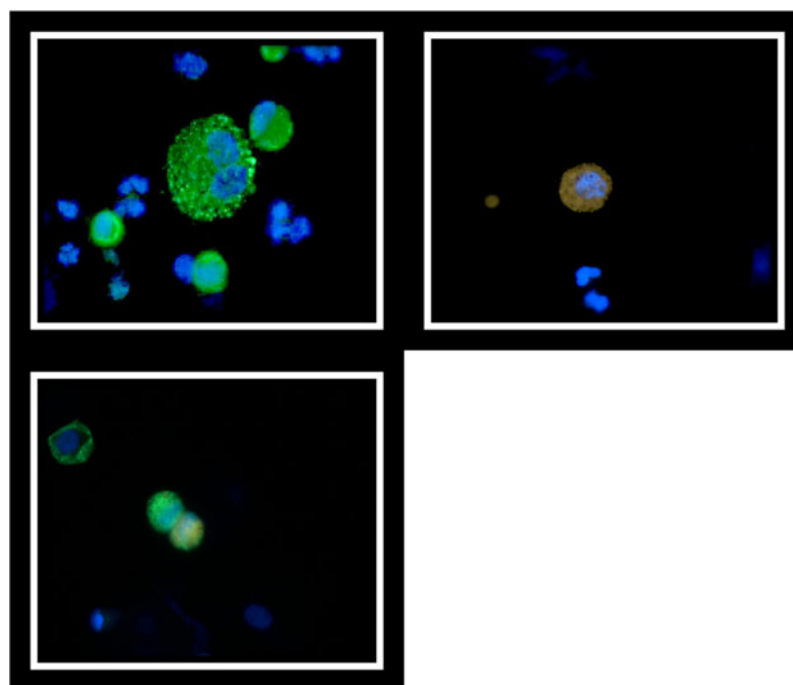


Figure 1. Macrophages with high brightness of CD206 were seen as green macrophages, cells with high brightness of one of the tested receptors (TSLPR or ST2) were seen as red macrophages, cells with positive reaction for both markers (with CD206 and one of the tested receptors) were visualized as orange macrophages.

Table 1. Comparison of clinical, biochemical and cytological data in controls and asthma patients.

	Control <i>n</i> = 23	Asthma <i>n</i> = 20	<i>p</i>
Age (years)	38 (31–49)	51.5 (45–56.5)	0.007
Gender (F/M)	13/10	16/4	0.101
BMI (kg/m ²)	25.85 (21.3–27.5)	26.9 (24–28.7)	0.227
Smoking status: never smokers/ex-smokers/current smokers (n)	20/3/0	14/4/2	0.132
Atopy (n)	11	15	0.065
FEV ₁ (% predicted)	95.5 (91–103)	75 (60.5–89.5)	0.0001
FEV ₁ /VC	81.24 (77.64–86.76)	69.22 (56.5–81.5)	0.004
IgE (IU/mL)	27.5 (6.5–53.5)	264.5 (43.5–410.5)	0.0012
CRP (mg/L)	1.95 (1.4–3.35)	1.6 (0.7–4.1)	0.517
Disease duration (months)	0	138 (84–204)	0.0000
IS epithelial cells (%)	23.5 (16–30)	24 (7–36)	0.865
IS macrophages (%)	44 (39–54)	43 (38–50)	0.392
IS lymphocytes (%)	6 (4–9)	4 (3–7)	0.095
IS neutrophils (%)	47 (36–55)	45.5 (40–50)	0.825
IS eosinophils (%)	0 (0–2)	2 (1–10)	0.006

Data presented as median and IQR. Fisher's exact test or Mann-Whitney U test. F: female; M: men; BMI: body mass index; ICS: inhaled corticosteroid; IgE: immunoglobulin E; CRP: C reactive protein; FEV₁: forced expiratory volume in one second; IS: induced sputum; VC: vital capacity.

Bold values for *p* < 0.05, statistically significant difference between asthma and control group.

variables were tested using Pearson Chi-square test and nonparametric Mann-Whitney U test, respectively. Results are given as median and interquartile range (IQR). Correlations between variables were analyzed with Spearman's rank test. Differences were considered statistically significant at *p* < 0.05.

Results

Baseline patient characteristics are presented in Table 1. Female gender dominated in asthma group compared to controls. Forty eight percent of controls and 75% of

asthmatics were atopic. Intermittent, mild-to-moderate and severe asthma was diagnosed in 3 (15%), 12 (60%) and 5 (25%) patients, respectively.

Seventeen patients (85%) were treated with a combination of inhaled corticosteroids (ICS) (median dose 1600 µg of budesonide or equivalent (1600–1600 µg)) and long acting β_2 -agonists (LABA), while 3 patients (15%) received only short-acting β_2 -agonists (SABA) as needed (newly diagnosed patients). Five patients with severe asthma were treated with systemic corticosteroids (methylprednisolone median dose 4 mg (1–26 mg)). Additionally, 11 patients received omalizumab therapy –

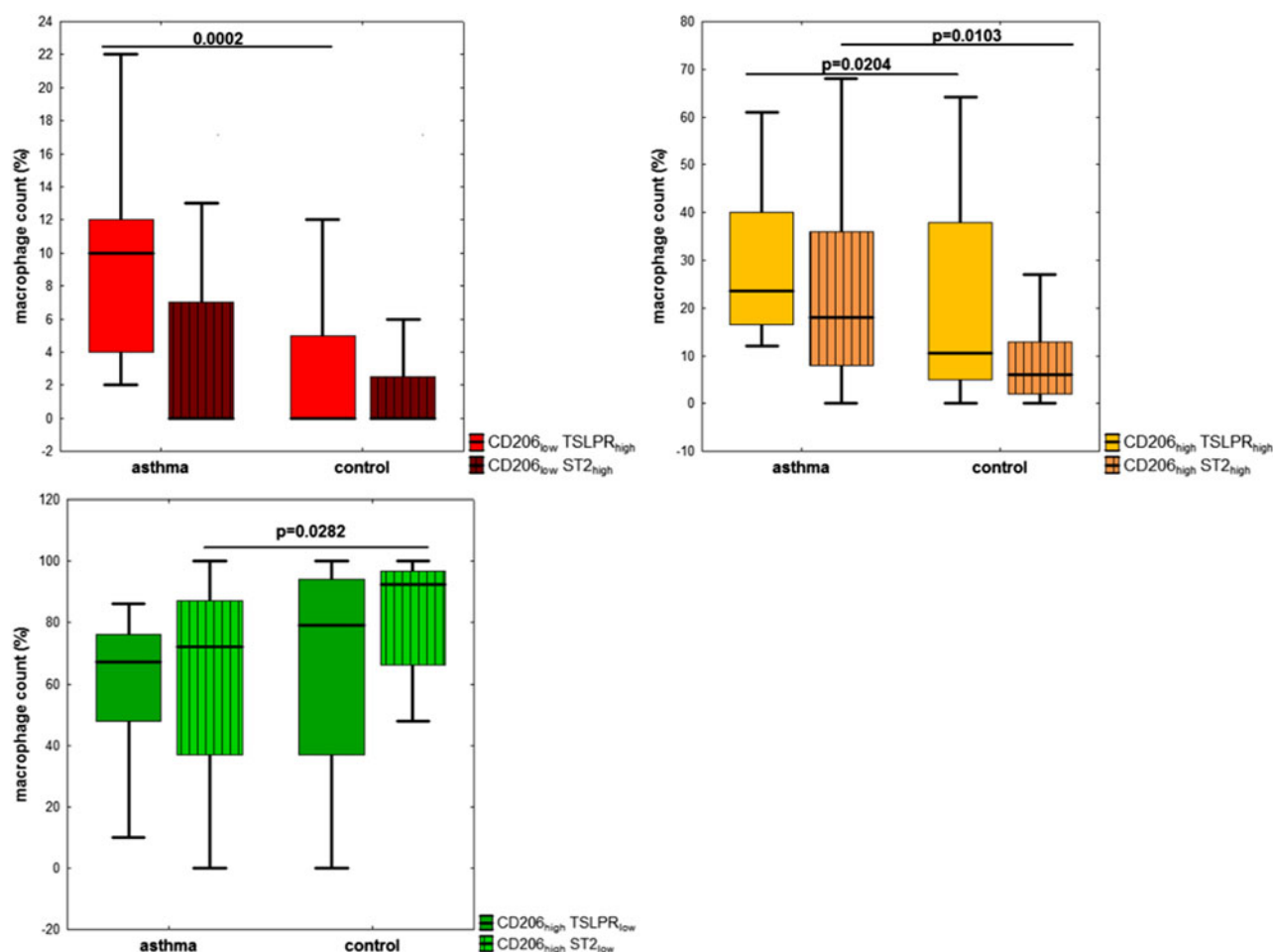


Figure 2. Sputum macrophage count (%) in immunofluorescence staining for TSLPR and ST2 in asthma and control subjects. Comparisons were performed using Mann-Whitney U-test, significant differences ($p < 0.05$) are shown.

the median duration of treatment was 24 months (12–36 months), the median dose was 450 mg (150–450 mg).

Clinical characteristics and cellular composition of IS in asthmatics and controls are shown in Table 1. The asthma patients were older and had significantly worse lung function than controls. The percentage of eosinophils was significantly higher in asthma group compared to healthy controls.

Immunofluorescence staining of sputum macrophages for TSLPR expression showed higher percentage of CD206_{low} TSLPR_{high} macrophages: 10% (4–12%) vs. 0% (0–5%) as well as CD206_{high} TSLPR_{high} macrophages: 23.5% (16.6–40%) vs. 10.5% (5–38%) in asthma group compared to controls, respectively. The evaluation of IL-33 receptor showed higher percentage of CD206_{high} ST2_{high} sputum macrophages in asthmatics: 18% (8–36%) vs. 6% (2–13%) and higher amount of CD206_{high} ST2_{low} macrophages in controls: 92.3% (66–96.6%) vs. 72% (37–87%) (Figure 2).

Subanalysis performed within the asthma group did not show any significant differences in terms of

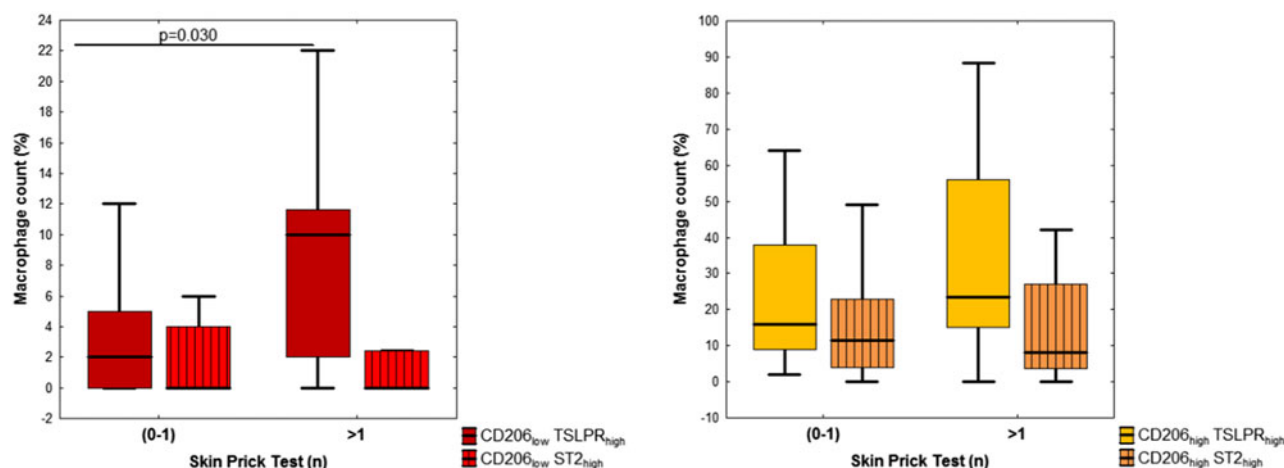
CD206_{high/low} TSLPR/ST2_{high/low} macrophage count. This refers to comparison of patients with and without atopy, patients with vs. without sputum eosinophilia, patients treated with systemic steroids vs. patients without systemic steroid treatment, patients treated with ICS vs. patients without ICS treatment, patients treated with omalizumab vs. patient not treated with omalizumab (Supplementary material, Tables S2 and S3). We also did not find any significant differences either in TSLPR or in ST2 expression between smoking and nonsmoking subjects in both asthma and control group. Since subanalyses among asthma group did not revealed any significant differences, we studied the relationship between the evaluated sputum macrophages and clinical data for the entire group.

Correlation analysis between clinical data and the macrophage count showed positive correlations between disease duration and CD206_{low} TSLPR_{high} macrophages, as well as CD206_{high} TSLPR/ST2_{high} macrophages with simultaneous negative correlations with CD206_{high} ST2_{low} macrophages. FEV₁/VC

Table 2. Correlations between disease duration, lung function data, total serum IgE and TSLPR, IL-33R stained sputum macrophage count of healthy controls and asthma patients.

	Disease duration (months)	FEV ₁ (% of predicted)	FEV ₁ /VC (percent)	Total IgE (IU/mL)
TSLPR				
CD206 _{low} TSLPR _{high}	$r = 0.520, p = 0.001$	$r = -0.410, p = 0.009$	$r = -0.409, p = 0.009$	$r = 0.568, p = 0.001$
CD206 _{high} TSLPR _{high}	$r = 0.338, p = 0.040$	$r = -0.253, p = 0.120$	$r = -0.468, p = 0.003$	$r = 0.275, p = 0.157$
CD206 _{high} TSLPR _{low}	$r = -0.244, p = 0.171$	$r = 0.169, p = 0.298$	$r = 0.416, p = 0.008$	$r = -0.139, p = 0.465$
ST2				
CD206 _{low} ST2 _{high}	$r = 0.130, p = 0.417$	$r = -0.125, p = 0.431$	$r = -0.062, p = 0.697$	$r = 0.186, p = 0.308$
CD206 _{high} ST2 _{high}	$r = 0.406, p = 0.014$	$r = -0.316, p = 0.546$	$r = -0.343, p = 0.035$	$r = 0.320, p = 0.104$
CD206 _{high} ST2 _{low}	$r = -0.411, p = 0.008$	$r = 0.182, p = 0.249$	$r = 0.275, p = 0.077$	$r = -0.082, p = 0.655$

Correlations were performed with Spearman's rank test.

**Figure 3.** Sputum macrophage count in immunofluorescence staining for TSLPR and ST2 in asthma and control subjects divided according to the atopic status. Comparisons were performed using Mann-Whitney U-test, significant differences ($p < 0.05$) are shown.

correlated negatively with CD206_{low} TSLPR_{high} and CD206_{high} TSLPR/ST2_{high} macrophages but not with CD206_{low} ST2_{high} macrophages. FEV₁/VC correlated positively with CD206_{high} TSLPR_{low} macrophages. Total serum IgE correlated positively with CD206_{low} TSLPR_{high} macrophages (Table 2).

Analysis of the relationships between TSLPR and ST2 macrophage expression and atopy status (subjects were classified according to the number of positive skin prick tests) showed an increased percentage of CD206_{low}TSLPR_{high} macrophages: 10% (2–11.6%) vs. 2% (0–5%) in patients with more than one positive SPT compared to patients with 0–1 positive SPT (Figure 3).

Discussion

Our study demonstrated an enhanced expression of TSLP and IL-33 receptors on sputum macrophages in patients with asthma. Although literature data concerning the presence of TSLP and IL-33 in the sputum of asthmatics are available, the interactions between these cytokines and BMs are unknown [30–32]. To our knowledge, this is the first study reporting the

new phenomenon that may be involved in airway inflammation in asthma. Our analysis showed that increased expression of TSLPR and ST2 was related to longer disease duration and impaired lung function. According to our results, only a part of CD206 positive macrophages have altered expression of TSLPR and ST2 and only a small percentage of CD206_{low} macrophages is capable of expressing these receptors. Thus, we believe our study points out a potential role of crosstalk between macrophages, TSLP but not IL-33 in allergic reactions in humans.

The results of previous animal studies showed that TSLP and IL-33 promote macrophage proliferation and M2 polarization [19,33]. The same epithelial cytokines contribute to allergic inflammation by stimulation of airway macrophages to increased production of cytokines related to Th2 type of response (mainly IL-13 and IL-5) [21]. An increased expression of TSLPR and ST2 on BMs and its correlation with asthma duration and worse spirometric results found in our study may suggest that TSLP and IL-33 mediate the macrophage activity not only at the early phase of allergic reaction but also during chronic asthmatic inflammation.

Our study showed that TSLPR and ST2 had the highest expression on CD206_{high} macrophages. However, not all CD206 macrophages had surface expression of TSLPR or ST2. We may hypothesize that time exposure to airway locally secreted cytokines is one of the factors affecting macrophage polarization. Thus, CD206_{high}TSLPR/ST2_{low} macrophages, observed in our study, could have been the population in the early stage of polarization.

Macrophage phenotype has large plasticity. The shift in M1/M2 polarization can be obtained *in vitro* within hours and macrophage can easily and frequently change their phenotype. After a polarization into M1 or M2 phenotypes, macrophages are able to revert to the uncommitted M0 state within 12 days in cytokine free medium. Moreover, after depolarization macrophages can switch their polarization, i.e., M1 macrophages switch to M2 and vice versa in 6 days [34]. Continuous changes in local mediator concentrations and their influence on macrophage polarization are factors responsible for lack of pure M1 or M2 populations in the human airways. Such simplified framework is possible to obtain in isolated and controlled *in vitro* environment only. As one of our aims was to evaluate the TSLPR and ST2 expression on M2like macrophage population, we selected CD206 as frequently used marker of M2 polarized macrophages. An interesting observation from this study is that some of CD206_{low} macrophages, which might be consider as M1 macrophages, are also capable of TSLPR and ST2 expression. This result is consistent with other studies which found that IL-33 amplifies both Th1 and Th2 type of response in human immune cell. Positive correlation of both CD206_{low}TSLPR_{high} and CD206_{high}TSLPR_{high} with disease duration may suggest important impact of this cytokine on asthma mechanism. It seems that in asthma pathobiology both CD206 positive and CD206 negative (M2like, M1like) macrophages are capable of responding to TSLP.

As almost one half of our patients received an anti-IgE therapy we were able to analyze a possible effect of biological treatment on TSLP and IL-33 receptors expression on BMs. However, we did not find any significant correlations between omalizumab treatment (dose and time) and the count of CD206/TSLPR or CD206/ST2 stained macrophages. To our knowledge, no data describing this issue are available. Some authors suggest that omalizumab decreases sputum eosinophilia due to inhibition of IL-5 mechanism which is associated with TSLP and IL-33 pathway [35]. In more detail, destruction of epithelial cells in

asthma leads to release of mediators (TSLP, IL-33) that stimulate ILC2, which subsequently secrete Th2 cytokines (e.g. IL-5), stimulating IgE production and eosinophil recruitment. More detailed future experiments are needed for evaluation this observation.

In our study asthma patients with more than one positive SPT showed increased percentage of CD206_{low}TSLPR_{high} sputum macrophages. Moreover, the number of these cells correlated positively with serum IgE level. We may suppose that these results indicate the crosstalk between macrophages and TSLP but not IL-33 in allergic reaction. Thus, our results may confirm a link between allergic inflammation and TSLP that has been reported by other authors. For example, Gauvreau et al. found that TSLP was necessary for the persistence of eosinophilic inflammation in subjects with mild allergic asthma [36].

The results of this study are partially consistent with various animal studies that showed a critical role of IL-33 in Th2 dependent inflammation. IL-33/ST2 was defined as an important mediator regulating events between mast cells and IgE-dependent inflammation, amplifier of IgE synthesis and inductor of an anaphylaxis in naïve mice [37,38]. Macrophage-derived IL-33 contributed to the exacerbation of IgE-mediated airway inflammation by mechanisms associated with macrophages and CD4+ cells, and airway remodeling through the activation of macrophages [39]. In the above context, it is important to realize that our study did not evaluate the direct role of TSLP and IL-33 in asthma and allergy but analyzed the associations between macrophages and receptors for these cytokines. Since we did not measure the level of cytokines in sputum it cannot be excluded that studied mediators are increased and associate with allergic inflammation in asthma or/and control subjects. Our results (positive correlation between CD206_{low}TSLPR_{high} with IgE and elevated level of CD206_{low}TSLPR_{high} in atopic patients) may indicate important role of TSLP-M1like macrophage interaction in the allergic reactions. The more detailed analysis of the interactions between TSLP/IL-33 and macrophage polarization in allergic inflammation might be an interesting plan for the future study.

Our study has several limitations. Firstly, due to the preliminary nature of our research the study group was relatively small. Different demographic variables such gender proportion and smoking status between controls and asthmatics subjects could affect the results of the study. This refers, in particular, to asthma subgroups that included asthmatics with different disease features and severity. Secondly, most

asthmatics received different treatment for example 85% of them were treated with high ICS doses that were necessary to maintain a proper disease control. However, it should be emphasized, that the exclusion of asthmatics treated with ICS would make the evaluation of severe asthma group, in which such treatment is necessary, impossible. The analysis of asthma patients classified according to ICS treatment (treated $n=17$ vs. not treated $n=3$) showed no significant differences in evaluated macrophage count. However, due to the small group of non-ICS asthma patients in our study, we did not analyze other parameters among ICS and non-ICS asthma patients. It is well documented that ICS inhibits eosinophilic inflammation and may affect the cellular composition of IS but has no or only weak anti-inflammatory effect on neutrophilic phenotype in asthma [40]. Corticosteroids do not change a total macrophage number in the airways but promote macrophage survival by inhibition of apoptosis. These drugs do not suppress macrophage effector functions, but influence on the differentiation of a specific anti-inflammatory phenotype [41]. It cannot be excluded that ICS suppressed the expression of analyzed receptors on macrophages in our study. However, an observed tendency (higher population of CD206_{high} TSLPR/ST2_{high} macrophages in asthma group) is pronounced and statistically significant for three evaluated receptors. Thirdly, in the immunofluorescent method used in this study, we were unable to evaluate CD206_{low} TSLPR/ST2_{low} macrophages. Such population can be identified in flow cytometric analysis only. Albeit immunofluorescent method has moderate sensitivity, it allows to visualize the distribution of assessed receptors and macroscopic features of immunopositive cells what gives us an interesting preliminary observations that need to be verified in the future work.

Conclusions

Our study demonstrated an increased expression of TSLP and IL-33 receptors on bronchial CD206 positive macrophages in asthma group. The same, except IL-33 receptor, was shown for CD206 negative macrophages. This phenomenon may be the feature of airway inflammation in asthma. The macrophage response during allergic reaction is likely to be connected with TSLP but rather not with IL-33 action. The results of our study warrant further evaluation of functional relationship between TSLP and IL-33 and various macrophage populations in asthmatic airways.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

Ethical approval

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Supplementary file:

Table 1. Skin Prick Test (SPT) allergens characteristic.

Allergens	Species
Grass/Rye Grass (meadow soft grass, cock's foot grass, perennial ryegrass, timothy-grass, smooth meadow-grass, meadow fescue), barley, common oat, rye grass, common wheat	Poales (<i>Holcus lanatus</i> , <i>Dactylis glomerata</i> , <i>Lolium perenne</i> , <i>Phleum pratense</i> , <i>Poa pratensis</i> , <i>Festuca pratensis</i>), <i>Hordeum vulgare</i> , <i>Avena sativa</i> , <i>Secale cereal</i> , <i>Triticum sativum</i>
Grass meadow soft grass, cock's foot grass, perennial ryegrass, timothy-grass, smooth meadow-grass, meadow fescue	<i>Holcus lanatus</i> , <i>Dactylis glomerata</i> , <i>Lolium perenne</i> , <i>Phleum pratense</i> , <i>Poa pratensis</i> , <i>Festuca pratensis</i>
Rye	<i>Secale cereale</i>
Birch	<i>Betula alba</i>
Alder	<i>Alnus glutinosa</i>
Hazel	<i>Corylus avellana</i>
Mugwort	<i>Artemisia vulgaris</i>
Ribwort plantain	<i>Plantago lanceolata</i>
<i>Dermatophagoides farinae</i>	<i>Dermatophagoides farinae</i>
<i>Dermatophagoides pteronyssinus</i>	<i>Dermatophagoides pteronyssinus</i>
Cat	Cat
Dog	Dog
Cladosporium	<i>Cladosporium herbarum</i>
Alternaria	<i>Alternaria alternata (tenuis)</i>
Aspergillus	<i>Aspergillus fumigatus</i>

Atopy was defined as the presence of at least one positive skin prick test to common allergens, with a diameter of 3 mm or greater than the negative control [1].

Table S2. The comparison of TSLPR, IL-33R stained sputum macrophage count in asthma patients with and without omalizumab treatment

Treatment	Without omalizumab (n=9)	With omalizumab (n=11)	p value
CD206 _{low} TSLPR _{high}	8.3 (2.2-13.5)	10 (5-11)	0.897
CD206 _{high} TSLPR _{low}	56 (36-81.9)	69.2 (62-74)	0.897
CD206 _{high} TSLPR _{high}	31.5 (15.8-50.5)	22.3 (17-25)	0.696
CD206 _{low} ST2 _{high}	0 (0-5)	1 (0-9.5)	0.331
CD206 _{high} ST2 _{low}	64 (10-76)	73.3 (52.3-92.8)	0.21
CD206 _{high} ST2 _{high}	23 (18-42)	15 (7.1-29)	0.193

Data presented as median and IQR. Mann-Whitney U test.

Table S3. The comparison of TSLPR, IL-33R stained sputum macrophage count in asthma patients with and without ICS treatment

Treatment	Without ICS (n=3)	With ICS (n=17)	p value
CD206 _{low} TSLPR _{high}	2.3 (2-12)	10.4 (5-15)	0.362
CD206 _{high} TSLPR _{low}	80.8 (48-86)	67.2 (24-74)	0.244
CD206 _{high} TSLPR _{high}	16.6 (12-40)	23.5 (17-61)	0.363
CD206 _{low} ST2 _{high}	0 (0-6)	0.5 (0-8)	0.634
CD206 _{high} ST2 _{low}	76 (64-82)	65.5 (17-87)	0.555
CD206 _{high} ST2 _{high}	18 (18-36)	23 (8-38)	0.900

Data presented as median and IQR. Mann-Whitney U test.

Reference:

- 1 Position paper: Allergen standardization and skin tests. The European Academy of Allergology and Clinical Immunology. Allergy 1993;48:48–82.

