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Elevated Expression of IL-33 and TSLP in the Airways of Human Asthmatics In Vivo: A Potential Biomarker of Severe Refractory Disease

Yan Li,^{*,1} Wei Wang,^{*,1} Zhe Lv,^{*} Yun Li,^{*} Yan Chen,^{*} Kewu Huang,[†] Chris J. Corrigan,[‡] and Sun Ying^{*,‡}

The epithelial cytokines IL-33, thymic stromal lymphopoietin (TSLP), and IL-25 have been implicated in asthma pathogenesis because they promote Th2-type cytokine synthesis, but their expression is relatively poorly documented in “real-life” human asthma. Using bronchoalveolar lavage fluid (BALF), we measured airway concentrations of these mediators and compared them with those of Th1- and Th2-type cytokines, airway infiltration of neutrophils and eosinophils, and lung function in a large group of asthmatic patients with a range of disease severity ($n = 70$) and control subjects ($n = 30$). The median BALF concentrations of IL-33, TSLP, IL-4, IL-5, IL-13, and IL-12p70, but not IL-25, IL-2, or IFN- γ , were significantly elevated in asthmatics compared with controls ($p < 0.05$). The concentrations of IL-33 and TSLP, but not IL-25, correlated inversely with the lung function (forced expiratory volume in the first second) of asthmatics (IL-33: $r = -0.488, p < 0.0001$; TSLP: $r = -0.565, p < 0.0001$) independently of corticosteroid therapy. When divided according to disease severity and corticosteroid therapy, all subgroups of asthmatics had elevated median numbers of eosinophils in BALF, whereas the patients with more severe disease who were treated with corticosteroids had higher numbers of neutrophils compared with milder asthmatics not so treated and control subjects ($p < 0.05$). The data implicate TSLP and IL-33 in the pathogenesis of asthma that is characterized by persistent airway inflammation and impaired lung function despite intensive corticosteroid therapy, highlighting them as potential molecular targets. *The Journal of Immunology*, 2018, 200: 000–000.

The prevalence of asthma continues to increase (1, 2), and the disease remains a significant cause of mortality, suffering, and health care costs. It is characterized by variable obstruction of the airways, which, in turn, reflects intrinsic bronchial smooth muscle hyperresponsiveness and narrowing of

the airway lumen by chronic inflammation of its mucosal lining. These may be partially or completely resistant to conventional therapy for asthma, resulting in the accelerated irreversible decline in airflow that sometimes accompanies the disease (3). Although Th2-type CD4⁺ T cells have conventionally been regarded as a principal source of the Th2-type cytokines that appear to drive this inflammation in the majority of cases, there has been increasing scrutiny of the possible role for resident mucosal group 2 innate lymphoid cells (ILC2s) as a potentially more prominent source, given their capacity to produce large quantities of these cytokines (4–6). Furthermore, it is now evident that the cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), produced by epithelial and other cells at mucosal surfaces in response to a wide range of environmental stimuli, play an important role in activating ILC2s to produce Th2-type cytokines (IL-33, IL-25) and in inducing Th2-type differentiation of CD4⁺ T cells directly or by suitable priming of Ag-presenting dendritic cells (DCs) (by TSLP) (7). These cytokines are likely involved in innate and adaptive immune responses initiated by the interaction of epithelial cells with the external environment in diseases including asthma and atopic dermatitis (4–8).

Local topical administration or lung-specific transgenic expression of TSLP, IL-25, or IL-33 alone is sufficient to induce a complete spectrum of asthma-associated pathogenetic features, including airway inflammation, eosinophil infiltration, goblet cell hyperplasia, and airway hyperresponsiveness, in the airways of animal surrogates of asthma (9–11). Conversely, targeting these cytokines or their receptors inhibits asthma-related phenomena (12–14). Elevated expression of these cytokines and their receptors has also been observed in bronchial biopsies of human asthmatic patients compared with controls (15–17). Increased concentrations of IL-33 and TSLP have also been documented in bronchoalveolar lavage fluid (BALF) of patients with a variety of chronic airway disorders, including asthma (18, 19).

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; DC, dendritic cell; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid; ILC2, group 2 innate lymphoid cell; LABA, long-acting β -agonist; SABA, short-acting β -agonist; TSLP, thymic stromal lymphopoietin.

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Despite this accruing evidence, there is still relatively little information about the expression of IL-33, IL-25, and TSLP in the airways of human asthmatic patients in a clinical setting and how this expression may be related to that of other biomarkers of asthma, as well as to disease severity. To address these gaps in knowledge, we hypothesized that, in asthmatic patients, concentrations of TSLP, IL-33, and IL-25 and other Th2-type, but not Th1-type, cytokines are elevated in BALF compared with controls; BALF concentrations of TSLP, IL-33, and IL-25 remain elevated in the face of increasing anti-asthma therapy, thus potentially contributing to corticosteroid-refractory disease; concentrations of these cytokines correlate inversely with airway airflow; concentrations of these cytokines correlate positively with those of the Th2 cytokines IL-4, IL-5, and IL-13, but not those of the Th1 cytokines IFN- γ , IL-2, and IL-12p70; and concentrations of these cytokines correlate positively with the numbers of BALF inflammatory effector cells.

Materials and Methods

Subjects

One hundred subjects (70 asthmatics and 30 normal controls) were recruited through the Department of Asthma, Allergy and Respiratory Science, King's College London School of Medicine. These subjects were recruited for a variety of departmental studies on asthma, and all provided written informed consent for their samples to be used in related approved studies, including the current study, which was independently approved by the Local Research Ethics Committee of King's College Hospital (London, U.K.). Asthmatics had a clear history of relevant symptoms, documented reversible airway obstruction (minimum 12% improvement in forced expiratory volume in 1 s [FEV₁], either spontaneously or after administration of inhaled β_2 agonist), and/or histamine provocation concentration causing a 20% decrease in FEV₁ < 8 mg/ml typically measured in the 2-wk period prior to sampling as part of the patient-screening process. FEV₁ measurements were recorded pre- and postbronchodilator after temporary withholding of any existing bronchodilator for an appropriate time period. None had ever smoked, and there was no history of other respiratory disease. All subjects were clinically free of respiratory infection for ≥ 1 mo prior to the study. Asthmatic patients were arbitrarily assigned to three groups based on their existing therapy at recruitment: No steroids (short-acting β -agonist (SABA) therapy or long-acting β -agonist (LABA)

therapy only), inhaled corticosteroid (ICS; 40–2000 μ g/d beclomethasone equivalent, with or without additional bronchodilator), and ICS + Oral (ICS plus oral prednisolone [35–50 mg/d] for ≥ 4 wk prior to the study). Some patients were also taking oral montelukast or theophylline preparations. Normal control subjects were healthy lifelong nonsmoking volunteers who had no history of lung disease. Atopy was recorded and defined as a positive skin prick test (wheal at 15 min > 3 mm in diameter in the presence of positive histamine and negative diluent controls) to one or more extracts of a panel of common local seasonal and perennial aeroallergens; 66 of 70 asthmatic patients and 4 of 30 controls were atopic by this definition. Among the atopic asthmatics, eight had allergic rhinoconjunctivitis, and two of these patients also suffered from atopic dermatitis.

Fiber optic bronchoscopy and bronchoalveolar lavage

Bronchoalveolar lavage, using warmed sterile saline (4 $\times$ 60 ml), was performed at fiber optic bronchoscopy, as described previously (15, 20). The volume of fluid recovered by aspiration was noted. BALF was passed through sterile gauze to remove any mucus. Aliquots of supernatants were stored at -80°C until analyzed. Total cell counts were measured using a hemocytometer, and cytopins were prepared for differential cell counting following H&E staining. The absolute numbers of eosinophils, neutrophils, lymphocytes, and monocytes/macrophages were quantified and expressed as 10^6 per milliliter.

Analysis of cytokines

BALF samples were concentrated using Amicon Ultra-15 filters (Millipore, Hertfordshire, U.K.), as previously described (21). A Meso Scale Discovery system (Meso Scale Diagnostics, Rockville, MD) (21) was used to measure concentrations of IL-33 (base catalog number K151WFK for human assays; dynamic range 0.59–10,300 pg/ml) and Th1/Th2 cytokines (base catalog number K15011A for IFN- γ , IL-2, IL-12 p70, IL-13, IL-4, and IL-5; dynamic range 0.08–10,000 pg/ml). TSLP and IL-25 concentrations in BALF were determined using in-house ELISA platforms developed by Novartis, with lower limits of detection of 1 and 2 pg/ml (8, 15).

Statistical analysis

Data were analyzed with the aid of a commercially available statistical package (Minitab for Windows Release 9.2; Minitab, Coventry, U.K.). Between-group ANOVA (Kruskal–Wallis) was followed by the Mann–Whitney *U* test with the Bonferroni correction. Correlation coefficients were obtained by the Spearman rank-order method with correction for tied values. For all tests, $p < 0.05$ was considered significant.

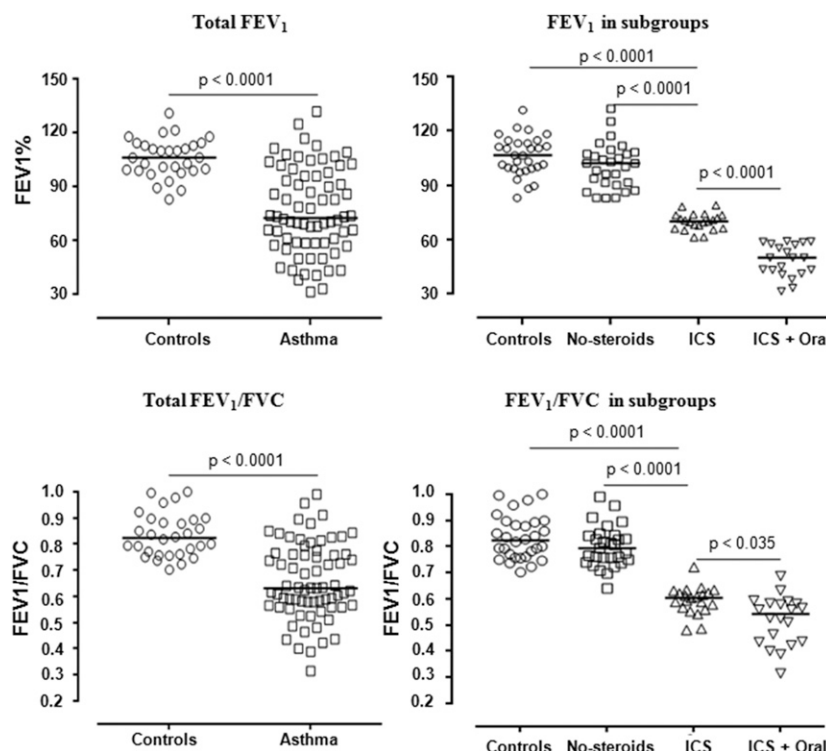


FIGURE 1. Lung function (FEV₁% and FEV₁/FVC) in the entire group of asthmatic patients ($n = 70$) and control subjects ($n = 30$) (left panels). FEV₁% and FEV₁/FVC in subgroups of asthmatics, including patients not treated with corticosteroids (No steroids, $n = 29$), those treated with ICS alone (ICS, $n = 21$), and those treated with oral corticosteroid in addition to ICS (ICS + Oral, $n = 20$) (right panels). The Mann–Whitney *U* test was used to determine statistical significance.

Results

Clinical data

The median prebronchodilator FEV₁ (percent predicted) and FEV₁/forced vital capacity (FVC) ratio of asthmatic patients (FEV₁: median 81.29%, range 31.54–131.00%; FEV₁/FVC: median 0.63%, range 0.314–0.98%) were significantly lower than those of controls (FEV₁: median 106.35%, range 80.0–133.2%; FEV₁/FVC: median 0.82%, range 0.702–0.99%) ($p < 0.0001$) (Fig. 1, Table I). Of the asthmatics, 29 were treated with bronchodilators only (no steroids), 21 of 70 were treated with regular ICS (beclometasone equivalent 400–2000 µg/d), and the remainder (20 of 70) were treated with regular inhaled and oral corticosteroids (prednisolone, 35–50 mg/d) (ICS + Oral). Patients in the latter two groups were also treated with bronchodilators; in addition, some were treated with montelukast and/or oral theophylline preparations. The median prebronchodilator FEV₁ and FEV₁/FVC ratios of the asthmatic patients in these three groups showed a progressive significant decline (Fig. 1, $p < 0.0001$). Furthermore, the median postbronchodilator FEV₁ of the asthma patients in the most severe group did not exceed 70% of the predicted value following bronchodilator, consistent with the presence of irreversible airway obstruction in at least a proportion of these patients.

Concentrations of Th2-promoting cytokines TSLP, IL-25, and IL-33 in BALF

The median concentration of IL-33 was significantly elevated in BALF from asthmatic patients compared with controls ($p = 0.0002$) (Fig. 2). Furthermore, it was significantly elevated in asthmatic patients treated with ICS, with or without additional oral corticosteroids, compared with those not so treated ($p = 0.0342$ and $p = 0.0012$, respectively), whereas it was slightly, but significantly, elevated in asthmatics not treated with steroids compared with control subjects ($p = 0.043$). A similar situation pertained to TSLP. The median concentration of TSLP was significantly elevated in BALF from the entire population of asthmatic patients compared with controls ($p = 0.009$) (Fig. 2), and it was significantly elevated in BALF of patients treated with ICS or without oral corticosteroid compared with those not so treated ($p = 0.0428$ and $p < 0.0001$, respectively). In addition, the median BALF concentration of TSLP was significantly elevated in asthmatics not treated with steroids compared with control subjects ($p = 0.0378$).

In contrast to IL-33 and TSLP, there were no significant differences in the median concentrations of IL-25 in BALF of the entire group of asthmatic patients compared with controls or among the subgroups of asthmatic patients (Fig. 2). Indeed, the concentrations of IL-25 in BALF of the majority of asthmatics and controls were below the limit of detection of the high-sensitivity assay used.

Concentrations of Th2- and Th1-type cytokines in BALF

As anticipated, a predominant Th2-type, but not Th1-type, profile of cytokine expression was evident in BALF of asthmatic patients compared with controls. The median concentrations of the Th2 cytokines IL-4, IL-5, and IL-13 were significantly greater in BALF from the entire population of asthmatic patients compared with controls ($p = 0.0007$, $p = 0.013$, and $p = 0.0003$, respectively) (Fig. 3). In addition, they were significantly elevated in ICS-treated asthmatic patients compared with asthmatics not treated with steroids ($p = 0.0188$, $p = 0.0034$, and $p = 0.0196$, respectively), although there were no significant differences in the median concentrations of these mediators in BALF of asthmatics treated with ICS only and those treated with ICS plus additional oral corticosteroids.

Table I. Clinical data on asthmatics and controls

Subjects	Gender (n; F:M)	Age (y)	Serum IgE (IU/ml)	FEV ₁ % (Pre)	FEV ₁ % (Post)	FEV ₁ /FVC (Pre)	SGRQ Score	Exacerbations (n; Past 12 mo)
Controls (n = 30)	11:19	26 (19–68)	21.4 (7.4–121.3)	106.35 (80.0–133.2)	110.6 (80.0–131.2)	0.82 (0.70–0.99)	0 (0.0–22.01)	N/A
Asthma (total, n = 70)	27:43	42 (21–73)	79.1 (24.1–246.2)*	81.29 (31.54–131.0)*	93.42 (44.94–135.7)***	0.63 (0.314–0.98)*	43.96 (0.0–92.13)	3 (0–8)
No steroids (n = 29) ^a	17:12	27 (21–57)	64.5 (29.1–187.3)*	102.0 (82.6–131.0)	106.52 (87.75–133.6)***	0.79 (0.64–0.98)	26.40 (0.0–59.65)	0
ICS (n = 21) ^b	8:13	46 (22–68)*	110.4 (33.6–211.3)*	70.0 (61.3–78.9)*	91.37 (79.28–102.74)***	0.60 (0.48–0.72)*	39.44 (27.5–71.29)*	3 (2–4)
ICS + Oral (n = 20) ^c	2:18	56 (31–73)***	135.9 (36.3–246.2)***	50.0 (31.54–59.1)***	70.17 (49.94–122.5)***	0.54 (0.31–0.68)***	64.05 (46.66–92.13)***	6 (5–8)

Data are expressed as median (range).

^aSABA and/or LABA only.

^bSABA/LABA (as before) with ICS (beclometasone dipropionate or fluticasone propionate, 400–1000 µg/d).

^cICS and SABA/LABA (as before) with oral corticosteroid (prednisolone, 35–50 mg/d).

* $p < 0.0001$ versus control or no steroid, ** $p < 0.01$ versus ICS, *** $p < 0.05$ versus pre FEV₁%, Mann–Whitney U test.

F, female; FEV₁% (post), postbronchodilator FEV₁ (% predicted); FEV₁% (pre), prebronchodilator FEV₁ (% predicted); M, male; N/A, not applicable; SGRQ: St. George's Respiratory Questionnaire (total score).

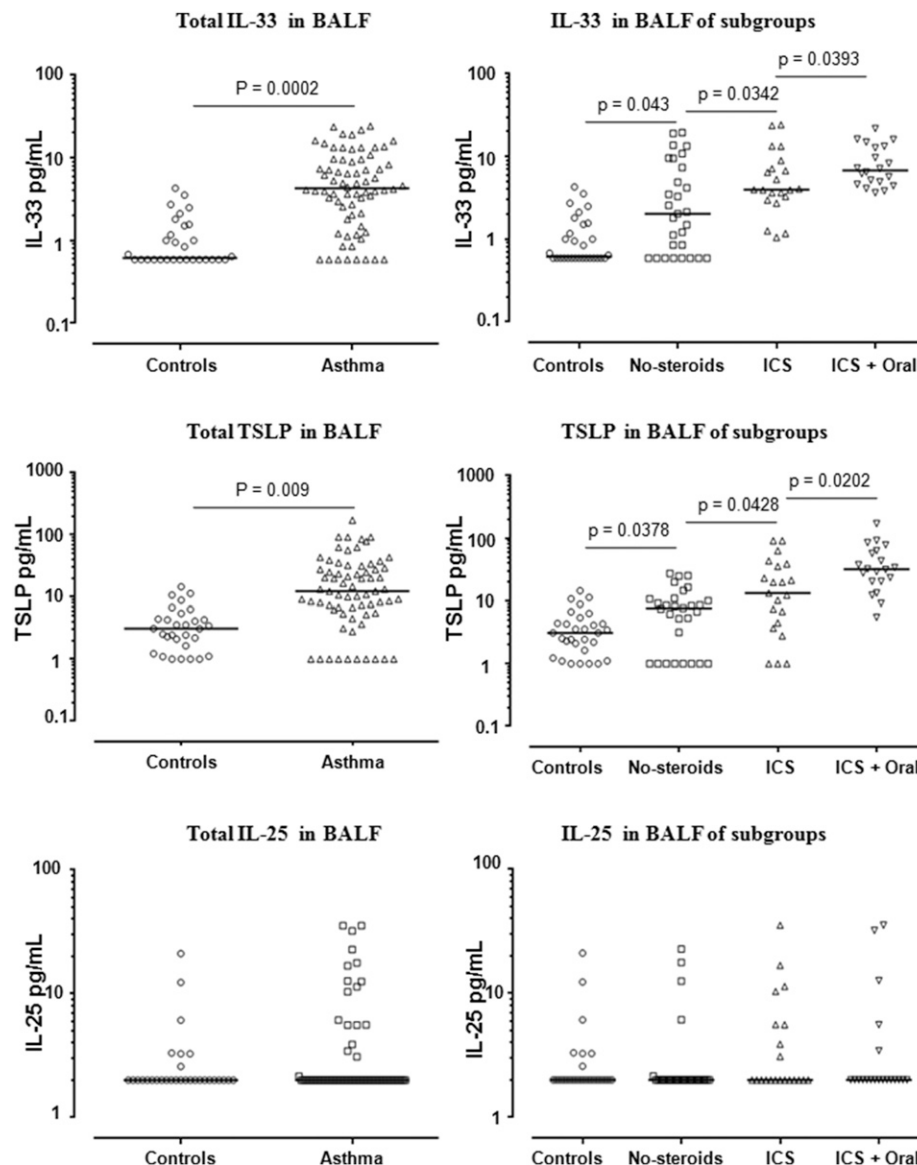


FIGURE 2. Concentrations of the Th2-promoting cytokines IL-33, TSLP, and IL-25 in BALF in the entire group of asthmatic patients ($n = 70$) and control subjects ($n = 30$) (left panels). Concentrations of the Th2-promoting cytokines IL-33, TSLP, and IL-25 in the subgroups of asthmatics, including patients not treated with corticosteroid (No steroids, $n = 29$), those treated with ICS alone (ICS, $n = 21$), and those treated with oral corticosteroid in addition to ICS (ICS + Oral, $n = 20$). The Mann–Whitney U test was used to determine statistical significance.

Furthermore, the median concentrations of IL-4 and IL-13, but not IL-5, in asthmatics not treated with steroids were significantly higher than in control subjects ($p = 0.029$ and $p = 0.0096$, respectively) (Fig. 3).

In contrast to these Th2-type cytokines, there were no significant differences in the median BALF concentrations of the Th1-cytokines IFN- γ and IL-2 when comparing all asthmatic patients and controls or the subgroups of asthmatics based on their antiasthma therapy, although the median concentration of IL-12p70 was slightly, but significantly, elevated in the entire group of asthmatic patients compared with controls (Fig. 4, $p = 0.0359$).

Relationships between concentrations of bronchial luminal Th2-promoting cytokines and Th2 cytokines and prebronchodilator FEV₁

BALF concentrations of IL-33 and TSLP, but not IL-25, in the entire group of asthmatic patients and the subgroups treated with ICS, with or without additional oral corticosteroids, correlated inversely with lung function (FEV₁%) (all asthmatics: IL-33 $r = -0.488$, $p < 0.0001$; TSLP $r = -0.565$, $p < 0.0001$; ICS: IL-33 $r = -0.744$, $p < 0.0001$; TSLP $r = -0.532$, $p = 0.013$; ICS + Oral: IL-33 $r = -0.742$, $p < 0.0001$; TSLP $r = -0.459$, $p = 0.042$) (Fig. 5).

With regard to the Th2- and Th1-type cytokines measured in BALF, IL-13 was the only cytokine whose concentration correlated inversely with FEV₁% ($r = -0.272$, $p = 0.023$) in the entire group of asthmatics but not the individual subgroups (data not shown).

Inflammatory cellular infiltration into BALF

Elevated median numbers of eosinophils and neutrophils were observed in BALF of the entire group of asthmatic patients compared with control subjects ($p < 0.0001$ in each case) (Fig. 6, Table II). Patients treated with ICS or ICS plus oral corticosteroids had higher median numbers of neutrophils compared with patients not so treated ($p \leq 0.0002$) (Fig. 6, Table II).

In individual asthmatic patients, the numbers of neutrophils, but not eosinophils, correlated inversely with lung function (FEV₁%) ($r = -0.556$, $p < 0.0001$) (Fig. 6).

Relationships between concentrations of bronchial luminal Th2 and Th2-promoting cytokines and inflammatory cellular infiltration

In asthmatic patients considered as a single group, BALF concentrations of IL-33 correlated significantly with those of TSLP ($r = 0.425$, $p < 0.0001$) (Fig. 7) but not IL-25 (data not shown). In addition, BALF concentrations of IL-33, but not TSLP, correlated

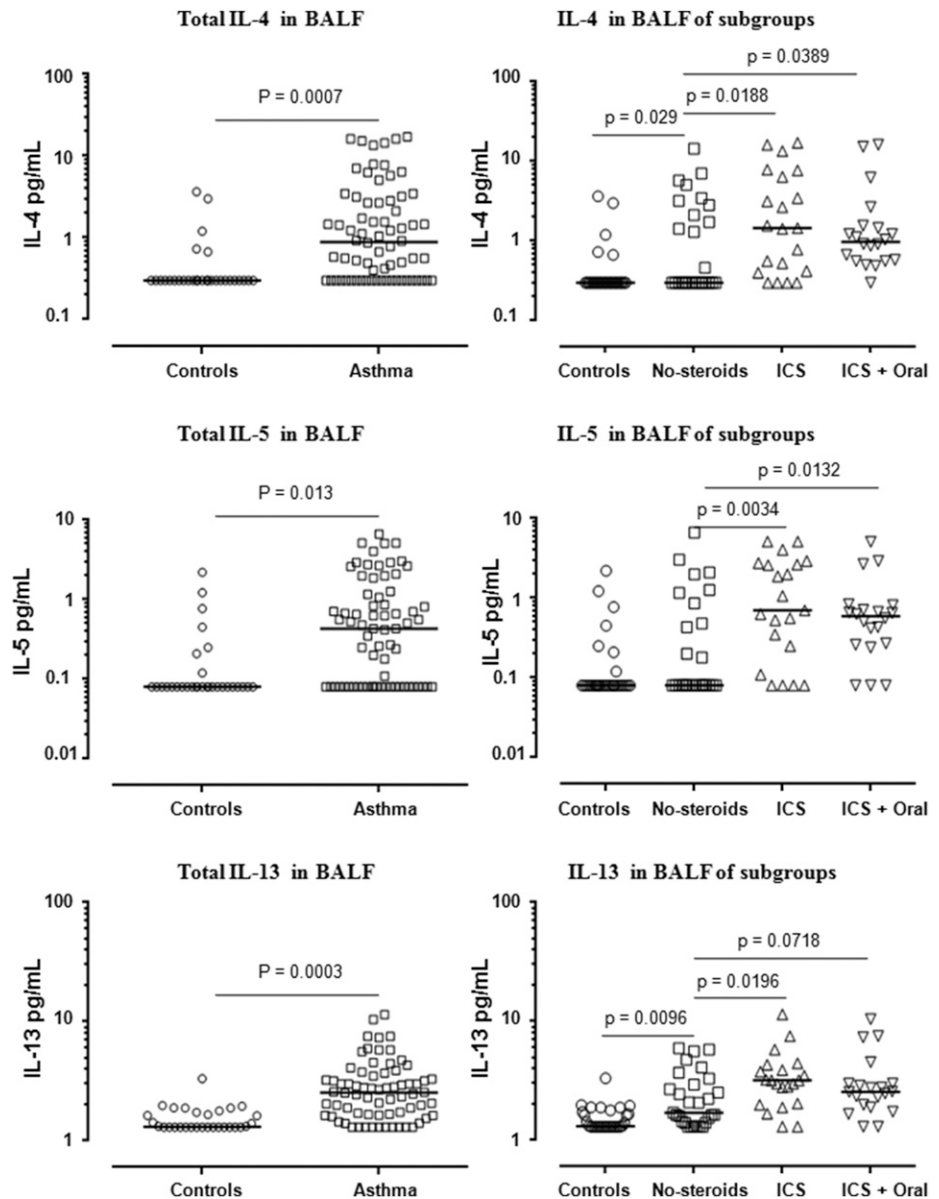


FIGURE 3. Concentrations of the Th2-type cytokines IL-4, IL-5, and IL-13 in BALF in the entire group of asthmatic patients ($n = 70$) and control subjects ($n = 30$) (left panels). Concentrations of the Th2-type cytokines IL-4, IL-5, and IL-13 in BALF from subgroups of asthmatics, including patients not treated with corticosteroid (No steroids, $n = 29$), those treated with ICS alone (ICS, $n = 21$), and those treated with oral corticosteroid in addition to ICS (ICS + Oral, $n = 20$). The Mann–Whitney U test was used to determine statistical significance.

positively with those of IL-13 ($r = 0.426$, $p < 0.0001$), but not IL-4 and IL-5, even though the concentrations of IL-4, IL-5, and IL-13 correlated positively and significantly with each other (IL-4 and IL-5: $r = +0.741$, $p < 0.0001$; IL-4 and IL-13: $r = +0.351$, $p = 0.003$; IL-5 and IL-13: $r = +0.336$, $p = 0.004$) (data not shown).

Surprisingly, among the cytokines measured, the concentration of TSLP alone correlated positively with the number of neutrophils ($r = +0.429$, $p < 0.0001$) (Fig. 7).

Discussion

Although theoretical, potential roles for the epithelial-derived cytokines IL-25, IL-33, and TSLP in regulating airway airflow and initiating and supporting Th2-type inflammation in asthma in vivo have been suggested by a number of existing studies in animal models and humans; however, we are not aware of any systematic study of their expression in parallel in the airways of human asthmatic patients in “real life.” In this study, we used BALF as a window to evaluate the concentrations of these cytokines in a relatively large cohort of human patients with asthma of wide-ranging severity and therapy, as well as in nondiseased controls. A proportion of our most severe asthmatics retained

significant airway obstruction, at least as shown by FEV₁, despite near-maximal conventional antiasthma therapy. Our data are clearly compatible with the hypothesis that IL-33 and TSLP, but not IL-25, play key roles in regulating airway airflow and, thereby, disease severity in asthma. In contrast, this was less apparent with the more “downstream” Th2-type cytokines IL-4, IL-5, and IL-13, of which only IL-13 expression correlated significantly with lung function. Although these findings may, to some extent, reflect the limitations of quantifying mediators satisfactorily in BALF, it is also compelling to speculate that they reflect additional effects of IL-33 and TSLP (other than their propensity to promote local Th2-type cytokine synthesis) on airway remodeling (collagen laydown and neoangiogenesis) and, consequently, the irreversible reduction in airway caliber and, therefore, airflow, as demonstrated by ourselves (10, 11) and other investigators (9, 12–14) in animal asthma models. Speculating that such changes may take some time to develop in the airways and mindful of studies suggesting variability in airway mucosal inflammation in asthmatic patients with increasing age, although it was not directly cogent to our hypotheses, we did look in retrospect for an influence of age on BALF cytokine concentrations in our asthmatic patients stratified

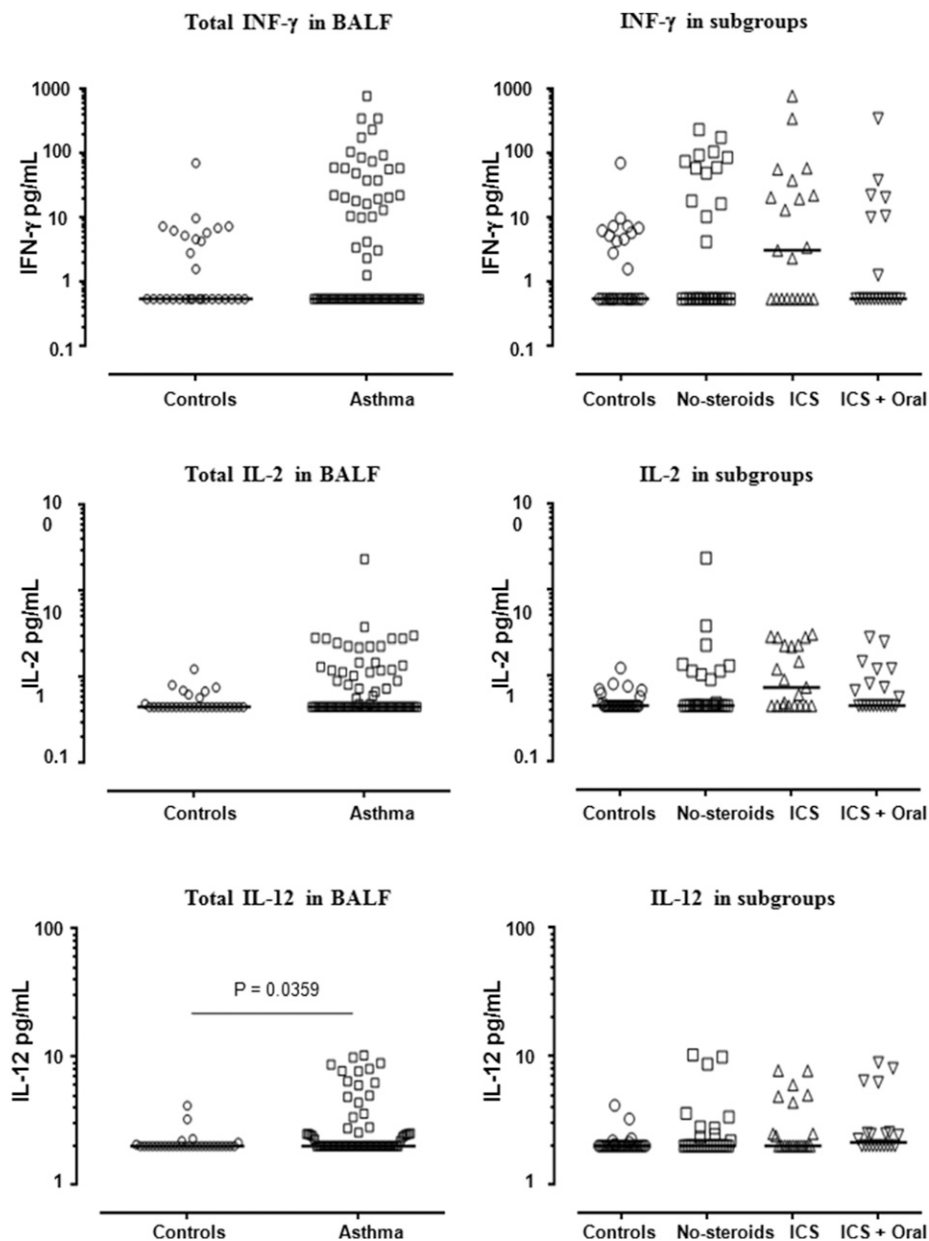


FIGURE 4. Concentrations of the Th1-type cytokines IFN- γ , IL-2, and IL-12p70 in BALF in the entire group of asthmatic patients ($n = 70$) and control subjects ($n = 30$) (left panels). Concentrations of the Th1-type cytokines IFN- γ , IL-2, and IL-12p70 in BALF in subgroups of asthmatics, including patients not treated with corticosteroids (No steroids, $n = 29$), those treated with ICS alone (ICS, $n = 21$), and those treated with oral corticosteroid in addition to ICS (ICS + Oral, $n = 20$). The Mann-Whitney U test was used to determine statistical significance.

by severity but found none. Similarly, we could not uncover any influence of patient gender (data not shown).

It is also noteworthy that the airway concentrations of IL-33 and TSLP were more prominently elevated in patients with more severe disease in the face of escalating antiasthma therapy, including systemic corticosteroids. This observation is compatible with the hypothesis that the production of IL-33 and TSLP is relatively insensitive to inhibition by corticosteroids in human asthma *in vivo* compared with that of the downstream Th2-type cytokines IL-4, IL-5, and IL-13.

Our data augment accruing evidence implicating TSLP in the pathogenesis of asthma, likely as a result of binding to its receptor expressed on the surface of adaptive immune cells (DC, T cells, and B cells) and innate immune cells (mast cells, eosinophils, basophils, and airway smooth muscle cells) (22). In addition to epithelial cells, TSLP may be expressed by other airway structural cells, including fibroblasts, smooth muscle cells, and endothelial cells, as well as by immune cells, including mast cells, monocytes/macrophages, granulocytes, and DCs (22). Mice expressing a TSLP transgene in lung epithelial cells developed spontaneous

airway inflammation with the cardinal features of human asthma, including airway hypersensitivity (23). Blockade of the TSLP receptor with a mAb reduced BALF total cells, eosinophils, and lymphocytes, IL-4 and IL-5 concentrations, and the expression of several surface markers on local pulmonary DCs in an OVA-induced murine asthma model (24). Our own previous studies have demonstrated that the numbers of cells expressing TSLP mRNA in the bronchial mucosa of asthmatics correlate inversely with lung function (FEV₁) (25); similarly, TSLP protein expression was significantly elevated in the airway epithelium, lamina propria, and induced sputum of asthmatic patients to a degree that correlated with the severity of airflow obstruction (26, 27). Liu et al. (18) also recently reported that concentrations of TSLP in BALF from patients with severe asthma were significantly elevated compared with controls and implicated TSLP in inducing steroid refractoriness in ILC2s through a MEK and STAT5-mediated pathway. In a double-blind clinical trial, Gauvreau et al. (28) found that pretreatment with AMG 157, a human anti-TSLP mAb reduced allergen-induced bronchoconstriction and indices of airway inflammation following allergen challenge. In a very recent

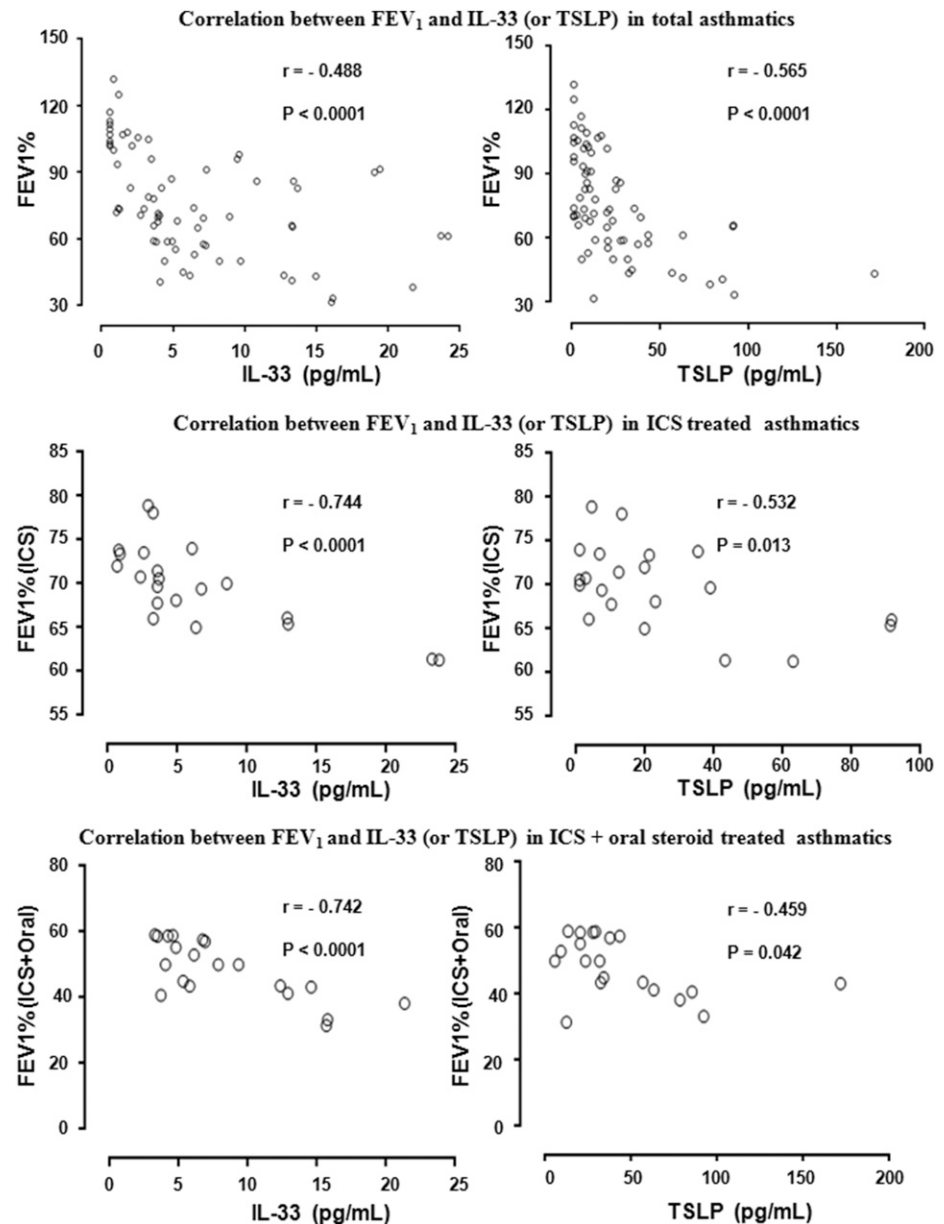


FIGURE 5. Correlations of the concentrations of IL-33 and TSLP in BALF with lung function (FEV₁% predicted) in the entire group of asthmatics and subgroups of patients treated with ICS alone (ICS, $n = 21$) and those treated with oral corticosteroid in addition to ICS (ICS + Oral, $n = 20$). Correlation coefficients were obtained by the Spearman rank-order method with correction for tied values.

study, Corren et al. (29) showed that “uncontrolled asthmatics” treated with this same Ab had lower rates of clinically significant asthma exacerbations than did placebo-treated controls, an effect that was independent of baseline blood eosinophil counts.

IL-33, a member of the IL-1 cytokine family, also promotes Th2-type cytokine production in vitro and acts on a wide range of cells expressing its receptor ST2, including Th2 T cells, mast cells, eosinophils, basophils, and ILC2s (30). Functionally, IL-33 is considered an epithelial environmental alarmin. Elevated expression of IL-33 and its receptor has been reported in the bronchial mucosa of patients with severe asthma (31, 32) and, congruent with our own findings, it has been implicated in therapy resistance and airway remodeling in pediatric asthmatic patients with severe corticosteroid-resistant disease (33), as well as in the pathogenesis of allergen-induced bronchial inflammation, airway hyperresponsiveness, and local IL-17A, IL-25, and TSLP expression in an animal model of atopic asthma (10). The detection of human IL-33 ex vivo is at a relatively developmental stage. One recent study (34) has highlighted the possibility that there is variability in the outcomes of a range of commercial assays, particularly when

IL-33 is measured in human serum, owing to the presence of various forms of the IL-33 molecule (pro- and mature IL-33), the influence of binding to its soluble receptor ST2 and other plasma-binding proteins, and evidence of cleavage products generated by the activities of proteases, such as cathepsins or elastase. More research is required to clarify and hopefully obviate this uncertainty if IL-33 measurement in serum ex vivo is to become an integrated feature of asthma management.

In contrast to the data implicating TSLP and IL-33, the evidence that IL-25 plays a key role in the regulation of asthma severity and therapy resistance is more ambiguous. We (16) showed that IL-25-immunoreactive cells are elevated in the bronchial mucosa of asthmatics to a degree that correlates negatively with FEV₁, whereas we (10) and other investigators (35) have directly implicated IL-25 in causing airway inflammation in animal models. In contrast, we were unable to demonstrate an elevated median BALF concentration of IL-25 in asthmatics in the current study compared with controls. This is congruent with a recent study by Bleck et al. (36), which used induced sputum epithelial cells and DCs as a “window” on airway inflammation and showed elevated

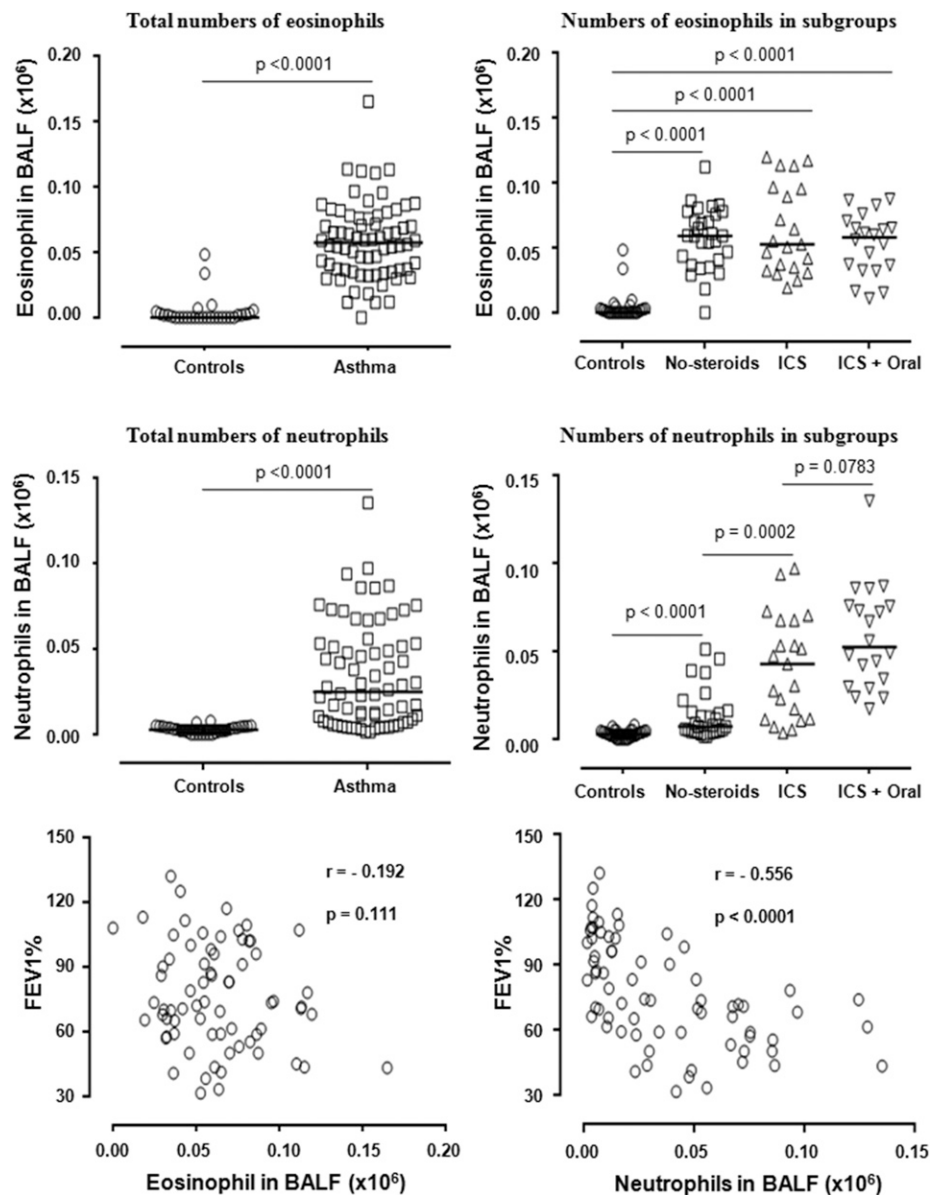


FIGURE 6. Numbers of eosinophils and neutrophils ($\times 10^6$) in BALF from the entire group of asthmatic patients ($n = 70$) and control subjects ($n = 30$) (top left and middle left panels) and in subgroups of asthmatics (top right and middle right panels), including patients not treated with corticosteroid (No steroids, $n = 29$), those treated with ICS alone (ICS, $n = 21$), and those treated with oral corticosteroid in addition to ICS (ICS + Oral, $n = 20$). The Mann-Whitney U test was used to determine statistical significance. Correlations between the numbers of eosinophils and neutrophils and lung function (FEV₁%) in the entire group of asthmatics (bottom panels). Correlation coefficients were obtained using the Spearman rank-order method with correction for tied values.

expression of TSLP and IL-33, but not IL-25, in a group of human asthmatics compared with controls. Another study reported by Glück et al. (37), in which statistically significantly elevated concentrations of IL-33 and TSLP, but not IL-25, were observed in exhaled breath condensate and serum samples from patients with asthma compared with controls, is also congruent with our findings. In contrast, Cheng et al. (38) reported elevated epithelial

IL-25 mRNA expression and mean serum IL-25 protein concentration in a subset of recently diagnosed asthmatics with relatively mild disease characterized by elevated Th2-type cytokine expression, elevated mean total serum IgE concentration, and “high” corticosteroid responsiveness, whereas Seys et al. (39), using induced sputum and cytokine mRNA expression as a window on airway inflammation in 66 adult asthmatics, described an “IL-5,

Table II. Blood eosinophils and cell counts in BALF

	Asthma			Controls ($n = 30$)
	No Steroids ($n = 29$)	ICS Only ($n = 21$)	ICS + Oral Steroids ($n = 20$)	
Blood eosinophils (cells/mm ³)	175 (89–374)*	198 (128–421)*	177 (109–341)*	62 (21–239)
BALF				
Total number ($\times 10^6$ /ml)	1.8 (1.0–5.8)	2.1 (0.9–3.3)	1.9 (0.9–5.8)	1.6 (0.8–3.1)
Neutrophils	0.008 (0.0016–0.16)	0.05 (0.0015–0.13)*	0.05 (0.017–0.14)***	0.015 (0.0–0.07)
Eosinophils	0.04 (0.0–0.112)*	0.05 (0.019–0.12)*	0.06 (0.03–0.16)*	0.0 (0.0–0.2)
Lymphocytes	0.09 (0.03–1.11)	0.13 (0.04–0.25)	0.09 (0.03–0.19)	0.1 (0.03–0.95)
Macrophages	1.64 (0.84–5.46)	1.93 (0.8–2.9)	1.76 (0.76–3.0)	1.44 (0.74–2.84)

Data are median (range).

* $p < 0.01$ versus control or no steroids, ** $p < 0.01$ versus ICS, Mann-Whitney U test.

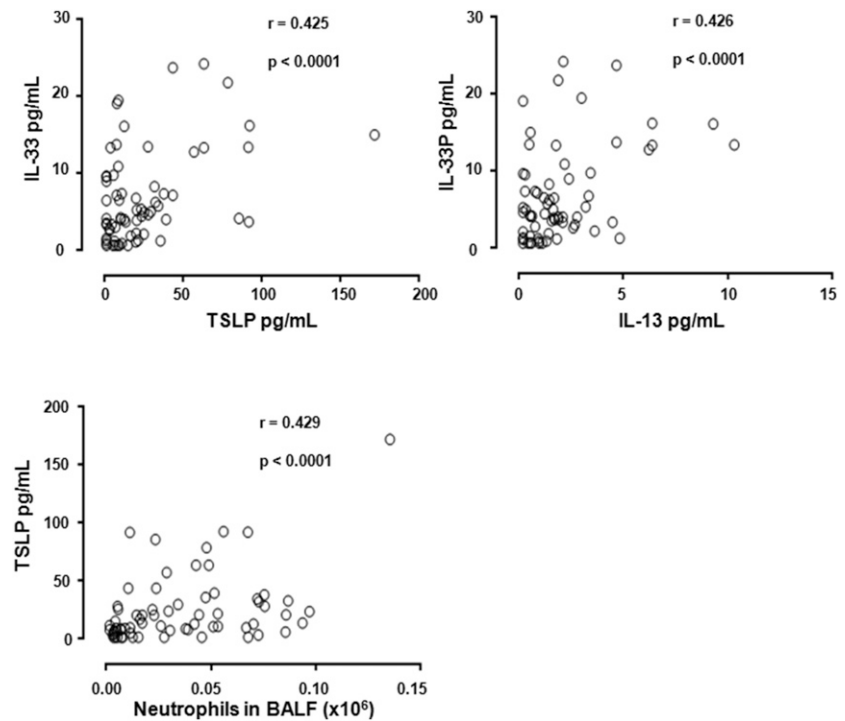


FIGURE 7. Correlations between the concentrations of Th2- and Th2-promoting cytokines and the numbers of neutrophils in BALF from the entire group of asthmatic patients ($n = 70$). Correlation coefficients were obtained using the Spearman rank-order method with correction for tied values.

IL-17A, IL-25 high” pattern of expression associated with more severe (what the investigators termed “uncontrolled”) asthma. Again, these discrepancies may reflect, at least in part, the particular window chosen through which to examine airway cytokine expression, the nature of the cells examined, and the methods of cytokine detection (mRNA, protein, immunoreactivity).

Finally, with regard to the inflammatory cellular counts in BALF from our asthmatic patients, although eosinophil numbers were clearly elevated in the group as a whole, we did not observe a clear correlation with the degree of airway obstruction. We speculate that this reflects the net effect of escalating corticosteroid therapy in inhibiting the production of corticosteroid-sensitive cytokines, such as IL-5, but not corticosteroid-resistant cytokines, such as IL-33, which may regulate tissue eosinophil infiltration. We observed a clearer elevation of neutrophil numbers with increasing exposure to corticosteroid therapy (which mirrored declining airway airflow), which may simply reflect the effects of corticosteroids in promoting blood and tissue neutrophilia. Alternatively, or in addition, neutrophil infiltration may contribute to airway inflammation, remodeling, and obstruction in some patients with asthma who do not have eosinophilic inflammation. It is interesting to note, for example, that, in the aforementioned study by Corren et al. (29), anti-TSLP Ab reduced exacerbations in asthmatic patients without blood eosinophilia, and TSLP expression in BALF correlated most closely with neutrophil infiltration in the present study.

In summary, our data clearly highlight TSLP and IL-33 as potential key biomarkers of established severe therapy-refractory asthma with eosinophilic and neutrophilic airway inflammation, with the corollary that they qualify as key potential therapeutic targets in this scenario. It should be noted that their overexpression does not necessarily reflect corticosteroid resistance of the individual patient but simply the fact that this alarmin axis is not susceptible to corticosteroid inhibition. If predictive biomarkers of IL-33- and TSLP-driven refractory airway inflammation with airway remodeling are to be identified, it will be necessary to characterize the triggers for, and natural history of, overexpression of these cytokines in the airways of individual patients. In this regard, it is interesting that TSLP and IL-33 have recently been

reported to promote the migration of hematopoietic stem cells in patients with atopic asthma (40) and to be involved in corticosteroid-resistant disease in vitro and in vivo in animal models (41).

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Disclosures

The authors have no financial conflicts of interest.

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