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TITLE PAGE

Title: Sputum cytokine mapping reveals an “IL-5, IL-17A, IL-25-high” pattern associated with poorly controlled asthma

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Keywords: airway inflammation, asthma phenotyping, cytokine, induced sputum, asthma control

ABSTRACT

Background and objective: Asthma is a heterogeneous disease with various clinical, inflammatory and molecular phenotypes. We studied sputum cytokine mRNA expression patterns in an unselected group of adult asthma patients in order to characterize the underlying inflammatory process.

Methods: Differential cell counts and cytokine mRNA (quantified by real-time PCR) were analyzed on sputum from 40 controls and 66 asthmatic adults. A “cytokine-high” profile was defined if mRNA levels for that particular cytokine

exceeded the 90th percentile value in the control population. Radar graphs were used to visualize cytokine profiles.

Results: Sputum mRNA analysis confirmed heterogeneity of cytokine patterns among patients. Thirty-six patients (55%) had a Th2 cytokine pattern: “IL-5-high” (n=13), “IL-4-high” (n=17) or “IL-4- and IL-5-high” (n=6). The “IL-5-high” asthma profile (n=13) coincided with the “IL-25-high” (10/13) and surprisingly also with the “IL-17A-high” (11/13) profile. The “IL-5-/IL-25-/IL-17A-high” profile was different from the “IL-4-high” pattern. Patients with the “IL-5, IL-17A, IL-25-high” pattern had significantly worse lung function parameters. Uncontrolled asthmatics (ACT<20) had higher sputum IL-5, IL-17A and IL-25 mRNA levels compared to controlled asthmatics (p=0.002; p=0.002; p=0.066) and uncontrolled asthma is more common among “IL-5- and IL-17A-high” asthmatics compared to “IL-5-, IL-17A-low” asthmatics (Chi²=3.7, p=0.027; relative risk: 1.8, 95% CI= 1.1-3.1).

Conclusions and clinical relevance: Patients with the “IL-5, IL-17A, IL-25-high” airway inflammatory pattern are often uncontrolled asthmatics, despite daily treatment. It seems worthwhile to evaluate whether measuring sputum cytokine levels might be used to assess the response to increased doses of steroids in patients with asthma.

Text

INTRODUCTION

Asthma is characterized by reversible airway obstruction, airway hyperreactivity (AHR) and chronic airway inflammation (1). Despite these common

characteristics, asthma is a heterogeneous disease with different underlying mechanisms and phenotypes. Asthma phenotyping has been performed based on the provocative triggers (allergens, infectious agents, exercise, aspirin and others) (2), on the asthma severity (3), on the characteristics of the underlying inflammation (4,5) or on the genetic expression profiles of bronchial cells (6).

Over the past 20 years, several studies have addressed cytokine and chemokine expression in the asthmatic airways (7-9). Increased gene expression or secretion of the Thelper (Th)2 cytokines interleukin (IL)-4 and IL-5 as well as increased expression of the Th2 associated cytokines IL-9 and IL-13, and of the Th2 signal transduction molecule Stat 6 were firmly established (10-13). The role of Th1 cytokines is a matter of debate. Decreased T-bet expression levels have been reported in asthmatic patients compared to controls (14). Several studies reported increased interferon (IFN)- γ expression in asthma patients, especially in severe asthmatics (11, 12, 15, 16). The functional role in asthma of IL-17A and IL-22, both produced - though not exclusively - by Th17 cells, remains unclear (17-19). We reported increased IL-17A mRNA levels in sputum of individuals with asthma (20), and other groups observed increased Th17 cells in the airways of asthmatics (21). Higher TNF concentrations are found in bronchoalveolar lavage (BAL) fluid and bronchial biopsies from patients with severe asthma compared to mild asthmatics (22,23). Murine and human data recently also revealed a prominent role for epithelial cell cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) in asthma (24,25).

Heterogeneity in cytokine expression might reflect different inflammatory subtypes of asthma. Brasier and coworkers identified three clinically distinct groups of asthma patients based on a decision tree model including BAL expression levels of IL-2, IL-4 and/or IL-5 (26). These expression levels were then linked to the degree of airway hyperreactivity (AHR). Woodruff and coworkers reported two distinct molecular asthma phenotypes based on analysis of three IL-13 inducible genes (periostin, CLCA1, and SERPINB2) in bronchial brushings: a “Th2-high” phenotype with mostly steroid sensitive patients and a “Th2-low” phenotype with a poor response to inhaled steroids (6).

In the present study, sputum cytokine mRNA expression was measured in a study population of unselected asthma patients, recruited at the outpatient clinic. Our aim was to evaluate airway cytokine mRNA expression using a non-invasive, clinically useful tool. Different cytokine mRNA profiles were identified and visualized by radar graphs. The relation between parameters of airway inflammation (sputum differential cell count, F_ENO level), airway obstruction and sputum cytokine patterns was also studied.

METHODS

Subjects

Asthmatic patients were recruited amongst those consecutively attending the outpatient clinic of pulmonary disease or allergology of the university hospital Leuven. Only patients with symptoms compatible with asthma were considered. Then, diagnosis of asthma was confirmed based on previous (<24 months) or

current proof of reversibility of $FEV_1 \geq 12\%$ after inhalation of salbutamol and/or a positive histamine provocation test ($PC_{20} < 8$ mg/ml). Healthy volunteers were recruited amongst students and university coworkers (n=59). Only stable asthmatics between 18 and 65 years were included (n=90). Patients with a respiratory infection one month prior to analysis or exacerbation three months before analysis were excluded. Patients who did not take inhaled steroids for at least 3 months were classified as steroid naive patients.

Lung function, F_{ENO} measurements and asthma control

F_{ENO} was measured prior to spirometry by means of a chemiluminescence analyzer (Niox Flex). Spirometry was performed according to ATS criteria, both before and after inhalation of salbutamol 400 µg. Different dynamic lung volumes were measured: FEV_1 (forced expiratory volume in one second), FVC (forced vital capacity), PEF (peak expiratory flow), $FEF_{25-75\%}$ (mean forced expiratory flow at 25%-75% interval). Asthma control was assessed by Asthma Control Test questionnaire.

Sputum induction and analysis

Sputum induction and processing was performed as described previously (11) (supplementary information). Sputum total and differential cell counts were obtained by cytopsin (Shandon cytocentrifuge). Cytokine mRNA levels (IL-4, IL-5, IL-6, IL-10, IL-17A, IL-22, IL-25, IL-29, IL-33, TSLP, IFN- γ and TNF) were measured by real-time (RT)-PCR. Patients were defined as being “cytokine-high”

if sputum mRNA expression levels of that particular cytokine exceeded the 90th percentile value of the control group.

Sputum supernatant IL-5 protein was measured by Enzyme-Linked ImmunoSorbence Assay (ELISA) according to the manufacturers' protocol (BD biosciences, Erembodegem, Belgium). A two-step ELISA was used to analyse the influence of DTT on a dilution series of IL-5 standard (with a known concentration of IL-5 protein), which was diluted in the buffer that was used to process the sputum (containing DTT; detection limit of 50 pg/ml) or in RPMI medium without DTT (detection limit 20 pg/ml). This study revealed limited interference of DTT with protein detection of IL-5.

Radar plots and statistics

Radar plots were generated by presenting the proportion of patients having a 'cytokine-high' mRNA expression profile as a set of points plotted along a set of axes radiating from a central point, and with the points connected by a line (27).

Statistical analyses were performed with Graphpad Prism V for Macintosh (Graphpad Software Inc., San Diego, USA) by use of Kruskal-Wallis, Dunn's multiple comparison test (as post-test) and Mann-Whitney U test when appropriate. Correlations were analyzed with the Spearman test. T-test was performed if data were normally distributed. Benjamini-Hochberg correction was used as a multiple comparison test. Normality was analyzed prior to between group analysis by Shapiro-Wilk test. A difference was considered to be significant when $p < 0.05$.

RESULTS

Subject characteristics

Subject characteristics are summarized in Table 1. In brief, 90 asthmatic patients and 59 healthy volunteers were recruited amongst student and university coworkers. Sixty-six of ninety patients and 40 out of 59 control subjects had sufficient amounts of sputum cDNA for further analysis. Asthmatic patients (median: 36.5 years) were slightly older compared to healthy individuals (median: 27.0 years; $p=0.02$; Table 1). Allergy was more common among asthmatics (69%) compared to control subjects (30%). Five patients reported to be a current smoker and nine were former smokers. All of the control subjects were non-smokers. Fourteen patients had neutrophilic asthma (24%), 13 had eosinophilic asthma (22%), 7 (12%) had mixed granulocytic asthma and the majority of patients ($n=24$; 41%) had paucigranulocytic asthma (definition: see supporting information).

Sputum cytokine mRNA expression in asthmatics

The median sputum cytokine expression levels in control subjects ($n=40$) and asthmatics ($n=66$) are listed in Table 2. mRNA levels in the asthmatic population were significantly higher for IL-4, IL-10, IL-17A and IL-25. As shown in Table 2, a “cytokine-high” profile (mRNA levels above the 90th percentile of the control population) was observed in more than 15% of patients for: IL-4 ($n=29$), IL-10 ($n=20$), IL-17A ($n=15$), IL-5 ($n=13$) and IFN- γ ($n=12$).

Four epithelial cytokines were included in our analysis (IL-25, TSLP, IL-33 and IL-29). Ten patients were identified as “IL-25-high”. Sputum TSLP mRNA levels tended to be higher in asthmatics compared to control subjects ($p=0.068$), but the difference was not statistically significant, and only one patient was identified as “TSLP-high”. Sputum IL-33 mRNA levels were not statistically different between asthmatic and control subjects ($p=0.17$) and only 3 patients were “IL-33-high”. Nine patients had an “IL-29-high” profile.

Among innate associated cytokines, we analyzed IL-6 and TNF. Sputum IL-6 mRNA levels were not statistically different between asthmatic and healthy individuals ($p=0.44$), but we identified a limited number of patients ($n=9$) with an “IL-6-high” profile. Surprisingly the total group of asthmatics had significantly lower TNF levels when compared to control subjects ($p=0.004$), but still 6 asthmatic patients were “TNF-high”.

Upon adjustment for multiple comparison, the increase in sputum IL-17A and IL-25 mRNA levels in asthmatics did no longer reach statistical significance (adjusted p -values; Table 2). This data hints that these cytokines are simultaneously expressed and are linked with each other, fitting with our radar plots.

Sputum Th2 cytokine mRNA expression in asthmatics

On the basis of either an “IL-4-high” or “IL-5-high” profile (or both), 36 patients could be classified as having a Th2 profile. Among the patients with low IL-4 as well as low IL-5 mRNA levels (n=30), 17 patients could be defined as “cytokine-high” for one or two of the following cytokines: “IL-6-high” (n=6), “IL-10-high” (n=7), “IL-17A-high” (n=3), “IL-22-high” (n=2), “IL-33-high” (n=2), “TNF-high” (n=4) and “IFN- γ -high” (n=2). Overlap of “cytokine-high” patterns among these 17 patients was limited (n=8). In 13 patients, we found no exceeding of the cut-off value for any of the cytokines that were measured (“cytokine-low” patients).

Sputum cytokine mRNA expression in relation to current treatment

Patients recruited in this study were allowed to continue their regular treatment. In order to assess the degree and type of airway inflammation present in treated patients, we studied cytokine mRNA radar plots in patients with different treatment regimens: steroid-naïve patients (n=25), patients on inhaled steroids (ICS) only (n=30) and patients on inhaled steroids (ICS) combined with oral steroids (oral CS) (n=11). Characteristics of these patient groups were shown in Table E1. Steroid-naïve patients have a cytokine radar plot characterized by a significant proportion of “IL-4-high” asthmatics (40%), “IL-6-high” asthmatics (37.5%) and/or “IL-22-high” asthmatics (37.5%) (Figure 1A). The proportion of patients with an “IL-17A-high” profile was low in steroid naïve patients (4%) but higher (27% and 55% respectively) in ICS treated patients and patients treated with ICS and oral steroids (Figure 1A-C). Many of the ICS treated patients were “IL-4-high” (40%) and/or “IL-10-high” (40%). Despite treatment with oral

steroids, more than 60% of these patients still were “IL-4-high” whereas an “IFN- γ -high” profile and an “IL-5-high” profile was detected in 27% and 36% of the patients respectively (Figure 1C). Absolute cytokine mRNA values were significantly higher in steroid-treated patients compared to steroid-naïve patients for: IL-5, IL-17A, IL-25, IL-29 and IFN- γ (Figure E1 in supporting information). Interestingly, sputum IL-22 mRNA levels were significantly higher in steroid-naïve patients compared to steroid-treated patients (Figure E1 in supporting information).

Association between individual sputum cytokine mRNA expression levels

Most patients had elevated mRNA levels for several cytokines combined. We used cytokine radar plots in order to reveal associations between the “cytokine-high” expression patterns (Figure E2). Only, a minority of “IL-5-high” patients (n=6) also was “IL-4-high”, and the majority of “IL-4-high” patients (17 out of 23) had an “IL-5-low” profile. “IL-5-high”, “IL-17A-high” and “IL-25-high” patterns were found to be largely overlapping (Table 3 and Figure E2 D, E and F), indicating that IL-5, IL-25 and IL-17A are often co-expressed. Sputum “IL-25-high” expression was restricted to patients with an “IL-5-high” profile (Table 3). Moreover, a nearly 1:1 correlation between sputum IL-5 and sputum IL-17A mRNA levels was found (Spearman $r=0.91$; $p<0.0001$). IL-5 protein levels in sputum supernatant correlated with IL-5 mRNA levels in patients with “IL-5-high” profile (Spearman $r=0.34$; $p=0.03$). Supernatant IL-5 protein levels were below detection limit in all healthy individuals (data not shown). In “IL-4-high” patients, “IFN- γ -high” patients and “IL-10-high” patients, the cytokine pattern

was different from each other, and also different from the “IL-5-, IL-25- and IL-17A-high” cytokine pattern (Table 3; Figure E2 A-F).

Clinical characteristics of patients associated with different cytokine-high patterns

We also assessed whether there was a link between the sputum cytokine patterns and the clinical status of the patient. Patients with the “IL-5-high, IL-17A-high and/or IL-25-high” pattern had significantly worse FEV1 % predicted (IL-5-high: mean= 77.6%, IL-17A-high: mean= 76.3%, IL-25-high: 80.4%) compared to patients in their respectively “cytokine-low” pattern (IL-5-low: mean= 97.5%, IL-17A-low: mean= 98.9%, IL-25-low: 96.9%; $p=0.001$, $p=0.0001$, $p=0.01$; Figure 2A). For sputum IL-5 mRNA levels, patients with FEV1 % predicted <80% were clearly separated from patients with FEV1 % predicted >80% ($p=0.018$; Figure E3A). The same pattern was found for sputum IL-17A mRNA levels ($p=0.024$; Figure E3B). No differences were seen in sputum IL-4 ($p=0.44$), IFN- γ ($p=0.25$) and IL-10 ($p=0.21$) mRNA levels *were found for the different groups of patients stratified by FEV1 % predicted* (Figure E3C-E). Furthermore, peak flow and FEF25-75% were significantly lower in patients with the “IL-5-high” pattern (data not shown). Along the same line, patients with IL-5 protein levels above 100 pg/ml had significantly lower FEV1 %pred. (mean= 88.7%) compared to patients with IL-5 levels below 100 pg/ml (mean= 102.0%, $p=0.019$; Figure 2B). The proportion of patients with FEV1% <90% is higher in patients with IL-5 >100 pg/ml compared to these with IL-5 <100 pg/ml ($\chi^2= 2.9$, $p=0.04$; relative risk= 2.4, 95% CI= 0.8-7.4). On the other hand, patients with an “IL-5-high” mRNA pattern had similar FeNO levels ($p=0.49$)

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and/or sputum eosinophil percentages ($p=0.39$) than “IL-5-low” patients. Patients with an “IL-4-high” pattern, on the contrary, had significantly higher FeNO levels ($p=0.02$) and sputum eosinophil percentages ($p=0.0005$) than “IL-4-low” patients. FEV1% pred. tended to be lower in this patient group compared to “IL-4-low” patients ($p<0.08$; data not shown). Sputum IL-4, IL-10 and IL-5 mRNA levels did not differ between allergic or non-allergic asthmatics ($p=0.13$; $p=0.13$ and $p=0.15$; data not shown).

Uncontrolled asthmatics (Asthma Control Test <20) had significantly higher sputum IL-5 and IL-17A mRNA levels and tended to have higher IL-25 mRNA levels ($p=0.002$, $p=0.002$, $p=0.066$; Figure 2C) than controlled asthmatics. Along the same line, uncontrolled asthma is more common among “IL-5- and IL-17A-high” asthmatics compared to “IL-5-, IL-17A-low” asthmatics ($\text{Chi}^2=3.7$, $p=0.027$; relative risk: 1.8, 95% CI= 1.1-3.1). Sputum IL-4, IL-10 or IFN- γ mRNA levels did not differ between uncontrolled and controlled asthmatics ($p=0.12$, $p=0.11$ and $p=0.089$; data not shown).

DISCUSSION

We studied the expression of a panel of twelve cytokines at the mRNA level in sputum of asthmatics and controls, in order to identify distinct cytokine patterns in a heterogeneous group of stable asthmatic patients. Patients were allowed to continue their regular treatment. A cytokine-high pattern for a particular cytokine was defined based on the 90th percentile value in control subjects.

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Radar plots of cytokine expression revealed that most patients with an “IL-5-high” pattern also have an “IL-25-high” and an “IL-17A-high” pattern. The “IL-25-high” expression is even restricted to those patients with an “IL-5-high” pattern, suggesting a close relation between both cytokines. Wang and coworkers demonstrated that IL-25 produced by epithelial cells, enhances the cytokine production by Th2 memory cells independently from IL-4 (24). The capacity of epithelial cell derived IL-25 to induce Th2 cytokine production in the airways, might be restricted to IL-5 only, and IL-5 production by airway Th2 cells might depend on costimulation by IL-25. Alternatively, other cell types, such as natural helper cells, might also be involved in IL-5 production in an IL-25 dependent way (28). The contribution of these cells and other recently identified innate cell types (e.g. nuocytes, multi-potent progenitor type 2 cells and innate type 2 helper cells) (29), in human asthma and particularly in the airways of asthmatics requires further study. We found a linear correlation between sputum IL-17A and IL-5 mRNA levels. The co-expression of IL-17 and IL-5 is unexpected but consistent with our earlier observations (20) and could potentially be explained by the findings of Wakashin and coworkers who showed that the IL-23/IL-17A axis enhances Th2 cytokine production in mice (30). Moreover, a novel subset of blood CD4⁺ Th2 cells that produces both IL-17A and IL-5 was identified (31). Our data support the existence of a similar cell type in the human airways.

In contrast to what was expected, only a small number of patients (n=6) were both “IL-4-high and IL-5-high”, whereas 17 patients had an “IL-4-high” pattern without high IL-5 expression. We however have to stress that in most sputum

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samples from patients with an “IL-5-low” profile, IL-5 mRNA was detectable but amounts were comparable to the amounts observed in control subjects. The correlation between sputum IL-5 mRNA and protein levels was not high (0.36), though significant. Several factors may explain this finding. It is likely that protein detection by ELISA is less sensitive (detection limit of the assay: 50 pg/ml) than mRNA detection by RT-PCR. In our hands, interference of DTT with IL-5 protein was limited. Other factors such as posttranslational modification may interfere with the transcription of IL-5 protein. The observation that IL-4 and IL-5 patterns are poorly correlated suggests production by different cell types, and, is in accordance with our earlier reports (32). Recently, two subpopulations of human Th2 cells were identified in blood based on their differentiation status: IL-5⁺ (highly differentiated) and IL-5⁻ (less differentiated) Th2 cells (33). Alternatively, Th2 cells might have different functional states depending on the stimulus. Furthermore, four Th2 subpopulations were defined in blood of asthmatics based on expression of L-selection (CD62L) and CXCR3 (34). All of these subpopulations produce IL-4 and IL-13, whereas only the population with CD62L^{lo} and CXCR3^{lo} expression produced IL-5. Whether these Th2 cell populations are indeed present in asthmatic airways requires further study. More recently, message for IL-4 and IL-13 but not IL-5 was detected in a type 2 myeloid population (IL-17RB⁺CD11b⁺GR-1^{mid}) and IL-5 but not IL-4 or IL-13 transcripts were found in the CD11b^{neg} fraction in lungs of mice upon intratracheal instillation with IL-25 (35). These results strengthen the hypothesis that two separate populations of cells were responsible for the early production of IL-4 and IL-13 on the one hand, and IL-5 on the other hand.

Achieving asthma control is fundamental in the management of asthma as highlighted in GINA guidelines (3). We evaluated cytokine profiles in controlled and uncontrolled asthmatics. Patients with IL-5 protein levels above 100 pg/ml had a 1.8 times greater chance to have uncontrolled asthma. Moreover, patients with an “IL-5-high” cytokine profile had worse lung function parameters compared to those with an “IL-5-low” pattern. We are aware of the limitations of this observational study. These data indeed only show a link between sputum cytokine measurements and the clinical status of the patient, without giving any proof of causality. Before using cytokine levels in a clinical setting, these cytokine values need to be assessed on a longitudinal basis to check whether gain or loss of asthma control is associated by relevant changes in sputum cytokine values. Despite lack of control in patients with high IL-5 levels (mostly steroid-treated patients), these patients had equally high percentages of sputum eosinophils as patients with low IL-5 levels. This might be explained by the fact that 13 “IL-5-low” patients were steroid-naïve, in contrast to only 1 “IL-5-high” patient. On the other hand, patients with high IL-4 levels (both steroid-treated and steroid-naïve patients) had higher sputum eosinophil levels than “IL-4-low” patients. Repeated sputum IL-5 measurements might therefore potentially be more useful in asthma guidance than repeated sputum eosinophil measurements.

Atopy did not influence the sputum Th2 related cytokine mRNA levels in asthmatics nor in healthy individuals. When only allergic control subjects were considered to define the sputum IL-4 or IL-5 cut-off value, no marked changes were seen (data not shown).

A sputum “IL-4-high” profile was seen in 40% of the asthma patients irrespective of their treatment with inhaled steroids and was the most prominent cytokine pattern in patients treated with both inhaled and oral steroids. In addition, the “IL-5-high, IL-17A-high and IL-25-high” pattern was most prevalent in the latter and almost absent in steroid naive asthmatics. We hypothesize that sputum cytokines may be more steroid resistant compared to sputum eosinophil counts and FeNO levels. It seems worthwhile to evaluate whether measuring sputum cytokine levels might be used to assess the response to increased doses of steroids in patients with asthma.

A study with anti-IL-5 mAb in refractory ‘eosinophilic’ asthma patients showed reduced asthma exacerbations and improved asthma quality of life scores (37), but not in an unselected population of mild asthmatics (38). However, the beneficial spectrum of anti-IL-5 therapy may be larger than eosinophilic asthma. Seventy percent of the “IL-5-high” patients in our study indeed did not have eosinophils exceeding 1.9%. Patients identified with an “IL-5-high” profile – under adequate steroid treatment and thereby having lowered sputum eosinophil percentages – may also benefit from anti-IL-5 mAb. The same might be true for anti-IL-17 (-receptor), anti-IL4R α or anti-TNF treatments in the respective groups of patients with elevated levels of these cytokines in their sputum.

Several validation steps now need to be performed to implement this non-invasive technique as a tool to assess the asthma profile in a single patient.

Enlarging the cohort of asthma patients and of the control group is essential for an unsupervised analysis of the sputum cytokine profiles. Several factors that may influence these profiles (e.g. treatment, sex, age) need to be identified to a larger extent. A longitudinal study is required to evaluate how these factors may affect the sputum cytokine profile. In addition, it remains to be elucidated how sputum cytokine profiling may help to predict response to biologicals in asthmatics.

Conflict of interest statement

All authors declare that no conflict of interest exists.

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Figure legends

Figure 1: Radar plots representing the proportion of patients (%) with increased sputum mRNA of a particular cytokine in subgroups of asthmatics stratified by medication use. *A: steroid naive asthmatics. B: asthmatic patients using inhaled corticosteroids only (ICS only). C: asthmatic patients using ICS in combination with oral corticosteroids (ICS and oral CS). Radar plots were generated by presenting the proportion of “cytokine-high” patients (IL-4, IL-5, IL-6, IL-10, IL-17A, IL-22, IL-25, IL-29, IL-33, IFN- γ , TNF and TSLP) as a set of points plotted along a set of axes radiating from a central point, and with the points connected by a line to visualize cytokine-high patients.*

Figure 2: Clinical characteristics (A: asthma control, B: lung function) in relation to sputum IL-5, IL-17A and IL-25 mRNA and IL-5 protein levels (C).

Cytokine mRNA was measured by RT-PCR and adjusted to β -actin. Sputum IL-5 protein levels were measured by ELISA. Patients were defined as uncontrolled asthmatics if Asthma Control Test (ACT) score was below 20/25 (39). Data were represented as median values with interquartile range. Differences between both groups were analyzed by Mann Whitney test.

TABLES

TABLE 1. SUBJECT CHARACTERISTICS

	Control	Asthma	p-value
Subjects (n=)	40	66	
Male/female	16/24	33/33	
Age (years)	27.0	36.5	0.01
Allergy	11 on 37 (30%)	37 on 54 (69%)	0.02
Smoking (C/E/N)	0/0/40	5/9/52	
FEV1, % pred. (mean $\pm$ SD)	109.4 $\pm$ 11.2	92.9 $\pm$ 20.1	<0.0001
Sputum eosinophils (%)	0.0- 0.0 -0.4	0.0- 0.7 -2.5	0.009
Sputum neutrophils (%)	17.8- 26.6 -42.5	20.3- 54.0 -74.5	0.003
Sputum macrophages (%)	45.4- 64.8 -76.4	16.5- 35.0 -66.7	0.001
Sputum lymphocytes (%)	1.4- 3.6 -5.2	0.8- 1.5 -3.7	0.002
Inhaled steroids (n=)	0	30	
Low dose		18	
Intermediate dose		7	
High dose		2	
Inhaled steroids and oral steroids (n=)	0	11	
Steroid naive (n=)	40	25	

Characteristics of controls and asthmatic patients with sufficient sputum RNA are shown. Smoking (C: current smoker, E: ex-smoker, N: never

smoker). Inhaled corticosteroid dose was unknown in 3 patients. Allergy status was assessed by skin prick test or immunocap test (timothee grass, reye grass, birch, house dust mite, cat, dog and Alternaria alternata) (not performed in 12 patients and 3 of the control subjects). Sputum cell percentages are expressed as medians with interquartile range. Number of patients with low, intermediate or high dose of inhaled steroids were calculated based on equivalent dose table (GINA report 2011).

TABLE 2. SPUTUM CYTOKINE mRNA VALUES ^a

	Control	Asthma	p-value ^b	Adjusted p-value ^c	"Cytokine-high" patients ^d
IFN- γ	0.4- 2.3 -7.2	0.4- 2.3 -15.8	0.31	0.31	12 (18%)
IL-10	1.0- 8.5 -25.0	2.3- 14.1 -50.4	0.007	0.007	20 (30%)
IL-4	0.0- 0.0 -0.0	0.0- 0.0 -44.8	0.0001	0.0001	29 (44%)
IL-5	12.9- 122 -1441	15.4- 282.5 - 2122	0.07	0.07	13 (20%)
IL-17A	5.3- 42.2 -457.9	3.7- 86.6 -772.7	0.03	0.03	15 (23%)
IL-22	0.0- 7.4 -75.7	0.0- 5.3 -58.8	0.25	0.25	4 (6.0%)
IL-25	2.9- 17.5 -345.7	2.9- 59.9 -563.3	0.03	0.03	10 (15%)
IL-33	0.0- 0.08 -1.0	0.0- 0.1 -1.0	0.17	0.17	3 (4.5%)
TSLP	0.1- 0.3 -1.2	0.0- 0.2 -0.8	0.068	0.068	1 (1.5%)
IL-29	3.9- 50.7 -1066	2.7- 248.1 - 2029	0.058	0.058	9 (14%)
IL-6	1.0- 14.6 -48.1	0.2- 12.7 -98.6	0.44	0.44	9 (14%)
TNF	110- 6413 - 51049	81.1- 1055 - 74485	0.004	0.004	6 (9.0%)

^a: mRNA was quantified by RT-PCR and normalized to β -actin. Representation of median and 10th-90th percentile values of control and asthmatic subjects.

^b: Comparison between control and asthma population.

^c: p-value after correction for multiple comparison (Benjamini-Hochberg correction).

^d: Patients were defined as having a “cytokine-high” profile if sputum mRNA expression levels of a particular cytokine exceed the 90th percentile value achieved in controls.

TABLE 3. ASSOCIATION OF INDIVIDUAL SPUTUM IL-4, IL-5, IL-10, IL17A, IL-25 and IFN- γ mRNA EXPRESSION

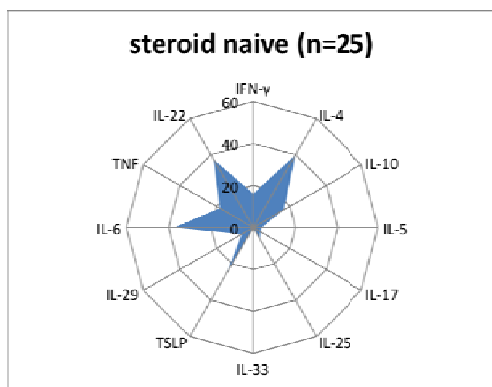
	“IL-4-high” (n=29)	“IL-5-high” (n=13)	“IL-10-high” (n=20)	IL-17A-high (n=15)	“IL-25-high” (n=10)	“IFN- γ -high” (n=12)
“IL-4-high”	-	6 (46.2%)	9 (36%)	6 (40.0%)	6 (60.0%)	8 (75%)
“IL-5-high”	6 (20.7%)	-	10 (40%)	11 (73.3%)	10 (100%)	6 (50%)
“IL-10-high”	9 (31%)	10 (77%)	-	9 (60%)	9 (90%)	6 (50%)
“IL-17A-high”	6 (20.7%)	11 (84.6%)	9 (36%)	-	9 (60%)	7 (58.3%)
“IL-25-high”	6 (20.7%)	10 (77.0%)	9 (36%)	9 (60%)	-	7 (58.3%)
“IFN- γ -high”	8 (27.6%)	6 (46.2%)	6 (30%)	7 (46.7%)	7 (70%)	-

Patients were defined to have a “cytokine-high” profile if sputum mRNA expression levels of a particular cytokine exceeds the 90th percentile value observed in controls. The absolute and relative numbers of “cytokine-high” patients were shown for each IL-4-, IL-5-, IL-10-, IL-17A-, IL-25-, and IFN- γ -high expression pattern.

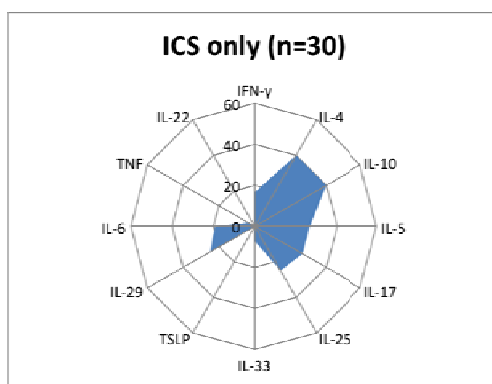
FIGURES

Figure 1

A.



B.



C.

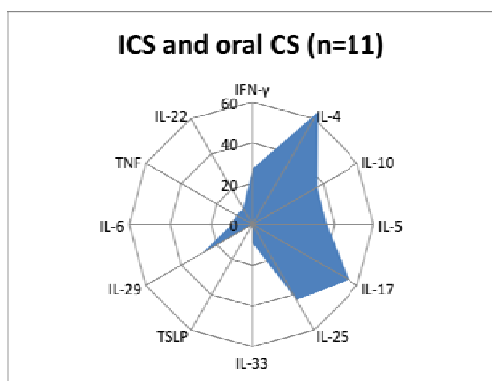
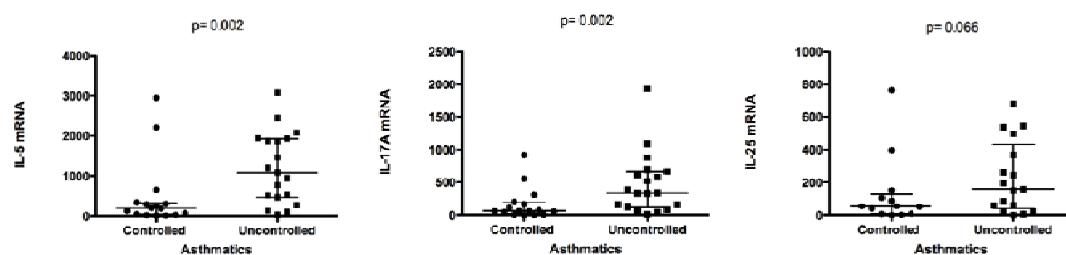
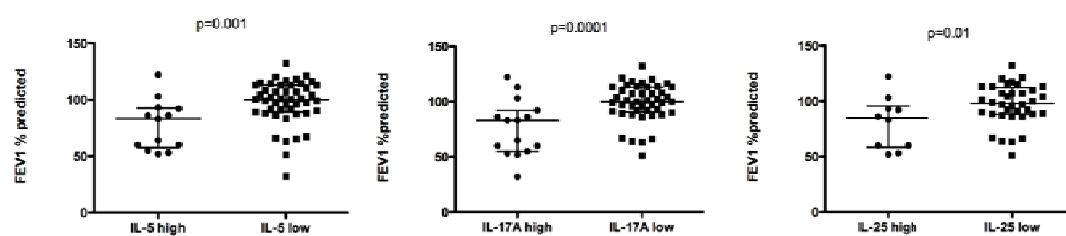


Figure 2

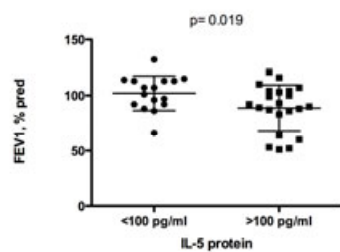
A.



B.



C.



Supporting information

Title: Sputum cytokine mapping reveals a pattern associated with poorly controlled asthma

Authors: Sven F. Seys^{1,2}, Marcin Grabowski^{3,6}, Wim Adriaensen⁴, Ann Decraene¹, Ellen Dilissen³, Jeroen A. Vanoirbeek⁵, Lieven J. Dupont¹, Jan L. Ceuppens³, Dominique M.A. Bullens²

Supplementary methods

Subject characteristics

Between September 2008 and June 2010, 90 asthmatic patients who gave informed consent were recruited at the outpatient clinic of the University Hospital Leuven. Of those, 66 asthmatics (72%) had sufficient amounts of sputum cDNA for further analysis. Similarly, 70% (n=40) of recruited healthy individuals (n=59) (students and university coworkers) had sufficient amounts of sputum cDNA. Healthy controls had normal spirometry, presented no clinical symptoms of upper or lower airway disease nor did they use any anti-asthmatic medication at present or in the past 5 years (Table 1). However, one control subject reported rhinoconjunctival symptoms and another reported atopic dermatitis. The study was approved by the local ethical committee of the Faculty of Medicine, Leuven.

Sputum induction and processing

Sputum induction and processing of the selected plugs was previously described (1, 2). Cytospins were air-dried and stained using Diff-Quik (Medion Diagnostics, Dürdingen, Switzerland). In each sample, 250 leukocytes were counted and the percentage of each specific cell type was determined. Cells were stored on lysis buffer from the Qiagen Mini Rneasy kit (Maryland, USA) at -80°C until use.

Primer and probe sequences

The primers and probes for IL-4, IL-5, IL-10, IL-17A, IL-29 and IFN- γ have been reported (1-3). All primers and probes were purchased from Applied Biosystems or Eurogentec S.A. The primer and probe sequence for β -actin, IL-22, IL-25, IL-33, TSLP and TNF were designed with Primer Express (Applied Biosystems):

β-actin FW 5' gga cat ccg caa aga cct gt 3'

β-actin RV 5' ctc agg agg agc aat gat ctt gat 3'

β-actin TP 5' ctg gcg gca cca cca tgt acc ct 3'

IL-22 FW 5' ttc atg ctg gct aag gag gc 3'

IL-22 RV 5' gca gcg ctc act cat act gac t 3'

IL-22 TP 5' agc ttg gct gat aac aac aca gac gtt cgt 3'

IL-25 FW 5' gga gat atg agt tgg aca gag act tg 3'

IL-25 RV 5' ctg tct gta ggc tga cgc agt g 3'

IL-25 TP 5' acc tgt acc acg ccc gtt gcc tg 3'

IL-33 FW 5' atg aat cag gtg acg gtg ttg a 3'

IL-33 RV 5' gcc tgg tct ggc agt ggt t 3'

IL-33 TP 5' cca aca aca agg aac act ctg tgg agc tc 3'

TSLP FW 5' agc tat ctg gtg ccc agg ct 3'

TSLP RV 5' gcc aca atc ctt gta att gtg aca 3'

TSLP TP 5' ttc gga aac tca gat aaa tgc tac tca ggc aat g 3'

TNF FW 5' tct tct cga acc ccg agt ga 3'

TNF RV 5' cct ctg atg gca cca cca g 3'

TNF TP 5' tag ccc atg ttg tag caa acc ctc aag ct 3'

IL-6 FW 5' cac cgg gaa cga aag aga ag 3'

IL-6 RV 5' ccc agg gag aag gca act g 3'

IL-6 TP 5' cct tct cca caa gcg cct tcg gt 3'

Lung function and $F_{E}NO$ measurement

The FEV1 was expressed as percentage of predicted (FEV1 % pred.) and reported in Table 1. FEV1 was measured at the day of sputum induction before bronchodilation with salbutamol. The Fraction exhaled Nitric Oxide ($F_{E}NO$) at a flow rate of 50 ml/sec was measured with a NIOX Flex chemiluminiscence analyser (Nosegem, Belgium).

Differential cell counts

'Eosinophilic' and 'neutrophilic' asthma were defined by an eosinophil count exceeding 1.9% and neutrophil count exceeding 65.3% of leukocytes on cytopsin respectively (4). Asthmatics with both eosinophils and neutrophils exceeding these percentages were defined as having a 'mixed inflammatory' phenotype. Patients with both eosinophils and neutrophils below these percentages were defined as having 'pauci-inflammatory' asthma.

References of supplementary methods

1. Truyen E, Coteur L, Dilissen E, Overbergh L, Dupont LJ, Ceuppens JL, e.a. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax*. 2006;61(3):202–8.
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4. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax*. 2002;57(10):875–9.

Supplementary results

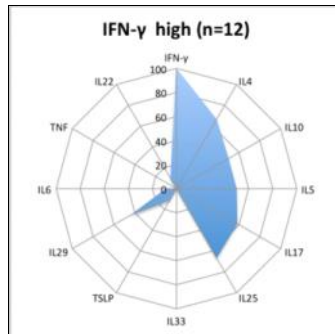
Sputum cytokine mRNA expression in relation to established markers of airway inflammation

We studied the cytokine mRNA expression pattern in the four classical inflammatory phenotypes as defined on the basis of the percentage of neutrophils and eosinophils in sputum (Methods section). The proportion of “IL-4-high” asthmatics is higher among patients with eosinophilic inflammation (54%) than among those with neutrophilic inflammation (36%) (Figure E4A, B) and is highest in patients with a mixed granulocytic phenotype (Figure E4C). To our surprise, only 23% of eosinophilic asthmatics could be identified as “IL-5-high” (n=3/13; Figure E4A). Among the patients with neutrophilic inflammation, 50% had elevated sputum IL-17A mRNA levels (Figure E4B). Only a small proportion of patients with paucigranulocytic asthma had elevated sputum cytokine levels (Figure E4D). As expected, patients with eosinophilic asthma had significantly higher FeNO levels (median: 34.3 ppb) than patients with neutrophilic asthma ($p<0.05$; Table E1). FEV1 (% pred.) was significantly different amongst the four groups ($p=0.038$) with the lowest FEV1 in the patients with a mixed granulocytic phenotype (median: 66.5%; Table E1). Significantly higher levels of sputum neutrophils were detected in patients taking ICS ($p<0.01$) and patients taking ICS and oral steroids ($p<0.05$) compared to steroid-naïve patients (Table E2). Sputum eosinophil percentages did not differ between the three groups of patients ($p=0.74$; Table E2).

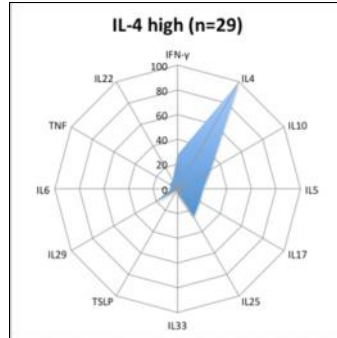
Supplementary figures

Figure E1

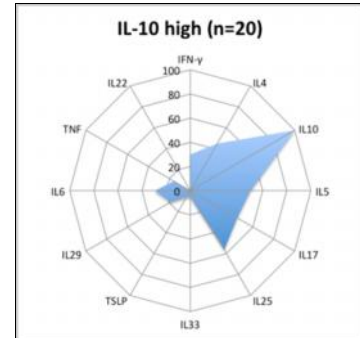
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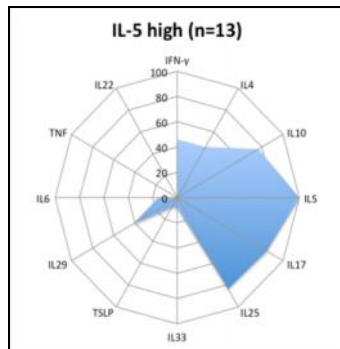
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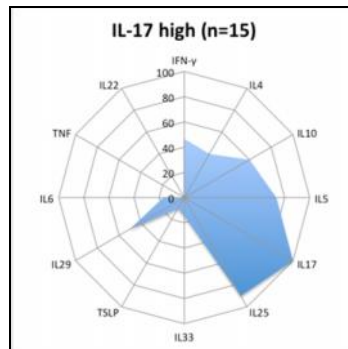
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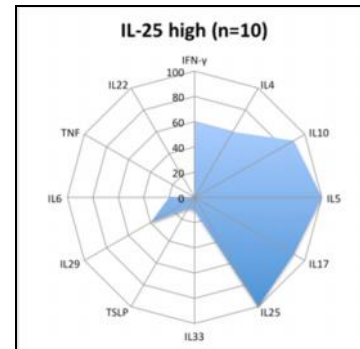
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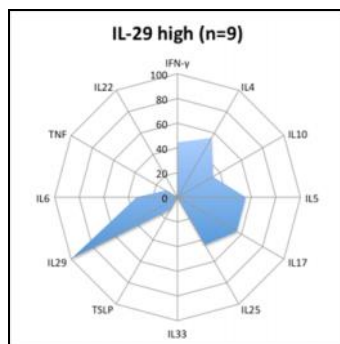
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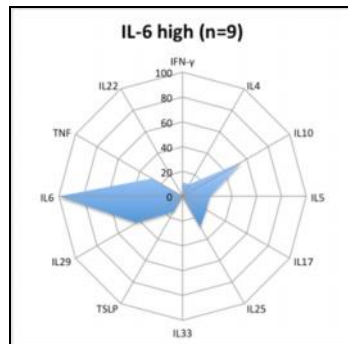
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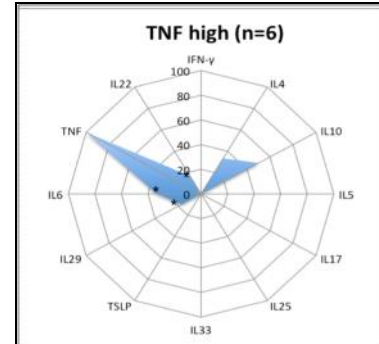
G.



H.



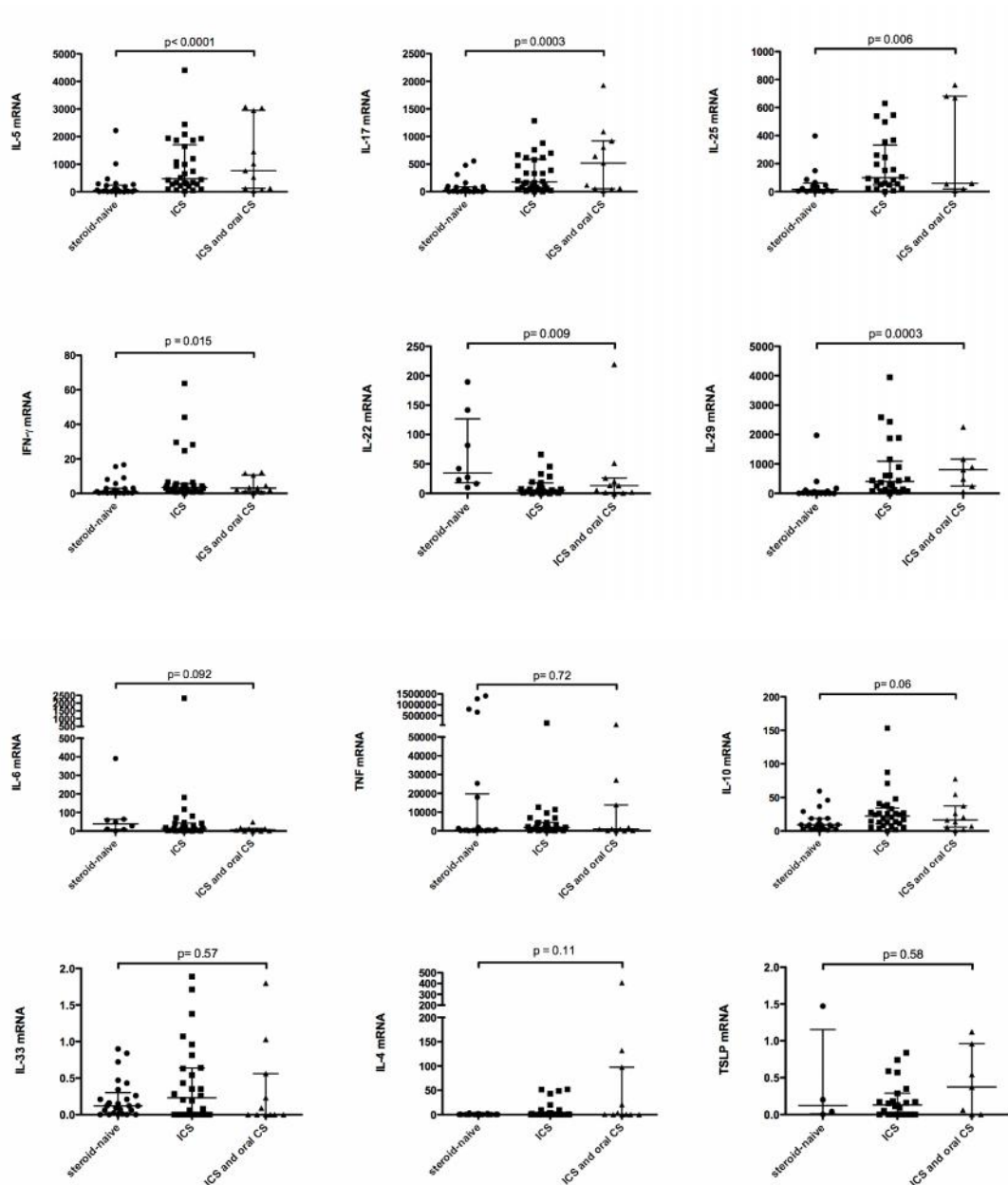
I.



Radar plots of cytokine-high patients. Radar plots were generated by presenting the proportion of “cytokine-high” patients (IL-4, IL-5, IL-6, IL-10, IL-17A, IL-22, IL-25, IL-29, IL-33, IFN- γ , TNF and TSLP) as a set of points plotted along a set of axes radiating from a central point, and with the points connected by a line to visualize

cytokine-high patients. *(radar plot TNF high): Missing values for IL-6 (4), IL-22 (2) and IL-29 (5).

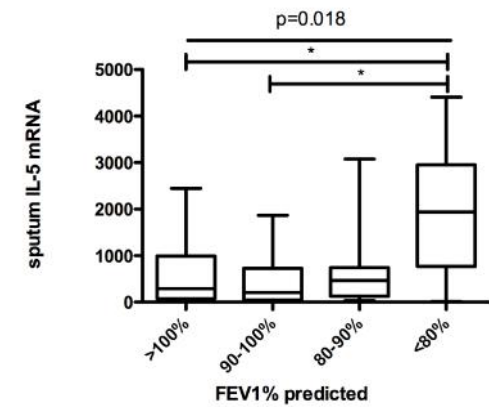
Figure E2



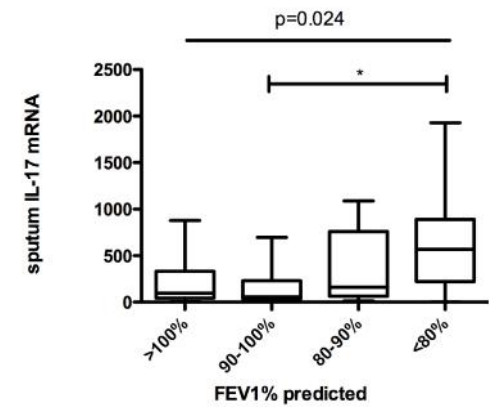
Sputum cytokine mRNA levels in three groups of asthmatic patients according to their medication use. Cytokine mRNA was measured by RT-PCR and adjusted to β -actin. Cut-off values for each cytokine (representing the 90th percentile value in control subjects) were visualized by a dotted line.

Figure E3

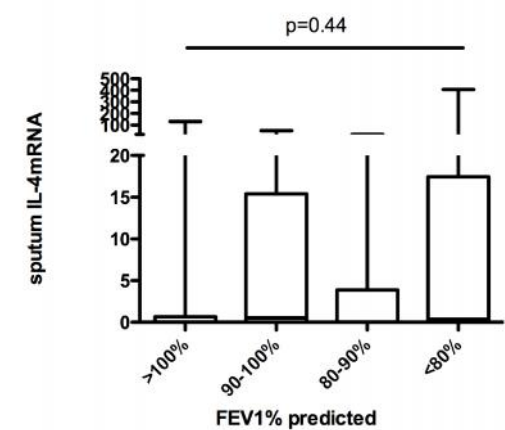
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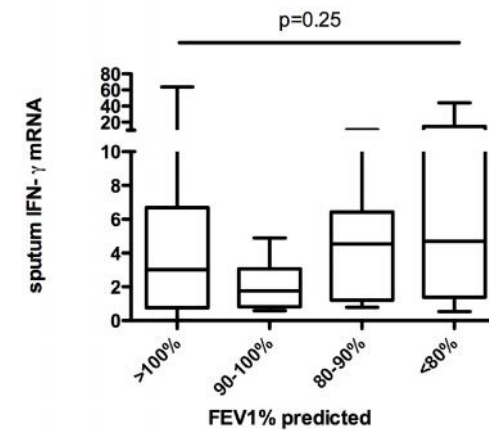
B.



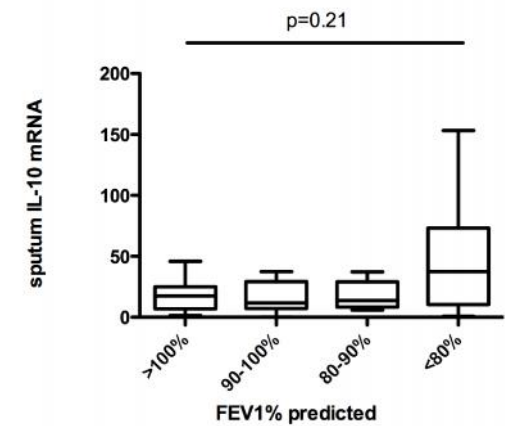
C.



D.



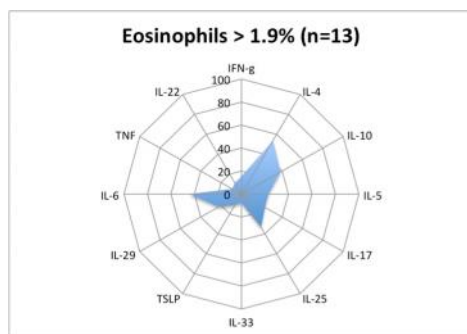
E.



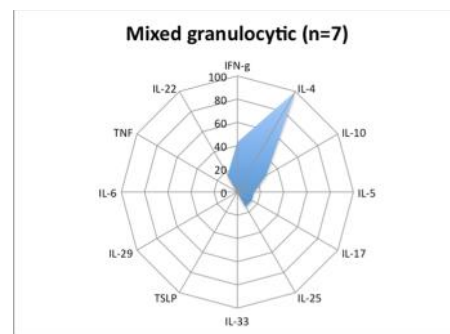
Sputum cytokine mRNA levels in four groups of asthmatic patients according to their lung function. Cytokine mRNA was measured by RT-PCR and adjusted to β -actin.

Figure E4

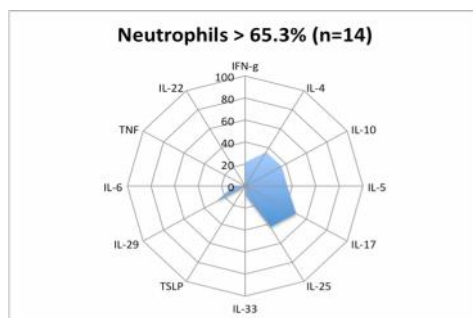
A.



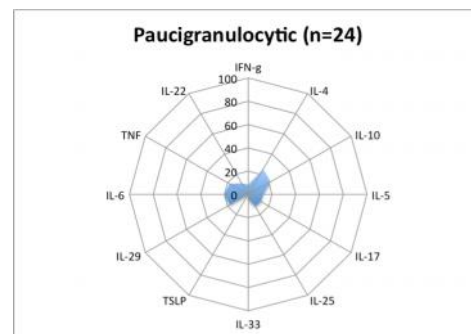
C.



B.



D.



Radar plots representing the proportion of patients with increased sputum mRNA of a particular cytokine in four inflammatory phenotypes. The four inflammatory phenotypes were identified based on the sputum eosinophil ($\leq 1.9\%$) and neutrophil ($\leq 65.3\%$) percentages (Method section). Radar plots were generated by plotting the proportion (as percentages) of “cytokine-high patients”, as defined in the method section, as a

set of points plotted along a set of axes each representing a particular cytokine (IL-4, IL-5, IL-6, IL-10, IL-17A, IL-22, IL-25, IL-29, IL-33, IFN- γ , TNF and TSLP), radiating from a central point. The values on each axis were connected to visualize the cytokine pattern.

Supplementary table

TABLE E1. CHARACTERISTICS OF PATIENTS WITH DIFFERENT SPUTUM INFLAMMATORY PHENOTYPES

	Eosinophilic (n=13)	Neutrophilic (n=14)	Mixed granulocytic (n=7)	Pauci-ganulocytic (n=24)	p-value*
FEV1, % pred.	60-92-108	65-88-102	62-67-112	89-102-114	0.04
FeNO (ppb)	23.9-34.3-83.8 [†]	5.0-10.9-27.63	10.8-18.1-73.9	10.6-15.8-25.2	0.03
Eosinophils ^a (%)	3.2-7.2-56.9	0.0-0.1-0.65	2-2.8-4.2	0.0-0.2-0.75	0.0001
Neutrophils ^a (%)	4.0-18.9-39.4	75.3-83.2-90.2	72.4-73.6-83.8	12.7-36.5-54.4	0.0001

*: Kruskal-Wallis test was used to compare four groups of inflammatory phenotypes. The four inflammatory phenotypes were identified based on the sputum eosinophil ($\leq 1.9\%$) and neutrophil ($\leq 65.3\%$) percentages (Method section). [†]: $p < 0.05$ compared to neutrophilic (Dunn's multiple comparison test). ^a: on cytospin of induced sputum.

TABLE E2. CHARACTERISTICS OF PATIENTS ACCORDING TO THEIR MEDICATION USE

	Steroid naive (n=25)	ICS only (n=30)	ICS and po CS (n=11)	p-value ^a
FEV1 % pred	92-101-107	86-96-113	55-83-100	0.09
FeNO (ppb)	13.2-28.8-86.7	9.4-17.2-23.2	13.2-33.2-68.0	0.06
Sputum eosinophils (%)	0.0-1.2-2.8	0.0-0.2-3.1	0.0-0.6-2.0	0.74
Sputum neutrophils (%)	6.3-22.5-57.5	41.2-64.8-76.9**	38.2-60.2-90.0*	0.003

Lung function (FEV1 % pred) was assessed by spirometry. Fraction of exhaled nitric oxide (FeNO) was measured by chemiluminiscence. Data are represented as median values with interquartile range. ^aKruskal-Wallis test. *: p<0.05 and **: p<0.01 (compared to steroid-naive patients; Dunns multiple comparison test).