



Low interleukin (IL)-18 levels in sputum supernatants of patients with severe refractory asthma

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KEYWORDS

Severe refractory asthma;
IL-18;
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Summary

Background: Severe refractory asthma (SRA) is characterized by persistent asthma symptoms, amplified airway inflammation despite treatment with high dose inhaled steroids and increased airway bacterial colonization. Interleukin (IL)-18 is a pleiotropic pro-inflammatory cytokine that modulates airway inflammation. Furthermore, as a product of the inflammasome, IL-18 is involved in host defence against viral and bacterial stimuli by modulating the immune response.

Objective: To determine IL-18 levels in sputum supernatants of patients with asthma and to investigate whether underlying severity affects its levels. Furthermore, possible associations with atopy and mediators and cells involved in the inflammatory process of the airways were examined.

Methods: Forty-five patients with mild intermittent asthma (21 smokers) and 18 patients with SRA in stable state were studied. All subjects underwent lung function tests, skin prick tests, and sputum induction for cell count identification. IL-18 and ECP levels were measured in sputum supernatants. Furthermore, sputum samples were examined for the commonest respiratory pathogens and viruses by real time polymerase chain reaction (RT-PCR).

Results: Patients with SRA had significantly lower IL-18 levels in sputum supernatants compared to mild asthmatics ($p < 0.001$). Twelve out of eighteen patients with SRA were

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colonized by viruses and/or bacterial pathogens. IL-18 levels correlated with the percentage of macrophages ($r = 0.635$, $p = 0.026$) and inversely correlated with the percentage of neutrophils in sputum ($r = -0.715$, $p = 0.009$). No correlations were found between IL-18, ECP and the percentage of eosinophils in the sputum of SRA.

Conclusions: In SRA IL-18 is possibly involved in chronic airway inflammation through an eosinophil independent pathway. The decreased levels of IL-18 in SRA support the hypothesis of deregulated inflammasome activation, justifying the susceptibility of these patients for bacterial colonization or infection.

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Introduction

Severe refractory asthma (SRA) is characterized by persistent respiratory symptoms and worse lung function compared to mild-moderate asthma, airway remodelling, and increased airway bacterial colonization which seems to modulate the host immune response [1].

Interleukin-18 (IL-18), along with interleukin-1 β (IL-1 β), is produced by inflammasomes when activated by a number of pathogen, environmental or host-derived danger signals [2]. Inflammasomes are innate immune regulatory protein complexes which seem to play a key role in the host immune response of asthmatic patients [3]. IL-18 is a pleiotropic cytokine known to influence the balance of Th1/Th2 immune response [4], and it is released from activated monocytes/macrophages, dendritic cells, and airway epithelial cells [5].

The involvement of IL-18 in the pathogenesis of asthma has been established in several studies showing its potent interferon (IFN)- γ inducing activity, its ability to increase serum IgE levels and to promote allergen-induced eosinophil influx into the airways [6,7]. Increased levels of IL-18 have been previously measured in asthmatic subjects in comparison to healthy subjects in serum [8], induced sputum [9,10], and lung tissue [11]. Furthermore, significantly higher serum IL-18 levels have been reported in patients with acute severe asthma [12], while IL-18 gene

polymorphisms are reported to be associated with asthma severity [13].

It is acknowledged that IL-18, as a product of the inflammasome, is involved in host defence against viral and bacterial stimuli by modulating the immune response [14]. Emerging data show that atopic patients with uncontrolled asthma exhibit defective innate immune responses, with a reduced inflammasome function resulting in a decreased inflammasome-dependent cytokine production and an increased susceptibility to viral or bacterial airway infections [15–17].

The aim of this study was to determine IL-18 levels in sputum supernatants of patients with asthma and to investigate whether the underlying severity affects its levels. Furthermore possible associations with atopy and mediators and cells involved in the inflammatory process of the airways were determined.

Methods

Subjects

Eighteen patients with SRA (aged 51.7 ± 14.6 , mean $\pm$ SD) and 45 patients with mild intermittent asthma (21 non-smokers and 24 smokers) (aged 45.2 ± 10.2 , and 49.4 ± 8.7 , mean $\pm$ SD, respectively) were enrolled in the study,

Table 1 Clinical characteristics of study subjects.

	Mild asthma Non smokers n = 21	Mild asthma Current smokers n = 24	SRA n = 18	p value
Age (yrs)	45.2 ± 10.2	49.4 ± 8.7	51.7 ± 14.6	0.019
Gender (F/M)	13/8	12/12	12/6	
Smoking (Pack-years)	—	39 ± 22	$12 \pm 20^{\#}$	0.001
Current smokers/former smokers	—	24/0	0/5	
Atopy	8 (38%)	7 (29%)	12 (67%)	0.044
FEV ₁ (% pred)	98 ± 16	$86 \pm 17^*$	$67 \pm 17^{\#}$	0.001
FVC (% pred)	104 ± 15	98 ± 15	$88 \pm 19^*$	0.001
FEV ₁ /FVC	79 ± 7	$72 \pm 10^*$	$64 \pm 8^{\#}$	0.001
DLCO (% pred)	103 ± 15	95 ± 13	$78 \pm 10^{\#}$	0.045
FRC (% pred)	85 ± 21	91 ± 20	$113 \pm 24^{\#}$	0.004

Values are expressed as mean $\pm$ SD; * $p < 0.05$ vs non-smoking mild asthma; $^{\#}p < 0.05$ vs smoking mild asthma.

Abbreviations: FEV₁ = forced expiratory volume in one second; FVC = forced vital capacity; DLCO = diffusion capacity; FRC = functional residual, capacity; SRA = severe refractory asthma.

Table 1. Patients were consecutively recruited from an open cohort of patients with asthma who were followed up in the asthma clinics of the 1st and 2nd Respiratory Medicine University Departments in Athens during the period May 2013 to December 2013. Diagnosis of asthma was established according to the Global Initiative for Asthma (GINA) guidelines [18]. SRA diagnosis was established according to American Thoracic Society criteria [19].

Patients with mild intermittent asthma were stratified according to smoking habit and were asymptomatic for at least 3 months before entering the study. Five out of eighteen SRA patients were former smokers, smoking patients with mild asthma were current smokers, and non smoking patients with mild asthma were never smokers, **Table 1**. Subjects with any other respiratory disease or any concomitant malignant, heart, renal, liver or collagen disease were excluded. Patients with a respiratory tract infection or asthma exacerbation in the past 8 weeks prior to study admission were also excluded.

Patients were in a stable clinical condition as demonstrated by the low daily variability (<15%) of peak flow measurements during the two weeks before the study and by the low variability in forced expiratory volume in one second (FEV₁) in the two days of the study.

Patients with SRA were all receiving >1200 mg budesonide/day or equivalent plus long-acting beta-2 agonists (LABA). Among patients with SRA, 11 were also receiving 5 mg prednisolone/day, one was receiving 10 mg prednisolone/day, while 3 were receiving 7.5 mg prednisolone/day. 3 patients were receiving omalizumab and they were all on their fourth month of anti-IgE treatment. Subjects with mild asthma were only taking inhaled short acting β -2 agonists on an as needed basis for their asthma and at the time of the study.

All subjects gave informed consent for participation in the study, which was approved by the Hospital ethics committees.

Study design

Measurements

Lung function tests (flow–volume curves, reversibility test, diffusing lung capacity for carbon monoxide (DLCO), skin prick tests, blood sampling and sputum induction) were performed in two different days.

Pulmonary function test

Pulmonary function (FEV₁, FEV₁/FVC) was measured with a dry wedge spirometer (Viasys Healthcare, Jaeger, Hoechst, Germany) according to standardized guidelines [20]. The diffusing lung capacity for carbon monoxide (DLCO) was measured by the single breath method at least twice (Masterscreen, Jaeger, Hoechst, Germany).

Sputum induction and processing

Sputum was induced by inhalation of hypertonic saline aerosol as previously described [21], and using all the modifications for safe measurements according to asthma severity [22]. Briefly, 15 min after salbutamol inhalation (200 μ g), normal saline 0.9% and then hypertonic saline

(3%, 4% and 5%) nebulized by an ultrasonic nebulizer (ULTRA-NEB 2000, DeVilbiss Healthcare INC, Somerset, USA) was inhaled for each concentration over a period of 7 min. Subjects were encouraged to cough deeply after the 7-min intervals. All subjects produced an adequate aliquot of sputum which was processed within 2 h after termination of the induction. Sputum was diluted threefold with phosphate buffer solution containing freshly prepared dithiothreitol (final concentration: 1 mmol/L) (Sigma Chemical Co, Poole, UK). The sample was vortexed briefly and incubated at 37 °C for 15 min with constant agitation. The suspension was filtered through monofilament filter cloth to remove mucus, then centrifuged at 790 g for 4 min (4 °C), and the pellet was resuspended. Total cell counts were determined with a Neubauer haemocytometer (VWR International Ltd, Poole, UK) using trypan blue exclusion to determine cell viability; dead cells and epithelial cells were excluded.

Cell smear preparations were made using cytocentrifuge at 500 g for 2 min (Cytospin 3, Shandon, INC, Pittsburgh, USA). Sputum cytospin slides were stained with May-Grunwald-Giemsa for differential cell counts. Counting of at least 400 non-squamous cells took place in a blinded way by one technician. Sputum samples containing >20% of squamous cells and with cell viability <70% were excluded from the analysis as an indication of poor cytospin quality. The supernatant was stored at –80 °C until measurement of cytokines using an enzyme-linked immunosorbent assay kit (ELISA). IL-18 was measured using an ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) with a detection limit of 12.5 pg/ml. ECP was measured using Unicap ECP kit (Pharmacia Diagnostics; Uppsala, Sweden) with a detection limit of 0.5 ng/ml. The intra-assay and inter-assay variability were assessed according to the manufacturers' instructions for both mediators measured and were within acceptable coefficient of variation (%).

Sputum induction was well tolerated by all patients.

Assessment of the atopic status

Atopic status was assessed by skin-prick testing using 13 common allergens applied to the forearm. The allergens tested (HAL allergen Lab B. V. Harlem) were house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), household pets (cat and dog), pollens (mixed grass, olive, mixed weed, and *Parietaria judaica*) and moulds (alternaria, and *Aspergillus fumigatus*). Histamine and glycerinated saline solution were used as positive and negative controls, respectively. A skin-prick test result was considered positive if the mean wheal diameter was at least 3 mm.

RT-PCR for the detection of bacteria and viruses in sputum supernatants

The sputum supernatants were examined for the commonest respiratory pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydiae*, *Mycoplasma hominis*). Primer Design™ kits (DNA 2.0, Inc., USA) were used to detect and quantify all the pathogens tested, (except for *Mycoplasma hominis*) following the manufacturer's instructions. The priming efficiencies were larger than 95% and the detection limit was less than 100 copies of target template.

Mycoplasma hominis was detected by Real-Time PCR (Light Cycler® Fast Start DNA Master Sybr GreenI-ROCHE), followed by melting analysis. The oligonucleotide primers for *M. hominis* were used as published (F-RNAH1 5'-CAATGGCTAATGCCGATACGC-3', R-RNAH2 5'-GGTACCGT-CAGTCTGCAAT-3'). In every reaction, 0.2 µM of each forward and reverse primer were used per 20 µl reaction, whereas, the MgCl₂ concentration was 1.5 mM. The PCR conditions were the following: 95 °C for 10 min (DENATURATION), 95 °C for 15 s, 62 °C for 30 s, 72 °C for 30 (50 cycles), 95 °C for 01 s, 72 °C for 10 s, 95 °C for 01 s, 40 °C for 30 s (COOLING). The sensitivity and specificity of the method exceeds 95%.

Virus detection was performed via RT-PCR (CLART®P-neumoVir, GENOMICA, S.A.U. Madrid, Spain) amplification of a specific 120–330 bp fragment of the viral genome. Viruses analyzed included: Adenovirus; Influenza virus A; Metapneumovirus (subtypes A and B); Parainfluenza virus; Rhinovirus; Respiratory Syncytial Virus type A and type B (VSR-A, VSR-B).

Statistical analysis

Data were expressed as mean ($\pm$ SD) or median values. Cytokine levels were expressed in median values and inter-quartile range. Differences between groups were initially assessed by Kruskal–Wallis test, and if significant, the Mann–Whitney rank test was then assessed. Correlations between inflammatory cells and mediators in sputum, smoking characteristics or lung function parameters were calculated with Spearman's rank correlation test. Statistical analysis was not influenced by values at the lower limits of detection since the non-parametric tests used were based on ranks of values.

A p value of less than 0.05 was considered significant.

Results

The clinical characteristics of the subjects included in the study are shown in Table 1.

IL-18 levels in induced sputum

IL-18 levels were significantly lower in the induced sputum of SRA patients [median value (inter-quartile range), 67 (38.5–196)], as compared to mild asthmatics (smoking and non-smoking) [median value (inter-quartile range), 256 (102–419), 283 (103–590), respectively, $p < 0.001$] (Fig. 1, Table 2).

Furthermore, sputum IL-18 levels were significantly lower in the induced sputum of atopic versus non-atopic asthma patients (median (inter-quartile range), 192 pg/ml (38.5–468) vs (335 pg/ml) (103–590), $p = 0.013$) (Fig. 2).

In SRA, IL-18 levels in induced sputum positively correlated with the degree of airflow limitation ($r = 0.760$, $p = 0.004$ for FEV₁ (% pred), and $r = 0.620$, $p = 0.032$ for FVC (% pred), and the percentage of sputum macrophages ($r = 0.632$, $p = 0.026$) and negatively correlated with neutrophils ($r = -0.715$, $p = 0.009$) (Table 3) (Fig. 3). No correlation was found between IL-18 and ECP levels or

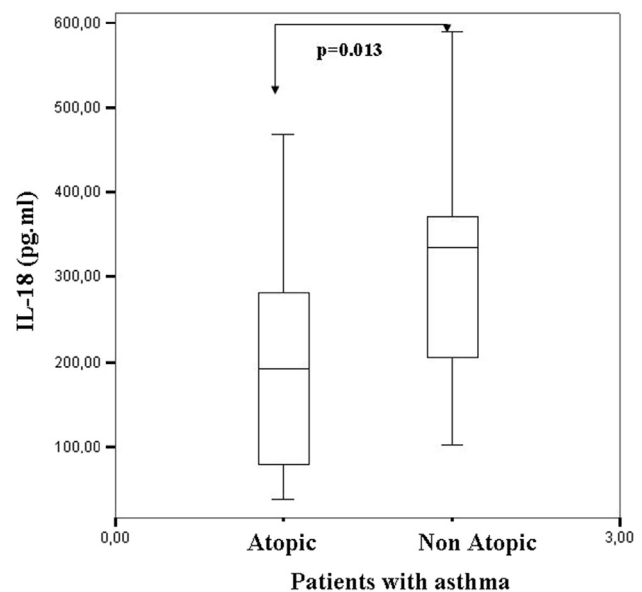


Fig. 1 IL-18 levels (pg/ml) in the induced sputum of patients with mild (smoking and non smoking) and severe refractory asthma.

eosinophil counts in induced sputum of SRA patients (Table 3).

Sputum cells and inflammatory mediators

Total number of cells in sputum per ml, % percentage of macrophages, neutrophils, lymphocytes, and eosinophils, as well as, eosinophilic cationic protein (ECP), and IL-18 levels in sputum are summarized in Table 2.

The median (inter-quartile range) total number of cells per ml in sputum was significantly higher in SRA compared to mild asthma (smoking and non-smoking, $p < 0.001$) (Table 2). SRA patients had a significantly higher percentage of sputum eosinophils and neutrophils compared to smoking and non-smoking mild asthmatics. ECP was significantly higher in patients with SRA as compared to mild asthma in smoking and in non-smoking asthmatic patients, (Table 2).

Viral and bacterial infection load in SRA patients

Twelve out of eighteen patients with SRA were found to be colonized by viruses and/or bacterial pathogens. RT-PCR results for the detection of bacteria and viruses in sputum supernatants are shown in Table 4.

Discussion

In this study, patients with severe refractory asthma (SRA) had significantly lower IL-18 levels in induced sputum as compared to mild asthmatics. Moreover, IL-18 levels were associated with both airflow limitation and sputum macrophages and neutrophils. No relationship was found between IL-18 and ECP and/or eosinophils in induced sputum. IL-18 levels were significantly lower in the presence of atopy.

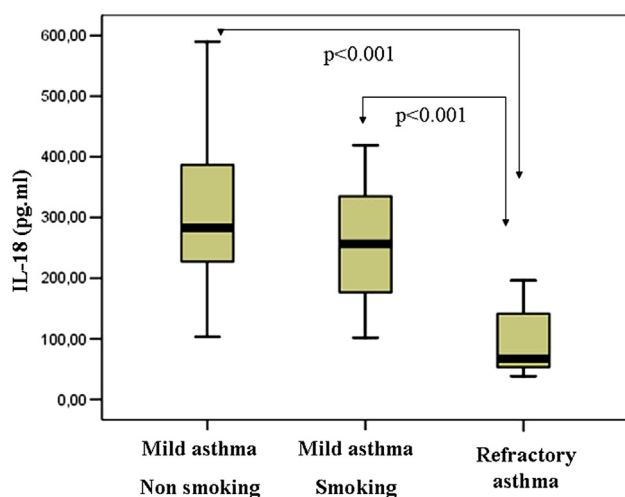
Table 2 Inflammatory variables of study subjects.

	Mild asthma Non smokers n = 21	Mild asthma Smokers n = 24	SRA n = 18	p value
Total number of cells ($\times 10^6$ /ml)	0.8 (0.6–1.1)	1 (0.7–1.6)	2.9 (0.9–3.5)*, #	0.64
Macrophages, %	69 (17.4–93.5)	44 (4.7–95)	50 (11–72) [§]	0.12
Neutrophils, %	22 (2–66)	35 (10–67)	50 (4–91) [¶]	0.04
Lymphocytes, %	1.3 (0.5–2.1)	1.5 (0.2–3)	2.7 (1–6)	0.08
Eosinophils, %	3.9 (1.5–18)	1.5 (0.25–21)	12.5 (2–51) ^{§, *}	0.001
ECP ($\times 10^2$ pg/ml)	760 (450–1070)	1450 (600–3040)*	2200 (580–5120) ^{¶, §}	0.04
IL-18 (pg/ml)	283 (103–590)	256 (102–419)	67 (38.5–196)*, #	0.03

Values are expressed as median (inter-quartile range) *p < 0.001 vs non-smoking mild asthma; #p < 0.001 vs smoking mild asthma;

[¶]p < 0.05 vs non-smoking mild asthma; [§]p < 0.05 vs smoking mild asthma.

Abbreviations: ECP = eosinophilic cationic protein; IL-18 = interleukin-18; SRA = severe refractory asthma.

**Fig. 2** IL-18 levels (pg/ml) in the induced sputum of atopic versus non atopic asthma patients.

To the authors' knowledge this is the first study examining IL-18 in the induced sputum of patients with SRA. Interestingly IL-18 levels were significantly lower in SRA compared to those in mild asthma. Previous studies have

shown increased IL-18 levels in the serum [8] and sputum of asthmatic subjects in comparison to healthy subjects [9–12], and significantly higher serum IL-18 levels in patients with acute severe asthma [12].

Our results are consistent with the newly expressed hypothesis of the reduced innate immune response in patients with poorly controlled asthma [15–17] which assumes that impaired inflammasome function results in a decreased inflammasome-dependent cytokine production and an increased susceptibility to viral or bacterial airway infections. Indeed, twelve out of eighteen patients with SRA in stable condition were colonized by viruses and/or bacterial pathogens in our study. This is in accordance with reports showing that patients with poorly controlled asthma have increased bacterial colonization of the airways, as indicated by either increased airway endotoxin levels or increased 16S rRNA load in bronchial samples [23,24]. Viral and bacterial mechanisms modify the host immune response in asthma as shown previously [17,25]. Newcomb et al. [23] showed decreased numbers of circulating CD81 lymphocytes, in association with deficient IFN- γ and IL-10 responses after experimental rhinoviral infection.

Atopy seems to be another determinant of low sputum IL-18 levels in sputum supernatants in our study. Atopic asthmatic patients had significantly lower IL-18 levels

Table 3 Correlations between IL-18 and inflammatory cells, mediators, and lung function in the study groups.

	Mild asthma Non smokers n = 21	Mild asthma Smokers n = 24	SRA n = 18
Total cells (3×10^6 /ml)	$r = 0.696, p = 0.004$	$r = -0.195, p = 0.373$	$r = -0.203, p = 0.527$
Macrophages, %	$r = -0.271, p = 0.328$	$r = 0.348, p = 0.112$	$r = 0.635, p = 0.026$
Neutrophils, %	$r = 0.112, p = 0.703$	$r = -0.295, p = 0.182$	$r = -0.715, p = 0.009$
Lymphocytes, %	$r = 0.083, p = 0.835$	$r = -0.800, p = 0.200$	$r = 0.277, p = 0.384$
Eosinophils, %	$r = -0.077, p = 0.794$	$r = -0.175, p = 0.448$	$r = -0.200, p = 0.533$
ECP ($\times 10^2$ pg/ml)	$r = 0.017, p = 0.966$	$r = -0.114, p = 0.613$	$r = 0.046, p = 0.888$
FEV ₁ (% pred)	$r = -0.357, p = 0.191$	$r = 0.627, p = 0.002$	$r = 0.760, p = 0.004$
FVC (% pred)	$r = -0.239, p = 0.390$	$r = 0.460, p = 0.031$	$r = 0.620, p = 0.032$
DLCO (% pred)	$r = 0.405, p = 0.320$	$r = 0.001, p = 0.997$	$r = 0.312, p = 0.324$
FRC (% pred)	$r = -0.690, p = 0.058$	$r = -0.471, p = 0.042$	$r = -0.526, p = 0.079$

Abbreviations: FEV₁ = forced expiratory volume in one second; FVC = forced vital capacity; DLCO = diffusion capacity; FRC = functional residual, capacity; ECP = eosinophilic cationic protein; SRA = severe refractory asthma. Statistical significance for p < 0.05.

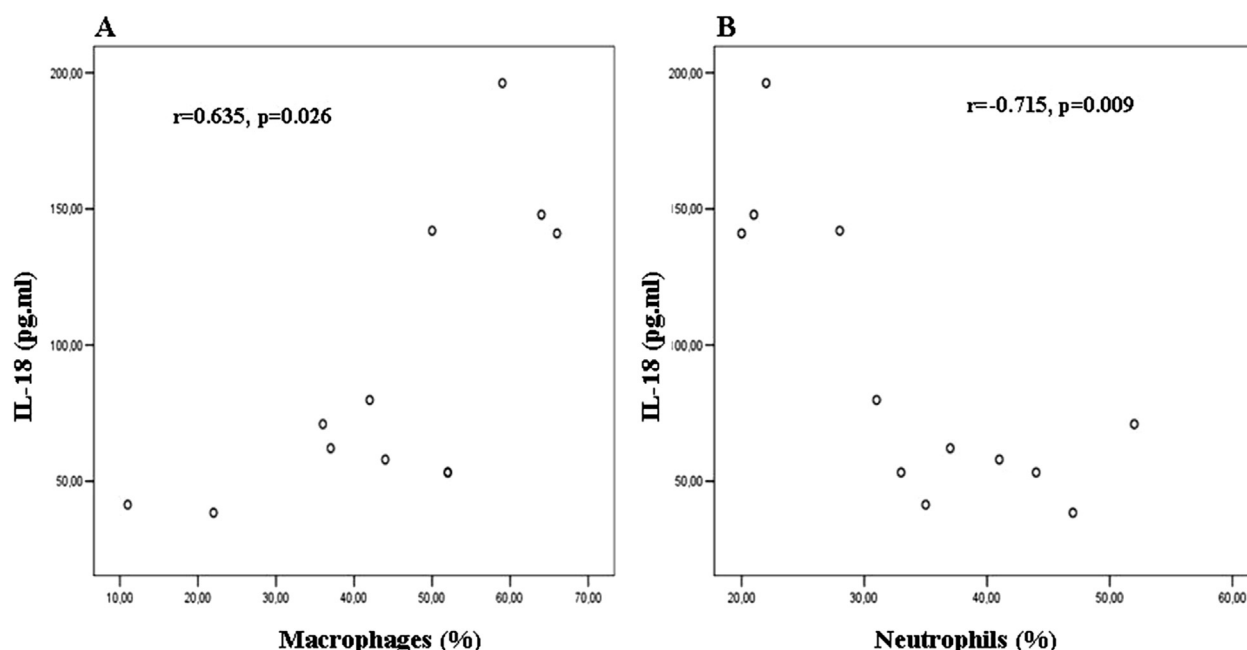


Fig. 3 Correlation between IL-18 levels in induced sputum and percentage of sputum macrophages (A) and neutrophils (B) in severe refractory asthma.

compared to non-atopic patients. The important role of inflammasomes in the modulation of the responses in allergic conditions has been previously reported [15,16,26]. Brickey et al. [15] showed that inflammatory cells from patients with allergic rhinitis and asthma have decreased inflammasome gene expression as compared to healthy non-atopic volunteers. Recently, Hernandez et al. [16] demonstrated that atopic asthmatic patients show reduced inflammatory cell recruitment after inhaled endotoxin challenge compared to healthy subjects and demonstrated muted IL-1 β and IL-18 responses to LPS.

We have previously shown reduced IL-18 levels in the induced sputum of asthmatic smokers as compared to non-smokers [9], while McKay et al. [10] demonstrated that the reduced IL-18 levels in smoking asthmatics were associated

with decreased IL-18mRNA expression in sputum cells. In this study there was a close correlation of IL-18 levels with sputum (macrophages and neutrophils). Several types of cells release IL-18 such as (activated monocytes/macrophages, dendritic cells, and airway epithelial cells). Although, eosinophils were prominent in our SRA patients as compared to mild asthma, they could not possibly represent the main source of IL-18. In the study of Guarda et al. [27] it was shown that eosinophils express inflammasome genes to a lower degree in comparison to macrophages and neutrophils. Previous studies in asthma patients, using immunocytochemistry localized IL-18 expression to sputum macrophages [9,10]. Macrophages may also be implicated in the decreased expression of IL-18 in SRA. It is known, that in atopic conditions, or in the presence of Th2

Table 4 Bacterial and viral detection in sputum supernatants using RT-PCT.

	Mild asthma Non smokers n	Mild asthma Smokers n	SRA n
Influenza virus A	—	—	2
Parainfluenza virus	—	1	1
Rhinovirus	1	—	2
Adenovirus	—	1	—
Streptococcus pneumoniae	—	—	4 (3831 copies/ml 1685 copies/ml 125 copies/ml 275 copies/ml)
Haemophilus influenzae	—	—	3 (1750 copies/ml 845 copies/ml 6974 copies/ml)

SRA = severe refractory asthma.

cytokines (especially IL-4 and IL-13) macrophages can be polarized to the M2 phenotype as compared to the M1 phenotype [28]. The M2 macrophage phenotype is associated with decreased inflammasome activity, resulting in low expression of IL-1 β and IL-18 [28]. Since our SRA patients were atopic to a greater proportion compared to mild asthmatics, and had significantly higher levels of ECP and eosinophils in sputum it is possible that the M2 phenotype of macrophages is dominant over the M1 phenotype.

SRA patients presented higher neutrophil counts in comparison to mild asthmatics. Increased neutrophils have been found in patients with severe, persistent asthma [29,30], and this may be partly attributed to corticosteroid treatment. Several studies have linked corticosteroid treatment in asthma with neutrophilic inflammation [31,32]. IL-18 secretion has been linked to neutrophilic inflammation [33–36]. IL-18 promotes neutrophil accumulation by increasing the production of chemo-attractants, such as IL-8 and chemokine ligand 1 (CXCL1) [34,35].

Eosinophilic inflammation was prominent in the SRA patients. However, there was no association between IL-18 and markers of eosinophilic inflammation. Our data suggest that IL-18 is probably implicated in the chronic inflammation of SRA via an eosinophil independent pathway. In favour of our results, Yamagata et al. [37] have demonstrated that IL-18-deficient mice had eosinophilic inflammation in their BALF after exposure to OVA, while Kodama et al. [38] showed that IL-18 deficiency enhanced pulmonary eosinophilia in an acute inflammation model. Further studies are needed in order to document inflammasome activation and its relation to remodelling in severe refractory asthma.

In conclusion, IL-18 levels were lower in the induced sputum of patients with SRA as compared to mild asthma supporting the hypothesis of reduced innate immune response in patients with poorly controlled atopic asthma, which in turn leads to increased susceptibility to viral and/or bacterial airway colonization or infections.

The correlation of IL-18 with airflow limitation and airway inflammation indicates its involvement in the pathogenesis of chronic inflammation in SRA, probably though, through an eosinophil independent pathway. Further studies are needed to explore the role of IL-18 in the phenotype of SRA and its potential use as a therapeutic target.

Conflict of interest

Authors have no conflict of interest to declare.

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