Interleukin-18 in induced sputum: Association with lung function in chronic obstructive pulmonary disease

Nikoletta Rovina a,*, Efrossini Dima a, Christina Gerassimou b, Androniki Kollintza b, Christina Gratziou c, Charis Roussos a, b, c

a Department of Respiratory Medicine, Medical School, University of Athens, "Sotiria" Hospital for Diseases of the Chest, 152 Mesogion Avenue, Athens GR-11527, Greece
b "M. Simos" Laboratories, Department of Critical Care and Pulmonary Services, Evangelismos Hospital, University of Athens, Athens, Greece
c Asthma and Allergy Centre, Pulmonary and Critical Care Department, Evgenidion Hospital, Medical School, University of Athens, Athens, Greece

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KEYWORDS
Chronic obstructive pulmonary disease; Interleukin-18; Biomarker in induced sputum; Pulmonary function

Summary
Background: It has been shown that interleukin (IL)-18 levels in induced sputum are reduced in asthmatic and healthy smokers. However, in chronic obstructive pulmonary disease (COPD) patients, recent data show an overproduction in the lungs and increased serum levels of IL-18, suggesting that IL-18 may be involved in the pathogenesis of COPD.
Method: In order to assess the relation of IL-18 with pulmonary function and airway inflammation in COPD, IL-18, tumour necrosis factor-α, and IL-8 levels were measured by ELISA in sputum supernatants obtained from patients with bronchitis type COPD (n = 28), and healthy subjects (18 smokers and 17 non-smokers). Cellular localization of IL-18 was assessed by immunocytochemistry.
Results: The levels of IL-18 were significantly higher in sputum supernatants of COPD patients compared to healthy smokers and non-smokers (p < 0.05). IL-18 production was localized to sputum macrophages. IL-18 levels were inversely correlated with FEV1 (% predicted) (r = −0.572, p = 0.002) and FEV1/FVC ratio in COPD smokers (r = −0.608, p = 0.001). No correlations were found between IL-18 levels and inflammatory markers studied in induced sputum obtained from COPD patients, healthy smokers and non-smokers.
Conclusion: In patients with COPD, increased levels of IL-18 in induced sputum were associated with airflow limitation, suggesting that IL-18 may be implicated in the pathogenesis of COPD.
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Introduction

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible, is usually progressive, and associated with an abnormal inflammatory response of the lungs. Long-term cigarette smoking is the most important risk factor that may initiate the disease, as a result of influx of inflammatory cells into the lung (leading to chronic airway inflammation), imbalance between proteolytic and anti-proteolytic activity, oxidative stress and apoptosis. However, there is no effective way as yet to predict the onset of the disease among smokers, and more importantly, to treat pulmonary inflammation in COPD, even when irreversible damage has not yet been established in the airways or parenchyma.

Cigarette smoking activates the inflammatory cells in the airways and airway epithelial cells to release inflammatory cytokines, such as interleukin (IL)-1β, IL-8, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, granulocyte-macrophage colony Stimulating factor, and IL-18. A pro-inflammatory cytokine originally identified as IL-1β converting enzyme, has recently been implicated in the pathogenesis of COPD induced by cigarette smoking, via IL-18 dependent signalling. Cigarette smoking induces epithelial apoptosis, stimulates proteases and chemokines and activates a variety of caspases via IL-18 dependent mechanisms. IL-18 is produced intracellularly in a wide range of cells from a biologically inactive precursor, pro-IL-18, by caspase-1. It plays an important role in Th1 polarization and various Th1-type diseases, inducing TNF-α, IL-1β, granulocyte-macrophage colony-stimulating factor and chemokine production by monocytes and T lymphocytes, whereas it is now appreciated to be an important regulator of innate and adaptive immunity.

In mouse models, it has been shown that constitutive IL-18 overproduction in the lungs induces emphysema and treatment with recombinant IL-18 plus IL-2 induces pulmonary inflammation and lung injury. The implication of IL-18 in COPD pathogenesis is further supported by the fact that IL-18 and its receptor are expressed in human lung in COPD and serum levels of IL-18 are significantly higher in patients with severe COPD than in healthy smokers or non-smokers. Most recently, it was demonstrated that serum levels of IL-18 are negatively correlated with FEV1 (% pred) in COPD patients, further supporting its implication in the pathogenesis of COPD. To our knowledge, there are no studies on IL-18 in sputum obtained from COPD patients.

Based on the above data, we aimed to identify and quantify IL-18 levels in sputum samples from healthy and COPD patients, and furthermore, to evaluate the associations between IL-18 in induced sputum, pulmonary function parameters and airflow inflammation.

Our results provide the first evidence that IL-18 can be measured in the sputum of COPD patients, and demonstrate the clinical relevance of the findings of the previously conducted animal studies in humans, further supporting the implication of IL-18 in the pathogenesis of COPD.

Methods

Subjects

Twenty-eight COPD smokers, 18 asymptomatic healthy current smokers and 17 non-smoking controls were included in the study. All healthy smokers were lifelong smokers (>15 pack-years), with no history of lung disease, no chronic respiratory symptoms, and normal spirometry. All COPD patients were current smokers (>15 pack-years), with chronic cough and sputum production over at least 3 months for 2 successive years, and irreversible airflow limitation (reversibility <10% predicted forced expiratory volume in 1 second (FEV1) after 200 μg of inhaled salbutamol). All patients satisfied the ERS criteria for COPD and were selected to have no evidence of emphysema, based on high-resolution computed tomographic scans of the lungs and the diffusing capacity of lung for carbon monoxide (DLCO).

All participants met the following criteria: no use of inhaled or oral corticosteroids for 6 months prior to sample collection, no atopy (negative skin prick tests for 10 common aeroallergens), and no respiratory tract infection 1 month prior to the study. None of the COPD patients was ever hospitalized due to an exacerbation of COPD. Patients with co-morbidities, such as bronchiectasis, asthma, interstitial diseases and heart failure, were excluded. None of the healthy non-smokers and smokers was using long-acting bronchodilators. Nine out of 28 COPD patients were under treatment with inhaled tiotropium and inhaled short-acting β-agonists per need, ten were receiving inhaled short-acting β-agonists or ipratropium per need, and none was receiving long-acting bronchodilators. All subjects gave informed consent for participation in the study, which was approved by the hospital ethics committee.

Measurements

Lung function tests (flow—volume curves, reversibility test, diffusing lung capacity for carbon monoxide (DLCO), measurement of arterial blood gases, skin prick tests, blood sampling and sputum induction were performed.

Lung function

Lung function (FEV1, FEV1/FVC) was measured with a dry wedge spirometer (Masterscreen, Jaeger, Hoechberg, Germany) according to standardized guidelines. Measurements were always performed by the same technician using the same spirometer. Reversibility test was performed 20 min after inhalation of 200 μg salbutamol via a metered dose inhaler. Subjects were not allowed to use short-acting bronchodilators 12 h, and tiotropium 48 h prior to pulmonary function testing. The diffusing lung capacity for carbon monoxide (DLCO) was measured by the single-breath method at least twice (Masterscreen, Jaeger, Hoechberg, Germany).

Sputum induction and processing

Sputum was induced by inhalation of hypertonic saline aerosol and processed as described previously. Briefly,
15 min after salbutamol inhalation (200 µg), normal saline 0.9% and then hypertonic saline (3%, 4% and 5%) nebulized by an ultrasonic nebulizer (ULTRA-NEB 2000, DeVibiss Healthcare Inc., Somerset, NJ, 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 samples were transferred to a Petri dish and the volume and macroscopic characteristics of the whole sample recorded. Sputum plugs were separated from contaminating saliva using sterile forceps. The plugs were placed in a pre-weighed tube and the weight recorded. The sputum then 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 re-suspended. 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 sputum preparations were made using cytocentrifuge at 500 × g for 2 min (Cytospin 3, Shandon Inc, Pittsburgh, PA, USA). Sputum cytospin slides were stained with May–Grünwald–Giemsa for differential cell counts. Counting of 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 analysis as indication of poor cytospin quality. The supernatant was stored at −80 °C for subsequent assay for IL-18, IL-8, and TNF-α concentration.

Measurement of IL-18, TNF-α, and IL-8

The concentration of IL-18 in sputum supernatants and serum was determined by an enzyme-linked immunosorbent assay kit (ELISA) (MBL International Corporation) following the manufacturer’s instructions. The sensitivity of the assay used was 12.5 pg/ml.

The concentrations of TNF-α and IL-8 were determined by ELISA as well, using kits purchased from R&D Systems (Minneapolis, MN, USA). In all cases, the assays were carried out according to the manufacturer’s recommendations. The sensitivities of the assays used were 1.6 pg/ml and 3.5 pg/ml, respectively.

Immunocytochemistry of IL-18 expression

IL-18 localization on sputum cell cytopreparations was performed as previously described.17 Freshly isolated sputum cells were centrifuged at 500 × g for 2 min (Cytospin 3, Shandon Inc, Pittsburgh, PA, USA), fixed with ice-cold para-formaldehyde solution 4% (Sigma Chemical Co, Steinheim, Germany) and stored at −20 °C until analysis. On the day of analysis, slides were thawed and then washed at room temperature with Tris-buffered saline three times. Following incubation with blocking buffer (1% BSA in TBS-0.1% Tween) for 1.5 h, IL-18 was localized using the mouse monoclonal antibodies IL-18 (1.51 E3E1):sc-13602 (Santa Cruz Biotechnology). Bound antibodies were detected with DakoCytomation Polyclonal Rabbit Anti-Mouse Immunoglobulins and APAAP. Immunoreactive cells were visualized by the addition of DAKO Fast Red Substrate after being lightly counterstained with Harris’ haematoxylin (DAKO, Glostrup, Denmark). All antibody incubations were conducted at 37 °C.

Statistical analysis

Data were expressed in mean (±SD) or median values. IL-18, IL-8, and TNF-α were expressed in median values and inter-quartile range. Differences between subjects’ 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 participated in the study are shown in Table 1. All smokers had similar mean values for age, smoking pack years, arterial oxygen tension, DLCO (% pred), FRC (% pred), RV (% pred), and TLC (% pred). However, FEV1, FVC, and FEV1/FVC were significantly lower (p < 0.05, Table 1) in COPD smokers compared to healthy smokers and healthy non-smokers.

Table 1. Subjects’ characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Healthy non-smokers</th>
<th>Healthy smokers</th>
<th>COPD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47 ± 2</td>
<td>49 ± 2</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>Smoking (pack-years)</td>
<td>0</td>
<td>45 ± 4</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>6/11</td>
<td>8/10</td>
<td>19/9</td>
</tr>
<tr>
<td>FEV1 (% pred)</td>
<td>105 ± 3*</td>
<td>100 ± 2*</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>FVC (% pred)</td>
<td>107 ± 2*</td>
<td>105 ± 1*</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>83 ± 2*</td>
<td>80 ± 18*</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>FEF25–75</td>
<td>92 ± 8*</td>
<td>80 ± 5*</td>
<td>30 ± 2</td>
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<tr>
<td>RV (% pred)</td>
<td>99 ± 5</td>
<td>93 ± 3</td>
<td>95 ± 12</td>
</tr>
<tr>
<td>TLC (% pred)</td>
<td>94 ± 3</td>
<td>94 ± 2</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>FRC (% pred)</td>
<td>94 ± 5</td>
<td>95 ± 4</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>DLCO</td>
<td>105 ± 6</td>
<td>83 ± 1</td>
<td>74 ± 15</td>
</tr>
<tr>
<td>Arterial PO2</td>
<td>95 ± 8</td>
<td>83 ± 6</td>
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</table>

COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; FEF25–75, forced expiratory flow 25–75; RV, residual volume; TLC, total lung capacity; FRC, forced residual capacity; DLCO, diffusing lung capacity for carbon monoxide; PaO2, partial pressure of oxygen, arterial.

Values are expressed as mean ± SE. *p < 0.05, asymptomatic smokers and healthy non-smokers vs COPD smokers.
**Sputum cells**

The median (inter-quartile range) total number of cells in COPD smokers was higher ($p > 0.05$) compared to healthy smokers and significantly higher ($p < 0.05$) compared to non-smokers (Table 2). Smokers with COPD had higher percentage of sputum neutrophils compared to healthy smokers ($p < 0.05$), and non-smokers ($p < 0.05$). In contrast, the percentage of sputum macrophages was significantly lower in COPD smokers compared to healthy smokers ($p < 0.05$) and non-smokers ($p < 0.05$) (Table 2).

**IL-18 levels in induced sputum**

IL-18 levels were higher in the induced sputum of healthy non-smokers compared to healthy smokers ($p = 0.059$). In the induced sputum samples of COPD smokers IL-18 levels were significantly higher compared to healthy smokers ($p = 0.038$) and healthy non-smokers ($p = 0.003$) (Fig. 1).

IL-18 levels in induced sputum inversely correlated with FEV$_1$ (% pred) ($r = -0.572$, $p = 0.002$) and FEV$_1$/FVC ($r = -0.608$, $p = 0.001$) in COPD patients (Fig. 2).

No significant correlations between sputum levels of IL-18 and pulmonary function parameters were observed in healthy smokers and non-smokers.

IL-8 levels in induced sputum of COPD smokers were significantly higher compared to healthy smokers ($p = 0.007$) and non-smoking subjects ($p = 0.002$), while TNF-$\alpha$ levels were significantly higher in the induced sputum of COPD smokers compared to healthy non-smokers ($p = 0.001$) (Table 3). No significant correlations between IL-18 levels and IL-8 and TNF-$\alpha$ levels were found in any of the studied groups.

**IL-18 localization in sputum cells by immunocytochemistry**

The cellular distribution of IL-18 synthesis was evaluated in sputum cytospin preparations by immunocytochemistry. Positive staining was observed in sputum macrophages (Fig. 3).

**Discussion**

This study is the first to examine IL-18 levels in the induced sputum obtained from patients with COPD. Like many patient-based clinical studies, this is an observational study, showing that IL-18 levels in the induced sputum of COPD patients are significantly higher compared to healthy smokers and non-smokers. The levels were correlated with airflow limitation in COPD patients.

The finding of elevated IL-18 levels in the sputum of COPD patients may not be surprising taking in account the results of previously published data on COPD. IL-18, a member of IL-1 family, was originally described as IFN-$\gamma$-inducing factor (IGIF) and is appreciated to be an important regulator of innate and adaptive immunity. Cigarette smoke is the major cause of COPD and it has a well-known ability to modulate innate immunity either by increasing the number of innate immune effectors such as neutrophils and macrophages in the lung or by regulating macrophage function and the expression of genes involved in innate immune responses. It is appreciated that innate immune response plays a particularly important role in the pathogenesis of the inflammatory and remodelling responses in CS-exposed individuals. Kang et al. demonstrated that the interaction of CS with inflammatory, remodelling, and apoptotic responses is associated with the early induction of type I IFNs and IL-18, later induction of IL-12/IL-23 p40 and IFN-$\gamma$, and the activation of PKR and eIF2$\alpha$ offering a new window of opportunity for a better understanding of the unique interaction.

| Table 2 | Sputum inflammatory cell numbers in healthy and COPD smokers as compared to healthy non-smokers. |
|---|---|---|
| | Healthy non-smokers | Healthy smokers | COPD patients |
| | $n = 17$ | $n = 18$ | $n = 28$ |
| Total no of cells $\times 10^4$ | 28 (8–43) | 37 (30–115) | 62 (18–233)* |
| Macrophages, % | 56 (13–86) | 48 (38–62)* | 32 (10–84)* |
| Neutrophils, % | 41 (12–79) | 48 (8–59)* | 62 (17–92)* |
| Lymphocytes, % | 0.6 (0.2–1) | 2 (0.6–5.5) | 2.5 (0.41–4.4) |
| Eosinophils, % | 0.9 (0.3–0.6) | 0.32 (0.1–1.9) | 1.2 (0.25–2.2) |

Values are median (inter-quartile range). *$p < 0.05$ vs healthy non smokers, *$p < 0.05$ vs healthy smokers.
between cigarette smoke and innate immune responses and the role of IL-18 in this procedure.

The most potent explanation to consider for the finding of the elevated IL-18 levels in induced sputum obtained from COPD patients might be the bacterial colonization of the bronchial tree. Bacterial colonization of distal airways is frequently found in patients with stable COPD and several risk factors have been found to influence it (smoking, airway obstruction severity, airway inflammation).24,25 It has recently been shown that *Haemophilus influenzae* is frequently present in the airways of COPD patients during clinically stable periods.26,27 forming biofilms on airway epithelia.28 These biofilms cause the release of proinflammatory cytokines and chemokines by airway epithelia. Previous studies reported that *H. influenzae* stimulates respiratory epithelial production of macrophage-inflammation proteins, IL-8, and TNF-α in cell culture and mouse models.28 These data support the idea that airway epithelia play key roles in innate immunity and may help to signal and coordinate both innate and adaptive immune responses. This scenario could probably explain the decreased levels of IL-18 found in induced sputum obtained from asthmatic and healthy smokers in contrast to COPD patients,29 and pose the distinct role IL-18 may play in COPD as compared to asthma.

Age as a factor up-regulating IL-18 levels in the sputum of COPD patients could not be the issue in this study since all the subjects enrolled did not differ significantly in age. Nevertheless, no correlation was found between IL-18 levels in sputum and the age of the subjects in any of the studied groups. To the authors’ knowledge there is no reference in the literature concerning the variation of IL-18 levels in sputum with age.

In the present study, as set in the inclusion criteria, COPD patients were stable, actually never hospitalized for an exacerbation, and were not on ICS treatment that modulates bronchial inflammation. All the above criteria, however, do not diminish the possibility of a bacterial colonization scenario to be the major factor contributing to the elevated levels of IL-18 in induced sputum. However, further analysis is needed to clarify whether airway bacterial colonization along with cigarette smoking or COPD per se is the cause for increased IL-18 levels in the induced sputum of COPD patients.

Immunocytochemistry localized IL-18 expression to sputum macrophages. It has been suggested by the authors that in line with the findings in the lung biopsies where IL-18 is strongly expressed in alveolar macrophages and CD8⁺ T lymphocytes, in the airways the majority of CD8⁺ T cells and activated macrophages produce IL-18.5

In this study, an inverse correlation was found between IL-18 levels and FEV₁ (% pred) and FEV₁/FVC ratio in COPD patients. This finding is additive to the first evidence shown by Imaoka and colleagues5 in severe COPD, where IL-18 levels in the sera were inversely correlated with % FEV₁. Our findings further support the association between increased IL-18 levels and airflow limitation in COPD patients, suggesting that IL-18 might be used as a new biomarker for disease activity and progression.

None of the COPD patients who participated in this study had been receiving inhaled or systemic steroid therapy. The

**Table 3** IL-8 and TNF-α levels in induced sputum.

<table>
<thead>
<tr>
<th></th>
<th>Healthy non smokers</th>
<th>Asymptomatic smokers</th>
<th>COPD smokers</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Sputum IL-8 (ng/ml)</td>
<td>0.7 (0.3–4.6)</td>
<td>1.3 (0.6–30.6)</td>
<td>6.2 (0.9–38)*</td>
</tr>
<tr>
<td>Sputum TNF-α (pg/ml)</td>
<td>14 (0.5–35)</td>
<td>21 (1.5–173)</td>
<td>53 (3.5–210)*</td>
</tr>
</tbody>
</table>

Values are median (inter-quartile range), *p < 0.05 vs non smokers, †p < 0.05 vs smokers.
treatment received by COPD patients consisted mainly of the use of bronchodilators, such as β2-agonists and anti-cholinergics which do not alter airway inflammation. Up to now, there is no effective therapy to reduce the persistent pulmonary inflammation in COPD patients and improve their prognosis. Therefore, the disease has to be targeted with new anti-inflammatory treatments. Our results further support the possibility raised by previous studies that blockade of the IL-18 pathway might be a feasible therapeutic target in COPD.

In conclusion, elevated levels of IL-18 in induced sputum of COPD smokers in combination with the close correlation of IL-18 levels with airflow limitation in bronchitis type COPD patients support the implication of IL-18 in the pathologic changes occurring in bronchial epithelium quite early in COPD patients’ airways. If this is proven to be true, then IL-18 levels in induced sputum could be used as a valid biomarker for disease activity and progression, and the IL-18 pathway could be considered as a potential therapeutic target for COPD.

Conflicts of interest statement

All authors of this paper declare that they have no financial or other potential conflicts of interest concerning the subject of this manuscript.

Authors’ contributions

N.R. and E.D. performed all the clinical measurements of the study. C.G. and A.K. analysed the sputum samples. C.G. provided intellectual input, writing and review of the data and paper. C.R. reviewed the paper. All authors read and approved the final manuscript.

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