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Original Research Article

# Increased innate and adaptive immune responses in induced sputum of young smokers

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ABSTRACT

*Background and objectives:* It is known that chronic obstructive pulmonary disease (COPD) development process is imperceptible and can be asymptomatic for 20 or more years. It is of great importance to diagnose early inflammatory changes that can lead to COPD in young asymptomatic cigarette smokers. The aim of our study was to analyze the cell spectrum of induced sputum (IS) of young cigarette smokers, with emphasis on T-regulatory cells.

*Materials and methods:* A total of 20 healthy nonallergic smokers, 20 nonsmokers and 20 COPD patients were enrolled in the study. After lung function measurements were taken, we performed sputum induction and analyzed sputum cells. We evaluated the cell count of FOXP3-positive, $CD4^+$ and $CD8^+$ T lymphocytes by immunocytochemistry staining, and the cell count of macrophages and neutrophils by May-Grünwald Giemsa staining.

*Results:* Induced sputum of smokers contained a higher absolute amount of macrophages and neutrophils when compared to nonsmokers. FOXP3-positive cells in the sputum of young smokers showed a statistically significant increase when compared to nonsmokers. Induced sputum of COPD patients contained an increased absolute amount of neutrophils and FOXP3-positive Treg cells when compared to nonsmokers. Regression analysis showed that the amount of FOXP-3 positive cells, neutrophils and macrophages in the induced sputum was increasing with the number of pack years.

*Conclusions:* This study demonstrates that young smokers have early inflammatory changes in their airways that not only initiate nonspecific mechanisms recruiting neutrophils, but also involve specific immune mechanisms with recruitment of T regulatory lymphocytes. The lymphocyte response is probably adaptive.



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## 1. Introduction

Cigarette smoking is a major risk factor for the development of COPD [1]. In addition, the clinical course of COPD can be asymptomatic for 20 or more years. The clinical symptoms due to smoking usually present themselves after approximately 40 years. The ''golden standard'' for diagnosis of COPD remains spirometry. The early diagnosis of COPD is of particular importance especially among young smokers [2].

There is an urgent need for identification of biological markers for the early stage, as well as new molecular targeting therapy for COPD. New potentially relevant biomarkers for an early noninvasive diagnosis of COPD are of particular importance and could be used as a screening tool for the populations at risk. Patients with COPD typically have at least a 10 pack-year history of smoking, but only few of the heavy smokers develop severe airflow limitation. This suggests COPD is not dependent upon the smoke exposure alone.

It is believed that the presence of inflammation in COPD together with the accumulation of components of innate immune system, such as activated macrophages and neutrophil leukocytes, is an important signifier in the disease development [1,3]. More recent studies have postulated that adaptive immune response also contributes to the pathophysiology of COPD [4]. An increased amount of neutrophils in induced sputum is a characteristic feature in patients with COPD [5].

Chronic cigarette smoke exposure increases numbers of alveolar macrophages in the airways lumen of smokers. Smokers' macrophages have an ability to inhibit effects on proliferation of lymphocytes and activities of natural killer (NK) cells [6].

An increased total number of circulating T-lymphocytes has been observed in smokers [6]. It was observed that lymphocytes and macrophages are the predominant cellular elements of the inflammatory infiltrates within the airway walls of patients with COPD. Other studies extended these observations by showing that the numbers of $CD8^+$ lymphocytes in COPD lung were directly related to the degree of airflow limitation [7]. Similarly, in the induced sputum of COPD patients there are higher levels of $CD8^+$ T-lymphocytes [8]. T-lymphocytes can cause tissue injury either by direct cytolytic activities or through the secretion of pro-inflammatory mediators that activate other immune cells. In addition to the generally potent pro-inflammatory effect of $CD4^+$ lymphocytes, a subset of these cells also may impact the progression of COPD by up-regulating the intensity of inflammatory cascades [9].

It is now clearly established that a forkhead box protein 3 (FOXP3) expressed by subset of $CD4^+$ $CD25^+$ T cells, also called regulatory T cells, is essential for the maintenance of self-tolerance and immune homeostasis [10].

$CD4^+$ $CD25^+$ T regulatory (Treg) cells are important in realizing peripheral immunological tolerance, down-regulation of persistent inflammation and prevention of autoimmune reactions by inhibition of other T cell responses [11]. Dysfunction of Treg cells can lead to autoimmune disease, allergy and chronic inflammatory diseases.

We hypothesized that T regulatory cells are involved in the pathogenesis of COPD and that assessment of the numbers of T regulatory cells in the induced sputum could serve as a biomarker for early diagnosis, prognosis and treatment of COPD.

## 2. Materials and methods

### 2.1. Ethics statement

The study was approved by the Ethics Committee of the Institute of Experimental and Clinical Medicine, University of Latvia.

### 2.2. Subjects

A total of 20 healthy nonallergic current smokers (mean age, $21.5 \pm 2.6$ years; smoking history, $3.03 \pm 3.0$ pack-years), asked to refrain from smoking at least 2 h before the sample collection, in order to exclude the acute effect, 20 nonsmokers (mean age, $22.4 \pm 2.6$ years) and 20 COPD patients (mean age, $62.3 \pm 2.6$ years; smoking history, $39.18 \pm 4.8$ pack-years) gave informed consent to participate in the study. None of the volunteers had experienced any airway infection at least within one month before the session.

### 2.3. Study design

All subjects had only one session. We performed lung function measurements, sputum induction, sputum immunocytochemistry, May-Grünwald Giemsa staining and sputum cell analyses. All subjects filled in a questionnaire about their smoking habits. Healthy volunteers were questioned about any complaints or symptoms that could be related with COPD.

Before inclusion in the trial all volunteers were informed about the study design and possible side effects, and signed an informed consent.

### 2.4. Lung function

Before sputum induction, all subjects underwent spirometry and bronchodilation test with a 200-μg salbutamol (Ventolin™, GlaxoSmithKline) inhalation. We used bronchodilators to make sputum induction easier. We repeated spirometry 15 min after inhalation of salbutamol, using MIR Spirobank II spirometer and following the American Thoracic Society (ATS) and European Respiratory Society (ERS) Spirometry standardization recommendations [12,13]. Repeated spirometry allowed us to ensure that none of the patients has asthma.

### 2.5. Sputum induction

Sputum induction was performed according to modified protocol validated by E. Pizzichini [14]. For inhalation we used constant concentration of 4% NaCl using an ultrasonic nebulizer (OMRON NE-U17, OMRON Matsusaka Co., Ltd., Japan) with a flow rate of 1 mL/min. Induction was performed for three times, each 5 min long. After each inhalation period volunteers were asked to rinse their mouth with water, to minimize contamination with saliva. Then they were asked to expectorate into a sterile container. Before each inhalation