Changes in sputum T-lymphocyte subpopulations at the onset of severe exacerbations of chronic obstructive pulmonary disease

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Summary CD8+ve T-cell responses play a primary role in chronic obstructive pulmonary disease (COPD), but there is little information regarding COPD exacerbations. Sputum induction is a relatively non-invasive and safe method to study airway inflammation. The aim of the study was to investigate changes in airway T-lymphocyte subpopulations at the onset of severe COPD exacerbations via analysis of sputum. Induced sputum samples were collected from 12 COPD patients aged (mean ± SD) 69 ± 7 years, ex-smokers (68 ± 23 pack-years), mean FEV\textsubscript{1} (%predicted) 40 ± 14 at the onset of an acute severe exacerbation requiring hospital admission and 16 weeks after remission of the exacerbation. Inflammatory cells and T-lymphocyte subpopulations (CD4, CD8, Tc1, Tc2) were measured using chemical and double immunocytochemical methods. Increased percentages of sputum neutrophils (\(P = 0.002\)) and decreased CD4/CD8 and CD8-IFN\textgamma/CD8-IL4+ve (Tc1/Tc2) cell ratios (\(P = 0.03, P = 0.02\), respectively) were found at the onset of exacerbation compared to stable state. We conclude that a CD8+ve type-2-mediated immune response is induced at the onset of severe COPD exacerbation.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation, associated with an abnormal inflammatory response of the lungs to noxious particles or gases, specifically cigarette smoke. Scientific evidence
supports a primary role for CD8+ve T-cell-mediated immunity in the development of inflammation in COPD. Increased CD8+ve T-lymphocyte numbers have been found in the airways and lung parenchyma of COPD patients in comparison to “healthy smokers” and healthy non-smokers. Moreover, a strong correlation has been found between severity of airflow limitation and the number of CD8+ve cells in bronchoalveolar lavage fluid (BALF), sputum and biopsies from COPD patients. CD8+ve cells are mainly divided into three subpopulations, depending on their cytokine profile. Type-1 cells (Tc1) produce interferon γ (IFN-γ), type-2 cells (Tc2) produce interleukin 4 (IL4), while type-0 (Tc0) cells produce both cytokines. Both Tc1 and Tc2 cells show comparable cytotoxicity. Which subpopulation of CD8+ve lymphocytes is mostly involved in COPD pathogenesis is currently under investigation. Some first evidence coming from blood and lung biopsy studies supports the predominance of type-1 T-cells.

COPD is a continuously deteriorating disease with periodic exacerbations and remissions. Patients suffering from a severe COPD exacerbation often require hospitalization. Hospital mortality of patients admitted for an acute exacerbation of COPD is approximately 10% and the long-term outcome is poor. Although increased inflammation has been presumably related to COPD exacerbations, there has been little information available on the type of inflammation at the onset of severe COPD exacerbations. This may be partly due to technical difficulties in studying such seriously diseased patients. Common methods to study inflammation on stable COPD patients, such as the examination of BALF, bronchial biopsies or surgical specimens, cannot be easily applied on the case of exacerbations. Sputum induction has been proven a safe, reliable and highly reproducible technique, which could be used for the analysis of inflammatory cell profiles on COPD exacerbations by ethically allowing further samples to be taken when patients are stable.

So far evidence suggests that sputum and tissue eosinophil numbers are increased during mild exacerbations, while tissue neutrophil numbers and neutrophilic markers during severe exacerbations. Although high tissue CD8+ve T-lymphocyte numbers have been reported in stable patients with COPD, there is no information regarding changes in T-lymphocyte populations and subpopulations on severe COPD exacerbations. Considering that infections are the most frequent cause of an exacerbation and infections trigger a T-cell-mediated immune response, we wished to test the hypothesis that an increase in sputum CD8+ cell numbers and a change in T-lymphocyte phenotypes occurs at the onset of severe COPD exacerbations. Furthermore, we wished to investigate whether there are any changes in neutrophil or eosinophil numbers associated with severe COPD exacerbations. For this reason we analyzed sputum inflammatory populations, including T-lymphocyte subpopulations, at the onset of a severe COPD exacerbation requiring hospital admission and on stable condition. Our results showed a statistically significant increase in sputum neutrophil percentages and decrease in CD4/CD8 and CD8-IFNγ/CD8-IL4+ve cell ratios on severe exacerbation relative to stable state, suggesting that a CD8+ve type-2-mediated immune response was induced at the time of the exacerbation.

Material and methods

Study subjects

Twelve patients with COPD were studied at the onset of an acute severe exacerbation and on stable condition. Diagnosis of COPD was made according to the GOLD criteria. In order to enter the study, the patient had to have satisfied the following inclusion criteria: (1) an ex-smoking history of at least 20 pack-years of smoking (2) fixed airflow limitation with salbutamol reversibility of <12% or <200 ml. Exclusion criteria included: (1) a history of allergy; (2) current smoking or history of having smoked within 2 years of assessment; (3) use of other medication than inhaled β2 agonists, anti-cholinergics and inhaled steroids; (4) history of a concomitant pulmonary or systemic disease that could predispose to severe exacerbations (e.g. bronchiectasis, heart failure); (5) long-term oxygen therapy.

Study design

Recruitment of COPD patients was made from those admitted for a severe COPD exacerbation in the Department of Thoracic Medicine in the University Hospital of Heraklion. Exacerbation was defined as severe if the patient fulfilled one of the following criteria: use of accessory respiratory muscles, paradoxical chest wall movements, respiratory failure breathing room air, requiring hospital admission. Between January and June 2002, a total of 156 admissions were recorded. Fourteen patients satisfied the inclusion and exclusion criteria and were enrolled in the study. All patients were under...
inhaled steroid treatment at the time of the enrollment.

Induced sputum samples were taken at the emergency room, where the patients referred for the treatment of the first signs of a severe exacerbation, before any therapeutic intervention was done. A chest radiograph and blood gases analysis was done. All patients were treated with systemic steroids until remission of the exacerbation and with inhaled steroids after remission of the exacerbation. No change in any of the patients regular medication was introduced after recovery from the exacerbation. Twelve out of the 14 patients were reassessed on a scheduled visit 16 weeks after remission of the exacerbation. Two patients were excluded from the study because they experienced another exacerbation and/or an acute respiratory tract infection within the 16 weeks period. Induced sputum samples were collected again at stable condition; spirometry, skin-prick reaction tests and blood gases analysis were also performed.

Induced sputum from exacerbation and stable state of the same patient was processed for analysis of inflammatory cells and T-lymphocyte subsets and sent to the microbiology department for detection of common microorganisms (gram stain and bacterial cultures for aerobes and anaerobes). An acute bacterial infection on exacerbation state was defined as demonstration of a new pathogenic organism cultured from sputum on the day of exacerbation, but not cultured at baseline. The Ethical Committee of the University Hospital of Heraklion in Crete, Greece, approved the protocol and all subjects gave their written consent.

Methods

Sputum induction and processing

Sputum was induced and processed based on the method of Pin et al., as previously described.23,24 Lymphocytes were stimulated by incubating the cell suspension in 24-well plates at a concentration of 2 × 10^6 cells/µl for 5 h, under 5% CO₂, at 37 °C, in RPMI-1640 at 10% FCS (Invitrogen Corporation Carlsbad, California, USA) in the presence of phorbol 12-myristate 13-acetate 25 ng/ml, ionomycin 1 µmol/L and brebeldin A 10 µg/ml (Sigma ST Louis, Missouri, USA). Cytospins were made using cytocentrifugation of 50 µl of a 3.5 × 10^6 cells/µl cell suspension and were stored at −80°C for immunocytochemical analysis later. Approximately 175,000 cells were cytospined on each slide, among which there were sufficient number of lymphocytes to stain.

Immunocytochemical analysis

After defrosting the cytospins they were fixed in acetone and rehydrated in TBS. The double immunocytochemical method was performed as previously described.21 Two investigators examined the slides under double visual and ultraviolet microscope and their results were averaged. The between-observer coefficient of variation was <15%. The visual filter was used to differentiate cells by morphology and the UV filter to count fluorescent lymphocytes in the same optical field. Three replicate measurements were performed by each observer in 10 slides. The within observer repeatability was tested using agreement analysis and the K coefficient was always >0.8. Results were expressed as ratio of CD4+ve to CD8+ve cells and of CD8+ve-IFNγ producing T-cells to CD8+ve-IL4 producing T-cells. For the estimation of each ratio 500 T-cells were counted for each patient, with more than one cytospin stained if necessary, because this number was sufficient to obtain a mean value per subject that remained constant after further increasing the number of cells counted.

Measurement of CD4+ ve and CD8+ ve T-cells: The primary anti-CD4 mouse anti-human monoclonal antibody (Caltag Burlingame, CA, USA) with secondary rabbit anti-mouse IgG-FITC antibody (Immunotech Marseille, France) and the primary anti-CD8 mouse anti-human monoclonal antibody (Caltag) with secondary rabbit anti-mouse IgG-phycocerythrin-conjugated (IgG-PE) antibody (Immunotech) were used. Five hundred CD4+ve and CD8+ve cells were counted to estimate CD4/CD8 ratio.

Measurement of CD8+ve-IFNγ producing and CD8+ve-IL4 producing T-cells: The primary anti-CD8 mouse anti-human monoclonal antibody with secondary rabbit anti-mouse IgG-FITC antibody and the primary anti-IFNγ; mouse anti-human monoclonal antibody (Caltag) with secondary rabbit anti-mouse IgG-phycocerythrin-conjugated (IgG-PE) antibody were used. Five hundred CD8+ve cells were counted to estimate the number of CD8-IFNγ+ve cells. The same method was applied for staining CD8+ve cells with anti-IL4 antibody (Caltag).

Statistical analysis

Data are presented as mean ± SD for normally and as median (range) for non-normally distributed variables. Differences between stable state and exacerbation state were tested using the Wilcoxon
signed-rank test for non-normally and the paired \( t \)-test for normally distributed variables. Normality was tested by the Shapiro–Wilk test. The statistical software StatsDirect (Camcode Cambridge, UK) was used for the entire analysis. A \( P \)-value of < 0.05 was considered statistically significant.

Due to significant comorbidities in the majority of COPD patients admitted for severe exacerbation, we were only able to recruit a relatively limited number of patients. The significant differences of all variables compared were evaluated against the criterion, alpha. According to the data and the results of the study: (a) number of patients (sample size), (b) differences obtained for all variables tested (effect size) and, (c) criterion \( \alpha = 0.05 \) the power was always greater than 80%. Therefore, it is unlikely that a type 1 error has occurred.

**Results**

**Subjects**

Demographic data, baseline and exacerbation characteristics of all subjects are given in Table 1. All had negative skin-prick reaction tests and serum IgE levels within normal range. All patients received the same medication at the onset of the exacerbation and on stable state. No subject was able to perform acceptable spirometry at the onset of exacerbation. A clear deterioration in cough, sputum and blood gases analysis was observed in all subjects on exacerbation. All had a \( P_O2 < 60 \text{mmHg} \) at the time of the exacerbation. Five patients had positive sputum cultures at the onset of exacerbation (three for \( S. \text{pneumoniae} \) and two for \( P.s. \text{aeroginosa} \)). Chest radiographs at the time of exacerbation did not reveal pneumonia or pneumothorax in any patient.

**Cellular composition**

Viability of cells recovered by sputum processing was 89(59–99)% median (range) and 89(63–98)% on exacerbation and on stable state, respectively. The percentage of squamous epithelial cells among total cells was 0.2(0.04–1)% on exacerbation and 1.3(0.1–4.7)% on stable conditions. No statistically significant difference was observed in cell viability or squamous cell contamination between exacerbation and stable state samples. Table 2 shows sputum cellularity in both states. Percentages of neutrophils were significantly higher on exacerbation (\( P = 0.002 \)). In contrast, percentages of macrophages were significantly lower at the onset of exacerbation (\( P = 0.003 \)). No statistically significant difference was observed in percentages of eosinophils, lymphocytes or total non-squamous cell count between exacerbation and stable state.

A lower CD4/CD8+ cell ratio (\( P = 0.03 \)) and a lower CD8-IFN/CD8-IL4+ cell ratio (\( P = 0.02 \)) were found at the onset of exacerbation (Figs. 1 and 2).

No difference was observed in any of the above cell counts or ratios between subjects with bacterial +ve or -ve cultures at the onset of exacerbation.

| Table 1 Demographic data of the subjects on stable state and at the onset of exacerbation. |
|---------------------------------------------|--------|
| Male                                       | 11     |
| Female                                     | 1      |
| Age                                        | 69 ± 7 yr |
| Smoking history                            | 68 ± 23 p/yr |
| Inh steroid intake                         | 500 (500–1000) mg fl or 400(400–600) mg bd |
| FEV\(_1\) (%pred)                          | 40 ± 14 |
| FEV\(_1\)/FVC (%pred)                      | 53 ± 11 |
| \( \Delta \)FEV\(_1\) (%baseline)          | \( -4.9 \) |
| \( P_O2 \)                                 | 72 ± 11 |
| \( P_CO2 \)                                | 39 ± 5  |
| Purulent sputum                            | 0/12   |
| Positive sputum cultures                   | 0/12   |
| Cough                                      | 4/12   |
| Dyspnea at rest                            | 3/12   |

Data are presented as mean ± SD for normally and as median (range) for non-normally distributed variables. Abbreviations: \( p/yrs \): pack/years, Inh: inhaled, fl: fluticasone, and bd: budesonide.

\(^*\) \( P < 0.05 \).

\(^{**}\) Subjects were not able to perform proper spirometric test at the onset of severe exacerbation.
**Discussion**

Although T-cell-mediated immunity has been associated with stable COPD, relatively little is known about the role of T-cells in severe exacerbations. This study examined changes in airway T-lymphocyte subpopulations at the onset of severe COPD exacerbations requiring hospital admission. Our results showed decreased CD4/CD8 and CD8-IFNg/CD8-IL4+ve cell ratios on exacerbation relative to clinically stable condition. This evidence suggests that an imbalance in T-lymphocyte subpopulations might be associated with the development of severe COPD exacerbations.
Sputum lymphocyte subpopulations were studied by immunocytochemical methods. Although the number of T-lymphocytes in sputum relative to other biological materials is low, by using a highly concentrated sample of sputum cells on the preparations of our slides, we achieved to get a good number of lymphocytes. Before immunocytochemistry T-lymphocytes were stimulated with PMA and ionomycin. Although after differentiation into Tc1 and Tc2 subtypes, T-lymphocytes show a stable cytokine pattern and rarely, if ever, switch to the opposite phenotype, this intervention might have altered the cytokine profile of the cells. However, differences found in T-cell subsets in vitro must represent real differences in vivo, since all samples were stimulated in the same way.

Considering important to achieve high repeatability of our results, we tried as much as possible to eliminate variability in sputum sampling. A standard saline concentration was always used (N/S) for all patients. Moreover, all sputum induction and processing were performed from the same investigator. By using a relatively high cell count per slide and by minimizing variability in sputum sampling, we achieved good agreement in the analysis of different sputum aliquots. Ten different slides from the same sputum sample of 5 patients were stained for T-lymphocyte subpopulations and counted. Intra-sample coefficient of variation was <7%. Repeatability of our results within interval of 2 days was tested and found to be >90% in the same patient.

Previous studies in patients with COPD have shown increased tissue CD8+ve T-lymphocytes and a close correlation between the number of these cells and severity of airflow limitation. We have recently shown that COPD patients demonstrate decreased sputum CD4/CD8+ve cell ratios and increased CD8+ve cell periforin expression and cytotoxic activity relative to "healthy smokers". However, the exact role of CD8+ve cells in the pathophysiology of COPD is not yet fully understood. Interesting evidence suggests that they can cause a TNF-α-mediated lung injury. Saetta et al. have suggested that an excessive recruitment of CD8+ve T-lymphocytes may occur in response to repeated viral infections in some smokers, and that this excessive response may damage the lungs of these subjects.

Our results showed similar sputum lymphocyte counts between exacerbation and stable state, but significantly decreased CD4/CD8+ve cell ratios at the onset of severe COPD exacerbation requiring hospital admission (P = 0.03) (Fig. 1). Therefore, a further shift towards the CD8+ve cell-mediated immune response was seen on severe COPD exacerbation. Such a response may have been induced by viral or bacterial respiratory tract infections, which are thought to be the most common triggers of acute COPD exacerbations. Indeed, 6 patients presented with purulent sputum at the onset of the exacerbation and 5 out of these 6 had positive cultures on exacerbation and negative on stable state (Table 1).

No difference was observed in CD4/CD8+ve cell ratios between patients with bacterial +ve or –ve exacerbation. Although the relatively limited number of patients included in the present study does not allow us to make safe conclusions, a plausible explanation is that viral infections triggered the CD8+ve response in patients with bacterial –ve exacerbations.

Despite the statistically significant difference in CD4/CD8+ve cell ratio between exacerbation and stable state, 4 patients showed either no change or slight increase in their ratio (Fig. 1). This phenomenon was observed irrespective of the presence of bacterial positive sputum cultures on exacerbation. COPD patients presenting with decreased CD4/CD8+ve cell ratio on exacerbation could represent an interesting phenotype related to accelerated airway inflammation and disease deterioration. On the other hand, some patients may not increase their CD8+ve cell numbers on exacerbation due to an already maximal increase in the CD8+ve response under stable conditions or because the triggering factor of their exacerbation is other than respiratory tract infection. Either hypothesis needs to be further investigated for any conclusions to be made safely.

Saetta et al. have shown increased CD3+ (T-cell) numbers in endobronchial biopsies from COPD patients on mild exacerbations, but no difference in CD4 or CD8+cell numbers. This seems to come in contrast to our findings of decreased CD4/CD8+ve cell ratios at the onset of severe COPD exacerbation. However, it is not safe to make comparisons between ratios and absolute numbers. Moreover, different results could be attributed to the fact that we have studied severe COPD patients on severe exacerbations or that we have examined sputum samples and not endobronchial biopsies.

We have gone further in evaluating this increase in CD8+ve T-lymphocytes and found significantly decreased CD8+IFNγ/CD8-IL4+ve cell ratios in COPD patients on severe exacerbation, irrespective of the presence of bacterial positive sputum cultures on exacerbation (P = 0.02) (Fig. 2). Since Tc0 cells are IFNγ as well as IL4+ve, lower CD8-IFNγ/CD8-IL4+ve cell ratio means lower Tc1/Tc2 ratio. Therefore, the Tc2-type reaction is enhanced on severe COPD exacerbations. Saetta et al. reports high
Tc1 numbers in peripheral airways of stable COPD patients when compared to healthy smokers or to non-smokers, while a type-1 cytokine phenotype has been also demonstrated in peripheral blood. The switch towards the Tc2 phenotype on acute severe exacerbations of COPD may be implicated in the inflammatory processes of severe exacerbations. Tc2 cells do not produce INF\textsubscript{\gamma} and reduced ability of producing INF\textsubscript{\gamma} prevents the elimination of pathogens.\textsuperscript{27,28} Reduced anti-viral activity of Tc2 cells in vivo may not always be due to INF\textsubscript{\gamma} secretion but to decreased expression or lower activity of adhesion molecules.\textsuperscript{29} Furthermore, Tc2 cells produce IL4, which down-regulates some steps of cell-mediated responses that are required for host protection.\textsuperscript{27,28} Therefore, an increase in Tc2 cells could lead to severe respiratory tract infection and therefore severe COPD exacerbation.

Although the overall difference between exacerbation and stable state was statistically significant, 4 patients showed almost no change in their sputum CD8-INF\textsubscript{\gamma}/CD8-IL4+ve cell ratio between exacerbation and stable state. This was not related to the presence of bacterial positive sputum cultures on exacerbation. Among those patients, 2 had also shown no change in CD4/CD8+ve cell ratio. Obviously, changes in lymphocyte subpopulations do not occur in all COPD patients at the onset of acute exacerbation, which supports the hypothesis of more than one phenotypic reaction on acute COPD exacerbation. Should this hypothesis be confirmed, it would be of interest to investigate the natural history of different exacerbation phenotypes.

Increased percentages of sputum neutrophils were demonstrated at the onset of bacterial +ve or –ve COPD exacerbations requiring hospital admission. Saetta et al.\textsuperscript{11} and Qiu et al.\textsuperscript{20} have reported a similar increase in neutrophil numbers in endobronchial biopsies taken from COPD patients on mild or severe exacerbation, respectively. Furthermore, the neutrophil chemoattractants CXCL8, CXCR1, CXCL5 and CXCR2 appear to play important roles in the airway neutrophilia characteristic of severe exacerbations. Taken together, these results and ours provide convincing evidence that neutrophils are implicated in the pathogenesis of COPD exacerbations. Neutrophils could be involved in the pathology of COPD exacerbations through the release of proteolytic enzymes and reactive oxygen species.\textsuperscript{30}

Aaron et al.\textsuperscript{18} showed that COPD patients exhibit prominent sputum neutrophilia during acute exacerbations, independent of a demonstrable viral or bacterial airway infection. On the contrary, Sethi et al.\textsuperscript{15} demonstrated increased airway inflammation in patients with H. influenza and M. catarhalis exacerbations when compared to H. parainfluenza and bacterial –ve exacerbations. It could be possible that Aaron et al. and we did not find a correlation between airway inflammation and bacterial +ve exacerbations due to the small number of patients included in both studies.

On contrary to neutrophils, no difference was found in sputum eosinophil percentages or numbers between severe COPD exacerbation and stable state. These results confirm previous evidence that eosinophils are involved in mild exacerbations, while neutrophils in severe COPD exacerbations.\textsuperscript{11,12,16,20}

In conclusion, COPD patients at the beginning of their hospitalization for a severe exacerbation demonstrated increased sputum neutrophil percentages and decreased CD4/CD8 and Tc1/Tc2 ratios. These findings suggest that a CD8+ve type-2 exacerbation phenotype may be associated with increasing severity of COPD exacerbations and indicate future therapeutic strategies aimed at regulating CD8+ve type-2-mediated immune responses on severe COPD exacerbations. However, further studies are needed before phenotypes of T-cell-mediated immune responses at the onset of acute exacerbations of COPD are well defined.

References

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