Tc2 Response at the Onset of COPD Exacerbations*

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Background: T lymphocytes and especially the subpopulations of CD8+ cells are believed to have a key role in COPD pathophysiology, but there are only few data regarding the role of these cells in COPD exacerbation.

Aim: We aimed to study prospectively changes of CD8+ T-lymphocyte subpopulations in the sputum of COPD patients at the onset of mild exacerbations and at a stable condition in order to provide further insight in the pathophysiology of the disease.

Methods: Induced-sputum samples were collected from 24 COPD patients with median age of 52 years (interquartile range [IQR], 44 to 58 years) and FEV1 percentage of predicted of 78.05% (IQR, 75.8 to 80.1%) at the onset of mild exacerbations not requiring hospitalization and when stable. Inflammatory cells and T-lymphocyte subpopulations (CD4+, CD8+, and cells producing interferon [IFN]-γ or interleukin [IL]-4) were measured using flow cytometry and immunocytochemical methods.

Results: A significant increase in sputum CD8+ T lymphocytes (p < 0.0001) and significant decreases in CD4+ T lymphocytes as well as in CD4+/CD8+ (p = 0.0001) and CD8+IFN-γ+/CD8+IL-4+ (p = 0.001), CD4+IFN-γ+/CD4+IL-4+ (p = 0.0003) sputum cells ratios were found decreased at the onset of exacerbations compared to stable condition. The changes in T-lymphocyte subpopulations were not associated with smoking history, demographic characteristics, or disease severity.

Conclusion: The findings of the present study suggest that CD8+ lymphocytes are increased and potentially polarized toward a Tc2 profile in the airways of COPD patients at the onset of COPD exacerbations with respect to stable condition. The clinical impact of the observed phenomenon requires further investigation.

Key words: COPD; exacerbations; lymphocytes

Abbreviations: FITC = fluorescein isothiocyanate; IFN = interferon; IL = interleukin; IQR = interquartile range; PE = phycoerythrin; sIFN-γ = supernatant interferon-γ; sIL-4 = supernatant interleukin-4; Th1 = T helper type-1

COPD is characterized by an abnormal airway inflammation.1 Previous studies2-3 have shown that airway inflammatory cells may be implicated in the complex pathogenesis of the disease, although the exact role of each cell is still under investigation. T lymphocytes are believed to orchestrate immune responses to exogenous stimuli.4,5 Previous investigations3,6 showed that the number of CD8+ T cells in the lungs and blood was increased compared to that of CD4+ T cells, and that the CD4+/CD8+ T-cell ratio was negatively correlated with lung function in COPD patients. However, little is known about CD8+ T-cell contribution to the pathology of COPD.

The role of these immune cells might be crucial not only in stable disease but also in COPD exacerbations that are caused primarily by infections, resulting in augmented inflammation, physiologic deterioration, and morbidity.7,8 CD8+ cells can produce interferon (IFN)-γ (Tc1), or interleukin (IL)-4 (Tc2), or possibly both (Tc0), and they are crucial in the response to infections.9 However, there is a lack of studies addressing this issue. A previous study10 reported a CD8+ type-2–mediated immune response at the onset of exacerbations. Nevertheless, only severe exacerbations requiring hospitalization were included in that investigation, while the popu-
ation consisted of patients with severe COPD. Evidence regarding T-lymphocyte subpopulation changes during mild exacerbations, which represent the majority of exacerbations in COPD, is limited.11–13 In the present investigation, we aimed to analyze changes of T-lymphocyte subsets in induced sputum of COPD patients at the onset of mild exacerbations not requiring hospitalization compared to stable disease.

**Materials and Methods**

**Subjects and Protocol**

A total of 24 subjects with COPD were examined at the onset of an exacerbation and in stable condition. Patients were recruited between from 2005 to 2006 by consecutive sampling from a community-based outpatient primary medical clinic. All patients who sought medical assistance in the clinic during the study period were asked about their COPD exacerbation-related symptoms medical history, and then they were included in the study if they satisfied all the following criteria: (1) COPD diagnosis according to the Global Initiative for Chronic Obstructive Lung Disease consensus statement1; (2) initiation of symptoms diagnostic for COPD exacerbation in the past 72 h; (3) abstention from any new therapeutic intervention; and (4) absence of any signs suggestive of severe exacerbation requiring hospitalization.1

Patients with asthma or other respiratory diseases were excluded from this study. Sputum was induced at the emergency department of the outpatient clinic both at exacerbation and in stable condition (at least 8 weeks after exacerbation) in the same patient; samples were processed for analysis of inflammatory cells and cytokines and microbiology. The definition of an exacerbation was based on the criteria described by Anthonisen et al,14 requiring either the presence of at least two major symptoms (increase in dyspnea, sputum production, purulence) or of one major symptom in addition to at least one minor symptom (wheeze, cough, nasal discharge, sore throat, fever) for 2 consecutive days. Exacerbations were categorized as type I or type II or type III, if all three, two, or one major symptoms were experienced, respectively. Postbronchodilator spirometry was performed with a computerized system (MasterLab 2.12; Jaeger; Wuerzburg, Germany) according to guidelines.15 The study was approved by the Research Ethics Committee of the hospital, and patients gave informed consent.

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**Sputum Induction and Processing**

Sputum was induced and processed as previously described16 (see online data supplement for details). Cell differential counts were expressed as percentage of nonsquamous cells.

**Antibody Labeling**

The following mouse anti-human monoclonal antibodies were used for labeling sputum cells: anti-CD3-fluorescein isothiocyanate (FITC), anti-CD8-phycocerythrin (PE), anti-IFN-γ, anti-IL-4, anti-CD4, anti-CD8, as well as rabbit anti-mouse IgG-FITC and IgG-PE were used (Immunotech; Marseille, France). Isotypic antibodies mouse-anti-mouse IgG were used as control subjects. For staining, immunocytochemistry was used after plating the cells on Superfrost Plus slides (Erie Scientific Company; Portsmouth, NH) by cytocentrifugation with the kit of DAKO (Dako; Glostrup, Denmark) according to instructions of the manufacturer. The method used has been previously described.16

**Immunocytochemical Analysis**

Immunocytochemical analysis and T-cell determination were performed in sputum cytospins. Sputum lymphocytes were stimulated in 24-well plates in RPMI-1640 at 10% fetal calf serum in the presence of phorbol 12-myristate 13 acetate, 25 ng/mL; ionomycin, 1 μmol; and Brefeldin A, 10 μg/mL (Sigma-Aldrich; St. Louis, MO). Cytospins were made using cytocentrifugation of sputum of COPD patients at the onset of mild exacerbations not requiring hospitalization compared to stable disease.

**Flow Cytometric Analysis**

Sputum samples were analyzed on a fluorescence-activated cytometer (EPICS ELITE; Coultronics; Louton, UK). The lymphocytes were tightly gated by volume and complexity on a forward (0°) and side light scattering (90°) mode. Phycocyanate-conjugated anti-human CD45 monoclonal antibodies (DAKO; Ely, UK) were used to exclude nonlymphocyte events by logical gating, and the QC-Combo Kit (FCSC; San Juan, Puerto Rico) was used for quantification of antibody binding (see data supplement for more details).

**Cytokine Assays**

IL-4 and IFN-γ in supernatant of the samples (supernatant IFN-γ [sIFN-γ] and supernatant IL-4 [sIL-4]) were measured using a high-sensitivity enzyme-linked immunosorbent assay (R&D Systems; Minneapolis, MN). The reproducibility of these assays was confirmed by performing repeated measurements on...
successive days. The intraclass coefficient (R) for both measurements was between 0.96 and 0.99. Investigators performing the cytokine assays were kept blinded to the sequence of the sputum specimens.

Statistical Analysis

Normality of the numerical parameters was tested using the Kolmogorov-Smirnov test. Wilcoxon signed-rank test for non-parametric outcomes and paired t test for parametric outcomes were used for comparison of data at two different time points (at stable condition and at exacerbation). Data and results presented refer to immunocytochemical analysis. Statistical software (SPSS version 11.0; SPSS; Chicago, IL) was used for analysis; p < 0.05 was considered statistically significant.

The analyses were repeated using the values obtained by flow cytometry. The results were very similar to the above and therefore are not presented in detail.

Results

Baseline characteristics of participants are presented in Table 1. According to the Global Initiative for Chronic Obstructive Lung Disease severity of COPD, 6 patients (25%) were stage 0–I, 13 patients (54%) were stage II, and 5 patients (21%) were stage III.

Sputum Induction

An adequate sputum sample was obtained from all but two subjects at exacerbation onset and by all subjects in stable condition. Hence, these two patients were excluded from the study leaving 22 patients for analysis. The median time interval between exacerbation and stable condition was 90 days (interquartile range [IQR], 55 to 145 days). The total cell count (×10^7 cells per gram) was significantly higher at the exacerbation onset than in stable condition: median, 3.3 (IQR, 2.5 to 4.2) vs median, 1 (IQR, 0.7 to 1.25) [p = 0.001]. The percentage viability of cells recovered by sputum induction was 85.5% (IQR, 82.4 to 95.5%) and 92.5% (IQR, 87 to 95.5%) at exacerbation and in stable condition, respectively. The percentage of squamous epithelial cells on differential counting was 15.2% (IQR, 12.4 to 18.1%) and 8.5% (IQR, 5.2 to 14.5%) at exacerbation and in stable condition, respectively. Significant differences between exacerbation and stable condition were observed in percentages of neutrophils and macrophages (p < 0.001) [Fig 1].

Sputum Lymphocyte Subpopulations and Inflammatory Indexes

The percentage of sputum CDS+ T lymphocytes among lymphocytes was found significantly increased at exacerbations compared to stable condition: 36% (IQR, 32 to 40%) vs 25% (IQR, 17.5 to 28%), p < 0.0001 (Fig 2). The percentage of sputum CD4+ T lymphocytes was found decreased at the onset of exacerbation compared to stable condition: 32.5% (IQR, 30 to 38%) vs 43.5% (IQR, 40 to 46%), p = 0.0002. Thus, sputum CD4+/CDS+ cell ratios were significantly lower at the onset of exacerbation.
Figure 2. Percentage of CD4+ and CD8+ cells among lymphocytes in induced sputum from COPD patients in stable state and at the onset of exacerbations as obtained by immunocytochemistry.

compared to stable condition: 0.9 (IQR, 0.78 to 1.06) vs 1.7 (IQR, 1.4 to 2.6), p = 0.0001.

Sputum CD8+IFN-γ+ cells percentage among lymphocytes was not significantly different between exacerbation and stable condition (21%; IQR, 12.5 to 24%; vs 20%; IQR, 12.5 to 22; p = 0.4), whereas sputum CD8+IL-4+ percentage was increased (1%; IQR, 0.3 to 14%; vs 0.1%; IQR, 0.1 to 0.3%; p = 0.0001). CD8+IFN-γ+/CD8+IL-4+ cell ratios were significantly lower at the onset of exacerbation compared to stable condition: 21 (IQR, 0.8 to 69) vs 45 (IQR, 112 to 215), p = 0.0003 (Fig 3).

Sputum CD4+IFN-γ+ cell percentages among lymphocytes at exacerbation and stable condition were 25.5% (IQR, 22.5 to 28.5%) and 32.5% (IQR, 30.5 to 35.5%) [p = 0.001], and CD4+IL-4+ cell percentages were 0.1% (IQR, 0.1 to 0.2%) and 0.1% (IQR, 0.1 to 0.2%) [p = 0.4], respectively. CD4+IFN-γ+/CD4+IL-4+ cell ratio was decreased at exacerbation onset (p = 0.001). sIL-4 and sIFN-γ (pg/mL) were found significantly increased at exacerbation compared to stable condition values: 2,475 pg/mL (IQR, 1,970 to 3,130 pg/mL) vs 32.2 pg/mL (IQR, 25 to 43.5 pg/mL), p < 0.001; and 24,100 pg/mL (IQR, 15,580 to 34,170 pg/mL) vs 11,600 pg/mL (IQR, 4,600 to 27,080 pg/mL), p < 0.001, respectively. sIFN-γ/sIL-4 ratios were decreased at exacerbation.

No statistically significant relationship was found between the change (expressed as exacerbation values/stable condition values) of sputum CD4+/CD8+ or CD4+, CD8+ cell subpopulation ratios and exacerbation type, age, sex, pack years, smoking status, FEV1 percentage of predicted condition, and FEV1 percentage of predicted change between exacerbation and stable condition.

Discussion

The present prospective study investigated the changes in airway T-lymphocyte subpopulations at the onset of mild exacerbations in COPD patients. A significant increase in sputum CD8+ and a significant decrease in CD4+/CD8+ and CD8+IFN-γ+/CD8+IL-4+ sputum cells ratios were demonstrated at exacerbation onset compared to stable condition. These changes were not associated with smoking history, demographic characteristics, or disease severity. The findings of the present investigation are complementary to the results of a previous study10 that reported a CD8+ type-2–mediated immune response at the onset of severe exacerbations requiring hospitalization and underline the potentially important role of T lymphocytes in COPD exacerbations.

The role of T lymphocytes—especially of CD8+ cells—in COPD pathobiology has been underlined by previous studies.3,17 However, it is not yet clear whether T cells are polarized toward a Tc1 T helper type-1 (Th1) or a Tc2 Th helper type-2 profile in stable COPD patients.18–20 Furthermore, there is little evidence with respect of the changes of T-cell subpopulations that occur during exacerbations. Our findings suggested that CD8+ lymphocytes are increased at the onset of COPD exacerbations, while a Tc2 type response predominated. Although a switch to Th helper type-2 response was also observed, the percentage of CD4+ cells among lymphocytes were not increased at exacerbation. Thus, the involvement of CD4+ cells in COPD was not obvious in our study. We acknowledge that we have studied patients initially at exacerbation and then at stable state, whereas the opposite might have provided an even better insight into COPD exacerbation pathogenesis and might have given us the opportunity to notify a consistency of our findings in a sequence of exacerbations. Nevertheless, our findings still provide useful information for the role of lymphocytes in COPD exacerbations.
The increased percentage of CD8+ T cells at the onset of exacerbations compared to that found in stable condition may represent a response to exogenous insults that possibly take place during exacerbations, such as infections. However, CD8+ cells (both Tc1 and Tc2) are also cytotoxic.21 Their increased numbers might cause active tissue damage via the release of lytic substances such as perforin and granzyme or through the Fas ligand pathway. Tumor necrosis factor-α-mediated lung injury.22,23 On this basis, these cells might provoke tissue damage and consequently development of emphysema during repeated infectious exacerbations of COPD.

In the present study the percentage of sputum CD8+IL4+ cells among sputum lymphocytes was significantly increased and CD8+IFN-γ+/CD8+IL-4+ ratios were found decreased at the onset of exacerbations (Fig 3). This suggests an enhanced Tc2 type reaction at the exacerbation onset that would favor bronchial hyperresponsiveness.24 Furthermore, any antigen getting into the major histocompatibility complex class I pathway (CD8+ cell activation) at the beginning of an immune response tends to produce a response with Th1 bias,25,26 which is critical for the elimination of pathogens.9 However, this was not observed in our COPD patients at exacerbation. Therefore, a suppressed Th1 response and an augmented Tc2 response at the onset of an infectious exacerbation might partly explain the recruitment of inflammatory cells in the airway tissue, the prolonged inflammation, and physiologic deterioration seen at COPD exacerbations.11,27

It should be underlined here that although our results suggest that a Tc2 response may be implicated in the inflammatory process of COPD exacerbations, few patients presented a shift toward Tc1 response (Fig 3). These patients did not differ to the rest of our patients in terms of baseline clinical characteristics, disease severity, or type of exacerbation experienced. A plausible explanation for this disparity could be that this phenomenon represents a different phenotype of the disease. It would be interesting to explore it in a future investigation and to see whether different immunophenotypes during exacerbations are associated with other parameters of the disease, such as exacerbation frequency or lung function decline, which were not taken into account in this study.

The definition of COPD exacerbation is still under discussion, and exacerbations can be categorized in terms of either clinical presentation or health-care resource utilization.28 In the present study, we used Antonisen’s criteria for exacerbation diagnosis. One might argue that Antonisen’s criteria depend on patient reports of symptoms, and in this respect are subjective, not “solid,” and therefore of low importance. However, these criteria are applied widely in medical practice playing important role in treatment decisions.13,29

Despite the fact that a definitive cause of exacerbations could not be established in our study, we analyzed exacerbations according to their clinical complexity.14,28 No significant association was found between the complexity of exacerbations and lymphocyte subpopulation changes, cell ratios, and sIFN-γ/sIL-4 ratios. Thus, it is likely that the shift toward a type 2 response is a phenomenon that occurs irrespectively to the clinical complexity of exacerbations. Considering that a similar response has been also observed in COPD patients with severe exacerbations,10 our findings suggest that this immune response might be present both in mild and severe exacerbations.

In the present study, we analyzed airway cells by using sputum induction, which is a valid method for assessing inflammation in the human airway in vivo, and is less invasive than BAL. We used previously accepted methods for induction and processing,17,20 and we aimed to eliminate variability in sampling by using the same induction time and solution for all patients. However, there are certain drawbacks in the sputum examination compared to other methods such as blood or BAL fluid examination. The low percentage of lymphocytes in sputum demands laborious work in order to obtain a sufficient number of cells for immunocytochemical analysis. Thus, for the estimation of each ratio in the study, many cytosprins have to be examined. In addition, dithiothreitol and various maneuvers of sputum processing may produce damage in cell membrane, although it seems that lymphocytes are relatively resistant to these maneuvers in respect to other sputum cells. We certainly acknowledge that these drawbacks in current sputum examination methods may be inevitable. However, they are likely to affect all samples at the same degree, allowing reliable comparisons of inflammatory indexes and, in this respect, our end point, which was CD8+ subpopulation changes at exacerbation onset and stable condition, could be assessed.

In conclusion, the findings of the present study suggest that CD8+ lymphocytes are increased and potentially polarized toward a Tc2 profile in the airways of COPD patients at the onset of COPD exacerbations, unlike stable condition. Thus, we hypothesize that during COPD exacerbations, the immune response targeted to the elimination of pathogens might be reduced and this could result in tissue damage. Further investigation is required to investigate how different causes of COPD exacerbation modify the immune response that occurs during
exacerbation and to determine the clinical impact of the observed phenomenon.

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