

Tregs and HLA-DR Expression in Sputum Cells of COPD Patients Treated with Tiotropium and Formoterol

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Abstract

Immune cells expressing the activation markers HLA-DR and regulatory T cells (Tregs) may be involved in the regulation of chronic inflammation in chronic obstructive pulmonary disease (COPD). In this study we analyzed native and activated cell profiles in sputum of 22 stable COPD patients receiving formoterol (F) or formoterol + tiotropium (F + T) for 3 months. Cells were isolated from induced sputum and were examined on Coulter flow cytometer using fluorescent antibodies specific for CD3, CD4, CD8, CD14, CD19, CD25, CD127, and HLA-DR antigens. Cell profiles and cell activation were assessed by analysis of HLA-DR, CD25, and CD127 co-expression in double-stained samples. Tregs were defined as CD4⁺CD25^{high} CD127^{low} cells. We found that the combined therapy significantly decreased the CD8⁺ cell number ($p < 0.01$). At baseline, HLA-DR was expressed in about 10 % of sputum T or B cells and a higher expression was found on monocytes. The HLA-DR expression on lymphocytes, but not monocytes, was significantly lower ($p < 0.01$) in patients treated with F + T. Fractions of activated [CD4⁺ CD25⁺] cells were also significantly lower in the combined therapy group, except for the subpopulation of CD4⁺CD25^{high} CD127^{low} cells which was not altered. We conclude that tiotropium in add-on therapy to formoterol affects Treg cell profiles and decreases HLA-DR expression in airway lymphocytes.

Keywords

Airway inflammation • Long acting beta adrenoceptor agonist • MHC class II cell surface receptor • Obstructive lung disease • Regulatory T cells

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1 Introduction

The incidence of chronic obstructive pulmonary disease (COPD) is constantly increasing and a high prevalence of the disease and its morbidity and mortality still present a challenging problem. The main reason for progressively reduced lung function in COPD is local and systemic inflammation which is not only resistant to steroids but persists despite smoking cessation (GOLD 2013; Viegi et al. 2007). Different cells are involved in inflammation in COPD, including macrophages, neutrophils, epithelial cells, T-cells, B-cells, fibroblasts, and also airway smooth muscle cells. Activated cells release cytokines, chemokines, and proteases producing specific inflammatory outline (GOLD 2013). Moreover, different immune mechanisms are involved in the pathomechanism of COPD and both clinical and experimental data indicate that inflammatory cell recruitment, T cell activation, and autoimmunity play a role at different stages of the disease (Faner et al. 2013a). Recent evidence points to the immune cells expressing the activation marker HLA-DR (Faner et al. 2013b) and regulatory T cells (Tregs) (Hou et al. 2013). HLA-DR is a marker of immune stimulation. The most important function of HLA-DR-positive cells is to present antigens to the immune system and to affect the response of suppressing T-cells. Treg cells are CD4⁺ T lymphocytes that highly express CD25 surface antigen but have low expression of CD127 antigen. They control the inflammatory process in pathology, but also play a role in autoimmunity as increased Tregs prevent the development of autoimmune diseases (Dasgupta and Saxena 2012). We have previously shown altered histone acetylation and modified expression of molecules relevant to inflammatory signaling in induced sputum cells of COPD patients on add-on tiotropium therapy (Holownia et al. 2010, 2013a, b) pointing to a possible role of the drug in the immune response. Inhibition of acetylcholine-mediated release of chemotactic molecules by tiotropium can also block neutrophil migration and inflammation (Bühling et al. 2007). Considering a major role of local inflammation in COPD, the aim of

the present study was to assess the Treg profiles and HLA-DR expression in sputum cells of COPD patients treated with tiotropium in add-on therapy to formoterol.

2 Methods

2.1 Subjects and Treatment

The study has been approved by the Ethics Committee for Human Research of Medical University in Białystok, Poland and informed consent was obtained from the participants after a full discussion of the nature of the study. Twenty two male patients of the mean age of 65 years were included into the study. All patients had stable COPD as defined according to GOLD (2013). They were characterized with respect to smoking history, COPD symptoms, co-morbidities, and medical treatment. No patient had symptoms or was treated for COPD exacerbation during at least 2 months before the day of inclusion. Exclusion criteria included other systemic diseases, other lung diseases apart from COPD, lung tumors, pulmonary infection, antibiotic treatment during 4 weeks before inclusion, or inhaled/oral glucocorticosteroids during 3 months before inclusion. Spirometry and lung volumes were performed using a body box (Elite DL, Medgraphics, USA) and standard protocols.

All patients underwent a 4-week washout therapy with salbutamol. After that, they were treated for 4 weeks with 12 µg formoterol b.i.d. and then subjected to sputum induction. Subsequently, all patients were treated for 3 months with add-on 18 µg tiotropium q.i.d. and the sputum was collected again.

2.2 Sputum Induction and Processing

Sputum was induced by inhalation of an aerosol of 4.5 % saline solution, generated by an ultrasonic nebulizer (Voyager, Secura Nova; Warsaw, Poland). Three flow volume curves were

performed before and after each inhalation, and the best FEV1 was recorded. Induction of sputum was stopped if the FEV1 value fell by at least 20 % from the baseline level or if troublesome symptoms occurred. Samples were processed within about 15 min after induction termination. They were solubilized in equal volumes of 0.1 % dithiotreitol (Sigma Chemicals, Poznan, Poland) in Hanks solution, and incubated for 15 min on ice. Cell suspension was then rinsed twice with Hanks solution, filtered by a nylon membrane, centrifuged (1,000 rpm) on Histopaque 1077, and isolated cells were further analyzed in a flow cytometer.

2.3 Flow Cytometry

Cells were diluted to a final concentration of 10^6 cells/ml. For each test, 10 μ l of a commercial antibody solution was added to 200 μ l of cell suspension and allowed to bind for 30 min at room temperature in darkness. The cells were then washed with PBS, fixed with CellFIX™ (Becton Dickinson, Oxford, UK) and run on an Epics XL flow cytometer (Coulter Electronics, High Wycombe, UK). Specific cell subpopulations were gated according to their forward and side-scatter profiles and 3,000 total events were collected per sample. To identify particular cell subtypes, double staining procedures were used with specific monoclonal Beckman-Coulter antibodies (Beckman-Coulter, Warsaw, Poland) conjugated to green, orange, or red fluorescent markers raised against human CD3, CD4, CD8, CD14, CD19, CD25, CD127, and HLA-DR antigens. The percentages of HLA-DR⁺ (cell fractions with 'high' and 'low' fluorescence) or Treg cells were determined out of the gated subpopulations of cells. Tregs were defined as CD4⁺CD25^{high}CD127^{low} cells.

2.4 Statistical Analysis

Data were expressed as means \pm SD. A non-parametric Wilcoxon test for paired samples

was employed for the analysis of differences. Statistical significance was defined as $p < 0.05$. Statistical elaboration was performed using a commercial Statsoft package (Cracow, Poland).

3 Results

Percentages of total lymphocytes (CD3⁺), lymphocytes T (CD4⁺ and CD8⁺), lymphocytes B (CD19⁺), monocytes (CD14⁺), and the corresponding fractions of HLA-DR expressing cells in induced sputum of COPD patients treated with Formoterol (F) or Formoterol + Tiotropium (F + T) are presented in Table 1. There were no significant differences in the total CD3⁺ and CD4⁺ lymphocytes, and monocytes between the two groups. However, the fraction of CD8⁺ lymphocytes was significantly lower in the F + T group compared with that in F-monotherapy ($p < 0.01$). Also, the ratio of CD4/CD8 cells in combined therapy was about fourfold greater. Concerning the HLA-DR expressing cells in the F + T group, significantly lower fractions were observed in lymphocytes T and B, but not in monocytes, compared with those in F-monotherapy ($p < 0.01$). The highest decrease (>80 %) was observed in CD4⁺ cells.

The percentages of CD4⁺ lymphocytes and the corresponding fractions of CD4⁺ lymphocytes with high expression of CD25 antigen and low expression of CD127 antigen (Treg cells) are presented in Table 2. The fraction of CD4⁺CD25⁺ cells was significantly lower (decreased by >55 %; $p < 0.01$) in the F + T-treated patients. Slightly less pronounced, but still a significant difference ($p < 0.05$), was observed in CD4⁺CD25^{high} cells, whose number was lower in the F + T-treated group by >40 % compared with that in F-monotherapy. Concerning Tregs, the percentages of cells expressing CD4⁺ and CD25⁺ or CD4⁺, and CD25^{high} were significantly lower after 3 months of add-on tiotropium, but there was no significant difference between the two groups in the cell subpopulations expressing CD4⁺CD25^{high}CD127^{low}.

Table 1 Lymphocyte and monocyte profiles and expression of HLA-DR in cells isolated from induced sputum of COPD patients treated with formoterol (F) or formoterol + tiotropium (F + T)

			F	F + T
Lymphocytes	Total	CD3 ⁺	68 ± 7	70 ± 9
		CD3 ⁺ HLA-DR ⁺	11 ± 2	5 ± 3**
	T	CD4 ⁺	51 ± 6	62 ± 6
		CD4 ⁺ HLA-DR ⁺	11 ± 3	7 ± 1*
		CD8 ⁺	10 ± 7	3 ± 1**
	B	CD8 ⁺ HLA-DR ⁺	24 ± 13	4 ± 2**
CD19 ⁺		13 ± 7	15 ± 6	
		CD19 ⁺ HLA-DR ⁺	12 ± 5	4 ± 2**
Monocytes	CD14 ⁺	17 ± 5	17 ± 8	
		CD14 ⁺ HLA-DR ⁺	58 ± 7	64 ± 6

*p < 0.05; **p < 0.01 for comparisons with the corresponding data from F-monotherapy

Table 2 Effects of add-on tiotropium therapy on CD25 and CD127 antigen expression in CD4⁺ cells isolated from induced sputum of COPD patients treated with formoterol (F) or formoterol + tiotropium (F + T) for 3 months. Tregs were defined as CD4⁺CD25^{high}CD127^{low} cells

	F	F + T
CD4 ⁺ CD25 ⁺	9 ± 2	4 ± 2**
CD4 ⁺ CD25 ^{high}	7 ± 2	4 ± 1*
CD4 ⁺ CD25 ^{high} CD127 ^{low}	15 ± 4	13 ± 4

*p < 0.05; **p < 0.01 for comparisons with the corresponding data from F-monotherapy

4 Discussion

The bronchodilatory drug tiotropium bromide produces respiratory benefits in COPD patients, but apart from reduced cholinergic signaling and altered cholinergic contractile tone, inflammatory pathways affected by the drug remain unknown in detail. Recently published data indicate that tiotropium may decrease airway inflammation and airway remodeling (Santus et al. 2012; Pera et al. 2011). We have previously shown that in patients treated with formoterol + tiotropium there are increased acetylated H3 and H4 histone levels (Holownia et al. 2010, 2013b). Histones are important in inflammatory signaling, because they are responsible for gene transcription and expression of inflammatory and anti-inflammatory proteins. Due to a significant role of local inflammation in COPD, it seems that

induced sputum analysis could provide relevant information regarding the intensity of inflammation and the immune mechanisms that are involved. Our data show that in combined therapy there is an inappreciable change in the number of monocytes, but the number of CD8⁺ cells decreases, resulting in a decrease in the CD4⁺/CD8⁺ ratio. It has been shown that a T lymphocyte imbalance is related to the inflammatory response of smokers with established COPD (Tzanakis et al. 2004). It has also been shown that increased CD8⁺ T cells are associated with COPD exacerbations and may contribute to COPD progression. Similar data have been observed in smoking asthmatics (Ravensberg et al. 2013). Since CD8⁺ subpopulations of T lymphocytes appear to play a significant role in COPD, a substantial decrease in CD8⁺ cells in the add-on tiotropium therapy should be considered beneficial. It remains to be established how long this decrease persists after the therapy end.

Concerning the activation phenotypes of sputum cells, we quantified HLA-DR antigen expression and subpopulation of Tregs. We show that HLA-DR is expressed in about 10 % of sputum lymphocytes and significantly higher levels are present in monocytes. In patients treated with F + T, HLA-DR expression on lymphocytes, but not on monocytes, was significantly lower. DR is a marker for immune stimulation and a lower antigen expression may indicate that immune functions related to the antigen presentation may be affected. On the

other hand, this change may also reflect an adaptative alteration related to decreased inflammation, when cell activation is no longer necessary. In COPD, airflow limitation has been found to correlate not only with an increased number of CD3⁺ T lymphocytes and CD8⁺ cells but also with increased expression of HLA-DR (O'Shaughnessy et al. 1997). Consequently, it seems that decreased HLA-DR may be related to a decrease in local inflammation.

Changes in the acquired immune system in COPD are less recognized. Recently, it has been shown that tiotropium increases apoptosis of CD8⁺ T cells (Profita et al. 2012) and also promotes the amplification of CD4⁺ T cells, lower expression of CD25⁺ T cells, and enhanced expression of CD8⁺ Tregs in the blood of stable COPD patients (Zhang et al. 2011). Treg cells are involved in the control of autoimmunity and increased Tregs may indicate either their dysfunction or resistance to suppression. It seems that our data reflect mostly local changes in cell profiles and cell activation, but they are similar to recently published blood data which confirm the decreased CD25 antigen expression in CD4⁺ lymphocytes and no change in Tregs (Zhang et al. 2011). It seems that monitoring the regulatory T cells may help assess changes in inflammation and tissue damage in patients with COPD. It is possible that the tiotropium effects on inflammatory cells are related to the main signaling pathway of the drug, the muscarinic receptor antagonism, since muscarinic receptors are present on lymphocytes, macrophages, and neutrophils (Verbout and Jacoby 2012; Reinheimer et al. 1997). The anti-inflammatory effects of tiotropium have also been confirmed in clinical studies on severe asthma, in which tiotropium has been more effective than inhaled corticosteroids (Tashkin and Ferguson 2013; Peters et al. 2013).

In conclusion, the present study indicates that tiotropium in add-on therapy to formoterol alters the lymphocyte profiles and affects lymphocyte activation, which confirms the modulatory role of tiotropium in the immune system of COPD patients. It is possible that changes in the immune cells may provide information about the patient's response to therapy.

Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.

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