

Effects of Oral Glucocorticoid Therapy on CD4+CD25+CD127- and CD4+CD25high T Cell Levels in Asthmatic Patients

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Abstract—Our purpose was to evaluate the effects of short-term oral glucocorticoid (GC) treatment on frequencies of T cells with putative regulatory phenotype (namely, CD4+CD25+CD127- and CD4+CD25high) in patients with asthma exacerbations. In addition, we sought to determine frequencies of above T cell subsets in adult asthmatic patients in relation to disease severity and different treatment regimens. The analysis was performed in 62 patients with different stages of asthma and ten healthy controls. Polychromatic flow cytometry was applied to delineate T cells with CD4+CD25+CD127- and CD4+CD25high phenotype. Exhaled nitric oxide analysis was used to assess allergic airway inflammation. Levels of neither CD4+CD25+CD127- nor CD4+CD25high T cells were significantly altered after 7-day oral GC treatment. Importantly, there were no detectable differences in frequencies of those cells among studied groups of asthmatics with different severity of disease and healthy controls. Moreover, levels of CD4+CD25+CD127- and CD4+CD25high T cells in asthmatic patients were not correlated to exhaled nitric oxide concentrations. Our data indicate that neither effects of average doses of oral GC treatment nor disease severity are related to changes in frequencies of CD4+CD25+CD127- and CD4+CD25high T cells in adult asthmatic patients.

KEY WORDS: regulatory T cells; CD127; asthma; exhaled nitric oxide; glucocorticoids.

INTRODUCTION

CD4+ regulatory T cells (T reg cells) play an important role in the modulation of immune responses including those underlying allergic inflammation [1–4]. From a clinician's point of view, allergic inflammation present in asthma and other allergic diseases is most efficiently suppressed by treatment with glucocorticoids (GC), either local or systemic [5]. To date, relatively few studies have attempted to characterize and quantify effects of GC treatment on levels and activity of T reg

cells in asthmatic patients. Karagiannidis *et al.* reported that asthmatics treated with GC present with upregulated Foxp3 expression as compared to healthy controls [6]. In addition, frequencies of memory CD4+CD25+ increased within 3 and 12 h after oral GC uptake. It is unclear, however, whether such daily fluctuations of T reg cells could subsequently lead to persistent increase of those cells in asthmatic patients treated with GC for longer time periods. To date, despite a number of recent reports, there is no detailed analysis of T cells with regulatory phenotype in all of the different groups of asthmatic patients in various stages of disease and treatment programs. In the current paper, we provide a quantitative time-course analysis of lymphocytes with T reg cell phenotype in asthmatic patients with exacerbations subject to treatment with oral GC. Importantly, we also demonstrate a comparative analysis of those T cells in five groups of asthmatics in relation to severity of the disease and different GC regimens.

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PATIENTS AND METHODS

Patients

Characteristics of 72 subjects included in the study are shown in Table 1. Sixty-two asthmatic patients were enrolled to the study. Asthma and severity of disease were classified according to Global Initiative for Asthma (GINA) criteria [5]. All patients from asthmatic groups were atopic as defined by at least two positive skin prick tests to common allergens. All patients were in the stable stage of disease. Current smokers, ex-smokers, and subjects with a history of respiratory infection during the previous 4 weeks were excluded from the study. Control group comprised of ten healthy nonatopic nonsmoking volunteers who indicated no history of asthma or of any respiratory disease and who had normal lung function.

In addition, 14 moderate asthmatic patients aged 36–54 years with exacerbation requiring rescue oral glucocorticoids were recruited. Exacerbation was defined by an increase in dyspnea (by at least 2 points in Medical Research Council dyspnoea score), at least three to fourfold increase in the use of rescue short-acting beta agonist, a fall of FEV1 by at least 20%, and an increase in exhaled nitric oxide level by at least 20% of baseline value. These patients had normal white blood cell counts, normal C-reactive protein and sedimentation rate levels, no clinical evidence of respiratory infection, and normal sputum culture results. Patients were hospitalized in the Department of Allergology and Internal Medicine, Medical University Hospital in Bialystok and were administered 30 mg of methylprednisolone for 7 days in addition to their current treatment. The study protocol was approved by the local ethics committee, and all subjects gave informed consent.

Exhaled Nitric Oxide Measurements

The concentration of NO in the exhaled air was assessed with the use of chemiluminescence analyzer NOA™ 280i (Sievers, USA) according to American Thoracic Society recommendations as described previously [7]. In brief, the analyzer was fitted with a biofeedback display unit to provide visual guidance for the subject to maintain the pressure and exhalation flow within a certain range. Subjects exhaled slowly from total lung capacity over 30 s with an exhalation flow of 50 ml/s. The mean of three separate measurements was used for analysis.

Immunostaining and Flow Cytometric Analysis

Fresh whole blood samples were incubated for 30 min at room temperature with 20 µl of mAbs: anti-CD4 FITC (BD PharMingen, Erembodegen, Belgium), anti-CD25 PE-Cy5 (IL-2R, BD PharMingen, Erembodegen, Belgium), anti-CD127 PE (IL-7R, Immunotech, Marseille, France), and anti-FoxP3 PE or Alexa Fluor 647 (BD PharMingen, Erembodegen, Belgium). The cells were washed twice with phosphate-buffered saline and fixed with 2% paraformaldehyde. Before staining with anti-FoxP3 antibodies, cells were incubated for 30 min at room temperature with permeabilizing buffer indicated by manufacturer. Flow cytometry analysis was performed with the use of a FACSCalibur cytometer (BD Immunocytometry Systems, San Jose, USA) as described elsewhere [8]. First, lymphocytes were gated by a combination of forward and side scatter. The gating of CD4+CD25+CD127low and CD4+CD25+ T cells was performed as described elsewhere with the use of isotype and fluorescence-minus-one controls [9–12] (Fig. 1). CD4+CD25high T cell population was defined such that it contained all CD25+ cells with mean fluorescence intensity (MFI) for CD4 less than 0.9 times CD4 MFI for

Table 1. Subject Characteristics

Subject	Age	Number	Atopy	Asthma history	ICS use	LABA use	% Predicted FEV1
Control	34.3 (SD±12.6)	10	No	No	No	No	105.4% (SD±11.2)
Mild asthmatic ICS (–)	38.2 (SD±9.7)	14	Yes	3.6 years (0–8 years)	No	No	99.3% (SD±19.4)
Mild asthmatic ICS (+)	40.1 (SD±8.8)	12	Yes	4.1 years (0–10 years)	200–400 µg of BUD	No	96.4% (SD±14.5)
Moderate asthmatics ICS (–)	43.4 (SD±8.1)	11	Yes	7.8 years (3–12 years)	No	Yes	78.2% (SD±21.1)
Moderate asthmatics ICS (+)	46.6 (SD±6.7)	12	Yes	9.3 years (6–18 years)	200–800 µg of BUD	Yes	79.6% (SD±14.5)
Severe asthmatics ICS (+)	48.4 (SD±14.4)	13	Yes	16.7 years (8–33 years)	800–1,600 µg of BUD	Yes	58.9% (SD±24.6)

Values are means ± standard deviation (SD)

ICS inhaled glucocorticosteroids, BUD budesonide, LABA long-acting beta-agonists, FEV1 forced expiratory volume in 1 s

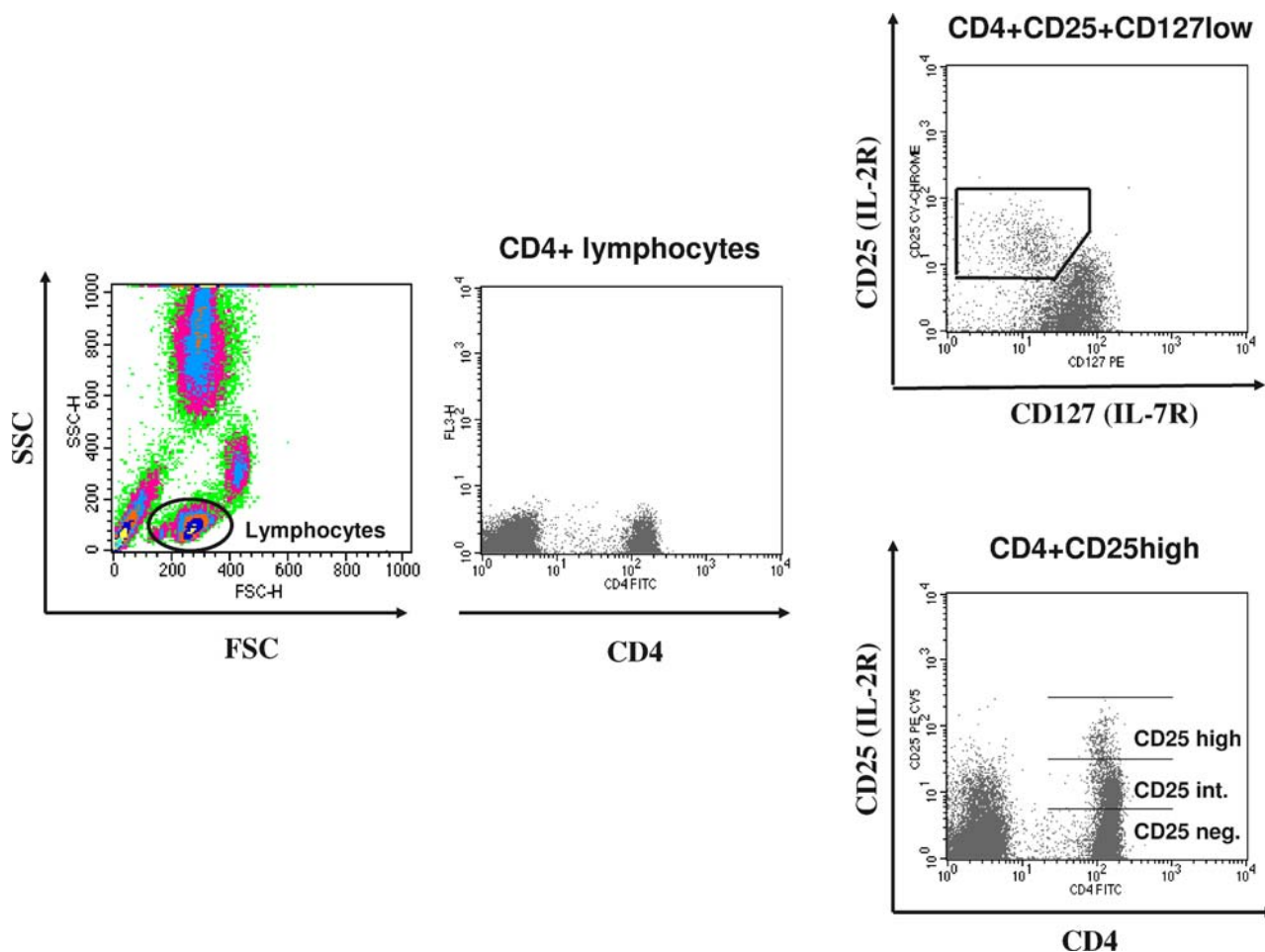


Fig. 1. Flow cytometric density and dot plots characteristic for studied individuals. Plots are gated for CD4+ T lymphocytes and CD25+CD127low, and CD4+CD25high lymphocytes are marked.

the region defined by the isotype-matched control (Fig. 1 bottom and [14]).

Statistical Analysis

We used Kruskal–Wallis ANOVA and Mann–Whitney nonparametric tests to analyze statistically significant differences among groups. The nonparametric Wilcoxon test was used to analyze the pre- and after-treatment values. The correlations were examined by the Spearman rank correlation coefficient. In all tests, statistically significant results were identified by a p value of <0.05 .

RESULTS

We performed a time-course analysis of changes in frequencies of CD4+CD25+CD127- and CD4+CD25high

T cells in asthmatics treated for 7 days with systemic GC due to asthma exacerbation. The median frequency of CD4+CD25+CD127- lymphocytes was 5.79% [interquartile range (IQR), 2.15%] before GC treatment and 5.22% [IQR, 2.08%] after cessation of GC administration. Out of 14 analyzed patients, a slight decrease in CD4+CD25+CD127- T cell frequencies was observed in nine subjects while remaining five subjects presented with insignificant increase of those cells (Fig. 2a, top). Altogether, there was no statistically significant difference between pre- and after-treatment values of CD4+CD25+CD127- T cell frequencies ($p>0.05$). Same pattern was seen for CD4+CD25high lymphocytes (Fig. 2a, bottom). Similarly, the analysis of available FoxP3 data in six studied patients revealed varying effects of 7-day GC treatment on the expression of this molecule. Taken together, the difference in FoxP3 expression before and after GC administration was not

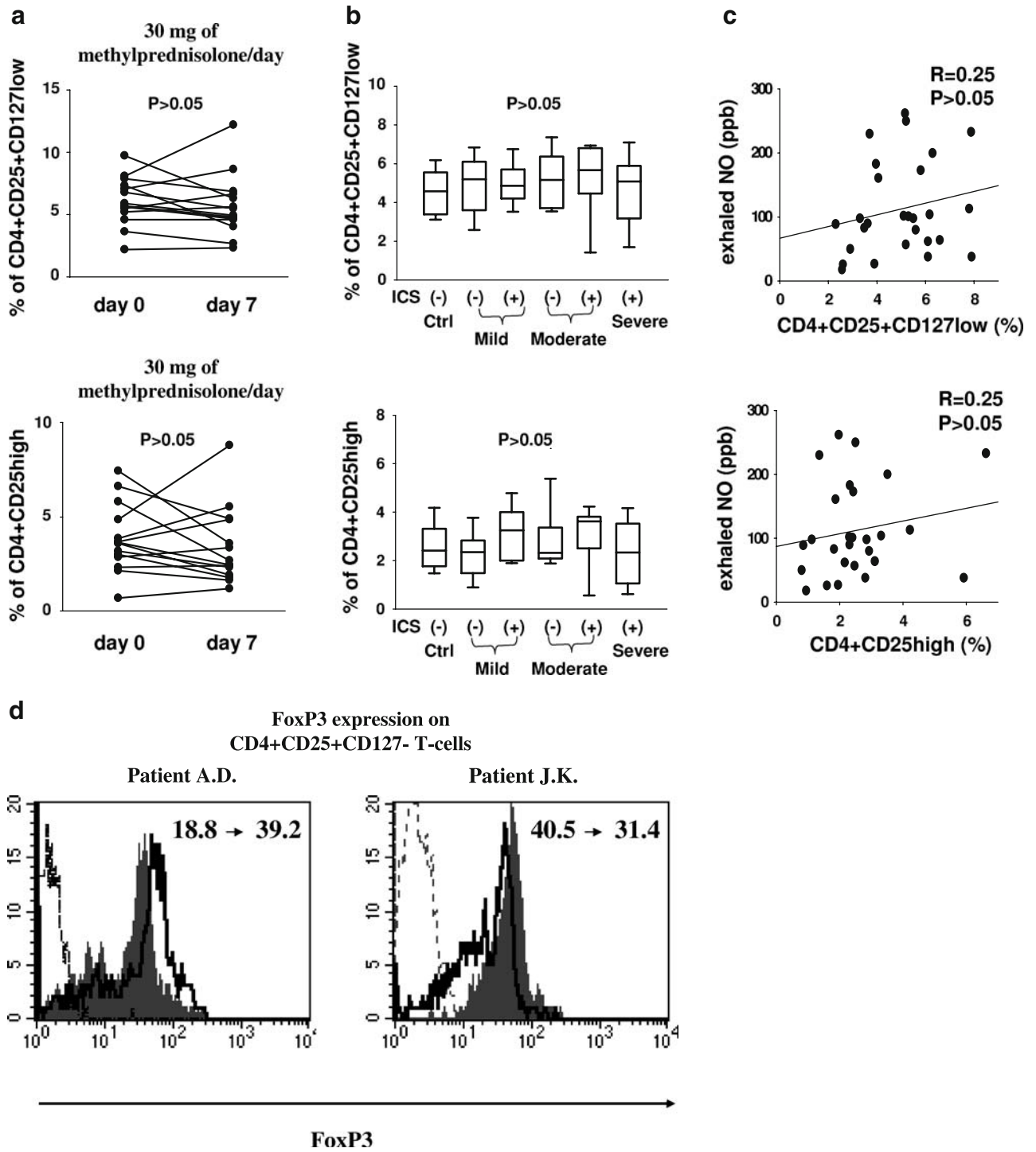


Fig. 2. **a** Detailed time-course analysis of frequencies of CD4+CD25+CD127low (*top panel*) and CD4+CD25high lymphocytes (*bottom panel*) before and after administration of methylprednisolone. **b** Frequency of CD4+CD25+CD127low lymphocytes (*top panel*) and CD4+CD25high lymphocytes (*bottom panel*) in healthy subjects and mild and moderate and severe asthmatics. Data are depicted as box plots. Medians are noted by *inside bars*, and the boxes span the 25th and 75th percentiles while whiskers represent the 5th and 95th percentiles. **c** Linear regression analysis of exhaled nitric oxide (eNO) levels and frequency of CD4+CD25+CD127low lymphocytes (*top panel*) and CD4+CD25high lymphocytes (*bottom panel*). **d** The effects of 7-day oral administration of 30 mg of methylprednisolone in asthmatic patients during exacerbation on FoxP3 expression. Flow cytometric histograms characteristic for studied patients before and after 7 days of treatment. Pre- and after-treatment staining with anti-FoxP3 monoclonal antibody is represented by *shaded areas* and *thick black line*, respectively. *Dashed lines* represent staining with fluorescence-minus-one controls. The values in histograms are for MFI of FoxP3 expression by CD4+CD25+CD127- T cells before and after methylprednisolone treatment, respectively.

statistically significant (median MFI—28.5 [IQR, 21.7] vs 31.4 [IQR, 16.5], before and after treatment, respectively; $p > 0.05$; Fig. 2d).

Importantly, frequencies of CD4+ T cells with putative T reg cell phenotype in stable asthmatic patients remained unchanged over a 7-day interval (7.70% [IQR, 2.55%] vs 7.1% [IQR, 2.0%], $p > 0.05$, and 2.42% [IQR, 2.46%] vs 2.20 [IQR, 0.42%], $p > 0.05$, for CD4+CD25+CD127- and CD4+CD25high T cells, respectively).

Next, we demonstrated that levels of lymphocytes with CD4+CD25+CD127- phenotype are similar in healthy subjects (median—4.58% [IQR, 2.16%]), steroid-naïve mild asthmatics (5.2% [IQR, 2.40%]), steroid-treated mild asthmatics (4.86% [IQR, 1.50%]), steroid-naïve moderate asthmatics (5.16% [IQR, 2.55%]), steroid-treated moderate asthmatics (5.67% [IQR, 1.27%]), and steroid-treated severe asthmatics (5.08% [IQR, 2.71%]; Fig. 2b, top). Again, same pattern was seen when analysis was performed for CD4+CD25high lymphocytes (Fig. 2b, bottom). In addition, we did not find difference in frequency of both CD4+CD25+CD127low and CD4+CD25high T cells between moderate asthmatics with exacerbations and those in stable stage of disease ($p > 0.05$; data not shown). Moreover, frequencies of both subsets in steroid-naïve asthmatic patients were not correlated with intensity of allergic airway inflammation measured by exhaled nitric oxide analysis ($R = 0.25$, $p = 0.207$ and $R = 0.25$, $p = 0.201$ for CD4+CD25+CD127low and CD4+CD25high, respectively; Fig. 2c).

DISCUSSION

In the current paper, we performed a comparative analysis of frequencies of lymphocytes with regulatory T cell phenotype in asthmatic patients before and after 7 days of oral GC treatment. We demonstrated that levels of both CD4+CD25+CD127low and CD4+CD25high lymphocytes are not significantly affected by short-term oral GC treatment. Our data indicate that despite previously reported enhancement of Foxp3 expression [6], oral GC in doses widely used in asthma exacerbations do not increase frequencies of putative T reg cells. Thus, anti-inflammatory effect of GC observed in asthma exacerbations does not seem to be related to changes in numbers of circulating lymphocytes with T reg cell phenotype. This is in contrast to our recent observation regarding monocytes: Equivalent doses of GC preferentially diminished amounts of peripheral proinflammatory monocytes as opposed to other monocyte subsets [13]. On the other hand, further investigations on the effects of GC treatment on T reg cells localized in compartments other than blood are still warranted.

Importantly, in our analysis of cohort of asthmatics treated with inhaled glucocorticoids, we did not find differences in CD4+CD25+CD127- and CD4+CD25high cell numbers as compared to steroid-naïve patients. To date, reports regarding T reg cells in asthmatics in comparison to healthy subjects brought conflicting data, i.e., increased Foxp3 expression in moderate–severe asthmatics [6] or similar frequencies of CD4brightCD25high lymphocytes in asthmatics [14] or decreased levels of CD4+CD25+ and CD4+CD25high lymphocytes in pediatric asthmatics [15]. Importantly, in two latter studies, steroid-naïve and steroid-treated patients were analyzed jointly. Most recently, Mamessier *et al.* found decreased levels of CD4+CD25+CD62L+CD45RO+ lymphocytes in patients with severe refractory asthma [16]. In fact, it is difficult to compare results from above studies as they come from patients of different age, with varying levels of severity of asthma and different treatment regimens. On the other hand, it is possible that some discrepancies regarding numbers of T reg cells in asthmatic patients might be related to different protocols used for T reg cell identification in various studies. In the current study, we went beyond “classical” approach to T reg cell separation and applied recently established method for delineation of well-separated T reg cell population, namely CD4+CD25+CD127 [9, 10, 12]. Having used the same approach, we demonstrated recently that frequencies of putative T reg cells in sensitized patients were

decreased after intrabronchial allergen challenge [11]. In the same study, baseline levels of T reg cells were similar between subjects that subsequently developed or not bronchoconstriction following allergen inhalation. That observation was supplemented in the current paper by notion of lack of differences in T reg cell numbers between asthmatics in stable stage of disease and those with exacerbation. Taken together, those data suggest that T reg cell level ought not to be regarded as potential predictive marker for development of bronchoconstriction in asthmatic subjects.

In summary, our data indicate that treatment with commonly used doses of both oral and inhaled GC in adult asthmatic patients does not lead to significant changes in frequencies of lymphocytes with regulatory T cell phenotype.

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