Original paper

Relationship between CCR5$^+$FoxP3$^+$ Treg cells and forced expiratory volume in 1 s, peak expiratory flow in patients with severe asthma

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Abstract

Introduction: Severe asthma is a special clinical problem. CD4$^+$CD25^{high}CD127^{low}Foxp3$^+$ Tregs play a role in maintaining appropriate immunological response. It is a known fact that Treg cells with CCR5 expression represent strong suppressive activity. It has been shown that a low number or altered function of FoxP3$^+$ Tregs is associated with the inflammatory process and airway obstruction in asthma.

Aim: To evaluate whether CCR5 Tregs expression and surface density on FoxP3$^+$ Treg cells depend on the severity of asthma.

Material and methods: The study included 50 patients with asthma (25 with severe and 25 with mild-to-moderate asthma). The control group comprised 25 healthy volunteers. The phenotype of CD4$^+$CD25^{high}CD127^{low}Foxp3$^+$CCR5$^+$ cells was evaluated by multicolour flow cytometry. The degree of airflow obstruction was assessed by spirometry as forced expiratory volume in 1 s (FEV_1) and peak expiratory flow (PEF).

Results: The absolute count of FoxP3$^+$ Treg cells in patients with severe asthma was significantly decreased in comparison with the control group. MFI (median fluorescence intensity) of CCR5 expression on FoxP3$^+$ Treg cells was significantly decreased in severe asthma compared to the mild-to-moderate asthma and control groups. CCR5 expression on FoxP3$^+$ Treg cells as MFI positively correlated with lung function parameters FEV_1% and PEF% in patients with severe asthma.

Conclusions: High CCR5 Tregs expression as MFI is associated with improved in lung function parameters: FEV_1% and PEF% in patients with severe asthma. The measurement of CCR5 expression on the surface of peripheral blood FoxP3$^+$ Treg cells as MFI could be an additional tool to estimate the severity of asthma.

Key words: asthma, Tregs, CCR5.

Introduction

Severe asthma is much less common than other types of asthma and affects about 5% of patients. It is a serious clinical problem because of the difficulty with its control [1].

Inflammation is the central feature of asthma pathogenesis and plays the main role in airway obstruction and hyperresponsiveness [2]. The mechanisms that regulate inflammatory immune responses in asthma include Foxp3$^+$ T regulatory cells suppressive function. These cells inhibit pro inflammatory effector cells. It has been shown that CCR5 chemokine receptors presented on CD4$^+$ lymphocytes are associated with airway hyperresponsiveness in asthma.

Treg CD4$^+$ cells are a subpopulation with immunosuppressive property. The major discovery related to CD4$^+$ Treg cells was the identification of transcription factor Foxp3. Transcription factor FoxP3 is believed to be essential for the differentiation, development and functioning of Tregs subset. However, CD25^{high} and CD127^{low} can be also useful markers in the identification Tregs subpopulation [3]. It is a very well-known fact that the CD4$^+$CD25^{high}FoxP3$^+$ cells demonstrate the greatest suppression activity.

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The ability of Tregs to access inflamed tissues is due to the wide array of receptors for inflammatory chemokines that they express. CCR5 chemokine receptors have been reported to play an important role in the lung T-cell homing pathway and may be potential targets for asthma therapy [4]. Human $CD4^+$ Tregs express higher levels of CCR5 receptors compared to effector $CD4^+$ T cells [5]. Treg cells can enter inflamed tissues and act directly at the sites of inflammation.

Activation of Treg cells leads to up-regulation of CCR5 receptors. It is a known fact that Treg cells with CCR5 expression represent strong suppressive activity [4]. The function of CCR5 receptors in the cellular immune response suggests that alteration in its sequence or expression may be associated with predisposition to hypersensitivity. Furthermore, data suggest that T cells CCR5 expression play a role in airway inflammation and accumulation of these cells in the airways [6]. It has been shown that FoxP3 and CCR5 chemokine receptors are associated with the inflammatory response in asthma.

Aim

The aim of the study was to evaluate whether the expression of these markers depend on the severity of asthma. We hypothesized that CCR5 Tregs expression and surface density on $FoxP3^+$ Treg cells are associated with the severity of asthma and lung function parameters as a forced expiratory volume in 1 s (FEV_1) and peak expiratory flow (PEF).

Material and methods

Study populations

We retrospectively enrolled 50 asthma patients (25 with severe and 25 with mild-to-moderate asthma) registered with the Asthma and Allergy outpatient clinic of the Medical University of Lodz. The control group comprised 25 healthy, non-smoking volunteers without respiratory tract infections for 4 weeks before the study. Healthy subjects did not have symptoms of other allergic diseases. This study enrolled non-smokers who were previously diagnosed and treated for asthma. Patients with cardiovascular disorders, respiratory infection, chest pain, and those who could not undergo spirometry have also been excluded from the study. Patients underwent clinical examination and spirometry with airways reversibility test before the blood samples collection. The degree of airflow obstruction was assessed by spirometry as FEV_1 and PEF. Lung function indexes namely FEV_1 and mean PEF were measured three times, and the best results were selected. Patients also underwent skin prick testing to common allergens and specific serum immunoglobulin E (IgE) was measured. Atopy was defined by either a positive skin test to at least one of a selected panel of allergens (Allergopharma), or detectable allergen-specific IgE. A paper and pencil version of Asthma Control Test (ACT) was used to assess the level of asthma control. The diagnosis of asthma was made based on the history of asthma symptoms, and reversible airflow limitation, in accordance with GINA 2017 recommendations. We used the ENFUMOSA criteria to assess disease severity [7].

Patients in the group with severe asthma (SA) required continuous treatment with high doses of inhaled corticosteroids (≥ 1600 μg/day of budesonide or beclomethasone, 800 μg/day of fluticasone or equivalent). In chronic oral steroid therapy the dosage amounted to 800 μg/day of budesonide or beclomethasone, 400 μg/day of fluticasone or equivalent. Also patients with severe asthma required long-term therapy: long-acting β-agonist (LABA, long-acting β-agonist, formoterol 9 μg/dose, twice per day or salmeterol of 50 μg/dose, twice per day) or oral theophylline (200–300 mg, 1–2 times per day). Asthma in these patients has not been fully controlled; in the year before our study these patients had at least one exacerbation. Patients with mild-to-moderate asthma (MA) were treated by 800 μg/day of budesonide or beclomethasone, 500 μg/day of fluticasone or equivalent, plus symptomatic treatment. Seventeen (68%) people with severe asthma received systemic oral steroids. Hypersensitivity to ASA occurred in one person with mild asthma (4%) and 9 with severe asthma (36%). FEV_1 value was 85.71 ±16.12% of the norm in patients with a mild form and 55.67 ±15.98% with a severe form. The characteristics of the tested groups are shown in Table 1.

An institutional review board approved the study protocol (RNN/133/11/KE) before study initiation. All participants provided written informed consent.

Evaluation of the absolute number of peripheral blood lymphocytes. Blood collection and processing

Venous blood was drawn into S-Monovette® blood collection tubes (Sarstedt, Germany) containing K3 EDTA. All samples were drawn around 8 a.m. and after 12 h of fasting. The absolute number and percentage of peripheral blood lymphocytes were assessed by haematological analyser 5 diff ABX Pentra DX. The absolute number of lymphocytes was obtained at each venous blood sampling and used to convert T cell subset frequencies obtained by flow cytometry. Absolute cell counts per μl were evaluated by assuming that the proportion of $CD3^+CD4^+$ T cells found in lymphocytes identified by forward scatter and side scatter in flow cytometry would equal that found in lymphocytes identified by WBC 5 DIFF.

PBMC isolation

PBMC (peripheral blood mononuclear cells) were purified from EDTA peripheral blood using density gradient centrifugation (400 g, 30 min) over Histopaque-1077 (Sigma-Aldrich). The percentage of viable isolated cells (PBMC) was determined on Pentra DX 120 analyser. The purity of the PBMC was more than 90%.

Table 1. Characteristics of the studied groups

Parameter	Severe asthma	Mild-moderate asthma	Control group	*P*-value
N	25	25	25	NS
Sex (F : M)	48% : 52%	48% : 52%	48% : 52%	NS
Ethnic origin	Caucasian (100%)	Caucasian (100%)	Caucasian (100%)	NS
Age [years] F ± SD : M ± SD	49 ±5.5 : 45 ±13.5	41 ±10.5 : 45 ±14.5	40 ±11.5 : 43 ±9.5	NS
Duration of disease [years] F ± SD : M ± SD	15 ±7.5 : 17 ±9.7	9 ±5.1 : 11 ± 6.3	–	< 0.05
Atopy (Y/N, F : M)	82% : 81%	90% : 87%	–	< 0.05
FEV_1 (%) F ± SD : M ± SD	66 ±13.0 : 62 ±9.2	75 ±9.5 : 80 ±8.2	93 ±5.5 : 94 ±3.4	< 0.01
PEF (%) F ± SD : M ± SD	65 ±11.2 : 66 ±12.3	80 ±11.8 : 82 ±12.2	90 ±9.2 : 91 ±7.5	< 0.01
ACT [points] F ± SD : M ± SD	9.5 ±2.60 : 9.7 ±2.80	22.5 ±3.00 : 23.0 ±2.00	–	< 0.01

BMI – body mass index, FEV_1 – forced expiratory volume in the first second as a percentage of predicted value, PEF – peak expiratory flow as a percentage of predicted value, ACT – asthma control test.

Positive selection of CD4+ T cells

The CD4+ T Cell Isolation Kit (Order no. 130-096-533) (Miltenyi Biotec) for magnetic beads was used for the positive selection of CD4+ T cells from the isolated total PBMC cells. The resulting cells were > 98% pure.

Immunostaining

The following antibodies were used for immunophenotyping of Treg cell subsets: anti-human CD3 conjugated with AmCyan, anti-human CD4 conjugated with Pacific Blue, anti-human CD25 conjugated with FITC, anti-human CD127 conjugated with PE, anti-human APC-Cy7 conjugated with CD195 (CCR5), and FoxP3 conjugated with Alexa 647. All antibodies were purchased from BD Pharmingen.

CD4+ T selected cells were washed twice in BD Pharmingen Stain Buffer and incubated with 10 µl anti-human CD3, anti-human CD4, anti-human CD25, anti-human CD127, anti-human CCR5, 20 min at room temperature, in the dark. Cells were re-suspended in wash buffer and then incubated with 2 ml of 1x Human FoxP3 Buffer A, 10 min in the dark, at room temperature. After incubation, cells were washed with 2 ml of Stain Buffer (FBS) and centrifuged. The pellets were washed and incubated with 0.5 ml of 1× Human FoxP3 Buffer C, 10 min in the dark, at room temperature. Then cells were washed with 2 ml Stain Buffer (FBS) and incubated with anti-Human FoxP3, 30 min in the dark, at room temperature. Finally, cells were re-suspended in 500 µl staining buffer, and fluorescent events were acquired by flow cytometry.

Flow cytometry detection of Tregs chemokine receptors

CCR5 receptor expression on FoxP3+ Treg cells was measured using 8-color flow cytometer BD FACS CANTO II. First lymphocyte gates were set based on forward scatter vs. side scatter. The threshold excluding cell debris was set. To analyse surface CD25, CD127, CCR5 and intracellular FoxP3 expression on isolated CD4+ T cells, cells were gated as $CD4^+CD25^{high}CD127^{low}$ events. We evaluated the subpopulation with phenotype $CD4^+CD25^{high}CD127^{low}FoxP3^+$ ($FoxP3^+$ Treg) and $CD4^+CD25^{high}CD127^{low}FoxP3^+CCR5^+$ ($CCR5^+FoxP3^+$Treg). A minimum of 30 000 events were captured for each sample. The expression of CCR5 chemokine receptors on Treg cells was further characterized as median fluorescence intensity (MFI). This parameter characterized the density of the CCR5 receptors on the Treg cells surface.

Histograms and Quadrants dot-plots were set for each antibody. Automatic compensation for spectral overlap was performed electronically to minimize fluorescence spillover, using antibody capture beads. Gating strategy was determined by comparison to Fluorescence Minus One (FMO) control. Gating was standardized within individual samples. Data were analysed using FACS Diva software version 6.1.2.

Statistical analysis

The results were analysed statistically with the Statistica v 12 PL software. Variables were assessed regarding the distribution and equality of variances. Quantitative variables were characterized with median and interquartile range (IQR). Comparisons of studied features between groups were performed with Mann-Whitney *U*-test. Correlations between parameters were assessed using the Spearman rank correlation coefficient. Statistical significance was set as $p < 0.05$.

Results

Absolute count of CD4+ T cells in asthma

There was no significant difference in the median CD4+ T cells absolute count in peripheral blood samples between asthmatic patients and the control group (Table 2).

Table 2. Variables (median, interquartile range (IQR)) describing the absolute count of CD4+T cells, FoxP3+ Tregs, CCR5+FoxP3+ Tregs in asthma

Parameters/absolute count	Severe asthma [cell/µl]	Mild-to-moderate asthma [cell/µl]	Control group [cell/µl]	*P*-value
CD4+ T cells	598 (509–772)	624 (468–950)	825 (708–980)	NS
FoxP3+ Treg	50 (37–60)*	54 (37–61)	62 (40–85)*	< 0.05
CCR5+FoxP3+ Treg	22 (14–30)	15 (11–25)	19 (16–21)	NS

**Statistically significant result between FoxP3+ Treg cells in severe asthma and control groups.*

Table 3. Variables (median, interquartile range (IQR)) describing MFI of CCR5 on FoxP3+ Treg cells and ratio CCR5^{high} Tregs/CCR5^{low} Tregs in patients with asthma

Parameters	Severe asthma	Mild-moderate asthma	Control group	*P*-value
CCR5+FoxP3+ Treg Median-MFI (range)	1143*/** (1019–1366)	1398** (1168–1846)	1812* (1416–2350)	< 0.01
CCR5^{high} Treg/CCR5low Treg (ratio)	0.44 (0.33–0.5)	0.28 (0.26–0.48)	0.31 (0.26–0.44)	NS

**Statistically significant result between CCR5+ FoxP3+ Treg cells in severe asthma and control groups. **Statistically significant result between CCR5+ FoxP3+ Treg cells in severe asthma and mild-to-moderate asthma groups.*

Absolute count of FoxP3+ Treg and CCR5+FoxP3+ Treg subpopulations in asthma

In our study we showed that the absolute count of FoxP3+ Treg cells in patients with severe asthma was significantly decreased in comparison with the control group, $p < 0.05$ (Table 2).

There were no differences in absolute counts of CCR5+FoxP3+ Tregs in severe asthma, mild-to-moderate asthma and control groups (Table 2).

MFI of CCR5 expression on FoxP3+ Treg cells, and ratio of CCR5^{high} Tregs/CCR5^{low} Tregs in asthma

MFI of CCR5 expression on FoxP3+Treg cells was significantly decreased in severe asthma compared to mild-to-moderate asthma and control groups, $p < 0.01$ (Table 3). There was no significant difference in the ratio of CCR5^{high} Tregs to CCR5^{low} Tregs between severe and mild-to-moderate asthma patients and the control groups.

Correlation between absolute counts of FoxP3+ Tregs, CCR5+FoxP3+ Tregs, CCR5+FoxP3+ Treg cells MFI and lung function parameters (FEV_1%, PEF%)

There was no significant correlation between absolute counts of FoxP3+ Treg cells and FEV_1% predicted values. The results showed a high positive correlation between absolute counts of CCR5+FoxP3+ Tregs and FEV_1% predicted values ($r = 0.72$, $p < 0.01$) in the severe asthma group. The present study showed a moderate positive correlation between MFI of CCR5+ on FoxP3+ Treg cells and FEV1% predicted values ($r = 0.60$, $p < 0.05$) in the severe asthma group.

We found no correlation between absolute counts of FoxP3+ Treg cells and peak expiratory flow (PEF%) values. Absolute counts of CCR5+FoxP3+ Tregs correlated moderately positively with PEF% ($r = 0.55$, $p < 0.05$) in the severe asthma group (Table 4). Furthermore, we observed a moderate positive association between MFI of CCR5+FoxP3+ Treg cells and PEF% ($r = 0.65$, $p < 0.05$).

Discussion

In this study we focused on Tregs CCR5 expression in relation to the severity of asthma. We demonstrated that CCR5 expression and surface density on circulating FoxP3+Treg cells is associated with lung function parameters FEV_1 and PEF. This disclosure supported the relevance of Treg cell dysfunction in asthma.

We found that the absolute count of FoxP3+ Tregs was significantly decreased in severe asthma. These results suggest that the decreasing number of FoxP3+ Tregs is connected with asthma severity.

Many authors evaluated the relative number of Treg cells and did not evaluate Tregs FoxP3 expression. Shi *et al*. *showed* that the percentages of circulating Treg cells in asthmatic patients and healthy subjects were not different. It has been noticed that the percentage of Treg cells increased only in the asthmatics during acute exacerbation [8]. Cohen *et al.* showed that obese, atopic women with childhood-diagnosed asthma demonstrate an increased Treg cell number [9].

These results indicated that peripheral FoxP3+ Tregs reflect the immune status with alterations in different diseases [10, 11].

It is well known that the basic clinical parameter used to assess disturbances of lymphocyte subpopulations is an absolute, and not relative value. Most studies focused on Tregs percentages instead of absolute counts [12]. Furthermore, FoxP3+ Treg cells act through the mechanism of cell contact with antigen presenting cells (APCs). The efficient function of Tregs depends on its proper number. Thus, regulatory T cells absolute count rather than the relative value must be considered as a parameter of these cells' disturbances.

Table 4. Correlation analysis between the absolute count of FoxP3$^+$ Tregs, CCR5$^+$FoxP3$^+$ Tregs, MFI of CCR5 on FoxP3$^+$ Tregs cells and lung function parameters FEV_1% of predicted value and PEF% of predicted value

Parameter		Severe asthma	Mild-to-moderate asthma
FEV_1% of predicted value/FoxP3$^+$ Treg cells/µl	*r*	0.31	0.29
	p-value	NS	NS
FEV_1% of predicted value/CCR5$^+$FoxP3$^+$ Treg cells/µl	*r*	0.72	0.61
	p-value	< 0.01	< 0.05
FEV_1% of predicted value/MFI of CCR5 on FoxP3$^+$ Treg cells	*r*	0.60	0.42
	p-value	< 0.05	NS
PEF% of predicted value/FoxP3$^+$ Treg cells/µl	*r*	0.41	0.31
	p-value	NS	NS
PEF% of predicted value/CCR5$^+$FoxP3$^+$ Treg cells/µl	*r*	0.55	0.40
	p-value	< 0.05	NS
PEF% of predicted value/MFI of CCR5 on FoxP3$^+$ Treg cells	*r*	0.65	0.48
	p-value	< 0.05	NS

r – correlation coefficient.

The interpretation of FoxP3$^+$ Treg cells results is clinically important. Patients with asthma are treated with glucocorticosteroids. It was shown that glucocorticosteroids are able to increase the number of FoxP3$^+$ Treg cells at the periphery [13].

CCR5 density on Treg cells but not an absolute count of CCR5$^+$Foxp3$^+$ T cells is decreased in patients with severe asthma.

One of the most important manifestations of allergic asthma is airway hyperresponsiveness (AHR). It is known that the degree of hyperresponsiveness of the airways is related to the severity of asthma. A crucial role of CCR5$^+$CD4$^+$T cells in the development of allergen-induced airway responses has been reported. In the absence of CCR5 receptors on T cells, the reduction in AHR was associated with a significantly lower number of CD4$^+$ cells in the airways [6].

We did not observe any changes in the absolute count of CCR5$^+$Foxp3$^+$ Treg cells in asthmatic patients. However in patients with severe asthma we found that FoxP3$^+$ Treg subset presented decreased CCR5 density compared to mild-to-moderate asthma and the control group. Decreased CCR5 expression on FoxP3$^+$ Treg cells as a MFI value, may indicate a decreased number of Tregs CCR5 receptors. In several studies it has been shown that binding chemokines – CCR5 receptors lead to their down-modulation. This phenomenon could be responsible for the decreasing number of Tregs CCR5 receptors and MIF [14].

Most of the reports characterized the expression of CCR5 receptors on T effector cells. However in some cases, CCR5$^+$ Treg cells have been shown to contribute to this subset. Kallinichi *et al.* showed that CCR5 receptors were expressed on T cells from BAL and no differences in the receptor density were observed [14]. It was shown that Treg cells deprived of CCR5 receptors on their surface were less effective in preventing mortality caused by graft versus host disease (GVHD) [15]. It has been investigated that Treg cells without CCR5 receptor expression were characterized by impaired migration to the pulmonary lesions [16].

Kallikourdis *et al.* demonstrated that based on the expression of the CCR5 receptor, Treg cells can be divided into highly suppressive CCR5$^+$ and less suppressive CCR5. CCR5$^-$ Treg cells were characterized by decreased efficiency in the maintenance of immune tolerance between mother and foetus [17].

It was suggested that CCR5 receptor was responsible for an enhanced recruitment of Treg cells that have already been activated by the antigen in the periphery [16].

Other studies indicated the immune-regulatory role of CCR5 receptor. Its reduced amount on Tregs surface may be associated with migration dysfunction of this population to the places of immune response. In opposite to this study our examination did not confirm that subpopulations with a higher expression of CCR5 were connected with the severity of asthma.

CCR5$^+$FoxP3$^+$ Treg subpopulations positively correlate with the lung function parameter: forced expiratory volume in the first second as a percentage of predicted value (FEV_1%) and peak expiratory flow as a percentage of predicted value (PEF). Main parameters of lung function, FEV_1% and PEF% are not equivalent in many patients, especially women and those with less severe airflow limitation, therefore we cannot use these two parameters interchangeably [18].

In our study we examined the relationship between these two measurements with FoxP3$^+$ Treg cells and surface CCR5 density separately. Some data indicate that most lung function parameters such as FEV_1 and PEF are not reliable in that they do not guarantee a thorough as-

sessment of the entire lower respiratory tract or are subject to intra-individual variability. In addition, some patients with mild or poorly controlled asthma have normal pulmonary physiological indexes. Therefore, new markers are sought for the assessment of airway obstruction.

Our results showed that FoxP3+ Treg cells surface CCR5 density rather than their absolute count is associated with the severity of asthma. Although CCR5 expression on FoxP3+ Treg cells correlated with lung function parameters (FEV_1 and PEF), but the absolute count of these subpopulations was not significantly decreased in contrast to surface CCR5 density on Treg cells.

Other authors showed a correlation between the density of CCR5 receptors expression on lymphocytes and the severity of the disease. Vallejo *et al*. showed that high levels of CCR5 density might contribute to progress of HIV1 infection and immunodeficiency [19]. Moreover Reynes *et al*. suggest that CCR5 density is a predictive factor of the treatment effectiveness [20].

Several studies demonstrated the association between inflammatory indicators and asthma severity [21, 22]. These data confirmed that blood eosinophil counts can be used to diagnose patients with severe eosinophilic asthma [23]. It has been described that fractional exhaled nitric oxide (FE_{NO}) correlated with airway hyperresponsiveness measured by lung function values in childhood asthma [24]. We suggest that FoxP3+ Treg cells surface CCR5 density can be an additional indicator in patients with severe asthma and probably has a prognostic value as a biomarker of severe asthma.

Further analyses are needed to evaluate quantity methods that characterized CCR5 receptors density on Treg cells. It is particularly important in routine practice because MFI of CCR5 on FoxP3+ Treg cells can be useful especially in patients with severe asthma.

Conclusions

High CCR5 Tregs expression as MFI is associated with improved lung function parameters FEV_1% and PEF% in patients with severe asthma. The measurement of CCR5 expression on the surface of peripheral blood FoxP3+ Treg cells as MFI could be an additional tool to estimate the severity of asthma.

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Conflict of interest

The authors declare no conflict of interest.

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