The CD8⁺CD25⁺⁺⁺FoxP3⁺ naturally occurring T regulatory cells in allergic asthma

CD8⁺CD25⁺⁺⁺FoxP3⁺ naturalnie występujące limfocyty T regulatorowe w astmie oskrzelowej

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Summary
Naturally occurring CD8⁺CD25⁺⁺⁺FoxP3⁺ nTreg cells were shown to play a role in maintaining immunologic tolerance however, their correlation with the severity of asthma remains unclear. The aim of our study is to explore the relevance of CD8⁺CD25⁺⁺⁺FoxP3⁺ nTreg cells and the mRNA FOXP3 expression to the severity of allergic asthma. We characterized CD8⁺CD25⁺⁺⁺FoxP3⁺ nTreg cells in the venous blood of patients with allergic asthma (AA) and normal controls (NC) after sorting using a multicolor flow cytometry. Our study demonstrated a decrease in the percentage of CD8⁺CD25⁺⁺⁺FoxP3⁺ nTreg cells in patients with allergic asthma compared with healthy controls. Moreover, we showed a decrease in the percentage of CD8⁺CD25⁺⁺⁺FoxP3⁺ nTreg cells in severe asthma compared with those with mild asthma (MA). The level of mRNA FOXP3 expression was significantly lower in severe asthma than in mild asthma. However, there was no significant difference between the mRNA FoxP3 expression in severe asthma (SA) patients and normal controls (NC). There was a higher FOXP3 mRNA expression in patients with middle asthma than in normal controls. We also showed a spearman rank correlation between the regulatory T cells transcription factor FOXP3 and the force expiratory volume in one second (FEV1) in allergic asthma patients. This correlation was significant in the severe asthma group and the mild asthma group. We suggested that the low frequency of CD8⁺CD25⁺⁺⁺FoxP3⁺ nTreg phenotype in AA patients and consequently the decrease in their suppressive capacity might play a role in the progression of allergic asthma whilst the expression of mRNA FOXP3 might determine asthma severity.

Streszczenie
Naturalne regulatorowe limfocyty T, CD8⁺CD25⁺⁺⁺FoxP3⁺ odgrywają podstawową rolę w utrzymaniu tolerancji immunologicznej, chociaż zależność pomiędzy funkcją komórek regulatorowych CD8⁺ i stopniem ciężkości astmy jest niejasna. W niniejszej pracy zbadano populację naturalnych regulatorowych limfocytów CD8⁺CD25⁺⁺⁺FoxP3⁺ we krwi obwodowej chorych na astmę alergiczną, (AA) z uwzględnieniem stopnia jej ciężkości. Badania przeprowadzono w grupach chorych z ciężką (SA) i łagodną astmą alergiczną (MA). Limfocyty nTreg CD8⁺ po izolacji wysortowano a immunofenotyp oceniono na cytometrze przepływowym. Wykazano, że odsetek limfocytów CD8⁺CD25⁺⁺⁺FoxP3⁺ był znamiennie obniżony u chorych z astmą ciężką w porównaniu zarówno do grupy kontrolnej jak i chorych z łagodną postacią astmy. Ekspresja mRNA dla genu FOXP3 u chorych z SA była również niższa, jednak bez znamienności statystycznej. Ekspresja mRNA dla genu FOXP3 u chorych z łagodną postacią astmy była wyższa w porównaniu do grupy kontrolnej, także bez znamienności statystycznej. Wydaje się, że wynika to z udokumentowanego wpływu terapii sterydowej na wzrost ekspresji mRNA dla genu FOXP3. Potwierdza to również stwierdzona dodatnia korelacja pomiędzy wielkością FEV1 u chorych na astmę alergiczną i ekspresją FOXP3 w regulatorowych limfocytach T, CD8⁺CD25⁺⁺⁺FoxP3⁺. Uzyskane wyniki badań sugerują istnienie zależności pomiędzy limfocytami regulatorowymi CD8⁺CD25⁺⁺⁺FoxP3⁺ i stopniem ciężkości astmy oskrzelowej.

Key words: allergic asthma, CD8⁺ regulatory cells, FOXP3

Słowa kluczowe: astma alergiczna, limfocyty regulatorowe CD8⁺, FOXP3

Introduction
Asthma is a chronic inflammatory disorder characterized by airway obstruction and hyper responsiveness [9]. The clinical manifestation of asthma namely wheezing, dyspnea, bronchial constriction; most of which occur during the night or at the earlier morning result from inflammatory changes in
the small and the large airways. Although severe or refractory asthma affects less than 10% of the asthmatic population, it mobilizes about 85% of the overall costs of asthma because it is difficult to treat and it is often prone to severe exacerbations [2]. Autopsy studies showed an increased inflammation and wall thickness in patients who died of severe asthma compared with mild asthma and healthy non-allergic subjects [4]. Clinical observation and epidemiological studies demonstrated that, the concept of the balance between Th1 and Th2 cells could not adequately explain the predisposition to allergies or autoimmune disorders [8]. Moreover, Th2-skewed parasitic helminthes infections rather protect against allergy and asthma whereas, Th1 cells and their secreted cytokines exacerbate allergic disorders such as allergic asthma [6]. These unmet needs of the hygiene paradigm suggests the existence of immune regulatory mechanisms that determine how the immune system functions and why it can either protect or injure the host. Of the various mechanisms that confer tolerance to self or allergens, T regulatory cells play a crucial role in maintaining peripheral tolerance against auto antigens and allergens [19]. Treg cells namely the naturally occurring subsets can suppress effector T cells including Th1 or Th2 phenotypes thus, their implication in the development of allergy or autoimmunity. Evidences suggest that several immune cells can exhibit regulatory function however; most regulatory T cells derive from the CD4+ or CD8+ T cells compartment. Although CD8+ Treg cells were the first described T cells with suppressor ability, they have received less attention compared with their CD4+ Treg cells counterparts [12]. T-regulatory (Treg) cells are mainly divided into the naturally occurring (nTreg) subsets that develop in the thymus and the inducible (iTreg) that are generated into the naturally occurring subsets can suppress effector T cells challenging. Most markers of Treg cells are also expressed by activated T cell which makes the differentiation between Treg cells and activated T cells challenging. The transcription factor gene FoxP3 is constitutively expressed at high level on regulatory T cells compared with conventional activated T cells. Mutations in the human FOXP3 gene causes severe immune deregulation with polyendocrinopathy and enteropathy X-linked syndrome, a disorder that shares with asthma some features such as eczema and high serum IgE levels. Specific immunotherapy exerts its effects by expanding the CD8+CD25highFoxP3+ Treg population [18]. Furthermore, naturally occurring CD8+CD25highFoxP3+ regulatory T cells were shown to play a role in maintaining immunologic tolerance however, their correlation with severe asthma remains uncertain. The aim of our study is to explore the relevance of CD8+CD25highFoxP3+ Treg cells and the mRNA FOXP3 expression to the severity of allergic asthma.

Material and methods

Patient’s characteristics

Peripheral blood samples were drawn from 48 patients with allergic asthma (AA) and 25 normal controls (NC). The diagnosis of asthma has been confirmed by pulmonologists based on evidence of variable airways obstruction according to The Global Initiative for Asthma (GINA 2008) guidelines. Patients were divided into the severe asthma (SA) (n=25) and mild-to-moderate asthma (MA) (n=23) based on European Network For Understanding Mechanisms Of Severe Asthma (ENFUMOSA) classification [13] [16]. All patients were non-smokers in stable clinical condition. Patients’ medical and demographic data were collected and the study has been approved by the ethic committee of the Medical University of Lodz (RNN/17/09/KE). Healthy participants had neither history nor symptoms of allergic or infectious diseases. Patients who had at least one exacerbation per year were included into the group of severe asthma. These patients must require continuous treatment with either high dose of inhaled steroids, long-acting β-agonists or oral theophylline for at least a year. To be included in the group of mild-to-moderate asthma, patients must have stable disease with minimal symptoms and no exacerbations or hospitalisations. They must be using daily inhaled steroids (800 μg/day budesonide or beclomethasone), in combination with symptomatic treatment. The average age of patients was 42 ± 13 years for mild asthma, and 48 ± 14 years for severe asthma. The average age of healthy individuals was similar to that in the group of patients (48, 5±14, 5). Hypersensitivity to ASA was noted in 1 person with mild asthma (4, 54 %) and 9 with severe asthma (50 %). Atopy was evaluated based on skin tests or evidence of increase specific serum IgE . Skin tests were performed against the ten common allergen extracts. Atopy was defined based on a wheal reaction of at least 3mm in diameter.

Assessment of serum levels of total IgE

Patients’ peripheral blood mixed with EDTA was centrifuged at 2000 rpm for 10 minutes and the serum was collected. The serum was kept at −70°C and dissolved just before processing. Total serum IgE level was determined using ELISA and according to the manufacturer’s instructions. Cell isolation and sorting

About 10 ml of venous blood was collected from all participants on EDTA tubes and processed immediately. Complete blood count was performed using the hematologic analyzer BC 5800 Mindray. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood sample dropped on a Ficoll-Hypaque (Sigma-Aldrich, St Louis, Missouri) by density gradient centrifugation run for 30 minutes at 1800 rpm. PBMCs were resuspended in PBS (heat-inactivated Fetal Bovine serum). Briefly CD8+ T lymphocytes were sorted from isolated lymphocytes using the BD™ IMag Human CD8 T Lymphocyte Enrichment Set and the cell Separation BD™ IMagnet (BD Biosciences). CD8+ T cells were purified using The CD8 T Lymphocyte Enrichment Cocktail containing the following biotin-conjugated monoclonal antibodies: anti-human CD4, anti-human CD11b/Mac-1(CR3), anti-human CD16, anti-human CD19, anti-human CD36, anti-human CD41a, anti-human CD56, anti-human CD123 (IL-3 Receptor α chain) , anti-human CD235a (Glycophorin A), anti-human γδ TCR. All steps were performed according to the manufacturer’s instructions. Naive CD8+ T cells were therefore negatively selected using the BD IMagnet magnetic field (BD Biosciences). Following immunoseparation of...
cells suspension aliquots, the labeled cells were retained in the magnetic field whereas the unlabeled naïve CD8+ T cells are retained in the enriched fraction. To increase the purity of the CD8+ T cells enrichment fraction, the negative selection was simultaneously repeated on the enriched fraction until the purity of the enrichment CD8+ T cells supernatant reaches 95%.

**Immunostaining**

1x10^5 Treg cells were stained with 10 μl of the following monoclonal antibodies: CD8 PerCP – Cy5.5, CD25 FITC, and FoxP3 Alexa APC. The monoclonal antibodies were purchased from BD Pharmingen, USA. We used Intracellular FoxP3 Staining Protocol of BD Pharmingen, USA.

**Flow cytometry analysis**

Each analysis was performed using at least 50,000 cells that were gated in the region of the lymphocyte population, as determined by light scatter properties (forward scatter vs. side scatter). Automatic compensation was later applied to minimize fluorescence spectrum overlap. Fluorescence-minus-one (FMO) control was later used to set positive/negative boundaries. CD8+CD25+FoxP3+ nTreg cells were gated on CD8+FoxP3+ cells and a “positive” gate was drawn to include fewer than 0,1% of cells in the FMO control. The percentage of cells from the fully stained sample in the resulting positive gate was taken as the proportion of positive cells.

**RNA Isolation and detection of FOXP3 mRNA in Tregs**

Total RNA was extracted from sorted CD8+ T lymphocytes with a NucliSENS Magnetic Extraction Reagents kit, BioMerieux. TaqMan reverse transcription (RT)-polymerase chain reaction (PCR) was performed with 1 μg of total RNA for cDNA synthesis [11]. Primers were used as follows: forward primer 18S rRNA 5'-AGT CCC TGC CCT TTG TAC ACA – 3’ reverse primer 18S rRNA 5’ – GAT CCG AGG TCA CTA AAC – 3’. For the generation of FOXP3 cDNA, a total of 41 cycles were run. The following conditions were used for FOXP3 RT-PCR: 10 min. 95°C, 30 sec. 95°C, 1 min. 58°C. For quantitative real-time PCR analysis, 2 μM of isolated RNA was transcribed into cDNA using random hexamers. Polymerase chain reactions were performed in duplicates according to the Brilliant II QPCR Master Mix procedure, Stratagene, USA and run on the Smart Cycler Sequence Detector System. The amount of FOXP3 mRNA expression was normalized to the 18S rRNA standard (primer TTA GAA GAG ACT CGG TAT AAA AGC AAA GTT GTT TT) by calibration curve. Relative quantification and calculation of the range of confidence was performed by using the comparative threshold cycle (DDCT) method, as previously described in the real-time Quantitative Nucleic Acid Sequence Based Amplification (real-time QT-NASBA) method [10]. The concentration of the FoxP3 calibrator is expressed in pmol/μl in this technique.

**Statistical analysis**

Statistical analysis was performed using Statistica in Windows System 8.0 PL and data were verified for Gauss distribution. Data (medians) from the phenotypic analysis are expressed as percentages and did not follow a normal distribution. Results were compared between severe asthmatics, mild asthmatics and healthy volunteers using the nonparametric Mann-Whitney U test and ANOVA. P values less than 0, 05 were considered statistically significant. The Spearman correlation test was also used.

**Results**

The mean FEV1, % of predicted value was 85, 71 ± 16, 12 % in mild and 55, 67 ± 15, 98 % in severe asthmatics.

**Increase concentration of total serum IgE in patients with allergic asthma.**

Patients with allergic asthma have higher concentration of IgE. Moreover patients with severe asthma had higher IgE concentration that those with mild asthma (Fig.1).

**Decrease in the percentage of CD8+CD25+FoxP3+ nTreg cells in patients with allergic asthma compare with healthy controls.**

The percentage of CD8+CD25+FoxP3+ nTreg cells was significantly lower in AA patients as compared with NC (p<0,0003) (Fig.2). We suggested that the low frequency of CD8+CD25+FoxP3+ nTregs phenotype in AA patients and consequently the decrease in their suppressive capacity might play a role in the progression of allergic asthma. The lowest frequency of CD8+CD25+FoxP3+ n Treg cells (5 %) were shown in patients with severe asthma compared to 13 % in middle asthma and 20 % in healthy controls. This finding suggests that the frequency of CD8+CD25+FoxP3+ n Treg cells depends on the severity of asthma.
Decrease in the percentage of CD8*CD25*FoxP3* nTreg cells in severe asthma. 

The percentage of CD8*CD25*FoxP3* nTreg cells was significantly lower in SA as compared with MA, \( p<0.04 \) (Fig.2). We concluded that the percentage of peripheral CD8*CD25*FoxP3* nTreg cells may depend on the severity of asthma. 

Low FOXP3 mRNA expression in patients with severe asthma. 

The level of mRNA FOXP3 expression was non significantly lower in severe asthma than in mild asthma (Fig.3). Moreover, there was no significant difference between the mRNA FoxP3 expression in severe asthma (SA) patients and normal controls (NC). This data suggest a correlation between the expression of mRNA FOXP3 and asthma severity. 

Higher FOXP3 mRNA expression in patients with middle asthma than in normal controls. 

The level of mRNA FOXP3 expression was significantly higher in middle asthma than in healthy subjects (Fig.3) whereas severe asthma group expressed non significantly lower FOXP3 mRNA than healthy controls group. 

Correlation between the FOXP3 and FEV. 

We found a spearman rank correlation \( p<0.05 \) between the FOXP3 and FEV. The level of mRNA FOXP3 expression was non significantly lower in severe asthma than in mild asthma (Fig.3). In addition, the total IgE concentration was lower in patients with severe asthma than those with the mild asthma. This finding is in concordance with previous studies that demonstrated lower levels of IgE in SA compared to MA. 

Lung function parameters such namely FEV(1) are one of the important criteria used to differentiate different asthma phenotypes. Problematic or refractory severe asthma is characterized by poor asthma control despite high-dose inhaled corticosteroid therapy. Although our severe patients were receiving corticosteroids, they had a low FEV1 which suggests poor asthma control in this group. We used sorted cells therefore the percentages of gated CD25* cells were high. Our study showed a very significant difference in the frequency of CD8*CD25*FoxP3* nTreg cells in patients with severe asthma compared to those with the mild asthma and the healthy subjects. The lower level of CD8*CD25*FoxP3* nTreg cells in patients with allergic asthma compared to healthy subjects suggests that the discrepancy in the number of CD8*CD25*FoxP3* nTreg cells hence, the impairment in their function influences the development of allergic asthma. In addition the lower prevalence of these cells in patients with severe asthma compared with those affected by mild asthma may lead to the suggestion that, the lowest the frequency of CD8*CD25*FoxP3* nTreg cells the highest the severity of asthma syndrome. Severe asthma may be differentiated from mild asthma based on the degree of bronchial hyper responsiveness, cellular inflammatory infiltrate in the bronchial submucosa; less responsiveness to current asthma drugs namely the glucocorticosteroids(GCs). Although CD8* Treg cells received less attention compared with their CD4* Treg cells counterparts, their contribution to the maintenance of immune tolerance represents the backbone of regulatory T cells response. One of the issues that hamper the investigation of CD8* Treg cells is their low abundance and the transient nature in pathologic milieu [3][9]. CD8* Treg cells expressing the transcription marker Foxp3 represents a small proportion of FOXP3* cells. Moreover FOXP3*CD8* Treg cells represent a small proportion of circulating CD8* T cells in human blood and it has been suggested that, Foxp3 expression does not always confer suppressor properties [14]. To check this assumption, FOXP3 mRNA expression was analyzed in healthy subjects and in patients with mild to moderate or severe asthma. FOXP3 mRNA expression is decreased in CD8*CD25*FoxP3* Treg cells of severe asthmatic patients compared with those with mild asthma and the healthy groups. Although this finding may be controversial, it may suggest Foxp3 defect or unresponsiveness to corticosteroid in patients with severe asthma. In addition to their side effects, current asthma therapies do not always
relieve the symptoms of refractory asthma. People affected by this severe asthma phenotypes are unresponsive to higher doses of anti-inflammatory drugs such as ICS. The therapeutic effect of these drugs depend on the expression of Foxp3. Defect in Foxp3 expression in severe asthma patients may be linked to drug responsiveness in these patients. The patients with moderate asthma were allowed to receive inhaled steroids but no systemic steroids. A significant increase in FOXP3 mRNA expression was observed in freshly isolated and non-stimulated CD8^+CD25^+FoxP3^+ Treg cells of steroid-treated patients with moderate asthma (2.10^-fold, P less than 0.05; Fig 2) compared with healthy controls subjects or severe asthma patients. Foxp3 is thought to be crucial in both the development and suppressor function of Treg cells [5]. Our study showed a lower frequency of CD8^+CD25^+FoxP3^+ Treg cells in patients with severe asthma compared to the moderate asthma and healthy subjects. The decrease in the Foxp3 mRNA expression in severe asthma group with lower CD8^+CD25^+FoxP3^+ Treg cells suggests Foxp3 expression defect in patients with severe asthma. The low percentage of naturally occurring Treg cells or the low expression of mRNA Foxp3 probably contributes to the development of allergic asthma and determines the severity of asthma. Glucocorticosteroids exert their therapeutic effect by interacting with the transcription factor Foxp3. The 20% of severe asthma patients affected by poorly controlled severe persistent asthma are often prone to life threatened exacerbations. These patients often require high-dose inhaled corticosteroids (ICS) in combination with long-acting β₂-agonists (LABAs) and other controller drugs [16]. Although there is a lower frequency of CD8^+CD25^+FoxP3^+ Treg cells in patients with severe asthma compared with the controls subjects (4-fold increase) there is no significant difference in FOXP3 mRNA expression between these two studied groups (P > 0.05; Fig 2). This finding suggests Foxp3 defect in patients with severe asthma. Our data suggest a correlation between the frequency of naturally occurring CD8^+ Treg subsets and the grade of asthma severity. Larger cohorts studies of patients with severe asthma will be needed to confirm the contribution of CD8^+CD25^+FoxP3^+ Treg cells in the pathological mechanism of allergic asthma and their relevance to the severity of the disease.

Reference:


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