Frequency and Activation of CD4+CD25^{high} FoxP3+ Regulatory T Cells in Peripheral Blood from Children with Atopic Allergy

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Key Words

Allergy · Children · Regulatory T cells · Flow cytometry

Abstract

Background: Atopic allergy is among the immune tolerance-related disorders resulting from a failure of the regulatory network. Regulatory T cells (Tregs) play a leading role in the development of homeostasis in the immune system. The aim of this study was to determine the role of Tregs in the pathogenesis of atopic diseases in children by exploring the relationship between Treg frequency, activation markers and the clinical manifestations of the disease. **Methods:** Twenty allergic and 50 healthy children were enrolled to the study. Peripheral blood mononuclear cells were stained with monoclonal antibodies (anti-CD25–CD4–CD127–FoxP3–CD69–CD71) and evaluated using flow cytometry. Tregs were identified as CD4+CD25^{+/high}FoxP3+CD127– T cells. **Results:** The percentage of Tregs in allergic patients (2.3%) was significantly decreased in comparison to healthy controls (4.6%, $p = 0.003$). The frequency of Tregs in patients with symptoms of atopic dermatitis and/or food allergy (1.7%) was significantly lower than in patients without these symptoms (2.9%, $p = 0.04$). A significant correlation between the percentage of Tregs and the sIgE serum concentra-

tion was observed ($p = 0.037$). Relative fluorescence intensities of FoxP3 expression in allergic patients were higher than in healthy controls ($p = 0.00004$). The frequency of CD4+CD25^{high}CD127–CD71+ cells did not differ between the groups. **Conclusions:** Tregs display substantial deficiencies in atopic children, especially in children with multiorgan involvement, compared to patients with single organ manifestations. Additionally, there is an association between Tregs and the sIgE serum concentration. Better identification and characterization of Tregs in allergy is needed as they limit responses to foreign antigens, thereby minimizing T cell-mediated immunopathology in allergic diseases.

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Introduction

Regulatory mechanisms are necessary to maintain peripheral tolerance by the immune system. Allergy is one of the immune tolerance-related disorders resulting from a failure of the regulatory network. The most important cells involved in the immune system regulation are CD4+CD25^{high} regulatory T cells (Tregs). Tregs play a major role in regulation of allergic reactions by inducing and maintaining immune tolerance to allergens. Tregs

should keep a state of tolerance to innocuous substances and limit incorrect or excessive immune responses. Several pathways allow Tregs to control and modify the development of allergic reactions. Tregs directly inhibit the activation of Th2 cells (they suppress the production of IL-4, IL-5, IL-9 and IL-13), block the migration of effector T cells into inflamed tissue, suppress the production of IgE, induce IgG4 in B cells and limit Th17-mediated inflammation (as recently demonstrated in mice) [1–3].

Tregs are divided into several populations. Naturally occurring Tregs, expressing the forkhead transcription factor (FoxP3), are found in thymus and in peripheral blood, where they account for 5–10% of CD4⁺ T cells. FoxP3 is a crucial factor responsible for the development of Tregs in the thymus. It is also needed to maintain the suppressive activity of mature peripheral Tregs and is a specific molecular marker of Tregs in human peripheral blood. Decreased FoxP3 expression causes conversion of Tregs into effector cells [4]. FoxP3 expression in Tregs plays a crucial role in maintaining immunological tolerance [5–7].

As has been suggested by many authors, FoxP3 expression is also crucial for the identification of T cells bearing suppressive activity [7]. However, FoxP3 alone is not sufficient to characterize this specific subpopulation. According to Seddiki et al. [8] and Liu et al. [9] CD127 is downregulated in Tregs and this population should be defined as CD4⁺CD25^{+/high}CD127^{-/low}. This phenotype provides the possibility to separate activated T cells, which are CD127⁺, from Tregs, which are CD127^{low} or CD127⁻. Hence, CD127 can be a useful marker used in purification of the CD4⁺CD25^{high}FoxP3⁺ T cell population [8–10].

The process of activation of Tregs remains unclear. It was widely proposed that CD69 was involved in the activation and proliferation of different leukocyte subsets. CD69 is a homodimeric leukocyte transmembrane protein that is transiently expressed upon cell activation and is detected on small subsets of T cells from healthy subjects. CD71 (transferrin receptor) is also considered as an activation-associated molecule on lymphocytes [11, 12]. Some authors report a lack of activation markers (e.g. CD69 or CD71) on the surface of Tregs [13], whereas others have documented their presence [14] or even increased CD69 expression after specific therapy [15].

Recently, different studies have started to highlight the role of Tregs in the course of allergy and the link between this subpopulation and specific immunotherapy. Nevertheless, the role of Tregs in the pathogenesis of pediatric allergic disorders is still far from being elucidated. The aim of this study is to provide new insights into the field

by exploring the role of Tregs in the pathogenesis of atopic disease in children, especially the relationship between Treg frequency and clinical manifestations of the disease as well as IgE level. Another aim is to evaluate the expression levels of cell surface molecules that may modulate the function of CD4 cells in the course of asthmatic inflammation.

Materials and Methods

Patients

Twenty children with atopic allergy (diagnosed and treated in the Department of Pediatric Pneumonology and Allergology, Medical University of Warsaw, Warsaw, Poland) and 50 healthy children were enrolled. All patients had characteristic symptoms of controlled asthma (CA), allergic rhinitis (AR) and allergic conjunctivitis (AC). Ten of them also had atopic dermatitis (AD) and/or food allergy (FA). The study was approved by the Independent Ethics Committee of the Medical University of Warsaw and the patients/parents gave informed consent for participation in the study.

The diagnosis of CA was based on the patient's medical history, a physical examination and lung function tests that demonstrated reversibility of airflow obstruction. The degree of reversibility in FEV₁ after inhalation of a rapid-acting bronchodilator (200 µg salbutamol) was ≥12% and ≥200 ml from the prebronchodilator value [16]. The diagnosis of AR was based on a typical history of allergic symptoms such as rhinorrhea, sneezing, nasal obstruction and pruritus, confirmed by the presence of allergen-specific IgE by skin prick tests and/or the detection of allergen-specific IgE in serum [17].

Table 1 shows the clinical characteristics of the children with allergy and the healthy controls. All patients were examined at the time of diagnosis of allergy and before allergen-specific immunotherapy. Patients treated with inhaled corticosteroids were excluded from the study because corticosteroids may affect lymphocyte functions and CD25 expression [18]. The patients and controls were age matched.

Cells

After routine diagnostic tests were performed in the children with allergy and the healthy controls, 0.5–1.0 ml of heparinized blood remained, which was used for this study. Peripheral blood mononuclear cells (PBMCs) were isolated using a standard Ficoll-Histopaque[®]-1077 (Sigma Aldrich Co., St. Louis, Mo., USA) gradient centrifugation according to the instruction manual. The cell concentration was adjusted to 1 × 10⁶/ml in PBS supplemented with 0.5% inactivated FBS. The viability of blood lymphocytes was determined by trypan blue staining and achieved approximately 98%.

Analysis of FoxP3 Expression in CD4⁺CD25^{high}CD127⁻ T Cells and the Assessment of CD4⁺CD25^{high} T Cell Activation

Freshly isolated 100 µl of 1 × 10⁶/ml PBMCs were stained with 5 or 10 µl of monoclonal antibodies (according to the manufacturer's instructions; BD Pharmingen, BD Biosciences, San Jose, Calif., USA) in 2 tubes.

Table 1. Clinical characteristics of allergic children and healthy controls

	Allergic children	Healthy children (control group)
Patients, n	20	50
Boys	17	21
Girls	3	29
Mean age $\pm$ SD, years	7.8 $\pm$ 2.63	7.0 $\pm$ 4.93
Type of allergy		
Pollen	20	N/A
Dust	6	
Clinical manifestations		
CA, AR, AC only	10	N/A
also AD and/or FA	10	
WBC, cells/ μ l		
Average $\pm$ SD	7,860 $\pm$ 2,461.8	7,113 $\pm$ 2,054.4
Median	8,300	6,890
Lymphocytes, cells/ μ l		
Average $\pm$ SD	3,245 $\pm$ 1,004.5	3,325 $\pm$ 1,739.9
Median	3,150	2,625
Eosinophils, cells/ μ l		
Average $\pm$ SD	361 $\pm$ 349.5 (4.7 $\pm$ 3.33%)	328 $\pm$ 159.6 (7.0 $\pm$ 4.93%)
Median	286 (4.4%)	270 (8.5%)
Serum-specific IgE to birch pollen, kU/l		
Average $\pm$ SD	34.0 $\pm$ 45.73 ¹	N/A
N/A = Not applicable. ¹ n = 14.		

Tube 1: anti-CD25 PE-Cy7 (clone M-A251), anti-CD4 PE-Cy-5, anti-CD127 PE.

Tube 2: anti-CD69 PE, anti-CD71 FITC, anti-CD25 PE-Cy7 (clone M-A251), anti-CD4 PE-Cy-5.

More accurate identification of activated Tregs in PMBCs from 6 patients was possible due to staining with the following antibodies: anti-CD71 FITC, anti-CD127 PE, anti-CD25 PE-Cy7 clone M-A251, anti-CD4 PE-Cy-5.

The tubes were incubated for 20 min in the dark at room temperature (RT). Subsequently, cells were washed twice with washing buffer (PBS supplemented with 0.5% inactivated FBS) for 5 min at 250 g.

FoxP3 intracellular staining was performed according to the manufacturer's instructions (BD Pharmingen). Briefly, cells were suspended in 2 ml of buffer A and incubated for 10 min at RT in the dark. After 5 min of washing with washing buffer at 500 g, cells were incubated for 30 min in 500 μ l of buffer C at RT in the dark. Subsequently, the cells were stained with 20 μ l of anti-FoxP3 monoclonal antibody (Alexa Fluor 488, BD Pharmingen) for 30 min at RT in the dark and washed twice.

Flow Cytometric Analysis

The appropriate isotype controls were included. The samples were analyzed within 24 h on a Cytomics FC500 flow cytometer (Beckmann Coulter, Brea, Calif., USA).

Statistical Analysis

The Shapiro-Wilk test was employed for quantitative variables to confirm nonparametric data distribution. Statistical analysis was performed using nonparametric Wilcoxon test for dependent samples and nonparametric Mann-Whitney U test for independent samples. Correlations between quantitative variables were studied by Spearman's test. The significance level considered was 0.05.

Results

Morphological and Cytometric Parameters of Leukocytes

A significantly higher number of white blood cells in children with allergy than in healthy controls [median (25 percentile; 75 percentile): 8,300 (5,900.0; 9,300.0) vs. 5,650 (3,500.0; 7,870.0), respectively, $p = 0.003$] was found. However, the relative number of lymphocytes and eosinophils as well as the total eosinophil count did not differ between controls and patients. No difference was found between total CD4+CD25+ T cell counts in samples from children with allergy and healthy controls

Fig. 1. Treg gating strategy: example of 1 patient from the control group. PBMCs were stained with a combination of surface antigens (anti-CD4, anti-CD25 and anti-CD127) and intracellularly with anti-FoxP3. For analysis, CD4+ cells from PBMCs were gated

(dot plot SS vs. CD4, gate B; **a**), and analyzed for CD25 and FoxP3 expression (dot plot CD25 vs. CD4FoxP3 for gate B; **b**), cells in gate H were considered as Tregs. **c** The expression of CD127 in the Treg population was analyzed (dot plot CD4 vs. CD127 for gate H).

(about 14–16% of CD4 T cells were CD25+ in both groups). The percentage of CD4+FoxP3+ cells did not differ between the examined populations either.

Percentages of CD4+CD25^{high}FoxP3+ Tregs in Peripheral Blood of Allergic Patients and Healthy Controls

Tregs in peripheral blood were identified as CD4+CD25^{+/high}FoxP3+CD127– T cells. The number of Tregs is expressed as a percentage of all CD4+ T cells. The gating strategy is displayed in figure 1. The percentage of Tregs in samples from allergic patients was significantly decreased in comparison to healthy controls [median (25 percentile; 75 percentile): 2.3 (1.63; 3.58) vs. 4.6 (3.69; 5.68), respectively, $p = 0.003$; fig. 2].

All allergic patients suffered from CA, AR and AC. AD and/or FA was also diagnosed in a subgroup of patients. We observed significantly lower percentages of Tregs in patients with symptoms of AD and/or FA than in patients free from those clinical presentations [median (25 percentile; 75 percentile): 1.7 (1.51; 2.04) vs. 2.9 (2.45; 3.70), respectively, $p = 0.043$; fig. 3].

In the subgroup of patients allergic to birch pollen, sIgE serum concentrations to birch allergen were correlated with the relative number of Tregs. A significant correlation between Treg percentage and sIgE serum concentration ($r = -0.56$, $p = 0.037$) was found (fig. 4). The

percentages of Tregs did not differ between subgroups of patients sensitized to few (1–2) and to multiple antigens (3 or more).

Relative fluorescence intensities (RFI) of FoxP3 expression were also compared between patients and controls. RFI was calculated using the following formula: experimental mean fluorescence intensity (MFI) / MFI with isotype control antibody, according to Dechant et al. [19]. Significantly higher RFI of FoxP3 expression in children with allergy than in healthy controls [median (25 percentile; 75 percentile): 11.4 (9.65; 13.53) vs. 6.8 (5.35; 8.94), respectively, $p = 0.00004$] was found. The RFI value was significantly lower in the subgroup of patients with CA, AR and AC compared to the group of patients with concomitant AD and/or FA [median (25 percentile; 75 percentile): 9.9 (6.56; 11.47) vs. 13.5 (11.26; 20.98), respectively, $p = 0.007$]. The results are shown in figure 5.

The Frequency of CD4+CD25^{high} T Cells with CD69 and CD71 Expression

Initially CD71 and CD69 expression on the whole population of CD4+CD25^{high} T cells was analyzed. CD69 expression on CD4+CD25^{high} was significantly higher in allergic patients ($n = 11$) compared to healthy controls [$n = 23$; median (25 percentile; 75 percentile): 6.8 (3.74; 9.68) vs. 3.1 (1.90; 4.34), respectively, $p = 0.013$]. Accord-

Fig. 2. The percentage of CD4+CD25^{high}FoxP3+CD127- Tregs in the CD4 T cell population in peripheral blood of patients with allergy (A; n = 20) and the control group (n = 50).

Fig. 3. The percentage of CD4+CD25^{high}FoxP3+CD127- Tregs in the CD4 T cell population in peripheral blood of patients only with CA, AR and AC (0; n = 10) and patients with concomitant FA and/or AD (1; n = 10).

Fig. 4. Correlation between the percentages of CD4+CD25^{high}FoxP3+CD127- Tregs in the CD4 T cell population in peripheral blood of patients and birch pollen sIgE serum concentrations (kU/l).

ingly, allergic patients had significantly higher expression of CD71 compared to nonallergic controls [median (25 percentile; 75 percentile): 4.2 (2.59; 8.20) vs. 2.8 (1.37; 3.76), respectively, $p = 0.021$]. The same analysis was conducted after elimination of activated T effector cells by

excluding CD4+CD25^{high}CD127+CD71+ cells. The frequency of CD4+CD25^{high}CD127-CD71+ cells did not differ between allergic children [$n = 6$; median (25 percentile; 75 percentile): 3.5 (2.59; 5.87)], and the control group [$n = 20$; 3.83 (1.82; 5.00)].

Fig. 5. Differences in RFI of FoxP3 expression in CD4+CD25^{high}-FoxP3+CD127⁻ Tregs in peripheral blood of patients with allergy (A; n = 20) and healthy controls (n = 40) according to the counting formula presented in the Results section.

Discussion

In the present study the frequency and phenotype of circulating Tregs in allergic children were analyzed. Tregs were defined as CD4+CD25^{high}FoxP3+CD127⁻ T cells. Recent studies have demonstrated that downregulation of CD127, a specific receptor for IL-7, can distinguish Tregs from activated T cells. We could demonstrate that the frequency of Tregs was lower in allergic children compared to age-matched healthy controls. This difference was statistically significant even though the number of allergic patients was relatively small. Recently, different studies have started to highlight the link between allergy and defective immunoregulation; however, the results are inconsistent. Lee et al. [20] and Xu et al. [21] described a reduction of the Tregs compartment in patients with allergy. Zhang et al. [22] found a significant decrease of Tregs only in acute exacerbation of asthma in comparison with chronic stable disease. The study by Adkis et al. [23] also corresponds with our findings as they described a significantly lower relative count of Tr1 cells (producing IL-10) in the peripheral blood of adult patients. Nevertheless, other scientists state that the frequency of Tregs does not differ between allergic and nonallergic populations [24–26]. The reasons given for this discrepancy remain speculative, but may be related to the clinical form of the disease, age of the patient, exposure to allergens and the

implemented therapy. A higher frequency of Tregs in patients treated with steroids or during exacerbation has been reported [20, 27, 28].

The discordant results may depend not only on the patient population but also on the way Tregs were characterized. Not all analyzed reports defined Tregs as CD4+CD25^{high}FoxP3+ T cells. In some studies Tregs were recognized as CD4+CD25^{high} cells. In addition, this type of analysis can be biased by different drug regimens of patients participating in clinical research studies or by inconsistent time points of specimen collection, etc. Nevertheless, most researchers agree that Tregs are downregulated in allergic patients and these observations correspond to our findings.

There were a few attempts to explain the decline in the percentage of Tregs in patients with allergy. The role of Tregs in asthma and allergic disease is consistent with the hygiene hypothesis, stating that exposure to bacterial products can result in a lower incidence of atopy and allergy/asthma through favoring the development of tolerance to antigens encountered at mucosal surfaces [29]. The altered immunological programming in early life by specific infections or inflammatory reactions may affect the balance between effector and regulatory cells. The antigens carried by the infectious agents induce both humoral and cellular Th1, Th2 and Th17 responses. Such responses can compete with allergic responses elicited by allergens, which are in the greater part weak antigens. Infections in childhood can alter the nature of innate/adaptive immunity interaction through toll-like receptors, which have a direct and an indirect impact on Tregs, for example, TLR2 ligation reduces FoxP3 expression by Tregs [30]. Another environmental factor contributing to the development of allergy is the intensity of the exposition to allergens [31].

In the course of autoimmune diseases such as rheumatoid arthritis, decline in the percentage of peripheral blood Tregs can be explained by their migration to the sites of inflammation, especially to the affected joints. Several authors have revealed increased infiltration of functional Tregs in synovial fluid aspirated from affected joints [3, 32, 33]. The issue of distribution and compartmentalization of Tregs in the course of allergic diseases is unknown. Nevertheless, significant differences were observed between allergic patients and healthy controls in terms of the absolute number or the percentage of Treg cells in the airways that could be explained by possible defects of chemokine CCL1 and XCL1 receptors in Tregs [34, 35]. Hartl et al. [36] showed that the percentage of Tregs and their function in bronchoalveolar lavage fluid

(BALF) obtained from asthmatic children are decreased in comparison to BALF from healthy children.

The relationship between the frequency of Tregs and disease severity has been reported in several studies. In 2004, Shi et al. [28] demonstrated that the number of Tregs increases during an exacerbation of asthma. In 2007, Lee et al. [20] showed that children with persistent AR had significantly higher percentages of Tregs compared to children with intermittent AR. Moreover, patients with moderate-to-severe bronchial asthma had higher percentages of Tregs than patients with mild bronchial asthma. In 2009, Meszaros et al. [37] found an association between symptom severity and the proportion of CD4+FoxP3+ cells.

These findings are consistent with our observations that the relative number of Tregs depends on the clinical manifestations of atopic disease. Children with respiratory tract atopy had significantly higher Tregs in their peripheral blood than children with coexisting gastrointestinal tract or skin involvement (AD and/or FA; fig. 2). The significance of this observation is currently unclear. The differences in immunoregulation may be reflected in differing phenotypes of atopy. A significant decrease in Treg number or function may predispose not only toward the development of CA and AR, but also toward other atopic conditions such as AD. In general, the clinical signs of FA and/or AD predate the development of CA and AR in the majority of affected patients, giving rise to the atopic march, which suggests that FA/AD is an initial step in the development of subsequent atopic diseases. As shown in a recent review by Oiso [38], the results obtained in different studies reveal many discrepancies regarding the role of Tregs in AD. Still, the number of Tregs in peripheral blood and infiltrating the skin in patients with AD is controversial. To the best of our knowledge, this is the first time patients with typical allergic symptoms were compared with patients who additionally developed FD/AD. We observed a greater decrease of Tregs in peripheral blood of FD/AD patients, which can be explained by the migration of Tregs to skin lesions [38].

Hartl et al. [36] did not find any association between total or specific IgE levels and the percentage of CD4+CD25^{high} T cells in BALF or peripheral blood in children with asthma. In our study we noticed a significant correlation between specific IgE serum levels and the percentage of Tregs in children with birch pollen allergy ($p = 0.036$). Our findings correspond with the observation that in allergic diseases Tregs suppress the production of allergen-specific IgE and induce IgG₄ production by direct effects on B cells [39]. We also found that the

different combination of markers used to define Tregs influences the interpretation and conclusions drawn from the results.

In addition, the expression of molecules associated with activation as CD69 and CD71 on Tregs was evaluated. We noticed higher expression of CD69 and CD71 on CD4+CD25^{high} T cells from children with allergy in comparison to healthy controls, but not on CD4+CD25^{high}CD127[–] cells. Some authors have described enhanced expression of CD69 on CD4+CD25⁺ T cells in children with allergy. Lee et al. [20] did not observe any differences in expression of this antigen between their control group and children with allergic airways disease. However, the majority of authors claim that CD69 is not expressed on Tregs and can serve as a marker to exclude activated effector T cells from the Treg population [13, 40].

CD69 is persistently expressed on leukocyte infiltrates of different chronic inflammatory diseases. This observation is very consistent with our findings as CD4+CD25^{high} cells represent effector rather than regulatory cells. However, recent *in vivo* data indicate that this molecule may also play a different role in the immune system. Some *in vitro* studies showed that triggering of CD69 induces the production of TGF- β , an immunoregulatory and anti-inflammatory cytokine [13–15]. Thus, these data strongly suggest that CD69 can be a negative regulator of allergic reactions although this molecule exerts its action on effector cells rather than on Tregs.

Interestingly, increased proportions of cells expressing CD71 were found on CD4+CD25^{high} T cells in atopic patients as compared to healthy controls, but no differences in the expression of CD71 on CD4+CD25^{high}CD127[–] Tregs in patients with allergy and in healthy children were found. Similarly to CD69, the possible explanation for this observation might be the presence of activated non-Treg T cells within the CD4+CD25^{high} T cell population. Additionally, in both examined groups the expression of both molecules on circulatory effector and regulatory T lymphocytes was very low.

Several studies have reported an increase in the expression of CD71 on differently defined Tregs in the course of different inflammatory diseases [33]. In the literature we did not find any study exploring the role of CD71 in allergy.

Another aim was to analyze the RFI of FoxP3 expression in the examined groups. Even though Tregs were significantly less frequent in the allergic population they had significantly higher expression of FoxP3. Similarly, patients with their allergy limited to the respiratory tract

and a higher percentage of Tregs had lower RFI of FoxP3. A very cautious hypothesis based on this observation may suggest that higher FoxP3 expression on decreased populations of Tregs can at least partially maintain their suppressive function. We did not find any explanation of this phenomenon in other works.

Taken together, using several types of analyses and linking all data obtained, we have provided evidence that CD4+CD25^{high}FoxP3+CD127^{low} Tregs display substan-

tial deficiencies in atopic patients as compared with healthy children. Moreover, this phenomenon is more deeply expressed in patients with additional symptoms, such as FA/AD. Although Treg deficiency is not the only abnormality of immune regulation in atopy, better quantitative and qualitative characterization of Tregs in allergy is needed because the decrease in the number and function of these cells may lead to downregulation of T cell tolerance and exacerbation of the disease.

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