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T Regulatory Cells in Children with Atopic Dermatitis

Atopik Dermatitli Çocuklarda Regülatör T Hücreleri

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Abstract

Objectives: The aim of this study was to examine peripheral blood CD4+CD25^{high}+FOXP3+ regulatory T (Treg) cell levels in children with atopic dermatitis (AD) and to evaluate their effects on the pathogenesis of the disease.
Materials and Methods: Forty one patients (age between 1-60 months) satisfied the criteria for AD (Hanifin and Rajka criteria) and 20 healthy children were included into the study. Intracytoplasmic FOXP3 expression in CD4+CD25+ cells was measured by flow cytometry (Beckman Coulter FC500, USA, Software CXP 1.2). The change in mean fluorescence intensity (MFI) of the FOXP3 molecule relative to the MFI of the isotypic control was based on evaluation.
Results: Children with atopic dermatitis have significantly lower levels of peripheral blood CD4+CD25^{high}+FOXP3+ regulatory T cells and higher levels of CD4+CD25^{high}+FOXP3+ regulatory T cell MFI than the control group ($p<0.001$).
Conclusion: Low levels of CD4+CD25^{high}+FOXP3+Treg cells in atopic dermatitis might be due to the accumulation of these cells on the skin. While the CD4+CD25^{high}+FOXP3+Treg cell levels were low, the high MFI levels of these cells suggests an attempt by Treg cells to regulate the immune response. Therefore, CD4+CD25^{high}+FOXP3+Treg cells might play a role in the pathogenesis of atopic dermatitis.
Key Words: Atopic Dermatitis, Regulatory T, FOXP3 Expression, Flow Cytometry, Mean Fluorescence Intensity Levels

Öz

Amaç: Bu çalışmanın amacı, atopik dermatitli (AD) çocuklarda periferik kanda CD4+CD25^{high}+FOXP3+ regülatör T (Treg) hücreleri ve bu hücrelerin hastalığın patogenezi üzerine etkilerini değerlendirmektir.
Gereç ve Yöntem: AD kriterlerini (Hanifin ve Rajka kriterlerini) karşılayan 41 AD'li hasta (yaş aralığı: 1-60 ay) ve 20 sağlıklı çocuk çalışmaya dahil edildi. CD4+CD25 + hücrelerde intrasitoplazmik FOXP3 ekspresyonu akım sitometrisi (Beckman Coulter FC500, USA, Software CXP 1.2) ile ölçüldü. Değerlendirmede FOXP3 molekülünün ortalama floresan yoğunluğunun (MFI) izotipik kontrolün MFI'ya göre değişimi esas alındı.
Bulgular: AD'li çocukların kontrol grubuna göre; periferik kan Treg hücre seviyeleri düşük ve CD4+CD25^{high}+FOXP3+Treg hücre MFI'ı daha yüksek bulundu ($p<0,001$).
Sonuç: AD'de düşük CD4+CD25^{high}+FOXP3+Treg hücre seviyeleri, bu hücrelerin deride birikmesine bağlı olabilir. CD4+CD25^{high}+FOXP3+Treg hücre sayıları düşük iken, bu hücrelerin yüksek MFI seviyelerinin olması Treg hücrelerinin immün yanıtlarını düzenlemek için bir girişim olduğunu düşündürmektedir. Bu nedenle CD4+CD25^{high}+FOXP3+Treg hücreleri AD patogenezinde rol oynayabilir.
Anahtar Kelimeler: Atopik Dermatit, Regülatör T, FOXP3 İfadesi, Akım Sitometri, Ortalama Floresan Yoğunluğu Düzeyleri

Introduction

Atopic dermatitis (AD) is a chronic, recurring inflammatory skin disease characterized by severe itching. AD primarily begins in childhood; it occurs in the first year of life in 60% of cases, and before age 5 in 85%. The disease affects 8%-12% of the population in industrialized countries, and, as with asthma, there has been an increase in AD frequency over the last 30 years (1-3).

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To date, several factors have been implicated in the pathogenesis of AD. The combination of genetic and acquired skin barrier dysfunctions, as well as distorted innate and adaptive immune responses, lead to the development of the characteristic clinical picture. In the last few years, a cellular immune response, particularly regulatory T cells (Treg cells), have been thought to play a key role in the pathogenesis of AD (4).

Treg cells are divided into three subgroups as follows: transforming growth factor-β (TGF-β) producing Th3 cells (adaptive Treg cells); Tr1 cells (adaptive Treg cells) which exert their activity through interleukin (IL)-10; and CD4+CD25+forkhead box P3 (FOXP3)+ Treg cells (natural Treg cells) that play a more prominent role in self-tolerance, prevention of autoimmunity, and regulation of immune response (4). The interactions between the subgroups of Treg cells and the nature of the regulatory process have not yet been fully elucidated. Differently from Th3 and Tr1 cells (adaptive Treg cells, effector T cells), and independently from the inhibitory effects of cytokines such IL-10 and TGF-β, CD4+CD25+FOXP3+Treg cells are believed to exert their effect through a cell-to-cell contact-dependent manner during the regulatory process.

CD4+CD25+ cells express FOXP3, which is a transcriptional regulatory protein. This protein is also the main regulator of development and function in Treg cells (5). A deficiency or insufficiency in FOXP3 expression leads to the developed of the IPEX syndrome (6,7).

The aim of this study was to evaluate whether natural Treg cells are involved in the development and pathogenesis of AD, a common condition among children, by measuring Treg cells levels in the peripheral blood during the active stage of the disease prior to treatment.

Materials and Methods

Selection of the Study Group

Forty-one children who were newly admitted to and/or were under follow-up at the our Department of Pediatric Immunology and Allergy, Ankara University Faculty of Medicine, between March 2009 and April 2010 were included in the study. The control group consisted of 20 healthy age- and sex-matched children.

The Hanifin and Rajka diagnostic criteria were used for AD diagnosis. Patients with active AD were not receiving local or systemic treatment (especially steroids) at the time of the study or at least 3 months prior to the study. Data collected during the study included gender, symptoms, physical examination findings, information regarding past medical and family history, exposure to cigarette smoke, complete blood count, total neutrophil count, lymphocyte and eosinophil counts, total immunoglobulin E (IgE) levels, Phadiatop values, skin prick test results (if previously performed), and specific IgE, and Treg cell levels.

Sample Collection

The study was initiated following its approval by the local ethics committee of Ankara University Faculty of Medicine. Written parental consents were obtained from the parents of all children. To evaluate the FOXP3 expression on T helper cells, peripheral blood samples initially obtained for complete blood cell count were utilized.

Methods

Three color flow cytometric analyses were performed on the whole blood samples collected in ethylenediamine tetraacetic acid (EDTA) containing tubes. Following this, 100 µL of whole blood with EDTA was added to each tube, and surface staining was performed using the CD4 (Beckman Coulter, Marseille, France) and CD25 (Beckman Coulter, Marseille, France) antibodies. The samples were then washed with Flow Cytometry Staining Buffer (eBioscience, Cat. 00-4222) or cold phosphate buffered saline. The cell pellets were resuspended with pulse vortex, and freshly prepared fixation/permeabilization working solution (eBioscience, Cat. 00-5123) was added to each sample.

Samples were incubated at 4 °C for 45 minutes in the dark. Samples were washed with 1x permeabilization buffer (eBioscience, Cat. 00-8333) and then centrifuged, after which the supernatant was decanted. This step was repeated once again, with cells being washed in 1× permeabilization buffer and centrifuged again, and the supernatant being decanted. Recommended volume of fluorochrome conjugated anti-human FOXP3 antibody (eBioscience, Cat. 12-4776.) or isotype control was added to 1× permeabilization buffer, and samples were incubated at 4 °C for at least 30 minutes in the dark. Cells were again washed in 1× permeabilization buffer and centrifuged, and the supernatant was decanted. Cells were resuspended in appropriate volume of Flow Cytometry Staining Buffer, and analyzed using a cytometer.

Three subsets of CD4+ T cells were defined according to CD25 staining: CD25-, $CD25^{low}$ and $CD25^{high}$.

Cells expressing $CD25^{high}$ were chosen and gated for the detection of FOXP3+ T cells. Beckman Coulter Cytomics FC500 (three-color flow cytometry with CXP software version 2.2 was used for analysis. All flow experiments and analysis were performed by the same staff of the laboratory to avoid inter-individual differences in technique.

Statistical Analysis

The statistical analysis was performed at the Biostatistics Department of Ankara University Faculty of Medicine using the

Statistical Package for the Social Sciences (version 15. SPSS Inc. Chicago, IL, USA). The nonparametric Mann-Whitney U test used for comparing FOXP3 levels and mean fluorescence intensity (MFI) values on CD4+CD25+ cells between the two groups. For comparing T regulatory cells with respect to age, gender, symptoms on admission, physical examination, laboratory findings and exposure to cigarette smoke; a non-parametric correlation test, the Spearman's rho, was used. A p value of <0.05 was considered statistically significant.

Results

The patient group consisted of 16 girls and 25 boys with ages ranging between 6 months and 36 months, and a median age of 7 months. The male/female ratio was 1.56. The control group, on the other hand, consisted of 8 girls and 12 boys with a median age of 12 months.

Evaluation of the patient complaints on admission revealed that dry skin was present in all patients. Erythema on the cheeks was observed in 21 (51.2%) of the patients. The IgE levels ranged between 0 and 324 kU/L (median, 36 kU/L), and the IgE levels were found to be higher compared to the same age group in 36% of the patients (Table 1).

CD4+CD25+FOXP3+Treg cells in the peripheral blood of the patient and control groups were measured as 2.89±0.95% and 6.97±1.57%, respectively. MFI of CD4+CD25+FOXP3+ expressing Treg cells was found to be 13.6±7.2 in the patient group (median, 11.9), and 6.1±2.0 (median, 5.8) in the control group. A significant difference was found between the two groups with respect to the CD4+CD25+FOXP3+Treg cells (%) and MFI of CD4+CD25+FOXP3+Treg cells (p<0.001) (Table 2). No significant relation was identified between the levels and MFI values of CD4+CD25+FOXP3+Treg cells, on one hand, and the parameters of age, gender, exposure to cigarette smoke, complaints on admission, physical examination findings, SCORAD scores white blood cell counts, total lymphocyte counts, total neutrophil counts, total eosinophil counts, total IgE levels and specific IgE levels, on the other (p>0.05). Only MFI values were found to be lower in the cigarette smoke exposed group compared to the non-exposed group (p<0.05, r=-0.35).

Table 1: Demographic, clinical and laboratory features of the patients and control groups

	AD group (n=41)	Control group (n=20)
Male/female-ratio	25/16-1.56	12/8-1.5
Age [min-max/median] (months)	1-60 (med: 7)	1-60 (med: 12)
Symptoms Dry skin Erythema on the cheeks Debris on the skin Itching No symptom	 100% 51.2% 24.4% 22% 4%	
SCORAD index points	21-62 (med: 36)	
White blood cell count (mm³)	6400-17500 (med: 11400)	
Total lymphocyte count (mm³)	2600-13000 (med: 6200)	
Total eosinophil count (mm³)	0-1300 (med: 400)	
Phadiotop (n=37)	11% positive	
IgE levels	0-360 (med: 42 kiU/L)	
Specific IgE levels (n=36) [fx (5)]	36.6% positive	
Prick test results (n=17)	27.7% positive	

min: Minimum, max: Maximum, IgE: Immunoglobulin E

Table 2: The percentage of CD4+CD25+FOXP3+Treg cells and the CD4+CD25+FOXP3+Treg cell mean fluorescence intensity values in the patient and control groups

	AD group (n=41)	Control group (n=20)
CD4+CD25+FOXP3+Treg cells levels in the peripheral blood (%) (mean ± SD)	2.89±0.95 p<0.001	6.97±1.57
Expression of FOXP3 on the CD4+CD25+^{high} cells (%) (mean ± SD)	43.7±8.2 p<0.001	92.2±4.4
MFI values of CD4+CD25+FOXP3+Treg cells (%) (mean ± SD)	13.6±7.2 p<0.001	6.1±2.0

Treg: Regulatory T, FOXP3: Forkhead box P3, AD: Atopic dermatitis, MFI: Mean fluorescence intensity, SD: Standard deviation

Discussion

AD is a frequently encountered chronic inflammatory disease in children. Despite the identification of the factors causing the disease and the increase in treatment options, the frequency of AD is gradually increasing around the world. Due to the costs associated with its treatment and follow-up, great efforts are being made to elucidate the etiopathogenesis of the disease and to seek new treatment options.

The main cause of inflammation in AD has not yet been determined and is still being investigated. Currently, the cellular immune response, and particularly Treg cells are thought to play a key role in AD pathogenesis.

There are many studies investigating the relationship between asthma and Treg levels. On the other hand, the number of studies evaluating Treg levels in AD is somewhat limited. In these studies, different subsets of Treg cells have generally been evaluated without making any distinction between pediatric/adult patients and Treg cells subsets in AD patients. To the best of our knowledge, there is no study in the literature evaluating

natural Treg levels solely in pediatric AD patients. Furthermore, the levels of CD4+CD25+FOXP3+Treg cells have been expressed as percent ages in all existing studies, and it is noteworthy that no evaluations have been performed regarding the MFI values.

In a study performed by Ito et al. (8) on 35 patients with AD with a mean age of 27.1±7.5 years and 36 controls with a mean age of 27.5±10.0 years, the levels of circulating CD4+CD25+FOXP3+Treg cells (%) were found to be significantly higher in the patient group compared to the control group (8). However, in the patients whose samples were analyzed after 1 month and 2 months, the levels of CD4+CD25+FOXP3+Treg cells (%) decreased as the patients skin lesions improved, regardless of medications used. The authors suggested that this dynamic change in Treg cell compartments in the peripheral blood and skin might play a role in the pathogenesis of AD. In the study of Ito et al. (8), FOXP3 expression was measured in all CD4+CD25+ cells (in other words, in high, medium and low CD4+CD25+ cells without making any distinction). FOXP3 expression in effector T cells was also measured (9). In the present study, Treg cell levels (of the CD4+CD25+FOXP3+ phenotype) were measured in the peripheral blood and in pediatric patients. Differently from the results of this study, we observed markedly lower percentages of FOXP3 and FOXP3 expression on CD4+CD25+Treg cells in the patient group. Along with these parameters, the MFI of natural Treg cells were also measured in the present study, which were determined to be significantly higher in the patient group compared to the control group.

A study of Szegedi et al. (9) investigating the number of CD4+CD25+FOXP3+Treg cells and Tr1 cells in the peripheral blood samples of AD patients was performed with 31 AD patients comprising both of children and adults with a mean age of 22.2 years (range: 6-46 years) and 40 healthy controls. In the said study, significantly elevated numbers of Tr1 cells were identified in the peripheral blood samples of the AD patients. However, no significant difference was found between the two groups with respect to the percentage of CD4+CD25+FOXP3+Treg cells. Moreover, biopsy specimens were obtained from AD patients with skin lesions, and when the measurements were performed following the separation process, increased number of CD4+CD25+FOXP3+Treg cells were found in the skin of patients with AD. In the current study, however, the levels of CD4+CD25+FOXP3+Treg cells were only measured in the peripheral blood of children with AD and found to be low as compared to the control group (9). This finding is not consistent with the results of the study conducted by Szegedi et al. (9) according to Szegedi et al. (9), an increased number of CD4+CD25+FOXP3+Treg cells were found in the skin of patients with AD, and there was no significant difference between the patient and control groups with respect to the number of CD4+CD25+FOXP3+Treg cells in the peripheral blood to the accumulation of these cells in the skin. In the present study, aside from the fact that the levels of CD4+CD25+FOXP3+Treg cells were not evaluated at the tissue level, lower percentages of FOXP3 and lower FOXP3 expression on CD4+CD25+ Treg cells in the peripheral blood of patients with AD might have resulted from the accumulation of these cells in the tissue. Therefore, the increased MFI value of CD4+CD25+FOXP3+Treg cells in our study might be a reflection of the functional activation of these cells to compensate the disorganized inflammatory response.

In a study of Ou et al. (10) the authors included 15 patients with AD, 14 healthy control subjects and 12 asthmatic patients. The patients with AD were found to have significantly higher number of CD4+CD25+ Treg cells as compared to the asthmatic patients and healthy control subjects. In that study, Treg cells were evaluated as cells expressing only CD4 and CD25. However, nowadays, this is no longer considered sufficient for describing Treg cells, since it is also necessary to detect intracytoplasmic FOXP3 expression in addition to cell-surface expression of CD4 and CD25. Moreover, in the abovementioned study, adult and pediatric patients were evaluated together, and the levels of CD4+CD25+FOXP3+Treg cells were not measured (10). In the present study, on the other hand, only pediatric patients were evaluated, and a different definition was used for the natural Treg cells.

In a study conducted by Verhagen et al. (11) on 15 healthy volunteers and 24 patients with AD aged 19-45 years, skin biopsy samples were obtained for the evaluation of Treg cells levels. Although Tr1 and IL-10 levels were higher in the patient group, no CD4+CD25+FOXP3+Treg cells were detected (11). This is because adaptive Treg cells (Tr1 and Th3) are known to express FOXP3, although to a lower extent than natural Treg cells (of the CD4+CD25+FOXP3+ phenotype). Verhagen et al. (11) concluded that the lack of Treg cells of the CD4+CD25+FOXP3+ phenotype might be a factor that plays a role in the development of AD. The findings of our study are not consistent with the results of the study conducted by Verhagen et al. (11) As we mentioned previously, there are important methodological and contextual differences between Verhagen et al.'s (11) study and our own.

One common feature of these studies is the high Tr1 levels observed in the peripheral blood or skin. However, this condition still seems to be insufficient in explaining the pathogenesis of AD, since it is known that natural Treg cells (of the CD4+CD25+FOXP3+ phenotype), and particularly FOXP3, play a more prominent role in the regulation of immune response and inflammation, and that FOXP3 sometimes mediates this process by regulating the adaptive Treg cell compartments (12). Furthermore, in the above-mentioned study as well as similar studies, the MFIs of CD4+CD25+FOXP3+Treg cells have not been investigated.

The gold standard of precise analysis of human Tregs is the measurement of the DNA methylation status TSDR (Treg specific demethylated region) in the FOXP3 locus. Using this method Roesner et al. (13) have not found the differences concerning the Tregs number between AD patients and controls. However, Treg density correlated with the severity of the disease defined by SCORAD. A high number of Treg cells could be observed only in severely affected patients, similar to our study (13). A study by Stelmaszczyk-Emmel et al. (14) in children with AD indicated that Tregs defined as CD4+CD25highCD127-CD71+ were significantly less frequent in comparison to healthy controls. The frequency of Tregs in patients with symptoms of AD and/or food allergy was lower than in patients without these symptoms (14).

A study performed by Hinz et al. (15) reported that maternal cytokines (IL-13, IL-17E and interferon-γ) and maternal smoking/exposure to tobacco smoke during pregnancy were also associated with decreased cord blood Treg numbers. Children with lower Treg numbers at birth had a higher risk to develop AD and sensitization to food allergens during the first year of life. We could not find a correlation between exposure to cigarette smoke with Treg cells but MFI values were found lower in the cigarette smoke exposed group than non-exposed group. We did not ask for prenatal cigarette exposure and did not measure tokine levels. There is a need for further study in this issue.

Conclusion

In the current study, lower percentages of FOXP3 in lymphocytes and lower levels of FOXP3 expression in CD4+CD25+ Treg cells in peripheral blood might have resulted from the accumulation of these cells in the skin of AD patients. On the other hand, it is possible that the accumulation of these cells in the skin might also have resulted from lower levels or decreased functions of CD4+CD25+FOXP3+Treg cells. In this context, the higher MFI values observed despite the lower percentage of CD4+CD25+FOXP3+Treg cells might be due to these cells exhibiting an active function in the peripheral blood in an effort to regulate the immune response. These findings suggest that CD4+CD25+FOXP3+Treg cells play a role in the pathogenesis of AD.

Ethics

Ethics Committee Approval: The study was initiated following its approval by the local ethics committee of Ankara University Faculty of Medicine.

Informed Consent: We obtained written informed consent from the family members and controls.

Authorship Contributions

Concept: Ö.Ç., A.İ., Design: Ö.Ç., A.İ., Data Collection or Processing: Ö.Ç., Z.Ş.H., D.G., F.Ç., A.Ç., Analysis or Interpretation: D.G., F.D., A.İ., K.K., Literature Search: Ö.Ç., Z.Ş.H., Writing: Ö.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

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