

Evaluation of Immune Indices and Serum Vitamin D Content in Children with Atopic Dermatitis

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Abstract

The influence of vitamin D on allergic diseases, including atopic dermatitis, is linked to the presence of vitamin D nuclear receptors in immune cells. The present study seeks to determine the possible relationship between serum vitamin D content and immune indices in children with atopic dermatitis. The study was conducted in 19 children with atopic dermatitis. The control consisted of 17 age-matched healthy children. A single significant finding was a distinctly lower number of serum regulatory T cells in atopic dermatitis compared with controls ($p < 0.00001$). There were no appreciable differences between the two groups concerning the immunological indices such as the phenotypes: CD3, CD4, CD8, CD4/CD8, CD19, CD16/56, natural killer T cells, and anti-CD3 human leukocyte antigen – antigen D related cell surface receptor (HLA-DR3), or the percentage of lymphocytes, eosinophils, and the IgE level. We also revealed an inverse association between the serum vitamin D and the percentage of CD8+ cells ($p < 0.05$; $r = 0.62$) in atopic dermatitis. In conclusion, the results point to a regulatory role of T cells in the pathogenesis of atopic dermatitis, but fail to substantiate the influence of vitamin D on the course of the disease.

Keywords

Allergy • Atopic dermatitis • Children • Regulatory T cells • Vitamin D

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1 Introduction

Atopic dermatitis is a chronic inflammatory skin disease related with epidermis and dermis dysfunction. It is characterized by a chronic and recurrent course and distinctive morphology and location of lesions. The concept of atopic dermatitis was introduced in 1933 by Wise and Sulzberger (Reitamo et al. 2008), while the diagnostic criteria were proposed in 1980 by Hanifin and Rajka (1980) and they are still valid (Darsow et al. 2010). The disease usually begins in early childhood; 60% of cases occur before one year of age and in up to 45% of cases the first symptoms appear before 6 months of age. The incidence of allergic diseases, including atopic dermatitis, increases in recent years, which is related to civilization development. Over the past 30 years, two- or even threefold increase in the morbidity of atopic dermatitis has been observed in industrialized countries (Bieber 2010). The incidence of the disease in the European population is estimated at 10–12% (Romański 1998), and it reaches 15–30% in pediatric patients (Bieber 2010). A higher percentage of cases is observed in urban environments, which highlights the influence of the surrounding environment on the development and course of the disease (Williams and Flohr 2006). Atopic dermatitis is often the first manifestation of allergic disease, which course has a form of so-called atopic march. This means that symptoms of atopic dermatitis can overtake the syndromes of asthma and allergic rhinitis (Kapoor et al. 2008). The main clinical symptoms of atopic dermatitis include erythematous papules, thickened skin with a tendency to lichenification, and severe pruritus.

The pathogenesis of atopic dermatitis is complex and not fully understood. The disease is a result of genetic, environmental, and immune disorders. Atopic dermatitis is a complex inflammatory process, in which T cells, macrophages, eosinophils, and immunoglobulin E (IgE) play an essential role. In the acute phase of the disease, there is an imbalance between Th1 and Th2-responses (Boguniewicz 2014; Leung

2000). Elevated Th2-expressing cells in the peripheral blood cause increases in IL-4, IL-5, and IL-13 concentration (Ong and Leung 2006). These cytokines stimulate B cells to excessive production of immunoglobulin E and enhance eosinophils proliferation. Furthermore they are capable of inhibition of the Th1 cells cytokine expression and maturation. However, in the chronic phase there is a domination of Th1-responses. Excessive activity of both pathways may be inhibited by the regulatory T lymphocytes (Treg, Fox P3), but their number and activity is markedly reduced in atopic dermatitis.

The pleiotropic effect of vitamin D in the human body is widely discussed. This vitamin also plays an important role in atopic dermatitis (Mesquita et al. 2013). Numerous studies have shown that the appropriate level of vitamin D can affect the T cells-activation and the number of regulatory T cells (Vassallo and Camargo 2010). Nowadays, deficiency of vitamin D in the European population is commonly observed. This is mostly caused by a change of people's lifestyle during last decades consisting of spending more time indoors, use of UV filters, and dietetic vitamin D deficiency.

The aim of the present study was to evaluate the percentage of natural T regulatory cells (nTreg, CD4⁺, CD25⁺, FoxP3⁺, and CD127⁻) and lymphocyte phenotypes (CD3⁺, CD4⁺, CD8⁺, CD4/CD8 ratio, CD19⁺ CD16⁺/56⁺, and CD3⁺ anty-HLA-DR⁺) in children with atopic dermatitis.

2 Methods

2.1 Patients

The study was approved by the Ethics Committees of the Military Institute of Medicine in Warsaw (permit no. 123/14) and the parents of all participants gave written informed consent. The study group consisted of 19 children with atopic dermatitis, with the median age of 3 years (IQR 6–2 years). The inclusion criteria were the age from 6 months to 15 years and the diagnosis

of atopic dermatitis as based on the criteria of Hanifin and Rajka (1980). A suspicion of other skin diseases excluded patients from the study group. Atopic dermatitis was evaluated by the SCORAD scale, an index of its severity, which takes into consideration the extent and severity of lesions, and the intensity of subjective symptoms such as itching and insomnia. The control group consisted of 17 children with no history of atopy and no skin lesions. The median age in this group was 3 years (IQR 5–2 years).

2.2 Blood Lymphocyte Phenotype Determination

Blood lymphocyte phenotypes were determined as previously described (Kalicki et al. 2013).

Briefly, peripheral blood samples were collected in EDTA-anticoagulated tubes. The lymphocyte immunophenotype was determined using BD Simultest™ – IMK Plus Kit (BD Biosciences, Warsaw, Poland). Blood samples (100 μL) were incubated for 20 min with appropriate antibodies and then erythrocyte lysis was performed in the dark at room temperature for 10 min (FACS Lysing Solution; BD Biosciences). Finally, cells were washed twice with 2 ml of phosphate buffered saline (PBS) and fixed in 200 μL of 1% paraformaldehyde in PBS. Then, cells were examined by flow cytometry (FACS Calibur; BD Biosciences). Additionally, percentage distribution of white blood cells (lymphocytes, monocytes, granulocytes, neutrophils, and eosinophils) was assessed using CD45 FITC and CD14 PE antibodies, and FSC/SSC

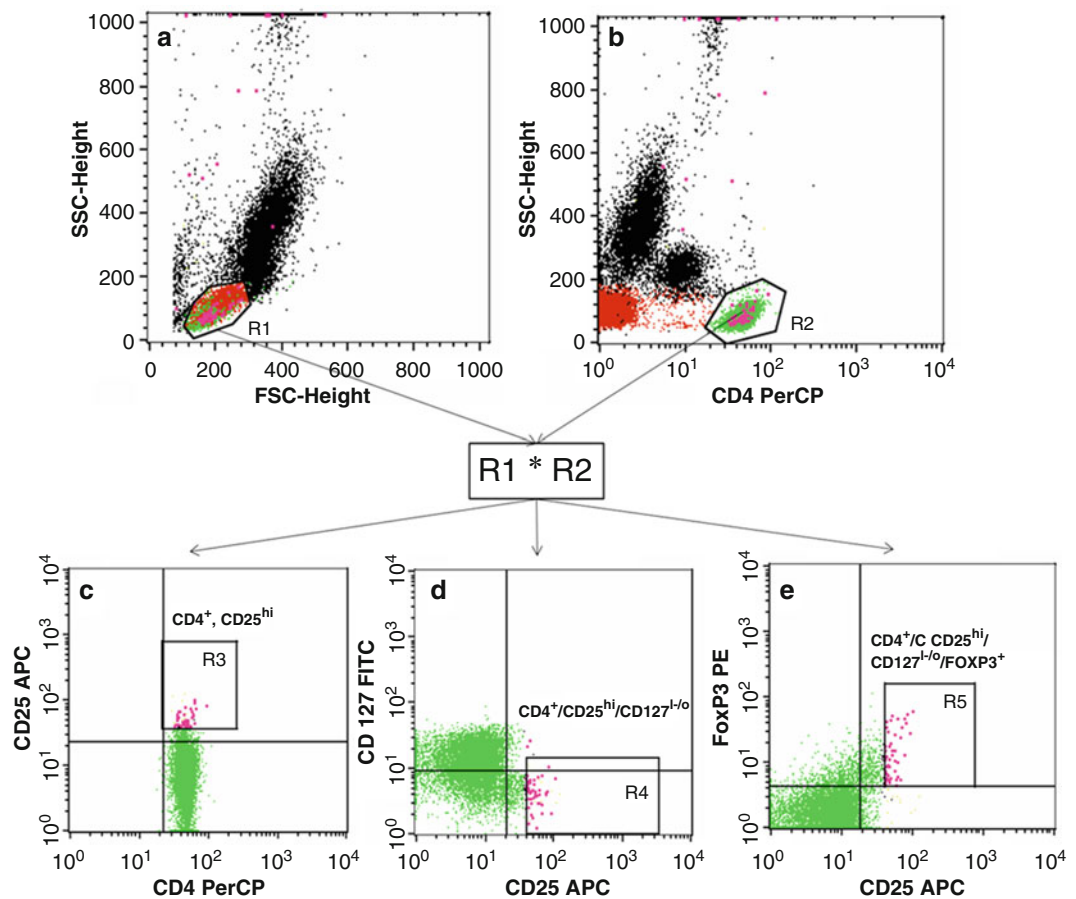


Fig. 1 Gating strategy in assessing nTreg populations (see phenotype determination for details)

determination. The results of lymphocyte phenotype assessment are presented as mean percentages of lymphocytes $\pm$ SD.

To characterize T regulatory cells, whole blood samples (100 μ L) were stained with the primary antibodies CD4-PerCP, CD25-APC, and CD127-FITC (BD Biosciences, Warsaw, Poland) or with an appropriate isotype control with the addition of CD4-PerCP antibody. Blood samples were incubated for 20 min/in the dark at room temperature and the erythrocyte lysis was performed (BD FACS Lysing Solution). After flushing the cells twice with PBS, they were fixated and permeabilized in fixaton/permeabilization buffer and stained with FoxP3 PE or isotype IgG1 kappa PE antibody (45 min in the dark at room temperature). Afterward, cells were washed twice with PBS, fixed in 300 μ L of 1% paraformaldehyde in PBS and were examined by flow cytometry. Ten thousand counts of CD4 PerCP positive cells finished the cytometric acquisition. In the flow cytometry data analysis, two gate restrictions were used: R1 – FSC/SSC lymphocytes and R2 – CD4 PerCP positive cells (Fig. 1a, b). Next, common parts of R1 and R2 (R1*R2) were used for the examination of the results obtained. CD4⁺/CD25^{high} was defined as R3 (Fig. 1c), CD4⁺/CD25^{high}/CD127^{low} as R4 (Fig. 1d), and finally CD4⁺/CD25^{high}/CD127^{low}/FoxP3⁺ (nTreg) as R5 (Fig. 1e). Lymphocyte phenotype determination was conducted with FACS Calibur and BD CellQuest™ PRO software (BD Biosciences, Poland).

2.3 IgE Determination

Total IgE concentration was determined in serum samples by a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle, calibrated with commercially available IgE standards. Total IgE values are expressed in IU/ml.

2.4 Vitamin D Determination

An Dia-Sorin Liaison[®] analyzer (Saluggia, Italy) was used to determine the concentration of total 25-hydroxyvitamin D. The apparatus measures the concentration range from 4.0 to 150 ng/ml (in the SI unit: ng/ml $\times$ 2.5 = nmol/l). Values below 4.0 ng/ml are recorded as <4 ng/ml. The highest recorded value without dilution is 150 ng/ml. This test is a direct competitive assay technology using chemiluminescent immunoassays (CLIA) to quantify the level of 25-hydroxyvitamin D in serum and plasma. During the first incubation, 25-hydroxy vitamin D is separated from the binding protein and combined with an antibody to a solid phase. Vitamin D marker is added after 10 min. Subsequently, there is an incubation for 10 s, whereupon unbound material is removed in the rinse cycle. Then, initiating factors, which immediately cause a chemiluminescent reaction, are added sequentially. A light signal is measured using a photomultiplier, in relative light units (RLU). The signal is inversely proportional to the concentration of 25-hydroxyvitamin D present in the calibrators, control, and samples.

2.5 Statistical Elaboration

Age of patients was given as medians with lower and upper quartiles. Other variables, such as vitamin D content, and percentages of lymphocytes and white blood cells, were presented as means $\pm$ SD. Differences between groups were evaluated with a *t*-test (values with normal distribution) or Kruskal-Wallis test (values with non-parametric distribution). An analysis of the correlation between immunological indices and vitamin D content or disease severity have also been done. A *p*-value < 0.05 was adopted to define the level of statistical significance.

3 Results

Mild atopic dermatitis was diagnosed in 18% of patients; 41% of children had an average course of disease and 41% had severe disease.

3.1 Lymphocytes

The mean percentages of lymphocytes (atopic dermatitis group: $44.7 \pm 16.2\%$, control group: $39.8 \pm 11.3\%$) and eosinophils (atopic dermatitis group: $6.2 \pm 4.5\%$, control group: $3.5 \pm 2.4\%$) tended to be greater in atopic dermatitis, but the differences failed to reach statistical significance ($p = 0.36$ and $p = 0.06$, respectively). Likewise, there was insignificant difference in total IgE concentration between the two groups due to a very large data scatter, although the IgE content tended to be greater in atopic dermatitis (atopic dermatitis: 2127.6 ± 6736.6 , control group: 70.3 ± 72.4 , IU/ml; $p > 0.05$). However, a significantly lower percentage of regulatory T cells was observed in the serum of children with atopic dermatitis compared with the control group ($p < 0.00006$) (Fig. 2) There were no significant differences concerning the other immunological parameters: phenotype CD3, CD4, CD8, CD4/CD8, CD19, CD16/56, natural killer T (NKT) cells, and anti-CD3 HLA -DR3 between

the atopic dermatitis and control groups (Table 1). The study did not reveal any association between disease severity and the percentage of immunological indices.

3.2 Vitamin D

The mean serum vitamin D level was similar in the atopic dermatitis and control groups; 24.3 ± 7.0 and 24.5 ± 9.1 ng/ml, respectively, which is close to the 20 ng/ml low side limit of what is considered adequate for healthy children. The study revealed an inverse association between vitamin D content and the percentage of CD8 cells ($p < 0.05$; $r = 0.62$) in atopic dermatitis. The ratio of CD4/CD8 was proportional to vitamin D content in atopic dermatitis ($p < 0.05$; $r = 0.66$). However, we failed to reveal any other associations between vitamin D content and the other immunological indices investigated in atopic dermatitis. Nor was there any association between vitamin D content and disease severity (Table 2)

4 Discussion

Numerous studies have focused on a significant role of regulatory T cells (Tregs) in the formation

Fig. 2 Regulatory T cells in children with atopic dermatitis and healthy controls

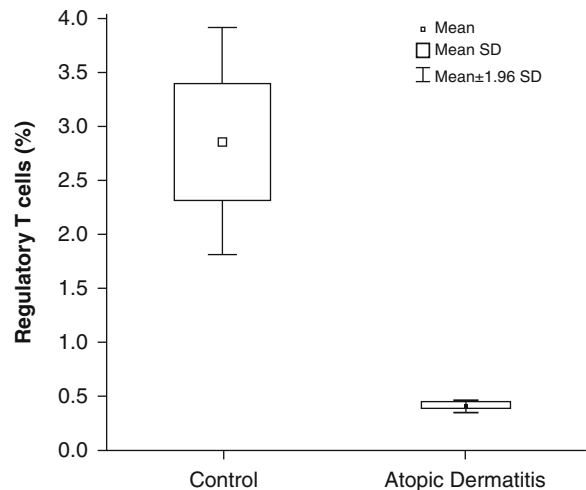


Table 1 Content of indices investigated in atopic dermatitis and control groups

	AD group	Control group	p-value
IgE total (IU/ml)	2127.6 ± 6736.6	70.3 ± 72.4	ns
CD3 (%)	65.6 ± 9.7	61.3 ± 10.1	ns
CD19 (%)	19.7 ± 9.2	26.7 ± 12.4	ns
CD4 (%)	38.7 ± 7.6	34.4 ± 8.4	ns
CD8 (%)	25.4 ± 5.6	24.8 ± 10.6	ns
CD4/CD8 ratio	1.6 ± 0.6	1.7 ± 1.1	ns
CD16/56 (%)	9.6 ± 5.0	9.2 ± 3.9	ns
NKT (%)	2.1 ± 1.8	1.4 ± 1.4	ns
CD3 anty-HLADR3 (%)	6.7 ± 3.3	5.0 ± 4.5	ns
Regulatory T cells (%) (CD4 ⁺ /CD25 ^{high} /CD127 ^{low} /FoxP3 ⁺)	0.41 ± 0.26	2.81 ± 2.19	0.00001
Lymphocytes (%)	44.7 ± 16.2	39.8 ± 11.3	ns
Monocytes (%)	7.4 ± 1.7	8.6 ± 2.2	ns
Granulocytes (%)	47.1 ± 16.2	50.9 ± 11.9	ns
Neutrophils (%)	40.7 ± 17.2	47.1 ± 12.2	ns
Eosinophils (%)	6.2 ± 4.5	3.5 ± 2.4	0.06

Data are means ± SD, *NKT* natural killer T cells, *ns* nonsignificant ($p > 0.05$)

Table 2 Associations between vitamin D content and immunological cellular indices in children with atopic dermatitis

	Vitamin D	p-value
CD3	−0.09	ns
CD19	0.39	ns
CD4	0.31	ns
CD8	−0.62	p < 0.05
CD4/CD8 ratio	0.66	p < 0.05
CD16/56	−0.42	ns
NKT	−0.39	ns
CD3 anty-HLA DR3	−0.43	ns
Tregs	0.36	ns

NKT natural killer T cells, *Tregs* T-regulatory cells, *ns* nonsignificant ($p > 0.05$)

and development of allergic diseases. Regulatory T cells CD4⁺CD25⁺ are a heterogeneous subpopulation of lymphocytes formed in both thymus and peripheral tissues. These cells show the ability to suppress a variety of target cells. There are a lot of proposed markers that could distinguish Tregs from the lymphocyte population. The most characteristic markers are the occurrence of CD25 (α chain of the IL-2 receptor), FOXP3 (forkhead box P3), CD152 (cytotoxic T cell antigen 4), GITR (glucocorticoid-induced TNFR family related gene) and the lack of CD127 (receptor of IL-7) protein. In the physiological

condition, Tregs have the ability to maintain immunological tolerance by modulation of CD4⁺ T cells, CD8⁺ T cells, macrophages, NK cells, and the dendritic cells function (Sledź-Gawrońska 2010; Bacchetta et al. 2007). In atopic dermatitis, a decreased amount and attenuated function of Tregs have been observed (Lesiak et al. 2012; Samochocki et al. 2012; Hijnen et al. 2009; Szegedi et al. 2009). Tregs' dysfunction may be caused by mutations of the nuclear factor Foxp3 (Lyons et al. 2015). Additionally, staphylococcal colonization of the skin of patients with atopic dermatitis may modulate Tregs' function (Boguniewicz and Leung 2011). The results of the present study confirm the participation of regulatory T cells in allergic diseases as the number of Tregs was significantly lower in children with atopic dermatitis.

It is well established that activated CD4 cells, producing Th2 cytokines such as IL-4, IL-5, and IL-13, play a key role in allergy. These cytokines lead to overproduction of IgE and eosinophils (Gittler and Shemer 2012; Brandt and Sivaprasad 2011). In the acute phase of atopic dermatitis, skin biopsies are marked by perivenular infiltrates, predominantly consisting of lymphocytes and occasional monocytes. Lymphocytes are primarily CD4⁺, CD45RO⁺, and CLA⁺, which suggests their previous contact

with antigens (Sampson 2001). Hamid et al. (1994) have shown the increased expression of IL-4 and IL-13, the cytokines produced by the CD4 cells, in skin specimens collected during the acute phase of atopic dermatitis. In the present study, the amount of CD4 cells also was greater in children with atopic dermatitis, although the increase failed to reach statistical significance.

Studies indicate a deficiency of IFN- γ , a component of the atopic dermatitis pathogenesis (Gros et al. 2011). It is known that CD8⁺ cells are a source of IFN- α (Machura et al. 2008). CD8 T lymphocytes and T helper cells have recently been divided into two groups: the cells secreting Th1-like cytokines, known as Tc1, and the cells secreting Th2 cytokines, known as Tc2 (Romagnani 1997). A reduced level of Tc1 cells, which have a high cytotoxic capacity, may be associated with enhanced propensity for skin infections in patients with atopic dermatitis (Lacour and Hauser 1993). In addition, Tc1 cell deficiency leads to the overproduction of IgE due to Tc1 capacity to regulate immunoglobulins. On the other hand, the number of Tc2 cells is not reduced in atopic dermatitis, as the production of Th2 cytokines, like IL-4 and IL-10, increases. Such quantitative and functional disorders of CD8⁺ T cells are conducive for IgE antibody excess and skin infections in atopic dermatitis patients (Novak and Leung 2016; Kemeny et al. 1994). In the present study, no significant difference in the level of CD8⁺ cells between the two groups was revealed, which may be due to the heterogeneity of this subpopulation of lymphocytes and the superiority of Tc2 over Tc1 cells in atopic dermatitis.

The influence of vitamin D on atopic dermatitis is related to the presence of a nuclear receptor for vitamin D in various immune-related cells. Bikle (2009) has shown that vitamin D might affect the expression of genes responsible for the proliferation of dendritic cells, macrophages, and other antigen presenting cells. In addition, vitamin D inhibits synthesis of IL-12 and IL-23, thereby reducing the number of Th1 cells (Baeke et al. 2010). It has also been shown that vitamin

D increases the expression of antiproliferative cytokines, IL-4, IL-5, and IL-10, and leads to the differentiation of CD4 regulatory T-cells, all of which plays a significant role in reducing allergic reactions. A number of studies have demonstrated the influence of vitamin D on the course of atopic dermatitis. The results, however, are often contradictory. Javanbakht et al. (2011) have demonstrated that vitamin D supplementation alleviates symptoms of atopic dermatitis compared with placebo, using the SCORAD index of disease severity. Likewise, a Japanese cohort study has shown a reduced risk of atopic dermatitis in early childhood in children of mothers being supplemented with vitamin D during pregnancy (Miyake et al. 2010). In contrast, Bäck et al. (2009) have shown that vitamin D supplementation in infancy is associated with increased risk of atopic dermatitis at 6 years of age.

In the present study, we did not observe a significant correlation between the serum vitamin D content and severity of atopic dermatitis. However, we noticed an appreciable enhancing effect of a higher content of vitamin D on the CD4/CD8 ratio. That effect may counter the notion of vitamin D protection against allergic diseases, which should be further explored in bigger population samples.

In conclusion, this study demonstrates that regulatory T cells were much reduced in children with atopic dermatitis compared with healthy population, which confirms these cells' role in the pathogenesis of allergy. On the other side, we failed to confirm the beneficial influence of vitamin D on the severity of atopic dermatitis.

Acknowledgements The authors thank Mr. Piotr Murawski, Head of ICT Department, of the Military Institute of Medicine for assistance in the implementation of statistical analysis. The authors also thank the study participants and their parents. Supported by grant 1/8865 (323) of the Military Institute of Medicine.

Conflicts of Interest The authors declare no competing interests in relation to this article.

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