

The Role of T-Regulatory Cells and Toll-Like Receptors 2 and 4 In Atopic Dermatitis

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Abstract

Regulatory T cells (Tregs), toll-like receptors (TLRs) and interleukin-17 (IL-17) play important role in inflammatory diseases; however, their relevance in atopic dermatitis (AD) pathogenesis is not clear. The aim of study was to evaluate the number of circulating Tregs and peripheral blood mononuclear cells (PBMC) expressing TLR2 and TLR4 receptors in patients with AD. PBMC and CD4⁺/CD25^{high} Tregs were isolated from the whole blood of 32 AD patients and 36 healthy volunteers. Expression of CD4⁺CD25⁺, TLR2 and TLR4 receptors and IL 17⁺ was assessed with the flow cytometry. In the peripheral blood of AD patients, the percentage of Tregs was significantly higher when compared with the controls ($P = 0.0003$). The number of TLR2+PBMC and TLR4+ PBMC in AD patients was significantly lower than in the controls ($P = 0.035$; $P = 0.001$, respectively). Also the percentages of Tregs with expression of both TLR2+ and TLR4+ in AD patients were significantly lower than in the control (3.85 versus 21.6, $P < 0.0001$; 2.2 versus 17.6, $P < 0.0001$, simultaneously). The percentage of CD4⁺/CD25^{high}+/FOXP3⁺ Treg lymphocytes with expression of IL-17 was significantly higher in AD group than in healthy subjects (0.3% versus 0.06%; $P = 0.011$). Distinct number of Tregs and various distribution of TLR2 and TLR4 expression on PBMC in AD patients suggest their contribution in the pathogenesis of AD.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease. New approach to AD pathogenesis includes the link between innate and adaptive immune response. The reduction in antimicrobial peptides, diminished recruitment of innate immune cells (PMNs, pDC, and NK cells) to the skin, epithelial barrier disruption, and toll-like receptors (TLRs), mainly TLR 2 defects, partially explain an increased susceptibility of AD patients to bacterial and viral infection [1, 2]. Interestingly, recent studies have shown that adaptive immunity may regulate innate response by diminishing innate inflammatory cell influx and in consequence lead to tissue damage. Recently, there is some evidence suggesting the role of regulatory T (Tregs) cells in the AD pathogenesis. They are heterogenic T cells population with different surface markers, able to suppress activated immune system, and prevent against autoimmunization [3, 4]. The best characterized Tregs subpopulation are Tr1, CD4⁺/CD25⁺,

Th3 cells and NK Treg cells. CD4⁺/CD25⁺ cells may induce anergy and protect against T effector cells proliferation via suppression of dendritic cells activity [5–7]. There are scarce data on Tregs role in inflammatory skin diseases including AD.

In AD skin biopsies, higher expression of Tr1 was shown while CD4⁺/CD25⁺/FoxP3⁺ Tregs were not detected [8]. In contrary, other authors revealed the presence of TCD4⁺/CD25⁺/FOXP3 Tregs in inflammatory lesions in AD [9–11]. Szegedi *et al.* [11] although found Tregs within skin lesions, they did not find increased number of these cells in peripheral blood. Discrepant results obtained by different authors suggest the necessity of performing further studies on the subject.

Toll-like receptors are human pattern recognition receptors expressed either on the cell surface (TLR1, 2, 4, 6, 10) or intracellularly (TLR3, 7–9) [12]. The main function of these receptors is the induction of inflammation, maturation of dendritic cells, and development of antibacterial defence by activation of Th1 or Th17 cells [13].

It was shown that defects in TLR-2 and TLR-4 signals may promote Th2 response and in consequence cause AD development [14–16].

Interleukin 17 (IL-17) plays an important role in immune and inflammatory responses as a pro-inflammatory regulator by inducing the expression of various cytokines, chemokines, adhesion molecules and growth factors [17–20].

Some data indicate that an increase in IL-17 level is closely associated with a range of inflammatory diseases including rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, psoriasis and also allergic diseases [21, 22].

Thus, the aim of this study was to evaluate the number of circulating T regulatory cells and the number of peripheral blood mononuclear cells (PBMC) expressing TLR2 and TLR4 receptors as well as IL-17 in patients with atopic dermatitis and in healthy controls.

Material and methods

Patients. Thirty-two patients (mean age 27.1 y.o.; 18 female, 14 male) with AD and 36 healthy controls, age- and sex-matched were enrolled into the study. All subjects from both groups were Caucasians living in Central Poland. AD was diagnosed according to criteria proposed by Hanifin and Rajka [23]. Clinical characteristics of the patients is presented in Table 1.

Each patient or his/her parents gave written informed consent before entering the study and all the experiments

Table 1 Clinical characteristics of AD patients.

Clinical feature	<i>n</i> = 32
Sex	
Female	18
Male	14
Age (y.o.)	27.1
Disease onset	
Before 6 month	15
6 month–2 years	14
Over 3 years	3
Dry skin	
Yes	29
No	3
Keratosis pilaris	
Yes	21
No	11
Hand and foot dermatitis	
Yes	6
No	26
Ichthyosis	
Yes	0
No	32
Nipple eczema	
Yes	0
No	32

Table 1 (Continued)

Clinical feature	<i>n</i> = 32
Cheilitis	
Yes	17
No	15
Recurrent conjunctivitis	
Yes	15
No	17
Denni–Morgan infraorbital fold	
Yes	25
No	7
Orbital darkening	
Yes	19
No	13
Pityriasis alba	
Yes	8
No	24
Anterior Neck folds	
Yes	5
No	27
White dermographism	
Yes	12
No	20
Palmar hyperlinearity	
Yes	6
No	26
Itch when sweating	
Yes	25
No	7
Intolerance to wool	
Yes	28
No	4
Course influence by environmental and emotional factors	
Yes	25
No	7
Family history of atopy	
Yes	22
No	10
Clinical remission	
Yes	16
No	16
Mean IgE concentration (IU/ml)	181.42
Mean % eosinophils	4.4

were approved by the local Ethics Committee. The investigations were carried out in accordance with Declaration of Helsinki. Before entering the study, the subjects underwent thorough physical examination and SCORAD (scoring atopic dermatitis) index was assessed [24]. The patients enrolled in the study had moderate AD (mean SCORAD index 22; range 15–39).

Assessment of Tregs, TLR2+ and TLR4+ cells, and IL17+ cells. Blood samples were taken in all AD patients and healthy controls.

T-reggs. Treg cells were assessed using Human Treg Flow Kit(FOXP3 AlexaFluor 488/CD4 PE-Cy5/CD25PE) (BioLegend, San Diego, CA, USA). The peripheral whole

blood sample (100 μ l) was treated according to standard 'lyze-no wash' procedure and then incubated with 20 μ l of CD4 PE-Cy5/CD25 PE cocktail for 20 min, at room temperature, in the dark. Afterward, cells were washed in BioLegend cell staining buffer and centrifuged (5 min/250 g). Cells were then re-suspended and incubated with for 20 min with BioLegendFIX/Perm buffer (at room temperature, in the dark). Next, cells were centrifuged (5 min/250 g) and re-suspended in BioLegend-FOX3 FIX/Perm buffer and incubated for 15 min (at room temperature, in the dark). This procedure was repeated twice. After spinning down the cells and discarding the supernatant, the sample was re-suspended again in FOX3 FIX/Perm. Then, 5 μ l Alexa Fluor 488 anti-human FOXP3 antibody was added and incubated for 30 min (at room temperature, in the dark). After that, cells were washed twice, re-suspended with cell staining buffer, and the fluorescence was measured using flow cytometry. Cells expressing CD4⁺/CD25^{high}+/FOXP3⁺ were determined as T-regs.

TLR 2+ and TLR4+ cells. Twenty microlitre of monoclonal antibodies anti-CD25 was added (FITC-conjugated; Becton Dickinson, San Jose, CA, USA), and anti-CD4 (APC-conjugated; Becton Dickinson) as well as anti-TLR2 or anti-TLR4 APC-conjugated antibody (both eBioscience; San Diego, CA, USA) was added to 50 μ l of the whole blood, and then, sample was incubated for 20 min at room temperature, in the dark. After incubation, 'lyze-no wash' procedure was performed. Then, cells were centrifuged (5 min/250 g) and, next, re-suspended in PBS. TLR2+ and TLR4+ cells were identified among CD4⁺/CD25^{high} T-regs or in peripheral blood mononuclear cell (PBMC) populations, using flow cytometry.

IL-17A+ cells. 20 μ l of monoclonal antibodies anti-CD25 was added (FITC-conjugated; Becton Dickinson, San Jose, CA, USA), and anti-CD4 (APC-conjugated; Becton Dickinson, San Jose, CA, USA) was added to 50 μ l of the whole blood, and then, sample was incubated for 20 min at room temperature, in the dark. After incubation, 'lyze-no wash' procedure was performed. Then, cells were washed in PBS and centrifuged (5 min/250 g). Next, they were incubated for 20 min with FIX/Perm buffer (at room temperature, in the dark).

Afterward, cells were washed in PBS and centrifuged (5 min/250 g). Then, they were re-suspended in FIX/Perm buffer and incubated for 15 min (at room temperature, in the dark). After washing up and re-suspension in PBS, 20 μ l of anti-IL-17 PE-conjugated monoclonal antibody was added and cells were incubated for 30 min (at room temperature, in the dark). After that, cells were washed twice, re-suspended with PBS, and the fluorescence was measured using flow cytometry. Cells with CD4⁺/CD25⁻/IL-17⁺ immunophenotype were recognized as IL-17+ lymphocytes.

Flow cytometry. All fluorescence measurements were performed by multicolour flow cytometry (FACScan; Becton-Dickinson), using standard emission filters, and the CellQuest software has been used to process the data. Ten thousand cells per sample were calculated routinely in all experiments.

Peripheral blood mononuclear cells were gated on the base of SCC versus FSC distribution. The gating strategy is shown on Fig. 1A, B.

Statistical analysis. Data were analysed using the Mann-Whitney *U* Test, and correlations coefficients were determined by using the Spearman rank correlation test. A *P* value of < 0.05 was considered as statistically significant.

Results

In the peripheral blood samples of patients with AD, the percentage of Treg lymphocytes was significantly higher when compared with the control group (3.9% versus 0.5%; *P* = 0.0003, Table 2). The percentages of all PBMC with expression of TLR2 or TLR4 in AD patients were significantly lower than in the control (1.05 versus 2.1, *P* = 0.035; 0.9 versus 2.4; *P* = 0.005). Also the percentages of Tregs with expression of both TLR2+ and TLR4+ in AD patients were significantly lower than in the control (3.85 versus 21.6, *P* < 0.0001; 2.2 versus 17.6, *P* < 0.0001, simultaneously). The percentage of CD4⁺/CD25^{high}+/FOXP3⁺ Treg lymphocytes with expression of IL-17 was significantly higher in AD group than in healthy subjects (0.3% versus 0.06%; *P* = 0.011) (Fig. 1A, B). We found no statistical significant correlations between number of analysed parameters and disease activity (measured by SCORAD), IgE level and percentage of eosinophiles (*P* > 0.05 for all comparisons).

Discussion

During the last decade, it was shown that regulatory T cells play a key role in immune regulation. The most known subpopulation includes a subset of naturally occurring T cells [25, 26]. These CD4⁺CD25⁺ T cells have been found in mice, rats and humans and also can be detected in the thymus, peripheral blood, lymphoid organs and also at sites of inflammation [26]. They are involved mainly in regulation, transplantation tolerance, antitumor immunity and in bacterial infections but also in autoimmune reactions [27, 28]. Till now their role in atopic diseases is not clear. It has been hypothesized that Tregs mediate tolerance to some antigens which pass by respiratory and intestinal tracts and that the defect of this tolerance may provoke atopic disorders development [29, 30].

The data on the potential role of CD4⁺CD25^{high} T cells in AD come from the patient suffering from genetically conditioned syndrome IPEX syndrome

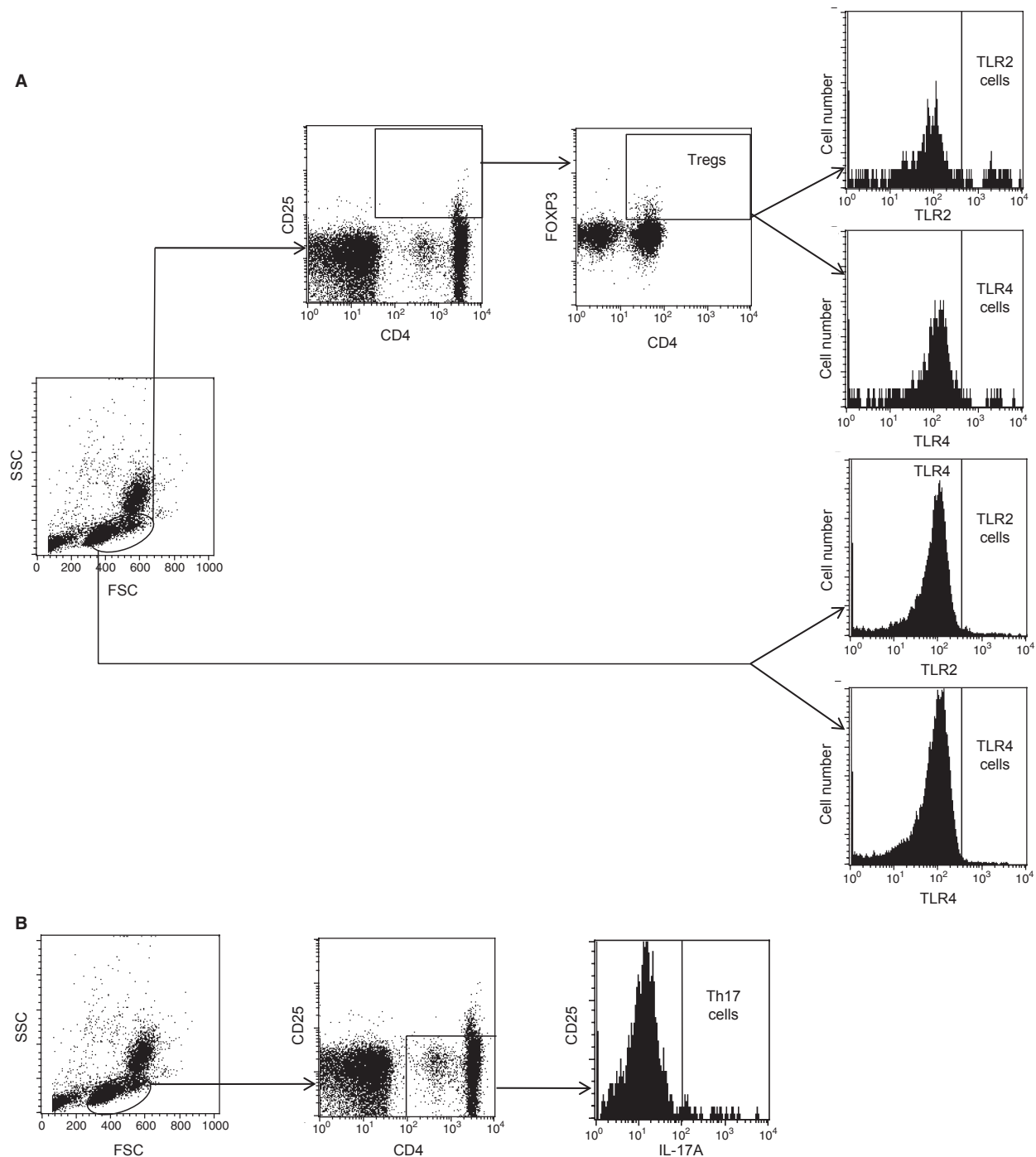


Figure 1 (A) Assessment of T regulatory cells (Tregs) by flow cytometry. Cells were stained with monoclonal antibodies against CD4, CD24 and FOXP3. Peripheral blood mononuclear cells (PBMCs) were gated based on side scatter (SSC) and forward scatter distribution (FSC). Then, CD4⁺/CD25^{high}/FOXP3⁺ cells were detected as Tregs. The percentage of toll-like receptor (TLR) 2 and 4 cells was calculated among both Tregs (upper panel) and PBMCs (lower panel). (B) Detection of interleukin-17A (IL-17A)-expressing cells. Cells were stained with monoclonal antibodies against CD4, CD24 and IL-17A. PBMC were discriminated based on SSC and FSC distribution. CD4⁺/CD25⁺/IL-17A⁺ cells were recognized as IL-17⁺ (Th17) cells.

(the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in whom the defect in Tregs function was found [31].

There are discrepant results on Tregs alteration in AD patients. Karlsson *et al.* [32] showed CD4⁺CD25^{high} T cells impairment in children with cow's milk allergy.

Table 2 The median (range) percentages of Tregs/TLR2+, TLR2+, TLR4+ and IL-17+ lymphocytes in blood samples from atopic dermatitis (AD) group (1) and healthy controls (2).

Cells (%)	AD patients	Control group	<i>P</i> = 1 versus 2
	1	2	
Tregs *	3.9 (0.8–6.9)	0.5 (0.07–3.1)	0.0003
TLR2+ *	1.05 (0.05–3.9)	2.1 (0.15–7.3)	0.035
Tregs/TLR2+	3.85 (3.5–14.9)	21.6 (6.1–28.3)	<0.0001
TLR4+ *	0.9 (0.07–5.15)	2.4 (0.2–17.1)	0.005
Tregs/TLR4+	2.2 (0.1–12.9)	17.6 (8.2–28.2)	<0.0001
CD4+/IL17+ *	0.3 (0.05–3.6)	0.06 (0.01–2.1)	0.011

* % of all PBMC; Tregs - CD4+ CD25^{high} cells.

In other studies, it was shown that Tregs from AD patients have decreased suppressive capacity when compared with healthy control [33]. In other studies, there was no differences in Tregs number between AD patients and healthy controls [11, 34, 35].

In the contrary, the study by Ou *et al.* [36] showed the enhanced number of CD4+CD25+ cells in patients with AD. Our results are in line with this study as we found significantly increased Tregs number in AD patients. Further investigations are needed to characterize the *in situ* immunosuppressive activity of CD4+ CD25+ Tregs and their relationship with other elements of epidermal immune response.

Toll-like receptors, mainly TLR2, recognize pathogen-associated molecular patterns of various Gram-positive and Gram-negative bacteria as well as fungi and even herpes viruses [37–39].

Recent papers showed that defects in toll-like receptors pathways, mainly including TLR2 pathway, are associated with AD patients' susceptibility to skin bacterial and viral infections [16]. Similar observation, however, was not found in regard to TLR4. The authors suggest that defects in TLR pathways observed in AD patients are responsible for promoting Th2 response in this group of patients. Most data on TLR in AD patients concern TLR2 receptors, especially their role in the development and infectious complications observed in patients with AD. Genetic polymorphisms in genes encoding TLR are probably one of the factors being responsible for this phenomenon [40, 41]; however, the data are discrepant as other authors did not show such a correlation [42].

In our study, we found significantly decreased number of both TLR4+ and TLR2+ PBMC when compared with the healthy patients. The obtained results confirm defects in TLR pathways in the pathogenesis of atopic dermatitis.

In summary, this work suggests that the TLR pathway may be impaired in AD patients on a genetic or acquired basis with possibilities that include altered TLR structure or altered expression/function of signalling proteins. Recently, a novel and unique subset of IL-17-producing

CD4+ cells was described. These 17+ cells are probably involved in protection against bacterial pathogens and in pathogenesis of Th1-mediated chronic inflammatory diseases. As AD is believed as a Th2-mediated disease, the role of IL-17 in its pathogenesis is still under investigation [43]. In the skin biopsy specimens recovered from acute and chronic skin lesions from patients with AD, IL-17 mRNA was preferentially associated with acute lesions [44]. In a recent study, the percentage of Th17+ cells was shown to be higher in the peripheral blood of AD patients and associated with the severity of AD. Immunohistochemically, IL-17+ cells infiltrated the papillary dermis of atopic eczema more markedly in acute lesions than in chronic lesions [45, 46]. However, the data are still discrepant, because recently it was shown by Hayshida *et al.* [47] that the number of Th17+ cells was decreased in AD patients and their number correlated with disease activity. Taking together, these findings show that Th17+ cells are elevated in peripheral blood and are present in the skin of AD. The role of these cells in acute eczema for the course of AD remains to be elucidated. In our study, we showed increased number of IL-17+ cells in AD patients what underlines its role in pathogenesis of disease. Concluding, our data suggest once again the link between innate and adaptive immunity [48].

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Conflict of interest

The authors declare no conflict of interest.

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