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Report

Circulating $CD4^+CD25^{high}FoxP3^+$ T-regulatory cells in patients with atopic dermatitis after narrowband-ultraviolet B phototherapy

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Abstract

Background Previous studies showed controversial results regarding $CD4^+CD25^{high}FoxP3^+$ T-regulatory cells (Tregs) in atopic dermatitis (AD) and effect of therapy.

Methods Circulating $CD4^+CD25^{high}FoxP3^+$ Tregs were assessed by flow cytometry in 20 controls and 20 patients with AD at baseline and after narrowband ultraviolet B with assessment of disease severity.

Results Patients showed higher pretreatment T-effector cells (Teffs) (%) and lower pretreatment Tregs FoxP3 expression% than controls ($P = 0.003$ and 0.01, respectively). Mild AD showed a lower Tregs/Teffs ratio compared to controls ($P = 0.013$), while moderate group showed higher Teffs%, and lower Tregs FoxP3 expression% and Tregs/Teffs compared to controls ($P = 0.016$, 0.007, and 0.009 respectively). The severe group had higher Tregs% and Teffs%, yet with a lower Tregs FoxP3 expression% compared to controls ($P < 0.001$, $P = 0.043$, $P = 0.044$, respectively). There was significant reduction of severity after narrowband ultraviolet B ($P = 0.007$), with overall significant elevation of Tregs FoxP3 expression% in patients ($P = 0.004$). All patients' post-treatment laboratory findings were statistically matched to each other and to controls whatever their previous severity or therapeutic response. The improvement of severity score correlated with the change in both Tregs% and Tregs/Teffs.

Conclusions Significant reduction in AD disease severity is correlated with the change in Tregs% and Tregs/Teffs.

Introduction

Regulatory T cells are a subset of T lymphocytes that play a central role in inducing and maintaining immunologic tolerance and in the termination of vigorous immune responses.[1] Thus, deficiency or dysfunction of these cells may lead to autoimmunity.[2] Two main subsets of regulatory T cells have been proposed that differ in terms of development, specificity, and mechanisms: naturally occurring regulatory T cells and adaptive regulatory T cells. The latter develop from mature T cells in peripheral tissues under certain conditions of antigen-specific stimulation. This subset includes the T-regulatory 1 (Tr1) and T-helper (Th) 3, which exert their suppressive function via the secretion of interleukin (IL)-10 and transforming growth factor-β, respectively.[3] On the other hand, naturally arising regulatory T cells (Tregs), which are induced in the thymus, display a constitutive high expression of CD25 (the alpha chain of the IL-2 receptor) and are generally referred to as $CD4^+CD25^{high}$ Tregs. FoxP3 (a member of the forkhead/winged-helix family of transcription factors) is considered the best marker for Tregs.[4] These natural Tregs, known as $CD4^+CD25^{high}$ $FoxP3^+$ Tregs, suppress other T cells by cell–cell contact in a cytokine-independent fashion.[5] They suppress the proliferation of naive T cells and their differentiation to effector and autoreactive T cells and are crucial for the maintenance of peripheral tolerance to self- and non-self-antigens.[6]

Atopic dermatitis (AD) is an eczematous highly pruritic chronic inflammatory relapsing skin disease. Its presentation varies from an acute eczematous relapsing eruption in early life to a characteristic lichenified dermatitis in older patients. Various studies indicate that AD has a complex etiology, with activation of multiple immunologic and inflammatory pathways.[7] These include an inappropriate balance between $CD4^+CD25^{high}$ Tregs and Th2 effector cells.[8] However, previous studies with regard to Tregs in AD showed controversial results. While

increased numbers of circulating $CD4^+CD25^{high}$ T cells and overexpression of FoxP3 were found in patients with AD compared with controls in some studies,[9–12] they were decreased in others.[13–15]

George *et al.*[16] first studied narrowband-ultraviolet B (NB-UVB) phototherapy for AD. Since then, several studies have been published that show its efficacy in AD.[17–22] In part, this is probably due to the induction of various regulatory cells, which are central to the concept of transferable UVB-induced tolerance.[23] Several types of UV-induced regulatory T cells have been described, including natural Tregs or antigen-specific $CD4^+CD25^+$ Tregs expressing the transcription factor FoxP3.[24] Yet, it is noteworthy that the number of skin Tregs did not change significantly after medium dose UVA1 radiation therapy in one study.[25]

The aim of this work was to study the frequency of circulating $CD4^+CD25^{high}FoxP3^+$ Tregs in AD in relation to disease severity and to compare these levels with those in normal controls as well as the levels after NB-UVB therapy.

Subjects and methods

In this pilot study, 20 patients with AD and 20 healthy controls (with no family history of atopy) were recruited from the outpatient dermatology clinic of Ain Shams University Hospitals over a period of one year, after signing an informed consent. The study was conducted according to the Declaration of Helsinki Principles and was approved by the medical ethical committee of Ain Shams University. Exclusion criteria included patients with photosensitivity, chronic systemic diseases, manifestations of other dermatological diseases (e.g., psoriasis), personal or family history of skin cancer or melanoma, history of diseases that probably affect Tregs (e.g., mycosis fungoides and rheumatoid arthritis), and any medical treatment that can possibly affect the study outcome three months before the study (e.g., steroids, immunosuppressive drugs, and phototherapy).

All patients were subjected to full history taking, including onset, course, duration, and previous treatments with response, history of asthma or hay fever in patients with AD and in their first-degree relatives, and possible environmental and/or emotional aggravating factors. Clinical examination was done including site, shape, number, and distribution of AD lesions together with evaluation of extent of involvement and approximate percentage of the body surface area involved. The clinical severity was visually scored according to the Leicester score[26] at baseline and at the end of phototherapy sessions. It assesses 10 body zones (face, neck, abdomen, back, elbows, antecubital fossae, dorsa of hands, palms and wrists, popliteal fossae, feet) for five signs, which are erythema, excoriation, dryness, cracking, and lichenification, giving a maximum score of 150. Patients were then graded as mild (grade 1) if their score was between 0 and 50, moderate (grade 2) if it was between 50 and 100, and severe (grade 3) if it was 100 and 150. Photographs were taken at baseline and after finishing NB-UVB sessions under standardized conditions for lightening, position, and focal length, using a Fujifilm finepix A 800 camera.

For assessment of circulating Tregs by flow cytometry, 2 ml of peripheral venous blood were collected from each patient (at baseline and at the end of phototherapy sessions) and control using a vacutainer containing anticoagulant (potassium ethylenediaminetetraacetic acid) in a final concentration of 1.5 mg/ml. Fifty microliters of whole anticoagulated blood was lysed using 1 ml IQ test lysing reagent (Beckman Coulter, Miami, FL, USA) followed by washing with phosphate-buffered saline (Oxoid, Hampshire, UK). After that, the cells were stained with combinations of the following antibodies (5 μl each): anti-CD25-PE, anti-CD4-FITC, and isotype controls (Isotype controls colors are FITC, PE, and PE-Cy5) (Beckman Coulter). The test-tubes were then incubated in the dark for 20 minutes followed by washing with phosphate-buffered saline. Intracellular staining FoxP3-PE-Cy5 (eBioscience, San Diego, CA, USA) was done according to the manufacturer's instructions with certain modifications according to Attia *et al.*[27]

Data acquisition and analysis were performed on EP-ICS XL flow cytometry using SYSTEM II version 3 software with a standard three-color filter configuration. A total of at least 5000 $CD4^+$ cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. Discrimination of $CD25^{high}$ Tregs, $CD25^{low}$ activated effector-memory T cells (Teffs) were acquired after gating the $CD4^+$ lymphocyte population as previously described by Zhang *et al.*[28] The $CD25^{high}$ population was determined relative to the low intensity of CD25 staining found on non-$CD4^+$ T cells; cells expressing CD25 at levels above those of the isotype control but at higher expression levels than the $CD25^{low}$ cells were considered as Tregs.

NB-UVB Waldmann full-body UV therapy system 100L with folding side parts (Herbert Waldmann GmbH & Co. KG, Villingen-Schwenningen, Germany) and 8 Philips TL-01 fluorescent lamps (Philips, Hamburg, Germany) with a radiation of 310–315 nm with a peak of 311 nm were used for phototherapy in the form of three sessions per week on non-consecutive days for six weeks. Initial doses and subsequent increments were given using ready calibrated tables supplied by the manufacturer. During treatment, the genital area was shielded in all cases, and UV-blocking goggles protected the eyes. All atopic lesions were carefully monitored, and repeated evaluations for clinical assessment were performed before each session and at the end of therapy.

Data were collected, coded, revised, and entered into the statistical package for social science (SPSS) – version 15 (SPSS Inc., Chicago, IL, USA). Qualitative data were presented as numbers and percentages, while quantitative data were presented as means, SDs, and ranges. The Kolmogrov–

Smirnov test was used to test for normality of the data distribution and was proven normally distributed. The comparisons between groups with qualitative data were done by using chi-squared test and Fisher exact test (was performed in tables containing values <5). The comparisons between two independent groups with quantitative data were done by using independent *t*-test, while the comparison between two paired groups was done by using the paired *t*-test. The comparisons between more than two groups were done by using the one-way ANOVA test. Correlations between parameters were done by using Pearson correlation coefficients. *P* was considered statistically non-significant if >0.05, significant if ≤0.05, and highly significant if ≤0.001.

Results

This study included 20 patients with AD (14 women [70%] and six men [30%]; age range 19–44, mean 29.9 years ± 8.05) and 20 healthy gender- and age-matched volunteers as controls (14 women [70%] and six men [30%], age range 19–40 years, mean 29 years ± 6.41). The patients were divided into three groups based on clinical severity according to Leicester score: group 1 (mild): included six patients (30%) (score >0 and ≤50); group 2 (moderate), which included nine patients (45%) (score 50–100); and group 3 (severe), which included five patients (25%) (score ≥100–150).

Mean patients' Tregs foxP3 was 54.53 ± 16.94, while mean Teffs foxP3 was 11.18 ± 7.21, and mean controls' Tregs foxP3 was 67.10 ± 12.15, while mean Teffs foxP3 was 20.87 ± 13.10. This ensured proper gating.

Regarding all pre- and post-treatment laboratory data, we observed statistically significant higher pretreatment Teffs% and lower pretreatment Tregs FoxP3 expression % in patients than controls ($P = 0.003$ and 0.01 respectively) (Table 1). Comparing pretreatment laboratory data among patients with different grades of severity, there was a highly significant difference in Tregs% and Tregs/Teffs ratio ($P \leq 0.001$) (Table 2). A *post-hoc* test was used to assess the significant difference between the three groups. There was statistically significant higher Tregs% and Tregs/Teffs ratio in severe group patients compared to each of mild and moderate groups ($P \leq 0.001$). Comparing pretreatment laboratory data among patients with different grades of severity and controls, there was a significant difference in Tregs%, Teffs %, Tregs FoxP3 expression%, and Tregs/Teffs ratio ($P < 0.05$) (Table 2). Comparing each grade of patients with controls revealed significantly lower pretreatment Tregs/Teffs ratio in the mild group compared to controls ($P = 0.013$), while significantly higher pretreatment Teffs % and significantly lower Tregs FoxP3 expression% and Tregs/Teffs ratio in moderate disease patients compared to controls ($P = 0.016$, 0.007, and 0.009, respectively). Regarding severe grade patients, we found significantly higher pretreatment Tregs% and Teffs% and lower Tregs FoxP3 expression% in patients compared to controls ($P < 0.001$, $P = 0.043$, and $P = 0.044$, respectively).

Table 1 Comparison between patients and controls regarding laboratory data

	Patients		Controls		Independent *t*-test	
Parameter	**Mean**	**SD**	**Mean**	**SD**	***t***	***P* value**
Tregs% before	2.99	2.37	2.59	1.54	−0.630	0.533
Tregs% after	2.91	1.01			−0.795	0.432
Teffs% before	10.53	2.40	7.16	4.11	−3.165	0.003*
Teffs% after	9.30	2.48			−1.993	0.054
Tregs FoxP3% before	54.53	16.94	67.10	12.15	2.696	0.01*
Tregs FoxP3% after	66.75	8.67			0.105	0.917
Tregs/Teffs ratio before	0.28	0.21	0.4	0.21	1.837	0.074
Tregs/Teffs ratio after	0.33	0.16			1.115	0.272

*$P \leq 0.05$ significant.

There was significant reduction of clinical severity grades according to the Leicester score after NB-UVB therapy ($P = 0.007$), with overall significant elevation of Tregs FoxP3 expression% in patients ($P = 0.004$) (Table 3). Comparing pre- and post-phototherapy data in each severity group separately, there was a statistically significant elevation of Tregs% and Tregs/Teffs ratio in mild grade patients ($P = 0.001$ and 0.047, respectively) and significant elevation of Tregs FoxP3 expression% and Tregs/Teffs ratio in the moderate group ($P = 0.014$ and 0.013, respectively). However, no significant change was detected in the severe group. There was no significant difference among the studied groups of patients (mild [group 1], moderate [group 2], severe [group 3]) and between any of them compared to the control group regarding laboratory data after NB-UVB ($P > 0.05$), i.e., all patients' post-treatment laboratory findings were matched to each other and to controls whatever their previous severity or therapeutic response (data not shown).

All pretreatment laboratory data did not show a significant difference in male patients versus females, in chronic versus relapsing AD, or in patients with a family history of atopy versus those without ($P > 0.05$) (data not shown). A significant positive correlation was found between Tregs% and each of Teffs% and Tregs/Teffs ratio ($r = 0.461$ and $P = 0.041$ and $r = 0.927$ and $P < 0.001$, respectively). The improvement of severity score was highly correlated with the change in both Tregs

Table 2 Comparison between patients with different grades of severity from pretreatment laboratory findings

Pretreatment parameter	Severity according to Leicester score before											
	Mild		Moderate		Severe		ANOVA		Controls		ANOVA	
	Mean	SD	Mean	SD	Mean	SD	*F*	*P* value	Mean	SD	*F*	*P* value
Tregs%	1.60	0.50	2.16	1.14	6.13	2.65	14.369	<0.001*	2.59	1.54	9.72	<0.001*
Teffs%	9.84	3.22	10.86	1.85	10.78	2.56	0.331	0.722	7.16	4.11	3.309	0.031**
Tregs FoxP3%	61.75	14.89	51.02	16.62	52.18	20.50	0.767	0.480	67.10	12.15	3.121	0.038**
Tregs/Teffs ratio	0.16	0.04	0.19	0.09	0.57	0.20	21.341	<0.001*	0.40	0.21	22.24	<0.001*

*$P \leq 0.001$ highly significant.
**$P \leq 0.05$ significant.

Table 3 Comparison between before and after treatment laboratory data

Parameter	Before		After		Paired *t*-test	
	Mean	SD	Mean	SD	*t*	*P* value
Tregs%	2.99	2.37	2.91	1.01	0.117	0.908
Teffs%	10.53	2.40	9.30	2.48	1.777	0.092
Tregs FoxP3%	54.53	16.94	66.75	8.67	−3.287	0.004*
Tregs/Teffs ratio	0.28	0.21	0.33	0.16	−0.872	0.394

*$P \leq 0.05$ significant.

% and Tregs/Teffs ratio ($r = 0.752$ and $P < 0.001$ and $r = 0.718$ and $P < 0.001$, respectively).

Discussion

Inappropriate balance between $CD4^+CD25^{high}$ Tregs and Th2 Teffs has been suggested in AD. This imbalance may result from a deficiency in suppression by Tregs or by strong activation signals that supersede the regulatory mechanism.[8] In our work, comparing between all patients and controls, pretreatment Teffs% was significantly higher than that of controls. In accordance, Verhagenl *et al.*[29] observed increased Teff cells in AD, reflecting the proinflammatory nature of the disease. We also found lowered pretreatment Tregs FoxP3 expression %. Similarly, Lin *et al.*[30] found decreased FoxP3 protein expression within $CD4^+CD25^{high}$ Tregs in their study. Many cytokines and factors negatively regulate FoxP3 gene transcription. Among these is IL-4, strongly associated with AD,[31] which upregulates GATA-3 expression (the master Th2 transcription factor) and in turn can bind to the FoxP3 promoter region and suppress gene expression.[32]

Comparing each severity grade patients with controls regarding the pretreatment laboratory data revealed a lower Tregs/Teffs ratio in the mild group compared with controls. This was in agreement with Reefer *et al.*[9] who found that the Tregs/Teffs ratio was decreased in atopic patients. As the Tregs population suppresses the proliferation of naive T cells and their differentiation to Teffs,[33] the low Treg/Teff ratio reflects the imbalance between these two populations in AD.

Similar to the overall comparison between patients and controls, there was a significantly higher Teffs% and significantly lower Tregs FoxP3 expression% in moderate disease patients compared to controls. When we compared between severe grade patients and controls, we found significantly higher Tregs% and Teffs% but a lower Tregs FoxP3 expression% for patients compared to controls. In accordance, Lesiak *et al.*[11] found that the percentage of $CD4^+CD25^{high}$Tregs was significantly higher in patients with AD compared to healthy subjects when $CD4^+CD25^{high}$Tregs were isolated from the whole blood of 32 patients with AD and 36 healthy volunteers. On the contrary, Verhagen *et al.*[29] observed increased Teffs cells in AD with decreased $CD4^+CD25^+FoxP3^+$ T cells. This discrepancy can be explained as follows: although some groups reported that Tregs were absent in AD,[29] others reported the existence of an increased pool of circulating Tregs in a group of patients with AD, yet with the lack of functional Tregs in the skin lesions.[34] In addition, the increment of circulating Tregs in a group of patients with AD could be a compensatory mechanism to severe inflammation. Elucidation of such phenomena necessitates future studies on frequency and function of Tregs and Teffs cells, both in the circulation and in lesional skin of larger populations of patients with AD with different grades of severity.

UVB irradiation source that emits mostly 311–312 nm radiation (NB-UVB) has been very successfully used in the treatment of inflammatory skin diseases such as AD and psoriasis.[35] Yet, no previous studies investigated the NB-UVB influence on Tregs in AD. In our study, comparing the clinical severity grades before and after

phototherapy, there was a significant reduction according to the Leicester score after NB-UVB therapy. Our results are in accordance with previous studies.[16–22] Following NB-UVB therapy, all laboratory data tended to be normalized. Comparison between before- and after-therapy laboratory data of all patients showed significant elevation of Tregs FoxP3 expression%. Furthermore, we found that there was a statistically significant elevation of Tregs% and the Tregs/Teffs ratio in mild grade patients and significant elevation of Tregs FoxP3 expression% and the Tregs/Teffs ratio in the moderate group. Despite that, there was no significant change in laboratory data of the severe group, and data were comparable to controls, i.e., normalized after therapy. The insignificant changes are probably due to the small sample size as well as the high pretreatment Tregs% in this group of patients, as a compensatory mechanism to severe inflammation and/or staphylococcal colonization, for further studies. Overall, there was no significant difference between any of the studied groups of patients and control group data following treatment. In agreement, Reynolds *et al.*[19] treated adults with moderate-to-severe AD with NB-UVB during a 12-week course and found no significant difference between patients and the control group after NB-UVB phototherapy regarding Tregs% and Tregs FoxP3 expression%. In addition, Orihara *et al.*[36] reported elevation of Tregs FoxP3 expression % after NB-UVB treatment in AD. Brandt *et al.*[13] also found that there was no difference in Tregs%, and the Tregs/Teffs ratio between the control group and atopic patients after NB-UVB phototherapy treatment. Moreover, after receiving treatment three times a week, Tregs% increased from a mean of 0.5% to a mean of 1.6% in another study by Milliken.[37] We found that the improvement in severity score was highly correlated with the elevation in Tregs%. This observation is matched with that of Morita.[38]

It is worth mentioning that imbalances in Th1/Th2 and Treg/Th17 have been found in atopic patients.[39] Thus, further research work should investigate whether the number of Tregs is related or not to other T-cell population levels (Th2, Th17) and how UVB irradiation alters the complete T-cell repertoire in peripheral blood. A quantitative polymerase chain reaction approach to define specific cytokine levels for the different T-cell subtypes may be an interesting issue to address the mechanisms on how Tregs could be a major checkpoint in regulating AD inflammation.

Conclusion

We concluded that the improvement of the AD Leicester severity score was highly correlated with the change in $CD4^{+}CD25^{high}FoxP3^{+}$ Treg% as well as the Treg/Teff ratio, denoting a control effect of Tregs on the activation of proinflammatory Teffs and the crucial role of Tregs in the maintenance of peripheral tolerance in AD. A limitation of this study is the size of the evaluated sample, which limits the significance of the observed results for further studies on larger population.

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