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RESEARCH ARTICLE

New insights in the pathogenesis of Atopic Dermatitis

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Abstract

Regulatory T cells (Tregs), are crucial for the maintenance of immunological tolerance and may directly prevent the activation and function of non-regulatory effector T cells, either Th1 or Th2 effector cells and/or Tregs may inhibit antigen presentation by dendritic cells (DCs) to effector T cells. Recently, there is some evidence suggesting the role of regulatory T cells in the AD pathogenesis. The aim of this work was to study the percentage levels of CD4+CD25+ Treg cells and analyze the FoxP3+T cells expression in the peripheral blood of patients complaining of atopic dermatitis and to evaluate the levels of IL-10 and IgE. Forty three children complain of atopic dermatitis showing that, the percentage of $CD4^{+}CD25^{+}$ found to be significantly elevated (11.3 ± 4.7) as compared to 10 non-atopic controls (3.7 ± 1.5, $p < 0.001$). The median value of IL-10 (2645 pg/ml) was higher than that of the control group (785 pg/ml). The median value of IgE was much higher in AD group (600 IU/ml) than in control group (46.6 IU/ml). The percentage levels Tregs and the levels of IL-10 and IgE in AD patients suggest their contribution in the pathogenesis of AD.

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INTRODUCTION

Atopic dermatitis (AD) is one of the most common skin disorders in infants and children. It is understood to be imbalance between the Th2 and Th1-type immune responses, with the cutaneous inflammatory response in AD being at least partially mediated by Th2 cytokines; IL4, IL13 and IL5. However, rather than Th1-type cytokines (INF-γ) down-regulating the allergic immune response **(Hansen et al., 1999).** There is a third group of cytokines, which includes TGF-B and IL10, have been found to have general immunosuppressive activity, blocking both Th1- and Th2 **(DaVeiga, 2012).**

Atopic dermatitis typically has three phases: the infantile phase from 0 to 2 years of age, the childhood phase between 2 and 12 years of age, and the adult phase. The typical locations during the infantile phase include the face and extensor sides, whereas during childhood, the flexural involvement dominates while the skin lesions in adults more frequently involve the hand, head and neck **(Mortz et al., 2014).**

Understanding the natural history of atopic dermatitis is complicated by the lack of diagnostic criteria useful in both children and adults. Many of the questionnaire-based criteria such as the UK working party criteria **(Williams et al., 1996)** may not perform well among adults **(Lan et al., 2009).**

The pathogenesis of AD is a complex interaction between genetic aberrations, skin barrier defects, abnormalities in innate and adaptive immunity, abnormalities of humoral and cellular immunity, environmental influences, and stress **(Mellor and Munn, 2011)**.

The immune system evolved to protect the host against the attack of foreign, potentially pathogenic microorganisms. It does so by recognizing antigens expressed by those microorganisms and mounting an immune response against all cells expressing them, with the ultimate aim of their elimination **(Anne and Paulo, 2004)**.

Various mechanisms have been reported to control and regulate the immune system to prevent or minimize reactivity to self- antigens or an overt response to a pathogen, both of which can result in damage to the host. Deletion of auto-reactive cells during T and B-cell development allows the immune system to be tolerant to most self-antigens. Peripheral tolerance to self was suggested several years ago to result from the induction of anergy in peripheral self-reactive lymphocytes **(Mellor and Munn 2011).**

Regulatory T cells (Tregs), formerly known as suppressor T cells. They are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus. Two major classes of CD4 Tregs have been described as naturally occurring Treg cells and adaptive T reg cells **(Hanabuchi et al., 2010).**

Tregs may directly prevent the activation and function of non-regulatory effector T cells, either Th1 or Th2 effector cells and/or Tregs may inhibit antigen presentation by dendritic cells (DCs) to effector T cells **(DaVeiga, 2012).**

There is some evidence suggesting the role of regulatory T cells in the AD pathogenesis. They are heterogenic T cells population with different surface markers, able to suppress activated immune system and prevent occurance of autoimmunization **(Werfel & Wittmann, 2008).** $CD4^+/CD25^+$ cells may induce anergy and protect against T effector cells proliferation via suppression of dendritic cells activity **(Elkord, 2006).**

Based on the phenotype and cytokine profile, Treg are accuratly identified as naturally arising fork head box protein 3 $(FoxP3)^+$ $CD4^+CD25^+$ Tregs (nTregs), IL10 producing $CD4^+$ Treg type1 (Tr1), TGF-β producing $CD4^+$ (Th3). The signature of the Tregs is their potent ability to suppress the effector cells **(Couper et al., 2008).**

Regulatory T cells reactivation of indolamine 2,3-dioxygenase (IDO) in dendritic cells (DCs). This is partially mediated via the interaction of cytotoxic T lymphocyte associated antigen 4 (CTLA-4) expressed on Tregs and CD80/86 expressed on DCs. IDO catalyzes the initial and rate-limiting step of tryptophan degradation, resulting in tryptophan deficiency. Because tryptophan is an essential proliferative stimulus for effector Tcells these cells undergo apoptosis in a tryptophan deprived manner **(Fallarino et al., 2003).**

Interleukin 10 (IL-10) is a homodimer with a molecular mass of 37KDa. Each monomer consists of 160 amino acids with a molecular mass of 18.5 KDa. (IL-10) is a key cytokine produced by a multitude of immune effector cells and possesses distinct regulatory effects on immune functioning in the skin. It can influence Th1/Th2 differentiation, antigen-presenting cell functioning (APC), APC-mediated T-cell activation & T-cell, B-cell and mast cell growth and differentiation that is aberrant in various disease processes **(Weiss et al., 2004).**

Aim of this work:

- In our study we attempted to study the percentage levels of $CD4^+CD25^+$ Treg cells and analyze the $FoxP3^+$T cells expression in the peripheral blood of patients complaining of atopic dermatitis.
- To evaluate the levels of IL-10 and IgE in patients complaining of atopic dermatitis.

Patient & method:

Forty three children complain of atopic dermatitis entering the outpatient clinic of the dermatology and venereology department of Zagazig University Hospitals in the period between January 2014 and March 2015, their ages range from 2-12 years, 10 healthy children as control group were enrolled in this study. Parents of each child gave written informed consent before entering the study and all the experiments were approved by the local Ethics Committee. The investigations were carried out in Clinical Pathology department Zagazig Universiy Hospitals. Before entering the study, the subjects undergo physical examination and the SCORAD (scoring atopic dermatitis) index was assessed **(oranje et al., 2007)**.

AD patients were diagnosed clinically based on UK Working Party's Diagnostic Criteria **(Williams et al., 1994)**, all patients were subjected to thorough history taking, general and dermatological examination. Severity of the disease was assessed in all patients by means of the SCORAD index. Total SCORAD is calculated with this equation: SCORAD = A/5 + 7B/2 + C. The higher score the more severe disease. Mild AD: SCORAD below 20, Moderate: 20-40, Severe: above 40 **(oranje et al., 2007)**.

The patients enrolled in the study had moderate AD (mean SCORAD index 28; range 20–40). This study included 53 children divided into two groups, Group (I) included 43 patients with atopic dermatitis, they were 19 males and 24 females. Group (II) included 10 healthy children.

All individuals of were subjected to:

- Full history taking, with stress on: exposure to allergen, age of onset, course, family history of atopy.
- Skin examination, distribution, clinical appearance.
- General examination: chest, ophthalmic, ENT examination for atopic manifestations.
- Laboratory investigations:

1. Complete blood picture with stress on eosinophilic percentage using Sysmex SF 3000 (Roche Diagnostic GmbH, Mannheim, Germany).
2. Serum IgE level by laser nephelometry.
3. Serum IL-10 level by enzyme-linked immunosorbent assay (ELISA).
4. Flowcytometric detection of T-regulatory cells using labelled monoclonal antibodies against CD4, CD25, FoxP3.

Methods:

- Sampling

Five ml blood volumes were withdrawn aseptically into a sterile disposable syringe from every patient and control then divided as follow: 2ml blood were collected in sterile vacutainer tube for flowcytometry analysis, 3 ml blood were collected in sterile vacutainer tube for cytokines assessment. Sera were separated and stored in -20 ^{0}C until used.

Flowcytometeric assay of $CD4^+CD25^{high}$ (Tregs) and FoxP3

- Lymphocyte Separation

Peripheral lymphocyte cells were purified from peripheral blood by Ficoll-Hypaque gradients (Biochrom, Berlin, Germany). Separated lymphocytes were washed twice with fluorescence-activated cell sorter (FACS) washing buffer (1% phosphate-buffered saline, 2% fetal calf serum, 0.05% sodium azide, 0.5M ethylenediamine tetra-acetic acid).

- Sample Preparation and Flowcytometry Analysis

Lymphocytes staining was performed using mouse anti-human monoclonal antibodies (mAbs) (anti-CD25) Phycoerythrin and (anti-CD4) Peridinine chlorophyll protein (Per-CP) conjugate (Dako). The surface staining was done by adding 10 ul of each mAbs to 100 ul of separated lymphocytes in the same tube, incubated for 30 min in the dark at 4 ^{0}C, then washed twice with FACS washing buffer. Finally, 0.5 ml of phosphate buffer saline (PBS) was added on the washed cells and samples were ready for the measurement of the CD4/CD25 using a FACSCalibur flowcytometry (Becton Dickinson, San Jose, CA).

- FoxP3 Staining

FoxP3 staining was performed according to the manufacture′s protocol {FITC antihuman FoxP3 Staining Set (eBioscience)}. Cells were first stained with surface mAb of interest (anti-CD4/CD25) followed by FoxP3 intracellular staining using permealizing solution. To avoid nonspecific Fc receptor staining, we used appropriate isotype controls of mouse anti-human mAbs.

With multigated analysis, the percentage of FoxP3 expression were determined on $CD4^+$ T-cells that are very high in $CD25^+$ to avoid contamination with other $CD4^+$ CD25 low/intermediate effector T-cells. FACS-acquisation and analysis were performed immediately with FACS CellQuest software (BD Biosciences).

Method of estimation of IL-10:

Quantitative determination of sIL-10 was done using enzme-linked immunosorbent assay (Quantikine; R&D systems, Minneapolis, USA) according to manufacturer′s recommendations. The minimum detectable limit of IL-10 was typically less than 3.9 pg/ml.

Method of measuring IgE:

Serum IgE level by laser nephelometry, using DADE Behring B.N. Prospeck. (DADE Behring New ark 4217 USA).

Polystyrene particles coated with antibodies specific to human IgE are aggregated when mixed with samples containing human IgE. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration. Reference curves are constructed by multipoint calibration. The results are evaluated automatically by mean of a logit-log function.

Results

Table (1) *Demographic data of the patients:*

Variable	Value	
Age (years): *Mean ± SD* *Range*	6.7±3.8 2 – 12	
	No(n=43)	**%**
Gender: *Male* *Female*	19 24	44.19 55.81

Table (2) Laboratory data of the groups:

	Group (I)	Group (II)	P
IL-10 (pg/ml) **Median** **Range**	2645 655-6100	785 595-1005	0.001 HS
IgE (IU/ml)	600 170-2375	46.6 10-89	0.001 HS
Esinophilis (%)	11 1-18	2 1-3	0.02 S

It was observed that the median value of IL-10 (2645 pg/ml) was higher than that of the control group (785 pg/ml). The level of IgE was elevated in all the patients in group I. The median value of IgE was much higher in group I (600 IU/ml) than in group II (46.6 IU/ml). The eosinophilic percentage normally represents 1-6% of the total leucocytic count. The median of eosinophilic percentage was higher in group I (11%) than group II (2%).

There were highly significant differences in the levels of IL-10 and IgE between group (I) & Group (II). Also there was significant difference in the level of esinophilis between Group (I) & Group (II).

Table (3) Correlation between IL-10 & other laboratory parameters of studied groups:

	Group (I)		Group (II)	
	R	**P**	**R**	**P**
IgE	o.179	0.315	0.149	0.667
Esinophilis	0.067	0.719	0.087	0.758

There was a non-significant correlation between the level of IL-10 & other laboratory parameters of studied groups.

Table (4) The percentage of T cells (mean ± SD) in the peripheral blood of AD patients:

Group	Subpopulations of T-lymphocytes		
	Total $CD4^+$	$CD4^+CD25^{+high}$	$CD4^+CD25^{high}FoxP3$
AD patients	31.7±5.2	11.3±4.7	9.2±3.7
Controls	43.7±5.1	3.7±1.5	2.8±1.2
P	< 0.001	< 0.001	< 0.001

The percentage of $CD4^+CD25^+$ found to be significantly elevated (11.3 ± 4.7) as compared to non-atopic volunteers (3.7 ± 1.5, $p < 0.001$). The $CD4^+CD25^{high}$ cells bearing FoxP3 receptor were highly increased in AD patients (9.2. ± 3.7 %) versus controls (2.8 ± 1.2 %) ($P< 0.001$).

Table (5): comparison between CD4+ CD25+ MFI among cases and controls

Parameters	Cases	Controls	T	P
CD4+CD25+ MFI				
-Range	43	10		
-Mean±SD	31.8-75.03	8.9-59.4	3.507	0.001(HS)
	54 ± 16.5	33.3±18.2		

MFI= Mean Fluorescent Intensity

This table showed comparative study of CD4+CD25+ MFI among cases and controls. It revealed that there was highly statistically significant difference between patients and controls.

Discussion

Autoreactive T cells are predominantly deleted in the thymus, but this process is not stringent. Thus, autoreactive T cells escape into the peripheral T cell pool, subsequent activation can result in autoimmune pathogenic conditions. $CD4^+CD25^+$ regulatory T cells, which comprise 5-10% of $CD4^+$ T cells are crucial for maintenance of peripheral tolerance **(Kristen et al., 2008)**.

The concept of AD implies Th2 cytokines in acute phase, becoming Th1 cytokines in chronic phase, and finally progressing to an autoimmune disease with IgE antibodies against autologous epidermal proteins. Th2 cells are characterized by IL3,-4,-5,-9 and -13 secretion. Th1 cells are important in inflammatory delayed hypersensitivity producing IFN-ᵞ, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-12 **(Lipozenčić and Wolf, 2007).**

The $CD4^+CD25^+$ regulatory T cells are among the most important cells in the immune system regulation. They can control auto-aggressive T cells and B cells, which escape negative selection in the thymus. They block activation and effector functions of autoreactive T cells maintaining peripheral self-tolerance and preventing the development of various inflammatory diseases through direct contact with effector immune cells and the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β **(Alfadhli, 2013).**

Both types of Tregs actively regulate Th2 responses to allergens in healthy donors, whereas their suppressive function is impaired in allergic individuals, and therefore Tregs could play a major role in maintaining a healthy immune response to allergen **(Robinson et al., 2004).**

In addition, **Taylor et al. (2007)** show that in 6-month-old infants the expression of FoxP3 receptor on $CD4^+CD25^{high}$ peripheral blood cells is higher in those patients who developed AD lesions later than others.

There are discrepant results on Tregs alteration in AD patients**. Karlsson et al. (2004)** showed that $CD4^+$ $CD25^{high}$ T-cells impairment in children with cow's milk allergy. Some authors, who do not report increased number of circulating $CD4^+CD25^{high}FoxP3^+$ cells have found their accumulation in the skin lesions **(Franz et al., 2007 & Schnopp et al. 2007).** However, this is not confirmed by **Verhagen et al., (2006).**

In a study done by **(Zbigniew et al., 2012)**, stated that the decreased percentage of $CD95^+$ on Tregs indicates that activated $CD3^+CD4^+CD25^{high}$ FoxP3 cells can prolong survival, which at least partly contributes to their increased percentage in the peripheral blood. The presence of surface T-regulatory receptors, i.e. CD152 (CTLA-4) and GITR that triggers the function of T-regulatory cells as well as enhanced co-expression of both CD62L and CD134 molecules argues for their activation.

In contrast to our findings **Szegedi et al. (2009)** have not found the percentage of FoxP3+ Tregs to be higher than in controls. These data indicate that neither the severity of the disease nor the total IgE level influences the number of FoxP3+ T-regulatory cells.

Valencia et al., (2006) also revealed that elevated tumor necrosis factor-alpha (TNF-α) may interfere with suppressive capacity of nTregs.

In other studies, it was shown that Tregs from AD patients have decreased suppressive capacity when compared with healthy control **(Ling et al., 2004)**, however **Szegedi et al. (2009)** stated that there were no differences in Tregs number between AD patients and healthy controls.

The idea that T cells may be affected by the inflammatory milieu is certainly not new. However, it is increasingly apparent that specific T cell types can exhibit plasticity depending on the microenvironment, and this appears to hold true for Tregs. Additionally, attenuation of Foxp3 expression results in conversion of Tregs to Th2 cells and this occurs even in a Th1-polarizing environment **(Joetham et al., 2008)**. Moreover, there is evidence to suggest that the conversion pathway is bi-directional. For example, antigen-specific Th2 cells can be re-differentiated into Foxp3+ Tregs by TGF-β in the presence of *all-trans* retinoic acid and rapamycin **(Kim et al., 2010).**

Notably, Tregs can also be converted to other T cell types including Th17 cells. This cell type is induced from naïve T cells by TGF-β and IL-6. However, Tregs producing TGF-β can differentiate into Th17 cells in the presence of IL-6 without the requirement for TGF-β **(Xu et al., 2007)**. These aspects of T cell plasticity may be highly relevant to disease processes in the skin. Numerous studies have demonstrated expression of a broad array of inflammatory factors in AD skin. These include IL-4, IL-5, IL-12, IL-13, IL-17, IFN-γ, GM-CSF, IL-31 and TNF-α. Thus, Tregs recruited to inflamed skin are exposed to a host of factors that could influence their ability to function (**Rachana et al., 2011)**

In our study we reported that there is an increase in the level of IL-10 in group (I), when compared with the control group. This increased level may be due to the trial of the immune system to control the allergic immune response. This finding comes in agreement with **(Yoshizawa et al., 2002 & Homey et al., 2006)** who concluded that immunosuppressive cytokines, i.e., IL-10 and TGF-β are reported to be found in AD patients, which are linked to relative prevalence of Th2 cells.

Akdis & Akdis (2003) reported that the levels of IL-10 were high in AD. An overexpression of IL-10 has been noted in peripheral blood mononuclear cells (PBMC) from AD patients. This may reflect the general immunologic imbalance towards a type 2 response. Type 2 cytokines (IL-4, IL-5 and IL-10) are considered to be crucial in AD **(Asadullah et al., 2003).**

Laouini et al., (2003), reported that IL-10 is overexpressed in the skin of AD patients. Interventions directed at downregulating IL-10 production in atopic individuals at the time of allergen exposure may provide a novel therapeutic modality for the prevention of allergic diseases.

In a study done by **(Zbigniew et al., 2012)** , they reported that concentrations of both serum IL-10 & TGF-β and production of these cytokines by lymphocyte cultures have been significantly decreased, which points out to insufficient suppression of the immunological response by these cytokines in severe AD. This is consistent with the data by **(Antiga et al. 2011)**, who report decreased serum levels of IL-10 and TGF-β in AD similar to the patients with lupus erythematosus.

Vakirlis et al. (2011) reported that IL-10 decrease in the patients with active phase of AD as compared to chronic AD cases and controls; however, these data are not correlated with SCORAD index. In addition, **Senevirate et al. (2006)** stated that severe atopic dermatitis is associated with reduced frequency of IL-10 producing allergen-specific CD4+ T cells.

The study of Tregs is progressing to a new level, where the need to demonstrate their existence has been replaced by the need to understand their biology so that therapeutic use will become a reality. The ability to induce or expand Tregs *in vitro* or *in vivo* may have important implications in the field of autoimmunity and inflammation.

Another point of view is that most immunosuppressive therapies may be used to reduce or eliminate activated T cells; however, such treatment may actually promote autoimmune responses by also depleting Tregs. An important advantage of using Tregs therapeutically would be that these cells can exert by stander suppression in an antigen nonspecific fashion, which means that Tregs do not necessarily need to recognize the antigen(s) that is the subject of immune attack.

Recent advances in the field of Treg biology have partially delineated the mechanisms involved in the in vivo generation of functional Treg. The identification of new molecules implicated in these processes is emerging. These aspects, together with a better understanding of the role that specific DC subsets play in the generation of functional Treg, will contribute to the design of more efficient and safer immunotherapy against allergic diseases in the near future.

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