T Regulatory Cells Response to Allergen Specific Immunotherapy in Patients with Allergic Airway Diseases: A Prospective Study

[1]Raghdaa A. Ramadan, [1]Emad A. Morad, [2]Mohamed El-Shabrawy

Departments of [1]Medical Microbiology & Immunology and [2]Chest Diseases, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Forkhead box P3 (FoxP3) T regulatory (Treg) cells modulate the immune system by blocking other types of T-cells. They maintain tolerance to self-antigens and help in inducing tolerance to foreign antigens. A deregulation of FoxP3 Tregs seems to play an important role in allergic disorders. The aim of this work was to study the response of FoxP3 Treg cells and their FoxP3 expression in patients, attending the Allergy Unit and the Chest Outpatient Clinic, Faculty of Medicine, Zagazig University, with allergic airway diseases, before and 1 year after receiving subcutaneous allergen specific immunotherapy (SIT). This prospective study was carried out on 25 patients with allergic airway diseases, confirmed by positive skin test, and that showed clinical improvement one year after SIT. All cases were subjected to total immunoglobulin E quantitation by ELISA. FoxP3 Treg cells frequency and FoxP3 relative fluorescence intensity, as an indicator of Tregs function, were assessed by flowcytometry. The results were compared before and after SIT. Twenty five age and sex matched apparently healthy volunteers were enrolled as controls. Our findings demonstrated that in comparison to the control group, the count of FoxP3 T regulatory cells was higher; however, the function was lower among the enrolled patients (P= 0.007 and P< 0.001, respectively). When FoxP3 Tregs were compared in the patients before and one year after SIT, it was found that both the count and FoxP3 expression showed statistically significant increase (P< 0.001). An inverse correlation was found between FoxP3 Tregs count and FoxP3 expression. It is concluded that patients with allergic airway diseases have increased number of FoxP3 Treg cells but with defective function. SIT plays a role in increasing the number of FoxP3 Tregs and improving their suppressive function, which leads to control of airway inflammation and thus clinical improvement.

Allergic respiratory diseases are considered a serious public health problem and have an increasing prevalence in all regions of the world, regardless of the economic and social development of these regions (Martinez, 2008). The increasing recognition over the last 50 years that allergic rhinitis and allergic asthma frequently co-exist and the strong pathophysiologic link between them has led to the concepts of 'united airways disease' (Passalacqua *et al.*, 2001), 'respiratory allergy' (Papadopoulos *et al.,* 2008) or 'combined allergic rhinitis and asthma syndrome' (WAO, 2016).

T regulatory (Treg) cells comprise a specialized subset of $CD4^+$ T cells with potent suppressive capabilities in vitro and in vivo, and are key mediators of tolerance and immune homeostasis (Bin Dhuban & Piccirilo, 2014). Among the Treg cells known subpopulations, the forkhead box P3 (FoxP3) Treg cells are essential master-switch in immune-regulation (Pellerin *et al.,* 2014).

FoxP3 Treg cells can be defined as follows: 1) thymus-derived Treg cells (tTregs) or called thymic-derived natural Tregs; 2) peripherally derived Treg cells (pTregs) which differentiate in the periphery (Abbas *et al.,* 2013). They could be identified as expressing CD4, high levels of the interleukin 2 (IL-2) receptor alpha chain (CD25), the cytotoxic T lymphocyte antigen 4 (CTLA4) and the FoxP3 transcription factor which is essential for their function (Sakaguchi *et al.,* 1995; Fontenot *et al.,* 2003; Sansom &

Walker, 2006). FoxP3 is a potent repressor of IL-2 production, but upregulates the expression of CD25 and CTLA4 (Wu *et al.,* 2006). It also induces the expression of anti-inflammatory cytokine IL-10 (Komatsu and Hori, 2007).

FoxP3 Tregs are known to play a chief role in maintaining the immune tolerance by preventing the T helper (Th)2 induction and Th2 cytokine release in response to allergens (Cottrez *et al.,* 2000). They use unique mechanisms to exert their immunosuppressive action: 1)- They use perforin and granzyme B to induce cytolysis of cytotoxic T lymphocytes, B and natural killer cells (Cao *et al.,* 2007; Loebbermann *et al.,* 2012), 2)- They can use the tumor necrosis factor-related apoptosis inducing ligand (TRAIL)/death receptor 5 (DR5) pathway to induce cytolysis of T cells (Ren *et al.,* 2007), 3)- They can also produce inhibitory cytokines such as IL-10, IL-35, and transforming growth factor beta (TGF-β) (Vignali *et al.,* 2008). A deregulation of FoxP3 Tregs seems to play an important role in allergic disorders (Thunberg *et al.,* 2010; Kinoshita *et al.,* 2014).

Allergen specific immunotherapy (SIT) is so far the only specific treatment of allergic disorders with a potential to modify the course of the disease and is considered the most effective therapeutic approach for deregulated immune response towards allergens, by enhancing immune tolerance mechanisms (Stelmaszczyk-Emmel *et al.,* 2015).

The aim of this work was to study the response of FoxP3 Treg cells to subcutaneous SIT by measuring their frequency and FoxP3 expression level, as an indicator of their suppressor function, in peripheral blood of patients with allergic airway diseases and to correlate this to clinical improvement.

Subjects and Methods

This prospective study was conducted in the Allergy Unit of Microbiology and Immunology Department, Chest Outpatient Clinic and the Flowcytometry Unit of Clinical Pathology Department, Faculty of Medicine, Zagazig University during period from December 2014 till January 2016. The study was approved by the Ethical Committee of Faculty of Medicine, Zagazig University. All subjects had given written informed consent before participation in the study.

Subjects

The study was conducted on 25 patients suffering from allergic airway disease and demonstrated positive intradermal skin test. Compliance to subcutaneous allergen specific immunotherapy (SIT) and clinical improvement one year after commencing therapy were inclusion criteria to patients' enrollment in this study. Excluded patients were those suffering from other immunological or chronic disorders or having received previous immunotherapy or treatment with immunomodulatory drugs including systemic corticosteroids. Twenty five apparently healthy volunteers were included in the study as controls. Asthmatic patients were clinically diagnosed by history of variable respiratory symptoms which are wheeze, shortness of breath, chest tightness, cough and evidence of variable expiratory airflow limitation. The severity of asthma was determined by breathless talking, alertness, respiratory rate, accessory muscles and suprasternal retractions, wheeze, heart rate, pulsus paradoxus, peak expiratory volume (PEF) after initial bronchodilator % predicted or% personal best, PaO_2 (on air) and/or $PaCO_2$, and SaO_2% (on air). Both asthma diagnosis and severity characterization were according to the definitions and guidelines of Global Initiative for Asthma (2015). Clinical diagnosis and severity categorization of allergic rhinitis patients were based on the criteria published by Allergic Rhinitis and Its Impact on Asthma (ARIA) (Bousquet *et al.,* 2008).

Methods

- Pulmonary function tests

They were done for asthmatic patients and were carried out using a portable spirometer "Vitalograph copd-6TM" apparatus. Expiratory flow-volume curve was performed before and 20 minute after inhalation of 4 puffs of 400 µg salbutamol metered dose inhaler. Forced expiratory volume in the first second (FEV1) was measured as absolute value and percentage of the predicted value (FEV1%)

- Blood samples

Four mL blood samples were taken from all patients (before and one year after SIT) and controls. Serum was separated from 2 mL for total immunoglobulin E (IgE) estimation by ELISA. The other 2 mL were added to EDTA tube for immunophenotypic analysis of

Tregs for expression of $CD4^+$, $CD25^{high}$ and FoxP3 by flowcytometry.

- Assessment of the atopic status

Intradermal skin test: Seven coca's extracted antigens (House dust, cigarette smoke, wool, cotton, mixed fungi, pollens and hay dust) were used from the Allergy Unit of Microbiology and Immunology department of Faculty of Medicine, Zagazig University. The intradermal skin test was performed as devised by Indrajana *et al.* (1971). Briefly, patients were instructed to stop antihistaminics for at least 7 days before testing. Skin test was performed on the volar surface of the forearm leaving 5 cm from the wrist and 3 cm from the elbow. The test sites were allocated at least 3 cm apart to avoid overlapping. Insulin syringes were used to intradermally deliver 0.05 mL of 1/1000 dilution of extracts. After 15 minutes, the tested sites were inspected for wheal and/or erythema reactions. If no result was observed after 15 minutes, the sites were inspected after 30 minutes. Physiological saline served as the negative control. A positive skin reaction was evident by a wheal and flare reaction. The diameter of wheal was recorded in two dimensions. A positive test was the one in which the mean of two wheal diameters was greater than negative control.

- Measurement of total serum IgE

Immunospec IgE Enzyme Immunoassay kit (IMMUNOSPEC Corporation, Canoga Park, CA) was used for the quantitation of total IgE in human serum. The assay was performed according to the manufacturer's instructions. The total IgE in a normal, allergy-free adult is less than 150 IU/mL. The sensitivity detection limit of this assay is 5.0 IU/mL.

- Allergen specific immunotherapy

Crude allergen extraction in the allergy unit was based on Tsay *et al.,* (1987):

1)- Homogenization or grinding of the source material, 2)- Defatting in acetone to remove lipophilic compounds then filtration. Defatting was repeated until acetone remains transparent after contacting the source material, 3)- Drying of the source material to remove acetone, 4)- Extraction of crude allergens from the source material using coca's solution (5 gram sodium chloride, 2.5 gram sodium bicarbonate, 5 gram phenol crystals and water for injection to make 1000 ml water) in a ratio of (1/10) for 6 hours, 5)- Separation of the crude allergen extracts dissolved in the liquid phase from the non-allergenic residue by centrifugation at 12.000 xg for 1 hour, 6)- Sterilization of the allergen extracts by membrane filtration, 7)- Standardization: the allergic potency of the crude allergen extracts was measured and standardized in weight/volume (w/v) unit i.e., weight of allergenic substances extracted per volume of extracting fluid. However, the initial dilution of extract, starting dose and progression of dosage have to be determined carefully on the basis of the patient's history and sensitivity tests, 8)- Storage of allergen extracts at 2-8°C.

Safety was completely ensured during all steps of crude allergen extraction through: 1)- Hand hygiene and wearing gloves, 2)- Sterilization of glassware in hot air oven at 180°C for 1 hour, 3)- Allergen extraction was done under laboratory safety cabinet class II A (Microflow, USA), 3)- Vial stoppers were disinfected by careful wiping with sterile 70% isopropyl alcohol swabs, 4)- Subcultures from all allergens stocks were done on a regular basis for bacterial or fungal growth and stocks were discarded once showed any sign of contamination.

For specific subcutaneous immunotherapy, patients received calculated twice weekly doses from gradually decreasing dilutions of allergen extracts (1/2000, 1/1000, 1/500, 1/250 and 1/125) over 6 months followed by a weekly maintenance dose of 1/125 dilution.

- Immunophenotyping by flowcytometry

Blood samples were processed within 24 hours of collection, being preserved at 4ºC. Briefly, 100 µL blood were mixed with 10 µL of each of fluorescein isothiocyanate (FITC) conjugated anti-Human CD4 and phycoerytherin (PE) conjugated anti-Human CD25 (Affymetrix, eBioscience, Inc., USA) in the test tube, while PE and FITC isotype controls (Affymetrix) in the control tube. Both tubes were incubated for 20 minutes in the dark at the room temperature. Two mL of fluorescence activated cell sorting (FACS) lysing solution (Becton Dickinson (BD), USA) were added and incubated for 10 minutes in the dark at the room temperature. The cell pellet was washed twice with 2 mL phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich Chemie Gmbh). For intracellular staining, 1 mL of fixation/permeabilization solution (Affymetrix) was added to the cell pellet and incubated for 30 minutes at 4ºC. The cell pellet was washed twice in 2 mL of permeabilization buffer. Ten µL of allophycocyanin (APC) conjugated anti-Human FoxP3 and APC isotype control (Affymetrix) were added to the cell pellet in the test and control tubes, respectively and incubated in the dark at the room temperature for 30 minutes. After washing twice in 2 mL of permeabilization buffer, the cells were suspended in 200 µL PBS for analysis by the flowcytometer (FACSCalibur, BD) using CELL Quest TM software.

Gating was done on lymphocytes using side scatter (SSC) and forward scatter (FSC) strategy. $CD4^+$ T cells expressing high levels of CD25 were further gated and analyzed for FoxP3. FoxP3 Tregs were defined by co-expression of $CD4^+$, $CD25^{high}$ and FoxP3. Relative fluorescence intensity (RFI) of FoxP3, an indicator of FoxP3 expression level, was calculated using the following formula: mean fluorescence intensity (MFI)/MFI with isotype control antibody (Stelmaszczyk-Emmel *et al.,* 2012).

Statistical Analysis

The data were coded, entered and checked using the Statistical Package for Social Science (IBM SPSS) software (version 22, Chicago, IL) and Epi-Info 7. Results were expressed as the arithmetic mean, standard deviation, median and range for continuous variables, number and percentage for categorical variables. Percent of categorical variables were compared using Chi-square test. Continuous variables were compared using t test. Wilcoxon signed-rank test was used to compare the variables of the patient group before and after immunotherapy. Correlation was used to find the association between two measurement variables and to see whether the two measurement variables covary. Correlation results were expressed in the form of correlation coefficient (r). $P< 0.05$ was considered statistically significant.

Results

The enrolled patients and controls were demographically matched as there was no statistically significant difference regarding their baseline characteristics. Out of the 25 patients, 19 and 21 had had allergic asthma and allergic rhinitis, respectively (table 1). Intradermal skin test revealed that 84% of studied cases were allergic to mixed pollens, 80% to each of smoke and hay dust, 68% to house dust, 20% to cotton, 16% to wool while none of them was allergic to mixed fungi.

The count of FoxP3 Tregs was higher in the patients' group compared to the control group in terms of statistical significance. Conversely, the intracellular FoxP3 expression, indicated by the FoxP3 RFI, was significantly higher in the control group (table 2).

When FoxP3 Tregs were compared in the patients before and one year after subcutaneous allergen specific immunotherapy (SIT), it was found that both the number and FoxP3 expression showed statistically significant increase after SIT, as indicated in table 3.

FoxP3 Tregs % dot plots and FoxP3 expression histograms of a case before and after SIT are depicted in figures 1 and 2, respectively.

Moreover, statistical comparison of other laboratory parameters showed significant decrease in the IgE level, eosinophil and lymphocyte counts and significant increase in the FEV1% after SIT (table 4).

As regards statistical correlation between FoxP3 Tregs and characteristics of the studied patients, it has been found that FoxP3 Tregs count was positively correlated with age and allergic airway disease severity, and negatively correlated with FoxP3 expression (table 5). As for FoxP3 expression, it was negatively correlated with FoxP3 Tregs%, disease severity and eosinophils count (table 6).

Table 1. Baseline demographic and clinical characteristics of the studied groups.

	Controls (n= 25)		Patients (n= 25)		*P* value
Demographic characteristics					
Age					
mean ± SD	30.8 ± 8.5		28 ± 9.8		NS
Range	18 – 50		18 – 52		
Sex	No.	%	No.	%	
Male	4	16	9	36	NS
Female	21	84	16	64	
Residence	No.	%	No.	%	
Rural	17	68	2	8	NS
Urban	8	32	23	92	
Clinical characteristics					
Smoking	No.	%	No.	%	
Yes	11	44	10	40	NS
No	14	56	15	60	
Heritance	No.	%	No.	%	
Yes	0	0	11	44	< 0.001
No	25	100	14	56	
Allergic asthma	(n= 0)		(n= 19)		
	No.	%	No.	%	
Mild	0	0	5	26.3	< 0.001
Moderate	0	0	11	57.9	
Severe	0	0	2	10.5	
Life threatening	0	0	1	5.3	
Allergic rhinitis	(n= 0)		(n= 21)		
	No.	%	No.	%	
Mild intermittent	0	0	1	4.7	< 0.001
Moderate/severe intermittent	0	0	1	4.7	
Mild persistent	0	0	2	9.6	
Moderate/severe persistent	0	0	17	81	

P>0.05 is not significant (NS).

Table 2. Baseline FoxP3 Tregs % and their FoxP3 expression among the studied groups.

	Controls (n= 25)	Patients (n= 25)	*P* value
FoxP3 Tregs %			
mean ± SD	2.6 ± 1.6	6.9 ± 4.8	
Median	3	6	0.007
Range	0.7 – 8	0.5 – 16	
FoxP3 RFI			
mean ± SD	18.6 ± 5.4	12.1 ± 2.2	
Median	19	12	<0.001
Range	9 – 25	9 – 18	

RFI: Relative fluorescence intensity. *P*<0.05 is significant..

Table 3. FoxP3 Tregs % and FoxP3 expression of the patients before and after immunotherapy.

	Before immunotherapy (n= 25)	After immunotherapy (n= 25)	*P* value
FoxP3 Tregs %			
mean± SD	6.9 ± 4.8	12.3 ± 7.8	<0.001
Median	6	9	
Range	0.5 – 16	2.5 – 29	
FoxP3 RFI			
mean± SD	12.1 ± 2.2	13.6 ± 5.1	<0.001
Median	12	14	
Range	9 – 18	5 – 23	

RFI: Relative fluorescence intensity. *P*<0.05 is significant.

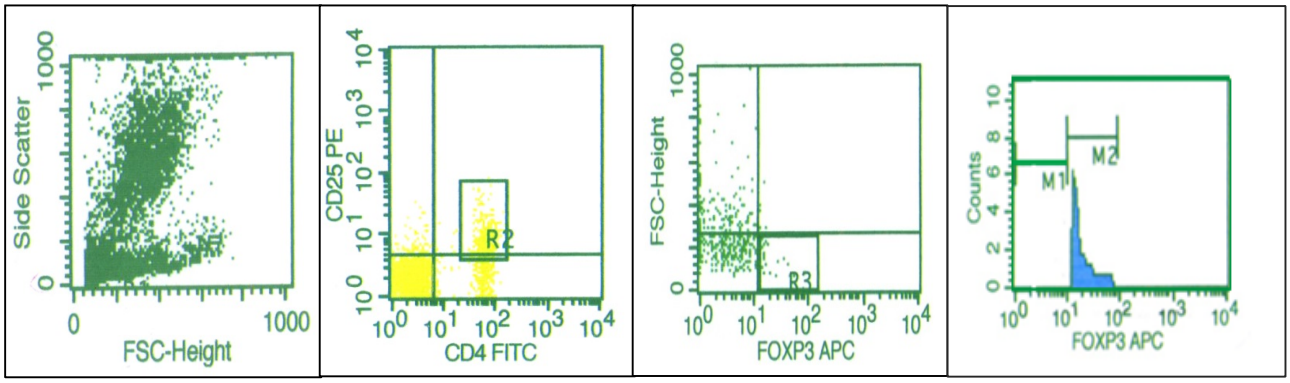

Figure 1. Flowcytometric data analysis showing dot plot of FoxP3 Tregs % and FoxP3 expression histogram of a recently diagnosed case with allergic airway disease.

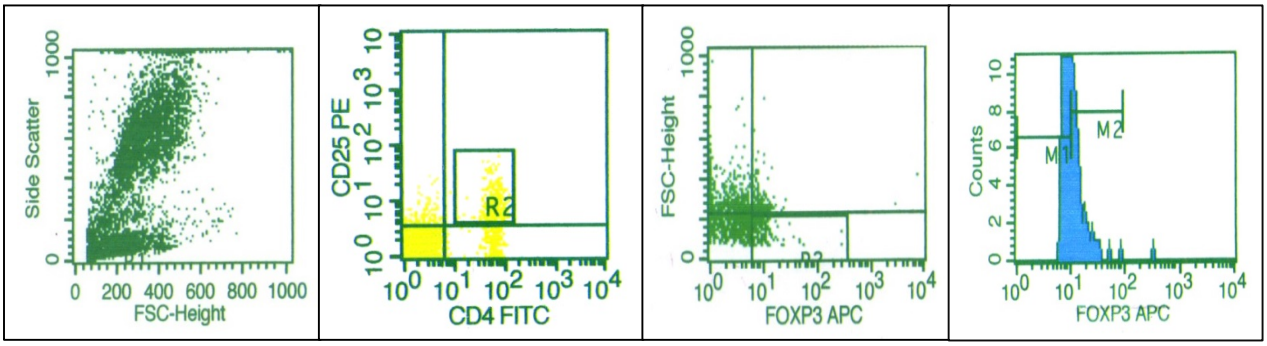

Figure 2. Flowcytometric data analysis showing dot plot of FoxP3 Tregs % and FoxP3 expression histogram of the case one year after immunotherapy.

Table 4. Laboratory characteristics and FEV1% of the patients before and after immunotherapy.

	Before immunotherapy (n= 25)	After immunotherapy (n= 25)	*P* value
IgE			
mean ± SD	143 ± 108.8	61.6 ± 63.3	<0.001
Median	140	36	
Range	10 – 402	5 – 218	
Relative eosinophil count (%)			
mean ± SD	4.1 ± 1.7	2.9 ± 1.8	<0.001
Median	4	2	
Range	1 – 6	1 – 8	
Lymphocyte count ($X10^3/mm^3$)			
mean ± SD	3.7 ± 2.5	3.2 ± 1	<0.001
Median	3.9	3.1	
Range	3 – 11	2 – 10	
FEV1% (n= 19)			
mean ± SD	75.4 ± 8.6	86.3 ± 2.7	<0.001
Median	78	87	
Range	59 – 89	82 – 89	

FEV1: Forced expiratory volume in the first second.

P<0.05 is significant.

Table 5. Correlation between FoxP3 Tregs % and characteristics of the studied patients.

	FoxP3 Tregs %			
	Before immunotherapy		After immunotherapy	
	r	*P* value	r	*P* value
Age	0.4	0.04	0.4	0.03
FEV1%	-0.002	NS	-0.2	NS
Eosinophils	0.3	NS	-0.2	NS
IgE	0.3	NS	0.1	NS
Severity grade	0.7	<0.001	-0.2	NS
FoxP3 RFI	-0.5	0.007	-0.7	<0.001

r: Correlation coefficient. RFI: Relative fluorescence intensity. FEV1: Forced expiratory volume in the first second.

P >0.05 is not significant (NS).

Table 6. Correlation between FoxP3 expression and characteristics of the studied patients.

	FoxP3 RFI			
	Before immunotherapy		After immunotherapy	
	r	*P* value	r	*P* value
Age	0.09	NS	-0.4	NS
FEV1%	-0.4	0.06	0.1	NS
Eosinophils	-0.01	NS	-0.4	0.02
IgE	0.2	NS	0.2	NS
Severity grade	-0.5	0.04	-0.6	0.009
FoxP3 Tregs %	-0.5	0.007	-0.7	<0.001

r: Correlation coefficient. RFI: Relative fluorescence intensity. FEV1: Forced expiratory volume in the first second.

P >0.05 is not significant (NS).

Discussion

FoxP3 Tregs have been emerging as key focus in the pathogenesis of allergy. It is recognized that acquired immunity is controlled by Tregs that suppress responses of effector T cells (Zhang *et al.,* 2014). Early Treg studies used CD25 as a marker to identify Tregs. However, Sakaguchi *et al.,* (2010) reported that CD25 is highly upregulated on conventional T cells upon stimulation, i.e., during inflammatory conditions. Later studies showed that $CD4^{+}$ cells expressing high levels of CD25 ($CD25^{high}$) should be used for Treg assessment as this CD25 expressing population expresses high levels of FoxP3 and exhibits a more

consistent suppressive ability exvivo (Bin Dhuban & Piccirilo, 2014). Therefore, FoxP3 Tregs have been qualified in our study by co-expression of CD4, CD25 ^{high} and FoxP3.

In this study and in concordance with Lin *et al.,* (2008) and Raedler *et al.,* (2015), patients with allergic airway diseases had significantly higher FoxP3 Tregs count than the control subjects. However, these findings do not comply with Provoost *et al.,* (2009) and Lou *et al.,* (2012) who reported non-significant difference between both groups. These findings are also not consistent with Huang *et al.,* (2013) and Stelmaszczyk-Emmel *et al.,* (2013) who found Tregs in allergic patients to be significantly lower. The increased number of FoxP3 Tregs in allergic patients, particularly patients with allergic asthma, may indicate a counter-regulatory mechanism that is yet not sufficient to control allergic inflammation.

Comparing FoxP3 Tregs count before and 1 year after subcutaneous SIT, in clinically improved patients, a statistically significant increase in FoxP3 Tregs count has been found. This finding agrees with that reported by Fujimura *et al.,* (2011), Lou *et al.,* (2012), Sorensen et al. (2013) and Suarez-Fueyo *et al.,* (2014). On the contrary, Ajduk *et al.,* (2008) and Stelmaszczyk-Emmel *et al.,* (2015) reported non-significant change in FoxP3 Tregs count among children with allergic asthma after 1 year of SIT. Similarly, Mobs *et al.,* (2010), Wei *et al.,* (2010) and Susanti *et al.,* (2013) reported non-significant deviations in FoxP3Tregs count after SIT. The discrepant response of FoxP3 Tregs % in the peripheral blood after SIT among studies might be explained by the divergence in the patients' demographics, disease severity, immunotherapy protocols and timing of blood sampling after SIT. Another explanation is the difference in the distribution of FoxP3 Tregs caused by their homing to the lungs, lymph nodes and secondary organs. In addition, the inconsistent definition of Tregs and the difference in methods used to identify this minor population of cells makes different studies not fully comparable.

In this study, FoxP3 expression, the indicator of FoxP3 Tregs suppressor function, was investigated by measuring FoxP3 relative fluorescence intensity (RFI). We found that controls had significantly higher values than patients. This means that although FoxP3 Tregs count was higher in allergic patients, they did have lower FoxP3 RFI. This was in agreement with Xu *et al.,* (2007) who found the expression level of FoxP3 mRNA in the nasal mucosa and peripheral blood mononuclear cells of patients with allergic rhinitis to be lower than the control group. Similarly, Provoost *et al.,* (2009) reported lower FoxP3 expression in asthma patients compared to controls. However, this finding was discrepant with Stelmaszczyk-Emmel *et al.,* (2013) and Raedler *et al.,* (2015) who reported significantly higher FoxP3 expression in children with allergy than in healthy controls. Such discrepancy may be attributed to the difference in age group as it was found that infections in childhood can alter the nature of innate/adaptive immunity interaction through toll-like receptors, which have a direct and an indirect impact on Tregs (Stelmaszczyk-Emmel *et al.,* 2013). Comparing FoxP3 RFI before and 1 year after SIT, in clinically improved patients, a statistically significant increase in FoxP3 RFI has been found, which reflects an improvement in Tregs function. This finding is discrepant with Stelmaszczyk-Emmel *et al.,* (2015) who observed decrease in the median RFI in the studied patients after SIT.

The report of Thunberg *et al.,* (2010) that the cells obtained from peripheral blood are not always indicative for the target organ and may be influenced by environment differences might explain the divergent results of the studies involving FoxP3 Tregs. We had the

opportunity to test peripheral blood but some authors used different specimens such as sublingual epithelium, bronchoalveolar lavage fluid or nasal mucosa. The former authors demonstrated increased expression of FoxP3 in bronchoalveolar lavage after allergen provocation without any respective alterations in peripheral blood.

In this study and in discrepancy with Abdel Gawad *et al.,* (2012), FoxP3 Tregs count was positively correlated with age and respiratory allergy severity. Correlation with age may be explained by enhanced immunoregulatory mechanisms in response to repeated exposure to different environmental and infectious agents throughout the patient life. As regards FoxP3 expression, it was negatively correlated with eosinophils count, disease severity and FoxP3 Tregs %. Such inverse correlation between FoxP3 expression and Foxp3 Tregs count might be explained by the assumption that persistent airway hyper-responsiveness due to Tregs dysfunction may have urged the immune system to increase FoxP3 Tregs in a way to correct the immune dysregulation, however, this waits to be explained in more studies investigating the underlying genetic and epigenetic control of FoxP3 expression in these patients. In the present study, FoxP3 Tregs % and FoxP3 expression did not correlate with IgE, while discrepantly, Provoost *et al.,* (2009) and Stelmaszczyk-Emmel *et al.,* (2013) reported an inverse correlation between FoxP3 expression and FoxP3 Tregs %, and total serum IgE, respectively.

In conclusion, FoxP3 Tregs count is elevated in respiratory allergic patients but their FoxP3 expression and thus their function are reduced which explains the development of dysregulated airway hyper-responsiveness in these patients. Allergen specific immunotherapy (SIT) leads to bilateral improvement in FoxP3 cells, i.e., count and function, which might lead to control of airway inflammation and thus clinical improvement. In addition, FoxP3 Tregs count and their FoxP3 expression are inversely correlated in respiratory allergic patients. We recommend that FoxP3 Tregs should be taken into consideration and investigated as potential candidates for future therapies of respiratory allergies. Airway samples, that may be more representative of the respiratory airways, should be considered, as possible, in future studies for better understanding of local immune response and for better optimization and personalization of immunotherapy protocols.

Acknowledgement

Deep thanks to Dr. Maha Atfy, Clinical Pathology Department, Faculty of Medicine, Zagazig University for her significant contribution in the immunophenotyping step of this study through operating and processing the flowcytometer.

References

1. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, Rudensky A, Sakaguchi S, Shevach EM, Vignali DA, Ziegler SF. (2013). Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol. 14(4):307-8.

2. Abdel Gawad TA, Al Sharkawy AA, Mansour A, Yousef A. (2012). Study of Treg FOXP3 in childhood bronchial asthma in relation to corticosteroid therapy. Egypt J Pediatr Allergy Immunol. 10(1):39-43.

3. Ajduk J, Marinic I, Aberle N, Rabatic S, Gagro A. (2008). Effect of house dust mite immunotherapy on transforming growth factor beta1-producing T cells in asthmatic children. Ann Allergy Asthma Immunol. 100(4):314-22.

4. Bin Dhuban K, Piccirillo CA. (2014). Markers for human FOXP3+ regulatory T cells: Current status and implications for immune monitoring in human disease. International Trends in Immunity. 2(4):162-65.

5. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, Zuberbier T, Baena-Cagnani CE, Canonica GW, van Weel C, Agache I, Ait-Khaled N, Bachert C, Blaiss MS, Bonini S,

Boulet LP, Bousquet PJ, Camargos P, Carlsen KH, Chen Y, Custovic A, Dahl R, Demoly P, Douagui H, Durham SR, van Wijk RG, Kalayci O, Kaliner MA, Kim YY, Kowalski ML, Kuna P, Le LT, Lemiere C, Li J, Lockey RF, Mavale-Manuel S, Meltzer EO, Mohammad Y, Mullol J, Naclerio R, O'Hehir RE, Ohta K, Ouedraogo S, Palkonen S, Papadopoulos N, Passalacqua G, Pawankar R, Popov TA, Rabe KF, Rosado-Pinto J, Scadding GK, Simons FE, Toskala E, Valovirta E, van Cauwenberge P, Wang DY, Wickman M, Yawn BP, Yorgancioglu A, Yusuf OM, Zar H, Annesi-Maesano I, Bateman ED, Ben Kheder A, Boakye DA, Bouchard J, Burney P, Busse WW, Chan-Yeung M, Chavannes NH, Chuchalin A, Dolen WK, Emuzyte R, Grouse L, Humbert M, Jackson C, Johnston SL, Keith PK, Kemp JP, Klossek JM, Larenas-Linnemann D, Lipworth B, Malo JL, Marshall GD, Naspitz C, Nekam K, Niggemann B, Nizankowska-Mogilnicka E, Okamoto Y, Orru MP, Potter P, Price D, Stoloff SW, Vandenplas O, Viegi G, Williams D. (2008). Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA^{2}LEN and AllerGen). Allergy. 63 Suppl 86:8-160.

6. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, Ley TJ. (2007). Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. Immunity. 27(4):635-46.

7. Cottrez F, Hurst SD, Coffman RL, Groux H. (2000). T regulatory cells 1 inhibit a Th2-specific response in vivo. J Immunol. 165(9):4848-53.

8. Fontenot JD, Gavin MA, Rudensky AY. (2003). Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nat Immunol. 4(4):330-6.

9. Fujimura T, Yonekura S, Horiguchi S, Taniguchi Y, Saito A, Yasueda H, Inamine A, Nakayama T, Takemori T, Taniguchi M, Sakaguchi M, Okamoto Y. (2011). Increase of regulatory T cells and the ratio of specific IgE to total IgE are candidates for response monitoring or prognostic biomarkers in 2-year sublingual immunotherapy (SLIT) for Japanese cedar pollinosis. Clin Immunol. 139(1):65-74.

10. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention (2015). Available from: URL:http://ginasthma.org/. Last accessed on 04-05-2016. (Archived by WebCite® at http://www.webcitation.org/6hFe1FqGf).

11. Huang X, Li P, Yang Q, Chen Y, Zhang G. (2013). The expression of IL-27, Th17 Cells and Treg cells in peripheral blood of patients with allergic rhinitis. International Journal of Otolaryng and Head & Neck Surgery. 2(4):138-42.

12. Indrajana T, Spieksma FT, Voorhorst R. (1971). Comparative study of the intracutaneous, scratch and prick tests in allergy. Ann Allergy. 29(12):639-50.

13. Kinoshita T, Baatjes A, Smith SG, Dua B, Watson R, Kawayama T, Larche M, Gauvreau GM, O'Byrne PM. (2014). Natural regulatory T cells in isolated early responders compared with dual responders with allergic asthma. J Allergy Clin Immunol. 133(3):696-703.

14. Komatsu N, Hori S. (2007). Full restoration of peripheral $Foxp3^+$ regulatory T cell pool by radioresistant host cells in scurfy bone marrow chimeras. Proc Natl Acad Sci U S A. 104(21):8959-64.

15. Lin YL, Shieh CC, Wang JY. (2008). The functional insufficiency of human $CD4^+CD25$ high T-regulatory cells in allergic asthma is subjected to TNF-alpha modulation. Allergy. 63(1):67-74.

16. Loebbermann J, Thornton H, Durant L, Sparwasser T, Webster KE, Sprent J, Culley FJ, Johansson C, Openshaw PJ. (2012). Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. Mucosal Immunol. 5(2):161-72.

17. Lou W, Wang C, Wang Y, Han D, Zhang L. (2012). Responses of $CD4^{(+)}$ $CD25^{(+)}$ $Foxp3^{(+)}$ and IL-10-secreting type I T regulatory cells to cluster-specific immunotherapy for allergic rhinitis in children. Pediatr Allergy Immunol. 23(2):140-9.

18. Martinez FD. (2008). Trends in asthma prevalence, admission rates, and asthma deaths. Respir Care. 53(5):561-5; discussion 565-7.

19. Mobs C, Slotosch C, Loffler H, Jakob T, Hertl M, Pfutzner W. (2010). Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. J Immunol. 184(4):2194-203.

20. Papadopoulos NG, Borres M, Gern J, Nieto A. (2008). New visions in respiratory allergy (asthma and allergic rhinitis): An iPAC summary and future trend. Pediatr Allergy Immunol. 19 Suppl 19:51-9.

21. Passalacqua G, Ciprandi G, Canonica GW. (2001). The nose-lung interaction in allergic rhinitis and

asthma: united airways disease. Curr Opin Allergy Clin Immunol. 1(1):7-13.

22. Pellerin L, Jenks JA, Begin P, Bacchetta R, Nadeau KC. (2014). Regulatory T cells and their roles in immune dysregulation and allergy. Immunol Res. 58(2-3):358-68.
23. Provoost S, Maes T, van Durme YM, Gevaert P, Bachert C, Schmidt-Weber CB, Brusselle GG, Joos GF, Tournoy KG. (2009). Decreased FOXP3 protein expression in patients with asthma. Allergy. 64(10):1539-46.
24. Raedler D, Ballenberger N, Klucker E, Bock A, Otto R, Prazeres da Costa O, Holst O, Illig T, Buch T, von Mutius E, Schaub B. (2015). Identification of novel immune phenotypes for allergic and nonallergic childhood asthma. J Allergy Clin Immunol. 135(1):81-91.
25. Ren X, Ye F, Jiang Z, Chu Y, Xiong S, Wang Y. (2007). Involvement of cellular death in TRAIL/DR5-dependent suppression induced by $CD4^{(+)}$ $CD25^{(+)}$ regulatory T cells. Cell Death Differ. 14(12):2076-84.
26. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 155(3):1151-64.
27. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. (2010). $FOXP3^{+}$ regulatory T cells in the human immune system. Nat Rev Immunol. 10(7):490-500.
28. Sansom DM, Walker LS. (2006). The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. Immunol Rev. 212:131-48.
29. Sorensen AE, Johnsen CR, Dalgaard LT, Wurtzen PA, Kristensen B, Larsen MH, Ullum H, Soes-Petersen U, Hviid TV. (2013). Human leukocyte antigen-G and regulatory T cells during specific immunotherapy for pollen allergy. Int Arch Allergy Immunol. 162(3):237-52.
30. Stelmaszczyk-Emmel A, Jackowska T, Rutkowska-Sak L, Marusak-Banacka M, Wasik M. (2012). Identification, frequency, activation and function of CD4+ CD25(high)FoxP3+ regulatory T cells in children with juvenile idiopathic arthritis. Rheumatol Int. 32(5):1147-54.
31. Stelmaszczyk-Emmel A, Zawadzka-Krajewska A, Szypowska A, Kulus M, Demkow U. (2013). Frequency and activation of $CD4^{+}CD25$ $FoxP3^{+}$ regulatory T cells in peripheral blood from children with atopic allergy. Int Arch Allergy Immunol. 162(1):16-24.
32. Stelmaszczyk-Emmel A, Zawadzka-Krajewska A, Glodkowska-Mrowka E, Demkow U. (2015). FoxP3 Tregs response to sublingual allergen specific immunotherapy in children depends on the manifestation of allergy. J Immunol Res. 2015:731381.
33. Suarez-Fueyo A, Ramos T, Galan A, Jimeno L, Wurtzen PA, Marin A, de Frutos C, Blanco C, Carrera AC, Barber D, Varona R. (2014). Grass tablet sublingual immunotherapy downregulates the TH2 cytokine response followed by regulatory T-cell generation. J Allergy Clin Immunol. 133(1):130-8.
34. Susanti N, Barlianto W, Kalim H, Kusuma HC. (2013). Asthma clinical Improvement and reduction in the number of $CD4^{+}CD25^{+}foxp3^{+}$Treg and $CD4^{+}IL\text{-}10^{+}$ cells after administration of immunotherapy house dust mite and adjuvant probiotics and/ or Nigella Sativa powder in mild asthmatic children. IOSR J Dental and Medical Sci. 7(3):50-9.
35. Thunberg S, Gafvelin G, Nord M, Gronneberg R, Grunewald J, Eklund A, van Hage M. (2010). Allergen provocation increases TH2-cytokines and FOXP3 expression in the asthmatic lung. Allergy. 65(3):311-8.
36. Tsay YG, Beigler MA, Calenoff E, Friesen GL, Nichols JL. (1987). Stable allergenic extracts and methods. Google Patents.
37. Vignali DA, Collison LW, Workman CJ. (2008). How regulatory T cells work. Nat Rev Immunol. 8(7):523-32.
38. Wei W, Liu Y, Wang Y, Zhao Y, He J, Li X, Shen K. (2010). Induction of CD4+CD25+Foxp3+IL-10+ T cells in HDM-allergic asthmatic children with or without SIT. Int Arch Allergy Immunol. 153(1):19-26.
39. World Allergy Organization (WAO) (2016). Available from: http://www.worldallergy.org/public/allergic_diseases_center/caras/. Last accessed: 08-05-2016. (Archived by WebCite® at http://www.webcitation. org/6hMYlEfK2).
40. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler

SF, Mathis D, Benoist C, Chen L, Rao A. (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell. 126(2):375-87.

41. Xu G, Mou Z, Jiang H, Cheng L, Shi J, Xu R, Oh Y, Li H. (2007). A possible role of $CD4^{+}CD25^{+}$ T cells as well as transcription factor Foxp3 in the dysregulation of allergic rhinitis. Laryngoscope. 117(5):876-80.

42. Zhang H, Kong H, Zeng X, Guo L, Sun X, He S. (2014). Subsets of regulatory T cells and their roles in allergy. J Transl Med. 12:125.