

Original article

Evaluation of regulatory T cells in obese asthmatic children

Background: Asthma is an airway inflammatory disorder with functional and structural changes. Regulatory T (T reg) cells are important in controlling immune responses. The study was aimed to investigate the frequency of Treg cells in obese asthmatic children, in comparison to non-obese asthmatics and healthy matched controls.

Methods: In addition to anthropometric and body mass index (BMI) assessment, peripheral blood samples from healthy control subjects (n = 30) and asthmatic obese (n=30) and asthmatic non-obese children (n=30) were examined for serum IgE, eosinophils counts, and flowcytometric measurement of CD4+CD25+CD127 low/neg- T cells. Pulmonary function testing was performed for asthmatic children.

Results: Obese asthmatics showed significantly higher levels of serum IgE and CD4+CD25+CD127 low/neg- T cells as compared to healthy controls ($p < 0.001$, 0.001 , respectively) while comparable numbers of T reg cells were found among obese and non-obese asthmatic children. Asthmatics receiving inhaled corticosteroids (ICS) showed higher percentages of CD4+CD25+CD127 low/neg- T cells than the non-receivers (median 11.8% vs 8.8%, $p < 0.001$). No significant correlations were found between Treg and age, eosinophil percentage, total serum IgE, pulmonary functions, or BMI and its Z score.

Conclusion: Our study demonstrates an increased frequency of Treg cells in asthmatic children compared to controls with possible association with the use of ICS but not with obesity. Small sample size and lack of obese non-asthmatic group are the main points of limitation in our study.

Keywords: asthma, natural regulatory T cells, FOXP3, obesity

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INTRODUCTION

Asthma is a heterogeneous disease characterized by chronic airway inflammation. It is identified by history of respiratory symptoms such as wheezes, shortness of breath, chest tightness and cough that differ over time and in intensity, together with variable expiratory airflow limitation.¹ Many trials have been done to classify asthmatics into different phenotypes that might predict disease sequence and treatment response.²

Obesity among children has been increased significantly in the last few years. It is defined as a body mass index (BMI) at or above the 95th percentile for children of the same age and gender.³ In recent years, the incidences of obesity and asthma have been rising with a parallel relationship. Obese asthma phenotype is supposed to be non-T helper type 2 (Th2) mediated in which low fractional exhaled nitric oxide, eosinophils and IgE levels were detected.⁴

In allergic diseases, the current concept suggests that inadequate Treg cell suppression of effector cells or diminished Treg cell numbers contributes to the development of allergic inflammation. The mechanisms underlying the association between obesity and asthma might be related to a decreased immunological tolerance induced by a defective function of Tregs.⁵

The frequency and significance of Treg cells in allergic obese and non-obese asthmatics remains poorly defined, especially in children where the initiation of allergic asthma most often occurs. Previously published studies analyzing human Treg cells have utilized several phenotypic markers including CD25, CD127, and Foxp3.⁶

In this study, we used multi-parameter flow cytometry to investigate the frequency of Treg cells (CD4⁺ CD25⁺ CD127 low/neg- T cells) in the peripheral blood of obese and non-obese asthmatic children, compared to healthy matched controls. The frequency of Treg cells was further analyzed in relationship to asthma severity, control and medication use.

METHODS

Patient samples

This is a controlled cross-sectional study involving 60 asthmatic cases (30 obese and 30 non-obese) and 30 healthy age and gender matched controls, with age ranging between 6 to 18 years. They were recruited from the Outpatient Clinic of Allergy, Respiratory and Clinical immunology unit of Mansoura University Children's Hospital (MUCH) during the period between February 2016 and February 2018. Bronchial asthma was diagnosed according to guidelines of Global Initiative for Asthma Management and Prevention (GINA 2016).⁷ Obesity was defined as having BMI at or above the 95th percentile for children of the same age and gender.³ Asthmatic patients with comorbidities such as chronic cardiopulmonary disease, concurrent pneumonia or nasal polyps were excluded from the study. Also, patients on allergen immunotherapy protocols or those receiving systemic steroids were excluded from the study due to possible effects on the T reg cells population.⁸ All patients' samples were obtained in accordance with the Declaration of Helsinki, with informed consent from parents/ caregivers of enrolled cases. The study was approved from Faculty of Medicine-Mansoura University Institutional Review Board (IRB) with the code number MS/16.01.45.

Methods

Enrolled subjects were subjected to the following clinical and laboratory evaluation:

Detailed clinical history taking and general and systematic examination. Patient's data included age, sex, residence, parental consanguinity, family history of bronchial asthma, associated allergic rhinitis, atopic dermatitis, allergic march, exposure to trigger, nutritional history and dietetic history. Asthma severity and control were assessed according to GINA, 2016.⁷

Physical examination included measurement of height and weight for calculation of body mass index (BMI = weight (Kg)/ height (m)²). Age- and sex-specific Z-score for BMI [standard deviation scores (SDS)] using Egyptian children and adolescents' reference data are calculated by the following equation:⁹

$$Z\text{-score} = (\text{observed value}) - (\text{median reference value of a population}) / \text{SD of reference population}$$

Pulmonary function tests (PFTs):

The top of three successive measurements of forced expiratory volume 1 (FEV1) was measured by a spirometer Spiro bank II (Roma, Italy). Reference standards were computed according to American Thoracic Society (ATS) of Acceptability and Reproducibility.¹⁰

Blood Collection and laboratory investigations

Peripheral blood (PB) samples were collected from patients and healthy controls and used as follows: Blood samples collected in plain tubes were used for measuring total serum IgE in serum by immunoassay techniques, by using IgE ELISA KITS from Aptech Services, following the manufacturer's instructions.

Blood samples collected in Ethylene diamine tetra acetic acid (EDTA) tube were used for measuring complete blood counts, automatically performed by Coulter LH 750 cell counter (Coulter, Electronics, Hialeah, FL, USA) then peripheral blood smears were stained with Leishman stain for manual differential white cell counts with special emphasis on eosinophils relative counts (EOS %).

Flow Cytometry, Antibodies, and Staining

Fresh EDTA samples were used also for evaluation of Treg cells (CD4⁺ CD25⁺ CD127 low/- T cells). Cells were stained with appropriate monoclonal antibodies (anti CD 4 phycoerythrin (PE), anti-CD25 PE-CY7-A and anti-CD127 fluorescein isothiocyanate (FITC) (Beckman Coulter, Kraemer Blvd. Brea, CA 92821 U.S.A.). They were used at

concentration recommended by their manufacturers. Cells were acquired on FACS CantoII (BD Biosciences). Data were analyzed using FACS DIVA software. A gate was drawn around the population of interest: CD4+ cells. The gate on double positive CD4+ CD25+ cells was set. Analysis of the fluorescence data from events in this gate was then performed to determine the CD127 low/neg-.

Statistical methods: SPSS software package version 22.0 was used to analyze the data. Qualitative data were represented as number and percent. Quantitative data were represented as range (minimum and maximum), mean, standard deviation and median. The used tests were paired t-test, Chi-square test, Kruskal-Wallis test, One Way ANOVA, and Mann Whitney test to match the groups. Statistical significance was identified as $p < 0.05$.

RESULTS

Studied groups

The Mean age was 9.87 ± 2.52 , 9.63 ± 2.89 and 9.87 ± 0.73 years and male represented 66.7 %, 70 % and 60 % among asthmatic obese, asthmatic non-obese and healthy control groups, respectively, with comparable results between the three groups ($F=0.079$, $p=0.924$; $\chi^2=2.34$, $p=0.31$).

Characteristics of asthma among obese and non-obese patients.

The mean duration of asthma was 7.7 ± 1.97 and 6.2 ± 1.56 years among the obese and non-obese asthmatics, respectively ($t=3.27$, $p=0.002$), with longer asthma duration among the obese group despite comparable ages. No significant difference was observed between both groups as regard the frequency of associated allergic rhinitis and atopic dermatitis ($\chi^2=1.09$, 0.88 and $p=0.29$, 0.35, respectively). Moderate persistent asthma was detected in 63.3% of asthmatic obese group and in 30% in asthmatic non-obese group ($\chi^2=6.69$, $p=0.01$). None of the participants had severe asthma. Controlled asthma comprised 60% of the obese cases versus 83.3% of the non-obese asthmatics

($\chi^2=4.02$, $p=0.04$). ICS represented the commonest controller medication for persistent asthma among our patients, used in 73.4% among obese and 53.3% among the non-obese asthmatics.

Comparison between obese and non-obese asthmatics in terms of their clinical characteristics, asthma severity, asthma control and controller medications are shown in table 1.

Laboratory findings among the studied groups:

Total serum IgE levels were significantly higher in both asthmatic obese and non-obese groups compared to healthy non-obese control group ($p < 0.001$), while eosinophil relative counts were comparable among the three studied groups ($F=1.07$, $p=0.34$) (table 2).

Peripheral blood (PB) Treg cell frequency among the studied groups

In our study, analysis of PB revealed a significantly higher percentage of CD4+CD25+CD127 low/neg- T cells, in both asthmatic obese and non-obese groups compared to healthy controls ($p < 0.001$). No significant difference was noted in CD4+CD25+CD127 low/neg- T cells percentages between obese and non-obese asthmatics ($p=0.75$) (Table 2).

The correlation between Treg and the clinical and laboratory data of asthmatic children

Our analysis revealed significant positive correlation between Treg and degree of asthma severity and asthma uncontrol ($r=0.418$, 0.292, $p=0.001$, 0.02, respectively). No significant correlation was found between Treg and age, eosinophil counts, total serum IgE levels, pulmonary functions parameters, BMI, and its Z score (table 3).

The relation between T reg cells and ICS treatment

T reg percentages were significantly higher ($Z=10.98$, $p < 0.001$) in patients on ICS treatment [median (range): 11.8 (5.3-18.2)] versus those who were not [median (range): 8.85 (5.4-14.3)].

Table 1. Clinical characteristics of the studied obese and non-obese asthmatic children

Variables	Asthmatic Obese group (n=30)		Asthmatic Non-obese group (n=30)		p-value
Duration of illness (years)	7.70±1.97		6.2±1.56		t=3.27
Mean ± SD					p=0.002
	N	%	N	%	
Degree of asthma severity					
Mild Persistent	11	36.7	21	70	$\chi^2=6.69$ p=0.01
Moderate persistent	19	63.3	9	30	
Associated atopic dermatitis					
Positive	5	16.7	8	26.7	$\chi^2=0.88$ p=0.35
Negative	25	83.3	22	73.3	
Associated allergic rhinitis					
Positive	19	63.3	15	50	$\chi^2=1.09$ p=0.29
Negative	11	36.7	15	50	
History of controller medications					
ICS	5	16.7	1	3.3	$\chi^2=4.43$ p=0.11
ICS & leukotriene modifier	17	56.7	15	50	
Leukotriene modifier	8	26.7	14	46.7	
Level of asthma control					
Controlled	18	60	25	83.3	$\chi^2=4.02$ p=0.04
Partially controlled	12	40	5	16.7	

ICS: inhaled corticosteroids; N: number of subjects; SD: standard deviation; t: student t – test; χ^2 : Chi square test

Table 2. Laboratory profile among the studied groups (asthmatic obese, asthmatic non-obese and non-obese healthy controls)

Variables	Asthmatic Obese group (n=30)	Asthmatic Non-obese group (n=30)	Healthy Control group (n=30)	p-value
eosinophil %	6.6 (4.3-9.6)	6.4 (4.2-9.2)	6.15 (4.4-9.2)	F=1.07 p=0.34
Total IgE (IU/ml)	162 (71.0-547.0)	233 (77.0-570.0)	82 (65.0-120.0)	F=18.95 p<0.001 p2<0.001 p3<0.001
CD4 %	32.2 (2.5-43.3)	33.95 (10.30-47.7)	31.5 (3.7-46.0)	F=0.77 p=0.35
CD4 +CD25 %	4.9 (1-9.4)	4.95 (1.9-12.5)	4.15 (0.5-10.5)	F=2.38 p= 0.06 p3=0.02
CD4+CD25+ CD127 low/neg-T cells %	10.8 (5.3-18.2)	10.4 (5.4-15.8)	8.1 (4.2-13.1)	F=10.98 p<0.001 p2=0.001 p3=0.001

Data is presented as median and range.

BMI: body mass index; F: One Way ANOVA; IgE: Immunoglobulin E; KW: Kruskal-Wallis test. n: number of subjects; SD: Standard Deviation

p1: difference between asthmatic obese and asthmatic non-obese,

p2: difference between asthmatic obese and healthy control,

p3: difference between asthmatic non-obese asthmatics and healthy controls

Table 3. Correlation between Treg and clinical and laboratory parameters in the studied asthmatic patients.

	age	FEV ₁ (L/s)	FVC (L)	FEV ₁ /FVC Ratio	Eosinophil %	IgE IU/mL	BMI Z score	Asthma severity	Asthma uncontrol	
T reg %	r	-0.097	-0.064	-0.055	0.027	0.181	0.146	0.029	0.418	0.292
	p	0.46	0.62	0.67	0.83	0.17	0.26	0.82	0.001	0.024

BMI: body mass index; FEV₁: forced expiratory volume in first second; FVC: forced vital capacity; IgE: Immunoglobulin E; r=Spearman's rank correlation; L: liter; Ls: Liter per second; p=probability

DISCUSSION

Asthma is one of the main reasons of chronic morbidity and mortality in the world. There is evidence that asthma prevalence has increased in the last 20 years.¹¹ In Egypt, prevalence of childhood was found to be 7.7% in the Delta region.¹² Obesity and asthma are the most common chronic morbidities in children in the developing and developed regions.¹³

The current study was conducted to evaluate T regulatory cells among obese asthmatic children in relation to their clinical characteristics including pulmonary functions, controller medications, inflammatory biomarkers (total serum IgE, peripheral eosinophil percentages) and in comparison to non-obese asthmatic and healthy matched controls.

In the current study, asthmatic children, whether obese or non-obese, had higher percentages of T reg cells CD4+CD25+CD127 low/neg- T cells compared to the healthy controls. T regulatory cells and its secreted anti-inflammatory cytokine IL-10 are essential to control the abnormally Th2 driven immune response in asthma, aiming to maintain the immune response within the normal range.¹⁴ Studies investigating the levels of these cells in asthmatic children have contradictory results. Concordant with our results, Smyth et al reported increased T reg cell counts in the bronchoalveolar lavage fluid (BALF) of asthmatic patients in comparison to healthy controls, with higher T reg cell counts among patients with moderate to severe versus those with mild asthma.¹⁵ Worth to note that in our study, Tregs correlated positively with asthma severity and the degree of asthma uncontrol. Some relevant studies reported similar findings.¹⁶⁻¹⁸

On the other hand, Hartl et al, reported lower levels of T reg cells together with their impaired function in the BALF of asthmatic children compared with values in matched controls, abnormalities that were reversed with the use of ICS.¹⁹ Also, Yang et al who investigated 150 asthmatic children, noticed significantly lower Treg cell counts with significantly lower Th1/Th2 ratio in the asthma group. The authors also reported negative correlation between the Treg cell counts and the severity of asthma, suggesting important role of Treg in pathogenesis of asthma.²⁰ Their findings were also reported by other investigators.²¹⁻²³

Different results between our study and the others might be attributed to several factors including different study designs and methods of evaluation of T reg cells whether using CD4+CD25+ only, CD4+ CD25+ FOXP3+ or

CD4+CD25+CD127 low/neg-Tcells. Also, we have no data about the baseline levels of T reg cells among the studied patients and demonstrating the changes in these levels with disease exacerbation might have provided clearer conclusions. We have no information about the patients' vitamin D status at enrollment and our patients were not steroid naive as well, and all of them were on controller medications at enrolment, and their dosing were not considered in the statistical analysis. All these factors in addition to the different sample sizes might explain the different study results.

In our patients, T reg cells percentages were significantly higher among patients on ICS versus those who were not. Singh et al, showed increasing Treg cell counts in asthmatic children that was related to ICS, and not severity of asthma, allergic sensitization, or the presence of atopy.⁶ Interestingly, an Egyptian study reported significantly increased expression of FOXP3 in asthmatic children on corticosteroids (either inhaled or systemic) in comparison to steroid naive asthmatics, confirming the promoting effect of corticosteroids on suppressive T reg cells, adding to the ICS anti-inflammatory effect.²⁴ In contrast to our study, Moniuszko et al, showed no significant difference in Treg cells between asthmatic patients with inhaled corticosteroids compared to steroid-naive patients groups.²⁵

The role of corticosteroids was postulated to shift the balance of adaptive immune responses toward Treg cell predominance.⁶ Also, it was reported that in vitro glucocorticoids induced FOXP3 expression in CD4+T cells. Karagiannidis et al reported that asthmatic patients receiving glucocorticoids had higher levels of Tregs and FOXP3 mRNA expression in peripheral blood compared with levels seen in untreated asthmatic patients.²⁶

Obesity increases the incidence of asthma by 2.0 folds in children and obese asthmatic patients are often described as severe and poorly controlled. Several factors are implicated including physiological and anatomical factors, decreased response to corticosteroids and the association of obesity with chronic low-grade systemic inflammation that is thought to enhance systemic complications. The association between obesity and asthma severity was also proposed to be related to decreased immunological tolerance induced by a defective function of Tregs in obese individuals. Improper clearance of apoptotic cells might contribute to development and progression of chronic inflammatory diseases such as asthma.²⁷ In our study, the obese asthmatic group showed higher

frequencies of moderate and partially uncontrolled asthma in comparison to the non-obese asthmatics. However, we could not find a significant difference between obese and non-obese asthmatic children in terms of the T reg cells percentages. There is some evidence on the anti-inflammatory role exerted by Treg cells in obesity via release of IL-10 aiming to suppress obesity-induced inflammation. On the other hand, high insulin levels in obesity has been suggested to exert an inflammatory role by decreasing IL-10 production and impairing Treg cell-induced suppression, owing to insulin receptors on T reg cells.²⁸ Treg cells are decreased in both obese mice and humans.²⁹ Furthermore, Adiponectin reduction has been observed in obese asthmatic children which is thought to be responsible for a diminished release of IL-10 by Treg cells, impairing the function of these cells and thus worsening the pro-inflammatory status.³⁰ In our work, asthmatic patients were on ICS and we did not measure IL-10 production or other anti- and pro inflammatory cytokines, which comprise limiting factors to our results and might have masked possible T reg cell abnormalities expected to be observed in obese subjects.

In contrast to our results, a study performed on 160 children comparing (40 obese, 40 asthmatic, 40 obese asthmatic, and 40 healthy controls) showed lower frequencies of Treg cells in obese, asthmatic and obese asthmatic children than normal controls.³¹ This is while, in another study that investigated asthmatic patients (12 obese and 10 non obese), the authors could not demonstrate a significant difference in Tregs counts between obese and control groups.³²

We did not find significant associations between IgE levels and eosinophil percentage and Treg cells, results that are matching to those reported by Hartl et al.¹⁹ We did not perform skin prick test to verify the atopic status of our studied patients, which is another limiting factor to our results.

In conclusion, our study demonstrates increased frequency of Treg cells in the peripheral blood of asthmatic obese and non-obese children compared to healthy controls. T reg cells percentages are also associated with the degree of asthma severity, and uncontrol with possible impact of ICS treatment on T reg cells. Added to the aforementioned factors, our findings are limited by small sample size, lack of obese non-asthmatic group, lack of baseline data and atopy verification, broad age range of enrolled subjects, non-consideration of the lifestyle, type of diet, exercising, muscle mass and vitamin D status; factors that warrant wider scale longitudinal studies

with assessment of anti- and pro-inflammatory cytokines production.

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