Original article

Study of Treg FOXP3 in childhood bronchial asthma in relation to corticosteroid therapy

Background: *T cells are considered the main cells responsible for production of suppressive cytokines, and play a key role in balancing the immune responses to maintain the peripheral tolerance against allergens.*
Objective: *The present study investigates T regulatory (Treg) forkhead-winged helix protein 3 FOXP3 expression in childhood asthma and its relation to corticosteroid therapy.*
Methods: *In this case control study, Treg $FOXP_3$ was measured in blood of 60 children using real time polymerase chain reaction (RT-PCR) technique. Two asthmatic groups were included, one on corticosteroid therapy (20 patients) and the other not on corticosteroid treatment (20 patients). They were compared to 20 healthy children as controls.*
Results: *$FOXP_3$ concentration was significantly elevated in asthmatic patients (90 ± 77.4) compared to healthy children (12.844 ± 10.6) (p= 0.000). FOXP3 was significantly more elevated in asthmatics on corticosteroids (161.158 ± 63.9) than steroid naive asthmatics (36.038 ± 23.4) (p=0.000). Levels of Treg FOXP3 in asthmatics with inhaled corticosteroids (mean 151.16 ± 53.79) were almost similar to FOXP3 in asthmatics with systemic corticosteroids (161.49±72.5) (p>0.05). FOXP3 levels did not differ with smoking, asthma severity or disease control and did not correlate with age, FEV1, blood lymphocytes percentage or eosinophils percentage.*
Conclusion: *Asthmatics have increased expression of $FOXP_3$, and corticosteroid therapy –whether oral or inhaled - enhances FOXP3 expression.*

Key words: FOXP3, Treg, Corticosteroids, Bronchial asthma, Transcription factors, Cytokines.

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INTRODUCTION

Asthma is a chronic inflammatory disease of the lung[1], hypothetically caused by autoreactive Th2 cells, whereas Th1 and regulatory T cells may be protective[2]. The role of specific T cells and their cytokines in allergic asthma is now well known[3]. Allergic asthma starts with influx of naive CD4+ T cells and eosinophils into the bronchial mucosa. The priming of the naïve CD4+ T cells to differentiate into proinflammatory Th2 cells - instead of the infection-fighting Th1 cells - by allergen activated dendritic cells (DCs) is an important proposed mechanism in occurrence of asthma[4]. Tregs have been implicated in playing a key role as immune negative regulators and key inducers of immune tolerance to inhaled allergens[5].

The development of Th subpopulations is dependent on the expression of Treg transcription factors[2]. Foxp3 is transcription factor (Foxp3) predominantly expressed by CD4+ CD25+ Treg cells, and may correlates with the suppressive activity of these cells[6]. The discovery of FOXP3 as a specific marker of Treg-cell[7] has recently led to an explosion of research in the biological properties of these T-cells which may open novel therapeutic strategies aimed at the induction of FOXP3 to convert conventional CD4+ T-cell to reverse aberrant Th2-mediated allergic asthmatic response[8]. So, this study aimed at investigating Treg FOXP3 expression in childhood asthma and its relation to corticosteroid therapy.

METHODS

This case control study was conducted on total 60 Egyptian children; 40 asthmatic children who attended Chest Clinic, Children's Hospital, Ain Shams University from October 2010 to February 2011 and 20 normal children (control subjects). Informed written consents were taken from all participants' parents. The study was approved by the local Ethics Committees. The initial diagnosis and grading of bronchial asthma was made in the presence of typical asthma manifestations, and presence of reversible obstructive pattern on spirometry[9] as recorded in the patient's file. Detailed medical history including asthma symptoms, details of corticosteroid treatment were obtained from patient's recording files. Chest examination (chest deformity, chest movement, air entry, wheezes, and additional sounds) was done to exclude the possibility of other causes of wheezy chest like cystic fibrosis, interstitial pulmonary disease, and others. Complete blood picture with differential count was done using cell counter (Coulter T660; Miami, USA).

Spirometry was performed using MIR spirobank, which is a multifunction pocket spirometer that can operate as a stand-alone pocket spirometer or connected with a personal computer. Parameters obtained from such spirobank included; forced vital capacity (FVC), forced expiratory volume in the 1st second (FEV1), and FEV1% (FEV1/FVC X 100). Data were corrected to patient's age, sex, weight and height. Obtained values of FEV1 and FEV1% were used to correlate the current FEV1% with blood levels of FOXP3 and to judge level of asthma control[9].

Asthmatics were allocated into 2 groups. **Group A**: included 20 asthmatic children (8 males and 12 females) with mean age 7.3±3.02 years (age range; 5 - 13 years) receiving regular corticosteroid (CS) inhalation therapy. **Group B**: included 20 children (15 males and 5 females) with mean age 6.25±1.77 years (age range 5 - 11) not on CS treatment. In addition, **Group C** included 20 matched healthy children (11 males and 9 females) with a mean age of 7.00±1.76 years (age range 6 - 11) enrolled as a control group.

Exclusion criteria included; children with any other chronic chest disease (cystic fibrosis, interstitial lung disease, congenital or developmental lung malformations) and children below 5 years (unable to perform spirometry).

Assessment of FOXP3 concentration

Samples: Lymphocytes were separated from heparinized blood samples and were processed and analyzed by multiplex RT-PCR analysis for semi-quantitative PCR analysis of FOXp3 gene expression, relative β actin gene expression. Total RNA was extracted according to TriFast™ Total RNA Purification Kits, based on a modified salt precipitation procedure in combination with highly effective inhibitors of RNase activity (PeQLab Biotechnologie GmbH Corporation, Erlangen, Germany). Semi-quantitative Multiplex RT-PCR was done on 2µg RNA from each sample using FOXp3 and β actin Primers. RNA was converted to cDNA using QIAGEN One Step RT-PCR Kit (QIAGEN, USA), Using FOXp3 primers 25 pm/ul : Sense primer: 5'-CCC ACT TAC AGG CAC TCC TC-3', and antisense primer 5'-CTT CTC CTT CTC CAG CAC CA-3'[10]. Housekeeping β actin primers 3 pm/ul: The sequences of these primers were chosen according to Smith[11]**.** First step of RT was at 60˚ C for 60 minutes, PCR activation was at 95˚ C for 15 minutes, then repeated 40 cycles of; denaturation at 95˚ C for 1 minute, annealing at 50˚ C for 1 minutes, extension at 72˚ C for 1 minutes and final extension at 72˚ C for 10 minutes. β actin products bands were used as reference bands for the semi-quantization of the FOXp3 RNA bands[12]**.** FOXP3 concentration was expressed as percentage of housekeeping.

Statistical Analysis

The data were expressed as mean ± standard deviation. Two-tailed unpaired t test was used to test the difference between different groups, where $p<0.05$ was considered statistically significant. All statistical analysis was performed with the software package SPSS for Windows, version 15.0 (SPSS Inc., Chicago,, Illinois).

RESULTS

Among group A (patients), family history of atopy was positive in 55% and 60% of these patients were passive smokers. According to GINA guidelines[9]; 25% of them had mild asthma, 25% had moderate asthma and 50% had severe asthma; the mean value of FEV1% was 79.75 ±5.03; 45% were well controlled and had FEV 1 > 80%. The mean values for blood eosinophil and lymphocyte percentage of the total leucocytic count were 5.65±2.99 and 41.9±14.8 respectively.

Among group B, family history of atopy was positive in 45%; 75% of these patients were passive smokers. All had mild asthma, the mean value of FEV1% was 83.65 ±3.71; 90% were well controlled and had FEV 1 > 80%. The mean values for blood eosinophil and lymphocyte percentage of the total leucocytic count were 6.15±3.11 and 32.52±12.55

respectively. Treg FOXP3 concentration was significantly higher in asthmatic patients (90 ± 77.4) compared to healthy controls (12.844 ± 10.6) (p<0.000) (table 2). Treg FOXP3 concentration in steroid naïve asthmatics (36.038 ± 23.4) was significantly higher compared to controls (12.844 ± 10.6) (p= 0.007) and it was more elevated in asthmatics on regular C.S (161.158 ± 63.9) (p<0.000) (table 3). Route of corticosteroid intake (whether inhaled or systemic) did not affect the concentrations of FOXP3 significantly. FOXP3 with inhaled corticosteroids (mean 151.16±53.79) was almost similar to FOXP3 with systemic corticosteroids (161.49±72.5) (p>0.05) (table 4). FOXP3 levels did not differ with passive smoking, disease severity, or disease control (p>0.05). FOXP3 was not correlated to age, FEV1 %, blood eosinophil or blood lymphocyte ratios (p>0.05).

Table 1. Clinical and laboratory data of enrolled subjects.

	Group A (n= 20)	**Group B (n= 20)**	**Group C (n= 20)**
Age (mean +SD)	7.3±3.02	6.55±1.57	7.00±1.76
Gender			
Male	8 (40%)	15 (75%)	6 (30%)
Female	12 (60%)	5 (25%)	14(70%)
Family history of asthma	10 (50%)	11 (55%)	-
Parental smoking	12 (60%)	15 (75%)	2 (10%)
Asthma grade			
Mild	5 (25%)	20 (100%)	-
Moderate	5 (25%)	-	-
Severe	10 (50%)	-	-
Asthma control			
Well controlled	9 (45%)	18 (90%)	-
Partly controlled	11 (55%)	2 (10%)	-
FOXP3 concentration%	161.158 ± 63.9	36.038 ± 23.4	12.844 ± 10.6
FEV1%	79.75 +5.03	83.65 +3.71	-
Lymphocytes %	41.9 + 14.8	32.52+12.55	-
Eosinophils %	5.65+2.99	6.15+3.11	-

Table 2. FOXP3 concentration % among asthmatics (Group A+Group B) versus control (Group C).

FOXP3 %	**Mean ± SD**	**Median**	**Range**	**P**
Group A+Group B (n = 40)	90 ± 77.4	34.5	12.2 - 229	0.000
Group C (n = 20)	12.844 + 10.6	32.6087	4.57-38.51	

SD= standard deviation

Table 3. FOXP3 concentration % among Group A, B and Group C.

FOXP3%	**Group A (n = 20)**	**Group B (n = 20)**	**Group C (n = 20)**	**P value A vs B**	**P value A vs C**	**P value B vs C**
Mean ±SD	161.158 ± 63.9	36.038 ± 23.4	12.844 ± 10.6	0.000 (HS)	0.000 (HS)	0.007 (S)

SD= standard deviation

Table 4. FOXP3 concentration % among Group A regarding route of administration of corticosteroid treatment.

FOXP3%	Patients on inhaled C.S (n =15)	Patients on inhaled & systemic C.S (n=5)	P Value
Mean±SD	151.16 ± 53.79	161.49 ± 72.5	0.308
Median (Range)	150 (28.91 -229.17)	160 (30.65 - 229.17)	(NS)

SD = standard deviation, CS= corticosteroids

DISCUSSION

In our study, FOXP3 concentration was significantly elevated in asthmatic patients compared to healthy controls (p= 0.000). Moreover, Foxp3 levels were significantly higher in asthmatic group receiving corticosteroids than the steroid naïve group. FOXP3 may be a natural anti-inflammatory secreted by Treg as an intrinsic body defense in hyperimmune states like bronchial asthma. One of the anti- inflammatory mechanisms exerted by the most ever known anti-inflammatory agent - which is cortisol- may be through FOXP3 production. Similarly Hori et al.[13] stated that FOXP3 was highly expressed in CD4+CD25+ Treg cells in asthmatics and they explained their finding that FOXP3 acts as anti-inflammatory agent in asthma. In their opinion, decreased Foxp3 expression causes immune disease by subverting the suppressive function of Treg cells and converting Treg cells into effector cells. Also, Wan and coworkers[14] reported increased FOXP3 gene expression in all asthmatics as a defense mechanism expressed by Treg cells against the inflammatory process occurring in asthma.

In contrast; Karengiannidis et al.[15] found no significant difference in FOXP3 level in both healthy and asthmatics. However, they agreed with our study in elucidating the upregulatory role of corticosteroids on Treg FOXP3. They recorded increased Treg activity and FOXP3 mRNA in unstimulated peripheral blood CD41 T cells of asthmatic patients treated with glucocorticoids, but not in patients with untreated asthma. In addition, they reported tight correlation between FOXP3 expression and IL10 mRNA expression, which is a well-known anti-inflammatory cytokine in bronchial asthma. Another study done by Piccirillo et al.[16] reported that there is increased expression of suppressive cytokines and transcription factor FOXP3 by Treg in response to corticosteroid treatment. The results of the present study revealed that FOXP was significantly increased in asthmatic patients receiving inhaled corticosteroid treatment, or systemic corticosteroid treatment, or both with no significant difference between them. Karengiannidis et al.[15], Gemoungesaeth et al.[17] and Kagoshima et al.[18] found increased FOXP3 level in asthmatic patients on CS with no significant correlation to the dose or route of treatment.

No data were found in the previous studies concerning the effect of clinical variables and other asthma medications on Treg FOXP3 expression. Our study revealed that Treg FOXP3 levels were not affected by or correlated to age, exposure to environmental tobacco smoke, disease control, asthma severity, lymphocyte percentage, eosinophil percentage or FEV1%. This finding may suggest that; expression of Treg FOXP3 in asthmatic patients is exclusively related to and affected by asthma pathogenesis and corticosteroid pharmacogenetic effects on Tregs. Karengiannidis et al.[15] investigated correlation between FOXP3 mRNA expression and many variables. They reported no difference in FOXP3 mRNA expression between asthmatic patients with moderate and severe disease, intrinsic and extrinsic cause. They also found no relation of FOXP3 expression to the microsatellite genotypes, which were described to be associated with type I diabetes[19]. They found only serum IgE levels to be correlated with FOXP3 expression, possibly because of the concomitant effect of glucocorticoids. Although Matsumoto et al.[20] emphasized the role of lung Foxp3+CD4+CD25+ T cells in the regulation of asthma phenotypes through an IL-10-mediated mechanism, they found no significant correlation between the frequency of Foxp3+CD4+CD25+ T cells and asthma severity.

Finally most of the studies including ours support that asthmatics express FOXP3 more than healthy, and corticosteroid therapy whatever - its route -markedly enhances FOXP3 expression. Thus, corticosteroid treatment is considered not only immunosuppressive and anti-inflammatory agent,

but also promotes or initiates differentiation towards TR1 cells (suppressive Treg) by a FOXP3-dependent mechanism. Therefore, strategies that convert transient glucocorticoid-induced Treg activity into a stable phenotype might improve allergy and asthma therapy.

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