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Role of Regulatory $CD4^+CD25^+$ Foxp3 T Cells in Bronchial Asthma in Egyptian Children

Salwa I Bakr, Manal Z Mahran, Dina A Soliman

Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

$CD4^+CD25^{+high}$ Foxp3 regulatory T (Treg) cells are known to play a key role in balancing immune response to maintain peripheral tolerance against harmless antigens or allergens. Defective immunological suppression by $CD4^+CD25^{+high}$ Foxp3 Treg cells can be a cause of the inflammation that leads to an allergic condition such as asthma. The aims of the study are to (1) determine $CD4^+CD25^{+high}$ Foxp3 Treg cells frequency in the peripheral blood of children with and without asthma; and (2) investigate the association between $CD4^+CD25^{+high}$ Foxp3 Treg cells frequency with disease severity and corticosteroid therapy. Sixty asthmatic children with varying disease severity (20 mild, 20 moderate and 20 severe) were enrolled in the study. Severe asthmatic children were further subdivided into two groups, one on corticosteroid therapy and the other was not on corticosteroid. Twenty age and sex matched healthy children were enrolled as controls. Number of circulating $CD4^+CD25^{+high}$ Foxp3 Tregs were measured using flowcytometry. Our finding demonstrates that children with asthma had a significant decrease of $CD4^+CD25^{+high}$ Foxp3 Treg cells and Tregs /T effectors ratio in peripheral blood compared to children without asthma. Patients with moderate asthma demonstrated lower frequency of $CD4^+CD25^{+high}$ Foxp3 Treg cells compared to mild and severe asthmatic patients. Those on corticosteroid therapy revealed significant increase in $CD4^+CD25^{+high}$ Foxp3 Treg cells and decrease in T effectors. It is concluded that asthmatic children have decreased number of $CD4^+CD25^{+high}$ Foxp3 Treg cells leading to increase in effectors cells which mediate inflammation in the airways. Corticosteroid therapy plays a role in elevating number of $CD4^+CD25^{+high}$ Foxp3 Treg cells and maintaining its suppressor function.

Asthma is a chronic inflammatory disease of the airways associated with airway hyper- responsiveness (AHR). The role of specific T cells and their cytokines in allergic asthma is well known (Ray *et al.,* 2010). Allergic asthma starts with influx of naïve $CD4^+$ T cells and eosinophils into the bronchial mucosa. The priming of the naïve $CD4^+$ T cells to differentiate into proinflammatory Th2 cells by allergen activated dendritic cells (DCs) is an important proposed mechanism in occurrence of asthma (Hammad *et al.,* 2008). $CD4^+CD25^{high}$ Foxp3 Tregs are known to play a key role in balancing immune responses to maintain peripheral tolerance against harmless antigens or allergens (Bacchetta *et al.,* 2006). $CD4^+CD25^{+high}$ Foxp3 Treg cells may block the transition from the early activation stage to the differentiated Th2 state, limit airway allergic inflammation and act to prevent inappropriate Th2 responses to environmental allergens (Lewkowich *et al.,* 2005; kearley *et al.,* 2008). The mechanism of immunological regulation by which $CD4^+CD25^{+high}$ Foxp3 Treg cells mediate their effect on other activated T cells is an area of investigation and ongoing controversy and may involve a spectrum of regulatory roles in several disease state. Suggested mechanisms include an essential role of cytokines, cell-cell interaction, modifications of antigen presenting cells by Treg cells and competition with naive T cells for specificity to the same antigen in adhesion to antigen presenting cells (Sakaguchi, 2004; Von Boehmer, 2005). Candidate molecules responsible for the immunosuppressive function of Treg cells include inhibitory cytokines IL-10 and transforming growth factor beta (TGF)-β, those of the B7 family interacting with cytotoxic T lymphocyte – associated antigen 4(CTLA-4), a negative co-stimulatory molecule, and the glucocorticoid – induced tumor necrosis factor family – related receptor (GITR) and its related signal transduction (Read *et al.,* 2000; Shimizu *et al.,* 2002). In allergic disease, the current concept suggests that inadequate $CD4^+CD25^{+high}$ Foxp3 Treg cells suppression of effectors cells or diminished Treg cells number contributes to the development and perpetuation of allergic inflammation (Singh *et al*., 2013). As Treg cells are important for suppressing immune response and inflammation, we hypothesized that children with asthma would have fewer $CD4^+CD25^{+high}$ Foxp3 Treg cells compared to unaffected children. The frequency of $CD4^+CD25^{+high}$ Foxp3 Treg cells was further analyzed in relationship to disease severity, and corticosteroid treatment.

Subjects and Methods

Subjects

Sixty well documented asthmatic children were enrolled in the present study , recruited from outpatient clinic of pediatric, Chest and Immunology Unit ,Ain Shams University Pediatric Hospital (28 male and 32 female children; age range (5-12 years); (mean± SD, 7.17±2.17years). The children were classified according to severity of asthma (Yawn, 2008) into: mild (n=20); moderate (n=20) and severe (n=20). Severe asthmatic children were further subdivided according to corticosteroid therapy into children who received corticosteroid (n=11) and children did not receive corticosteroid (n=9). A written informed consent was taken from all participants. Ain Shams Medical Research Ethics Committee approved this study. Twenty age and sex matched healthy children 9 female, 11 male with age range (5-12 years old), (mean±SD; 7.95 ± 2.02) were studied as a control group.

Methods

- Blood samples

Five ml of venous blood were withdrawn from every subject and were divided in three tubes: EDTA tube for estimation of $CD4^+CD25^{+high}$ Foxp3 Treg cells by flowcytometry and for CBC, citrated tube for erythrocyte sedimentation rate (ESR) and the third tube was plain to obtain serum which was separated into two aliquots one for CRP estimation and the other for IgE assay.

Analytical methods

The following investigations were performed to all controls and patients:

1-CBC was measured using Beckman Coulter Counter (HmX Hematology Analyzer, Coulter Corporation, Miami, FI 33116-9015).

2-ESR was measured by Western green method.

3-CRP levels were assayed using turbidimetric method (Turbiquant, Behringwerke Diagnostics-Marburg, Germany).

4-Serum total IgE was assayed by quantitative Enzyme linked immunosorbent assay (ELISA) using a human IgE ELISA quantitation kit (Bethyl, Montgomery, TX, USA).

5-Determination of $CD4^+CD25^{+high}$, Foxp3 Tregs was done by detection of the combined expression of $CD4^+$, $CD25^{high}$ and intracellular Foxp3 on peripheral blood lymphocytes using three colors EPICS XL flow cytometer (Coulter Electronics, Florida, USA).

The test was done on lysed whole blood using the following monoclonal antibodies conjugated with different fluorescent dyes: Fluorescein isothiocyanate (FITC)-conjugated anti-humanCD4 , Phycoerythrin (PE)-conjugated anti-human CD25, Phycoerythrin- Cyanine 5 (PE-Cy5)-conjugated anti-human Foxp3, PE-Cy5 isotype control and PE isotype control, all supplied by (eBioscience, USA). Two plastic test tubes were used; one for test and the other for isotypic control. According to manufacture instruction:

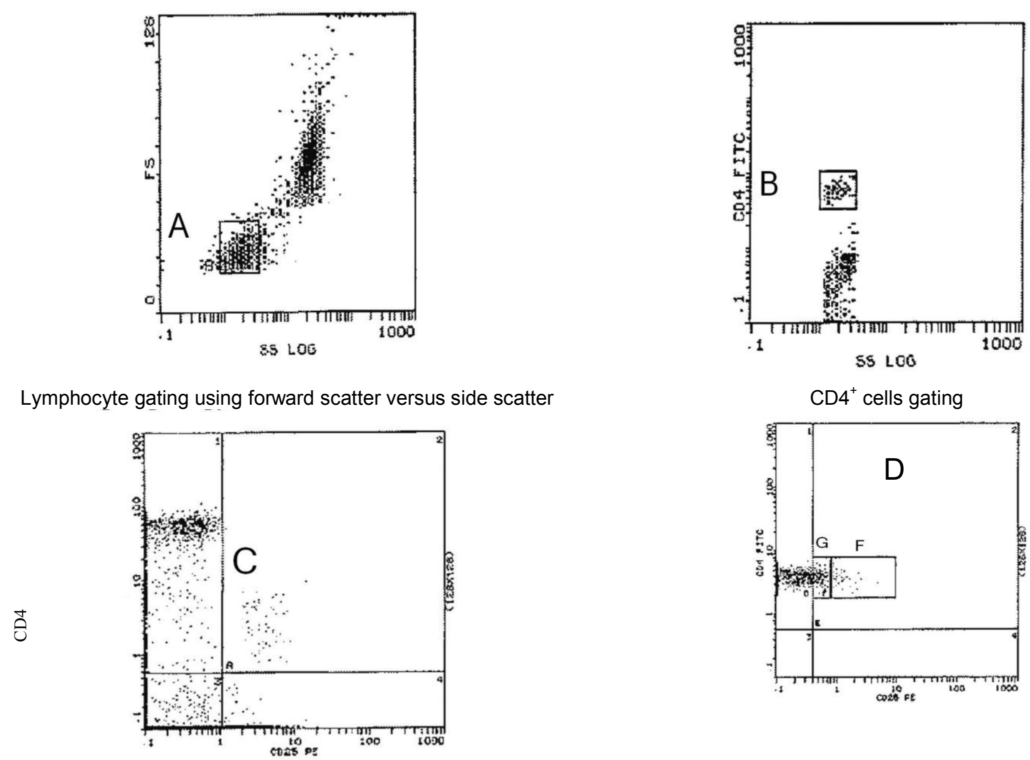
Fifty µL of EDTA anticoagulated whole blood was added to each tube which contain 1 mL lysing solution followed by wash with PBS, then cell pellet was stained with combinations of the following antibodies (five µl each): anti-CD25-PE, anti-CD4-FITC in the test tube, while PE isotype control and anti-CD4-FITC in the control tube. Both tubes were then incubated in dark at room temperature for 20 minutes followed by single wash with PBS. The cell pellet was resuspended in 0.5 ml of freshly prepared fixation/permealization working solution and incubated for 30 minutes at 4°C in dark. This was followed by washing once with PBS then washing once again with 1ml of 1X permealization buffer. Ten µL of anti-Foxp3-PE-Cy5 and PE-Cy5 isotype control were added in test tube and control tube respectively, and incubated for 30 minutes at 4°C in dark. This was followed by washing once with PBS then resuspended in 0.5 ml PBS for analysis.

Lymphocytes were gated according to their forward and side scatter properties, and CD4 positive T cells were gated out of total lymphocytes. Tregs were identified by combined expression of $CD4^+$, $CD25^{high}$ and Foxp3, while the T effectors cells were identified by the combined expression of $CD4^{+,}$ $CD25^{low}$ and low expression of Foxp3.

Data acquisition and analysis were performed on EP-ICS XL flowcytometry using SYSTEM II version 3 software with a standard three-color filter configuration. Isotype-matched controls were used to set up the gating. Lymphocytes were identified according their size and complexity on the forward and side scatter plot Fig. (1-A). $CD4^+$T cells were identified based on $CD4^+$ expression Fig. (1-B). PE isotype control was used to set gates separating $CD4^+CD25^+$cells from $CD4^+CD25^-$ cells. CD25 ^{high} were identified relative to the intensity of CD25 on $CD4^-$ cells dividing it into two parts on the plot, the dim area ($CD4^+CD25^{low}$) Fig. (1-G) and the bright one is ($CD4^+CD25^{high}$) Fig. (1-F). PE-Cy5 isotype control was used to set the gates for separating the Foxp3 $^+$cells from Foxp3 $^-$cells.

Statistical Method

Analysis of data was done using SPSS (statistical program for social science version 12). Quantitative values were presented as, median, 25^{th} and 75^{th} percentiles. Qualitative variables (gender) were presented as number and percentage. Chi-square test was used to compare qualitative variables between groups. Unpaired t-test and Mann-Whitney "U" test were used to compare quantitative variables. Binary correlation was carried out by Spearman correlation test. Results were expressed in the form of correlation coefficient (R) and *P*-values. *P* value <0.05 was considered as significant, and > 0.05 as non significant (Miller & Knapp, 1992).



Lymphocyte gating using forward scatter versus side scatter

$CD4^+$ cells gating

CD25 expression on $CD4^+$ cells

$CD4^+$ CD25 ^{high} (region F), $CD4^+$ CD25 ^{low} (region G)

Figure 1. Representative dot plots of flowcytometry and the gating strategy used (A to D): (A) show forward and side scatter to gate lymphocytes. (B) Show CD4 $^+$ cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. (C), (D) Gating approach for $CD25^+$ cells and for discrimination between $CD25^{high}$, $CD25^{low}$ on $CD4^+$ cells. The gates for the $CD25^{high}$ and $CD25^{low}$ populations were set by comparing the CD25 expression levels of $CD4^-$ cells.

Results

Results of the present study are illustrated in tables 1 - 4. Statistical comparison between asthmatic children and control as regard demographic and laboratory parameter were illustrated in (Table 1).

Comparative statistic of $CD4^+CD25^{+high}$ Foxp3 Treg cells, T effectors, Foxp3 between asthmatic children and control revealed a highly significant decrease in $CD4^+CD25^{+high}$ Foxp3 Treg cells, Tregs/ T eff ratio, Foxp3 determination, and highly significant increase in T eff ($P< 0.001$) in asthmatic children, while no statistically significant difference ($P>0.05$) could be detected between both as regard $CD4^+$ cells and $CD4^+CD25^+$ of all lymphocytes (Table 2).

$CD4^+CD25^{+high}$ Foxp3 Treg cells, T effectors and Foxp3 were compared in asthmatic subgroup which proved significant decrease of $CD4^+CD25^{+high}$ Foxp3 Treg cells and Tregs/ T eff ratio in children with moderate asthma than mild and severe asthma, (Table 3). When severe asthmatic children were statistically compared based on inhaled corticosteroid , percentage of $CD4^+CD25^{+high}$ Foxp3 Treg cells, Foxp3 and Tregs/ T eff ratio showed highly significant increase and a highly significant decrease in T effectors in children received corticosteroid than those who did not receive (Table 4).

Table 1. Statistical comparison between control and asthmatic children as regard demographic and laboratory parameters.

	Control (n=20)		Cases (n = 60)		*P* value
	n	%	n	%	
Female	9	45	32	53.3	NS
Male	11	55	28	46.7	
	Median	25^{th} and 75^{th} percentiles	Median	25^{th} and 75^{th} percentiles	*P* value
Age (y)	7.70	6.33- 9.08	6.70	5.18-9	NS
ESR (mm/ hr)	4.50	4-5	19.50	13.25-28	<0.001
CRP(mg/dl)	3.50	3-5	19.00	13-27	<0.001
TLC (10^9/L)	6.85	5.40-8	5.95	4.93-6.90	0.038
Absolute lymphocytic count (10^9/L)	3.05	2,05-5.30	2.30	1.90-3.50	NS
Absolute eosinophilic count (10^9/L)	0.20	0.10-0.30	0.60	0.50-0.60	<0.001
FEV_1 (%)	87.00	84.25-90.00	70.50	55.50-82.72	<0.001
IgE (IU/dl)	26.50	19.75-31	657	220.50-874.25	<0.001

P>0.05 is not significant (NS).

Table 2. Statistical comparison between control and asthmatic children as regard $CD4^{+}CD25^{+high}$ Foxp3 Treg cells ,T effectors, Tregs /T effectors ratio.

	Controls n =20		Cases n =60		*P* value
	Median	25^{th} and 75^{th} percentiles	Median	25^{th} and 75^{th} percentiles	
% of $CD4^{+}$ of all lymphocytes	39.75	29.20-49.40	36.50	23.92-48.08	NS
% of $CD4^{+}CD25^{+}$of all lymphocytes	8.00	7.02-9.57	8.74	7.03-9.84	NS
% of $CD4^{+}CD25^{high}$ Foxp3(Tregs)	5.43	3.86-5.80	2.70	1.40-3.90	<0.001
% of $CD4^{+}CD25^{low}$(T eff)	3.25	2.23-3.80	5.90	4.13-7.20	<0.001
Tregs/ Teff ratio	1.52	1.17-2.39	0.47	0.23-0.80	<0.001
Foxp3% on Tregs	66.55	61.30-73.60	52.70	42.25-61.50	<0.001

P>0.05 is not significant (NS).

Table 3. Statistical Comparison between control and asthmatic groups as regard $CD4^{+}CD25^{+high}$ Foxp3 Treg cells ,T effectors, Tregs /T effectors ratio.

	Mild vs. Control	Mod vs. Control	Severe vs. Control	Mod vs. Mild	Severe vs. Mild	Severe. vs. Mod
% of $CD4^{+}CD25$ ^{high} Foxp3 of all $CD4^{+}$(Tregs)	3.80	1.98	2.84	1.98	2.84	2.84
	5.43	5.43	5.43	3.80	3.80	1.98
P value	<0.001	<0.001	<0.001	<0.001	NS	NS
% of $CD4^{+}CD25^{low}$ (T eff)	5.19	6.85	4.68	6.85	4.68	4.68
	3.25	3.25	3.25	5.19	5.19	6.85
P value	<0.001	<0.001	0.010	NS	NS	NS
Tregs / T eff ratio	0.73	0.25	0.66	0.25	0.66	0.66
	1.52	1.52	1.52	0.73	0.73	0.25
P value	<0.001	<0.001	<0.001	<0.001	NS	NS
Foxp3 % on Tregs	55.55	48.60	56.95	48.60	56.95	56.95
	66.55	66.55	66.55	55.55	55.55	48.60
P value	0.002	<0.001	0.015	NS	NS	NS

P>0.05 is not significant (NS).

Table 4. Comparative statistics between severely asthmatic children who received corticosteroid and those who didn't as regard $CD4^+CD25^{+high}$ Foxp3 Treg cells, T effectors, Tregs/T effectors ratio.

	No inhaled corticosteroids (n = 9)			Inhaled corticosteroids (n =11)			*P* value
	Median	25^{th} and 75^{th} percentiles		Median	25^{th} and 75^{th} percentiles		
% of $CD4^+$ of all lymphocytes	44.23	31.30	62.15	34.12	19.60	46.30	NS
% of $CD4^+CD25^+$ of all lymphocytes	8.78	7.89	10.73	7.00	6.21	8.00	0.011
% of $CD4^+CD25^{high}$ $Foxp3^+$ (Tregs)	0.80	0.50	0.91	3.98	3.00	4.54	<0.001
%of $CD4^+CD25^{low}$(T eff)	7.98	7.22	9.97	3.01	2.34	4.23	<0.001
Tregs/ T eff Ratio	0.08	0.05	0.10	1.32	0.89	1.70	<0.001
Foxp3 % on Tregs	35.00	30.00	46.30	65.20	58.30	75.30	0.003

P>0.05 is not significant (NS).

Discussion

Airway inflammation in asthma is characterized by activation of Th2 cells, IgE production and eosinophilia (Belkaid, 2007). $CD4^+CD25^{high}$ Foxp3 Tregs are known to play a key role in balancing immune responses to maintain peripheral tolerance against harmless antigens or allergens (Bacchetta *et al.,* 2006).

In the present study, $CD4^+CD25^{+high}$ Foxp3 were used to define Treg cells. Our finding revealed a highly significant decrease in Treg cells in peripheral blood of asthmatic children when compared with healthy control subjects. These results were in agreement with Mohammed *et al.* (2005), Lee *et al.* (2007), and Hartl *et al.* (2007) who investigated the role of Treg cells in childhood asthma. They found a significant decrease in circulating Treg cells in peripheral blood of diseased subject than controls. These findings were explained by many researches done on the role of Treg cells in allergic diseases especially in asthma, they showed that Treg cells can inhibit proliferation of Th2 cells leading to decrease the proinflammatory cytokines which mediate AHR. In allergic disease Treg cells are either inadequate in number due to genetic defect during development or defect in one or more chemokines as CCL17 and CCL22 which maintain and recruit Treg cells at site of inflammation, also some inflammatory mediators like IL-6 can suppress Treg cells capacity of modulating the immune response (Wernik *et al.,* 2009). On contrary Provoost *et al. (*2009) reported no difference in the number of circulating Treg cells in control subjects versus asthmatic patients. This difference may be related to difference in methods used in assessment of Tregs. They used $CD4^+$ $CD25^+$ population to measure Treg and did not use the specific marker for Treg cells as Foxp3. Similarly, our results were comparable to Provoost *et al. (*2009) when measured $CD4^+$ $CD25^+$ only. Cosmmani *et al.* (2011) showed that $CD4^+CD25^+$ not only represent Treg cells, but it could involve T effectors cells also, so any decrease in Treg cells when accompanied with increase in T effectors cells make no significant change in $CD4^+CD25^+$ population.

In the present study measurement of Foxp3 in addition to $CD4^+CD25^{high}$ renders Tregs identification more accurate and reliable. This was supported also in our result by finding a positive correlation between Treg cells and percentage of Foxp3 on Tregs; so decreased Tregs in asthma is associated with decreased

Foxp3. These finding were in agreement with Hori *et al.* (2003) who revealed that Foxp3 serves as a master regulator for Tregs development and functions. On the other hand Wan *et al.* (2009) and Thorburn & Hansbro (2010) reported a significant increase in Foxp3 in asthmatic patient than in healthy controls. They mentioned that Foxp3 gene expression in asthmatic patient was considered as a defense mechanism against inflammation, and increase Foxp3 expression stimulates the Treg cells development, but they stressed on that these Treg cells are functionally impaired that can not affect the proliferation of T effectors cells due to absence one or more of some co-stimulatory molecules such as CTLA4, OX40 or due to cytokine present at site of inflammation render Treg cells are less active such as IL6.

In the present study there was a significant increase in T effectors cells in cases group when compared with control healthy group. Our findings are in agreement with Hartl *et al.* and Horoni *et al.* (2007) who investigated the role of Treg cells in suppressing T effectors which cause allergy, they found a significant increase in T effectors in asthmatic children than healthy group. They attributed their results to the fact that; IL-10-producing cells were able to suppress human cytokine production of IL-4-producing effectors cells in non atopic subjects, while it was not capable to suppress effectors cells in atopic patient either due to Treg cells dysfunction or T effectors cells resistant to Treg cells, and this led to elevation in T effectors cells. Moreover in the present study Tregs/T eff ratio was highly significantly decreased in asthmatic children, and there was an inverse correlation between Tregs and T effectors. These findings was consistent with a study done by Mohammed *et al.* (2005) who found an inverse correlation between Tregs and T effectors cells in asthmatic patient this could be explained by the fact that Treg cells have an inhibitory function on T effectors so increase in number of Treg cells can suppress T effectors cells proliferation. Also Mestin *et al.* (2012) added that the inverse correlation between Tregs and T effectors cells is a good prognostic value if the frequency of Treg cells is increasing, this indicate that Treg cells are capable to inhibit cytokine production of T effectors, limit its proliferation and control the immune response. In atopic patient there is deficiency in the role of Tregs in suppression the inflammation through its different mechanisms; cell to cell contact, and cytotoxic mediators, by these mechanisms Treg cells can inhibit the proliferation of T effectors result in decrease its number. So adequate number and function of Treg cells are always associated with normal or even suppressed T effectors, it had also been proved that impairment of number of Treg cells elevate T effectors cells number (Merh *et al.,* 2009). This was explained by different theories, some of them that decrease in Treg cells lead to decrease CD25 (IL2α R) which may elevate the level of IL2 and stimulate proliferation of Th2 (Ashine, 2008). Also the decreased in Tregs number could be associated with a decrease in IL-10, and TGFβ level which play an important role in immune modulation (Jerk *et al.,* 2008). These findings of the current work could fit with the hypothesis that in asthma, which is characterized by an unwanted inflammatory reaction there is an underlying relative deficiency in Tregs allowing high number of T effectors cells to develop (Barnes, 2008).

In the present study the percentage of Treg cells were significantly decrease in children with moderate asthma than mildly asthmatic children. However, difference was not significant when comparing mild versus severe asthmatic children, or moderate versus severe asthmatic children. Marcon *et al.* (2011) had carried out their study on 30 asthmatic children classified to mild, moderate, severe asthma, they found that increase the severity and duration of the allergic disease did not decrease the frequency of Treg cells but it has shown that Treg cells decreased in mild to moderate asthma but return to normal in moderate to severe asthma. They reported that Treg cells have no significant change in classification of asthma severity and they referred the normalized Treg cells number in severe asthma to the fact that at site of inflammation some of $CD4^{+}$ cells converted to induced Treg (iTreg) cells, this might increase the total Treg cells percentage. As it is well known that at site of inflammation, under the effect of cytokines such as IL-10 and TGFβ some of $CD4^{+}CD25^{-}$ acquired $CD25^{+}$ and converted to iTreg cells. Additionally, Wan *et al.* (2009) reported that there were no difference between Tregs and T effectors cells on comparing mild, moderate, and severe asthma. They suggested that it is difficult to depend on Treg cells percentage in classification of severity of asthma due to many reasons; 1) Treg cells are affected by many other factors rather than allergy such as autoimmune disease, co presence infection, or total number of T lymphocytes. 2) Adequate number of Treg cells does not exclude the presence of allergy, as the normal function of Treg cells and its ability to inhibit proinflammatory cells is also attribute the results. 3) There was many different factors affect the course of bronchial asthma not only the severity. Duration of the disease, treatment received by the patient, age, and the state of the disease whether stable or acute exacerbation, all these factors affect Treg cells in the peripheral blood. These findings render the depending on Treg cells in classification of asthma severity still questionable.

In the present study the effect of inhaled corticosteroid (ICO) on percentage of Tregs, T effectors and Foxp3 surface expression in severely asthmatic children showed highly significant increase in Treg cells and Foxp3 and decrease in T effectors. This was in accordance with Rogi *et al.,* (2009) and Provoost *et al.* (2009). Additionally, Hartl *et al.* (2007) who reported that Foxp3 expression is significantly decrease in asthmatic patient when compared with control group. However, after 4 weeks of treatment with inhaled glucocorticoids there were an increase percentage of Foxp3 messenger RNA expression levels in peripheral blood and BALF. Kerini *et al.* (2011) suggested that treatment with corticosteroid in asthmatic, might increase the Foxp3 expression in T regs, which play an important role in Treg cells development, also it render Treg cells more functioning by increase its co-stimulatory molecules expression such as GIRT; In addition corticosteroids had anti-inflammatory role which suppress the unwanted allergic reaction, and inhibit the proliferation of T effectors, by antagonizing its cytokines, this lead to reverse the Treg cells function at site of inflammation (Omert *et al.,* 2008). In agreement with a study done by Singh *et al.* (2013) who found that increased Treg cells frequency in children with asthma was most directly associated with inhaled corticosteroid use, and not asthma severity, allergic sensitization, or atopic status of the asthma and he suggested that inhaled corticosteroids may work by shifting the balance of adaptive immune response toward Treg cells predominance. On contrary Sang *et al. (*2006) had done a study on 30 allergic patients, 12 of them were receiving corticosteroid and they revealed no significant difference between both groups. In their study they emphasized up on the fact that the severity of inflammation not the corticosteroid therapy had a greater affect on number of Tregs in severely asthmatic patient, these results were supported by an earlier study was done by Wittness *et al.* (2003) on 15 asthmatic children. The samples were taken from the subjected group during acute exacerbation and were compared with that in stable condition. It was found that Treg cells are markedly increased during the acute exacerbation of asthma regardless the severity of asthma or the corticosteroid. Much of this muddle is probably due to methodological differences in various laboratories, given that the precise definition of human regulatory T cells has changed several times in recent years, and most of these studies have not distinguished between potentially distinct regulatory T cell subsets (Ziegler & Nepom, 2010). Interestingly, Liu *et al.* (2006) postulate that T regulatory cells can be defined only by the expression of CD4 and CD127, not necessarily by $CD25^{high}$ expression. The majority of $CD4+CD127^{low}$ T cells express Foxp3, are anergic and possess strong suppressive function. However, according to the authors of that report the best combination of cell surface markers for T regulatory cells separation is $CD4^{+}CD25^{high}CD127^{low}$, which accounts for about 80% of $Foxp3^{+}$ cells (Liu *et al*. 2006).

In conclusion, asthmatic children have decreased number of Treg cells leading to proliferation of T effectors which conduct more inflammation in airway. Depending on Treg cells in classifying asthma groups is not reliable. Treatment of corticosteroid has a highly effective role on elevating number of Treg cells, promote Foxp3 surface expression, and maintain the suppressor function of Tregs on T effectors. This indicate that Treg cells play an important role in the anti-inflammatory effect of this medication. The percentages of T regulatory cells in children with asthma need to be confirmed in larger studies and extended to delineate the defect in function.

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