Original article

Can montelukast correct immune dysregulation in preschool children with mild persistent asthma?

Background: *Asthma is the most common inflammatory disorder among preschool and school-age children. Regulation of immune cells and their cytokines is essential to control asthma. Montelukast is a leukotriene receptor antagonist that suppresses inflammatory cell proliferation, and reduces cytokines and mediator secretion.* ***Objective:*** *The research team's goal was to study the immunological parameters among mild asthmatic patients before and after the treatment with Montelukast.* ***Methods:*** *Forty preschool children with mild persistent asthma and twenty healthy, non-allergic children were included in the study. Blood eosinophil count, total IgE, serum IL-4, IL-10, and IL-13 levels were assessed. T helper (CD3+CD4+) and T regulatory (CD4+CD25+) cell counts were measured using flow cytometry; for mild asthmatics before and after six weeks of treatment with Montelukast and for the control group.* ***Results:*** *Asthmatic children have shown a significant elevation of serum levels of IgE, IL4 and IL13, and also an increase of eosinophils, total lymphocyte T cells and T helper cell count. However; serum levels of IL10 and Treg cell count was lower in asthmatics compared to control. Following six weeks of Montelukast treatment, all immunological parameters improved. There was a significant elevation of serum levels of IL10 and Treg cell count, with a decrease in serum levels of IgE, IL4 and IL13; eosinophil counts, and helper T cells.* ***Conclusion:*** *Montelukast treatment improves the impaired immunological balance of mild asthmatic children through the increase of serum IL-10, T regulatory cell counts that have anti-inflammatory and immunoregulatory effects. It also decreases T helper cells and their proinflammatory cytokines.*

Keywords: Montelukast; Asthma; Children; Egypt.

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INTRODUCTION

Asthma is a complex and chronic inflammatory disorder associated with airway hyper-responsiveness, chronic mucosal inflammation, and tissue remodeling of the airway structure mediated by Th2 (CD4+) cells.[1]

It is the most common chronic disease of childhood. Its morbidity among preschoolers results in higher emergency department visits, more sleep disturbances, and more restriction of family activities,[2] while among school children there are more school absences and more hospitalizations than any other chronic illness.[3]

Asthma incidence is higher during childhood.[4] Asthma prevalence is about 8.6% of children, and the greatest rate is in preschool children.[3] However, more than half of all cases of persistent asthma start before the age of 3 years.[5]

Cytokines play a key role in airway inflammatory disease of asthma by recruiting and activating multiple inflammatory cells in the respiratory tract, and hence modifies and determines the severity of the inflammatory responses in asthma.[6]

In asthmatic patients, there is an increase in the number of $CD4^+$ T helper cells in the airways, which are predominantly of the Th2 subtype, which are characterized by secretion of Interleukin (IL-4, IL-13, IL-5 and IL-9).[7] these cytokines increase IgE, eosinophils, and develop airway hyperresponsiveness.[8]

Interleukin-4 (IL-4) mediates proinflammatory functions in asthma. it is important for Th2 cell differentiation from uncommitted Th0 cells,[6] induction of the IgE isotype, switching of B cells, and promotion of eosinophil transmigration across endothelium and mucous secretion. [9]

IL-13 expression levels are increased in the airways of asthmatic patients[10]. IL-13 is a critical Th2 lineage cytokine. Being associated with asthma exacerbations[11], IL-13 mimics IL-4 in inducing IgE secretion, and causes structural changes in the airways. [12] It also induces inflammation through stimulating the expression of chemoattractants CCL11 (eotaxin) from epithelial cells, and induces airway hyperresponsiveness (AHR) and mucous hypersecretion.[13] Excessive IL-4 and IL-13 production results in the production of Th2 clones, which enhances inflammation.[14]

Interleukin (IL10) is an important immunoregulatory and anti-inflammatory cytokine that is reduced in asthmatic airways.[14] It inhibits the synthesis of inflammatory proteins, cytokines (such as TNF-α, GM-CSF, IL-5, and several chemokines) that are over-expressed in asthma, and also inhibits antigen presentation.[15]

Interleukin IL-10 modulates cells involved in the allergic response, including TH2-cell activation, mast-cell,[16] eosinophilic function, [17] and IgG to IgE ratios.[18] It promotes IgG4 production in B cells, also downregulates eosinophil survival, and inhibits IgE-mediated activation of mast cells. Moreover, it impairs dendritic cell (DC) maturation and reduces Th1 and Th2 cells stimulation.[19]

Regulatory T cells (CD4+CD25+ Tregs) suppress harmful immune responses in human beings,[20] as well as in animal models.[19] Two subsets of Treg (naturally occurring Treg cells and IL-10-producing Tregs) have the potentials to suppress pathogenic Th2 responses. The airways of asthmatic patients show reduced expression of Foxp3 and deficiency of $CD25^{high}$ Treg-suppressive function. [20-21]

Montelukast is a potent, specific leukotriene receptor antagonist. Administered once daily in a tablet form, Montelukast reduces the signs and symptoms of chronic asthma in adults and children with a tolerability profile similar to that of placebo.[22-23]

Montelukast has a safety profile that can be used as a monotherapy in children because of the suboptimal delivery of inhaled medication, well-controlled asthma, poor adherence to inhaled corticosteroids (ICS) and considered as an add-on therapy to patients insufficiently controlled by ICS, especially children with decelerated growth.[24] The drug also attenuates exercise-induced bronchoconstriction. [25, 26]

Stepwise approach to managing asthma in children 0-4 years recommends Montelukast in step 2 (mild persistent asthma) as an alternative for inhaled corticosteroids (ICS), and in step 3&4, as an add-on therapy. [24, 27]

The aim of this study is to determine the effect of Montelukast in patients with mild persistent asthma by analyzing serum IL-13, IL-4 , IL-10, T-helper cell and T-regs (CD4/CD25) before and after six weeks of therapy.

METHODS

This is a prospective controlled study conducted among sixty children, aged 2–5 years, who came to the Pediatric Outpatient Clinic in Ismailia University Hospital in the period from August 2015 to August 2016. Forty children with asthma presented to pediatric outpatient clinic in acute exacerbation were evaluated with a screening questionnaire based on the Pediatric Asthma Quality of Life and were diagnosed as mild persistent asthma. The questionnaire includes both daytime and nocturnal asthma symptom diary scales. [28, 29]

Asthma severity is determined according to the *Global initiative of Asthma (GINA).* [30]Twenty children matched for age and sex, with no history of allergic or respiratory disease, were chosen as the control group. Children were excluded if they have received a long-term controller therapy or systemic corticosteroids, or had concomitant infections, or any chronic inflammatory disorders.

A full clinical assessment, through history taking and physical examination, for each participant in both patients and control groups of the study.

Blood Samples were withdrawn from patients (before starting Montelukast)and controls for the following baseline hematological and immunological assessments:

Complete blood count, eosinophils counts measured by an automated hematology analyzer Coulter LH 750 (Beckman Coulter, USA) and expressed as the number of cells per cubic mm.

Measurement of serum level of interleukin-4(IL-4), IL-10 IL-13, using an ELISA kit (R & D systems) (Minneapolis, MN, USA).

Flow cytometric assessment of T helper and T regulatory cells using a flow cytometer (FACs Calibur, BD, Bectom Dickinson).

After diagnosis of mild persistent asthma, asthmatic children were started on once-daily 4 mg of Montelukast (chewable tablet). They were followed up every two weeks for six weeks through

history taking and clinical examination for acute exacerbation and need for rescue medications. All the above investigations were repeated at the end of six weeks of Montelukast therapy .

Flow cytometry procedure:

Staining of (CD3, CD4, and CD25) cell markers was conducted according to the protocol of the manufacturing company of flow cytometer (FACS Calibur, BD, Bectom Dickinson (BD), USA); using conjugated monoclonal antibodies (MCAB) with phycoerythrin (PE), fluorescent isothyocyanate (FITC) and Allophycocyanin (APC).

Immunophenotyping for T-regulatory cell:

The reagents for color analysis of T cell surface marker (CD3+ CD4+) consist of CD3-FITC and CD4-PE; for T-regulatory (cell surface staining CD4+CD25+ intracellular staining for FoxP3) the reagents consisted of FITC conjugated anti-human CD4; PE conjugated anti-human CD25, and APC-conjugated anti-human FOXP3[31].

Ethical consideration:

An informed written consent consistent with the ethical principles of the International Conference of Harmonization guideline and Good Clinical Practice (ICH-GCP)[32] was obtained from the guardians of all the cases. The study was approved by the local Ethics Committee of the Faculty of Medicine, Suez Canal University, Egypt.

RESULTS

Table 1 shows the demographic and laboratory data which demonstrate no statistical difference between patients and controls regarding the mean age, sex, BMI, and blood picture.

The serum levels of IgE, IL4 and IL13 (Figs 1, 2). And the counts of eosinophils, total lymphocytes, T cells (CD3+) and helper T cells (CD3+CD4+) were all increased with a statistically significant difference in children with asthma more than the healthy control children. However, the serum levels of IL10 and Treg cells ($CD4^+CD25^+FoxP3^+$) counts were significantly lower in asthmatic children (Table 2) and (Figs 3,4).

Montelukast treatment resulted in a significant decrease in serum levels of IgE, IL4 and IL13, whereas the serum level of IL10 significantly increased (Figs 1, 2, 3). The eosinophils cell count, total lymphocytes count, T cells (CD3+) and helper T cells count (CD3+CD4+) significantly decreased after treatment with Montelukast while Treg cell ($CD4^+CD25^+$ $FoxP3^+$) count significantly increased in asthmatic children after treatment (Table 3) and (Fig4).

In this study, we assessed the correlation between the cytokines and IgE and immunological cells. There was a significant positive correlation of IgE with IL4 and IL13, whereas there was a significant negative correlation between IgE and IL10. T lymphocytes had a significant positive correlation with IL13, while significant negative correlation with IL10 and no correlation with IL4. Helper T cells had a significant positive correlation with IL4 and IL13 however, it showed no correlation with IL10. T regulatory cells had no correlation with the studied cytokines (Table 4).

Table1. Demographic and laboratory characteristics of the studied population

	Asthmatic children	**Control children**	**p-value**
Age Mean (±SD)	3.68±0.94	3.98±1.21	0.47
BMI kg/m^2 Mean ±SD	18.11±2.4	19.03±1.9	0.14
Gender (n%*) Male Female	26 (65%) 14 (35%)	11 (55%) 9 (45%)	0.57
Hb gm/dl Mean (±SD)	11.8±58	12.1±.55	0.06
WBCs $x10^3/\mu l$ Mean (±SD)	7.32±1.52	7.6±1.69	0.47
Platelets $x10^3/\mu l$ Mean (±SD)	261.6±81.93	239.2±73.37	0.07

BMI = Body Mass Index; Hb= hemoglobin; WBC= White Blood cell count

Table 2. Assessment of cytokine levels and immunological cell counts between asthmatic children before treatment and healthy control.

	Asthmatic children	Healthy control	P value
Total IgE IU/ml	384.60±128.92	59.45±15.95	<0.001*
IL4 ng/l	161.99±31.43	44.17±10.4	<0.001*
IL10 ng/l	4.87±.44	7.65±.93	<0.001*
IL13 ng/l	102.07±35.01	17.04±2.57	<0.001*
Lymphocyte x10^3/µl	4.434 ±0.6294	3.639±0.445	<0.001*
Esinophil (cell/mm^3)	403.8038±93.9107	179.51±41.69	<0.001*
CD3+ (cell/mm^3)	2180.28±484.12	1674.784±233.87	0.01*
CD3+%	48.9±5.8	45.4±3.3	<0.001*
CD3+CD4+ (cell/mm^3)	1620.5± 249.29	1124.1±211.25	<0.001*
CD3+CD4%	36.65± 3.36	30.86±3.99	<0.001*
CD^{+4} CD25+ FoxP3$^+$ (cell/mm^3)	185.5± 25.7	201.7±32.8	<0.001*
CD4$^+$ CD25+ FoxP3$^+$%	0.401± .09144	0.5321±.06819	<0.001*

Statistical Significance at $p < 0.05$; *Statistically-significant

Table 3. The Impact of Montelukast treatment on cytokine levels and immunological cell counts in asthmatic children

	Before treatment	After treatment	P value
Total IgE IU/ml	384.60±128.92	168.55±34.244	<0.001*
IL4 ng/l	161.99±31.43	130±29.486	<0.001*
IL10 ng/l	4.87±.44	6.9273±.86039	<0.001*
IL13 ng/l	102.07±35.01	53.64±12.03	<0.001*
Lymphocyte (Cell/mm^3)	4.434 ±0.6294	3.887±0.5970	<0.001*
Esinophil (cell/mm^3)	403.8038±93.9107	266.848±35.20512	<0.001*
CD3+ count (Cell/mm^3)	2180.2828±484.122	1897.42±494.875	0.012*
CD3+%	48.9±5.8	43.5±1.2	0.01*
CD3+CD4+ (Cell/mm^3)	1620.5±249.29	1342±306	<0.001*
CD3+CD4%	36.65±.03365	31.1±.081	<0.001*
CD4$^+$ CD25$^+$ FoxP3$^+$% (Cell/mm^3)	185±25	207.42±31.9	<0.001*
CD4$^+$ CD25$^+$ FoxP3$^+$ %	0.401±.0914	0.468±.038	<0.001*

Statistical Significance at $p < 0.05$; *Statistically-significant

Table 4. Correlation between serum levels of cytokines with IgE, T lymphocytes and its subtypes in asthmatic children

Cytokines	IgE		T lymphocytes CD3+		Helper T lymphocytes CD3+CD4+		T regulatory CD4+CD25+	
	r#	P value	r#	P value	r#	P value	r#	P value
IL4	0.673	<0.001*	0.112	0.321	0.264	0.018*	0.019	0.864
IL10	-0.716	<0.001*	-0.262	0.019*	-0.077	0.638	0.108	0.342
IL13	0.686	<0.001*	0.292	0.009*	0.38	0.001*	-.0193	0.087

#Pearson Correlation; *Statistically-significant

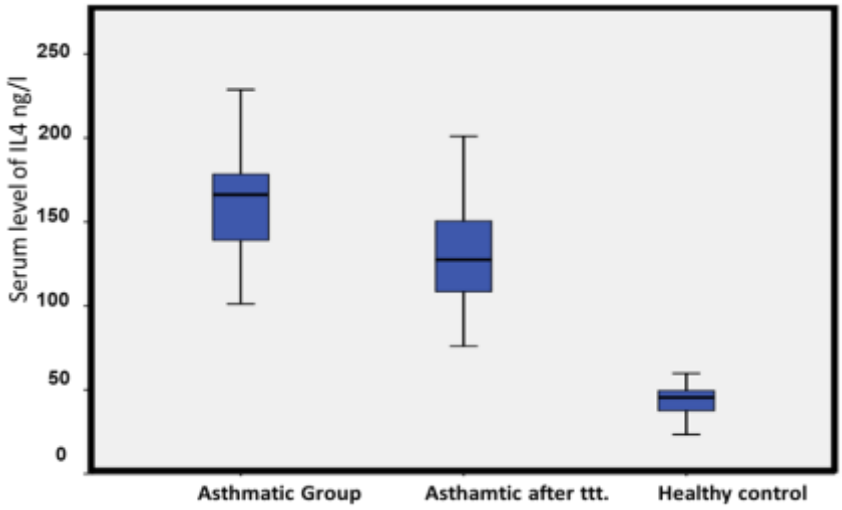

Figure 1. Serum levels of IL 4 in healthy control and asthmatic children before and after treatment.

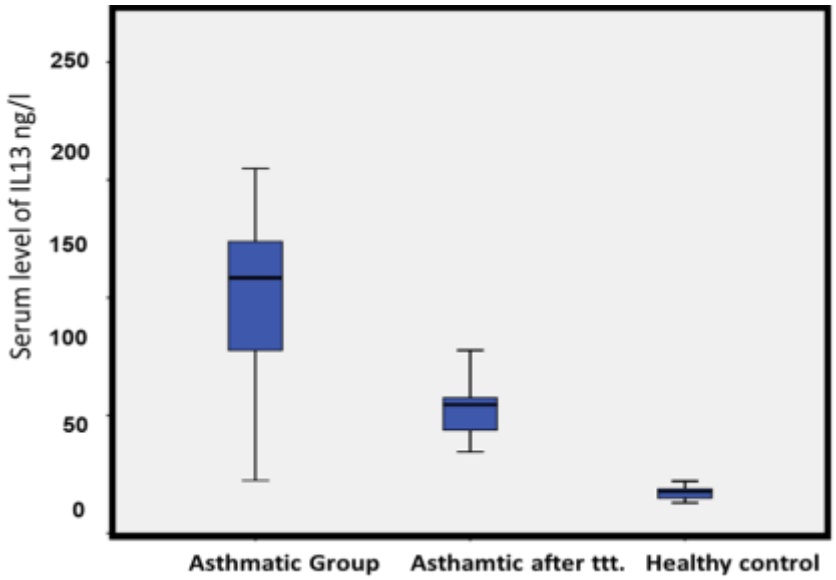

Figure 2. Serum levels of IL 13 in healthy control and asthmatic children before and after treatment.

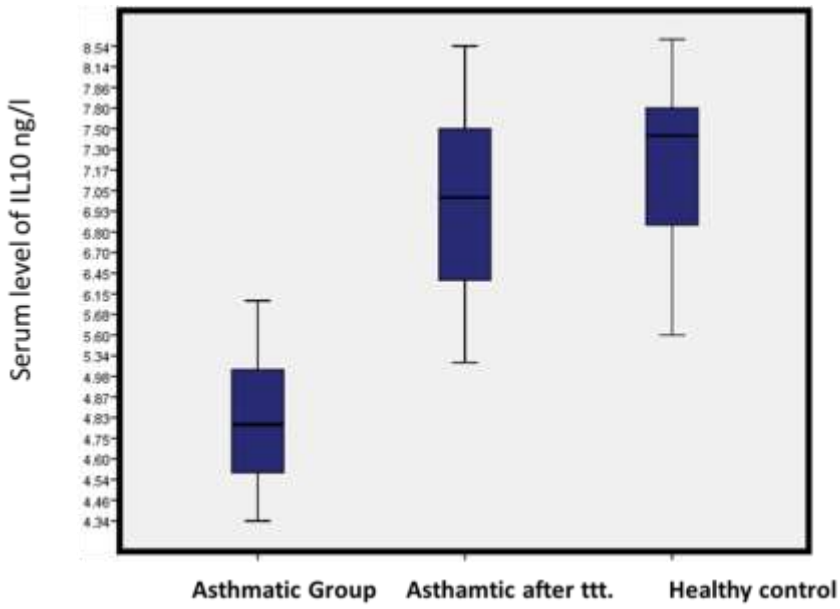

Figure 3. Serum levels of IL 10 in healthy control and asthmatic children before and after treatment.

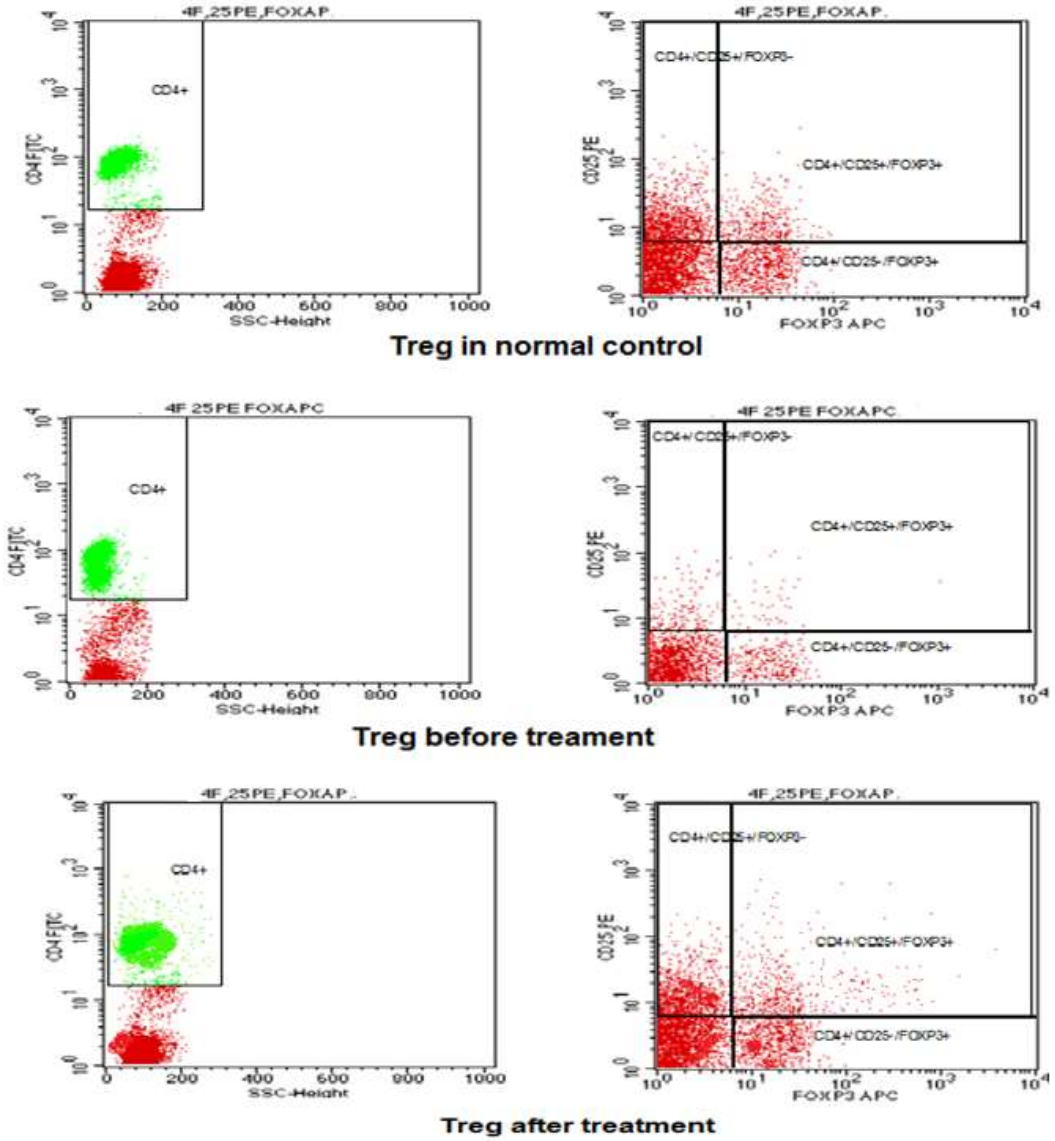

Figure 4. Assessment of T helper and T regulatory cells by flow cytometer. CD4 positive events gated within the lymphoid region (T helper) and dot plot was made with cells from this region showing CD4, CD25 and Foxp3 positive cells to assess the CD4+/CD25+/FoxP3+ coexpression (T regulatory) from the T cells.

DISCUSSION

In our study serum L4 and IL13 and eosinophil count were high in all asthmatic children, findings which agree with those of other studies, which suggested that airway inflammation is the key to reversible airway obstruction in asthma.[33] TH2 cytokines (IL-4 and IL-13) play a great role in eosinophilic inflammation of asthmatic airways.[34] Correspondingly, Wills-Karp et al. [35] reported the role of TH2 cytokines in IgE synthesis by B cells; mucus production, bronchial fibrosis and airway hyper-responsiveness (AHR) in asthma. Levels of IL-13 and eosinophils numbers were high in sputum and bronchial biopsies from asthmatic patients.[36] Furthermore, eosinophil percentages were (29% and 23%) in severe and mild cases.[37]

IgE acts as a mediator of the allergic response in asthma.[38] Our results confirmed that all asthmatic children have increased IgE levels than their control counterparts. In a previous study, IgE level has been shown to be high in severe and mild asthma (43% and 50% respectively).[36-37] Specific IgE triggers eosinophils production and activates mast cells that differentiate T helper cells into Th2 cells which secrete IL-4, IL-5, IL-10 and IL-13 cytokines.[39-40] This explains the increased total lymphocyte count, and eosinophils cell count in our asthmatic children as compared to control.

IL-10 controls immune tolerance to allergens. Asthma severity is inversely correlated with IL-10 levels, Which inhibits Th1 and Th2 cell activation and acts as an anti-inflammatory cytokine.[40] Also, it

inhibits eosinophilia by suppression of IL-5 and GM-CSF, direct effects on eosinophil apoptosis, and effects on cell proliferation through down-regulation of IL-1.[41]

Our results show decreased serum levels of IL10 in asthmatic children compared to healthy children. Diminished levels of IL-10 and elevated levels of proinflammatory IL-17F and IL-33 were found in asthmatics.[42,43] Patients with asthma had significantly fewer IL-10 producing cells as compared to normal controls.[44]

IL-10 is secreted by monocytes and T regulatory cells under the control of IL- 2 in response to allergens.[41] Our low levels of IL10 might be due to the decrease of T regulatory cells (Tregs) count. However, both steroid therapy and allergen specific immunotherapy are known to elevate endogenous IL-10 levels as a target for treatment of asthma, due to its ability to inhibit IFN-γ and IL-2 production in Th2 cells.[41] Akdis et al., reported the IL-10 reduction and increased IL-4 allergen-responsive T cells in allergic patients compared to non-atopic people. [45]

Our results show Treg cell (CD4+ CD25+ FoxP3+) count reduction, an increase of T cells (CD3+) and helper T cell (CD3+CD4+) counts in asthmatic children as compared to controls. Our findings agree with den Otter [46] who showed a strong correlation between high CD4+ cells (from bronchial biopsies) of asthmatic and a decline in its long-term lung function.

Asthmatic patients have increased levels of IL-13 and type 2 cytokines, reduction of bronchoalveolar lavage regulatory T-cell numbers and increase mast cell mediator levels.[47] The inadequate production of immunoregulatory cytokines (e.g., transforming growth factor-b and IL-10 and conversion of regulatory T cells (Tregs) to Th2 effector cells all contribute in asthma pathogenesis. [48-50]

Function and numbers of CD4+CD25 T cell as well as Foxp3 mRNA in the lung (but not peripheral blood) were lower in asthmatic children as compared to non-asthmatics with cough,[51] also Foxp3 protein expression was reduced among asthmatic patients.[52]

Montelukast decreases the bronchial tree hyper responsiveness in asthma. Sputum and peripheral blood eosinophils are reduced by treatment with Montelukast.[53] After treatment with Montelukast, we observed a decreased eosinophil, total lymphocyte, T cells (CD3+) and helper T cell (CD3+CD4+) counts.

In-vitro studies showed that Montelukast given in high doses reduces IL-4, IL-5 and IL-13 levels in the lung.[54] Montelukast was found to decrease IL-4 mRNA expression in the lungs after challenge with allergens.[55] Scientists found that allergic rhinitis children treated with Montelukast had a significant decrease of IL-4 and IL-13 and a significant increase of IFN-γ levels in their nasal lavage.[56] In the current study, a decrease in the serum levels of IgE, IL4 and IL13, and elevation of the serum levels of IL10 after Montelukast usage was observed.

T regulatory inhibits the immune responses progression, and limits its duration through IL-10 and TGF-β.[57] Montelukast can suppress the proliferation of inflammatory cells and decrease cytokines and inflammatory mediators while inhibiting airway remodeling or fibrosis.[58]

The existing study investigates the role of Treg cells in the pathogenesis of mild asthma in children by detecting the levels of Tregs and cytokines in the peripheral blood. The serum levels of IL10, and Treg cells count decreased in asthmatic children as compared to the control group. Treatment with Montelukast has increased the Treg cell count in asthmatic children. This suggests that Treg cells are crucial in asthma, which may help future studies of asthma pathogenesis and provides a reliable guide for treatment.

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