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Research Article

Immune Dysregulation in Children with Allergic Asthma: A Close Relationship Between IL-17 but not IL-4 or IFN-γ, and Disease Severity

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Abstract

Introduction: Allergic asthma is a chronic airway inflammatory disease often determined with degrees of inflammation, hypersensitivity, bronchial constriction, and airway changes. Th1, Th2, and Th17 cells are the main cells involved in asthma pathophysiology.

To evaluate Th1, Th2, and Th17 functions by assessing INF-γ, IL-4, and IL-17 gene and protein levels in asthma patients and healthy controls.

Materials and Methods: In total, 44 individuals of Iranian ethnicity including 24 patients with allergic asthma and 20 healthy controls were enrolled. Peripheral blood mononuclear cells of all participants were isolated and cDNA was synthesized following RNA extraction. Gene expression and protein levels of INF-γ, IL-4, and IL-17 were evaluated by real-time polymerase chain reaction and sandwich ELISA, respectively.

Results: The results of this study showed that the gene expression of IL-4 and IL-17 in patients was increased significantly compared to the control group ($p = 0.046$ and 0.03, respectively) whereas that of IFN-γ was significantly decreased in the group of patients ($p = 0.021$). Compared to the healthy controls, serum levels of IL-17 and IL-4 were significantly increased in asthma patients ($p = 0.015$ and 0.03, respectively).

Conclusion: Higher IL-17 and IL-4 mRNA expression and serum levels in asthma patients than healthy controls highlight the role of Th2 and Th17 cells in asthma pathogenesis and their potential as therapeutic targets.

Keywords: Allergic asthma, IL-4, IL-17, IFN-γ, Immune dysregulation

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1. Introduction

Allergic asthma is a chronic inflammatory disease of the airway that is often determined with degrees of inflammation, hypersensitivity, bronchial constriction, and airway changes (1,2). In the past decade, prevalence of allergic diseases such as asthma has increased greatly and approximately 10 percent of people in developed countries suffer from asthma (3-4). Based on the World Asthma report published in 2007, the prevalence of asthma in Iran is about 13.14% of all population which is higher than the global average (5). Inflammation and other clinical symptoms of asthma are caused by the activation of diverse cells such as lymphocytes, macrophages, neutrophils, and more importantly, mast cells and eosinophils. Asthma disease is a result of T cell activation after exposure to allergens such as pollen, pet hair, wool, and dust. T-cells differentiate to Th1, Th2, Th17, and other cells after antigen exposure and receptors stimulation. Th2 cells producing IL-4, IL-5, and IL-13 have a central role in creating inflammatory conditions and development of asthma symptoms, but the role of other T cell subsets remains controversial. IL-4 increases the expression of inflammatory cytokines in the lung, increases the differentiation of Th0 to Th2 cells and eventually stimulates the production of immunoglobulin E (IgE). IL-5 is responsible for differentiation, activation, and recruitment of eosinophils. IL-13 plays an important role in goblet cell hyperplasia and mucus secretion (6).

IFN-γ is predominantly produced by Th1 cells, and activates phagocytes, stimulates TNF-α secretion, and increases the turnover and migration of inflammatory cells to the site of inflammation (7). Studies on the role of IFN-γ in asthma pathogenesis show inhibitory effects of IFN-γ in Th2 differentiation (8). Because the balance between Th1 and Th2 and their secreted cytokines in asthma is important, an increase in the IFN-γ/IL-4 ration can decrease the asthma symptoms in patients. However, some studies have reported increased (9,10) as well as decreased (11,12) IFN-γ secretion in asthma. Notably, in some cases of asthma with high levels of IFN-γ, there is a greater degree of airway hyperresponsiveness and lung function impairment (13,14).

Th17 cells play a crucial role in the exacerbation of asthma symptoms by the secretion of proinflammatory IL-17A and IL-17F cytokines, which play important roles in neutrophilic inflammation and induction of inflammatory cytokine production from epithelial, endothelial, and fibroblast cells (15-17). Stimulation of bronchial epithelial cells and increased cytokine and chemokine production by IL-17 in allergic asthma is one of the causes of mucus gland hyperplasia, corticosteroid resistance, and airway deformation (18-22).

Since Th2 and Th17 have major roles in asthma pathophysiology, these two pathways are recently under profound investigation as targets of therapeutic strategies against

asthma (23). However, the exact roles of these cells in asthma pathophysiology are unknown. In order to take a close look at immune dysregulation in allergic asthma, we evaluated Th1, Th2, and Th17 cell functions by assessing the INF-γ, IL-4, and IL-17 gene and protein levels in asthma patients and healthy volunteers as controls.

2. Materials and Methods

2.1. Study population

This case-control study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Research Ethics Committee at the Mazandaran University of Medical Sciences, Sari, Iran. Written informed consent was obtained from each individual or their parents/relatives. In total, 44 participants including 24 patients with allergic asthma and 20 unrelated healthy controls, were enrolled between April 2016 and November 2016. The mean age of the patients was 12.46 $\pm$ 7.9 years. The diagnostic criteria for allergic asthma were according to the last revised Global Initiative on Asthma (GINA) guidelines (24). Allergic asthma was confirmed by skin prick test and eosinophilia. Clinical and laboratory data in addition to the results of spirometric evaluation were recorded in individual questionnaires. Patients with emphysema, COPD, and viral and bacterial infections were excluded from the study. Asthma severity was defined by the GINA Guidelines and was classified as mild, moderate, and severe persistent.

The control group comprised healthy volunteers without any underlying disease, including allergic, autoimmune, and infectious diseases. The asthma patients and healthy controls were matched according to age, sex, localization, and ethnicity

2.2. Blood sampling and cytokine measurement

Peripheral blood samples (5 ml) were collected from all patients and were kept in two separate test tubes either without anticoagulant or containing 50 mM ethylene diamine tetraacetic acid (EDTA). Serum was extracted from collected blood by centrifugation at 1500 $\times$ *g* for 15 min and was stored at -80◦C for further analysis.

Serum levels of IL-4, IL-17, or IFN-γ were quantified with a quantitative sandwich enzyme immunoassay using a commercial ELISA kit (R&D, CA). Briefly, the plates were coated overnight with the appropriate goat anti-human IL-4, IL-17, or IFN-γ specific antibody as the capture antibody at room temperature. Subsequently, 100 µl of appropriate standards or sera were added and the procedure was performed according to the manufacturer's instructions. Reference concentrations of the cytokines were used for

assay calibration. Absorbance was determined with an ELISA reader (Biotek ELX800, USA) at 450 nm. The sample concentrations were interpolated from standard curves and expressed in pg/ml. Inter- and intra-assay coefficients of variation were below 10%. To avoid any bias, all samples were analyzed blindly without any knowledge of the clinical status. All samples were run in duplicate with the appropriate standards on Nunc MaxiSorb 96-well micro plates (Sigma-Aldrich, Germany).

2.3. Isolation of peripheral blood mononuclear cells (PBMCs) and RNA extraction

Blood samples were obtained from the antecubital vein in test tubes containing 50 mM EDTA anticoagulant and then subjected PBMC isolation by the Ficoll–Hypaque density gradient (SEROMED; Biochrom KG, Berlin, Germany). Briefly, 3 ml of blood sample was mixed with an equal amount of sterile PBS, gently layered over 2 ml of Ficoll-Hypaque 1077, and centrifuged at 2500 rpm for 30 min at room temperature. The PBMC layer was carefully removed, and washed twice with PBS. Total RNA was isolated using a commercial column-based RNA extraction kit (Cinapure, Cinagene, Iran) according to the manufacturer's recommendations. The resulting RNA was resuspended in 300 µl DEPC water and stored at −80°C until use. Integrity of the purified RNA was verified by visualization of the 28S/18S banded pattern upon 1% agarose electrophoresis. The quantity of PBMC-derived RNA was determined using a WPA nanospectrophotometer (Biochrom. England).

2.4. cDNA synthesis and cytokine gene expression

RNA (1 µg) was transcribed to complementary DNA (cDNA) using a commercially available RevertAid^{TM} First-Strand accessible Easy cDNA synthesis kit (Parstous, Tehran, Iran) following the manufacturer's instructions. To obtain the maximum cDNA yield, oligo (dT) primers and random hexamers were used in equal amounts in a two-step reverse transcriptase reaction. The primer pairs for IL-4, IL-17, and IFN-γ were designed using AlleleID software. To inhibit reaction with the genomic DNA, the cytokine primer pairs were designed to span exon-exon junctions and possessed mRNA/cDNA specificity. Efficiency of all pairs of primers was evaluated by the dilution method as well as by temperature gradient reaction from 55°C to 65°C. Interestingly, all primer-sets had the best specificity at 60◦C. Quantitative gene amplification was performed using SYBR Premix Ex Taq (Takara, Japan) on the TaqiQ5 Cycler system (Bio-Rad, Ca, USA). To

reveal any non-specific amplification or primer dimer formation, final PCR products were subjected to melting curve analysis.

Relative gene expression was assessed using the $2^{-\Delta\Delta Ct}$ method in which the expression of each target gene in asthma patients was compared to that in the healthy controls (23). The data analysis was based on at least 3 independent experiments. To normalize any variation in gene expression, we used elongation factor-1 (EF-1) as a house-keeping gene and subtracted the cycle threshold (Ct) value of the target gene from the Ct of EF-1.

2.5. Statistical analysis

Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS) software version 19.0 (SPSS Inc., Chicago, IL, USA). The Kolmogrov-Smirnov test was used to ensure that the data were normally distributed. Quantitative data were evaluated using an independent Student's *t*-test or one-way analysis of variance (ANOVA) and qualitative data were assessed by applying the Chi-square or Fisher's exact test, as appropriate. Tukey's *post hoc* test was used for multiple comparisons. Correlations between cytokine concentrations and other parameters were made using Spearman's rank correlation tests. All tests were performed with a confidence level of 95% and a p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics and demographic parameters of the study population

The clinical characteristics and demographic parameters of the study population are presented in Table 1. The mean age of the patients and controls was not significantly different (p = 0.379). In addition, the two compared populations were matched in gender variables (p = 0.529). The prevalence of a family history of asthma was higher in the asthma patients compared to that in the controls (25% *vs*. 10%). Eosinophil counts and respiratory function parameters were significantly altered in the asthma patients than in the controls.

3.2. Relative gene expression and secretory levels of cytokines

In order to evaluate immune dysregulation in asthma, we compared the profile of IL-4, IL-17, and IFN-γ at the mRNA expression and secretory levels in asthma patients with those

Table 1: Anthropometric and clinical findings of study population.

	Healthy controls	Asthma patients	p-value
Age	15.1 ± 7.7	12.46 ± 7.9	0.379
Gender			
Male – n (%)	12 (60)	13 (54.2)	0.529
Female – n (%)	8 (40)	11(45.8)	
Eosinophils (/mm^3)	219.1 ± 66.9	829.1 ± 666.9	0.008
Family history of asthma	1 (10)	6 (25)	0.005
FEV1 (%), median (IQR), % predicted	101 (99-103)	72.4 (55-91)	0.024
FVC (%)	96 (94–98)	74.9 (55-92)	0.038

Table 2: Relative gene expression of cytokines in asthma patients compared to those in healthy controls.

Cytokine	Healthy controls	Asthma patients	p-value
IL-4	0.83 ± 0.24	2.5 ± 0.51	0.046
IL-17	0.94 ± 0.38	3.17 ± 0.61	0.03
IFN-γ	2.39 ± 0.92	0.86 ± 0.15	0.021

in the healthy controls. As shown in Table 2, the expression of IL-4 and IL-17 mRNA was increased by more than three-fold in asthma patients than in the controls. However, IFN-γ mRNA was dramatically decreased in the asthma patients. In addition, the secretory levels of IFN-γ were significantly lower in asthma patients than in the controls (13.45 ± 2.3 *vs*. 19.65 ± 1.6, p = 0.03). As shown in Fig 1, IL-4 and IL-17 levels were profoundly higher in asthma patients compared to those in the controls (p = 0.033 and 0.015, respectively).

The gene expression of cytokines in PBMCs from asthma patients and healthy controls was measured by quantitative PCR. All values are expressed as the mean ± SD. All values were normalized to the expression of EF-1 as the housekeeping gene.

3.3. Risk factors associated with asthma severity

In order to obtain a better image of the effect of cytokine variations and specific T helper (Th) polarization on asthma severity, the asthma patients were categorized as mild (6), moderate (12), and severe persistent (6) based on the GINA recommendation. As shown in Table 3, there are no significant differences between the three subpopulations according to age, sex, a positive family history of asthma, duration of the disease, and eosinophil count. However, lung function tests revealed significant differences among the three asthma subpopulations. To assess the relation between asthma severity and Th1, Th2, and Th17 cell variations, the mRNA expression and soluble levels of IFN-γ, IL-4, and IL-17 were compared among cases of mild, moderate, and severe asthma. As shown in Table 3, IL-4 mRNA expression had an increasing trend in mild to severe

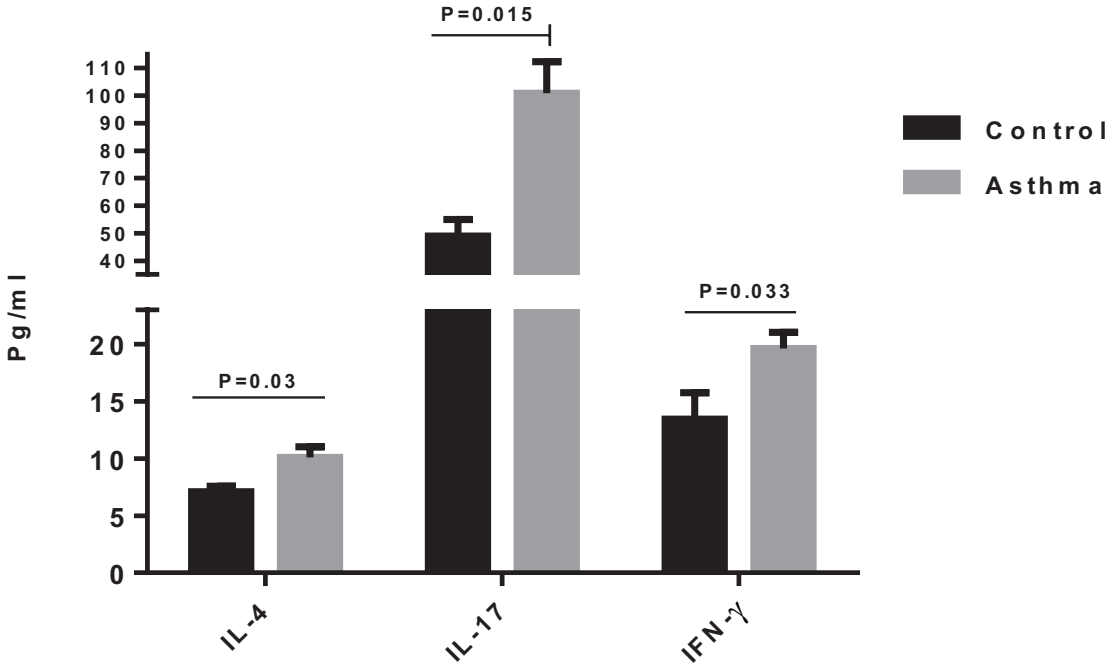

Figure 1: Serum was extracted from patient and control blood samples and soluble levels of IL-4, IL-17, and IFN-γ were quantified using a quantitative sandwich ELISA. As shown, the levels of IFN-γ were decreased significantly in the asthma patients than in the controls (p = 0.03), whereas the levels of IL-4 and IL-17 were significantly higher than those in the controls (p = 0.033 and 0.015, respectively).

persistent asthma but this fluctuation was not statistically significant. This situation was also observed for serum levels of IL-4. The variation of IFN-γ, at mRNA expression or serum levels, was not significant among the stages of asthma. However, IL-17 mRNA expression was significantly different among the different stages of asthma severity (p = 0.029). Tukey's *post hoc* test revealed a significant difference between mild and severe persistent asthma (p = 0.022). On the other hand, the expression of IL-17 mRNA was prominent in severe asthma compared to that in mild asthma. The soluble levels of IL-17 also showed an increasing trend among the asthma stages but statistical analysis did not confirm this variation (p = 0.555).

3.4. Correlation between disease characteristics and the cell function profiles

To evaluate any association between asthma criteria and immune dysregulation, we compared the eosinophil counts, and the relative mRNA expression of IFN-γ, IL-4, and IL-17 in asthma patients. As shown in Figure 2, the eosinophil count was reversely related with the FEV/FVC ratio in patients (r = 0.469, p = 0.032,). Interestingly, Figure 3 shows a close direct relationship between IL-17 and IL-4 mRNA expression in asthma patients (r = 0.46, p = 0.024).

TABLE 3: Risk factors associated with asthma severity.

	Mild persistence	Moderate persistence	Severe persistence	p-value
Age (year)	11.29 ± 7.5	11.0 ± 4.7	16.5 ± 12.3	0.37
Sex- M:F	3:4	7:4	3:3	0.67
Family history of asthma	3 (42.9)	2 (18.2)	1 (16.7)	0.431
Disease duration (year)	5.79 ± 2.2	4.41 ± 3.2	7.0 ± 10.4	0.653
Eosinophils (/µl)	783.4 ±463.3	933.2 ± 748.7	743.67 ± 541.3	0.862
IL-4 (fold change)	2.0 ± 1.12	2.11 ±0.49	3.8 ± 1.41	0.357
IL-17 (fold change)	1.0 ± 0.77	3.13 ± 0.76	5.45 ± 1.4	0.029
IFN-γ (fold change)	3.2 ± 2.0	2.13 ± 0.76	3.03 ± 1.5	0.889
IL-4 (pg/ml)	9.28 ± 1.1	8.96 ± 1.4	12.76 ± 1.8	0.175
IL-17 (pg/ml)	85.49± 34.47	101.92 ± 7.37	123.73 ± 19.67	0.555
IFN-γ (pg/ml)	16.0 ± 1.83	11.0 ± 1.65	14.31 ± 2.44	0.187

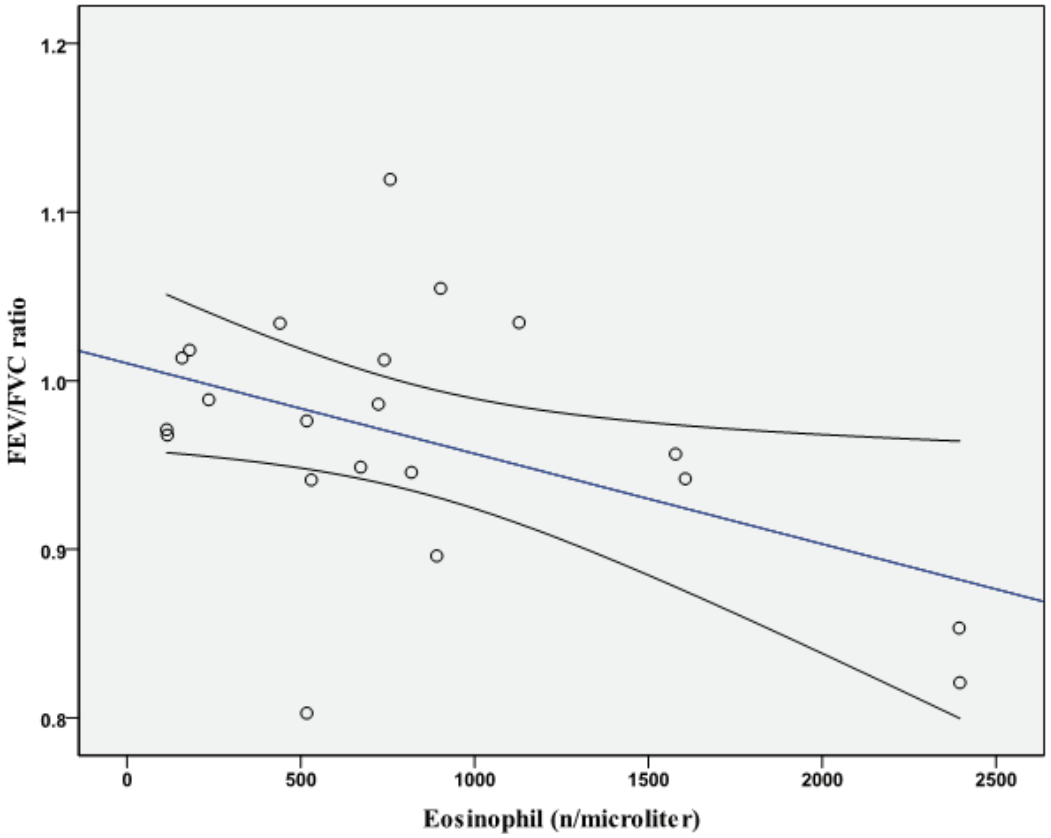

Figure 2: Relationship between the FEV/FVC ratio and eosinophil counts in asthma patients. There was a significant reverse relationship in asthma patients (r = -0.469, p = 0.032). Scatter lines show the 95% confidence interval of the mean.

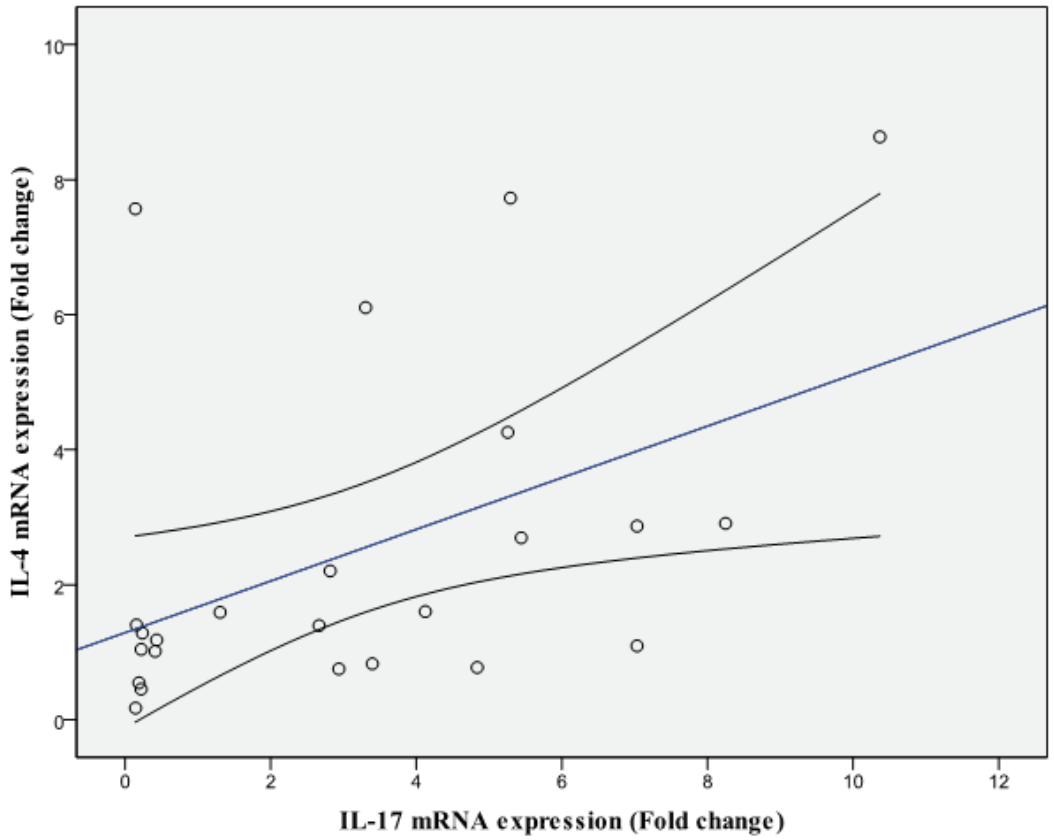

Figure 3: Relationship between IL-17 and IL-4 mRNA expression in asthma patients. A direct close association was seen between IL-17 and IL-4 fold changes in asthma (r = 0.460, p = 0.024). Scatter lines show the 95% confidence interval of the mean.

4. Discussion

The main findings of this study were increased expression of IL-4 and IL-17 gene and protein expression and decreased expression of IFN-γ in children with allergic asthma compared to that in the controls. We evaluated the mRNA expression and serum levels of IFN-γ, IL-4, and IL-17 as restricted candidates of Th1, Th2, and Th17 cytokines. A direct positive correlation between IL-17 and IL-4 mRNA expression in asthma patients represents a cohesiveness of the Th2 and Th17 cell functions in asthma development. In addition, high expression of IL-17 mRNA in severe asthma emphasizes the critical role of Th17 cells in asthma pathogenesis. Allergic asthma is a set of disorders with different degrees of manifestation including bronchostenosis, hypersensitivity, airway inflammation, and infiltration of lymphocytes and eosinophils into the bronchial tubes. T lymphocytes, eosinophils, and mast cells play a basic role in this disorder (26). The disease often begins in childhood and serum levels of total IgE and specific IgE are elevated (27).

The incidence and severity of asthma can be affected by various environmental and genetic factors. Furthermore, viruses, allergens, and occupational exposure also can change the disease course (28, 29). We have shown that people with a family history of asthma are more than twice susceptible to develop asthma than are healthy people, as was shown in other studies as well. However, some studies have shown that the prevalence of asthma in immature boys is higher than that in immature girls (30); however, this is different from the observations in adults, as the disease is more prevalent in women than in men, due to the impact of hormones such as estrogen and progesterone and the shift of the immune system to Th2 type (31). Since the majority of our patients were children with an average age under 12 years, the slight increase in male patients is not surprising.

In line with other studies (32), we have shown high eosinophilia in asthma patients compared to that in the controls. On the other hand, a reverse significant relationship between eosinophil count and lung function test parameter emphasized the role of eosinophils in asthma. Activated eosinophils release toxic proteins such as major basic protein, cationic proteins, and peroxidase. These proteins might disturb respiratory function via destruction of airway epithelial cells and therefore, decrease the respiratory discharge volume in patients (33,34). Eosinophils are prominent cellular effectors in Type 2 inflammatory responses and have a pivotal role in the maintenance of long term inflammation in asthma patients compared to that in healthy controls. A reverse significant correlation between eosinophils and forced expiratory volume (FVC) in asthma patients emphasized the role of eosinophils in the pathophysiology of asthma.

Increased expression of IL-17 mRNA/cDNA in asthma patients compared to that in the controls, may indicate a pivotal role for Th17 cells in asthma severity and progression. Further, a significantly higher expression of IL-17 in the severe form of asthma is thought to contribute to airway hyperresponsiveness and bronchial inflammation. Th17 cells are defined by the production IL-17A, IL-17F, IL-21, and IL-22 which have been identified in bronchial biopsies from patients with severe asthma (15-17, 21, 35-36). IL-17 is known to be associated with mucus hypersecretion and epithelial hyperplasia in the airways of asthma patients (37). We showed a direct correlation between IL-17 mRNA expression and eosinophils in asthma patients (unpublished data). In line with our findings, Guerra et al. have recently reported that eosinophils are the main source of IL-17E production in a murine model of asthma challenged with *Aspergillus fumigatus*. They showed that depletion of eosinophils correlates with decreased airway hypersensitivity and mucus production (38). The upregulation of IL-17 in severe asthma may affect the eosinophils and neutrophils which induce inflammatory conditions and tissue remodeling in allergic asthma. Increased coordination of IL-4 and IL-17 expression in asthma patients has

two speculated mechanisms: synergistic production of IL-4 and IL-17 by eosinophils or T lymphocytes, and infiltration and activation of Th2 and Th17 cells. In line with our findings, Irvin *et al* reported an influx of double positive Th2/Th17 cells in BAL from asthma patients compared to that in the controls (39).

Overall, the results of this study suggest high expression of IL-17 and IL-4 mRNA along with prominent serum levels of these cytokines in asthma patients compared to those in healthy controls, highlighting the role of Th2 and Th17 cells in asthma pathogenesis. Increased IL-17 expression/production in the severe form of asthma indicates Th17 cells as potential targets d direct therapeutic strategies.

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Conflict of Interest

The authors declare no conflict of interest.

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