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Elevated Th17 cell frequencies and Th17/Treg ratio are associated with airway hyperresponsiveness in asthmatic children

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ABSTRACT

Introduction: The elevation of T helper (Th)17 cell frequencies and the imbalance of Th17/regulatory T (Treg) cells occur in asthma pathogenesis. Airway hyperresponsiveness (AHR) is a cardinal feature of asthma, and Th17 responses can promote AHR. We hypothesized that changes in Th17 cells and the Th17/Treg ratio correlate with AHR in asthmatic children.

Methods: Twenty asthmatic children and twenty healthy children were included in the study. The peak expiratory flow (PEF) % pred, forced expiratory volume in 1 s (FEV₁) % pred and the FEV₁/forced vital capacity (FVC) ratio were measured in all subjects. Methacholine challenge test (MCT) was performed in asthmatic children. Flow cytometric analysis was used to determine the proportions of Th17 and Treg cells in peripheral blood mononuclear cells. ELISA was used to assess serum levels of interleukin (IL)-17A and IL-10.

Results: Th17 cell frequencies ($2.272 \pm 0.207\%$ in asthmatics, $1.193 \pm 0.131\%$ in controls, $P < 0.01$) and Th17/Treg ratios (0.371 ± 0.0387 in asthmatics, 0.183 ± 0.020 in controls, $P < 0.01$) were significantly increased in asthmatic children compared to controls. In asthmatic children, the MCT grade had positive correlations with the Th17 cell frequencies [$r = 0.718$, $P < 0.01$], serum IL-17A level [$r = 0.753$, $P < 0.01$] and Th17/Treg ratio [$r = 0.721$, $P < 0.01$], while the log₁₀PD20-FEV₁ value was negatively correlated with the Th17 cell frequencies [$r = -0.654$, $P < 0.01$], serum IL-17A level [$r = -0.652$, $P < 0.01$] and Th17/Treg ratio [$r = -0.625$, $P < 0.01$].

Conclusion: Th17 cell, IL-17A and Th17/Treg ratio were positively correlated with AHR in asthmatic children. It may be helpful to monitor Th17 cells and the Th17/Treg ratio as indicators of AHR in clinical practice.

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Introduction

Asthma is currently a serious public health concern. In 2013, 8.3% of children worldwide suffered from asthma (1). Asthma appears as a recurrent cough, wheeze or chest tightness, causing substantial psychological pressure and financial burdens for asthmatic children and their families. Airway hyperresponsiveness (AHR), which is one of the defining characteristics of asthma, is mainly responsible for these symptoms. Therefore, measuring AHR is of great importance for evaluating severity, monitoring progression and observing treatment effects in asthma patients in clinical practice. Methacholine challenge test (MCT) is a highly specific examination of AHR (2). However, some young asthmatic children cannot

cooperate with the testing procedure, which increases the difficulty of establishing a definitive diagnosis. Thus, it is worth exploring more convenient targets for measuring AHR.

Asthma involves different T cell-driven inflammatory processes, and CD4⁺ T cells remain at the forefront of asthma pathogenesis (3). In previous studies, imbalances of T helper (Th)1 and Th2 cells have been widely acknowledged as a main cause of asthmatic disease and considered central to the classic immune theory in asthma (4). Th17 cells have been reported to produce the cytokine interleukin (IL)-17 and enhance Th2 cell-mediated eosinophilic airway inflammation, which plays a vital role in the pathogenesis of asthma (5,6). It is universally acknowledged that Th17

Table 1. The demographic characteristics and pulmonary function of control and asthma group.

	Control(<i>n</i> = 20)	Asthma(<i>n</i> = 20)	<i>P</i>
Sex (male/female)	12/8	15/5	0.311
Age (years)	9(6.75, 10.25) ^a	8.5(6.75, 10)	0.682
PEF % pred (%)	92.85 ± 11.26 ^b	79.14 ± 12.24	<0.01
FEV ₁ % pred (%)	105.62 ± 10.82	85.38 ± 11.01	<0.01
FEV ₁ /FVC (%)	87.22 ± 4.17	87.71 ± 7.26	0.797

^aMedian(interquartile range).^bMean ± SD; PEF: peak expiratory flow; FEV₁: forced expiratory volume in 1 s.

responses and IL-17 can promote AHR (7–10). Regulatory T (Treg) cells, which secrete IL-10 and transforming growth factor (TGF)- β , mediate the anti-inflammatory response and suppress overactive Th2 responses (11). Previous studies revealed that Tregs can restrain AHR (12–14). It is noteworthy that Treg cells can also inhibit Th17 cell secretion of IL-17 and suppress Th17 differentiation (12,15). Interactions between Th17 and Treg cells have been proposed to regulate the evolution of autoimmune and allergic reactions (16). Jiang H (17) and colleagues found an imbalance of Th17 and Treg cells in asthmatic mice. Our previous studies confirmed the presence of the Th17/Treg imbalance in asthmatic children (18). These results showed that the imbalance of Th17 and Treg cells participates in the immune milieu of asthma. However, no report has described the association between Th17/Treg imbalance and AHR in asthma.

Thus, the purpose of this study was to explore the association of Th17 cells, Treg cells and the imbalance of Th17 and Treg cells with AHR. Therefore, we examined Th17 and Treg cells in all subjects. We also performed pulmonary function test and MCT to examine AHR. We hypothesized that the imbalance of Th17 and Treg cells might be correlated with AHR in asthmatic children.

Methods

Study subjects

Twenty children with IgE-mediated allergic asthma and twenty healthy children were included in the study. Table 1 summarizes the demographic characteristics of these subjects. The diagnosis of asthma was made according to the criteria of the Global Initiative for Asthma (GINA). In addition, all asthmatic patients were clinically allergic to dust mites and had been found to have a positive test for dust mite-specific IgE. None of the patients had been administered corticosteroids or other immunosuppressants within one month before inclusion in the study. All allergic asthmatic children had no other chronic diseases, and the

healthy children had no chronic diseases, including allergic disease. None of the subjects suffered from acute infection in the week before they were recruited for this study. The study was approved by the Ethical Committee of the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University in China.

Pulmonary function test and MCT

For all subjects, forced expiratory volume in 1 s (FEV₁), forced vital capacity (FVC) and peak expiratory flow (PEF) were measured with a JAEGER MasterScope Body automated system (JAEGER, Germany) (19). Due to ethical requirements, we only performed MCT in asthmatic children. In MCT, cumulative doses of methacholine of 0.4, 1.6, 6.4 and 12.8 μ mol were administered by inhaling the corresponding concentrations of methacholine diluent (6.25, 25 or 50 mg/ml) in sequence (20,21). Asthmatic children inhaled incremental doses of methacholine until the occurrence of a 20% reduction of FEV₁. MCT was considered positive for the cumulative dose of inhaled methacholine that caused a 20% decrease in FEV₁ from baseline. For statistical analysis, the response was denoted as grade 0 when MCT yielded a negative result after the patient inhaled the largest cumulative dose (12.8 μ mol) of methacholine; MCT positivity was recorded when a positive result was obtained with a 12.8, 6.4, 1.6 or 0.4 μ mol cumulative dose of inhaled methacholine, which was denoted as grade 1, 2, 3, or 4, respectively. The provocation dose of methacholine that produced a 20% decrease in FEV₁ (PD20-FEV₁) was calculated for asthmatic children who had positive results on the MCT. For asthmatic children who had negative results on the MCT, the PD20-FEV₁ value was reported as ">12.8 μ mol" (22,23).

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by human peripheral blood lymphocyte separation fluid (TBD Science, China). CD4⁺IL-17A⁺ Th17 cells were tagged with FITC anti-human CD4 and PE anti-human IL-17A antibodies (BD Bioscience, USA). First, the PBMCs were cultured for 5 h with PMA/Ionomycin/BFA/Monensin Mixture (Multi Science, China). Second, the PBMCs were stained with a FITC anti-human CD4 antibody after culture. Third, the PBMCs were stimulated with a Fix and Perm cell permeabilization kit (Multi Science, China) for intracellular staining. Then, the PBMCs were stained with a PE-labeled anti-

Table 2. The result of methacholine challenge test in asthmatic subjects.

MCT	Asthmatic subjects	
	Positive (n = 17)	Negative (n = 3)
MCT grade (0/1/2/3/4)	0/6/8/3/0	3/0/0/0/0
PD20-FEV ₁ (μmol)	3.86(2.24, 7.10) ^a	>12.8
Log ₁₀ PD20-FEV ₁	0.59(0.35, 0.85)	NA

^aMedian(interquartile range); MCT: methacholine challenge test; PD20-FEV₁: the provocation dose of methacholine that produced a 20% decrease in FEV₁; NA: not applicable.

human IL-17A antibody. For CD4⁺CD25⁺CD127^{low} Treg cell analysis, the PBMCs were stained with FITC anti-human CD4, APC anti-human CD25 and PE anti-human CD127 antibodies (BD Bioscience, USA). Matching fluorescence-conjugated antibodies were selected as the isotype controls. The samples were analyzed with an FC500 flow cytometer (Beckman Coulter, USA). Background fluorescence was assessed by the corresponding isotype control. KALUZA 2.0 software (Beckman Coulter, USA) was used to analyze the data.

Quantitative real-time PCR (QRT-PCR)

Total RNA in PBMCs was extracted by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA was evaluated and quantified by a Thermo Scientific Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). For mRNA detection, total RNA was reverse transcribed using PrimeScript RT Master Mix (TaKaRa, Japan). The expression of mRNA was detected using LightCycler 480 SYBR Green I Master Mix (Roche, Switzerland) on a LightCycler 480 System (Roche, Switzerland). GAPDH was used as an internal control. The following gene-specific primers were used: GAPDH, 5'-GGACCTGACCTGCCG TCTAG-3' (forward) and 5'-GTAGCCCAGGATG CCCTTGA-3' (reverse); retinoic acid-related orphan receptor γ t (ROR γ t) ROR γ t, 5'-AATCTCATCCTCGG AAAAGTG-3' (forward) and 5'-CAATGGAAGTGGTG CTGGTTA-3' (reverse); and Foxp3, 5'-TGGAGG AACTCTGGGAATGTG-3' (forward) and 5'-AGG CTTCATCTGTGGCATCAT-3' (reverse). The Ct value of each sample was normalized to the level of GAPDH, and relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-17A and IL-10 in the serum were determined using ELISA following the manufacturer's instructions. The ELISA kit was purchased from Boyun (Shanghai, China).

Statistical analysis

Pearson's Chi-square test and independent-samples *t*-tests were used to analyze differences between groups. The correlation coefficient *r* was generated using Spearman's rank correlation. A *p* values of less than 0.01 was considered statistically significant.

Results

Characteristics of the study subjects

Table 1 shows that the two groups were similar in composition in terms of age and sex.

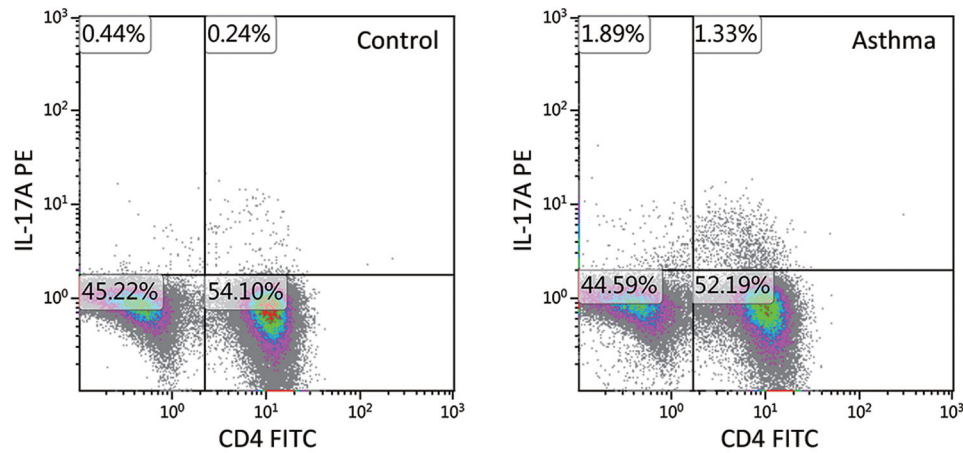
Pulmonary function test and MCT

Asthmatic subjects had lower PEF % pred ($79.14 \pm 12.24\%$ in asthma patients, $92.85 \pm 11.26\%$ in controls, $P < 0.01$) and FEV₁% pred ($85.38 \pm 11.01\%$ in asthma patients, $105.62 \pm 10.82\%$ in controls, $P < 0.01$) compared to healthy subjects. FEV₁/FVC was not significantly different between asthma patients ($87.71 \pm 7.26\%$) and healthy controls ($87.22 \pm 4.17\%$) ($P = 0.797$) (Table 1). Among asthmatic children, seventeen subjects had positive results on the MCT, and three subjects had negative MCT results (Table 2).

Increased Th17 cell frequencies and Th17/Treg ratios in peripheral blood from asthmatic patients

The proportions of Th17 and Treg cells among CD4⁺ cells in peripheral blood from the study subjects were determined by flow cytometric analysis (Figure 1). Th17 cell frequencies were significantly increased in asthmatic children ($2.272 \pm 0.207\%$) compared to those in healthy subjects ($1.193 \pm 0.131\%$) ($P < 0.01$) (Figure 2a). However, Treg cells did not show a significant difference between asthma patients ($6.341 \pm 0.261\%$) and healthy controls ($6.680 \pm 0.267\%$) ($P = 0.369$) (Figure 2b). As shown in Figure 2c, the Th17/Treg ratio tended to be higher in the asthma group (0.371 ± 0.0387) than in the control group (0.183 ± 0.020) ($P < 0.01$). Using QRT-PCR analysis, we found that the mRNA expression of the Th17 lineage-specifying transcription factor ROR γ t was significantly upregulated in the asthma group (1.559 ± 0.184) compared to that in the control group (0.952 ± 0.103) ($P < 0.01$) (Figure 2d), while the mRNA expression of the transcription factor Foxp3 in Treg cells showed no significant difference between asthma patients (1.236 ± 0.101) and healthy controls (1.207 ± 0.167) ($P = 0.882$) (Figure 2e). The QRT-PCR results were consistent with the results of flow cytometric analysis.

(a)



(b)

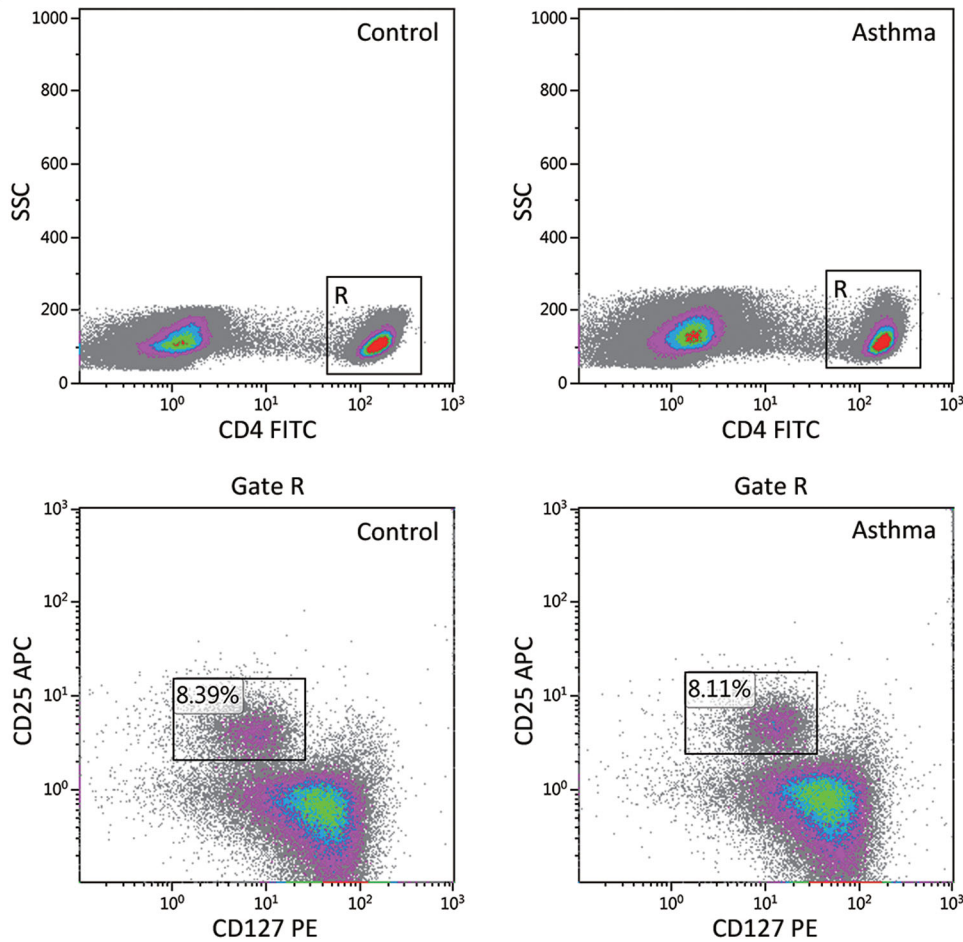


Figure 1. Representative Flow cytometric analysis figures of CD4⁺IL-17A⁺Th17 cells (a) and CD4⁺CD25⁺CD127^{low}Treg cells (b) from individual of each group. The percentage of positive cells is shown in each panel.

Altered production of Th17 and Treg cytokines

IL-17A and IL-10 are the dominant cytokines produced by Th17 and Treg cells, respectively. Using ELISA, we found that the level of IL-17A was significantly higher in the asthma group [(61.931 ± 4.387)

pg/ml] than in the control group [(37.137 ± 1.772) pg/ml] ($P < 0.01$) (Figure 2f. However, serum levels of IL-10 did not show a significant difference between asthma patients [(173.187 ± 16.125) pg/ml] and controls [(165.992 ± 16.959) pg/ml] ($P = 0.76$) (Figure 2g).

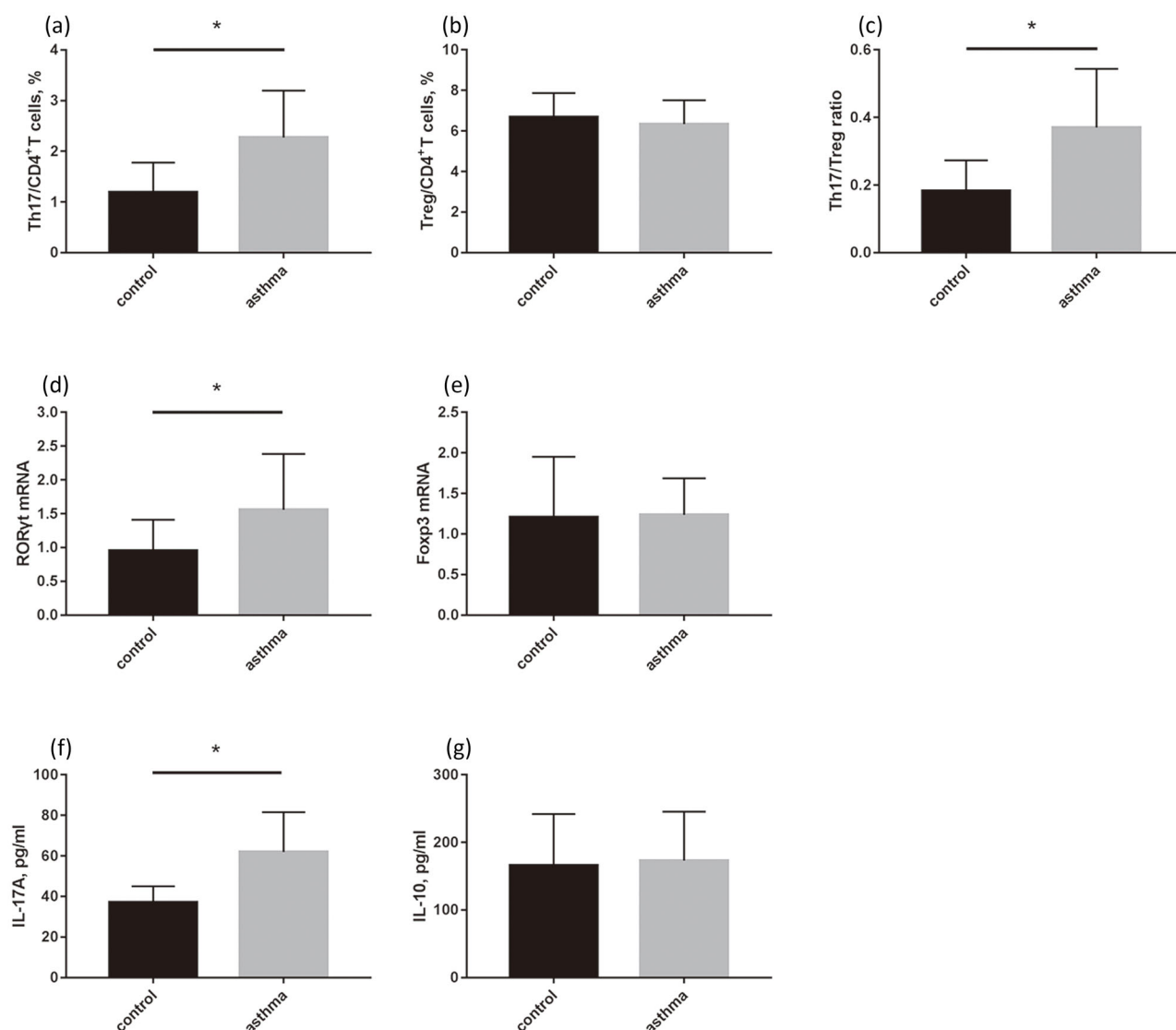


Figure 2. The percentage of Th17 in CD4⁺ cells (a), the percentage of Treg in CD4⁺ T cells (b), Th17/Treg ratio (c), RORγt mRNA (d), Foxp3 mRNA (e), IL-17A (f) and IL-10 (g) from individual of each group. * $P < 0.01$.

Correlations of Th17 cell frequencies with pulmonary function and MCT in the asthma group

PEF % pred, FEV₁% pred and FEV₁/FVC are common pulmonary function surveillance indicators used in the assessment of asthma. As shown in Figure 3a, b, and c, PEF % pred, FEV₁% pred and FEV₁/FVC had no significant correlations with the percentage of Th17 cells. Similarly, PEF % pred, FEV₁% pred and FEV₁/FVC had no significant correlations with IL-17A expression (Figure 3d, e, and f). It is worth noting that Th17 cell frequencies [$r = 0.718$, 95% CI (0.392 to 0.884), $P < 0.01$] (Figure 3g) and IL-17A levels [$r = 0.753$, 95% CI (0.445 to 0.900), $P < 0.01$] (Figure 3i) were positively correlated with MCT grade. The Th17 cell frequencies [$r = -0.654$, 95% CI (-0.867 to -0.239), $P < 0.01$] (Figure 3h) and serum IL-17A level [$r = -0.652$, 95% CI (-0.866 to -0.235),

$P < 0.01$] (Figure 3j) were negatively correlated with the log₁₀PD20-FEV₁ value.

Correlation of Treg cell frequencies with MCT grade in the asthma group

There was no significant correlation between MCT grade and Treg cell frequencies (Figure 3k). Similarly, MCT grade was not correlated with IL-10 levels (Figure 3l).

Correlations of the Th17/Treg ratio with pulmonary function and MCT in the asthma group

FEV₁% pred and FEV₁/FVC had no significant correlation with the Th17/Treg ratio (Figure 4b and c). However, PEF % pred and the Th17/Treg ratio showed a trend toward a negative correlation [$r = -0.430$, 95%

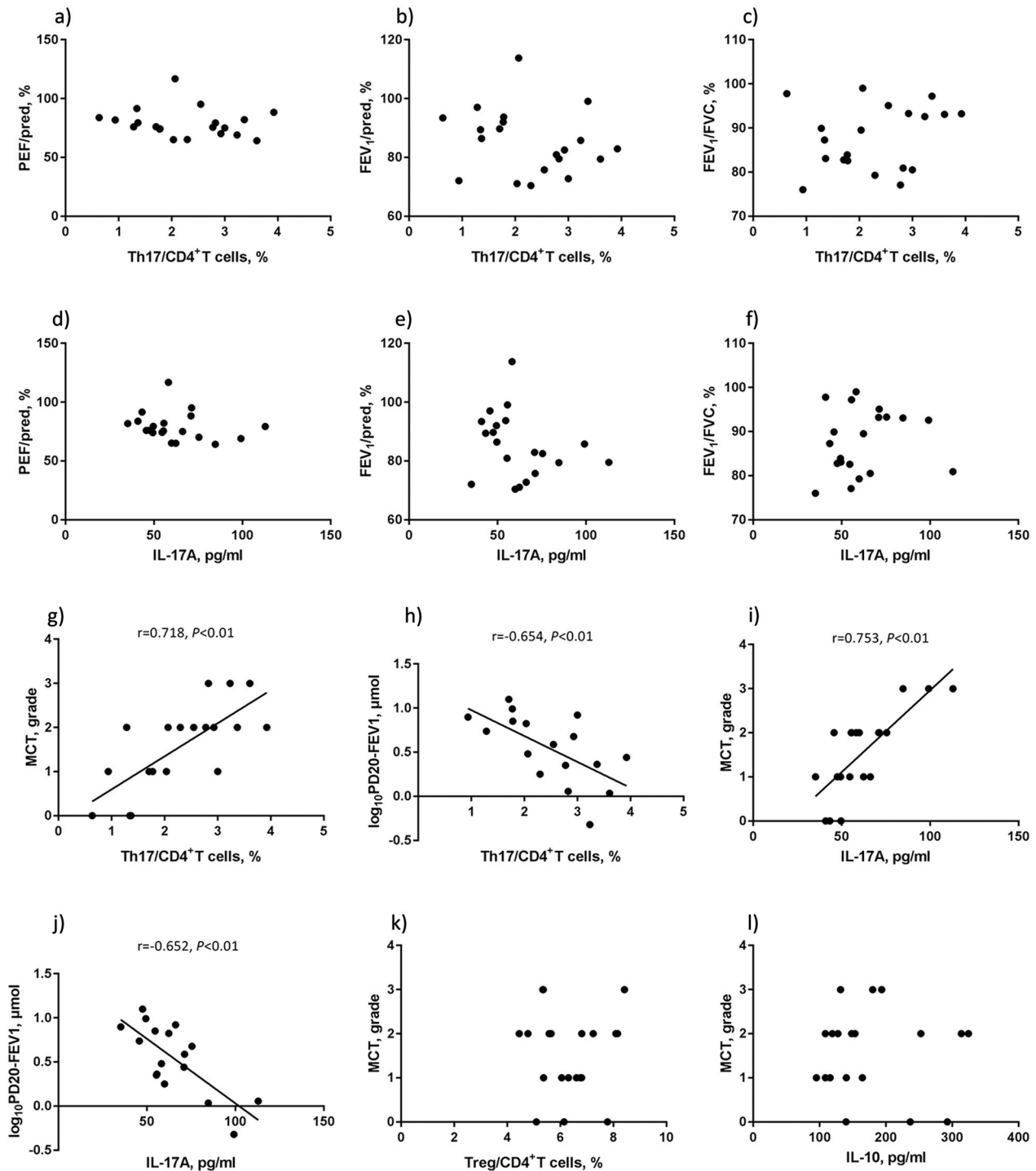


Figure 3. Correlations between the percentage of Th17 in CD4⁺ T cells with PEF%pred (a), FEV₁%pred (b), FEV₁/FVC (c), MCT grade (g) and log₁₀PD20-FEV₁ (h) in asthmatic children. Correlations between IL-17A with PEF%pred (d), FEV₁%pred (e), FEV₁/FVC (f), MCT grade (i) and log₁₀PD20-FEV₁ (j) in asthmatic children. Correlations between the percentage of Treg in CD4⁺ T cells (k) and IL-10 (l) with MCT grade in asthmatic children.

CI (−0.749 to 0.030), $P=0.059$] (Figure 4a), although the correlation was not statistically significant. Furthermore, the Th17/Treg ratio was positively correlated with MCT grade [$r=0.721$, 95% CI (0.397 to 0.885), $P<0.01$] (Figure 4d), and was negatively correlated with the log₁₀PD20-FEV₁ value [$r=-0.625$, 95% CI (−0.625 to −0.191), $P<0.01$] (Figure 4e).

Discussion

In our study, we confirmed the elevation of Th17 cell frequencies and the Th17/Treg ratio in asthmatic children compared to controls. We found that circulating Th17 cells, IL-17A levels and the Th17/Treg ratio were positively correlated with MCT grade, while

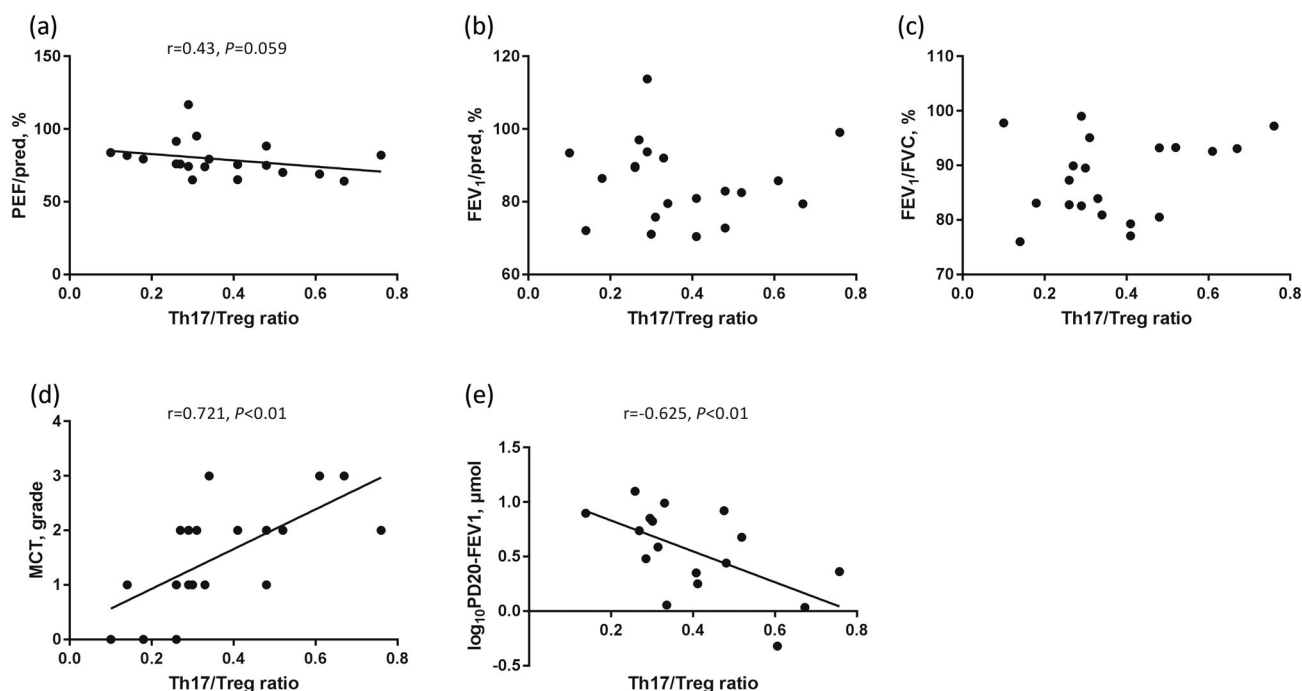


Figure 4. Correlations between Th17/Treg ratio with PEF%pred (a), FEV₁%pred (b), FEV₁/FVC (c), MCT grade (d) and log₁₀PD20-FEV₁ (e) in asthmatic children.

Th17 cells, IL-17A levels and the Th17/Treg ratio were negative correlated with the log₁₀PD20-FEV₁ in asthmatic children. These findings suggested that Th17 responses and Th17/Treg imbalance are associated with AHR.

Th17 cells, which express the characteristic transcription factor ROR γ t and produce IL-17, play a critical role in the pathogenesis of asthma. Evidence shows that the elevation of Th17 cells and IL-17 in asthma participates in the development of AHR. It was reported that chronic obstructive pulmonary disease (COPD) patients have an elevation of Th17 cells in the blood, and the level of blood Th17 cells had a negative association with FEV₁% (24). In COPD patients, the quantity of CD4⁺IL-17⁺ cells in the small airways exhibited a positive correlation with airflow limitation (25). This evidence suggests an association between Th17 cells and airflow obstruction. However, this association between Th17 and airflow obstruction targets, such as PEF % pred, FEV₁% pred and FEV₁/FVC, was not confirmed in asthmatic subjects in our study. Our previous works showed that IL-17A and Th17 cells were positively correlated with airway resistance after inhalational methacholine challenge in an obese asthmatic mouse model (26). Similarly, we found that the proportion of Th17 cells in CD4⁺ T cells and the level of serum IL-17A were positively correlated with AHR in asthmatic children.

Treg cells play a critical role in normal immune responses, mainly in terms of maintaining immunological tolerance and limiting excessive immune responses (27). In asthma, Treg cells suppress inflammatory cells, including Th2 and Th17 cells (12,28), thus inhibiting airway inflammation and AHR (11). Previous studies have shown that inhaled corticosteroid (ICS) treatment, which is the standard drug treatment for asthma patients, elevated the level of Treg cells (29,30). Lee JH (31) found that children with moderate to severe asthma had higher Treg cell levels than children with mild asthma. Therefore, the severity of asthma, previous anti-asthma treatment and asthma control influenced the overall level of Treg cells in asthma patients. Several different opinions about Treg levels in asthma have been reported. Our study found no significant difference in Treg cell levels between asthmatic patients and healthy children. Similarly, Furukawa T (32) found that the percentage of Treg cells was not significantly different between the asthma group and the control group. Interestingly, Hartl D (30) found that Treg cells decreased in patients with allergic asthma, but Singh AM (29) found that asthmatic children had higher Treg levels than those in the controls. It is definitely worthwhile to investigate the association between Treg cells and AHR in view of Treg suppressing Th2 and Th17 cells which contribute to AHR in asthma. Previous studies suggested that infusion of Treg cells and depletion of

Treg cells alleviated and aggravated the degree of AHR, respectively (12,14). In mouse models of allergic airway disease, AHR can be alleviated by intravenous immunoglobulin (IVIg), which is associated with a significant increase in Treg cells. After depleting Treg cells, IVIg failed to attenuate AHR (33). These findings suggest that Treg cells are essential mediators of immune homeostasis that suppress AHR, although we did not find a significant linear correlation between Treg cell frequencies and MCT grade in asthmatic children.

It is universally acknowledged that the balance between Th17 and Treg cells participates in the pathogenesis of immune disorders. The Th17/Treg balance trends toward the proinflammatory Th17 side in several autoimmune disorders, including rheumatoid arthritis and multiple sclerosis (34), as well as in allergic diseases, including atopic dermatitis, asthma and allergic rhinitis (35,36). The balance of Th17 and Treg cells embodies the interrelation between the Th17 and Treg responses. Therefore, it is also worth considering the impact of Th17/Treg imbalance on AHR. The Foxp3/ROR γ t ratio was positively correlated with lung function in COPD patients (37). In this study, the Th17/Treg ratio and PEF % pred showed a trend toward a negative correlation. Th17/Treg imbalance might be associated with airflow limitation. It is noteworthy that the Th17/Treg ratio was positively correlated with the MCT grade in asthmatic children. In addition, ICS is a conventional treatment for asthma and can decrease AHR (38). Previous studies have shown that fluticasone can reduce Th17 cells and increase Treg cells in a dose-dependent manner (16). The percentages of Treg cells in peripheral blood and bronchoalveolar lavage fluid were increased in asthmatic children after ICS treatment (30). These findings verified that the imbalance of Th17 and Treg cells is associated with AHR in asthmatic subjects. In addition, restoration of the Th17/Treg imbalance generated by ICS treatment may contribute to the reduction of AHR. Taken together, these findings suggest the capacity of the Th17/Treg ratio to reflect the degree of AHR.

According to our study, Th17 cell frequencies and the Th17/Treg ratio in the peripheral blood were positively correlated with AHR and might be auxiliary targets for measuring AHR. These targets warrant thorough investigations to affirm their use in clinical applications. Even though this is a small sample study, we believe that our work provides connections between fundamental animal research, clinical research and clinical applications.

Conclusion

The evidence presented in our study confirmed the imbalance of Th17 and Treg cells in asthmatic children. Furthermore, Th17 cell frequencies, IL-17A levels and the Th17/Treg ratio were positively correlated with AHR in asthmatic children. The Th17 cell level and the Th17/Treg ratio appeared to be useful for monitoring the immune state and measuring AHR in asthma patients, which might be a promising approach for facilitating diagnosis, assessing the efficacy of pharmacologic interventions and investigating the pathophysiology of asthma.

Declaration of interest

No potential conflict of interest was reported by the author(s).

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