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## **Imbalance of Peripheral Th17 and Regulatory T Cells in Children with Allergic Rhinitis and Bronchial Asthma**

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### **ABSTRAT**

The purpose of the present study is to investigate the prevalence of Th17 and regulatory T (Treg) cells in children with allergic rhinitis (AR) accompanying with bronchial asthma (BA).

24 children with AR, 22 children with BA, 18 children with AR accompanying with BA, and 20 healthy controls were recruited. The prevalence of peripheral blood Th17 and Treg cells were determined by flow cytometry. mRNA expression of retinoid-acid receptor-related orphan receptor (ROR)- $\gamma$ t and forkhead box P3 (Foxp3) were determined by realtime polymerase chain reaction. Cytokine expressions in plasma were determined by enzyme linked immunosorbent assay.

The frequency of Th17 cells, ROR- $\gamma$ t mRNA expression, and the plasma levels of IL-17 were significantly higher, while Treg cells and Transforming growth factor (TGF)- $\beta$ 1 were significantly lower in children with AR accompanying with BA compared with those in children with AR or BA alone or control subjects. In children with allergic airway disease, total IgE levels were positively correlated to the frequency of Th17 cells ( $r=0.607$ ,  $p<0.01$ ), plasma IL-17 levels, and negatively correlated to the frequency of Treg cells ( $r=-0.429$ ,  $p<0.01$ ) and TGF- $\beta$ 1 levels ( $r=-0.224$ ,  $p<0.01$ ). While Forced expiratory volume in one second (FEV1) (% predicted) was negatively correlated to the frequency of Th17 cells ( $r=-0.602$ ,  $p<0.01$ ), plasma IL-17 levels ( $r=-0.577$ ,  $p<0.01$ ), and positively correlated to the frequency of Treg cells ( $r=0.504$ ,  $p<0.01$ ) and TGF- $\beta$ 1 levels ( $r=0.231$ ,  $p<0.05$ ).

Our results demonstrate that the imbalance of peripheral Th17/Treg cells plays an important role in the pathogenesis of AR accompanying with BA.

**Keywords:** Allergic Rhinitis; Bronchial Asthma; Th17 cells; Treg cells

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## INTRODUCTION

Allergic diseases, including allergic rhinitis (AR) and bronchial asthma (BA), are chronic inflammatory disorders characterized by an aberrant immune response to harmless environmental antigens, such as house dust mites.<sup>1</sup> The inhalation of allergens leads to hyperreactivity, recruitment of eosinophils, mast cells and lymphocytes in the upper and lower airways, triggering the inflammatory cascade and generating local and systemic inflammatory responses.<sup>2</sup>

Experimental and clinical data have indicated that the balance between the Th1 and Th2 cells responses are central to the pathogenesis of allergic airway inflammation.<sup>3</sup> It is widely accepted that Th2 cytokines such as interleukin (IL)-4, IL-5, IL-9 and IL-13 play critical roles in orchestrating and amplifying allergic inflammation, while the Th1 cytokines, such as interferon- $\gamma$ , is thought to prevent this process.<sup>4</sup> However, in recent years, it was recognized that Th1/Th2 imbalance does not fully explain the aetiology of asthma, reversing the Th1/Th2 imbalance does not fully control asthmatic symptoms in humans.<sup>5,6</sup> Th17 and regulatory T (Treg) cells have been recently described as two distinct subsets from within the Th1 and Th2 T cell population. Th17 cells and their effector cytokine IL-17 have been showed to play pro-inflammatory roles in the development of autoimmune disorders and chronic inflammation,<sup>6</sup> while Treg cells play an anti-inflammatory role and maintain tolerance to self-components by producing anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)- $\beta$ .<sup>7,8</sup> A number of recent studies have evidenced that both an excess in Th17 function, or increased numbers of Th17 cells, and a defect in Treg function or reduced numbers of Treg cells, trigger the development and progression of allergic diseases, including allergic asthma and rhinitis.<sup>5,9,10</sup> Thus, the balance between Th17 and Treg cells may be important in the development of allergic diseases.

It is broadly recognized that allergic rhinitis and bronchial asthma commonly occur together, and rhinitis is a major risk factor for the development of asthma.<sup>11</sup> The reported prevalence of allergic rhinitis in children with asthma ranges from 6.2% to 95%.<sup>12</sup> Previous studies often examined the prevalence and pathogenic roles of Th1/Th2, or Th17/Treg balance in children with allergic asthma or rhinitis alone.<sup>9,13,14</sup> However, rare studies were seen focusing on

this balance in children with both BA and AR. Thus, the aim of this study was to describe the prevalence of Th17/Treg cell subsets in children with AR accompanying with BA, compared with children with AR or BA alone.

## MATERIALS AND METHODS

### Study Groups

64 children aged 6-15 years with allergic airway symptoms were recruited to the study between February and December 2013, including 24 with AR, 22 with BA and 18 with AR accompanying with BA. 20 age-matched, healthy control subjects without allergy were recruited as normal control subjects. BA diagnosis and assessment of severity were performed according to Global Initiative for Asthma (GINA) guidelines.<sup>15</sup> AR diagnosis was performed according to Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines.<sup>16</sup> This study was approved by the Ethical Committee of our hospital. Written informed consent was obtained from all parents/guardians of all children and control subjects.

### Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs of heparinized peripheral blood from the study subjects were isolated by Ficoll density gradient centrifugation (HAO YANG, Tianjin, China). The cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 100 U/ml penicillin and 100 U/ml streptomycin, 2 mM glutamine, and 10% FBS (Gibco, USA).

### Intracellular Staining of ROR- $\gamma$ t and Foxp3

$2 \times 10^6$  PBMCs were harvested, washed and stained with FITC-conjugated anti-human CD3 and PercpCy5.5-conjugated anti-human CD4 for 30min on ice in the presence of FcR-block (eBioscience, San Diego, CA). After surface staining, cells were fixed and permeabilized using the human forkhead box P3 (FoxP3) Buffer Set according to the manufacturer's instruction (eBioscience, San Diego, CA). Finally, the cells were stained with PE-conjugated anti-human retinoid-acid receptor-related orphan receptor (ROR)- $\gamma$ t for Th17 detection and APC-conjugated anti-human Foxp3 for Treg detection at 4°C for 30min. Isotype controls were used to enable correct compensation and confirm antibody specificity. Data were acquired on a FACS Calibur (BD Bioscience, San

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Diego, CA) and analyzed using Flowjo software (FlowJo, Ashland, OR).

### Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total mRNA from PBMCs was extracted using TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized from 1 µg mRNA using reverse transcriptase (Fermentas, Glen Burnie, MD) and oligo(dT) primers. Quantitative real-time PCR was performed using the ABI 7300 Sequence Detection System with primer pairs and SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences used were as follows: ROR- $\gamma$ t forward: 5'-GCTGGTTAGGATGTGCCG-3' and reverse: 5'-GGATGCTTTGGC GATGA-3'; Foxp3 forward: 5'-ATGCGACCCCT TTCACCTAC-3' and reverse: 5'-TGGCGGATGGCGTTCTTC-3';  $\beta$ -actin forward: 5'-CACCATTGGCAATGAGCGGTTCC-3' and reverse: 5'-GTAGTTTCGTGGATGCCACAGG-3'. The mRNA expression was normalized to the expression of the  $\beta$ -actin housekeeping gene using the  $2^{-\Delta Ct}$  (comparative threshold cycle, or CT) method.

### Enzyme Linked Immunosorbent Assays (ELISA)

The concentrations of Interleukin (IL)-6, IL-10, IL-17, IL-23 and TGF- $\beta$ 1 in plasma which were collected from EDTA blood samples were measured by ELISA in accordance with the manufacturer's instructions (eBioscience, San Diego, CA). The concentrations of total IgE were also measured by ELISA. All samples were measured in duplicate.

### Statistical Analysis

SPSS 16.0 (SPSS Inc., USA) was used for statistical analysis. Data were expressed as the mean  $\pm$  SD. Statistical analysis was performed using Kruskal Wallis test and the differences between groups were evaluated by nonparametric Mann Whitney test. The correlation

coefficient was generated by using Spearman's rank correlation. Statistical significance was defined as  $p < 0.05$ .

## RESULTS

### Characteristics of Study Participants

The clinical characteristics of the study participants are summarized in Table 1. There were no significant differences in terms of age or gender among the three subgroups of children with allergic diseases and the normal control group. Total IgE levels were significantly higher in all children with allergic disease than in the normal control group. Children with AR accompanying with BA had total IgE levels significantly higher than those of children with BA or AR alone.

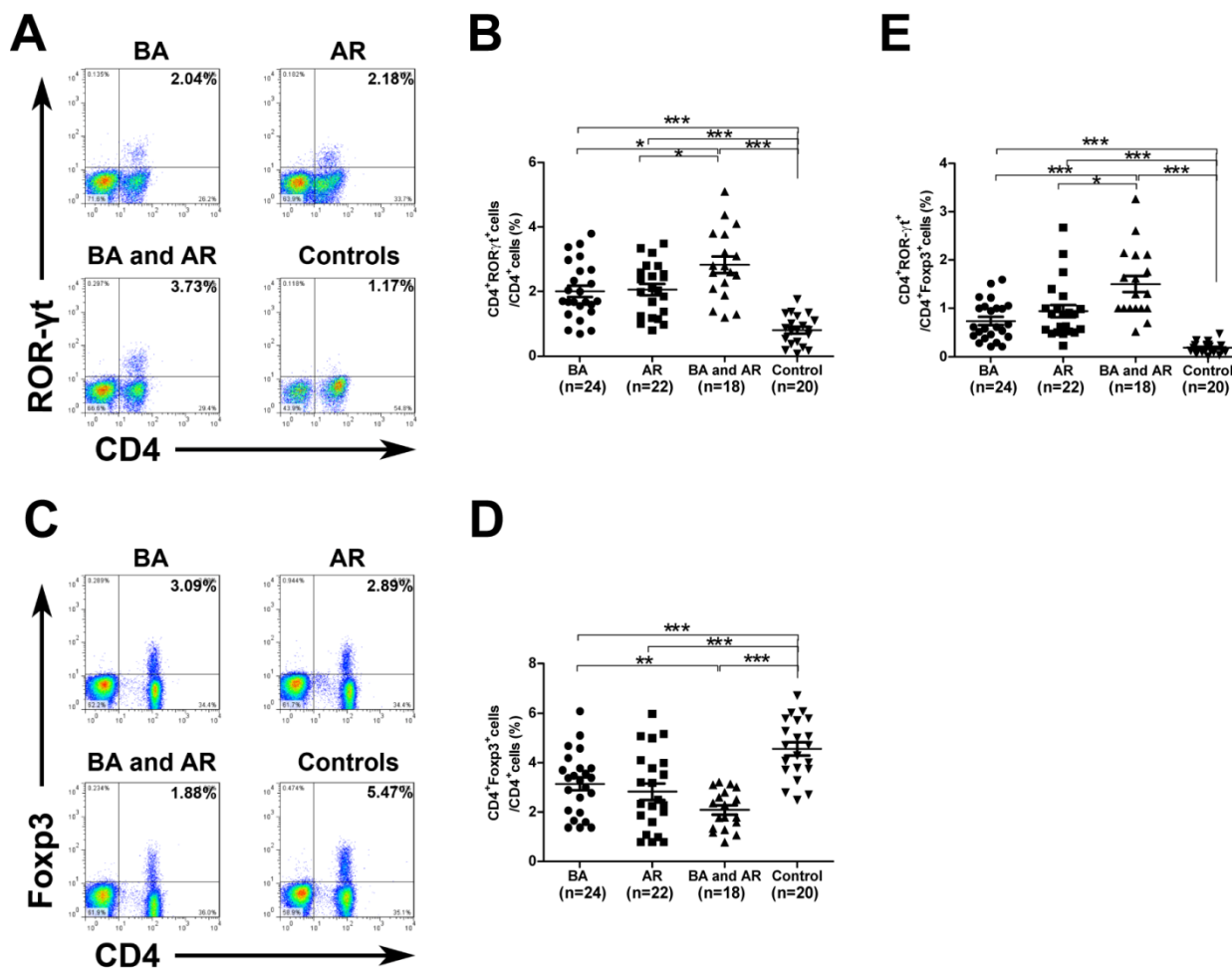
### Prevalence of Th17 and Treg Cells in the Peripheral Blood of Children with Allergic Disease

PBMCs in children with allergic diseases and control subjects were examined for the prevalence of Th17 cells (CD4<sup>+</sup>ROR- $\gamma$ t<sup>+</sup>) and Treg cells (CD4<sup>+</sup>Foxp3<sup>+</sup>). As shown in Figure 1, the frequency of Th17 cells were significantly increased (Figures 1A and B), while Treg cells were markedly decreased (Figures 1C and D) in children with allergic diseases in comparison with those in control subjects. However, there was no significant difference in the prevalence of Th17 cells and Treg cells between the BA and AR children. Interestingly, children with AR accompanying with BA had highest level of Th17 cells and lowest level of Treg cells among children with allergic diseases. Compared with control subjects, the ratio of Th17/Treg cells was also higher in children with allergic diseases (Figure 1E). Furthermore, the ratio of Th17/Treg cells was significantly higher in children with AR accompanying with BA than in children with AR or BA alone (Figure 1E).

**Table 1. Clinical characteristics of study participants (mean  $\pm$  SD)**

Characteristics	BA (n=24)	AR (n=22)	BA and AR (n=18)	Controls (n=20)
Age (years)	9.1 $\pm$ 2.7	9.7 $\pm$ 3.3	9.4 $\pm$ 2.7	9.2 $\pm$ 3.5
Gender (male/female)	12/12	14/8	12/6	10/10
Total IgE (IU/ml)	204.5 $\pm$ 57.9**	137.5 $\pm$ 53.7**	271.7 $\pm$ 45.3***†	44.3 $\pm$ 16.6
FEV1(% predicted)	88.5 $\pm$ 8.2	91.4 $\pm$ 10.3	81.6 $\pm$ 7.7	99.4 $\pm$ 8.7

BA, bronchial asthma; AR, allergic rhinitis; BA and AR, bronchial asthma accompanying AR. FEV1, forced expiratory volume in 1 second. Data are expressed as mean  $\pm$  SD. \*\*  $P < 0.01$  versus control subjects; †  $P < 0.05$  versus BA or AR.



**Figure 1.** The frequencies of Th17 and Treg cells relative to CD4<sup>+</sup>T cells in the peripheral blood of children with allergic disease and normal subjects were evaluated by flow cytometry. (A) Representative dot plots of CD4<sup>+</sup>ROR-γt<sup>+</sup> cells from a single children in each group. (B) Frequencies of CD4<sup>+</sup>ROR-γt<sup>+</sup> cells in each group. (C) Representative dot plots of CD4<sup>+</sup>Foxp3<sup>+</sup> cells from a single children in each group. (D) Frequencies of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in each group. (E) The ratio of Th17/Treg cells in each group. Data shown are the means±SD. \* *P*<0.05, \*\* *P*<0.01, \*\*\* *P*<0.001. BA, bronchial asthma; AR, allergic rhinitis; BA and AR, bronchial asthma accompanying with AR.

### Expression of ROR-γt and FoxP3 mRNA Levels in Children with Allergic Disease

mRNA expression of specific transcription factors of Th17 and Treg cells in PBMCs were also compared between children with allergic diseases and control subjects. As shown in Figure 2A, the expression of ROR-γt mRNA was markedly higher in children with allergic diseases than in the control group, and children with AR accompanying with BA had higher mRNA expression of ROR-γt than that of children with AR or BA. However, there was no significant difference of Foxp3 mRNA expression among the four groups, but a downward trend was observed across children with AR

accompanying with BA to children with AR or BA alone. The ratio of ROR-γt/Foxp3 mRNA was significantly higher in children with AR accompanying with BA than the other three groups (Figure 2B).

### Plasma Cytokine Levels in Children with Allergic Disease

Levels of the Th17 and Treg cell effector cytokines IL-6, IL-17, IL-23, IL-10 and TGF-β1 in the plasma were measured. As shown in Table 2, the plasma levels of IL-6, IL-17 and IL-23 were significantly higher while TGF-β1 level was significantly lower in children with allergic diseases than those in control subjects.

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Furthermore, the level of IL-17 was significantly higher in children with AR accompanying with BA than in children with AR or BA alone, and the level of TGF- $\beta$ 1 was significantly lower in children with AR accompanying with BA than in children with AR or BA alone. However, the level of IL-10 in the plasma of allergic disease patients was similar to those of the control subjects. There were no differences in the expression of these cytokines between the two subgroups of children with allergic diseases.

### Correlation Analysis in Children with Allergic Airway Disease

In children with allergic airway disease, the frequency of peripheral blood Treg cells was negatively correlated to the percentage of Th17 cells ( $r=-0.579$ ,

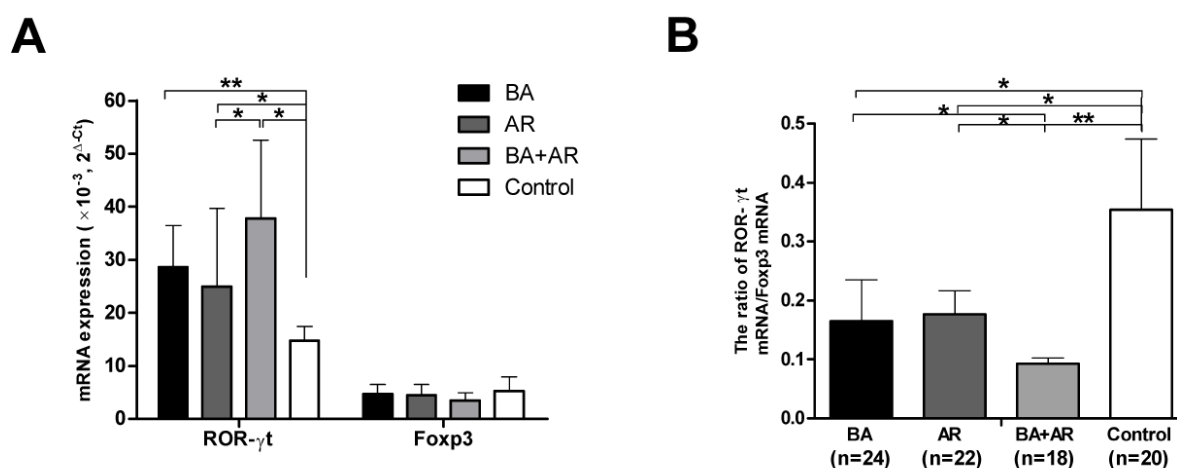
$p<0.01$ ) and plasma IL-17 levels ( $r=-0.337$ ,  $p<0.01$ ). Total IgE levels were positively correlated to the frequency of Th17 cells ( $r=0.607$ ,  $p<0.01$ ), plasma IL-17 levels ( $r=0.513$ ,  $p<0.01$ ) and IL-23 levels ( $r=0.127$ ,  $p<0.05$ ), and negatively correlated to the frequency of Treg cells ( $r=-0.429$ ,  $p<0.01$ ) and TGF- $\beta$ 1 levels ( $r=-0.224$ ,  $p<0.01$ ). Forced expiratory volume in one second (FEV1) (% predicted) in children with allergic airway disease was negatively correlated to the frequency of Th17 cells ( $r=-0.602$ ,  $p<0.01$ ), plasma IL-17 levels ( $r=-0.577$ ,  $p<0.01$ ), IL-6 levels ( $r=-0.117$ ,  $p<0.05$ ) and IL-23 levels ( $r=-0.334$ ,  $p<0.01$ ) and positively correlated to the frequency of Treg cells ( $r=0.504$ ,  $p<0.01$ ) and TGF- $\beta$ 1 levels ( $r=0.231$ ,  $p<0.05$ ).

**Table 2. Plasma IL-6, IL-10, IL-17, IL-23 and TGF- $\beta$ 1 levels (mean  $\pm$ SD, pg/ml)**

Cytokine	BA (n=4)	AR (n=36)	BA and AR (n=30)	Controls (n=40)
IL-6	78.4 $\pm$ 28.3*	82.1 $\pm$ 21.5*	85.2 $\pm$ 31.7**	24.8 $\pm$ 14.3
IL-10	24.6 $\pm$ 16.3	21.3 $\pm$ 12.7	22.1 $\pm$ 15.9	19.2 $\pm$ 8.1
IL-17	79.5 $\pm$ 17.7*	71.3 $\pm$ 24.6*	107.3 $\pm$ 22.4**†	21.8 $\pm$ 6.5
IL-23	197 $\pm$ 42.5*	211.3 $\pm$ 39.2*	224.9 $\pm$ 78.9**	97.5 $\pm$ 28.3
TGF- $\beta$ 1	367.2 $\pm$ 158.8*	401.7 $\pm$ 201.7*	221.4 $\pm$ 109.3**†	1037.7 $\pm$ 243.7

BA, bronchial asthma; AR, allergic rhinitis; BA and AR, bronchial asthma accompanying AR. FEV1, forced expiratory volume in 1 second. Data are expressed as mean  $\pm$ SD. IL, Interleukin; TGF, transforming growth factor. Data are expressed as mean  $\pm$ SD.

\* $P<0.05$ , \*\*  $P<0.01$  versus control subjects; † $P<0.05$  versus BA or AR.



**Figure 2. Expression of ROR- $\gamma$ t and FoxP3 mRNA levels in children with allergic disease and normal subjects were examined by real-time PCR. (A) Relative mRNA expression of ROR- $\gamma$ t and FoxP3 in children with allergic disease and normal subjects, the relative expression was quantified as  $2^{-\Delta C_t}$  with respect to the housekeeping gene  $\beta$ -actin. (B) The ratio of ROR- $\gamma$ t/Foxp3 mRNA in allergic disease and normal subjects. Data shown are the means  $\pm$  SD. \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ . BA, bronchial asthma; AR, allergic rhinitis; BA and AR, bronchial asthma accompanying with AR.**

## DISCUSSION

In the present study, the results showed for the first time that the proportion of peripheral Th17 cells in AR accompanying with BA was significantly higher than those of children with AR or BA alone and the controls, accompanied by an increase in the expression of ROR- $\gamma$ t mRNA and serum levels of IL-6, IL-17 and IL-23. Additionally, the proportion of Treg cells in peripheral blood of AR accompanying with BA children was significantly lower than that of children with AR or BA alone and the controls, accompanied by the reduction of serum TGF- $\beta$ 1 level. Furthermore, the ratio of Th17/Treg cells in peripheral blood and ROR- $\gamma$ t/Foxp3 mRNA expression were also significantly higher in children with AR accompanying with BA than in children with AR or BA alone. These findings suggest that Th17/Treg imbalance plays an important role in the pathogenesis of AR accompanying with BA.

Th17 cells are characterized by the production of effector cytokine IL-17, and ROR- $\gamma$ t is a key nuclear transcription factor of Th17 cells. Previous studies have demonstrated that the serum level of IL-17 in AR patients was significantly increased when compared with controls,<sup>17,18</sup> and that the proportion of IL-17 positive cells in peripheral blood and nasal mucosa of AR patients was also markedly higher than that in healthy subjects.<sup>10,19</sup> The serum IL-17 level was positively related to the severity of symptoms of AR and positively associated with the number of peripheral eosinophils.<sup>17,20</sup> The increased Th17 cells and IL-17 expression have also been found in asthmatic patients.<sup>9,13,21</sup> As a minor fraction of approximately 5–10% of CD4<sup>+</sup>T cells, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells play a critical role in preventing autoimmunity and maintaining self-tolerance. Treg cells exert their function partly by the secretion of anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ 1.<sup>22</sup> In human allergic patients, Treg cell numbers decrease in peripheral blood and BALF,<sup>23</sup> and cannot suppress the cell proliferation and cytokine production of allergen-stimulated CD4<sup>+</sup>T cells.<sup>24,25</sup> A recent study by Zhang *et al.* showed that the proportion of Treg cells and serum TGF- $\beta$ 1 levels in AR patients were dramatically reduced compared with healthy controls.<sup>10</sup> In addition, several murine studies have demonstrated that the induction of Treg cells function reverses airway hyper-responsiveness and protects against experimentally induced asthma.<sup>26,27</sup>

The balance of Th17 and Treg cells is crucial for the maintenance of the immune tolerance.<sup>28</sup> In the present study, the results demonstrated that the frequency of peripheral blood Treg cells was negatively correlated to the percentage of Th17 cells and IL-17 level in children with allergic airway disease. In addition, the increased Th17 cells and plasma IL-17 level were positively correlated to total serum IgE levels and negatively correlated to FEV1 (% predicted), while the decreased Treg cells and plasma TGF- $\beta$ 1 levels were negatively correlated to IgE levels and positively correlated to FEV1 (% predicted). FEV1 (% predicted) is an important marker for the evaluation of lung function, and total serum IgE levels could also be used to assess the hypersensitive and allergic reactions in allergic patients. A decline in FEV1 (% predicted) and increased IgE levels are associated with worsening of allergic inflammation. Therefore, we believe that the imbalance in Th17/Treg cells is associated with worsening of allergic airway disease.

In conclusion, the present study demonstrated that a Th17/Treg functional imbalance also exists in children with AR accompanying with BA, suggesting a potential pathogenic role for this imbalance in the development of the condition. Further research in the Th17/Treg balance may provide a novel strategy for the treatment of AR accompanying with BA.

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