

Association of imbalance of effector T cells and regulatory cells with the severity of asthma and allergic rhinitis in children

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ABSTRACT

Purpose: This study investigated the frequency and absolute numbers of different subsets of peripheral blood cells, including CD4⁺ T cells and CD19⁺ interleukin (IL) 10⁺ B regulatory cells (Breg) and their potential association with clinical laboratory measurements in children diagnosed with simple asthma or asthma plus allergic rhinitis (AR).

Method: The frequency and numbers of peripheral blood CD4⁺ interferon (IFN) gamma⁺ T-helper (Th) type 1, CD4⁺ IL-4⁺ Th2, CD4⁺ IL-17A⁺ Th17, CD4⁺CD25⁺ Forkhead Box P3⁺ regulatory T cells (Treg) and CD19⁺ IL-10⁺ Bregs in 22 children with asthma, 17 children with asthma and AR, and 25 healthy controls were determined by flow cytometry. The levels of serum cytokines were determined by enzyme-linked immunosorbent assay.

Results: In comparison with healthy controls, patients with asthma alone had significantly increased numbers of Th1, Th2, and Th17 cells, and their cytokines but decreased numbers of Tregs and Bregs, and the cytokines IL-10 and transforming growth factor beta 1. This imbalance between effector and regulatory cells and their cytokines further increased in patients with asthma and AR. The ratios of percentage effector T cells (Th1 + Th2 + Th17) to regulatory cells (Treg + Breg) were positively correlated with fractional exhaled nitric oxide but negatively correlated with forced expiratory volume in 1 second in patients with asthma or asthma plus AR.

Conclusion: The imbalance of effector T cells and regulatory cells contributed to the pathogenesis of airway inflammation of asthma and AR in children.

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Asthma and allergic rhinitis (AR) are common allergic diseases in children, and, in recent years, the incidence of simultaneous asthma and AR is increasing worldwide. Actually, 70–90% of patients with asthma develop AR, and 40–50% of patients with AR have asthma. AR can promote the progression of asthma; similarly, asthma control can also alleviate the severity of AR. However, the immunopathologic similarity and difference between asthma alone and asthma plus AR have not been clarified. Effector T cells, such as T-helper (Th) type 2, Th1, and Th17, are central to the pathogenesis of allergic airway inflammation. Th2 cells play critical roles in

orchestrating and amplifying allergic inflammation by secreting interleukin (IL) 4, IL-5, and IL-13. Th1 cells promote neutrophil infiltration in the lung by secreting interferon (IFN) γ , which enhances production of neutrophil-attracting chemokines.

Th17 cells enhance the development of airway chronic inflammation by producing IL-17A, Th17F and IL-21. These inflammatory actions can be negatively regulated by regulatory T cells (Treg) and their inhibitory cytokines, IL-10 and transforming growth factor (TGF) β 1. Recent studies indicate that impairment of Treg responses is associated with the development and progression of asthma and AR.^{1,2} In addition, CD19⁺ IL-10⁺ regulatory B cells (Breg) can inhibit effector T-cell-mediated inflammation and may enhance Treg responses.^{3,4} Although previous studies reveal the effect of Tregs on effector T cells in children with asthma or AR alone,^{2,5} little is known on how different subsets of T cells and Bregs are present in patients with both asthma and AR, and how they are associated with disease severity and lung function in children with asthma and AR.

In this study, we characterized the frequency and absolute numbers of peripheral blood immune cells, including subsets of CD4⁺ T cells as well as CD19⁺ IL-10⁺ Bregs in children with asthma alone or asthma

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and AR and compare them with gender- and age-matched healthy controls. Subsequently, we analyzed the potential relationship among different measures. Analysis of our data indicated that an imbalance of effector T cells and regulatory cells existed in patients with asthma and AR and was associated with disease severity and poor lung function in this population.

METHODS

General Data

From October 2014 to February 2016, 22 children with asthma, 17 children with asthma and AR, and 25 age- and gender-matched healthy controls were recruited sequentially at the department of pediatrics and physical examination center, in the First Hospital of Jilin University. Children with asthma were diagnosed and assessed according to the guidelines of Global Initiative for Asthma.⁶ Children with AR were diagnosed according to the guidelines of AR and its Impact on Asthma.⁷ The patient inclusion criteria included ages 2–16 years old, without a history of another system disease, and had not received immunosuppressive drugs during the past 6 months. All of the healthy controls had no abnormal symptoms and signs, and no history of asthma and rhinitis. The experimental protocol was approved by the human ethics committee of Jilin University, China. All procedures performed in studies that involved human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from the guardians of the patients.

Clinical Data

Individual patients were measured for the concentrations of fractional exhaled nitric oxide (FeNO) by using a NIOX MINO system (Aerocrine AB, Solna, Sweden) according to the manufacturers' instruction. They were evaluated for forced expiratory volume in 1 second (FEV₁) by spirometry (APS; Jaeger, Wurzburg, Germany). White blood cell (WBC), peripheral blood mononuclear cell (PBMC), and blood eosinophil counts were determined, and the levels of serum total immunoglobulin E (IgE), specific IgE against house-dust mites, fungi, pollens, and other allergens were determined by using specific kits (ImmunoCAP System; Phadia, Uppsala, Sweden).

Flow Cytometry

Peripheral blood samples were collected from individual subjects, and PBMCs were prepared by density gradient centrifugation. PBMCs (2×10^6 cells/tube) were stained with fluorescein isothiocyanate-conju-

gated anti-CD19, and stimulated in duplicate with 25 ng/mL phorbol myristate acetate and 0.25 μ g/mL ionomycin (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C in 5% CO₂ and cultured for another 2 hours in the presence of Brefeldin A (BD Biosciences, Franklin Lakes, NJ). The cells were then stained with phycoerythrin-conjugated anti-IL-10, or isotype control. The frequency of CD19⁺ IL-10⁺ Bregs was analyzed by flow cytometry. All the antibodies were from eBioscience (San Diego, CA).

Furthermore, PBMCs (2×10^6 cells/tube) were stained in duplicate with fluorescein isothiocyanate-conjugated anti-CD4, allophycocyanin-conjugated anti-CD25, and allophycocyanin-conjugated anti-CD3 (eBioscience). Some cells were washed, fixed, permeabilized, and stained with PE-conjugated anti-Forkhead Box P3 (Foxp3) or isotype control. The frequency of CD3⁺ CD4⁺ CD25⁺ Foxp3⁺ Tregs was determined by flow cytometry. In addition, the remaining stained cells were stimulated in duplicate with phorbol myristate acetate and ionomycin for 2 hours and cultured for another 3 hours in the presence of 2 nmol/mL of monensin. The cells were fixed, permeabilized, and stained with PE-conjugated anti-IFN- γ , anti-IL-4, and anti-IL-17A, or isotype control (eBioscience). The frequency of Th1 (CD4⁺ IFN- γ ⁺), Th2 (CD4⁺ IL-4⁺), and Th17 (CD4⁺ IL-17A⁺) was determined by flow cytometry. All data were analyzed by using FlowJo software (Version X, Ashland, OR).

Enzyme-linked Immunosorbent Assay

The concentrations of serum IL-10, TGF- β 1, IFN- γ , IL-4, IL-5, IL-13, and IL-17 in individual subjects were analyzed with enzyme-linked immunosorbent assay by using specific kits according to the manufacturer's instructions (eBioscience).

Statistical Analysis

All data are expressed as mean $\pm$ standard deviation. The difference between the groups was analyzed by using Student's *t*-test, the Mann-Whitney *U* test, or the Fisher exact test when applicable. The relationship between two variants was determined by the Spearman rank correlation test. All statistical analyses were performed by using the SPSS software window 17. A *p* value of <0.05 was considered statistically significant (SPSS, Inc, Chicago, IL).

RESULTS

Patient Characteristics

To determine the potential function of different subsets of CD4⁺ T cells in the pathogenesis of asthma and AR, 22 children with asthma alone, 17 with asthma and AR, and 25 healthy controls were recruited. Their demographic and clinical characteristics are shown in

Table 1 The clinical characteristics of the patients

Characteristics	Healthy Controls (n = 25)	Patients with Asthma (n = 22)	Patients with Asthma + AR (n = 17)
Age, mean $\pm$ SD, y	6.2 $\pm$ 1.9	6.8 $\pm$ 2.2	6.5 $\pm$ 2.4
No. men/women	12/13	12/10	8/9
Asthma duration, mean $\pm$ SD, y		2.3 $\pm$ 0.5	2.1 $\pm$ 0.6
AR duration, mean $\pm$ SD, y			2.7 $\pm$ 0.9
FeNO level, mean $\pm$ SD, Parts Perbillion	12.2 $\pm$ 3.3	33.3 $\pm$ 11.9*	42.8 $\pm$ 15.3*#
FEV ₁ value, mean $\pm$ SD%	96.9 $\pm$ 8.7	72.4 $\pm$ 13.2*	67.2 $\pm$ 13.6*
WBC count, mean $\pm$ SD, $\times 10^3/\mu\text{L}$	7.35 $\pm$ 2.23	8.78 $\pm$ 2.59*	9.17 $\pm$ 3.28*
PBMC, mean $\pm$ SD, $\times 10^3/\mu\text{L}$	3.79 $\pm$ 1.75	3.95 $\pm$ 1.22	4.21 $\pm$ 1.46
Blood eosinophils, mean $\pm$ SD, $\times 10^3/\mu\text{L}$	0.12 $\pm$ 0.08	0.23 $\pm$ 0.11*	0.35 $\pm$ 0.19*#
Total IgE, mean $\pm$ SD, $\mu\text{g/mL}$	42.5 $\pm$ 18.4	235.2 $\pm$ 48.9*	283.6 $\pm$ 52.3*#
Specific IgE, no. positive cases (%)			
House-dust mites	0	10 (45.5)	14 (82.4)*
Fungi	0	3 (13.6)	5 (29.4)
Pollens	0	5 (22.7)	6 (35.3)
Other allergens	0	5 (22.7)	8 (47.1)

AR = Allergic rhinitis; SD = standard deviation; FeNO = fractional exhaled nitric oxide; FEV₁ = forced expiratory volume in 1 second; WBC = white blood cell; PBMC = peripheral blood mononuclear cell; IgE = immunoglobulin E.

* $p < 0.05$ vs the healthy controls.

$p < 0.05$ vs the asthma alone.

Table 1. There was no significant difference in the distribution of age and gender as well as the numbers of PBMCs among these groups of subjects. The patients with asthma or with asthma and AR had significantly greater numbers of WBCs but lower FEV₁ values than that of the healthy controls. The numbers of blood eosinophils and the levels of FeNO and total IgE in patients with asthma alone were significantly higher than that in the healthy controls but significantly lower than that in those patients with asthma and AR in this population. In addition, the frequency of house-dust mite specific IgE was significantly higher in the patients with both asthma and AR than those with asthma alone. Hence, patients with both asthma and AR have more severe inflammation.

Detection of Peripheral Blood Different Subsets of CD4⁺ T Cells by Flow Cytometry

The percentages of CD4⁺ IFN γ ⁺, CD4⁺ IL-4⁺, and CD4⁺ IL-17A⁺ T cells in total CD4⁺ T cells in individual subjects were determined by flow cytometry (Fig. 1 A). The percentages and numbers of peripheral blood Th1 cells in the patients with asthma alone or with both asthma and AR were significantly higher than that in the healthy controls (Fig. 1 B), and there was no significant difference in the frequency of Th1 cells between the patients with asthma alone and those with both asthma and AR. Furthermore, the percentages and numbers of Th2 and Th17 cells in the patients with asthma alone were significantly higher than that in the

healthy controls but significantly lower than that in those with both asthma and AR (Fig. 1 B).

In addition, PBMCs from individual subjects were stained with anti-CD4, fixed, and permeabilized, followed by intracellularly staining with anti-Foxp3. The percentages and numbers of peripheral blood CD4⁺ CD25⁺ Foxp3⁺ Tregs in total CD4⁺ T cells were determined by flow cytometry (Fig. 1 C). The percentage of peripheral blood CD4⁺ CD25⁺ Foxp3⁺ Tregs in total CD4⁺ T cells were significantly lower in patients with asthma alone than in the healthy controls. However, this percentage was higher in the asthma alone group than in the group with both asthma and AR ($p < 0.05$ for both). Similarly, the numbers of peripheral blood Tregs in the patients with asthma alone were significantly less than that in the healthy controls but significantly greater than that in those with both asthma and AR ($p < 0.05$ for all). Together, patients with asthma and AR had increased numbers of peripheral blood inflammatory Th1, Th2, and Th17 cells, and decreased numbers of Tregs.

Characterization of Peripheral Blood CD19⁺ IL-10⁺ Bregs by Flow Cytometry

Next, the percentages and numbers of CD19⁺ IL-10⁺ Bregs in individual subjects were determined by flow cytometry. As shown in Fig. 2, the percentages of CD19⁺ IL-10⁺ Bregs in total B cells in patients with asthma alone were significantly lower than that in the healthy controls but significantly higher than

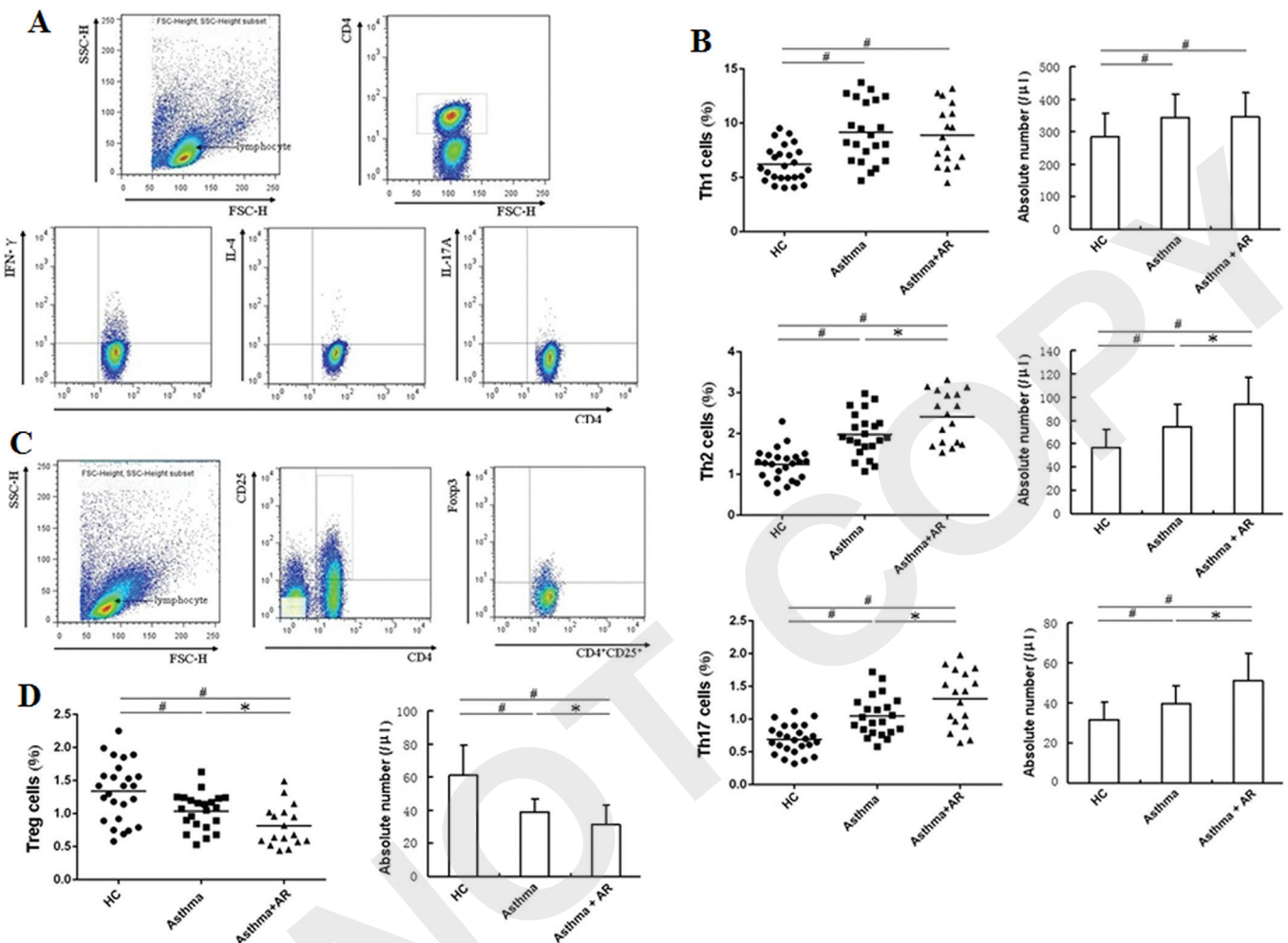


Figure 1. Flow cytometry analysis of different subsets of CD4⁺ T cells. The percentages of CD4⁺CD25⁺ Forkhead Box P3⁺ (Foxp3⁺) regulatory T cells (Treg) were determined by flow cytometry. The cells were first gated on living lymphocytes and then on CD4⁺ CD25⁺ cells for analysis of Tregs. The numbers and frequency of each type of CD4⁺ T cells were calculated, according to the total numbers of peripheral blood mononuclear cells (PBMC). Data are representative flow cytometry charts or expressed as the mean individual values or mean $\pm$ standard deviation (SD) of each group of subjects ($n = 25$ for the healthy controls [HC]; $n = 22$ for patients with asthma; and $n = 17$ for patients with asthma and allergic rhinitis [AR]). (A) Representative fluorescence-activated cell sorting (FACS) charts of different subsets of effector T cells. (B) Increased percentages and absolute numbers of T-helper (Th) type 1, Th2, and Th17 cells in patients with asthma, and further increased Th2 and Th17 cells in patients with asthma and AR. (C) Representative FACS charts of Tregs. (D) Decreased percentages and absolute numbers of Tregs in patients with asthma and further decreased Tregs in patients with asthma and AR. # $p < 0.05$ versus the HCs; * $p < 0.05$ versus the asthma alone.

those with both asthma and AR ($p < 0.05$ for all) (Fig. 2, A and B). A similar pattern of the numbers of Bergs was detected in different groups of subjects (Fig. 2 C). Hence, decreased numbers of peripheral blood Bergs were seen in patients with asthma and were associated inversely with the disease severity in this population.

Further analysis of serum cytokines indicated that the levels of serum IL-10 and TGF- β 1 in the patients with asthma alone were significantly lower than that in the healthy controls but significantly higher than that in those with both asthma and AR ($p < 0.05$ for all) (Fig. 3). Furthermore, the levels of serum IFN- γ and

IL-5 in the patients with asthma alone or with both asthma and AR were significantly higher than that in the healthy controls ($p < 0.05$ for all), but there was no significant difference in the levels of those cytokines between the patients with asthma alone and with both asthma and AR ($p > 0.05$). In addition, the levels of serum IL-4, IL-13, and IL-17A in the patients with asthma alone were significantly higher than that in the healthy controls but significantly lower than that in those with asthma and AR ($p < 0.05$ for all). The imbalance of pro- and anti-inflammatory cytokine responses existed in patients with asthma and higher levels of serum IL-4, IL-13, and IL-17A

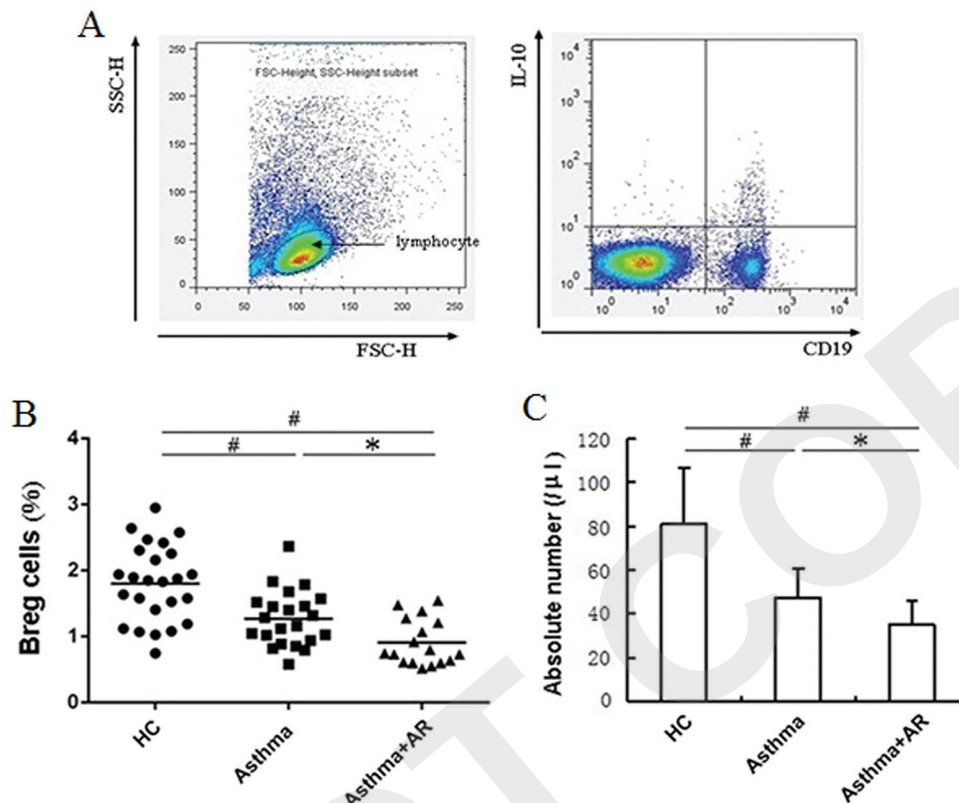


Figure 2. Flow cytometry analysis of peripheral blood regulatory B cells (Breg). Peripheral blood mononuclear cells (PBMC) (2×10^6 cells/tube) from individual subjects were stained with fluorescein isothiocyanate–anti-CD19 and stimulated in duplicate, with phorbol myristate acetate and ionomycin for 4 hours in the presence of Brefeldin A in the last 2-hour incubation. The cells were stained intracellularly with phycoerythrin–anti-interleukin (IL) 10, and the frequency of CD19⁺ IL-10⁺ Bregs in total CD19⁺ B cells was determined by flow cytometry. The cells were gated first on living lymphocytes and then on CD19⁺ cells. The numbers of Bregs per microliter were calculated according to the numbers of PBMCs and the percentages of CD19⁺ IL-10⁺ Bregs. Data are representative charts or are expressed as individual values of mean percentage or mean $\pm$ standard deviation (SD) of each group of subjects. (A) Representative fluorescence-activated cell sorting charts of CD19⁺ IL-10⁺ Bregs. (B) Decreased percentages of peripheral blood Bregs in patients with asthma and further reduced percentages of Bregs in patients with asthma and Bregs. (C) Decreased numbers of Bregs in patients with asthma and further reduced numbers of Bregs in patients with asthma and allergic rhinitis (AR). #*p* < 0.05 versus the healthy controls (HC); **p* < 0.05 versus the patients with asthma.

were associated with the development of AR in patients with asthma.

Relationship among FeNO, FEV₁, and Cellular Immune Imbalance

Also, we analyzed the potential association among cellular immune imbalance, FeNO, and FEV₁. We found that the ratios of percentages of T effectors (Th1 + Th2 + Th17) to regulatory cells (Tregs + Bregs) were positively correlated with the values of FeNO in patients with asthma alone or patients with both asthma and AR (Fig. 4 A). In contrast, the ratios of the percentages of T effectors to regulatory cells were negatively correlated with the values of FEV₁ in the patients with asthma or with both asthma and AR (Fig. 4 B). Collectively, the degrees of imbalance of effector T cells to regulatory cells were correlated positively with disease severity but negatively with the lung function in patients with asthma.

DISCUSSION

In this study, we characterized subsets of CD4⁺ T cells and CD19⁺ IL-10⁺ Bregs in patients with asthma alone or with both asthma and AR as well as the healthy controls. In comparison with healthy controls, significantly increased numbers of WBCs, eosinophils, total IgE levels, and FeNO values but lower levels of FEV₁ were detected in patients with either asthma or asthma and AR. These findings supported the notion that both asthma and AR are inflammatory diseases. The patients with both asthma and AR had the most severe inflammation, which impairs lung function. It was notable that a significantly higher percentage of patients with both asthma and AR exhibited elevated IgE levels against house-dust mites compared with patients with asthma alone. Our data were consistent with previous observations^{8–10} and indicated that house-dust mites may be important allergens for inducing AR.

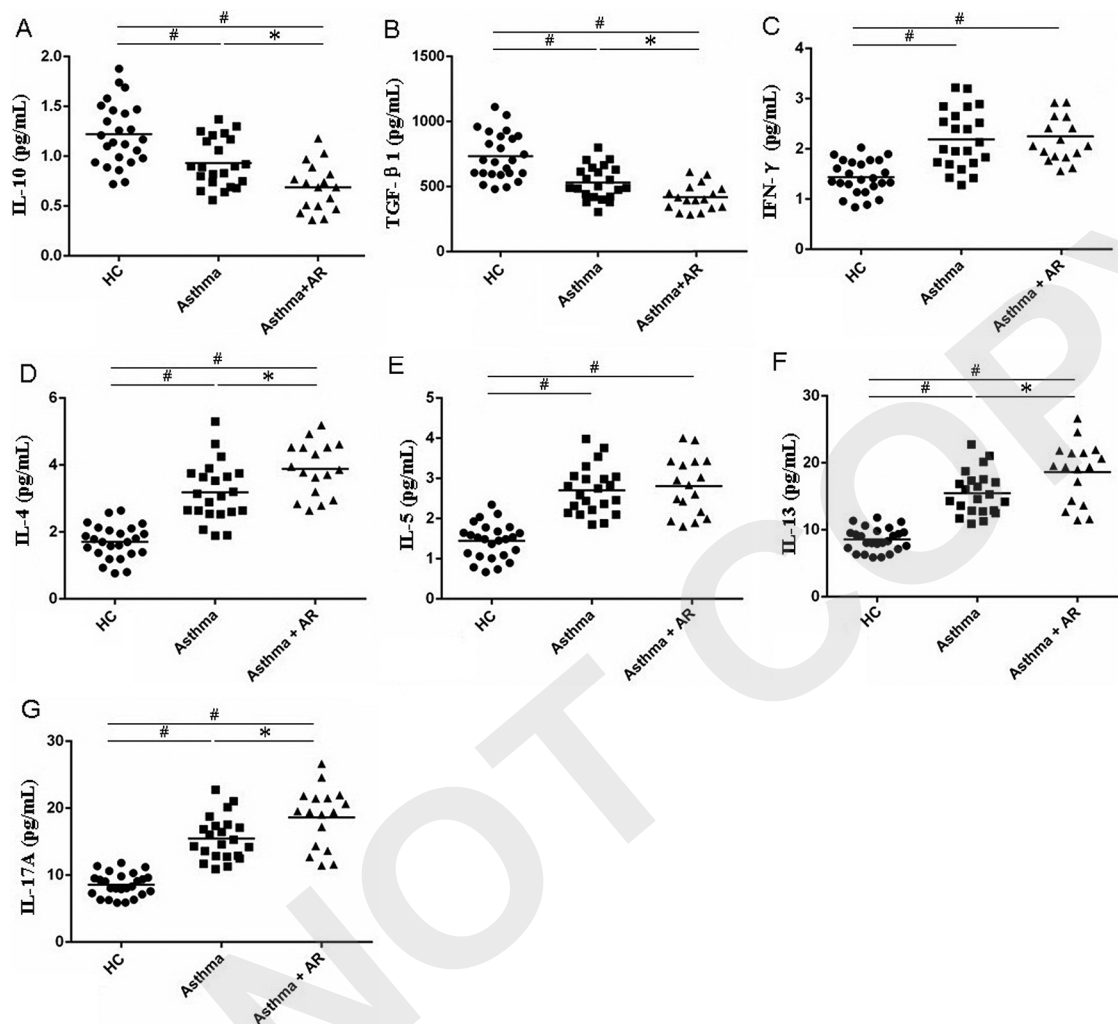


Figure 3. Enzyme-linked immunosorbent assay (ELISA) analysis of the levels of serum cytokines. (A) The levels of serum interleukin (IL) 10; (B) transforming growth factor (TGF) β 1; (C) interferon (IFN) γ ; (D) IL-4; (E) IL-5; (F) IL-13; and (G) IL-17A in individual subjects were analyzed by ELISA and the different groups of sera in triplicate were tested simultaneously. Data are expressed as the mean values of individual subjects and the lines represent the median values for different groups. # $p < 0.05$ versus the healthy controls (HC); * $p < 0.05$ versus the asthma alone.

Previous studies showed that imbalance of effector T cells and regulatory cells, such as Tregs, is associated with the development of asthma.^{5,11} During the pathogenic process of allergic airway inflammation, aberrant Th2 responses and their secreted cytokines, such as IL-4, IL-5, and IL-13, can promote eosinophil infiltration and mast cell activation, and enhance allergen-specific IgE production and the smooth-muscle contraction.¹² Th1 and Th17 cytokines, including IFN- γ , IL-17A, IL-17F, and IL-21, can recruit neutrophil and macrophage infiltration, which contributes to allergic airway inflammation.^{13,14} Furthermore, Tregs and their inhibitory IL-10 and TGF- β 1 can attenuate the activity of Th1, Th2, and Th17 cells, and inhibit allergic airway inflammation.¹⁵⁻¹⁷

In this study, we found that, in comparison with that in the healthy controls, significantly greater numbers

of Th1, Th2, and Th17 cells, and significantly fewer numbers of Tregs were detected in patients with asthma alone or with both asthma and AR, which indicated that the imbalance of effector CD4⁺ T cells and Tregs was associated with the development of asthma and AR. Interestingly, significantly greater numbers of Th2 and Th17, and higher levels of IL-4, IL-13, and IL-17A, but significantly fewer numbers of Tregs and lower levels of IL-10 and TGF- β 1 were observed in patients with both asthma and AR, relative to those with asthma alone. These data were partly similar to previous findings that Th2 inflammation is involved in AR pathogenesis and IL-10 has potential as an inhibitor of AR.^{18,19} Furthermore, other studies indicate that Th2 and Th17 responses may be crucial for the pathogenesis of AR in patients with asthma.^{20,21} Hence, Th2 and Th17 cells may provide therapeutic

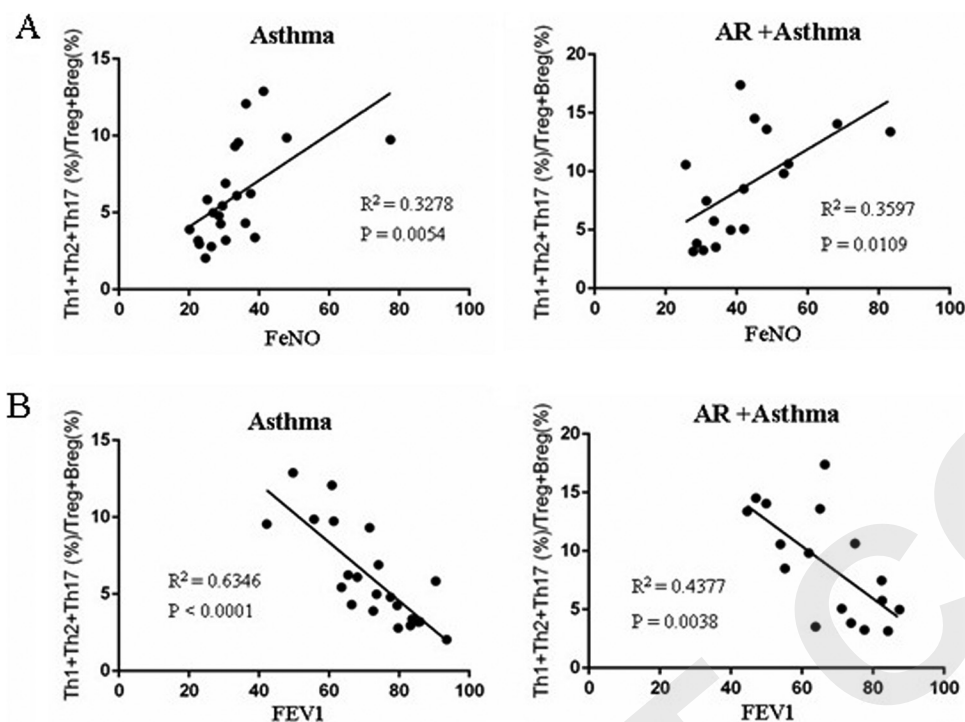


Figure 4. Correlation analyses. The potential correlation between the percentage ratios of effector CD4⁺ T cells (T-helper [Th] type 1 + Th2 + Th17), percentage to regulatory cells (regulatory T [Treg] + regulatory B [Breg]), percentages and values of (A) fractional exhaled nitric oxide (FeNO) or (B) forced expiratory volume in 1 second (FEV₁) were analyzed in each group of patients. Data are the ratios versus the values of FeNO or FEV₁ in the different group.

targets for intervention in patients with AR and asthma.

Previous studies mainly focused on the immune relationship between effector T cells and Tregs in either asthma or AR.^{5,14,17,20} There are few studies on the role of Bregs in the pathogenesis of human asthma and rhinitis.^{22,23} In particular, a comprehensive study of the function of effector T cells, Tregs, and Bregs in children with asthma and AR is rare. In this study, we centered on the relationship among effector T cells, Tregs, and Bregs in children with asthma and AR because Bregs can directly inhibit effector T-cell function and promote Treg differentiation by converting CD4⁺ CD25⁻ T cells into CD4⁺ CD25⁺ Foxp3⁺ Tregs through secreted TGF- β 1.^{3,4} Furthermore, adoptive transfer of Bregs enhance Treg responses and inhibit pathogenic T-cell activation.^{22,23} In addition, Bregs can secrete IL-10 and limit chronic inflammation.^{24–26} Adoptive transfer of CD19⁺ IL-10⁺ Bregs attenuates contact hypersensitivity and autoimmune diseases in a IL-10-dependent manner.^{27–29} In this study, we found significantly fewer numbers of peripheral blood Bregs and lower levels of IL-10 and TGF- β 1 in patients with asthma alone or with both asthma and AR, relative to that in the healthy controls. These data were consistent with a previous report that relative decrease in Breg cells is a exaggerating factor of AR to bronchial asthma.³⁰ Hence, Bregs may be important inhibitors of pathogenic T-cell responses during the process of AR. The decreased numbers of Bregs may impair Treg responses and contribute to the pathogenesis of AR in patients with asthma.

To understand the importance of effector T cells and regulatory cell imbalance during the pathogenesis of asthma and AR, we analyzed the potential association between the percentage ratios of all effector CD4⁺ T cells (Th1 + Th2 + Th17) to regulatory cells (Tregs + Bregs) and the values of FeNO and FEV₁ in patients with asthma. We found that the percentage ratios of effector CD4⁺ T cells to regulatory cells were positively correlated with the values of FeNO but negatively with the values of FEV₁ in children either with asthma alone or with asthma and AR in this population. The percentage ratios of effector T cells to regulatory cells revealed the degrees of immune imbalance of inflammatory and anti-inflammatory responses in patients with asthma.

Given that increased values of FeNO and decreased FEV₁ reflected severity of asthma and poor lung function, analysis of our novel data indicated that the degrees of immune imbalance of inflammatory and anti-inflammatory responses were positively correlated with the severity of disease and the impairment of lung function in children with asthma and AR. Therefore, if validated, the measure may be valuable for evaluating the disease severity and therapeutic responses in patients with asthma and AR. We recognized that our study had limitations, including small sample size and lack of longitudinal research and mechanistic investigation. Therefore, further studies in a larger population are needed to validate the findings and investigate the impact of common therapies on the imbalance of effector CD4⁺ T cells and regulator cells as well as the

mechanisms by which Tregs and Bregs regulate the pathogenesis of asthma and AR in children.

CONCLUSION

Analysis of our data indicated significantly higher numbers of Th1, Th2, and Th17 but fewer numbers of Tregs and Bregs in patients with asthma, and this imbalance of effector T cells and regulatory cells was worse in children with both asthma and AR. Furthermore, the percentage ratios of effector CD4⁺ T cells to regulatory cells were positively correlated with the values of FeNO but negatively correlated with the values of FEV₁ in children with asthma and in those with both asthma and AR. Analysis of these novel data indicated that the imbalance of effector CD4⁺ T cells and regulatory cells contributed to the pathogenesis of asthma and AR. Therefore, our findings may provide new insights into the pathogenesis of AR in children with asthma and aid in the design of new strategies for intervention of asthma and AR.

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