Original article

An increased ratio of Th2/Treg cells in patients with moderate to severe asthma

SHI Yu-heng, SHI Guo-chao, WAN Huan-ying, AI Xiang-yan, ZHU Hai-xing, TANG Wei, MA Jia-yun, JIN Xiao-yan and ZHANG Bo-ying

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Background Recent studies have shown that T helper type-2 (Th2) cells can induce the apoptosis of CD4+CD25+ Treg cells or resist the immunosuppressive effect of Treg cells. We hypothesize that an imbalance of Th2/Treg is present in patients with allergic asthma.

Methods Twenty-two patients with mild asthma, 17 patients with moderate to severe asthma, and 20 healthy donors were enrolled. All patients were allergic to house dust mites. The proportion of peripheral blood CD4+CD25+ Treg cells and Th2 cells were determined by flow cytometry. The concentration of interleukin (IL)-10, transforming growth factor (TGF)-β and IL-4 in plasma was determined by enzyme linked immunosorbent assay. In these subjects, peripheral blood mononuclear cells from 17 mild asthmatic patients, 13 moderate to severe asthmatic patients and 14 healthy donors were acquired and expression of forkhead box P3 (Foxp3) and GATA-3 mRNA was detected by reverse-transcriptase polymerase chain reaction.

Results Compared with healthy donors and patients with mild asthma, the percent of CD4+CD25+ Treg cells and plasma IL-10 levels were decreased in patients with moderate to severe asthma. There were no significant differences in Foxp3 mRNA expression among three groups, but a downward trend seen among patients with asthma. However, the percent of Th2 cells, IL-4 levels and expression of GATA-3 mRNA was markedly higher in patients with mild and moderate to severe asthma than in the control group. The ratio of Th2/Treg and their cytokines was increased in allergic asthma, especially for moderate to severe asthma. The ratio of GATA-3/Foxp3 mRNA was also increased in allergic asthma. In patients with moderate to severe asthma, the percentage of peripheral blood Treg cells was negatively correlated to the percentage of Th2 cells and IL-4 levels.

Conclusions The decline of CD4+CD25+ Treg cells in patients with moderate to severe asthma may play an important role in progress of the disease. Furthermore, the deficiency of CD4+CD25+ Treg cells was associated with the over-expression of Th2 response.

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Asthma is a chronic inflammatory disorder of the airways, which involves several inflammatory cells (such as eosinophils, mast cells, T lymphocytes and neutrophils), structural cells (such as airway smooth muscle cells and airway epithelial cells) and multiple mediators that result in characteristic pathophysiological changes.[1,2] In asthma, activated T helper type-2 (Th2) cells and their secreted cytokines promote the development of eosinophilia, airway hyper-responsiveness and high immunoglobulin E response. It has been recognized that the imbalance in Th1/Th2 expression are central to the pathogenesis of asthma. Immunoregulatory therapies that initiate a shift from Th2 to Th1 responses have been also explored, however, these approaches have had limited success in clinical trials.[3] It has been found that the Th1/Th2 imbalance paradigm does not fully explain the etiology of asthma and other T cell subsets may play a role in asthma, especially regulatory T (Treg) cells.[4]

CD4+CD25+ Treg cells, which continually express the transcription factor forkhead box P3 (Foxp3), are major regulators of autoimmunity and critical for the control of antigen specific inflammation.[5,6] CD4+CD25+ Treg cells exert their influence through direct cell to cell contact or via cytokines such as interleukin (IL)-10 and transforming growth factor beta (TGF-β). CD4+CD25+ Treg cells can suppress Th2 maturation,[7] possibly by inhibiting IL-4 production.[8] Recently, it has been found that the Th2 cells response can induce Treg cell apoptosis and inhibit the actions of Treg cells.[9,10] So we hypothesized that a Th2/Treg

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Department of Pulmonary Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China (Shi YH, Shi GC, Wan HY, Ai XY, Zhu HX and Tang W)
Department of Pulmonary Medicine, Shanghai Third People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201900, China (Shi YH and MA JY)
Department of Pulmonary Medicine, Changning District Centre Hospital, Shanghai 200336, China (Jin XY)
Department of Pulmonary Medicine, Luwan Branch of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200020, China (Zhang BY)
Corresponding to: Dr. SHI Guo-chao, Department of Pulmonary Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China (Tel: 86-21-64370045 ext. 360803. Email: shiguochao@hotmail.com)
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imbalance existed in patients with asthma and may play an important role in progress of the disease.

METHODS

Study population

Thirty-nine patients with chronic persistent asthma, based on positive results of allergy tests to house dust mites (UniCap, Pharmacia, Stockholm, Sweden), were enrolled. According to the Global Institute for Asthma guidelines,[11] the 39 patients with allergic asthma could be divided into two subgroups: 22 had mild asthma and 17 had moderate to severe asthma. None of the patients had been treated with systemic glucocorticoids within one month before the study and had never been treated with other immunosuppressive agents or specific immunotherapy. All of the patients denied a history of smoking. Twenty healthy donors, nonsmokers with normal pulmonary function and negative allergy tests, were selected as a normal control group. There were no significant differences in terms of age or gender among the two subgroups of patients with asthma and the normal control group. This study was approved by the Research Ethics Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from all individuals.

Sample preparation

Ten milliliters of heparinized peripheral venous blood were collected from each participant. Plasma was isolated from peripheral blood and stored at –80°C until used to measure the concentrations of cytokines. Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood by Ficoll-Hypaque density centrifugation at 2500 r/min for 20 minutes at room temperature). PBMCs were suspended at a density of 2×10^6 cells/ml in RPMI 1640 with GlutaMAX supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat inactivated, and fetal calf serum (Gibco, USA).

Flow cytometric analysis

For analysis of Treg cells, the cell suspension was transferred into tubes and washed once in PBS. The cells were stained with fluorescein isothiocyanate (FITC) antihuman CD4 and allophycocyanin (APC) antihuman CD25 at 4°C for 30 minutes. The cells were then incubated with phycoerythrin (PE) antihuman Foxp3 after fixation and permeabilization according to the manufacturer's instruction. All of antibodies and reagents were from eBioscience, USA.

For analysis of Th2 cells, the cell suspensions were stimulated with 20 ng/ml phorbol 12-myristate-13-acetate (PMA) (Sigma-Aldrich, USA), 1 μg/ml ionomycin (Sigma-Aldrich) and 2 mmol/ml monensin (Sigma-Aldrich) in 24-well plates. After four hours of culture (37°C and 5% CO_2), the cells were transferred to tubes and washed once in phosphate buffered saline (PBS). The cells were then incubated with phycoerythrin-cy5.5 (PE-CY5.5) antihuman CD4 (BD Pharmigen, USA) at 4°C for 30 minutes. After surface staining, the cells were fixed and permeabilized according to the manufacturer's instruction, and then stained with PE antihuman IL-4 (BD Pharmigen).

Isotype controls were used to correct nonspecific binding. All stained cells were analyzed by flow cytometry.

Enzyme linked immunosorbent assays (ELISA)

The concentrations of IL-10, TGF-β1 and IL-4 in plasma were measured by ELISA in accordance with the manufacturer's instructions (R&D Systems, USA). All samples were measured in duplicate.

Quantitative polymerase chain reaction (PCR)

PBMCs from 17 mild asthmatic patients, 13 moderate to severe persistent asthmatic patients, and 14 healthy donors were acquired. PBMCs were cultured with 20 ng/ml PMA for four hours, and ribonucleic acid (RNA) was extracted using Trizol reagent (Invitrogen, USA). The isolated RNA was reverse transcribed with oligo dT primer in a reverse transcriptase procedure in a total volume of 20 μl (Progema, USA). Quantitative real-time PCR was performed using the ABI 7900 Sequence Detection System with primer pairs and SYBR Green PCR Master Mix (Biotnt, China). The sequence used were as follows: Foxp3 forward 5′-ATG CGA CCC CCT TTC ACC TAC-3′ and reverse 5′-TGG CGG ATG GCG TTC TTC-3′, GATA-3 (GATA binding protein 3) forward 5′-GAG ATG GCA CGG GAC ACT AC-3′ and reverse 5′-GTG GTT GTG GTG GTC TGA CAG T-3′. Quantification of the PCR signals was performed by comparing the cycle threshold (Ct) value of the gene of interest with the Ct value of the reference gene β-actin.

Statistical analysis

SPSS 11.0 (SPSS Inc., USA) was used for statistical analysis. Homogeneity of variance in three groups was tested first. If each group showed homogeneity, further analysis was done using one way analysis of variance (ANOVA) followed by the Student Newman Keuls test. When heteroscedasticity was present in each group, the data were analyzed using Mann-Whitney test and were expressed as medians (interquartile range). Pearson's correlation analysis was used to analyze the relationship among the expression of CD4+CD25+ Treg and Th2 cells, including their cytokines. Statistical significance was defined as $P<0.01$.

RESULTS

Percent of Treg, Th2 cells and ratio of Th2/Treg in allergic asthma

The percent of Treg cells (CD4+CD25+Foxp3+ cells) was significantly lower in peripheral blood of patients with moderate to severe allergic asthma ((2.73±1.64)%) than in patients with mild asthma ((4.59±1.90)%, $P<0.05$) or the control group ((4.10±2.38)%, $P<0.05$) (Figure 1). This indicates that the severity of asthma is negatively correlated with the percent of Treg cells. The percent of Th2 cells (CD4+IL-4+ cells) was markedly higher in patients with

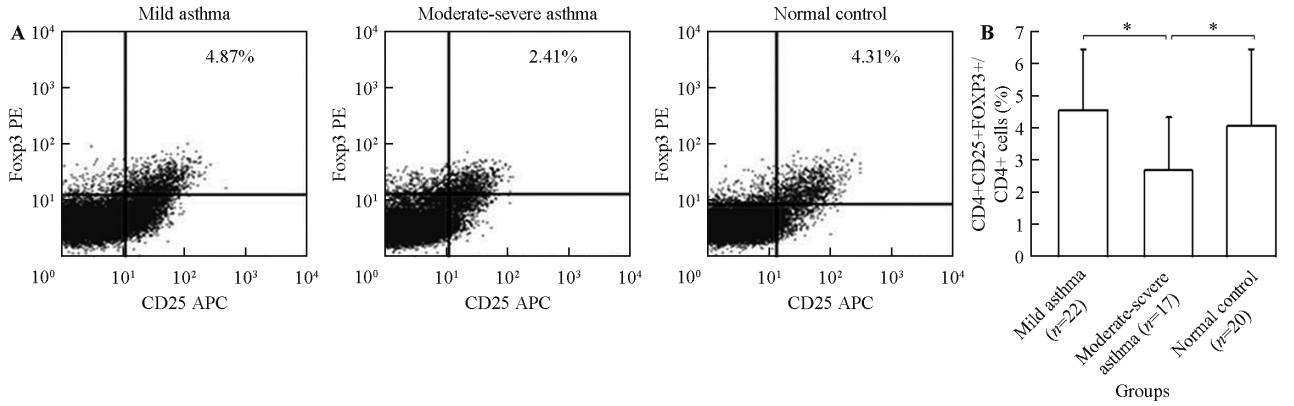

Figure 1. The frequencies of CD4+CD25+Foxp3+ Treg cells relative to blood CD4+ cells among patients with asthma and normal subjects were examined by flow cytometry. **A:** Representative plots are from a single patient in each group. **B:** Frequencies of Treg cells in each group. $^{*}P$ <0.05.

mild asthma (3.59%, range 2.76%–5.72%), and moderate to severe asthma (4.49%, range 2.52%–6.49%) than in the control group (1.71%, range 1.19%–2.63%, P <0.01). (Figure 2)

Compared with normal subjects (0.51, range 0.28–0.88), the ratio of Th2/Treg cells was higher in patients with mild (0.73, range 0.51–1.68, P <0.05) and moderate to severe asthma (1.72, range 1.01–2.78, P <0.01). Furthermore, the ratio of Th2/Treg cells was significantly higher in patients with moderate to severe asthma than in patients with mild asthma (P <0.05) (Figure 3).

Plasma IL-10, TGF-β1 and IL-4 levels

The plasma IL-10 level was significantly lower in patients with moderate to severe asthma than in patients with mild asthma and in the normal control group. But plasma IL-4 levels were significantly higher in patients with mild and moderate to severe asthma than in the control group. In patients with moderate to severe asthma, the levels of TGF-β1 were lower than those in the control group, but were not significantly different to those in patients with mild asthma (Table 1).

Compared with normal subjects and the mild asthma group, the ratio of IL-4/IL-10 was markedly higher in the moderate to severe asthma group. However, there is no significant difference between the mild asthma group and normal controls. The ratio of IL-4/TGF-β1 was significantly higher in patients with mild and moderate to severe asthma than in the control group (Table 1).

Expression of Foxp3, GATA-3 messenger RNA (mRNA) and their ratios in allergic asthma

The expression of GATA-3 mRNA was markedly higher in patients with mild ($(17.24\pm9.88)\times10^{-3}$), and moderate to severe asthma ($(15.99\pm9.83)\times10^{-3}$) than in the control group ($(4.97\pm1.61)\times10^{-3}$, P <0.01). There was no significant difference of Foxp3 mRNA expression among the mild asthma, moderate to severe asthma, and the normal control groups, $(1.06\pm1.63)\times10^{-3}$ vs. $(0.67\pm0.42)\times10^{-3}$ vs. $(1.43\pm1.9)\times10^{-3}$ (P >0.05), but a downward trend was seen across patients with mild and moderate to severe asthma. The ratio of GATA-3 mRNA/Foxp3 mRNA was

Table 1. Plasma IL-10, TGF-β1, IL-4 levels and ratios of IL-4/IL-10, IL-4/TGF-β1

Cytokine	Mild asthma (n=22)	Moderate to severe asthma (n=17)	Normal control (n=20)
IL-10 (pg/ml)	63±36	38±18*§	61±36
TGF-β1 (ng/ml)	29±18	21±15*	36±21
IL-4 (pg/ml)	65* (37–102)	81† (48–98)	38 (22–49)
IL-4/IL-10 (ratio)	1.12 (0.42–2.02)	2.32†‡ (1.29–3.37)	0.69 (0.34–1.25)
IL-4/TGF-β1(ratio)	0.12* (0.08–0.20)	0.18† (0.10–0.34)	0.06 (0.02–0.17)

$^{*}P$ <0.05 vs. normal control; $^{†}P$ <0.01 vs. normal control; $^{‡}P$ <0.01 vs. mild asthma; $^{§}P$ <0.05 vs. mild asthma.

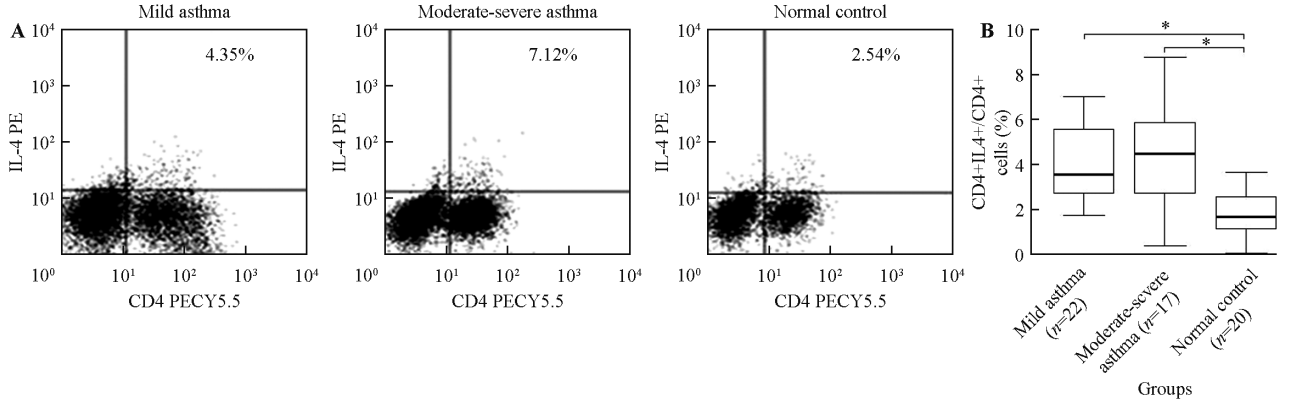

Figure 2. The frequencies of Th2 cells relative to blood CD4+ cells among patients with asthma and normal subjects were examined by flow cytometry. **A:** Representative plots are from a single patient in each group. **B:** Frequencies of Th2 cells in each group. $^{*}P$ <0.01.

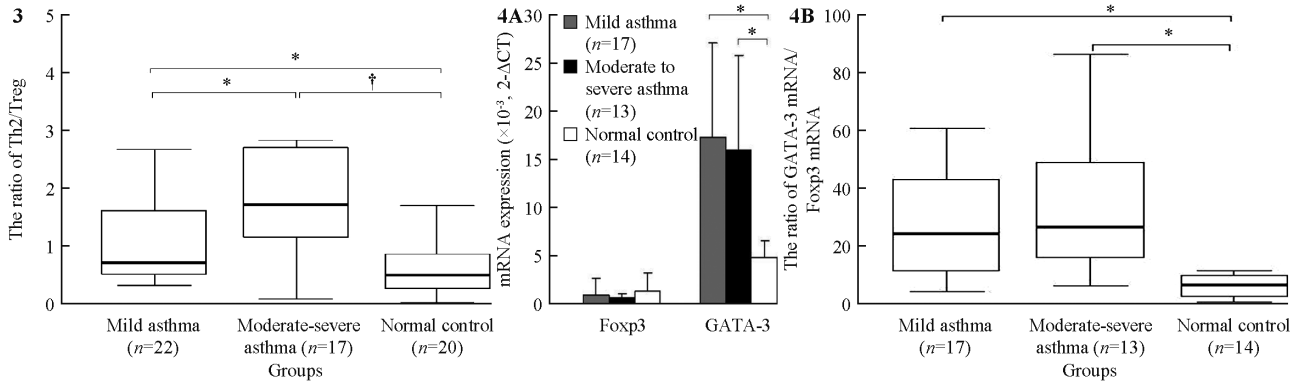

Figure 3. Ratio of Th2/Treg among patients with asthma and normal subjects. $^{*}P$ <0.05, $^{\dagger}P$ <0.01.
Figure 4. Expression of Foxp3 and GATA-3 mRNA in three groups. **4A:** Expression of Foxp3 and GATA-3 mRNA in patients with asthma and normal subjects were examined by real-time PCR. **4B:** The ratio of GATA-3/Foxp3 mRNA in asthma and normal. $^{*}P$ <0.01.

significantly higher in patients with mild and moderate to severe asthma than in normal subjects (24.32, range 11.33–47.22 vs. 26.62, range 12.56–54.20 vs. 6.64, range 2.50–10.09 (P <0.01) (Figure 4).

Correlation analysis in patients with allergic asthma
In patients with moderate to severe asthma, the frequency of peripheral blood CD4+CD25+ Treg cells were negatively correlated to the percentage of Th2 cells (r=–0.729, P <0.01) and IL-4 levels (r=–0.675, P <0.01).

DISCUSSION

In the present study, we demonstrated that the decline of CD4+CD25+ Treg cells in patients with moderate to severe asthma may play an important role in progress of the disease and that the deficiency of Treg cells was associated with the over-expression of Th2 response in asthma.

Th2 cells are believed to augment the inflammatory response observed in asthma by expressing multiple cytokines such as IL-4, IL-5, IL-9 and IL-13, each of which could potentially augment the eosinophilic inflammation in asthma. We also proved that the Th2 response was increased both in patients with mild and moderate to severe asthma at the cell, cytokine, and RNA levels. Th2 cells are central to the pathogenesis of asthma as has been described in most data.[12] However some studies suggest that other T subsets, including Th1 cells,[13] Th17 cells[14,15] and Treg cells also play a role in asthma. These data indicate that the immune mechanisms in asthma are very complex.

In healthy individuals, CD4+CD25+ Treg cells play an essential role in modulating and regulating immune responses by promoting tolerance, counterbalancing aggressive inflammatory reactions, and maintaining homeostasis. The effector function of all T helper cells, Th1, Th2 and Th17 cells, is regulated by Treg cells.[16,17] Several independent studies have shown that the number and function of Treg cells was impaired or altered in allergic patients compared with healthy individuals.[18-20] But the relationship between Treg and Th2 cells in patients with asthma is unclear.

Whether the expression of CD4+CD25+ Treg cells in patients with asthma is increased or decreased is still controversial. Some studies indicated increased Treg cells in asthma subjects,[21,22] one study reported that the CD4+CD25^{high} T-cell proportion is not reduced in allergic asthma,[23] but more studies confirmed that there is a population decline or functional defect of Treg cells in asthma.[18-20,24,25] Our study showed that the percent of CD4+CD25+ Treg cells was decreased in moderate to severe asthma. Matsumoto et al[26] suggested that the percent of CD4+CD25+Foxp3+ T cells was inversely correlated with bronchial hyperresponsiveness and allergen-specific IgE levels in the serum of asthmatic mice. Abdulamir et al[24] reported that expression of Treg cells was extensively diminished in patient with severe asthma. These studies confirmed CD4+CD25+ Treg cells were associated with the severity of asthma.

As well as reductions in numbers, CD4+CD25+ Treg cells from individuals with atopy have a significantly reduced capacity to suppress effector T cells and Th2 cytokines.[3] The cytokines most commonly implicated in Treg-mediated suppression of allergic asthma are TGF-β and IL-10.[27] IL-10 and TGF-β were secreted not only by CD4+CD25+ Treg cells but other regulatory T cells, for example, IL-10 secreted by type 1 regulatory T cells and TGF-β secreted by Th3 cells. Some studies suggest that CD4+CD25+ Treg play an important role in allergic asthma, through IL-10 or TGF-β mediated mechanism.[18,26,28,29] So levels of IL-10 or TGF-β can partly reflect the function of CD4+CD25+ Treg. In the present study, we demonstrated remarkable reduced IL-10 and TGF-β levels in the blood of patients with moderate to severe asthma.

The transcription factor, Foxp3, is essential for the suppressive activity, survival, and stability of CD4+CD25+ Treg cells. Persistent expression of Foxp3 is critical to the immunosuppression by CD4+CD25+ Treg cells. Curotto de Lafaille et al[30] demonstrated Foxp3+ Treg cells play an essential role in allergic inflammation and mucosal tolerance in mice. In the present study, the lower level of Foxp3 mRNA expression could be observed in patients with moderate to severe asthma, though the Foxp3 mRNA

expression in the asthma group was not significantly different with that in the control group.

Not only the decline of IL-10 and TGF-β but also the decreased expression of Foxp3 may imply functional deficiency of CD4+CD25+ Treg cells in moderate to severe asthma. The asthmatic patients with fewer and less functional Treg cells may have further uncontrolled effector cell responses.[3]

Furthermore, our study clarified that the deficiencies in Treg cells were inversely correlated with the percentage of Th2 cells and IL-4 levels. We found ratios of Th2/Treg, IL4/TGF-β and GATA-3/Foxp3 were higher in allergic asthma. In particular, ratios of Th2/Treg were notably increased in moderate to severe asthma. This means the relative deficiencies of Treg cells regarding Th2 response was correlated with the severity of asthma.

These results above indicated severe Th2 inflammation may affect the expression of Treg cells or their function. Mechanisms of this phenomenon are still unclear. It is clear that both Th2 and Treg cells coexist at the site of allergic inflammation and Th2-cytokines, like IL-4, can promote Th2 but block TGF-β-induced Treg cell development.[31] STAT6 is activated by IL-4 in naïve CD4+ T cells and upregulates GATA-3 expression, which is the master regulator of Th2 cell differentiation. Both STAT6 and GATA-3 can bind to the Foxp3 promoter to inhibit Foxp3 gene transcription.[32,33] So the percent of CD4+CD25+ Treg cells was significantly lower in moderate to severe asthma, presumably through the increase of the IL4/TGF-β or GATA-3/Foxp3 ratios. Further studies are needed to explain the exact ratios of factors that can lead to the decrease of Treg cell generation. It is recently proven that Treg cells can be converted into Th2 cells under the influence of an inflammatory microenvironment. Tang et al[9] showed that increased numbers of Th2 cells can compete with Treg cells by combined interleukin-2 in inflammatory parts of the pancreas of mice with type 1 diabetes. Phillemer et al[33] confirmed that T helper cells were resistant to Treg cells in a mouse model of asthma.

Our earlier study indicated that elevated Th17 cell responses and an imbalance in Th17/Treg levels were associated with moderate to severe asthma.[25] However, in this study, we confirmed that an imbalance in Th2/Treg levels also play an important role in progress of asthma. It suggests that Th2/Treg and Th17/Treg imbalance, beyond the Th1/Th2 paradigm, have important and possibly complementary roles in asthma.

In summary, we found a deficiency of CD4+CD25+ Treg cells in moderate to severe asthma, and it was associated enhanced Th2 response. So we confirmed that the Th2/Treg imbalance existed in patients with moderate to severe asthma, which may be involved in the pathogenesis of allergic asthma.

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