

Analysis of CD4⁺CD25⁺ Regulatory T Cells and Foxp3 mRNA in the Peripheral Blood of Patients with Asthma^{*}

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Summary: The changes of CD4⁺CD25⁺ regulatory T cells (CD4⁺CD25⁺ Treg) and Foxp3 mRNA in peripheral blood mononuclear cells (PBMCs) from patients with asthma were investigated in order to elucidate the possible roles of CD4⁺CD25⁺ Treg in the development of asthma. The peripheral blood samples were collected from 29 healthy controls (normal control group) and 78 patients with asthma which included 30 patients in exacerbation group, 25 patients in persistent group, and 23 patients in remission group. By using flow cytometry and RT-PCR, the CD4⁺CD25⁺ Treg ratio and Foxp3 mRNA in PBMCs were detected. The CD4⁺CD25⁺ Treg ratio and Foxp3 mRNA in PBMCs of exacerbation and persistent groups were lower than that of remission and normal control groups ($P < 0.05$). Although the CD4⁺CD25⁺ Treg ratio and Foxp3 mRNA of remission group were also lower than that of normal control group, there was no significant difference between them ($P > 0.05$). As compared with persistent group, exacerbation group had lower CD4⁺CD25⁺ Treg ratio and Foxp3 mRNA ($P < 0.05$). It was indicated that the decrease of CD4⁺CD25⁺ Treg ratio and its function in PBMCs may be responsible for pathogenesis of asthma.

Key words: asthma; peripheral blood mononuclear cells; CD4⁺CD25⁺ regulatory T cells; Foxp3 mRNA

CD4⁺CD25⁺ regulatory T cells (CD4⁺CD25⁺ Treg) which have immunosuppression function play an important role in maintenance of immunological homeostasis, immune tolerance, and prevention of autoimmune diseases. Transcription factor Foxp3 is specially overexpressed in CD4⁺CD25⁺ Treg, plays a key role in the development and function of CD4⁺CD25⁺ Treg, and can reflect the number and function of CD4⁺CD25⁺ Treg to some extent^[1-3]. Asthma characterized by chronic airway inflammation, involves a number of different inflammatory cells, inflammatory mediators, and cytokines. Recently, many researches indicated that the activation of effector CD4⁺ T cells, especially Th2 cells which could produce many inflammatory mediators and cytokines played a key role in the pathogenesis of asthma. CD4⁺CD25⁺ Treg probably could inhibit the development of asthma through inhibiting the activation of Th2 cells. In this study, we investigated the changes of CD4⁺CD25⁺ Treg and Foxp3 mRNA in peripheral blood mononuclear cells (PBMCs) from patients with asthma, so as to elucidate the possible roles of CD4⁺CD25⁺ Treg in the development of asthma and provide the method for immune treatment of asthma.

1 MATERIALS AND METHODS

1.1 Subjects

Seventy-eight patients with asthma in Department of Respiratory Diseases, Tongji Hospital, from October 2005 to March 2006, were enrolled in this study. All patients met the criteria in prevention and treatment guidance of asthma set by Asthma Group, Respiratory Sub-association, Chinese Medical Association in 2003^[4]. There were 30 patients in exacerbation group including 16 males and 14 females with their age ranging from 25 to 58 years old (mean 40.56 ± 5.79 years), 25 patients in persistent group including 13 males and 12 females with age ranging from 28 to 57 years old (mean 42.12 ± 5.23 years), and 23 patients in remission group including 13 males and 10 females with age ranging from 22 to 61 years old (mean 41.25 ± 6.15 years). At the same time, 29 healthy controls with no record of recent infection and autoimmune diseases were selected, including 15 males and 14 females with age ranging from 26 to 56 years old (mean 40.87 ± 5.11 years). All patients received no special treatment before samples collection. Six mL venous blood was taken from each subject and anti-coagulated with heparin (12 U). The sex and age between all groups had no significant difference ($P > 0.05$).

1.2 Major Reagents

Human FITC-conjugated CD4 monoclonal antibody (McAb) and PE-conjugated CD25 McAb were obtained

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from BD PharMingen Co. (USA). Trizol was purchased from Gibco BRL Co. (USA). RT-PCR kit was got from Promega Co. (USA). Foxp3 primers were synthesized by Bioasia Co. (China).

1.3 Detection of CD4⁺CD25⁺ Treg Ratio in PBMCs by Flow Cytometry

PBMCs were separated with human Ficoll solution, then washed twice in phosphate-buffered saline (PBS), and adjusted to a density of 1×10^7 cells/mL. PBMCs (1×10^6 cells) were incubated with FITC-conjugated CD4 McAb and PE-conjugated CD25 McAb at 4 °C for 30 min. After washed for three times and resuspended with 200 μ L PBS, cell fluorescent levels were analyzed by flow cytometry.

1.4 Detection of the Foxp3 mRNA Expression in PBMCs by RT-PCR

PBMCs (1×10^7 cells) were washed twice in PBS, then total RNA isolated by Trizol and quantified by ultraviolet spectrophotometer. RT-PCR was performed as follows: 5 μ g total RNA was reversely transcribed into cDNA in the presence of oligo-dT and M-MLV reverse transcriptase, and amplification was performed using cDNA as template. Primer sequences used for Foxp3 amplification were as follows: sense, 5'-CTACGCCACGCTCATCCGCTGG-3'; antisense, 5'-GTAGGGTTGGAACACCTGCTGGG-3'^[5]. The size was 261 base pairs (bp). Primer sequences for the control GAPDH were: sense, 5'-CCACCCATGGCAAATTCCATGGCA-3'; antisense, 5'-TCTAGACGGCAGGTCAGGTCCAC-3'. The size was 598 bp. Amplification was performed with 32 cycles of pre-denaturation (94 °C, 5 min), denaturation (94 °C, 1 min), annealing (60 °C, 2 min), and extension (72 °C, 2 min). The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and were analyzed by GDS8000 Image Evaluation System (UVP Co., England). Relative levels of Foxp3 mRNA expression were got by calculating Foxp3/GAPDH absorbance (A) of the same sample.

1.5 Statistical Analysis

All data were dealt with SPSS 10.0 software. The results in different groups were presented as $\bar{x} \pm s$ and analyzed by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

2 RESULTS

2.1 The CD4⁺CD25⁺ Treg Ratio in PBMCs of Patients with Asthma

As shown in table 1, the CD4⁺CD25⁺ Treg ratio in exacerbation and persistent groups was lower than that in remission and normal control groups ($P < 0.05$). Although the CD4⁺CD25⁺ Treg ratio in remission group was also lower than that in normal control group, there was no significant difference between them ($P > 0.05$). As compared with persistent group, exacerbation group had lower CD4⁺CD25⁺ Treg ratio ($P < 0.05$).

2.2 The Foxp3 mRNA Expression in PBMCs of Patients with Asthma

As shown in fig. 1 and table 2, The Foxp3 mRNA expression in exacerbation and persistent groups was lower than that in remission and normal control groups ($P < 0.05$). Although the Foxp3 mRNA expression in re-

mission group was also lower than that in normal control group, there was no significant difference between them ($P > 0.05$). As compared with persistent group, exacerbation group had lower Foxp3 mRNA expression ($P < 0.05$).

Table 1 The CD4⁺CD25⁺ Treg ratio in PBMCs of patients with asthma ($\bar{x} \pm s$)

Groups	<i>n</i>	CD4 ⁺ CD25 ⁺ Treg (%)
Exacerbation	30	3.01±0.76 ^{*△}
Persistent	25	4.25±0.98 [*]
Remission	23	5.14±1.15
Normal control	29	5.79±1.22

^{*} $P < 0.05$ vs the normal control group and remission group,

[△] $P < 0.05$ vs the persistent group

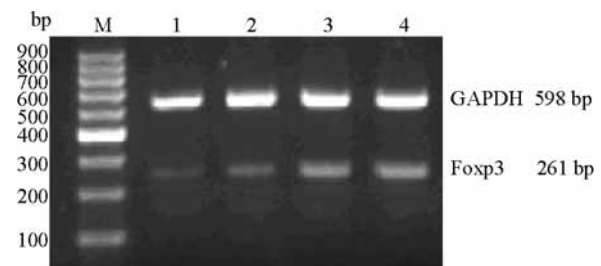


Fig. 1 The Foxp3 mRNA expression in PBMCs of patients with asthma

M: Marker; 1: Exacerbation group; 2: Persistent group; 3: Remission group; 4: Normal control group

Table 2 Relative levels of Foxp3 mRNA expression (A ratio, $\bar{x} \pm s$)

Groups	<i>n</i>	Foxp3mRNA (Foxp3/GAPDH)
Exacerbation	30	0.07±0.01 ^{*△}
Persistent	25	0.13±0.02 [*]
Remission	23	0.31±0.05
Normal control	29	0.32±0.04

^{*} $P < 0.05$ vs the normal control group and remission group,

[△] $P < 0.05$ vs the persistent group

3 DISCUSSION

According to its surface marker, mechanism and cytokines production, regulatory T cells can be classified as CD4⁺CD25⁺ Treg, Th3 cells, Treg1 cells, NKT cells, etc. As the most important Treg, CD4⁺CD25⁺ Treg is a small subpopulation of CD4⁺ T cells and first described by Sakaguchi *et al*^[6], whose ratio is about 5%—10% of circulating CD4⁺ T cells. CD4⁺CD25⁺ Treg is low responsive to antigen and has the ability of immunosuppression. The immuno-hyporesponsiveness is that many stimulators such as IL-2 of high concentration and anti-CD3 McAb all can promote proliferation of CD4⁺ T cells, but not CD4⁺CD25⁺ Treg. Immunosuppression is the most important function of CD4⁺CD25⁺ Treg, through which CD4⁺CD25⁺ Treg can inhibit proliferation and activation of effector CD4⁺ T cells^[7,8]. CD4⁺CD25⁺ Treg exerts immunosuppression mainly through cell-to-cell contact dependent on cytotoxic T lymphocyte antigen 4^[9]. Depletion of CD4⁺CD25⁺ Treg can induce

autoimmune diseases such as thyroiditis, type I diabetes mellitus, inflammatory bowel disease, and so on.

Foxp3 (forkhead/winged helix transcription factor) is specially overexpressed in CD4⁺CD25⁺Treg^[10]. Hori *et al*^[3] discovered that CD4⁺CD25⁺T cells transfected by Foxp3 gene could acquire the phenotype and function of CD4⁺CD25⁺Treg. Scurfy mice lost the ability of immunosuppression and had lymphocytic proliferative diseases because of deficiency of CD4⁺CD25⁺Treg caused by Foxp3 gene mutation^[11]. The diseases were mediated by CD4⁺ cells which could produce excessive cytokines and were in state of high activation *in vivo* and *in vitro*. Human X-linked autoimmunity-allergic dysregulation syndrome characterized by severe eczema, high IgE level, lots of eosinophils, and severe food allergy is also due to deficiency of CD4⁺CD25⁺Treg caused by Foxp3 gene mutation^[12]. So, Foxp3 is crucial to production and function of CD4⁺CD25⁺Treg.

With immunologic development, many researches indicated that cellular immune disturbance, especially Th1 cells/Th2 cells imbalance played a key role in asthma pathogenesis. Because the cellular immune disturbance is in touch with abnormal immunoregulation, the patients with asthma probably have CD4⁺CD25⁺Treg abnormality. Our study revealed that the CD4⁺CD25⁺Treg ratio and Foxp3 mRNA in PBMCs of exacerbation and persistent groups, especially exacerbation group, were lower than that in remission and normal control groups, indicating that CD4⁺CD25⁺Treg participated in the pathogenesis of asthma. The decrease of immunosuppression in asthma patients caused by few CD4⁺CD25⁺Treg led to the activation of effector CD4⁺T cells, especially Th2 cells which could produce many inflammatory mediators and cytokines such as IL-4, IL-5, IL-13, and so on, followed by asthma exacerbation or persistence. After relief through treatment or spontaneous remission, CD4⁺CD25⁺Treg number and Foxp3 mRNA expression in asthma patients were increased, close to the normal level, then CD4⁺CD25⁺Treg exerted its function of immunosuppression, inhibited Th2 cell activation, rectified the imbalance of Th1 and Th2 cells and finally promoted asthma remission.

To sum up, the results of this study discovered that the decrease of CD4⁺CD25⁺Treg number and function in PBMCs of patients with asthma, leading to the activation of effector CD4⁺T cells, especially Th2 cells which could produce many inflammatory mediators and cytokines was a key factor in asthma pathogenesis. So, finding an efficient way to interfere with the CD4⁺CD25⁺

Treg number and function is helpful to control the development of asthma.

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