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Altered expression of regulatory T and Th17 cells in murine bronchial asthma

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Abstract. Alteration of the careful balance of the ratio of Th1/Th2 cell subsets impacts immune function and plays an important role in the pathogenesis of asthma. There is little research on the impact of changes on the balance of the regulatory T (Treg)/Th17 subset ratio and its possible repercussions for asthma. This investigation used a murine model of asthma to measure the expression levels of Treg and Th17 cells and the levels of their transcription factors Foxp3 and retinoic acid receptor-related orphan nuclear receptor (ROR)γt in bronchial asthma while assessing indexes of airway inflammation. Thirty female SPF BALB/c mice were divided into three equally numbered groups: a normal control, an asthma and a dexamethasone treatment group. All the airway inflammation indexes measured were more prominent in the asthma group and less so in the control group. The percentage of the lymphocyte subset $CD4^+CD25^+Foxp3^+$ cells in the $CD4^+$ cells in the asthma group was significantly lower than that in the normal control group ($P<0.01$). The percentage of the lymphocyte subset $CD4^+IL\text{-}17^+$ cells in the $CD4^+$ cells in the asthma group was significantly higher than that in the normal control group ($P<0.01$). The ratio of $CD4^+CD25^+Foxp3^+$ cells/$CD4^+IL\text{-}17^+$ cells in the asthma group decreased compared with that in the normal control group ($P<0.01$). The expression level of Foxp3 of the mice in the asthma group was significantly lower than that in the control group ($P<0.01$). The expression intensity of RORγt in the asthma group was higher than that in the normal control group ($P<0.01$). Finally, the Foxp3/RORγt protein expression ratio in the asthma group was significantly lower than that in the normal control group ($P<0.01$). The Foxp3/RORγt protein expression ratio and the airway responsiveness were negatively correlated. The average levels of inflammation markers in the dexamethasone group were intermediate between the other groups. During the course of bronchial asthma the unbalanced expression of Treg and Th17 affects mostly the expression of Foxp3/RORγt, leading to inflammation of the airways. Dexamethasone may inhibit airway inflammation by regulating the balance between Treg and Th17.

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Introduction

Bronchial asthma is a chronic inflammatory disease involving multiple inflammatory cells such as mastocytes, eosinophilic granulocytes and T lymphocytes. The characteristic airway inflammation, tissue damage and airway dysfunction are a result of local aggregation of inflammatory cells in the airways and release of inflammatory mediators and cytokines (1). The T lymphocytes play a core role in asthma airway inflammation (2). Bronchial asthma is a chronic inflammatory disease mediated by excessively activated Th2 cells. In contrast, a Th1 reaction is considered a protective factor of allergic diseases including asthma. In addition, the immunotherapy for allergic diseases aims at transforming the Th2 cytokine phenotype into a Th1 phenotype (3). Thus, the idea of a Th1/Th2 imbalance in asthma is widely accepted. Nevertheless, there still are experimental and clinical phenomena related to asthma that remain unexplained by the altered Th1/Th2 model. Research has found that the Th1 cells can exacerbate allergies and asthma. The Th1 cytokine IFN-γ is associated with airway hyper-responsiveness induced by antigens and infiltration of eosinophilic granulocytes (4). The regulatory T (Treg) cells cannot only inhibit the Th1 cells but can inhibit the Th2 cells as well (5). Therefore, research on Treg cells has become a priority to better understand the pathogenesis of asthma.

The Th17 cells mediate the incidence and progression of inflammatory reactions, autoimmune diseases, tumors and transplant rejection (6). The major biological function of the secreted IL-17 is to promote inflammatory reactions. IL-17 is a pre-inflammatory cytokine and also an early activating factor of the inflammatory reactions induced by the T cells (7). It can amplify inflammatory reactions by promoting the release of other pre-inflammatory cytokines. It can also recruit neutrophils, promote release of inflammatory factors by multiple cells, promote secretion of mucus by the mucous glands, and strengthen the airway hyper-responsiveness. IL-17 is closely

associated with the incidence and progression of the chronic inflammatory diseases related to the airways (8). In the pulmonary tissues of asthma model rats, the IL-17 secreted by Th17 cells is significantly and positively correlated with an increase in the eosinophilic granulocytes in the bronchoalveolar lavage fluid (BALF). Thus, the Th17 cells and IL-17 may induce the expression of eosinophilic granulocytes in the eosinophilic asthma.

Moreover, research surveys in recent years have shown that allergic asthma accounts only for ~41% of all asthma attacks (9) and the remaining 59% of episodes are associated with neutrophils, indicating that approximately half of the attacks are not attributable to allergens or atopy (10). Given this complex pathogenic scenario, the roles of neutrophils and their powerful recruiting factor, IL-17, remain to be clarified. For example, activation in ovalbumin (OVA)-sensitized mice leads to increased expression of IL-17 mRNA in the pulmonary tissue and significant neutrophil infiltration (11). The high expression of IL-17 in BALF of asthma patients (12) indicates that the Th17 cells may participate in the onset of asthma.

Transcription factors are a class of nucleoproteins that identify and are bound to specific DNA regulatory sequences thereby stimulating or inhibiting transcription. Any changes in their quantity or activity may lead to abnormal expression of genes critical to cellular growth and differentiation.

Among the transcription factors promoting the differentiation of Treg and Th17, Foxp3 and retinoic acid receptor-related orphan nuclear receptor (ROR)γt are the most important ones (13). RORγt is a critical transcription factor that controls differentiation in Th17 cells. Research indicates that the level of RORγt mRNA increases gradually when the initial T cells are differentiating into Th17 (14). T cells with a deletion of the RORγt are not able to differentiate into Th17 in *in vitro* experiments. There is a complex mutual relationship between the Treg and the Th17 cells. They are correlated during differentiation but display antagonistic functions (13). Other cytokines such as IL-6, and TGF-β can determine whether the immune response of the Treg or that of the Th17 is dominant. The TGF-β produced by the immune system inhibits proliferation of the effector T cells, and induces differentiation of the naive T cells into the Treg cells (15). Treg cells secrete TGF-β and express Foxp3, inhibit inflammatory responses, maintain the immune tolerance of the organism, and prevent the incidence of autoimmune diseases (16) when the immune system is in a steady state or subject to no inflammatory injury. However, the acute stage protein IL-6 will be substantially produced when infection or inflammation occur. IL-6 inhibits proliferation of the Treg cells. IL-6 and TGF-β jointly induce differentiation of Th17 cells and secrete IL-17 and IL-6. Experiments have demonstrated that once the Th17 cells are produced and IL-17 is secreted, a positive feedback develops. It induces IL-6, further producing Th17, mediates pre-inflammatory responses, and participates in autoimmune diseases (17).

It has been established that the Th1 and Th2 cell subsets play important roles in the pathogenesis of bronchial asthma. However, the function of other cell subsets like the Treg and Th17, and the significance of the changes to their ratio are the subject of research in our study. We believe a probe into the balance of Treg/Th17 will supplement and improve the current understanding of the immunological pathogenesis of bronchial asthma and aid in the search for more effective treatment strategies. To this end, we focused on the specific transcription factors of Foxp3 and RORγt that determine the differentiation of Treg/Th17.

Materials and methods

Experimental animals. SPF inbred strain female BALB/c mice were purchased from the Laboratory Animal Center of Cheeloo College of Medicine, Shandong University and raised in a laboratory animal center. Mice aged 6-8 weeks were selected for the experiments, and were divided into three groups according to a random number table method: normal control, asthma, and dexamethasone treatment group (10 mice in each group). The mice were raised in separate cages and fed with special food containing no allergens. This study was approved by the Animal Ethics Committee of Binzhou People's Hospital.

Sensitization and activation in mice. Each mouse was sensitized 3 times, at 0, 7, and 14 days of the experiments, as follows. Each mouse in the asthma group was subjected to intraperitoneal and bilateral femoral subcutaneous injections of a total of 200 μl sensitization solution containing 20 mg OVA and 20 mg $Al(OH)_3$ (ICN Biomedicals, Inc., Costa Mesa, CA, USA). The mice in the dexamethasone treatment group (Sanyao Science & Technology Development Co., Beijing, China) were sensitized in the same way. Finally, the mice in the normal control group were injected with normal saline for sensitization at the same sites and dosage as those of the other groups.

Mice in the asthma group were placed in an in house aerosol inhalation box and subjected to 5% (m/v) OVA aerosol inhalation activation for 7 consecutive days for 45 min daily from the 21st day after the experiment. A Paxiboy high-frequency atomizer (PE Applied Biosystems, Foster City, CA, USA) was used. The asthma attacks in mice were observed and recorded. The mice in the dexamethasone treatment group were injected intraperitoneally with dexamethasone (1 mg/kg) (Sanyao Science & Technology Development Co.) 30 min before being subjected to aerosol inhalation of 5% OVA for activation from days 21 to 27. The mice in the normal control group had normal saline instead of OVA atomization for activation.

Determination of airway responsiveness. The Buxco mouse plethysmograph (Thermo Fisher Scientific, Inc., Waltham, MA, USA), flow sensor, and atomization device were properly connected. The connection between the sensor and the amplifier and that between the amplifier and the computer were checked. All interfaces were well sealed. The software BioSystem XA analysis module (version 10.2; Tree Star, Inc., Ashland, OR, USA) was established. Each experimental mouse was placed in a non-invasive plethysmograph box. The %ΔPenh value for each mouse was recorded continuously after activation with atomization of normal saline and acetylcholine at different concentrations. The experimental results were collected and saved. The mice were returned to the cage.

Counting and classification of leukocytes. A total of 100 ml of cold PBS were used to dilute the BALF cell sediment.

Thirty milliliters were taken and the cells were counted with a cell counting chamber (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). A 50 ml sample of the cell sediment smear was dried and fixed. Two hundred cells were counted under the microscope for differential counting. The cells were divided into eosinophilic granulocytes, neutrophils, and lymphocytes based on morphological characteristics.

Detection of IL-10 and IL-17 in BALF and serum. The BALF blood sample from the mice was not diluted. The standard was diluted as required. The sample or standard was added to the ELISA plate pre-embedded with 100 ml/well anti-IL-10 and IL-17 antibodies (EMD Millipore, Billerica, MA, USA). A total of 100 ml/well of biotinylated antibodies IL-10 and IL-17 (Polysciences, Inc., Warrington, PA, USA) were added. The plates were incubated for 2 h at room temperature. Next, each plate was washed 3 times. Then, 100 ml streptavidin horseradish peroxidase solution (Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China) was added to each well. The plate was incubated for 1 h at room temperature. Then, the plate was washed 3 times. The TMB substrate solution was added as per 100 ml/well (Wuhan Boster Biological Engineering Co., Ltd.). It was left to develop for 15 min in the dark at room temperature. A total of 100 ml of the stop buffer were finally added. The OD value was measured at an absorption wavelength of 450 nm (Thermo Fisher Scientific, Inc.). A standard curve was plotted to obtain the content of IL-10 and IL-17 in the sample.

Pathological sections of pulmonary tissue. Paraffin sections of mouse pulmonary tissue were subjected to xylene dewaxing, and immersed into gradient grade ethanol 3 times for 5 min each. The sections were stained with hematoxylin (Beifang Biotechnology Research Institute, Beijing, China) for 10 min following standard procedures and then with eosin stain. The sections were routinely dehydrated, cleared with xylene, mounted with neutral gum, and observed under an optical microscope (Olympus Corp., Tokyo, Japan).

Flow cytometry. The spleen was removed from sacrificed mice under sterile conditions, placed in a sterile plate containing RPMI-1640 culture medium (Invitrogen Life Technologies Inc., Carlsbad, CA, USA), cut into 1-2 mm pieces with curved scissors, and ground gently with the inner core of a 2 ml injection syringe. The ground spleen cells were washed with 5-6 ml of the RPMI-1640 culture medium containing 2% FBS (Invitrogen Life Technologies, Inc.), and centrifuged for 10 min at 4˚C at 1,350 x g to discard the supernatant. A total of 7-8 ml of the erythrocytes lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) were added and the sample was allowed to stand still for 4-5 min. Twenty-five milliliters of the RPMI-1640 culture medium containing 2% FBS (Hyclone, Logan, UT, USA) were added. The sample was centrifuged at 850 x g for 10 min at 4˚C and the supernatant was discarded. Next, 15 ml of the culture medium was added. Twelve milliliters of the upper cells were re-suspended and centrifuged at 850 x g for 10 min at 4˚C. Five milliliters of the RPMI-1640 complete medium was added and the cells were re-suspended. A total of 20 ng/ml PMA (Sigma-Aldrich), 1 mg/ml inonmycin (Sigma-Aldrich), and 10 mg/ml BFA (Sigma-Aldrich) were added. After the appropriate fluorescence-labeled monoclonal antibodies, anti-CD4-FITC and anti-CD25-PE (BD Biosciences, Heidelberg, Germany) were added to the 100 ml samples, each sample was well mixed gently, incubated for 20 min at room temperature, and then further incubated for 10 min in the dark at room temperature. The samples were centrifuged at 850 x g for 5 min and the supernatants were discarded. Two milliliters of cell staining buffer solution was added and well mixed before centrifuging again at 850 x g for 5 min. The supernatant was discarded. A total of 0.5 ml of cell fixation/membrane rupture buffer solution (Sigma-Aldrich) was added to each sample. The samples were then incubated for 20 min in the dark at room temperature. The appropriate fluorescence labeled intracellular cytokine antibodies anti-IL-17-PE and anti-Foxp3-APC (BD Biosciences, Mountain View, CA, USA) were added. The solutions were well mixed and incubated for 20 min in the dark at room temperature. A total of 0.5 ml of the fixation buffer solution was added to re-suspend the cells. A flow cytometer (BD Biosciences, San Jose, CA, USA) was used for detection and analysis.

Immunohistochemical method. Sections were routinely dewaxed and dehydrated. Then the endogenous oxidases were removed. Sections were subjected to microwave antigen retrieval and incubated for 10 min at room temperature. The sections were washed 3 times for 3 min each with PBS. Next, 50 ml of non-immune animal serum (Bioss Biological Technology Co., Beijing, China) were added, the samples were incubated for 10 min at room temperature, and washed once with the buffer solution. Then after addition of 50 ml of dilute antibody Foxp3, RORγt (Abcam, Cambridge, UK), each section was incubated overnight at 4˚C. The next day, each section was washed 3 times for 3 min each with PBS. Fifty milliliters of the biotin-marked secondary antibody (Wuhan Boster Biological Engineering Co., Ltd.) was added to the sections and they were then incubated for 10 min at room temperature. The sections were washed 3 times with PBS for 3 min each. After addition of 50 μl streptavidin-peroxidase solution, each section was incubated for 10 min at room temperature (Wuhan Boster Biological Engineering Co., Ltd.). The sections were washed 3 times for 3 min each with PBS. Finally, each section was observed for 3-10 min under the microscope after addition of 100 ml of freshly prepared DAB solution. Then, each section was washed with water, lightly stained with hematoxylin (Beifang Biotechnology Research Institute), washed with water, dehydrated with gradient ethanol, dried, and mounted with neutral gum. The Image-Pro Plus microscope image analysis software (version 12.0; Tree Star, Inc.) was used for analysis of the expression intensity of the proteins studied. Five fields of view were determined for each section. The count of the positive cells in each field of view was computed and expressed by an average.

Western blot analysis. One milligram of whole pulmonary tissue protein sample was subjected to SDS-PAGE. After completion of SDS-PAGE, two pieces of gel were removed carefully. The spongy cushion, filter paper, gel, film, filter paper, and spongy cushion were set up in sequence for preparation of the gel transfer interlayer. After expelling the bubbles

with a glass rod, the sandwich was placed in an electroblotting tank and transferred for 60-90 min at 100 V (350 mA). After completion of membrane transfer, the membrane was transferred with tweezers to a plate containing 25 ml blocking buffer and shaken gently for 2 h decoloration on a shaking table at room temperature. After addition of the primary antibodies of Foxp3 and RORγt (Abcam), the membranes were incubated overnight at 4˚C. The next day, each membrane was washed 3 times for 5 min each with TBST, on a shaking table, at room temperature. After addition of the secondary antibody (Wuhan Boster Biological Engineering Co., Ltd.), each membrane was incubated on a shaking table for 1 h at 37˚C. The membranes were washed with TBST, 3 times for 5 min each. A developer was added (Thermo Fisher Scientific, Inc.) for observation.

Statistical analysis. SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical processing. The data are expressed with mean ± SD. The one-way analysis of variance and multiple comparisons were used for comparative analysis in various groups. The SNK method was used for multiple comparisons in the case of homogeneity of variance. The Welch's method was used for analysis and the Dunnett's T3 method was used for multiple comparisons in the case of heterogeneity of variance. A difference was considered significant at P<0.05.

Results

Airway responsiveness of the mice in various groups. The results indicated that the level of %ΔPenh in various groups increased with the amount of the acetylcholine concentration and there were significant differences among the mice with different concentrations (F=893.456, P<0.01). The level of %ΔPenh of the mice in the asthma group was significantly higher than that in the normal control group (F=265.558, P<0.01). There were no significant differences between the normal saline asthma group and normal control group (F=1.817, P=0.182). %ΔPenh in the asthma group was high in the case of other concentrations of acetylcholine (P<0.01). An interaction effect existed between different acetylcholine concentrations and different groups (F=54.160, P<0.001) (Table I).

Morphological changes in pulmonary tissue and differential count of BALF leukocytes. Based on the pathological sections of pulmonary tissues, we observed infiltration of a large number of inflammatory cells around the bronchiole and concomitant vessels in the mice of the asthma group. The infiltration by a large number of inflammatory cells was dominated by eosinophilic granulocytes and lymphocytes in the airway wall and pulmonary tissue, additionally there was a thickened bronchial wall, luminal stenosis, mucous plugs in the bronchial lumens, reduced mucosa plicae, goblet cell hyperplasia, widened alveolar septum, partial rupture, and development of emphysema.

In the dexamethasone treatment group, we observed orderly arranged bronchiole cilia, round lumens, occasional infiltration of eosinophilic granulocytes in the vessel lumens, alveolar cavities, and pulmonary interstitium, infiltration of a small number of lymphocytes, and no thickened basilar membrane.

Table I. %ΔPenh values of the BALB/c mice in various groups under different concentrations of acetylcholine (mean ± SD).

	%ΔPenh								
		Acetylcholine concentration (mg/ml)							
Group	Normal saline	3.125	6.25	12.5	25	50	Sum	F	P-value
Asthma group	13.25±2.56	106.39±10.25	198.14±33.45	156.43±52.39	585.87±50.27	887.09±85.70	348.34±35.97	469.478	<0.001
Dexamethasone group	12.59±3,87	27.43±10.53	84.34±35.53	328.57±50.44	336.78±54.98	568.16±55.76	187.93±20.91	265.347	<0.001
Normal control group	12.58±3.77	21.38±5.32	59.58±15.19	125.68±30.46	196.67±51.35	384.46±3.35	135.38±34.54	112.967	<0.001
Sum	12.45±3.36	51.47±36.21	113.49±64.47	214.35±105.38	205.27±102.98	370.56±168.53	613.46±226.57	236.438[a]	<0.001[a]
F	1.776	244.57	55.34	61.47	135.46	113.57	266.639[a]	F=55.469[b]	
P-value	0.156	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001[a]	P<0.001[b]	

[a]F statistics and P-value of the main effect. [b]F statistics and P-value of the interaction effect.

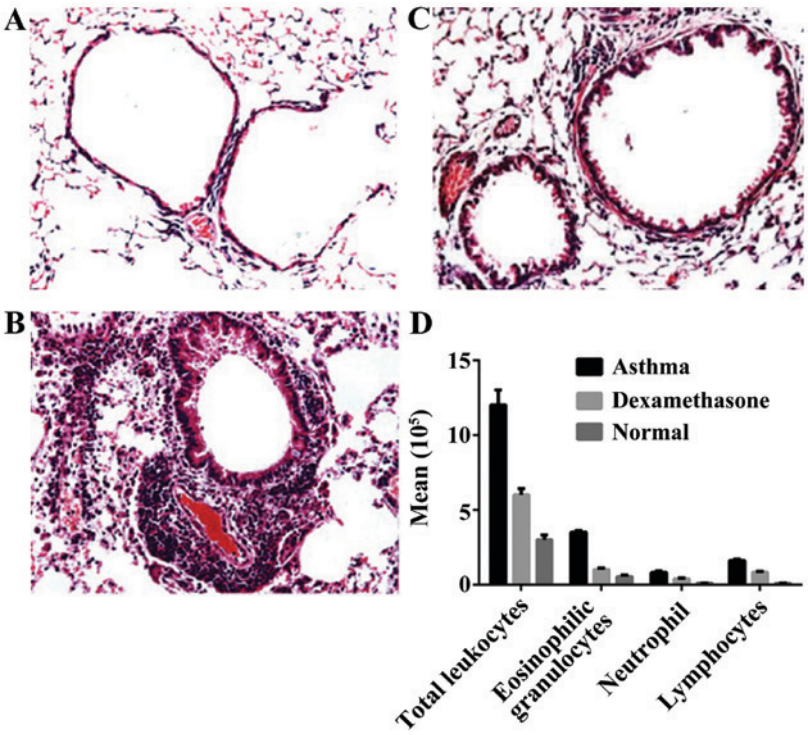

Figure 1. Morphological changes in the pulmonary tissue and differential counting of BALF leukocytes. H&E staining for the pulmonary tissue (A) in the normal control group, (B) in the asthma group and (C) in the dexamethasone treatment group. (D) The total count of leukocytes and the levels of eosinophilic granulocytes, neutrophil, and lymphocytes in the BALF of the mice in the three groups. BALF, bronchoalveolar lavage fluid.

The total count of cells in BALF and the count of eosinophilic granulocytes of mice in the asthma group were significantly larger than that in the normal control group, indicating that significant inflammation dominated by eosinophilic granulocytes occurred locally in the airway of the mice in the asthma group. The total counts of cells and the count of neutrophils, eosinophilic granulocytes, and lymphocytes increased significantly when compared with those in the normal control group (P<0.01). The counts of neutrophil, eosinophilic granulocytes, and lymphocytes in the dexamethasone treatment group decreased significantly when compared with those in the asthma group (P<0.01). The counts of neutrophils, eosinophilic granulocytes, and lymphocytes in the dexamethasone treatment group were still large when compared with those in the normal control group and the differences were significant (P<0.01) (Fig. 1).

Changes in the content of IL-10 and IL-17 in the BALF and serum, and the proportion of the $CD4^+CD25^+Foxp3^+$ and $CD4^+IL\text{-}17^+$ cells of the lymphocyte population in the $CD4^+$ T cells. Based on the determination of content of the IL-10 and IL-17 cytokines in BALF and serum, it was found that the level of IL-17 in BALF and serum of the mice in the asthma group were increased significantly. There were significant differences between the asthma group, and the dexamethasone treatment group and the normal control group (P<0.01). The level of IL-17 in BALF and serum in the dexamethasone treatment group decreased slightly when compared with that in the asthma group but it was still higher when compared with that in the normal control group. The level of IL-10 in BALF and serum of the mice in the asthma group was the lowest. There were significant differences between the asthma group, and the dexamethasone treatment group and the normal control group (P<0.01). The level of IL-10 in BALF and serum in the dexamethasone treatment group was increased slightly when compared with that in the asthma group (Fig. 2A-D). The lymphocyte subset $CD4^+CD25^+Foxp3^+$ cells/$CD4^+$ T cells in the asthma group were significantly fewer than those in the normal control group. The $CD4^+CD25^+Foxp3^+$ cells/$CD4^+$ cells in the dexamethasone treatment group were increased slightly when compared with those in the asthma group, but were still fewer than those in the normal control group (P<0.01). The lymphocyte subset $CD4^+IL\text{-}17^+$ cells/$CD4^+$ T cells in the asthma group were higher than those in the normal control group. The ratio of $CD4^+IL\text{-}17^+$ cells/$CD4^+$ cells in the dexamethasone treatment group was decreased slightly when compared with that in the asthma group, but was still higher when compared with that in the normal control group. There were statistically significant differences between the two groups (P<0.01). The ratio of $CD4^+CD25^+Foxp3^+$ cells/$CD4^+IL\text{-}17^+$ cells in the asthma group (50.4±0.45) was decreased when compared with that in the normal control group (50.4±0.45). The ratio of $CD4^+CD25^+Foxp3^+$ cells/$CD4^+IL\text{-}17^+$ cells in the dexamethasone treatment group was increased slightly when compared with that in the asthma group but was still lower than that in the normal control group. There were statistically significant differences between the two groups.

Expression of the mRNAs of the transcription factors Foxp3 and RORγt in pulmonary tissue of the mice. The expression of the mRNA of RORγt in pulmonary tissue of the mice in the asthma group was increased significantly when compared with that in the normal control group. The expression of the RORγt mRNA in the pulmonary tissue of the mice in

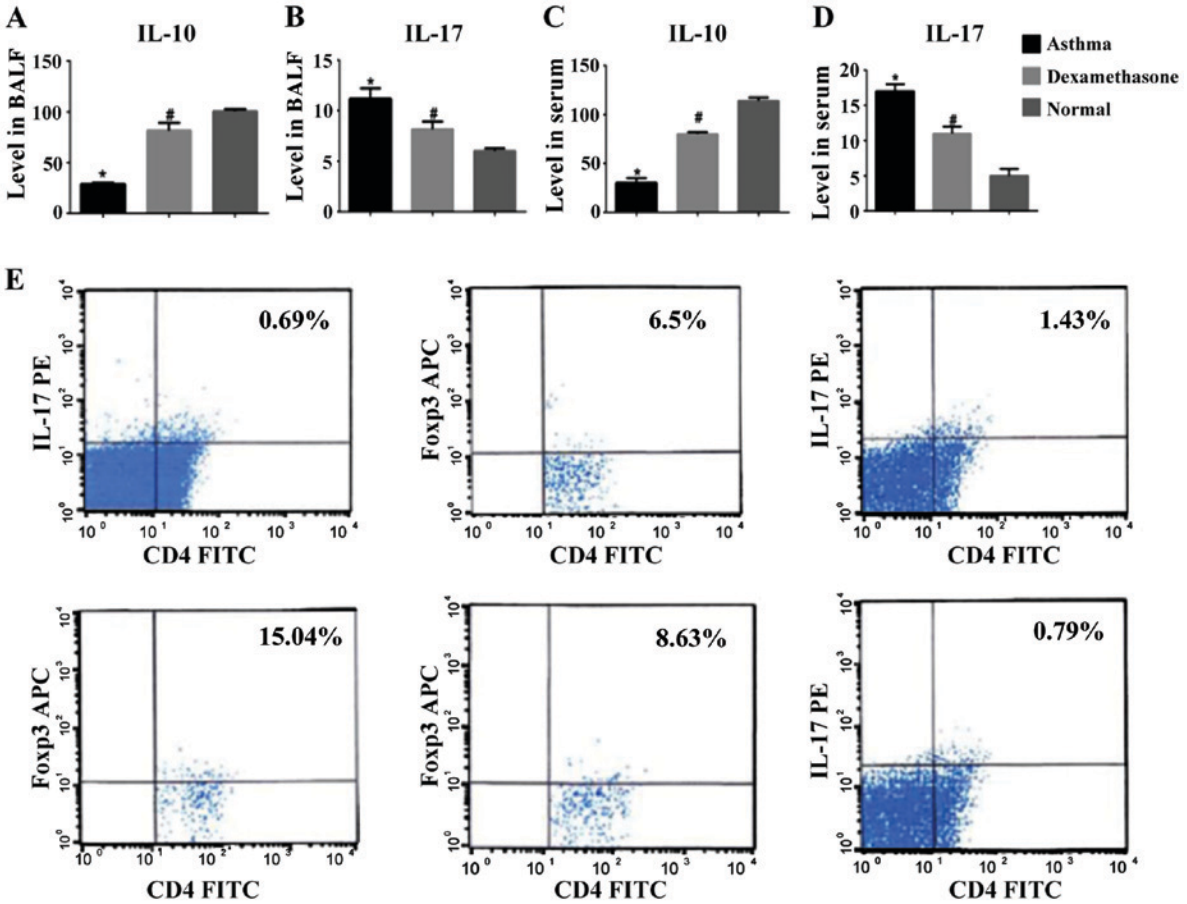

Figure 2. Content of IL-10 and IL-17 in the BALF and serum and the ratio of lymphocyte populations $CD4^+CD25^+Foxp3^+$ and $CD4^+IL\text{-}17^+$. Levels of (A) IL-10 and (B) IL-17 in the BALF of the mice in various groups. Levels of (C) IL-10 and (D) IL-17 in the serum of the mice in various groups. (E) Flow cytometer analysis of the $CD4^+CD25^+Foxp3^+$ cells in various groups. BALF, bronchoalveolar lavage fluid. *Compared with normal group, p<0.01; #compared with normal group, p<0.05.

Table II. Expression of IL-17, RORγt, IL-10, and Foxp3 mRNA (mean ± SD) in the pulmonary tissue of the mice.

Group	n	IL-17	IL-10	RORγt	Foxp3	Foxp3/RORγt
Asthma group	10	0.30±0.05[a,b]	0.22±0.07[a,b]	0.64±0.08[a,b]	0.11±0.05[a,b]	0.18±0.05[a,b]
Dexamethasone group	10	0.16±0.04[a]	0.58±0.08[a]	0.52±0.07[a]	0.31±0.08[a]	0.59±0.14[a]
Normal control group	10	0.09±0.04	0.74±0.08	0.28±0.10	0.53±0.15	1.97±0.58
F		65.306	129.192	42.954	42.311	72.437
P-value		0.000	0.000	0.000	0.000	0.000

[a]Comparison with the normal control group P<0.01. [b]Comparison with the dexamethasone group P<0.01. ROR, retinoic acid receptor-related orphan nuclear receptor.

the dexamethasone treatment group was decreased and was significantly lower than that in the asthma group. However, it was still higher than that in the normal control group. There were statistically significant differences between the two groups (P<0.01). The expression of the mRNA of Foxp3 in the pulmonary tissue of mice in the asthma group was decreased significantly when compared with that in the normal control group. The expression of Foxp3 mRNA in the pulmonary tissue of the mice in the dexamethasone treatment group was increased slightly when compared with that in the asthma group but was still lower than that in the normal control group. There were statistically significant differences between the two groups (P<0.01) (Table II).

Expression of the pulmonary transcription factors of Foxp3 and RORγt. The transcription factors of Foxp3 and RORγt expressed in the pulmonary tissue of the mice in the normal control group were located in the cytoplasm and nuclei of the cells such as lymphocytes, around the airways and in the pulmonary tissue. The expression intensity of the transcription factor Foxp3 of the mice in the asthma group was significantly lower and the expression range decreased. The

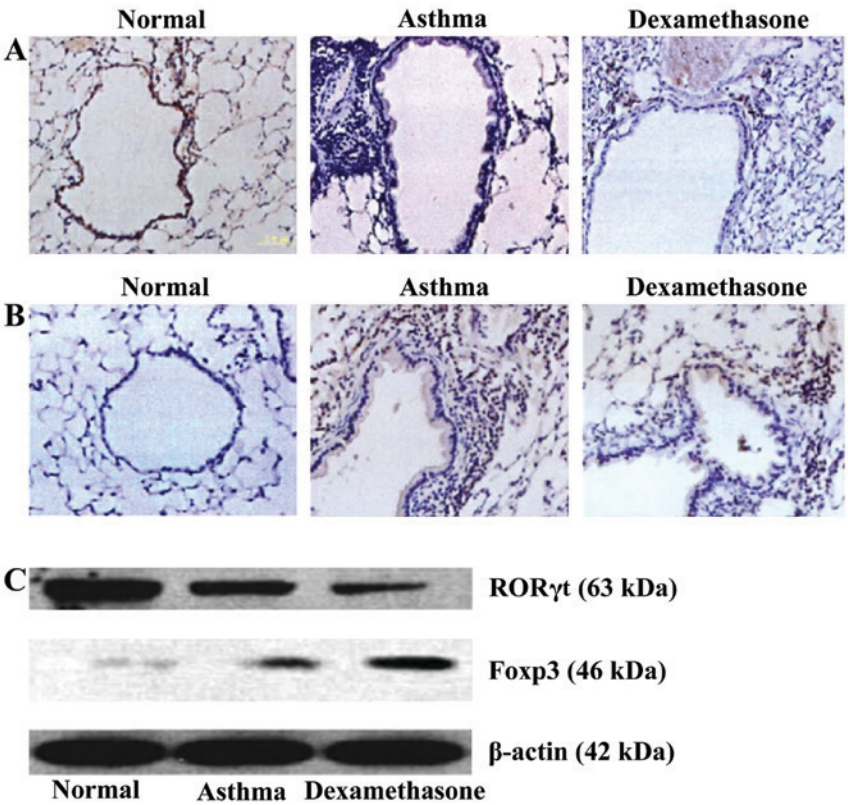

Figure 3. Expression of the transcription factors Foxp3 and RORγt in pulmonary tissue. (A) Immunohistochemistry for Foxp3. Expression intensity and range of the transcription factor Foxp3 in lung tissues representative of the three experimental groups. (B) Immunohistochemistry for RORγt. Expression intensity and range of RORγt. (C) Western blot analysis for Foxp3 and RORγt. Differences between the two groups were statistically significant (P<0.01). ROR, retinoic acid receptor-related orphan nuclear receptor.

Table III. Analysis of the correlation between the Foxp3/RORγt protein expression ratio and the airway inflammation.

Item	r	P-value
%ΔPenh		
3.125	-0.739	<0.001
6.25	-0.752	<0.001
12.5	-0.733	<0.001
25	-0.854	<0.001
50	-0.867	<0.001
Count of eosinophilic granulocytes	-0.815	<0.001
Count of lymphocytes	-0.865	<0.001
Count of neutrophil	-0.867	<0.001
Serum IL-17	-0.898	<0.001
BALF IL-17	-0.854	<0.001
Serum IL-10	-0.717	<0.001
BALF IL-10	-0.819	<0.001

ROR, retinoic acid receptor-related orphan nuclear receptor.

expression intensity and range of the transcription factor of the mice in the dexamethasone treatment group was slightly higher than that in the asthma group (P=0.015). The intensity and range were still decreased when compared with those in the normal control group. There were statistically significant differences between the two groups (P<0.01). The expression intensity and expression range of the positive cells of RORγt of the mice in the dexamethasone treatment group was decreased slightly when compared with that in the asthma group and the differences were statistically significant (P<0.01).

The western blot analysis indicated that the transcription factor Foxp3 protein was clearly expressed in the pulmonary tissue of the mice in the normal control group but only mildly expressed (or even not expressed) in the asthma group and the differences were statistically significant (P<0.01). Following the dexamethasone intervention, the expression of the Foxp3 protein in the pulmonary tissue of the mice was increased and was higher than that in the asthma group (P<0.01) (Fig. 3).

In contrast, the transcription factor protein RORγt was lowly expressed in the pulmonary tissue of the mice in the normal control group and highly expressed in the asthma group (P<0.01). After dexamethasone intervention treatment, the expression of the RORγt protein decreased and was lower than that in the asthma group. However, it was still higher than that in the normal control group. The differences between the two groups were statistically significant (P<0.01).

Analysis of the correlation between the Foxp3/RORγt protein expression ratio and airway inflammations. An analysis was conducted for the correlation between Foxp3/RORγt protein expression ratio and airway inflammation indexes. The result indicated that the Foxp3/RORγt protein expression ratio

and airway responsiveness (%ΔPenh) were negatively correlated (Table III).

When different concentrations (3.125, 6.25, 12.5, 25, and 50 mg/ml) of acetylcholine were used for activation, the correlation coefficients of the expression of the appropriate airway responsiveness (%ΔPenh) and Foxp3/RORγt protein expression ratio were respectively: -0.739, 0.752, 0.733, 0.854, and 0.867 ($P<0.01$). The Foxp3/RORγt protein expression ratio and the counts of eosinophilic granulocytes and lymphocytes and neutrophils were negatively correlated (r=-0.815, -0.865, -0.867, $P<0.01$). The Foxp3/RORγt protein expression ratio and serum and the content of IL-17 in BALF were negatively correlated (r=-0.898, -0.854, $P<0.01$). They were positively correlated with the content of IL-10 in the serum and BALF (r=-0.717, -0.819, $P<0.01$).

Discussion

Bronchial asthma is a chronic allergic reaction disease displaying increased responsiveness of the airways to multiple stimulating factors involving inflammatory cells such as eosinophilic granulocytes, lymphocytes and mastocytes (18).

After OVA stimulation, the %ΔPenh value of asthma model mice increased significantly. Based on pathological sections of pulmonary tissue, we observed infiltration of a larger number of inflammatory cells around the bronchioles and concomitant vessels, eosinophilic granulocytes within the pulmonary mesenchymal and alveolar cavity, mucous plugs within the bronchial lumen, decreased mucosa plicae, hyperplasia of goblet cells, thickened basilar membrane, widened alveolar septum, partial rupture, and formation of emphysema. The total count of leukocytes in BALF and the count of eosinophilic granulocytes increased significantly. The IL-5 of the Th2 cytokines in BALF increased significantly as the Th1 cytokines decreased. The immune abnormalities discovered are consistent with a state of Th2 hyperfunction and Th1 hypofunction.

In recent years, increasing research shows that Treg cells affect the incidence and progression of asthma and allergic diseases (19-22). The $CD4^+CD25^+$ Treg cells have become one of the research highlights as they can inhibit Th1, Th2, and $CD8^+$ T cells. Substantial research has found that the quantity and functions of Treg cells are decreased in autoimmune diseases (23). In our study, we found that the proportion of $CD4^+CD25^+Foxp3^+$ cells from the $CD4^+$ cells is decreased significantly when compared with the same proportion in the mice of the normal control group. In addition, the levels of IL-10 in BALF and serum in the asthma group are decreased significantly when compared with those in the normal control group. The level of IL-10 and the proportion of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ cells are positively correlated. This indicates that the amount and functions of Treg cells in asthma are decreased significantly when compared with those in the normal control group, finding consistent with other researchers results. The conclusion is that Treg cells seem to protect the organism in asthma. The decline in the numbers and functions of the Treg cells is closely associated with the incidence and progression of asthma.

The Th17 cells are another new subtype of $CD4^+$ T cells different from Th1 and Th2. The Th17 cells play an important role in asthma. Studies indicate that the levels of mRNA and protein IL-17 in pulmonary tissue, BALF, and serum in asthma patients increase significantly and are positively correlated with airway hyper-responsiveness (24,25). Studies have shown the infiltration of pulmonary eosinophilic granulocytes, the level of Th2-like cytokines, and the airway hyper-responsiveness of mice with deletion of the IL-17 gene decrease significantly (26). Another study found that the eosinophilic granulocytes increase when IL-17 is secreted during the onset of asthma. In our experiments, we found that the proportion of the $CD4^+IL\text{-}17^+$ T cells out of the $CD4^+$ cells in the asthma group was increased significantly when compared with that in the normal control group. The level of the Th17-related cytokine IL-17 in BALF and serum was increased significantly when compared with that in the normal control group, suggesting that Th17 participates in the onset of asthma.

The number of $CD4^+CD25^+Foxp3^+$ cells in the asthmatic mice was decreased significantly. There were statistically significant differences between the mice in the asthma group and those in the normal control group. The $CD4^+IL\text{-}17^+$ cells were fewer in the normal mice but highly expressed in the asthma mice. Our research also found that the count of $CD4^+CD25^+Foxp3^+$ cells and that of $CD4^+IL\text{-}17^+$ cells varied, while the levels of their respective cytokines, IL-10 and IL-17, were changing.

Once asthma occurs in the mice, the $CD4^+IL\text{-}17^+$ cells are increased and the $CD4^+CD25^+Foxp3^+$ cells decrease. Disruption of the normal immune balance occurs in the $CD4^+CD25^+Foxp3^+$ (Treg) and the $CD4^+IL\text{-}17^+$ cells, (Th17). The ratio of the $CD4^+CD25^+Foxp3^+/CD4^+IL\text{-}17^+$ cells is closely associated with various airway inflammation indexes. A smaller ratio of $CD4^+CD25^+Foxp3^+/CD4^+IL\text{-}17^+$ cells leads to a larger value of %ΔPenh activated by acetylcholine. A smaller ratio of $CD4^+CD25^+Foxp3^+/CD4^+IL\text{-}17^+$ cells leads to larger count of eosinophilic granulocytes, neutrophils, and lymphocytes in BALF. Also, a smaller ratio leads to a higher content of IL-17 in the serum and BALF and higher expression of the IL-17 gene and protein in the pulmonary tissues. The same ratio is negatively correlated with IL-17. This means that an altered Treg/Th17 ratio may be one of the important causes of airway inflammation in bronchial asthma.

The transcription factors are a class of nucleoproteins that identify and are bound to specific DNA regulatory sequences and stimulate or inhibit transcription. Any abnormalities in their quantity or activity lead to abnormal expression of the genes critical to cellular growth and differentiation. Foxp3 and RORγt are key transcription factors regulating differentiation of Treg and Th17 cells, respectively. Based on our results, RORγt is highly expressed in the pulmonary tissues of asthma mice, indicating that epithelial cells, mononuclear macrophages and other cell types in the pulmonary tissues secrete substantial amounts of TGF-β and IL-6 in response to allergic stimulation. Locally released TGF-β and IL-6 result in a high expression of the RORγt gene and protein in the $CD4^+$ T cells, thus inducing differentiation of the naive T cells towards Th17. In addition, RORγt can also upregulate the expression of the IL-23 receptor on the surface of $CD4^+$ T cells, promote binding between IL-23 and its receptor, maintain the survival of Th17, promote proliferation of Th17, and secrete IL-17. IL-17, in turn, is bound to the IL-17 receptor

on the surface of such inflammatory cells as epithelial cells, neutrophils, and eosinophilic granulocytes, thereby allowing for release of various chemotactic factors and inflammatory mediators, inducing aggregation of substantial inflammatory cells towards the inflammatory site, and further promoting the incidence of the pulmonary tissue inflammation. Our results indicate that the transcription factors Foxp3 and RORγt in the normal mice are uniformly expressed in an immunologic balanced state. Once asthma in mice occurs, both high expression of RORγt and low expression of the Foxp3, result in an imbalanced expression of the Foxp3/RORγt ratio. Our research has also found that during asthma, while the expression of the transcription factors Foxp3 and RORγt changes, the levels of the cytokines IL-10 and IL-17 are also changing in a manner consistent with a dominant differentiation of Th0 towards Th17 creating a special immunological picture for asthma.

In conclusion, the imbalanced expression of the transcription factors Foxp3/RORγt is closely associated with the airway inflammations of asthma. This indicates that, in addition to the known change in the Th1/Th2 ratio that occurs in the pathogenesis of asthma, there is also a deregulation of the Treg/Th17 ratio. At the same time, the levels of the corresponding transcription factors T-bet/GATA3 and Foxp3/RORγt also reflect the immunologic balance of Th1/Th2 and Treg/Th17, respectively. Changes in the state of the Th1/Th2 and Treg/Th17 ratios play important roles in the immunological mechanisms of asthma and probably provide good novel targets for intervention strategies against asthma.

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