Analysis of the levels of Th9 cells and cytokines in the peripheral blood of mice with bronchial asthma

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Abstract. The purpose of the study was to detect the level of T-helper type 9 (Th9) cells and the cytokine interleukin-9 (IL-9) in peripheral blood of mice with bronchial asthma, and to explore the relationship between the expression of Th9 cells and the pathogenesis of asthma. Thirty female-specific pathogen-free (SPF) Bagg' albino (BALB)/c mice were selected and randomly divided into the control group (n=15) and the bronchial observation group (n=15). Mice in the bronchial observation group were treated with ovalbumin (OVA) for sensitization and induction of a mouse model of asthma. The airway reactivity of mice was measured by a mouse pulmonary function apparatus using the non-invasive pulmonary impedance method. The proportions of Th9 cells in peripheral blood of mice in the two groups were detected using flow cytometry. Digital polymerase chain reaction (dPCR), enzyme-linked immunosorbent assay (ELISA) and western blot analysis were applied to detect the levels of IL-9 messenger ribonucleic acid (mRNA) and proteins in peripheral blood and lung tissues of mice in the two groups, respectively. Compared with that in the control group, the expression level of Th9 cells in the peripheral blood of mice in the observation group was significantly elevated ($P<0.05$), the expression level of IL-9 proteins in the peripheral blood was significantly increased ($P<0.05$), and the levels of IL-9 mRNA and proteins in lung tissues were significantly increased ($P<0.05$). The results show that the levels of Th9 cells and their cytokine IL-9 in the peripheral blood of mice with bronchial asthma are significantly increased, suggesting that Th9 cells play important roles in the pathogenesis of asthma.

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Introduction

Bronchial asthma is a type of disease with symptoms of bronchial hyper-responsiveness and airway obstruction. It is characterized by chronic airway inflammation and bronchial asthma. A relatively more unified view reveals that asthma is a development process, in which epithelial cells, fibroblasts, dendritic cells, eosinophils, mastocytes, T lymphocytes and other inflammatory cells interact with constituent cells of the airway, and the secreted inflammatory mediators participate in the development of asthma (1-3).

According to available data, the prevalence, mortality and treatment costs of asthma clearly show the rising trends globally. The prevalence of asthma is increased by approximately 50% every 10 years (4). Findings have shown that, the T-helper type 2 (Th2) subgroup in the cluster of differentiation 4 $(CD4)^+$T cells plays a key role in the occurrence of asthma, and it mediates the occurrence of II inflammatory responses by secreting a large number of cytokines, thus greatly promoting the occurrence of asthma (5,6). Interleukin-9 (IL-9) has long been considered a Th2 cytokine, that plays an important role in the pathogenesis of asthma, parasitic infection in the body and the occurrence process of tuberculosis (7,8). Newly identified Th9 cells are $CD4^+$ T effector cells and are different from Th2, which are differentiated by the combined stimulation of transforming growth factor β (TGF-β) and IL-4. They can secrete IL-9 and IL-10, albeit the former is the main type, and participate in asthma and parasitic infection-mediated immune responses such as Th2 cells (9-11).

In the present study, we established a mouse model of bronchial asthma to observe the roles of Th9 cells and their cytokine IL-9 in the pathogenesis of bronchial asthma.

Materials and methods

Reagents and instruments. Thirty female-specific pathogen-free (SPF) Bagg' albino (BALB)/c mice weighing 20.65±2.35 g on average were included in the present study. Ovalbumin (OVA) was purchased from Sigma (St. Louis, MO, USA); aluminum hydroxide gel was purchased from Pierce (Rockford, IL, USA); IL-9 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China); Roswell Park Memorial Institute (RPMI)-1640 medium was purchased from Gibco (Grand

Island, NY, USA); fluorescein isothiocyanate (FITC)-labeled anti-human CD4 monoclonal antibodies, phycoerythrin (PE)-labeled anti-human IL-9 monoclonal antibodies and 2-SYBR® Green Real-time Polymerase Chain Reaction (PCR) Master Mixes were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). β-actin mouse monoclonal antibodies and goat anti-mouse horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Zhongshan Golden Bridge Biological Technology Co., Ltd. (Beijing, China); phorbol 12-myristate 13-acetate (PMA), ionomycin and monensin were purchased from Sigma. The microplate reader was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and the flow cytometer was purchased from Becton-Dickinson (Franklin Lakes, NJ, USA).

The present study was approved by the Ethics Committee of Children's Hospital of Zhengzhou (Dongsan Street Hospital).

Establishment of a mouse model of bronchial asthma. OVA was used for sensitization and induction of a mouse model of bronchial asthma. Fifteen mice in the observation group were intraperitoneally injected with 0.1 ml saline solution containing 50 μg OVA and 2 mg aluminum hydroxide (at the concentration of 10%) on days 0 and 14. From day 21, the mice were placed in a closed box and atomized with 2.5% OVA solution for 7 days. In the control group, 15 mice were treated with sterile saline instead of OVA solution in the sensitization and induction phases. The usage and dosage were consistent with those in the observation group. The mice were sacrificed at the last 24 h after the atomization.

Detection of non-invasive pulmonary functions of mice. A Buxco non-invasive pulmonary function instrument (Buxco Respiratory Products, Data Sciences International) was connected, and the standard value was set. The airway responsiveness of mice in the two groups was detected. Then the two groups of mice were placed into a non-invasive pulmonary function instrument box. After the mice adapted to the environment for 5 min, 20 μl mean corpuscular hemoglobin (MCH) was added at concentrations of 0, 6.25, 12.50 and 25.00 mg/ml for induction atomization, respectively. MCH at each concentration was used for atomization for 1 min, and the results were recorded for 3 min. At the end of the experiment, data and statistical results were output automatically. The ratio of specific airway resistance (sRaw) represented the level of airway responsiveness.

Detection of Th9 lymphocytes in peripheral blood of mice. Blood from abdominal aorta of mice in the two groups was extracted and mononuclear cells were isolated by lymphocyte separation medium. RMPI-1640 complete culture medium was used for cell suspension, and the cell concentration was adjusted to $2x10^6$/ml. The cells were then inoculated into 6-well plates with 1 ml per well. The medium was added mixed with PMA (25 ng/ml), ionomycin (1 μg/ml) and monensicillin (1.7 μg/ml), and cultured in an incubator with 5% CO_2 at 37˚C for 6 h. Cells were collected, placed in the flow tube (testing tube and control tube), and centrifuged for 5 min at rate of 2,600 x g. The supernatant was then discarded, and 100 μl phosphate-buffered saline (PBS) was added for resuspension. FITC-CD4 antibodies (10 μl) were added in the testing and control tubes and incubated in the dark at room temperature for 15 min. The tissues were washed twice with PBS, 100 μl rupture agent was added, placed at room temperature in the dark for 10 min, and centrifuged at the rate of 2,600 x g for 5 min. The supernatant was then discarded, 2 μl PE-IL-9 antibodies were added to the testing tube, and 2 μl of the same antibodies were added in the control tube for control. The antibodies were incubated at room temperature for 30 min. After being washed twice, the cells were resuspended in 0.5 ml PBS. The percentage of $CD4^+IL\text{-}9^+$ T cells in $CD4^+$ T cells was detected.

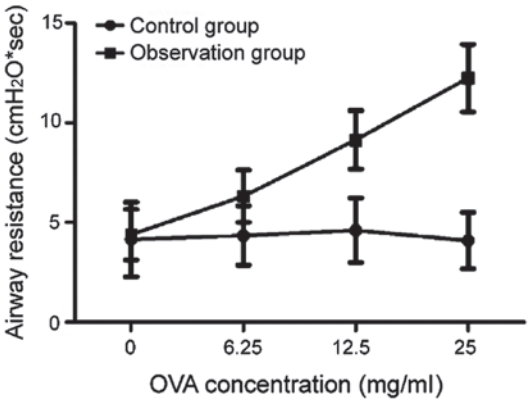

Figure 1. Comparison of the airway reactivity between the two groups. After the motivation of MCH, the sRaw of mice in the observation group is significantly higher than that of the control group, and the difference is statistically significant ($P<0.05$). With the increase of MCH concentration, the sRaw of mice in the observation group increased gradually.

Detection of the level of IL-9 messenger ribonucleic acid (mRNA) in lung tissues of mice. Lung tissues of mice in the two groups were placed in diethyl pyrocarbonate (DEPC)-treated 1.5 ml Eppendorf (EP) tubes, respectively. TRIzol (1 ml) (Invitrogen) was added, and an ultrasonic cell crusher was used for tissue homogenate. Chloroform (200 μl) was added, thoroughly mixed with tissues and left to stand at room temperature for 3 min. Tissues were then centrifuged at the rate of 9,100 x g at 4˚C for 15 min. The supernatant was transferred to another 1.5 ml EP tube, and tissues were added and mixed thoroughly with 500 μl isopropyl alcohol and left to stand at room temperature for 10 min. Subsequently the tissues were centrifuged at the rate of 5,800 x g at 4˚C for 5 min. The supernatant was discarded, and 50 μl ribonuclease (RNase)-free water-soluble liquid was added to obtain the total RNA. The concentration and optical densitity (OD)260/OD80 ratio were measured. The total RNA with (OD) 260/OD80 ratio between 1.8 and 2.0 were used for reverse transcription. A 20 μl reverse transcription reaction system was established to obtain complementary deoxyribonucleic acid (cDNA), RNA was reversely transcribed into single-stranded cDNA according to the protocol of the reverse transcription kits (Takara Biomedical Technology Co., Ltd., Dalian, China). IL-9 primers used were: F: 5'-GTGACATACATCCTTGCCTC-3' and R: 5'-GTGGTACAACAGTTGGG-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were: 5'-CTCTG CTCCTCCTGTTCGAC-3', and 5'-GCGCCCAATACGA CCAAATC-3'. A 20 μl reverse transcription quantitative PCR

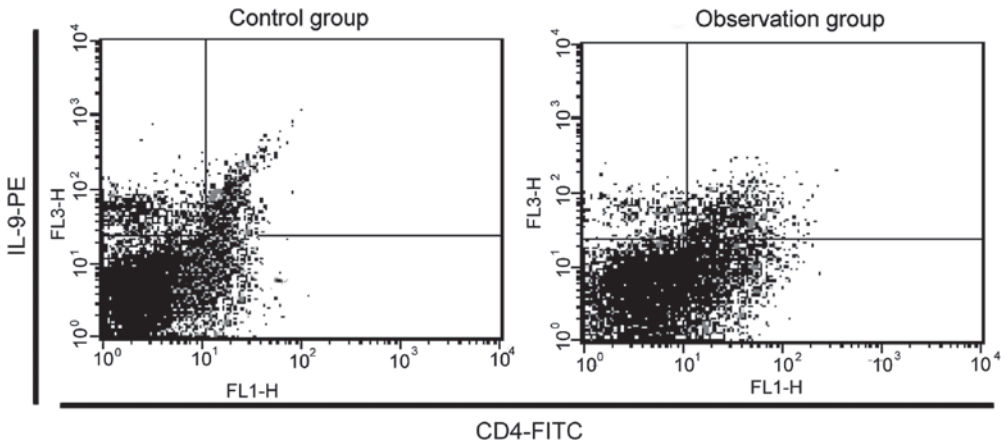

Figure 2. Comparison of Th9 cell subgroups of mice between the two groups. The proportion of Th9 cells in peripheral blood of mice in the control and observation groups was analyzed using flow cytometry. The proportion of Th9 cells in peripheral blood of mice in the observation group is significantly increased compared with that in the control group.

system (Vazyme Biotech Co., Ltd.) was established. The reaction conditions for the PCR were: denaturation at 95˚C for 90 sec annealing; at 95˚C for 15 sec extension; at 57˚C for 20 sec and further elongation; at 66˚C for 30 sec; with a total of 35 cycles. Data were measured, and quantitative analysis was conducted (ΔΔCq method).

Determination of the level of IL-9 proteins in peripheral blood and lung tissues of mice. The level of IL-9 in peripheral blood was detected using ELISA kits, in strict accordance with the protocol of the kit. IL-9 in lung tissues was analyzed by western blot analysis. Lung tissues (5 mg) of mice in the two groups were taken, respectively, and placed in a homogenizer for full grinding. The cell suspension was transferred into a 1.5 ml EP tube, and tissues were centrifuged at the rate of 2,600 x g at 4˚C for 10 min. The supernatant was discarded, and the suspension was added with radioimmunoprecipitation assay (RIPA) lysate (Beyotime Biotechnology, Shanghai, China). The mixed liquor was vibrated for full mixing and then left to stand on ice for 30 min and tissues were centrifuged at the rate of 9,100 x g at 4˚C for 10 min. The bicinchoninic acid assay (BCA) kit (Beyotime Biotechnology) was used to detect the content of the proteins. After the sodium dodecyl sulfate polyacrylamide gel (10%) electrophoresis (SDS-PAGE) was performed, the gel was transferred to polyvinylidene difluoride (PVDF) membranes. After specific blocking in bovine serum albumin (BSA) for 1 h, the tissues were washed with phosphate-buffered saline supplemented with Tween-20 (PBST) 3 times for 5 min each time. Mouse anti-human IL-9 monoclonal antibodies (diluted at 1:500; cat. no. 564255) were incubated at 4˚C for 12 h, and tissues were washed with PBST 3 times for 5 min each time. Rabbit anti-mouse IgG monoclonal antibodies (1:1,000; cat. no. ZB-2305) were then incubated at room temperature for 1 h, and the tissues were washed with PBST 3 times for 5 min each time. Membranes were coated with luminescent liquid for development with β-actin as the internal reference.

Statistical analysis. The experimental results were expressed as mean ± standard deviation, and SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

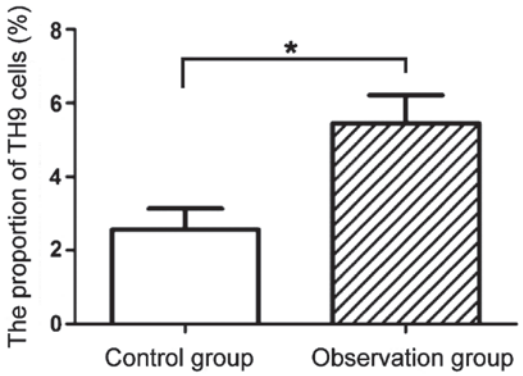

Figure 3. Comparison of Th9 cell subgroups between the observation and control groups. The histogram shows the proportion of TH9 cells in peripheral blood of mice in the control and observation groups. *P<0.05.

Comparisons between groups were performed using LSD test. P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of mouse behavioral manifestations and pulmonary functions. After the induction of OVA, varying degrees of symptoms emerged, such as forelimbs scratching the nose, dysphoria, lying prostrately without motion, depression, cyanosis, tachypnea, abdominal convulsions, urinary and fecal incontinence. After induction of MCH, the sRaw of mice in the observation group was significantly higher than that in the control group, and the difference was statistically significant (P<0.05). With the increase of MCH concentration, the sRaw of mice in the observation group was increased gradually, indicating that mice in the observation group had bronchial hyper-responsiveness. The results of the analysis of mouse behavioral manifestations and pulmonary function verified that the mouse model was established successfully in the observation group (Fig. 1).

Comparison of Th9 cell subgroups of mice between the two groups. The results of flow cytometry are shown in Figs. 2 and 3.

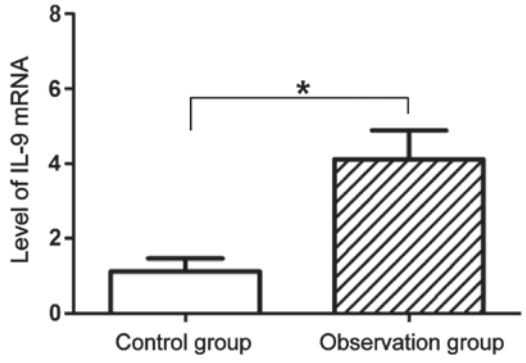

Figure 4. Comparison of the level of IL-9 mRNA of mice between the two groups. The expression level of IL-9 mRNA in lung tissues of mice in the control and observation groups was analyzed by qPCR. The expression level of IL-9 mRNA of mice in the observation group is significantly higher than that in the control group. *P<0.05.

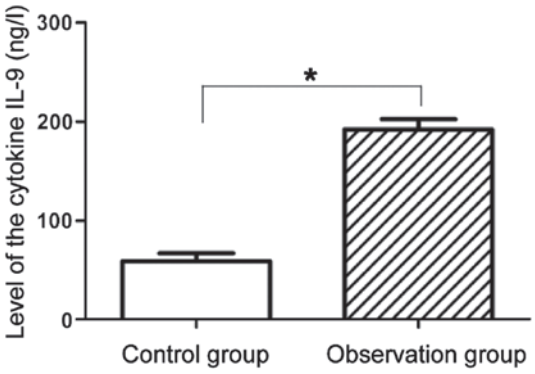

Figure 5. Comparison of the level of the cytokine IL-9 in peripheral blood between the two groups. The expression level of the cytokine IL-9 in peripheral blood of mice in the control and observation groups was analyzed by ELISA. The expression level in the observation group is significantly higher than that in the control group. *P<0.05.

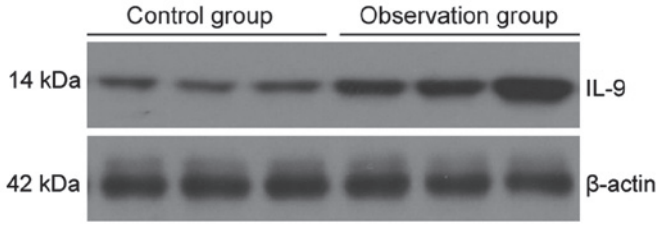

Figure 6. Comparison of the level of IL-9 proteins in lung tissues of mice between the two groups by western blot analysis. The level of IL-9 proteins in lung tissues of mice in the control and observation groups was analyzed by western blot analysis. The expression level in the observation group is significantly higher than that in the control group.

Compared with the proportion of Th9 cells in peripheral blood of mice in the control group (2.56±0.57), that in the observation group (5.45±0.76) was significantly increased (P<0.05).

Comparison of the level of IL-9 mRNA of mice between the two groups. The expression level of IL-9 mRNA in lung tissues was analyzed by qPCR. As shown in Fig. 4, the expression level of IL-9 mRNA in lung tissues of mice in the observation group (4.12±0.76) was significantly higher than that in the control group (1.12±0.34), and the difference was statistically significant (P<0.05).

Comparison of the level of IL-9 proteins in peripheral blood and lung tissues of mice between the two groups. Results of ELISA are shown in Fig. 5. Compared with the expression level of IL-9 proteins in peripheral blood of mice in the control group 59.43±7.91 ng/l, that in the observation group 192.33±10.23 ng/l was significantly increased, and the difference was statistically significant (P<0.05). Results of western blot analysis of the level of IL-9 proteins in lung tissues indicated that the expression level of IL-9 proteins in lung tissues of mice in the observation group was significantly higher than that in the control group, and the difference was statistically significant (P<0.05) (Fig. 6).

Discussion

Bronchial asthma is one of the common chronic diseases of the respiratory system. There are approximately 230 million individuals with chronic asthma worldwide (12). The morbidity and mortality rates of asthma worldwide have gradually increased in recent years, which poses a great threat to human health (13). Virus infection, allergens and air pollution can induce the occurrence of acute asthma. Chronic non-specific airway inflammation and increased airway responsiveness are the most important features of asthma (14,15). At present, the imbalance of the number and function of Th1/Th2 in $CD4^+$ T lymphocyte subgroups is closely related to the pathogenesis of asthma. The incidence of asthma is mainly affected by Th2 cell-mediated immune responses (16). Previous findings showed that IL-9 plays an important role in asthma, parasitic infections and other Th2-related diseases (17). However, it has also been found that the function of IL-9 is not completely identical to that of other Th2-related cytokines. Therefore, $CD4^+$ Th effector cells secreting IL-9 are a class of independent cell subgroups, namely Th9 cells (9,18). Due to the important role of IL-9 in asthma, the action mechanism of Th9 cells, the main cells secreting IL-9, in bronchial asthma has drawn increasing attention.

In the present study, the mechanism of Th9 cells and the cytokine IL-9 in the study of pathogenesis of asthma was studied by establishing a mouse model of bronchial asthma. The flow cytometry analysis results of Th9 lymphocytes mouse in the model of bronchial asthma revealed that the level of Th9 cells in mice with asthma was significantly increased compared with that in normal mice, suggesting that Th9 cells play significant roles in the occurrence and development of asthma. IL-9 can act on a variety of inflammatory and tissue cells and play an important role in inflammation and allergic reactions, constituting a pathological factor of asthma (19). Researchers suggested that the level of IL-9 in patients with asthma can be used as one of the indicators for determining disease severity (20). In this study, both RT-PCR and western blot results indicated that the levels of IL-9 mRNA and proteins in lung tissues of mice with asthma were significantly higher than those in normal mice, which were consistent with those of Th9 cells.

In summary, Th9 cells, as a kind of important effector T cells, promote the occurrence and development of bronchial

asthma together with their main cytokine IL-9, and may become an important indicator for determining the condition of asthma, thus providing a certain guidance for the clinical treatment of asthma.

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