

Original Article

Changes in levels of IL-9, IL-17, IFN- γ , dendritic cell numbers and TLR expression in peripheral blood in asthmatic children with *Mycoplasma pneumoniae* infection

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Abstract: *Mycoplasma pneumoniae* (MP) infection in children with asthma resulted in a more severe allergic state compared with a non-MP infected group. The infection rate of children with asthma was higher than that of the other groups, suggesting that being asthmatic may be a predisposing factor for MP infection and that the infection itself is an important co-factor in the disease progression of asthma. The number of dendritic cells (DCs) and the expression of TLR2 and TLR4 were compared in 22 asthmatic patients with MP infection, 22 asthmatic patients without MP infection, and 17 normal children as controls. The percentages of DCs in the peripheral blood of the three groups showed significant differences between asthmatic children with MP infection and controls, and asthmatic children without MP and controls ($P < 0.05$), whereas no difference was found between asthmatic children with and without MP infection. The asthmatic children with MP infection group showed increased expression of TLR-2 and TLR-4 on DCs ($P < 0.01$). Asthmatic patients infected with MP showed that DCs and TLRs (TLR-2, TLR-4) might play an important role in asthma pathogenesis with MP infection. The cytokines produced by the T-cell subsets in asthmatic children with MP infection showed a significant increase in IL-9 ($P < 0.01$) and a decrease in IFN- γ ($P < 0.05$) levels post-MP infection, while the IL-17 level remained stable ($P > 0.05$), indicating a shift towards Th1/Th9 in the presence of MP infection.

Keywords: Childhood asthma, *Mycoplasma pneumoniae* infection, dendritic cell, Toll-like receptor, T-helper cell, cytokines

Introduction

The relationship between infectious disease and bronchial asthma has been attracting increasing attention in recent years. Clinical observations have shown that the incidence of bronchial asthma and the degree of acute exacerbation episodes increase after infections with atypical pathogens, fungi, and some bacteria [1, 2], particularly *Mycoplasma pneumoniae* (MP) [3-5]. MP is an atypical pathogen and is a common cause of respiratory infection among children. In particular, recent clinical studies have reported the presence of atypical pathogens, including MP and *Chlamydia pneumoniae*, in patients with chronic stable asthma and acute exacerbations. Moreover, clinical studies have shown that MP has a 3-64% infec-

tion rate [6-8] in children of various age groups with acute asthma or chronic stable asthma. Thus, MP is recognized as a co-factor in both the stable phase of bronchial asthma and bronchial asthma exacerbations [9-11]. Use of macrolide antibiotics for the treatment of children with MP infection can significantly improve lung function and relieve respiratory symptoms.

Over the past two decades, the relationship between MP infection and bronchial asthma has been a topic of research and debate. Bronchial asthma is a common childhood respiratory disease, the incidence of which is increasing. The causes of asthma are complex and include genetic factors, immunological factors and environmental factors. Asthma is a type of chronic allergic airway inflammation that

involves various immune cells [12]. These include lymphocytes, eosinophils, basophils, mast cells and dendritic cells (DCs), all of which play important roles in the pathogenesis of bronchial asthma.

Over the past decade, cell-based immune response theory, focusing on the function of T helper 9 cells (Th9) and T helper 17 cells (Th17) [13] has received increasing attention [14-20]. DCs, as antigen-presenting cells (APCs), which express Toll-like receptors (TLRs), provide a new direction for research on the immunological pathogenesis of asthma [21-24].

This study focused on the number of DCs and the expression level of TLR-2 and TLR-4 as well as the numbers of, and the cytokines secreted by Th9, Th17, and Th1 cells. It also analyzed the role of DCs and the possible shifts in T-cell populations during asthma pathogenesis and MP infection. Moreover, the role of T-cell shifts and DCs expressing TLRs in bronchial asthma with MP infection was evaluated; the findings provide a theoretical basis for possible future immunological therapy.

Materials and methods

Subjects

Children with bronchial asthma from the Pediatric Outpatient Department of Ren Ji Hospital were investigated. Peripheral blood samples from 76 children were collected from January 2011 to December 2013. The diagnostic criteria used were those of the diagnosis and prevention guidelines for bronchial asthma in children (published in the *Journal of Pediatrics*, October 2008, Volume 46, No. 10). All admitted patients were older than 5 years with a clinically stable history of asthma. The patients consisted of 51 males and 25 females, with an average age of 7.29 ± 1.62 years. In total, 78 patients (35 males, 43 females; average age of 8.05 ± 1.34 years) with other upper respiratory infections and bronchopneumonia were also included. The control group comprised children selected from the Urology Department of our hospital and undergoing normal check-ups. These children had neither a history nor a family history of allergy. They had no sign of respiratory disease at the time of selection. All children were tested for serum MP

IgM antibodies. Children from the asthma group and other respiratory infection group were also tested for eosinophil cationic protein (ECP), total IgE, and mite-specific IgE. Among the 44 patients in the asthma group, 22 (15 males, 7 females) patients with an average age of 7.17 ± 2.05 years were infected with MP; the remaining 22 patients (17 males, 5 females), with an average age of 7.17 ± 2.05 years, were not. Seventeen children (15 males, 2 females) from the control group with an average age of 8.82 ± 3.41 years were tested for the number of DCs and TLR-2 and TLR-4 expression in their peripheral blood using flow cytometry (FCM).

Reagents and FCM methods for TLR-2 and TLR-4 detection

The following reagents were used: erythrocyte lysis buffer (Maidueer Biotechnology Co. Shanghai, China), PE mouse anti-human CD1a (Material No.: 555807; BD Biosciences, USA), APC-H7 mouse anti-human HLA-DR (Material No.: 561358; BD Biosciences), V450 mouse anti-human CD86 (Material No.: 560357; BD Biosciences), monoclonal anti-human TLR2-PerCP (Catalog No.: FAB2616C; R&D Systems, USA), anti-human CD284 (TLR4) PE-Cy7 (Catalog No.: 25-9917; eBioscience, USA), monoclonal anti-human TLR5-Alexa Fluor 488 (Catalog No.: FAB6704G; R&D Systems), anti-human CD286 (TLR6) Biotin (Catalog No.: 13-9069; eBioscience), and streptavidin APC (Catalog No.: 17-4317; eBioscience). A BD FACSCanto II FCM instrument and FCM software were also used. FCM procedures are follows: (1) The following monoclonal antibodies were added to the tube according to the manufacturer's protocol: PE mouse anti-human CD1a (20 μ L); APC-H7 mouse anti-human HLA-DR (5 μ L); Monoclonal anti-human TLR2-PerCP (10 μ L); Anti-human CD284 (TLR4) PE-Cy7 (5 μ L); Monoclonal anti-human TLR5-Alexa Fluor 488 (5 μ L); Anti-human CD286 (TLR6) Biotin (1 μ L); (2) Complete blood (100 μ L) was added and incubated in the dark for 20 min; (3) Newly prepared erythrocyte lysis buffer (1 mL) was added, fully mixed, and stored in the dark for 10 min to lyse red blood cells; (4) The mixture was centrifuged (2500 rpm, 5 min) to pellet the cells; (5) The cells were resuspended in media or other buffer and again centrifuged; (6) The sample was analyzed using the FCM instrument.

Serum MP IgM antibody

An ELISA was used; a diagnosis was made if the MP-IgM titer was higher than 1:160 or if a paired serum sample showed a fourfold increase or decrease within 2 weeks. Serological methods remain in use for clinical diagnosis because they are convenient and rapid and have high specificity and sensitivity [25].

ECP

We used the Pharmacia UniCAP system.

Serum total IgE and mite-specific IgE levels

Serum total IgE and mite-specific IgE levels were determined by radioimmunoassay (RIA).

Skin allergen test

The diagnosis was made using a skin prick test. The hive area created by the allergen was assessed and compared with that created by histamine (positive control). The diagnostic criteria were as follows: no reaction (-), area of allergen hive/area of histamine hive $> \frac{1}{4}$ (+), area of allergen hive/area of histamine hive $> \frac{1}{2}$ (++) , two areas are equal (+++), and allergen area is twice the size of the histamine area or the allergen area has a blister bleb (++++).

Determination of interleukin 9 (IL-9), interleukin 17 (IL-17) and interferon- γ (IFN- γ) levels in the peripheral blood in asthmatic children with MP infection

The following reagents were used: Maibio MHK-0027-96T human IL-17 96T, Maibio MHK0017-96T human IFN- γ 96T, Maibio MHK0005-96T human IL-4 96T, and RayBiotech ELH-IL9-001 human IL-9 ELISA 96T. A Thermo Scientific Microplate reader MK3 was used. An ABC-ELISA was carried out as follows. Microtiter plates were coated with anti-IFN- γ , -IL-9, and -IL-17 antibodies. The patient samples were then added to the plates. Biotinylated anti-human-IFN- γ , -IL-9, and -IL-17 antibodies were also added, resulting in formation of immune complexes. Streptavidin was added to react with biotin, and the solute then turned blue. OD values at 450 nm were assessed because they were proportional to the levels of IFN- γ , IL-9, and IL-17. The concentrations were determined using standard curves.

Statistical analysis

Normally distributed data are expressed as means $\pm$ standard deviation, and t-tests were conducted to compare differences among groups. Non-normally distributed data were expressed using medians (interquartile range), and a simple ANOVA (homogeneity of variances) or rank sum test (heterogeneity of variances) was used to compare the differences among groups. The Kruskal-Wallis H-test was conducted to compare differences among multiple groups. Count information or rating information was presented as frequency or percentage (%) using the χ^2 test. All data were analyzed using the SPSS software (ver. 16.0). A two-tailed test was used for all *P* values. *P* values < 0.05 were considered to indicate statistical significance.

Results

MP infection rate in asthmatic children, children with other respiratory infections, and normal controls

Of 76 asthmatic children, 34 were positive for serum anti-MP IgM, a 44.74% infection rate. Among the 78 children with other respiratory infections, 18 were positive for anti-MP IgM, a 23.08% infection rate. Three of the forty-two normal controls cases were positive for anti-MP IgM (7.14% infection rate). Asthmatic children showed a significantly higher MP infection rate than the other respiratory infection group ($P < 0.05$) and the control group ($P < 0.001$).

Comparison of total serum IgE, mite-specific IgE, and ECP in asthmatic children with and without MP infection

The total serum IgE levels in asthmatic children with and without MP infection were 977.4 ± 101.6 and 332.2 ± 69.15 IU/mL, respectively, a significant difference ($P < 0.0001$). Mite-specific IgE levels in asthmatic children with MP infection and asthmatic children without MP infection were 39.93 ± 7.14 and 9.72 ± 3.65 IU/mL, respectively, a significant difference ($P < 0.0005$). The ECP levels in asthmatic children with MP infection and asthmatic children without MP infection were 87.83 ± 14.43 and 41.57 ± 6.41 μ g/L, respectively. This difference was also statistically significant ($P < 0.01$).

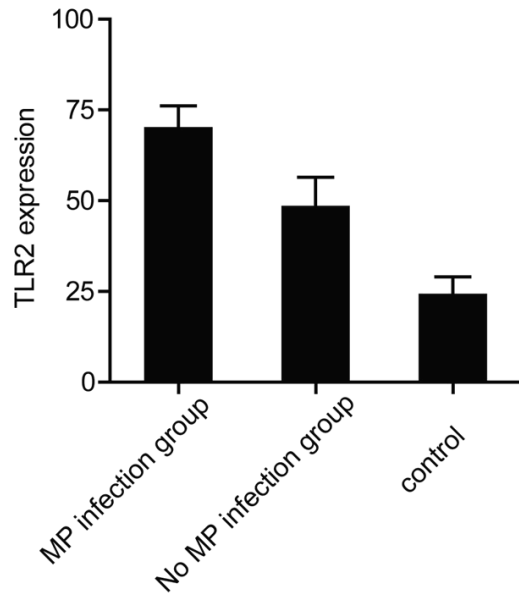


Figure 1. Comparison of TLR-2 expression among MP infection group, no MP infection group and the control group.

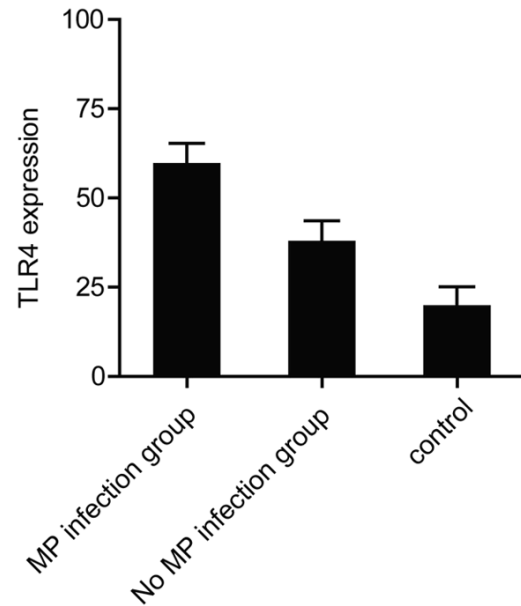


Figure 2. Comparison of TLR-4 expression among MP infection group, no MP infection group and the control group.

Skin tests for major allergens in asthmatic children with and without MP infection

The common mite was used as the allergen for skin tests. The results in the MP-infected group were 54.55% (++++), and 45.45% (++) , and those in the non-MP infected group were 68.18% (++++), and 31.82% (++) . These results were not significantly different ($P > 0.05$).

Percentage of DCs (CD1a⁺ HLA-DR⁺) in peripheral blood

The percentages of DCs in asthmatic children with MP infection, asthmatic children without MP infection, and the control group were $0.82 \pm 0.13\%$, $0.79 \pm 0.11\%$, and $0.40 \pm 0.09\%$, respectively. A statistically significant difference was observed between asthmatic children with or without MP infection and the control group ($P < 0.05$).

TLR2 expression in DCs

The ratio of TLR-2 expression in the DCs of asthmatic children with MP infection, asthmatic children without MP infection and the control group were $70.30 \pm 5.84\%$, $48.59 \pm 7.86\%$ and $24.35 \pm 4.70\%$, respectively (**Figure 1**). The differences between asthmatic children with/without MP infection ($P < 0.05$), asthmatic chil-

dren with MP infection and the normal control group ($P < 0.0001$), and asthmatic children without MP infection and the normal control group ($P < 0.05$) were statistically significant.

TLR4 expression in DCs

The ratio of TLR-4 expression in the DCs of asthmatic children with MP infection, asthmatic children without MP infection, and the control group were $54.97 \pm 5.65\%$, $38.08 \pm 5.56\%$, and $19.98 \pm 5.13\%$, respectively (**Figure 2**). The differences between asthmatic children with/without MP infection ($P < 0.008$), asthmatic children with MP infection and normal control group ($P < 0.0001$), and asthmatic children without MP infection and normal control group ($P < 0.02$) were statistically significant.

Levels of IFN- γ , IL-9 and IL-17 in peripheral blood

The IFN- γ levels in asthmatic children with MP infection, asthmatic children without MP infection, and normal control group were 19.20 ± 4.47 , 22.90 ± 3.85 , and 93.05 ± 37.55 pg/mL, respectively (**Figure 3A**). The difference between the asthmatic children with/without MP infection groups was not significant, but the levels in both groups were significantly different compared with the normal control group ($P < 0.05$).

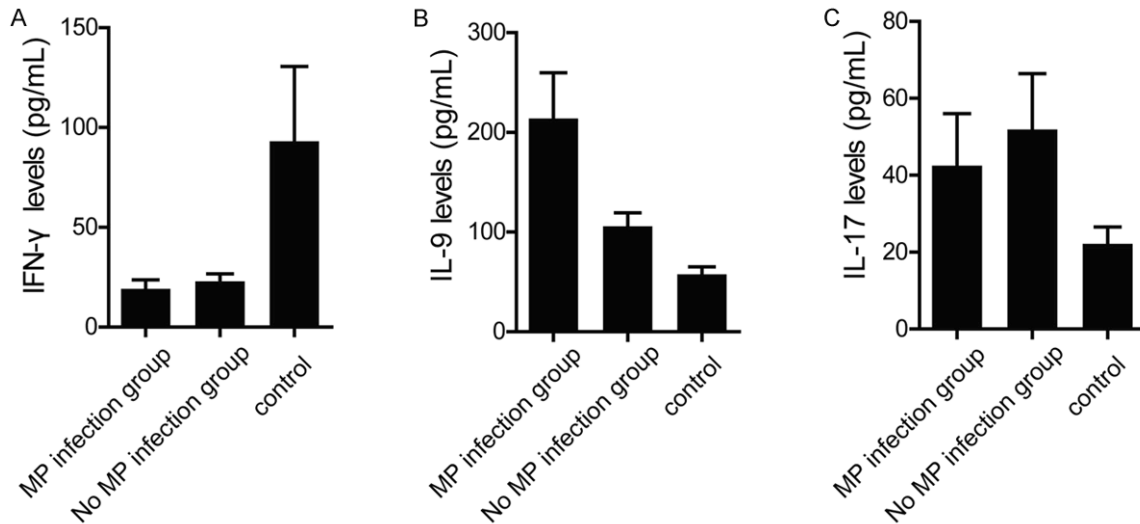


Figure 3. The levels of IFN-γ (A), IL-9 (B) and IL-17 (C) of peripheral blood in the asthmatics with or without MP infection group and the control group.

The IL-9 levels in asthmatic children with MP infection, asthmatic children without MP infection, and the normal control group were 213.8 ± 46.04 , 105.9 ± 13.50 , and 57.65 ± 7.50 pg/mL, respectively (**Figure 3B**). The levels in the asthmatic children with/without MP infection groups were significantly different ($P < 0.03$), and both groups were significantly different compared with the normal control group ($P < 0.005$ and < 0.006 , respectively).

The IL-17 levels in asthmatic children with MP infection, asthmatic children without MP infection, and the normal control group were 42.50 ± 13.52 , 51.90 ± 14.55 , and 22.16 ± 4.39 pg/mL, respectively (**Figure 3C**). The difference between the asthmatic children with/without MP infection groups was not significant, and neither group showed a significant difference versus the normal control group ($P > 0.05$).

Discussion

Asthma is a common respiratory disease in children. The disease is regarded as infectious by some scholars [26] because the onset and exacerbation of asthma in children have been observed to be associated with pathogen infection in clinical practice. Asthma is especially related to colonization by atypical pathogens, such as *M. pneumoniae* and *C. pneumoniae*, in the airway of asthma patients during acute exacerbations and the chronic stable phase

[27-29]. Moreover, 3-64% of pediatric asthma patients in the chronic stable phase are infected with *M. pneumoniae*. Thus, MP is a known aggravating factor of asthmatic symptoms in both acute exacerbations and the chronic stable phase. This is further demonstrated by the alleviation of respiratory system symptoms and improvement of pulmonary function after use of macrolides to treat MP infection [30]. The current study evaluated serum anti-MP IgM levels, and used clinical symptoms and other clinical examinations to diagnose MP. Consistent with previous reports, the results indicated a high MP infection rate (44.74%) in children with asthma, which was significantly higher than the control group and the other respiratory infection group.

We also tested allergic markers in asthma associated with airway inflammation that can be used as follow-up measures of allergic status and treatment effectiveness. Significant differences existed in the levels of total IgE and sIgE against mites and ECP between the MP-infected and non-MP-infected groups. We suggest that infection with MP alters the production of cytokines associated with IgE in particular ways. Airway inflammation may predispose an individual to infection with MP. MP is an atypical pathogen that adheres tightly to the plasma membrane of host cells by means of specialized structures and damages host cells by releasing toxic metabolites. Thus, severe air-

way inflammation results in a reduced ability of the airway to eliminate pathogens. Moreover, the levels of cytokines related to airway inflammation will increase. All of these factors can predispose the airway to the adhesion of further MP and thus establish a vicious cycle. Hassan et al. assessed the serum levels of several cytokines in pediatric asthma patients with upper or lower respiratory infection with MP. The cytokines included IL-1 α and IL-6; Th1-subtype cytokines, such as IL-2, IFN- γ , and IL-12; Th2-subtype cytokines, such as IL-4, IL-5, and IL-10; chemokines, such as IL-8; and other factors, such as IL-1 β , TNF- α , and GM-CSF. The results showed that the cytokines whose levels were elevated in the serum of patients with URI/LRI of MP were mainly of the Th2 subtype. Moreover, the total IgE levels in these patients were also higher than that in the control group, indicating that asthma pathogenesis involves cytokines that are released by immune cells after MP infection [31, 32].

Although the rate of MP infection in asthmatic children was high, and although clinical symptoms and allergic markers are relevant to the infection, the following questions must be addressed: (1) why does asthma predispose patients to MP infection, and (2) what is the immunological mechanism that explains the connection between asthma pathogenesis and MP infection? These questions have been the subject of much research in recent years. The roles of DCs and TLRs in the pathogenesis of asthma have received considerable attention.

DCs are the most potent APCs and play an important role in the pathogenesis of bronchial asthma [33, 34]. In vivo, the DC undergoes a development process, from naïve to mature. During this process, differentiation antigens are expressed, including CD1a, major histocompatibility complex II (MHC-II), human leukocyte antigen-DR, CD80, CD83, and CD86. These surface antigens are used as markers of DCs in peripheral blood and bone marrow. In this study, we deemed CD1a and HLA-DR double-positive cells to be peripheral blood DCs. No significant difference in the ratio of peripheral blood DCs was observed between the MP-infected asthma group and non-MP-infected asthma group. However, a significant difference existed between both of these groups and the normal control group. Both the MP-infected asthma group and the non-MP-infected asthma group had a higher DC ratio than the normal

control group. This indicates that in bronchial asthma or asthma accompanied by MP infection, numbers of DCs are higher in the peripheral blood, but no significant difference existed between the infected and non-infected groups.

DCs are involved in antigen presentation in bronchial asthma and can secrete cytokines associated with trachea inflammation in asthma. DCs play an important role in initiating the immune response, which involves the skin, gut, and bronchial mucosa in the form of a strong immune surveillance network. The number of DCs within the bronchial epithelium in patients with allergic diseases has been confirmed to be increased markedly. Our results suggest that the percentages of DCs in both the MP-infected asthma group and the non-MP-infected asthma group were higher than that in the normal control group; thus, our findings are consistent with existing research. No difference was observed in the percentage of peripheral blood DCs between the MP-infected group and the non-infected group. This result suggests that MP infection may have no significant effect on the number of DCs. However, the expression of several DC surface receptors is involved in the occurrence of asthma. TLRs are pattern-recognition receptors that are expressed on the surface of many immune cells, such as DCs. Some researchers have reported that the immune-regulatory function of DC is associated with surface TLR expression [35]. TLRs 1, 2, 3, 4, 5, 6, 7 and 9 can be expressed by DCs. Various antigens are recognized by the TLRs. Then, intracellular signal transduction pathways are initiated and an inflammatory reaction, as well as changes in cytokine networks, participate in the development of various allergies and neoplasms [36, 37]. Currently, the study of allergic diseases (e.g., asthma) is focused mainly on TLRs 2, 4, and 5 [38, 39]. Our results showed that a considerably greater number of cells expressed TLRs 2 and 4 in the asthma MP infection group than in the non-MP infection group and the normal control group. Additionally, the number of cells expressing TLRs 2 and 4 was markedly higher in the non-MP infection group than the normal control group, suggesting that TLRs 2 and 4 play important roles in both asthma and asthma accompanied by MP infection [38, 40, 41].

The chronic airway allergic inflammation of asthma is related to Th1, Th2, Th9, and Th17 T

cell subtypes and their cytokines, IFN- γ , IL-4, IL-9, and IL-17 [42]. In recent years, Th9 and Th17 cells, as well as their cytokines, IL-9 and IL-17, have received much attention [43]. Several preclinical studies have shown that IL-9 is a core substance involved in the development of allergic inflammation in asthma. IL-9 was first identified in 1980 and causes release of immune mediators by mast cells and lymphocytes [44]. IL-9 is regarded as a Th2-subtype cytokine [45], and is involved in the pathogenesis of several inflammatory diseases [46]. Some studies report that Th9 cells in OVA-sensitized animal models can lead to airway hypersensitivity and that this can be relieved by IL-9 blockers, as demonstrated by alleviation of airway hypersensitivity, eosinophilic infiltration, and hyperplasia of goblet cells. Other studies show the relevance of sIgE and IL-9; IL-9-releasing Th9 cell numbers are also related to sIgE levels in asthmatic patients. However, the results of other studies contradict these findings; thus the situation remains unclear. In this study, total sIgE and specific sIgE levels in pediatric asthma patients were not obviously related to IL-9 levels in the peripheral blood in either the MP-infected or the non-MP-infected group. Further investigations involving a greater number of cases are warranted. The IL-9 gene and allergic inflammation are closely associated in chronic asthma patients. The airways of transgenic rats, in which the IL-9 gene is overexpressed in the lung, show inflammation characterized by the infiltration of eosinophils and lymphocytes. IL-9 has also been shown to, directly or indirectly, cause airway remodeling in chronic asthma patients. Th9 cells from patients with atopic disease tend to release more IL-9 than those from patients without atopic disease. Moreover, patients with atopic disease have a greater number of Th9 cells. These findings suggest the presence of a Th1/Th9 shift in asthma and indicate the core role of IL-9 in the pathogenesis of chronic asthma. In the present study, IL-9 levels in the peripheral blood of MP-infected asthma patients was significantly higher compared with those in non-MP-infected asthma patients and the control group. The peripheral blood IFN- γ levels in MP-infected asthma patients were significantly lower compared with those in non-MP-infected asthma patients and the control group. These results indicate that IL-9 secretion increased, whereas that of IFN- γ decreased, after MP

infection, suggesting that the Th1/Th9 balance shift after MP infection. This result is consistent the changes in the cytokine network; changes in cytokine levels after MP infection have been reported, demonstrating primarily that MP infection may induce asthma by stimulating the release of inflammatory mediators. Changes in IL-9 levels, airway hypersensitivity, and eosinophilic infiltration are also observed in MP-infected patients without asthma. Hassan et al. tested the levels of 12 cytokines in patients with MP infection, including pre-inflammatory factors, Th1 and Th2 cytokines, and the chemokine IL-8. Increases in pre-inflammatory factors, such as IL-1a and IL-6, and other cytokines, such as IL-10, were found. This indicates that MP infection can cause Th2 cell reactions and disequilibrium of Th1/Th2 cells. In the present study, the IL-9 level increased but that of IFN- γ decreased in the peripheral blood of MP-infected asthma patients. These results show that cell-induced immune reactions caused by MP infection and cytokine release are related to the pathogenesis and acute exacerbations of asthma [31, 47]. A recent study showed that various MP surface complexes not only aid attachment to respiratory epithelial cells and cause damage to host cells but also trigger abnormal immune reactions in the host [47]. Many harmful components of MP, including LPS, are major components of MP cell membranes. These components can be recognized by pattern-recognition receptors (PRRs), such as TLRs, and induce immune reactions, stimulating cells to synthesize cytokines that are involved in the pathogenesis of asthma. This result is in agreement with the changes in DC numbers and TLR2 and TLR4 receptor expression, indicating that cytokine changes, such as in IL-9, induced by serial immune reactions are mediated by TLRs on the DC surface after MP infection.

In conclusion, the MP infection rates differed among groups of asthmatic children, children with other respiratory infections and a normal control group. The levels of serum total IgE, mite-specific IgE, and ECP in the peripheral blood were significantly higher in MP-infected children than in MP-uninfected children, suggesting that MP influences the serum IgE level, possibly through the effects of several cytokines. The numbers of DCs in both groups with asthma were higher than in the normal control

group. The number of DCs was similar in asthmatic children with and without MP infection. This result suggests that MP affects the expression of important DC surface receptors rather than altering the number of DCs. We also assessed TLR-2 and TLR-4 expression by DCs. DC TLR-2 and TLR-4 expression was higher in asthmatic children with than without MP infection and compared to the normal control group. The asthmatic children without MP infection also showed greater TLR-2 and TLR-4 expression than the normal control group. These findings suggest that MP infection affects children with asthma through TLR-2 and TLR-4 expressed by DCs. IL-9 is considered a Th2-cell cytokine and is involved in many inflammatory diseases. This study found that IL-9 levels increased and IFN- γ levels decreased in asthmatic children with MP infection. This result suggests a possible shift in the Th1/Th9 balance following MP-induced cell-mediated immune response and cytokine release.

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Disclosure of conflict of interest

None.

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