

Mycoplasma pneumoniae induces allergy by producing P1-specific immunoglobulin E



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ABSTRACT

Background: Our previous study found that most *Mycoplasma pneumoniae* (MP) pneumonia (MPP) patients had elevated serum total immunoglobulin E (IgE) levels.

Objective: To determine components of MP that can cause an IgE increase in children, and to clarify its specific mechanism.

Methods: The components of MP cells were isolated by serum IgE from patients with MP pneumonia. These components obtained through the prokaryotic expression were used as allergens to detect the proportion of allergen-specific IgE produced in MPP patients, and the clinical characteristics and related immune parameters of these patients who produced this allergen-specific IgE were also analyzed. In addition, a cell experiment was used to verify the biological effect of these components in vitro.

Results: P1-specific IgE was detected in serum of MPP children. An approximately 24-kDa polypeptide of P1 protein was obtained through prokaryotic expression purified by nickel agarose affinity chromatography. Approximately 9.2% of MPP patients produced IgE against this polypeptide of P1 protein, which was more likely to be produced in MPP patients with no history of allergies or family history of allergy-related diseases. P1-specific IgE-positive MPP patients had more severe clinical symptoms, with excessive secretion of interleukin (IL)-4 and IL-5 and overdifferentiation of Th0 cells into Th2 cells. Tests also demonstrated that the P1 protein stimulated excessive secretion of IL-4 and IL-5 in peripheral blood mononuclear cells from the peripheral blood of healthy donors.

Conclusion: *Mycoplasma pneumoniae* is not only an infectious agent but also an allergen for certain individuals. The P1 protein of MP can induce the production of P1-specific IgE.

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Introduction

Mycoplasma pneumoniae (MP) was first identified as a cause of atypical pneumonia in the 1940s by Eaton and colleagues¹ and eventually was identified as a pleuropneumonia-like organism and cultured by Chanok and colleagues in the 1960s.² MP is one of the major pathogens that causes respiratory tract infections in children, with 10% to 40% of community-acquired pneumonia cases in children caused by MP.³ In 1970, Berkovich et al first reported a connection between asthma attacks and MP.⁴ Since then, the relationship between MP infection and asthma has become a primary focus of researchers worldwide.^{5,6} Accumulating evidence suggests that MP infection

is closely related to asthma attacks, and a recent large-scale retrospective study in Taiwan further confirmed this relationship.⁷ However, no direct evidence exists to explain this phenomenon. Considerable controversy surrounds the possible causal relationship between MP infection and asthma, and the exact underlying mechanism is unclear. The current study investigates these issues.

Methods

This is a prospective study of subjects selected from children with MP pneumonia (MPP) who were hospitalized in the Affiliated Children's Hospital of Zhejiang University School of Medicine from May 2016 to May 2017. Conditions of enrollment were as follows: (1) younger than 16 years of age; (2) symptoms compatible with the diagnosis of pneumonia and MP-RNA-positive serum determined by simultaneous amplification and testing; (3) elimination of other bacteria, viruses, or chlamydia infections; (4) no treatment with hormones or immunomodulators 2 weeks before the collection of specimens. Peripheral blood samples were collected from all enrolled patients before treatment to detect cytokines and T cell subsets, and detailed medical and family histories including history of allergy or family history of allergy-related disease were ob-

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tained by active inquiry. The definition of having no history of allergy was that the patient had no history of eczema, asthma, allergic rhinitis, and so forth. The definition of having no family-history of allergy related disease was that their parents had no allergic diseases, such as asthma, allergic rhinitis, or measles. All patients were followed up by telephone within 7 to 14 days after discharge; signs and symptoms were recorded daily. This study was approved by the Ethical Committee of the Affiliated Children's Hospital of Zhejiang University School of Medicine, and all families of patients involved in the study provided written informed consent.

Cytokine Determination

Blood samples were centrifuged at 1,000g at 20°C for 20 minutes after clotting. The serum was carefully harvested, and interleukin (IL)-4, IL-5, and interferon gamma (IFN- γ) levels were measured immediately by 320 flow cytometry with BD cytometric bead array human cytokine kit (BD Biosciences, San Jose, California).^{8,9}

Measurement of Th1/Th2 Cell Subsets and Total Immunoglobulin E

Blood was collected directly and resuspended at a concentration of 1×10^6 cells/mL in Roswell Park Memorial Institute medium (Biomed, Poland). Flow cytometric immunophenotyping was performed by using flow cytometry with double staining (BD Biosciences, San Jose, California). Th1 cells were characterized as CD3+, CD4+, CCR6-, and CXCR3+, and Th2 cells were characterized as CD3+, CD4+, CCR6-, and CXCR3-.¹⁰ The data were collected on a Beckman Navios and analyzed with Cell Quest software (Kaluza V1.5, Beckman Coulter, Paris, France). Immunoglobulin E (IgE) serum levels were obtained in an ELECSYS 2010 apparatus with a ruthenium-labeled anti-IgE monoclonal antibody (Roche, Diagnostic Corp, Indianapolis, Indiana). Reactions were developed according to the manufacturer's instructions.

Extraction of MP Cell Protein

The MP strain was cultured in mycoplasma broth consisting of mycoplasma broth base CM403 (OXIOD, Hampshire, United Kingdom), mycoplasma-selective supplement G SR59 (OXIOD), 0.5% glucose, and 0.002% phenol red at 37°C under 5% (v/v) humidified CO₂. The MP culture broth was packed in 6 round-bottom centrifuge tubes and centrifuged in a high-speed refrigerated centrifuge at 12,000 rpm for 30 minutes at 37°C. The supernatants were discarded, and the samples were washed 5 times by adding phosphate-buffered saline to each tube, vortexed to resuspend, and transferred to eppendorf tubes, followed by centrifugation in a high-speed refrigerated centrifuge at 12,000 rpm for 30 minutes at 37°C. After the final centrifugation, the supernatants were discarded, and 500 μ L of lysis buffer with phenylmethylsulfonyl fluoride (radioimmuno-precipitation assay buffer = 20 μ L: 1.3 mL) was added to each tube and mixed well by pipetting, after which samples were placed at -60°C for 30 minutes, incubated at 37°C for 10 minutes, and vortexed to resuspend. This procedure was repeated twice. The tubes were then centrifuged in the high-speed centrifuge at 12,000 rpm for 30 minutes at 37°C. The supernatants were collected, and protein concentrations were determined using the bicinchoninic acid assay. The samples were then stored at -80°C for later use.

Western Blotting

Western blotting of the MP strain was performed as described previously, with minor modifications.⁹ Briefly, 50- μ g cell lysates were denatured, separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burling-

ton, Massachusetts). For detection, the membranes were incubated overnight with pooled patient sera at 4°C, followed by the addition of anti-human IgE antibody (Sunlong Biotech Co, Ltd, Hangzhou, China [#ABIN363691]) for 1 hour at room temperature and color-substrate solution for 15 minutes at room temperature. The membranes were then scanned using an imaging system.

Proteomic Analysis by Liquid Chromatography Coupled with Tandem Mass Spectrometry

The target gel band (the band that reacts with the MP-specific IgE antibody) was excised from the gel and subjected to in-gel digestion. Peptides were dried to completion and resuspended in 0.1% formic acid and directly loaded onto a reverse-phase analytical column. Peptide separation was performed using a gradient consisting of an increase from 4% to 35% solvent B (0.1% formic acid in 98% acetonitrile) over 22 minutes, 35% to 80% in 5 minutes, and holding at 80% for the last 3 minutes, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system (Thermo Fisher Scientific, Waltham, Massachusetts). The resulting peptides were analyzed on a Q ExactiveTM plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The resulting tandem mass spectrometry (MS/MS) data were processed using the mascot search engine. Tandem mass spectra were searched against an MP database. Trypsin/P was specified as the cleavage enzyme, allowing up to 2 missed cleavages. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys was specified as fixed modification and oxidation on Met, and acetylation on protein N-term was specified as variable modifications. The peptide ion score was set to 20 or greater.

Prokaryotic Expression and Purification of P1

The P1 protein gene was cloned into the pET28a vector. Then, 1 μ L of recombinant pET28a vector was transformed into BL21 (DE3) cells by heat shock for 90 seconds at 42°C, followed by incubation on ice for 2 minutes and plating on LB agar medium containing 30 μ g/mL kanamycin. Single colonies of expression strain BL21 were picked and inoculated into triangular flasks containing LB medium (30 μ g/mL kanamycin) for overnight culture at 37°C, 220 rpm. Overnight cultures were then inoculated into 3 L of LB medium at a ratio of 1:100, with kanamycin added to a final concentration of 30 μ g/mL for culture at 37°C, 220 rpm. When the optical density value reached 0.6, isopropyl β -D-1-thiogalactopyranoside was added to a final concentration of 0.5 mM for overnight induction 20°C or 4 hours at 37°C, 220 rpm, along with a negative control without isopropyl β -D-1-thiogalactopyranoside induction. The cells were collected by centrifugation at 4,000 rpm for 10 minutes. The supernatants were discarded, and the cells were suspended in 500 μ L of phosphate-buffered saline (pH 7.4) buffer and sonicated for 6 minutes, stopping for 1.5 seconds every 0.5 second. The supernatants and pellets were collected by centrifugation. The pellets were dissolved in 500 μ L of inclusion body solubilization buffer (8 M urea, 50 mM Tris-HCl, 300 mM NaCl, pH 8.0). Then, 40 μ L of sample and 10 μ L of 5 $\times$ protein loading buffer were mixed and boiled for 10 minutes, followed by centrifugation at 12,000 rpm for 20 minutes at 4°C. The supernatants were purified by nickel agarose affinity chromatography, the collected components were analyzed by SDS-PAGE, and the target protein was dialyzed into 1 $\times$ phosphate-buffered saline (pH 7.4; 1.0 mg/mL, purity > 90%, 1 mL/tube) and stored at -80°C.

Western Blotting of P1-Specific IgE

The P1 protein (200 μ g/mL) was spotted onto a nitrocellulose membrane (0.8- μ m pore size, manufactured by Sartorius, Germany), using a chip sampling apparatus (model: AD1500, manufactured

by BioDot), and biotin-labeled mouse anti-human immunoglobulin G by Thermo Fisher, and 56°C-inactivated serum were also spotted onto the nitrocellulose membrane as positive and negative controls, respectively. The nitrocellulose membrane spotted by P1 protein was soaked in the 5% bovine serum albumin sealing solution for 1 hour (The buffer system is Tween–Tris-buffered saline with a pH of 7.4.), and then dried in the oven. The nitrocellulose membrane was then fixed in the groove of a polyvinyl chloride assay plate. Then, 300 μ L of MPP patient serum was added to the groove of the assay plate. After incubation and washing with Tris buffer 5 times, we then added 300 μ L of biotin anti-human IgE antibody complex to the assay plate. After incubation and washing, we subsequently washed the assay plate with running water and read it with a scanner.

Isolation and Culture of Mononuclear Cells

Mononuclear cells from the peripheral blood of healthy individuals were isolated by density gradient centrifugation and inoculated into Roswell Park Memorial Institute medium–containing 6-well culture plates at a density of 1.5×10^6 cells/well; we then added 4 concentrations (0 g/mL, 10 μ g/mL, 50 μ g/mL, and 100 μ g/mL) of P1 protein and cultured the cells in a CO₂ incubator (at 37°C; saturated humidity, 50 mL/L). The supernatants were collected for cytokine detection at 3 points (day 1, day 2, and day 3) after culture.

Statistics

All the data in this study were analyzed using SPSS 11.5 statistical software (IBM Corp, Armonk, New York), and the results of normally distributed data were expressed as the mean $\pm$ standard deviation. The data of the two groups were in good agreement with the normality and variance homogeneity test. A *t*-test was used. One-way analysis of variance was used to compare independent samples. A least significant difference test was used to compare the two groups. *P* < .05 was considered statistically significant. Graphs were plotted using GraphPad Prism 6.0 (GraphPad Software, La Jolla, California).

Results

The P1 Protein of MP Cells Can React with IgE Antibodies in MPP Patient Serum

A total of 98 strains of clinical isolates obtained from MPP patients' alveolar lavage fluid, as well as the standard MP strains ATCC29342 and ATCC15531, were cleaved with cell lysates. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using total bacterial protein samples obtained by cleavage. After electrophoresis, Coomassie brilliant blue staining showed that the electrophoretic bands of the isolates were not completely identical, but they all had 6 bands with the highest protein content. These protein bands were transferred to PVDF membranes, and mixed serum from 10 MPP patients was then added as a primary antibody, which was hybridized to the protein bands on the PVDF membranes, followed by the addition of anti-human IgE antibody as a secondary antibody for testing. The results showed that the 170-kDa protein bands from all strains were able to react with mixed serum from MPP patients. These protein bands were cut from the corresponding SDS-PAGE gel for mass spectrometry analysis, which indicated that the protein was the P1 protein of MP (Fig 1). These results were further confirmed by the binding of anti-P1 protein antibody to the protein bands, clearly demonstrating that this protein was the P1 protein of MP.

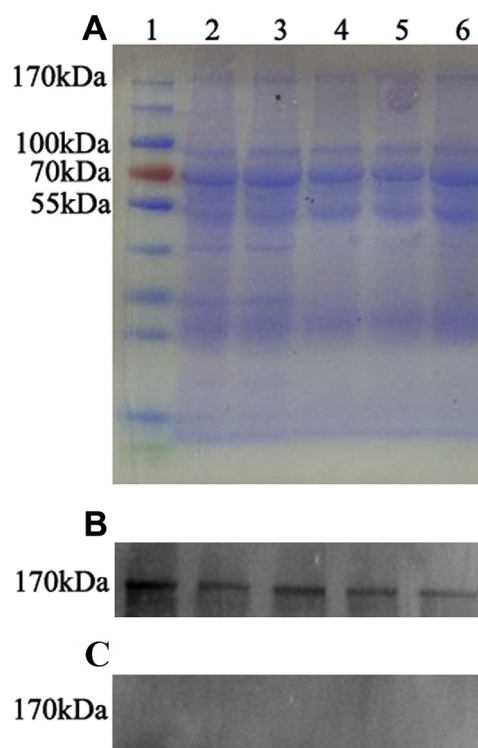


Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of mycoplasma pneumoniae lysates and western blotting of P1-specific immunoglobulin E. A, After sodium dodecyl sulfate polyacrylamide gel electrophoresis, Coomassie brilliant blue staining showed that the electrophoretic bands of the isolates were not completely identical, but they all had 6 bands with the highest protein content. Lane 1: marker; lane 2: ATCC29342; lane 3: ATCC15531; lanes 4–6: clinical mycoplasma pneumonia strains. B, The protein bands in the sodium dodecyl sulfate polyacrylamide gel electrophoresis gel were transferred to a polyvinylidene difluoride membrane and probed with mixed serum from *Mycoplasma pneumoniae* pneumonia patients as a primary antibody, followed by the addition of anti-human immunoglobulin E antibody as a secondary antibody. The 170-kDa protein bands from all of the *Mycoplasma pneumoniae* strains reacted with mixed serum from *Mycoplasma pneumoniae* pneumonia patients. C, Negative control probed with serum from healthy children as a primary antibody, and other experimental steps are the same as in B.

P1-Specific IgE is Produced in 9.2% of MPP Patients

To determine the proportion of P1-specific IgE produced in MPP patients, the P1 protein gene was cloned into the pET28a vector. The recombinant plasmid was then transformed into *Escherichia coli* BL21 cells for prokaryotic expression and purification of the P1 protein. The purified P1 protein was then used as an allergen to detect P1-specific IgE in MPP patient serum by western blotting. P1-specific IgE was found in the serum of 48 of 520 MPP patients, for a positive rate of 9.2% (Fig 2).

P1-Specific IgE was more Likely to Be Produced in MPP Patients with No History of Allergy or Family History of Allergy-Related Diseases

Of the 520 MPP patients, 344 had a history of allergy, and the remaining 176 had no history of allergy. The prevalence of MP infection was greater in atopic than nonatopic children (*P* < .05). The positive rate of P1-specific IgE in the 344 patients with an allergic history was 4.7% (16 cases), and the positive rate of P1-specific IgE in the 176 patients with no allergic history was 18.2% (63 cases), and this difference was statistically significant (*P* < .001). These 520 MPP patients were further analyzed to determine whether they had a family history of allergy-related diseases. The results showed that 76 patients had a family history of allergy-related diseases, and the

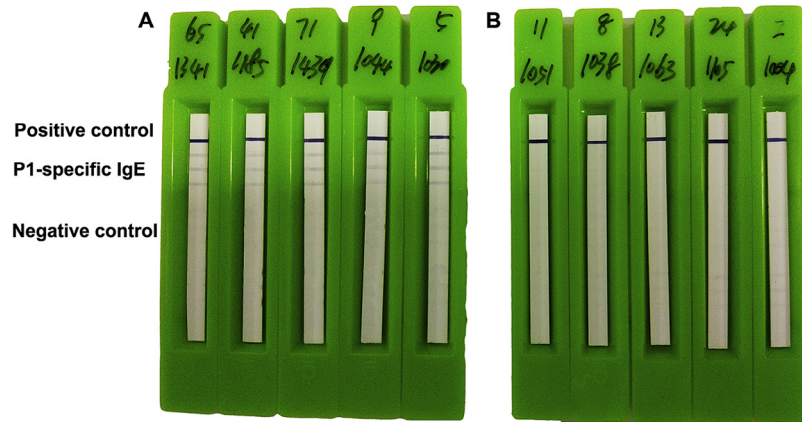


Figure 2. Western blotting of P1-specific immunoglobulin E. Purified P1 protein was used as an allergen to detect P1-specific immunoglobulin E in the serum of 520 *Mycoplasma pneumoniae* pneumonia patients by western blotting, with a positive rate of 9.2%.

remaining 444 patients had no family history of allergy-related diseases. The positive rate of P1-specific IgE in MPP patients with a family history of allergic-related illness was 0.0%, whereas the positive rate of P1-specific IgE in the 444 patients with no family history of allergy-related diseases was 10.8% (48 cases). A statistically significant difference was seen between the positive rates of P1-specific IgE between these 2 groups ($P < .001$; see Fig 3 for details). Details and basic information for these 520 MPP patients are shown in Table 1.

MPP Patients with P1-Specific IgE Had More Severe Clinical Symptoms

To eliminate the influence of the history of allergy and the family history of allergy-related diseases in this study, of the 48 P1-specific IgE-positive MPP patients, 32 patients with no history of allergy or family history of allergy-related diseases were enrolled in a cohort study, and 32 age- and sex-matched MPP patients without P1-specific IgE and no history of allergies or family history of allergy-related diseases were randomly selected as controls. The analysis of the case-control study was prospective. The incidence of wheezing in P1-specific IgE-positive MPP patients was 71.9%, which was significantly higher than that in P1-specific IgE-negative MPP patients (43.8%, $P < .01$). The forced expiratory volume in 1 second expressed as a percentage of the forced vital capacity of P1-specific IgE-positive MPP patients was significantly lower than that of P1-specific IgE-negative MPP patients ($68\% \pm 7\%$ vs $81\% \pm 8\%$,

$P < .001$), both of which were significantly lower than those of healthy controls ($89\% \pm 8\%$). In addition, P1-specific IgE-positive MPP patients had significantly longer hospital stays than P1-specific IgE-negative MPP patients (7.21 ± 1.8 vs 6.12 ± 1.5 days, $P < .01$). See Table 1 for details.

P1-Specific IgE-Positive MPP Patients Had Higher Serum Levels of IL-4 and Total IgE

Serum levels of IL-4, IFN- γ , and total IgE in the 2 groups were further measured. Compared with P1-specific IgE-negative MPP patients, P1-specific IgE-positive MPP patients exhibited higher serum levels of IL-4 (3.81 ± 0.12 pg/mL vs 2.8 ± 0.29 pg/mL, $t = 18.2$, $P < .001$), but lower serum levels of IFN- γ (7.62 ± 0.25 pg/mL vs 17.6 ± 1.51 pg/mL, $t = 36.89$, $P < .001$), leading to a remarkable increase in the IL-4/IFN- γ ratio (0.57 ± 0.04 vs 0.21 ± 0.03 , $t = 40.73$, $P < .001$); serum total IgE levels also increased significantly (160.52 ± 43.3 IU/mL vs 73.3 ± 6.67 IU/mL, $t = 11.26$, $P < .001$; Fig 4).

The Percentage of Th2 Cells in the P1-Specific IgE-Positive MPP Patients was Significantly Reduced after Disease Recovery

The percentages of Th1 and Th2 cells in peripheral blood CD4+T cells from 6 P1-specific IgE-positive MPP patients in the acute phase and 3 months after recovery were analyzed. The percentage of Th1 cells among CD4+T cells was significantly increased after recovery from MPP ($14.73\% \pm 2.10\%$ vs $20.15\% \pm 4.01\%$, $t = 3.02$, $P = .03$),

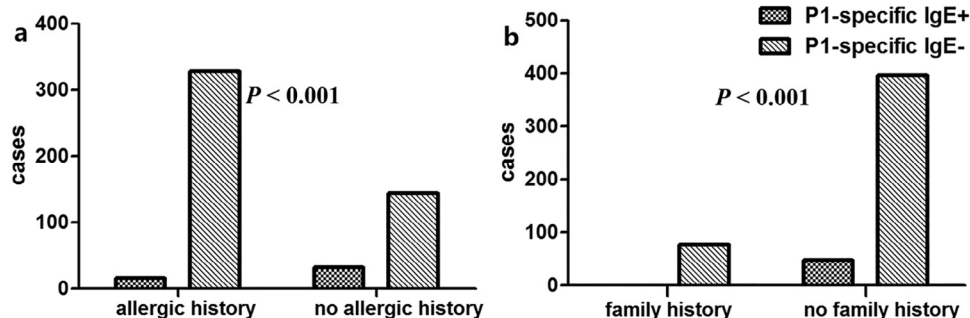


Figure 3. The positive rate of P1-specific immunoglobulin E in *Mycoplasma pneumoniae* pneumonia patients with and without a history of allergy or a family history of allergy-related diseases. A, The positive rate of P1-specific immunoglobulin E in 344 patients with an allergic history was 4.7% (16 cases); the positive rate of P1-specific immunoglobulin E in 176 patients with no allergic history was 18.2% (63 cases). B, The positive rate of P1-specific immunoglobulin E in *Mycoplasma pneumoniae* pneumonia patients with a family history of allergic-related illness was 0.0%; the positive rate of P1-specific immunoglobulin E in the 444 patients with no family history of allergy-related diseases was 10.8% (48 cases).

Table 1
Characteristics of Children With *Mycoplasma pneumoniae* Pneumonia

Feature	MPP (n = 520)	P1-IgE + MPP (n = 32)	P1-IgE- MPP (n = 32)	P value
Age, years (Mean $\pm$ SD)	3.8 $\pm$ 3.5	5.8 $\pm$ 2.6	5.0 $\pm$ 3.4	NS
Sex (male/female)	284/236	19/13	19/13	NS
Clinical symptoms				
Fever	458 (88.1%)	31 (96.9%)	27 (84.4%)	NS
Cough	520 (100.0%)	32 (100.0%)	32 (100.0%)	NS
Tachypnea	59 (11.3%)	10 (31.3%)	3 (9.4%)	<.01
Chest tightness	6 (1.2%)	1 (3.1%)	0 (0.0%)	NS
Wheezing	230 (44.2%)	23 (71.9%)	14 (43.8%)	<.01
Chill	10 (1.9%)	6 (1.9%)	5 (1.6%)	NS
Extrapulmonary complications	56 (10.8%)	3 (9.4%)	4 (12.5%)	<.05
Physical examination, n (%)				
Rales	369 (71.0%)	23 (71.9%)	21 (65.6%)	NS
Decreased unilateral lung sound	78 (15.0%)	8 (25.0%)	5 (15.6%)	NS
Length of fever, days	7.53 $\pm$ 1.6	11.42 $\pm$ 1.9	7.34 $\pm$ 1.7	<.01
Hospital stay, days	6.21 $\pm$ 1.3	8.21 $\pm$ 1.8	6.12 $\pm$ 1.5	<.01
Management				
Length of antibiotic therapy days	10.89 $\pm$ 1.8	16.15 $\pm$ 2.3	11.01 $\pm$ 1.7	<.01
Oxygen therapy, n (%)	28 (5.4%)	9 (28.1%)	1 (3.1%)	<.05
Intensive care unit, n (%)	11 (2.1%)	1 (3.1%)	0 (0.0%)	NS
Others				
FEV ₁ %	-	68 $\pm$ 7	81 $\pm$ 8	<.01

Abbreviations: FEV₁%, forced expiratory volume in 1 second; IgE, immunoglobulin E; MPP, *Mycoplasma pneumoniae* pneumonia; NS, not significant. Data are presented as number (percentage) or mean $\pm$ SD. P value: P1-IgE + MPP vs P1-IgE- MP.

whereas the percentage of Th2 cells among CD4 + T cells was significantly reduced ($80.58\% \pm 1.80\%$ vs $70.92\% \pm 5.16\%$, $t = 5.19$, $P = .0035$; Fig 5).

P1 Protein Stimulates Excessive IL-4 and IL-5 Secretion by Peripheral Blood Mononuclear Cells From the Peripheral Blood of Healthy Donors

To further validate the function of the P1 protein, peripheral blood mononuclear cells (PBMCs) from the peripheral blood of healthy

donors were collected for culture and then stimulated by adding different concentrations of P1 protein (0 μ g/mL, 10 μ g/mL, 50 μ g/mL, and 100 μ g/mL). The cell culture supernatants were collected after 1 day of culture to determine the levels of IL-4, IFN- γ , and IL-5. The levels of IL-4 and IL-5 increased with increasing P1 protein concentrations, and their levels were significantly higher than those of blank controls (without P1 protein; $P < .05$). IFN- γ levels decreased with increasing concentrations of P1 protein and were significantly reduced compared with the blank control ($P < .05$; Fig 6). Next, 10 μ g/mL P1 protein was added to PBMCs for 3 days of

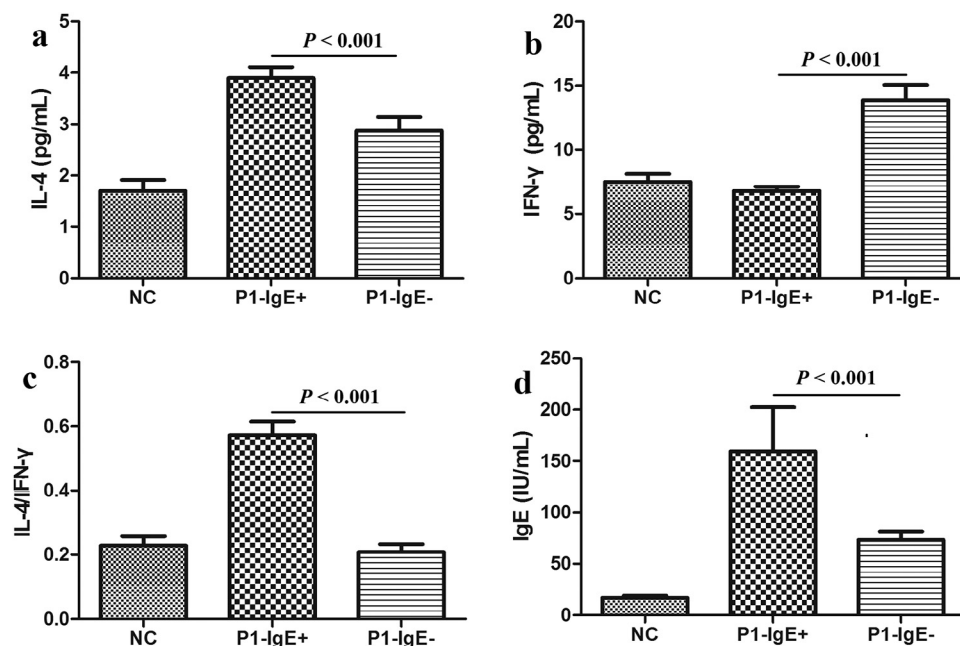


Figure 4. Serum levels of interleukin-4, interferon-gamma, and total immunoglobulin E in P1-specific immunoglobulin E-positive *Mycoplasma pneumoniae* pneumonia patients. Compared with P1-specific immunoglobulin E-negative *Mycoplasma pneumoniae* pneumonia patients, serum levels of interleukin-4 in P1-specific immunoglobulin E-positive *Mycoplasma pneumoniae* pneumonia patients were higher (3.81 ± 0.12 pg/mL vs 2.8 ± 0.29 pg/mL, $t = 18.2$, $P < .001$), but serum interferon-gamma levels were lower (7.62 ± 0.25 pg/mL vs 17.6 ± 1.51 pg/mL, $t = 36.89$, $P < .001$), leading to a remarkable increase in the interleukin-4/interferon-gamma ratio (0.57 ± 0.04 vs 0.21 ± 0.03 , $t = 40.73$, $P < .001$). Serum total immunoglobulin E levels also increased significantly (160.52 ± 43.3 IU/mL vs 73.3 ± 6.67 IU/mL, $t = 11.26$, $P < .001$). A, Interleukin-4. B, Interferon-gamma. C, Interleukin-4/interferon-gamma. D, Serum total immunoglobulin E.

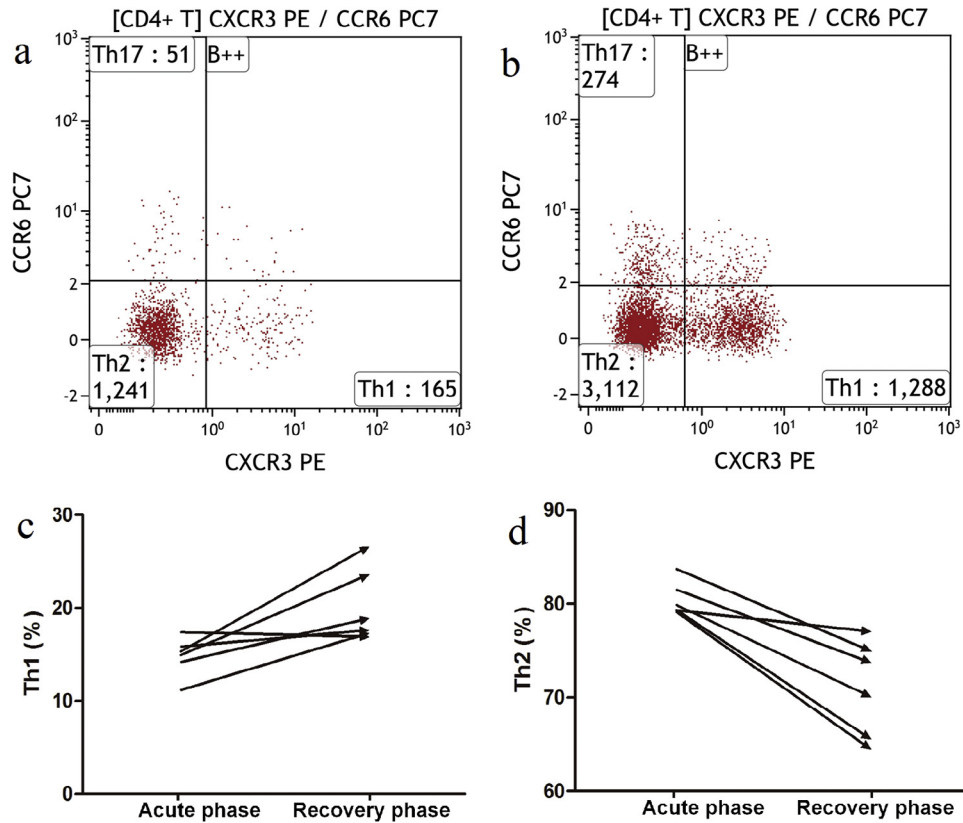


Figure 5. The percentage of Th1 and Th2 cells in *Mycoplasma pneumoniae* pneumonia patients with P1-specific immunoglobulin E in the acute phase and after disease recovery. The percentage of Th1 cells among CD4 + T cells was significantly increased after recovery from mycoplasma pneumonia (14.73% ± 2.10% vs 20.15% ± 4.01%, $t = 3.02$, $P = 0.03$), whereas the percentage of Th2 cells among CD4 + T cells was significantly reduced (80.58% ± 1.80% vs 70.92% ± 5.16%, $t = 5.19$, $P = .0035$). A, The percentage of Th1 and Th2 cells in *Mycoplasma pneumoniae* pneumonia patients with P1-specific immunoglobulin E in the acute phase. B, The percentage of Th1 and Th2 cells in *Mycoplasma pneumoniae* pneumonia patients with P1-specific immunoglobulin E after disease recovery. C, The percentage of Th1 cells in *Mycoplasma pneumoniae* pneumonia patients with P1-specific immunoglobulin E in the acute phase and after disease recovery. D, The percentage of Th2 cells in *Mycoplasma pneumoniae* pneumonia patients with P1-specific immunoglobulin E in the acute phase and after disease recovery.

continuous culture; the culture medium was changed every day, and the supernatants were collected for detection. Interleukin-4 and IL-5 secretion reached the highest levels on the second day of P1 protein stimulation, whereas IFN- γ secretion decreased (Fig 7).

Discussion

One of our previous studies also found that 61.76% of 680 MPP patients had elevated serum total IgE levels (>100 IU/mL), whereas only 20.0% of 650 patients with pneumonia caused by other factors had increased IgE levels.¹¹ Therefore, MP is not only a source of infection but also an allergen for some people. To clarify the allergic components of MP cells, SDS-PAGE was performed using total bacterial protein samples obtained by MP cleavage; the protein bands then were transferred to PVDF membranes and probed with mixed serum from MPP patients as a primary antibody, followed by the addition of anti-human IgE antibody as a secondary antibody for testing. The 170-kDa protein bands from all the MP strains reacted with the mixed serum from patients with MP infection. The protein was identified as the P1 protein of MP by mass spectrometry. In addition, western blotting showed that these bands were able to bind to P1 protein antibody, confirming the identities of these proteins as the P1 protein of MP.

To determine the proportion of P1-specific IgE produced in MPP patients, recombinant P1 protein was expressed in bacteria, and the purified P1 protein was then used as an allergen to detect P1-specific IgE in MPP patient serum by western blotting. The P1-specific IgE was found in the serum of 48 of 520 MPP patients, and

the positive rate was 9.2%, which showed that the P1 protein of MP was an allergen in some people. Other studies have also suggested that MP infection can increase the risk of asthma attacks,⁷ and many studies have argued that possible causes include inflammation caused by allergies that make the body more susceptible to MP¹² and the combination of allergy-related genetic background and MP infection exacerbating asthma attacks.¹³ However, our results show that the positive rate of P1-specific IgE was 18.2% in MPP patients without allergic history, which was significantly higher than the rate in MPP patients with an allergic history (4.7%). Similarly, the positive rate of P1-specific IgE in MPP patients without a family history of allergy-related diseases was 10.8%, whereas the positive rate of P1-specific IgE in the 76 MPP patients with a family history of allergy-related diseases was 0%. Therefore, compared with patients with allergies and allergy-related genetic backgrounds, healthy individuals infected with MP are more likely to experience asthma and other allergic diseases. This conclusion is consistent with reports that MP infection can cause asthma in people with no allergy-related diseases⁷ but is inconsistent with the aforementioned prevailing academic view that the combination of allergy-related genetic background and MP infection exacerbates asthma attacks. A recent big-data analysis by Jun-Jun Yeh also showed that the risk of asthma attacks in MPP patients without atopy syndrome (aHR, 3.57; 95% confidence interval, 2.52–5.05) was greater than that in those with atopy syndrome (aHR, 3.46; 95% confidence interval, 2.65–4.53).⁷ The causal link between MP infection and asthma remains controversial. Several researchers believe that patients who suffer from asthma are more susceptible to MP, their

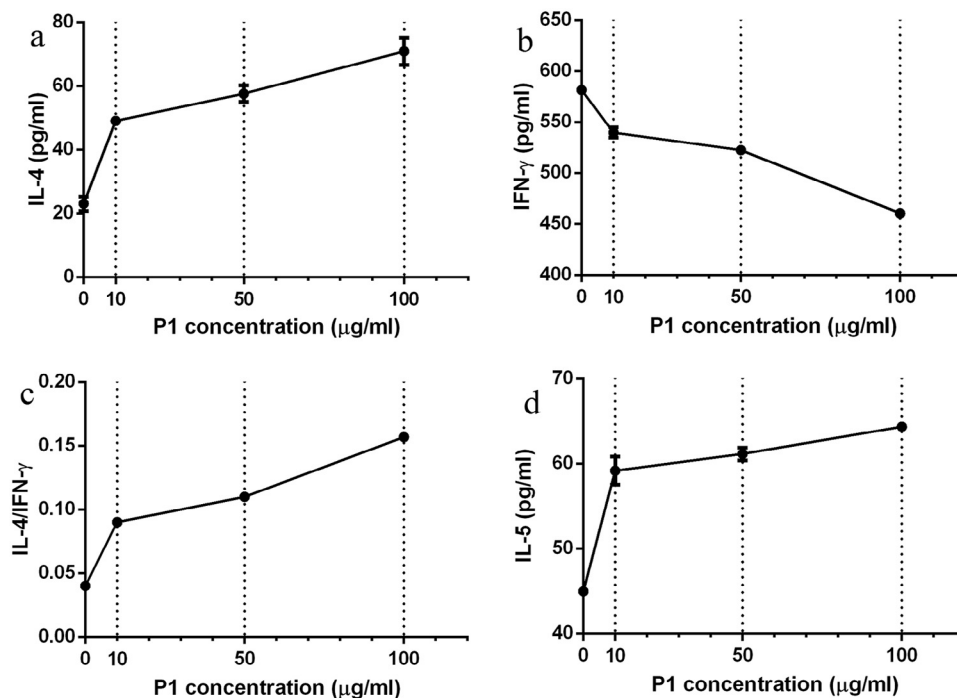


Figure 6. Interleukin-4, interferon-gamma, and interleukin-5 levels after stimulation with different concentrations of P1 protein. Peripheral blood mononuclear cells from the peripheral blood of healthy donors were collected for culture and then stimulated with different concentrations of P1 protein (0 µg/mL, 10 µg/mL, 50 µg/mL, and 100 µg/mL). Interleukin-4 and interleukin-5 levels increased with increasing P1 protein concentrations, whereas interferon-gamma levels decreased with increasing P1 protein concentrations. The results were described by mean and standard error of the mean. A, Interleukin-4. B, Interferon-gamma. C, Interleukin-4/interferon-gamma. D, Interleukin-5.

evidence coming from observations that asthma patients have a high carrier rate of MP.^{12–14} However, data from the current study showed that MP infection was the cause of asthma rather than an outcome and was an independent risk factor for asthma attacks. This con-

clusion is also supported by many studies.^{15–18} Biscardi et al¹⁹ reported that 50% of children experiencing their first asthma attack had an onset during the course of MP infection. Mok et al²⁰ performed a two-and-a-half-year follow-up study of children with acute

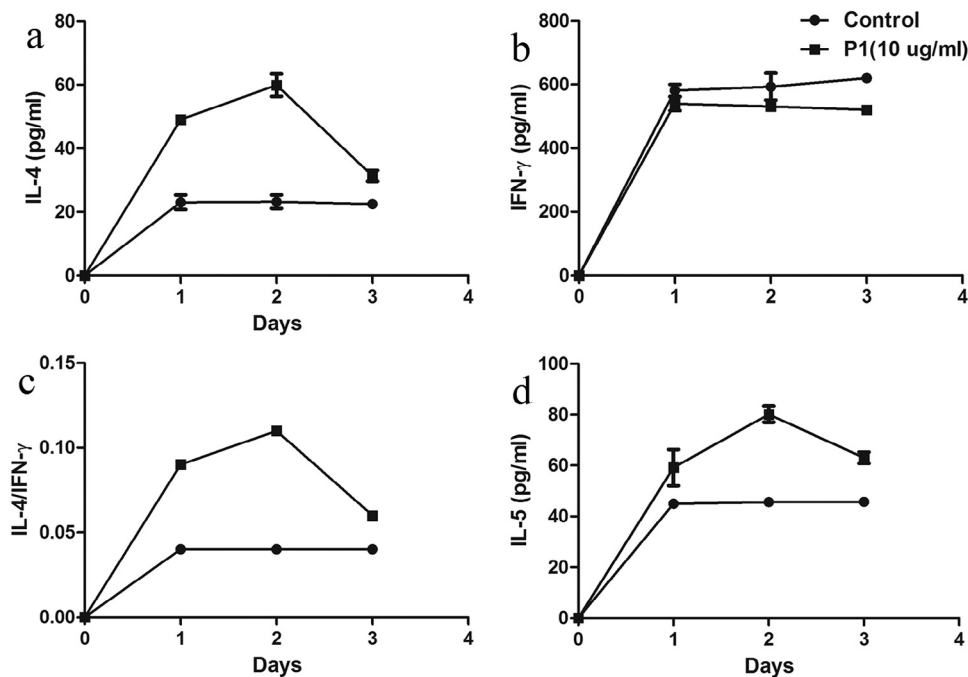


Figure 7. Interleukin-4, interferon-gamma, and interleukin-5 levels after P1 protein was added to peripheral blood mononuclear cells for 3 days of continuous culture. A total of 10 µg/mL P1 protein was added to peripheral blood mononuclear cells for 3 days of continuous culture. The culture medium was changed every day to collect the supernatants for analysis. Interleukin-4 and interleukin-5 secretion peaked on the second day of P1 protein stimulation, whereas interferon-gamma secretion was relatively steady. The results were described by mean and standard error of the mean. A, Interleukin-4. B, Interferon-gamma. C, Interleukin-4/interferon-gamma. D, Interleukin-5.

MPP and found that 12% of them subsequently experienced their first asthma attack. These clinical observations and studies have also suggested that MP infection is involved in the initiation of asthma. However, because of the limited number of specimens in this study, the reliability of this conclusion remains to be validated in large-sample, multicenter follow-up studies.

To eliminate the influence of the history of allergy and a family history of allergy-related diseases on this study, of the 48 P1-specific IgE-positive MPP patients, 32 patients with no history of allergies or family history of allergy-related diseases were enrolled in a cohort study along with 32 age- and sex-matched MPP patients without P1-specific IgE and no history of allergies and family history of allergy-related diseases as controls. Our study shows that the incidence of wheezing and the length of hospital stays of P1-specific IgE-positive MPP patients were significantly higher than those of P1-specific IgE-negative MPP patients; the forced expiratory volume in 1 second expressed as a percentage of the forced vital capacity of P1-specific IgE-positive MPP patients was also significantly lower than that of P1-specific IgE-negative MPP patients. Sabato et al.²¹ also found that the incidence of wheezing in MP children in the acute phase of lower respiratory tract infection was as high as 40%. Esposito et al.²² reported that, among non-antibiotic-treated children, MP-positive children with an acute episode of wheezing showed a recurrence of wheezing of 69.2%, significantly higher than that of MP-negative children (30.6%). These studies suggest that MP is an important pathogen that causes acute wheezing, especially for repeated wheezing in children, and are consistent with our findings. These findings provide a theoretical basis for novel clinical treatment strategies for MP infection, especially for refractory MP infection.

To further study the causes of P1-specific IgE production in some MPP patients, the levels of cytokines and T cells were examined in these patients. Compared with P1-specific IgE-negative MPP patients, serum levels of IL-4 in P1-specific IgE-positive MPP patients were increased, whereas serum IFN- γ levels were lower, leading to a remarkable increase in the IL-4/IFN- γ ratio; serum total IgE levels also increased significantly. Therefore, the increase in the IL-4/IFN- γ ratio might lead to an imbalance in Th1 and Th2 cells in these patients,²³ causing overactivation of Th2 cells and promoting the synthesis of P1-specific IgE; the P1-specific IgE may trigger allergic diseases such as asthma.^{10,24} To verify this mechanism, the percentages of Th1 and Th2 cells among peripheral blood CD4 + T cells from 6 P1-specific IgE-positive MPP patients were also analyzed, both in the acute phase and 3 months after recovery. The percentage of Th1 cells among CD4 + T cells was significantly increased after recovery from MPP, whereas the percentage of Th2 cells among CD4 + T cells was significantly reduced. Furthermore, to verify whether P1 protein stimulates the production of Th2 cytokines (such as IL-4), PBMCs from the peripheral blood of healthy donors were collected for culture and then stimulated with different concentrations of P1 protein. The IL-4 and IL-5 levels both increased with increasing P1 protein concentrations, whereas IFN- γ levels decreased; IL-4 and IL-5 secretion peaked on the second day of P1 protein stimulation. Interleukin-5 can promote the growth and differentiation of eosinophils and has been identified as the most important cytokine that causes wheezing in acute MP infection.^{19,25} Therefore, we believe that IL-5 also plays a very important role in this process. From these experimental data, we conclude that MP infection leads to excessive secretion of Th2 cytokines (IL-4) and overdifferentiation of Th0 cells into Th2 cells, resulting in a Th1 and Th2 cell imbalance.^{26,27} Excessive secretion of Th2 cytokines from Th2 cells can cause antibody class switching to produce P1-specific IgE. The MP-induced P1-specific IgE can mediate type 1 hypersensitivity and is a potential risk factor for the onset of bronchial asthma.

In summary, several patients were also allergic to the P1 protein of MP, thereby inducing the production of P1-specific IgE, which may mediate the occurrence and development of asthma.

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