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Original Article

Altered cytokine levels in bronchoalveolar lavage fluids from patients with *mycoplasma pneumonia* infection

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Abstract: Many reports have demonstrated that *Mycoplasma pneumonia* (*M. pneumoniae*) infection may cause the release of multiple cytokines into sputum or serum samples. However, studies focusing on bronchoalveolar lavage fluid (BALF) samples are scarce. The aim of this study was to identify the cytokines associated with *M. pneumoniae* infection, and explore the possible factors affecting their secretion during acute lower respiratory tract infection. 60 children with confirmed *M. pneumoniae* infection complicated with atelectasis, according to chest X-ray or CT scan data, and 20 children with confirmed foreign bodies in the airways (control group) were enrolled. Bronchoalveolar lavage (BAL) samples were obtained by flexible bronchoscopy, and assessed for the presence of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. Interestingly, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, and IFN-γ amounts were increased in *M. pneumonia* infected children compared with the control group. Multiple univariate analyses of variance and multiple linear regressions showed that fever during bronchoscopy markedly affected IL-1β, IL-4, IL-10 and IFN-γ concentrations. In conclusion, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10 and IFN-γ levels were increased in BALF from patients with *M. pneumonia*. Increased levels of secreted IL-1β, IL-4, IL-10 and IFN-γ were associated with fever in these patients.

Keywords: *Mycoplasma pneumonia*, bronchoalveolar lavage, children, cytokines, fever

Introduction

Mycoplasma pneumonia (*M. pneumoniae*) is one of the most common pathogens that cause lower respiratory tract infections, especially in preschool children. *M. pneumoniae* infection is a self-limited disease, but its symptoms vary in different age groups [1]. Older children are generally more severely affected than younger ones, in contrast to other respiratory pathogens like respiratory syncytial virus (RSV) and *Streptococcus pneumonia* [2]. Although airway damage plays an important role in *M. pneumonia* induced inflammation [3], an inappropriate immune response also accounts for pneumonia pathogenesis [1, 4].

Cytokines are intracellular signaling proteins mainly produced by immuno-competent cells. Their primary function is the regulation and coordination of immune responses [5]. Cytokine release depends on special mechanisms induced by the microorganism itself [6, 7] or common reactions like fever [8], during infection. The former provide some insights regarding the pathogen, while the latter suggest which reactions should be taken into consideration during investigations aiming to assess how cytokine changes may help understand the pathogenesis of *M. pneumoniae* infection. Although multiple studies have reported IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, and IFN-γ [5, 7, 9-11] changes during *M. pneumoniae* infection, consistent data were not obtained [8, 12, 13].

Fever is the most common reaction to infection. It can be induced by a multitude of substances administered peripherally, ranging from inorganic to organic compounds, or microbial and mammalian proteins; the existence of a common endogenous fever mediator has been postulated [5]. IL-1 receptor antagonist (IL-1ra), IL-1β, IL-6, and IL-10 are considered endogenous pyrogens, while IL-1ra and IL-10 have antipyretic effects [5]. In addition, fever limits the production of the fever-inducing IL-1β, and influences the adaptive immune response, favoring Th2 cytokine production [8]. Therefore, fever is

an important reaction that should be taken into consideration in cytokine research in humans or animal models.

Glucocorticoids (GCs) are agents that down-regulate the cell-mediated immune response, which may be reflected by cytokine levels [6]. For many severe clinical *M. pneumoniae pneumonia* cases that cause excessive immune response, GCs are effective in reducing the immune response and avoiding complications [14, 15]. Like fever and cytokines, GCs and cytokines interact with each other. GCs down-regulate pro-inflammatory cytokines while the latter limit GC effects [16]. Therefore, treatment with GCs should also be taken into account as it may affect cytokine secretion.

Refractory *M. pneumoniae pneumonia* (RMPP) is a severe pneumonia characterizing cases with clinical and radiological deterioration despite appropriate antibiotic therapy for 7 days or more [17]. Its exact mechanism is unclear and overreaction of the immune response may account for this ailment, as macrolides combined with corticosteroids are more effective than macrolides alone for treatment [17, 18]. Some cytokines [19] were even considered useful predictors of refractory or severe *Mycoplasma pneumoniae* pneumonia.

Fiberoptic bronchoscopy (FOB) is an important technology for the examination and treatment of lower respiratory tract disorders in children. As the lower respiratory tract is believed to be sterile and close to lesions, detection of pathogens and cytokines is more reliable in BALF than sputum, cerebrospinal fluid, or serum. Multiple studies have reported varying cytokine secretion levels in *M. pneumoniae* infection, while assessing serum or cerebrospinal fluid samples [9]. However, such studies focusing on bronchoalveolar lavage fluid (BALF) specimens are scarce, although BALF is close to lesions, since FOB is invasive and only applied to treat atelectasis or foreign bodies.

Although animal models are helpful in reducing sampling error, many symptoms and treatment outcomes will not be reflected in them, which contribute to disparate conclusions among studies. Therefore, appropriately defined conditions are meaningful in this investigation. We selected BALF as test sample close to lesions, in patients complicated with atelectasis, an indication for bronchoalveolar lavage (BAL). We also took into consideration the three most important conditions (fever, glucocorticoids treatment, and RMPP), according to previous reports.

Methods

Study design

Between February 2012 and February 2013, children were eligible for enrollment if diagnosed with *M. pneumoniae* pneumonia, complicated with atelectasis (X-ray or CT scan data), or confirmed presence of foreign bodies. Finally, 60 patients with clinically confirmed *M. pneumoniae pneumonia* complicated with atelectasis and 20 with foreign bodies were recruited at Children's Hospital, Zhejiang University School of Medicine, Hangzhou, China. No patients had severe underlying diseases such as bronchial asthma, chronic bronchitis, and nasosinusitis, which might affect the clinical course. Nasopharyngeal swabs and blood samples were obtained from all patients upon admission. Then, the swabs were sent for sputum culture and *M. pneumonia* detection by real time PCR. The presence of respiratory syncytial virus (RSV), human metapneumovirus (HMPV), influenza virus types A and B, parainfluenza virus types 1-3, and adenovirus was assessed by direct immunofluorescence. Serum samples were sent for blood culture (BioMerieux, France). All 80 patients underwent BAL 3 days after admission. IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, and IFN-γ levels in BALF samples were determined by sandwich enzyme linked immunosorbent assay (ELISA) with specific kits (Diaclone, Besancon Cedex, France) according to the manufacturer's instructions. The limits of detection were 6.5, 7.0, 0.7, 2.0, 29.0, 5.0, 20.0, 8.0, and 5.0 pg/mL for IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, and IFN-γ, respectively. BALF samples were also sent for the assessment of *M. pneumoniae* load. Clinical parameters (age, gender, and clinical manifestations such as fever state during BAL, GC treatment before BAL, and fever duration), laboratory examinations (routine blood test, C-reaction protein levels, lactate dehydrogenase [LDH] amounts, and type B ultrasound for pleural effusion if needed), other complications (rash, cephalomeningitis, and thrombotic thrombocytopenic purpura), and prognosis data (relief durations of fever, cough, and chest ra-

Table 1. Demographic data and cytokines of M. pneumoniae pneumonia and foreign body patients[a]

Variables	M. pneumoniae Pneumonia (n=60)	Foreign Body (n=20)	*p*-value
Age, years	5.23±2.42	1.79±0.54	<0.001
Male	36 (60%)	16 (80%)	0.175
Length of stay, days	9 (7-12)	3 (2-4.75)	<0.001
IL-1β (pg/ml)	117.66 (27.79-584.79)	7.05 (4.96-10.80)	<0.001
IL-2 (pg/ml)	9.54±0.86	9.19±0.62	0.09
IL-4 (pg/ml)	6.15 (0.19-12.96)	0.03 (0-0.17)	<0.001
IL-6 (pg/ml)	25.84 (6.40-72.73)	2.00 (0.49-8.21)	<0.001
IL-8 (pg/ml)	1614.22±1169.45	125.34±172.81	<0.001
IL-10 (pg/ml)	7.23 (3.49-21.86)	3.10 (1.73-4.08)	<0.001
IL-12 (pg/ml)	32.74 (25.73-52.78)	26.23 (21.87-33.50)	0.033
TNF-α (pg/ml)	12.23 (0.67-73.18)	11.60 (1.00-32.87)	0.379
IFN-γ (pg/ml)	11.11 (3.61-103.31)	2.12 (1.60-4.50)	<0.001

[a]Results are presented as the mean ± standard deviation, number (percentage), or median (25th-75th percentile).

diograph abnormalities) were collected. Informed consent was obtained from the parents of all patients, and the study protocol was approved by the Ethics Committee of Zhejiang University School of Medicine.

Criteria for the determination of M. pneumoniae infection, fever, GC treatment, and foreign body presence

M. pneumoniae infection was characterized by: (i) detection of *M. pneumoniae* DNA in the bronchoalveolar lavage fluid (BALF) sample by PCR; (ii) positive serological results, that is, IgG titer >1:320 and IgM titer >1.1 in the same serum sample, detected with a commercially available ELISA kit (EUROIMMUN, Germany), according to the manufacturer's instructions. A febrile state was defined as body temperature exceeding 37.5°C [20]. GC treatment was considered after patients had received three-day methylprednisolone at 2 mg/kg/d intravenously within 7 days of BAL [15]. RMPP indicated cases with clinical and radiological deterioration despite appropriate antibiotic therapy for 7 days or more [17]. Fever duration was recorded from the first febrile day after onset. Foreign bodies were identified based on clinical history, endoscopic inspection, and, occasionally, histopathological examination [21].

Statistical analysis

Independent-samples t test and one-way analysis of variance (ANOVA) with post hoc LSD were used to compare various groups, and outcomes were presented as mean ± standard deviation (SD) for normally distributed data. For non-normally distributed data, the Mann-Whitney U test was used for comparison, and outcomes were presented as median with interquartile range (25^{th}-75^{th}). Multiple univariate ANOVA and multiple linear regressions were used to analyze fever, GC treatment, and RMPP for their correlations with cytokines. P<0.05 was considered statistically significant.

Results

Demographic data and cytokine levels in patients with M. pneumoniae pneumonia and foreign bodies

Of the 80 children with *M. pneumoniae* pneumonia complicated with atelectasis or foreign bodies, 52 (65.0%) were male. Mean patient age was 4.37 years, ranging from 0.87 to 11.5 years. *M. pneumoniae* infection was confirmed by serology and real-time PCR in serum and BALF samples. No child required admission to the intensive care unit during hospital stay, and all patients were treated with macrolides beginning at the outpatient clinic or after admission.

Regarding the demographics and cytokine levels in patients with *M. pneumoniae* pneumonia and foreign bodies, children with *M. pneumoniae* pneumonia were older than those with foreign bodies but no difference in gender distribution was found between the two groups. Regardless of conditions, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10 and IFN-γ levels were higher in the *M. pneumoniae* pneumonia group compared with patients with foreign bodies (**Table 1**).

Fever, RMPP, and GC treatment in M. pneumoniae pneumonia patients

Multi-factor analysis of variance of fever, RMPP, and GC treatment showed that only febrile state during FOB was correlated with IL-1β, IL-4, IL-10, and IFN-γ. RMPP and GC treatment or combination between the three parameters did not show significant correlations (**Table 2**).

Table 2. Multiple factor analysis of variance of fever, GCs treatment and RMPP[a]

Cytokines/Factors	Fever	RMPP	GCs	Fever+RMPP	Fever+GCs	RMPP+GCs
IL-1β (pg/ml)	0.002	0.812	0.741	0.841	0.751	0.680
IL-2 (pg/ml)	0.118	0.323	0.817	0.225	0.232	0.064
IL-4 (pg/ml)	0.005	0.614	0.946	0.852	0.160	0.622
IL-6 (pg/ml)	0.434	0.577	0.829	0.914	0.890	0.538
IL-8 (pg/ml)	0.107	0.589	0.327	0.210	0.974	0.191
IL-10 (pg/ml)	0.016	0.613	0.362	0.161	0.142	0.968
IL-12 (pg/ml)	0.991	0.843	0.993	0.768	0.983	0.301
TNF-α (pg/ml)	0.244	0.083	0.370	0.260	0.804	0.105
IFN-γ (pg/ml)	0.003	0.728	0.873	0.754	0.761	0.910

[a]Results are presented as the *p*-value of each factor and combination in univariate multiple factor analysis of variance.

Table 3. Multiple linear regressions of fever, RMPP and GCs treatment[a]

Cytokines/Factors	R	Regression Sig	Fever	RMPP	GCs
IL-1β (pg/ml)	0.452	0.005	0.001	0.908	0.908
IL-2 (pg/ml)	0.351	0.059	0.018	0.834	0.803
IL-4 (pg/ml)	0.419	0.012	0.002	0.625	0.599
IL-6 (pg/ml)	0.128	0.816	0.454	0.686	0.631
IL-8 (pg/ml)	0.191	0.550	0.216	0.987	0.674
IL-10 (pg/ml)	0.318	0.110	0.034	0.788	0.283
IL-12 (pg/ml)	0.088	0.931	0.815	0.534	0.716
TNF-α (pg/ml)	0.161	0.687	0.486	0.235	0.755
IFN-γ (pg/ml)	0.479	0.002	0.001	0.740	0.745

[a]Results are presented as the *p*-value of the coefficient of each factor in multiple linear regressions.

Multiple linear regression analysis of fever, RMPP, and GC treatment showed that only febrile state during FOB was significantly correlated with IL-1β, IL-4, and IFN-γ levels. IL-2 and IL-10 were related to fever but the associations were not significant (P=0.059 and P=0.110, respectively). RMPP and GC treatment showed no significant associations with cytokine levels (**Table 3**).

Clinical profiles and cytokine levels in various groups

Clinical profiles and cytokine levels in patients with fever, no-fever, and foreign bodies: A total of 60 patients with *M. pneumoniae* pneumonia were subdivided into fever and no-fever groups. The fever group showed longer fever duration, fever duration after macrolides, and higher C-reactive protein levels. As for cytokine amounts, the fever group showed significantly higher IL-2, IL-4, IL-10, and IFN-γ levels; IL-1β was also higher but did not reach significance level compared with the no-fever group. In turn, the no-fever group showed higher levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, and IFN-γ, compared with the foreign body group (**Table 4**).

Clinical profiles and cytokine levels in the RMPP, no-RMPP and foreign body groups: A total of 60 patients with *M. pneumoniae* pneumonia were divided into RMPP and no-RMPP groups. Interestingly, the RMPP group showed longer fever duration and fever duration after macrolides. Regarding cytokines, RMPP patients showed no statistically significant difference compared with the no-RMPP group except for IFN-γ (**Table 5**).

Clinical profiles and cytokines in the GCs, no-GCs, and foreign body groups: The 60 patients with *M. pneumoniae* pneumonia were subdivided into GC and no-GC treatment groups. For clinical profiles, the GC group showed longer duration of fever and fever duration after macrolides. As for cytokines, the GC and no-GC groups showed similar values (**Table 6**).

Discussion

The present study aimed to analyze the potential effects of three conditions (fever, GC treatment, and RMPP) on cytokine secretion into BALF according to previous reports, assessing 9 cytokines in patients with *M. pneumoniae* pneumonia and foreign bodies, respectively. Interestingly, we found that fever was more powerful than the other two conditions, especially in altering IL-1β, IL-4, IL-10, and IFN-γ secretion.

Table 4. Clinical profiles and cytokines of fever, no fever and foreign body groups[a]

Variables	Fever (n=30)	No Fever (n=30)	Foreign Body (n=20)	*p*-value (Fever vs No Fever)	*p*-value (Fever vs Foreign Body)	*p*-value (No Fever vs Foreign Body)
Age, years	5.37±2.35	5.09±2.51	1.79±0.54	0.607	<0.001	<0.001
Male	14 (46.7%)	22 (73.3%)	16 (80%)	0.064	0.022	0.740
Length of stay, days	10.93±4.74	9.57±4.59	3 (2-4.75)	0.213	<0.001	<0.001
Fever duration, days	14.37±5.72	10.37±4.69		0.004		
Fever duration after macrolides, days	5.72±5.52	4.00±3.98		0.006		
Duration of cough, days	7.00 (5.75-10.00)	8.00 (4.75-10.00)		0.687		
Peripheral leukocyte count, $\times 10^9$/l	8.55±4.03	8.82±4.52		0.810		
C-reactive protein, mg/l	61.79±45.12	22.48±22.75		<0.001		
Lactate dehydrogenase, IU/l	542.47±188.68	551.27±300.02		0.892		
IL-1β (pg/ml)	484.36 (105.62-843.56)	41.03 (15.94-201.31)	7.05 (4.96-10.80)	0.081	<0.001	<0.001
IL-2 (pg/ml)	9.84±0.88	9.25±0.74	9.19±0.62	0.004	0.004	0.784
IL-4 (pg/ml)	11.72 (3.45-16.36)	0.43 (0.04,7.23)	0.03 (0-0.17)	<0.001	0.040	0.040
IL-6 (pg/ml)	51.86±63.40	42.70±43.91	2.00 (0.49-8.21)	0.460	0.002	0.011
IL-8 (pg/ml)	1825.84±1234.68	1402.61±1079.50	125.34±172.81	0.109	<0.001	<0.001
IL-10 (pg/ml)	9.32 (4.57-31.73)	5.12 (2.88-10.51)	3.10 (1.73-4.08)	0.011	0.001	0.229
IL-12 (pg/ml)	32.24 (26.48-42.99)	35.20 (23.94-61.22)	26.23 (21.87-33.50)	0.969	0.063	0.069
TNFα (pg/ml)	12.23 (0.92-79.76)	17.40 (0.44-84.26)	11.60 (1.00-32.87)	0.809	0.073	0.114
IFN-γ (pg/ml)	79.06 (12.23-247.45)	4.43 (1.88-10.26)	2.12 (1.60-4.50)	<0.001	<0.001	0.686

[a]Results are presented as the mean ± standard deviation, number (percentage), or median (25th-75th percentile).

Table 5. Clinical profiles and cytokines of RMPP, no RMPP and foreign body groups[a]

Variables	RMPP (n=38)	NRMPP (n=22)	Foreign Body (n=20)	*p*-value (RMPP vs NRMPP)	*p*-value (RMPP vs Foreign Body)	*p*-value (NRMPP vs Foreign Body)
Age, years	5.18±2.11	5.32±2.94	1.79±0.54	0.806	<0.001	<0.001
Male	22 (57.9%)	14 (63.6%)	16 (80%)	0.787	0.146	0.315
Length of stay, days	11.13±4.74	8.73±4.24	3 (2-4.75)	0.033	<0.001	<0.001
Fever duration, days	14.82±5.38	8.14±2.53		<0.001		
Fever duration after macrolides, days	6.00 (4.00-8.00)	3.00 (1.00-5.00)		0.001		
Duration of cough, days	8.00 (6.75-10.00)	6.5 (4.00-10.00)		0.687		
Peripheral leukocyte count, $\times 10^9$/l	8.36±3.62	9.25±5.21		0.437		
C-reactive protein, mg/l	49.03±44.96	30.23±28.88		0.054		
Lactate dehydrogenase, IU/l	588.79±246.57	474.45±240.29		0.086		
IL-1β (pg/ml)	205.80 (38.77-655.96)	45.75 (18.99-458.66)	7.05 (4.96-10.80)	0.118	<0.001	0.023
IL-2 (pg/ml)	9.66±0.92	9.35±0.72	9.19±0.62	0.151	0.036	0.519
IL-4 (pg/ml)	8.92±9.14	7.09±8.96	0.03 (0-0.17)	0.390	<0.001	0.006
IL-6 (pg/ml)	24.54 (7.77-64.05)	35.94 (5.03-103.19)	2.00 (0.49-8.21)	0.797	0.004	0.005
IL-8 (pg/ml)	1693.86±1199.24	1476.67±1130.10	125.34±172.81	0.431	<0.001	<0.001
IL-10 (pg/ml)	7.86 (3.87-23.99)	5.06 (2.79-17.29)	3.10 (1.73-4.08)	0.301	0.007	0.119
IL-12 (pg/ml)	32.74 (25.73-53.44)	35.20 (23.94-53.59)	26.23 (21.87-33.50)	0.561	0.034	0.157
TNFα (pg/ml)	9.73 (0.67-71.99)	23.42 (0.44-133.97)	11.60 (1.00-32.87)	0.266	0.166	0.029
IFN-γ (pg/ml)	18.64(4.92-137.75)	5.96 (1.75-25.93)	2.12 (1.60-4.50)	0.031	0.002	0.353

[a]Results are presented as the mean ± standard deviation, number (percentage), or median (25th-75th percentile).

Table 6. Clinical profiles and cytokines of GCs, no GCs and foreign body groups[a]

Variables	GCs (n=22)	No GCs (n=38)	Foreign Body (n=20)	*p*-value (GCs vs No GCs)	*p*-value (GCs vs Foreign Body)	*p*-value (No GCs vs Foreign Body)
Age, years	4.62±2.25	5.58±2.47	1.79±0.54	0.094	<0.001	<0.001
Male	11 (50.0%)	25 (65.8%)	16 (80%)	0.280	0.058	0.037
Length of stay, days	11.68±4.97	9.42±4.35	3 (2-4.75)	0.045	<0.001	<0.001
Fever duration, days	14.73±7.23	11.00±3.81		0.033		
Fever duration after macrolides, days	6.27±5.64	5.55±4.81		0.576		
Duration of cough, days	9.50 (6.75-10.00)	7.00 (5.00-10.00)		0.175		
Peripheral leukocyte count, $\times 10^9$/l	9.96±5.46	7.94±3.21		0.124		
C-reactive protein, mg/l	42.49±40.79	41.93±41.02		0.960		
Lactate dehydrogenase, IU/l	562.50±215.92	537.82±267.95		0.714		
IL-1β (pg/ml)	107.52 (19.65-606.56)	117.66 (27.96-565.44)	7.05 (4.96-10.80)	0.925	0.002	0.001
IL-2 (pg/ml)	9.59±0.96	9.52±0.81	9.19±0.62	0.753	0.114	0.142
IL-4 (pg/ml)	7.29±7.97	8.80±9.67	0.03 (0-0.17)	0.478	0.005	<0.001
IL-6 (pg/ml)	41.66±45.35	50.53±59.15	2.00 (0.49-8.21)	0.491	0.021	0.001
IL-8 (pg/ml)	1702.13±1183.83	1563.33±1173.94	125.34±172.81	0.651	<0.001	<0.001
IL-10 (pg/ml)	8.30 (3.96-27.99)	6.85 (3.37-21.63)	3.10 (1.73-4.08)	0.245	0.006	0.050
IL-12 (pg/ml)	32.24 (23.42-53.44)	33.25 (2.83-53.59)	26.23 (21.87-33.50)	0.804	0.112	0.045
TNFα (pg/ml)	5.22 (0.41-105.31)	23.25 (2.29-70.86)	11.60 (1.00-32.87)	0.978	0.111	0.080
IFN-γ (pg/ml)	13.10 (4.40-129.06)	9.89 (2.94-92.34)	2.12 (1.60-4.50)	0.666	0.023	0.033

[a]Results are presented as the mean ± standard deviation, number (percentage), or median (25th-75th percentile).

Fever is the multiphasic response of elevation and decline of the body core temperature, regulated by central thermoregulatory mechanisms localized in the preoptic area of the hypothalamus. The proinflammatory cytokines IL-1, IL-6, and TNF-α as well as anti-inflammatory cytokines interleukin 1 receptor antagonist (IL-1ra) and IL-10 have been widely investigated for their pyrogenic or antipyretic activities [5]. IL-1β is a member of the IL-1 family, and a prototypic multifunctional cytokine present in almost every cell type [7]. IL-1β and IL-10 levels increase in the febrile state, and account for their pyrogenic effects. IL-4 and IFN-γ are representative factors for T helper type 2 (TH2) and T helper type 1 (TH1) immune responses, respectively. High levels of IL-4 and IFN-γ indicate intense inflammatory and anti-inflammatory reactions, which may be reflected by persistent fever, as IL-4 and IFN-γ showed higher levels in the fever group compared with the no-fever group. IL-6, a representative proinflammatory cytokine and major endogenous pyrogen [22], did not significantly increase in the febrile state, as shown above. A possible explanation is that fever limits the production of fever-inducing proinflammatory cytokines [8]. Increased IL-1β but not elevated IL-6 levels during fever may be attributed to different cytokine responses at various phases of fever, and the temperature level might be involved as well.

GCs are widely used in multiple severe infectious pneumonia, shortening fever duration, reducing complications, and down-regulating the cell-mediated immune response [15, 23, 24]. In this study, however, GC treatment did not alter cytokine profiles in patients with *M. pneumoniae* pneumonia. Although Remmelts HH [6] and Tamura A [17] reported an obvious improvement in clinical outcome after administration of dexamethasone or methylprednisolone, their use at high dosage has not been proven safe enough, and our routine dose for severe infection is only 1-2 mg/kg/day. Low dosage GC treatment has distinct effects on different patients [18], and some may need high dosage [25]. Therefore, individual differences in both GC dosage and sensitivity may be important [26].

RMPP is a severe state in *M. pneumoniae* infection, and Wang M and colleagues [27] reported that serum TNF-α and IFN-γ levels are higher in the RMPP group compared with non-refractory *M. pneumoniae* pneumonia (NRMPP) patients. Since fever is vital to cytokine secretion in BALF as mentioned above, it may also affect cytokine distribution in serum or other body fluids. Previous studies did not classify patients according to fever state, and most of them may have been carried out with fever persistence, as RMPP is usually defined by long fever duration. Unlike our outcome in BALF, different sample types and other unbalanced conditions like fever may affect the data.

We also found higher IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10 and IFN-γ levels in *M. pneumonia* infected children without fever compared with the 20 children confirmed with foreign bodies. These data indicate that *M. pneumoniae* itself and other unknown factors caused the cytokine changes observed in BALF samples. In addition, significantly higher IL-4/IFN-γ ratios (0.0853 [0.0291-0.5514]) were found in patients with *M. pneumoniae* pneumonia compared with the foreign body group (0.0057 [0.0000-0.0676]). The cytokine profiles described above suggest that *M. pneumoniae* infection causes an unbalanced Th1/Th2 immune response, which may contribute to allergic disorders, as reported by Koh YY and colleagues [28]. They may also help distinguish *M. pneumoniae* pneumonia from similar diseases such as pneumococcal pneumonia.

Age is an important parameter shown above to be significantly different between patients with *M. pneumoniae* pneumonia and the foreign body group. Foreign bodies and atelectasis are two major indications of FOB. Most foreign bodies are found in toddlers, while atelectasis caused by *M. pneumoniae* pneumonia often affects preschool children. However, there is not enough evidence to confirm the correlation between age and cytokine levels. We also divided the 20 foreign body group into two subgroups, including the young (10 youngest children) and old (10 oldest ones) subgroups, and no significant differences were found in cytokine levels between these two groups. These findings suggest that there is no correlation between age and cytokine levels in BALF.

A limitation of this study is that pneumonia complicated with atelectasis is not representative of *M. pneumoniae* infection as a whole.

However, it is a common manifestation, and required for BAL; in addition, it can be considered a severe *M. pneumoniae* pneumonia to some extent. The relatively small sample size also limited the experimental conditions to be considered.

In conclusion, fever affects the secretion into BALF of cytokines, especially IL-1β, IL-4, IL-10, and IFN-γ. *M. pneumoniae* pneumonia causes increased IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, and IFN-γ levels in BALF despite a febrile state. The exact mechanism needs to be further investigated.

Disclosure of conflict of interest

None.

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