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Clinical Significance and Correlation of CXCL8 and its mRNA in the Children with *Mycoplasmal pneumonia*

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ABSTRACT

The acute inflammation in bronchial and pulmonary interstitial are regarded as the main pathological injury of *Mycoplasma pneumoniae* pneumonia (MPP). However, the exact mechanism of CXCL8 and its mRNA in the children with Mycoplasmal pneumonia needs to be further clarified. The concentration of the CXCL8 in serum and the level of CXCL8 mRNA in PBMCs of forty-eight children were dynamically measured by ELISA and PCR. The ratio of lgcDNA/lgGAPDH was regarded as the extreme level of CXCL8 mRNA. The serum level of CXCL8 and expression of CXCL8 mRNA in PBMCs in MPP children were (298.917±51.860) pg/mL and (1.848±0.525) lgcDNA/lgGAPDH. There were significant differences between the trial groups and normal controls (P<0.05). Further observation showed that the levels of CXCL8 mRNA in peripheral blood of the children with severe illness were significantly higher than those in light cases (P<0.05). Intravenous infusion of Erythromycin was provided in the acute phase for seven to ten days, and followed by the use of sequential therapy of Azithromycin for about three to four weeks, the children's condition were gradually from acute stage to recovery stage. CXCL8 and its mRNA levels in peripheral blood of the sick children were all significantly decreased comparing with those in the acute stage (P<0.05). Further analysis showed that CXCL8 and its mRNA had a significant correlation in the acute phase, and it was related to the severity of the disease and the course of the disease. The correlation between CXCL8 and its mRNA was significantly decreased in the recovery phase. There was only a weak correlation between serum CXCL8 level and serum Anti-MP level in both acute and convalescent stages (r= -0.2917, P=0.0891; r=-0.2783, P=0.1055). The level of CXCL8 and its mRNA was increased in the peripheral blood of the sick children with mycoplasma pneumonia, and also correlated with the severity of the disease. The level of CXCL8 in peripheral blood was strongly correlated with its mRNA and weakly correlated with anti-MP level. The content of CXCL8 in serum of the sick children can be reduced by Azithromycin *via* the pathway of inhibiting the proliferation of MP.

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Authors' Contribution
JW designed the study, performed experimental work and analyzed the data. LS, XB and ZK collected and analyzed the clinical data. XL helped in laboratory examinations. JW, LS and XB wrote the article.

Key words
***Mycoplasma pneumoniae* (MP), *Mycoplasma pneumoniae* pneumonia (MPP), Peripheral blood mononuclear cells (PBMCs), CXCL8, mRNA, Azithromycin, Sequential therapy**

INTRODUCTION

Mycoplasma pneumoniae pneumonia (MPP), also known as primary atypical pneumonia, is caused by *Mycoplasma pneumoniae* (MP) and common in infants and children. The acute inflammation in bronchial and pulmonary interstitial are regarded as the main pathological injury. The data of new research have been verified that the infection rate of MP was increased to 12.3%, and quarterly positive rates of MP samples ranged from 1.5% to 27.3% (Dumke *et al*., 2015). The infection rate of MP in the infants and young children in our country is also gradually increased (Sun *et al*., 2015), and has the central easy recurrence and others. The exact mechanism needs to feature as follows, such as lower onset age, longer course, be further clarified. The present study has been shown that mycoplasma pneumonia is regarded not only as a pathogen, but also as one of the most important allergens, which can trigger the immune system of the hosts, and subsequently induce strong immune responses. The cellular immune might play an important regulatory role in pathological damages of pulmonary interstitial inflammation in the sick children. CXCL8, an important proinflammatory factor, is one of a vital member of the CXC chemokine subfamily and has made great contributions to the immune regulation of inflammatory responses (Wang *et al*., 2017). In order to explore the effect of CXCL8 and the immune response during the prognosis of MPP, the levels of CXCL8 and its mRNA in the peripheral blood of some typical cases were detected. The detailed results

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would be as follows.

MATERIALS AND METHODS

Clinical data

Forty-eight typical cases (male 28, female 20) with MPP were recruited from october 2014 to march 2016 in the Maternal and Child Health-Care Hospital of Huainan. The age of the victims ranged from four months to ten years. Among them, the age from three to twelve months in 6 cases, the age from one to three years in 18 cases and from three to ten years in 24 cases. The diagnosis of MPP was based on the criteria set out by the guidelines for the management of children with community acquired pneumonia (2013 revision) (Chinese medical science branch of science and society). All the sick children were selected to meet the following diagnostic criteria: the positive of anti-MP-IgM in serum, the negative of sputum culture, without immune system disease, without long-term use of corticosteroids. According to the children's condition, the cases were divided into light disease (36 cases) and severe cases (12 cases). According to the course of the disease, the patients were divided into acute stage and recovery stage. The thirty children (male 15, female 15, aged from five months to fifteen years) with normal physical examination in the same period were considered as normal controls. All of the patients and their parents provided verbal permission for their data and records to be used in this study.

Reagents and instruments

The ELISA test kits used for the quantitative measurement of CXCL8 was purchased from DIACLONE, France. The fetal bovine serum was purchased from Bio Basic Inc, USA. Ficoll-Hypaque (1.077±0.001) was purchased from the Second Reagent Factory of Shanghai. Trizol reagent was purchased from Invitrogen Co., USA. RPMI 1640 complete culture medium was purchased from Sigma, USA. The extraction kits of the first strand of AMV cDNA were purchased from Shanghai Shenneng Gambling Co., China. Polymerase chain reaction (PCR) kits were purchased from Sangon Biotech (Shanghai) Co., China. The synthesis kits of script cDNA were purchased from Beijing Tiangeng biochemical technology Co., China. Light Cycler Fast Start DNA Master SYBR Green I reagent was purchased from Roche Co., Germany. Mycoplasma pneumoniae antibody diagnostic kits were purchased from Zhuhai Lizhu Reagent Co., Ltd. The automatic microplate washer (EXL-50X) and the automatic microplate-reader for ELISA (EXL-808) were all purchased from Bio-Tek Co., USA. The high-speed centrifuge (5415D) was purchased from Eppendorf® Co., Germany. The ultraviolet visible spectrophotometer (UV-5800PC) was purchased from Shanghai Element Analysis Instrument Co., China. The fluorescence microscope (Nikon E-400) was purchased from Nikon Co., Japan. Gradient PCR instrument (TP600) was purchased from TaKaRa Co., Japan. The iCyclez real-time quantitative PCR instrument was purchased from Bio-Rad Co., USA. Molecular Imager ChemiDoc™ XRS$^+$ analysis system was purchased from Bio-Rad Co., USA.

Collection of specimens

The total volume of 5 ml inguinal venous blood or peripheral venous blood were collected from the sick patients with MPP, and quickly stored in two heparin anticoagulant tubes and other two sterile Eppendorf test tubes, respectively. The former tubes were used for separation of the peripheral blood mononuclear cells (PBMCs) and extraction of the total RNA, and follow-up measurement of CXCL8 mRNA in the PBMCs. The latter tubes were used for measurement of free CXCL8 in serum. All samples were detected in our laboratory as soon as possible.

Detection of CXCL8 in serum

Solid phase sandwich ELISA method was used to detect in our studies. The standard curve was drawn by 1:2 dilution of the standard dilution solution provided by the reagent company. The groin venous blood or peripheral venous blood of the cases was collected in batches and then used to isolate the fresh serum. The control groups of CXCL8 markers with two blank pores, two negative pores two positive pores were made in each test. Every titer was measured twice by ELISA analyzer at 450 nm and the final average OD of titer was then calculated. The positive threshold was ≥2.1, which was the rate of average OD of sample to the average titer OD of negative control. The minimum detectable dose of human CXCL8 was usually less than the concentration of 19 pg/mL, and its detective value was range from 78 pg/mL to 5,000 pg/mL. The dilutions of the standard were taken as the abscissa and the OD data were taken as the ordinate in standard curves, so that the concentrations of CXCL8 in serum samples were calculated from these curves using the standard samples in the kits.

Detection of Mycoplasma pneumoniae *antibody*

The serum was routinely separated and determined by Passive Agglutination method. When the titer of *Mycoplasma pneumoniae* antibody with more than or equal to 1:80, it could be considered positive.

Extraction, isolation and purification of PBMCs

After total volume of 1 mL heparin anticoagulant

blood mixed with the equal volume Hank's liquid without Ca^{2+} and Mg^{2+}, and then layered the diluted blood over the surface of the isolation medium carefully. Conventionally centrifuge at 500 RCF for 35 min at 20~25°C. The blood should separate out into four distinct bands, such as plasma, PBMCs, isolation media, and the red blood cell pellet. Pipette the layer of PBMCs carefully and the place the solution into another clean centrifuge tube. Take the diluted the PBMCs solution to 5 mL with Hanks Balanced Salt Solutions (HBSS) without Ca^{2+} and Mg^{2+} and then invert the tubes several times to suspend the cells. The PBMCs solution was centrifuged at 350 RCF for 10 minutes so that large of PBMCs pellet should be present at the bottom of the tube. Remove the supernatant with a pipette carefully so that the pellet was not disturbed. Continued to centrifuge the tubes at 250 RCF for 5 min and removed the supernatant with pipette as soon as possible. PBMCs were resuspended in complete HBSS without Ca^{2+} and Mg^{2+} at a density calculated to yield a final concentration of (1~2) $\times 10^6$ cells per mL in each tube. The results had been shown that the yield samples and the viability of PBMCs was more than ninety-five percent. A small amount of cell suspension was randomly selected and taken by the blue stain, and the cellular activity was examined under the microscope after Trypan blue exclusion assay.

Extraction of total RNA from PBMCs, and identification of its concentration and purity

The concentration of PBMCs was diluted to (1~2) $\times 10^6$/mL with RPMI 1640 complete culture fluid and their total RNA was extracted by Trizol reagent. The total RNA 3 μL was mixed with bromophenol blue 1 μL, and taken in 1.2% TAE agarose gel (0.51 EB g/mL) electrophoresis for 25 min. Three fluorescent bands (28S, 18S, 5.8S) could be visible under the nucleic acid gel image analyzer and the density scanning camera was performed under the background of ultraviolet radiation. The density of 28S band was more than the twice of that of 18S band. The results indicated that the total RNA was good, which could be satisfied with the requirements of the following experiments (Fig. 1a). The total RNA template was diluted in the proportion 1 in 250 and the value of OD_{260}/OD_{280} was measured by ultraviolet spectrophotometry. When the value was ranged from 1.8 to 2, the total RNA purity was better.

The reverse transcription of total RNA

The total RNA was extracted from PBMCs of the sick children and health controls, which were all reversed transcription into cDNA under the help of random primers. The reaction system was as follows: no RNase water 2 μL, 5×RT buffer 4μL, dNTP Mix (10m mol/L) 2μL, RNase inhibitor (20U/μL) 1μL, Oligo (dT)18 (0.5μg/μL) 1μL, total RNA 8μL, AMV RT (10U/μL) 2μL, total volume 20μL. After 37°C for 60 min and 70°C for 10 min, and followed by instantaneous centrifuge for a few seconds. The cDNA was carefully packed and frozen in the refrigerator at -20°C.

Detection of CXCL8 mRNA

According to the sequence of human CXCL8 mRNA in GeneBank, the gene specific primers and probes were designed by software of Primer Express, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was regarded as internal reference. The primer sequences were designed based on Table I. Total RNA of PBMCs was isolated by Trizol reagent and then reverse-transcribed to cDNA by oligo $(dT)_{18}$ primers. Positive and negative controls were all set up at the same time for comparison in each test. PCR amplification of cDNA was carried out with 1.25 units of Taq DNA polymerase in 25 μL reaction mixture containing 10 pmol of specific primers, 2.5 mmol of $MgCl_2$, Ex-Taq 1.25 U, 2 mmoL/L of dNTPs, 1×PCR buffer (500 mmoL/L KCl, 100 mmoL Tris, 20 mg/mL gelatin, pH 8.3), 2×SYBR™ Green Ⅰ and 2 μL of a standard substance or cDNA. The cDNA template was diluted in the proportion 1 in 5. The sequences for CXCL8 and GAPDH and its specific amplified products were also shown in Table I. The cycling conditions for GAPDA and CXCL8 were the same namely, preheated for 5 minutes at 94°C, followed by 30 cycles of heating at 94°C for 30 seconds, 55°C for 40 seconds, 72°C for 30 seconds, and a final elongation for 5 minutes at 72°C, then cooling to 4°C until electrophoresis.

Statistical analysis

The ratio of lgcDNA/lgGAPDH was regarded as the extreme level of CXCL8 mRNA and the data were expressed as means ± SD. Subsequently, the product was diluted to seven graded concentrations (10^6, 10^5, 10^4, 10^3,

Table I. The primer sequences of MP, CXCL8, GAPDH.

Gene	Forward Primer (5'→3')	Reverse Primer (3'→5')	Product size (bp)
MP	AAG GAC CTG CAA GGG TTC GT	CTC TAG CCA TTA CCT GCT AA	277
CXCL8	CTT TGT CCA TTC CCA CTT CTG A	TCC CTA ACG GTT GCC TTT GTA T	306
GAPDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452

10^2, 10, and 0 copies/μL), which were used to plot the standard curve. Differences between groups were assessed by the Student's t test or t' test and the relationship was considered statistically significant when P value was lower than 0.05. The correlation between CXCL8 and mRNA was analyzed and then drawn pictures by use of SPSS16.0 statistical software. The standard of correlation analysis was at $0 < |r| \leq 0.3$ for the weak correlation, $0.3 < |r| \leq 0.5$ for the low correlation, $0.5 < |r| \leq 0.8$ for the significantly relation and $0.8 < |r| \leq 1$ for the high relation.

RESULTS

The levels of CXCL8 and its mRNA in the peripheral blood of the forty-eight sick children were higher than those of the normal controls. There was a significant difference between the two groups (P<0.05).

Expression of CXCL8 in serum of the children with Mycoplasma pneumoniae

The levels of CXCL8 in the serum of the forty-eight sick children were higher than those of the normal controls. There was a significant difference between the two groups (P<0.05). Further studies conclusively showed that there was no significant difference of CXCL8 level between the light diseases and severe cases (P>0.05). However, the significant difference of CXCL8 was found between in acute and convalescent phase sera of mycoplasma pneumonia in children (P<0.05). See Figure 1b and Figure 2.

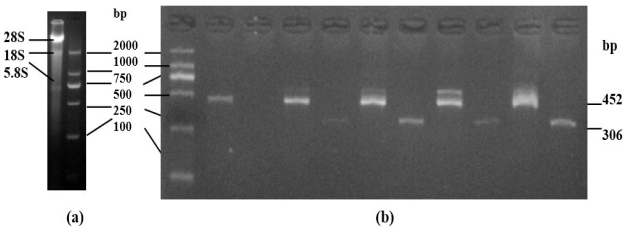

Fig. 1. (a). The electrophorogram of total RNA from PBMCs. (b). Expression of CXCL8 mRNA in the PBMCs of mycoplasmal pneumonia. Note: M: DL2000; 1, 2 for GAPDHC and CXCL8 of normal control; 3, 4, 5, 6 for GAPDH and CXCL8 of mycoplasmal pneumonia (light 1 and light 2); 7, 8, 9, 10 for GAPDH and CXCL8 of mycoplasmal pneumonia (severe 1 and severe 2).

Level of CXCL8 mRNA in PBMCs of the children with Mycoplasma pneumoniae

The total levels of CXCL8 mRNA in PBMCs of the forty-eight sick children were higher than those of the normal controls (P<0.05). Further dynamic observation showed that the high significant differences of CXCL8 mRNA were existed in the light cases and severe cases (P<0.05). The exciting was that the levels of CXCL8 and its mRNA in peripheral blood were significantly lower than those in the acute phase (P<0.05). With the success of sequential therapy, the level of CXCL8 mRNA was significantly decreased and gradually down-regulated to normal level. The significant difference of CXCL8 mRNA was still identified between in acute and recovery phase of mycoplasma pneumonia in children (P<0.05). See Figure 3. The amplified cDNA spectrum band for CXCL8 mRNA by agarose electrophoresis in peripheral blood of severe children was measured under ultraviolet transilluminator. See Figure 1b.

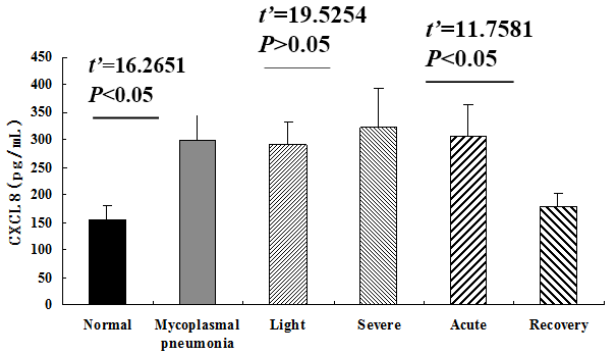

Fig. 2. Levels of CXCL8 in serum of *Mycoplasma pneumoniae*. The high concentration of CXCL8 in peripheral blood was explored in the children with *Mycoplasma pneumoniae*, and was mostly occurred in the acute stage. There was no significant difference of the CXCL8 level in the patients between the light and severe.

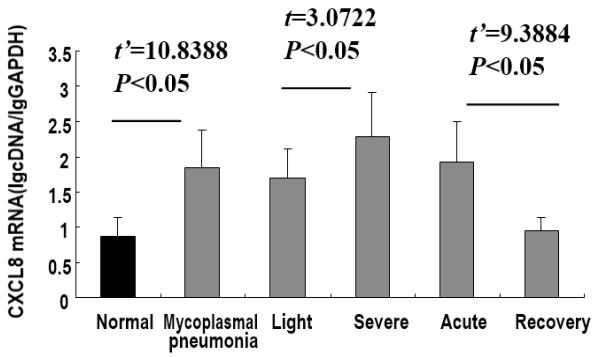

Fig. 3. Levels of CXCL8 mRNA in PBMCs of *Mycoplasma pneumoniae*. The loads of CXCL8 mRNA in PBMCs were significantly increased in children with *Mycoplasma pneumoniae*, and were more common in acute stage and the severe cases.

Chest X-ray characteristic changes in the sick children

The different X-ray manifestations were discovered in the different case groups. Before the sequential treatment, the obvious inflammatory infiltration accompanied with local patchy fuzzy shadow was found in the middle lobe of the right lung of light cases (see Fig. 4A). The patchy and fuzzy shadows could be seen in left hilar and left lower lung of the children with moderate mycoplasma pneumonia (see Fig. 4C). A large amount of interstitial inflammation was showed in double pulmonary and the clear atelectasis was also visual in the middle lobe of the right lung of severe cases (see Fig. 4E). With development of sequential treatment continuously, the improvement of adverse symptoms was noticeable, a wide range of invasive lesions with vague edge and patchy shadows all had been obviously absorbed (see Figs. 4B, D and F).

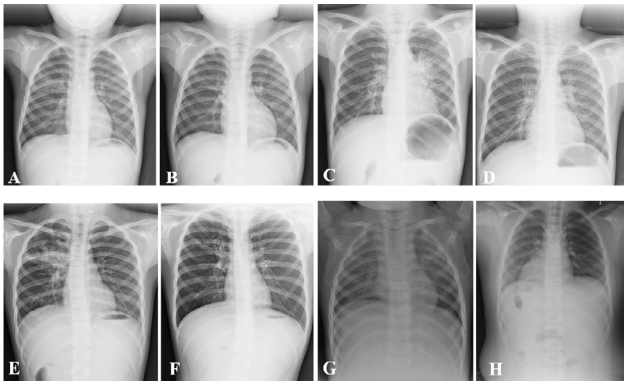

Fig. 4. The Iconography features of *Mycoplasma pneumoniae* before and after treatment. **A** and **B**, Before and after the treatment of the patient (Light). C and D: Before and after the treatment of the patient (Severe). **A**, The obvious inflammatory infiltration accompanied with local patchy fuzzy shadow was found in the middle lobe of the right lung. **B**, The slight inflammation was residue after treatments. **C**, The patchy and fuzzy shadows could be seen in left hilar and left lower lung of the children with moderate mycoplasma pneumonia. **D**, Most fuzzy shadows in left hilar and left lower lung were markedly relieved. **E**, The interstitial inflammation was showed in double pulmonary and the clear atelectasis was also visual in the middle lobe of the right lung. **F**, Most of the interstitial inflammation was reduced, and the atelectasis in the middle lobe of the right lung was obviously disappeared. **G** and **H** were both as normal controls.

Relationship between expression of CXCL8 in serum and the level of CXCL8 mRNA in PBMCs of sick children

The level of serum CXCL8 in was closely related to the content of CXCL8 mRNA in the PBMCs of the children with mycoplasma pneumonia (r=0.5815, P<0.0001), and the correlation was high in the acute phase (r=0.5982, P=0.0001). After the sequential therapy of Erythromycin and Azithromycin for three to four weeks, the disease gradually entered the recovery period, both still had a good correlation at that time, but the correlation coefficient had been significantly reduced (r=0.4060, P=0.0155). There was no significant increase in the level of CXCL8 in serum of normal controls, and no significant correlation was found between the level of CXCL8 in serum and loads of CXCL8 mRNA in PBMCs (r=0.0083, P=0.9655). (see Fig. 5).

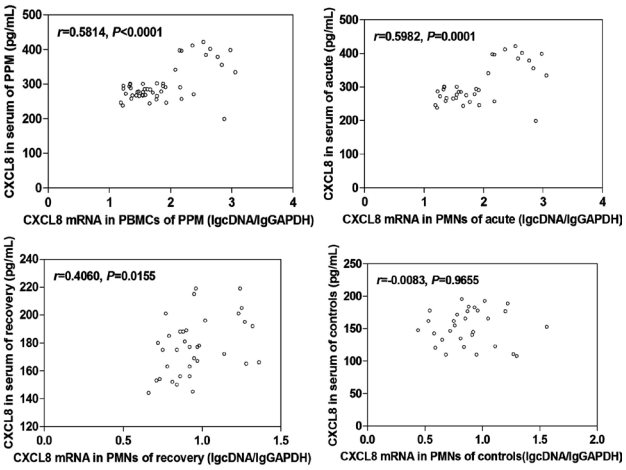

Fig. 5. The correlation between CXCL8 content and its mRNA expression in different course of mycoplasma pneumonia in children. The level of CXCL8 was positively correlated with the expression of its mRNA in the children. With the sequential treatment of erythromycin and azithromycin, the correlation between them decreased significantly.

The relationship between serum CXCL8 and serum Anti-MP in children

There was no significant correlation between serum CXCL8 level and serum Anti-MP level in the children with mycoplasma pneumonia (r=0.1709, P=0.1228), and there was only a weak correlation between them in acute and convalescent stages (r=-0.2917, P=0.0891; r=-0.2783, P=0.1055). (see Fig. 6).

DISCUSSION

The major pathological changes, such as interstitial inflammatory infiltration and acute bronchiolitis, have been demonstrated in the pathogenic process of Mycoplasma pneumonia. The special terminal structure of surface proteins P1 (170kDa) and P30 (32kDa) in Mycoplasma, as an important adhesion molecule, can be conglutinated to the surface of host epithelial cells and colonized in the

intercellular space, and further lead to the host cells injury (Hausner *et al*., 2013; Chang *et al*., 2011). A large number of toxic metabolites, such as neurotoxin, phospholipase C, hydrogen peroxide, can inhibit the movement of ciliated cells in the hosts, and even lead to the disappearance of cilia cells, and cause the secondary injury in mucosal epithelial cell of the host. With the development of inflammatory infiltration around the bronchial and pulmonary vessels, the lesions will be up to the alveolar space and further involved the alveolar, so that the extensive interstitial infiltration are set up in the lobuli pulmonum and alveolar septum (Deng *et al*., 2018).

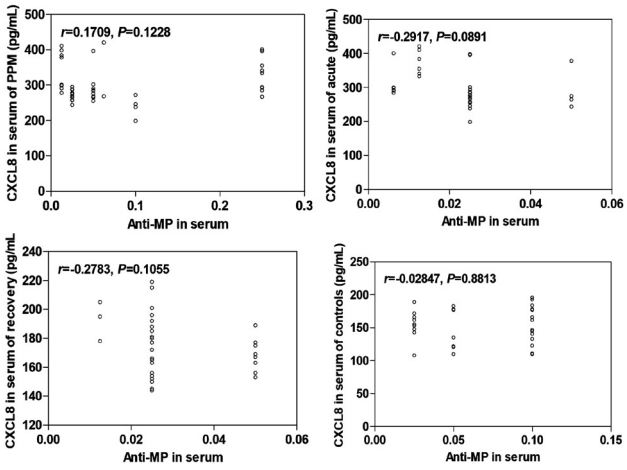

Fig. 6. The correlation between serum CXCL8 level and serum Anti-MP level in children with mycoplasma pneumonia in different course of disease. The weak correlations between the levels of CXCL8 and Anti-MP all had been explored in the acute and convalescent stages.

The cellular immunity has very important clinical significance to control the development and process of lungs injury in mycoplasma pneumoniae pneumonia (Wang *et al*., 2008, 2010). After infected by MP, the immune system in the hosts will be triggered, and a variety of chemokines can be induced, such as CXCL8, TNF-alpha and others (Chen *et al*., 2016), and then actively participate in the local and systemic inflammatory reaction. Appropriate inflammatory response is very beneficial to the human, for example, killing pathogens, limiting infection and repairing the tissue damage, etc. However, the excessive inflammatory response can cause immune pathological damage, and is not conducive to the recovery of the disease. CXCL8 (also known as IL-8), a kind of multi-cell origin of chemokine with positive of ELR (Glu-Leu-Arg) and a major chemotactic agent for neutrophils and lymphocytes, may be derived from a variety of immune cells, such as monocytes, macrophages, neutrophils, lymphocytes, vascular endothelial cells and hepatocytes, etc (Wang *et al*., 2018). It can attract many kinds of inflammatory cells to accumulate in the lung tissues, and release the vasoactive substances, and ulteriorly cause obvious tissue damage in the lungs. The current studies have been shown that the infiltrating cells in the lungs of the patients with mycoplasma pneumonia are mainly mononuclear macrophages, lymphocytes and neutrophils (Rodman *et al*., 2018).

CXCL8, a neutrophil chemotactic and a highly active small molecule polypeptide with molecular weight about 8~10kd (Wang *et al*., 2019), is a kind of proinflammatory cytokine derived from multiple immune cells (Joseph *et al*., 2018). Our clinical data showed that the levels of CXCL8 in peripheral blood of the children with mycoplasma pneumonia were significantly higher than those in normal controls. It was found that the MP colonized in tracheal and bronchial epithelial cells could release toxic metabolic product, which caused local epithelial cell injury, bronchial edema and inflammatory infiltration, and the injury continuously involved in alveolar septum and alveolar cells along the bronchial and pulmonary vessels so that the interstitial inflammation might be induced in the position of pulmonary lobule and alveolar septum. In addition, mycoplasma pneumonia could also trigger the immune system of the children, and prompted a lot of CXCL8 secretion from mononuclear macrophage, T lymphocytes, neutrophils and epithelial cells. Partial CXCL8 originated in different immune cells released into the peripheral blood, so that the levels of CXCL8 in the serum were obviously increased. High levels of CXCL8 could be helpful to resist the MP further intrusion (Yuan *et al*., 2016). However, over high levels of CXCL8 might chemotactically attracted a large number of inflammatory cells in the focus on the accumulation of infiltration, and mediated immune injury in pulmonary, tracheal and bronchial epithelial cells, so that the children had fever, cough, chest pain and other symptoms. Further observation showed that the levels of CXCL8 in the peripheral blood of the severe cases were no significantly higher than those of the light cases. Although CXCL8 was involved in the pathological damages caused by, it had no significant relationship with the degree of immune injury. MP adhesion to epithelial cells promoted the target cells secreting more CXCL8, and further attracted mononuclear macrophage, lymphocyte to lesions aggregation, so that multiple pathological symptoms could be induced, such as airway smooth muscle contraction, glandular secretion, the higher airway reactivity. The above factors might continuously induce a strong inflammatory response and serious tissue injury (Zhang *et al*., 2017). Notably, under the treatment of macrolides antibiotics in clinical trials, the

combination of corticosteroids or / and intravenous human immunoglobulin can inhibit the excessive immune tissue damages for the severe cases.

The mRNA is a critical marker that can reflect the transcription of genes in cells, which may further understand the gene regulation ability in a specific condition. CXCL8 mRNA, as a key control indicator of transcription and translation (Fig. 7), can directly affect the free expression of CXCL8 in the peripheral blood (Jundi and Greene. 2015; Gottipati *et al*., 2015). Pneumonia mycoplasma lack cell walls and rich in cholesterol in the cell membrane. The special structure at the end (terminal structure) can promote adhesion and colonization of pneumonia mycoplasma on the surface of respiratory epithelium cells so as to induce the multiple cellular immune damages. PBMCs are rich in many kinds of immune cells, which can play a variety of anti infection effects through the pathways of specific immune and non-specific immune. Our results had shown that the levels of CXCL8 mRNA in PBMCs of the children with mycoplasma pneumonia were higher than those of normal controls. It verified that the cellular immunity was actively involved in the process of inflammation induced by Mycoplasma, and could up-regulate the level of gene transcription, and chemotactically attracted a large number of mononuclear macrophage and neutrophil cells, and promoted the immune cells to secrete more CXCL8 into the peripheral blood (Wang and Lu, 2008). The limitation on inflammatory infiltration was beneficial to promote the phagocytosis and kill pathogenic mycoplasma. Unfortunately, the high levels of CXCL8 mRNA were still in a few of the severe cases, and excessive up-regulation of CXCL8 mRNA was not favorable to exclude the intrusion of the mycoplasma pneumonia. It could further activate inflammatory factor signal transduction pathway, and released more inflammatory factors, so that the lung, bronchial and other local inflammation should be additionally aggravated (Tsivkovskii *et al*., 2011).

The correlation between CXCL8 and CXCL8 mRNA can be regarded as a sensitive indicator of CXCL8 expression from the related with the levels of transcription and translation. The results of comparative analysis the correlation of CXCL8 and its mRNA showed that the positive relevance was obvious in the children with mycoplasmal pneumonia (Arae *et al*., 2011). The clinical data suggested that it might be related to the patient's condition and the course of the disease. The more closely correlation between CXCL8 and mRNA in the acute stage than that in the recovery period was further evaluated in the course of clinical treatments. Because of the continuous stimulation of MP, the levels of mRNA were up-regulated in multiple cytes, such as monocytes, macrophages, lymphocytes and bronchial epithelial cells, so that the secretion of CXCL8 was also increased. When the course of diseases into the recovery period, the stimulative effects caused by the lower concentration of pneumonia mycoplasma on a variety of immune cells and bronchial epithelial cells were decreased, and were also advantageous to reduce the secretion of CXCL8 and the correlation coefficient between the two markers.

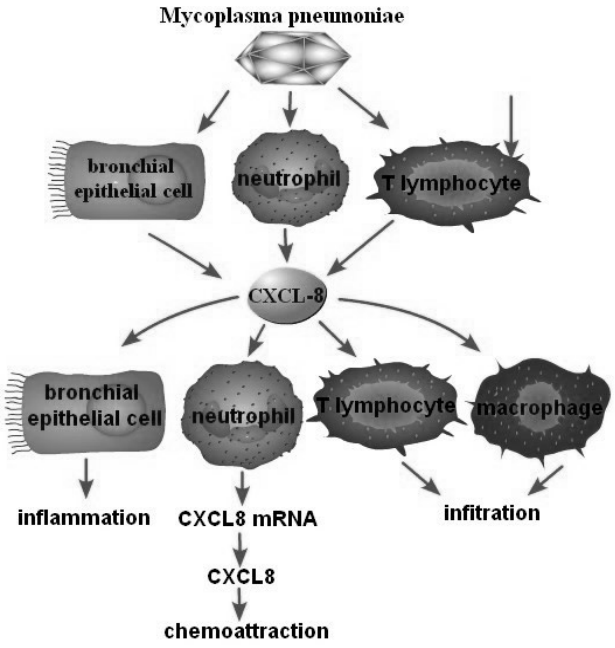

Fig. 7. CXCL8 expression induced by *Mycoplasma pneumoniae*. CXCL8 can be induced by *Mycoplasma pneumoniae* via the pathway of signal transduction in bronchial epithelial cells and neutrophils.

Azithromycin belongs to the second generation of the macrolides antibiotics, which is based on molecular reconstruction of erythromycin, such as basic structural rearrangement, expansion, reduction and N-methylation and other molecular remodeling, and has better advantage on tissue permeability with a very good record of safety (Koštrun *et al*., 2017). The high concentration of Azithromycin in local lesions cells can be kept by transmembrane transport of macrophages so that the therapeutic effect will be improved (Fleming *et al*., 2016). Intravenous infusion of Erythromycin was provided in the acute phase for seven to ten days, so that the children's condition could be significantly controlled obviously, and the symptoms of pulmonary inflammation were also relieved. Followed by use of the sequential therapy of Azithromycin for nearly three to four weeks, the children's condition were gradually from acute stage to recovery stage. Happily, the levels of CXCL8 and its mRNA in the

peripheral blood of the patients were significantly decreased in the recovery period than those in the acute phase (P<0.05). It had been suggested that Azithromycin could not only inhibit the proliferation of MP, reduced pathogen inflammatory effects, suppressed the inflammation, relieved immune injury, and also could down regulate the expression of CXCL8 mRNA, reduced CXCL8 secretion, so that the immune injury recovered gradually (Beigelman *et al*., 2015). Unfortunately, the levels of CXCL8 or/and CXCL8 mRNA in our clinical trials were still high in some children in the recovery period. In order to prevent the recurrence of the disease, we have to continue to consolidate the treatment with great patience, and as soon as possible to improve the clinical symptoms of the children.

Chest X-ray accessory examination is important for diagnosis of lung and airway diseases in the sick children, and advantageous to evaluate the severity and prognosis of the disease (Tanaka *et al*., 2015). The results of our researchers showed that the most of the sick children with MPP were belong to light cases, and the common features of chest X-ray were spotted shadows and patchy shadows in the right lung. During the acute stage, a major clinical feature was a mixed ventilation dysfunction, which not only had restricted ventilation dysfunction, but also had obstructive ventilation dysfunction. It was interesting that the different X-ray manifestations were discovered in the different case groups. The high patchy density shadow in the pulmonary lobules were easily visible and could easily invade the two lung in the children under the age of three years old, and the parenchymal infiltration lesions of pulmonary segment or lobe were regarded as salient characteristic, and unilateral lesions were common in the elder children. The most lesions were especially located in the right lower lung and in the middle lobe of the right lung, and accompanied by vague edge and patchy shadows, which might be related to the long slender anatomic structure in the middle lobe of the right lung. With development of sequential treatment, the adverse symptoms were gradually improved and their diseases were also gradually into the recovery period. However, the airway hyperreactivity, relatively slow lung compliance and slower recovery might be presented in some sick children. It should be particularly strengthened on airway inflammation targeted therapy, and promote interstitial disease recovery via the way of large respiratory training (Shu *et al*., 2012; Ma *et al*., 2014). A wide range of invasive lesions were established in the children with severe mycoplasma pneumonia and often associated with bad complications, such as atelectasis, pulmonary consolidation, ardent fever and others (Zhang *et al*., 2016). For these children, the postural drainage and inhaled adrenal hormone and aerosol inhalation bronchodilator could be taken to significantly contribute the efficiency of anti-inflammatory and respiratory secretions discharge. Simultaneously, the airway hyper-responsiveness also could be reduced and obviously improved the absorption of pneumonia, so that the cough symptoms in children should be alleviated.

In summary, MPP is a common respiratory tract disease in the children. MP as a critical pathogen and start factor of inflammation play a key role in immune inflammatory response. CXCL8, as one of proinflammatory factors, energetically participates in the pathogenesis of MPP and plays an important role in the process of inflammatory infiltration and immune pathological reaction. The expression level of CXCL8 and its mRNA in peripheral blood of children with MP is increased, which has a good correlation with each other, and has a certain correlation with the severity of the disease, but has a weak correlation with the level of anti-MP. It can be used as a laboratory auxiliary diagnostic index to monitor the prognosis of the disease. Azithromycin can reduce the concentration of CXCL8 in serum of the sick children, and also down regulate the expression of mRNA, so that the immune injury mediated by MP may be gradually inhibited.

CONCLUSIONS

The expression level of CXCL8 and its mRNA in peripheral blood of children with mycoplasma pneumonia can decrease gradually from acute stage to convalescent stage, and also is positively correlated with the severity of the disease. Sequential treatment with Azithromycin may not only directly inhibit the proliferation of Mycoplasma pneumoniae in the ciliary epithelial cells of respiratory tract, but also reduce the content of CXCL8 in serum, down-regulate the expression of CXCL8 mRNA and inhibit the immune damage mediated by overaggregation of CXCL8.

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Statement of conflict of interest

We declare no conflicts of interest in this study.

REFERENCES

Arae, K., Hirata, M., Kurata, S., Kamiya, S. and Taguchi, H., 2011. *Mycoplasma pneumoniae* induces interleukin-8 production via the epidermal growth factor receptor pathway. *Microbiol. Immunol*, **55**: 748-750. https://doi.org/10.1111/j.1348-0421.2011.00375.x

Beigelman, A., Isaacson-Schmid, M., Sajol, G., Baty, J., Rodriguez, O.M., Leege, E., Lyons, K., Schweiger, T.L., Zheng, J., Schechtman, K.B., Castro, M. and Bacharier, L.B., 2015. Randomized trial to evaluate azithromycin's effects on serum and upper airway IL-8 levels and recurrent wheezing in infants with respiratory syncytial virus bronchiolitis. *J. Allergy. clin. Immunol*, **135**: 1171-1178.e1. https://doi.org/10.1016/j.jaci.2014.10.001

Chang, H.Y., Jordan, J.L. and Krause, D.C., 2011. Domain analysis of protein P30 in *Mycoplasma pneumoniae* cytadherence and gliding motility. *J. Bact.*, **193**: 1726-1733. https://doi.org/10.1128/JB.01228-10

Chen, Z., Shao, X., Dou, X., Zhang, X., Wang, Y., Zhu, C., Hao, C., Fan, M., Ji, W. and Yan, Y., 2016. Role of the *Mycoplasma pneumoniae*/Interleukin-8/Neutrophil axis in the pathogenesis of pneumonia. *PLoS One*, **11**: e0146377. https://doi.org/10.1371/journal.pone.0146377

Chinese medical science branch of science and society, 2013. The guidelines for the management of children with community acquired pneumonia (2013 revision) (I). *Chin. J. Pediatr*, **51**: 745-752.

Chinese medical science branch of science and society, 2013. The guidelines for the management of children with community acquired pneumonia (2013 revision) (I). *Chin. J. Pediatr.*, **51**: 856-862.

Deng, H., Rui, J., Zhao, D. and Liu, F., 2018. *Mycoplasma pneumoniae* 23S rRNA A2063G mutation does not influence chest radiography features in children with pneumonia. *J. Int. Med. Res.*, **46**: 150-157. https://doi.org/10.1177/0300060517716312

Dumke, R., Schnee, C., Pletz, M.W., Rupp, J., Jacobs, E., Sachse, K., Rohde, G. and Capnetz Study Group., 2015. *Mycoplasma pneumoniae* and *Chlamydia* spp. infection in community-acquired pneumonia, Germany, 2011-2012. *Emerg. Infect. Dis.*, **21**: 426-434. https://doi.org/10.3201/eid2103.140927

Fleming-Dutra, K.E., Friedman, C.R. and Hicks, L.A., 2016. Early azithromycin treatment to prevent severe lower respiratory tract illnesses in children. *J. Am. med. Asst.*, **315**: 2121-2122. https://doi.org/10.1001/jama.2016.0918

Gottipati, K.R., Bandari, S.K., Nonnenmann, M.W., Levin, J.L., Dooley, G.P., Reynolds, S.J. and Boggaram, V., 2015. Transcriptional mechanisms and protein kinase signaling mediate organic dust induction of IL-8 expression in lung epithelial and THP-1 cells. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **308**: L11-L21. https://doi.org/10.1152/ajplung.00215.2014

Hausner, M., Schamberger, A., Naumann, W., Jacobs, E. and Dumke, R., 2013. Development of protective anti-Mycoplasma pneumoniae antibodies after immunization of guinea pigs with the combination of a P1-P30 chimeric recombinant protein and chitosan. *Microb. Pathog.,* **64**: 23-32. https://doi.10.1016/j.micpath.2013.07.004

Joseph, P.R.B., Spyracopoulos, L. and Rajarathnam, K., 2018. Dynamics-Derived Insights into Complex Formation between the CXCL8 Monomer and CXCR1 N-Terminal Domain: An NMR Study. *Molecules*, **23**: pii: E2825. https://doi.org/10.3390/molecules23112825

Jundi, K. and Greene, C.M., 2015. Transcription of Interleukin-8: How altered regulation can affect cystic fibrosis lung disease. *Biomolecules*, **5**: 1386-1398. https://doi.org/10.3390/biom5031386

Koštrun, S., Munic, Kos, V., Matanović, Škugor, M., Palej, Jakopović, I., Malnar, I., Dragojević, S., Ralić, J. and Alihodžić, S., 2017. Around the macrolide-impact of 3D structure of macrocycles on lipophilicity and cellular accumulation. *Eur. J. med. Chem.*, **133**: 351-364. https://doi.org/10.1016/j.ejmech.2017.03.056

Ma, X., Ding, M.J., Zhao, X.X., Sun, J., Yang, J.Z. and Han, Y.L., 2014. Features of lung dysfunction in children with Mycoplasma pneumoniae pneumonia with different chest imaging findings. *Zhongguo. Dang. Dai. Er. Ke. Za. Zhi.*, **16**: 997-1000.

Rodman, Berlot, J., Krivec, U., Praprotnik, M., Mrvič, T., Kogoj, R. and Keše, D., 2018. Clinical characteristics of infections caused by *Mycoplasma pneumoniae* P1 genotypes in children. *Eur. J. clin. Microbiol. Infect. Dis.*, **37**: 1265-1272. https://doi.org/10.1007/s10096-018-3243-5

Shu, L.H., Shang, Y.X., Cai, X.X., Zhang, H., Li, M., Zhou, Q.L., Zong, Z.H., Meng, X.N., Li, X. and Liu, F., 2012. Changes to surfactant proteins in the bronchoalveolar lavage fluid and serum of children with *Mycoplasma pneumoniae* pneumonia. *Zhongguo. Dang. Dai. Er. Ke. Za. Zhi*, **14**: 928-932.

Sun, H., Chen, Z., Yan, Y., Huang, L., Wang, M. and Ji, W., 2015. Epidemiology and clinical profiles

of *Mycoplasma pneumoniae* infection in hospitalized infants younger than one year. *Respir. Med.*, **109**: 751-757. https://doi.org/10.1016/j.rmed.2015.04.006

Tanaka, N., Emoto, T., Suda, H., Matsumoto, T. and Matsunaga, N., 2015. Community-acquired pneumonia: a correlative study between chest radiographic and HRCT findings. *Jpn. J. Radiol.*, **33**: 317-328. https://doi.org/10.1007/s11604-015-0420-7

Tsivkovskii, R., Sabet, M., Tarazi, Z., Griffith, D.C., Lomovskaya, O. and Dudley, M.N., 2011. Levofloxacin reduces inflammatory cytokine levels in human bronchial epithelia cells: Implications for aerosol MP-376 (levofloxacin solution for inhalation) treatment of chronic pulmonary infections. *FEMS. Immunol. med. Microbiol*, **61**: 141-146. https://doi.org/10.1111/j.1574-695X.2010.00755.x

Wang, J., Cai, W.J., Sun, L., Cao, W.Y., Li, C.D. and Han, Z.Y., 2018. Occult HBV infection in peripheral blood neutrophils of chronic hepatitis b and its effects on expression of CXCR1, CXCR2, CXCL8 and CD16. *Nanosci. Nanotech. Lett.*, **10**: 244-251. https://doi.org/10.1166/nnl.2018.2608

Wang, J., Cao, W.Y., Cai, W.J., Li, C.D. and Sun, L., 2017. Structure of Toll-like receptors, signal transduction, and regulation. *Nanosci. Nanotech. Lett.*, **9**: 624-639. https://doi.org/10.1166/nnl.2017.2374

Wang, J., Liu, Z., Xu, S., Hu, M.F., Liu, X.R. and Cai, W.J., 2019. The effect of hemodialysis on the expression of CXCL8 and its mRNA in neutrophils of the patients with chronic renal failure. *Pakistan J. Zool.*, **51**: 655-666. https://doi.org/10.17582/journal.pjz/2019.51.2.655.666

Wang, J. and Lu, Q., 2008. Expression of T subsets and mIL-2R in peripheral blood of newborns with hypoxic ischemic encephalopathy. *World J. Pediatr.*, **4**: 140-144. https://doi.org/10.1007/s12519-008-0028-4

Wang, J., Wang, P.P., Xiang, G.J. and Hu, X.B., 2010. Relationship between the expression of IP-10 and IP-10 mRNA in peripheral blood and HBV DNA level in patients with cirrhosis. *Hepatobil. Pancreat. Dis. Int.*, **9**: 280-286.

Wang, J., Zhao, J.H., Wang, P.P. and Xiang, G.J., 2008. Expression of CXC chemokine IP-10 in patients with chronic hepatitis B. *Hepatobil. Pancreat. Dis. Int.*, **7**: 45-50.

Yuan, Z.F., Chen, B., Mao, S.S., Shen, J., Yu, Y.L., Gao, F. and Xia, Z.Z., 2016. Reversible bilateral striatal lesions following *Mycoplasma pneumoniae* infection associated with elevated levels of interleukins 6 and 8. *Brain Dev.*, **38**: 149-153. https://doi.org/10.1016/j.braindev.2015.04.008

Zhang, J., Yin, Y., Lin, X., Yan, X., Xia, Y., Zhang, L. and Cao, J., 2017. IL-36 induces cytokine IL-6 and chemokine CXCL8 expression in human lung tissue cells: Implications for pulmonary inflammatory responses. *Cytokine*, **99**: 114-123. https://doi.org/10.1016/j.cyto.2017.08.022

Zhang, Y., Zhou, Y., Li, S., Yang, D., Wu, X. and Chen Z., 2016. The clinical characteristics and predictors of refractory *Mycoplasma pneumoniae* pneumonia in children. *PLoS One*, **11**: e0156465. https://doi.org/10.1371/journal.pone.0156465