Characteristics of Inflammatory Storm in BALF of Severe Pneumonia in Children: A Case Control Study

Fang Deng
Department of Respiratory, Children's Hospital of Chongqing Medical University
Hui ling Cao
Department of neonatology, Children's Hospital of Chongqing Medical University
Xiao hua Liang
Clinical Epidemiology and Biostatistics Department, Children's Hospital of Chongqing Medical University
Qu bei Li
Department of Respiratory, Children's Hospital of Chongqing Medical University
Yang Yang
Department of Respiratory, Children's Hospital of Chongqing Medical University
Zhi hua Zhao
Department of Respiratory, Children's Hospital of Chongqing Medical University
Jun jie Tan
Clinical Molecular Medical Center, Children's Hospital of Chongqing Medical University
Guo Fu
Clinical Molecular Medical Center, Children's Hospital of Chongqing Medical University
Chang Shu (400361@hospital.cqmu.edu.cn)
Department of Respiratory, Children's Hospital of Chongqing Medical University

Research

Keywords: cytokines, inflammatory cells, BALF, children, severe pneumonia

Posted Date: January 5th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-138549/v1

Abstract

Background: It is difficult to collect bronchoalveolar lavage fluid (BALF) from children. There are few studies on inflammatory storms in BALF of children with pneumonia. Therefore, we will retrospectively investigate characteristics of inflammatory storms in BALF of children with pneumonia in our hospital.

Methods: This retrospective study included 161 children with pneumonia. Pediatric pneumonia was divided into two groups: mild and severe pneumonia, according to the clinic diagnosis. All clinic information and data came from our hospital's database. Blood and BALF samples were collected and measured by Clinical laboratory and Clinical Molecular Medical Center. Flow cytometry was performed to detect the levels of cytokines, including IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ. We performed receiver operator characteristic curve (ROC) and spearman correlation to evaluate the role of BALF inflammatory cytokines and cells in pneumonia.

Results: 1) The levels of IL-6, IL-10, IL-17A, and TNF-α, and ratios of IL-6 to IL-10 and TNF-α to IL-10 in BALF of severe pneumonia group were higher than those of mild pneumonia group. 2) The number of inflammatory cells and the percentage of neutrophils in BALF of severe pneumonia group increased, and the percentage of macrophages decreased, compared with the mild pneumonia group. 3) ROC analysis showed that the levels of IL-6, IL-10 and TNF-α, and ratios of IL-6 to IL-10 and TNF-α to IL-10, the number of inflammatory cells and neutrophils, as well as the percentage of neutrophils exceeded the cut-off value (60.37pg/mL, 2.54pg/mL, 13.07pg/mL, 29.30, 1.57, 3140.00*10^9/L, 768.00*10^9/L, 43.00%, respectively) could be used to distinguish children with severe pneumonia from children with mild pneumonia. While the percentage of macrophages in BALF decreased to below the cut-off value (36.00%) also had the discrimination ability. 4) The BALF levels of IL-6, IL-10, TNF-α and IFN-γ were correlated, positively with the inflammatory cell count and the percentage of neutrophils, and negatively with the percentage of macrophages.

Conclusion: In severe pediatric pneumonia, BALF inflammatory cytokines and inflammatory cell infiltration are more intense, especially above the cut-off value. This study demonstrates that these inflammatory signals are potential biomarkers for predicting the severity of pediatric pneumonia.

Trial registration: This study approved by the Medical Research Ethics Committee of Children's Hospital of Chongqing Medical University, registered in http://www.chictr.org.cn/, No. ChiCTR2000034048(registration date, June 22, 2020).

Backgrounds

Pneumonia remains a significant cause of morbidity and mortality among children in the world despite the great progress made in medicine. Previous studies have confirmed that there are absent or only a few cytokines in a healthy body, and a series of cytokines are involved in the occurrence of acute inflammation[1–5]. After pathogenic microorganisms invaded the lungs, alveolar macrophages activate and produce related cytokines. Appropriate cytokine response is essential for controlling infection.

Cytokine deficiency can lead to serious and fatal infections[4,6]. On the other hand, excessive cytokine storm can also cause lung damage[5,7].

C-reactive protein (CRP) and procalcitonin (PCT) are often used to reflect the severity of pneumonia, but their application in clinical practice is limited due to their poor specificity and delayed response to inflammation. Studies have shown that the cytokine signals in patients with severe and mild pneumonia are different [8,9]. Studies have also pointed out that in children and adults with pneumonia, serum cytokine signals (mainly IL-6 and IL-10) help to identify the pathogen[10,11], evaluate the severity and prognosis of pneumonia [12–14]. Tocilizumab, an IL-6 receptor (IL-6R) antagonist, is effective in treating patients with severe 2019 coronavirus disease (COVID-19) whose serum IL-6 is significantly elevated[15]. Previous researchers have found that in patients with pneumonia, certain immune function genes are up-regulated in lung aspirate cells and down-regulated in blood mononuclear cells[16], and the serum cytokines cannot fully and truly reflect the local immune responses[17]. The affected lung may be the source of cytokines[18]. Studies have pointed out that the levels of cytokines in bronchoalveolar lavage fluid (BALF), such as IL-17A, are closely related to the severity of childhood mycoplasma pneumoniae (MP) pneumonia[19]. Therefore, we hypothesized that the cytokines and inflammatory cells in the lungs of children with severe pneumonia have specific changes, and local anti-inflammatory therapy and anti-cytokine receptor therapy may be suitable for children with severe pneumonia.

This study retrospectively analyzed the levels of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ) and inflammatory cells in BALF in children with pneumonia. We evaluated the predictive value of cytokine levels and inflammatory cell levels in BALF for children with severe pneumonia. We also analyzed the relationship between cytokines and inflammatory cells in BALF in children with pneumonia. The results of the study will help us better understand the inflammatory storm in the lungs, and help us to diagnose and treat pediatric patients with severe pneumonia early.

Methods

Patients

Children with pneumonia, who met the inclusion criteria and were hospitalized at the Children's Hospital of Chongqing Medical University from March 2019 to February 2020, were included. Inclusion criteria: 1) meeting diagnostic criteria for pneumonia; and the diagnosis of pneumonia, according to British Thoracic Society guidelines for the management of community acquired pneumonia in childhood[20], was based on the presence of respiratory symptoms, with or without fever, and inflammation of the lungs confirmed by chest X-ray or CT examination; 2) aged 29 days to 18 years old. We collected the clinical and laboratory data of 161 children with pneumonia from our hospital's database. All children underwent bronchoalveolar lavage during hospitalization. Children were divided into severe and mild pneumonia groups, according to the guideline for diagnosis and treatment of community-acquired pneumonia in children (2019 version)[21] and British Thoracic Society guidelines for the management of community

acquired pneumonia in childhood[20] (Table 1). Exclusion criteria: 1) suffering from asthma, tuberculosis and other respiratory diseases, 2) suffering from severe diseases of important organs, tumors, immune system diseases or malnutrition, 3) ever used immunosuppressants or glucocorticoids before admission.

Table 1
Evaluation of the severity of pneumonia in children#.

	Mild	Severe
General situation	Good	Pale, depression, poor response
Disorder of consciousness	No	Yes
Hypoxemia	No	Cyanosis; tachypnea, respiratory rate (RR) ≥60 breaths/min(age 0–2 months), RR≥50 breaths/min(age 2–12 months); RR≥40 breaths/min(age 1–5 years); RR≥20 breaths/min(age > 5 years);grunting, nasal flaring, retractions (suprasternal, intercostals or subcostal); apnea; pulse oximetry measurement<92%
Fever	Inadequate severity	Ultra-high fever, persistent high fever > 5 days
Dehydration	No	Decreased in body weight > 3%, skin elasticity poor, mucous membranes dry, tears decreased, oliguria (urine volume < 1 ml/kg.h)
Chest X-ray / CT	Inadequate severity	Pleural effusion, pneumothorax, atelectasis, lung necrosis, lung abscess
Extrapulmonary complications	No	Meningitis, central nervous system abscess, pericarditis, endocarditis, septic arthritis, sepsis, hemolytic uremic syndrome
Standard	All of the above	Any of the above

#: The standard based on British Thoracic Society guidelines for the management of community acquired pneumonia in childhood[20]and the guideline for diagnosis and treatment of community-acquired pneumonia in children (2019 version)[21].

Flow cytometry

The levels of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ) were determined by flow cytometry, using commercial human Th1/Th2/Th17 test kits (SaiKi Biotechnology, Jiangxi, China). 2.5 pg/mL was the lowest detection limit (LODL) of the cytokines. And the upper limits of normal reference values of IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ were 9.80 pg/mL, 3.00 pg/mL, 16.60 pg/mL, 4.90 pg/mL, 14.80 pg/mL, 5.20 pg/mL and 17.30 pg/mL, respectively.

qt-PCR

Pathogen nucleic acids were determined by SLAN-96P Real-time quantitative Polymerase Chain Reaction (*qt*-PCR) instrument (Shanghai Hongshi, Shanghai, China), using DNA diagnostic kit (Sansure Biotech,

Hunan, China) or RNA diagnostic kit (Rendu Biotechnology, Shanghai, China).

Immunofluorescence assay

Nasopharyngeal aspirates (NPAs) and BALF virus antigens were determined by immunofluorescence assay, using D^3 ultra DFA respiratory virus screen & ID kit (Diagnostic Hybrids, Shanghai, China).

Passive particle agglutination

Serum specific antibodies of MP or chlamydia pneumoniae (CP) were determined by passive particle agglutination, using diagnostic kits for measurement of antibodies (SERODIA-MYCO II, Guangzhou, China).

Culture

The bacteria culture was carried out according to the National Guide to Clinical Laboratory Procedures version 4, and semi-quantitative culture was used. Qualified specimens of NPAs and BALF were inoculated to Columbia blood agar plate and chocolate blood agar plate (Antobio, Zhengzhou, China), and then incubate in an HF90/HF240 incubator (Heal Force Bio-Meditech, Shanghai, China) at 35℃, 7% CO2.

Definition of pathogenic microorganisms

The definition of pathogenic microorganisms was as follows[20–23]: 1) the pathogenic bacteria were cultured in pleural effusion or BALF (≥ +), 2) the NPAs obtained through artificial airway cultured dominant bacteria (≥ ++), 3) the NPAs culture had the growth of dominant bacteria (≥ +++), 4) the bacterial nucleic acid test of NPAs or BALF were positive, 5) the detection of virus antigen or virus PCR of NPAs, BALF or pleural effusion was positive, or the serum virus IgM-specific antibody was elevated (≥ 4 folds), 6) the serum MP IgM-specific antibody was positive (≥ 1/160), the PCR of MP in NPAs, BALF or pleural effusion was positive, 7) the serum CP IgM-specific antibody was positive (≥ 1/40), the PCR of CP in NPAs, BALF or pleural effusion was positive.

Statistics

Statistical software SPSS version 25.0, MedCalc version 19.1.3 and GraphPad Prism version 8.0.1 were used for analyzing data. Data exceeded the LODL were represented as 1/2 of LODL for statistical processing. Non-normal data were expressed as median (interquartile range) and normal data were expressed as mean ± standard deviation. Chi-square test or Fisher's exact test was performed for classified variables, and Mann-Whitney U test was performed for non-parametric variables. The correlation between biomarkers was performed by Spearman correlation coefficient (r). The prediction accuracy of biomarkers was analyzed by receiver operator characteristic curve (ROC). A ROC can create a performance standard that best predicts a clinically significant outcome of interest. The area under curve (AUC), represented the predictive value, and optimal cutoff values were calculated. And the higher the AUC, the higher the predictive value of biomarkers. The difference was statistically significant with double-tailed *P*-value < 0.05.

Results

Patients Characteristics

This study retrospectively analyzed the data of 161 patients with pneumonia including 100 cases in the severe pneumonia group and 61 cases in the mild pneumonia group (Table 2). There were no significant differences in age, gender and weight between the two groups. The days of fever and hospitalization in the severe group were significantly longer than those in the mild group.

Table 2
Patients' features and laboratory data of blood.

	Mild pneumonia (n = 61)	**Severe pneumonia (n = 100)**	***P*-value**
Age, years	5.11 ± 3.00	4.84 ± 2.78	= 0.562
Male/female	1.10(32/29)	1.33(57/43)	= 0.574
Body weight, kg	18.00(14.00 ~ 21.50)	17.00(13.00 ~ 22.38)	= 0.605
Days of fever, days	4.00(1.00 ~ 7.50)	8.00(5.00 ~ 11.00)	< 0.001
Days of hospitalization, days	6.00(3.50 ~ 7.00)	8.00(6.00 ~ 9.00)	< 0.001
WBC, (*10^9/L)	7.40(6.29 ~ 9.71)	7.35(5.91 ~ 9.85)	= 0.716
Platelet, (*10^9/L)	404.00(302.00 ~ 489.00)	391.00(290.50 ~ 468.25)	= 0.414
RBC, (*10^{12}/L)	4.55(4.28 ~ 4.80)	4.41(4.01 ~ 4.73)	= 0.022
Hemoglobin, g/L	125.05 ± 9.46	119.29 ± 11.56	= 0.001
Neutrophil, %	0.54 ± 0.14	0.57 ± 0.14	= 0.092
CRP, mg/L	4.00(4.00 ~ 10.50)	4.00(4.00 ~ 12.75)	= 0.153
PCT, ng/mL	0.07(0.04 ~ 0.17)	0.13(0.07 ~ 0.27)	= 0.003
Albumin, g/L	44.57(42.90 ~ 46.35)	42.05(38.23 ~ 44.68)	< 0.001
ALT, U/L	17.00(14.00 ~ 22.00)	19.50(15.00 ~ 26.75)	= 0.022
LDH, U/L	311.00(269.50 ~ 348.50)	364.25(288.75 ~ 442.75)	= 0.003
Fibrinogen, g/L	3.40 ± 1.10	3.91 ± 1.17	= 0.007
D-dimer, mg/L	0.50(0.24 ~ 0.72)	0.98(0.51 ~ 2.37)	< 0.001

Data on age, hemoglobin, neutrophil, fibrinogen were expressed as mean ± standard deviation, and data except age, hemoglobin, neutrophil, fibrinogen were expressed as median (interquartile range). Abbreviations: white blood cell count (WBC), red blood cell count (RBC), C-reactive protein (CRP), Procalcitonin (PCT), Alanine aminotransferase (ALT), lactate dehydrogenase (LDH).

The red blood cell count (RBC) and hemoglobin level of the severe group decreased significantly, and the white blood cell count (WBC), platelet count and neutrophil percentage were not significantly different between the two groups. There was no significant difference in CRP levels between the two groups, while PCT levels of the severe group were significantly increased.

And in the severe group, the levels of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) increased significantly, while the levels of albumin were decreased significantly. The levels of fibrinogen and D-dimer in the severe group were also increased significantly.

Among children with severe pneumonia, 51 cases progressed to respiratory failure, 1 case developed acute respiratory distress syndrome (ARDS), 24 cases had pleural effusion, and 28 cases had pulmonary necrosis. 34 cases were treated with non-invasive continuous positive airway pressure (CPAP) and 1 case was treated with invasive mechanical ventilation (endotracheal intubation). The microorganisms of children with pneumonia were shown in Table 3. The pathogen detection rate of severe pneumonia group was higher than that of mild pneumonia group (100% vs. 85.24%). The pathogen detection of bacteria, virus, MP and CP showed no statistical difference between mild and severe pneumonia groups ($P > 0.05$, Table 3). The mild pneumonia group with mixed infection was infected with two microorganisms simultaneously, and severe pneumonia group with mixed infection was infected with two or three kinds of microorganisms.

Table 3
Distribution of pathogenic microorganisms in BALF.

	Mild pneumonia (n = 61)	Severe pneumonia (n = 100)	*P*-value
Bacteria	6	15	= 0.471
Streptococcus pneumoniae	4	8	= 0.771
Staphylococcus aureus	0	2	= 0.526
Haemophilus influenzae	2	2	= 1.000
Stenotrophomonas maltophilia	0	1	= 1.000
Moraxella catarrhalis	0	2	= 0.526
Mycoplasma pneumoniae (MP)	48	89	= 0.109
Chlamydia pneumoniae (CP)	0	1	= 1.000
Virus	10	27	= 0.128
Adenovirus	1	21	= 0.001
Respiratory syncytial virus	7	2	= 0.027
Rhinovirus	0	1	= 1.000
Influenza virus	2	1	= 0.558
Parainfluenza virus	0	1	= 1.000
Boca virus	1	2	= 1.000
Mixture (≥2 pathogenic microorganisms)	11	30	= 0.098
Negative	9	0	0.001

Both pro-inflammatory and anti-inflammatory cytokines are elevated in BALF of children with severe pneumonia

We used flow cytometry to detect the cytokines levels in BALF. The results showed that the levels of IL-6 (190.89 pg/mL, 70.23 ~ 512.57 pg/mL), IL-10 (5.09 pg/ml, 1.25 ~ 11.57 pg/mL), IL-17A (4.80 pg/mL, 1.25 ~ 22.16 pg/mL) and TNF-α (8.28 pg/mL, 1.25 ~ 26.27 pg/mL) in BALF of children with severe pneumonia were significantly higher than those of children with mild pneumonia (Fig. 1A). The levels of IL-2, IL-4 and IFN-γ in BALF were not significantly different between the two groups ($P > 0.05$, Fig. 1A).

The ratios of pro-inflammatory/anti-inflammatory cytokine in BALF of severe group are higher than that of mild group

It is important to maintain a balance between pro-inflammatory and anti-inflammatory cytokines, so we calculated the ratio of proinflammatory cytokines to anti-inflammatory cytokines in BALF of children with pneumonia. In our study, the IL-6/IL-10 ratio (34.48, 13.82 ~ 66.67) and TNF-α/IL-10 ratio (0.91, 1.16 ~ 2.64) in BALF of severe group were significantly higher than those of mild group (Fig. 1B). There was no significant difference in the IL-17A/IL-10 ratio between the two groups ($P > 0.05$, Fig. 1B).

There are more inflammatory cells in BALF of severe group than that of mild group

Next, we analyzed the levels of inflammatory cells in BALF of children with pneumonia. The inflammatory cell count (2525.00*10^6/L, 1187.50 ~ 5100.00*10^6/L), neutrophil count (1157.70*10^6/L, 440.90 ~ 2726.54*10^6/L), macrophage count (488.00*10^6/L, 243.53 ~ 849.90*10^6/L) and lymphocyte count (128.20*10^6/L, 128.20 ~ 861.90*10^6/L) (Fig. 1C) in BALF of children with severe group were significantly higher than those of children with mild group. And, the percentage of neutrophils (51.00%, 29.00 ~ 65.00%) (Fig. 1D) in BALF of children with severe pneumonia also increased significantly. Interestingly, the percentage of macrophages (21.00%, 11.00 ~ 34.00%) (Fig. 1D) in BALF of children with severe pneumonia was significantly reduced. No significant difference was found in the percentage of lymphocytes in BALF between the two groups ($P > 0.05$, Fig. 1D).

Analysis of Receiver operator characteristic curves (ROCs)

The ROCs of the cytokines and inflammatory cells in BALF were performed (Table 4, Fig. 2). In our study, ROC analysis showed that if IL-6 > 60.37 pg/mL, IL-10 > 2.54 pg/mL, TNF-α > 13.07 pg/mL, IL-6/IL-10 ratio > 29.03, TNF-α/IL-10 ratio > 1.57, total number of inflammatory cells > 3140.00*10^6/L, neutrophil count > 768.00*10^6/L, the percentage of neutrophils > 43.00%, the percentage of macrophages ≤ 50.00% (Fig. 2), which indicated that the child may be a patient with severe pneumonia. The other cytokines and inflammatory cells in BALF in our study had no significant predictive effect (Table 4).

Table 4
ROCs of cytokines and inflammatory cells in BALF to distinguish severe pneumonia from mild pneumonia.

	AUC (95%CI)	Cut-off value	Sensitivity (%)	Specificity (%)	*P*-value
Pro-inflammatory cytokines					
IL-2, pg/mL	0.530(0.437 ~ 0.622)	> 1.25	29.00	78.69	*= 0.529*
IL-17A, pg/mL	0.590(0.501 ~ 0.678)	> 10.69	40.00	83.60	= 0.057
IFN-γ, pg/mL	0.585(0.495 ~ 0.674)	> 6.47	49.00	70.49	= 0.072
Anti-inflammatory cytokines					
IL-4, pg/mL	0.534(0.443 ~ 0.625)	> 2.8	18.00	90.16	*= 0.465*
Pro-inflammatory/anti-inflammatory cytokine ratios					
IL-17A/IL-10 ratio	0.496(0.406 ~ 0.585)	≤ 0.724	34.00	80.33	= 0.926
Inflammatory cells					
Macrophage, (*10^6/L)	0.575(0.483 ~ 0.667)	> 273.60	73.00%	42.62%	= 0.111
Lymphocyte, (*10^6/L)	0.554(0.463 ~ 0.645)	> 810.00	31.00%	86.89%	= 0.249
Lymphocyte, %	0.460(0.365 ~ 0.555)	≤ 36.00%	90.00	26.23	= 0.555

Spearman Correlation Analysis of cytokines, inflammatory cells in BALF of children with pneumonia

We used Spearman correlation analysis to further understand the relationship between cytokines and inflammatory cells in BALF of children with pneumonia, (Fig. 3). The levels of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ) in BALF of children with pneumonia were significantly positively correlated in pairs. As shown in Fig. 3, IL-6 levels were significantly positively correlated with the levels of IL-10, IL-17A, TNF-α, IFN-γ (r = 0.791, r = 0.466, r = 0.810, r = 0.650, respectively). Similarly, IL-10 levels were also significantly correlated with the levels of IL-17A, TNF-α and IFN-γ (r = 0.450, r = 0.765, r = 0.701, respectively).

The total number of inflammatory cells in BALF was significantly positively correlated with the levels of IL-6, IL-10, TNF-α and IFN-γ (r = 0.576, r = 0.507, r = 0.481, r = 0.341, respectively). There was no

statistically significant correlation between the total number of inflammatory cells and the levels of IL-2, IL-4 and IL-17A ($P > 0.05$). The percentage of neutrophils in BALF was significantly positively correlated with the levels of IL-6, IL-10, IL-17A, TNF-α and IFN-γ (r = 0.517, r = 0.417, r = 0.281, r = 0.383, r = 0.287, respectively). There was no statistically significant correlation between the percentage of neutrophils and the levels of IL-2 and IL-4 ($P > 0.05$). On the contrary, the percentage of macrophages was significantly negatively correlated with the levels of IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ (r= -0.169, r= -0.157, r= -0.525, r= -0.451, r= -0.396, r= -0.462, r= -0.345, respectively). The correlations between the levels of cytokines and the percentage of lymphocytes were not significant ($P > 0.05$). The total number of inflammatory cells in BALF was directly proportional to the percentage of neutrophils (r = 0.603), and inversely proportional to the percentage of lymphocytes (r= -0.182) and macrophages (r= -0.441). In addition, the percentage of neutrophils and macrophages were negatively correlated (r= -0.750).

Discussion

At present, pneumonia is still one of the reasons that threaten the health of children in the world. The clinical manifestations of children with pneumonia are very complicated due to the infection by a variety of pathogenic microorganisms. As we all known, inflammation is not only related to the invasion of pathogenic microorganisms, but also related to the body's inflammatory immune response[24]. We should look for a detection or treatment method for the inflammatory immune response of pneumonia[25], so that we can quickly identify the critically ill children and take more effective treatments. The intense inflammatory storm in patient with severe pneumonia has aroused widespread concern. And our study further demonstrates that the cytokines and inflammatory cells in BALF are effective tools for assessing the severity of pneumonia.

Our study shown that compared with children with mild pneumonia, children with severe pneumonia had longer days of fever and hospitalization. The significant decrease of RBC and hemoglobin level in children with severe pneumonia also indicated a decrease of oxygen carrying capacity, which was related to more severe hypoxia symptoms. The inflammatory markers (PCT, LDH) in children with severe pneumonia also increased significantly. And the abnormal liver function and blood coagulation function in children with severe pneumonia suggested that severe patients had damage external lung organ.

Children with severe pneumonia had abnormal inflammatory immune responses. In our study, the levels of IL-6, IL-10, IL-17A, TNF-α and the number of inflammatory cells (including neutrophils, macrophages and lymphocytes) in BALF of children with severe pneumonia were significantly higher than those of children with mild pneumonia. These results were similar to previous study[8,26]. Interestingly, compared with children with mild pneumonia, the percentage of macrophages in BALF of children with severe pneumonia was decreased relatively, while the percentage of neutrophils increased significantly. It suggests that undue infiltration of neutrophils is an important mechanism of lung inflammation in children with severe pneumonia.

The levels of IL-6 are associated with the severity of infection and inflammation[27–30],as well as the severity of adult pneumonia[31]. In this study, compared with children with mild pneumonia, children with severe pneumonia had more severe inflammatory cell infiltration in lungs. Immune cells, such as activated alveolar macrophages and lymphocytes, and damaged endothelial cells, could secrete IL-6 and chemokines that elicit neutrophils migration into the lungs[32,33]. Early recruitment of neutrophils was the key to initiating effective host defenses[34]. IL-6 level in BALF of children with pneumonia was positively correlated with the percentage of neutrophils. On the one hand, IL-6 could promote the survival of neutrophils[35], enhance the bactericidal activity of neutrophils, and expand the inflammatory effect to eliminate pathogens[36]. However, undue release of IL-6 caused excessive inflammatory damage, which in turn leads to lung damage and aggravation of the disease[37].

IL-10 is a cytokine with anti-inflammatory effect[38]. IL-10 could inhibit the effect of activated alveolar macrophages on T helper cells, reduce the release of pro-inflammatory cytokines, and relieve the body's inflammatory response[38]. There was a significant positive correlation between IL-6 level and IL-10 level, which may be due to that IL-6 itself can induce IL-10 expression. In this study, IL-10 level in BALF of children with severe pneumonia were significantly increased, suggesting that IL-10 is also one of the indicators for evaluating the severity of pediatric pneumonia. Studies had shown that IL-10 could inhibit the overproduction of neutrophils[39]. However, in our study, IL-10 level in BALF of children with pneumonia was positively correlated with the percentage of neutrophils. It might be because IL-6 promotes the expression of IL-10 and also promotes the aggregation of neutrophils. So, the level of IL-10, the number and the percentage of neutrophils in BALF can reflect the intensity of the body's inflammation. Our results also showed that IL-10 level in BALF was negatively correlated with the percentage of macrophages, which might be related to the inhibition of macrophages by IL-10[38].

The level of TNF-αwas also positively correlated with the level of IL-6, IL-10, and the percentage of neutrophils. TNF-α seems to have the same effect as IL-6 in amplifying the inflammatory response. TNF-α, secreted by activated alveolar macrophages, is also the major factor affecting the migration of neutrophils to inflammatory sites. Under the synergistic effect of IL-17A and TNF-α, neutrophils migrated rapidly to the inflammation site and persist[40]. Blocking TNF-α could significantly down-regulate inflammatory[41]. Like IL-6 and IL-10, TNF-α is also an indicator of the severity of inflammation in the body. TNF-α was a promoter of lung pathological damage[42]. TNF-α could induce macrophage and neutrophils to release chemokines[43]. And TNF-α could stimulate the release of platelet activating factors to cause vasodilation, and it could promote the adhesion and migration of leukocytes to inflammatory sites[36]. It could lead to more severe inflammatory damage in lung tissue. Therefore, the level of TNF-αmay also indicate the severity of pediatric pneumonia.

We speculate that the lungs of children with severe pneumonia are relatively insufficient in anti-inflammatory cytokines, which is well demonstrated in our study by the levels of anti-inflammatory cytokines. The ratios of IL-6 to IL-10 and TNF-α to IL-10 in BALF of children with severe pneumonia were significantly elevated, indicating the excessive release of pro-inflammatory cytokines and the relative

insufficiency of anti-inflammatory cytokines in the lungs. The imbalance of pro-inflammatory and anti-inflammatory effects exacerbated lung damage. Other studies supported this finding[13,44,45].

In addition, in our study, an increase in the number of inflammatory cells in BALF was also found in children with severe pneumonia. Pro-inflammatory cytokines could help extend the inflammatory response by increasing the total number of inflammatory cells, especially neutrophils[46,47]. Compared with children with mild pneumonia, the percentage of macrophages in BALF in children with severe pneumonia decreased significantly. This decline was relative, because the number of macrophages in children with severe pneumonia was also increased significantly. While, a decrease in the activity of macrophages was also found in bacteria-infected mice, and this change was closely related to abnormal cytokine signals[48]. Microbial infection could change the function of macrophages and impaired their phagocytic and killing ability[50]. In our study, the undue cytokines might inhibit the proliferation and activation of macrophages to avoid more harmful inflammation. Because the levels of cytokines in BALF were negatively correlated with the percentage of macrophages. Both undue accumulation of neutrophils and relative insufficiency of macrophages were associated to lung injury. Unfortunately, due to the lack of data of macrophage activity in BALF in this study, it is not clear whether the decrease in the percentage of macrophages is accompanied by a decrease in macrophage activity.

ROCs of cytokines and inflammatory cells in BALF were used to distinguish severe pneumonia from mild pneumonia. We found that in BALF the levels of IL-6, IL-10 and TNF-α, the ratio of IL-6 to IL-10, the ratio of TNF-α to IL-10, the number of inflammatory cells and neutrophils, the percentage of neutrophils, increased above the threshold could predict severe pediatric pneumonia. And the percentage of macrophage decreased exceeding the threshold could also predict pediatric severe pneumonia. As a result, the signals of inflammatory cytokines and cells in BALF are important biomarkers for predicting the severity of pediatric pneumonia, helping to identify critically ill children more accurately and choose individualized treatment regimens.

And, suppressing of inflammatory storm and regulating of cytokine imbalance should be one of the new therapeutic targets. If the condition of children with pneumonia permits, bronchoalveolar lavage is a promising treatment option to remove and reduce undue cytokines and inflammatory cells for alleviating lung damage. For severe pediatric patients, appropriate anti-inflammatory treatments (including glucocorticoids and immunoglobulins) should be used as an option. Meduri,G.U and colleagues' study[51] showed that long-term use of glucocorticoids could reduce systemic inflammatory response in adults patients with ARDS. Local anti-inflammatory treatments are also worth considering. And inhaled hormones may be one of the important anti-inflammatory treatments, because aerosol inhalation of budesonide could effectively prevent the occurrence of pneumonia after thoracotomy in adults[52]. Of course, the use of glucocorticoids should be more personalized and discreet to achieve greater benefits for children with pneumonia. Other studies reported that intravenous IL-6R antagonists are beneficial for COVID-19 patients[53,54], so local anti-cytokine receptor therapy may be applicable for critical pediatric pneumonia.

Our data demonstrated that the cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ) in BALF of children with pneumonia were positively pairwise correlated. It was also supported by previous studies[55–57], showing a cascade of cytokine responses. Moreover, the levels of cytokines (IL-6, IL-10, TNF-α, IFN-γ) in BALF in children with pneumonia were positively correlated with the number of inflammatory cells and the percentage of neutrophils. This indicated that the pro-inflammatory effect of cytokines was associated with the increase of the total number of inflammatory cells in the lungs, especially the accumulation of neutrophils. Previous studies indicated that excessive accumulation and continuous activation of neutrophils could lead to greater cytokine storms and harmful inflammatory responses[24,46].

There are some limitations in this study. Previous studies[10,11] found that cytokine profiles also played a role in the identifying of pathogens. However, due to individual differences and few children with pneumonia with pathogen mono-infection in our study, no specific profiles of pathogen-related cytokines were found. In addition, because of the ethical restrictions on collecting BALF from healthy children, and it is difficult to determine that relatively healthy children who underwent bronchoscopy and collected BALF had no lung inflammation at all, this study did not set up a healthy child control group.

Conclusion

In summary, our data shows that the signals of cytokines and inflammatory cells in BALF have a unique role in evaluating the severity of inflammatory and pneumonia in children. The undue release of cytokines and inflammatory cells in BALF of children with severe pneumonia are more serious than that of children with mild pneumonia. And both undue accumulation of neutrophils and insufficiency of macrophage are associated with lung injury. ROC analysis showed that the levels of IL-6, IL-10 and TNF-α, the ratios of IL-6 to IL-10 or TNF-α to IL-10, the number of inflammatory cells and neutrophils, the percentage of neutrophils increased over the threshold could be used to distinguish children with severe pneumonia from children with mild pneumonia. Moreover, the decrease in the percentage of macrophages in BALF below the threshold also has predictive effect on severe pediatric pneumonia. Consequently, the signals of cytokines and inflammatory cells in BALF are potentially important biomarkers for predicting the severity of pneumonia. And suppressing of inflammatory storms and regulating of cytokine imbalance (such as, anti-inflammatory therapy, anti-cytokine receptor therapy) may be new treatment chooses for children with pneumonia.

Abbreviations

C-reactive protein (CRP), procalcitonin (PCT), bronchoalveolar lavage fluid (BALF), IL-6 receptor (IL-6R), 2019 coronavirus disease (COVID-19), mycoplasma pneumoniae (MP), Nasopharyngeal aspirates(NPAs), lowest detection limit (LODL), interleukin (IL), tumor necrosis factor (TNF), interferon (IFN), polymerase chain reaction (PCR), chlamydia pneumoniae (CP), receiver operator characteristic curve (ROC), area under curve (AUC), Alanine aminotransferase (ALT), lactate dehydrogenase (LDH), red blood cell count (RBC), white blood cell count (WBC), acute respiratory distress syndrome (ARDS), continuous positive airway pressure (CPAP).

Declarations

Ethics approval

This study approved by the Medical Research Ethics Committee of Children's Hospital of Chongqing Medical University, registered in http://www.chictr.org.cn/, No. ChiCTR2000034048(registration date, June 22, 2020).

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

There is no conflict of interest among authors.

Funding

No funding was received.

Authors' contributions

CS participated in the study design and article revision. QL, YY, YW, XT and SL collected and analyzed the data, FD wrote this manuscript. XH and HC provided technical support. All authors read and approved the final manuscript.

Acknowledges

We acknowledge Xiao li Liu for her help in writing and reviewing the article. And we are grateful for Xiao bo Tu, Yu min Wei and Shuang chun Lei in data management.

References

1. Leonard EJ, Yoshimura T. Neutrophil attractant/activation protein-1 (NAP-1 [interleukin-8]). Am J Respir Cell Mol Biol. 1990;2:479–86.
2. Maus U, Rosseau S, Knies U, Seeger W, Lohmeyer J. Expression of pro-inflammatory cytokines by flow-sorted alveolar macrophages in severe pneumonia. Eur Respir J. 1998;11:534.
3. Seki M, Yanagihara K, Higashiyama Y, et al. Immunokinetics in severe pneumonia due to influenza virus and bacteria coinfection in mice. Eur Respir J. 2004;24:143–9.

4. Spörri R, Joller N, Albers U, Hilbi H, Oxenius A. MyD88-Dependent IFN-γ Production by NK Cells Is Key for Control ofLegionella pneumophila Infection. J Immunol. 2006;176:6162–71.
5. Bordon J, Aliberti S, Fernandez-Botran R, et al. Understanding the roles of cytokines and neutrophil activity and neutrophil apoptosis in the protective versus deleterious inflammatory response in pneumonia. Int J Infect Dis. 2013;17:e76–83.
6. Shinozawa Y, Matsumoto T, Uchida K, Tsujimoto S, Iwakura Y, Yamaguchi K. Role of interferon-gamma in inflammatory responses in murine respiratory infection with Legionella pneumophila. J Med Microbiol. 2002;51:225–30.
7. Mizgerd JP. Inflammation and Pneumonia: Why Are Some More Susceptible than Others? Clin Chest Med. 2018;39:669–76.
8. Yang M, Meng F, Gao M, Cheng G, Wang X. Cytokine signatures associate with disease severity in children with Mycoplasma pneumoniae pneumonia. Sci Rep. 2019;9:17853.
9. Fan H, Lu B, Yang D, Zhang D, Shi T, Lu G. Distribution and Expression of IL-17 and Related Cytokines in Children with Mycoplasma pneumoniae Pneumonia. Jpn J Infect Dis. 2019;72:387–93.
10. Menendez R, Sahuquillo-Arce JM, Reyes S, et al. Cytokine activation patterns and biomarkers are influenced by microorganisms in community-acquired pneumonia. Chest. 2012;141:1537–45.
11. Siljan WW, Holter JC, Nymo SH, et al. Cytokine responses, microbial aetiology and short-term outcome in community-acquired pneumonia. Eur J Clin Invest. 2018;48:e12865.
12. Nguyen TDT, Pham NA, Craig TJ, Duong-Quy S. Clinical characteristics and cytokine changes in children with pneumonia requiring mechanical ventilation. J Int Med Res. 2017;45:1805–17.
13. de Brito RC, Lucena-Silva N, Torres LC, Luna CF, Correia JB, Da SG. The balance between the serum levels of IL-6 and IL-10 cytokines discriminates mild and severe acute pneumonia. Bmc Pulm Med. 2016;16:170.
14. Yende S, D'Angelo G, Kellum JA, et al. Inflammatory markers at hospital discharge predict subsequent mortality after pneumonia and sepsis. Am J Respir Crit Care Med. 2008;177:1242–7.
15. Zhang C, Wu Z, Li JW, Zhao H, Wang GQ. Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. Int J Antimicrob Agents. 2020;55:105954.
16. Arankalle VA, Lole KS, Arya RP, et al. Role of host immune response and viral load in the differential outcome of pandemic H1N1 (2009) influenza virus infection in Indian patients. Plos One. 2010;5:e13099.
17. Nagaoka Y, Nosaka N, Yamada M, et al. Local and Systemic Immune Responses to Influenza A Virus Infection in Pneumonia and Encephalitis Mouse Models. Dis Markers. 2017;2017:1–7.
18. Boutten A, Dehoux MS, Seta N, et al. Compartmentalized IL-8 and elastase release within the human lung in unilateral pneumonia. Am J Respir Crit Care Med. 1996;153:336–42.
19. Yang M, Meng F, Wang K, et al. Interleukin 17A as a good predictor of the severity of Mycoplasma pneumoniae pneumonia in children. Sci Rep. 2017;7:12934.

20. M H, J C, N C, et al. British Thoracic Society guidelines for the management of community acquired pneumonia in children: update 2011. Thorax. 2011;66:i1-i23. DOI:10.1136/thoraxjnl-2011-200598.
21. National Health Commission Of The People'S Republic Of China SAOT. Guideline for diagnosis and treatment of community-acquired pneumonia in Children (2019 version). Vol. 12: Chin J Chin Infect Dis; 2019. DOI:10.3760/cma.j.issn.1674-2397.2019.01.002.
22. Kragsbjerg P, Jones I, Vikerfors T, Holmberg H. Diagnostic value of blood cytokine concentrations in acute pneumonia. Thorax. 1995;50:1253–7.
23. Emergency Physicians Branch CMDA. Expert consensus on the diagnosis and treatment of adult community-acquired pneumonia in the emergency department. CHINESE JOURNAL OF CRITICAL CARE MEDICINE. 2011;31:961–7. DOI:10.1093/cid/cir531.
24. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. Semin Immunopathol. 2017;39:529–39.
25. Matsuse H, Yanagihara K, Mukae H, Tanaka K, Nakazato M, Kohno S. Association of plasma neutrophil elastase levels with other inflammatory mediators and clinical features in adult patients with moderate and severe pneumonia. Respir Med. 2007;101:1521–8.
26. Schutte H, Lohmeyer J, Rosseau S, et al. Bronchoalveolar and systemic cytokine profiles in patients with ARDS, severe pneumonia and cardiogenic pulmonary oedema. Eur Respir J. 1996;9:1858–67.
27. Hagau N, Slavcovici A, Gonganau DN, et al. Clinical aspects and cytokine response in severe H1N1 influenza A virus infection. Crit Care. 2010;14:R203.
28. Doughty LA, Kaplan SS, Carcillo JA. Inflammatory cytokine and nitric oxide responses in pediatric sepsis and organ failure. Crit Care Med. 1996;24:1137–43.
29. Michelow IC, Katz K, McCracken GH, Hardy RD. Systemic cytokine profile in children with community-acquired pneumonia. Pediatr Pulmonol. 2007;42:640–5.
30. Haugen J, Chandyo RK, Brokstad KA, et al. Cytokine Concentrations in Plasma from Children with Severe and Non-Severe Community Acquired Pneumonia. Plos One. 2015;10:e138978.
31. Paats MS, Bergen IM, Hanselaar WE, et al. Local and systemic cytokine profiles in nonsevere and severe community-acquired pneumonia. Eur Respir J. 2013;41:1378–85.
32. Strieter RM, Chensue SW, Basha MA, et al. Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor-alpha, lipopolysaccharide, and interleukin-1 beta. Am J Respir Cell Mol Biol. 1990;2:321–6.
33. N N. T K. Interleukin 6: from bench to bedside. Nat Clin Pract Rheumatol. 2006;2:619–26.
34. Qiu H, KuoLee R, Harris G, Chen W. High susceptibility to respiratory Acinetobacter baumannii infection in A/J mice is associated with a delay in early pulmonary recruitment of neutrophils. Microbes Infect. 2009;11:946–55.
35. X G, J Y. H W, et al. Streptococcus pneumoniae IL-6 During Influenza- Co-Infected Pneumonia-A Protector. Front Immunol. 2019;10:3102. DOI:10.3389/fimmu.2019.03102.

36. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis. 1990;141:471–501.
37. Gubernatorova EO, Gorshkova EA, Polinova AI, Drutskaya MS. IL-6: Relevance for immunopathology of SARS-CoV-2. Cytokine Growth Factor Rev. 2020;53:13–24.
38. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A Pillars Article:IL-10 Inhibits Cytokine Production by Activated Macrophages. J. Immunol. 1991. 147: 3815–3822. J Immunol. 2016;197:1539–1546.
39. Cox G. IL-10 enhances resolution of pulmonary inflammation in vivo by promoting apoptosis of neutrophils. Am J Physiol. 1996;271:L566–71. DOI:10.1152/ajplung.1996.271.4.L566.
40. X S, Y Q. The activation and regulation of IL-17 receptor mediated signaling. Cytokine. 2013;62:175–182.
41. Shi X, Zhou W, Huang H, et al. Inhibition of the inflammatory cytokine tumor necrosis factor-alpha with etanercept provides protection against lethal H1N1 influenza infection in mice. Crit Care. 2013;17:R301.
42. Du TLNL. L, et al. Tumor necrosis factor-α plays an initiating role in extracorporeal circulation-induced acute lung injury. Lung. 2013;191:207–14.
43. JD RM. L, DL L. Anti-TNFα therapy in inflammatory lung diseases. Pharmacol Therapeut. 2017;180:90–8.
44. Ding S, Wang X, Chen W, et al. Decreased Interleukin-10 Responses in Children with Severe Mycoplasma pneumoniae Pneumonia. Plos One. 2016;11:e146397.
45. Saghafian-Hedengren S, Mathew JL, Hagel E, et al. Assessment of Cytokine and Chemokine Signatures as Potential Biomarkers of Childhood Community-acquired Pneumonia Severity: A Nested Cohort Study in India. Pediatr Infect Dis J. 2017;36:102–8.
46. Chen Z, Shao X, Dou X, et al. Role of the Mycoplasma pneumoniae/Interleukin-8/Neutrophil Axis in the Pathogenesis of Pneumonia. Plos One. 2016;11:e146377.
47. Guo L, Liu F, Lu MP, Zheng Q, Chen ZM. Increased T cell activation in BALF from children with Mycoplasma pneumoniae pneumonia. Pediatr Pulmonol. 2015;50:814–9.
48. Moser EK, Field NS, Oliver PM. Aberrant Th2 inflammation drives dysfunction of alveolar macrophages and susceptibility to bacterial pneumonia. Cell Mol Immunol. 2018;15:480–92.
49. Nolan TJ, Gadsby NJ, Hellyer TP, et al. Low-pathogenicity Mycoplasma spp. alter human monocyte and macrophage function and are highly prevalent among patients with ventilator-acquired pneumonia. Thorax. 2016;71:594–600.
50. Meduri GU, Tolley EA, Chrousos GP, Stentz F. Prolonged methylprednisolone treatment suppresses systemic inflammation in patients with unresolving acute respiratory distress syndrome: evidence for inadequate endogenous glucocorticoid secretion and inflammation-induced immune cell resistance to glucocorticoids. Am J Respir Crit Care Med. 2002;165:983–91.

51. Li G, Wang JS, Qin SD, et al. [Clinical study of the oxygen drive aerosol inhalation with budesonide and ambroxol in the prevention of adult post-thoracotomy pneumonia]. Beijing Da Xue Xue Bao Yi Xue Ban. 2018;50:840–4.
52. De Luna G, Habibi A, Deux JF, et al. Rapid and severe Covid-19 pneumonia with severe acute chest syndrome in a sickle cell patient successfully treated with tocilizumab. Am J Hematol. 2020;95:876–8.
53. Toniati P, Piva S, Cattalini M, et al. Tocilizumab for the treatment of severe COVID-19 pneumonia with hyperinflammatory syndrome and acute respiratory failure: A single center study of 100 patients in Brescia, Italy. Autoimmun Rev. 2020;19:102568.
54. Wang H, Zhou Q, Dai W, et al. Lung Microbiota and Pulmonary Inflammatory Cytokines Expression Vary in Children With Tracheomalacia and Adenoviral or Mycoplasma pneumoniae Pneumonia. Front Pediatr. 2019;7:265.
55. Glynn P, Coakley R, Kilgallen I, Murphy N, O'Neill S. Circulating interleukin 6 and interleukin 10 in community acquired pneumonia. Thorax. 1999;54:51–5.
56. Zhang Y, Mei S, Zhou Y, Huang M, Dong G, Chen Z. Cytokines as the good predictors of refractory Mycoplasma pneumoniae pneumonia in school-aged children. Sci Rep. 2016;6:37037.

Figures

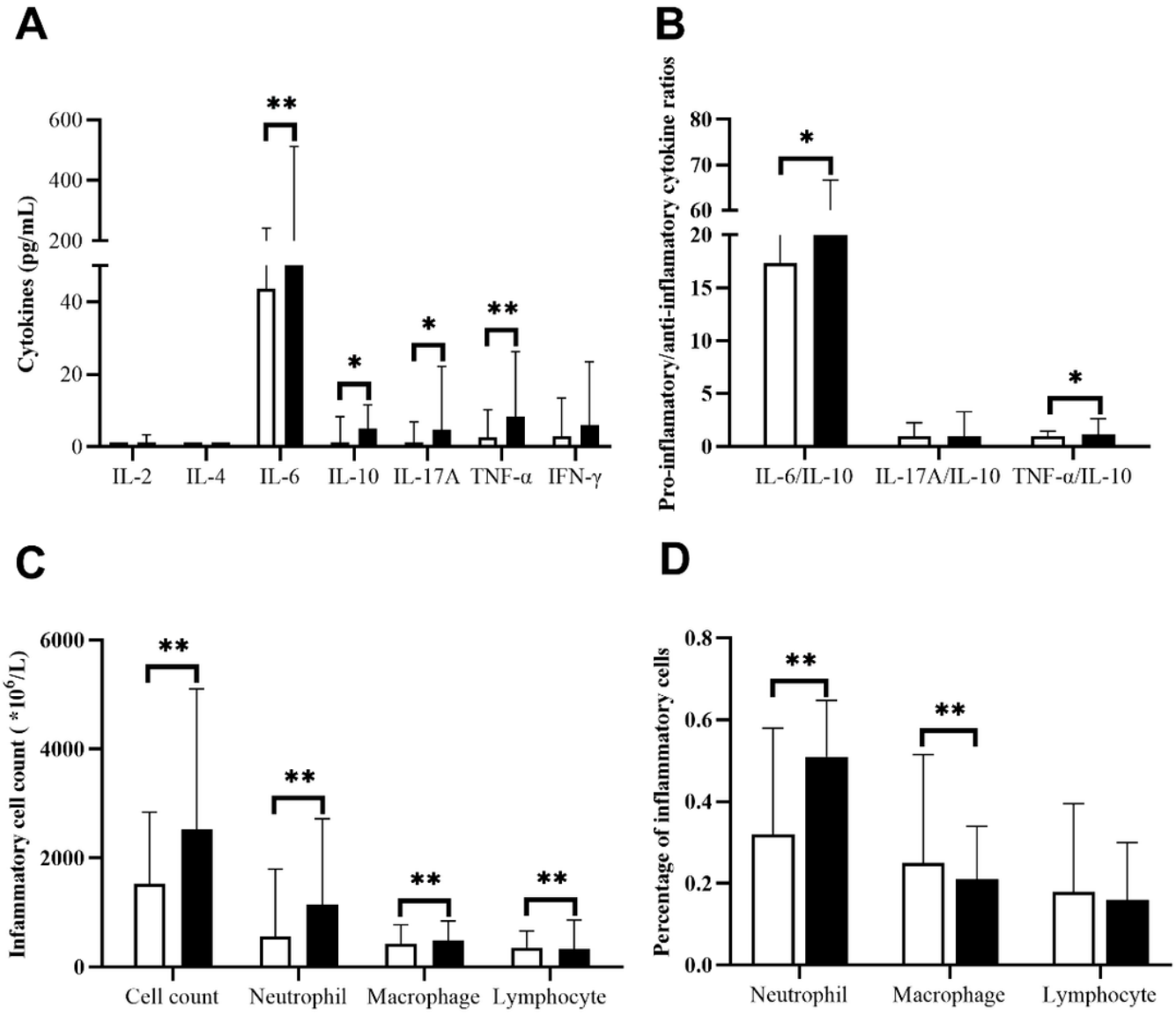

Figure 1

Cytokines and inflammatory cells in BALF. BALF was collected from children with mild (white bar) and severe pneumonia child (black bar). (A) Quantitation of flow cytometry assay for cytokines, including IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ. (B) Ratios of IL-6 to IL-10, IL-17A to IL-10, and TNF-α to IL-10. (C) Number of inflammatory cells in BALF. (D) Percentage of inflammatory cell in total inflammatory cell count. The bars were represented by median and interquartile range. *P<0.05, **P<0.01, mild pneumonia group vs. severe pneumonia group.

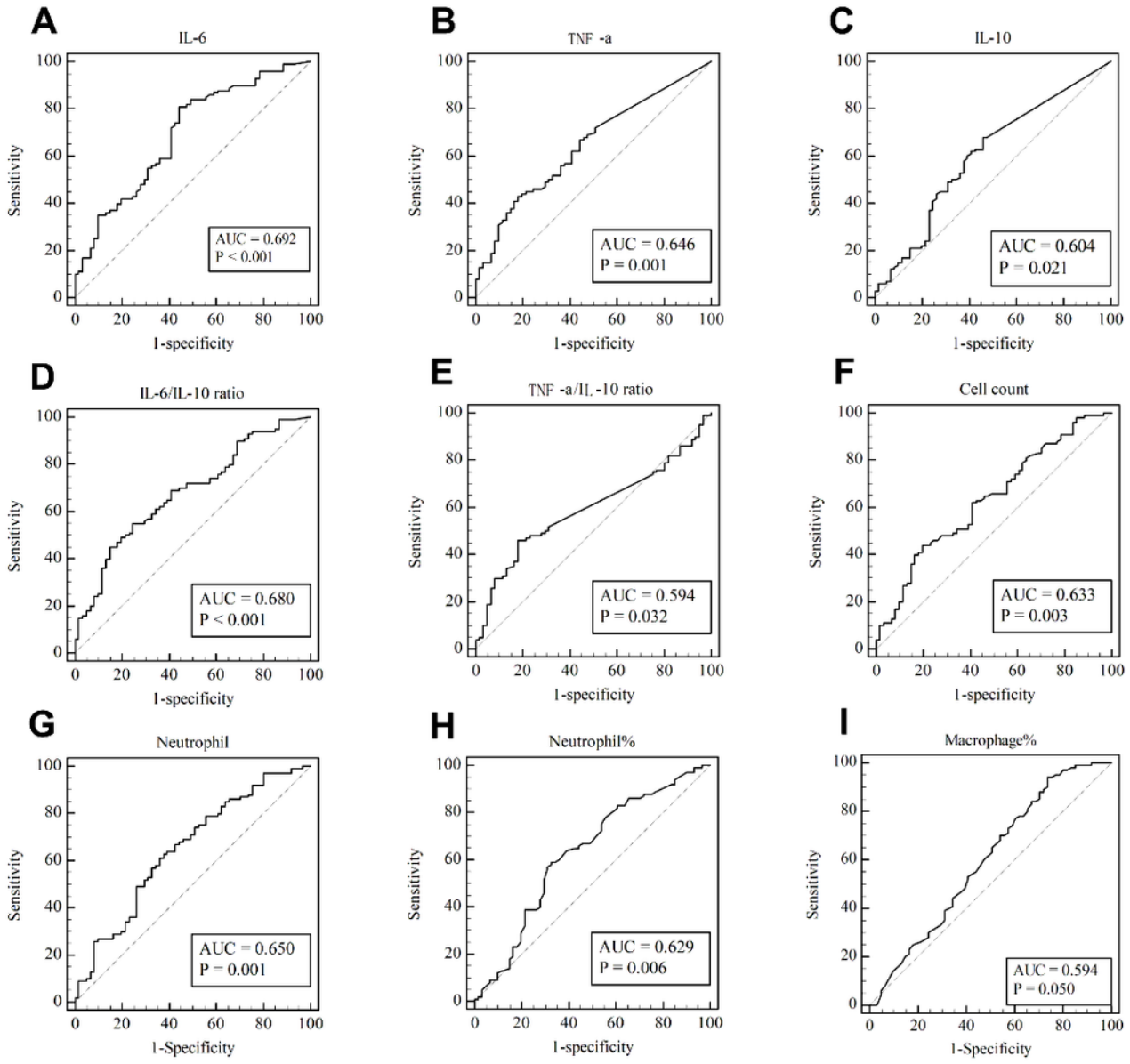

Figure 2

ROCs of cytokines and inflammatory cells in BALF to distinguish severe pneumonia from mild pneumonia. The area under curve (AUC) of ROC represented the predictive value of biomarker, and the higher the AUC, the higher the predictive value of biomarker. P<0.05 suggests that the predictive value is significant. (A) IL-6. (B) TNF- α. (C) IL-10. (D) Ratio of IL-6 to IL-10. (E) Ratio of TNF-α to IL-10. (F) Number of inflammatory cells. (G) Percentage of neutrophils. (H) Percentage of macrophages.

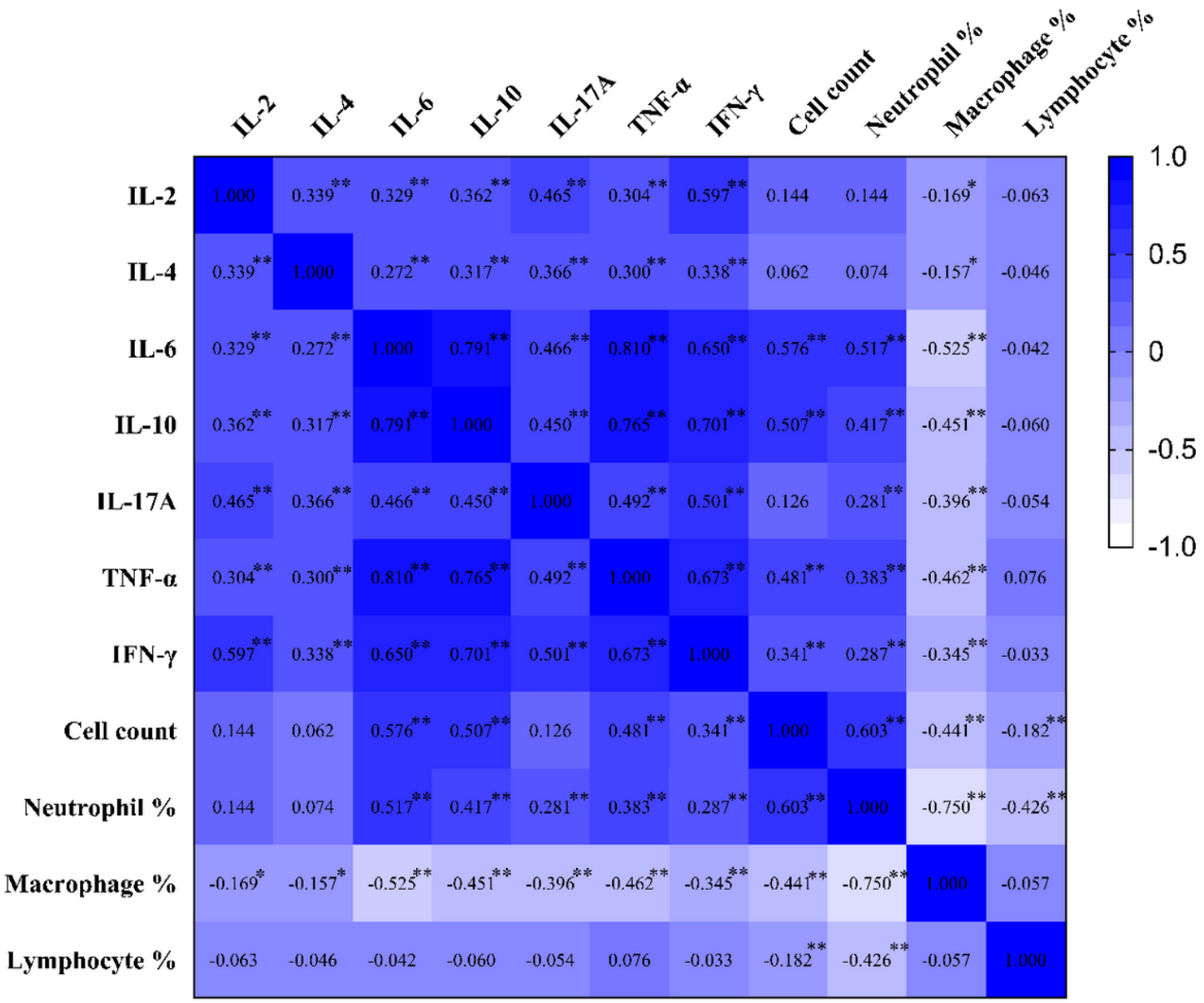

Figure 3

Heatmap of cytokines and inflammatory cells in BALF from pneumonia children. The bar represented the range of the correlations between indicators, from -1 (white) to 1(dark blue). The greater the absolute value, the stronger the correlation, and minus mean sign indicts the negative correlation. *P<0.05, **P<0.01, and it suggests that the correlation is significant.