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The profile of peripheral blood lymphocyte subsets and serum cytokines in children with 2019 novel coronavirus pneumonia

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SUMMARY

Objectives: The study was aimed at investigating the characteristics of peripheral blood lymphocyte subsets and serum cytokines in children with 2019 novel coronavirus (2019-nCoV) pneumonia.

Methods: Children with 2019-nCoV pneumonia or with respiratory syncytial virus (RSV) pneumonia were included. Data including lymphocyte subsets and serum cytokines were collected and analyzed.

Results: 56 patients were included in the study, 40 children with 2019-nCoV pneumonia and 16 children with RSV pneumonia. Compared with children with RSV pneumonia, patients with 2019-nCoV pneumonia had higher count of CD3+8+ lymphocyte, higher percentages of CD3+, CD3+8+ lymphocytes and a lower percentage of CD19+ lymphocyte. The serum IL-10 level was significantly higher in children with RSV pneumonia. One 2019-nCoV pneumonia child who was with an obvious increase of IL-10 developed severe pneumonia.

Conclusions: Immune response played a very important role in the development of 2019-nCoV pneumonia. The effective CD8+ T cell response might influence the severity of 2019-nCoV pneumonia. The adaptable change in IL-10 level might contribute to the relatively mild pneumonia symptoms in children with 2019-nCoV pneumonia and bacterial co-infection might be a risk factor of severe 2019-nCoV pneumonia.

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Introduction

2019 novel coronavirus (2019-nCoV) was first detected in Wuhan, China. It was reported in adults that the virus could cause severe respiratory illness and 2019-nCoV pneumonia was more likely to be fatal to the elderly with comorbidities.^{1,2} Humans of all ages are susceptible to the virus.

Researches proved that host immune response played a pivotal role in clearing viral infections from the lung.³ Children are more susceptible to infections as their immune system is developing. Some viruses, such as respiratory syncytial virus (RSV), mainly cause severe pneumonia in children less than 5 years of age. Theoretically, when children were infected with 2019-nCoV, the prognosis of infected children might be poorer than adults. However, research concerning children with 2019-nCoV infection reported that the clinical manifestations of 2019-nCoV infection in pedi-

atric patients were mild.⁴ In our clinical practice, we noticed that the severity of respiratory symptoms in children with 2019-nCoV pneumonia was also different from adults with 2019-nCoV pneumonia. Children with 2019-nCoV pneumonia seldom developed dyspnea. Based on the unique clinical features in children who were infected with 2019-nCoV, we hypothesized that there might be some characteristic immune responses that help children avoid suffering from severe 2019-nCoV pneumonia.

Researches about the function of lymphocyte subsets in viral diseases were conducted for decades.⁵ Studies showed that Cytotoxic CD8+ T cells are important cells in viral clearance,⁶ however, they can also cause severe damage to the lung if they were not well regulated.

In the present study, we retrospectively investigated the characteristics of blood lymphocyte subsets and serum cytokines in children with 2019-nCoV pneumonia and tried to find some clues in immune response for the treatment of severe 2019-nCoV pneumonia. As the city is blocked during the study period and blood samples from healthy children could not be obtained, lymphocyte subsets and cytokines in children with 2019-nCoV pneumonia were compared with those in children with RSV pneumonia, a relatively well-studied viral pneumonia of children.

Abbreviations: 2019-nCoV, 2019 novel coronavirus; RSV, respiratory syncytial virus; Tregs, regulatory T cells; Breg, regulatory B lymphocytes.

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Table 1
The patients' demographic data and laboratory findings.

characteristics	with 2019-nCoV pneumonia	with RSV pneumonia	P value
number of patients (n)	40	16	–
age (years) [mean (sd)]	5.09(4.71)	1.36(0.85)	0.000 ^a
gender (male/ female) (n)	23/17	10/6	0.731 ^c
body weight(kg) [median(IQR)]	14.50(9.48–29.75)	10.00(8.63– 12.25)	0.009 ^b
height(cm) [mean (sd)]	106.58(38.27)	79.19(13.09)	0.000 ^a
ALT (U/L) [mean (sd)]	24.25(18.09)	29.88(19.47)	0.308 ^a
serum creatinine ($\mu\text{mol/L}$) [median(IQR)]	30.0(23.2–40.5)	24.0(20.1–27.0)	0.007 ^b
CRP (mg/L) [median(IQR)]	1.86(0.75–4.80)	2.6(0.81–7.66)	0.483 ^b
PCT (ng/ml) [median(IQR)]	0.065(0.050–0.090)	0.105(0.090–0.292)	0.002 ^b
white blood cell count ($\times 10^9/\text{L}$) [mean (sd)]	6.86(2.89)	7.10(2.69)	0.780 ^a
neutrophil% [mean (sd)]	39.65(15.23)	40.93(19.69)	0.795 ^a
lymphocyte% [mean (sd)]	50.69(15.58)	49.12(18.72)	0.748 ^a
concomitant pathogens			
mycoplasma	13	7	0.427 ^c
influenza A or B virus	3	1	–
adenovirus	1	0	–
streptococcus pneumonia	1	0	–

^a Normally distributed continuous variables were reported as means with standard deviations (SD) and compared with Student's t-test.

^b Non-normally distributed continuous variables were reported as medians with inter-quartile range (IQR) and compared with Mann-Whitney U test.

^c Categorical data were reported as proportions(n with%)and compared using Chi-square test.

Patients and methods

Patients

Subjects of the study were children with either confirmed 2019-nCoV pneumonia (admitted between 24 January and 22 February 2020) or RSV pneumonia (admitted between 10 December 2019 and 22 February 2020) in Wuhan Children's hospital and patients who underwent the detection of peripheral blood lymphocyte subsets were included in the study. 2019-nCoV infection was confirmed by real-time RT-PCR. All the 2019-nCoV pneumonia patients were diagnosed according to the interim protocol of diagnosis and treatment of 2019 novel coronavirus-associated pneumonia (the fifth revised version).⁷ Previously healthy children were included in the study, and children receiving chemotherapy, treatment of glucocorticoids or immunosuppressant before the diagnosis of the pneumonia were not included in the study as their immune response to viral infections might be different, and should be analyzed separately. Children with 2019-nCoV infections were admitted to isolation wards and treated separately from children with other diseases. The study was approved by the Research Ethics Board of Wu Han Children's Hospital. Consent of the patients' legal guardians was obtained.

Children admitted to ICU were defined as having severe disease, while those treated in the general wards were defined as with mild to moderate disease.

Demographic data, clinical manifestations, laboratory findings (including C-reactive protein (CRP), procalcitonin (PCT), serum creatinine (Scr), alanine aminotransferase (ALT), lymphocyte subsets, cytokines (IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ)) and treatments were recorded from the medical records.

Lymphocyte subsets and cytokines were detected by flow cytometry analysis.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS version 21.0, SPSS Inc., Chicago, IL, USA). Normally distributed continuous variables were presented as means with standard deviations (SD) and compared with a Student's t-test. Non-normally distributed continuous variables data were presented as medians with inter-quartile range (IQR) and compared with a Mann-Whitney U test. Categorical data were presented as proportions and compared using a Chi-square test. Vari-

ables with a two-tailed p value < 0.05 were considered statistically significant.

Results

40 children with 2019-nCoV pneumonia (2019-nCoV pneumonia group) and 16 children with RSV pneumonia (RSV pneumonia group) were included in the study. Symptoms at onset of 2019-nCoV pneumonia were fever (52.5%), cough (67.5%), fatigue (10%), diarrhea (5%), sore throat (5%), and a runny nose (5%). One child with severe 2019-nCoV pneumonia suffered from acute kidney injury on admission. Beside 2019-nCoV or RSV, some of the studied children were concomitantly infected with other pathogens. The patients' clinical characteristics, details of the concomitant pathogens were presented in Table 1. There were significant differences in age, height, body weight, serum creatinine and PCT between the two groups. The age of 2019-nCoV pneumonia patients ranged from 16 days to 14.2 years, while RSV mainly infected children under 5 years of age. Decreased lymphocyte was found in only 1 child with 2019-nCoV pneumonia. There were no significant differences in white blood cell (WBC) count, neutrophil (NEU)% and lymphocyte (LYM)% between the two groups.

All patients had a chest CT test within 3 days after the admission to hospital. Of the 40 2019-nCoV pneumonia children, on chest CT, 13 children were with unilateral involvement, 26 children had bilateral lesions, and no lesion was shown in 1 child. Of the RSV pneumonia children, 3 children were with unilateral damage, 12 children were with bilateral lesions and no lesion was shown in 1 child.

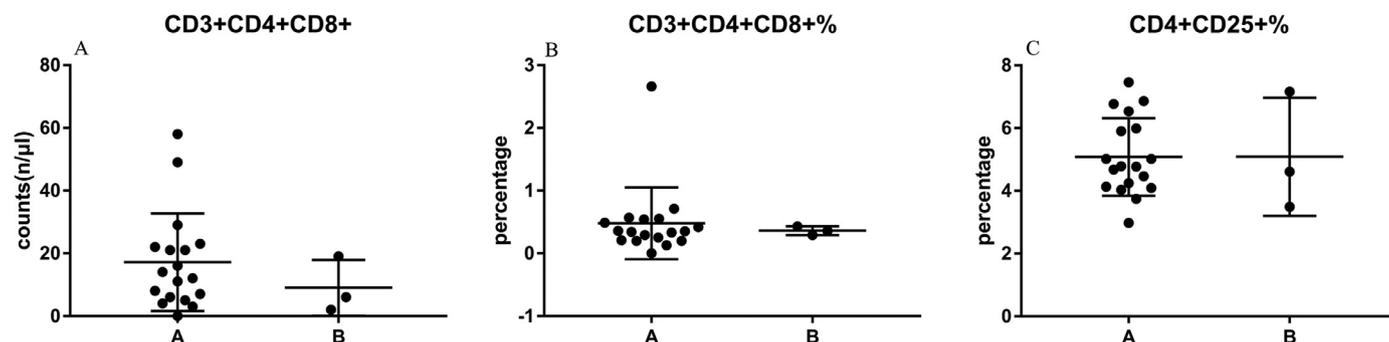
49 children (40 in 2019-nCoV pneumonia group and 9 in RSV pneumonia group) received the treatment of interferon- α nebulization. 20 patients with 2019-nCoV pneumonia received oral oseltamivir. 5 children (4 in 2019-nCoV pneumonia group and 1 in RSV pneumonia group) received intravenous immunoglobulin. Intravenous glucocorticoids were used in 7 children (3 in 2019-nCoV pneumonia children and 4 in RSV pneumonia children). 20 children (13 in 2019-nCoV pneumonia children and 7 in RSV pneumonia children) with concomitant infection of mycoplasma were treated with oral azithromycin.

Of all children, 3 patients developed severe pneumonia, 1(2.5%) in 2019-nCoV pneumonia children and 2(12.5%) in RSV pneumonia group. No children in the study died. As the numbers of patients with severe pneumonia were small, when data were analyzed, the patients were not stratified according to the severity degree

Table 2

Blood lymphocyte subsets of children with 2019-nCoV pneumonia and children with RSV pneumonia.

Variables	with 2019-nCoV pneumonia	with RSV pneumonia	P value
number of patients (n)	40	16	–
CD3+(n/ μ l) [mean (sd)]	2594(1270)	2029(1278)	0.140 ^a
CD3+% [mean (sd)]	67.77(7.71)	58.09(10.57)	0.000 ^a
CD3+CD4+(n/ μ l) [mean (sd)]	1469(872)	1187(821)	0.271 ^a
CD3+CD4+% [mean (sd)]	37.05(7.62)	33.20(7.41)	0.091 ^a
CD3+ CD 8+(n/ μ l) [mean (sd)]	933(421)	675(426)	0.044 ^a
CD3+ CD 8+% [mean (sd)]	25.80(6.59)	20.36(5.23)	0.005 ^a
CD4+/CD8+ [mean (sd)]	1.57(0.69)	1.73(0.57)	0.424 ^a
CD16+CD56+ (n/ μ l) [median(IQR)]	309(197–595)	349(167–521)	0.856 ^b
CD16+CD56+% [mean (sd)]	11.80(5.77)	12.02(4.97)	0.893 ^a
CD19+ (n/ μ l) [mean (sd)]	727(460)	938(718)	0.197 ^a
CD19+% [mean (sd)]	18.68(7.98)	27.83(10.74)	0.001 ^a

^a Normally distributed continuous variables were reported as means with standard deviations (SD) and compared with Student's t-test.^b Non-normally distributed continuous variables were reported as medians with inter-quartile range (IQR) and compared with Mann-Whitney U test.**Fig. 1.** Profile of CD3+CD4+CD8+ and CD4+CD25+ lymphocytes in children with 2019-nCoV pneumonia and children with RSV pneumonia

A 2019-nCoV pneumonia children

B RSV pneumonia children

The variables in group A were normally distributed and were presented as means with standard deviations.

of illness. The proportion of severe pneumonia was higher in the RSV pneumonia group. However, the difference in the severity of pneumonia between the two groups was not significant which made the data from the two groups of children comparable.

A thirteen months of age child with 2019-nCoV pneumonia developed dyspnea and was admitted to ICU. The boy recovered after being supported by mechanical ventilation and treated with interferon- α -1b nebulization (day1-day4), intravenous immunoglobulin (day1-day4), glucocorticoids (methylprednisolone, day1-day6; hydrocortisone, day9), antibacterial agents (meropenem, day1-day9; linazolamide, day1-day12; piperacillin tazobactam, day14-day19) and ribavirin(day9-day17), and was discharged on the 17th day when the 2019-nCoV nucleic acid was proved to be negative for two consecutive tests.

Lymphocyte subsets

CD3+, CD3+CD4+, CD3+CD8+, CD16+CD56+ and CD19+ lymphocytes were detected in all children. CD3+CD 4+CD 8+, CD4+CD25+ lymphocytes were detected in 18 children with 2019-nCoV pneumonia, and in 3 children with RSV pneumonia.

All the blood samples for the first detection of lymphocyte subsets were obtained within 3 days after admission to hospital. For children who received the treatment of intravenous glucocorticoids, the blood samples for the first detection of lymphocyte subsets were obtained before the first administration of glucocorticoids.

The details of CD3+, CD3+CD4+, CD3+CD8+, CD16+CD56+ and CD19+ lymphocytes were presented in Table 2. Compared with RSV pneumonia children, patients with 2019-nCoV pneumo-

nia had higher count of CD3+CD8+ lymphocyte, higher percentage of CD3+ and CD3+CD8+ lymphocytes and lower percentage of CD19+ lymphocyte. There were no significant differences in CD4+/CD8+, the counts of CD3+, CD3+CD4+, CD16+CD56+ and CD19+ lymphocytes and the percentage of CD3+CD4+ and CD16+CD56+ lymphocytes between the two groups.

CD3+CD4+CD8+ and CD4+CD25+ lymphocytes were shown in Fig. 1. It seemed that the CD3+CD4+CD8+ and CD4+CD25+ lymphocytes were similar in RSV pneumonia children and 2019-nCoV pneumonia children.

Cytokine concentrations

Serum cytokines IL-2, IL-4, IL-6, IL-10, TNF- α and IFN- γ were detected in 30 children with 2019-nCoV pneumonia, and in 5 children with RSV pneumonia within 3 days after admission to hospital.

IL-10 was significantly higher in children with RSV pneumonia while there were no significant differences in IL-2, IL-4, IL-6, TNF- α and IFN- γ (Fig. 2).

In children with 2019-nCoV pneumonia IL-6 ranged from 1.74 to 120.31 pg/ml and rose in 6 children according to the normal reference range provided by the test reports. IL-10 ranged from 2.29 to 34.91pg/ml in children with 2019-nCoV pneumonia, and increased in 10 children, among whom 6 children were with a slight increase of IL-10 (less than twice the value of maximal normal reference), and 2 children were with an obvious increase (more than three times the value of maximal normal reference). One of the two children who were with an obvious increase in IL-10 developed severe 2019-nCoV pneumonia.

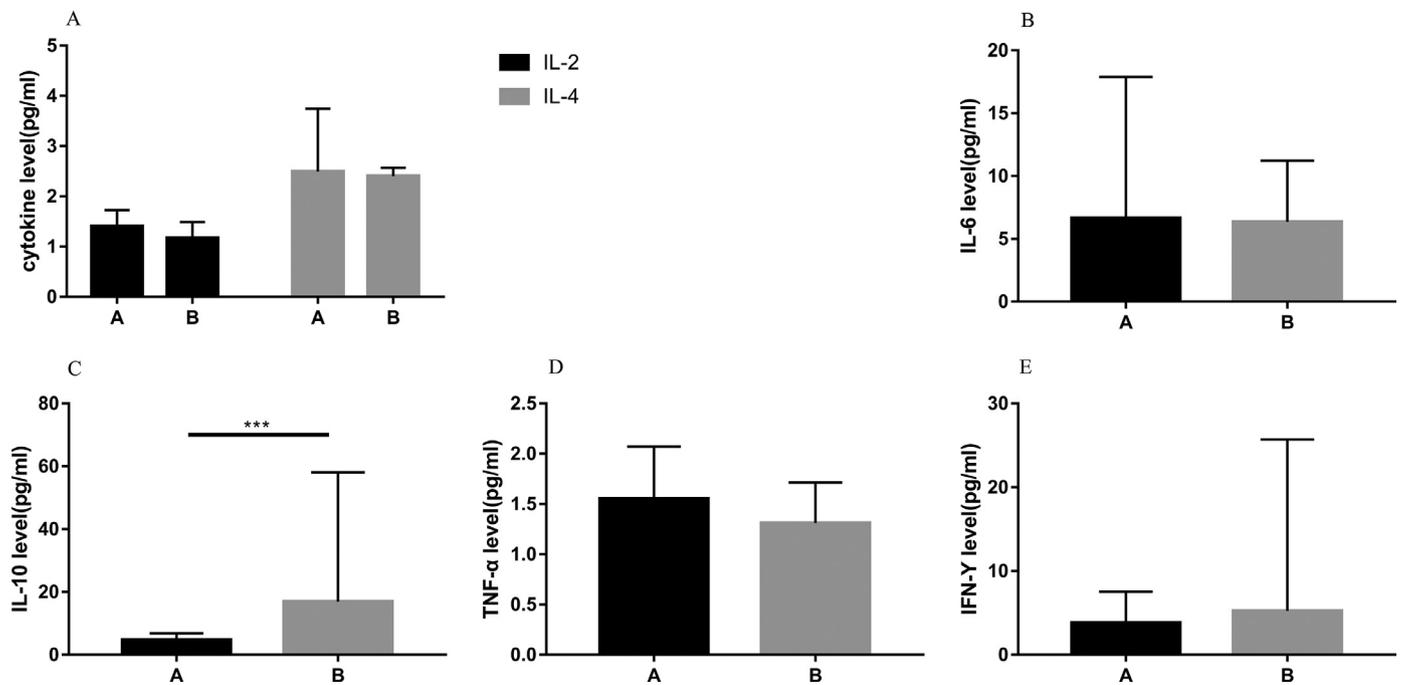


Fig. 2. Serum cytokine profile of children with 2019-nCoV pneumonia and children with RSV pneumonia

A 2019-nCoV pneumonia children

B RSV pneumonia children

Serum cytokine values were non-normally distributed and were presented as medians with inter-quartile range (IQR) and compared with Mann-Whitney U test.

***p = 0.003.

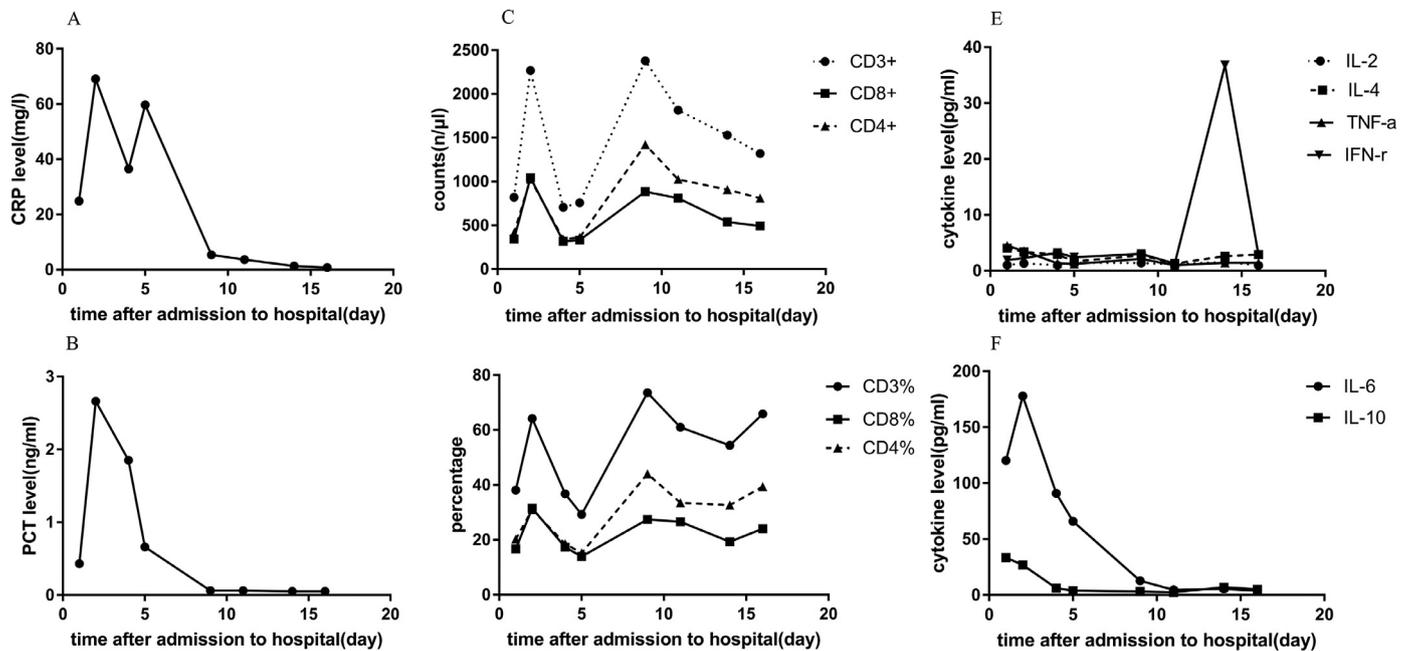


Fig. 3. The fluctuation of CRP, PCT, lymphocyte subsets and serum cytokine concentrations of the child with severe 2019-nCoV pneumonia

The blood samples for the detection were collected on day1, day2, day4, day5, day9, day11, day14 and day16 after admission to hospital.

In the child with severe 2019-nCoV pneumonia who underwent consecutive surveillance of the lymphocyte subsets, the first blood sample was obtained on day1 before the first administration of glucocorticoids, and the following blood samples were collected in the early morning of day2, day4, day5, day9, day11, day14 and day16 after admission to hospital. The fluctuations of CRP, PCT, lymphocyte subsets and serum cytokine concentrations of the child were shown in Fig. 3. IL-6 level rose rapidly together with the increased CRP and PCT levels, and gradually returned to normal

range concomitantly with CRP and PCT levels after powerful antibiotic therapy. The IL-10 increased on admission and gradually decreased during the convalescent period. The counts of CD3+, CD3+CD4+ and CD3+CD8+ lymphocytes, the percentage of CD3+, CD3+CD4+ and CD3+CD8+ lymphocytes decreased in the acute phase of the disease and increased when the child recovered from the disease. The changes of IL-2, IL-4, TNF- α , IFN- γ were slight in the acute phase of the disease. In the convalescent period, the level of IFN- γ increased obviously.

Discussion

The initiation and regulation of innate and adaptive immune responses played a pivotal role in controlling viral infections and mitigating immune-mediated injury. The function of lymphocyte subsets and cytokines in 2019-nCoV infections remains unclear now. Knowledge about the characteristics of lymphocyte subsets and cytokines in 2019-nCoV infections can help clinicians to make effective strategies in clearing the virus and avoiding pulmonary injuries caused by extensive immune responses.

Studies conducted in animal models have proven the prominent role of CD8⁺ T cells in respiratory viral clearance.⁸ Research investigating the immune responses in patients with Middle East respiratory syndrome coronavirus (MERS-CoV) infection found that the early CD8⁺ T cell response was associated with the severity of the disease.⁹ Effective T cell responses could help clear MERS-CoV efficiently and avoid a high level of plasma pro-inflammatory cytokines which can cause strong inflammatory and eventually lead to lung tissue injury. It seemed that CD8⁺ T cell response was also relevant to the severity of COVID-19 infections. In an adult with severe COVID-19 infection, the counts of CD4 and CD8 T cells in peripheral blood were found to decrease substantially.¹⁰ In this study, the child with severe 2019-nCoV pneumonia had low CD3⁺CD8⁺ lymphocyte count and low CD3⁺CD8⁺ lymphocyte percentage on admission, while the counts of CD3⁺CD8⁺ lymphocytes, percentages of CD3⁺, CD3⁺CD8⁺ lymphocytes were higher in 2019-nCoV pneumonia group compared with those in RSV pneumonia group. RSV is a common pathogen of infantile pneumonia, and can cause severe lower respiratory tract infection. CD8⁺ T cell counts in children with RSV infection were found significantly lower compared with healthy children.¹¹ Effective CD8⁺ T cell activation might be one of the reasons why the symptoms of pneumonia in most children with 2019-nCoV pneumonia were mild to moderate. We speculated that the effective CD8⁺ T cell response had an important role in the development of 2019-nCoV pneumonia and CD8⁺ T cell could be used as a biomarker to predict the prognosis of COVID-19 infection.

CD4⁺ regulatory T cells (Tregs) are important in efficiently activating the CD8⁺ T cell response to acute respiratory virus infection in the early stage of the infection.¹² CD4⁺Treg was detected in part of the children studied. It seemed that the CD4⁺Treg was similar in children with 2019-nCoV pneumonia and children with RSV pneumonia which indicated that 2019-nCoV had no unique influence on CD4⁺Treg compared with RSV.

IL-10 is an immune regulatory cytokine and has been proven to be able to limit immune responses in the lung. The anti-inflammatory effects of IL-10 can help the host tolerate infection and avoid pulmonary injury caused by an extensive immune response. However, on the other hand, the inhibition of protective immunity by IL-10 can dampen the clearance of pulmonary pathogens.¹³ IL-10 could be secreted by regulatory B lymphocytes (Breg). A study about neonatal-specific regulatory B (nBreg) cells in human neonates with RSV infection showed that the RSV-infected nBreg increased the severity of the infection by producing IL-10 and weakening Th1 cell responses.¹⁴ In this study, Breg was not detected. However, the higher percentage of CD19⁺ lymphocyte might be one of the reasons for the higher IL-10 levels in children with RSV pneumonia and influenced the severity of the RSV pneumonia. In adults with 2019-nCoV infection, IL10 was found to be higher in ICU patients than non-ICU patients.¹ In this study, of 30 children with 2019-nCoV pneumonia who underwent the detection of cytokines, IL-10 level obviously increased in 2 children, one of who developed severe 2019-nCoV pneumonia. It seemed that IL10 was also relevant to the severity of the 2019-nCoV infection and the adaptable change in IL-10 level contributed to the relatively slight pneumonia symptoms in children. However, as the

number of severe children with 2019-nCoV pneumonia was small in the study, the result needed to be verified in a study with a larger number of patients.

IL-6 is a pro-inflammatory cytokine and increases in patients with bacterial infections. Its value in the diagnosis of bacterial sepsis has been widely studied in adults.¹⁵ The difference in IL-6 level was not found between children with 2019-nCoV pneumonia and children with RSV pneumonia. In the child with severe 2019-nCoV pneumonia, the trend of IL-6 level was similar to those of CRP and PCT levels, and IL-6 level returned to normal range concomitantly with CRP and PCT levels after powerful antibiotic therapy. The increased IL-6 level might be attributed to bacterial co-infection and bacterial co-infection might be a risk factor of severe 2019-nCoV pneumonia.

There were two main limitations in this study. Firstly, the study was aimed to find some characteristics of the immune response in children with 2019-nCoV pneumonia. As no blood samples could be obtained from healthy children, we could not get direct evidence about the role of the studied lymphocyte subsets and cytokines in the development of 2019-nCoV pneumonia by comparing with healthy children. However, we tried to find some profile of the immune response by comparing the lymphocyte subsets and cytokines in 2019-nCoV pneumonia group with those in the RSV pneumonia group and made some deduction from what has already known about RSV pneumonia. The results found in the study could be used as clues of the immune response in 2019-nCoV pneumonia in further study. Secondly, the number of patients in the study was very limited. As there was only one child who suffered from severe 2019-nCoV pneumonia during the study period, the exact reason for severe 2019-nCoV pneumonia in children could not be confirmed by only one case.

Conclusion

Immune response played a very important role in the development of 2019-nCoV pneumonia. The effective CD8⁺ T cell response might influence the severity of 2019-nCoV pneumonia. The adaptable change in IL-10 level might contribute to the relatively mild pneumonia symptoms in children with 2019-nCoV pneumonia and bacterial co-infection might be a risk factor of severe 2019-nCoV pneumonia.

Declaration of Competing Interest

No authors have financial interests that could create a conflict of interest about the work.

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