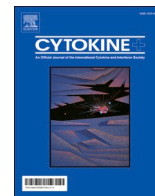Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

An inter-correlated cytokine network identified at the center of cytokine storm predicted COVID-19 prognosis

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ABSTRACT

The hyper-inflammatory response is thought to be a major cause of acute respiratory distress syndrome (ARDS) in patients with COVID-19. Although multiple cytokines are reportedly associated with disease severity, the key mediators of SARS-CoV-2 induced cytokine storm and their predictive values have not been fully elucidated. The present study analyzed maximal and early (within 10 days after disease onset) concentrations of 12-plex cytokines in plasma. We found consistently elevated plasma levels of IL-6, IL-8 and IL-5 in patients who were deceased compared with those who had mild/moderate or severe disease. The early plasma concentrations of IFN-a and IL-2 positively correlated with the length of the disease course. Moreover, correlation network analysis showed that IL-6, IL-8, and IL-5 located at the center of an inter-correlated cytokine network. These findings suggested that IL-8, IL-6, IL-5 might play central roles in cytokine storms associated with COVID-19 and that the early detection of multiple plasma cytokines might help to predict the prognosis of this disease.

1. Introduction

By the end of October 2020, more than 45 million people worldwide had been infected with SARS-CoV-2. Its high transmission capability and variable clinical manifestations have resulted in a pandemic that is difficult to control. Although most patients with COVID-19 develop only mild to moderate symptoms, accumulating data suggest that the death rate is >2% [1,2], which is much higher than that of pandemic influenza [1]. Without an effective therapy, current management of COVID-19 consists of supportive treatment [3]. Under such circumstances, the prognosis of COVID-19 is determined mostly by the outcome of a battle between SARS-CoV-2 and the human immune system.

While appropriate immune responses are necessary to contain viral infections, a hyperactivated immune system might result in serious consequences [4]. Uncontrolled inflammatory responses, known as a cytokine storm, are associated with several infectious and non-infectious diseases [4]. Similar to SARS-CoV and MERS-CoV, infection with SARS-CoV-2 can also fiercely activate inflammatory responses [5–8], which is the main cause of acute respiratory distress syndrome (ARDS) [9,10].

Among TNF-α, IL-1β, IL-6, IL-7, IFN-γ, IL-2, IL-10, plasma levels of which are elevated during cytokine storms, blocking IL-6, TNF-α and IL-1β were thought to be beneficial [6,11]. The effects of the anti-IL-6R antibody (Tocilizumab) are now under clinical investigation [1]. Although IL-6 levels might significantly increase in critically ill patients with COVID-19 [12], its role in SARS-CoV-2 induced cytokine storm, and its prognostic value have not been fully elucidated.

This retrospective study using correlation network analysis showed that multiple cytokines formed an inter-correlated network and predicted the prognosis of patients with COVID-19.

2. Materials and methods

2.1. Data collection

Peripheral lymphocyte counts and ratios (%), plasma cytokine concentrations and the disease course of 52 patients with COVID-19 (47 recovered, 5 deceased) were analyzed. Among the 47 patients who recovered, 37 and 10 had mild/moderate, and severe disease,

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respectively (Table 1). The clinical criteria for diagnosis and discharge followed the standards for the "Diagnosis and Treatment Scheme of New Coronavirus Infected Pneumonia" (trial version 6, National Health Commission of the People's Republic of China). All patients had been hospitalized between January and April 2020, in Shanghai Public Health Clinical Center affiliated to Fudan University. Peripheral lymphocyte counts and ratios (%) were determined immediately after admission and monitored every 3 days during hospitalization. Plasma concentrations of IL-5, IFN-α, IL-2, IL-6, IL-1β, IL-10, IFN-γ, IL-8, IL-17, IL-4, IL-12p70, and TNF-α were regularly measured from February 11, 2020.

2.2. Ethics statement

Written, informed consent was obtained from all patients to participate in the present study. The Research Ethics Review Committee of the Shanghai Public Health Clinical Center approved the study (2020-Y025-01).

2.3. Peripheral blood lymphocyte count

Peripheral blood lymphocytes in BD Trucount™ tubes with TBNK reagents (Cat# 337166) were detected, and their physical and chemical characteristics were measured using a BD FACSCanto™ II flow cytometer (both from Becton Dickinson and Co., Franklin Lakes, NJ, USA) as described by the manufacturer. Briefly, TBNK detection reagent (20 μL) was gently mixed with fresh anticoagulated whole blood (50 μL) in the Trucount tubes and left for 15 min at room temperature in darkness. Red cell lysis buffer (450 μL) was added, gently mixed, and the tubes were placed for another 15 min in darkness. Thereafter, the cells were assessed by cytometry.

2.4. Detection of plasma cytokine concentration

Plasma concentrations of IL-5, IFN-α, IL-2, IL-6, IL-1β, IL-10, IFN-γ, IL-8, IL-17, IL-4, IL-12p70 and TNF-α were determined in fresh plasma samples using 12-plex microsphere array kits (Qingdao Raisecare Biotechnology Co., Ltd., Shandong, China) as described by the manufacturer. Briefly, 25 μL each of experimental buffer, clarified plasma, capture antibodies, and detection antibodies were consecutively added to the tubes and shaken (400 – 500 rev/min) at room temperature for 2 h. Thereafter, SA-PE (25 μL) was added to each tube and incubated as described above for 30 min. Diluted wash buffer (500 μL) was added to the tubes and briefly vortex mixed. The mixtures were separated by centrifugation at 300*g* for 5 min. The supernatant was discarded, and clustered microspheres were resuspended in diluted washing buffer (200 μL). Fluorescence intensity was determined by flow cytometry using the BD FACSCanto™ II and cytokine concentrations were calculated as described by the manufacturer.

2.5. Statistical analysis

All data were statistically analyzed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Multiple groups were compared using non-parametric one-way ANOVA. Correlations were analyzed using Spearman rank correlations and correlation networks were analyzed using UCINET 6 software. Heatmap clustering was analyzed using Heatmapper (www.heatmapper.ca). Values with P < 0.05 were considered statistically significant.

3. Results

3.1. The maximal concentrations of several cytokines were significantly elevated in patients who died compared with those who had mild/moderate and severe disease

Cytokine storm is associated with a poor disease prognosis [9,10]. We monitored plasma concentrations of a panel of cytokines in 52 hospitalized patients with COVID-19. We initially compared maximal plasma cytokine concentrations among patients with mild/moderate and severe disease, and those who died. Heatmaps showed obviously different cytokine responses between patients who died, and those with mild/moderate and severe disease who lived (Fig. 1). In contrast, differences between patients with mild/moderate and severe disease were not clear. Further comparisons of each cytokine among the three groups of patients showed that the maximal concentrations of IL-5, IL-2, IL-6, IL-10, IFN-γ, IL-8, IL-17 and IL-12p70 among the 12 cytokines were significantly higher among the deceased patients (Fig. 2) but did not significantly differ between those with mild/moderate and severe symptoms.

We also assessed the correlations between maximum cytokine concentrations and the length of the disease course using Spearman correlation test. Maximum plasma cytokine concentrations did not significantly correlate with disease course (Supplementary Table 1).

3.2. Early detection of plasma cytokine concentrations predicted prognosis

Plasma cytokines were measured within 10 days after disease onset in 21 (mild moderate symptoms, n = 11; severe symptoms, n = 6; deceased, n = 4) of the 52 patients. We assess the ability of early plasma cytokine detection to predict disease prognosis by comparing early cytokine concentrations among these 21 patients. Heatmap results showed substantially different cytokine values between patients who died and those who had mild/moderate and severe disease (Fig. 3). Further investigations showed that early IL-5, IL-6 and IL-8 values were significantly higher in deceased patients (Fig. 4), but these did not correlate with the length of disease course. In contrast, those of IFN-α and IL-2 significantly correlated with the length of disease course (Fig. 5).

3.3. Early plasma cytokines clustered into two inter-correlated groups

The results of correlation network analysis revealed inter-correlations among early (Fig. 6) and maximal (Supplementary Fig. 1) plasma cytokine concentrations. Compared with the intensively correlated maximum cytokine responses (Supplementary Fig. 1), the early cytokine responses notably clustered into two inter-correlated groups (Fig. 6): IL-5, IL-6, IL-8, IFN-α, IL-12p70, and IL-1β in one group; IL-10, IL-2, IFN-γ, IL-17, IL-4 and TNF-α in the other group. Further analysis showed that IL-6 and IL-8 negatively correlated with the relative ratios (%) of CD3^{+} T cells (Fig. 7), thus indirectly proving that IL-6 and IL-8 correlates with disease severity.

Table 1
Demographical descriptions of the patients enrolled in this study.

Variables	All patients	Mild/moderate symptom	Severe symptom	Deceased	P-value
	(N = 52)	(N = 37)	(N = 10)	(N = 5)	
Age (year)	55.9 ± 17.0	51.7 ± 16.2	67.0 ± 8.9	65.6 ± 23.2	0.0135
Gender (%)					
Men	28(53.8)	19(51.4)	6(60)	3(60)	0.8516
Women	24(46.2)	18(48.6)	4(40)	2(40)	
Disease course (Day)	29.8 ± 11.8	27.2 ± 8.8	31.4 ± 8.1	45.8 ± 23.5	0.0025

Fig. 1. Heatmap clustering analysis of the maximum concentrations of plasma cytokines. The maximum plasma cytokine concentrations of 52 COVID-19 patients during hospitalization were analyzed using an online heatmapping tool (www.heatmapper.ca, clustering method: Single-linkage). D1 ~ D5, deceased cases; S1 ~ S10, severe cases; M1-M37, mild/moderate cases.
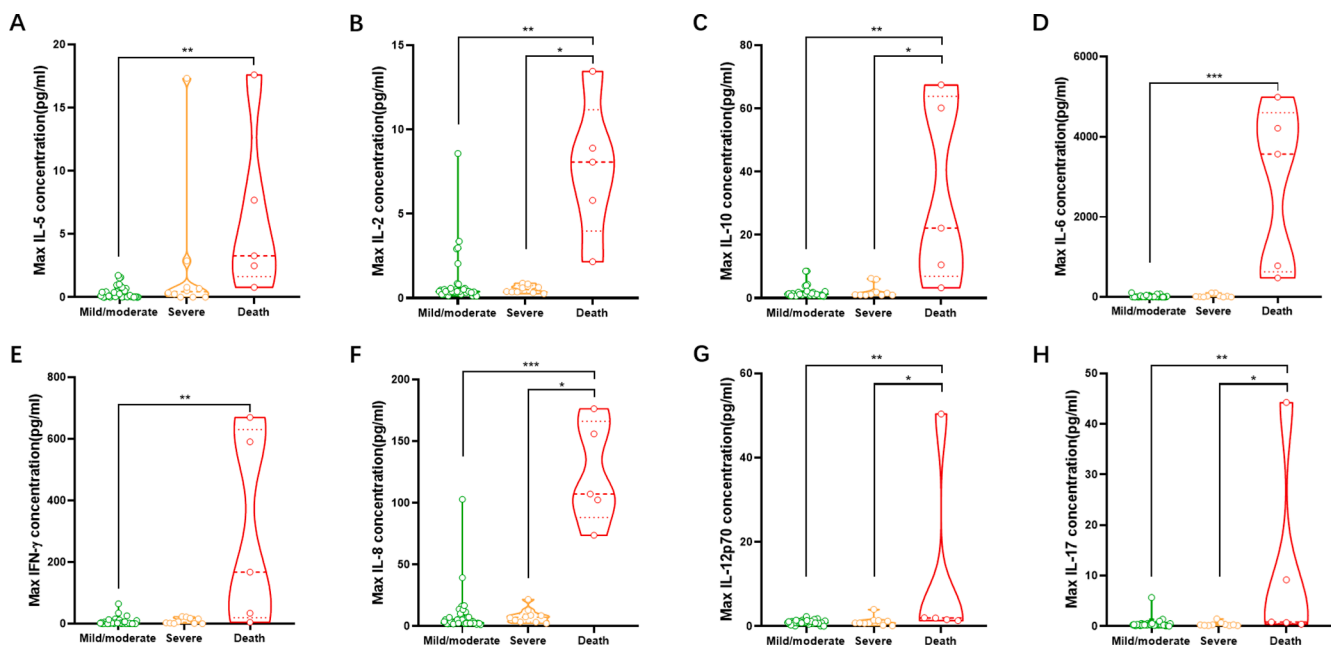

Fig. 2. Comparisons of maximum plasma cytokine concentrations among mild/moderate, severe and deceased patients. The maximum plasma cytokine concentrations were compared by non-parametric one-way ANOVA. Levels of IL-5 (A), IL-2 (B), IL-10 (C), IL-6 (D), IFN-γ (E), IL-8 (F), IL-12p70 (G) and IL-17 (H) were observed to be significantly elevated in deceased patients.

4. Discussion

The hyper-secretion of several cytokines is thought to be associated with poor disease outcomes [13], but relationships between cytokine responses and disease progression are not fully understood. We found that the maximal concentrations of 12 cytokines differentiated patients who died from those who had mild/moderate and severe disease but recovered. Concentrations of IL-5, IL-2, IL-6, IL-10, IFN-γ, IL-8, IL-17, and IL-12p70 were significantly elevated in deceased patients. These findings were generally consistent with those of other studies suggesting that inflammatory cytokine values are associated with disease severity [5,10,14]. It is noteworthy that maximal plasma cytokine values did not

correlate with the length of disease course. Because the length of the disease course reflects the amount of time required for virus clearance, the above findings suggested that maximal plasma cytokine levels (including IFN-γ) do not correlate with virus clearance. These findings are similar to those of previous studies indicating that the SARS-CoV-2 load does not correlate with symptomatic severity [5,15].

We investigated whether plasma cytokine concentrations within 10 days of disease onset could predict disease prognosis. Our data showed significantly higher plasma levels of IL-5, IL-6 and IL-8 in patients who died, compared with those who had mild/moderate and severe disease. Therefore, these cytokines might serve as early markers for predicting disease severity even though they did not correlate with the length of the
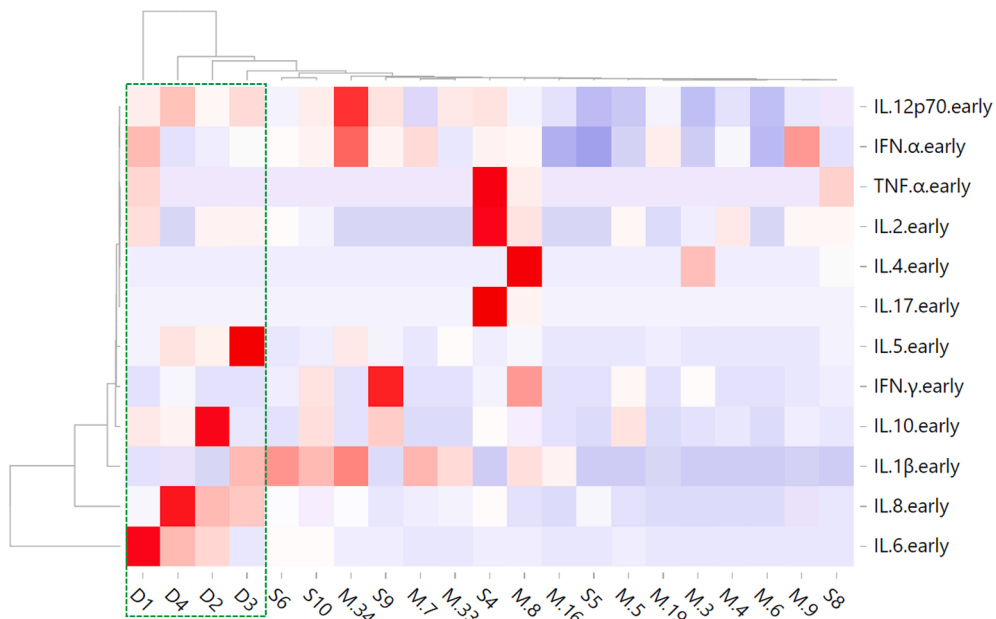
Fig. 3. Heatmap clustering analysis of the plasma cytokine concentrations early after disease onset. Plasma cytokine detection was initiated within 10 days after disease onset for 21 out 52 COVID-19 patients, including 4 deceased patients (D1-D4), 6 severe cases (S4, S5, S6, S8, S9, S10) and 11 mild/moderate cases (M3, M4, M5, M6, M7, M8, M9, M16, M19, M33, M34). Heatmapping tool and clustering method were the same with the analysis of maximum cytokine levels.
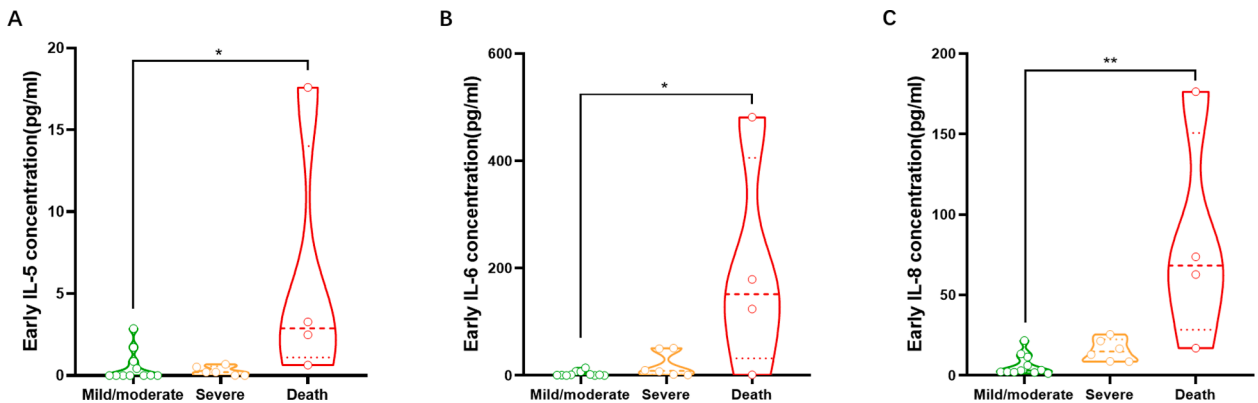

Fig. 4. Comparisons of early plasma cytokine concentrations among the mild/moderate, severe and deceased patients. The early plasma cytokine concentrations were compared by non-parametric one-way ANOVA. Levels of IL-5 (A), IL-6 (B) and IL-8 (C) were observed to be significantly elevated in the deceased patients.
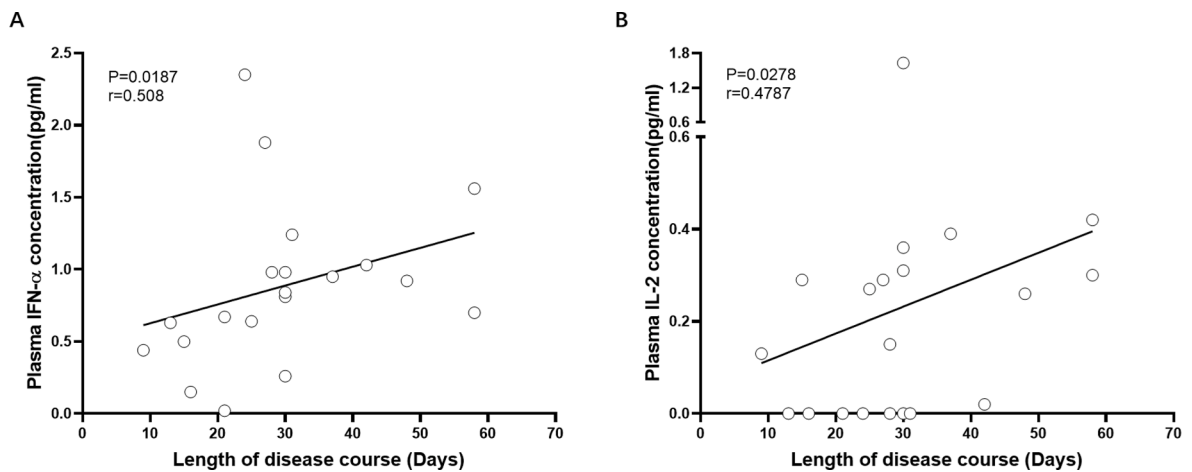

Fig. 5. Correlation analyses between early plasma cytokine concentrations and the length of disease course. Correlation analyses showed that the early plasma concentrations of IFN-α (A) and IL-2 (B) positively correlated with the length of disease course.
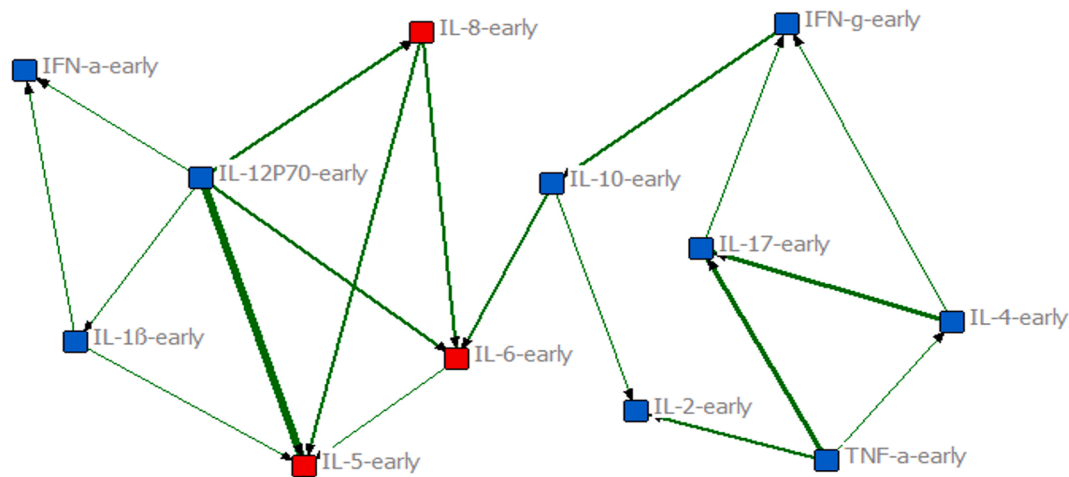
Fig. 6. Correlation network analysis of plasma cytokine concentrations early after disease onset. Correlation network analysis was employed to investigate the inter-relationships among different cytokines. Significant correlations (P < 0.05) were shown by the green connecting lines and line width reflected the actual P value. The wider the line is, the smaller is the P value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
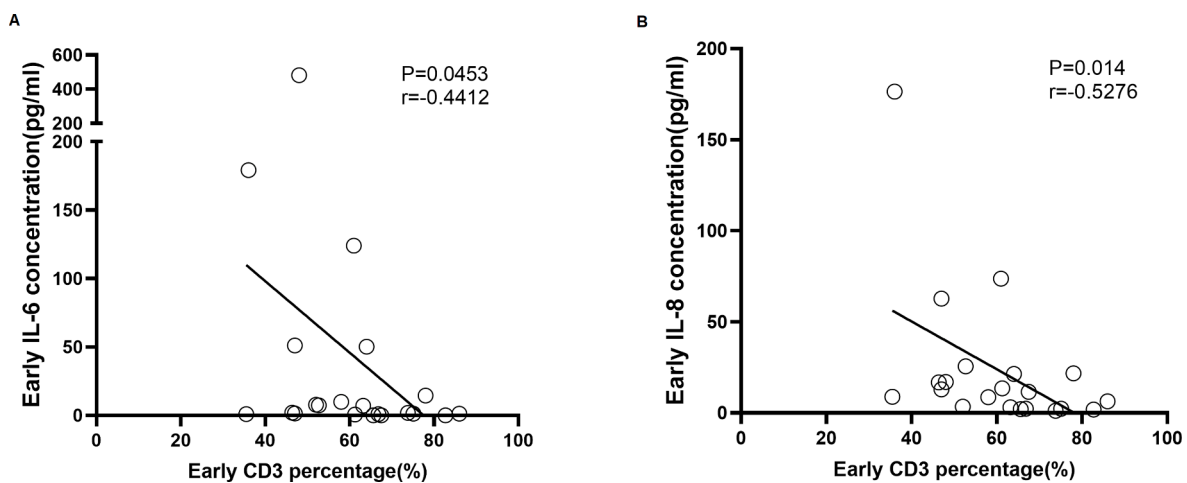

Fig. 7. Early plasma concentrations of IL-6 and IL-8 negatively correlated with the number and percentage CD3^{+} T cells in peripheral blood. Correlations between early plasma cytokine concentrations and peripheral lymphocyte percentages were analyzed by the method of Spearman correlation. IL-6 (A) and IL-8 (B) were found to negatively correlate with the percentages of CD3^{+} T cells in CD45^{+} lymphocytes.

disease course. We also found that early plasma IFN-α and IL-2 levels positively correlated with the length of disease course. In addition to our findings, recent studies suggested that CXCL10 played a crucial role in COVID-19 related immune pathology [7], which might serve as a predictor for disease progression [16]. However, CXCL10 was not included in our detection panel, so it is still not clear whether CXCL10 and many other potential factors might also serve as early indicators for disease progression. A more comprehensive analysis of early innate immune responses is needed to clarify this question and find out the driving force of COVID-19 related cytokine storm.

Defining the key mediators of cytokine storms has been vigorously investigated, primarily in gene knockout mice [17–20]. In this study, we found that both the early and the maximal IL-5, IL-6, and IL-8 levels were significantly elevated in deceased patients with COVID-19. Therefore, we suspected that they might play central roles in SARS-CoV-2 induced cytokine storm. The present retrospective study did not allow a cause-and-effect analysis, so we probed major cytokines using correlation network analysis. Our results showed that early levels of plasma cytokines clustered into two connected groups. Interleukin-6 was located at the center of this cytokine network, which inter-correlated with IL-5 and IL-8. Moreover, plasma concentrations of IL-6 and IL-8 negatively correlated with the ratios of CD3^{+} T cells in

peripheral blood. As decreased peripheral lymphocytes are associated with severe clinical symptoms of COVID-19 [14,20,21], this finding indicated that IL-6 and IL-8 might be associated with disease severity. Overall, our data implied that IL-6, IL-8, and IL-5 are central players in COVID-19-related cytokine storm.

Despite lacking the power to reach a conclusion about cause and effects, our comprehensive correlation analyses showed that IL-6 was located at the center of a cytokine network with IL-8 and IL-5. The early detection of IL6, IL-8, IL-5, IFN-α and IL-2 might be able to predict the prognosis of patients with COVID-19.

Author contributions

ZZ and YW designed the experiments. YL conducted most experiments and analyses. DCh, JH, HL, and DCa assisted with some experiments. MGu, YL, MGa and YZ assisted with collecting samples and information from patients. ZZ wrote the manuscript and YW revised the manuscript. All authors have read and approved the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2020.155365.

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