Changes of peripheral lymphocyte subset in patients with SARS-CoV-2 infection during the whole course of disease

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ABSTRACT

Background: Little is known about changes in lymphocyte subsets after SARS-CoV-2 infection.

Methods: Clinical data of 580 COVID-19 patients hospitalized in Zhongnan Hospital of Wuhan University from 20 December 2019, to 8 March 2020, were retrospectively analyzed. The relation of lymphocyte subsets and severity or prognosis of disease were analyzed.

Results: At 2–3 weeks after the onset of symptoms, lymphocyte subsets decreased to the lowest levels. The levels of lymphocyte subsets in asymptomatic patients were close to healthy persons, except for CD8+ T lymphocyte cells. The levels of lymphocyte subsets in patients with severe illness were lower than that in patients with mild-to-moderate illness (P < 0.01). Similarly, among patients with severe illness, lower levels of lymphocyte subsets were found in dead patients compared to survivors (P < 0.001). Moreover, by comparing the results of the same patients at different stages of the disease, we found levels of lymphocyte subsets were lower in the acute phase compared to that in convalescent-phase (P < 0.001). However, the levels of lymphocyte subsets in patients who had SARS-CoV-2 viral load >5000 copies/ml and 500–5000 copies/ml were at similar levels.

Conclusions: Lymphocyte subsets are a good biomarker to assess the severity and prognosis of the disease at 2–3 weeks after the onset of symptoms.

1. Introduction

The study is interesting and provides timely information COVID-19 dynamics of immune cells. Since the global outbreak of COVID-19, although its etiological and clinical characteristics have been more fully understood [1–3], studies on the clinical impact of immune therapy for COVID-19 and strategy for circumventing or limiting the emergence of viral escape mutants were still rare [4,5]. Lymphocytes play an important role in the maintenance of immune system function. Although the clinical characteristics of lymphopenia in COVID-19 patients have been recognized earlier [3,6–9], the changes pattern of lymphocyte subsets throughout the course of disease development and their value in evaluating clinical treatment and prognosis remains to be elucidated. In this study, we aimed to clarify the characteristics and clinical significance of peripheral lymphocyte subset alteration in COVID-19, which might help elucidate the pathogenesis and develop novel biomarkers and therapeutic strategies for COVID-19.

2. Methods

2.1. Study population

In this retrospective study, patients with COVID-19 were admitted to Affiliated Hospitals of Wuhan University, which were designated to treat COVID-19 patients, from 20 December 2019, to 8 March 2020. All the 580 patients were diagnosed as COVID-19 based on the positive real-time RT-PCR tests for oropharyngeal swab samples. Simultaneously, we tested 120 healthy blood donors to establish inter-laboratory reference ranges for various parameters. These reference values were used to provide data for the healthy controls in this study.

A waiver of the requirement for documentation of informed consent was granted for analyzing existing data without interfering with patient treatment.

2.2. Data collection and definitions

This is a retrospective study. Epidemiological, clinical, laboratory and radiological characteristics and treatment and outcome data were obtained with data collection forms from electronic medical records. The data were reviewed by a trained team of physicians.

Asymptomatic patients in this study are those who have no fever, dry cough, fatigue, myalgia, or any other clinical symptoms throughout the whole course of SARS-CoV-2 infection.

Severe COVID-19 was defined as satisfying at least one of the following criteria: (i) respiratory rate ≥30/min; (ii) pulse oximeter oxygen saturation (SpO2) <93% at rest on room air (iii) ratio of the partial pressure of arterial oxygen (PaO2) to a fraction of inspired oxygen (FiO2) <300 mmHg (1 mmHg = 0.133 kPa).

The period in which SARS-CoV-2 nucleic acid was positive or suspiciously positive or fever, and respiratory symptoms associated with SARS-CoV-2 infection were not relieved, was...
defined as the acute phase. Otherwise, it was defined as a convalescent phase.

2.3. Detection of lymphocyte subsets

Samples of EDTA anticoagulated peripheral blood (2 mL) was collected from patients confirmed with SARS-CoV-2 infection on admission and every 1 week during hospitalization. All samples were tested within 6 hours of being obtained. Briefly, CD3+/CD4+/CD8 + T-cell, CD19 + B-cell, and CD16 + CD56 + NK-cell counts (cells/μL) were measured by multicolor flow cytometry with human monoclonal anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-phycocerythrin (PE), anti-CD8-allophycocyanin (APC), anti-CD19-PE, anti-CD16-APC, and anti-CD56-PE antibodies (BD Multitest) according to the manufacturer’s instructions. The cells were analyzed on a BD FACS Canto II flow cytometry system (BD Biosciences).

2.4. Statistical analysis

All statistical analyses were performed using SPSS Statistics version 23.0 software. Categorical variables were described as frequency rates and percentages, and continuous variables were described using mean, median, or interquartile range (IQR) values. χ² analysis was used to examine the categorical variables. Means for continuous variables was compared using independent group t tests. Bivariate correlation analysis was used to statistically analyze the relation of lymphocyte subset counts and total lymphocyte counts. P < 0.05 was considered statistically significant.

3. Result

3.1. Baseline characteristics of 580 inpatients with SARS-CoV-2 infection

Five hundred and eighty inpatients with SARS-CoV-2 infection were analyzed in this study. The mean age was 56.5 years, and 52.1% (302/580) were male. Comorbidities accounted for 30.9% (179/580) of the total number. The proportions of asymptomatic patients, COVID-19 patients with mild/moderate illness, and COVID-19 patients with severe illness were 10.3% (60/580), 64.5% (374/580), and 25.2% (146/580), respectively. Twenty-six patients died during hospitalization, and the mortality was 4.5% (26/580). The blood test results are shown in Table 1.

3.2. Changes of various lymphocyte subsets at different times during the course of COVID-19

The change trends of various lymphocyte subsets at different times during the course of COVID-19 are shown in Figure 1. During 6-week monitored course, CD4+ T lymphocyte cells and CD19+ B cells decreased to the lowest level at 2 weeks after the onset of symptom, while CD8+ T lymphocyte cells and CD16+CD56+ NK cells decreased to the lowest level at 3 weeks after the onset of symptom. Since then, levels of CD4+ T lymphocyte cells, CD8+ T lymphocyte cells, and CD19+ B cells steadily increased.

3.3. The relation of lymphocyte subsets and the severity or prognosis of COVID-19 patients

All the lymphocyte subsets were measured in the acute phase. Compared with healthy controls, SARS-CoV-2 infection patients had lower levels of CD4+ T lymphocyte cells (P = 0.002), CD8+ T lymphocyte cells (P < 0.001), CD19+ B cells (P < 0.001) and CD16+CD56+ NK cells (P < 0.001). Moreover, these symptomatic patients were divided into patients with severe illness and patients with the mild-to-moderate illness. All kinds of lymphocyte subsets in patients with severe illness were lower than those with mild-to-moderate illness. Likewise, among those
patients with severe illness, all kinds of lymphocyte subsets in dead patients were lower than survivors. The levels of lymphocyte subsets with the severity, prognosis, and time course of COVID-19 patients are shown in Table 2.

It was interesting that, except for CD8+ T lymphocyte cells, other lymphocyte subsets in asymptomatic infections and healthy controls were at similar levels (Table 2). Among SARS-CoV-2 infections, the level of CD8+ T lymphocyte cells in symptomatic and asymptomatic patients was at similar levels (P = 0.590). On the other hand, the levels of CD4+ T lymphocyte cells, CD19+ B cells, and CD16+ CD56+ NK cells were significantly different (Table 2).

### 3.4. The levels of SARS-CoV-2 viral load with lymphocyte subsets, the severity, mortality and time course of COVID-19 patients

In Zhongnan Hospital of Wuhan University, the level of SARS-CoV-2 viral load above 5000 copies/ml detected by Polymerase Chain Reaction (PCR) was defined as positive, while 500–5000 copies/ml for 2 times was defined as suspiciously positive. The relationship between SARS-CoV-2 viral load of the upper respiratory tract and levels of various lymphocyte subsets are shown in Figure 2. The difference of levels of CD4+ T lymphocyte cells (P = 0.109), CD8+ T lymphocyte cells (P = 0.240), CD19+ B cells (P = 0.468) and CD16+CD56+ NK cells (P = 0.565) in COVID-19 patients had VL>5000 copies/ml and 500–5000 copies/ml were not statistically significant.

Moreover, the levels of viral load with the severity, mortality, and time course of COVID-19 patients are further analyzed in Figure 3. The levels of SARS-CoV-2 viral load were converted to Log. The log value in patients with severe illness and mild-to-moderate illness was 4.903 ± 0.194 and 4.353 ± 0.129, respectively (P < 0.001). However, among COVID-19 patients with severe illness, the log value of SARS-CoV-2 viral load in death patients were similar to those in survivors (5.124 ± 0.250, and 4.874 ± 0.154, P = 0.106). Not surprisingly, but still important was that the level of SARS-CoV-2 viral load in the acute phase was higher than that in the convalescent-phase (4.510 ± 0.063 and 3.641 ± 0.075, P < 0.001).

### 3.5. The relation of lymphocyte subsets and disease phases after SARS-CoV-2 infection

In acute phase after SARS-CoV-2 infection, the levels of CD4+ T lymphocyte cells, CD8+ T lymphocyte cells, CD19+ B cells, and CD16+CD56+ NK cells were 436 ± 23 cells/µl, 313 ± 21 cells/µl, 147 ± 8 cells/µl, and 168 ± 10 cells/µl, respectively, while in convalescent-phase was 668 ± 20 cells/µl, 428 ± 13 cells/µl, 213 ± 9 cells/µl and 272 ± 11 cells/µl, respectively. The levels of lymphocyte subsets in the acute phase were lower than that in convalescent-phase (P < 0.001). The result is shown in Figure 4.

### 3.6. The relation of levels of pro-inflammatory biomarkers and CD4+ T lymphocyte counts

CD4+ T lymphocyte cells <200/µl is a recognized threshold for people living with HIV to be prone to various opportunistic infections. In this study, the relation of levels of pro-inflammatory biomarkers and CD4+ T lymphocyte counts is shown in Table 3. The levels of CRP, IL-6 and TNF-α in COVID-19 patients had CD4+...
Table 2. The relation of lymphocyte subsets and the severity of or prognosis of COVID-19 patients.

| Lymphocyte Subset | All-ill patients (n=580) | Healthy control (n=120) | Asymptomatic infections (n=350) | Symptomatic patients (n=160) | Severe patients (n=160) | Survivor (n=100) |
|-------------------|--------------------------|-------------------------|--------------------------------|-----------------------------|------------------------|----------------|
| CD4+ T lymphocyte | Mean±SD                  | P           | Mean±SD                  | P           | Mean±SD                  | P           | Mean±SD                  | P           | Mean±SD                  | P           | Mean±SD                  | P           |
|                   | 695±300                  | <0.001      | 695±300                  | <0.001      | 695±300                  | <0.001      | 695±300                  | <0.001      | 695±300                  | <0.001      | 695±300                  | <0.001      |
|                   | 398±355                  | <0.001      | 398±355                  | <0.001      | 398±355                  | <0.001      | 398±355                  | <0.001      | 398±355                  | <0.001      | 398±355                  | <0.001      |
|                   | 116±115                  | <0.001      | 116±115                  | <0.001      | 116±115                  | <0.001      | 116±115                  | <0.001      | 116±115                  | <0.001      | 116±115                  | <0.001      |

T lymphocyte cells <200/ul was higher than in those had CD4+ T lymphocyte cells ≥200/ul (67.1 ± 5.7 vs 18.8 ± 2.1 mg/l, P < 0.001; 121.46 ± 51.09 vs 45.13 ± 30.74 pg/ml, P = 0.035; 101 ± 37 vs 0.55 ± 0.18 pg/ml, P = 0.033). However, there were no statistically significant differences in IFN-γ, IL-2, IL-4 and IL-10 between COVID-19 patients who had CD4+ T lymphocyte cells <200/ul and those who had CD4+ T lymphocyte cells ≥200/ul.

4. Discussion

COVID-19 has spread worldwide as an emerging infectious disease. Lymphocytopenia is common after SARS-CoV-2 infection [3,6-8]. Lymphocytes consist of various lymphocyte subsets, including CD4+ T lymphocyte cells, CD8+ T lymphocyte cells, CD19+ B cells, and CD16+CD56+ NK cells. The results in this study showed that levels of lymphocyte subsets decreased in patients with SARS-CoV-2 infection: However, the effect was reversible, as shown by gradually increasing lymphocyte subsets during the course of disease.

Asymptomatic patients, who were defined as those who have no fever, dry cough, fatigue, myalgia, or any other clinical symptoms throughout the whole course of SARS-CoV-2 infection, were a sub-population after SARS-CoV-2 infection. Similar to many other viral infections, the asymptomatic disease is present in a significant but currently unknown fraction of the affected individuals. Asymptomatic patients seemed to account for approximately 40–45% of the SARS-CoV-2 infections [10]. In this study, only CD8+ T lymphocyte cell counts decreased in this population, while other lymphocyte subsets were similar to healthy controls. This interesting finding may have implications for further research on the pathogenic mechanism and cellular immunity of SARS-CoV-2.

Lymphocytes play a decisive role in maintaining immune homeostasis and inflammatory response throughout the body. The results in a previous study suggested that lymphopenia as a predictor of prognosis in COVID-19 patients [11]. A Time-LYM% model (TLM) was established and LYM% was considered to be a reliable indicator to classify the moderate, severe, and critical ill types independent of any other auxiliary indicators [11]. In this study, the relation of lymphocyte subsets and the severity of the disease after SARS-CoV-2 infection was further observed. Among SARS-CoV-2 infections, we found that levels of lymphocyte subsets were closely related to clinical symptoms, as shown by lower levels of lymphocyte subsets in symptomatic patients compared to that in asymptomatic patients. Moreover, the severity of the disease can also affect the levels of lymphocyte subsets, as shown by lower levels of lymphocyte subsets in patients with severe illness compared to that in patients with mild-to-moderate illness in this study. Further, the levels of lymphocyte subsets were associated with prognosis for COVID-19 patients with severe illness, as shown by lower levels of lymphocyte subsets in dead patients than that in survivors. The results were consistent with previous studies, which reported that the decrease of CD4+ and CD8+ T lymphocyte was closely correlated with the severity of illness and prognosis of COVID-19 patients [12,13]. Taken together, we believe that lymphocyte subsets can be used as another indicator when evaluating the
condition of COVID-19 patients or predicting the prognosis of patients with severe illness.

After SARS-CoV-2 infection, levels of lymphocyte subsets were dynamic [14]. According to the results shown in this study, CD4+ T lymphocyte cells and CD19+ B cells decreased to the lowest level at 2 weeks after the onset of symptoms. Differently, the period of lowest levels of CD8+ T lymphocyte cells and CD16+CD56+ NK cells was 1 week later. Various lymphocyte subsets were involved in the immune activity of recognizing and helping to clear virus-infected cells when SARS-CoV-2 as a foreign virus invades the body [15]. The cost of the immune activity was lymphocyte subset depletion, but they can gradually recover after SARS-CoV-2 was cleared away. The time-point of the lowest levels of lymphocyte subsets was exactly consistent with the duration of SARS-CoV-2 shedding as in previous reports [16,17]. We believe that 2–3 weeks after the onset of symptoms may be a good time to detect lymphocyte subsets because more accurate reflection on the degree of damage to lymphocytes caused by SARS-CoV-2 could be acquired then.

It was not difficult to understand that for those patients with SARS-CoV-2 infection in the convalescent phase, their levels of lymphocyte subsets increased along with the improvement of lung symptoms. What was interesting in this study was that the difference of levels of lymphocyte subsets in COVID-19 patients with a high and low viral load of SARS-CoV-2 was not statistically significant. Several mechanisms leading to lymphocyte deficiency were speculated by Tan L et al. [11]. Although lymphocytes
express the coronavirus receptor ACE2 and may be a direct target of SARS-CoV-2 [18], other possible mechanism that tumor necrosis factor (TNF)α, interleukin (IL)-6, and other pro-inflammatory cytokines induces lymphocyte deficiency could not be ignored [19]. In this study, we found the levels of CRP, IL-6, and TNF-α in patients who had CD4⁺ T lymphocyte <200/ul was higher than in those who had CD4⁺ T lymphocyte ≥200/ul. Although the hypothesis that lymphocyte apoptosis is associated with elevated inflammatory cytokines needs to be confirmed in the future, the results in this clinical study could provide study clue for further basic studies. In this study, we found that there was no correlation between the level of lymphocyte subsets and the viral load of SARS-CoV-2. It suggested that in addition to the direct effects of SARS-CoV-2 on lymphocytes, multiple mechanisms mentioned above or beyond might work together to cause lymphopenia, and further research is needed.

In addition to exploring mechanisms of SARS-CoV-2 infection, studies on immunology associated with SARS-CoV-2 infection may also provide clues for further treatment strategies against COVID-19. For patients infected with SARS-CoV-2, pathological roles associated with host defense factors was an important factor affecting disease progression and prognosis, which would provide a theoretical basis for future treatment strategies. By analyzing immune responses in 113 patients with moderate or severe COVID-19, Lucas et al. found that immune profiling revealed an overall increase in innate cell lineages, with a concomitant reduction in T cell number [9]. They proposed four signatures of immune response profiles that more accurately divide patients into distinct

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**Figure 4.** The relation of lymphocyte subsets counts and disease phases after SARS-CoV-2 infection.

**Table 3.** The relation of levels of pro-inflammatory biomarkers and CD4⁺ T lymphocyte counts.

|                      | CD4⁺ T lymphocyte <200/ul (n = 180) | CD4⁺ T lymphocyte ≥200/ul (n = 400) | Test  | P    |
|----------------------|-------------------------------------|-------------------------------------|-------|------|
| CRP (mg/l)           | 67.1 ± 5.7                          | 18.8 ± 2.1                          | 9.540 | <.001|
| IL-6 (pg/ml)         | 121.46 ± 51.09                      | 45.13 ± 30.74                       | 2.114 | 0.035|
| TNF-α (pg/ml)        | 1.01 ± 0.37                         | 0.55 ± 0.18                         | 2.143 | 0.033|
| IFN-γ (pg/ml)        | 1.90 ± 0.39                         | 1.68 ± 0.20                         | 1.308 | 0.192|
| IL-2 (pg/ml)         | 1.60 ± 0.10                         | 1.32 ± 0.11                         | 1.420 | 0.161|
| IL-4 (pg/ml)         | 1.33 ± 0.22                         | 1.06 ± 0.30                         | 0.962 | 0.339|
| IL-10 (pg/ml)        | 7.18 ± 0.81                         | 6.33 ± 0.82                         | 0.696 | 0.487|
COVID-19 disease courses. Further, they raise the possibility that early immunological interventions that target inflammatory markers that are predictive of worse disease outcome would be more beneficial than those that block late-appearing cytokines. The targeted treatment of patients with COVID-19 based on early cytokine markers is expected to be the future direction of disease treatment.

There were several limitations in our study which might make some potential bias. First, as a retrospective study, we were unable to provide information about change trend of naïve and memory CD4+ T lymphocyte cells, although the balance between the two cells is crucial for maintaining an efficient immune response. Second, this was a single-center study with limited sample size. Thus, a multicenter design with a larger cohort will be warranted in the future. Third, due to the sudden outbreak of COVID-19, the examination and treatment of COVID-19 patients were implemented based on the physicians' previous clinical work experience. Laboratory test of ferritin was not provided for COVID-19 patients during the epidemic of COVID-19 in Wuhan. Therefore, the relation of levels of ferritin and lymphocyte counts was not analyzed in this study. Even so, we recognize that ferritin testing is an indicator that is overlooked by many clinicians but deserves attention.

In conclusion, we believed that the levels of lymphocyte subsets were associated with the severity of illness and prognosis of COVID-19 patients. The most appropriate time to detect lymphocyte subsets was 2–3 weeks after the onset of symptoms.

Declaration of interest
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Authors’ contributions
Rongrong Yang and Xingxia Yu drafted and supervised the manuscript. All authors were responsible for summarizing all data related to this study.

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