Changes of lymphocyte subsets in patients with COVID-19 and clinical significance: a case-control observational study
Guang Yang, Fan Feng, Xue Li, Tian Zhang, Xiang Li, Boan Li*

Abstract
Objective: To investigate and analyze changes of T lymphocyte and other lymphocyte subsets in the peripheral blood of patients with coronavirus disease 2019 (COVID-19), with the goal of improving clinical understanding and the value of research applications.

Methods: General data of 66 confirmed COVID-19 patients admitted to the Fifth Medical Center of Beijing PLA General Hospital from January 2 to March 23, 2020 were collected in this retrospective case-control observational study, and they were divided into mild (n = 26), mid-grade (n = 19), and severe/critical disease groups (n = 21) according to disease severity. Neutrophils, lymphocytes, neutrophil/lymphocyte ratios, CD4 absolute counts, CD4/CD8 expression ratios of peripheral whole blood among the three patient groups were compared. The study protocol was approved by the Ethics Committee of the Fifth Medical Center, General Hospital of Chinese PLA (approval No. 2020-69-D) on May 5, 2020.

Results: Among the 66 COVID-19 patients examined, 38 were male and 28 were female, with an average age of 53 ± 17 years. Among patients, 26 cases were mild, 19 cases were mid-grade, and 21 cases were severe/critical. Neutrophils, neutrophil/lymphocyte ratios, and CD4+/CD8+ ratios of the severe/critical group were significantly higher compared with mild and mid-grade groups (P < 0.01); however, there was no obvious difference between mid-grade and mild groups (P > 0.05). Lymphocytes, CD4 absolute counts, and CD8+ absolute counts of the severe/critical group were significantly lower compared with mild and mid-grade groups (P < 0.01); however, there was no significant difference between mid-grade and mild groups (P > 0.05).

Conclusion: Counts of lymphocytes and T lymphocytes in severe/critically ill patients were decreased, which is of great significance for the identification of severe and critical COVID-19 patients.

Keywords: clinical significance, COVID-19, lymphocytes, neutrophils, T lymphocyte

Introduction
The main routes of transmission of coronavirus disease 2019 (COVID-19) are respiratory droplets and contact transmission. The main clinical manifestations are fever, cough, and fatigue. In severe cases, respiratory distress syndrome may occur, accompanied by sepsis.[1,2] Pneumonia infections caused by COVID-19 are rapidly increasing, which seriously endanger not only people’s safety and lives, but also international economic development.[3,4] Therefore, it is of great significance to perform COVID-19-related basic theoretical research, especially to clarify the impact of COVID-19 on the human immune system.

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COVID-19 presents with different manifestations that can be asymptomatic, mild, moderate or severe. The mortality rate of severe patients is high and treatment remains difficult. Thus, predicting the direction of pneumonia (“-mild-”–“-severe-”–“-critical”) and effect of the current treatment plan as early as possibly are important measures to improve the cure and survival rates of patients.[5,6] There is an urgent need to accelerate relevant research support to fully understand the disease characteristics of COVID-19.[7] To this end, we here report laboratory data and cellular immune function of patients with COVID-19, and explore correlations of immune function and cells with the clinical grade of COVID-19 infection pneumonia.

Subjects and methods

Subjects
The protocol of this study and the usage of human-related materials were approved by the Ethics Committee of the Fifth Medical Center, General Hospital of Chinese PLA, China (approval No. 2020-69-D) on May 5, 2020 with the written informed consent from patients. The experiments were performed according to the Declaration of Helsinki. From January 2 to March 23, 2020, a total of 66 COVID-19 patients were admitted to the Fifth Medical Center of PLA General Hospital. According to classification criteria of the New Coronavirus Pneumonia Diagnosis and Treatment Plan (Trial Sixth Edition)[8] the patient cohort included 26 mild cases (39.4%), 19 mid-grade cases (28.8%), 19 severe cases (28.8%), and critically
ill type 2 cases (3%). After dividing patients into three groups (mild, mid-grade, and severe/critical), we compared peripheral blood and lymphocyte subsets of the three groups.

The sample size (a total number of 66 patients) used in the presence work has adequate power to detect a prespecified effect size (the 1–β: 0.8; α/2: 0.025; P < 0.05). The original hypothesis was that there was no significant difference in the tested indicators (for example, neutrophil/lymphocyte ratios) among different patient groups; whereas the alternative hypothesis was that there was a significant difference in the tested indicator (for example, neutrophil/lymphocyte ratios) among different patient groups.

### Eligible criteria

The selected 66 patients all met the diagnostic criteria of the New Coronavirus Pneumonia Diagnosis and Treatment Plan (Trial Sixth Edition)[10] combined with patient clinical manifestations, laboratory examinations, and imaging.

The inclusion criteria for selection of the patients: 1. Epidemiological history, (1) Travel or residence history in Wuhan City and surrounding areas, or other communities with case reports within 14 days before the onset of illness; (2) A history of contact with a person infected with the novel coronavirus (a person with a positive nucleic acid test) within 14 days before the onset of illness; (3) Contact with patients with fever or respiratory symptoms from Wuhan City and surrounding areas, or communities with case reports within 14 days before the onset of illness; (4) Cluster disease; 2. Clinical manifestations, (1) Fever and/or respiratory symptoms; (2) There are imaging features of the above-mentioned novel coronavirus pneumonia; (3) The total number of white blood cells is normal or decreased in the early stage of the disease, and the lymphocyte count is decreased; 3. Real-time fluorescent RT-PCR detection of new coronavirus nucleic acid positive; 4. Viral gene sequencing, highly homologous with known new coronaviruses. Additionally, the patient’s respiratory tract samples (throat swabs, nasal swabs, or sputum samples) tested positive for the new coronavirus nucleic acid by quantitative real-time polymerase chain reaction. The exclusion criteria: patient information or patient specimens are missed, or some patients have unclear diagnosis.

### Laboratory procedures

Neutrophil counts (N) and lymphocyte counts (L) of peripheral blood were measured using a blood routine analyzer (Sysmex XT5000, SYSMEX Corporation, Kobe City, Japan) to evaluate the number of neutrophils and lymphocytes. Neutrophil counts (N) and lymphocyte counts (L) of peripheral blood were measured using a blood routine analyzer (Sysmex XT5000, SYSMEX Corporation, Kobe City, Japan) to evaluate the number of neutrophils and lymphocytes.

Flow cytometry was used to identify and sort peripheral blood lymphocyte subsets of patients, as shown in Figure 1. Compared with patients in mild and mid-grade groups, neutrophil counts and neutrophil/lymphocyte ratios (NLR) of the severe group were significantly increased ($F = 24.25, P < 0.001$ compared with mid-grade group); $F = 38.87, P < 0.001$ (compared with mild group); whereas NLRs of patients in the mild group were not significantly different from the mid-grade group. Moreover, lymphocyte counts of patients in the severe group were significantly decreased compared with mid-grade and mild groups ($F = 61.85, P < 0.001$); whereas lymphocyte counts of patients in the mild group were not significantly different from the mid-grade group (Table 1). Therefore, NLR and lymphocyte counts were valuable indicators of severe disease.

### Statistical analysis

SPSS19.0 statistical software (IBM Corp., Armonk, NY, USA) was used for data analysis and processing. Measurement data is expressed as mean ± standard deviation, and comparisons of the three groups of means were performed by a one-way analysis of variance with Tukey’s post hoc test. $P < 0.05$ indicates a statistically significant difference.

### Results

#### General patient information

There were 66 patients, including 38 males and 28 females, with an average age of 53 ± 17 years. There were 26 mild cases [16 males (72%) and 10 females (38%)], 19 mid-grade cases [12 males (63%) and 7 females (37%)], and 21 severe/critical cases [11 males (52%) and 10 females (48%)] (Table 1).

#### Relationship between blood routine testing and disease severity in the three patient groups

Flow cytometry was used to identify and sort peripheral blood lymphocyte subsets of patients, as shown in Figure 1. Compared with patients in mild and mid-grade groups, neutrophil counts and neutrophil/lymphocyte ratios (NLR) of the severe group were significantly increased ($F = 24.25, P < 0.001$ compared with mid-grade group); $F = 38.87, P < 0.001$ (compared with mild group); whereas NLRs of patients in the mild group were not significantly different from the mid-grade group. Moreover, lymphocyte counts of patients in the severe group were significantly decreased compared with mid-grade and mild groups ($F = 61.85, P < 0.001$); whereas lymphocyte counts of patients in the mild group were not significantly different from the mid-grade group (Table 1). Therefore, NLR and lymphocyte counts were valuable indicators of severe disease.

#### Relationship between lymphocyte subsets in the three patient groups and disease severity

The results described above mainly focused on total lymphocyte-related features. To further examine relationships between lymphocyte subsets in the three groups of patients and the severity of COVID-19, T-cell subsets were also examined. As shown in Table 3, the results indicated that compared with patients in mild and mid-grade groups, CD4+ (Th cells) and CD8+ (cytotoxic T lymphocytes [CTL]) counts of patients in the severe group were significantly reduced ($F = 16.34, P < 0.001$ for CD4+ count; $F = 33.36, P < 0.001$ for CD8+ count). Moreover, CD4+/CD8+ ratios were significantly increased ($F = 9.57, P < 0.001$) in patients with severe disease compared with the other two groups (Table 3). Therefore, decreased CD4+ or CD8+ counts, and increased CD4+/CD8+ ratios could indicate severe disease.

#### Discussion

The emerging coronavirus pneumonia is an acute respiratory infectious disease caused by a new β-coronavirus closely related to severe acute respiratory syndrome coronavirus 2 and middle...
east respiratory syndrome coronavirus.\textsuperscript{[11-13]} The main source of infection is patients infected by the new coronavirus, and it mainly spreads by respiratory droplets or close contact. CD4+ T cells are a subgroup of T cells that exert immune regulation, while CD8+ T cells are effector cells of specific cellular immunity that exert immunosuppressive and antiviral effects.\textsuperscript{[14]} Results of the present study indicate that as the course of the disease progresses, CD4+ and CD8+ T cells significantly decline in critically ill and severe patients, making them predictors of mid-grade, light, and severe/critical conditions. Early warning signs of severe disease and related indicators can well-reflect disease progression and be used to guide clinical treatment.\textsuperscript{[15,16]}

The mechanism by which COVID-19 mediates lymphopenia is still unclear.\textsuperscript{[15,16]} Some recent publications reported that excessive T cell activation after infection is related to excessive Th17, which explains observations of immune damage in patients.\textsuperscript{[17]} Pathological Th1 cell activation increases interleukin 6, which causes inflammatory monocytes to activate and proliferate.\textsuperscript{[17]} Inhibition of granulocyte-macrophage colony-stimulating factor or interleukin 6 is expected to inhibit

### Table 2

<table>
<thead>
<tr>
<th>Lymphocyte subsets</th>
<th>Mid-grade group (n=19)</th>
<th>Mild group (n=26)</th>
<th>Severe/critical group (n=21)</th>
<th>F values</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEU (x10^9/L)</td>
<td>3.79±2.35</td>
<td>3.48±1.16</td>
<td>5.72±3.58</td>
<td>24.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LYM (x10^9/L)</td>
<td>1.45±0.53</td>
<td>1.84±0.53</td>
<td>0.98±0.60</td>
<td>61.85</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NLR</td>
<td>2.74±1.87</td>
<td>2.04±0.90</td>
<td>8.20±7.26</td>
<td>38.87</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±SD and were analyzed using one-way analysis of variance with Tukey post hoc test. COVID-19 = coronavirus disease 2019, LYM = lymphocytes, NEU = neutrophils, NLR = neutrophils/lymphocytes ratio.

### Table 3

<table>
<thead>
<tr>
<th>T-cell subsets</th>
<th>Mid-grade group (n=19)</th>
<th>Mild group (n=26)</th>
<th>Severe/critical group (n=21)</th>
<th>F values</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+T (cells/µL)</td>
<td>517.23±257.17</td>
<td>629.95±254.50</td>
<td>372.9±283.63</td>
<td>16.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD8+T (cells/µL)</td>
<td>404.67±181.39</td>
<td>492.80±186.36</td>
<td>240.59±196.31</td>
<td>33.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>1.43±0.79</td>
<td>1.32±0.50</td>
<td>2.09±1.67</td>
<td>9.57</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±SD and were analyzed using one-way analysis of variance with Tukey post hoc test. COVID-19 = coronavirus disease 2019.
immunopathological damage, which is a possible mechanism involved in disease deterioration. Indeed, release of granulocyte-macrophage colony-stimulating factor and interleukin 6 by T lymphocytes and monocytes may be the key to COVID-19-induced cytokine storm.\textsuperscript{19,21} The limitations of the study may include: (1) the study only observed changes in lymphocytes, neutrophils and NLR, and T-cell subsets; (2) the study focused on the relationship between disease diagnosis and peripheral blood counts of lymphocytes, but failed to analyze the effect of different treatment strategies on peripheral blood lymphocyte counts in such patients; (3) there was no follow-up visit after discharge.

In summary, by analyzing the laboratory results of 66 patients with new coronary disease, we identified a positive correlation between the degree of T lymphocyte subpopulation reduction and severity of COVID-19. This finding has guiding value for early identification and diagnosis of critically ill patients, which is necessary to reduce mortality and improve prognosis. At present, COVID-19 related research has entered a new stage. With the improvement of testing capabilities, the detection rate of COVID-19 patients continues to increase, and their overall mortality rate is gradually decreasing.\textsuperscript{19–21} At the same time, a variety of vaccines in China, the United States and Europe have been approved for marketing.\textsuperscript{19,21} Therefore, future research will turn to the immune response of patients after vaccination, especially how to find indicators of the immune protection effect of patients after vaccination. In addition, this study initially revealed the relationship between the disease of COVID-19 patients and the immune cells in blood samples. In the future, we will continue to dig deeper to explore more detailed subgroup analysis such as T cells and B cells.

Acknowledgments
None.

Author contributions
BL designed and implemented the study, and participated in data analysis. GY and FF participated in data processing, data analysis, data validation and manuscript drafting. FF and XL participated in visual analysis. TZ and XL were responsible for trial supervision. BL and FF participated in manuscript reviewing and editing. All authors approved the final version of the manuscript.

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None.

Institutional review board statement
This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Fifth Medical Center, General Hospital of Chinese PLA (approval No. 2020-69-D) on May 5, 2020.

Declaration of patient consent
The authors certify that they have obtained the patient consent forms. In the form, patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity.

Conflicts of interest
The authors declare that they have no conflicts of interest.

References