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Clinical and immunologic features in severe and moderate Coronavirus Disease 2019

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BACKGROUND. Since December 2019, an outbreak of Coronavirus Disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, and is now becoming a global threat. We aimed to delineate and compare the immunologic features of severe and moderate COVID-19.

METHODS. In this retrospective study, the clinical and immunologic characteristics of 21 patients (17 male and 4 female) with COVID-19 were analyzed. These patients were classified as severe (11 cases) and moderate (10 cases) according to the Guidelines released by the National Health Commission of China.

RESULTS. The median age of severe and moderate cases was 61.0 and 52.0 years, respectively. Common clinical manifestations included fever, cough and fatigue. Compared to moderate cases, severe cases more frequently had dyspnea, lymphopenia, and hypoalbuminemia, with higher levels of alanine aminotransferase, lactate dehydrogenase, C-reactive protein, ferritin and D-dimer as well as markedly higher levels of IL-2R, IL-6, IL-10, and TNF-α. Absolute number of T lymphocytes, $CD4^+$T and $CD8^+$T cells decreased in nearly all the patients, and were markedly lower in severe cases (294.0, 177.5 and 89.0 × 10^6/L) than moderate cases (640.5, 381.5 and 254.0 × 10^6/L). The expressions […]

Clinical and immunologic features in severe and moderate Coronavirus Disease 2019

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Abstract

Background: Since December 2019, an outbreak of Coronavirus Disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, and is now becoming a global threat. We aimed to delineate and compare the immunologic features of severe and moderate COVID-19.

Methods: In this retrospective study, the clinical and immunologic characteristics of 21 patients (17 male and 4 female) with COVID-19 were analyzed. These patients were classified as severe (11 cases) and moderate (10 cases) according to the Guidelines released by the National Health Commission of China.

Results: The median age of severe and moderate cases was 61.0 and 52.0 years, respectively. Common clinical manifestations included fever, cough and fatigue. Compared to moderate cases, severe cases more frequently had dyspnea, lymphopenia, and hypoalbuminemia, with higher levels of alanine aminotransferase, lactate dehydrogenase, C-reactive protein, ferritin and D-dimer as well as markedly higher levels of IL-2R, IL-6, IL-10 and TNF-α. Absolute number of T lymphocytes, $CD4^+$T and $CD8^+$T cells decreased in nearly all the patients, and were markedly lower in severe cases (294.0, 177.5 and 89.0 × 10^6/L) than moderate cases (640.5, 381.5 and 254.0 × 10^6/L). The expressions of IFN-γ by $CD4^+$T cells tended to be lower in severe cases (14.1%) than moderate cases (22.8%).

Conclusion: The SARS-CoV-2 infection may affect primarily T lymphocytes particularly $CD4^+$T and $CD8^+$ T cells, resulting in decrease in numbers as well as IFN-γ production. These potential immunological markers may be of importance due to their correlation with disease severity in COVID-19.

Trial registration: This is a retrospective observational study without a trial registration number.

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57 Keywords: SARS-CoV-2; COVID-19; cytokines; lymphocytes; pneumonia

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Introduction

Coronaviruses (CoV) are a large family of respiratory viruses that can cause diseases ranging from the common cold to the Middle-East Respiratory Syndrome (MERS) and the Severe Acute Respiratory Syndrome (SARS) (1, 2), both of which are zoonotic in origin and induce fatal lower respiratory tract infection as well as extrapulmonary manifestations. The new coronavirus, officially designated as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a member of Beta-CoV lineage B, which was first identified in Wuhan by the Chinese Center for Disease Control and Prevention (CDC) (3, 4). Recent reports have provided evidence for person to person transmission of the SARS-CoV-2 in family and hospital settings (5, 6). As of February 27, 2020, the number of SARS-CoV-2 cases globally had eclipsed 82567, largely exceeding the total number of SARS cases during the 2003 epidemic, and more than 2810 people had now died. The outbreak of SARS-CoV-2-induced Coronavirus Disease 2019 (COVID-19) has put health authorities on high alert in China and across the globe.

It has been revealed that SARS-CoV-2 has a genome sequence 75% to 80% identical to the SARS-CoV and has more similarities to several bat coronaviruses (7). Both clinical and epidemiological features of patients with COVID-19 have recently been reported, demonstrating that the SARS-CoV-2 infection can cause clusters of severe respiratory illness with clinical presentations greatly resembling SARS-CoV, leading to intensive care unit (ICU) admission and high mortality (8). Clinical manifestations have included fever, fatigue, dry cough, shortness of breath, and acute respiratory distress syndrome. Additionally, a study of the first 41 laboratory-confirmed cases with COVID-19 showed that 63% of patients had lymphopenia, and cytokine storm could be associated with disease severity. However, information on immunologic features between severe and moderate COVID-19 is scarce (8).

In this study, we performed a comprehensive evaluation of characteristics of 21 patients with COVID-19 admitted to Tongji Hospital, Wuhan. We aimed to compare the clinical and immunologic features between severe cases and moderate cases. These findings may help us extend our understanding of the risk factors associated with disease severity in the SARS-CoV-2 infection.

Results

Patient demographics and baseline characteristics of severe and moderate COVID-19

As of January 27, 2020, a total of 21 admitted hospital patients with pneumonia were identified as laboratory-confirmed SARS-CoV-2 infection at Wuhan Tongji hospital. Of these patients, only four patients including a familial cluster of three confirmed cases had direct exposure to Huanan seafood market. According to the Guidelines for diagnosis and management of COVID-19 (6^{th} edition, in Chinese) issued by the National Health Commission of China (9), 11 (52.4%) patients with percutaneous oxygen saturation (SpO2)≤93% or respiratory rates ≥30 per min on room air who required high-flow nasal cannula or non-invasive mechanical ventilation using the Bilevel Positive Airway Pressure (BiPAP) mode to correct hypoxemia, were classified as having severe COVID-19, whereas 10 (47.6%) patients not reaching criteria of severe COVID-19 were considered as moderate. There were more male patients in both severe cases and moderate cases. The median age of the severe cases (61.0 years) was significantly older than moderate cases (52.0 years) (Table 1). More severe cases had comorbidity. The median time from onset of symptoms to first hospital admission was 8.0 days in severe cases and 7.0 days in moderate cases.

Four of eleven severe cases died at an average of 20 days after the onset of the illness. Of these four deceased patients, all of them were male and aged 50 years and older, with two cases having hypertension. Median age of deceased cases was 64.0 years old. Three of the deceased cases had arterial oxygen tension (PaO2) over inspiratory oxygen fraction (FiO2) (PaO2/FiO2) ratio ≤ 100 on admission.

Excluding one patient without a clear history due to the disorder of consciousness (coma) (classified as severe case), the most common clinical manifestations at onset of illness include fever, cough, fatigue and myalgia. Less common symptoms include sputum production, diarrhea, headache and hemoptysis. Compared to moderate cases, chest tightness tended to be more common in severe cases. In addition, tachypnea and dyspnea were only developed in severe cases. All the severe cases developed dyspnea, and nine of them with SpO2≤93% showed no improved SpO2 even with high-flow nasal cannula, who were then ventilated using the BiPAP mode to treat hypoxemia. Arterial blood gas (ABG) test was performed in 10 patients

on admission (six severe and four moderate cases). Of them, PaO2/FiO2 ratio was significantly lower in severe cases (104.8) than moderate cases (371.7), with 3 out of 6 severe patients below 100.

Laboratory findings and CT scans of severe and moderate COVID-19

Compared with normal range, the whole blood count on admission of three (30%) moderate cases showed mild leucopenia, while white blood cell (WBC) counts were normal or slightly increased above the upper limit of normal (ULN) in all the severe cases (Table 2). Both WBC and neutrophil counts were significantly higher in severe cases than moderate cases. Whereas lymphocyte counts were significantly lower in severe cases (0.7×10^9/L) than moderate cases (1.1×10^9/L). Lymphopenia (lymphocyte count $<0.8 \times 10^9$/L) was developed in 8 (72.7%) severe cases and only 1 (10.0%) moderate cases (p=0.008). Overall, severe cases have increased WBC count (p = 0.003), but lower lymphocyte count (p = 0.049).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly higher in severe cases than moderate cases. Albumin concentrations were significantly lower in severe cases than moderate cases, and hypoalbuminemia (albumin<32g/L) was more frequent in severe cases (Table 2). Levels of lactate dehydrogenase (LDH), concentrations of serum high-sensitivity C-reactive protein (hsCRP), ferritin and D-dimer levels were markedly higher in severe cases than moderate cases. Besides, serum levels of procalcitonin tended to be higher in severe cases than in moderate cases. These results suggest an increased level of systemic inflammation in severe cases.

Interstitial lung abnormalities were observed in chest computed tomography (CT) scans of all patients on admission. Of the 21 patients, 10 (90.9%) severe cases and 7 (70%) moderate cases had bilateral involvement on admission (Table 2). The typical findings of chest CT images of severe COVID-19 on admission showed bilateral ground glass opacity and subsegmental areas of consolidation (Figure 1A), then progressed rapidly with mass shadows of high density in both lungs (Figure 1B). Whereas the representative chest CT images of moderate COVID-19 showed bilateral ground glass opacity (Figure 1C). Later chest CT images revealed bilateral ground-glass opacity had been resolved (Figure 1D).

Immunologic features of severe and moderate COVID-19

We detected the plasma cytokine levels to examine the presence of cytokine storm in these patients. Evaluation of serum cytokines on admission revealed that levels of interleukin (IL)-2R, IL-6, IL-10, and tumor necrosis factor-α (TNF-α) were markedly higher in severe cases than in moderate cases (Figure 2, Supplementary Table 1). IL-1β concentrations were undetectable (<5pg/mL) in nearly all the patients with either severe or moderate COVID-19. Overall, we found that macrophage-related proinflammatory cytokines, particularly IL-6, IL-10 and TNF-α, are significantly increased in majority of severe cases. Of note, IL-6 levels were increased in both moderate and severe cases.

We next examined the proportions and effector functions of immune cells in peripheral blood (Figure 3, Table 3). Preliminary analysis of circulating immune cells subsets as shown in Table 3 demonstrated that absolute numbers of total T lymphocytes, $CD4^+$T cells and $CD8^+$T cells were reduced below the lower limit of normal (LLN) in the vast majority of patients with either severe or moderate COVID-19, and they were reduced more profoundly in severe cases (294.0, 177.5 and 89.0 × 10^6/L) than in moderate cases (640.5, 381.5 and 254.0 × 10^6/L) (Figure 3A, 3B).The proportion of B cells was significantly higher in severe cases (20.2%) than in moderate cases (10.8%). This could be partly due to the more significant decrease of T lymphocytes in severe cases. In addition, six (75.0%) of eight severe cases showed a broad, significant decrease in all the lymphocyte subsets excluding B cells, with total T lymphocytes counts below 400 × 10^6/L, $CD8^+$T cells counts below 150 × 10^6/L, and NK cells counts below 77 × 10^6/L. Of these six patients, three (50%) eventually died.

Moreover, the frequencies of regulatory T cells (Tregs) ($CD4^+CD25^+CD127^{low+}$) and $CD45RA^+$Tregs were reduced (below LLN) in nearly all the severe and moderate cases, with $CD45RA^+$Tregs proportion was markedly lower in severe cases (0.5%) than in moderate cases (1.1%). The reduced expressions of interferon-γ (IFN-γ) by $CD4^+$T, $CD8^+$T and NK cells below LLN were observed in some patients with severe (50%, 16.7% and 16.7%) or moderate COVID-19 (14.3%, 0% and 14.3%). The expressions of IFN-γ by $CD4^+$T cells tended to be lower in severe cases (14.1%) than moderate cases (22.8%) (Table 3, Figure 2C). However,

there was no significant difference in terms of mean fluorescence intensity of IFN-γ production by $CD4^+$T, $CD8^+$T or NK cells (data not shown). Overall, we found a significant reduction in $CD4^+$ T cell count and a borderline reduction in IFN-γ expression in severe cases.

Complications and clinical outcomes of COVID-19

With regards to complications as shown in Supplementary Table 2, common complications observed in severe cases included acute respiratory distress syndrome (100.0% of patients with available ABG data), respiratory failure (83.3%). Less common complications among the severe cases included secondary infection (27.3%), acute cardiac injury (9.1%), and hypoxic encephalopathy (18.2%), acute kidney injury (18.2%), shock (9.1%) and acute liver injury (9.1%), most of which were not developed in any recovered cases.

All the severe and moderate cases were given empirical antimicrobial treatment (moxifloxacin and/or cephalosporin, etc.). 7 (63.6%) severe cases and all moderate cases received antiviral therapy (oseltamivir and/or ganciclovir). In Addition, all severe and moderate cases were administered corticosteroids (methylprednisolone) during the course of hospitalization. Nine (81.8%) severe cases and no moderated case required non-invasive mechanical ventilation. As of February 2, 2020, 4 (36.4 %) of 11 severe cases and none (0.0 %) of the moderate cases died, the median days from illness onset to death was 20 days. One severe and one moderate case recovered. Patients were transferred to the designated hospital after being identified as having laboratory-confirmed SARS-CoV-2 infection.

Discussion

This is the first preliminary study evaluating descriptively the immunologic characteristics of patients with laboratory-confirmed SARS-CoV-2 infection. Both clinical and epidemiological features of patients with COVID-19 have recently been reported(5, 6, 8, 10). However, there is insufficient knowledge of pathophysiological parameters particularly immunologic indicators to understand the mechanism involved in COVID-19. Consistent with previous reports(8), this present study showed that a male predominance in the incidence of COVID-19 has been noted similar to that of SARS-CoV, indicating males are more susceptible to SARS-CoV-2 infection than females. Older males (>50 years old), particularly those with underlying comorbidities may be more likely to develop severe COVID-19. The most common clinical manifestations at onset of illness included fever, cough, fatigue and myalgia. Severe cases more frequently had dyspnea and developed acute respiratory distress syndrome. In terms of laboratory findings, leukocytosis ($\geq 10\times 10^9$/L) but lymphopenia ($<0.8\times 10^9$/L) were more common in severe cases than in moderate cases. ALT, LDH, D-dimer and inflammatory markers including hsCRP and ferritin were significantly higher in severe cases than in moderate cases. Serum concentrations of both pro-inflammatory cytokines and anti-inflammatory cytokines, including IL-2R, IL-6, TNF-α and IL-10 increased in the majority of severe cases and were markedly higher than did those in moderate cases, suggesting cytokine storms might be associated with disease severity. Similarly, SARS was also characterized by exuberant inflammatory responses and lung damage. A study using mice model of SARS demonstrated that rapid kinetics of SARS-CoV replication and delay in IFN-I signaling promoted inflammatory monocyte-macrophage accumulation, resulting in elevated lung cytokine/chemokine levels, vascular leakage, and suboptimal T cell responses (11). The underlying the cellular origin and mechanism involving cytokine accumulation in COVID-19 warrants further exploration.

Additionally, we noted that SARS-CoV-2 infection can cause a significant reduction in circulating lymphocytes and T cell subsets. Although the proportions of T cells subsets in peripheral blood remained within the normal range in most patients, decreased $CD4^+$T cell and $CD8^+$T cell counts below LLN was considerably frequent in both severe and moderate cases. More importantly, the number of $CD4^+$T cells and $CD8^+$T cells was markedly lower in severe

cases than moderate cases. In contrast, both the proportion and number of B cells were not reduced in most patients, with 75.0% of severe cases showing increased proportion of B cells. This could be partly due to the more significant decrease of T lymphocytes in these patients. It is notable that six out of eight severe cases and none of moderate cases with available immunologic data exhibited a broad, significant decline in all the lymphocyte subsets excluding B cells. Of these six patients, three eventually died. Moreover, the production of IFN-γ by $CD4^+$T cells but not $CD8^+$T cells or NK cells tended to be lower in severe cases than moderate cases. These data suggest that SARS-CoV-2 infection induces lymphopenia, particularly $CD4^+$T and $CD8^+$T cells, as well as suppressed IFN-γ production by $CD4^+$T cells, which correlates with disease severity of COVID-19.

Although the total Tregs proportion was comparable between severe cases and moderate cases, severe cases showed a significantly lower proportion of $CD45RA^+$ naive Tregs (nTregs) and a bit higher proportion of their memory counterparts $CD45RO^+$ memory Tregs (mTregs). nTregs might be activated in the periphery by antigen and subsequently converted to mTregs, and thus is thought to represent precursor cells of antigen -experienced mTregs and possess an equivalently strong suppressive capacity as compared with mTregs (12). It is reported that peripheral homeostatic mechanisms are crucial in the control of Tregs diversity and concomitantly in the maintenance of immune tolerance in healthy individuals. Disturbances within these mechanisms may have detrimental consequences and could contribute to the development of certain diseases particularly autoimmune diseases (12). Whether altered Treg proportion observed in this current study accounts for the severity of COVID-19, or correlates to the viremia, warrants further investigation.

$CD4^+$ T cells play a pivotal role in regulating immune responses, orchestrating the deletion and amplification of immune cells, especially $CD8^+$ T cells. $CD4^+$ T cells facilitate virus-specific antibody production via the T-dependent activation of B cells (13). However, $CD8^+$ T cells exert their effects mainly through two mechanisms, cytolytic activities against target cells or cytokines secretion, including IFN-γ, TNF-α, and IL-2 as well as many chemokines (14). The production of IFN-γ is essential for the resistance against infection of various pathogens such as virus, bacteria, and parasite (15). As a major source of IFN-γ, the ability of T cells to respond

to infection is part of the adaptive immune response and takes days to develop a prominent IFN-γ response.

In this study, albeit decreased numbers of $CD8^+$ T cells in severe cases, the proportion of $CD8^+$HLA-DR^+ T cells was slightly greater than that in moderate cases, which was in agreement with a recent case report (16). Circulating $CD8^+$T cells were found to harbor high concentrations of cytotoxic granules, including perforin and granulysin (16). Besides, a "cytokine storm" was exhibited in nearly all these populations, the only current available histological examination of a severe case who died of SARS-CoV-2 demonstrated lung interstitial mononuclear inflammatory infiltrates, dominated by lymphocytes, and multinucleated syncytial cells with atypical enlarged pneumocytes in the intra-alveolar spaces (16). These findings suggested that overactivation of cytotoxicity $CD8^+$T cells, along with over production of pro-inflammation cytokines, might account for, at least in part, the immunogenicity of COVID-19. Nevertheless, the cellular source (T cells, dendritic cells or macrophages) of these cytokines remains to be determined.

The roles of T cell responses in the context of SARS-CoV and MERS-CoV infection have been previously studied. Likewise, patients who survived SARS-CoV and MERS-CoV infections usually had better immune responses than those who did not (17). The immune system plays an important role in both diseases, but it is differentially affected by the two viruses (18). A study in SARS-CoV mice model has shown that depletion of $CD8^+$ T cells at the time of infection does not affect viral replication or clearance. However, depletion of $CD4^+$ T cells leads to an enhanced immune-mediated interstitial pneumonitis and delayed clearance of SARS-CoV from the lungs, demonstrating the vital role of $CD4^+$ T but not $CD8^+$ T cells in primary SARS-CoV infection (19). A Chinese study in SARS-CoV-infected patients has demonstrated that the majority of infiltrative inflammatory cells in the pulmonary interstitium are $CD8^+$ T cells that play an important role in virus clearance as well as in immune-mediated injury (20). After comparing T cell-deficient mice and B cell-deficient mice, it is found that T cells are able to survive and kill virus-infected cells in the MERS-CoV infected lung (21). These data highlight the importance of T lymphocytes, $CD4^+$ T cells in particular, but not B cells in controlling and finetuning the pathogenesis and outcomes of SARS-CoV and MERS-CoV infection. However,

a cohort study investigating adaptive immune responses to SARS-CoV infection revealed that despite no significant correlation between the total T cell responses and disease progression, the disease severity correlates strongly with high level $CD4^+$ T cell responses but not the memory $CD8^+$ T cell response (22). It is noteworthy that the immune responses evaluated in this study were in patients who recovered fully, thus whether these responses contribute to recovery or disease progression remains unclear (22).

Hin Chu et al demonstrated that MERS-CoV but not SARS-CoV can efficiently infect T cells from the peripheral blood and from human lymphoid organs, and induce apoptosis in T cells, which involves the activation of both the extrinsic and intrinsic apoptosis pathways. This may partly explain the lymphopenia observed in MERS-CoV-infected patients (23). SARS-CoV can also significantly decrease peripheral $CD4^+$ and $CD8^+$ T lymphocyte subsets and it was related to the onset of illness (24). Several potential mechanisms may be involved, including the development of auto-immune antibodies or immune complexes triggered by viral infection, directly infecting and promoting the growth inhibition and apoptosis of hematopoietic stem and progenitor cells. The use of glucocorticoids may also account for the decrease of lymphocytes in some SARS patients (25). At present little is known about mechanism underlying the lymphopenia caused by SARS-CoV-2 infection. In this study we could not exclude the possibility that some of the lymphopenia may be worsen due to the use of steroids during hospitalization. Further research is required to determine the effects of corticosteroid on lymphocytes in the context of COVID-19.

Our study has some limitations. First of all, we mainly evaluated the number of T cell subsets and NK cells as well as their IFN-γ production, the function of these cells, as well as the role of activated macrophages and lymphocytes infiltrating pulmonary interstitium remains to be elucidated. Second, this study only included a small number of patients, thus the results should be interpreted with caution, and statistical non-significance may not rule out difference between severe and moderate cases. Third, since data regarding the viremia profile of SARS-CoV-2 are not available, further studies are needed to investigate the correlation between the virus load kinetics and the dynamics of cellular immune responses. Clarification of these questions will allow further dissection of the complex SARS-CoV-2 pathogenesis, with potential implications

for the development of therapeutics and vaccines.

In conclusion, the SARS-CoV-2 infection induced cytokine storm and lymphopenia, particularly decrease in $CD4^+$T and $CD8^+$T cells counts, as well as suppressed IFN-γ production by $CD4^+$T cells, which might be correlated with disease severity of COVID-19. Gaining a deeper understanding of the factors that affect lymphocytes particularly T lymphocytes count and their association between disease severity in patients with SARS-CoV-2 infection is of importance for clinical management of COVID-19.

Methods

Study participants

From late December 2019 to January 27, 2020, a total of 21 cases who initially presented with fever or respiratory symptoms, with pulmonary infiltrates on chest CT scans in isolation ward of Department of Infectious Disease, Tongji hospital were later confirmed to be infected with SARS-CoV-2 by the local health authority. Four cases had a history of exposure to the Huanan seafood market.

We retrospectively evaluated and analyzed the medical history, physical examination, and hematological, biochemical, radiological, microbiological and immunological evaluation results obtained from these 21 patients with COVID-19. Epidemiological, clinical, laboratory, and radiological characteristics and treatment as well as outcomes data were obtained from electronic medical records. The data collection forms were reviewed independently by two researchers.

Clinical classifications and complication definitions

According to the Guidelines for diagnosis and management of COVID-19 (6^{th} edition, in Chinese) released by National Health Commission of China (9), the clinical classifications of COVID-19 are as follows:

Mild cases: The clinical symptoms are mild and no pneumonia manifestation can be found in imaging;

Moderate cases: Patients have symptoms like fever and respiratory tract symptoms, etc. and pneumonia manifestation can be seen in imaging;

Severe cases: Meeting any of the following: Respiratory distress, respiratory rates ≥30 breaths/min; The SpO2 ≤93% at a rest state; PaO2/FIO2 ratio ≤300; Patients with >50% lesions progression within 24 to 48 hours in pulmonary imaging should be treated as severe cases.

Critical ill cases: Meeting any of the following: Respiratory failure occurs and mechanical ventilation is required; Shock occurs; Complicated with other organ failure that requires monitoring and treatment in ICU.

Acute respiratory distress syndrome and shock were defined according to the interim guidance of WHO for SARS-CoV-2 (26).

Hypoxemia was defined as PaO2/FiO2 ratio of less than 300.

Acute kidney injury was identified and classified on the basis of the highest serum creatinine level or urine output criteria according to the kidney disease improving global outcomes classification.

Acute liver injury was defined as jaundice with a total bilirubin level of ≥ 3 mg/dl and an acute increase in alanine aminotransferase of at least five times the upper limit of the normal range and/or an increase in alkaline phosphatase of at least twice the upper limit of the normal range.

Cardiac injury was diagnosed if serum levels of cardiac biomarkers (e.g. troponin I) were > the 99th percentile upper reference limit, or new abnormalities were shown in electrocardiography and echocardiography.

Secondary infection including bacteria and fungus was diagnosed if the patients had clinical symptoms or signs of nosocomial pneumonia or bacteremia, and was combined with a positive culture of a new pathogen from a respiratory tract specimen or from blood samples taken ≥48 h after admission.

Laboratory measurements

Real-Time reverse transcription polymerase chain reaction assay for SARS-CoV-2

Respiratory specimens were collected by local CDC and then shipped to designated authoritative laboratories to detect the SARS-CoV-2. The presence of SARS-CoV-2 in respiratory specimens was detected by real-time RT-PCR methods. The primers and probe target to envelope gene of CoV were used and the sequences were as follows: forward primer 5′-TCAGAATGCCAATCTCCCCAAC-3′; reverse primer 5′-AAAGGTCCACCCGATACATTGA-3′; and the probe 5′CY5-CTAGTTACACTAGCCATCCTTACTGC-3′BHQ1. Conditions for the amplifications were 50°C for 15 min, 95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s.

Clinical laboratory measurements

Initial clinical laboratory investigation included a complete blood count, serum biochemical test (including liver and renal function, creatine kinase, lactate dehydrogenase, and electrolytes), coagulation profile, as well as immunological test (including serum cytokines, peripheral immune cells subsets and the expression of IFN-γ by immune cells). Respiratory specimens,

including nasal and pharyngeal swabs, or sputum were tested to exclude evidence of other virus infection, including influenza, respiratory syncytial virus, avian influenza, parainfluenza virus and adenovirus. Routine bacterial and fungal examinations were also performed.

Cytokine measurement

To explore the influence of SARS-CoV-2 infection on the secretion of cytokines, cytokines including IL-1β, IL-2R, IL-6, IL-8 (also known as CXCL8), IL-10, and TNF-α were assessed in serum samples drawn shortly after hospital admission by Chemiluminescence Immunoassay (CLIA) performed on a fully automated analyzer (Immulite 1000, DiaSorin Liaison, Italy or Cobas e602, Roche Diagnostics, Germany) for all patients according to the manufacturer's instructions. IL-1β kit (#LKL11), IL-2R kit (#LKIP1), IL-8 kit (#LK8P1), IL-10 kit (#LKXP1), and TNF-α kit (#LKNF1) were purchased from DiaSorin (Vercelli, Italy). IL-6 kit (#05109442 190) was purchased from Roche Diagnostics, Germany.

Evaluation of peripheral blood immunological indicators

The proportions and numbers of NK, $CD4^+$T, $CD8^+$T, Treg and B cells, and the expression of cell surface markers as well as IFN-γ expression by $CD4^+$T, $CD8^+$T and NK cells were studied in these patients with laboratory-confirmed SARS-CoV-2 infection. Flow cytometry antibodies against human surface and intracellular molecules are commercially available. The following antibodies were used: anti-CD28 (CD28.2, PE, #555729), anti-CD8 (RPA-T8, PE-Cy7, #557746), anti-CD45 (2D1, PerCP, #347464), anti-HLADR (G46-6, APC, #560744), anti-CD3 (SK7, APC-Cy7, #557832), anti-CD4 (RPA-T4, V450, #560345); anti-CD45RA (HI100, FITC, #555488), anti-CD45RO (UCHL1, PE, #5618898), anti-CD127 (HIL-7R-M21, PE-Cy7, #560822), anti-CD45 (2D1, PerCP, #347464), anti-CD25 (M-A251, APC, #561399), anti-CD3 (SK7, APC-Cy7, #557832), anti-CD4 (RPA-T4, V450, #560345); anti-CD3 (UCHT1, FITC, #561806), anti-CD8 (RPA-T8, PE, #555367), anti-CD56 (B159, PE-Cy7, #557747), anti-IFN-γ (4S.B3, APC, #551385), anti-CD4 (RPA-T4, APC-Cy7, #557871). All reagents were purchased from Becton, Dickinson, and Company (BD, Franklin Lakes, USA). All samples were detected by BD FACS Canto II Flow Cytometry System and analyzed with the BD FACS Diva Software.

The steps of intracellular staining for IFN-γ in immune cells were as follows, cell cultures were

added BD GolgiStop (BD Biosciences, #554724) and stimulated for 4 hours and then resuspended in FACS buffer for flow cytometry antibody staining. Peripheral blood mononuclear cells (PBMCs) were stained for surface antibody at 4°C for 30 minutes and were washed with FACS buffer followed by fixation/permeabilization (BD Cytofix/Cytoperm, #554722) at 4°C for 20 minutes in the dark. Then fixed/permeabilized cells were washed twice with Perm/Wash buffer (BD Biosciences, #554723), then thoroughly resuspended in 50 μL of Perm/Wash buffer containing a pre-determined optimal concentration of a fluorochrome-conjugated anti-IFN-γ antibody or appropriate negative control and incubated at 4°C for 30 minutes in the dark. Cells were washed twice with Perm/Wash buffer and resuspended in FACS buffer prior to flow cytometric analysis.

Statistics

Continuous variables were expressed as median (IQR) and compared with the unpaired 2-sided Student's t test; categorical variables were expressed as number (%) and compared by $\chi 2$ test or Fisher's exact test between moderate and severe case groups. A two-sided α of less than 0.05 was considered statistically significant. Statistical analyses were done using the SPSS (version 19.0).

Study approval

The study was performed in accordance with Good Clinical Practice and the Declaration of Helsinki principles for ethical research. The study protocol was approved by the Institutional Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (located at Wuhan, China). Written informed consent was waived due to the rapid emergence of this infectious disease.

Author contributions QN and DW designed the study and had full access to all data in the study and take responsibility for the integrity of data and the accuracy of the data analysis.

GC and DW contributed to patient recruitment, data collection, data analysis, data interpretation, literature search, and writing of the manuscript.

WG and YC had roles in patient recruitment, data collection, and clinical management.

DH, HW, TW, XZ, HC, HY, XZ, MZ, SW, JS, TC, MH, SL, XL, and JZ had roles in the patient management, experiments, data collection, data analysis, and data interpretation.

All authors contributed to data acquisition, data analysis, or data interpretation, and reviewed and approved the final version of the manuscript.

GC, DW, WG and YC share first authorship; DH, HW, TW and XZ are co-second authors; and the order in which they are listed was determined by workload.

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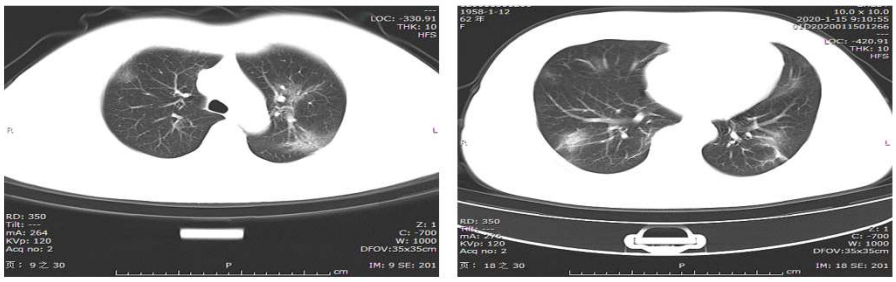
Figure legends

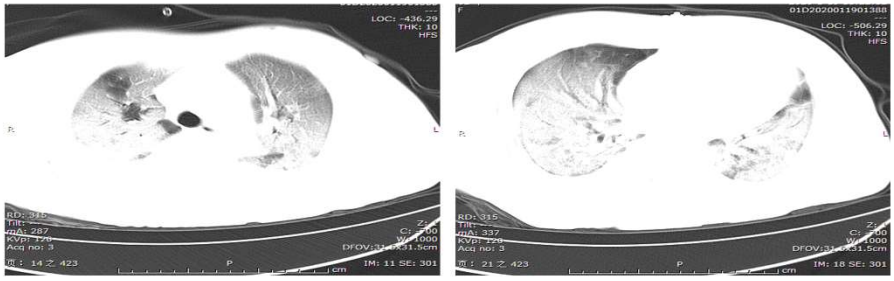
Figure 1: Computed tomography of the chest of patients with COVID-19

Chest CT axial view lung window from a 62-year-old female with severe COVID-19 showing bilateral ground-glass opacity and subsegmental areas of consolidation on day 6 after symptom onset (A), and typical presentation of a "white lung" appearance with bilateral multiple lobular and subsegmental areas of consolidation on day 8 after symptom onset (B). Chest CT axial view lung window from a 32-year-old male with moderate COVID-19 showing bilateral ground-glass opacity on day 7 after symptom onset (C), and resolved bilateral ground-glass opacity on day 11 after symptom onset (D).

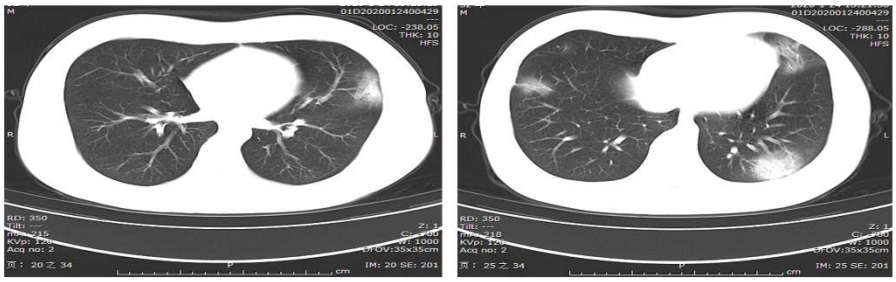
A Case 1 first chest CT



B Case 1 second chest CT



C Case 2 first chest CT



D Case 2 second chest CT

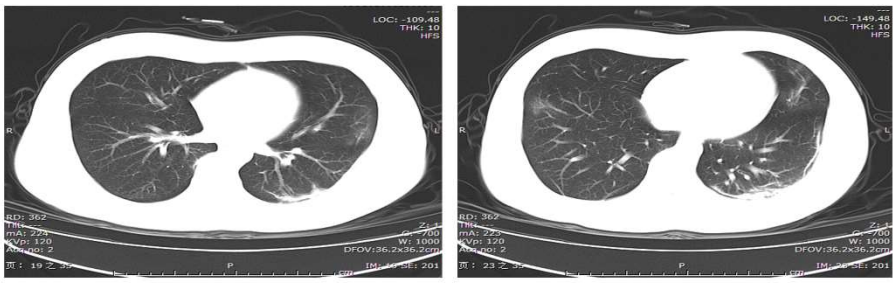


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Figure 2: Plasma cytokines levels in patients with COVID-19

Series of comparisons of plasma cytokines levels between severe cases (n=9) and moderate cases (n=7). All data represent mean ± SEM. Differences were tested using unpaired 2-sided Student's t test.

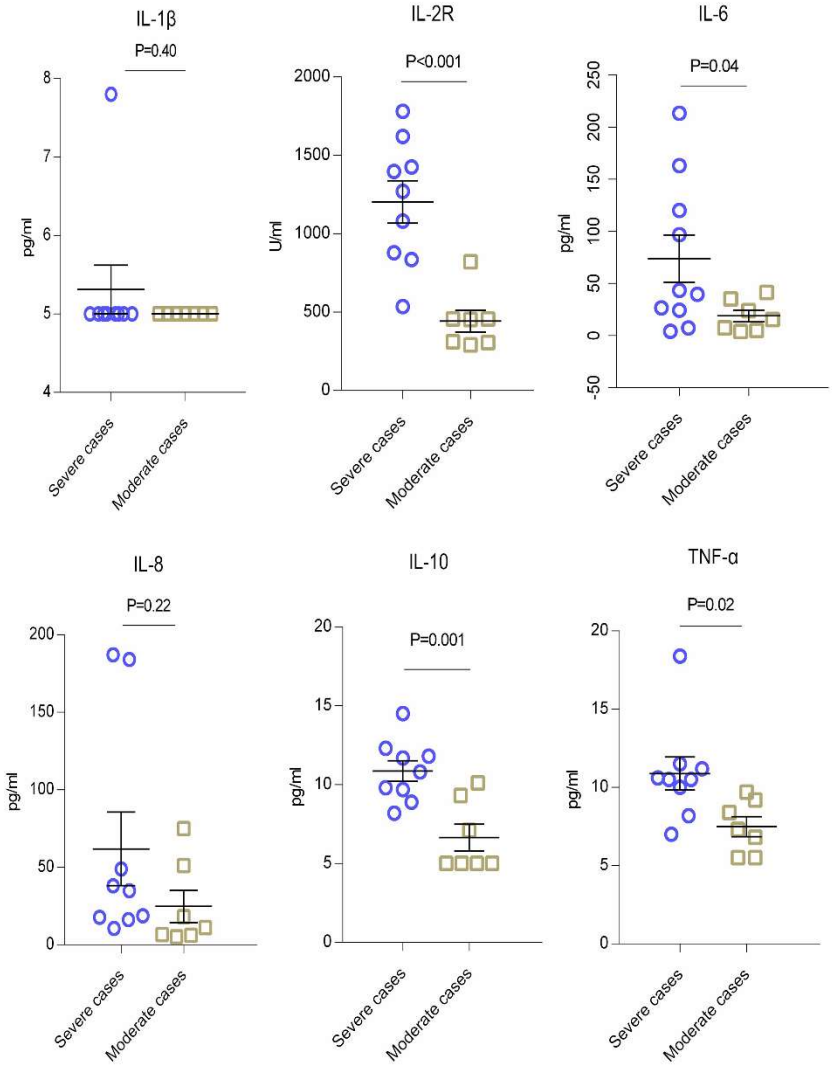
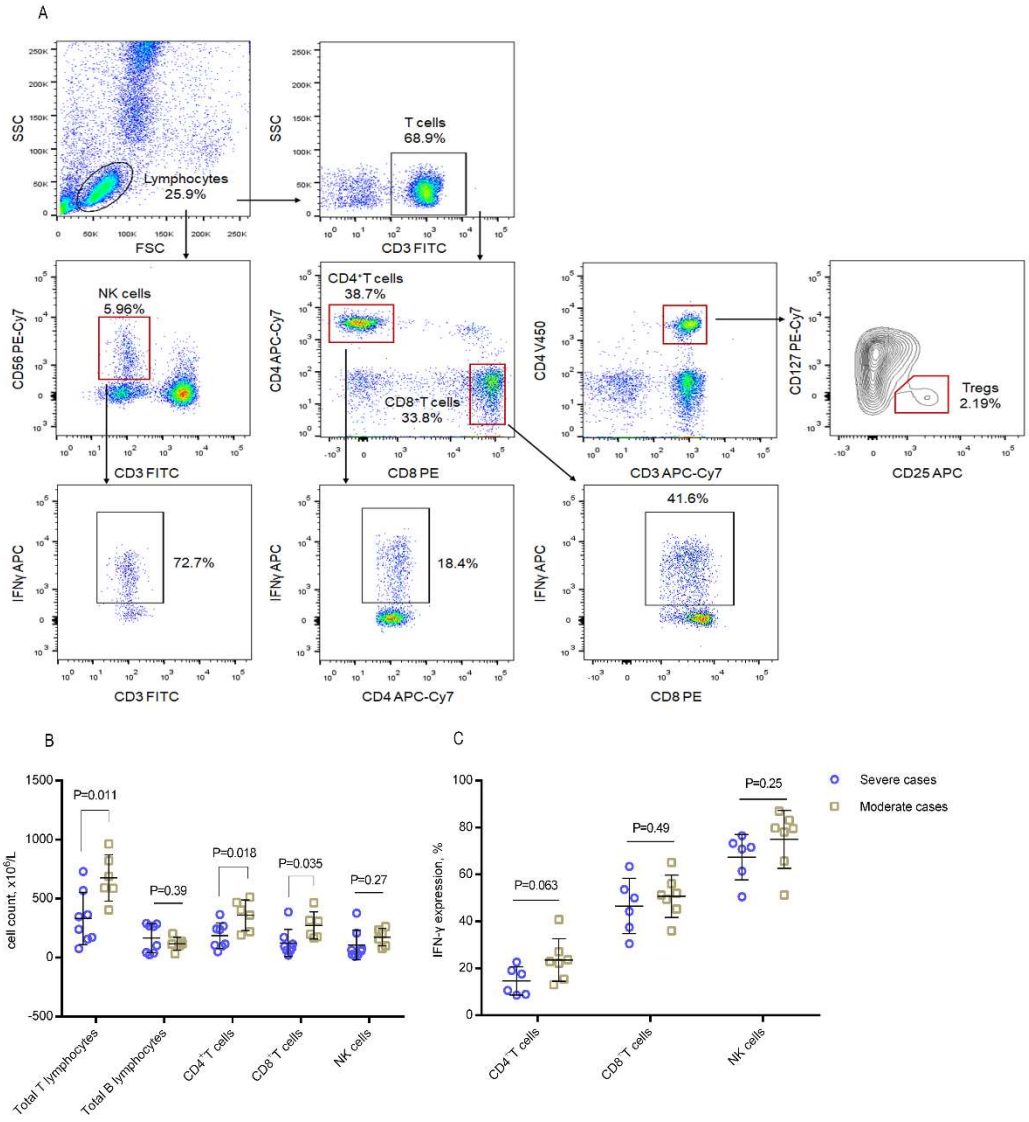


Figure 3: Number of immune cell subsets and proportion of IFN-γ expression in patients with COVID-19

(A) Flow cytometry staining of natural killer (NK) cells, $CD4^+$T cells, $CD8^+$T cells and Tregs as well as production of IFN-γ by $CD4^+$T cells, $CD8^+$T cells and NK cells from a representative patient.

(B) A series of comparisons of absolute number of total T&B lymphocytes, $CD4^+$T cells, $CD8^+$T cells and NK cells between severe cases (n=8) and moderate cases (n=6). All data represent mean ± SEM. Differences were tested using unpaired 2-sided Student's t test.

(C) A series of comparisons of production of IFN-γ by $CD4^+$T cells, $CD8^+$T cells and NK cells between severe cases (n=6) and moderate cases (n=7). All data represent mean ± SEM. Differences were tested using unpaired 2-sided Student's t test.


A
SSC
FSC
Lymphocytes
25.9%
T cells
68.9%
CD3 FITC
NK cells
5.96%
CD56 PE-Cy7
CD4⁺T cells
38.7%
CD8⁺T cells
33.8%
CD4 APC-Cy7
CD8 PE
CD4 V450
CD3 APC-Cy7
CD127 PE-Cy7
Tregs
2.19%
CD25 APC
IFNγ APC
72.7%
18.4%
41.6%
B
cell count, x10⁶/L
P=0.011
P=0.39
P=0.018
P=0.035
P=0.27
Total T lymphocytes
Total B lymphocytes
CD4⁺T cells
CD8⁺T cells
NK cells
C
IFN-γ expression, %
P=0.063
P=0.49
P=0.25
Severe cases
Moderate cases

545

546

547 **Table 1 Demographics and baseline characteristics of patients with COVID-19**

	All patients (n=21)	severe cases (n=11)	moderate cases (n=10)	P value
Characteristics				
Males, n (%)	17 (81.0%)	10 (90.9%)	7 (70.0%)	0.31
Age, yrs	56.0 (50.0-65.0)	61.0 (56.5-66.0)	52.0 (42.8-56.0)	0.043
>50	15 (71.4%)	10 (90.9%)	5 (50.0%)	0.043
Huanan seafood market exposure, n (%)	4 (19.0%)	1 (9.1%)	3 (30.0%)	0.31
Any comorbidity, n (%)	7 (33.3%)	5 (45.5%)	2 (20.0%)	0.36
Hypertension, n (%)	5 (23.8%)	4 (36.4%)	1 (10.0%)	0.31
Diabetes, n (%)	3 (14.3%)	2 (18.2%)	1 (10.0%)	1.00
Signs and symptoms				
Fever, n/N (%)	20/20 (100%)	10/10 (100%)	10/10 (100%)	NA
Highest temperature, ºC	38.7 (38.5-39.1)	38.6 (38.4-39.3)	38.8 (38.6-39.0)	0.87
38.1-39.0 ºC, n/N (%)	12/19 (63.2%)	5/9 (55.6%)	7/10 (70.0%)	0.52
>39.0 ºC, n/N (%)	7/19 (36.8%)	4/9 (44.4%)	3/10 (30.0%)	..
Cough, n/N (%)	16/20 (80.0%)	7/10 (70.0%)	9/10 (90.0%)	0.58
Fatigue, n/N (%)	17/20 (85.0%)	10/10 (100.0%)	7/10 (70.0%)	0.21
Myalgia, n/N (%)	8/20 (40.0%)	5/10 (50.0%)	3/10 (30.0%)	0.65
Sputum production, n/N (%)	5/20 (25%)	2/10 (20.0%)	3/10 (30.0%)	1.00
Headache, n/N (%)	2/20 (10.0%)	1/10 (10.0%)	1/10 (10.0%)	1.00
Diarrhea, n/N (%)	4/20 (20.0%)	1/10 (10.0%)	3/10 (30.0%)	0.58
Chest tightness, n/N (%)	11/20 (55.0%)	8/10 (80.0%)	3/10 (30.0%)	0.07
Coma, n (%)	1 (4.8%)	1 (9.1%)	0 (0.0%)	1.00
Dyspnea, n (%)	11 (52.4%)	11 (100.0%)	0 (0.0%)	0.000
Days from illness onset to dyspnea	8.0 (7.0-10.0)	8.0 (7.0-10.0)	NA	NA
Systolic pressure, mm Hg	122.0 (109.0-135.0)	124.0 (118.5-145.5)	120.0 (107.5-134.0)	0.17
>140mmHg, n (%)	4 (19.0%)	4 (36.4%)	0 (0.0%)	0.09
Heart rate, bpm	89.0 (78.0-106.0)	95.0 (77.0-108.0)	89.0 (85.5-96.0)	0.90
Respiratory rate, per min	21.0 (20.0-25.0)	25.0 (22.5-31.0)	20.0 (20.0-20.8)	0.005
≥30, n (%)	5 (23.8%)	5 (45.5%)	0 (0.0%)	0.035
Percutaneous oxygen saturation ≤93 % on room air	11 (52.4%)	11 (100.0%)	0 (0.0%)	0.000
PaO2/FiO2	172.0 (102.1-350.0)	104.8 (94.6-119.0)	371.7 (350.0-422.7)	0.001
>300, n/N (%)	3/10 (30.0%)	0/6 (0.0%)	4/4 (100.0%)	0.007
200-300, n/N (%)	2/10 (20.0%)	1/6 (16.7%)	0/4 (0.0%)	..
100-200, n/N (%)	2/10 (20.0%)	2/6 (33.3%)	0/4 (0.0%)	..
≤100, n/N (%)	3/10 (30.0%)	3/6 (50.0%)	0/4 (0.0%)	..

548 Abbreviations: COVID-19, Coronavirus Disease 2019; FiO2, inspiratory oxygen fraction; IQR,
549 interquartile range; PaO2, arterial oxygen tension; SARS-CoV-2, severe acute respiratory syndrome

coronavirus 2. Data are median (IQR), n (%), or n/N (%), where N is the total number of patients with available data. p values comparing severe cases and moderate cases are from χ^2 test, Fisher's exact test, or unpaired 2-sided Student's t test.

554 **Table 2 Laboratory findings and chest CT images of patients with COVID-19**

	Normal range	All patients (n=21)	severe cases (n=11)	moderate cases (n=10)	P value
White blood cell count, × 10⁹/L	3.5-9.5	5.7 (4.6-8.3)	8.3 (6.2-10.4)	4.5 (3.9-5.5)	0.003
<4, n (%)		3 (14.3%)	0 (0.0%)	3 (30.0%)	0.017
4-10, n (%)		15 (71.4%)	8 (72.7%)	7 (70.0%)	..
≥10, n (%)		3 (14.3%)	3 (27.3%)	0 (0.0%)	..
Neutrophil count, × 10⁹/L	1.8-6.3	4.8 (2.8-6.9)	6.9 (4.9-9.1)	2.7 (2.1-3.7)	0.002
Lymphocyte count, × 10⁹/L	1.1-3.2	0.9 (0.7-1.1)	0.7 (0.5-0.9)	1.1 (1.0-1.2)	0.049
<0.8, n (%)		9 (42.9%)	8 (72.7%)	1 (10.0%)	0.008
Hemoglobin, g/L	130-175	137.0 (127.0-147.0)	136.0 (125.5-144.5)	139.5 (132.8-146.0)	0.78
Platelet count, × 10⁹/L	125-350	160.0 (137.0-189.0)	157.0 (134.0-184.5)	175.6 (148.3-194.0)	0.88
<100, n (%)		1 (4.8%)	0 (0.0%)	1 (10.0%)	0.48
Alanine aminotransferase, U/L	≤41	26.0 (16.0-42.0)	42.0 (32.5-50.0)	16.0 (13.3-21.8)	0.000
Aspartate aminotransferase, U/L	≤40	27.0 (21.0-47.0)	47.0 (28.0-74.5)	24.0 (21.5-26.5)	0.014
>40, n (%)		6 (28.6%)	5 (45.5%)	0 (0.0%)	0.035
Albumin, g/L	35.0-52.0	33.7 (29.6-37.4)	29.6 (28.6-33.0)	37.2 (35.8-38.8)	0.013
<32 g/L, n (%)		8 (38.1%)	7 (63.6%)	1 (10.0%)	0.024
Total bilirubin, mmol/L	≤26	8.8 (6.8-10.3)	8.8 (7.9-10.5)	7.8 (6.4-9.5)	0.24
Blood urea nitrogen, mmol/l	3.1-8.0	5.1 (4.1-6.4)	6.1 (5.2-9.1)	4.0 (3.4-4.8)	0.015
Creatinine, μmol/L	59-104	81.0 (67.0-85.0)	82.0 (67.5-91.5)	76.5 (63.3-81.0)	0.21
Creatine kinase, U/L	≤190	73.0 (63.0-287.0)	214.0 (90.0-329.0)	64.0 (57.5-83.5)	0.16
Lactate dehydrogenase, U/L	135-225	336.0 (221.0-537.0)	537.0 (433.5-707.5)	224.0 (200.3-251.8)	0.001
>300 U/L, n (%)		11 (52.4%)	10 (90.9%)	1 (10.0%)	0.000
Prothrombin time, seconds	11.5-14.5	13.7 (13.0-14.5)	14.3 (13.6-14.6)	13.4 (12.8-13.7)	0.15
Activated partial thromboplastin time, seconds	29.0-42.0	39.4 (33.6-44.5)	33.7 (32.1-38.4)	44.0 (42.6-47.6)	0.002
D-dimer, μg/mL	<0.5	0.5 (0.4-1.8)	2.6 (0.6-18.7)	0.3 (0.3-0.4)	0.029
Procalcitonin, ng/mL	0.02-0.05	0.11 (0.05-0.24)	0.18 (0.13-0.81)	0.05 (0.04-0.06)	0.059
<0.1, n/N (%)		7/18 (38.9%)	0/10 (0.0%)	7/8 (87.5%)	0.002
0.1-0.25, n/N (%)		6/18 (33.3%)	6/10 (60.0%)	0/8 (0.0%)	..
0.25-0.5, n/N (%)		2/18 (11.1%)	1/10 (10.0%)	1/8 (12.5%)	..
≥0.5, n/N (%)		3/18 (16.7%)	3/10 (30.0%)	0/8 (0.0%)	..
High-sensitivity C-reactive protein, mg/L	<1	108.4 (28.0-139.5)	139.4 (86.9-165.1)	22.0 (14.7-119.4)	0.003
>60, n/N (%)		14/20 (70%)	11/11 (100.0)	3/9 (33.3%)	0.002
Ferritin, μg/L	30-400	1424.6 (337.4-1780.3)	1598.2 (1424.6-2036.0)	337.4 (286.2-1275.4)	0.049
>800, n/N (%)		12/19 (63.2%)	9/9 (100.0%)	3/10 (30.0%)	0.003
Bilateral involvement of chest computed tomography scan on admission		17/21 (81.0%)	10/11 (90.9%)	7/10 (70.0%)	0.31

555 Abbreviations: COVID-19, Coronavirus Disease 2019; IQR, interquartile range; SARS-CoV-2, severe
556 acute respiratory syndrome coronavirus 2. Data are median (IQR) or n (%), or n/N (%), where N is the

557 total number of patients with available data. p values comparing severe cases and moderate cases are
558 from χ^2, Fisher's exact test, or unpaired 2-sided Student's t test.
559

Table 3 Immunological features of patients with COVID-19

	All patients (n=21)	severe cases (n=11)	moderate cases (n=10)	P value	Normal range
Total T lymphocytes (%)	60.5 (54.4-70.3)	55.1 (52.2-60.5)	68.8 (64.7-75.2)	0.020	50-84
Total T lymphocytes count, × 10^6/L	486.5 (267.0-664.8)	294.0 (169.3-415.3)	640.5 (588.3-789.5)	0.011	955-2860
decreased, n/N (%)	13/14 (92.9%)	8/8 (100.0%)	5/6 (83.3%)	0.43	
<400, n/N (%)	6/14 (42.9%)	6/8 (75.0%)	0/6 (0.0%)	0.010	
Total B lymphocytes (%)	16.9 (10.8-22.4)	20.2 (17.6-39.5)	10.8 (10.3-12.4)	0.025	5-18
increased, n/N (%)	7/14 (50.0%)	6/8 (75.0%)	1/6 (16.7%)	0.10	
Total B lymphocytes count, × 10^6/L	115.5 (57.8-249.3)	184.0 (42.8-273.3)	115.5 (102.8-133.5)	0.35	90-560
decreased, n/N (%)	4/14 (28.6%)	3/8 (37.5%)	1/6 (16.7%)	0.58	
$CD4^+$T cells, (%)	36.7 (32.0-40.0)	36.7 (30.7-37.3)	36.4 (32.0-40.6)	0.56	27-51
$CD4^+$T cells count, × 10^6/L	241.5 (135.0-363.8)	177.5 (104.0-249.8)	381.5 (255.0-451.0)	0.018	550-1440
decreased, n/N (%)	14/14 (100.0%)	8/8 (100.0%)	6/6 (100.0%)	NA	
$CD8^+$T cells, (%)	22.2 (15.7-26.9)	17.4 (14.7-23.4)	25.2 (22.8-34.2)	0.093	15-44
$CD8^+$T cells count, × 10^6/L	169.5 (86.0-281.5)	89.0 (61.5-130.3)	254.0 (183.3-312.8)	0.035	320-1250
decreased, n/N (%)	12/14 (85.7%)	7/8 (87.5%)	5/6 (83.3%)	1.00	
<150, n/N (%)	6/14 (42.9%)	6/8 (75.0%)	0/6 (0.0%)	0.010	
NK cells, (%)	14.8 (10.3-21.9)	14.7 (7.5-21.0)	15.1 (11.6-22.8)	0.62	7-40
NK cells count, × 10^6/L	89.0 (58.8-207.0)	60.5 (27.5-109.0)	180.5 (115.0-228.0)	0.27	150-1100
decreased, n/N (%)	8/14 (57.1%)	6/8 (75.0%)	2/6 (33.3%)	0.28	
<77, n/N (%)	6/14 (42.9%)	6/8 (75.0%)	0/6 (0.0%)	0.010	
$CD28^+CD4^+$T cells/ $CD4^+$T, %	98.3 (96.8-98.8)	97.5 (96.8-98.7)	98.6 (97.2-99.0)	1.00	84.11-100.00
$CD28^+CD8^+$T cells/ $CD8^+$T, %	64.8 (44.6-75.9)	44.6 (37.5-73.1)	70.3 (63.3-76.9)	0.20	48.04-77.14
HLA-DR^+CD8^+T cells/ $CD8^+$T, %	42.3 (30.9-48.2)	46.2 (42.3-48.2)	28.6 (25.4-37.9)	0.19	20.73-60.23
$CD45RA^+CD4^+$T cells/ $CD4^+$T, %	32.8 (31.7-40.3)	32.8 (31.8-36.4)	36.0 (29.3-40.5)	0.54	29.41-55.41
$CD45RO^+CD4^+$T cells/ $CD4^+$T, %	67.2 (59.7-68.3)	67.2 (63.6-68.2)	64.0 (59.5-70.7)	0.54	44.44-68.94
Treg, %	4.1 (3.5-4.9)	4.7 (2.6-5.4)	3.9 (3.6-4.3)	0.92	5.36-6.30
$CD45RA^+$ Treg, %	0.8 (0.5-1.1)	0.5 (0.3-0.7)	1.1 (1.0-1.3)	0.020	2.07-4.55
$CD45RO^+$Treg, %	3.3 (2.4-3.8)	3.8 (1.9-4.9)	2.9 (2.5-3.4)	0.59	1.44-2.76
IFN-γ expressing $CD4^+$T cells, %	19.1 (13.0-22.8)	14.1 (9.4-18.8)	22.8 (18.8-25.4)	0.063	14.54-36.96
IFN-γ expressing $CD8^+$T cells, %	50.1 (44.2-53.6)	47.2 (39.2-52.7)	51.2 (47.3-54.1)	0.49	34.93-87.95
IFN-γ expressing NK cells, %	73.3 (65.7-79.7)	71.2 (63.8-72.9)	79.7 (71.9-81.5)	0.25	61.2-92.65

Abbreviations: COVID-19, Coronavirus Disease 2019; IQR, interquartile range; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. Data are median (IQR) or n/N (%), where N is the total number of patients with available data. p values comparing severe cases and moderate cases are from χ^2, Fisher's exact test, or unpaired 2-sided Student's t test.