Different T cell related immunological profiles in COVID-19 patients compared to healthy controls

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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.

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ABSTRACT

In various pathological conditions, cellular immunity plays an important role in immune responses. Among immune cells, T lymphocytes pdomote cellular and humoral responses as well as innate immunity. Therefore, careful investigation of these cells has a significant impact on accurate knowledge in COVID-19 disease pathogenesis. In current research, the frequency and function of various T lymphocytes involved in immune responses examined in SARS-CoV-2 patients with various disease severity compared to normal subjects. In order to make an accurate comparison among patients with various disease severity, this study was performed on asymptomatic recovered cases (n = 20), ICU hospitalized patients (n = 30), non-ICU hospitalized patients (n = 30), and normal subjects (n = 20). To precisely evaluate T cells activity following purification, their cytokine secretion activity was examined. Similarly, immediately after purification of Treg cells, their inhibitory activity on T cells was investigated. The results showed that COVID-19 patients with severe disease (ICU hospitalized patients) not only had a remarkable increase in Th1 and Th17 but also a considerable decrease in Th2 and Treg cells. More importantly, as the IL-17 and IFN-γ secretion was sharply increased in severe disease, the secretion of IL-10 and IL-4 was decreased. Furthermore, the inhibitory activity of Treg cells was reduced in severe disease patients in comparison to other groups. In severe COVID-19 disease, current findings indicate when the inflammatory arm of cellular immunity is significantly increased, a considerable reduction in anti-inflammatory and regulatory arm occurred.

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1. Introduction

In December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was the leading reason for the COVID-19 disease, first identified in Wuhan, china [1]. On the 30th of January 2020, the World Health Organization (WHO) proclaimed the prevalence of COVID-19 as an international pandemic, and to date, about 136 million people worldwide were infected with the disease, and more than 2,900.000 people died [2]. SARS-CoV-2 is common in animals and humans, belongs to the *Coronaviridae* family, known for its high mutagenic capacity, growth under epidemiological conditions, high ability to infect hosts, and crossing species barriers [3–5]. The SARS-CoV-2 virus uses the angiotensin-converting enzyme 2 (ACE2) as a receptor to enter various cells such as the oral mucosa, bladder urothelium, myocardial cells, ileum enterocytes, esophageal epithelium, and lung epithelium [6–8]. The severity of COVID-19 varies from person to person and can range from an asymptomatic form to severe respiratory failure (SRF). The most common manifestation of COVID-19 is pneumonia which is associated with dyspnea, dry cough, and fever. Other symptoms include neurological disorders, skin rashes, gastrointestinal symptoms, sore throats, and headaches [9–11].

Cellular immunity plays a vital role in inducing immune responses against viral and bacterial infections and has a high potential to regulate humoral immune function as well as innate immunity [12]. Immunologically, impaired cellular immune function, especially T $CD8^+$ and T $CD4^+$ cells, leads to overexpression of inflammatory cytokines, immune system imbalances, and lymphopenia [13]. In addition to cellular immunity, B lymphocytes, NK cells, macrophages, and neutrophils have crucial roles to fight against SARS-CoV-2 virus. However, SARS-CoV-2 by affecting immune cells' function, disrupts the immune responses and promotes disease by indirect induction of severe inflammation [14,15].

In viral infections such as SARS-CoV-2, the immune system shifts responses to the Th17 and Th1 phenotypes, which induce both inflammatory cells and cytokines to neutralize the infection. Th1 cells by expressing IFN-γ and activating $CD8^+$ cells and Th17 cells by expressing TNF-α, IL-22, and IL-17 cytokines have an antiviral role in the immune system [14,16,17]. In contrast, Treg cells, known as immunosuppressive cells, by producing TGF-β, IL-35, and IL-10 as anti-inflammatory cytokines can have a crucial role in the immune hemostasis [18,19]. Studies have also shown that excessive increase in inflammatory responses and antiviral activity causes overproduction of MCP-1, TNF-α, MIP-1A, IFN-γ, G-CSF, GM-CSF, IL-8, IL-2, IL-6, and IL-1β cytokines, creating a "cytokine storm", enhances the recruitment of monocytes, neutrophils, and other immune cells which results in damage to the respiratory system, hyper-inflammation, and disease progression [20,21]. All these signaling pathways induce an inflammatory condition in order to eliminate SARS-CoV-2. Over the past year, various studies conducted to evaluate cellular immunity in COVID-19 patients, facing conflicting results which encourage us to design more investigations in this subject. Partly because of these discrepancies, previous studies had some shortcomings. In many experiments, no proper classification of patients was done. However, in our study, different types of patients with different disease levels were evaluated. This research investigated the frequency and function of various T lymphocytes involved in immune responses in normal, ICU hospitalized (severe disease), non-ICU hospitalized (mild disease), and asymptomatic recovered cases. To accurately evaluate T cells' activity in COVID-19 patients, Th17, Th2, Th1, Treg cells, and exhausted T cells were analyzed. Notably, in our study, Treg cells were purified and then their immunosuppressive function evaluated.

2. Material and method

2.1. Patients and study design

In this research, 60 patients with COVID-19 (30 ICU hospitalized and 30 non-ICU hospitalized patients) admitted to Imam Reza Hospital affiliated to Tabriz University of Medical Sciences as well as 20 asymptomatic recovered donors, were examined compared to 20 normal subjects. Of note, SARS CoV-2 positivity was approved by using RT–PCR test in duplicate. About the sampling, it was taken by an expert staff from the upper respiratory tract. However, in order to accurately diagnose the disease, a combination of RT-PCR test and clinical signs of patients especially CT images was used.

Patient groups and control were randomly selected with the age ranging from 23 to 71 years. Written consent was taken from all healthy individuals and patients. This research confirmed in the Research Ethics Committee of Maragheh University of Medical Sciences (IR.MARAGHEHPHC.REC.1399.022). In the present research, the inclusion criteria were laboratory evaluation (positive RT–PCR test for SARS CoV-2), etiological features and clinical criteria. In contrast, COVID-19 patients with a history of allergic disease, cancer (lymphoma and leukemia), and infectious diseases (HIV, brucellosis, and hepatitis) were excluded from the study. In addition, the inclusion criteria for normal individuals were no underlying diseases, no liver and kidney failure, and no infectious diseases.

The progressive respiratory failure and extensive alveolar damage were seen in ICU hospitalized patients. In this group, due to severe shortness of breath and pneumonia, a significant reduction in oxygen levels occurred which led to using a ventilator to facilitate breath. Moreover, this group exhibited more severe weakness and organ defects compared to non-ICU hospitalized (mild) patients, associated with profound lymphopenia. In this group, the average hospital stay of patients was about 11 (8–15) days. Most of the patients in the non-ICU hospitalized (mild) group, without viral pneumonia and hypoxia, had a mild illness. Therefore, there is no necessity for hospitalization and it was even possible to receive treatment at home with the necessary care and attention. In this group, the average hospital stay of patients was about 5 (4–8) days. The asymptomatic recovered donors were hospital staff; whose IgG test was positive for the SARS-CoV-2 without any symptoms. In order to match the results, blood samples were taken from the patients of the two hospitalized groups on the admission time.

The clinical characteristics of patients and normal subjects are shown in Table 1.

2.2. Blood sampling, PBMCs isolation, and cell culture

For cellular and molecular tests, about 10 ml of blood from each normal subject and patient was collected. To use density-gradient method, Ficoll (density 1.080 g/ml) (MedChem Express, USA) was used to isolate PBMC, and then it was centrifuged for 40 min and finally washed twice using PBS (Santa Cruz, CA, USA). In the next step, isolated

Table 1
The clinical characteristics of COVID-19 patients and normal subjects.

Gene name	Forward	Reverse
T-bet	5′-CGG CTG CAT ATC GTT GAG GT-3′	5′-GTC CCC ATT GGC ATT CCT C-3′
GATA-3	5′-TCA TTA AGC CCA AGC GAA GG-3′	5′-GTC CCC ATT GGC ATT CCT C-3
ROR-γt	5′-ACTCAAAGCAGGAGCAATGGAA-3′	5′-AGTGGGAGAAGTCAAAGATGGA-3′
FoxP3	5′-TCATCCGCTGGGCCATCCTG-3′	5′-GTGGAAACCTCACTTCTTGGTC-3′
β-actin	5′-GGTCATCACTATTGGCAACG-3′	5′-ACGGATGTCAACGTCACACT-3′

PBMCs in a culture medium consisting of 250 ml L-glutamine (eBioscience), 15 ng/ml of PMA, 150 U/ml of penicillin, and 10% FBS were cultured, and then incubated for 48 h in 5% CO_2 and temperatures of 37 °C. Finally, the RT-PCR, the flow cytometry technique, and ELISA method were used to measure genes expression, count frequency of cells, and cytokines secretion, respectively.

Until death or discharge, the patients were followed up by obtaining demographic information, laboratory data, and clinical manifestations. According to previous studies [22], the SOFA score (Sequential Organ Failure Assessment), were collected and calculated by physicians upon admission, which combines data based on kidneys, nervous, coagulation, liver, respiratory and cardiovascular system status.

2.3. Flow cytometry

Flow cytometry was applied to assess the frequency of Treg, Th17, Th2, Th1, and exhausted T cells in patients with COVID-19 and normal subjects [23]. In brief, 1×10^6 cells were twice washed with washing buffer (PBS 0.15 M, 0.5% BSA, 0.1% NaN3) and then suspended in 100 μl of this buffer. Then, the cells were incubated with optimized amounts of fluorochrome-conjugated monoclonal antibodies for 40 min in the dark at 4° C. Detection of Treg cells was performed by cytoplasmic expression of FoxP3. For this purpose, after staining the cell surface with anti-CD4, CD3, and CD25 monoclonal antibodies, cells were fixed and permeabilized by using the BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences). After twice washing, the cells were incubated with the optimized amount of anti-FoxP3 antibody for 40 min in the dark at 4° C. Finally, the cells were washed and scanned with a flow cytometer. To identify Th1, Th2, and Th17, cells were incubated with PMA (20 ng/mL) (Sigma), ionomysin (450 ng/mL) (Sigma), and Brefeldin A (1 μl) (Sigma) for 4 h. Then, cells were twice washed and cell surface stained with anti-CD3 and CD4 antibodies for 40 min in the dark at 4° C. Intracellular staining for IFN-γ, IL-4, and IL-17 was performed using the same protocol as used for FoxP3. Finally, FlowJo software (Becton Dickinson, CA, USA) and FACS caliber flow cytometer (BD Biosciences), was used to count the number of stained cells.

2.4. Cell separation by MACS

At first, $CD3^+$ T cells were separated from PBMCs of the COVID-19 patients and normal subjects (n = 10 in each group) through depletion process of non-$CD3^+$ T cells called as a negative selection by utilizing human $CD3^+$ T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany). Based on flow cytometry analysis, $CD3^+$ T cells' purity was typically more than 95%.

Also, $CD4^+CD25^-$ effector T cells and $CD4^+CD25^+$ Tregs were separated from PBMC of patients and normal subjects (n = 5 in each group) by utilizing $CD4^+CD25^+$ regulatory T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Summarily, non-$CD4^+$ cells, by labeling with a cocktail of biotin-conjugated mAbs against non-$CD4^+$ cells and then with anti-biotin mAbs conjugated to microbeads, were negatively selected. Finally, $CD25^+$ cells, by labeling of purified $CD4^+$ cells with anti-CD25-coupled microbeads, were positively selected. Based on flow cytometry analysis, Treg cells' purity was typically more than 90% [24].

2.5. Real-Time PCR

By using real-time PCR, the expression of T-bet (Th1), ROR-γt (Th17), GATA-3 (Th2), and FoxP3 (Treg) transcription factors was evaluated in patient groups with COVID-19 (n = 10 subjects in each group) and control. By utilizing RNX-PLUS solution (Pastor institute, Iran), total RNA was obtained from $CD3^+$ T cells in patient groups and control, and then by using Reverse Aid ™ reverse transcriptase kit (Santa Cruz, CA, USA) and random hexamer primer, cDNA was synthesized. In the next step, the qPCR was applied with following programs: the initial denaturation at 95 °C for 10 min, repeated by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 35 s). In order to plot standard curves, standards were provided in six different concentrations with 10-fold serial dilutions [25]. Also, the electrophoresis assessment was applied to prove gene amplification, using Bio systems (Seqlab, Germany) with 2% agarose gel as previously described [26]. Finally, the formula $2^{-\Delta\Delta Ct}$ was applied to analyze the data compared to control genes based on following equation [27]. The β-actin gene was used as an internal control. The primers' sequences are given in Table 2.

ΔCT = CT (a target gene) - CT (a reference gene)

$2^{-\Delta\Delta CT}$ = ΔCT (a target sample) – ΔCT (a reference sample)

2.6. ELISAxxx

ELISA kits (MyBioSource) were used to evaluate the levels of cytokines secreted by Treg (IL-10), Th17 (IL-17), Th1 (INF-γ), and Th2 (IL-4) in supernatants of MACS-mediated purified $CD3^+$ T cells, stimulated with PHA (10 μg/ml) for 36 h, in patient groups with COVID-19 (n = 10 subjects in each group) and control. Briefly, the ELISA plate was first coated with antibody (150 μl) and incubated for 24 h. The plate was washed with PBS (Tween-20, 0.05 ٪) and then incubated with a blocking buffer for 2 h. After adding 150 μl of standard and sample, it was followed by incubation, and then the plate was washed and incubated for 2 h after adding 150 μl of biotinylated antibody. Then 150 μl of the avidin–biotin-peroxidase solution was added, and incubated for 1 h. Finally, 150 μl of TMB was added and incubated for 1 h. To evaluate the adsorption values, reactions were stopped and then detected by an ELISA reader (Medgenix, BP-800; Biohit) at 460 nm [28].

2.7. Suppression assay

To evaluate the suppressive function of Treg cells, $CD4^+CD25^+$ cells, were purified from the PBMC of normal subjects and COVID-19 patients (n = 5 in each group). Briefly, 10×10^4 $CD4^+CD25^-$ effector T cells separated by magnetic beads were seeded and stimulated by PHA (10 μg/ml) for 36 h. Additionally, $CD4^+CD25^-$ T cells were also isolated by MACS technique and seeded in 96-well plate and stimulated with PHA for 36 h. After 36 h, effector T cells were co-cultured with $CD4^+CD25^+$ Tregs in different Treg/T cell ratios, including 1:6, 1: 3, and 1: 1 [29]. The suppressive effect of Tregs on effector T cells proliferation was studied by using CFSE assay through flow cytometry.

2.8. Statistical analysis

SPSS PC Statistics Software (version 18.0; SPSS Inc.) was applied for statistical analysis. The Shapiro–Wilk test regulated the data proportion to standard ranges. Furthermore, the Mann–Whitney *U* test and unpaired t-test were used to compare the abnormally distributed and standard data among the COVID-19 patients and healthy controls, respectively. To examine the relationship between quantitative variables, Spearman analysis was applied. Fisher's exact test was utilized to examine the correlation between the two variables. Also, to visualize and calculate the relationship among variables, the R function *ggcorrplot* was applied, the color of Red and green indicates a negative and a positive relationship, respectively. Correlations with values $P > 0.05$ left blank and were considered insignificant. Also, data was shown as mean ± SD, and $p < 0.05$ was considered statistically significant. To plot the graphs, GraphPad Prism (version 8.10; GraphPad Software; www.graphpad.com) was utilized.

Table 2
Primer sequences.

	Normal subjects (n = 20)	Asymptomatic recovered donors (n = 20)	Non-ICU hospitalized patients (n = 30)	ICU hospitalized patients (n = 30)
Age, Years	25–69 (58.5 ± 10.6)	23–66 (56.4 ± 11.6)	24–67 (57.3 ± 10.6)	25–71 (61.2 ± 10.6)
Sex				
Men	10 (50%)	10 (50%)	15 (50)	15 (50)
Women	10 (50%)	10 (50%)	15 (50)	15 (50)
White blood cell count × 109/L				
<4	1 (5%)	1 (5%)	4 (13.3%)	1 (%) (3.3%)
4–10	17 (85%)	18 (90%)	22 (73.4%)	24 (80%)
>10	2 (10%)	1 (5%)	4 (13.3%)	5 (16.7%)
Lymphocyte count × 109/L				
<1·0	3 (15%)	2 (10%)	8 (26.6%)	16 (53.3%)
≥1·0	17 (85%)	18 (90%)	22 (73.4%)	14 (46.7%)
Platelet count				
<100	1 (5%)	2 (10%)	8 (26.6%)	12 (40%)
≥100	19 (95%)	18 (90%)	22 (73.4%)	18 (60%)
Fever	–	–	24 (80%)	28 (93.3%)
Sore throat	–	–	14 (46.7%)	22 (73.4%)
Dyspnea	–	–	15 (50%)	23 (76.6%)
Cough	–	–	22 (73.4%)	25 (83.3%)
Lactate dehydrogenase, U/L				
≤245	18 (90%)	19 (95%)	11 (36.7%)	8 (26.6%)
>245	2 (10%)	1 (5%)	19 (63.3)	22 (73.4%)
C-reactive protein ≥ 10 mg/L	–	2 (10%)	16 (53.3%)	18 (60%)
Creatinine, μmol/L				
≤133	19 (95%)	19 (95%)	6 (20%)	2 (6.7%)
>133	1 (5%)	1 (5%)	24 (80%)	28 (93.3%)
Bilateral involvement of chestradiographs	–	–	14 (46.6%)	29 (96.6%)

3. Results

3.1. Distribution of white blood cells in COVID-19 patients and normal subjects

In current research, the distribution of white blood cell components such as lymphocytes, monocytes, and granulocytes was investigated in COVID-19 patients and normal subjects. Findings showed that the lymphocyte population in both hospitalized patient groups was significantly reduced compared to asymptomatic recovered donors and normal subjects. (Fig. 1a and Table 3). In contrast, monocytes and granulocytes' frequency in both hospitalized patient groups was significantly increased compared to asymptomatic recovered donors and normal subjects (Fig. 1b-c and Table 3).

3.2. The frequency of Th1 and Th2 cells in COVID-19 patients and normal subjects

The flow cytometry method was utilized to examine the Th1 and Th2 cells' frequency in both normal subjects and COVID-19 patients (Fig. 2a). Results indicated as Th1 was significantly increased in both hospitalized patient groups, there was no significant change in the asymptomatic recovered donors compared to normal subjects (Fig. 2b and Table 3). In contrast, the frequency of Th2 cells was markedly decreased in both hospitalized patient groups compared to the asymptomatic recovered donors and normal subjects (Fig. 2c and Table 3). Also, The Th1/Th2 ratio was markedly increased in both hospitalized patient groups compared to the asymptomatic recovered donors and normal subjects (Fig. 2d and Table 3). It should be noted that ICU-hospitalized patients showed the most significant increase in Th1 frequency and Th1/Th2 ratio and the most considerable decrease in Th2 frequency.

The lineage-specific transcription factors of GATA-3 (Th2) and T-bet (Th1) were examined using qPCR. To improve the sensitivity and specificity of qPCR, the expression of the specific transcription factors of T cells was done on purified cells, using MACS technique (n = 10 subjects in each group). Notably, similar to the Th1 and Th2 cells' frequency, T-bet and GATA-3 were increased and decreased with disease progression, respectively (Fig. 2e). The ratio of T-bet/GATA-3 was also markedly increased with disease progression (Fig. 2f).

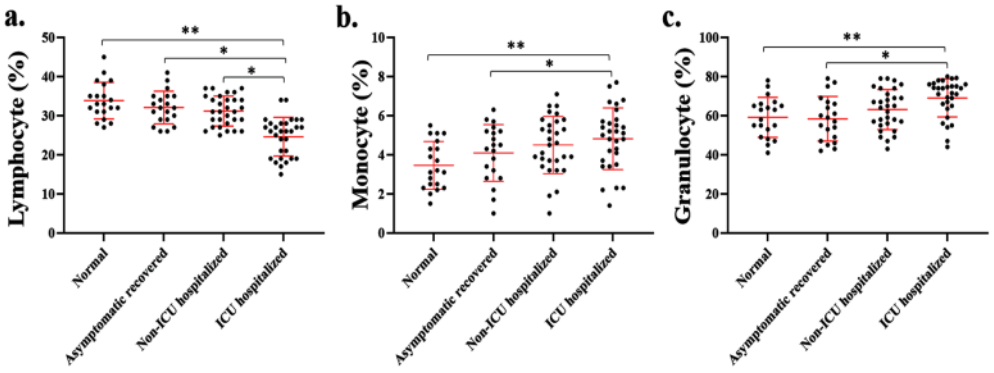

Fig. 1. Dot plots demonstrate distribution of lymphocytes (a), monocytes (b), and granulocytes (c) as components of white blood cells.

Table 3
Mean percentages of the immune cells in peripheral blood of normal subjects and the COVID-19 patients.

		Normal subjects (n = 20)	Asymptomatic recovered donors (n = 20)	Non-ICU hospitalized patients (n = 30)	ICU hospitalized patients (n = 30)
Lymphocytes	Percentage	33.85 ± 4.68	32.1 ± 4.19	31.2 ± 3.8	24.6 ± 2.9
	Absolute count ($\times10^{-3}$/ml)	2.6 (0.9–3.1)	2.45 (0.85–3)	2.3 (0.8–2.4)	0.91 (0.3–1.9)
Monocytes	Percentage	3.26 ± 1.22	4.09 ± 1.45	4.13 ± 1.3	4.82 ± 1.57
	Absolute count ($\times10^{-3}$/ml	0.45 (0.13–0.68)	0.51 (0.12–0.7)	0.56 (0.12–0.72)	0.6 (0.13–0.73)
Granulocytes	Percentage	59.15 ± 10.18	58.4 ± 11.35	63.13 ± 10.23	69.07 ± 9.68
	Absolute count ($\times10^{-3}$/ml	6.7 (1.6–8.7)	6.8 (1.7–8.5)	7.1 (1.57–8.45)	7.4 (1.62–8.53)
Th1 (%)	22.95 ± 4.91	23.96 ± 5.36	25.6 ± 3.72	27.72 ± 3.33	
Th2 (%)	5.25 ± 1.05	4.91 ± 1.88	3.76 ± 1.36	6.31 ± 0.82	
Th1/Th2 (%)	4.64 ± 1.14	4.94 ± 0.83	5.66 ± 1.02	4.25 ± 0.94	
Treg (%)	6.32 ± 1.78	6.84 ± 1.47	5.53 ± 1.38	4.85 ± 1.3	
Th17 (%)	3.7 ± 1.29	3.11 ± 1.06	5.26 ± 1.72	5.51 ± 1.51	
Th17/Treg (%)	0.47 ± 0.15	0.41 ± 0.08	0.96 ± 0.12	1.1 ± 0.15	
$CD4^+PD\text{-}1^+$ (%)	4.82 ± 0.74	3.53 ± 0.85	4.62 ± 1.14	4.94 ± 1.02	
$CD8^+PD\text{-}1^+$ (%)	2.82 ± 0.65	2.91 ± 0.79	3.28 ± 0.64	3.6 ± 0.71	
$CD4^+LAG\text{-}3^+$ (%)	2.22 ± 0.72	2.3 ± 0.61	2.88 ± 0.74	3.12 ± 0.84	
$CD8^+LAG\text{-}3^+$ (%)	2.66 ± 0.61	2.8 ± 0.79	3.03 ± 0.88	3.2 ± 0.81	

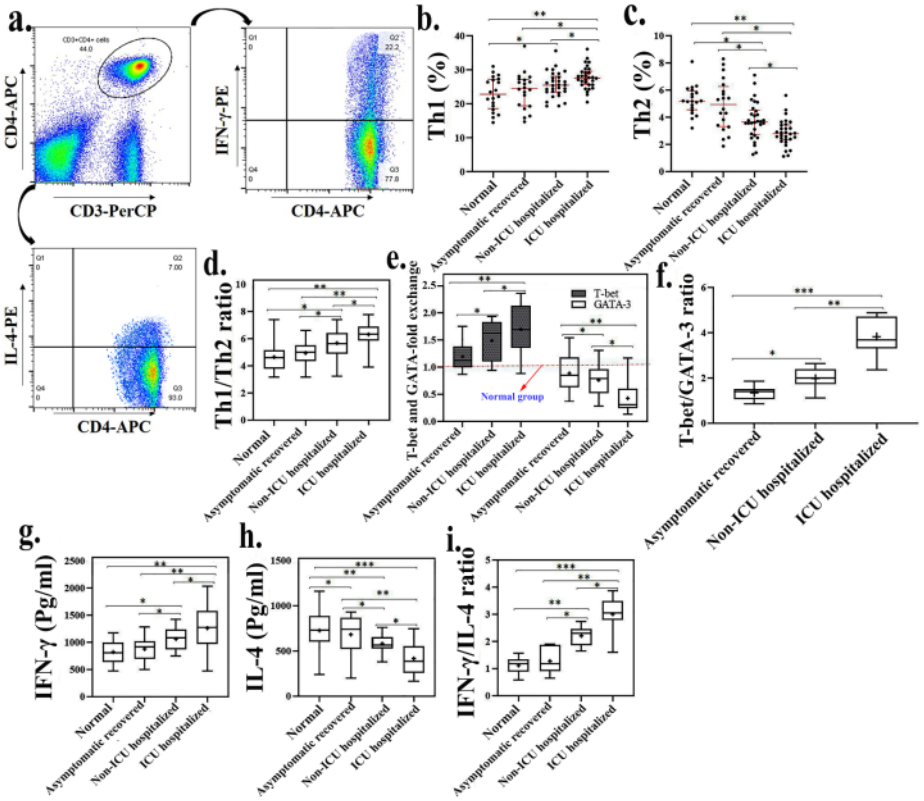

Fig. 2. Progressive COVID-19 disease is correlated with the upregulation of Th1 and downregulation of Th2. Dot plots demonstrate an analysis method used for evaluation of Th1 and Th2 cells (a). Th1 was significantly increased in both hospitalized patient groups (b). Inversely, the frequency of Th2 cells was markedly decreased in both hospitalized patient groups (c). The Th1/Th2 ratio in both hospitalized patient groups was markedly increased (d). The mRNA levels of T-bet was increased, while GATA-3 was decreased with disease progression. The red dashed line indicates the normal group (e). Also, the ratio of T-bet/GATA-3 was markedly increased with disease progression (f). The secretion levels of IL-4 and IFN-γ were examined by using the ELISA assay in the supernatant of cultured T cells (g and h). ICU hospitalized patients had the highest IFN-g/IL-4 ratio compared to other groups (i). The box plots show an oblong box describing the middle 50% of the data. The lines describing the 25% lower and upper of the distribution, the + symbol indicates the average values, and the horizontal line inside the box describing the median values.

Also, to assess Th1 and Th2 cells' function, $CD3^+$ T cells were purified from PBMC of normal subjects and COVID-19 patients by MACS method (n = 10 subjects in each group). Subsequently, the secretion of IL-4 and IFN-γ was examined, using ELISA assay. The results shown that ICU hospitalized patients showed the highest IFN-γ (Fig. 2e and Table 4) and the lowest IL-4 (Fig. 2g-h and Table 4) in secretion level compared to other groups. Similarly, ICU hospitalized patients had the highest IFN-γ/IL-4 ratio compared to other groups (Fig. 2i and Table 4).

3.3. Evaluation of Treg and Th17 cells in COVID-19 patients and normal subjects

The flow cytometry method was applied to evaluate Treg and Th17 cells' frequency in normal subjects and COVID-19 patients (Fig. 3a). In ICU hospitalized patients compared to other groups, the frequency of Treg was markedly decreased (Fig. 3b and Table 3), whereas the frequency of Th17 and Th17/Treg ratio significantly increased. Interestingly, asymptomatic recovered donors exhibited a similar frequency of Th17 and Treg to normal subjects (Fig. 3c-d and Table 3).

The qPCR method was also performed to evaluate the expression of lineage-specific transcription factors of FoxP3 (Treg) and ROR-γt (Th17) genes. Like Treg and Th17 cells' frequency, ROR-γt and FoxP3 were increased and decreased with disease progression, respectively (Fig. 3e). Therefore, the ratio of ROR-γt/FoxP3 was also markedly increased with disease progression (Fig. 3f).

In order to investigate Treg and Th17 cells' function, $CD3^+$ T cells were purified by the MACS method (n = 10 subjects in each group). Subsequently, the secretion of IL-10 and IL-17 was evaluated by using the ELISA test. Notably, ICU hospitalized patients showed the lowest IL-10 (Fig. 3g and Table 4), and the highest IL-17 (Fig. 3h and Table 4). So, the ratio of IL-17/IL-10 was the highest compared to other groups (Fig. 3i and Table 4).

Moreover, the CFSE assay was applied to evaluate the inhibitory activity of Treg cells on the proliferation of $CD4^+CD25^-$ T cells. As shown in Fig. 3j, Treg cells' activity was significantly decreased in ICU hospitalized patients compared to other groups.

3.4. Evaluation of exhausted T cells in normal subjects and COVID-19 patients

The flow cytometry assay was used to evaluate exhausted T cells in normal subjects and COVID-19 patients (Fig. 4a). The data analysis indicated that $CD4^+$ $PD\text{-}1^+$ (Fig. 4b), $CD8^+$ $PD\text{-}1^+$ (Fig. 4c), $CD4^+$ $LAG\text{-}3^+$ (Fig. 4d), and $CD8^+$ $LAG\text{-}3^+$ (Fig. 4e) exhausted T cells were increased in ICU hospitalized patients compared to other groups. In mild patients (non-ICU hospitalized patients), the increase in frequency of cells was also significant compared to asymptomatic recovered donors and normal individuals, but there was no significant difference between asymptomatic recovered donors and normal subjects. The mean percentages of these cells are indicated in Table3.

3.5. The frequency of different T cell populations in COVID-19-dead patients and improved ones

Different T cell populations were investigated among COVID-19-dead patients and improved ones in ICU hospitalized patients. Of note, out of 30 ICU hospitalized patients, 7 cases were dead and 23 improved. The results demonstrated that dead patients had higher Th1, lower Th2 (Fig. 5a), higher Th1/Th2 ratio (Fig. 5b), higher IFN-γ, lower IL-4 (Fig. 5c), higher IFN-γ/IL-4 ratio (Fig. 5d), lower Treg, higher Th17 (Fig. 5e), higher Th17/Treg ratio (Fig. 5f), lower IL-10, higher IL-17 (Fig. 5g), higher IL-17/IL-10 ratio (Fig. 5h), and higher exhausted T cells (Fig. 5i). Mean percentages of the different immune cell populations and their secreted cytokines are indicated in Tables 5 and 6.

3.6. Immunological and biochemical parameters associated with COVID-19 infection

We applied a correlation map to examine the relationship among leukocyte populations, biochemical parameters, and clinical features (Fig. 6). The results have shown a positive correlation between increasing the percentage of lymphocytes such as Th17, Th1, CD4, and CD3 and biochemical inflammatory parameters such as D-dimer, LDH, CRP, fibrinogen, and ferritin. In contrast, it has been demonstrated that they had a negative correlation with Th2 and Treg cells. In addition, a negative relationship between neutrophils and lymphocytes was observed.

The results also showed that there was a negative relationship between the expression of Tbet, ROR-γt, IFN-γ, and IL-17 with the expression of GATA3, Foxp3, IL-4, and IL-10. It was shown that age had a negative relationship with neutrophil and whole blood count, whereas it had a positive relationship with Th17, Th1, CD8, and CD4 lymphocyte counts. Also, the length of hospital stay showed a reverse relationship with Treg and Th2 cells, and a direct correlation with Th17, Th1, CRP, and fibrinogen. Finally, the SOFA score was directly related to CRP, troponin I, LDH, D-dimer, age, and length of hospital stay, whereas it was negatively correlated with the T-cell subset and lymphocytes.

4. Discussion

Due to an increase in the prevalence of COVID-19, more investigations are required to identify the pathogenic mechanisms, the role of immune system in pathogenesis, and factors affecting disease progression [30,31]. Clinical studies have shown that inflammation caused by COVID-19 can result in damage to the gastrointestinal tract, nervous system, kidneys, and liver [32]. Studies have reported that the risk of COVID-19 is significantly increased in patients with underlying diseases such as viral infections, cancers, immunodeficiency, autoimmunity, diabetes, cardiovascular disease, hypertension, and obesity [33,34].

Infected epithelial cells with the SARS-CoV-2 recruit and activate the innate immune cells, including neutrophils and macrophages by producing the cytokine IL-8, as well as adaptive immune cells to complete the immune responses [35,36]. Basically, antiviral responses increase through the production of inflammatory cytokines and immune cells' activation [37–39]. Th17, Th1, and cytotoxic T cells (CTLs) play a significant role in fighting against viral infections via the production of inflammatory cytokines and the perforin/granzyme mechanism, respectively [40,41]. Due to the upregulation of Th17 and Th1, a reduction in Th2 and Treg cell counts, and immune system imbalances in

Table 4
Mean concentration (pg/ml) of T cells-derived cytokines.

	Normal subjects (n = 10)	Asymptomatic recovered donors (n = 10)	Non-ICU hospitalized patients (n = 10)	ICU hospitalized patients (n = 10)
IFN-γ	822 ± 223.4	1311.7 ± 213.9	842 ± 136.5	182.5
IL-4	726 ± 258.7	681.6 ± 233.5	579.4 ± 105	416.9 ± 464.6
IFN-γ/IL-4	1.11 ± 0.29	1.27 ± 0.46	2.21 ± 0.35	3 ± 0.62
IL-10	495.3 ± 86.8	522.4 ± 84.4	450.3 ± 67.4	414 ± 53.2
IL-17	1357.1 ± 103.1	1317 ± 94.6	1499.6 ± 118.3	1557 ± 136.7
IL-17/IL-10	2.6 ± 0.51	2.57 ± 0.34	3.2 ± 0.25	3.62 ± 0.29

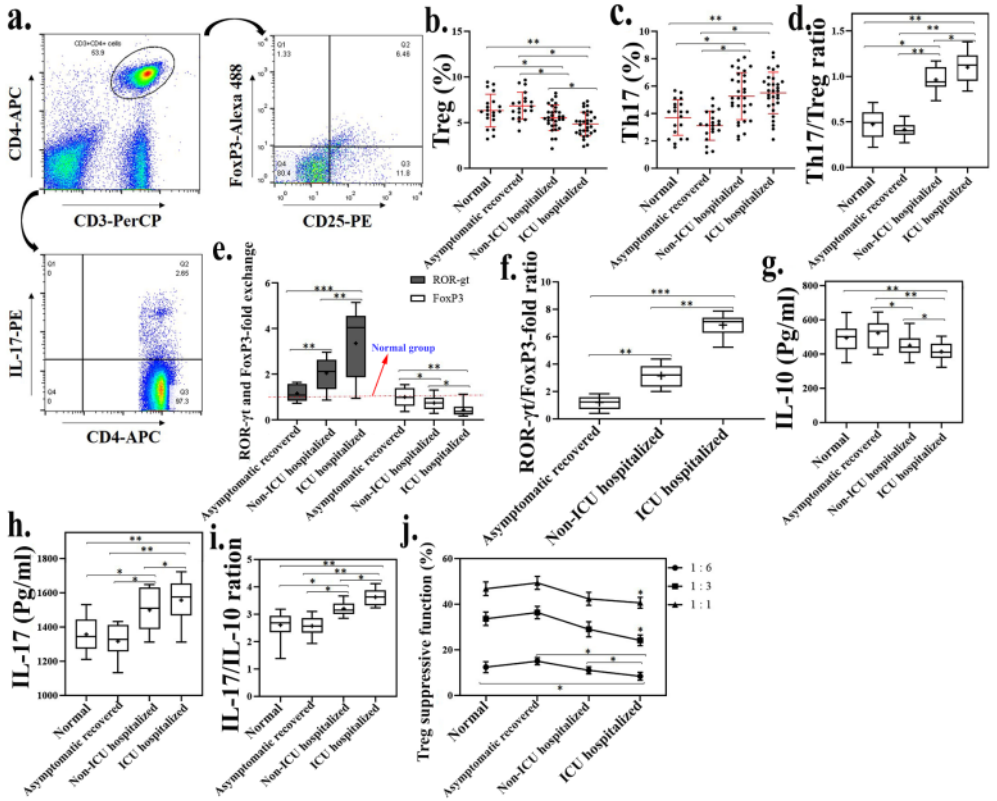

Fig. 3. COVID-19 progression is associated with decreased Treg and increased Th17 cells. Dot plots demonstrate the analysis method used for evaluation of Treg and Th17 cells (a). ICU hospitalized patients showed the lowest Treg (b), the highest Th17 (c), and the greatest Th17/Treg ratio (d) in comparison with other groups. The mRNA levels of ROR-γt was increased, while FoxP3 was decreased with disease progression. The red dashed line indicates the normal group (e). The ratio of ROR-γt/FoxP3 ratio was also markedly increased with disease progression (f). To examine the Treg and Th17 cells activity, the secretion of IL-10 and IL-17 was evaluated by using the ELISA test in the supernatant of cultured T cells. (g-i). Moreover, to evaluate the suppressive function of Treg cells, $CD4^{+}CD25^{+}$ Treg cells (n = 5 in each group) were cultured with $CD4^{+}CD25^{-}$ T cells and proliferation of these cells was studied by using CFSE assay (j). The box plots show an oblong box describing the middle 50% of the data. The lines describing the 25% lower and upper of the distribution, the + symbol indicates the average values, and the horizontal line inside the box describing the median values.

COVID-19 disease, the levels of inflammatory cytokines such as MIP-1A, GM-CSF, TNF-α, IFN-γ, G-CSF, IL-2, IL-8, and IL-1β are increased, which lead to cytokine release syndrome (CRS), which in turn causes damage to the respiratory system and disease progression [9,38].

In order to assess the activity and function of immune cells involved in the cell mediated immunity, T cell activity was investigated following separation of these cells. In fact, T cell isolation may lead to the departure of these cells from their physiological conditions. However, in relation to cytokine secretion, it should be considered that it is not just T cells producing cytokines, but also each cytokine can be produced by several different cells in the blood. In current study, it was very important to evaluate cytokine secretion, such as IFN-γ, from only T cells associated with cellular immunity, but not other cells such as NK cells.

In our experiment, the frequency of white blood cell components such as lymphocytes, monocytes, and granulocytes was investigated in COVID-19 patients and normal cases. It has been shown that although lymphocytes were decreased during disease progression, neutrophils and monocytes were increased. Consistently, Zhang and colleagues have manifested that the number of granulocytes and monocytes abnormally increased in patients with COVID-19. Also, it was confirmed that the neutrophil to lymphocyte ratio (NLR) increased in these patients, and enhancing this ratio can be a useful predictor for COVID-19 patients [42]. By studying 286 patients with COVID-19, Qin and colleagues showed that the neutrophils, neutrophil–lymphocyte-ratio (NLR), and leukocytes were dramatically increased, whereas the percentages of lymphocytes, basophils, eosinophils, and monocytes decreased [43]. Besides, Zhang and colleagues reported that the number of non-classical and intermediate monocytes increased in COVID-19 patients compared to healthy donors even though the number of classical monocytes significantly reduced [40].

Studies reported that Th1 population plays a crucial role in activating macrophage-dependent inflammation and cell-induced immunity [44]. Our results showed that although Th1 cells and their related cytokines, and transcription factor were significantly increased in both hospitalized patient groups, there was no significant change in the asymptomatic recovered donors compared to normal subjects. Therefore, it can be concluded that Th1 cells increase inflammation in patients with COVID-19 by INF-γ production. Consistent with these results, it has been reported that the SARS-CoV-2 virus induces polyclonal Th1 cells, that the rate of these responses varies from patient to patient based on the immune system status and history of the underlying disease [45]. In another study, Elizaldi and colleagues indicated that Th1 cell responses dramatically increased after infection with the SARS-CoV-2 virus [46]. It has been reported that in patients with severe COVID-19, the Th1 cell population, IFN-γ, and T-bet were dramatically increased [47]. In another study, Liu and colleagues reported cytokines including IP-10,

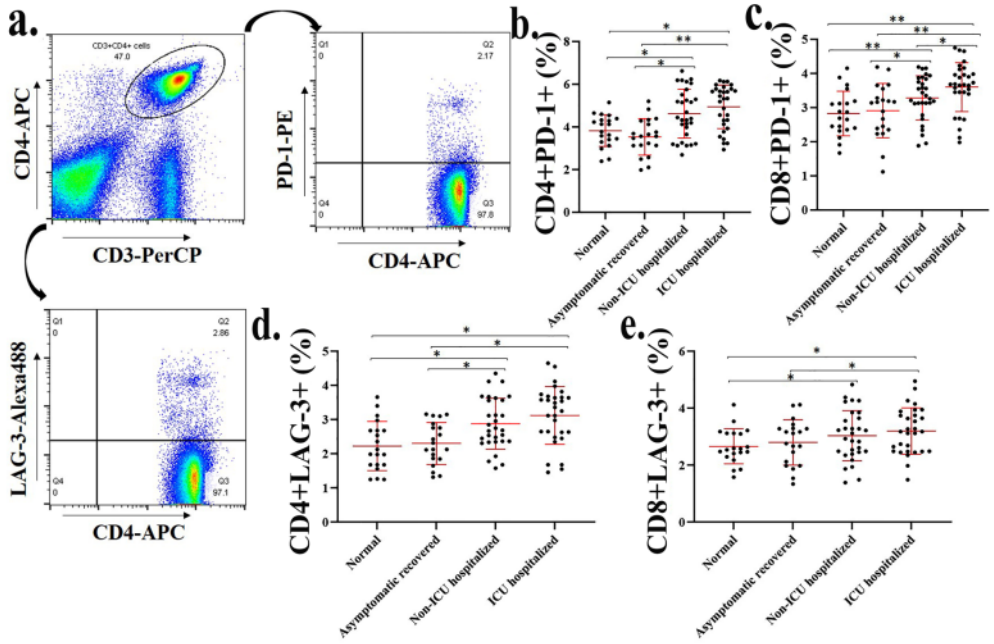

Fig. 4. Exhausted T cells are increased in severe COVID-19 disease. Dot plots indicate an analysis method used for evaluation of exhausted T cells (a). $CD4^+$ $PD\text{-}1^+$ (b), $CD8^+$ $PD\text{-}1^+$(c), $CD4^+$ $LAG\text{-}3^+$(d), and $CD8^+$ $LAG\text{-}3^+$(e) exhausted T cells were increased in ICU hospitalized patients compared to other groups.

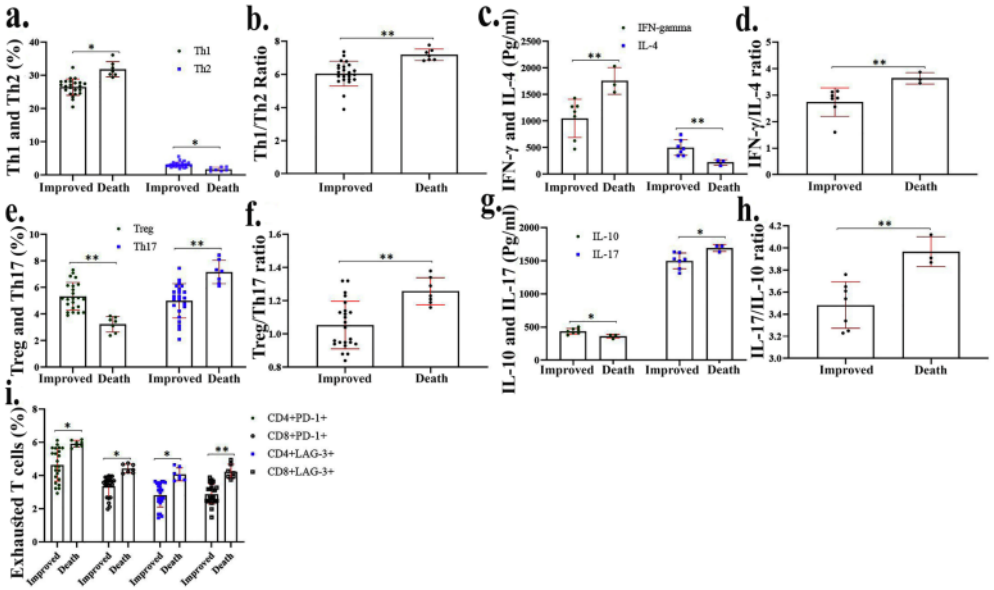

Fig. 5. Different T cell populations are present in COVID-19 dead patients and improved ones. Dead patients (n = 7) compared to improved ones (n = 23) had higher Th1, lower Th2 (a), higher Th1/Th2 ratio (b), lower IL-4, higher IFN-γ (c), higher IFN-γ/IL-4 ratio (d), lower Treg, higher Th17 (e), higher Th17/Treg ratio (f), lower IL-10, higher IL-17 (g), higher IL-17/IL-10 ratio (h), and higher exhausted T cells (i).

IFN-γ, M-CSF, G -CSF, IFN-α2, IL-12, IL-7, IL-2, and TNF-α were significantly increased in COVID-19 patients. They suggested that an increase in Th1 population and their cytokines plays an important role in the progression of SARS-CoV-2 pathogenesis [48]. In contrast, studies reported that the population of Th1 and related cytokines were significantly decreased in COVID-19 patients compared to normal subjects [23,49].

In our investigation, the data indicated that the frequency of Th2, IL-4 secretion level, and GATA-3 was dramatically reduced in both hospitalized patient groups compared to the normal subjects and asymptomatic recovered donors. To our knowledge, no studies have been reported a reduction in Th2 cell population in COVID-19 patients. In

Table 5
Mean percentages of the different immune cells in peripheral blood of improved and death COVID-19 patients.

	Improved patients (n = 23)	Death patients (n = 7)
Th1	26.47 ± 2.48	31.85 ± 2.31
Th2	2.45 ± 0.63	4.14 ± 0.75
Th1/Th2	6.05 ± 0.74	7.18 ± 0.35
Treg	5.34 ± 1.04	3.24 ± 0.57
Th17	5 ± 1.28	7.17 ± 0.88
Th17/Treg	1.05 ± 0.14	1.25 ± 0.08
CD4 + PD-1+	4.64 ± 0.98	5.92 ± 0.2
CD8 + PD-1+	3.35 ± 0.61	4.42 ± 0.26
CD4 + LAG-3+	2.83 ± 0.72	4.07 ± 0.36
CD8 + LAG-3+	2.88 ± 0.6	4.24 ± 0.43

Table 6
Mean concentration (pg/ml) of T cells-derived cytokines.

	Improved patients (n = 7)	Death patients (n = 3)
IFN-γ	1051.1 ± 359.7	1754 ± 64.8
IL-4	500.5 ± 79	222 ± 52.4
IFN-γ/IL-4	2.73 ± 0.53	3.63 ± 0.21
IL-10	437.4 ± 42.1	359.3 ± 32.34
IL-17	1498.8 ± 118.3	1692.6 ± 52.5
IL-17/IL-10	3.48 ± 0.2	3.96 ± 0.13

contrast, Huang et al. manifested that the population of Th2 cells and related cytokines such as IL-4 was increased in 41 admitted hospital patients with COVID-19 compared to normal subjects. Also, their data demonstrated that plasma levels of IL-1β, IL-4, IL-7, IL-8, IL-9, IL-10, IL-13, IL-15, Eotaxin, and RANTES were higher in both hospitalized patients compared to healthy adults. It was suggested that that inflammation was inhibited by an increase in Th2 population which primarily triggered with Th17 and Th1 cells [9]. Carli *et al.* also reported that an increase in Th2 cell populations in severe COVID-19 patients could be a protective against the SARS-CoV-2 pathogenesis by regulating interferon-dependent responses and allergic reactions [50].

In the progression of inflammatory and autoimmune diseases, the role of Th17 cells is well known [51,52]. In the current study, the results showed that the frequency of Th17, ROR-γt expression, and IL-17 secretion levels were significantly increased in both hospitalized groups compared to control. Accordingly, Orlov and colleagues indicated that the frequency of Th17 cells was highly increased in SARS-CoV-2 patients and these cells promoted disease by producing cytokines including IL-17. It was suggested the Th17/IL-17A axis could be a plausible therapeutic target for COVID-19 that should be used to investigate in clinical trials [53]. In the PBMC of a patients with COVID-19, it was reported that Th17 cells were significantly increased, leading to cytokine storm, which in turn enhanced the disease progression. Their results imply that over-activation of Th17 cells and high cytotoxicity of $CD8^+$ T cells, partly accounts for the severe immune injury in these patients [54]. Also, Sadeghi and colleagues investigated the frequency of Th17 and Treg cells, the levels of specific gene expression, and the secretion levels of cytokines by flow cytometry, RT-PCR, and ELISA, respectively. Their data indicated that the population of Th17 cells and their related factors such as IL- 23, RORγt, and IL-17 were dramatically increased in 40 ICU-admitted patients with COVID-19 compared to 40 healthy controls. Also, it was suggested that inflammation triggered by Th17 cells is a significant factor in disease progression [13]. Recent studies have also reported that Th17 cells are responsible for inflammatory responses, which in turn enhance neutrophil migration, play an essential role in causing edema, pneumonia, and severe respiratory damage in COVID-19 patients [55,56]. In contrast, Gutiérrez-Bautista

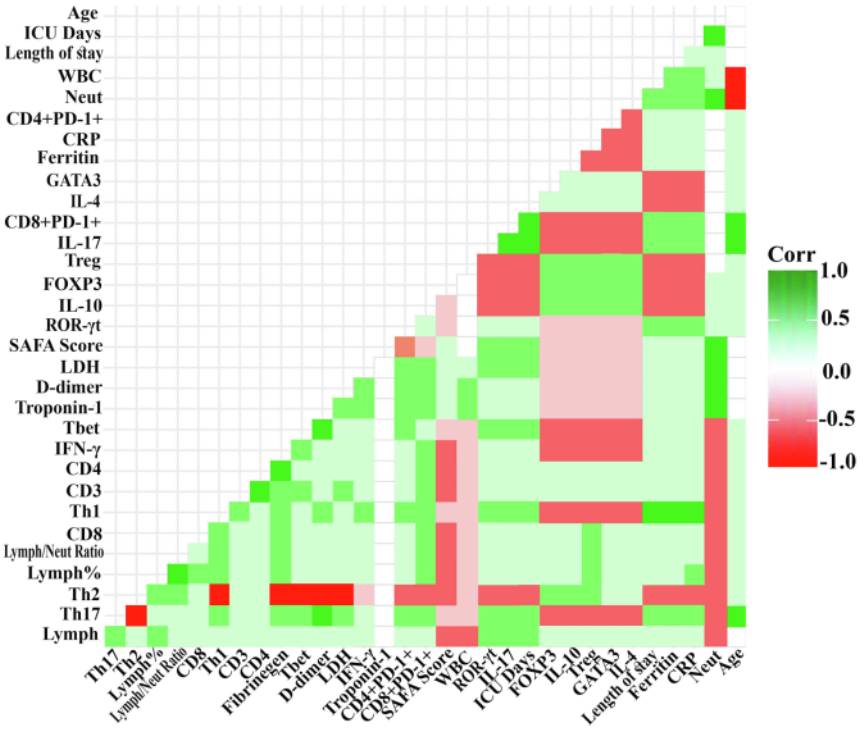

Fig. 6. Spearman association heat-map of all assessed cell populations, clinical and biochemical parameters. The color of Red and green indicates a negative and a positive relationship, respectively.

et al. by studying on 144 COVID-19 patients using multi-parametric flow cytometry analysis, showed that the frequency of Th17 was dramatically reduced in COVID-19 patients. They also identified that the neutrophil/lymphocyte ratio, PD-1$^+$ CD4$^+$/ CD8$^+$ T cells, and HLA-DR$^+$ CD4$^+$ T cells could be utilized as useful factors to predict critical illness and fatal outcome in COVID-19 patients [23].

Also, we evaluated the frequency of Treg, the expression levels of Foxp3, and secretion levels of IL-10 cytokine in COVID-19 patients and normal subjects. Basically, Tregs are classified as immunosuppressive cells involving in homeostasis and tolerance during inflammatory and autoimmune diseases [57]. Our data have shown that the population of Treg, Foxp3 expression, and IL-10 secretion levels were significantly decreased in both hospitalized patient groups. Consistently, Meckiff *et al.* reported that Treg cells were significantly reduced, whereas cytotoxic T helper cells (CD4-CTLs) and cytotoxic follicular helper cells were significantly increased in hospitalized COVID-19 patients as investigated by single-cell transcriptomic analysis of >100,000 viral antigen-reactive CD4$^+$ T cells from 40 COVID-19 patients [58]. More importantly, in distinct disease severities, their study provides great insight into the patterns of gene expression in SARS-CoV-2-reactive CD4$^+$ T cells. It is also demonstrated that Treg cell population, FoxP3 expression levels, IL-10 and TGF-β secretion levels were significantly reduced in 40 patients with COVID-19 compared to 40 healthy controls [13]. Consistently, Qin and colleagues indicated that Treg cells and their suppressive activity were considerably decreased in 450 patients with severe COVID-19, although naive Th cells were significantly increased [59]. Studies have also shown that upregulation of inflammatory cytokines, TNF-α and IL-6, can decrease the suppressive function of Treg by inhibiting TGF-β and Foxp3 expression levels in COVID-19 patients [60,61]. In the study on 40 COVID-19 patients, it is reported that the number of Tregs and related factors including TGF-β, FoxP3, and IL-10 significantly decreased [13]. In contrast, another research by studying on 144 COVID-19 patients, reported that the frequency of Treg was dramatically enhanced in COVID-19 patients [23]. Yang *et al.* reported that in the early stages of asymptomatic SARS-CoV-2 infection, the Treg population increased which led to a reduction in CD8$^+$ T cell and CD4$^+$ T cell functions in COVID-19 patients upon admission at days of 7, 13, 22, and 28 as investigated by flow cytometry analysis. In the asymptomatic SARS-CoV-2 infection, the data indicated that the T cell activation is considerably reduced but also an impairment in Th1/Th17 and CD8$^+$ T cell functions were seen. Based on these results, it can be concluded that an early increase in Tregs may be involved in both the activation and T cell function in asymptomatic SARS-CoV-2 infection [62].

Various studies have reported that the SARS-CoV-2 virus can cause T lymphocytes to be exhausted by constant stimulation and to suppress the response of immune system against it [63]. Our data indicated that CD8$^+$ LAG-3$^+$, CD4$^+$ LAG-3$^+$, CD4$^+$ PD-1$^+$, and CD8$^+$ PD-1$^+$, as exhausted T cells, were increased in ICU hospitalized patients compared to normal and asymptomatic recovered donors. However, some studies have indicated that the SARS-CoV-2 infection can inhibit CD4$^+$ and CD8$^+$ T cell functions by eliminating the production of IL-21 and IFN-γ and that is why the exhaustion of these cells occurred [63,64]. By polychromatic flow cytometry technique, De biasi and colleagues reported that CD4$^+$ CD57$^+$ PD-1$^+$ exhausted T cells were significantly increased in 39 COVID-19 patients compared to normal subjects [65]. In another study, Zheng and colleagues, indicated that TIGIT$^+$ CD8$^+$ and CD8$^+$ PD-1$^+$ T cells were dramatically reduced, but that exhausted T cells were increased during disease progression in 16 COVID-19 patients group compared to control, as evaluated by flow cytometry technique. It can be concluded that homeostasis of the immune system plays an important role in the development of COVID-19 pneumonia [66]. Also, Mazzoni *et al.* reported that the number of T, B, and NK cells decreased in the peripheral blood, and also had exhausted CD8$^+$ T cells phenotype in COVID-19 patients. It was reported that IL-6 levels were significantly increased in these patients and this cytokine was a major cause of cytotoxic lymphocyte dysfunction and exhaustion. They suggested that Off-label treatment with tocilizumab restores the cytotoxicity of NK cells and lymphocytes. It can be concluded that SARS-CoV-2 virus not only causes excessive induction of CD8$^+$ T cells but also disrupts the activity of CD4$^+$ T cells [67]. In contrast, in a study comparing the levels of exhausted lymphocyte populations, Gutie'rrez-Bautista *et al.* reported that the levels of TIGIT$^+$ CD8$^+$, PD-1$^+$ CD8$^+$, and PD-1$^+$ CD4$^+$ T cells were significantly decreased in COVID-19 patients compared to healthy donors [23].

Our results showed that biochemical inflammatory parameters such as D-dimer, LDH, CRP, fibrinogen, and ferritin were significantly increased in COVID-19 patients. Consistently, Feng and colleagues demonstrated that inflammatory parameters, including CRP, neutrophil–lymphocyte ratio, WBC, and neutrophil were dramatically increased in patients with COVID-19 [68]. In another study, Zho *et al.* reported that inflammatory parameters such as IL-6, CRP, LDH, and ferritin were dramatically increased in COVID-19 patients, leading to progression and worsening of the disease [69]. More importantly, increased inflammatory status can also enhance neutrophil activity through the production of G-CSF [70]. Basically, these inflammatory parameters can also explain the positive relationship between acute-phase proteins and neutrophils [71]. Based on ROC curve data, the concentrations of LDH, CRP, D-dimer, and Troponin-I could be used as predictors of disease progression during hospitalization. Also, the measurement of lymphocytes and PD-1$^+$ CD4$^+$ T cells can be useful to predict disease progression [23].

As discussed above, in COVID-19 patients, various studies have found many inconsistencies regarding the abundance of different cells in cellular immunity. These contradictions can have various reasons, some of which are mentioned here. One of the most important reasons for observing these contradictions can be related to performing studies on patients with different conditions and degrees of disease. In some studies, patients are not divided into different groups, which can have a significant impact on the results. Different studies on different races may have different results. The immune system and its relationship with the virus and the genetic background of patients can have a great impact on the results of various studies. The timing of blood sampling from patients is also very important. Whether blood sampling was done before, after, or during treatment can have completely different results. The type of treatment is also very important. For example, treating acute patients with anti-inflammatory drugs has a direct effect on immune cells. Finally, the type of technique used to evaluate immune cells and related cytokines can also have some effect on the results.

Our study had also limitations, perhaps the most important of which was the sample size. Using a larger sample size might lead to more accurate results. Another limit of this study is that the specific immune response towards SARS-CoV-2 has not been investigated. Although in this study, specific immune response towards SARS-CoV-2 has not been investigated, other studies have confirmed the concept that a higher magnitude of cell-mediated and humoral immune responses are associated with unfavorable outcomes and that virus-reactive T cells express immune-checkpoint molecules [72].

Therefore, based on the data obtained from our study as well as studies of other researchers in the field of SARS-CoV-2, it can be suggested whether decreasing Treg and Th2 populations or increasing Th1 and Th17 populations can result in activating inflammatory responses and progression of COVID-19 disease.

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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