Reduced frequency of T helper 17 and T helper 1 cells and their association with critical coronavirus disease 2019

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There is very little knowledge about the immune responses, particularly cellular immunity to coronavirus disease 2019 (COVID-19). The main objective of this study was to evaluate the frequency of T helper (Th) cell subtypes, including Th1, Th17, and Treg cells, in moderate-to-severe and critical COVID-19 patients compared to healthy controls. Twenty-nine moderate-to-severe and 13 critical patients confirmed for COVID-19, and 15 healthy subjects were included in this study. Interferon-γ (IFN-γ)-producing Th1 and interleukin-17A-producing Th17 and Treg cells in peripheral blood were measured with flow cytometry. The frequency of Th1 and Th17 was significantly decreased in critical patients compared to healthy subjects (aMD: 2.76 and 2.34) and moderate-to-severe patients (aMD: 1.89 and 1.89), respectively (p < 0.05). Differences were not significant between moderate-to-severe patients and healthy subjects for both Th1 (p = 0.358) and Th17 (p = 0.535), respectively. In contrast, significant difference was not observed between study subjects regarding the frequency of Treg cells. Patients with critical COVID-19 had a markedly lower Th1/Treg and Th17/Treg ratios compared with the controls and moderate-to-severe cases. Our study showed a dysregulated balance of Th1 and Th17 cells and its relation to the severity of COVID-19.

Key words: Coronavirus disease 2019; T helper 1; T helper 17; Treg.

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New coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has recently become pandemic [1,2]. At present, although several anti-viral drugs have been seriously tested, no effective prophylactic or therapeutic is available, and current treatments are largely based on symptomatic treatment. Therefore, there

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is a demand for the urgent development of vaccines and anti-viral drugs for the prevention and treatment of COVID-19.

Infected patients may develop acute respiratory distress and failure, which are the leading causes of death in patients; moreover, damage to other organs and systems, such as hepatic, renal, cardiac, and hemorrhage, can also lead to death [3,4]. The damage may be triggered by an indirect cytokine storm caused by the immune system activation and direct attack from SARS-CoV-2 [5].

The previous studies showed that SARS-COV-2 infection can activate innate and adaptive immune responses [6]. However, uncontrolled immune response to SARS-CoV-2 at innate and adaptive immunity may lead to harmful tissue damage, both locally and systemically [7]. Many COVID-19 patients develop acute respiratory distress syndrome (ARDS), which leads to pulmonary edema and lung failure, as well as liver, heart, and kidney damages [8,9]. Severe proinflammatory response or a cytokine storm causes ARDS and dysfunction of several organs. It has also been suggested that inflammatory response is very different in adults and children. Aging is associated with an increase in proinflammatory cytokines, which control neutrophil functions, and is correlated with the severity of ARDS [10]. According to the results of epidemiological studies, older male patients with comorbidity are more likely to die.

We need to understand this new virus better and find ways to control its spread. Knowing the host immune system response and how the virus may evade such host responses can help us to understand the pathogenesis of COVID-19 and improve clinical strategies against the disease. The importance of innate and adaptive immunity in the defense against SARS-CoV-2 needs to be urgently determined.

Many respiratory viruses, including coronaviruses, suppress innate immune responses to gain a window of opportunity for efficient virus replication and establishment of infection. The consequences for the host’s immune response are often incomplete and delayed/reduced or show a very strong induction that may cause tissue damage [11]. The impaired innate immune response also affects subsequent adaptive responses, and therefore, viral innate immune evasion often weakens completely protective immunity. Cellular responses are initiated days after innate immunity. Recent studies have shown that there is a reduced count of CD4+ and CD8+ T cells in COVID-19 patients compared with healthy controls [12–14]. However, there is poor information regarding the concomitant role of Th1 and Th17 cells, which are two inflammatory subsets of CD4+ T cells. In this regard, this study aimed to evaluate the frequency of T cell subtypes (Th helper 1 [Th1], T helper 17 [Th17], and T regulatory [Treg] cells) in moderate-to-severe and critical COVID-19 patients compared to healthy controls.

**MATERIALS AND METHODS**

All samples were obtained with informed consent. The study was approved by the Ethics Committee of Babol University of Medical Sciences (reference number: IR.MUBABOL.REC.1399.084) and was carried out in accordance with the Helsinki Declaration.

**Study population and data collection**

We performed a hospital-based, multicenter case–control study of 42 patients with newly diagnosed COVID-19 and 15 control subjects without infection. All patients were recruited from three hospitals in Babol city, including Shahid Yahya Nezhad, Ayatollah Rouhani, and Shahid Beheshti Hospitals. Patients of COVID-19 were diagnosed with chest x-ray or chest computed tomography (CT) and confirmed with a positive reverse transcription-polymerase chain reaction (RT-PCR) test of respiratory secretions. Patients who did not meet the above inclusion criteria were excluded from the study.

The control group consisted of 15 subjects without a history of COVID-19, who were seen for clinical manifestations in the same hospitals from which the case patients were enrolled. None of the controls showed any clinical manifestations of COVID-19 at the time of blood sample collection. All of the healthy subjects have negative molecular testing of respiratory secretions.

Approximately 5 mL of heparinized venous blood was obtained from each subject, and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation (350 g for 20 min at room temperature) with Ficoll Histopaque density gradient (Biowest, Nuaille, France) according to the manufacturer’s directions. The medical records of participants were abstracted for baseline demographic and clinical features. All variables were documented by the clinicians caring for the patient when staying in the hospital. The medical records of all subjects were abstracted by one of the authors by using a standardized data collection tool.

**Flow cytometry**

PBMCs at the concentration of $1 \times 10^6$ cells/mL were stimulated with phorbol myristate acetate (PMA; 20 ng/mL; Sigma, St. Louis, USA) and ionomycin (1 µg/mL; Sigma, St. Louis, USA) for 1 h and then incubated in the presence of GolgiPlug (1 µL/mL; BD, San Jose, CA, USA) for 4 h. The stimulated cells were collected and washed twice with staining buffer (phosphate-buffered saline [PBS] + 0.5% bovine serum albumin [BSA]) and then stained with FITC anti-human CD4 (clone SK3) and APC anti-human CD25 (clone...
BC96) for 15 min on ice in the dark. For intracellular staining, the True-Nuclear™ Transcription Factor Buffer Set (BioLegend, San Diego, CA, USA) was used. After staining for cell surface antigens, the cells were washed twice with staining buffer and fixed in fixation buffer. After 45 min of fixation at room temperature in the dark, the cells were permeabilized with permeabilization buffer without washing. Finally, the cells were stained with PE anti-human IFN-γ for Th1 cells and PerCP-CY5.5 anti-human interleukin-17A for Th17 cells. All fluorochrome-conjugated antibodies were purchased from BioLegend Company. The stained cells were read by a FACS Calibur flow cytometer (BD, San Jose, CA, USA), and the findings were analyzed using CellQuest (BD, San Jose, CA, USA) and FlowJo (Tree Star, Ashland, OR, USA) software. A minimum of 10^5 events in lymphocyte gate were read.

Statistical analysis

Continuous variables were described by mean ± standard deviation (SD). Categorical variables were described by counts and percentages (%). The distribution of the cell frequency and other continuous variables were compared by using the independent sample t-test. The categorical variables were analyzed by using the Chi-squared test. We estimated the adjusted mean difference (MD(adj)) and 95% confidence intervals (CIs) via linear mixed-effects models. The models were adjusted for the following potential confounders: age, sex, existing medical problems, smoking, white blood cell (WBC), lymphocyte, and CD4. Also, statistical analyses were performed on Stata 16.0 (Stata Corp, College Station, TX, USA). All statistical tests were two tailed at the significance level of p < 0.05.

RESULTS

Clinical findings in patients infected with the SARS-CoV-2

A total of 42 patients (29 with moderate-to-severe and 13 with critical conditions), infected with the SARS-CoV-2, were consecutively enrolled in this study. The median age was 58 years (range: 14–88), and 20 patients were male (47.61%, 95% CI [32.51, 62.72]). Four patients were current smokers (9.52%, 95% CI [0.64, 18.40]), and 23 patients had at least one comorbidity (54.76%, 95% CI [39.70, 69.81]). Patients with critical COVID-19 had higher frequency of comorbidities (84.61%) compared to moderate-to-severe patients (54.76%, 95% CI [39.70, 69.81]). Compared with moderate-to-severe patients, patients with critical COVID-19 had a higher respiratory rate (MD: 7.10, 95% CI [4.17, 10.03]; p < 0.001) and lower level of oxygen saturation (MD: −11.55, 95% CI [−15.49, −7.61]; p < 0.001). The mean level of ALT in critical COVID-19 patients was higher than healthy controls (MD: 141.23, 95% CI [11.58, 270.89]; p = 0.034). Moreover, the mean level of ALT in moderate-to-severe COVID-19 patients was higher than healthy controls (MD: 21.34, 95% CI [5.35, 37.33]; p = 0.010). No significant difference in the level of AST between the three groups was observed (p > 0.05). Also, the D-dimer (µg/mL) data of some patients were available. Seven patients out of the 13 critical COVID-19 patients and 15 patients out of the 37 moderate-to-severe patients had D-dimer data. Critical COVID-19 patients had significantly higher D-dimer (mean ± SD, 4.1 ± 3.2) compared with moderate-to-severe patients (1.4 ± 2.2, p = 0.03). The biochemical and demographic characteristics of participants are shown in Table 1.

Lower frequency of Th1 cells in critical COVID-19 patients

Flow cytometric analysis showed that IFN-γ-producing (Th1) cells were notably decreased in patients with critical COVID-19 compared with healthy adults (MD: −2.36, 95% CI [−4.48, −0.25]; p = 0.029) and patients with moderate-to-severe COVID-19 (MD: −2.74, 95% CI [−4.67, −0.81]; p = 0.006), while there was no difference between the moderate-to-severe and the control group (MD = −0.37, 95% CI [−1.60, 2.36]; p = 0.703; Figs 1A and 2). After adjusting for potential confounders, the difference remained significant for critical patients and healthy adults (aMD: −2.76, 95% CI [−5.13, −0.39]; p = 0.022) and nonsignificant for moderate-to-severe patients and controls (aMD: −0.87, 95% CI [−2.72, 0.98]; p = 0.358). Also, the difference remained significant for critical and moderate-to-severe patients (aMD: −1.89, 95% CI [−3.58, −0.20]; p = 0.028; Table 2).
Lower frequency of Th17 cells in critical COVID-19 patients

Similar to Th1 cells, the frequency of interleukin-17A-producing (Th17) cells was dramatically lower in patients with critical COVID-19 compared with moderate-to-severe patients (MD: −2.54, 95% CI [−4.04, −1.04]; p = 0.006) and healthy adults (MD: −2.38, 95% CI [−4.01, −0.75]; p = 0.006), whereas there was no difference between the moderate-to-severe patients and the control group (MD: 0.16, 95% CI [−1.46, 1.79]; p = 0.839; Figs 1B and 2). In a separate linear mixed-effects model, after adjusting for potential confounders, we observed a significant difference between the critical patients and the healthy adults (aMD: −2.34, 95% CI [−4.18, −0.50]; p = 0.012). Moreover, the difference remained significant for critical and moderate-to-severe patients (aMD: −1.89, 95% CI [−3.20, −0.57]; p = 0.005) and nonsignificant for moderate-to-severe patients and controls (aMD: −0.45, 95% CI [−1.89, 0.98]; p = 0.535; Table 2).

Same Treg cell frequency in study subjects

In a bivariate analysis, the mean frequency of CD4+ CD25high (Treg) cells [17, 18] did not differ between patients with critical COVID-19 and healthy adults (MD: 0.84, 95% CI [−0.14, 1.82]; p = 0.090). Also, there was no difference between the moderate-to-severe patients and the other two groups (with critical patients: MD: 0.07, 95% CI [−0.93, 1.09]; p = 0.876) (with controls: MD: 0.76, 95% CI [−0.10, 1.63]; p = 0.085; Figs 1C and 2). In multivariate analyses, compared with healthy adults (aMD: 1.82, 95% CI [0.78, 2.86]; p = 0.001) and moderate-to-severe patients (aMD: 0.96, 95% CI [0.17, 1.75]; p = 0.017), critical patients had higher Treg cells. Also, there was a significant difference between the moderate-to-severe patients and the control group (aMD: 0.96, 95% CI [0.17, 1.75]; p = 0.017; Table 2).

Lower frequency of Th1/Treg cell ratio in critical COVID-19 patients

As shown in Fig. 1D, patients with critical COVID-19 had a markedly lower Th1/Treg cell ratio than the controls (MD: −1.29, 95% CI [−1.90, −0.67]; p < 0.001) and moderate-to-severe patients (MD: −1.02, 95% CI [−1.83, −0.20]; p = 0.015). There were no differences between moderate-to-severe and healthy subjects (MD: −0.26, 95% CI [−1.04, 0.50]; p = 0.487). Linear mixed-effects model, further, proved that the critical COVID-19 infection is a strong prognosticator for the reduced level of a Th1/Treg cell ratio with an adjusted MD per one standard deviation of −1.95 (95% CI [−2.89, −1.01]; p < 0.001). After adjusting for potential confounders, moderate-to-severe COVID-19 infection was associated with a lower Th1/Treg cell ratio (aMD: −0.90, 95% CI [−1.64, −0.17]; p = 0.015). In addition, the ratio of Th1/Treg cells was significantly lower in the critical group than in the moderate-to-severe group (aMD: −1.04, 95% CI [−1.76, −0.33]; p = 0.004; Table 2).

Lower ratio of Th17/Treg cells in critical COVID-19 patients

Bivariate analysis showed that Th17/Treg cell ratios were notably decreased in critical cases compared with healthy adults (MD: −1.24, 95% CI [−1.72, −0.77]; p < 0.001) and moderate-to-severe patients (MD: −0.89, 95% CI [−1.41, −0.37]; p = 0.001), while there was no difference between the moderate-to-severe patients and the control group (MD: −0.35, 95% CI [−0.89, 0.19]; p = 0.202; Fig 1E). After adjusting for potential confounders, the difference remained significant for critical patients versus healthy adults (aMD: −1.42, 95% CI [−2.07, −0.78]; p < 0.001) and critical patients versus moderate-to-severe patients (aMD: −0.73, 95% CI [−1.22, −0.24]; p = 0.003). Also, the difference convert to significant for moderate-to-severe patients versus controls (aMD: −0.69, 95% CI [−1.19, −0.19]; p = 0.007; Table 2).

DISCUSSION

Recently, a serious respiratory syndrome associated with pneumonia triggered by a new coronavirus (SARS-CoV-2) was announced in China at the end of 2019 [19]. The underlying immune responses to SARS-CoV2 remained unclear. Thus, understanding of the immunological basis of the disease is urgently demanded.

One of the common characteristics of SARS-CoV-2 infection is lymphopenia [1,2]. A significant reduction in the frequency of lymphocytes suggests that SARS-CoV-2 may deplete immune cells and prevent host cellular immunity. SARS-CoV-2, like the older sister of this family (i.e., Middle East respiratory syndrome coronavirus [MERS-CoV]), may able to activate the apoptosis pathways and promote apoptosis of T lymphocytes [19]. However, no evidence shows the direct infection of T cells by SARS-CoV-2. Moreover, apoptosis of T cells, which was observed in the spleens and lymph nodes of patients who had died from COVID-19, was related to increased FAS expression [20,21]. Apoptosis of T cells might be a key element, leading to
exacerbations of infected subjects [22]. A similar mechanism could occur in COVID-19 patients. Moreover, the much more increase in D-dimer in the critical COVID-19 patients could be considered as a sign of thrombosis and pulmonary embolism in these patients [23]. CD4+ T cells consist of several subpopulations, such as Th1, Th17, and Treg cells, and a better

Table 1. Background characteristics of participants

|                        | Critical cases (n = 13) | Moderate/severe cases (n = 37) | Healthy controls (n = 15) | P-value
|------------------------|------------------------|-------------------------------|--------------------------|---------|
| Age (year)             | 52.69 (18.55)          | 58.34 (18.82)                | 38.20 (9.12)             | 0.002   |
| Sex                    |                        |                               |                          |         |
| Male                   | 7 (53.84)              | 15 (51.72)                   | 11 (73.34)               | 0.367   |
| Female                 | 6 (46.16)              | 14 (48.28)                   | 4 (26.66)                |         |
| WBC                    |                        |                               |                          |         |
| 2800–11,000            | 11 (84.62)             | 23 (79.31)                   | 15 (100)                 | 0.171   |
| 11,001–19,300          | 2 (15.38)              | 6 (20.69)                    | 0 (0)                    |         |
| Lymphocyte             |                        |                               |                          |         |
| 720–1000               | 6 (46.15)              | 5 (17.24)                    | 15 (100)                 | 0.008   |
| 1001–4830              | 7 (53.85)              | 24 (82.76)                   | 0 (0)                    |         |
| C-reactive protein     |                        |                               |                          |         |
| 2–10                   | 0 (0)                  | 7 (24.14)                    | 15 (100)                 | <0.001  |
| 10–493                 | 13 (100)               | 22 (75.86)                   | 0 (0)                    |         |
| Comorbidities          |                        |                               |                          |         |
| Yes                    | 11 (84.61)             | 12 (41.37)                   | 1 (6.66)                 | <0.001  |
| No                     | 2 (15.39)              | 17 (58.63)                   | 14 (93.34)               |         |
| BUN                    | 39.54 (28.30)          | 21.36 (19.88)                | 15.01 (3.62)             | 0.007   |
| AST                    | 120.33 (60.75)         | 51.58 (12.46)                | 21.38 (4.03)             | 0.076   |
| ALT                    | 180.58 (0.41)          | 39.34 (27.98)                | 18.01 (6.75)             | 0.027   |
| NLR                    | 8.71 (5.08)            | 4.01 (2.36)                  | 2.16 (0.60)              | <0.001  |
| PLR                    | 208.78 (130.93)        | 185.88 (109.67)              | 114.31 (40.78)           | 0.046   |

ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; NLR: neutrophil to lymphocyte ratio; PLR: platelet to lymphocyte ratio; WBC: white blood cells.

1Continuous variables compared with independent t-test, categorical variables compared with Chi-squared test or Fisher’s exact test.

Fig. 1. The percent of Th1 (A), Th17 (B), and Treg (C) as well as the ratios of Th1/Treg (D) and Th17/Treg (E) are displayed for all groups in different graphs.

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Fig. 2. Gating strategy for identification of Th1, Th17, and Treg cells. The lymphocytes were gated and separated based on CD4 marker. CD4⁺ lymphocytes were analyzed based on IL-17A and IFN-γ to achieve Th17 and Th1 cells, respectively. Treg cells were gated based on CD4⁺ and CD25^{High} concomitant expression. A, healthy control group, B, moderate/severe group, C, critical group.
understanding of different subpopulations of CD4+ T cells could better manage the patients with COVID-19. Sadeghi et al. showed that critical COVID-19 patients had a higher frequency of Th17 and Th17/Treg cell ratio compared with healthy adults [24]. In contrast, we demonstrated that critical COVID-19 patients had significantly lower Th1 and Th17/Treg cell ratios compared with both healthy and moderate/severe groups. Similar results were also observed for the Th1 and Th1/Treg cell ratio in our study subjects. In consistent with our study, Biasi et al. reported a lower frequency of Th17 cells in the COVID-19 patients compared with healthy adults [25].

In general, in this study, we found that the frequency of proinflammatory Th1 and Th17 cells was substantially reduced in the critical COVID-19 patients and not in the moderate/severe patients. It seems that proinflammatory Th1 and Th17 lymphocytes migrate from blood to the lung of critical COVID-19 patients and cause the reduced frequency of these immune cells in peripheral blood. The shift to proinflammatory T helper cells (Th1, Th17) in the lungs gives rise to diffuse alveolar destruction with abundant neutrophils and macrophages [25,26]. In acute respiratory distress syndrome, IL-17 promotes the damage of the lung by several mechanisms such as inappropriate neutrophil recruitment, apoptosis inhibition through stimulating the production of granulocyte colony-stimulating factor (G-CSF), and induction of proinflammatory mediators [26].

Moreover, recruited Th1 cells to the lung will trigger macrophage activation syndrome, which results in cytokine storm in COVID-19 patients. In support of this concept, a recently published study has shown higher proportions of neutrophils and macrophages in bronchovascular lavage fluids (BALFs) of patients with severe-to-critical SARS-CoV-2 infection [27].

It seems that Th1 and Th17 cells have the main role to increase the inflammatory condition of critical patients. T helper cells (Th1, Th17) perform their effector functions through producing inflammatory cytokines including IFN-γ and IL-17, respectively. In this regard, it seems that inhibition of IFN-γ or IL-17 may improve the clinical status of critical patients. In support of this concept, some studies showed that critical patients had an increased amount of IFN-γ or IL-17 compared with moderate patients or healthy control subjects [28–32]. Moreover, studies reported that patients with ankylosing spondylitis or rheumatic diseases who were on IL-17 inhibitor showed better clinical improvement [33]. It could be concluded that early inhibition of IL-17 may contribute to the better clinical status.

Now we know that clinical manifestations of SARS-CoV-2 infection are not limited to the respiratory system. Some other organs can be affected by SARS-CoV-2 infection. For example, evidence indicates that the brain can be targeted by SARS-CoV-2. SARS-CoV-2 may infect the brain through two pathways; (i) ACE2 receptor-dependent pathway in which SARS-CoV-2 binds to ACE2, which is expressed in the brain and (ii) ACE2 receptor-independent pathway in which SARS-CoV-2 infect the brain via the olfactory bulb upon inhalation. As a consequence of brain infection, neurological complications may appear in patients, including encephalopathy, encephalitis, seizures, and cerebrovascular events. Critical patients are more likely to have neurological complications compared with moderate patients. Thus, a better understanding of the immunological characteristics of patients may improve the clinical status of them and reduce the complications related to other organs [34–36].

Moreover, identifying biological pathways and transcriptomic profiles which were altered during SARS-CoV-2 infection may help to characterize new targets for controlling the COVID-19. By using computational analysis, studies suggested that mitogen-activated protein kinase (MEK), serine-threonine kinase (AKT), mammalian target of rapamycin (mTOR) and I kappa B Kinase (IKK) were new targets for controlling the COVID-19.

### Table 2. Association between COVID-19 infection and the frequency of CD4+ cell subsets

| Analyte     | Critical patients vs. healthy controls | Moderate/severe patients vs healthy controls | Critical patients vs Moderate/severe patients |
|-------------|----------------------------------------|---------------------------------------------|---------------------------------------------|
|             | MDadj 95% CI                            | MDadj 95% CI                                 | MDadj 95% CI                                 |
| Th1 cells   | −2.76, −5.13, −0.39                     | −0.87, −2.72, 0.98                          | −1.89, −3.58, −0.20                          |
| Th17 cells  | −2.34, −4.18, −0.50                     | −0.45, −1.89, 0.98                          | −1.89, −3.20, −0.57                          |
| Treg cells  | 1.82, 0.78, 2.86                        | 0.96, 0.17, 1.75                           | 0.96, 0.17, 1.75                             |
| Th1/Treg ratio | −1.95, −2.89, −1.01                    | −0.90, −1.64, −0.17                         | −1.04, −0.76, −0.33                         |
| Th17/Treg ratio | −1.42, −2.07, −0.78                   | −0.69, −1.19, −0.19                         | −0.73, −1.22, −0.24                         |

**MDadj**: adjusted mean difference; 95% CI: 95% confidence interval

Mean difference estimated directly from linear mixed effect model. The final multivariable models were adjusted for the following risk factors: age, sex, existing medical problems, smoking, white blood cells, lymphocyte, and CD4.

Significant at P value < 0.05.

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inhibitors could be considered in patients with COVID-19 [37,38].

Our study has several limitations, that is, the small number of subjects, heterogeneous patients, lack of lymphocyte subset analysis from alveolar lavage fluid, and lack of patient monitoring before and after infection. Therefore, more studies regarding to the role of these immune cells in the pathogenesis of COVID-19 are required.

CONCLUSION

Taken together, our study indicated a comprehensive decrease in proinflammatory Th1 and Th17 lymphocytes in the critical COVID-19 patients. The reduced frequency of these lymphocytes may result from their migration to the lung of critical patients, which increases lung inflammation and deteriorates patients’ status. Another possibility is that these lymphocytes become apoptotic and die.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All samples were obtained with informed consent. The study was approved by the Ethics Committee of Babol University of Medical Sciences (reference number: IR.MUBABOL.REC.1399.084).

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