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Genetic Regulation of Interleukin-6 and Interleukin-10 in COVID-19 Infection

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Abstract

Background: The role and regulation mechanisms of the interleukin-6 and 10 (IL6 and IL-10) serum levels and the interaction between CD4+ and CD8+ lymphocytes with SARS-COV-2 IgM and IgG in the context of COVID-19 infection are not fully understood.

Methods: This study was conducted on 45 COVID-19 patients and 45 healthy individuals. The IL-6 and IL-10 promoter methylation, IL-6 and IL-10 gene expression, SARS-COV-2 IgM, and IgG antibodies and CD4+ and CD8+ lymphocytes were studied by qMSP-PCR, Real-time PCR, ELISA, and flow cytometry techniques, respectively.

Results: The male ratio and mean age of critically ill patients' group were significantly higher in compared to controls (P< 0.05). IL-6 gene expression and serum levels were significantly increased in patients compared to controls (P=0.002, 0.001), but IL-6 promoter methylation was not significantly decreased in patients (P=0.835). The IL-10 promoter methylation and expression were not different between cases and controls (0.326, 0.455), but serum IL-10 levels were higher in patients (P< 0.001). The CD4+ and CD8+ lymphocytes decreased (P< 0.001) and mean SARS-COV-2 IgG increased (P=0.002) in the patients compared to controls.

Conclusions: The COVID-19 disease result in severe complications in men and elderly. The serum levels of interleukin-6 and 10 increases in COVID-19 infection, and the gene expression of these two interleukins underlying in this increase. The serum levels of IL-6, IL-10 and SARS-COV-2 IgG as well as CD4+ and CD8+ lymphocyte counts should be investigated to monitor patients and predict the course of disease.

Keywords: COVID-19, Gene Expression, Interleukin-6 (IL-6), Interleukin-10 (IL-10), SARS-COV-2, Promoter Methylation.

Introduction

The world has so far experienced outbreaks of SARS-CoV in 2002-2003 and MERS-CoV in 2011, both of which, related to the causal agents from coronavirus family (1). At the end of 2019, another novel coronavirus, SARS-CoV-2, causing COVID-19, emerged from Wuhan, China and spread worldwide which led to the global crisis in health care systems and many aspects of human life (2, 3). The clinical course of the COVID-19 varies depending on the individuals' characteristics and medical status. Extensive spectrum of clinical manifestations can be seen in COVID-19 patients ranging from mild symptoms (e.g., cough, fever, headache, sore throat, malaise, muscle pain, loss of taste and smell, nausea,

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vomiting, and diarrhea) resolving in a few days, to life-threatening critical illnesses with acute respiratory distress syndrome (ARDS), and multiple organ failure (4). Although, many COVID-19-related clinical features including transmission rate, mortality, and clinical symptoms have been largely determined by now, more time is still needed to elucidate the immune response against the infection (2, 5).

Cytokines hold key roles in regulating immunological and inflammatory responses and cytokine storm is one of the possible mechanisms for rapid disease progression and mortality in COVID-19 patients (5). Interleukin-6 (IL-6) gene, located at 7p21 and spanning 5 kb, contains four introns and five exons. IL-6 protein is a four-helix cytokine containing 184 amino acids, secreted by many cell types in response to infection, cancer, and inflammation (6). IL-6 plays a key role in cytokine storm and can effectively regulate B and T cell responses and coordinate activities of both the innate and adaptive immune systems (1). Interleukin-10 (IL-10) is a key anti-inflammatory cytokine, encoded by IL-10 gene located at 1q31. IL-10 plays a critical role in the resolution of peripheral inflammation and is produced by a variety of immune cells including activated macrophages, Th1, Th2, Th17, and T-reg cells (7). Many studies have proven the increase of IL-6 and IL-10 proteins in COVID-19 patients, however, the exact mechanisms of this increase remain to be discovered (4, 8).

DNA methylation is a biological process which can modulate the activity of a DNA segment without changing the sequence. In this process, chemical tags called methyl groups attach to promoter, a particular location within DNA sequence, where they switch a gene on or off (9). The methylation can alter gene expression, thereby regulating the production of proteins encoded by the respective genes (10). It has been demonstrated that promoter methylation of different cytokine genes plays an important role in the clinical course of various autoimmune and infectious diseases (11). We hypothesized that hypomethylation of IL-6 and IL-10 gene promoters in COVID-19 patients are associated with increased gene expression which ultimately leads to elevated serum level of IL-6 and IL10 proteins. In this study, we assessed the methylation status, gene expression and serum levels of IL-6 and IL-10 and their relationship with SARS-COV-2 IgM and IgG antibodies as well as CD4+ and CD8+ lymphocyte counts in individuals infected with COVID-19.

Materials and Methods

Study population

This study was conducted between January and March 2022 at Golestan Hospital, Kermanshah, Iran. The hospital information system was reviewed, and 45 patients referred to the emergency department with COVID-19 infection confirmed by real time polymerase chain reaction (RT-PCR) on nasopharyngeal swab samples, were included in the study. In addition, 45 COVID-19-negative individuals without diseases were randomly selected as the control group. The health status of control subjects was confirmed through clinical examination and self-administered questionnaire. The hospital laboratory software was used to retrieve the results of gender, age and paraclinical test results. All the patients were divided into three groups based on the clinical symptoms, low oxygen saturation, radiological pneumonia, shock, and any kind of organ failure. The assigned groups were as follow: mild/moderate: no need for hospitalization; severe: hospitalization in the general ward, and critical: required hospitalization in the intensive care unit (ICU) (12). Informed consent was obtained from all study participants. This study was performed in line with the principles of the Declaration of Helsinki and approved by the ethics committee of Kurdistan University of Medical Sciences with the ethics ID IR.MUK.REC.1401.030.

DNA extraction and bisulfite modification

Venous blood samples in EDTA-containing tubes were collected from patients and control individuals. The genomic DNA was extracted with the QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's

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instructions. Methylation status of IL-6 (Gene ID: 3569) and IL-10 (Gene ID: 3586) gene promoters were assessed by bisulfite treatment of DNA, as previously described by Goldenberg et al (13). With the bisulfite treatment, unmethylated cytosines of DNA are converted to uracil, whereas methylated cytosines remain unmodified. Briefly, 2 µg of genomic DNA was denatured by incubation with 2 mL of NaOH 3 mol/L for 20 minutes at 50 °C. After adding sodium bisulfite (2.5 mol/L) and hydroquinone (1 mol/L), the samples were incubated for 16 h at 55 °C in the dark. The samples were then purified using PCR purification columns and recovered samples were desulphonated by 0.3 M NaOH treatment for 15 min at 37 °C. Modified DNA was washed by ethanol and resuspended in 50 µL of elution buffer.

Quantitative Methylation Specific PCR

Sodium bisulfite-treated genomic DNA was amplified using methylation specific real-time PCR using SYBR green qPCR Master Mix (Pars Tous, Iran). The methylation status of the IL-6 and IL-10 genes was examined using *Alu* as the internal control for DNA quantification, it contains no CpG dinucleotides and is not affected by sodium bisulfite treatment. Sequence of primers used in the assay were as follow:

IL-6: forward primer 5'-ATATTATATAGACGGATTATAGTGTACG GT-3', reverse primer 5'-CGTAAACACTCCTAAACCAAATTCTCT-3';
IL-10: forward primer 5'-AAGGTATTTCGGAGATTTC-3', reverse primer 5'-AACTCAACACTACTCTATTAC-3';
Alu: forward primer 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3'; reverse primer 5'-AACCAATAAAACCTACTCCTCCCTTAA-3'.

For each sample, 25 ng of bisulfite-treated DNA was added to 2X Master Mix in a 25 µl PCR volume and amplified using LightCycler 98 Real-Time System (Roche, Germany). Thermocycling conditions were 95 °C for 10 min (polymerase activation), followed by 40 cycles of 95 °C for 10 s (denaturation) and 60 °C for 30 s (annealing and extension). The methylation of IL-6 and IL-10 for each sample was assessed using this formula:

$\mathrm{Cq}\left[\frac{\mathrm{IL}}{\mathrm{CqAlu}}\right] = \frac{[\mathrm{Cq\ IL}]}{[\mathrm{CqAlu}]}$ (14, 15).

Flow cytometry

Flow cytometry was performed using antibodies to CD4 (FITC) and CD8 (PE) (ED7052, Exbio, USA). 100 µL of whole blood samples in EDTA-containing tubes were added to 20 µL of adjacent antibody and incubated in the dark for 20 min. Following the lysis of red blood cells and dilution with isotonic solution, about 10,000 leukocytes of each sample were evaluated with the flow cytometer and the results were analyzed using FlowJo v10.7.1 (BD Life Sciences).

ELISA

Human IL-6 ELISA kit (Demeditec, Germany), Human IL-10 ELISA kit (Demeditec, Germany), SARS-CoV-2 IgM kit (Pishtazteb, Iran) and SARS-CoV-2 IgG kit (Pishtazteb, Iran) were used to analyze the serum concentrations of IL-6, IL-10, SARS-CoV-2 IgM, and SARS-CoV-2 IgG antibodies, respectively, according to the manufacturer's instructions. A microplate reader (Stat Fax 4200, USA) was used to evaluate the optical density (OD) values and sample concentrations were calculated based on the standard solution concentration and corresponding OD value.

IL-6 and IL-10 gene expression

Total RNA was extracted from whole blood using Trizol reagent (SinaClon, Iran), and the complementary DNA was synthesized from 1 mg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions. The transcriptional expression of IL-6 and IL-10 was analyzed by Lightcycler 96 Real-time PCR (Roche, Germany), using SYBR green qPCR Master Mix (Pars Tous, Iran). Amplification was performed in a total volume of 25 µL, containing 0.5 mM of cDNA, 0.5 mM of each primer and 2X SYBR green. The primer sequences used in the experiments were

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as follow; IL-6: 5'-CAAATTCGGTACATCCTC-3' (forward) and 5'-CTGGCTTGTTCCTCACTA-3' (reverse), IL-10: 5'-TGAGAACAGCTGCACCCACTT-3' (forward) and 5'-TCGGAGATCTCGAAGCATGTTA-3' (reverse), β-actin: 5'-CGCGAGAAGATGACCCAGAT-3' (forward) and 5'-GCACTGTGTTGGCGTACAGG-3' (reverse). Thermal cycling conditions included activation at 95 °C (10 min) followed by 40 cycles of denaturation at 95 °C (15 s) and annealing/elongation at 60 °C (1 min). Each sample was analyzed in triplicate with β-actin as housekeeping gene, and the mean values of IL-6 and IL-10 were calculated. The cycle threshold (Ct) was used to calculate relative amounts of IL-6 and IL-10. The resulting data were normalized for β-actin levels using the $2^{-\Delta\Delta Ct}$ method (16, 17).

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 22.0 software. Kolmogorov-Smirnov test was used to assess the normal distribution of the data. Categorical variables were described as number (percentage) and difference in proportion was analyzed using Chi-square test. Normally distributed numerical variables were described as mean (standard deviation). Comparisons of the RBC, HCT, HCO3 and promoter methylation of IL-6 and IL-10 between cases and controls were performed by independent sample t-test and one way-ANOVA. Comparisons of other variables were performed by non-parametric tests. A P value of < 0.05 was considered as statistically significant.

Results

In the current study, 15 COVID-19 patients have mild/moderate symptoms, 15 patients showed severe disease and 15 patients affected by critical form of disease leading to hospitalization in ICU. The patients and the controls were matched by gender and age. However, when subgroups of patients were compared with the controls, the mean age and number of male patients in critical COVID-19 subgroup were significantly higher than controls ($P< 0.05$). Table 1 compares the demographic and laboratory parameters between patients and controls. P value was calculated for all the patients as a group. Dyspnea (42.2%), cough (17.7%), fever (16.6%) and lethargy (10.0%) were the most common clinical findings recorded in the hospital information system.

Table 1. The comparison of measured variables between patients and controls.

Parameter	Controls *n* = 45	Patients *n* = 45			P value
		Mild/moderate (n=15)	Severe (n=15)	Critical (n=15)	
Gender					
Male Female	22(48.9%) 23(51.1%)	8(53.3%) 7(46.7%)	6(40.0%) 9(60.0%)	9(60.0%) 6(40.0%)	0.954
Age (years)	57.4±16.5	59.1±20.6	57.3±19.9	71.3±10.9	0.073
COVID-19 IgM (ng/ml)	0.097±0.067	0.139±0.095	0.096±0.075	0.161±0.149	0.38
COVID-19 IgG (ng/ml)	0.236±0.190	0.603±0.362	0.482±0.381	0.631±0.446	0.002
WBC ($\times10^9$/L)	6.70±1.16	8.63±3.99	9.16±4.60	10.41±5.17	0.014
Neutrophil ($\times10^9$/L)	3.79±0.97	6.57±3.71	7.02±3.89	8.79±4.92	<0.001
Lymphocyte ($\times10^9$/L)	2.46±0.42	1.38±0.68	1.28±0.82	0.99±0.57	<0.001
Neut/Lymph	1.59±0.44	5.92±4.71	6.94±5.92	11.16±8.45	<0.001
CD4+ (cells/mm^3)	1206/8±187.2	401.5±361.8	467.4±890.7	175.0±230.3	<0.001
CD8+ (cells/mm^3)	716.4±267.3	308.7±253.4	278.8±296.2	136.6±157.7	<0.001
Monocyte ($\times10^9$/L)	0.43±0.21	0.40±0.25	0.46±0.30	0.39±0.26	0.399
Eosinophil ($\times10^9$/L)	0.14±0.06	0.17±0.14	0.10±0.13	0.21±0.18	0.758
Basophil ($\times10^9$/L)	0.01±0.02	0.03±0.03	0.02±0.03	0.03±0.03	0.227
RBCs ($\times10^{12}$/L)	4.78±0.44	4.73±0.69	4.24±0.47	4.52±0.83	0.037
HB (g/dL)	14.30±1.30	13.98±1.80	12.55±1.16	13.14±2.23	0.009
Platelet ($\times10^9$/L)	254±78	209±75	226±113	206±92	0.024

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In the COVID-19 patients, the mean serum level of IL-6 sharply increased and in parallel, the mean IL-6 gene expression was increased 4.8 times; nevertheless, the mean IL-6 methylation ratio reduced by only about 2% compared to the controls. The mean serum level of IL-10 was higher in the COVID-19 patients compared to controls, the mean IL-10 gene expression was also increased 1.81 times, however, it was not statistically significant. Also, the mean IL-10 methylation ratio was not different between groups (Table 2).

Table 2. The mean methylation ratio, gene expression and serum levels of IL-6 and IL-10 in COVID-19 patients and controls groups.

		Controls, n=90, Mean ± SD	Cases, n=90, Mean ± SD	P value
IL-6	Methylation	0.997±0.217	0.979±0.212	0.835
	Expression	0.010±0.033	0.048±0.133	0.002
	Protein	5.53±6.80	292.4±527.9	<0.001
IL-10	Methylation	0.963±0.197	1.043±0.199	0.326
	Expression	2.794±5.747	5.075±11.749	0.455
	Protein	9.82±2.34	18.37±7.65	<0.001

The results showed an increased IL-10 promoter methylation ratio was associated with reduced serum levels of IL-10 in the control group (Figs. 1 and 2).

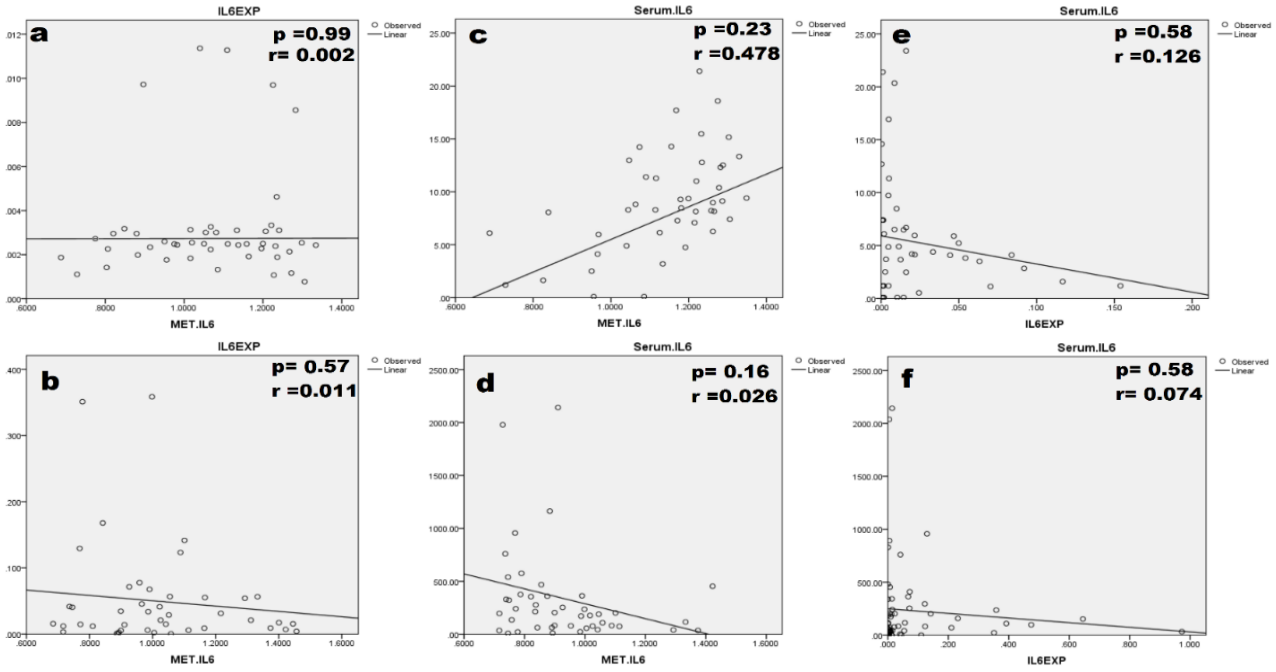

Fig. 1. The relationship between promoter methylation and gene expression (a, b), promoter methylation and serum levels (c, d) as well as gene expression and serum levels (e, f) of IL-6 in controls and patients, respectively. As table 1 shows, the number of CD4+ and CD8+ lymphocytes drastically reduced in patients with COVID-19 compared to the controls. Moreover, the number of these cells decreased further with the increase in the severity of the disease.

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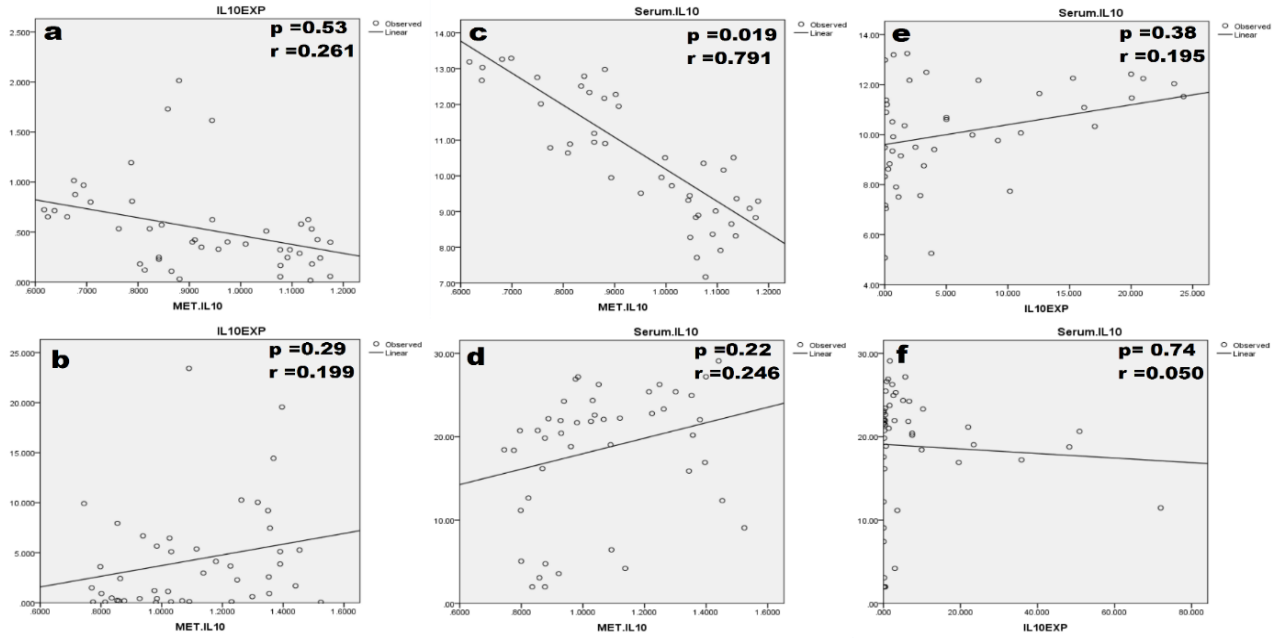

Fig. 2. The relationship between promoter methylation and gene expression (a, b), promoter methylation and serum levels (c, d) as well as gene expression and serum levels (e, f) of IL-10 in controls and patients, respectively.

Discussion

This study was conducted between January and March 2022 which was coincided with the sixth wave of COVID-19 in Iran. The Omicron variant of COVID-19 was the predominant variety in this wave of the COVID-19 (18). In our study, the male ratio and mean age of critically ill patients' group were significantly higher in compared to control group (Table 1). Many studies have demonstrated that the elderly patients were more vulnerable and showed more severity and higher mortality compared with younger patients. Also men are more likely to contract the disease (5, 19). A possible explanation for this finding may be because of the natural biological diversity of the immune system and genetic factors, which make males more likely to contract the infection than females (2). In addition, characteristic male type lifestyle such as less activity and being less strict to social distancing measures increases the risk of morbidity in men (1). Consistently, Bwire et al. reported that women were more responsible than men for the COVID-19 crisis (19).

Our literature review revealed few studies focusing on the possible role of epigenetic factors in pathogenesis of COVID-19 infection. In the current study, increased serum levels of IL-6 and IL-10 was noticed in the patients, as expected. In line with this finding, the mean levels of IL-6 and IL-10 gene expression in patients increased 4.8 and 1.81 times, compared to controls. In addition to confirming the previous findings about the role of serum IL-6 and IL-10 in the pathogenesis of COVID-19, these results indicating that the transcription of IL-6 and IL-10 genes is one of the mechanisms of IL-6 and IL-10 serum levels regulation in COVID-19 infection (4, 7).

The cells show complex transcriptional changes upon infection, and DNA methylation is one of the mechanisms responsible for gene expression regulation (10). While DNA methylation was considered as a relatively stable epigenetic modification, recent evidence indicates that DNA methylation patterns can be changed rapidly upon exposure with infection (10, 20). Infectious agents can alter DNA methylation indirectly by mediators of inflammation induced during the infection and/or directly through repressing or inducing DNA methylation enzymes (21). Some studies reported that septicemia is associated with changes in patterns of DNA methylation in

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white blood cells of critically ill patients which correlated with increased IL-6 and IL-10 levels (20, 22). Similar results have been reported in other studies (11, 23, 24). However, in our study, the mean methylation ratio of IL-6 and IL-10 were not significantly changed between groups, and there was no significant relationship between the IL-6 and IL-10 promoter DNA methylation changes and their respective serum levels in COVID-19 patients.

Besides epigenetic regulation, several mechanisms may contribute to increased serum levels of these interleukins. IL-6 and IL-10, for instance, may be produced from internal or peripheral sources around the involved tissue and enter into the blood stream (25). A variety of posttranslational mechanisms such as protein glycosylation, folding, protein–protein interactions and protease inhibitor production, lead to an increase in the half-life of these cytokines in the blood (26). It has been shown that the half-life of IL-6 and IL-10 in blood is about 1 hour. The increase in the types of interleukin-producing cells, can be considered as another mechanism (8).

In this study, along with the increase in the number of CD4+ lymphocytes, the mean serum levels of IL-6 also significantly increased in patients (Fig. 2). In Luo et al. study, the CD8+ lymphocyte counts reduced in COVID-19 patients hospitalized in the ICU, and the number of CD4+ and CD8+ lymphocytes showed a negative relationship with serum level of IL-6 (27). IL-6 prolongs the survival of CD4+ lymphocytes by retaining Bcl-2 expression (27). It has also been shown that IL-6 protects CD4+ lymphocytes from cell death by inhibition of Fas/FasL expression (28). In return, IL-6 inhibit the CD8+ lymphocyte activation and cytokine production by inducing downregulation of STAT4 phosphorylation and upregulation of STAT3 phosphorylation (29). Moreover, TLR stimulation with IL-6 blockade during viral infection, led to enhanced immunity of virus-specific CD8+ lymphocytes and improved viral replication control (30).

In our study, increased serum levels of IL-10 and remarkably reduced number of CD4+ and CD8+ lymphocytes were observed in patients. *In vitro* studies have shown that, IL-10 inhibits the proliferation of CD4+ cells and the production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α cytokines in these cells, mainly affecting the Th1 (30, 31). Also, IL-10 induced during infection can reduce the antigen sensitivity of antigen specific CD8+ lymphocytes enabling the virus to overcome the immune system and cause disease (32). Previous studies indicated that T cells, macrophages and monocytes are initiators of the cytokine storm. However, more recent studies suggest that hyperactive lymphocytes may play a role in the cytokine storm in early stages of the disease (30, 33). Most immune cells such as macrophages, lymphocytes, and neutrophils can produce IL-10, which suppresses the immune system and prevents the cytokine storm (29).

In our study, the mean serum levels of SARS-COV-2 IgG were significantly higher in ICU-admitted patients compared to controls. Many studies have reported an association between higher levels of SARS-COV-2 IgG antibody and COVID-19 disease severity, although there are reports to the contrary (34-36). These conflicting results can possibly be explained by the dual role of antibodies during viral infections (37). The neutralizing antibodies are generally beneficial for viral clearance, however, the prior existence or early production of non-neutralizing antibodies can facilitate the antibody-mediated viral entry and induce an acute inflammatory response (38). This study showed that patients with mild/moderate symptoms produced less anti SARS-COV-2 IgG antibody. Some studies indicate that patients with mild disease may not be able to maintain long term immunity and patients with primary humoral defects and reduced antibody production, do not develop severe COVID-19 (34, 37).

The lack of accurate recording of the clinical symptoms of the patients, was one of the limitations of this study. There was also no

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information on vaccines possibly used by the study participants.

In conclusion, The COVID-19 disease caused by the SARS-CoV-2 virus can result in severe complications in men and elderly individuals. The serum levels of IL-6 and IL-10 increases in COVID-19 infection, and the gene expression of these two interleukins underlying in this increase. However, changes in the promoter DNA methylation, did not show any roles in gene expression and subsequently serum levels of IL-6 and IL-10 in patients. On the other hand, a reduction in the number of CD4+ and CD8+ lymphocytes and an increase of SARS-COV-2 IgG antibodies were revealed to be associated with COVID-19 disease severity. Finally, according to the results of this study, it is suggested that the serum levels of IL-6, IL-10 and SARS-COV-2 IgG as well as CD4+ and CD8+ lymphocyte counts should be investigated to monitor patients and predict the course of disease.

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Conflicts of interest

The authors declare no conflicts of interest.

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