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Identification of lymphocyte subgroups with flow cytometry in COVID-19 patients

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ABSTRACT

Objective: We aimed to determine lymphocyte subgroups and activation status of flow cytometry in COVID-19 patients and examine their relationship with disease stage and length of hospital stay.

Material and Method: Forty patients were analyzed in this study and compared with the age and sex-matched 40 healthy controls. COVID-19 patients have split as early and advanced-stage diseases. Flow cytometry assay was performed to determine the counts of lymphocyte subsets and activation status. Total lymphocyte count was calculated and CD45 (cluster of differentiation), CD3, CD4, CD8, CD19, CD27, CD38, CD56, CD57, and IgD were studied on lymphocyte gate. T helper / T cytotoxic rates and length of hospital stay were recorded.

Results: The patients' CD3(+)CD4(+) (T helper) count and CD27 expression on T cells counts were significantly lower, and CD57 expression on CD3(+)CD8(+) T cytotoxic cells were significantly higher ($p<0.05$) than the control group. When the patients were divided into early and advanced stages, it was observed that CD38 expression on T cells was significantly lower in advanced-stage patients ($p< 0.05$) Total lymphocyte count and CD3(+) T lymphocyte count were negatively correlated with the duration of hospitalization as statistically significant ($p <0.05$).

Conclusion: Our data showed that the SARS-CoV-2 primarily affects T lymphocytes. It was thought that this effect occurred by impairment of development and activation of T lymphocytes. There are some discordances among the studies on T lymphocytes in the literature.

Keywords: COVID-19, flow cytometry, lymphocytes, SARS-CoV-2, T cells

INTRODUCTION

Coronaviruses (CoV) are a large family of viruses that cause a diversity of diseases varying from common gribal infections to more serious diseases. The new Coronavirus Disease was first identified in China (1,2). The disease caused by a 2019-new coronavirus (advanced acute respiratory syndrome coronavirus 2 (SARS-CoV-2)) was officially named COVID-19 by the World Health Organization (WHO) (3). While most of the infected patients recovered, some patients experienced the infection at a serious and vital level (4,5).

The severity of the disease is related to many clinical features, such as age and the presence of co-morbidities, like diabetes, obesity, heart disease, and laboratory parameters such as elevated procalcitonin, lactate dehydrogenase, D-dimer, C-reactive protein, neutrophil, lymphocyte counts, and pro-inflammatory cytokines like interleukin-6, respectively (6,7,8). Both innate and adaptive immune responses are critical for the control of viral infections. Lymphocytes in the blood, which are an important part of the immune response, participate in

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various host defense mechanisms against viral infections. Although changes in major lymphocyte subsets (CD3, CD4, CD8, CD19, CD3(-)CD56(+)) and lymphocyte activation status have been observed in patients infected with SARS-CoV-2, the results of the studies differ from each other. Therefore, we aimed to determine lymphocyte subgroups and activation status in COVID-19 patients with flow cytometry and examined the effects of these changes on the disease stage and duration of hospital stay.

MATERIAL AND METHOD

The study was carried out with the permission of the İnönü University Faculty of Medicine Clinical Researches Ethics Committee (Date: 30.06.2020, Decision No: 101). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

Patients

The data of COVID-19 patients diagnosed with reverse transcriptase-polymerase chain reaction (PCR) at Malatya İnönü Training and Research Hospital between June 01, 2020, and July 01, 2020, were retrospectively evaluated. Informed consent from patients was provided. Forty COVID-19 patients and forty disease-free control groups were included in this study. Disease-free controls were selected from hospital staff of similar age and sex to the patient group. To assess the effect of infection on lymphocyte subgroups and activation status of each patient at a patient/control ratio of 1: 1. Patients were divided into as early and advanced stages, according to the severity of COVID 19. Age, gender, lymphocyte subgroups, and activation status of the patient and disease-free control groups were compared. The relationship between these findings of all patients and hospitalization was examined.

Patients were divided into 4 stages from early to advanced according to clinical severity. Stages 1 and 2 were considered as early-stage patients, and stages 3 and 4 were considered advanced-stage patients (9,10).

Flow Cytometry Analysis

We made the flow analyses in BD Diva software. Blood was collected in an Ethylenediaminetetraacetic acid (EDTA) tube early in the morning from all patients, and all tests were performed within 2 hours. Six-colour, three tube analyses were performed with the different fluorochromes labeled monoclonal antibodies (CD45, CD3, CD4, CD8, CD19, CD24, CD27, CD38, CD56, CD57, IgD). BNII fluorochromes were conjugated. CD3, CD4, CD8, CD19, CD27, CD38, CD45 were studied in the first tube, CD57, CD16, CD3, CD45, CD19 in the second tube, and CD24, CD19, CD45, and CD38 in the third tube. The lymphocyte gate was determined according to CD45 and side scatter intensity curve. The lymphocyte subtypes: CD3(+) (/microL), CD3(+)CD4(+) (/microL), CD3(+) CD8(+) (/microL), and CD3(+)CD4(+)/ CD3(+) CD8(+) ratio studied. Moreover, the expressions of CD27, CD57, CD38, and IgD were studied to demonstrate the activation status of T cells, and B cells. Markers indicating the activation status of the cells were expressed in percent.

Statistical Analysis

Statistical analyzes were performed using IBM SPSS v25 software. p-value ≤ 0.05 was contemplated statistically significant. Variables evaluated for normal distribution were analyzed with the Kolmogorov Smirnov test. Categorical variables were analyzed with the chi-square test. Numerical variables were compared with the Mann-Whitney U test.

Roc analysis was applied to find a cutoff point for the different morphological conditions between early and advanced patients. Spearman's rho correlation coefficient (rs) was used to investigate the relationships between quantitative variables.

RESULTS

The patients included in the study were compared with the age and sex-matched healthy controls (**Table 1**). The patients' CD3(+) CD4(+) (T helper) count (**Figure 1**) and CD27 expression on T cells counts were significantly lower (**Figure 2**), and CD57 expression on CD3(+) CD8(+) T cytotoxic (**Figure 3**) cells were significantly higher ($p<0.05$) than the control group. While the CD3(+) and CD8(+) counts of the patients were low, the difference was not statistically significant when compared control group. No statistically significant difference was found in the expression of IgD, CD27, and CD38 on CD19+ B cells, showing the activation status of B cells. Moreover, B cell count is not different between groups.

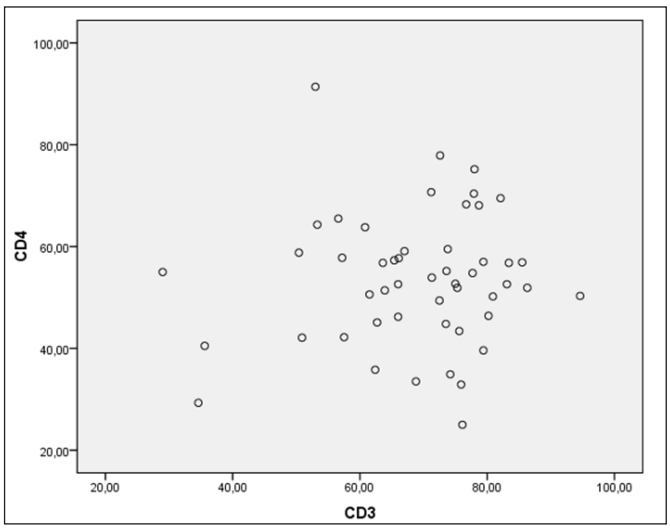

Figure 1. CD3(+)CD4(+) (T helper) count of the patient group

Table 1. Clinical features and laboratory values of all the patients.

Characteristics and laboratuvar values (Normal range)	All patients median (min-max)	Control median (min-max)	p
Total number of patients	40	40	
Median Age (year)	38 (12-88)	38.5 (21-47)	0.802
Gender			
Female (number/percent)	17 (42.5)	16 (40)	1.000
Male (number/percent)	23 (57.5)	24 (60)	
Total lymphocyte count (/microL)	1.42 (0.39-4.56)	1.83 (1.04-2.84)	0.125
CD3(+) (/microL)	0.98 (0.20-3.13)	1.34 (0.89-2.25)	0.087
CD3(+)CD4(+) (/microL)	0.58 (0.09-1.33)	0.77 (0.44-1.3)	0.039
CD3(+)CD8(+) (/microL)	0.43 (0.01-2.06)	0.52 (0.15-1.19)	0.264
Th/Ts (CD3(+)CD4(+)/ CD3(+)CD8(+))	1.19 (0.34-79)	1.34 (0.67-4)	0.264
CD3(+)CD27(+) (%)	45.5 (20.4-71.3)	59.1 (50.9-68.1)	0.001
CD3(+)CD57(+) (%)	15.35 (2.5-37.4)	11.15 (2.2-20)	0.144
CD3(+)CD38(+) (%)	29.8 (9.2-57.3)	25.75 (14.5-39.1)	0.827
CD3(+)CD8(+)CD57(+) (%)	40.8 (14.2-74.3)	30.6 (8.2-43.7)	0.030
CD3(-)CD56(+) (/microL)	0.22 (0.07-0.92)	0.26 (0.08-0.98)	0.126
CD19(+) (/microL)	0.16 (0.05-1.10)	0.25 (0.11-0.32)	0.144
CD19(+)IgD(+) (%)	8 (1.7-26.2)	7.8 (5-17)	0.765
CD19(+)CD24(+) (%)	10.3 (1.7-31.1)	10.25 (6.5-17.6)	0.896
CD19(+)CD27(+) (%)	4.05 (0.8-38.4)	4.85 (2.6-9.5)	0.369
CD19(+)CD38(+) (%)	7.8 (3-25.8)	7.35 (5.4-14.6)	0.693
Length of stay in Hospital (day)	6 (1-17)		

When the patients were divided into early and advanced stages, they were similar in terms of gender, while advanced stage patients were found to be significantly older. In lymphocyte subgroup analysis, it was observed that CD38 expression on T cells was significantly lower in advanced-stage patients ($p<0.05$) (**Table 2, Figure 4**).

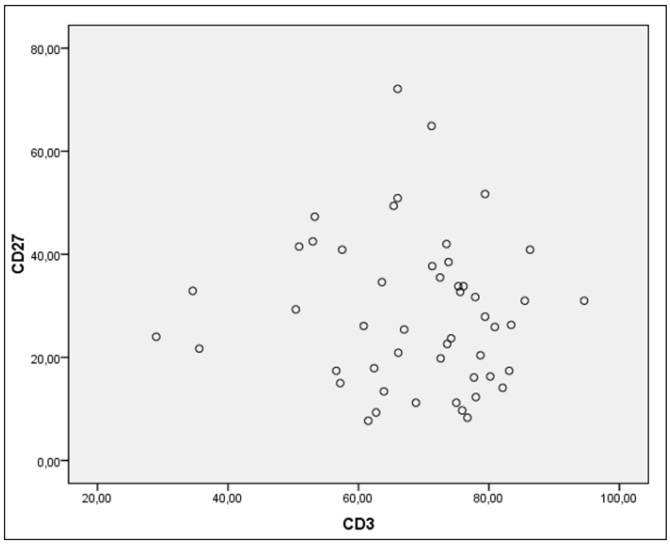

Figure 2. CD27 expression on T cells of the patient group

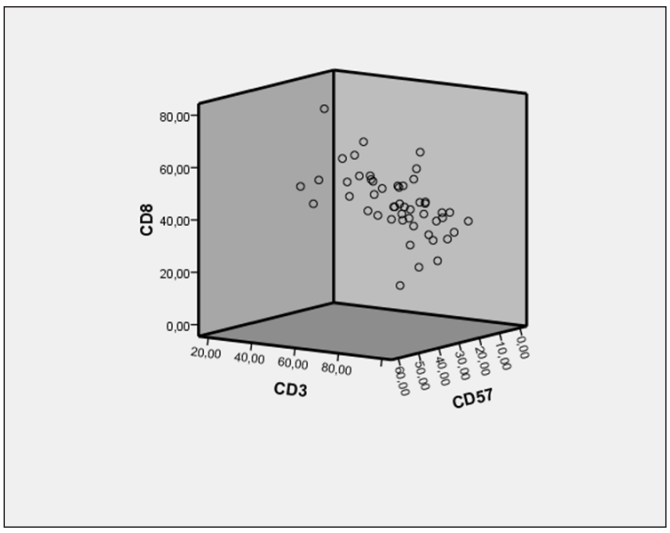

Figure 3. CD57 expression on CD3(+)CD8(+) T cytotoxic cells of the patient group

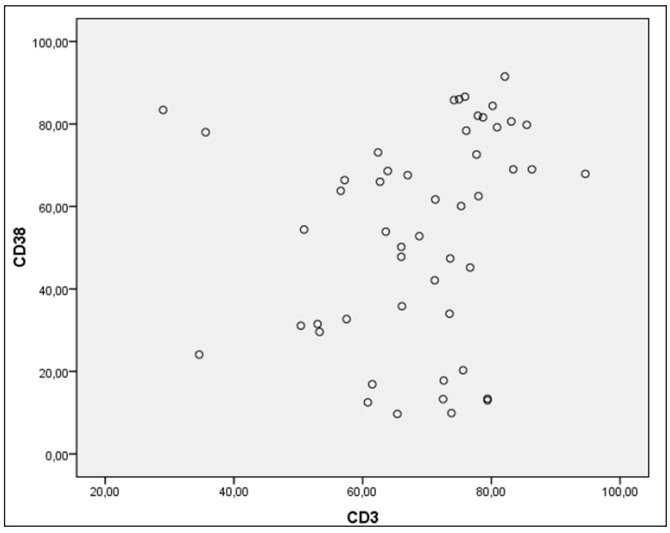

Figure 4: CD38 expression on T cells in advanced-stage patients

Table 2. Comparison of laboratory values and clinical features of mild and severe stage patients

Characteristics and laboratuvar values (Normal range)	Mild Stage Median (min-max)	Severe Stage Median (min-max)	p
Total number of patients	23	17	
Median Age (year)	28 (12-52)	58 (31-88)	<0.001
Gender			
Female (number/percent)	9 (39.1)	8 (47.1)	0.859
Male (number/percent)	14 (60.9)	9 (52.9)	
Stage (Number, (%)			
Mild			
Stage I	4 (17.4)		
Stage II	19 (82.6)		
Severe			
Stage III		15 (88.2)	
Stage IV		2 (11.8)	
Total lymphocyte count (/microL)	1.38 (0.79-4.56)	1.44 (0.39-2.32)	0.342
CD3(+) (/microL)	1.03 (0.26-3.13)	0.96 (0.20-1.74)	0.191
CD3(+)CD4(+) (/microL)	0.64 (0.14-1.33)	0.54 (0.09-1.19)	0.149
CD3(+)CD8(+)(/microL)	0.46 (0.10-2.06)	0.35 (0.01-0.81)	0.401
Th/Ts (CD3(+)CD4(+)/ CD3(+)CD8(+))	1.11 (0.34-2.43)	1.40 (0.36-79)	0.120
CD3(+)CD27(+) (%)	52.6 (20.4-71.3)	41.2 (25.5-67.6)	0.191
CD3(+)CD57(+) (%)	15.1 (5-37.4)	15.6 (2.5-35.7)	0.607
CD3(+)CD38(+) (%)	37.2 (10.2-57.3)	22.95 (9.2-40.4)	0.007
CD3(+)CD8(+)CD57(+) (%)	39 (15.7-74.3)	42.6 (14.2-72)	0.957
CD3(-)CD56(+) (/microL)	0.22 (0.07-0.92)	0.21 (0.09-0.45)	0.967
CD19(+) (/microL)	0.16 (0.05-1.10)	0.14 (0.05-0.55)	0.342
CD19(+)IgD(+) (%)	8 (3.3-18.9)	8 (1.7-26.2)	0.892
CD19(+)CD24(+) (%)	10.5 (5.3-28.2)	9.2 (1.7-31.1)	0.315
CD19(+)CD27(+) (%)	4.2 (0.8-27.2)	3.5 (1-38.4)	0.464
CD19(+)CD38(+) (%)	8.7 (4.6-24.2)	6.6 (3-25.8)	0.058

Total lymphocyte count and CD3(+) T lymphocyte count were negatively correlated with the length of hospital stay as statistically significant ($p<0.05$). The strength of the relationships between hospitalization days with lymphocyte count and CD3(+) T were found to be weak but close to moderate (**Table 3, Figure 5**).

Table 3. Relationship of patients' laboratory parameters with the length of stay

		All patients (n=40)
Lymphocyte	rs	-0.395
	p	0.012
CD3 (+) (/microL)	rs	-0.358
	p	0.023
CD3 (+) CD4 (+) (/microL)	rs	-0.305
	p	0.056
CD3 (+) CD8 (+) (/microL)	rs	-0.184
	p	0.255
Th / Ts	rs	-0.218
	p	0.176
CD3 (+) CD27 (+)	rs	-0.062
	p	0.706
CD3 (-) CD56 (+)	rs	-0.018
	p	0.936
CD3 (+) CD57 (+)	rs	0.096
	p	0.556
CD3 (+) CD38 (+)	rs	-0.226
	p	0.257
CD3 (+) CD8 (+) CD57(+)	rs	0.137
	p	0.399
CD19 (+) (/microL)	rs	-0.083
	p	0.611
CD19 (+) IgD (+)	rs	0.169
	p	0.297
CD19(+) IgM(+)	rs	-0.155
	p	0.490
CD19(+) CD24 (+)	rs	0.008
	p	0.960
CD19 (+) CD27 (+)	rs	0.095
	p	0.560
CD19 (+) CD38 (+)	rs	0.037
	p	0.821

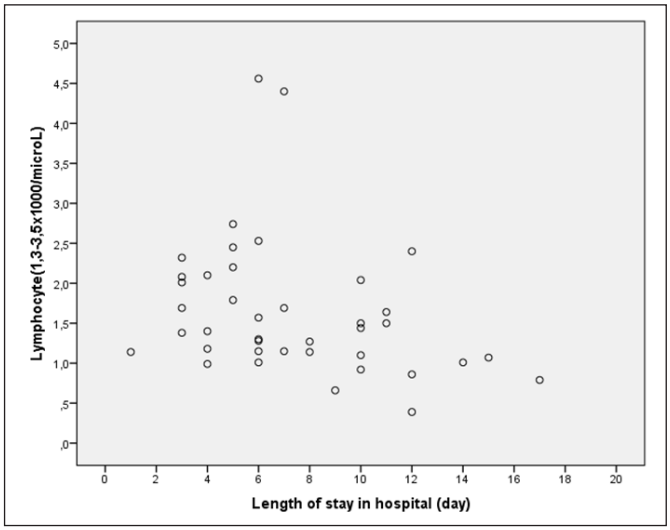

Figure 5A. Relationship between total lymphocyte count and length of hospital stay.

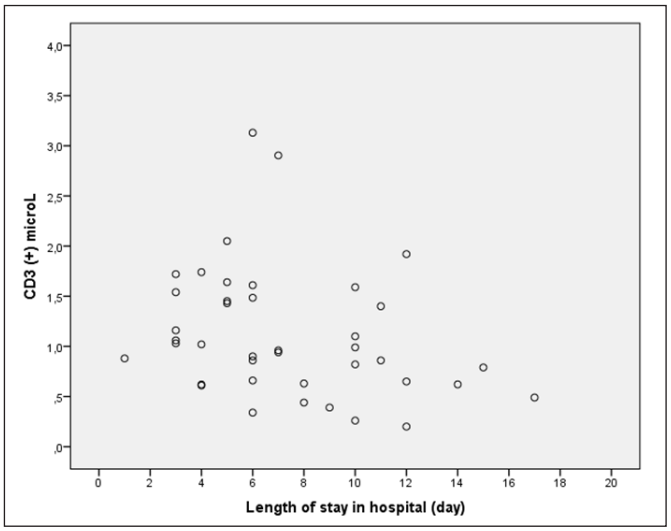

Figure 5B. Relationship between CD3 (+) lymphocyte count and length of hospital stay**.**

DISCUSSION

Our study showed that T helper count and CD27 expression on T cells are significantly decreased, and CD57 expression on cytotoxic T cells is significantly increased in COVID-19 patients at the time of diagnosis ($p < 0.05$).

T helper (CD3(+)CD4(+)) cells are the mediator cells in the immune response. They proliferate and release cytokines that regulate or assist effector lymphocyte function when activated. They are one of the targets of many viral infections and the reduction in CD4+ T cells facilitates the emergence of virus-related diseases (11). In our study, T helper counts were significantly decreased in the patient group. While CD27 was highly expressed in central memory and stem cell memory cells, its expression was decreased in effector T cells (12). In our study, it was observed that the CD27 expression on T cells was significantly decreased in the patient group. The CD57 antigen is routinely used to identify terminally differentiated 'senescent' cells with reduced proliferative capacity and altered functional properties (13). We also found that CD57 expression on cytotoxic T cells increased significantly in the patient group.

When the patients were divided into early and advanced stages, the CD38 on T cells was found to be significantly lower in an advanced stage. CD38 is expressed at a low rate in mature naive T lymphocytes, but CD38 expression in T lymphocytes is upregulated with mitogenic activation (14). This finding may suggest that T lymphocytes may lose their active state as the COVID-19 infection progresses. The total lymphocyte count and CD3(+) T cell count of the patients were negatively correlated with the length of hospital stay (15). When this result is evaluated together with the other results obtained from our study, it shows that the COVID-19 virus may change the number and activation status of T lymphocytes, especially in the advanced stage.

Jiang et al. (15) showed the count and immune status of lymphocytes by flow cytometry in 32 COVID-19 patients and 18 healthy individuals. They observed total T (CD3(+)), CD3(+)CD8(+) and NK (CD3(-) CD56(+)) cells in COVID-19 patients decreased significantly as compared with disease-free group. Similarly, we have observed that T cells decreased in patients. Jiang et al. (15) showed that a sustained decrease of total T and NK cells in the critical group was observed, and CD8+ T cell count in the critical group was significantly decreased as compared with healthy individuals and the early group. In our study, when compared with the early disease group, the total T and NK cell number was lower in patients with advanced disease, although it was not statistically significant. Nonetheless, no significant difference in CD3(+) CD4(+) and B cell (CD19(+)) count was observed between patients and disease-free individuals in the same study (15).

Kazancioglu et al. (16) reported that B, T lymphocytes, and NK and natural killer T (NKT) cells were found to be decreased in patients with advanced COVID-19. Wang et al. (17) showed that CD4+ and CD8+ T cells, B cells, and NK cells decreased in COVID-19 patients, and advanced cases had lower total lymphocytes than early cases. In our study, a significant decrease was not observed in the B lymphocyte count, but there was a statistically significant decrease in the CD4(+) T lymphocyte counts. Jiang et al. (15) found that circulating CD8(+) T cells from COVID-19 patients had higher expression of CD38 compared to healthy individuals, while the expression of CD38 in CD8(+) T cells was not significantly different among the severity of the disease. In our study, we studied CD38 on total T lymphocytes (CD3(+)). Although it was not statistically significant CD38 counts were higher in the patient group compared to the control group. In addition, the level of CD3(+) CD38(+) was interpreted as significantly higher in early patients.

Almeida et al. (18) reported that patients who received antiretroviral therapy, those with high CD 38 expression in CD8 and CD4 T lymphocytes measured from peripheral blood after 1-year treatment, had a better response to treatment. When evaluated together with the data of this study, the high mortality reported in the literature in advanced-stage patients can be explained by the low level of CD38 in T lymphocytes in these advanced-stage patients. Kang et al. (19) compared cell-mediated immune responses between advanced and early COVID-19 cases. They also examined frequencies of CD38 as makers of activated T cells. Although CD4(+) CD38(+) and CD8(+) CD38(+) tended to be higher in the patient groups than in the healthy control group, but not statistically significant. We could not find any significant difference in flow cytometry evaluations in early and advanced-stage patients,

except for lower CD3(+) CD38(+) levels in early patients at the time of diagnosis. Mazzoni et al. (20) reported that CD3(+), and CD19(+) cell counts were significantly lower in COVID-19 patients than in healthy subjects. In addition, among CD3(+) cells, they found a significant reduction of CD4(+), and CD8(+) cells in COVID-19 patients. In our study, the number of CD3(+), CD19(+), CD3(+)CD4(+), CD3(+) CD8(+) cells decreased compared to the healthy group, but this decrease was only observed significantly in CD3(+)CD4(+) cells. Also, they showed that the CD4(+)/ CD8(+) T cell ratio in COVID19 patients was significantly higher than in healthy subjects.

Kazancioglu et al. (16) observed that the CD4+/CD8+ ratio was not significantly different between patients with COVID-19 and healthy controls. Wang et al. (17) found that CD4+ /CD8+ ratio showed no significant association with the severity of the disease. No statistically significant difference was found between the patient and the healthy group in the CD4(+)/CD8(+) T cell ratio in our study. On the other side, in our study CD57(+) expression on cytotoxic T cells was significantly higher in COVID-19 patients when compared to healthy subjects. Huang et al. (21) reported that CD4(+) T cell, CD8(+) T cell, B cell, NK cell, and total lymphocyte cell counts statistically significant were lower in patients with advanced/critical COVID-19 than in early/moderate disease. Although it was not statistically significant in our study, the number of these cells was found to be low in advanced-stage patients.

Jiang et al. (17) reported that in receiver operator characteristics (ROC) curve analysis, total lymphocytes and CD3(+) lymphocytes also had good value in distinguishing critical COVID19 patients with a total area under the curve (AUC) of 0.865 and 0.826, respectively. Wang et al reported that CD8+ T cells tended to be an independent predictor for COVID-19 severity and treatment efficacy. In our study, there was a significant negative correlation between total lymphocyte and total T lymphocyte count and length of hospital stay by the data of this study. In some studies, multivariate analysis has shown that Th/Ts (CD3(+) CD4(+)/ CD3(+)CD8(+)) are independent predictors of patient outcomes (21). In our study, the Th / Ts ratio was not statistically significant when evaluated together with the length of hospital stay.

The strengths of our study are that at the time of diagnosis, blood samples of all patients were taken before any COVID-19 treatment and the flow cytometry study was performed by the same person. The limitations of our study are the small patient group included in the study, the inability to associate the data with disease mortality due to this small patient group, and the inability to monitor cell number and activation status with intermittent evaluations during patient follow-up.

CONCLUSION

The evaluated data showed that the SARS-CoV-2 primarily affects T lymphocytes. It was thought that this effect occurred by impairment of development and activation of T lymphocytes. There are some discordances among these studies on T lymphocytes in the literature. Studies with more patients are needed to make this information more reliable.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of the İnönü University Faculty of Medicine Clinical Researches Ethics Committee (Date: 30.06.2020, Decision No: 101).

Informed Consent: Because the study was designed retrospectively, no written informed consent form was obtained from patients.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version

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