Official Journal of Erciyes University Faculty of Medicine

DOI: 10.14744/cpr.2024.14622
J Clin Pract Res 2024;46(1):67–75

Responses of Helper T Cell Subsets at Diagnosis and After Discharge in Patients with Non-Severe COVID-19: A Prospective Observational Study

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Cite this article as:
Karaselek MA, Duran T, Kuccukturk S, Vatansev H. Responses of Helper T Cell Subsets at Diagnosis and After Discharge in Patients with Non-Severe COVID-19: A Prospective Observational Study. J Clin Pract Res 2024; 46(1): 67–75.

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Submitted: 22.11.2023
Revised: 01.01.2024
Accepted: 07.02.2024
Available Online: 16.02.2024

Erciyes University Faculty of Medicine Publications - Available online at www.jcpres.com

ABSTRACT

Objective: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus responsible for Coronavirus Disease 2019 (COVID-19), elicits a strong immune response similar to that seen in other viral infections. The predominant cell type in this immune response also influences the disease prognosis. This study, conducted between 2022 and May 2023, aimed to evaluate the transcription factors and cytokine expressions of Th (helper T) cell subsets at the time of diagnosis and after discharge in patients with non-severe COVID-19.

Materials and Methods: Forty-eight patients with non-severe COVID-19 were included in the study. Transcription factor and cytokine expressions of Th cell subsets were evaluated using the quantitative polymerase chain reaction (qPCR) method, and the results were compared at the time of diagnosis and after discharge.

Results: It was determined that the cytokines and transcription factors of T helper 1 (Th1) cells (T-box expressed in T cells [T-bet], 2.71-fold, p<0.001; Interferon-gamma [IFN-γ], 1.42-fold, p=0.010) and T helper 17 (Th17) cells (RAR-related orphan receptor gamma [RORγt], 1.06-fold, p=0.946; Interleukin-22 [IL-22], 1.01-fold, p=0.599) decreased, whereas the expression of T helper 2 (Th2) cells (GATA binding protein 3 [GATA3], 2.56-fold, p<0.001; Interleukin-4 [IL-4], 1.34-fold, p=0.012; Interleukin-5 [IL-5], 1.02-fold, p=0.649; Interleukin-13 [IL-13], 2.06-fold, p=0.0119) and regulatory T (Treg) cells (Forkhead box P3 [FoxP3], 3.56-fold, p<0.001; Transforming growth factor-beta [TGF-β], 1.03-fold, p=0.670; Interleukin-10 [IL-10], 1.40-fold, p=0.010) increased.

Conclusion: Our study in non-severe COVID-19 patients demonstrated significant changes in the transcription factor and cytokine expressions of Th cell subsets at the time of diagnosis compared to discharge. We think that even if the patients do not exhibit severe clinical and laboratory findings, Th cell immune responses may be strong, warranting careful consideration.

Keywords: COVID-19, Th1, Th2, Th17, Treg.

INTRODUCTION

At the end of 2019, a disease caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) beta coronavirus emerged, leading to a pandemic. This disease has been named COVID-19 (Coronavirus Disease 2019).[1] While patients with a non-severe form of COVID-19 may experience non-serious clinical symptoms such as fever, muscle pain, and cough, severe cases can lead to Acute Respiratory Distress Syndrome (ARDS) as well as liver, heart, and kidney damage.[2] ARDS, especially in severe COVID-19 patients, is closely related to Cytokine Release Syndrome (CRS), characterized by high levels of proinflammatory cytokines and chemokines in the plasma.[3]

Most COVID-19 patients exhibit decreased absolute levels of B, helper T (Th) cells, cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and lymphopenia, although an increase in monocytes and granulocytes has been reported.[4] In addition to low total T cell counts, decreased levels of Th subsets and CTLs have been associated with the severity of the infection and the need for intensive care.[5] T helper 1 (Th1), T helper 2 (Th2), and T helper 17 (Th17), the major subsets of Th cells, coordinate immune responses. Th1 cells aid in the activation of CTLs and NK cells through interleukin-2 (IL-2) and interferon-gamma (IFN-γ). Th2 cells, in addition to coordinating humoral immunity, activate basophils, eosinophils, and mast cells through cytokines (IL-4 and IL-6).[6] It is believed that dominance by Th1 cells is associated with a good prognosis, while Th2 cell dominance is linked to a poor prognosis in COVID-19 patients.[7] The role of Th17 cells in COVID-19 is still uncertain, indicating that further research is necessary.[8] Although studies in COVID-19 patients have reported increased and/or decreased regulatory T (Treg) cells, the situation with these cells is not clear.[9–11] Therefore, evaluating Th cell subsets and the cytokines they secrete from these subsets is important for understanding COVID-19 and resolving confusion in the literature.

Studies of COVID-19 and Th subsets in the literature generally consist of research conducted using flow cytometric analysis in patients with severe COVID-19. The transcription factors of these cells and the cytokines of the microenvironment play a crucial role in the functioning of Th cells by secreting cytokines at an optimal level. Thus, this study aimed to evaluate the major cytokines and transcription factor expressions of Th cells at the time of diagnosis and after discharge in non-severe COVID-19 patients.

MATERIALS AND METHODS

Patients and Data Collection

This study was conducted prospectively at Necmettin Erbakan University, Faculty of Medicine, Department of Chest Diseases in May 2023. The studies reported herein were approved by the Necmettin Erbakan University Scientific Research Ethics Committee (27.01.2023, 2023/035). Written informed consent was obtained from all participants. Forty-eight COVID-19 patients (36 males and 12 females) who were non-severe were included in the study. The median age of all patients was 56.56±18.68 years (males: 58±21.64; females: 58.42±18.10). The flowchart of the study is shown in Figure 1.

All patients had SARS-CoV-2 positivity confirmed by the quantitative polymerase chain reaction (qPCR) method at the time of diagnosis. Patients without SARS-CoV-2 positivity confirmed by the qPCR method were excluded from the study. Biochemical (C-reactive protein (CRP), lactate dehydrogenase (LDH), ferritin, lactate, amylase, lipase, procalcitonin) hematological (absolute lymphocyte count (ALC)), and coagulation tests (D-dimer, fibrinogen) were conducted on all patients. Additionally, these tests were repeated after treatment. The treatment of the patients was carried out in accordance with the Ministry of Health's Adult Patient Treatment Guide. Using the qPCR method, blood samples were evaluated for transcription factors and cytokine expressions of Th cell subsets [Th1 (T-bet, Signal Transducer and Activator of Transcription 1 (STAT1), IFN-γ), Th2 (STAT6, GATA Binding Protein 3 (GATA3), IL-4, IL-5, IL-13), Th17 (RAR-related orphan receptor gamma (RORγt), STAT3, IL-17, IL-21, IL-22, IL-6) and Treg (Forkhead box P3 (FoxP3), STAT5, IL-10, Transforming Growth Factor-beta (TGF-β))]. Blood samples were collected into Potassium Ethylenediaminetetraacetic Acid (K3-EDTA) tubes during routine examinations.

Ribonucleic Acid (RNA) Isolation, Complementary Deoxyribonucleic Acid (cDNA) Synthesis, and qPCR Analysis

Lymphocytes were isolated using Ficoll-Hypaque (Sigma–Aldrich, Steinheim, Germany) for total RNA isolation. Subsequently, the cells were washed with Roswell Park Memorial Institute (RPMI) medium (Sigma–Aldrich, Steinheim, Germany) and carefully resuspended in 500 µl of QIAzol (Qiagen, India). cDNA synthesis was performed according to the procedure using the cDNA Synthesis Kit (A.B.T.™, Türkiye).

Primers for the genes to be analyzed by qPCR were designed using Integrated DNA Technologies (IDT) Primer Quest (Appendix 1). The qPCR reaction was performed using SYBR Green Master Mix (Hibrigen, 2x SYBR Green Master Mix) with the QuantStudio 3 qPCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The qPCR mix components and PCR profile are depicted in Figure 1.

Statistical Analysis

For normalization, the Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) housekeeping gene was used. Gene

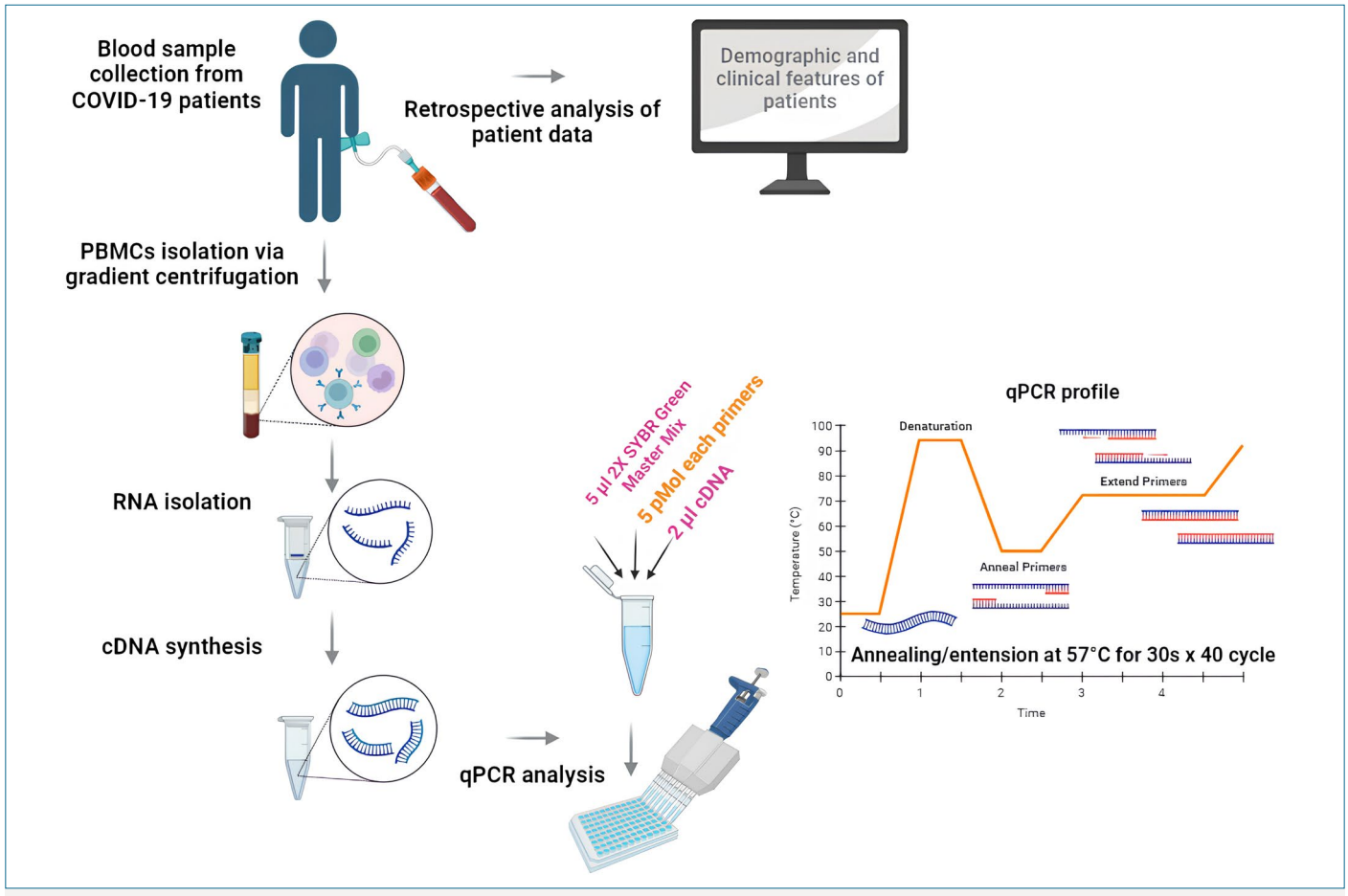


Figure 1. The flowchart of the study.

expression was analyzed using the Comparative Livak's $2^{-\Delta\Delta CT}$ method. After normalization, ΔCt values were employed in the analyses. Comparisons of gene expression before and after treatment were conducted using the paired t-test. VolcaNoseR was utilized to generate volcano plots that display only unique and/or up-regulated features. Correlation analysis between Th cell transcription factors and cytokines was performed using Pearson Correlation. Laboratory parameters of the patients included in the study at diagnosis and after treatment were analyzed with the Wilcoxon Signed Rank Test. Data are presented as the median and lower/upper quartile, [median (Q1–Q3)]. Tests were considered significant at a baseline level of $p<0.05$. All analyses were performed with the Statistical Package for the Social Sciences software, version 21 (IBM SPSS Corp.; Armonk, NY, USA).

RESULTS

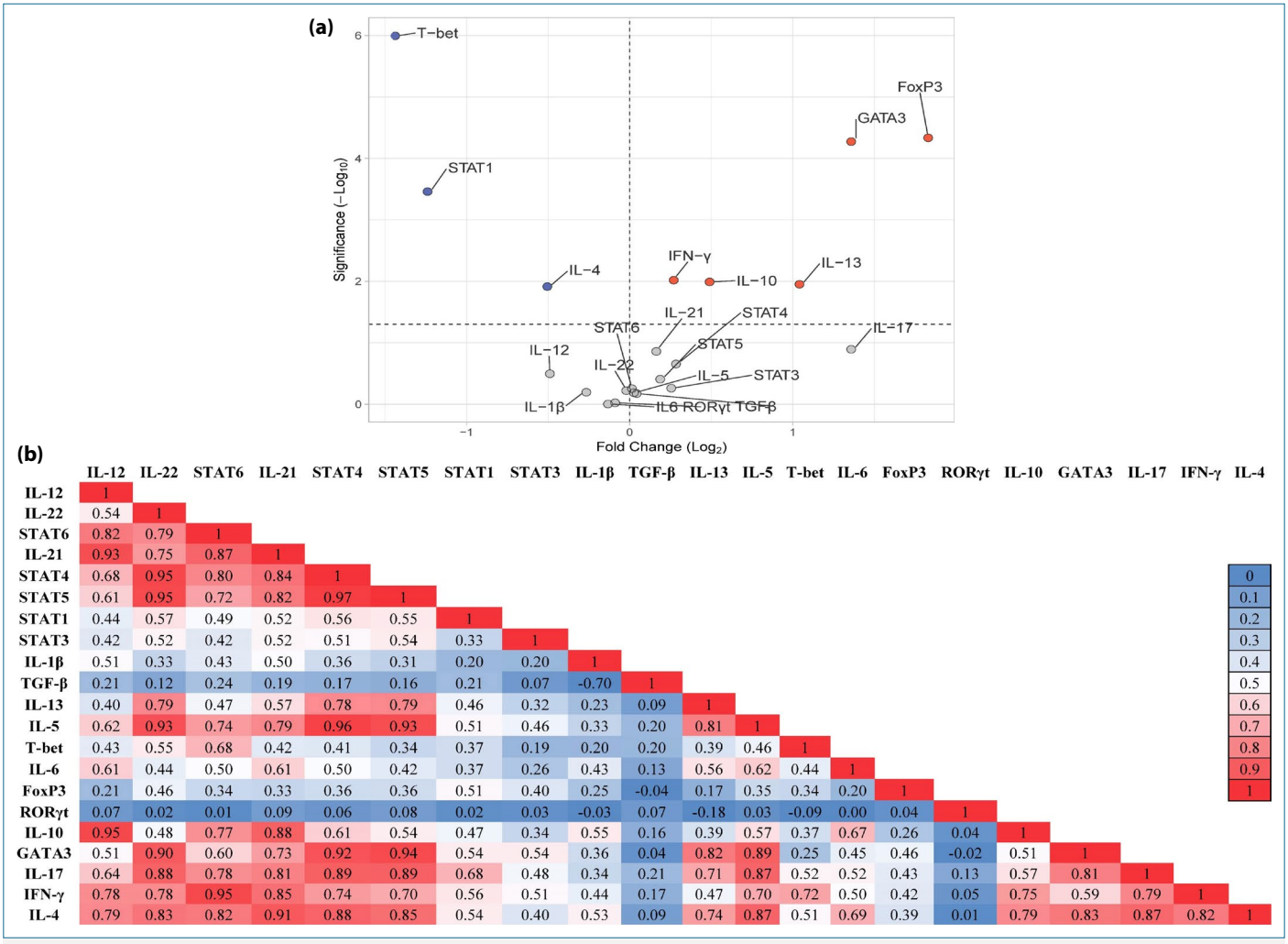
Clinical and Laboratory Results of Patients

The oxygen saturation of the patients was >88% on room air, and none required invasive or non-invasive mechanical ventilation support. In lung chest X-ray findings, patients exhibited mild or moderate lung involvement. Sixteen patients had comorbidities, primarily diabetes (n=11) and hypertension (n=6). Additionally, two patients had laryngeal cancer, and two had chronic obstructive pulmonary disease.

The routine laboratory analyses of the patients before (at diagnosis) and after treatment are presented in Table 1. At diagnosis, patients exhibited lymphopenia and increased values of amylase/lipase, ferritin, procalcitonin, CRP, fibrinogen, and LDH. Changes in parameters, other than lactate and D-dimer, between diagnosis and discharge were statistically significant (Table 1). Based on the clinical and laboratory findings, patients were evaluated as having non-severe COVID-19. All patients in the study tested negative for SARS-CoV-2 by qPCR at the time of discharge.

Transcription Factor and Cytokine Analysis of Th Subsets

The major transcription factor of the Th1 cell, T-bet, and the major cytokine of the Th1 cell, IFN-γ, were found to be



	IL-12	IL-22	STAT6	IL-21	STAT4	STAT5	STAT1	STAT3	IL-1β	TGF-β	IL-13	IL-5	T-bet	IL-6	FoxP3	RORγt	IL-10	GATA3	IL-17	IFN-γ	IL-4
IL-12	1																				
IL-22	0.54	1																			
STAT6	0.82	0.79	1																		
IL-21	0.93	0.75	0.87	1																	
STAT4	0.68	0.95	0.80	0.84	1																
STAT5	0.61	0.95	0.72	0.82	0.97	1															
STAT1	0.44	0.57	0.49	0.52	0.56	0.55	1														
STAT3	0.42	0.52	0.42	0.52	0.51	0.54	0.33	1													
IL-1β	0.51	0.33	0.43	0.50	0.36	0.31	0.20	0.20	1												
TGF-β	0.21	0.12	0.24	0.19	0.17	0.16	0.21	0.07	-0.70	1											
IL-13	0.40	0.79	0.47	0.57	0.78	0.79	0.46	0.32	0.23	0.09	1										
IL-5	0.62	0.93	0.74	0.79	0.96	0.93	0.51	0.46	0.33	0.20	0.81	1									
T-bet	0.43	0.55	0.68	0.42	0.41	0.34	0.37	0.19	0.20	0.20	0.39	0.46	1								
IL-6	0.61	0.44	0.50	0.61	0.50	0.42	0.37	0.26	0.43	0.13	0.56	0.62	0.44	1							
FoxP3	0.21	0.46	0.34	0.33	0.36	0.36	0.51	0.40	0.25	-0.04	0.17	0.35	0.34	0.20	1						
RORγt	0.07	0.02	0.01	0.09	0.06	0.08	0.02	0.03	-0.03	0.07	-0.18	0.03	-0.09	0.00	0.04	1					
IL-10	0.95	0.48	0.77	0.88	0.61	0.54	0.47	0.34	0.55	0.16	0.39	0.57	0.37	0.67	0.26	0.04	1				
GATA3	0.51	0.90	0.60	0.73	0.92	0.94	0.54	0.54	0.36	0.04	0.82	0.89	0.25	0.45	0.46	-0.02	0.51	1			
IL-17	0.64	0.88	0.78	0.81	0.89	0.89	0.68	0.48	0.34	0.21	0.71	0.87	0.52	0.52	0.43	0.13	0.57	0.81	1		
IFN-γ	0.78	0.78	0.95	0.85	0.74	0.70	0.56	0.51	0.44	0.17	0.47	0.70	0.72	0.50	0.42	0.05	0.75	0.59	0.79	1	
IL-4	0.79	0.83	0.82	0.91	0.88	0.85	0.54	0.40	0.53	0.09	0.74	0.87	0.51	0.69	0.39	0.01	0.79	0.83	0.87	0.82	1

Figure 2. (a) Changes in the expression of genes involved in Th cell subsets at diagnosis and after discharge. **(b)** Correlation analysis of changes in transcription factor and cytokine expressions of Th cell subsets at diagnosis and discharge. (Red dots represent significantly increased expression. In the volcano plots, the right side of the "0" point on the fold-change (Log2) axis signifies increased expression, and the left side indicates decreased expression. A value of "1.3" on the significance (-Log10) axis denotes p<0.05 above this line and p>0.05 below it).

down-regulated at diagnosis compared to after treatment (2.71-fold and 1.42-fold, respectively), with these reductions being statistically significant (p<0.001 and p=0.010, respectively). STAT1 expression was also down-regulated and statistically significant after comparison (2.36-fold, p<0.001). It was observed that the major transcription factor of the Th2 cell, GATA3, and Th2 cytokines IL-4, IL-5, and IL-13 were upregulated at diagnosis (2.56-fold, 1.34-fold, 1.02-fold, and 2.06-fold, respectively), with these expressions being statistically significant except for IL-5 (p<0.001, p=0.012, p=0.649, and p=0.011, respectively). Although STAT6 expression was upregulated, it was not statistically significant (1.01-fold, p=0.556). RORγt, the transcription factor of the Th17 cell, and IL-22, a Th17 cell cytokine, were down-regulated after treatment (1.06-fold, p=0.946; 1.01-fold, p=0.599, respectively). IL-17 and IL-21 expressions were upregulated (1.21-fold, p=0.137; 1.12-fold, p=0.127, respectively). Expression changes in Th17 cells were not statistically significant. STAT3 expression was upregulated, but this was not statistically significant (1.19-fold, p=0.546). Treg cell transcription factor FoxP3 (3.56-fold, p<0.001), TGF-β (1.03-fold, p=0.670), and IL-10 (1.40-fold, p=0.010), secreted by Treg cells, were found to be upregulated before treatment. The increase in FoxP3 and IL-10 expression was statistically significant. STAT5 expression was upregulated, but it was not statistically significant (1.14-fold, p=0.389) (Fig. 2a).

Table 1. Laboratory parameters before and after hospitalization (Q1: 25th percentiles; Q3: 75th percentiles)

Laboratory parameters	At the time of diagnosis Median (Q1–Q3) (n=48)	At the time of discharge Median (Q1–Q3) (n=48)	p
Lymphocyte (10^3/mL)	1.50 (1–2.15)	2.17 (1.98–2.56)	0.005
Lactate (g/L)	1.9 (1.6–2.6)	2.26 (1.8–2.26)	0.917
Amylase (g/L)	53 (45.6–60)	81 (60–81)	<0.001
Lipase (g/L)	28 (23–30)	80 (65–80)	<0.001
LDH (g/L)	238 (183–279)	190 (181–195)	0.002
Procalcitonin	0.08 (0.07–0.08)	0.05 (0.05–0.09)	0.028
CRP (mg/L)	24.62 (7.76–71.86)	10 (3.36–15)	0.003
Ferritin (ng/mL)	100.7 (39.78–241)	220 (185.9–220)	0.023
D-dimer (ng/mL)	141 (85–449)	200 (98–200)	0.191
Fibrinogen	436 (311–485)	300 (287–300)	0.001

Q: Quartile; LDH: Lactate dehydrogenase; CRP: C-reactive protein.

Correlation analysis was performed on the changes in Th1, Th2, Th17, and Treg cell transcription factor and cytokine expressions at the time of diagnosis and discharge (Fig. 2b). There was a strong correlation between IFN-γ and T-bet and STAT4 (r=0.719, p<0.001; r=0.555, p=0.005, respectively). There was a strong correlation between GATA3 and IL-4 and IL-5 (r=0.828, p<0.001; r=0.890, p<0.001 respectively), and a moderate correlation with IL-13 (r=0.828, p<0.001). There was a moderate correlation between IL-17 and IL-6 (r=0.524; p=0.009). IL-17, IL-21, and IL-22 were moderately correlated with STAT3 (r=0.475, p=0.019; r=0.518, p=0.009; r=0.522, p=0.009, respectively). IL-10 was strongly correlated with FoxP3 and STAT3 (r=0.663, p=0.013; r=0.539, p=0.007, respectively).

DISCUSSION

In this study, the expression of transcription factors and cytokines of Th cell subsets was investigated molecularly in non-severe COVID-19 patients, and the expression results were compared at the time of diagnosis and discharge. Our study, conducted with non-severe COVID-19 patients, revealed that Th cell responses were molecularly similar to those in severe COVID-19 patients.

Th1 cells, which produce IFN-γ, are very important for viral infections. Although there are studies involving Th1 cells, especially in severe COVID-19 patients, the situation with Th1 cells is not clear. In the initial immune response to COVID-19 disease, the proliferation of SARS-CoV-2 spike protein-specific T cells increases, and IFN-γ is produced by these cells.[12] These cytokines secreted by Th1 cells are aimed at controlling the infection through macrophages and CTLs.[13] In patients with severe COVID-19, increased serum levels of IL-12 cytokine, which plays an important role in Th1 differentiation, have been impaired, and elevated levels of IFN-γ have been detected.[14,15] In our study, expressions of IL-12 and IFN-γ were found to be lower at the time of diagnosis compared to discharge. Since the patients included in the study were non-severe COVID-19 cases, it was hypothesized that the low expression of IL-12 was due to the low viral load. The IFN-γ level was also found to be low, due to the low level of IL-12. T-bet, the main regulatory transcription factor of Th1 cells, is activated by STAT4.[16] Decreased expressions of T-bet and STAT4, along with the low expressions of IL-12 and IFN-γ detected in our study, support this coordination in Th1 cells.

Differentiation of Th cells towards Th2 is controlled by the STAT6-activated transcription factor, GATA3. Upon its activation, IL-4, IL-5, and IL-13 are produced by Th2 cells.15 It has been reported that levels of IL-4, IL-5, IL-13, and IFN-γ are increased in patients diagnosed with COVID-19 pneumonia.[17] In a study involving severe COVID-19 patients, only a marginal increase in serum IFN-γ level was detected, and no change in Th2 cell cytokines was reported.[18] Generally, although Th2 cell responses are dominant in severe COVID-19 patients and associated with poor prognosis, our study revealed that Th2 cell responses were also predominant in mild COVID-19 cases, indicating a non-poor prognosis. Although Th2 responses were dominant in severe COVID-19 patients, our study revealed that Th2 responses were also predominant in non-severe patients. Considering the literature data and our findings, it is noteworthy that Th1 and Th2 responses vary in COVID-19. However, current data suggest that the balance between Th1 and Th2 cell responses in COVID-19 is closely related to the outcome of the

disease. If the coordination of the immune response during infection is disrupted and the Th response shifts towards Th2, poor prognosis and CRS may occur.[7,19] Therefore, although the mechanisms of Th cell differentiation in COVID-19 patients are not fully understood, it is evident that the dominant Th cell profile plays an important role in the prognosis of COVID-19.

IL-17, IL-21, and IL-22 cytokines are secreted by Th17 cells, and these cytokines recruit neutrophils and monocytes to the site of infection. Furthermore, Th17 cells play a crucial role in the pathogenesis of autoimmunity.[16] Th17 cell differentiation is controlled by the STAT3-activated transcription factor RORγt.[16] Recent studies have suggested that Th17 cells may largely contribute to lung damage in COVID-19 patients with severe lung involvement.[20,21] In our study, cytokines and transcription factors related to Th17 cell were found to be low in non-severe COVID-19 patients at the time of diagnosis. Contrary to our findings, Th17 cells are elevated in patients with lung involvement. Although there is evidence in the literature indicating dominant Th17 responses in severe COVID-19 patients, the findings of our study support this hypothesis. Additionally, an increase in Th17 cells in the peripheral blood was reported using flow cytometry in severe COVID-19 patients compared to non-severe patients. Therefore, it is suggested that the use of therapeutic agents targeting the IL-17 cytokine, secreted by Th17 cells, may be beneficial in preventing ARDS.[12,17,22,23] However, it is emphasized that more research is needed in this area. The patients in our study did not present with ARDS, and Th17 cytokines were low in these patients. Hence, our findings support the hypothesis of a link between Th17 and the development of ARDS. Th17 responses are supported by IL-6 and IL-1β. It has been reported in the literature that levels of IL-1β and IL-6 are elevated in severe patients compared to non-severe patients.[24] In our study, expressions of IL-1β and IL-6 were found to be low at the time of diagnosis, which aligns with the literature.

Treg cells are a subset of Th cells that inhibit pathogenic activity and maintain immune homeostasis.[25] Treg cells are generally identified based on the expression of FoxP3. Therefore, determining FoxP3 expression provides important insights into Treg cells. In studies involving COVID-19 and Treg cells, it was reported that the Treg population increased during active infection.[26] Conversely, the literature suggests that in severe COVID-19 patients, Treg cells are suppressed.10 Although there are variable findings in a limited number of studies, it is clear that more detailed research on this topic is needed. In our study, the expression of FoxP3, evaluated at the time of diagnosis, was found to be higher compared to discharge. Given the functions of Treg cells, high FoxP3 expression at diagnosis appears logical in terms of preventing the worsening of the patients' clinical condition by providing immune regulation. Inhibition of FoxP3 usually leads to inflammation, which is undesirable during infection. Moreover, suppression of FoxP3 expression results in ineffective T cell responses and adverse outcomes such as immune-mediated tissue damage.[25,27,28] It has also been reported that disruption in FoxP3 expression in severe COVID-19 patients leads to autoimmune-like aberrant T cell responses to self-antigens.[29] Hypotheses in the literature suggest that either Th17 cells and/or Treg cells are responsible for worsening the disease state. The results of our study indicate that FoxP3 expressions are high in non-severe COVID-19 patients. When it is acknowledged that FoxP3 expressions are high in severe patients according to the literature, it supports the hypothesis that Treg cells may be responsible for the exacerbation of the disease. Therefore, determining FoxP3 expression in patients, whose disease severity is considered to be worsening based on laboratory and clinical findings, may serve as an important indicator for preventing the progression of disease severity.

Although the mechanism behind the decrease in FoxP3 expression in severe COVID-19 patients has not been fully elucidated, IL-6 is thought to contribute to this reduction.[30] The decreased IL-6 expression detected in our study supports this hypothesis. Treg cell suppressive functions are mediated by IL-10 and TGF-β.10 Our study found that IL-10 and TGF-β expressions were high, which aligns with this understanding. The elevated IL-10 expression at diagnosis, which suppresses T cell proliferation, could explain the lymphopenia observed in COVID-19 patients.

Numerous reports have indicated a relationship between the prognosis of COVID-19 infection and the balance of Th subsets. However, available data do not clearly elucidate the relationship between disease prognosis and Th cell subsets. Based on current and our study data, at the onset of a viral infection, optimal Th1 cell responses can clear the infection without leading to severe symptoms. Conversely, if Th1 cell responses are inadequately organized, Th2 responses, which are associated with poor prognosis, may prevail and predispose individuals to cytokine storm syndrome. The role of Th17 cells in influencing the prognosis of the disease as either good or poor is variable. In our study, cytokines and transcription factors associated with Th1 and Th17 cells were suppressed, while those related to Th2 and Treg cells were upregulated. Although literature mentions variations in these cell types at different stages of COVID-19, definitive evidence remains lacking. Notably, the upregulation of the Treg cell transcription factor FoxP3 was considered a promising marker for predicting the course of the disease in patients. Moreover, most studies on this topic have focused on severely ill patients. Identifying the dominant cell type at the initial stage of the disease, rather than in severely ill patients, is crucial for aiding in preventing disease progression.

The same patients were included in the study both at diagnosis and at discharge. However, some patients, despite the disappearance of clinical findings, could not be included in the study if SARS-CoV-2 positivity persisted. Therefore, the number of patients represents a limitation of the study.

CONCLUSION

In conclusion, based on this data, we believe that our study, which evaluates the changes in T cell subsets at the time of diagnosis at the expression level, will make a significant contribution to the literature.

Ethics Committee Approval: The Erbakan University Clinical Research Ethics Committee granted approval for this study (date: 27.01.2023, number: 2023/035).

Author Contributions: Concept – HV, MAK; Design – MAK, TD, SK; Supervision – HV; Resource – HV; Materials – HV; Data Collection and/or Processing – HV, MAK, TD, SK; Analysis and/or Interpretation – MAK, TD, SK; Literature Search – HV, MAK, TD, SK; Writing – MAK; Critical Reviews – HV.

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

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Use of AI for Writing Assistance: Not declared.

Financial Disclosure: The study was supported by Necmettin Erbakan University Scientific Research Projects Coordination Unit with project number 201218010.

Peer-review: Externally peer-reviewed.

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Appendix 1. Genes used in qPCR analysis and primers

Genes	Primers (5'.....3')	Genes	Primers (5'.....3')
IFN-γ	F:GGCAAGGCTATGTGATTACAAGG	IL-17	F:TCCCACGAAATCCAGGATGC
	R:CATCAAGTGAAATAAACACACAACCC		R:GGATGTTCAGGTTGACCATCAC
T-bet	F:GTCCAACAATGTGACCCAGAT	IL-21	F:TAGAGACAAACTGTGAGTGGTCA
	R:ACCTCAACGATATGCAGCCG		R:GGGCATGTTAGTCTGTGTTTCTG
STAT1	F:TGGGTGCATCATGGGCTTCA	IL-22	F:GCTTGACAAGTCCAACTTCCA
	R:CGCATGGAAGTCAGGTTCGC		R:GCTCACTCATACTGACTCCGT
STAT4	F:GCTTAACAGCCTCGATTTCAAGA	RORγt	F:CTGCTGAGAAGGACAGGGAG
	R:GAGCATGGTGTTCATTAACAGGT		R:AGTTCTGCTGACGGGTGC
IL-12	F:CCTTGCACTTCTGAAGAGATTGA	STAT3	F:ACCAGCAGTATAGCCGCTTC
	R:ACAGGGCCATCATAAAAGAGGT		R:GCCACAATCCGGGCAATCT
IL-6	F:CCTGAACCTTCCAAAGATGGC	IL-10	F:TCAAGGCGCATGTGAACTCC
	R:TTCACCAGGCAAGTCTCCTCA		R:GATGTCAAACTCACTCATGGCT
IL-4	F:CGGCAACTTTGTCCACGGA	TGF-β	F:CCCAGCATCTGCAAAGCTC
	R:TCTGTTACGGTCAACTCGGTG		R:GTCAATGTACAGCTGCCGCA
IL-5	F:AAGAGACCTTGGCACTGCTTTC	FoxP3	F:GTGGCCCGGATGTGAGAAG
	R:GGAACAGGAATCCTCAGAGTCTCA		R:GGAGCCCTTGTCGGATGATG
IL-13	F:GAGGATGCTGAGCGGATTCTG	STAT5	F:ACGGGGTGATGGAGGTGTTG
	R:CACCTCGATTTTGGTGTCTCG		R:TCAGGTTCCACAGGTTGCGT
STAT6	F:TTGGGCTTGAGGTTCCTGGG	GATA3	F:GCCCCTCATTAAGCCCAAG
	R:TGCTGTTCTCCAAGGGCACA		R:TTGTGGTGGTCTGACAGTTCG
GAPDH	F:CCGTCTAGAAAAACCTGCC		
	R:GGAGGAGTGGGTGTCGCTGT		

qPCR: Quantitative polymerase chain reaction.