EFFECtor memory T cell subset CD45RA−CCR7−CD27−CD28− EM3 increases in direct proportion to the disease severity of COVID-19

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Abstract
COVID-19, which emerged in December 2019 and continues to wreak havoc, has led to the death of many people around the world. In this study, we aimed to uncover the variables underlying the exacerbation of the disease by considering the changes in T cell subsets in adults and juveniles with different disease severity of COVID-19. Peripheral blood samples of 193 patients (128 adults and 65 juveniles) diagnosed with COVID-19 were evaluated in a flow cytometer, and a broad T cell profile was revealed by examining T cell subsets in terms of exhaustion and senescence. We found remarkable differences in the effector memory (EM; CD45RA−CCR7−) cell subsets of severe pneumonia cases. The frequencies of EM2 CD4+ T, EM3 CD4+ T, EM3 CD8+ T, EM2 DN T and EM3 DN T cells were found to increase in severe pneumonia cases. Consistently, these cells were found in juveniles and uncomplicated adults in similar or lower proportions to healthy controls. The findings of our study provide a view of the T cell profile that may underlie differences in the course of COVID-19 cases in juveniles and adults and may provide new insights into the development of effective treatment strategies.
1 | INTRODUCTION

SARS-CoV-2 can trigger uncontrolled inflammatory responses leading to pro-inflammatory cytokine release, lymphopenia, lymphocyte dysfunction, granulocyte and monocyte abnormalities. A broad clinical picture emerges in COVID-19, ranging from asymptomatic cases to severe pneumonia requiring intensive care. The immunological mechanisms underlying the occurrence of the various clinical pictures have not yet been adequately elucidated.

Lymphocytes and their subsets play an important role in protecting immune system functions. CD4+ T cells play a role in protective immunity against viruses by infection or vaccination. Double-negative T cells (DN T, CD3+ CD4− CD8−) are cells that can elicit antigen-specific regulatory responses in transplantation, autoimmune and infectious diseases. While most of the cytokines and chemokines produced by DNTs cause proinflammatory responses, they can also suppress T cell responses by producing anti-inflammatory cytokines such as IL-10. Due to these immunomodulatory properties, they form a current research group in studies of viral infections.

T lymphocytes are classified into four main groups based on their expression of the leukocyte common antigen isoform CD45RA and the chemokine receptor CCR7 (CD197): Naive (CD45RA+ CCR7+), central memory (CM; CD45RA− CCR7+), effecter memory (EM; CD45RA− CCR7+) and terminally differentiated memory RA (TEMRA; CD45RA+ CCR7−). Memory T cells are heterogeneous and contain distinct populations based on surface markers and effector functions. Based on the expression profiles of the TNF family co-stimulatory receptor CD27 and the B7 family receptor CD28, different subsets have been defined. Naive T cells and CM T cells are generally identified as CD27+ CD28+, while other populations can be differentiated into EM T cells and TEMRA T cells. EM T cells are analysed into four subsets, EM1 (CD27+ CD28+), EM2 (CD27+ CD28−), EM3 (CD27− CD28−) and EM4 (CD27− CD28+). TEMRA T cells, on the other hand, are studied in three different subsets: Pre-effector 1 (pE1; CD27+ CD28+), pre-effector 2 (pE2; CD27+ CD28−) and effector cells (E; CD27− CD28−). Koch and colleagues have shown that differentiation of CD4+ and CD8+ T cells occurs towards N > C M > EM1 > EM2 > pE1 > pE2 > EM4 > EM3 > E and eventually proliferative capacity is lost.

The current literature on T cell subsets and depletion in the immune response to SARS-CoV-2 virus is partially contradictory. These inconsistencies are generally due to the small patient populations and the lack of grouping by disease severity in some studies. In addition, there is insufficient information on T cell subsets in juvenile patients. Therefore, it is aimed to reveal the potential roles of CD3+ CD4+, CD3+ CD8+ and CD3+ CD4− CD8− T cell subsets and exhaustion and senescence of these cells in the course of COVID-19. The immunological data obtained from this study may reveal new ideas in the development of COVID-19 treatment approaches as well as for the diagnosis and follow-up of patients.

2 | MATERIAL AND METHODS

2.1 | Study design and participants

A total of 128 adult patients (58 females and 70 males) and 65 juvenile patients (34 girls and 31 boys) diagnosed with COVID-19 who applied and/or were followed up to Bursa Uludag University Faculty of Medicine, Department of Infectious Diseases and Department of Pulmonology and University of Health Sciences, Bursa Yuksek Ihtisas Training Research Hospital, Department of Pediatric Infectious Diseases and Department of Infectious Diseases, were included in the study. The diagnoses of all patients included in the study were made by physicians specialized in infectious diseases based on the evaluation of laboratory and radiographic findings and the reverse-transcriptase polymerase chain reaction (RT-PCR) (Bio-Speedy Direct RT-qPCR SARS-CoV-2 nucleic acid detection kit) (Bioeksen). The adult patients were divided into three groups according to the criteria of the guideline WHO (March 13, 2020): uncomplicated, mild and severe pneumonia. The uncomplicated group included patients who did not have pneumonia but had some non-specific symptoms (fever, cough, loss of appetite, etc.). Patients with pneumonia who did not meet the criteria of respiratory rate >30 breaths/min, severe dyspnoea or SpO2≤93% on room air were included in the mild pneumonia group, while patients with at least one of these criteria were included in the severe pneumonia group.

Clinical data from the first wave of the pandemic suggest that the disease is generally mild and appears to affect juveniles less than adults. Since all of the juvenile patients included in the study had a mild or asymptomatic infection in this sense, it was not possible to classify them according to the severity of pneumonia. While about 50% of the immune system develops by the early years of adolescence, this development is generally complete by the end of adolescence. For this reason, the juvenile patients in our study were divided into two
groups, the 0- to 12-year olds and the 13- to 18-year olds, to investigate the effects of COVID-19 before and after puberty. In addition, 5 patients in the adult group and 28 patients in the juvenile group were followed up. These patients whose symptoms completely resolved and who had negative PCR results were included in the recovered follow-up groups (~15-28 days).

Thirteen individuals who had applied to Bursa Uludağ University, Faculty of Medicine, Raşit Durusoy Blood Donor Centre, as donor candidates and had no known or diagnosed disease were included in the study as a healthy control group by obtaining informed consent. In addition, 15 healthy children who applied to the Health Sciences University, Bursa Yuksek İhtisas Training and Research Hospital, for reasons other than infection as healthy control group (growth and development control, etc.) were included in the study.

The clinical and demographic data of all patients participating in the study are shown in Table 1. The times from onset of disease symptoms to sample collection in adult and juvenile patient groups are listed in Table S1. In addition, some laboratory findings of the patients can be found in Table 2. All patients and healthy controls both juveniles and adults were not vaccinated against COVID-19. It should also be noted that all patients were infected with wild-type SARS-CoV-2.

2.2 | Flow cytometry

Flow cytometry method was used to study T cell subsets in peripheral blood samples from patients and healthy controls. One flow cytometry tube was used for isotypic control and two for T cell subsets-specific markers. 100μl of peripheral blood samples with EDTA were transferred to the tubes. In this stage, CD3 as T cell marker, CD4 as T helper cell marker, CD8 as cytotoxic T cell marker and CD16 and CD56 as NKT marker were selected. Also, for T cell subsets, CD45RA and CCR7 were used as markers of naive, effector, central memory, effector memory and TEMRA. CD27 and CD28 were used for effector memory and TEMRA subsets. CD57 and PD-1 were used to assess T cell senescence and exhaustion respectively. According to the designated panel, mentioned monoclonal antibodies were pipetted into the suitable tubes and following vortexed, incubated for 15 minutes at room temperature, protected from light. After incubation, 500μl of VersaLyse Lysing Solution (Beckman Coulter) was added to all tubes to lyse the erythrocytes. The final incubation was, again, carried out for 15 minutes at room temperature protected from light. After incubation, the samples were evaluated on the flow cytometer (Novious EX, Beckman Coulter). T cell subsets were analysed using Kaluza software (Beckman Coulter). Fluorescence minus one (FMO) controls were used to distinguish between negative and positive populations (Figure S1). The fluorescent colours selected for the conjugated monoclonal antibody panel used in the study to reveal possible changes in T cell subset profiles in COVID-19 patients are as follows: CD45RA-FITC, CCR7-PE, CD28-ECD, CD27-PC5.5, CD4-APC, PD-1-PC7, CD8-APC-A700, CD3-APC-A750, CD57-PB, CD45-KrO and CD3-FITC/CD(16+56)-PE (Beckman Coulter). Detailed information on the monoclonal antibodies used in this study can be found in the Table S2.

2.3 | Statistical analysis

In this study, all statistical analyses were performed using IBM SPSS Statistics Software version 28.0. (for Windows Version 28.0., IBM Corp.) The conformity of the values to the normal distribution was evaluated using visual (histogram and probability charts) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). In adults, multigroup statistics were performed with the Kruskal-Wallis test and the post hoc Dunn test. In juvenile patients, the Mann-Whitney U test and Bonferroni correction were used for comparisons between groups. Patients who were followed up after recovery were examined with the Wilcoxon signed-rank test. Categorical variable values were compared using the χ²-test or Fisher’s exact test. Correlations were determined using a Spearman’s correlation test. In all analyses, P<.05 was considered statistically significant.

3 | RESULTS

In this study, we assessed CD4⁺, CD8⁺ and DN T cell subsets (naive, CM, EM, TEMRA, EM₁, EM₂, EM₃, EM₄, pE₁, pE₂ and E cells) as well as PD-1 and CD57 expression and NKT cells by flow cytometry in 193 COVID-19 patients. All of our flow cytometric results are expressed as percentages (%), except for the CD4⁺/CD8⁺ ratio. The gating strategies used for the analysed cells are shown in Figure 1. To mention, the study groups consisting of SARS-CoV-2-infected juvenile and adult patients were compared with healthy control groups matched for age and sex. Moreover, in the juvenile patients, patients aged 0-12 years were compared with patients aged 13-18 years, whereas in the adult patients, the groups were compared according to the severity of pneumonia (uncomplicated, mild and severe pneumonia). However, the classification into groups for adults and juveniles was based on different criteria (situation of pneumonia and age), so adults and juveniles were not compared.
**TABLE 1** Demographic and clinical picture in adult and juvenile patient groups of COVID-19 (a uncomplicated vs severe, b uncomplicated vs mild c COVID-19 vs HC and d COV-0-12 vs COV 13-18). Statistically significant values are shown in bold.

| **Adult patients** | **Total illness** | **Uncomplicated** | **Mild pneumonia** | **Severe pneumonia** | **Healthy controls** | **P value** |
|--------------------|-------------------|-------------------|-------------------|---------------------|---------------------|-------------|
| **n = 128**        |                   |                   |                   |                     |                     |             |
| **n = 33**         |                   |                   |                   |                     |                     |             |
| **n = 41**         |                   |                   |                   |                     |                     |             |
| **n = 54**         |                   |                   |                   |                     |                     |             |
| **n = 13**         |                   |                   |                   |                     |                     |             |
| **Sex**            |                   |                   |                   |                     |                     |             |
| Male (%)           | 70 (54.6)         | 17 (51.5)         | 23 (56.0)         | 30 (55.6)           | 6 (46.1)            | NS          |
| Female (%)         | 58 (45.4)         | 16 (48.5)         | 18 (44.0)         | 24 (44.4)           | 7 (53.9)            |             |
| Age median year (min-max) | 49 (19-89) | 41 (19-80) | 48 (26-80) | 59 (24-89) | 42 (24-64) | < .01 a |
| **Comorbidity**    |                   |                   |                   |                     |                     |             |
| Hypertension (%)   | 22 (17.1)         | 7 (21.2)          | 4 (9.7)           | 11 (20.4)           | -                   | NS          |
| Diabetes mellitus (%) | 24 (18.7)      | 5 (15.2)          | 5 (12.2)          | 14 (25.9)           | -                   | NS          |
| Cardiovascular disease (%) | 12 (9.3) | 3 (9.1)          | 3 (7.3)           | 6 (11.1)            | -                   | NS          |
| Chronic pulmonary disease (%) | 10 (7.8) | 4 (12.1)         | 1 (2.4)           | 5 (9.2)             | -                   | NS          |
| Other (%)          | 28 (21.8)         | 7 (21.2)          | 12 (29.2)         | 9 (16.6)            | -                   | NS          |
| No comorbidity (%) | 38 (29.6)         | 8 (24.2)          | 18 (43.9)         | 12 (22.2)           | -                   | NS          |
| **Symptoms at admission** |                   |                   |                   |                     |                     |             |
| Fever (%)          | 40 (31.2)         | 11 (33.3)         | 11 (26.8)         | 18 (33.3)           | -                   | NS          |
| Cough (%)          | 65 (51.6)         | 11 (33.3)         | 28 (68.2)         | 28 (51.9)           | -                   | .023 b     |
| Dyspnoea (%)       | 28 (22.2)         | 6 (18.2)          | 5 (12.8)          | 17 (31.5)           | -                   | NS          |
| Myalgia (%)        | 26 (20.6)         | 5 (15.2)          | 6 (15.4)          | 15 (27.8)           | -                   | NS          |
| Sore throat (%)    | 21 (16.4)         | 6 (18.2)          | 7 (17.1)          | 8 (14.8)            | -                   | NS          |
| Headache (%)       | 16 (12.7)         | 9 (27.3)          | 4 (10.3)          | 3 (5.6)             | -                   | .012 a     |
| Anosmia (%)        | 5 (4)             | 1 (3)             | 3 (7.7)           | 1 (1.9)             | -                   | NS          |
| Diarrhoea (%)      | 14 (11.1)         | 3 (9.1)           | 3 (7.7)           | 8 (14.8)            | -                   | NS          |
| Fatigue (%)        | 31 (23.8)         | 8 (24.2)          | 11 (28.2)         | 12 (20.4)           | -                   | NS          |
| Smoker (%)         | 7 (5.6)           | 3 (9.1)           | 2 (5.1)           | 2 (3.7)             | -                   | NS          |
| Need to mechanical ventilation (%) | 27 (21.4) | 0 (0)            | 0 (0)             | 27 (50)             | N/A                 |             |

| **Juvenile patients** | **Total illness** | **0-12 age group** | **13-18 age group** | **Healthy control** | **P value** |
|-----------------------|-------------------|--------------------|---------------------|---------------------|-------------|
| **n = 65**            |                   | **n = 29**         | **n = 36**          | **n = 15**          |             |
| **Sex**               |                   |                     |                     |                     |             |
| Male (%)              | 34 (52.3)         | 13 (44.8)          | 15 (41.7)          | 6 (40)              | NS          |
| Female (%)            | 31 (47.7)         | 16 (55.2)          | 21 (58.3)          | 9 (60)              |             |
| Age. median year (min-max) | 10 (0-18) | 4 (0-12)        | 15 (13-18)         | 9 (0-17)            | NS          |
| **Comorbidity**       |                   |                     |                     |                     |             |
| There is no comorbid disease in juvenile patients |                   |                     |                     |                     |             |
| **Symptoms at admission** |                   |                     |                     |                     |             |
| Fever (%)             | 38 (58.4)         | 19 (65.5)          | 19 (52.7)          | -                   | NS          |
| Cough (%)             | 18 (28.1)         | 7 (24.1)           | 11 (31.4)          | -                   | NS          |
| Dyspnoea (%)          | 2 (3.1)           | 1 (3.4)            | 1 (2.9)            | -                   | NS          |
| Myalgia (%)           | 2 (3.1)           | -                  | 2 (5.7)            | -                   | NS          |
| Sore throat (%)       | 5 (7.8)           | 1 (3.4)            | 4 (11.4)           | -                   | NS          |
3.1 | Evaluation of T cells in patients infected with SARS-CoV-2

3.1.1 | T cell profile in adult patients

As a result of the evaluation using the T cell surface marker CD3 revealed a significant decrease in the percentages of T cells in adult patients with severe pneumonia compared with mild pneumonia (ps vs m = .041; Figure 2A). In adult patients, the decrease in T cell frequency was found to be inversely associated with disease severity. A statistically non-significant trend towards an increase in CD3+CD4+ T cells was observed in patients compared with healthy controls, and this increase was inversely proportional to disease severity (Figure 2B). DN T cells were significantly decreased in patients with mild and severe pneumonia compared with healthy controls (pm vs h = .016, ps vs h = .019; Figure 2D). There was no statistically significant difference between the healthy control group and adult patient groups in terms of CD3+CD8+ T cells, NKT cells and CD4/CD8 ratio (Figure 2C,E,F,G).

3.1.2 | T cell profile in juvenile patients

When the juvenile patient groups were evaluated, it was found that the percentage of CD3+ T cells significantly decreased in the age group 0-12 years compared with the age group 13-18 years (pcov 0-12 vs cov 0-13 < .002; Figure 2A). A slight downward trend in CD3+CD4+ T cells was observed in juvenile patients, in contrast to the increase observed in adult patients (Figure 2B). In contrast to the decrease in CD3+CD8+ T cells in adult patients with severe pneumonia, an increasing trend was observed in juveniles compared with healthy controls. In addition, significantly more CD3+CD8+ T cells were found in the 13-18 age group than in the 0-12 age group (Figure 2C). There was no statistically significant difference between the healthy control group and the juvenile patient groups with regard to CD3+CD8+ T cells, NKT cells and CD4/CD8 ratio (Figure 2D,E,F,G).

3.2 | Circulating CD4+ T cells are in the final stage of differentiation in severe cases

3.2.1 | CD4+ T cell subset profile in adult patients

Effector memory CD4+ cells represent the highest percentage of adult CD4+ T cell subsets (Figure 3A). The percentage of EM3 CD4+ T cells was significantly increased in severe pneumonia cases compared with uncomplicated cases (pun vs s = .002; Figure 4G). In patients with pneumonia, the distribution within TEMRA CD4+ T cell subsets changed from pE1 CD4+ T cells to E CD4+ T cells (Figure 3C). The percentage of pE1 CD4+ T cells was significantly decreased in cases with severe pneumonia compared with uncomplicated cases (pun vs s = .005; Figure 4I). E CD4+ T cells were significantly increased in severe pneumonia cases compared with uncomplicated cases (pun vs s = .004; Figure 4K). The percentage of CD4+ T cells exhibiting PD-1 was significantly increased in all adult patient groups compared with healthy controls (pun vs h = .015, pm vs h = .021, ps vs h = .002; Figure 4L). Although CD4+CD57+ T cells showed an increasing trend in severe cases, no statistically significant results were found (Figure 4M). On the other hand, this increase observed in severe cases decreased significantly after follow-up.

3.2.2 | CD4+ T cell subset profile in juvenile patients

It was found that naive CD4+ T cells had the highest percentage of CD4+ T cell subsets until adolescence, but this situation changed towards adulthood and EM CD4+ T cells gradually increased (Figure 3A). Moreover, EM CD4+ T cells were significantly increased, whereas naive CD4+ T cells decreased in the 13-18 age group compared with the 0-12 age group (pcov 0-12 vs cov 13-18 < .001; Figure 4A; pcov 0-12 vs cov 13-18 < .001; Figure 4C). Another finding is that EM1 CD4+ T cells were significantly decreased in...
the 13-18 age group compared with the 0-12 age group ($P_{\text{cov} \, 0-\text{12} \text{ vs } \text{cov} \, 13-\text{18}} < .001$; Figure 4E), whereas EM$_1$ CD4$^+$ T cells increased ($P_{\text{cov} \, 0-12 \text{ vs } \text{cov} \, 13-18} < .001$; Figure 4H). In the 13- to 18-year-old age group, both PD-1$^+$ cells ($P_{\text{cov} \, 13-\text{18} \text{ vs } h \, 13-\text{18}} = .017$) and CD57$^+$ cells ($P_{\text{cov} \, 13-\text{18} \text{ vs } h \, 13-\text{18}} = .011$) were significantly increased compared with healthy controls (Figure 4L,M). There was no significant difference between the 0- to 12-year-old group and the age-matched healthy control group in terms of CD4$^+$ T cell subsets. There were no significant changes in either juvenile patient group after follow-up.

### Table 2

| Laboratory findings in adult and juvenile patient groups of COVID-19 (‘severe pneumonia vs mild pneumonia, uncomplicated vs severe pneumonia and ‘COV-0-12 vs COV 13-18). Statistically significant values are shown in bold. |
|---------------------------------------------------------------|
| **Adult patients**                                            | Normal range | Uncomplicated cases | Mild pneumonia | Severe pneumonia | All patients | $P$ value |
| White blood cell count (K/μL)                                 | 4.5-11       | 5.8 (3.3-14.7)      | 6.2 (2.3-15.2) | 6.4 (1.0-26.1)  | 6.2 (1.0-26.1) | .017*     |
| Neutrophil count (K/μL)                                       | 2-6.9        | 3.5 (1.2-15.3)      | 3.6 (1.3-12.6) | 4.3 (2.0-23.6)  | 3.9 (1.2-23.6) | <.001*    |
| Lymphocyte count (K/μL)                                       | 1.3-3.8      | 1.6 (0.5-7.1)       | 1.3 (0.4-4.3)  | 1.1 (0.4-7.8)   | 1.3 (0.4-7.8)  | .01*      |
| Platelet count (K/μL)                                         | 145-400      | 240 (101-686)       | 227 (103-497)  | 257 (24-774)    | 240 (24-774)   | NS        |
| Monocyte count (K/μL)                                         | 0.2-0.95     | 0.7 (0.05-1.3)      | 0.5 (0.07-0.6) | 0.4 (0.08-1.4)  | 0.5 (0.05-1.4) | NS        |
| CRP (mg/L)                                                    | <5           | 8 (2.0-190)         | 10 (2.0-194)   | 75 (2.2-234)    | 37 (2.0-234)   | <.001*    |
| Procalcitonin (ng/mL)                                         | <0.08        | 0.01 (0-0.04)       | 0.02 (0.01-0.04) | 0.06 (0.02-1.79) | 0.03 (0-1.79) | .006*     |
| ALT (U/L)                                                     | 9-57         | 18 (5-132)          | 19 (5-124)     | 30 (5-145)      | 27 (5-145)     | .014*     |
| AST (U/L)                                                     | 13-30        | 21 (11-90)          | 24 (11-98)     | 37 (9-184)      | 29 (9-184)     | <.001*    |
| Lactic acid dehydrogenase (LDH) (U/L)                         | 125-243      | 229 (84-695)        | 285 (121-707)  | 372 (130-664)   | 312 (84-707)   | <.001*    |
| Ferritin (μg/L)                                               | 15-260       | 72 (4.2-770)        | 118 (2.8-1268) | 346 (12.3-2000) | 204 (2.8-2000) | <.001*    |
| D-dimer (μg/mL)                                               | <0.55        | 0.5 (0-1.4)         | 0.4 (0-1.6)    | 0.6 (0-1.6)     | 0.5 (0-1.6)    | <.001*    |
| Troponin I (ng/L)                                             | <34.2        | 1.16 (0.6-6.1)      | 3.4 (1.3-25.4) | 19.75 (2.1-127.9)| 9.8 (0.6-127.9) | NS        |

| **Juvenile patients**                                         | Normal range | Normal range     | Normal range     |
|---------------------------------------------------------------|---------------|------------------|------------------|
| **0-12 age**                                                  | 4.5-13.5      | 4.5-13.5         | 7.9 (3.5-16)     | 5.1 (2.8-9.3)   | 6.1 (2.8-16)   | <.001*    |
| **13-18 age**                                                 | 1.5-8         | 2-6.9            | 3.3 (1-11)       | 2.6 (1.3-6.3)   | 2.9 (1-11)     | NS        |
| **Platelet count (K/μL)**                                     | 200-450       | 145-400          | 281 (154-593)    | 246 (156-440)   | 259 (154-593)  | .03*      |
| **Monocyte count (K/μL)**                                     | 0.2-1.0       | 0.2-1.0          | 0.6 (0.3-7.2)    | 0.4 (0.2-0.7)   | 0.5 (0.2-7.2)  | .01*      |
| **CRP (mg/L)**                                                | <5            | <5               | 3.1 (1-257)      | 3.1 (3-25.9)    | 3.1 (1-257)    | NS        |
| **ALT (U/L)**                                                 | 5-30          | 5-30             | 16 (6-41)        | 12 (8-156)      | 14 (6-156)     | NS        |
| **AST (U/L)**                                                 | 8-60          | 8-48             | 30 (15-70)       | 21 (7-52)       | 23 (7-70)      | <.001*    |
| **Lactic acid dehydrogenase (LDH) (U/L)**                     | 143-345       | 105-283          | 288 (180-548)    | 212 (163-285)   | 239 (163-548)  | .018*     |
| **Ferritin (μg/L)**                                           | 7-140         | 7-260            | 44.1 (9-267)     | 25 (6-63)       | 36 (0-267)     | NS        |
| **D-dimer (μg/mL)**                                           | 0.09-0.5      | <0.55            | 0.22 (0.06-1.6)  | 0.37 (0.01-1)   | 0.3 (0.01-1.6) | NS        |
3.3 SARS-CoV-2 infection predisposes circulating CD8+ T cells to a more cytolytic phenotype

3.3.1 CD8+ T cell subset profile in adult patients

Evaluation of EM CD8+ T cell subsets revealed significant differences in cases with severe pneumonia (Figure 3E). In severe pneumonia, the frequency of EM1 CD8+ T cells was significantly decreased compared with other adult patient groups and healthy controls ($p_s$ vs $h$ = .002, $p_s$ vs unc = .042; $p_s$ vs m = .037; Figure 5E). It was observed that the percentage of EM3 CD8+ T cells was significantly increased in patients with severe pneumonia compared with patients with uncomplicated and mild pneumonia ($p_s$ vs unc = .036, $p_s$ vs m = .018; Figure 5G). No statistical significance was observed in TEMRA CD8+ T cells between adult patient groups in all three subsets based on surface expression of CD27 and CD28 (Figure 5I-K). A significant increase in PD-1 expression was observed in CD8+ T cells in all adult patient groups compared with healthy controls ($p_{unc}$ vs $h$ = .034, $p_m$ vs $h$ = .046, $p_s$ vs $h$ = .015; Figure 5L). A tendency to decrease PD-1 levels was observed in CD8+ T cells of severe pneumonia cases followed up after recovery.
3.3.2 | CD8+ T cell subset profile in juvenile patients

The frequency of naive, EM and TEMRA CD8+ T cells appears to change after adolescence. Naive CD8+ T cells and EM2 CD8+ T cells were significantly increased in the 0-12 years age group compared with the 13-18 years age group (Figures 4F and 5A). It was found that the frequency of EM CD8+ T cells was increased in the age group of 0-12 years compared with healthy controls in the same age group (Figure 5C). On the other hand, it was found that the frequency of EM4 CD8+ T cells was significantly lower in the age group of 0-12 years than in those of 13-18 years (p cov 0-12 vs cov 13-18 = .001; Figure 5H). It was observed that the frequency of ECD8+ T cells was significantly increased in the 13-18 age group compared with both age-matched healthy controls and the 0-12 age group (p cov 0-12 vs cov 13-18 = .004, p cov 13-18 vs h 13-18 = .020; Figure 5K). In addition, it was found that pE2 CD8+ T cells were significantly increased in the 0-12 age group compared with the 13-18 age group (p cov 0-12 vs cov 13-18 = .001; Figure 5I). A significant increase in PD-1+ CD8+ T cell frequency was observed in the age group 0-12 years compared with healthy controls (p cov 0-12 vs h 0-12 = .001; Figure 5L). Both juvenile patient groups had a slightly increased percentage of CD57+ CD8+ T cells compared with healthy controls, whereas a significant increase in CD57 expressing CD8+ T cells was found in direct proportion to age (p cov 0-12 vs cov 13-18 = .038; Figure 5M).

3.4 | SARS-CoV-2 infection alters the frequency of circulating DN T cell subsets

3.4.1 | DN T cell subset profile in adult patients

Effector memory DN T cells were significantly reduced in severe pneumonia compared with uncomplicated cases (p unc vs s = .008; Figure 6C). Whereas the percentage of EM1 DN T cells decreased significantly (p vs h = .009; Figure 6E), the percentage of EM3 DN T cells increased in patients with severe pneumonia compared with healthy controls (p s vs h = .018; Figure 6G). Notably, significant differences were observed in adults TEMRA DN T cell subsets in cases with pneumonia (Figure 3I). Compared with uncomplicated cases, pE3 DN T cells were significantly decreased in severe pneumonia cases (p unc vs s = .017; Figure 6I), whereas E DN T cells were increased
In addition, the percentage of CD57+ DN T cells was increased in severe pneumonia compared with uncomplicated cases ($p_{\text{unc vs s}} = .004$; Figure 6M).

### 3.4.2 DN T cell subset profile in juvenile patients

The frequency of CM DN T cells was significantly higher in the 0-12 age group than in age-matched healthy controls ($p_{\text{cov 0-12 vs h 0-12}} = .046$; Figure 6B). Compared with the 0-12 age group, EM DN T cells increased significantly in the 13-18 age group ($p_{\text{cov 0-12 vs cov 13-18}} = .021$), whereas TEMRA DN T cells decreased ($p_{\text{cov 0-12 vs cov 13-18}} = .038$; Figure 6C,D). In addition, compared with patients aged 0-12 years, the percentages of EM$_2$ DN T cells decreased significantly in the age group 13-18 years ($p_{\text{cov 0-12 vs cov 13-18}} = .039$), whereas EM$_3$ ($p_{\text{cov 0-12 vs cov 13-18}} = .026$) and EM$_4$ DN T cell ($p_{\text{cov 0-12 vs cov 13-18}} < .001$) percentages increased (Figure 6F-H). In the 13-18 age group, there was a significant decrease in pE$_2$ DN T cells compared with the 0-12 age group and healthy controls ($p_{\text{cov 0-12 vs cov 13-18}} = .002$, $p_{\text{cov 13-18 vs h 13-18}} = .002$) and an increase in E DN T cells noted ($p_{\text{cov 0-12 vs cov 13-18}} = .020$; Figure 6J,K). PD-1+ DN T cells were significantly increased in both juvenile patient groups compared with healthy controls ($p_{\text{cov 0-12 vs cov 13-18}} = .002$, $p_{\text{cov 13-18 vs h 13-18}} = .001$). PD-1+ DN T cells were significantly increased in the 0-12 age group compared with the 13-18 age group ($p_{\text{cov 0-12 vs cov 13-18}} = .001$; Figure 6L). The increased PD-1 expression in DN T cells in the age group 0-12 years significantly decreased after follow-up ($p_{\text{follow-up d. cov 0-12 vs follow-up s. cov 0-12}} = .022$). In the juvenile patient groups, the frequency of CD57-expressing DN T cells increased statistically significantly with age ($p_{\text{cov 0-12 vs cov 13-18}} < .001$; Figure 6M).
3.5 | Association of EM₃ T cells and clinical parameters in severe pneumonia cases

In adult patients, correlation analyses were performed between clinical parameters and EM₃ T cells, which showed differences according to disease severity in flow cytometry. In severe pneumonia cases, a positive correlation was found between EM₃ CD₄⁺ T and EM₃ CD₈⁺ T cells and ferritin, C-reactive protein (CRP), lactate dehydrogenase (LDH) and D-dimers, whereas a negative correlation was found between lymphocytes. Moreover, in severe pneumonia cases, a positive correlation was found between EM₃ DN T cells and CRP and LDH levels and a negative correlation with lymphocytes. However, in uncomplicated cases, EM₃ T cells were not statistically significant in the correlation analysis between clinical parameters (Figure 7). Interesting correlation results were also obtained between clinical manifestations and EM₃ cells. In severe pneumonia cases, EM₃ CD₈⁺ T cells were positively correlated with fever and cough, whereas a positive correlation was found between EM₃ DN T cells and myalgia. Furthermore, a positive correlation between EM₃ CD₈⁺ T cells and fever was found in uncomplicated cases (Figure 8C).

4 | DISCUSSION

SARS-CoV-2 infection presents with different clinical pictures in humans. Most adult patients are characterized
by an uncontrolled release of cytokines called a ‘cytokine storm’. Due to the excessive inflammatory and immune responses, acute respiratory distress (ARDS) and multiple organ failure are observed.\textsuperscript{16} Despite these severe clinical pictures in adult patients, a milder course of the disease is generally observed in juvenile patients.\textsuperscript{17,18} The cytokine storm seen at COVID-19 indicates the presence of uncontrollable, irregular responses in the host immune system.\textsuperscript{19}

It is well known that functional T cell responses are critical in clearing acute viral infections and preventing viral persistence. In this context, our study planned an in-depth phenotypic analysis of T cell subsets in peripheral blood samples from COVID-19 patients. Previous studies generally consisted of a small study group and limited COVID-19 disease groups. In our study, different groups ranging from uncomplicated cases to severe pneumonia were investigated. At the same time, we aimed to clarify the possible T cell subset profiles underlying this difference in disease course by including juvenile patients with a milder course compared with adults. Thus, disease severity and T cell differences between adult and juvenile patients were jointly assessed.

Li et al.\textsuperscript{20} showed in a study of 32 COVID-19 cases that T cells decreased in severe cases compared to healthy controls and mild patients. Similarly, a study showed that in 10 COVID-19 patients with moderate-to-severe ARDS treated in the intensive care unit (ICU), the percentage of T cells
in the patients' peripheral blood was decreased compared to the healthy control group. In a study of 61 COVID-19 patients, it was shown that CD3⁺ T, CD4⁺ T, CD8⁺ T, DN and NKT cells were significantly reduced compared to healthy controls. In the same study, a significant decrease was observed only in CD3⁺ T and NKT cells in severe cases compared to mild cases. In our study, it was observed that the percentage of T cells decreased significantly in patients with severe pneumonia compared to patients with mild pneumonia. Although the percentage of T cells decreased in juvenile patients compared with healthy controls, there was no statistical significance. Although no significant changes in CD3⁺ T cells were detected in patients compared with healthy controls, our detailed studies demonstrate that significant changes in subsets may play an important role in the immunopathogenesis of COVID-19.

According to the results of our study, EM₂ CD4⁺ T cells were significantly increased in severe pneumonia cases compared with uncomplicated cases. Okada and colleagues showed that within the EM CD4⁺ T cell subsets, EM₂ CD4⁺ T cells produce significantly more IFN-γ than others. These data suggest that differentiation in EM CD4⁺ T cell subsets has a critical role in COVID-19 disease severity. When TEMRA CD4⁺ T cell subsets were examined, an increase in pE₂ and E CD4⁺ T cells was observed in severe pneumonia cases, whereas an increase in pE₁ CD4⁺ T cells was observed in uncomplicated cases (Figure 3C). Although no significant change in CD4⁺ T
cell subsets was observed between the 0-12 age group and healthy controls in juveniles, the fact that both EM and TEMRA CD4$^+$ T cell subsets are in the final stages of differentiation in severe pneumonia cases in adults indicates an irregularity in CD4$^+$ T cell subsets.

CD8$^+$ T cells are cells involved in the clearance of pathogens after many acute viral infections in the lung. CD8$^+$ T cells utilize multiple effector mechanisms to control viral replication. Studies are showing that memory CD8$^+$ T cells protect against recurrent infections caused by respiratory viruses such as influenza and SARS. Some studies have been published showing that naive CD8$^+$ T and CM CD8$^+$ T cells are decreased and TEMRA CD8$^+$ T cells are increased in COVID-19 patients. Intensive care patients have been shown to have a significant decrease in EM CD8$^+$ T cells and an increase in TEMRA CD8$^+$ T cells compared to non-intensive care patients. In our study, a decrease in naive CD8$^+$ T cells was observed in juvenile and adult patients. A significant increase in EM CD8$^+$ T cells was observed in patients in the age group of 0-12 years compared with age-matched healthy controls. It has been shown that EM CD8$^+$ T cells with a strong proliferative capacity can migrate into peripheral tissues and rapidly exert effector functions. The significant increase observed in EM CD8$^+$ T cells in the 0-12 age group may indicate remarkable protective immunity in juveniles in this age group. It has been reported that TEMRA CD8$^+$ T cells have high perforin concentrations with decreased proliferation and functional capacity. Of all the patient groups who participated in our study, the cases with severe pneumonia had the highest levels of TEMRA CD8$^+$ T cells. This suggests that in severely ill patients, the ability to proliferate may gradually decrease due to the high proliferation of CD8$^+$ T cells.

Our study is the first to investigate EM CD8$^+$ T cell subsets in COVID-19. EM$_1$ CD8$^+$ T cells in patients with severe pneumonia cases were significantly increased compared with patients with uncomplicated and mild pneumonia. In patients with severe pneumonia, EM$_1$ CD8$^+$ T cells are markedly reduced, whereas EM$_3$ CD8$^+$ T cells are increased (Figure 3E). Compared with EM$_1$ CD8$^+$ T cells, EM$_3$ CD8$^+$ T cells are known to have a stronger cytolytic activity. There was no significant change in EM CD8$^+$ T cell subsets (EM$_1$, EM$_2$, EM$_3$ and EM$_4$) in juvenile patients compared with healthy controls. The distribution of EM CD8$^+$ T cells in juvenile patients is maintained, but the differentiation of EM CD8$^+$ T cells from EM$_1$ to EM$_3$ in
patients with severe pneumonia cases (Figure 3E) seems to attract attention in studies to prevent the worsening of COVID-19 disease.

The percentage of DN T cells was decreased in all adult patient groups compared with healthy controls. No significant change was observed in juvenile cases. One of the studies has shown that DN T cells exert immunosuppressive effects and have a negative correlation with T cell activation. While another study found a significant decrease in DN T cells in adult patients, the lack of significant change in juvenile cases suggests that this may be a major cell group involved in the pathogenesis of adult COVID-19. When DN T cells were examined based on their CD45RA and CCR7 expression profiles, it was found that EM and TEMRA DN T cells can produce IFN-γ in psoriasis. In our study, TEMRA DN T cells were dominant in cases with severe and mild pneumonia, whereas EM DN T cells occupied this position in uncomplicated cases (Figure 3G). While the CM DN T cell frequency decreased as the disease severity increased in adult patients, an increase was found in the 0-12 age group compared to the healthy control group in juveniles. Regarding EM DN T cell subsets, no significant difference was observed in juvenile patients compared with healthy control subjects, and EM1 DN T cells decreased and EM1 DN T cells increased in patients with severe pneumonia compared with healthy adult controls (Figure 3H). These data suggest that circulating EM DN T cells tend to be more differentiated depending on the severity of the disease. While uncomplicated cases showed a similar profile to the healthy control group in terms of TEMRA DN T cell subsets, cases with severe pneumonia showed a decrease in pE1 DN T cells and an increase in E DN T cells. In the uncomplicated cases, no change was observed in the TEMRA DN T subsets (pE1, pE2 and E), but interestingly, the pE1 and pE2 DN T cells decreased and the E DN T cells increased in the age group 13-18 years (Figure 3I). This irregularity in the TEMRA DN T cell subsets in the 13-18 age group may be tolerated by the low frequency of TEMRA DN T cells in these patients. In addition, DN T cells are reduced by half in all adult
patient groups compared with the healthy control group due to SARS-CoV-2 infection. This significant reduction in peripheral blood may be the result of two conditions. First, there may be a disruption of DN T cell production. Second, DN T cells from the peripheral blood might migrate to tissues where they could be involved in inflammation (especially in the lungs, heart, etc.). With further experiments to be performed, this obscurity can be elucidated and considerable results obtained for the pathogenesis of COVID-19.

Programmed cell death protein 1 (PD-1) is one of the well-characterized inhibitory receptors associated with T cell exhaustion, and these cells are characterized by decreased cytokine production and proliferative capacity.33 The expression of PD-1 was increased in all CD4+ T, CD8+ T and DN T cells in all our patient groups, which consisted of juveniles and adults, and tended to decrease in patients followed up after recovery. These findings we have made are compatible with and at the same time extend the literature. The senescence of T cells is characterized by increased expression of CD57. These cells are characterized by shortened telomeres and replicative senescence, resulting in decreased proliferative capacity and the inability to eliminate infections.33–35 We observed higher expression of CD57 with increasing disease severity in our adult patient groups. This could be the result of high T cell proliferation in severe pneumonia cases.

On the basis of this study, statistically significant differences were found between severe pneumonia and uncomplicated cases, particularly with respect to EM3 T cells. Correlation analyses with these cell groups also yielded interesting results in severe pneumonia cases. Many studies have reported significant changes in lymphocyte count, LDH, CRP, ferritin and D-dimers with disease severity.36,37 Consistent with these studies, in our study, we not only observed that laboratory parameters and clinical manifestations varied with disease severity but also obtained considerable evidence that these changes were related to EM3 cells. These results suggest that EM3 cells are a candidate biomarker that could indicate the disease severity of COVID-19.

Finally, we are aware that our work has some limitations. First, the major limitation of this study is the very small number of inpatients who were followed up. As a result of our phenotypic analyses, we found an increase in T cell subsets that are thought to have potent cytolytic activity in severe pneumonia. However, we do not have additional experiments showing the functional activities of these cells (perforin, CD107a degranulation, granzyme B, etc.). The fact that all juvenile patients included in the study had asymptomatic/presymptomatic infections complicated the classification of disease groups in juveniles. Further research is needed to determine the impact of T cell subsets in adult and juvenile COVID-19 patients and their influence on disease severity.

Overall, the results of our study at different disease severity levels suggest that there are significant changes, particularly in EM T cell subsets. However, it was found that the EM T cell subsets in juvenile patients generally had a similar profile to healthy controls. In severe pneumonia cases, the frequency of EM1 CD8+ T and EM1 DN T cells producing low levels of effector mediators decreased, whereas the frequency of EM1 CD4+ T, EM2 CD8+ T and EM3 DN T cells with stronger cytolytic activity and effector properties increased. These data we have obtained show that EM T cells play a crucial role in the COVID-19 severity of the disease. Our research will provide further insight into studies that may explain why COVID-19 can cause death in adult patients, whereas it is generally mild in juveniles. Exploring and regulating the mechanisms of T cell differentiation in COVID-19 is crucial for establishing a successful immune response against SARS-CoV-2.

**AUTHOR CONTRIBUTIONS**

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**CONFLICT OF INTEREST**

The authors declare that there are no conflicts of interest.

**DATA AVAILABILITY STATEMENT**

Data collected and/or analysed in the current study are available from the corresponding author upon reasonable request.
ETHICS APPROVAL AND CONSENT TO PARTICIPATE
This study protocol was approved by the Ethics Committee of Bursa Uludag University, Faculty of Medicine ( Permit number: 2020-9/22) Bursa, Turkey, and all subjects gave written informed consent.

CONSENT FOR PUBLICATION
All authors have reviewed the manuscript and agreed to its publication.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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