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Pronounce expression of Tim-3 and CD39 but not PD1 defines CD8 T cells in critical Covid-19 patients

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ABSTRACT

Background: During viral infection, inhibitory receptors play a key role in regulating CD8 T-cell activity. The objective of this research was to investigate programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), and CD39 exhaustion markers in CD8 T cells of new coronavirus disease-2019 (COVID-19) patients.

Methods: A total of 44 patients with COVID-19 (17 subjects in a critical group and 27 patients in a non-critical group) and 14 healthy controls, who were admitted to Hospitals in Babol, were recruited to the study. In subjects' peripheral blood mononuclear cells (PBMCs), we compared the phenotype of CD8 T lymphocytes, expressing PD-1, TIM-3, or CD39, both alone and in various combinations.

Results: The findings showed that the percentage of $CD8^+$ cells was significantly lower in patients. Critical and non-critical patients were more likely than healthy controls to have an escalated frequency of $CD8^+$ $TIM\text{-}3^+$, $CD8^+$ $CD39^+$, and $CD8^+$ $TIM\text{-}3^+$ $CD39^+$ cells. No significant differences were observed between all groups in the $CD8^+$ $PD\text{-}1^+$ cell counts. There was also no difference between three groups regarding the counts of $CD8^+$ $TIM\text{-}3^+$ $PD\text{-}1^+$, $CD8^+$ $PD\text{-}1^+$ $CD39^+$, and $CD8^+$ $TIM\text{-}3^+$ $PD\text{-}1^+$ $CD39^+$ cells. The counts of non-exhausted cells were significantly lower in critical and non-critical individuals compared to the healthy individuals' value.

Conclusion: Patients, infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), altered exhausted CD8 T lymphocytes with CD39 and TIM-3 exhaustion markers, which may account the dysregulated immune response found in COVID-19.

1. Introduction

Today, an outbreak of new coronavirus disease-2019 (COVID-19) due to RNA virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is threatening the public health [1]. The World Health Organization (WHO) has listed the outbreak of COVID-19 as a Public Health Emergency of International Concern (PHEIC) [2,3].

Most of the infected patients have mild disease, diagnosed with shared signs and symptoms such as dry cough, fever, fatigue, dyspnea, and myalgia [3]. Some of the patients especially older ones with comorbidities, such as diabetes, hypertension, chronic obstructive pulmonary disease (COPD), cardiomyopathy, chronic renal failure, and weak immunity, seemed to be at a higher risk of developing a critical illness [4].

Tools for the early prediction of critical illness are very important for prognosis and treatment of COVID-19 patients. The development of these tools depends on the knowledge of the disease pathogenesis and helps to discover new treatment and develops vaccine strategies.

Several studies showed that the adaptive immune system responds to coronavirus, as well as to SARS-CoV-2, and is necessary for effective clearance of the virus [5,6]. The reduced numbers of CD4 and CD8 T cells were seen in patients infected with SARS-CoV-2, suggesting impaired cellular immunity, especially in critical patients [7,8].

It has been found that, during chronic viral infections, virus-specific

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CD8 T cells, which gradually lose effector functions, can eventually become exhausted [9]. Several studies have identified the presence or high-level expression of a number of inhibitory receptors such as programmed death protein 1 (PD-1), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), T cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), CD39 *(ENTPD1)*, Lymphocyte-activation gene 3 (LAG3), and 2B4 (CD244) on exhausted T cells [9–12]. However, there is poor information regarding the exhausted CD8 T cells in COVID-19. Therefore, we aimed to study exhaustion markers of PD-1, CD39, and TIM-3 on CD8 T cells of patients infected with SARS-CoV-2.

2. Materials and methods

Informed consent was obtained from all subjects. The study was approved by the Ethics Committee of Babol University of Medical Sciences (reference number: IR.MUBABOL.REC.1399.178) and was done in accordance with the Helsinki Declaration.

2.1. Study population and data collection

Forty-four patients with newly diagnosed COVID-19 (17 subjects in a critical group and 27 patients in a non-critical group) and 14 healthy control subjects were included in the study. COVID-19 patients were diagnosed with typical clinical symptoms (i.e., dry cough, fever, and shortness of breath) and radiological characteristics (i.e., chest X-ray or chest computed tomography [CT]). Then, the patients with positive SARS-CoV-2 real-time reverse transcription-polymerase chain reaction (RT-PCR) test of respiratory secretions were continuously recruited. Fourteen healthy adults were enrolled as a control group. These subjects were considered healthy in that they had no known history of COVID-19 manifestations. All of the healthy subjects had negative SARS-CoV-2 real-time RT-PCR result of respiratory secretions.

The medical records of all cases were collected for demographic characteristics and clinical features. All variables were recorded by the healthcare specialists caring for the patients in the hospitals. The medical records of all patients were abstracted by using a hospital data collection instrument.

2.2. Peripheral blood mononuclear cell isolation

In this research, 5 mL of heparinized venous blood was collected from each person, and the Ficoll-Hypaque density gradient centrifugation procedure was used for isolation of the peripheral blood mononuclear cells (PBMCs). In brief, the whole blood samples were centrifuged for 5 min at 350 g, and the plasma was discarded. The phosphate-buffered saline (PBS) was added at a 1:1 ratio to the packed cells. Then, the tubes were centrifuged for 20 min at 400 g in RT, and a buffy coat ring was collected. Human TruStain FcX (Biolegend, USA) was used for preventing background dye signals, caused by FCγRs of lymphocyte and monocytes. This treatment allows the antibodies specifically attach to the given markers.

2.3. Flow cytometry

To analyze exhausted CD8 lymphocytes, 10^6 of PBMCs were washed twice with staining buffer (PBS containing 0.5% bovine serum albumin [BSA]) and then stained with FITC-labeled anti-human CD8 (Clone SK1), PD-1-PerCp-Cy5.5 (Clone EH12.2H7), CD39-PE (Clone A1), and APC-labeled anti-human TIM-3 (Clone F38-2E2) (all from Biolegend, San Diego, CA, USA) for 20 min on ice in the dark. The conjugated antibodies dissolved in 50 μL staining buffer and were added to each test tube. After another two washes with staining buffer, cells were resuspended in 1 mL of staining buffer. Cell samples were analyzed on a BD FACSCalibur flow cytometer.

Each subset of the exhausted cells was then detected according to the antibody staining. Resultant data were assessed by using the BD CellQuest Pro software and analyzed via FlowJo software version 7.6.1 (Tree Star, USA). Lymphocytes were gated based on their forward and side scatter properties. After gating, the proportions of each exhausted subpopulation were determined (Figs. 1 and 2).

2.4. Statistical analysis

Continuous variables were described by mean ± standard deviation (*SD*). Categorical variables are shown as numbers and percentages. We compared the distributions of cytotoxic T cells across the groups initially by using appropriate univariate methods ($\chi 2$ test, one-way analysis of variance (ANOVA), and Student's t-test). Subsequently, linear mixed-effects models were employed to estimate the adjusted mean difference (MD_{adj}) between groups. The models were adjusted for the following potential confounders: age, sex, existing medical problem, smoking, white blood cell (WBC), and lymphocyte. Statistical analyses were performed on Stata 16.0 (Stata Corp, College Station, TX, USA). All statistical tests were two-tailed at the significance level of $p < 0.05$.

3. Results

3.1. Clinical findings

Seventeen patients in critical and 27 patients in non-critical groups consecutively enrolled in this case-control study. The mean age was 53.43 years (95% confidence intervals [CI] [48.88, 57.97]; range: 14–88), and 23 patients (52.27%, 95% CI [37.51, 67.03]) were male. Twenty-three patients (54.76%, 95% CI [39.70, 69.81]) had at least one comorbidity. At least one comorbidity was seen more commonly in critical cases than in non-critical cases (76.47% vs. 44.44%; $p = 0.037$) and healthy controls (76.47% vs. 6.66%; $p < 0.001$). On admission, a total of seven (15.90%, 95% CI [5.10, 26.71]) patients showed leukocytosis (WBC count $>11 \times 10^9$/L), 11 patients (25.01%, 95% CI [12.20, 37.79]) showed lymphopenia (lymphocyte count $<1.0 \times 10^9$/L), and 38 patients (86.36%, 95% CI [76.22, 96.50]) showed increasing in C-reactive protein (CRP) (>10 mg/L). Critical cases were more likely to have increased CRP (100% vs. 77.78%; $p = 0.036$) compared with non-critical cases. There was no difference between the two groups regarding the prevalence of leukocytosis (11.76% vs. 18.52%; $p = 0.551$) and lymphopenia (64.71% vs. 81.48%; $p = 0.211$). As compared with moderate patients, the respiratory rate (94.12% vs. 37.04%; $p < 0.001$) and decreased oxygen saturation (100% vs. 85.19%; $p = 0.096$) were higher among critical patients (see Table 1).

3.2. Distribution of $CD8^+$ lymphocytes

The count of $CD8^+$ cells in controls and two case groups is shown in Figs. 1 and 3. There was a significant difference between the critical cases and healthy controls in terms of $CD8^+$ cell counts (*MD*: 13.42, 95% CI [-18.13, −8.71]; $p < 0.001$). However, no significant difference was observed between the critical and non-critical cases in this regard (*MD*: 3.28, 95% CI [-8.71, 2.13]; $p = 0.228$). Also, the percentage of $CD8^+$ cell counts was significantly lower in non-critical patients compared to controls (*MD*: 10.13, 95% CI [-15.40, −4.87]; $p < 0.001$) (Figs. 1 and 3). The difference remained significant for critical patients and healthy adults (*aMD*: 6.74, 95% CI [-13.38, −0.11]; $p = 0.046$) and non-significant for critical and non-critical patients (*aMD*: 0.90, 95% CI [-5.69, 3.88]; $p = 0.711$) after adjusting for potential confounders. Also, the difference remained significant for non-critical patients and healthy controls (*aMD*: 5.83, 95% CI [-11.40, −0.27]; $p = 0.040$) (Table 2).

3.3. Tim-3 and CD39 but not PD1 expression on CD8 T cells increase in critical covid-19 patients

In the univariate analysis, critical patients were more likely to have

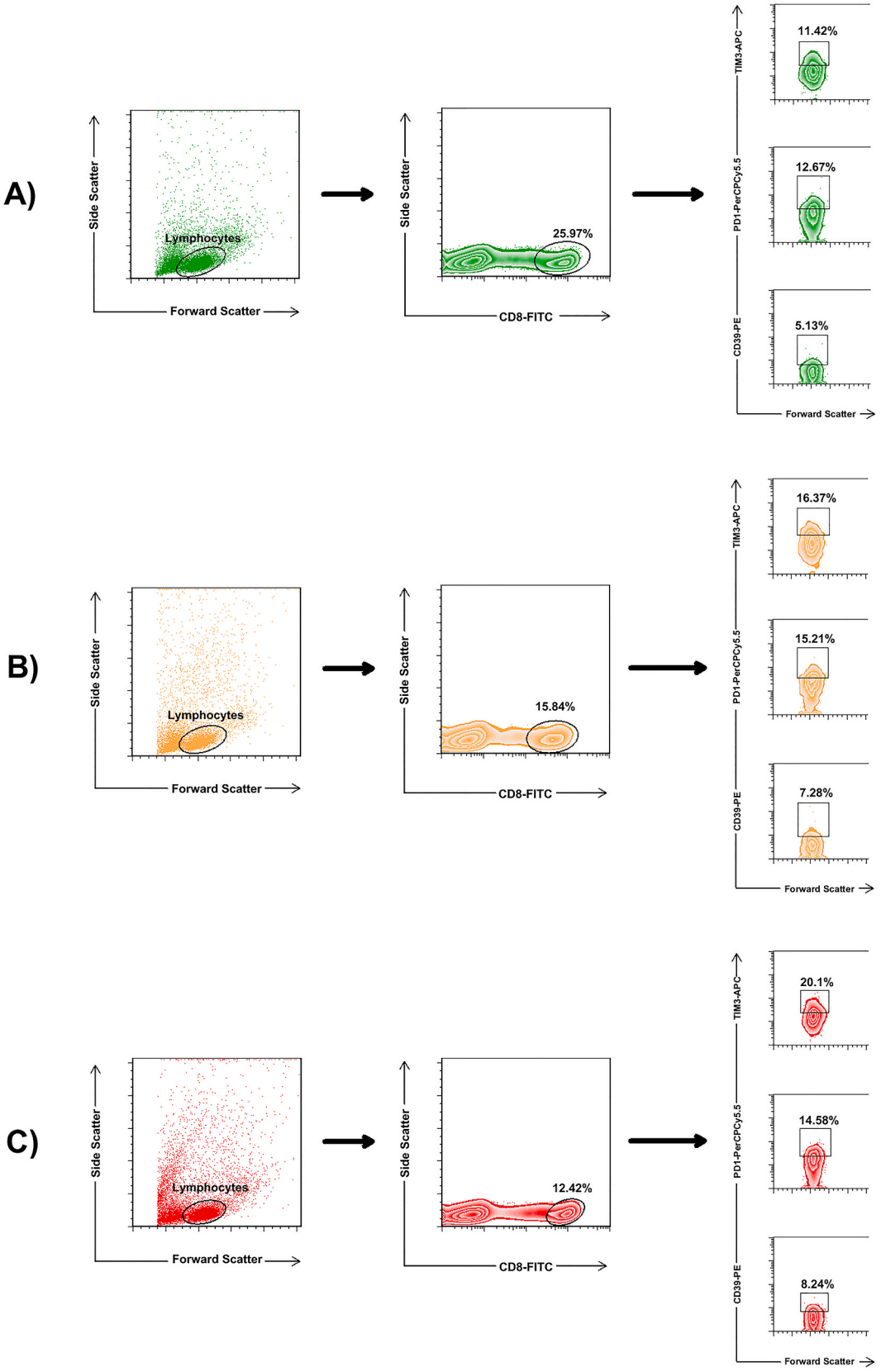

Fig. 1. The high expression of TIM-3 and CD39 on CD8 T cells of patients with SARS-CoV-2 infection. *Note.* PBMCs were stained with CD8-FITC, PD-1-PerCp-Cy5.5, CD39-PE, and TIM-3-APC conjugated monoclonal antibodies and analyzed with FACSCalibur flow cytometer. Lymphocytes were gated based on forward and side scatters; the expression levels of exhaustion markers, including TIM-3, CD39, and PD-1 in CD8 T cells, were then detected according to the antibody staining. A: the healthy control group; B: the non-critical group; C: the critical group; PBMCs: peripheral blood mononuclear cells.

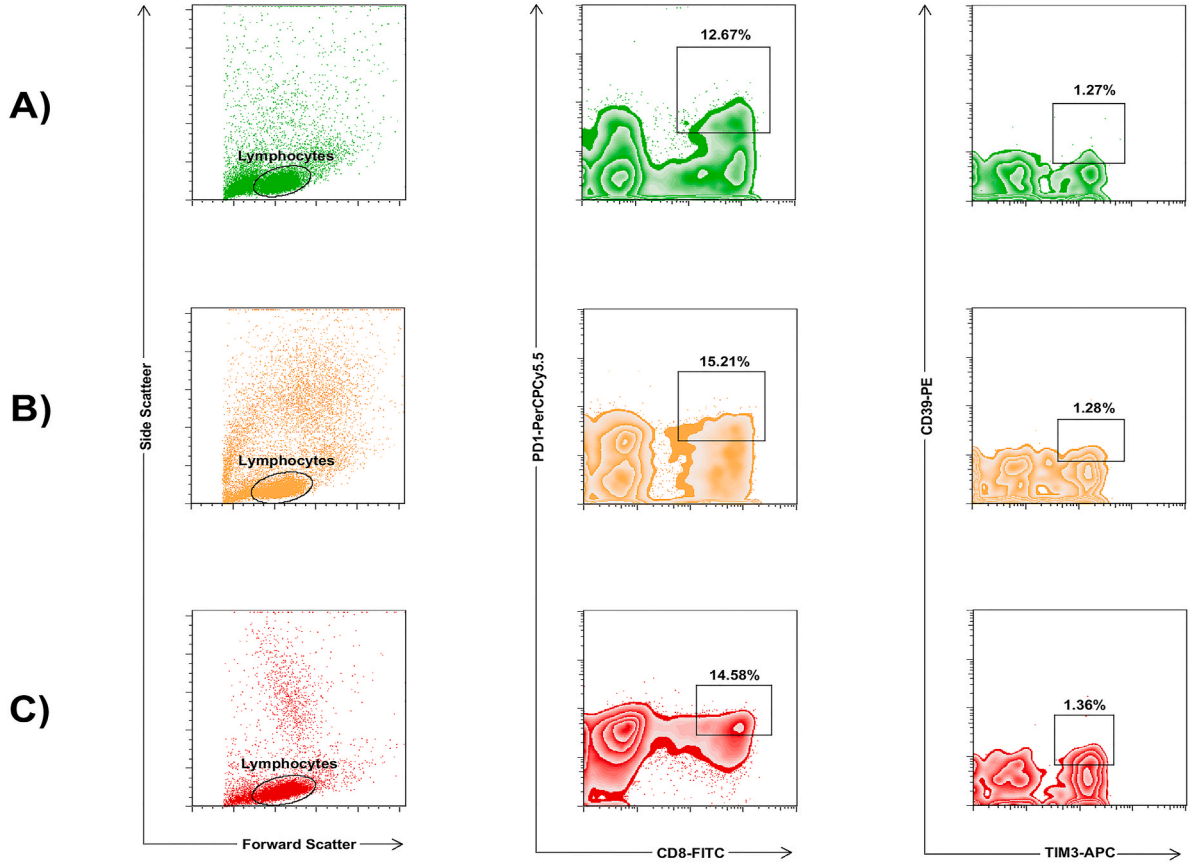

Fig. 2. The concomitant expression levels of TIM-3 and CD39 were significantly higher in CD8$^+$ cells of COVID-19 patients. *Note.* PBMCs were stained with CD8-FITC, PD-1-PerCp-Cy5.5, CD39-PE, and TIM-3-APC conjugated monoclonal antibodies and analyzed with FACSCalibur flow cytometer. CD8 T cells were initially gated from the lymphocyte population in order to analyze the obtained graphs, and then the concomitant expression levels of exhaustion markers were determined in the CD8$^+$ cell populations. A: the healthy control group; B: the non-critical group; C: the critical group; PBMCs: peripheral blood mononuclear cells.

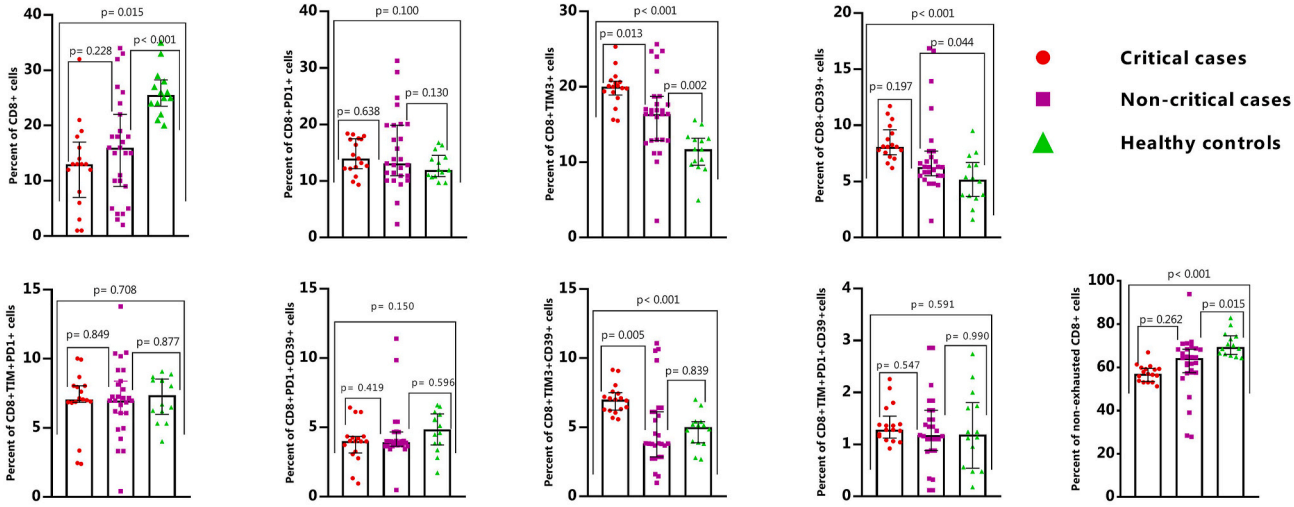

Fig. 3. The subset analysis of the exhausted CD8$^+$ cells in patients with COVID-19 and healthy subjects. *Note.* (A) The percentage of each subset was calculated in CD8$^+$ cells. Each dot represents a single patient or a healthy subject. $P < 0.05$ was considered statistically significant.

escalated CD8$^+$ TIM-3$^+$ cell counts compared to healthy controls (*MD*: 8.41, 95% CI [6.51, 10.31]; $p < 0.001$) and non-critical patients (*MD*: 3.46, 95% CI [0.76, 6.15]; $p = 0.013$). Also, the percentage of CD8$^+$ TIM-3$^+$ cell was significantly higher in non-critical patients compared to controls (*MD*: 4.95, 95% CI [1.95, 7.94]; $p = 0.002$) (Figs. 1 and 3). The multivariable analysis indicated that the mean of CD8$^+$ TIM-3$^+$ cell counts is significantly higher in critical patients compared to controls (*aMD*: 12.05, 95% CI [8.83, 15.26]; $p < 0.001$) and non-critical patients (*aMD*: 5.37, 95% CI [3.02, 7.72]; $p < 0.001$). Also, the difference remained significant for non-critical patients and controls (*aMD*: 6.67, 95% CI [4.01, 9.33]; $p < 0.001$) (Table 2).

No significant differences in the CD8$^+$ PD-1$^+$ cell counts were

Table 1
Background characteristics of participants.

	Critical cases (n = 17)	Non-critical cases (n = 27)	Healthy controls (n = 14)	P-value[a]
Age (year)	54.23 (16.46)	60.03 (17.40)	39.71 (8.79)	<0.001
Sex				
Male	9 (52.94)	14 (51.85)	10 (71.43)	0.451
Female	8 (47.06)	13 (48.15)	4 (28.57)	
WBC				
2800–11000	15 (88.24)	22 (81.48)	14 (100)	0.225
11001–19300	2 (11.76)	5 (18.52)	0 (0)	
Lymphocyte				
490–1000	6 (35.29)	5 (18.52)	0 (0)	0.008
1001–4400	11 (64.71)	22 (81.48)	14 (100)	
C-reactive protein				
0–10	0 (0)	6 (22.22)	13 (92.86)	<0.001
10–493	17 (100)	21 (77.78)	1 (7.14)	
Comorbidities				
Yes	13 (76.47)	12 (44.44)	1 (6.66)	0.001
No	4 (23.53)	15 (55.56)	14 (93.34)	
BUN	39.81 (24.53)	22.09 (20.43)	15.01 (3.62)	<0.001
AST	122.87 (180.47)	40.81 (27.38)	21.38 (4.03)	<0.001
ALT	182.18 (298.79)	37.77 (26.19)	18.01 (6.75)	0.027
NLR	8.18 (4.52)	4.12 (2.38)	2.16 (0.60)	<0.001
PLR	198.92 (116.24)	188.60 (109.67)	114.31 (40.78)	0.001

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

[a] Continuous variables compared with independent *t*-test, categorical variables compared with chi-square test or Fisher exact test.

observed between all groups (Figs. 1 and 3). The adjusted *MD* did not change substantially and remained non-significant for critical patients and healthy controls (*aMD*: 4.09, 95% CI [-0.18, 8.36]; $p = 0.061$), critical and non-critical patients (*aMD*: 0.12, 95% CI [-3.01, 3.24]; $p = 0.940$), as well as non-critical patients and controls (*aMD*: 3.97, 95% CI [0.42, 7.51]; $p = 0.028$) (Table 2).

The percentage of $CD8^+$ $CD39^+$ cell in healthy controls was 5.13 ± 2.17, which was lower than that of the critical patients (8.47 ± 1.55; $p < 0.001$) and non-critical patients (7.28 ± 3.52; $p = 0.044$) (Figs. 1 and 3). The three groups differed in respect of $CD8^+$ $CD39^+$ cell counts; the critical patients had more cells than the healthy controls (*aMD*: 5.37, 95% CI [3.12, 7.62]; $p < 0.001$) and non-critical patients (*aMD*: 2.23, 95% CI [0.59, 3.88]; $p = 0.008$). Also, the non-critical patients had more cells compared with the healthy controls (*aMD*: 3.13, 95% CI [1.26, 4.99]; $p = 0.001$).

3.4. Increased frequency of $CD8^+$ TIM-3^+ $CD39^+$ cells in critical covid-19 patients

The percentage of $CD8^+$ TIM-3^+ $CD39^+$ cell was observed significantly more frequently in critical cases than in controls (*MD*: 2.24, 95% CI [1.41, 3.08]; $p < 0.001$) and non-critical patients (*MD*: 2.08, 95% CI [0.65, 3.51]; $p = 0.005$), but there was no difference between non-critical patients and controls (*MD*: 0.16, 95% CI [-1.43, 1.75]; $p = 0.839$) (Figs. 2 and 3). In a separate linear mixed-effects model, after adjustment for potential confounders, we observed a significant difference between critical patients and healthy adults (*aMD*: 3.72, 95% CI [2.01, 5.42]; $p < 0.001$), as well as between critical patients and non-critical patients (*aMD*: 2.80, 95% CI [1.56, 4.05]; $p < 0.001$). Moreover, the difference remained non-significant for critical patients and healthy controls (*aMD*: 0.91, 95% CI [-0.49, 2.32]; $p = 0.204$) (Table 2). In the crude and multivariate analysis, there was no difference between the three groups regarding the counts of $CD8^+$ TIM-3^+ PD-1^+, $CD8^+$ PD-1^+ $CD39^+$, and $CD8^+$ TIM-3^+ PD-1^+ $CD39^+$ (Table 2).

3.5. Frequency of $CD8^+$ TIM-3^- PD-1^- $CD39^-$ cells

In the bivariate analysis, the counts of $CD8^+$ TIM-3^- PD-1^- $CD39^-$ cells were significantly lower in critical (*MD*: 13.46, 95% CI [-16.99, −9.93]; $p < 0.001$) and non-critical individuals (*MD*: 9.63, 95% CI [-17.28, −1.98]; $p = 0.015$) compared to the healthy individuals' value without differences between critical and non-critical individuals (*MD*: 3.82, 95% CI [-10.61, 2.96]; $p = 0.262$). In the multivariate model, significant differences were observed between the critical patients and controls (*aMD*: 21.51, 95% CI [-29.31, −13.70]; $p < 0.001$), non-critical patients and controls (*aMD*: 13.77, 95% CI [-20.24, −7.30]; $p < 0.001$), as well as critical and non-critical patients (*aMD*: 7.73, 95% CI [-13.43, −2.03]; $p = 0.008$).

4. Discussion

T cells, especially CD8 lymphocytes, are pivotal for the host's cellular immune response against viral infection, as they can kill virus-infected cells directly [13,14]. Therefore, CD8 T lymphocytes are crucial to generate an appropriate antiviral response in patients infected with SARS-CoV-2 [15] and also for viral clearance subsequent acute viral infections [16]. The acute phase of SARS-CoV2 infection in humans was correlated with a severely decreased numbers of T cells in the blood, including a considerable loss of CD8 T cells [4,17].

In the present study, the frequency of CD8 T lymphocytes was considerably reduced in patients with SARS-CoV-2 infection in both the critical and non-critical groups, which is in accordance with previous reports and demonstrates that the frequency of lymphocytes could be used as a diagnostic indicator of COVID-19 in the clinic [4,18]. A single

Table 2
Association between COVID-19 infection and subsets of exhausted cells.

	Critical patients vs. healthy controls		Non-critical patients vs. healthy controls		Critical patients vs. Non-critical patients	
Cells	MD_{adj}	95% CI	MD_{adj}	95% CI	MD_{adj}	95% CI
$CD8^+$	−6.74	−13.38, −0.11	−5.83	−11.40, −0.27	−0.90	−5.69, 3.88
CD8 + TIM3 +	12.05	8.83, 15.26	6.67	4.01, 9.33	5.37	3.02, 7.72
CD8 + PD1 +	4.09	−0.18, 8.36	3.97	−0.42, 7.51	0.12	−3.01, 3.24
CD8 + CD39 +	5.37	3.12, 7.62	3.13	1.26, 4.99	2.23	0.59, 3.88
CD8 + TIM3 +$CD39^+$	3.72	2.01, 5.42	0.91	−0.49, 2.32	2.80	1.56, 4.05
CD8 + TIM3 + PD1 +	0.20	−1.79, 2.19	0.46	−1.18, 2.11	−0.26	−1.72, 1.18
CD8 + PD1 +$CD39^+$	−0.29	−1.80, 1.21	−0.16	−1.41, 1.08	−0.12	−1.23, 0.97
CD8 + TIM3 + PD1 +$CD39^+$	0.23	−0.31, 0.79	0.11	−0.35, 0.57	0.12	−0.27, 0.53
Non-exhausted cells	−21.51	−29.31, −13.70	−7.73	−13.43, −2.03	−13.77	−20.24, −7.30

MD_{adj}: adjusted mean difference; 95% CI: 95% confidence interval.
†Mean difference estimated directly from linear mixed effect model. The final multivariable models were adjusted for the following risk factors: age, sex, existing medical problems, smoking, white blood cells, lymphocyte, and CD_4.
*Significant at P value < 0.05.

marker does not properly identify exhausted cells. Therefore, we evaluated the level of three exhaustion molecules, including PD-1, TIM-3, and CD39 in CD8 T cells by flow cytometry. CD8 T cells of COVID-19 patients (notably critical ones) showed increased exhaustion markers, i.e., CD39 and TIM-3, which suggests that impaired antiviral immunity may play a key role in the severity and pathogenesis of SARS-CoV-2 infection.

In our study, the frequency of $CD8^+$ $TIM\text{-}3^-$ $PD\text{-}1^-$ $CD39^-$ cells was significantly lower in the COVID-19 cases (especially in critical ones), which shows that SARS-CoV-2 triggers an early over-activation and high cytotoxicity of lymphocytes in the onset of the disease, continued by exhaustion of them. Thus, it is suggested that the excessive exhaustion of CD8 T lymphocytes weakens cellular immunity to SARS-CoV-2 in critical patients.

Several studies have demonstrated that exhausted CD8 T cells with CD39 and TIM-3 exhaustion markers increased in the peripheral blood of patients with a variety of viral infections caused by RNA viruses, such as HBV, HCV, HIV, and LCMV [10,19–21]. In addition, the interaction of TIM-3 on T cells with its ligand galectin-9 results in apoptosis of CD8 T lymphocytes in viral infections [22,23]. In this study, the TIM-3 exhaustion marker was upregulated in CD8 T lymphocytes of COVID-19 patients, which describes the observed lymphopenia. Additionally, CD39 is extensively expressed by virus-specific CD8 T lymphocytes, and its expression correlates with viral load in patients infected with HIV and HCV [11]. The elevated expression level of CD39 in COVID-19 patients, notably in the critical group, suggests that high viral antigen load may lead to exhaustion in the virus-specific pool of CD8 T lymphocytes by increasing CD39 expression.

However, there are some limitations in this study. First, the sample size was relatively small in our study. Second, we only studied peripheral blood CD8 lymphocytes in the context of exhaustion. A further study of CD8 cells from alveolar lavage fluid is demanded. Third, an additional analysis of lymphocytes with other exhaustion markers, such as CTLA-4, 2B4, and LAG-3, helps to better understand the disease immunopathology. Fourth, the study of exhaustion in SARS-CoV-2 specific CD8 T cells gives a precise concept.

In summary, our study suggested the appearance of TIM-3 and CD39 as exhaustion markers in CD8 lymphocytes of SARS-CoV-2 patients. Critical patients have more frequency of $CD8^+$ $TIM\text{-}3^+$ and $CD8^+$ $TIM\text{-}3^+$ $CD39^+$ lymphocytes than non-critical patients, implying that immunological dysfunction (T cell exhaustion) may exacerbate the patients' status. Thus, preventing T cell exhaustion may contribute to better function of the immune system against COVID-19 patients.

Author statement

M-MA, M-M, and M-SH drafted the manuscript. M-MA, M-B, Z-M, M-R, M-J, M-S, and M-SH performed the experiments. M-SH and M-MA Supervisioned the experiments. All authors participated in data analysis and revision of the manuscript and approved the final version.

Declaration of competing interest

The authors declare no competing financial interests. The authors alone are responsible for the content and writing of the paper.

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References

[1] N. Zhu, D. Zhang, W. Wang, X. Li, B. Yang, J. Song, et al., A novel coronavirus from patients with pneumonia in China, 2019, N. Engl. J. Med. 382 (8) (2020) 727–733.
[2] W.H. Organization, Statement on the Second Meeting of the International Health Regulations (2005) Emergency Committee Regarding the Outbreak of Novel Coronavirus (2019-nCoV), 2005.
[3] C. Huang, Y. Wang, X. Li, L. Ren, J. Zhao, Y. Hu, et al., Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China, Lancet 395 (10223) (2020) 497–506.
[4] N. Chen, M. Zhou, X. Dong, J. Qu, F. Gong, Y. Han, et al., Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study, Lancet 395 (10223) (2020) 507–513.
[5] M.Z. Tay, C.M. Poh, L. Rénia, P.A. MacAry, L.F. Ng, The trinity of COVID-19: immunity, inflammation and intervention, Nat. Rev. Immunol. (2020) 1–12.
[6] R. Channappanavar, J. Zhao, S. Perlman, T cell-mediated immune response to respiratory coronaviruses, Immunol. Res. 59 (1–3) (2014) 118–128.
[7] J. Liu, S. Li, J. Liu, B. Liang, X. Wang, H. Wang, et al., Longitudinal characteristics of lymphocyte responses and cytokine profiles in the peripheral blood of SARS-CoV-2 infected patients, EBioMedicine (2020) 102763.
[8] B. Diao, C. Wang, Y. Tan, X. Chen, Y. Liu, L. Ning, et al., Reduction and functional exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19), Front. Immunol. 11 (827) (2020).
[9] E.J. Wherry, T cell exhaustion, Nat. Immunol. 12 (6) (2011) 492–499.
[10] H.T. Jin, A.C. Anderson, W.G. Tan, E.E. West, S.J. Ha, K. Araki, et al., Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection, Proc. Natl. Acad. Sci. U. S. A 107 (33) (2010) 14733–14738.
[11] P.K. Gupta, J. Godec, D. Wolski, E. Adland, K. Yates, K.E. Pauken, et al., CD39 expression identifies terminally exhausted CD8+ T cells, PLoS Pathog. 11 (10) (2015), e1005177.
[12] H. Mohammadizad, M. Shahbazi, M.R. Hasanjani Roushan, M. Soltanzadeh-Yamchi, M. Mohammadnia-Afrouzi, TIM-3 as a marker of exhaustion in CD8+ T cells of active chronic hepatitis B patients, Microb. Pathog. 128 (2019) 323–328.
[13] S. Rosendahl Huber, J. van Beek, J. de Jonge, W. Luytjes, D. van Baarle, T cell responses to viral infections - opportunities for Peptide vaccination, Front. Immunol. 5 (2014) 171.
[14] M.E. Schmidt, S.M. Varga, The CD8 T cell response to respiratory virus infections, Front. Immunol. 9 (678) (2018).
[15] M. Zheng, Y. Gao, G. Wang, G. Song, S. Liu, D. Sun, et al., Functional exhaustion of antiviral lymphocytes in COVID-19 patients, Cell. Mol. Immunol. 17 (5) (2020) 533–535.
[16] A. Retamal-Díaz, C. Covián, G.A. Pacheco, A.T. Castiglione-Matamala, S.M. Bueno, P.A. González, et al., Contribution of resident memory CD8+ T cells to protective immunity against respiratory syncytial virus and their impact on vaccine design, Pathogens 8 (3) (2019) 147.
[17] D. Wang, B. Hu, C. Hu, F. Zhu, X. Liu, J. Zhang, et al., Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China, Jama 323 (11) (2020) 1061–1069.
[18] F. Wang, H. Hou, Y. Luo, G. Tang, S. Wu, M. Huang, et al., The laboratory tests and host immunity of COVID-19 patients with different severity of illness, JCI insight 5 (10) (2020) 137799.
[19] L. Golden-Mason, B.E. Palmer, N. Kassam, L. Townshend-Bulson, S. Livingston, B. J. McMahon, et al., Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells, J. Virol. 83 (18) (2009) 9122–9130.
[20] R.B. Jones, L.C. Ndhlovu, J.D. Barbour, P.M. Sheth, A.R. Jha, B.R. Long, et al., Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection, J. Exp. Med. 205 (12) (2008) 2763–2779.
[21] H. Mohammadizad, M. Shahbazi, M.R.H. Roushan, M. Soltanzadeh-Yamchi, M. Mohammadnia-Afrouzi, TIM-3 as a marker of exhaustion in CD8+ T cells of active chronic hepatitis B patients, Microb. Pathog. 128 (2019) 323–328.
[22] J.A. Mengshol, L. Golden-Mason, T. Arikawa, M. Smith, T. Niki, R. McWilliams, et al., A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C infection, PloS One 5 (3) (2010).
[23] S. Sehrawat, P.B.J. Reddy, N. Rajasagi, A. Suryawanshi, M. Hirashima, B.T. Rouse, Galectin-9/TIM-3 interaction regulates virus-specific primary and memory CD8 T cell response, PLoS Pathog. 6 (5) (2010) e1000882-e.