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Increased percentage of apoptotic and CTLA-4 (CD152) expressing cells in CD4+/CD8+ cells in COVID-19 patients

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Abstract

Coronavirus infectious disease 2019 (COVID-19) confirmed cases are characterized by T lymphopenia. Total apoptotic and cytotoxic T-lymphocyte antigen-4 (CTLA-4) expressing cells among CD4+/CD8+ cells were analyzed in 24 COVID-19 patients (16 out-patients and 8 in-patients) and 18 healthy volunteers using flow cytometry to detect their possible role in T lymphopenia. Hospitalized patients did not show significant difference compared to non-hospitalized patients. While the percentage and absolute count of CD4+/CD8+ cells were significantly reduced in COVID-19 cases compared to healthy control ($P < .05$), the proportion of apoptotic and CTLA-4 expressing CD4+/CD8+ cells were significantly up-regulated in COVID-19 patients ($P < .05$). In addition, apoptotic and CTLA-4+/CD4+ cells were directly related to dyspnea duration, chest CT score, ferritin, and C-reactive protein and inversely correlated with platelet count in COVID-19 patients. While apoptotic and CTLA-4+/CD8+ cells were directly related to lymphocyte count in COVID-19 patients. The apoptotic and CTLA-4+ cells were directly related to each other in CD4+/CD8+ cells ($P < .05$). White blood cells (WBCs) ($\times 10^3$/L), eosinophils (ratio and count), lymphocyte ratio, neutrophil ratio, neutrophil/lymphocyte ratio, neutrophil/CD4 ratio, neutrophil/CD8 ratio, CD4+ cells ratio, and CTLA-4+ cells percentage), and CD8+ cells (ratio, count, total apoptotic cell, and CD152 + cells) were all found to be significantly altered in association with COVID-19. Total lymphopenia and depletion of CD4+/CD8+ cells are characterizing COVID-19 patients. Increased apoptosis and CTLA-4 expression in CD4+/CD8+ cells in COVID-19 and their correlations with reduced cell count and severity indicators as CRP and ferritin can be used for diagnosis and follow up of the clinical severity. Our current study proposes promising future diagnostic and therapeutic targets.

Abbreviation: CD4+ = cluster of differentiation 4+, CD8+ = cluster of differentiation 8+, COVID-19 = coronavirus infectious disease 2019 novel coronavirus, CRP = C reactive protein, CTLA-4 = cytotoxic T-lymphocyte antigen-4, FITC = fluoroisothiocyanate, PI = propidium iodide, RT-PCR = reverse transcriptase-polymerase chain reaction, SARS-CoV-2 = severe acute respiratory syndrome coronavirus-2.

Keywords: apoptosis, COVID-19, CTLA-4 (CD152), infection, SARS-CoV-2

1. Introduction

Coronavirus infectious disease 2019 (COVID-19) is an ongoing pandemic caused by a novel coronavirus, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Most of the infected cases presented either with no or mild to moderate symptoms. Reports of severe cases were associated with a dysregulated immune response.[1–4] Attention is being paid to developing

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Institutional Review Board (IRB), Qena Faculty of Medicine, Qena, Egypt approved this study. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was gathered from all participants.

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novel therapeutic modalities, including those modulating the host immune system. Studying the influences of COVID-19 on immune cell specificity and functionality can produce clues to understanding the mechanism of the disease and suggest biomarkers that can be used as therapeutic targets in the COVID-19 setting.

Wuhan study assessed the immune response induced by COVID-19 in 452 laboratory-confirmed patients and showed CD4 lymphopenia more often in severe cases, with intact cytotoxic T cells and B cells. Later on, a number of reports came to support the same concept of increased neutrophil count at the expense of lymphocyte count that may be exhausted and damaged by COVID-19.[4–10] Lessons from previous viral epidemics and animal experiments in the current pandemic show possible protective T cell responses against SARS-CoV-2 infection.[11,12]

Lymphocyte exhaustion may be due to the dysfunction of apoptotic pathways of the host cell and/or the immune system that interacts with these pathways. Previous reports from other coronaviruses revealed that these viruses induce apoptosis during their replication.[13–15] An increase in apoptosis was reported among mononuclear cells from COVID-19 patients as well, which was more pronounced in severe cases.[16]

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) (CD152) and CD28 are homologous receptors expressed by both $CD4^+$ and $CD8^+$ T cells, which mediate opposing functions in T-cell activation. Both receptors share a pair of ligands, CD80 and CD86, expressed on antigen-presenting cells' surface. CTLA-4 interacts with both ligands with higher affinity and avidity than CD28[17] and acts as an antagonist of CD28-mediated co-stimulation.[18] Robins et al showed the attribution of T cell exhaustion to the elevation of the CTLA-4 marker on the peripheral blood mononuclear cells of critically ill COVID-19 patients.[19] Data on the factors standing behind lymphopenia and T cell exhaustion in COVID-19 patients remains limited. We aimed to assess the $CD4^+/CD8^+$ apoptosis and the expression of CTLA-4 in COVID-19 patients and their relation to disease severity.

2. Patients and Methods

2.1. Patient selections

A prospective case-control study included 24 patients who were admitted to Qena University hospital, Upper Egypt during the period from August 2020 to September 2020. With a confirmed positive result for SARS-CoV-2 using reverse transcriptase-polymerase chain reaction (RT-PCR) from an upper respiratory tract (nose/throat) swab tested in accredited laboratories.

The patients were 21 males (87.5%) and 3 females (12.5%). Their mean age was 30.6 ± 14. The data was compared with that of 18 age and sex-matched healthy individuals, their mean age was 34.7 ± 12.5 ($P = .326$) whose SARS-CoV-2 RT-PCR test showed negative results. The study was done in accordance with Helsinki guidelines, considered, and accepted by the Institutional Review Board (IRB), Qena Faculty of Medicine, Qena, Egypt, [SVU-MED-CHT019, 4-20-8-64], and all participants gave informed consent to participate in the study.

The complete history and clinical symptoms or signs were evaluated. Laboratory assessments included complete blood count and D-dimer, C-reactive protein (CRP), and ferritin levels. A Chest CT was done to diagnose COVID-19 pneumonia. Peripheral blood samples were collected from all participants in ethylinediaminetetraacetate acid vacutainer tubes for the flow cytometry.

2.2. Chest CT

Chest CT was done to diagnose COVID-19 pneumonia which revealed multisegmental consolidative areas with a score 18 c or multisegmental peripheral ground glass opacities with a score 3 A according to the modified total severity scoring system "Supplemental Digital Content (Figure S1, Supplemental Digital Content 1, http://links.lww.com/MD/H352)."

2.3. Flow cytometric detection of apoptosis

Peripheral blood samples were collected from all participants in ethylinediaminetetraacetate acid vacutainer tubes for the flow cytometry.

2.3.1. Flow cytometric detection of apoptosis of $CD4^+$/$CD8^+$ cells. Apoptosis was measured by the Annexin V- (AV) fluoroisothiocyanate (FITC) binding assay according to the manufacturer's instructions (BD Biosciences, San Jose, CA). A 100 µL of whole blood was stained with 10 µL of peridinium-chlorophyl-protein-conjugated CD8 and APC-conjugated CD4, washed twice with 2 mL of phosphate-buffered saline and red blood cells were lysed. The cells were washed and re-suspended in 100 µL of the AV-conjugate binding buffer, to which 10 µL of FITC-conjugated AV and 10 µL of Propidium iodide (PI) were added. After incubation for 15 minutes, 400 µL of the binding buffer was added. Analysis was done using FACSCalibur flow cytometry (BD Biosciences, San Jose, CA). Anti-human IgG was used as an isotype-matched negative control with each sample. A forward and side scatter histogram was used to identify the lymphocyte population. Then, the percentages of $CD4^+$ and $CD8^+$ cells were assessed in the lymphocyte population, and the total percentage of apoptotic cells [AV^+PI^- early apoptotic and AV^+PI^+ late apoptotic cells][20,21] was then calculated among the $CD4^+$ and $CD8^+$ cells "Supplemental Digital Content (Figure S2, Supplemental Digital Content 2, http://links.lww.com/MD/H353)."

2.3.2. $CD4^+/CD8^+$ expression of CTLA-4 (CD152) A total of 10 µL peripheral blood was stained with 10 µL of FITC conjugated CD152, peridinium-chlorophyl-protein-conjugated-CD8, and APC conjugated- CD4 (Becton-Dickinson, San Jose, CA). Isotype controls were included for each sample. A total of 50,000 events were acquired for each sample using a FACSCalibur flow cytometer. Then, the percentages of CD152 were assessed in the $CD4^+$ and $CD8^+$ cells.

2.4. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 21. The data were tested for normality using Shapiro–Wilk test based on $P < .05$ Mann–Whitney U test was used to compare continuous variables, and Chi-square tests were used to compare categorical, and ordinal variables between the studied groups and for $P > .05$ Student t test was used. Correlations were performed via the ***Pearson*** correlation coefficient. To calculate the odd's ratio, a logistic regression model was used for factors associated with infection. To start with, a univariable logistic regression was done and variables significant at a $P > .2$ were included in the multivariable model. Statistical significance was set at $P < .05$

The sample size in this study was calculated by "EBI" program at power 80%, with confidence 95.0%, α 0.5 equal 42 patients divided in 2 groups.

3. Results

3.1. Demographic data of the studied group

The study included 24 patients with a confirmed positive test for SARS-CoV-2. The patients were 21 males (87.5%) and 3 females (12.5%). Their mean age was 30.6 ± 14. The data was compared with that of 18 age and sex-matched healthy individuals, their mean age was 34.7 ± 12.5 ($P = .326$) whose SARS-CoV-2 RT-PCR test showed negative results.

3.2. Clinical and laboratory finding in COVID-19 patients

We studied 24 COVID-19 patients, 25% gave a clear history of exposure to a confirmed COVID-19 case. Most of them had fever (87.5%), cough (70.8%), and dyspnea (50%), and a few were complaining of anosmia (20.8%) and diarrhea (16.7%). Chest CT showed minimal ground glass (<25%) in 3 patients (12.5%), 3 others showed moderate ground glass (>25%), and 5 patients (20.8%) had acute respiratory distress syndrome. Patients were further stratified according to their hospital status into in-patients (No. 8) and out-patients (No. 16) to address any clinical or laboratory differences among them. The results revealed that dyspnea was commoner among in-patients than out patients (P = .014), in addition worse CT findings were commoner among in-patients than out patients (P = .001) (Table 1).

Laboratory finding did not significantly vary among COVID-19 patients based on their hospital status, ferritin level was higher in in-patients than in out-patients (P = .018) (Table 1).

3.3. Baseline laboratory features of COVID-19 infected patients

On comparing COVID-19 cases with healthy control, the mean white blood cell (WBC) count significantly decreased in COVID-19 patients (5.6, confidence interval [CI] (4.6, 6.7) and 7.3, CI (4.6, 6.7), P = .01). Moreover, mean lymphocyte count was considerably lower in COVID-19 patients (1.04, CI (0.88, 1.2) and 3.1, CI (2.6, 3.7), P < .0001), no significant alteration was detected in the neutrophil count between patients and healthy controls. The CRP mean level in COVID-19 patients was 15.8, CI (9.2, 22). Additionally, the mean D-dimer level was 422.9 ± 53 mg/L, and that of ferritin was 45.24, CI (280.9, 627.5) ng/mL (Table 2).

Interestingly, a marked decrease in the mean neutrophil/lymphocyte ratio, neutrophil/CD4 ratio, neutrophil/CD8 ratio but not CD4/CD8 ratio was found in COVID-19 patients when compared with that of healthy controls (Table 2).

Table 1

Clinical and laboratory characteristics of 24 COVID-19 patients.

	In-patients	Out-patients	
	No. 8	No. 16	*P* value
Clinical characteristics			
Fever (No. 21 (87.5%))	7	14	.723
Anosmia (No. 5 (20.8%))	5	0	.103
Dyspnea (No. 12 (50%))	7	5	.014
Cough (No. 17 (70%))	10	7	.218
GIT disturbances (No. 4 (16.7%))	2	2	.165
Chest CT			.001
Free (No. 13 (54.2%))	1	12	
Minimal ground glass <25% (No. 3 (12.5%))	0	3	
Moderate ground glass >25% (No. 3 (12.5%))	2	1	
>Ground glass or ARDS (No. 5 (20.8%))	5	0	
Laboratory characteristics			
WBCs (×10^3/μL)	6, 95% CI (4. 7.9)	5.5, 95% CI (4.1, 6.9)	.601
Eosinophils ratio	2, 95% CI (0.5. 3.5)	2.6, 95% CI (1.3, 3.9)	.551
Eosinophils count (×10^3/μL)	0.1, 95% CI (0.04. 0.1)	0.1, 95% CI (0.08, 0.2)	.383
Lymphocyte ratio	21, 95% CI (11, 30)	20.7, 95% CI (11.5, 30)	.996
Lymphocyte count (×10^3/μL)	1.1, 95% CI (0.7, 1.5)	1, 95% CI (0.8, 1.2)	.339
Neutrophil ratio	68, 95% CI (59, 78)	68, 95% CI (62, 74)	.944
Neutrophil count (×10^3/μL)	4.2, 95% CI (2.5, 5.9)	3.9, 95% CI (2.7, 5)	.714
Hemoglobulin g/dL	14.7, 95% CI (13, 16)	14, 95% CI (13, 15)	.55
Platelet count	296, 95% CI (193, 398)	318, 95% CI (283, 353)	.507
D dimer (mg/L)	514, 95% CI (211, 817)	377, 95% CI (236, 518)	.202
CRP (mg/dL)	18.4, 95% CI (3.7, 33)	14.5, 95% CI (6.2, 22.7)	.924
Ferritin (ng/mL)	667, 95% CI (201, 1134)	348, 95% CI (196, 499)	.018
Neutrophil/lymphocyte ratio	0.3, 95% CI (0.1, 0.5)	0.3, 95% CI (0.2, 0.5)	.891
Neutrophil/CD4 ratio	0.1, 95% CI (0.04, 0.17)	0.1, 95% CI (0.06, 0.14)	.811
Neutrophil/CD8 ratio	0.07, 95% CI (0.03, 0.1)	0.06, 95% CI (0.04, 0.09)	.861
CD4/CD8 ratio	1.4, 95% CI (1.4, 1.8)	1.6, 95% CI (1.4, 1.9)	.712
CD4$^+$ cells			
Absolute count (×10^9/L)	0.4, 95% CI (0.2, 0.5)	0.3, 95% CI (0.2, 3)	.187
Relative to lymphocyte count (%)	47, 95% CI (44, 49)	48, 95% CI (43, 52)	.805
Total apoptotic CD4$^+$ cells (%)	16, 95% CI (14, 19)	16, 95% CI (13, 18)	.395
Total apoptotic CD4$^+$ cells count	0.06, 95% CI (0.04, 0.08)	0.04, 95% CI (0.03, 0.05)	.137
CD4$^+$ CTLA-4$^+$ cells (%)	13.2, 95% CI (10.6, 15.8)	11.7, 95% CI (10.2, 13.3)	.355
CD4$^+$ CTLA-4$^+$ cells count	0.04, 95% CI (0.03, 0.06)	0.03, 95% CI (0.03, 0.04)	.09
CD8$^+$ cells			
Absolute count (×10^9/L)	0.2, 95% CI (0.2, 0.3)	0.2, 95% CI (0.1, 0.2)	.103
Relative to lymphocyte count (%)	30, 95% CI (28, 32)	30, 95% CI (27, 33)	.882
Total apoptotic CD8$^+$ cells (%)	8.7, 95% CI (8, 9)	9.8, 95% CI (8.6, 11.1)	.19
Total apoptotic CD8$^+$ cells count	0.02, 95% CI (0.1, 0.03)	0.02, 95% CI (0.01, 0.02)	.35
CD8$^+$ CTLA-4$^+$ cells (%)	8.3, 95% CI (5.2, 11.5)	8.2, 95% CI (6.4, 10.1)	.925
CD8$^+$ CTLA-4$^+$ cells count	0.02, 95% CI (0.01, 0.03)	0.01, 95% CI (0.01, 0.02)	.251

ARDS = acute respiratory distress syndrome, CD4$^+$ = cluster of differentiation 4$^+$, CD8$^+$ = cluster of differentiation 8$^+$, CI = confidence interval, COVID-19 = coronavirus infectious disease 2019 novel coronavirus, CRP = C-reactive protein, CT = computed tomography, CTLA-4$^+$ = cytotoxic T-lymphocyte antigen-4$^+$, GIT = gastrointestinal tract, WBCs = white blood cells.

Table 2

Baseline laboratory features of COVID-19 infected patients.

Features	Patients (n = 24)	Controls (n = 18)	*P* value
WBCs (×10³/μL)	5.6, CI (4.6, 6.7)	7.3 CI (4.6, 6.7)	.011
Eosinophils ratio	2.4, CI (1.5, 3.3)	4 CI (3.2, 4.8)	.003
Eosinophils count (×10³/μL)	.12, CI (.08,.15)	.29 CI (.22,.36)	<.0001
Lymphocyte count (×10³/μL)	1.04, CI (.88, 1.2)	3.1 CI (2.6, 3.7)	<.0001
Neutrophil count (×10³/μL)	4, CI (3.1, 4.8)	3.3 CI (2.8, 3.8)	.426
D dimer (mg/L)	422.9,CI (297.7, 548)	–	–
CRP (mg/dL)	15.8,CI(9.2,22)	–	–
Ferritin (ng/mL)	454.24, CI (280.9, 627.5)	–	–
Neutrophil/ lymphocyte ratio	4, CI (3.2,.4.8)	1.2, CI (.95, 1.4)	<.0001
Neutrophil/CD4 ratio	13.6, CI (10.7, 16.5)	3.1, CI (2.5, 3.8)	<.0001
Neutrophil/CD8 ratio	21.1, CI (16.9, 25.3)	5.4, CI (4.2, 6.7)	<.0001
CD4/CD8 ratio	1.62, CI (1.43,1.82)	1.81, CI (1.5, 2.13)	.46

Results expressed as mean, 95% confidence interval for mean (CI), Mann–Whitney *U* test, a *P* value is significant if <.05.

CD4 = cluster of differentiation 4, CD8 = cluster of differentiation 8, COVID-19 = coronavirus infectious disease 2019 novel coronavirus, CRP = C-reactive protein, WBCs = white blood cells.

3.4. Percentages of the total apoptotic and CD152 expressing CD4⁺/CD8⁺ cells in COVID-19 patients and healthy controls

As presented in Figure 1A, both the CD4⁺ cells percentage (a) and count (b) showed marked reduction in COVID-19 patients when compared with the control group (50.3 ± 9.4 vs 56.8 ± 7.8, $P = .014$ and 0.3 ± 0.14 vs 1.2 ± 0.51, $P < .0001$, respectively). Meanwhile, a marked increase was detected in the levels of both the total apoptotic CD4⁺ cell percentage (c) and absolute count (d) (16.5 ± 3.7 vs 1.3 ± 0.3, $P < .0001$ and 0.06 ± 0.03 vs 0.02 ± 0.01, $P < .0001$, respectively). An increase in the CD152 expressing CD4⁺ cell percentage (e) was also seen in the COVID-19 patients in comparison with the controls (12.2 ± 2.8 vs 6.1 ± 3.2, $P < .0001$), while the absolute count of CD152 expressing CD4⁺ cells could not reach a significant increase due to CD4 lymphopenia in COVID-19 patients (0.04 ± 0.02 vs 0.07 ± 0.03, $P = .003$) (f) (Fig. 1A).

Likewise in Figure 1B, both the CD8⁺ cells percentage (a) and absolute count showed an obvious fall in COVID-19 patients than in the control group (28.7 ± 5.4 vs 33.6 ± 7.2, $P = .02$ and 0.2 ± 0.07 vs 0.7 ± 0.4 0.4, $P < .0001$, respectively). Contrariwise, a marked increase was noticed in the levels of both the total apoptotic CD8⁺ cell percentage (c) and absolute count (d) (9.8 ± 2 vs 2.2 ± 2.7, $P < .0001$ and 0.019 ± 0.01 vs 0.01 ± 0.01, $P = .002$, respectively). The proportion of CD152 expressing CD8⁺ cell (e) was higher in the COVID-19 patients in comparison with the controls (8.2 ± 3 vs 4 ± 2.3, $P < .0001$), while the calculated absolute CD152 expressing CD8⁺ cell count significantly reduced in COVID-19 patients as it is affected by CD8 lymphopenia (0.02 ± 0.01 vs 0.03 ± 0.01, $P = .003$) (Fig. 1B).

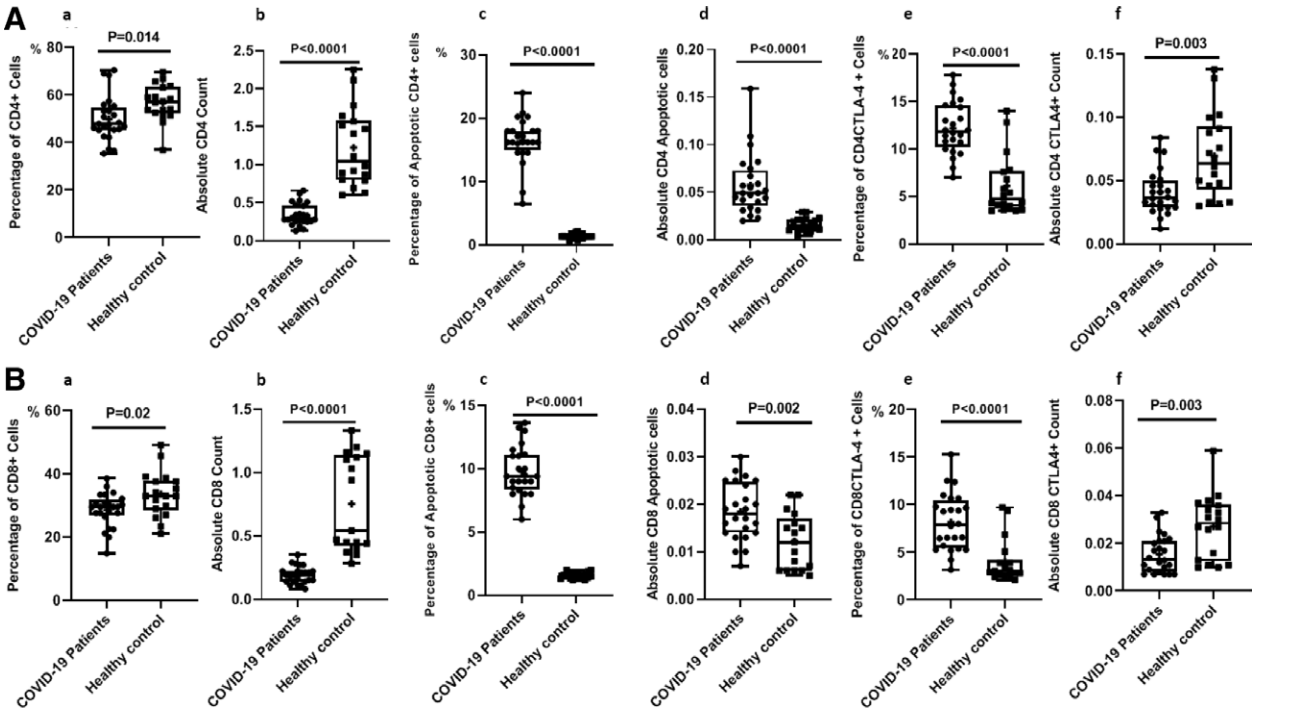

Figure 1. Percentages of the total apoptotic and CD152 expressing CD4⁺ and CD8⁺ cells in COVID-19 patients and healthy controls. (A) CD4⁺ cells percentage (a), CD4⁺ count (b), CD4⁺ total apoptotic cell percentage (c), CD4⁺ total apoptotic absolute count (d), CD4⁺ CD152 ⁺ ratio (e), and CD4⁺ CD152 absolute count (f) in the COVID-19 patients and healthy controls. (B) CD8⁺ cells percentage (a), CD8⁺ count (b), CD8⁺ total apoptotic cell percentage (c), CD8⁺ total apoptotic absolute count (d), CD8⁺ CD152 ⁺ ratio (e), and CD8⁺ CD152 absolute count (f) in the COVID-19 patients and healthy controls. CD4⁺ = cluster of differentiation 4⁺, CD8⁺ = cluster of differentiation 8⁺, COVID-19 = coronavirus infectious disease 2019 novel coronavirus.

3.5. Correlations of total apoptotic cells and clinical/laboratory parameters

Table 3 shows that in COVID-19 patients: the total CD4+ apoptotic cell count negatively correlated with platelet count and increased with higher ferritin and CRP ($R^2 = 0.269$, $P = .009$; $R^2 = 0.276$, $P = .008$ and $R^2 = 0.219$, $P = .021$, respectively). In the control group: the total CD4+ apoptotic cell count proportionally correlated with WBCs and lymphocyte count ($R^2 = 0.444$, $P = .003$ and $R^2 = 0.218$, $P = .05$, respectively) and negatively correlate with neutrophil/lymphocyte ratio ($R^2 = 0.333$, $P = .012$).

Interestingly, while WBCs proportionally correlate with CD8+ apoptotic cell count in healthy control. CD8+ apoptotic cell count proportionally correlated with lymphocyte count in both healthy control and COVID-19 patients (WBCs: $R^2 = 0.537$, $P = .001$ and $R^2 = 0.117$, $P = .002$, respectively; lymphocyte count: $R^2 = 0.699$, $P < .0001$ and $R^2 = 0.601$, $P < .0001$). Neutrophil/lymphocyte ratio negatively correlated with CD8+ apoptotic cell count in health control only ($R^2 = 0.355$, $P = .012$) (Table 3).

3.6. Correlations of CD4+/CD8+ expressing CTLA-4 (CD152) cells and laboratory parameters

Table 3 shows that CD4+ CTLA-4+ cell count negatively correlated with platelet count and positively correlate with CRP in COVID-19 patients ($R^2 = 0.283$, $P = .008$ and $R^2 = 0.164$, $P = .05$, respectively).

Interestingly, CD8+ CTLA-4+ cell count proportionally correlated with lymphocyte count ($R^2 = 0.346$, $P = .014$ in COVID-19 patients, and proportionally correlate with WBCs ($R^2 = 0.465$, $P = .002$), neutrophil count ($R^2 = 0.354$, $P = .009$) and lymphocyte count ($R^2 = 0.234$, $P = .014$) in healthy control (Table 3). Both apoptotic and CTLA-4+ CD4+ cells were directly related to dyspnea duration, chest CT score in COVID-19 cases (Apoptotic CD4+: dyspnea duration: $R^2 = 0.375$, $P = .001$ and

Table 3

Correlations of total apoptotic or CTLA-4 expressing cells in (CD4+/CD8+ cell) and laboratory value in COVID-19 patients versus healthy controls.

	COVID-19 patients		Healthy controls	
	r-factor	*P* value	*r*-factor	*P* value
Total apoptotic CD4+ cell count				
WBCs count	0.09	.66	0.47	.05
Eosinophil count	−0.26	.26	0.25	.31
Neutrophil count	0.22	.35	0.67	.003
Lymphocyte count	0.29	.17	0.003	.98
Neutrophil/lymphocyte count	−0.24	.3	−0.58	.01
Platelet count	−0.52	.009	–	–
Ferritin level	0.52	.008	–	–
D-dimer	0.21	.32	–	–
CRP	0.47	.02	–	–
HbA1C	0.39	.08	–	–
Total apoptotic CD8+ cell count				
WBCs count	0.34	.1	0.73	.001
Eosinophil count	−0.11	.62	0.15	.62
Neutrophil count	0.26	.26	0.25	.33
Lymphocyte count	0.78	<.001	0.84	<.001
Neutrophil/lymphocyte count	0.29	.21	0.6	.012
Platelet count	0.09	.66	–	–
Ferritin level	0.08	.72	–	–
D-dimer	0.07	.69	–	–
CRP	0.04	.84	–	–
HbA1C	0.08	.84	–	–
Total CD4+ CTLA-4 cell count				
WBCs count	0.13	.55	0.31	.2
Eosinophil count	0.4	.39	0.35	.15
Neutrophil count	0.22	.35	0.003	.98
Lymphocyte count	0.4	.05	0.45	.06
Neutrophil/lymphocyte count	0.29	.34	0.003	.98
Platelet count	−0.53	.008	–	–
Ferritin level	0.32	.13	–	–
D-dimer	0.17	.41	–	–
CRP	0.4	.05	–	–
HbA1C	0.35	.12	–	–
Total CD8+ CTLA-4 cell count				
WBCs count	0.1	.64	0.68	.002
Eosinophil count	−0.13	.58	0.31	.22
Neutrophil count	0.003	.98	0.59	.009
Lymphocyte count	0.59	.01	0.48	.014
Neutrophil/lymphocyte count	−0.22	.35	0.003	.98
Platelet count	0.003	.9	–	–
Ferritin level	0.19	.38	–	–
D-dimer	0.29	.16	–	–
CRP	−0.18	.38	–	–
HbA1C	0.21	.36	–	–

CD4 = cluster of differentiation 4, CD8 = cluster of differentiation 8, COVID-19 = coronavirus infectious disease 2019 novel coronavirus, CTLA-4 = cytotoxic T-lymphocyte antigen-4, CRP = C-reactive protein, WBCs = white blood cells.

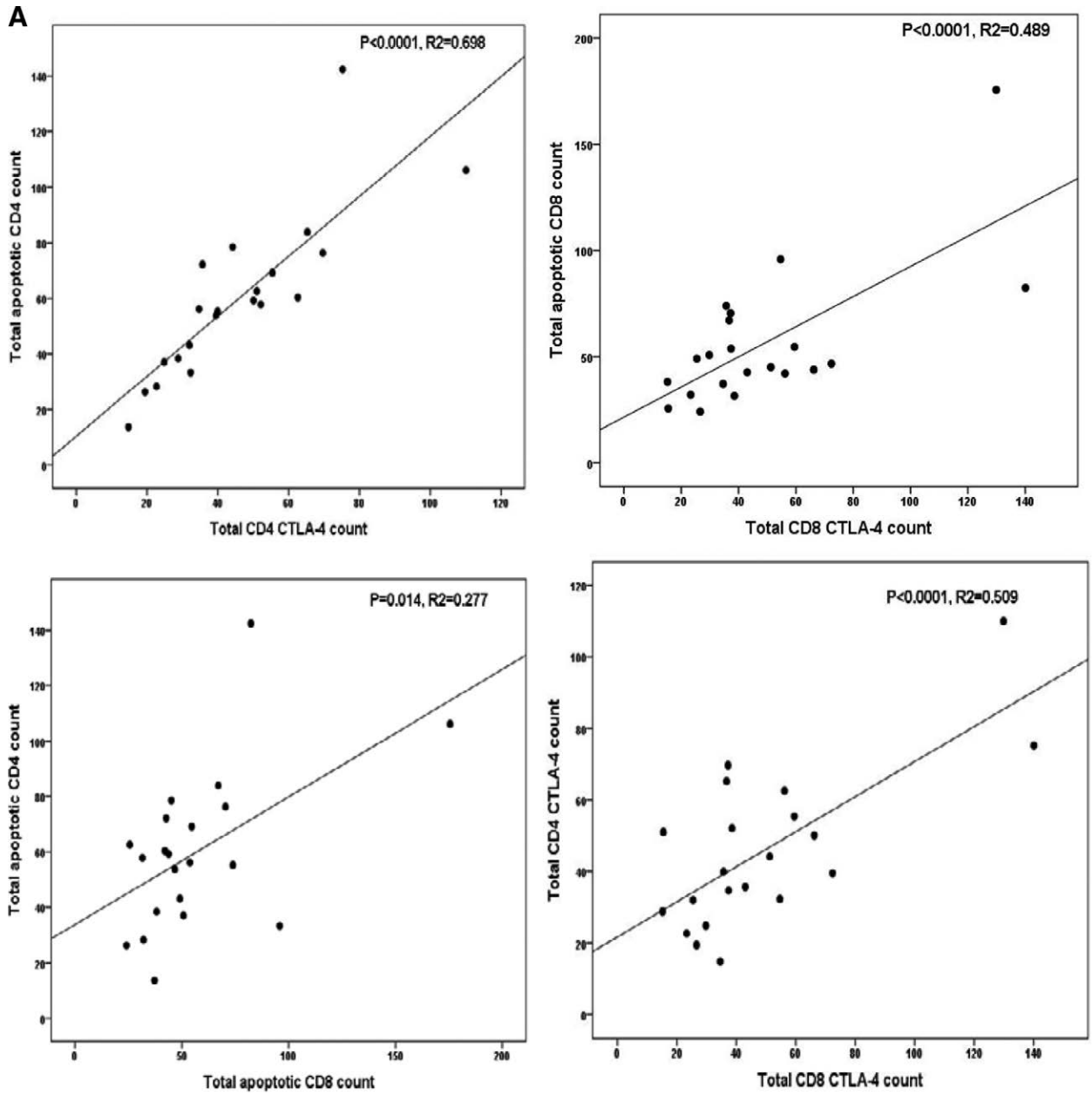

Figure 2. Correlations of total apoptotic and CD152 expressing CD4 and CD8 cells and laboratory parameters in COVID-19 patients (A) and healthy controls (B). CD4 = cluster of differentiation 4, CD8 = cluster of differentiation 8, COVID-19 = coronavirus infectious disease 2019 novel coronavirus.

chest CT score: $R^2 = 0.258$, $P = .015$, respectively; CD152 $CD4^+$: dyspnea duration: $R^2 = 0.375$, $P = .002$ and chest CT score: $R^2 = 0.261$, $P = .012$, respectively).

3.7. Relation between apoptotic and CTLA-4 (CD152) expressing $CD4^+/CD8^+$ cells in COVID-19 patients

Total apoptotic cells in $CD4^+/CD8^+$ cells proportionally correlated with their CTLA-4, furthermore, both total apoptotic and CTLA-4^+ $CD4^+$ cells proportionally correlate with that of $CD8^+$ cells (Fig. 2).

3.8. Regression analysis for COVID-19 patients and healthy controls

Regression analysis for COVID-19 revealed that WBCs ($X10^3$/μL), eosinophils (ratio and count), lymphocyte ratio, neutrophil ratio, neutrophil/lymphocyte ratio, neutrophil/CD4 ratio, neutrophil/CD8 ratio $CD4^+$ cells ratio, and CTLA-4^+ cells percentage) and $CD8^+$ cells (ratio, count, total apoptotic cells and $CD152^+$ cells) were significantly associated with COVID-19 using univariable analysis, but none of them was detected as an independent factor using multivariable analysis (Table 4).

4. Discussion

Current data on the contribution of T cells in COVID-19 pathogenesis is limited and inconsistent. Some reports attribute lung injury to T cells' high cytotoxic activity rather than a defective T cell response. An autopsy study proposed that direct infection of ACE2-expressing macrophages may result in activation-induced T cell apoptosis.[22] On the other hand, lymphopenia in COVID-19 patients may contribute to the severe disease being a probable cause of functional T cell exhaustion, principally in elderly and critically ill patients.[8]

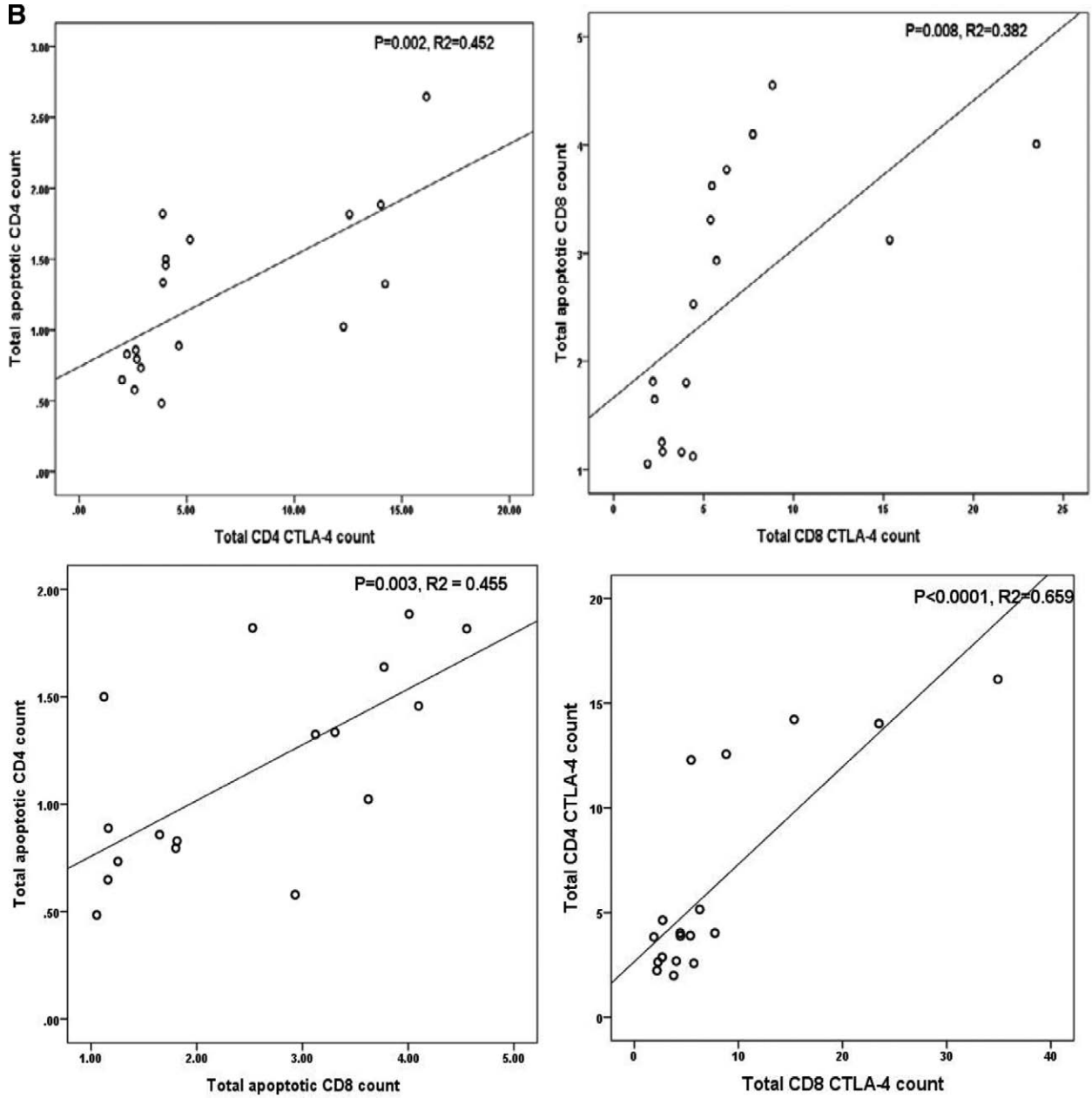

Figure 2. Continued

We analyzed the total apoptotic cells and CTLA-4 expression among CD4 and CD8 cells in patients with confirmed symptomatic COVID-19 and compared this data with that of normal individuals. Consistent with previous studies,[8,23,24] our results showed that both the $CD4^+$ and $CD8^+$ absolute cell counts and percentages showed a marked reduction in COVID-19 patients compared to healthy controls. A higher neutrophil/lymphocyte ratio is also reported in those critically ill patients during the critical stages, reflecting the serious disturbance of their internal environment.

Virus-induced direct cytopathic effects are believed to play a major role in disease severity.[25] A rapid innate immune response is crucial against viral infections. However, if it is not well coordinated, excessive inflammation and even death can occur.[3] In addition, pronounced $CD3^+$ and $CD4^+$ lymphopenia were observed in severe cases of COVID-19 cases, which may be the reason for aggravated inflammatory responses, cytokine storm production, and tissue damage. Although not conclusive, correlative evidence from those severe COVID-19 patients with lymphocyte reduction suggests a role for dysregulated immune responses in disease pathogenesis. Our results are also consistent with Hu et al, who reported reduced $CD3^+$ T cells and their $CD4^+$ and $CD8^+$ cell subsets in the dead pneumonic group compared with the surviving group, suggesting the activation and depletion of T cells in the antiviral process.[24] Similarly, Channappanavar et al, and others reported up to 100% of COVID-19 patients showing marked CD4 lymphopenia[3,8] and 87%, 76%, and 55% showing CD8, B cells, and NK cell decrease, respectively.[3] Consistent with Wang,[7] except for the CD4/CD8 ratio, we did not find statistically significant differences between the 2 groups. Studies with bigger sample size are needed to better understand lymphocyte subset dysregulation and CD4/CD8 ratio roles in COVID-19 pathogenesis and their therapeutic implications.

Further analysis of COVID-19 cases enrolled in our study revealed that total apoptotic $CD4^+$ and $CD8^+$ cells as well as

Table 4

Univariable regression analysis for COVID-19 patients and healthy controls.

Variable	*P* value
WBCs (×10³/μL)	.017
Eosinophils ratio	.018
Eosinophils count (×10³/μL)	.002
Lymphocyte ratio	.001
Lymphocyte count (×10³/μL)	.122
Neutrophil ratio	.001
Neutrophil count (×10³/μL)	.206
Neutrophil/lymphocyte ratio	.001
Neutrophil/CD4 ratio	.001
Neutrophil/CD8 ratio	.002
CD4/CD8 ratio	.276
Lymphocyte subset	
CD4+ cells	
Absolute count (×10⁹/L)	.083
Relative to lymphocyte count (%)	.025
Total apoptotic CD4⁺ cells (%)	.994
Total apoptotic CD4⁺ cells count	.009
CD4⁺ CTLA-4⁺ cells (%)	<.0001
CD4⁺ CTLA-4⁺ cells count	.006
CD8+ cells	
Absolute count (×10⁹/L)	.015
Relative to lymphocyte count (%)	.027
Total apoptotic CD8+ cells (%)	<.0001
Total apoptotic CD8+ cells count	.006
CD8+ CTLA-4+ cells (%)	.001
CD8+ CTLA-4+ cells count	.004

CD4⁺ = cluster of differentiation 4⁺, CD8⁺ = cluster of differentiation 8⁺, CTLA-4⁺ = cytotoxic T-lymphocyte antigen-4⁺, CRP = C-reactive protein, WBCs = white blood cells.

CTLA-4 expressing CD4⁺ and CD8⁺ cells are elevated in our cases. Although the absolute count is decreased in CD4⁺ and CD8⁺ this is possible due to the marked reduction in total CD4⁺ and CD8⁺ counts. The Total apoptotic CD4⁺ count increased, the higher ferritin level, and the raised CRP level, and both of them indicate inflammation and severity. On the other hand, the total apoptotic CD4⁺ count decreased, the higher platelet count. Interestingly, total apoptotic CD4⁺ increased the higher WBCs and lymphocyte count in healthy control but not in COVID-19 patients. In contrast to total apoptotic CD4⁺, we discovered that total apoptotic CD8⁺ increased the higher lymphocyte count and do not correlate with other severity markers. This indicates that although apoptotic cells were up regulated in CD4⁺ and CD8⁺ cells, they did not behave in the same way. Closer to the previous finding CD4⁺ CTLA-4⁺ was found to positively correlate with CRP and inversely correlate with platelet count. CD8⁺ CTLA-4⁺ positively correlate with lymphocyte count in COVID-19 patients. The level of apoptotic CD4⁺/CD8⁺ cells was significantly proportionate to the level of expression of CTLA-4 CD4⁺/CD8⁺ cells in both patients and control groups. This finding can explain the state of lymphopenia that characterized the COVID-19 setting.

In line with our finding, Taghiloo and colleagues showed the increase in apoptosis of mononuclear cells from COVID-19 patients, which was more prominent in severe cases. They attributed this to the possible production of autoantibodies due to the viral infection and the hematopoietic cell growth inhibition that can also reduce T cell generation and differentiation.[16]

CTLA-4 is known to deliver a robust co-inhibitory signal, leading to negative regulation of T cell activation and proliferation.[25] While the TCR and CD28 are ligand-activated, CTLA-4 activation can still inhibit cell cycle progression and cause T cell proliferation arrest by suppressing IL-2 production.[22] Furthermore, some authors reported CTLA-4 induced apoptosis as early as 24 hs after T cell activation.[26] Together with our previous findings, this may indicate the critical role of CTLA-4 in eliciting apoptosis in CD4⁺ T cells but not CD8⁺ T cells in the context of COVID-19, causing the profound T cell lymphopenia observed in those patients.

Even though in normal individuals, activated CD8 killer T cells express CTLA-4, higher levels are expressed by CD4⁺ T cells.[27] The primary physiologic role of CTLA-4 appears to be via its preferential effects on CD4⁺ T cell subsets.[28] This suggests that targeting CTLA-4 favorably affects the CD4⁺ T-cell function.[27,28]

Jeannet and others reported that CD4 T cells showed increased CTLA-4 and programmed death-1 (PD1) expression levels in severe COVID-19 patients, while CD8 T cells, which persistently expressed PD-1, didn't show a significant change in CTLA-4 expression.[29] In addition, An earlier study on HIV stated that unlike CD4⁺ T cells, CTLA-4 expression by CD8⁺ T cells was always low and that CD8 impaired responses to B7 co-stimuli were due to CD28 downregulation rather than increased CTLA-4 expression.[30]

Patients with severe COVID-19 exhibit lymphocytopenia and suffer from T-cell exhaustion. Based on observations that stemmed from the use of immune-checkpoint inhibitors in medical oncology, antibodies blocking the PD-1/PD ligand-1 (PD-L1) axis have given new hope for restoring T-cell competence and efficiently counteracting the SARS-Cov2 infection. Several clinical trials are currently open to examine the efficacy of anti-PD-1 antibody in COVID-19 individuals.[31]

Moreover, our results showed that ferritin and CRP levels had positive relations with the levels of the total apoptotic CD4⁺ cells. And These relations may reflect the presence of a possible link between apoptotic T cell levels and the extensive inflammatory reaction observed in severe cases. In addition, negative correlation observed between CD4⁺ CD152⁺ cell count and platelet count and CRP in COVID-19 patients can increase the chances of the possible anti-inflammatory effect of CD152 marker.

Strengths and limitations: our study is the first to investigate the relation between apoptosis and CD152 expression among CD4 and CD8 cells in COVID-19 and their relation with some severity indicators. However, further evaluation in larger studies, with a further investigation for other markers of apoptosis as CD95 and its mediators, may result in a better understanding of COVID19 pathogenesis and complications, which would help in tailoring a more effective therapy.

5. Conclusion

The present study showed increased apoptosis and CTLA-4 expression by CD4⁺ and CD8⁺ cells in COVID-19 patients. Besides, CTLA-4 and total apoptotic CD4⁺/CD8⁺ cell correlation that is related to clinical severity, proposes that CTLA-4 and total apoptotic cells can be used as reliable markers for diagnosis and prognosis of COVID-19 and can further be promising future targets for immune therapy.

The limitations of our study the small sample size and inability to measure other inflammatory markers as IL-6, to reduce the expenses in the epidemic times, no exclusion of natural killer cells inside CD4⁺ and CD8⁺ due to the limited gating stratiges in FACsCalibur.

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