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Changes in PD-1- and CTLA-4-bearing Blood Lymphocytes in ICU COVID-19 Patients Treated with Favipiravir/*Kaletra* or *Dexamethasone/Remdesivir*: A Pilot Study

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ABSTRACT

COVID-19, caused by SARS-CoV-2, requires new approaches to control the disease. Programmed cell death protein (PD-1) and cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) play important roles in T-cell exhaustion in severe COVID-19. This study evaluated the frequency of whole blood lymphocytes expressing PD-1 and CTLA-4 in COVID-19 patients upon admission to the intensive care unit (ICU) (i.e., severe) or infection ward (i.e., moderate) and after 7 days of antiviral therapy.

COVID-19 patients were treated with either favipiravir or Kaletra (FK group, 11 severe and 11 moderate) or dexamethasone plus remdesivir (DR group, 7 severe and 10 moderate) for 7 days in a pilot study. Eight healthy control subjects were also enrolled. The frequency of PD-1$^+$ and CTLA-4$^+$ lymphocytes in whole blood was evaluated by flow cytometry.

Patients on DR therapy had shorter hospital stays than those on FK therapy. The frequency of PD-1$^+$ lymphocytes in the FK group at baseline differed between COVID-19 patients and healthy controls, while the frequency of both PD-1$^+$ and CTLA-4$^+$ cells increased significantly 7 days of FK therapy. The response was similar in both moderate and severe patients. In contrast,

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the frequency of PD-1$^+$ and CTLA-4$^+$ lymphocytes varied significantly between patients and healthy controls before DR treatment. DR therapy enhanced PD-1$^+$ but not the CTLA-4$^+$ frequency of these cells after 7 days.

We show that the frequency of PD-1 and CTAL-4-bearing lymphocytes during hospitalization was increased in Iranian ICU COVID-19 patients who received FK treatment, but that the frequency of CTLA-4$^+$ cells was higher at baseline and did not increase in patients who received DR. The effectiveness of DR treatment may reflect differences in T-cell activation or exhaustion status, particularly in CTLA-4-expressing cells.

Keywords: Antiviral therapy; COVID-19; Cytotoxic T-lymphocyte–associated protein 4; Cytokine storm; Program cell death 1; T cells

INTRODUCTION

In December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes COVID-19, spread worldwide. The World Health Organization (WHO) officially declared COVID-19 a pandemic on 12 March 2020. Over 80% of patients have symptoms similar to the common cold and pneumonia; 14% have severe respiratory inflammation; and 5% present with respiratory failure, septic shock, organ dysfunction, or multiple organ failure.[1-3] This is associated with the release of proinflammatory cytokines following lung inflammation and organ failure in COVID-19 patients.[4,5] SARS-CoV-2 is a single-stranded RNA virus with crown-shaped projections (spikes or S proteins) that protrude from its coat. The viral S protein binds to angiotensin-converting enzyme-2 (ACE-2) receptors on the surface of target cells and thus attacks the cell.[3,6]

T-cell lymphopenia is usually seen in the severe form of the disease, which may be due to the infiltration of leukocytes into the airways or excessive T-cell activity resulting in the expression of proapoptotic molecules.[7-10] In animal models of COVID-19, lymphopenia causes overactive T cells.[11] It is possible that overactive T cells in COVID-19 cause lymphocyte loss. Patients with severe disease and evidence of a cytokine storm[3] may possess blood lymphocytes expressing cell surface markers of overactivation and exhaustion. These markers include programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3), lymphocyte-activation gene 3 (LAG3), cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), and the natural killer cell receptor (NKG2A) or CD39.[10,12] Therefore, the phenotype and function of lymphocytes can play an important role in the disease process and treatment.[11,13]

Little information is available regarding the impact of antiviral therapy on the phenotype of lymphocytes in COVID-19 patients. We hypothesized that antiviral therapy would alter the lymphocyte phenotype according to the clinical response. Thus, we measured the frequency of peripheral blood lymphocytes expressing PD-1 and CTLA-4 cell surface molecules in COVID-19 patients upon admission to the intensive care unit (ICU) and after 7 days of therapy with either favipiravir and Kaletra (FK group; from March to July 2020) or dexamethasone and remdesivir (DR group; from February to June 2021).

MATERIALS AND METHODS

Patients and Controls

All patients were diagnosed with COVID-19 according to the World Health Organization interim guidance[14]. Severe COVID-19 disease was confirmed based on previously defined criteria.[2,4] All participants were enrolled in the study upon admission to Masih Daneshvari Hospital of Shahid Beheshti Medical University, Tehran, Iran. Two treatment groups were selected for this investigation, and data were collected at baseline and after 7 days of treatment. The first group (FK group) wereas treated with favipiravir or Kaletra and included 22 confirmed COVID-19 patients: 11 with severe disease and 11 with moderate disease. These patients were enrolled in the study between March and July 2020. The treatment protocol for the FK group was the same as described earlier.[4] The DR group was treated with dexamethasone (100 mg/day) and remdesivir (200 mg on the first day and 100 mg/day for the following 7 days) and was composed of 7 severe and 10 moderate

COVID-19 patients enrolled in the study between 5 February and June 2021. The healthy control group was selected based on negative RT-PCR for SARS-CoV-2 and normal serum erythrocytre sedimentation rate (ESR) and C-reactive protein (CRP) ranges. We used the same control for the two groups and found no differences by analysis.

Data Collection

Clinical, laboratory, radiology, treatment, and outcome data were obtained from electronic medical records. The data were interpreted by the clinical research group within the Department of Critical Care Medicine, Masih Daneshvari Hospital of Shahid Beheshti University.

Flow Cytometric Assay

Whole blood (3 mL in EDTA tubes) was obtained at admission (Day 0) and after 7 days of treatment. The surface expression of PD-1 and CTLA-4 in lymphocytes was examined by staining whole blood cells with mouse anti-human PD1-FITC (eBioscience, catalog number: 11-9969-42, San Diego, CA, USA) and CTLA-4-APC (eBioscience, catalog number: 17-1529-42) antibodies for 30 min at 4°C. Isotype-matched control antibodies were applied in parallel to all samples. FACSCalibur (BD Biosciences, CA, USA) was used to count 10,000 events as standard. Data were processed using Flow Jo software version 8 (USA). Whole blood, rather than $CD3^+$ T cells, was studied to capture any other T-cell population that may have shown changes in CTLA-4 or PD-1 expression.

Statistical Analysis

Results were presented as the mean±standard deviation (SD). The data between the 2 groups were analyzed using Student's *t* test for parametric analysis and the Mann–Whitney U test for nonparametric analysis. Differences between the 3 groups were compared using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test. Data normality was assessed using the Kolmogorov-Smirnov test before and after comparisons of treatment using a paired *t* test for normal data and Wilcoxon's test for nonparametric data where numbers were too low to ensure normality. Pearson's chi-square and logistic regression were used to analyze the likelihood of early discharge from the hospital. Data analysis was performed using the SPSS program version 16.0 (SPSS, Inc. Chicago, USA) and GraphPad Prism software version 8 (GraphPad Software, Inc.). $p<0.05$ was considered statistically significant.

RESULTS

Demographic and Clinical Characteristics of Patients

The demographic information of the participants is presented in Table 1.

Two subjects died in the FK group, while none died in the DR group, and the duration of hospitalization (days vs. days) was lower in the DR subjects compared with the FK group (*t* test, $p=0.036$; Pearson's chi-square $p=0.002$; odds ratio [OR]=1.4, $p=0.056$, 95% confidence interval [CI]=0.99–2.00). Patients were discharged in either group when the respiratory rate was less than 24 breaths per minute, and the oxygen saturation values were higher than 88% in the absence of external oxygen therapy.

Frequencies of $PD\text{-}1^+$ and $CTLA\text{-}4^+$ Lymphocytes Before and After Treatment

The percentages of $PD\text{-}1^+$ and $CTLA\text{-}4^+$ lymphocytes were determined in the whole blood from COVID-19 patients and healthy controls. The gating strategy is shown in Figure 1; representative plots for a single healthy subject (Figure 1A), a COVID-19 subject before (Figure 1B) and after (Figure 1C) FK treatment for 7 days, and a COVID-19 patient before (Figure 1D) and after (Figure 1E) DR treatment for 7 days are shown.

The frequency of $PD\text{-}1^+$ but not $CTLA\text{-}4^-$ lymphocytes at baseline in the FK-treated combined COVID-19 group was significantly different from that of the healthy control group ($p<0.001$) (Table 2 and Figures 2A and 2B). FK treatment for 7 days significantly enhanced the percentages of both $PD\text{-}1^+$ and $CTLA\text{-}4^+$ cells (Table 2, Figures 2C and 2D).

In contrast, the frequency of $PD\text{-}1^+$ ($p<0.0001$) and $CTLA\text{-}4^+$ ($p<0.0001$) lymphocytes was significantly higher in all DR-treated COVID-19 patients than in healthy controls at baseline (Table 2 and Figures 3A and 3B). In addition, the frequency of $PD\text{-}1^+$ ($p<0.02$) but not of $CTLA\text{-}4^+$ lymphocytes was elevated after DR therapy (Table 2 and Figures 3C and 3D).

Baseline $CTLA\text{-}4^+$ frequencies were significantly higher in the DR group (6.8±3.3) compared to the FK group (0.53±0.61) at baseline ($p<0.0001$) and remained significantly higher after therapy (Table 2).

Table 1: Demographics, blood biochemical and clinical analysis, and ESR in moderate and severe COVID-19 patients at baseline and after antiviral therapy

	Moderate		Severe		All		*p*			
	BT	**AT**	**BT**	**AT**	**BT**	**AT**	**BTvs. AT (S)**	**BTvs. AT (M)**	**BTvs. AT (All)**	**FKvs. DR at BT**
FK Group										
Age in Years	57.7±11.9, N=11		59.3±13.3, N=11		58.54±12.4, N=22		-	-	-	0.85
Male, N (%)	5 (45.5)		6 (54.5)		11(50)		-	-	-	-
Respiratory Rate	19.6± 2.5 N=11	17 ±0.94 N=11	23.4±4.2 N=10	18.7±2.4 N=10	21.4±3.8 N=21	17.8±1.9 N=21	0.002	0.007	0.0001	0.76
O_2 Saturation (%)	89.5±5.1 N=11	94±1.7 N=11	77.6±7.4 N=10	85.1±7.5 N=10	83.8±8.6 N=21	89.8±6.9 N=21	0.002	0.01	0.0001	0.08
CT Scan (%)	18.1±8.4 N=11	-	66±10.7 N=10	-	40.9±26.2	-	-	-	-	0.57
Hospitalization Duration (Day)	7.1±0.98 N=11		10.3±4 N=10		8.6±3.2 N=21		-	-	-	0.036
ESR (mm/hr)	48.7±27.8 N=10	29.7±20.8 N=8	64.8±29.7 N=9	27.8±23.2 N=9	56.3±29.1 N=19	28.7±21.4 N=17	0.02	0.01	0.001	0.005
CRP (mg/L)	59.6±22.2 N=11	13.5±10.9 N=9	49.7±24.1 N=10	17.2 ±12.3 N=9	54.9±23.10 N=21	15.3±11.5 N=18	0.005	0.01	0.0001	0.041
LDH (U/L)	639±210 N=11	527±17.5 N=3	762 ±257 N=10	1179±547 N=6	698.1±236.8 N=21	961.8±542.2 N=9	0.11	0.10	0.46	0.98
CPK (U/L)	91±103 N=8	44±14.1 N=2	109± 68.4 N=7	93 N=1	99.5±86.2 N=15	60.3±30.0 N=3	-	0.18	0.16	0.27
Lymphocytes %	25.6±7.4 N=9		13.7±12.7 N=12		18.8±12.1 N=21					0.26
DR Group										
Age in Years	56.5±9.9, N=10		63.2±15.9,, N=7		59.2±12.7, N=17		-	-	-	-
Male, N (%)	4 (40)		7 (100)		11 (64.7)		-	-	-	-
RR	17.6±0.7 N=9	16.4±0.52 N=9	25.6±9.6 N=6	18±1.6 N=6	20.8±7.07 N=15	17.0±1.3 N=15	0.04	0.02	0.059	-
O_2 Saturation (%)	93.3±2.5 N=9	96±1.6 N=9	81.8±6.9 N=6	92.3±2.3 N=6	88.7±7.4 N=15	94.5±2.6 N=15	0.04	0.01	0.004	-
CT Scan (%)	17.7±7.1 N=9	-	63.3±12.1 N=6	-	36±24.7 N=15	-	-	-	-	-
Hospitalization Duration (Day)	5.0 ±0.0, N=9		8.8±2.9, N=6		6.53±2.61, N=15		-	-	-	-
ESR (mm/hr)	28 N=1	18 N=1	28.7±14 N=4	11±2.3 N=5	28.6±12.1 N=5	12.1±3.5 N=6	0.04	-	0.04	-
CRP (mg/L)	40 ±1.4 N=2	13.5 ±3.5 N=2	43.5±8.8 N=4	30.2±17.2 N=4	42.3±7.0 N=6	24.6±16.0 N=6	0.06	-	0.008	-
LDH (U/L)	434, N=1	430, N=1	766±348 N=4	595±148 N=3	700±336.8 N=5	553.7±146.7 N=4	0.71	-	0.8	-
CPK (U/L)	80, N=1	110, N=1	442±521 N=	85.3±5.5	370.2±480 N=5	91.5±13.1 N=4	0.46	-	0.40	-
Lymphocytes %	26.2±8.1 N=8		17.5±6 N=4		23.3±8.3 N=12					

Comparisons between the groups were performed using the Student's *t* test for the variables with a normal distribution and the nonparametric Mann-Whitney U test for non-normally distributed data. The comparisons between BT and AT used Wilcoxon's paired test. Comparisons between before and after treatment were analyzed using a Wilcoxon's paired *t* test.

AT: after treatment, BT: before treatment, CPK: creatine phosphokinase, CRP: C-reactive protein, CT: computerized tomography, DR: dexamethasone/remdesevir, ESR: erythrocyte sedimentation rate, FK: favipiravir/Kaletra, LDH: lactate dehydrogenase, M: moderate, RR:Respiratory rate,, S: severe.

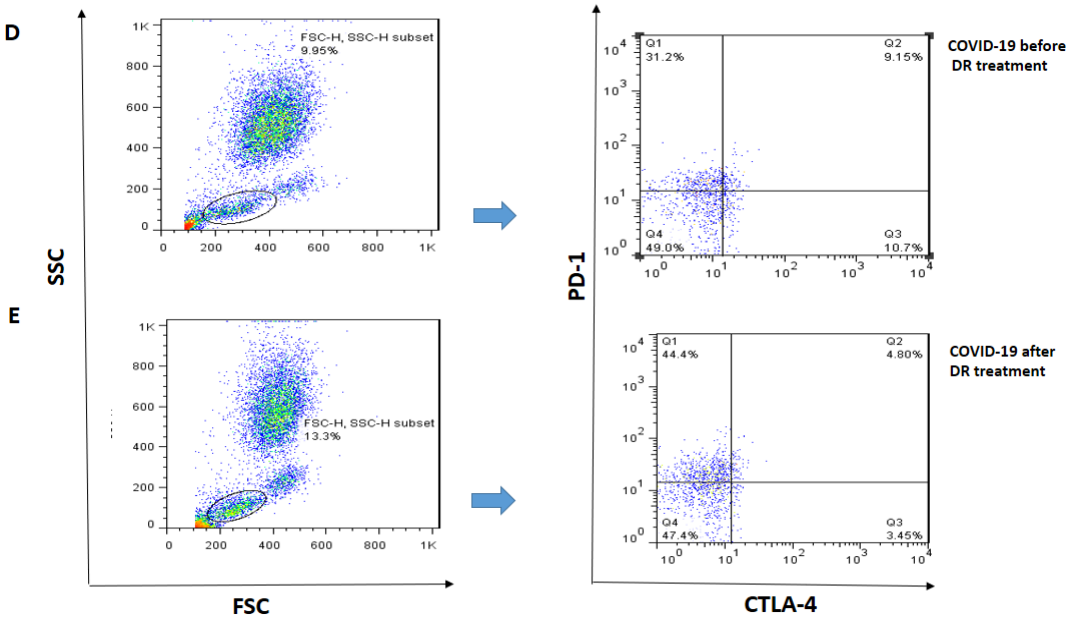

Figure 1. Representative flow cytometry data to indicate the gating strategy for detection of PD-1$^+$ and CTLA-4$^+$ surface marker expression on peripheral blood lymphocytes from COVID-19 patients and healthy control subjects.
Whole blood was stained with PD-1$^+$, CTLA-4$^+$, or isotype control antibodies. The lymphocyte population was gated on forward (FSC) and side scatter (SSC), and the frequency of PD-1$^+$ and CTLA-4$^+$ cells was calculated. Representative plots for a single healthy control subject (A), a COVID-19 subject before (B) and after (C) favipiravir/Kaletra treatment for 7 days, and a COVID-19 patient before (D) and after (E) dexamethasone/remdesevir treatment for 7 days.

Table 2. Comparing the frequency of PD-1$^+$ and CTLA-4$^+$ lymphocytes in all COVID-19 patients at baseline and after antiviral therapy with healthy controls.

	HC	BT	AT	BT/HC *p*	BT/AT *p*
FK Group					
PD-1$^+$ Lymphocytes (%)	5.8±3.3 n=8	15.05±8.03 n=22	25.2±15.1 n=22	0.001	0.007
CTLA-4$^+$ Lymphocytes (%)	0.26±0.14 n=8	0.53±0.61 n=22	1.09±0.41 n=22	0.8	0.001
DR group					
PD-1$^+$ Lymphocytes (%)	5.8±3.3 n=8	24.5±12.8 n=17	34.38±16.5 n=17	0.0001	0.02
CTLA-4$^+$ Lymphocytes (%)	0.26±0.14 n=8	6.8±3.3 n=17	6.8±3.8 n=17	0.0001	0.99

Comparisons between the groups were performed using the Student's *t* test for the variables with a normal distribution and the nonparametric Mann-Whitney U test for nonnormally distributed data. Comparisons between before and after treatment were analyzed using a paired *t* test.

AT: after treatment, BT**:** before treatment, CTLA-4: cytotoxic T-lymphocyte antigen 4, DR: dexamethasone/remdesevir, FK: favipiravir/Kaletra, HC: healthy controls, M: moderate, PD-1: programmed cell death protein 1, S: severe.

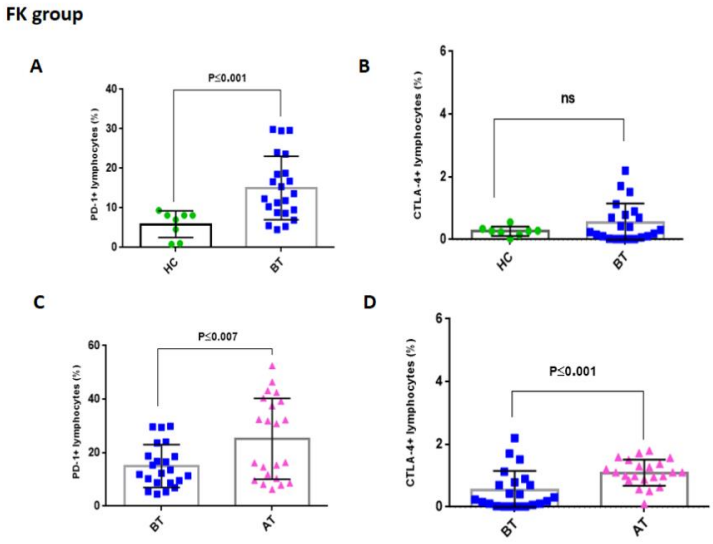

Figure 2. Profile of PD-1$^+$ and CTLA-4$^+$ frequencies in whole blood cells of healthy control subjects (HC) and COVID-19 patients before and after FK treatment. The frequency of PD-1$^+$ and CTLA-4$^+$ lymphocytes in HC (n=8) and COVID-19 subjects (n=22) before (BT) and after (AT) favipiravir/Kaletra (FK) treatment. Results are plotted as means±SD of individual patient data. NS: not significant. (Comparisons between the groups were performed using the Student's *t* test for the variables with a normal distribution and the nonparametric Mann-Whitney U test for nonnormally distributed data.)

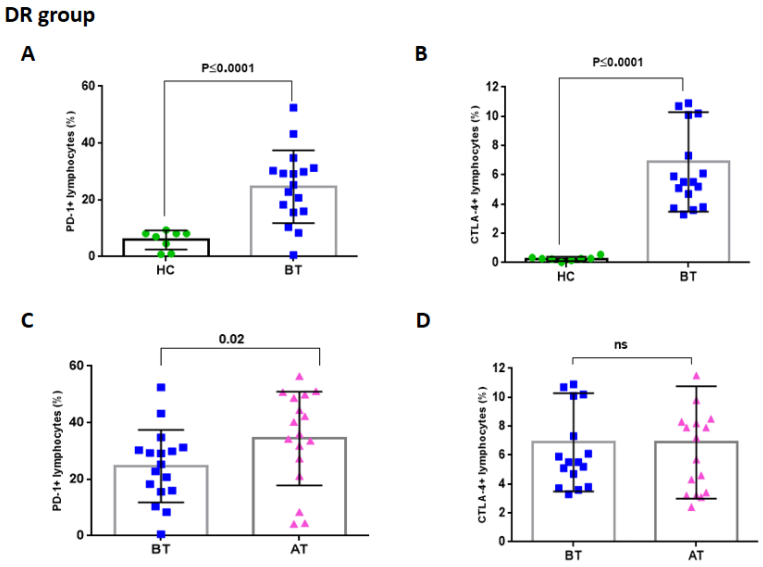

Figure 3. Profile of PD-1$^+$ and CTLA-4$^+$ frequencies in whole blood cells of healthy control subjects (HC) and COVID-19 patients before and after DR treatment. The frequency of PD-1$^+$ and CTLA-4$^+$ lymphocytes in COVID-19 subjects (n=17) before (BT) and after (AT) dexamethasone/remdesevir (DR) treatment. Results are plotted as means±standard deviation of individual patient data. NS: not significant. (Comparisons between the groups were performed using the Student's *t* test for the variables with a normal distribution and the nonparametric Mann-Whitney U test for nonnormally distributed data.)

A comparison of healthy control subjects with COVID-19 patients stratified according to severity and treatment groups was also undertaken (Table 3). In contrast to HC, PD-1$^+$ lymphocyte frequencies were significantly elevated at baseline in moderate (p<0.004) and severe (p<0.005) COVID-19 patients treated with FK (p<0.0001) and DR (p<0.02) (Table 3). In contrast, while baseline levels of CTLA-4$^+$ cells were significantly elevated in DR-treated severe (p<0.001) and moderate (p<0.0001) subjects, there was no significant difference in frequencies in the FK-treated subjects at baseline (Table 3). Subjects were also analyzed according to COVID-19 severity before and after treatment. The percentage of PD1$^+$ cells and CTLA-4$^+$ cells was elevated after treatment in the FK group in severe patients (p<0.01 and p<0.05, respectively). In addition, the percentage of CTLA-4$^+$ cells was elevated in patients with moderate COVID-19 treated with FK (P<0.008). In contrast, while DR treatment did not significantly affect the percentage of PD-1$^+$ cells in either the moderate or severe groups, there was a significant decrease in CTLA-4$^+$ cells in the severe group (P≤0.05) (Table 3).

Data were also analyzed according to COVID-19 severity within each treatment group. There was no significant difference in BT or AT's PD-1$^+$ or CTLA-4$^+$ lymphocyte frequencies between patients with moderate or severe COVID-19, apart from a significantly reduced frequency of CTLA-4$^+$ cells (p<0.001) in the severe DR-treated patients compared with patients with moderate disease (Table 4).

Table 3. Frequency and Mean Fluorescence Intensity(MFI) of PD-1$^+$ and CTLA-4$^+$ lymphocytes in patients at baseline compared to healthy subjects stratified on severity and response to therapy.

		BT					AT			
	HC	M	M/HC *p*	S	S/HC *p*	HC/M/S *p*	M	BT/AT *p*	S	BT/AT *p*
FK Group										
PD-1$^+$ Lymphocytes (%)	5.8±3.3 N=8	15.7±8.6 N=11	0.004	14.4±7.7 N=11	0.005	0.01	26.2±13.9 N=11	0.26	24.1±16.8 N=11	0.01
CTLA-4$^+$ Lymphocytes (%)	0.26±0.14 N=8	0.51±0.73 N=11	0.29	0.55±0.51 N=11	0.10	0.25	1.1±0.36 N=11	0.008	1.0±0.47 N=11	0.05
DR Group										
PD-1$^+$ Lymphocytes (%)	5.8±3.3 N=8	25.2±11.7 N=10	0.0001	15.1±5.7 N=7	0.02	0.002	33.5±13.9 N=10	0.06	35.5±20.9 N=7	0.16
CTLA-4$^+$ Lymphocytes (%)	0.26±0.14 N=8	6.5±3.6 N=10	0.0001	7.3±3.2 N=7	0.001	0.0001	9.1±3.4 N=10	0.051	3.6±1.0 N=7	0.050

The Student's *t* test was used to compare the two groups for variables with a normal distribution and the nonparametric Mann-Whitney U test for nonnormally distributed data. Differences between the 3 groups were compared using one-way analysis of variance followed by post hoc Dunnett's test. The effect of treatment (AT vs. BT) was compared using Wilcoxon's test.

AT: after treatment, BT: before treatment, CTLA-4: cytotoxic T-lymphocyte antigen 4, DR: dexamethasone/remdesevir, FK: favipiravir/Kaletra, HC: healthy controls, M: moderate, PD-1: programmed cell death protein 1, S: severe.

Table 4. Frequency of PD-1$^+$ and CTLA-4$^+$ lymphocytes at baseline and after antiviral therapy in severe compared to moderate COVID-19 patients

	BT (M)	BT (S)	*p*	AT (M)	AT (S)	*p*
FK treated group						
PD-1$^+$ Lymphocytes (%)	15.7±8.6 N=11	14.4±7.7 N=11	0.71	26.2±13.9 N=11	24.1±16.8 N=11	0.75
CTLA-4$^+$ Lymphocytes (%)	0.51±0.73 N=11	0.55±0.51 N=11	0.88	1.1±0.36 N=11	1.0±0.47 N=11	0.42
DR group						
PD-1$^+$ Lymphocytes (%)	25.2±11.7 N=10	23.7±15.1 N=7	0.82	33.5±13.9 N=10	35.5±20.9 N=7	0.81
CTLA-4$^+$ Lymphocytes (%)	6.5±3.6 N=10	7.3±3.2 N=7	0.66	9.1±3.4 N=10	3.6±1.0 N=7	0.001

The Student's *t* test was used to compare the 2 groups for variables with a normal distribution and the nonparametric Mann-Whitney U test for nonnormally distributed data. AT: after treatment, BT: before treatment, CTLA-4: cytotoxic T-lymphocyte antigen 4, DR: dexamethasone/remdesevir, FK: favipiravir/Kaletra, HC: healthy controls, M: moderate, PD-1: programmed cell death protein 1, S: severe.

DISCUSSION

We show that, at baseline, COVID-19 patients had a higher percentage of PD-1$^+$ and CTLA-4$^+$ lymphocytes than healthy control subjects, except for COVID-19 patients in the FK group. The percentage of CTLA-4$^+$ lymphocytes was highest in COVID-19 patients who received DR therapy. In COVID-19 patients who received the FK treatment, the frequency of PD-1$^+$ and CTLA-4 $^+$ lymphocytes after treatment increased. These effects are likely due to the FK therapy rather than a side effect of COVID-19 and need to be elucidated in future studies. The response to treatment was similar in both moderate and severe patients.

In contrast, the percentage of CTLA-4$^+$ cells significantly decreased with DR therapy in severe patients but was not significantly altered in moderate or all COVID-19 patients. Both treatments significantly reduced CRP and ESR baseline levels but not LDH or CPK, whereas DR therapy was associated with a shorter hospital stay and lower mortality. Overall, the baseline CTLA-4$^+$ status was associated with a good clinical response to DR in patients in terms of mortality and hospitalization time with severe disease.

Viral-induced dysregulation of the innate and adaptive immune responses may contribute to SARS-CoV-2 immunopathology.[15] Indeed, severe COVID-19 is associated with acute respiratory distress syndrome, lymphocytopenia, and lymphocyte exhaustion, which contribute to increased patient mortality rates.[16] Peripheral blood lymphocytes become activated during the acute phase of COVID-19 disease, despite the low frequency of T cells in the systemic circulation.[16] In severe SARS-CoV-2 infection, the early activation of systemic T-cells progresses to an exhaustion phenotype over time.. [15]As a consequence, T cells may express both activation (CTLA-4, CD69, CD38, and CD44) and exhaustion (mucin-3, PD-1, NKG2A) markers.[15,17]

Other markers such as soluble T-cell immunoglobulin and mucin domain 3 (sTim-3), soluble CD27, soluble lymphocyte activating gene-3 (sLAG-3), sPD-1 and its ligand PD-L2, sCD28, sCTLA-4, soluble B and T lymphocyte attenuator (sBTLA), CD270, and sCD80 are also expressed on these cells (18). Moreover, the expression of GITR, Tim-3, CD27, PD-1, and LAG-3 on the surface of CD4$^+$ and CD8$^+$ T cells increases in severe COVID-19 patients compared to patients with mild disease.[18,19]

PD-1 expression on the surface of T cells may cause suppression of appropriate immune responses .[19] Enhanced PD-1 expression on the surface of T-cells during the early stage of SARS-CoV-2 infection induces CD8$^+$ cell dysfunction and the secretion of perforin and granzymes.[19,20] Furthermore, PD-1$^+$ lymphocytes have a higher proportion of interferon-gamma (IFN-γ)-producing cells than PD-1$^-$ lymphocytes, suggesting that PD-1$^+$ cells actively participate in SARS-CoV-2 infection by releasing IFN-γ and possibly other key

mediators.[20] We have previously reported that FK treatment for 7 days reduced CT, CRP, and LDH levels but enhanced serum levels of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α), suggesting a possible differential effect on systemic and lung inflammation.[44]

The overexpression of PD-1 on lymphocytes in COVID-19 patients likely results from the cytokine storm, which leads to $CD8^+$ T-cell exhaustion. In severe COVID-19 patients, blood levels of proinflammatory cytokines, such as IL-6, IL-17, and TNF-α, are significantly elevated together with increased activity of the host immune system.[3] These mediators are able to trigger an up-regulation of PD-L1 on the surface of immune cells via phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) and nuclear factor-κB (NF-κB) pathways that induce apoptosis.[21]

Immune cell exhaustion may be important in severe COVID-19 since the anti–IL-6 monoclonal antibody tocilizumab increases $CD8^+$ T-cell frequency and function in COVID-19 patients,[22] in addition, blocking PD-1/CTLA-4 enhances the antiviral capacity of T cells in COVID-19 patients.[23] Theoretically, reactivation of exhausted T cells by PD-1/PD-L1 or CTLA-4 blockade may further trigger inflammatory cytokine release,[23] although PD-1 blockade did not affect COVID-19 severity in patients with lung cancer and COVID-19.[24]

Kaletra and IFN-β reduced systemic inflammation in a clinical trial of COVID-19 patients[25] Favipiravir partially, but not completely, either. downregulated the expression of inflammatory mediators or improved respiratory status in mechanically ventilated COVID-19 patients.[26] However, due to the side effects of Favipiravir, such as the long-inducing elevation of hepatic enzymes, ECG changes, and possible drug interactions, this treatment is no longer recommended by the WHO.

Dexamethasone has general anti-inflammatory effects and inhibits cytokines, chemokines, and adhesion molecules. In a large trial study of over 6000 patients with COVID-19, dexamethasone plus standard care significantly reduced 28-day mortality compared with standard care alone (22.9% vs. 25.7%; $p<0.001$). This was more evident in subjects on mechanical ventilation (29.3% vs. 41.4%) and with a greater duration of symptoms.[27] This effect of dexamethasone may be associated with attenuation of the COVID-19-associated cytokine storm.[28] Remdesivir inhibits the activation of IFN regulatory factor 3 and the NF-κB pathways and decreases the expression and release of IL-18, IL-6, IL-1β, and TNF-α.[29] In vitro studies also indicate that remdesivir suppresses SARS-CoV-2 replication in human liver cells[30] which may account for its clinical efficacy in severe COVID-19 patients.[31] Remdesivir has a reversible effect on enhancing the expression of transaminases and several other side effects. [32] However, the combination of remdesivir and dexamethasone was highly effective in treating COVID-19 patients [33,34] and resulted in reduced mortality and shorter hospitalization than with FK in our study.

A strength of our data is the identification of overexpressed PD-1 and CTLA-4 in lymphocytes from FK-treated COVID-19 patients and linking this to patient survival and duration of hospitalization. The duration of hospitalization was lower in the DR-treated subjects compared with the FK-treated patients, as shown by the odds ratio. However, this study has several limitations, including its being a small, single-center study and failure to study PD-1 and CTLA-4 expression in specific T-cell subsets. Furthermore, there were significant differences in baseline $CTLA\text{-}4^+$ cells between the FK- and DR-treated patients, which may affect the results. Moreover, we do not have a control standard of care comparator group as all patients were treated with these drugs as initial clinical trial data suggested their utility. We cannot, therefore, rule out that the differences seen here regarding T cell surface marker expression would not have occurred over time, irrespective of treatment. In mitigation, we believe this is unlikely as the results for FK and DR were distinct. Further studies are required to confirm these results in a larger multicenter study.

This study has some limitations, including the single treatments of patients with each medicine, which we could not use as a single treatment due to ethical points. The healthy control group selection during the pandemic period of COVID-19 was difficult since the spreading virus, even in its mild form, does not show any signs of disease. Thus, our control group was selected based on negative PCR reactions and routine biochemical and hematological tests such as ESR and complete blood counts. In conclusion, our study indicated heightened activation and exhaustion of lymphocytes, characterized by differentially elevated frequencies of $PD\text{-}1^+$ and $CTLA\text{-}4^+$ cells at baseline, possibly activated by a cytokine storm. DR treatment reduces the cytokine storm, which may prevent the increase in $PD\text{-}1^+$ and $CTLA\text{-}4^+$ cell frequencies seen with FK treatment.

These studies need to be confirmed in a larger multicenter study.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Masih Daneshvari Hospital, Tehran, Iran (IR.SBMU.NRITLD.REC.1399.223). An informed consent form from all participants was obtained before collecting the samples. All methods were performed in accordance with the relevant guidelines and regulations.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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