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ORIGINAL ARTICLE

Role of T Lymphocyte Activation Profile in Predicting SARS-CoV-2 Severity: Experience from Tertiary Care Centre of North India

Anshul Gupta[1] · Archit Pandharipande[1] · Mansi Gupta[2] · Zia Hashim[2] · Sanjeev[1] · Priyanka Chauhan[1] · Ruchi Gupta[1] · Dinesh Chandra[1] · Manish Kumar Singh[1] · Rajesh Kashyap[1] · Khaliqur Rahman[1]

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Abstract

Background Immune dysregulation plays a key role in determining COVID-19 disease severity. We aimed to analyze the T cell activation profile in COVID – 19 cases and its predictive role in disease severity and outcome.

Material & methods This was a prospective observational pilot study from a tertiary care COVID-19 hospital. Peripheral blood samples obtained between the fifth and seventh day of COVID-19 illness, were subjected to lymphocyte subset analysis using multicolor flowcytometry using a single tube, 8 antibodies (CD45, CD19, CD3, CD4, CD8, CD38, HLADR, and CD56) analysis. Correlation between lymphocyte subset analysis and clinical profile was determined.

Results 26 patients including 11 with mild disease and 15 with severe disease were enrolled. The median age was 58 years (range: 33–81), with a male: female ratio of 1.36:1. Significant lymphopenia was observed in the severe group compared to the mild group ($p < 0.02$). The absolute numbers of CD3+, CD4+, CD8 + T cells, B cells, and NK cells were significantly reduced in the severe group as compared to the mild group ($p < 0.05$). In patients with severe disease, the proportion of CD8 + and CD4 + T cells were significantly higher than those in patients with mild disease ($p = 0.0372$). Using ROC analysis, a CD4:8 T cell ratio of ≥ 2.63 and an activated (CD38 + HLA-DR+) CD8 T cell proportion of > 15.85% of the total CD8 T cell population, significantly determined the severe disease category.

Conclusions Severe COVID-19 is associated with severe lymphopenia, altered CD4/CD8 ratio and markedly increased CD8 T cell activation profile.

Keywords COVID-19 · T lymphocyte activation · Severity · Outcome · T cell Activation profile · Disease severity

Background

Ever since the first case of a novel coronavirus emerged in Wuhan, China, in December 2019, the virus has infected more than 323 million people worldwide, leading to a

✉ Khaliqur Rahman
drkhaliq81@gmail.com

Anshul Gupta
anshulhaemat@gmail.com

Archit Pandharipande
archit10000@gmail.com

Mansi Gupta
mansig@sgpgi.ac.in

Zia Hashim
ziahashim@sgpgi.ac.in

Sanjeev
drsanjeev_ranchi@gmail.com

Priyanka Chauhan
priyanka.chauhan1785@gmail.com

Ruchi Gupta
ruchipgi@yahoo.co.in

Dinesh Chandra
dinesh3224@gmail.com

Manish Kumar Singh
einstein24mks@gmail.com

Rajesh Kashyap
drkhaliq81@gmail.com

[1] Department of Hematology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareli Road, 226014 Lucknow, UP, India

[2] Department of Pulmonary Medicine, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareli Road, 226014 Lucknow, UP, India

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global pandemic [1]. Contrary to similar coronaviruses, such as SARS-CoV & MERS-CoV, which are associated with severe disease in the majority, SARS-CoV-2 is associated with a very high infectivity rate and mild respiratory illness in 80% of infected cases [2–3]. However, the remaining 20% of patients typically need hospital care with intensive life support, as they present with moderate to severe pneumonia and acute respiratory distress syndrome (ARDS). Although certain risk factors, such as age (> 60 years)-associated comorbidities (diabetes, hypertension, cancer, chronic renal failure), are frequently associated with severe disease, a significant proportion of patients without any such risk factors have also been reported to develop severe disease. [4] This heterogeneity in clinical presentation has led researchers to think about the putative role of a dysregulated immune response to SARS-CoV-2 infection in severe disease. It has been found that patients with severe disease often have a dysregulated early antiviral type I interferon (IFN) response by resident alveolar macrophages (AMs) and epithelial cells in the lungs, which is primarily responsible for clearing the virus and resolving inflammation [5–6]. SARS-CoV-1, and likely SARS-CoV-2, evades our immune system by inhibiting multiple viral sensing pattern recognition receptors (PRRs) and downstream signaling pathways, thereby leading to blunted early antiviral type I IFN. 5 Furthermore, this leads to the initiation of a dysregulated inflammatory cascade that can lead to ARDS and systemic inflammation [5–6]. Recently, Yao et al. elegantly demonstrated that the transcriptomic profile of peripheral blood mononuclear cells (PBMCs) from severe COVID-19 patients with ARDS had defective antigen presentation and interferon (IFN) response in monocytes and higher response to IFN signaling in lymphocytes. [7] In addition, they also noticed altered cytotoxic T cell function and humoral immunity in a severe disease subset, thereby leading to the perpetuation of inflammation and causing a "cytokine storm". Thus, understanding these B & T cell immune dysfunction pathways is of prime importance in identifying patients who are prone to develop severe disease. This also provides us with an opportunity to develop a bedside tool/investigation that can help in identifying these patients with poor prognosis at the time of initial COVID-19 diagnosis so that appropriate management strategies may be adopted. Thus, in the present study, we aimed to analyze the T cell activation profile by immunophenotyping in patients with COVID-19 to predict disease severity and outcome.

Materials and methods

Patients

This was a prospective observational pilot study from a COVID-19 tertiary care hospital in northern India. The study cohort comprised 26 consecutive adults (> 18 years) with SARS-CoV-2-positive cases diagnosed in our hospital from 31st March 2021- 10th April 2021. The study was approved by the institutional ethics committee (IEC no. 2021–6- IMP-154), and written informed consent was obtained from all the enrolled patients. The study inclusion criteria included patients who were diagnosed SARS-CoV-2 positive by qualitative real-time PCR (qRT–PCR) performed on nasal and/or oropharyngeal swab samples and had less than seven days of onset of illness determined by the earliest reported symptom by the patient. The enrolled study patients needed to have a minimum follow-up 14 days from disease onset. The details about the demographic profile, nature of comorbidities, disease symptoms along with duration, and any concomitant medications for every patient was recorded in the predesigned proforma. Patients meeting the inclusion criteria were recruited irrespective of the severity of COVID-19 or setting of treatment (home isolation, outpatient treatment, or hospitalization). The study exclusion criteria included patients on any immunosuppressive medications, chronic renal disease, duration of illness > 7 days, follow-up of < 14 days and failure to receive consent for study participation. Genomic surveillance of the SARS-CoV-2 virus was not performed in our study. All patients diagnosed with COVID-19 were classified by disease severity (mild, moderate & severe) as per the WHO (World Health Organization) guidelines and managed with uniform therapy as per the standardized institutional protocol (based on the latest national guidelines for the treatment of COVID-19, Ministry of Health and Family Welfare, India). [8] The follow-up (in days) period was taken from the date of the first positive qRT–PCR report for SARS-CoV-2 to the last follow-up date or the date of death. All patients had a planned follow-up until day 56 with a minimum follow-up of 14 days.

Investigations Peripheral venous blood samples were obtained between the fifth and seventh day of COVID-19 illness, which was confirmed by SARS-CoV-2-positive qRT–PCR. The baseline laboratory-based evaluation in every COVID-19 patient included complete blood counts, coagulation profile, kidney function tests, liver function tests, quantitative d-dimer levels, C-reactive protein levels (CRP), serum ferritin levels, serum procalcitonin levels, serum lactate dehydrogenase (LDH) levels, and a digital chest X-ray. A high-resolution CT (HRCT) scan of the chest

was only performed for severe cases. In addition to these routine investigations, a 2.5 ml peripheral venous sample was collected for immunophenotyping (lymphocyte subset analysis) by multicolor flow cytometry. For the purpose of analysis, 07 venous blood samples were collected from healthy controls.

Flow cytometry Lymphocyte subset analysis was performed using multicolor flow cytometry and a single tube 7 color analysis. Briefly, 200 µl of ethylene diamine tetraacetic acid (EDTA) anticoagulated peripheral blood was incubated with a pretitrated cocktail of antibodies (as detailed in Supplementary Table 1) for 20 min in the dark. This was followed by red cell lysis using the commercial lysing agent FACS Lyse (BD Biosciences, San Jose, USA), two washes using 2 ml PBS (Gibco, Life Technologies, New York) and a final suspension in 0.5 ml of 0.5% paraformaldehyde for acquisition on a flow cytometer. The samples were acquired on BD FACS Lyric equipment using FACS Suite software. The analysis was also carried out using FACS suite software version 1.4.0. The different lymphoid subsets analyzed were CD3 + T cells, CD3+/CD8 + cytotoxic T cells, CD3+/CD4 + helper T cells, CD38+/HLADR + highly activated T cells, CD19 + B cells, and CD56 + NK cells. Both the proportion and absolute count per microliter were obtained.

Statistical analysis Statistical analyses were carried out using IBM SPSS version 21, and p values less than 0.05 were considered statistically significant. The continuous variables included in the study were represented using the mean with standard deviation or median with interquartile range and compared using the Mann–Whitney U test to determine any statistically significant difference between them. The categorical variables were represented using the frequencies with corresponding percentages and were compared using Fisher's exact test or chi-square tests to analyze the differences in the distribution between individual parameters. Receiver operating curves (ROCs) were generated to derive optimal cutoff points for different lymphocyte subsets in patients with mild, severe COVID-19 and healthy controls by choosing the threshold with the highest specificity without compromising the sensitivity (the point closest to the upper left corner). Outcomes were defined as death at the day 28 and day 56 timepoints post qRTPCR positivity, and cure was defined as two successive negative qRTPCR reports more than 24 h apart. Univariate and multivariate analyses (Cox proportional hazard regression model) were also performed to consider each potential prognostic factor.

Results

Demographics and COVID-19 disease characteristics

There were 26 patients who met our study inclusion criteria and were enrolled and categorised as per the WHO disease severity criteria. At the time of patients' recruitment, these patients qualified as mild (n = 11), moderate (n = 2) and severe (n = 13) COVID disease categories. However on follow up, we noted that the 02 patients who were initially categorized as moderate disease progressed to severe disease requiring ventilatory support. Hence we clubbed these 02 cases that were initially grouped under moderate category to severe disease category only. So, at the time of data analysis, only to disease categories, mild (n = 11) and severe (n = 15) cases were compared. Supplementary Table 2 shows the demographics and baseline disease characteristics of all patients included in the study. The median age was 58 (range: 33–81), with a male:female ratio of 1.36:1. There was no significant difference between the median age of patients in the mild and severe disease groups (p value = 0.9; Table 1). The median duration between the onset of symptoms and admission was 7.5 days (IQR 4.0–8.0), with 13 (50%) patients consulting the health care facility within 7 days of onset of illness. Of the 15 patients with severe disease, 12 (80%) had a duration of COVID symptoms for more than 7 days (P value = 0.02; Table 1). Fever was the most common symptom reported in 24 (92.3%) patients, followed by myalgia or fatigue (88.4%), shortness of breath (46.1%), cough (61.5%), and diarrhoea (15.3%). Twelve (46.1%) patients had diabetes mellitus as the most common coexisting comorbidity, followed by cardiovascular disease, which was present in 8 (30.7%) patients. The COVID-19 viral load was analyzed in all patients by determining the RT–PCR cycle threshold, with 17 (65.4%) patients having a cycle threshold between 20 and 30. Baseline HRCT chest showed ground glass opacity and consolidation involving multiple lobes in most patients, with a CT severity score between 10 and 15 in the majority (18/26; 69.2%). In our cohort, there was no significant difference in the CT severity score between the mild and severe disease groups (p value = 0.09; Supplementary Table 2). The detailed comparison of clinical and laboratory characteristics and treatment outcomes of COVID-19 patients in the mild and severe categories is shown in Table 1.

Table 1 Clinical & lab characteristics of COVID-19 cases

Variable	All pts [n = 26]	Mild [n = 11]	Severe [n = 15]	P value
Age, years	58(33–81)	2(33.3)	4(66.7)	0.90
Median (Range)	6 (26.1)	8(37.5)	5(62.5)	
18–40	13(34.8)	1(44.4)	6(55.6)	
41–60	7(39.1)			
> 60				
Duration of COVID symptoms at diagnosis	13(47.8)	10(72.7)	3(27.3)	0.02
< 7 days	13(52.2)	1(8.3)	12(91.7)	
≥ 7days				
CT severity index **at ICU admission**	2(9.0)	2(18.3)	0	0.095
< 10	18(69.2)	8(72.7)	10(45.5)	
10–15	3(27.3)	1(9)	2(45.5)	
16–20	3(4.6)	0	3(9.0)	
> 20				
COVID-19 Viral Load at Diagnosis (in terms of cycle threshold)	08(30.4)	1(14.3)	7(85.7)	0.155
< 20	17(65.2)	9(46.7)	8(53.3)	
20–30	1(4.3)	1(100)	0	
> 30				
Laboratory findings, median (IQR)	5200(3000–33,825)	8000(4600–10,950)	5000(4350–10,500)	0.48
WBC (/µl)	2100(1150–5450)	7360(3555–9645)	4777(3735–10,184)	0.9
Absolute Neutrophil Count (/µl)	416(152-848.8)	848.8(592.5-1138.5)	484.8(88-623.8)	0.02
Absolute Lymphocyte count (/µl)	1.19(0.42–1.82)	1.24(1.19–1.72)	0.81(0.36–1.92)	0.207
Platelets (/µl)	11.3(8.9–12.5)	12.7(10.7–13.4)	10.35	0.01
Hb (gm%)	0.56(0.23–2.1)	0.23(0.15–0.39)	(6.77–11.65)	0.001
D-dimer	0.11(0.03–0.33)	0.03(0.02–0.09)	1.65(0.34–2.75)	0.06
Procalcitonin	18(3–64)	7(1.25–66.75)	0.18(0.08–0.345)	0.313
CRP	856(198–3400)	158(75–348)	25(9–64)	0.001
Serum ferritin	425(287–540)	461(380–529)	2090(1251–5075)	0.208
Serum Fibrinogen	410(350–864)	350(302–393)	325(226–578)	0.008
LDH			807(376–1057)	
Co-infections	04(17.4)	0	04(100)	0.09
Bacterial	05(21.7)	0	05(100)	0.07
Fungal				
Complications	6(26.1)	0	6(100)	0.022
ARDS	04(17.4)	0	4(100)	0.043
Acute Renal Failure	05(21.7)	0	5(100)	0.045
Sepsis with MODS	4(17.4)	0	4(100)	0.078
Secondary HLH				
Treatment	16(69.6)	02(12.5)	14(87.5)	0.001
Steroids	14(60.9)	0	14(100)	0.001
Remdesivir	03(13)	0	03(100)	0.253
Convalescent plasma	12(52.1)	0	12(100)	0.002
LMWH	15(60.9)	0	15(100)	0.001
NIV	6(26.1)	0	6(42.9)	0.048
Tociluzumab	06(26.1)	0	06(42.9)	0.048
Mechanical Ventilation				

Laboratory findings in COVID-19 patients

Among the various hematological parameters, we noted that there was significant lymphopenia in the severe group compared to the mild group ($p < 0.02$). The severe group also showed statistically significant differences in various biochemical parameters, including D-dimer ($p = 0.001$,

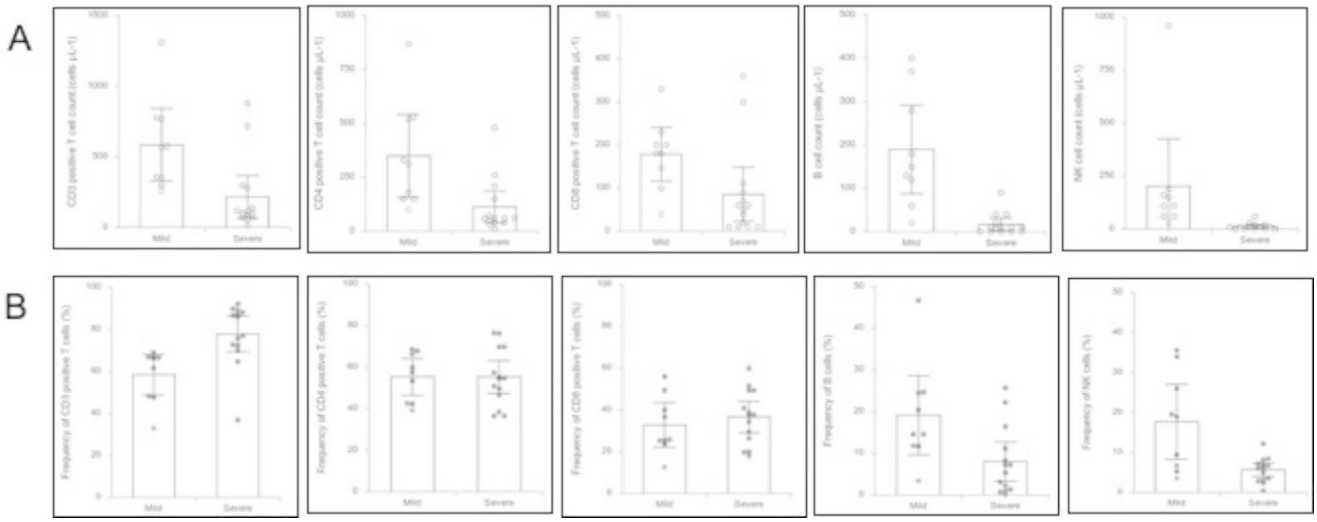

Fig. 1 Absolute cell counts and percentages of peripheral lymphocyte subsets in SARS-CoV-2 infected patients. (a) Absolute cell counts and (b) Percentages of CD3 + T cells, CD4 + T cells, CD8 + T cells, B cells, and NK cells in the mild (n = 11) and severe (n = 15) groups. Data expressed as mean ± SD.*p < 0.05 by two-tailed Mann–Whitney U-test

Table 2 Lymphocyte subset analysis stratified according to COVID-19 disease severity

Variable	All pts [n = 26]	Mild [n = 11]	Severe [n = 15]	P value
Laboratory findings, median (IQR)	5200(3000–33,825)	8000(4600–10,950)	5000(4350–10,500)	0.48
WBC (/µl)	2100(1150–5450)	7360(3555–9645)	4777(3735–10,184)	0.9
Absolute Neutrophil Count (/µl)	416(152-848.8)	848.8(592.5-1138.5)	484.8(88-623.8)	0.02
Absolute Lymphocyte count (/µl)	284(99.6-583.3)	567.8(322.2-770.5)	288.8(71.8-368.7)	0.04
Absolute T cell count (/µl)	145.1(55.6-312.1)	312.09(150.2–526)	156.5(42.5-225.5)	0.04
Absolute CD4 count(/µl)	1.9(0.86–4.20)	1.6(0.8–2.8)	2.83(1.1-6.425)	0.01
% activated CD4 count ($CD4^{38/\ HLA\ DR+ve}$)	88.5(36.9–195.0)	181.4(121.2-215.24)	54.3(13.6–92.9)	0.05
Absolute CD8 count(/µl)	10.6(3.0-30.625)	4.8(1.3–9.8)	20.7(5.9–35.6)	0.02
% activated CD8 count ($CD8^{38/\ HLA\ DR+ve}$)	2.3(1.01–3.85)	1.34(0.76–2.69)	2.64(1.37–4.23)	0.001
Median CD4/CD8 ratio(/µl)	96.8(16.2-156.3)	123.8(83.2-164.1)	56.4(12.3–96.2)	
Absolute B cell count (/µl)	43.2(3.7–161)	105(41–161)	13.2(3.7–21.8)	
Absolute NK cell count (/µl)				

two-tailed Mann–Whitney U test), serum ferritin (p = 0.001) and LDH values (p = 0.009).

The absolute numbers of CD3+, CD4+, CD8 + T cells, B cells, and NK cells were significantly reduced in the severe group compared to the mild group, which was attributable to significant lymphopenia in the former compared to the latter subgroup, as shown in Fig. 1a. However, no significant differences were observed in the relative frequencies (%) of CD3 + T cells, CD4 + T cells, or CD8 + T cells between the mild and severe groups, except for that of B cells and NK cells, which were significantly decreased in the severe patient cohort (p = 0.01 and p = 0.001, respectively) (Fig. 1b). The median absolute counts of different lymphocyte subsets (CD3+, CD4 + and CD8 + T cells, B cells, and NK cells) in the mild and severe COVID-19 groups are shown in Table 2.

Furthermore, we evaluated the degree of T-cell activation among the two T cell subsets (CD4 & CD8) in our cohort. The levels of T-cell activation, as depicted by *CD38 + HLA DR* + CD8 & CD4 T cells, were significantly higher in SARS-CoV-2-infected patients than in healthy controls (HCs) (Fig. 2). The frequencies of CD38 + HLA-DR + CD8 + T cells and HLA-DR + CD8 + T cells correlated positively with time after disease onset (R = 0.482, p = 0.01 and R = 0.465, p = 0.02, respectively, (Pearson correlation test). There was no difference in the absolute number of CD38 + HLA-DR + CD8 + T and CD38 + HLA-DR + CD4 + T cells between the mild and severe groups. (Supplementary Fig. 1A)

T cell subset analysis and its correlation with COVID-19 severity:

The absolute CD 4 and CD8 cell counts of all our COVID-19 patients were analyzed as categorical variables by observing an arbitrary cutoff (based on previous studies) of ≥ 250 cells/µl for CD 4 T cells and ≥ 100 cells/µl for CD8 T cells based on previously published reports. We observed that of the 11 patients in the mild disease category, only 02 (18.2%) had a CD 4 count < 250 cells/µl, while in the severe disease

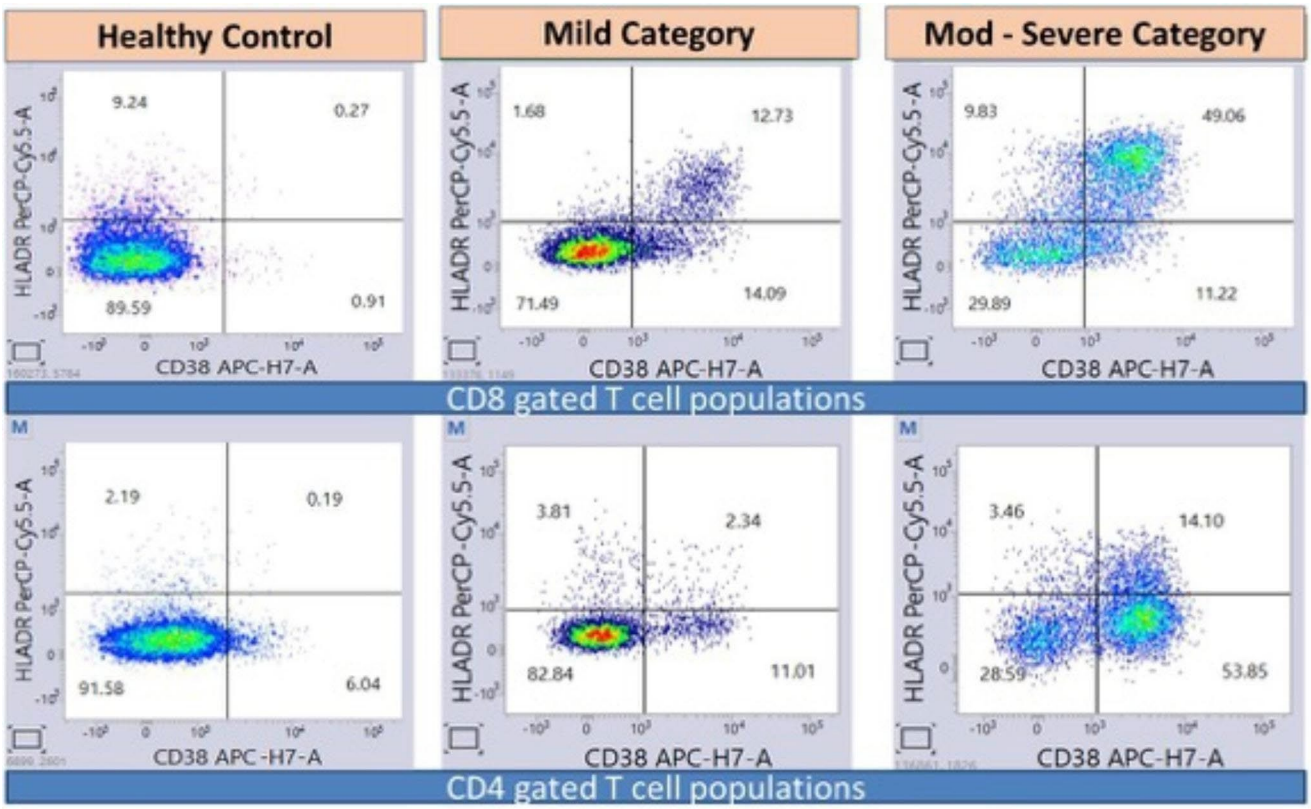

Fig. 2 Flowcytometry plots showing activated T cell profile (*CD38 + HLA DR+*) in CD8 & CD 4 + T cells in healthy controls, mild & severe COVID-19

category, 13 of 15 patients (86.6%) had a CD 4 count < 250 cells/µl (p = 0.001). Similarly, in the severe disease group, 10 (66.6%) patients had a CD8 T cell count < 100 cells/µl, while only 01 patients in the mild disease group had a critically low CD8 T cell count (p value 0.003).

Receiver operating curves (ROCs) were generated comparing patients with mild and severe COVID-19 infection along with healthy controls. The optimal cutoff points were determined for the CD4/8 T cell ratio and frequency of activated CD8 T cells (%) by choosing the threshold with the highest specificity without compromising the sensitivity (the point closest to the upper left corner). We observed that a CD4/8 T cell ratio of ≥ 2.63 had sensitivity = 100% and specificity = 78.9% for determining COVID disease severity (area under the curve 91.7%; p = 0.001) (Fig. 2b). Similarly, the frequency of an activated (CD38 + HLA-DR+) CD8 T cell count > 15.85% of the total CD8 T cell population had sensitivity = 100% and specificity = 80.8% in determining severe COVID-19 infection (area under the curve 92.3%; p = 0.001) (Fig. 2d).

Survival analysis

When the data were censored on June 9, 2021, 7 (26.9%) patients succumbed to their infection at a median follow-up of 31 days (range: 17–76 days). The 28-day and 56-day mortality rates of our cohort were 19.2% and 26.9%, respectively. Of the 7 deaths, 5 patients died due to severe ARDS with multiorgan dysfunction, while 2 patients succumbed due to rhinocerebral mucormycosis with secondary HLH & sepsis. Survival at the 60-day time point for mild versus.

COVID-19 infection was 100% versus 53.3% (log rank p = 0.01), respectively. Survival analysis of the groups based on the cut-off proportion of CD4+, CD8 + and CD4:CD8 ratio is shown in Supplementary Fig. 2A-D.

Univariate & multivariate analysis of the prognostic factors

Univariate analysis of potential prognostic factors predicting mortality in patients with SARS-CoV infection showed absolute lymphocyte count ≤ 1000 cells/µl (HR = 1.9, 95% CI: (1.02–9.86); p = 0.04), absolute CD4 T cell count ≤ 250 cells/µl (HR = 2.3, 95% CI (1.23–18.32); p = 0.01), absolute CD8 T cell count ≤ 100 cells/µl (HR = 3.5, 95% CI

Table 3 Univariate & multivariate analysis of prognostic factors determining survival in COVID-19 patients

S.No	Variable	Univariate Analysis		Multivariate Analysis for	
		HR (95%CI)	**P value**	**HR (95%CI)**	**P value**
1.	**Age** ≥ 60 years vs. < 60 years	1.2(0.42–6.66)	0.12	-	-
2.	**Presence of medical comorbidities** Present vs. Absent	1.62(0.56–13.2)	0.09	1.2 (0.55–5.37)	0.12
3.	**Time to hospital reporting after COVID-19 symptoms** ≥ 7 days vs. < 7 days	1.4(0.86–9.43)	0.21	-	-
4.	**Absolute Lymphocyte count** ≤ 1000 cells/µL vs. > 1000 cells/ µL	1.9(1.02–9.86)	**0.04**	1.1(0.67–1.96)	0.60
5.	**CD 4 T cell count** ≤ 250 cells/µL vs. > 250 cells/ µL	2.3 (1.23–18.32)	**0.01**	-	-
6.	**CD 8 T cell count** ≤ 100 cells/µL vs. > 100 cells/ µL	3.5 (1.1–36.5)	**0.001**	2.2(1.03–5.68)	**0.01**
7.	**CD4/CD8 ratio** ≥ 2.5 vs. ≤ 2.5	2.1 (1.02–19.34)	**0.04**	1.8(1.16–10.35)	0.07
8.	**% Activated CD 8 T cell** ≥ 15% vs. < 15%	3.9(2.78–16.8)	**0.001**	2.1(2.45–21.3)	**0.023**
9.	**Serum Ferritin** ≥ 2000 ng/ml vs. < 2000 ng/ml	1.3 (0.25–23.2)	0.2	-	-
10.	**D Dimer** ≥ 1500 ng/ml vs. < 1500 ng/ml	1.23(0.62–8.96)	0.45	-	-
11.	**C-Reactive Protein** ≥ 5 x normal vs. < 5 x normal	1.32(0.89–11.2)	0.16		
12.	**Duration of ICU stay** < 14 days vs. > 14 days	1.23(0.23–3.46)	0.41	-	-
13.	**Mechanical Ventilation** Yes vs. No	5.2(3.45–26.76)	**0.001**	2.3(1.8-18.43)	**0.01**

(1.1–36.5); p = 0.001), CD4/CD8 ≥ 2.5 (HR = 2.1, 95% CI (1.02–19.34); p = 0.04), > 15% activated CD8 T cell count (HR = 3.9, 95% CI (2.78–16.8); p = 0.001) and need for mechanical ventilation (HR = 5.2, 95% CI (3.45–26.76); p = 0.001) to be associated with poor survival.(Table 3) Of these variables, absolute CD8 T cell count ≤ 100 cells/µl (HR = 2.2, 95% CI (1.03–5.68); p = 0.01), > 15% activated CD8 T cell count (HR = 2.1, 95% CI (2.45–21.3); p = 0.023) and need for mechanical ventilation (HR = 2.3, 95% CI 1.8-18.43; p = 0.01) retained their prognostic significance with respect to survival on multivariate analysis, as shown in Table 3.

Discussion

In the present study, we analyzed the clinicopathological features along with CBC findings and flow cytometric lymphoid subset analysis of COVID-19 patients for the prediction of disease severity. We demonstrated that there are clinically significant differences in the cellular immune response (comprising CD4 + and CD8 + T cell subsets) of the body to COVID-19 infection with mild and severe infection associated with distinct T cell immune activation profiles that can be utilized bedside in predicting disease severity at the initial COVID-19 diagnosis. Furthermore, we also observed that in addition to lymphopenia, which is known to have prognostic relevance, a ratio of CD4/CD8% > 2.7 and $CD8^+$ $CD38^+HLA$ DR^+ T cell activation > 15% were associated with severe disease and poor outcome.

COVID-19 infection poses a unique challenge, as it incites our body's inflammatory and immune responses that play a major role in the expression of the clinical spectrum of COVID-19 from asymptomatic/mild disease to severe ARDS resulting from a cytokine storm due to immune activation. Several studies have demonstrated that severe COVID-19 is characterized by dysregulated immune responses that cause lung damage, ARDS, and systemic inflammation. [9–12].

Lymphopenia is one of the characteristic hematological features of COVID-19 infection reported uniformly by several collaborative research groups. [13–16] In the initial two reports from Wuhan, China, the incidence of lymphopenia was reported to be 63% and 70.3%, respectively. [2–4] Similarly, Mathew et al., in their deep immune profiling of 149 COVID-19 patients, demonstrated that approximately half of the COVID-19 patients had lymphopenia at diagnosis (absolute lymphocyte count < 1000/µl).[17] Furthermore,

research groups have also shown that the degree of lymphopenia correlates with disease severity and poor outcome. [18–19] In their series of 41 COVID-19 infection-positive cases, Song et al. demonstrated that COVID-19 severe disease (N = 12) resulted in a statistically significant decline in the absolute number of CD3+, CD4+, CD8 + T cells and NK cells compared to patients with mild disease (N = 26; $p < 0.0001$). [20] It has been postulated by researchers that increased lymphocytic activation and exhaustion/apoptosis in response to the body's defense against COVID-19 might contribute to this finding. Furthermore, migration of lymphocytes from peripheral circulation to pulmonary tissue due to COVID-19 may also be an additive factor leading to lymphopenia [21–22].

Another key finding noticed in our cohort was that in addition to absolute lymphopenia, the proportionate decrease in absolute CD4 and CD8 counts (surrogate marker of increased T cell apoptosis) also holds prognostic significance in COVID-19 infection. Interestingly, we demonstrated that CD 4 count < 250/µl and CD 8 count < 100/µl were associated with significantly poor outcome in COVID patients, thereby highlighting the fact that severity of infection is directly proportional to increased apoptosis of T lymphocytes (CD4 & CD 8 positive) as a result of the type 1 interferon antiviral response of the body. Similar to our results, Lanetta et al. demonstrated the prognostic significance of absolute T cell subset analysis (CD3-, CD4-, and CD8-positive T cells) in defining outcomes in COVID-19 patients. They demonstrated that peripheral T lymphocyte counts (CD3 + < 733 cells/µl; CD3 + CD4 + < 426 cells/µl; CD3 + CD8 + < 262 cells/µ) were consistently associated with poor outcome. [23] Similarly, Bornheimer et al., in their cohort of 160 COVID-19 patients, demonstrated that patients with low CD4 (< 250/µl) and CD8 (< 100/µl) counts had at least an ~ 5 to 6 times higher relative risk of mechanical ventilation and an ~ 4.5 times higher relative risk of mortality. [24] We observed that a CD4/8 T cell ratio of ≥ 2.63 had sensitivity = 100% and specificity = 78.9% for determining COVID disease severity (area under the curve 91.7%; $p = 0.001$). This needs to be validated in a larger cohort of patients. Recently, Mathew et al. demonstrated in their single-cell RNA sequencing experiments that severe COVID infection leads to increased expression of apoptotic gene pathways and defective cytotoxic function in CD4 and CD8 T cells and NK cell populations, thereby leading to delayed viral clearance in severe disease. [17].

Next, we also analyzed the percentage of activated CD4 + and CD8 + T cells (CD38 + HLA-DR+) in healthy controls and mild and severe COVID-19 patients and noted that the percentage of activated CD4 + and CD8 + T cells was directly proportional to COVID-19 severity. Furthermore, we observed that the percentage of activated CD8 T cells ($CD38^+HLA\ DR^+$) > 15% also has prognostic significance. Based on these results, we hypothesize that the severity of COVID-19 (which in turn is proportional to SARS-CoV load) determines the intensity of the type 1 interferon response of the body to infection, with mild infection causing modest activation and severe infection causing intense T cell activation, thereby causing severe lung injury and ARDS due to cytokine release. Furthermore, intense T cell activation is followed by activation of T lymphocytic exhaustion/apoptosis pathways, which is reflected by the severe lymphopenia that ensues in severe cases. This observation is further supported by Song et al., who demonstrated intense CD4 + and CD8 + T cell infiltrates, with strong granzyme B expression in the postmortem lung specimen of one of their severe COVID-19 patients. [20] Mathew et al. also demonstrated that the type 1 interferon response is significantly upregulated in patients with severe disease, thereby leading to increased activation profiles of CD4 & CD8 cells correlating with viral clearance and disease. [17] Based on our findings, we hypothesize that lymphopenia along with a markedly skewed CD4/CD8 T cell ratio and increased CD8 cell activation profile early in disease course (day 5–7 of illness) may point toward severe COVID-19 disease and should be managed aggressively. One of the main shortcomings of this study was that the T cell exhaustion/apoptotic pathways were not analyzed in this study, which could have significantly impacted our results. Additionally, our study population was small, and hence, our hypothesis needs to be validated in a large multicenter prospective study.

Considering the likelihood of clinical application, lymphocyte subset analysis along with T cell activation and exhaustion immunophenotypes by flow cytometry can be a useful tool in preemptively identifying severe COVID-19 cases at onset, which has a significant bearing on effective patient management strategies and could also be utilized to predict disease outcomes and evaluate new interventions for COVID-19 patients.

Electronic supplementary material

Supplementary Fig. 1

(A) Expression of activation markers (*CD38 + HLA DR+*) assessed by flow cytometry on $CD4^+$ T cells in mild (n = 11), severe (n = 15) SARS-CoV-2 patients and healthy control (n = 7) population. (B) ROC analysis of CD4/CD8 ratio of > 2.77 in predicting survival in COVID-19 patients. (C) Increased expression of activation markers (CD38 and HLA-DR) in CD8 + T cells in PBMCs of HC (n = 7), mild

(n = 11) and severe (n = 15) SARS-COV-2 patients. (D) ROC analysis of % activated CD8 $^{38/DR+ve}$ T cells ≥ 15% in predicting survival in predicting disease severity in COVID-19 patients. Data are expressed as median (IQR). *p < 0.05, by two tailed Mann–Whitney U-test.

Supplementary Fig. 2

Shows the hazard ratios of all prognostic factors determining overall survival on multivariate analysis.

Supplementary Fig. 3

Kaplan Meier survival analysis plots for (a) all COVID positive patients (b) patients above and below the CD4+ T-cell cutoffs at hospital admission (c) patients above and below the CD8+ T-cell cut-offs at hospital admission (d) patients above and below the CD4/CD 8 T-cell ratio cut-offs at hospital admission.

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Data Availability The data that support the findings of this study are available on request from the corresponding author, Dr. Khaliqur Rahman.

Declarations

Conflict of interest disclosure None.

Ethics approval The study has been approved by Institution Ethics Committee (IEC no 2021-6-IMP-154).

Patient consent statement Written informed consent has been taken from all patients included in the study.

Permission to reproduce material from other sources This is the original research and all figures, diagrams, schemes, tables, and text in this manuscript are our own, original, unpublished work.

Competing interests and Source of Funding None Declared.

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