Cureus

Open Access Original Article

DOI: 10.7759/cureus.40556

Sequential Shifting in T-helper and T-cytotoxic Subset Cell Population in Mild and Severe COVID-19 Patients Infected With Variant B.1.61

Review began 05/25/2023
Review ended 06/13/2023
Published 06/17/2023

Jyotsna Agarwal [1] , Namrata P. Awasthi [2] , Shivani Singh [3] , Vandana Tiwari [3]

1. Microbiology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, IND 2. Pathology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, IND 3. Biochemistry, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, IND

Corresponding author: Vandana Tiwari, drvandana2166@yahoo.com

Abstract

Aim: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) modulates antiviral immunity via T cells, but whether these cells are active or abundant in coronavirus disease 2019 (COVID-19) patients is unknown. The present study aimed to investigate the temporal shifting in the T-cell population and their subsets, T-Helper (Th) cell (CD4) and T-Cytotoxic (Tc) cell (CD8) in COVID-19 patients.

Method: Thirty confirmed COVID-19 patients (nasal swab reverse transcription-polymerase chain reaction (RT-PCR) confirmed) were enrolled. On the basis of oxygen saturation (SpO2) levels, patients were stratified into two categories: (i) mild (n=11) having fever and SpO2 level >95%, and (ii) severe (n=19) on the ventilator, and in the intensive care unit (ICU) as per the Indian Council of Medical Research (ICMR) guidelines. Thirty age-sex-matched controls without infectious diseases unrelated to COVID-19 were also enrolled in the study. Patients with inflammatory diseases and severe comorbidities that compromise immunity were excluded from the study. Immunophenotyping flow cytometry assay was used to evaluate T-cell viability, Th, and Tc cells population in mild and severe COVID-19 patients on day 1 (at admission) and day 4 (decreasing the infection load) in the second COVID-19 wave (variant: B.1.61).

Categorical variables were expressed as frequency and percentage and p-values were calculated by Chi-square test. All the variables were represented in median and Q1 (25 percentile) and Q3 (75 percentile). The Mann-Whitney test was used to compare the study groups. The Δ mean differences were calculated by using the Paired samples t-test. The statistically significant level was taken as $p<0.05$.

Results: Hemoglobin, total leukocyte count (TLC), lymphocytes, monocytes, and eosinophils were significantly reduced in patients ($p<0.05$). A significant decrease of CD4 and CD8 cells in severe COVID-19 patients vs. controls (CD4, median 49; CD8, 40.12; $p>0.05$) was seen. Th-EM (effector memory)-Tim-3 (T-cell immunoglobulin domain and mucin domain 3)+ was significantly higher ($p=0.002$) however, Tc-EMRA (effector memory cells re-expressing)-Tim-3+, Tc-Naive-Tim-3+, Tc-EM-PD1+ and Tc-CM (central memory)-Tim-3+ significantly reduced ($p<0.05$) in mild COVID-19 patients than controls. Similarly, in severe COVID-19 patients, Th-EMRA-Tim-3+, Th-Naive-PD1+, Th-EM-PD1+, Th-EM-Tim 3+ and Th-CM-Tim-3+ showed a significant reduction ($p<0.05$) and Tc-EMRA-Tim-3+, Tc-Naive-Tim-3+, Tc-EM-PD1+, and Tc-CM-Tim-3+ showed similar results.

In mild vs. severe group, decreased T-cells ($p=0.001$), Th-EMRA-Tim-3+ ($p=0.024$), and Th-Navie-Tim-3+ ($p=0.005$), and significantly increased ($p<0.05$) Tc-Naive-Tim3+ ($p=0.001$), Tc-EM-Tim-3+ ($p=0.031$), and Tc-CM-Tim-3+ ($p=0.08$) were observed. Severe COVID-19 patients showed a significant increase in Th-Naive-Tim3+ (day 4-day 1; δ43, $p=0.019$), Th-EM-Tim3+ (δ 16.24, $p=0.033$), and Th-CM-Tim3+ (δ 13.57, $p=0.041$).

Conclusion: T-cell populations and CD8 subset help to differentiate the mild and severe COVID-19 patients. Monitoring T cells, especially CD8 subset changes, has important implications for diagnosing and treating mild and severe patients being critically ill.

Categories: Infectious Disease, Pulmonology, Hematology
Keywords: disease severity, flowcytometry, t-cytotoxic cells, t-helper cells, covid-19

Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in nearly 800 million confirmed cases and seven million deaths worldwide as of May 2023 [1,2].

Even amid an active infection, the immune system invests in the host's future by selecting activated T cells to become memory progenitors. If the primary response successfully wards off the infection, the organism

will, in most cases, go on to preserve part of that immune response in the form of memory T cells. Because of the successful resolution of the prior infection, these memory T cells are a known quantity and are thus preserved at an increased frequency throughout the body, ready to mediate an enhanced and accelerated response to reinfection [3]. However, T-cell memory is not only important as a source of new effector T cells; memory Th responses can enhance memory B cell responses upon rechallenge, which becomes particularly important in the context of pathogen evolution to evade antibody recognition. For these reasons, the major goal of all vaccines against SARS-CoV-2 should be to elicit T-cell memory in both the circulation and tissues. In the context of both natural SARS-CoV-2 infection and vaccination, tracking the stability of circulating and tissue-resident T-cell memory over months and years in humans and animal models will be crucial.

Although work on SARS-CoV-2 suggests that T-cell memory to SARS-CoV-2 is likely long-lived, further research and more time are required to assess the immunity to SARS-CoV-2 fully [4]. Furthermore, whether pre-existing SARS-CoV-2-reactive T cells in naive individuals provide beneficial immunity, promote an ineffective response by biasing the responding population, or cause immunopathology remains unanswered, but these populations will likely have a role in the development of anti-SARS-CoV-2 memory response.

T cells have a pivotal role in antiviral immunity. Effector T cells eradicate virus-infected cells, contribute to the innate antiviral response, and promote B cell responses, culminating in the generation of virus-specific antibodies [5]. Conventional T cells, defined by the expression of the cell-surface receptors CD4 and CD8, are characterized by their expression.

CD8 cells can recognize SARS-CoV-2 antigen in a proportion of healthy individuals and COVID-19 patients [6-8]. Furthermore, CD8 cells frequently display depleted characteristics in this disease and drastically reduce cell numbers in certain severe patients [9]. This raises questions about the inability of CD8T cells to mediate cellular protection during the peak of the infection [10].

Similar to CD8 cells, evidence is that CD4 cells exhibit functional impairment and elevated expression of activation and/or exhaustion markers in COVID-19 patients [11]. Human peripheral CD4+ T cells can be characterized as naive (CCR7+CD45RA+), central memory (CCR7+CD45RA-), and effector-memory (CCR7-, CD45RA-) cells that respond differently during antigen re-exposure [12].

Most acute viral infections, including SARS-CoV-2 infection in humans, activate and alter T cells. Hence, quantitative and/or qualitative shifts in the T cells and their subsets (CD4 and CD8 cells) have been associated with SARS-CoV-2 infection [13]. Various studies have observed an increase in the frequency of activated T cell phenotypes and the expression of T cell exhaustion-related surface markers, such as programmed cell death 1 (PD-1) and T-cell immunoglobulin mucin domain 3 (TIM3) [14]. COVID-19 is highly contagious and its pathologic mechanism has attracted much attention [2,15]. Most patients recovered after careful treatment, but some developed severe and critical illnesses [16]. The detailed mechanisms underlying severe respiratory syndrome caused by the COVID-19 coronavirus remain unclear [14]. There is no effective treatment to rescue severe patients who might be turned into patients with critical illnesses [15]. Therefore, clarifying the pathological differences between moderate, severe, and critical patients is urgent. Thus, the current research aimed to identify the status of T-cell subsets (CD4 and CD8) surface markers status (populations) to differentiate disease severity and dynamic change in the cell population at day 1 (infection) and day 4 (viral load).

Materials And Methods

Study population

In the current study, 60 subjects were enrolled during the second wave of COVID-19 (variant B.1.61), which included 30 confirmed COVID-19 patients (nasal swab reverse transcription-polymerase chain reaction (RT-PCR) confirmed) admitted to the COVID-19 ward at Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India, and 30 normal healthy subjects (age-sex matched with cases) enrolled as the control group in the study. Furthermore, on the bases of oxygen saturation (SpO2) levels, the 30 COVID-19 patients were stratified into two categories: (i) mild (n=11) with fever and SpO2 level >95%, and (ii) severe (n=19) on the ventilator and in intensive care unit (ICU) are required as per the Indian Council of Medical Research (ICMR) guidelines. COVID-19 patients with mild diabetes and hypertension as comorbidity were included in the study. The exclusion criteria were: age <18 years of age, pregnant women, other lung disease, renal disease, malignancy, inflammatory diseases, and severe comorbidities that compromised immunity.

Blood samples were taken at the time of admission (day 1) and then on day 4, irrespective of disease progression or recovery in clinical symptoms, lab results, or arterial oxygen saturation after obtaining consent from the patient's guardian/attendant. The ethical approval was obtained from the Institutional Ethics Committee of Dr. Ram Manohar Lohia Institute of Medical Sciences (approval number: IEC-113/20).

Reagents and panel

For the immunophenotyping study (total T cells, viable T cell, and T-cell subset analysis), the following fluorescently labeled anti-human monoclonal antibodies with fluorochrome and viability dye were used

from BD Biosciences (San Jose, California, United States): anti-CD3 V500c, CD4 APC- R700, CD8 Per CP, CD197 PE (2LI-A), CD45RA FITC (HI100), CD279 BV605 (EH12.1), CD366 Alexa647 (TD3), CD25 BV421 (MA251), CD127 PE-Cy7 (HIL-TRMZ1), and fixable viability stain (FVS) 780 in both the samples of each enrolled study subject for measurements by flow cytometry.

Laboratory investigations

Hematological Parameters

Complete blood count was analyzed using five-part white blood cells (WBC) differential using advanced multi-angle polarized scattered separation (MAPSS) technology (Abbott Laboratories, Chicago, Illinois, United States).

T Cell and Subsets Analysis by Flowcytometry

Peripheral blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacutainer vials on days 1 and day 4 and mononuclear cells were separated. In brief, 300 µL of blood was mixed with 700 µL of sheath fluid and centrifuged at 300xg for five minutes. The supernatant was discarded, the cell pellet was resuspended in 100 µL of sheath fluid, 1 µL of FVS dye was added, and incubated for 15 minutes in the dark at room temperature. The cell suspension was washed twice with 2 ml of stain buffer by centrifugation at 300xg for five minutes. Finally, the cell pellet was resuspended into 100 µl stain buffer, and 50 µL brilliant stain buffer was added after that; antibodies were added to the cell suspension and vortexed. The tube was incubated for 20 minutes in the dark at room temperature, followed by adding 2 mL of 1X FACS lyse solution in each tube, vortexed, and incubated for 10-12 minutes in the dark at room temperature. The cell suspension was further centrifuged at 250 g for five minutes. The supernatant was discarded carefully, and the pellet was broken. Again 2 mL sheath fluid was added, and the cell suspension was centrifuged at 300 g for five minutes.

The final cell pellet was resuspended in a 300 µL sheath to acquire stained cells in BD FACSCanto™ flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States) with a BVR (blue-violet-red) laser.

Gating Strategy

Singlet gating was used to exclude doublets. Further, sequential gating on forward and side scatter followed by CD3-based gating was applied to study CD4 and CD8 T cells and their subsets. One million cells were acquired and analyzed using BD FACSDiva™ Software (Becton, Dickinson and Company).

Statistical analysis

Categorical variables were expressed as frequency and percentage and the p-values were calculated by Chi-square test. All the variables were represented in Median and Q1 (25 percentile) and Q3 (75 percentile). The Mann-Whitney test was used to compare the study groups. The Δ mean differences were calculated by using the paired samples t-test. The statistically significant level was taken as $p<0.05$. All data were analyzed by using IBM SPSS Statistics for Windows, Version 21.0 (Released 2012; IBM Corp., Armonk, New York, United States).

Results

General and hematological characteristics of study population

The baseline characteristics of the study groups are listed in Table *1*. Patients had symptoms like cough, fever, sore throat, rhinorrhea, shortness of breath, and comorbidities. There was no significant difference between the age and sex of the mild and severe groups of the COVID-19 patients (p=0.109 and 0.416, respectively).

Variables		Category		p-value
		Mild, n (%)	Severe, n (%)	
Age (Years)	31-45	2 (18.2)	9 (47.4)	0.109
	46-66	9 (81.8)	10 (52.6)	
	Mean±SD	54.3 1±14.66	48.85±12.40	0.242
Sex	Male	8 (72.7)	11 (57.9)	0.416
	Female	3 (27.3)	8 (42.1)	
Cough	Yes	6 (54.5)	11 (57.9)	0.858
	No	5 (45.5)	8 (42.1)	
Fever	Yes	7 (63.6)	10 (52.6)	0.557
	No	4 (36.4)	9 (47.4)	
Sore Throat	Yes	8 (72.7)	19 (100)	NA
	No	3 (27.3)	0	
Rhinorrhea	Yes	6 (54.5)	11 (57.9)	0.85
	No	5 (45.5)	8 (42.1)	
Shortness of Breath	Yes	5 (45.5)	12 (63.2)	0.260
	No	6 (54.5)	7(36.8)	
Co-morbidities	Yes	8 (72.7)	15 (78.9)	0.69
	No	3 (27.3)	4 (21.1)	

TABLE 1: General characteristics of COVID-19 patients

Applied Chi-square test/Fisher exact test as appropriate

COVID-19: coronavirus disease 2019

Table *2* represents the hematological parameters in which lymphocytes, monocytes, and eosinophils were significantly decreased ($p<0.05$); in contrast, neutrophils and platelets were significantly increased ($p<0.05$) in COVID-19 patients compared to the control group.

Variables	COVID-19 Patients (N=30), Median (Q1-Q3)	Controls (N=30), Median (Q1-Q3)	p-value
Hb (g/dL)	11.90 (9.10-12.90)	12.70 (11.95-13.10)	0.124
Hct (%)	35.50 (29.10-38.90)	38.75 (35.30-40.50)	0.082
RBCs (×10^6/uL)	4.16 (3.49-4.67)	4.29 (3.84-4.66)	0.684
MCV (fL)	87.70 (78.70-92.80)	88.35 (85.65-94.05)	0.268
MCH (pg)	28.00 (26.80-31.00)	28.90 (28.00-31.75)	0.075
MCHC (%)	33.30 (31.80-34.00)	32.45 (32.10-33.20)	0.871
WBC count (× 10^3/uL)	9.50 (7.00-12.30)	7.23 (6.56-9.41)	0.075
Neutrophil (%)	85.00 (77.00-86.00)	58.00 (52.00-66.00)	<0.0001*
Lymphocyte (%)	11.00 (8.00-19.00)	28.50 (21.50-35.50)	<0.0001*
Monocyte (%)	2.00 (1.00-4.00)	7.00 (6.00-10.00)	0.001*
Eosinophil (%)	2.00 (1.00-2.00)	3.00 (2.00-4.00)	<0.0001*
Platelet count (×10^3/uL)	228.00 (160.00-289.00)	155.50 (129.00-199.00)	0.023*

TABLE 2: Comparison of Hematological Parameters in COVID-19 Patients and Controls.

Data were represented in Median, lower Quartile (Q1) and upper Quartile (Q3).; the Mann-Whitney U test was used to calculate the p-value.

*p-value <0.05 considered as statistically significant.

Hb: hemoglobin; Hct: hematocrit; RBCs: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cells; COVID-19: coronavirus diseae 2019

The overall T-cell viability was reduced in severe COVID-19 patients followed by mild COVID-19 patients as compared to the control (p<0.0001) (Figures *1*-*4*).

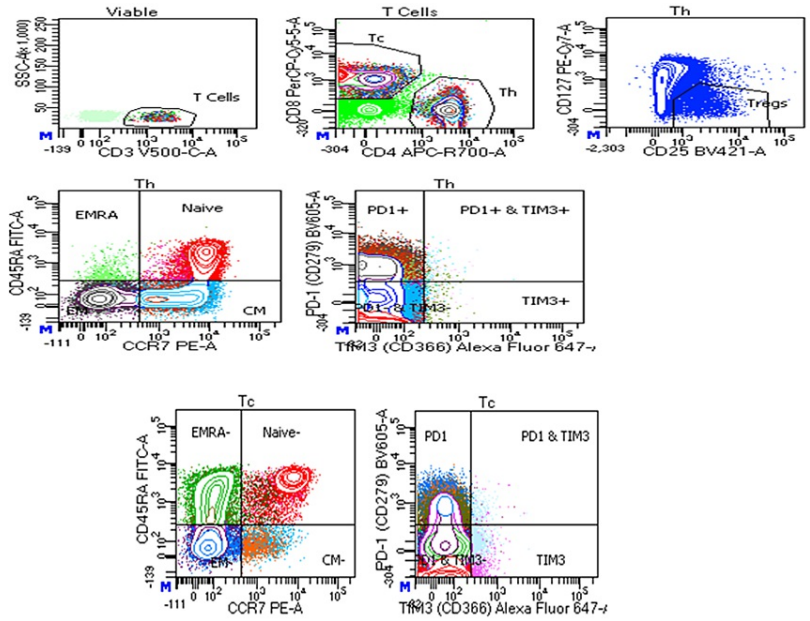


FIGURE 1: Flow cytometric analysis of T cells (Th and Tc) viability and their subsets in controls

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; EMRA: effector memory cells re-expressing; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin mucin domain 3

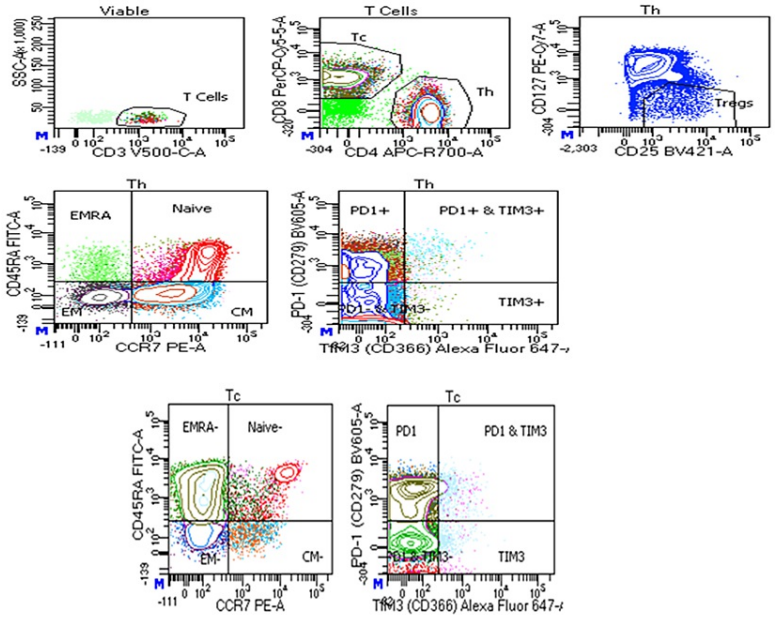


FIGURE 2: Flow cytometric analysis of T cells (Th and Tc) and their subsets in mild COVID-19 patients

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; EMRA: effector memory cells re-expressing; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin mucin domain 3

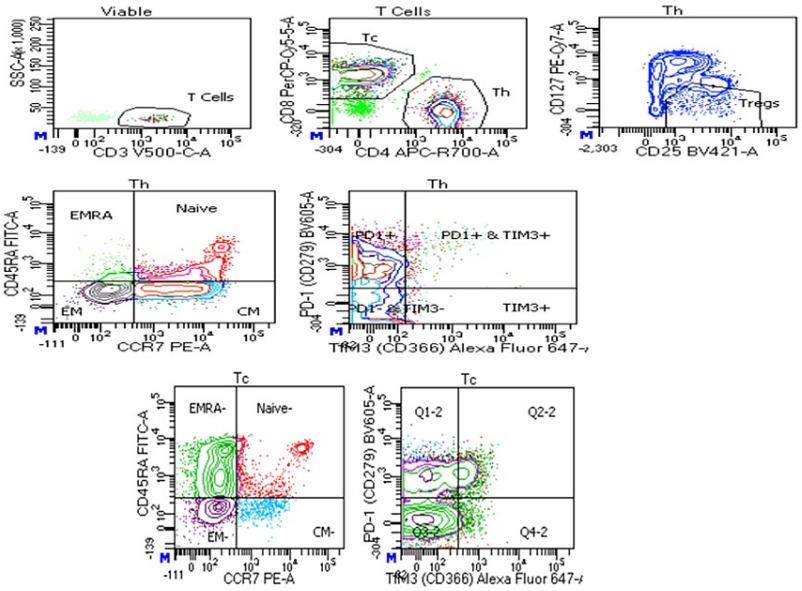


FIGURE 3: Flow cytometric analysis of T cells (Th and Tc) and their subsets in severe COVID-19 patients

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; EMRA: effector memory cells re-expressing; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin mucin domain 3

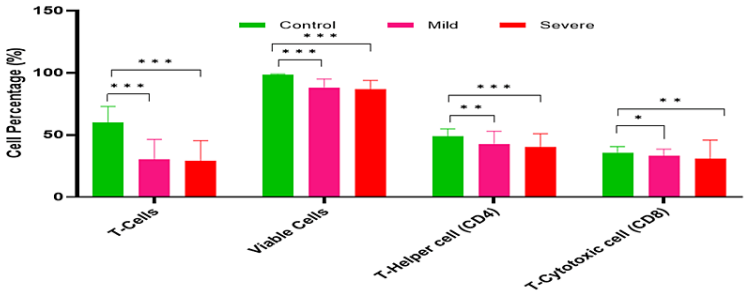


FIGURE 4: Comparative analysis of T cells, viability, and their subsets (Th and Tc) in controls, and mild and severe COVID-19 patients.

*- p-value <0.05, **- p-value<0.01, ***- p-value<0.001

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019

Status of Th (CD4) and Tc (CD8) subset cells in mild COVID-19 patients

In mild COVID-19 patients' CD4 subset, Th EM-Tim-3+ (Median 3.3; Q1-Q3 2.45-5.6.3) population was significantly higher as compared to controls (Median 0.5; Q1-Q3 0.3-0.85; p=0.002). However, CD8 subsets; Tc EMRA (effector memory cells re-expressing)-Tim-3+ (Median 8.4; Q1-Q3-4 25-20.75 vs. Median 2.75; Q1-Q3 1.1-5.65, p=0.003), Tc Naive-Tim-3+ (Median 13.4; Q1-Q3 -7.8-15.55 vs. Median 3.3; Q1-Q3 -2.3-38.75;

p<0.0001), Tc EM-PD1+ (Median 59.2; Q1-Q3 44.65-61.6 vs. Median 40.9; Q1-Q3 -26.2-54.1; p=0.036) and Tc-CM-Tim-3+(Median 3.0; Q1-Q3 -2.4-5.7 vs. Median 1.6; Q1-Q3 1.1-2.25; p=0.002) showed a significant increase as compared to controls (Figures *1*-*3*, *5*, *6*).

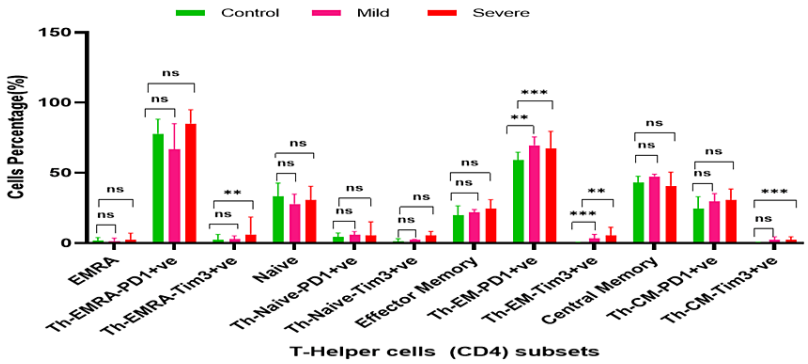


FIGURE 5: Representative graph of T-helper cells and their subsets in mild and severe cases of COVID-19 and controls.

*- p-value <0.05, **- p-value<0.01, ***- p-value<0.001* and ns-non significant.

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; EMRA: effector memory cells re-expressing; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin mucin domain 3; CM: central memory; EM: effector memory

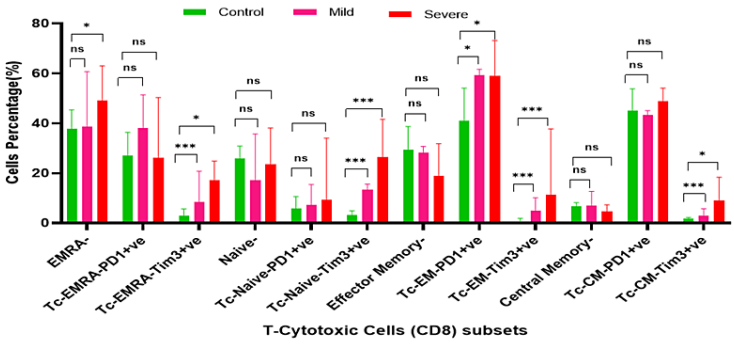


FIGURE 6: Representative graph of T-cytotoxic cells and their subsets in mild and severe cases of COVID-19, and controls.

*- p-value <0.05, **- p-value<0.01, ***- p-value<0.001

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; EMRA: effector memory cells re-expressing; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin mucin domain 3; CM: central memory; EM: effector memory

Status of Th (CD4) and Tc (CD8) subset cells in severe COVID-19 patients

The CD4 subset including Th EMRA-Tim-3+ (Median 6.2; Q1-Q3 -3.7-18.6 vs. Median 2.35; Q1-Q3 0.65-6.15; p=0.004), Th Naive-PD1+ (Median 5.45; Q1-Q3 -1.9-152 vs Median 4.4; Q1-Q3 2.4-7.2; p<0.0001), Th-EM (effector memory)-PD1+ (Median 67.45; Q1-Q3 -59.2-79.6 vs. Median 59.15; Q1-Q3 45.1-64.8; p<0.0001), Th EM-Tim 3+ (Median 5.65; Q1-Q3 -4.0-11.3 vs. Median 0.5; Q1-Q3 0.3-0.85; p=0.009), and Th CM (central memory)-Tim-3+ (Median 2.4; Q1-Q3 -1.3-4.5 vs. Median 0.5; Q1-Q3 -0.3-0.85; p<0.0001) showed a significantly higher in severe COVID-19 patients as compared to control. Similarly, CD8 subset cells including Tc EMRA-Tim-3+ (Median 17.2; Q1-Q3 -10.5-24.8 vs. Median 2.75; Q1-Q3 1.1-5.65; p<0.0001), Tc Naive-Tim-3+ (Median 26.4; Q1-Q3 -20.3-41.6 vs. Median 3.3; Q1-Q3 -2.3-4.95; p<0.0001), Tc EM-PD1+ (Median 58.85; Q1-Q3 -44.3-73.2 vs. Median 40.9; Q1-Q3 -26.2-54.1; p=0.011) and Tc CM-Tim-3+ (Median 9.0; Q1-Q3-5.7-18.3 vs. Median 1.6; Q1-Q3 1.1-2.25; p<0.0001) showed a significant increment as compared to controls (Figures *5*-*6*).

Patterns of Th (CD4) and Tc (CD8) subset cells in mild and severe COVID-19 patients

The T cell population was significantly decreased in COVID-19 patients (Severe: Median 30; Q1-Q3 46.4-18.5 vs. mild Median: 35.7; Q1-Q3 -40.8-21.35; p=0.001). The CD4 subsets were Th EMRA-Tim-3+ (Median 6.2; Q1-Q3 -3.7-18.6 vs. Median 2.8; Q1-Q3 -1.5-5.15; p=0.024), and Th Navie-Tim-3+ (Median 5.35; Q1-Q3 -3.5-8.4 vs. Median 2.5; Q1-Q3 -1.95-2.65; p=0.005). Severe COVID-19 patients demonstrated a significant decrease compared to the mild group. Similarly, the CD8 subsets Tc Naive-Tim3+ (Median 26.4; Q1-Q3 20.3-41.6 vs.Median 13.4; Q1-Q3 -7.8-15.5; p=0.001), Tc EM-Tim-3 + (Median 11.3; Q1-Q3 -6.2-37.8 vs. Median 4.9; Q1-Q3 3.65-10.1; p=0.031) and Tc CM-Tim-3+ (Median 9.0; Q1-Q3 -5.7-18.3 vs. Median 3.0; Q1-Q3 2.4-5.7; p=0.08) were significantly increased in severe patients as compared to mild patients (p<0.05). However, other CD4 and CD8 subsets did not show significant differences between the mild and severe groups (Figures *5*-*6*).

Dynamic changes (day 1 and day 4) in Th (CD4) subset cells in mild and severe COVID-19 patients

There was no significant difference in CD4 and CD8 subsets cell populations on day 1 and day 4 in the mild group of COVID-19 patients (Figure *7*). While severe COVID-19 patients showed a significant increase in Th-Naive-Tim3+ (day 4: Mean±SD 26.30±31.60- 7.87±8.94; day1: Δ18.43; p=0.019), Th-EM-Tim3+ (day 4: Mean±SD 25.21±30.68-8.97±8.90; day 1: Δ16.24; p=0.033), and Th-CM-Tim3+ (day4: Mean±SD 18.70±26.22-5.13±9.63; day 1: Δ 13.57; p=0.041) (Table *3*, Figure *8*).

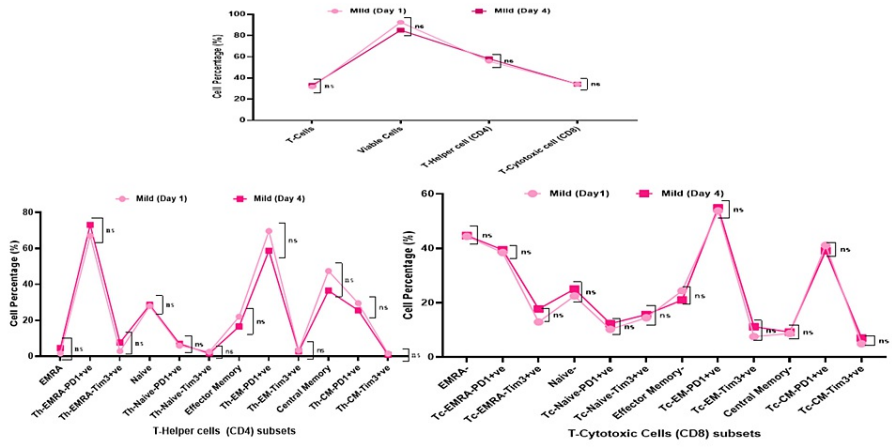


FIGURE 7: Relationship between the T cells, T cell viability, Th (CD4) cell, Tc (CD8) cell, and their subsets in Mild COVID-19 patients on day 1 and day 4.

*- p-value <0.05, **- p-value<0.01, ***- p-value<0.001

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; EMRA: effector memory cells re-expressing; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin mucin domain 3; CM: central memory; EM: effector memory; ns: not significant

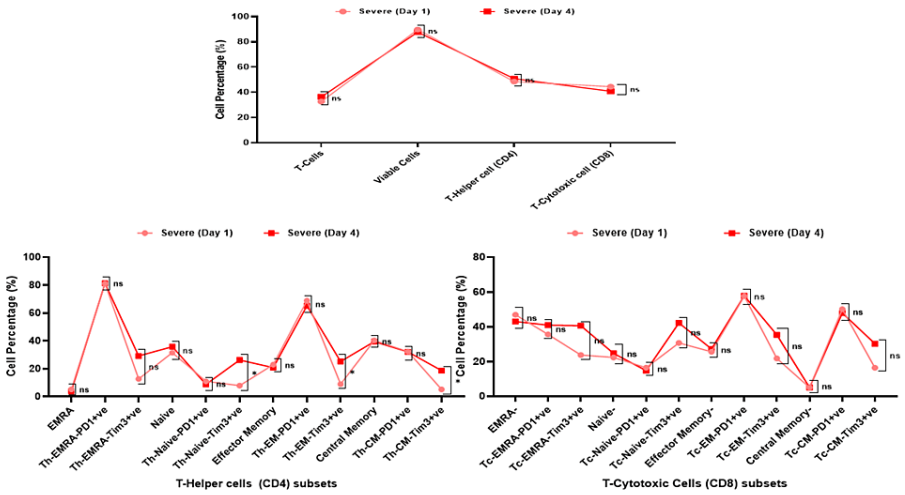


FIGURE 8: Relationship between the Tcells, T-cell viability, Th (CD4) cells, Tc (CD8) cells, and their subsets in severe COVID-19 patients on day 1 and day 4.

*- p-value <0.05, **- p-value<0.01, ***- p-value<0.001

Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; EMRA: effector memory cells re-expressing; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin mucin domain 3; CM: central memory; EM: effector memory; ns: not significant

Categories	Variables	Δ Mean Difference	p-value
Mild	T Cells	1.44	0.861
	Viable Cells	-7.46	0.111
	Th cell (CD4)	2.35	0.687
	EMRA	0.93	0.809
	Th-EMRA-PD1+	-0.32	0.963
	Th-EMRA-Tim3+	3.60	0.293
	Naive	5.83	0.387
	Th-Naive-PD1+	3.45	0.156
	Th-Naive-Tim3+	2.37	0.472
	Effector Memory	-5.29	0.175
	Th-EM-PD1+	-7.32	0.177
	Th-EM-Tim3+	1.00	0.675
	Central Memory	-1.50	0.778
	Th-CM-PD1+ve	-4.55	0.116
	Th-CM-Tim3+ve	-0.57	0.701
	T-Cytotoxic cell (CD8)	-0.01	0.998
	EMRA-	0.31	0.974
	Tc-EMRA-PD1+	1.03	0.878
	Tc-EMRA-Tim3+	4.79	0.428
	Naive-	2.57	0.706

	Tc-Naive-PD1+	2.10	0.561
	Tc-Naive-Tim3+	1.17	0.809
	Effector Memory	-3.37	0.518
	Tc-EM-PD1+	1.11	0.817
	Tc-EM-Tim3+	3.67	0.375
	Central Memory-	0.50	0.838
	Tc-CM-PD1+	-2.00	0.536
	Tc-CM-Tim3+	2.12	0.567
Severe	T-Cells	3.78	0.578
	Viable Cells	-2.18	0.308
	T-Helper cell (CD4)	2.29	0.605
	EMRA	-1.28	0.516
	Th-EMRA-PD1+	0.18	0.974
	Th-EMRA-Tim3+	16.50	0.058
	Naive	4.29	0.468
	Th-Naive-PD1+	-2.21	0.570
	Th-Naive-Tim3+	18.43	0.019*
	Effector Memory	-2.15	0.550
	Th-EM-PD1+	-3.67	0.437
	Th-EM-Tim3+	16.24	0.033*
	Central Memory	-0.85	0.862
	Th-CM-PD1+	-0.19	0.953
	Th-CM-Tim3+	13.57	0.041*
	T-Cytotoxic cell (CD8)	-3.58	0.447
	EMRA-	-3.92	0.468
	Tc-EMRA-PD1+	5.32	0.514
	Tc-EMRA-Tim3+	16.84	0.079
	Naive-	2.32	0.690
	Tc-Naive-PD1+	-1.75	0.701
	Tc-Naive-Tim3+	11.47	0.165
	Effector Memory	1.60	0.793
	Tc-EM-PD1+	0.19	0.976
	Tc-EM-Tim3+	13.53	0.158
	Central Memory	-0.01	0.992
	Tc-CM-PD1+	-2.17	0.633
	Tc-CM-Tim3+	13.88	0.147

TABLE 3: Comparision between day 1 and day 4 altered flow cytometry profiling in COVID-19 patients based on severity.

Paired t-test were used to calculate the difference between Day 1 and Day 4

*p-value <0.05 is considered as statistically significant.

EMRA: effector memory cells re-expressing, PD1: programmed cell death protein 1; Th: T-helper; Tc: T-cytotoxic; COVID-19: coronavirus disease 2019; TIM3: T-cell immunoglobulin mucin domain 3; ns: not significant

Discussion

The present pilot study analyzed the immune cells population in mild and severe COVID-19 patients and compared them with normal controls. The lymphocytes, monocytes, and eosinophils were significantly decreased in severe patients as compared to mild patients. In contrast, neutrophils were increased in both groups of COVID-19 patients. The present study concluded that thrombocytopenia and lymphopenia may indicate COVID-19 disease. The effects of viral pneumonia on the immune system include decreasing leukocyte and lymphocyte counts [16,17].

Our results demonstrated that T cells, viable cells, T regulatory cells, Th (CD4) cells, and Tc (CD8) cells were significantly reduced in severe patients as compared to mild. There are two layers of adaptive antiviral responses from an immunological perspective. First, CD8 T cell response is initially programmed to prevent the disease from progressing to a severe phase. Second, Th cells are poised to stimulate and program B cells to generate neutralizing antibodies against specific antigens [18], conferring durable humoral immunity [19]. Th cells (CD4) are crucial for adaptive immunological responses [20]. Th (CD4) cells may develop into various subsets in response to antigen presentation [21].

Moreover, Tc (CD8) cells restricted by class I major histocompatibility complex molecules are essential for developing immunity to the influenza virus because they identify internal viral proteins that are conserved across diverse viral strains [22,23]. The novel coronavirus is highly comparable to SARS and Middle East Respiratory Syndrome (MERS); both severe respiratory viruses are controlled by CD4 and CD8 [24]. However, modest CD4 and CD8 reductions have been documented in COVID-19 patients [7,25].

Furthermore, the present investigation endeavors to elucidate unresolved inquiries concerning the memory of CD8+ T cells in response to SARS-CoV-2 infection in humans through tracking individual memory CD8+ T cell clones. Similar to previous research, our study observed a noteworthy reduction in CD4 and CD8 cells among severe patients compared to controls [10,26].

The categorization of immune CD4+ and CD8+ T cells into four primary subsets is determined by the surface expression of CCR7 and CD45RA. The entities mentioned above exhibit varying degrees of maturation and differentiation of T cells, which are characterized by unique functional properties. The various subsets that are present include the naïve CD4+ T cell subsets (CCR7+CD45RA+), central memory CD4+ T cells (CCR7+CD45RA−), effector memory CD4+ T cells (CD45RA+), revertant effector memory cells (CCR−CD45RA+) (T_{EMRA}) [27,28]. The current investigation involved an analysis of various CD4 and CD8 subsets, including Th EMRA-Tim-3+, Th Navie-Tim-3+, Tc Naïve-Tim3+, Tc EM-Tim-3+, and Tc CM- Tim-3+. Furthermore, it has been reported that CD27-CD28-T cells possess a high level of effector functionality that is comparable to that of the terminal effector T cells T_{EMRA} subset, as demonstrated by Romero et al. [28] and Koch et al. [29]. The data presented in our study indicates a statistically significant reduction in the T cell population among patients with both severe and mild conditions. The CD4 subsets, namely Th EMRA-Tim-3+ and Th Navie-Tim-3+, are the subject of discussion. The group of patients with severe COVID-19 exhibited a notable reduction compared to the cohort with mild symptoms. The study found a statistically significant increase in the CD8 subsets Tc Naïve-Tim3+, Tc EM-Tim-3+, and Tc CM-Tim-3+ in severe patients compared to mild patients.

In contrast, the naive T cell is primarily responsible for activation and proliferation, as Okada et al. [28] and Koch et al. [30] noted. The CD27+CD28+ subset of T cells, commonly referred to as naïve T cells, play a critical role in mounting an effective immune response against novel viral infections such as COVID-19 or in response to vaccination. The study found no statistically significant variation in the population of CD4 and CD8 subsets of cells between day 1 and day 4 among COVID-19 patients with mild symptoms. Severe COVID-19 patients exhibited a notable elevation in Th-Naive-Tim3+, Th-EM-Tim3+, and Th-CM-Tim3+.

A phenotypic transition of CD8+ T cells from T effector/T_{EM} cells to T_{EMRA} cells has been observed, with a notable prevalence of T_{EMRA} cells among SARS-CoV-2-specific CD8+ T cells as reported in studies [31,32]. T lymphocytes differentiate into either CD4+ or CD8+ T cells, which exit the thymus and migrate to secondary lymphoid organs. These cells play a crucial role in the adaptive cellular immune response, aiding in eradicating infections [33-34].

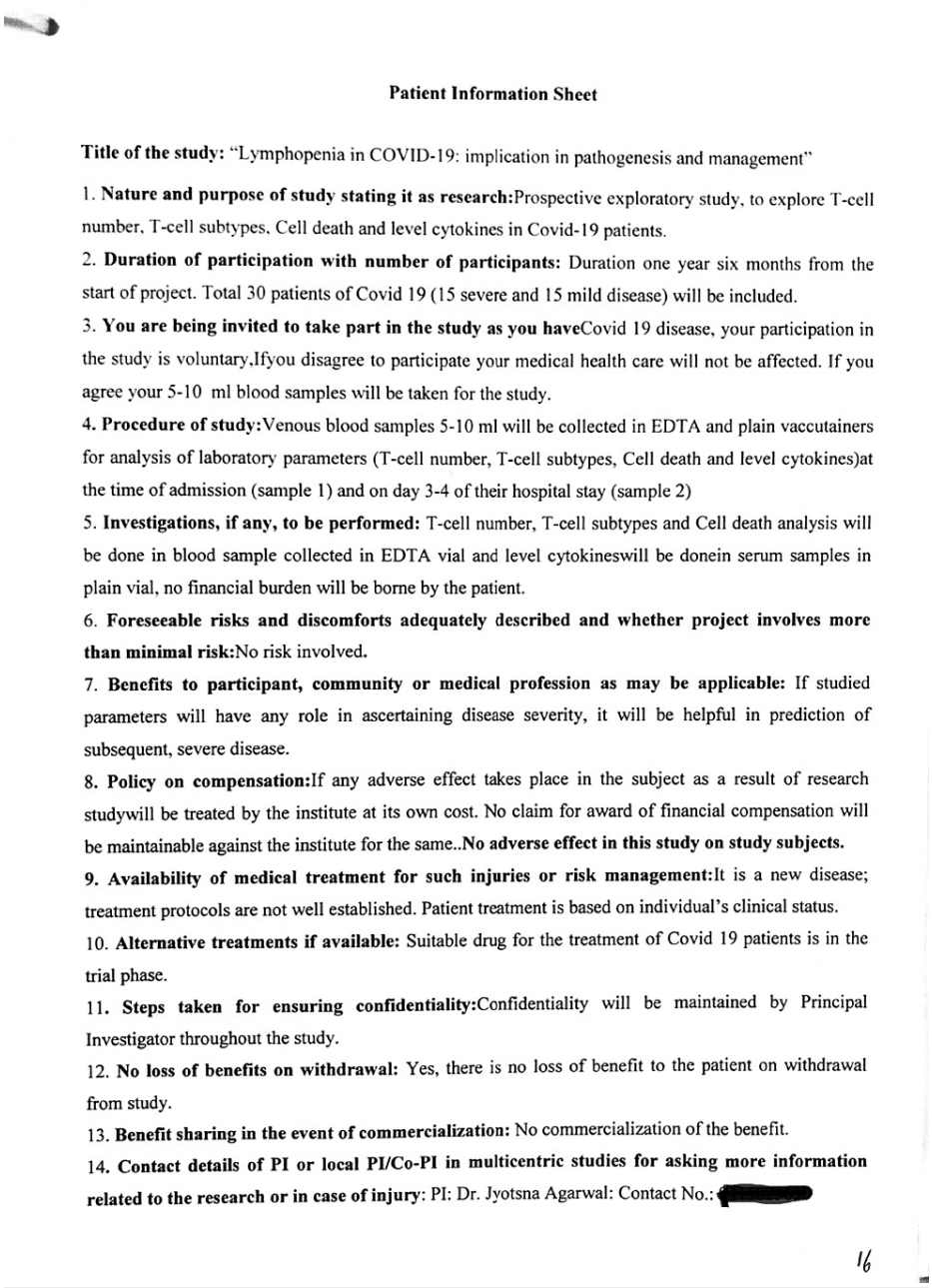
De Biasi et al. compared the immune system of hospitalized patients exhibiting mild to moderate disease and revealed a reduced count of total CD4+ and CD8+ T cells, along with their respective naive and T memory subsets, in the patient group [11,35].

A relatively small sample size remains the main limitation of this study. In addition, combining this standardized data with a more in-depth investigation of SARS-CoV-2-specific immune responses could provide a complete picture of immune responses to COVID-19. Additional research is required, preferably with follow-up and immune response evaluation after vaccination.

Conclusions

This study revealed that lymphocytes, monocytes and eosinophils were significantly reduced in COVID-19 patients. A significant decrease of CD4 and CD8 cells in severe patients was observed. T cell populations and CD8 subset help to differentiate the mild and severe COVID-19 patients. Monitoring T cells, especially CD8 subset changes, has important implications for diagnosing and treating mild and severe patients. The higher percentages of Th and Tc memory cells in the mild group of recovered individuals may serve as a prognostic indicator and reinforces the potential involvement of T cells in COVID-19 and in the development of immunological memory subsequent to recovery.

Appendices



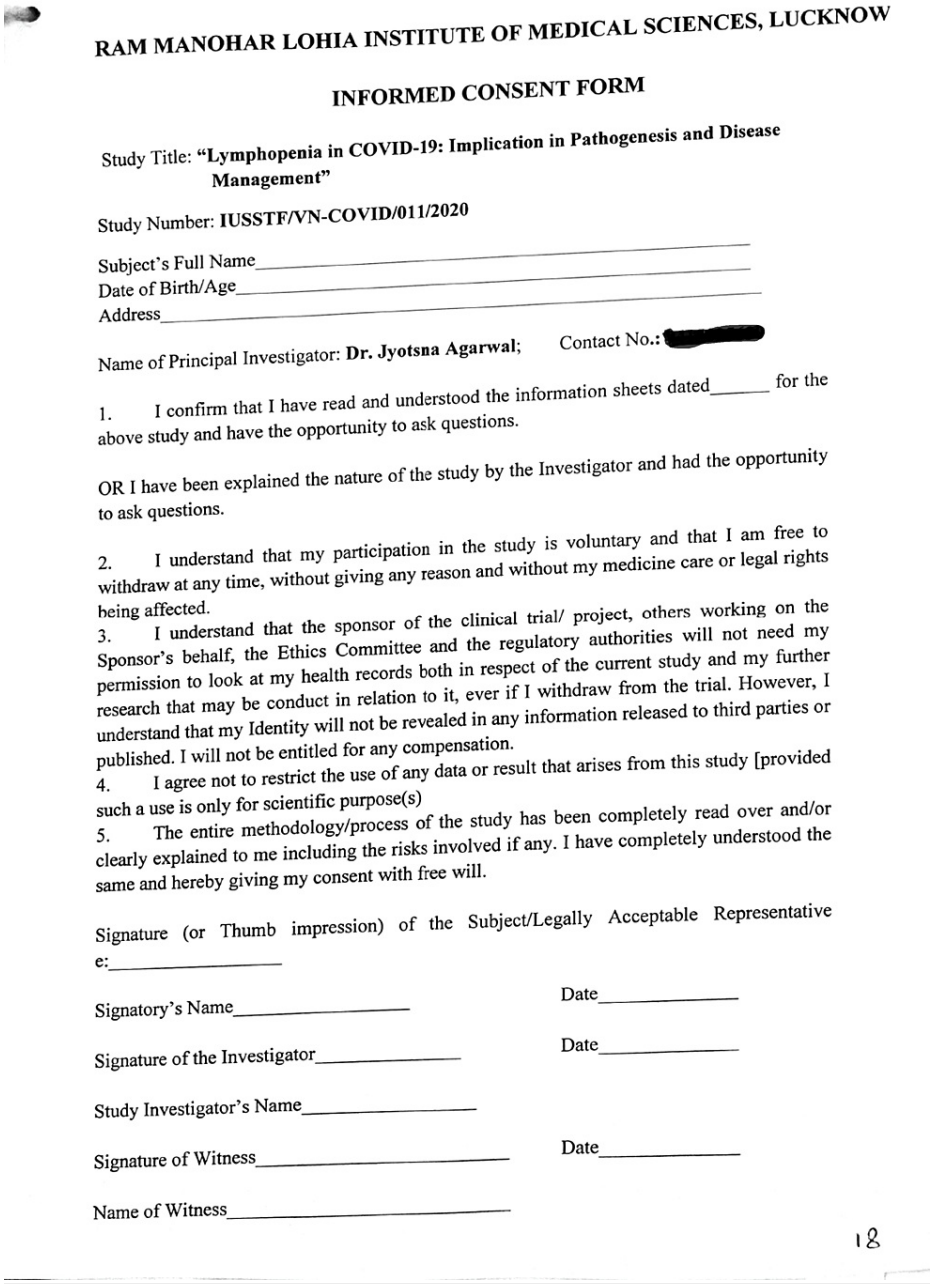
Patient Information Sheet

Title of the study: "Lymphopenia in COVID-19: implication in pathogenesis and management"

1. **Nature and purpose of study stating it as research:**Prospective exploratory study, to explore T-cell number, T-cell subtypes, Cell death and level cytokines in Covid-19 patients.
2. **Duration of participation with number of participants:** Duration one year six months from the start of project. Total 30 patients of Covid 19 (15 severe and 15 mild disease) will be included.
3. **You are being invited to take part in the study as you have**Covid 19 disease, your participation in the study is voluntary,Ifyou disagree to participate your medical health care will not be affected. If you agree your 5-10 ml blood samples will be taken for the study.
4. **Procedure of study:**Venous blood samples 5-10 ml will be collected in EDTA and plain vaccutainers for analysis of laboratory parameters (T-cell number, T-cell subtypes, Cell death and level cytokines)at the time of admission (sample 1) and on day 3-4 of their hospital stay (sample 2)
5. **Investigations, if any, to be performed:** T-cell number, T-cell subtypes and Cell death analysis will be done in blood sample collected in EDTA vial and level cytokineswill be donein serum samples in plain vial, no financial burden will be borne by the patient.
6. **Foreseeable risks and discomforts adequately described and whether project involves more than minimal risk:**No risk involved.
7. **Benefits to participant, community or medical profession as may be applicable:** If studied parameters will have any role in ascertaining disease severity, it will be helpful in prediction of subsequent, severe disease.
8. **Policy on compensation:**If any adverse effect takes place in the subject as a result of research studywill be treated by the institute at its own cost. No claim for award of financial compensation will be maintainable against the institute for the same..**No adverse effect in this study on study subjects.**
9. **Availability of medical treatment for such injuries or risk management:**It is a new disease; treatment protocols are not well established. Patient treatment is based on individual's clinical status.
10. **Alternative treatments if available:** Suitable drug for the treatment of Covid 19 patients is in the trial phase.
11. **Steps taken for ensuring confidentiality:**Confidentiality will be maintained by Principal Investigator throughout the study.
12. **No loss of benefits on withdrawal:** Yes, there is no loss of benefit to the patient on withdrawal from study.
13. **Benefit sharing in the event of commercialization:** No commercialization of the benefit.
14. **Contact details of PI or local PI/Co-PI in multicentric studies for asking more information related to the research or in case of injury**: PI: Dr. Jyotsna Agarwal: Contact No.:

16

FIGURE 9: Patient information sheet for participation in the study



RAM MANOHAR LOHIA INSTITUTE OF MEDICAL SCIENCES, LUCKNOW

INFORMED CONSENT FORM

Study Title: **"Lymphopenia in COVID-19: Implication in Pathogenesis and Disease Management"**

Study Number: **IUSSTF/VN-COVID/011/2020**

Subject's Full Name_____
Date of Birth/Age_____
Address_____

Name of Principal Investigator: **Dr. Jyotsna Agarwal**; Contact No.: [redacted]

1. I confirm that I have read and understood the information sheets dated_____ for the above study and have the opportunity to ask questions.

OR I have been explained the nature of the study by the Investigator and had the opportunity to ask questions.

2. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason and without my medicine care or legal rights being affected.
3. I understand that the sponsor of the clinical trial/ project, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and my further research that may be conduct in relation to it, ever if I withdraw from the trial. However, I understand that my Identity will not be revealed in any information released to third parties or published. I will not be entitled for any compensation.
4. I agree not to restrict the use of any data or result that arises from this study [provided such a use is only for scientific purpose(s)
5. The entire methodology/process of the study has been completely read over and/or clearly explained to me including the risks involved if any. I have completely understood the same and hereby giving my consent with free will.

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative e:_____

Signatory's Name_____ Date_____

Signature of the Investigator_____ Date_____

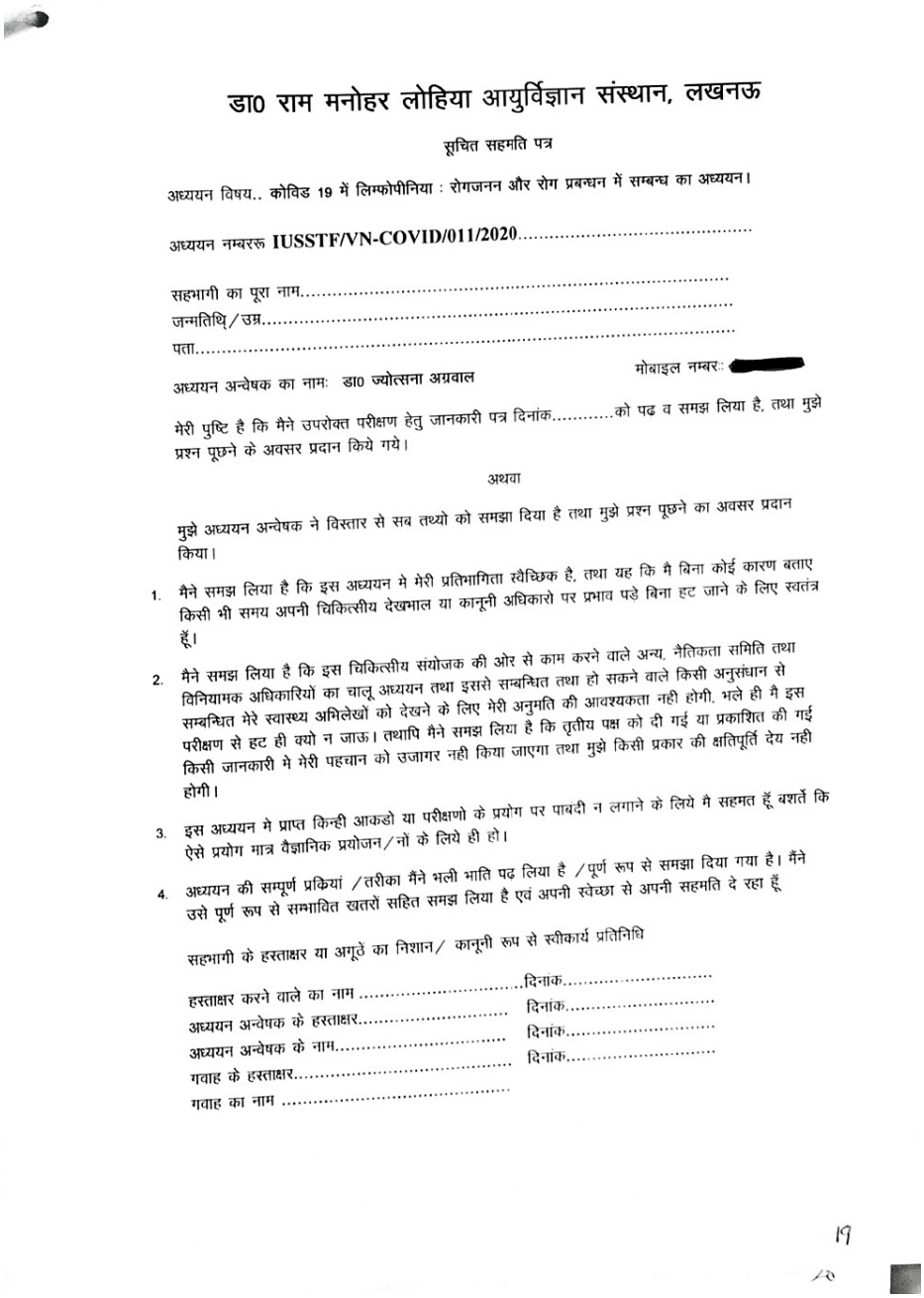
Study Investigator's Name_____

Signature of Witness_____ Date_____

Name of Witness_____

18

FIGURE 10: Consent form in English



डा0 राम मनोहर लोहिया आयुर्विज्ञान संस्थान, लखनऊ

सूचित सहमति पत्र

अध्ययन विषय.. कोविड 19 में लिम्फोपीनिया : रोगजनन और रोग प्रबन्धन में सम्बन्ध का अध्ययन।

अध्ययन नम्बररू IUSSTF/VN-COVID/011/2020.....

सहभागी का पूरा नाम.....

जन्मतिथि/उम्र.....

पता.....

अध्ययन अन्वेषक का नाम: डा0 ज्योत्सना अग्रवाल मोबाइल नम्बर:: [redacted]

मेरी पुष्टि है कि मैने उपरोक्त परीक्षण हेतु जानकारी पत्र दिनांक.....को पढ व समझ लिया है, तथा मुझे प्रश्न पूछने के अवसर प्रदान किये गये।

अथवा

मुझे अध्ययन अन्वेषक ने विस्तार से सब तथ्यो को समझा दिया है तथा मुझे प्रश्न पूछने का अवसर प्रदान किया।

1. मैने समझ लिया है कि इस अध्ययन मे मेरी प्रतिभागिता स्वैच्छिक है, तथा यह कि मै बिना कोई कारण बताए किसी भी समय अपनी चिकित्सीय देखभाल या कानूनी अधिकारो पर प्रभाव पडे बिना हट जाने के लिए स्वतंत्र हूँ।
2. मैने समझ लिया है कि इस चिकित्सीय संयोजक की ओर से काम करने वाले अन्य, नैतिकता समिति तथा विनियामक अधिकारियों का चालू अध्ययन तथा इससे सम्बन्धित तथा हो सकने वाले किसी अनुसंधान से सम्बन्धित मेरे स्वास्थ्य अभिलेखों को देखने के लिए मेरी अनुमति की आवश्यकता नही होगी, भले ही मै इस परीक्षण से हट ही क्यो न जाऊ। तथापि मैने समझ लिया है कि तृतीय पक्ष को दी गई या प्रकाशित की गई किसी जानकारी मे मेरी पहचान को उजागर नही किया जाएगा तथा मुझे किसी प्रकार की क्षतिपूर्ति देय नही होगी।
3. इस अध्ययन मे प्राप्त किन्ही आकडो या परीक्षणो के प्रयोग पर पाबंदी न लगाने के लिये मै सहमत हूँ, बशर्ते कि ऐसे प्रयोग मात्र वैज्ञानिक प्रयोजन/नों के लिये ही हो।
4. अध्ययन की सम्पूर्ण प्रकियां /तरीका मैंने भली भाति पढ लिया है /पूर्ण रूप से समझा दिया गया है। मैंने उसे पूर्ण रूप से सम्भावित खतरो सहित समझ लिया है एवं अपनी स्वेच्छा से अपनी सहमति दे रहा हूँ

सहभागी के हस्ताक्षर या अगूठें का निशान/ कानूनी रूप से स्वीकार्य प्रतिनिधि

हस्ताक्षर करने वाले का नाम .....दिनांक.....

अध्ययन अन्वेषक के हस्ताक्षर..... दिनांक.....

अध्ययन अन्वेषक के नाम..... दिनांक.....

गवाह के हस्ताक्षर..... दिनांक.....

गवाह का नाम .....

19

FIGURE 11: Consent form in Hindi

Additional Information

Disclosures

Human subjects: Consent was obtained or waived by all participants in this study. Institutional Ethics Committee of Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India issued approval IEC-113/20. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** This work was funded by the Indo-U.S. Science & Technology Forum (IUSSTF) (No. IUSSTF Virtual Networks for COVID-19 Networks/Ref: IUSSTF/VN-COVID/011/2020) to the Indian Partner, Prof. Jyotsna Agarwal (PI) and Prof. Vandana Tiwari (Co-PI). **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

References

1. WHO Coronavirus (COVID-19) Dashboard. (2023). Accessed: May, 2023: https://covid19.who.int/.
2. Johns Hopkins University of Medicine: COVID-19 dashboard . (2023). Accessed: May 2023: https://coronavirus.jhu.edu/map.html.
3. Jarjour NN, Masopust D, Jameson SC: T cell memory: understanding COVID-19. Immunity. 2021, 54:14-8. 10.1016/j.immuni.2020.12.009
4. Cohen KW, Linderman SL, Moodie Z, et al.: Longitudinal analysis shows durable and broad immune memory after SARS-CoV-2 infection with persisting antibody responses and memory B and T cells. Cell Rep Med. 2021, 2:100354. 10.1016/j.xcrm.2021.100354
5. Cervia C, Nilsson J, Zurbuchen Y, et al.: Systemic and mucosal antibody responses specific to SARS-CoV-2 during mild versus severe COVID-19. J Allergy Clin Immunol. 2021, 147:545-557.e9. 10.1016/j.jaci.2020.10.040
6. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A: Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999, 401:708-12. 10.1038/44385
7. Sallusto F, Geginat J, Lanzavecchia A: Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol. 2004, 22:745-63. 10.1146/annurev.immunol.22.012703.104702
8. Grifoni A, Weiskopf D, Ramirez SI, et al.: Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. Cell. 2020, 181:1489-1501.e15. 10.1016/j.cell.2020.05.015
9. Ni L, Ye F, Cheng ML, et al.: Detection of SARS-CoV-2-specific humoral and cellular immunity in COVID-19 convalescent individuals. Immunity. 2020, 52:971-7.e3. 10.1016/j.immuni.2020.04.023
10. Zheng HY, Zhang M, Yang CX, et al.: Elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may predict severe progression in COVID-19 patients. Cell Mol Immunol. 2020, 17:541-3. 10.1038/s41423-020-0401-3
11. De Biasi S, Meschiari M, Gibellini L, et al.: Marked T cell activation, senescence, exhaustion and skewing towards TH17 in patients with COVID-19 pneumonia. Nat Commun. 2020, 11:3434. 10.1038/s41467-020-17292-4
12. Chen G, Wu D, Guo W, et al.: Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest. 2020, 130:2620-9. 10.1172/JCI137244
13. Mathew D, Giles JR, Baxter AE, et al.: Deep immune profiling of COVID-19 patients reveals distinct immunotypes with therapeutic implications. Science. 2020, 369:8511. 10.1126/science.abc8511
14. Yang X, Yu Y, Xu J, et al.: Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020, 8:475-81. 10.1016/S2213-2600(20)30079-5
15. Li R, Pei S, Chen B, Song Y, Zhang T, Yang W, Shaman J: Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV-2). Science. 2020, 368:489-93. 10.1126/science.abb3221
16. Wu Z, McGoogan JM: Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese centre for disease control and prevention. JAMA. 2020, 323:1239-42. 10.1001/jama.2020.2648
17. Wang J, Jiang M, Chen X, Montaner LJ: Cytokine storm and leukocyte changes in mild versus severe SARS-CoV-2 infection: review of 3939 COVID-19 patients in China and emerging pathogenesis and therapy concepts. J Leukoc Biol. 2020, 108:17-41. 10.1002/JLB.3COVR0520-272R
18. Azkur AK, Akdis M, Azkur D, et al.: Immune response to SARS-CoV-2 and mechanisms of immunopathological changes in COVID-19. Allergy. 2020, 75:1564-81. 10.1111/all.14364
19. Shi Y, Wang Y, Shao C, et al.: COVID-19 infection: the perspectives on immune responses . Cell Death Differ. 2020, 27:1451-4. 10.1038/s41418-020-0530-3
20. Iwasaki A, Yang Y: The potential danger of suboptimal antibody responses in COVID-19 . Nat Rev Immunol. 2020, 20:339-41. 10.1038/s41577-020-0321-6
21. Zhou H, Liu F: Regulation, communication, and functional roles of adipose tissue-resident CD4(+) T cells in the control of metabolic homeostasis. Front Immunol. 2018, 9:1961. 10.3389/fimmu.2018.01961
22. Zhu J, Paul WE: CD4 T cells: fates, functions, and faults . Blood. 2008, 112:1557-69. 10.1182/blood-2008-05-078154
23. Jansen JM, Gerlach T, Elbahesh H, Rimmelzwaan GF, Saletti G: Influenza virus-specific CD4+ and CD8+ T cell-mediated immunity induced by infection and vaccination. J Clin Virol. 2019, 119:44-52. 10.1016/j.jcv.2019.08.009
24. Liu Q, Zhou YH, Yang ZQ: The cytokine storm of severe influenza and development of immunomodulatory therapy. Cell Mol Immunol. 2016, 13:3-10. 10.1038/cmi.2015.74
25. Lu R, Zhao X, Li J, et al.: Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet. 2020, 395:565-74.
26. Diao B, Wang C, Tan Y, et al.: Reduction and functional exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19). Front Immunol. 2020, 11:827. 10.3389/fimmu.2020.00827
27. Okada R, Kondo T, Matsuki F, Takata H, Takiguchi M: Phenotypic classification of human CD4+ T cell subsets and their differentiation. Int Immunol. 2008, 20:1189-99. 10.1093/intimm/dxn075
28. Romero P, Zippelius A, Kurth I, et al.: Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. J Immunol. 2007, 178:4112-9. 10.4049/jimmunol.178.7.4112
29. Koch S, Larbi A, Derhovanessian E, Ozcelik D, Naumova E, Pawelec G: Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people. Immun Ageing. 2008, 5:6. 10.1186/1742-4933-5-6
30. Dan JM, Mateus J, Kato Y, et al.: Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. Science. 2021, 371:10.1126/science.abf4063
31. Jung JH, Rha MS, Sa M, et al.: SARS-CoV-2-specific T cell memory is sustained in COVID-19 convalescent

patients for 10 months with successful development of stem cell-like memory T cells. Nat Commun. 2021, 12:4043. 10.1038/s41467-021-24377-1

32. Noulsri E, Lerdwana S, Fucharoen S, Pattanapanyasat K: Phenotypic characterization of circulating CD4/CD8 T-lymphocytes in β-thalassemia patients. Asian Pac J Allergy Immunol. 2014, 32:261-9. 10.12932/AP0426.32.3.2014
33. Waschbisch A, Sammet L, Schröder S, Lee DH, Barrantes-Freer A, Stadelmann C, Linker RA: Analysis of CD4+ CD8+ double-positive T cells in blood, cerebrospinal fluid and multiple sclerosis lesions. Clin Exp Immunol. 2014, 177:404-11. 10.1111/cei.12345
34. Nascimbeni M, Shin EC, Chiriboga L, Kleiner DE, Rehermann B: Peripheral CD4(+)CD8(+) T cells are differentiated effector memory cells with antiviral functions. Blood. 2004, 104:478-86. 10.1182/blood-2003-12-4395
35. Kusnadi A, Ramírez-Suástegui C, Fajardo V, et al.: Severely ill COVID-19 patients display impaired exhaustion features in SARS-CoV-2-reactive CD8(+) T cells. Sci Immunol. 2021, 6:10.1126/sciimmunol.abe4782