T-cell responses in COVID-19 survivors six to eight months after infection: a longitudinal cohort study in Pune

POONAM SURYAWANSHI[1], Bhagyashri Patil-Takbhate[1], Prachi Athavale[1], Shubhangi Kanitkar[1], Sachin Kisan Shivnitwar[1], M. S. Barthwal[1], Sachinkumar Dole[1], Hanumant Chavan[1], Priyanka Jali[1], Sujata Pawale[1], Dhanashree Kakad[1], Arjun Kakrani[1], Jitendra Bhawalkar[1], Madhura Ajitkumar Gandhi[1], Sarika Chaturvedi[2], Mahesh Karandikar[1], and Srikant Tripathy[1]

[1]Dr D Y Patil Medical College Hospital and Research Centre
[2]Dr D Y Patil Vidyapeeth

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Abstract

The SARS-CoV-2 immune response is crucial for disease management, although diminishing immunity raises the possibility of reinfection. In matched samples collected at one month and six to eight months after infection, we examined the immunological response to SARS-CoV-2 in a group of convalescent critically ill COVID-19 patients. The PBMCs were isolated from enrolled study participants and flow cytometry analysis was done to assess the lymphocyte subsets of naive, effector, central memory, and effector memory CD4+ or CD8+ T cells in COVID-19 patients at one month and six to eight months after infection. Immunophenotypic characterization of immune cell subsets was performed on individuals who were followed longitudinally for one month (n=44) and up to 6-8 months (n=25) after recovery from COVID infection. We observed that CD4+ T cells in hospitalized SARS-CoV-2 patients tended to decrease, whereas CD8+ T cells steadily recovered after one month, while there was a sustained increase in the population of effector T cells and effector memory T cells. Furthermore, COVID-19 patients showed persistently low B cells and a small increase in the NK cell population. In conclusion, our findings show that T cell responses were maintained at 6-8 months after infection. This opens new pathways for further research into the long-term effects in COVID-19 immunopathogenesis.

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Authors: - Poonam Suryawanshi[1], Bhagyashri Patil-Takbhate[1], Prachi Athavale[2,]Shubhangi Kanitkar[3], Sachin Shivnitwar[3] , M. S. Barthwal[4], Sachin Dole[4] , Hanumant Chavan[5,]Priyanka Jali[1], Sujata Pawale[1], Dhanashree Kakad[1] , Arjun Kakrani[3], Jitendra Bhawalkar[6] , Madhura Gandhi[1] , Sarika Chaturvedi[7], Mahesh Karandikar[1] , Srikanth Tripathy [1#]

Affiliation

1. Central Research Facility, Dr D.Y. Patil Medical College, Hospital and Research Centre, Dr D.Y. Patil Vidyapeeth, (deemed to be University), Pimpri, Pune
2. Department of Microbiology, Dr D.Y. Patil Medical College, Hospital and Research Centre, Dr D.Y. Patil Vidyapeeth, (deemed to be University), Pimpri, Pune
3. Department of General Medicine, Dr D.Y. Patil Medical College, Hospital and Research Centre, Dr D.Y. Patil Vidyapeeth, (deemed to be University), Pimpri, Pune
4. Department of Respiratory Medicine, Dr D.Y. Patil Medical College, Hospital and Research Centre, Dr D.Y. Patil Vidyapeeth, Pimpri, (deemed to be University), Pune

5. Dr D.Y. Patil Medical College, Hospital and Research Centre, Dr D.Y. Patil Vidyapeeth, (deemed to be University), Pimpri, Pune
6. Department of Community Medicine, Dr D.Y. Patil Medical College, Hospital and Research Centre, Dr D.Y. Patil Vidyapeeth, (deemed to be University), Pimpri, Pune
7. Dr D.Y. Patil Vidyapeeth, (deemed to be University), Pimpri, Pune

 # Corresponding Author

Dr Srikanth Tripathy

Director Medical Research

Dr D.Y. Patil Medical College, Hospital and Research Centre,

Sant Tukaram Nagar, Pimpri, Pune-411018

Email: - director.medicalresearch@dpu.edu.in

Phone- +91 20 27805100/27805101/67116499

Fax: - +91 020 27805217

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The SARS-CoV-2 immune response is crucial for disease management, although diminishing immunity raises the possibility of reinfection. We examined the immunological response to SARS-CoV-2 in a cohort of convalescent critically ill COVID-19 patients in matched samples collected at one month and six to eight months after infection. The PBMCs were isolated from enrolled study participants and flow cytometry analysis was done to assess the lymphocyte subsets of naive, effector, central memory, and effector memory CD4+ or CD8+ T cells in COVID-19 patients at one month and six to eight months after infection. Immunophenotypic characterization of immune cell subsets was performed on individuals who were followed longitudinally for one month (n=44) and up to 6-8 months (n=25) after recovery from COVID infection. We observed that CD4+ T cells in hospitalized SARS-CoV-2 patients tended to decrease, whereas CD8+ T cells steadily recovered after one month, while there was a sustained increase in the population of effector T cells and effector memory T cells. Furthermore, COVID-19 patients showed persistently low B cells and a small increase in the NK cell population. In conclusion, our findings show that T cell responses were maintained at 6-8 months after infection. This opens new pathways for further research into the long-term effects in COVID-19 immunopathogenesis.

Keywords: SARS-CoV-2, flow cytometry, COVID-19 survivors, coronavirus, lymphocyte subsets

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infection that led to the coronavirus disease in 2019 (COVID-19) is still a hazard to the general public's health. Since the beginning of the pandemic, there have been more than 5.3 million recorded deaths and over 275 million confirmed cases (WHO weekly epidemiologic update). The predominant symptom of COVID-19 is a respiratory illness with symptoms ranging from asymptomatic or moderate infection to severe symptoms necessitating intensive care unit (ICU) hospitalization [1].

To control and eradicate viral infections, a unique adaptive immune response must be developed. More specifically, virus-specific T and B cells are stimulated, grow, and eventually develop into effector cells in response to infection. Neutralizing antibodies and memory B and T cells, which are specific to the viral antigen survive long after the infection has been eradicated. This memory immune response, which is

activated during vaccination, is crucial in the prevention of reinfection. To comprehend the emergence and persistence of such protective immunity, it is crucial to characterize in detail the extent of specific adaptive immune responses in convalescent COVID-19 patients with varying degrees of severity. For foreseeing and controlling potential future waves of infections in the general population, a deeper understanding of the mechanisms driving the development of protective immunological memory in recovered individuals is of paramount importance for public health.

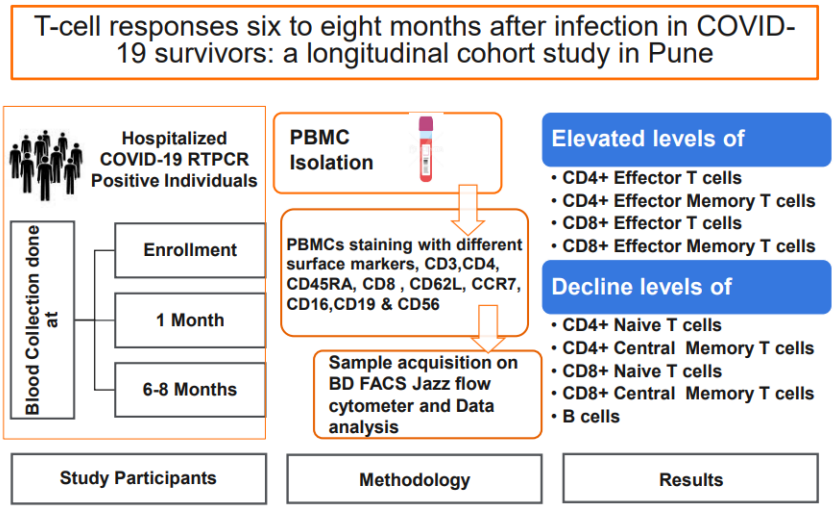
The early response to SARS-CoV-2 infection in severely ill COVID-19 patients is marked by significant immunological dysfunctions linked to a systemic inflammatory response and the emergence of altered innate and adaptive immune responses [1, 2]. More particular, T cell response is significantly altered in critically ill COVID-19 patients, and the most severe COVID-19 patients have been reported as having severe lymphopenia, phenotypic, and functional T cell alterations [3]. Therefore, it is still uncertain whether these critically ill patients can develop a strong and long-lasting SARS-CoV-2 specific T cell response despite the presence of significant immunological changes during the stay in the hospital.

Considering this, the objective of the current investigation was to monitor the immunological response, including memory T cells specific to SARS-CoV-2, in samples obtained 1 month and 6–8 months after infection from a cohort of convalescent critically ill COVID-19 patients.

Methodology

Study design

The present study was approved by the Institutional Ethics Committee of Dr D Y Patil Vidyapeeth (Ref No-DYPV/EC/634/2021 dated 25 February 2021) and recruited hospitalized patients with COVID-19 during March 2021 to February 2022 at Dr. D. Y. Patil Medical College, Hospital and Research Centre, Pune. The participants were [?]18-years-old male and female subjects who provided written informed consent before enrolment. The study was carried out in compliance with good clinical practices, including the International Conference on Harmonization Guidelines and the Declaration of Helsinki. COVID-19 patients were enrolled into the study during the period of hospitalization and the first blood samples were drawn for immunological investigations, which was followed by two additional samples collected at 1 month and 6-8 months, respectively after inclusion in the study (Graphical Abstract).



Graphical Abstract:- Graphical abstract showing study participants, study methodology and results

Patient characteristics

The COVID-19 patients were administered an approved questionnaire upon enrolment to collect information regarding COVID-19-related symptoms prior to hospitalization and smoking behaviours. The date of COVID-19 diagnosis, highest level of care received, the maximum amount of oxygen supplementation required (unit, oxygen need 5L/min supplemented by High Flow Nasal Oxygen (HFNO) or Continuous Positive Airway Pressure (CPAP)), and the critical care provided (intensive care unit, with or without mechanical ventilation) for COVID-19 illness were all gathered from digital medical records.

PBMC Isolation

EDTA anticoagulated peripheral blood (5 mL) was collected from each individual. All samples were tested within 6 hours of blood collection. The peripheral blood mononuclear cells

(PBMCs) and plasma were separated by density gradient centrifugation according to standard protocols and the plasma was stored at -80^{0}C till further use.

Flow cytometry for Immunophenotyping

For analysis of surface markers, fresh PBMCs were incubated for 30 minutes at room temperature in Stain Buffer (BD Biosciences, San Jose, CA) with optimal concentrations of the fluorochrome conjugated antibodies. Lymphocyte subsets were determined using the monoclonal antibodies as follows- CD3 APCH7 (Clone - SK-7), CD4 BV480 (Clone - SK-3), CD8 FITC (Clone - RPA-T8), CD62L APC (Clone - Dreg 56), CCR7 PE (Clone - 2-L1-A), CD45RA BV421 (Clone - 5H9), CD56 BV480 (Clone - NCAM 16.2) and CD16 FITC (Clone - 3G8) (BD Biosciences, San Jose, CA). The cells were further washed with stain buffer, fixed with 1% Formaldehyde in PBS and acquired to obtain 100000 gated lymphocyte events on FACS Jazz (BD Biosciences, San Jose, CA). The data was analysed using FACS Flow Jo software V10.7 (BD Biosciences, San Jose, CA).

Statistical analysis

SPSS (Statistical Package for Social Sciences) version 26.0, IBM, USA, was used to analyse the data. The Shapiro-Wilk Test was used to assess the distribution of the data set. Total counts (frequency), percentages, means, and standard deviations were generated as part of descriptive statistics for patient demographics. For continuous variables, an independent sample t-test / Mann Whitney U-test was employed, and for categorical connections, a Chi-square or Fisher exact test was utilised. Spearman's rank correlation was used for the correlation analysis, and a p value of < 0.05 was considered statistically significant.

Results

Clinical characteristics of COVID-19 patients

During an 18-month period (March 2021 to September 2022), blood samples were collected from hospitalized COVID-19 patients. A subset of donors was followed longitudinally at one month (n=44) and up to 6-8 months (n=25). Our analysis focused on the effect of COVID-19 on various T cell subsets and B cell immune responses (Graphical abstract). The clinical and demographic characteristics of the hospitalized COVID-19 cohort are summarized in Table 1. Our hospitalized COVID-19 cohort represents the infection in wider society in terms of biological sex distribution, encompassing 25 males and 19 females, and a mean age of 51.4 years (Table 1). Table 2 provides the biochemical laboratory parameters for the participants in the study. The disease categorization for the patients were moderate or severe as per the guidelines given by the National Institutes of Health [4].

Table 1- Demographic and clinical characteristics of the enrolled study participants

	Total (n=44)
Age (Yrs.)	51.4 ± 13.2
Sex	**Sex**
Male n (%)	25 (56.81%)

	Total (n=44)
Female n (%)	19 (43.19%)
Height Cm	158.5 ± 13.4
Weight kg	67.9 ± 13.3
BMI kg/m2	27.1 ± 5.4
Comorbidity n (%)	**Comorbidity n (%)**
Diabetes Mellitus	12 (27.9%)
Hypertension	16 (37.2%)
Liver failure	1 (2.3%)
Kidney disease	3 (7%)
Cancer	0
COPD	0
Signs and Symptoms at admission – n (%)	**Signs and Symptoms at admission – n (%)**
Rigors (shaking chills)	10 (23.3%)
Diarrhoea ([?]3 stools/24hrs)	8 (18.6%)
Cough	30 (69.8%)
Breathing difficulty	36 (83.7%)
Dysuria	3 (7%)
Abdominal pain	2 (4.7%)
Myalgia	27 (62.8%)
Arthralgia	3 (7%)
Sore throat	10 (23.3%)
Anosmia	4 (9.3%)
Nausea/vomiting	6 (14%)
Altered mental status	0
Headache	9 (20.9%)
Rash	1 (2.3%)
Loss of smell and/or taste	11 (25.6%)

Table 2 –Biochemical laboratory parameters for the enrolled study participants

Biochemical test	**Total (n=44)**	**Reference range**
HB (g/dL)	12.4 ± 2.0	11.6-15.0
Total Leukocyte Count (cells/μL)	6885 ± 3263	4000-10000
Neutrophils %	74.9 ± 11.9	40-80
lymphocytes %	18.2 ± 10.7	20-40
Monocytes %	5.9 ± 3.1	2-10
Eosinophils %	0.8 ± 1.1	1-6
Platelets(cells/μL)	210609 ± 85071	150000-410000
Creatinine mg/dL	1.4 ± 1.7	0.6-1.35
Total bilirubin mg/dL	0.5 ± 0.3	0.22-1.20
SGOT U/L	124.0 ± 258.9	8-43
SGPT U/L	87.0 ± 220.6	7-45
CRP mg/dL	62.1 ± 47.8	<10
ESR mm/hr	48.7 ± 22.2	0-20
Sr. Ferritin	599 ± 525	4.63-274.66
D Dimer ng/ml	851 ± 862	0-500

Values given as mean+ SD

Hospitalized SARS-CoV-2 patients had a persistent decline in CD4+ T cells, whereas CD8+ T cells gradually recover after one month

To assess the longitudinal effect of COVID-19 on T cell populations, we sampled PBMCs from patients with COVID-19 over a period of 6-8 months. PBMCs were analyzed by flow cytometry to investigate immunophenotype across the various T cell compartments.

The lymphocyte populations recovered, with a substantial increase in cell counts at 1 month (P=0.0003) that remained consistent until 6-8 month (P=0.0003) (Figure 1A). Total T cells (CD3+T cells) increased significantly after one month (P0.0001) and then dropped slightly at 6-8 months (P=0.022) but remained significantly higher than at the time of enrolment (P=0.021) (Figure 1B) (Table 3).

When comparing CD4+T cells to enrolment, a constant and significant reduction was observed at one month (P=0.022) and continued until 6-8 months (P=0.0016) (Figure 1C). In relation to CD8+ T cells, the increase was significantly higher at 6 months compared to that at enrolment (P=0.017) and at one month (P=0.012) (Figure 1D) (Table 3).

Together, these data are reflective of the alterations in the levels of various immune cell types during COVID-19, in line with observations made by others [5-9].

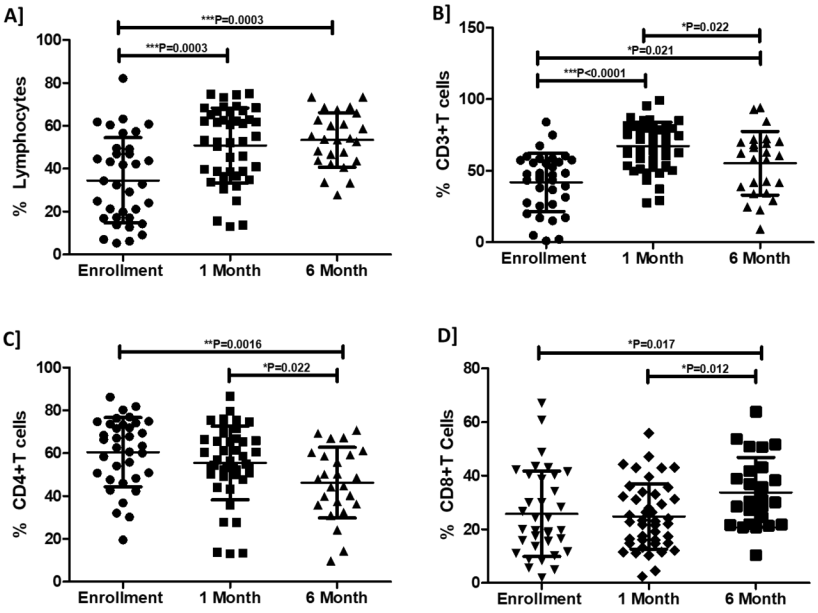


Figure 1- Flow Cytometry analysis of T cells subsets – PBMCs from COVID-19 infected were stained and acquired on flow cytometer. The bar graphs represent the comparison of percentages of immune cells and their subpopulation at different time point A] Lymphocytes B] CD3+ T cell profile C]CD4+ T cells and D] CD8+T cells profile Data are represented as median with interquartile range. *P<0.05, **P<0.001 and *** P <0.0001 significant.

COVID-19 patients have a sustained, elevated Effectors T cells and Effector Memory T cells population

The environment established by an infection, both locally and systemically, can cause changes in general memory T cell populations [10]. We looked at how COVID-19 affected naive, effector, and memory CD4+ and CD8+ T cells. Over time, distinct variations in the CD4+ and CD8+ T populations of COVID-19 patients were detected (Table 3). The fraction of CD4+ naive T cells and CD4+ central memory cells decreased significantly with time when compared to the time of enrolment (Figure 2A & 2C). At 6-7 months

after discharge from hospital, there was a considerable increase in the CD4+ effector and CD4+ effector memory T cell fractions compared to results obtained at the time of hospitalization (Figure 2B & 2D).

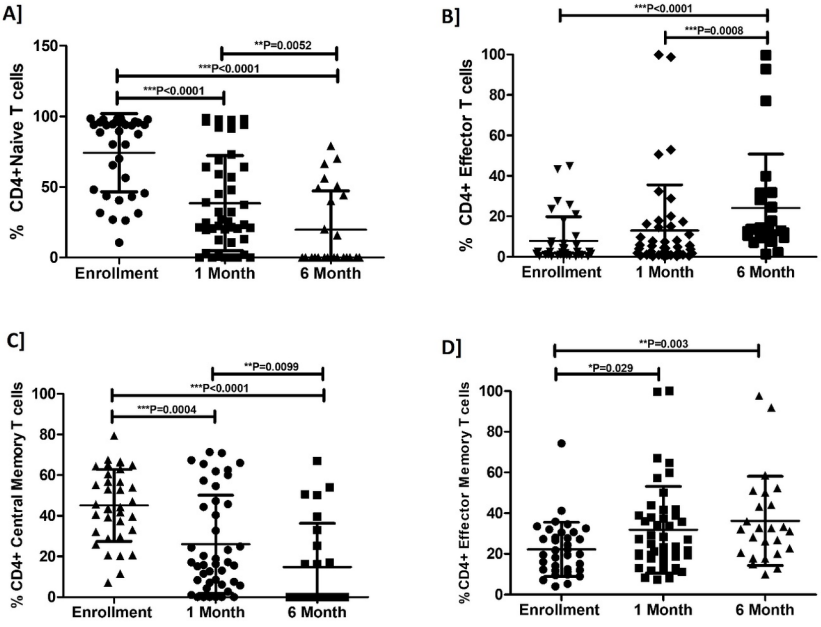


Figure 2- Flow Cytometry analysis of CD4+T cells subsets-the bar graphs represent the comparison of percentages of immune cells and their subpopulation at different time point A] CD4+ Naïve T cells B] CD4+ Effectors T cells C]CD4+ Central Memory T cells and D] CD4+ effector Memory T cells profile. Data are represented as median with interquartile range. *P<0.05, **P<0.001 and *** P <0.0001 significant.

We observed that CD8+ T cells showed more sustained changes in their naive and central memory from one month to 6-8 months after enrolment (Figure 3A & 3C). The frequencies of CD8+ effectors and CD8+ T Effector Memory, on the other hand, increased from one month to 6-8 months (Figure 3B & 3D). Overall, we found alterations in the memory T cell pool, most notably in the CD4+ and CD8+ effector memory subsets, where the changes persisted until the end of the study.

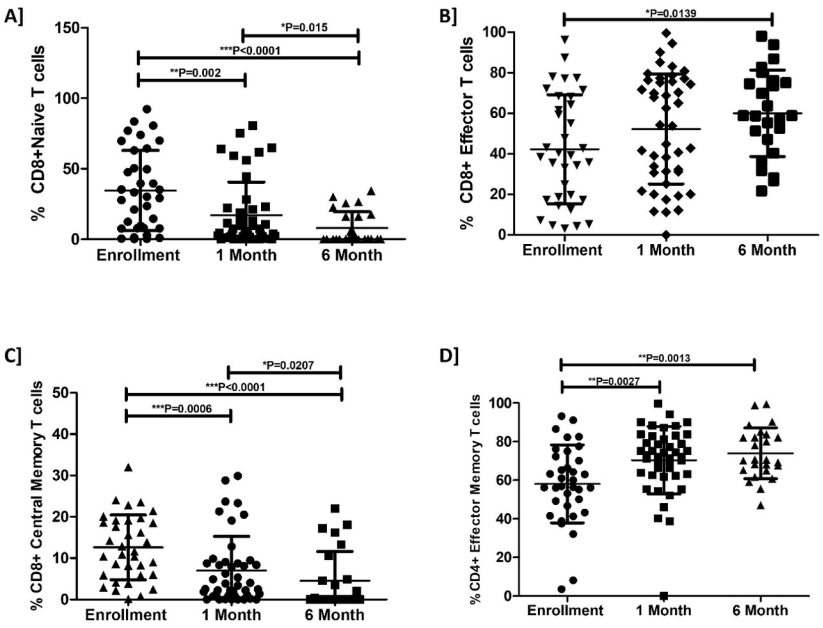


Figure 3- Flow Cytometry analysis of CD8+T cells subsets- The bar graphs represent the comparison of percentages of immune cells and their subpopulation at different time point A] CD8+ Naïve T cells B] CD8+ Effectors T cells C]CD8+ Central Memory T cells and D] CD8+ effector Memory T cells profile. Data are represented as median with interquartile range. *P<0.05, **P<0.001 and *** P <0.0001 significant.

Patients with COVID-19 had a persistently low B cells and minimal increase in NK cell population

We further assessed B cells, NK cells, and NKT cells in peripheral blood to better understand lymphocyte attrition. When compared to hospitalization, there was a substantial decline in B cells from one month (P=0.043) to 6-7 months (P=0.0034) (Fig 4A). The cytotoxic and regulatory fractions of NK cells increased slightly but not significantly with time compared to enrolment (Fig 4B & 4C). Despite this, no significant changes in NKT cells were observed over time (Fig 4D).

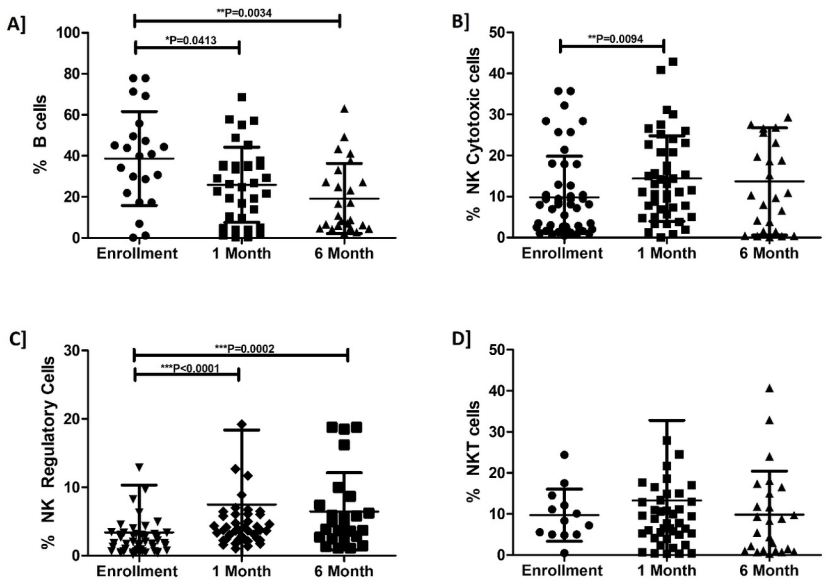


Figure 4- Flow Cytometry analysis of B cells & NK cells subsets- The bar graphs represent the comparison of percentages of immune cells and their subpopulation at different time point A] B cells B] NK Cytotoxic cells C] NK Regulatory cells and D] NKT cells profile. Data are represented as median with interquartile range. *P<0.05, **P<0.001 and *** P <0.0001 significant.

Table 3 Percentages of different immune cell subsets at different time points

	Enrolment (n=44)
Lymphocytes	**32.8 (7.06-82.1)**
CD3+ T cellls	**45.8 (0.92-84.1)**
CD4+ T Cells	**63.4 (19.5-86.2)**
CD4+ NAÏVE T Cells (CD4+CD45RA+CCR7+CD62L+)	**89.6 (10.5-98.9)**
CD4+ EFFECTORS T Cells (CD4+CD45RA+CCR7-CD62L-)	**2.41 (0.2-44.9)**
CD4+ CENTRAL MEMORY T Cells (CD4+CD45RA-CCR7+CD62L+)	**44.3 (7.14-79.4)**
CD4+ EFFECTOR MEMORY T Cells (CD4+CD45RA-CCR7-CD62L-)	**19.9 (4.03-74.3)**
CD8+ T Cells	**20 (1.96-67.2)**
CD8+ NAÏVE T Cells (CD8+CD45RA+CCR7+CD62L+)	**30.2 (0.19-92.3)**
CD8+ EFFECTORS T Cells (CD8+CD45RA+CCR7-CD62L-)	**39.4 (3.2-96.2)**
CD8+ CENTRAL MEMORY T Cells (CD8+CD45RA-CCR7+CD62L+)	**11.6 (0.11-32)**
CD8+ EFFECTOR MEMORY T Cells (CD8+CD45RA-CCR7-CD62L-)	**56.9 (3.55-93.2)**
B Cells (CD3-CD19+)	**40.4 (0.11-77.9)**
NK REGULATORY Cells (CD3-CD16-CD56+)	**1.84 (0.15-47.7)**
NK CYTOTOXIC Cells (CD3-CD16+CD56+)	**7.98 (0.47-35.7)**
NKT Cells (CD3+CD56+)	**8.34 (0.49-24.4)**
All Values are given in Median with Range	**All Values are given in Median**

Discussion: -

We have profiled T cell populations during COVID-19 in a longitudinal study cohort, where we followed

them from inclusion up to 6-8 months post-recovery, using spectral flow cytometry. We found wide-ranging alterations to the T cell compartment including a rise in effectors and effector memory T cells that lasted for a period of 6 months after discharge from the hospital.

Lymphopenia was observed in many of the COVID-19 patients in our cohort at inclusion (i.e., at hospitalization [11], and is a common clinical observation [12-15] that may be attributed to the cells relocating or dying at this stage of the disease. Indeed, a highly inflammatory form of cell death, i.e., pyroptosis, induced in infected and uninfected cells, appears to be a major contributing factor for the onset of strong inflammatory responses seen globally in many individuals with COVID-19 [8, 16-19]. Furthermore, we and others have shown that the T cell compartment is affected to a higher degree than other immune cells such as B or NK cells [20-23] , which implies that T cell subsets play a paramount role in COVID-19 pathogenesis. Of note, reductions in circulating NKT cells as illustrated by us and others [24], have also been correlated to severe COVID-19 disease and poor outcome [25-26].

Memory T cells, both general and antigen-specific, in the context of SARS-CoV-2 infection have been widely studied, notwithstanding often for shorter time-periods [27-30] with fewer long-term studies, i.e. 8-9 months [31]. Evidence from the SARS-CoV outbreak in 2005 suggests that anti-SARS-CoV antibodies fell below detection limits within two years [32], and SARS-CoV-specific memory T cells were detectable 11 years after the SARS outbreak [33]. Memory T cells are an important and diverse subset of antigen experienced T cells that are sustained long term, and when needed are converted into effector cells during reinfection/ exposure [34, 35]. Depending on their cellular programming and phenotype they are classified into different central and effector memory subtypes. The effector subsets contain the CD45RA+CCR7- TEMRA, which are essentially TEM that re-express CD45RA after antigen stimulation [36]. Not much is known about the functionality of this population, but CD4+ TEMRA are implicated in protective immunity [36].

Furthermore, elevated levels of virus specific CD8+ effectors are maintained after dengue vaccination [37]. We found an elevation of both (CD4+ and CD8+T) effectors T cells and effectors memory T cells that lasted throughout the study, i.e., 6-7 months which contrasted with the CD4+ effectors that remained unaltered by COVID-19 in previous study [38]. Previous studies have shown the CD8+ TEMRA population to be increased at hospitalization [39,40] and sustained for 6 weeks [39]. Currently, the exact role of CD8+ effectors in COVID-19 remains largely ambiguous, but Cohen et al. [31] found an increase in SARS-CoV-2-specific CD8+ effectors over time. In our case, we have explored the whole expanded CD8+ effectors and CD8+ effector memory T cells population and cannot confirm if there was a larger fraction of antigen-exposed, i.e., SARS-CoV-2-specific T cells, among the population.

We found that all COVID-19 patients developed effectors T cells and effector memory T cells, which increased over time. Our findings are in accordance with other studies that have shown that SARS-CoV-2 infection results in increased expansion of antigen-specific CD4+ and CD8+ T cell subsets [41,42]. It is still unclear if the lower antigen-specific responses seen at one month compared to 6-7 months are due to an overall immunosuppression [43] or a natural development of the immune response over time [44].

At present, all immunocompetent individuals develop SARS-CoV-2-specific antibodies, which is also evident in our study. Some studies provide clear evidence that these antibodies are detected only for a few months after infection [45,46], whereas others support the detection for a minimum of 6 months [47,48]. In our cohort, we found that the increase in B cells lasted for 6-8 months post infection, even though there was a drastic decline at some time point after 6 weeks. Our findings are like those by Björkander et al. [42], who have reported that the antibody responses lasted up to 8 months among young adults. Even if the antibody levels are waning, the affinity maturation will continue and the SARS-CoV-2-specific humoral immunity will have the ability to provide protection against severe disease, and these antibodies could have increased potency to neutralize the virus [49].

With the continued burden of the current COVID-19 pandemic on the population, there is still a need for more insight into the SARS-CoV-2-specific immune response elicited during infection. Despite having multiple approved and licensed vaccines, the emergence of variants with multiple mutations [50], and the

long list of long-term symptoms following a natural SARS-CoV-2 infection [51-53], are still cause for great concern. Further, given the likely impact of inter-human variations in clinical parameters, more data is needed regarding the durability and sustenance of SARS-CoV-2-specific antibodies and T cells generated during

COVID-19 and their contribution to the quality of immune responses and what is the lasting effect on the immune cell compartment in individuals who that have recovered from COVID-19.

Despite the immensely challenging conditions during the pandemic, we do believe that the cohort presented in this investigation is well characterized and of high quality and value.

Altogether, this study highlights the alterations in the immune response which occur during hospital treated SARS-CoV-2 infection and convalescence. These novel longitudinal data illustrate the substantial changes to the T cell landscape lasting for more than 6 months. Our findings, in combination with others, are valuable in providing insight into SARS-CoV-2 cellular and humoral immunity, and open new avenues to be explored for improved understanding of the long-term alterations described herein in COVID-19 immunopathogenesis.

Conflicts of Interest

The authors declare no conflicts of interest.

CTRI ID-CTRI/2021/03/032028

Author Contribution

PS contributed to execution of the study, performed the flow cytometry experiments, data collection, analysis of data, interpretation of results and writing the manuscript. BT contributed to the study methodology and data collection. PA, SK, SS, MB, SD, HC, PJ, SJ and Dk contributed to the enrolment of study participants in the study. MG contributed to statistical data analysis of manuscript. AK, JB, SC, MK and ST contributed to conceptualize the study, study coordination and data collection. ST contributed to conceptualization of study, writing of the project proposal, obtaining regulatory approvals, and funding for carrying out the study, study design, execution of the study, data curation, review, and finalization of manuscript. All authors have read and approved the final manuscript.

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Data Availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Ethics Review Board of Dr D. Y. Patil Vidyapeeth, (Deemed to be University) Pune. The patients/participants provided their written informed consent to participate in this study.

Abbreviations-

CD	-	Cluster of Differentiation
COVID-19	-	Coronavirus disease in 2019
CPAP	-	Continuous Positive Airway Pressure
CTRI	-	Clinical Trials Registry- India
HFNO	-	High Flow Nasal Oxygen
ICU	-	Intensive Care Unit
NK	-	Natural Killer cells
NKT	-	Natural Killer T Cells
PBMCs	-	Peripheral Blood Mononuclear Cells
SARS	-	Severe acute respiratory syndrome
SARS-CoV-2	-	Severe Acute Respiratory Syndrome Coronavirus 2
SPSS	-	Statistical Package for Social Sciences
TEM	-	T Effector Memory
TEMRA	-	Terminally differentiated Effector Memory cells Re-expressing CD45RA
WHO	-	World health Organization

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