Journal of Research in Applied and Basic Medical Sciences 2025; 11(1): 1-10

Original Article

RABMS Journal of Research in Applied and Basic Medical Sciences

Assessment of immune response in ICU admitted SARS-CoV-2 patients from Kashmir, North India

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Abstract

***Background & Aims*:** The present study aimed to investigate the lymphocyte subpopulation counts (Th, Tc, B-cell, and NK cell) in SARS-CoV-2 patients (admitted to the ICU) to gain greater insight into the dysregulated immune response found in COVID-19.

***Materials & Methods*:** A total of 30 SARS-CoV-2 patients recruited in the intensive care unit (ICU) of Chest Disease (CD) and DRDO hospitals in Srinagar were investigated for lymphocyte subpopulation counts (T cell, B cell, and NK cell) by FACS analysis of blood samples (using the Beckman Coulter (Navios) and FACS Diva software).

***Results*:** Correlation analysis of lymphocyte subpopulation counts revealed, in comparison to normal healthy controls, an overall decreased mean T cell subset count, i.e., T-helper (CD3+, CD4+) (1422.5 cell/ul vs 662.4 cell/ul); T-cytotoxic (CD3+CD8+) (973.7 cell/ul Vs 629.2 cell/ul) and B cell count (CD45+CD19+) (442.1 cell/ul Vs. 144.4 cell/ul) with CD4+/CD8+ ratio (1.4 Vs. 1) in SARS-CoV-2 patients. On comparison, Stage 3 Vs Stage 2 patients, the mean T cytotoxic lymphocyte count i.e. CD3+CD8+, was lower (518.9 cells/ul Vs. 849.9 cell/ul) and the T-helper cell count, i.e. CD3+CD4+, was higher (764.9 cell/ul Vs. 457.3 cell/ul) with CD4+/CD8+ = 1.4 Vs. 0.5. Furthermore, the antibody immune response reflected by B-cell count was lower (i.e., CD19+131.9 cell/ul) in Stage 3 patients compared to Stage 2 patients (i.e., CD19+=162.9 cell/ul). ROC analysis with disease outcome revealed raised CD3+CD4+ count (T-helper cell response: AUC = 0.70) and decreased CD19+ count (humoral immune response: AUC = 0.80) and increased NLR (AUC = 0.65) as predictors of poor disease outcome.

***Conclusion*:** In conclusion, the study identified increased NLR, T-cell activation (increased T-helper cells), T-cytotoxic exhaustion (decreased T cytotoxic cells), and decreased humoral immune response (decreased CD19+ B cells) as predictive markers of severity and poor disease outcome in ICU-admitted patients with SARS-CoV-2.

***Keywords*:** COVID-19, Cytokines, Kashmir, SARS-CoV-2

Received 01 May 2024; accepted for publication 16 October 2024

Introduction

As of April 2024, the COVID-19 pandemic has caused over 6.7 million fatalities globally and affected the lives of millions more people. There is a wide variety of potential symptoms and an unpredictable clinical course for the illness caused by SARS-CoV-2 infection. Thus, it is now more crucial than ever to use early markers of severity to aid in therapeutic care. By promptly identifying instances that have the potential to deteriorate clinically, hospitals may be able to better prioritize care and distribute vital resources like ventilators and intensive care units (1).

In COVID-19 patients, routine investigations including complete blood count (CBC) and other biochemical markers have been widely used in conjunction with clinical information to monitor disease progression (2). A poor prognosis may be indicated by lymphopenia, which is regarded as a determinant in determining the severity of the disease in hospitalized patients (3,4). The neutrophil-to-lymphocyte ratio (NLR) is a crucial comparative metric since these individuals frequently have relative neutrophilia in addition to a significant decrease in lymphocyte count (5, 6).

A more comprehensive analysis of the CBC, including the evaluation of lymphocyte subpopulations (CD4+ and CD8+ T cells, B cells, NK cells, etc.), could provide more detailed information about the underlying immune response and serve as indicators of disease severity and convalescence when considered in conjunction with other clinical parameters (7, 8). An increasing number of publications indicate that the assessment of lymphocyte subset counts provides prognostic information for COVID-19 disease severity (9).

Recent immune profiling of SARS-CoV-2-infected patients has identified potential novel biomarkers that could serve as new treatment targets. However, it has been observed that individuals from geographically diverse regions with different ethnicities and genetic backgrounds respond differently to SARS-CoV-2 infection. While some populations (from the United States and Brazil) were found to be more susceptible to and more likely to succumb to the disease, others (such as Tuvalu and Nauru) were more resistant (10, 11). Some individuals from the same geographic region but of different ethnicities have shown varying responses to the severity of COVID-19. For example, populations in Kishtwar, Jammu and Kashmir, and Nagaland in India were more resistant to SARS-CoV-2. This could be due to differences in genetics, lifestyle, or environmental factors, etc (www.investindia.gov.in). Furthermore, there is evidence suggesting that SARS-CoV-2 may modulate host innate immune responses to evade immune detection and weaken host defences. To elucidate these population-specific differences, it is imperative to assess the immune profile of SARS-CoV-2 patients across diverse populations. Therefore, this study investigated the immune profile (using flow cytometry) of ICU-admitted SARS-CoV-2 patients to gain greater insight into the dysregulated immune response underlying the disease. The goal is to potentially provide novel biomarkers of prognostic, predictive, and/or therapeutic significance, at least specific to the Kashmiri population.

Materials & Methods

The study was conducted in the Department of Clinical Hematology, SKIMS (Sher-e-Kashmir Institute of Medical Sciences) between March 2022 and July 2022. A total of 30 ICU-admitted patients (with RT-PCR-confirmed SARS-CoV-2-positive status) and 10 control subjects (RT-PCR-confirmed SARS-CoV-2-negative) were included in this cohort study. The severity /staging of the patients was determined according to the standard WHO/NIH criteria for COVID-19 (2020) (https://pubmed.ncbi.nlm.nih./gov/32150360/ and https://iris.who.int). Stage 2 (moderately ill) individuals had evidence of lower respiratory disease and SpO2 ≥ 94%. Stage 3 patients included both severely ill individuals (with SpO2 < 94%, PaO2/FiO2 < 300 mm Hg, a respiratory rate > 30 breaths/min, or

lung infiltrates > 50%) and critically ill individuals (who had respiratory failure, septic shock, and/or multiple organ dysfunction). The patients had a mean age of 68.3 ± 11.7 years, were of urban origin, and included an equal number of males and females. Pneumonia was the most common clinical presentation. All patients were on oxygen support in the ICU and were receiving antiviral, corticosteroid, and anticoagulant therapy. The study was initiated only after obtaining approval from the Ethical Committee of Government Medical College, Srinagar (IEC/GMC-Sgr/27, dated 19th December 2021). Written informed consent and a standardized questionnaire were obtained from both patients and healthy controls, and the documentation adhered to hospital protocol.

Patients were followed up on days 14 and 28 post-admission, with the primary outcomes being either death or discharge. Fresh blood samples from critically ill patients were collected for initial CBC analysis, followed immediately by flow cytometry (FACS) analysis to estimate immune cell counts (T-helper cells, T cytotoxic cells, B cells, and NK cells).

Multiparameter flow cytometry (MFC) was performed using a Beckman Coulter Navios flow cytometer, and data were analyzed with FACS Diva software. Whole blood samples were lysed using BD FACS Lysing Solution (1:10 dilution). The BD Multitest™ (BD Biosciences, San Jose, USA) was used to identify T-cell subsets. This reagent includes cell surface markers CD4 (FL6-APC), CD8 (FL8-APC-AF750), CD3 (FL4-PC5.5), and CD19 (FL5-PC7). Fifty thousand events per tube were acquired.

The manufacturer's guidelines for instrument performance, compensation, and daily median fluorescence intensity (MFI) controls were followed. Software called Infinicyt 2.0 was used to analyze the data. After debris was removed as part of the analytical procedure, cells were first recognized by their low forward scatter (FSC) and side scatter (SSC) characteristics. T-helper and cytotoxic T-cells were discriminated using CD3+, CD4+, and CD8+ antibodies, while B-cells were identified by CD19+ staining. NK cells were identified as CD3-CD16+ cells.

Statistical analysis: Data were analyzed using STATA software version 17 (standard edition). Descriptive statistics were performed, and data were presented as frequency (N) and percentage (%). Continuous data were presented as mean and standard deviation. The Mann-Whitney U test and one-way ANOVA were used to compare proportions between groups, as deemed appropriate by the statistical expert. Receiver Operating Characteristic (ROC) analysis was conducted to determine the prognostic value of T-helper, T cytotoxic, B-cell, and NK cell counts. A *P-value* of less than 0.05 was considered statistically significant.

Results

Patient Characteristics:

Between March 2022 and July 2022, 30 critically ill patients were recruited from the Intensive Care Unit (ICU) of the Chest Disease (CD) and DRDO hospitals in Srinagar, all with RT-PCR-confirmed SARS-CoV-2 infection. All patients exhibited imaging findings and clinical characteristics consistent with COVID-19. The median age of the SARS-CoV-2 patients was 68.3 ± 11.7 years. Most of the patients were of urban origin, with nearly equal numbers of males and females. Pneumonia was the most common clinical presentation. Based on clinical characteristics and the use of mechanical ventilation (MV), as suggested by the WHO, the confirmed SARS-CoV-2 group was divided into moderate (n = 10; 33%) and severe (n = 20; 67%) cases. The mortality rate among the patients in this study was 55%. Most patients who recovered completely and were discharged from the hospital had moderate disease severity (33%).

Correlation analysis of lymphocyte subpopulation counts (T, B, NK) with disease severity/stages in ICU-admitted SARS-CoV-2 patients (n = 30):

Correlation analysis of lymphocyte subpopulation counts (i.e., CD19+, CD3+, CD4+, CD8+, and CD16+) compared to healthy controls revealed an overall decrease in the mean counts of T-cell subsets, though

this difference was not statistically significant. Specifically, the T-helper (CD3+CD4+) count was lower in patients (1422.5 cells/µl vs. 662.4 cells/µl). The T-cytotoxic (CD3+CD8+) count was also reduced (973.7 cells/µl vs. 629.2 cells/µl). The B-cell (CD45+CD19+) showed a similar decline (442.1 cells/µl vs 144.4cells/µl). The CD4+/CD8+ ratio was reduced (1.4 vs. 1.0) (Figure1, Table 1).

Table 1. Correlation of lymphocyte subpopulation counts in ICU-admitted SARS-CoV-2 patients compared to controls.

Alc	cd16	cd19	cd3	cd4	cd8
30	30	30	30	30	30
225.6	10.67	24.82	101.29	66.24	31.63
399	41.33	68.7	257.6	99.5	165.9
1177.6	149.1	134	666.52	229.19	621.1
1930.6	783.9	205.4	1637.2	1043.4	854.4
3762	944.7	323	2836.17	2255.3	1749.7
1531.33	354.92	144.40	1024.72	662.41	629.29
1227.985	400.881	95.246	945.364	745.594	540.955
10	10	10	10	10	10
2551	488.1	147.7	1255	997	773.21
2551	488.1	147.7	1255	997	773.21
2551.8	514.19	476.1	1303.8	1609.4	801
2551.85	1047.5	702.7	1509.1	1661.2	1347.1
2551.85	1047.5	702.7	1509.1	1661.2	1347.1
2551.55	683.26	442.17	1355.97	1422.53	973.77
0.477	315.708	279.052	134.843	369.432	323.612
0.2091	0.2091	0.0636	0.7273	0.1455	0.2818

When comparing severe and moderate patients, the mean T-cytotoxic lymphocyte count (CD3+CD8+) was lower in severe patients (518.9 cells/µl) than in moderate patients (849.9 cells/µl). In contrast, the T-helper cell count (CD3+CD4+) was higher in severe patients (764.9 cells/µl) compared to moderate patients (457.3 cells/µl), with a CD4+/CD8+ ratio of 1.4 in severe patients versus 0.5 in moderate patients. Additionally, the humoral immune response, as indicated by the B-cell count (CD19+), was lower in severe patients (131.9 cells/µl) than in moderate patients (162.9 cells/µl) (Table 2).

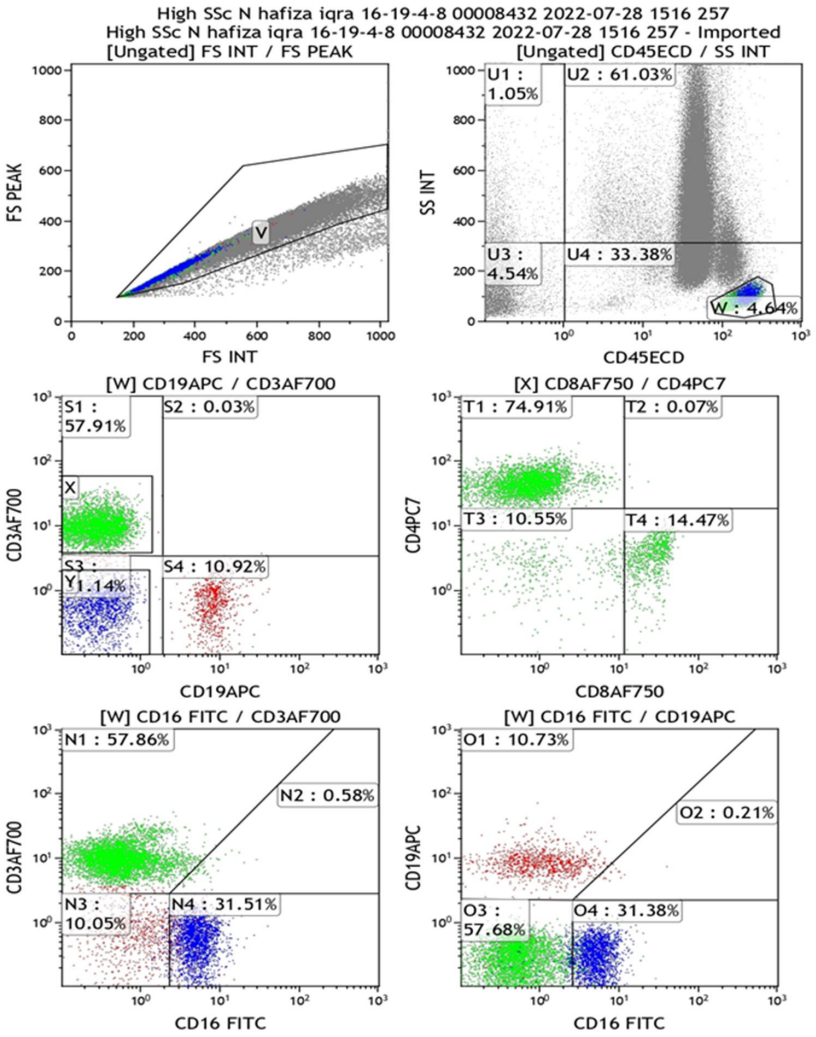

Fig. 1. Gated percentages of total lymphocyte and lymphocytes subsets, including: CD3+ vs. CD19+, CD3+CD4+Th vs. CD3+CD8+ Tc; CD3+ vs. NK cell; and CD19+ B cells vs. CD16+ NK cell counts

Table 2. Correlation of lymphocyte subpopulation counts with disease severity/stages in ICU-admitted SARS-CoV-2 patients

Group	ALC	CD16	CD19	CD3	CD4	CD8	LYM	NEU	NLR
Stage 2									
N	10	10	10	10	10	10	10	10	10
Minimum	1118	68.7	68.7	265.1	99.5	674.2	12.8	68.5	4
1st quartile	1147.8	171	137	465.8	164.3	764.3	15	69.7	4
Median	1177.6	273.3	205.4	666.5	229.1	854.4	17.2	71	4
3rd quartile	1554.1	528.6	219.5	1151.8	636.2	937.8	18.4	76.3	5

Group	ALC	CD16	CD19	CD3	CD4	CD8	LYM	NEU	NLR
Max	1930.6	783.9	233.7	1637.2	1043.4	1021.3	19.7	81.7	6
Mean	1408.7	375.3	169.2	856.3	457.3	849.9	16.5	73.7	4.6
SD	452.9	368.3	88.2	705.4	511.6	173.5	3.4	7	1.1
Stage 3									
N	20	20	20	20	20	20	20	20	20
Minimum	225.6	10.6	24.8	101.2	66.24	31.6	1.6	44	1
1st quartile	366.3	149.1	70.1	240.3	93.8	123.8	3.7	56.7	2.5
Median	1101	95.2	123	870.7	546.7	300.7	12.8	79	8.5
3 rdquartile	2708.8	711.6	139.7	1676	1060.5	574.5	23.2	91.5	29.5
Max	3762	944.7	323	2836.7	2255.3	1749.7	45	97	61
Mean	1592.6	344.7	131.9	1108.9	764.9	518.9	16.6	74	19.3
SD	1522.2	449.9	104.1	1097.8	864.2	642.3	16.6	22.2	24
Control									
N	10	10	10	10	10	10	10	10	10
Minimum	2551	488.1	147.7	1255	997	773.21	35	55	1
1st Quartile	2551	488.1	147.7	1255	997	773.21	36	57.5	1.5
Median	2551.8	514.19	476.1	1303.8	1609.4	801	37	60	2
3 Quartile	2551.85	1047.5	702.7	1509.1	1661.2	1347.1	38.5	62.5	2
Maximum	2551.85	1047.5	702.7	1509.1	1661.2	1347.1	40	65	2
Mean	2551.55	683.26	442.17	1355.97	1422.53	973.77	37.30	60.00	1.60
SD	0.477	315.708	279.052	134.843	369.432	323.612	2.500	5.000	0.500
P-value (one way anova)	0.31	0.5	0.05	0.79	0.2	0.42	0.09	0.5	0.33

ROC analysis of lymphocyte subpopulation counts with disease outcome in ICU admitted SARS-CoV-2 patients:

To determine the predictive value of lymphocyte subpopulations, ROC analysis of lymphocyte subpopulation counts (i.e., CD19+, CD3+, CD4+, CD8+, and CD16+) with disease outcome (death/discharge) revealed an elevated CD3+CD4+ count (T-helper cell response: AUC = 0.70, with 80% sensitivity and 75% specificity), a decreased CD19+ count (B-cell/humoral immune response: AUC = 0.80, with 60% sensitivity and 50% specificity), and an increased NLR (AUC = 0.65, with 60% sensitivity and 75% specificity). These were identified as predictors of poor disease outcomes (Figure 2, Table 3).

Table 3. ROC analysis of lymphocyte subpopulation counts with disease outcome in ICU admitted SARS-CoV-2 patients

Area under the curve					
Test result variable(s)	Area	Std. error[a]	Asymptotic Sig.[b]	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
ALC	.500	.213	1.000	.082	.918

Area under the curve					
Test result variable(s)	Area	Std. error[a]	Asymptotic Sig.[b]	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
CD16	.300	.192	.327	.000	.676
CD19	.800	.183	.142	.441	1.000
CD3	.450	.215	.806	.029	.871
CD4	.700	.191	.327	.326	1.000
CD8	.200	.159	.142	.000	.511
NLR	.650	.196	.462	.265	1.000

The test result variable(s): NLR has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

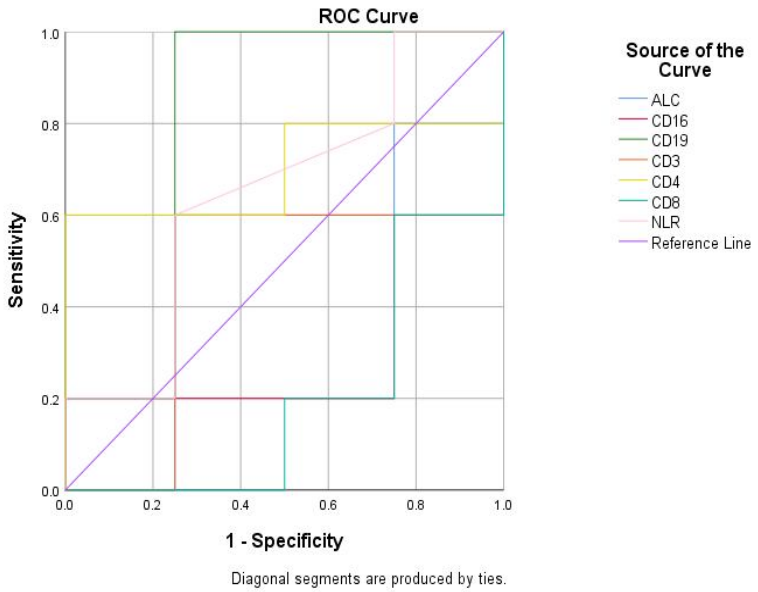

Fig. 2. ROC analysis of lymphocyte subpopulation count and NLR with disease outcome in SARS-CoV-2 patients

Discussion

With the emergence of new SARS-CoV-2 variants showing enhanced immune evasion posing difficulties in predicting the clinical course, it has become increasingly necessary to determine and implement early markers of dysregulated immune response, such as altered lymphocyte subset population counts as indicators of disease severity, especially in critically ill patients. Rapidly identifying cases that might worsen clinically could help hospitals allocate critical resources, including ventilators and intensive care unit beds, and prioritize treatment. In this study, we found increased NLR, T-cell activation (increased T-helper cells), T-cytotoxic exhaustion (decreased T-cytotoxic cells), and a decreased humoral immune response (decreased CD19+ B cells) as markers of disease

severity and poor outcomes in ICU- admitted SARS-CoV-2 patients.

The increase in NLR with disease severity observed in these patients proved to be a valuable early indicator for identifying patients who require ICU admission with respiratory monitoring and supportive care. The neutrophilia seen in these patients could be due to the activation and migration of neutrophils from the venous system to the immunological sites of infection. Additionally, inflammatory factors associated to viruses, including as TNF-α, G-CSF, IL-6, IL-8, and INF-γ factors, which are synthesized by lymphocytes and endothelial cells, can activate neutrophils. (12). The increase in neutrophils also led to an elevated the expression of circulating vascular endothelial growth factor (VEGF). which markedly contributed to tissue and organ damage observed in these ICU-admitted patients (13).

Although T-cells are necessary for controlling the infection and lowering the viral load, excessive T-cell activation (Th1, Th17) leads to ARDS by producing cytokines and chemokines that exaggerate the T-cell-mediated immune response. In the present study low CD3+CD8+ and increased CD3+CD4+ counts have been found as markers of poor disease outcome in these patients, which is consistent with worldwide reports in which the severity of COVID-19 has been associated with CD4+T-cell abnormalities and CD8+T-cell exhaustion. This could possibly be due to enhanced and persistent expression of several immune checkpoint proteins, notably PD-1, PDL-1, CTLA-4, CD39, and Tim-3, common features of T-cell exhaustion or dysregulation found in severe viral infections (14, 15). Exhausted T-cells, by releasing a variety of cytokines, including IFN-gamma, TNF-alpha, and IL-2, inhibit T-cell clonal growth and B-cell priming, which contributes to the weakened adaptive immune response observed in these patients (16, 17).

Several human mAbs to counteract these immune checkpoints and restore T-cell functionality with improved disease outcomes have been reported (18). Although there are studies that have shown improved clinical outcomes with elevated levels of CD8+ cells, high levels of T-cell activation lead to host cell damage by secreting molecules like granzyme B and NKG2D, which stimulate NK cells (19). In addition, consistent with worldwide reports, an age-dependent reduction in T-cells in patients > 60 years and immunological depression due to pre-existing chronic conditions like diabetes, COPD, cancer, etc., were found to be risk factors for COVID 19 severity (20). Similar to various other studies, this study found a decline in CD19+ cell count, which correlated with increasing disease severity. Among the lymphocyte subpopulations, the highest predictive value was obtained for the CD19+ count (AUC = 0.80), suggesting a low activation status of B-cells that preceded the decline in specific antibody responses particularly in severe cases of SARS-CoV-2.

The study, apart from providing predictive markers such as increased NLR, T-cell activation (increased T-helper cells), T-cytotoxic exhaustion (decreased T-cytotoxic cells), and decreased humoral immune response (decreased CD19+ B cells), suggests the adoption of therapeutic strategies such as immunotherapies for T-cell exhaustion (anti-PDL1, PDL-1, CTLA-4, CD39, and Tim-3) and vaccination drives/plasma therapy (for improving B cell response) for managing severe cases of SARS-CoV-2. Recognizing specific markers of T-cell exhaustion (e.g., PD-1, CTLA-4, Tim-3) can help identify patients who are more likely to benefit from immune checkpoint inhibitors. Insights into the stages of T-cell exhaustion could guide the timing of therapies. For instance, administering checkpoint inhibitors early in the disease process may prevent the development of exhaustion, leading to a more effective response. However, the current study was limited by the single-centricity and small sample size. As a consequence, comparable multicentric research from other regions of our nation and even from other nations with greater sample sizes should support the study's findings.

Conclusion

In conclusion, the study identified increased NLR, T-cell activation (increased T-helper cells), T-cytotoxic exhaustion (decreased T-cytotoxic cells), and

decreased humoral immune response (decreased CD19+ B cells) as markers of disease severity and poor outcomes in ICU-admitted patients with SARS-CoV-2. The lymphocyte sub population count, especially T and B-cell counts guides clinicians in selecting appropriate therapeutic strategies such as immunotherapies (in the form of immune checkpoint inhibitors like anti-PDL1, PDL-1, CTLA-4, CD39, and Tim-3) for overcoming T-cell exhaustion and vaccination drives/plasma therapy for improving B-cell response while managing severe cases of SARS-CoV-2. These findings also contribute to a deeper understanding of COVID-19 immunopathology.

Acknowledgments

Multi-disciplinary Research Unit (MRU), GMC Srinagar.

Department of Biochemistry, Bio safety Level II Lab (SMHS associated hospital of GMC Srinagar).

Ethical statement

The work was ethically approved by the ethical committee of Govt. Medical College and associated SMHS Hospital. Approval No: Ref No. IEC-GMC-Sgr/27.

Data availability

None declared.

Conflict of interest

The authors declare no conflict of interest.

Funding/support

The work was conducted with the research funding received by the Multi-disciplinary Research Unit (MRU) GMC Srinagar from ICMR (Indian Council of Medical Research).

Authorship contributions

Authors IF and RE contributed to the design and implementation of the study. Authors IF and IH carried out the data collection. Authors IF and IH performed the formal analysis. Authors RE and MT investigated the work. Authors IF, SF, RE, SH, SS, and SM wrote the original draft and edited the manuscript. The final manuscript was read and approved by all authors.

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