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LETTER TO THE EDITOR

Peripheral lymphocyte subset alterations in COVID-19 patients

Dear Editors,

The novel coronavirus SARS-CoV-2 which causes the acute respiratory illness known as COVID-19 has been characterized by the World Health Organization (WHO) as a global pandemic since March 13, 2020. Despite a lower case-fatality rate, COVID-19 has resulted in significantly more deaths than SARS in 2003, largely in part due to its higher infectivity. As of May 21, 2020, more than 4,800,000 cases of SARS-CoV-2 infection have been confirmed worldwide, with more than 321,000 fatalities. Singapore confirmed its first case of COVID-19 on January 23, 2020. Since then, we have had a total of 29,342 cases and 22 fatalities. Majority of the hospitalized cases were treated at our center, the National Centre of Infectious Diseases (NCID).

Recent studies have shown a decrease in peripheral lymphocytes in COVID-19 patients.[1-3] We sought to assess the lymphocyte subset alterations in COVID-19 patients, in particular the differences between critically ill patients admitted to the intensive care unit (ICU) and general ward patients. As the number of SARS-CoV-2 infections increases throughout the world, healthcare systems are being overwhelmed. Hence, it is increasingly important to appropriately triage COVID-19 patients and identify those who would need ICU care early.

We retrospectively analyzed 129 samples of ethylenediamine tetraacetic acid (EDTA) anticoagulated peripheral blood samples from a total of 75 patients warded at the NCID, between February 24, 2020, and March 28, 2020. Lymphocyte subsets namely CD3+/CD4+/CD8+T-cell, CD19+B-cell, and CD16+CD56+ NK cell counts (cells/μL) were measured by multicolor flow cytometry, using the following reagents: human monoclonal anti-CD3-fluorescein isothiocyanate (FITC), anti-CD8-phycoerythrin (PE), anti-CD4-allophycocyanin (APC), anti-CD19-APC, anti-CD16+CD56 PE, and anti-CD45-peridinin-chlorophyll-protein (PerCP) antibodies. Cells were analyzed on a Becton Dickinson FACSCanto™ II Flow analyzer. A complete blood count (CBC) and differential count were also performed on a Beckman Coulter DxH800 analyzer. All non-ICU patients had CBC, differential counts, and lymphocyte subsets performed on the day of admission. The aforementioned tests were also performed on ICU patients, with the first sample taken within the first week of admission for most (17 out of 20) of the ICU patients. These laboratory markers were repeated in ICU patients during their hospitalization to monitor their progress. In addition, we had previously performed the above tests on 19 healthy subjects for comparison. Information such as age, sex, gender, race, and clinical history was obtained from the electronic medical records.

Most of our patients were Chinese (69.3%) and male (66.7%), with a median age of 50. Leucopenia was seen in 16.0% of patients, lymphopenia in 48.0%, neutrophilia in 14.7%, and thrombocytopenia in 4.0%. 20 out of 75 COVID-19 patients (26.7%) had severe illness requiring admission to the ICU. Criteria for admission to the ICU were based on the clinical assessment of patients by the ICU team. All patients were transferred to the ICU for ventilatory support or for severe metabolic acidosis. All 20 ICU patients had chest X-ray changes in keeping with pneumonia, 19 out of 20 required supplemental oxygen, and 16 out of 20 patients were intubated and mechanically ventilated.

ICU patients were older by 20 years (median age of 64 compared to 44), with significantly higher leucocytes (8.0×10^9 vs 4.6×10^9), higher neutrophils (6.65×10^9 vs 2.93×10^9), higher neutrophil-lymphocyte ratio (11.20 vs 2.03), and lower hemoglobin (10.9 g/dL vs 14.5 g/dL) compared to non-ICU patients.

Looking at lymphocyte subsets, compared to healthy subjects, COVID-19 patients in general had significantly lower total lymphocytes (1072 vs 1787 cells/μL, $P < .001$), CD4+cells (408 vs 662 cells/μl, $P < .001$), CD8+cells (278 vs 562 cells/μL, $P < .001$), B cells (119 vs 308 cells/μL, $P < .001$), and NK cells (182 vs 258 cells/μL, $P = .017$). CD4+/8+ratio was not significantly different.

Compared to non-ICU patients, ICU patients had significantly lower total lymphocytes (514 vs 1352 cells/μl, $P < .001$), CD4+T cells (190 vs 464 cells/ul, $P < .001$), CD8+T cells (86 vs 355 cells/μL, $P < .001$), NK cells (68 vs 205 cells/μL, $P < .001$), and B cells (97 vs 142 cells/μL, $P = .010$). CD4/8 ratio was also significantly higher in the ICU patients (1.80 vs 1.28, $P = .037$).

Categorical variables were compared using either Pearson's chi-square or Fischer's exact tests. Nonparametric comparative tests (Kruskal-Wallis or Mann-Whitney) were used to compare continuous variables across groups. All statistical analyses were conducted either using SPSS or GraphPad Prism with significance defined as $P < .05$.

In our study, total lymphocytes, T cells, B cells, and NK cells were significantly lower in COVID-19 patients as compared to healthy subjects, suggesting a correlation between lymphocyte subset alteration and the pathogenesis of SARs-CoV-2. These findings were similarly seen in patients with SARS.[4]

Among COVID-19 patients, ICU patients had significantly lower total lymphocytes, CD4+ T cells, CD8+T cells, and B cells. In the study by Wong RS et al on SARS,[5] they similarly found that low CD4 and CD8 counts at presentation were associated with adverse outcomes. The significantly lower CD8+ T cells and higher CD4/8 ratio seen in our group of ICU patients indicate that CD8+ T cells are

TABLE 1 Comparisons of demographic and clinical characteristics between icu and non-icu patients

	Non-ICU Patients (n = 55)		ICU Patients (n = 20)			Overall (n = 75)	
	Median (IQR)	No. (%)	Median (IQR)	No. (%)	*P*-value	Median (IQR)	No. (%)
Demographic Characteristics at Admission							
Age (years)	44 (27-56)		64 (57-71)		<0.001	50 (30-62)	
Ethnicity							
Chinese		37 (67.3)		15 (75.0)	0.768		52 (69.3)
Malays		4 (7.3)		2 (10.0)			6 (8.0)
Indians		1 (1.8)		0 (0.0)			1 (1.3)
Others		13 (23.6)		3 (15.0)			16 (21.3)
Gender							
Males		34 (61.8)		16 (80.0)	0.140		50 (66.7)
Females		21 (38.2)		4 (20.0)			25 (33.3)
Hematologic parameters							
Hb (g/dL)	14.5 (13.2-15.3)		10.9 (8.7-12.4)		<0.001	13.9 (12.1-15.0)	
WBC ($x10^9$/L)	4.6 (4.0-5.7)		8.0 (6.0-9.8)		<0.001	5.2 (4.3-6.5)	
WBC ($x10^9$/L) <4 ≥4		10 (18.2) 45 (81.8)		2 (10.0) 18 (90.0)	0.497		12 (16.0) 63 (84.0)
ALC ($x10^9$/L)	1.31 (0.89-1.66)		0.49 (0.38-0.70)		<0.001	1.03 (0.71-1.55)	
ALC ($x10^9$/L) <1.0 ≥1		18 (32.7) 37 (67.3)		18 (90.0) 2 (10.0)			36 (48.0) 39 (52.0)
ANC ($x10^9$/L)	2.93 (2.21-3.36)		6.65 (4.13-7.99)		<0.001	3.17 (2.34-4.14)	
ANC ($x10^9$/L) <1.0 ≥1.0		4 (7.3) 51 (92.7)		0 (0.0) 20 (100.0)	0.568		4 (5.3) 71 (94.7)
ANC ($x10^9$/L) ≤6.6 >6.6		55 (100.0) 0 (0.0)		9 (45.0) 11 (55.0)	>0.001		64 (85.3) 11 (14.7)

(Continues)

TABLE 1 (Continued)

	Non-ICU Patients (n = 55)		ICU Patients (n = 20)			Overall (n = 75)	
	Median (IQR)	No. (%)	Median (IQR)	No. (%)	*P*-value	Median (IQR)	No. (%)
Platelets (x10^9/L)	233 (182-272)		205 (127-269)		0.104	222 (168-272)	
Platelets (x10^9/L) <100 ≥100		0 (0.0) 55 (100.0)		3 (15.0) 17 (85.0)	0.017		3 (4.0) 13 (17.3)
Neutrophil to Lymphocyte Ratio	2.03 (1.66-3.34)		11.20 (6.92-22.85)		<0.001	2.57 (1.73-5.72)	
Lymphocyte subsets by flow cytometry							
Total Lymphocytes (cells/ul)	1352 (939-1704)		514 (407-707)		<0.001	1072 (665-1511)	
CD4/CD8 ratio	1.28 (0.95-1.85)		1.80 (1.45-2.38)		0.037	1.38 (1.01-2.01)	
CD3 (cells/μL)	840 (618-1166)		307 (186-498)		<0.001	740 (435-1055)	
CD19 (cells/μL)	142 (76-241)		97 (55-132)		0.010	119 (72-199)	
CD4 (cells/μL)	464 (358-643)		190 (123-290)		<0.001	408 (239-572)	
CD8 (cells/μL)	355 (241-561)		86 (61-172)		<0.001	278 (129-446)	
NK (cells/μL)	205 (156-313)		68 (32-113)		<0.001	182 (112-273)	

TABLE 2 Comparison of lymphocyte subsets between healthy subjects and COVID patients in general

	Healthy Controls (N = 19)	COVID-19 Patients (N = 75)	
	Median (IQR)	Median (IQR)	*P*-value
Total Lymphocytes (cells/ul)	1787 (1616-2521)	1072 (665-1512)	<.001
CD4/CD8 ratio	1.16 (0.89-1.60)	1.38 (1.01-2.01)	.200
CD3 (cells/ μL)	1221 (1032-1032)	740 (435-1055)	<.001
CD19 (cells/μL)	308 (227-393)	119 (72-199)	<.001
CD4 (cells/μL)	662 (524-764)	408 (239-572)	<.001
CD8 (cells/μL)	562 (365-733)	278 (129-446)	<.001
NK (cells/μL)	258 (168-347)	182 (112-273)	.017
Neutrophil to Lymphocyte Ratio	1.71 (1.45-2.27)	2.57 (1.73-5.72)	.002

reduced to a greater extent than CD4+ T cells in ICU patients, and this could be a predictor of disease severity.

CD8+ T cells are critical in viral clearance after acute respiratory infections such as respiratory syncytial virus (RSV) or influenza A virus (IAV) infections. In mice, adoptive transfer of CD8+ T-cell clones reduced viral titers in the lung following these infections.[6] In mice depleted of CD8+ T cells, RSV replication in the nose appears to be prolonged.[7] In humans, RSV infection was more severe in immunocompromised children with T-cell defects, and they experienced prolonged viral shedding compared to immunologically normal children.[8] Following natural H7N9 infection, the reduction in IFN-γ^+ CD8 T cells correlated with clinical recovery.[9]

Additionally, we found that NK cells were significantly reduced in ICU patients compared to non-ICU patients and could also be a useful predictor of clinical severity.

NK cells play an important role in our immune defense against viral infections. NK cells respond to early viral infections by killing virally infected cells through the production of cytokines particularly interferon gamma (IFN-γ), the secretion of cytolytic granules such as perforin and granzymes, and the use of death receptor-mediated cell apoptosis.[10] Mice and humans deficient in IFN-γ or interferon gamma receptors (IFN-γR) are more sensitive to viruses such as human simplex virus (HSV).[11]

Our study echoes the findings by Wang F et al,[12] where they described the lymphocyte subsets in COVID-19 and found significantly lower total lymphocytes, CD4+ T cells, CD8+ T cells, and B cells in "severe cases" compared to "mild cases." However, in contrast to our study, the CD4/8 ratio and NK cells were not found to be significantly different between the two groups. Wan S et al[13] showed only statistically significant differences in CD4+ and CD8+ T cells between the "severe group" and "mild group," without any significant differences with regard to B cells, NK cells, and CD4/8 ratio. Lymphocyte subsets were monitored over time for all 20 ICU patients in our study. In three patients who were transferred out of the ICU to the general wards, clinical recovery and transfer to the general ward was associated with increasing CD4, CD8, and NK cell counts. On the other hand, in the three patients who died from COVID-19, CD4, CD8, and NK cell counts remained low throughout ICU admission.. This further shows that clinical severity of COVID-19 is related to lymphocyte subsets alterations.

The US Food and Drug Administration (FDA) has currently not approved any drugs or other therapeutics to treat COVID-19. A variety of treatment options are under investigation, such as Remdesivir, interleukin-6 inhibitors, and convalescent plasma. A new drug recently cleared by the FDA for trials is CYNK-001, a cryopreserved allogeneic NK cell therapy that aims to identify and destroy SARS-CoV-2 infected cells.

There are several limitations in our study. Firstly, as it is a retrospective study, blood tests were performed sporadically throughout the admission and not at specified time points. Secondly, we acknowledge that treatments administered such as steroids can influence lymphocyte subsets, and this could potentially confound our results. In conclusion, we found statistically significant differences in lymphocyte subsets between ICU and non-ICU patients. We also found that peripheral lymphocyte subset alterations are associated with the clinical severity of SARS-CoV-2 infection. Possible predictors of severity include reduction in CD4+ T cells, CD8+ T cells, and NK cells, as well as an increased CD4/8 ratio.

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CONFLICT OF INTEREST

The authors have no competing interests. This study was approved by the National Healthcare Group Domain Specific Review Board (DSRB).

AUTHORS' CONTRIBUTIONS

SSWC, BEF, and TGB contributed to the conception and design of the project. TGB performed all flow cytometry tests and provided data for Tables 1 and 2. CYL conducted all statistical analyses, and SSWC and DC interpreted the data. SSWC drafted the manuscript with critical review by DC, VCLC, and BEF. OKH supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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Stephrene S. W. Chan[1,2]
Dheepa Christopher[1,2,3]
Guat B. Tan[4]
Vanessa C. L. Chong[1]
Bingwen E. Fan[1,2,3]
Clement Y. Lin[1]
Kiat H. Ong[1,2,3]

[1]*Department of Haematology, Tan Tock Seng Hospital, Singapore*
[2]*Lee Kong Chian School of Medicine, Singapore*
[3]*Department of Laboratory Medicine, Khoo Teck Puat Hospital, Singapore*
[4]*Department of Laboratory Medicine, Tan Tock Seng Hospital, Singapore*

Correspondence
Stephrene Seok Wei Chan, Tan Tock Seng Hospital, 11 Jln Tan Tock Seng, Singapore 308433.
Email: Seok_Wei_Stephrene_Chan@ttsh.com.sg

ORCID

Stephrene S. W. Chan https://orcid.org/0000-0003-4828-6303
Bingwen E. Fan https://orcid.org/0000-0003-4367-5182

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