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RESEARCH PAPER

Peripheral Blood Lymphocyte Immunophenotypes among COVID-19 Patients with Different Severity

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Abstract

Background: The COVID-19 pandemic poses an urgent need to understand the role of host immunity in viral infections. Peripheral blood lymphocytes play an important role in the immune system. The disrupted immune response can lead to alterations of lymphocyte counts among COVID-19 patients.

Objective: The study aimed to quantify and compare the peripheral blood lymphocytes, including CD3+ T cells, CD4+ T cells, CD8+ T cells, B cells and Natural killer cells in COVID-19 patients according to clinical severity.

Methods: The cross-sectional study was conducted from March 2020 to January 2021 in the Department of Microbiology and Immunology, Bangladesh Medical University. A total of 103 RT-PCR confirmed COVID-19 patients and 20 healthy subjects were enrolled. COVID-19 patients were clinically categorized into asymptomatic (n=18), mild-moderate (n=38) and severe-critical (n=47) groups. Peripheral venous blood samples were collected from study groups and immunophenotyping was done by flow cytometry.

Results: Compared to the healthy group, COVID-19 patients had significantly decreased percentages of total peripheral lymphocytes and T cells (P < .0001 and P= .002, respectively). The percentage of B cells increased (P=.001) while NK cells remained statistically unchanged in COVID-19 patients. In accordance with clinical severity, the severe-critical group had a decrease in total lymphocytes and T cells than the asymptomatic and mild-to-moderate groups (P<.001). Furthermore, CD4+ and CD8+ T cell counts were decreased in severe-critical COVID-19 patients compared to asymptomatic and mild-moderate patients (P<.001).

Conclusion: Patients with severe COVID-19 showed a significant decrease in total lymphocytes, including T cells, CD4+ T cells and CD8+ T cells. Therefore, lymphocyte immunophenotyping could be an important laboratory approach in monitoring COVID-19 patients.

Key Words: COVID-19, Peripheral blood lymphocyte, Flow cytometry, Lymphocyte immunophenotyping.

Introduction

The pandemic Coronavirus disease 2019 (COVID-19) is caused by newly emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is genetically related to previous SARS-CoV and MERS-CoV.[1] The common clinical manifestation of COVID-19 patients are fever, cough, sore throat, shortness of breath, fatigue, ARDS, even multi organ failure. Considerable rate of ICU admission and mortality are also reported.[2, 3] Clinically COVID-19 is categorized into mild, moderate, severe and critical cases based on severity.[4] However, there is increasing evidence that COVID-19 can remain as asymptomatic infection, which might be the source of transmission of the virus in community. [5, 6] Variation in clinical manifestations and disease progression is largely determined by the host immune response to SARS-CoV-2. [7]

It is considered that innate and adaptive immune system activation and regulation are crucial for effective viral clearance.[8] The components of the immune system including T lymphocytes, B lymphocytes and natural killer cells play major role in antiviral immunity. Among immune cells, T lymphocyte count consists of CD4+T lymphocyte and CD8+ T lymphocyte count. The CD4+T lymphocytes help B cells to produce virus specific antibody, whereas CD8+ T lymphocytes and natural killer cells decrease viral load by killing virus infected cells or producing virus specific antibody[7,9].

In COVID-19, intense inflammatory response and excessive pro-inflammatory mediator production can contribute exacerbation of immune response resulting

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immune dysregulation.[7, 8] Consequently, lymphopenia and cytokine storm are observed in COVID-19 patients, and mostly in severe cases.[9] Impairment of immune system was also evident in previously emerged SARS-CoV with significant alteration of peripheral blood lymphocytes. [10] Notably, lymphocyte count is considered as predictor of severity and clinical outcomes in SARS-CoV patients. [11]

Marked reduction of total lymphocyte count in COVID-19 patients is reported from different countries in world including China, Italy, Spain, Iran and United state. [12-19] Moreover, further evaluation on lymphocyte subsets has also revealed that CD4+ T cell, CD8+ T cell, B cell and NK cell count are altered between severe and non severe COVID-19 cases. [20-22] As the immune patterns are related with disease progression [7], it is important to understand the immune responses generated by COVID-19 and to identify the most severe and risky patients by early detection of the lymphocyte immune phenotype.

In Bangladesh, supportive investigations like complete blood count, C-reactive protein, procalcitonin, serum ferritin, D-dimer are carried out in diagnosed cases of COVID-19, which provide limited information on the host immune response. Considering the facts, the present study was designed to compare the peripheral blood lymphocyte subsets between COVID-19 patients and healthy subjects and to evaluate the peripheral blood lymphocyte subsets alteration among COVID-19 patients, which might help to monitor the severity of disease and to stratify patients in clinical setting for early therapeutic intervention.

Materials and Methods

This cross-sectional study was conducted between March 2020 to January 2021 in the Department of Microbiology and Immunology, Bangladesh Medical University. A total of 103 RT-PCR confirmed COVID-19 patients were enrolled in this study from COVID Unit and Intensive Care Unit of BSMMU. Twenty healthy subjects were included, who were RT-PCR negative for COVID-19. Healthy subjects and patients with immunosuppressive drug, chemotherapy and immunodeficiency disorders were excluded.[15, 17]

Informed written consent was obtained from all subjects. Relevant data were collected in a data collection sheet and confidentiality was maintained.

Categorization of patients and blood sample collection:

COVID-19 patients were categorized into asymptomatic, mild– moderate and severe–critical groups according to National Guidelines on Clinical Management of Coronavirus Disease 2019, Bangladesh. [23] On admission, patient's venous blood was collected for complete blood count and 3ml blood was taken in a BD Vacutainer EDTA tube (Ref. 367856) for cytometry. The complete blood count report was collected on the subsequent day for total WBC count. Blood Collection, transport and processing were performed with proper biosafety.[24]

Flow cytometric analysis: Cytometry was done according to manufacturer's instructions with following reagents.

1. For each sample, 50ìl anticoagulated blood was pipetted into two 12x75 FACS tubes separately, labeled as panel A and panel B.
2. A 5ìl of AntiCD45-ECD, AntiCD3-FITC, AntiCD8-PC7, AntiCD19- PE flurochrome conjugated monoclonal antibodies was added to panel A to detect CD45+ total lymphocytes, CD45+CD3+ T cells, CD45+CD3+CD8+ cytotoxic T cell and CD3" CD45+CD19+ B cells.
3. A combination of AntiCD45-ECD, AntiCD3-FITC, AntiCD4- PE, AntiCD56- PE/CY5 antibodies was added to panel B to detect CD45+ CD3+CD4+helper T cells and CD3" CD45+CD56+ NK cells.
4. Incubation was done for 10-15 minutes in dark at room temperature and 200ìl of Lysing solution (OptiLyse C, Beckman Coulter, USA) was added to tubes to lyse RBC.
5. After incubation, 3ml of sheath fluid (IsoFlow, Beckman Coulter, USA) was added. Centrifugation was done for 5 minutes at 300g and the supernatant was discarded. Cells were re-suspended in 50ìl of sheath fluid.
6. The tubes were run on a precalibrated flow cytometer (Beckman Coulter Cytomics FC 500) and data were analyzed by CXP software. For each sample, 10,000 events were counted.

Interpretation of results:

The lymphocytes gating strategy included use of dot plots of CD45 expressions versus side scattering. The

total lymphocyte count was calculated by multiplying the percentages of total lymphocyte with the total WBC count. Subsequently, Absolute counts of lymphocyte subsets were calculated by multiplying the percentages of lymphocyte subsets with total lymphocyte count.

Quality control:

The cytometer was calibrated daily by running controls before test and minimum PMT voltage setting for each detector was adjusted. Any spectral overlap between multiple dyes was corrected by AutoSetup compensation software.

Data analysis:

Descriptive analysis of all relevant variables was performed with SPSS software version-27(Strata Corporation, College Station, Texas) and GraphPad Prism 9.0 software. Continuous variables were expressed as median (interquartile range) or mean ± standard deviation depending on whether data conformed to normal distribution and categorical variables as frequency and percentage. Mann-Whitney test or One-way ANOVA was used for statistical analysis according to the data distribution. P-value <0.05 was considered statistically significant.

Results

In this study, 103 laboratory-confirmed COVID-19 patients were enrolled and divided into asymptomatic (n=18), mild-moderate (n=38) and severe-critical (n=47) group according to the disease severity. Most of the COVID-19 patients of severe-critical group (19, 40.43%) and mild-moderate group (17, 44.47%) were in age group of 61-70 years and 51-60 years, respectively. (Figure 1)

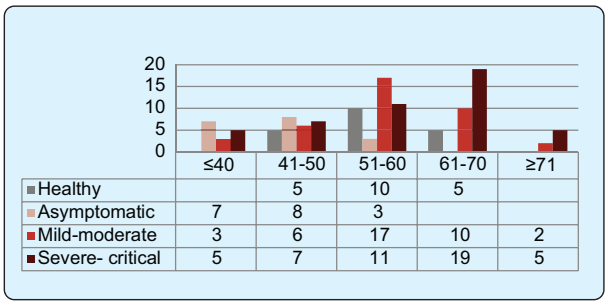

Figure 1: Age distribution of the study groups (N=123).

Peripheral blood lymphocytes were measured by flow cytometry and compared between COVID-19 patients and healthy group. As shown in the Table I, percentages of total lymphocytes and T cells were significantly decreased in COVID-19 patients (P < 0.0001 and P=0.002, respectively), as well as B cell percentage was increased (P=0.001) in COVID-19 patients compared to healthy group. There was no statistical difference in NK cell percentage (P=0.734).

Table I: Comparison of peripheral blood lymphocytes among COVID-19 patients and healthy group

	Median (IQR)		P value
	Healthy (n=20)	COVID-19 patients (n=103)	
Total lymphocytes,%	29 (27-34)	13 (7-24)	<0.0001
T cells,%	70 (68-75)	65 (52-70)	0.002
B cells,%	18 (10-20)	23(15-35)	0.001
NK cells,%	12 (10-14)	11 (9-15)	0.734

P<0.05 indicated statistical significance. P value was calculated by Mann- Whitney test to compare between two groups.

Peripheral lymphocytes percentages were further analyzed among COVID-19 patients according to clinical severity (Table II). The mean percentage of total peripheral lymphocytes were significantly decreased in the severe-critical group (7.13) compared to those in the asymptomatic group (31.55, P<0.001) and mild-moderate group (20.78, P<0.001), respectively. The total peripheral lymphocyte percentage was also significantly lowered (P < 0.01) in the mild-moderate group compared to asymptomatic group. The T cell percentage was decreased significantly in the severe-critical group (53.77) compared to asymptomatic (72.44, P<0.001) and mild-moderate group (66.95, P<0.001), whereas no significant difference was found between asymptomatic and mild-moderate group.

Regarding B cells, mean percentage was increased significantly in severe-critical group compared to asymptomatic group (P=0.01). The percentages of NK cells were decreased both in the severe-critical and mild-moderate group compared to asymptomatic group (P=0.006 and P=0.03, respectively). No significant change was found between severe-critical and mild-moderate group.

Further, T cell subsets (CD4+ helper cells and CD8+ cytotoxic T cells) were compared among the COVID-19 groups. Figure 2 has shown that a significant decrease (P<0.001) was observed in the absolute

Table II: Comparison of peripheral blood lymphocytes among different severities of COVID-19 patients

	Mean ± SD			P value
	Asymptomatic (n=18)	Mild -moderate (n=38)	Severe-critical (n=47)	
Total lymphocytes,%	31.55 ± 6.84	20.78 ± 8.13	7.13 ± 4.18	<0.001[ab] <0.01[c]
T cells,%	72.44 ± 6.2	66.95 ± 8.68	53.77 ± 13.33	<0.001[ab]
B cells,%	25.44 ± 6.60	28.21 ± 9.64	31.53 ± 13.33	0.01[a]
NK cells,%	14.33 ± 3.72	10.21 ± 3.77	10.51 ± 6.43	0.006[a] 0.03[c]

a - Asymptomatic vs severe-critical, b - Mild-moderate vs severe-critical, c- Asymptomatic vs mild-moderate, P<0.05 indicated statistical significance. P value was calculated by One-way ANOVA to compare among different groups.

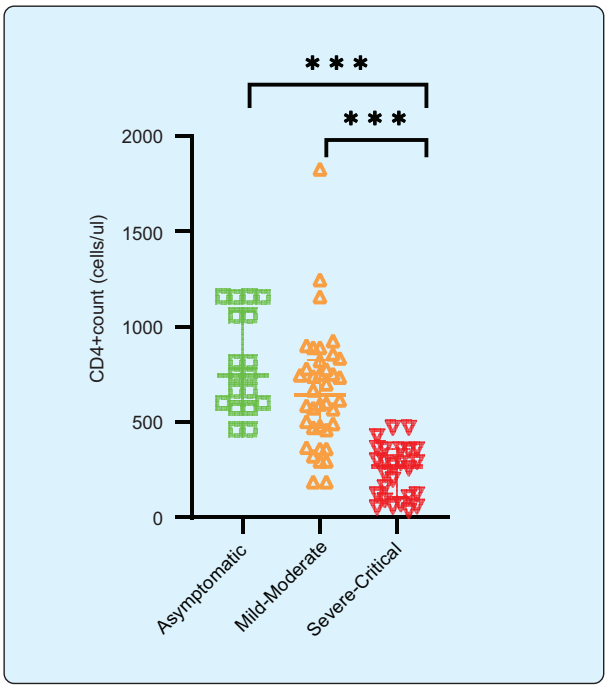

Figure 2: The comparisons of CD4+ T cells in peripheral blood of asymptomatic group, mild-moderate and severe-critical COVID-19 groups are showed with scatter plot graph. The longer horizontal line in graph indicates the median value for each group. *** indicates P < 0.001.

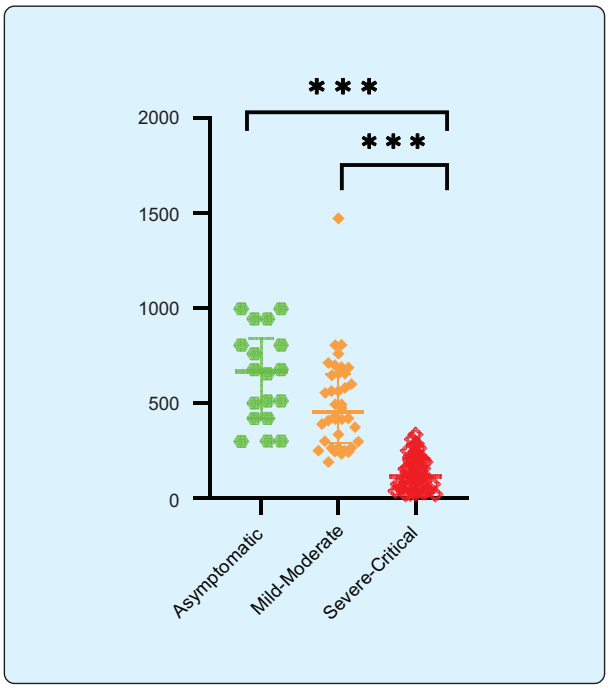

Figure 3: Absolute counts of CD8+ T cells in asymptomatic group, mild-moderate and severe-critical COVID-19 groups are showed with scatter plot graph. The longer horizontal line in graph indicates the median value for each group. *** indicates P<0.001.

count of CD4+ T cells in the severe-critical group (204.03/uL) compared to those in the asymptomatic (804.42/uL) and mild-moderate group (667.07/uL).

In Figure 3, the severe-critical group showed significant decrease (P<0.001) in the absolute count of CD8+ T cell (134.08/uL) compared to the asymptomatic (623.66/uL) and mild-moderate group (498.08/uL). There was no statistical difference in T cell subsets between asymptomatic group and mild-moderate group.

Discussion

The complex interaction between viral infection and host immune response might determine the clinical severity in COVID-19 patients [8]. The purpose of this study was to evaluate and compare peripheral blood lymphocyte subsets in COVID-19 patients.

This study included total 103 laboratory confirmed COVID-19 patients, consisting of 18 asymptomatic, 38 mild-moderate, and 47 severe-critical cases where severe-critically ill patients were mostly in age group

of 61–70 years. In previous studies, the severity of COVID-19 correlated significantly with older age [18].

In the present study, the total lymphocyte and T lymphocyte percentages were significantly decreased in COVID-19 patients compared to healthy group. Several studies have reported that the number of lymphocytes is significantly decreased in most COVID-19 patients [13, 17, 25].

In the comparison among COVID-19 patients with different severity, percentage of total lymphocyte and T lymphocyte were found significantly lower in severe-critical group. Similar findings were reported from several studies where lymphocyte and T lymphocyte count was decreased significantly in severe-critical patients than in mild-moderate and asymptomatic cases [12,14,22,25]. The decrease in lymphocyte count could be due to increased lymphocyte infiltration in the lungs of COVID-19 patients or direct SARS-CoV-2 invasion [26]. Furthermore, in response to SARS-CoV-2 infection, cytokine production increases. As a result, anti-inflammatory cytokines such as IL-4 and IL-10 can inhibit T cell activation [27]

In this study, further evaluation on lymphocyte subsets revealed that the CD4+ T cell and CD8+ T cell count were significantly decreased in severe-critical group compared with the asymptomatic and mild-moderate group, which is consistent with previous reports. [12,13,17,22,25] Moreover, studies recommended CD8+ T cell count as an independent predictor of disease severity.[21, 26] In contrast, other studies found that the percentage of CD4+ T cell was unchanged between mild-moderate and severe-critical group.[12,28] CD4+ T cell depletion in peripheral blood is thought to be caused by their migration to the lungs [21]. Lymphopenia could also result from spleen atrophy and lymph node necrosis, as SARS-CoV-2 virus has the potential to destroy spleen and lymph node [12, 13, 21].

Regarding B cells, the mean percentage was significantly higher in the COVID-19 patients as well as in severe-critical patients. Previously, similar findings were reported in other COVID-19 studies. The possible cause of the relative increase of B cells percentage could be due to the significant decrease of T cells in these patients [17, 29].

Although the peripheral NK cell percentage was statistically unchanged between COVID-19 and healthy group, NK cells were decreased significantly both in the severe-critical group and mild- moderate group compared to asymptomatic group. Xu et al and Sun et al reported a significant reduction in NK cell population in the severe groups in comparison to moderate group, whereas Liu et al showed no difference in between the severe and moderate groups [21, 22, 13]. Increased MCP-1 and IP-10 levels in COVID-19 patients promote NK cell migration from blood to the lung, resulting lower NK cell population [30].

Altogether, this study found that severe-critical COVID-19 patients had significantly lower peripheral blood lymphocytes and lymphocyte subsets, such as CD3+ T lymphocytes, CD4+ helper T cells, and CD8+ cytotoxic T cells. This suggests that the T cell subsets were markedly suppressed according to the severity of COVID-19.

Conclusion

Depletion of peripheral blood lymphocytes, including lymphocyte subsets, is observed in severe- critical COVID-19 patients. Lymphocyte immunophenotyping might be carried out to detect the alterations of lymphocyte subsets for monitoring COVID-19 patients in a clinical setting. The role of lymphocyte subsets as a predictor of disease severity needs to be evaluated by further large-scale study.

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Ethical Clearance: The study was approved by Institutional Review Board of Bangladesh Medical University (BMU)

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