Dynamics of Peripheral Blood T-lymphocytes Have Predictive Values for the Clinical Outcome of COVID-19 Patients in Intensive Care Unit

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ABSTRACT

BACKGROUND: Coronavirus disease 2019 (COVID-19) patients with severe disease had a high mortality rate. It's imperative to identify risk factors associated with disease progression and prognosis. Immune responses played an important role in the host's defense against the virus. We studied the dynamics of peripheral blood lymphocytes (PBLs) in relation to the clinical outcome in COVID-19 patients in intensive care unit (ICU).

DESIGN: This cohort included 342 COVID-19 patients who were admitted to ICU between February 1 and May 30, 2020, with 178 having follow-up PBL analysis. The patients were divided into a group that survived and an expired group. PBL analysis was performed by flow cytometry.

RESULTS: At time of initial flow analysis, there were no statistically significant differences in lymphocyte, T-cell and subsets, B-cell or natural killer (NK) cell counts between the 2 groups. However, during the ICU course, the surviving group demonstrated a full recovery of CD3+ T-cells, CD4+ T-cells, and CD8+ T-cells, with no significant change in B-cells, and a slight upward trend in NK-cells. In contrast, the expired group showed no recovery in T-cells (and subsets) and no significant changes in B-cells and NK-cells. We identified the earliest time points and cut-off values for T-cell subsets that predict clinical outcomes.

CONCLUSION: The results of this study suggest that evaluation of PBL in COVID-19 patients could be valuable in the study of the immune responses to the disease and the prognostication of outcome.

KEYWORDS: Coronavirus, COVID-19, severe acute respiratory syndrome coronavirus 2, flow cytometry, peripheral blood lymphocytes

Introduction

The clinical manifestations of COVID-19 vary from asymptomatic to life-threatening acute respiratory distress syndrome and multi-organ failure. COVID-19 patients who were critically ill, especially those requiring intensive unit care, had a high mortality rate.[1-3] It is imperative to identify risk factors associated with disease progression and prognosis in order to reduce the mortality in COVID-19. Immune responses play an important role in the host's defense against virus and pathological damage to the host.[4,5] Study of the immune responses in COVID-19 patients will help our understanding of the immunological mechanisms of SARS-CoV-2 infection so as to develop effective preventive and therapeutic strategies. We conducted a retrospective study on peripheral blood lymphocytes (PBLs) and their dynamics in the COVID-19 patients admitted to intensive care unit (ICU) in relation to the clinical outcomes.

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Materials and Methods

Patient consent statement

This study received approval for all experimental protocols by the Institutional Review Board (IRB) of the Human Research Protection Program licensing committee at Northwell Health. All methods were carried out in accordance with all guidelines and regulations. Patient consent was not required by the institutional review board (IRB) committee due to the retrospective nature of the study (Northwell Health IRB number: 20-0200).

Patient and data collection

This is a retrospective study of 342 patients who were diagnosed with COVID-19 and treated in intense care unit in the period between February 1 and May 31, 2020 at the Northwell Health System, New Hyde Park, New York, USA. The patients were divided into 2 groups based on the clinical outcomes: the alive group (171 patients) and the expired group (171patients). Infection with SARS-COV-2 was confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR)

performed on nasopharyngeal swab specimens. All the patients were admitted to the ICUs in our 2 tertiary hospitals –North Shore University Hospital and Long Island Jewish Medical Center. The demographic information and relevant laboratory findings were retrieved from Northwell laboratory quality assurance databases. The retrieved laboratory data at admission and along the disease course included white blood cell (WBC), lymphocyte and subsets, neutrophil, monocyte, eosinophil, basophil, red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), serum lactate dehydrogenase (LDH), activated partial thromboplastin time (aPTT), prothrombin time (PT), fibrinogen, D-dimer.

Flow Cytometry Analysis of PBL

Flow cytometry was performed to quantitate PBL and subsets. Cells were stained with a panel of fluorescently labeled, mouse anti-human monoclonal antibodies including CD3, CD4, CD8, CD19, CD16, and CD56. The analysis was performed using FACSDiva software (Becton-Dickenson Biosciences, San Jose, CA). Initial point was defined as a time when the first flow cytometry analysis was conducted while end point was defined as a time when the last flow cytometry analysis was conducted. All the cases had at least 1 set of T-cell subset value. Of the 342 cases, 178 cases had at least 2 sets of flow cytometry data for CD3+ T-cell, CD4+ T-cell, CD8+ T-cell at different time points of the clinical course. For T-cell subsets, we compiled enough cases to perform dynamics analysis for 15 days, with all but two of the time points including at least 20 cases for the alive group and at least 10 cases for the expired group. We performed dynamic analysis for 10 days on 75 cases (41 alive cases and 34 expired cases) with CD19+ B-cell count and 75 cases (41 alive cases and 34 expired cases) with NK cell count

Statistical Analysis

Data generated from the study were analyzed with GraphPad Prism and expressed as medians and interquartile ranges (IQR) or mean ± standard error (SE), as appropriate, and compared using independent group Student's *t*-test. A *p* value of <.05 was considered statistically significant. The receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to assess the sensitivity and specificity of variables in the prediction of clinical outcome in COVID-19 patients. Cox regression uni- and multi- factor analysis were performed to identify risk factors associated with outcomes. The ROC, AUC, and Cox regression were performed on IBM SPSS Version 27, 27.0.1.0.

Results

Clinical and laboratory characteristics of the COVID-19 ICU patients

The clinical and relevant laboratory characteristics at ICU admission are summarized in Table 1. The mean age was

62.5 years for the whole cohort, 66.0 years for the expired group, and 59.0 years for the alive group. The male to female ratio was 1.1:1 for the whole cohort, 0.9:1 for the alive group, and 1.4:1 for the expired group. There was no significant correlation between age and clinical outcome ($p > .05$). The 2 groups all had prolonged PT, prolonged aPTT, elevated levels of fibrinogen, D-dimer and LDH. The expired group also had leukocytosis and neutrophilia. The mean lymphocyte count was within normal reference range but less than 1500/uL in both groups. Statistical analysis revealed that the expired group had significantly higher levels of WBC ($p < .01$), aPTT ($p < .03$), LDH ($p < .03$), D-dimer ($p < .04$), and significantly lower level of HCT ($p < .01$) than the alive group. But there were no significant differences in neutrophil, lymphocyte, monocyte, eosinophil, basophil, RBC, Hb, PT, and fibrinogen levels between the 2 groups.

PBL alterations during ICU hospitalization in the COVID-19 patients

There were 178 cases (100 alive and 78 expired) with at least 2 sets of flow cytometry data for T-cell subsets at different time points including the initial point and the endpoint. At the initial point, in both groups, the mean CD3+ T-cell and CD4+ T-cell counts were both below the reference ranges, whereas the mean CD8+ T cell, B cell, and NK cell counts were all within the reference ranges. There was no significant difference in initial CD3+ T-cell, CD4+ T-cell, CD8+ T-cell, B-cell, or NK cell between the 2 groups. At the endpoint, the alive group had significantly higher number of CD3+ T-cell ($p < .01$), CD4+ T-cell ($p < .01$), and CD8+ T-cell ($p < .01$) than the expired group (Figure 1). In the alive group, there was significant increase in CD3+ T-cell ($p < .01$), CD4+ T-cell ($p < .01$), or CD8+ T-cell ($p < .01$) at the end point compared to the initial point (Figure 1).

Dynamics of PBLs during the ICU hospitalization of COVID-19 patients

The dynamics of PBLs during the ICU hospitalization of COVID-19 patients were analyzed. In the alive group, CD3+ T-cell started increasing at day 2, reached the normal level at day 8, peaked at day 10, and then gradually decreased and plateaued at a normal level (Figure 2a). CD4+ T-cell displayed a similar pattern in the early days but did not reach the normal range until day 10. After day 10, CD4+ T-cell showed a continuous decrease before rebounding at day 13 to 15 (Figure 2b). CD8+ T-cell was above the normal range over the clinical course, showed a significant increase after day 7, and peaked at day 10 followed by a decrease (Figure 2c). In the expired group, CD3+ T-cell and CD4+ T-cell were consistently below the normal ranges over the clinical course while CD8+ T-cell count was slightly below or close to the

Table 1. Clinical and laboratory characteristics of the COVID-19 ICU patients at admission.

LAB VALUES	ALIVE	EXPIRED	p VALUE	REFERENCE
	MEAN ± SEM	MEAN ± SEM		RANGE
Gender			.453*	
Female	91	71		
Male	80	100		
Age	61.50 ± 0.97	66.40 ± 1.20	.179	
RBC count	4.35 ± 0.08	4.22 ± 0.18	0.280	$3.85\text{-}5.20 \times 10^6$/uL
Hemoglobin	11.90 ± 0.19	10.70 ± 0.63	**.002**	11.5-15.5 g/dL (F)
				13.0-17.0 g/dL (M)
Hematocrit	35.07 ± 7.46	31.07 ± 8.99	.210	34.5-45.0% (F)
				39.0-50.0% (M)
White blood cells	9.56 ± 0.71	17.68 ± 4.13	**.001**	3.80-10.50 K/uL
Neutrophils#	6.87 ± 0.45	11.39 ± 0.69	.280	1.80-7.40 K/uL
Lymphocytes#	1.43 ± 0.07	3.44 ± 2.55	**.001**	1.00-3.30 K/uL
Monocytes#	0.70 ± 0.59	0.76 ± 0.64	**.014**	0.00-0.90 K/ul
Eosinophils#	0.19 ± 0.02	0.09 ± 0.02	**.042**	0.00-0.50 K/uL
Basophils#	0.05 ± 0.01	0.03 ± 0.00	.424	0.00-0.20 K/uL
PT	15.32 ± 0.38	18.29 ± 1.18	**.001**	10.6-13.6 s
PTT	38.09 ± 1.13	59.51 ± 4.15	**.001**	27.0-36.3 s
Fibrinogen	719.69 ± 23.16	779.04 ± 38.16	.140	290-520 mg/dL
D-dimer	2016.67 ± 396.80	3709.58 ± 731.50	**.028**	≤229 ug/mL
LDH	446.00 ± 43.71	660.00 ± 4.90	**.030**	135-225 U/L

Abbreviations: #, absolute count; LDH, lactate dehydrogenase; PT, prothrombin time; PTT, partial thromboplastin time; RBC, red blood cell; SEM: standard error of the mean.
*The statistic test for gender is a Chi-square test. *T*-test was used for the rest of parameters.
The bold *p* values are those < 0.05.

normal level. There was a transient increase in CD4+ T-cell between day 11 and day 15 in the expired group (Figure 2a-c). Statistical analysis revealed that the dynamics started to show significant difference between the 2 groups at day 7 for CD3+ T cell and CD8+ T and at day 8 for CD4+ T-cell. At day 2, there were statistically significant differences in CD3+ T-cell, CD4+ T-cell, and CD8+ T-cell counts between the 2 groups (Figure 2a-c). The CD19+ B-cell count was initially elevated and then became relatively stable in the alive group but showed fluctuation with 2 peaks (at day 4 and day 7) in the expired group (Figure 2d). Overall, the CD19 dynamics showed no significant difference between the 2 groups. NK cell levels displayed a slight upward trend with a peak at day 5 in the alive group, but lower and more stable in the expired group (Figure 2e). However, the overall dynamics were not significantly different between the 2 groups.

Six individual cases with more than 20 days' follow-up, including 3 alive patients and 3 expired patients were also identified. The dynamics of CD3+ T-cell, CD4+ T-cell, and CD8+ T-cell were significantly different between the alive patients and the expired patients. All the alive patients had complete recovery of CD3+ T cell, CD4+ T cell, and CD8+ T cell at discharge, whereas the expired patients failed to recover in all 3 subsets.

AUC-ROC analysis and Cox-regression for survival analysis

Univariate analysis of the initial variables did not identify any risk factors among CD3+ T-cell, CD4+ T-cell, CD8+ T-cell, CD19+ T-cell, NK cell, and WBC. However, multivariate analysis identified initial CD4+ T-cell (hazard ratio
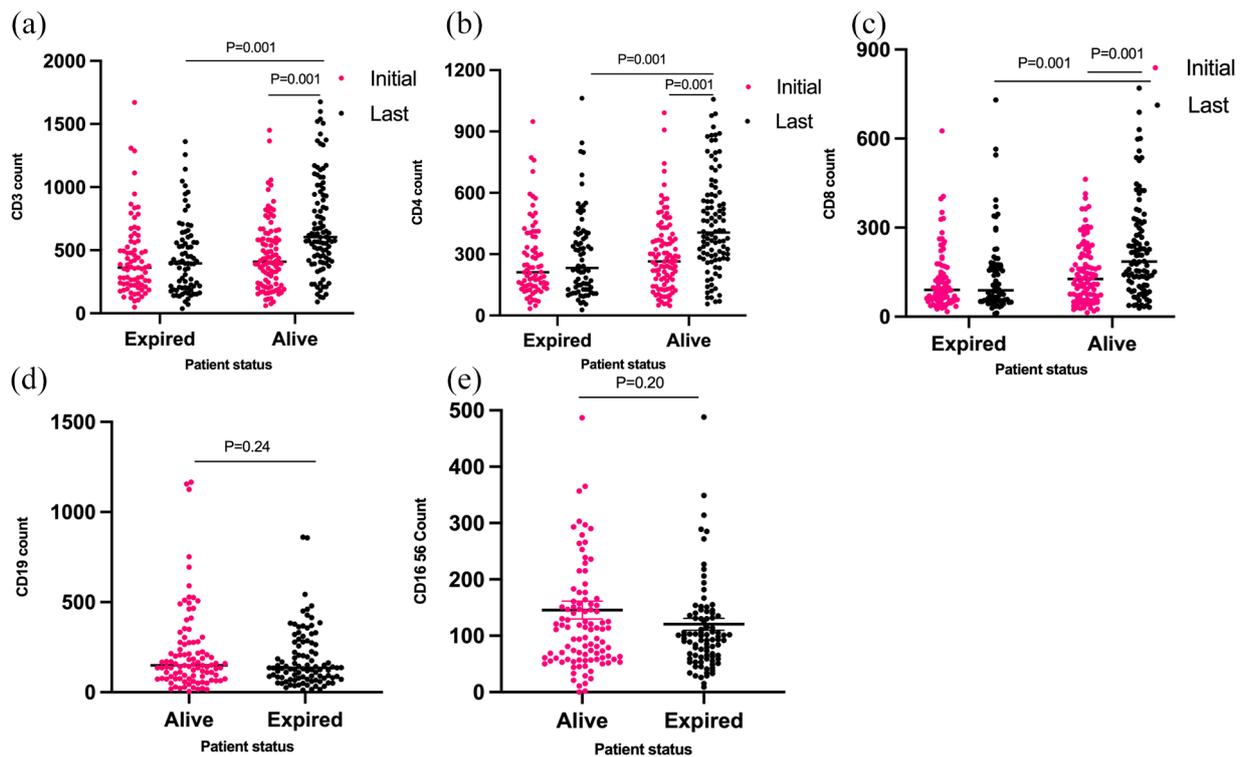
Figure 1. Flow cytometry analysis of peripheral blood lymphocytes at the initial point and the end point. Means with independent *T*-test were used for the statistic test: (a) CD3+ T-cell, *p* value = .001 between the initial and end points in the alive group, and between the alive and expired groups at the end point, (b) CD4+ T-cell, *p* value = .001 between the initial point and the end point in the alive group, and between the alive and expired groups at the end point, (c) CD8+ T-cell, *p* value = .001 between the initial and end points in the alive group, and between the alive and expired groups at the end point (d) CD19+ B-cell, *p* value = .24 between the alive and expired groups at the initial point, and (e) CD56 + CD16+ NK cell, *p* = .20, between the alive and expired groups at the initial point.
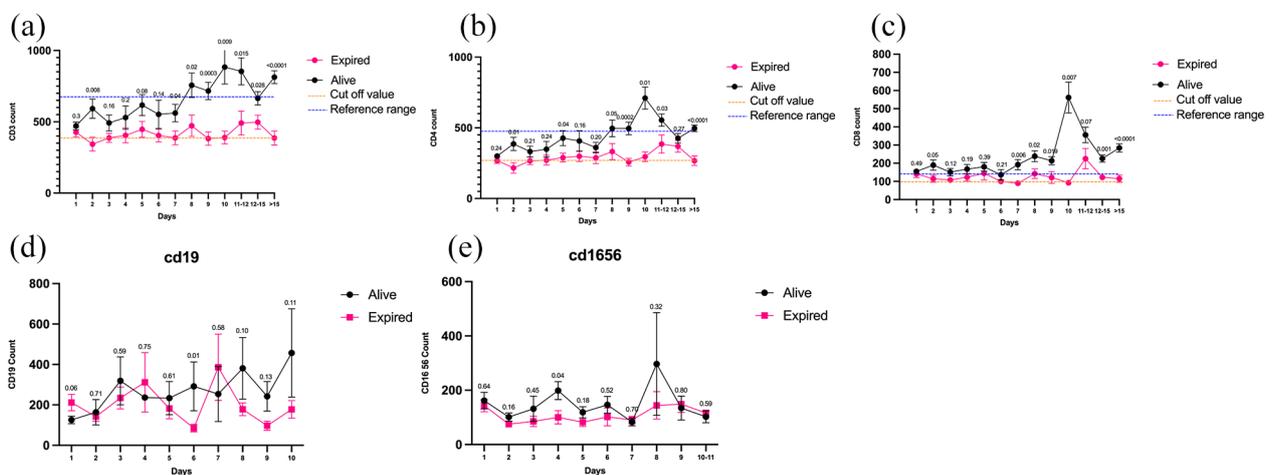

Figure 2. Dynamic analysis of peripheral blood lymphocytes (mean, standard error, and *P* value) during the ICU hospitalization in the alive and expired groups. There are a total of 178 patients (alive n = 101, expired n = 77). The daily case numbers for T-cell subsets in the alive group are as follows: day 1 (101), day 2 (26), day 3 (49), day 4 (29), day 5 (31), day 6 (14), day 7 (26), 8 (25), day 9 (22), day 10 (18), after day 10, combined values of day 11 and day 12 (27), combined values of day 13 and day 14 (25), and combined values of >15 days (57). The daily case numbers for T-cell subset in the expired group is as follows: day 1 (77), day 2 (20), day 3 (33), day 4 (30), day 5 (25), day 6 (21), day 7 (18), day 8 (20), day 9 (15), day 10 (10), after day 10, combined values of day 11 and day 12 (16), combined values of day 13 and day 14 (15), and combined values of >15 days (27): (a) CD3+ T-cell, (b) CD4+ T-cell, (c) CD8+ T-cell, (d) CD19 + B-cell, and (e) CD56 + CD16+ NK cell.

[HR] 1.079 [1.003-1.162], $p < .05$), NK cell (HR 1.025 [1.003-1.047], $p < .05$), and WBC (HR 1.319 [1.017-1.712], $p < .05$] as risk factors for overall survival (Table 2). While performing the Cox-regression analysis for day 7/8 flow variables on CD3+ T-cells, CD4+ T-cell, and CD8+ T-cell, the univariate analysis shows increase in CD3 (HR 0.998 [0.997-0.999], $p < .05$), and CD8 (HR 0.995 [0.991-0.999], $p < .05$) T-cells significantly associated with better

Table 2. Cox-regression survival predication of the initial flow.

VARIABLE	NUMBER	UNIVARIATE HR	CI	*p* VALUE	MULTIVARIATE HR	CI	*p* VALUE
CD3	178	1.000	0.999-1.001	.636	0.934	0.871-1.003	.059
CD4	178	0.999	0.998-1.001	.241	**1.079**	**1.003-1.162**	**.042**
CD8	178	1.000	0.999-1.002	.609	1.029	0.967-1.095	.374
CD19	54	1.001	0.999-1.003	.261	1.008	1.000-1.017	.054
NK cells	54	1.000	0.999-1.002	.697	**1.025**	**1.003-1.047**	**.029**
WBC	117	0.710	0.202-2.50	.594	**1.319**	**1.017-1.712**	**.037**

Abbreviations: CI, confidence interval; HR, hazard ratio.
The bold values are those with *p* < 0.05.

Table 3. Cox-regression survival predication of day 7/8.

VARIABLE	NUMBER	UNIVARIATE HR	CI	*p* VALUE	MULTIVARIATE HR	CI	*p* VALUE
CD3	89	**0.998**	**0.997-0.999**	**0.028**	0.972	0.944-1.001	.057
CD4	89	0.999	0.997-1.000	0.119	1.029	0.999-1.060	.054
CD8	89	**0.995**	**0.991-0.999**	**0.011**	1.024	0.994-1.055	.112

Abbreviations: CI, confidence interval; HR, hazard ratio.
The bold values are those with *p* < 0.05.

Table 4. Area under the curve (AUC) comparison for variables.

VARIABLES	NUMBER	AUC INITIAL FLOW	CI	*p* VALUE	DAY 7/8	AUC DAY 7/8	CI	*p* VALUE	CUT OFF VALUE	REFERENCE RANGE
CD3	178	0.593	0.472-0.713	.142	89	**0.691**	**0.580-0.803**	**.002**	393.5	672-1870 cell/uL
CD4	178	0.542	0.420-0.664	.506	89	**0.656**	**0.539-0.773**	**.014**	263.00	489-1457 cell/uL
CD8	178	**0.644**	**0.527-0.760**	**.023**	89	**0.737**	**0.632-0.842**	**<.001**	97.00	142-740 cell/uL

Abbreviation: CI, confidence interval.
The bold values are those with *p* < 0.05.

outcomes. An increase of 100 cells/uL correlates to 20%, 10%, and 50% decrease in mortality for CD3+T-cells, CD4+T-cells, and CD8+ T-cells, respectively (Table 3).

AUC-ROC analysis revealed that initial data were less sensitive and specific than day 7 data in predicting clinical outcome. Among the initial variables, only CD8+ T-cell had predictive value for prognosis while the 3 variables at day 7 (CD3+ T-cell, CD4+ T-cell, and CD8+ T-cell) showed predictive values.

For CD3+ T cell, the initial flow set showed a predictive sensitivity of 50% (no predictive significance); the day 7/8 flow set showed a predictive sensitivity of 69% (*p* = .002). The cut off value for prognosis was 393.5 cells/uL based on day 7/8 flow results where the predictive sensitivity was 80% and the specificity was 50%. For CD4+ T cell, the initial flow set showed a predictive sensitivity of 50% (no predictive significance); the day 7/8 flow showed a predictive sensitivity of 66% (*p* = .0014). The cut off value for prognosis was 263.0 cells/uL based on the day 7/8 flow results where the predictive sensitivity was 81%

and the specificity was 54%. For CD8+ T cell, the initial flow set showed a predictive sensitivity of 64% (*p* = .023); the day 7/8 flow results showed a predictive sensitivity of 74% (*p* < .001). The cut off value for prognosis was 97.0 cells/uL based on the day 7/8 flow results where the predictive sensitivity was 78% and the specificity was 54% (Table 4).

Discussion
COVID-19 patients with severe disease had a higher mortality rate than those with mild disease. The definition of severe disease has not been standardized in COVID-19. It is recently recommended that severe disease in COVID-19 should apply only to those requiring ICU care.[6] This is one of the largest cohort studies so far on COVID-19 ICU patients. In our cohort, the mortality rate was 50% and we did not find any association between age and clinical outcome. Consistent with the reported laboratory characteristics of severe COVID-19 disease, our patients had prolonged PTT, prolonged PT, and elevated levels of fibrinogen,

D-dimer and LDH.[6,7] WBC, PTT, D-dimer, and LDH were found to be associated with prognosis in our cohort. Multivariate analysis revealed that higher WBC count was a risk factor for mortality. It was reported that COVID-19 patients often developed neutrophilia during the ICU hospitalization.[8,9] We observed isolated neutrophilia in the expired group, which did not correlate with prognosis. In our cohort, the mean lymphocyte count at admission was <1500/uL. A recent meta-analysis study suggested that lymphopenia defined by <1500/uL was a practical parameter for predicting disease severity in COVID-19 patients.[10]

Adaptive immune responses against viral infection are characterized by earlier development of antigen-specific T cells and subsequent production of neutralizing antibodies.[11] Acute SARS-CoV-2 infection results in broad immune cell reduction and functional impairment. Studies have shown that SARS-CoV-2 can inhibit T-cell responses through restraining antigen presentation, inducing cytotoxic CD8+ T cell exhaustion, and reducing memory CD4+ T-cell and T regulatory cells. It can also induce CD4-biased T-cell responses which results in proinflammatory cytokine storm leading to pathological damage in the host. The immune features of SARS-CoV-2 infection are characterized by rapid generation of neutralizing antibodies and delayed production of antigen-specific T-cells.[12-22]

Small cohort studies have shown that lymphocyte, CD4+ T-cell, CD8+ T-cell, and B-cell were significantly decreased in COVID-19 ICU patients as compared to non-ICU patients.[8,23] Limited studies suggested that the trending of PBL during SARS-CoV-2 infection may have predictive values for clinical outcomes.[22,24,25] We studied the dynamics of PBLs in a large cohort of COVID-19 ICU patients and evaluated their prognostic values. The results of the present study showed that among the PBLs, only CD3+ T-cell and CD4+ T-cell were below normal ranges at the initial point. Low CD4+ T-cell count has been reported to be an independent risk factor for ICU admission.[26] Cox-regression analysis in this study indicated that low CD4+ T-cell count was a risk factor for poor survival in the COVID-19 ICU patients. Low CD8+ T-cell count was reported to be a predictor for high mortality and illness severity.[17,27,28] In our cohort, the initial CD8+ T cell counts were within normal range in both groups, however, the failure of increase in CD8+ T cell counts within day7/8 is a strong predictor for mortality. Moreover, the AUC-ROC analysis of the initial and day 7 variables showed that CD8+ T-cell had predictive values for prognosis. Although there was no significant difference in the initial value of CD3+ T-cell, CD4+ T-cell, or CD8+ T-cell between the 2 groups, their dynamic patterns showed significant difference. The difference in dynamics became statistically significant for CD3+ T cell and CD8+ T-cell after day 7 and for CD4+ T-cell after day 8. By analyzing the day7/8 data, we found that CD3+ T-cell, CD4+ T-cell, and CD8+ T-cell all had predictive values for prognosis. In contrast to the expired group, CD3+ T-cell,

CD4+ T-cell, and CD8+ T-cell were fully recovered after 1 week in the alive group. Interestingly, there were significant differences in the CD3+T-cell, CD4+ T-cell, and CD8+ T-cell count at day 2 between the 2 groups, which indicate that the day 2 data can provide the earliest information for timely intervention. Based on the AUC-ROC data, we derive the cut-off values for T-cell subsets that have predictive value for prognosis. These cut-off values can be used to identify patients who are at risk for poor outcome and need close monitoring and timely interventions. Furthermore, we observed an elevation of CD4+ T-cell between day 12 and day 15 in the expired group. Given that CD4-biased T-cell responses were associated with proinflammatory cytokine storm, our finding suggests that the transient increase of CD4+ T-cell in the late clinical course may contribute to higher mortality in these patients.

There was no significant difference in CD19+ B-cell counts at initial and end points as well as in the dynamics of CD19+ B-cell between the 2 groups. CD19+ B-cell level was initially elevated and then became relatively stable during the clinical course in the alive group but it showed fluctuation with 2 peaks observed at day 5 and day 7 in the expired groups. These results suggest that the ICU patients had the ability to develop humoral immune responses against the virus but the responses vary among the patients. In support of our observation, previous studies have shown that COVID-19 ICU patients often developed a faster and higher level of neutralizing antibody responses, but the antibody responses were heterogeneous.[29,30] The initial NK cell counts were normal in the 2 groups. In the alive group, the NK cell displayed a slight upward trending with a peak observed at day at day 5, whereas it was relatively steady at a lower level in the expired group. Multivariate analysis also identified low NK cell as a risk factor for poor survival.

Conclusions
This study retrospectively evaluated the relationship between survival and the T cell response. It is possible that there were other factors that were responsible for this association that were not considered. The presence of other bacterial or viral infections could have influenced the outcome of this association. The study was also done prior to the routine use of corticosteroids. Therefore, the use of steroids or other medications that may be used more often in critically ill patients was not considered. In addition, this study may have potential biases because of the different subsets of data in the different statistical models. Our findings on PBL dynamics, however, suggest that T lymphocytes, especially low CD8+ T-cell count may be used to predict clinical outcomes in COVID-19 patients in ICU. They also provide insights into the host immune responses against SARS-CoV-2 infection. The development of high titers of neutralizing antibodies is seen with Covid infection, but its relationship with T cell function is unclear, and should be of interest in future studies.

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Supplemental Material

Supplemental material for this article is available online.

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