

Comparative evaluation of memory T cells in COVID-19 patients and the predictive role of CD4⁺CD8⁺ double positive T lymphocytes as a new marker

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SUMMARY

BACKGROUND: The COVID-19 pandemic has affected the entire world, posing a serious threat to human health. T cells play a critical role in the cellular immune response against viral infections. We aimed to reveal the relationship between T cell subsets and disease severity.

METHODS: 40 COVID-19 patients were randomly recruited in this cross-sectional study. All cases were confirmed by quantitative RT-PCR. Patients were divided into two equivalent groups, one severe and one nonsevere. Clinical, laboratory and flow cytometric data were obtained from both clinical groups and compared.

RESULTS: Lymphocyte subsets, CD4⁺ and CD8⁺ T cells, memory CD4⁺ T cells, memory CD8⁺ T cells, naive CD4⁺ T cells, effector memory CD4⁺ T cells, central memory CD4⁺ T cells, and CD3⁺CD4⁺ CD25⁺ T cells were significantly lower in severe patients. The naive T cell/CD4⁺ + EM T cell ratio, which is an indicator of the differentiation from naive T cells to memory cells, was relatively reduced in severe disease. Peripheral CD4⁺CD8⁺ double-positive T cells were notably lower in severe presentations of the disease (median DP T cells 11.12 μ L vs 1.95 μ L; $p < 0.001$).

CONCLUSIONS: As disease severity increases in COVID-19 infection, the number of T cell subsets decreases significantly. Suppression of differentiation from naive T cells to effector memory T cells is the result of severe impairment in adaptive immune functions. Peripheral CD4⁺CD8⁺ double-positive T cells were significantly reduced in severe disease presentations and may be a useful marker to predict disease severity.

KEYWORDS: COVID-19, Lymphocyte Subsets, Adaptive Immunity.

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INTRODUCTION

Most patients with COVID-19 are asymptomatic or have only mild symptoms¹. Symptomatic cases mostly suffer from mild fever, cough, shortness of breath, muscle pain, headache, and diarrhea^{2,3}. COVID-19 can lead to severe pneumonia, acute respiratory distress syndrome (ARDS), multiple organ failure, and, eventually, death in patients with advanced age and severe comorbid diseases^{2,4}. One of the most implicated mechanisms in severe disease is immune system alterations⁵⁻⁷.

The pathophysiology of COVID-19 and the underlying mechanisms in severe cases are the most important challenges of the ongoing studies. Several studies have highlighted that coronaviruses such as SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus), MERS-CoV (Middle East Respiratory Syndrome-Coronavirus), SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) leads to a severe reduction in T lymphocyte subsets, which are not common in other viral infections^{8,9}. Studies have focused on revealing the immune system changes to explain pathogenesis. In severe cases of the disease, memory T cell and regulatory T cell changes may be responsible for the uncontrolled inflammatory response and the lack of specific immunity.

METHODS

Study Design and Participants

This study was designed as a cross-sectional study. The diagnosis was confirmed by quantitative RT-PCR. A total of 40 patients over 18 years old were recruited randomly in the study. The patients were divided into two equivalent groups, according to clinical and laboratory findings, i.e., severe and nonsevere. Written informed consent was obtained from all participants. Patients using convalescent plasma, tocilizumab, and systemic steroids were excluded.

Definitions

The Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) (Released by the National Health Commission & State Administration of Traditional Chinese Medicine on March 3, 2020) was used to define the severity of the disease¹⁰. Patients were classified as mild/moderate/severe/critical according to this protocol. We defined mild/moderate cases as nonsevere and severe/critical cases as severe. Cases with respiratory distress (< 30 breaths/

min), oxygen saturation $\leq 93\%$ at rest, arterial partial pressure of oxygen (PaO₂) fraction of inspired oxygen (FiO₂) > 300 mmHg (1 mmHg = 0.133 kPa), and all cases requiring mechanical ventilation support or having any organ failure due to COVID-19 were included in the severe group; cases that did not meet these criteria were included in the nonsevere group.

Data Collection

Demographic data and laboratory findings including complete blood count, routine serum biochemical tests, acute phase and infection indicators, and coagulation parameters were collected from inpatient records. Lymphocyte subsets were analyzed from fresh blood samples by flow cytometry. We used the dual-platform flow cytometric method to measure (DP FCM) the lymphocyte subsets^{11,12}. All other clinical and laboratory data were collected simultaneously with a flow cytometric analysis.

Flow Cytometry

On the same day of the analysis, 4-5 ml of peripheral blood samples were taken from the tubes containing EDTA and sent to the microbiology laboratory of our hospital without waiting. Peripheral blood samples in tubes containing EDTA were labeled using monoclonal antibodies. For this purpose, the number of cells was calculated to be 1×10^6 cells per mL. Lymphocyte subsets were analyzed by flow cytometry as previously described in the literature.¹³ Subsets were determined using antibodies as follows: CD3 (FITC) / CD4 (PeCY7) / CD8 (APC Cy7) / CD45ROPE / CD45RA (APC) / CD197 (PerCpCy5,5) / CD25 (APC Cy7) (BD Biosciences, AB). The tubes were incubated for 20 minutes at room temperature in the dark. At the end of the incubation, the red blood cells in the samples were removed by adding 2-3 mL of Lysing Solution (Becton Dickinson, San Jose, CA 95131 USA). Following washing with Lysing Solution, they were washed with 2 mL of PBS (Phosphate Buffer Saline), the cells were suspended with 500 μ L of PBS containing 1% paraformaldehyde and kept in the dark at 2-8 °C until the time of analysis. The cells were analyzed with the FACSCantoII (Becton Dickinson, Immunocytometry Systems, San Jose, CA 95131 USA) model flow cytometry device using the BD FACSDiva program.

Statistical Analysis

Statistical analysis was performed with SPSS Statistics (IBM Corporation, Somers, NY) software,

version 22). The normality of the distribution of continuous variables was determined using the Kolmogorov–Smirnov test. The continuous variables were expressed as mean and standard deviation or as median and interquartile range, depending on the normality of their distribution. Categorical variables are interpreted by frequency tables. The Mann–Whitney U test was used to compare the variables that were not normally distributed. On the other hand, the Student's t-test was used to compare the variables with a normal distribution. Categorical features and relationships between the groups were assessed using an appropriate chi-square test. A p-value of <0.05 was accepted as statistically significant.

RESULTS

Demographic and Basic Clinical Features of Severe and Nonsevere COVID-19 Patients

The demographic features, basic clinical and laboratory characteristics of the 40 patients are presented in Table 1. While there was no significant difference in the women and men ratio, patients in the severe disease group were significantly older (mean age of severe patients: 71.9 ± 11.2; nonsevere patients: 55.4 ± 17.0, $p < 0.001$). There were 3 patients with concomitant

malignancy and 20 patients with a comorbid chronic metabolic disease, and the differences between the two groups were not significant, but the number of patients was insufficient for interpretation.

Biochemical and Inflammatory Markers, Blood Cell Counts Representing Disease Severity

The inflammatory and biochemical markers (C-reactive protein, erythrocyte sedimentation rate, procalcitonin, d-dimer, ferritin, fibrinogen, and lactate dehydrogenase) were found to be significantly elevated in the severe disease group (Table 1). In the severe disease group, the white blood cell count (WBC) and absolute neutrophil count (ANC) increased considerably, whereas a critical decrease in the absolute lymphocyte count (ALC) was noticed, with no distinct difference in platelet counts (WBC 8.86 ± 3.51 vs 5.89 ± 2.30 $10^3/\text{mm}^3$; $p = 0.003$, ANC 7.72 ± 3.41 vs 3.67 ± 2.01 $10^3/\text{mm}^3$; $p < 0.001$, ALC 0.61 vs 1.39 $10^3/\text{mm}^3$; $p < 0.001$, platelet count 221.6 ± 123.8 vs 199.5 ± 100.3 $10^3/\text{mm}^3$; $p = 0.538$ respectively). The neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) were increased significantly in the severe disease group (NLR 10.55 vs 2.21 ; $p < 0.001$, PLR 319.5 vs 119.5 ; $p < 0.001$).

TABLE 1. DEMOGRAPHIC FEATURES OF PATIENTS AND COMPARISON OF INFLAMMATORY MARKERS AND BLOOD CELL COUNTS ACCORDING TO THE CLINICAL SEVERITY OF THE DISEASE.

	Nonsevere patients (n=20)	Severe patients (n=20)	P-value
Age, years	55.4 ± 17.0	71.9 ± 11.2	0.001
Gender, F/M (%)	13/7 (65/35)	7/13 (35/65)	0.056
Chronic diseases			
No (%)	12 (60)	5 (25)	0.081
*Other chronic diseases(%)	7 (35)	13 (65)	
Malignancy (%)	1 (5)	2 (10)	
C-reactive protein (CRP), mg/L	12.10 (3.31-38.80)	167.50 (85.55-190.0)	<0.001
Sedimentation, mm/1 hr	43.11 ± 29.08	79.15 ± 23.27	<0.001
Procalcitonin, ng/mL	0.062 (0.034-0.079)	0.726 (0.197-3.232)	<0.001
Albumin, gr/L	36.4 ± 3.5	23.8 ± 3.6	<0.001
D-dimer, ng/mL	663.5 (346.5-1205)	2120 (1612.5-4300)	<0.001
Ferritin, ng/mL	120.9 (44.3-427.4)	864.5 (460.4-2000.0)	<0.001
Fibrinogen, mg/dL	348.0 (294.5-381.0)	477.5 (390.8-609.8)	<0.001
Lactate dehydrogenase (LDH), IU/L	223.5 (211-262.5)	395 (308-502.5)	<0.001
White blood cell count, $10^3 / \text{mm}^3$	5.89±2.30	8.86±3.51	0.003
Absolute neutrophil count, $10^3 / \text{mm}^3$	3.67±2.01	7.72±3.41	<0.001
Absolute lymphocyte count, $10^3 / \text{mm}^3$	1.39 (1.16-2.25)	0.61 (0.38-0.97)	<0.001
Platelet count, $10^3 / \text{mm}^3$	199.5±100.3	221.6±123.8	0.538
Neutrophil lymphocyte ratio (NLR)	2.21 (1.38-2.94)	10.55 (6.56-22.63)	<0.001
Platelet lymphocyte ratio (PLR)	119.5 (94.1-155.4)	319.5 (184.0-604.7)	<0.001

*Due to the low number of patients, patients with diabetes mellitus, hypertension, coronary heart disease, chronic kidney disease, and chronic inflammatory diseases were not demonstrated in the chronic metabolic disease group.

Comparison of Lymphocyte Subsets and Memory T cells According to Disease Severity

Lymphocyte subsets were compared according to disease severity and are presented in Table 2. CD8+ cytotoxic T cells and CD4+ helper T cells were significantly lower in patients with severe disease (CD8+ T cells 192.00 vs 504.15 μ L; $p < 0.001$, CD4+ T cells 395.45 vs 958.83 μ L; $p < 0.001$); however, there was no significant difference in the CD4/CD8 ratio in both groups (CD4/CD8 ratio 1.81 vs 1.57; $p = 0.738$). Memory T cells showed a remarkable decrease in the severe disease group (CD4+ memory T cells 304.99 \pm 204.75 vs 682.38 \pm 269.26 μ L; $p < 0.001$, CD8+ memory T cells 87.61 vs 288.33 μ L; $p < 0.001$). The naive, EM, and CM CD4+ T cells were notably reduced in the severe disease group (Naive T cells 175.06 vs 502.68 μ L; $p < 0.001$, EM 0 vs 14.61 μ L; $p = 0.015$, CM 475.74 vs 1051.68 μ L; $p < 0.001$). Furthermore, the naive CD4+ T cell/CD4+ effector memory T cells ratio was significantly impaired in patients with severe disease (median 0 vs 35.10; $p < 0.001$). The CD3+CD4+CD25+ T cell subset was significantly lower in the severe disease group (374.42 vs 965.96 μ L; $p < 0.001$). As a new finding, CD4+CD8+ double-positive (DP) T lymphocytes in peripheral blood decreased significantly in the severe disease group (DP T cells 1.95 vs 11.12 μ L; $p < 0.001$, respectively). The comparison of lymphocyte subsets is presented schematically in Figure 1.

DISCUSSION

Many studies have shown that advanced age and concomitant diseases are risk factors for severe COVID-19. In severe COVID-19 cases, adaptive

immunity cannot overcome the disease and the virus spreads through the blood and damages tissues. In addition to virus-induced direct cytopathic damage, the development of organ injuries secondary to the uncontrolled release of cytokines (called cytokine storm) is believed to be responsible for the pathogenesis of the disease in severe cases^{5,14,15}.

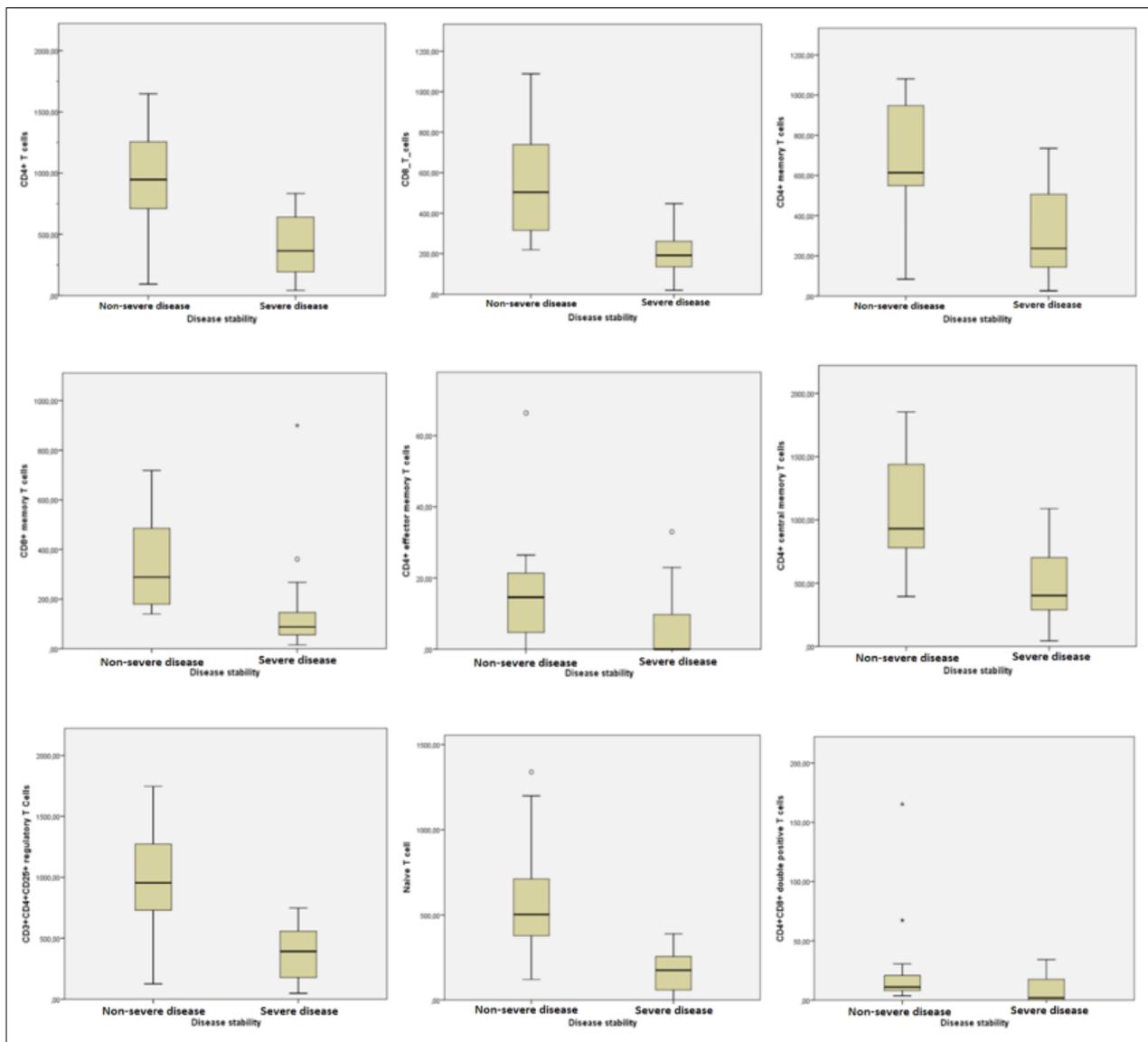
Previous publications have reported that the neutralizing Ig-G antibodies last over 1-2 years in SARS-CoV and MERS-CoV infections¹⁶⁻¹⁹. However, recent studies have reported that neutralizing antibodies begin to decrease after 2-3 months in convalescent COVID-19 patients and many of them become negative in a short time¹⁹⁻²¹. The short-term reduction and disappearance of neutralizing antibodies in convalescent COVID-19 patients may suggest that the virus causes deeper and permanent impairments to the immune system's memory functions.

In this study, we aimed to contribute to the explanation of the pathogenesis of the disease by comparing T lymphocyte subsets in severe and nonsevere COVID-19 patients and revealing disorders of the T-cell-mediated immune response. Thus, we analyzed CD4+ T helper cells, CD8+ cytotoxic T cells, memory T cells (CD4 and CD8 positive), CD3+CD4+CD25+ T cells, CD4+CD8+ double-positive (DP) T cells, which are important components of T-cell-mediated immunity.

The severe cases of the disease were significantly older (Table 1). The production of naive T cells and memory T cells decreases with aging²². The weakened adaptive immune response in the elderly can explain the increased severity of the disease with age. We found that CRP, ESR, procalcitonin level, which show

TABLE 2. COMPARISON OF LYMPHOCYTES SUBSETS BY CLINICAL SEVERITY OF THE DISEASE.

	Nonsevere patients (n=20)	Severe patients (n=20)	P-value
CD4+ T cells, μ L	958.83 \pm 416.24	395.45 \pm 237.59	<0.001
CD8+ T cells, μ L	504.15 (313.61 – 786.22)	192.00 (135.52 – 261.80)	<0.001
CD4+ memory T cells, μ L	682.38 \pm 269.26	304.99 \pm 204.75	<0.001
CD8+ memory T cells, μ L	288.33 (178.94 – 492.92)	87.61 (52.15 – 148.52)	<0.001
CD4+ effector memory T cells, μ L	14.61 (4.34 – 21.47)	0 (0 – 12.15)	0.015
CD4+ central memory T cells, μ L	1051.68 \pm 427.16	475.74 \pm 298.57	<0.001
CD3+CD4+CD25+ T Cells, μ L	965.96 \pm 416.58	374.42 \pm 224.00	<0.001
Naive CD4+ T cell, μ L	502.68 (358.26 – 746.01)	175.06 (58.20 – 259.61)	<0.001
CD4+CD8+ double positive T cells μ L	11.12 (7.61 – 21.71)	1.95 (0 -17.66)	<0.001
CD4/CD8 lymphocyte ratio	1.57 (1.38-2.85)	1.81 (1.16-3.13)	0.738
Naive CD4+ T cell/ CD4+ memory T cells ratio	0.92 \pm 0.40	0.80 \pm 0.82	0.583
Naive CD4+ T cell/ CD4+ central memory T cells ratio	0.59 \pm 0.31	0.59 \pm 0.96	0.998
Naive CD4+ T cell/ CD4+ effector memory T cells ratio	35.10 (15.83-102.20)	0 (0-14.14)	<0.001

FIGURE 1. LYMPHOCYTE SUBTYPES ACCORDING TO THE CLINICAL SEVERITY OF THE DISEASE DEMONSTRATED SCHEMATICALLY.

Note. The data defined on the Y axis shows the absolute cell count per 1 microliter.

the severity of the infection and reflect the inflammatory response, were significantly high in patients with severe disease (Table 1). LDH, fibrinogen, d-dimer, ferritin, which are biochemical indicators of inflammation, were higher in patients with severe disease, and albumin was significantly lower in severe disease cases (Table 1). Lymphopenia is the first indicator of impairment in T-cell-mediated immunity. The prognostic ratios such as NLR and PLR increased notably in patients with severe disease as a result of decreased ALC (Table 1).

CD4+ T helper cells activate other immune cells and help B cells in the production of antibodies, while CD8+ cytotoxic T cells kill virus-infected cells directly with their granules. Persistent stimulation

of viral infection and increased inflammatory cytokines lead to a depletion of T cells²³. In addition, the suggestion that SARS-CoV-2 infects T cells through spike-protein and increases apoptosis of T cells is still under investigation²⁴. Our study demonstrated that helper and cytotoxic T cells were significantly reduced in patients with severe disease. CD4/CD8 ratio has a crucial role in the management of some diseases, especially in HIV infection. However, we did not find a remarkable difference in the CD4/CD8 ratio between the two groups, therefore this is not a useful marker in COVID-19 management.

Normally, some of the naive T cells differentiate into long-lived memory cells after contact with a pathogen. Thus, they guarantee a much faster and

stronger response when the same pathogen is encountered again. We have demonstrated that CD4+ memory T cells, CD8+ memory T cells, naive T cells, CD4+ EM T cells, and CD4+ CM T cells are critically reduced in severe disease cases (Table 2). We would like to point out that the naive T cell/CD4+ EM T cell ratio was impaired. As far as we know, a similar finding has been previously reported only by Chuan Qin et al., who found that the naive-to-memory CD4+ T cell ratio was impaired in patients with severe disease⁵. These results suggest that the differentiation from naive T cells to EM T cells is also impaired.

We encountered a new finding to predict disease severity that has not been reported in previous studies. CD4+CD8+ double-positive (DP) T lymphocytes were remarkably lower in severe disease. DP T cells are present in peripheral blood in small numbers. Their roles in the pathogenesis of autoimmune diseases, viral infections, and cancers are under ongoing debate²⁵⁻²⁷. Some studies have suggested that DP T cells are developing T cells released from the thymus, while others suggest that they are differentiated effector memory cells and have anti-viral effects^{27,28}. Michelina Nascimbeni et al. have offered that peripheral DP T cells are involved in the adaptive immune response to viral pathogens²⁷. The prominent reduction of peripheral DP T cells suggests that the adaptive immune response is seriously impaired in severe disease. DP T cells can be an important marker to predict severity if supported by larger studies.

Tregs regulates the immune response by suppressing the activation, proliferation, and cytokine production of CD4+ T cells and CD8+ T cells and are thought to suppress B cells and dendritic cells^{29,30}. Otherwise, excessive immune response and inflammation damage all tissues. As disease severity increases in COVID-19 infection, inflammation caused by uncontrolled cytokines becomes more and more severe, leading to organ failure. This well-known entity is called a cytokine storm. In our study, we compared the subset of CD3+ CD4+CD25+ T cells and found it to be significantly lower in patients with

severe disease. Doubtless, CD25+ T cells do not fully represent Tregs, but they also contain them. Possibly the impairment in the regulatory functions is one of the leading pathologies that cause cytokine storms in patients with severe disease.

Our study has several limitations. First, this is a cross-sectional and single-center study with 40 patients. A prospective study with more participants and successive flow cytometric analysis would undoubtedly provide more valuable information. However, the high statistical significance in our results makes our study precious. Second, we could not compare all lymphocyte subsets due to the limited antibody supply and we couldn't identify Tregs exactly since CD127/FOX-P3 was not available.

Despite the limitations, we obtained precious data regarding the adaptive immune response. In particular, we have demonstrated the importance of memory T cells and DP T cells in severe disease.

CONCLUSION

We have demonstrated that CD4+ helper T cells, CD8+ cytotoxic T cells, and memory T cells are significantly reduced in severe disease. The relative impairment in naive T cell/CD4+ effector memory T cell refers to deeper disorders in adaptive immune functions of patients with severe disease. The decrease in DP T cells is a new and useful marker for predicting disease severity.

Conflicts of interest

All authors declare that there is no potential conflict of interest relevant to this article.

Author's Contribution

Writing - Review and Editing: Yasin Kalpakci; Validation: Tuba Hacibekiroglu; Supervision: Gulay Trak; Resources: Cengiz Karacaer; Methods: Taner Demirci; Data curation: Havva Kocayigit; Conceptualization: Cenk Sunu; Visualization: Ceyhun Varim; Investigation: Mesude Falay.

RESUMO

OBJETIVO: A pandemia de COVID-19 tem afetado o mundo todo, constituindo uma ameaça grave para a saúde humana. As células T desempenham um papel crítico na imunidade celular contra infecções virais. Procuramos desvendar a relação entre sub

GRUPOS DE CÉLULAS T E A SEVERIDADE DA DOENÇA.

MÉTODOS: Um total de 40 pacientes com COVID-19 foram aleatoriamente recrutados para o presente estudo transversal. Todos os casos foram confirmados por RT-PCR quantitativo. Os pacientes foram divididos em dois grupos equivalentes, um grave e um não-grave. Os dados da avaliação clínica, laboratorial e da citometria de fluxo foram obtidos para ambos os grupos e comparados.

RESULTADOS: Os subconjuntos de linfócitos, células T CD4+ e CD8+, células T de memória CD4+, células T de memória CD8+, células T CD4+ virgens, células T efetoras CD4+, células T de memória central CD4+ e células T CD3+ CD4+ CD25+ estavam significativamente mais baixas nos pacientes graves. A razão células T virgens/células T efetoras TCD4+, que é um indicador da diferenciação entre células T virgens e células de memória, estava relativamente reduzida em casos graves da doença. As células T duplo-positivas CD4+CD8+ periféricas estavam notavelmente mais baixas em casos graves da doença (mediana das células T DP: 11,12 µL vs. 1,95 µL; $p < 0,001$).

CONCLUSÃO: Conforme aumenta a gravidade da doença nos casos de COVID-19, o número de subconjuntos de células T diminui significativamente. A supressão da diferenciação de células T virgens para células T efetoras é o resultado do comprometimento grave das funções imunológicas adaptativas. As células T duplo-positivas CD4+CD8+ periféricas estavam notavelmente mais baixas em casos graves da doença e podem ser um marcador útil para prever a severidade da doença.

PALAVRAS-CHAVE: COVID-19, Subconjuntos de linfócitos, Imunidade adaptativa.

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