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Diagnostic and Prognostic Significance of Studying the Immunophenotype of Patients with Moderate and Severe COVID-19

D. K. Shirinov, M. I. Shamsutdinova, Z. M. Tadjieva

Tashkent Medical Academy, "Specialized Hospital "Zangiota No. 1" for Treatment Patients with Coronavirus Infection", Tashkent, Uzbekistan

Abstract **The aim** of the study was to determine the immunophenotype of patients with moderate and severe COVID-19. **Background.** January 12, 2020 The World Health Organization (WHO) has named the latest virus as the cause of a new coronavirus infectious disease of 2019 (COVID-19). The outbreak was declared by WHO on January 31 as a public health emergency of international importance, and then on March 11 as a pandemic Protective antiviral immunity depends on the activation of CD8+ T cells and the destruction of infected cells, an increase in the number and function of T-lymphocytes in patients with COVID-19, which is necessary for the effective recovery of patients. In this study, we presented clinical data, the main criteria for immunophenotyping, including T-helper cells, cytotoxic T-cells, NK-cells, B-cells in patients with COVID-19. **Material and methods.** 100 patients with confirmed COVID-19 (54 men and 46 women) were examined, who were treated at the "Specialized Hospital "Zangiota 1" for the treatment of patients with coronavirus infection " and 25 practically healthy of the same sex and age to compare the results of immunophenotyping. Severe patients were defined according to the following criteria: respiratory rate ≥24 times/min; pulse oximeter oxygen saturation (SpO2) ≤85% at rest; partial pressure of oxygen (PaO2) <60 mmHg. Data acquisition by flow cytometry was performed on the BD Accury C6 flow cytometer system. Flowjo (Treestar) software was used to calculate events and results. **Results.** Patients were divided into moderate and severe stages of disease severity based on several criteria, including respiratory rate, SpO2, PaO2, PaCO2. Although the mean age of patients with severe clinical stage was greater than those with moderate clinical stage, the difference was not statistically significant (p =0.1). Due to a significant decrease in CD4+ and CD8+ T lymphocytes, the percentage of CD19+ B cells in patients with COVID-19 was higher than in the control group, which was statistically significant (p=0.04). Our results showed that a decrease in the number of CD4+ T-cells, CD8+ T-cells and NK-cells correlates with the course and severity of the disease in patients with COVID-19. **Conclusion.** Lymphopenia and neutropenia were detected upon admission to the hospital in the moderate and severe course of COVID-19. CT revealed a greater involvement of the lungs in the pathological process in the severe course of the disease. The content of CD4+ T-lymphocytes, CD8+ T-cells, NK-cells is markedly suppressed in patients with COVID-19, especially in severe cases. In patients with severe COVID-19, a significant decrease in lymphocytes was observed due to increased CD95+ expression.

Keywords Cellular immunity, Immunophenotyping of lymphocytes, COVID-19, Coronavirus infection, T-lymphocytes, Severity of the course

1. Introduction

SARS-CoV-2 is a new type of coronavirus not previously identified in humans [1,2,3,5]. January 12, 2020 The World Health Organization (WHO) has named the latest virus as the cause of a new coronavirus infectious disease of 2019 (COVID-19). Currently, cases of COVID-19 infection have been registered in many countries of the world [5,8,9,11]. The outbreak was declared by WHO on January 31 as a public health emergency of international importance, and then on March 11 as a pandemic [6,7,9,11]. It is already known that the clinical symptoms of COVID-19 include fever, shortness of breath, dry cough, muscle pain, fatigue, diarrhea and pneumonia [9,10,14,17]. In patients with severe clinical manifestations, pneumonia caused by the virus leads not only to severe acute respiratory syndrome, but also to multiple organ failure and, ultimately, to death [11,14,16,20,21]. Efficient indicators of the disease severity, the outcome of the disease and the therapeutic response have not been fully studied yet.

Protective antiviral immunity depends on the activation of CD8+ T cells and the destruction of infected cells, an increase in the number and function of T-lymphocytes in

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patients with COVID-19, which is necessary for the effective recovery of patients [11,14,15,17,20]. Thus, it was shown that in addition to lymphopenia, cytokine release syndrome was also observed in patients with COVID-19, especially in severe cases, which clearly indicates that the immune components of the host are involved in the development of COVID-19 [13,17]. Both cytokine release syndrome and lymphopenia may indicate a poor prognosis of the disease, which was also shown in severe cases of the 2009 flu pandemic [11,14]. Interestingly, in severe patients with initial SARS-CoV, lymphopenia and cytokine release syndrome were also evident [13,15]. It is known that these characteristics in patients with COVID-19 correlate with the severity of the disease course, impaired regulation of the immune response and increased mortality [7,11,15,17,22]. However, the factors that can cause lymphopenia and activation of cytokine regulation of immunity in patients with COVID-19 remain not fully understood. However, the factors that can cause lymphopenia and activation of cytokine regulation of immunity in patients with COVID-19 remain not fully understood. We hope that these results will be an important step in determining the immunopathogenesis of the disease and the state of the immune response in patients with COVID-19.

The aim of the study was to determine the immunophenotype of patients with moderate and severe COVID-19.

2. Material and Methods

100 patients with confirmed COVID-19 (54 men and 46 women) were examined, who were treated at the "Specialized Hospital "Zangiota 1" for the treatment of patients with coronavirus infection " and 25 practically healthy of the same sex and age to compare the results of immunophenotyping. The diagnosis of COVID-19 was based on the current protocols of the Ministry of Health of the Republic of Uzbekistan using a combination of clinical symptoms, assessment of the severity of the disease, computed tomography (CT) and laboratory data. All patients had laboratory confirmed positive for SARS-CoV-2 using real-time polymerase chain reaction (RT-PCR) throat swab samples. The patients were divided into 3 groups: Group I - 25 practical healthy patients (the average age was 52 years); Group II - 50 patients with moderate COVID-19; Group III - 50 patients with severe COVID-19. The mean age of patients with COVID-19 was 56 years.

The exclusion criteria for the healthy control group were: active respiratory infection, infection with other infectious agents (HIV, syphilis, tuberculosis, influenza, adenovirus infection and other respiratory viral infections), severe systemic diseases, malignant neoplasms and other chronic diseases, including hematological disorders, cachexia, active bleeding, malnutrition, cardiovascular, renal, impaired lung and liver function. Informed consent letters were obtained from all healthy individuals.

Severe patients were defined according to the following criteria: respiratory rate ≥24 times/min; pulse oximeter oxygen saturation (SpO2) ≤85% at rest; partial pressure of oxygen (PaO2) <60 mmHg.

Immunological studies included: the frequency and number of CD4+ T cells, CD8+ T cells, CD19+ B cells, CD16+/CD56+ NK cells were measured by flow cytometry using human monoclonal antibodies - CD4-phycoerythrin (PE; Cytognos), anti-CD8 -fluorescein isothiocyanate (FITC; eBioscience), anti-CD16-PE (BD Biosciences), anti-CD19-PE (BD Biosciences), anti-CD56-PE (Immunostep), and CD95+ antibodies according to manufacturer's instructions. Appropriate isotype-matched negative controls were also used for each sample, to subtract background staining. After incubation of 100 µl of whole blood with an appropriate amount of various monoclonal antibodies (at 4°C for 30 min in the dark), erythrocyte lysis (RBC) was performed using the RBC Lysis Solution kit based on the manufacturer's protocol (Eskanteb Asia). Data acquisition by flow cytometry was performed on a BD Accury C6 flow cytometer system. Events and results were counted using Flowjo software (Treestar).

All statistical analysis and plot preparation was performed using GraphPad Prism version 8.0 software. (GraphPad Software Inc.). Categorical variables were presented as frequencies or percentages, and continuous variables were shown as means ± SD or medians with interquartile ranges (IQR). Parametric two-tailed Student's t-test and non-parametric Mann-Whitney U-test were used to calculate the mean difference between groups. Categorical variables were compared using Fisher's exact test. P-values less than 0.05 were considered significant.

3. Results

All patients infected with SARS-CoV-2 were confirmed by PCR with a throat swab sample. Most patients had the following comorbidities: diabetes mellitus, arterial hypertension and chronic cardiovascular diseases. Patients were divided into moderate and severe stages of disease severity based on several criteria, including respiratory rate, SpO2, PaO2, PaCO2. Although the mean age of patients with severe clinical stage was greater than those with moderate clinical stage, the difference was not statistically significant ($p = 0.1$). Compared with moderate cases, the proportion of some comorbidities was higher in severe cases, but the difference was not statistically significant. Clinical signs and symptoms did not differ between moderate and severe cases, with the exception of fever, which was more prominent in severe patients. As expected, chest CT showed that patients with severe clinical stage had more lung involvement than those with moderate stage ($p = 0,04$).

Significant differences were observed in laboratory data of patients with moderate and severe COVID-19, including platelet count ($p<0.0001$), total lymphocyte percentage ($p<0.0001$), neutrophil percentage ($p<0.0001$), monocyte

percentage (p=0.0003).

Immunophenotyping of lymphocytes showed that the frequency of peripheral blood lymphocyte subpopulations was studied both in the COVID-19 groups and in the control group using the flow cytometry method. As we expected, the percentage of lymphocytes was significantly lower in patients with COVID-19 than in the control group (p<0.0001). The results of immunophenotyping showed a significant decrease in the percentage of CD4 + and CD8 + T-lymphocytes in patients with COVID-19 in compare with the control group (p<0.0001 and p=0.01, respectively). The frequency of CD16+ NK-cells and CD56+ NK-cells did not differ statistically in the patient groups and the control group (p=0.5, p=0.3 and p=0.05, respectively). Due to a significant decrease in CD4+ and CD8+ T lymphocytes, the percentage of CD19+ B cells in patients with COVID-19 was higher than in the control group, which was statistically significant (p=0.04). We were unable to calculate the absolute number of peripheral blood lymphocyte subpopulations for our normal group, but 90 cells/μl for NK and 147 cells/μl for CD19+B cells were found in patients with COVID-19 that were below their normal range in a healthy population. There was a significant difference in the CD4+/CD8+ ratio between the patient groups and the control group, which confirms the suppression of lymphocytes number and, moreover, a significant suppression of the CD4+ subpopulation in patients with COVID-19. The frequency of lymphocyte subpopulations in moderate to severe clinical stages in patients with COVID-19 showed the presence of significant changes in the following immunological markers in the severe stage of the disease: significant suppression of CD3+ of the total pool of T-lymphocytes, a significant suppression of CD4+ T-helpers/inducers, the same values for CD8+ T-cytotoxic lymphocytes, a significant decrease in CD16+/56+ natural killers, naturally a decrease in the immunoregulatory index (IRI).

Due to the importance of lymphopenia in severe forms of COVID-19 and the possibility of its prediction, we summarized the analyzed data in patients with COVID-19 depending on the severity of the process. Thus, the percentage and absolute number of lymphocytes was significantly reduced in patients with COVID-19 at the severe clinical stage, even compared with patients at the moderate stage of the disease (p=0.0001 and p=0.001, respectively). Besides, the frequency and absolute number of CD4+ T-lymphocytes and CD8+ T-lymphocytes were also significantly reduced in severe patients in compared with other cases (p<0.0001, p<0.0001, respectively). The percentage of CD16+ and CD56+ NK-cells was the same in both groups of patients (p=0.7 and p=0.5, respectively), but the absolute numbers of CD16+ and CD56+ NK-cells were slightly increased. As expected, and due to a decrease in the frequency of CD4+ and CD8+ T-cells in severe patients, the percentage of CD19+ B-cells was slightly increased in severe cases than in other patients, but the absolute number did not differ between the groups (p=0.5). And as we have already mentioned, there was a significant difference in the ratio of CD4+/CD8+ between the studied groups of patients, and in severe cases there was a significant suppression of the immunoregulatory index.

An important aspect of the study of immunophenotyping is the explore of the apoptosis marker CD95+ using flow cytofluorimetry. Thus, we investigated apoptosis of lymphocytes against the background of severe COVID-19, which was evaluated and compared with the control group using the annexin V-FITC/PI detection method based on flow cytofluorometry. Our results showed that the percentage of apoptotic cells was significantly increased in patients with COVID-19 in compare with the control group (p<0.0001). In addition, the frequency of apoptotic cells was significantly higher in patients with severe COVID-19 compared even with the moderate course of the disease, which confirms reliable intense lymphopenia in severe cases of the disease (p=0.04). Our studies have shown that the percentage of CD95+ in the norm was 22.3 ± 1.02%, in a moderate course - 31.5 ± 2.1%, and in a severe course of the disease - 35.8 ± 1.6%. All values between the studied groups were reliably distinguishable, which had important diagnostic and prognostic significance. The results obtained were significantly distinguishable, which reflects the importance of the studies conducted, the results of which were comparable with the clinical course of the disease.

We know that coronavirus infection can cause prolonged reactions of cytokines and chemokines, which leads to immune defects and death of some patients. The host's antiviral immunity to this virus depends on the interaction between the innate and adaptive immune response, and all components of the host's immune system, including CD4+ T-cells, CD8+ T-cells, NK-cells and B-cells, play a vital role in the successful antiviral protection and elimination of the virus.

Thus, lymphopenia was detected in patients with COVID-19. Knowledge of the structure of lymphocyte subpopulations is crucial and can provide a new understanding of the mechanisms of the immune system, as well as the immunopathogenesis of the virus. Consequently, in the current study we demonstrated that the majority of infected patients had lymphopenia upon admission to the hospital, and the frequency of CD4+ and CD8+ T-cells was markedly suppressed in patients with COVID-19. It is important to note that the number of CD4+ T-cells, CD8+ T-cells and NK-cells was reduced to a greater extent in patients in the severe clinical stage. Thus, the morbidity of COVID-19 and its development depend on the interaction between the virus and the immune response of infected patients. It has been established that T-cells directly kill virus-infected cells in order to destroy the virus and trigger the production of cytokines that enhance the antiviral immunity of T-cells and other immune cells such as B-cells, NK-cells and macrophages. Like many other viruses, this pathogen has a protective function of both humoral and cellular immunity. Although lymphocytes, and especially T-cells, are necessary for an antiviral immune response, several studies have shown that patients with COVID-19

suffer from lymphopenia, and the number of lymphocytes in most patients is significantly reduced. Based on these findings, the researchers suggest that SARS-CoV-2 affects lymphocytes, especially T-cells, and then defects in the immune system occur during the disease.

In this study, flow cytometry was performed to determine the frequency of lymphocyte subpopulations in the peripheral blood of patients with COVID-19. Although a marked decrease in CD4+ and CD8+ T-cells was found in patients with COVID-19, there was no significant difference in the percentage of NK-cells compared to the normal group. Various publications indicated that the number of CD4+ T-cells, CD8+ T-cells, B-cells and NK-cells in the peripheral blood of patients with COVID-19 had a lower level than that of the control group. Similarly, a decrease in the frequency of immune cells was previously observed in patients with pneumonia caused by SARS-CoV and MERS-CoV. Reports on the lymphocyte subpopulation in SARS-CoV have shown that lymphopenia occurs in 84% of affected patients, and the frequency of reduction in the number of CD4+ T-cells, CD8+ T-cells, NK-cells and B-cells is 100%, 87%, 55% and 76%, respectively. However, we have not been able to find answers to many questions yet. It still remains to be found out which factors contribute to a decrease in lymphocytes in patients with COVID-19.

Thus, some authors have suggested a direct cytotoxic activity of SARS-CoV-2 on T-cells to clarify the depletion of lymphocytes that has been reported in severe cases of COVID-19. [5,9,12,13,22]. Other studies have shown that host factors, including aging, diabetes, hypertension, cardiovascular, cerebrovascular, and chronic diseases, can also cause lymphopenia in these cases, and can also be caused by viral attachment or, indirectly, by immune damage of inflammatory mediators. In addition, the mechanisms of T-cell deletion may be associated with direct cytopathic invasion of SARS-COV-2, which was previously reported and confirmed in MERS-CoV infection [1,4,5,9,15,17,20].

Thus, patients with severe COVID-19 had a significant decrease in the number of lymphocytes, which was consistent with other reports on COVID-19. Further evaluation of lymphocyte subpopulations also showed that the number of all lymphocyte subpopulations, including CD4+ T-cells, CD8+ T-cells, CD16+ and CD56+ NK-cells, was significantly lower in severe patients compared to lighter cases, which are consistent with the results of previous studies of SARS-CoV and SARS-CoV-2. Consequently, lymphocytes and their subpopulations may be potential predictors of clinical efficiency and severity of the disease in COVID-19. In patients with COVID-19, the CD4+/CD8+ ratio was lower depending on the disease severity. Both CD4+ and CD8+ T-lymphocytes have been shown to be susceptible targets for SARS-CoV-2. Our results also showed that the percentage of apoptotic cells was significantly higher in COVID-19 patients than in controls, which was more evident in severe cases. The production of autoantibodies caused by viral infection can contribute to apoptosis and inhibition of hematopoietic cell growth, which can also reduce the formation and differentiation of T-cells and other lymphocytes. Based on previous data, SARS-CoV-2 may have the ability to induce internal and external apoptosis pathways and stimulate T-cell apoptosis, as it has also been observed in MERS-CoV cases [5,9,11,17,19].

In conclusion, we evaluated the clinical and laboratory features of COVID-19 and their relationship with changes in the lymphocyte subpopulation. Our results showed that a decrease in the number of CD4+ T-cells, CD8+ T-cells and NK-cells correlated with the course and severity of the disease in patients with COVID-19. Immune cell frequency can be used as a potential indicator and biomarkers for predicting severity and prognosis in patients with COVID-19. The results may help to explain the immunopathogenesis of SARS-CoV-2 and the development of new biomarkers and therapeutic strategies for this disease.

4. Conclusions

Lymphopenia and neutropenia were detected upon admission to the hospital in the moderate and severe course of COVID-19.

CT revealed a greater involvement of the lungs in the pathological process in the severe course of the disease.

The content of CD4+ T-lymphocytes, CD8+ T-cells, NK-cells is markedly suppressed in patients with COVID-19, especially in severe cases.

In patients with severe COVID-19, a significant decrease in lymphocytes was observed due to increased CD95+ expression.

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