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Original article

CHANGES IN THE EXPRESSION OF REGULATORY RECEPTORS ON CD8 T CELLS AS RISK FACTORS FOR THE FORMATION OF LONG COVID IN PATIENTS AFTER A SEVERE COURSE OF COVID-19

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Objective. Long COVID is a syndrome that develops after SARS-CoV-2 infection and is associated with immune dysregulation. The aim of this study was to investigate the state of $CD8^+$ T cells and the expression of immune exhaustion–related markers in patients with long COVID.

Materials and Methods. The study included 64 adult patients aged 18–65 years with long COVID. Patients were stratified into three groups according to disease severity: mild (n = 21), moderate (n = 22), and severe (n = 21). In addition, 20 healthy individuals were included as a control group. Multiparametric flow cytometry analysis was performed using peripheral blood samples. We assessed the expression of the inhibitory receptor/ligand PD-1/PD-L1, the inhibitory receptor TIM-3, and the regulatory receptor CD38 on the surface of $CD8^+$ T cells in patients who had recovered from mild, moderate, and severe COVID-19.

Results. PD-L1 ($CD274^+$) expression was significantly higher in patients after moderate ($p = 0.011$) and severe ($p = 0.003$) courses of COVID-19 compared with the control group. PD-1 ($CD279^+$) expression in patients after a severe course of COVID-19 was significantly lower than in patients after a mild course of the disease ($p = 0.009$) and in the control group ($p = 0.041$). TIM-3 ($CD366^+$) expression in patients after a severe course of COVID-19 was significantly lower compared to patients after mild and moderate courses of the disease ($p = 0.043$) as well as to the control group ($p = 0.046$). $CD38^+$ expression in patients after a severe course of COVID-19 was significantly higher compared to the control group ($p = 0.042$), but did not differ significantly from that in patients after mild and moderate courses of COVID-19.

Discussion. Our findings are consistent with previously published data demonstrating long-lasting cytotoxic alterations in $CD8^+$ T cells in patients after COVID-19, suggesting that recovery after severe COVID-19 is associated with an increased risk of autoimmunity. We hypothesize that persistent immune dysregulation in patients with long COVID after severe disease leads to a shift toward Th1 lymphocytes, upregulation of activation and inhibitory receptors, and increased CD38 expression. Together, these alterations may contribute to pathological outcomes, including autoimmunity and other forms of immunopathology following COVID-19.

Conclusions. In patients with long COVID after a severe course of COVID-19, we observed: (1) a decreased number of $CD8^+$ T cells ($p < 0.05$); (2) an imbalance in the PD-1/PD-L1 system ($p = 0.003$; $p = 0.041$); and (3) decreased TIM-3 expression ($p = 0.046$) together with increased CD38 expression ($p = 0.042$). These findings indicate that patients who experienced severe COVID-19 may have a higher risk of autoimmunity due to impaired immunoregulatory function of activation and inhibitory receptors on $CD8^+$ T cells.

***Keywords*:** long COVID, cell exhaustion, CD8+ T cells, PD/PD1L system, TIM-3 receptor, activatory receptor CD38**.**

Introduction

The following symptoms are characteristic of infection with SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2): fever, fatigue, and dry cough, as well as cognitive, neurovegetative, and behavioral disturbances [1]. Long COVID is a syndrome that develops after SARS-CoV-2 infection and involves persistent symptoms affecting multiple organ systems [2]. According to the World Health Organization, post-COVID-19 condition refers to symptoms that occur no earlier than 12 weeks after infection, persist for at least 8 weeks, and cannot be explained by an alternative diagnosis [3]. The term long COVID, established to describe patients with complex and prolonged symptoms that persist or emerge after 4 weeks after the acute infection and may last for months, is used in this paper—taking into account revised terminology and clinical approaches—to denote persistent symptoms following SARS-CoV-2 infection with no other identifiable cause [1-5].

The pathogenesis of long COVID is based on immune dysfunction resulting from the long-term persistence of SARS-CoV-2 [2; 6]. It is also characterized by persistent inflammation, immune dysregulation and an increased risk of autoimmunity [7; 8; 9]. An important clinical feature of severe COVID-19 is progressive lymphopenia [10], reflecting early and profound involvement of the adaptive immune system, particularly the T-cell compartment [11].

Several authors have reported chronic activation of a subset of $CD8^+$ T cells in patients with long COVID persisting for up to 8 months after SARS-CoV-2 infection. This is reflected in increased expression of immune exhaustion markers on $CD8^+$ T cells due to persistent antigenic stimulation by SARS-CoV-2 [2; 3]. Severe COVID-19 is characterized by hyperactivated and exhausted $CD8^+$ cytotoxic T lymphocytes (CTLs) [12]. These CTLs are virus-specific $CD8^+$ T cells recognizing SARS-CoV-2 antigens. The immune response of innate and adaptive components in patients after COVID-19 includes the release of pro-inflammatory cytokines, recruitment of inflammatory cells and the generation of virus-specific CTLs. Acute immune exacerbations are often observed in critically ill COVID-19 patients [6].

The induction of apoptosis represents the primary effector function of cytotoxic T lymphocytes (CTLs) [12]. One of the mechanisms for triggering apoptosis is the release of cytotoxic molecules, such as perforin and granzymes, leading to disruption of the infected cell membrane. The second mechanism involves receptor–ligand interactions between CTLs and infected cells via specific receptors on the infected cells' surfaces [13]. The receptors listed below are particularly important.

PD-1 (Programmed cell death protein-1), also known as PDCD1 or CD279, is an inhibitory receptor on T cells that helps regulate undesirable immune responses. It prevents T-cell overactivation and potential tissue damage [14]. PD-1 is a transmembrane protein belonging to the CD28/CTLA-4 superfamily that is expressed during immune responses [15]. PD-1 is most commonly expressed on activated T lymphocytes and NK cells, and inhibits their cytotoxic potential. Thus, PD-1 may exert both inhibitory and regulatory effects [16; 17].

PD-1, upon binding to its ligand, known as programmed cell death 1 ligand (PD-L1), negatively regulates the immune response [15]. The PD-1 glycoprotein predominantly interacts with PD-L1. Although PD-1 is found on T-cells, its ligand PD-L1 is often found on infected cells. The regulation of PD-L1 expression involves non-immune and immune-related mechanisms. Non-immune mechanisms include genomic abnormalities. Immune-related mechanisms involve activation of inflammatory signaling pathways triggered by immune cells [17]. Overexpression of PD-L1 inhibits T lymphocyte activity [18].

An inhibitory signal is generated by the interaction between PD-1 and PD-L1, which maintains immune homeostasis [15]. These inhibitory immune checkpoint molecules balance the immune response, preventing the accumulation of self-reactive T cells [14]. Under physiological conditions, PD-1/PD-L1 signaling suppresses self-reactive T cells and promotes regulatory immune mechanisms, thereby limiting inflammation and preventing autoimmune processes [14; 15]. The binding of PD-1 to its ligand PD-L1 leads to decreased T cell activity and reduced release of cytotoxic mediators from T cells [17]. The PD-1/PD-L1 pathway inhibits T cell activation [16]. The number of $PD\text{-}1^+CD8^+$ T cells in the peripheral blood of patients with autoimmune diseases decreases during disease remission. However, overexpression of these negative co-stimulatory molecules, which inhibit T-cell responses, is associated with persistent activation of autoreactive T cells. Depletion of $PD\text{-}1^+CD8^+$ T lymphocytes

significantly facilitates the progression of autoimmune disease. Prolonged stimulation of the PD-1/PD-L1 pathway leads to T-cell exhaustion [15].

The PD-1/PD-L1 pathway sends inhibitory signals that suppress immune defense against infections. This interaction leads to inhibition of T cell receptor signaling, reduced receptor-mediated cytotoxicity, impaired $CD8^+$ T cell proliferation, and eventual apoptosis of activated T cells [14]. This pathway is a key factor for regulating T cell exhaustion in patients with SARS-CoV-2. Therefore, it is very important to study whether immune checkpoint molecules are involved in immune dysregulation and the severity of COVID-19 [10].

In addition to PD-1, TIM-3 (T-cell immunoglobulin and mucin domain 3), a transmembrane protein that acts as a negative modulator of immune cell activity, is excessively expressed on $CD8^+$ T cells in COVID-19. It plays a vital role in preventing hyperactive immune responses and the development of autoimmune diseases. TIM-3 suppresses CTL function, which is essential for fighting infections. In chronic viral infections, changes in TIM-3 expression are used as a marker of dysfunctional T cells [19].

The receptor CD38 is expressed on different immune cells (e.g., T lymphocytes, NK cells). This receptor regulates the traffic of cells to the sites of inflammation and thus affects innate and adaptive immune responses. CD38 overexpression resulted in the growth of apoptosis, and also reduced the intracellular NAD^+ level. In patients with viral infections, loss of CD38's functional relevance was observed [20]. CD38 is regulated by different nuclear factors, first and foremost NF-κB, which promotes T-helper type 1 polarization [21].

For a long time, researchers have sought reliable markers of immune dysfunction associated with long COVID. Investigated parameters included differential blood cell counts, particularly $CD8^+$ T cells, as well as the expression of activation markers (CD38) and inhibitory receptors (PD-1, TIM-3) [22]. However, the clinical relevance of T-cell activation and inhibition during the course of COVID-19 has received relatively little attention in the literature to date [3]. Researchers have renewed their focus on identifying laboratory tests that most accurately assess functional changes in cytotoxic lymphocytes.

The aims of our study were (1) to investigate the expression of the inhibitory immune checkpoint receptor–ligand system PD-1/PD-L1, regulatory-activating receptor CD38, and inhibitory receptor TIM-3 on CD8+ T cells in patients with long COVID; and (2) to evaluate interdependence between mild, moderate and severe course of COVID-19 and expression of PD-1/PD-L1, CD38 and TIM-3 as risk factors for the development of immunopathology in patients after COVID-19.

Materials and methods

The study took place in 2023-2024 at the Department of Clinical Immunology and Allergology of the State Non-Profit Enterprise Danylo Halytsky Lviv National Medical University. The study included patients (mainly residents of the western region of Ukraine) who applied for a clinical appointment with internists, family doctors, immunologists, pulmonologists, and rheumatologists at the Lviv Regional Clinical Diagnostic Center, the Lviv Regional Clinical Hospital, and other medical and preventive institutions in Lviv region. The study was approved by the Commission on Bioethics of Scientific Research, Experimental Developments, and Scientific Works of the Danylo Halytsky Lviv National Medical University (approval protocol No. 1, January 23, 2023).

Long-COVID was diagnosed according to the UK National Institute for Health and Care Excellence COVID-19 rapid guideline: managing the long-term effects of COVID-19, 2020 [22]. The forms of COVID-19 were determined by criteria in the Clinical Management of COVID-19: living guideline, August 18, 2023 [23].

This study included 64 adults aged.18-65 with long COVID, comprising 43 women (67%) and 21 men (33%). Patients were stratified into mild (n = 21), moderate (n = 22), and severe (n = 21) disease groups. In addition, 20 healthy individuals were included as a control group.

Patients included in the study had a history of previous COVID-19. They had existing symptoms that first appeared after the COVID-19 and were not explained by alternative diagnoses for an extended period after COVID-19: increased fatigue 64 (100.0%); sleep disturbance, constant fatigue and increased sweating in 54 (84.4%); mobility impairment, headaches, and loss of smell in 51 (79.7%); cough and memory and attention impairment in 45 (70.3%) patients. Half of the patients (50.0%) experienced apathy, anxiety and depressive thoughts, to a lesser

extent, complaints of chest tightness, loss of appetite, loss of taste, hair loss, arthralgia, myalgia, skin rash, etc. were less common (15.0-45.0%). Depending on the course of COVID-19 in the anamnesis, it was divided into mild (asymptomatic course of the disease with mild symptoms of acute respiratory viral infection and a positive PCR result for SARS-CoV-2), moderate (pneumonia without respiratory failure) and severe (dyspnea, hypoxia, and significant lung damage on imaging studies). According to the course of COVID-19 in the anamnesis, patients were divided into groups: 1—patients after a mild course of COVID-19 (21); 2—patients after a moderate course of COVID-19 (22); 3—patients after a severe course of COVID-19 (21). Informed consent was signed with each patient. Exclusion criteria: patients with a history of chronic comorbidities, including systemic and organ-specific autoimmune diseases. Children and pregnant women were excluded from this study. The control group consisted of 20 healthy participants (without a history of COVID-19, confirmed by serological tests).

Multiparametric flow cytometry analysis was performed on EDTA-anticoagulated peripheral blood samples collected from 64 patients who had recovered from COVID-19 and 20 healthy controls using a BD FACSCalibur™ flow cytometer (Becton Dickinson, CA, USA). The cytometer was used to measure fluorescence intensity, which was analyzed using CellQuest™ software (Becton Dickinson Biosciences, San Jose, CA). Cell staining was performed with the following antibodies: FITC-conjugated mouse anti-human CD8 mAb (BD Pharmingen™, California, USA), PE-conjugated Mouse Anti-human CD274 mAb (BD Pharmingen™, California, USA), APC-conjugated mouse anti-human CD279 mAb (BD Pharmingen™, California, USA), APC-conjugated mouse anti-human CD38 mAb (BD Pharmingen™, California, USA) and PE-conjugated mouse anti-human CD366 (TIM-3) mAb (BD Pharmingen™, California, USA). 20 μL of the appropriate mAb was added to each of the two tubes. The first tube was used to analyze $CD8^+CD274^+CD279^+$ cells, and the second tube was used to analyze $CD8^+CD38^+CD366^+$ cells. Erythrocytes were lysed with BD.Pharm Lyse™ Lysing Buffer working solution. 20,000 $CD8^+$ events per tube were recorded. The percentages of CD274, CD279, CD38, and CD366 (TIM-3) expression in CD8+ cells were analyzed. Isotype controls were used to set gates for positive CD274, CD279, CD38, and CD366 (TIM-3) events. Statistical processing was performed using the MedCalc Software Ltd (version 23.0.2).

Results

At the beginning of the study, the percentage of $CD8^+$ T cells in the peripheral blood of patients after mild, moderate, and severe COVID-19 was determined. The percentage of $CD8^+$ effector cells was significantly lower in patients with severe COVID-19 than in healthy individuals ($p = 0.008$). In contrast, patients after mild and moderate COVID-19 showed a trend toward decreased values of this parameter; however, the differences were not statistically significant ($p = 0.205$ and $p = 0.107$, respectively) (Fig. 1).

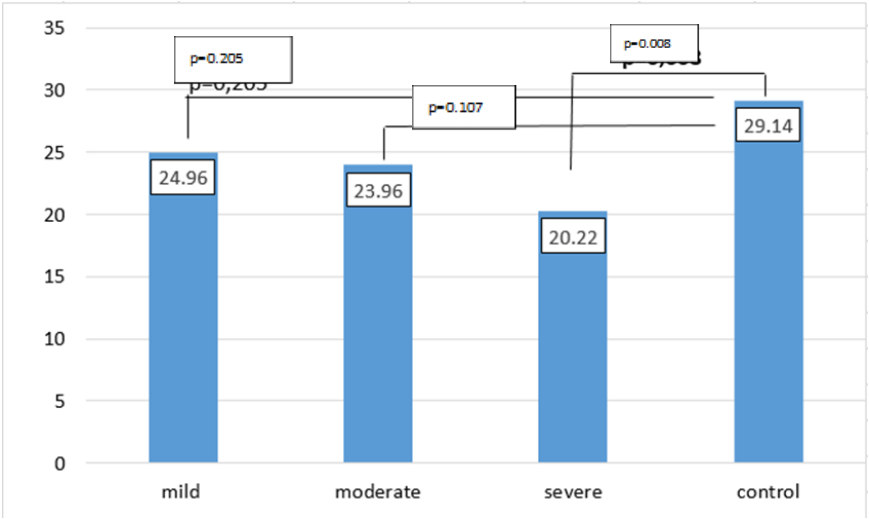

Figure 1. The percentage of CD8+ T cells from the peripheral blood of patients after mild, moderate and severe courses of COVID-19

Several studies have reported that CD8+ T cells from patients after COVID-19 exhibit reduced cytokine production upon viral stimulation. In contrast, other studies have shown that $CD8^+$ T cells from patients after COVID-19 display enhanced effector functions, including increased production of pro-inflammatory cytokines such as IL-2

and IL-17A, as well as elevated expression of the degranulation marker CD107a [4, 6]. However, data on changes in the cytotoxic function of these cells remain limited.

Therefore, the next stage of the study was to investigate the expression of the inhibitory receptors PD-1 and PD-L1, the inhibitory receptor TIM-3, and the regulatory/activation marker CD38 on the surface of $CD8^+$ T cells in patients after mild, moderate, and severe COVID-19. The results showed that PD-L1 ($CD274^+$) expression was significantly higher in patients after moderate COVID-19 ($p = 0.011$) and severe COVID-19 ($p = 0.003$) compared with the control group. The expression level of PD-1 ($CD279^+$) in patients after severe COVID-19 was significantly lower compared to patients after mild COVID-19 ($p = 0.009$) and the control group ($p = 0.041$). TIM-3 ($CD366^+$) expression in patients after severe COVID-19 was significantly lower than in patients after mild or moderate COVID-19 ($p = 0.043$) and the control group ($p = 0.046$). CD38 expression in patients after severe COVID-19 was significantly higher compared to the control group ($p = 0.042$). However, the differences between patients after severe COVID-19 and those after mild and moderate COVID-19 did not reach statistical significance ($p = 0.950$ and $p = 0.353$, respectively) (Table 1).

Table 1

The expression of inhibitory receptors PD1L/PD-1, TIM-3 and regulatory-activatory marker CD38+ on CD8+ T cells of patients after mild, moderate and severe courses of COVID-19

Receptor/ marker	After a mild COVID-19 n=21	After a moderate COVID-19 n=22	After a severe COVID-19 n=21	Control n=20	Statistically significant differences between groups, "p"					
Groups	1	2	3	4	1 vs 4	2 vs 4	3 vs 4	1 vs 2	1 vs 3	2 vs 3
$CD274^+$ (PD-1L)	3.22±1.34	3.76±1.58	4.34±2.11	2.49±1.44	0.105	**0.011**	**0.003**	0.251	0.306	0.065
$CD279^+$ (PD-1)	9.42±4,22	7.86±3,62	6.35±2,71	9.10±5.14	0.831	0,383	**0.041**	0.217	**0.009**	0.144
$CD366^+$ (TIM-3)	9.23±4.84	6.74±3.77	6.38±3.73	9.30±5.12	0.964	0.079	**0.046**	0.077	**0.043**	0.673
$CD38^+$	4.88±2.65	5.65±3.09	6.71±3.20	4.83±2.39	0.950	0.354	**0.042**	0.403	0.950	0.353

Discussion

In patients after COVID-19, cytotoxic T lymphocytes (CTLs) appear to be activated; however, the absolute number of $CD8^+$ T cells is reduced. This may result from alterations in multiple immune checkpoint receptors expressed on hyperactivated and exhausted $CD8^+$ T lymphocytes [24]. In our study, we demonstrated a decrease in $CD8^+$ T cells, particularly in patients with moderate or severe COVID-19 ($p < 0.05$). In contrast, other authors have reported increased numbers of activated $CD8^+$ T cells three months after recovery from mild, moderate, and severe COVID-19 [6].

Viral infection can induce upregulation of PD-1 expression. Increased expression of the exhaustion receptor PD-1 has been reported in patients after COVID-19 [14; 24; 25]. T cells from patients who have recovered from COVID-19 become insensitive to stimulation, and this impaired responsiveness is accompanied by increased PD-1 expression [25]. In patients after severe and critical COVID-19, T cells shift from a state of hyperactivation to exhaustion while simultaneously expressing increased levels of PD-1 [11]. In patients with long COVID, a significantly reduced number of PD-1–expressing $CD8^+$ T cells was observed three months after recovery from COVID-19 compared with healthy controls [6]. In contrast, patients with a critical course of COVID-19 exhibited increased numbers of $CD8^+$ T cells with elevated PD-1 expression [10]. Our results demonstrated reduced PD-1 expression in patients after severe COVID-19 compared to those after mild COVID-19 ($p = 0.009$) and with the control group ($p = 0.041$), consistent with published data and supporting the tendency toward the development of long COVID in such patients.

The overexpression of PD-L1 leads to the inhibition of T-lymphocyte activity, as PD-L1 suppresses peripheral T-cell function [18]. The level of PD-L1 expression can predict the course of COVID-19, as decreased PD-L1 expression on T cells in patients after COVID-19 indicates T-cell hyperactivation, which may lead to immunopathology [11]. Overexpression of PD-1 and PD-L1 has been reported on various immune cells in patients with autoimmune diseases [14]. In our study, we demonstrated increased PD-L1 expression on $CD8^+$ T cells in patients with moderate and severe COVID-19 ($p = 0.011$ and $p = 0.003$, respectively) compared to the control group. In our opinion, this finding indicates a high degree of $CD8^+$ T-cell exhaustion and a loss of their cytotoxic capacity toward infected cells. In our patients, exhaustion resulted from chronic stimulation by viral antigens.

In addition to the hyperactivated/exhausted phenotype of $CD8^+$ T cells, a characteristic feature of COVID-19 is increased TIM-3 receptor expression, particularly in patients with a severe course of the disease [26, 27]. TIM-3 expression is associated with Gal-9/TIM-3–induced cell death. The lectin galectin-9 (Gal-9) can interact with PD-1 on the surface of T cells, contributing to the persistence of $PD\text{-}1^+TIM\text{-}3^+$ T cells and attenuating Gal-9/TIM-3–mediated apoptosis [14]. Similar to TIM-3, Gal-9 serves as a trigger of intracellular signaling pathways leading to apoptosis. Its proapoptotic activity is manifested by increased Annexin V binding on the outer leaflet of the cell membrane during early apoptosis, which serves as a marker of this process [14; 28].

The authors reported higher TIM-3 expression on CD8+ T cells in patients with a critical course of COVID-19 compared to those with a mild course [10, 11]. Increased TIM-3 expression on CD8+ T cells is associated with T-cell activation and closely correlates with the degree of disease activity [27]. Our results showed that TIM-3 expression in patients after a severe course of COVID-19 was significantly lower than in patients after mild or moderate courses of the disease ($p = 0.043$) and in the control group ($p = 0.046$). Since TIM-3 is considered a potential diagnostic marker of COVID-19 severity, the observed decrease in its expression on CD8+ T cells may indicate the development of their functional exhaustion.

Previous studies reported a significantly higher number of $CD8^+CD38^+$ cells in non-survivors of COVID-19 compared with survivors, while recovery was associated with a significant decrease in their numbers [22]. Circulating $CD8^+$ T cells in patients after severe COVID-19 exhibit increased CD38 expression [27]. The level of CD38 expression plays a central role in immune alterations after COVID-19 [29]. Our results showed that CD38 expression in patients after a severe course of COVID-19 was significantly higher than in the control group ($p = 0.042$); however, differences compared with patients after mild or moderate COVID-19 did not reach statistical significance. Thus, increased CD38 expression on $CD8^+$ T cells in patients after severe COVID-19 appears to reflect enhanced immune exhaustion.

The results of our study are consistent with data reported by other authors, indicating long-lasting cytotoxic alterations of $CD8^+$ T cells after COVID-19. Residual excessive inflammation following infection manifests as changes in receptor expression on $CD8^+$ T cells, reflecting inflammatory and exhaustion processes. Due to their cytotoxic activity, autologous cells are destroyed, leading to the release of autoantigens and the production of autoantibodies against them [3; 30]. Long-term immune dysregulation and chronic inflammation after COVID-19 may lead to pathological consequences, including autoimmunity [32] and other forms of immunopathology [30; 31; 33].

Conclusions: We identified in the patients with long COVID after a severe course of COVID-19: (1) a reduced number of $CD8^+$ T cells ($p < 0.05$); (2) an imbalance of the PD-1/PD-L1 system ($p = 0.003$; $p = 0.041$); (3) decreased TIM-3 expression ($p = 0.046$) and increased CD38 expression ($p = 0.042$). We demonstrated that patients after a severe course of COVID-19 have a higher risk of autoimmunization, as activating and inhibitory receptors on $CD8^+$ T cells lose their capacity for immune regulation.

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