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Original article

MARKERS OF ACTIVATION AND DYSFUNCTION OF CYTOTOXIC IMMUNE CELLS IN PATIENTS WITH LONG COVID AND DURING AGEING

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Background. Ageing is a process of gradual age-related changes in which molecular disorders accumulate under the influence of environmental factors, including air pollution, smoking, and poor nutrition.
Objective: To evaluate the level of expression of activation markers and dysfunction of the immune system in patients with Long Covid and in ageing.
Results. During our study, we found significant correlations between the study groups and the control group. However, we did not observe a statistically significant difference (p=0.4969) in CD38+ expression among CD8-positive cells between elderly patients (5.12±2.40%) and the post-COVID group (4.84±1.27%). CD366 on cytotoxic CD8 T cells between the group of elderly patients (5.12±2.67%) and the group of post-COVID patients showed weak statistical significance (6.55±3.28%, P=0.0470). No statistically significant difference (p=0.1247) was observed between the group of elderly patients (5.70±2.30%) and the group of post-COVID patients (4.88±2.38%) in terms of CD38+ expression on CD16/56-positive cells. No significant difference was observed in the expression of the exhaustion marker CD366 on cytotoxic $CD56^+/CD16^+$ NK cells between elderly patients (5.92 ± 2.61%) and post-COVID patients (6.97 ± 3.21%) (p = 0.1338).
Conclusions.
1. Elevated levels of CD38 and CD366 expression on cytotoxic lymphocytes are a biological reflection of immune ageing, which has profound clinical implications.
2. CD38 is a central regulator of metabolic dysfunction and NAD+ deficiency, which underlie age-related inflammation and metabolic disorders.
3.CD366 is a critical marker of immune tolerance and exhaustion, contributing to the evasion of immune surveillance in ageing and chronic viral infections.

Keywords: Long COVID, NK cells, cytotoxic T cells, Immune Markers CD38, CD366 (TIM3), cellular ageing, ageing.

Introduction

Ageing is an inevitable process in which our body's functions decline over the years, leading to a deterioration in mental and physical functions and an increased risk of disease. As a result, we become less adaptable to change, recover from injuries more slowly, and lose our ability to reproduce. This occurs because damage accumulates in the cells and molecules of the human body (internal factors), including cell wear and tear, disruption of apoptosis processes, and depletion of stem cell reserves. The body is also affected by external factors, including air pollution, smoking, alcohol use, poor nutrition, and exposure to ultraviolet radiation [1].

The adaptive immune system suffers more than the innate immune system [2]. Immunosenescence negatively affects immune responses to infections and the development of long-term immune memory. The natural ageing process causes ageing. It is accompanied by profound changes in the immune system, leading to reduced immune defence and increased susceptibility to severe viral infections, neoplasms, and autoimmune diseases [3].

Cells with functional and phenotypic changes in key lymphocyte populations, including cytotoxic lymphocytes: NK cells (CD16/CD56) and CD8+ T cells, play a key role in this decline. The markers CD38 and CD366 (TIM-3) play an essential role in regulating the activation, differentiation, and exhaustion of these cells. CD38 and CD366 (TIM-3) markers on cytotoxic CD8+ T cells and NK cells are not only indicators of immunosenescence but also critical factors in the development and progression of age-related pathologies [4]. High and sustained expression of these molecules on lymphocytes in older adults reflects chronic inflammation, metabolic dysfunction, and immune exhaustion, with direct clinical consequences [5].

CD38 is a multifunctional transmembrane glycoprotein with enzymatic activity (ADPR cyclohydrolase) that functions as a receptor and enzyme. Its expression is a common indicator of lymphocyte activation and differentiation.

In young, healthy individuals, CD38 is a marker of activation and is intensely expressed on CD8+ T cells involved in acute immune responses. However, with ageing, CD38 expression increases significantly in highly differentiated, senescent subpopulations (groups of cells that have reached a state of cellular ageing (senescence) rather than dying as programmed), including CD8+ T cells, especially in CD8+EMRA T cells (T-effector memory CD45RA+). These cells are often characterised by increased CD38 expression and a pro-inflammatory phenotype [6; 7]. High CD38 expression in ageing CD8+ T cells correlates with chronic inflammation and metabolic dysfunction, often observed in age-related diseases [8]. CD38 is a constitutive marker on NK cell subpopulations and serves as an important marker for identifying and assessing NK cell function [9]. In the context of ageing, CD38 expression on NK cells may reflect their differentiation status. Studies show that ageing is associated with changes in NK cell subpopulation composition (an increase in CD56dim/CD16high cells) and may be accompanied by increased CD38 expression on highly differentiated NK cells, especially in conditions of chronic stimulation or infection [8]. A high level of CD38 expression on NK cells is also associated with metabolic changes and decreased NAD+ levels, which are essential factors in ageing [6].

CD366 (T-cell immunoglobulin and mucin domain-containing protein 3, TIM-3) is an inhibitory immune checkpoint receptor that was initially identified as a marker of exhausted TH1 cells. CD366 is one of the most important markers of T-cell exhaustion along with PD-1. Its expression on CD8+ T-cells is low or absent in healthy young individuals. However, in chronic viral infections (e.g., HIV, HCV) or cancer, CD366 is highly expressed, indicating functional dysfunction [3; 9]. During ageing, CD366 expression increases on CD8+ T cells, reflecting the accumulation of senescent and exhausted cells that are unable to perform their functions effectively [3]. CD366+ CD8+ T cells in older adults may have reduced cytotoxic activity and cytokine production [9]. CD366 is also expressed on NK cells. Commonly, CD366+ NK cells may be associated with regulatory functions or a specific stage of maturation. However, as with T cells, a high level of CD366 expression in NK cells is a sign of dysfunction or depletion, especially in conditions of chronic stimulation, such as chronic viral infections and cancer [10]. Although the direct effect of ageing on CD366 expression in NK cells has been studied less extensively than in T cells, its appearance suggests that NK cells are also subject to phenotypic exhaustion during immunosenescence [10].

This publication is a continuation of the scientific research "Methods and means of studying markers of ageing and their impact on post-COVID effects for prolonging the working period", funded by the National Research Fund of Ukraine (registry No. 2021.01/0103) [11; 12].

The aim of the study is to assess expression levels of markers of immune system activation and dysfunction in patients with Long Covid and in ageing.

Patients and methods. The study was carried out in compliance with the Declaration of Helsinki (7th revision, 2013), the Council of Europe Convention on Human Rights and Biomedicine, and the relevant legislation of Ukraine. The study protocol was reviewed and approved by the Ethics Committee for Scientific Research, Experimental Development, and Scientific Activities of Danylo Halytsky Lviv National Medical University (Protocol No. 10, dated 9 October 2023).

The inclusion criteria were 50 adults of both sexes aged 18 to 65 (63.9% women and 36.1% men, average age 49.4±12.7) with a history of COVID-19 and persistent post-COVID symptoms lasting longer than 12 weeks that could not be attributed to an alternative diagnosis; these individuals constituted the first study group. [13,14]. During the clinical examination of patients with long COVID-19, the most frequently reported symptoms included increased fatigue, observed in 50 patients (100%); sleep disturbances, persistent fatigue, and excessive sweating in 42 patients (84%); mobility impairment, headaches, and anosmia in 40 patients (80%); and cough as well as memory and attention deficits in 35 patients (70%).

Neuropsychiatric symptoms, including apathy, anxiety, and depressive thoughts, were reported by half of the patients (50%). Additionally, between 15% and 45% of patients reported chest tightness, loss of taste, hair loss, and other complaints. The second study group comprised 30 elderly patients aged 55–70 who had no history of COVID-19 or had experienced a mild form of the disease without developing post-COVID symptoms. Their average age was 62.7±23.5, and 68% were women and 32% were men. The third group (control) consisted of 40 practically healthy middle-aged individuals (40-55 years old), with an average age of 45.2±18.4, including 55% women and 45% men. All participants involved in this study signed an informed consent form [13].

The NICE criteria (COVID-19 rapid guideline: managing the long-term effects of COVID-19. NICE guideline [15]) were used to verify long COVID-19.

The study was conducted at the Department of Clinical Immunology and Allergology of Danylo Halytsky Lviv National Medical University and commenced in October 2024. The study was performed using flow cytometry (BD FACSCalibur, USA) with CellQuest Pro software to study markers on cytotoxic lymphocytes [12].

Statistical analysis was performed using Student's t-test for normally distributed data. For non-normally distributed variables, the non-parametric Mann–Whitney U test was applied. Data are presented as mean ± standard deviation (M ± SD). Differences between groups were considered statistically significant at $p < 0.05$.

Results

To conduct our study, we examined in detail the regulatory changes in the immune system. In the study groups, we determined the expression of CD38 and CD366 receptors on cytotoxic CD8 and NK cells. The results are presented in Table 1.

Table 1

Comparative analysis of CD38+ and CD366 activation markers on cytotoxic CD8+ and CD56/16 cells in the study groups

	Post-COVID patients	Elderly patients	Control	Statistically significant differences between groups, "p"		
	1st n=50	2nd n=30	3rd n=40	1 vs 2	1 vs 3	2 vs 3
M±SD						
CD38/ CD8	4.84±1.27	5.12±2.40	4.04±2.10	0.4969	**0.0281**	**0.0492**

CD366/ CD8	6.55±3.28	5.12±2.67	4.05±1.95	**0.0470**	**0.0001**	**0.0423**
CD38/ CD56/16	4.88±2.38	5.70±2.30	3.63±1.89	0.1247	**0.0066**	**0.0001**
CD366/ CD56/16	6.97±3.21	5.92±2.61	2.84±1.55	0.1338	**<0.0001**	**<0.0001**

As shown in Table 1, a statistically significant difference in CD38+ expression among CD8-positive cells was observed between the post-COVID patient group (4.84±1.27%, p=0.0281) and the control group of middle-aged individuals (4.04±2.10%). A significant difference (4.04±2.10% vs 5.12±2.40%) in the activation marker was also observed in the control group of middle-aged individuals and elderly patients (p=0.0492). No significant statistical difference (p=0.4969) was observed between elderly patients (5.12±2.40%) and the group of post-COVID patients (4.84±1.27%).

Based on the assessment of CD366 expression in cytotoxic CD8 T cells, a statistically significant difference was observed across all study groups. Thus, between the group of elderly patients (5.12±2.67%) and the group of post-COVID patients (6.55±3.28%, P=0.0470); in the studied group of post-COVID patients (6.55±3.28%) compared to the control group (4.05±1.95%), a significant difference was also observed (P=0.0001); between the groups of elderly patients (5.12±2.67%) and the control group of middle-aged individuals (4.05±1.95%, P=0.0423).

As shown in Table 1, regarding the expression of the studied markers, a significant difference in CD38+ expression was observed between elderly patients (5.70±2.30%) and the control group of middle-aged individuals (3.63±1.89%; P=0.0001). There was also a significant difference between the post-COVID group (4.88±2.38%) and the middle-aged control group (3.63±1.89%; P=0.0066). No significant difference (p=0.1247) was observed between the group of elderly patients (5.70±2.30%) and the group of post-COVID patients (4.88±2.38%).

Based on the results of determining the expression of the CD366 exhaustion marker on cytotoxic CD56/16 NK cells, a statistically significant difference was found among study groups. Thus, no significant difference was observed between elderly patients (5.92±2.61%) and the group of post-COVID patients (6.97±3.21%, P=0.1338); in the study group of post-COVID patients (6.97±3.21%) compared to the control group of middle-aged individuals (2.84±1.55%), a significant difference was observed (p<0.0001); there was a statistically significant difference between the groups of elderly patients (5.92±2.61%) and the control group of middle-aged individuals (2.84±1.55%, P<0.0001).

Discussion

During our study, we found probable correlations between the study groups and the control group. However, no significant difference (p=0.4969) in CD38+ expression among CD8-positive cells was observed between elderly patients (5.12±2.40%) and the post-COVID group (4.84±1.27%). Regarding the expression of the exhaustion marker CD366 on cytotoxic CD8 T cells, there was a weak statistical difference between elderly patients (5.12±2.67%) and post-COVID patients (6.55±3.28%; P=0.0470). No statistically significant difference (p=0.1247) was observed between the group of elderly patients (5.70±2.30%) and the group of post-COVID patients (4.88±2.38%) in terms of CD38+ expression on CD16/56-positive cells. Regarding the expression of the exhaustion marker CD366 on cytotoxic CD56/16 NK cells, no significant difference was observed between elderly patients (5.92±2.61%) and the group of post-COVID patients (6.97±3.21%, P=0.1338).

Thus, we found that the exhausted T cell phenotype closely overlaps with that of ageing T cells. With age, there is a progressive loss of the naive T cell pool and an accumulation of highly differentiated cells, often with signs of senescence and/or exhaustion. In most patients who have fully recovered from mild or moderate COVID-19, the expression of CD38 and TIM-3 markers on cytotoxic cells (CD8+ T cells and NK cells) decreases to levels close to normal or to those corresponding to their age (background ageing). However, in patients with severe COVID-19 and in those with long COVID, persistently elevated expression of these markers is often observed [16; 17].

Patients who have survived severe COVID-19 have been found to show signs of accelerated immune ageing. Analysis revealed an increased frequency of exhausted CD8+ T/EMRA lymphocytes, a feature typical of

senescence and correlated with persistent low-grade inflammation and chronic fatigue [17; 18]. This suggests that severe viral infections can exacerbate or accelerate age-related immune system defects.

CD38 expression on CD8+ T cells generally increases with age. CD38 is often used as a marker of T cell activation. It is also associated with a state of exhaustion or metabolic dysfunction (e.g., in the context of chronic viral infection or cancer). The increase in CD38 in old age may contribute to chronic inflammation ("inflammaging") because CD38 consumes NAD+, which can lead to metabolic changes and cell dysfunction. A high level of CD38 expression on CD8+ T cells in older people is associated with decreased proliferative capacity, despite a hyperactivated phenotype, a sign of immune imbalance [7].

TIM-3 expression on CD8+ T cells also increases with age, especially in a subset of cells exhibiting an exhaustion phenotype. TIM-3 is one of the key inhibitory receptors (immune checkpoints), and its upregulation on CD8+ T cells indicates an accumulation of dysfunctional cells that respond poorly to antigen stimulation, exhibit proliferation defects, and exhibit altered cytokine production (e.g., increased IL-10). This is likely a sign of immunosenescence. A high level of TIM-3 expression is a classic sign of T-cell exhaustion, a state of cellular dysfunction in which cells lose their ability to effectively fight pathogens or tumours. Receptors similar to TIM-3 (e.g., PD-1) are often referred to as immunoregulatory because they suppress cell activity. Still, they do so as part of the exhaustion/immune suppression mechanism rather than as direct cellular regulators that maintain homeostasis (such as Treg cells).

Thus, an increased level of CD38 and CD366 expression on cytotoxic CD8 lymphocytes is a biological reflection of immune ageing, which has profound clinical consequences. CD38 is a central regulator of metabolic dysfunction and NAD+ deficiency, which underlie age-related inflammation and metabolic disorders. CD366 is a critical marker of immune tolerance and exhaustion, contributing to the evasion of immune surveillance in cancer and chronic viral infections. This increase is a sign of immune ageing (immunosensitivity) and chronic activation/exhaustion of the immune system, contributing to the development of age-related inflammation (inflammaging) and a decrease in the effectiveness of the immune response [19].

There is evidence that CD38 expression on NK cells also increases with age. CD38 is involved in the activation of NK cells, stimulating cytotoxic response and secretion of granzymes and perforins. TIM-3 is also expressed on NK cells, is considered a marker of their maturation, and is associated with cytokine production. Although there are fewer direct studies on the clear relationship between age and TIM-3 expression on NK cells alone, the general trend of immunosensitivity indicates an accumulation of terminally differentiated or functionally altered NK cells, which may include increased expression of inhibitory receptors such as TIM-3 [19; 20].

CD38 and CD366 are important indicators of the state of cytotoxic lymphocytes during ageing: CD38 increases with ageing on highly differentiated CD8+ T cells and NK cells, reflecting chronic activation and metabolic dysfunction [5]. CD366 (TIM-3) serves as a marker of CD8+ T-cell exhaustion, whose expression increases with age, thereby decreasing their cytotoxic efficacy [9].

Thus, joint analysis of CD38 and CD366 expression provides a better understanding of the phenotypic changes and dysfunction of cytotoxic lymphocytes that underlie immunosenescence.

Conclusions

1. An increased level of CD38 and CD366 expression on cytotoxic lymphocytes is a biological reflection of immune ageing, which has profound clinical implications.

2. CD38 is a central regulator of metabolic dysfunction and NAD+ deficiency, which underlie age-related inflammation and metabolic disorders.

3. CD366 is a critical marker of immune tolerance and exhaustion, contributing to the evasion of immune surveillance in ageing and chronic viral infections.

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