Медицинская иммунология
2021, Т. 23, № 4,
стр. 865-870

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2021, Vol. 23, № 4, pp. 865-870

ОСОБЕННОСТИ КОЛИЧЕСТВЕННОЙ ЭКСПРЕССИИ РЕЦЕПТОРОВ CHECKPOINT-МОЛЕКУЛ PD-1 И TIM-3 НА CD4$^+$ И CD8$^+$Т-КЛЕТКАХ ПРИ РАКЕ МОЛОЧНОЙ ЖЕЛЕЗЫ РАЗНОЙ СТЕПЕНИ ПРОГРЕССИИ

Шевченко Ю.А.[1], Кузнецова М.С.[1], Хантакова Ю.Н.[1], Облеухова И.А.[1], Христин А.А.[2], Сидоров С.В.[3], Сенников С.В.[1]

[1] ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии», г. Новосибирск, Россия
[2] ГБУЗ Новосибирской области «Городская клиническая больница № 1», г. Новосибирск, Россия
[3] ФГАОУ ВО «Новосибирский национальный исследовательский государственный университет», г. Новосибирск, Россия

Резюме. Во время течения хронических вирусных инфекций или роста опухоли из-за постоянного присутствия антигена и воспаления наступает дисфункциональное состояние Т-клеток, называемое истощением (exhaustion). К факторам, связанным с истощением Т-клеток, относят повышение экспрессии различных ингибиторных рецепторов, известных также как checkpoint-молекулы, которое приводит к подавлению пролиферации и продукции таких медиаторов, как IL-2, IFNγ и TNFα.

Рецептор TIM-3 представляет собой иммунорегуляторный рецептор, открытый в 2002 г., который экспрессируется на различных иммунных клетках, включая дендритные клетки, макрофаги и Т-клетки, и опосредует супрессивную активность на иммунных клетках. Устойчивая экспрессия рецептора PD1 на Т-лимфоцитах также ассоциирована с фенотипом истощения, при этом остается неясным, как экспрессия данных ингибиторных рецепторов в норме отличается от таковой в патологических состояниях организма, для которых характерно увеличения числа истощенных Т-клеток.

Целью настоящего исследования стало определение относительного и абсолютного количества Т-клеток, экспрессирующих PD-1 и Tim-3, а также количества молекул PD-1 и Tim-3 на поверхности CD4$^+$ и CD8$^+$Т-клеток у здоровых людей и пациентов с раком молочной железы (РМЖ). Группа пациентов с РМЖ была условно разделена на две группы в зависимости от степени прогрессии заболевания на пациентов с первичным (отсутствие метастаз) и прогрессирующим РМЖ (наличие метастаз).

В результате исследования показано, что у больных раком молочной железы наблюдается увеличение абсолютного количества PD-1-позитивных CD4$^+$Т-клеток. Абсолютное количество молекул на клетку также выше у пациентов с РМЖ по сравнению со здоровыми донорами. Для больных показана тенденция к увеличению абсолютного количества TIM3-позитивных CD4$^+$Т-клеток по сравнению со здоровыми донорами и в ряду от первичного заболевания к прогрессирующему РМЖ.

Адрес для переписки:

Сенников Сергей Витальевич
ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии»
630099, Россия, г. Новосибирск, ул. Ядринцевская, 14.
Тел.: 8 (383) 222-19-10.
E-mail: sennikovsv@gmail.com

Address for correspondence:

Sennikov Sergey V.
Research Institute of Fundamental and Clinical Immunology
630099, Russian Federation, Novosibirsk,
Yadrintsevskaya str., 14.
Phone: 7 (383) 222-19-10.
E-mail: sennikovsv@gmail.com

Образец цитирования:

Ю.А. Шевченко, М.С. Кузнецова, Ю.Н. Хантакова, И.А. Облеухова, А.А. Христин, С.В. Сидоров, С.В. Сенников «Особенности количественной экспрессии рецепторов checkpoint-молекул PD-1 и TIM-3 на CD4$^+$ и CD8$^+$Т-клетках при раке молочной железы разной степени прогрессии» // Медицинская иммунология, 2021. Т. 23, № 4. С. 865-870.
doi: 10.15789/1563-0625-QEF-2239

For citation:

Yu.A. Shevchenko, M.S. Kuznetsova, Yu.N. Khantakova, I.A. Obleukhova, A.A. Khristin, S.V. Sidorov, S.V. Sennikov "Quantitative expression features of PD-1 and TIM-3 checkpoint molecule receptors on CD4$^+$ and CD8$^+$T-cells in breast cancer of varying progression degrees", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2021, Vol. 23, no. 4, pp. 865-870.
doi: 10.15789/1563-0625-QEF-2239

DOI: 10.15789/1563-0625-QEF-2239

Таким образом, различия в характере экспрессии checkpoint-молекул TIM-3 и PD1 наблюдаются при сравнении нормы и злокачественной патологии молочной железы, и могут стать важным маркером функционального состояния Т-лимфоцитов у больных РМЖ.

Ключевые слова: PD-1, TIM-3, рак молочной железы, рецепторы checkpoint-молекул, истощенные Т-лимфоциты, количественная экспрессия рецепторов

QUANTITATIVE EXPRESSION FEATURES OF PD-1 AND TIM-3 CHECKPOINT MOLECULE RECEPTORS ON $CD4^+$ AND $CD8^+$ T-CELLS IN BREAST CANCER OF VARYING PROGRESSION DEGREES

Shevchenko Yu.A.[a], Kuznetsova M.S.[a], Khantakova Yu.N.[a], Obleukhova I.A.[a], Khristin A.A.[b], Sidorov S.V.[c], Sennikov S.V.[a]

[a] *Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*
[b] *City Clinical Hospital No. 1, Novosibirsk, Russian Federation*
[c] *Novosibirsk National Research State University, Novosibirsk, Russian Federation*

Abstract. During the course of chronic viral infections or tumor growth, due to the constant presence of antigen and inflammation, a dysfunctional state of T-cells called exhaustion occurs. Factors associated with T-cell exhaustion include an increase in the expression of various inhibitory receptors, also known as checkpoint molecules, which leads to inhibition of the proliferation and production of mediators such as IL-2, IFNγ, and TNFα.

The TIM-3 molecule is expressed on a variety of immune cells, including dendritic cells, macrophages, and T-cells, and mediates suppressive activity on immune cells. Sustained expression of the PD-1 receptor on T-lymphocytes is also associated with the exhaustion phenotype, while it remains unclear how the expression of these inhibitory receptors normally differs from that in pathological conditions of the body, which are characterized by an increase in the number of exhausted T-cells.

The aim of the study was to determine the relative and absolute number of T-cells expressing PD-1 and TIM-3, as well as the number of PD-1 and TIM-3 molecules on the surface of $CD4^+$ and $CD8^+$T-cells in healthy donors and breast cancer (BC) patients. Group of BC patients were conditionally divided into two groups depending on the degree of disease progression into patients with primary (without metastases) and metastatic BC.

As a result of the study, it was shown that an increase in the absolute number of PD-1^+CD4^+T-cells is observed in breast cancer patients. The absolute number of molecules per cell is also higher in BC patients compared to healthy donors. For patients, a tendency towards an increase in the absolute number of TIM-3^+CD4^+T-cells was shown in comparison with healthy donors and in a row from primary disease to metastatic BC.

Thus, differences in the expression pattern of TIM-3 and PD-1 checkpoint molecules are observed when comparing the norm and malignant pathology of the breast, and can become an important marker of the functional state of T-lymphocytes in BC patients.

Keywords: PD-1, TIM-3, breast cancer, checkpoint molecule receptors, exhausted T-lymphocytes, quantitative expression of receptors

Introduction

Currently, an important role in the development of immune-mediated diseases, including malignant neoplasms, is assigned to suppressor populations of immunocompetent cells (Shou et al., 2016). There are many mechanisms for malignant tumor generated and maintained immunosuppressive state. Thus, a malignant neoplasm attracts as well as promotes activation and differentiation of immunocompetent cells with a regulatory – immunosuppressive phenotype in its microenvironment. In addition, tumor cells express a variety of membrane-bound and soluble molecules that alter the functional state of infiltrating

immune cells to act as tumor-supporting cells (Ager et al., 2021).

The TIM-3 receptor is an immunoregulatory receptor, discovered in 2002, that is expressed on a variety of immune cells, including dendritic cells, macrophages, and T-cells. TIM-3 mediates suppressive activity on immune cells through its ligands, including phosphatidylserine, CEACAM-1, and galectin-9 ligand (Li et al., 2021). The signaling triggered by it on cytotoxic T-cells leads to the development of an exhaustion phenotype. The programmed cell death protein 1 (PD-1) is also expressed on the surface of activated T-cells and regulates the immune response at the effector stage. PD-1 plays an important role in the balance of protective immunity and immunopathology, homeostasis and tolerance; however, in case of tumor growth, immunosuppression becomes the main PD-1 activity. The effect that PD-1 and TIM-3 molecules have on cells depend on many parameters, such as the level of soluble mediators, the percentage of cells carrying receptors, the ratio between the percentage and density subpopulations, and intracellular proteins of the target cell. Currently, close attention of both fundamental science and clinical immunology have been paid to such an important factor for the regulation of the biological properties of immunoregulatory proteins such as the expression density of surface receptors (Sennikov et al., 2019). For an informative assessment of the expression level, it is necessary to accurately count the number of binding sites on the cell surface.

Thus, it can be assumed that impaired cell functional activity resulting from increased expression of PD-1 and TIM-3 may be associated not only with increased number of positive cells, but also with the number of cognate receptors on the cell surface, so that if a certain level of receptors is exceeded, then cell loses the ability to execute effector functions.

The aim of the study was to determine the relative and absolute number of T-cells expressing PD-1 and TIM-3, as well as the number of PD-1 and TIM-3 molecules on the surface of normal and breast cancer $CD4^+$ and $CD8^+$T-cells. Assessing the number of PD-1 and TIM-3 molecules will help to determine the threshold level of exhaustion for cell functional properties and impairment of cytotoxic functions in patients with breast cancer (BC).

Materials and methods

Donors and patients

Peripheral venous blood cells were collected from 25 patients with primary breast cancer, 15 patients with metastatic breast cancer (presence of metastases), as well as healthy women without clinically and instrumentally diagnosed breast pathology (25 people). The study was conducted with the informed consent of all healthy donors and patients. The study was approved by the local ethics committee of RIFCI.

Venous blood from patients with primary breast cancer (T 1-3 N 0-2 M 0) was collected before surgery (radical mastectomy or radical breast resection) or from patients with metastases before the course of chemotherapy. Blood sampling from donors and patients was carried out in vacuum tubes added with anticoagulant EDTA (Improvacuter, China). Peripheral blood mononuclear cells (PBMCs) were isolated under sterile conditions using a Ficoll-Urographin gradient ($\rho = 1.077$ g/l) using a standard method.

Cell phenotyping and counting TIM-3 and PD-1 molecules

The phenotypic characteristics of T-lymphocytes were assessed by flow cytometry on a FACSVerse cytometer (BD, USA) using monoclonal antibodies (Biolegend, USA): CD3-PerCP-Cy5. (clone OKT3, Cat # 317336), CD4-PE-Cy7 (clone RPA-T4, Cat # 300512), CD8-FITC (clone RPA-T8, Cat # 301006), TIM-3-PE (clone F38-2E2, Cat # 345006), PD-1-PE (clone NAT105, Cat # 367404). A BD QuantiBRITE PE kit (BD Biosciences, USA) was used to create a calibration curve and convert the fluorescence intensity values of cells expressing the corresponding marker into absolute values of the receptor number, containing 4 fractions of lyophilized beads, each of which carries a different level of phycoerythrin.

The study of the expressed TIM-3 and PD-1 was carried out with the same parameters of the voltage of the photomultiplier tube according to the PE detector as in the analysis of the calibration beads, which allowed to convert the values of the fluorescence intensity into the number of PE molecules per cell. Further, the number of PE molecules per cell was converted to the number of antibody molecules per cell using the known ratio of PE molecules per antibody, equal to 1:1. Cytometer settings were checked weekly using Cytometer Setup and Tracking (CS&T) beads (BD Biosciences, USA).

Statistical methods

Results are presented as median and interquartile range. For statistical data processing, ANOVA and Tukey's test were used in GraphPadPrism 6 software. Differences were considered significant at $p < 0.05$. Correlations between the studied parameters were established using the Spearman correlation coefficient (at $p \leq 0.05$).

Results and discussion

Determining amount of PD-1- and TIM-3- positive cells in peripheral blood

To determine the number of PD-1- and $TIM\text{-}3^+$ cells, populations of $CD4^+$ and $CD8^+$T-lymphocytes were isolated from the $CD3^+$T-lymphocyte popu-

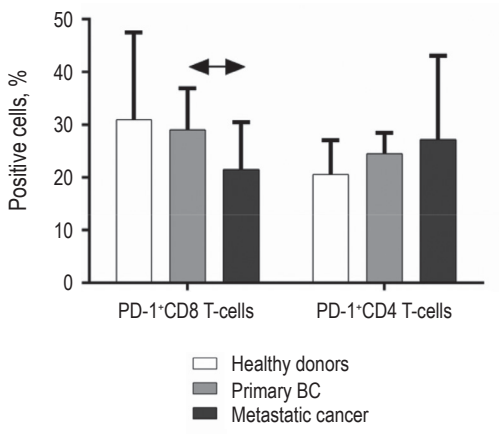

Figure 1. Relative number of PD-1$^+$-cells in healthy donors (n = 25), patients with primary breast cancer (n = 25), and patients with metastatic breast cancer (n = 15)

Note. Data are presented as median and range of quartiles. Arrows indicate statistically significant differences, $p < 0.05$.

lation, in which the relative and absolute number of target cells were determined. For the index of PD-1$^+$ cells, significant differences were obtained between the groups of CD8$^+$T-lymphocytes from patients with primary and metastatic breast cancer (Figure 1). For TIM-3, no significant differences were found.

Assessing absolute number of cells expressing the markers examined showed significant differences between the groups of patients with primary and metastatic breast cancer, respectively, in the number of PD-1$^+$CD8$^+$ T-cells, as well as differences between the cells of healthy donors and patients in the number of PD-1$^+$CD4$^+$ T-cells (Figure 2A). For TIM-3$^+$ cells, significant differences in the absolute number of cells were found only for CD4$^+$-cells: between the groups of apparently healthy donors and patients with metastatic breast cancer, as well as between groups of patients with primary and metastatic breast cancer (Figure 2B).

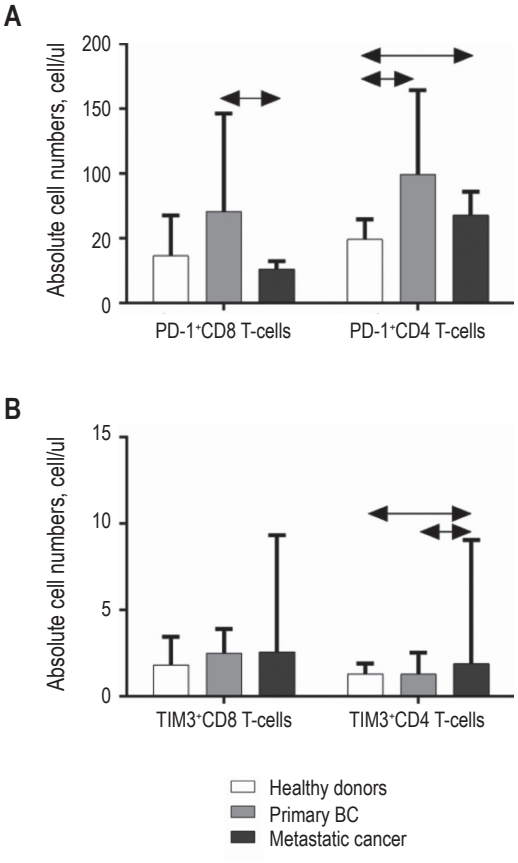

Figure 2. Absolute number of (A) PD-1- and (B) TIM-3$^+$-cells in healthy donors (n = 25) and patients with primary (n = 25) and metastatic breast cancer (n = 15)

Note. As for Figure 1.

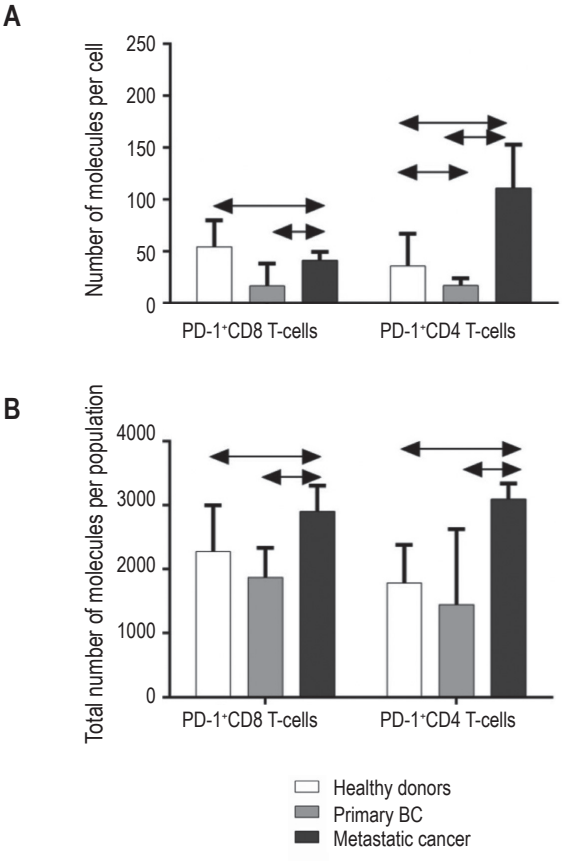

Figure 3. (A) Total number of PD-1 molecules on the surface of CD4 and CD8 T-cells and (B) the number of PD-1 molecules per 1 cell on the surface of CD4 and CD8 T-cells in healthy donors (n = 25), patients with primary breast cancer (n = 25) and patients with metastatic breast cancer (n = 15)

Note. As for Figure 1.

Determining the number of PD-1 and TIM-3 molecules in peripheral blood T-cells

While studying the total number of molecules on T-lymphocytes, it was shown that T-lymphocytes from both healthy donors and breast cancer patients carry approximately the same number of TIM-3 molecules, i.e. showing no significant differences. The total number of PD-1 molecules on $CD4^+$ and $CD8^+$ T-cells was increased in patients with metastatic breast cancer compared with the other two groups (Figure 3A).

In addition to the total number of molecules expressed on the surface of total $CD4^+$ and $CD8^+$ T-cells, we also calculated the parameter corresponding to the number of PD-1- and TIM-3 molecules per one cell for each population. With regard to the PD-1 molecule, it was found that the number of molecules in terms of the number of $CD8^+$ T-cells in patients with metastatic breast cancer is significantly lower than in healthy donors. At the same time, the opposite trend is observed for $CD4^+$-cells from these two groups – the number of PD-1 per cell for $CD4^+$ T-cells in patients with advanced cancer was significantly higher than that for CD4-cells from healthy donors (Figure 3B). The number of PD-1 molecules per cell in the group of patients with primary breast cancer was significantly lower both in $CD4^+$ and $CD8^+$ T-cells than that in the group of patients with metastases.

To clarify the mutual influence of the studied subpopulations, we carried out a correlation analysis for each of the parameters. To calculate the correlations, the Spearman coefficient and the p-level of significance were used. The presence of a relationship between the values was considered significant at $p < 0.05$, which corresponded to Spearman's coefficient from 0.4 to 1 (moderate and high tightness of the relationship).

In healthy donors, the maximum number of correlations is determined between PD-1- and TIM-3-positive $CD4^+$ T-cells (for parameters, the absolute and relative number of cells, total number of molecules on the cells) and PD-1- and TIM-3-positive $CD8^+$ T-cells (for the parameters, the absolute number of cells, the total number of molecules on the cells, the number of molecules per cell), there are also correlations between PD-1- and TIM-3 in different subpopulations (correlations between the relative numbers of $PD\text{-}1^+CD8^+$ and $TIM\text{-}3^+CD4^+$, absolute number of $PD\text{-}1^+CD4^+$ and $TIM\text{-}3^+CD8^+$-cells). In breast cancer patients, fewer correlations were observed particularly being found between the absolute number of $PD\text{-}1^+CD8^+$ and $TIM\text{-}3^+CD8^+$-cells, $PD\text{-}1^+CD4^+$ and $TIM\text{-}3^+CD8^+$-cells, between the total number of molecules per cell for $PD\text{-}1^+CD8^+$ and $TIM\text{-}3^+CD8^+$-cells.

Thus, changes in the absolute number of PD-1 and TIM-positive cells and the number of molecules are shown in breast cancer patients. In particular, in breast cancer patients compared with healthy people, the absolute number of $CD4^+$-cells expressing PD-1 is higher, and the absolute number of PD-1 molecules per cell also increases, while the most pronounced differences between health and pathology are found while comparing healthy donors with a group of patients with metastatic breast cancer. Also, patients tended to increase absolute number of $TIM\text{-}3^+CD4^+$ T-cells compared to healthy donors. At the same time, a correlation was shown in the expression pattern of the studied markers between PD-1- and TIM-3-positive $CD4^+$ T-cells, as well as between PD-1- and TIM-3-positive $CD8^+$ T-cells, which also indicates an important prognostic role for evaluating the expression and co-expression of such checkpoint molecules and the need for further studies regarding an influence of T-cell phenotype on related function in the course of breast cancer.

The development of methods for analyzing the features of the expressed checkpoint molecules on peripheral blood T-cells can serve as the basis for the development of minimally invasive methods that might allow with a high probability to assess the prognosis of oncological disease without using histological archival material. It is assumed that it is the expression of suppressor co-stimulating molecules on antigen-presenting cells that fundamentally differs in healthy donors and patients with breast cancer and could accouint for isolating factors to maximize the overexpression of suppressor molecules: soluble mediators, tumor-associated antigens, or contact interaction of immunocompetent and tumor cells.

References

1. Ager C.R., Obradovic A.Z., Arriaga J.M., Chaimowitz M.G., Califano A., Abate-Shen C., Drake C.G. Longitudinal immune profiling reveals unique myeloid and T-cell phenotypes associated with spontaneous immunoediting in a prostate tumor model. *Cancer Immunol. Res., 202, canimm.0637.2020.* doi: 10.1158/2326-6066.CIR-20-0637.

2. Li F., Chen Y., Pang M., Yang P., Jing H.. Immune checkpoint inhibitors and cellular treatment for lymphoma immunotherapy. *Clin. Exp. Immunol., 2021.* doi: 10.1111/cei.13592.

3. Sennikov S.V., Alshevskaya A.A., Zhukova J., Belomestnova I., Karaulov A.V., Lopatnikova J.A. Expression density of receptors as a potent regulator of cell function and property in health and pathology. *Int. Arch. Allergy Immunol., 2019, Vol. 178, no. 2, pp. 182-191.*

4. Shou J., Zhang Z., Lai Y., Chen Z., Huang J. Worse outcome in breast cancer with higher tumor-infiltrating FOXP3$^+$ Tregs: a systematic review and meta-analysis. *BMC Cancer, 2016, Vol. 16, no. 1, 687.* doi: 10.1186/s12885-016-2732-0.

Авторы:

Шевченко Ю.А. *— к.б.н., старший научный сотрудник лаборатории молекулярной иммунологии ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии», г. Новосибирск, Россия*

Кузнецова М.С. *— к.б.н., научный сотрудник лаборатории молекулярной иммунологии ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии», г. Новосибирск, Россия*

Хантакова Ю.Н. *— к.м.н., научный сотрудник лаборатории молекулярной иммунологии ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии», г. Новосибирск, Россия*

Облеухова И.А. *— к.б.н., старший научный сотрудник лаборатории молекулярной иммунологии ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии», г. Новосибирск, Россия*

Христин А.А. *— врач 3-го онкологического отделения ГБУЗ Новосибирской области «Городская клиническая больница № 1», г. Новосибирск, Россия*

Сидоров С.В. *— д.м.н., заведующий кафедрой хирургии ФГАОУ ВО «Новосибирский национальный исследовательский государственный университет», г. Новосибирск, Россия*

Сенников С.В. *— д.м.н., заведующий лабораторией молекулярной иммунологии ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии», г. Новосибирск, Россия*

Authors:

Shevchenko Yu.A., *PhD (Biology), Senior Research Associate, Laboratory of Molecular Immunology, Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*

Kuznetsova M.S., *PhD (Biology), Research Associate, Laboratory of Molecular Immunology, Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*

Khantakova Yu.N., *PhD (Medicine), Research Associate, Laboratory of Molecular Immunology, Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*

Obleukhova I.A., *PhD (Biology), Senior Research Associate, Laboratory of Molecular Immunology, Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*

Khristin A.A., *Physician, Oncology Department No. 3, City Clinical Hospital No. 1, Novosibirsk, Russian Federation*

Sidorov S.V., *PhD, MD (Medicine), Head, Surgery Department, Novosibirsk National Research State University, Novosibirsk, Russian Federation*

Sennikov S.V., *PhD, MD (Medicine), Head, Laboratory of Molecular Immunology, Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*

Поступила 10.03.2021
Принята к печати 03.06.2021

Received 10.03.2021
Accepted 03.06.2021