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ПЕНТОКСИФИЛЛИН СПОСОБСТВУЕТ ПОВЫШЕНИЮ *IN VITRO* ПРОТИВООПУХОЛЕВОЙ ЦИТОТОКСИЧНОСТИ Т-КЛЕТОК БОЛЬНЫХ РАКОМ МОЛОЧНОЙ ЖЕЛЕЗЫ

Кузнецова М.С.[1], Шевченко Ю.А.[1], Хантакова Ю.Н.[1], Христин А.А.[2], Облеухова И.А.[1], Силков А.Н.[1], 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. Транскрипционный фактор NF-κB контролирует экспрессию генов, ответственных за реализацию клеточного цикла, апоптоза и ряда других иммунорегуляторных функций. После обнаружения возможности блокирования опухолевого роста через подавление активности NF-κB был обнаружен ряд неспецифических ингибиторов NF-κB, применение которых, однако, было осложнено множественными побочными эффектами, такими как системное воспаление, вызванное чрезмерной экспрессией интерлейкина-1β, или не связанными с иммунитетом осложнениями, которые могут быть следствием ингибирования субъединицы p65 NF-κB, играющей центральную роль в органогенезе и воспалении. Ингибирование же субъединицы c-Rel приводит к ограничению роста опухоли за счет модуляции свойств Т-регуляторных клеток.

В 2017 году Grinberg-Bleyer и соавт. проверили гипотезу о том, что субъединицу c-Rel можно избирательно ингибировать пентоксифиллином, регулируя тем самым активность Treg в ходе опухолевого роста. Авторы показали, что пентоксфиллин действительно может вызывать избирательную деградацию c-Rel, не влияя на p65, и предположили, что подобное воздействие может быть эффективным для подавления опухолевого роста. В связи этим нами была поставлена цель исследовать *in vitro* влияние пентоксифиллина на функции и противоопухолевый цитотоксический потенциал Т-клеток пациентов со злокачественными опухолями.

В качестве объекта исследования использовались мононуклеарные клетки периферической венозной крови 25 пациенток с первичным РМЖ (отсутствие метастаз), 15 пациенток с прогрессирующим РМЖ (наличие метастаз), а также 25 условно здоровых женщин без диагностированной патологии молочных желез. Исследование проводилось с добровольного информированного согласия доноров и пациентов. Исследование одобрено локальным этическим комитетом НИИФКИ.

В результате исследования было показано, что использование пентоксифиллина для ингибирования транскрипционного фактора NF-κB *in vitro* усиливает проапоптотический и цитотоксический

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

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противоопухолевый ответ, приводя к повышению экспрессии TRAIL на Т-лимфоцитах, в основном у здоровых доноров и пациентов с метастатическим раком молочной железы, как на интактных Т-клетках, так и в ответ на клетки опухолевой линии карциномы молочной железы человека ZR-75-1. У здоровых доноров в присутствии пентоксифиллина появляется популяция высокоэкспрессирующих TRAIL CD4 и CD8 Т-клеток.

Ключевые слова: пентоксифиллин, ингибиторы NF-κB, цитотоксичность, рак молочной железы, регуляция иммунного ответа, Т-лимфоциты

PENTOXIFYLLINE ENHANCES *IN VITRO* T-CELL ANTITUMOR RESPONSE IN BREAST CANCER PATIENTS

Kuznetsova M.S.[a], Shevchenko Yu.A.[a], Khantakova Yu.N.[a], Khristin A.A.[b], Obleukhova I.A.[a], Silkov A.N.[a], 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. The NF-κB transcription factor controls the expression of genes responsible for cell cycle, apoptosis, and other immunoregulatory functions. Some nonspecific NF-κB inhibitors were found after discovering the possibility of blocking tumor growth through suppression of NF-κB activity, but their use was complicated by multiple side effects, such as interleukin-1β-related systemic inflammation or non-immune-related complications, which may be due to inhibition of the p65 NF-κB subunit that plays a central role in organogenesis and inflammation. Inhibition of the c-Rel subunit leads to tumor growth restriction by modulating the T-regulatory cell activity.

In 2017, Grinberg-Bleyer and co-authors checked the hypothesis that selective inhibition of the c-Rel subunit can be performed using pentoxifylline and will effectively regulate Treg activity during tumor growth. The authors showed that pentoxphylline, an FDA-approved drug, could indeed induce selective degradation of c-Rel without affecting p65, and suggested that such an effect could be effective in suppressing tumor growth. In this regard, we aimed to investigate *in vitro* how pentoxifylline affect the functional activity and antitumor cytotoxic potential of T-cells in cancer patients.

The objects of the study were peripheral blood mononuclear cells from 25 patients with primary breast cancer (no metastases), 15 patients with metastatic breast cancer, and 25 healthy women without breast pathology. Informed consent was obtained from all donors and patients. The study was approved by the local ethics committee.

Here we showed that pentoxifylline treatment *in vitro* enhances the pro-apoptotic and cytotoxic antitumor response via increasing the expression of TRAIL on T-lymphocytes, mainly in healthy donors and patients with metastatic breast cancer, both on intact T-cells and in response to the cells of the tumor line of human breast carcinoma ZR-75-1. In healthy donors, in the presence of pentoxifylline, a population of highly expressing TRAIL CD4 and CD8 T-cells appears.

Keywords: pentoxifylline, NF-κB inhibitors, antitumor cytotoxicity, breast cancer, regulation of the immune response, T-lymphocytes

Introduction

The principle of the complex interplay between tumor and immune system is currently described by the theory of immunoediting, formulated at the beginning of the 21st century, according to which the immune system is directly related to the processes of malignant growth, performing not only antitumor surveillance, but also participating in the processes of maintaining tumor growth. During development, a malignant tumor enables many different strategies to maintain its own growth and metastasis, prevent recognition processes and elimination by the immune system. One of the main tumor mechanisms in immune evasion is based on the interaction of immunosuppressive receptors on effector T-cells with cognate ligands expressed in the tumor microenvironment. New approaches to cancer immunotherapy are aimed at overcoming these tolerance mechanisms and promoting a productive antitumor immune response.

The blocking of immune control molecules (checkpoint molecules) using monoclonal antibodies in clinical practice and experimental studies has been shown to be effective in inhibiting tumor growth, which was rightfully confirmed by awarding the 2018 Nobel Prize to researchers who proposed inhibition of CTLA-4 and PD-1 molecules as an effective method for cancer immunotherapy. However, despite a significant breakthrough in this field, it remains obvious that a thorough study of other mechanisms of cancer immunoediting, which promote tumor evasion from the immune response, and methods of modulating these mechanisms are required. Despite the fact that the emergence of cancer immunotherapy methods based on blocking immune control molecules (immunoregulatory receptors or checkpoint molecules) has become a real breakthrough in the treatment of malignant tumors, there is a significant number of tumors whose therapy does not lend itself to such methods (Sharma and Allison, 2015). One possible explanation for this resistance is that such tumors involve other immunosuppressive mechanisms, such as the accumulation of $CD4^+FoxP3^+$T-regulatory cells (Treg) suppressing antitumor responses (Nishikawa and Sakaguchi, 2010; Tang and Bluestone, 2008).

The NF-κB protein, discovered more than 30 years ago, is a transcription factor that controls the expression of genes responsible for executing cell cycle processes, apoptosis, and a number of other immunoregulatory functions. After discovering an opportunity for blocking tumor growth through suppression of NF-κB activity, it was actively searched for nonspecific NF-κB inhibitors, but their use was complicated by multiple side effects, such as systemic inflammation due to overexpressed interleukin-1β or non-immune-related complications, which may be due to inhibition of the p65 subunit of NF-κB, which plays a central role in organogenesis and inflammation. Inhibition of the c-Rel subunit leads to restriction of tumor growth by modulating the properties of T-regulatory cells.

A number of studies have shown that the transcription factor NF-κB particularly the c-Rel subunit, is critical for the expression of FoxP3 and the development of T-regulatory cells in the thymus (Isomura et al., 2009). The literature detailed the role of the transcription factor NF-κB in the initiation, proliferation, and spread of tumors. This led several groups to test whether global inhibition of NF-κB affects tumor growth. As a result, the use of nonspecific NF-κB inhibitors has been complicated by multiple side effects such as systemic inflammation resulting from overexpressed interleukin (IL)-1β or non-immune complications, which may result from inhibition of the p65 NF-κB subunit playing a central role in organogenesis and inflammation. Inhibition of the c-Rel subunit leads to restriction of tumor growth by modulating the properties of T-regulatory cells.

In a 2017 study, a group of researchers headed by Grinberg-Bleyer tested the hypothesis that inhibition of c-Rel may be a viable method of selectively modulated Treg activity in cancer and that pentoxphylline, an FDA-approved drug, can induce selective degradation of c-Rel without affecting p65 (Grinberg-Bleyer et al., 2017). In this regard, we set out to select conditions for inhibiting the transcription factor NF-κB by pentoxifylline and functional assessment of the cytotoxic potential of human T-cells in normal conditions and in a malignant tumor process.

Materials and methods

Object of study

Mononuclear cells (PBMCs) from peripheral venous blood of 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) were examined. The study was conducted with the voluntary informed consent from 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) and 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).

PBMCs were isolated under sterile conditions by a standard method in a Ficoll-Urographin gradient (ρ = 1.077 g/l). PBMCs of peripheral blood and tumor cells of the breast carcinoma line ZR-75-1 were cultured at 10:1 ratio. Pentoxifylline treatment was carried out by adding it to cell cultures (500 μg/ml) for 15 minutes at 37 °C. For the final culture, a number of functional tests were performed to assess the cytotoxic activity.

Assessment of cytotoxic activity

To assess the PBMC antitumor cytotoxic activity, we used the non-radioactive cytotoxic test CytoTox96 (Promega, USA), based on measuring amount of lactate dehydrogenase (LDH). Human mammary ductal carcinoma cell line ZR-75-1 (Bank of cell cultures, Institute of Center of the Russian Academy of Sciences, Russia) were used as target cells. The procedure was carried out according to the manufacturer's protocol. The level of cytotoxicity was estimated as the ratio of the optical density in a sample with a mixture of effectors and targets to the optical density in a sample with completely lysed targets,

expressed as a percentage. The corrections for the presence of LDH in the medium and the spontaneous release of LDH from effectors and target cells were taken into account.

In addition to the non-radioactive cytotoxic test CytoTox96, the level of tumor cell apoptosis was assessed using Annexin V and the level of TRAIL expression on $CD4^+$ and CD8 T-cells was analyzed. T-cells were stained with antibodies CD3-APC-Cy7 (clone HIT3a, Cat # 300318), CD4-PE-Cy7 (clone RPA-T4, Cat # 300512), CD8-APC (clone RPA-T8, Cat # 301049), TRAIL-PE (clone RIK-2, Cat # 308206) (Biolegend, USA). The analysis was performed on a BD FACS Verse flow cytometer.

Statistical methods

Statistical data processing was carried out using GraphPadPrism 6. The results are presented as median and interquartile range; for statistical processing of the data, ANOVA with post-hoc Tukey test was used.

Results and discussion

To assess the effect of pentoxifylline on the functional activity of T-lymphocytes, we investigated spontaneous and target cell-induced protein expression of the tumor necrosis factor family members TRAIL on CD4 and CD8 T-cells treated/untreated with pentoxifylline. The analysis identified such parameters as percentage of $TRAIL^+$CD4 and CD8-cells, the percentage of CD4 and CD8 T-cells with high TRAIL expression ($TRAIL^{high}$ cells) and the mean fluorescence intensity (MFI, mean fluorescence intensity) for medium and highly expressing TRAIL-cells.

The study of the spontaneous TRAIL expression by T-cells showed that pentoxifylline affects the level of $TRAIL^+$CD4 and CD8 T-cells in the group of healthy donors and patients with metastatic breast cancer (Figure 1A). Moreover, in the group of healthy donors, pentoxifylline led to decreased TRAIL expression, while in the group of patients with metastatic breast cancer, on the contrary, it was increased after treatment. At the same time, analysis of TRAIL MFI on positive cells showed a significant increase in the fluorescence intensity of $TRAIL^+$CD8 T-cells in the group of patients with primary breast cancer after treatment with pentoxifylline (Figure 1B).

After culture of T-lymphocytes with ZR-75-1 target cells, a significant increase in TRAIL expression on CD4 and CD8 T-cells was observed in all study groups. In this case, the appearance of cells with medium and high intensity of TRAIL expression was found. In patients with primary breast cancer, no significant effect of pentoxifylline on TRAIL expression on CD4 and CD8 T-cells was shown in the presence of ZR-75-1 cells. The percentage of $TRAIL^+$CD4 and CD8 T-cells in healthy donors increased significantly after treatment with pentoxifylline in the presence of ZR-75-1 cells (Figure 2A).

The fluorescence intensity of $TRAIL^+$CD4 and CD8 T-cells, which is an indirect indicator of molecule quantity, decreased after exposure to pentoxifylline in the presence of ZR-75-1 cells in patients with metastatic breast cancer, remaining unchanged for other groups (Figure 2B). It was also shown that percentage of highly expressing TRAIL CD4 T-cells treated with pentoxifylline in the presence of ZR-75-1 cells was increased in patients with metastatic breast cancer, whereas level of highly expressing TRAIL CD8 T-cells in same patients slightly decreased (Figure 2C). The fluorescence intensity of highly expressing TRAIL CD4 T-cells under the action of pentoxifylline in the presence of ZR-75-1 cells was increased only in healthy donors and patients with metastatic breast cancer (Figure 2D).

A study of the tumor cell apoptosis level after culture with the effector T-cells (using fluorescently labeled annexin V) showed that T-cells from healthy donors and patients with primary breast cancer significantly stronger stimulate apoptosis of tumor cells in coculture compared with patients with metastatic breast cancer. After adding pentoxifylline, the level of apoptosis significantly increased in healthy donors and patients with metastatic breast cancer, while in patients with breast cancer the level of apoptosis reached that of healthy donors, which leads to no significant differences between the groups (Figure 3A).

When studying the cytotoxic activity of mononuclear cells against ZR-75-1 tumor cells, it was shown that addition of pentoxifylline leads to increased release of LDH from tumor cells in all studied groups (Figure 3B).

Thus, it was shown that the use of pentoxifylline as an inhibitor of the transcription factor NF-κB to suppress the functions of Treg by upregulating expression of TRAIL mainly in healthy donors and patients with metastatic breast cancer both on intact T-cells and in response to ZR-75 tumor cells. In healthy donors, in the presence of pentoxifylline, a population of highly expressing TRAIL CD4 and CD8 T-cells emerged. The cytotoxic activity and the level of apoptosis induced by T-cells differ in breast cancer patients and healthy donors, but while using an inhibitor of the transcription factor NF-κB, the antitumor activity in breast cancer patients is stimulated as high as in healthy donors. The data obtained on the stimulation of the cytotoxic response against tumor cells upon hindering activation of the transcription factor NF-κB would allow to combine the mechanisms of blockade and activation in the most effective way while creating models for inducing antitumor response.

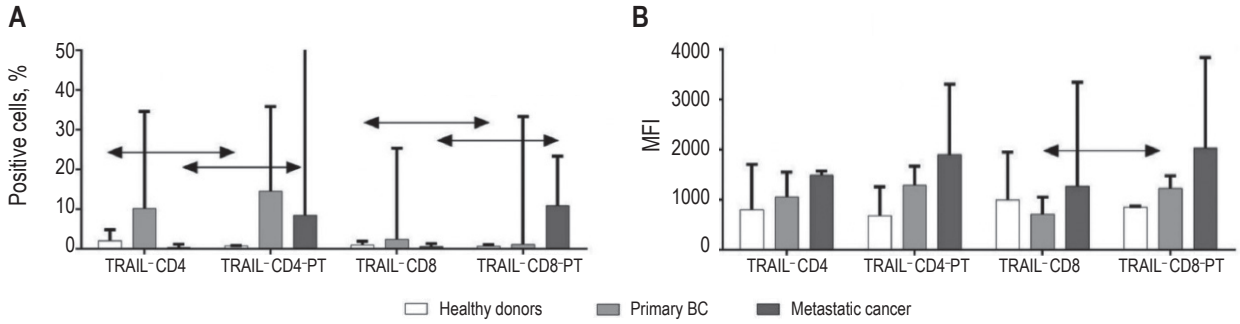

Figure 1. Influence of pentoxifylline on (A) spontaneous expression of TRAIL and on (B) the intensity of TRAIL fluorescence on CD4 and CD8 T-cells of healthy donors (n = 25), patients with primary breast cancer (n = 25) and metastatic breast cancer (n = 15)

Note. Data are presented as median and interquartile range. Arrows indicate statistically significant differences at $p < 0.05$.

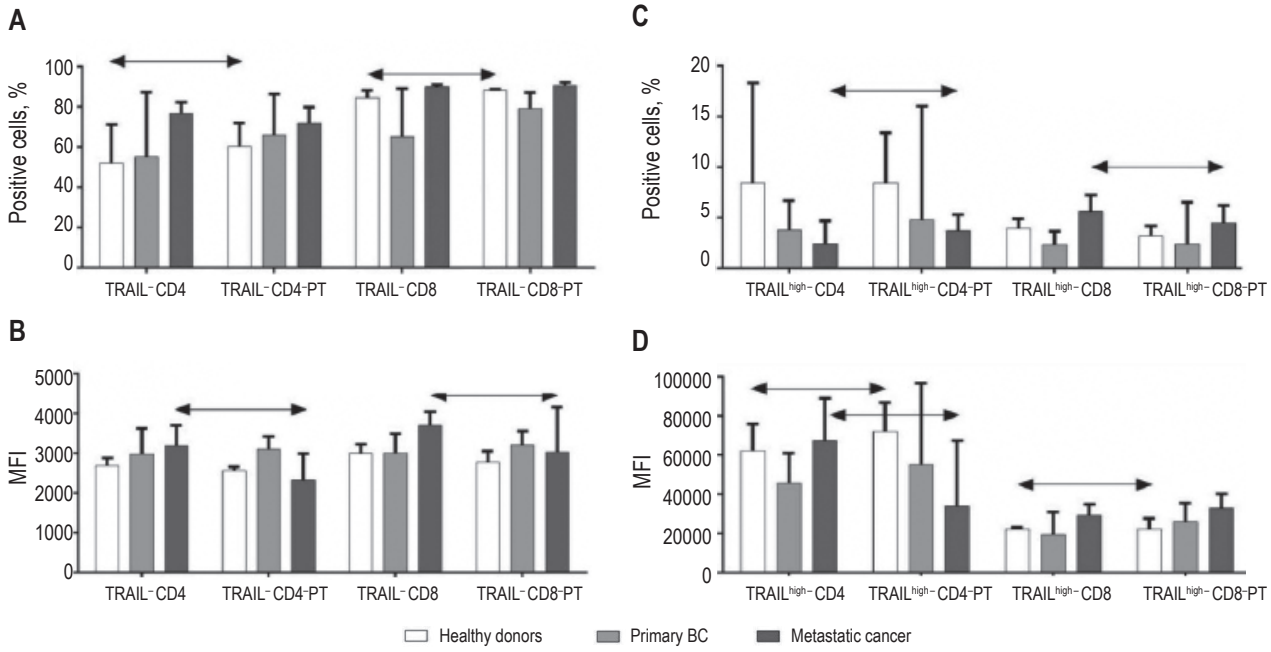

Figure 2. Influence of pentoxifylline on the surface expression of TRAIL CD4 and CD8 by T-lymphocytes of healthy donors (n = 25), patients with primary breast cancer (n = 25) and metastatic breast cancer (n = 15) when cultured with cells of the tumor line ZR-75-1

Note. As for Figure 1. A, the content of TRAIL⁺CD4 and CD8 T-cells. B, fluorescence intensity of TRAIL⁺CD4 and CD8 T-cells. C, content of TRAILhigh CD4 and CD8 T-cells. D, intensity of fluorescence of TRAILhigh CD4 and CD8 T-cells.

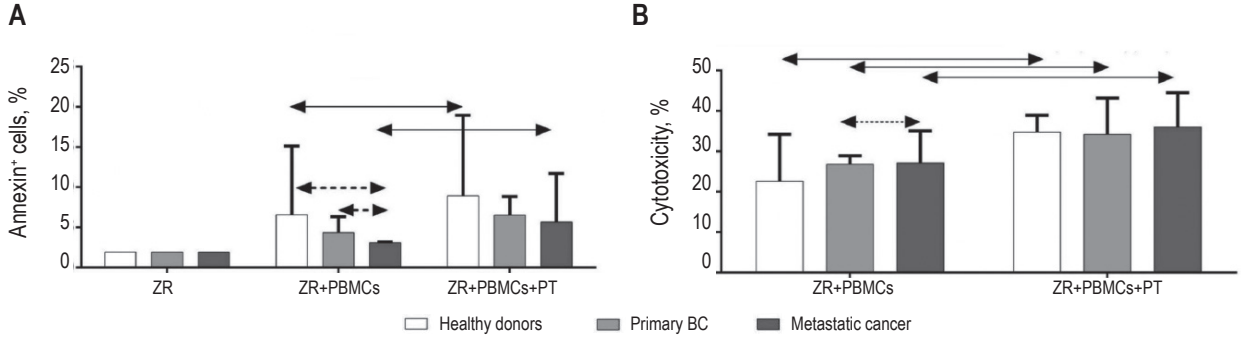

Figure 3. Study of the effect of pentoxifylline on the level of T-cell cytotoxicity using (A) cytometric analysis of the level of apoptosis of ZR-75-1 tumor cells labeled with Annexin V and (B) determination of LDH release in the CytoTox96 test in healthy donors (n = 25), patients with primary breast cancer (n = 25) and progressive breast cancer (n = 15) after cultivation with cells of the tumor line ZR-75-1

Note. As for Figure 1.

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Авторы:

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

Шевченко Ю.А. *— к.б.н., старший 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*

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

Силков А.Н. *— д.б.н., директор ФГБНУ «Научно-исследовательский институт фундаментальной и клинической иммунологии», 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:

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

Shevchenko Yu.A., *PhD (Biology), Senior 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*

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

Silkov A.N., *PhD, MD (Biology), Director, Research Institute of Fundamental and Clinical Immunology, 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*

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