ISSN 1563-0625 (print)
ISSN 2313-741X (online)

Том 25, № 3. С. 427-722

2023

Официальный журнал
Санкт-Петербургского Регионального Отделения
Российской Ассоциации
Аллергологов и Клинических Иммунологов

# МЕДИЦИНСКАЯ ИММУНОЛОГИЯ

*Данный выпуск реализован при финансовой поддержке Правительства Санкт-Петербурга*

**ИЛЛЮСТРАЦИИ К СТАТЬЕ «ВЛИЯНИЕ РАСТВОРИМЫХ ФАКТОРОВ МАКРОФАГОВ, ПОЛЯРИЗОВАННЫХ ЭФФЕРОЦИТОЗОМ, НА НЕЙРОНАЛЬНУЮ ПЛОТНОСТЬ ВО ФРОНТАЛЬНОЙ КОРЕ И ГИППОКАМПЕ МЫШЕЙ В МОДЕЛИ СТРЕСС-ИНДУЦИРОВАННОЙ ДЕПРЕССИИ» (АВТОРЫ: РАЩУПКИН И.М., АМСТИСЛАВСКАЯ Т.Г., МАРКОВА Е.В., ОСТАНИН А.А., ШЕВЕЛА Е.Я. [с. 521-526])**

ILLUSTRATIONS FOR THE ARTICLE "EFFECT OF SOLUBLE FACTORS OF MACROPHAGES POLARIZED BY EFFEROCYTOSIS ON NEURONAL DENSITY IN THE FRONTAL CORTEX AND HIPPOCAMPUS OF MICE IN A MODEL OF STRESS-INDUCED DEPRESSION" (AUTHORS: RASHCHUPKIN I.M., AMSTISLAVSKAYA T.G., MARKOVA E.V., OSTANIN A.A., SHEVELA E.YA. [pp. 521-526])

Frontal cortex

CA1

CA3

**Figure 1. Neuronal density in the pyramidal layer of the frontal cortex, ca1 and ca3 zones of the hippocampus of depression-like mice**

Note. (A) Intranasal administration of rpmi-1640. (B) Intranasal administration of M2(LS) conditioned media. (C) Relative neuronal density (%) in mice brain (n = 4-5). Intact, intact group of animals; depr, depressive-like mice (control of model); depr + rpmi, intranasal administration of rpmi-1640 (control of treatment); depr + M2(LS), intranasal administration of M2(LS) conditioned media (experimental group). Data presented as the percentage of an area of interest occupied by the nissl stained cells (Me; IQR). P, significance of differences (the Mann–Whitney non-parametric U test).

**ИЛЛЮСТРАЦИИ К СТАТЬЕ «МОРФОФУНКЦИОНАЛЬНЫЕ ИЗМЕНЕНИЯ МИКРОГЛИИ У МОЛОДЫХ И СТАРЫХ КРЫС WISTAR» (АВТОРЫ: СЕНТЯБРЕВА А.В., МЕЛЬНИКОВА Е.А., МИРОШНИЧЕНКО Е.А., ЦВЕТКОВ И.С., КОСЫРЕВА А.М. [с. 527-532])**

ILLUSTRATIONS FOR THE ARTICLE "MORPHOFUNCTIONAL CHANGES OF MICROGLIA IN ADULT AND OLD WISTAR RATS" (AUTHORS: SENTYABREVA A.V., MELNIKOVA E.A., MIROSHNICHENKO E.A., TSVETKOV I.S., KOSYREVA A.M. [pp. 527-532])

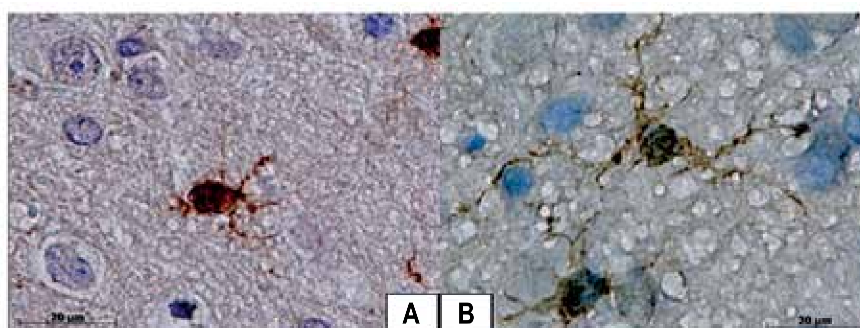


**Figure 1. Morphological characteristics of microglia cells with thin and short processes in adult Wistar rats (A) and with spheroidal swelling, hypertrophic, beaded, and tortuous processes in old Wistar rats (B)**

Note. Iba-1 antibody + HRP secondary antibody IHC and hematoxylin staining, ×1600.

САНКТ-ПЕТЕРБУРГСКОЕ РЕГИОНАЛЬНОЕ ОТДЕЛЕНИЕ
РОССИЙСКОЙ АССОЦИАЦИИ АЛЛЕРГОЛОГОВ И КЛИНИЧЕСКИХ ИММУНОЛОГОВ
(СПб РО РААКИ)

# **МЕДИЦИНСКАЯ** ИММУНОЛОГИЯ

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**2023, том 25** **№ 3**

Основан в марте 1999 года

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д.м.н. Чухловин А.Б.

*Редактор электронной версии:*
Ерофеева В.С.



**Редакция:** тел./факс (812) 233-08-58
**Адрес для корреспонденции:**
197101, Санкт-Петербург, а/я 130.
**Электронная версия:** www.mimmun.ru; www.elibrary.ru


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Журнал зарегистрирован Северо-Западным региональным управлением Государственного комитета РФ по печати 26 марта 1999 г.
Свидетельство о регистрации № П 3612.
Министерством РФ по делам печати, телерадиовещания и средств массовых коммуникаций 30 июня 2003 г.
Свидетельство о регистрации ПИ № 77-15892.
Федеральной службой по надзору в сфере связи, информационных технологий и массовых коммуникаций (Роскомнадзор)
Свидетельство о регистрации средства массовой информации ПИ №ФС77-60436 30 декабря 2014 г.


Данный материал распространяется по лицензии Creative Commons Attribution 4.0 License.


Издательство «Человек»
199004, Россия, Санкт-Петербург, Малый пр. В.О., 26, оф. 3.
E-mail: mail@mirmed.ru
Тел./факс: (812) 325-25-64.

Подписано в печать 22.05.2023 г. Формат 60 x 90 1/8. Печать офсетная.
Усл. печ. л. 37. Тираж 2000 экз. (1-й завод – 1000 экз.) Заказ № 032
Напечатано в ООО «АРТЕМИДА».
199178, Санкт-Петербург, 8-я линия В.О., 83, корп. 1, Литер А
Тел.: (812) 950-10-99.

***С 2001 года журнал «Медицинская иммунология» регулярно входит в «Перечень ведущих рецензируемых научных журналов и изданий, в которых должны быть опубликованы основные научные результаты диссертации на соискание ученой степени доктора наук», рекомендованных ВАК Министерства образования и науки РФ.***

***С 2016 года журнал «Медицинская иммунология» включен в международную базу SCOPUS.***

RUSSIAN ASSOCIATION OF ALLERGOLOGISTS AND CLINICAL IMMUNOLOGISTS,
ST. PETERSBURG REGIONAL BRANCH
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May-June
**2023, volume 25** **No. 3**
Published since March 1999

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**Address for correspondence:**
197101, St. Petersburg, P.O. Box 130.
**Electronic version:** www.mimmun.ru; www.elibrary.ru



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The Journal is registered at the North Western Regional Administration for the Press Affairs of the Russian Federation, March 26, 1999.
Certificate of registration PI № 77-15892 by the Ministry of Press, Television, Broadcasting and Mass media of the Russian Federation, June 30, 2003.
Federal Service for Supervision of Communications, Information Technology and Mass Media (ROSKOMNADZOR)
Certificate on registration of mass media PI №FS77-60436, December 30, 2014



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Chelovek Publishing House
199004, Russian Federation, St. Petersburg, Malyi ave., Vasilevsky Island, 26, office 3.
E-mail: mail@mirmed.ru
Phone/fax: (812) 325-25-64.

Passed for printing 22.05.2023. Print format 60 x 90 1/8. Offset printing.
Printed sheets 37. Circulation 2000 copies. (1st edition – 1000 copies.)

Print in LLC «ARTEMIDA»
199178, Russian Federation, St. Petersburg, 8 line of Vasilievsky Island, 83/1-A
Phone: (812) 950-10-99

***Since 2001, the Medical Immunology Journal is admitted to the Index of leading peer-reviewed scientific Journals intended for publication of key research results of MD Theses, as recommended by the Higher Attestation Commission of the Russian Ministry of Education and Science.***

***Since 2016, the Medical Immunology Journal is included into international SCOPUS database.***

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Медицинская иммунология
2023, Т. 25, № 3,
стр. 435-440

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 435-440

# СЛОЖНАЯ ИММУНОБИОЛОГИЯ ФАКТОРА НЕКРОЗА ОПУХОЛЕЙ И НОВАЯ АНТИ-TNF ТЕРАПИЯ

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**Резюме.** Фактор некроза опухолей (TNF) был открыт почти 50 лет назад как в «сывороточный фактор» у мышей после инфекций или инъекции бактериального липополисахарида (ЛПС) и обладающий ярким противоопухолевым эффектом. Молекулярное клонирование установило, что этой активностью обладает небольшой белок (17 кДа), принадлежащий к широкому множеству цитокинов. В силу особенности организации кодирующей последовательности TNF в геноме, все клетки, продуцирующие растворимый TNF, несут на своей поверхности и мембранно-связанный цитокин. Физиологические эффекты TNF опосредованы передачей сигналов через два типа высокоспецифичных рецепторов. Несмотря на гомеостатические и защитные функции TNF, в случае его избыточной системной или локальной продукции могут развиваться различные патологии – от септического шока до хронического воспаления. Поэтому в практической иммунотерапии нашли свое применение не агонисты TNF (от которых ожидали противоопухолевых эффектов), а антагонисты-блокаторы, которые оказались эффективными при лечении целого ряда аутоиммунных заболеваний с воспалительным компонентом. Наши исследования на мышах, основанные на технологиях обратной генетики и экспериментальных моделях заболеваний, выявили парадоксальное свойство TNF, состоящее в том, что одни клеточные источники этого цитокина (такие как миелоидные клетки) способствовали развитию заболеваний, а другие клетки (например, Т-лимфоциты) производили защитную форму того же цитокина. Имеется несколько возможных механистических объяснений этому явлению. Одно из них предполагает, что «патогенный» цитокин продуцируется в растворимом виде и может оказывать системные эффекты, действуя через TNFR1. При этом защитные эффекты связаны с мембранно-связанным TNF, который действует через TNFR2. Известно, что системная антицитокиновая терапия сопровождается нежелательными побочными эффектами, которые гипотетически могут быть объяснены нейтрализацией «полезных» функций конкретного цитокина. На основании этих соображений нами были разработаны прототипы блокаторов TNF, которые ограничивают биодоступность этого

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**Образец цитирования:**
*С.А. Недоспасов «Сложная иммунобиология фактора некроза опухолей и новая анти-TNF терапия» // Медицинская иммунология, 2023. Т. 25, № 3. С. 435-440.*
*doi: 10.15789/1563-0625-COI-2860*

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**For citation:**
*S.A. Nedospasov “Complexity of immunobiology of tumor necrosis factor and novel anti-TNF therapy”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 435-440.*
*doi: 10.15789/1563-0625-COI-2860*

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**DOI:** 10.15789/1563-0625-COI-2860

цитокина только из его главного «патогенного» источника – миелоидных клеток. Эти блокаторы, называемые MYSTI, представляют собой биспецифичные миниантитела, лишенные Fc-домена и связывающие как TNF, так и поверхностный маркер миелоидных клеток. MYSTI удерживает вновь синтезированный TNF на поверхности клетки-продуцента, а затем интернализует его. Этот новый тип иммунотерапевтических препаратов уже показал эффективность в ряде экспериментальных заболеваний.

*Ключевые слова: цитокины, антицитокиновая терапия, биспецифичные антитела, мышиные модели, гуманизация*

# COMPLEXITY OF IMMUNOBIOLOGY OF TUMOR NECROSIS FACTOR AND NOVEL ANTI-TNF THERAPY

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**Abstract.** Tumor Necrosis Factor (TNF) was discovered almost 50 years ago as “serum factor” detected in mice following infections or administration of bacterial lipopolysaccharide (LPS), with a remarkable anti-tumor effect. Molecular cloning showed that this activity is mediated by a small protein (17 kDa), which belongs to a wide plethora of cytokines. Due to the particular organization of the TNF gene coding sequence, all cells producing soluble TNF also carry a membrane-bound cytokine on their surface. The physiological effects of TNF are mediated by signaling through two types of highly specific receptors. Despite established protective and homeostatic functions of TNF, when overproduced systemically or locally, it can trigger pathologies ranging from septic shock to autoimmune diseases. Therefore, in clinical immunotherapy there were not the TNF agonists, which were expected to induce anti-tumor effects, but rather the antagonistic blockers, that proved effective in a wide range of autoimmune diseases with an inflammatory component. Our studies in mice based on the technologies of reverse genetics and experimental disease models, revealed a paradoxical feature of TNF: some cellular sources of this cytokine (such as myeloid cells) promoted diseases, while other cell types (such as T lymphocytes) produced a protective form of the same cytokine. There are several possible mechanistic explanations for this phenomenon. On the one hand, the "pathogenic" cytokine is produced in a soluble form and can exert systemic effects via broadly expressed TNFR1. On the other hand, protective functions are mediated by the membrane-bound TNF via TNFR2. Systemic anti-cytokine therapy is known to be accompanied by undesirable side effects, which can hypothetically be explained by the neutralization of these protective functions. Thus, we developed prototypes of TNF blockers which limit the bioavailability of this cytokine only from its main “pathogenic” source – myeloid cells. This type of inhibitors, called MYSTI, represent bispecific mini-antibodies binding both TNF and a surface marker on myeloid cells and lacking the Fc domain. MYSTI retain newly synthesized TNF on the surface of the producing cell and then internalize it. This novel type of immunotherapy drug has already shown efficacy in a number of experimental disease models.

*Keywords: cytokines, anti-cytokine therapy, bispecific antibodies, mouse models, humanization*

This study is supported by Russian Science Foundation, grant #19-75-30032.

## Introduction

Tumor Necrosis Factor (TNF) was discovered due to its anti-tumor effect in mice [3]. Molecular cloning identified a small protein (17 kDa) that can recapitulate TNF activity *in vivo* [14]. It was later found that TNF is the founding member of a family of cytokines that play an important role in the immune regulation [9].

TNF gene is linked to class III region of the MHC [11, 15] and is flanked by two related lymphotoxin genes, the other members of TNF family [12]. TNF is transcriptionally regulated in response to stimulation of various receptors of the innate immune system, such as TLR4. The primary product of TNF gene is a 26 kDa membrane-bound form of this cytokine. Analysis of TNF-deficient mice ruled out TNF as an enigmatic player in anti-tumor defense, but at the same time uncovered several unexpected functions, including proinflammatory activity [10, 13]. These functions are mediated by two distinct receptors, TNFR1 (the main receptor for the soluble TNF that is promiscuously expressed) and TNFR2 (responding to membrane-bound TNF and expressed on hematopoietic cells) [9]. Therefore, TNF signaling turned out to be more complex than originally proposed and includes three distinct signals: by soluble TNF through TNFR1, by membrane-bound TNF through TNFR2 and the "reverse signaling" by membrane-bound TNF into TNF-expressing cell (Figure 1). Unexpectedly, TNF was identified as an obligatory mediator of a number of disease states, including septic shock [2] and several autoimmune diseases with an inflammatory component. As a result, anti-TNF therapy was established as an effective treatment for rheumatoid arthritis [6] and several other diseases. However, such therapy cannot be free from unwanted side effects due to TNF role in protection from infections and its several homeostatic functions.

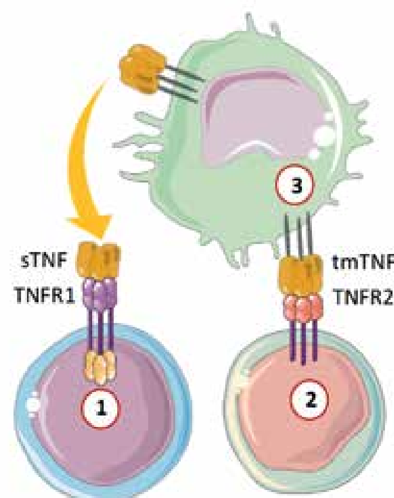


**Figure 1. Complexity of signaling patterns induced by TNF**

Note. Signal 1 is triggered by soluble TNF through TNFR1, signal 2 is triggered by membrane-bound TNF through TNFR2. Signal 3 is triggered through intracellular part of transmembrane TNF inside the producing cell

## Materials and methods

**Mice.** TNF-humanized hTNFKI mice (6-8 weeks) were housed in SPF conditions at the Animal Facility of the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, EIMB RAS. All manipulations with animals were carried out in accordance with the protocols approved by the Bioethics Committee of the EIMB RAS.

**Primary cultures of bone marrow-derived macrophages (BMDM) and TNF-retention *in vitro*.** Bone marrow was isolated from tibias and femurs of hTNFKI mice. For BMDMs differentiation bone marrow cells were cultured on non-treated cell culture plates in DMEM containing 30% L929 conditioned medium as the source of M-CSF, 20% FBS (Capricorn Scientific), L-glutamine and antibiotics (Pen/Strep, ThermoFisher). To analyze TNF production, cells were activated with LPS 10 ng/mL (O111:B4) for 4 hrs. Cytokine release in culture supernatants of stimulated BMDM, as well as BMDM preincubated with MYSTI for TNF-retention experiments, was measured by enzyme-linked immunosorbent assay (ELISA) using ready-made commercial kits "Human TNFalpha ELISA Ready-SET-Go" (ThermoFisher) according to the manufacturer's protocol.

**Administration of MYSTI and Infliximab.** Anti-TNF reagents were administered i.p. at doses 1.5-3 mcg/g of body weight every day for MYSTI and every three days for Infliximab.

**Germinal center (GC) formation.** Induction of full-scope immune response was carried out by immunizing mice with $2 \times 10^6$ SRBC via i.p. injection in 200 mcl of PBS. Assessment of germinal center B cells and GC formation was carried out on day 8 following immunization by flow cytometry and IHC.

**LPS/D-gal hepatotoxicity model.** Mice were injected with MYSTI or Infliximab 30 min or 12-48 hours prior to of acute hepatotoxicity, which was induced by i.p. administration of 400 ng/g LPS (Sigma-Aldrich, L2630) and 800 μg/g D-Galactosamine (Sigma-Aldrich, G1639). Control group received vehicle buffer only (PBS). Mice were observed for 800 min after LPS/D-Gal injection. Moribund animals were euthanized, and time of death was noted.

**Collagen antibody-induced arthritis.** For induction of arthritis with arthritogenic antibodies, a cocktail containing five monoclonal antibodies against collagen type II and LPS from *E. coli* O111:B4 as adjuvant were injected in accordance with the manufacturer's protocol (Chondrex, USA, cat #53040). Clinical assessment for each paw was determined based on the scale as previously reported [8].

**DSS-induced colitis.** Mice were subjected to 5% DSS in drinking water for five consecutive days. Body weight changes and survival was measured during the three weeks following colitis induction. Colon length, weight and spleen index were measured upon termination of the experiment.

**Experimental autoimmune encephalomyelitis.** Mice were s.c. immunized with 50 μg of $MOG_{35-55}$ peptide (Gene Script) emulsified in complete Freund's adjuvant (CFA) supplemented with 5 mg/mL *Mycobacterium tuberculosis* (Difco), followed by 150 ng of Pertussis toxin (List Biological Laboratories) administration on day 0 and 2. Mice were scored daily, and clinical signs were assessed according to standard protocol.

## Results and discussion

Systemic anti-cytokine therapy may have undesirable side effects due to neutralization of the "beneficial" functions of a particular cytokine, which underlie its evolutionary selection, in particular, the role in protection against infections.

In this study we used engineered mice with humanization of the TNF system to compare the efficacy of two types of TNF inhibitors in several experimental disease models. We also addressed possible homeostatic effects of TNF inhibitors on microarchitecture of peripheral lymphoid tissues, known to be TNF-dependent. Earlier we used a panel of engineered mice with TNF deletions in specific cell types [7, 8] to define cellular sources of "deleterious" TNF in several experimental diseases. Surprisingly, in experimental arthritis "pathogenic" TNF is produced by myeloid cells, such as macrophages, while TNF produced by T lymphocytes provides protection [8]. Based on this unexpected observation we proposed a new type of anti-TNF therapy that relies on pharmacological neutralization of TNF from myeloid cells – the main source of pathogenic TNF. This therapeutic strategy is based on the administration of bispecific mini-antibodies that retain newly synthesized soluble TNF on the surface of macrophages and prevent it from diffusing out and triggering systemic TNFR1-mediated events [4, 5]. Such reagents, called MYSTI (from myeloid cell-specific TNF inhibitors), lead to retention and subsequent TNF internalization *in vitro* in the model of bone marrow-derived macrophages activated with LPS [4]. Side-by-side comparison with the efficacy of chimeric full-size therapeutic antibody, Infliximab, and MYSTI *in vivo* was performed throughout this study (Figure 2).

Specifically, both types of inhibitors protected mice from lethal hepatotoxicity and ameliorated experimental arthritis. However, only MYSTI was protective in experimental colitis and, unlike Infliximab, did not interfere with germinal center formation in SRBC-immunized mice. The lack of

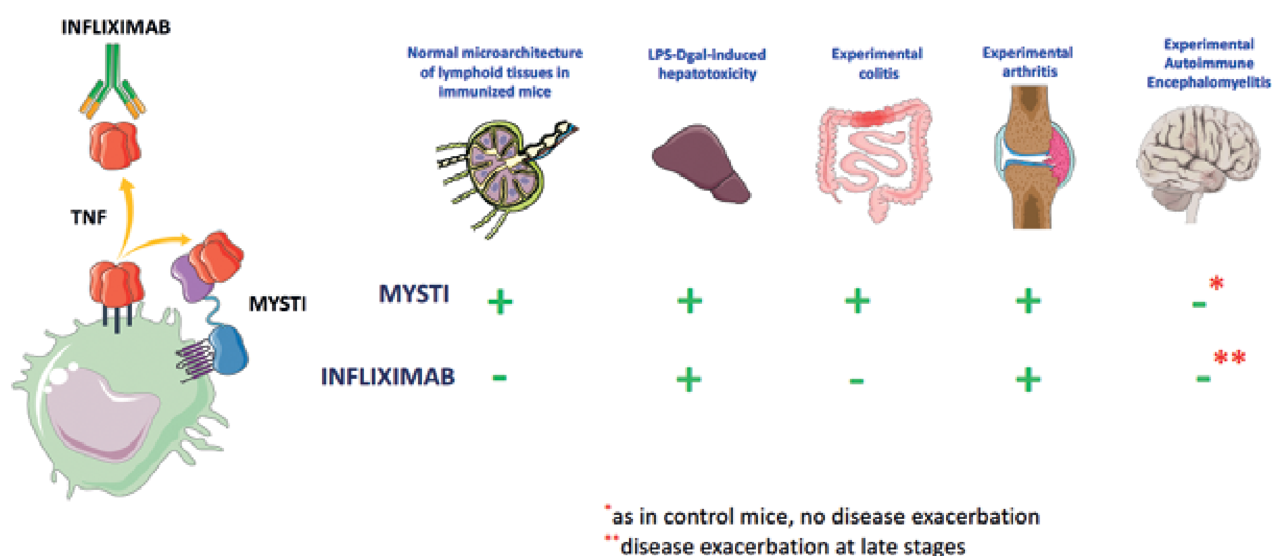


**Figure 2. Comparison of the efficacy of systemic and myeloid cell-restricted TNF neutralization after immunization and in several disease models in humanized mice**

staining for germinal center markers were previously noted in patients undergoing systemic anti-TNF therapy [1]. In EAE, a mouse model for multiple sclerosis (MS), both reagents failed to protect mice from disease development, but only Infliximab caused exacerbation at the late stage of the disease, reminiscent of the effects in patients that led to termination of a clinical trial with another systemic blocker in MS.

## Conclusion

MYSTI represents a promising platform for further preclinical and clinical drug development.

## Acknowledgments

I am greatly indebted to my colleagues Andrei Kruglov, Marina Drutskaya and Grigory Efimov, as well as to other members of my laboratory.

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*Поступила 24.04.2023*
*Принята к печати 30.04.2023*

*Received 24.04.2023*
*Accepted 30.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 441-446



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 441-446

# ЭКСПАНСИЯ NK-КЛЕТОК *IN VITRO* СОПРОВОЖДАЕТСЯ ПОТЕРЕЙ ЭКСПРЕССИИ ИНГИБИРУЮЩИХ РЕЦЕПТОРОВ KIR

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**Резюме.** NK-клетки – лимфоциты врожденного иммунитета, которые способны эффективно элиминировать измененные клетки организма, что делает перспективным их применение в иммунотерапии вирусных заболеваний и опухолей. Популяция NK-клеток отличается высоким фенотипическим и функциональным разнообразием. В частности, в пуле высокодифференцированных NK-клеток в присутствии цитомегаловируса (HCMV) может формироваться популяция адаптивных клеток, которые отличаются высокой продолжительностью жизни и высокой цитотоксичностью. Однако для осуществления цитотоксической реакции NK-клетке необходимо пройти процесс обучения, в ходе которого она приобретает экспрессию рецепторов NKG2A и KIR. Ингибирующий сигнал от этих рецепторов предотвращает цитотоксическую реакцию против здоровых клеток организма. В настоящий момент существует множество эффективных методов накопления NK-клеток для последующего применения в терапии, один из них – стимуляция IL-2 и фидерными клетками K562-mbIL21. Высокодифференцированные NK-клетки с адаптивно-подобным фенотипом способны отвечать экспансией на такую стимуляцию. Однако показано, что в ходе активной пролиферации может динамически изменяться фенотип NK-клеток. Потеря экспрессии ингибирующих рецепторов KIR в ходе интенсивной пролиферации NK-клеток в ответ на стимул может негативно сказаться на их цитотоксическом потенциале и способности элиминировать мишени. В этой работе показано, что высокодифференцированные NK-клетки $CD56^{dim}NKG2C^{+}$ HCMV-серопозитивных индивидов отличаются высокой долей клеток $KIR2DL2/3^{+}$. Это может свидетельствовать о высокой стабильности экспрессии рецепторов KIR в этой популяции. Нами было показано, что клональные культуры $CD56^{dim}NKG2C^{+}$, полученные при стимуляции IL-2 и K562-mbIL21, отличаются высокой стабильностью экспрессии KIR2DL2/3 по сравнению с NKG2C-негативными и менее дифференцироваными $CD56^{bright}NKG2C^{+}$. Также в гетерогенных культурах предшественников адаптивных NK-клеток $CD57^{-}CD56^{dim}NKG2C^{+}$ наблюдался более высокий уровень экспрессии KIR2DL2/3 в сравнении с NKG2C-негативными

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**Образец цитирования:**
*Н.А. Алексеева, М.О. Устюжанина, М.А. Стрельцова, М.В. Гречихина, Г.В. Луценко, Е.И. Коваленко «Экспансия NK-клеток in vitro сопровождается потерей экспрессии ингибирующих рецепторов KIR» // Медицинская иммунология, 2023. Т. 25, № 3. С. 441-446.*
*doi: 10.15789/1563-0625-NCE-2845*

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**For citation:**
*N.A. Alekseeva, M.O. Ustyuzhanina, M.A. Streltsova, M.V. Grechikhina, G.V. Lutsenko, E.I. Kovalenko “NK cell expansion in vitro is followed by loss of inhibitory KIR expression”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 441-446.*
*doi: 10.15789/1563-0625-NCE-2845*

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**DOI:** 10.15789/1563-0625-NCE-2845

культурами CD57$^-$CD56$^{dim}$NKG2C$^-$. Таким образом, накопление NK-клеток при стимуляции IL-2 и фидерными клетками K562-mbIL2 может приводить к потере экспрессии рецепторов KIR и снижению их функциональной активности. Однако культурам высокодифференцированных NK-клеток HCMV-серопозитивных индивидов CD56$^{dim}$NKG2C$^+$, а также культурам предшественников адаптивных NK-клеток CD57$^-$CD56$^{dim}$ NKG2C$^+$ свойственна большая стабильность экспрессии KIR2DL2/3 в сравнении с культурами NKG2C-негативных и менее дифференцированных NK-клеток. Как следствие, стимуляцию IL-2 и фидерными клетками K562-mbIL21 можно применять для накопления адаптивно-подобных клеток и их предшественников, и при этом цитотоксический потенциал полученных культур, как и экспрессия ингибирующих рецепторов KIR, будет стабилен.

*Ключевые слова: NK-клетки, натуральные киллеры, ингибирующие рецепторы KIR, экспансия NK-клеток, иммунотерапия, цитомегаловирус, HCMV*

# NK CELL EXPANSION *IN VITRO* IS FOLLOWED BY LOSS OF INHIBITORY KIR EXPRESSION

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**Abstract.** NK cells are innate lymphocytes that are able to eliminate altered cells, which makes them promising for the immunotherapy of viral diseases and tumors. The NK cell population is characterized by high phenotypic and functional diversity. In particular, in the pool of highly differentiated NK cells in the presence of cytomegalovirus (HCMV), a population of adaptive cells can be formed, characterized by a high lifespan and high cytotoxicity. However, in order to carry out a cytotoxic reaction, a NK cell must undergo a licensing process, during which it acquires the expression of NKG2A and KIRs. Currently, there are many effective methods of NK cell accumulation for subsequent use in therapy, one of them is the stimulation with IL-2 and K562-mbIL21 feeder cells. Highly differentiated adaptive-like NK cells are able to expand in respond to such stimulation. However, the phenotype of actively expanding NK cells dynamically changes. Loss of inhibitory KIR expression during intense proliferation of NK cells may adversely affect their cytotoxic potential. This work shows that highly differentiated CD56$^{dim}$NKG2C$^+$ NK cells from HCMV-seropositive individuals have a high proportion of KIR2DL2/3$^+$ cells. This may indicate a high stability of KIR receptor expression in this population. We have shown that CD56$^{dim}$NKG2C$^+$ clonal cultures obtained by stimulation with IL-2 and K562-mbIL21 are characterized by high stability of KIR2DL2/3 expression compared to NKG2C-negative and less differentiated CD56$^{bright}$NKG2C$^+$. Also, in heterogeneous cultures of adaptive NK cells precursors CD57$^-$CD56$^{dim}$NKG2C$^+$, a higher expression level of KIR2DL2/3 was observed in comparison with NKG2C-negative cultures of CD57$^-$CD56$^{dim}$NKG2C$^-$. Thus, the accumulation of NK cells upon stimulation with IL-2 and K562-mbIL2 feeder cells can lead to loss of expression of KIR receptors and a decrease in their functional activity. However, cultures of highly differentiated NK cells of HCMV-seropositive individuals CD56$^{dim}$NKG2C$^+$, as well as cultures of precursors of adaptive NK cells CD57$^-$CD56$^{dim}$NKG2C$^+$, are characterized by a greater stability of KIR2DL2/3 expression. As a result, stimulation with IL-2 and K562-mbIL21 feeder cells can be used to accumulate adaptive-like cells and their progenitors with stable inhibitory KIR expression and high cytotoxic potential.

*Keywords: NK cells, natural killers, inhibitory KIR, NK cells expansion, immunotherapy, HCMV*

The study was supported by the Russian Science Foundation grant # 22-15-00503.

## Introduction

NK cells are innate lymphocytes that are capable of exhibiting cytotoxic activity against altered cells without prior sensitization. They are characterized by high phenotypic and functional diversity. In NK cell population, there are both regulatory cytokine-producing cells and effector cells with a high density of cytolytic granules. The population diversity of human NK cells is resulted from both their interaction with the internal environment of the organism and by

external factors, including a wide range of pathogens. Pathogens can influence the differentiation and maturation of NK cells, as well as promote the formation of a pool of so-called adaptive NK cells in the subset of highly differentiated CD56$^{dim}$NK cells. These adaptive NK cells are distinguished by a high lifespan and specialized functional activity.

Among adaptive NK cells the best studied are cytomegalovirus (HCMV)-specific ones, which have high expression levels of the NKG2C activating receptor [8], capable of recognizing the HCMV-derived peptide presented on the surface of infected cells in context of HLA-E [2, 6] engagement of inhibitory killer immunoglobulin-like receptors (KIR. However, these cells show cytotoxic activity only after the licensing, when inhibitory receptors KIR or NKG2A begin to be expressed on the surface of NK cells. These receptors are able to recognize HLA-I on surrounding cells, which allows NK cells to inhibit the cytotoxic response towards healthy cells. NKG2A expression is typical of poorly differentiated cells and decreases during maturation, while the proportion of KIR-positive cells increases during differentiation. In addition, the NKG2A receptor, in contrast to KIR, is more sensitive to the expression level of HLA-I molecules than to the presented peptide repertoire [3]. Thus, KIR receptors can enhance the specificity of the cytotoxic pathogen response of NK cells.

NK cells are currently used in cell therapy of several diseases. The lifespan along with the high level of antibody-dependent cytotoxicity of adaptive NK cells make them promising for the immunotherapy of various diseases, both viral and oncological. Therefore, the search for effective approaches to the accumulation of adaptive NK cells with a stable phenotype is especially important. For *ex vivo* expansion NK cells are stimulated with cytokines in the presence of feeder cells, made from modified and irradiated cell lines. K562 with membrane-bound interleukin 21 (K562-mbIL21) cells have shown their effectiveness and can induce an active proliferation of NK cells and increase their functional activity [5]. However, during cultivation, the cell phenotype changes dynamically. For example, when cultured with K562-mbIL21 feeder cells, NK cells are able to acquire the expression of the terminal differentiation marker CD57 or the NKG2A inhibitory receptor *de novo* [11] we analyzed the phenotype and growth of human NK cell clones obtained by the stimulation of individual NK cells with IL-2 and gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21).

In this work, we analyzed the stability of KIR2DL2/3 expression in cultures of NK cell subsets which differ in the differentiation stage and the level of NKG2C expression. The stability of KIR2DL2/3 expression in the subset of potential adaptive NK cell progenitors [7] was evaluated as well. NK cell *in vitro* cultures were obtained by stimulation with IL-2 and K562-mbIl21 feeder cells.

## Materials and methods

NK cells for following experiments were isolated from peripheral blood mononuclear cells (PBMC) by negative magnetic separation (NK cell isolation kit, MiltenyiBiotec, Bergisch Gladbach, Germany). PBMC were previously obtained by gradient centrifugation from blood samples of healthy donors, who gave their informed consent. *Ex vivo* NK cells were stained with anti-human monoclonal antibodies: CD56-BrilliantViolet (clone HCD56), NKG2C-AlexaFluor (AF) 488 (clone 108724), KIR2DL2/3-PE-Vio615 (clone REA1006) (Miltenyi Biotec, Germany) and CD57-PE-Vio770 (clone REA769) and then sorted into wells of 96-well plate. Heterogeneous cultures were obtained via planting by 100 cells into each well, clonal cultures by planting a single cell into each well. Sorted NK cell subsets differed in differentiation stage and NKG2C expression level: CD56$^{bright}$NKG2C$^{-}$, CD56$^{bright}$NKG2C$^{+}$, CD56$^{dim}$NKG2C$^{-}$, CD56$^{dim}$NKG2C$^{+}$. Subsets of adaptive NK cells precursors CD57$^{-}$CD56$^{dim}$NKG2C$^{+/-}$ were sorted as well.

These sorted cells were cultivated in medium for clones (80% DMEM medium (PanEco, Moscow, Russia), 20% x-vivo medium (Lonza, Swiss), 2 mM L-glutamine, 2 mM sodium pyruvate (PanEco, Moscow, Russia), 2 mM antibiotic-antimycotic (Sigma-Aldrich, St. Louis, MO, USA)) with 100 U/ml IL-2 and feeder cells K562-mbIL21 (37 °C, 5% CO2), for 2 weeks (heterogeneous cultures) or 3 weeks (clonal cultures). *Ex vivo* NK cells and NK cell cultures obtained after the stimulation were stained with following anti-human monoclonal antibodies: NKG2C-AF 488, KIR2DL2/3-PE-Vio615 and CD57-PE-Vio770, CD56-APC, KIR2DL2/3-PE (clone DX27). Then flow cytometry data was obtained on MACSQuant 10 cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with 405 nm, 488 nm, and 635 nm lasers. Flow cytometer data was processed in FlowJo software version X (TreeStar Williamson Way, Ashland, OR, USA). Statistical analysis was performed using the GraphPad Prism 7 software (StatSoft Inc., Tulsa, OK, USA). Student's t-test was applied for data that passed the Shapiro–Wilk normality test and the Mann–Whitney test for data not normally distributed. Means ± SEM are presented throughout the paper (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

## Results and discussion

Adaptive NK cell population belongs to the pool of highly differentiated CD56$^{dim}$ cells and is characterized by a high expression level of the terminal differentiation

marker CD57 and the NKG2C activating receptor. To study the relationship between the expression of KIR in highly differentiated NK cells, the individual HCMV serological status and proportion of NKG2C-expressing cells, the KIR2DL2/3 expression in newly isolated $CD56^{dim}NKG2C^{+/-}$ NK cells was analyzed. In the highly differentiated cell subset of HCMV-seronegative individuals, the proportion of $KIR2DL2/3^{+}$ cells was independent of NKG2C expression (Figure 1A), while HCMV-seropositive donors had a higher proportion of $KIR2DL2/3^{+}$ cells in the $CD56^{dim}NKG2C^{+}$ NK cell subset (Figure 1B). This indicates that not only the presence of a pathogen contributes to the accumulation of licensed cells and the emergence of a pool of adaptive ones, but also that these two processes are linked together.

It is known that cytomegalovirus infection induces not only the expansion of the $NKG2C^{+}$ cells, but also the expansion of cells expressing inhibitory KIRs specific to the individual's own HLA-I [4]. This phenomenon is more pronounced in subsets of $NKG2C^{+}$ cells [1]. We have shown that this process is most notable in the subset of highly differentiated $CD56^{dim}NKG2C^{+}$ cells. The role of inhibitory KIR in the formation of adaptive NK cells is currently poorly understood. It is possible that the recognition of viral peptides in HLA-I by inhibitory KIR receptors also leads to the expansion of $KIR^{+}$ cells within the subpopulation of adaptive cells. High proportion of $KIR2DL2/3^{+}$ cells in the subset of adaptive NK cells

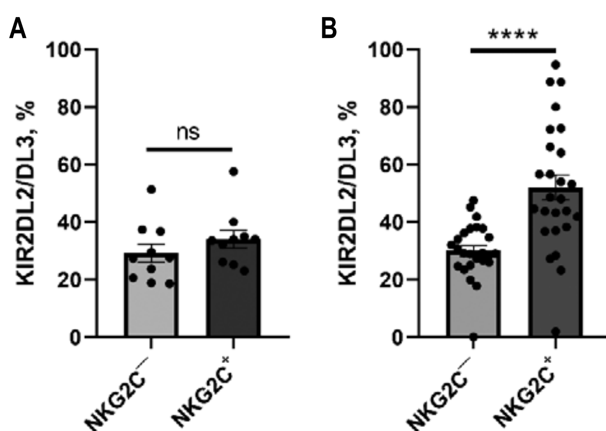


**Figure 1. Proportion of $KIR2DL2/3^{+}$ cells in subsets of highly differentiated $CD56^{dim}$ *ex vivo* NK cells differing in the level of NKG2C expression**

Note. (A) Proportion of $KIR2DL2/3^{+}$ cells in subsets of highly differentiated $CD56^{dim}$ NK cells differing in the level of NKG2C expression in HCMV seronegative individuals (n = 10). (B) Proportion of $KIR2DL2/3^{+}$ cells in subsets of highly differentiated $CD56^{dim}$ NK cells differing in the level of NKG2C expression in HCMV seropositive individuals (n = 26). Statistical analysis was performed using a paired t-test (**** $p < 0.0001$). Columns represent means ± SEM.

may be also due to the high expression stability of these receptors in the presence of infection.

To assess the stability of KIR expression by NK cells of HCMV-seropositive individuals at different stages of differentiation and with distinct NKG2C expression levels, freshly isolated cells with the phenotype $CD56^{bright}NKG2C^{-}$, $CD56^{bright}NKG2C^{+}$, $CD56^{dim}NKG2C^{-}$ and $CD56^{dim}NKG2C^{+}$ were sorted. Cells were cultured under stimulation with IL-2 and K562-mbIL21 feeder cells, and after two weeks of cultivation, KIR2DL2/3 expression was detected on the surface of cells from the resulting cultures. In all KIR2DL2/3-positive subsets, a decrease in the proportion of $KIR2DL2/3^{+}$ cells was detected. There was a tendency towards a higher proportion of $KIR2DL2/3^{+}$ cells in NKG2C-positive subsets regardless of the level of differentiation (Figure 2A).

Further, to assess the stability of KIR surface expression on the NK cells of HCMV seropositive individuals, clonal cultures of $KIR2DL2/3^{+}$ subsets differing in NKG2C expression and differentiation stage were obtained. The cells were cultured for 3 weeks with stimulation of IL-2 and K562-mbIL21, thereafter KIR2DL2/3 expression on the surface of NK cells in the obtained cultures was evaluated by flow cytometry.

One individual (Figure 2C) showed a high stability of expression of inhibitory KIR2DL2/3 receptors in the $CD56^{dim}NKG2C^{+}$ population compared to NK cells from a less differentiated $CD56^{bright}NKG2C^{+}$ subset and compared to $CD56^{dim}$ cells not expressing the NKG2C receptor. Another individual showed the same tendency (Figure 2B). This observation does not contradict the data obtained in the previous experiment when cultivating heterogeneous, not clonal cultures. Thus, a high proportion of KIR2DL2/3-positive cells in the $CD56^{dim}NKG2C^{+}$ population may be due to the high stability of this receptor when co-expressed with NKG2C. In addition, the differentiation stage can also affect the stability of the expression of the KIR2DL2/3 receptors.

Adaptive NK cells are able to respond by intense proliferation to a repeated encounter with a pathogen; however, adaptive NK cells obtained from the blood of healthy individuals also have a low expression level of the adapter molecule FcεRIγ. Low expression of FcεRIγ is a feature of NK cells with poor proliferative activity [10]. Therefore, the accumulation of adaptive NK cells in the absence of HCMV infection appears to be a challenging task. Nevertheless, the precursors of adaptive NK cells with the $CD57^{-}CD56^{dim}NKG2C^{+}$ phenotype retain many properties of adaptive cells, while being highly proliferative [7]. It has been previously shown that NK cells with the $CD57^{-}CD56^{dim}KIR^{+}NKG2C^{+}$ phenotype are able to respond with intense expansion

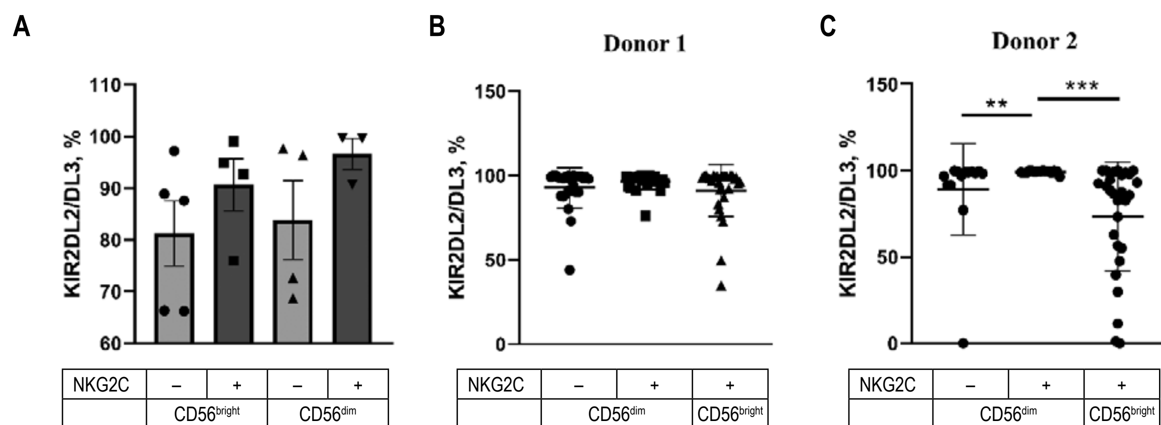


**Figure 2. Stability of KIR2DL2/3 expression in heterogeneous and clonal cultures of KIR2DL2/3⁺ NK cells of HCMV-seropositive individuals differing in differentiation stage and the level of NKG2C expression**

Note. (A) Proportion of KIR2DL2/3⁺ cells in cultures derived from KIR2DL2/3⁺ NK cell subsets differing in differentiation stage and NKG2C expression level. The number of individuals N = 5. Cultivation time t = 2 weeks. (B) and (C) Proportion of KIR2DL2/3⁺ cells in NK cell clones of two individuals after cultivation under stimulation with IL-2 and K562-mbIL21. Statistical analysis was performed using an unpaired Mann–Whitney (** $p < 0.01$, *** $p < 0.001$). Columns represent means ± SEM.

to stimulation by IL-2 and K562-mbIL21 feeder cells [9]. To evaluate the stability of KIR2DL2/3 expression in the adaptive NK cell progenitor subpopulation, heterogeneous CD57⁻CD56^dim^ cultures of KIR2DL2/3-positive subsets were obtained. NK cells were cultured for 2 weeks, after which KIR2DL2/3 expression was detected. As in cultures of highly differentiated CD56^dim^ cells, there was a tendency towards greater stability of KIR2DL2/3 expression in the NKG2C-positive subset (Figure 3A). Also, in the CD57⁻CD56^dim^NKG2C⁺ subset, a higher level of KIR2DL2/3 expression was observed, compared to the CD57⁻CD56^dim^NKG2C⁻ subset (Figure 3B).

## Conclusion

Earlier studies have shown that KIRs have a high expression stability [11] we analyzed the phenotype and growth of human NK cell clones obtained by the stimulation of individual NK cells with IL-2 and gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21. However, we found that NK cells, when expanded in response to stimulation by feeder cells, are able to lose KIR expression. Although stimulation with K562-mbIL21 feeder cells allows efficient accumulation of NK cells in large numbers with high expansion ratios, it is important for proliferating cells to retain their original phenotype during the expansion. Inhibitory KIR expression is necessary for the functional activity of cells, since it is associated with the accumulation of a large amount of cytotoxic granules near the center of microtubule organization. Loss of KIR expression may lead to a decrease in the effector function of NK cells. In this work, we have shown that highly differentiated cells with the phenotype of adaptive NK cells and their progenitors, CD56^dim^NKG2C⁺ and CD57⁻CD56^dim^NKG2C⁺, are characterized by high stability of expression of inhibitory KIR receptors, which makes possible to use the IL-2 stimulation method in combination with K562-mbIl21 feeder cells for accumulation of adaptive-like NK cells with a stable phenotype for subsequent immunotherapy.

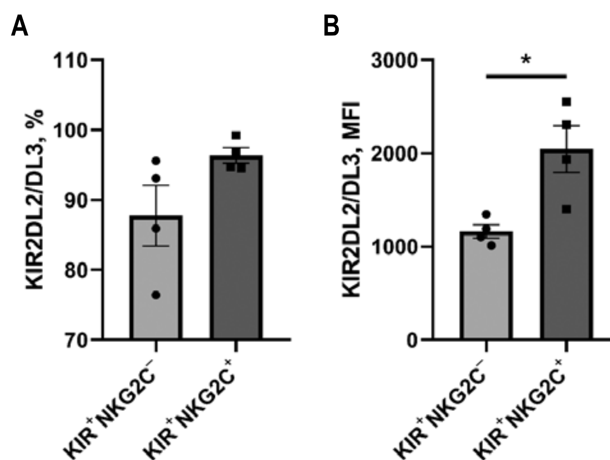


**Figure 3. Proportion of KIR2DL2/3⁺ cells in cultures from the pool of HCMV-specific adaptive NK cells precursors CD57⁻CD56^dim^ differing in the level of NKG2C expression and derived from HCMV-seropositive individuals**

Note. (A) Proportion of KIR2DL2/3⁺ cells in cultures derived from populations of KIR2DL2/3-positive adaptive NK cell precursors with the CD57⁻CD56^dim^ phenotype, differing in the level of NKG2C expression. N = 4, t = 2 weeks. (B) Expression level of KIR2DL2/3 in cultures derived from populations of KIR2DL2/3-positive adaptive NK cell precursors with the CD57⁻CD56^dim^ phenotype, differing in the level of NKG2C expression. N = 4, t = 2 weeks. Statistical analysis was performed using an unpaired Mann–Whitney (* $p < 0.05$). Columns represent means ± SEM.

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*Поступила 16.04.2023*
*Отправлена на доработку 25.04.2023*
*Принята к печати 27.04.2023*

*Received 16.04.2023*
*Revision received 25.04.2023*
*Accepted 27.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 447-452

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 447-452

# NK-КЛЕТКИ ЧЕЛОВЕКА ИНТЕРНАЛИЗУЮТ РЕКОМБИНАНТНЫЙ ОСНОВНОЙ СТРЕСС-ИНДУЦИРУЕМЫЙ БЕЛОК HSP70

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**Резюме.** Одной из основных функций белков теплового шока семейства 70 кДа (HSP70) является защита внутриклеточных протеинов от повреждающих воздействий стрессирующих факторов различной природы. Наряду с этим HSP7070 играют важную роль в жизнедеятельности клеток и в нормальных физиологических условиях, выполняя вспомогательные, так называемые шаперонные функции. Эти упомянутые функции реализуются во внутриклеточном пространстве; однако в некоторых случаях эти белки также обнаруживаются на клеточной поверхности и во внеклеточной среде. Причины и механизмы такой транслокации на клеточную поверхность и секреции HSP70 во внеклеточное пространство еще недостаточно изучены. Вместе с тем показано, что такая необычная внеклеточная локализация HSP70 активирует клетки иммунной системы. Поверхностные HSP70 и их внеклеточный пул стимулируют цитотоксическую активность, в том числе NK-клеток. Однако прямых экспериментальных доказательств возможности интернализации белков HSP70 NK-клетками еще не было продемонстрировано. В данной работе представлены результаты взаимодействия внеклеточного пула HSP70 с NK-клетками периферической крови здоровых доноров. Результаты исследования подтвердили возможность интернализации экзогенных молекул HSP70 NK-клетками. С этой целью нами были получены флуоресцентно меченые молекулы рекомбинантного стресс-индуцируемого HSP70 человека. О связывании HSP70 с флуорохромом свидетельствовала флуоресценция полученного конъюгата при воздействии освещения с длиной волны 488 нм. Данные электрофореза свидетельствовали об отсутствии деградации белка в процессе мечения, чистоте и стабильности модифицированного белка. Для оценки взаимодействия HSP70 с NK-клетками флуоресцентно меченый HSP70 добавляли к культуре NK-клеток *in vitro*, выделенных методом магнитной сепарации из мононуклеарной фракции периферической крови, и анализировали с помощью конфокальной микро-

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**Образец цитирования:**
*М.А. Шевченко, Д.Г. Гарбуз, А.И. Давлетшин, А.А. Бойко, М.Б. Евгеньев, А.М. Сапожников «NK-клетки человека интернализуют рекомбинантный основной стресс-индуцируемый белок HSP70» // Медицинская иммунология, 2023. Т. 25, № 3. С. 447-452.*
*doi: 10.15789/1563-0625-HNC-2837*

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**For citation:**
*M.A. Shevchenko, D.G. Garbuz, A.I. Davletshin, A.A. Boyko, M.B. Evgen'ev, A.M. Sapozhnikov "Human NK cells internalize recombinant major stress protein HSP70", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 447-452.*
*doi: 10.15789/1563-0625-HNC-2837*

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**DOI:** 10.15789/1563-0625-HNC-2837

скопии. Этот анализ показал, что живые NK-клетки интернализуют внеклеточные HSP70, которые локализуются как в лизосомах, так и в фагосомах. Наши эксперименты впервые иллюстрируют процесс проникновения внеклеточных HSP70 в эти клетки. Полученные результаты позволяют предположить, что активация NK-клеток под действием экзогенного HSP70 может быть связана в том числе и с интернализацией этих белков.

*Ключевые слова: HSP70 – белок теплового шока 70 кДа, NK-клетки, интернализация, флуоресцентное мечение, флуоресцентная визуализация, конфокальная микроскопия*

# HUMAN NK CELLS INTERNALIZE RECOMBINANT MAJOR STRESS PROTEIN HSP70

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**Abstract.** Heat shock proteins 70 kDa (HSP70) protect intracellular proteins from the damaging effects of stress factors of various natures. Moreover, HSP70 play an important role in the vital activity of cells under normal physiological conditions, performing chaperone functions. These functions are realized in the intracellular space; however, in some cases, these proteins are also found on the cell surface and in the extracellular environment. The causes and mechanisms of HSP70 translocation to the cell surface and secretion into the extracellular space have not yet been well understood, but such an unusual localization of HSP70 activates the immune system. The surface HSP70 and their extracellular pool stimulate the cytotoxic activity of NK cells. However, direct experimental evidence for the internalization of HSP70 molecules by NK cells has not yet been demonstrated. This paper presents the results of the interaction of the extracellular HSP70 pool with NK cells from the peripheral blood. The results demonstrated the confirmation of the internalization of exogenous HSP70 molecules by NK cells. To this end, fluorescently labeled recombinant stress-inducible human HSP70 were obtained. The electrophoretic data indicated the absence of protein degradation during the labeling process, the purity and stability of the modified protein. To assess the interaction of HSP70 with NK cells, the fluorescently labeled HSP70 was added to an *in vitro* culture of NK cells isolated by magnetic separation from the peripheral blood mononuclear fraction and analyzed by confocal microscopy. This analysis indicated that living NK cells internalize extracellular HSP70 with localization both in lysosomes and in phagosomes. Our experiments illustrated for the first time the process of penetration of the extracellular form of HSP70 into these cells. The results suggest that the activation of NK cells under the action of exogenous HSP70 could be associated with the internalization of these protein molecules.

*Keywords: HSP70 – heat shock protein 70 kDa, NK cells, internalization, fluorescent labeling, fluorescent imaging, laser confocal microscopy*

The study was funded by Russian Science Foundation, grant numbers 23-15-00472 and 19-14-00167.

## Introduction

The 70 kDa heat shock proteins (HSP70) belong to the large family of heat shock proteins, HSPs. One of the main functions of these molecules is the protection of intracellular proteins from the damaging effects of stress factors of various nature. Along with this, HSPs play an important role in the cells under normal physiological conditions, interacting with a wide range of intracellular proteins and performing auxiliary, so-called chaperone functions. These HSP functions are realized in the intracellular space, however, in some cases, these proteins are also found on the cell surface and in the extracellular environment and behave as cytokines ("chaperokines"). This unusual localization is most characteristic of members of the HSP70 family, which are found on the surface of tumor, virus-infected, and stressed cells [8]. It has been established that transport of HSP70 to the cell surface is carried out with the help of a non-classical,

independent mechanism of the Golgi apparatus. It was also found that the translocation of HSP70 to the cell surface is enhanced at the final stages of apoptosis and is probably aimed at stabilizing the membrane of dying cells.

The phenomenon of HSP70 secretion in cell cultures of different tissues was demonstrated [11], in particular, in the culture of human peripheral blood mononuclear cells [6]. The possibility of HSP70 exocytosis in vivo is indicated by data on the presence of HSP70 in blood serum in normal subjects and in various pathologies [14]. The causes and mechanisms of HSP70 translocation to the cell surface and secretion of these proteins into the extracellular space have not yet been studied, but it has been shown that such an unusual localization of HSP70 is a “danger signal” and activates the immune system. Thus, it is now well known that surface HSP70 stimulates the cytotoxic activity of NK cells [9], while extracellular HSP70 enhances cytokine production and accelerates the maturation of antigen presenting dendritic cells [13]. At the organism level, extracellular HSP70 also perform immunoregulatory functions. In particular, these proteins are considered by many authors as an evolutionarily formed alarm signal for the immune system, indicating the emergence and localization of the focus of stress development in the body [7].

Previously, we demonstrated that recombinant human HSP70 after intranasal administration can penetrate various brain regions of mice in its native form and subsequently undergo rapid degradation. It was also shown that labeled HSP70 added to culture medium of different human and mouse cell lines enters the cells with strikingly different kinetics, which positively correlates with the basic levels of membrane bound Toll-like receptors (TLR) that are characteristic of these cell lines [15]. Our preliminary data also point to the possibility of receptor-independent internalization of extracellular HSP70 by lymphoid cells. It can be assumed that this pathway of exogenous HSP70 internalization is associated with the demonstrated ability of these proteins to interact with plasma membrane phospholipids [1].

There are currently several hypotheses regarding the physiological functions of the extracellular pool of HSP70. In cell culture models, it was shown that these proteins are adsorbed on the cell surface and internalized fairly quickly [5]. Moreover, absorbed HSP70 retain their protective activity and prevent the development of apoptosis in cell cultures [4]. Perhaps, at the cellular level, one of the functions of extracellular HSP70 produced during stress is aimed at protecting the entire population of cells from stress-mediated damage. Obviously, circulating serum HSP70 can have similar activity at the organism level, aimed at preventing cell damage [10].

Characteristically, using RNA-Seq, we identified a lot of differentially expressed genes in the hippocampus of a late-onset model of hereditary Alzheimer’s disease compared with those of nTg mice. Most importantly, we observed that recombinant human HSP70-induced upregulation of multiple genes participating in antigen processing and presentation especially the members of major histocompatibility complex (class I and II) in the brains of old Tg animals, suggesting that recombinant human HSP70 executes its beneficial role via activation of adaptive immunity [2]. The totality of these data indicates a significant, but poorly understood, role of extracellular HSP70 in the functioning of the immune system.

This paper presents the results of our study of the interaction of the HSP70 extracellular pool with NK cells, in particular, the experimental confirmation of the internalization of exogenous HSP70 molecules by these cells.

## Materials and methods

In this study, we used NK cells, isolated from human periphery blood. Blood samples were collected from healthy donors, who gave their informed consent (approved by the local ethics committee of the Pirogov Russian National Medical University). The fraction of peripheral mononuclear blood cells was obtained by gradient centrifugation in 1.077 density Ficoll solution (PanEco, Russia), then NK cells were isolated by negative magnetic separation using the NK cell isolation kit (MiltenyiBiotec, Germany) in accordance with the manufacturer recommendations. NK cells were incubated overnight at 37 °C and 5% $CO_2$, transferred to 18 well polymer μ-Slide (Ibidi, Germany), and underwent a vital staining with CellMasck Deep Red and LysoTracker Red DND-99 (all from ThermoFisher, USA) in accordance with the manufacturer recommendations. Nuclei were counterstained with Hoechst33342 (PanEco, Russia).

To investigate HSP70-NK cell interactions, we obtained recombinant HSP70 using expression construct of human HSP70 (HSPA1A, P0DMV8 HS71A_HUMAN) and pET14a (Novagen, USA) as described previously [3]. The obtained protein contained 6-His-Tag on the N-terminus. HSP70 was purified using Ni-NTA-Sepharose (Amersham Biosciences, UK) and subjected for dialysis against DPBS (PanEco). Endotoxin was removed using High Capacity Endotoxin Removal Resin (ThermoFisher) in accordance with the manufacturer recommendations. The protein was characterized quantitatively using Coomassie Plus (Bradford) Assay Kit (ThermoFisher). SDS-PAGE and Western-blot with monoclonal antibodies HSC70/HSP70 monoclonal antibody (BB70) (Enzo Life Sciences) were used to estimate the purity of HSP70.

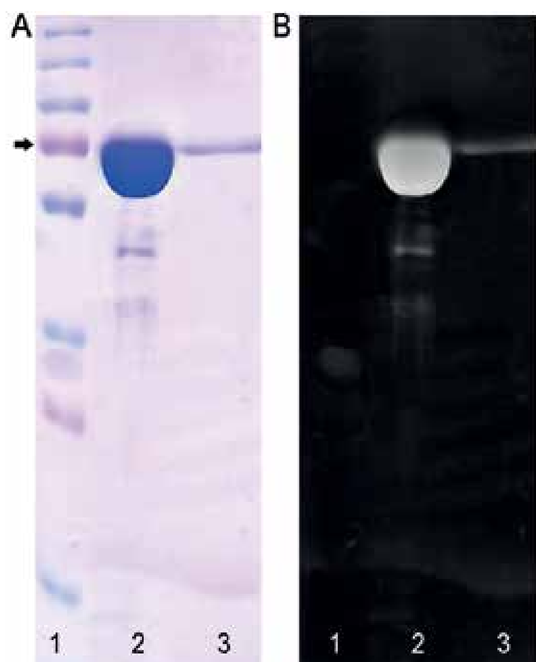


**Figure 1. Labelling of Hsp70 with AlexaFluor488**

Note. (A) Electrophoretic separation of Hsp70 proteins in SDS-PAGE. Sample explanation: 1, molecular weight marker (70 kDa band is indicated with arrow); 2, Hsp70-AlexaFluor488 in concentration 10 μg; 3, Hsp70-AlexaFluor488 in concentration 100 ng. (B) 488 nm laser-excited image of the gel presented in A.

HSP70 was labelled with AlexaFluor488 NHS Ester (ThermoFisher) in accordance with manufacturer recommendations. Unreacted labelling reagent was removed using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-50 regenerated cellulose membrane (Merck, Germany). The concentration of labelled HSP70 was tested with Protein assay (Bio-Rad, Germany) using BSA as a referent protein.

SDS-PAGE was made to prove the absence of HSP70 degradation during the labelling. Fluorescent imaging of the SDS-PAGE gel was made to confirm the HSP70-AlexaFluor488 conjugation.

AlexaFluor488-labelled HSP70 was added in excessive concentration of 10 μg/mL to NK cell culture. Cells were incubated in the presence of HSP70 at 37 °C and 5% $CO_2$. 30 min after, cells were washed three times with DPBS (PanEco) and subjected to imaging using Zeiss LSM980 (Carl Zeiss, Germany) equipped with 100 oil objective and 405, 488, and 633 nm lasers. Image analysis was performed using Imaris 9.8 (Oxford Instruments, UK).

## Results and discussion

For labelling, HSP70 in concentration of 10 mg/mL was incubated with AlexaFluor488 NHS, and 0.65 mg of HSP70-Alexa488 was yielded from each

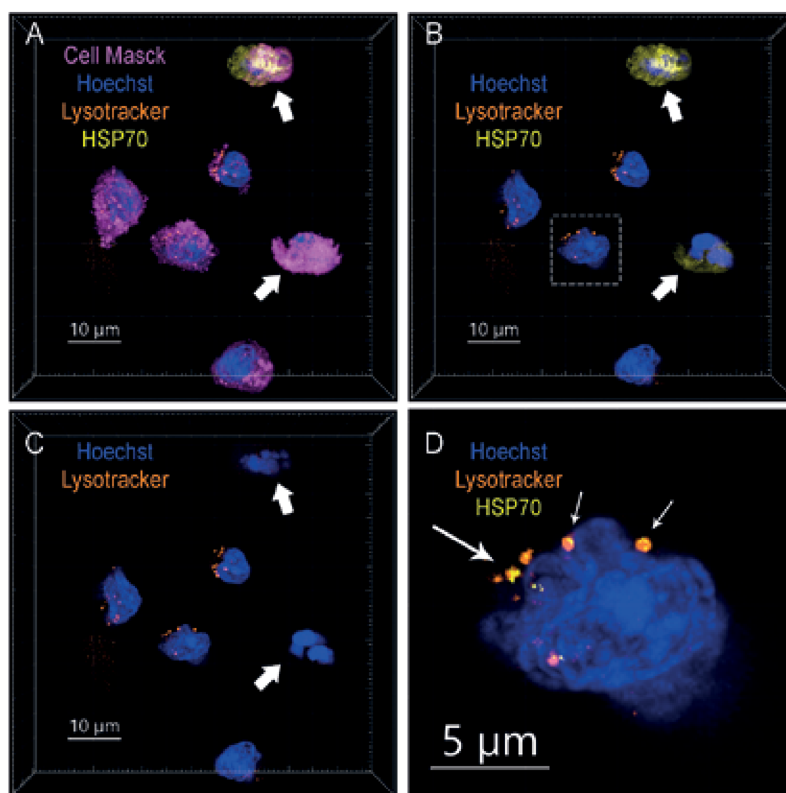


**Figure 2. Hsp70-NK cell interactions**

Note. (A-C) Representative image of NK cells that were stained with CellMask (magenta), LysoTracker (orange), and Hoechst (blue) and cultivated for 30 min in presence of Hsp70 (yellow). Cells covered with Hsp70 are indicated with bold arrows. Scale Bar 10 μm. (D) Enlarged image of the cell framed in B. Lysosomes with Hsp70 are indicated with small arrows, Hsp70 outside lysosome is indicated with a large arrow. Scale bar 5 μm.

1 mg of HSP70. The purity and stability of the protein was confirmed by electrophoresis (Figure 1A). The AlexaFluor488-conjugated state of the protein was confirmed by the fluorescent imaging of the protein (Figure 1B).

Freshly isolated NK cells were not adhesive; however, on a specifically treated surface of slide they attached to the slide bottom. Thus, within 30 min at 37 °C and 5% $CO_2$ NK cells were mostly detected at the slide bottom (Figure 2A). Upon supplying to the NK cell culture, HSP70 dissolved and steadily sedimented, and 30 min after the administration, large amount of AlexaFluor488-labelled HSP70 can be identified in the slide bottom (data not shown).

Thirty minutes after the supplementation, we detected HSP70-AlexaFluor488 both covering NK cells (Figure 2A-C, bold arrows) and internalized by NK cells (Figure 2A-D). Precise analysis indicated that in the cases when HSP70 covered NK cells, the cells were mostly dying. The morbid status of these cells was confirmed by the chromatin disaggregation in the nuclei (Figure 2C, bold arrows).

In the cases of the internalization of HSP70 by NK cells, HSP70 was detected both inside (Figure 2D, small arrows) and outside the lysosomes (Figure 2D, large arrow). LysoTracker dye allows the visualization of acidic cell compartments, such as lysosomes or phagolysosomes, but not phagosomes. Thus, we can assume that internalized AlexaFluor488-labelled HSP70 located outside the lysosomes was in the phagosomes that at the investigated time point were still not fused with the lysosomes.

Thus, to test the possibility of internalization of HSP70 by human NK cells, we obtained fluorescently labeled molecules of recombinant protein HSP70, the inducible member of the human HSP70 family. The electrophoretic data indicated the absence of protein degradation during the labeling process and the purity and stability of the resulting fluorescent protein. The binding of HSP70 to fluorochrome was evidenced by the fluorescence of the obtained conjugate when exposed to illumination with a wavelength of 488 nm.

To assess the interaction of HSP70 with NK cells, the resulting fluorescently labeled protein was added to an *in vitro* culture of NK cells isolated by magnetic separation from the peripheral blood mononuclear fraction of healthy donors. Samples of NK cells incubated with HSP70 were analyzed by laser confocal microscopy using additional fluorescent probes. This analysis indicated that living NK cells internalize extracellular HSP70 molecules, and the absorbed proteins can be located both in lysosomes and in phagosomes.

It should be noted that currently there are data in the literature on the internalization of exogenous HSP70 by different cell types [15]. At the same time, as regards the interaction of NK cells with the extracellular pool of this protein, there is much evidence of the activating effect of HSP70 and fragments of this protein on NK cells [12], however, direct experimental evidence of the internalization of HSP70 molecules by NK cells has not yet been demonstrated.

## Conclusion

Our experiments illustrated for the first time the process of penetration of the extracellular form of HSP70 into these cells. The results obtained indicate that the well-characterized activation of NK cells under the action of exogenous HSP70 is associated with the internalization of molecules of this protein.

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*Поступила 15.04.2023*
*Отправлена на доработку 22.04.2023*
*Принята к печати 26.04.2023*

*Received 15.04.2023*
*Revision received 22.04.2023*
*Accepted 26.04.2023*

# ФИБРОГЕННЫЙ И ФИБРОЛИТИЧЕСКИЙ ПОТЕНЦИАЛ РАЗЛИЧНО АКТИВИРОВАННЫХ МАКРОФАГОВ ЧЕЛОВЕКА

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**Резюме.** Макрофаги участвуют в регуляции фиброгенеза и процессе синтеза/деградации внеклеточного матрикса. Одним из способов реализации данной функции является продукция ими фиброгенных и фибролитических факторов, включая фибронектин, ламинин, коллаген, а также протеазы внеклеточного матрикса. Продукция большинства из них хорошо изучена в экспериментальных моделях на животных, однако в отношении макрофагов человека все еще остается много неясностей. Поэтому целью настоящего исследования являлось изучение содержания протеаз внеклеточного матрикса (MMP-2 и MMP-9, катепсина L), их ингибиторов (TIMP-1) и коллагена (I типа) в супернатантах различно активированных макрофагов человека. Нами было проведено сравнение макрофагов, дифференцированных M-CSF или GM-CSF и далее поляризованных в M1 липополисахаридом, в M2a – IL-4 и в M2c – дексаметазоном. Макрофаги получали из моноцитов периферической крови условно здоровых доноров. Содержание MMP, TIMP, катепсина и коллагена определяли с помощью соответствующих наборов иммуноферментного анализа. Согласно полученным результатам, дифференцировочные факторы играют более важное значение для продукции вышеперечисленных веществ по сравнению с поляризующими стимулами (липополисахарид, IL-4, дексаметазон). При этом макрофаги, дифференцированные M-CSF, проявляли преимущественно антифиброгенную активность благодаря выраженной продукции MMP, тогда как GM-CSF-индуцированные культуры, напротив, характеризовались профиброгенными свойствами за счет высокого уровня TIMP-1 и коллагена I типа. M1, M2a и M2c, индуцированные M-CSF, различались только по уровню продукции MMP-2, причем M2a активнее продуцировали данную металлопротеиназу по сравнению с другими подтипами. Среди GM-CSF-дифференцированных макрофагов более высокий уровень продукции TIMP-1 и, в меньшей степени, коллагена I типа был характерен для M1, тогда как супернатанты M2c отличались минимальной концентрацией указанных факторов. Что касается уровня продукции катепсина L, то он был относительно постоянным и не зависел от условий генерации макрофагов (дифференцировочных и поляризующих сигналов). Таким образом, полученные нами данные помогают идентифицировать подтипы макрофагов с анти- или профиброгенным потенциалом и могут быть полезны для разработки клеточной терапии заболеваний, связанных с нарушением регуляции фиброгенеза.

*Ключевые слова: макрофаги, матричные металлопротеиназы, коллаген, катепсин, фиброз, антифиброгенная активность*

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**Образец цитирования:**
*А.А. Максимова, Л.В. Сахно, А.А. Останин «Фиброгенный и фибролитический потенциал различно активированных макрофагов человека» // Медицинская иммунология, 2023. Т. 25, № 3. С. 453-458. doi: 10.15789/1563-0625-FAF-2713*

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**For citation:**
*A.A. Maksimova, L.V. Sakhno, A.A. Ostanin "Fibrogenic and fibrolytic potential of differently activated human macrophages", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 453-458. doi: 10.15789/1563-0625-FAF-2713*

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**DOI:** 10.15789/1563-0625-FAF-2713

# FIBROGENIC AND FIBROLYTIC POTENTIAL OF DIFFERENTLY ACTIVATED HUMAN MACROPHAGES

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**Abstract.** Macrophages are involved in the regulation of fibrogenesis and turnover of the extracellular matrix. One way to perform this function is through the production of profibrotic and fibrolytic factors including fibronectin, laminin, collagen, and extracellular matrix proteases. The production of most of them has been well studied in experimental models; however, much remains unclear regarding human macrophages. Therefore, the aim of this study was to study the content of extracellular matrix proteases (MMP-2 and MMP-9, cathepsin L), their inhibitors (TIMP-1), and collagen (type I) in supernatants of differently activated human macrophages. We compared macrophages differentiated by M-CSF or GM-CSF and further polarized in M1 with lipopolysaccharide, in M2a with IL-4, and in M2c with dexamethasone. Macrophages was obtained from peripheral blood monocytes. The content of MMPs, TIMP, cathepsin, and collagen was determined using appropriate ELISA kits. The results obtained demonstrate that differentiation factors are more important for the production of the above factors compared to polarizing stimuli (lipopolysaccharide, IL-4, dexamethasone). Moreover, macrophages differentiated by M-CSF showed predominantly antifibrotic activity because of pronounced MMPs production, while GM-CSF-induced cultures, on the contrary, were characterized by profibrotic properties due to the high level of TIMP-1 and type I collagen. M1, M2a, and M2c, induced by M-CSF, differed only in MMP-2 production, and M2a produced this metalloproteinase more than other subtypes. In the case of GM-CSF-differentiated cells, a higher level of production of TIMP-1 and, to a lesser extent, type I collagen was characteristic of M1, whereas M2c have minimal concentration of them among GM-CSF-induced macrophage subtypes. Concerning the level of cathepsin L production was relatively constant and did not depend on the generation conditions (differentiation and polarizing signals). Thus, the data obtained help to identify macrophage subtypes with anti- or profibrotic potential and may be useful for the development of cell therapy for diseases associated with fibrogenesis dysregulation.

*Keywords: macrophages, matrix metalloproteinase, collagen, cathepsin, fibrosis, anti-fibrotic activity*

This work was financed from the budget of Research Institute of Fundamental and Clinical Immunology (reg. number 122011800324-4).

## Introduction

Macrophages are the central players in the innate immunity. Due to amazing plasticity, these cells are involved in the regulation of many processes in the human body, including regeneration and tissue remodeling. The ability to produce various biologically active substances allows macrophages to directly influence the turnover of the extracellular matrix (ECM) through the synthesis and secretion of metalloproteinases (MMPs) and other enzymes for ECM degradation. Moreover, it has recently been shown that macrophages directly contribute to the heart regeneration and fibrosis by collagen production [13].

However, there are still many unexplored questions regarding human macrophages. First, animal and human macrophages differ significantly, e.g., in the spectrum and expression level of mRNA of MMPs and TIMPs [10], so the data obtained on experimental models require confirmation in the human. Second, most human macrophage research has been done on classically and alternatively activated macrophages (generally termed M1 and M2a), whereas other subtypes are still incompletely described (M2b, M2c, M2d, etc.) [3, 11]. Finally, many researchers use different differentiation factors to obtain M1 and M2 cells (GM-CSF and M-CSF, respectively), while the conditions of differentiation largely influence the macrophage functional phenotype [5].

Thus, **the aim of our study** was to study how differentiation and polarization signals affect the production of fibromodulatory factors by macrophages, including matrix metalloproteinases (MMP-2 and MMP-9), tissue inhibitors of metalloproteinases (TIMP-1) and collagen, and to determine macrophage phenotypes with a more profibrotic or fibrolytic potential.

## Materials and methods

The study included 63 healthy donors of both sexes aged 22-60 years. Mononuclear cells were isolated by centrifugation of heparinized venous blood in a ficoll-verografin density gradient and then cultured in an amount of $4\text{-}5 \times 10^6$/mL in 12-well plates (TPP, Switzerland) in RPMI-1640 medium (BioloT, Russia) supplemented with 10% fetal bovine serum (Biowest, USA) and 50 ng/mL recombinant M-CSF or GM-CSF (Sigma-Aldrich, USA). After 1 hour, the non-adherent fraction of cells was removed, and the adhesive one continued to be cultured for 7 days. On the 5th day, appropriate polarizing stimuli were added to the cultures: 10 μg/mL LPS (*E. coli* 0114:B4, Sigma-Aldrich, USA) to obtain M1; 20 ng/mL IL-4 (Sigma-

Aldrich, USA) – to M2a; 50 ng/mL dexamethasone (Dex) (KRKA, Slovenia) – to M2c. The supernatants were collected, centrifuged, cryopreserved and stored at -80 °C.

The level of production of MMP-9, MMP-2, TIMP-1, cathepsin L, and type I collagen in supernatants of 7-day-old macrophage cultures was assessed using enzyme immunoassay. To determine the concentration of MMP-2, MMP-9, and TIMP-1, the corresponding ELISA kit (all R&D System kits, USA) was used in accordance with the manufacturer's instructions. Human Cathepsin L Platinum ELISA (Invitrogen, USA) was used to determine the level of cathepsin L production in accordance with the manufacturer's instructions. The level of collagen production was determined by the concentration of the α1-chain of type I collagen using the Human COL1A1 (Collagen Type I Alpha 1) ELISA Kit (FineTest, China) in accordance with the manufacturer's instructions. The data were recalculated per 100,000 cells.

The significance of statistical differences between the compared groups was assessed using nonparametric Wilcoxon matched pair test and Mann–Whitney U test; the differences were considered significant at $p < 0.05$.

## Results and discussion

Initially, we analyzed the content of ECM proteases in the supernatants of M- and GM-CSF-differentiated macrophages. MMP-2 and MMP-9 were chosen for assessment because they are important for fibrogenesis and are the most well produced by human macrophages. Figure 1A shows that the level of MMP-2 in the supernatants of M-CSF-differentiated cells was significantly higher compared to GM-CSF-differentiated analogs. For example, M1(LPS) in the presence of M-CSF produced MMP-2 at a level of 3050 pg/mL, while that in the presence of GM-CSF reached only 33 pg/mL. The content of MMP-2 in M-CSF-differentiated M2a(IL-4) supernatants was more than 100 times higher than that of GM-CSF-induced analogs (5450 and 48 pg/mL, respectively, $p_U < 0.01$), and in M2c(Dex) was more than 70 times higher (2730 and 40 pg/mL, respectively, $p_U < 0.01$). At the same time, among M-CSF-differentiated macrophages, M2a(IL-4) were characterized by a higher MMP-2 value compared to M1(LPS) and M2c(Dex) ($p_W < 0.05$ and $p_W = 0.06$, respectively). No significant differences were found among GM-CSF-differentiated M1(LPS), M2a(IL-4), and M2c(Dex).

Next, we determined the content of MMP-9 (Figure 1B). In general, human macrophages produced this metalloproteinase more actively than MMP-2 (6500-11,000 *vs* 30-5500 pg/mL, respectively). At the same time, the level of MMP-9 in cultures of M-CSF-differentiated macrophages was on average 1.5-1.7 times higher compared to cells differentiated by GM-CSF. M-CSF- and GM-CSF-induced M1(LPS) (10170 *vs* 6770 pg/mL) and M2c(Dex) (11000 *vs* 6540 pg/mL) differed significantly from each other ($p_U < 0.05$). As for M2a(IL-4), despite the fact that in general, M-CSF-induced cells produced more MMP-9 compared to GM-CSF (9330 *vs* 6770 pg/mL), no significant differences were found between subtypes. At the same time, polarizing stimuli apparently had a less significant effect on the level of MMP-9 production, since statistical differences were not found among M1(LPS), M2a(IL-4) and M2c(Dex), differentiated by the same factor.

TIMPs are specific inhibitors of metalloproteinases. M-CSF induced the cells with equally low TIMP-1-producing activity for all subtypes (Figure 1C) (at the lower sensitivity level of the method, ≤ 80 pg/mL). However, GM-CSF-differentiated macrophages quite actively produced this inhibitor (1450-3800 pg/mL). TIMP-1 production by M1(LPS) was the highest (3080 pg/mL) and significantly exceeded M2c(Dex) and M2a(IL-4) at the trend level (1450 and 2100 pg/mL, respectively, $p_W = 0.004$ and 0.06). On the contrary, M2c(Dex) were characterized by a minimal level of TIMP-1 compared to other subtypes of GM-CSF-differentiated cells ($p_W < 0.05$).

Cathepsin L is the one of the proteases for which ECM is a substrate. Figure 1D demonstrates that macrophages produced cathepsin L at relatively the same level, regardless of the differentiation and polarizing stimuli. Thus, M-CSF-differentiated M1(LPS) did not differ significantly from M2a(IL-4) and M2c(Dex), although Cathepsin L concentration in the M2c(Dex) supernatants was slightly higher compared to the other macrophage subtypes (2350 pg/mL *vs* 1650 and 1510 pg/mL, respectively). GM-CSF-differentiated M1(LPS), M2a(IL-4), M2c(Dex) also did not differ in cathepsin L production (1600, 1480, and 1330 pg/mL, respectively).

Finally, the production of type I collagen by human macrophages was investigated. It turned out that all the studied macrophage subtypes produced collagen I at a well-detectable level (Figure 1E). In general, except M2c(Dex), M-CSF-differentiated macrophages had a significantly lower level of collagen I production compared to GM-CSF analogs. Actually, the concentration of collagen in M-CSF-differentiated M1(LPS) supernatants was more than 2.5 times lower than that in GM-CSF-induced M1(LPS) cultures (3350 *vs* 8100 pg/mL, respectively; $p_U = 0.05$). M2a(IL-4) differentiated M-CSF also had lower levels of collagen I compared to GM-CSF analogs (4740 *vs* 7000 pg/mL, $p_U < 0.05$). However, no significant differences were found between M1(LPS), M2a(IL-4), and M2c(Dex) differentiated by M-CSF factor.

MMPs and TIMPs are the most important mediators of ECM turnover, so the ability of macrophages to produce these factors at high concentrations is believed to reflect their ability to maintain tissue homeostasis through ECM remodeling [15]. MMPs degrade ECM proteins, predominantly collagen, while TIMPs are specific inhibitors of MMPs.

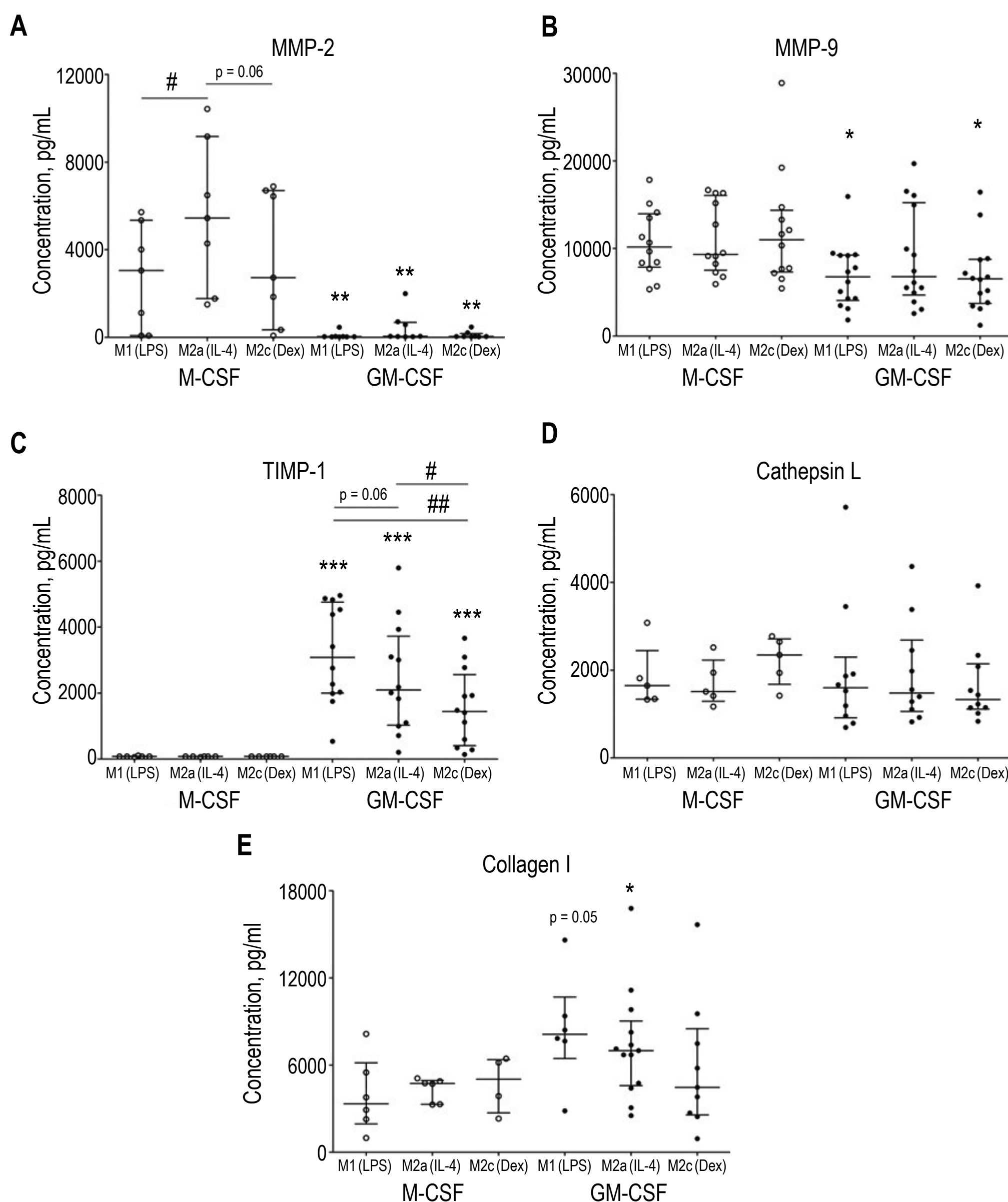


**Figure 1. Production of profibrotic and fibrolytic factors by differently activated human macrophages**

Note. (A) Production of MMP-2 by macrophage subtypes, n = 7-8. (B) Production of MMP-9 by macrophage subtypes, n = 12-14. (C) Production of TIMP-1 by macrophage subtypes, n = 6-12. (D) Production of cathepsin L by macrophage subtypes, n = 5-10. (E) Production of collagen I by macrophage subtypes, n = 4-13. Data are presented as individual values, median and interquartile range; *, $p_U < 0.05$; **, $p_U < 0.01$; ***, $p_U < 0.001$ statistical difference with M-CSF-induced cells. The line indicates the differences between the indicated macrophage subtypes; #, $p_W < 0.05$; ##, $p_W < 0.01$.

The expression of MMPs and TIMPs in cells and tissues is specific and depends on many factors. The synthesis of MMPs and TIMPs is controlled by various microenvironmental signals such as cytokines and growth factors. Moreover, the expression of various MMPs can be stimulated or suppressed by integrin signals, ECM proteins, and cell shape changes. In general, the level of MMP production outside of pathology is quite low, but it significantly increases in tissues during wound healing, repair, and remodeling [10].

Human macrophages produce the wide spectrum of MMPs, as well as TIMPs, and their production depends on the stage of maturation/cell differentiation and the polarization state of macrophages. In general, the expression of most MMPs (MMP-2, MMP-7, MMP-9, etc.) is significantly increased during the differentiation of monocytes into macrophages [10]. At the same time, differentiation factors, such as M-CSF or GM-CSF, can affect the spectrum of MMPs and TIMPs produced. Here we demonstrate that in relation to MMP-2 and MMP-9 production, M-CSF increases MMP levels more than GM-CSF, and this is consistent with the study of Aristorena et al., which indicated that mRNA MMP-2 and

MMP-9 were more strongly expressed in M-CSF-stimulated macrophages (although the differences were not too pronounced) [1].

In addition, various activation signals (LPS, IFNγ, TNFα, IL-1β, IL-4, IL-10) influence the MMP and TIMP profile [6, 7]. However, the data obtained are often contradictory. So, Huang et al. showed that the polarization of macrophages towards M1 phenotype increases the mRNA expression of MMP-1 and MMP-12 along with decrease in MMP-2 expression. As for MMP-9 and TIMP-1, there is no difference between M1 and M2 cells [6]. On the contrary, Jager et al. revealed an increase in MMP-1, MMP-9, MMP-12 and TIMP-1 expression after M2 polarization, while M1 and M2 cells did not differ in the MMP-2 expression [7]. Most likely, these differences are due to the different protocols for the generation of macrophages. Of note, the authors utilized M-CSF for macrophage generation. Along with Huang's study, we also found higher levels of MMP-2 in M-CSF-induced M2a(IL-4) cultures compared to M1(LPS), and showed that there is no significant difference on MMP-9 production between M1(LPS), M2a(IL-4), and M2c(Dex).

TIMP-1 production reflects the profibrotic capacity of cells, and high values of this inhibitor were found in progressive fibrosis in humans and in experimental animals. On the contrary, during the resolution of fibrosis, there is a rapid decrease in the TIMP level and a change in the overall balance of MMP/TIMP, accompanied by an increase in the rate of ECM degradation [2]. In addition, TIMPs can influence fibrogenesis by regulating the growth of various cell types, in particular, by stimulating proliferation of fibroblasts [9]. According to our results, GM-CSF directs macrophage differentiation to a more profibrotic phenotype compared to M-CSF. In contrast to Jager et al., who showed a higher level of TIMP-1 in M-CSF- differentiated macrophages with the M2 phenotype at the level of mRNA expression [7], we did not reveal any differences between M1 and M2 at the protein level. But this discrepancy may be insubstantial, since the level of production often is not in line with the level of gene expression.

Here, we also studied for the first time the production of such an important ECM protease as cathepsin L by differently activated human macrophages. Our data show that macrophages produce cathepsin L at a well-detectable level (1300-2300 pg/mL), which is relatively constant regardless of microenvironmental conditions.

Collagen is the predominant component of ECM playing an important structural role and largely determines the mechanical properties, organization, and structure of tissues. An increase in the collagen amount is associated with the development of fibrosis. It is believed that fibrillar collagens, and especially type I collagen, are of paramount importance. Thus, type I collagen has been shown to be involved in the pathogenesis of pulmonary and hepatic fibrosis, systemic scleroderma, and hypertrophic scars [8].

It was previously thought that all collagens are secreted exclusively by fibroblasts, but it is now known that certain types of collagens can be produced by numerous epithelial cells as well as by macrophages. Several studies in animal models have demonstrated that macrophages express collagen mRNA and isoforms of collagen-associated genes, and are capable of producing various types of collagens, in particular, type I collagen [13, 14]. Moreover, by producing collagen, macrophages directly contribute to the development of cardiac fibrosis [13]. Recent studies suggest that collagen production by macrophages is an important link in the pathogenesis of fibrosis and regeneration [14].

In humans, the ability of macrophages to produce collagen has also been demonstrated. Indeed, Schnoor et al. showed that monocyte-derived macrophages expressed mRNA of all collagen types (except XIII and XXII) are capable of producing type VI collagen [12]. In another study, the high level of expression of mRNA of various types of collagens (IV, VI, VIII, etc.) was also confirmed [4].

Here we first demonstrate that human macrophages are capable of producing type I collagen. Moreover, the level of production of this protein is largely determined by the conditions of macrophage differentiation (M-CSF or GM-CSF), while polarizing stimuli have a less significant effect. Differentiation stimuli can act directly on collagen mRNA expression and its further production or indirectly through the regulation of the MMP/TIMP balance, which in turn affects collagen degradation.

## Conclusion

In summary, M-CSF-differentiated macrophages in general exhibit more fibrolytic properties due to the higher level of production of MMP-2 and MMP-9, along with a low value of TIMP-1 and collagen I, while GM-CSF, on the contrary, promotes profibrotic activity of macrophages. At the same time, polarization in the M1, M2a, M2c direction in the presence of M-CSF slightly affects the production of the studied factors (differences were found only for MMP-2 production). In the case of GM-CSF-differentiated cells, the differences were also not very noticeable. However, in general, it can be concluded that a higher level of production of TIMP-1 and, to a lesser extent, collagen I was characteristic of M1(LPS). In contrast, M2c(Dex) have minimal concentration of them among GM-CSF-induced macrophage subtypes. Thus, the results obtained help to identify macrophage subtypes with anti- or profibrotic potential and may be useful for the development of cell therapy for diseases associated with fibrogenesis dysregulation.

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*Поступила 11.04.2023*
*Принята к печати 11.04.2023*

*Received 11.04.2023*
*Accepted 11.04.2023*

# ОСОБЕННОСТИ ИЗМЕНЕНИЙ УРОВНЯ ЭКСПРЕССИИ РЕЦЕПТОРОВ TNFα И ФУНКЦИОНАЛЬНОГО ОТВЕТА КЛЕТОЧНЫХ ЛИНИЙ ПРИ СТИМУЛЯЦИИ РАЗЛИЧНЫМИ ДОЗАМИ ЦИТОКИНА

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**Резюме.** TNFα является провоспалительным цитокином, передача сигналов которого осуществляется через рецепторы типа 1 (TNFR1) и типа 2 (TNFR2). TNFR1 обычно опосредует апоптоз, выживание клеток и секрецию цитокинов, в то время как TNFR2 избирательно опосредует выживание клеток и секрецию цитокинов. В некоторых случаях при активации рецепторов происходит изменение функционального ответа клеток на противоположный. Активация сигнальных путей имеет свои пусковые механизмы, отличающиеся при взаимодействии между разными формами цитокина и разными формами рецепторных комплексов, а также при изменении соотношения различных типов рецепторов. Изучение механизмов регуляции в системе лиганд-рецептор является приоритетной задачей многих исследований. В данной работе показано дозозависимое влияние TNFα на экспрессию цитокиновых рецепторов и изменение функционального ответа опухолевых клеточных линий различного происхождения. Для этого в исследовании проводили сравнительную оценку экспрессии и ко-экспрессии рецепторов, фаз клеточного цикла и апоптоза клеточных линий без стимуляции и стимулированных TNFα в концентрациях 5 и 50 нг/мл. Было выявлено, что для клеточной линии K562 характерны более выраженные изменения ко-экспрессии рецепторов, которые наблюдались при концентрации TNFα 50 нг/мл по сравнению как с контрольной группой, так и с группой 5 нг/мл. Снижение относительного содержания клеток, экспрессирующих только TNFR1, сочеталось со снижением процента клеток в апоптозе, что подтверждает литературные данные о роли данного рецептора в развитии апоптоза. При этом изменений плотности экспрессии для этой клеточной линии не наблюдалось. Для клеточной линии ZR75-1 наибольшее количество эффектов было выявлено также для концентрации TNFα 50 нг/мл. Увеличение относительного со-

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*Ю.А. Лопатникова, Ю.В. Жукова, А.А. Альшевская, И.А. Облеухова, Ф.Д. Киреев, И.А. Беломестнова, С.В. Сенников «Особенности изменений уровня экспрессии рецепторов TNFα и функционального ответа клеточных линий при стимуляции различными дозами цитокина // Медицинская иммунология, 2023. Т. 25, № 3. С. 459-464.*
*doi: 10.15789/1563-0625-FOC-2718*

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**For citation:**

*Yu.A. Lopatnikova, Yu.V. Zhukova, A.A. Alshevskaya, I.A. Obleukhova, F.D. Kireev, I.A. Belomestnova, S.V. Sennikov “Features of changes in the expression level of TNFα receptors and the functional response of cell lines upon stimulation with various doses of cytokine”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 459-464.*
*doi: 10.15789/1563-0625-FOC-2718*

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**DOI:** 10.15789/1563-0625-FOC-2718

держания клеток, экспрессирующих только TNFR2, сочеталось с увеличением апоптоза, однако плотность экспрессии данного типа рецептора была низкой, что могло повлиять на переключение сигнальных путей в сторону проапоптотических. Таким образом, наше исследование позволило выявить особенности изменений экспрессии и ко-экспрессии рецепторов TNFα, характерных для клеточных линий различного происхождения, а также изменение функционального ответа клеток в ответ на стимуляцию различными дозами цитокина. Все это позволяет расширить представления о регуляторных механизмах в системе цитокин-рецептор.

*Ключевые слова: фактор некроза опухоли альфа, клеточные линии, рецепторы фактора некроза опухоли 1-го типа, рецепторы фактора некроза опухоли 2-го типа, апоптоз, плотность экспрессии рецепторов*

# FEATURES OF CHANGES IN THE EXPRESSION LEVEL OF TNFα RECEPTORS AND THE FUNCTIONAL RESPONSE OF CELL LINES UPON STIMULATION WITH VARIOUS DOSES OF CYTOKINE

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**Abstract.** TNFα is a pro-inflammatory cytokine that is signaled through type 1 (TNFR1) and type 2 (TNFR2) receptors. TNFR1 normally mediates apoptosis, cell survival, and cytokine secretion, while TNFR2 selectively mediates cell survival and cytokine secretion. But in some cases, when receptors are activated, the functional response of cells changes to the opposite. Activation of signaling pathways has its own triggers, which differ in the interaction between different forms of cytokine and different forms of receptor complexes, as well as changes in the ratio of different types of receptors. The study of the mechanisms of regulation in the ligand-receptor system is a priority task for many studies. This work shows the dose-dependent effect of TNFα on the expression of cytokine receptors and changes in the functional response of tumor cell lines of various origins. For this, a comparative assessment of the expression and co-expression of receptors, cell cycle phases and apoptosis of cell lines without stimulation and stimulated with TNFα at concentrations of 5 and 50 ng/mL was carried out. It was found that the K562 cell line was characterized by more pronounced changes in receptor co-expression, which were observed at a TNFα concentration of 50 ng/mL compared to both the control group and the 5 ng/mL group. The decrease in the relative content of cells expressing only TNFR1 was combined with a decrease in the percentage of cells in apoptosis, which confirms the literature data on the role of this receptor in the development of apoptosis. At the same time, no changes in expression density were observed for this cell line. For the ZR75-1 cell line, the largest number of effects was also found for a TNFα concentration of 50 ng/mL. An increase in the relative content of cells expressing only TNFR2 was combined with an increase in apoptosis; however, the expression density of this type of receptor was low, which could affect the switching of signaling pathways towards proapoptotic ones. Thus, our study allowed us to reveal the features of changes in the expression and co-expression of TNFα receptors characteristic of cell lines of various origins, as well as changes in the functional response of cells in response to stimulation with different doses of cytokine. All this allows us to expand our understanding of the regulatory mechanisms in the cytokine-receptor system.

*Keywords: tumor necrosis factor alpha, cell lines, tumor necrosis factor receptor 1, tumor necrosis factor receptor 2, apoptosis, receptor expression density*

The study was supported by a grant from the Russian Science Foundation (project No. 20-75-10051).

## Introduction

TNFα is a pro-inflammatory cytokine that is signaled through type 1 (TNFR1) and type 2 (TNFR2) receptors. TNFR1 is ubiquitously expressed on most cell types. TNFR2 expression has been predominantly described for immune cells. TNFR1 and TNFR2 have structurally different intracellular domains that activate different signaling pathways. TNFR1 normally mediates apoptosis, cell survival, and cytokine secretion, while TNFR2 selectively mediates cell survival and cytokine secretion [4]. However, in some cases, when the receptors are activated, the functional response of the cell changes to the opposite. These processes depend on several possible variants of signaling, which are currently being actively studied [7]. The triggering of signaling pathways, in turn, has its own triggers. One of the activation mechanisms can be the interaction between different forms of cytokine and different forms of receptor complexes, as well as a change in the ratio of different types of receptors [2, 3, 5].

Another mechanism is conformational changes in the receptor, which lead to signaling switching [6]. Finally, regulation may depend on changes in the ligand-receptor ratio. When the threshold level of receptor expression density is reached, switching of signaling pathways is possible [8]. Despite a large number of hypotheses, there is no common understanding of how the functional response of TNF receptors is mediated. The study of the mechanisms of regulation in the ligand-receptor system is a priority for many studies, but there are still many questions regarding the effect of cytokine on the distribution of various types of receptors and their expression density on cells, as well as the functional response to stimulation with various doses of the cytokine. Thus, **the aim of our study** was to study the effect of stimulation with different concentrations of TNF on the co-expression and absolute number of receptors on cells in combination with the assessment of apoptosis and cell cycle phases.

## Materials and methods

The object of the study is the culture of human cell lines: ZR-75-1 (breast carcinoma, ascitic fluid); and K562 (chronic myelogenous leukemia, pleural fluid). All cell lines were provided by the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg, Russia).

Cell cultivation was carried out in culture flasks (TPP, Switzerland) using RPMI-1640 medium containing 10% fetal bovine serum (FBS) (HyClone, USA), 2 mM L-glutamine (BioloT Ltd., Russia), $5 \times 10^{-4}$ M 2-mercaptoethanol (Sigma-Aldrich, USA), 80 μg/mL gentamicin (KRKA, Slovenia), 10 mM HEPES buffer (Sigma-Aldrich, USA), 100 μg/mL of benzylpenicillin (JSC “Biosintez”, Russia), in an incubator in a humid atmosphere at 37 °C and $CO_2$ concentration of 5%.

**Evaluation of the dose-dependent effect of TNFα**

To assess the dose-dependent effect of TNFα, cells were cultured for 72 hours in the absence and presence of recombinant TNFα at concentrations of 5 and 50 ng/mL in plates (TPP, Switzerland) at the optimal density for each cell line. Cells were cultured in an incubator in a humid atmosphere at 37 °C and 5% $CO_2$.

**Estimation of expression density and co-expression of receptors**

Quantitative expression of TNFR1 and TNFR2 receptors was assessed by flow cytometry using a commercial kit of BD QuantiBRITE PE calibration particles (BD Biosciences, USA).

Two combinations of monoclonal antibodies were used to assess co-expression of TNFα type 1 and 2 receptors (TNFR1 and TNFR2) on cells:

TNFR1-Phycoerethrin (R&D System, USA) and TNFR2-Allophycocyanin (R&D System, USA); and TNFR1-Allophycocyanin (R&D System, USA) and TNFR2-Phycoerethrin (R&D System, USA).

The relative abundance of double-negative ($TNFR1^-TNFR2^-$), double-positive ($TNFR1^+TNFR2^+$) and cells expressing only TNFR1 ($TNFR1^+TNFR2^-$) and TNFR2 ($TNFR1^-TNFR2^+$) cells was determined as the average between the two experiments. The analysis was performed on a FacsVerse flow cytometer (BD, USA).

**Assessment of cell cycle phases and apoptosis**

For intracellular DNA labeling, 7-aminoactinomycin D (7-ADD) (BD Biosciences, USA) was used. The analysis was performed on a FacsVerse flow cytometer (BD, USA).

**Statistical analysis**

Statistical processing of the results was carried out using the GraphPad Prism version 8 application package and Microsoft Excel version 2010.

Kruskal–Wallis rank analysis of variance was used to assess the difference between groups. Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

**Co-expression of receptors**

The study evaluated the dose-dependent effect of TNFα on receptor co-expression. Cell lines were incubated with TNFα at doses of 5 and 50 ng/mL and without TNFα. Evaluation of changes in co-expression was carried out after 72 hours.

For the cell line ZR 75-1 (Figure 1), a statistically significant decrease in the percentage of double-positive cells was observed for all concentrations of

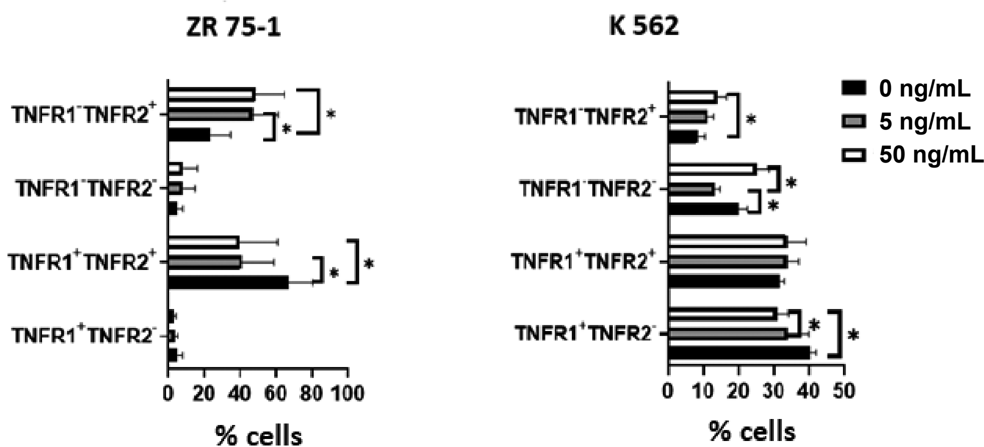


**Figure 1. Co-expression of receptors on K562 and ZR 75-1 cell lines upon stimulation with TNFα at a dose of 5 and 50 ng/mL and without addition of TNFα**

Note. Data are presented as medians normalized to mean values. *, indicate significant ($p < 0.05$, Kruskal–Wallis test for multiple comparisons).

TNFα compared with the control group from 66.9% to 41.2% at a concentration of 5 ng/mL (p = 0.0039) and up to 39.9% at 50 ng/mL (p = 0.013). Also, this cell line was characterized by an almost two-fold increase in the percentage of cells expressing only TNFR2 from 23.7% to 46.7% at a concentration of 5 ng/mL (p = 0.002) and up to 48.7% at a concentration of 50 ng/mL (0.0062) compared to the control group. At the same time, the indicator between groups with a concentration of 5 and 50 ng/mL did not have significant differences.

For the K562 cell line (Figure 1), more pronounced changes were detected. The percentage of cells expressing only TNFR1 decreased for a concentration of 50 ng/mL from 40% to 30% (p = 0.0189) compared with the control, while the percentage of cells expressing only TNFR2 increased from 8.6% to 13.9% (p = 0.0098). The percentage of cells with double-negative cells was lower at 5 ng/mL compared to controls (13.3 and 20%, respectively) (p = 0.0096) and 50 ng/mL (25.1%) (p = 0.0046). Further, the study evaluated the change in the expression density of TNFR1 and TNFR2 receptors on cells with the addition of TNF at concentrations of 5 and 50 ng/mL and without TNF.

Changes in receptor expression density were significant only for the ZR75-1 cell line (Figure 2). There was a significant decrease in the amount of TNFR2 in the 5ng/mL group, both compared with the control (2661 and 6788, respectively (p = 0.002) and compared with the 50 ng/mL group (6184 p = 0.003). For the K562 cell line, statistically significant differences were not found. Next, the functional response of cells was evaluated, during which changes in the relative numbers of cells in various phases of the cell cycle and apoptosis were evaluated.

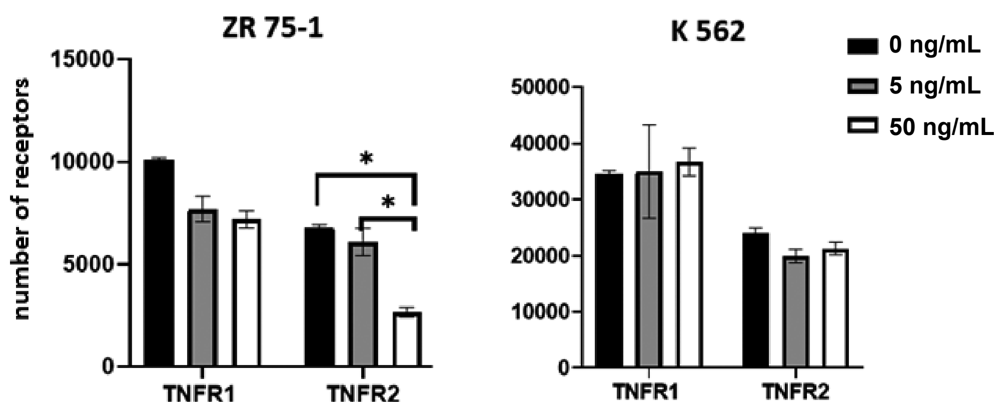


**Figure 2. Density of receptor expression on K562 and ZR75-1 cell lines upon stimulation with TNFα at a dose of 5 and 50 ng/mL and without addition of TNFα**

Note. Data are presented as medians. *, indicate significant ($p < 0.05$, Kruskal–Wallis test for multiple comparisons).

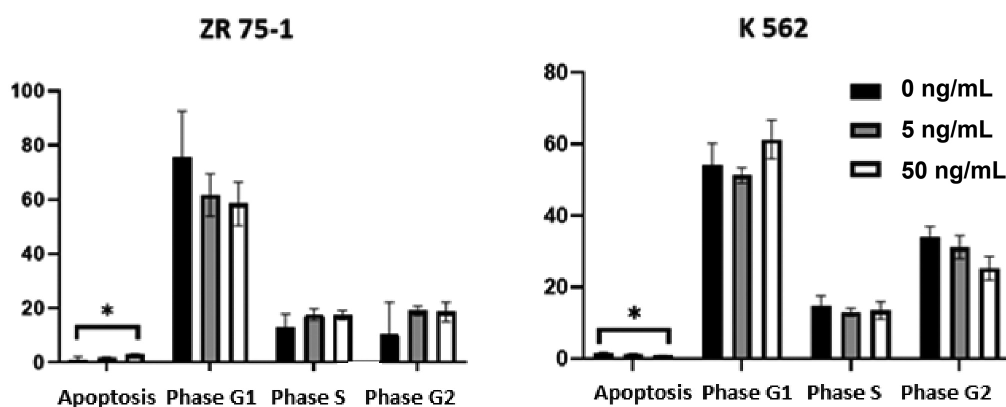


**Figure 3. Phases of the cell cycle and apoptosis in K562 and ZR75-1 cell lines upon stimulation with TNFα at a dose of 5 and 50 ng/mL and without the addition of TNFα**

Note. Data are presented as medians. *, indicate significant ($p < 0.05$, Kruskal–Wallis test for multiple comparisons).

The cell line ZR75-1 (Figure 3) was characterized by an increase in the percentage of cells in apoptosis from 1.1% to 2.8% ($p = 0.0224$) for the group with 50 ng TNFα stimulation compared to the control. For the K562 cell line, the opposite picture was observed; in the 50 ng group, the percentage of cells in apoptosis decreased compared to the control from 1.7 to 0.8% ($p = 0.0003$). As a result of the study, it was found that the K562 cell line is characterized by more pronounced changes in receptor co-expression, which were observed at a TNFα concentration of 50 ng/mL compared to both the control group and the 5 ng/mL group. The decrease in the relative content of cells expressing only TNFR1 was combined with a decrease in the percentage of cells in apoptosis, which confirms the literature data on the role of this receptor in the development of apoptosis [8]. At the same time, no changes in expression density were observed for this cell line.

For the ZR75-1 cell line, the largest number of effects was also found for a TNFα concentration of 50 ng/mL. An increase in the relative content of cells expressing only TNFR2 was combined with an increase in apoptosis; however, the expression density of this type of receptor was low, which could affect the switching of signaling pathways towards proapoptotic ones [1].

## Conclusion

Our study allowed us to reveal the features of changes in the expression and co-expression of TNFα receptors characteristic of cell lines of various origins, as well as changes in the functional response of cells in response to stimulation with different doses of cytokine. All this allows us to expand our understanding of the regulatory mechanisms in the cytokine-receptor system.

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*Поступила 11.04.2023*
*Отправлена на доработку 14.04.2023*
*Принята к печати 16.04.2023*

*Received 11.04.2023*
*Revision received 14.04.2023*
*Accepted 16.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 465-468

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 465-468

# МЕЛАТОНИН В ДИФФЕРЕНЦИРОВКЕ Th17/Treg: ВКЛАД СОБСТВЕННОЙ ПРОДУКЦИИ ГОРМОНА Т-ЛИМФОЦИТАМИ

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**Резюме.** Гормон мелатонин обладает широким спектром биологических эффектов и регулирует работу практически всех органов и систем организма. В иммунной системе важнейшей мишенью мелатонина являются основные эффекторы адаптивного иммунитета – Т-лимфоциты: они экспрессируют специфические мелатониновые рецепторы – мембранные, МТ1 и МТ2, и ядерный, RORα (все с разной аффинностью к гормону), а также ряд внутриклеточных молекул, неспецифически связывающих мелатонин в высоких концентрациях. Более того, в исследованиях *in vitro* многими авторами показана собственная продукция мелатонина Т-лимфоцитами в ответ на поликлональную активацию, а также участие такого эндогенного мелатонина в качестве аутокринного или паракринного фактора в индукции синтеза Т-клетками IL-2 и IL-2-рецептора (IL-2R), причем в реализацию данных эффектов были вовлечены как мембранные, так и ядерный рецепторы для мелатонина. Поскольку IL-2/IL-2R-зависимый сигнал является ключевым событием в индукции пролиферативного ответа Т-лимфоцитов, собственный мелатонин, по-видимому, напрямую задействован как минимум в клональной экспансии этих клеток. Мы в настоящей работе исследовали вклад Т-клеточного мелатонина в регуляцию следующего этапа активации Т-лимфоцитов, а именно, в дифференцировку Т-хелперных популяций Th17 и Treg. Показано, что блокада и мембранных, и ядерного мелатониновых рецепторов не вызывает статистически значимых изменений в дифференцировке Th17, хотя тенденция к снижению фиксировалась. В то же время, уровень $CD4^+FoxP3^+$Т-клеток снижался на фоне неселективной блокады мембранных рецепторов для гормона, а концентрация соответствующего Treg-ассоциированного цитокина TGF-β в супернатантах активированных культур снижалась как в случае неселективной блокады МТ1/МТ2, так и при селективной блокаде МТ2. Полученные данные свидетельствуют о том, что мелатонин, продуцируемый Т-лимфоцитами в культуре, может вносить вклад в контроль дифференцировки наивных $CD4^+$Т-клеток в Treg *in vitro*, причем действие гормона опосредуется мембранными мелатониновыми рецепторами. Наличие у Т-лимфоцитов большого количества разноаффинных мишеней для мелатонина определяет ключевую роль концентрации гормона в его эффектах в отношении этих клеток. Поэтому важно учитывать собственную продукцию гормона лимфоцитами, поскольку Т-клеточный мелатонин может маскировать эффекты экзогенного гормона или препятствовать его действию за счет конкурентного связывания с гормональными рецепторами.

*Ключевые слова: мелатонин, мелатониновые рецепторы, Т-лимфоциты, Th17, Treg, дифференцировка*

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**Образец цитирования:**
*Н.С. Глебездина, Е.М. Куклина, И.В. Некрасова «Мелатонин в дифференцировке Th17/Treg: вклад собственной продукции гормона Т-лимфоцитами» // Медицинская иммунология, 2023. Т. 25, № 3. С. 465-468.
doi: 10.15789/1563-0625-MIT-2798*

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**For citation:**
*N.S. Glebezdina, E.M. Kuklina, I.V. Nekrasova "Melatonin in Th17/Treg differentiation: the contribution of the hormone's own production by t lymphocytes", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 465-468.
doi: 10.15789/1563-0625-MIT-2798*

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**DOI:** 10.15789/1563-0625-MIT-2798

# MELATONIN IN Th17/Treg DIFFERENTIATION: THE CONTRIBUTION OF THE HORMONE'S OWN PRODUCTION BY T LYMPHOCYTES

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**Abstract.** The hormone melatonin is involved in regulation of functioning of almost all organs and systems of the organism. In the immune system, T lymphocytes are an important target of melatonin: they express specific melatonin receptors with different affinities – membrane MT1 and MT2 and nuclear RORα, as well as intracellular molecules that nonspecifically bind melatonin at high concentrations. Moreover, many *in vitro* studies reveal their own production of melatonin by T lymphocytes in response to polyclonal activation and its involvement as autocrine or paracrine factor in the induction of IL-2 and IL-2 receptor (IL-2R) synthesis by T cells, with melatonin receptors involvement in implementation of these effects. Since IL-2/IL-2R-dependent signal is a key event in T lymphocytes proliferative response induction, intrinsic melatonin seems to be directly involved at least in the clonal expansion of these cells. In this work, we investigated the contribution of T cells' melatonin to regulation of the next stage of T lymphocyte activation, namely, the differentiation of T helper populations Th17 and Treg. It was shown that blockade of both membrane and nuclear melatonin receptors did not cause statistically significant changes in Th17 differentiation, although the trend was fixed for a decrease. Simultaneously, $CD4^+FoxP3^+T$ cells level decreased under the nonselective blockade of membrane hormone receptors, and Treg-associated cytokine TGF-β concentration in activated cultures supernatants decreased both in case of MT1/MT2 nonselective blockade and MT2 selective blockade. The data indicate that melatonin produced by T lymphocytes in culture can contribute to the control of naive $CD4^+T$ cell differentiation into Treg *in vitro*, and the hormone effects are mediated by membrane melatonin receptors. The presence of a large number targets with different affinities for melatonin in T lymphocytes determines the key role of the hormone concentration in its effects on these cells. And when interpreting data on melatonin-dependent regulation of Treg, it is important to take into account the hormone's own production by lymphocytes, since T cells' melatonin can mask the exogenous hormone effects or interfere with its action due to competitive binding to hormone receptors.

*Keywords: melatonin, melatonin receptors, T lymphocytes, Th17, Treg, differentiation*

The study was carried out with the financial support of the Russian Science Foundation and the Perm Territory within the framework of scientific project No. 22-25-20121.

## Introduction

The hormone melatonin, synthesized at the system level mainly by the pituitary gland, has multifunctional biological and pharmacological effects, including antioxidant, antitumor, anti-inflammatory, antiviral, antibacterial, neuroprotective ones [5]. It determines the functioning of the nervous and endocrine systems of the organism, and is also able to effectively regulate immune responses due to receptor-dependent and receptor-independent mechanisms [5]. The obvious target of the hormone in the immune system is the main effectors of the adaptive immunity, namely, T lymphocytes: they express at least two high-affinity membrane receptors for melatonin, MT1 and MT2 [15], and for the T helper population Th17, as well as for some regulatory T lymphocytes (Treg), the presence of a nuclear receptor RORα is shown [11].

Furthermore, at micromolar concentrations, melatonin is able to bind other intracellular targets, such as quinone reductase 2, identified as another cytoplasmic receptor for melatonin, MT3 [1], or calmodulin [13] – both factors are also present in T lymphocytes and are involved in the activation signaling. Consequently, melatonin effectively regulates T lymphocytes; its action has been convincingly demonstrated both for the early stages of activation of these cells, including IL-2 production, proliferative response [2, 6, 8] and apoptosis [3], as well as for the functional differentiation of T helper populations, such as Th1, Th2 [6, 14], Th17 and Treg [4, 7, 9].

Moreover, *in vitro* studies, many authors have shown the production of melatonin by T lymphocytes themselves in response to polyclonal activation, in amounts exceeding its upper physiological level, as well as the involvement of such endogenous melatonin as an autocrine or paracrine factor in the induction of IL-2 synthesis and IL-2 receptor (IL-2R) expression by T cells: blockade of hormone synthesis in the cell with a tryptophan hydroxylase inhibitor caused a decrease in the level of both factors, which was reversed by the addition of exogenous melatonin [2, 10, 12]. The involvement of both membrane (MT1/MT2)

and nuclear (RORα) receptors for melatonin in the implementation of these effects has been shown [2]. Since the IL-2/IL-2R-dependent signal is a key event in the induction of T lymphocyte proliferative response, intrinsic melatonin seems to be directly involved at least in the early stages of these cells' activation. And given the ability of the hormone to effectively regulate the functional differentiation of T lymphocytes, in particular, the development of T helper populations, it was important to evaluate the possible involvement of own T cell's melatonin in this process.

**The aim of this work** is to determine the contribution of intrinsic melatonin produced by T lymphocytes to the differentiation of Th17 and Treg cells.

## Methods and materials

We used leukocytes from healthy donors (n = 10, mean age 38.30±1.95 years). All donors signed the informed consent form for participation in the study. The study was approved by the Ethics Committee of the IEGM Ural Branch of the Russian Academy of Sciences (protocol No. 15 dated May 20, 2022) and conducted in accordance with the provisions of the Declaration of Helsinki on research involving humans. Leukocytes were isolated from heparinized venous blood by density gradient centrifugation in ficoll-verografin ($\rho$ = 1.077 g/cm$^3$). We used naive $CD4^+T$ lymphocytes fractionated with commercial isolation systems (BioLegend, USA). $CD4^+T$ cells ($1 \times 10^6$ cells/mL) were cultured for 48 hours in RPMI 1640 medium (Gibco, UK) with 1 mM HEPES (Sigma-Aldrich, USA), 2 mM L-glutamine (Serva, Germany), and 40 U/ml gentamicin (Pharmacia, Sweden) at 37 °C and 5% $CO_2$ without activator (spontaneous variant) and under the polyclonal activation (activation system based on monoclonal antibodies to CD3/CD28, Invitrogen, USA).

The contribution of specific melatonin receptors to the implementation of the effects of the hormone was determined by inhibitory analysis, using the corresponding antagonists for membrane receptors – non-selective (luzindole – for MT1/MT2) and selective (4-P-PDOT – for MT2; both Tocris Bioscience, USA), and for the nuclear melatonin receptor RORα, small interfering RNAs (siRNA, OriGene, USA: three types of RORα-specific siRNA and scrambled siRNA as a negative control). siRNA transfection was carried out using lipofectamine (Invitrogen, USA), and the effectiveness of their action was confirmed by assessing the expression of RORα in cells, both at the mRNA level, by polymerase chain reaction (RT-qPCR), using the SingleShot™ SYBR® Green One- Step Kit (Bio-Rad, USA) and at the protein level by flow cytometry using anti-RORα*PE monoclonal antibodies (R&D Systems, USA).

Cell expression of transcription factors RORγt (Th17 differentiation marker) and FoxP3 (Treg marker) was determined at the end of 48-hour cultivation (by flow cytometry using monoclonal antibodies: anti-CD4*FITC, anti-RORγt*PerCP, anti-FoxP3*PE (Novus Biologicals, R&D Systems, BioLegend, USA)). The synthesis of the key cytokines of the studied subpopulations, IL-17A and TGF-β, was assessed by their level in culture supernatants (enzymatic immunoassay, R&D Systems, BioLegend, USA). Statistical analysis was carried out using STATISTICA 10.0 software. Significance of differences between groups was assessed using Student's t-test. The results are presented as the mean and its standard error (M±m). The reported percentages of Th17 and Treg cells, as well as the concentration of IL-17A and TGF-β in culture supernatants, are normalized with respect to the corresponding control (unstimulated or stimulated).

## Results and discussion

Blockade of specific membrane receptors for melatonin did not reveal statistically significant changes in the differentiation of Th17 lymphocytes when assessing both the content of $CD4^+ROR\gamma t^+T$ cells in culture and the level of IL-17 in culture supernatants, although a downward trend was recorded. At the same time, the level of $CD4^+FoxP3^+T$ cells decreased in the presence of non-selective hormone membrane receptor antagonist (percentage of $CD4^+FoxP3^+T$ cells normalized to control: luzindole – 0.72±0.07 *vs* control – 1.0; $p < 0.05$), but only in a spontaneous variant. The concentration of the corresponding Treg-associated cytokine TGF-β in the supernatants of cultures of activated $CD4^+T$ cells decreased as in the case of non-selective blockade of MT1/MT2 (the level of TGF-β in the supernatants of $CD4^+T$ cell cultures, normalized with respect to control: luzindole – 0.90±0.02 *vs* control – 1.0; $p < 0.05$), and with selective blockade of MT2 (the level of TGF-β concentration in supernatants of $CD4^+T$ cell cultures, normalized with respect to control: 4-P-PDOT – 0.89±0.03 *vs* control – 1.0; $p < 0.05$). Under the blockade of intracellular melatonin receptor RORα using siRNA specific to RORα-mRNA, no statistically significant differences in the percentage of $CD4^+ROR\gamma t^+$ and $CD4^+FoxP3^+$ T cells in culture were found.

## Conclusion

We have shown that melatonin produced by T lymphocytes in culture can contribute to the control of differentiation of naive $CD4^+T$ cells into Treg *in vitro*, and the effects of the hormone are mediated by membrane melatonin receptors. The presence in lymphocytes of a large number of targets for melatonin with different affinities for the hormone indicates that the key factor determining the presence and direction of hormone effects in these cells is its concentration. And when interpreting the results, it is important to take into account self production of melatonin by lymphocytes, which increases upon activation and exceeds its physiological levels in experiments *in vitro* [2, 10, 12], since melatonin synthesized by lymphocytes can mask the effects of both an endogenous hormone secreted by the pineal gland and an exogenous one.

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*Поступила 14.04.2023*
*Отправлена на доработку 20.04.2023*
*Принята к печати 21.04.2023*

*Received 14.04.2023*
*Revision received 20.04.2023*
*Accepted 21.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 469-476



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 469-476

# ВЛИЯНИЕ ГАЛЕКТИНА-9 НА ЭКСПРЕССИЮ МОЛЕКУЛЫ TIM-3 В РАЗНЫХ СУБПОПУЛЯЦИЯХ НАТУРАЛЬНЫХ КИЛЛЕРОВ

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**Резюме.** Галектин-9 является β-галактозид-связывающим лектином и обладает выраженной иммунорегуляторной активностью. Во время беременности галектин-9 вырабатывается клетками трофобласта и модулирует функции естественных киллеров (NK) на границе мать-плод посредством связывания с молекулами Tim-3 (Т-клеточный Ig и белок 3, содержащий домен муцина). NK-клетки периферической крови экспрессируют молекулы Tim-3. Концентрация галектина-9 повышается в периферической крови во время физиологической беременности. При беременности фенотип и функции периферических NK-клеток изменяются для поддержания толерантности иммунной системы матери к генетически чужеродному плоду. Периферические NK-клетки мигрируют к границе раздела мать-плод и трансформируются в децидуальные NK-клетки. Концентрация галектина-9 снижается у женщин с осложненной беременностью и выкидышами. Однако влияние галектина-9 на различные субпопуляции NK-клеток периферической крови не изучено. Поэтому целью работы являлось изучение влияния галектина-9 на трансформацию фенотипа и экспрессию Tim-3 NK-клетками, выделенными из периферической крови здоровых небеременных фертильных женщин. $CD56^+$NK-клетки получали методом иммуномагнитной сепарации и культивировали *in vitro* в течение 72 часов с цитокинами (IL-2 и IL-15), галектином-9 (5 нг/мл). Концентрация галектина-9 соответствует его уровню в периферической крови в первом триместре физиологической беременности. Количество регуляторных NK ($CD16^-CD56^{bright}$), цитотоксических NK ($CD16^+CD56^{dim/-}$) клеток и экспрессию Tim-3 на них оценивали методом проточной цитометрии. Показано, что Tim-3 экспрессировался на всех субпопуляциях NK-клеток периферической крови ($CD16^-CD56^{bright}$NK, $CD16^+CD56^{dim}$NK, $CD16^+CD56^-$NK). Инкубация с галектином-9 увеличивала экспрессию Tim-3 на регуляторных клетках $CD16^-CD56^{bright}$NK и не влияла присутствие Tim-3 на цитотоксических $CD16^+CD56^{dim/-}$NK-клетках. Галектин-9 снижал процент цитотоксических $CD16^+CD56^{dim}$NK в культуре, но не влиял на количество регуляторных

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**Образец цитирования:**
*Е.Г. Орлова, О.А. Логинова, О.Л. Горбунова, С.В. Ширшев «Влияние галектина-9 на экспрессию молекулы Tim-3 в разных субпопуляциях натуральных киллеров» // Медицинская иммунология, 2023. Т. 25, № 3. С. 469-476.*
*doi: 10.15789/1563-0625-GIT-2778*

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**For citation:**
*E.G. Orlova, O.A. Loginova, O.L. Gorbunova, S.V. Shirshev "Galectin-9 influences the Tim-3 molecule expression in natural killer different subpopulations", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 469-476.*
*doi: 10.15789/1563-0625-GIT-2778*

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**DOI:** 10.15789/1563-0625-GIT-2778

$CD16^-CD56^{bright}$ NK и цитотоксических $CD16^+CD56^-$NK-клеток. Таким образом, галектин-9 регулирует экспрессию молекулы Tim-3 и соотношение субпопуляций NK-клеток в культуре *in vitro*.

*Ключевые слова: галектин-9, Tim-3, цитотоксические NK, регуляторные NK, беременность, in vitro*

# GALECTIN-9 INFLUENCES THE TIM-3 MOLECULE EXPRESSION IN NATURAL KILLER DIFFERENT SUBPOPULATIONS

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**Abstract.** Galectin-9 is a β-galactoside binding lectin with expressed immunoregulatory activity. During pregnancy galectin-9 is produced by trophoblast cells and regulates the function of natural killer (NK) cells at the maternal-fetal interface via binding to Tim-3 (T-cell Ig and mucin domain-containing protein 3) molecules. Natural killer (NK) lymphocytes belong to the innate lymphoid cells, which have a cytotoxic effect on target cells and are capable of producing a large number of regulatory factors (cytokines, chemokines). Decidual NK have a tolerant phenotype and play a leading role in the regulation of invasive trophoblast growth and provide peripheral immune tolerance in the area of uteroplacental contact. Peripheral NK cells express Tim-3 molecules. Galectin-9 concentration is increased in peripheral blood during physiologic pregnancy. At pregnancy phenotype and functions of peripheral NK cells are changed to maintain the maternal—fetal immune tolerance. Peripheral NK cells migrate to the maternal-fetal interface and are transformed into a decidual NK-like phenotype cells. Galectin-9 concentration is decreased in women with a complicated pregnancy and miscarriage. However the galectin-9 effects on different NK cell subpopulations of peripheral blood are not investigated. Therefore, we studied the galectin-9 influence on phenotype transformation and Tim-3 expression of NK cells isolated from peripheral blood of healthy non-pregnant fertile women. $CD56^+$NK cells were obtained by immunomagnetic separation and cultivated *in vitro* during 72 hours with cytokines (IL-2 and IL-15). Galectin-9 (5 ng/mL) and anti-Tim-3 (10 mg) antibodies were added to the NK cultures. Galectin-9 concentration is corresponded to its level during first trimester of physiologic pregnancy. The number of regulatory NK ($CD16^-CD56^{bright}$), cytotoxic NK ($CD16^+CD56^{dim/-}$) cells and Tim-3 expression on different NK subpopulations were assessed by flow cytometry. It was found that Tim-3 was expressed on all subpopulations of peripheral blood NK cells ($CD16^-CD56^{bright}$NK, $CD16^+CD56^{dim}$NK, $CD16^+CD56^-$NK). Incubation with galectin-9 increased the expression of Tim-3 on regulatory $CD16^-CD56^{bright}$NK cells and did not change on cytotoxic $CD16^+CD56^{dim/-}$NK cells. Galectin-9 reduced the percentage of cytotoxic $CD16^+CD56^{dim}$NK in culture, but did not influence the number of regulatory $CD16^-CD56^{bright}$ NK and cytotoxic $CD16^+CD56^-$NK cells. Thus, galectin-9 regulates Tim-3 molecule expression and NK cell subpopulation distributions *in vitro* culture.

*Keywords: galectin-9, Tim-3, cytotoxic NK, regulatory NK, pregnancy, in vitro*

This work was supported by the Russian Science Foundation, Project No. 22-25-00694.

## Introduction

Galectin-9 is a β-galactoside binding lectin with expressed immunomodulatory activity produced by many type of cells. Galectin-9 participates in regulation of many physiological processes such as cell growth, differentiation, adhesion, communication and death [6]. During pregnancy trophoblast cells secrete galectin-9 and regulate the function of decidual natural killer (NK) cells at the maternal-fetal interface via binding to Tim-3 (T cell Ig and mucin domain-containing protein 3) receptor molecules [9]. Natural killer (NK) lymphocytes belong to the innate lymphoid cells, which have a cytotoxic effect on target cells and are capable of producing a large number of regulatory factors (cytokines, chemokines). NK eliminate virus-infected and tumor cells by releasing cytotoxic granules containing granzyme B or by engaging death receptors that initiate caspase cascades.

During pregnancy, the NK cytotoxic potential in peripheral blood decreases due to a diminish in the percentage of cytotoxic $CD16^{+}CD56^{dim}$NK and elevation of regulatory $CD16^{-}CD56^{bright}$NK cells [4]. At the same time, the increased number of $CD16^{+}56^{dim}$NK in the peripheral blood of women is associated with spontaneous pregnancy loss, because $CD16^{+}56^{dim}$NK are able to lyse of trophoblast cells [4]. Recent studies have shown that there is also a subpopulation of cytotoxic exhausted $CD16^{+}CD56^{-}$ NK cells in peripheral blood, which are characterized by reduced cytotoxic and secretory activity [3]. The number of $CD16^{+}CD56^{-}$NK increases in severe viral infections and is associated with a decrease in antiviral immunity, but changes in their number and functions during physiological pregnancy have not been studied [3]. It is generally accepted that the peripheral blood $CD56^{bright}CD16^{-}$NK cells migrate to the uterus and transform into decidual NK cells during early pregnancy [4]. Decidual NK have a tolerant phenotype and play a leading role in the regulation of invasive trophoblast growth and provide peripheral immune tolerance in the area of utero-placental contact [4]. However, the mechanisms of the NK phenotype shift from cytotoxic towards the tolerant during pregnancy remain poorly understood.

Tim-3 is a marker of NK cells activation and maturation and plays a critical role in the NK function regulation including degranulation, cytotoxicity, cytokine production, fetal trophic functions [5]. Galectin-9/Tim-3 signaling suppress decidual NK cells cytotoxicity and regulate cytokine productions [8]. Tim-3-positive decidual NK cells display higher interleukin (IL)-4 and lower tumor necrosis factor (TNF)-$\alpha$ and perforin production [8]. Tim-3 blockade on decidual NK results in fetal loss in mice [10]. In contrast, other reports have provided evidence that Tim-3 functions as a NK-cell coreceptor to enhance interferon-gamma production, which has important implications for control of infectious disease and cancer [5]. Other authors have shown that increased Tim-3 expression on NK cells leads to NK cell dysfunction in chronic virus infections, such as hepatitis B and HIV infection [9]. However, the functions of Tim-3 in peripheral blood NK function regulation during pregnancy are not elucidated. NK cells of peripheral blood constitutively express Tim-3 molecules [9]. Galectin-9 concentration is gradually increased in peripheral blood during physiologic pregnancy, but the decrease of galectin-9 level is associated with a complicated pregnancy and miscarriage [1]. All mentioned above emphasizes the important galectine-9/Tim-3 role in NK cells phenotype and function regulation associated with successful pregnancy. However the galectin-9 effects on different NK cell subpopulations of peripheral blood are not investigated. Therefore **the aim of this work** was studied the galectin-9 influence on phenotype and Tim-3 expression of peripheral blood NK cells.

## Materials and methods

Peripheral blood of healthy non-pregnant fertile women (in follicular phase of the menstrual cycle) aged 21-29 years was studied (n = 12). The inclusion criteria were the absence of acute and chronic somatic, endocrine, autoimmune, genetic diseases; compliance with diet, treatment with contraceptive and hormonal, anti-inflammatory or antibacterial drugs. This study was approved by the local ethics committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences in accordance with the Helsinki Declaration. Written informed consent was received from all participants.

Peripheral blood samples were collected in sodium heparin vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by ficoll-verografin (1.077g/$cm^3$) density gradient centrifugation.

NK cells were obtained from PBMC by the method of negative immunomagnetic separation (depletion of the PBMC population from T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells, using the NK cell isolation kit (Miltenyi Biotec, USA). The purity of the isolated $CD56^{+}CD3^{-}$NK was more than 95% (estimated as the number of $CD56^{+}$ in a gate of $CD3^{-}$ cells measured by flow cytometry with CD56-BV605 and CD3-PE (Figure 1). Purified NK cells (1 $10^5$) were cultured in 0,3 mL of complete media (RPMI-1640 (Gibco, UK); with 10% fetal bovine serum (Biolot, Russia); 1 мM HEPES (Biolot, Russia); 2 мM L-glutamine (ServiceBio, China), penicillin G (100 U/mL) – streptomycin (0.1 mg/mL) (Biolot, Russia) in 96-well cell culture plates (Eppendorf, USA) during 72 hours at 37 °C in a humid atmosphere with 5% $CO_2$. Cultures were supplemented with IL-15 (10 ng/mL) and IL-2 (500 ng/mL) (Cloud-Clone Corp, USA) [2]. The purified NK cells were subjected to galectin-9 (5 ng/mL; Cloud-Clone Corp, USA) and anti-human Tim-3 (CD366) (10 mg/mL; ultra-LEAFO blocking antibodies, BioLegend, USA) [8]. Galectin-9 concentration was corresponded to its level during first trimester of physiologic pregnancy [11]. The NK cells were harvested for flow cytometry after 72 h of incubation.

NK cell phenotype was assessed by flow cytometry using the following antibodies: CD3 (PE anti-human CD3, clone OKT3, eBioscience), CD56 (Brilliant Violet 605™ anti-human CD56 (NCAM), clone HCD56, BioLegend), CD366 (APC anti-human CD366 (Tim-3), clone F38-2E2, BioLegend). Cells were labeled with Zombie UV™ (Zombie UV™

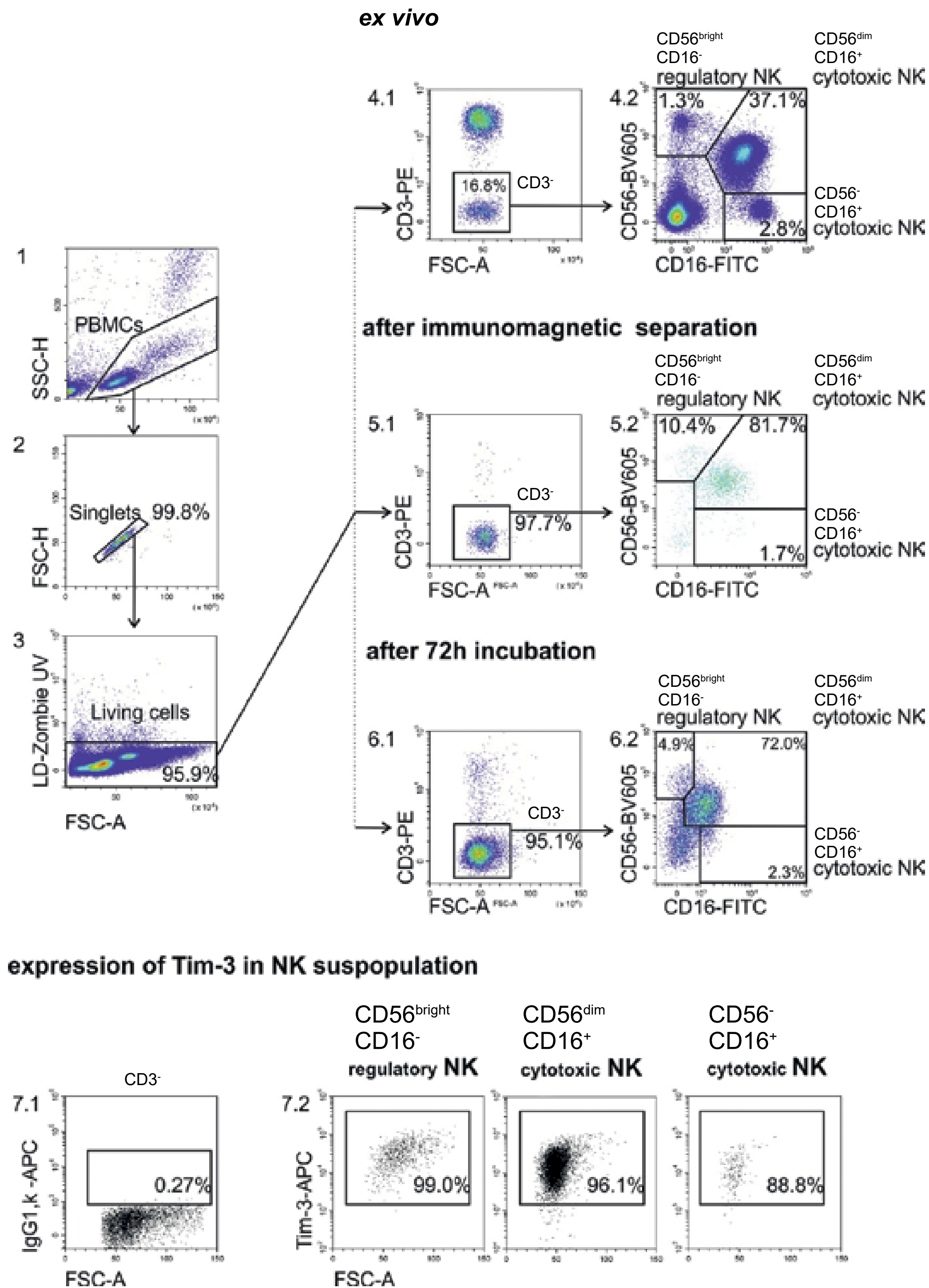


**Figure 1. Gating strategy for NK cell phenotype and Tim-3 expression assessment**

Note. 1, PBMC gate selection according to the forward (FSC-A) and side (SSC-H) scattering parameters; 2, discrimination of doublets according to the FSC-A/FSC-H parameters; 3, discrimination between dead and live cells by LIVE/DEAD-ZOMBIE UV stained; 4.1, selection of $CD3^-$ and $CD3^+$ cells in the peripheral blood PBMC living cell gate; 4.2, the number of the regulatory subpopulation of NK was determined as a percentage of $CD16^-CD56^{bright}$ and cytotoxic subpopulation of NK as a percentage of $CD16^+CD56^{dim}$ and $CD16^+CD56^-$ in the gate of CD3-negative PBMC (*ex vivo* – before immunomagnetic separation; 5.1, selection of $CD3^-$ and $CD3^+$ cells in the living cell PBMC gate after immunomagnetic separation; 5.2, the percentage of the NK cell different subpopulations according to CD16 and CD56 coexpression in the CD3-negative gate in after immunomagnetic separation; 6.1, selection of $CD3^-$ and $CD3^+$ cells in the living cell PBMC gate after immunomagnetic separation and 72 h cultivation; 6.2, the percentage of the NK cell different subpopulations according to CD16 and CD56 coexpression in the CD3-negative gate after immunomagnetic separation and 72 h cultivation; 7.1, the level of non-specific binding was determined using isotyping mAbs (isotype) for assessment of Tim-expression in the studied subpopulations (7.2).

Figure 1 shows histograms of one representative experiment.

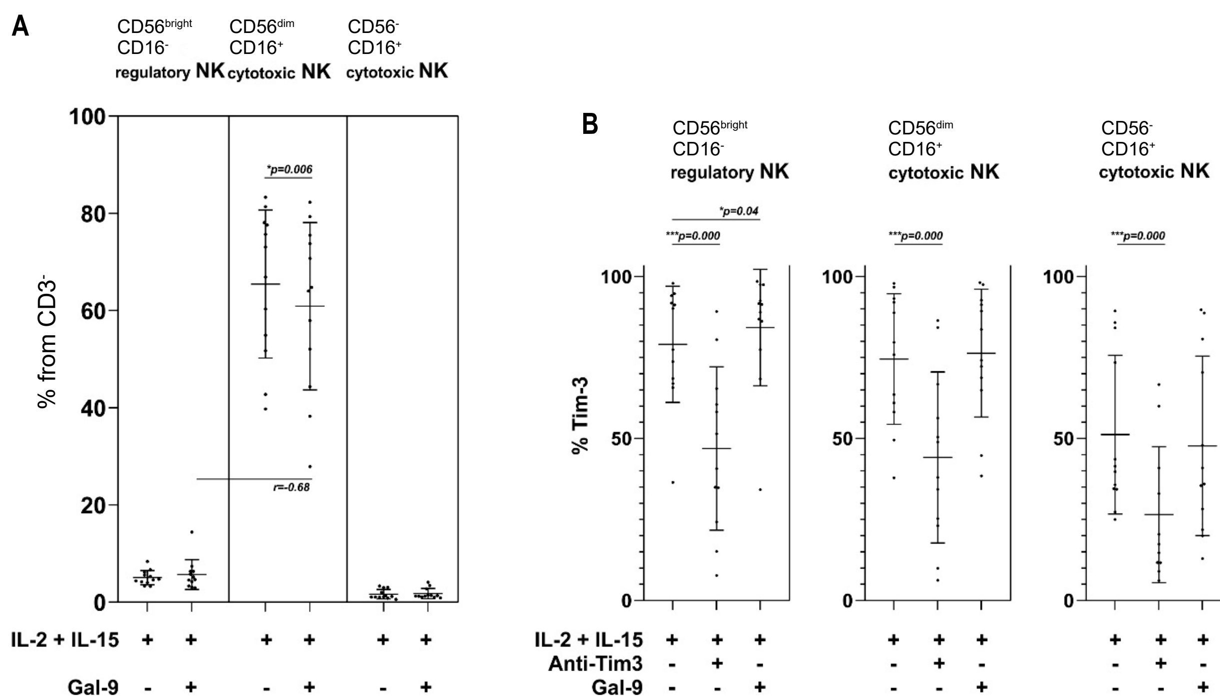


**Figure 2. Assessment of the NK subpopulations**

Note. (A) regulatory NK ($CD3^-CD16^-CD56^{bright}$) and cytotoxic NK ($CD3^-CD16^+CD56^{dim}$) and ($CD3^-CD16^+CD56^-$) after 72 h incubation *in vitro* with cytokines (IL-15, IL-2) and galectin-9 (Gal-9). (B) Assessment of Tim-3 expressions in different NK subpopulations – regulatory NK ($CD3^-CD16^-CD56^{bright}$) and cytotoxic NK ($CD3^-CD16^+CD56^{dim}$) and ($CD3^-CD16^+CD56^-$) after 72h incubation *in vitro* with cytokines (IL-15, IL-2) and galectin-9 (Gal-9).

In Figures 2A and 2B, data are presented as median and the lower and upper quartiles, Me ($Q_{0.25}$-$Q_{0.75}$); *, p value by two-tailed paired t-test in comparison to the NK cell cultures treated with cytokines only; r, Pearson's correlation coefficient.

Fixable Viability Kit, BioLegend) to assess cell viability.

NK subpopulations were determined by the co expression of CD56 and CD16 molecules in the gate of CD3-negative mononuclear cells: regulatory NK – $CD16^-CD56^{bright}$, cytotoxic NK – $CD16^+CD56^{dim}$ and $CD16^+CD56^-$. Gating strategy was presented in Figure 1.

The data were presented as median and the lower and upper quartiles, Me ($Q_{0.25}$-$Q_{0.75}$). Statistical analyses were performed using "GraphPad Prism version 8.01" (StatSoft, USA). The Kolmogorov-Smirnov test was used for verifying normal distribution. The significance of the difference between two groups was determined using the two-tailed paired t-test. Spearman rank correlation (r) test was used for correlation analysis. The differences were considered as significant at $p < 0.05$.

## Results and discussion

To investigate the galectin-9 role in the phenotype regulation of the peripheral blood NK cells, PBMC were isolated from a peripheral blood of healthy fertile non-pregnant women. $CD56^+$NK cells were isolated by immunomagnetic separation. The purity of $CD56^+$NK cells was more than 95% confirmed using flow cytometric analysis after magnetic bead sorting (Figure 1B). Purified $CD56^+$NK were incubated with IL-15 and IL-2 during 72h. IL-15 and IL-2 have been reported to have stimulating effects on NK cell survival *in vitro* [2]. NK cell viability was assessed by Zombie staining in cultures and was more than 95% after incubation with IL-15, IL-2 and less than 50% without cytokines (data not shown).

Purified NK cells were cultured with galectin-9 in concentration corresponding to its level during first trimester of physiologic pregnancy [11]. It is known that in first trimester of pregnancy NK cytotoxic potential in peripheral blood decreases due to a diminish in the percentage of cytotoxic $CD16^+CD56^{dim}$NK and elevation of regulatory $CD16^-CD56^{bright}$NK cells [4]. At the same time, peripheral blood $CD56^{bright}CD16^-$NK cells migrate to the uterus and transform into decidual NK cells during early pregnancy [4]. The percentage of regulatory $CD16^-CD56^{bright}$ NK, cytotoxic $CD16^+CD56^{dim}$ NK and $CD16^+CD56^-$ NK were estimated in galectine-9-primed NK cultures. The results showed that galectin-9 reduced the percentage of cytotoxic $CD16^+CD56^{dim}$NK in cultures, but did not influence the percentage of regulatory $CD16^-CD56^{bright}$NK and cytotoxic $CD16^+CD56^-$NK cells (Figure 2A).

According to literature, the percentage of cytotoxic $CD16^+CD56^{dim}$NK may reduce due to their transformation into $CD16^-CD56^{bright}$NK or cytotoxic $CD16^+CD56^-$NK cells or undergo apoptosis *in vitro* [7]. Therefore the percentage of Zombie-negative $CD16^+CD56^{dim}$NK cells was assessed after galecting-9 incubation. Zombie UV™ is a fluorescent dye that is non-permeant to live cells, but permeant to the cells with compromised membranes that help to discriminate apoptotic/necrotic cells from live cells. There were no significant differences in the percentage of Zombie-negative $CD16^+CD56^{dim}$NK cells in cultures with cytokines (control) and in cultures with galecting-9 (% of Zombie-negative $CD16^+CD56^{dim}$NK cells in control = 79.04 (69.0-88.3); in cultures with galecting-9 = 82.4 (70.5-87.5); $p > 0.05$). We suggested that galecting-9 influence NK phenotype transformation due to CD16 and CD56 expressions regulation *in vitro* cultures. This suggestion is confirmed by the correlation analysis. The percentage of $CD16^+CD56^{dim}$NK cells decreased proportionally to $CD16^-CD56^{bright}$ NK increased after galectin-9 incubation ($r = -0.68$; $p < 0.05$) (Figure 2A). Thus, galectin-9 in concentration at first trimester of pregnancy regulates peripheral blood NK cell subpopulation distributions *in vitro* culture. Other authors had shown that during pregnancy trophoblast cells secrete galectin-9 and reduce cytotoxicity, TNFα and perforin production by decidual NK cells at the maternal-fetal interface [8, 9].

It is known that galectin-9 realizes immunomodulatory activity due to Tim-3 interactions on NK cells [8, 9]. The Tim-3 expression was analyzed on different subpopulations of peripheral blood NK cells after 72h incubation with cytokines *in vitro*. It was found that Tim-3 was expressed in all subpopulations of peripheral blood NK cells ($CD16^-CD56^{bright}$NK, $CD16^+CD56^{dim}$NK, $CD16^+CD56^-$NK) (Figure 2B). Incubation with galectin-9 increased the percentage of Tim-3-positive regulatory $CD16^-CD56^{bright}$NK cells and did not influence on cytotoxic Tim-3-positive $CD16^+CD56^{dim}$NK and $CD16^+CD56^-$NK cells (Figure 2B). It should be noted that obtained data in agreement with the results of another authors that galectin-9 affects the formation of a regulatory phenotype of decidual NK with increased Tim-3 expression [8, 9]. The Tim-3 blockade by anti-Tim-3 antibodies expected decreased Tim-expression on all investigated subpopulations of peripheral blood NK cells ($CD16^-CD56^{bright}$, $CD16^+CD56^{dim}$, $CD16^+CD56^-$) (Figure 2B).

## Conclusion

Thus, galectin-9 regulates the expression of Tim-3 molecules on $CD16^-CD56^{bright}$NK cells and NK cell subpopulation distributions *in vitro* culture. The obtained results are important for understanding the underlying mechanism of immune dysfunctions during pregnancy and could have significant value in treating reproductive disorders associated with NK cells, including intrauterine growth restriction and repeated miscarriages.

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*Поступила 14.04.2023*
*Отправлена на доработку 19.04.2023*
*Принята к печати 21.04.2023*

*Received 14.04.2023*
*Revision received 19.04.2023*
*Accepted 21.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 477-482



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 477-482

# ИТАКОНАТ РЕГУЛИРУЕТ ПРОДУКЦИЮ ЦИТОКИНОВ ЗА СЧЕТ ПОДАВЛЕНИЯ СУКЦИНАТДЕГИДРОГЕНАЗЫ В МОДЕЛИ ЛПС-ИНДУЦИРОВАННОГО ВОСПАЛЕНИЯ

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**Резюме.** Итаконат – это иммунорегуляторный метаболит, продуцируемый миелоидными клетками и играющий ключевую роль в регуляции иммунного ответа. Итаконат, с одной стороны, способен подавлять активность сукцинатдегидрогеназы (СДГ), тем самым внося существенный вклад в метаболическое репрограммирование клетки. С другой стороны, итаконат может регулировать активность ряда транскрипционных факторов и регуляторов транскрипции, тем самым влияя на экспрессию генов. В большинстве экспериментальных работ итаконат охарактеризован преимущественно как противовоспалительное вещество. В частности, итаконат, продуцируемый активированными макрофагами, подавляет продукцию цитокинов TNF, IL-1β, IL-6, IL-10. Тем не менее некоторые данные свидетельствуют и о провоспалительной роли итаконата в ряде мышиных моделей заболеваний. Так, делеция гена *Acod1*, ответственного за продукцию итаконата, приводит к подавлению продукции TNF и IL-6 в модели мышиного полимикробного сепсиса, а значит, в контексте воспаления *in vivo* итаконат может выступать как индуктор провоспалительных цитокинов. Механизм регуляции итаконатом продукции цитокинов при системном воспалении остается неизученным. В этой работе мы показали, что инъекция итаконата и его производного диметилитаконата мышам с последующей индукцией воспаления бактериальным липополисахаридом (ЛПС) приводит к изменению содержания цитокинов в крови. Интересно, что системная продукция IL-6 и IL-10 в ответ на итаконат увеличивается, вопреки результатам, ранее полученным на клеточных культурах. При этом продукция IFNγ, наоборот, подавляется. По-видимому, итаконат регулирует продукцию цитокинов *in vivo* за счет подавления активности СДГ. Инъекция ингибитора СДГ, диметилмалоната, с последующей индукцией воспаления у мышей, приводит к аналогичным изменениям содержания цитокинов в крови, наблюдаемым в ответ на итаконат: повышению продукции IL-6, IL-10 и подавлению продукции IFNγ. Наоборот, добавление сукцината, субстрата СДГ и, соответственно, ее активатора, приводит к противоположному эффекту на продукцию цитокинов. Таким образом, можно предположить, что наблюдаемые эффекты

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**Образец цитирования:**
*Д.Э. Анисов, М.С. Друцкая, М.А. Носенко, С.А. Недоспасов «Итаконат регулирует продукцию цитокинов за счет подавления сукцинатдегидрогеназы в модели ЛПС-индуцированного воспаления» // Медицинская иммунология, 2023. Т. 25, № 3. С. 477-482.*
*doi: 10.15789/1563-0625-IMI-2841*

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**For citation:**
*D.E. Anisov, M.S. Drutskaya, M.A. Nosenko, S.A. Nedospasov "Itaconate-mediated inhibition of succinate dehydrogenase regulates cytokine production in LPS-induced inflammation", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 477-482.*
*doi: 10.15789/1563-0625-IMI-2841*

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**DOI:** 10.15789/1563-0625-IMI-2841

итаконата на продукцию цитокинов в модели ЛПС-индуцированного воспаления опосредованы его способностью ингибировать СДГ. Эти результаты помогают понять неоднозначную роль итаконата при воспалении и проливают свет на не описанную ранее взаимосвязь работы СДГ и продукции цитокинов в воспалении *in vivo*.

*Ключевые слова: иммунометаболизм, врожденный иммунитет, итаконат, макрофаги, воспаление, сукцинатдегидрогеназа*

# ITACONATE-MEDIATED INHIBITION OF SUCCINATE DEHYDROGENASE REGULATES CYTOKINE PRODUCTION IN LPS-INDUCED INFLAMMATION

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**Abstract.** Itaconate is an immunoregulatory metabolite produced by myeloid cells and plays a key role in the regulation of the immune response. Itaconate, on the one hand, is able to suppress the activity of succinate dehydrogenase (SDH), thereby making a significant contribution to the metabolic reprogramming of the cell. On the other hand, itaconate can regulate the activity of a number of transcription factors and transcription regulators, thereby affecting gene expression. In most experimental studies, itaconate has been characterized predominantly as an anti-inflammatory agent. In particular, itaconate produced by activated macrophages inhibits the production of cytokines TNF, IL-1β, IL-6, IL-10. However, some evidence suggests a pro-inflammatory role for itaconate in a number of mouse disease models. Thus, the deletion of the *Acod1* gene responsible for the production of itaconate leads to the suppression of the production of TNF and IL-6 in the mouse polymicrobial sepsis model, which means that in the context of inflammation *in vivo*, itaconate can act as an inducer of pro-inflammatory cytokines. The mechanism of itaconate regulation of cytokine production in systemic inflammation remains unexplored. In this work, we have shown that injection of itaconate and its derivative dimethyl itaconate into mice, followed by induction of inflammation by bacterial lipopolysaccharide (LPS), leads to changes in the content of cytokines in the blood. Interestingly, the systemic production of IL-6 and IL-10 in response to itaconate is increased, contrary to the results previously obtained in cell cultures. At the same time, IFNγ production, on the contrary, is suppressed. Apparently, itaconate regulates the production of cytokines *in vivo* by suppressing the activity of SDH. Injection of the SDH inhibitor, dimethylmalonate, followed by induction of inflammation in mice, results in similar changes in blood cytokines observed in response to itaconate: increased production of IL-6, IL-10 and suppression of IFNγ production. On the contrary, the addition of succinate, a SDH substrate, leads to the opposite effect on cytokine production. Thus, it can be assumed that the observed effects of itaconate on cytokine production in the model of LPS-induced inflammation are mediated by its ability to inhibit SDH. These results help to understand the controversial role of itaconate in inflammation and shed light on a previously undescribed relationship between SDH and cytokine production in inflammation *in vivo*.

*Keywords: immunometabolism, innate immunity, itaconate, macrophages, inflammation, succinate dehydrogenase*

This work was supported by RSF grant No. 19-75-30032.

## Introduction

Immune cell activation is closely related to alterations in the intensity of metabolic pathways. These alterations lead to accumulation of individual metabolites, which, in addition to their main function in biosynthetic and bioenergetic processes, act as immune effector molecules which strongly affects the phenotype of immune cells. In the light of this paradigm, we can call them immunometabolites [12].

One of such immunometabolites is itaconate. Itaconate is obtained via decarboxylation of cis-aconitate, the Krebs cycle intermediate, by aconitate decarboxylase 1 (also known as Irg1). Itaconate can directly inhibit the enzymatic activity of succinate dehydrogenase (SDH), the key enzyme of the

Krebs cycle, because of its structural similarity with succinate [3]. It is equally important that itaconate can also alkylate cysteine residues on multiple proteins and thereby regulate the activity of a number of enzymes and pathways. Itaconate affects KEAP1-NRF2 axis [5], which regulates the expression of antioxidant proteins and ATF3-IκBζ axis, which coordinates the second wave of the transcriptional response to TLR stimulation [1]. Itaconate can also suppress the work of glycolysis enzymes [7], NLRP3 inflammasome activation [2] and NADPH oxidase functioning [9].

Since the discovery of the immunoregulatory properties of itaconate in LPS-activated bone marrow derived macrophages (BMDM), it has been shown to mediate anti-inflammatory effects. Itaconate administration led to downregulation of inflammatory marker genes and production of inflammatory mediators, including pro-inflammatory cytokines. TNF, IL-1β, IL-6, IL-12p40, IL-18 production was suppressed in macrophage cultures in response to a range of PAMPs/DAMPs. Anti-inflammatory properties of itaconate were also shown in a number of experimental disease models in mice, such as sepsis [4], psoriasis [1], peritonitis [2], pulmonary fibrosis [6].

However, itaconate effects on cytokine production in experimental disease models in mice differ from study to study. Systemic IL-6 production was suppressed in the mouse model of hepatic ischemia-reperfusion injury [11]. At the same time there was also evidence suggesting a pro-inflammatory role for itaconate in inflammation *in vivo*. It was shown that myeloid-specific *Irg1* deletion is sufficient to suppress systemic IL-6 levels in the mouse model of polymicrobial sepsis, which means that itaconate can also enhance IL-6 production [10]. Here we report that itaconate increases systemic production of IL-6 and IL-10 and decreases IFNγ systemic production in the mouse model of LPS-induced inflammation via inhibition of the SDH activity.

## Materials and methods

**Mice**

C57Bl/6 mice (6-8 weeks) were housed in SPF conditions at the Animal Facility of the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, EIMB RAS (under contract No. 075-15-2021-1067 with the Ministry of Science and Higher Education of the Russian Federation). All manipulations with animals were carried out in accordance with the protocol approved by the Bioethics Committee of the EIMB RAS (Protocol No. 3 from 27/10/22).

***In vivo* model of endotoxin-induced sepsis**

Neutral itaconate and succinate solutions were prepared by adding 10 M NaOH to itaconate or succinate solutions (100 mg $mL^{-1}$) in PBS to neutral pH. Wild type C57Bl/6 mice were treated intraperitoneally (i.p.) with itaconate (1 g $kg^{-1}$, Sigma), succinate (1 g $kg^{-1}$, Sigma) or dimethyl itaconate (1 g $kg^{-1}$, Sigma) and dimethyl malonate (0,3 or 0,5 g $kg^{-1}$, Sigma) in corn oil (Sigma) or vehicle control for 2 h before stimulation with LPS (*E. coli* O55:B5; 2.5 mg $kg^{-1}$, Sigma) i.p., 4 h later mice were euthanized, blood and organ samples were collected.

**Cytokine detection**

Cytokines in blood serum were analyzed by enzyme-linked immunosorbent assay (ELISA) using commercial kits “Mouse IL-6 ELISA Ready-SET-Go”, “Mouse IL-10 ELISA Ready-SET-Go”, “Mouse IFN gamma ELISA Ready-SET-Go” (Thermo-Fisher) according to the manufacturer’s protocol or measured using Luminex xMAP multiplex technology and MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel kit according to the manufacturer’s standard protocol (Merck).

**RNA isolation and real time quantitative PCR**

Organ samples were homogenized using PowerLyzer 24 Homogenizer (Qiagen) in ExtractRNA reagent (Evrogen). Reverse transcription and sample preparation were carried out using RevertAid kit according to the manufacturer’s recommendations (Thermo Scientific). qPCR was performed using qPCRmix-HS SYBR (Evrogen) according to the manufacturer’s instructions. Quantitative PCR was performed using Quant Studio 6 (Applied Biosystems).

**Statistical analysis**

Data were analyzed using GraphPad Prism 8 software. Data were analyzed using one-way ANOVA test followed by Tukey’s post-test analysis for multiple comparisons. Results are displayed as mean ± SEM. Multiplex data are displayed as z-score transformed (heatmap). Differences were considered significant when p values were below 0.05.

## Results and discussion

**ITA and DI regulate systemic production of IL-6, IL-10 and IFNγ in a mouse model of LPS-induced inflammation**

To investigate itaconate effect on cytokine production in inflammation, mice were pre-treated with ITA or DI, and then inflammation was induced with a non-lethal dose of LPS. Blood cytokine screening revealed a similar pattern of changes in the levels of cytokines in the blood (Figure 1A). Despite our expectations, IL-6 and IL-10 levels were significantly increased in the blood in response to itaconate (Figure 2B, C). Itaconate also dramatically decreased IFNγ levels in the blood (Figure 2D). We also observed the similar effects of itaconate on cytokine production at the gene expression level. DI increased *Il6* and

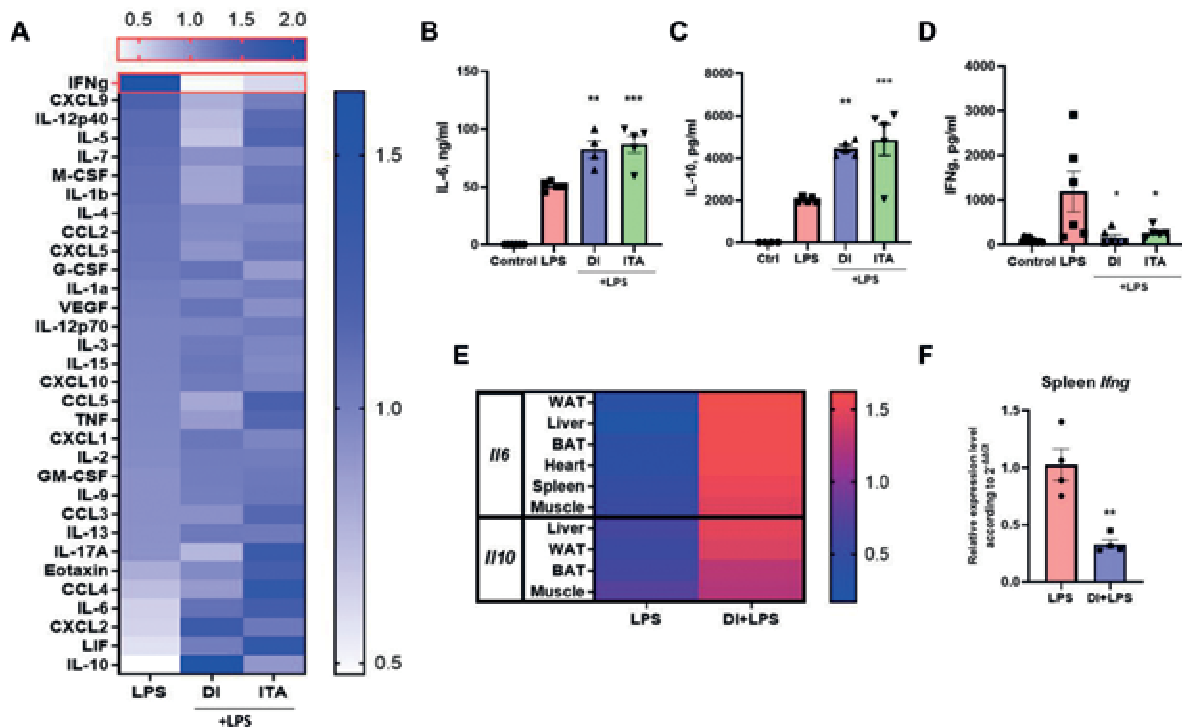


**Figure 1. ITA and DI regulate systemic production of IL-6, IL-10 and IFNγ in a mouse model of LPS-induced inflammation**

Note. (A) Mice were treated with 50 ug of LPS for 4 h (added at 2 h of 1 g $kg^{-1}$ itaconate (ITA) or dimethyl itaconate (DI) pre-treatment). Blood cytokine levels were measured using multiplex assay. (B-D) Blood IL-6 (B), IL-10 (C) and IFNγ (D) levels were measured by ELISA. (E) *Il6* and *Il10* relative expression in organs. WAT, white adipose tissue; BAT, brown adipose tissue. (F) *Ifng* relative expression in spleen. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

*Il10* expression in organs actively expressing these genes under LPS-induced inflammation (Figure 1E).

Being a lymphocyte-specific cytokine, *Ifng* expression level was decreased in the spleen in response to DI, which also agrees with blood level changes of IFNγ in our model. Altogether, these data demonstrate that itaconate regulates systemic production of IL-6, IL-10 and IFNγ during inflammation *in vivo* and, at the same time, IL-6 and IL-10 blood levels were changed in a different way than it was initially shown in macrophage cell cultures [1, 8].

**Itaconate regulates cytokine production via SDH inhibition**

To investigate the mechanism which mediates the observed effects of itaconate on systemic cytokine production in the mouse model of LPS-induced inflammation, we next used dimethyl malonate (DM), a classical SDH-inhibitor. DM pre-treatment leads to the similar pattern of changes in IL-6, IL-10 and IFNγ production: blood IL-6 and IL-10 levels were increased and IFNγ levels were decreased as compared to LPS-control group both after DI and after DM pre-treatment (Figure 2A). The similarity between DI and DM effects on IL-6 levels were also observed in peritoneal lavage (Figure 2B). We observed the same effects of DM at the *Il6* and *Il10* gene expression. In addition, succinate, the SDH substrate and activator, regulated IL-6 and IFNγ levels in the blood in the opposite way to DI and DM: succinate administration suppressed systemic IL-6 levels and enhanced IFNγ production. Altogether, these data suggest that itaconate-mediated changes in IL-6, IL-10 and IFNγ production are due to its ability to inhibit the SDH.

Itaconate has been initially characterized as an important immunoregulatory metabolite. It is synthesized under inflammatory conditions and regulates the production of a number of cytokines, including IL-6. Here we show that both itaconate and dimethyl itaconate increase the systemic levels of IL-6 and IL-10 during inflammation *in vivo*, which does not correlate with their effects previously described in experiments *in vitro* [1, 8]. The role of itaconate in the regulation of cytokine production *in vivo* varies depending on the experimental setting. Itaconate was not studied in the model of LPS-induced inflammation, however, our results agree with Wu et al., which demonstrated that myeloid-specific *Irg1* deletion leads to decreased IL-6 systemic levels in the mouse model of polymicrobial sepsis [10]. At the same time, we for the first time report that itaconate and dimethyl itaconate decrease systemic IFNγ production *in vivo*. This result is in agreement

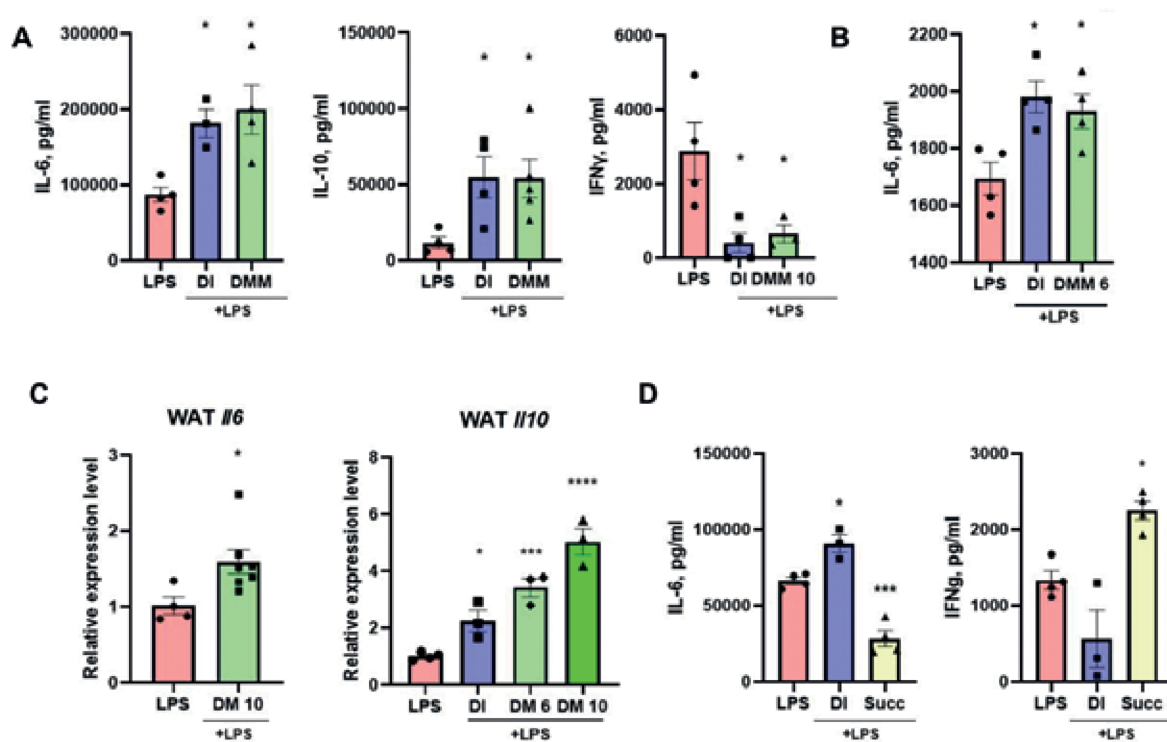


**Figure 2. Itaconate regulates cytokine production via SDH inhibition**

Note. (A) Blood IL-6, IL-10 and IFNγ levels were measured by ELISA. DI, dimethyl itaconate; DMM, dimethyl malonate. (B) IL-6 concentration in peritoneal lavage. (C) *Il6* and *Il10* relative expression in white adipose tissue (WAT). (D) Blood IL-6 and IFNγ levels. Succ, succinate. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

with Zhao et al., which demonstrated that itaconate suppressed IFNγ in CD8+T cells [13].

Itaconate is known as an inhibitor of SDH. Itaconate rediscovery as an exogenous SDH suppressor by Lampropoulou et al. shed light on its role in the regulation of the immune response of macrophages [3]. However, the effects of itaconate on macrophage cytokine production *in vitro* are related to the regulation of transcriptional regulators such as IκBζ [1] or TNFAIP3 [14].

## Conclusion

We suppose that itaconate effects on cytokine production *in vivo* are related to its ability to suppress SDH activity. This seems to be dispensable in the context of regulation of IL-6 in macrophages *in vitro* [1], however, during inflammatory reaction *in vivo*, the itaconate-SDH axis appears to be a significant contributor to the course of inflammation.

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*Поступила 15.04.2023*
*Отправлена на доработку 24.04.2023*
*Принята к печати 26.04.2023*

*Received 15.04.2023*
*Revision received 24.04.2023*
*Accepted 26.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 483-488



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 483-488

# ГОРЯЧИЕ ТОЧКИ МУТАЦИЙ ВО ВНЕКЛЕТОЧНЫХ ДОМЕНАХ MICA/MICB

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**Резюме.** MICA и MICB – это неклассические молекулы MHC, которые являются индикаторами клеточного стресса. Они выполняют функцию лигандов рецепторов NKG2D NK-клеток, вызывая цитотоксический ответ против поврежденных, инфицированных или трансформированных клеток. Образование растворимых форм MICA/MICB происходит путем расщепления их внеклеточных доменов. Экспрессия молекул MICA/MICB на опухолевых срезах или уровни их растворимых форм в крови могут быть использованы при диагностике онкологических заболеваний. Они могут предсказывать важные клинические параметры онкологических больных, такие как общая и безрецидивная выживаемость. Однако их обширный молекулярный полиморфизм затрудняет разработку моноклональных антител (МКАТ) для использования в диагностике. Ввиду этого диагностическая ценность анализов на основе МКАТ может меняться в зависимости от частоты аллельных вариантов в локальных человеческих популяциях. Мы изучили аминокислотные последовательности экстраклеточных доменов более 280 аллельных вариантов MICA и 50 аллельных вариантов MICB. Кроме того, мы выявили 172 и 58 однонуклеотидных полиморфизмов, расположенных в кодирующих областях соответствующих генов и приводящих к аминокислотным заменам. Наиболее частые аминокислотные замены (> 10%) в экстраклеточных доменах происходят в 11 и 4 сайтах MICA и MICB, соответственно. Мы обнаружили, что частоты однонуклеотидных полиморфизмов в выявленных горячих точках выраженно коррелируют друг с другом в различных популяциях человека, несмотря на разнообразие частот аллельных вариантов. Известна функциональная роль только одного сайта. Замена валина на метионин в положении 152 повышает сродство MICA к связыванию с рецептором NKG2D. Поскольку «горячие точки» распределены по всей последовательности экстраклеточных доменов, они могут играть иную роль, нежели модуляция аффинитета взаимодействия с рецептором NKG2D. Мы рекомендуем, чтобы наборы антигенов для валидации МКАТ к MICA и MICB, отвечали двум критериям. Во-первых, они должны включать аллели как MICA, так и MICB, поскольку их аминокислотные по-

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**Образец цитирования:**
*А.Ю. Столбовая, И.В. Смирнов «Горячие точки мутаций во внеклеточных доменах MICA/MICB» // Медицинская иммунология, 2023. Т. 25, № 3. С. 483-488.*
*doi: 10.15789/1563-0625-MHS-2680*

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**For citation:**
*A.Yu. Stolbovaya, I.V. Smirnov "Mutation hot spots in MICA/MICB extracellular domains", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 483-488.*
*doi: 10.15789/1563-0625-MHS-2680*

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**DOI:** 10.15789/1563-0625-MHS-2680

следовательности схожи между собой. Во-вторых, аллели должны покрывать вариабельность, наблюдаемую в выявленных «горячих точках».

*Ключевые слова: MICA, MICB, полиморфизм, рак, диагностика, моноклональные антитела*

# MUTATION HOT SPOTS IN MICA/MICB EXTRACELLULAR DOMAINS

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**Abstract.** MICA and MICB are non-classical MHC molecules that indicate cellular stress. They act as ligands for NKG2D receptors found on NK cells, thereby triggering a cytotoxic response against damaged, infected, or transformed cells. The production of soluble forms of MICA/MICB occurs via the cleavage of their extracellular domains (ECDs). The expression of MICA/MICB molecules in tumor sections or the levels of their soluble forms in blood have potential as diagnostic tools for cancer. They can predict important clinical outcomes for cancer patients, such as overall and recurrence-free survival. However, their extensive molecular polymorphism complicates the development of monoclonal antibodies (mAbs) for diagnostic use. Therefore, the diagnostic value of mAb-based assays may vary depending on the frequencies of allelic variants in local human populations. We examined the ECD amino acid sequences of more than 280 MICA and 50 MICB allelic variants. Additionally, we identified 172 and 58 single nucleotide polymorphisms (SNPs) located in the coding regions of the respective genes and resulting in amino acid replacements. The most frequent amino acid replacements (> 10%) in the ECD occur at 11 and 4 sites of MICA and MICB, respectively. We found that the frequencies of SNPs in the identified hot spots strongly correlate with each other in different human populations, despite the diversity of allelic variant frequencies. The functional role of only one site is known. The replacement of valine with methionine at position 152 enhances the affinity of MICA to NKG2D receptor. As the hot spots are dispersed throughout the entire ECD sequences, they may play a role other than modulating affinity with the NKG2D receptor interaction. We recommend that Ag sets used to validate anti-MICA/MICB mAbs meet two criteria. First, they should include both MICA and MICB alleles, as these genes have highly similar sequences. Second, the alleles should cover the variability observed in the identified hot spots.

*Keywords: MICA, MICB, polymorphism, cancer, diagnostics, monoclonal antibodies*

This research was funded by Russian Science Foundation (grant No. 21-15-00021).

## Introduction

MICA and MICB are non-classical MHC molecules found on the cell membranes of stressed, virus-infected, or transformed cells [2]. Their extracellular domains (ECDs) consist of three immunoglobulin domains. Unlike classical MHC molecules, MICA/MICB do not associate with β2-microglobulin and do not present antigen (Ag) peptides. NK cells recognize their expression via NKG2D receptors and eliminate damaged or dangerous cells. Two domains (α1 and α2) are involved in the interaction with NKG2D receptors. Soluble forms of MICA/MICB are produced as a result of ECD cleavage in the α3 domain. Sequence homology of MICA/MICB molecules is high and reaches 84%.

Similar to MHC molecules, MICA/MICB demonstrate an extensive molecular polymorphism [5]. As of 2023, the IMGT database comprises over 500 sequences that encode in excess of 290

MICA proteins, and more than 200 sequences that encode more than 50 MICB proteins. Human populations significantly differ from each other in frequencies of MICA/MICB allelic variants [2]. The variability of MICA molecules is found in all three functional parts: ECD, transmembrane region, and cytoplasmic tail. The variability in the MICA transmembrane region is well studied and occurs due to short tandem repeats in exon V. Variants A4, A5, A6, A7, A9, and A10 possess a corresponding number of GCT triplets encoding alanine resides. MICA-A9 is more frequent in patients with psoriasis [4], nasopharyngeal carcinoma [12], gastric adenocarcinoma [7], acute lymphoblastic and myeloid leukemias [1]. Variants having an inserted G after five GCT repeats (A5.1 variants) form a premature stop-codon and possess short cytoplasmic tails. Patients having these MICA alleles are more likely to develop pancreatic cancer [9], oral cancer [11], breast cancer [6], and atypical forms of celiac disease [8]. Synonymous or nonsynonymous single nucleotide polymorphisms (SNPs) are found in exons encoding ECD. The role of variability in ECD is less understood. The replacement of valine with methionine at position 152 (or 129 if counting starts from E24) increases the affinity of MICA and NKG2D receptor binding [10]. Patients with the Val/Val phenotype at this position have a significantly higher frequency of multiple myeloma relapse [14] and are predisposed to nasopharyngeal cancer [3]. MICB molecular variability is also due to SNPs, but the functional consequences are yet to be established.

The expression of MICA/MICB molecules in tumor sections, or levels of their soluble forms in blood, could be used for cancer diagnosis. These parameters have been shown to predict important clinical outcomes for cancer patients, including overall and recurrence-free survival [13]. However, the polymorphic nature of MICA/MICB ECDs may significantly affect the way monoclonal antibodies (mAbs) used in diagnostic assays bind to these Ags. Therefore, the diagnostic value of these assays may vary depending on the frequencies of allelic variants in local human populations. We suggest that mAbs used in immunohistochemistry and sandwich ELISA should be validated using sets of allelic MICA/MICB variants. By analyzing publicly available sequence and SNP data, we found that the most frequent amino acid replacements in the ECDs are located in 11 and 4 sites of MICA and MICB, respectively.

## Materials and methods

The genetic sequences of MICA/MICB allelic variants for this study were retrieved from the IMGT website (https://imgt.org/). We numbered amino acids starting from the first methionine residue in the leader peptide. ECDs were defined as amino acid sequences from E24 to S297. Multiple alignment of the amino acid sequences was performed using the ClustalW algorithm. Information regarding SNPs located in MICA/MICB genes and their frequencies (“1000 Genomes” project) was obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/snp/). Data analysis was carried out using custom R scripts.

## Results and discussion

We carried out multiple alignments of 280 and 50 amino acid sequences of MICA and MICB ECD allelic variants, respectively, excluding those with premature stop codons or partial sequences. We identified 63 MICA and 16 MICB alleles with identical sequences in their extracellular parts. Most MICA variants exhibited differences in amino acid sequence at 11 sites, whereas MICB alleles differed at 4 positions (Table 1).

To investigate the frequency of MICA/MICB mutations in human populations, we identified 172 and 58 SNPs located in the coding regions of the respective genes resulting in amino acid replacements. To our surprise, mutations with high frequencies (> 10%) in the global population were located in the previously defined positions (Table 1). Interestingly, we found a high correlation in the frequencies of identified SNPs among different local human populations (Table 2).

We recommend that Ags sets used to validate anti-MICA and anti-MICB mAbs meet two criteria. First, they should include both MICA and MICB alleles as these genes have highly similar sequences. Second, the alleles should cover the variability observed in the identified hot spots. It may also be useful to include variants with replacements in positions that have lower frequency rates (< 10%). mAbs, or combinations thereof, that cover this variability and exhibit high specificity to either MICA or MICB could be used for diagnostic purposes.

**TABLE 1. SNPs IN MICA AND MICB GENES LEADING TO THE MOST FREQUENT AMINO ACID REPLACEMENTS. SEQUENCES OF THE MOST COMMON ALLELES (MICA*008 AND MICB*005) WERE USED AS REFERENCES**

| Ag | Position | Amino acid variation | Frequencies | | | | |
|---|---|---|---|---|---|---|---|
| | | | Europe | East Asia | South Asia | Africa | America |
| MICA | 37 | W > G | 0.193 | 0.179 | 0.237 | 0.38 | 0.339 |
| | 59 | Y > C | 0.314 | 0.301 | 0.299 | 0.479 | 0.408 |
| | 145 | L > V | 0.195 | 0.122 | 0.273 | 0.272 | 0.212 |
| | 152 | V > M | 0.314 | 0.301 | 0.299 | 0.479 | 0.408 |
| | 196 | E > K | 0.314 | 0.300 | 0.300 | 0.480 | 0.405 |
| | 198 | G > S | 0.282 | 0.422 | 0.41 | 0.285 | 0.350 |
| | 229 | S > G | 0.314 | 0.300 | 0.299 | 0.477 | 0.406 |
| | 233 | R > W | 0.314 | 0.300 | 0.299 | 0.477 | 0.406 |
| | 236 | I > T | 0.509 | 0.422 | 0.572 | 0.75 | 0.618 |
| | 238 | T > S | 0.314 | 0.300 | 0.299 | 0.477 | 0.406 |
| | 274 | R > Q | 0.509 | 0.423 | 0.572 | 0.752 | 0.62 |
| MICB | 75 | D > N | 0.232 | 0.094 | 0.122 | 0.154 | 0.135 |
| | 80 | K > E | 0.263 | 0.275 | 0.241 | 0.383 | 0.19 |
| | 121 | I > M | 0.09 | 0.068 | 0.039 | 0.119 | 0.045 |
| | 136 | D > N | 0.262 | 0.274 | 0.241 | 0.383 | 0.19 |
| N: | | | 1006 | 1008 | 978 | 1322 | 694 |

**TABLE 2. CORRELATION MATRIX OF FREQUENCIES OF SNPs IN IDENTIFIED POSITIONS ACROSS DIFFERENT HUMAN POPULATIONS**

| | MICA | | | | | MICB | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Europe | East Asia | South Asia | Africa | America | Europe | East Asia | South Asia | Africa | America |
| Europe | 1 | 0.811 | 0.899 | 0.941 | 0.967 | 1 | 0.774 | 0.909 | 0.778 | 0.977 |
| East Asia | | 1 | 0.8 | 0.623 | 0.789 | | 1 | 0.967 | 1 | 0.891 |
| South Asia | | | 1 | 0.758 | 0.837 | | | 1 | 0.969 | 0.977 |
| Africa | | | | 1 | 0.966 | | | | 1 | 0.893 |
| America | | | | | 1 | | | | | 1 |

We found that the frequencies of SNPs in the identified hot spots strongly correlate with each other in different human populations, despite the diversity of allelic variant frequencies. This may indicate that selective pressure maintains SNP frequencies at similar levels in different populations. As mentioned earlier, the functional role of only one replacement is known. The functions of all others are yet to be established. Because the hot spots are scattered throughout the entire ECD sequences, they may play a role other than modulating affinity with the NKG2D receptor interaction.

## Conclusion

We conclude that the polymorphic nature of MICA and MICB molecules should be taken into account during the development of mAb-based immunoassays. Antigen sets covering the variability in amino acid replacement hotspots could be used for their validation. Revealing the functional role of these replacements may shed light on the pathogenic mechanisms leading to disease development associated with certain MICA/MICB alleles.

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*Поступила 28.03.2023*
*Отправлена на доработку 31.03.2023*
*Принята к печати 05.04.2023*

*Received 28.03.2023*
*Revision received 31.03.2023*
*Accepted 05.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 489-494

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 489-494

# ВЛИЯНИЕ CFP-10/ESAT-6 СЕКРЕТОРНЫХ ПРОТЕИНОВ НА ДОЛГОВРЕМЕННУЮ НЕСПЕЦИФИЧЕСКУЮ ИММУНОЛОГИЧЕСКУЮ ПАМЯТЬ В МАКРОФАГАХ МЫШЕЙ

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**Резюме.** Клетки врожденного иммунитета (моноциты/макрофаги, NK) могут также развивать иммунную память, что означает, что эти клетки обучаются после их первой встречи с патогенами, так что они проявляют неспецифический иммунологический ответ на этот же или другой патоген. Бацилла Кальметта–Герена (БЦЖ) индуцирует во врожденных иммунных клетках неспецифическую врожденную память (тренированный иммунитет). Исследовали неспецифическую врожденную память в макрофагах мышей BALB/c в ответ на микобактерии, имеющие или не имеющие в геноме RD1 область. Мышей иммунизировали вакциной БЦЖ, на 7-й день выделяли перитонеальные макрофаги и стимулировали их бактериальным липополисахаридом, CFP-10 или ESAT-6. Кроме этого, мышей иммунизировали вакциной *Mycobacterium tuberculosis* уро-BCG ($RD1^-$) и штаммом *Mycobacterium tuberculosis* H37Rv ($RD1^+$) подкожно или внутривенно, на 4-й день выделяли перитонеальные макрофаги и стимулировали липополисахаридом. Альвеолярные макрофаги получали из эксплантатов легких мышей инфицированных *Mycobacterium tuberculosis* штамма H37Rv мышей, наращивали до конфлуэнтности 70-80% и далее стимулированы их липополисахаридом. В кондиционированной среде макрофагов исследовали уровень лактата, цитокинов и глюкозы. Показано, что перитонеальные макрофаги от мышей, праймированных вакциной БЦЖ, в ответ на CFP-6 и ESAT-10 увеличили уровень продукции IL-1β, TNFα и лактата ($p < 0{,}05$). Необходимо отметить тот факт, что липополисахарид также увеличивал продукцию IL-1β, TNFα и потребление глюкозы праймированными вакциной БЦЖ перитонеальными макрофагами ($p < 0{,}05$). Показано, что перитонеальные макрофаги, праймированные Уро-БЦЖ, увеличивали спонтанную продукцию IL-1β и снижали спонтанную продукцию TNFα ($p < 0{,}05$). В случае праймирования макрофагов подкожным или внутривенным способом введения *Mycobacterium tuberculosis* штамм H37Rv по-разному влияли на продукцию цитокинов – снижали продукцию IL-1β и увеличивали TNFα и IL-10. В ответ на липополисахарид перитонеальные макрофаги увеличивали продукцию IL-1β, TNFα, IL-10 и потребление глюкозы ($p < 0{,}05$). Способ праймирования макрофагов *Mycobacterium tuberculosis* штамм H37Rv также вел к разнонаправленному

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**Образец цитирования:**
*А.П. Лыков, С.Н. Белогородцев, Е.К. Немкова, А. Ветлугина, Т.М. Терехова, Я.Ш. Шварц «Влияние CFP-10/ESAT-6 секреторных протеинов на долговременную неспецифическую иммунологическую память в макрофагах мышей» // Медицинская иммунология, 2023. Т. 25, № 3. С. 489-494.*
*doi: 10.15789/1563-0625-EOE-2663*

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**For citation:**
*A.P. Lykov, S.N. Belogorodtsev, E.K. Nemkova, A. Vetlugina, T.M. Terekhova, Ya.Sh. Schwartz "Effect of CFP-10/ESAT-6 secretory proteins on long-term non-specific immunological memory in mouse macrophages", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 489-494.*
*doi: 10.15789/1563-0625-EOE-2663*

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**DOI:** 10.15789/1563-0625-EOE-2663

уровню продукции цитокинов. Было показано, что альвеолярные макрофаги сохраняли тренированный иммунитет, так, они продуцировали повышенные уровни IL-1β, TNFα, IL-10 ($p < 0,05$). Таким образом, макрофаги мышей сформировали фенотип тренированного иммунитета в ответ на различные типы микобактерий, который сохраняется длительное время после первичного контакта с патогеном, в частности в альвеолярных макрофагах.

*Ключевые слова: вакцина БЦЖ, штаммы Mycobacterium tuberculosis, цитокины, лактат, глюкоза, оксид азота*

# EFFECT OF CFP-10/ESAT-6 SECRETORY PROTEINS ON LONG-TERM NON-SPECIFIC IMMUNOLOGICAL MEMORY IN MOUSE MACROPHAGES

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**Abstract.** Innate immune cells (monocytes/macrophages, NK) can also develop immune memory, which means that these cells are trained after their first encounter with pathogens so that they exhibit a nonspecific immunological response to the same or another pathogen. Bacilli Calmette–Gu rin (BCG) induces nonspecific innate memory (trained immunity) in innate immune cells. We examined nonspecific innate memory in macrophages of BALB/c mice in response to mycobacteria with or without the RD1 region in the genome. Mice were immunized with BCG vaccine, and peritoneal macrophages were isolated on day 7, and then stimulated with bacterial lipopolysaccharide, CFP-10, or ESAT-6. In addition, mice were immunized with *Mycobacterium tuberculosis* uro-BCG vaccine ($RD1^-$) and *Mycobacterium tuberculosis* strain H37Rv ($RD1^+$) subcutaneously or intravenously; peritoneal macrophages were isolated and stimulated with lipopolysaccharide on day 4. Alveolar macrophages were obtained from lung explants of mice infected with *Mycobacterium tuberculosis* strain H37Rv mice, were expanded to confluence 70-80% and further stimulated with lipopolysaccharide. Lactate, cytokines, and glucose levels were examined in conditioned macrophage medium. Peritoneal macrophages from mice primed with BCG vaccine were shown to increase IL-1β, TNFα, and lactate production in response to CFP-6 and ESAT-10 ($p < 0.05$). Of note is the fact that lipopolysaccharide also increased production of IL-1β, TNFα, and also increased glucose uptake by peritoneal macrophages primed with BCG vaccine ($p < 0.05$). Peritoneal macrophages primed with Uro-BCG were shown to increase spontaneous production of IL-1β and decrease spontaneous production of TNFα ($p < 0.05$). When macrophages were primed by subcutaneous or intravenous administration of *Mycobacterium tuberculosis* strain H37Rv differentially affected cytokine production, by decreasing IL-1β production and increasing TNFα and IL-10, was observed. In response to lipopolysaccharide, peritoneal macrophages increased IL-1β, TNFα, IL-10 production and glucose consumption ($p < 0.05$). The mode of priming of macrophages with *Mycobacterium tuberculosis* strain H37Rv also led to multidirectional levels of cytokine production. Alveolar macrophages were shown to retain trained immunity, as they produced elevated levels of IL-1β, TNFα, and IL-10 ($p < 0.05$). Thus, mouse macrophages formed a trained immunity phenotype in response to different types of mycobacteria, which persists for a long time after primary contact with the pathogen, particularly in alveolar macrophages.

*Keywords: BCG vaccine, strain of Mycobacterium tuberculosis, cytokines, lactate, glucose, NO*

## Introduction

Macrophages (Mf) are the main host cells of *Mycobacterium tuberculosis* (MBT), which could suppress the functional activity of these cells [2]. The BCG vaccine strain is able, through the induction of epigenetic reprogramming and metabolic changes in innate immune cells, to elicit trained immunity (TI). TI capable developed highest response to secondary exposure to homologous or heterologous pathogens, and protect from tuberculosis [14]. MBT virulence is associated with the presence of the RD1 region in the genome encoding CFP-10 and ESAT-6 [1, 3, 6, 7, 11]. It was obtained increased production of IFNγ, TNFα and IL-10 by lymphocytes in response to CFP-10/ESAT-6 stimulus [1, 15].

**The aim of the study** was to evaluate the severity of the induction of trained immunity taking into account the presence or absence of the RD1 region in the

mycobacterial genome in the resident macrophages of BALB/c mice.

## Materials and methods

The study was performed in compliance with Directive 2010/63/EU of the European Parliament and Council on the protection of animals used for scientific purposes. Experiment (1) BALB/c mice were immunized with hot-inactivated BCG vaccine (0.1 mg intraperitoneally, Microgen, RF), and then peritoneal macrophages (pMf) were isolated on day 7. $10^6$ pMf/well in RPMI-1640 medium (Paneco, Russia) supplemented with 2 mM L-glutamine (Sigma, USA), 5 mM HEPES-buffer (Sigma, USA), 10% FCS (Gibco, USA) and LPS (0 and 0.5 μg/mL lipopolysaccharide, Sigma, USA), CFP-10 and ESAT-6 (0 and 10 μg/mL, Oxford Immunotech, UK) were incubated in 24-well plates (TPP, Switzerland) for 24 hours in a $CO_2$ incubator (Sanyo, Japan).

Experiment (2) BALB/c mice were immunized with MBT uro-BCG medac strain RIVM ($RD1^-$) or H37Rv ($RD1^+$) subcutaneously (s.c.) or intravenously (i.v.). On day 4, $10^6$ pMf/well in RPMI-1640 medium supplemented with 2 mM L-glutamine, 5 mM HEPES buffer, 10% FCS, and LPS (0 and 0.5 μg/mL) were incubated in 24-well plates for 24 hours in a $CO_2$ incubator (Sanyo, Japan).

Experiment (3) Alveolar macrophages (aMf) were isolated from BALB/c mice infected with MBT strain H37Rv by migration of cells from the explants, and expanded to achieve 70-80% of confluence. Then $10^6$ aMf/well in RPMI-1640 medium supplemented with 2 mM L-glutamine, 5 mM HEPES buffer, 10% FCS, and LPS (0 and 0.5 μg/mL) were incubated in 24-well plates for 24 hours in a $CO_2$ incubator (Sanyo, Japan). Lactate, NO, cytokines, and glucose levels were determined in conditioned Mf media. The level of stable nitric oxide (NO)-nitrite (NO2-) metabolites was assessed using the Griess reagent. Lactate using the Lactate-Novo kit, IL-1β, IL-10, and TNFα (Vector-BEST, Russia) were assessed according to the manufacturer's instructions, and glucose was assessed using the Glucose-Novo kit (Vector-BEST, Russia).

The data was statistically processed using Statistica 10.0 for Windows. The normality of the distribution of the data obtained was assessed using the Shapiro–Wilk's w-criterion. Data in the table 1 were presented as mean and standard deviation (M±SD); statistical significance of intergroup differences was assessed by ANOVA with Bonferroni post hoc test and was accepted at $p < 0.05$.

## Results and discussion

As shown in the Table 1, CFP-10 and ESAT-6 increased the production of IL-1β, TNFα, and lactate by BCG-primed pMf ($p < 0.05$). In response to the second stimulus, pMf increased production of IL-1β, TNFα, NO, and lactate compared to BCG-primed cells alone ($p < 0.05$). The combination of CPF-10, ESAT-6, CFP-10/ESAT-6 with LPS increased pMf IL-1β production and decreased TNFα production ($p < 0.05$). The non-virulent strain of MBT increased IL-1β, TNFα, and IL-10 production ($p < 0.05$). At the same time, immunization s.c. with a virulent strain of MBT increased IL-1β, IL-10 production and decreased TNFα production, while i.v. MBT administration resulted in increased TNFα production and decreased IL-1β, IL-10 production compared to basal levels ($p < 0.05$).

LPS stimulation of pMf primed *in vivo* in the group of mice immunized with the non-virulent MBT strain increased IL-1β and TNFα production compared to the same indices in intact animals ($p < 0.05$). pMf from the mice primed with the virulent strain of MBT (s.c. injection) responded to the LPS stimulus with an increase in TNFα and IL-1β production, and a decrease in TNFα and IL-10 production ($p < 0.05$) when immunized by the i.v. Comparing the effects of the vaccine administration route on the induction of trained immunity in the pMf of mice without a second stimulus, we noted the fact that the production of IL-1β and TNFα was higher with i.v. immunization with the non-virulent MBT strain ($p < 0.05$).

In the group of mice immunized with the virulent strain of MBT, multidirectional production of IL-1β and TNFα was shown. The second stimulus in the case of pMf from mice immunized with the i.v. contributed to the increase in the levels of all cytokines, while the maximum increase in the production of only TNFα ($p < 0.05$) was observed when s.c. immunization with the virulent strain of MBT was used.

Intravenous immunization with the virulent strain promoted an increase in the production of pMf IL-1β and a decrease in the production of TNFα and IL-10 in response to LPS, while b/c immunization showed an increase in the production of only TNFα ($p < 0.05$). It seemed legitimate to evaluate the effect of mycobacteria on the expression of trained immunity *in vivo* in mice. Thus, aMf in response to LPS stimulus responded with increased production of IL-1β, TNFα, IL-10, NO ($p < 0.05$).

Tuberculosis is considered to be a global emergency worldwide [8]. The BCG vaccine is primarily a tuberculosis vaccine that also has a protective effect against leprosy, Buruli ulcer, and other non-tuberculosis mycobacterioses as a consequence of epigenetic and metabolic reprogramming of innate immunity cells [2, 14]. We observed an increase in pro-inflammatory cytokine production by BCG vaccine-primed pMf in response to CFP-10, ESAT-6, but no such pattern was found in response to the second stimulus (LPS). It is known that cytokines are involved in the pathogenesis of tuberculosis: their levels change with the stage of the pathological process,

**TABLE 1. CYTOKINE PRODUCTION BY MACROPHAGES OF BALB/c MICE DURING INDUCTION OF TRAINED IMMUNITY *IN VITRO* AND *IN VIVO* (M±SD)**

| Parameter | IL-1β, pg/mL | TNFα, pg/mL | IL-10, pg/mL | NO, μM/mL | Lactate, mM | Glucose consumption, mM |
|---|---|---|---|---|---|---|
| **Experiment 1** | | | | | | |
| **Without second stimulus** | | | | | | |
| **Basal** | 72.3±3.1 | 85.0±7.1 | 71.7±1.9 | 4.76±0.62 | 2.590±0.001 | 6.27±0.22 |
| **CFP-10** | 106.0±3.5* | 106.6±1.2* | 61.5±8.3 | 4.28±0.48 | 3.07±0.11* | 6.04±0.09 |
| **ESAT-6** | 111.2±6.3* | 193.3±4.4* | 71.9±5.3 | 4.37±0.90 | 3.04±0.13* | 6.15±0.21 |
| **CFF-10/ESAT-6** | 61.0±0.6* | 174.3±3.2* | 80.4±6.1 | 5.12±0.52 | 2.94±0.13* | 5.77±0.21 |
| **In response to LPS** | | | | | | |
| **LPS** | 80.3±1.9* | 132.1±1.9* | 69.1±3.8 | 6.83±0.13* | 2.80±0.02 | 6.12±0.22 |
| **CFP-10** | 83.4±3.9 | 145.3±5.4# | 68.0±1.9 | 4.08±0.21# | 2.90±0.24 | 5.93±0.19 |
| **ESAT-6** | 141.4±1.5# | 120.1±5.0# | 67.3±3.5 | 4.56±0.50# | 3.22±.2.00# | 5.71±0.22 |
| **CFP-10/ESAT-6** | 107.3±1.8# | 130.3±1.8 | 65.7±2.1 | 4.07±0.06# | 2.89±0.03# | 4.33±0.21# |
| **Experiment 2** | | | | | | |
| **Without second stimulus** | | | | | | |
| **Untreated** | 61.8±3.1 | 123.4±0.8 | 61.5±0.4 | 2.83±0.05 | 2.29±0.11 | 3.33±0.14 |
| **Uro-BCG, s.c.** | 77.6±0.3* • | 95.1±1.8* • | 127.9±9.6* • | 2.80±0.02 | 2.96±0.97 | 2.95±0.26 |
| **Uro-BCG, i.v.** | 84.9±0.6* | 114.3±0.4* | 76.1±3.6* | 2.85±0.08 | 3.04±0.73 | 2.01±0.28* |
| **H37Rv, s.c.** | 92.0±0.7* • | 61.7±1.5* • | 66.0±0.8* • | 2.80±0.03 | 2.76±0.24 | 1.65±0.40* |
| **H37Rv, i.v.** | 54.9±0.9* | 153.5±3.9* | 57.4±1.3* | 2.87±0.03 | 2.44±0.15 | 1.75±0.04* |
| **In response to LPS** | | | | | | |
| **Untreated** | 58.5±1.4 | 74.4±1.9 | 73.8±4.6 | 6.90±0.12 | 2.58±0.03 | 3.84±0.12 |
| **Uro-BCG, s.c.** | 74.6±0.7# • | 218.3±4.2# • | 67.6±2.9 | 6.05±0.07# | 2.88±0.31 | 3.43±0.13# |
| **Uro-BCG, i.v.** | 139.1±1.7# | 153.3±2.5# | 131.7±0.4# | 6.08±0.12# | 2.92±0.13# | 2.41±0.09# |
| **H37Rv, s.c.** | 62.6±7.4 | 275.7±1.9# • | 57.5±2.6# | 5.41±0.06# • | 3.00±0.21# | 2.24±0.29# |
| **H37Rv, i.v.** | 73.7±0.7# | 48.8±0.5# | 60.9±2.6# | 4.91±0.10# | 2.71±0.10 | 2.06±0.11# |
| **Experiment 3** | | | | | | |
| **Basal** | 88.75±0.66 | 74.94±3.58 | 56.16±2.02 | 6.97±0.83 | 5.54±0.15 | 1.23±0.35 |
| **LPS** | 116.35±2.09* | 105.26±2.05* | 73.36±4.13* | 9.71±1.81* | 5.58±0.08 | 1.32±0.32 |

Note. $p < 0.05$ *, with basal; #, with LPS; •, with i.v. administration.

polychemotherapy and can serve as an indicator of the effectiveness of treatment [9].

Our data point to the fact that we have partially succeeded in reproducing the phenomenon of trained immunity in pMf in response to stimulation with BCG vaccine. Thus, BCG vaccine-primed pMf in response to the second stimulus (LPS) increased cytokine and nitric oxide production, but did not increase lactate production or glucose consumption. Our findings on the effect of mycobacterial proteins on the secretory potential of primed BCG vaccine pMf are interesting. On Mf derivatives of THP-1 (cultured with phorbol-12-myristate-13-acetate), ESAT-6, CFP-10 and ESAT-6/CFP-10 were shown to significantly reduce NO and reactive oxygen species (ROS) production [12]. The RAW264.7 cell line, in response to ESAT-6, CFP-10 and ESAT-6/CFP-10 stimulation, reduced spontaneous and LPS-stimulated reactive oxygen species (ROS) production [5]. In the murine monocyte line ANA-1, CFP-10/ESAT-6 was shown to stimulate NO and IL-12 production in response to IFNγ stimulation, which is abolished when the cells are treated with AG490, a selective inhibitor of the JAK/STAT signaling pathway [7].

At the same time, recombinant CFP-10/ESAT-6 promotes dose-dependent increase of TNFα production by human monocytes and THP-1 cells, enhances CD80 and CD40 expression, increased IFNγ-induced TNFα production and HLA-DR expression [4]. Our data indicates stimulation of IL-1β, TNFα production by pMf after priming them with BCG vaccine, but we did not observe changes in

cellular production of NO. The differences detected with the literature data may be a consequence of the use of pMf in our work rather than monocytic cell lines and peripheral blood monocytes or macrophages derived from human or animal bone marrow. MBT secretory proteins encoded by the RD-1 region play an essential role in mycobacterial virulence, as they can inhibit ROS production [5].

Mycobacterial virulence also depends on the ability of mycobacteria to inhibit the IL-1β-dependent pro-inflammatory response, the suppression of apoptosis, delayed recruitment and activation of adaptive immunity cells [2]. On this basis, we investigated the effect of the method of mycobacterial administration and their virulence on the expression of trained immunity in Mf. Thus, vaccination with uro-BCV enhances the production of IL-1β by primed pMf. The pMf from mice vaccinated with MBT strain H37rv by s.c. administration produced higher levels of IL-1β, while in mice vaccinated by i.v. administration – TNFα. The pMf primed with non-virulent or virulent strains of MBT increased production of IL-1β, TNFα, and NO in response to LPS.

Consequently, MBT virulence and mode of immunization are not essential in the induction of trained immunity in the Mf. The vaccine strain of Mycobacterium BCG differs from virulent strains of MBT in the pattern of dissemination from aMf to other myeloid cells, mainly neutrophils and recruiting macrophages, which plays an essential role in MBT dissemination [10]. In addition, the aerosol route of BCG administration has been shown to develop resistance to *Streptococcus pneumonia* infection in mice. NRF2 (immune response regulator) aMf from knockout mice has been shown to initiate a significant pro-inflammatory response as early as day 10 of infection with MBT [13]. On this basis, we obtained aMf from mice with tuberculosis process induced by i.v. injection of MBT strain H37Rv. In response to LPS, aMf increased the production of both IL-1β, TNFα, IL-10, and NO. Consequently, taking into account the time required to obtain a primary culture of aMf (2-3 weeks), we can judge about the persistence of priming of these cells to MBT.

## Conclusion

Thus, MBT vaccination irrespective of virulence and method of administration induces trained immunity in Mf. Moreover, mycobacterial secretory proteins CFP-10, ESAT-6 may act as substrates capable of influencing trained immunity. Judging by the increased production of aMf cytokines from MBT-infected mice, trained immunity in cells *in vitro* is preserved for a long-time.

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*Поступила 08.03.2023*
*Отправлена на доработку 10.03.2023*
*Принята к печати 26.03.2023*

*Received 08.03.2023*
*Revision received 10.03.2023*
*Accepted 26.03.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 495-500

*Краткие сообщения*
*Short communications*

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 495-500

# ИССЛЕДОВАНИЕ ФЕНОТИПИЧЕСКИХ И ЦИТОТОКСИЧЕСКИХ СВОЙСТВ ЭРИТРОИДНЫХ КЛЕТОК СЕЛЕЗЕНКИ ПРИ ГЕМОПОЭЗ-СТИМУЛИРУЮЩИХ ВОЗДЕЙСТВИЯХ

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**Резюме.** В последние годы исследования выявили большое разнообразие функций эритроидных клеток, в том числе в модуляции врожденного и адаптивного иммунного ответа. Анемический или гипоксический стресс стимулирует физиологический ответ в виде стрессового эритропоэза, направленного на увеличение доставки кислорода к тканям. При стрессовом эритропоэзе активируются клетки-предшественники и используются механизмы, которые отличаются от стационарного эритропоэза костного мозга. Для рассмотрения роли эритроидных клеток в регуляции гемопоэза были смоделированы гемопоэз-активирующие состояния: химически индуцированная гемолитическая анемия, острая кровопотеря, гипоксия. Серию экспериментов проводили на мышах-гибридах первого поколения CBA C57Bl6. Выделение эритроидных клеток проводили с помощью магнитной сепарации по маркеру CD71. Стадии дифференцировки эритроидных клеток определяли по сочетанию экспрессии маркеров TER-119, CD71 и параметров прямого светорассеяния в популяции как CD45-позитивных, так и CD45-негативных клеток селезенки. Для изучения иммунорегуляторной активности эритроидных клеток мы исследовали опосредованную цитотоксичность спленоцитов против опухолевых клеток линии мышиной меланомы B78 после культивирования с кондиционными средами селезенок после различных гемопоэз-стимулирующих воздействий. При различных гемопоэз-стимулирующих воздействиях происходит реорганизация количественного и качественного состава клеток селезенки в зависимости от компенсаторного механизма для восстановления гомеостаза. Анализ клеточного состава селезенки показал, что при гемопоэз-стимулирующих воздействиях происходит перераспределение популяций с маркером CD45: при гипоксии резко снижается количество CD45-негативных клеток и повышается количество CD45-позитивных клеток. Популяция базофильных эритробластов наименее подвержена количественному изменению при всех гемопоэз-стимулирующих воздействи-

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**Образец цитирования:**
*Ю.А. Шевченко, К.В. Назаров, С.В. Сенников «Исследование фенотипических и цитотоксических свойств эритроидных клеток селезенки при гемопоэз-стимулирующих воздействиях» // Медицинская иммунология, 2023. Т. 25, № 3. С. 495-500.*
*doi: 10.15789/1563-0625-SOP-2730*

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**For citation:**
*Yu.A. Shevchenko, S.V. Sennikov, K.V. Nazarov “Study of phenotypic and cytotoxic properties of erythroid cells of the spleen under hematopoiesis-stimulating effects”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 495-500.*
*doi: 10.15789/1563-0625-SOP-2730*

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**DOI:** 10.15789/1563-0625-SOP-2730

ях. При гипоксии наблюдается наиболее заметное изменение клеточного состава селезенки за счет повышенного накопления CD45-позитивных эритроидных клеток в селезенке. Медиаторы эритроидных клеток селезенки мышей после гипоксии не приводят к усилению цитотоксического проапоптотического действия спленоцитов на опухолевые клетки в отличие от эритроидных клеток нормальной селезенки, селезенки при анемии и кровопотере. Таким образом, именно тканевая гипоксия является процессом, который не только стимулирует эритропоэз, но и приводит к максимальному изменению супрессивных свойств окружающих клеток. Мы предполагаем, что реализация компенсаторных механизмов при исследованных гематопоэз-стимулирующих воздействиях направлена на активацию механизмов врожденного иммунитета и локальной иммуносупресии для предотвращения местного воспаления, накопления питательных веществ и привлечения клеточных элементов в очаг гемопоэза для восстановления гомеостатических функций.

*Ключевые слова: эритробласты, селезенка, анемия, гипоксия, острая кровопотеря, терминальная дифференцировка*

# STUDY OF PHENOTYPIC AND CYTOTOXIC PROPERTIES OF ERYTHROID CELLS OF THE SPLEEN UNDER HEMATOPOIESIS-STIMULATING EFFECTS

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**Abstract.** In recent years, research has revealed a wide variety of erythroid cell functions, including modulation of innate and adaptive immune responses. Anemic or hypoxic stress stimulates a physiological response in the form of stress erythropoiesis, aimed at increasing oxygen delivery to tissues. Stress erythropoiesis activates progenitor cells and uses mechanisms that differ from stationary bone marrow erythropoiesis. To consider the role of erythroid cells in the regulation of hematopoiesis, hematopoiesis-activating states were modeled: chemically induced hemolytic anemia, acute blood loss, hypoxia. A series of experiments was carried out on first-generation hybrid mice CBA C57Bl6. Isolation of erythroid cells was performed using magnetic separation for the CD71 marker. The stages of differentiation of erythroid cells were determined by the combination of expression of TER-119 and CD71 markers and direct light scattering parameters in the population of both CD45-positive and CD45-negative spleen cells. To study the immunoregulatory activity of erythroid cells, we investigated the mediated cytotoxicity of splenocytes against tumor cells of the mouse melanoma B78 line after cultivation with conditioned spleen media after various hematopoiesis-stimulating effects. With various hemopoiesis-stimulating effects, the quantitative and qualitative composition of the spleen cells is reorganized depending on the compensatory mechanism for restoring homeostasis. An analysis of the cellular composition of the spleen showed that under hematopoiesis-stimulating effects, a redistribution of populations with the CD45 marker occurs: during hypoxia, the number of CD45-negative cells sharply decreases and the number of CD45-positive cells increases. The population of basophilic erythroblasts is the least susceptible to quantitative changes under all hematopoiesis-stimulating effects. During hypoxia, the most noticeable change in the cellular composition of the spleen is observed due to the increased accumulation of CD45-positive erythroid cells in the spleen. Mediators of erythroid cells of the spleen of mice after hypoxia do not lead to an increase in the cytotoxic proapoptotic effect of splenocytes on tumor cells, in contrast to the erythroid cells of the normal spleen, spleen with anemia and blood loss. Thus, it is tissue hypoxia that is the process that not only stimulates erythropoiesis, but also leads to the maximum change in the suppressive properties of surrounding cells. We assume that the implementation of compensatory mechanisms under the studied hematopoiesis-stimulating effects is aimed at activating the mechanisms of innate immunity and local immunosuppression to prevent local inflammation, accumulate nutrients, and attract cellular elements to the focus of hematopoiesis to restore homeostatic functions.

*Keywords: erythroblasts, spleen, anemia, hypoxia, acute blood loss, terminal differentiation*

The study was carried out at the expense of State Assignment No. 122011800108-0.

## Introduction

In recent years, studies have revealed a wide variety of functions of erythroid cells, including modulation of the innate and adaptive immune response [8]. In research practice, the study of erythropoiesis for a long time took place separately from other lines of hematopoiesis, however, in the process of ontogenesis, myeloerythropoiesis occurs simultaneously with the development of lymphoid precursors, so their mutual influence during development is natural [10]. An important part of the functionality of immature erythroid cells is associated with their ability to produce cytokines or other immunomodulatory molecules with diverse, often opposite functions [11, 12, 13], which can be explained by the source of origin, influences, and the general heterogeneity of the erythroid cell population during terminal differentiation. Stationary bone marrow erythropoiesis maintains erythroid homeostasis throughout life. Anemic or hypoxic stress stimulates a physiological response in the form of stress erythropoiesis, aimed at increasing oxygen delivery to tissues. Stress erythropoiesis uses progenitor cells and signals that differ from stationary bone marrow erythropoiesis [1]. In this regard, the study of the phenotype of nucleated erythroid cells and their role in interaction with other cells, which manifest themselves during stimulation of hematopoiesis in pathological conditions, is relevant and significant today.

## Materials and methods

In the work, we used mice hybrids of the first generation CBA C57Bl6 (3-5 months). The animals were kept in the NIIFKI vivarium under standard conditions with free access to food and drink. The study was carried out in accordance with the principles set out in the Declaration of Helsinki. Mice were removed from the experiment using cervical dislocation. To consider the role of erythroid cells in the regulation of hematopoiesis, hematopoiesis-activating states were modeled: chemically induced hemolytic anemia, acute blood loss, hypoxia. Similar intact mice were used as controls. Hemolytic anemia was induced by 3-fold (1.2 mg/mouse, 0.6 mg/mouse, 0.6 mg/mouse with 12-hour interval between injections) intraperitoneal administration of phenylhydrazine, which causes erythrocyte lysis. On the 4th day after the start of the experiment, the bone marrow and spleen were taken.

To simulate acute blood loss in mice under isoflurane anesthesia, blood was taken from the retroorbital sinus in a volume of ~ 0.5-0.7 mL, which corresponded to a loss of 12-14% of the circulating blood volume (average acute blood loss). On the 4$^{th}$ day after the start of the experiment, the bone marrow and spleen were taken. Hypoxic conditions were simulated in a pressure chamber (staying for 16 hours in a cage with bedding, food and water), where a pressure of ~ -46 kPa was created, which corresponded to an ascent to a height of 4200 m. At the end of exposure, mice were returned to standard vivarium conditions. Spleen sampling was carried out on the third day after the start of the experiment. Isolation of erythroid cells from a cell suspension purified on a ficoll-urografin density gradient ($\rho$ = 1.119 g/cm$^3$) was performed using magnetic separation using monoclonal antibodies to CD71.

The stages of erythroid cell differentiation were determined by the combination of expression of TER-119 and CD71 markers and FSC forward light scatter parameters in the population of both CD45-positive and CD45-negative spleen cells. The analysis was performed on an Attune NxT Flow Cytometer (ThermoFisher Scientific, USA). To study the immunoregulatory activity of erythroid cells, we investigated the mediated cytotoxicity of splenocytes against tumor cells of the mouse melanoma B78 line after cultivation with conditioned spleen media after various hematopoiesis-stimulating effects. To do this, splenocytes were preliminarily cultured with a conditioned erythroblast medium for 24 hours, and then planted with B78 mouse melanoma cells for another 24 hours. The cytotoxic effect was determined by the expression of the apoptosis marker annexin.

Statistical analysis of the obtained data was performed using GraphPad Prism 8 software using ANOVA and Tukey's multiple comparison test. Data were presented as median and interquartile range – Me ($Q_{0.25}$-$Q_{0.75}$). Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

Analysis of the cellular composition of the spleen showed that during hypoxia, the number of CD45-negative cells sharply decreases, and the number of CD45-positive cells increases. In addition, there are significant differences in the cellular composition of the spleen between the states of anemia and hypoxia. For the total content of erythroid cells in the splenocyte population, the same trend is observed: a decrease in the number of CD45-negative erythroblasts and an increase in the number of CD45-positive erythroblast cells during hypoxia (Table 1).

Determining the stages of terminal differentiation of erythroblasts showed that among CD45-negative cells, polychromatophilic erythroblasts normally predominate, while their number decreases with anemia, blood loss and hypoxia, and the content of orthochromatophilic erythroblasts increases with the same effects. In the population of CD45-positive erythroblasts, the predominant stage of differentia-

**TABLE 1. STRUCTURE OF THE SPLENOCYTE POPULATION IN MICE UNDER HEMATOPOIESIS-STIMULATING EFFECTS, Me ($Q_{0.25}$-$Q_{0.75}$)**

| | Intact mouse (n = 7) | Anemia (n = 7) | Acute blood loss (n = 7) | Hypoxia (n = 11) |
|---|---|---|---|---|
| **CD45-negative splenocytes** | 83.73% (78.26-84.87) | 75.09% (52.03-88.49)# | 66.07% (24.14-68.32) | 20.88% (16.25-66.79)* |
| **CD45-positive splenocytes** | 16.27% (15.34-21.74) | 25.47% (11.62-48.13)# | 34.24% (31.93-71.86)* | 78.66% (33.13-83.22)* |
| **CD45-negative erythroblasts** | 42.54% (34.45-59.94) | 41.29% (29.03-51.52)# | 27.68% (13.86-41.33) | 13.38% (12.01-43.63)* |
| **CD45-positive erythroblasts** | 9.863% (8.314-13.240) | 19.48% (5.661-32.670)# | 17.58% (15.79-32.41)* | 47.73% (20.78-58.55)* |

Note. *, compared with the Intact mouse; #, compared with hypoxia. Differences were considered statistically significant at $p < 0.05$.

tion is basophilic erythroblasts both in intact mice and under hematopoiesis-stimulating effects. The content of polychromatophilic erythroblasts significantly increases during hypoxia compared to intact erythroblasts, and the content of orthochromatophilic erythroblasts in anemia is significantly higher than in hypoxia. In the total fraction of erythroblasts of the spleen, there is a decrease in the content of polychromatophilic erythroblasts and an increase in the content of otrochromatophilic erythroblasts under all types of exposure (Figure 1).

Since erythroblasts can play an important role in regulating the functions of other cells, we studied the effect of their conditioned media on the effector functions of splenocytes, namely, cytotoxic activity against melanoma B 78 tumor cells, i.e., cytotoxic effect on tumor cells (Figure 2).

In this work, it is shown that under various hematopoiesis-stimulating effects, the quantitative and qualitative composition of spleen cells is reorganized. Each of these effects requires a separate compensatory mechanism to restore homeostasis. With hemolytic anemia, it is necessary to restore the destroyed pool of erythrocytes, with hypoxia, it is necessary to increase the number of erythroid cells relative to the already existing baseline, and with acute blood loss, it is necessary to restore not only erythrocytes, but also lymphoid cells, as well as the circulating blood volume.

The population of CD45-positive cells traditionally includes T, B, NK cells, monocytes, macrophages, however, this molecule is also present on early-stage erythroblasts [4] and in foci of extramedullary erythropoiesis in pathological

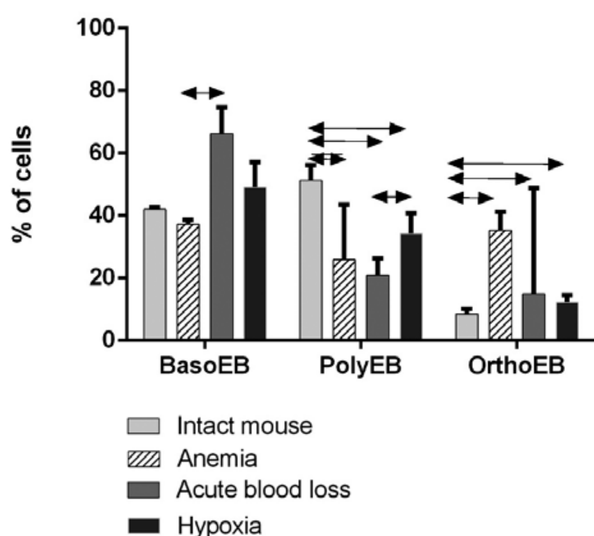


**Figure 1. Determination of the stages of terminal differentiation of erythroid cells of the spleen under hematopoiesis-stimulating effects**

Note. Data are presented as median and interquartile range – Me ($Q_{0.25}$-$Q_{0.75}$). Differences were considered statistically significant at $p < 0.05$.

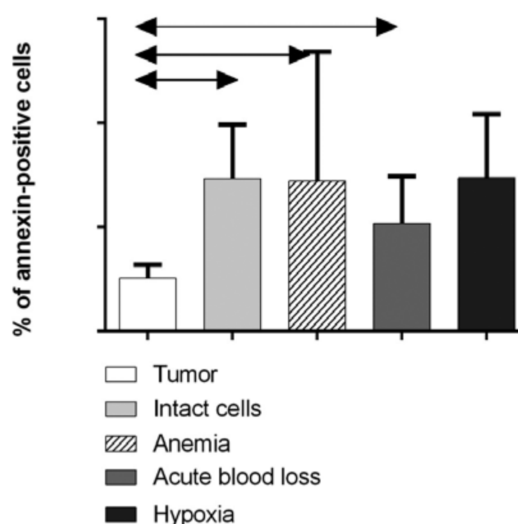


**Figure 2. Cytotoxic effect of splenocytes treated with conditioned erythroblast media obtained under various hematopoiesis-stimulating effects against tumor line B 78 cells (n = 8)**

Note. As for Figure 1.

conditions, for example, in the spleen of mice in transplantable tumor models [5]. During erythroid development, the CD45 marker arrests progenitors of CD71$^+$TER119$^+$ cells at undifferentiated stages, and its expression is a hallmark of early progenitors [7]. The population of basophilic erythroblasts is the least susceptible to quantitative changes under all hematopoiesis-stimulating effects. During hypoxia, the most noticeable change in the cellular composition of the spleen is observed, possibly due to the increased accumulation of CD45-positive erythroid cells in the spleen. CD45$^+$ erythroblasts are capable of greater suppressive activity compared to other cells [3]. In our work, we investigated the mediated effect of mediators that produce erythroid cells of the spleen on the cytotoxic activity of splenocytes. Mediators of erythroid cells of the spleen of mice after hypoxia do not lead to an increase in the cytotoxic proapoptotic effect of splenocytes on tumor cells, in contrast to the erythroid cells of the normal spleen, spleen with anemia and blood loss. It can be assumed that mediators secreted by erythroid cells under hypoxic conditions have the maximum suppressive effect on effector cells in the splenocyte population.

Tissue hypoxia is accompanied by the expression of the hypoxia factor HIF1a and the expression of the erythropoietin receptor, which protects cells from deep hypoxic damage [6]. Erythropoietin under conditions of stimulation of hematopoiesis leads to an increase in the production of erythrocytes, but an increase in the number of erythroid precursors and the priority of erythroid differentiation is compensated by a decrease in the number of precursors for other lines [14]. HIF induces a number of aspects of host immune function, from increasing the antibacterial capacity of phagocytes to stimulating T cell differentiation and cytotoxic activity [9]. Thus, it is tissue hypoxia that is the process that not only stimulates erythropoiesis, but also leads to the maximum change in the suppressive properties of surrounding cells.

## Conclusion

We assume that the implementation of compensatory mechanisms under the studied hematopoiesis-stimulating effects is aimed at activating the mechanisms of innate immunity and local immunosuppression to prevent local inflammation, accumulate nutrients, and attract cellular elements to the focus of hematopoiesis to restore homeostatic functions.

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*Поступила 12.04.2023*
*Отправлена на доработку 13.04.2023*
*Принята к печати 15.04.2023*

*Received 12.04.2023*
*Revision received 13.04.2023*
*Accepted 15.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 501-506



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 501-506

# РЕГУЛЯЦИЯ КИССПЕПТИНОМ-54 АКТИВНОСТИ ИНДОЛАМИН-2,3-ДИОКСИГЕНАЗЫ И АПОПТОЗА ЛИМФОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ

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**Резюме.** Беременность представляет собой феномен естественной полуаллогенной трансплантации, поскольку плод наполовину чужероден в силу экспрессии отцовских антигенов. Установлено, что гипоталамический гормон кисспептин в период беременности вырабатывается синцитиотрофобластом плаценты и участвует в формировании нового специфического гормонального фона. В крови беременных женщин циркулируют несколько форм гормона: кисспептин-10, кисспептин-14 и кисспептин-54 (по количеству аминокислотных остатков в молекуле гормона), однако основной активной формой является кисспептин-54. Основным механизмом формирования иммунной толерантности во время беременности является индукция экспрессии фермента индоламин-2,3-диоксигеназы (IDO) антигенпрезентирующими клетками периферической крови, вследствие чего происходит катализ триптофана (Trp) до кинуренинов (KYN), блокирующих активацию и вызывающих апоптоз цитотоксических $CD8^+$T-лимфоцитов в зоне соприкосновения материнских иммунных клеток с антигенами плацентарно-фетального комплекса. Кроме этого, в период беременности важная роль отводится процессу апоптоза, поскольку активированные клетки могут быть потенциально опасными для развивающегося плода. Иммунокомпетентные клетки крови экспрессируют специфический мембранный рецептор кисспептина (KISS-1R). Поскольку кисспептин-54 поступает в системный кровоток только во время беременности, то гормон оказывает действие на иммунные клетки только в этот период.

Целью данной работы была оценка влияния кисспептина-54 в концентрациях, сопоставимых с его уровнем во время физиологической беременности, на активность IDO и апоптоз лимфоцитов периферической крови.

В качестве объекта исследования использовались мононуклеарные клетки периферической крови (PBMC) полученные от 10 здоровых небеременных женщин репродуктивного возраста (от 23 до 32 лет). Апоптоз лимфоцитов оценивали в суспензии PBMC путем окрашивания аннексином-V и йоди-

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**Образец цитирования:**

*О.Л. Горбунова, С.В. Ширшев «Регуляция кисспептином-54 активности индоламин-2,3-диоксигеназы и апоптоза лимфоцитов периферической крови» // Медицинская иммунология, 2023. Т. 25, № 3. С. 501-506.
doi: 10.15789/1563-0625-ROK-2751*

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**For citation:**

*O.L. Gorbunova, S.V. Shirshev "Regulation of kisspeptin-54 activity of indolamine-2,3-dioxygenase and apoptosis of peripheral blood lymphocytes", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 501-506.
doi: 10.15789/1563-0625-ROK-2751*

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**DOI:** 10.15789/1563-0625-ROK-2751

стым пропидием. Определение количества клеток на ранней и поздней стадиях апоптоза проводили в изолированном гейте лимфоцитов. Активность IDO в PBMC определяли спектрофотометрически по изменению концентрации KYN – первого стабильного метаболита пути распада Trp.

Выявлено, что кисспептин-54 в концентрации 4,6 pM, соответствующей II триместру беременности, достоверно усиливает активность IDO, увеличивает количество клеток, находящихся в ранней и поздней стадиях апоптоза. Таким образом, кисспептин-54 является важным механизмом контроля этих процессов в период беременности, направленным на защиту полуаллогенного плода от неблагоприятных иммунных реакций матери и благоприятным развитием беременности.

*Ключевые слова: кисспептин-54, беременность, апоптоз, индоламин-2,3-диоксигеназа, мононуклеарные клетки периферической крови, лимфоциты*

# REGULATION OF KISSPEPTIN-54 ACTIVITY OF INDOLAMINE-2,3-DIOXYGENASE AND APOPTOSIS OF PERIPHERAL BLOOD LYMPHOCYTES

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**Abstract.** Pregnancy is a phenomenon of natural semi-allogeneic transplantation, since the fetus is half alien due to the expression of paternal antigens. It was found that the hypothalamic hormone kisspeptin during pregnancy is produced by the syncytiotrophoblast of the placenta and participates in the formation of a new specific hormonal background. Several forms of the hormone circulate in the blood of pregnant women: kisspeptin-10, kisspeptin-14 and kisspeptin-54 (according to the number of amino acid residues in the hormone molecule), but the main active form is kisspeptin-54. The main mechanism for the formation of immune tolerance during pregnancy is the induction of the expression of the enzyme indolamine-2,3-dioxygenase (IDO) by antigen-presenting cells of peripheral blood, resulting in the catalysis of tryptophan (Trp) to kynurenins (KYN) blocking the activation and causing apoptosis of cytotoxic $CD8^{+}T$ lymphocytes in the zone of contact of maternal immune cells with placental-fetal complex antigens. In addition, during pregnancy, an important role is assigned to the process of apoptosis, since activated cells can be potentially dangerous for the developing fetus. Immunocompetent blood cells express a specific membrane receptor of kisspeptin (KISS-1R). Since kisspeptin-54 enters the systemic circulation only during pregnancy, the hormone has an effect on immune cells only during this period.

The aim of this work was to evaluate the effect of kisspeptin-54 in concentrations comparable to its level during physiological pregnancy on IDO activity and apoptosis of peripheral blood lymphocytes.

Peripheral blood mononuclear cells (PBMC) obtained from 10 healthy non-pregnant women of reproductive age (from 23 to 32 years) were used as the object of the study. Lymphocyte apoptosis was assessed in PBMC suspension by staining with annexin-V and propidium iodide. The determination of the number of cells in the early and late stages of apoptosis was carried out in the isolated gate of lymphocytes. IDO activity in PBMC was determined spectrophotometrically by changes in the concentration of KYN, the first stable metabolite of the Trp decay pathway.

It was found that kisspeptin-54 at a concentration of 4.6 pM corresponding to the second trimester of pregnancy significantly enhances the activity of IDO, increases the number of cells in the early and late stages of apoptosis. Thus, kisspeptin-54 is an important mechanism for controlling these processes during pregnancy, aimed at protecting the semi-allogeneic fetus from adverse immune reactions of the mother and the favorable development of pregnancy.

*Keywords: kisspeptin-54, pregnancy, apoptosis, indolamine-2,3-dioxygenase, peripheral blood mononuclear cells, lymphocytes*

This work was carried out within the framework of the state task, the state topic registration number: AAAA-A19-119112290007-7.

## Introduction

Pregnancy is a phenomenon of natural semi-allogeneic transplantation since the fetus is half foreign due to the expression of paternal antigens [12]. During pregnancy, the mother's immune system is restructured due to the formation of a specific immune tolerance aimed at preserving the fetus from adverse immune reactions of the mother and, simultaneously, protecting the mother and fetus from pathogens [8]. The expression of indolamine-2,3-dioxygenase (IDO) by antigen-presenting peripheral blood cells is one of the mechanisms for the formation of peripheral tolerance during pregnancy. IDO catalyzes tryptophan (Trp) to kynurenines (KYN), which block the activation and cause apoptosis of cytotoxic $CD8^{+}$T lymphocytes in the zone of contact of maternal immune cells with placental-fetal complex antigens [8]. Also during pregnancy, an important role is assigned to the process of apoptosis, programmed cell death of cells. In addition to the elimination of altered – damaged, defective, mutant or infected cells, through apoptosis, the processes of differentiation and morphogenesis are realized during the formation of tissues and organs, the cellular homeostasis of an already formed organism is maintained, as well as its protection from pathogens during the implementation of protective reactions [15].

Pregnancy hormones play an important role in this restructuring, having a regulating effect on the cells of the mother's immune system [10]. It has recently been established that the hypothalamic hormone kisspeptin is also produced by the placental syncytiotrophoblast [5], and can have systemic effects on the leukocytes of a pregnant woman since they express a specific membrane kisspeptin receptor (KISS–1R) [9]. KISS-1R is a membrane protein that belongs to the class of Gaq-associated receptors (GPCR) [4]. However, there are few data on the immunomodulatory effect of kisspeptin on the cells of the immune system. We have previously shown that the interaction of kisspeptin-54 with $CD4^{+}$T lymphocytes causes their transformation into suppressor type regulatory cells (Treg) with simultaneous inhibition of Th17 differentiation and their functional activity [3].

**The aim of this work** was to evaluate the effect of kisspeptin-54 in concentrations comparable to its level during physiological pregnancy on IDO activity and apoptosis of peripheral blood lymphocytes.

## Materials and methods

**Hormone**

Kisspeptin (Kisspeptin-54, Metastin, Synthetic, CALBIOCHEM, USA) was used in physiological concentrations corresponding to its level in peripheral blood in the first, second and third trimesters of pregnancy: 1,3 pM, 4,6 pM and 9,6 pM, respectively [5].

**Objects of research**

The object of the study was peripheral blood mononuclear cells (PBMC), as well as separated monocytes and neutrophils obtained from 10 healthy non-pregnant women of reproductive age (from 23 to 32 years). Venous peripheral blood was collected in the follicular phase of the menstrual cycle (day 5-11) since the expression of KiSS-1R has a maximum during this period [2]. The study was conducted in accordance with the Helsinki Declaration of the BMA of 2000 and the Protocol of the Council of Europe Convention on Human Rights and Biomedicine of 1999. The approval was received from the Ethics Committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences. Voluntary informed consent to the examination was required to be included in the study. The individuals taking hormonal drugs were excluded from participation.

**Isolation and cultivation of cells**

PBMC of blood was obtained by centrifugation at 350×g for 40 minutes on a Ficoll-Urographin density gradient (1,077 $g/cm^3$) (Pharmacia, Sweden; Bayer Schering Pharma AG, Germany). After that, the cells were washed with RPMI 1640 (Sigma-Aldrich, USA). Then PBMC were divided into two parts. The first part was used to determine the activity of IDO. The second part was used to determine apoptosis of lymphocytes. The viability of PBMC determined by the inclusion of the vital dye eosin (0,01%) (Sigma, USA) was 95-98%.

Culturing of PBMC ($10^6$ cell/mL) with kisspeptin-54 was performed in a complete nutrient medium containing RPMI 1640 (Sigma-Aldrich, USA) with the addition of 10% FBS (Sigma, USA), 10 mM Hepes (ICN Pharmaceuticals, USA), 2 mM L-glutamine (ICN Pharmaceuticals, USA) and 30 μg/mL gentamicin (KRKA, Slovenia) at 37 °C and 5% $CO_2$ for 1 h. A hormone solvent (0,9% NaCl) was added to the control samples.

**Determination of lymphocyte apoptosis**

Lymphocyte apoptosis was evaluated in PBMC suspension by staining with annexin-V (AnV-FITC, Caltag, USA) and propidium iodide (PI, eBioscience, USA). This method makes it possible to identify cells in the early ($AnV^{+}/PI^{-}$) and late ($AnV^{+}/PI^{+}$) stages of apoptosis [13]. Dexamethasone ($10^{-6}$ M, "KRKA", Slovenia) was used to induce apoptosis, which was introduced into cultures 30 minutes before the hormone. The control was samples to which only an apoptosis inducer was added. Incubation was carried out for 24 hours in a full nutrient medium, at 37 °C and 5% $CO_2$. The results were taken into account

**TABLE 1. EFFECT OF KISSPEPTIN-54 ON LYMPHOCYTE APOPTOSIS**

| Experimental impact | Apoptosis, % | | |
|---|---|---|---|
| | n | $An^+Pr^-$ | $An^+Pr^+$ |
| **Control** | 10 | 10.60±0.82 | 15.69±0.96 |
| **Kisspeptin-54, 1,3 pM** | 10 | 12.65±0.50 p < 0.05 | 18.12±0.18 p < 0.05 |
| **Kisspeptin-54, 4,6 pM** | 10 | 17.88±1.23 p < 0.05 | 17.20±0.56 p < 0.05 |
| **Kisspeptin-54, 9,6 pM** | 10 | 15.48±0.84 p < 0.05 | 19.71±0.99 p < 0.05 |

on a FACSCalibur flow cytofluorimeter (Becton Dickinson, USA). The determination of the number of cells in the early and late stages of apoptosis was carried out in the isolated gate of lymphocytes.

**Determination of IDO enzymatic activity**

IDO activity in PBMC was determined spectrophotometrically by changes in the concentration of KYN, the first stable metabolite of the Trp degradation pathway [1]. For this purpose, PBMC stimulated with lipopolysaccharide (LPS) (100 ng/mL, Sigma, USA) was cultured in HBSS containing 100 μM of Trp (Sigma, USA) for 4 hours. Then, 50 μL of 30% $C_2HCL_3O_2$ was added to 100 μL of the cellular supernatant, shaken and centrifuged for 5 min. Then 75 μL the resulting reaction mixture was added to the wells of the 96-well plate, mixed with an equal volume of Ehrlich reagent (100 mg of p-dimethylbenzaldehyde, 50 mL $C_2H_4O_2$). Optic density was measured at 492 nm using a microplate reader (Synergy H1, BioTek, USA).

**Statistical analysis**

The obtained experimental data were processed using variational statistics. For the variables representing the analyzed sample, the arithmetic mean and the error of calculating the average (M±m) were calculated. For statistical verification of compliance with the law of normal distribution, the Fisher criterion was used. Given that the sample distribution was normal in all tests, the reliability of the differences between the mean values was evaluated according to the Student's paired t-test.

## Results and discussion

**The effect of kisspeptin-54 on lymphocyte apoptosis**

Kisspeptin-54 increases the number of cells in the stage of early apoptosis in concentrations characteristic of the II and III trimesters of pregnancy. Introduction of kisspeptin-54 into PBMC culture, regardless of concentration, leads to an increase in

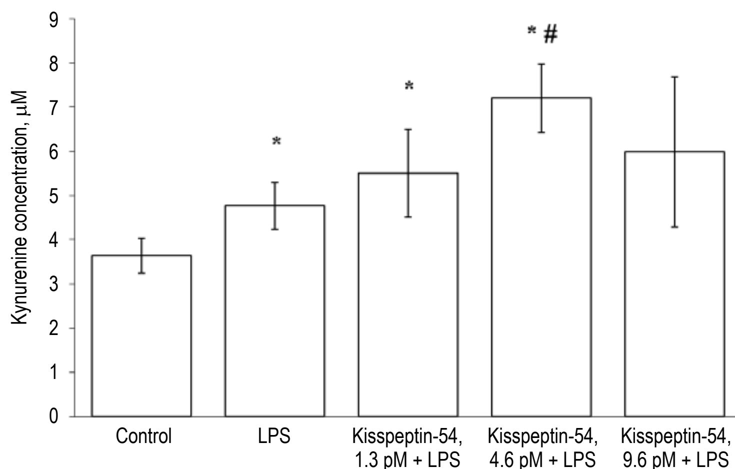


**Figure 1. Kisspeptin-54 influence on IDO activity**

Note. *, p < 0.05 compared with control, pair Student's t-test. #, p < 0.05 compared with LPS, pair Student's t-test.

the percentage of cells in the late stage of apoptosis (Table 1).

**Modulation of IDO activity by kisspeptin-54**

When evaluating the LPS-induced activity of IDO into PBMC, it was found that kisspeptin-54 at a concentration of 4,6 pM corresponding to the second trimester of pregnancy significantly increases the activity of IDO (Figure 1).

Thus, the hormone initiates immune tolerance at the level of adaptive immune responses. It is important to emphasize that the concentration of kisspeptin-54, observed only in the second trimester of pregnancy, the most vulnerable to immunocompromising conditions, has such plasticity of the regulatory potential [11]. Considering that IDO is produced only by antigen presenting cells, which are mainly represented by monocytes in PBMC, it can be assumed that kisspeptin-54 enhances LPS-stimulating signaling, leading to the expression of active IDO in these cells.

Summarizing the results obtained, it can be argued that kisspeptin-54 is directly involved in the regulation of PBMC apoptosis, which is obviously an important mechanism for controlling the activation of these cells during pregnancy. Stathaki, M. and co-authors also showed that kisspeptin-54 induces apoptosis of lymphocytes *in vitro* [14]. In addition, we have shown that kisspeptin-54 promotes the formation of peripheral tolerance by stimulating IDO activity. In turn, kininurinins – IDO products block activation and cause apoptosis of cytotoxic $CD8^{+}T$ lymphocytes [6]. Thus, it can be assumed that kisspeptin-54 acts on cytotoxic $CD8^{+}T$ lymphocytes, causing their apoptosis, through increased production of IDO by antigen-presenting cells.

It was found that at the beginning of physiological pregnancy, activation of apoptosis of peripheral lymphocytes and monocytes, a shift in the differentiation of T helper cells towards Th2 cells is observed. At the end of pregnancy, the process of apoptosis stabilizes, but the high level of cells in its later stages remains [7]. Most likely, the elimination of activated Th2 cells increases in the placenta at the beginning of pregnancy, and cytotoxic lymphocytes accumulate in the later stages of pregnancy. Apparently, the elimination of activated cell clones due to apoptosis is a protective mechanism, since activated cells can be potentially dangerous for the developing fetus.

## Conclusion

The data obtained by us indicate that kisspeptin-54 is directly involved in the control of these processes during pregnancy, which is aimed at protecting the semi-allogeneic fetus from adverse immune reactions of the mother and the favorable development of pregnancy.

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*Поступила 13.04.2023*
*Отправлена на доработку 14.04.2023*
*Принята к печати 17.04.2023*

*Received 13.04.2023*
*Revision received 14.04.2023*
*Accepted 17.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 507-512



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 507-512

# ЭКСПРЕССИЯ АРГИНАЗЫ-1 И ТИРОЗИНКИНАЗЫ MER МОНОЦИТАМИ КРОВИ В ДИНАМИКЕ ФИЗИОЛОГИЧЕСКОЙ БЕРЕМЕННОСТИ

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**Резюме.** При беременности иммунная система матери должна сохранять толерантность к отцовским антигенам, обладая при этом способностью элиминировать патогены, что достигается ослаблением адоптивного иммунитета и активацией врожденного иммунитета, в частности моноцитов. Однако вопрос о функциональном фенотипе моноцитов, обладающих не только провоспалительной, но и противовоспалительной активностью, остается открытым. В настоящей работе методом проточной цитофлюориметрии исследована экспрессия ассоциированных с М2-фенотипом супрессорных маркеров Arg1 и MerTK в субпопуляциях моноцитов в динамике неосложненной беременности. В исследование были рекрутированы 53 беременных с неосложненной гестацией, включая 14 беременных в первом триместре, 20 — во 2-м и 19 — в 3-м триместре беременности. Группу сравнения составили 15 фертильных небеременных без отягощенного соматического анамнеза, имеющих в анамнезе не менее одних родов. Полученные результаты показали, что в группе небеременных циркулирующие Мо экспрессируют Arg1 и MerTK, и наибольшее относительное содержание $Arg1^+$ и $MerTK^+$ клеток сосредоточено в промежуточных и неклассических моноцитах. При беременности экспрессия исследуемых молекул в моноцитах достоверно возрастает. Усиление экспрессии MerTK проявляется одновременным увеличением содержания $MerTK^+$ клеток и средней интенсивности флюоресценции данного маркера; наблюдается в 1-м и 2-м триместре и регистрируется во всех трех субпопуляциях моноцитов. В то же время усиление экспрессии Arg1 проявляется либо увеличением доли $Arg1^+$ клеток, либо возрастанием плотности рецепторов, регистрируется на протяжении всей беременности, включая 3-й триместр, и максимально выражено в классических моноцитах. Между содержанием $Arg1^+$ и $MerTK^+$ клеток в промежуточных моноцитах имеется прямая корреляционная связь, которая усиливается по мере прогрессии беременности и в 3-м триместре выявляется также в классических и неклассических моноцитах. В целом, выявленное усиление экспрессии моноцитами Arg1 и MerTK

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**Образец цитирования:**
*Е.Я. Шевела, Н.Г. Бухтуева, М.А. Тихонова, Л.В. Сахно, Н.М. Пасман, Е.Р. Черных «Экспрессия аргиназы-1 и тирозинкиназы Mer моноцитами крови в динамике физиологической беременности» // Медицинская иммунология, 2023. Т. 25, № 3. С. 507-512.*
*doi: 10.15789/1563-0625-EOA-2728*

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**For citation:**
*E.Ya. Shevela, N.G. Bukhtueva, M.A. Tikhonova, L.V. Sakhno, N.M. Pasman, E.R. Chernykh “Expression of arginase 1 and tyrosine kinase Mer by blood monocytes in the dynamics of physiological pregnancy”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 507-512.*
*doi: 10.15789/1563-0625-EOA-2728*

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**DOI:** 10.15789/1563-0625-EOA-2728

свидетельствует о возрастании противовоспалительного потенциала моноцитов при беременности и участии моноцитов в регуляции воспалительного процесса на системном уровне. При этом особенности экспрессии Arg1 и MerTK в различных субпопуляциях моноцитов и в динамике беременности позволяют предполагать, что экспрессирующие Arg1 и MerTK моноциты могут опосредовать различные механизмы иммунной адаптации в ходе беременности.

*Ключевые слова: субпопуляции моноцитов, беременность, иммунная адаптация, M2-поляризация, аргиназа-1, тирозинкиназа Mer*

# EXPRESSION OF ARGINASE 1 AND TYROSINE KINASE MER BY BLOOD MONOCYTES IN THE DYNAMICS OF PHYSIOLOGICAL PREGNANCY

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**Abstract.** During pregnancy, the maternal immune system must maintain tolerance to paternal antigens, at the same time being able to eliminate pathogens, which is achieved by the weakening of adoptive immunity and the activation of innate immunity, in particular, monocytes. However, the question about the functional phenotype of monocytes, having not only pro-inflammatory, but also anti-inflammatory activity, remains open. In the given work, we have investigated the expression of M2-associated suppressive markers Arg1 and MerTK in monocyte subpopulations during uncomplicated pregnancy. Fifty-three pregnant women with uncomplicated gestation were recruited, including 14 pregnant in the 1st trimester, 20 – in the 2nd and 19 – in the third pregnancy trimester. The comparison group consisted of 15 fertile unpregnant women without aggravated somatic anamnesis, with a history of at least one childbirth. The findings showed that in the unpregnant group circulating Mo express Arg1 and MerTK, and the most relative number of Arg1+ and MerTK+ cells is concentrated in intermediate and nonclassic monocytes. During pregnancy the expression of researched molecules in monocytes reliably increases. An increase in MerTK expression is manifested by a simultaneous increase in the number of MerTK+ cells and the mean fluorescence intensity of this marker; it is observed in the 1st and 2nd trimesters and registered in all three monocyte subpopulations. At the same time, an increase in Arg1 expression is manifested either by an enhancement of Arg1+ cells, or an increase in receptor density; it is registered throughout pregnancy, including the 3rd trimester, and is maximally expressed in classic monocytes. There is a direct correlation between the number of Arg1+ and MerTK+ cells in intermediate Mo, which increases with the progression of pregnancy, and in the 3rd trimester is also detected in classical and non-classical Mo. In general, the revealed increase in the expression of Arg1 and MerTK by monocytes indicates an increase in the anti-inflammatory potential of monocytes during pregnancy, and the involvement of monocytes in the regulation of the inflammatory process at the system level. Moreover, the features of Arg1 and MerTK expression in various monocyte subpopulations during pregnancy suggest that monocytes expressing Arg1 and MerTK can mediate different mechanisms of immune adaptation during pregnancy.

*Keywords: monocyte subsets, pregnancy, immune adaptation, M2 polarization, arginase 1, Mer tyrosine kinase*

The work was funded by the federal budget for fundamental scientific research (project No. 122011800324-4).

## Introduction

Normal pregnancy requires the suppression of aggressive reaction of the maternal immune system to paternal antigens while simultaneously being able to eliminate pathogens. This, at first glance, contradictory condition is reached with the help of the fundamental reconstruction of maternal immune system, which is called immune adaptation [1]. Immune reconstruction involves the weakening of adoptive immunity and the strengthening of innate one. The suppression of lymphocyte-meditated cytotoxic and autoimmune reactions is targeted at

fetus protection. Arising deficiency of lymphocyte anti-infective protection is compensated by the activation of innate immunity cells [11], the most important of them are monocytes.

Human circulating monocytes (Mo) are represented by heterogenic population, in which by the expression of two markers (CD14 and CD16) three subpopulations are distinguished – classical (cMo; $CD14^{++}CD16^{-}$), intermediate (iMo; $CD14^{++}CD16^{+}$) and non-classical (nMo; $CD14^{+}CD16^{++}$) Mo, which represent consecutive stages of differentiation and possess different functions [3]. During pregnancy the Mo total number enhances mainly due to iMo, and these cells are characterized by the increased expression of activation markers [1, 4, 11]. Considering the fact that iMo are effective producers of anti-inflammatory cytokines and their number grows during inflammatory diseases, it was initially considered that during pregnancy Mo have pro-inflammatory phenotype [4]. However, it was later discovered that iMo can have both pro- and anti-inflammatory properties [12], and the functional phenotype depends on activating signal. For example, activation by classic way inducts pro-inflammatory (M1) phenotype, and by alternative way – anti-inflammatory (M2) one [10, 13].

Another important aspect of immune adaptation is dynamics of immune changes, by which three immunological phases of pregnancy are distinguished. Moreover, when the second one, during which the fetus grows and the maximum protection from maternal cytotoxic cells is required, is anti-inflammatory, the first and third phases, corresponding respectively to blastocyte invasion and expulsion of fetus, are inflammatory processes. The research of local immunity has shown that on the mother-fetus border an important role in inflammatory regulation is played by M2 macrophages [5]. At the same time, the question whether monocytes take part in the regulation of inflammatory reaction on the system level remains open.

We hypothesized that from beginning to end of pregnancy the pro-inflammatory activity of Mo is balanced by an increase in their regulatory (immunosuppressive / anti-inflammatory) properties, the expressiveness of which regulates the intensity of inflammation during different stages of pregnancy. To verify this hypothesis, we aimed to study the immunosuppressive / anti-inflammatory potential of various Mo subpopulations during pregnancy. As markers of the regulatory activity of Mo associated with immune-suppressive / anti-inflammatory activity was assigned to the expression of arginase 1 (Arg1) and tyrosine kinase Mer (MerTK) [9, 15]. Expression of arginase 1 (Arg1) and tyrosine kinase Mer (MerTK) was evaluated as markers of monocyte regulatory activity associated with immunosuppressive / anti-inflammatory activity.

## Materials and methods

Fifty-three pregnant women with uncomplicated gestation at the age of from 18 to 41, including 14 women in the first trimester of pregnancy, 20 in the second one and 19 in the third one, took part in this research. The primigravidae in the researched group were accounted for by 37.7% (20 women), 16.9% (9 women) had high pregnancy parity (from 3 to 7). Pregnancies in the researched group went uncomplicated. Medial durations of gestation at the moment of research were correspondingly, 9, 18.3 and 38.5 weeks. The comparison group consisted of 15 fertile unpregnant women without aggravated somatic anamnesis at the age of from 23 to 42, having no less than one delivery in anamnesis. Blood collection took place on the 4-7$^{th}$ day of menstrual cycle. The research was carried out after obtaining written informative agreement.

Mononuclear cells (MNC) were isolated by the method of centrifugation of heparinized blood in ficoll-verografin density gradient (p = 1.078). The estimation of classical (cMo, $CD14^{++}CD16^{-}$), intermediate (iMo, $CD14^{++}CD16^{+}$) and non-classical (nMo, $CD14^{+}CD16^{++}$) monocytes was carried out by flow cytometry with the use of PerCP-, FITC- and PE-marked monoclonal anti-HLA-DR, anti-CD14 and anti-CD16 antibodies correspondingly (BD PharMingen, USA). The relative number of $MerTK^{+}$ cells in Mo subpopulations was estimated with the use of AlexaFluor 647 anti-MerTK (BioLegend) antibodies. To estimate the intracellular expression of arginase 1, the cells were treated with permeabilizing solutions (Transcription Factor Buffer Set, BD Pharmingen) and marked with APC-conjugated anti-Arg 1 antibodies (RD Systems).

The statistic processing of the results obtained was carried out using the Statistica 6.0 software package. The data are presented as median values (Me) and quartile range ($Q_{0.25}$-$Q_{0.75}$). The nonparametric Mann–Whitney U-test was used to identify significant differences in the compared parameters. Correlation analysis was performed using Spearman's rank correlation (Rs). Differences were considered significant at a significance level of $p < 0.05$.

## Results and discussion

The comparison of relative number of three Mo subpopulations in the groups of unpregnant and pregnant women (Table 1) showed a significant increase in cMo in the 1$^{st}$ trimester and iMo in the 2$^{nd}$ trimester compared to unpregnant women. The proportion of nMo did not change significantly, but in the 1$^{st}$ and 2$^{nd}$ trimesters it was significantly lower than in the 3$^{rd}$ trimester. The highest expression of

**TABLE 1. EXPRESSION OF M2-ASSOCIATED MARKERS IN SUBPOPULATIONS OF MONOCYTES IN THE DYNAMICS OF PREGNANCY**

| Parameter | Unpregnant | Pregnant women | | |
|---|---|---|---|---|
| | | 1st trimester | 2nd trimester | 3rd trimester |
| **cMo %** | 88 (86-90) | 92 (88-93)* | 91 (86.5-92.0) | 88 (84-90)& |
| **iMo %** | 2.9 (2-5) | 3.3 (2.7-4.4) | 4.9 (4.0-6.5)* & | 3.9 (2.5-6.8) |
| **nMo %** | 3 (2.2-4.0) | 2.3 (1.5-2.9) | 2.1 (2.0-2.9) | 2.9 (2.5-4.9)& # |
| **MerTK** | | | | |
| **cMo %** | 50 (43-55) | 70 (64-77)* | 69 (55-83)* | 64 (37.0-76.2) |
| **MFI** | 550 (260-640) | 815 (769-879) | 843 (798-912)* | 635 (517-750)& # |
| **iMo %** | 71 (66-87) | 88.5 (80-91)* | 88 (74.0-91.5)* | 84 (74-90) |
| **MFI** | 840 (540-1107) | 1129 (1037-1383)* | 1351 (1130-1542)* | 990 (890-1164)& # |
| **nMo %** | 75 (56-88) | 92 (88-93)* | 86 (79.0-89.2)& | 89 (56.0-92.1) |
| **MFI** | 750 (550-1020) | 1176 (1066-1284)* | 1381 (1128-1738)* | 880 (696-1030)& # |
| **Arg 1** | | | | |
| **cMo %** | 18 (15-24) | 20 (17-28) | 22.5 (18-28) | 22 (19-33)* |
| **MFI** | 605 (563-654) | 805 (673-949)* | 688 (652-742)* | 714 (635-749)* & |
| **iMo %** | 61 (48-68) | 57.5 (50-66) | 50.5 (39.5-60.5) | 61 (44-73) |
| **MFI** | 773 (753-815) | 1030 (921-1206)* | 850 (744-1070) | 891 (854-1307)* |
| **nMo %** | 74 (53-78) | 68 (59-77) | 63 (51.5-74.0) | 77 (53-83)# |
| **MFI** | 799 (756-882) | 1020 (863-1184) | 892 (809-996) | 820 (762-1100) |

Note. *, significance of differences with the unpregnant; &, with the pregnant in the 1st trimester; #, with the pregnant in the 2nd trimester (Mann–Whitney U criterion). MFI, mean fluorescence intensity.

MerTK and Arg1 in the unpregnant group was found in subpopulations of iMo and nMo. The relative number of $MerTK^+$ and $Arg1^+$ cells and the mean fluorescence intensity (MFI) of specified markers in the given subpopulations was reliably higher than in cMo. This was most pronounced in relation to $Arg1^+$ cells, the number of which in iMo (Me 61%) and nMo (Me 74%) was more than 3 times higher than their number in cMo (Me 18%).

In comparison with the control group, the expression of MerTK in Mo of pregnant women was increased. The significant increase in $MerTK^+$ cells and MFI MerTK values in the 1st trimester was registered in all subpopulations and persisted into the 2nd trimester. At the same time, in the 3rd trimester the expression of this marker decreased. So, the MFI MerTK in all Mo subpopulations were significantly lower than in the 2nd and 1st trimesters, and the relative number of $MerTK^+$ cells in all three Mo subpopulations was the same as the similar indicator of unpregnant women.

Expression of Arg1 was also found to be increased on Mo of pregnant women. However, unlike MerTK, which was elevated only in the 1st and 2nd trimesters, an increase in Arg1 expression was observed in all trimesters. In the 1st trimester we observed the significant increase in Arg1 MFI in cMo and iMo and – as a trend – in nMo. In the 2nd trimester these changes weakened a little, as evidenced by a moderate decrease in Arg1 MFI in cMo and iMo compared to the 1st trimester. The values of MFI Arg1 during these periods were still significantly higher than in the unpregnant group, however, the differences in MFI Arg1 compared to unpregnant in iMo no longer reached statistical significance.

In the 3rd trimester the increase of $Arg1^+$ cells in cMo reached statistical significance, and MFI Arg1 level in this subpopulation continued to be increased. The increase in MFI Arg1 in iMo was again statistically significant, and in nMo population, we observed the significant increase in the proportion of $Arg1^+$ cells compared to the 2nd trimester. Thus, the increase in Arg1 expression was mostly observed in the 1st and 3rd trimesters and had the highest expression in cMo subpopulation.

Analysis of a relationship between variables demonstrated that in the unpregnant group the direct correlation between $MerTK^+$ and $Arg1^+$ cells was observed in iMo population ($Rs = 0.52$; $p = 0.048$) and in nMo ($Rs = 0.49$; $p = 0.074$). In pregnant women, there was no significant relationship between the specified subpopulations in the 1st trimester, whereas in the 2nd one it was registered only in iMo ($Rs = 0.49$; $p = 0.027$), and in the 3rd one – in all three subpopulations, including cMo ($Rs = 0.49$; $p = 0.035$), iMo ($Rs = 0.69$; $p = 0.001$) and nMo ($Rs = 0.76$; $p = 0.0002$).

The adaptation of immune system during pregnancy is connected to the weakening of adoptive immune response and the compensative activation of acquired immunity cells, among which the most important part is played by Mo. However, Mo is endowed not only with an effector, but also with a regulatory function [8]. Moreover, if the pro-inflammatory properties of Mo are actively studied, then the research of Mo anti-inflammatory and immunosuppressive activity remains in the background. This prompted us to concentrate on researching Arg1 and MerTK expression – as suppressive markers – in Mo subpopulation in the dynamics of gestation.

The results obtained lead to the conclusion that Mo of fertile unpregnant women express Arg1 and MerTK, and the expression of the specified markers manifests mostly in iMo and nMo subpopulations and is minimal in cMo subpopulation. During pregnancy, the expression of MerTK and Arg1 in Mo increases. Notably, increased MerTK expression is manifested by a simultaneous enhancement in the number of $MerTK^+$ cells and MFI level; this takes place in the 1st and 2nd trimesters and is registered in all three Mo subpopulations. Unlike merTK, the increase in Arg1 expression becomes manifest either by the increase in the $Arg1^+$ cell numbers or in MFI; it is registered throughout pregnancy, including the 3rd trimester, and is maximally expressed in cMo. Thus, in spite of the fact that both markers are associated with M2 phenotype, the changes of their expression in monocytes during pregnancy have their special characteristics, in particular, they differ in expressiveness, association with subpopulation and expression dynamics.

According to literature data, the expression of arginase 1 and tyrosine kinase Mer in macrophages is associated with immunosuppressive and anti-inflammatory activity of these cells and is viewed as functional markers of M2 phenotype [2, 7]. At the same time, the data on their expression by blood monocytes and association with Mo subpopulations amounts to practically nothing. In this aspect, we have shown for the first time that MerTK and Arg1 are expressed by human circulating monocytes; the expression of these markers, especially Arg1, is considerably higher in iMo and nMo, and there is a direct correlation between the number of $MerTK^+$ and $Arg1^+$ monocytes.

Our findings have also shown for the first time, that beginning from the 1st trimester the expression of markers responsible for immune suppression and the suppression of inflammatory response in Mo is increased. Despite the fact that the immune adaptation during pregnancy is connected firstly to tolerance induction, the starting and finishing stages of pregnancy are connected to inflammatory processes, which on local level are controlled by M2 macrophages [5]. Our findings concerning the increase in expression of MerTK and Arg1 by pregnant women's circulating Mo show that monocytes / macrophages take part in the regulation of inflammatory response not only on the local level, but also on the system one. That is, typical for pregnancy moderately expressed Mo inflammatory activity [6] is controlled by the enhancing their anti-inflammatory properties.

Interesting, in our opinion, are the data on a decrease in the expression of MerTK in monocytes in the 3rd trimester with retaining high Arg1 expression. It is known that the arginase effect is connected firstly with the suppression of T cells, because arginine, metabolized by Arg1, is responsible for supporting the proliferation of T lymphocytes [7]. In its turn, MerTK has mostly an anti-inflammatory effect on the cells of innate immunity, being a negative regulator of TLR-mediated immune response [14]. Possibly, the weakening of Mo expression of MerTK in the 3rd trimester simplifies the start of inflammatory reaction necessary to prepare for childbirth. At the same time, the increased Arg1 expression restricts the functions of cytotoxic T cells, the activity of which progressively decreases during the gestation [6].

## Conclusion

The enhancement of the correlation between Arg1 and MerTK in iMo during pregnancy, which we found, may indicate an increase in the proportion of cells coexpressed the specified markers, during gestation period. The appearance of such a correlation in cMo and nMo in the 3rd trimester may display the possible role of Mo expressing Arg1 and MerTK in preventing preterm labour. However, further studies are required to verify this assumption.

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*Поступила 12.04.2023*
*Принята к печати 15.04.023*

*Received 12.04.2023*
*Accepted 15.04.2023*

# ВЛИЯНИЕ ТРОФОБЛАСТИЧЕСКОГО β1-ГЛИКОПРОТЕИНА НА ДИФФЕРЕНЦИРОВКУ МИЕЛОИДНЫХ СУПРЕССОРНЫХ КЛЕТОК

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**Резюме.** Миелоидные супрессорные клетки (MDSC) – гетерогенная клеточная популяциия, угнетающая функции, преимущественно, Т-лимфоцитов при здоровой беременности и патологиях. MDSC считаются одними из ключевых регуляторов иммунных реакций, поиск способов управления которыми крайне актуален для терапии рака, аутоиммунных заболеваний, невынашивания беременности и посттрансплантационных осложнений. Механизмы иммуносупрессии MDSC связаны с экспрессией молекул CD73, ADAM17, PD-L1, продукцией аргиназы 1 (Arg 1), индуцибельной синтазы оксида азота (iNOS), индоламин-2,3-диоксигеназы (IDO) и цитокинов IL-10 и TGF-β1.

Трофобластический β1-гликопротеин (ТБГ) – гликопротеин беременности. Описаны его модулирующие эффекты в отношении дендритных клеток и макрофагов, опосредующие сдвиг фенотипов Т-клеток в сторону Th2 и Treg. Ранее нами было показано что нативный ТБГ подавляет дифференцировку Th17 и продукцию ими провоспалительных цитокинов. Кроме того, этот гликопротеин стимулировал продукцию IDO моноцитами и дифференцировку Treg.

Так как функции и специфичность нативных и рекомбинантных белков отличаются, а рекомбинантные белки более доступны и перспективны, необходимо исследовать оба вида препаратов.

Учитывая иммуномодулирующие свойства ТБГ, а также ключевую роль MDSC в патологиях, целью нашей работы стала оценка влияния нативного и рекомбинантного ТБГ на дифференцировку MDSC *in vitro*.

MDSC дифференцировали из $CD11b^+$ клеток периферической крови. Клетки культивировали 7 дней, поэтапно добавляя GM-CSF, IL-1β и LPS. Нативный (н) (1,10 и 100 мкг/мл) и рекомбинантный (р) (1 и 10 мкг/мл) ТБГ вносили в культуры за три дня до окончания инкубации. Методом проточной цитометрии определяли процент MDSC ($Lin^-HLA\text{-}DR^-CD11b^+CD33^+$) от клеток в культуре, а также проценты M- ($Lin^-HLA\text{-}DR^-CD11b^+CD33^+CD14^+CD66b^-$), PMN- ($Lin^-HLA\text{-}DR^-CD11b^+CD33^+CD14\text{-}CD66b^+$) и e-MDSC ($Lin^-HLA\text{-}DR^-CD11b^+CD33^+CD14^-CD66b^-$) от общего количества MDSC.

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*В.П. Тимганова, К.Ю. Шардина, М.С. Бочкова, С.В. Ужвиюк, Д.И. Усанина, С.А. Заморина «Влияние трофобластического β1-гликопротеина на дифференцировку миелоидных супрессорных клеток» // Медицинская иммунология, 2023. Т. 25, № 3. С. 513-520.
doi: 10.15789/1563-0625-EOP-2838*

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**For citation:**

*V.P. Timganova, K.Yu. Shardina, M.S. Bochkova, S.V. Uzhviyuk, D.I. Usanina, S.A. Zamorina "Effect of pregnancy-specific β1-glycoprotein on myeloid-derived suppressor cell differentiation", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 513-520.
doi: 10.15789/1563-0625-EOP-2838*

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**DOI:** 10.15789/1563-0625-EOP-2838

Обнаружено, что нТБГ не влиял на процент MDSC в культурах. Однако рТБГ (1 мкг/мл) увеличивал процент этих клеток по сравнению с контролем. нТБГ (1 и 10 мкг/мл) и рТБГ (10 мкг/мл) увеличивали процент M-MDSC. Кроме того, рТБГ (10 мкг/мл) угнетал дифференцировку CD11b⁺ клеток в PMN-MDSC. Процент e-MDSC под действием ТБГ не изменялся.

Можно сделать вывод, что цитокиновый фон в культурах CD11b⁺клеток способствовал дифференцировке преимущественно M-MDSC, сходно с опухолевым микроокружением, а нативный и рекомбинантный ТБГ усиливали этот эффект.

Таким образом, нТБГ и рТБГ обладают способностью модулировать дифференцировку MDSC, увеличивая их количество, преимущественно за счет моноцитарной субпопуляции. Этот факт открывает перспективы для новых исследований, касающихся направленного манипулирования клетками MDSC с целью применения клеточных технологий в науке и медицине.

*Ключевые слова: миелоидные супрессоры, иммуносупрессия, трофобластический β1-гликопротеин, моноцитарные миелоидные супрессоры, гранулоцитарные миелоидные супрессоры, CD11b⁺CD33⁺ клетки*

# EFFECT OF PREGNANCY-SPECIFIC β1-GLYCOPROTEIN ON MYELOID-DERIVED SUPPRESSOR CELL DIFFERENTIATION

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**Abstract.** Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population that primarily suppress T lymphocytes in healthy pregnancies and pathologies. MDSCs are one of the key regulators of immune responses. Finding ways to control them is important for the treatment of cancer, autoimmune diseases, miscarriage, and post-transplant complications. The mechanisms of immune suppression by MDSC are: expression of CD73, ADAM17, PD -L1, production of Arg 1, iNOS, IDO, IL -10 and TGF-β1.

Pregnancy-specific β1-glycoprotein (PSG) has modulatory effects on dendritic cells and macrophages that mediate the shift of T cell phenotypes toward Th2 and Treg. We have previously shown that native PSG suppresses Th17 differentiation and cytokine production, stimulates the production of IDO by monocytes and the differentiation of Tregs.

Considering the immunomodulatory properties of PSG and the key role of MDSCs in pathologies, the aim of our work was to investigate the effect of native and recombinant PSG on the differentiation of MDSCs *in vitro*.

MDSCs were differentiated from CD11b⁺ peripheral blood cells. Cells were cultured for 7 days and received stepwise GM-CSF, IL-1β, and LPS. Native (n) (1; 10 and 100 μg/mL) and recombinant (r) (1 and 10 μg/mL) PSG were introduced into the cultures three days before the end of incubation. Flow cytometry was used to determine the percentage of MDSC among the cells in culture and the percentage of M-, PMN-, and e-MDSC among the total number of MDSCs.

It was found that rPSG (1 μg/mL) increased the percentage of MDSCs in culture. Both nPSG (1 and 10 μg/mL) and rPSG (10 μg/mL) increased the proportion of M-MDSC, whereas rPSG (10 μg/mL) decreased the number of PMN-MDSC.

Thus, the cytokine background in CD11b⁺ cell cultures favored the differentiation of predominantly M-MDSC, similar to the tumor microenvironment, whereas native and recombinant PSG enhanced this effect.

Thus, nPSG and rPSG are able to modulate the differentiation of MDSCs by increasing their number, mainly due to the monocytic subpopulation. This fact opens perspectives for new research on targeted manipulation of MDSCs.

*Keywords: myeloid-derived suppressor cells, immunosuppression, pregnancy-specific β1-glycoprotein, CD11b⁺CD33⁺ cells, M-MDSC, PMN-MDSC*

This research was supported by RSF (project No. 22-25-00378).

## Introduction

Myeloid-derived suppressor cells (MDSCs) are a small (usually less than 1% in blood) heterogeneous population composed of immature neutrophils and monocytes capable of suppressing innate and acquired immune responses, including those against tumors [3]. The number of MDSCs increases in healthy pregnancy [8] and in many pathological conditions, including inflammation, sepsis, traumatic shock, autoimmune diseases, and cancer [15].

The main mechanisms of immunosuppressive activity of MDSC are associated with the expression of a number of surface markers (CD73, ADAM17, PD-L1), intracellular expression of arginase 1 (Arg 1), iNO synthase (inducible nitric oxide synthase, iNOS), indoleamine 2,3-dioxygenase (IDO) and production of a number of cytokines (IL-10, TGF-β1) [1]. Due to the large arsenal of suppressive mechanisms, MDSCs are currently considered one of the most important regulators of immune responses. Finding ways to control them is extremely relevant from the perspective of therapy of all diseases and conditions in which these cells are involved. In addition, MDSCs may become a successful pharmacological target for solving problems related to immune rejection of both semi-allogeneic embryos and transplanted organs or tissues.

One of the factors that provide immune tolerance during pregnancy is pregnancy-associated proteins. Pregnancy-specific β1-glycoprotein (PSG) is a dominant fetoplacental protein produced by cyto- and syncytiotrophoblast cells and has immunoregulatory properties. In humans, the dominant expression product is PSG-1, which was discovered and identified in 1970 by a group of Russian researchers [11]. In pregnancy dynamics, the level of PSG gradually increases, reaching of 200-400 μg/mL in the third trimester, while its level in fetal blood serum does not exceed 1-2 μg/L [4]. It is known that PSG level in blood serum decreases in spontaneous abortion, ectopic pregnancy, intrauterine growth retardation, preeclampsia and fetal hypoxia [5]. In 2020, it was confirmed that circulating levels of PSG (PSG1) were significantly reduced in women with preeclampsia compared to healthy pregnant women. Thus, this protein is extremely important for the successful development of pregnancy.

The complex structure and multiple forms of PSG lead to certain difficulties in obtaining its pure, native active ingredient. Only recombinant forms of PSG are available for research, which have their drawbacks (structural differences, incomplete folding, uneven post-translational modification, etc.). Our team of authors has its own patented method for obtaining a native human PSG preparation [10], which ensures priority in research. Thus, in the last 5 years, we have demonstrated the effects of a native human PSG preparation obtained by the authors' method in terms of expression of IDO by antigen-presenting cells, regulatory T lymphocytes, Th17 cells, immune memory T cells, as well as regulation of the cytokine profile of these cells [13]. At the same time, it is obvious that for the practical application of the drug, it is necessary to study the recombinant forms of PSG in detail. The key point of our work is the fact that the role of PSG in regulating the differentiation and functional activity of MDSC has not yet been investigated.

Therefore, **the aim of this work** is to investigate the role of native and recombinant PSG in the regulation of MDSC differentiation in human peripheral blood.

## Materials and methods

Peripheral blood from volunteer donors was collected by venipuncture (n = 4). The study was conducted in accordance with the WMA Declaration of Helsinki 2000 and the Protocol of the Council of Europe Convention on Human Rights and Biomedicine 1999; approval of the Ethics Committee of the IEGM Ural Branch of the Russian Academy of Sciences (IRB00010009) dated 15 February 2022 was obtained for the experimental scheme used. Written informed consent was obtained from all patients. The authors adhered to all relevant ethical standards.

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation ($\rho$ = 1.077 g/cm$^3$, Diakoll, Dia-M, Russia). CD11b$^+$ cells were isolated from PBMC by positive immunomagnetic separation (MACSiBeads™, LS columns, (MiltenyiBiotec, Germany)). The resulting cells at a concentration of 1 × 10$^6$ were plated into a 96-well plate containing complete culture medium (RPMI-1640, 10% FBS, 10 mM Hepes (ICN Ph., USA), 2 mM-Glutamine (ICN Ph.) and 100 μg/mL penicillin-streptomycin-amphotericin (100 μL per 10 mL medium, BI, Israel)). GM-CSF (MiltenyiBiotec, Germany) was added to the cultures at a concentration of 20 ng/mL. Cells were then incubated for three days in a humidified atmosphere in a $CO_2$ incubator at 5% $CO_2$ and 37 °C. After completion of the first phase, the culture medium was replaced by addition of the cytokine IL -1β (20 ng/mL, MiltenyiBiotec, Germany) and LPS (0.1 μg/mL, Sigma Aldrich, USA) to activate the cells. The next day, the studied proteins were added: native PSG (RF Patent No. 2367449,

Raev M.B.) at concentrations of 1, 10, and 100 μg/mL and recombinant PSG (recombinant E. Coli-derived human pregnancy-specific beta-1-glycoprotein 1, PSG 1, Cusabio, China) at concentrations of 1 and 10 μg/mL. The choice of concentrations was based on their correspondence to the different trimesters of pregnancy. Cells were then cultured for an additional three days. After a total of 7 days of incubation, the cells were transferred to test tubes for flow cytometry. The cells were removed from the plate using a reagent containing enzymes that enhance cell detachment from the plastic (Accutase®, Capricorn Scientific, Germany), and the wells were additionally washed with ice-cold DPBS (Thermo Fisher Scientific, USA). Cells were then stained for viability with Zombie Aqua dye (BioLegend, USA) according to the manufacturer's protocol. After washing, the cells were incubated with fluorochrome-labeled antibodies to determine the MDSC phenotype using a flow cytometer. The following group of antibodies was used for this purpose: anti HLA-DR-Alexa Fluor 750, anti CD33-APC, anti CD11b-Alexa Fluor 405, anti CD66b-PE, anti CD14-PerCP (R&D Systems, USA). To exclude the presence of lymphocytes and NK cells in the target gate, three types of antibodies with the same fluorescent labeling were used: anti CD19-AF700, anti CD56-AF700, anti CD3-AF700 (designated as Lin). FMO samples (fluorescence minus one) and isotype controls were used as controls to determine negative populations.

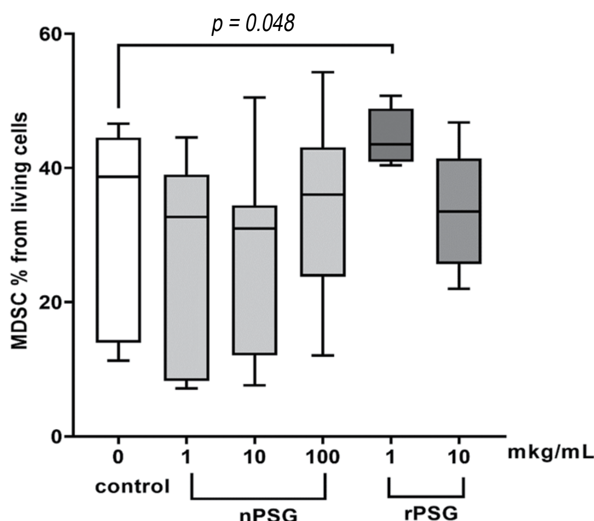


**Figure 1. Effect of native (nPSG) and recombinant (rPSG) PSG on the percentage of MDSC in CD11b⁺ cell cultures**

Note. n = 4; x-axis is the type and concentration of PSG; y-axis is the percentage of MDSC in the live cell gate. Medians (horizontal lines), interquartile ranges (rectangles), maximum and minimum values ("whiskers") are shown. Significant differences ($p < 0.05$) compared with control are indicated.

Samples were then analyzed using a CytoFLEX S flow cytometer (Beckman Coulter, USA). Cells were first gated on a dot plot of side scatter (SSC) and forward scatter (FSC), then live cells that were not stained with Zombie Aqua dye were gated. Next, a region of $Lin^-$HLA-DR cells was selected in the live cell gate and then plotted on a two-parameter CD11b/CD33 plot. The live $Lin^-HLA\text{-}DR^-CD33^+CD11b^+$ cells thus gated were plotted on a two-parameter CD14 and CD66b dot plot to determine M- (monocytic), PMN- (polymorphonuclear), and e-MDSC ("early," $Lin^-HLA\text{-}DR^-CD33^+CD11b^+CD14^-CD66b^-$) MDSC subpopulations, respectively.

Flow cytometry data were processed using CytExpert software (Beckman Coulter, USA).

Statistical data processing was performed with GraphPad Prizm 8.0.1 software using the Friedman test and Dunn post hoc test for multiple comparisons. Results are presented as median, lower quartile, and upper quartile: Me ($Q_{0.25}$-$Q_{0.75}$). The significance level was set at 0.05.

## Results and discussion

Viability in cultures of CD11b cells ranged from 95-98%. No statistically significant changes were observed.

Regarding the effects of the study proteins, it was found that the native PSG preparation did not affect the percentage of MDSC in the live cell gate. However, the recombinant protein preparation at a concentration of 1 μg/mL increased the percentage of these cells compared with the control (Figure 1).

The next task of our study was to determine the effects of native and recombinant PSG on the composition of MDSC subpopulations. To this end, we analyzed the percentage of $CD14^+$M-MDSC, $CD66b^+$ PMN-MDSC, and $CD14^-CD66b^-$e-MDSC in the total $CD11b^+CD33^+$ MDSC subset.

Native PSG at concentrations of 1 and 10 μg/mL and recombinant PSG at a concentration of 10 μg/mL were found to increase the percentage of M-MDSC (Figure 2).

Interestingly, recombinant PSG had a suppressive effect on the differentiation of $CD11b^+$ cells into CD $66b^+$ PMN-MDSC and reduced the proportion of these cells to as low as 0% in some cultures (Figure 3).

The native glycoprotein had no pronounced effect on the differentiation of this subpopulation.

Native and recombinant PSG did not alter the percentage of e-MDSC in CD11b cell cultures (data not shown).

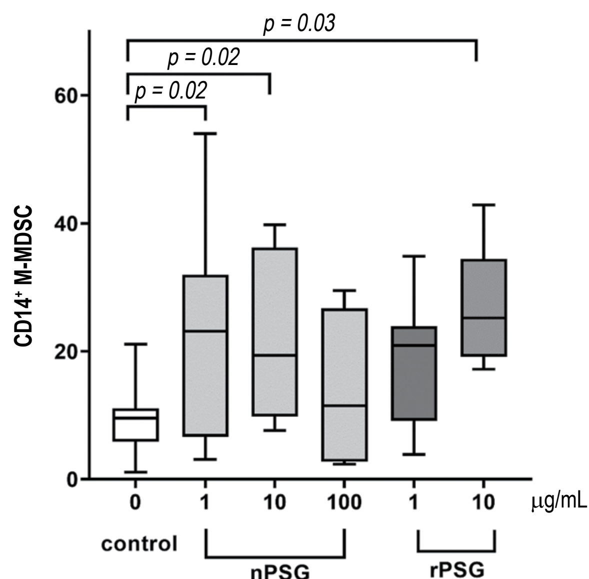


**Figure 2. Effect of native (nPSG) and recombinant (rPSG) PSG on the percentage of CD14+ M-MDSC in CD11b+ cell cultures**

Note. n = 4; x-axis is the type and concentration of PSG; y-axis is the percentage of CD14+M-MDSC in the MDSC gate. Medians (horizontal lines), interquartile ranges (rectangles), maximum and minimum values ("whiskers") are shown. Significant differences ($p < 0.05$) compared with control are indicated.

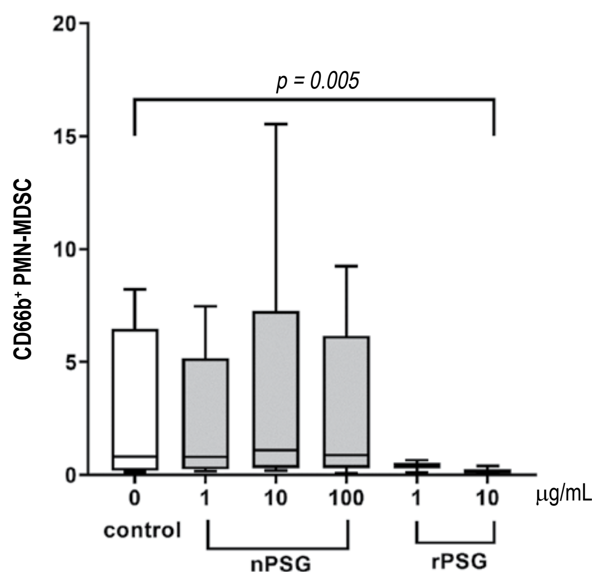


**Figure 3. Effect of native (nPSG) and recombinant (rPSG) PSG on the percentage of CD66b+ PMN-MDSC in CD11b+ cell cultures**

Note. n = 4; x-axis is type and concentration of PSG; y-axis is percentage of CD66b+ PMN-MDSC in MDSC gate. Medians (horizontal lines), interquartile ranges (rectangles), maximum and minimum values ("whiskers") are shown. Significant differences ($p < 0.05$) compared with control are indicated.

Thus, recombinant PSG at a concentration of 1 μg/mL was shown to increase the percentage of MDSC in CD11b+ cell cultures. This effect may be due to the fact that MDSCs exert a fetoprotective function during pregnancy by suppressing the maternal immune response to paternal antigens [15].

A specific receptor for PSG has not yet been found on human cells. However, it has been shown that PSG1 can exert its immunoregulatory functions by activating latent TGF-β1 and TGF-β2 [2]. TGF-β is considered one of the factors contributing to tumor progression and can "collaborate" with MDSCs from the tumor microenvironment [7]. Cells that cease to suppress T lymphocytes and acquire enhanced antigen-presenting and costimulatory capabilities. In addition, cells derived in this manner have acquired antitumor activity, which is very promising in terms of developing cell therapy against cancer [6].

The differences in the effects of native and recombinant proteins may be due to various factors. It should be emphasized that the native PSG preparation contains some quantity of PSG3, PSG7, PSG9 and some of their isoforms and precursors in addition to PSG1, whereas the recombinant protein contains only the PSG1. Moreover, the main difference between native proteins is their glycosylation, which determines their structure, functions, and stability, as well as the specificity of their interactions with the receptor.

As mentioned earlier, the distinguishing feature of MDSCs is their heterogeneity. The names of the main MDSC subpopulations, monocytic (M) and polymorphonuclear (PMN), are based on the morphological similarity of these cells to monocytes and neutrophils, which is also supported by the expression of the corresponding surface molecules CD14 and CD15/CD66b. In addition, PMN-MDSCs, whose major suppressive mechanism is the production of ROS, are known to require close intercellular contact with T cells, whereas M-MDSCs, which increase NO, arginase, and suppressive cytokines, do not require direct contact to suppress the T cell response. with cells. In addition, there is evidence that M-MDSCs are more effective per cell than PMN-MDSCs. Another difference is that in most tumor models, PMN-MDSCs accumulate in peripheral lymphoid organs, whereas M-MDSCs, in contrast, predominate directly in the tumor site. Presumably, hypoxia, low pH and other tumor microenvironmental factors do not support survival PMN-MDSC [15]. Interestingly,

PMN-MDSC predominates in decidua and blood during pregnancy [9].

In our study, both types of PSG (1 μg/mL nPSG and 10 μg/mL nPSG and rPSG) increased the percentage of M-MDSC in the general MDSC population, and recombinant PSG at a concentration of 10 μg/mL significantly inhibited the formation of PMN-MDSC. It should be noted that in our experiment, the ratio between the number of M-MDSCs and the number of PMN-MDSCs indicated a strong predominance of the former over the latter. In the cultures that served as controls, the number of M-MDSCs was 60-fold greater than that of PMN-MDSCs, and when both types of PSG were added, this number increased to 200.

This suggests that the cytokine background in the CD11b cell cultures promotes differentiation of predominantly M-MDSCs, similar to the tumor microenvironment, rather than PMN-MDSCs, as in the decidual membrane. Native and recombinant PSG likely enhanced this effect through TGF-β.

## Conclusion

Thus, recombinant and native PSG have the ability to modulate the differentiation of MDSCs by increasing their number, which is mainly due to the monocytic subpopulation of these cells. This fact opens the prospect of new research on targeted manipulation of MDSCs with the aim of applying these technologies in science and medicine.

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*Поступила 15.04.2023*
*Отправлена на доработку 21.04.2023*
*Принята к печати 26.04.2023*

*Received 15.04.2023*
*Revision received 21.04.2023*
*Accepted 26.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 521-526

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 521-526

# ВЛИЯНИЕ РАСТВОРИМЫХ ФАКТОРОВ МАКРОФАГОВ, ПОЛЯРИЗОВАННЫХ ЭФФЕРОЦИТОЗОМ, НА НЕЙРОНАЛЬНУЮ ПЛОТНОСТЬ ВО ФРОНТАЛЬНОЙ КОРЕ И ГИППОКАМПЕ МЫШЕЙ В МОДЕЛИ СТРЕСС-ИНДУЦИРОВАННОЙ ДЕПРЕССИИ

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**Резюме.** В последние десятилетия наблюдается неуклонный рост депрессивных расстройств, занимающих важное место в структуре причин нетрудоспособности. В основе патогенеза депрессии лежит нейровоспаление, ассоциированное с нарушением взрослого нейрогенеза. Важно отметить, что нейровоспаление, является частично обратимым, при этом ведущая роль в запуске и регуляции нейровосстановительных процессов отводится макрофагам/микроглии, характерными свойствами которых является гетерогенность и пластичность. При этом оппозитными состояниями активации макрофагов являются классически активированные M1 и альтернативно активированные M2-макрофаги, характеризующиеся, соответственно, про- и противовоспалительной активностью. Смещение баланса в сторону макрофагов с M2-фенотипом рассматривается в последние годы в качестве новой терапевтической стратегии в коррекции психо-неврологических расстройств. Одним из индукторов M2-фенотипа макрофагов является эффероцитоз. Ранее нами был разработан оригинальный протокол генерации макрофагов человека в условиях дефицита ростовых / сывороточных факторов, в котором ключевым моментом формирования M2-фенотипа является эффероцитоз. Получаемые таким образом макрофаги (M2(LS), LS – Low Serum) экспрессируют M2-ассоциированные маркеры и характеризуются активной продукцией ростовых и проангиогенных факторов (IGF-1, VEGF, BDNF, EGF, FGF-basic и др.), способных подавлять воспаление и стимулировать нейрорегенерацию/нейропластичность. В модели стресс-индуцированной депрессии был показан антидепрессантный

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**Образец цитирования:**
*И.М. Ращупкин, Т.Г. Амстиславская, Е.В. Маркова, А.А. Останин, Е.Я. Шевела «Влияние растворимых факторов макрофагов, поляризованных эффероцитозом, на нейрональную плотность во фронтальной коре и гиппокампе мышей в модели стресс-индуцированной депрессии» // Медицинская иммунология, 2023. Т. 25, № 3. С. 521-526.*
*doi: 10.15789/1563-0625-EOS-2731*

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**For citation:**
*I.M. Rashchupkin, T.G. Amstislavskaya, E.V. Markova, A.A. Ostanin, E.Ya. Shevela "Effect of soluble factors of macrophages polarized by efferocytosis on neuronal density in the frontal cortex and hippocampus of mice in a model of stress-induced depression", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 521-526.*
*doi: 10.15789/1563-0625-EOS-2731*

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**DOI:** 10.15789/1563-0625-EOS-2731

эффект растворимых факторов указанных М2-макрофагов, проявляющийся в снижении депрессивно-подобного поведения и снижении уровня провоспалительных цитокинов в отдельных структурах головного мозга. Однако влияние факторов M2(LS) на нейрогенез оставалось неизученным. В настоящей работе, которая является продолжением вышеупомянутого исследования, проанализировали влияние интраназального введения факторов M2(LS) макрофагов на нейрональную плотность в различных областях мозга – фронтальной коре и гиппокампе мышей в модели стресс-индуцированной депрессии. Полученные результаты показали, что нейрональная плотность во фронтальной коре, а также CA1 и CA3 зонах гиппокампа после терапии растворимыми факторами M2(LS) была значимо выше, чем у депрессивноподобных животных и сопоставима с таковой у интактных животных. Полученный результат может свидетельствовать о нейрорегенеративной активности M2(LS) макрофагов в модели стресс-индуцированной депрессии, который опосредуется через растворимые факторы и проявляется в повышении плотности нейронов во фронтальной коре и гиппокампе.

*Ключевые слова: макрофаги, М2-фенотип, мыши, депрессия, нейрорегенерация, нейрогенез*

# EFFECT OF SOLUBLE FACTORS OF MACROPHAGES POLARIZED BY EFFEROCYTOSIS ON NEURONAL DENSITY IN THE FRONTAL CORTEX AND HIPPOCAMPUS OF MICE IN A MODEL OF STRESS-INDUCED DEPRESSION

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**Abstract.** Recently, there has been a steady increase in depressive disorders, which occupy an important place in the structure of the causes of disability. In the pathogenesis of depression, an important role is played by neuroinflammation, which is associated with impaired adult neurogenesis. Notably, neuroinflammation is partially reversible, and the leading role in the initiation and regulation of neuroregeneration is given to macrophages. Opposite states of macrophage activation are classically activated M1 and alternatively activated M2 macrophages, characterized, respectively, by pro- and anti-inflammatory activity. A balance shift towards M2 macrophages has been considered as a new therapeutic strategy of psycho-neurological disorders. One of the inducers of the M2 phenotype is the efferocytosis. We have previously developed an original protocol for the generation of human macrophages under conditions of deficiency of growth / serum factors, in which M2 phenotype is formed through efferocytosis. Macrophages (M2(LS), LS – Low Serum) obtained according to this protocol express M2-associated markers, and are characterized by high production of growth and pro-angiogenic factors (IGF-1, VEGF, BDNF, EGF, FGF-basic, etc.), which can suppress inflammation and stimulate neuroregeneration / neuroplasticity. In the model of stress-induced depression, the antidepressant effect of soluble factors of M2(LS) macrophages was shown, accompanied by a decrease in the level of pro-inflammatory cytokines in certain brain structures. However, the effect of M2(LS) factors on neurogenesis remained unexplored. In the present work, which is a continuation of the aforementioned study, we analyzed the effect of intranasal administration of M2(LS) soluble factors on neuronal density in different brain areas – the frontal cortex and hippocampus – of depression-like mice. The results obtained showed that neuronal density in the frontal cortex, CA1 and CA3 zones of the hippocampus, was significantly higher in mice with intranasal administration of M2(LS) conditioned medium than in depression-like mice, and reached the level of neuronal density in intact animals. These results may indicate the neuroregenerative activity of M2(LS) macrophages in the model of stress-induced depression, which is mediated through soluble factors and manifests itself in an increase in the density of neurons in the brain.

*Keywords: macrophages, M2 phenotype, mice, depression, neuroregeneration, neurogenesis*

The work was funded by the federal budget for fundamental scientific research (project No. 122011800324-4).

## Introduction

Macrophages (Mϕ) are one of the most important components of the innate immunity system. In the central nervous system (CNS) Mϕ are represented by microglia (resident macrophages) and Mϕ of bone marrow origin recruited from peripheral blood. The proportion of the latter increases significantly in the presence of a tissue damage or/and inflammation. Mϕ/microglia is characterized by pronounced plasticity and heterogeneity. The extreme opposite states of Mϕ activation are classically activated M1 and alternatively activated M2 cells [7]. M1 exhibit proinflammatory properties, while M2 have a pronounced anti-inflammatory activity: they promote resolution of inflammation, produce anti-inflammatory cytokines and growth factors. The role of M1 and M2 Mϕ in the CNS pathology is being actively studied. For example, the role of various Mϕ phenotypes in a model of spinal cord injury has been described in detail, the participation of activated (M1) microglia in neuroinflammation in Alzheimer's disease has been shown [9], the protective effect of M2 was found in the model of oxygen-induced retinopathy. However, most of the studies in this field of research are devoted to two opposite Mϕ phenotypes: classically activated M1 and alternatively activated M2 (i.e., M2a). At the same time, it is currently well known that Mϕ are a heterogeneous population of cells and are able to form many transition states between the described extreme states of activation in response to various stimuli from the microenvironment. In particular, toll-like receptor ligands and immune complexes are inducers of the M2b, TGF-beta, IL-10, glucocorticosteroids promote the formation of M2c, and IL-6 and adenosine contribute to M2d phenotype formation [7].

Moreover, one of the important inductors of M2 phenotype in pathology is efferocytosis – an uptake of apoptotic material [10]. Considering this, we previously developed an original protocol for the generation of M2-like Mϕ under conditions of efferocytosis [2]. This protocol is based on the cultivation of human peripheral blood monocytes under growth / serum factors deficiency, which leads to deprivation apoptosis and subsequent engulfment of apoptotic cells by Mϕ. M2(LS) (LS – Low Serum) obtained according to this protocol are characterized by low antigen-presenting and pro-inflammatory activity, and a high level of production of various neurotrophic, neuroprotective and angiogenic factors (BDNF, IGF-1, FGF-basic, EPO, VEGF and etc.) [2]. Given these data, we hypothesized that M2(LS) may exhibit neuroprotective and neuroregenerative activity. Indeed, further studies showed the stimulating effect of M2(LS) on the proliferation and differentiation of NSC-like cells *in vitro* [13].

In recent decades, there has been a worldwide steady increase in mental illness associated with behavioral disorders, among which depression is one of the most significant medical and social problems. Numerous studies have associated depression with a decrease in biomarkers of adult hippocampal neurogenesis, including a decrease in the number of hippocampal neurons, neural stem / progenitor cells and the hippocampal volume as well as a reduction of neurogenic niche vascularization. In turn, depression treatment is correlated with an increase in these hippocampal biomarkers suggesting a link between adult hippocampal neurogenesis and depression [12].

Limited access to brain tissues in humans makes it difficult to directly study neurogenesis and increases the significance of these studies in models of CNS pathology. One of the widely used models of chronic stress in mice that reproduces a depression-like state is a social defeat stress. Anhedonia, decreased exploratory behavior, and social avoidance are observed in animals in this model. We have previously shown that intranasal administration of soluble factors derived from M2(LS) Mϕ to depression-like mice ameliorates behavioral pattern of animals, which was accompanied by a decrease in the level of pro-inflammatory cytokines in the brain [11]. The present study continues this line of work and focuses on the effect of M2(LS) Mϕ on neuronal density in the frontal cortex and hippocampus of depression-like mice.

## Materials and methods

M2(LS) Mϕ were generated from human peripheral blood mononuclear cells (PBMC), as described earlier [2]. Briefly, 3-5 × $10^6$/mL PBMCs were incubated in RPMI-1640 (Biolot, St. Petersburg, Russia) supplemented with 0.05 mM 2-mercaptoethanol, 2 mM sodium pyruvate 0.3 mg/mL L-glutamine, 1% nonessential amino acids, 100 μg/mL gentamycin, 2% autoplasma and 50 ng/mL recombinant human GM-CSF (R&D Systems, USA). After 18 hours, the non-adherent cells were removed, and the adhesive cells were cultured for 7 days. The conditioned media (CM) of Mϕ was collected, centrifuged, and stored at -80 °C.

This study was performed in male mice (CBAxC57Bl/6)F1 aged 3.5-4 months, weighing 25-30 g (obtained from the husbandry of the Institute of Pharmacology and Regenerative Medicine of the Russian Academy of Sciences). The animals were kept on a standard diet with free access to water and under normal light conditions in cages of 10/cage for at least 2 weeks prior to experiments. All procedures were approved by the Bioethics Commissions of Scientific Research Institute of Neurosciences and Medicine

and Scientific Research Institute of Fundamental and Clinical Immunology.

Depression-like behavior was induced in mice (n = 17) by repeated social defeat experience in daily (20 days) agonistic interactions (sensory contact model) [6]. Then the depression-like mice were individually placed in cages to exclude agonistic interactions and subdivided into experimental and control groups. The animals from experimental group received M2(LS) CM (30 μl in each nasal passage, twice a day) for 7 days. The control group of mice received RPMI-1640 medium.

Cryosections of the frontal cortex and hippocampus of mice were obtained using a cryotome, the thickness of each slice was 30 μm. The cryosections were then stained according to the Nissl staining method. The image was captured and analyzed by semi-automatic method using a microscope (ZEISS AXioskop2, Germany) and Image Pro Plus Software 6.0 (Media Cybernetics, CA, USA). Since neurons in the studied areas (pyramidal layer of the frontal cortex, CA1 and CA3 zones of the hippocampus) are tightly packed, especially in the hippocampus, the density of cells was measured to estimate the number of neurons. The neuronal density was calculated as the percentage of an area of interest (136036 $\mu m^2$ for frontal cortex and 145907 $\mu m^2$ for hippocampus) occupied by the Nissl-stained cells.

Statistical analysis was performed using the STATISTICA software version 8.0 (StatSoft. Inc., USA). The data are presented as median (Me) and interquartile range ($Q_{0.25}$-$Q_{0.75}$). To reveal a significant difference of the values compared, the Mann–Whitney nonparametric U test was used. Values of $p < 0.05$ were considered statistically significant.

## Results and discussion

Analysis of the relative neuronal density in the pyramidal layer of the *frontal cortex* showed that the depressive-like state was accompanied by a significant decrease in the number of pyramidal neurons compared with mice in the intact group: 16.8 (IQR 15.8-17.5%) *vs* 18.4 (IQR 17.1-21.2%) ($p < 0.05$) (Figure 1, see 2nd page of cover). Intranasal administration of RPMI-1640 medium had no effect on neuronal density, which remained significantly reduced in this area, not differing from the "depressive" control (16.8; 14.9-18.6%; $p < 0.05$). At the same time, in mice of the experimental group, intranasal therapy with soluble factors M2(LS) led to an increase in the number of neurons, reaching the level of intact animals – 19.8 (18.9-21.2%) ($p < 0.05$). In this group, neuronal density was significantly higher compared to both depression-like animals (model control) and RPMI-1640-treated mice (treatment control).

In both studied *hippocampal* zones, *CA1* and *CA3*, similar changes were observed (Figure 1, see 2nd page of cover). The development of a stress-induced depression-like state was associated with a statistically significant decrease in the number of neurons from 35.6 (29.8-41.5%) to 31.7 (26.7-34.6%) in the CA1 zone and from 41.8 (38.5-45.6%) to 34.5 (31.4-36.5%) – in the CA3 zone of the hippocampus ($p < 0.05$). Administration of RPMI-1640 failed to enhance neuronal density. In contrast, intranasal administration of M2(LS) CM led to an increase in neuronal density in both CA1 (33.6; 28.4-37.3%) and CA3 (40.3; 36.3-45.7%) areas of the hippocampus ($p < 0.05$). At the same time, the number of neurons was comparable to that in intact animals and significantly exceeded the values in the RPMI-treated mice.

Thus, the results obtained indicate that: 1) the development of a depression-like state in mice in the model of stress-induced depression is accompanied by a decrease in neuronal density in the frontal cortex and hippocampus; and 2) soluble factors of M2(LS) macrophages increase neuronal density in the studied areas of the mice brain.

The current study aims to evaluate the effect of secretory products of M2(LS) macrophages, polarized by interaction with apoptotic cells, on neuronal density in the frontal cortex and hippocampus of depression-like mice. The relative neuronal density is widely used to estimate the number of neurons in different areas of the brain, because in many areas the neurons are packed so tightly that it is often not possible to visualize individual cells. The neuronal density is an integral indicator that reflects the intensity of at least two interrelated processes: neurogenesis, on the one hand; and neuroinflammation and neurodegeneration, on the other. Accordingly, a decrease in the intensity of neuroinflammation and neurodegeneration, along with an increase in reduced neurogenesis, represent targets for therapeutic interventions in CNS pathology, in particular, in depression. An analysis of the properties of M2(LS) macrophages allows us to suggest that both of the above mechanisms may underlie the neuroregenerative activity of these cells.

Previously, we have shown a high level of production of growth and neurotrophic factors by M2(LS) Mϕ [2]. In particular, M2(LS) actively produce IGF-1, and the level of IGF-1 production by these macrophages is dozens of times higher than that of classical M1 and M2a cells [2]. The neuroprotective effects of IGF-1 have been demonstrated both *in vitro* and *in vivo* [1]. Moreover, a number of studies report the involvement of IGF-1 in the pathogenesis of depression, although the data are ambiguous. For example, Kuang et al. showed that IGF-1 injections contribute to the correction of depression-like behavior in mice in a model of chronic stress [8]. In patients with depression, the level of IGF-1 in the cerebrospinal fluid increased after antidepressant

therapy, which may indirectly indicate an IGF-1-mediated effect of antidepressants.

On the other hand, Levada et al. report a decrease in initially elevated serum IGF-1 levels in patients with major depressive disorder during antidepressant therapy, and a positive correlation between a decrease in IGF-1 and an improvement in clinical symptoms [14]. Thus, despite the lack of an unambiguous view of the role of IGF-1 in the pathogenesis of depression, there is no doubt that this factor has a significant impact on the disease course. It is very likely that it is IGF-1 in the composition of the secretory product of macrophages M2(LS) that makes a significant contribution to the antidepressant effect of macrophages observed in the present and our previous studies [11].

Another factor produced by M2(LS) at a significantly higher level than M1 and M2a Mϕ is VEGF [2]. Several recent studies demonstrate that VEGF is an angiogenic protein with neurotrophic and neuroprotective effects. Moreover, accumulating evidence has implicated VEGF in the major depression disorder pathophysiology. In particular, rodent models of stress-induced depressive-like states show significant decreases in VEGF and BDNF levels in the prefrontal cortex and hippocampus. In addition, VEGF can potentially mediate the antidepressant effects of typical antidepressants [15]. In another study, Greene et al. in a rat model of chronic stress showed that antidepressant therapy leads to an increase in the level of VEGF in the hippocampus and in peripheral blood. In addition to VEGF, IGF-1, and BDNF, other soluble factors, such as FGF-basic, EPO, EGF, etc., may also play a role in mediating the neuroregenerative effects of M2(LS) Mϕ, which is subject to further study.

Chronic neuroinflammation, which plays an important role in the pathogenesis of depression, leads to an increase in neurodegenerative processes (damage to neurons and their subsequent apoptosis) and a decrease in the intensity of neurogenesis [3]. Indeed, an increased level of pro-inflammatory cytokines (IL-1β, IFNγ, IL-6, TNFα), as well as reactive oxygen species in the brain, leads to numerous disorders of neurotransmitter metabolism, contributing to the development of depressive symptoms (anhedonia, sleep disturbance, decreased motor activity, etc.). Zhang et al. showed that intracerebral injections of IFNγ reduced neurogenesis in the mouse hippocampus and contributed to the development of depression-like behavior. At the same time, microglia isolated from the hippocampus of such mice suppressed the proliferation of neural stem cells and stimulated apoptosis of immature neurons *in vitro*.

Fan et al. showed that chronic stress leading to the depression-like behavior is accompanied by an increase in the IL-1β concentration in the prefrontal cortex of the mouse brain. Moreover, the introduction of a virus with overexpression of IL-1β into the brain of mice not subjected to chronic stress also led to a shift in behavioral patterns (anhedonia, decreased motor activity). This indicates a key role of pro-inflammatory cytokines in the pathogenesis of depression. In addition, regardless of the method of inducing a depression-like state (chronic stress or IL-1 β overexpressing virus), an increased level of neuronal apoptosis in the prefrontal cortex was recorded in mice [4]. These data indicate a close relationship between a depression-like state and neuronal apoptosis.

In a number of studies, in models of chronic stress and depression-like states in rodents, neurodegenerative changes were shown in neurons in various areas of the brain (in particular, the hippocampus and prefrontal cortex): a decrease in the density of synapses, a reduction in the length and branching of dendrites, and cell loss. Hill et al. showed a direct correlation between anxiety / depression and adult neurogenesis. Activation of neurogenesis through inactivation of the pro-apoptotic gene Bax in Nestin-positive cells contributed to the reduction of anxiety and depressive behavior caused by chronic corticosterone treatment [5]. Finally, there is ample evidence of increased neurogenesis after antidepressant therapy in rodent models [12].

## Conclusion

Previously, we revealed the ability of M2(LS) Mϕ to enhance neurogenesis *in vitro* through stimulation of proliferation, differentiation and survival of NSC-like cells of the SH-SY5Y line [13]. Along with this, we observed a decrease in the concentration of pro-inflammatory cytokines (IL-1β and TNFα) in the brain of depression-like mice during intranasal therapy with soluble factors of M2(LS) [11], which indicates the anti-inflammatory activity of M2(LS) Mϕ. Taken together, our data obtained earlier and presented in the current study indicate the neuroregenerative potential of macrophages polarized into the M2 phenotype by efferocytosis.

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*Received 12.04.2023*
*Revision received 13.04.2023*
*Accepted 15.04.2023*

*Received 12.04.2023*
*Revision received 13.04.2023*
*Accepted 15.04.2023*

*Медицинская иммунология 2023, Т. 25, № 3, стр. 527-532*

*Краткие сообщения*
*Short communications*

*Medical Immunology (Russia)/ Meditsinskaya Immunologiya 2023, Vol. 25, № 3, pp. 527-532*

# МОРФОФУНКЦИОНАЛЬНЫЕ ИЗМЕНЕНИЯ МИКРОГЛИИ У МОЛОДЫХ И СТАРЫХ КРЫС WISTAR

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**Резюме.** Болезнь Альцгеймера (БА) является одним из наиболее распространенных нейродегенеративных заболеваний, приводящих к деменции. На сегодняшний день не существует эффективных методов лечения этого заболевания, так же как и единого мнения относительно механизмов, лежащих в основе его патогенеза. Получение данных об этих механизмах *in vivo* возможно только путем моделирования нейродегенерации у лабораторных животных. Среди различных теорий инициации нейродегенерации активно изучается влияние микроглии, а также инфламэйджинг – хроническое системное низкоуровневое возраст-ассоциированное воспаление. Оно проявляется увеличением числа стареющих клеток с секреторным фенотипом, ассоциированным со старением (SASP). В конечном итоге это приводит к манифестации и прогрессированию возраст-зависимых заболеваний, в том числе БА. Целью исследования была оценка возрастных изменений микроглии, про- и противовоспалительных цитокинов в головном мозге, а также субпопуляций лимфоцитов в периферической крови. В работе использовали самцов крыс Wistar двух возрастных групп – старых (возраст 24 месяца) и половозрелых (возраст 3 месяца) при отсутствии какого-либо дополнительного воздействия. В гиппокампе оценивали морфологические изменения микроглии на препаратах, окрашенных антителами к Iba1. В префронтальной коре головного мозга с помощью ПЦР-РВ исследовали уровень экспрессии провоспалительных – IL-6 и TNFα, противовоспалительных – IL-10 и TGF-β, цитокинов, а также маркеров активации микроглии – iNOS и MMP-9. В периферической крови оценивали содержание основных субпопуляций лимфоцитов с помощью проточной цитофлуорометрии. Показано, что по сравнению с половозрелыми крысами старые животные характеризуются значительными изменениями морфологии микроглии, увеличением уровня экспрессии провоспалительных и снижением противовоспалительных цитокинов, повышением маркеров активации микроглии. При старении

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**Образец цитирования:**
*А.В. Сентябрева, Е.А. Мельникова, Е.А. Мирошниченко, И.С. Цветков, А.М. Косырева «Морфофункциональные изменения микроглии у молодых и старых крыс Wistar» // Медицинская иммунология, 2023. Т. 25, № 3. С. 527-532.*
*doi: 10.15789/1563-0625-MCO-2757*

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**For citation:**
*A.V. Sentyabreva, E.A. Melnikova, E.A. Miroshnichenko, I.S. Tsvetkov, A.M. Kosyreva “Morphofunctional changes of microglia in adult and old Wistar rats”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 527-532.*
*doi: 10.15789/1563-0625-MCO-2757*

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**DOI:** 10.15789/1563-0625-MCO-2757

наблюдалось снижение процентного содержания моноцитов и В-клеток в периферической крови. Полученные данные свидетельствуют о развитии инфламэйджинга, который проявляется в виде активации микроглии, смещения баланса продукции цитокинов в сторону провоспалительных и, как следствие, активации миграции моноцитов и В-лимфоцитов из крови в ткани. Таким образом, исследование роли воспаления в развитии БА целесообразно выполнять на старых животных, физиологическое состояние которых соответствует таковому у людей. Дальнейшие исследования в этой области позволят расширить понимание механизмов инициации и прогрессирования нейродегенерации, необходимое для разработки новых и эффективных терапевтических подходов к лечению БА.

*Ключевые слова: нейродегенерация, старение, микроглия, инфламэйджинг, воспаление, иммуносенесценция*

# MORPHOFUNCTIONAL CHANGES OF MICROGLIA IN ADULT AND OLD WISTAR RATS

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**Abstract.** Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases leading to dementia. There is no effective treatments for this disease so far, as well as a consensus concerning the mechanisms of its pathogenesis initiation. Obtaining data on them *in vivo* is possible only by modeling neurodegeneration in laboratory animals. Among the various theories of the initiation of neurodegeneration, the impact of microglia is vigorously studied recently, as well as inflammaging, which is a term for chronic age-related low-grade systemic inflammation. It manifests in the increasing number of senescent cells with senescence-associated secretory phenotype (SASP). Eventually, it leads to manifestation and progression of age-related diseases, such as AD. The aim of the study was to evaluate age-related changes in microglia, pro- and anti-inflammatory cytokines expression levels in the brain, as well as ones of microglial activation, and also subpopulations of lymphocytes in peripheral blood. We used male Wistar rats of two age groups, which were composed of old (age 24 months) and adult (age 3 months) rodents, without any additional exposure. In the hippocampus, morphological changes in microglia were assessed on preparations stained with antibodies to Iba1. In the prefrontal cortex, RT-qPCR was used to study the level of expression of pro-inflammatory IL-6 and TNFα, anti-inflammatory IL-10 and TGF-β cytokines, as well as microglial activation markers iNOS and MMP-9. In the peripheral blood, the relative numbers of the main subpopulations of lymphocytes and monocyte were measured by flow cytometry. It was shown that, compared with adult rats, old animals are characterized by significant changes in the morphology of microglia, an increase in the level of expression of pro-inflammatory and a decrease in anti-inflammatory cytokines, and an increase in microglia activation markers. With aging, a decrease in the percentage of monocytes and B cells in peripheral blood was observed. These data indicate the development of inflammaging, which displays itself in microglia activation, a shift in the balance of cytokine production towards pro-inflammatory ones, and, as a result, activation of the migration of monocytes and B lymphocytes from the blood into tissues. Thus, it is justified to study the role of inflammation in the development of AD in old animals whose physiological state corresponds to that in humans. Further research in this area will expand the understanding of the mechanisms of initiation and progression of neurodegeneration, which is necessary for the development of novel and effective therapeutic approaches to the treatment of AD.

*Keywords: neurodegeneration, aging, microglia, inflammaging, inflammation, immunosenescence*

Number of state registration of research, development, and technological work for civil purposes – 122030200530-6.

## Introduction

Alzheimer's disease (AD) is one of the most prevalent types of neurodegenerative diseases leading to dementia. Today there are around 50 million patients with AD worldwide [10], and this number will triple by 2050 [11]. AD remains a great burden for not only patients and people who provide the care of them. It also makes an economic load on health care systems of many countries worldwide, creating an annual global cost of almost 1 trillion USD. Another challenge associated with AD is an absence of any effective treatment so far. Existing and approved drugs can only faintly relieve the symptoms of AD.

There is no consensus concerning the initial mechanisms of AD pathogenesis [1]. The impact of inflammaging, or chronic age-related low-grade systemic inflammation [3] is one of the most perspective and intensively studying hypotheses so far [5]. According to recent data microglia, which are resident immune cells in the central nervous system (CNS), might play the key role of AD development as well [8]. Cellular senescence manifesting with senescence-associated secretory phenotype (SASP) leads to microglia activation and is associated with the consistent increasing of pro-inflammatory mediators' production [9] forming a vicious circle of the disease. It is also worth noting that microglia cells, as well as other types of immune cells, undergo the process of cellular senescence themselves, which affects and probably disrupts their function.

Although neurodegenerative diseases, such as AD, belong to the group of age-related pathologies but their modeling is still being conducted only on adult rodents, whereas experiments on old animals can provide more relevant data. **The purpose of this work** was to study morphofunctional changes of microglia in adult Wistar rats in comparison with old ones to reveal age-related alterations.

## Material and methods

The work was performed on adult (n = 10, age 3 months) and old (n = 10, age 24 months) male Wistar rats, euthanized by overdose (15 mg/kg) of Zoletil (Vibrac Sante Animale). The study was approved by the Bioethical Commission of the Avtsyn Research Institute of Human Morphology of Federal state budgetary scientific institution "Petrovsky National Research Centre of Surgery" (Protocol No. 36 (12) March, 28, 2022). All experimental work involving animals was carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986).

For ICH-P study, frontal histological sections of brains (6.0 mm posterior relative to bregma) were stained with antibodies Iba1 (1:100; P4C288Ra01, Cloud Clone) and secondary HRP Donkey-anti-Rabbit antibodies (1:500; 416035, Novex Life Technologies). The pictures were captured with the Leica microscope (DM 2500 Leica Microsystems) on magnification 1600.

The expression of IL-6, IL-10, TNFα, TGF-β, iNOS, and MMP-9 mRNA was assayed by real-time qPCR in tissue fragments of the prefrontal cortex. The levels of all aforementioned mRNA expression relative to GAPDH expression level as a reference were determined using a qPCRmix-HS SYBR (Eurogen, Russia) containing fluorescent intercalating dye SYBR Green I. Amplification with detection and digital analysis of fluorescence in real time was carried out on DT-96 Real-Time PCR Cycler (DNA-Technology JSC, Moscow, Russia) in a standard mode at 95 °C for 5 minutes followed by 95 °C for 15 seconds, 62 °C for 10 seconds + reading and 72 °C for 20 seconds 45. All the primers sequences were picked up by on-line soft Primer-BLAST.

Absolute and relative numbers of lymphocytes various subpopulations were counted using flow cytometry (Beckman Coulter, USA) in peripheral blood. The following antibodies (eBioscience, USA) were used for immune phenotypic analysis: anti-rat CD3-PE for total T lymphocyte population, anti-rat CD4-FITC for $CD3^+CD4^+$ for T helpers, anti-rat CD8-PE-Cy5 for $CD3^+CD8^+$ for T cytotoxic cells, anti-rat CD45R-FITC for $CD45R^+B$ cells, and anti-rat CD43-PE for $CD43^+$ monocyte. Erythrocytes were lysed with the OptiLyse C solution (eBioscience, USA). The results were analyzed by Statistica 8.0 software (StatSoft, Inc.) using Mann–Whitney U test.

## Results and discussion

In a morphological study, adult rats' microglia had a regular size and thin ramified processes, which are features of resting functional status (Figure 1A, see 2nd page of cover). At the same time, old rats' microglia had an increased size and spheroidal swelling, hypertrophic, beaded, and tortuous processes (Figure 1B, see 2nd page of cover).

The result of qPCR showed that IL-6 and TNFα expression levels were higher in old rats than in adult rats by 1.5-fold and 3-fold accordingly. Also iNOS, which is a marker of M1 activated microglia, was

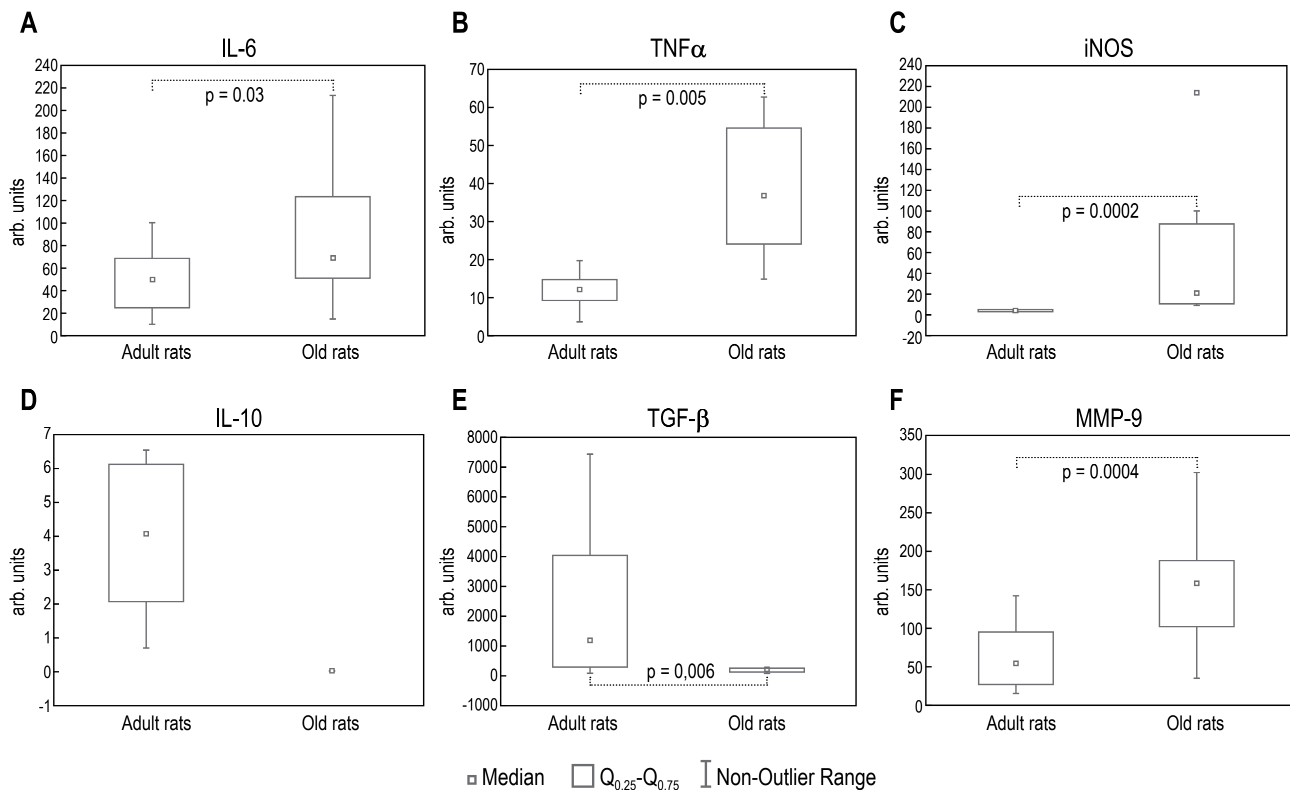


**Figure 2. mRNA expression levels of IL-6 (A), TNFα (B), iNOS (C), IL-10 (D), TGF-β (E), and MMP-9 (F) in the prefrontal cortex of adult (left bar) and old (right bar) Wistar rats**

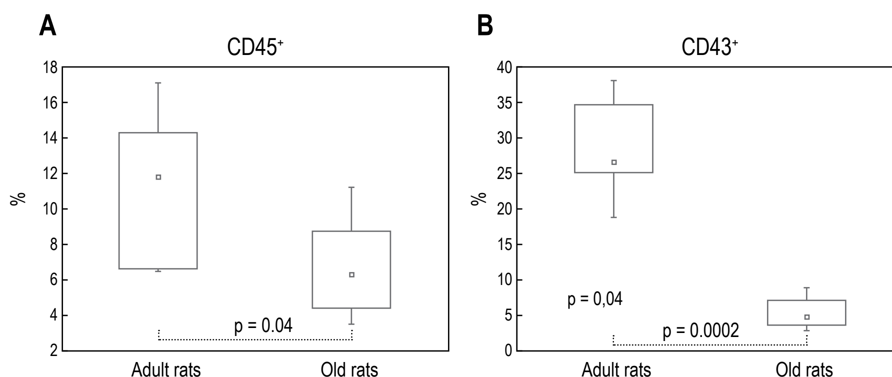


**Figure 3. Percentage of $CD45R^{+}B$ cells (A) and $CD43^{+}$ monocyte (B) in peripheral blood of adult (left bar) and old (right bar) Wistar rats**

5-fold higher in old rats in comparison with adult rats. Old rats group showed no IL-10 expression at all, whereas the expression level of TGF-β was 7.25-fold higher in adult rats. At the same time *MMP9*, which is a marker of M2 activated microglia, was 3-fold higher in old rats group (Figure 2).

Additionally, the immune phenotypic analysis of monocyte and lymphocytes subpopulations was performed to estimate the impact of aging on the number of various immune cells in peripheral blood. Flow cytometry data demonstrated no statistically significant differences in the relative numbers of lymphocytes ($CD3^{+}$), including $CD3^{+}CD4^{+}T$ helpers and $CD3^{+}CD8^{+}T$ cytotoxic cells, between the groups. However, the percentage of $CD45R^{+}B$ cells and $CD43^{+}$ monocyte was almost 2-fold and 6.5-fold higher in adult rats in comparison with old rats (Figure 3).

Hence, we observed morphological changes of microglial cells, the decreasing of anti-inflammatory cytokines expression levels and the increasing of pro-inflammatory ones as well as microglia activation markers, and the reduction of monocyte and B cells' numbers in old rats due to aging.

Our data are consistent with latter results obtained from humans. It was shown that identical changes of

microglia appeared in healthy human with aging [6]. Shahidehpour et al. described age-related changes of microglia morphology as hypertrophic and dystrophic and stated there were a strong evidence that dystrophic microglia are disease-associated one. It is generally accepted that the CNS resident immune cells have so called "resting" and activated states. Microglia activation, just as macrophages' one, leads cells to pro-inflammatory M1 polarization or anti-inflammatory M2 polarization. But also there is a continuum of different intermediate phenotypes between M1 and M2, and microglia can take turns from one state to another depending on microenvironment [4]. So, it is not a surprising found of presence both M1 and M2 microglia in healthy adult and old rats.

At the same time, the significant rise of iNOS and MMP9 expression levels in old rats implies there is a higher number of activated immune cells caused by aging itself due to the development of SASP. Being secretory active and likely senescent as well, these microglial cells might not only fail their surveillance and clearance functions, but also take part in aggravation of already existing neuroinflammation with unpleasant consequences. Revealing exact mechanisms to prevent them is a task yet to come.

We showed the reduction of monocyte and B cells' numbers in peripheral blood of old rats. In recent research concerning lymphopoiesis in both human and mouse aging revealed a link between the level of TNFα, consistently produced by peripheral B cells themselves during aging, and restraining of B cell lymphopoiesis in the bone marrow [2]. Meanwhile, Snodgrass et al. showed a decreasing of circulating monocyte pool due to aging in humans [7]. It could happen due to abating of monocytopoiesis in bone marrow as well as B cells' or cells destruction caused by pro-inflammatory background or other reasons; but it also could be related with an intensification of monocyte migration to different tissues, including brain parenchyma, since inflammaging is a system condition involving the whole organism.

## Conclusion

Hence, this study confirms a great deal of differences between adult and old rodents' physiological state. Obviously, their reactions to the same exposure of various factors will be quite different as well. Since old rats demonstrate age-related changes in immune system's cells similar to humans', using aged animals for modeling of neurodegeneration is justified. Further investigations in this search field will provide more essential data for future inventions of novel and efficacious therapeutic approaches.

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*Поступила 13.04.2023*
*Отправлена на доработку 14.04.2023*
*Принята к печати 18.04.2023*

*Received 13.04.2023*
*Revision received 14.04.2023*
*Accepted 18.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 533-538

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 533-538

# ИММУНОМОДУЛИРУЮЩИЕ СВОЙСТВА КОФЕИНА И ОБРАБОТАННЫХ КОФЕИНОМ ИММУНОКОМПЕТЕНТНЫХ КЛЕТОК ПРИ ДЕПРЕССИВНО-ПОДОБНОМ СОСТОЯНИИ

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**Резюме.** Депрессия является одной из ведущих глобальных проблем здравоохранения во всем мире. Значительное увеличение распространенности среди трудоспособного населения, а также высокая коморбидность, частичная или полная фармакорезистентность трети пациентов определяет необходимость разработки новых подходов к терапии депрессии. Нарушение взаиморегуляции основных гомеостатических систем играет важнейшую роль в патогенезе депрессии; психо- и иммунопатология тесно взаимосвязаны: патологические изменения в функционировании обеих систем происходят одновременно и взаимообусловлены. Это определяет перспективность методов лечения депрессии, основанных на иммунологических подходах. Кофеин – препарат, известный своими психонейромодулирующими свойствами является антагонистом аденозиновых рецепторов с выраженным дозозависимым эффектом. Аденозиновые рецепторы экспрессируются как клетками ЦНС, так и клетками иммунной системы, что обуславливает его иммуномодулирующие свойства. Сходство как фенотипов, так и функций клеточных элементов иммунной и нервной систем, а также однонаправленное влияние большинства психоактивных препаратов на ЦНС и иммунную систему определяет интерес к изучению иммуномодулирующих свойств кофеина для направленного воздействия на функциональную активность иммунных клеток, с целью их последующего использования в качестве модельных объектов для нормализации нарушенных при депрессивном состоянии нейроиммунных регуляторных связей. Ранее нами впервые была продемонстрирована возможность редактирования депрессивно-подобного поведения прекультивированными с кофеином иммунокомпетентными клетками и показаны центральные механизмы этого эффекта, направленные на стимуляцию процессов нейропластичности и снижение нейровоспаления. Целью настоящего исследования было оценить функциональный фенотип иммунокомпетентных клеток депрессивно-подобных животных после обработки клеток *in vitro* кофеином, а также эффекты трансплантации прекультивированных с кофеином иммунокомпетентных клеток на параметры функциональной активности иммунной системы сингенных депрессивно-подобных реципиентов. В результате проведенного исследования было показано, что кофеин в низкой концентрации повышает спонтанную и индуцированную митогенами пролифера-

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**Образец цитирования:**
*Е.В. Маркова, М.А. Княжева «Иммуномодулирующие свойства кофеина и обработанных кофеином иммунокомпетентных клеток при депрессивно-подобном состоянии» // Медицинская иммунология, 2023. Т. 25, № 3. С. 533-538.
doi: 10.15789/1563-0625-IPO-2666*

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**For citation:**
*E.V. Markova, M.A. Knyazheva “Immunomodulatory properties of caffeine and caffeine-treated immune cells in depression-like state”, Medical Immunology (Russia)/ Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 533-538.
doi: 10.15789/1563-0625-IPO-2666*

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**DOI:** 10.15789/1563-0625-IPO-2666

тивную активность спленоцитов депрессивно-подобных самцов-мышей (CBA x C57BL/6)F1 *in vitro*; при этом изменяется спонтанная и митоген-стимулированная продукция этими клетками цитокинов TNFα IL-1β, IFNγ, IL-2 и IL-10. После внутривенного введения прекультивированных с кофеином спленоцитов депрессивно-подобных доноров сингенным депрессивно-подобным реципиентам у последних наблюдалась стимуляции гуморального иммунного ответа, оцененного по увеличению как относительного, так и абсолютного числа антителообразующих клеток селезенки. Была зарегистрирована также стимуляция спонтанной пролиферативной активности лимфоцитов культуре спленоцитов. Полученные данные свидетельствуют о выраженном эффекте кофеина *in vitro* на функциональную активность иммунокомпетентных клеток, равно как и о позитивном иммуномодулирующем эффекте прекультивированных с кофеином клеток при депрессивно-подобном состоянии *in vivo*.

*Ключевые слова: кофеин, депрессивно-подобное состояние, иммунный ответ, иммунокомпетентные клетки, пролиферация, цитокины*

# IMMUNOMODULATORY PROPERTIES OF CAFFEINE AND CAFFEINE-TREATED IMMUNE CELLS IN DEPRESSION-LIKE STATE

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**Abstract.** Depression is one of the leading global health problems worldwide. A significant increase in prevalence among the working-age population, as well as high comorbidity, partial or complete drug resistance in a third of patients determines the need to develop new approaches to the treatment of depression. Violation of mutual regulation of the main homeostatic systems plays an important role in the pathogenesis of depression. Psycho- and immunopathology are closely interrelated: pathological changes in the functioning of both systems occur simultaneously and are interdependent. This determines the prospects for the treatment of depression based on immunological approaches. Caffeine, a drug known for its psychoneuromodulatory properties, is an adenosine receptor antagonist with a pronounced dose-dependent effect. Adenosine receptors are expressed by both CNS cells and cells of the immune system, which determines its immunomodulatory properties. The similarity of both phenotypes and functions of the cellular elements of the immune and nervous systems, as well as the unidirectional effect of most psychoactive drugs on the central nervous system and the immune system, determines the interest in studying the immunomodulatory properties of caffeine for a targeted effect on the functional activity of immune cells, with a view to their subsequent use as model objects for the normalization of neuroimmune regulatory connections disturbed in a depressive state. Previously, we first demonstrated the possibility of editing depression-like behavior by immune cells precultivated with caffeine and showed the central mechanisms of this effect aimed at stimulating neuroplasticity processes and reducing neuroinflammation. The aim of this study was to evaluate the functional phenotype of immune cells in depressive-like animals after *in vitro* treatment of cells with caffeine, as well as the effects of transplantation of caffeine-precultured immune cells on the parameters of the functional activity of the immune system of syngeneic depressive-like recipients. As a result of the study, it was shown that low concentrations of caffeine increase the spontaneous and mitogen-induced proliferative activity of splenocytes of depression-like male mice (CBA x C57BL/6)F1 *in vitro*; this changes the spontaneous and mitogen-stimulated production of cytokines TNFα IL-1β, IFNγ, IL-2, and IL-10 by these cells. After intravenous administration of the precultured with caffeine depression-like donor's splenocytes to syngeneic depression-like recipients, stimulation of the humoral immune response was observed in the latter, assessed by an increase in both the relative and absolute number of antibody-forming spleen cells. Stimulation of spontaneous proliferative activity of lymphocytes in splenocyte culture was also registered. The data obtained indicate a positive effect of caffeine *in vitro* on the immune cell's functional activity, as well as a positive immunomodulatory effect of the immune cells precultured with caffeine in a depression-like state *in vivo*.

*Keywords: caffeine, depression-like state, immune response, immune cells, proliferation, cytokines*

This work was supported by the Russian federal budget allocated for the basic scientific research at the Scientific Research Institute of Fundamental and Clinical Immunology (theme No. 122011800324-4 (2021-2023)).

## Introduction

Depression is one of the urgent medical and social problems of our time due to its wide prevalence among various contingents, including among the working population throughout the world. There is still no consensus on the contribution of genetic, environmental, social factors to the etiology and pathogenesis of this disease. The heterogeneous nature, high comorbidity, and pronounced drug resistance of major depressive disorder (MDD) determine the search and development of new highly effective treatment approaches. In patients with major depressive disorder (MDD), a violation of the functional activity of the immune system and its cellular elements was found, which prevents a favorable prognosis, including the response to antidepressants. Altered innate and adaptive immunity involving a pronounced increase in the number of neutrophils, alterations in the numbers of monocytes, in the relative abundance of T cell subtypes, reduced numbers of the regulatory T (Treg) and B (Breg) cells, increased Th1/Th2 ratio and the Th17 cells, a decrease in the number of B-cells, decrease in the proliferative activity of lymphocytes and the intensity of the immune response has been found in depressed patients and in stress-induced depression-like behavior in animal models [2, 4, 9]. There is also evidence of changes in the levels of cytokines IL-1β, IL-2, IL-4, IL-6, IL-12, IL-18, IL-8, IL-13, TNFα, IFNγ, TGF-β1, and IL-10 [6, 7, 8, 9].

Among the known molecular pathways activated in MDD are JAK-STAT and PI3K/Akt/RAS/MAPK [3, 5, 15]. The main pharmacological agents for the treatment of depression include drugs aimed at the monoamine and melatonin systems. Positive effects are also provided anti-cytokine treatment [7, 14], but the issue of toxicity and safety of the use of such drugs is not well understood.

Caffeine, a drug known for its psychoneuromodulatory properties, is an adenosine receptor (AR) antagonist with a pronounced dose-dependent effect [1, 13, 15]. Adenosine receptors are not only expressed by CNS cells, they are also present in most immune cells and are involved in the regulation of various cell functions. The similarity of both phenotypes and functions of the cellular elements of the immune and nervous systems, as well as the unidirectional effect of most psychoactive drugs on the central nervous system and the immune system, determines the interest in studying the immunomodulatory properties of caffeine for a targeted effect on the functional activity of immune cells, with a view to their subsequent use as model objects for the normalization of neuroimmune regulatory connections disturbed in a depressive state and alleviation of depressive symptoms. Previously, we first demonstrated the possibility of editing depression-like behavior by immune cells precultured with caffeine and showed the central mechanisms of this effect aimed at stimulating neuroplasticity processes and reducing neuroinflammation [10, 11, 12].

In connection with the above, **the aim of this study** was to evaluate the functional phenotype of immune cells in depressive-like animals after *in vitro* treatment of cells with caffeine, as well as the effects of transplantation of caffeine-precultured immune cells on the parameters of the functional activity of the immune system of syngeneic depressive-like recipients.

## Materials and methods

Male mice (CBA × C57BL/6)F1 at the age of 4 months, weighing 25-30 g, obtained from the animal facility of the E.D. Goldberg Scientific Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center of the Russian Academy of Sciences (Tomsk, Russia) were used. The animals were housed in groups of ten per cage in a laboratory vivarium, at least two weeks prior the start of the experiment under standard conditions on a standard diet, with free access to water and a natural light regime. All experimental procedures were in compliance with the European Communities Council Directive (Strasburg, 1986) and were approved by the Institutional Animal Care and Use Committees of the Scientific Research Institute of Fundamental and Clinical Immunology. Every effort was made to minimize the number of animals used and their suffering.

A depression-like state was formed in passive mice ($n = 36$) as a result of repeated experience of defeat in agonistic interactions with an aggressive partner during 20 days, as described earlier [10, 11]. The depression-like phenotype was characterized in the forced swimming test, open field and plus maze using a modern hardware and software complex EthoVision XT (Noldus, Netherlands). Anhedonia was assessed by the sucrose preference test using an automated system for behavioral and cognitive phenotyping IntelliCage (TSE systems, Germany). Then depression-like mice were isolated into individual cages to avoid agonistic interaction and were used as donors and recipients of immune cells.

Immune cells were obtained under sterile conditions from a suspension of splenocytes, precultured *in vitro* with caffeine at the dose of 100 μg per $15 \times 10^6$ cells with 3% FCS (Hyclone) for 25 minutes,

as we described earlier [10, 11], with subsequent assessment of the cells spontaneous and mitogen-induced proliferative activity by a standard method for the inclusion of a radioactive label in nucleoprotein fractions ($H^3$-thymidine), as well as the production of a number of cytokines by splenocytes by determining their quantitative content in samples of culture supernatants by ELISA, using specific test-systems for the determination of IFNγ, IL-6 (eBioscience, BenderMedSystems, Austria) and for the determination of IL-1β, IL-10, TNFα (R&D Systems Inc, UK) in accordance with the manufacturer's instructions.

At the second stage of the study, splenocytes precultured with caffeine were administered intravenously to syngeneic depression-like recipients. Splenocytes precultured under similar experimental conditions, but without caffeine, as well as a group of depressed recipients who were transplanted with these cells served as controls. In syngeneic depression-like recipients, the intensity of the humoral immune response was assessed by the relative and absolute number of antibody-forming spleen cells in response to the T-dependent antigen (sheep erythrocytes). The intensity of the cellular immune response was assessed by the severity of the delayed-type hypersensitivity reaction (DTH).

The results were statistically processed using the Mann–Whitney paired test (Statistica for Windows 10.0 software). Results are presented as the mean ± SEM. Differences were considered significant at $p \leq 0.05$.

## Results and discussion

As a result of the study, it was found that caffeine *in vitro* stimulates the proliferative activity of depression-like mice splenocytes, which is expressed in an increase in spontaneous and Con-A-induced proliferation of lymphocytes within splenocytes (Table 1), apparently through the direct action of caffeine on A1AR and A2AR receptors present on spleen immune cells. The binding of caffeine to these receptors leads to a change in the chain of intracellular events that affect, among other things, the synthesis and production of cytokines [1, 3, 13, 15]; therefore, the production of a number of cytokines by caffeine-treated depression-like mouse splenocytes was evaluated.

It has been shown that after *in vitro* treatment with caffeine at a low concentration, the spontaneous production of IL-1β, IFNγ and TNFα decreases with a significant increase in the production of IL-2 and IL-10. In the study of mitogen-stimulated production of cytokines, a decrease in the production of IL-1β, IFNγ and an increase in IL-10 was found (Table 2). The obtained results are consistent with the data of other researchers, in particular, on the negative regulation of TNFα secretion by caffeine in various cells, including splenocytes, peripheral blood mononuclear cells, and mast cells [13, 15]. A likely mechanism for changing the proliferative activity of splenocytes and the production of a number of pro- and anti-inflammatory cytokines by cells is an increase in cAMP accumulation as a result of *in vitro* binding of caffeine at a low concentration used to A1AR and A2AR, followed by prevention of NF-κB activation through the cAMP/PKA pathways [1, 3, 15]. It is assumed that activated A2AR promotes the differentiation of $CD4^+$T cells towards regulatory T cells, due to an increase in the level of IL-2 and a decrease in the level of IL-6, which is one of the activators of the JAK-STAT signaling pathway [4, 5].

**TABLE 1. PROLIFERATIVE ACTIVITY OF DEPRESSION-LIKE MALES (CBA × C57BL/6) F1 SPLENOCYTES AFTER *IN VITRO* TREATMENT WITH CAFFEINE AND DEPRESSION-LIKE RECIPIENT'S SPLEEN CELLS AFTER TRANSPLANTATION OF SYNGENEIC CAFFEINE-TREATED SPLENOCYTES, M±SD**

| Cell group | Splenocyte proliferative activity (cpm) | | |
|---|---|---|---|
| | spontaneous | Con A | LPS |
| **Splenocytes of depression-like mice precultured without caffeine (control)** | 372.7±73.2 | 30854.4±3095.8 | 3848.7±491.4 |
| **Splenocytes of depression-like mice precultured with caffeine (experimental)** | 861.4±104.1** | 50437.1±7237.8* | 4117.0±342.1 |
| **Depression-like recipient's splenocytes (control)** | 401.2±75.6 | 32589.2±4161.7 | 4198.7±396.8 |
| **Depression-like recipient's splenocytes (experimental)** | 623.4±71.6** | 43964.3±6172.8 | 4569.3±281.5 |

Note. Control cell's group – splenocytes of depression-like mice precultured without caffeine or splenocytes of depression-like recipients after transplantation of these cells. Experimental cell's group – splenocytes of depression-like mice precultured with caffeine or splenocytes of depression-like recipients after transplantation of these cells. *, $p < 0.05$; **, $p < 0.01$ relative to the corresponding indicator in the control group.

**TABLE 2. CYTOKINE PRODUCTION (pg/mL) BY *IN VITRO* CAFFEINE-TREATED SPLENOCYTES OF DEPRESSION-LIKE MALES (CBA × C57BL/6)F1, M±SD**

| Cell group | IL-1β | IL-4 | IL-6 | IL-10 | IL-2 | IFNγ | TNFα |
|---|---|---|---|---|---|---|---|
| | Spontaneous production | | | | | | |
| Splenocytes of depression-like mice precultured without caffeine (control) | 11.3±1.5 | 6.4±1.5 | 201.0±8.1 | 13.1±7.3 | 10.8±2.3 | 28.0±5.8 | 32.5±3.8 |
| Splenocytes of depression-like mice precultured with caffeine | 7.5±2.7* | 5.3±1.4 | 191.0±7.1 | 31.9±4.1** | 34.2±6.5* | 12.2±4.2** | 24.5±2.4** |
| Mitogen-stimulated production | | | | | | | |
| Splenocytes of depression-like mice precultured without caffeine (control) | 192.1±5.6 | 31.3±5.9 | 1081.0±108.3 | 52.9±10.2 | 48.3±3.4 | 115.1±10.4 | 159.4±8.9 |
| Splenocytes of depression-like mice precultured with caffeine | 61.1±7.5** | 32.2±2.3 | 971.0±95.1 | 91.0±14.1** | 57.1±2.2* | 42.2±7.1** | 148.9±5.4 |

Note. *, p < 0.05; **, p < 0.01 compared with the corresponding indicator in the control group of cells.

**TABLE 3. INTENSITY OF THE HUMORAL AND CELLULAR IMMUNE RESPONSE IN DEPRESSION-LIKE RECIPIENTS (CBA × C57BL/6)F1 AFTER TRANSPLANTATION OF SYNGENEIC SPLENOCYTES MODULATED *EX VIVO* BY CAFFEINE, M±SD**

| Parameter | Recipient group | |
|---|---|---|
| | Control group | Experimental group |
| Relative number of antibody-forming spleen cells /$10^6$ | 332.2±74.7 | 553.6±57.1* |
| Absolute number of antibody-forming spleen cells | 69515.4±7678.6 | 87821.2±6118.6* |
| DTH reaction (%) | 10.2±2.3 | 12.1±1.4 |

Note. Control group of recipients – depression-like recipients after transplantation of syngeneic splenocytes precultured without caffeine. Experimental group of recipients – depression-like recipients after transplantation of syngeneic splenocytes precultured with caffeine. *, p < 0.01 relative to the corresponding indicator in the control group.

Previously, we demonstrated the depression-like behavior editing by the transplantation of caffeine-treated immune cells [10, 11]. Taking into account the above-mentioned important role of disturbances in the functional state of the immune system and its cells in the pathogenesis of depression, including the formation of depression-like behavior, in this study, the intensity of the main components of the immune response and the proliferative activity of lymphocytes of depression-like recipients were assessed after transplantation of syngeneic splenocytes treated *in vitro* with caffeine. As a result of the studies, stimulation of the humoral immune response was registered in depressive-like recipients, which was assessed by an increase in both the relative and absolute number of antibody-forming spleen cells (Table 1). At the same time, a significant stimulation of the spontaneous proliferative activity of splenocytes was also shown (Table 3).

There were no significant changes in the DTH response in depressive-like recipients. The demonstrated stimulation of the functional activity of the immune system of depression-like recipients occurs along with the modulation of the structural and functional parameters of their nervous system, including editing depression-like behavior, reduction of neuroinflammation and stimulation of neuroplasticity [11]. Taking into account the fact that si-

milar effects are also observed during antidepressant therapy [4, 9, 14], adoptive immunotherapy with *in vitro* caffeine-modulated immune cells should be considered as a possible promising method in the treatment of depression, excluding the negative side effects observed with the direct use of this psychoactive drug substances.

## Conclusion

Thus, the data obtained indicate a pronounced effect of caffeine *in vitro* on the functional activity of immune cells, as well as a positive immunomodulatory effect of the immune cells precultured with caffeine *in vivo* in a depression-like state.

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*Поступила 12.03.2023*
*Принята к печати 26.03.2023*

*Received 12.03.2023*
*Accepted 26.03.2023*

# ТУЧНЫЕ КЛЕТКИ ТИМУСА КАК КОМПОНЕНТ НЕЙРО-ЭНДОКРИННО-ИММУННЫХ ВЗАИМОДЕЙСТВИЙ ПРИ СТРЕССЕ

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**Резюме.** Тучные клетки являются обязательным компонентом микроокружения тимуса, за счет выработки ряда цитокинов они влияют на межклеточные взаимодействия и проницаемость гемато-тимического барьера. Предполагают, что тимус является местом образования и депонирования тучных клеток. Тучные клетки, находясь под сложным комплексным нейроэндокринным контролем, при формировании стресс-реакции могут играть важную роль в процессе временной трансформации тимуса, влияя в том числе на экстратимическую миграцию клеток. Цель данного исследования – оценить функциональную вовлеченность тучных клеток в процесс временной трансформации тимуса при различных гипер- и гиподинамических воздействиях на фоне формирования стресс реакции и без нее.

Эксперимент проведен на самцах крыс линии Wistar. В качестве стресс-факторов применяли физическую нагрузку (плавание) разной интенсивности и иммобилизацию (у животных с сохраненными и удаленными надпочениками), как два полярных состояния динамического стресса. На гистологических препаратах тучные клетки типировали и рассчитывали коэффициент дегрануляции и средний гистохимический коэффициент (синтетическую активность).

В группах с сохраненными надпочечниками после воздействий отмечается достоверное снижение коэффициента массы тимуса, что свидетельствует об ослаблении его функциональной активности в ответ на развитие стресса. При этом тучные клетки тимуса довольно быстро реагируют на нейроэндокринные факторы, выделяемые при стрессе, и вовлекаются в общую реакцию: их активность проявляется в синхронном снижении синтеза гранул в цитоплазме и усиленном выбросе активных веществ, накопленных ранее. В группах с удаленными надпочечниками, напротив, после иммобилизации масса и структура тимуса остаются неизменными, не выявляются и изменения морфофункциональных показателей тучных клеток. Эксперименты с гипо- и гипердинамической нагрузкой животных с сохраненными и удаленными надпочечниками свидетельствуют, что реакция тучных клеток во многом определяется гипоталамо-гипофизарно-надпочечниковой осью эндокринной системы. Удаление надпочечников (невозможность выброса глюкокортикоидов) приводит к отсутствию функционального ответа со стороны тучных клеток тимуса. Стимулирующее влияние глюкокортикоидов надпочечников на тучные клетки при стрессе осуществляется в комплексе с другими нейроэндокринными факторами (катехоламинами, кортикотропин-релизинг-гормоном, адренокортикотропным гормо-

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**Образец цитирования:**

*О.С. Арташян, Ю.С. Храмцова «Тучные клетки тимуса как компонент нейро-эндокринно-иммунных взаимодействий при стрессе» // Медицинская иммунология, 2023. Т. 25, № 3. С. 539-544.
doi: 10.15789/1563-0625-TMC-2774*

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**For citation:**

*O.S. Artashyan, Yu.S. Khramtsova “Thymus mast cells as a component of neuro-endocrine-immune interactions under stress”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 539-544.
doi: 10.15789/1563-0625-TMC-2774*

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**DOI:** 10.15789/1563-0625-TMC-2774

ном) и при формировании полноценной стресс-реакции организмом, они активно вовлекаются в процесс временной трансформации тимуса через выделение ряда цитокинов, что является важным условием для выработки адаптационных механизмов со стороны иммунной системы.

*Ключевые слова: тимус, тучные клетки, стресс, иммунные клетки, адаптация, надпочечники*

# THYMUS MAST CELLS AS A COMPONENT OF NEURO-ENDOCRINE-IMMUNE INTERACTIONS UNDER STRESS

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**Abstract.** Mast cells (MCs) are a required component of the thymus microenvironment. They affect intercellular interactions and permeability of the hematothymic barrier through cytokine production. There is speculation that the thymus is the site of MCs formation and deposition. MCs are under complex neuro-endocrine control and they can play an important role in the process of acute transformation of the thymus in the formation of a stress reaction, affecting the extrathymic migration of cells. The purpose of this study is to assess the functional involvement of MCs in the process of the thymus acute transformation at various hyper- and hypodynamic effects during the formation of stress response and without it.

The study was conducted on male Wistar rats. The stress factors were physical activity (swimming) of different intensities and immobilization, which represent two opposite states of dynamic stress. MCs were classified on histological preparations; a degranulation coefficient and a mean histochemical coefficient (synthetic activity) were calculated.

In groups with preserved adrenal glands after exposure a significant decrease in the thymus mass coefficient is noted, which indicates a weakening of its functional activity in response to the development of stress. At the same time, MCs of the thymus quickly respond to neuro-endocrine factors under stress. These cells are involved in a general reaction: their activity consists in a synchronous decrease of the synthesis of granules in the cytoplasm and an increased release of active substances accumulated earlier. The mass and structure of the thymus remain unchanged in groups with removed adrenal glands after immobilization. No changes in morphofunctional indicators of mast cells were detected either. Experiments with hypo- and hyperdinamic loading of animals with preserved and removed adrenal glands indicate that the MCs response is largely determined by the hypothalamic-pituitary-adrenal axis of the endocrine system. Removal of the adrenal glands (inability to release glucocorticoids) leads to a lack of functional response from the thymus MCs. The stimulating effect of adrenal glucocorticoids on MCs under stress is carried out in combination with other neuro-endocrine factors (catecholamines, corticotropin-releasing hormone, adrenocorticotropic hormone). When this axis is activated and a full-fledged stress reaction is formed by the body, MCs are actively involved in the process of acute transformation of the thymus through cytokine secretion. These is an important condition for the development of adaptation mechanisms by the immune system.

*Keywords: thymus, mast cells, stress, immune cells, adaptation, adrenal glands*

The work was carried out partly within the framework of the IIF UB RAS themes No. 122020900136-4.

## Introduction

Today, cells of the thymic microenvironment are being actively studied as they play an important role in the differentiation, selection, and migration of T-lymphocytes, macrophages, epithelial, dendritic, and mast cells (MCs). The question of MCs' functions in the thymus continues to be open. There is evidence that MCs are normally involved in the processes of updating the extracellular matrix of the thymus in organogenesis, thymopoiesis, T-lymphocyte selection and angiogenesis [2, 5, 8, 9, 11]. Despite the intensive study of MCs, studies on the features of thymus mast cell population under the action of stress factors on the body and in the development of induced organ involution are rare. Acute thymus involution is known to be a manifestation of adaptation syndrome in response to stress. Under stress, the level of thymus polypeptides increases, which limits the stress-damaging effect of hypothalamic-pituitary-adrenal axis excessive activity [12]. In this case, the content of lymphoid cells in the thymus is reduced, and the number of epithelioreticulocytes and degenerating cells is increased. The researchers noted such a reaction of this organ under completely different extreme influences. It is known that MCs affect intercellular

interactions, permeability of the hematothymic barrier, and migration of thymus lymphocytes through the production of IL-1, IL-2, IL-3, IL-4, IL-6, TNFα, GM-CSF, NGF [4, 6, 10]. Additionally, it is assumed that thymus is the place of formation and deposit of MCs [8, 12]. In this regard, MCs can play an important role in the process of acute transformation of the thymus, affecting the extratimic migration of cells during the development of the stress reaction.

**The purpose of this study** is to assess the functional involvement of MCs in the process of acute transformation of the thymus at various hyper- and hypodynamic effects during the formation of stress response and without it.

## Materials and methods

The study was conducted on male Wistar rats weighing 150-200 g. The conditions of animal housing and treatment of animals used in the experiment were in accordance with the directive of the European Community of 80/609/EEC and were approved by the local ethics committee of the IIF UrO RAS (protocol No. 10 of 03.06.2016).

The stress factors were physical activity (swimming) of different intensities and immobilization, which represent two opposite states of dynamic stress. The animals were divided into seven experimental groups, and each group consisting of five animals (n = 5).

Physical activity (PA) consisted of rats being forced to swim in a special pool under different regimens for 4 weeks: 1) moderate physical activity (MPA) – daily swimming for 2 hours, and 2) maximum physical activity (MaxPA) – 6-day swimming with a load of 20% of their body weight, according to the following scheme: 5 sets of 60 seconds with 3 minutes of rest in between. The rats' weight was measured weekly, and the weight of the load was adjusted accordingly. After the completion of all swimming batches, the animals were removed from the experiment, and their thymi were extracted.

Immobilization stress (IS) was simulated by inducing neuromuscular tension, achieved by immobilizing the animals on their back for 6 hours once on an operating table. Two series of experiments were performed. In the first series, intact animals were subjected to neuromuscular stress, while in the second series, both adrenal glands were removed (AE) in animals 48 hours prior to immobilization stress. The animals were then removed from the experiment and their thymi were extracted. The thymi were extracted in two groups – one with preserved adrenal glands and one with removed adrenal glands – within two periods: immediately after the end of immobilization (groups IS 6 h and IS + AE 6 h), and 48 hours after immobilization (groups IS 48 h and IS + AE 48 h).

Histological preparations of the thymus were stained with hematoxylin-eosin to assess structural changes in the gland. Preparations were also stained with toluidine blue (pH = 2.0) to detect MCs by highlighting acidic sulfated glycosaminoglycans in a blue-violet color, a characteristic feature of metachromasia. MCs were then stained with alcian blue – safranin to assess maturity. Safranin binds to highly sulfated glycosaminoglycans, such as heparin, staining them red or pink. The presence of such granules is an indication of mature MCs. Alcian blue, on the other hand, binds to slightly sulfated glycosaminoglycans, mainly heparin precursors, staining them blue. The presence of such granules is an indication of immature MCs or MCs at an intermediate stage of maturation.

MCs were classified based on the content of granules in the cytoplasm to assess their functional activity. "0" – MCs with diffuse arrangement of granules in the cytoplasm and a clearly visible nucleus, "+1" – cells with a denser arrangement of granules in the cytoplasm and a contoured nucleus, "+2" – cells with a high degree of granularity in the cytoplasm, the nucleus is not clearly visible, the granules are tightly adjacent to each other, "+3" – cells with a very dense content of granules in the cytoplasm, the nucleus and individual granules are not detected. Based on these data, the mean histochemical coefficient was calculated: MHCC = (0 × "0" + 1 × "+1" + 2 × "+2" + 3 × "+3") / Σ all cell types. Cells were divided into three groups based on signs of degranulation: "DG" – actively degranulating MCs with a violation of membrane integrity and observation of granules outside the cell; "PDG" – partially degranulating MCs with a large number of granules outside; "NDG" – non-degranulating MCs with integral membrane and no granules outside the cell. Based on these data, the degranulation coefficient was calculated: DC = ((DG + PDG) / Σ all cell types) × 100%.

Statistical analysis was performed using the Statistica 6.1 program. Group comparisons were made using non-parametric methods, specifically the Mann-Whitney test. Differences were considered significant at $p < 0.05$.

## Results and discussion

MCs are obligatory cells of the thymus gland and are more often located in the peripheral part of the lobes around blood vessels, among the premedullary row of cells and accompany nerve fibers. The production of biogenic amines is a constant function of MCs of the thymus both normally and in pathological processes. MCs facilitate contact and transfer of stem cells and lymphocytes into tissues during circulation and promote migration of the formed lymphocytes

by secreting mediators for vascular permeability (histamine, serotonin, catecholamines, etc.).

Normally, the MCs of the thymus is quite large, of different shapes, more often rounded or oval, located in the capsule and connective branch walls (septa) of the gland, the content in 1 $mm^2$ is 117.9±3.62. Typing thymus MCs according to the degree of maturity, based on sulfation of glycosaminoglycans in granules, showed that 62% were mature cells and 38% were immature. Thus, more cells that have completed the synthesis, accumulation, and maturation of granules with biologically active substances are ready to show their activity by degranulating in response to endo- or exogenous stimuli. TC degranulation is regulatory because granule isolation is generally directed towards target cells (Figure 1, see 3rd page of cover).

In experimental groups with preserved adrenal glands under different PA regimens and 48 hours after IS, a significant decrease in the thymus mass coefficient is observed, on average by 1.5 times. This indicates a weakening of its functional activity (acute transformation) in response to the development of a stress reaction (from 1.394±0.088 mg/g in intact animals to 0.952±0.097 × mg/g in the MaxPA group, and to 1.006±0.156 × mg/g in the IS group 48 hours).

The thymus capsule is part of the fascial skeleton of the mediastinum, directly involved in the blood supply of the organ. There were no significant changes after different types of PA and IS in the capsule thickness and in the ratio of the cortex and the medula of the thymus of rats.

The results of the morphometric analysis of thymus mast cells showed that there were no significant differences in the amount of mast cells present in the gland stroma after exposure to all types of hyperdynamic and hypodynamic stress. It is expected that MCs functional activity will change in groups with preserved adrenal glands. In this case, MCs probably play a role in the temporal involution of the thymus, as they are involved in the formation of the adaptation syndrome during the development of stress.

The assessment of synthetic activity and degranulation activity revealed a decrease in the mean histochemical coefficient and, on the contrary, an increase in the coefficient of MC degranulation in the thymus in all experimental groups with preserved adrenal glands (Figure 2).

In accordance with the data presented in the figures, it can be stated that thymus MCs quickly respond to neuro-endocrine factors and are involved in a stress response, where their activity is manifested in a synchronous decrease in the synthesis of granules in the cytoplasm and an increased release of active substances accumulated earlier. The short-term early response of MCs is confirmed by experiments with immobilization of rats. Furthermore, experiments on continuous PA of different intensities demonstrate the preservation of this trend until the end of the stressor action.

In experimental groups with removed adrenal glands after IE, the thymus mass coefficient and gland structure remain unchanged. No changes in morphofunctional MC parameters were detected in the thymus of adrenalectomized animals after IS. Their amount in the organ, synthetic and degranulatory activity, remained at the level of the intact group (Figure 2).

A generalizing scheme can be presented to illustrate the neuro-endocrine-immune interactions involved in the development of a stress reaction, as well as the role of MCs in this process (Figure 3).

In response to extreme effects in the formation of stress reactions, MCs are influenced by various factors. The main pathway of MC activation under stress remains unclear. First, catecholamines produced by activation of the sympathoadrenal system directly affect MCs through adrenergic receptors [12], and thymus MCs are known to establish connections with catecholaminergic nerve terminals. Second, corticotropin-releasing hormone produced by the hypothalamus affects MC by binding to the KRG-Rl receptor, resulting in the release of histamine, which leads to increased permeability and vasodilation [1]. Third, adrenocorticotropic hormone activates MC secretion via melanocortin receptors [7]. Thus, degranulation of the thymus MCs during the development of stress under the influence of these factors can lead to gland atrophy. Conflicting questions remain about the effects of glucocorticoids, which are known to be cell membrane stabilizers and anti-inflammatory hormones that suppress the activity of MCs in allergies. However, the mechanism of their action on MCs during stress is not fully understood. Our experiments with hypo- and hyperdynamic stress on animals with preserved and removed adrenal glands indicate that the MC response is largely determined by the hypothalamic-pituitary-adrenal axis of the endocrine system. Thus, the removal of adrenal glands, and therefore the inability to release glucocorticoids, leads to the absence of a functional response from the thymus MCs. The stimulating effect of adrenal glucocorticoids on MCs under stress is likely mediated by their interaction with the factors listed above.

## Conclusion

Thus, the response of MCs thymus to extreme factors is unidirectional, expressed by the activation of degranulation and the suppression of their synthetic activity. This indicates that the MC response in impaired homeostasis caused by various factors is nonspecific and serves as an integral component in the development of a stress response during the body's adaptation to changing conditions. The peculiarities of

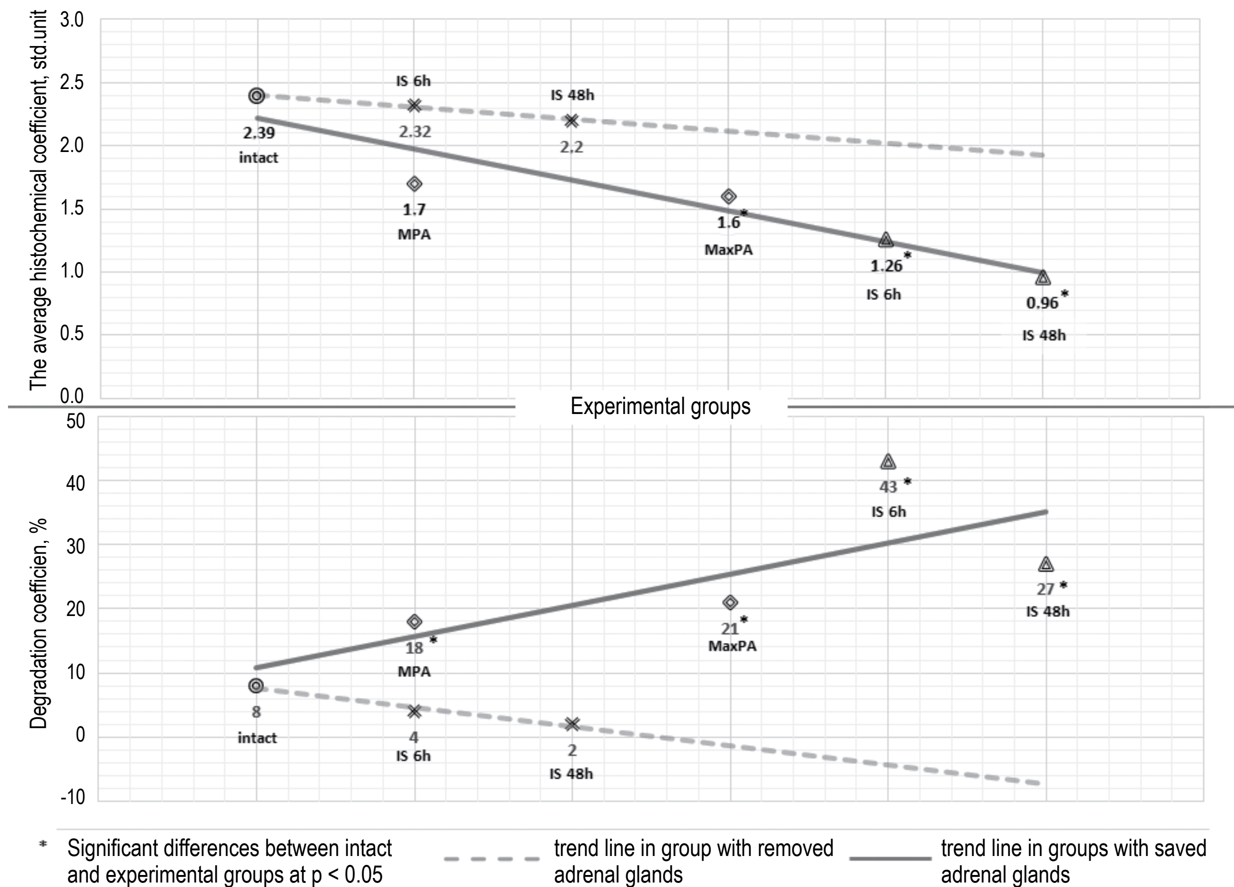


**Figure 2. Change in the synthetic and degranulatory activity of thymus mast cells under stress**

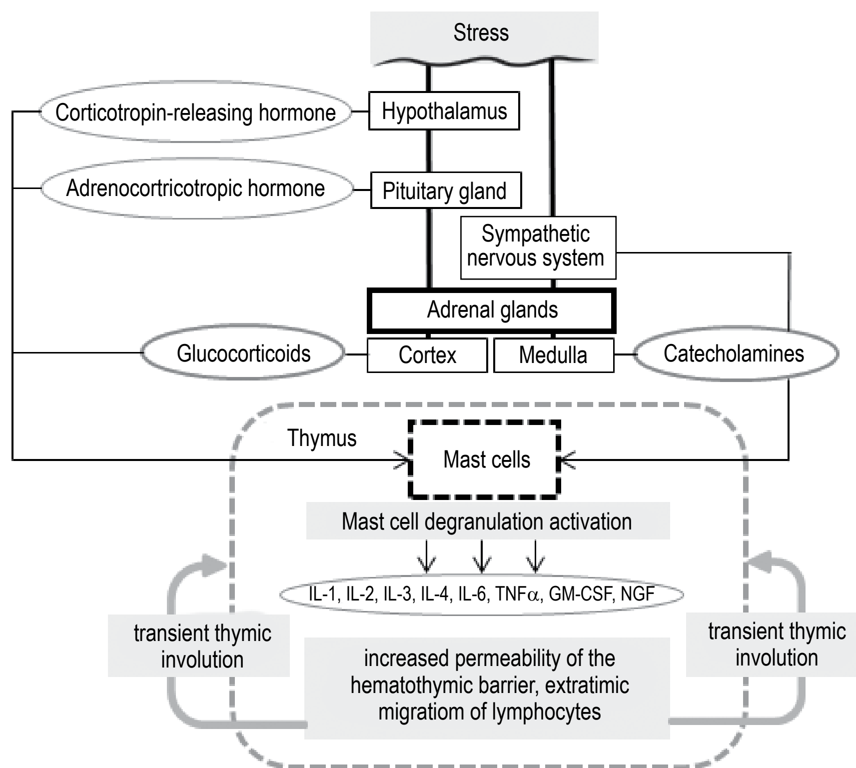


**Figure 3. Thymus mast cells as a component of neuro-endocrine-immune interactions under stress (scheme)**

the MCs thymus response at hypo-and hyperdinamic load confirm that their reaction is largely determined by the hypothalamic-pituitary-adrenal axis of the endocrine system. When this axis is activated and a full-fledged stress reaction is formed by the body, MCs are actively involved in the process of acute transformation of the thymus through cytokine secretion. This is an important condition for the development of adaptation mechanisms by the immune system, including the thymic-lymphatic apparatus. Under the influence of stress factors, the immune system triggers a variety of regulatory effects aimed at changing the functions of immune cells and redistributing them through the compartments of the body. This process is crucial for maintaining homeostasis and promoting resilience in the face of stress [3].

## Acknowledgments

This work was performed using the equipment of the Shared Research Center of Scientific Equipment SRC IIP UB RAS

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*Поступила 14.04.2023*
*Отправлена на доработку 18.04.2023*
*Принята к печати 20.04.2023*

*Received 14.04.2023*
*Revision received 18.04.2023*
*Accepted 20.04.2023*

# ВЛИЯНИЕ ЭНДОМОРФИНОВ НА ГУМОРАЛЬНЫЙ ИММУННЫЙ ОТВЕТ, ПРОДУКЦИЮ Th1/Th2/Th17-ЦИТОКИНОВ И АПОПТОЗ CD4$^+$, CD8$^+$ ЛИМФОЦИТОВ *IN VIVO*

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**Резюме.** Эндогенные опиоидные пептиды представляют собой большую группу физиологически активных соединений с выраженным сродством к рецепторам опиоидного типа, способную проявлять выраженную анальгетическую активность, а также оказывать дополнительные эффекты на периферии, ввиду своего широкого распространения на клетках многих органов и тканей. Мало изученными представителями этой группы являются эндоморфины, которые, благодаря своей структуре и свойствам, способны производить сильное антиноцицептивное воздействие после центрального введения, а значит, в перспективе, они могут рассматриваться как потенциальные заменители низкомолекулярных опиатов. Цель данного исследования заключается в том, чтобы оценить влияние эндоморфинов на гуморальный иммунный ответ, продукцию Th1/Th2/Th17-цитокинов и апоптоз CD4$^+$, CD8$^+$ лимфоцитов *in vivo*. В качестве объекта исследования использовали спленоциты белых мышей самцов породы Swiss. Оценку количества антителообразующих клеток в селезенке проводили с использованием метода локального гемолиза в геле агарозы по Jerne. Количественное определение цитокинов проводили методом твердофазного иммуноферментного анализа с помощью наборов (R&D, США) согласно методике, предложенной производителем. Апоптоз оценивали при помощи реагентов Annexin V-FITC/7-AAD kit (Beckman Coulter, США) в соответствии с инструкцией производителя методом проточной цитометрии на проточном цитофлюориметре CytoFLEX S (BeckmanCoulter, США). В ходе исследования установлено, что эндоморфины усиливают антителогенез селезенки, а предварительная блокада опиатных рецепторов налоксоном приводила к отмене стимулирующего влияния пептидов. Эндоморфины не влияли на продукцию спленоцитами IL-2, IL-4, IFNγ, однако введение эндоморфина-2 налоксоннезависимо усиливало индуцированную продукцию IL-17. Оценка влияния эндоморфинов на апоптоз спленоцитов 24 ч культур показала, что эндоморфин-2 в нестимулированных культурах налоксонзависимо увеличивал процент позднего апоптоза CD8$^+$ лимфо-

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**Образец цитирования:**
*Я.А. Кадочникова, С.В. Гейн «Влияние эндоморфинов на гуморальный иммунный ответ, продукцию Th1/Th2/Th17-цитокинов и апоптоз CD4$^+$, CD8$^+$ лимфоцитов in vivo» // Медицинская иммунология, 2023. Т. 25, № 3. С. 545-550.*
*doi: 10.15789/1563-0625-EOE-2783*

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**For citation:**
*Ya.A. Kadochnikova, S.V. Gein “Effect of endomorphins on humoral immune response, Th1/Th2/Th17 cytokine production and CD4$^+$, CD8$^+$ lymphocyte apoptosis in vivo”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 545-550.*
*doi: 10.15789/1563-0625-EOE-2783*

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**DOI:** 10.15789/1563-0625-EOE-2783

цитов, однако в индуцированных культурах оба эндоморфина повышали апоптотическую активность CD8⁺ лимфоцитов уже независимо от предварительной блокады опиоидных рецепторов. Подводя итог, можно сказать, что в системе *in vivo* эндоморфины оказывают широкий спектр разнонаправленных иммуномодулирующих эффектов, которые в дальнейшем могут представлять большой интерес для практического использования.

*Ключевые слова: эндоморфины, спленоциты, антителообразующие клетки, апоптоз, цитокины, налоксон*

# EFFECT OF ENDOMORPHINS ON HUMORAL IMMUNE RESPONSE, Th1/Th2/Th17 CYTOKINE PRODUCTION AND $CD4^+$, $CD8^+$ LYMPHOCYTE APOPTOSIS *IN VIVO*

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**Abstract.** Endogenous opioid peptides are a large group of physiologically active compounds with a pronounced affinity for opioid-type receptors, capable of showing pronounced analgesic activity, as well as having additional effects on the periphery, due to their wide distribution on the cells of many organs and tissues. Little studied representatives of this group are endomorphins, which due to their structure and properties, are capable of producing a strong antinociceptive effect after central administration, which means that, in the future, they can be considered as potential substitutes for low molecular weight opiates. The aim of this study is to evaluate the effect of endomorphins on the humoral immune response, the production of Th1/Th2/Th17 cytokines and apoptosis of $CD4^+$, $CD8^+$ lymphocytes *in vivo*. The splenocytes of Swiss white mice were used as the object of the study. The number of antibody-forming cells in the spleen was assessed using the method of local hemolysis in agarose gel according to Jerne. Quantitative determination of cytokines was carried out by enzyme-linked immunosorbent assay using kits (R&D, USA) according to the method proposed by the manufacturer. Apoptosis was assessed using Annexin V-FITC/7-AAD kit reagents (Beckman Coulter, USA) according to the manufacturer's instructions by flow cytometry on a CytoFLEX S flow cytometer (Beckman Coulter, USA). In the course of the study, it was found that endomorphins enhance the antibody genesis of the spleen, and the preliminary blockade of opiate receptors with naloxone led to the cancellation of the stimulating effect of peptides. Endomorphins didn't affect splenocyte production of IL-2, IL-4, and IFNγ, however, the introduction of endomorphin-2 naloxone-independent enhanced the induced production of IL-17. Evaluation of the effect of endomorphins on apoptosis of splenocytes in 24-h cultures showed that endomorphin-2 in unstimulated cultures of naloxone-dependently increased the percentage of late apoptosis of $CD8^+$ lymphocytes, however, in stimulated cultures, both endomorphins increased the apoptotic activity of $CD8^+$ lymphocytes, regardless of the preliminary blockade of opioid receptors. In summary, we can say that in the *in vivo* system, endomorphins have a wide range of multidirectional immunomodulatory effects, which may be of interest for practical use in the future.

*Keywords: endomorphins, splenocytes, antibody-forming cells, apoptosis, cytokines, naloxone*

The research was carried out within the framework of a state task: state registration number of the topic No. AAAA-A19-119112290007-7.

## Introduction

Endogenous opioid peptides are a large group of physiologically active compounds with a pronounced affinity for opioid-type receptors, capable of showing pronounced analgesic activity, as well as having additional effects on the periphery, due to the wide expression of opioid receptors on the cells of many organs and tissues [3, 4, 13]. It is well known that μ-opioid receptor agonists are the most effective means of relieving severe pain. Their antinociceptive action is due to activity at the supraspinal, spinal and peripheral levels [6]. Endomorphins are tetrapeptides that are highly affine selective agonists of the μ-receptor. Based on their structure, these peptides

represent a potential "substitute" for low-molecular-weight opiates [9, 12]. They act like morphine, but presumably have fewer side effects caused by low-molecular-weight opiates.

From a practical point of view, the introduction of endogenous antinociceptive ligands can be accompanied by a number of advantages, such as rapid enzymatic degradation and low toxicity [6, 7, 10, 11]. Therefore, in the literature, selective μ-receptor ligands are considered as promising compounds, and their wide distribution in the immune system suggests an active participation in the modulation of the functions of innate and adaptive immunity cells [6, 9, 15].

**The aim of the study** was to evaluate the effect of endomorphins on the humoral immune response, the production of Th1/Th2/Th17 cytokines and apoptosis of $CD4^+$, $CD8^+$ lymphocytes *in vivo*.

## Materials and methods

Experimental studies were carried out on white Swiss male mice with a body weight of 20±2 g. All experiments were conducted strictly in accordance with the recommendations and ethical standards specified in the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986), and also comply with the ethical standards approved by the legal acts of the Russian Federation.

Endomorphins (Sigma, USA) were administered intraperitoneally to mice at a dose of 100 μg/kg, control animals were injected with 0.9% NaCl in the appropriate volume. Naloxone hydrochloride (Moscow Endocrine Factory, Russia) was injected subcutaneously at a dose of 0.2 μg/kg 20 min before the administration of endomorphins.

To assess the effect of endomorphins on the formation of antibody-forming cells (AFC), the spleens of animals were immunized with ram erythrocytes (ER, NPO Microgen, Russia) intraperitoneally ($10^8$ cells in 0.2 mL of 0.9% NaCl) after 60 minutes from the moment of administration of endomorphins. On the 5th day, the animals were taken out of the experiment by decapitation under ether anesthesia, and the amount of AFC was determined by local hemolysis in an agarose gel according to Jerne [8].

To study the effects of endomorphins on the production of IL-2, IL-4, IL-17, and IFNγ, mice were removed from the experiment 1 hour after the introduction of peptides and their spleen was extracted, followed by the isolation of splenocytes. Cells were cultured in 96-well plates ($1 \times 10^7$cells/1 mL of complete nutrient medium, which was prepared on the basis of RPMI 1640 medium (Gibco, UK) with the addition of 10 mM HEPES (Sigma, USA), 2mM Glutamax (Sigma-Aldrich, USA), 100 units/mL gentamicin and 10% embryonic calf serum (Capricorn scientific, Germany)) at 37 °C for 24 hours. ConA (MPBiomedicals, France) at a concentration of 10 μg/mL was used as an inducer. Culture supernatants were collected, frozen and stored at -20 °C. The determination of IL-2, IL-4, IL-17, IFNγ was carried out by enzyme-linked immunosorbent assay using R&D kits (USA) according to the method proposed by the manufacturer.

The study of the effect of endomorphins on the apoptotic activity of $CD4^+$, $CD8^+$ lymphocytes was performed on the day of the experiment and in 24 h cultures. Animals were removed from the experiment 1 hour after the administration of the peptide; the isolated splenocytes were stained with monoclonal antibodies PE anti-mouse $CD4^+$ and PE anti-mouse $CD8^+$ (BioLegend, USA) for 20 minutes in accordance with the instructions provided by the manufacturer. Apoptosis was assessed using Annexin V-FITC/7-AAD kit reagents (Beckman Coulter, USA). The results were recorded by flow cytometry on a CytoFLEX S flow cytometer (Beckman Coulter, USA). To assess the effect of endomorphins on apoptosis of cell cultures, isolated splenocytes were cultured in 96-well plates ($4 \times 10^6$ cells/mL of complete nutrient medium, which was prepared on the basis of RPMI 1640 medium (Gibco, UK) with the addition of 10 mM HEPES (Sigma, USA), 2 mM Glutamax (Sigma-Aldrich, USA), 100 units/mL gentamicin and 20% embryonic calf serum (Capricorn scientific, Germany), 10 μM 2-mercaptoethanol (Gibco, USA)) at 37 °C for 24 h in the presence of ConA 10 μg/mL.

Statistical data processing was carried out using the Student's unpaired t-test. The differences were considered significant at $p < 0.05$. The results are presented in the form of the arithmetic mean and its standard error (M±m).

## Results and discussion

Evaluation of the effect of endomorphins on the amount of AFC showed that intraperitoneal administration of peptides significantly increased both the absolute and relative number of antibody-forming cells compared with the control (Table 1). Preliminary blockade of opiate receptors with naloxone led to the abolition of the stimulating effect of endomorphins.

Analysis of the effect of endomorphins on cytokine production showed that neither the peptides themselves nor their administration against the background of opioid receptor blockade had any effect on spontaneous and stimulated production of IL-2, IL-4, IFNγ (data not provided). At the same time, endomorphins increased the stimulated production of IL-17 (Figure 1); this effect was not canceled by the administration of naloxone.

In experiments to study the effects of endomorphins on $CD4^+$, $CD8^+$ lymphocyte apoptosis, no statistically significant effects were detected imme-

**TABLE 1. EFFECT OF ENDOMORPHIN-1, ENDOMORPHIN-2, AND NALOXONE ON THE ABSOLUTE AND RELATIVE NUMBER OF AFC IN THE SPLEEN**

| Effect | $Log_{10}$ AFC/million | $Log_{10}$ AFC/organ |
|---|---|---|
| **Control (n = 10)** | 1.46±0.20 | 3.87±0.22 |
| **Endomorphin-1 (n = 6)** | 1.96±0.08* | 4.63±0.14* |
| **Endomorphin-2 (n = 8)** | 2.02±0.11* | 4.47±0.16* |
| **Endomorphin-1 + naloxone (n = 6)** | 1.36±0.09 | 3.78±0.07 |
| **Endomorphin-2 + naloxone (n = 6)** | 1.81±0.13 | 4.17±0.13 |
| **Naloxone (n = 8)** | 1.91±0.08 | 4.23±0.17 |

Note. *, $p < 0.05$ compared to the control. AFC, Antibody-forming cells.

diately after the animals were removed from the experiment (data not provided). Evaluation of the effect of endomorphins on apoptosis of splenocytes in 24-hour cultures showed that endomorphin-2 in unstimulated cultures increased the percentage of late apoptosis of $CD8^+$ lymphocytes, and the preliminary administration of naloxone led to the abolition of this effect (Table 2). In stimulated cultures, both endomorphins also significantly increased the apoptotic activity of $CD8^+$ lymphocytes, however, in this case, the effect of endomorphins on the background of naloxone administration did not change.

Thus, endomorphins *in vivo* naloxone-dependent stimulate antibody formation and enhance $CD8^+$ lymphocyte apoptosis and IL-17 production independently of opioid receptor blockade. Previously, we have shown that *in vivo* administration of β-endorphin to mice at doses of 1; 0.01 and 0.0005 μg/kg statistically significantly increased the amount of AFC in the spleen, however, this effect was not reversed by naloxone [5]. In the *in vitro* system, the addition of endomorphins to splenocytes of mice led to naloxone-independent inhibition of the production of antibodies against sheep erythrocytes, at the same time the effect was leveled by the introduction of monoclonal antibodies to endomorphins [1].

Despite the modulation of the humoral immune response by endomorphins, the effects of peptides on the synthesis of Th1/Th2 cytokines (IL-2, IL-4, IFNγ) could not be identified, however, an effect on the synthesis of IL-17, a cytokine that plays a key role in protecting organism from extracellular bacterial and fungal infections. Previously, data on the participation of endomorphins in the regulation of IL-17 production were not mentioned in the literature. In addition, endomorphins naloxone-independently enhanced late apoptosis of $CD8^+$ lymphocytes. It can be assumed that the implementation of inhibitory signals in relation to the effector link of cellular immunity can be mediated by stimulation of the μ-receptor. It is known that $CD8^+$T lymphocytes are more sensitive to the apoptosis-inducing action of the peptides studied by us, compared with $CD4^+$ cells [2].

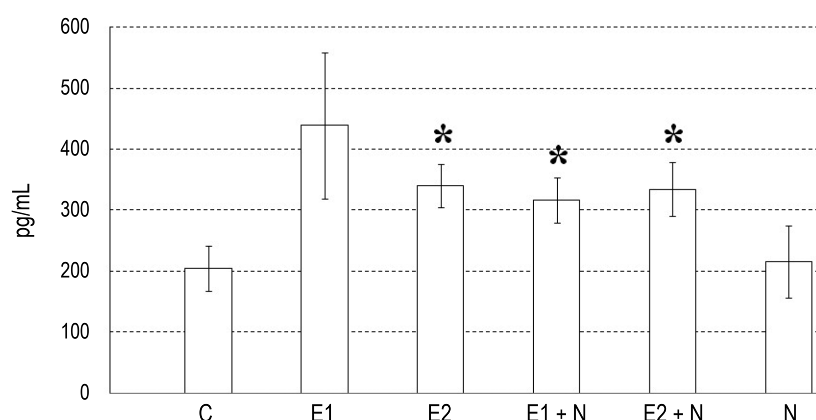


**Figure 1. Effect of endomorphins-1, 2 and naloxone on IL-17 production in stimulated splenocytes**

Note. *, $p < 0.05$ compared to the control (n = 9). C, Control; E1, Endomorphin-1; E2, Endomorphin-2; N, Naloxone; E1 + N, Endomorphin-1 + naloxone; E2 + N, Endomorphin-2 + naloxone.

**TABLE 2. EFFECT OF ENDOMORPHINS-1, 2 AND NALOXONE ON EARLY AND LATE APOPTOSIS OF CD4$^{+}$ AND CD8$^{+}$T LYMPHOCYTES AFTER 24 h CULTIVATION**

| Effect | CD4 | | CD8 | |
|---|---|---|---|---|
| | Early | Late | Early | Late |
| **Without inductor** | | | | |
| **Control** | 6.93±0.45 | 18.86±3.01 | 3.63±0.33 | 19.36±1.77 |
| **Endomorphin-1** | 8.73±1.44 | 20.38±2.11 | 3.68±0.48 | 21.76±3.35 |
| **Endomorphin-2** | 7.21±0.82 | 20.06±1.22 | 4.28±0.45 | 25.49±1.95* |
| **Endomorphin-1 + naloxone** | 8.67±1.07 | 21.01±2.79 | 3.93±0.24 | 27.98±3.39* |
| **Endomorphin-2 + naloxone** | 6.85±1.35 | 25.22±2.27 | 4.18±0.53 | 23.03±2.22 |
| **Naloxone** | 7.75±0.48 | 23.34±2.38 | 4.66±0.46 | 23.71±2.75 |
| **ConA** | | | | |
| **Control** | 8.44±1.19 | 35.61±11.2 | 4.76±0.45 | 18.17±1.34 |
| **Endomorphin-1** | 7.41±0.46 | 29.82±5.15 | 4.74±1.29 | 34.26±6.36* |
| **Endomorphin-2** | 8.48±0.53 | 19.63±1.99 | 3.83±0.47 | 28.58±2.82* |
| **Endomorphin-1 + naloxone** | 7.78±0.49 | 22.39±1.91 | 4.65±0.44 | 32.59±4.55* |
| **Endomorphin-2 + naloxone** | 5.43±1.15 | 31.28±6.11 | 6.30±0.69 | 44.34±4.13* |
| **Naloxone** | 8.34±0.83 | 17.76±3.58 | 4.75±0.57 | 21.45±0.99 |

Note. *, $p < 0.05$ compared to the control (n = 6); ConA, concanavalin A.

Naloxone, unlike endomorphins, is a complete antagonist of the μ-receptors, displacing mainly morphine derivatives from the binding site within the receptor, thereby eliminating their effect on the body. However, the blockade of opioid receptor may not always lead to the abolition of the action of agonists, especially of a peptide nature. The reversal of the effect of agonists by naloxone may depend on a number of factors, such as ligand concentration, experimental model, age and gender. Thus, it was shown that cold stress-induced analgesia was naloxone-dependent in females and naloxone-independent in males [14].

## Conclusion

In summary, we can say that in the *in vivo* system, the effects of endomorphins are comparable in nature to the immunomodulatory effects of other opioid peptides, in particular endorphins, and have little in common with the effects of morphine, which has a pronounced inhibitory effect on the functions of innate and adaptive immunity cells. This proves that there are significant differences in the mechanism of immunomodulatory action between opioid agonists of a peptide and non-peptide nature.

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*Поступила 15.04.2023*
*Отправлена на доработку 19.04.2023*
*Принята к печати 20.04.2023*

*Received 15.04.2023*
*Revision received 19.04.2023*
*Accepted 20.04.2023*

# ФАГОЦИТАРНАЯ АКТИВНОСТЬ МОНОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ В УСЛОВИЯХ *IN VIVO* И *IN VITRO* ГИПОКСИИ У ВЫСОКОУСТОЙЧИВЫХ И НИЗКОУСТОЙЧИВЫХ К НЕДОСТАТКУ КИСЛОРОДА КРЫС

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**Резюме.** Известно, что существуют индивидуальные различия устойчивости к гипоксии, которые могут определять предрасположенность к развитию и тяжесть течения различных заболеваний, в том числе инфекционно-воспалительных и опухолевых. Стандартизованных способов оценки устойчивости к гипоксии экспериментальных животных и людей без гипоксического воздействия не существует. Поиск молекулярно-биологических маркеров, позволяющих выявить людей с различной устойчивостью к дефициту кислорода в условиях нормоксии или при умеренном гипоксическом воздействии, несомненно целесообразен. Возможно, что оценка исходной устойчивости к гипоксии позволит прогнозировать развитие и тяжесть течения заболеваний, механизмы которых связаны с кислородной недостаточностью. Одним из способов оценки устойчивости организма к гипоксии без воздействия в барокамере или в условиях гор может быть моделирование гипоксии *in vitro*. Цель исследования – охарактеризовать фагоцитарную активность моноцитов периферической крови у высокоустойчивых и низкоустойчивых к гипоксии крыс Wistar в условиях нормоксии, а также после гипоксического воздействия *in vitro* и *in vivo*. Устойчивость крыс к гипоксии определяли по «времени жизни» животных «на высоте» 11 500 м в барокамере. Через месяц после определения устойчивости к гипоксии одну группу крыс помещали в барокамеру на высоту 5000 м на 1 час для моделирования гипоксического состояния *in vivo*, а у другой группы крыс получали кровь из хвостовой вены для моделирования гипоксического состояния *in vitro* в условиях 1% кислорода в течение 1 часа. Проводили оценку фагоцитарной активности моноцитов периферической крови методом проточной цитофлуориметрии. Показано, что в условиях нормоксии у исходно высокоустойчивых и низкоустойчивых к гипоксии крыс фагоцитарная активность моноцитов не различалась. Фагоцитарная активность моноцитов после *in vitro* и *in vivo* гипоксического воздействия была выше у высокоустойчивых к гипоксии животных по сравнению с низкоустойчивыми. Увеличение фагоцитарной активности моноцитов по сравнению с условиями нормоксии наблюдалось только у высокоустойчивых крыс в условиях *in vitro* гипоксического воздействия. Полученные результаты свидетельствуют о том, что высокоустойчивые

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**Образец цитирования:**
*Д.Ш. Джалилова, А.М. Косырева, И.С. Цветков, О.В. Макарова «Фагоцитарная активность моноцитов периферической крови в условиях in vivo и in vitro гипоксии у высокоустойчивых и низкоустойчивых к недостатку кислорода крыс» // Медицинская иммунология, 2023. Т. 25, № 3. С. 551-556. doi: 10.15789/1563-0625-PAO-2779*

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**For citation:**
*D.Sh. Dzhalilova, A.M. Kosyreva, I.S. Tsvetkov, O.V. Makarova "Phagocytic activity of peripheral blood monocytes under in vivo and in vitro hypoxia conditions in tolerant and susceptible to oxygen deficiency rats", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 551-556. doi: 10.15789/1563-0625-PAO-2779*

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**DOI:** 10.15789/1563-0625-PAO-2779

и низкоустойчивые к гипоксии организмы различаются по фагоцитарной активности моноцитов в условиях недостатка кислорода, что может определять течение воспалительных и опухолевых заболеваний. При поиске маркеров устойчивости организма к гипоксии целесообразно использовать моделирование гипоксии *in vitro*.

*Ключевые слова: моноциты, фагоцитоз, in vitro, устойчивость к гипоксии, фагоцитарная активность, крысы*

# PHAGOCYTIC ACTIVITY OF PERIPHERAL BLOOD MONOCYTES UNDER *IN VIVO* AND *IN VITRO* HYPOXIA CONDITIONS IN TOLERANT AND SUSCEPTIBLE TO OXYGEN DEFICIENCY RATS

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**Abstract.** It is known that there are individual differences in resistance to hypoxia, which can determine the predisposition to the development and severity of various diseases, including infectious, inflammatory and tumor. There are no standardized methods for assessing resistance to hypoxia in experimental animals and humans without hypoxic exposure. The search for molecular-biological markers, identifying people with different resistance to oxygen deficiency under normoxic conditions or under moderate hypoxic exposure is undoubtedly efficient. It is possible that the assessment of the basic resistance to hypoxia can help to predict the development and severity of the course of diseases, the mechanisms of which are associated with oxygen deficiency. One of the methods to assess organism resistance to hypoxia without exposure in a decompression chamber or in highland conditions can be modeling hypoxia *in vitro*. The aim of the study was to characterize the phagocytic activity of peripheral blood monocytes in tolerant and susceptible to hypoxia Wistar rats under normoxic conditions, as well as after hypoxic exposure *in vitro* and *in vivo*. The resistance of rats to hypoxia was determined by the gasping time at an altitude of 11.500 m in a decompression chamber. A month after determining the resistance to hypoxia, one group of rats was placed in a decompression chamber at an altitude of 5,000 m for 1 hour to simulate the hypoxic state *in vivo*. Blood from the tail vein of the other group of rats was placed in 1% oxygen for 1 hour to simulate the hypoxic state *in vitro*. The phagocytic activity of peripheral blood monocytes was assessed by flow cytometry. It was demonstrated that phagocytic activity of monocytes did not differ in tolerant and susceptible to hypoxia rats under normoxic conditions. The phagocytic activity of monocytes after *in vitro* and *in vivo* hypoxic exposure was higher in tolerant to hypoxia animals in comparison to susceptible ones. An increase in the phagocytic activity of monocytes compared to normoxia conditions was observed only in tolerant rats under *in vitro* conditions of hypoxic exposure. The obtained results indicate that tolerant and susceptible to hypoxia organisms differ in the phagocytic activity of monocytes under conditions of oxygen deficiency, which can determine the course of inflammatory and tumor diseases. The data obtained will be the basis for further experimental investigations organism hypoxia resistance markers.

*Keywords: monocytes, phagocytosis, in vitro, resistance to hypoxia, phagocytic activity, rats*

The work was carried out and financed under the budgetary topic 122030200530-6 “Cellular and molecular biological mechanisms of inflammation in the development of socially significant human diseases”.

## Introduction

Hypoxia, or oxygen deficiency, can develop in any pathological condition of the body, including infectious diseases, systemic inflammatory response, sepsis, acute respiratory distress syndrome, and the new coronavirus infection [4]. The typical cell response to oxygen deficiency is based on the activation of the signaling pathway of the hypoxia inducible transcription factor HIF (Hypoxia-Inducible Factor). According to literature, both animals and humans differ in individual sensitivity to oxygen deficiency, as well as in the content of HIF-1 [3, 8, 15]. Individual resistance to hypoxia can determine the severity of infectious and inflammatory diseases. We have previously demonstrated that the systemic inflammatory response is more severe in susceptible to hypoxia rats than in tolerant animals [3].

Currently, there are no standardized methods for assessing resistance to hypoxia in experimental animals and people without hypoxic exposure. One of the most common methods for determining resistance to oxygen deficiency is a model that reproduces the conditions of hypobaric hypoxia in decompression chambers in which volunteers, pilots, astronauts or experimental animals are placed [3, 6, 7, 9, 12]. The common way to assess the human resistance to hypoxia is the height exposure of several thousand meters and the division of subjects into resistant and sensitive to hypoxia, depending on their susceptibility to acute mountain sickness and high altitude pulmonary edema [9, 12].

Due to the fact that sensitivity to $O_2$ deficiency determines physiological reactions, adaptive processes, and, obviously, the course of a number of diseases, the search for markers for screening the body for resistance to oxygen deficiency without hypoxic exposure, placement in a decompression chamber or in highland conditions, is an urgent task. Determination of molecules that provide resistance or sensitivity to stress factors such as hypoxia plays an important role in assessing adaptive capabilities under hypoxic exposure of varying severity. Taking this into consideration, the search for molecular biological markers that make it possible to determine individual resistance to oxygen deficiency in humans under normoxic conditions or under moderate hypoxic exposure is undoubtedly expedient. One of the ways to assess resistance to hypoxia without exposure in a decompression chamber or in highlands can be modeling hypoxia *in vitro*.

Oxygen deficiency and activation of HIF-1 regulates glycolysis and energy metabolism, which affects the functioning of innate and adaptive immunity cells, in particular, promotes the inhibition of apoptosis, an increase in phagocytic activity and the migration of neutrophils and macrophages [11, 13].

Since, as mentioned above, organisms with different resistance to hypoxia are characterized by different levels of HIF-1 activation, it is likely that the functional activity of neutrophils and monocytes also differs. Features of the functional activity of monocytes in animals with different resistance to hypoxia can affect the severity of inflammatory and alterative damage in infectious and inflammatory diseases, in particular, in a systemic inflammatory response. However, data on the phagocytic activity of monocytes in tolerant and susceptible to oxygen deficiency rats under conditions of normoxia, as well as hypoxia, are not presented in the literature.

**The aim of the study** was to characterize the phagocytic activity of peripheral blood monocytes in tolerant and susceptible to hypoxia rats under normoxic conditions and after hypoxic exposure *in vivo* and *in vitro*.

## Materials and methods

The study enrolled adult male Wistar rats (3 months old, body weight 220-250 g, n = 50) and was approved by Bioethics Committee at the Avtsyn Research Institute of Human Morphology (Protocol No. 36 (12), March 28, 2022). All efforts were made to decrease suffering and possible stress for the animals and performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The reporting in the manuscript follows the ARRIVE guidelines.

Hypoxia resistance was assessed through response to oxygen deprivation using decompression chamber test as described previously [3, 8]. The animals were exposed, one at a time, to simulated hypobaric hypoxia equivalent to 11,500 m altitude (180 mmHg) using a mercury barometer-coupled decompression chamber. All decompressions and recompressions were achieved gradually at a rate of 600 m ($\approx$ 40 mmHg)/min to exclude effects of sharp changes, at 2 L/min airflow and 40-50% relative humidity. Time length till the first sign of characteristic hyperventilatory response (“gasping time”) was recorded using an electronic stopwatch. Based on gasping time, the animals were assigned to three groups: “susceptible” (< 80 s, n = 15), “normal” (80-240 s, n = 23) and “tolerant” (> 240 s, n = 12). The “normal” group was excluded from subsequent experiments. All rats survived the decompression chamber test and resumed their normal activity without signs of trauma.

A month after determining the resistance to hypoxia [3, 8], in order to simulate the hypoxic state *in vivo*, one group of rats was placed in a decompression chamber at an altitude of 5,000 m for 1 hour, then blood was taken from the tail vein under Zoletil anesthesia (Virbac Sante Animale, France). Blood from the tail vein of the other group of rats was placed in 1% oxygen for 1 hour to simulate the hypoxic state *in vitro*. The phagocytic activity of peripheral blood monocytes was assessed after *in vivo* and *in vitro* exposures by flow cytometry using the IngoFlowEx kit (Exbio Diagnostics, Czech Republic).

Statistica 8.0 software was used for statistical processing of the obtained data. Data were expressed as median and interquartile range Me ($Q_{0.25}$-$Q_{0.75}$). Since the data were not normally distributed, the Mann–Whitney, Kruskal–Wallis, and Dunn non-parametric tests were used to establish the significance of differences between the indicators. Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

Under normoxic conditions, the phagocytic activity of monocytes did not differ in tolerant and susceptible to hypoxia rats (Table 1). The phagocytic

**TABLE 1. PHAGOCYTIC ACTIVITY OF MONOCYTES UNDER NORMOXIC CONDITIONS, AFTER *IN VIVO* AND *IN VITRO* HYPOXIC EXPOSURE IN ANIMALS WITH DIFFERENT OXYGEN DEFICIENCY RESISTANCE, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Phagocytic activity of monocytes, % | Tolerant | Susceptible | p |
|---|---|---|---|
| **Normoxia** | 4.4 (4.2-5.7) | 4.5 (3.8-7.3) | 0.89 |
| ***In vivo* hypoxic exposure** | 7.0 (6.2-8.8) | 2.7 (1.6-3.5) | 0.02 |
| ***In vitro* hypoxic exposure** | 17.7 (9.7-18.8)* | 3.5 (1.6-8.0) | 0.015 |

Note. p, statistical significance of differences between tolerant and susceptible to hypoxia rats, Mann–Whitney test; *, statistically significant differences compared with normoxia, Kruskal–Wallis and Dunn test.

activity of monocytes after *in vivo* and *in vitro* hypoxic exposure was higher in tolerant to hypoxia animals compared to susceptible ones. The functional activity of monocytes is largely determined by the activation of the HIF-1 [11, 13]. We have previously demonstrated that after acute hypoxic exposure, the content of HIF-1 in the blood serum and its expression in the liver are more pronounced in tolerant to hypoxia rats compared to susceptible ones, which ensures adaptation to hypoxia [2]. Probably, in monocytes of tolerant to hypoxia rats, under conditions of oxygen deficiency, a more pronounced activation of HIF-1 occurs, which leads to an increase in their phagocytic activity. Differences in the phagocytic activity of peripheral blood monocytes after hypoxic exposure can determine the course of the systemic inflammatory response, in which innate immune cells play a key role.

It should be noted that a statistically significant increase in the phagocytic activity of monocytes compared to normoxia conditions was observed only in tolerant rats under *in vitro* conditions of hypoxic exposure. Thus, *in vitro* hypoxic exposure using lower oxygen concentrations has a more pronounced effect on blood cells compared to *in vivo* hypoxia and avoids acute hypoxic effects on the body. The pronounced effect of hypoxic exposure *in vitro* is probably due to lower oxygen concentrations, as well as the absence of adaptive changes on the part of the whole organism, such as the release of hormones, activation of the antioxidant defense system, etc. The use of *in vitro* hypoxic exposure on blood cells can be a promising method for determining the organism's resistance to oxygen deficiency. Therefore, when searching for markers of resistance to hypoxia, it is advisable to use hypoxia modeling *in vitro*.

It was demonstrated that short-term (within 2 hours) exposure to systemic hypoxia (5.500 m) on the body of healthy volunteers increases the phagocytic activity of neutrophils, but not monocytes [5]. However, in this work, individual resistance to hypoxia and gender characteristics were not taken into account; both men and women were included in the studies. Later, it was demonstrated that exposure to hypoxia (0.5-3% oxygen) *in vitro* during 24 hours does not affect the phagocytic activity of neutrophils in healthy volunteers [14]. At the same time, an increase in the phagocytic activity of monocytes under hypoxic conditions was demonstrated by many researchers [1, 10].

However, these studies did not take into account individual resistance to hypoxia. The revealed differences in the functional activity of peripheral blood phagocytes in animals with different resistance to hypoxia should be taken into account when developing new approaches to the prevention and treatment of infectious and inflammatory diseases.

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*Поступила 14.04.2023*
*Отправлена на доработку 18.04.2023*
*Принята к печати 20.04.2023*

*Received 14.04.2023*
*Revision received 18.04.2023*
*Accepted 20.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 557-562



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 557-562

# ФАГОЦИТОЗ И ОКИСЛИТЕЛЬНАЯ АКТИВНОСТЬ НЕЙТРОФИЛОВ ПРИ ВЗАИМОДЕЙСТВИИ С БИОПЛЕНКАМИ УРОПАТОГЕННЫХ *ESCHERICHIA COLI*

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**Резюме.** Развитие рецидивирующих инфекций мочевыводящих путей (ИМП) связано, в первую очередь, со способностью *Escherichia coli* образовывать биопленки. Взаимодействие нейтрофилов, факторов врожденного иммунитета с микроорганизмами в биопленках затруднено по сравнению с планктонными формами из-за отсутствия прямого контакта, а также из-за антифагоцитарного действия внеклеточного матрикса биопленок. Цель данного исследования – оценка фагоцитарной и окислительной активности нейтрофилов при взаимодействии с биопленками уропатогенных *E. coli* (UPEC) DL82 и R44. Нейтрофилы периферической крови здоровых мужчин выделяли с помощью двухградиентного фиколла-урографина, инкубировали в течение 1 ч с клетками бактерий из биопленок или их супернатантами, после чего оценивали функциональную активность лейкоцитов. Фагоцитарную активность нейтрофилов определяли по степени гашения биолюминесценции светящегося штамма *E. coli* K12 TG1 $lux^+$ (pXen) при их поглощении нейтрофилами. Продукцию внеклеточных активных форм кислорода (АФК) анализировали по интенсивности люминолзависимой хемилюминесценции в спонтанном и стимулированном клетками *E. coli* K12 вариантах. Достоверность различий определяли с помощью критерия Стьюдента при $p < 0,05$. Установлено, что взаимодействие нейтрофилов с клетками или супернатантами биопленки UPEC не влияло на фагоцитарную активность. Супернатанты *E. coli* DL82 снижали спонтанную продукцию АФК нейтрофилами по сравнению с контролем и клетками биопленок. Супернатанты *E. coli* R44 с низким вирулентным потенциалом не влияли на продукцию АФК нейтрофилами, в то время как клетки биопленки ее стимулировали. При оценке стимулированной продукции АФК воздействие супернатантов штамма R44 не вызывало снижения способности нейтрофилов к активации в ответ на внешний раздражитель (клетки *E. coli* K12). Предварительный контакт нейтрофилов с бактериями *E. coli* R44 приводил к высокому и длительному уровню продукции АФК по сравнению с контролем. Взаимодействие нейтрофилов с клетками DL82 приводило к более высокому уровню АФК по сравнению с супернатантами, однако наблюдалось по-

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**Образец цитирования:**
*И.Л. Масленникова, И.В. Некрасова, М.В. Кузнецова «Фагоцитоз и окислительная активность нейтрофилов при взаимодействии с биопленками уропатогенных Escherichia coli» // Медицинская иммунология, 2023. Т. 25, № 3. С. 557-562.*
*doi: 10.15789/1563-0625-PAO-2773*

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**For citation:**
*I.L. Maslennikova, I.V. Nekrasova, M.V. Kuznetsova “Phagocytosis and oxidative activity of neutrophils after interaction with uropathogenic Escherichia coli biofilms”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 557-562.*
*doi: 10.15789/1563-0625-PAO-2773*

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**DOI:** 10.15789/1563-0625-PAO-2773

следующее быстрое истощение окислительного потенциала нейтрофилов. Таким образом, клетки и супернатанты биопленок UPEC могут определять активацию нейтрофилов.

*Ключевые слова: нейтрофилы, активные формы кислорода, фагоцитоз, биопленки, Escherichia coli, UPEC*

# PHAGOCYTOSIS AND OXIDATIVE ACTIVITY OF NEUTROPHILS AFTER INTERACTION WITH UROPATHOGENIC *ESCHERICHIA COLI* BIOFILMS

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**Abstract.** Recurrent urinary tract infections (UTIs) are associated primarily with the ability of *Escherichia coli* to form biofilms. The interaction of neutrophils, factors of innate immunity, with microorganisms in biofilms is difficult compared to planktonic forms due to the lack of direct contact, as well as due to the antiphagocytic action of the extracellular matrix of biofilms. The purpose of this study was evaluation of neutrophils phagocytic and oxidative activity during interaction with biofilms of uropathogenic *E. coli* (UPEC) DL82 and R44. Peripheral blood neutrophils from healthy men were isolated using ficoll-urographin double gradient, incubated for 1 h with bacterial cells from biofilms or their supernatants, then leukocytes functional activity was evaluated. Phagocytic activity of neutrophils was determined by the degree of bioluminescence inhibition of bioluminescent strain *E. coli* K12 TG1 lux$^+$ (pXen) upon their absorption by neutrophils. Production of extracellular reactive oxygen species (ROS) was analyzed by the intensity of luminol-dependent chemiluminescence in spontaneous and stimulated by *E. coli* K12 variants. Significance of differences was determined using Student's t-test at $p < 0.05$. It was found that neutrophils interaction with UPEC biofilm cells or supernatants did not affect the phagocytic activity. *E. coli* DL82 supernatants reduce neutrophils spontaneous ROS production compared to control and biofilm cells. *E. coli* R44 supernatants with a low virulence potential did not affect ROS production, while biofilm cells stimulated it. When assessing stimulated ROS production, exposure to R44 strain supernatants did not cause a decrease in neutrophils activation in response to an external stimulus (*E. coli* K12 cells). Preliminary contact of neutrophils with *E. coli* R44 bacteria resulted in a high and prolonged level of ROS production compared to the control. Neutrophils interaction with DL82 cells resulted in a higher level of ROS compared to supernatants, however a subsequent rapid depletion of neutrophils oxidative potential was observed. Thus, cells and supernatants of UPEC biofilms can determine the activation of neutrophils.

*Keywords: neutrophils, reactive oxygen species, phagocytosis, biofilms, Escherichia coli, UPEC*

The study was carried out within the framework of the state task AAAA-A19-119112290009-1 and with the financial support of the Russian Foundation for Basic Research and the Perm krai, No. 19-44-590014 r_a.

## Introduction

During urinary tract infections (UTI), *Escherichia coli* must overcome several lines of defense [3], among which neutrophils are the most effective [6]. Counteraction to antimicrobial mechanisms of host innate immunity may depend on the virulent potential of "biofilm" bacteria [8] and be achieved by enhanced synthesis of structural and functional proteins of *E. coli* cell wall, allowing the integrity of the bacterial cell to be maintained.

Neutrophils play a key role in the fight against pathogenic microorganisms in biofilm during the infection by performing phagocytosis, releasing toxic enzymes and reactive oxygen species (ROS) from

granules, and extracellular traps (NETs) [7]. However, the interaction of neutrophils with microorganisms in biofilms is difficult compared to planktonic forms due to the lack of direct contact between neutrophils and bacteria in biofilm [2] as well as the antiphagocytic effect of extracellular matrix of biofilms [9].

The current research aims are to estimate phagocytosis and oxidative activity of neutrophils after interaction with biofilms of uropathogenic *E. coli* (UPEC) DL82 and R44.

## Materials and methods

Neutrophils from peripheral blood of healthy men (n = 3-6) were isolated using double gradient Fikoll–Urografin (1.077 and 1.112 g/mL) centrifugation at 400 × g. Cell purity and viability of neutrophils were 97% (Trypan Blue assay).

*Escherichia coli* DL82 was a reference strain from Ex culture collection of the University of Ljubljana, Slovenia. *E. coli* R44 was isolated from patients with UTI in Perm, Russian Federation. Bacteria ($10^6$/mL) were grown in 96-well plates in LB (Sigma-Aldrich, USA) for 24 h. Cell-free supernatants (CFS) were collected from the wells and sterilized by filtration (pore diameter 0.22 μm). Bacteria were released from biofilms by ultrasound (Elma Ultrasonic 30S; 5 times for 1 min) and resuspended in RPMI-1640. In the "neutrophil-bacteria" system, neutrophils (250 μL in RPMI; $10^6$ cells/mL) were cultured with UPEC biofilm cells (100 μL of suspension) for 1 h. Then the supernatant was removed, and the pellet was resuspended in 400 μL of colorless Hanks' Balanced Salt Solution (HBSS). In the "neutrophil – CFS" system, neutrophils (250 μL in RPMI; $10^6$ cells/mL) were cultured with UPEC CFS (250 μL) for 1 h, and then supernatants were removed and the pellet was resuspended in 400 μL of HBSS.

Analysis of the phagocytic activity of neutrophils was performed as described previously [4]. Lyophilized bioluminescent *E. coli* K12 TG1 lux$^+$ (pXen) [1] were rehydrated in cold 0.89% NaCl for 30 min at 4°C and then for 30 min at 20°C. A reaction mixture consisted of 20 μL of *E. coli* K12 TG1 lux$^+$ ($10^8$ CFU/mL), 20 μL of serum (50% solution in 0.89% NaCl), and neutrophils (160 μL, $10^6$ cells/mL) in 96-well white plates. The control was 20 μL *E. coli* K12 TG1 lux$^+$ ($10^8$ cells/mL), 180 μL of HBSS or 20 μL *E. coli* K12 TG1 lux$^+$ ($10^8$ cells/mL), 20 μL 50% serum, 160 μL HBSS. Bioluminescence was measured at 37 °C for 40 min on a microplate reader (Synergy H1, BioTek, USA). Phagocytic activity was calculated as the degree of bioluminescence inhibition: (Ik – Io)/Ik × 100%, where Ik, Io are the bioluminescence of *E. coli* K12 TG1 lux$^+$ without/with neutrophils, respectively.

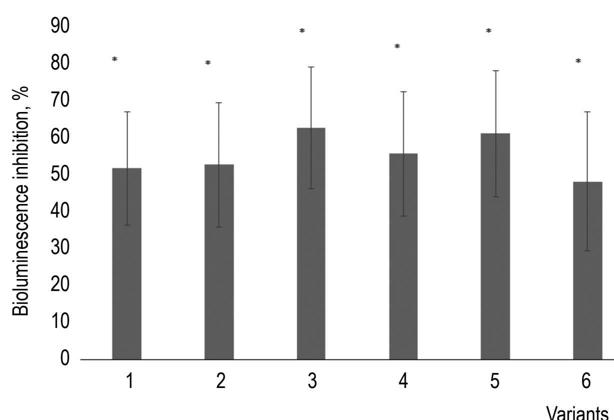


**Figure 1. Phagocytosis of neutrophils exposed to CFS (2, 3) and biofilm bacteria (5, 6) of UPEC DL82 (2, 5) and R44 (3, 6)**

Note. *, significant difference with the control without neutrophils. 1, LB; 4, 0.89% NaCl.

The production of extracellular reactive oxygen species (ROS) was analyzed as described previously [4]. To 20 μL of neutrophils ($10^6$ cells/mL) was added 220 μL of the reaction mixture to assess either spontaneous level of ROS production (180 μL of 1 mM luminol sodium salt (Sigma, USA), 40 μL of HBSS) or stimulated ROS production (180 μL of 1 mM luminol sodium salt, 20 μL *E. coli* K12 ($10^8$ CFU/mL), 20 μL serum pool (50% solution in 0.89% NaCl)) in a 96-well plate. Luminescence was assessed for 60 min at 37 °C using a microplate reader (Synergy H1, BioTek, USA).

The results were processed statistically using Microsoft Office XP Excel. The data are presented as M±SD. The significance of differences was determined with a Student's t-test at $p < 0.05$.

## Results and discussion

The strains of *E. coli* DL82 and *E. coli* R44 were characterized by the same biofilm biomass (OD570: 0.90-1.14), but *E. coli* DL82 had a wide range of virulence factors (*fimH, papC, papGII, sfaDE, hlyA, usp, fyuA, iucD, iroCN, iroN*) as opposed to R44 [5].

Figure 1 suggests that inhibition of *E. coli* TG1 lux$^+$ luminescence was observed by 51-62% in all variants with neutrophils. Thus, pretreatment of neutrophils with CFS of *E. coli* DL82 and *E. coli* R44 or their interaction with UPEC biofilm cells did not affect neutrophil phagocytosis.

We observed that CFS of *E. coli* DL82 strain reduced spontaneous ROS production of neutrophils compared to LB and biofilm cells (Figure 2A, B). The CFS of *E. coli* R44 with a low virulence potential had no effect on ROS production of neutrophils, while the biofilm cells stimulated it (Figure 2A, B). This, apparently, may indicate about more effective

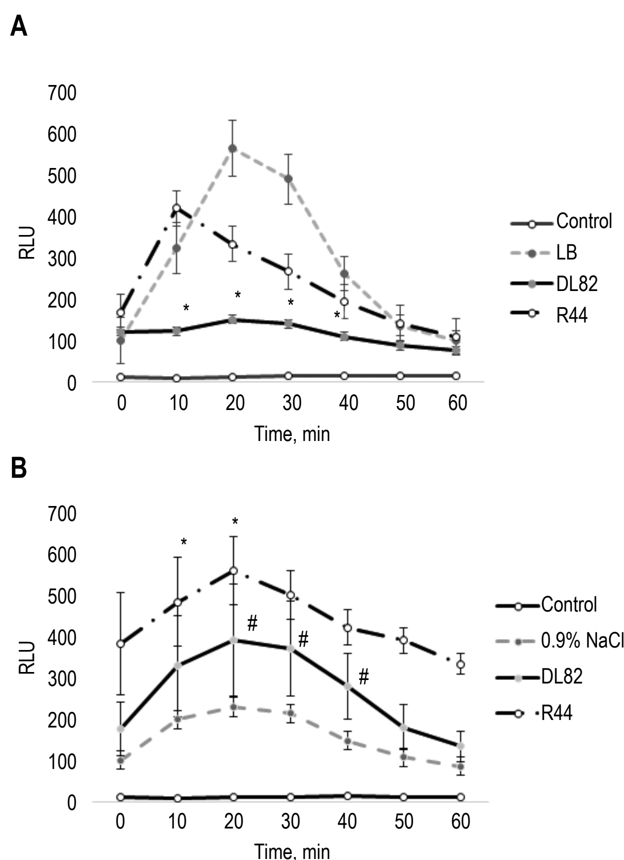


**Figure 2. Extracellular spontaneous ROS production of neutrophils exposed to CFS (A) and biofilm cells (B) of *E. coli* DL82 and *E. coli* R44 strains**

Note. *, significant differences compared to the control; #, significant differences between biofilm cells and CFS effect. RLU, relative light units.

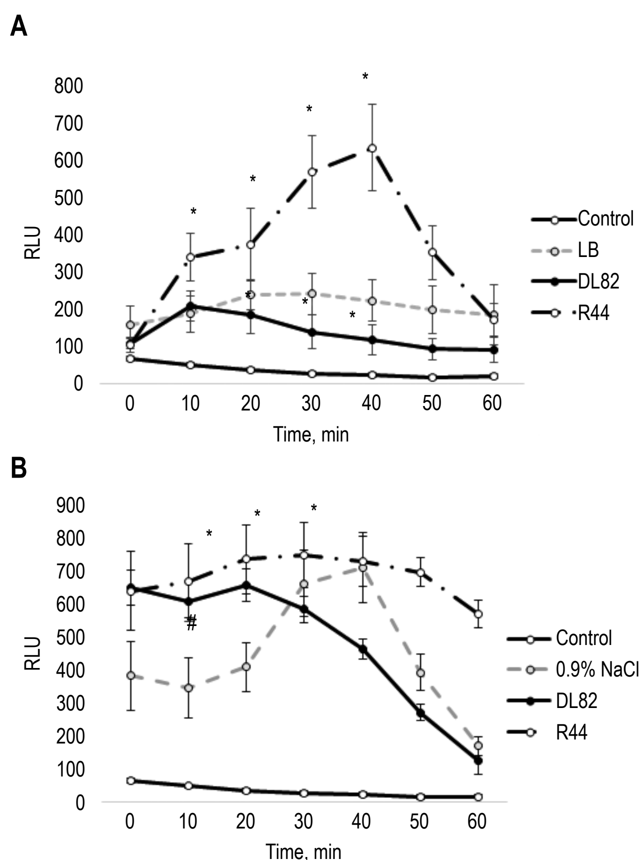


**Figure 3. Extracellular stimulated ROS production of neutrophils exposed to CFS (A) and biofilm cells (B) of *E. coli* DL82 and *E. coli* R44 strains**

Note. *, significant differences compared to the control ; #, significant differences between biofilm cells and CFS effect.

elimination of UPEC pathogens with a low virulence potential from the inflammation sites.

In the system of stimulated ROS release, exposure to CFS of the R44 strain did not cause a decrease in the ability of neutrophils to be activated in response to an external stimulus (*E. coli* K12 cells) (Figure 3A). The low activation of neutrophils by CFS of DL82, comparable to control, is probably associated with the damaging effect of "biofilm" supernatants on neutrophils [5].

Preliminary contact of neutrophils with bacteria of *E. coli* R44 resulted in a high and prolonged level of ROS production compared to the control (Figure 3B). The interaction of neutrophils with DL82 cells led to a higher level of ROS compared to CFS, but the subsequent rapid depletion of the oxidative potential of neutrophils was observed.

## Conclusion

Thus, the virulence potential of UPEC strains can determine neutrophil activation upon direct contact with both bacteria cells and their "biofilm" supernatants. It is possible that the severity of UTI may be determined by the functional activity of neutrophils, namely, the features of their interaction with bacterial biofilm structures.

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*Поступила 14.04.2023*
*Принята к печати 18.04.2023*

*Received 14.04.2023*
*Accepted 18.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 563-568

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 563-568

# ХРОНОБИОЛОГИЧЕСКИЙ ПОДХОД К ИЗУЧЕНИЮ ФИЗИОЛОГИЧЕСКОЙ АКТИВНОСТИ *CANDIDA* SPECIES

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**Резюме.** Изучение биологических свойств в течение суток позволяет выявить ритмометрические маркеры, которые можно использовать для дифференциальной диагностики возбудителей при разных состояниях пациента. Данные закономерности изучены на примере клинических изолятов *C. albicans*, *C. tropicalis* и *C. krusei*, выделенных из вагинальной микробиоты при кандидозном дисбиозе. Контролем служили эталонные штаммы из американской коллекции типовых культур (ATCC). Физиологические свойства детально изучены на примере биопленкообразования дрожжевых патогенов. Биологическую активность пленкообразования *Candida* sp. смотрели в течение двух суток с 4-часовым интервалом, в зимнее время года. Для экспериментов использовали суточные культуры, что соответствовало максимальной адгезии их на поверхности стекла. Хронометраж исследований подразумевал получение по оцениваемой функции 6 измерений в сутки с 3-5-кратным повторением условий эксперимента. Для выявления цикличности изучаемого параметра, данные статистически обработаны по Стьюденту, непараметрическими методами с применением критерия Манна–Уитни и методу наименьших квадрантов.

В ходе экспериментов выявлено наличие пленкообразующей активности грибов в течение суток ($p < 0,05$) и обнаружить общие закономерности проявления свойств у представителей всех изучаемых видов. Доказано, что основными ритмометрическими параметрами имеющие диагностическое значение являются период ритма и амплитудно-фазовая стабильность. Установлено, что суточная динамика биопленкобразования C. albicans 24433 ATCC характеризовалась ультрадианным (около 12-часовым) вкладом ритма в утреннее – 04:00 и вечернее время – 16:00. У *C. non-albicans* ATCC выявлены достоверные циркадианные (околосуточные) ритмы активности адгезии к поверхности стекла. У клинических изолятов дрожжей, выделенных из женского репродуктивного тракта при кандидозной патологии, динамика биопленкообразования характеризовалась достоверными ультрадианными (около 12-часовыми) гармониками, что имеет важное биологическое значение, определяющее устойчивость к внешним воздействиям и способность к адаптивному ответу на периодические раздражители.

Использование хронобиологического метода, на наш взгляд, открывает новые перспективы при изучении физиологии *Candida* sp., так как дает возможность прогнозировать динамику состояния

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**Образец цитирования:**

*М.В. Николенко, Н.В. Барышникова, О.И. Малишевская, Е.М. Васева «Хронобиологический подход к изучению физиологической активности Candida species» // Медицинская иммунология, 2023. Т. 25, № 3. С. 563-568.*
*doi: 10.15789/1563-0625-CAT-2750*

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**For citation:**

*M.V. Nikolenko, N.V. Baryshnikova, O.I. Malishevskaya, E.M. Vaseva “Chronobiological approach to study the physiological activity of Candida species”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 563-568.*
*doi: 10.15789/1563-0625-CAT-2750*

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**DOI:** 10.15789/1563-0625-CAT-2750

микроорганизма и учитывать особенности срочной и долговременной адаптации к разным факторам внешней среды. Выявление суточных ритмов биопленкообразующей активности у различных штаммов *Candida* sp., открывает возможность управлять жизнеспособностью бактериально-грибковых ассоциаций и прогнозировать их устойчивость к различным антимикробным средствам.

*Ключевые слова: хроном, биоритмы, грибы рода Candida, биопленки, физиологическая активность, амплитудно-фазовая стабильность*

# CHRONOBIOLOGICAL APPROACH TO STUDY THE PHYSIOLOGICAL ACTIVITY OF *CANDIDA* SPECIES

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**Abstract.** Rythmometric markers can be identified within a day during the study of biological characteristics in order to be used for differential diagnostics of pathogens of different patients' physical condition. These principles are based on analysis of clinical isolates *C. albicans*, *C. tropicalis* and *C. krusei* allocated from the vaginal microbiota at *Candida* dysbiosis condition. Control examples were the master samples from the American Type Culture Collection (ATCC). Detailed research was conducted on physiological characteristics through the formation of biofilms by yeast pathogens. Biological activity of *Candida* sp. biofilming was observed within 2 days with 4 hours interval in winter. Daily cultures were used for the experiment to correspond to their maximum adhesion to the glass surface. It was important to obtain 6 measurements per day with 3-5 times repetition of experiment conditions during the specified timeline. In order to determine the periodicity of the parameters studied, the data was statistically processed by Student's t-test, using Mann–Whitney criteria and nonparametric method of least square method.

It was found out that biofilming activity during 24 hours ($p < 0.05$) of fungi exists and that all species have many principles in common. It was attested that the main rhythmometric parameters of diagnostic significance are the rhythm period and amplitude-phase stability. It was found that the daily dynamics of C. albicans 24433 biofilm formation from American Type Culture Collection was characterized by an ultradian (about 12-hours) contribution of the rhythm in the morning – 4 A.M and in the evening – 4 P.M. Significant circadian (approx. daily) rhythms of adhesion glass surface activity were revealed in *C. non-albicans* from American Type Culture Collection. The dynamic of biofilm formation isolates of yeast from female reproductive organs with *Candida* pathology was characterized by reliable ultradian (about 12-hour) harmonics which biological significance defines resistance to external impact and the ability to adaptively respond to periodic stimuli.

To sum up, implementation of the chronobiological approach has opened up new prospects for studying the physiology of *Candida* sp., as it enables us to predict the dynamics of microbial states and takes into account the specificity of emergency and long-term adaptation to different environmental factors. The detection of the circadian rhythm of biofilm formation activity of different *Candida* sp. strains provides the possibility to manage the vitality of the Society of Bacteria and Fungi and predicts its resistance to various antibiotics.

*Keywords: chronome, biorhythms, Fungi of the genus Candida, biofilms, physiological activity, amplitude-phase stability*

## Introduction

Biofilm formation is one of the main survival strategies of bacteria. Bacterial-fungal microsymbiocenosis acquires protection from various physical, chemical, and biological antimicrobial factors. Therefore, we can consider a biofilm as one of the forms of persistence of microorganisms [3]. Up to 80% of infectious diseases [15] and polyantibiotic resistance of microorganisms [5] are associated with biofilm formation. A number of papers review the importance of biofilms for various fields of scientific research and applications, showing an increased interest in this problem [11], as well as information on a large number of methods for studying, culturing, and indicating biofilms in vivo and in vitro [9].

In recent years, dynamic methods for studying the formation of biofilms have been widely used, namely, the Robinson apparatus in its various modifications, laboratory fermenters, and the flow method [6]. The maximum approximation to the conditions of living

systems is the main advantage of dynamic methods of biofilm formation [13]. Fluorescence, confocal scanning laser and electron microscopy are innovative technologies that revealed the heterogeneous structure of bacterial-fungal biofilms. The genes encoding adhesion proteins of *Candida* sp. [12] were studied and the phases of biofilm formation were described: adhesion, formation of the extracellular matrix, and formation of a mature biofilm consisting of single cells and mycelium [4]. In their works, Al-Fattani M. et al. [1] studied the chemical composition of the *C. albicans* and *Candida* tropicalis (*C. tropicalis*) biofilm matrix. The main structural components were proteins, carbohydrates, hexosamine, and phosphorus.

In the last decade, biofilms in bacterial-fungal populations have been studied by the method of fluorescent hybridization to determine the location of mRNA in the cells that form biofilms. It was used to identify persister cells responsible for the survival of the population under the influence of factors of various nature [2].

The methods used in scientific research to study biofilm formation are complex and require specialized equipment, a vivarium, which creates difficulties in real clinical practice in identifying biofilms and results in a false impression of the low frequency of their formation.

Another group of methods is based on the creation of static conditions for the cultivation of microorganisms, which are convenient, highly productive and visual. The authors proposed a modification of the method for dynamic studying biofilm formation on plastic dishes during the day [14]. From this point of view, the chronobiological approach acts both as a methodological principle and as a technique [7].

**Aim:** studying the physiological activity of *Candida* species using chronobiological approaches.

## Materials and methods

Seventeen isolates of C. albicans, *C. tropicalis* and *C. krusei* isolated from the reproductive tract of women diagnosed with candidal dysbiosis were the object of the study. Repository reference strains of *Candida* sp. obtained from the American Type Culture Collection (ATCC) C. albicans 24433, *C. tropicalis* 750, and *C. krusei* 6258 were used as a control. These types of fungi were chosen as a model since in the vast majority of cases (85-90%) the causative agent of candidiasis is C. albicans, the most pathogenic and significant species in clinical practice. Among other species of *Candida* sp., predominantly *C. tropicalis* with 3-5% and *C. krusei* with 1-3% are of clinical significance. Recently, the number of diseases caused by *C. non-albicans* fungi has increased [1].

The fungi were identified by a set of features: cultural properties, sensitivity to antifungal drugs by the disk diffusion method, chlamydospore formation, morphogenesis ability, type of filamentation, and saccharolytic and biochemical activities [8].

The paper presents a modified method for assessing biofilm formation proposed by O'Toole G.A. et al. [12]. At the first stage of the study, 24-hour cultures were obtained by growing them on a Sabouraud nutrient medium with tellurite at a temperature of 37 °C, which corresponded to the beginning of the stationary phase of fungal development. To obtain a biofilm, glass test tubes P-2-14-120 washed with a chromium mixture were used. The initial 0.5 McFarland concentration of microorganisms was prepared using a densitometer. To obtain a working concentration of $1.5 \times 10^3$ CFU/mL, the cultures were titrated with physiological saline, after which they were added to test tubes with Sabouraud Dextrose Broth and incubated in a thermostat at a temperature of 37 °C. After incubation, to remove the planktonic phase of the culture, the contents of the tubes were taken with a pipette without touching the walls of the vessel and gently washed twice with distilled water. To quantify the biofilm volume, 2 mL of gentian violet was added. After 45 minutes, the dye was carefully poured off and the walls of the test tube were repeatedly washed with distilled water. To extract the dye, 2 mL of 96% ethanol was added to each test tube for 15 minutes.

The kinetics of biofilm formation was studied by changes in the light transmittance and optical density on a KFK-3-01 spectrophotometer at a wavelength of 540 nm; measurements were taken after 24, 36, 48, 72 and 96 hours. 96% ethanol was used as a control. Gentian violet extracted with ethanol from the biofilm was placed into disposable plastic macro cuvettes $10 \times 10 \times 45$ mm in size, 4 mL in volume (optical path length was 10 mm). The temperature of the measured liquids corresponded to room temperature. To obtain statistically reliable data, the experiment was repeated five times. Experimental data were statistically processed in Primer of Biostatics Version 4.03 by Stanton A. Glantz 1998, Microsoft Office Excel 2010, with a given certainty $p < 0.05$.

In the second series of experiments, the authors studied the biofilm formation activity of *Candida* sp. within two days with a 4-hour interval, the fourth lunar phase, in the winter season. A 48-hour culture of fungi was used, which corresponded to their maximum adhesion on the glass surface. Eleven experiments were performed; 486 measurements were obtained.

The data were processed by the least squares method with a given significance of certainty $p < 0.05$ [10]. The main parameters of the rhythms were determined: the rhythm contribution (T = 24; T = 12), mesor, amplitude and acrophase of the rhythm.

The Mann–Whitney test compared the differences between unrelated samples (experiment – control, collection and hospital cultures). The analyzed differences were considered significant at $p < 0.05$.

## Results and discussion

All *Candida* sp. isolates had a characteristic growth rate. The adhesive properties of fungi of the *Candida* genus depended on the phase of their development. The glass surface of the test tube in the nutrient medium was covered with primary biofilm after 12 hours of culturing. The first (logarithmic) section (12-24 hours) of the kinetic curve corresponded to the stage of reversible and irreversible microbial adhesion, the duration of which did not depend on the nature of the microorganism. It was experimentally found that 48 hours was the most optimal time for studying the biofilm formation: the light transmittance was the highest, since the fungal cell walls inside the biofilm sorbed the dye, gentian violet, to the maximum extent. Therefore, the ability of micromycete cells to form conglomerates is significantly higher in the stationary growth phase than in the logarithmic one ($p < 0.05$). The cultivation period of 72-96 hours was characterized by a decrease in the activity of biofilm formation by fungi. Apparently, their cells lost their mobility and began to intensively secrete extracellular polymers, forming a polymer matrix [11]. Lisovskaya S.A., 2008, obtained similar results when observing 1-4 day cultures using the technique of growing biofilms on a nitrocellulose surface [8].

The chronobiological method used by the authors in the second series of experiments revealed the presence of biofilm-forming activity of fungi during the day ($p < 0.05$) and found common patterns in the manifestation of properties in representatives of all studied species.

The daily dynamics of *C. albicans* biofilm formation was characterized by an ultradian (about 12-hour) rhythm with an acrophase in the morning and evening. Significant circadian rhythms of adhesive activity were found for *C. non-albicans*. The maximum values of the indicator for *C. tropicalis* were recorded in the evening, and for *C. krusei* at night. The presence of circadian rhythms in the spectral composition of microorganisms simultaneously with ultradian rhythms indicates an increase in the adaptive capabilities of microorganisms. Average daily indicators (mesor) of biofilm formation did not exceed 1.5±0.05, which made it possible for us to infer that the activity of all the studied cultures was low (Table 1).

For all clinical isolates of *Candida* sp. isolated from the reproductive tract of women diagnosed with candidal dysbiosis, the dynamics of biofilm formation was characterized by significant ultradian variations, with 8 and 12-hour fluctuations. The maximum values were recorded in the early morning from 03.09 to 04.18 and in the evening from 18.45 to 20.20 (Table 2). On the contrary, ultradian rhythms characterize the variability of periods, which determines the ability

**TABLE 1. RHYTHMOMETRIC PARAMETERS OF BIOFILM FORMATION OF COLLECTION STRAINS OF C. SPECIES**

| Cultures | Period (day) | Rhythm contribution, % | Mesor M±m | Amplitude | Acrophase, hour |
|---|---|---|---|---|---|
| | | 24-hour<br>12-hour | | 24-hour<br>12-hour | 24-hour<br>12-hour |
| *C. albicams* 24433 | 1 | 34.3<br>58.6* | 1.50±0.04 | 0.30±0.04<br>0.50±0.01 | 04.18<br>16.40 |
| | 2 | 38.0<br>60.8* | 1.40±0.07 | 0.20±0.09<br>0.40±0.05 | 05.35<br>17.03 |
| *C. tropicalis* 750 | 1 | 69.0*<br>13.4 | 1.40±0.05 | 0.40±0.05<br>0.10±0.02 | 17.10<br>06.00 |
| | 2 | 86.4*<br>9.3 | 1.50±0.04 | 0.20±0.01<br>0.10±0.01 | 16.02<br>04.04 |
| *C. krusei* 6258 | 1 | 78.5*<br>2.6 | 1.30±0.05 | 0.30±0.04<br>0.10±0.01 | 20.00<br>04.03 |
| | 2 | 54.7*<br>34.1 | 1.30±0.06 | 0.40±0.03<br>0.30±0.04 | 20.00<br>03.18 |

Note. *, $p < 0.05$.

TABLE 2. RHYTHMOMETRIC PARAMETERS OF BIOFILM FORMATION OF C. SPECIES ISOLATES FROM BIOLOGICAL MATERIAL OF WOMEN WITH CANDIDAL DYSBIOSIS

| Culture | Period (day) | Rhythm contribution, % | Mesor M±m | Amplitude | Acrophase, hour |
|---|---|---|---|---|---|
| | | 24-hour<br>12-hour | | 24-hour<br>12-hour | 24-hour<br>12-hour |
| *C. albicans* | 1 | 11.5<br>81.8* | 2.60±0.01* | 0.50±0.02<br>2.30±0.19* | 18.45<br>03.12 |
| | 2 | 8.3<br>90.4* | 2.40±0.01* | 0.20±0.01<br>1.90±0.39* | 20.00<br>07.54 |
| *C. tropicalis* | 1 | 17.8<br>78.3* | 1.7±0.3 | 0.7±0.2<br>1.5±0.3 | 04.00<br>20.25 |
| | 2 | 25.3<br>65.2* | 1.7±0.2 | 0.3±0.1<br>1.0±0.3* | 03.34<br>19.20 |
| *C. krusei* | 1 | 30.2<br>53.7* | 1.7±0.1 | 0.5±0.1<br>1.5±0.1* | 04.18<br>20.12 |
| | 2 | 26.8<br>54.8* | 1.6±0.4 | 0.9±0.2<br>1.3±0.4 | 04.06<br>19.40 |

Note. *, $p < 0.05$.

to adaptively respond to periodic stimuli and resist external influences [7].

It has been experimentally established that the average daily biofilm formation rates for C. albicans are significantly higher than for *C. non-albicans* ($p < 0.05$). The revealed regularity is consistent with the data obtained in experiments on silicone models. Confocal scanning laser and electron microscopy shows that C. albicans forms a quantitatively larger and structurally more complex biofilm than *Candida* non-albicans [14]. The extracellular matrix of *C. albicans* consisted of 57.0% glucose, while the biofilm of *C. tropicalis* was dominated by hexosamine. Biofilm formations of C. albicans were more easily separated from plastic surfaces by treating them with the beta-1,3-glucanase enzyme than that of *C. tropicalis* [8].

Analysis of the chronoinfrastructure of the studied function of clinical isolates and reference strains of *Candida* sp. showed a change in the contribution of rhythm, mesor and amplitude. The activity of biofilm formation increased in the direction "reference strains – clinical isolates". The Mann–Whitney test was 29 for C. albicans, 26 for *C. tropicalis*, and 30 for *C. krusei* ($p < 0.05$). The chronobiological approach expands the understanding of yeast physiology. In the course of the study, we found that the contribution of rhythm and the amplitude-phase characteristic of biofilm formation are rhythmometric markers of strain pathogenicity. These criteria are stationary and distinguish pathological disorders from adaptive changes [7].

## Conclusion

The proposed macrometric method solves the problem of accelerating and simplifying the quantitative assessment of the process of biofilm formation and increases sensitivity as it eliminates errors associated with the use of polystyrene material. The ease of its implementation makes it accessible to any laboratory.

In our opinion, the chronobiological method opens up new perspectives in the study of the physiology of *Candida* sp., as we can predict the dynamics of the biological activity of a microorganism and take into account the features of immediate and long-term adaptations to various environmental factors. The detection of daily rhythms of biofilm formation activity in various strains of *Candida* sp. opens up the possibility of controlling the viability of bacterial-fungal associations and predicting their resistance to various antimicrobial agents.

## Acknowledgments

The research was funded by a grant from Tyumen State Medical University in the field of biomedical and biopharmaceutical technologies No. 8210053 dated August 12, 2021. The team of authors would like to thank Tyumen State Medical University for their support in conducting the research.

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*Поступила 13.04.2023*
*Отправлена на доработку 14.04.2023*
*Принята к печати 17.04.2023*

*Received 13.04.2023*
*Revision received 14.04.2023*
*Accepted 17.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 569-572



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 569-572

# ВЛИЯНИЕ МИКРОБНЫХ ПОЛИАМИНОВ НА ПРОДУКЦИЮ IL-10 ЛЕЙКОЦИТАМИ ПЕРИФЕРИЧЕСКОЙ КРОВИ ЗДОРОВЫХ ДОНОРОВ

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**Резюме.** Как известно, полиамины бактериального происхождения, к которым относятся кадаверин и путресцин, способны разносторонне влиять на активность иммунокомпетентных клеток. В частности, такая ситуация наблюдается при длительно текущих воспалительных заболеваниях, особенно при интенсивном размножении микроорганизмов, способных к продукции полиаминов. Представляет интерес изучение продукции одного из основных противовоспалительных цитокинов – IL-10 – под влиянием бактериальных полиаминов. Для проведения исследований из периферической крови практически здоровых доноров путем градиентного центрифугирования выделяли популяцию мононуклеарных лейкоцитов. Клеточную суспензию помещали в круглодонный планшет с предварительно внесенными полиаминами в концентрациях 5, 25, 50, 75, 100 ммоль/л. В качестве контроля использовали лунки не содержащие полиаминов, по окончании инкубации в течение 72 ч при 37 °C и 5% $CO_2$ супернатанты стягивали и использовали для определения концентрации IL-10. В работе использовали набор для определения концентрации IL-10 с помощью иммуноферментного метода (Россия). Статистический анализ проводили с помощью программного пакета Statistica 6.0. В случае распределения приближенного к нормальному использовали критерий Стьюдента, в остальных – применяли критерий Манна–Уитни для оценки значимости различий. В ходе проведения исследований показано, что лейкоциты в присутствии конканавалина А продуцируют IL-10 в концентрации 17,13±6,08 пг/мл. Установлено, что под влиянием полиаминов бактериального происхождения продукция IL-10 усиливается только если путресцин и кадаверин в концентрациях 50 ммоль/л и выше. При низких концентрациях полиаминов значимого увеличения продукции IL-10 не выявлено. Поскольку IL-10 является противовоспалительным цитокином, для которого известен в том числе и противоболевой эффект, следует ожидать, что при увеличении его концентрации в очаге инвазии условно патогенных бактерий воспалительный процесс будет развиваться латентно, когда симптомы слабо выражены. В целом можно ожидать, что бактерии-продуценты полиаминов будут способствовать поддержанию малосимптомного воспаления.

*Ключевые слова: бактериальные полиамины, IL-10, лейкоциты, конканавалин А, человек, воспаление*

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**Образец цитирования:**
*А.П. Годовалов, И.А. Морозов «Влияние микробных полиаминов на продукцию IL-10 лейкоцитами периферической крови здоровых доноров» // Медицинская иммунология, 2023. Т. 25, № 3. С. 569-572.*
*doi: 10.15789/1563-0625-IOM-2842*

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**For citation:**
*A.P. Godovalov, I.A. Morozov “Influence of microbial polyamines on IL-10 production by peripheral blood leukocytes of healthy donors”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 569-572.*
*doi: 10.15789/1563-0625-IOM-2842*

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**DOI:** 10.15789/1563-0625-IOM-2842

# INFLUENCE OF MICROBIAL POLYAMINES ON IL-10 PRODUCTION BY PERIPHERAL BLOOD LEUKOCYTES OF HEALTHY DONORS

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**Abstract.** As is known, bacterial polyamines, which include cadaverine and putrescine, are capable of influencing the activity of immunocompetent cells in many ways. In particular, this situation is observed in long-term inflammatory diseases, especially with intensive reproduction of microorganisms capable of producing polyamines. It is of interest to study the production of one of the main anti-inflammatory cytokines, IL-10, under the influence of bacterial polyamines. For research, a population of mononuclear leukocytes was isolated from the peripheral blood of healthy donors by gradient centrifugation. The cell suspension was placed in a round-bottomed plates with preliminarily added polyamines at concentrations of 5, 25, 50, 75, and 100 μmol/L. Wells not containing polyamines were used as a control. After incubation for 72 h at 37 °C and 5% $CO_2$, the supernatants were harvested and used to determine the concentration of IL-10. We used a kit for determining the concentration of IL-10 using the enzyme immunoassay method (Russia). Statistical analysis was performed using the Statistica 6.0 software package. In the case of a distribution close to normal, Student's t-test was used; in the rest, the Mann–Whitney test was used to assess the significance of differences. Studies have shown that leukocytes in the presence of concanavalin A produce IL-10 at a concentration of 17.13±6.08 pg/mL. It has been established that under the influence of polyamines of bacterial origin, the production of IL-10 is enhanced only if putrescine and cadaverine are at concentrations of 50 μmol/L and higher. At low concentrations of polyamines, no significant increase in IL-10 production was detected. Since IL-10 is an anti-inflammatory cytokine, for which the analgesic effect is also known, it should be expected that with an increase in its concentration in the focus of invasion of opportunistic bacteria, the inflammatory process will develop latently, when the symptoms are mild. In general, it can be expected that polyamine-producing bacteria will contribute to the maintenance of few symptomatic inflammation.

*Keywords: bacterial polyamines, IL-10, leukocytes, concanavalin A, human, inflammation*

## Introduction

Recently, more and more chronic forms of inflammatory diseases have been recorded, while the clinical picture is characterized by mild symptoms. This situation creates the main problem in reproduction, for example, in infertile couples, asymptomatic bacteriospermia occurs in more than 30% of cases [2]. The absence of symptoms leads to untimely diagnosis of such conditions, which further exacerbates the problem of infertility. A long-term inflammatory process ensures the persistence of microorganisms [4] with a less pronounced response of the immune system.

We have previously shown that a large variety of microorganisms is recorded in the genital tract in chronic inflammatory diseases [4]. For successful establishment in the biotope, microorganisms change the reactivity of the immune system, for example, through the production of polyamines. Thus, polyamines of bacterial origin can have a versatile effect on eukaryotic cells. Among the cytokines regulating the immune system, the study of IL-10 is of interest. IL-10 has anti-inflammatory effect, affecting mast cells, B lymphocytes, neutrophils, NK-cells and macrophages.

**The aim of investigation** was to evaluate the effect of bacterial diamines cadaverine and putrescine on the production of interleukin-10 in a culture of human mononuclear leukocytes.

## Materials and methods

The object of the study was peripheral venous blood leukocytes obtained from 18 healthy male volunteers (mean age 24.0±0.6 years). The non-inclusion criterion was female gender, since the activity of human leukocytes is under the influence of female sex hormones, the level of which changes cyclically. The isolation of leukocytes was carried out from heparinized blood by gradient centrifugation using a ficoll-verografin mixture with a density of 1.077 g/$cm^3$. After collecting the interphase part, the cell suspension was stirred and washed three times.

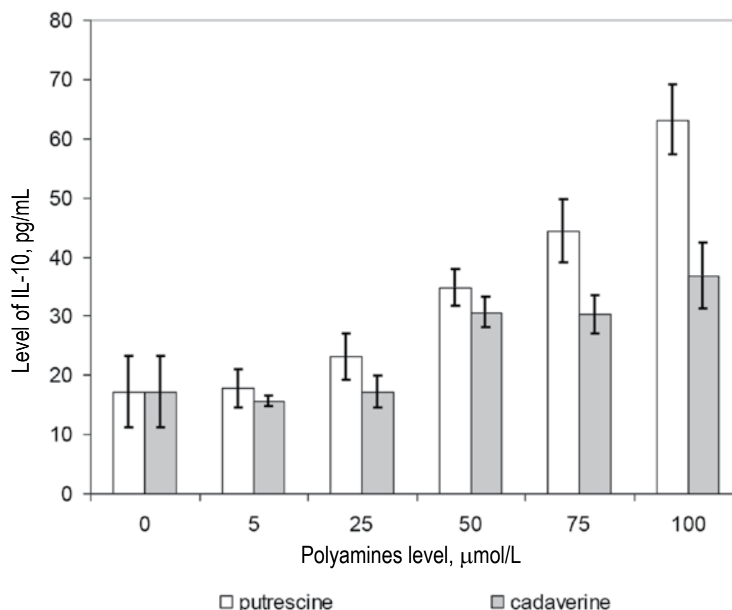


**Figure 1. Influence of bacterial polyamines on the production of IL-10 by human leukocytes**

The micromethod and plastic round-bottom 96-well plates were used for culturing lymphocytes. Each culture contained 2 × $10^5$ cells in complete culture medium. The latter was prepared *ex tempore* based on medium 199 supplemented with 2 μM L-glutamine, 10 μM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), 100 μg/mL gentamicin sulfate, and 10% plasma. Concanavalin A at a concentration of 5 μg/mL was used as a T-cell mitogen. Polyamines were used at final concentrations of 5, 25, 50, 75, and 100 μM/L. Cultivation was carried out in a humid atmosphere with 5% $CO_2$ at 37 °C for 72 hours. At the end of the incubation period, the culture liquid was pulled together and frozen for subsequent determination of the concentration of interleukin-10. Reagent kits for enzyme immunoassay for determining the concentration of IL-10 (Russia) were used.

Statistical analysis was carried out using the Statistica 6.0 software package. The arithmetic mean (M) and the standard error of the arithmetic mean (m) were calculated. The Shapiro–Wilk test was used to check the normality of the distribution. In the case of a distribution close to normal, Student’s t-test was used. In the rest, the Mann–Whitney test was used to assess the significance of differences. The critical level of significance (p) when testing statistical hypotheses was taken equal to 0.05.

## Results and discussion

When studying the production of IL-10 by leukocytes in the presence of Concanavalin A, it was shown that the average level of IL-10 production was 17.13±6.08 pg/mL. Under the influence of putrescine and cadverine at concentrations up to 25 μmol/L, the secretion of IL-10 does not change significantly (Figure 1). With an increase in the concentration of polyamines, an increase in the synthesis of IL-10 is observed. At the same time, putrescine at a concentration of 100 μmol/L stimulates the secretion of IL-10 2 times more than cadaverine at the same concentration.

The results obtained partly indicate a possible mechanism for the development of low-symptomatic chronic inflammatory diseases. As is known, in the focus of invasion of microorganisms, there is an increase in the concentration of polyamines, putrescine and cadaverine [1], which, by acting on the cells of the immune system, provide an increase in the concentration of IL-10. It is known that IL-10 suppresses the production of proinflammatory cytokines and nitric oxide, which significantly reduces the antimicrobial effect of leukocytes [3]. And this, in turn, is a condition for the persistence of microorganisms. Moreover, since IL-10 is an inhibitor of inflammation and the cytokine cascade, an asymptomatic clinical picture of inflammatory diseases caused by polyamine-producing microorganisms should be expected.

## Conclusion

Thus, polyamines of bacterial origin, putrescine and cadaverine, affect not only the microbial community, but also immunocompetent human cells, for example, by changing the production of IL-10.

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*Поступила 15.04.2023*
*Отправлена на доработку 21.04.2023*
*Принята к печати 26.04.2023*

*Received 15.04.2023*
*Revision received 21.04.2023*
*Accepted 26.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 573-580



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 573-580

# СКООРДИНИРОВАННАЯ ЭКСПРЕССИЯ МАРКЕРОВ NK-КЛЕТОК И ОТВЕТА IgG ПРИ ИНФЕКЦИИ hCMV

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**Резюме.** Цитомегаловирус человека (hCMV) является распространенным вирусом, поражающим большую часть населения во всем мире. Естественные клетки-киллеры (NK) представляют собой иммунные клетки, которые играют решающую роль в борьбе с инфекцией hCMV. Несмотря на широкое распространение hCMV-инфекции, данных о взаимосвязи врожденного и адаптивного иммунитета до сих пор недостаточно. В этом исследовании изучалась взаимосвязь между экспрессией NK-клеточных маркеров и гуморальным иммунитетом во время инфекции hCMV. Было проанализировано 33 образца, полученных от здоровых волонтеров. Титр anti-CMV IgG антител измерялся в образцах сыворотки крови, а экспрессия NKG2C, HLA-DR, CD57, KIR2DL2/DL3 и KIR2DL1 на поверхности NK-клеток ($CD56^+CD3^-$) исследовалась в образцах PBMC методом проточной цитометрии. Для анализа процентного содержания различных субпопуляций NK-клеток в зависимости от титра IgG предварительно была проведена кластеризация всех полученных данных, по результатам которой было выделено 4 основных кластера. Выделенные кластеры продемонстрировали зависимость от уровня антител к hCMV, по которой были сгруппированы кластеры, соответствующие серонегативным и низко положительным образцам. Исследование показало, что инфицирование hCMV приводит к увеличению популяций NK-клеток, экспрессирующих маркер NKG2C, что коррелирует с более высокими уровнями ответа IgG на hCMV. Интересно, что мы выявили повышение $HLA\text{-}DR^+$ и снижение $KIR2DL1^+$NK-клеток со средним уровнем титра IgG к hCMV по сравнению с образцами, полученными от серонегативных и низко положительных доноров. Кроме того, была обнаружена статистически значимая отрицательная корреляция между NK-клетками $KIR2DL1^+$ и титром анти-hCMV IgG антител, в то время как положительная корреляция между HLA-DR и уровнем антител была отмечена только без кластера, соответствующего высокому уровню анти-hCMV IgG. Однако в данном исследовании не было обнаружено связи между экспрессией KIR2DL3 и CD57 на NK-

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**Образец цитирования:**
*М.О. Устюжанина, Ю.Д. Вавилова, Н.А. Алексеева, Г.В. Луценко, Д.М. Чудаков, Е.И. Коваленко «Скоординированная экспрессия маркеров NK-клеток и ответа IgG при инфекции hCMV» // Медицинская иммунология, 2023. Т. 25, № 3. С. 573-580. doi: 10.15789/1563-0625-CON-2844*

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**For citation:**
*M.O. Ustiuzhanina, J.D. Vavilova, N.A. Alekseeva, G.V. Lutsenko, D.M. Chudakov, E.I. Kovalenko "Coordination of NK cell marker expression and IgG response in hCMV infection", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 573-580. doi: 10.15789/1563-0625-CON-2844*

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**DOI:** 10.15789/1563-0625-CON-2844

клетках и уровнями IgG-ответа на hCMV инфекцию. Это указывает на то, что разные субпопуляции NK-клеток могут выполнять различные роли в регуляции гуморального иммунитета к hCMV. В целом результаты этого исследования дают ценную информацию о координации экспрессии маркеров NK-клеток и ответа IgG при инфекции hCMV.

*Ключевые слова: NK-клетки, hCMV, IgG, NKG2C, HLA-DR, CD57, KIR2DL2/DL2, KIR2DL1, кластерный анализ, корреляционный анализ*

# COORDINATION OF NK CELL MARKER EXPRESSION AND IgG RESPONSE IN hCMV INFECTION

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**Abstract.** Human cytomegalovirus (hCMV) is a prevalent virus that affects a large proportion of the population worldwide. Natural Killer (NK) cells are essential immune cells that play a crucial role in controlling hCMV infection. Despite the wide spread of hCMV infection, there is still not enough data related to the association between innate and adaptive immunity. This study investigated the coordination between some of the NK cell markers expression and humoral immune response during hCMV infection. Thirty-three samples obtained from different healthy donors were investigated. The anti-hCMV IgG antibody titer was measured in serum samples, and expression of NKG2C, HLA-DR, CD57, KIR2DL2/DL3, and KIR2DL1 were analyzed in $CD56^{+}CD3^{-}$ cells in PBMC samples by flow cytometry. To evaluate the dependence of proportions of different NK cell subsets on IgG titers, cluster analysis was first performed on all the obtained data, resulting in the identification of four main clusters. The identified clusters demonstrated a dependence on the levels of hCMV antibodies, according to which clusters corresponding to seronegative and low-positive were grouped. The results confirmed that hCMV infection leads to an expansion of NK cell populations expressing the NKG2C marker, which correlates with higher levels of IgG response to hCMV. Besides, we identified increased $HLA\text{-}DR^{+}$ and decreased of $KIR2DL1^{+}$ NK cells proportions in the middle anti-CMV-IgG level group compared to samples obtained from seronegative and low-positive donors. Moreover, the statistically significant negative correlation was found between $KIR2DL1^{+}$NK cell percentage and anti-CMV IgG antibody titer, while the positive correlation between $HLA\text{-}DR^{+}$NK cell proportion and the IgG level was noticed only without the cluster corresponded to high level of anti-hCMV IgG. In this cohort, we did not find any association between KIR2DL3 and CD57 expression in NK cells and levels of IgG response to hCMV. This may indicate that different subsets of NK cells may have distinct roles in regulating humoral immunity to hCMV. Overall, the results of the study provide valuable insights into the coordination of NK cell marker expression and IgG response in hCMV infection.

**Keywords:** *NK cells, hCMV, IgG, NKG2C, HLA-DR, CD57, KIR2DL2/DL2, KIR2DL1, clusterization assay, correlation assay*

The study was supported by the Russian Science Foundation grant No. 22-75-00135.

## Introduction

NK cells are able to perform an immune response against virus-infected and tumor cells without prior sensitization [9]. Many activating and inhibitory receptors are involved in the recognition of pathogens and altered cells [7]. The pool of NK cells is characterized by significant phenotypic and functional diversity. However, the phenotype of NK cells changes during differentiation and activation. This process is regulated by the interaction of NK cell receptors with their ligands. Phenotypic and functional diversity is formed not only by the cellular and cytokine environment, but also by the pathogens

that the organism encounters during life. Human cytomegalovirus infection (hCMV) occupies a special place among such pathogens.

hCMV is a ubiquitous herpesvirus that infects approximately 60% of adults worldwide. While usually asymptomatic in healthy individuals, hCMV can cause severe disease and even death in immunocompromised patients, such as transplant recipients or those with HIV/AIDS [3]. Despite its prevalence and clinical significance, the immune response to hCMV remains poorly understood.

NK cells play an important role in immune defense against human cytomegalovirus infection (hCMV). NK cells express various activating and inhibitory receptors, which allow them to recognize and eliminate virus-infected cells while sparing healthy cells. Recently, a special subpopulation of “adaptive” NKG2C$^+$NK cells was identified that reacts to hCMV infection and possesses some properties of immunological memory [2]. The term “immunological memory” implies an enhanced or in some way reprogrammed, prolonged and antigen-specific response. Adaptive NKG2C$^+$NK cells are highly differentiated; they express carbohydrate antigen CD57 and the KIR family receptors [5]. The HLA-DR as an essential activating receptor on the surface of NK cells [1] may also characterize the response of NK cells to hCMV. In addition, hCMV-specific antibodies, particularly of the IgG isotype, are crucial for controlling hCMV replication and preventing disease progression [10].

However, the exact interplay between NK cell activation and the humoral immune response in hCMV infection is not well understood. Understanding this connection could be crucial for developing more effective therapies against hCMV. Therefore, in this study, we investigate the potential coordination of NK cell markers expression and IgG response in hCMV infection. By exploring the connection between these two arms of the immune system, we hope to provide new insights into the immune response to hCMV.

## Materials and methods

### 1. Samples

Peripheral blood mononuclear cells (PBMC) and plasma samples of volunteer healthy adults of different genders with median age 31 years were collected. Oral informed consent to participate in the study was received from each donor. The participant cohort included 33 subjects, the main characteristics of which are listed in Table 1.

**TABLE 1. CHARACTERISTICS OF HEALTHY VOLUNTEERS**

| Donor | Gender | Age | IgG to hCMV |
|---|---|---|---|
| 1 | female | 53 | 13.448 |
| 2 | female | 24 | 0 |
| 3 | female | 38 | 3.388 |
| 4 | female | 61 | 3.558 |
| 5 | female | 54 | 1.958 |
| 6 | male | 57 | 3.498 |
| 7 | female | 22 | 0.792 |
| 8 | male | 42 | 2.448 |
| 9 | female | 69 | 3.832 |
| 10 | female | 24 | 4.894 |
| 11 | male | 45 | 1.364 |
| 12 | female | 23 | 1.358 |
| 13 | female | 26 | 8.954 |
| 14 | male | 28 | 0 |
| 15 | female | 18 | 9.616 |
| 16 | female | 49 | 12.732 |
| 17 | male | 65 | 0 |
| 18 | male | 23 | 0 |
| 19 | female | 30 | 1.272 |
| 20 | female | 25 | 8.763 |
| 21 | female | 27 | 0.398 |
| 22 | male | 59 | 7.942 |
| 23 | female | 27 | 0 |
| 24 | female | 31 | 12.656 |
| 25 | male | 27 | 8.672 |
| 26 | female | 30 | 0.359 |
| 27 | female | 48 | 3.515 |
| 28 | female | 53 | 9.985 |
| 29 | male | 27 | 2.82 |
| 30 | female | 29 | 8.788 |
| 31 | female | 41 | 5.731 |
| 32 | female | 39 | 7.582 |
| 33 | female | 59 | 9.935 |

**2. ELISA**

The hCMV-specific IgG levels in plasma samples of the healthy volunteers were measured using hCMV IgG Fluorescent Immunoassay kit (Vector-Best, Novosibirsk, Russia).

**3. Phenotype analysis**

PBMC samples were stained with the following fluorescent-labeled antibodies: CD3-PerCP (clone HIT3a, Sony Biotechnology San Jose, CA, USA), CD56-APC-Vio770 (clone REA196, Miltenyi Biotec, Bergisch Gladbach, Germany), NKG2C-FITC (clone REA205, Miltenyi Biotec, Bergisch Gladbach, Germany), HLA-DR-PE-Vio770 (clone REA332, Miltenyi Biotec, Bergisch Gladbach, Germany), KIR2DL2/L3-APC (clone DX27, Sony Biotechnology, San Jose, CA, USA), CD57-Vio-Blue (clone TB03, Miltenyi Biotec, Bergisch Gladbach, Germany), KIR2DL1-PE (clone HP-MA4, Sony Biotechnology, San Jose, CA, USA). Samples were analyzed using a MACSQuant 10 flow cytometer (Miltenyi Biotec, Germany) equipped with lasers $\lambda = 405$ nm, $\lambda = 488$ nm, $\lambda = 635$ nm.

**4. Statistical analysis**

The data was analyzed using FlowJo, GraphPad Prism X 10.0.7r2, and R language. The clusterization analysis was performed via construction of a heatmap using the library (pheatmap), which constructs a heatmap based on the numeric values in the sample matrix. The “row Z-score” scaling method was used to scale each row to have a mean of 0 and a standard deviation of 1. The analysis of percentages was carried out with a nonparametric Kruskal-Wallis test. Correlation analysis was done using Spearman correlation for nonparametric samples. The value of $p < 0.05$ was considered statistically significant.

## Results and discussion

**1. Clusterization of samples with following phenotypic analysis**

Surface expression levels of NKG2C, HLA-DR, CD57, and KIR2DL2/DL3 in NK cells were analyzed in PBMC samples obtained from 33 volunteer healthy adults by flow cytometry. NK cells were gated as $CD56^{+}CD3^{-}$ cells. The hCMV serological status was determined by ELISA kit to hCMV-specific IgG level. Pre-log normalized data on anti-hCMV IgG antibody titers together with the percentages of the NKG2C, HLA-DR, CD57, and KIR2DL2/DL3 were used for clustered heatmap analysis with hierarchical relationships between samples in order to divide the donor into groups (Figure 1A). The four main clusters were identified (Figure 1A) and anti-hCMV IgG antibody titer to hCMV among clusters was analyzed. The highest anti-hCMV IgG antibody titers were found in cluster 4, which significantly differed from the cluster 1 and cluster 2 IgG levels (Figure 1B). The cluster 3 also showed higher anti-hCMV IgG levels compared to the cluster 1. The cluster 1 corresponded to the sero-negative status of donors, and the cluster 2 represented the low-seropositive hCMV status. Taking into account that no differences were observed between the clusters 1 and 2 (Figure 1B), for further analysis of relationships of the IgG levels and NK cell subset proportions the clusters 1 and 2 were combined.

The highest percentage of $NKG2C^{+}$NK cells was observed in cluster 4 (Figure 1C), which has been noticed by different research groups previously [4, 6, 8]. Another important thing in antiviral response is the activation state of the cells. The HLA-DR is an essential activation marker of NK cells [1]. The HLA-DR expression was increased in samples of cluster 3 compared to the cluster 1 and 2, while there were no differences between cluster 4 and other clusters (Figure 1D). We revealed that the percentage of $HLA\text{-}DR^{+}$NK cells was increased in cluster 3 with middle anti-hCMV IgG antibody titers compared to undetectable and low anti-hCMV IgG antibody titers, which indicated that the NK cells were activated in cluster 3. At the same time, in cluster 4 containing samples with high anti-hCMV IgG antibody titers the percentage of $HLA\text{-}DR^{+}$NK cells did not differ significantly compared to samples from clusters 1 and 2 (Figure 1D). These findings may indicate that higher adaptive B cells immune response is associated with lower NK cell activation level and vice versa the high NK cells response indicates lower adaptive B cells response. We did not find significant differences in the percentages of both $KIR2DL2/DL3^{+}$ and $CD57^{+}$NK cells between all studied groups (Figure 1E, F). Additionally, we decided to analyze the KIR2DL1 expression in NK cells in part of the donors. The level of $KIR2DL1^{+}$NK cells showed the tendency to decrease in cluster 3 compared to the group that united clusters with undetectable and low anti-hCMV IgG antibody levels (Figure 1G).

**2. Correlations between NK cell markers and anti-hCMV IgG antibody titers**

The Spearman correlation assay of $NKG2C^{+}$, HLA- $DR^{+}$, $CD57^{+}$, $KIR2DL2/DL3^{+}$, and $KIR2DL1^{+}$NK cell percentages vs anti-hCMV IgG antibody titers

was performed. Positive correlation was identified between NKG2C+ cells and hCMV-specific IgG levels (Figure 2A). It was shown earlier that the NKG2C expression is mostly associated with CD57 expression in hCMV infection [4]. However, we have not observed any dependence of CD57 expression on anti-hCMV IgG antibody titers (Figure 2C). By contrast, a negative correlation was observed between KIR2DL1 expression and anti-hCMV IgG antibody titer (Figure 2B). No correlations were found for KIR2DL2/DL3 as well as HLA-DR to IgG to CMV (Figure 2D, E). However, further analysis of HLA-DR expression on the samples from clusters 1, 2 and 3 revealed the positive correlation between HLA-DR

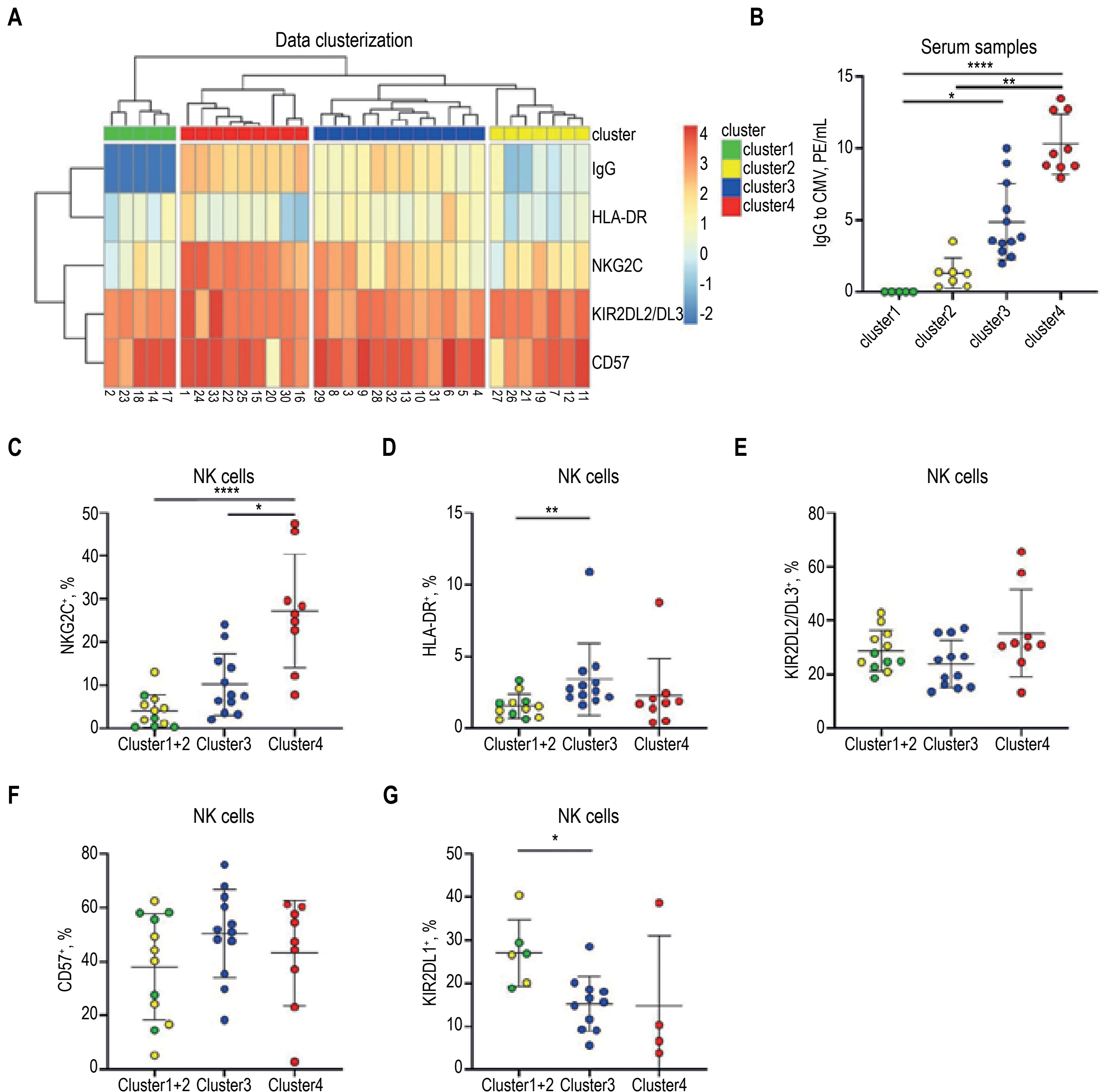


**Figure 1. Clustering analysis of the NK cells phenotype in hCMV infection**

Note. (A) Hierarchical clustering of log-normalized anti-hCMV IgG antibody titers and percentage of HLA-DR, NKG2C, CD57 and KIR2DL2/DL3 NK cells for 33 samples using heatmap reveals 4 discrete sample-level clusters. (B) anti-hCMV IgG antibody titers to hCMV in PE/ml among 4 clusters. (C) NKG2C expression on NK cells among 4 clusters. (D) HLA-DR expression on NK cells among 4 clusters. (E) KIR2DL2/DL3 expression on NK cells among 4 clusters. (F) CD57 expression on NK cells among 4 clusters. (G) KIR2DL1 expression on NK cells among 4 clusters. Data are presented as the mean (± SD). Kruskal–Wallis statistical tests for nonparametric samples were used. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

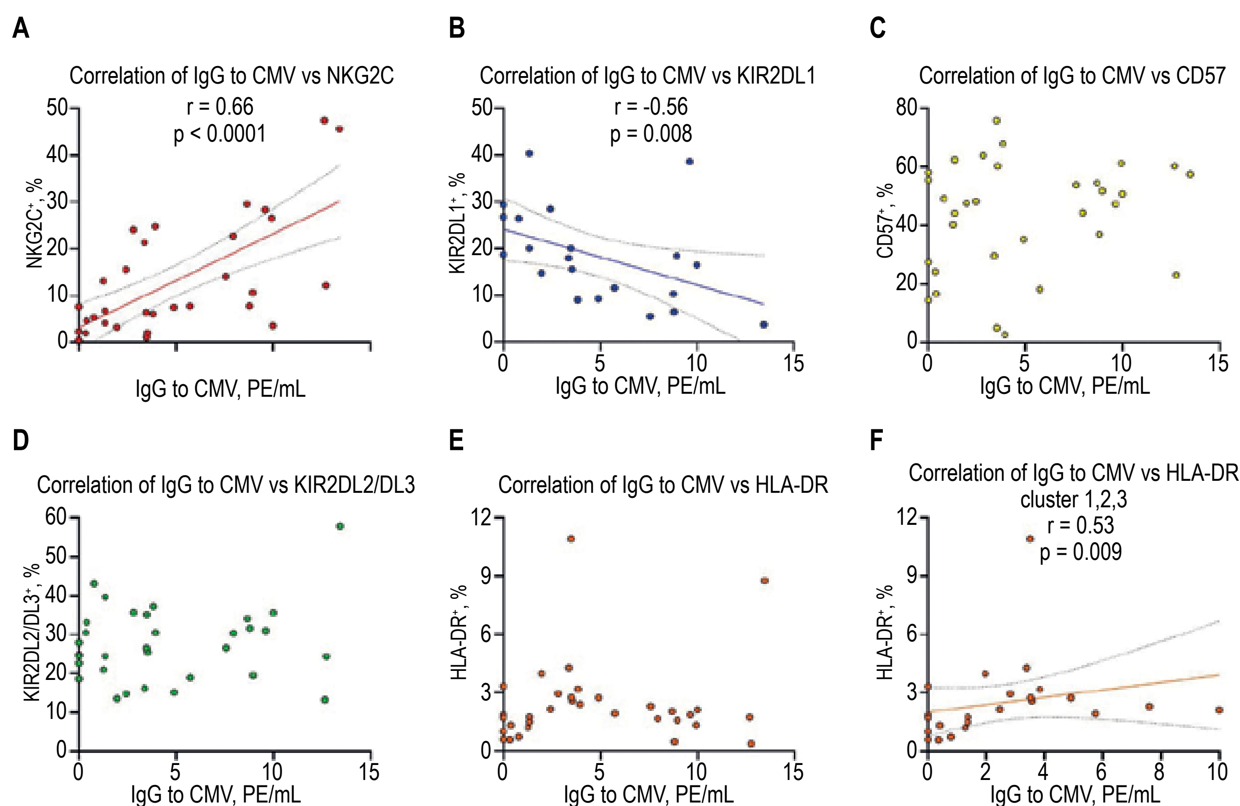


**Figure 2. Correlation assay of NK cells in hCMV infection**

Note. (A) Spearman correlation between the percentage of NKG2C and IgG to CMV in PE/mL. (B) Spearman correlation between the percentage of KIR2DL1 and IgG to CMV in PE/mL. (C) Spearman correlation between the percentage of CD57 and IgG to CMV in PE/mL. (D) Spearman correlation between the percentage of KIR2DL/2DL3 and IgG to CMV in PE/mL. (E) Spearman correlation between the percentage of HLA-DR and IgG to CMV in PE/mL. (F) Spearman correlation between the percentage of HLA-DR and IgG to CMV in PE/mL among clusters 1, 2, and 3.

and anti-hCMV IgG antibody titers (Figure 2F). What may possibly indicate that the high adaptive B cells immune response is associated with lower degree of NK cells activation, while the high NK cells response corresponds to the indicated lower adaptive B cells response.

## Conclusion

Further research is needed to fully understand the role of NK cells in the complex immune response to hCMV infection. Taken together, our results indicate the coordination of NKG2C, HLA-DR, and KIR2DL1 expression in hCMV infection.

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*Поступила 16.04.2023*
*Отправлена на доработку 25.04.2023*
*Принята к печати 27.04.2023*

*Received 16.04.2023*
*Revision received 25.04.2023*
*Accepted 27.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 581-586

Краткие сообщения
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Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 581-586

# ВЛИЯНИЕ СУПЕРНАТАНТА *BIFIDOBACTERIUM BIFIDUM* НА МОРФОФУНКЦИОНАЛЬНЫЕ СВОЙСТВА ФИБРОБЛАСТОВ ЧЕЛОВЕКА В ДИНАМИКЕ В ЭКСПЕРИМЕНТЕ *IN VITRO*

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**Резюме.** В настоящее время активно идет поиск экзогенных стимуляторов процессов репарации и регенерации. В последние десятилетия накоплены данные об иммунотропной активности бифидобактерий. Ключевая же роль в восстановлении дефектов в области раны принадлежит фибробластам за счет секреции компонентов внеклеточного матрикса, метаболитов, сигнальных факторов для окружающих клеток и регуляции тканевого метаболизма. В работе приведены результаты исследования влияния супернатанта *Bifidobacterium bifidum* (10 мкл/мл) на морфофункциональные свойства фибробластов человека в динамике в эксперименте *in vitro*. В работе использовали эталонный штамм *Bifidobacterium bifidum* 791 (Всероссийская коллекция промышленных микроорганизмов ФГУП ГосНИИ «Генетика», № депонента АС-1247), использующийся при производстве пробиотика «Бифидумбактерин» (ЗАО «Экополис», г. Ковров) и фибробласты взрослого человека (линия клеток ЛЭЧ-4(81)) (лаборатория клеточных культур ЕНИИВИ, г. Екатеринбург). Структурно-функциональные исследования проводили на 1-е, 3-и, 7-е, 14-е, 21-е, 28-е сутки сокультивирования. Продукты вторичного метаболизма B. bifidum оказывают стрессовое воздействие на морфофункциональное состояние фибробластов в первые сутки. Стимулируют процессы пролиферации в культуре в опыте 2,67±0,24 в сравнении с контролем 0,75±0,15 ($p < 0,01$), не блокируя при этом апоптоз в клетке. Это приводит к усилению продукции белков внеклеточного матрикса, как коллагена (пг/мл) (400±19 против 110±25 в контроле), так и эластина (нг/мл) 395±30 и 125±29). Сокультивирование фибробластов в опытном образце в течение суток приводит к массивному «сбрасыванию» рецептора CD44 ($p < 0,05$), в отличие от контроля, которое подтверждается фенотипическими изменениями ($r = 0,66$). На 1 и 3 сутки наблюдается снижение $CD105^+$, $CD44^+$ рецепторов ($p < 0,05$), по сравнению с контрольной группой и увеличение экспрессии $CD29^+$ ($p < 0,05$). Активированные фибробласты обладают измененным секреторным фенотипом, продуцирующим цитокины различной направленности TGF-β ($r = 0,78$), IL-6 ($r = 0,57$), IL-1β ($r = 0,75$), IL-8 ($r = 0,63$). Максимальная адаптация клеток в опытной систе-

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**Образец цитирования:**
*А.А. Марков, Е.Г. Костоломова, Т.Х. Тимохина, Г.С. Соловьев, Я.И. Паромова, Е.Д. Полянских, К.А. Воронин «Влияние супернатанта Bifidobacterium bifidum на морфофункциональные свойства фибробластов человека в динамике в эксперименте in vitro» // Медицинская иммунология, 2023. Т. 25, № 3. С. 581-586.*
*doi: 10.15789/1563-0625-EOB-2720*

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**For citation:**
*A.A. Markov, E.G. Kostolomova, T.Kh. Timokhina, G.S. Solovyev, Ya.I. Paromova, E.D. Polyanskikh, K.A. Voronin "Effect of Bifidobacterium bifidum supernatant on the morphological and functional characteristics of human fibroblasts in real time during an in vitro experiment", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 581-586.*
*doi: 10.15789/1563-0625-EOB-2720*

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**DOI:** 10.15789/1563-0625-EOB-2720

ме регистрируется на 7-е сутки, что коррелирует с морфометрическим (r = 0,59) и цитометрическим (r = 0,71) исследованиями. Полученные данные способствуют пониманию механизмов иммунорегуляторного влияния нормобиоты (на модели бифидобактерий) на процессы репарации и регенерации.

*Ключевые слова: фибробласты, Bifidobacterium bifidum, цитокины, коллаген, эластин, проточная цитометрия*

# EFFECT OF *BIFIDOBACTERIUM BIFIDUM* SUPERNATANT ON THE MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF HUMAN FIBROBLASTS IN REAL TIME DURING AN *IN VITRO* EXPERIMENT

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**Abstract.** Currently, there is an active search for exogenous stimulators of repair and regeneration processes. In the recent decades, some data on the immunotropic activity of bifidobacteria have been accumulated. The key role in healing of wound defects belongs to fibroblasts due to the secretion of the extracellular matrix components, metabolites, signal factors for the surrounding cells, and tissue metabolism regulation. The paper presents the results of the study of the effect of *Bifidobacterium bifidum* supernatant (10 μl/mL) on the morphological and functional properties of human fibroblasts in real time during the *in vitro* experiment. In our work, we used the reference strain *B. bifidum* 791 (All-Russian Collection of Industrial Microorganisms of the State Research Institute for Genetics and Selection of Industrial Microorganisms “Genetika”, Deposit No. AS-1247) used in the production of the probiotic product “Bifidumbacterin” (ZAO “Ecopolis”, the city of Kovrov), and adult human fibroblasts (cell line LECH-4 (81)) (laboratory of cell cultures ENIIVI, the city of Yekaterinburg). Structural and functional studies were conducted on co-culture days 1, 3, 7, 14, 21, and 28. The products of *B. bifidum* secondary metabolism have a stressful effect on the morphological and functional state of fibroblasts on the first day. The processes of proliferation are stimulated in the culture in the experiment (2.67±0.24) compared with the control group (0.75±0.15) ($p < 0.01$) without blocking apoptosis in the cell. This leads to the increase in the production of extracellular matrix proteins, both collagen (pg/mL) (400±19 against 110±25 in the control group), and elastin (ng/mL) 395±30 and 125±29). Co-culture of fibroblasts within 24 hours in the experimental sample leads to a massive “release” of the CD44 receptor ($p < 0.05$), compared to the control group which is confirmed by phenotypic changes (r = 0.66). The decrease of $CD105^+$, $CD44^+$ receptors ($p < 0.05$), compared with the control group and the increase of $CD29^+$ expression ($p < 0.05$) is observed on days 1 and 3. Activated fibroblasts have an altered secretory phenotype that produces cytokines of various types such as TGF-β (r = 0.78), IL-6 (r = 0.57), IL-1β (r = 0.75), IL-8 (r = 0.63). The maximum adaptation of cells in the experimental system is registered on the 7th day, which correlates with morphometric (r = 0.59) and cytometric (r = 0.71) studies. The received data contribute to understanding of the mechanisms of the immunoregulatory influence of normal biota (in the bifidobacteria model) on the repair and regeneration processes.

*Keywords: fibroblasts, Bifidobacterium bifidum, cytokines, collagen, elastin, flow cytometry*

## Introduction

Wound healing is a highly dynamic process, which involves complex interactions of extracellular matrix molecules, soluble mediators, and various resident cells [11]. The key role in wound repair belongs to fibroblasts that provide collagen to the new extracellular matrix and produce a number of cytokines, chemokines and growth factors in response to tissue damage [12]. Currently, there is an active search for exogenous stimulators of repair and regeneration processes. In the recent decades, some data on the immunotropic activity of bifidobacteria have been accumulated [8]. Our pilot study confirms the immunomodulatory function of *Bifidobacterium bifidum*, which regulates the balance between Th1 and Th2 immune response [5]. The published studies have shown that probiotics, especially the *Bifidobacterium bifidum* genus, can stimulate fibroblast migration and proliferation as well as the formation of new blood vessels during the wound healing process [7]. In view of the above, the interest in further research into the reparative and regenerative properties of probiotic bacteria is obvious.

**The aim of the research** was to study the effect of *Bifidobacterium bifidum* (*B. bifidum*) supernatant on the morphological and functional characteristics of human fibroblasts in real time during the *in vitro* experiment.

## Materials and methods

The reference strain *B. bifidum* 791 (All-Russian Collection of Industrial Microorganisms of the State Research Institute for Genetics and Selection of Industrial Microorganisms "Genetika", Deposit No. AS-1247) utilized in the production of "Bifidumbacterin" probiotic was used in our study. The *B. bifidum* supernatant was obtained by the method described previously [13].

Adult human fibroblasts (LECH-4(81) cell line) (Laboratory of Cell Cultures, Yekaterinburg) were cultured in DMEM/F12 medium containing 2 mM L-glutamine and 10% fetal calf serum. Fibroblasts were seeded on Petri dishes at a density of $1 \times 10^6$ cells/cm$^2$ and placed in standard conditions of $CO_2$ incubator B 52 (BINDER GmbH, Germany). Live imaging of fibroblasts was performed using a Nikon Ts2 inverted microscope (Nikon Corp, Japan) using NIS-Elemnts D 5.30.00 (Assembly 1531) 64-bit software with a Nikon Ts2 inverted microscope (Nikon Corp, Japan), and NIS-Elements D 5.30.00 (assembly 1531) 64-bit software.

The proliferative activity was studied according to the time of cell population doubling and the state of their cell cycle [6]. The expression of specific surface cell markers was assessed by fibroblasts immunophenotyping using the CytoFLEX flow cytometer. For this purpose, the cells were removed from the surface of the plastic using a 0.25% trypsin-EDTA solution with Hanks salts (PanEco, Russia), washed out of the culture medium and the enzyme, and then their number was counted.

The monoclonal antibodies used in this work were as follows: CD105-FITC (endoglin, a coreceptor to transforming growth factors (TGF) β1 and β3), CD90-FITC (thymocyte differentiation antigen 1), CD45-FITC (common leukocyte antigen), CD34-FITC (intercellular adhesion molecule), HLA-DR FITC (major histocompatibility complex DR molecule), CD14 (membrane glycosyl-phosphatidylinositol-related protein expressed on the surface of monocytes), CD44-FITC (transmembrane glycoprotein), and CD29-FITC (β1 integrin subunit) (BD Bioscience, USA). The cell suspension was incubated with antibodies in the dilutions recommended by the manufacturer for 30 min at room temperature. After that, it was washed from the antibodies not bound to the antigen by the CellWash solution (Becton Dickinson, USA). Cell viability was determined using the colorimetric analysis of MTT 3-(4.5-dimethylthiazol-2-yl)-1.5-diphenyltetrazolium bromide [1].

Fibroblast supernatants were selected on incubation days 1, 3, 7, 14, 21, and 28, passed through a filter with a pore diameter of 0.22 μm (GE Osmonics, USA), and stored at -80 °C. The concentration of elastin, collagen type I (COL1), and soluble CD44var (v6) was determined by enzyme immunoassay (ELISA) using Invitrogen test systems, Thermo Fisher, USA), according to the manufacturer's method. The determination of cytokines (IL-1β, IL-6, IL-8, IL-10, TGF-β, IL-18 and IL-19) was conducted by ELISA method: IL-1β, IL-6, IL-8, IL-10, TGF-β, IL-18 and IL-19 using a standard set of reagents by "Protein Contur" Ltd (Russia) in accordance with the manufacturer's instructions. The results were registered in a Multiskan photometer (Labsystems, Finland).

**Preparation of fibroblast culture for experimental conditions**

The following factors influence the growth rate and properties of fibroblasts in culture: the number of passages, method of cultivation, type of media and sera used. The obtained cellular population is heterogeneous (small spindle-shaped progenitor cells; larger spindle-shaped maturing cells; large mantle-like mature fibrocytes).

The main pool of cells is in G0-phase, 9.5 to 19% of culture is in the process of constant division, the DNA index is 1.96 on the average, which corresponds to the characteristics of differentiated fibroblasts.

An important feature that characterizes the functional state of the cell population is the synthesis of extracellular matrix proteins. Fibroblasts lose this ability during long cultivation, and the risk of accumulation of chromosomal abnormalities in the cells arises [9]. Considering the above, the fibroblasts were taken into the experiment after 5-6 passages [2].

MTT analysis was carried out for the determination of the optimal dose of *B. bifidum* supernatant. The results obtained by co-culturing of fibroblasts with *B. bifidum* supernatant showed that the maximum cell proliferation was at a supernatant concentration of 10 μl/mL ($p = 0.011$). Each experiment was replicated 3 times with duplication of analytical measurements (n-3).

The design of the experiment:

1. Control group;
2. Co-culture of fibroblasts with *B. bifidum* supernatant at a concentration of 10 μl/mL.

Data distribution was evaluated using the Kolmogorov–Smirnov test (one-sample and two-sample tests). The data were expressed as mean value ± standard deviation, and analyzed using a t-test for the paired sample. $p < 0.05$ was considered statistically significant. The SigmaPlot software (version 12.0) was used for representation of data.

## Results and discussion

Fibroblasts *in vitro* are characterized by plasticity and a variety of forms (oval, polygonal, fusiform, dendritic) [4]. The morphology of fibroblasts after 24-hour co-culture with *B. bifidum* supernatant was different from cells cultivated under normal conditions. The cells were in both suspended (dead) and adherent states. The number of viable cells in

the experiment was 48% ±9, and 90% ±3 was in the control group, respectively. Microscopy showed small rounded cells: prefibroblasts with a high proliferative potential. The population in the control group was heterogeneous (small spindle-shaped progenitor cells and larger spindle-shaped maturing cells; large mantle-like mature fibrocytes). After 3 days of co-culturing, spindle-shaped cells, with outflowing thin long processes, and spindle-shaped actively dividing progenitor cells were observed. Both large spindle-shaped maturing cells and mature spindle-shaped fibroblasts were observed after 7 days of co-culturing. From the 14th day of cultivation in the experimental and control samples, cells were spindle-shaped with a large ellipsoidal nucleus.

The proliferation index increased after 24-hour co-culture in the experiment (2.67±0.24) compared with the control group 0.75±0.15 ($p < 0.01$), then gradually decreased by the 7th day (1.13±0.09) that corresponded to the control group (1.03+0.1). The decrease of the proliferation index in the compared groups was observed from the 21st day reflecting the morphological and functional state of fibroblasts. The high proliferation index as well as the increased production of extracellular matrix components can presumably be explained by the mechanisms of cell adaptation to co-culture conditions [3]. Apparently, at the initial stage of co-culturing, the culture medium when supernatant is added is “stressful” for the fibroblasts, and the state of the cells themselves during this period is shocking. Then, some time is spent for the preparation and carrying out of adaptive “measures” due to which it is possible not only to avoid complete death of cells, but also to create conditions for stimulation of proliferative process, reducing high, at first obviously “cytotoxic” effect.

The functions of fibroblasts are as follows: the synthesis of extracellular matrix proteins (collagen of several types), the main component of the intercellular substance (elastin), and the metabolism of hyaluronic acid, as evidenced by the concentration of soluble CD44.

The reliable increase in the concentration of both collagen and elastin in the supernatant when compared with the control group is observed during the first day of co-culturing (Table 1). The increase in the synthesis of extracellular matrix materials by the fibroblasts in the control group and their decrease in the experimental group to the level of control indices is observed by the 3rd day. The decrease in the synthesis of collagen and elastin to the minimum indices is registered on the 28th day and observed in both studied groups during subsequent cultivation (7, 14, 21 days). The results obtained show the aging of the fibroblast culture in the studied groups, which is confirmed by morphological changes [10]. Co-culturing fibroblasts in the experimental sample for a day leads to a massive “release” of the CD44 receptor (Table 1), in contrast to the control, which is confirmed by phenotypic changes ($r = 0.66$) (Figure 1). However, on the 3rd day we observed a decrease in the studied marker in the supernatant, which reflects, in our opinion, the adaptation of the fibroblast culture to the new conditions of cultivation.

Maximum cell adaptation in the experimental system was recorded on day 7, which correlates with morphometric ($r = 0.59$) and cytometric ($r = 0.71$) studies. Aging of fibroblast culture was detected starting from the 21st day of cultivation, which was confirmed by a sharp increase in the concentration of soluble receptor CD44 ($r = 0.88$). The stimulation by the supernatant (10 μl/mL) leads to significantly higher levels of cytokine release in the first day of co-culture compared to unstimulated cells. Among the tested 7 pro- and anti-inflammatory cytokines, the results of only IL-1β, IL-6, IL-8, and TGF-β were significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The immunophenotype of fibroblasts allows evaluating their antigenic profile. The subpopulation composition of the fibroblast culture is characterized by the expression of mesenchymal (CD44, CD29, CD90, CD105) and the absence of epithelial, hematopoietic leukocyte and activation markers (CD14, CD34, CD45, and HLA-DR) [9].

All mentioned above has been confirmed by the analysis of the expression of specific cell surface markers during co-culture with *B. bifidum* supernatant. A decrease of $CD105^+$, $CD44^+$ receptors ($p < 0.05$), compared with the control group, and an increase of $CD29^+$ expression ($p < 0.05$) was observed on the

**TABLE 1. CHANGES IN THE CONCENTRATION OF TYPE I COLLAGEN, ELASTIN, SOLUBLE CD44 BY FIBROBLAST CULTURE UNDER CONDITIONS OF CO-CULTURE BY *B. bifidum* SUPERNATANT**

| | | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 7 | 14 | 21 | 28 |
| **Type I collagen, pg/mL** | Experiment | 400±19* | 362±24# | 148±13* # | 54±10# | 17±4# | 5.0±0.2* # |
| | Control | 110±25 | 364±25# | 210±21# | 49±11# | 18±2# | 0# |
| **Elastin, ng/mL** | Experiment | 395±30* | 190±17* # | 92±19# | 32±5# | 10±3# | 3.0±0.2* # |
| | Control | 170±19 | 262±24# | 108±13# | 34±10# | 12±2# | 0* # |
| **Soluble CD44, ng/mL** | Experiment | 19±5* | 7.0±1.2* # | 4.0±0.02# | 9.0±1.2# | 18.0±1.9# | 25.0±4.2# |
| | Control | 5.0±0.4 | 3.00±0.03# | 3.0±0.06 | 10.0±1.4# | 15.0±2.2# | 29.0±5.4# |

Note. *, reliable differences between control and experimental groups, $p < 0.05$, $n = 15$. #, reliable differences from days 1 to 28 in experimental and control groups, $p < 0.05$, $n = 15$.

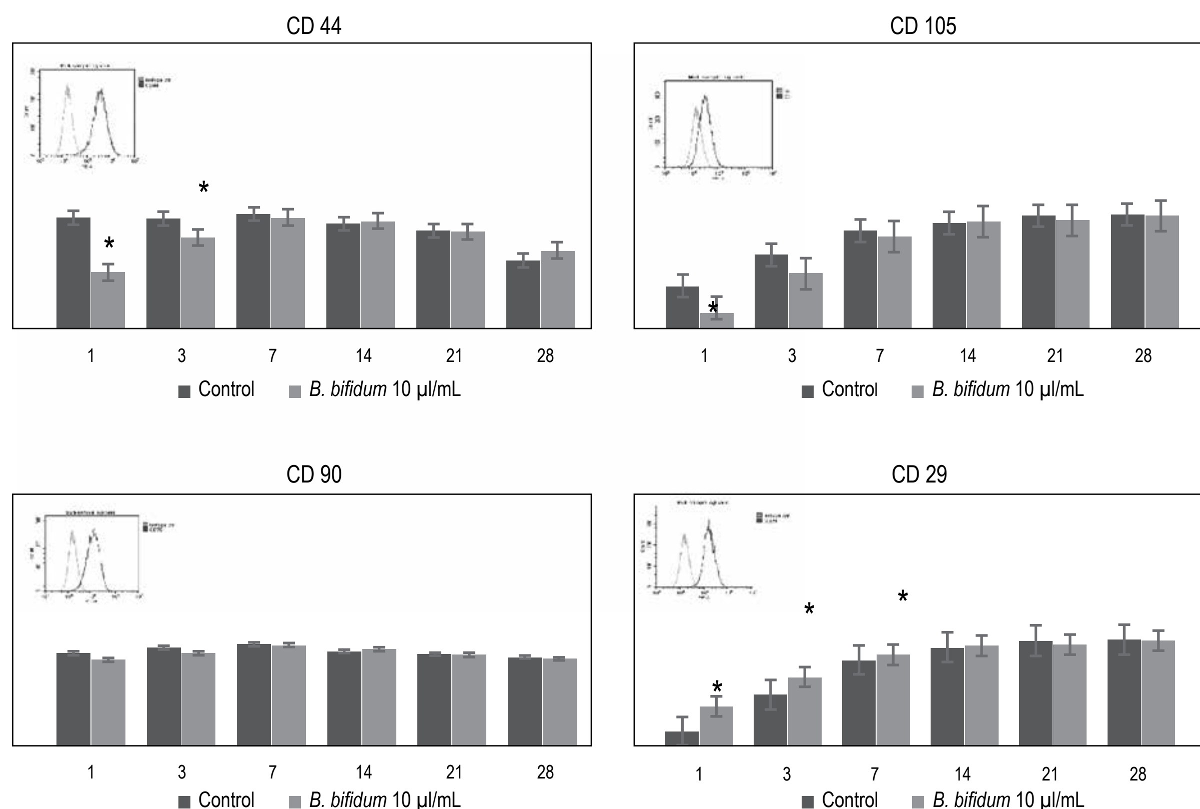


**Figure 1. Expression of CD90 CD29 CD44 CD105 markers during co-culture with *B. bifidum* supernatant**

Note. The dependence of the expression level of mesenchymal markers over fibroblasts on the time of incubation of fibroblasts with *B. bifidum* (10 μl/mL) supernatant. Isotype control group is represented by cells treated only by secondary fluorescently labeled antibodies only. Control group does not contain *B. bifidum* supernatant. The results of the average value from three independent experiments are presented; error bars correspond to the standard error of the mean. Statistical reliability of differences between the experimental and control groups was tested using Student's t-test, *corresponding to $p < 0.05$.

$1^{st}$ and $3^{rd}$ days. Recovery of the number of $CD105^+$, $CD44^+$, $CD29^+$ receptors was observed by the $7^{th}$ day of co-culturing of fibroblasts with the supernatant (Figure 1). Activated fibroblasts have an altered secretory phenotype producing cytokines TGF-β ($r = 0.78$), IL-6 ($r = 0.57$), IL-1β ($r = 0.75$), IL-8 ($r = 0.63$), which promote proliferation and mediate the recruitment of other cell types to the damaged tissue. Fibroblasts classically function in acute wound healing by becoming "reversibly" activated, secreting extracellular matrix to heal the wound.

## Conclusion

The products of the secondary metabolism of *B. bifidum* have a stressful effect on the morphological and functional state of fibroblasts on the first day of co-culturing. They stimulate proliferation processes; do not block apoptosis in the cell, which leads to increased production of extracellular matrix proteins and cytokines of different action, apparently potentiating the processes of tissue repair and regeneration.

**Conflicts of interest**

The authors have no conflicts of interest to declare.

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*Поступила 11.04.2023*
*Отправлена на доработку 12.04.2023*
*Принята к печати 18.04.2023*

*Received 11.04.2023*
*Revision received 12.04.2023*
*Accepted 18.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 587-594

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 587-594

# МОДУЛЯЦИЯ *LACTOBACILLUS JOHNSONII* ДЕНДРИТНЫХ КЛЕТОК КОСТНО-МОЗГОВОГО ПРОИСХОЖДЕНИЯ У МЫШЕЙ, НЕСУЩИХ НУЛЕВУЮ МУТАЦИЮ В ГЕНЕ MUC2

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**Резюме.** В кишечнике обитает более триллиона бактерий, которые нарабатывают до 60% метаболитов хозяина. Поэтому кишечный микробиом играет важную роль в регуляции иммунного ответа хозяина. В настоящее время получено много данных не только о влиянии пробиотических штаммов бактерий на развитие патологий, связанных с дисбиозами и нарушениями метаболического обмена, но и о важной роли бактерий в лечении воспаления, онкологии и нейродегенеративных нарушений. Изучение влияния пробиотических штаммов на лечение различных патологий проводят на экспериментальных животных с нарушением работы генов, приводящих к данной патологии. Для понимания механизма прямого действия пробиотиков используют клетки здоровых мышей или перевиваемые культуры клеток в *in vitro* исследованиях. Однако проводится довольно мало исследований эффекта пробиотических штаммов на клетки, полученные от животных с патологией. В данной работе мы исследовали фенотип дендритных клеток (ДК) мышей Muc2$^{-/-}$ с признаками хронического воспаления кишечника и оценивали какой эффект *L. johnsonii* оказывает на функциональную активность ДК. Известно, что ключевыми признаками всех экспериментальных моделей воспалительных заболеваний кишечника (ВЗК) являются истончение защитного муцинового слоя в кишечнике и изменение кишечной микрофлоры. В нашей работе мы сравнили эффективность созревания и активации ДК, полученных из костного мозга мышей с мутацией в гене Muc2, и ДК, полученных от здоровых мышей линии C57BL/6 свободных от специфических видовых патогенов. А также оценили экспрессию костимуляторных молекул, пролиферативный индекс и возможность активации Т-регуляторного ответа ДК, которые которые были стимулированы пробиотическими бактериями *L. johnsonii*.

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**Образец цитирования:**
*Е.А. Блинова, Е.П. Гончарова, Г.В. Калмыкова, Н.И. Акулова, Е.А. Литвинова «Модуляция Lactobacillus johnsonii дендритных клеток костно-мозгового происхождения у мышей, несущих нулевую мутацию в гене Muc2» // Медицинская иммунология, 2023. Т. 25, № 3. С. 587-594.*
*doi: 10.15789/1563-0625-LJM-2831*

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**For citation:**
*E.A. Blinova, E.P. Goncharova, G.V. Kalmykova, N.I. Akulova, E.A. Litvinova "Lactobacillus johnsonii modulation of bone marrow-derived dendritic cells generated from mice with null mutation of the Muc2 gene", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 587-594.*
*doi: 10.15789/1563-0625-LJM-2831*

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**DOI:** 10.15789/1563-0625-LJM-2831

Методом проточной цитометрии оценивали экспрессию клеточных маркеров дендритных и Т-клеток с помощью антител к вне- и внутриклеточным белкам. Пролиферативную активность спленоцитов оценивали с помощью WST теста.

В работе было показано, что дендритные клетки, полученные из костного мозга мышей с нулевой мутацией гена Muc2 имели незрелый фенотип по основным маркерам ДК. Дендритные клетки Muc2$^{-/-}$ мышей не могли эффективно стимулировать пролиферацию аллогенных и сингенных Т-клеток. Пробиотический штамм *L. johnsonii* был способен не только стимулировать созревание дендритных клеток, полученных от Muc2$^{-/-}$мышей, но и повышать экспрессию FoxP3 на CD25$^{+}$ Т-клетках, которые ко-культивировали с дендритными клетками.

Таким образом, мы полагаем, что данный пробиотический штамм бактерий может снижать признаки воспаления и уменьшать проявление патологических нарушений у мышей с признаками развития ВЗК.

*Ключевые слова: дендритные клетки, Lactobacillus, Т-регуляторные клетки, Muc2, воспаление, кишечник*

# *LACTOBACILLUS JOHNSONII* MODULATION OF BONE MARROW-DERIVED DENDRITIC CELLS GENERATED FROM MICE WITH NULL MUTATION OF THE MUC2 GENE

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**Abstract.** The gut is inhabited by a trillion bacteria that produce up to 60% of the host's metabolites. The gut microbiome plays an important role in regulating host immune function. A lot of research concerned the effect of probiotic on the pathologies associated not only with dysbiosis and metabolic disorders, but there is breakthrough in the treatment of inflammation, oncology and neurodegenerative disorders. Animals with mutation of the genes leading to pathology used to assay probiotic effect. To understand direct action of probiotics, cells derived from control mice or cell culture of tumor genesis *in vitro* studies are used. However, there is little research of the probiotic effect on cells derived from mice with pathology. In this study, we assessed the phenotypes of dendric cells derived from Muc2$^{-/-}$ mice with chronic inflammation and assessed the effect of *L. johnsonii* on the dendric cells. It is known that the key features of IBD models are thinning of mucin layer and changes in the intestinal microbiome. We compared the efficiency of maturation and activation of dendric cells derived from the bone marrow of Muc2$^{-/-}$ mice and dendric cells obtained from healthy C57BL/6 mice free from specific species pathogens. We evaluated the expression of co-stimulatory molecules, the proliferative index, and the ability to trigger the T regulatory response of dendric cells, which were stimulated with the probiotic *L. johnsonii*.

Markers of dendritic and T cells were assessed by flow cytometry using antibodies to extra- and intracellular proteins. The proliferative activity of splenocytes was assessed using the WST test.

It was shown that dendric cells derived from the Muc2$^{-/-}$ had an immature phenotype. Dendric cells of Muc2$^{-/-}$ mice could not effectively stimulate the proliferation of allogeneic and syngeneic T cells. *L. johnsonii* was able not only to stimulate the maturation of dendric cells derived from Muc2$^{-/-}$ mice, but also to increase the expression of FoxP3 on CD25$^{+}$ T cell that were co-cultured with DCs.

Thus, we believe that this probiotic bacterium can reduce signs of inflammation and reduce pathological processes in animals of an experimental model of IBD *in vivo*.

*Keywords: dendritic cells, Lactobacillus, T regulator cells, Muc2 gene, inflammation, intestine*

Laboratory animals were obtained with the support of the Budgetary funding for basic scientific research No. 122042700001-9 and DC cultivation and FC analysis were supported by the Russian Science Foundation (RSF) grant No. 20-64-47020.

## Introduction

It is widely accepted that the gut microbiota plays an important role in multiple functions of the host. The human gut is inhabited by a trillion of bacteria, represented by $10^4$ various species. The number of intestinal bacteria genes by 150 times exceeds the one of human genes, and its metabolites account for 60% of all blood metabolites in human body [3]. The microbiome actively participates in regulation of the host homeostasis, has a systemic effect on the populations of cells of innate and adaptive immunity, and provides the supply of certain essential nutritional components both to the host, as well as to its symbionts in the microbial community. In a healthy state, these relationships are well balanced, but disruption of this balance can contribute to various conditions, including inflammatory bowel disease (IBD) and atopy [7].

An association between the gut microbiota misfunctioning and the risk or presence of specific human diseases has been published recently [1]. In order to restore the balance, food supplements containing some beneficial bacteria species, such as of *Lactobacillus* and *Bifidobacterium* genera, are widely used as probiotics. For example, *Lactobacillus johnsonii* is one of the lactobacilli species, which is used as a probiotic drug. It alleviates the symptoms of various diseases, such as type I diabetes [8]. However, the mechanisms by which these individual probiotics modulate host response and immunity are diverse and often strain-specific. It has previously been shown that some lactic acid bacteria can have a direct effect on DCs, monocytes and tissue macrophages and, to a lesser extent, on B cells [6].

Currently, studies of the effect of probiotic drugs on the immune system are carried out on healthy mice with SPF status, but probiotics are most widely used to correct the composition of the microbiota in IBD patients. It is known that key signatures of all IBD models are the thinning of the protective mucin layer in the intestine and changes in the intestinal microflora [2]. One of the IBD model are Muc2-/- mice carrying a mutation in the Muc2 gene, which leads to disruption of the protective mucin layer in the small and large intestines. In our work, we compared the efficiency of maturation and activation of DCs obtained from the bone marrow of mice with a mutation in the Muc2 gene and DCs obtained from healthy C57BL/6 mice with SPF status. We also evaluated the expression of co-stimulatory molecules, the proliferative index, and the ability to trigger the T regulatory response of DCs, which were stimulated with the probiotic *L. johnsonii*.

## Materials and methods

**Generation of bone-marrow-derived dendritic cells**

Bone marrow-derived DCs were generated from bone marrow cells isolated from the femur and tibia of female 7-8-week-old C57BL/6 and Muc2-/- mice by flushing out the cells with PBS using a sterile syringe. The harvested cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 50 μg/mL gentamicin, 100 μg/mL penicillin-streptomycin (Gibco, China) (complete culture medium). Then, the cells were pooled and plated on 25 $cm^2$ flasks at a density of $1 \times 10^6$ cell/mL. Subsequently, growth factors GM-CSF (BioLegend, USA) and IL-4 (BioLegend, USA) were added into the medium to a final concentration of 20 ng/mL. The cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$. The culture medium was replaced on day 3 with fresh growth factors. On day 7, the cells were gently collected by cell scraper from the plate. The cells were plated into 24-well plates for priming by *L. johnsonii* in proportion 1:10 (MOI 0.1) for 2 days in duplicates.

**Flow cytometry analysis of DCs phenotype**

The cells were collected after incubation, washed with PBS, $1 \times 10^6$ cells were taken into the analysis. Dead cells were stained with Zombie Violet dye (BioLegend, USA) diluted in PBS (1:400). Then, cells were labeled with monoclonal antibodies conjugated with fluorochromes to surface antigens (BioLegend, USA): anti-CD11c-APC, anti-CD80-FITC, anti-CD86-APC/Cy7, anti-CD83-PE/Cy7, anti-I-Ab-PerCP/Cy5.5. Flow cytometry was performed using FACS CantoII flow cytometer (Becton Dickinson, USA), and the data were analyzed with the BD FACSDiva 6.0 software.

**Mixed lymphocyte reaction**

The effect of DCs, primed by *L. johnsonii*, on the proliferation of syngeneic and allogeneic splenocytes was assessed by WST-1 method. We obtained splenocyte suspensions from the spleens of C57BL/6, $Muc2^{-/-}$ and BALB/c mice. The immature DCs were collected on day 7 and plated on 96-well plate in a concentration $2 \times 10^5$ cells/mL in RPMI-1640 complete culture medium, supplemented IL-4 (20 ng/mL), GM-CSF (20 ng/mL). Then, bacteria were added at MOI 0.1, and DCs were incubated for 2 days at 37 °C in a humidified atmosphere containing 5% $CO_2$.

After culturing, DCs were treated with 50 ng/mL mitomycin C (Roche Diagnostics GmbH, Germany) for 1h. Then, DCs were washed with RPMI-1640 complete culture medium. 2 × $10^5$ splenocytes in RPMI-1640 complete culture medium were added to DCs. The ratio of DCs to splenocytes was 1:10. Splenocytes without DCs were used as a control. To measure the proliferation of syngeneic cells we used splenocytes from C57BL/6 and $Muc2^{-/-}$ respectively, to measure the proliferation of allogeneic cells – splenocytes from BALB/c for C57BL/6 and $Muc2^{-/-}$ DCs. Cells co-cultured in 96-well plate at 37 °C for 72 h in a humidified atmosphere containing of 5% $CO_2$. Next, WST-1 solution (Roche Diagnostics GmbH, Germany) was added to each well for an additional 6 h incubation. The optical density was measured at 450 nm with reference at 620 nm by reader Infinite® F50 (Tecan, Switzerland).

***In vitro* generation of Treg from naive CD4⁺T cells**

Naive $CD4^+CD44^-T$ cells were isolated from the spleens of C57BL/6 and $Muc2^{-/-}$ mice using a naive $CD4^+T$ cells isolation kit (Miltenyi Biotec Inc., Germany). The immature DCs (2 × $10^4$ cells/well) cultured with or without *L. johnsonii* at MOI 0.1 in RPMI-1640 complete culture medium, supplemented IL-4 (20 ng/mL), GM-CSF (20 ng/mL) for 2 days at 37 °C containing of 5% $CO_2$. Then, culture medium was gently discarded from the wells, and syngeneic purified naive $CD4^+T$ cells (2 × $10^6$ cells/mL) added to DCs for 72 h in a culture medium containing 100 ME/mL of recombinant human IL-2. Brefeldin A (5 μg/mL; BioLegend, USA) was added to the wells 20 hours before cell harvested to evaluate $IL\text{-}10^+$Tregs.

**Flow cytometry analysis of Treg**

After co-culturing, cells were collected, washed twice with PBS, then cells were stained with monoclonal antibodies against mouse surface (BioLegend, USA) CD3-FITC, CD4-APC, CD25-PerCP and intracellular markers (BioLegend, USA) FoxP3-PE, IL-10-APC/Cy7. For intracellular staining, cells were fixed and permeabilized using True-Nuclear™ Transcription Factor Buffer Set (BioLegend, USA). Subsequent analyses were performed by FACS CantoII (Becton Dickinson, USA).

Statistical distribution had not normally distributed data; the analysis of the data was performed by non-parametric two-way PERMANOVA test with post-hoc Bonferroni test. All data are presented as mean ± standard diversity.

## Results and discussion

To analyze the phenotype of bone-marrow DCs from mice with intestinal inflammation and investigate the effect of co-culture DCs with probiotic bacteria we measured expression of co-stimulated molecules of DCs by flow cytometry. Percentage of $CD80^+$DCs generated from born-marrow of $Muc2^{-/-}$ mice were significantly lower compared to C57BL/6 mice DCs (Two-way PERMANOVA test $F(1.17) = 39.33$ $p < 0.0001$) (Figure 1A). At the same time, DCs priming by *L. johnsonii* and interaction of two factors (genotypes and bacteria primering) did not affect the percentage of $CD80^+$DC (Two-way PERMANOVA test $F(1.17) = 3.59$ ND and $F(1.17) = 0.99$ ND respectively). Culturing DC with *L. johnsonii* significant increased percentage of $CD83^+$DCs (Figure 1B) (Two-way PERMANOVA test $F(1.17) = 8.60$ $p = 0.01$). However, percentage of $CD83^+$DC did not depend on mice genotype and interaction two factors (Two-way PERMANOVA test $F(1.17) = 0.53$ ND and $F(1.17) = 1.35$ ND respectively).

As for another DC co-stimulatory molecule, $CD86^+$, it depended both on mice genotype and DCs priming by *L. johnsonii* (Two-way PERMANOVA test $F(1.17) = 22.55$ $p < 0.001$ and $F(1.17) = 26.84$ $p < 0.001$). Thus, higher level of $CD86^+$DCs were observed in cell obtained from C57BL/6 mice than from $Muc2^{-/-}$ mice and it was independent on culturing with or without *L. johnsonii*. DCs priming by bacteria led to enhanced CD86 expression (Figure 1C). Percentage of MHC I-$A^b$ DCs depended on presence of *L. johnsonii* in cell culture and was similar between $Muc2^{-/-}$ and C57BL/6 mice as well as interaction of two factors (Two-way PERMANOVA test $F(1.17) = 11.62$ $p < 0.01$ and $F(1.17) = 0.36$ ND and $F(1.17) = 0.05$ ND respectively). Thus, addition of bacteria increased percentage of MHC I-$A^b$ DCs of both genotypes of mice. After priming by *L. johnsonii* percentage of $CD86^+$DC became higher in C57BL/6 mice compared to $Muc2^{-/-}$ mice (Figure 1D).

To investigate the functional capacity of DCs treated with *L. johnosonii* to stimulate allogeneic and syngeneic T cell proliferation we used WST assay. Proliferation index depended on mice genotype but not on treatment of probiotic and interaction of two factors (Two-way PERMANOVA test $F(1.23) = 35.08$ $p < 0.0001$ and $F(1.23) = 0.46$ ND and $F(1.23) = 0.41$ ND respectively). Thus, T cell proliferation activity of DCs generated from C57BL/6 mice was higher than DCs from $Muc2^{-/-}$ mice and it was independent of priming by bacteria. However, only proliferation index of DCs from C57BL/6 mice was higher than proliferation activity of intact BALB/c splenocytes (Figure 2A). There was no effect of DCs from $Muc2^{-/-}$ mice to stimulate allogenic T cell proliferation.

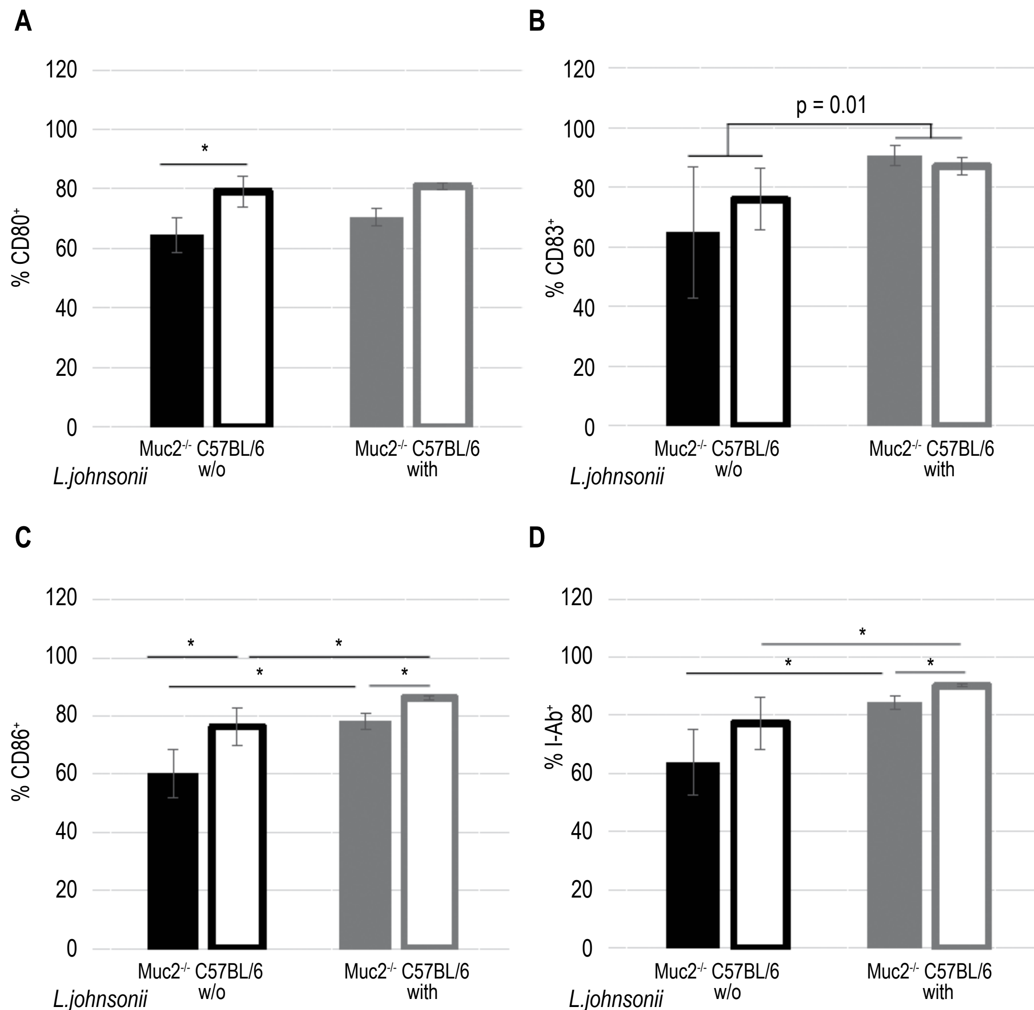


**Figure 1. Bone-marrow derived dendric cells from C57BL/6 and Muc2$^{-/-}$ cultured with and without *L. johnsonii***

Note. (A) Percentage of CD80$^{+}$DCs gated on CD11b$^{+}$ cells. (B) Percentage of CD83$^{+}$DCs gated on CD11b$^{+}$ cells. (C) Percentage of CD86$^{+}$DCs gated on CD11b$^{+}$ cells. (D) Percentage of MHC I-Ab$^{+}$ DCs gated on CD11b$^{+}$ cells. *, $p < 0.05$ Post-hoc Bonferroni test; $p < 0.01$ Effect primed with *L. johnsonii* Two-way PERMANOVA test.

On the contrary, syngeneic proliferation index was dependent on probiotic treatment and interaction of two factors but not on mice genotype (Two-way PERMANOVA test $F(1.23) = 6.49$ $p < 0.05$ and $F(1.23) = 33.88$ $p < 0.001$ and $F(1.23) = 0.90$ ND respectively). Immature DC from C57BL/6 mice demonstrate more proliferation activity than DC of Muc2$^{-/-}$ mice but treatment with probiotic reversed it and significantly decreased the index. Cell proliferation stimulated by mature and immature DCs was compared to splenocytes proliferation in steady state without addition of DCs (Figure 2B).

To understand whether DCs are capable of stimulating T regulatory response, we co-cultured splenocytes with DCs and assessed the percentage of CD25$^{+}$FoxP3$^{+}$ and IL-10$^{+}$ cells. High impact of culturing DCs with *L. johnsonii* was detected on percentage of CD25$^{+}$FoxP3$^{+}$ cells only (Two-way PERMANOVA test $F(1.23) = 10.99$ $p < 0.001$) Mice genotype and interaction of two factors did not affect the percentage of CD25$^{+}$FoxP3$^{+}$ cells (Two-way PERMANOVA test $F(1.23) = 0.60$ ND and $F(1.23) = 0.24$ ND respectively). Splenocytes became tolerogenic (enhanced expression CD25 and FoxP3) only during co-culture with mature DCs of Muc2$^{-/-}$ mice that were stimulated with *L. johnsonii*. Immature and mature DC of C57BL/6 did not stimulate activation of T regulatory cells (Figure 2C). At the same time, there were no effects of any factors to the percentage of IL-10$^{+}$T cell (Two-way PERMANOVA test $F(1.23) = 2.98$ ND and $F(1.23) = 2.38$ ND and $F(1.23) = 0.66$ ND respectively) (Figure 2D).

Thus, priming by *L. johnsonii* had no impact on DCs ability to induce proliferation of allogeneic splenocytes. However, in Muc2$^{-/-}$ mice functional capacity of DCs was reduced compared to DCs of

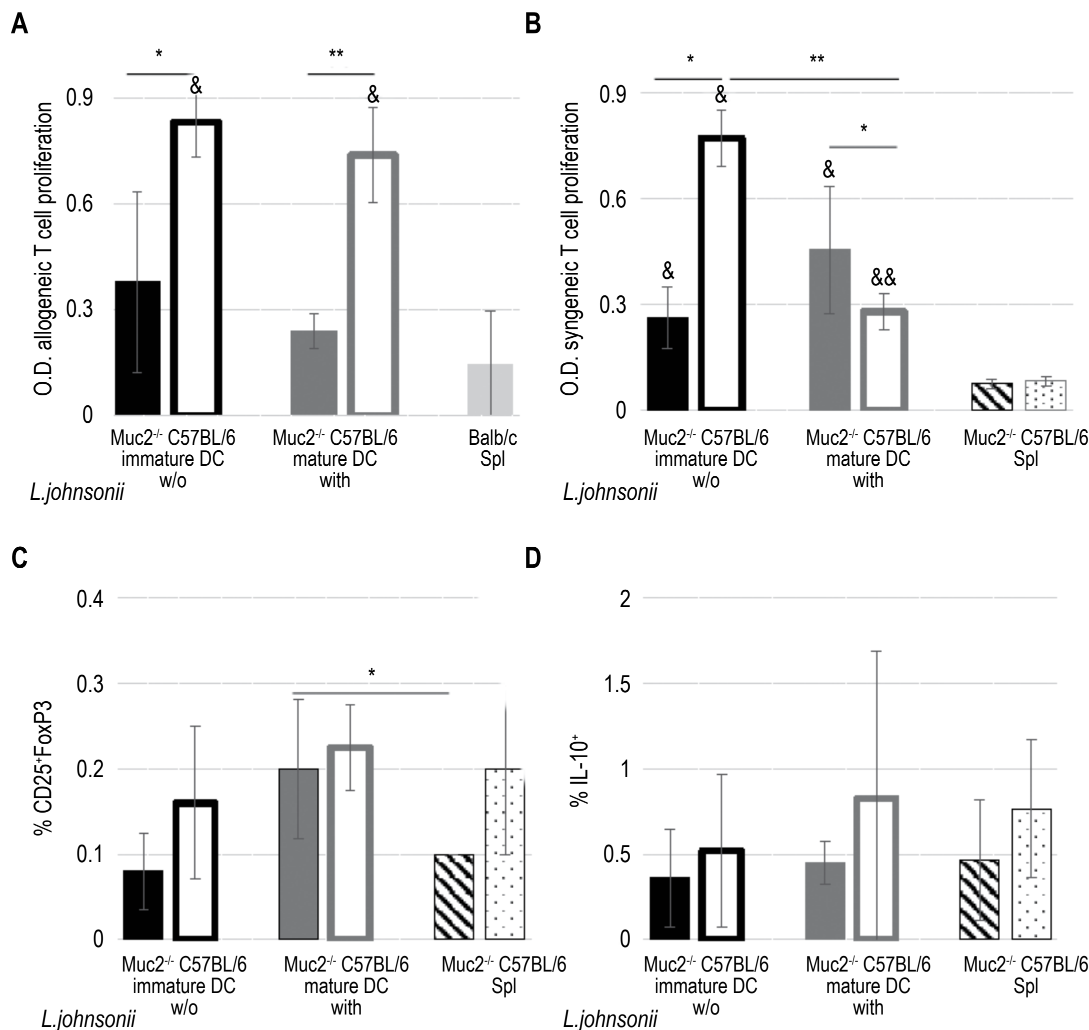


**Figure 2. Co-cultivation of splenocytes and born-marrow derived DCs from C57BL/6 and Muc2$^{-/-}$ mice cultured with and without *L. johnsonii***

Note. (A) Proliferation index of allogeneic T cells of BALB/c mice co-cultured with DCs. (B) Proliferation index of syngeneic T cell co-cultured with DCs. (C) Percentage of CD25$^+$FoxP3$^+$T cells co-cultured with DCs. (D) Percentage of IL-10$^+$T cells co-cultured with DCs. *, $p < 0.05$; **, $p < 0.01$ Post-hoc Bonferroni test; $^{\&}$, $^{\&\&}$, Mann–Whitney test: difference from mono cultivation of splenocytes respectively.

C57Bl/6 mice. This effect is consistent with immature phenotype of DCs from Muc2$^{-/-}$ mice, which have lower expression of co-stimulatory molecules CD80, CD86 and CD83 compared to DCs from C57BL/6 mice. Moreover, DC from Muc2$^{-/-}$ culture with *L. johnsonii* stimulated tolerogenic cells that could be associated with immature phenotype of DC.

We have shown that Muc2$^{-/-}$ mice that model IBD generate an immature DC phenotype compared to control animals. This effect was shown for the first time. Previous studies have analyzed functions of DCs from animals without inflammation [4]. The addition of probiotic bacteria to DC culture stimulated maturation, but the genotype of mice from which cells were generated had no impact on maturation. The immature phenotype of DCs from Muc2$^{-/-}$ mice shows a low potential for T cell proliferation, both allogeneic and syngeneic. Whereas C57BL/6 mature DCs primed with the probiotic stimulated proliferation of foreign T cells and reduced the proliferative index of their own splenocytes. It has been shown that IL-10 is able to reduce proliferative activity, but in our experiment, the percentage of IL-10$^+$ cells was not changed [10]. At the same time, the immature phenotype DCs from Muc2$^{-/-}$ mice in combination with the probiotic showed a significant increase in proliferation of tolerogenic cells. Previously, it was shown that *L. johnsonii* causes an increase in TGF and IL-10 production of $CaCo_2$ cells [5]. However, effect on DCs generated from mice with chronic inflammation was shown for the first time. In the literature is discussed that immature

DCs are able to stimulate tolerogenic response more effectively [9].

## Conclusion

We have demonstrated impaired growth of DCs from the bone marrow of mice with a null mutation of the Muc2 gene, which did not stimulate the proliferation of allogeneic and syngeneic T cells. *L. johnsonii* was able not only to stimulate DC maturation, but also to increase FoxP3 expression on $CD25^+$ cells when cultured with DC. It can be assumed that the probiotic will have a beneficial effect on inflammation and reduce pathological processes in animals *in vivo*.

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*Поступила 15.04.2023*
*Отправлена на доработку 21.04.2023*
*Принята к печати 26.04.2023*

*Received 15.04.2023*
*Revision received 21.04.2023*
*Accepted 26.04.2023*

*Медицинская иммунология*
*2023, Т. 25, № 3,*
*стр. 595-600*

*Краткие сообщения*
*Short communications*

*Medical Immunology (Russia)/*
*Meditsinskaya Immunologiya*
*2023, Vol. 25, № 3, pp. 595-600*

# ИЗМЕНЕНИЯ СОСТАВА МИКРОБИОТЫ КИШЕЧНИКА, АССОЦИИРОВАННЫЕ С ДЕФИЦИТОМ IL-6

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**Резюме.** Интерлейкин-6 (IL-6) – цитокин широкого спектра действия, который участвует в иммунной, нервной и эндокринной регуляции многих биологических процессов. IL-6 выполняет как гомеостатические, так и патогенные функции, в том числе он является одним из ключевых участников цитокинового шторма при COVID-19, а также контролирует выработку белков острой фазы при воспалении. IL-6 вовлечен в поддержание кишечного гомеостаза и играет ключевую роль как в индукции воспаления, так и в восстановлении кишечника после повреждения. В свою очередь, комменсальная микробиота – населяющие кишечник организма-хозяина эукариоты, прокариоты и вирусы – представляет собой один из ключевых факторов, модулирующих иммунный ответ в кишечнике. Так, преобладание определенных групп организмов связывают с развитием воспаления кишечника, а пробиотики и антибиотики успешно применяются как поддерживающая терапия при воспалительных заболеваниях кишечника. IL-6 необходим для поддержания барьерной функции кишечника, поскольку передача сигнала от данного цитокина модулирует пролиферацию клеток кишечника, что необходимо для их своевременного обновления как в гомеостазе, так и при воспалении. Установлено, что генетическая инактивация *IL6* способствует развитию кишечного воспаления, при этом вклад IL-6 в регуляцию состава микробиоты остается неясным. Для изучения этого вопроса был проведен анализ образцов стула наивных мышей дикого типа и мышей, дефицитных по *IL6* (IL-6 KO), полученных на генетической основе C57Bl/6. Установлено, что у нокаутных мышей на фоне дефицита IL-6 наблюдаются значительные изменения в представленности отдельных таксономических групп, которые, предположительно, и обеспечивают чувствительность IL-6 KO к развитию колита. Было обнаружено, что у IL-6 KO мышей по сравнению с мышами дикого типа наиболее существенно снижается относительное содержание *Firmicutes* и *Clostridiales* и повышается – *Bacteroides.* Полученные нами данные о снижении представленности *Firmicutes* у мышей с дефицитом IL-6, а также о снижении представленности *Lactobacillaceae* и других крупных таксонов говорят о том, что композиция микробиоты IL-6 KO мышей отчасти похожа на композицию микробиоты, характерную для хронического воспаления кишечника. Настоящая работа представляет основу для дальнейших исследований вклада IL-6-опосредованных изменений микробиоты в поддержание гомеостаза кишечника и развитие воспаления.

*Ключевые слова: IL-6, микробиота, воспаление кишечника, маркеры воспаления, мышиные модели, кохаузинг*

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**Образец цитирования:**
*Е.О. Губернаторова, А.И. Полинова, Т.Р. Юракова, С.А. Недоспасов, М.С. Друцкая «Изменения состава микробиоты кишечника, ассоциированные с дефицитом IL-6» // Медицинская иммунология, 2023. Т. 25, № 3. С. 595-600.*
*doi: 10.15789/1563-0625-CIT-2797*

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**For citation:**
*E.O. Gubernatorova, A.I. Polinova, T.R. Yurakova, S.A. Nedospasov, M.S. Drutskaya “Changes in the composition of the intestinal microbiota associated with IL-6 deficiency”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 595-600.*
*doi: 10.15789/1563-0625-CIT-2797*

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**DOI:** 10.15789/1563-0625-CIT-2797

# CHANGES IN THE COMPOSITION OF THE INTESTINAL MICROBIOTA ASSOCIATED WITH IL-6 DEFICIENCY

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**Abstract.** Interleukin-6 (IL-6) is a broad-spectrum cytokine involved in the immune, nervous, and endocrine regulation of many biological processes. IL-6 performs both homeostatic and pathogenic functions. It is one of the key factors in the cytokine storm in COVID-19, and it also controls the production of acute phase proteins during inflammation. IL-6 is involved in the maintenance of intestinal homeostasis and is required for both the induction of inflammation and the repair of the injured intestinal tissue. In turn, the commensal microbiota, represented by eukaryotes, prokaryotes, and viruses, is one of the key factors modulating the immune response in the gut. The predominance of certain groups of commensal microorganisms is associated with the development of intestinal inflammation, while probiotics and antibiotics are successfully used to control inflammatory bowel disease. IL-6 is also necessary to maintain the barrier function of the intestine by modulating the proliferation of intestinal cells, which is necessary for their timely renewal both in homeostasis and inflammation. It has been established that the genetic inactivation of *IL6* contributes to the development of intestinal inflammation, while the involvement of IL-6 in the control of the gut microbiota composition remains unclear. To investigate this issue, we analyzed stool samples from wild-type naive mice and mice deficient in *IL6* (IL-6 KO) generated on the C57Bl/6 genetic background. It has been determined that IL-6 KO shows significant changes in some taxonomic groups of commensals, which may explain the sensitivity of IL-6 KO to the development of colitis. Interestingly, the relative contents of *Firmicutes* and *Clostridiales* are significantly reduced, whereas *Bacteroides* are increased in IL-6 KO as compared with wild-type mice. Our data on the reduction of *Firmicutes, Lactobacillaceae*, and other large taxa in IL-6 deficient mice suggest that the microbiota composition of IL-6 KO mice is somewhat similar to that of mice with chronic intestinal inflammation. Our study serves as a perspective for further research on the contribution of IL-6-mediated changes in the microbiota composition to the maintenance of intestinal homeostasis and the development of chronic gut inflammation.

*Keywords: IL-6, microbiota, intestinal inflammation, inflammatory markers, mouse models, cohousing K*

This work was supported by the Russian Science Foundation grant 22-25-00534.

## Introduction

The commensal microbiota is comprised of eukaryotes and prokaryotes inhabiting the body of a host organism, including fungi and protozoa, archaea, bacteria, as well as viruses and bacteriophages. The maternal microbiota plays a key role in shaping the commensal microbial community of the child. With age, the composition of the microbial community undergoes significant changes and is regulated by many internal and external factors. Microbes inhabit both external and internal surfaces of the human body, including the gastrointestinal, respiratory and urogenital tracts, skin, oral mucosa and conjunctiva, but the main contribution to the total microbial biomass is made by the microbiota of the large intestine. Qualitative and quantitative composition varies significantly between different individuals. However, it is assumed that the human microbiota is represented by the core bacteria, in particular, belonging to the genera *Faecalibacterium, Ruminococcus, Eubacterium* and *Dorea* (*Firmicutes*), *Bacteroides* and *Alistipes* (*Bacteroidetes*) and *Bifidobacterium* (*Actinobacteria*), found in most people and constituting a significant proportion of the total number of microbial cells in the intestine [14].

In the process of co-evolution of mammals and their microbial symbionts, stable mutualistic relationships were formed between them. As a result, the microbiota began to participate in many physiological processes of the host organism. In particular, it is necessary for the development and normal functioning of the immune system [12]. Disturbances in the immune system can trigger inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease. Disruption in the control of microbiota by the host organism is one of the prerequisites for the development of inflammatory bowel disease [8]. For example, genomic mutations associated with an increased risk

of intestinal inflammation encode genes involved in recognizing and destroying microorganisms, as well as in maintaining the normal functioning of the intestinal barrier [11].

Mouse models significantly contributed to our understanding of the physiological and inflammatory functions of intestinal commensals. It was demonstrated that the microbiota is necessary for the development of intestinal inflammation, and the use of antibiotics facilitates the course of the disease [13]. In addition, transfer of fecal microbiota from mice with colitis results in intestinal inflammation in healthy recipient mice [6]. Thus, the study of the microbiota associated with intestinal inflammation is of particular interest. However, in addition to studies of changes in the microbiota that occur during intestinal inflammation, studies aimed at searching for prognostic markers of inflammation are also relevant.

One of the key factors that maintains the barrier function of the gut is IL-6. IL-6 deficiency is associated with increased susceptibility to inflammation in a mouse model of DSS-induced colitis [7], and pharmacological blockade of IL-6 signaling in humans is associated with an increased risk of intestinal perforations [10]. This effect is attributed to the ability of IL-6 to stimulate the proliferation of epithelial cells and to prevent their apoptosis. Despite the interest in the molecular mechanisms mediating the protective functions of IL-6 in the context of intestinal inflammation, the effect of this cytokine on modulating the composition of the intestinal microbiota has not yet been addressed. In addition to identification of taxonomic groups composing gut microbiota, the search for predictive markers of intestinal inflammation, is also of particular interest for fundamental science and medicine. The present work focuses on the identification of bacterial groups, which may serve as indicators of colitis development by studying IL-6-deficient mice that are highly sensitive to intestinal inflammation.

## Materials and methods

**Mice**

The work was carried out on C57Bl/6 wild-type and *IL-6* knockout (IL-6 KO) mice, generated on C57Bl/6 genetic background. Mice at the age of 6-8 weeks were obtained from the SPF animal facility of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. They were housed in individually ventilated Tecniplast EM500 cages with hygienic dust-free bedding at a constant temperature of 21±3 °C, humidity 40±10%, and 12-hour day/night cycles with food and water ad libitum at the SPF animal facility of EIMB RAS

**Collection of stool samples and DNA isolation**

Stool samples were collected in 2 mL tubes, DNA was isolated on the same day using the Qiagen QIAamp Fast DNA Stool Mini Kit according to the manufacturer's protocol. Briefly, 1 mL of InhibitEX buffer was added to the tube and the contents were triturated with a plastic swab. Next, the sample was incubated for 10 min at 70 °C and 5 min at 95 °C. It was vigorously mixed on a vortex for 15 seconds and centrifuged at 4 °C 14000 g for 1 minute. 600 μL of the supernatant was transferred to a new tube containing proteinase K, after which 600 μL of AL buffer was added, incubated for 10 min at 70 °C, and 600 μL of 96% ethanol was added. After that, 600 μL of the sample was transferred to a DNA extraction column and centrifuged, the liquid was removed from the liquid collection tube and the application was repeated until the sample was completely loaded on the column. Then the column was washed with buffers AW1 and AW2 with centrifugation for 3 min. After that, the liquid collection tube was replaced with a clean tube, 50 μL of ATE buffer was applied to the column, centrifuged for 1 min to elute the DNA. The DNA concentration was measured on an Implen NanoPhotometer N50 spectrophotometer, the samples were stored at -80 °C.

**Real-time quantitative PCR (qRT-PCR)**

To analyze the composition of the intestinal microbiota, DNA samples were diluted with mQ to a concentration of 10 ng/μL. The reaction mix for one sample consisted of 4 μL qPCR premix (Evrogen qPCRmix-HS SYBR+LowROX), 1 μL each of forward and reverse 10 μM primers (Evrogen) (Table 1), 12 μL mQ and 2 μL DNA. Each reaction was run in duplicate in 96-well Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate, qRT-PCR was performed on a QuantStudio 6 Pro amplifier.

In stool samples, the representation of bacterial taxa was assessed (Table 1). The reference group was Eubacteria (Barman et al., 2008). The amplification mode was the same for all primers: 95 °C 5 min, 40X (95 °C 15 sec, 60 °C 30 sec, 72 °C 30 sec).

Analysis of the relative abundance (A) of the bacterial taxon (x) in the intestinal microbiota was performed as follows:

$A(x) = 2^{Ct(\mathit{Eubacteria}) - Ct(x)}$, where

Ct is the amplification cycle averaged over two repetitions, at which the content of the product in the reaction mixture reaches the threshold value (Kruglov et al., 2013). Statistical processing of the results was carried out using the GraphPad Prism 8 software. The compliance of the sample with a normal distribution was checked using the Shapiro–Wilk test. If the data were normally distributed, statistical significance was assessed using a t-test. A p-value less than 0.05 was considered statistically significant.

## Results and discussion

To determine the role of IL-6 in maintaining the normal composition of the gut microbiota, DNA was isolated from stool samples and the content of bacterial taxa in IL-6 KO naive and wild-type

**TABLE 1. PRIMER SEQUENCES FOR DETERMINING THE COMPOSITION OF THE INTESTINAL MICROBIOTA WITH qRT-PCR**

| Target | Primer type | Sequence |
|---|---|---|
| ***Actinobacteria*** | F | TGTAGCGGTGGAATGCGC |
| | R | AATTAAGCCACATGCTCCGCT |
| ***Bacteroides*** | F | GGTTCTGAGAGGAAGGTCCC |
| | R | GCTGCCTCCCGTAGGAGT |
| ***Clostridiales*** | F | ACTCCTACGGGAGGCAGC |
| | R | GCTTCTTAGTCAGGTACCGTCAT |
| ***Enterobacteriaceae*** | F | GTGCCAGCAGCCGCGGTAA |
| | R | GCCTCAAGGGCACAACCTCCAAG |
| ***Epsilonproteobacteria*** | F | AGGCTTGACATTGATAGAATC |
| | R | CTTACGAAGGCAGTCTCCTTA |
| ***Eubacteria*** | F | ACTCCTACGGGAGGCAGCAGT |
| | R | ATTACCGCGGCTGCTGGC |
| ***Firmicutes*** | F | GGAGYATGTGGTTTAATTCGAAGCA |
| | R | AGCTGACGACAACCATGCAC |
| ***Lactobacillaceae*** | F | AGCAGTAGGGAATCTTCCA |
| | R | CACCGCTACACATGGAG |

mice was studied (Figure 1). For analysis, we selected groups of bacteria with relative content changes during intestinal inflammation, namely the kingdom *Firmicutes* [15], its family *Lactobacillaceae* [9], and the order *Clostridiales* [1]; *Bacteroides* – a large genus of the kingdom *Bacteroidetes* [3]; kingdoms of *Actinobacteria*; *Epsilonproteobacteria* and *Betaproteobacteria* – classes of the kingdom *Proteobacteria* [15] – and *Enterobacteriaceae* – families of the class *Deltaproteobacteria* of the same kingdom [9].

We found that the relative content of *Firmicutes* and *Clostridiales* was significantly reduced while the content of *Bacteroides* was increased in IL-6 KO as compared with wild-type mice (Figure 1A, B, C). A less pronounced decrease was observed in IL-6

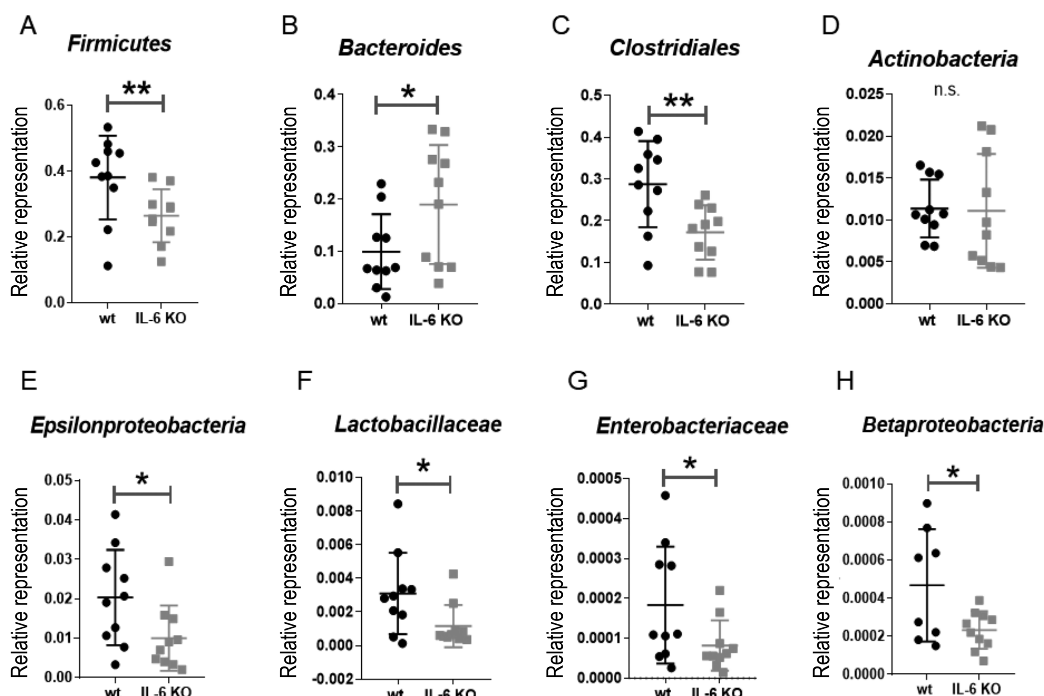


**Figure 1. IL-6 deficiency is associated with altered gut microbiota composition in naive mice compared to wild-type control mice**

Note. Relative representation of *Firmicutes* (A), *Bacteroides* (B), *Clostridiales* (C), *Actinobacteria* (D), *Epsilonproteobacteria* (E), *Lactobacillaceae* (F), *Enterobacteriaceae* (G) и *Betaproteobacteria* (H) in stool samples of naïve IL-6 KO mice and wild-type mice. *, $p < 0.05$; **, $p < 0.01$. Data are presented as mean and standard deviation.

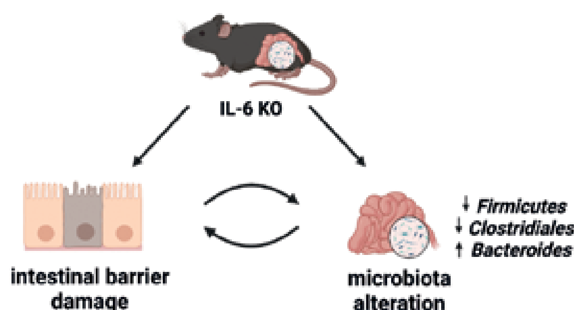


**Figure 2. Altered microbiota of IL-6 KO mice may impact predisposition to intestinal inflammation**

KO mice in relation to the *Epsilonproteobacteria*, *Lactobacillaceae*, *Betaproteobacteria*, and *Enterobacteriaceae* (Figure 1E, F, G, H). There were no differences in the relative content of Actinobacteria in mice of different genotypes (Figure 1D).

Inflammatory bowel disease is associated with a disturbance in the composition of the intestinal microbiota, which results both in a decrease in its overall diversity and in a change in the representation of individual taxa of microorganisms [4]. In particular, the gut microbiota of patients with chronic inflammation is enriched in the *Proteobacteria* and *Actinobacteria* kingdoms, while the relative content of the *Firmicutes* kingdom is, on the contrary, reduced [5]. Among the *Clostridiales*, both pathogenic and protective microorganisms are found; at the same time, the decrease in *Lactobacillaceae* in most studies is associated with the development of inflammation [2]. Despite an increase in protective *Bacteroides* [3], a significant decrease in *Firmicutes* in IL-6 deficient mice, as well as a decrease in *Lactobacillaceae* and other large taxa, suggests that the microbiota composition of IL-6 KO mice is somewhat similar to the microbiota composition characteristic of chronic intestinal inflammation.

## Conclusion

Our results indicate that IL-6 is involved in the control of the intestinal microbiota, while IL-6 deficiency is associated with changes in its composition. How exactly this shift in the composition of the gut microbiota affects the functionality of the immune system remains unclear and needs to be further addressed. At the same time, our data show that the microbiota of IL-6 KO differs from that of wild-type mice, supporting our hypothesis that mice predisposed to gut inflammation may have an altered microbiota (Figure 2). We plan to identify specific representatives of the microbiota, the manipulation of which may have a therapeutic effect in the context of intestinal inflammation and colorectal cancer. Determination of taxa, the change in the representation of which can serve as a marker of future inflammation, is of particular interest and requires further research.

## Acknowledgments

The authors are especially grateful to Dr. Andrey Kruglov for a critical discussion of the data, Ekaterina Gorshkova for help at all stages of the work and Ekaterina Bulekova for help with text editing.

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*Поступила 14.04.2023*
*Отправлена на доработку 20.04.2023*
*Принята к печати 24.04.2023*

*Received 14.04.2023*
*Revision received 20.04.2023*
*Accepted 24.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 601-604

**Краткие сообщения**
**Short communications**

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 601-604

# ЭКСПЕРИМЕНТАЛЬНОЕ ИЗУЧЕНИЕ ИММУНОТРОПНЫХ СВОЙСТВ МЕТАБОЛИТОВ ШТАММА *BACILLUS SUBTILIS B-9909*, ПЕРСПЕКТИВНОГО В КОНСТРИРОВАНИИ НОВОГО ГЕПАТОПРОТЕКТОРА

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**Резюме.** Настоящее краткое сообщение посвящено вопросам экспериментального изучения иммуннотропной активности нового соединения – метабиотика, на основе метаболитов (биологически активных веществ, БАВ), продуцируемых сапрофитным и безопасным стандартизированным штаммом ВКПМ *Bacillus subtilis B-9909*. Цель исследования – экспериментальная оценка иммунотропного действия метаболитов, продуцируемых пробиотическими микроорганизмами рода Bacillus культуры пробиотических микроорганизмов ВКПМ *Bacillus subtilis* В-9909 на лабораторных животных при моделировании у них токсического поражения. Метаболиты выделяли из культуральной жидкости бактериальной культуры *Bacillus subtilis*, штамм ВКПМ В-9909, при его глубинном культивировании в среде, состоящей из соляно-кислотного гидролизата соевой муки или панкреатического гидролизата казеина. Культура в это время находилась в конце экспоненциальной фазы роста (16-18 часов культивирования). Исследование показателей гуморального статуса у экспериментальных групп животных при оценке терапевтической эффективности экспериментального образца метабиотика, по отношению к группе лабораторных животных, получавших препарат сравнения урсосан, проводили путем определения таких количественных показателей сыворотки крови, как титры иммуноглобулинов M, G, A, E, титр α-интерферона и концентрации циркулирующих иммунных комплексов. Поражение печени изучали путем моделирования острого токсического гепатита у белых крыс. Экспериментальный токсический гепатит моделировали на лабораторных животных – белых крысах. Внутрижелудочно вводили 40%-ный раствор $CCl_4$ в вазелиновом масле в течение 2 недель из расчета 0,2 г.кг$^{-1}$. Полученные результаты экспериментальных исследований свидетельствуют, что возникновение иммунного воспалительного синдрома в значительной степени было в контрольной группе подопытных животных с воспроизведенным токсическим гепатитом. В группе, в которой лабораторным животным были назначены метаболиты (БАВ), патологический процесс был существенно менее выражен, чем в группе, получавшей препарат сравнения. Важно отметить тот факт, что по окончании срока наблюдения (30-е сутки), в группе лабораторных животных, получавших метаболиты отмечали нормализацию всех исследуемых показателей, в отличие от группы с урсосаном, в которой показатели воспалительного иммунного синдрома не были до конца восстановлены. Таким образом, проведенные исследования по изучению гуморального статуса лабораторных животных, получавших метаболиты,

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**Образец цитирования:**
*Н.А. Забокрицкий «Экспериментальное изучение иммунотропных свойств метаболитов штамма Bacillus subtilis B-9909, перспективного в конструировании нового гепатопротектора» // Медицинская иммунология, 2023. Т. 25, № 3. С. 601-604.*
*doi: 10.15789/1563-0625-ESO-2772*

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**For citation:**
*N.A. Zabokritskiy "Experimental study of immunotropic properties of metabolites of Bacillus subtilis B-9909 strain, promising in the formulation of a new hepatoprotector", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 601-604.*
*doi: 10.15789/1563-0625-ESO-2772*

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**DOI:** 10.15789/1563-0625-ESO-2772

продуцируемых пробиотическими микроорганизмами рода *Bacillus subtilis* B-9909 на лабораторных животных при моделировании у них токсического поражения, дают основания выполнить заключение о наличии у испытуемого образца метабиотика существенного иммуномодулирующего эффекта, в сравнении с урсосаном. Данное обстоятельство позволяет рекомендовать данное соединение, как перспективное для создания нового лекарственного кандидата гепатопротектора с иммунотропным эффектом.

*Ключевые слова: метабиотик, Bacillus subtilis, метаболиты, пробиотик, иммунотропная активность, гуморальный иммунитет, гепатотоксичность, гепатопротектор*

# EXPERIMENTAL STUDY OF IMMUNOTROPIC PROPERTIES OF METABOLITES OF *BACILLUS SUBTILIS B-9909* STRAIN, PROMISING IN THE FORMULATION OF A NEW HEPATOPROTECTOR

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**Abstract.** This brief report is devoted to the experimental study of the immunotropic activity of a new compound, a metabiotic, based on metabolites (biologically active substances, BAS) produced by the saprophytic and safe standardized strain of *Bacillus subtilis B-9909*. The aim of the study was to experimentally evaluate the immunotropic effect of metabolites produced by probiotic microorganisms of the genus Bacillus of the culture of probiotic microorganisms of the *Bacillus subtilis B-9909* on laboratory animals when modeling their toxic lesion. Metabolites were isolated from the culture fluid of the bacterial culture *Bacillus subtilis*, strain RNCIM (Russian National Collection of Industrial Microorganisms) *B-9909*, during its deep cultivation in a medium consisting of hydrochloric acid hydrolysate of soy flour or pancreatic hydrolysate of casein. The study of humoral status indicators in experimental groups of animals in assessing the therapeutic efficacy of an experimental metabiotic sample, in relation to the group receiving the ursosan comparison drug, was carried out by determining such quantitative serum parameters as the titers of immunoglobulins M, G, A, E, the IFN$\alpha$ titer and the concentration of circulating immune complexes. Liver damage was studied by modeling acute toxic hepatitis in white rats. Thus, the studies conducted to study the humoral status of laboratory animals receiving metabolites produced by probiotic microorganisms of the genus Bacillus of the culture of probiotic microorganisms of the *Bacillus subtilis B-9909* on laboratory animals when modeling toxic damage in them, give grounds to conclude that the test sample of BAS has a significant immunomodulatory effect, in comparison with ursosan. This circumstance allows us to recommend this compound as promising for the creation of a new hepatoprotector drug candidate with an immunotropic effect.

*Keywords: metabiotic, Bacillus subtilis, metabolites, probiotic, immunotropic activity, humoral immunity, hepatotoxicity, hepatoprotector*

## Introduction

Modern domestic and foreign scientists agree that it is very advisable to create new medical immunological preparations. It is especially important that such drug candidates implement specific pharmacological mechanisms in a variety of biological models. All this will allow in the future to extrapolate the obtained results to practical public health.

Today it is necessary to take into account that the human body is exposed to adverse factors, such as: ecology, climatic and geographical features, social, professional. In combination with a bad epidemiological situation, they cause various pathological disorders of both individual tissues and organs, and entire systems of the human body. All this affects the morbidity and mortality of the population of the country.

In connection with the foregoing, the creation and use of preparations of microbiological origin is especially promising. This pharmacological group is probiotics in various pharmacological dosage forms.

This confirms the fact that many probiotics, such as bactisubtil, biosporin, sporobacterin, etc. already implemented in practical healthcare. The proportion of the use of probiotics based on non-pathogenic bacteria of the genus Bacillus and their metabolites is increasing. These metabolites are considered biologically active substances (BAS). And drugs based on metabolites are called metabiotics [1, 3, 5, 7].

Thus, metabiotics derived from biologically active substances of bacteria of the genus Bacillus are of considerable scientific interest [3, 8].

**The aim of the study** was to experimentally evaluate the immunotropic effect of metabolites produced by

probiotic microorganisms of the genus Bacillus of a culture of probiotic microorganisms RNCIM *Bacillus subtilis B-9909* on laboratory animals when modeling their toxic damage.

## Materials and methods

Metabolites of microorganisms of the RNCIM strain *Bacillus subtilis B-9909* were used in the work.

The complex of biologically active substances was obtained under laboratory conditions according to the recommendations currently available in the scientific literature [2, 5, 6, 8].

Metabolites were isolated from the cultural liquid of the bacterial culture of *Bacillus subtilis*, strain RNCIM *B-9909*, during its deep cultivation in a medium consisting of hydrochloric acid hydrolysate of soy flour or pancreatic hydrolysate of casein. The culture at that time was at the end of the exponential growth phase (16-18 hours of cultivation) [2, 5].

Cultivation was carried out in 250.0 mL flasks on a temperature-controlled plant for growing microorganisms UVMT-12-250. To obtain the culture liquid in large volumes, a BIOR-0.1 fermenter was used.

Subsequently, the culture liquid was subjected to the following technological operations:

– centrifugation (8000 rpm-1 for 15 minutes) or, with large volumes of culture liquid, separation (to separate the cell mass) using an ASG-3MB separator;

– ultrasonic disintegration (to destroy the remaining bacterial cells of *Bacillus subtilis*) for which an ultrasonic disperser UZD2-0.1/22 was used [2, 4, 8];

– sterilizing ultrafiltration using membrane filters "Millipor" with a pore diameter of 0.22 μm and "Sartorius" with a pore diameter of 0.3 μm;

– freeze-drying (to a residual moisture level of 3-5%) in a laboratory freeze-drying unit LSS-2. The yield of freeze-dried BAS complex, freed from cell biomass (from 1 liter of centrate liquid), was 10-15 g.

The qualitative and quantitative content of metabolites was determined by high performance liquid chromatography. Separation was performed at room temperature using a SupelcosilTM LC-18 column (250 × 4.6 mm, particle size 5 μm).

The study of indicators of the humoral status in experimental groups of animals in assessing the therapeutic efficacy of the experimental sample, in relation to the comparison drug ursosan, was carried out by determining such quantitative indicators of blood serum as the titers of immunoglobulins M, G, A, E, the titer of α-interferon and the concentration of circulating immune complexes.

Liver damage was studied by modeling acute toxic hepatitis in albino rats.

Experimental toxic hepatitis was modeled on laboratory animals, white rats. A 40% solution of $CCl_4$ in vaseline oil was injected intragastrically for 2 weeks at the rate of $0.2\ g.kg^{-1}$.

The humoral status in the experimental groups of animals in assessing the therapeutic efficacy of the test preparations was determined by studying such quantitative indicators of blood serum as:

– titer of immunoglobulins M, $mg{\cdot}cm^{-3}$;

– titer of immunoglobulins G, $mg{\cdot}cm^{-3}$;

– titer of immunoglobulins A, $mg{\cdot}cm^{-3}$;

– titer of immunoglobulins E, $mg{\cdot}cm^{-3}$;

– titer of α-interferon (IFNα), $pg{\cdot}cm^{-3}$;

– concentration of circulating immune complexes (CIC), opt. units

Quantitative determination of the concentration of CEC was carried out using the precipitation method with a 3.5% solution of polyethylene glycol. Quantitative determination of immunoglobulins (IgM, IgG, IgA, IgE) and α-interferon in blood serum was performed using enzyme immunoassay. The sampling of material for research (blood serum) was carried out on the 1st and 7th days after the start of treatment with experimental samples of the tested drugs.

The results were statistically analyzed using Microsoft Office Excel 2010 and Statistica 12.0 software packages. In this case, the method of dispersion analysis (ANOVA) was used. The normality of the distribution of the obtained data was assessed using the Kolmogorov–Smirnov method. Statistical assessment of the significance of intergroup differences was performed using Fisher's parametric test, depending on the normality of the data distribution. Statistical hypotheses were evaluated at the critical significance level $p < 0.05$.

## Results and discussion

The experimental data obtained indicate that in the group of experimental animals with a reproduced model of acute toxic hepatitis, compared with the control group of animals, on the third day of the experiment, there is a significant increase in the values of humoral immunity indicators that are directly involved in the immediate hypersensitivity reaction. Thus, the IgM titer increased by 3.5 times, the IgE titer – by 2 times, and, accordingly, the CIC concentration – by 3.2 times. This immunosuppressive effect is caused by toxicity of carbon tetrachloride on immunity, its effect on sIgA and IgG, as well as a decrease in the titer of IFNα – 2.5 times in the experimental group with a reproduced model of acute toxic hepatitis, compared with the control.

Further observations show us that on the third day in the groups of animals that, against the background of the development of acute toxic hepatitis, intragastrically administered metabolites (BAS) and the reference drug ursosan saw the development of an immunoinflammatory syndrome. It is important to note that only small changes in humoral immunity parameters were observed in the group of animals treated with metabolites in relation to animals that were prescribed ursosan.

The study of the immunoinflammatory syndrome on the eighth day of the experiment showed a decrease in its severity in all groups of experimental animals.

It was found that the concentration of IgM and IgE remained quite high (1.8 times increase). At the same time, an increase in the concentration of IgA and IgG was also noted. In addition, in all groups of experimental animals with toxic hepatitis, the CIC titer remained without correlation changes. It is important to note that we were able to establish an increase in the concentration of α-interferon in the group of experimental animals treated with BAS (41.6±2.8 pg·cm$^{-3}$), a 2-fold increase compared to the control group (20.4±1.7 pg·cm$^{-3}$). At the same time, in the group of experimental animals treated with the reference drug ursosan, an increase in the concentration of IFNα correlated with an increase in its concentration in the group without treatment, respectively, 18.8±1.5 pg·cm$^{-3}$ and 16.6±1.5 pg·cm$^{-3}$. The increase in CIC titer persisted in all groups of animals with acute toxic hepatitis.

Further observations on the fourteenth day of the experiment showed a return to the normal studied parameters of humoral immunity. In all experimental groups, the concentration of IgM remained 2-2.5 times higher than in the control group of animals. Other studied indicators were also increased by 1.6 – 1.8 times. A higher concentration of IFNα was registered in the group with BAS – 34.2±1.8 pg·cm$^{-3}$, compared with the ursosan group – 19.1±1.5 pg·cm$^{-3}$ and in the group without treatment – 18,7±1.5 pg·cm$^{-3}$.

The thirtieth day of observation was the final one. It was found that in the group with BAS there was a restoration of all indicators of humoral immunity to the initial values. In the ursosan group, the IgM titer and CIC concentration remained elevated. All other indicators of humoral immunity in this group corresponded to the upper limit of normal.

The obtained results of experimental studies prove that the occurrence of the immune inflammatory syndrome was largely in the control group of experimental animals with reproduced toxic hepatitis. In the group in which metabolites (BAS) were prescribed to laboratory animals, the pathological process was significantly less pronounced than in the group receiving the reference drug. It is important to note that at the end of the observation period (day 30), in the group with BAS, normalization of all studied parameters was noted, in contrast to the group with ursosan, in which the indicators of the inflammatory immune syndrome were not fully restored.

## Conclusion

Thus, the conducted studies on the study of the humoral status of laboratory animals treated with metabolites produced by probiotic microorganisms of the genus Bacillus of the culture of probiotic microorganisms RNCIM *Bacillus subtilis B-9909* on laboratory animals when modeling their toxic damage, give grounds to conclude that the test sample has significant BAS immunomodulatory effect, in comparison with ursosan.

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*Поступила 14.04.2023*
*Отправлена на доработку 25.04.2023*
*Принята к печати 26.04.2023*

*Received 14.04.2023*
*Revision received 25.04.2023*
*Accepted 26.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 605-610



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 605-610

# ЭКСПРЕССИЯ ГЕНОВ *PD-L1* И *PD-L2* В КЛЕТКАХ ГЛИОБЛАСТОМ ЧЕЛОВЕКА, РЕЗИСТЕНТНЫХ К ХИМИО- И ЛУЧЕВОЙ ТЕРАПИИ

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**Резюме.** Мембранные молекулы PD-L1 и PD-L2, лиганды рецептора PD1 Т-лимфоцитов, выполняют иммунорегуляторные функции. Их связывание с рецептором приводит к ингибированию пролиферации, снижению продукции цитокинов, цитотоксической реакции и апоптозу Т-лимфоцитов. Клетки многих опухолей, вне зависимости от гистогенеза, экспрессируют молекулы PD-L1, тем самым ограничивая развитие противоопухолевой иммунной реакции. Глиобластомы – высоко злокачественные рецидивирующие опухоли центральной нервной системы. Основным источником рецидивов глиобластом служат резистентные опухолевые клетки, имеющиеся исходно в гетерогенных по клеточному составу глиомах, а также формирующиеся в процессе терапии. Увеличение дозы цитостатиков или облучения при терапии рецидивов оказывается не эффективным. В отношении ряда опухолей, в том числе рака яичников и немелкоклеточного рака легкого, показано, что препараты, предотвращающие взаимодействие PD-L1/PD1, оказываются эффективными при лечении новообразований, резистентных к химио- и радиотерапии. Иммунотерапию, в частности с использованием препаратов, ингибирующих связывание молекул PD-L с рецептором, рассматривают в качестве способа преодоления резистентности глиобластом к терапии. Цель работы состояла в оценке уровня экспрессии генов *PD-L1* и *PD-L2* в резистентных клетках глиобластом линий A172, R1, T2 и T98G, возобновивших пролиферацию после действия максимальных для каждой линии сублетальных доз цитостатиков (фотемустина и темозоломида), а также фракционированного или одноразового гамма-облучения. Линия A172 относится к числу глиобластом, высокочувствительных к использованным воздействиям, T98G – высокорезистентная линия; линии R1 и T2 занимают в этом ряду промежуточное положение. В интактных клетках глиобластом A172, R1 и T2 уровень экспрессии генов

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**Образец цитирования:**
*А.А. Пиневич, Н.Л. Вартанян, Л.Н. Киселева, И.И. Бодэ, И.Ю. Крутецкая, А.В. Карташев, В.Е. Макаров, Т.Е. Понежа, И.В. Смирнов, М.П. Самойлович «Экспрессия генов PD-L1 и PD-L2 в клетках глиобластом человека, резистентных к химио- и лучевой терапии» // Медицинская иммунология, 2023. Т. 25, № 3. С. 605-610. doi: 10.15789/1563-0625-PLA-2693*

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**For citation:**
*A.A. Pinevich, N.L. Vartanyan, L.N. Kiseleva, I.I. Bode, I.Yu. Krutetskaya, A.V. Kartashev, V.E. Makarov, T.E. Ponezha, I.V. Smirnov, M.P. Samoilovich “PD-L1 and PD-L2 gene expression in human glioblastoma cells resistant to chemo- and radiotherapy”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 605-610. doi: 10.15789/1563-0625-PLA-2693*

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**DOI:** 10.15789/1563-0625-PLA-2693

*PD-L1* и *PD-L2* был одинаково высоким, в клетках T98G он был достоверно меньшим. Воздействие цитостатиков или облучения на глиобластомы линий A172 и R1 существенно не изменяло экспрессии генов *PD-L1* и *PD-L2*, свойственной интактным клеткам. В клетках глиобластомы T2, и в особенности в клетках T98G, выявлено значительное увеличение уровня экспрессии этих генов, наиболее выраженное для гена *PD-L2*. Это усиление экспрессии может свидетельствовать об увеличенной злокачественности резистентных клеток T2 и T98G. Высокая экспрессия генов, отвечающих за продукцию PD-L1 и PD-L2, ограничивающих цитотоксическую реакцию организма против опухолевых клеток, является предпосылкой для использования лекарственных препаратов, мишенями которых являются эти белки, для элиминации резистентных к терапии клеток при глиобластомах.

*Ключевые слова: глиобластомы, PD-L1, PD-L2, резистентные клетки, фотемустин, темозоломид, Гамма-нож, фракционированное облучение, A172, T98G, T2, R1*

# *PD-L1* AND *PD-L2* GENE EXPRESSION IN HUMAN GLIOBLASTOMA CELLS RESISTANT TO CHEMO- AND RADIOTHERAPY

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**Abstract.** Membrane molecules PD-L1 and PD-L2, ligands of T lymphocytes PD1 receptor, perform immunoregulatory functions. Their binding to the receptor leads to inhibition of proliferation, reduction of cytokine production, cytotoxic response, and apoptosis of T lymphocytes. The cells of many tumors, regardless of their histogenesis, express PD-L1 molecules, thus limiting the development of an anti-tumor immune response. Glioblastomas are highly malignant recurrent tumors of the central nervous system. The main sources of glioblastoma recurrence are resistant tumor cells initially present in gliomas with heterogeneous cellular composition, as well as resistant cells that are formed during therapy. Increasing the dose of cytostatic drugs or radiation during relapse therapy is not effective in glioblastomas. It has been shown for a number of tumors, including ovarian cancer and non-small cell lung cancer, that drugs preventing PD-L1/PD1 interaction are effective in the treatment of neoplasms resistant to chemo- and radiotherapy. Immunotherapy using drugs that inhibit the binding of PD-L molecules to their receptor is considered as a way to overcome the resistance of glioblastomas to therapy. The aim of this work was to assess the level of *PD-L1* and *PD-L2* gene expression in resistant glioblastoma cells lines A172, R1, T2 and T98G, which resumed proliferation after exposure to the maximum for each line, sublethal doses of cytostatic drugs (fotemustine and temozolomide), fractionated or single gamma irradiation. A172 line belongs to glioblastomas that are highly sensitive to these influences, T98G is a highly resistant cell line, while R1 and T2 lines occupy an intermediate position. In intact glioblastoma A172, R1, and T2 cells the level of *PD-L1* and *PD-L2* gene expression was equally high, while in T98G cells it was significantly lower. Exposure of A172 and R1 glioblastoma lines to cytostatic drugs or irradiation did not significantly change the level of *PD-L1* and *PD-L2* genes expression typical for intact cells. In T2 glioblastoma cells, and especially in T98G cells, a significant increase in expression of these genes was found, most pronounced for *PD-L2* gene. This increase in expression may indicate an enhanced malignancy of resistant T2 and T98G cells. High expression of the genes responsible for the production of PD-L1 and PD-L2, which limit the cytotoxic response against tumor cells, is a prerequisite for the use of drugs targeted against PD-L1 and PD-L2 for the elimination of resistant cells in glioblastoma.

*Keywords: glioblastoma, PD-L1, PD-L2, resistant cells, fotemustine, temozolomide, Gamma Knife, fractionated irradiation, A172, T98G, T2, R1*

The work was performed according to Government Order "Study of resistant tumor cells on glioblastoma cultures in the simulation of stereotactic radiosurgery of recurrent glioblastoma" at the A. Granov Russian Research Center for Radiology and Surgical Technologies (St. Petersburg, Russia).

## Introduction

Membrane molecules PD-L1 and PD-L2, ligands of T lymphocytes PD1 receptor, perform immunoregulatory functions. Binding of these molecules to the receptor leads to proliferation inhibition, reduction of cytokine production, cytotoxic response, and apoptosis of T lymphocytes. Many tumor cells, regardless of histogenesis, express PD-L1 molecules, thus restricting the development of an anti-tumor immune response. In addition to PD1, PD-L1 molecules bind to costimulatory molecules CD28, CD80, and CTLA-4. PD-L2 expression in tumors is detected much less frequently than PD-L1 expression, and PD-L2 molecules are only able to bind to PD1. Preventing PD-L1 and PD-L2 from interacting with their receptors can abolish T cell unresponsiveness to tumor antigens.

Glioblastomas are highly malignant tumors of the central nervous system. Despite the ongoing aggressive treatment, including surgical removal of the tumor and chemoradiotherapy, glioblastomas recur, the prognosis remains unfavorable, and the life expectancy of patients is measured in months. The main source of glioblastoma recurrence are resistant tumor cells initially present in gliomas that are heterogeneous in cell composition, as well as tumor cells whose resistance is formed during therapy. Increasing the doses of radiation or cytostatic drugs can't help to overcome the resistance of such glioma cells. Alternative ways of elimination of tumor cells resistant to chemoradiotherapy are being developed. For a long time, it was believed that the central nervous system was an immunologically privileged system. However, the discovery of the antigen-presenting function of microglia, possible ways of immune cell penetration through the blood-brain barrier, and increased vessel permeability in tumors changed the primary understanding. The issue of glioblastoma immunotherapy possibilities came up for discussion [11].

Most glioblastomas, especially of the mesenchymal subtype, are known to express PD-L1 [2, 7, 10]. Little information is available about the presence of PD-L2 in glioblastomas. However, a high expression level of this biomarker has been shown to correlate with an unfavorable prognosis [9]. High expression of PD-L1 and PD-L2 on tumor cells is a prerequisite for the use of drugs directed against PD1 ligands [1]. There are single indications that temozolomide-resistant glioblastoma cells have a high level of PD-L1 expression [8]. At the same time, there are no data about the level of expression of these target molecules in resistant cells of different glioblastoma lines that resume proliferation after cytostatic treatment, fractionated or single dose gamma irradiation.

**The aim of the present work** was to assess the expression levels of *PD-L1* and *PD-L2* genes in human glioblastoma cells resistant to chemotherapy and radiation therapy.

## Materials and methods

Glioblastoma A172, R1, T2 and T98G cell lines with different properties, including various responses to irradiation and chemotherapy, were selected as research objects [3, 4]. Cells were cultured in plastic plates or ventilated vials at +37 °C, 6% $CO_2$, and 100% humidity, in α-MEM growth medium containing 5% fetal calf serum. Trypsin-EDTA solution was used for cell detachment.

Glioblastoma cells were treated with 0.1-5 mM temozolomide (Temodal pharmaceutical) for 24 h in growth medium, washed twice and cultured for 56 days under normal conditions. Every 3-4 days, half of the growth medium was replaced.

Glioblastoma cells were also treated with fotemustine (Mustophoran pharmaceutical) at drug concentrations of 0.5-750 μg/mL. Cells were incubated for 1 h in serum-free α-MEM medium containing fotemustine, washed three times, and cultured for two months more in standard growth medium.

Fractionated irradiation was performed on an Elekta Precise Treatment System™ Linear Accelerator (6 MeV, 460 cGy/min dose rate). Cells were irradiated with a single dose of 2 or 3 Gy/day or twice with 30 Gy, until a total dose of 36 or 51 Gy was reached. After irradiation, cells were cultured for two months.

Single stereotactic irradiation of glioblastomas with doses of 6-16 Gy was performed on a Leksell Gamma Knife® with 201-focused $^{60}Co$ radiation source at a dose rate of 3.236 Gy/min, 1.17 MeV energy, using a specially designed device for fixation and positioning of cell cultures during precision irradiation (patent for invention No. 2778859). The cells were then cultured for one month under normal conditions and their number was counted weekly.

Gene activity was studied by real-time polymerase chain reaction as described previously [4]. *PD-L1* gene was amplified using direct primers: TGGCATTTGCTGAACGCATTT and reverse primers: TGCAGCCAGGTCGTAATTGTTTT. The nucleotide sequences used for *PD-L2* amplification were ACCGTGAAAGAGCCCCACTTTG (forward) and GCGACCCCATAGATGATTATGC (reverse). The level of gene expression in intact cells was represented as the difference (ΔCt) between the threshold cycle of the studied gene and the compa-

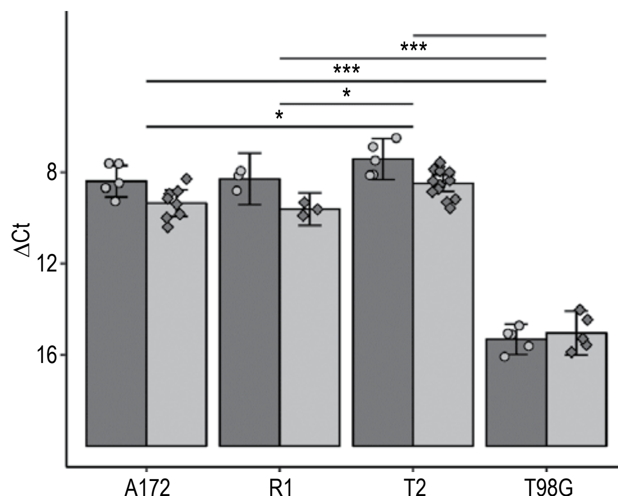


**Figure 1. *PD-L1* and *PD-L2* gene expression in intact glioblastoma cells**

Note. Horizontally – glioblastoma lines. Vertically – ΔCt, difference between threshold cycle of the studied gene and *GAPDH* housekeeping gene. Dark columns represent *PD-L1*, light columns represent *PD-L2*. Dots indicate individual values; bars denote the 95% confidence intervals. *, differences are significant at p < 0.05; ***, differences are significant at p < 0.001.

rison gene *GAPDH*. ΔΔCt value, the difference in the gene expression level in resistant and intact cells, served as an indicator of the difference in gene expression in the cells before and after the exposures. R language (version 4.1.2) was used for data processing and visualization. Statistical analysis was performed by creating linear regression models.

## Results and discussion

*PD-L1* and *PD-L2* gene expression was detected in all four tumor lines, which confirms the previous data about the activity of these genes in glioblastomas [9, 10]. In A172, R1 and T2 glioblastomas the level of *PD-L1* and *PD-L2* gene expression was equally high. In T98G, despite being considered the most aggressive and resistant to chemotherapy or radiation cell line, the level of *PD-L1* and *PD-L2* gene expression was significantly lower than in the other three lines (Figure 1) [5].

To date, there is no consensus which cells are considered resistant, and that causes certain difficulties when comparing data from different publications, especially in cases when the method of obtaining resistant cells is not specified [6].

In this work, the resistant A172, R1, T2, and T98G glioblastoma cells obtained in our laboratory earlier were examined. These cells were descendants of single cells that recovered their proliferative capacity after exposure to the highest sublethal doses of chemotherapeutic drugs or irradiation. The doses of exposures used to produce resistant cells (Table 1)

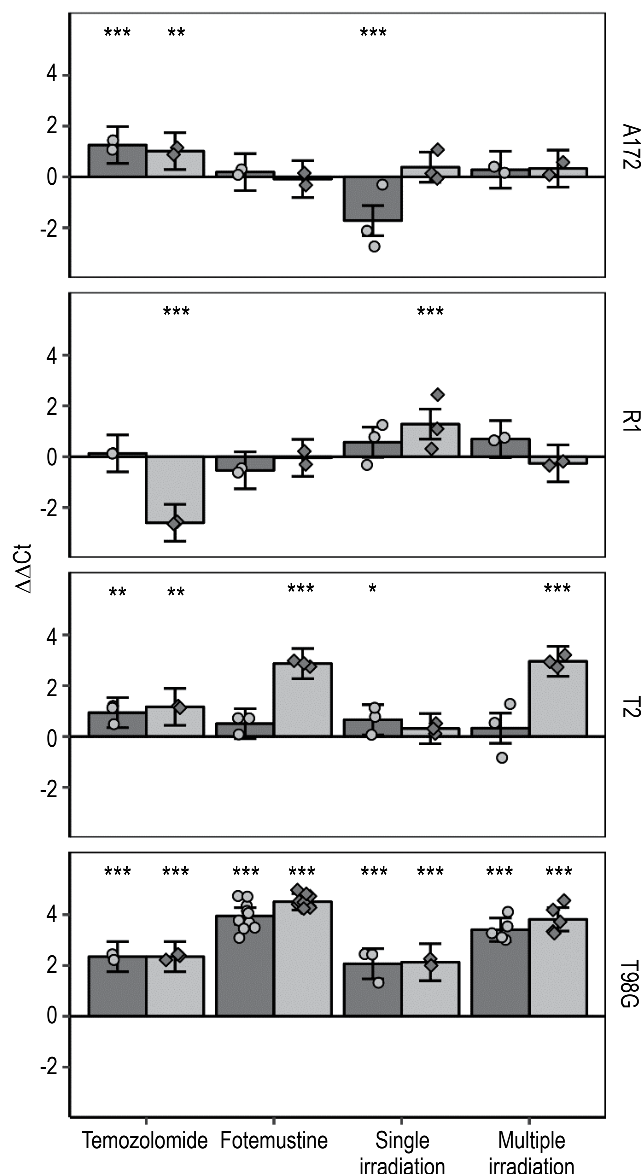


**Figure 2. Changes in *PD-L1* and *PD-L2* gene expression in A172, R1, T2, and T98G glioblastoma cells resistant to chemotherapy and irradiation.**

Note. Horizontally – effect on the cells, vertically – ΔΔCt, the difference in the gene expression level in resistant and intact cells. Horizontal lines correspond to the level of gene expression in intact cells. Dots indicate individual values; bars denote the 95% confidence intervals. *, differences are significant at p < 0.05; **, differences are significant at p < 0.01; ***, differences are significant at p < 0.001.

varied significantly, and were determined by the individual sensitivity of each glioblastoma cell line.

In resistant populations of A172 and R1 glioblastoma lines, which are among the more sensitive to the action of cytostatic drugs and irradiation, changes in *PD-L1* and *PD-L2* gene activity proved to be minimal. Resistant A172 cells that underwent single Gamma Knife irradiation had decreased *PD-L1* gene activity compared to intact cells, and proliferating cells after temozolomide action had slightly increased

**TABLE 1. DOSES OF EXPOSURE TO CHEMOTHERAPY OR RADIATION AT WHICH RESISTANT CELL POPULATIONS WERE ISOLATED**

| Exposure | Cell lines | | | |
|---|---|---|---|---|
| | A172 | R1 | T2 | T98G |
| **Temozolomide, mM** | 0,1 | 1 | 1 | 5 |
| **Fotemustine, μg/mL** | 100 | 100 | 300 | 300 |
| **Fractionated irradiation, Gy** | 50 (2)* | 36 (2) | 51 (3) | 60 (30) |
| **Single irradiation with Gamma Knife, Gy** | 9 | 11 | 12 | 14 |

Note. *, total dose of fractionated irradiation; single dose of irradiation in brackets.

activity of the same gene. Some changes in *PD-L2* gene expression were detected in resistant R1 cells. *PD-L2* gene activity decreased in R1 cells exposed to temozolomide and slightly increased after a single irradiation. In T2 and T98G lines, which are considered to be more resistant to chemo- and radiotherapy and differ from A172 and R1 lines in their ability to form "dormant" polyploid cells, the resistant proliferating cells showed increased expression of the studied genes [5]. In T2 glioblastoma the increase in activity affected predominantly *PD-L2* gene, while in T98G line there was a significant increase in the expression of both genes (Figure 2).

## Conclusion

The results indicate that glioblastoma cells resistant to alkylating antitumor drugs and irradiation actively express *PD-L1* and *PD-L2* genes. High levels of PD-L1 and PD-L2 molecules are unfavorable prognostic factors. At the same time, this indicator allows to consider immunotherapy with drugs that block PD-L1 and PD-L2 binding to their receptor as a possible means of elimination of therapy-resistant tumor cells [7, 8]. Several antitumor drugs with therapeutic effect based on inhibition of PD-L1 ligand binding to the receptors have been registered. These include Atezolizumab, Durvalumab, and Avelumab. All of these monoclonal antibody-based drugs block the interaction between PD-L1 and its receptors (PD1 and B7), but do not affect the interaction between PD1 and PD-L2.

For several other tumors, such as urothelial carcinoma, ovarian cancer, and non-small cell lung cancer, drugs that inhibit PD-L1/PD1 interaction have been shown to be effective in the treatment of neoplasms resistant to chemotherapy and radiotherapy. High expression of the genes responsible for the production of PD-L1 and PD-L2, which limit the cytotoxic response against tumor cells, is a prerequisite for the use of drugs targeted against PD-L1 and PD-L2 for the elimination of resistant cells in glioblastoma.

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*Поступила 04.04.2023*
*Принята к печати 06.04.2023*

*Received 04.04.2023*
*Accepted 06.04.2023*

# СЕКРЕЦИЯ IL-6 И IL-8 КЛЕТКАМИ ГЛИОБЛАСТОМ ЧЕЛОВЕКА, ПРОЛИФЕРИРУЮЩИМИ ПОСЛЕ ОБЛУЧЕНИЯ НА АППАРАТЕ ГАММА-НОЖ

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**Резюме.** Одним из современных методов лечения больных с первичными и рецидивирующими опухолями головного мозга является радиохирургическое облучение на аппарате Гамма-нож, позволяющее за 1-2 сеанса подвести терапевтическую дозу к опухоли, не превышающей 2,5 см в диаметре. Клетки опухоли на периферии этого объема получают меньшие дозы облучения, могут возобновлять пролиферацию и служить источником рецидивов. Увеличение дозы облучения чревато образованием некрозов и ухудшением прогноза. Свойства клеток глиобластом, выживающих и возобновляющих пролиферацию после облучения на установке Гамма-нож, до настоящего времени мало известны. Цель работы состояла в оценке экспрессии IL-6 и IL-8 клетками глиобластом линий A172, R1, T2 и T98G, которые возобновили пролиферацию после сублетального стереотаксического облучения. Клетки облучали однократно в дозах от 6 до 16 Гр, затем культивировали в течение 40 суток, подсчитывая еженедельно количество клеток и определяя таким образом летальную и сублетальную дозы для каждой линии глиобластом. В культурах, возникших в результате пролиферации единичных наиболее радиорезистентных клеток, методом ИФА определяли количество интерлейкинов (нг), секретированных за 96 часов в расчете на 1000 клеток. Клетки всех четырех линий глиобластом секретировали IL-6 и IL-8 в среду культивирования. Максимально высокой продукцией цитокинов, которая ранее не была известна для глиобластом, отличалась линия R1. Высокой продукцией обладала также глиобластома T2. Контраст этим линиям представляла глиобластома A172, наиболее чувствительная к действию цитостатиков и облучения, секреция IL-6 в которой была в 30 раз ниже, чем в клетках R1. Глиобластома T98G, известная своей высокой устойчивостью к действию химиопрепаратов и облучения, также обладала низкой продукцией интерлейкинов. Клетки глиобластом R1, T2 и T98G, возобновившие пролиферацию после облучения, обладали усиленной секрецией IL-6 и, в меньшей

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**Образец цитирования:**
*М.П. Самойлович, А.А. Пиневич, И.В. Смирнов, Н.Л. Вартанян, И.Ю. Крутецкая, Л.Н. Киселева, В.Е. Макаров, А.В. Карташев «Секреция IL-6 и IL-8 клетками глиобластом человека, пролиферирующими после облучения на аппарате Гамма-нож» // Медицинская иммунология, 2023. Т. 25, № 3. С. 611-616.
doi: 10.15789/1563-0625-IAI-2717*

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**For citation:**
*M.P. Samoilovich, A.A. Pinevich, I.V. Smirnov, N.L. Vartanyan, I.Yu. Krutetskaya, L.N. Kiseleva, V.E. Makarov, A.V. Kartashev "IL-6 and IL-8 secretion by human glioma cells proliferating after Gamma Knife irradiation", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 611-616.
doi: 10.15789/1563-0625-IAI-2717*

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**DOI:** 10.15789/1563-0625-IAI-2717

мере, IL-8. Зависимость увеличения продукции цитокинов от дозы облучения для этих клеток не носила линейного характера. Клетки A172 под действием облучения, наоборот, снизили секрецию IL-6 и IL-8. Разнонаправленные изменения в продукции IL-6 и IL-8 клетками разных линий глиобластом были долговременными и сохранялись более месяца. Представленные результаты ставят под сомнение возможность использования показателей продукции IL-6 и IL-8 клетками глиобластом в качестве потенциальных биомаркеров для ранней диагностики, мониторинга терапии, а также в качестве прогностических маркеров течения заболевания.

*Ключевые слова: глиобластомы, IL-6, IL-8, Гамма-нож, A172, R1, T2, T98G*

# IL-6 AND IL-8 SECRETION BY HUMAN GLIOMA CELLS PROLIFERATING AFTER GAMMA KNIFE IRRADIATION

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**Abstract.** One of the modern methods of treating patients with primary and recurrent brain tumors is radiosurgical irradiation using Gamma Knife, which allows therapeutic doses to be delivered to tumors not exceeding 2.5 cm in diameter in 1–2 sessions. Tumor cells on the periphery of this tissue volume that receive lower radiation doses can resume proliferation and serve as a source of recurrence. The increase of radiation dose may cause necroses formation and a worsening prognosis. The properties of glioblastoma cells that survive and resume proliferation long after stereotactic irradiation are still poorly known. The aim of the work was to evaluate the expression of IL-6 and IL-8 by glioblastoma A172, R1, T2, and T98G cell lines that resumed proliferation after sublethal Gamma Knife irradiation. Cells were irradiated once at doses ranging from 6 to 16 Gy, and then cultured for 40 days. Cell number was counted weekly; lethal and sublethal irradiation doses for each glioblastoma cell line were determined. In cultures descendant from proliferation of single most resistant cells, the level of IL-6 and IL-8 secretion after 96 hours cultivation (ng/1000 cells) was determined by ELISA. The cells of all four glioblastoma lines secreted IL-6 and IL-8 into culture medium. The highest production of cytokines, never before demonstrated for glioblastomas, was discovered in R1 cells. Glioblastoma T2 also had high interleukin production levels. In contrast to these lines, glioblastoma A172 (highly sensitive to the action of cytostatic drugs and radiation) secreted IL-6 at 30 times lower level than R1 cells. Glioblastoma T98G (highly resistant to the action of cytostatic drugs and radiation) also exhibited low interleukins production level. R1, T2, and T98G glioblastoma cells that resumed proliferation after irradiation had increased secretion of IL-6 and, to a lesser extent, IL-8. The dependence of cytokine production increase on irradiation dose for these cells was not linear. In contrast, A172 cells reduced IL-6 and IL-8 secretion under irradiation. The multidirectional changes in IL-6 and IL-8 production by cells of different glioblastoma lines were long-term and persisted for more than a month. The presented results cast doubt on the possibility to use IL-6 and IL-8 production by glioblastoma cells as potential biomarkers for early diagnosis, therapy monitoring as well as prognostic markers of the disease course.

*Keywords: glioblastoma, IL-6, IL-8, Leksell Gamma Knife, A172, R1, T2, T98G*

The work was performed according to Government Order “Study of resistant tumor cells on glioblastoma cultures in the simulation of stereotactic radiosurgery of recurrent glioblastoma” at the A. Granov Russian Research Center for Radiology and Surgical Technologies (St. Petersburg, Russia).

## Introduction

Primary tumors of the central nervous system account for about 2% of all human tumors. Among them, glioblastomas are the most malignant and have the worst prognosis. The formation of tumor cells resistant to therapy is the main reason for glioblastoma recurrence. One of the most modern methods of treatment of patients with primary and recurrent brain tumors is Gamma Knife radiosurgery which allows delivering a full therapeutic dose to the tumor center in 1-2 sessions. The distinctive feature of this radiotherapy method is focusing the photon beam on a small tissue volume not exceeding 2.5 cm in diameter. Tumor cells located on the periphery of this volume receive lower radiation doses. In consequence they

can resume proliferation and serve as an additional source of recurrence. At the same time, increasing the radiation dose can lead to the formation of necroses and a worsening prognosis. The properties of glioblastoma cell populations that survived and resumed proliferation after Gamma Knife irradiation are still poorly known. We are aware of only one work which contains information about the properties of these cells [3].

It was shown that glioblastoma cells that underwent Gamma Knife irradiation have a higher level of integrin beta-1 expression and consequently a higher migratory activity than intact cells. The increased ability of tumor cells to grow by continuation, as well as to form metastases, is precisely associated with the increase in their migratory activity.

Interleukins IL-6 and IL-8 play an important role in the process of carcinogenesis, including gliomagenesis [4]. Immunohistochemical studies have shown that glioblastoma cells can express these cytokines [4, 12]. High IL-8 expression was demonstrated in approximately 80% glioblastoma tissue samples. At the same time IL-8 has been shown to enhance glioma growth by binding to CXCR1 receptor on the cell surface in an autocrine manner [12]. IL-6 has been shown to be directly related not only to tumor growth, but also to manifestations of therapy resistance, in particular, multidrug resistance [5, 7, 14]. The possibility of using inhibitors of these cytokines (antibodies, anti-sense RNA) as drugs to overcome radio- and chemoresistance in tumors of different histogenesis is on the agenda [7, 10]. The aim of the work was to evaluate IL-6 and IL-8 expression by glioblastoma cells that resumed proliferation after sublethal Gamma Knife irradiation.

## Materials and methods

Glioblastoma A172, R1, T2, and T98G cell lines were cultured in 12.5 cm$^2$ plastic vials and 24-well plates at 37 °C, 6% $CO_2$, and 100% humidity, in α-MEM medium growth medium supplemented with 5% fetal calf serum and 0.5% gentamicin. Cells were cultured until reaching 70-90% confluency and reseeded using 0.25% trypsin-EDTA solution. Glioblastoma cells were irradiated once on a Leksell Gamma Knife® with 201-focused $^{60}$Co radiation source at a dose rate of 3.236 Gy/min, 1.17 MeV energy, using a specially designed device for fixation and positioning of cell cultures during precision irradiation (patent for invention No. 2778859). Two hours after irradiation, cells were disseminated into 96-well plates and cultured under normal conditions. During this period the number of live glioblastoma cells in tissue cultures was counted weekly; $LD_{100}$ and sublethal dose values for each cell line were determined.

IL-6 and IL-8 production was evaluated in cell cultures which were progeny of cells that survived and resumed proliferation after sublethal irradiation. Culture medium samples for analysis were collected 30-56 days after irradiation depending on irradiation dose, resumption time, and proliferation rate of surviving cells in different cell lines.

IL-6 and IL-8 secretion was assessed by determining cytokines concentration in the culture medium using Interleukin-6-IFA-BEST and Interleukin-8-IFA-BEST kits (Vector-Best, Russia) according to the manufacturer's recommendations. Cells were disseminated into 24-well plates and after 96 h cultivation culture medium was collected for analysis. Then the number of cells in each well was determined, and the average amount of cytokines secreted into culture medium in ng per thousand cells was calculated.

The data were processed and visualized using R language tools (version 4.1.2). Statistical analysis was performed by creating linear regression models.

## Results and discussion

Cells of all glioblastoma lines secreted IL-6 and IL-8 into the culture medium. R1 glioblastoma cells produced the maximum amount of cytokines – 22.3 ng of IL-6 and 9.0 ng of IL-8 per 1000 cells in 96 hours. The secretion of these cytokines by R1 glioblastoma cells has not been previously evaluated, and we are not aware of any studies related to glioblastoma cell lines with such high levels of IL-6 and IL-8 production. T2 glioblastoma has also been tested for the first time and showed high levels of both cytokines secretion. In contrast to these two lines, A172 glioblastoma secreted only 0.06 ng of IL-6 and 1.31 ng of IL-8 per 1000 cells. These data are consistent with the data from other researchers [15]. T98G glioblastoma cells produced average levels of IL-6 and IL-8 – 3.8 ng and 1.25 ng per 1000 cells correspondingly (Figure 1).

The balance of IL-6 and IL-8 production for intact R1, T2, and T98G lines was biased towards IL-6, while IL-8 secretion prevailed in low-secreting A172 line.

Sublethal irradiation of glioblastoma lines R1 and T2 resulted in increased production of IL-6 and IL-8 typical of intact cells. Glioblastoma A172 showed decreased secretion of IL-8 after 9 Gy irradiation ($LD_{100}$ for A172 was 11 Gy), while initially this cell line had the lowest production of both cytokines. Nonlinear dependence of cytokine secretion from radiation dose was observed in R1 and T98G glioblastomas. Thus, for R1 line maximum level of IL-6 and IL-8 was detected in cultures originated from cells irradiated at a dose of 8 Gy. At sublethal dose irradiation of 11 Gy ($LD_{100}$ for R1 cells was 12 Gy) cytokine secretion was lower than after irradiation dose of 8 Gy and did not differ from its level in intact cells. A similar trend was revealed for T98G cells; the peak of IL-6 secretion detected after irradiation dose of 11 Gy. After 14 Gy irradiation, the level of IL-6 secretion by T98G cells

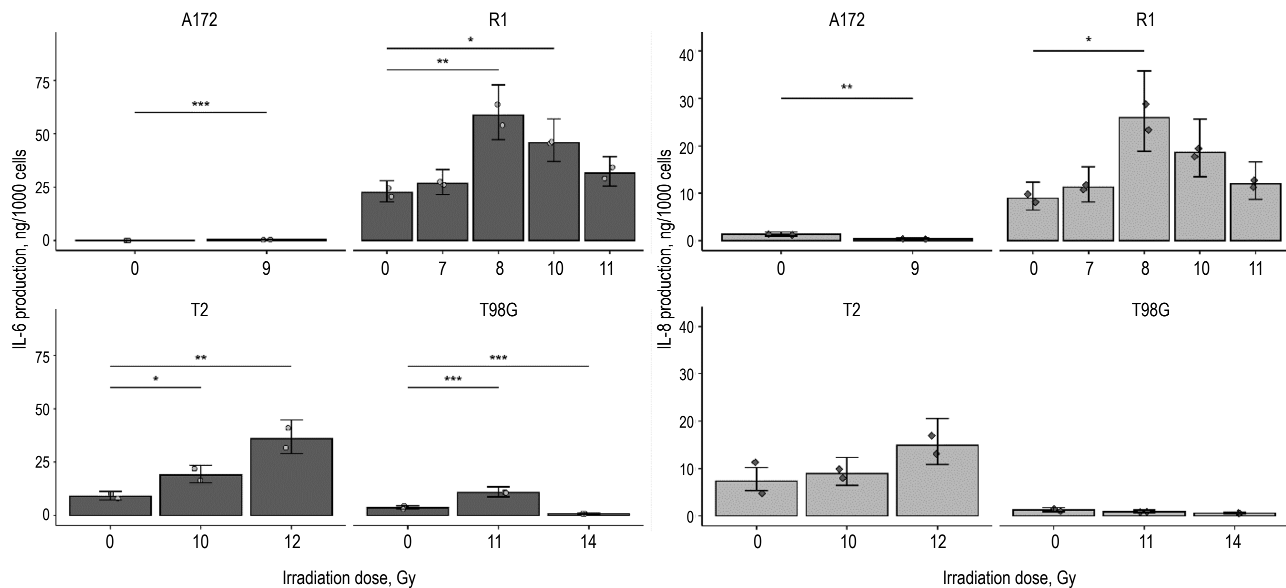


**Figure 1. IL-6 and IL-8 level secreted during 96 h cultivation by intact glioblastoma cells A172, R1, T2, T98G, and by cells that resumed proliferation after sublethal Gamma Knife irradiation**

Note. Horizontally – irradiation dose (Gy), vertically – amount of interleukins (ng), secreted by cells in 96 hours into the culture medium per 1000 cells. Dots indicate individual values; bars denote the 95% confidence intervals. *, differences are significant at $p < 0.05$; **, differences are significant at $p < 0.01$; ***, differences are significant at $p < 0.001$.

decreased and 16 Gy irradiation caused a complete stop of cell proliferation.

The secretion of IL-6 by T2 glioblastoma cell descendants that survived irradiation was many times higher than that by intact T2 cells. No decrease in cytokine production was observed when irradiation dose was increased up to 12 Gy. The ability of T2 cells to restore proliferation was abolished irradiation dose of 14 Gy.

It can be noted that three out of four studied glioblastoma cell lines, that resumed proliferation after sublethal Gamma Knife irradiation, demonstrated increased interleukin secretion although this effect was not equally dependent on irradiation dose.

The level of IL-6 and IL-8 expression is thought to correlate with the degree of gliomas malignancy [1, 11] but many questions still remain unresolved. In particular, our results suggest that two glioblastoma cell lines, A172 and T98G, have low IL-6 and IL-8 secretion. At the same time, according to the criteria of sensitivity to cytostatic drugs and irradiation, these lines are completely different. Glioblastoma A172 does not express MGMT, is highly sensitive to temozolomide, fotemustine, and radiation, while T98G line actively express *MGMT* gene, multiple drug resistance genes, and is highly resistant to chemo- and radiotherapy [8, 9]. The reasons for these discrepancies are not yet clear. It should be emphasized that in the course of present research, the relapse between glioblastoma irradiation and the assessment of IL-6 and IL-8 production took at least 30 days. Thus, we can conclude that increased cytokine secretion by cells was not due to a short-term effect of irradiation, but persisted for a long time.

## Conclusion

Currently, IL-6 and IL-8 are considered as potential biomarkers for early diagnosis and therapy monitoring, as well as prognostic markers of the disease course [2, 6, 13]. From this point of view, the results obtained in the present study on four glioblastoma cell lines exposed to Gamma Knife irradiation serve as a warning against making simple conclusions about the possibility of using IL-6 and IL-8 production as biomarkers for monitoring and prognosis of glioblastoma therapy.

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*Поступила 11.04.2023*
*Принята к печати 11.04.2023*

*Received 11.04.2023*
*Accepted 11.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 617-624

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 617-624

# ИММУНОМЕТАБОЛИЧЕСКИЕ ИЗМЕНЕНИЯ МАКРОФАГОВ В ОТВЕТ НА ЭКСТРАКТ ПЫЛЕВОГО КЛЕЩА

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**Резюме.** Молекулярные механизмы, лежащие в основе патогенеза астмы, одного из наиболее распространенных хронических заболеваний дыхательной системы, остаются не до конца изученными. Отсутствие специфичной и высокоэффективной терапии для некоторых подтипов этого гетерогенного заболевания требует поиска новых подходов к лечению. Один из таких подходов может заключаться в воздействии на иммунометаболические функции миелоидных клеток. Этот подход уже нашел свое применение в терапии рака и других заболеваний, в патогенезе которых важную роль играют макрофаги. Ранее было показано, что патогенез аллергической астмы, возникающей в ответ на распространенный сложный аллерген клеща домашней пыли, обусловлен метаболическим TNF-опосредованным перепрограммированием альвеолярных макрофагов. Это дает основания предполагать, что влияние на процесс продукции TNF или метаболические адаптации с помощью специфичных блокаторов могут также привести к ослаблению симптомов течения заболевания в целом. В данной работе было экспериментально проверено, сохраняется ли ранее описанный фенотип, возникающий у макрофагов в ответ на экстракт клеща домашней пыли (house dust mite, HDM) при культивировании в стандартной среде DMEM, в более физиологичных условиях – в среде близкой по составу к плазме крови. Также нами были проанализированы открытые базы данных секвенирования РНК из альвеолярных макрофагов, полученных от пациентов с астмой или из легких мышей в экспериментальной модели HDM-индуцированной астмы, с целью поиска и сравнения происходящих метаболических изменений. Было показано, что при культивировании в условиях близких к физиологическим, как и на классической среде, макрофаги в ответ на активацию HDM одновременно увеличивают показатели дыхания и гликолиза, а также продуцируют TNF. Наблюдаемый фенотип согласуется с данными транскриптомного анализа, выполненного на образцах человека и мыши, в которых было выявлено увеличение экспрессии генов, относящихся к гликолизу, окислительному фосфорилированию и сигнальному пути TNF. Таким образом, данные подтверждают релевантность

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**Образец цитирования:**
*Т.Р. Юракова, Е.А. Горшкова, М.А. Носенко, Е.О. Губернаторова, М.С. Друцкая «Иммунометаболические изменения макрофагов в ответ на экстракт пылевого клеща» // Медицинская иммунология, 2023. Т. 25, № 3. С. 617-624. doi: 10.15789/1563-0625-ICI-2827*

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**For citation:**
*T.R. Yurakova, E.A. Gorshkova, M.A. Nosenko, E.O. Gubernatorova, M.S. Drutskaya "Immunometabolic changes in macrophages in response to house dust mite extract", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 617-624. doi: 10.15789/1563-0625-ICI-2827*

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**DOI:** 10.15789/1563-0625-ICI-2827

фенотипа, полученного *in vitro*, изменениям происходящим в системах *in vivo*. На следующем этапе потребуется также функциональная верификация на уровне продуцируемых метаболитов, белков и изменений метаболической активности. Кроме того, в дальнейшем предстоит установить, как блокировка отдельных метаболических путей влияет на особенности функционального фенотипа макрофагов, возникающего в ответ на HDM, и способно ли данное воздействие облегчать симптомы астмы.

*Ключевые слова: макрофаги, иммунометаболизм, HDM, аллергическая астма, TNF, поляризация макрофагов*

# IMMUNOMETABOLIC CHANGES IN MACROPHAGES IN RESPONSE TO HOUSE DUST MITE EXTRACT

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**Abstract.** To date, much remains unclear about the pathogenesis of asthma, one of the most common chronic and highly heterogenic diseases of the respiratory system. The lack of specific and highly effective therapy in case of certain asthma subtypes requires the search for new approaches to treatment. One possible approach would be to influence the metabolism and immune functions of myeloid cells. This approach finds its application in the treatment of cancer and other diseases in the pathogenesis of which macrophages play an important role. It was shown that the pathogenesis of allergic asthma in response to one of the most common allergens, house dust mite, is due to a metabolic TNF-mediated reprogramming of alveolar macrophages. This suggests that influencing the process of TNF production or metabolic adaptations with specific blockers may also lead to a reduction in the symptoms of the course of the disease as a whole. In this work, we experimentally tested whether the previously obtained phenotype that occurs in macrophages in response to HDM cultured in DMEM is preserved if cells are cultured under more physiologically relevant conditions: in a medium closely related in composition to blood plasma. We also analyzed open databases of alveolar macrophages sequencing obtained from patients with asthma or from the lungs of mice in an HDM-induced asthma model in order to correlate specific immunometabolic changes. It was found that macrophages cultured under conditions close to physiological, simultaneously increase the rates of respiration and glycolysis, and also produce TNF in response to HDM. The observed phenotype is consistent with transcriptomic analyzes performed on human and mouse samples, which revealed an increase in the expression of genes related to glycolysis, oxidative phosphorylation, and the TNF signaling pathway. Thus, the data confirm the relevance of the phenotype obtained *in vitro* to the changes occurring in the *in vivo* system. However, functional verification at the level of metabolites, proteins and changes in metabolic activity is also required. In addition, it remains to be established how the blocking of individual metabolic pathways affects the features of the functional macrophage phenotype that occurs in response to HDM, and whether this effect can alleviate asthma symptoms.

*Keywords: macrophages, immunometabolism, HDM, allergic asthma, TNF, macrophage polarization*

Our work is supported by the Russian Science Foundation grant 19-75-30032.

## Introduction

Bronchial asthma is one of the most common chronic respiratory diseases. The commonly used steroid-based therapy is not always effective in asthma due to the heterogeneity of the disease. In this regard, it remains relevant to study the mechanisms underlying the pathogenesis of asthma and to search for new therapeutic strategies. In recent studies the role of the innate immune system in the development and course of asthma was highlighted. Lung tissue-

resident macrophages are the first to encounter antigens that enter the respiratory tract, including common allergens such as household dust. House dust mite (HDM) causing allergic asthma in humans, and, in addition, it is used to model asthma in mice *in vivo*. It was demonstrated that the release of TNF, a key proinflammatory cytokine, by macrophages results in formation of innate immune memory and is crucial for HDM-induced asthma development in mice. Moreover, macrophage cellular metabolism affects TNF production in the context of HDM-induced asthma [4].

In our previous study we established that HDM induces distinct inflammatory cytokine profiles in bone marrow derived macrophages, dendritic cells and alveolar macrophages. In addition, we determined that these myeloid cell types acquire a specific metabolic phenotype characterized by a simultaneous increase in glycolysis and respiration in response to HDM activation *in vitro* associated with the alterations in the morphology of mitochondria [7]. In order to translate the relevance of our observations *in vitro* to future development of novel immunometabolism-based therapies of asthma in humans, we analyzed available transcriptomic data of human and mouse HDM-activated alveolar macrophages [1, 6]. Moreover, we confirmed HDM-induced macrophage metabolic profiling using cell culture medium that mimics human sera. In conclusion, **the purpose of this work** is to further elucidate the mechanisms underlying the metabolic reprogramming of macrophages in response to HDM.

## Materials and methods

**Mice**

C57Bl/6 mice (6-8 weeks) were housed in SPF conditions at the Animal Facility of the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, EIMB RAS (under contract № 075-15-2021-1067 with the Ministry of Science and Higher Education of the Russian Federation). All manipulations with animals were carried out in accordance with the protocol approved by the Bioethics Committee of the EIMB RAS (Protocol No. 3 from 27/10/22).

**Primary cultures of bone marrow-derived macrophages (BMDM)**

Bone marrow was isolated from tibias and femurs of C57Bl/6 mice. For BMDMs differentiation bone marrow cells were cultured on non-treated cell culture plates in Plasmax medium [2] containing 30% L929 conditioned medium as the source of M-CSF, 2% FBS (Capricorn Scientific), and antibiotics (Pen/Strep, ThermoFisher). Cells were harvested and passaged in fresh medium on day 5 and used for subsequent experiments on day 6.

**BMDM activation**

To analyze cytokine production, cells were seeded on a 96-well plate at a density of $10^5$ cells per well in 100 μL of Plasmax medium 1 hour prior to the activation. Cells were activated for 4 hrs with LPS 10 ng/mL (Lipopolysaccharides from *Escherichia coli* O111:B4, catalog number L2630-100MG, Sigma) or HDM 10 μg/mL (*Dermatophagoides pteronyssinus* extract FD, catalog number 15G10, Citeq biologics).

**Cytokine production**

Cytokine release in culture supernatants of stimulated BMDM was measured by enzyme-linked immunosorbent assay (ELISA) using ready-made commercial kits “Mouse TNFalpha ELISA Ready-SET-Go” (ThermoFisher) according to the manufacturer’s protocol.

**Analysis of cellular metabolism**

Cell metabolism experiments were carried out on Seahorse XFe24 Analyzer (Agilent) using an in-house validated protocol. The day prior to the experiment, cells were seeded in Seahorse 24-well plates at a density of $1{,}5 \times 10^5$ cells per well in 1 mL of complete Plasmax medium. Plates were placed overnight in an incubator 37 °C, 5% $CO_2$. Upon activation, cells were washed and incubated in Seahorse Assay Medium supplemented with 1 mM sodium pyruvate and 2 mM L-Glutamine at 37°C without $CO_2$ for 30-45 min. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured during sequential addition of 10 mM glucose; 1 μM oligomycin; 2 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP); and combination of 1 μM rotenone, 1 μM antimycin A and 50 mM 2-deoxyglucose (2-DG). SeaHorse data were analyzed in accordance with the recommended protocol sing the Wave software (Agilent).

**Statistical analysis**

All experiments were performed at least 2 times. Data were analyzed using GraphPad Prism 7 software. Data were first tested for Gaussian distribution with the D’Agostino & Pearson omnibus normality test and then analyzed using Mann–Whitney test, 2-way ANOVA tests, followed by Sidak’s or Tukey’s post-test analysis for multiple comparisons. Results are displayed as mean ± SEM. Differences were considered significant when p values were below 0.05.

**Differential gene expression analysis**

Transcriptomic analysis of publicly available datasets (human data: GSE144576 [6]; mouse data:

GSE148590 [1]) was conducted and visualized using Phantasus resource (https://artyomovlab.wustl.edu/phantasus/). Data were log-transformed and genes below threshold expression level (median < 2) were excluded. Differential expression analysis was performed using limma algorithm. Genes from specific metabolic pathways were identified using Gene Ontology Browser from The Jackson Laboratory (https://www.informatics.jax.org/vocab/gene_ontology). P-values, adjusted for multiple comparisons, below 0.05 were considered statistically significant.

## Results and discussion

**Bone marrow-derived alveolar macrophages demonstrate HDM-specific metabolic phenotype**

To examine whether HDM-induced immunometabolic phenotype observed under standard culture medium conditions (high glucose levels, lack of physiologic carbon sources) can be reproduced under more physiological conditions *in vitro*, BMDM were grown in Plasmax medium which is similar in composition to blood plasma. After six days in culture cells were activated with HDM or LPS followed by assessment of TNF production, as well as glycolysis and respiration rates (Figure 1A). BMDM grown in a physiological medium maintained a metabolic response to allergens similar to cells grown in classical medium. Activation of BMDM with LPS resulted in accelerated glycolysis (Figure 1E, F), while HDM promoted both glycolysis and oxidative phosphorylation (Figure 1C, D).

Interestingly, Plasmax-grown BMDM produced less TNF in response to HDM and LPS activation than DMEM-grown macrophages (Figure 1B). Despite the fact that the composition of the culture medium affected the TNF levels in activated macrophages, the overall immunometabolic phenotype in response to HDM was preserved. Therefore, we hypothesized that HDM-induced immunometabolic phenotype of alveolar macrophages observed *in vitro* can be translated *in vivo*.

**Transcriptomic profiles of alveolar macrophages show increase in expression of a subset of 'Glycolysis', 'Electron transport chain' and 'TNF signaling' pathway genes**

The study of specific expression profiles of cells derived from patients is a powerful tool for uncovering pathological mechanisms. To establish if metabolic pathways are altered in alveolar macrophages (AM) in response to HDM, we analyzed the expression of genes related to several metabolic pathways (glycolysis, Krebs cycle, electron transport chain (ETC), fatty acid oxidation (FAO) and synthesis (FAS), tryptophan metabolism, glutamine metabolism, and the pentose phosphate pathway) in open databases of Bulk RNA sequencing. Our analysis was based on two previously published studies. One study compared AMs, harvested via bronchoscopy from twelve patients with intermittent-to-mild asthma and HDM asthma and challenged with saline or HDM extract combined with LPS [6]. Another study compared AMs from C57Bl/6 mice during experimental allergic airway disease driven by repeated HDM inhalation and compared them to control AMs from na ve mice [1].

We found that in response to HDM in AMs, both from patients with allergic asthma and those obtained in mouse asthma model, the expression of glycolysis (Figure 2A, B) and ETC (Figure 2C, D) genes increased. In particular, there is an increase in expression of genes encoding 6-phosphofructokinase 1 (PFKM, Pfk1, Pfkp), enolase (ENO1, Eno2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (Pgk1) and hexokinase (Hk1). In addition, the expression of a number of genes encoding the synthesis of complex I (NDUFS8, NDUFA9, NDUFB11, NDUFS7, NDUFS5, NDUFA3, Ndufc1, Ndufa13, Ndufb6), complex III (UQCRC1, Uqcrc10, Uqcrq, CYC1) and cytochrome c (COX8A, COX7A2, COX6A1, Cox7b, Cox5b, Cox7a2) increased. These data are consistent with the previously observed functional increase in glycolysis and respiration in HDM-activated macrophages [7].

In addition, HDM stimulates an increase in the genes involved in fatty acid synthesis and the production of prostaglandins, which play an important role in the pathogenesis of asthma. Our search did not reveal a significant differential expression of genes associated with Krebs cycle, FAO, tryptophan metabolism, glutamine metabolism, or the pentose phosphate pathway (data not shown). TNF-induced metabolic reprogramming plays an important role in HDM dependent asthma [4]. According to evidence of TNF participation in asthma pathogenesis, analyzed data also shown an increase in genes involved in the TNF signaling pathway (Figure 2E, F). However, it was previously shown that changes in immunometabolic functions can procced independently of transcriptional programs, since the regulation of many metabolism-associated factors rely on posttranslational modifications or substrates availability [3]. Thus, the multi-omics approach will allow learning more about the relationship between

immunity and metabolism in patients or *in vivo* animal models.

Altogether, the HDM-induced phenotype of alveolar macrophages observed *in vitro* (Figure 1) and *in vivo* (Figure 2) may suggest a major reorganization of metabolism in response to activation. On the other hand, it remains unclear, how simultaneous upregulation of glycolysis and oxidative phosphorylation influence the severity of asthma. We can hypothesize that high levels of respiration may promote the production of mitochondrial ROS, thus ROS play an important role in serve asthma pathogenesis. The activity of OXPHOS is associated with the Krebs cycle, which uses not only pyruvate as substrates, but also fatty acid oxidation products and glutamine. In this regard, it is important to study which substrates are involved in the development of increased respiration in macrophages activated by HDM.

The search for specific metabolic pathways involved in the formation of metabolic adaptations and TNF production by macrophages in response to HDM and their subsequent blocking seems to be a promising and interesting direction for finding new approaches to asthma therapy. For example, TNF blocking was effective in the context of a mouse model of asthma [4]. A deeper understanding of the TNF-dependent metabolic rearrangement will allow to improve this approach and make therapy more specific. Given that metabolic changes can be associated not only with the transcriptional level, RNAseq data have limited predictive value for the analysis of functional changes. However, our analysis allowed us to confirm the existence of general trends at different levels: *in vitro*, *in vivo* mouse models and clinical specimens, and the significance of the observed macrophage immunometabolic phenotype for HDM-induced asthma (Figure 3).

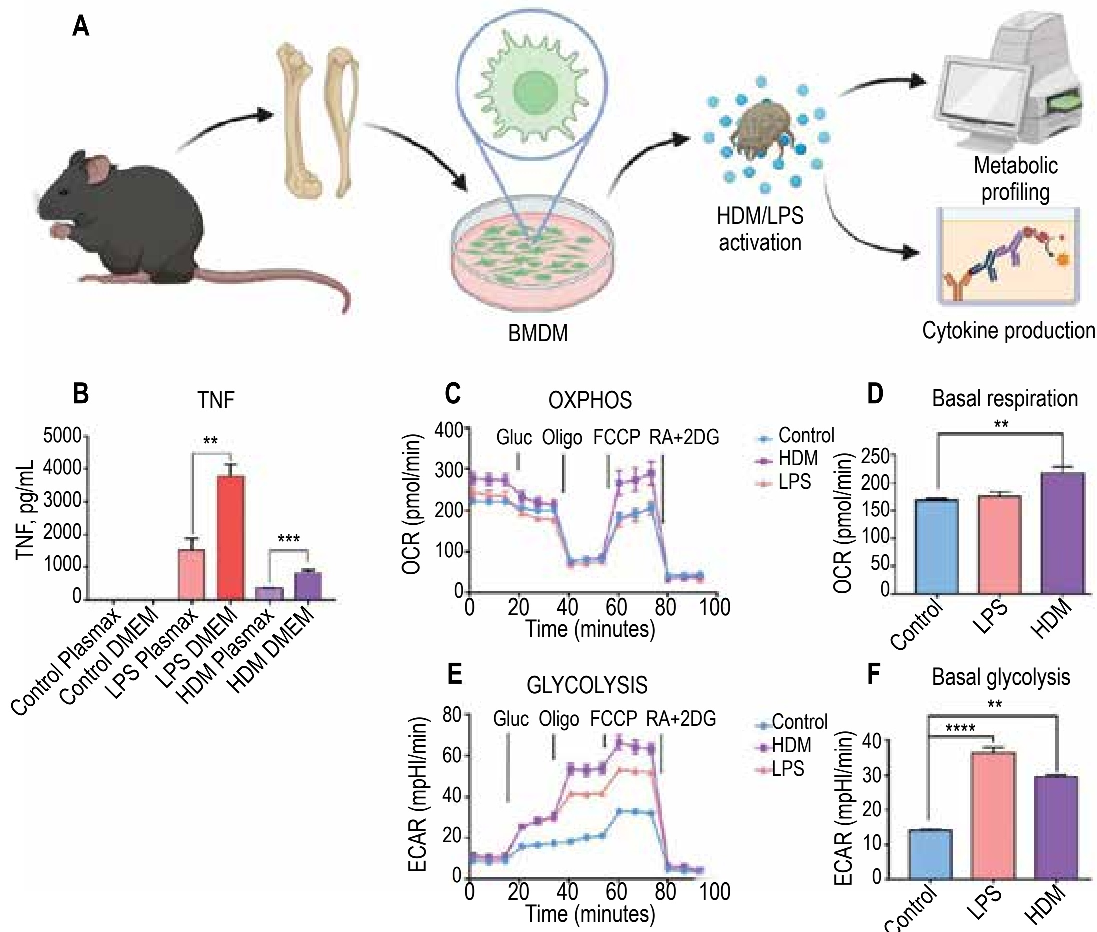


**Figure 1. Bone marrow-derived alveolar macrophages demonstrate HDM-specific metabolic phenotype in Plasmax**

Note. Bone marrow cells were differentiated in DMEM or Plasmax into macrophages with L929-conditioned medium; stimulated with HDM, LPS or unstimulated control for 24 h (A). Concentration of TNF in supernatants was measured by ELISA (B). OCR and ECAR of BMDM (C and E) were analyzed using Seahorse. Basal respiration was calculated based on OCR (D). Basal glycolysis was calculated based on ECR (F). Gluc, glucose; Oligo, oligomycin; FCCP, fluoro-carbonyl cyanide phenylhydrazone; RA + 2DG, combination of rotenone, antimycin A and 2-deoxy-glucose. Data are presented as mean ± SEM. TNF production data was analyzed using Mann–Whitney test. Seahorse data were analyzed using two-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

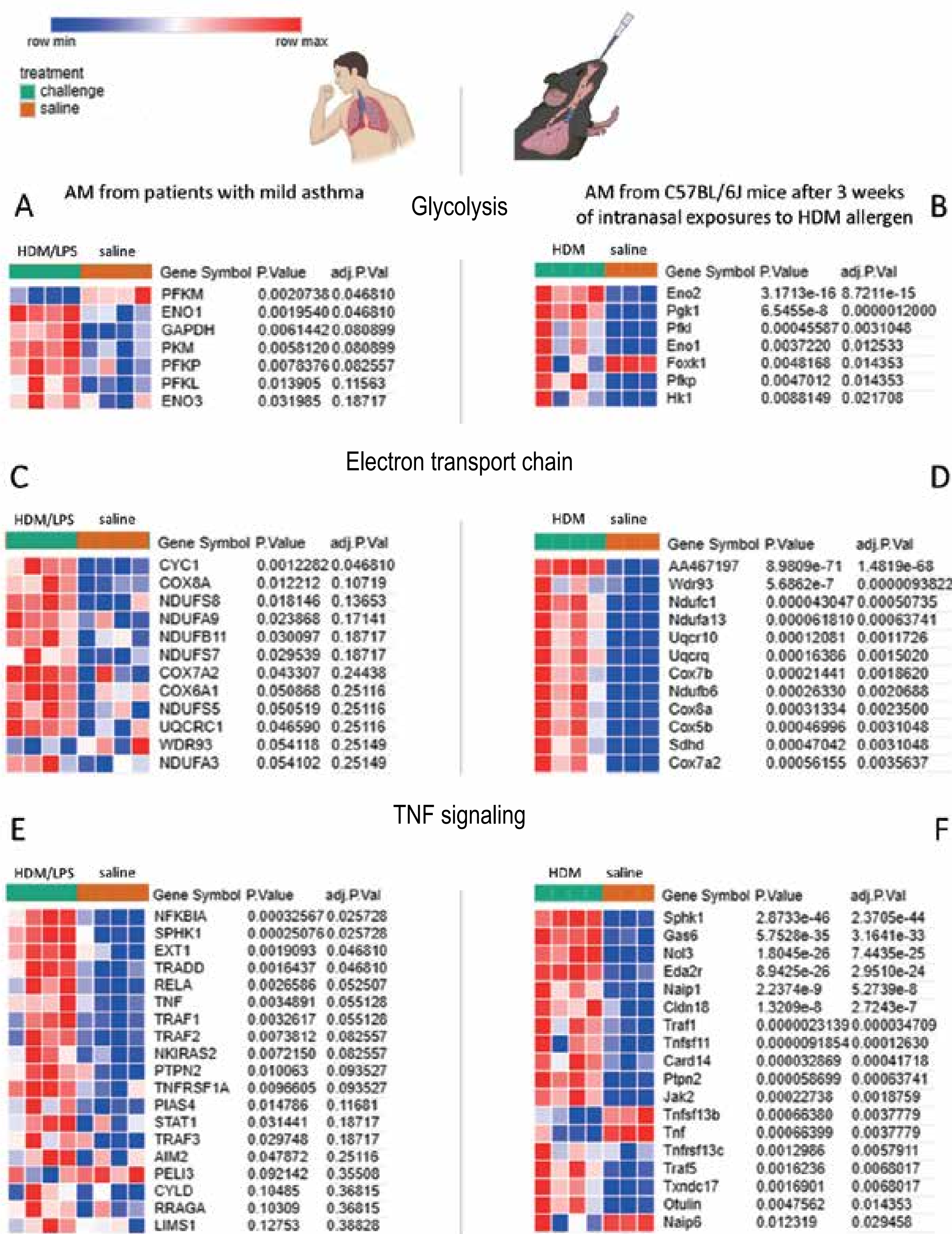


**Figure 2. HDM-induced increase in expression of genes, associated with glycolysis and cellular respiration in human and murine alveolar macrophages**

Note. Increased expression of representative genes from "Glycolysis" pathway in human AMs HDM/LPS compared to control AMs (A) and in mice AMs HDM compared to control AMs (B). Expression of representative genes for "Oxidative Phosphorylation and electron transport chain" subset in human AMs HDM/LPS compared to control AMs (C) and in mice AMs HDM compared to control AMs (D). Decreased expression of representative genes from "TNF signaling" pathway in human AMs HDM/LPS compared to control AMs (E) and in mice AMs HDM compared to control AMs (F). Heatmaps show row-centred gene expression values (n = 4 patients, PBS; n = 4 patients, HDM/LPS; n = 3 mice, PBS; n = 4 mice, HDM).

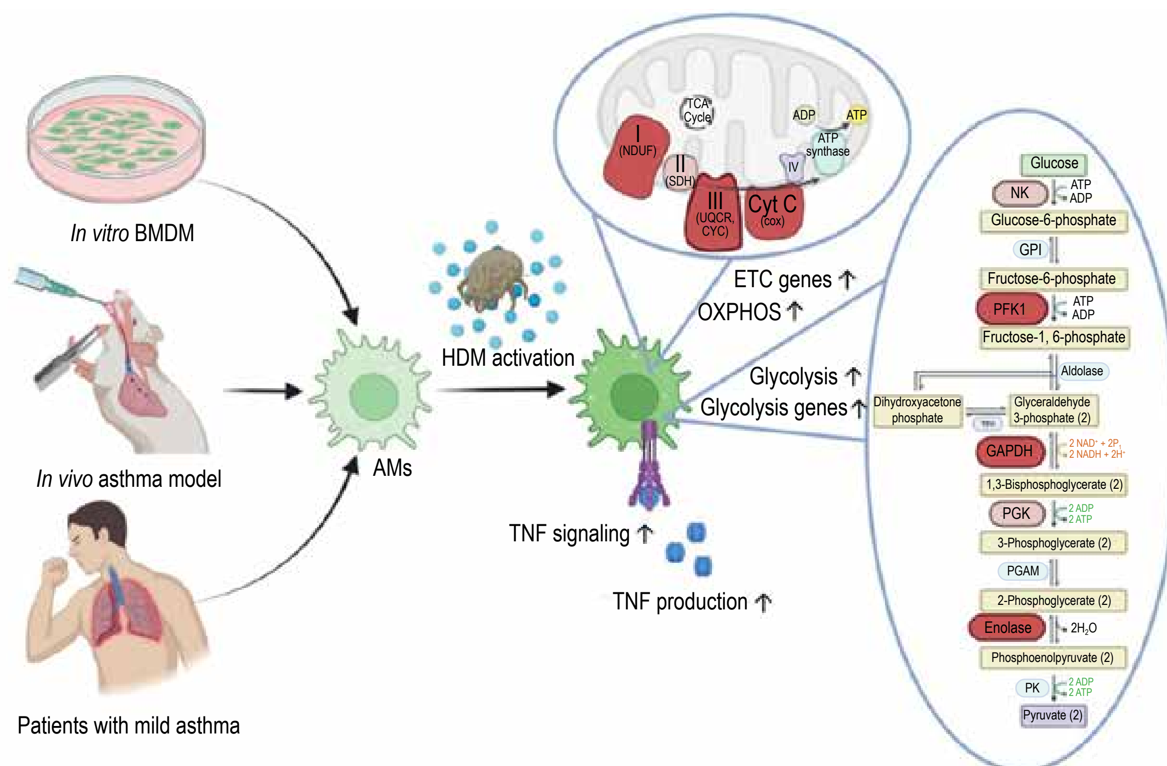


**Figure 3. Development of novel immunometabolism-based therapies of asthma in humans**

## Conclusion

The strategy for immunometabolism intervention is currently being studied predominantly in the context of cancer, but there is growing evidence suggesting that it may have wider applications. Although targeting metabolic pathways is a promising strategy for the treatment of inflammatory diseases, this approach has many limitations, including the low specificity of metabolic inhibitors. Many questions remain to be addressed, however, a comprehensive view on the metabolic pathway system, an identification of molecules associated with dynamic changes in macrophage activation, and a better understanding of their interactions are critical for understanding the molecular basis of disease progression and for development of new therapeutic strategies targeting macrophages.

## Acknowledgements

The authors thank Dr. Alexander Ivanov and Mikhail Golikov for critical comments and reagents.

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*Поступила 15.04.2023*
*Отправлена на доработку 22.04.2023*
*Принята к печати 24.04.2023*

*Received 15.04.2023*
*Revision received 22.04.2023*
*Accepted 24.04.2023*

# ПРОСТРАНСТВЕННОЕ РАСПОЛОЖЕНИЕ НЕЙТРОФИЛОВ И ЭОЗИНОФИЛОВ В СЛИЗИСТОЙ ОБОЛОЧКЕ ГЛАВНОГО БРОНХА ПРИ ИНДУЦИРОВАННОМ АЛЛЕРГИЧЕСКОМ ВОСПАЛЕНИИ ДЫХАТЕЛЬНЫХ ПУТЕЙ У МЫШЕЙ

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**Резюме.** В ответ на антигены, ежедневно попадающие с током воздуха в респираторный тракт человека, в слизистой оболочке дыхательных путей формируется клеточных иммунный ответ. Аллергическое воспаление дыхательных путей характеризуется эозинофил-опосредованным иммунным ответом, однако, в случаях тяжелой астмы, в респираторном тракте также присутствуют нейтрофилы. Для исследования механизмов, регулирующих развитие воспаления по тому или иному пути, широко используют модели с использованием мышей. Данные о пространственном расположении нейтрофилов и эозинофилов в респираторном тракте необходимы как для понимания механизмов развития аллергической реакции в дыхательных путях, так и для оценки потенциала лекарственных препаратов, однако недостаточно изучены. В данной работе была разработана и охарактеризована модель, позволяющая наблюдать активацию аллергического воспаления в дыхательных путях в ответ на введение экстракта гриба *Aspergillus fumigatus* на ранней стадии. Адекватность модели была подтверждена путем оценки доли эозинофилов в крови и в бронхоальвеолярных смывах. С использованием иммуногистохимического окрашивания тотального препарата главного бронха мыши и лазерной сканирующей конфокальной микроскопии было охарактеризовано пространственное расположение нейтрофилов и эозинофилов: на обращенной в просвет дыхательного пути стороне эпителиального барьера, в стенке главного бронха или в подслизистом слое в непосредственной близости от гладкой мускулатуры. Активацию аллергического иммунного ответа определяли по достоверному увеличению эозинофилов в периферической крови мышей по сравнению с интактными мышами. В этот период времени количество эозинофилов в бронхоальвеолярном смыве было также достоверно выше, чем у интактных мышей. Как в бронхоальвеолярных смывах, так и в слизистой главного бронха в исследуемый интервал времени эозинофилы были преобладающей клеточной популяцией по сравнению с нейтрофилами. Анализ пространственного расположения клеток в слизистой главного бронха выявили преимущественное расположение эозинофилов в подслизистом слое, меньше в стенке глав-

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**Образец цитирования:**
*М.А. Шевченко, Д.Е. Мурова, Е.А. Сервули «Пространственное расположение нейтрофилов и эозинофилов в слизистой оболочке главного бронха при индуцированном аллергическом воспалении дыхательных путей у мышей» // Медицинская иммунология, 2023. Т. 25, № 3. С. 625-630. doi: 10.15789/1563-0625-SCO-2830*

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**For citation:**
*M.A. Shevchenko, D.E. Murova, E.A. Servuli “Spatial characteristics of neutrophils and eosinophils in conducting airway mucosa of mice with induced allergic airway inflammation”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 625-630. doi: 10.15789/1563-0625-SCO-2830*

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**DOI:** 10.15789/1563-0625-SCO-2830

ного бронха и еще меньше – на обращенной в просвет стороне эпителия. В то же время нейтрофилы были в основном обнаружены в просвете дыхательного пути и в подслизистом слое, но не в стенке дыхательного пути. Полученные результаты свидетельствуют о том, что в ответ на последующие ингаляции аллергена мигрировать через стенку дыхательного пути в просвет будут с большей вероятностью эозинофилы, чем нейтрофилы. Таким образом, эозинофилы могут быть ответственны за повреждение эпителия в процессе развития аллергического воспаления дыхательных путей. При этом расположенные в непосредственной близости от клеток гладкой мускулатуры нейтрофилы наряду с эозинофилами могут быть вовлечены в индукцию бронхоспазма.

*Ключевые слова: нейтрофилы, эозинофилы, аллергическое воспаление дыхательных путей, слизистая оболочка дыхательных путей, Aspergillus fumigatus, модель на мышах*

# SPATIAL CHARACTERISTICS OF NEUTROPHILS AND EOSINOPHILS IN CONDUCTING AIRWAY MUCOSA OF MICE WITH INDUCED ALLERGIC AIRWAY INFLAMMATION

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**Abstract.** Daily inhaled antigens induce cellular immune response in the airways. In case of allergens, allergic airway inflammation is usually represented by eosinophils, however, neutrophil infiltration is also observed during severe asthma. Animal models contribute to investigation of the mechanisms that involve the switching to eosinophil- or neutrophil-mediated inflammation. Data about the spatial location of eosinophils and neutrophils in the airways are necessary for both the understanding of allergic airway inflammation mechanisms and the drag potential estimation, however, not completely investigated. In the present study, we characterized the model of *Aspergillus fumigatus* extract-induced allergic airway inflammation that allowed investigating the early stage of inflammation development. The model adequacy was confirmed according to the blood and bronchoalveolar lavage eosinophilia. Using immunohistochemical staining of conducting airway as a whole-mount and confocal laser scanning microscopy, we estimated neutrophil and eosinophil spatial location: in the luminal side of the epithelium, in the airway wall or in the submucosal compartment close to the smooth muscle layer. An allergic airway response activation was detected upon significant elevation of blood eosinophil percentage compared to intact mice. Simultaneously, the number of eosinophils in the bronchoalveolar lavage was also significantly increased compared to the intact mice. At this time point, eosinophils predominated both in bronchoalveolar lavages and in conducting airway mucosa compared to neutrophils. Spatial location of conducting airway mucosal cell analysis demonstrated that eosinophils mostly located in the submucosal compartment, in a lesser extent in the airway wall, and a few eosinophils were detected in the luminal side of the epithelium. Neutrophils mainly infiltrated the luminal side of the epithelium, and a few neutrophils were detected in the submucosal compartment, while no neutrophils were detected in the airway wall. The data suggests that in response to the further allergen challenge, evidently eosinophils but not neutrophils will migrate through the airway wall to the airway lumen. Thus, eosinophils can be expected to damage airway epithelium in allergic airway inflammation development. Simultaneously, neutrophils located in close proximity to the smooth muscle layer together with eosinophils can contribute to bronchoconstriction induction.

*Keywords: neutrophils, eosinophils, allergic airway inflammation, airway mucosa, Aspergillus fumigatus, mouse model, confocal microscopy*

The study was funded by Russian Science Foundation, grant number 23-25-00258.

## Introduction

Asthma is a complex disease that comprises of different mechanisms that result in the common symptoms such as dyspnea, cough, and respiratory stress [8]. Traditionally, two forms of asthma have been assumed: “T helper 2 (Th2)-high” and “Th2-low” [4]. The former is mainly, but not always, associated with airway eosinophilia and responsiveness to steroid-treatment [4]. Th2-low or severe asthma is mostly characterized with neutrophil infiltration and resistance for steroid therapy; however, some research indicates neutrophilic asthma as a subtype of Th2-low asthma endotype [1, 2]. Interestingly, in eosinophilic asthma, patients have about 2-3 % of eosinophils in the sputum, while no less than 40-60 % of sputum neutrophils contribute for neutrophilic asthma diagnosis [1].

To investigate the mechanisms of asthma, animal models, particularly, mouse models are required. Mouse models almost simulate the Th2-high asthma characteristics; however, eosinophil and neutrophil recruitment is dependent on the mouse strain [5, 10]. Recent studies demonstrated that in response to single provocation with *Alternaria alternata* extract, C57BL/6 mice exhibited eosinophil-mediated immune response, while in BALB/c mice the response was characterized by neutrophil infiltration [13]. Besides genetic factors, the environmental factors play important role in the type of immune response formation, and multiple allergen supplementation contributes to eosinophil-mediated response [5]. The effect of eosinophils and neutrophils on the further disease development is not identical, thus neutrophils can induce acute airway inflammation, while eosinophils are associated with chronic long-lasting disease [5, 7, 10]. In this view, the spatial distribution of neutrophils and eosinophils in asthma is important, but not completely investigated aspect.

In the present study, using immunohistochemistry of whole-mount conducting airways of BALB/c mice with *Aspergillus fumigatus* extract-induced allergic airway inflammation and confocal laser scanning microscopy we detected neutrophils and eosinophils in conducting airway mucosa. We characterized the precise position of the cells: in the luminal side of the airway epithelium, in the airway wall, and in the submucosal compartment. The observations helped to expect potential role of eosinophils and neutrophils in asthma manifestation.

## Materials and methods

In this study, we used BALB/c mice (Pushchino, Russian Federation) 18-20 g, 10-12 weeks. Mice were housed in the animal facility of IBCH RAS. Animal experiments were performed under the protocol 308/2020 approved by the Institutional Animal Care and Use Committee IBCH RAS. The animals were given standard food and tap water *ad libitum* and housed under regular 12 h dark:light cycles at 22 °C.

Mice were treated with 4 μg / mouse / application of *A. fumigatus* extract (Greer, USA) oropharyngeally with the 72 h intervals between the applications. Before each application, the peripheral blood was collected from the tail vein. For the cell analysis, blood was collected to the test-tubes with heparin, washed twice with hemolysis buffer and transferred to DPBS via centrifugation for 5 min at 380 g. 72 h after the 5$^{th}$ extract application, mice were euthanized and bronchoalveolar lavages (BALs) were obtained using DPBS and a cannula (Abbocath, EU). The samples were centrifuged for 5 min at 500 g. Blood and BAL cells were sediment to the glass slides using centrifuge Cytospin 2 (Shandon, UK), and stained using DiffQuick (Abris, Russia). Differential cell count was performed using stereomicroscope Zeiss Primo Star (Carl Zeiss, Germany) and objective 40×.

After BAL obtaining, lungs were inflated-fixed with 2% paraformaldehyde and stored at +4 °C overnight. The main axial airways of the left and right lower lobes were cut out using a stereomicroscope (Carl Zeiss) and micro-scissors (BBraun, Germany).

For immunohistochemistry, specimens were washed with DPBS at 150 rpm using orbital shaker (ApexLab, Russia), permeabilized with 0.3% Triton X-100. Non-specific antibody binding was blocked with 4% normal donkey serum in 0.5% BSA-DPBS. Specimens were stained with rat anti-mouse Ly6G-AlexaFluor488, and rat anti-mouse SiglecF-APC antibodies (all BioLegend, USA, in dilution 1:50) at +4 °C overnight. Specimens were washed with 0.3% Triton X-100 for 5 × 1 h, and stained with Phalloidin-Atto425 (Sigma Aldrich, USA, in dilution 1:50). Specimens were placed on slide glasses, and covered with Prolong Gold mounting medium (ThermoFisher, USA), and stored at -20 °C until use.

Three-dimensional images were obtained using an inverted confocal laser scanning microscope Zeiss LSM980 (Carl Zeiss). A 63× oil objective was used to image the region of interest for the further quantification. At least 4 z-stacks were acquired from each specimen. 405, 488 and 633 nm lasers and respective beam splitters were used. Fluorescence was detected using Lambda mode with the following spectral unmixing using the ZEN software (Carl Zeiss).

The image stacks were analyzed using Imaris 9.8 (Oxford Instruments, UK). The surfaces of epithelium and smooth muscles were created based on Phalloidin-Atto425 signal. Neutrophil and eosinophil surfaces were created based on Ly6G-Alexa488 and SiglecF-APC signals, respectively. The surfaces were created via three-dimensional surface rendering using volume and intensity mean thresholds. Cell surfaces were quantified automatically using intensity max in phalloidin channel and Z-position filters. Images were shown as maximal intensity projections. The final image processing was performed using Adobe Photoshop CS version 5 (Adobe Systems, USA).

Statistical analysis was carried out using GraphPad Prism software. The data were presented as medians and interquartile ranges. The data were analyzed using the Mann–Whitney U test. The difference in values at $p \leq 0.05$ was considered significant.

## Results and discussion

Neutrophils are known to be a dominant population in the peripheral blood in steady state, however, upon allergen application, eosinophil infiltration was detected (Figure 1A, B). Thus, five *A. fumigatus* extract applications resulted in significant elevation of eosinophil percentage compared to that in intact mice – before the 1$^{st}$ application (Figure 1A, B). The percentage of periphery blood neutrophils subsequently decreased (Figure 1A, B). Thus, the time point at 72 h after the 5$^{th}$ application was selected as the time of switching to eosinophil-mediated response (Figure 1A, B, dotted line).

Mice were sacrificed at 72 h after the 5$^{th}$ extract application (Figure 1A, B, dotted line), and the BAL cell number was detected. At this time point,

eosinophil number increased significantly compared to intact mice, while just a small number of neutrophils was detected in BALs (Figure 1C).

To detect the presence of neutrophils and eosinophils in conducting airway mucosa, analysis of the high-resolution images of the airway whole-mounts was made. The analysis demonstrated that at this time point, both neutrophils ($Ly6G^+$ cells with typical to segmented granulocyte morphology) and eosinophils ($SiglecF^+$ cells with typical to segmented granulocyte morphology) were detected in conducting airway mucosa and submucosal compartment of mice with induced allergic airway inflammation (Figure 2A). Phalloidin staining allowed to visualized actin-rich fibers of the epithelial barrier and smooth muscle layer, and to detect airway wall – the compartment bordered by the epithelium and smooth muscles (Figure 2A).

Epithelium, smooth muscles, and eosinophils were built via surface rendering (Figure 2B, C). Using thresholds on Z-position and intensity maximum in Actin channel, the number of eosinophils in the luminal side of the epithelium (Figure 2D, red), in the airway wall (Figure 2D, yellow), and in the submucosal compartment (Figure 2D, orange) were detected. Due to irregular structure of the submucosal compartment, only attached to the smooth muscle layer submucosal cells were considered for quantitative analysis and the cells that laid deep in the tissues were exclude from the estimation (Figure 2D, magenta). The same principle was used for the neutrophil position characterization and estimation (data not shown).

Quantitative analysis demonstrated that both eosinophil and neutrophil numbers were significantly higher in the airways of mice with induced allergic airway inflammation compared to intact mice (Figure 2E). Eosinophils dominated in conducting airway mucosa of mice with induced allergic airway inflammation compared to neutrophils (Figure 2E). Simultaneously, eosinophils mostly located in the submucosal compartment, less in the airway wall and only a few eosinophils were observed in the luminal side of the epithelium (Figure 2F). Opposite to eosinophils, neutrophils were mostly located in the luminal side of the epithelium, little to no neutrophils were detected in the airway wall, and a small number was detected in the submucosa close to the smooth muscle (Figure 2G). Some neutrophils were detected deep in the submucosa, however, it was not possible to conclude, whether these neutrophils were in the lung vessels or in the tissue (data not shown).

Thus, five applications of *A. fumigatus* extract led to the blood eosinophil percentage elevation and eosinophil domination above neutrophils both in BAL and in the conducting airway, including conducting airway mucosa, and in the submucosal compartment. In conducting airways, eosinophils mostly located in the submucosal area close to the smooth muscles, while neutrophils migrate to the airway lumen.

Spatial aspects of cellular immune response in sites of inflammation is important and intensively investigated area of immunology [6]. Eosinophil-intraepithelial dendritic cell interactions were characterized in the tracheal mucosa of mice with ovalbumin-induce allergic airway inflammation [14]. Together with eosinophils, neutrophils also play important role in the local immune response to allergens. Thus, ovalbumin-sensitized and challenged mice reacted with enhanced neutrophil infiltration in response to *A. fumigatus* conidia application [12]. Moreover, neutrophils mig-

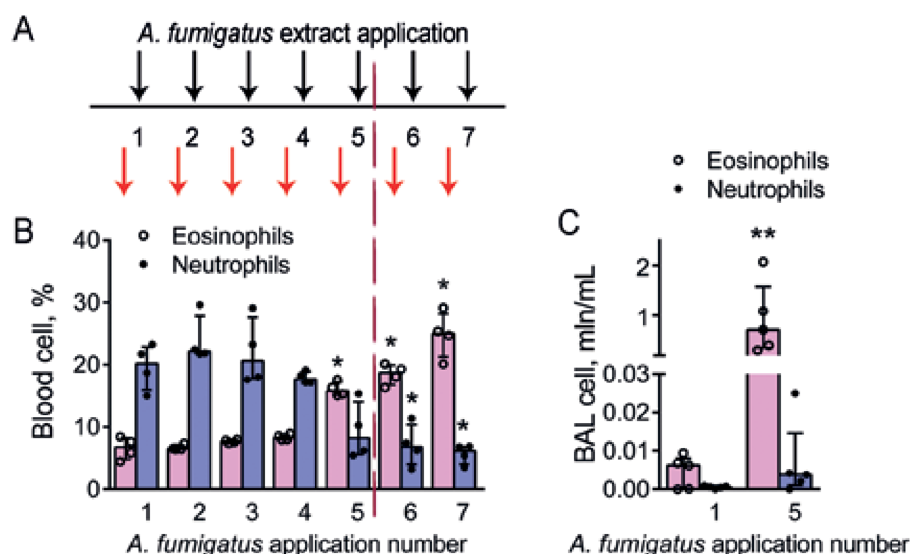


**Figure 1. Blood and BAL cell redistribution in allergic airway inflammation**

Note. A. The scheme of allergic airway inflammation induction via o.ph. applications of *A. fumigatus* extract (black arrows). Blood samples were collected before (T0), 72 h after the first (T2), and 72 h after the fifth (T6) extract applications (red arrows). B. Percentage of blood eosinophils (rose bars) and neutrophils (blue bars) at 72 h after the respective *A. fumigatus* extract application. C. Quantitative analysis of BAL eosinophils (rose bar) and BAL neutrophil (blue bar) before the 1st and before the 6th *A. fumigatus* extract application. Data are shown as median and i.q.r., significant difference between indicated groups: * $p \leq 0.05$, ** $p \leq 0.01$.

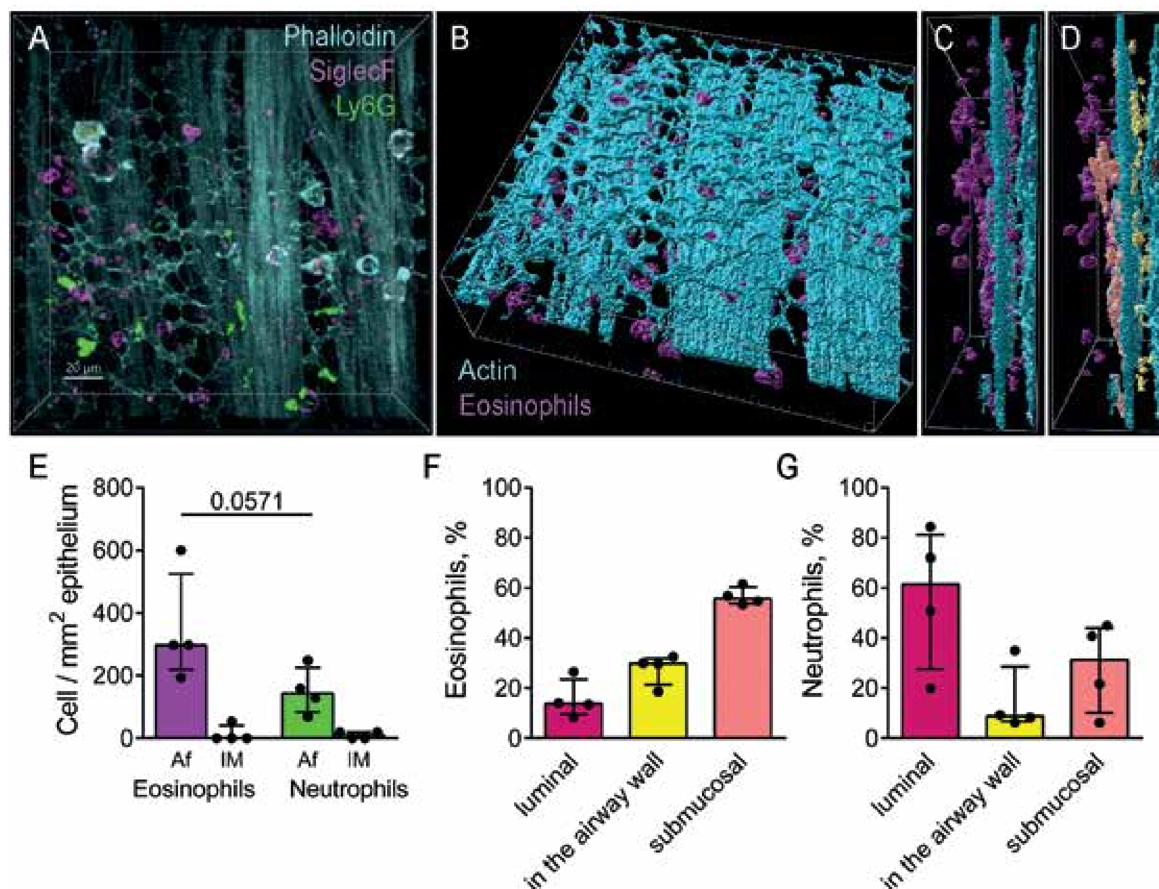


**Figure 2. Eosinophil and neutrophil distribution in conducting airway mucosa**

Note. A. Representative image of the region of conducting airway mucosa of mouse with induced allergic airway inflammation. Actin–rich epithelial and smooth muscle fibers (cyan), neutrophils (green) and eosinophils (magenta) are represented as maximum intensity projections. Scale bar 20 μm. B. Epithelium and smooth muscles, and eosinophils presented in A are shown via surface rendering. C. Side view of the image presented in B demonstrating eosinophils in relation to the airway wall (epithelium and smooth muscles). D. The sample of analysis of the eosinophil distribution: in the submucosal area that attached to smooth muscles (orange), in the airway wall (yellow), and in the luminal side of the epithelium (red). E. Quantitative analysis of eosinophils and neutrophils in conducting airway mucosa of mice with *A. fumigatus*-induced allergic airway inflammation (Af) and intact mice (IM). F, E. Percentage of eosinophils (F) and neutrophils (E) in the luminal side of epithelium (red bar), in the airway wall (yellow bar), and in the submucosal compartment (orange bar).

rated to the luminal side of the airway epithelium and internalized fungal conidia [12].

While *A. fumigatus* conidia applied to mice induce strong neutrophil-mediated response, the regular applications of small doses of *A. fumigatus* conidia or extract result in the eosinophil recruitment to the airways [11, 13, 14]. The data obtained in the present study demonstrate that despite the adherence of BALB/c mice to neutrophil-mediated response, multiple airway challenge with relatively low doses of the allergen – *A. fumigatus* extract, induced eosinophil recruitment.

Opposite to neutrophils that were mostly represented in the airway lumen, eosinophils infiltrated airways and accumulated near by the smooth muscles, from the side of submucosal compartment. From the one side, in response to the further allergen challenges eosinophils are more likely than neutrophils migrate through the airway wall to the airway lumen. In this case, upon migration, eosinophils can contact the smooth muscles that can trigger bronchoconstriction induction [5]. Migrating through the airway wall, eosinophils can also induce the damage of the airway epithelium that can activate the cascade of inflammatory response. From the other side, accumulation of eosinophils in the submucosal compartment may indicate the inability of eosinophils to migrate to the airway lumen due to the absence of the necessary signals or triggers and such inability can explain the protective effect of eosinophils in acute lung injury [5, 7].

## Conclusion

Thus, in the present study we traced the redistribution of neutrophils and eosinophils in blood and airways of mice upon allergic airway inflammation development. We identified microanatomical location of the myeloid effector cells (neutrophils and eosinophils) in conducting airway mucosa and submucosal compartment. The data can explain implication of neutrophils and eosinophils to distinct asthma symptoms.

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*Поступила 15.04.2023*
*Отправлена на доработку 21.04.2023*
*Принята к печати 26.04.2023*

*Received 15.04.2023*
*Revision received 21.04.2023*
*Accepted 26.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 631-636

*Краткие сообщения*
*Short communications*

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 631-636

# ИЗУЧЕНИЕ ЛОКАЛЬНОЙ ЭКСПРЕССИИ ГЕНОВ ИНФЛАММАСОМНОГО КОМПЛЕКСА ПРИ МОДЕЛИРОВАНИИ ДЕГЕНЕРАЦИИ СЕТЧАТКИ *IN VIVO*

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**Резюме.** Нейродегенеративная офтальмопатология является одной из основных причин необратимой слепоты и инвалидности в мире. В патогенезе заболеваний данной группы в последнее время все большее внимание уделяется роли локального воспаления, обусловленного активацией врожденного иммунитета и механизмам его генетической регуляции. В последние годы в области экспериментальной офтальмологии появились работы, которые продемонстрировали возможность сборки инфламмасомных комплексов NLRP1, NLRP3 при действии гипергликемии, влиянии кислородной депривации клеток сетчатки, а также при моделировании компрессионного стресса, подобного таковому при глаукоме, однако механизм участия инфламмасом в развитии нейродегенеративных заболеваний глаз остается невыясненным.

Целью работы явилось изучение локальной экспрессии генов, кодирующих белки инфламмасомного комплекса NLRP3 (NLRP3, CASP-1) в экспериментальной модели дегенерации сетчатки на кроликах.

Исследования выполнены в образцах тканевого комплекса (ТК) сетчатка/ретинальный пигментный эпителий (РПЭ), выделенного из глаз 14 кроликов породы новозеландских альбиносов, на которых моделировалось дегенеративное поражение сетчатки путем однократного субретинального введения 0,01 мл 0,9% раствора хлорида натрия, и 7 здоровых кроликов без поражения глаз. Оценка уровней экспрессии генов NLRP3 и CASP-1 в ТК сетчатка/РПЭ проводилась методом полимеразной цепной реакции с обратной транскрипцией (ОТ-ПЦР). По результатам проведенного исследования, отмечалось статистически значимое увеличение экспрессии гена NLRP3 ($p < 0,001$) в ТК сетчатка/РПЭ глаз экспериментальных животных, что, возможно, свидетельствует об участии компонентов инфламмасомы NLRP-3 в развитии нейродегенеративного поражения сетчатки. В то же время экс-

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**Образец цитирования:**
*Н.В. Нероева, О.А. Свитич, В.В. Нероев, А.Р. Кинкулькина, Н.В. Балацкая, Е.С. Сорожкина «Изучение локальной экспрессии генов инфламмасомного комплекса при моделировании дегенерации сетчатки in vivo» // Медицинская иммунология, 2023. Т. 25, № 3. С. 631-636.*
*doi: 10.15789/1563-0625-IOL-2780*

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**For citation:**
*N.V. Neroeva, O.A. Svitich, V.V. Neroev, A.R. Kinkulkina, N.V. Balatskaya, E.S. Sorozhkina "Investigation of local expression of NLRP3 inflammasome complex genes in modeling retinal degeneration in vivo", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 631-636.*
*doi: 10.15789/1563-0625-IOL-2780*

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**DOI:** 10.15789/1563-0625-IOL-2780

прессия гена, кодирующего CASP-1, обнаружена только в ТК сетчатка/РПЭ опытных глаз и, вероятно, обусловлена локальными воспалительными механизмами в ткани сетчатки.

Высокий уровень мРНК NLRP3, CASP-1, определявшийся во всех образцах ТК сетчатка/РПЭ опытных глаз на поздних сроках (3 и 6 мес.) эксперимента, позволяет предположить формирование механизмов (например, активированного глиального фенотипа), поддерживающих воспаление в ткани ретины, что должно учитываться при активно разрабатываемых в настоящее время трансплантационных методиках лечения ретинальной дегенерации.

*Ключевые слова: нейродегенерация, сетчатка, компоненты инфламмасомного комплекса, NLRP3, CASP-1, экспрессия генов, ОТ-ПЦР*

# INVESTIGATION OF LOCAL EXPRESSION OF NLRP3 INFLAMMASOME COMPLEX GENES IN MODELING RETINAL DEGENERATION *IN VIVO*

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**Abstract.** Neurodegenerative ophthalmopathology is one of the main causes of irreversible blindness and disability in the world. In the pathogenesis of diseases of this group, more and more attention has recently been paid to the role of local inflammation caused by the activation of innate immunity and the mechanisms of its genetic regulation. In recent years, works have appeared in the field of experimental ophthalmology that have demonstrated the possibility of NLRP1, NLRP3 inflammasome complexes assembling when exposed to hyperglycemia, oxygen deprivation of retinal cells, as well as modeling compressive stress similar to that in glaucoma [15]. However, the mechanism of inflammasome involvement in the development of neurodegenerative eye diseases remains unclear. The aim of the study was to investigate the local expression of genes encoding proteins of the NLRP3 inflammasome complex (NLRP3, CASP-1) in an experimental model of retinal degeneration in rabbits. The studies were performed on samples of tissue complex (TC) of the retina/retinal pigment epithelium (RPE) (retina/RPE TC), isolated from the eyes of 14 New Zealand albino rabbits, in which degenerative retinal lesion was modeled by a single subretinal injection of 0.01 mL of 0.9% sodium chloride solution, and 7 healthy rabbits without eye damage. The formation of retinal degeneration was judged on the basis of changes in morphofunctional parameters obtained during specialized ophthalmological research methods (optical coherence tomography, fundus autofluorescence, electroretinography) at follow-up periods of 1, 3 and 6 months. The level of expression of NLRP3 and CASP-1 genes in the retina/RPE TC was evaluated by reverse transcription polymerase chain reaction (RT-PCR). According to the results of the study, a statistically significant increase in NLRP3 gene expression ($p < 0.001$) was noted in the retina/RPE TC of experimental animals, which may indicate the involvement of NLRP-3 inflammasome components in the development of neurodegenerative retinal lesions. At the same time, the expression of the gene encoding CASP-1 was detected only in the retina/RPE TC of experimental eyes and is probably due to local inflammatory mechanisms in the retinal tissue.

The high level of NLRP3, CASP-1 mRNA, detected in all retina/RPE TC samples of experimental eyes at late stages of the experiment (3 and 6 months), allows us to assume the formation of mechanisms (for example, activated glial phenotype) that support inflammation in retinal tissue. This should be taken into account in actively developing transplantation methods for the treatment of retinal degeneration.

*Keywords: neurodegeneration, retina, components of the inflammasome complex, NLRP3, CASP-1, gene expression, RT-PCR*

## Introduction

Neurodegenerative eye pathology is a heterogeneous group that includes age-related macular degeneration (AMD), hereditary retinal dystrophy, primary open-angle glaucoma (POAG), diabetic retinopathy (DR), and some other forms. These diseases have different etiologies, but their common characteristic is a progressive destructive-degenerative process that affects all cellular elements of the neural retina with a violation of its integrity and leads to irreversible loss of visual functions, blindness and disability [4].

Significant visual impairment occurs at the advanced stages of neurodegenerative eye pathology: in this case, the implementation of therapeutic measures is limited, associated with certain difficulties (requires specialized invasive interventions) and does not always lead to positive functional results, and for some forms, such as, "geographic atrophy" and hereditary retinal degenerations, there is no treatment [12]. Diseases of this group are multifactorial, the pathogenesis of which remains completely unexplored: currently, in addition to the main factors of their development and progression – age (AMD, POAG), increased intraocular pressure (POAG), metabolic (AMD, DR), endocrine (DR), hemodynamic disorders (systemic and local), an increasing number of attention is paid to the role of local inflammation caused by activation of the innate immune response [8, 10].

In the retina, microglia macrophages are cells of innate immunity that recognize and form a responsive reaction to patterns associated with pathogens and damage (PAMPs and DAMPs) [1]. Being located in the inner and outer plexiform layers of a healthy retina, normally moderately activated microglia phagocytoses cellular debris and regulates neurovascular interactions [11, 14].

A number of inducing factors lead to the formation of an activated M1 phenotype of glial macrophages that implement a protective response, which is neutralized after a short-term adverse effect [5]. Prolonged activation of M1-microglia can cause neurotoxicity due to the dominance of excessive inflammatory response [2, 5, 6].

Indeed, *in vitro* experiments have shown that DAMPs released during trauma (including compression, surgical), prolonged exposure to UV light, toxic products of oxidative modification of biomolecules, etc., bind to pattern-recognition receptors (PRRs), cause activation of microglia macrophages and its transition to the M1 phenotype, secreting high levels of pro-inflammatory cytokines TNFα, IL-1β, IL-6, nitric oxide and reactive oxygen species, contributing to the maintenance of inflammation and damage to the neural retina [4, 7].

In recent years, works have appeared in the field of experimental ophthalmology that have demonstrated the possibility of assembling inflammasome complexes NLRP1, NLRP3 under the influence of hyperglycemia, the effect of oxygen deprivation of retinal cells, as well as when modeling compression stress similar to that in glaucoma [15]. However, the mechanism of inflammasome involvement in the development of neurodegenerative eye diseases remains unclear. **The purpose of the research** was to study the local expression of genes encoding NLRP3 inflammasome proteins (NLRP3, CASP-1) in an experimental model of retinal degeneration in rabbits.

## Materials and methods

The study was carried out in compliance with the international principles of the Helsinki Declaration on Humane Treatment of Animals, the principles of humanity set out in the European Community Directive (86/609/EC), and the "Rules for conducting work using experimental animals". Degenerative retinal damage was modeled in 14 New Zealand albino rabbits (experimental group: age 2.5-3.0 months, weight 2.0-2.5 kg) by a single subretinal injection at a distance of 1-1.5 mm below the optic nerve disc of 0.01 mL of 0.9% sodium chloride solution with the formation of a subretinal bubble according to the methodology developed in the Department of retina and optic nerve pathology of the Helmholtz National Medical Research Center of Eye Diseases [13]. Preoperative preparation of animals included intramuscular administration of painkillers 0.3 mL of zoletil 50 and 0.55 mL of 2% xylazine; also before surgery 0.3 mL of 0.4% dexamethasone and 0.5 mL of dicinone were administered.

The formation of retinal degeneration was judged on the basis of changes in morphofunctional parameters obtained during specialized ophthalmological research methods (optical coherence tomography, fundus autofluorescence, electroretinography) at follow-up periods of 1, 3 and 6 months. At these times, animals were removed from the experiment by air embolism after anesthesia (according to the order of the Ministry of Higher Education of the USSR No. 724 of November 13, 184), and then eye enucleation was performed.

The control group included 7 somatically healthy rabbits without ocular pathology. The study protocols were approved at a meeting of the local ethics committee of the Helmholtz National Medical Research Center of Eye Diseases [13].

Twenty-eight samples of retina/RPE TC were used as a material for molecular biological study, isolated from the eyes of experimental animals according to standard protocols. The biomaterial was transferred to cryotubes and stored at a temperature of -70 °C until the research was carried out.

Using the Vector NTI program (Invitrogen), nucleotide sequences (5'-) for rabbit genes NLRP3,

CASP1 and reference GAPDH were selected and subsequently synthesized (DNA Synthesis, LLC, Russia):

CASP1-F CTGAATGTCAACACCATCTTTGAG;

CASP1-R CTATTCCTTGGTTTTCACCACGA;

CASP1-probe FAM-CCCAAGGTGATCATCATCCAAGCC-BHQ1;

NLRP3-F TGCAGATGTTGGAGTTAGACAGC;

NLRP3-R GCAGGTCAGGATCGTGCAG;

NLRP3-Probe FAM-CCTCACCTCGCACTGCTGCTGG-BHQ1;

GAPDH-F GCTGGTCATCAACGGGAAGG;

GAPDH-R GGTGAAGACGCCAGTGGATT;

GAPDH-Probe FAM-CTTCCAGGAGCGAGATCCCGCC-BHQ1

Samples of retina/RPE TC were homogenized, and mRNA extraction was performed using the Gene JET RNA Purification Kit (Thermo Scientific, USA) in accordance with the manufacturer's instructions. Reverse transcription reaction was performed using the OT-1 kit from "Syntol" company (Russia). The obtained cDNA fragments were amplified by real-time polymerase chain reaction (RT-PCR) using a DT-96 thermocycler from DNA Technology (Russia). For PCR-RV, kits for determining the expression of the NLRP3, CASP-1, GAPDH genes were used (DNA synthesis, Russia).

To determine the relative amount of cDNA in the sample, the method of normalized expression of ΔΔCt was used. The results were expressed in relative units (rel. Units): the ratio of the threshold cycle value of amplification of the studied gene to the threshold cycle value of amplification of the reference gene (the "housekeeping" gene) GAPDH: ΔΔCt = (ΔCt of the sample) / (ΔCt GAPDH).Statistical processing of the results was carried out using the StatTech v. 3.1.1 software package (Stattech, Russia). Quantitative indicators were evaluated for compliance with a normal distribution (Shapiro–Wilk criterion). In the absence of a normal distribution, quantitative data were described using the median (Me) and the lower and upper quartiles ($Q_{0.25}$-$Q_{0.75}$); the Mann–Whitney U test was applied when comparing groups. The critical level of significance in testing statistical hypotheses was $p < 0.05$.

## Results and discussion

The results of the study are presented in Table 1 and Figures 1, 2.

In the retina of 43% of healthy animals' eyes (n = 6) and in all cases of the experimental group (n = 14), expression of the gene encoding NLRP3 was detected. The level of NLRP3 mRNA in the TC of experimental animals with retinal degeneration was statistically significantly higher than that in normal tissue, with a median expression of 35.0 rel. Units (Table 1, Figure 1)

An individual analysis revealed that in the vast majority of cases (70%), the degenerative process in the retina was associated with a significant increase in the expression of the NLRP3 gene, the indicators of which exceeded the upper limit of the established normal range. In about one-third of experimental eyes, its transcriptional activity was within normal values (from 1.9 to 2.1 rel. units).

Changes in NLRP3 gene expression in the TC of experimental eyes were the same at all follow-up periods (at 1, 3 and 6 months).

The expression of the gene encoding CASP-1 was studied in the retina/RPE TC of animals in the main group and control (Table 1, Figure 2). None of the studied samples of healthy retina showed expression of the CASP-1 gene, unlike the material from experimental animals, in which the median expression activity in retina/RPE TC was 38.0 rel. units (p = 0.00).

The pattern of changes in mRNA levels of CASP-1, as well as NLRP3, in the retina/RPE TC of experimental eyes was the same at all observation periods (at 1, 3 and 6 months) in the group with retinal neurodegeneration.

NLRP3 is a member of the PRRs superfamily and belongs to the NOD-like receptor (NLR) family

**TABLE 1. ANALYSIS OF GENE EXPRESSION OF COMPONENTS OF THE NLRP3 INFLAMMASOME COMPLEX (NLRP3, CASP-1) IN GROUPS OF RABBITS**

| Group | Expression level (rel. units) | | | | | |
|---|---|---|---|---|---|---|
| | NLRP3 | | | CASP-1 | | |
| | Me | ($Q_{0.25}$-$Q_{0.75}$) | p | Me | ($Q_{0.25}$-$Q_{0.75}$) | p |
| Control group (n = 14) | 0.0 | 0.0-23.0 | < 0.001* | 0.0 | – | < 0.001* |
| Retinal degeneration (n = 14) | 35.0 | 10.0-37.0 | | 38.0 | 13.0-39.0 | |

Note. *, differences considered significant at p value of 0.005 or less.

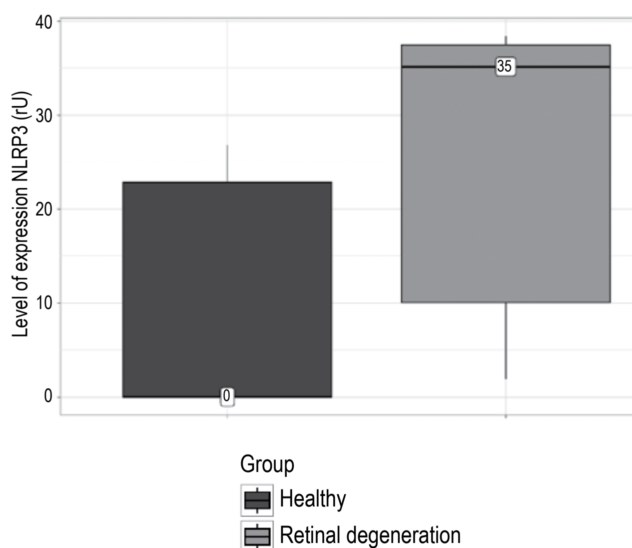


**Figure 1. Comparative analysis of expression levels (rel. units) of the NLRP3 gene in the TC retina/RPE of animals without ophthalmopathology (norm) and with degenerative retinal changes**

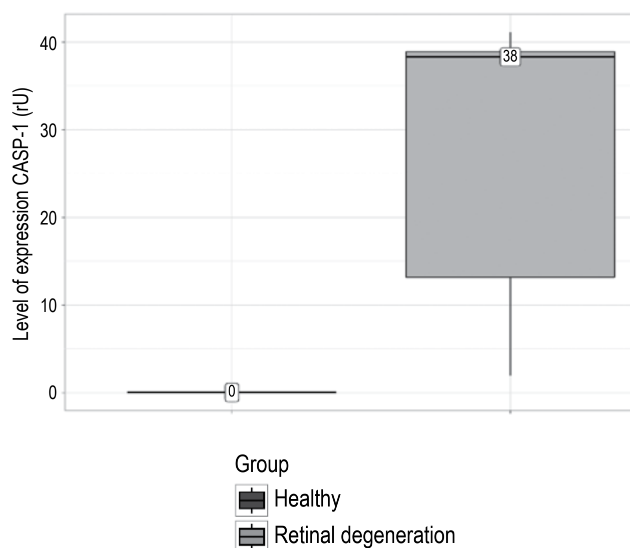


**Figure 2. Comparative analysis of expression levels (rel. units) of the CASP-1 gene in the TC retina/RPE in control groups (healthy without ophthalmopathology) and with degenerative retinal changes**

localized in the cell cytoplasm. After recognizing danger signals, NLRs are activated, oligomerized, and initiate the assembly of an inflammasome complex with the activation of caspases and maturation of cytokine precursor molecules pro-IL-1β and pro-IL-18 into biologically active forms. In addition, activated caspases are capable of initiating pyroptosis: a programmed proinflammatory form of cell death through the proteolytic protein gazdermine [9]. A significant increase in the level of NLRP3 mRNA ($p < 0.001$) in the retina/RPE TC of the eyes of experimental animals detected in our study indicates the involvement of this receptor in the development of retinal neurodegeneration.

The expression of the gene encoding CASP-1, found only in the retina/RPE TC of experimental eyes and absent in controls, suggests the presence of special local mechanisms regulating the activity of this protein (and therefore the inflammatory reaction) in healthy retina tissue.

Our results are consistent with data from a number of studies (mainly performed *in vitro* or in models on small rodents), which have demonstrated the ability of individual retinal cell elements (astrocytes, Muller cells, microglia) and RPE to actively produce pro-inflammatory mediators, including the IL-1 family, in response to danger signals: ischemia, photo-oxidative damage, hypertension, ATP stimulation. It has been shown that the production of these cytokines is controlled by the activation of an inflammasome complex formed in the cells of the pigment epithelium, retina and vascular membrane of the eye [3].

## Conclusion

In our study, special attention was drawn to the preservation of the local response from the studied NLRP3 components: high expression activity of protein genes (NLRP3 and CASP-1) was determined in all retina/RPE TC's of experimental eyes at late stages (3 and 6 months) of the experiment. This circumstance suggests the formation of an M1 activated glial phenotype that supports inflammation in the retinal tissue, which requires further research and should be taken into account in actively developing transplantation methods for the treatment of retinal degeneration in the future.

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*Поступила 14.04.2023*
*Отправлена на доработку 19.04.2023*
*Принята к печати 20.04.2023*

*Received 14.04.2023*
*Revision received 19.04.2023*
*Accepted 20.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 637-642



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 637-642

# ИММУНИЗАЦИЯ АПОПРОТЕИНАМИ ЛПВП ИНДУЦИРУЕТ ОПОСРЕДОВАННОЕ Т-ЛИМФОЦИТАМИ ВОСПАЛЕНИЕ АОРТЫ И ВЕН

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**Резюме.** В последнее время все большее распространение получает гипотеза о том, что атеросклеротические процессы в большей степени обусловлены иммунными (аутоиммунными) механизмами. В то же время аутоиммунная гипотеза атерогенеза не стала общепринятой и требует дополнительных доказательств. Ранее нам удалось вызвать изменения в стенке аорты крысы, подобные изменениям на ранних стадиях атеросклероза человека, а также вызвать висцеральное ожирение у нормохолестеринемических крыс Wistar путем однократной иммунизации нативными липопротеинами высокой или низкой плотности человека. Также нами было обнаружено, что иммунный ответ на нативные ЛПВП человека вызывает атеросклерозоподобные поражения в аорте кролика, такие как адипоцитарная и хондроцитарная метаплазия, отложения протеогликанов, лейкоцитарная инфильтрация. Изменения в стенке аорты кроликов и крыс, иммунизированных нативными липопротеинами, были получены на фоне нормального уровня холестерина крови. Таким образом, иммунный ответ против ЛПВП или ЛПНП может быть независимой причиной атерогенеза. Цель данного исследования состояла в том, чтобы проверить, будет ли иммунизация человеческими апопротеинами ЛПВП (белками апоА1 и апоЕ) вызывать атеросклерозоподобные поражения в аорте нормохолестеринемических крыс Wistar. Апопротеины ЛПВП выделяли из плазмы человека или крысы. Для иммунизации апопротеинами ЛПВП человека использовали крыс Wistar (n = 5) в возрасте 2 месяцев. Апопротеины ЛПВП вводили однократно в виде внутрикожной инъекции по 100 мкг на крысу в неполном адъюванте Фрейнда. Контрольным крысам вводили подкожно неполный адъювант Фрейнда (n = 5). Крыс вскрывали через 25 недель после иммунизации. Срезы аорты окрашивали гематоксилином и эозином для световой микроскопии. Для определения инфильтрации Т-лимфоцитами проводили иммуногистохимическое окрашивание FITC-мечеными антителами, специфичными к крысиному CD3. $CD3^+$Т-лимфоциты выявляли с помощью флуоресцентного микроскопа Olympus BX53. Уровень антител к апопротеинам человека и крысы определяли методом непрямого твердофазного иммуноферментного анализа. Иммунизация апопротеинами ЛПВП вызвала опосредованный Т-лимфоцитами иммунный ответ, без выработки аутоантител к апопротеинам ЛПВП. Интима и адвентиция аорты были инфильтрированы Т-лимфоцитами у крыс, иммунизированных апопротеинами ЛПВП. Неожиданным было обнару-

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**Образец цитирования:**
*А.Ю. Сидоров, К.В. Фомина, Л.В. Бедулева «Иммунизация апопротеинами ЛПВП индуцирует опосредованное Т-лимфоцитами воспаление аорты и вен» // Медицинская иммунология, 2023. Т. 25, № 3. С. 637-642.*
*doi: 10.15789/1563-0625-HAI-2699*

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**For citation:**
*A.Yu. Sidorov, K.V. Fomina, L.V. Beduleva “HDL apoprotein immunization induces T cell-mediated venulitis and inflammation in aorta”, Medical Immunology (Russia)/ Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 637-642.*
*doi: 10.15789/1563-0625-HAI-2699*

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**DOI:** 10.15789/1563-0625-HAI-2699

жение сильной Т-лимфоцитарной инфильтрации всех слоев стенки вен у крыс, иммунизированных апопротеинами ЛПВП человека. Таким образом, посредством иммунизации апопротеинами нам не удалось вызвать у крыс изменения в стенке аорты характерные для продвинутых стадий атеросклероза. Однако иммунизация апопротеинами ЛПВП вызвала опосредованное Т-лимфоцитами сильное воспаление аорты и вен.

*Ключевые слова: воспаление сосудов, апопротеины ЛПВП, аутоиммунное воспаление, Т-лимфоцитарная инфильтрация, экспериментальная модель*

# HDL APOPROTEIN IMMUNIZATION INDUCES T CELL-MEDIATED VENULITIS AND INFLAMMATION IN AORTA

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**Abstract.** The hypothesis that atherosclerotic processes are mostly caused by immune (autoimmune) mechanisms has recently been gaining attraction. At the same time, the autoimmune hypothesis of atherogenesis has not become generally accepted and requires additional evidence. Previously, we were able to induce changes in the aortic wall similar to those observed in the early stages of human atherosclerosis, and also to produce visceral obesity in normocholesterolaemic Wistar rats by a single immunization with human native high- or low-density lipoproteins. We also found that the immune response to native human HDL causes atherosclerosis-like lesions in the rabbit aorta, such as adipocyte and chondrocyte metaplasia, proteoglycan deposits, and leukocyte infiltration. Atherosclerosis-like lesions developed in the aorta of hnHDL-immunized rabbits against a background of normal blood LDL-cholesterol level. Thus, an immune response against HDL or LDL may be an independent cause of atherogenesis. The aim of this study was to test whether immunization with human HDL apoproteins (apoA1 and apoE proteins) would induce atherosclerosis-like lesions in the aorta of normocholesterolemic Wistar rats. HDL apoproteins were isolated from human or rat plasma. Wistar rats (n = 5) aged 2 months were used for immunization with human HDL apoproteins. HDL apoproteins were administered as a single intradermal injection of 100 μg per rat in incomplete Freund's adjuvant. Control rats were injected subcutaneously with incomplete Freund's adjuvant (n = 5). Rats were dissected 25 weeks after immunization. Rat aorta sections were stained with hematoxylin and eosin for light microscopy. T lymphocytes infiltration was determined by immunohistochemical staining with FITC-labeled antibodies specific to rat CD3. $CD3^+$T lymphocytes were detected using an Olympus BX53 fluorescent microscope. The level of antibodies to human and rat HDL apoproteins was determined by indirect enzyme-linked immunosorbent assay. Immunization with HDL apoproteins induced a T cell mediated immune response without production of autoantibodies to HDL apoproteins. The aortic intima and adventitia were infiltrated with T lymphocytes in rats immunized with HDL apoproteins. Pronounced T lymphocytic infiltration was found in all layers of the vein wall in rats immunized with human HDL apoproteins. Thus, immunization with HDL apoproteins causes T cell mediated inflammation of the aorta and venulitis.

*Keywords: vascular inflammation, HDL apoproteins, autoimmune inflammation, T lymphocytic infiltration, experimental model*

This work was supported by the Ministry of Science and Higher Education of the Russian Federation (project number 0827-2020-0012).

## Introduction

A high level of total cholesterol or low-density lipoprotein cholesterol is considered the main cause of atherosclerosis and cardiovascular disease [11]. However, the hypothesis that atherosclerotic processes are mostly caused by immune (autoimmune) mechanisms has recently been gaining attraction [6]. Over the lengthy period during which the autoimmune hypothesis of atherosclerosis has been developed and strengthened, the role of the immune response against oxidized LDL, the heat shock proteins (hsps) of microorganisms, apolipoprotein A-1 (main protein constituent of high-density lipoprotein [HDL]) and vessel wall antigens have been studied [8]. Measurement of circulating autoantibodies directed against native and malondialdehyde (MDA)-modified epitope p210 of apoB-100 (IgG-p210nat and IgM-p210MDA) in relation to early atherosclerosis in a large, European longitudinal cohort study of healthy high-risk individuals provides evidence of involvement of autoantibodies against native and MDA-modified apoB-100 peptide 210 in cardiovascular disease

in humans [7]. Previously, we were able to induce changes in the aortic wall similar to those observed in the early stages of human atherosclerosis, and also to produce visceral obesity in normocholesterolaemic Wistar rats by a single immunization with human native HDL (hnHDL) or hnLDL. Rats immunized with hnHDL or hnLDL that as a result produce antibodies against native lipoproteins were found to have pericardial fat, increased visceral adipose tissue volume, inflammation in the aortic wall as identified by the accumulation of leukocytes therein, and destruction of the intima and disruption of the media structure [4]. Immune response to hnHDL was found to cause atherosclerosis-like lesions in the rabbit aorta such as adipocytic and chondrocytic metaplasia, proteoglycan deposits, leukocytic infiltration. Atherosclerosis-like lesions developed in the aorta of hnHDL-immunized rabbits against a background of normal blood LDL-cholesterol level. Thus, immune response against HDL or LDL may be an independent cause of atherogenesis, and HDL is the potential target of the immune attack that leads to atherosclerosis [5].

Epitopes of apoproteins are considered to be the atherogenic component of lipoproteins. Antibodies against ApoA1 demonstrated positive correlations with atherogenesis [8] and were identified as biomarker with a high potential to predict increased cardiovascular disease risk [13]. High level of anti-Apo A-1 autoantibodies in patients with acute coronary syndrome was revealed [14]. Anti-ApoA-1 IgG might be associated with increased atherosclerotic plaque vulnerability in humans and mice. Passive immunization of atherosclerosis-prone apoE-/- mice with anti-apoA-1 IgG increased both atherosclerotic lesion size [9]. At the same time, the autoimmune hypothesis of atherogenesis has not become generally accepted and requires more evidence.

**The aim of this study** was to test whether immunization with HDL apoproteins would induce atherosclerosis-like lesions in aorta of normocholesterolaemic Wistar rats.

## Materials and methods

**Rats and Ethics statement**

Female Wistar rats were obtained from the Rappolovo breeding facility (Rappolovo, Russia). Animal experiments were performed in accordance with the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act, 1986, and EU Directive 2010/63/EU for animal experiments. The protocol and procedures employed were ethically reviewed and approved by the Bioethics Committee of Udmurt State University (Date 25/04/2022/No. 2202).

**HDL apoproteins isolation from human or rat plasma**

Healthy human sera were obtained at the Republic Blood Transfusion Station (Izhevsk, Russia). Precipitation HDL apoproteins were performed as previously published [1]. Briefly, to serum was added 10% dextran sulfate to final concentrations 0.05% and 1 M MnCl2 to final concentrations 0.05 M, mixing for 10 minutes. With these concentrations, the LDL and VLDL are completely and selectively precipitated. The precipitate is then removed by centrifugation for 10 min at 6000 g. to the LDL- and VLDL-free supernatant are added 10% dextran sulfate to final concentrations 0.65% and 1 M MnCl2 to final concentrations 0.2 M. Precipitation beings immediately and is complete after 2 hr. The mixture is centrifuged at 15 000 g for 30 minutes. The supernatant is removed and the precipitate is washed in Tris-HCl buffer containing 0.1% dextran sulfate and 0.1 M $MnCl_2$, pH = 7.6. The washed precipitate which contains the HDL is dissolved by stirring the suspension by added Tris-HCl buffer containing 0.2% sodium citrate, 1% NaCl, 0.05 M EDTA and 0.05% Twin-20, pH = 8.2. The mixture is centrifuged at 10 000 g for 5 minutes to remove the white precipitate of manganese oxide.

Delipidation HDL-enriched fraction were performed as previously published [2]. Briefly, an equal volume butanol-diethyl ether (40:60, V/V) was added to HDL-enriched fraction and intensively stirred for 30 minutes. Organic phase was removed by centrifugation at 10 000 g for 10 minutes. Apoproteins containing fraction was applied to Superdex 200 10/300 column equilibrating with Tris-HCl buffer containing 0.05% Twin-20, pH = 8.2. Three fractions were obtaining, first – IgG, second – serum albumin, third – apoproteins. However, third fraction besides apoproteins contains IgG and albumin up to 20% of total protein. Purification apoproteins was performed on Albumin&IgG Depletion column. The purity of the isolated HDL apoproteins was tested by SDS-PAGE. The purity of the preparation was 95.2±3.7%. Isolation and purification of HDL apoproteins was performed on a chromatograph AKTA pure 25 M, provided by the Center for the Collective Use of Scientific Equipment, Udmurt State University.

**Immunization**

At 2 months of age, Wistar rats (n = 5) were immunized with human HDL (hHDL) apoproteins. hHDL apoproteins were administered as a single intradermal injection of 100 μg per rat in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich). Control rats received a subcutaneous injection of IFA (n = 5).

**ELISA of antibodies to rat or human HDL apoproteins (apo-HDL)**

Plates (Corning-Costar, Acton, MA, USA) were coated overnight at 4 °C with rat or human apo-HDL (20 μg/ml) in 0.15 mol/L PBS. Plates were blocked with 150 μL of 0.15 mol/L PBS/0.05% BSA/Tween-20. Serum samples were added in serial dilution with PBS/Tween-20 and incubated for 1 h at RT. The plates were then incubated for 1 h at RT with 100 μL of goat anti-rat Ig (IgG, IgM, IgA) conjugated to horseradish peroxidase (IMTEC, Russia). Then the substrate

mixture (5 mL citrate buffer solution [pH 5.0]/3 mg ortho-phenylenediamine/15 mL 3% $H_2O_2$) was added. Absorbances were read after 15 min at 492 nm.

**Tissue preparation and histology**

Rats were dissected 25 weeks after immunization, Rat aortic specimens were fixed for 24 hours in immunofix and embedded in paraffin for light microscopy. Cross-sections, 5-μm thick, were stained with hematoxylin and eosin.

**Immunohistochemistry**

The glass-mounted aorta sections were dewaxed and rehydrated by the standard procedure. For the antigen unmasking sections were autoclaved at 120 °C in a Tris-HCL buffer/0.01% sodium borohydride, pH 7.6, for 20 minutes. After autoclaving, the sections were cooled and treated for 5 minutes with a solution of Sudan black B to prevent autofluorescence caused by lipofuscin. The sections were washed three times in the PBS/Twin-20. Further, the sections were treated with a blocking solution containing 5% milk powder in PBS with Twin-20. Then the sections were incubated with FITC labeled antibodies specific to rat CD3 (Mybiosource, USA) overnight at +4 °C, washed three times in the PBS/Twin-20. $CD3^+$T lymphocytes were detected using an Olympus BX53 fluorescence microscope (Japan) provided by the Center for the Collective Use of Scientific Equipment, Udmurt Federal Research Center of the Ural Branch of the Russian Academy of Sciences.

## Results and discussion

Human HDL apoproteins used for rat immunization consisted of apoA1 and apoE proteins. Histological analysis of the aorta of Wistar rats immunized with hHDL apoproteins revealed leukocyte infiltration of the aortic wall (Figure 1A) and veins (Figure 1B). In rats injected with IFA, no leukocytes were found in the aortic wall (Figure 1C) or other vessels.

Immunohistochemical analysis showed that the intima and adventitia of the aorta were infiltrated with T lymphocytes in rats immunized with hHDL apoproteins (Figure 2A, B). Severe T lymphocytic infiltration were found in the all layers of the vein wall in hHDL apoprotein immunized rats (Figure 2C).

In the hHDL apoprotein immunized rats the production of antibodies to the immunogen was

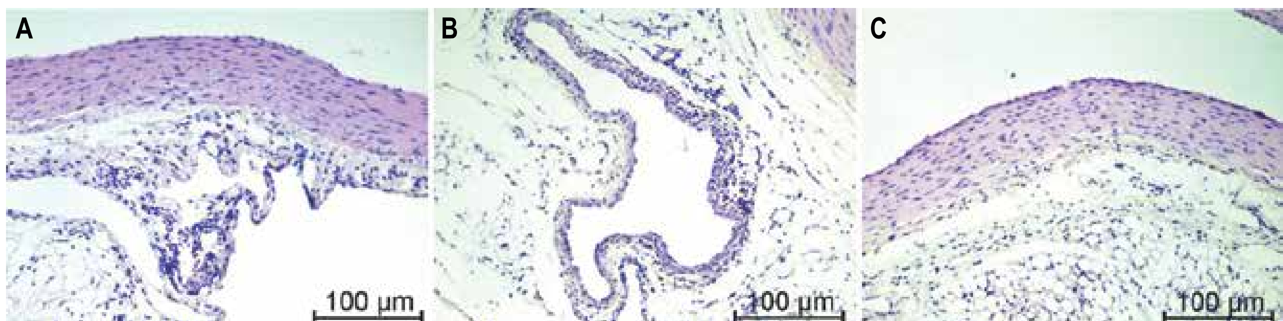


**Figure 1. Representative histological specimens of aorta and periaortic tissue of Wistar rats**

Note. H&E. A, aorta of rat immunized with hHDL apoproteins. B, leukocyte infiltration of vein of rat immunized with hHDL apoproteins. C, aorta of rat injected with IFA.

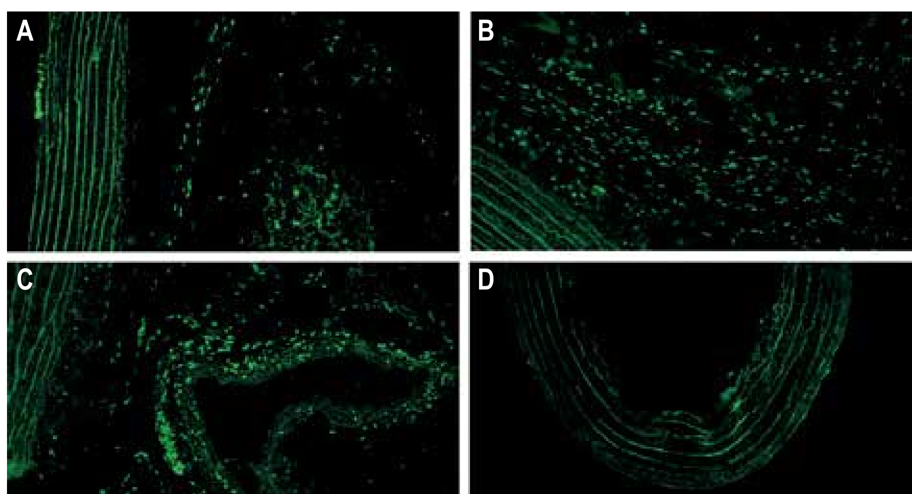


**Figure 2. Representative histological specimens of aorta and periaortic tissue stained with FITC anti-rat CD3 antibodies**

Note. T lymphocyte infiltrates were found in the intima (A), adventitia (B) of the aorta and in the all layers of the vein wall (C) of rats immunized with hHDL apoproteins. T lymphocytes are absent in the aorta (D) of rats injected with IFA.

observed (Figure 3). Their level continued to remain high 25 weeks after immunization. Autoantibodies to HDL apoproteins were not detected in rats immunized with hHDL apoproteins (Figure 3).

Thus, immunization with human HDL apoproteins resulted in T lymphocytic infiltration of the aortic wall and veins of Wistar rats.

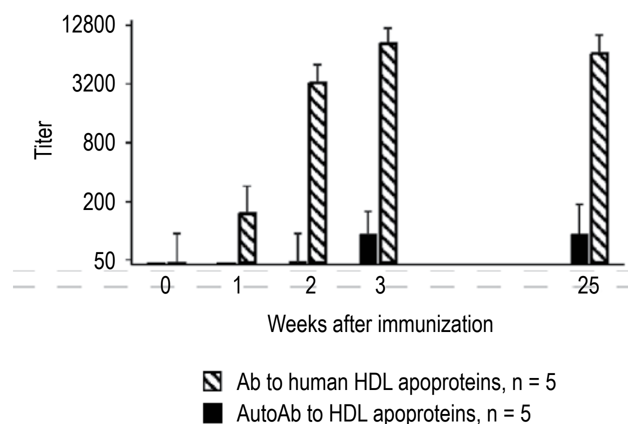


**Figure 3. Antibodies to human HDL apoproteins and autoantibodies to HDL apoproteins in Wistar rats immunized with hHDL apoproteins**

Note. Data are presented as mean ± SD.

## Conclusion

Lymphocytic infiltration of the aorta wall and vein wall is a sign of their inflammation. Since lymphocytic infiltration was detected 25 weeks after immunization, it can be assumed that autoimmune inflammation has become chronic. As is known, T lymphocytic infiltration is the first stage of autoimmune tissue damage in T cell mediated autoimmune diseases [12, 15]. Autoreactive T lymphocytes cause tissue inflammation, damage, or complete tissue destruction [3, 10]. Venulitis in hHDL apoprotein immunized rats was unexpected. Thus, human HDL apoprotein Immunization induces T cell-mediated inflammation in aorta and venulitis.

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*Поступила 15.04.2023*
*Отправлена на доработку 25.04.2023*
*Принята к печати 26.04.2023*

*Received 15.04.2023*
*Revision received 25.04.2023*
*Accepted 26.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 643-648

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 643-648

# ТРОМБОЦИТЫ ПРИ ХРОНИЧЕСКОЙ РЕАКЦИИ «ТРАНСПЛАНТАТ ПРОТИВ ХОЗЯИНА»: СВЯЗЬ С Th1/Th2-СООТНОШЕНИЕМ

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**Резюме.** Как обнаружено в клинических и лабораторных исследованиях, тромбоциты не только играют ключевую роль в процессах коагуляции и тромбообразования, но и способны активно участвовать в других патофизиологических процессах, в том числе и в развитии иммунных реакций. В частности, показано, что изменения в иммунной системе, приводящие к заболеванию системной красной волчанкой (СКВ), нередко сопровождаются изменениями числа тромбоцитов и их активности в периферической крови больных СКВ, коррелирующими с выраженностью клинических проявлений болезни. В предыдущие годы нами была детально исследована одна из стандартных экспериментальных моделей СКВ, основанная на индукции хронической реакции «трансплантат против хозяина» (хРТПХ) в полуаллогенной системе DBA/2 → (C57Bl/6 x DBA/2)$F_1$. Однако участие тромбоцитов в этом иммунопатологическом процессе исследовано не было, и в литературе нет данных о поведении тромбоцитов при хРТПХ или о связи их с состоянием Th1/Th2-баланса, хотя по аналогии с развитием СКВ у человека можно ожидать, что и в использованной нами экспериментальной модели количество тромбоцитов изменяется в соответствии с развитием хРТПХ. Поэтому целью данной работы было определение числа тромбоцитов в крови у мышей с Th1- и Th2-зависимыми вариантами хРТПХ.

В экспериментах были использованы самки мышей линий DBA/2 и гибридов (C57Bl/6 × DBA/2) $F_1$. Хроническую РТПХ в полуаллогенной системе индуцировали вводя спленоциты мышей DBA/2 мышам-гибридам B6D2$F_1$: по 60-70 × $10^6$ клеток в/в двукратно с интервалом в 6 дней. Исследуемые параметры оценивали через три месяца после начала эксперимента и формирования люпус-подобного гломерулонефрита у животных с Th2-зависимым вариантом хРТПХ.

Снижение количества эритроцитов и гемоглобина, уменьшение показателей гематокрита и параллельное увеличение количества ретикулоцитов в крови мышей с хРТПХ хорошо согласуется с ранее сделанным нами выводом о наличии у этих животных аутоиммунной гемолитической анемии. Было

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**Образец цитирования:**

*О.П. Колесникова, Е.В. Гойман, И.А. Орловская, Е.Н. Демченко, Н.Н. Вольский, Е.Д. Гаврилова «Тромбоциты при хронической реакции «трансплантат против хозяина»: связь с Th1/Th2-соотношением» // Медицинская иммунология, 2023. Т. 25, № 3. С. 643-648.*
*doi: 10.15789/1563-0625-BPI-2708*

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**For citation:**

*O.P. Kolesnikova, E.V. Goiman, I.A. Orlovskaya, E.N. Demchenko, N.N. Volsky, E.D. Gavrilova "Blood platelets in chronic graft-versus-host disease: association with Th1/Th2 ratio", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 643-648.*
*doi: 10.15789/1563-0625-BPI-2708*

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**DOI:** 10.15789/1563-0625-BPI-2708

обнаружено, что в отличие от других форменных элементов крови тромбоциты существенно возрастают при развитии хРТПХ, но о вторичном тромбоцитозе в данной модели СКВ можно говорить только в отношении Th2-зависимого варианта этого процесса, в то время как в группе с Th1-зависимым вариантом хРТПХ среднее количество тромбоцитов в крови не отличается от контрольной группы.

*Ключевые слова: хроническая реакция трансплантат против хозяина, системная красная волчанка, экспериментальная модель, тромбоциты, Th1/ Th2-баланс*

# BLOOD PLATELETS IN CHRONIC GRAFT-VERSUS-HOST DISEASE: ASSOCIATION WITH Th1/Th2 RATIO

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**Abstract.** As found in clinical and laboratory studies, platelets not only play a key role in the processes of coagulation and thrombosis, but are also able to actively participate in other pathophysiological processes, including the development of immune reactions. It has been shown that changes in the immune system leading to systemic lupus erythematosus (SLE) are often accompanied by changes in the number of platelets and their activity in the peripheral blood of SLE patients, which correlate with the severity of the clinical manifestations of the disease. Earlier we have studied the standard experimental model of SLE in detail, based on the induction of chronic graft-versus-host disease (cGVHD) in the semi-allogeneic system DBA/2 → $(C57Bl/6 \times DBA/2)F_1$. However, the participation of platelets in this immunopathological process has not been studied. There are no data in the literature on the behavior of platelets in cGVHD or on their relationship with the state of Th1/Th2 balance. It can been expected that the platelet count changes according to the development of cGVHD in the used experimental model by analogy with the development of SLE in humans.

In the experiments, we used female mice of the DBA/2 strain and $(C57Bl/6 \times DBA/2)F_1$ hybrids. Chronic GVHD in a semi-allogeneic system was induced by injecting DBA/2 mouse splenocytes into $B6D2F_1$ hybrid mice: $60\text{-}70 \times 10^6$ cells intravenously twice with an interval of 6 days. The studied parameters were evaluated three months after the start of the experiment and the formation of lupus-like glomerulonephritis in animals with Th2-dependent cGVHD variant.

A decrease in the number of erythrocytes and hemoglobin, a decrease in hematocrit and a parallel increase in the number of reticulocytes in the blood of mice with cGVHD are in good agreement with our earlier conclusion that these animals have autoimmune hemolytic anemia. It was found that, platelets increase significantly with the development of cGVHD unlike other blood cells. Secondary thrombocytosis is observed in the case of the Th2-dependent variant of cGVHD in this model of SLE, while in the group with the Th1-dependent variant of cGVHD, the average number of platelets in the blood does not differ from the control group.

*Keywords: chronic graft-versus-host disease, systemic lupus erythematosus, experimental model, platelets, Th1/Th2 balance*

The investigation was financed from the federal budget to fulfill the state assignment on the research work "The study of the immunopathogenesis of phenotypes of socially significant human diseases, polymorbid diseases, the development of new methods for their personalized diagnosis and treatment" (state registration No. 122012000366-9).

## Introduction

The results of numerous clinical and laboratory studies convincingly indicate that platelets, blood cells that are well studied in relation to their direct participation in the processes of coagulation and thrombosis, can play a significant role in other pathophysiological processes, including the development of immune reactions [4, 7]. In particular, it has been found that changes in the immune system leading to systemic lupus erythematosus (SLE) are often accompanied by changes in the number of platelets and their activity in the peripheral blood of SLE patients, which correlate with the severity of the clinical manifestations of the disease [1, 8, 12].

In previous years, we have studied in detail one of the standard experimental models of SLE, based on the induction of chronic graft-versus-host disease (cGVHD) in the semi-allogeneic system DBA/2 → (C57Bl/6 x DBA/2)F1 [3, 5]. It was found that the immunopathological process caused by the transfer of lymphocytes from one of the parental lines to hybrid mice can proceed both according to the Th2-dependent developmental variant with the formation of lupus-like immunocomplex glomerulonephritis, and according to the Th1-dependent developmental variant – without nephritis, but with manifestations of severe immunodeficiency. As we have described earlier [5, 11], an essential pathological component of cGVHD in this system is the presence of autoimmune hemolytic anemia caused by the production of large amounts of autoantibodies to own erythrocytes by activated B cells. However, the participation of platelets in this immunopathological process has not been studied, and there are no literature data on the behavior of platelets in cGVHD or on their relationship with the state of Th1/Th2 balance.

Based on the above-mentioned data obtained in clinical studies of patients with SLE, it can be expected that in our experimental model the number of platelets changes in accordance with the development of cGVHD.

Therefore, **the aim of present work** was to determine the number of platelets in the blood of mice with Th1- and Th2-dependent developmental variants of the immunopathological process.

## Materials and methods

In the experiments, we used female mice of the DBA/2 strain and (C57Bl/6 × DBA/2)F1 hybrids obtained from the SPF vivarium of the Institute of cytology and genetics SB RAS (Novosibirsk). The animals were kept under standard vivarium conditions in accordance with the rules adopted by the European Convention for the Protection of Animals Used for Experimental Purposes (Strasbourg, 1986). The study was approved by the RIFCI ethics committee (protocol No. 92 dated November 10, 2015).

Chronic GVHD in the semi-allogenic system was induced according to the standard regimen by inoculating splenocytes from DBA/2 mice to B6D2F1 hybrid mice: 60-70 × $10^6$ cells intravenously twice with an interval of 6 days [6]. The outcome of the immunopathological process in individual animals was determined 3 months after the induction of cGVHD by measuring the level of protein in the urine: with proteinuria of 3 mg/mL or more, mice were classified as a Th2-dependent variant of cGVHD, leading to the formation of glomerulonephritis, in the presence of protein in urine less than 3 mg/mL – to a Th1-dependent variant of the disease.

The cellular composition of peripheral blood, hematocrit and the content of hemoglobin was assessed using a PCE-90 hematological analyzer (ERMA Inc., Japan). Statistical processing of the results was carried out by nonparametric statistics using the Mann-Whitney test.

## Results and discussion

The results of determining the indicators characterizing the state of blood cells in mice with Th1- and Th2-dependent variants of the development of cGVHD are presented in Table 1.

A decrease in the number of erythrocytes and hemoglobin, a decrease in hematocrit and a parallel increase in the number of reticulocytes in the blood of mice with cGVHD is in good agreement with our earlier conclusion that these animals have autoimmune hemolytic anemia [5, 11]. In our previous studies, this conclusion was also confirmed by high Coombs test values, indicating the production of a large number of anti-erythrocyte antibodies during the development of cGVHD, and an increase in the amounts of erythroid precursors in the bone marrow, which

**TABLE 1. PERIPHERAL BLOOD PARAMETERS IN MICE WITH TH1- AND TH2-DEPENDENT CGVHD VARIANTS 3 MONTHS AFTER DONOR CELL TRANSFER**

| Group | Leucocytes, $10^6$/mL | Erythrocytes, $10^9$/mL | Hemoglobin, g/100 mL | Hematocrit, % | Reticulocytes, pro mille |
|---|---|---|---|---|---|
| **Control (intact $BDF_1$) (n = 12)** | 11.1 | 7.13 | 17.9 | 49.5 | 10.2 |
| **Th1-dependent variant of cGVHD (n = 11)** | 8.4* | 7.04 | 16.6 | 46.4 | 15.9* |
| **Th2-dependent variant of cGVHD (n = 13)** | 13.8# | 5.9* # | 15.0* # | 40.5* # | 21.3* # |

Note. *, significant difference from the control group ($p < 0.01$); #, significant difference from Th1-dependent variant of cGVHD ($p < 0.02$).

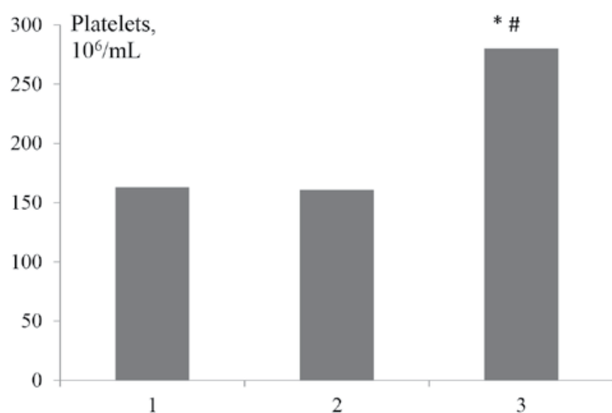


**Figure 1. Number of platelets in the blood of mice with cGVHD**

Note. 1, control (intact $BDF_1$) (n=12); 2, Th1-dependent variant of cGVHD (n = 11); 3, Th2-dependent variant of cGVHD (n = 13). *, statistically significant difference from the control group (p < 0.001); #, statistically significant difference from Th1-dependent variant of cGVHD (p < 0.02).

directly indicates the stimulation of erythropoiesis in these animals [5].

The data in Table 1 also shows that the manifestations of autoimmune hemolytic anemia are noticeably more pronounced in the group of mice with Th2-dependent variant of the development of cGVHD, compared with animals in which a relatively greater Th1 lymphocytes influence was observed on the development of the immunopathological process. This was to be expected, since it is Th2 cells that have a stimulating effect on the polyclonal activation of B lymphocytes and, accordingly, on the mass production of anti-erythrocyte autoantibodies.

Platelets increase significantly with the development of cGVHD unlike other blood cells. The data obtained in our experiments on the increased amount of platelets in the blood of experimental mice are shown in Figure1.

It can be seen that the presence of secondary thrombocytosis in cGVHD can be said only in relation to the Th2-dependent variant of this process, while in the group with Th1-dependent variant of cGVHD, the average number of platelets in the blood does not differ from the control group.

In a literature review based mainly on data from clinical studies [9], among the common causes leading to the occurrence of secondary thrombocytosis, autoimmune hemolytic anemia and chronic inflammation are indicated. It is precisely these manifestations of the pathological process that are characteristic signs of cGVHD in the case studied by us, which allows us to assume their pathogenetic role in the formation of thrombocytosis in this experimental model of SLE. As mentioned above, the anemia severity is significantly higher in the group with Th2-dependent variant of the development of cGVHD, which may be the cause of thrombocytosis in these mice.

In addition, the same group is characterized by the development of an active inflammatory process, lupus-like glomerulonephritis, which does not occur in mice with Th1-dependent cGVHD. Previously, we found that in the serum of mice with Th2-dependent variant of cGVHD, the concentration of IL-6 was doubled (compared to control), while in animals with Th1-dependent variant, this figure was even lower than in controls [6]. Since this cytokine is one of the main stimulators of platelet formation in the bone marrow [9], it may be the most important factor in the onset of thrombocytosis in our model.

It is assumed that an increase in the number of platelets in the blood of animals in our experimental model is a by-product of the main immunopathological processes that determine the development of cGVHD. However, it cannot be ruled out that changes in the number of these cells and their activity may also play a certain pathogenetic role in the formation of an SLE-like syndrome in this model. Many researchers point to the participation of platelets in immunopathological processes. Thus, in their work [2], performed on a large group of patients with SLE, the authors, on the basis of statistical processing of the obtained results, argue that the amount of platelet microparticles present in the blood of patients, which form immune complexes with IgG, correlates with the clinical manifestations of the disease and can be used to prediction of its course. A review of the literature on the role of platelets in the development of immune-mediated inflammatory diseases such as SLE or rheumatoid arthritis describes a variety of pathophysiological mechanisms that link platelet activity with cells of the immune system and with immune reactions [10].

Another recent work [8] describes the release by platelets from patients with SLE of microparticles containing extracellular mitochondrial DNA, which, according to the authors of the article, can play the role of an autoantigen in the development of this disease. Similar phenomena can be expected in the SLE model we studied, since, as we showed earlier, the concentration of extracellular DNA in the blood of mice with Th2-dependent variant of chronic GVHD noticeably increases [3].

## Conclusion

Thus, the observed increase in the number of platelets in the blood of mice with cGVHD can be a starting point for a deeper study of the pathogenesis of immune system dysfunctions in this experimental model of SLE.

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*Поступила 10.04.2023*
*Отправлена на доработку 13.04.2023*
*Принята к печати 26.04.2023*

*Received 10.04.2023*
*Revision received 13.04.2023*
*Accepted 26.04.2023*

*Медицинская иммунология 2023, Т. 25, № 3, стр. 649-654*

*Краткие сообщения Short communications*

*Medical Immunology (Russia)/ Meditsinskaya Immunologiya 2023, Vol. 25, № 3, pp. 649-654*

# МИКРОСКОПИЧЕСКИЙ И ЭЛЕМЕНТНЫЙ АНАЛИЗ КОСТНОЙ ТКАНИ ЧЕЛЮСТИ ПРИ ТРАВМЕ

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**Резюме.** Поскольку костная ткань челюсти подвергается травматическому воздействию на этапе внедрения металлического дентального имплантата, актуальной остается проблема развития воспалительных осложнений, приводящих к срыву остеоинтеграции. Представляют интерес иммунологические механизмы развития воспалительного процесса при эмиссии наноразмерных металлосодержащих частиц, а также механизмы его стихания после удаления металлического объекта из костной ткани. В работе проведен микроскопический и элементный анализ сегмента костной ткани нижней челюсти крысы линии Wistar после искусственной травматизации. В ходе эксперимента моделировали процесс нахождения металлического инородного тела в костном ложе. Для этого в соединительнотканное соединение нижней челюсти крысы вводили инсулиновую иглу, с последующим ее извлечением через семь дней. Микроскопический анализ костной ткани проводили методом растровой электронной микроскопии с помощью прибора Tesscan Vega 4 с системой EDX Oxford Xplore 30. По данным электронной микроскопии шлифа челюсти крысы в области лунок нижних резцов при малом увеличении в прямой проекции определяются: поверхность кортикального слоя альвеол зубов, хрящевое и соединительнотканное соединение челюстей с разрывом, расслоение хрящевого слоя. В области хрящевого и соединительнотканного соединения альвеолярных отростков челюстей при большем увеличении в прямой проекции находятся плотноструктурные кристаллические включения, очаги некротизации. Элементный состав костной ткани был получен методом атомно-эмиссионной спектроскопии (прибор – эмиссионный спектрометр iCAP 6300 Duo). В исследуемом образце количественное соотношение кальция и фосфора составило 1,68, что незначительно превышает оптимальное, равное 1,67. Изменение данного соотношения в сторону увеличения говорит о

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**Образец цитирования:**

*Н.Н. Катаева, Н.Г. Саркисян, Н.С. Чумаков, К.А. Хлыстова, О.М. Медведева, А.С. Шмыгалев «Микроскопический и элементный анализ костной ткани челюсти при травме» // Медицинская иммунология, 2023. Т. 25, № 3. С. 649-654.*
*doi: 10.15789/1563-0625-MAE-2738*

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**For citation:**

*N.N. Kataeva, N.G. Sarkisyan, N.S. Chumakov, K.A. Khlystova, O.M. Medvedeva, A.S. Shmygalev “Microscopic and elemental analysis of jaw bone tissue in injury”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 649-654.*
*doi: 10.15789/1563-0625-MAE-2738*

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**DOI:** 10.15789/1563-0625-MAE-2738

снижении уровня фосфора в костной ткани, что можно интерпретировать как локальный остеопороз. Кроме того, обнаружены элементы: Bi, Ga, Pb, Ti, Zn в количестве 0,03-0,06 массовых процентов. Перечень этих элементов соответствует химическому составу инсулиновой иглы, что свидетельствует о проникновении металлических частиц в ткани костного ложа. Эмиссия наноразмерных частиц и их последующее объединение до микро- и субмикронных размеров, их персистенция, а также биокоррозия в зонах активного костеобразования могут являться пусковым механизмом для развития асептического воспалительного процесса. Этот эффект обусловлен как прямым повреждающим фактором, так и опосредованным воздействием, через специфичные сигнальные молекулы, вырабатываемые в ответ на повреждение тканей.

*Ключевые слова: периимплантит, имплантация, наноразмерные частицы, воспаление, РЭМ-микроскопия, ремоделирование кости*

# MICROSCOPIC AND ELEMENTAL ANALYSIS OF JAW BONE TISSUE IN INJURY

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**Abstract.** Since the jawbone tissue is injured at the stage of metal dental implant introduction, the problem of inflammatory complication development leading to a breakdown in osseointegration remains relevant. Of interest are the immunological mechanisms of inflammatory process development during the emission of metal nanoparticles, as well as the mechanisms of its subsidence after the removal of a metal object. Microscopic and elemental analysis of the bone tissue of the Wistar rat lower jaw after artificial traumatization was carried out. During the experiment, the situation of presence of a metal foreign body in the bone bed was simulated. An insulin needle was injected into the connective tissue of the lower jaw, followed by its removal after seven days. Microscopic analysis of bone tissue was performed using a Tesscan Vega 4 scanning electron microscope. The teeth alveoli cortical layer surface, connection of the jaws with a gap, stratification of the cartilaginous layer were determined at low magnification in direct projection. Using higher magnification in the direct projection there are visible dense-structural crystalline inclusions, foci of necrosis in the area of junctions of the jaws alveolar processes. The elemental composition of bone tissue was obtained by atomic emission spectroscopy by iCAP 6300 Duo. In the test sample, the quantitative ratio of calcium and phosphorus was 1.68, which slightly exceeds the optimal value of 1.67. An upward change in this ratio indicates a decrease in phosphorus level, which can be interpreted as local osteoporosis. In addition, the following elements were found: Bi, Ga, Pb, Ti, Zn in the amount of 0.03-0.06 mass percent. The list of these elements corresponds to the chemical composition of an insulin needle, indicating the penetration of metal particles into bone bed tissues. The emission of nanoparticles and their subsequent association to micro- and submicron sizes, their persistence, as well as biocorrosion in areas of active bone formation can be a trigger for the development of an aseptic inflammatory process. This effect is due to both a direct damaging factor and an indirect effect through specific signal molecules produced in response to tissue damage.

*Keywords: periimplantitis, dental implantation, nanoparticles, inflammation, scanning electron microscope, bone remodeling*

## Introduction

Traumatization of tissue structures in the body, in particular bone tissue, is always accompanied by the development of an inflammatory process. The study of post-traumatic bone changes is an important step in assessing, among other things, the immunological mechanisms of inflammation. In this case, the nature of the mechanical action plays an important role.

Dental implantation is a surgical intervention that involves the formation of a mechanical bone defect, followed by the introduction of a metal implant into the injured bone. Currently the causes of chronic inflammatory complications in the long-term functioning of dental implants are being actively studied. [4]. One of the reasons for the development of such complications is probably the release of nanoparticles of the oxide layer of the dental implant in the tissue of the bone bed [2, 7]. The accumulation and conglomeration of such particles, as well as their biocorrosion, can probably be a personalized cause of the development of mucositis and peri-implantitis, which is associated with the inability of the cells of the immune system to timely utilize these particles, preventing their conglomeration, and their early death.

It is of interest to study the immunological aspect of bone repair after removal of a metal foreign body against the background of an inflammatory process. Particularly interesting are the immunological mechanisms of the development of the inflammatory process during the emission of nanoparticles, as well as the mechanisms of its remitting after the removal of a metal object from the bone tissue.

The purpose of the study was to study electron microscopy and elemental composition of the jaw bone tissue after traumatization with a metal implant in order to identify the causes of the development of a persistent inflammatory process, as well as the likelihood of participation of nanoparticles in triggering the immunological mechanisms of implant rejection.

## Materials and methods

The first stage of the research work was carried out on the basis of the Institute of Immunology and Physiology of Ural Branch, the Russian Academy of Sciences, Yekaterinburg. There was simulated the situation of presence a metal foreign body in the bone bed to assess the state of the bone tissue after a mechanical injury. To do this, an insulin needle 12 mm long was inserted into the connective tissue joint of the lower jaw of the Wistar rat, followed by its removal after seven days. A month after the removal of the foreign body, rats were taken out of the experiment to isolate the studied bone segment. All painful procedures were performed in accordance with the WMA Declaration of Helsinki on animal use in biomedical research.

Further, the study of the bone tissue sample was carried out in the laboratory of the Center for Collective Use of the Institute of High Temperature Electrochemistry of Ural Branch, the Russian Academy of Sciences, Yekaterinburg. Microscopic analysis was performed by scanning electron microscopy (SEM microscopy) using a Tesscan Vega 4 scanning microscope with an EDX Oxford Xplore 30 system. The bones were preliminarily poured into Struers Epofix epoxy resin, then a slice was prepared on an Allied metprep 4 grinding and polishing machine. The elemental composition of bone tissue was obtained by atomic emission spectroscopy (instrument – emission spectrometer iCAP 6300 Duo, Thermo Scientific, UK). Range of determined elements: Li – N, Na – S, K – Se, Rb – Mo, Ru – I, Cs – Nd, Sm – Bi, Th, U, Pu.

## Results and discussion

Figures 1-3 show the data of SEM microscopy of a segment of the bone tissue of the lower jaw of a rat in different projections and scales.

In the studied segment of bone tissue, 67 chemical elements were determined by atomic emission spectroscopy. The highest mass fraction belongs to the following elements: calcium (22.13%), phosphorus (13.14%), magnesium (0.69%), sodium (0.58%), sulfur (0.16%) because these are the basic elements of any mineralized bone tissue, including jaw bone tissue [6]. The skeletal system actively responds to the influence of various environmental and endogenous factors. It is known that the state of the body's immune defense also affects the morphogenesis of the skeletal system. Calcium and phosphorus are the main elements of any bone tissue, the ratio of which is an indicator of the quality of mineralization. For bones, the optimal ratio of calcium and phosphorus is 1.67, which indicates a normal degree of bone mineralization. In the studied sample of bone tissue, this ratio is 1.68. Even such a slight change in the Ca/P balance indicates the occurrence of pathological processes leading to the impossibility of normal repair. An upward change in this ratio indicates a decrease in the level of phosphorus in the bone tissue, which can be interpreted as local osteoporosis. This state of the peri-implant area prevents normal bone tissue remodeling and provokes the development of bone tissue resorption processes.

In addition, elemental analysis showed the presence of foreign elements in the bone tissue, the amount of which corresponds to the range from 0.01% to 0.06%. Basically, these are elements that form simple substances, metals: bismuth, gallium,

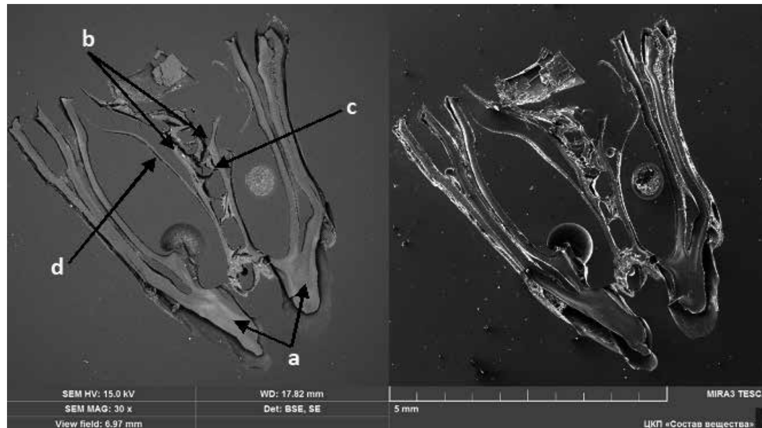


**Figure 1. SEM microscopy of a slice of a rat jaw in the area of the holes of the lower incisors at low magnification in direct projection seven days after the needle was inserted into the connective tissue joint of the jaws**

Note. There are determined the surface of the cortical layer of the alveoli of the teeth (a), cartilaginous connection (b) and connective tissue connection (c) of the jaws with a gap, stratification of the cartilaginous layer (d).

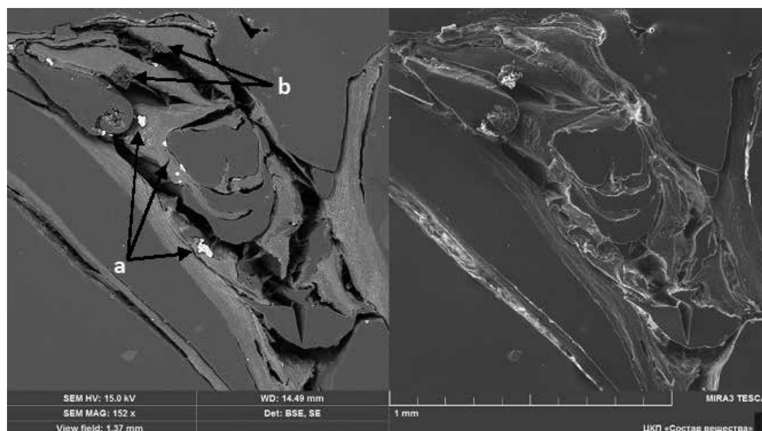


**Figure 2. SEM microscopy of a slice of a rat jaw in the area of the cartilaginous and connective tissue junction of the alveolar processes of the jaws at a higher magnification in the direct projection**

Note. There are determined indurated inclusions (a), foci of necrotization (b).

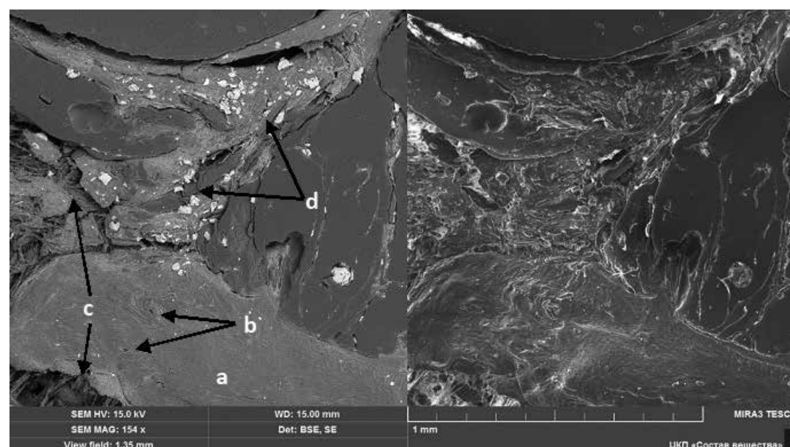


**Figure 3. SEM microscopy of a section of a rat jaw in the area of the socket of the lower incisor at a higher magnification in the side projection**

Note. There are determined part of the remaining cortical layer of the alveolus (a), pores (b), areas of cancellous bone (c), small and large indurated inclusions (d).

lead, iridium, titanium, zinc, mercury, molybdenum. The list of these metals corresponds to the chemical composition of the insulin needle in contact with bone tissue during injury. Insulin needles are made of steel, which includes alloying elements: Si, S, P, Bi, Pb, Ti, Zn and others. Alloy steel has improved technical characteristics: corrosion resistance, refractoriness, high hardness, etc.

Since the chemical composition of the jaw bone depends on many factors, for example, the quality of nutrition, the degree of mechanical stress, the physiological state of the body [1, 5], in addition to the indicated elements, the content of other elements in the range of $10^{-3}$-$10^{-4}$ mass percent was identified in the test sample.

It should be taken into account that any surgical intervention, in particular dental implantation, triggers a cascade of immunological mechanisms induced by immunocompetent cells due to their contact with the implant surface. The severity and duration of the immune response affects the quality of osseointegration. Isolation of inflammatory factors by immunocompetent cells determines the composition of the microenvironment and, as a consequence, the vector of development of the immune response.

Particular attention is drawn to the mechanisms of maintaining the immunological balance in the area of implantation. In particular, the degree of migration of neutrophils, thrombocytes and macrophages producing growth factors, cytokines and chemokines that promote the migration of other immune cells involved in the processes of phagocytosis and stimulation of the reparative process. There are two macrophage phenotypes: M1 – pro-inflammatory, producing IL-1β, IL-6 and TNF and inducing differentiation of osteoclasts; and M2 – anti-inflammatory, producing IL-4, IL-10, IL-13 and TGF-β, affecting the reactions of healing and bone tissue remodeling. It is likely that the emission and biocorrosion of nanoparticles into the area around the implant can cause a shift in the immunological balance towards the chronicity of the inflammatory process, which may be due to provoking the death of immunocompetent cells, incomplete processing of particles, the release of pro-inflammatory cytokines and the formation of a vicious circle.

The presence of microparticles in the bone tissue in contact with a metal foreign body indicates the possibility of provoking the development of an inflammatory process by nanoparticles and their conglomerates. The formation of complexes (conglomerates) of nanoparticles and their enlargement to micro- or submicron sizes, with the inability of immunocompetent cells to timely utilization and subsequent migration, can lead to their accumulation and provoke the occurrence of chronic aseptic inflammation. Achievement of CDNanoMP (Critical Dose of NanoMetal Particles) leads to early death of the immune system's own cells [3]. In this case, microbial contamination takes second place as an etiological factor in the occurrence of a breakdown in previously achieved osseointegration, i.e., tissue homeostasis provided by immunotolerance.

## Conclusion

The emission of nanoparticles and their subsequent association to micro- and submicron sizes, their persistence, as well as biocorrosion in the areas of bone tissue remodeling can be a trigger for the development of an aseptic inflammatory process. This effect is due to both a direct damaging factor and an indirect effect through specific signal molecules produced in response to tissue damage.

## Acknowledgments

The authors are grateful to the director of Immunology and Physiology of Ural Branch, the Russian Academy of Sciences, Doctor of Physics and Mathematics Solovyova O.E. for providing the opportunity to conduct research on the basis of the institution.

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*Поступила 12.04.2023*
*Отправлена на доработку 14.04.2023*
*Принята к печати 17.04.2023*

*Received 12.04.2023*
*Revision received 14.04.2023*
*Accepted 17.04.2023*

*Медицинская иммунология
2023, Т. 25, № 3,
стр. 655-664*

*Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 655-664*

***Краткие сообщения
Short communications***

# КЛИНИКО-ИММУНОЛОГИЧЕСКИЙ АНАЛИЗ ЭФФЕКТИВНОСТИ ЛОКАЛЬНОГО ПРИМЕНЕНИЯ ВИТАМИНА $D_3$ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ КОЛИТЕ

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**Резюме.** Патогенез воспалительных заболеваний кишечника до конца не изучен, а используемые средства терапии имеют побочные эффекты, ограничивающие их применение.

Целью данного исследования является проведение клинико-иммунологического анализа эффективности применения витамина $D_3$ в составе оригинальных ректальных суппозиториев при экспериментальном колите (ЭК).

ЭК моделировали оксазолоном. Оригинальные суппозитории с витамином $D_3$ в 3-й группе, и 5-АСК в 4-й группе применяли *per rectum*. Клинику оценивали по шкале Disease activity index. В очаге повреждения толстой кишки определяли экспрессию МРО и TNFα, содержание нейтрофилов, лимфоцитов, эозинофилов, гистиоцитов, плазмоцитов, фибробластов, язвенный дефект, tissue damage index. Исследование проводили на 2-е, 4-е и 6-е сутки.

При ЭК на все сутки повышается DAI, в очаге повреждения увеличивается МРО и TNFα, фиксируется язвенный дефект, нейтрофильно-лимфоцитарная инфильтрация, увеличивается TDI. При сравнении морфометрических параметров зоны альтерации при ЭК в условиях применения витамина $D_3$ в отличие от применения 5-АСК выявлено на 2-е сутки снижение количества лимфоцитов, увеличение фибробластов, на 4-е сутки уменьшение количества плазмоцитов и увеличение фибробластов, на 6-е сутки увеличение количества гистиоцитов и фибробластов. Диаметр язвенного дефекта и индекс TDI не имеют значимых различий между сравниваемыми группами. При сравнении эффективности применения витамина $D_3$ в отличие от применения с 5-АСК содержание МРО выше на 6-е сутки, содержание TNFα – на 4-е сутки.

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**Образец цитирования:**

*М.С. Бойко, М.В. Осиков, А.А. Федосов, И.В. Грекова «Клинико-иммунологический анализ эффективности локального применения витамина $D_3$ при экспериментальном колите» // Медицинская иммунология, 2023. Т. 25, № 3. С. 655-664.
doi: 10.15789/1563-0625-CAI-2685*

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**For citation:**

*M.S. Boyko, M.V. Osikov, A.A. Fedosov, I.V. Grekova “Clinical and immunological analysis of the effectiveness of local application of vitamin $D_3$ in experimental colitis”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 655-664.
doi: 10.15789/1563-0625-CAI-2685*

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**DOI:** 10.15789/1563-0625-CAI-2685

При ЭК эффекты применения ректальных суппозиториев с витамином $D_3$ на клинические признаки, размер язвенного дефекта, содержание MPO и TNFα в очаге повреждения сопоставимы с эффектами от применения ректальных суппозиториев с 50 мг 5-АСК; более выражены в отношении динамики клеточного состава очага повреждения толстой кишки.

*Ключевые слова: экспериментальный колит, витамин $D_3$, индекс активности болезни, MPO, TNFα, 5-аминосалициловая кислота*

# CLINICAL AND IMMUNOLOGICAL ANALYSIS OF THE EFFECTIVENESS OF LOCAL APPLICATION OF VITAMIN $D_3$ IN EXPERIMENTAL COLITIS

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**Abstract.** The pathogenesis of inflammatory bowel diseases has not been fully studied, and the therapies used have side effects that limit their use.

The purpose of this study is to conduct a clinical and immunological analysis of the effectiveness of vitamin $D_3$ in the original rectal suppositories in experimental colitis (EC).

EC was modeled with oxazolone. Original suppositories with vitamin $D_3$ in group 3 and 5-ASA in group 4 were used *per rectum*. The clinic was evaluated on the Disease activity index scale. The expression of MPO and TNFα, the content of neutrophils, lymphocytes, eosinophils, histiocytes, plasmocytes, fibroblasts, ulcerative defect, tissue damage index were determined in the focus of colon injury. The study was carried out on days 2, 4 and 6.

With EC, DAI increases for the entire day, MPO and TNFα increase in the lesion, ulcerative defect is fixed, neutrophil-lymphocytic infiltration increases, and TDI increases. When comparing the morphometric parameters of the alteration zone in EC under the conditions of vitamin $D_3$ use, in contrast to the use of 5-ASA, a decrease in the number of lymphocytes, an increase in fibroblasts was revealed on day 2, a decrease in the number of plasmocytes and an increase in fibroblasts on day 4, an increase in the number of histiocytes and fibroblasts on day 6. The diameter of the ulcerative defect and the TDI index have no significant differences between the compared groups. When comparing the effectiveness of vitamin $D_3$, in contrast to the use of 5-ASA, the MPO content is higher on day 6; the TNFα content is higher on day 4.

In EC, the effects of using rectal suppositories with vitamin $D_3$ on clinical signs, the size of the ulcerative defect, the content of MPO and TNFα in the lesion are comparable to the effects of using rectal suppositories with 50 mg of 5-ASA; more pronounced with respect to the dynamics of the cellular composition of the lesion of the colon.

*Keywords: experimental colitis, vitamin $D_3$, disease activity index, MPO, TNFα, 5-aminosalicylic acid*

## Introduction

Inflammatory bowel disease (IBD) is a long–term, chronic, inflammatory-destructive and progressive lesion of the gastrointestinal tract that occurs under the influence of trigger factors on a genetically predisposed organism, which requires constant therapy during exacerbation and maintenance therapy in remission.

The urgency of the problem is caused by the defeat of IBD of young, able-bodied people (the first age peak is 20-30 years old), as well as people of pre-retirement age (the second age peak is 50-60 years old) and the associated temporary or complete disability [5]. In recent years, there has been a tendency in a number of countries to increase the incidence of IBD in childhood and among people over 65 years of age [7]. The severity of IBD is determined by severe complications: intestinal (toxic megacolon, massive bleeding, perforation of the intestinal wall, colorectal cancer) and extra-intestinal (anemia, arthritis, sacroiliitis, hepatitis, cirrhosis of the liver, gangrenous

pyoderma, iritis, uveitis, episcleritis, thrombosis, etc.) [5].

In the pathogenesis of IBD, both Th2-dependent reactions involving IgM, IgG, and Th1-dependent reactions with increased production of IL-8, TNFα, etc. are important in the destruction of the intestinal wall. cytokines, activation of chemotaxis, absorption, killing activity of neutrophils, monocytes/macrophages, production of reactive oxygen species (ROS) and nitrogen. These changes at the morphological level lead to damage to the distal parts of the colon, destruction of intestinal crypts, hyperplasia of goblet cells, ulceration in the mucous membrane, fibrosis, which is clinically manifested by tenesmus, changes in stool consistency, admixture of blood in feces, body weight deficiency, and other symptoms, including intestinal and extra-intestinal complications [6].

IBD requires constant pharmacocorrection and lifelong administration of medications to maintain remission, many of which (derivatives of 5-aminosalicylic acid, glucocorticosteroids, immunosuppressors, genetically engineered biological drugs) have severe side effects, which reduces compliance in patients, leads to intolerance and ineffectiveness of therapy in at least 30% of patients [14]. Vitamin $D_3$, which has pleiotropic properties, including antioxidant, anti-inflammatory, immunomodulatory, etc., is of particular interest as a new therapeutic agent [13]. The use of vitamin $D_3$ in multiple sclerosis and psoriasis limits the severity of the inflammatory process and clinical manifestations due to an increase in IL-10 production and the amount of Treg in the blood, a change in the Th1/Th2 balance towards a Th2-dependent immune response [6]. In rheumatoid arthritis, vitamin $D_3$ inhibits Th17 activity and IL-17 production [1].

These facts are a prerequisite for the use of vitamin $D_3$ in IBD [4]. At the moment, there are no dosage forms with vitamin $D_3$ in the Russian Federation for local use *per rectum*, taking into account the impact on the focus of inflammation and the damaged area of the colon in IBD. We have developed original rectal suppositories with vitamin $D_3$ based on a 10% aqueous solution of vitamin $D_3$ (Patent 20.05.2019). We have previously demonstrated that vitamin $D_3$ in the original rectal suppositories in experimental colitis (EC) has a local antioxidant effect by limiting the formation of POL and OMB products; a systemic immunotropic effect by reducing the number of neutrophils, restoring the absorption and HCT-reducing activity of neutrophils, reducing the number of lymphocytes, including $CD3^+$ and $CD45RA^+$, reducing the concentration of IgM, IgG, IL-6 and IL-8 [8]. We believe that the systemic immunotropic effects of vitamin $D_3$ in the composition of the original rectal suppositories in EC are due to its local protective effect in the focus of damage to the colon.

**The aim of the study** was a clinical and immunological analysis of the effectiveness of vitamin $D_3$ in the original rectal suppositories in experimental colitis.

## Materials and methods

The study was performed on mature male rats weighing 220-230 g of the Wistar line obtained from the federal state budgetary institution “Nursery of Laboratory Animals “Rappolovo” (SIC “Kurchatov Institute” – PLZH “Rappolovo”), under supervision in the experimental biological clinic of the Federal State Educational Institution of the Ministry of Health of the Russian Federation in compliance with the rules of good laboratory practice (order of the Ministry of Health of the Russian Federation No. 199n of 01.04.2016), Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, with free access to food and water, on a standard diet. The organization of the study was approved by the Ethics Committee of the South Ural State Medical University, Protocol No. 11 of 27.12.2017, Protocol No. 3 of 14.03.2022.

Using simple randomization, 70 animals were divided into groups: I-I (n = 7) – intact control, II-I (n = 21) – animals with experimental colitis (EC), III-I (n = 21) – animals with EC under conditions of rectal administration of vitamin $D_3$ every 12 hours before withdrawal from the experiment on day 6, IV (n = 21) – animals with EC, under conditions of rectal use of 5-aminosalicylic acid (5-ASA) every 12 hours before withdrawal from the experiment on day 6.

EC was modeled by two-stage application of a 3% alcohol solution of oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-oh). At the first stage, cutaneous sensitization was carried out by applying 675 μl/kg to the interscapular area, at the second – rectal injection of 675 μl/kg into the colon to a depth of 8 cm [8].

The animals were removed from the experiment according to the recommendations under the influence of anesthesia with the drug “Zoletil 100” (INN: tiletamine hydrochloride) (Virbac Sante Animale; France) at a dose of 20 mg/kg.

EC verification was carried out by assessing the clinical picture and morphology of the lesion site in the colon. Suppositories with vitamin $D_3$ were prepared on the basis of a 10% aqueous solution of vitamin $D_3$, a mixture of polyethylene glycols with different molecular weights, emulsifier T-2, cremophore RH-40 and coliphore were used as

auxiliary substances. The vitamin $D_3$ content in each suppository was 1500 IU [9]. Rectal suppositories containing 50 mg of 5-ASA were prepared on the basis of rectal suppositories "Salofalk" (INN: Mesalazine, "Doctor Falk Pharma GmbH", Germany). The size and shape of the suppositories corresponded to the structural features of the distal colon of rats, the final weight of each suppository was 300 mg. The studies were carried out on days 2, 4 and 6.

To assess the clinical status, a Disease activity index (DAI) adapted for rats was used, including such indicators as body weight, stool consistency and blood admixture in feces [10]. The calculation of indicators on a 5-point scale from 0 to 4 was carried out daily, the maximum possible value of DAI was 12. Fragments of the distal part of the colon were fixed in a 10% neutral formalin solution; serial sections were stained with hematoxylin and eosin. The expression of myeloperoxidase (MPO) and TNFα in the colon mucosa was evaluated by immunohistochemical method using sets of rat-specific antibodies ("Cloud. Clon. Corp.", China) and highly adhesive glasses with a positively charged surface (Super Frost Plus); the result was expressed in units/mm$^2$. The formulation of the reaction was carried out in the immunohistostainer "Bench Mark XT" (Ventana, USA) in compliance with the protocol of the study. The "Ultra VIEW Universal DAB" system (Ventana, USA) and a complex of secondary antibodies and chromogen were used for visualization. In ten randomly selected fields of view on the microscope "Leica DMRXA" (Germany), at an increase of x400, the numbers of neutrophils (NF), lymphocytes (LC), eosinophils (EF), histiocytes (HZ), plasma cells (PC), fibroblasts (FB) were calculated by 1 mm$^2$, at an increase of x100, the diameter of the ulcer was determined defect (in microns). Morphometry was performed using the ImageScope M program (Russia). Colon tissue damage was assessed on a scale from 0 to 6 with determination of the relative area of damage, intestinal wall thickness, angiogenesis, loss of goblet cells, severity of leukocyte infiltration and calculation of tissue damage index (tissue damage index, TDI).

Statistical processing of the results was carried out using the IBM SPSS Statistics 19 program. The characteristics of the samples are presented in the format "Me ($Q_{0.25}$-$Q_{0.75}$) [Min-Max]", where Me is the median, $Q_{0.25}$ and $Q_{0.75}$ are the values of the lower and upper quartile, respectively, Min is the minimum value of the sample, and Max is the maximum. The significance of the differences between the groups was assessed using the criteria of Kruskal–Wallis, Mann–Whitney.

## Results and discussion

With EC on the 2nd day of observation, a body weight deficit is detected, defecation increases, the consistency of feces changes to liquid, the admixture of blood is determined both with a benzidine test and visually. Animals spend less time grooming, and fewer approaches to food and water are recorded. On the 4th and 6th days, the clinical signs become heavier. The DAI index increases significantly on days 2, 4 and 6, its value is 6 days higher ($p > 0.01$), compared with days 4 and 2 (Table 1).

On the 2nd day of EC, during histological examination of the colon wall, ulcers are detected in the lesion, the bottom of which is located in its own plate of the mucosa and in the superficial parts of the submucosal layer, there is also cellular infiltration with edema of the interstitial tissue, venous and capillary fullness, the crypt epithelium in a state of protein dystrophy (Figure 1A, see 3rd page of cover). On the 4th day of EC, ulcerative defects, swelling of the interstitial tissue, fullness with leukostasis and leukodiapedesis, plasma impregnation and swelling of the vascular walls, stroma infiltration persisted (Figure 2A, see 3rd page of cover). In the depth of ulcerative defects, the proliferation of preserved cambial cells of the intestinal glands. On the 6th day of EC, ulcerative defects with cellular detritus, edema and loosening of the interstitial tissue, vascular

**TABLE 1. CLINICAL PICTURE IN EC, Me ($Q_{0.25}$-$Q_{0.75}$) [MIN-MAX]**

| Indicator | Group 1 Intact (n = 7) | Group 2 EC | | | Group 3 EC + $VD_3$ | | | Group 4 EC + 5-ASA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) |
| DAI, c. u. | 0 | 8.00 (3.00-8.00) [3.00-10.00] * | 9.00 (7.00-11.00) [6.00-11.00] * | 12.00 (12.00-12.00) [8.00-12.00] * | 6.00 (6.00-6.00) [5.00-8.00] * | 5.00 (5.00-6.00) [5.00-6.00] * # | 5.00 (5.00-5.00) [3.00-5.00] * # | 6.00 (5.00-6.00) [5.00-6.00] * | 5.00 (5.00-6.00) [5.00-8.00] * # | 5.00 (3.00-5.00) [3.00-5.00] * # & && |

Note. *, statistically significant ($p < 0.05$) differences with group 1; #, with group 2, $, with group 3; &, with group 4 on day 2; &&, with group 4 for 4 days. EC, experimental colitis; $VD_3$, vitamin $D_3$; 5-ASA, 5-aminosalicylic acid.

fullness are visible (Figure 3A, see 3rd page of cover). Between the infiltration sites, proliferation of young fusiform fibroblasts and the initial phenomena of neoangiogenesis are visible, with pronounced epithelization along the edges of ulcerative defects.

Morphometric assessment of the cellular composition of the infiltrate in the focus of colon injury allowed us to establish that on the 2nd, 4th and 6th days of EC, the content of neutrophils (NF), lymphocytes (LC), eosinophils (EO), plasmocytes (PC), histiocytes (HC) and fibroblasts (FB), the area of the ulcerative defect and TDI significantly increases (Table 2). In the dynamics of EC, the number of NF is less for 4 days ($p < 0.01$) than for 2 days, and for 6 days less ($p < 0.01$) than for 2 days; the number of LC is greater for 6 days ($p < 0.01$) than for 2 and 4 days; the number of EF, HZ and PC on the 4th and 6th days is more ($p < 0.01$) than on the 2nd day; the amount of FB on the 4th day is more ($p < 0.01$) than on the 2nd day, on the 6th day more ($p < 0.01$) than on the 2nd and 4th days. The area of the ulcerative defect is larger on days 4 and 6 ($p < 0.01$) than on day 2. As can be seen, the maximum severity of quantitative representation in the NF focus was recorded on day 2, EF, HZ, PC and FB – on day 2 and 4, LC – on day 6 EC.

The content of MPO and TNFα in the colon tissue increases on the 2nd, 4th and 6th days of the experiment. In the dynamics of EC, the MPO content is 6 days lower than on days 2 and 4; the TNFα content is 6 days lower than on days 2 and 4 of the experimental study. The maximum content of MPO and TNFα in the colon in EC is noted on the 2nd day of the experiment.

Under conditions of local application of vitamin $D_3$ in EC, a change in the clinical picture in animals is observed. Animals become more active, approaches to food and water increase, animals devote time to grooming. Body weight does not decrease, fecal masses are denser, blood in fecal masses is determined only in a benzidine sample. DAI significantly decreases on days 4 and 6 (Table 1). In the dynamics of the study, the values of DAI for the entire day have no differences. DAI on days 2, 4 and 6 significantly differs from DAI in the group of intact animals, which indicates only a partial recovery of the indicator.

In EC, under the conditions of vitamin $D_3$ use, during histological examination of the colon wall in the lesion on day 2, ulcerative defects were recorded in the own plate of the mucous membrane and the submucosal layer with venous and capillary fullness, the mucous membrane is moderately edematous, the crypts are shortened, expanded, their epithelium is in a state of granular dystrophy (Figure 1B, see 3rd page of cover). On day 4, completely epithelized areas of replacement of ulcerative defects of the mucous membrane with the initial formation of intestinal glands and crypts, focal infiltration by granulocytes, proliferation of young fibroblasts were revealed (Figure 2B, see 3rd page of cover). On day 6, complete epithelialization of ulcerative defects, focal infiltration and extensive fields of proliferating fibroblasts, newly formed connective tissue fibers and vessels in large quantities are observed (Figure 3B, see 3rd page of cover).

Morphometric assessment of the cellular composition of the infiltrate in the focus of colon damage in EC with the use of vitamin $D_3$ allowed us to establish that on day 2 the number of NP, LC, EF and PC significantly decreases, and the number of HZ and FB increases. On day 4, the number of NF, LC, EF and PC significantly decreases, and the number of FB increases. On day 6, the number of NF, LC, EF and PC significantly decreases, and the number of HZ and FB increases. On days 2, 4 and 6, the area of ulcerative lesions decreases; on days 4 and 6, TDI decreases (Table 2). All morphometric parameters during the whole day of the experiment did not reach the values of the group of intact animals; they were partially restored. In the dynamics of EC, the number of EF and HZ is greater for 4 days ($p < 0.01$) than for 2 days, for 6 days more ($p < 0.01$) than for 2 days, the number of FB is greater for 4 days ($p < 0.01$) than for 2 days, for 6 days more ($p < 0.01$), than on the 4th and 2nd days. TDI on the 4th and 6th days is less ($p < 0.01$) than on the 2nd day. All morphometric parameters during the whole day of the experiment did not reach the values of the group of intact animals, they were partially restored.

Against the background of the use of vitamin $D_3$ in EC, on the 4th and 6th days of the experiment, the concentrations of TNFα and MPO in the homogenate of the zone of alteration of the colon mucosa decrease, not reaching values in the group of intact animals (Table 3). In dynamics, MPO expression is 4 days less ($p < 0.01$) than on day 2, 6 days less ($p < 0.01$) than on day 4 and 2; TNFα expression is 6 days less ($p < 0.01$) than on day 4 and 2.

Against the background of the use of 5-ASA in EC, its well-known anti-inflammatory properties were recorded. Animals willingly engage in mutual grooming, approaches to food and water are becoming more frequent. On days 2, 4 and 6, the DAI index decreases: body weight stabilizes, animals become more active, diarrhea is replaced by feces, and blood in feces is determined only in a benzidine sample. In the dynamics of EC, the DAI parameter is 6 days lower ($p < 0.01$) than on the 2nd and 4th days of the experiment.

The DAI parameter in the group of animals under the conditions of use of 5-ASA and in the group of animals under the conditions of use of vitamin $D_3$

TABLE 2. MORPHOMETRIC INDICATORS IN THE FOCUS OF DAMAGE TO THE LARGE INTESTINE IN EC, Me ($Q_{0.25}$-$Q_{0.75}$) [MIN-MAX]

| Indicator | Group 1 Intact (n = 7) | Group 2 EC | | | Group 3 EC + $VD_3$ | | | Group 4 EC + 5-ASA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) |
| Neutrophils, un/mm² | 204.56 (189.71-223.57) [147.45-231.92] | 2651.41 (2558.85-2813.85) [1827.41-3529.41] * | 1518.48 (1121.49-2100.00) [1100.11-2727.28] * | 1333.33 (1213.34-1608.04) [1008.06-3366.33] * | 873.78 (666.67-925.92) [654.21-931.67] * # | 550.45 (370.37-1006.03) [198.02-1078.43] * # | 654.21 (582.53-804.83) [458.71-873.78] * # | 926.81 (571.42-1063.83) [502.51-2824.85] * # | 803.57 (737.71-1592.92) [634.92-1872.14] * # | 833.07 (706.520-983.607) [645.160-1875.00] * # |
| Lympho-cytes, un/mm² | 338.99 (305.14-368.35) [284.55-368.95] | 1104.48 (947.67-1333.34) [931.67-1396.04] * | 1004.55 (880.09-1238.11) [841.12-1408.45] * | 1667.02 (1302.62-2038.84) [1209.67-2685.18] * | 680.07 (511.78-849.32) [427.81-864.63] * # | 710.67 (495.06-733.95) [462.96-784.31] * # | 642.19 (582.61-891.11) [467.29-904.52] * # | 1229.83 (983.61-1259.84) [489.13-2480.00] * $ | 796.46 (555.56-1095.89) [531.91-1967.21] * | 596.51 (452.26-1034.48) [400.00-1129.94] * # & |
| Eosinophils, un/mm² | 146.91 (120.83-176.18) [105.49-187.66] | 852.34 (839.46-857.45) [757.57-1456.31] * | 2671.29 (2352.95-3553.31) [1313.13-4476.19] * | 2380.11 (2110.11-2613.05) [1415.57-3333.33] * | 467.29 (304.57-611.13) [297.03-716.47] * # | 852.89 (635.86-1094.25) [198.12-1308.41] * # € | 805.12 (685.42-867.49) [594.05-901.81] * # € | 807.65 (514.28-1105.52) [451.97-1483.51] * | 1587.31 (707.960-2232.143) [655.73-2792.79] * # | 726.42 (483.87-1475.41) [217.39-1574.81] * # |
| Histiocytes, un/mm² | 13.47 (13.42-13.65) [12.72-13.86] | 571.94 (198.02-750.26) [93.45-757.57] * | 1197.11 (1049.31-1614.91) [818.83-1714.28] * | 1006.03 (970.87-1009.17) [807.26-1262.13] * | 913.31 (759.37-1102.82) [710.66-1187.12] * # | 1395.36 (1313.13-1600.00) [1207.24-1682.24] * € | 1617.79 (1512.09-1809.04) [1202.41-1962.61] * # € | 698.91 (351.75-862.06) [338.98-904.25] * | 821.91 (655.73-1415.92) [476.19-1648.93] * | 1097.96 (800.01-1338.58) [543.47-1696.42] * $ |
| Plasmo-cytes, un/mm² | 13.42 (12.87-13.56) [12.72-13.64] | 673.13 (549.12-704.52) [535.91-748.66] * | 804.02 (713.06-910.01) [605.44-1708.54] * | 810.13 (804.82-1210.12) [707.07-1800.00] * | 480.81 (370.37-560.74) [366.97-582.52] * # | 401.06 (372.67-411.77) [186.91-471.68] * # | 373.83 (297.03-545.56) [275.22-588.23] * # | 495.84 (201.01-549.45) [169.49-571.42] * # | 530.97 (446.42-540.54) [409.83-593.61] * # $ | 517.52 (480.01-655.73) [163.04-714.28] * # |
| Fibroblasts, un/mm² | 22.66 (13.56-26.82) [13.42-27.28] | 512.77 (281.37-711.65) [198.02-748.66] * | 1146.77 (866.81-1358.22) [857.44-2285.71] * | 1685.27 (1523.84-2057.07) [1388.88-2079.21] * | 1821.02 (1817.34-1845.66) [1467.89-1962.61] * # | 2353.94 (2311.23-2401.00) [2112.67-2952.38] * # € | 2467.89 (2413.88-3047.61) [2304.61-3517.58] * # € €€ | 856.07 (790.96-1318.68) [653.26-2011.49] * # $ | 1506.84 (1250.01-1858.41) [1031.74-1981.98] * $ | 2048.21 (1612.91-2240.01) [1086.95-3214.28] * $ & |

*Таблица 2 (окончание)*
*Table 2 (continued)*

| Indicator | Group 1 Intact (n = 7) | Group 2 EC | | | Group 3 EC + $VD_3$ | | | Group 4 EC + 5-ASA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) |
| **Diameter of the ulcerative defect, mcm** | 0 | 575.00 (305.00-780.60) [290.33-791.65] * | 735.00 (635.52-976.50) [600.75-983.42] * | 753.00 (372.00-882.50) [359.42-894.39] * | 294.00 (197.00-357.00) [187.35-372.35] * # | 242.00 (151.00-539.00) [143.65-537.25] * # | 238.50 (169.00-299.00) [161.35-301.95] * # | 250.82 (211.35-284.64) [200.75-299.42] * # | 492.26 (267.16-564.21) [243.75-574.32] * # | 213.49 (183.47-241.85) [181.75-245.75] * # |
| **TDI, c. u.** | 0 | 3.71 (3.00-4.00) [2.00-5.00] * | 3.57 (3.00-4.00) [2.00-6.00] * | 3.42 (3.00-4.00) [3.00-5.00] * | 3.00 (3.00-4.00) [2.00-5.00] * | 2.17 (1.00-3.00) [1.00-4.00] * # € | 2.12 (1.00-3.00) [1.00-4.00] * # € | 3.61 (3.00-4.00) [2.00-4.00] * | 2.00 (1.00-3.00) [1.00-4.00] * # | 1.81 (1.00-2.00) [1.00-3.00] * # |

Note. *, statistically significant ($p < 0.05$) differences with group 1; #, with group 2; $, with group 3; €, with group 3 on day 2; €€, with group 3 on day 4; &, with group 4 on day 2; &&, with group 4 for 4 days. EC, experimental colitis, $VD_3$, vitamin $D_3$; 5-ASA, 5-aminosalicylic acid, TDI, tissue damage index.

**TABLE 3. CONTENT OF MPO AND TNF$\alpha$ IN THE FOCUS OF DAMAGE TO THE LARGE INTESTINE IN EC, Me ($Q_{0.25}$-$Q_{0.75}$) [MIN-MAX]**

| Indicator, un/mm$^2$ | Group 1 Intact (n = 7) | Group 2 EC | | | Group 3 EC + $VD_3$ | | | Group 4 EC + 5-ASA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) | 2nd day (n = 7) | 4th day (n = 7) | 6th day (n = 7) |
| **MPO** | 16.28 (7.66-26.81) [5.74-28.73] | 1241.37 (967.04-1486.59) [729.88-1708.81] * | 938.69 (770.15-1109.19) [760.53-1206.89] * | 775.86 (766.28-814.17) [722.22-1427.21] * | 1053.64 (977.01-1091.95) [957.85-1206.89] * | 498.08 (478.92-593.86) [421.45-670.49] * # € | 287.35 (268.19-287.35) [210.70-344.82] * # € €€ | 984.67 (773.94-1112.83) [634.09-1170.11] * | 555.55 (478.92-574.71) [440.61-593.89] * # & | 249.04 (210.72-249.04) [191.57-325.67] * # $ & && |
| **TNF$\alpha$** | 52.68 (44.06-63.21) [26.81-76.62] | 1321.83 (919.54-1475.09) [720.31-1551.72] * | 1302.68 (1264.36-1321.83) [1245.21-1360.15] * | 752.87 (703.06-814.17) [670.49-950.19] * | 766.28 (670.49-957.85) [632.18-1053.64] * | 727.96 (727.97-766.28) [632.18-785.44] * # | 210.72 (172.41-229.88) [134.09-268.19] * # € €€ | 766.28 (545.97-967.43) [498.08-996.16] * | 402.29 (344.82-440.61) [306.51-478.92] * # $ & | 191.57 (172.41-191.57) [172.41-229.88] * # & && |

Note. *, statistically significant ($p < 0.05$) differences with group 1; # – with group 2; $ – with group 3; € – with group 3 on day 2; €€ – with group 3 on day 4; & – with group 4 on day 2; && – with group 4 for 4 days. EC, experimental colitis; $VD_3$, vitamin $D_3$; 5-ASA, 5-aminosalicylic acid.

has no statistically significant differences on the 2nd, 4th and 6th days of observation, which indicates the comparability of the effects of vitamin $D_3$ and 5-ASA with respect to clinical signs of EC (Table 1).

The clinical picture of EC in the conditions of 5-ASK application is reflected in the morphology of the alteration zone. On day 2, ulcerative defects are located in the superficial parts of the submucosal and the proper plate of the mucous membrane of the colon wall, infiltration in the projection of the defect, protein dystrophy of the glands (Figure 1C, see 3rd page of cover). On day 4, defects are more often found in the own plate of the mucosa, newly formed connective tissue fibers, signs of neoangiogenesis and epithelization of surface defects are revealed (Figure 2C, see 3rd page of cover). On day 6, the depth of defects varies from the superficial to the submucosal layers, infiltration with an admixture of histiocytes and plasmocytes was detected in the submucosal layer, fibrillogenesis, neoangiogenesis, epithelialization and proliferation of cambial cells of intestinal glands were recorded (Figure 3C, see 3rd page of cover).

Morphometric analysis of the colon alteration zone in EC under conditions of local application with 5-ASA found that on day 2 the number of neutrophils and plasmocytes decreases, the number of fibroblasts increases; on day 4 the number of neutrophils, eosinophils and plasmocytes decreases; on day 6 – neutrophils, lymphocytes, eosinophils and plasmocytes (Table 2). The diameter of the ulcerative defect decreases by 2, 4 and 6 days; the TDI indicator decreases by 4 and 6 days. When assessing the content of TNFα and MPO in the cell populations of colon tissue under conditions of local application of 5-ASA, it was revealed that these parameters decrease on days 4 and 6 (Table 3). In the dynamics of EC, the content of MPO and TNFα is 6 days lower than on the 2nd and 4th days of the experiment.

So, with EC, in the conditions of using rectal suppositories with vitamin $D_3$, unlike 5-ASA, the repair of the ulcerative defect is fixed earlier, the cellular infiltration of the inflammatory focus decreases. When comparing the morphometric parameters of the colon alteration zone in EC under the conditions of rectal suppositories with vitamin $D_3$, in contrast to the use of 5-ASA, a decrease in the number of lymphocytes, an increase in the number of fibroblasts was revealed on day 2, a decrease in the number of plasmocytes and an increase in the number of fibroblasts on day 4, an increase in the number of histiocytes and fibroblasts on day 6. The diameter of the ulcerative defect and the integral parameter of intestinal tissue damage, the TDI index, have no significant differences between the compared groups. When comparing the effectiveness of rectal suppositories with vitamin $D_3$, in contrast to the use of rectal suppositories with 5-ASA, the MPO content is higher on day 6, the TNFα content is higher on day 4.

Thus, the analysis performed in EC allows us to talk about the positive effect of the use of original rectal suppositories with vitamin $D_3$ on clinical manifestations according to the DAI indicator (stabilization of body weight, change in stool consistency to a more decorated one, absence of rectal bleeding) and the morphological picture of the lesion area (reduction of infiltration of the colon wall by neutrophils, lymphocytes, eosinophils and plasmocytes, involved in the alteration of the intestinal wall, an increase in the number of histiocytes and fibroblasts – regulators and participants of the repair, respectively, as well as a decrease in the diameter of the ulcerative defect, a decrease in the TDI index).

The clinical and morphological picture of colon lesions in EC corresponds to changes in IBD in humans and allows the oxazolone model of colitis to be used to study the pathogenesis and test the effectiveness of new therapeutic approaches in IBD [14].

We believe that the results obtained are related to several mechanisms of action of vitamin $D_3$ in EC. Firstly, the immunotropic effect of vitamin $D_3$ is realized by the action of the active metabolite of vitamin $D_3$ calcitriol on the proliferation and differentiation of T lymphocytes, a decrease in Th1, Th17 and an increase in – Treg due to a decrease in the synthesis of IL-1, IL-2, IL-6, IL-12, IL-17, IFNγ and TNFα, increased IL-10 synthesis. Vitamin $D_3$ inhibits the migration of macrophages and their release of IL-1, IL-6, IL-8, IL-12, chemotaxis and accumulation of neutrophils [11]. Vitamin $D_3$ interferes with the expression of TLR, CD40, CD80, CD83 and CD86 on the surface of dendritic cells, reduces their secretion of IL-2 and IFNγ, increases the synthesis of IL-10 [12]. This limits the activity of the inflammatory process and the alteration of tissues in the colon [2].

Secondly, vitamin $D_3$ accelerates repair in the focus of colon damage in EC. When interacting with specific nuclear receptors of colon epithelial cells (VDR), vitamin $D_3$ increases the expression of vinculin, zonulin, occludin, claudin – proteins involved in the formation of epithelial cells [12]. An increase in the number of histiocytes and fibroblasts in the lesion site indicates the activity of repair processes in the intestinal wall. In addition, the restriction of vascular exudative and leukocyte reactions due to anti-inflammatory and antioxidant effects accelerates the repair under conditions of vitamin $D_3$ use.

## Conclusion

Thus, the use of vitamin $D_3$ in EC in the composition of original rectal suppositories in a total dose of 18,000 IU reduces the severity of clinical signs, the

representation of cells involved in tissue destruction in the colon wall, increases the representation of cells mediating repair, reduces the content of MPO and TNFα in the colon alteration zone.

The effects of using rectal suppositories with vitamin $D_3$ on clinical signs, the size of the ulcerative defect, the content of MPO and TNFα in the alteration zone are comparable to the effects of using rectal suppositories with 50 mg of 5-ASA; they are more pronounced with respect to the dynamics of the cellular composition of the colon alteration zone.

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*Поступила 06.04.2023*
*Принята к печати 07.04.2023*

*Received 06.04.2023*
*Accepted 07.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 665-672

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 665-672

# РЕЗУЛЬТАТЫ ЭКСПЕРИМЕНТАЛЬНОГО ПРИМЕНЕНИЯ МИКРОВЕЗИКУЛ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК НА МОДЕЛИ ОСТРОЙ ПОЧЕЧНОЙ НЕДОСТАТОЧНОСТИ У МЫШЕЙ

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**Резюме.** Важную роль в восстановлении поврежденных органов и тканей играют мезенхимальные стволовые клетки (МСК) и продуцируемые ими микровезикулярные частицы (МВ). Они могут быть источником цитокинов, антиапоптозных и стимуляторных ростовых факторов. Кроме того, МВ осуществляют транспорт мРНК, микроРНК и сигнальных белков в поврежденные ткани. Это повышает способность клеток к регенерации, ингибирует апоптоз, способствует ангиогенезу и стимулирует пролиферацию клеток. Целью исследования было изучение иммунорегулирующих и прорегенераторных свойств микровезикул мезенхимальных стволовых клеток (МСК-МВ) на модели глицерол-индуцированной острой почечной недостаточности (ОПН) у мышей. Эксперименты проводились на мышах линии CBA возрастом 3-4 месяца. ОПН индуцировали однократным внутримышечным введением 50% глицерола. МСК получали из костного мозга здоровых животных, культивировали в стандартных условиях. Микровезикулы получали путем центрифугирования при 12000 g супернатанта МСК после индукции их апоптоза путем культивирования в условиях депривации кислорода и в бессывороточной среде. МСК-МВ вводили внутривенно в ретроорбитальный синус через сутки после индукции ОПН. Дозу МВ рассчитывали как эквивалентную (полученную из) 1 млн МСК, что составляло 100 мкл на мышь. Животных выводили из эксперимента на 4-е и 11-е сутки после инъекции МСК-МВ. Забирали плазму крови для определения уровня креатининна, мочу – для анализа альбумина, почки – для гистологического иссследования. Показано, что МВ, подуцируемые МСК,

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**Образец цитирования:**
*И.П. Иванова, Г.В. Селедцова, В.И. Селедцов, Т.С. Хабалова, А.Б. Доржиева «Результаты экспериментального применения микровезикул мезенхимальных стволовых клеток на модели острой почечной недостаточности у мышей» // Медицинская иммунология, 2023. Т. 25, № 3. С. 665-672. doi: 10.15789/1563-0625-EAR-2716*

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**For citation:**
*I.P. Ivanova, G.V. Seledtsova, V.I. Seledtsov, T.S. Khabalova, A.B. Dorzhieva “Experimental application results of mesenchymal stem cell microvesicles in the mouse model of acute renal failure”, Medical Immunology (Russia)/ Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 665-672. doi: 10.15789/1563-0625-EAR-2716*

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**DOI:** 10.15789/1563-0625-EAR-2716

дозозависимо стимулировали пролиферацию спленоцитов как в спонтанном, так и Кон-А индуцированном тесте. Добавление МВ вызывало снижение доксорубицин-индуцированного апоптоза селезеночных лимфоцитов у мышей. Вероятно, в этом случае, продуцируемые МСК-МВ оказывали иммуностимулирующее и антиапоптотическое действие. Также МВ оказывали положительный эффект на восстановление структуры и функции почек в модели острой почечной недостаточности у мышей. Использование МСК-МВ в лечении ОПН, индуцированной однократным введением 50% глицерола способствовало снижению уровня альбумина в моче и восстановлению уровня креатинина в сыворотке крови животных. Морфологические исследования показали уменьшение высоты клеток и диаметра собирательных трубочек в мозговом веществе и уменьшение наибольшего поперечного диаметра суперфициальных клубочков в корковом веществе почек больных мышей. Таким образом, полученные результаты свидетельствуют о значительных терапевтических и прорегенеративных свойствах МСК-МВ, которые требуют дальнейшего изучения.

*Ключевые слова: мезенхимальные стволовые клетки, микровезикулы, терапевтическое применение микровезикул, регенерация, острая почечная недостаточность, болезни почек*

# EXPERIMENTAL APPLICATION RESULTS OF MESENCHYMAL STEM CELL MICROVESICLES IN THE MOUSE MODEL OF ACUTE RENAL FAILURE

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**Abstract.** An important role in restoration of damaged organs and tissues is played by mesenchymal stem cells (MSCs) and microvesicular particles (MV) produced by them. They can be a source of cytokines, anti-apoptotic and growth stimulating factors. In addition, MVs carry out transport of mRNA, miRNA, and signal proteins into damaged tissues. This increases the ability of cells to regenerate and to inhibit apoptosis, promote to angiogenesis and stimulate cell proliferation. The aim of our research was to study the immunoregulatory and pro-regenerative properties of mesenchymal stem cell microvesicles (MSC-MV) in a model of glycerol-induced acute renal failure (ARF) in mice. The experiments were carried out on CBA mice aged 3-4 months. AKI was induced by a single intramuscular injection of 50% glycerol. MSCs were obtained from the bone marrow of healthy animals and cultivated under standard conditions. Microvesicles were obtained by centrifugation at 12000g of MSC supernatant after induction of their apoptosis by culturing under oxygen deprivation conditions and in serum-free medium. MSC-MV was injected intravenously into the retroorbital sinus one day after induction of ARF. The MV dose was calculated as equivalent to (derived from) 1 million MSCs, which was 100 μL per mouse. Animals were taken out of the experiment on days 4 and 11 after MSC-MV injection. Blood plasma was taken to determine the level of creatinine, urine – for albumin analysis, kidneys – for histological examination. It has been shown that MVs induced by MSCs dose-dependently stimulated splenocyte proliferation in both spontaneous and Con-A induced tests. The addition of MV caused a decrease in doxorubicin-induced apoptosis of splenic lymphocytes in mice. Probably, in this case, MV produced by MSCs had an immunostimulatory and antiapoptotic effect. Also, MVs had a positive impact on the restoration of structure and function kidneys in a model of ARF in mice. The use of MSC-MV in treatment of acute renal failure induced by a single injection of 50% glycerol contributed to decrease albumin level urine and restoration of creatinine level in blood serum of animals. Morphological studies have shown decrease in the height cell and collecting duct diameter in the medulla and a decrease in the largest transverse diameter of superficial glomeruli in the renal cortex of sick mice. Thus, the obtained results indicate significant therapeutic and pro-regenerative properties of MSC-MV, which require further study.

*Keywords: mesenchymal stem cells, microvesicles, therapeutic use of microvesicles, regeneration, acute renal failure, kidney disease*

## Introduction

Increasing evidence indicates important role of mesenchymal stem cells (MSCs) in restoration damaged organs and tissues. Their cytoprotective effect is due not only for processes homing and differentiation, but also stimulation endogenous regeneration resident cells [8]. This occurs due to cytokines secretion, anti-apoptotic and growth factors, production of microvesicular particles (exosomes and microvesicles) (MSC-MV), which purposefully deliver mRNA, microRNA, and signal proteins for damaged tissues [3, 5].

A number of experimental studies have shown introduction of microvesicular particles (MV) largely repeats therapeutic effect of MSCs in various experimental models of tissue damage, for example, in acute damage of tubular epithelium induced by intramuscular glycerol administration or acute ischemia-reperfusion [2, 5, 6, 10]. Thus, in a model with glycerol-induced acute renal failure (ARF), it was shown that MVs carry on their surface CD29, CD44, CD73, specific markers of MSCs, which provide targeted MVs transport to the injury site. It was also shown that MSC-MVs carry out horizontal transport of mRNA MSCs and involve in control of transcription, proliferation, and immunoregulation. Once in the lesion, MV inhibit apoptosis and stimulate proliferation of tubular epithelial cells, thus significantly to reduce manifestations ARF in laboratory animals [2]. Similar results were obtained in experimental model kidney ischemia-reperfusion, where it was also shown that MV reduce manifestations postischemic nephrosclerosis and developing chronic renal failure [6]. In our study, we analyzed the effects of exposure MVs derived from MSCs on changes in immunological, biochemical, and morphological parameters during glycerol-induced acute renal failure in mice.

## Materials and methods

The experiments were carried out on linear CBA mice aged 3-4 months. Acute renal failure was induced by single intramuscular injection of 50% glycerol at a dose of 8.6 mg/kg. Resulting rhabdomyolysis has mixed (ischemic, toxic, retention) effect on kidneys. In preliminary experiments, we showed that such an impact causes acute necrosis of proximal renal tubules epithelial cells accompanied by a rather significant increase in the level of blood serum urea. All experiments on animals were carried out in accordance with the “Rules for carrying out work using experimental animals” (Appendix to the order of the Ministry of Health of the USSR No. 755 of 1977).

Mesenchymal stromal cells were obtained from bone marrow syngeneic mice by the method of adhesion to cultural plastic. Bone marrow derived from femurs and tibias was suspended in RPMI with 10% FCS. Bone marrow stroma was mechanically destroyed with a glass homogenizer, washed twice, and placed in culture dishes in complete medium based on RPMI 1640. Non-adherent fraction of bone marrow cells was removed at culture medium changes starting from day 3. MSCs had classic fibroblast-like phenotype and formed a continuous monolayer by 4$^{th}$ week of cultivation. MSCs were removed from plastic with 0.25% versene-trypsin solution, after which cells were washed twice from culture medium and resuspended in physiological saline.

After reaching monolayer, $1 \times 10^6$/mL of MSCs were subjected to apoptosis by culturing under oxygen deprivation conditions and in a serum-free medium for 1-3 days (the culture flask was placed in container, a candle was lit nearby, and the lid was hermetically closed). Under these conditions, cells enter state of apoptosis, and their production of microvesicular particles, especially fractions of 100-1000 nm, increases significantly. At the end of cultivation, 1 million cells were centrifuged at 2000 g for 15 minutes to remove cell debris. The supernatant was additionally centrifuged at 12000 g for 60 minutes at 4 °C. The resulting pellet was resuspended in 100 μL of saline. Thus, standard for the amount of MV was their preparation from 1 million cells and dilution in 100 μL of 0.9% NaCl solution. In some experiments, protein content was determined in the obtained MVs by the Bradford method, and MV size was estimated by cytofluorimetry. The MV size was 100 nm-1000 nm, which was confirmed by cytofluorimetry using the FACS-CytoFLEX instrument.

MV was administered intravenously into the mouse retroorbital sinus one day after induction of acute renal failure. The MV dose was calculated as equivalent to 1 million MSCs, which was 100 μL per mouse. Animals with induced acute renal failure were withdrawn from the experiment on days 4 and 11 after MV injection. Blood plasma was collected to determine level of creatinine, urine – for albumin analysis, kidneys for histological examination, splenocytes for evaluation of proliferation and cell cycle.

Effect of microvesicular particles on splenocyte proliferation was studied in the 3H-thymidine incorporation test. Splenocytes were obtained by homogenizing the syngeneic mice spleens and then cultured in a 96-well plate at 300,000 cells per well in complete medium with or without addition MV for 3 days. The dose MV was calculated from the protein by Bradford method and added at 10 μg/mL, 30 μg/mL and 90 μg/mL. H3-thymidine was added 16 hours before finish culture. The results were evaluated on β-scintillation counter.

Splenocytes from CBA mice were cultured 2 days in a 6-well plate at 5 million cells per well in complete medium in absence or presence of microvesicles at 90 μg/mL for determine effect microvesicular particles

on resistance lymphocytes for apoptosis. Then, for induce apoptosis, doxorubicin was added at a dose of 0.1 μmol/l for a period of 24 hours. Next, cells were removed, fixed with 0.5% paraformaldehyde, stained with propidium iodide. The percentage of cells in the state of apoptosis was evaluated on a FACSCalibur flow cytometer. Splenocytes from healthy mice and splenocytes from mice with glycerol-induced renal failure were used in the experiment. Serum creatinine was measured using BioVision, Creatinine (Mouse) ELISA Kit rev 03/18, Catalog Number E4369-100 (Abcam); urinary albumin was measured using Mouse Albumin ELISA Kit, Catalog Number ab108792 (Abcam).

For histological examination mice kidneys were fixed in 4% formalin solution, then dehydrated according to standard procedure and embedded in paraffin. Paraffin sections 4-5 μm thick were obtained using a rotary microtome HM 340E (Carl Zeiss, Germany), stained with hematoxylin and eosin, Sirius red and Mallory. Light-optical examination and microphotography were carried out using an Axioskop 40 microscope (Carl Zeiss, Germany). Morphometric analysis of kidney structures was performed on paraffin sections. The values of the following morphometric parameters were determined: – diameters superficial renal glomeruli (renal corpuscles); diameters collecting ducts and height their cells, measured in the middle third renal medulla. Morphometric calculation was performed in a field of view with an eyepiece 10×/25 and a lens 63×. The data obtained during the study were processed using the one-way ANOVA method. Values were calculated using GraphPad Prism 8 software. The results were analyzed using the computer program Graph prism 8. Student's T-test was used to assess the significance of differences.

## Results and discussion

Figure 1 shows the results splenocyte proliferation and their apoptosis levels after MSC-MV exposure. We found a significant dose-dependent stimulatory activity of MSC-MV in both spontaneous and Con-A induced proliferative tests. The proliferative activity of splenocytes increased 3-fold after addition of 10 μg/mL MSC-MV, cultivation in presence of 30 and 90 μg/mL MSC-MV had an even more pronounced effect (Figure 1A, B). Also, MV addition caused decrease level of splenocyte apoptosis (Figure 1C). Thus, microvesicles produced by MSCs had an immunostimulatory effect and showed a high potential in maintaining and stimulating cell growth.

Mice were induced with ARF. Three experimental groups were formed for the study: intact animals; mice with ARF; mice with ARF treated with MSC-MV. Functional and biochemical parameters of excretory system and morphological changes in structure kidneys were studied in this groups on days 4 and 11 after intravenous administration of MSC-MV. After intramuscular injection of 50% glycerol on day 4 the level of urine albumin increased 3 times in experimental group mice compared with intact animals. MSC-MV introduction led to decrease in albumin (Table 1). The blood serum creatinine concentration mice with acute renal failure on day 4 did not differ from the values of intact mice, only on day 11 we recorded a significant increase in this indicator by 1.5 times. The MSC-MV restored the level of creatinine to control values in these experiments (Table 1). Thus, MVs had a positive effect on restoration of glomerular apparatus and excretory function of kidneys.

According to the results of morphological analysis, it can be said that in the group of mice with induced ARF on 4th day studies was enlarged (compared to intact group) superficial glomeruli are detected in the renal cortex. Cell height and collecting duct diameter was increased in the medulla (Figure 2A). Blood rheological properties in the papillae kidney manifested in the form of plasma proteins sweating and erythrocyte sludge. The urinary space of the glomeruli of cortical substance is practically not determined in the group ARF study kidney on 11th day. It was probably caused by reduced an amount

**TABLE 1. BIOCHEMICAL PARAMETERS IN URINE AND BLOOD SERUM OF MICE WITH ACUTE RENAL FAILURE ON DAYS 4 AND 11 AFTER EXPOSURE TO MSC MICROVESICLES.**

| Albumin concentration (ng/mL) in mice urine | | | |
|---|---|---|---|
| | **Intact** | **ARF** | **ARF + MSC-MV** |
| **4th day** | 98.41±15.63 | 298.61±51.55 | 172.60±74.39 |
| **11th day** | | 243.42±19.72 | 132.10±38.22 |
| **Creatinine concentration (ng/mL) in mice blood** | | | |
| | **Intact** | **ARF** | **MSC+ MSC-MV** |
| **4th day** | 2.24±0.16 | 2.379±0.090 | 2.65±0.12 |
| **11th day** | | 3.114±0.390 | 1.91±0.12* |

Note. * $p < 0.05$, significance of differences in comparison with the ARF group.

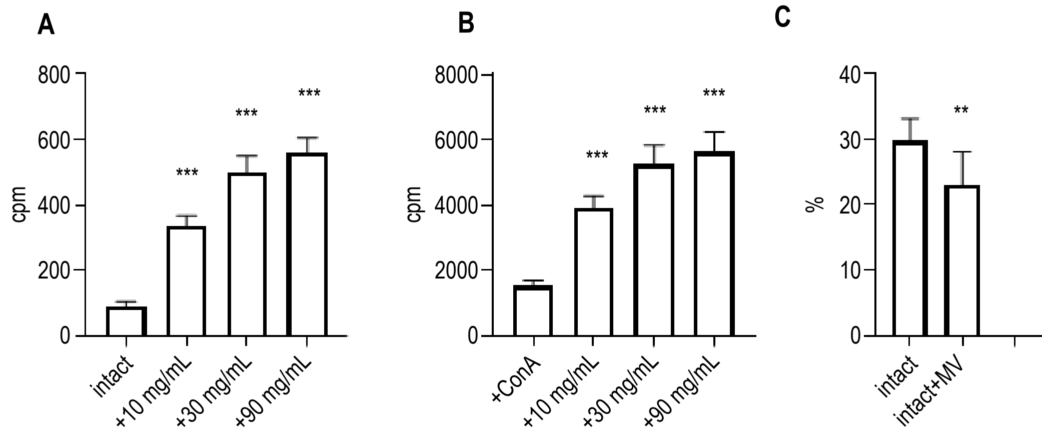


**Figure 1. Proliferative response (A – in absence or B – in presence of Kon A) and splenocyte apoptosis (C) of mice in response to MSC-MV exposure**

Note. 10 μg/mL, 30 μg/mL, 90 μg/mL, MV concentration in culture liquid. ** $p < 0.01$; *** $p < 0.001$, significance of differences compared to lymphocytes without addition of MV.

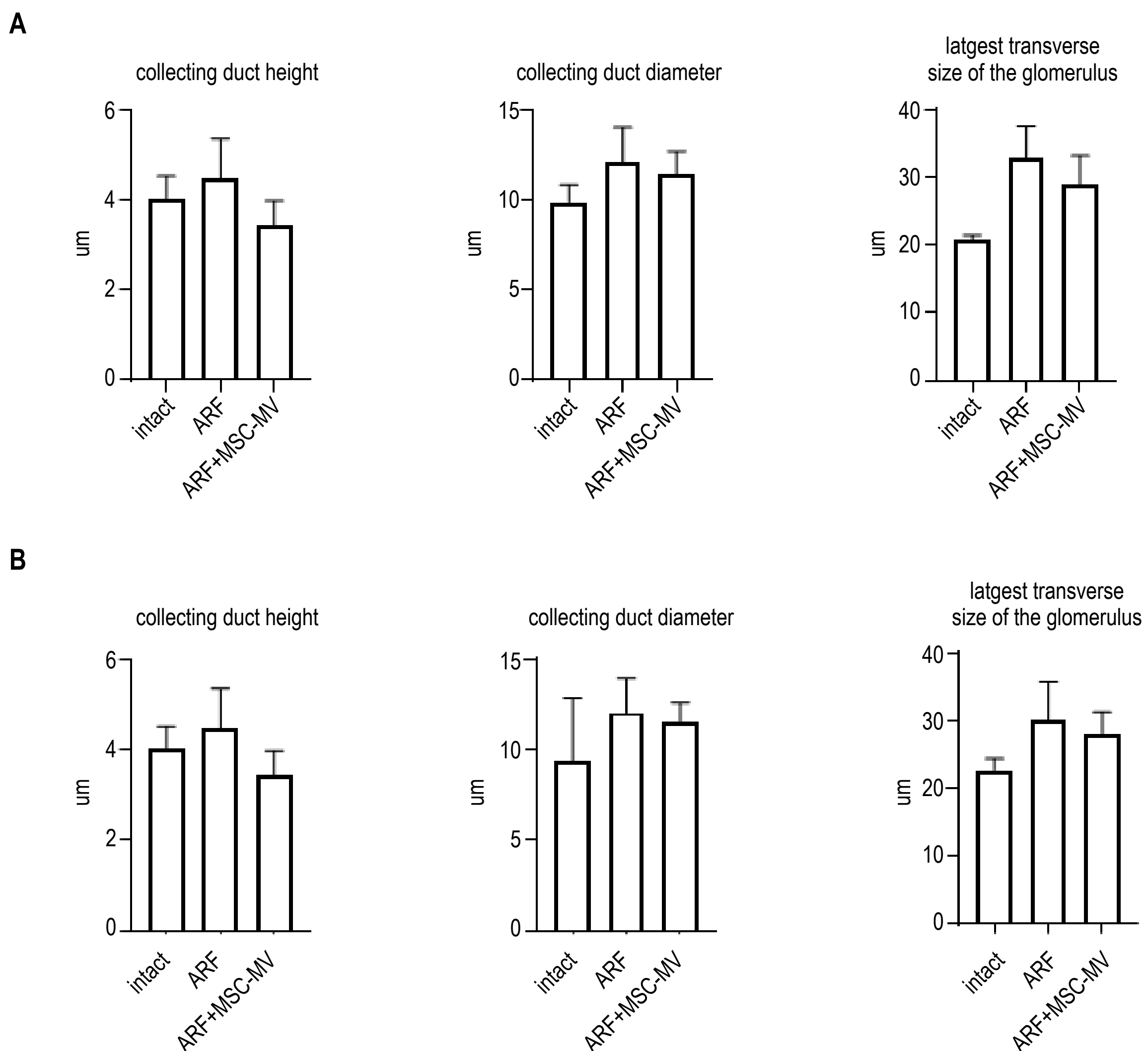


**Figure 2. Morphological changes of kidney structure in acute renal failure on day 4 (A) and day 11 (B) of the study**

of filtered urine. There is a sharp degeneration of glomerular cells (the size of the mesangial cells, the intercellular substance is reduced), the transverse size of glomeruli themselves is increased (micrographs of morphological data are not presented).

Morphological kidney changes were less pronounced in the group of mice with injected MSC-MV compared to the group ARF mice on research day 4. The height cells and collecting duct diameter are reduced in renal medulla, while we recorded increase the Henle loops lumen and decrease degeneration of the Henle loops cells. There is also insignificant decrease in the largest transverse diameter superficial glomeruli (Figure 2A). Partial spontaneous restoration structure kidneys occurred on the research 11th day, however, the MSC-MV introduction accelerates this process. There are minimal changes in the size glomeruli kidneys, no dystrophic changes were registered in the cortex (Figure 2B). Thus, in the model ARF, pronounced regenerative effect of microvesicles obtained from MSCs is noted. This effect was confirmed both morphologically and functionally by determining of albumin level in urine and creatinine in blood serum.

Microvesicles have structures 100-1000 nm. Their membrane separates from cytoplasmic membrane cells and carries various enzymes, transcription factors, mRNA molecules. MV formation is characteristic for many types of cells – leukocytes, erythrocytes, stem and tumor cells. At present, their participation in development of many cardiovascular and neurological diseases, invasion and metastasis tumor cells has been established [12]. The importance of extracellular vesicles lies in their ability to transfer biologically active molecules and genetic information to other target cells, influencing their functions [3]. Stem cell-derived MVs promote tissue repair and reduce inflammation in various models of ARF [4].

The hallmark of ARF is a rapid decline in kidney function in parallel with loss of tubular cells, leading to an increase in urea and creatinine plasma. MSC-MVs from a bone marrow accelerate to repair of damaged tubular cells, promoting cell proliferation and protecting cells from apoptosis [2]. The effects of MV are mainly associated with horizontal transfer of genetic material [5]. For example, MSC-MV from a bone marrow carry specific mRNAs that to stimulate damaged recipient cells to re-enter a cell cycle [2]. There is also evidence that transfer of human IGF-1 receptor mRNA (present in bone marrow MSC-MV) to tubular cells is a fundamental event to initiate kidney recovery [13]. In models of toxic acute renal failure induced by cisplatin and gentamicin, MSC-MV improved kidney function and reduced classical histological signs of the disease [15]. Similar results were obtained using umbilical cord blood MSC-MV, human liver stem cells, which showed high clinical efficacy in experimental conditions of acute renal failure.

## Conclusion

In our work, we found MVs derived from MSCs are able to enhance proliferation splenocytes and reduce level of their apoptosis in an experimentally induced system. Also, on the ARF model, we recorded an improvement in excretory function of kidneys, assessed by normalization of biochemical parameters in urine and blood (albumin, creatinine) and restoration of morphological structure already on the 4th day after introduction of MSC-MV. At the same time, MSCs have immunoregulatory properties [9]. Immune system contribution in processes damage and restoration renal tissue has been shown in a number of works. In acute kidney injury, regardless of genesis, there is an increased activity of many its components, an enhancement in local production of cytokines number, growth and colony-stimulating factors [7, 11]. There are also data on a negative role of T, B lymphocytes and activated macrophages in the pathogenesis of renal failure. [11, 14]. At the same time, T regulatory cells number increasing contributes anti-inflammatory effect [1, 11]. Considering the above, we believe it appropriate to further study immunoregulatory properties not only MSCs, but also microvesicles produced by them.

## Acknowledgments

We express our gratitude for pathomorphology assessment of samples to PhD Prokopieva Elena Alexandrovna, Geletina Nadezda Sergeevna; to Belogorodtsev Sergey Nikolaevich for help with models.

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*Поступила 12.04.2023*
*Принята к печати 26.04.2023*

*Received 12.04.2023*
*Accepted 26.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 673-678

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 673-678

# ОЦЕНКА ДЛИТЕЛЬНОСТИ СОХРАНЕНИЯ Т-КЛЕТОК ПАМЯТИ У МЫШЕЙ ПОСЛЕ ИММУНИЗАЦИИ ЖИВОЙ ТУЛЯРЕМИЙНОЙ ВАКЦИНОЙ

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**Резюме.** Вакцинный штамм *F. tularensis* 15 НИИЭГ индуцирует длительный клеточный иммунитет, однако проявляет определенную реактогенность и генетическую нестабильность. Прогресс в разработке новых противотуляремийных вакцин с улучшенными характеристиками затруднен из-за недостатка знаний о механизмах формирования и поддержания протективного иммунитета. Мыши линии BALB/c являются наиболее подходящей моделью при экспериментальной туляремии из-за относительно низкой стоимости, хорошо охарактеризованной генетики, доступных иммунологических инструментов и наиболее близко имитируют инфекционный процесс при заражении вирулентными штаммами *F. tularensis*.

Известно, что $CD4^+$ и $CD8^+$T-клетки необходимы для формирования защитного иммунитета, однако роль определенных субпопуляций Т-клеток памяти в длительной защите от вирулентных штаммов *F. tularensis* не установлена. Мы предположили, что защитный иммунитет зависит от центральных ($T_{CM}$) и эффекторных Т-клеток памяти ($T_{EM}$) и их функциональной активности. В данной работе был изучен Т-клеточный иммунный ответ у мышей BALB/c через 30, 60 и 90 дней после подкожной вакцинации *F. tularensis* 15 НИИЭГ.

Анализ иммунного ответа спленоцитов проводили методом многопараметрической проточной цитометрии, стимулируя клетки *in vitro* антигеном *F. tularensis*. $T_{EM}$ клетки идентифицировали как $CD3^+CD4^+CD44^+CD62L^-$ и $CD3^+CD8^+CD44^+CD62L^-$, $T_{CM}$ клетки как $CD3^+CD4^+CD44^+CD62L^+$ и $CD3^+CD8^+CD44^+CD62L^+$, соответственно. Функциональную активность Т-клеток памяти оценивали по следующим параметрам: уровню экспрессии маркера активации CD69 и цитокин-продуцирующей активности путем окрашивания внутриклеточных цитокинов IFNγ и TNFα.

Таким образом, для разработки новой вакцины требуется выявление иммунологических критериев оценки протективного иммунитета, которые присутствуют не только в раннюю фазу после вакцина-

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**Образец цитирования:**

*А.С. Карцева, М.В. Силкина, Г.М. Титарева, Т.И. Комбарова, Р.И. Миронова, В.В. Фирстова «Оценка длительности сохранения Т-клеток памяти у мышей после иммунизации живой туляремийной вакциной» // Медицинская иммунология, 2023. Т. 25, № 3. С. 673-678.
doi: 10.15789/1563-0625-EOT-2746*

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**For citation:**

*A.S. Kartseva, M.V. Silkina, G.M. Titareva, T.I. Kombarova, R.I. Mironova, V.V. Firstova "Evaluation of the long-term memory T cell in mice after immunization with a live tularemia vaccine", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 673-678.
doi: 10.15789/1563-0625-EOT-2746*

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**DOI:** 10.15789/1563-0625-EOT-2746

ции, но после окончания эффекторной фазы иммунного ответа. Показано, что для поддержания длительного протективного иммунитета, инициированного вакцинацией *F. tularensis* 15 НИИЭГ, требуется наличие антиген-специфических $CD4^+$ и $CD8^+$Т-клеток памяти, продуцирующих IFNγ и TNFα и экспрессирующих маркер активации CD69. В отдаленные сроки после вакцинации было выявлено снижение количества и угасание функциональной активности субпопуляций $CD8^+T_{EM}$ и $CD8^+T_{CM}$. Выявленные параметры функциональной активности Т-клеток памяти могут служить критериями оценки протективного иммунитета в отношении вирулентных штаммов *F. tularensis*.

*Ключевые слова: Fransicella tularensis, вакцинный штамм, Т-клетки памяти, CD69, IFNγ, TNFα, клеточный иммунитет*

# EVALUATION OF THE LONG-TERM MEMORY T CELL IN MICE AFTER IMMUNIZATION WITH A LIVE TULAREMIA VACCINE

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**Abstract.** The vaccine strain *F. tularensis* 15 NIIEG induces long-lived cell-mediated immunity but exhibits a certain reactogenicity and genetic instability. Progress in development of a vaccine against tularemia has been limited by a lack of information regarding the mechanisms required to protect against this disease. The BALB/c mouse is the most commonly used animal to study tularemia due to its relatively low cost, well-characterized genetics, available immunological tools and mouse infection with virulent *F. tularensis* recapitulates human disease.

$CD4^+$ and $CD8^+$T cells are known to be critical for the formation of protective immunity but the relative roles of memory T cell subpopulations in long lived protection against virulent strains of *F. tularensis* are not well established. We hypothesized that this immunity depends on central ($T_{CM}$) and effector memory ($T_{EM}$) T cells and their functional activity. In this study we have dissected the T cell immune response in BALB/c mice 30, 60 and 90 days after subcutaneous vaccination with 15 NIIEG.

Multiparametric flow cytometry were used to characterize *in vitro* recall responses of splenocytes to *F. tularensis* antigen. $T_{EM}$ cells were identified as $CD3^+CD4^+CD44^+CD62L^-$ and $CD3^+CD8^+CD44^+CD62L^-$, $T_{CM}$ cells as $CD3^+CD4^+CD44^+CD62L^+$ and $CD3^+CD8^+CD44^+CD62L^+$, respectively. The functional activity of memory T cells was assessed by the following parameters: the level of expression of the activation marker CD69 and cytokine-producing activity by staining with the intracellular cytokines IFNγ and TNFα.

Thus, development of a long-lived vaccine directed against *F. tularensis* is dependent on identifying not only the correlates of immunity present early after vaccination, but also those that persist in the host after the effector phase has ended. The maintenance of long-term protective immunity initiated by vaccination with *F. tularensis* strain 15 NIIEG has been shown to require the presence of antigen-specific $CD4^+$ and $CD8^+$ memory T cells producing IFNγ and TNFα and expressing the activation marker CD69. A decrease in count and functional activity of $CD8^+T_{CM}$ and $CD8^+T_{EM}$ was detected in the long term after vaccination. The detected parameters of functional activity of memory T cells can be used as criteria for evaluation of protective immunity against virulent strains of *F. tularensis*.

*Keywords: Fransicella tularensis, vaccine strain, memory T cells, CD69, IFNγ, TNFα, cellular immunity*

## Introduction

The live tularemia vaccine used in the Russian Federation based on the *F. tularensis* 15 strain NIIEG was developed empirically with limited understanding of its immunological mechanisms. The *F. tularensis* 15 NIIEG vaccine strain provides effective immunity against tularemia but exhibits a certain reactogenicity and genetic instability [2]. $CD4^+$ and $CD8^+$T cells are known to play a key role in the formation of protective immunity against tularemia [5] but the contribution of central ($T_{CM}$) and effector ($T_{EM}$) memory T cells subpopulation in the formation of long-term protective immunity remains poorly understood. The detailed knowledge of the mechanisms of long-term

immunity would allow an objective assessment of the immunogenic and protective properties of the new tularemia vaccine strains under development with improved properties.

The mouse is the most commonly used animal to study tularemia due to available immunological tools that allow a detailed study of the T cell immune response [5]. The study of the mechanisms of protective immunity to *F. tularensis* and the identification of immunological criteria for its assessment in a mouse model is an important step in research on the development of new vaccine strains and the improvement of laboratory methods for the evaluation of the T cell immunity.

**The purpose of the present study** was to investigate the subpopulation composition and functional activity of memory T cells in BALB/c mice in the long time after vaccination with *F. tularensis* strain 15 NIIEG.

## Materials and methods

All animal experiments were performed in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Specific-pathogen-free, 6-8-week-old BALB/c mice ($H2^d$) were purchased from Laboratory Animal Breeding Facility (Pushchino, Moscow Region).

Vaccine strain of F. tularensis 15 NIIEG was obtained in the SRCAMB collection (Obolensk, Moscow Region). Mice were vaccinated subcutaneously with *F. tularensis* 15 NIIEG cells in PBS (30 CFU per mouse) in the inner part of the upper thigh. A group of unvaccinated mice served as a control.

At 30, 60 and 90 days after immunization animals were euthanized, spleens were sterilely extracted and lymphocyte cultures were obtained according for evaluation of T cell immunological memory as previously described [4]. Lymphocytes were plated at $2 \times 10^5$ cells/well into a 96-well plate and incubated with 10 μg/mL *F. tularensis* antigen obtained by the published procedure [7].

Subpopulation composition and functional activity of memory T cells were detected by flow cytometry using commercial monoclonal antibodies (MAbs) (BD Bioscience, USA) conjugated with the different fluorochromes: CD3 APC and CD3 BV421 (clone 17A2); CD4 BB700 (clone RM4-5); CD8 APC (clone 53-6.7); CD62L FITC (clone MEL14); CD44 PE (clone IM7); CD69 BV421 (clone H1.2F3).

Central ($CD4^+T_{CM}$) and effector ($CD4^+T_{EM}$) memory T helper cells were identified as $CD3^+CD4^+CD44^+CD62L^+$ and $CD3^+CD4^+CD44^+CD62L^-$ subpopulations, respectively; cells with $CD3^+CD8^+CD44^+CD62L^+$ and $CD3^+CD8^+CD44^+CD62L^-$ phenotypes were identified as central ($CD8^+T_{CM}$) and effector ($CD8^+T_{EM}$) memory T cells. The functional activity of the above memory T cell subpopulations was assessed based on the expression level of the CD69 molecule on their surface.

Memory T cells were stained intracellular MAbs against IFNγ APC (clone XMG1.2) and TNFα APC (clone MP6-XT22) to analyze cytokine-producing activity as previously described [3]. Cells were acquired and analyzed using a FACSAria III (BD Biosciences, USA) flow cytometer and BD FACSDiva Software v. 8.0.1.

Statistical processing of the results was performed using GraphPad Prism 6.00 for Windows (GraphPad Prism Software Inc., USA). Cytometry data were presented as a percentage of the target T cell subpopulation and provided as a median and interquantile range (Me ($Q_{0.25}$-$Q_{0.75}$)). The two experimental groups were compared using the Mann–Whitney U test with the significance set at $p < 0.05$.

## Results and discussion

In our earlier study, we demonstrated that the long-term protective immunity against tularemia in vaccinated mice depends on the subspecies infecting strain: infection with the virulent *F. tularensis* 503 of the same *holarctica* subspecies as the vaccine strain *F. tularensis* 15 NIIEG provides 100 % protection for 180 days after vaccination; infection with the virulent *F. tularensis* Schu subspecies of *tularensis* leads to a decrease in protection with an increase in the postvaccination period to 60 days [4].

The major objective of this study was to identify immunological parameters characterizing the duration of protection against virulent *F. tularensis* strains. Antigen-specific $CD4^+$ and $CD8^+$ memory T cells are heterogeneous in composition and during immune response are differentiated in $T_{EM}$ and $T_{CM}$ [6]. We performed a comparative analysis of the dynamics of changes in subpopulation composition and functional activity of $CD4^+$ and $CD8^+$ memory T cells by flow cytometry at 30, 60 and 90 days after immunization with *F. tularensis* 15 NIIEG vaccine strain. A fluorescent-labelled monoclonal antibody panel to CD4, CD8, CD44 and CD62L surface antigens allows the identification of $T_{EM}$ ($CD44^+CD62L^-$) and $T_{CM}$ ($CD44^+CD62L^+$) in the $CD4^+$ and $CD8^+$ lymphocyte pool. Expression levels of the lymphocyte activation marker CD69 [8] and cytokine production of IFNγ and TNFα were analyzed to assess the antigen reactivity of memory T cell subpopulations. The dynamics of changes in the subpopulation composition and functional activity of memory T cells as a function of the time after vaccination is presented in Figure 1.

Comparative analysis of the data has shown that the percentage of functionally active $CD4^+T_{CM}$ did not change and was comparable in all studied periods after immunization: the cells expressed on their surface the

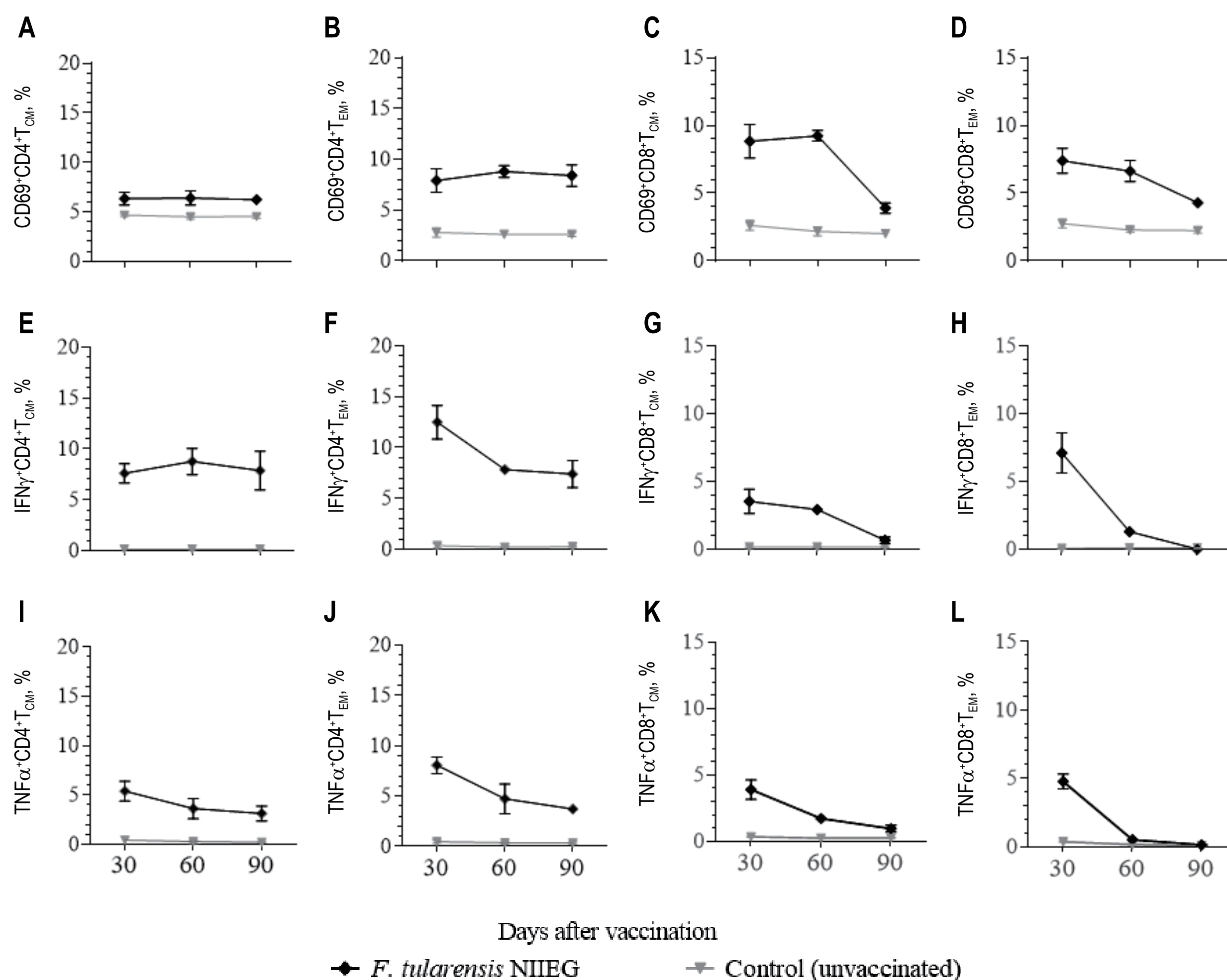


**Figure 1. Subpopulation composition and functional activity of $T_{CM}$ and $T_{EM}$ in the pool of $CD4^+$ and $CD8^+$T cells in the spleen of BALB/c mice during early and late time points after immunization with live tularemia vaccine**

Note. (A, B, C, D) T cell subpopulations expressing the activation marker CD69. (E, F, G, H) IFNγ-producing memory T cell subpopulations. (I, K, L, M) TNFα-producing memory T cell subpopulations.

activation marker CD69 (Figure 1A) and produced IFNγ (Figure 1E) and TNFα (Figure 1I). After the effector phase has ended (30 days) we observed a significant decrease in the number of cells with $IFN\gamma^+CD4^+T_{EM}$ (Figure 1F) and $TNF\alpha^+CD4^+T_{EM}$ (Figure 1J) phenotype at day 60 after vaccination; at day 90 no change was detected compared to the previous post-vaccination period. Therefore, no significant differences in the percentage and functional activity of $CD4^+$ memory T cells were detected in the long-term after vaccination with the studied *F. tularensis* strains. Antigen-specific $CD4^+$ memory T cells with $T_{CM}$ and $T_{EM}$ phenotypes produced cytokines TNFα and IFNγ and expressed activation marker CD69 even 90 days after immunization with the tularemia vaccine strain.

An important difference was found for $T_{CM}$ and $T_{EM}$ $CD8^+$ with phenotypes memory T cells at a long-term post-vaccination period. Comparative analysis of $CD69^+$ memory T cell subpopulations has shown to increasing post-vaccination time from 60 to 90 days was observed a decrease in $CD8^+T_{CM}$ (Figure 1C) and $CD8^+T_{EM}$ (Figure 1D) in immunized mice. A similar tendency was observed in the $CD8^+T_{CM}$ (Figure 1G, K) and $CD8^+T_{EM}$ (Figure 1H, L) cytokine-producing activities: at 60 days after immunization with *F. tularensis* 15 NIIEG strain, the cytokine-producing activity of the $CD8^+T_{CM}$ and $CD8^+T_{EM}$ subpopulations was of a low profile; at 90 days after immunization there was no statistically significant difference in the number of all cytokine-producing ($IFN\gamma^+$ and $TNF\alpha^+$) $CD8^+$ memory cells compared to the intact group.

One explanation for the waning of protective immunity over time against intracellular infections is the decreasing pool of long-lived $T_{CM}$ [1]. Our results are consonant with earlier published data about to the end of the effector phase of the T cell immune response most $T_{EM}$ undergo apoptosis and for formation long-term protection it is necessary for the vaccine to induce the generation of $T_{CM}$

capable of rapidly proliferating and differentiating into $T_{EM}$, which, in turn, successfully eliminate the pathogen [6]. Therefore, it is possibly that exhaustion of the $CD8^{+}T_{CM}$ pool with increasing post-vaccination period results in decreased functional activity of $CD8^{+}T_{EM}$ on days 60 and 90 after vaccination with *F. tularensis* 15 NIIEG.

## Conclusion

In summary, antigen-specific $T_{CM}$ and $T_{EM}$ with $CD4^{+}$ and $CD8^{+}$T cell phenotypes are producing IFNγ and TNFα and expressing CD69 are required to maintain long-term protective immunity initiated by vaccination with live tularemia vaccine. The obtained data on the significant functional activity of $T_{CM}$ and $T_{EM}$ $CD4^{+}$ memory T cells at all post-vaccination times explain the mechanism of protection up to 90 days after vaccination of mice who have been infected with the virulent strain *F. tularensis* 503 subsp. *holarctica* [4]. In turn, the decreased of functional activity of the $T_{CM}$ and $T_{EM}$ $CD8^{+}$ memory T cells is correlated with the weakening of protection against virulent *F. tularensis* Schu subsp. tularensis during late time points after vaccination with *F. tularensis* 15 NIIEG [4].

We recommend the functional activity of $T_{EM}$ and $T_{CM}$ by the expression level of the activation marker CD69 and the synthesis of cytokines IFNγ and TNFα as immunological criteria to evaluate the effectiveness of immunization with candidate vaccine strains of *F. tularensis*.

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*Поступила 13.04.2023*
*Отправлена на доработку 17.04.2023*
*Принята к печати 20.04.2023*

*Received 13.04.2023*
*Revision received 17.04.2023*
*Accepted 20.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 679-684



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 679-684

# ИССЛЕДОВАНИЕ АДЪЮВАНТНЫХ СВОЙСТВ ПРЕПАРАТОВ, СОДЕРЖАЩИХ РЕКОМБИНАНТНЫЙ ГРАНУЛОЦИТАРНО-МАКРОФАГАЛЬНЫЙ КОЛОНИЕСТИМУЛИРУЮЩИЙ ФАКТОР ЧЕЛОВЕКА

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**Резюме.** Актуальность поиска новых вакцинных адъювантов растет вместе с ростом количества новых вакцинных препаратов, особенно созданных на основе белков либо нуклеиновых кислот. Известно, что некоторые цитокины обладают адъювантными свойствами. Представленная работа посвящена изучению адъювантной активности рекомбинантного гранулоцитарно-макрофагального колониестимулирующего фактора человека (rhGM-CSF) и конструкций на его основе. Ранее нами была разработана технология выделения и очистки rhGM-CSF, а также технология получения конъюгатов полиглюкин-спермидин с рекомбинантным rhGM-CSF. Для получения молекулярных конструкций на основе конъюгата rhGM-CSF использовали двуспиральную РНК. Для сборки конструкций соотношение компонентов рассчитывали таким образом, чтобы в одной дозе препарата содержалось 5-40 мкг белка с rhGM-CSF и 100 мкг двуспиральной РНК. Эффективность сборки молекулярной конструкции оценивали в 1%-ном агарозном геле по снижению подвижности двуспиральной РНК. Эффективность полученных адъювантов определяли измерением титров специфических антител в сыворотках мышей методом ИФА с использованием в качестве антигенов овальбумина либо рекомбинантного рецептор-связывающего домена поверхностного белка коронавируса SARS-CoV-2 (вариант B.1.617.2 (Delta)). В работе использовали самцов мышей линии BALB/c массой 16-18 г в количестве 100 особей. Иммунизацию проводили двукратно с интервалом 14 суток, внутримышечной инъекцией по 200 мкл на животное. Рекомбинантный рецептор-связывающий домен поверхностного белка коронавируса SARS-CoV-2 вводился в дозе 50 мкг на животное, овальбумин в двух дозах – 1 и 5 мкг на животное. В качестве положительного контроля использовали соответствующий антиген. В качестве отрицательного контроля – физиологический раствор. Показано, что максимальный эффект был достигнут при иммунизации конструкцией на основе конъюгата полиглюкин-спермидин с rhGM-CSF с двуспиральной РНК, использование в качестве адъюванта конъюгата без двуспиральной РНК так

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**Образец цитирования:**
*Т.И. Есина, Е.А. Волосникова, Н.В. Волкова, О.Н. Каплина, Е.Д. Даниленко «Исследование адъювантных свойств препаратов, содержащих рекомбинантный гранулоцитарно-макрофагальный колониестимулирующий фактор человека» // Медицинская иммунология, 2023. Т. 25, № 3. С. 679-684.*
*doi: 10.15789/1563-0625-SOT-2833*

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**For citation:**
*T.I. Esina, E.A. Volosnikova, N.V. Volkova, O.N. Kaplina, E.D. Danilenko "Study of the adjuvant properties of preparations containing recombinant human granulocyte-macrophage colony stimulating factor", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 679-684.*
*doi: 10.15789/1563-0625-SOT-2833*

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**DOI:** 10.15789/1563-0625-SOT-2833

же приводило к усилению гуморального ответа. Использование нативного с рекомбинантным гранулоцитарно-макрофагальным колониестимулирующим фактором человека не привело к повышению показателей титров специфических антител. Таким образом, установлено, что rhGM-CSF в составе конъюгата с полисахаридом либо молекулярной конструкции обладал способностью усиливать гуморальный иммунный ответ на белковые антигены.

*Ключевые слова: адъювант, ГМКСФ, иммунный ответ, S-белок, овальбумин, иммунизация*

# STUDY OF THE ADJUVANT PROPERTIES OF PREPARATIONS CONTAINING RECOMBINANT HUMAN GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR

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**Abstract.** The relevance of the search for new vaccine adjuvants is growing along with the increase in the number of current vaccine preparations, especially those developed on the basis of proteins. Some cytokines are known to exert adjuvant properties. The present work is devoted to the study of adjuvant activity of recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) and constructs based on it. Earlier, we developed a technology for isolation and purification of GM-CSF from the E. coli SG20050/p280_2GM producer strain, as well as a technology for conjugating polyglucin:spermidine complexes with rhGM-CSF. Double-stranded RNA was used to obtain molecular constructs on the basis of rhGM-CSF conjugate. To assemble constructs, the ratio of the components was calculated for one dose of the preparation to contain 5-40 μg of rhGM-CSF and 100 μg of double-stranded RNA. The effectiveness of the formation of molecular constructs was evaluated by dsRNA electrophoretic mobility shift in a 1% agarose gel. The effectiveness of the resulting adjuvants was determined in ELISA assays by measuring the titers of specific antibodies in mouse sera against ovalbumin or recombinant receptor-binding domain of the surface S protein of the severe acute respiratory syndrome coronavirus 2 (Delta variant (B.1.617.2). The experiments were carried out in 100 male BALB/c mice weighing 16-18 g. Mice were immunized twice, with a 14-day interval, by intramuscular injection of 200 μL per animal. Recombinant receptor-binding domain of the surface protein of SARS-CoV-2 was administered at a dose of 50 μg/animal, ovalbumin – at two doses – 1 μg or 5 μg/animal. Corresponding antigen was used as a positive control, a saline solution – as a negative control. It was shown that the maximum effect was achieved by immunization with a construct based on double-stranded RNA and rhGM-CSF conjugated to polyglucin-spermidine. The use of a conjugate without double-stranded RNA as an adjuvant also improved humoral response. The use of native rhGM-CSF did not increase the titers of specific antibodies. Thus, it was found that rhGM-CSF being a part of a polysaccharide conjugate or a molecular construct exerted an ability to enhance the humoral immune response to protein antigens.

*Keywords: adjuvant, GM-CSF, immune response, S protein, ovalbumin, immunization*

The work was supported by the State Assignment 1/22 of the State Research Center of Virology and Biotechnology "Vector", Rospotrebnadzor, "Search and pharmaco-toxicological study of new vaccine adjuvants".

## Introduction

The relevance of the search for new vaccine adjuvants is growing along with the increase in the number of new vaccine preparations, especially those developed on the basis of proteins (subunit or peptide vaccines) or nucleic acids (RNA-, DNA-vaccines). Some cytokines are known to exert adjuvant properties [1, 14], which allows them to be considered as promising adjuvants of current vaccines.

Preventive vaccines based on GM-CSF elicited strong antitumor and antiviral immune responses in preclinical experiments [1, 10, 14]. However, in clinical studies, these effects were not always reproduced, moreover, at times they contradicted the

results of animal studies [15]. One of the reasons for such ambiguous results may probably be the rapid degradation of cytokine in the bloodstream and, as a consequence, the use of its high doses, which stimulate hematopoiesis, but at the same time inhibit the immune response [2, 9, 10]. In this regard, it is relevant to search for the ways to stabilize GM-CSF, which is possible, in particular, by its conjugation with various carriers or inclusion in the composition of corpuscular structures.

Earlier, an original system for depositing and transporting proteins was designed at the State Research Center of Virology and Biotechnology "Vector", which is a molecular construct containing yeast double-stranded RNA (dsRNA) in the central part, protected by an envelope of polyglucin-spermidine conjugate [8]. The introduction of dsRNA into the core of the construct, on the one hand, solves the problem of particles self-assembly, and on the other – potentiates the activity of the protein component due to immunomodulating activity of polynucleotide complex. Double-stranded RNAs used as the "core" of constructs were shown to be able to enhance the immunogenicity of vaccines [3, 7].

**The aim of the present work** was to obtain preparations containing recombinant human GM-CSF as part of conjugates or molecular constructs, and to study their adjuvant activity.

## Materials and methods

Earlier, we developed a technology for the isolation and purification of GM-CSF from the *E. coli SG20050/p280_2GM* producer strain [5], as well as a technology for conjugating polyglucin: spermidine (PGS) complexes with rhGM-CSF [6], used herein. The resulting rhGM-CSF-PGS conjugates were analyzed using electrophoresis in 15% PAAG, the concentration of the protein within the conjugate was determined by the Lowry method.

To obtain molecular constructs based on rhGM-CSF-PGS conjugates, the substance of the Ridostin drug (Sodium salt of double-stranded ribonucleic acid, FSP R No. 002021/01 – 07 04 20090769-08), containing 21% of dsRNA (produced by the Institute of medical biotechnology of the State Research Center of Virology and Biotechnology "Vector" of Rospotrebnadzor) was used. To assemble constructs, the ratio of the components was calculated for one dose of the preparation to contain 5-40 μg of rhGM-CSF and 100 μg of dsRNA. The effectiveness of the formation of molecular constructs was evaluated by dsRNA electrophoretic mobility shift in a 1% agarose gel.

Adjuvant activity of the obtained conjugates and constructs was assayed in ELISA and evaluated by changing the titers of specific antibodies in blood sera of mice immunized with ovalbumin (OVA, cat. No. A5503-5G, Sigma) or receptor-binding domain (RBD) of the surface S-protein of the severe acute respiratory syndrome coronavirus 2 (Delta variant (B.1.617.2).

The experiments were carried out in 100 male BALB/c mice weighing 16-18 g. The animals were kept and manipulated in compliance with the principles of humane treatment of laboratory animals in accordance with Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS № 123, Strasbourg, 1986). All the experimental procedures were approved by the Bioethics Committee of the State Research Center of Virology and Biotechnology "Vector" (SRC VB Vector/September 10, 2020, approved by the protocol of Bioethics Committee No. 5 as of October 1, 2020). BALB/c mice were immunized twice, with a 14-day interval, by intramuscular injection of 200 μL per animal. RBD was administered at a dose of 50 μg/animal, OVA – at a dose of 5 μg/animal, adjuvants – at a dose of 40 μg (GM-CSF). Corresponding antigen was used as a positive control, a saline solution – as a negative control.

On day 10 after the second immunization, blood samples were collected from the retro-orbital sinus of mice in a volume of 0.5 ml using a Pasteur pipette as described in [4]. Blood sera were obtained by standard methods and stored at (6 ± 2) °C for no more than 7 days. For longer storage, sera were frozen at -20 °C.

After blood sampling, mice were euthanized by cervical dislocation as described in [11]. Before the analysis, sera from 6 mice in the group were thawed and combined into one sample (total sera). The level of specific antibodies in the sera of immunized mice was determined by ELISA assays. In the 96-well plates, antigens (100 μL/well) were sorbed in phosphate-buffered saline (PBS) (pH 7.4) for 2 hrs at 37 °C and for 16 hrs at 4 °C. The unbound antigen solution was removed, and 200 μL of the blocking buffer (1% solution of bovine serum albumin (BSA) in PBS, pH 7.4) was added to the wells. The plates were incubated for 2 hrs at 37 °C, and then washed three times with 350 μL/well of PSBT (PBS supplemented with 0.05% Twin-20). 200 μL of PBS was added to each well of row A, and 100 μL of PSBT supplemented with 0.5% BSA – to each well of rows B – H. 12 samples of total sera of immunized animals were added to the wells of row A (1-12 strips), and the titration in the vertical rows was performed. To control the conjugate, a buffer dilution solution (single PBS (pH 7.4) supplemented with 0.05% Twin-20 and 0.5% BSA) was added to two wells, without sera samples. The plates were incubated at 310 rpm in a thermoshaker PST-60HL-4 for 1.5 hrs at 37 °C. After incubation, the wells were washed four times, as described above. Next, 100 μL of goat anti-mouse IgG-HRP antibodies conjugate

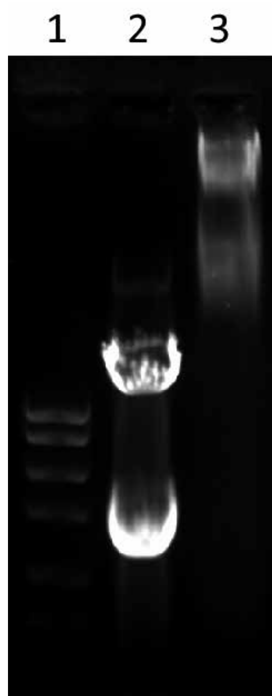


**Figure 1. Electrophoresis of preparations containing dsRNA in 1% agarose gel, staining with ethidium bromide**

Note. Lanes: 1, DNA ladder 250-2000 bp; 2, dsRNA (100 μg); 3, dsRNA (100 μg) incubated for 60 minutes with GM-CSF-PGS conjugate (40 μg of protein).

solution (Sigma, USA), diluted at 1:5000 in a buffer solution for dilution, was added to each well, and the plates were incubated at 310 rpm in a thermoshaker for 1 hr at 37 °C. The contents of the wells were then removed and washed five times, as described above. To produce a color reaction, the plates were coated with chromogen 3,3’,5,5’-tetramethylbenzidine solution (a one-component ready-made solution), 100 μL per well, and incubated for 30 min at RT protected from direct light. The reaction was stopped by adding 50 μL of “stop reagent” (1M solution of sulfuric acid) to each well. Absorbance was measured at 450 nm using a Varioskan Lux multimode microplate reader (Thermo Fisher Scientific, USA). The results were processed using GraphPad Prism 6.0 software.

## Results and discussion

The need to use adjuvants in current subunit vaccines is often associated with low immunogenicity or side effects at high doses. Therefore, ways to increase the immunogenicity of such vaccines remain urgent task in the development of new vaccine preparations.

We evaluated adjuvant properties of native GM-CSF and GM-CSF preparations. To stabilize GM-CSF, conjugates with a polysaccharide matrix (PGS complex) were synthesized according to [12],

**TABLE 1. GEOMETRIC MEAN TITERS OF SPECIFIC ANTIBODIES AGAINST OVA IN THE BLOOD SERUM OF MICE AFTER A TWO-TIME IMMUNIZATION WITH OVA, OR OVA + GM-CSF PREPARATIONS**

| Preparation | OVA | OVA + GM-CSF | OVA + GM-CSF-PGS conjugate | OVA + (GM-CSF-PGS + dsRNA) construct |
|---|---|---|---|---|
| Dose, μg/mouse | 5 | 5 μg (OVA)<br>40 μg (GM-CSF) | 5 μg (OVA)<br>40 μg (GM-CSF) | 5 μg (OVA)<br>40 μg (GM-CSF)<br>100 μg (dsRNA) |
| GMT | 500 | 125 000 | 500 000 | 3 000 000 |
| N | | 250 | 1 000 | 6 000 |

Note. GMT, geometric mean titer of specific antibodies against OVA; N, multiplicity of titers of antibodies against OVA in the blood serum of mice immunized with OVA combined with a preparation in comparison with OVA alone.

**TABLE 2. GEOMETRIC MEAN TITERS OF SPECIFIC ANTIBODIES AGAINST RBD IN THE BLOOD SERUM OF MICE AFTER A TWO-TIME IMMUNIZATION WITH RBD, OR RBD + GM-CSF PREPARATIONS**

| Preparation | RBD | RBD + GM-CSF | RBD + GM-CSF- PGS conjugate | RBD + (GM-CSF-PGS + dsRNA) construct |
|---|---|---|---|---|
| Dose, μg/mouse | 50 | 50 μg (RBD)<br>40 μg (GM-CSF) | 50 μg (RBD)<br>40 μg (GM-CSF) | 50 μg (RBD)<br>40 μg (GM-CSF)<br>100 μg (dsRNA) |
| GMT | 3 000 | 625 000 | 1 000 000 | 15 625 000 |
| N | | 208 | 333 | 5 208 |

Note. GMT, geometric mean titer of specific antibodies against RBD; N, multiplicity of titers of antibodies against RBD in the blood serum of mice immunized with RBD combined with a preparation in comparison with RBD alone.

and molecular constructs containing GM-CSF-PGS conjugate were obtained as in [6, 13].

The constructs based on dsRNA molecules and conjugate molecules assembled due to the formation of ionic bonds between positively charged spermidine and negatively charged dsRNA. Therefore, the effectiveness of the constructs formation was evaluated by dsRNA electrophoretic mobility decrease in 1% agarose gel as a result of formation of a polyglucin complex, which demonstrated in Figure 1.

The results of studying adjuvant properties of the obtained preparations (Table 1) indicate that a two-time immunization with OVA in combination with various adjuvants containing GM-CSF and dsRNA led to the appearance of specific antibodies in the blood of animals in titers from 125 000 to 3 000 000, while the values for immunization with OVA alone did not exceed 1:500.

The administration of GM-CSF in combination with OVA increased the titer of specific antibodies by 250 times, compared to the comparison group (OVA). The preparation of GM-CSF with PGS conjugate increased antibody titers by 1000 times compared to the mice immunized with OVA; the preparation of a molecular construct containing GM-CSF-PGS and dsRNA – by 6000 times.

Table 2 shows the values of geometric mean titers of specific antibodies against RBD protein. As one can see, the immunization of mice with RBD in combination with GM-CSF and dsRNA preparations led to a significant increase in titers of specific antibodies, similarly as in case with OVA. The GMT of antibodies after administration of GM-CSF increased by 208 times, GM-CSF-PGS conjugate – by 333 times. The administration of GM-CSF as part of a dsRNA construct led to even more pronounced stimulation. The titers of specific antibodies increased by 5208 times compared to RBD, by 15.6 and 25.0 times, compared to GM-CSF-PGS conjugate and GM-CSF, respectively.

## Conclusion

Thus, the experimental data obtained confirm that GM-CSF preparations possess the ability to enhance humoral immune response to immunization with various antigens (OVA, RBD). The use of dsRNA as a component of the adjuvant construct additionally contributes to the effectiveness of vaccination.

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*Поступила 15.04.2023*
*Отправлена на доработку 21.04.2023*
*Принята к печати 24.04.2023*

*Received 15.04.2023*
*Revision received 21.04.2023*
*Accepted 24.04.2023*

# РАЗРАБОТКА ВАКЦИННОГО АДЪЮВАНТА НА ОСНОВЕ СКВАЛЕНА И ИЗУЧЕНИЕ ЕГО АДЪЮВАНТНЫХ СВОЙСТВ

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**Резюме.** Использование современных субъединичных вакцин предполагает введение в их состав адъювантов. В настоящее время активно ведется поиск новых и усовершенствование существующих адъювантных систем. Адъюванты на основе сквалена известны и разрешены в ряде стран для клинического применения в составе вакцин против гриппа. Наша работа посвящена разработке адъюватной композиции, содержащей в своем составе сквален. Полученные нами адъювантные композиции представляли собой масляную эмульсию, содержащую гидрофильную и гидрофобную фазу. Стабильности эмульсии добивались путем обработки ее ультразвуком с частотой 22 кГц. Оценку размеров частиц полученных эмульсий проводили с помощью электронного микроскопа. Показано, что размер частиц большинства частиц (84%) составил от 50 до 80 нм. Оценку адъювантной активности проводили на 100 самцах мышей линии BALB/c массой 16-18 г. Для оценки гуморального иммунного ответа иммунизацию проводили двукратно с интервалом 14 суток, внутримышечной инъекцией объемом 200 мкл на животное. В качестве антигена использовали рецептор-связывающий домен поверхностного белка коронавируса SARS-CoV-2 (вариант B.1.617.2 (Delta)) либо овальбумин из куриных яиц. Рецептор-связывающий домен поверхностного белка коронавируса SARS-CoV-2 вводили в дозе 50 мкг на животное, овальбумин – 1 и 5 мкг на животное. В качестве положительного контроля использовали антиген с гидроксидом алюминия. В качестве отрицательного контроля – физиологический раствор. Эффективность полученных адъювантов определяли измерением титров специфических антител в сыворотках мышей методом ИФА с использованием рекомбинантного рецептор-связывающего домена поверхностного белка коронавируса SARS-CoV-2 (вариант B.1.617.2 (Delta)) либо овальбумин из куриных яиц. В ходе работы показано, что использование адъювантов на основе сквалена позволило увеличить иммуногенность антигенов. В случае с рецептор-связывающим доменом поверхностного белка коронавируса SARS-CoV-2 средние титры специфических антител в опытной группе в 4 раза превышали титры контрольной группы, иммунизированной антигеном с гидроокисью алюминия. Повышение иммуногенности антигена с добавлением сквалена наблюдали

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*Е.А. Волосникова, Д.Н. Щербаков, В.В. Ермолаев, Н.В. Волкова, О.Н. Каплина, М.Б. Боргоякова, Е.Д. Даниленко «Разработка вакцинного адъюванта на основе сквалена и изучение его адъювантных свойств» // Медицинская иммунология, 2023. Т. 25, № 3. С. 685-690.*
*doi: 10.15789/1563-0625-DOA-2824*

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**For citation:**

*E.A. Volosnikova, D.N. Shcherbakov, V.V. Ermolaev, N.V. Volkova, O.N. Kaplina, M.B. Borgoyakova, E.D. Danilenko “Development of a vaccine adjuvant based on squalene and study of its adjuvant properties”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 685-690.*
*doi: 10.15789/1563-0625-DOA-2824*

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**DOI:** 10.15789/1563-0625-DOA-2824

в опытной группе наблюдали и в случае с овальбумином. Таким образом, показано, что разработанная адъювантная система на основе сквалена является альтернативой традиционным адъювантам на основе солей алюминия.

*Ключевые слова: адъювант, сквален, иммунный ответ, S-белок, овальбумин, иммунизация*

# DEVELOPMENT OF A VACCINE ADJUVANT BASED ON SQUALENE AND STUDY OF ITS ADJUVANT PROPERTIES

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**Abstract.** The use of modern subunit vaccines involves adjuvant introduction into their composition. Currently, the search for new and improvement of existing adjuvant systems is actively underway. Squalene-based adjuvants are well-known and approved in a number of countries for clinical use in influenza vaccines. Our study was devoted to the development of an adjuvant composition on the basis of squalene. The resulting adjuvants were composed in a form of oil emulsion containing a hydrophilic and hydrophobic phase. The stability of the emulsion was achieved by treating it with ultrasound at a frequency of 22 kHz. Particle sizes of the obtained emulsions were examined with the use of an electron microscope. The particle size was calculated to be 50-80 nm for the majority of particles (84%). Adjuvant activity was evaluated in 100 male Balb/C mice, weighing 16-18 g. To assess the humoral immune response, immunization was performed twice, with a 14-day interval, by intramuscular injection of 200 μL per animal. The receptor-binding domain (RBD) of the surface S protein of the severe acute respiratory syndrome coronavirus 2 (Delta variant (B.1.617.2)) or ovalbumin (OVA) from chicken eggs were used as antigens. RBD was administered at a dose of 50 μg/animal; OVA was administered at two doses (1 μg or 5 μg/animal). An antigen with aluminum hydroxide was used as a positive control; a saline solution was used as a negative control. The effectiveness of the obtained adjuvants was determined by measuring the titers of specific antibodies in mouse sera in ELISA assays using the recombinant RBD of SARS-CoV-2 S-protein or ovalbumin from chicken eggs. It was shown that the use of squalene-based adjuvants increased the antigens' immunogenicity. The average titers of specific antibodies against RBD in the experimental group were 4 times higher than in the group immunized with RBD adjuvanted with aluminum hydroxide. An increase in immunogenicity of the antigen adjuvanted with squalene was also observed in the experimental OVA-group. Thus, it was shown that the developed squalene-based adjuvant compositions could be an alternative to the traditional adjuvants based on aluminum salts.

*Keywords: adjuvant, squalene, immune response, S protein, ovalbumin, immunization*

The work was supported by the State Assignment 1/22 of the State Research Center of Virology and Biotechnology "Vector", Rospotrebnadzor, "Search and pharmaco-toxicological study of new vaccine adjuvants".

## Introduction

Subunit vaccines continue to play an important role in the vaccination to prevent infectious diseases. One of the recent examples is the WHO approval of the subunit Mosquirix vaccine against malaria [6]. Long-term studies show that subunit vaccines, while possessing an unsurpassed safety profile, ease of production and high stability, nevertheless have relatively low immunogenicity. Therefore, adjuvants are an obligatory component of the vaccine preparations based on recombinant proteins. Through the use of adjuvants, it can be possible not only to increase immunogenicity, but also to induce mucosal immunity or trigger/stimulate cellular response mechanisms [4].

The most commonly used adjuvants in vaccines nowadays are aluminum salts. Their mechanisms of action are still not completely clear [1]. One of the main versions is the induction of a local inflammatory

process at the site of vaccine administration, which can elicit the development of a pronounced local and systemic reaction [5].

In addition to aluminum compounds, among those officially approved, adjuvants based on *Quillaja saponaria* saponins, CpG and squalene are being currently used [7]. The latter, in the form of MF59 adjuvant, has been successfully used in influenza vaccines for more than 15 years, with its high efficacy and safety being confirmed [2].

**The purpose of the present work** was to develop an adjuvant composition based on squalene and to study its effect on the vaccination effectiveness.

## Materials and methods

**Adjuvant composition assembly**

The formulation of adjuvant compositions was calculated for the final preparation to have squalene at the concentration of 4.3 or 8.6% of the volume. The hydrophobic phase consisted of phospholipids dissolved in squalene (0.5 – 1% of the final volume), the hydrophilic phase – the Twin 80 emulsifier dissolved in PBS (pH 7.6). The emulsion was obtained by treating the combined hydrophilic and hydrophobic phases with ultrasound with a frequency of 20-60 kHz. The resulting emulsion was sterile filtered through 0.22 μm bacterial filters.

For the control, an incomplete adjuvant was obtained, which comprised all the components of the composition described above except squalene.

**Animal immunization**

Ovalbumin (OVA) from chicken eggs or the receptor-binding domain (RBD) of the surface S-protein of the severe acute respiratory syndrome coronavirus 2 (Delta variant (B.1.617.2), obtained at the SRC VB "Vector" using CHO-K1 cells, were chosen as antigens.

The study of the adjuvant properties of the drug was carried out in male Balb/C mice, weighing 18-22 g, aged 6-8 weeks, obtained from the Nursery of the SRC VB "Vector" of Rospotrebnadzor, Koltsovo, Novosibirsk Region. Animal experiments were approved by the Bioethics Committee of the State Research Center of Virology and Biotechnology "Vector" (SRC VB Vector/September 10, 2020, approved by the protocol of Bioethics Committee No. 5 as of October 1, 2020). Mice of the positive control groups were administered intramuscularly twice, with a 14-day interval, with 1 and 5 μg OVA or 50 μg RBD in a volume of 200 μL/mouse (100 μL in each hind paw).

Mice of the experimental groups were administered with OVA at the doses of 1 and 5 μg/mouse or RBD at the doses of 25 and 50 μg/mouse (in a volume of 200 μL/mouse) in combination with the obtained adjuvant composition comprising 4.3 or 8.6% squalene. The mice of the negative control group were injected with an equivalent volume of the saline solution. Mice of the comparison groups were immunized intramuscularly twice, with a 14 day interval, with 50 μg RBD mixed with aluminum hydroxide or with 1 and 5 μg OVA mixed with aluminum hydroxide or an incomplete adjuvant.

Blood sampling was performed on day 7 after the second immunization as described in [3].

To detect the titer of specific antibodies in the sera of immunized mice, the 96-well plates were coated with 100 μL of OVA (5 μg/mL) or RBD (1 μg/mL) in phosphate-buffered saline (PBS), pH 7.4-7.5. The plates were incubated for 2 hrs at 37 °C and then for 16 hrs at 4 °C. OVA (or RBD) solution was removed by shaking, followed by adding to the wells 200 μL of blocking buffer (1% BSA solution in PBS, pH 7.4, supplemented with 0.05% Twin-20). After the incubation (2 hrs at 37 °C) and three washes with the washing buffer, 100 μL of 5-fold diluted sera (from 1:200 to 1:15625) were added to the wells. A diluting solution for serums was used to control the conjugate. The plates were incubated at 310 rpm in a thermoshaker (1.5 hrs at 37 °C), and then washed four times as described above. Next, the plates were incubated with a conjugate solution of goat anti-mouse IgG-HRP antibodies (Sigma, USA), (100 μL/well), at a dilution of 1:5000 at 37°C for 1 hr and washed five times as described above. For the color reaction manifestation, a solution of chromogen-TMB (3,3',5,5'-tetramethylbenzidine liquid substrate, slow kinetic form, for ELISA, SIGMA) was used. The plates were incubated for 30 min at RT protected from light. The reaction was stopped using a stop solution (1M sulfuric acid) in a volume of 50 μL/well. Absorbance was measured at 450 nm using a Varioskan Lux multimode microplate reader (Thermo Fisher Scientific, USA). The results were processed using GraphPad Prism 6.0 software.

## Results and discussion

Currently, among the adjuvants approved for use, the leading position is still occupied by preparations based on aluminum salts. At the same time, there is a tendency to use new, more efficient and safer compositions. One of the actively developing directions is the production of adjuvant systems in the form of emulsions based on squalene [4]. The use of squalene as the basis for adjuvant compositions has undeniable advantages, such as a high safety profile and biodegradability.

To evaluate immunogenicity, a recombinant S-protein RBD of SARS-CoV-2 (Delta variant

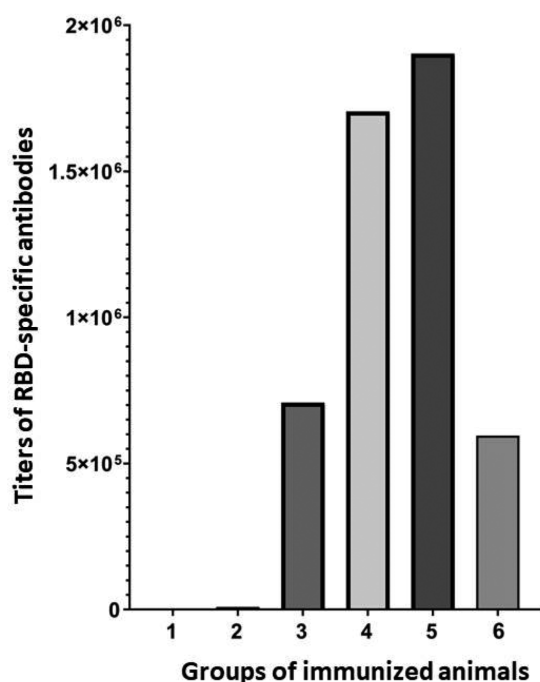


**Figure 1. Reciprocal titers of RBD-specific antibodies in the blood serum of immunized BALB/c mice**

Note. 1, saline solution; 2, RBD (50 μg); 3, RBD (50 μg) + aluminum hydroxide; 4, RBD (50 μg) + squalene (4.3%); 5, RBD (50 μg) + squalene (8.6%); 6, RBD (25 μg) + squalene (4.3%), respectively. The data are presented as GMTs. The graphics were made using GraphPad Prism 8.0.

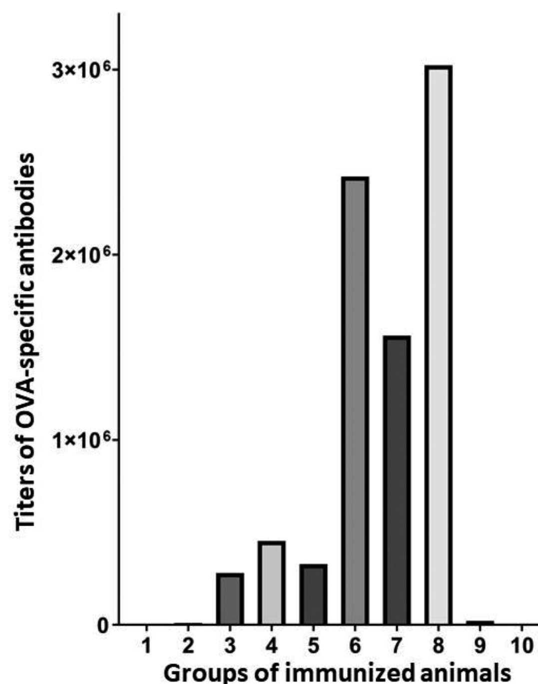


**Figure 2. Reciprocal titers of OVA-specific antibodies in the blood serum of immunized BALB/c mice**

Note. 1, OVA (1 μg); 2, OVA (5 μg); 3, OVA (1 μg) + aluminum hydroxide; 4, OVA (5 μg) + aluminum hydroxide; 5, OVA (1 μg) + squalene ( 4.3%); 6, OVA (5 μg) + squalene (4.3%); 7, OVA (1 μg) + squalene (8.6%); 8, OVA (5 μg) + squalene (8.6%); 9, OVA (1 μg) + incomplete adjuvant; 10, OVA (5 μg) + incomplete adjuvant. The data are presented as GMTs. The graphics were made using GraphPad Prism 8.0.

(B.1.617.2)) was used as one of the model antigens in our study. Analysis of mouse blood sera collected a week after the second immunization showed that the use of squalene emulsion elicited significant increase of antigens immunogenicity. The average titers of specific antibodies in the experimental group were 4 times higher than those in the group immunized with RBD with aluminum hydroxide (Figure 1). It is important to note that in the group of mice injected with a reduced dose of antigen (25 μg, group 6), the average indicator value was equivalent to the level of antibodies in the group immunized with a dose of 50 μg, but with aluminum hydroxide (group 3, Figure 1). A higher dose of squalene (8.6%) slightly increased the titer level of specific antibodies compared to its lower dose.

In many ways, similar patterns were observed in the other model, with the use of ovalbumin as an antigen. The two doses of OVA, 5 and 1 μg per mouse, were used for immunization.

Analysis of mouse blood sera after immunization showed that the use of a higher dose of adjuvant (8.6% squalene), regardless of the antigen dose (groups 7 and 8), can significantly, almost by an order, increase the immune response compared with aluminum hydroxide. Squalene (4.3%) significantly increased the level of antibody titers in mice immunized with OVA (5 μg) (Figure 2). It is important to note that the administration of an experimental adjuvant allowed to induce high titers of specific antibodies (more than 1:1000000), even with the use of a small dose of antigen (1 μg).

## Conclusion

Thus, we have obtained and evaluated an experimental adjuvant composition based on squalene. It has been shown that immunization of mice either with RBD or OVA proteins in combination with the obtained adjuvants, makes it possible to elicit a high level of humoral immune response exceeding the values achieved with the use of a classical adjuvant – aluminum hydroxide.

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*Поступила 15.04.2023*
*Отправлена на доработку 22.04.2023*
*Принята к печати 24.04.2023*

*Received 15.04.2023*
*Revision received 22.04.2023*
*Accepted 24.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 691-696



Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 691-696

# ПРИМЕНЕНИЕ СТВОЛОВЫХ КЛЕТОК В НАПРАВЛЕННОЙ РЕГЕНЕРАЦИИ КОСТНОЙ ТКАНИ

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**Резюме.** Современный уровень медицины позволяет все больше изучать и разрабатывать материалы и методики восстановительного лечения, которые бы опирались на иммунологические механизмы костной репарации. Одним из перспективных направлений в направленной костной регенерации является применение мезенхимальных стволовых клеток. Интерес в применении МСК связан с их способностью регулировать воспалительный процесс, и участвовать в формировании новых костных структур, тем самым обеспечивая воспроизведение процессов естественной репарации. Эффекторное влияние МСК на воспалительный процесс обусловлен, прежде всего, их способностью формировать специфическое микроокружение. Низкая экспрессия MHC-II и CD80/CD86 определяет их низкую иммуноконфликтность, продукция PGE2 и NO обеспечивает иммуносупрессию в месте заселения МСК, а продукция TGF- 1, IDO и IL-10 оказывает иммуномоделирующее действие. Более того, особое внимание к себе привлекает способность этих клеток дифференцироваться в остеогенный фенотип. Данный сложный многостадийный процесс сопровождается выделением ряда биологически активных веществ, влияющих на костную репарацию. Синтез ALP, BSP и в последующем Gla-protein и OPN обуславливают синтез внеклеточного матрикса и его последующую минерализацию. Регуляция данного процесса обеспечена действием Runx2, который активирует дифференцировку МСК по остеогенному пути. Данные эффекты МСК были взяты за основу в процессе разработки новой методики лечения атрофий костной ткани. Для выполнения поставленных задач была проведена разработка модели атрофии костной ткани, выполнена разработка препарата, содержащего в своем составе МСК, а также проведено экспериментальное исследование для оценки эффективности разработанной методики. В качестве основных критериев оценки качества проведенного лечения были взяты данные клинического и лабораторного исследований. Учитывались визуальные изменения исследуемого участка, по сравнению с аналогичным участком в разработанной модели атрофии, оценивались

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**Образец цитирования:**
*Н.С. Чумаков, К.А. Хлыстова, Н.Г. Саркисян, Ф.А. Фадеев, М.М. Мамедов «Применение стволовых клеток в направленной регенерации костной ткани» // Медицинская иммунология, 2023. Т. 25, № 3. С. 691-696.*
*doi: 10.15789/1563-0625-AOS-2724*

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**For citation:**
*N.S. Chumakov, K.A. Khlystova, N.G. Sarkisyan, F.A. Fadeev, M.M. Mamedov “Application of stem cells in guided bone regeneration”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 691-696.*
*doi: 10.15789/1563-0625-AOS-2724*

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**DOI:** 10.15789/1563-0625-AOS-2724

параметры ОАК, отражающие интенсивность протекания воспалительной реакции. Выполненное экспериментальное исследование позволяет определить разработанную методику лечения как способную в полной мере воссоздать условия процессов костной репарации, с учетом оптимизации иммунных реакций организма и процессов репарации, без дополнительного влияния извне, получить предсказуемые и контролируемые результаты. Имеющиеся данные исследования позволяют определить эффективность разработанной модели и методики лечения, а также дальнейший вектор проведения исследований.

*Ключевые слова: стволовые клетки, атрофия, репарация, костная регенерация, костный дефект*

# APPLICATION OF STEM CELLS IN GUIDED BONE REGENERATION

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**Abstract.** Modern medicine allows us to study and develop materials and methods of restorative treatment that would be based on the immunological mechanisms of bone repair. One of the promising directions in guided bone regeneration is the use of mesenchymal stem cells. Interest in MSCs is associated with their ability to regulate the inflammatory process, and directly participate in the formation of new bone structures, thereby providing a physiological repair process. The effector impact of MSCs on the inflammatory process due to their ability to form a specific microenvironment. Low expression of MHC-II and CD80/CD86, the production of PGE2 and NO determines their low immunoconflict, and the production of TGF-β1, IDO and IL-10 has an immunomodulating effect. The ability of MSCs to differentiate into an osteogenic phenotype is accompanied with the synthesis of ALP, BSP and, subsequently, Gla-protein and OPN determine the synthesis of the extracellular matrix and its subsequent mineralization. This process is provided by the action of Runx2, which activates the differentiation of MSCs along the osteogenic pathway. These effects of MSCs were taken as the basis for the development of a new method for the treatment of bone atrophy. To accomplish the task set, a model of bone tissue atrophy and a drug containing MSCs was developed, and an experimental study was conducted to evaluate the effectiveness of the developed methodology. As the main criteria, data from clinical and laboratory studies were taken. Visual changes in the studied area were taken into account, compared with a similar area in the developed model of atrophy, the parameters of the complete blood count (CBC) were evaluated. The performed study allows us to determine the developed treatment method as capable of fully recreating the conditions of bone repair processes, taking into account the optimization of the body's immune reactions and repair processes, without additional external influence, to obtain predictable and controllable results.

*Keywords: stem cells, atrophy, repair, bone regeneration, bone defect*

## Introduction

One of the most difficult issues of restorative dentistry we are facing these days is atrophy of the alveolar processes of the jaws [4]. Particular attention is drawn to the immunological mechanisms of the development of this pathology, as well as the possibility of optimizing the immunological aspects of bone repair [6]. The significance of these issues is due to complications that are associated with the development of bone loss, including a significant difficulty in restorative orthopedic and implant treatment, and the need for complex preparatory operations with a greater progression of pathology.

The development of modern medicine and the medical industry offers a variety of materials and techniques for guided bone tissue regeneration. The use of autogenous and artificial materials has good long-term results, but they are not able to fully

compensate for the processes of natural bone tissue regeneration [10]. This promotes the development of new treatment methods that have an effect on all parts of bone reparation.

One of such directions is cellular technologies. In recent years, the possibility of using mesenchymal stem cells (MSCs) has been actively studied. Interest in these cells is due to their ability to differentiate into cells of bone metabolism, as well as to produce a number of biologically active substances, among which growth factors, cytokines and various mediators are determined, thereby creating a microenvironment that determines the course of bone repair processes [8]. All this brings us as close as possible to the conditions of physiological regeneration with the formation of our own bone tissue.

Literature describes many examples of the successful use of MSCs in the restorative treatment of degenerative diseases of bone tissue [3, 9]. Methods for the treatment of articular cartilage necrosis, correction of post-traumatic facial deformities, treatment of bisphosphonate-associated osteonecrosis of the jaw bones is mentioned. In the treatment of bisphosphonate-associated osteonecrosis of the jaw, isolated and cultured culture of MSCs is preferred [5]. The effectiveness of treatment with this method is proved by a large number of publications in domestic and foreign literature. Many authors report that clinical success in the treatment of bisphosphonate-associated osteonecrosis of the jaw with MSCs has been observed even in patients receiving concomitant immunosuppressive therapy.

However, there is little data in the literature on the possibility of using MSCs for guided regeneration of the alveolar processes of the jaws. The low development of this topic dictates the need to pay more attention to the issues of stimulating endogenous regeneration, as well as the possibility of conducting direct regulation of this process. Thus, the issues of prevention and correction of bone tissue atrophy, in particular the atrophy of the alveolar processes of the jaws, remain promising areas and require the search for modern methods for correcting these conditions.

## Materials and methods

The study was conducted at the Federal State Budget Institution of Science "Institute of Immunology and Physiology" of the Ural Branch of the Russian Academy of Sciences, and included several successive stages:

1. Development and implementation of a model of bone tissue atrophy;
2. Cultivation of MSCs (conducted at the State Autonomous Healthcare Institution of the Sverdlovsk Region "Institute of Medical Cellular Technologies");
3. Conducting an experimental study.

Before developing the model, the main criteria were formulated, the observance of which would make it possible to assess its effectiveness:

– Maximum proximity to the real conditions for the development of atrophy;
– Formation of a sufficient amount of atrophy for an adequate assessment of the expected and obtained results;
– Non-development of adverse reactions, prevention of distortion of long-term results;
– No need for external influence and ease of implementation.

At its core, the model of bone tissue atrophy is similar to traumatic tooth extraction (an extraction of a tooth, in which an intentional and accidental additional bone defect is formed, in the form of a break of the interradicular septum / cortical plate in the area of the socket of the extracted tooth), and does not require additional manipulations in the postoperative period. The process of reproducing the model consists of the following steps:

1. Under general anesthesia (Diethyl ether), an incision is made in the mucous membrane, from the lower incisor, along the most protruding part of the alveolar ridge, approximately 0.7-1 cm long;
2. The mucoperiosteal flap is separated from the bone;
3. Using sharp wire cutters, a part of the incisor and alveolar ridge is cut off, approximately 3-5 mm deep;
4. After that, the edges of the wound are reduced (additional suturing of the wound is not required) and a dynamic observation of the individual is established.

According to the results of clinical and laboratory studies, it was found that the developed model meets all the above criteria and gives the expected results.

At the second stage, MSCs were cultivated. Cells were extracted from a 4 × 3 × 3 mm gingival tissue sample. The sample was crushed to fragments no larger than 1 $mm^3$, the resulting tissue mass was incubated in 2 mL of collagenase I solution (1000 U/mL) (Sigma-Aldrich, USA) at 37 °C for 2.5 hours with continuous gentle stirring. At the end of the incubation, 10 mL of Hank's solution with 10% fetal bovine serum (FBS) (Biosera) was added to the tissue mass tube to neutralize the enzyme, after which the tissue was dissociated by actively mixing the contents of the tube.

The resulting cell suspension was transferred to a new tube, the cells were pelleted by centrifugation (1100 rpm, 10 min), resuspended in growth medium, and transferred to a T25 culture flask (Nunc, Denmark). Cells were grown in DMEM + F-12 growth medium (PanEco, Russia) supplemented with 10% FBS, glutamine (0.03%), and gentamicin (50 μg/mL medium). Cells were grown in a $CO_2$ incubator at 37 °C in an atmosphere with 5% $CO_2$.

On the 3rd day after inoculation of the primary culture, dividing cells were observed; on the 6th day, sections of the monolayer began to form. Cell reseeding was performed after the monolayer reached 80% confluence. Cells were removed from plastic using 0.25% TrypLE solution (Gibco, Thermo Fisher Scientific, USA).

Fibroblasts were used for administration to rats after the 2nd reseeding. Cells were removed from plastic, washed twice to remove TrypLE residues with Hank's solution (on the first wash with 10% FBS), after that the cells were resuspended in saline at a concentration of 1.65 million cells/mL.

The third stage included the direct conduct of an experimental study. For this, the developed model of atrophy was reproduced and the drug based on MSCs was administered according to the following steps:

1. Interrupted sutures were applied to the wound surface;
2. The introduction of the filler of the drug was carried out through the reduced edges of the wound;
3. An insulin syringe was used to administer a suspension containing MSCs in an amount of 200 μL.

## Results and discussion

To evaluate the results of the experimental study and the course of the inflammatory reaction in particular, a complete blood count (CBC) was conducted. As the main indicator for evaluation, the indicators of the number of leukocytes (WBC) were taken, which were compared with the average indicators of the level of leukocytes in the intact group. Based on the results obtained, a graph was compiled (Figure 1), which shows that both in the atrophy model group and in the experimental group, a significant increase in the level of leukocytes is determined relatively to the average norm on the first day of the experiment. This indicates the development of an active inflammatory reaction. Normalization of the level of leukocytes occurs by the end of the second week of observations.

An interesting fact is that the level of leukocytes in the experimental group is higher than in the group of the developed model. This can be determined by a more intense inflammatory response mediated by the effector influence of stem cells on innate and adaptive immunity.

According to the objective examination data, it was found that visible bone loss was determined in the atrophy model group. At the same time, it was determined that the developed model does not cause the death of the neurovascular bundle of the lower incisor and, accordingly, is not capable of provoking the development of pathological processes in the tissues of the tooth and in periodontal structures (Figure 2).

By the end of 3 months, there is a significant thickening of the alveolar process of the lower jaw in the experimental group. Objectively, the thickening is dense, without pathological noises during palpation (Figure 3).

MSCs play an important role in the regeneration of tissues and organs due to the ability to differentiate and renew themselves. Due to these abilities, MSCs are the main contenders for work in the field of tissue engineering. Besides that, MSCs have a number of other features that improve implant survival. This is the ability to stimulate osteogenesis due to the release of growth factors, the immunomodulatory effect due to the production of inflammatory factors. Particular attention should be paid specifically to the immunomodulatory effect, since MSCs are able to not only prevent the immunological rejection of the graft, but also activate immune responses directly in the bone.

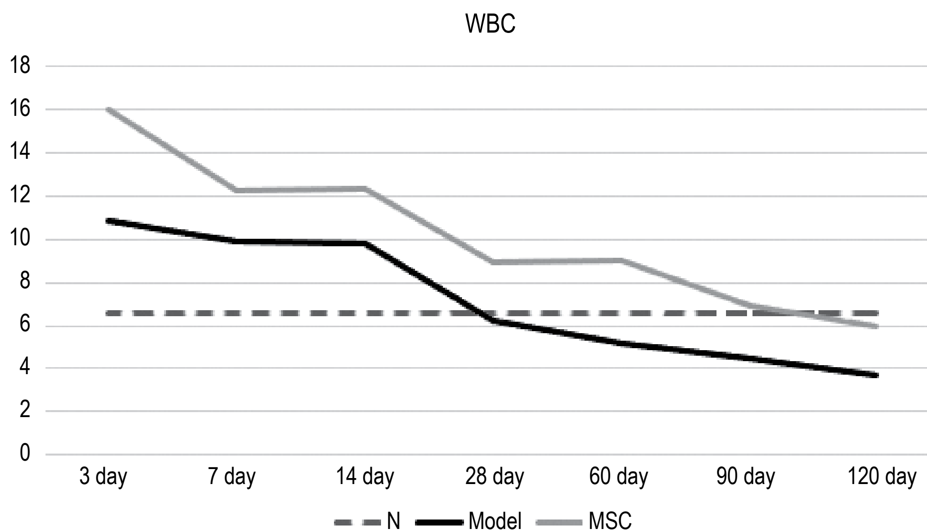


**Figure 1. WBC level graph**

**Figure 2. Alveolar bone atrophy**

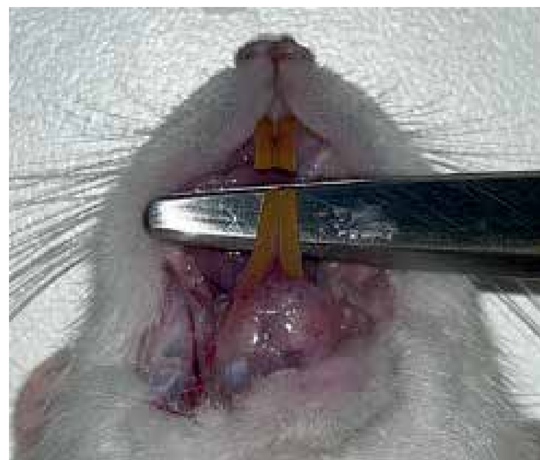

**Figure 3. Newly-formed bone area**

In recent years, many scientific papers have appeared, the authors of which propose methods for reconstructing bone defects by using grafts populated with stem cells or impregnated growth factors. Oftentimes, the variant of colonization by mesenchymal multipotent stem cells is described, since they have a low expression of MHC class II and CD80/CD86 molecules, due to which they have low immunoconflict and the possibility of using both autologous and allogeneic cultures. MSCs exhibit immunomodulatory effects by influencing inflammatory responses and optimizing immune cell responses. By inhibiting the proliferation and production of cytokines by antigen-presenting cells (APCs) and T lymphocytes, MSCs regulate the immunological background of bone tissue remodeling. Also, MSCs produce prostaglandin E2 (PGE2) and nitric oxide (NO), which has an immunosuppressive effect and, as a result, reduces the risk of transplant rejection. Stem cells, through a number of mechanisms, affect T cells, reducing their viability and ability to proliferate, and stimulate the proliferation of T regulatory (Treg) cells. Predominantly, this effect is mediated by CD274 molecules that are activated by IFNγ. In addition to that, MSCs suppress the proliferation of B lymphocytes, maturation of dendritic cells, and reduce the proliferation and cytotoxicity of NK cells, which together have effects on both innate and acquired (adaptive) immunity. The mediators secreted by MSCs also affect the immunomodulatory effect of these cells. These are predominantly transforming growth factor β1 (TGF-β1), prostaglandin E2 (PGE2), indolamine-pyrrole-2, 3-dioxegenase (IDO), nitric oxide (NO), and interleukin-10 (IL-10). Their produce is regulated by IFNγ, IFNα, IL-1, IL-1β [7].

MSCs are of particular value due to their antimicrobial effects. This is associated with direct and indirect mechanisms. Direct effects are directly related to the release of antimicrobial peptides by MSCs, such as cathelicidins, lipocalin-2, and β-defensins. Cathelicidins, similarly to other peptides, are active against all types of bacteria, both $Gram^+$ and $Gram^-$, against some viruses, fungi, and protozoa. To a large degree, these effects are due to the incorporation of peptides into the structure of the bacterial wall and the formation of a pore in it. In addition, this peptide functions as a chemotactic agent for neutrophils, monocytes, and T cells. With the development of the infectious process, MSCs also have an effector effect on the innate link of immunity, particularly on neutrophils and monocytes, increasing their migration to the infectious focus and enhancing their antimicrobial activity [1].

The ability of MSCs to differentiate along the osteogenic pathway has a beneficial effect on bone repair processes. This process is regulated by a number of signaling pathways, including Wnt, TGF-β, PI3K/Akt. In addition, BMP-2, BMP-6, BMP-7, and BMP-9 are important triggers for osteogenic differentiation of MSCs [2].

All of the above effects of MSCs favourably affect the regulation of the immunological mechanisms of inflammatory and regenerative processes.

## Conclusion

There are many more questions about the use of MSCs as a material for guided bone tissue regeneration that require detailed study. However, the results obtained indicate the prospects for the use of this technology. Influence on the links of innate and adaptive immunity, processes of bone metabolism, allows you to fully activate the natural processes of bone repair by creating a specific microenvironment and the ability of MSCs to transform into highly differentiated cells. It is the increase in the volume of bone tissue and not the accelerated healing of the bone defect that makes it possible to conclude that atrophy is prevented.

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*Поступила 11.04.2023*
*Отправлена на доработку 12.04.2023*
*Принята к печати 18.04.2023*

*Received 11.04.2023*
*Revision received 12.04.2023*
*Accepted 18.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 697-702

**Краткие сообщения**
**Short communications**

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 697-702

# СУПРАМОЛЕКУЛЯРНЫЕ КОМПЛЕКСЫ НА ОСНОВЕ КУКУРБИТ[7]УРИЛА И СОЕДИНЕНИЙ ПЛАТИНЫ ВЛИЯЮТ НА ЭКСПРЕССИЮ МОЛЕКУЛЫ CTLA-4 НА Т-РЕГУЛЯТОРНЫХ КЛЕТКАХ

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**Резюме.** Опухоли занимают лидирующее место по частоте встречаемости в популяции. Не все противоопухолевые лекарственные препараты первой линии позволяют адекватно и эффективно излечивать пациентов. Для некоторых препаратов, например, цитостатиков, характерны широкий спектр побочных эффектов и резистентность опухолей к проводимой ими терапии. На сегодняшний день описаны механизмы действия таких препаратов и предполагаются наиболее вероятные причины резистентности. Для минимизации побочных эффектов и преодоления резистентности используется система доставки лекарственных препаратов на основе кукурбит[7]урила (CB[7]), которая образовывает супрамолекулярные комплексы с оксалиплатином и карбоплатином.

Важно принимать во внимание, что большой вклад вносит иммунная система, соединения платины способны оказывать иммуномодулирующее действие на иммунокомпетентные клетки и все больше данных говорит о том, что цитотоксический ответ в отношении опухолевых клеток связывают и с этими свойствами. Опухоль создает специфическое микроокружение, в котором сосредотачивается огромное количество супрессорных клеток. $FoxP3^+$T-регуляторные клетки рекрутируются опухолью, увеличенное количество этих клеток и повышенные уровни экспрессии CTLA-4 и PD-1 способствуют прогрессированию опухолевого процесса. Данные показатели коррелируют с плохой выживаемостью пациентов. Поэтому необходимо, чтобы противоопухолевые агенты обладали влиянием на данную субпопуляцию клеток и их функциональную активность. В данном исследовании оценивалось влияние кукурбит[7]урила, соединений платины и супрамолекулярных комплексов на регуляторные Т-клетки и экспрессию молекул иммунных контрольных точек.

В исследовании использовались клетки периферической крови условно здоровых доноров (n = 8, средний возраст 29,0±2,4). Полученные стандартным путем мононуклеары инкубировали 72 часа в концентрациях 0,3 мМ и 0,1 мМ для карбоплатина и оксалиплатина соответственно, а также комплексами и CB[7] в эквивалентных дозировках, затем пробы окрашивали моноклональными антителами для определения фенотипа и экспрессии иммунных чекпоинт-молекул.

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**Образец цитирования:**
*А.А. Актанова, М.В. Быкова, О.С. Боева, Е.А. Пашкина, Л.В. Гришина, В.А. Козлов «Супрамолекулярные комплексы на основе кукурбит[7]урила и соединений платины влияют на экспрессию молекулы CTLA-4 на Т-регуляторных клетках» // Медицинская иммунология, 2023. Т. 25, № 3. С. 697-702.*
*doi: 10.15789/1563-0625-CBS-2752*

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**For citation:**
*A.A. Aktanova, M.V. Bykova, O.S. Boeva, E.A. Pashkina, L.V. Grishina, V.A. Kozlov “Cucurbituril-based supramolecular complexes with platinum compounds influence expression of CTLA-4 on regulatory T cells”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 697-702.*
*doi: 10.15789/1563-0625-CBS-2752*

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**DOI:** 10.15789/1563-0625-CBS-2752

Мы получили следующие результаты: комплекс CB[7]-карбоплатин в стимулированной и нестимулированной культурах достоверно снижал количество FoxP3⁺T-регуляторных клеток по сравнению с контролем. При этом карбоплатин и комплекс CB[7]-карбоплатин снижали экспрессию CTLA-4 в нестимулированной культуре по сравнению с CB[7].

Комплексы кукурбит[7]урилов с соединениями платины являются перспективным противоопухолевым средством с иммуномодулирующими свойствами.

*Ключевые слова: кукурбитурилы, макроциклические комплексы, карбоплатин, оксалиплатин, Т-регуляторные клетки, контрольные точки иммунного ответа, проточная цитометрия*

# CUCURBITURIL-BASED SUPRAMOLECULAR COMPLEXES WITH PLATINUM COMPOUNDS INFLUENCE EXPRESSION OF CTLA-4 ON REGULATORY T CELLS

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**Abstract.** Tumors are a leading pathology in the population. Chemotherapy cannot provide adequately and effectively to cure patients. Some medicine, such as cytostatic, are characterized by a wide range of side effects and resistance of solid tumors to chemotherapy by these medicines. In recent research, the mechanisms of action of cytotoxic agents have been described, and the most appropriate causes of resistance have been suggested. Drug delivery system based on Cucurbit[7]uril (CB[7]) was used to minimize side effects and overcome resistance. CB[7] has ability to form host-guest supramolecular complexes with oxaliplatin and carboplatin.

It is important to consider the immune system maintain to a great role, and platinum compounds are able to have an immunomodulatory effect on immunocompetent cells. There is convincing evidence about the cytotoxic response against tumor cells is also associated with immunomodulating properties. A specific immune microenvironment with high frequency of suppressor cells is made by tumors. FoxP3⁺ regulatory T cells are recruited by the tumor, an increased number of these cells and expression levels of CTLA-4 and PD-1 on them contribute to the progression of the tumor process. These markers correlate with recurrence and poor survival of the patients. Therefore, it is necessary that antitumor therapy agents have an effect on a subpopulation of regulatory T cells and their functional activity. This study evaluated the effects of cucurbit[7]uril, platinum compounds, and supramolecular complexes on FoxP3⁺ regulatory T cells and the expression of immune checkpoint molecules.

In this study peripheral blood cells from volunteers (n = 8, average 29.0±2.4) were used. Mononuclear cells obtained in the standard protocol were incubated for 72 h at concentrations of 0.3 and 0.1 mM for carboplatin and oxaliplatin, respectively, as well as complexes and CB[7] in equivalent dosages. Next, the samples were labeled with monoclonal antibodies to determine the phenotype and expression of immune checkpoint molecules by flow cytometry.

We obtained the following results: The CB[7]-carboplatin complex in stimulated and non-stimulated cultures significantly reduced the number of FoxP3⁺ regulatory T cells compared to the control. At the same time, carboplatin and the CB[7]-carboplatin complex reduced the expression of CTLA-4 in an non-stimulated culture compared to CB[7].

Complexes of Cucurbit[7]urils with platinum compounds are a perspective antitumor agent with immunomodulatory properties.

*Keywords: cucurbiturils, macrocyclic complexes, carboplatin, oxaliplatin, Tregs, immune checkpoint molecules, flow cytometry*

This study was supported by Russian Science Foundation according to the research project No. 19-15-00192.

## Introduction

Nowadays tumors are the leading pathology among the population. There are many treatment regimens for different tumors, including those based on platinum agents (drugs include cisplatin, oxaliplatin, carboplatin, etc.). However, platinum agents have a lot of specific and common side effects from hematotoxicity, ototoxicity to neurotoxicity, cardiotoxicity, and there are some tumors as ovarian cancer, melanoma, and colon which resistant to platinum treatment [2, 6]. There are several mechanisms of resistance, and an important role is

played by the low accumulation of platinum in cells and detoxification components, which don't allow to bind DNA by platinum. Multiple transporters for the active transport of platinum-based antitumor agents also may be responsible for platinum resistance [13]. Therefore, it is necessary to isolate platinum from enzymes and use drug delivery system to accumulate platinum into cells. CB[7]-based drug delivery system was used for this. Cucurbiturils are nanomolecular system for binding with cations, small peptide etc. with forming host-guest complexes. CB[7] is known to protect the drugs from biodegradation [5] and to cross the cell membrane [7]. The mechanisms of cellular cytotoxicity for a large amount of antitumor drugs have been well described. Also, the effects of cytostatic against cells of the immune system have been characterized. For example, oxaliplatin selectively depleted the pool of myeloid suppressors, directed the differentiation of myeloid cells towards a mature phenotype and diminished their immunosuppressive activity [4]. Carboplatin reduced the number of cells with immunosuppressive activity and increased the level of IFNγ [12]. It should also be noted that the effect of chemotherapy has not considered as effect associated to the immunological component. Although there are a lot of data not about the direct cytotoxic effect of drugs for chemotherapy on tumors, but about a greater effect on the immune response.

It is necessary to considered that the immune system plays a key role in the development of the tumor process and its maintenance or progress tumor growth. It is known, the cells of the immune system are recruited by tumor for creation a specific immunosuppressive environment [3]. At the same time, the role of T-regulatory cells $FoxP3^+$ in tumor microenvironment have been uncertain. It is described that tumor cells recruit Tregs $FoxP3^+$ and the amount of these cells' high increases with tumor growth. Also, patients with increased levels of expression of CTLA-4 and PD-1 have a worse prognosis compare to patients with low levels of expression these molecules. High levels of expression CTLA-4 and PD-1 can promote of metastasis and malignancy [8].

CTLA-4 and PD-1 on TREG are immune response suppressor molecules influencing through different signal ways. Applying of checkpoint inhibitors improves survival with some solid tumors [10]. Since cells of the immune system contribute significantly to the antitumor response, it is necessary to study an ability of potential and well-known chemotherapy agents to influence the immune response. **The aim of the study** was to evaluate the effect of cucurbit[7]uril, platinum compounds and supramolecular complexes on regulatory T-cells and expression of immune checkpoint molecules on Tregs.

## Materials and methods

Heparinized peripheral blood was isolated from 8 volunteers (average 29±2.4 years) after signing informed consent form. Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation in a density gradient ficoll-urografin according to protocol. Isolated PBMCs at a quantity of $1 \times 10^6$ cells/mL were incubated with CB[7], platinum compounds (carboplatin, oxaliplatin) and supramolecular complexes (CB[7]-carboplatin, oxaliplatin-CB[7]) using the culture medium RPMI-1640, supplemented with 10% FCS, 50 mg/mL gentamicin and 25 mg/mL thienam in 48-well plate (TPP, Switzerland) during 72 h in a standard culture conditions with 37 °C, 5% $CO_2$. Anti-CD3 monoclonal antibodies (aCD-3) and IL-2 were used as stimulants for cells. Carboplatin and complex CB[7]-carboplatin were added at the concentrations of 0.3 mM, while oxaliplatin and complex oxaliplatin-CB[7] at 0.1 mM. CB[7] was used as control for platinum compounds, complexes and respectively added at equivalent concentrations. Also, we used control with only culture medium as negative control.

After 72 h incubation, the treated cells were labeled by fluorochrome-conjugated antibodies to determine phenotypes cells. Regulatory T cell was determined as $CD3^+CD4^+CD25^+FoxP3^+$ (BioLegend, USA). The intracellular expression of FoxP3 was estimated according FoxP3-staining protocol using True-Nuclear™ Transcription Factor Buffer Set (BioLegend, USA). Expression of immune checkpoint molecules was performed by cell staining with fluorochrome-conjugated monoclonal antibodies specific to CD279(PD-1), CD274(PD-L1), CD152 (CTLA-4).

Samples were analyzed by flow cytometry using cytometer FACS Canto II (BD, Franklin Lakes, NJ, USA) with Diva 6.0 software (BD).

Statistical analysis was performed using GraphPad Prism software (version 9.0.0). Differences between groups were determined using non-parametric Friedman test. P value of $< 0.05$ was considered statistically significant. Data are presented as median ±interquartile range with $n = 8$.

## Results and discussion

We evaluated the effect of complexes Cucurbit[7]uril and platinum compounds on the number of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs $FoxP3^+$). The CB[7]-carb complex was significantly reduced the amount of Tregs $FoxP3^+$ compared to the control in stimulated and non-stimulated culture. Also, the complexes CB[7] with carboplatin and oxaliplatin were significantly decrease the number of Tregs $FoxP3^+$ compared to CB[7] at an equivalent dosage in both culture (Figure 1). Significant differences in the expression of immune check-point molecules such as PD-1 and PDL-1 on Treg were not found (Figure 2). At the same time, carboplatin and the CB[7]-carb complex were reduced the expression of CTLA-4 compared to CB[7] in the non-stimulated culture. Significant difference in expression of CTLA-

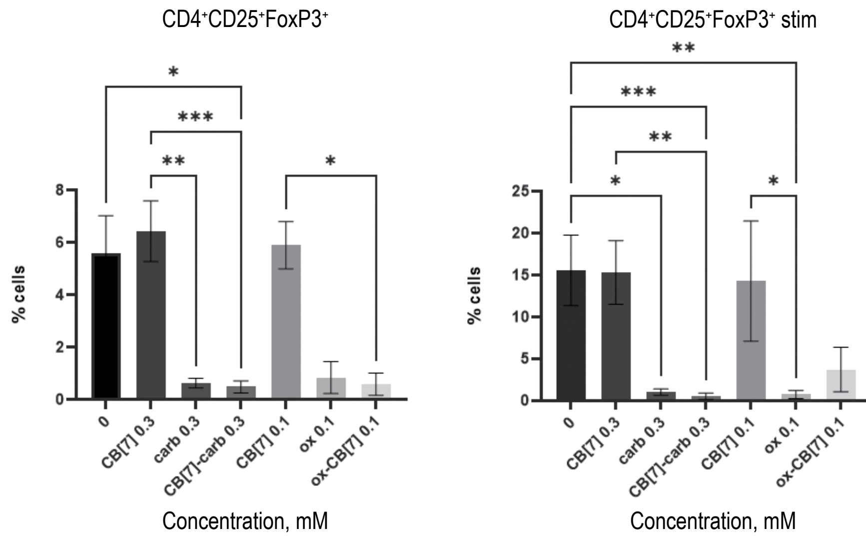


**Figure 1. Number of $CD4^+CD25^+FoxP3^+$ regulatory T cells after 72 h treatment with Cucucrbit[7]uril and complexes: CB[7]-carboplatin complex, oxaliplatin-CB[7] complex in stimulants (aCD3-antibody and IL-2) and non-stimulants cultures**

Note. Data are presented as median ± interquartile range with n = 8. *, significant differences are $p < 0.05$ by employing one-way ANOVA, Friedman test.

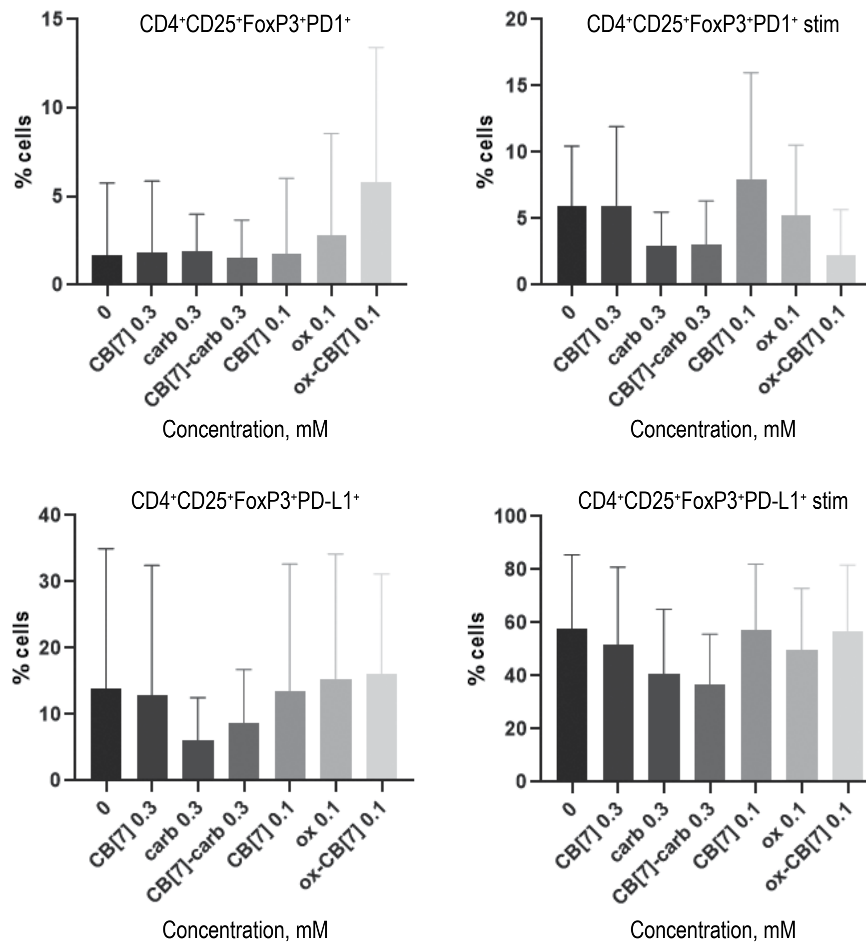


**Figure 2. Expressions of PD-1 and PDL-1 immune checkpoint molecules on $CD4^+CD25^+FoxP3^+$ regulatory T cells after 72 h treatment with Cucucrbit[7]uril and complexes: CB[7]-carboplatin complex, oxaliplatin-CB[7] complex in stimulants (aCD3-antibody and IL-2) and non-stimulants cultures**

Note. As for Figure 1.

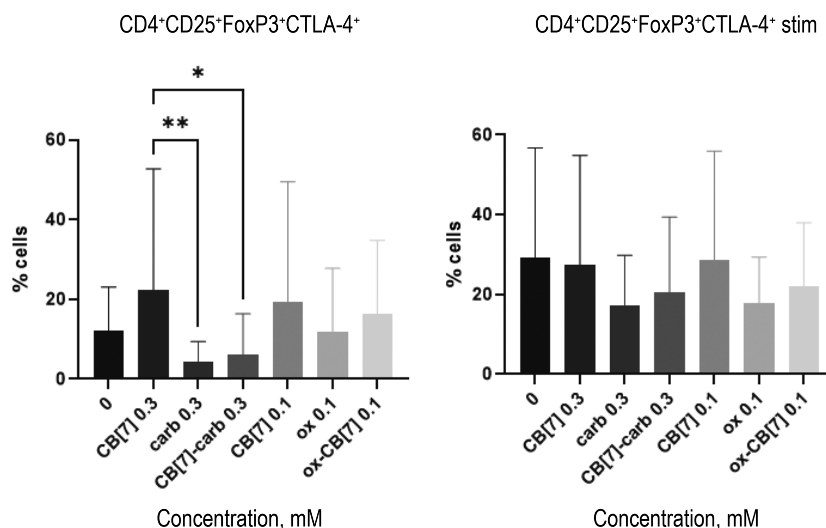


**Figure 3. Expressions of CTLA-4 immune checkpoint molecules on CD4+CD25+FoxP3+ regulatory T cells after 72 h treatment with Cucucrbit[7]uril and complexes: CB[7]-carboplatin complex, oxaliplatin-CB[7] complex in stimulants (aCD3-antibody and IL-2) and non-stimulants cultures**

Note. As for Figure 1.

4 between control and treated groups were not found (Figure 3).

It is known, Tregs FoxP3+ have high frequency infiltration into tumor and suppress the antitumor immune response. At the same time, a decrease in the expression of CTLA-4 and the number of Tregs FoxP3+ improve the prognosis in a tumor [11]. It has also been reported that increased Treg infiltration into tumor has been associated with low common survival and relapse-free survival. Also, patients with high Treg levels had worse outcomes in tumor [9]. These results demonstrate that it is required to reduce the expression of check point molecules and the amount of Tregs in a tumor, but at the same time check point inhibitors have not to use, because it increases the risk of developing autoimmune pathologies, or their application may be effectless and non-selective, since it is necessary to observe correct balance of PD-1 expression on Tregs and CD8+ [1].

## Conclusions

Complexes Cucurbit[7]urils with platinum compounds are a perspective antitumor agent with immunomodulatory properties. Furthermore, applying these complexes may provide a treatment with the greatest efficiency and fewer side effects. However, further research is needed to confirm the effectiveness of this kind of therapy in patients.

## Acknowledgements

This study was supported by Russian Science Foundation according to the research project No. 19-15-00192.

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*Поступила 13.04.2023*
*Принята к печати 16.04.2023*

*Received 13.04.2023*
*Accepted 16.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 703-708

# Краткие сообщения
# Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 703-708

# АНТОЦИАНИНЫ КАК ФАКТОР АЛИМЕНТАРНОГО ВОССТАНОВЛЕНИЯ КЛЕТОЧНОГО ИММУНИТЕТА ПРИ ИНДУЦИРОВАННОМ ДИЕТОЙ ОЖИРЕНИИ У КРЫС

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**Резюме.** В статье представлены результаты исследования влияния антоцианинов на клеточный иммунитет у крыс на модели алиментарного ожирения. Целью исследования являлось изучение влияния рациона, обогащенного антоцианинами, на клеточный иммунитет при индуцированном диетой ожирении у крыс. Работа выполнена на крысах самцах линии Wistar с исходной массой тела 108±2 г. Животные были рандомизированы по массе тела на 3 группы (по 8 крыс в группе). В течение 12 недель крысы 1-й (контрольной) группы получали полноценный модифицированный рацион AIN93M; крысы 2-й группы потребляли высококалорийный холинодефицитный рацион (ВКХДР), содержание жира в котором составляло 45%, фруктозы – 20% от энергетической ценности рациона; крысы 3-й группы получали ВКХДР с добавлением стандартизованных экстрактов черники и черной смородины (30% антоцианинов) в суточной дозе 11 мг антоцианинов/кг массы тела. Животных содержали по 2 особи в пластиковых клетках на подстилке из древесных стружек при искусственном освещении с равной продолжительностью ночного и дневного периодов и не ограничивали в употреблении воды. Экспрессию дифференцировочных маркеров лимфоцитов периферической крови крыс определяли методом проточной цитофлуориметрии. В результате исследования установлено, что у крыс 2-й группы с алиментарным ожирением повышено ($p < 0,05$) в периферической крови относительное содержание Т-хелперов ($CD3^+CD4^+$) (75,75±1,11% против 70,07±0,49% – 1-я группа, 72,14±0,91% – 3-я группа) и снижено ($p < 0,05$) содержание Т-цитотоксических лимфоцитов ($CD3^+CD8^+$) (22,54±1,14% против 28,09±0,72% – 1-я группа, 26,07±0,87% – 3-я группа). Соотношение CD3/CD4 у крыс 2-й группы превысило ($p < 0,05$) данный показатель у крыс 1-й и 3-й групп

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**Образец цитирования:**
*Э.Н. Трушина, О.К. Мустафина, И.В. Аксенов, В.А. Тутельян «Антоцианины как фактор алиментарного восстановления клеточного иммунитета при индуцированном диетой ожирении у крыс» // Медицинская иммунология, 2023. Т. 25, № 3. С. 703-708.*
*doi: 10.15789/1563-0625-AAA-2719*

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**For citation:**
*E.N. Trushina, O.K. Mustafina, I.V. Aksenov, V.A. Tutelyan “Anthocyanins as a factor in the alimentary restoration of cellular immunity in diet induced obesity in rats”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 703-708.*
*doi: 10.15789/1563-0625-AAA-2719*

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**DOI:** 10.15789/1563-0625-AAA-2719

(3,44±0,25 против 2,47±0,09 – 1-я группа, 2,79±0,13 – 3-я группа). Обогащение ВКХДР экстрактами черники и черной смородины привело к нормализации указанных параметров клеточного иммунитета. Число В-лимфоцитов (CD45R⁺), Т-лимфоцитов (CD3⁺) и NK-клеток (CD161⁺) в периферической крови крыс всех экспериментальных групп не имело статистически достоверных различий. Результаты исследования клеточного иммунитета у крыс с алиментарным ожирением свидетельствуют о наличии метавоспаления. Добавление в рацион крыс антоцианинов обеспечило восстановление изученных показателей адаптивного клеточного иммунитета до уровня крыс контрольной группы. Полученные результаты свидетельствуют о перспективе применения биологически активных веществ – антоцианинов в диетотерапии больных ожирением и другими алиментарно-зависимыми заболеваниями.

*Ключевые слова: алиментарное ожирение, клеточный иммунитет, лимфоциты, NK-клетки, антоцианины, метавоспаление*

# ANTHOCYANINS AS A FACTOR IN THE ALIMENTARY RESTORATION OF CELLULAR IMMUNITY IN DIET INDUCED OBESITY IN RATS

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**Abstract.** The article presents the results of a study of the effect of anthocyanins on cellular immunity in rats on a model of alimentary obesity. The aim of the study was to study the effect of an anthocyanin-enriched diet on cellular immunity in diet induced obesity in rats. The study was carried out on male Wistar rats with an initial body weight of 108±2 g. The animals were randomized by body weight into 3 groups (8 pcs. in group). For 12 weeks, rats of the 1st (control) group received a complete modified diet of AIN93M; rats of the 2nd group consumed a high-calorie choline-deficient diet (HCChDD), the fat content of which was 45%, fructose – 20% of the energy value of the diet; rats of the 3rd group received HCChDD with the addition of standardized blueberry and blackcurrant extract (30% anthocyanins) at an average daily dose of 11 mg anthocyanins/kg body weight. The expression of differentiation markers of peripheral blood lymphocytes was carried out by flow cytofluorimetry. As a result of the study, it was found that in rats of the 2nd group with alimentary obesity, the relative content in the peripheral blood of T helpers (CD3⁺CD4⁺) was increased ($p < 0.05$) (75.75±1.11% *versus* 70.07±0 49% – group 1, 72.14±0.91% – group 3) and reduced ($p < 0.05$) content of T cytotoxic lymphocytes (CD3⁺CD8⁺) (22.54±1.14% *versus* 28.09±0.72% – 1st group, 26.07±0.87% – 3rd group). The CD3/CD4 ratio in rats of the 2nd group exceeded ($p < 0.05$) this index in rats of the 1st and 3rd groups (3.44±0.25 *versus* 2.47±0.09 – 1st group, 2.79±0.13 – 3rd group). Enrichment of the HCChDD with the blueberry and blackcurrant extract led to the normalization of these parameters of cellular immunity. The number of B lymphocytes (CD45R⁺), T lymphocytes (CD3⁺) and NK cells (CD161⁺) in the rat peripheral blood of all experimental groups had no statistically significant differences. The results of the study of cellular immunity in rats with alimentary obesity indicate the presence of metainflammation. The received data indicate the prospect of using biologically active substances, anthocyanins, in the diet therapy of patients with obesity and other alimentary-dependent diseases.

*Keywords: alimentary obesity, cell immunity, lymphocytes, NK cells, anthocyanins, metainflammation*

## Introduction

Obesity is a multifactorial widespread disease that is a consequence of excess intake of calories and insufficient degree of their utilization. Obesity is accompanied by chronic inflammation and is a pathogenetic basis for the development of cardiovascular pathology, metabolic syndrome, type 2 diabetes mellitus, and insulin resistance [10]. Obesity causes dysregulation throughout the immune system, affecting the balance and levels of cytokines, adipokines and innate and adaptive immunity [2, 5]. Currently, the problem of metainflammation, which is metabolic in nature, chronic, associated with moderate expression of pro-inflammatory mediators and accompanied by modification of the structure of metabolic tissues with infiltration by immune cells, is being actively studied [4, 10]. In the genesis of the disease, the main role is played by oxidative stress and chronic inflammation in metabolically active tissues: adipose tissue, liver, intestines, muscles, pancreas, and others [9]. There are increases in pro-inflammatory cytokines and organ infiltration by increased numbers of tissue macrophages, B lymphocytes, T lymphocytes and mast cells with decreased numbers of regulatory T lymphocytes, MAIT cells (mucosal-associated invariant T cells), ILC2 (innate lymphoid cell) and invariant NKT cells and accompanied by changes in immune cell content in peripheral blood [5]. The problem of immunometabolism is being actively studied on rats and mouse models of diet induced obesity.

Excessive accumulation of body fat, disorders in the insulin-dependent signaling pathway, and hyperlipidemia lead to tissue hypoxia and the development of oxidative stress [12]. One of the components of therapy for insulin resistance, immunodeficiency in obesity has been the use of natural and synthetic antioxidants [3]. Currently, the effectiveness of the use of anthocyanins, a water-soluble subclass of flavonoids, has been proven in the treatment of a number of metabolic disorders, including glucose tolerance, insulin resistance, abdominal obesity, dyslipidemia, and arterial hypertension [7]. It has been established that they have vasoprotective properties, have antioxidant, anti-inflammatory, antiatherogenic and vasodilating effects [4].

**The aim of the study** was to study the effect of an anthocyanin-enriched diet on cellular immunity in diet induced obesity in rats.

## Materials and methods

The study was carried out on male Wistar rats with an initial body weight of 108±2 g. Rats were obtained from the nursery of the “Stolbovaya” branch of the Federal State Budgetary Institution of Science “Scientific Center for Biomedical Technologies of the FMBA”. The study was approved by the Ethics Committee of the “Federal Research Center for Nutrition and Biotechnology” (meeting No. 11 dated December 15, 2021) and was carried out in accordance with the recommendations of GOST 33216-2014 “Guidelines for accommodation and care of animals. Species-specific provisions for laboratory rodents and rabbits”.

The animals were randomized by body weight into 3 groups (8 pcs. in group). For 12 weeks, rats of the 1st (control) group received a complete modified diet of AIN93M [13]; rats of the 2nd group consumed a high-calorie choline-deficient diet (HCChDD), the fat content of which was 45%, fructose – 20% of the energy value of the diet; rats of the 3rd group received HCChDD with the addition of standardized blueberry and blackcurrant extract (30% anthocyanins, Healthberry 865, Evonik Nutrition & Care GmbH, Germany) at an average daily dose of 11 mg anthocyanins/kg body weight. Animals were kept in 2 individuals in plastic cages on a bed of wood shavings under artificial lighting with equal duration of the night and day periods and were not limited in the use of water. Withdrawal from the experiment was carried out by decapitation with a preliminary (16 hours) weaning of feed. Expression of CD45R, CD3, CD4, CD8a, CD161 receptors on rat peripheral blood lymphocytes and negative control IgG1/IgG2a was determined by direct immunofluorescent staining of whole blood cells using a panel of monoclonal antibodies conjugated to fluorescein: APC, FITC, PE (manufactured by “Miltenyi Biotec GmbH”, Germany). The samples were analyzed by flow cytometry using Cytomics FC 500 and CXP software (“Beckman Coulter”, USA). The leukocyte subsets were defined by forward- and side-scatter pattern. The negative control value was determined by a fluorescence background and antibody-nonspecific staining. The statistical analysis was performed to assess differences between groups using 1-way ANOVA. The hypothesis about the difference in the distribution function of data in the compared groups was additionally tested using the nonparametric Mann-Whitney test. Differences were considered significant at $p < 0.05$. The calculations

**TABLE 1. LYMPHOCYTE SUBPOPULATIONS IN RAT PERIPHERAL BLOOD (M±m, %)**

| Group number | CD45R+ B lymphocytes | CD3+ T lymphocytes | CD3+CD4+ T helpers | CD3+CD8+ T cytotoxic lymphocytes | CD4/CD8 ratio of CD4/CD8 | CD161+ NK cells |
|---|---|---|---|---|---|---|
| 1st | 22.63±1.77 | 67.43±2.10 | 70.07±0.49 | 28.09±0.72 | 2.47±0.09 | 3.26±0.29 |
| 2nd | 22.15±1.33 | 62.10±1.99 | 75.75±1.11* ** | 22.54±1.14* ** | 3.44±0.25* ** | 3.15±0.49 |
| 3rd | 23.66±1.75 | 62.39±3.34 | 72.14±0.91 | 26.07±0.87 | 2.79±0.13 | 3.30±0.58 |

Note. 1st gr., control, diet AIN 93M; 2nd gr., high-calorie choline-deficient diet (HCChDD), 3rd gr., HCChDD+ blueberry and blackcurrant extract. * $p < 0,05$, compared with the control group; ** $p < 0,05$, compared with the 3rd group.

were performed using the SPSS 20.0 software package. Data are presented as M±m.

## Results and discussion

The study results of lymphocyte subpopulations in the rat peripheral blood are presented in the Table 1. Our analysis demonstrated that in the rats of the 2nd gr with diet induced obesity an increase in the relative content of T helpers ($CD3^+CD4^+$) and a decrease in the percentage of T cytotoxic ($CD3^+CD8^+$) lymphocytes were found relative to these subpopulations in rats of the control group and the 3rd group ($p < 0.05$). The ratio of CD3/CD4 in rats of the 2nd group exceeded this indicator in rats of control group and the 3rd group ($p < 0.05$). Enrichment of the HCChDD with the blueberry and blackcurrant extract led to the normalization of the relative content of T helpers and T cytotoxic lymphocytes, as well as the ratio of CD4/CD8 in relation to the control group ($p < 0.05$). The number of B lymphocytes ($CD45R^+$) lymphocytes, T lymphocytes ($CD3^+$) and NK cells ($CD161^+$) in the rat peripheral blood of all experimental groups had no statistically significant differences (Table 1).

The currently obtained results of studies of the mechanisms of metabolic disorders in obesity convincingly show that the immune system takes an active part in the regulation of metabolism. On the one hand, this is the preservation of the integrity of organs and tissues that control metabolism, on the other hand, the influence of the metabolic status of the body on the effector abilities of the immune cells themselves [6]. This study demonstrates, that antigen-specific lymphocytes which are fundamental to immune function, providing for the nature of the immune response ($CD3^+CD4^+$) and direct cytotoxicity ($CD3^+CD8^+$) are affected by obesity. The increased amount of T helpers ($CD3^+CD4^+$) and ratio of CD3/CD4 increase testify to the development metainflammation. The presence of metainflammation in obesity rats is confirmed in our previous work, which demonstrated significant increase in plasma levels of pro-inflammatory cytokines IFNγ, MIP-3α, and RANTES and a decrease in the content of most immunoregulatory cytokines [14].

With the development of the inflammatory process, cells innate immunity: leukocytes, macrophages, dendritic cells, mast cells and others enhance the production and release of reactive oxygen species through "respiratory burst". Activated lymphocytes in adaptive immunity generate cytokines, chemokines, growth factors and other inflammatory mediators that stimulate signal transduction cascades in addition to alterations in transcription factors. Cellular stress reactions are mediated by changes in the expression of nuclear factor of activated T cells, hypoxia-inducible factor-1α (HIF1-α), nuclear factor kappa B (NF-κB), activator protein-1, NF-E2 related factor-2. In addition, an important role in the development of stress-induced metainflammation is assigned to changes in the expression of specific microRNAs, initiation of cyclooxygenase-2 (COX-2), inducibility of nitric oxide synthase (iNOS) [3].

Antioxidants have been used in the diet therapy of obesity and other nutritionally dependent diseases for quite a long time. Anthocyanins are a water-soluble subclass of flavonoids with endogenous antioxidant properties [15]. It has been established that anthocyanins reduce cellular oxidative damage

through a number of cellular mechanisms, including the Keap1/Nrf2/ARE redox-sensitive signaling system, the expression of antioxidant enzymes, such as superoxide dismutase, catalase [1, 11], and suppress the expression of the transcription factor NF-kB in activated cells and activator protein AP-1 [8]. Thus, the efficiency of consumption of anthocyanin-rich foods is due to the activation of various cellular pathways that contribute to the creation of a dynamic cellular antioxidant/anti-inflammatory microenvironment capable of responding to fluctuations in redox potential.

## Conclusions

Based on the study of cellular immunity in diet induced obesity in rats, the presence of meta-inflammation was established. Enrichment of high-calorie choline-deficient diet rats with anthocyanins in the composition of blueberry and blackcurrant extracts ensures the restoration of the studied indicators of adaptive cellular immunity to the level of control group rats. The received data indicate the prospect of using biologically active substances – anthocyanins in the diet therapy of patients with obesity and other alimentary-dependent diseases.

## Acknowledgments

The authors express their deep gratitude to the staff of the Federal Research Centre of Nutrition, Biotechnology and Food Safety: Guseva G.V., Trusov N.V., Avreneva L.I. and Balakina A.S. for help with research.

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*Поступила 11.04.2023*
*Отправлена на доработку 13.04.2023*
*Принята к печати 18.04.2023*

*Received 11.04.2023*
*Revision received 13.04.2023*
*Accepted 18.04.2023*

# ИММУНОМОДУЛИРУЮЩАЯ И НЕЙРОТРОПНАЯ АКТИВНОСТИ СИНТЕТИЧЕСКИХ ПЕПТИДОВ НА МОДЕЛИ ЧЕРЕПНО-МОЗГОВОЙ ТРАВМЫ У КРЫС

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**Резюме.** Лечение последствий черепно-мозговой травмы (ЧМТ) остается одной из актуальных проблем современной медицины. Для повышения эффективности лечения посттравматических осложнений рекомендуют различные препараты, обладающих нейропротекторной и нейрорепаративной активностью, в том числе пептид с нейромодуляторной активностью Семакс (Semax).

Цель настоящего исследования – определить наличие нейро- и иммунопротекторных свойств у синтетического пептида PR5, составленного из фрагментов пролин-богатых антимикробных пептидов.

Работа выполнена на крысах-самцах породы Wistar массой 300-350 г. В качестве модели механической травмы головного мозга использовали модель «падающего груза», в собственной модификации, вызывающую в основном диффузное повреждение мозга. Использовали синтезированный пептид PR5, составленный из фрагментов известных пролин-богатых пептидов нейтрофилов животных, и пептидный препарат Semax в виде 1%-ного водного раствора. Препараты вводили интраназально через 1 час после ЧМТ, затем 2 раза в день в течение 4 дней в дозе 100 мкг/кг массы тела. Контрольные животные, которые получали физиологический раствор в том же режиме, что и пептидные препараты.

ЧМТ приводила к значимому снижению массы тела на 14-е сутки после ЧМТ, однако у крыс, получавших пептидный препарат Semax, падение массы тела было существенно меньшим, чем у контрольных животных, а препарат PR5 полностью предотвращал падение массы тела после ЧМТ. На 7-е сутки после ЧМТ угнеталась пролиферативная активность лимфоцитов и снижалась цитотоксичность NK-клеток. У животных, пролеченных пептидными препаратами Semax и PR5, существенного угнетение цитотоксичности NK-клеток не наблюдалось, а пролиферативная активность лимфоцитов восстанавливалась до показателей контрольных животных к 14-м суткам после ЧМТ. Оба использованных пептидных препарата способствовали более высокой локомоторной активности на 7-е сутки., а у животных, пролеченных пептидом PR5, к 14-м суткам этот вид активности достигал параметров контрольных животных. Снижение продолжительности фризинга в группах, пролеченных пептидными препаратами, свидетельствует о наличии седативного эффекта.

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**Образец цитирования:**
*Н.Б. Серебряная, С.Н. Шанин, Т.А. Филатенкова, Е.Е. Фомичева, А.С. Комлев, О.В. Шамова «Иммуномодулирующая и нейротропная активности синтетических пептидов на модели черепно-мозговой травмы у крыс» // Медицинская иммунология, 2023. Т. 25, № 3. С. 709-714.*
*doi: 10.15789/1563-0625-IAN-2754*

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**For citation:**
*N.B. Serebryanaya, S.N. Shanin, T.A. Filatenkova, E.E. Fomicheva, A.S. Komlev, O.V. Shamova “Immunomodulatory and neurotropic activities of synthetic peptides in a model of brain injury in rats”, Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 709-714.*
*doi: 10.15789/1563-0625-IAN-2754*

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**DOI:** 10.15789/1563-0625-IAN-2754

Пептидный препарат PR5 был активен в данной серии экспериментов, показав иммунотропную и нейропротекторную активность, сопоставимую с препаратом Semax. Дальнейшие исследования, направленные на подтверждение выявленных видов активности пептидного препарат PR5, могут обосновать его перспективы для клинического использования как нового ноотропного агента.

*Ключевые слова: регуляторные пептиды, черепно-мозговая травма, стресс, активность лимфоцитов, поведенческие тесты, нейротропный эффект*

# IMMUNOMODULATORY AND NEUROTROPIC ACTIVITIES OF SYNTHETIC PEPTIDES IN A MODEL OF BRAIN INJURY IN RATS

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**Abstract.** Treatment of consequences of traumatic brain injury (TBI) remains one of the current problems of medicine. To increase the effectiveness of treatment of post-traumatic complications, various drugs are recommended, including the peptide with neuromodulatory activity Semax.

The present study aims to determine the presence of neuro- and immunoprotective properties of the synthetic peptide PR5, composed of fragments of proline-rich antimicrobial peptides.

The work was performed on male Wistar rats weighing 300-350 g. The “falling weight” model of mechanical brain injury was used, which mainly causes diffuse brain damage. The synthesized peptide PR5, composed of fragments of known proline-rich peptides of animal neutrophils, and the peptide preparation Semax in the form of a 1% aqueous solution were used. The drugs were administered intranasally 1 hour after TBI, then twice a day for 4 days at a dose of 100 μg/kg body weight. Control animals received physiological saline in the same regimen as the peptide preparations.

TBI led to a significant decrease in body weight, but in rats receiving the peptide preparation Semax, the decrease in body weight was significantly less than in control animals, and the PR5 preparation completely prevented the decrease in body weight after TBI. After TBI, the proliferative activity of lymphocytes was suppressed and the cytotoxicity of NK cells decreased. In animals treated with peptide preparations, there was no significant suppression of NK cell cytotoxicity, and the proliferative activity of lymphocytes was restored to the level of control animals by day 14 after TBI. Both peptide preparations used contributed to higher locomotor activity, and in animals treated with the PR5 peptide, this type of activity reached the parameters of control animals. The reduction in freezing duration in groups treated with peptide preparations indicates the presence of a sedative effect.

The peptide preparation PR5 was active in this series of experiments, showing immunotropic and neuroprotective activity comparable to the Semax preparation. Further studies aimed at confirming the identified types of activity of the peptide preparation PR5 may justify its prospects for clinical use as a new nootropic agent.

*Keywords: regulatory peptides, traumatic brain injury, stress, lymphocyte activity, behavioral tests, neurotropic effect*

## Introduction

Treatment of consequences of traumatic brain injury (TBI) remains one of the pressing problems of modern medicine. Patients with brain injuries of varying severity often suffer from physical and cognitive impairments for months and years, and there are no standard methods of therapy for these conditions. Various drugs with neuroprotective and neurorepair activity, including regulatory peptides, are used as correctors of post-traumatic complications. Regulatory peptides belong to a group of biologically active substances of peptide nature, having signs of polyfunctionality. Some authors classify regulatory peptides as histohormones, based on their relatively short half-life and participation in regulation processes at the local tissue level [1]. One of the peptides synthesized by the Ashmarin I.P. academician group, Semax, a synthetic analog of the ACTH4-10 fragment, has proven itself as a neuromodulator that stimulates a range of brain functions and is currently approved for the treatment of consequences of strokes, optic nerve atrophy, and some other neurological diseases [2].

Previously, it has been shown that natural antimicrobial peptides such as defensins and protegrins have corticostatic activity and can reduce stress-induced or ACTH-induced increases in corticosterone levels in blood, thereby exhibiting stress-protective effects. The current study focuses on analyzing the effects of a new synthetic peptide in order to identify its neuro- and immunoprotective properties.

**The study aims** to investigate the regulatory capabilities of the synthetic peptide PR5 compared to Semax on immune cell activity and behavioral parameters before and after traumatic brain injury in experimental rats.

## Materials and methods

The study was conducted on male Wistar rats weighing 300-350 g. The animals were housed in vivarium conditions at room temperature with a 12-hour light/dark cycle, free access to water and food, and fed a standard diet in accordance with laboratory animal maintenance standards.

The model of mechanical traumatic brain injury used was the "falling weight" model, also known as the "impact acceleration" model, in our own modification, which mainly causes diffuse brain damage [6]. A weight (460 g) with a blunt surface fell inside a hollow tube (inner diameter of 20 mm) from a height of 60 cm. The distance between the end of the tube and the animal's head was 7 cm. Prior to the injury, the animals received ether anesthesia at a rate of 3-5 mL of medical ether per 1 kg of body weight in a mixture with atmospheric air. The impact zone was located in the central part of the temporal area. The device for applying TBI was assembled at the Department of General Pathology and Pathophysiology of the Institute of Experimental Medicine on the basis of literature data on devices for the "falling weight" model [3].

After the injury, the animals were transferred to a special plastic cage, where they were observed until the restoration of normal behavioral patterns. The experiments were carried out in accordance with the National Standard of the Russian Federation GOST R-53434-2009 "Principles of Good Laboratory Practice" and the Order of the Ministry Health of the Russian Federation dated April 1, 2016 No. 199n "On Approval of the Rules of Good Laboratory Practice". All manipulations performed on animals were reviewed and approved at a meeting of the bioethical commission of the Institute of Experimental Medicine

The PR5 peptide sequence was composed of fragments of known proline-rich peptides of animal neutrophils, including PR-39 (domestic pig neutrophils) and bactenecin ChBac5 (domestic goat neutrophils), with an additional glyproline (Pro-Gly-Pro) at its C-terminus. The peptide was produced via the Fmoc solid phase synthetic approach on a Symphony X peptide synthesizer (Protein Technologies, USA) using a standard synthesis protocol. The purity of the peptide as determined by analytical chromatography was 96-98%. The molecular weight was confirmed by MALDI TOF mass spectrometry. Peptides were dissolved in physiological saline for experiments. Semax, a 1% aqueous solution of peptide, was purchased from a pharmacy.

In the series of experiments investigating the effects of PR5 peptide on TBI, the following experimental groups were formed, each consisting of 6-8 animals: 1 – control animals; 2 – animals subjected to TBI; animals subjected to TBI receiving PR5 peptide (group 3); and animals receiving Semax peptide after TBI (group 4). All peptides were administered intranasally 1 hour after TBI, followed by twice daily administration for 4 days at a dose of 100 μg/kg body weight. Material for analysis was collected on days 7 and 14 after TBI. Animals receiving physiological saline in the same regimen as the peptide groups were used as control.

The cytotoxic activity of spleen lymphocytes against K-562 cells and their ability to proliferate were used as functional parameters of immune cells to assess the effects of TBI. The intensity of lymphocyte proliferation was evaluated using the blast transformation reaction upon addition of Concanavalin A (ConA) and recombinant IL-1β (Betaleukin, produced by the Research Institute Highly Pure Biopreparations, St. Petersburg).

To analyze changes in the functional state of the CNS in animals after TBI, behavioral reactions were recorded using the "Open Field" test and VideoMot 2 software (TSE Systems, Germany). Motor activity was evaluated by changes in total distance traveled and average speed, exploratory activity by the animal's location in different sectors of the open field, vertical motor activity, and anxiety level by the number of grooming and freezing acts. The duration of behavior recording for each animal in the test was 5 minutes.

Statistical analysis of the results was performed using the STATISTICA 7 software package with the Mann–Whitney pairwise comparison test. Differences were considered significant at $p < 0.05$.

## Results and discussion

Traumatic brain injury (TBI) was a significant stressor event, as evidenced by the previously shown sharp fluctuations in the level of corticosterone (Cs) in the blood of injured animals [5]. Considering that experienced stress is associated with weight loss [4], the nature of changes in body weight in the studied groups was traced.

The results presented in Figure 1 indicate that TBI led to a significant decrease in body weight, however, in rats receiving the peptide preparation Semax, the decrease in body weight was significantly less than

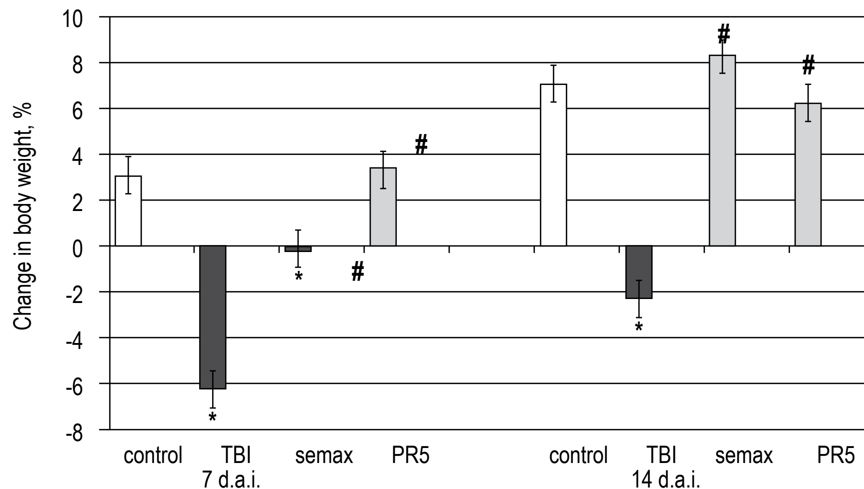


**Figure 1. Changes in body weight of animals at 7 days after TBI and administration of the peptide preparations**

Note. *, $p < 0.05$ compared to control animals. #, $p < 0.05$ compared to animals after TBI.

in untreated animals at 7 days post-TBI, and the PR5 preparation completely prevented the decrease in body weight in injured animals (Figure 1). In the presented series of experiments, a decrease in body weight of injured animals continued for up to 14 days after TBI. The Semax preparation contributed to the normalization of body weight of injured animals at 14 days post-TBI, while the PR5 peptide prevented stress-induced weight loss at both 7 and 14 days post-TBI.

The impact of traumatic brain injury (TBI) on the functional parameters of immune cells was assessed by means of evaluating the cytotoxic activity of spleen lymphocytes against K-562 cells and their ability to proliferate. The obtained data indicate that after TBI, the cytotoxicity of NK cells decreased at 7 days post-TBI and returned to baseline levels by 14 days post-TBI. In animals treated with the peptide preparations Semax and PR5, there was no significant suppression of NK cell cytotoxicity at 7 days post-TBI (Table 1). Additionally, at 7 days post-injury, there was a significant inhibition of lymphocyte proliferative activity stimulated by suboptimal doses of Con A and enhanced activation of cells by Con A in combination with IL-1β. Administration of Semax and PR5 prevented the inhibition of lymphocyte proliferative

**TABLE 1. CYTOTOXIC AND PROLIFERATIVE ACTIVITY OF LYMPHOCYTES AT 7 AND 14 DAYS POST-TBI AND ADMINISTRATION OF PEPTIDE PREPARATIONS**

| Groups | Cytotoxic activity, % | Proliferative activity, stimulation index | |
|---|---|---|---|
| | | Stimulation with Concanavalin A | Stimulation with Concanavalin A and IL-1β |
| **Control** | 19.8 (18.1-22.8) | 1.73 (1.66-2.40) | 2.70 (1.90-2.80) |
| **TBI, 7th day** | 13.3 (8.6-14.3)* | 1.28 (0.66-1.56)* | 1.50 (0.90-1.66)* |
| **TBI + Semax, 7th day** | 16.3 (15.7-17.9)# | 1.75 (1.45-2.20) | 2.20 (1.91-2.50)# |
| **TBI + PR5 7th day** | 18.8 (16.6-20.0)# | 1.66 (1.66-1.93) | 2.66 (2.15-2.88)# |
| **TBI, 14th day** | 18.9 (15.9-19.5) | 1.55 (1.35-2.05) | 1.67 (1.57-2.30)* |
| **TBI + Semax 14th day** | 22.0 (19.3-24.0) | 1.69 (1.45-2.25) | 2.75 (1.96-3.40)# |
| **TBI + PR5 14th day** | 18.5 (16.1-20.1) | 1.66 (1.45-1.88) | 1.72 (1.67-2.50)# |

Note. *, $p < 0.05$ compared to control animals at the same time of observation. #, $p < 0.05$ compared to injured animals at the same time of observation.

**TABLE 2. HORIZONTAL MOTOR ACTIVITY, EXPLORATORY BEHAVIOR RESPONSE (ebr), NUMBER OF GROOMING ACTS, AND DURATION OF FREEZING AT 7 AND 14 DAYS POST-TBI AFTER ADMINISTRATION OF PEPTIDE PREPARATIONS**

| Groups | Horizontal locomotor activity | | EBR | Number of grooming acts | Freezing duration, sec |
|---|---|---|---|---|---|
| | distance, m | speed, cm/s | | | |
| Control 7th day | 17.1 (12.3-22.1) | 5.85 (3.55-7.52) | 23 (18-27) | 8 (4-12) | 6 (0-9) |
| TBI, 7th day | 9.6 (7.1-13.5)* | 3.16 (2.40-4.40)* | 18 (15-23) | 9 (6-12) | 35 (17-82)* |
| TBI + Semax, 7th day | 10.9 (8.8-13.5)* | 3.65 (2.95-4.30) | 18 (16-51) | 11 (6-16) | 16 (7-21)# |
| TBI + PR5 7th day | 14.4 (10.3-18.6)# | 4.82 (3.43-6.21) | 26 (14-38)# | 9 (5-13) | 18 (10-24)# |
| Control 14th day | 11.6 (9.6-12.3) | 3.78 (3.40-4.11) | 25 (23-28) | 8 (5-10) | 66 (47-84) |
| TBI, 14th day | 8.9 (6.5-10.7) | 2.96 (2.15-3.56) | 15 (8-123) | 3 (1-10) | 102 (58-185)* |
| TBI + Semax 14th day | 9.9 (4.7-16.3) | 3.32 (1.57-5.45) | 19 (8-38)# | 8 (3-11) | 54 (37-87)# |
| TBI + PR5 14th day | 12.3 (9.6-19.0)# | 4.07 (3.22-6.33) | 13 (7-30) | 4 (1-9) | 75 (37-105) |

Note. As for Table 1.

activity in injured animals. In untreated animals, inhibition of lymphocyte proliferative activity was observed at 14 days post-TBI when using combined stimulation with Con A and IL-1β, while in animals treated with Semax and PR5, the stimulation index was higher than that in injured animals and did not differ from control animal values. The obtained data indicate the immunoprotective effects of both peptide preparations.

Study of animal behavior in the “Open Field” test showed (Table 2) that horizontal motor activity in terms of length and running speed significantly decreased in injured animals at 7 days post-TBI compared to control animals. However, in animals treated with the PR5 peptide, motor activity was preserved and remained significantly higher than in untreated injured rats. By 14 days post-TBI, horizontal motor activity in animals treated with the PR5 peptide was significantly higher than in injured rats and did not differ from control animal values. The running speed in rats treated with both peptides did not differ significantly from that in control rats.

The exploratory behavior response (EBR) of injured rats was assessed by the number of peeks into “nests” vertical posts (vertical motor activity), and the number of exits into the center of the field.

It was shown that at 7 days post-TBI, the EBR in animals treated with the PR5 peptide was higher than in injured animals, and at 14 days post-TBI, the EBR index was higher in rats treated with the Semax peptide compared to injured animals.

The level of hidden anxiety in rats was judged by the number of grooming acts and the duration of freezing (immobility reaction). There were no changes in the number of grooming acts in the studied groups, however, injured rats significantly differed from control animals in the duration of freezing at 7 days post-TBI. Animals treated with Semax and PR5 showed an increase in the duration of freezing compared to control animals, but the episodes of immobility were significantly less prolonged compared to injured animals. By 14 days post-TBI, the duration of freezing in all peptide-treated animals had returned to control animal parameters, while it increased in injured animals.

## Conclusion

Based on the obtained data, it can be concluded that Semax and PR5 preparations prevent post-traumatic weight loss after TBI, restore cytotoxic and proliferative activity of spleen lymphocytes when stimulated with Concanavalin A and IL-1β at 7 days after TBI. Both peptide preparations contribute to higher locomotor activity at 7 days after TBI, and in animals treated with the PR5 peptide, this type of activity reached the parameters of control animals by day 14. The reduction in freezing duration at 7 days after TBI in groups treated with peptide preparations indicates the presence of sedative activity, which is manifested for the Semax preparation at day 14 as well. Thus, the peptide preparation PR5 was active in this series of experiments, showing immunotropic and neuroprotective activity. Further studies aimed at confirming the identified types of activity of the peptide preparation PR5 may justify its prospects for clinical use as a new nootropic agent.

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*Поступила 13.04.2023*
*Отправлена на доработку 18.04.2023*
*Принята к печати 20.04.2023*

*Received 13.04.2023*
*Revision received 18.04.2023*
*Accepted 20.04.2023*

Медицинская иммунология
2023, Т. 25, № 3,
стр. 715-720

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 715-720

# ИММУНОЛОГИЧЕСКИЕ АСПЕКТЫ ПРИМЕНЕНИЯ МЕЛАТОНИНА ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ТЕРМИЧЕСКОЙ ТРАВМЕ

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**Резюме.** Распространенность термической травмы, высокий риск инфекционных и неинфекционных краткосрочных и долговременных осложнений, ограниченная эффективность применяемых терапевтических подходов являются предпосылкой для поиска и патогенетического обоснования новых средств терапии, среди которых внимание привлекает эндогенный регулятор гомеостаза с плейотропными свойствами мелатонин.

Цель работы – исследовать иммунологические аспекты эффективности внутрибрюшинного применения мелатонина (МТ) при экспериментальной термической травме (ТТ).

Работа выполнена на 158 крысах линии Wistar, ТТ IIIA степени и относительной площадью 3,5% моделировали погружением кожи в воду при 98-99 °С на 12 с. МТ применяли внутрибрюшинно ежедневно в дозе 10 мг/кг в течение 5 суток. Количественный состав клеток крови оценивали на гематологическом анализаторе. Концентрацию в плазме IL-4, TNFα, IFNγ, СРБ определяли на автоматическом иммуноферментном анализаторе с использованием специфических для крыс тест-систем, МТ – методом капиллярного электрофореза.

При экспериментальной ТТ на фоне прогрессивного от 5 к 20 суткам увеличения количества в крови лейкоцитов за счет нейтрофилов, моноцитов, базофилов, снижается количество лимфоцитов. При ТТ в сыворотке на 5 и 10 сутки возрастает концентрация СРБ, на 5-е, 10-е и 20-е сутки возрастает содержание TNF-α, IL-4 при отсутствии значимых изменений концентрации IFNγ. Концентрация сывороточного МТ значимо не изменяется. Внутрибрюшинное применение МТ при ТТ приводит к частичному восстановлению в крови количества лимфоцитов на 5-е сутки. Оценка цитокинового профиля в сыворотке выявила снижение концентрации TNFα на 10-е и 20-е сутки, значимых изменений концентрации IL-4 и IFNγ не зафиксировано, концентрация СРБ снижается на 5-е сутки. Концентрация сывороточного МТ увеличивается на 5-е сутки.

При ТТ на 5-е, 10-е, 20-е сутки эксперимента в крови увеличивается количество нейтрофилов, моноцитов, базофилов, снижается – лимфоцитов, в сыворотке возрастает содержание СРБ, TNFα, IL-4, содержание IFNγ и мелатонина не изменяется. Внутрибрюшинное применение МТ при ТТ частично восстанавливает в крови количество лимфоцитов, концентрацию СРБ, TNFα. Снижение концентрации в сыворотке TNFα и СРБ при ТТ в условиях применения МТ позволяют говорить об ограниче-

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**Образец цитирования:**
*М.В. Осиков, А.А. Агеева, М.С. Бойко, Ю.И. Агеев «Иммунологические аспекты применения мелатонина при экспериментальной термической травме» // Медицинская иммунология, 2023. Т. 25, № 3. С. 715-720.*
*doi: 10.15789/1563-0625-IAO-2706*

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**For citation:**
*M.V. Osikov, A.A. Ageeva, M.S. Boyko, Yu.I. Ageev "Immunological aspects of the use of melatonin in experimental thermal trauma", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 715-720. doi: 10.15789/1563-0625-IAO-2706*

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**DOI:** 10.15789/1563-0625-IAO-2706

нии острофазового ответа как следствие антиоксидантного, противовоспалительного действия МТ, что может способствовать ускорению заживления и уменьшению площади очага повреждения ТТ.

*Ключевые слова: термическая травма, мелатонин, СРБ, TNFα, IL-4, IFNγ*

# IMMUNOLOGICAL ASPECTS OF THE USE OF MELATONIN IN EXPERIMENTAL THERMAL TRAUMA

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**Abstract.** The prevalence of thermal trauma, the high risk of infectious and non-infectious short- and long-term complications, and the limited effectiveness of the therapeutic approaches used are prerequisites for the search and pathogenetic justification of new therapies, among which the endogenous homeostasis regulator with pleiotropic properties melatonin attracts attention.

The aim of the work is to investigate the immunological aspects of intraperitoneal use of melatonin (MT) in experimental thermal trauma (TT).

The work was performed on 158 rats of the Wistar line, grade III TT and a relative area of 3.5% were simulated by skin immersion in water at 98-99 °C for 12 s. MT was administered intraperitoneally daily at a dose of 10 mg/kg for 5 days. The quantitative composition of blood cells was evaluated on a hematological analyzer. Plasma concentrations of IL-4, TNFα, IFNγ, and CRP were determined on an automatic enzyme immunoassay using rat-specific test systems, and MT by capillary electrophoresis.

With experimental TT, against the background of a progressive increase in the number of leukocytes in the blood from 5 to 20 days due to neutrophils, monocytes, basophils, the number of lymphocytes decreases. With TT, the concentration of CRP increases in serum on days 5 and 10. The content of TNFα, IL-4 increases on days 5, 10 and 20 in the absence of significant changes in the concentration of IFNγ. The concentration of serum MT does not change significantly. Intraperitoneal use of MT in TT leads to a partial restoration of the number of lymphocytes in the blood on day 5. Evaluation of the cytokine profile in serum revealed a decrease in the concentration of TNFα on days 10 and 20, no significant changes in the concentration of IL-4 and IFNγ were recorded, the concentration of CRP decreased on day 5. The concentration of serum MT increases by 5 days.

With TT on the 5th, 10th, 20th day of the experiment, the number of neutrophils, monocytes, basophils in the blood increases, decreases – lymphocytes, the serum content of CRP, TNFα, IL-4 increases, the content of IFNγ and melatonin does not change. Intraperitoneal use of MT in TT partially restores the number of lymphocytes in the blood, the concentration of CRP, TNFα. A decrease in serum concentrations of TNFα and CRP in TT under the conditions of MT use suggests a limitation of the acute phase response as a consequence of the antioxidant, anti-inflammatory effect of MT, which can accelerate healing and reduce the area of the lesion of TT.

*Keywords: thermal injury, melatonin, CRP, TNFα, IL-4, IFNγ*

## Introduction

Burns are one of the important medical and social issues. Notwithstanding significant progress in kombustiology, slow healing and attachment of infection are major problems in patients with burns, which cause emotional distresses, prolonged hospitalization, decreased quality of life [1]. Thermal trauma (TT) obstacles are mostly associated with infections, primarily urinary tract infections and pneumonia. The infectious complication (sepsis) after TT is connected with immunosuppressive reactions as compensation of lasting stable proinflammatory response, especially in connection with excessive synthesis of overproduction of TGF-β, IL-10, IL-4 [2]. Immune reactions play a pivotal role in the TT pathogenesis and its complications at each stage. Understanding of the burn's pathophysiology is a precondition for the pathogenetically reasonable therapies development, as also methods of wound suturing and safe necrectomy [3]. In this attitude, endogenous homeostasis controllers are of specific interest. They can influence the increasing immune reaction to TT and be involved as immunocorrect agents.

In previous studies, we have demonstrated the healing-accelerating effects of the use of erythropoietin (locally and systemically), just as the local administration of EGF at TT expedite the burns healing and the immune status reconstruction in experimental and clinical stipulations [4, 5]. It is

known that melatonin (MT) is a homeostasis adjuster. At the same time, it has pleiotropic effects: regulation of the sleep-wake cycle, changing the redox factor due to the realization of the antioxidant effect, modulation of immunity and inflammation with its suppression and stimulation, influence on cellular differentiation, proliferation and apoptosis [7]. One of the sources of MT is epidermocytes. Also, in the study of keratinocytes, melanocytes, skin fibroblasts, MT metabolites were found in them [8]. According to research data, receptors for MT (MT1, MT2, RORa) are found in skin fibroblasts, keratinocytes, hair follicle cells and dermal blood vessels, as well as in melanocytes [9].

It is known that pharmaceutical forms of MT for topical use in case of skin damage are not accessible in the Russian Federation. There are no indications in the official recommendations for the use of oral forms of MT in TT.

**The aim of the study** is to explore the impact of the effect of intraperitoneal use of melatonin in experimental thermal trauma on some indicators of immune status.

## Materials and methods

Experimental studies were performed on 158 Wistar rats weighing 240-250 g in the vivarium of the Federal State Budgetary Educational Institution of the Southern State Medical University of the Ministry of Health of Russia. The experiment was conducted in strict accordance with the experimental animals' care and maintenance requirements, as also their elimination from the experiment with subsequent utilization in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes (ETS No. 123 of 03/18/1986, Strasbourg), Recommendations of the European Commission 2007/526/EC of June 18, 2007, Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes. The study organization was confirmed by the ethics commission of the Federal State Budgetary Educational Institution of the Ministry of Health of the Russian Federation (Protocol No. 10 of 15.11.2019, Protocol No. 13 of 12/28/2020).

Experimental rats were randomly separated into groups: group 1 (n = 33) – intact control, group 2 (n = 67) – animals with TT and daily aseptic dressing, group 3 (n = 58) – animals with TT with aseptic dressing and intraperitoneal administration of melatonin ("Flamma Sp", Italy) in a daily single dose of 10 mg/kg for 5 days. To simulate grade III TT and a relative area of 3.5%, an isolated interscapular area of rat skin was immersed in purified water at 98-99 °From to 12 s. The depth of the burn was verified by morphological methods. For anesthesia, the drug "Zoletil-100" ("Virbac SanteAnimale", France) was used at a dose of 20 mg/kg. On the 5th, 10th and 20th days from the moment of TT induction in groups 2 and 3, blood was taken under anesthesia after thoracotomy by heart puncture in the left ventricle into vacuum tubes "Vacuette" ("Greiner BioOne", Austria). The absolute number of leukocytes was evaluated on an automatic hematological analyzer for veterinary medicine, calibrated for rats, "VS-2800Vet" ("Mindray", China). The leukocyte formula was calculated in blood smears stained with azur II-eosin (Gemstandart-R, Russia). Plasma concentrations of IL-4, TNF$\alpha$, IFN$\gamma$, CRP were determined on an automatic enzyme immunoassay analyzer "Personal LAB" (Italy) using rat-specific test systems "Cloud-Clone Corp." (China) the results were expressed in pg/ml. The concentration of MT in plasma was determined by capillary electrophoresis on the device "Kapel-105M" (Lumex, St. Petersburg), the results were expressed in pg/ml (Kim Y.O. et al., 1999). Statistical processing of the results was carried out using the IBM SPSS Statistics 19 program. The characteristics of the samples are presented in the format "Me ($Q_{0.25}$-$Q_{0.75}$)", where Me is the median, Q1 and Q3 are the values of the lower and upper quartile, respectively. The significance of the differences between the groups was assessed using the criteria of Kruskal–Wallis, Mann–Whitney.

## Results and discussion

Primary and secondary alteration of the skin in the lesion of TT leads to systemic changes in homeostasis, among which the key position is occupied by the reactions of the immunobiological surveillance system aimed at ensuring the inflammatory process in the lesion, maintaining its sterility, repair, but can lead to excessive tissue damage in situ and at removal with hyperergia. On the 5th day of TT, the total number of leukocytes in the blood increases due to rod-shaped and segmented neutrophils, monocytes, the absolute number of lymphocytes decreases (Table 1). On the 10th day of TT, the total number of leukocytes in the blood does not change, the absolute number of rod-shaped and segmented neutrophils increases, the absolute number of lymphocytes decreases. On the 20th day of TT, no significant changes in the number of leukocytes were detected. In the dynamics of observation on day 10, the number of rod-shaped and segmented neutrophils, the total number of neutrophils, the number of monocytes was less than on day 5 TT (Table 1). On the 20th day, the total number of leukocytes, neutrophils, and rod–shaped neutrophils in the blood was less than on the 5th day of TT, the number of monocytes was less than on the 5th and 10th days of TT (Table 1).

On the 5th day of TT, the concentration of CRP in serum increases by about 10 times in the median relative to the values in the group of intact animals, and by about 2 times on the 10th day (Table 2). On day 20, there were no significant changes in the

concentration of CRP in serum, since it belongs to the reactants of the acute phase of the first echelon, the concentration of which reaches a maximum on the first day after tissue damage. In the dynamics of TT, the concentration of CRP in serum on the 10th day is significantly lower than on the 5th day, on the 20th day – lower than on the 5th and 10th days. Note that when studying the concentration of serum MT, we found no significant changes on the 5th, 10th and 20th days of the experimental TT. On the 5th, 10th and 20th days of TT, an increase in the concentration of TNFα, IL-4 was found in the absence of significant changes in IFNγ (Table 2). In the dynamics of TT, the concentration of TNFα and IL-4 on the 10th day of TT was higher than on the 5th day and did not differ from the values on the 20th day, which allows us to talk about the maximum level of TNFα and IL-4 on the 10th day.

With intraperitoneal administration of melatonin in TT, it was found that the quantitative composition of leukocytes in the blood on the 10th and 20th days of observation did not significantly change relative to the comparison group (Table 1). In dynamics, the total number of leukocytes, the total number of neutrophils, rod-shaped neutrophils, monocytes on the 10th and 20th days are less than on the 5th day, the total number of neutrophils on the 20th day is less than on the 10th day, the number of lymphocytes on the 10th day is more than on the 5th day, and on the 20th day is more than 10 a day. Relative to the group of intact animals, significant differences remain on day 5 with the total number of leukocytes, the total number of neutrophils, rod-shaped neutrophils, segmented neutrophils, lymphocytes, monocytes; on day 10 – with the total number of neutrophils, segmented neutrophils, lymphocytes, monocytes; on day 20, there were no significant differences with the group of intact animals.

Evaluation of the cytokine profile in serum revealed a decrease in the concentration of TNFα on days 10 and 20; no significant changes in the concentration of IL-4 and IFNγ were recorded (Table 2). At all follow-up periods, the concentration of TNFα and IL-4 in serum was higher than in the group of intact animals. The use of MT in TT leads to an increase in the concentration of MT in serum on day 5, without significant changes in this indicator on days 10 and 20; the concentration of MT in serum on day 5 is significantly higher than in the group of intact animals (Table 2). The concentration of CRP

**TABLE 1. EFFECT OF SYSTEMIC USE OF MT ON THE QUANTITATIVE COMPOSITION OF LEUKOCYTES IN THE BLOOD DURING TT, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Indicator | Group 1 Intact (n = 33) | Group 2 TI + AsD | | | Group 3 TI + MT | | |
|---|---|---|---|---|---|---|---|
| | | 5 days (n = 22) | 10 days (n = 26) | 20 days (n = 19) | 5 days (n = 25) | 10 days (n = 15) | 20 days (n = 18) |
| **White blood cells, × 10⁹/L** | 5.70 (4.00-6.70) | 6.30 (5.10-8.50) * | 5.50 (4.30-7.20) | 4.60 (4.30-6.20) ^ | 6.70 (6.40-7.30) * | 5.40 (4.90-6.00) & | 5.70 (4.30-5.90) & |
| **Basophils, × 10⁹/L** | 0 | 0 (0.00-0.31) * | 0 (0.00-0.10) * | 0 | 0 (0.00-0.06) | 0 (0.00-0.05) | 0 |
| **Eosinophils, × 10⁹/L** | 0.22 (0.04-0.39) | 0.15 (0.05-0.25) | 0.22 (0.09-0.30) | 0.16 (0.06-0.17) | 0.12 (0.06-0.24) | 0.12 (0.09-0.23) | 0.12 (0.06-0.12) & |
| **Rod – shaped neutrophils, × 10⁹/L** | 0 (0.00-0.04) | 0.27 (0.00-0.58) * | 0.04 (0.00-0.29) ^ | 0 | 0.27 (0.10-0.32) * | 0 (0.04-0.05) & | 0.04 (0.00-0.06) & |
| **Segmented neutrophils, × 10⁹/L** | 1.65 (1.09-1.88) | 2.78 (2.05-4.55) * | 2.48 (1.31-3.65) * ^ | 2.05 (1.27-2.94) ^ | 2.92 (2.52-3.32) * | 2.59 (2.34-2.90) * | 1.99 (1.89-2.56) & |
| **Neutrophils, × 10⁹/L** | 1.70 (1.09-1.88) | 2.98 (2.43-5.39) * | 2.58 (1.31-3.88) * ^ | 2.05 (1.27-2.94) ^ | 3.15 (2.77-3.57) * | 2.59 (2.35-2.93) * & | 2.02 (1.94-2.71) & && |
| **Lymphocytes, × 10⁹/L** | 3.40 (2.32-3.77) | 2.49 (2.00-2.86) * | 2.27 (2.00-3.69) * | 2.79 (2.05-3.92) | 2.71 (2.51-2.96) * # | 2.11 (1.91-2.34) * & | 2.69 (2.26-2.94) && |
| **Monocytes, × 10⁹/L** | 0.28 (0.21-0.41) | 0.71 (0.29-0.83) * | 0.33 (0.22-0.60) ^ | 0.22 (0.17-0.29) ^ ^^ | 0.67 (0.53-0.76) * | 0.54 (0.34-0.54) * & | 0.35 (0.24-0.57) & |

Note. *, significant ($p < 0.01$) differences with group 1; ^, with group 2 on day 5; ^^, with group 2 on day 10; #, with group 2 on the corresponding day; &, with group 3 on day 5, &&, with group 3 on day 10.

**TABLE 2. EFFECT OF SYSTEMIC USE OF MT ON SERUM CONCENTRATIONS OF MELATONIN AND CRP IN EXPERIMENTAL TT, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Indicator | Group 1 Intact (n = 33) | Group 2 TI + AsD | | | Group 3 TI + MT | | |
|---|---|---|---|---|---|---|---|
| | | 5 days (n = 10) | 10 days (n = 12) | 20 days (n = 10) | 5 days (n = 10) | 10 days (n = 8) | 20 days (n = 8) |
| MT, pg/mL | 24.08 (11.56-28.00) | 26.32 (13.36-33.88) | 22.35 (13.70-28.81) | 21.57 (15.27-28.15) | 32.14 (23.47-39.08) * # | 24.61 (18.31-27.98) | 23.78 (15.26-27.79) |
| CRP, pg/mL | 1.29 (0.89-1.66) | 12.14 (11.67-15.32) * | 2.37 (1.42-5.32) * ^ | 1.46 (1.38-1.73) ^ ^^ | 9.23 (7.02-10.05) * # | 1.98 (1.98-2.31) * & | 1.29 (1.18-1.35) & && |
| TNFα, pg/mL | 1.18 (0.84-1.96) | 2.25 (2.02-2.92) * | 3.42 (2.72-6.14) * ^ | 3.48 (1.77-5.05) * | 2.33 (1.85-3.03) * | 2.69 (2.19-3.03) * # | 1.91 (1.35-3.03) * # |
| IFNγ, pg/mL | 0.83 (0.29-1.24) | 0.73 (0.12-1.15) | 0.98 (0.59-1.35) | 1.06 (0.65-1.74) ^ | 0.84 (0.51-1.49) | 0.76 (0.52-1.08) | 1.08 (0.66-1.22) |
| IL-4, pg/mL | 0.79 (0.50-1.75) | 1.93 (1.36-2.51) * | 3.26 (2.58-5.65) * ^ | 2.97 (1.57-4.97) * ^ | 2.11 (1.79-2.86) * | 2.79 (2.43-3.36) * & | 2.18 (1.79-3.01) * |

Note. As for Table 1.

in serum decreases on day 5 and does not change on days 10 and 20 relative to the group of rats without the use of MT; on days 5 and 10, the concentration of CRP in serum is higher than in intact animals. In the dynamics of TT under the conditions of MT application, the concentration of CRP in serum on days 10 and 20 is less than on day 5, and on day 20 is less than on day 10 of observation.

We believe that the changes in hematological parameters detected by us during experimental TT are a reflection of tissue alteration and the development of the inflammatory process in the skin. In response to thermal damage, inflammatory mediators (including proinflammatory cytokines, arachidic acid cascade products, catecholamines, etc.) are released in the TT focus, which stimulate the myeloid bone marrow sprout, including CFU-HME, cause demargination of the parietal neutrophil pool and are associated with an increase in the number of neutrophils, monocytes, platelets in the blood, infiltration foci of neutrophils and monocytes. Lymphocytopenia in TT is registered after 48 hours and persists for up to 4 weeks, mainly due to the effects of TNFα mediated by the TIPE-2 protein (part of the TNFα-induced protein family) and an increase in the activity of caspase-3, caspase-8, caspase-9, cytochrome C, a decrease in the membrane potential of mitochondria, which initiates cell death by apoptosis [6]. A decrease in the number of lymphocytes in the blood and the focus of TT limits their participation in wound repair: secretion of growth factors, restriction of vascular exudative reactions, activation of angiogenesis, etc. In TT, a decrease in the number of lymphocytes in the blood, among other things, is associated with the activation of their death in conditions of increased TNFα production, changes in the acid-base state, and other factors [6].

Changes in gene expression, synthesis and secretion of cytokines in the focus of TT have been recorded by many researchers in relation to pro-inflammatory and anti-inflammatory cytokines depending on the depth, area and localization of burns, age, concomitant pathology, and other factors [7, 10]. The source of cytokines are activated neutrophils, macrophages, dendritic cells, lymphocytes, endotheliocytes, etc. The entry of cytokines from the TT focus into the systemic circulation leads to the activation of circulating leukocytes, platelets, endotheliocytes – additional sources of cytokines. Dysfunction of these cells, violation of the change of their phenotype leads to contradictory results in different researchers on the concentration of cytokines in serum and in the lesion of TT.

A decrease in serum concentrations of TNFα and CRP suggests a decrease in the severity of the acute phase response due to a decrease in destructive events in the focus of TT with intraperitoneal administration of MT. With accelerated healing and a decrease in the area of the TT focus, less lymphocytes enter it from the bloodstream. The suppressive effect of TNFα on lymphopoiesis decreases. An increased number of lymphocytes in the blood can result in an increase in the Ig concentration in the blood serum.

These changes make it possible to state to a certain extent a decrease in the severity of the acute phase response in TT in the conditions of systemic use of MT. This fact is a reflection of the events in the TT focus aimed at limiting the oxidative destruction of lipids and proteins, reducing the zone of secondary alteration, infiltration of the focus by neutrophils and lymphocytes, accelerating the burn healing. MT receptors are found in skin fibroblasts, keratinocytes, cells of hair follicle, eccrine glands, skin vessels,

melanocytes [7]. *In vitro*, MT has a protective effect on skin cells damaged by ultraviolet radiation due to the regulation of redox status and bioenergetic homeostasis, activation of DNA repair, NRF2-dependent pathways [10]. The MT anti-inflammatory effect is associated with limiting restriction of NF-kB-dependent pathways and the expression of pro-inflammatory factors (TNFα, COX-2, iNOS, etc.) [10].

## Conclusions

Primary and secondary skin alteration in the lesion of TT leads to changes in the immunobiological surveillance system aimed at ensuring the inflammatory process in the lesion. During experimental TT, we recorded on the 5th, 10th, 20th day of the experiment, the number of monocytes, basophils, neutrophils in the circulating blood increases, the number of lymphocytes decreases, the content of CRP, TNFα, IL-4 increases in the serum, the content of IFNγ and melatonin does not change.

Intraperitoneal use of MT in TT partially restores the number of lymphocytes in the blood, the concentration of CRP, TNFα. A decrease in serum concentrations of TNFα and CRP in TT under the conditions of MT use suggests a limitation of the acute phase response as a consequence of the MT anti-inflammatory, antioxidant effect, which can accelerate healing and reduce the area of the lesion of TT.

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*Поступила 11.04.2023*
*Принята к печати 13.04.2023*

*Received 11.04.2023*
*Accepted 13.04.2023*

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**ИЛЛЮСТРАЦИИ К СТАТЬЕ «ТУЧНЫЕ КЛЕТКИ ТИМУСА КАК КОМПОНЕНТ НЕЙРО-ЭНДОКРИННО-ИММУННЫХ ВЗАИМОДЕЙСТВИЙ ПРИ СТРЕССЕ» (АВТОРЫ: АРТАШЯН О.С., ХРАМЦОВА Ю.С. [с. 539-544])**

ILLUSTRATIONS FOR THE ARTICLE "THYMUS MAST CELLS AS A COMPONENT OF NEUROENDOCRINE-IMMUNE INTERACTIONS UNDER STRESS" (AUTHORS: ARTASHYAN O.S., KHRAMTSOVA YU.S. [pp. 539-544])

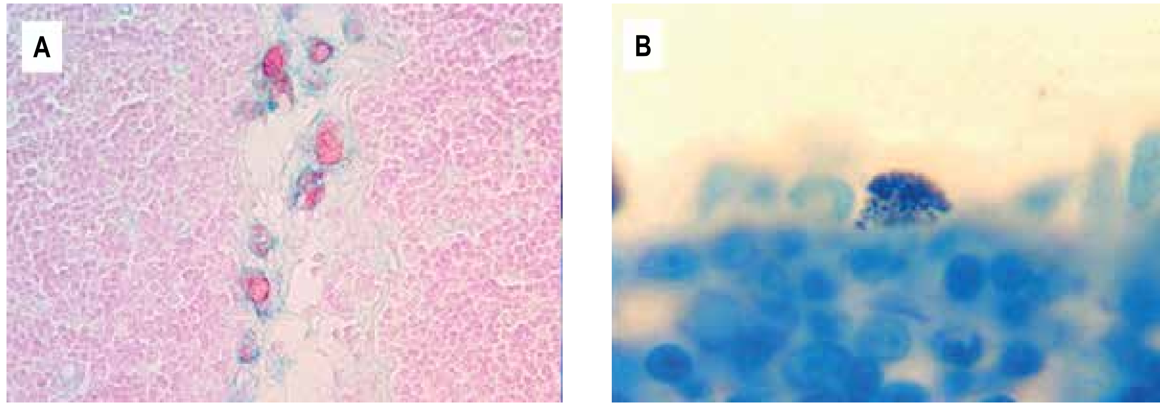


**Figure 1. Mast cells of the thymus in connective tissue: A, blue alcian – safranin, 400×; B, toluidine blue, 5000×**

**ИЛЛЮСТРАЦИИ К СТАТЬЕ «КЛИНИКО-ИММУНОЛОГИЧЕСКИЙ АНАЛИЗ ЭФФЕКТИВНОСТИ ЛОКАЛЬНОГО ПРИМЕНЕНИЯ ВИТАМИНА $D_3$ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ КОЛИТЕ» (АВТОРЫ: БОЙКО М.С., ОСИКОВ М.В., ФЕДОСОВ А.А., ГРЕКОВА И.В. [с. 655-664])**

ILLUSTRATIONS FOR THE ARTICLE "CLINICAL AND IMMUNOLOGICAL ANALYSIS OF THE EFFECTIVENESS OF LOCAL APPLICATION OF VITAMIN $D_3$ IN EXPERIMENTAL COLITIS" (AUTHORS: BOYKO M.S., OSIKOV M.V., FEDOSOV A.A., GREKOVA I.V. [pp. 655-664])

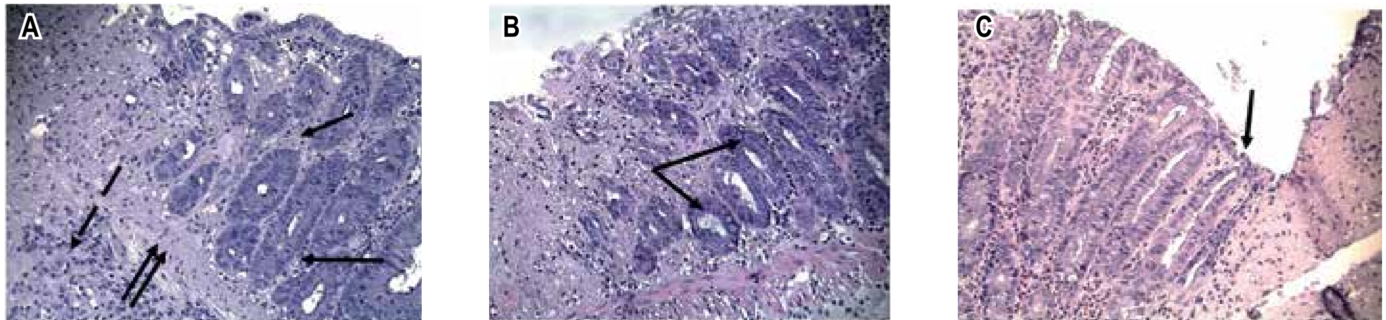


**Figure 1. Morphological changes in the area of colon injury on day 2 of EC**

Note. Hematoxylin-eosin stain, 200× magnification. (A) Group II; neutrophil-lymphocytic infiltration of the interstitial tissue (arrows), thickening of the own plate of the mucosa (double arrow), infiltration of the submucosal layer (dotted arrow). (B) Group III; shortened crypts. (C) Group IV; proliferation of the integumentary columnar epithelium.

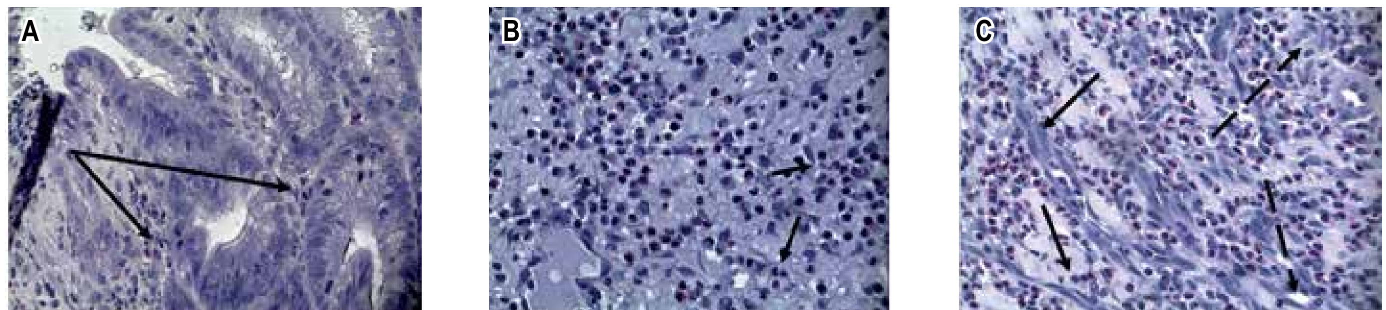


**Figure 2. Morphological changes in the area of colon injury on day 4 of EC**

Note. Hematoxylin-eosin stain, 400× magnification. (A) Group II; neutrophil – lymphocytic infiltration of the stroma with a large number of eosinophils. (B) Group III; proliferating fibroblasts. (C) Group IV; formation of a pseudopolype.

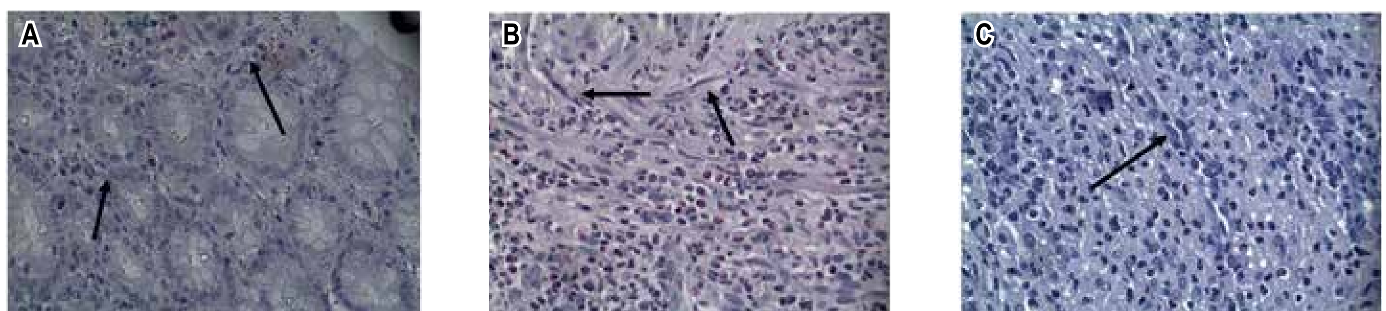


**Figure 3. Morphological changes in the area of colon injury on day 6 of EC**

Note. Hematoxylin-eosin stain, 400× magnification. (A) Group II; neutrophil infiltration of connective tissue layers. (B) Group III; fibroblast proliferation and fibrillogenesis. (C) Group IV; newly formed capillary.

**ПОДПИСНОЙ ИНДЕКС:**
УРАЛ-ПРЕСС – 42311

ISSN 1563-0625
9 771563 062002 >