ORIGINAL INVESTIGATIONS

Predicting the efficacy of tofacitinib therapy based on gene expression of proinflammatory cytokines and proteases in cultured blood cells of patients with rheumatoid arthritis

Markova G.A., Chetina E.V., Satybaldyev A.M.

V.A. Nasonova Research Institute of Rheumatology, Moscow
34A, Kashirskoe Shosse, Moscow 115522, Russia

The effectiveness of personalized therapy for rheumatoid arthritis (RA) is associated with the correct choice of the drug and the ability to predict its effect before starting the treatment.

Objective: *to study in patients with RA the relationship between results of therapy and initial expression of genes responsible for bone and articular cartilage resorption (matrix metalloproteinase 9 – MMP9, – cathepsin K) and inflammation (tumor necrosis factor α – TNFα – and interleukin 1β – IL1β) in mononuclear cells of peripheral blood (PBMC), cultured with tofacitinib (TOFA).*

Patients and methods. *We examined 12 patients with RA who had not previously received TOFA. The average age of the patients was 51 years, the average duration of the disease was 37.6 months. After 3 months of TOFA therapy, 6 patients achieved remission, while the rest had high and moderate disease activity.*

PBMC were isolated before therapy using a Ficoll density gradient and cultured in the presence of 100 nM TOFA for 48 h. Total RNA obtained from these cells was used to analyze the expression of MMP9, cathepsin K, IL1β, and TNFα genes using a real-time quantitative reverse transcription polymerase chain reaction.

Results and discussion. *TOFA is able to modify gene expression in cultured PBMC from RA patients compared to control cells. The initial expression of all the studied genes was significantly increased in cultured with TOFA cells of patients with persistent high and moderate disease activity during therapy, while TNFα gene expression was significantly reduced in patients who achieved remission.*

Conclusion. *In patients with RA who have not previously received TOFA, a decrease in TNFα gene expression in blood cells cultured with this drug before the start of therapy may be a prognostic biomarker for achieving remission during TOFA therapy.*

Keywords: *rheumatoid arthritis; tofacitinib; gene expression; cultured peripheral blood mononuclear cells; predictive biomarkers; tumor necrosis factor-α.*

Contact: *Elena Vasilievna Chetina;* ***etchetina@mail.ru***

For reference: *Markova GA, Chetina EV, Satybaldyev AM. Predicting the efficacy of tofacitinib therapy based on gene expression of proinflammatory cytokines and proteases in cultured blood cells of patients with rheumatoid arthritis. Sovremennaya Revmatologiya=Modern Rheumatology Journal. 2022;16(5):22–27.* ***DOI:*** *10.14412/1996-7012-2022-5-22-27*

Rheumatoid arthritis (RA) is the most common autoimmune rheumatic disease, which is a systemic multifactorial inflammatory process affecting the joints [1, 2]. The synovial membrane plays an important role in the pathogenesis of RA, since activated fibroblast-like synoviocytes produce prostanoids, cytokines, chemokines, matrix-degrading enzymes, angiogenic factors, and adhesion molecules that are involved in tissue activation [3]. In addition, synovial tissue is invaded by macrophages and activated lymphocytes. T-lymphocytes are capable of producing pro-inflammatory cytokines, predominantly of the tumor necrosis factor (TNF) and interleukin (IL) superfamilies, as well as growth factors. B-lymphocytes are associated with the production of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) [1]. Subsequently, the penetration of degrading enzymes, primarily matrix metalloproteinases (MMPs) and cathepsins activated by pro-inflammatory cytokines, into articular cartilage and bone tissue leads to the destruction of these tissues [4]. To prevent structural damage, to suppress disease activity, inflammation and pain in RA, non-steroidal anti-inflammatory drugs and genetically engineered biological drugs (GEBDs) are used. However, not all patients respond equally to treatment, and clinical remission is observed only in 30–60% of cases [5]. Therefore, predicting an individual's response to therapy before its onset is of great importance.

Tofacitinib (TOFA) is a Janus kinase (JAK) 1/3 inhibitor that inhibits the activity of intracellular tyrosine kinases [6]. It has been shown that TOFA is significantly more effective than methotrexate in the treatment of naive patients or patients with an inadequate response to GEBD [7]. However, some adverse events that may develop with the use of this drug (infections or thromboembolic disorders) limit its use in a number of patients [8]. In this regard, it seems important to predict the response to TOFA therapy even before its initiation.

Since peripheral blood is the most accessible biomaterial, and gene expression is the earliest cellular response of the body to environmental changes [9], gene expression analysis of peripheral blood mononuclear cells (PBMCs) can be used to assess the patient's response to therapy. Data from our previously published study showed that the achievement of clinical remission in patients with RA treated with TOFA was associated with lower baseline expression of genes involved in energy production (pyruvate kinase and succinate dehydrogenase) compared with the rest of the patients, while the

lack of response to TOFA was accompanied by high basal expression of these genes [10].

Another approach to predict response to therapy may be to evaluate the response of the patient's cultured blood cells in vitro. Since TOFA inhibits the signalling pathways of pro-inflammatory cytokines, which are further involved in the production of MMPs and the activation of osteoclasts involved in the destruction of cartilage and bone [11], it was suggested that in patients with RA, analysis of gene expression in blood cells cultured with TOFA before therapy may be useful for personalized prediction of treatment efficacy.

Patients and methods. The study included 12 patients with RA who met the 2010 ACR (American College of Rheumatology) criteria and had not previously received TOFA. The average age of the patients was 51.0±14.2 years, the average duration of the disease was 37.6 months (from 4 to 180 months). Initially, 11 patients had stage II according to Steinbroker, 1 patient had stage III. The majority of patients (91.7%) were seropositive for ACPA, and 58.3% were seropositive for RF. Initially, 7 patients had high RA activity (DAS28 >5.1), and 5 had moderate activity (3.2<DAS28<5.1).

The indication for the prescription of TOFA was refractoriness to methotrexate (at a dose of 20–25 mg/week), including 3 patients who received it in combination with methylprednisolone (8 mg/day) and 1 patient who received it with hydroxychloroquine (200 mg/day). All patients received TOFA (5–10 mg twice daily) for 3 months of follow-up. After 3 months of TOFA treatment, 6 patients achieved remission, the rest had high (n=3) or moderate (n=3) disease activity. Remission was defined using the ACR criteria for clinical remission based on a simplified 28-joint score (DAS28<2.6).

The study was conducted in accordance with the Declaration of Helsinki. The study protocol (No. 13 dated June 4, 2015) was approved by the local ethics committee, all patients voluntarily signed an informed consent.

At baseline and after 3 months, RA activity was assessed using DAS28-ESR. Nephelometric analysis was performed on a BN-100 analyzer (Dade Bering, Germany) to determine the concentrations of serum CRP (threshold value, 5 mg/l) and IgM RF (standard threshold value, 15 mU/l). ACPA was measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's recommendations (cut-off level 5 U/mL for positive antibodies, Axis Shield Diagnostics Limited, UK).

Fractionation of blood cells. Peripheral blood (10 ml) was collected in Vacutainer tubes containing EDTA (BDH, UK). Blood samples were taken in a standardized manner in the morning (between 07:00 and 09:00). Whole blood fractionation was performed using a Ficoll density gradient. PBMCs were collected and washed twice with phosphate-buffered saline.

Cell viability test. The viability of blood cells in RA patients was assessed by staining with 0.2% trypan blue 48 h after TOFA treatment. Cell viability was considered satisfactory if the same number of such cells was observed in the control (cells cultured without TOFA) or in PBMCs cultured in the presence of TOFA. Both TOFA concentrations tested (100 or 500 nM) were non-toxic as they did not affect cell viability compared to untreated samples. In all experiments, 100 nM of TOFA was used.

Cultivation of blood cells. 10^6 cells/mL were seeded into a 96-well plate and then cultured in RPMI-1640 medium (Gibco, Life Technologies, Inc., UK) containing 25 mmol/L of HEPES buffer, pH 7.4, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FBS, Gibco, Life Technologies, Inc., UK), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 150 μg/mL of gentamicin sulfate at 37°C in the presence of 5% CO_2 for 48 h. After changing the nutrient medium, cells were starved for 24 h in the same medium without serum, and then the medium was replaced with fresh medium without serum, which contained either 100 nM of TOFA citrate (Sigma-Aldrich) or the same medium without TOFA (control).

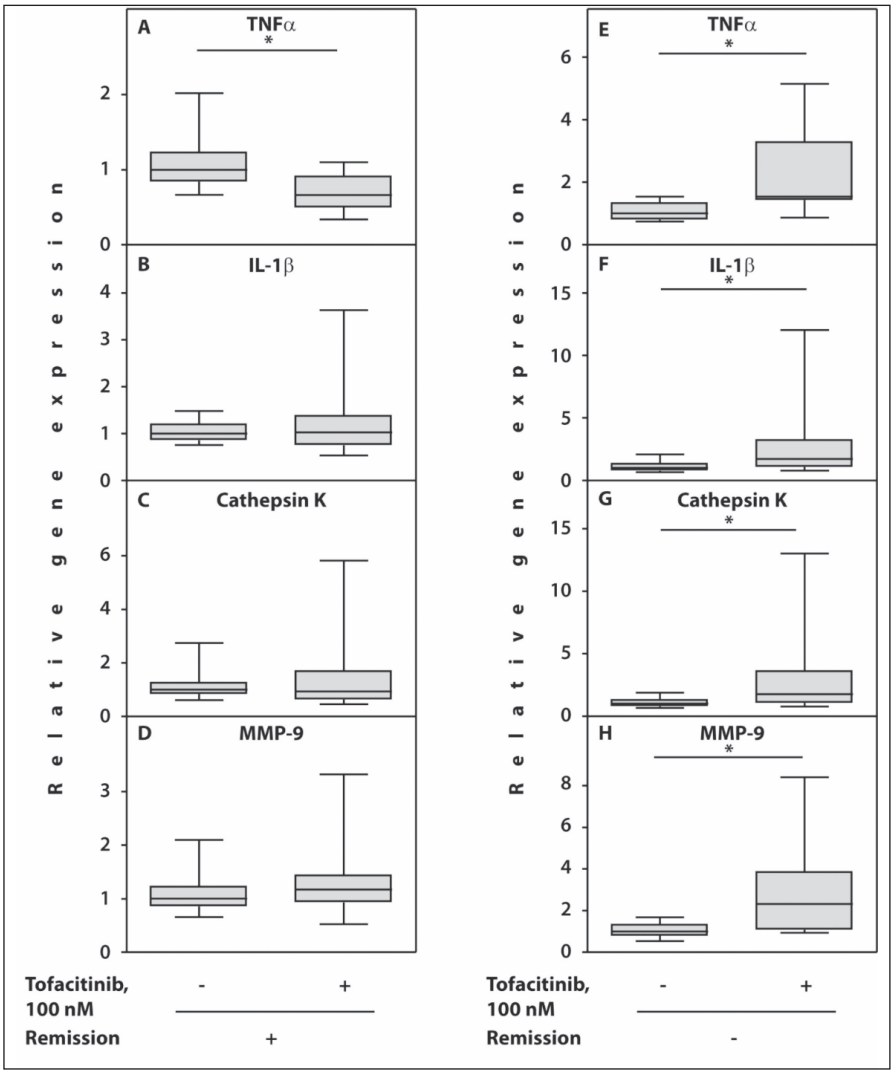

Fig. 1. *Relative gene expression in cells of RA patients cultured with TOFA for 48 hours before the start of therapy: a, b, c, d – patients who achieved remission (n=6); e, f, g, h – patients who did not achieve remission (n=6). * – significant differences with control cells cultured without TOFA (Wilcoxon test)*

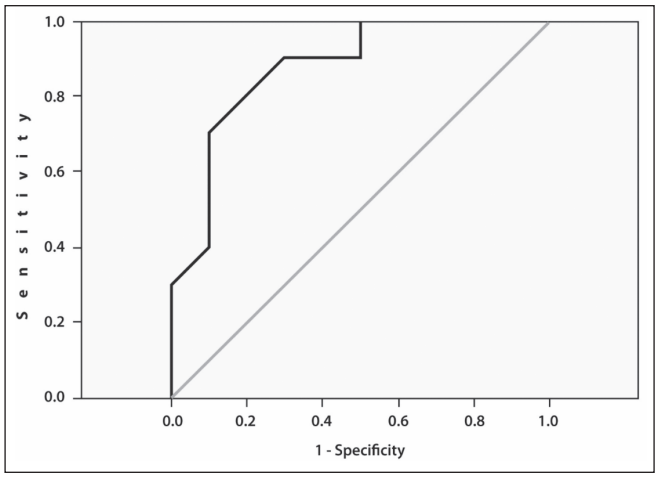

Fig. 2. *Area under the curve (AUC), demonstrating the relationship between the initial level of TNFα gene expression in blood cells of RA patients cultured with TOFA for 48 hours before the start of therapy, with the likelihood of remission during TOFA therapy*

Table. Clinical and laboratory characteristics of patients before and after TOFA therapy, Me [25th; 75th percentile]

Characteristic	Group 1 (n=6)		p	Group2 (n=6)		p	p Prior to therapy
	Prior to therapy (n=6)	After 3 mo of therapy (n=6)		Prior to therapy (n=6)	After 3 mo of therapy (n=6)		
Age, years#	46.5 [29.5; 59.5]			60 [41.5; 69]			0.18
Disease duration, mo#	30 [16.5; 108]			21 [6; 39]			0.42
CRP, mg/mL	18.6 [2.4; 98]	3.9 [0.25; 8.6]	0.06	17.5 [8.7; 83.2]	5.2 [1; 28.5]	0.31	0.93
ESR, mm	30 [14; 74.5]	13 [8; 18]	0.12	35.5 [25.5; 40]	48.0 [12; 75]	0.43	0.69
DAS28	4.85 [3.6; 6.2]	1.95 [1.3; 2.2]	0.03*	6.2 [5.3; 7.0]	4.8 [3.9; 5.4]	0.03*	0.09
ΔDAS28#		2.77 [1.9; 4.2]			1.4 [0.9; 2.1]	0.01*	
NSJ	5.5 [3; 11.5]	0 [0; 0.5]	0.03*	12.5 [7; 17]	2 [1; 8.5]	0.03*	0.06
NPJ	4 [2.5; 10.5]	0 [0; 0]	–	13.5 [7; 20.5]	6.5 [1.5; 15.5]	0.06	0.04#

Note. Significant differences between groups of patients: * – according to the Wilcoxon test, # – according to the Mann–Whitney U-test.

After 1 hour of incubation, 10% (v/v) FBS was added to each well and cells were incubated for another 48 hours. Cells were harvested and total RNA was isolated immediately. All experiments were carried out in triplicate.

Isolation of total RNA and reverse transcriptase reaction (RT). Total RNA was isolated from each well using the Extract RNA reagent (Evrogen, Russia). The RT reaction was performed using a kit containing M-MLV reverse transcriptase, random hexanucleotide primers and total RNA, according to the manufacturer's recommendations.

Quantitative polymerase chain reaction (PCR) in real time. The following ready-made primers and probes (Applied Biosystems, USA) were used for TaqMan analysis: TNFα (Hs00174128_m1), IL1β (Hs00174097_m1), MMP9 (Hs00234579_m1), and cathepsin K (Hs00987255_m1). β-actin served as endogenous control. Gene expression was quantified using the Quant Studio 5 real-time PCR system (Applied Biosystems, USA) as described previously [12]. For this purpose, 1 μL of the RT product was subjected to real-time PCR in a total reaction mixture (15 μL) containing 7.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 900 nM of sense and antisense primers, 50 nM of probe, and complementary matrix DNA. After a single step at 50°C for 2 min and an initial activation at 95°C for 10 min, the reaction mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min for annealing and elongation at 60°C). Relative gene expression was determined by the ΔΔCT method in accordance with the manufacturer's recommendations (Applied Biosystems, USA). The ΔCT value was calculated by subtracting the CT value for the housekeeping gene from the CT value for each sample. The ΔΔCT value was then calculated by subtracting the ΔCT value of each control from the ΔCT value observed in each TOFA-treated sample.

Statistical analysis. Statistical data processing was carried out using the Statistica software package (version 12.0 StatSoft Inc., USA). Quantitative data are expressed as median and interquartile range (Me [25th; 75th percentile]). The results were statistically processed using the Mann–Whitney and Wilcoxon tests. The value of $p \leq 0.05$ was considered statistically significant.

Results. Between patients who achieved (group 1) and did not achieve (group 2) remission, there were no significant differences in most baseline characteristics (see Table). However, initially, patients of the 2nd group had a greater number of painful joints – NPJ (p=0.04) compared with patients of the 1st group.

After 3 months of observation the number of swollen joints (NSJ) significantly decreased in patients of both groups (p=0.03), but the absence of joint pain was observed only in patients who achieved remission (see Table). All patients responded to treatment (ΔDAS28 >1.2). However,

the value of ΔDAS28 in patients of the 1st group was significantly higher than in the participants of the 2nd group.

Analysis of gene expression in blood cells cultured with TOFA before therapy. Significant activation of the expression of all the studied genes was observed in PBMCs cultured with TOFA, compared with control cells of RA patients (n=6), who retained high or moderate disease activity after 3 months of treatment (Fig. 1, *e, f, g, h*). In particular, in 5 out of 6 patients, an increase in the expression of the MMP9, TNFα and IL1β genes was detected, while an increase in the expression of the cathepsin K gene was found in all patients of the 2nd group.

On the contrary, in all examined patients of the 1st group (n=6) who achieved remission after 3 months of observation, a significant decrease in TNFα gene expression in PBMCs cultured with TOFA was noted compared to control cells (see Fig. 1, a) . The expression of other studied genes varied. For example, the expression of IL1β, MMP9, and cathepsin K genes in cells cultured with TOFA was reduced in 3 out of 6 patients, while an increase in their expression was demonstrated in another 3 patients (see Fig. 1, *b, c, d*) .

To assess the prognostic value of TNFα gene expression, ROC analysis was performed (Fig. 2), which confirmed the presence of significant associations of this gene expression with the likelihood of remission during TOFA therapy. Threshold values for TNFα gene expression were 1.17: area under the curve (AUC) = 0.875; 95% CI (0.718–1.000); p=0.005.

Discussion. The number of available GEBDs and targeted synthetic basic anti-inflammatory drugs is constantly growing [13]. Clinical studies show that different drugs are not equally effective in the treatment of RA, so it is necessary to distinguish between responders and non-responders [14].

The obtained results demonstrated that the achievement of remission in patients with RA on the background of TOFA treatment is associated with a significant decrease in the expression of the TNFα gene in PBMCs cultured with this drug before therapy onset. On the contrary, in the PBMCs of patients who did not go into remission after 3 months of using TOFA, a significant increase in the baseline expression of the TNFα, IL1β, MMP9, and cathepsin K genes was revealed. An increase in MMP9 expression in response to inhibition of Janus kinases was previously determined in macrophage cell cultures [15]. Our data are supported by previous animal studies, which showed that TOFA blocks TNFα production in cultured blood cells [16]. At the same time, our results explain the absence of differences in TNFα expression in RA patients who responded or did not respond to therapy, which was observed in studies with T-lymphocytes cultured with TOFA [17], since TNFα gene expression was suppressed only in patients who achieved remission. From the point of view of molecular mechanisms, it can be assumed that in blood cells TOFA preferentially inhibits signaling from cytokines, which is regulated by the JAK1/3-STAT signaling pathways, although TNFα does not directly signal through the JAK-STAT pathway [18]. However, it was previously demonstrated that TOFA can interfere with the response of cells to TNFα by suppressing the autocrine loop TNF-interferon β -JAK-STAT1 [19].

Conclusion. We have proposed an original approach to predicting the efficacy of therapy in RA patients by assessing changes in gene expression in blood cells cultured in vitro in the presence of a drug prior to treatment. In particular, a significant decrease in the expression of the TNFα gene in blood cells cultured with TOFA for 48 hours before the start of therapy can probably serve as a prognostic biomarker for achieving remission of the disease, while a significant increase in the expression of the TNFα, IL1β, MMP9, and cathepsin K genes will indicate a less pronounced response to the drug.

REFERENCES

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med*. 2011 Dec 8;365(23):2205-19. doi: 10.1056/NEJMra1004965.
2. Komatsu N, Takayanagi H. Inflammation and bone destruction in arthritis: synergistic activity of immune and mesenchymal cells in joints. *Front Immunol*. 2012 Apr 13;3:77. doi: 10.3389/fimmu.2012.00077. eCollection 2012.
3. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunol Rev*. 2010 Jan;233(1):233-55. doi: 10.1111/j.0105-2896.2009.00859.x.
4. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. *Lancet*. 2016 Oct 22;388(10055):2023-38. doi: 10.1016/S0140-6736(16)30173-8. Epub 2016 May 3.
5. Atzeni F, Sarzi-Puttini P, Gorla R, et al. Switching rheumatoid arthritis treatments: an update. *Autoimmun Rev*. 2011 May;10(7):397-403. doi: 10.1016/j.autrev.2011.01.001. Epub 2011 Jan 22.
6. Winthrop KL. The emerging safety profile of JAK inhibitors in rheumatic disease. *Nat Rev Rheumatol*. 2017 May;13(5):320. doi: 10.1038/nrrheum.2017.51. Epub 2017 Mar 31.
7. Strand V, Kremer JM, Gruben D, et al. Tofacitinib in combination with conventional disease-modifying antirheumatic drugs in patients with active rheumatoid arthritis: Patient-reported outcomes from a phase III randomized controlled trial. *Arthritis Care Res (Hoboken)*. 2017 Apr;69(4):592-8. doi: 10.1002/acr.23004.
8. Gadina M, Le MT, Schwartz DM, et al. Janus kinases to Jakinibs: from basic insights to clinical practice. *Rheumatology (Oxford)*. 2019 Feb 1;58(Suppl 1):i4-i16. doi: 10.1093/rheumatology/key432.
9. Tsuchiya H, Fujio K. The current status of the search for biomarkers for optimal therapeutic drug selection for patients with rheumatoid arthritis. *Int J Mol Sci*. 2021 Sep 2;22(17):9534. doi: 10.3390/ijms22179534.
10. Tchetina EV, Satybaldyev AM, Markova GA, et al. Putative association between low baseline gene expression in the peripheral blood and clinical remission in rheumatoid arthritis patients treated with tofacitinib. *Life (Basel)*. 2021 Dec 11;11(12):1385. doi: 10.3390/life11121385.
11. Furst DE, Emery P. Rheumatoid arthritis pathophysiology: update on emerging cytokine and cytokine-associated cell targets. *Rheumatology (Oxford)*. 2014 Sep;53(9):1560-9. doi: 10.1093/rheumatology/ket414. Epub 2014 Jan 8.
12. Tchetina EV, Poole AR, Zaitseva EM, et al. Differences in mammalian target of rapamycin gene expression in the peripheral blood and articular cartilages of osteoarthritic patients and disease activity. *Arthritis*. 2013;2013:461486. doi: 10.1155/2013/461486. Epub 2013 Jun 25.
13. Scherer HU, Dorner T, Burmester GR. Patient-tailored therapy in rheumatoid arthritis: an editorial review. *Curr Opin Rheumatol*. 2010 May;22(3):237-45. doi: 10.1097/BOR.0b013e328337b832.

14. Verweij CL. Transcript profiling towards personalised medicine in rheumatoid arthritis. *Neth J Med*. 2009 Dec;67(11):364-71.
15. Kothari P, Pestana R, Mesraoua R, et al. IL-6-mediated induction of matrix metalloproteinase-9 is modulated by JAK-dependent IL-10 expression in macrophages. *J Immunol*. 2014 Jan 1;192(1):349-57. doi: 10.4049/jimmunol.1301906. Epub 2013 Nov 27.
16. Ghoreschi K, Jessson MI, Li X, et al. Modulation of innate and adaptive immune response by tofacitinib (CP-690,550). *J Immunol*. 2011 Apr 1;186(7):4234-43. doi: 10.4049/jimmunol.1003668. Epub 2011 Mar 7.
17. Isailovic N, Ceribelli A, Cincinelli G, et al. Lymphocyte modulation by tofacitinib in patients with rheumatoid arthritis. *Clin Exp Immunol*. 2021 Aug;205(2):142-9. doi: 10.1111/cei.13609. Epub 2021 May 28.
18. Meyer DM, Jesson MI, Li X, et al. Anti-inflammatory activity and neutrophil reductions mediated by the JAK1/JAK3 inhibitor, CP-690,550, in rat adjuvant-induced arthritis. *J Inflamm (Lond)*. 2010 Aug 11;7:41. doi: 10.1186/1476-9255-7-41.
19. Yarilina A, Xu K, Chan C, Ivashkiv LB. Regulation of inflammatory responses in tumor necrosis factor-activated and rheumatoid arthritis synovial macrophages by JAK inhibitors. *Arthritis Rheum*. 2012 Dec;64(12):3856-66. doi: 10.1002/art.37691.

Received/Reviewed/Accepted
18.04.2022/20.06.2022/25.06.2022

Conflict of Interest Statement

The work was carried out with the financial support of the Ministry of Science and Higher Education of Russia (Project № 1021062512064-0).

There are no conflicts of interest. The authors are solely responsible for submitting the final version of the manuscript for publication. All the authors have participated in developing the concept of the article and in writing the manuscript. The final version of the manuscript has been approved by all the authors.

Markova G.A. https://orcid.org/0000-0001-5946-5695
Chetina E.V. https://orcid.org/0000-0001-7312-2349
Satybaldyev A.M. https://orcid.org/0000-0002-1508-0854