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BIOMARKERS FOR MODELING OF CANCER-SPECIFIC TUMOR-ASSOCIATED MACROPHAGES *EX VIVO*

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Abstract

Introduction. Tumor-associated macrophages (TAMs) are essential innate immune cells in the tumor microenvironment. TAMs can stimulate cancer cell proliferation and primary tumor growth, angiogenesis, lymphangiogenesis, cancer cell invasiveness in vessels and metastatic niche formation as well as support chemotherapy resistance. TAMs are phenotypically diverse both in various cancer localizations and in intratumoral heterogeneous compartments. Tumor-specific modeling of TAMs is necessary to understand the fundamental mechanism of pro- and anti-tumor activity, to test their interaction with existing therapies, and to develop TAM- targeted immunotherapy. **Aim of study:** To investigate cancer-specific transcriptomic features of *ex vivo* human TAM models. **Material and Methods.** Here we compared transcriptomic profiles of TAMs for breast, colorectal, ovarian, lung, and prostate cancers *ex vivo*. Human monocytes were isolated from buffy coats, and then stimulated by the tumor cell conditioned medium *ex vivo*. Using real-time PCR, we quantified the expression of key TAM biomarkers including inflammatory cytokines, scavenger-receptors, angiogenesis-regulating genes, and matrix remodeling factors. **Results.** PCR analysis allowed revealing cancer-specific expression profiles of modeled TAMs. By comparing the existing knowledge about TAM phenotypes in human tumors *in vivo* with the collected data, we discuss the advantages and limitation of *ex vivo* TAM models derived from human blood monocytes. **Conclusion.** Monocytes-derived macrophages stimulated with cancer cell-conditioned medium can, to a certain extent, allow modeling of cancer-specific programming of TAMs. Our model system is valuable to examine agents reprogramming key TAM pro-tumoral activities, and for the reproducible analysis of mechanistic events that program tolerogenic status of TAMs towards cancer cells.

Key words: tumor-associated macrophages, M1/M2 classification, cytokines, scavenger-receptors.

БИОМАРКЕРЫ МОДЕЛЬНЫХ ОПУХОЛЕАССОЦИИРОВАННЫХ МАКРОФАГОВ *EX VIVO*

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Аннотация

Введение. Опухولةассоциированные макрофаги (ОАМ) являются важными клетками врожденного иммунитета в микроокружении опухоли. ОАМ могут стимулировать пролиферацию опухолевых клеток и рост первичной опухоли, ангиогенез, лимфангиогенез, инвазию опухолевых клеток и образование метастатических ниш, а также влиять на чувствительность опухоли к химиотерапии. ОАМ – фенотипически разнообразная популяция клеток, и их гетерогенность наблюдается в зависимости как от локализации рака, так и от их внутриопухолевого расположения. Моделирование ОАМ в системах *ex vivo* необходимо для понимания молекулярных механизмов их про- и противоопухолевой активности, тестирования их взаимодействия с существующими противоопухолевыми препаратами или для разработки ОАМ-таргетной иммунотерапии.

Цель исследования – изучить специфичные для рака транскриптомные особенности модельных ОАМ человека *ex vivo*. **Материал и методы.** Проведено сравнение транскриптомных профилей ОАМ рака молочной железы, колоректального рака, рака яичников, рака легких и рака простаты *ex vivo*. Моноциты человека выделяли из лейкоцитарной пленки, а затем стимулировали средой, кондиционированной опухолевыми клетками. Используя ПЦР в режиме реального времени, количественно оценивалась экспрессия ключевых биомаркеров ОАМ, включая воспалительные цитокины, скавенджер-рецепторы, регуляторы ангиогенеза и факторы ремоделирования внеклеточного матрикса. **Результаты.** ПЦР-анализ позволил выявить специфичные для рака профили экспрессии модельных ОАМ. При сравнении накопленных сведений о фенотипах ОАМ в опухолях человека *in vivo* с собранными данными были обсуждены преимущества и ограничения нашей модели ОАМ *ex vivo*, полученной из моноцитов крови человека. **Выводы.** *Ex vivo* система макрофагов, полученных из моноцитов, стимулированных средой, кондиционированной раковыми клетками, может в определенной степени позволить моделировать специфическое для разных опухолей программирование ОАМ. Наша модельная система может быть полезна для изучения механизмов действия таргетных агентов, перепрограммирующих ключевые проопухолевые активности ОАМ.

Ключевые слова: опухолеассоциированные макрофаги, M1/M2 макрофаги, цитокины, скавенджер-рецепторы.

Introduction

Macrophages are essential components of the innate immune system playing crucial role in inflammation and cancer [1]. Macrophages are generally divided into two polarization states: classically activated pro-inflammatory macrophages 1 (M1) and alternatively activated anti-inflammatory macrophages 2 (M2) [2, 3]. In tumor microenvironment (TME), M1 macrophages are classified as antitumor and are able to recognize and kill cancer cells, while M2 macrophages promote tumor growth [3]. M1 and M2 polarization is triggered by diverse stimuli from the microenvironment, including growth factors, cytokines, chemokines, components of extracellular matrix (ECM), and other molecules [4, 5]. M1 macrophages express nitric oxide, reactive oxygen species, number of pro-inflammatory factors (IL-1 β , IL-6, IL-12, IL-23, CXCL9, CXCL10, CXCL11, and TNF- α), and surface

receptors including molecules of the main histocompatibility complex [MHC], CD68, CD80, CD86, and MARCO. Oppositely, M2 macrophages abundantly express anti-inflammatory factors, including IL-10, TGF- β , CCL14, CCL17, CCL18, CCL22, CCL24, arginase-1, CD206, CD204, and CD163 [4, 5].

Tumor-associated macrophages (TAMs) stimulate angiogenesis, metastasis, tumor cell immunity evasion, tumor cell proliferation and tumor growth, which has been shown in numerous *in vitro* and *in vivo* studies [1, 3, 6]. The predominance of M2 macrophages in the TME correlates with poor prognosis in lung cancer [7, 8], colorectal cancer [9], breast cancer [6], ovarian cancer [10], and other cancers. In the TME, M2 macrophages contribute to the survival of cancer cells and reduce their apoptosis [11], while M1 macrophages, on the contrary, promote apoptosis of cancer cells [8].

Many studies performed using cancer patient material demonstrate more complex diversity of TAMs inside the tumor that doesn't conform to generally accepted M1/M2 *in vitro* classification [2, 12]. Moreover, state-of-the-art technologies of single cell and spatial analysis open the perspectives to distinguish novel subpopulations of TAMs in different cancers [13, 14]. Despite the fact that *in vitro* studies of TAM activities have been performed for many years, recent advances allowed improvement and modify such models.

In our study, we generated TAMs for breast, colorectal, ovarian, lung, and prostate cancers using CD14+ human monocytes by stimulating them with cancer cell conditioned medium. We analyzed major TAM biomarkers, indicating pro-tumor activity, widely used for the analysis *in vitro* and *in vivo*. The advances and limitations of our models were demonstrated and discussed.

Aim of study: To investigate cancer-specific transcriptomic features of *ex vivo* human TAM models.

Material and Methods

Cell cultures

Tumor cell lines were obtained from the bioresource collection of vertebrate cell cultures of the Institute of Sciences of the Russian Academy of Sciences (St. Petersburg, Russia) and from the collection of The Global Bioresource Center, the American Type Culture Collection (ATCC, USA).

The following tumor cell lines were used for the study: MCF7 (breast adenocarcinoma, subtype LumA), BT474 (breast adenocarcinoma, subtype LumB), MDA-MB-231 (breast adenocarcinoma, triple-negative cancer), SKBR3 (breast adenocarcinoma, expressing human epidermal growth factor receptor 2 (HER2) cancer), SW837 (rectal adenocarcinoma), SKOV3 (ovarian serous cystadenocarcinoma), PC3 (prostate adenocarcinoma), and A549 (lung adenocarcinoma) [15]. Each cell line was cultured in recommended growth medium supplemented with 1 % antibiotic penicillin/streptomycin (PanEco, Russia) and 10 % fetal bovine serum (FBS) (Capricorn, Germany). Before use, the FBS was inactivated at 56 °C for 30 min. MCF7, MDA-MB-231 and A549 cell lines were grown in DMEM medium (Gibco, USA); BT474, SKBR3, SKOV3, and PC3 cell lines were grown in RPMI1640 medium (Gibco, USA); SW837 cell line was cultivated in Leibovitz's medium (Gibco, USA). Conditioned cancer cell supernatants were collected and filtered through sterile syringe filters with low protein binding ability having a 0.22 µm pore size (Millipore, USA). Filtered tumor cell line conditioned media were frozen at -80 °C until the stimulation of freshly isolated monocytes.

Monocyte isolation

Human monocytes were obtained from the Siberian Federal Scientific and Clinical Center of the Federal Medical and Biological Agency (Seversk, Russia). Monocytes were isolated from the buffy coats of ten healthy donors using magnetic sorting (MACS)

with CD14-positive selection. The buffy coats were diluted with PBS free of Ca²⁺ and Mg²⁺ (Gibco, ThermoFisher) at a ratio of 1:1. The resulting suspension (30 ml) was layered on 15 ml ficoll density 1.077 (PanEco, Russia). The mononuclear fraction after centrifugation was collected from the ficoll/serum interphase, transferred to fresh tubes, and washed twice with PBS. The washed cells were resuspended in 5 ml PBS and applied to a percoll-based continuous gradient consisting of 13.5 ml percoll (Sigma, USA), 1.5 ml 10x Earl's minimum basic medium (Sigma, USA) and 15 ml Spinner's medium (Sigma, USA). After centrifugation, the fraction in the upper part of the gradient enriched in monocytes up to 60–80 % was collected and washed 3 times with PBS. The monocyte-enriched fraction was used for magnetic sorting of cells using human CD14 MicroBeads (Miltenyi Biotech, USA). The purity was 95–98 % that was monitored by analysis of the surface expression of the monocyte marker CD14 by flow cytometry (CytoFLEX, Beckman Coulter, USA).

Modeled system

for tumor-associated macrophages

A model system for tumor-associated macrophages (TAMs) was obtained by the stimulation of primary human monocytes with cytokines (M-CSF 10 ng/µl [RP01221, ABclonal, China] and IL-4 10 ng/µl [RP00995, ABclonal, China]), and Dexamethasone 10⁻⁸M (D4902, Sigma, USA) and cancer cell conditioned media as has been described in our recent study [16]. Monocytes were cultivated at a concentration of 1x10⁶ cells/ml. Tumor cell supernatants were added to freshly isolated monocytes in an amount of 20 % of the total volume of the medium. As a control, monocytes were incubated with 20 % of empty cancer cell culture medium (DMEM, Leibovitz's or RPMI1640). Cells were cultured for 6 days at 37°C. After 6 days of cell incubation, the medium was removed, the plates with cells were placed on ice, and lysis buffer was added. Cell lysates were used for RNA isolation and further cDNA synthesis.

Quantitative real time PCR

RNA isolation from TAM samples was carried out according to the standard method using RNeasy Mini Kit (#74004, Qiagen, USA). cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (#K1621, Thermo Fisher Scientific, USA) with random hexanucleotide primers in accordance with the instructions for the kit. Gene expression was measured using Taqman technology and normalized to housekeeping gene expression for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 2^{-ΔΔCT} algorithm was used to calculate the expression of genes of interest. First, the difference in threshold cycle between the target and reference genes was calculated:

$$\Delta CT = CT(\text{a target gene}) - CT(\text{a reference gene}).$$

Second, the difference between the target and reference samples was calculated:

$$\Delta\Delta CT = \Delta CT(\text{a target sample}) - \Delta CT(\text{a reference sample}).$$

Finally, to work out the gene expression we did 2 to the power of negative $\Delta\Delta Ct$:

$$\text{Fold gene expression} = 2^{-(\Delta\Delta Ct)}.$$

Primers were designed using Vector NTI Advance 11.5.4 (Vector NTI, RRID:SCR_014265) and NCBI database. The primers were synthesized by DNA Synthesis (Moscow, Russia). qRT-PCR was performed using AriaMx Real-Time PCR thermocycler (RRID:SCR_019469, Agilent Technologies, USA).

The expression of genes that functionally refer to the pro- and antitumor TAM polarization was analyzed: *IL1 β* , *CXCL8*, *IL6*, *MRC1*, *CD163*, *MARCO*, *CXCL10*, *CCL2*, *MMP9*, *SPP1*, *SPARC*, *S100A4*, *IL10*, *Stab1*, *CHID1*, *CHI3L2*, *CHI3L1*, *CCL18*, *VEGFA*, *TGF β* , *SOC3*, *CCL2*, and *GATA3* (Table 1).

Statistical analysis

Statistical analysis was performed using Statistica 8.0 for Windows and GraphPad Prism 8.4.2. Shapiro–Wilk test was used for normality. The Mann–Whitney test for independent groups was implemented. Results of real-time PCR were presented using GraphPad Prism 8.4.2 software. Results were considered to be significant with $p < 0.05$.

Results

The major aim of the present study was to find significant differences in the phenotype of TAMs activated by the stimuli from the conditioned medium (CM) of different cancer cell lines: breast cancer (luminal A, luminal B, triple-negative breast cancer

Table 1/Таблица 1

Sequences of primers with original design Последовательности праймеров оригинального дизайна

Gene/ Ген	Sequence/Последовательность	Gene/ Ген	Sequence/Последовательность
<i>GAPDH</i>	F 5'- GCCAGCCGAGCCACATC-3'	<i>IL6</i>	F 5'- CTGGATTCAATGAGGAGACTTG-3'
	R 5'- GGCAACAATATCCACTTTACCAGA-3'		R 5'- GCTTGTCTCTCACTACTCTCAAA-3'
	FAM-CGCCCAATACGACCAATCCG-BHQ1		FAM-TCATCACTGGTCTTTTGAGTTTGA-BHQ1
<i>CCL2</i>	F 5'- CTCGCTCAGCCAGATGC-3'	<i>IL10</i>	F 5'- GCTGGAGGACTTTAAGGGTTA-3'
	R 5'- ACTTGCTGCTGGTGATTCTTC-3'		R 5'- TGTCTGGGTCTTGGTTCTCA-3'
	FAM-CAATGCCCCAGTCACCTGCTGT-BHQ1		FAM-AGCCTTGTCTGAGATGATCCAGTTT-BHQ1
<i>CCL18</i>	F 5'-CCAAGCCAGGTGTCATCCT-3'	<i>MARCO</i>	F 5'- CAGGGAGTGAAAGGATCTTCT-3'
	R 5'-CTTCAGGTCGCTGATGTATTCT-3'		R 5'- GGACACTGAGTTTTCACCTCTT-3'
	FAM-CCACTTCTATTGGGGTCAGCACAGATC-BHQ1		FAM-AGCAAGGAGTAAAGGGAGAAAAAGG-BHQ1
<i>CCRL2</i>	F 5'- GGCACCAGAGGATGAATATGATG-3'	<i>MMP9</i>	F 5'- CTTCCAGTACCGAGAGAAAGC-3'
	R 5'- GCGTCATACTTGTACATTGC-3'		R 5'- GCAGGATGTCATAGGTCACG-3'
	FAM-CATAGAAGGTGAAGTGGAGAGCGATGAG-BHQ1		FAM-TTCTGCCAGGACCGCTTCTACTG-BHQ1
<i>CD163</i>	F 5'-TAGTGAGTGTGGGCACAAG-3'	<i>MRC1</i>	F 5'- CTGCGACAGTAAACGAGGCT-3'
	R 5'-CCGACTGCAATAAAGGATGA-3'		R 5'- TGTTGAATCGTTGCTGGAGG-3'
	FAM-CACAACAGGTCGCTCATCCCG-BHQ1		FAM-TGCCAGACACGATCCGACCCTTCC-BHQ1
<i>CHID1</i>	F 5'- CCAGGTGGCAAAGAACCA-3'	<i>SOC3</i>	F 5'- GATTCTACTCTGTGCCTCTGA-3'
	R 5'- TGGGTGAGCATGTGGATGA-3'		R 5'- GCTGAGTATGTGGCTTTCCTATG-3'
	FAM-CGATGGCTTCGTGGTGGAGGT-BHQ1		FAM-AGAGATTCGCCTTAAATGCTCCCTGTCC-BHQ1
<i>CHI3L1</i>	F 5'- CTCAAGAACAGGAACCCCAA-3'	<i>SPP1</i>	F 5'- ACCTGACATCCAGTACCCTGA-3'
	R 5'- GGCTATCTTGGAAAATCTTTGAG-3'		R 5'-ACGGCTGTCCCAATCAGAAG-3'
	FAM-TGTGCTGTGCGAGGATGGAACCT-BHQ1		FAM-TGGAAAGCGAGGAGTTGAATGTCATACA-BHQ1
<i>CHI3L2</i>	F 5'- AACAACAAGGTTATCATCAAGGAC-3'	<i>SPARC</i>	F 5'- CTGTGACCTGGACAATGACAA-3'
	R 5'- TTTGGGATTCTTGGTTTGTAG-3'		R 5'- ATCCTTGTGCGATATCTCTGCTTG-3'
	FAM-AGTGAAGTGATGCTCTACCAGACCAT-BHQ1		FAM-TACATCGCCCTGGATGAGTGGG-BHQ1
<i>CXCL8</i>	F 5'- CACTGTGTGTAAACATGACTTCC-3'	<i>STAB1</i>	F 5'- TCCGCCTCTGGAATATAAG-3'
	R 5'- GGTGGAAGGTTTGGAGTATGT-3'		R 5'- CAGCTCATCCTGCGACAG-3'
	FAM-CAGTTTTTGCCAAAGGAGTGCTAAAGAACT-BHQ1		FAM-TTCACCATCTTCGTGCCGCA-BHQ1
<i>CXCL10</i>	F 5'- AGTGGCATCAAGGAGTACCT-3'	<i>S100A4</i>	F 5'- TCTTGGTTTGATCCTGACTGC-3'
	R 5'- CTAAAGACCTTGGATTAACAGGTT-3'		R 5'- CACCCTCTTTGCCCGAGTA-3'
	FAM-TGATGCAGGTACAGCGTACAGTTCTA-BHQ1		FAM-CATGGCGTGCCCTCTGGAGAA-BHQ1
<i>GATA3</i>	F 5'- CCTCATTAAGCCCAAGCGAA-3'	<i>TGFβ</i>	F 5'- CCTGGACACCAACTATTGCTT-3'
	R 5'- AGAGTGTGGTTGTGGTGGT-3'		R 5'- TCCTTGCGGAAGTCAATGTAC-3'
	FAM-CAGCCAGGAGAGCAGGACG-BHQ1		FAM-TCCACGGAGAAGAACTGCTGCG-BHQ1
<i>IL1β</i>	F 5'-TGGCTTATTACAGTGGAATG-3'	<i>VEGFA</i>	F 5'- GAATCATCACGAAGTGGTGAA-3'
	R 5'-TGGAAGGAGCACTTCATCTC-3'		R 5'- TGGCTTGAAGATGTAATCGAT-3'
	FAM-CCATCAGCTTCAAAGAACAAGTCA-BHQ1		FAM-CGAGACCTGGTGGACATCTT-BHQ1

Note: created by the authors.

Примечание: таблица составлена авторами.

Table 2/Таблица 2

Differential expression of macrophage-related genes in models of human *ex vivo* TAMs
Дифференциальная экспрессия генов в модельных ОАМ человека *ex vivo*

Genes	MCF-7	BT-474	MDA-MB-231	SKBR-3	SW-837	SKOV-3	PC-3	A-549
Inflammatory cytokines/Воспалительные цитокины								
<i>Il1b</i>	0.0708	0.6829 p=0.0185	1.1886 p=0.0264	0.7584 p=0.0185	0.0301	0.0556	0.304 p=0.0433	0.7093
<i>IL6</i>	0.642	4.018	15.284 p=0.0653	15.354	-5.558	1.058	2.651	11.284 p=0.0947
<i>CXCL8</i>	0.0891	0.184	0.5903	0.9206 p=0.0052	0.5997 p=0.0753	0.2799 p=0.0524	0.5866	0.7385
<i>IL10</i>	0.2717	0.0953	0.868 p=0.035	0.2802	-0.3514	-0.228	0.2129	-0.0962
Macrophage recruitment and polarization/Рекрутирование и поляризация макрофагов								
<i>CCL2</i>	3.561	2.52	6.063 p=0.0435	8.249 p=0.0355	1.008	1.369	10.849 p=0.0115	5.981 p=0.0101
<i>CCRL2</i>	0.0668	-0.0964	-0.1146	0.4802	-0.046	0.2832 p=0.0892	0.1275	-0.0833
<i>GATA3</i>	0.3042	-0.0948	2.0198 p=0.0814	0.5335	0.1705	-0.1682	0.2665	0.6828
<i>SOCS3</i>	0.05546	0.19895	0.24187	0.21025	-0.03503	0.00763	0.02615	0.01563
<i>CXCL10</i>	-2.3641	0.5281	-2.1778	9.3161 p=0.043	-0.1853	0.9031	3.6161 p=0.0748	-2.674
<i>CHID1</i>	0.1138	-0.0456	-0.10158	-0.017	-0.0513	-0.0697	0.0218	-0.13091
Scavenger receptors/Скавенджер-рецепторы								
<i>STAB1</i>	-0.0851	0.1487	-0.0062	0.0135	-0.2077	-0.3165	0.7053	-0.1652
<i>CD163</i>	-0.0022	0.469	0.4436	-0.1097	-0.1522	-0.1923	0.347	0.1672
<i>MRC1</i>	-0.718	0.053	0.709	-0.122	-0.753	-0.393	1.029 p=0.0787	-0.517
<i>MARCO</i>	0.09016	0.2859	0.46816	0.0458	-0.1616	-0.002	0.244	0.00527
Angiogenesis-regulating proteins/Белки-регуляторы ангиогенеза								
<i>SPP1</i>	-0.1136	-0.2487	-0.2316	-0.6193	1.8967	2.269	0.025	-0.2781
<i>SPARC</i>	-0.1954	-0.1845 p=0.0856	-0.381 p=0.001	-0.3367 p=0.0719	0.5231 p=0.0753	0.0771	-0.3005 p=0.022	-0.3865 p=0.0056
<i>S100A4</i>	-0.6318 p=0.0435	-0.3758 p=0.0606	-0.6588 p=0.0041	-0.282 p=0.0504	-0.1381	-0.2738 p=0.0416	-0.3761 p=0.0122	-0.627 p=0.0764
<i>VEGFA</i>	-0.1784	0.1331	-0.0798	0.4883 p=0.0892	0.3102 p=0.0753	-0.018	0.0415	0.2266
<i>CHI3L1</i>	0.0719	-0.2876	-0.0873	-0.2283	0.0471	-0.2348	-0.38	-0.2332
<i>CHI3L2</i>	-0.1279	0.2445	0.3945	0.2328	-0.1195	0.08	0.0608	0.0507
Matrix remodeling and invasion/Ремоделирование матрикса и инвазия								
<i>MMP9</i>	-0.0091	0.2479	0.3284	0.5292 p=0.0892	0.0717	0.2582	0.2861	-0.0266
<i>TGFb</i>	-0.0806	0.117	-0.1939	0.0345	-0.1822	-0.0232	-0.0405	-0.2692
<i>CCL18</i>	1.251	4.623	4.593	-2.241	-0.182	-2.633	-2.269	4.122

Notes: fold changes are indicated for all stimulations in comparison with control; statistically significant changes are marked in bold; values with minus indicate decreased expression compared to control, values without minus – increased expression; created by the authors.
Примечания: кратность изменений для всех стимуляций указана по сравнению с контролем; значимые различия выделены жирным шрифтом; значения с минусом указывают на снижение экспрессии по сравнению с контролем, без минуса – на повышение; таблица составлена авторами.

[TNBC], and HER2-positive subtypes), colorectal cancer, prostate cancer, lung cancer, and ovarian cancer. The comparative analysis was performed in groups of CM-stimulated TAMs versus TAMs stimulated by empty media.
The most activated state was observed in TAMs modeled for breast cancer. Statistically significant up-regulation of pro-inflammatory cytokines *IL1b* and

CXCL8 as well as anti-inflammatory cytokine *IL10* was found in breast cancer-modeled TAMs compared to control (Table 2, Fig. 1). The mRNA expression of pro-migratory factors *CCL2* and *CXCL10* were elevated in modeled TAMs (Table 2, Fig. 2). In contrast, downregulation of angiogenesis-regulating genes *SPARC* and *S100A4* was detected in breast cancer TAMs compared to control media (Table 2, Fig. 1 supplementary).

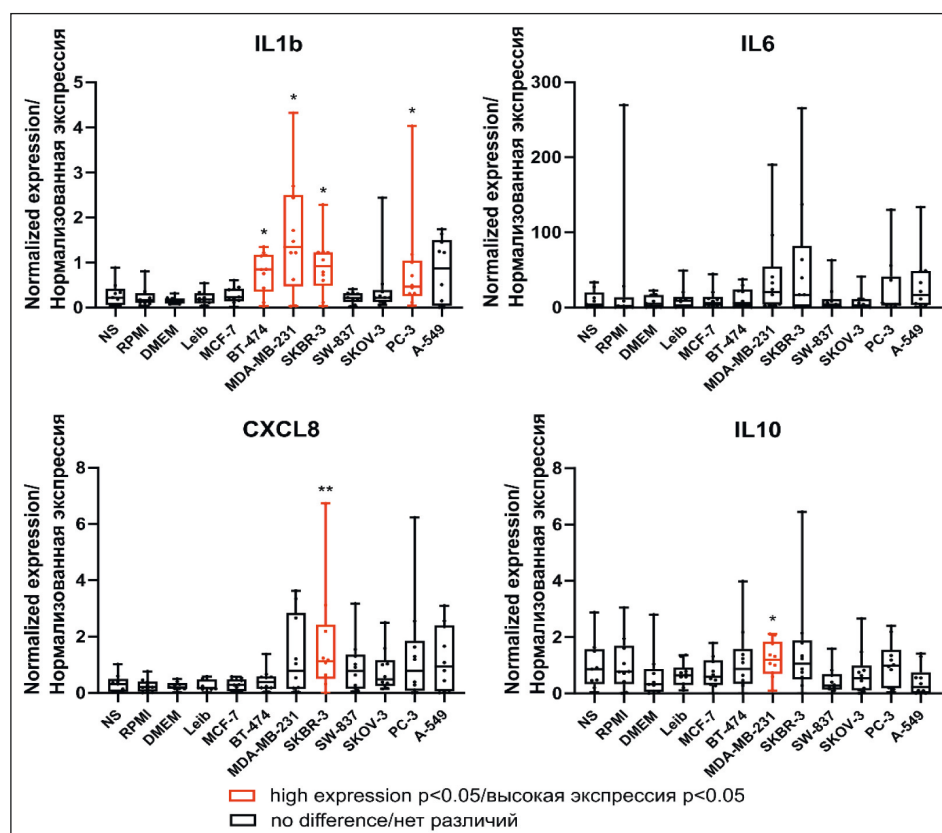


Fig. 1. The mRNA expression of inflammation-related genes in human modeled TAMs.
Notes: * – $p < 0.05$, ** – $p < 0.01$; created by the authors
Рис. 1. Экспрессия мРНК факторов, связанных с воспалением, в модельных ОАМ человека. Примечания: * – $p < 0.05$, ** – $p < 0.01$; диаграммы выполнены авторами

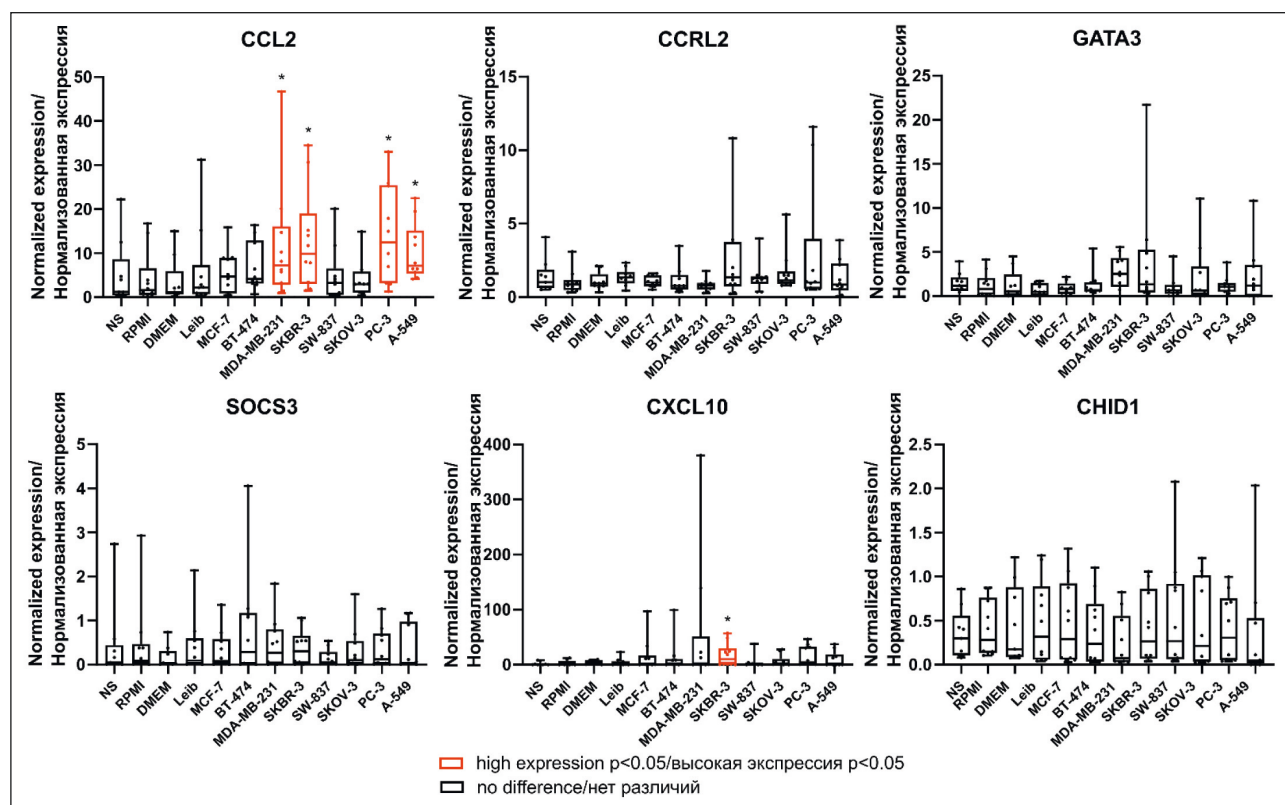


Fig. 2. The mRNA expression of pro-migratory factors in human modeled TAMs.
Notes: * – $p < 0.05$; created by the authors
Рис. 2. Экспрессия мРНК про-миграционных факторов в модельных ОАМ человека. Примечания: * – $p < 0.05$; диаграммы выполнены авторами

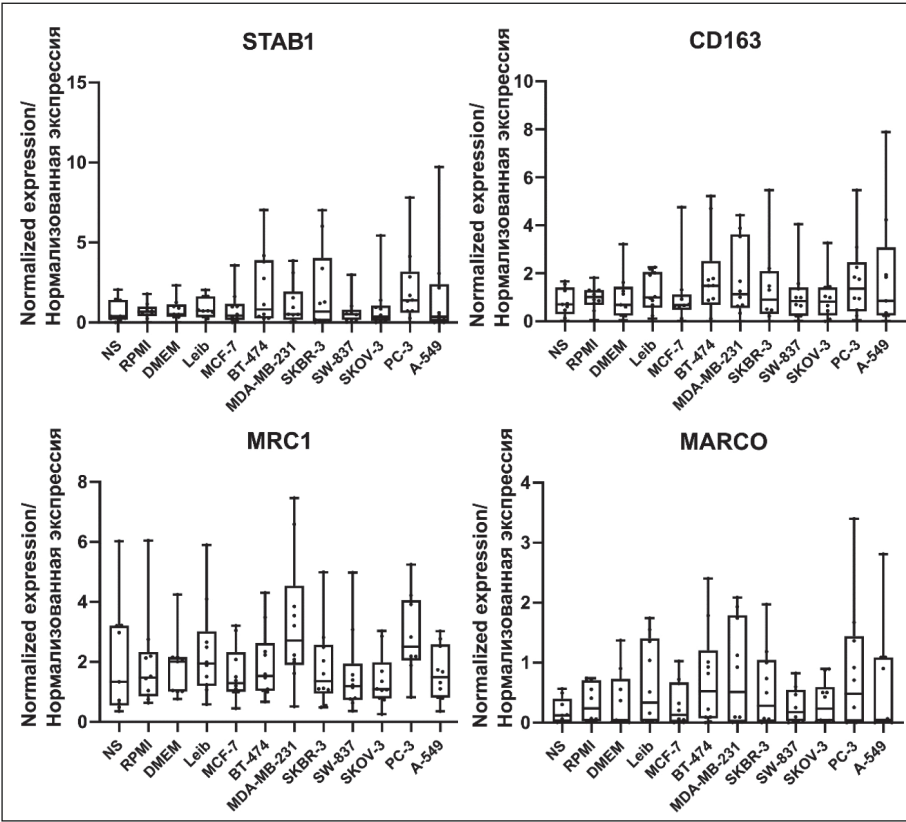


Fig. 3. The mRNA expression of scavenger receptors in human modeled TAMs.
Note: created by the authors
Рис. 3. Экспрессия мРНК сквенджер-рецепторов в модельных ОАМ человека. Примечание: диаграммы выполнены авторами

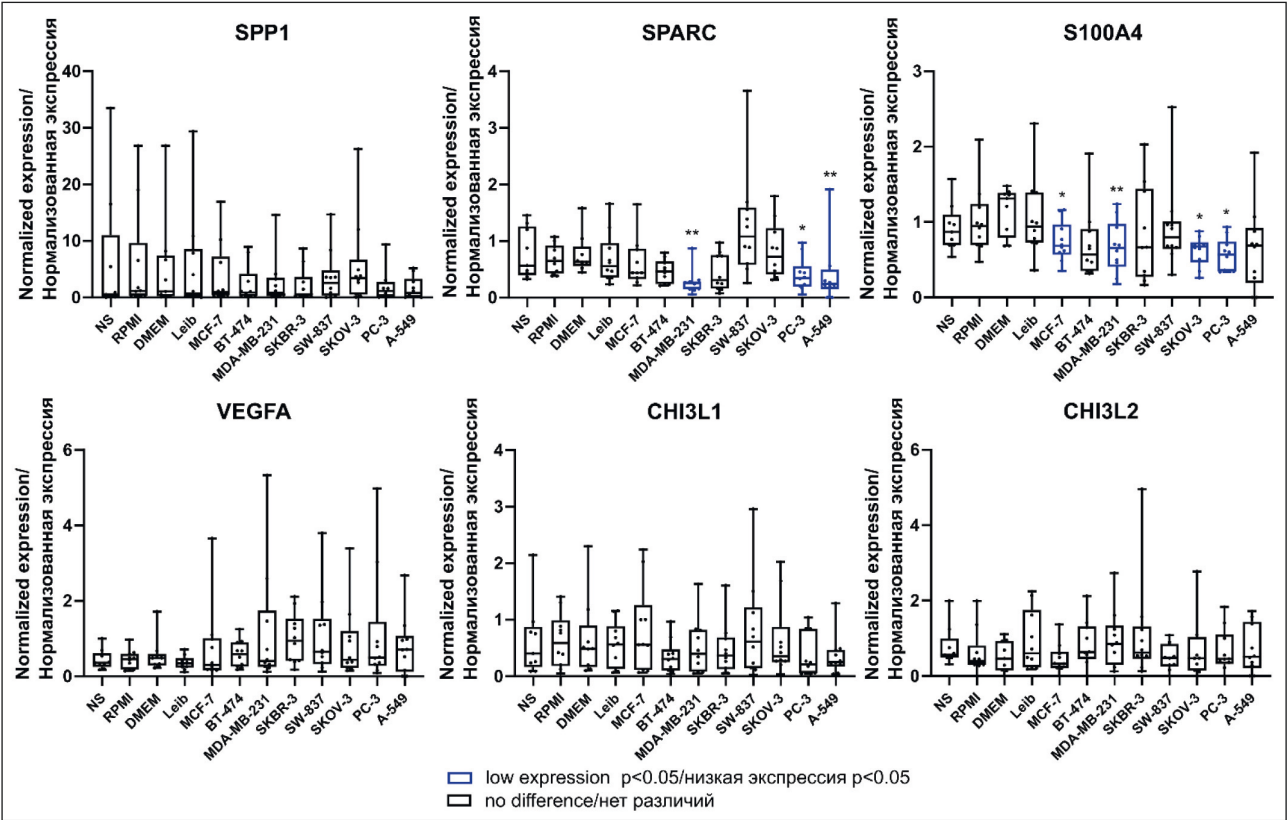


Fig. 1 supplementary. The mRNA expression of angiogenesis-regulating genes in human modeled TAMs.
Notes: * – $p < 0.05$, ** – $p < 0.01$; created by the authors
Рис. 1, дополнительный. Экспрессия мРНК факторов, регулирующих ангиогенез, в модельных ОАМ человека.
Примечания: * – $p < 0.05$, ** – $p < 0.01$; диаграммы выполнены авторами

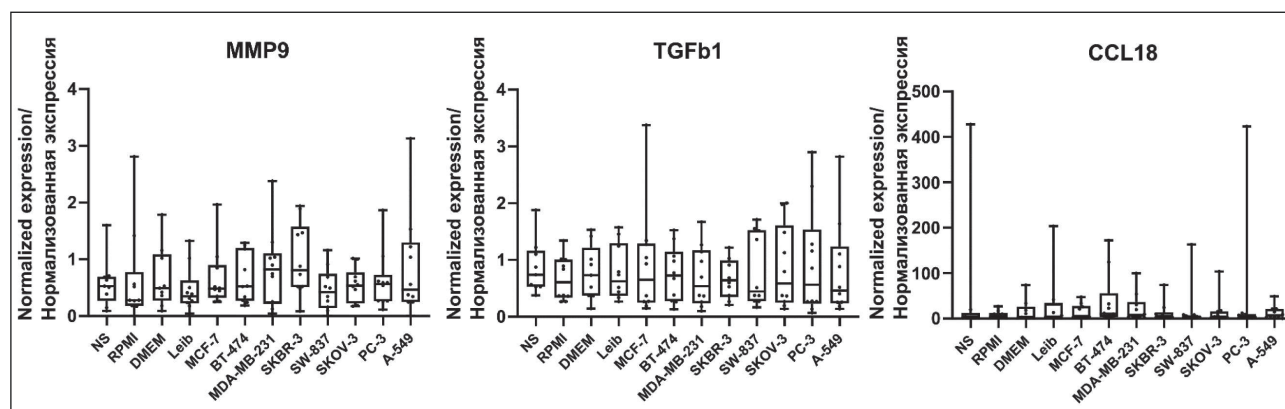


Fig. 2 supplementary. The mRNA expression of matrix remodeling and invasion factors in human modeled TAMs.

Note: created by the authors

Рис. 2, дополнительный. Экспрессия мРНК факторов ремоделирования матрикса и инвазии в модельных ОАМ человека.

Примечание: диаграммы выполнены авторами

The elevated expression of *IL1b* and *CCL2* was observed in TAMs stimulated by CM of HER2-positive (SKBR-3) (0.920 [0.480; 1.226] vs 0.162 [0.102; 0.318], $p=0.018$ for *IL1b* and 9.870 [3.012; 19.04] vs 1.621 [0.526; 6.573], $p=0.035$ for *CCL2*) as well as triple-negative (MDA-MB-231) (1.342 [0.472; 2.501] vs 0.1534 [0.083; 0.208], $p=0.026$ for *IL1b* and 7.208 [2.811; 16.07] vs 1.145 [0.638; 5.957], $p=0.043$ for *CCL2*) cell lines, correspondingly, but not of hormone-dependent luminal subtypes (Table 2, Fig. 1, Fig. 2). Accumulating evidence demonstrated the differences in triple-negative breast cancer (TNBC), or basal-like subtype, compared to luminal subtypes [17, 18]. In our model, *CCL2* expression was also elevated in TAMs of non-luminal subtypes. Up-regulation of pro-inflammatory cytokines *CXCL8* (1.122 [0.497; 2.421] vs 0.201 [0.051; 0.404], $p=0.005$) and pro-migratory factor *CXCL10* (9.913 [0.826; 30.06] vs 0.596 [0; 6.626], $p=0.043$) was detected in TAMs stimulated by CM of HER2-positive (SKBR-3) cell lines, but up-regulation of anti-inflammatory cytokine *IL-10* (1.190 [0.694; 1.846] vs 0.322 [0.057; 0.877], $p=0.035$) was observed only in TAMs stimulated by CM of triple-negative (MDA-MB-231) cell lines (Table 2, Fig. 1, Fig. 2). In addition, in our model, angiogenesis-regulating genes (*SPARC*, *S100A4*) [19] were downregulated in TAMs stimulated by CM of luminal A (MCF-7) (0.683 [0.569; 0.967] vs 1.315 [0.794; 1.385], $p=0.043$ for *S100A4*) and triple-negative (MDA-MB-231) cell lines (0.251 [0.146; 0.285] vs 0.632 [0.556; 0.902], $p=0.001$ for *SPARC* and 0.656 [0.406; 0.979] vs 1.315 [0.794; 1.385], $p=0.004$ for *S100A4*) (Table 2, Fig. 1 supplementary).

Similar to breast cancer-stimulated macrophages, the expression of *IL1b* (0.466 [0.258; 1.044] vs 0.162 [0.102; 0.318], $p=0.043$), *CCL2* (12.47 [3.164; 25.44] vs 1.621 [0.526; 6.573], $p=0.011$), *SPARC* (0.345 [0.190; 0.555] vs 0.645 [0.427; 0.921], $p=0.022$), *S100A4* (0.570 [0.343; 0.741] vs 0.9463 [0.695; 1.241], $p=0.012$) was statistically significant differed in TAMs of prostate cancer, compared to the

control macrophages (Table 2, Fig. 1, Fig. 2, Fig. 1 supplementary).

In modeled lung cancer-associated TAMs, *CCL2* was also upregulated compared (7.126 [5.382; 15.14] vs 1.145 [0.638; 5.957], $p=0.010$) to control media-stimulated TAMs (Table 2, Fig. 2). *CCL2*-CCR2 axis, which is a prominent activator of monocyte/macrophage recruitment, facilitate lung cancer progression [20, 21]. In addition, *S100A4* was downregulated (0.688 [0.194; 0.924] vs 1.315 [0.794; 1.385], $p=0.005$) in TAMs stimulated by CM of lung cancer cell line (A-549) (Table 2, Fig. 1 supplementary).

However, tumor-induced macrophages in our model didn't show any changes in the expression of general markers of TAMs, that belong to scavenger-receptors (*CD163*, *MRC1*, *MARCO*, *Stab1*) [22] (Table 2, Fig. 3) and markers associated with matrix remodeling (Table 2, Fig. 2 supplementary).

Discussion

It is well known that the TME can determine the polarization state of TAMs [4, 23]. M1/M2 paradigm has existed for more than 30 years and it reflects two extreme states of macrophage polarization – pro-inflammatory M1 and anti-inflammatory M2 [24]. In the context of tumor microenvironment, these states were defined as anti-tumor and pro-tumor, consequently [2, 24]. Recently, with the introduction of many advanced technological methods such as single-cell and spatial analysis we can look deeper into tumor composition to dissect multiple cell phenotypes with a distinct functional activity [25, 26]. Following that, a large diversity of dynamic cell subpopulations within a tumor has been revealed. Most recently collected single-cell data suggest that up to 10 subsets of TAMs may be distinguished, depending on the cancer type and localization [27, 28]. These studies allowed identifying new macrophage markers. Interestingly, generally accepted markers of scavenger-receptor macrophages [2] are not always in the top genes of TAM subsets, or aren't even present in the gene

signature [29]. Nevertheless, researchers continue to use them in many *in vitro* and *in vivo* studies as differential markers of M1 and M2 macrophages.

Well-known existing *in vitro* models for human macrophage analysis include either stable macrophage cell lines (e.g. THP-1 cells) or monocyte-derived macrophages [30]. However, most of the methods are based on the use of limited differentiation and polarization factors which determine the monocyte to macrophage transition [31]. It becomes clear that macrophages obtained *in vitro* do not reflect that intricate phenotypic diversity which develops under the influence of tumor environment [2, 12]. This can explain the limitations of existing macrophage targeting and inability of multiple approved clinical trials to overcome therapeutic failure [32, 33]. Thus, translatable and reproducible *in vitro* models for human macrophages are under intensive investigation [31].

In our study, we used an experimental *ex vivo* model of TAMs, which, in our opinion, can most accurately reflect the influence of tumor factors on macrophages. For this purpose, we treated human monocyte-derived macrophages with M2-polarizing cytokines and conditioned tumor supernatants. Generation of monocyte-derived TAMs subjected to the tumor-conditioned media presented more features that are similar to *in vivo* TAMs, compared to macrophages generated by cytokine stimulation alone [34]. The main question we raised was whether this model can adequately reflect the diversity and real functional activity of TAMs in the TME. The most pronounced up-regulation of macrophage-associated markers was detected in breast cancer TAMs. Accumulating evidence demonstrated the differences in triple-negative breast cancer (TNBC), or basal-like subtype, compared to luminal subtypes [17, 18]. Basal-like breast cancer cells determine more prominent differentiation of THP-1 cells compared to luminal cancer cells [35]. *In vitro* stimulation of macrophages with basal-like cells induced mixed M1/M2 phenotype [35] that is similar to observations made by us. However, gene expression analysis did not show differences in the expression of the main markers including scavenger-receptors (*CD163*, *MRC1*, *MARCO*, *Stab1*) or markers associated with matrix remodeling, although, elevated expression of these markers was observed in human tumor tissue TAMs and was significantly correlated with outcome of cancer patients [2, 19, 36].

Different studies show the variety among the induced phenotypes of TAMs. This depends on both the type of the model system and the differentiating and polarizing factors that are used to skewer macrophages into a pro-tumor phenotype. For instance, authors revealed that IL-34 and CXCL4 have similar functions to M-CSF and activate the same pathway for differentiation of macrophages *in vitro* [31]. Monocyte-derived macrophages stimulated by IL-34 acquire macrophage-like morphology and express CD45 and CD68. But, IL-34-treated macrophages show increased phagocytic capacity and higher IL-10

and CCL-17 production after stimulation, and CXCL4-activated macrophages have more pro-inflammatory features compared to M-CSF-stimulated ones [31]. Authors of another study determined that CD40 and CD64 are the most distinctive makers for human M1 macrophage activation and CD163 and MR for M2 macrophage activation *ex vivo* [37]. They also revealed that adherent and floating macrophages could have different activation state. Thus, the expression of MR and CD40 was indicative for adherent cells compared to the floating cells. Distinct activation states of human monocyte-derived macrophages were found when they matured with M-CSF or GM-CSF [37]. Monocyte-derived macrophages (MDMs) differentiated under serum-free conditions displayed greater phagocytic properties and tumor promoting activity for M2 macrophages in contrast to MDMs differentiated in the presence of serum [38]. Monocyte isolation method can also affect the phenotype of resulting macrophages *ex vivo* [39]. Expression of CD163 and CD14 were significantly lower on monocytes isolated by CD14-positive selection and by simple plastic adhesion, compared to untouched peripheral blood mononuclear cells. MDMs from plastic adhesion were M1-skewed (CD80^{high} HLA-DR^{high} CD163^{low}), whereas MDMs obtained by negative selection were M2-skewed (CD80^{low} HLA-DR^{low} CD163^{high}) [39].

Moreover, in the majority of studies insufficient attention is given to the components of culture media used for cancer cell passaging that can contain diverse growth factors affecting macrophage phenotype. In our *ex vivo* models, we provide the necessary control and clearly differentiate between the effects of empty cancer cell culture medium and conditioned cancer cell culture medium.

It becomes clear that traditional *in vitro* models cannot reproduce the genetic, phenotypical and functional features of macrophage environment *in vivo*. In recent review, authors provided the idea of new model systems where macrophages are first generated according to their ontogenetic identity and then induced to obtain their tissue-specific entity. One of such methods is generating macrophages from induced pluripotent stem cells (iPSCs) [30]. The use of 3D organotypic tumor models combining components of tumor microenvironment including macrophages has also become more popular [40, 41]. Authors of recent study developed 3D multicellular tumor spheroid (MCTS) model comprising of cancer cells, fibroblasts, and human monocyte-derived macrophages [42]. They demonstrated the importance of TAM-containing models in drug screening of anti-cancer immunotherapies targeting macrophages [42].

Conclusion

In summary, monocyte-derived macrophages stimulated with cancer cell-conditioned medium can, to a certain extent, allow modeling of cancer-specific programming of TAMs. This model enables analysis

of the differential expression of genes that are involved in the major regulatory activities of TAMs that support primary tumor growth and its metastatic potential. These gene groups cover inflammatory cytokines, regulators of macrophage recruitment and polarization, scavenger receptors, regulators of angiogenesis and extracellular matrix remodeling, as well as cancer cell invasion. Our model system is valuable to examine agents reprogramming key TAM pro-tumoral activities, and for the reproducible analysis of mechanistic events that program tolerogenic status of TAMs towards

cancer cells. However, in order to achieve the full specificity of TAM phenotypes 3D modelling is needed to create the most physiologically relevant context for macrophage interactions with the extracellular matrix, cancer cells and other cells of tumor microenvironment. In this regard, the rapidly developing field of organoids is highly promising direction which will allow to recreate three-dimensional multicellular composition of tumor tissue, and also to model not only cancer-specific but also patient-specific TAM phenotypes and study their functions.

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Conflict of interests

Prof. Kzhyshkowska J.G. is a member of the editorial board of Siberian Journal of Oncology. The authors are not aware of any other potential conflicts of interest related to this manuscript.

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Все авторы одобрили финальную версию статьи перед публикацией, выразили согласие нести ответственность за все аспекты работы, подразумевающую надлежащее изучение и решение вопросов, связанных с точностью и добросовестностью любой части работы.

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