ISSN 1990-519X, Cell and Tissue Biology, 2018, Vol. 12, No. 5, pp. 351–358. © Pleiades Publishing, Ltd., 2018.
Original Russian Text © L.V. Marchenko, A.D. Nikotina, N.D. Aksenov, L.V. Smagina, B.A. Margulis, I.V. Guzhova, 2018, published in Tsitologiya, 2018, Vol. 60, No. 5, pp. 357–364.

Phenotypic Characteristics of Macrophages and Tumor Cells in Coculture

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Received January 17, 2018

Abstract—Immunosuppressive activity of a tumor is provided by constituents of its microenvironment (TME), immune cells, particularly macrophages, which, instead of protecting the organism, render cancer cells more aggressive and metastatically active. A solid tumor is a spacious structure; a naïve macrophage, on its way through the tumor, encounters newer cell populations; and its functioning can be improved. In our study, we employed the method of sequential cocultivation of monocyte-like THP-1 cells with A431 human epidermoid cancer cells and explored the changes in the behavior of both cell counterparts depending on cocultivation stage, cytokine profiles, and metalloproteinase 2 and 9 (MMP) activity. We found that the first contact of the monocyte with tumor cells already increased the tumor cell proliferation and migration, release of protumorigenic cytokines, and monocyte polarization into M2 phenotype. One of the most abundant cytokines induced by the cocultivation was tumor necrosis factor, TNF-alpha, a potent inducer of apoptosis. Its release led to the massive death of THP-1 cells, so that at the other stages of cocultivation, a weakened population of macrophages could not affect the behavior of tumor cells. In conclusion, the macrophage function in TME strongly depends on its cytokine content and the data reported here elucidate one scenario of interaction between stromal and cancer cells in a real tumor.

Keywords: tumor microenvironment, macrophage, cytokine, A431, THP-1

DOI: 10.1134/S1990519X18050036

INTRODUCTION

The viability of tumor cells is mostly supported by their microenvironment (TME) composed from stromal and immune cells, proteins, growth factors, RNA molecules, vesicular particles, microelements, and other components (Witz, 2008). TME cells that come into contact with a tumor lose their protective function and become protumor. As a result, malignant tumors escape the host's immune defense, although they exhibit all the features necessary to activate the immune response. Tumor cells frequently have mutant or incorrectly processed proteins able to provoke an organism's immune response. Inflammation often develops in the tumor area (Munn and Bronte, 2016).

Tumors are also known to evade the host's immune defense due to inhabiting T lymphocytes, macrophages, dendrite cells and other cells of the immune system. In tumor tissue these cells synthesize immunosuppressive cytokines that increases the number of myeloid-derived suppressor cells (MDSC) in tumor, activation of $Fox3^+$ regulatory T-lymphocytes (Tregs), M2-like polarization of tumor-associated macrophages and inhibition of T-cell proliferation (Razavi et al., 2016). Tumor-associated macrophages secrete growth-promoting cytokines, in addition to maintaining the local inflammatory process, which, in turn, favors the genetic instability of tumor cells (Mantovani et al., 2008; Hanahan and Weinberg, 2011).

Interleukins IL-1β, IL6, IL-8, and IL-10, as well as TGF-β and TNF-α factors, are cytokines the concentration of which is increased in TME. Proinflammatory cytokine IL-1β activates IL-1β/IL-1RI/β-catenin signaling and facilitates the epithelial–mesenchymal transition (EMT) of tumor cells (Jiménez-Gardiño et al., 2017). Proinflammatory cytokine IL-1β, when activating IL-1β/IL-1RI/β-catenin signaling, provokes the epithelial–mesenchymal transition (EMT) of tumor cells. Another proinflammatory cytokine IL-6 is a resident of TME in various tumors, such as non-small-cell lung cancer (Abulaiti et al., 2013), breast cancer (Chang et al., 2013), prostate cancer (Giri et al., 2001), and pancreas cancer (Lesina et al., 2011). IL-6, through its receptor and Janus-

Abbreviations: TME—tumor microenvironment, MMP—matrix metalloproteinases, TNF-α–tumor necrosis factor, IL—interleukin, EMT—epithelial–mesenchymal transition, GFP—green fluorescent protein, TRAIL—TNF-related apoptosis-inducing ligand.

kinases, activates STAT3, which stimulates tumor cell proliferation. This results in tumor cells that are resistant to anticancer drugs, induction of EMT, and increased metastasis properties (Mace et al., 2013; Domingues et al., 2017).

IL-8 is also registered in TME of various tumors. It is released from tumor-associated fibroblasts, as well as tumor cells (Domingues et al., 2017). IL-8 recruits novel naïve cells of the immune system and stimulates angiogenesis in the tumor (Suarez-Carmona et al., 2015) and tumor cell EMT (Sanmamed et al., 2014).

Another factor that regulates TME is the pleiotropic cytokine TNF-α. It is a key regulator of apoptosis, tumor angiogenesis, inflammation, and immunity. TNF-α plays both pro- and antitumor roles (Ham et al., 2016). It is produced by TME cells and stimulates expression of other cytokines, intermediate metabolites of active oxygen species, nitrogen oxide, and prostaglandins (Balkwill, 2009). TNF-α is implicated in almost all stages of the carcinogenesis process and fulfils different functions depending on the tumor type and TME. High concentrations of TNF-α correlate with tumor regression, whereas its low amount for a long period facilitates the expression of other growth factors and cytokines (Balkwill, 2009).

Any solid tumor is a spatial structure. A naïve macrophage migration inside the tumor encounters novel population of tumor cells. To understand what happens with macrophages during their moving inside tumor, how their phenotype and TME cytokine profile change, and how the tumor cells respond to these processes was the aim of this paper.

MATERIALS AND METHODS

Cells. Human epidermal carcinoma A431 and monocytic leukemia THP-1 (suspension culture) cell lines were obtained from the Russian collection of vertebrate cells (Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia). Both cell lines were maintained in DMEM medium with 4.5% glucose (Gibco, United States), 2 mM glutamine, 10% fetal bovine serum (Gibco, United States), and 0.05 mg/mL gentamicin (Biolot, Russia) at 37°C and 6% CO_2. Monocytic cells THP-1 are commonly used as a model of undifferentiated macrophages (Starikova et al., 20005).

The process of a macrophage moving inside the tumor tissue and possible gaining of a novel phenotype was modeled by macrophage "education" stage by stage. An amount of 6×10^5 A431 cells were plated in 10-cm Petri dishes. After the cells were attached to the dish surface the cultures were supplemented with THP-1 cells in a ratio of 1 : 1. The cells were cocultivated for 24 h. "Educated" macrophage (stage 1) were transferred to fresh A431 cells seeded before in a ratio of 1 : 1. To maintain the ratio of 1 :1 at the first stage, cells were cocultured in three Petri dishes; at the second stage, in two; and, at the third stage, in one. Suspension THP-1 cells were collected by pipetting. At each transfer, the number of dead cells was counted in a Neubauer camera after cell staining with Trypan blue. Conditioned media from A431, THP-1, and coculture were collected separately after each stage, frozen, and stored at –80°C. Each stage was repeated six times.

IL-1β, IL-6, IL-8, and TNF-α cytokine content in the conditioned medium from each of three stages of cocultivation was evaluated with magnetic bead-based multiplex immunoassay and MilliPlex technology (Merck, Germany) according to the manufacturer's recommendations. A Milliplex Human Cytokine Magnetic Panel HCYTOMAG-60K was kindly provided by Merk. Two independent measurements of the cytokine profile in the culture medium were performed.

MMP activity in cocultivated A431 and THP-1 cells was assayed by zymography. The cells from cocultures at each of the three stages were thoroughly washed with PBS and placed into serum-free medium for 18 h. The medium was collected, centrifuged at 3000 g for 5 min and mixed with SDS-containing buffer. Acrylamide gel for electrophoresis was polymerized with 1 mg/mL gelatin. Samples were subjected to electrophoresis. After electrophoresis gels, were cleansed of SDS with 2.5% Triton X-100 for 30 min twice, incubated with the reaction buffer (50 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 10 mM $CaCl_2$, and 0.05% Brij 35) for 12 h, and stained with Coomassie blue R-250 to identify unstained bands with molecular weight corresponding to MMP-2 and MMP-9.

Expression of tumor-associated macrophage marker F4/80. Naïve THP-1 cells and THP-1 cells collected after the cocultivation (stage 1) were stained with antibodies to F4/80 (Cell Signaling, United States) at 4°C for 2h, washed with cold PBS, fixed with 4% formalin, and stained with Alexa-647-conjugated secondary antibodies (Invitrogen, United States). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, United Sates). In another experiment, THP-1 cells were transduced with lentivirus carrying green fluorescent protein GFP to identify contacts between A431 and THP-1 (considered as macrophages in our experiments). A431 cells (6×10^4 cells/mL) were seeded on coverslips in 24-well plates. The next day, THP-1-GFP cells were added into these wells at a ratio of 1 : 1. The cells were cocultivated for 24 h. The coverslips were carefully washed with PBS and stained with Alexa 633-conjugated phalloidin (Life Technology, United States). The nuclei were stained with DAPI. Samples were assayed with a TSL SP 5 confocal microscope (Leica, Germany) using 405-, 488-, and 647-nm lasers.

Flow cytometry. Naïve THP-1 and THP-1 collected from cocultures (stages 1–3) were incubated in the complete growth medium with antibodies to F4/80

(Abcam, United Kingdom) for 2 h at 4°C, antimouse secondary Alexa 488 labeled antibodies, and Annexin-V Alexa Fluor TM 633 reagent (Life Technology, United States) to identify apoptotic cells. Cells were analyzed with a CytoFlex Flow cytometer (Beckman Coulter, United States) using 488- and 647-nm lasers.

Immunoblotting. To assay differentiation markers of tumor-associated macrophages, "educated" THP-1 cells were collected after stages 1 and 2, centrifuged, and washed with PBS three times. A dry cell pellet was supplemented with the lysing buffer High RIPA (20 mM TrisHCl pH7.5, 150 mM NaCl, 0.1% Triton X-100, 0.5% SDS, 1% DOC, 2 mM EDTA, 100x mammalian inhibitor protease cocktail) (Sigma). After three freeze–thaw cycles, the lysate was centrifuged at 10 000 rpm. Protein concentration was determined with a Bradford protein assay (Bradford, 1976). Cell lysates (20 μg per well) were used for electrophoresis and immunoblotting. The membrane was subsequently incubated with antibodies to arginase (Arg-1, a marker of M2 macrophages) and secondary antimouse antibodies labeled with horseradish peroxidase (Abcam, United Kingdom). Antibodies to GAPDH (Abcam, United Kingdom) were used for loading control.

Migration and proliferation of A431 cells after cocultivation with THP-1 cells were monitored using in the real time xCELLigence RTCA DP instrument (Biosciences, Inc.). The cells after stages 1, 2, and 3 of cocultivation (2×10^5 cells/mL) were placed in 16-well E-plates with wells deposited with gold electrodes. The electrode resistance varied depending on the area of cell contacts with well surface. The impedance was measured every 10 min for 36 h. Migration properties of A431 cells after coculturing with THP-1 cells (stages 1–3) were assayed with Boyden chambers or plates with two compartments separated by semi-permeable membrane. The bottom had a gold deposited electrode (for real-time monitoring of cell migration). A431 cells (10×10^5 cells/mL) after cocultivation with "educated" THP-1 cells were seeded either in the upper compartment of the plate well or the upper part of the Boyden chamber. The bottom part was filled with the culture medium containing 10% fetal bovine serum. The impedance on the bottom part was measured every 10 min for 24 h. The results were analyzed using RTCA equipment with Software 1.2. The cells in the Boyden chamber were stained with Giemsa. Cells from the inner compartment were removed with a cotton swab. Cells retained on the outer part were photographed with an Axia Zeiss CamERK5 camera (Zeiss, Germany).

Statistical treatment. Results were treated with the Microsoft Excel 2010 software for small samplings. Data are presented as mean ± SD. The difference was statistically significant at $P < 0.05$. Each experiment was repeated two to four times.

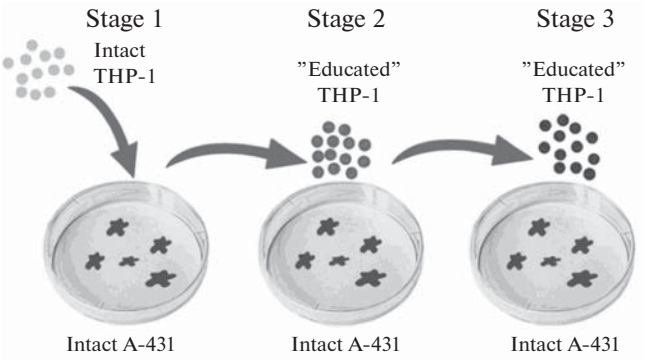

Fig. 1. Sequential cocultivation of A431 human epidermoid cancer cells with THP-1 cells imitating macrophages in our study. Monocytic intact THP-1 cells were added to A431 cell culture in a ratio of 1 : 1 and cocultivated for 24 h. "Educated" THP-1 cells were collected and plated to intact 431 cells. The procedure was repeated twice.

RESULTS

Modified phenotype of monocyte-like THP-1 cells resulted from their cocultivation of A431 cells. THP-1 morphology was assayed with confocal microscopy to elucidate the tumor cell impact on these cells modulating macrophages in our study. It was found that THP-1 cells changed their spherical morphology and became spread cells with adhesive contacts (Fig. 2a). It is an indication on the macrophage differentiation as monocytes circulating in blood have a round, ball-like morphology (Eligini et al., 2013).

The cells were stained with antibodies to M2 macrophage marker F4/80 and Annexin-V, an apoptotic marker, to test if morphologically modified cells showed a protumor phenotype using confocal microscopy and flow cytometry. It allowed the number of THP-1 cells with a protumor phenotype that died by apoptosis to be determined.

A small number of intact THP-1 cells express F4/80 marker of M2 phenotype on their surface (Figs. 2b, 2c). Their number increased to 61.12% after the first stage of cocultivation. However, 42.04% cells were committed to apoptosis (Fig. 2c). Opposite to our expectations, THP-1 cells considered as macrophages in our study lost rather than acquired their protumor phenotype during their "education." After the second education stage, 52.28% cells were F4/80 positive and 67.02% of them underwent apoptosis (Fig. 2c). At the third stage. only 17.01% cells of expressed F4/80 and 76.03% of them were Annexin-V positive (Fig. 2c).

The data on THP-1 cell loss of macrophage protumor phenotype M2 during the cell cultivation with aggressive tumor cells were confirmed by expression of Arg-1, another protumor macrophage marker. Under normal conditions, THP-1 had low Arg-1 expression (Fig. 2d). In cocultures with A431 cells, Arg-1 expression in THP-1 cells drastically increased at, stage 1,

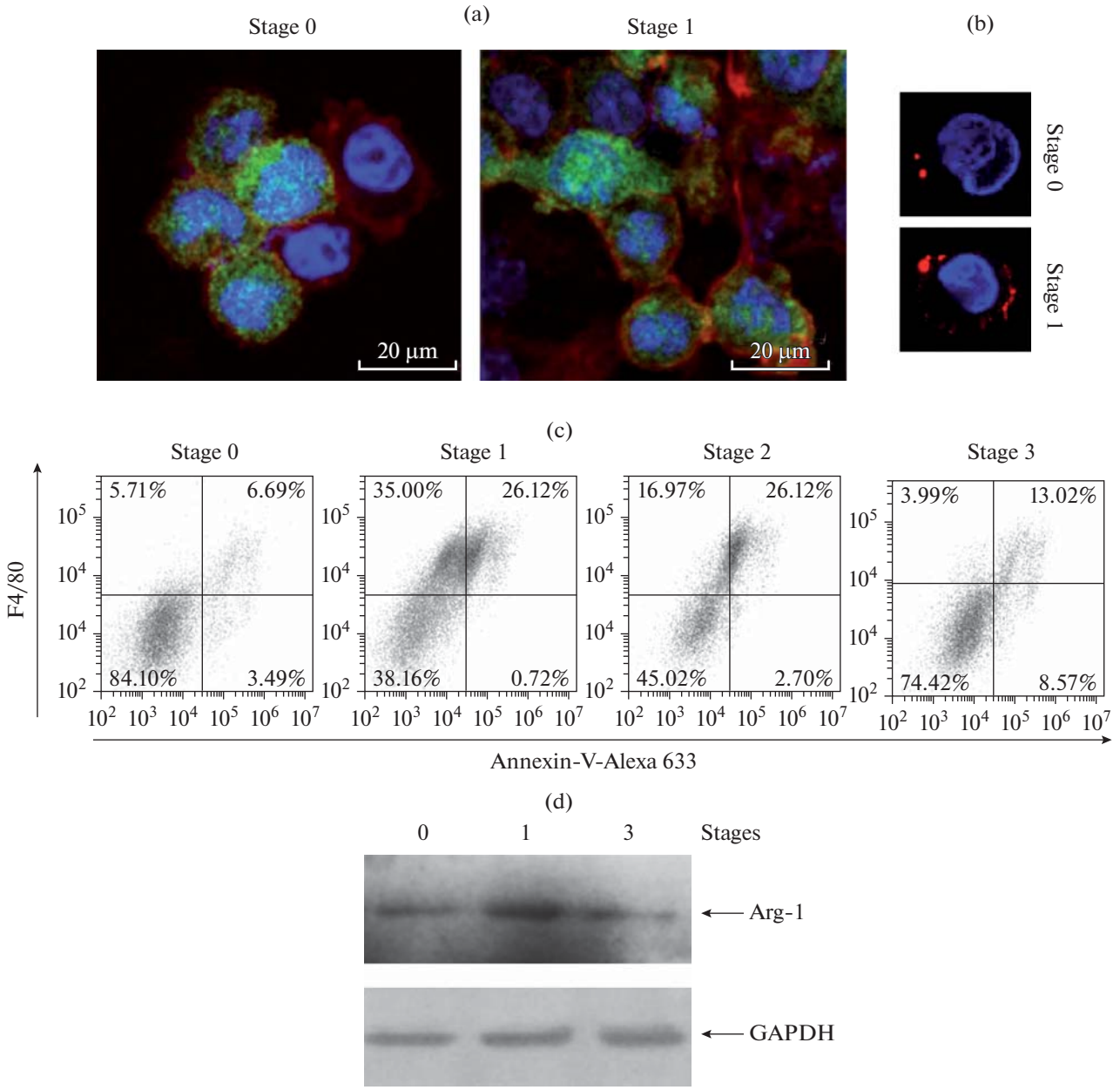

Fig. 2. Protumor of features THP-1 cells acquired during cocultivation with tumor A431 cells. (a) THP-1-GFP (*green*, infected with the gene of green fluorescent protein) cocultivated with A431 cells for 24 h were fixed and stained with Alexa 633-conjugated phalloidin (red); (b) THP-1 cells after cocultivation with A431 cells (stage 1) were stained with antibodies to protumor phenotype marker F4/80 (*red*); nuclei were stained with DAPI (a, b, blue); (c) flow cytometry assay of THP-1 cells collected after cocultivation with A431 cells (stages 1, 2, 3) and stained with antibodies to protumor phenotype marker F4/80 and Annexin-V reagent (the THP-1 number with F4/80 marker increased after stage 1 and then declined due to F4/80-positive cells by apoptosis; (d) immunoblotting of THP-1 cell lysate after cocultivation with A431 cells (stages 1, 2) with antibodies to protumor marker Arg-1.

but it approached the control level during the following stages (Fig. 2d).

Cocultivation of A431 and THP-1 cells alters cytokine secretome. Cytokine profiles (IL-1β, IL6, IL-8, and TNF-α) in TME were determined with magnetic-bead-based multiplex immunoassay and MilliPlex technology. It was found that the encounter of a tumor cell with naïve THP-1 cells (stage 1) markedly increased the amount of all four cytokines in the coculture medium. At stage 2, the cytokine level fell to the control level and later did not increase.

The key players in TME, in addition to cytokines, are enzymes involved in degradation of protein components in the extracellular matrix. Excessive degra-

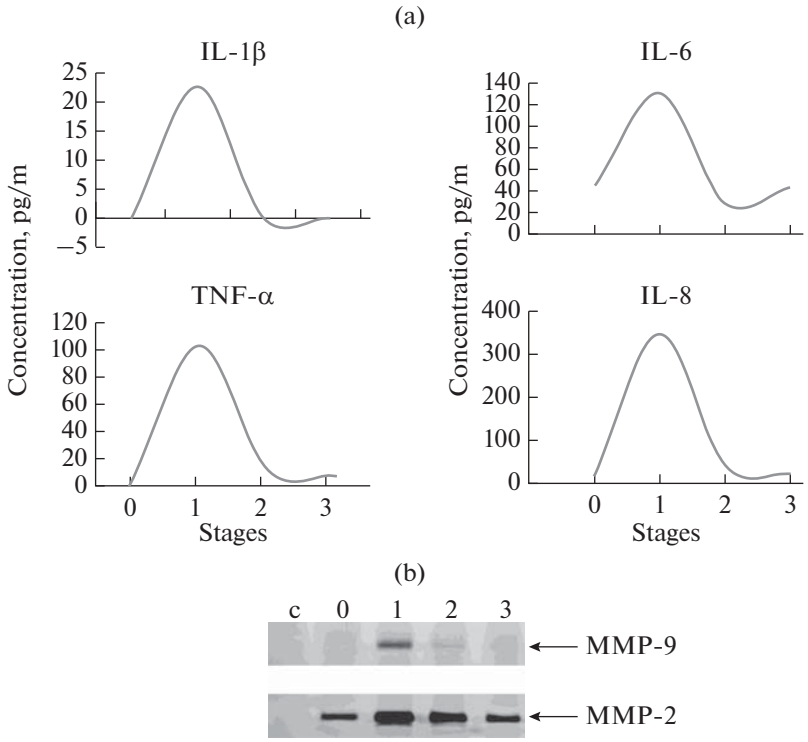

Fig. 3. (a) Cytokine concentration and (b) MMP level in medium of cocultivated A431 and THP-1 cells. (a) Conditioned medium from A431 cells collected after cocultivation with THP-1 cells (stages 1, 2, 3) analyzed with magnetic-bead-based multiplex immunoassay and MilliPlex technology; (b) zymography analysis after cell cocultivation (stages 1, 2, 3), C, control medium; 0, medium from intact A431 cells.

dation of the extracellular matrix is a common feature of the metastatic process. MMP secretion is basically regulated by proinflammatory cytokines produced by activated macrophages, neutrophils, and fibroblasts, as well as endothelial and tumor cells (Berezhnaya, 2009). The activity of MMP-2 and MMP-9 in the medium from A431 and THP-1 coculture was assayed with the zymography technique. It was shown that MMP-2 and MMP-9 in this medium were drastically increased at stage 1, then gradually decreasing during the "education" process (Fig. 3b).

Cocultivation of A431 and THP-1 cells enhances the proliferative and migration properties of tumor cells. A431's proliferative and migration properties during the three stages of cultivation with THP-1 cells (Fig. 1) were evaluated to learn the influence of "educated" macrophage on these cells.

It was found that the cell proliferation index of A431 cells in coculture with naïve THP-1 cells notably increased (Fig. 4a), but, at stage 2 and 3, it was similar to the proliferation rate of intact A431 cells (Fig. 4a).

Naïve THP-1 cells had a greater effect on A431 cell migration properties than did THP-1 cells from stages 2 and 3 of cocultivation (Fig. 4b), The data obtained with application of a Boyden chamber (Fig. 4c) showed that, after cocultivation of A431 cells with naïve THP-1 cells (stage 1), more cells passed through the pores of the permeable membrane and occurred at the bottom (Fig. 4c), which confirmed the results found with application of the xCELLigence instrument.

DISCUSSION

Tumor-associated macrophages are not uniformly spread in the tumor, as has been recently shown for breast cancer (Tashireva et al., 2017). Macrophage localization in a tumor has an effect on the cancer cell behavior and correlates with increased metastatic nodes in patients (Tashireva et al., 2017). Macrophage functioning also depends on their localization in TME (Budakov et al., 2017). This means that macrophage behavior and differentiation are determined by the cytokine profile, in particular, part of the tumor node (Stakheyeva et al., 2017). In our experiments, we attempted to reproduce in vitro events that happened to macrophages in TME as they moved inside the tumor.

We evaluated the changes in the macrophage phenotype, tumor cell properties, and cytokine content in the culture medium in cocultures of A431 and THP-1 cells (Fig. 1). It was found that the first contact of

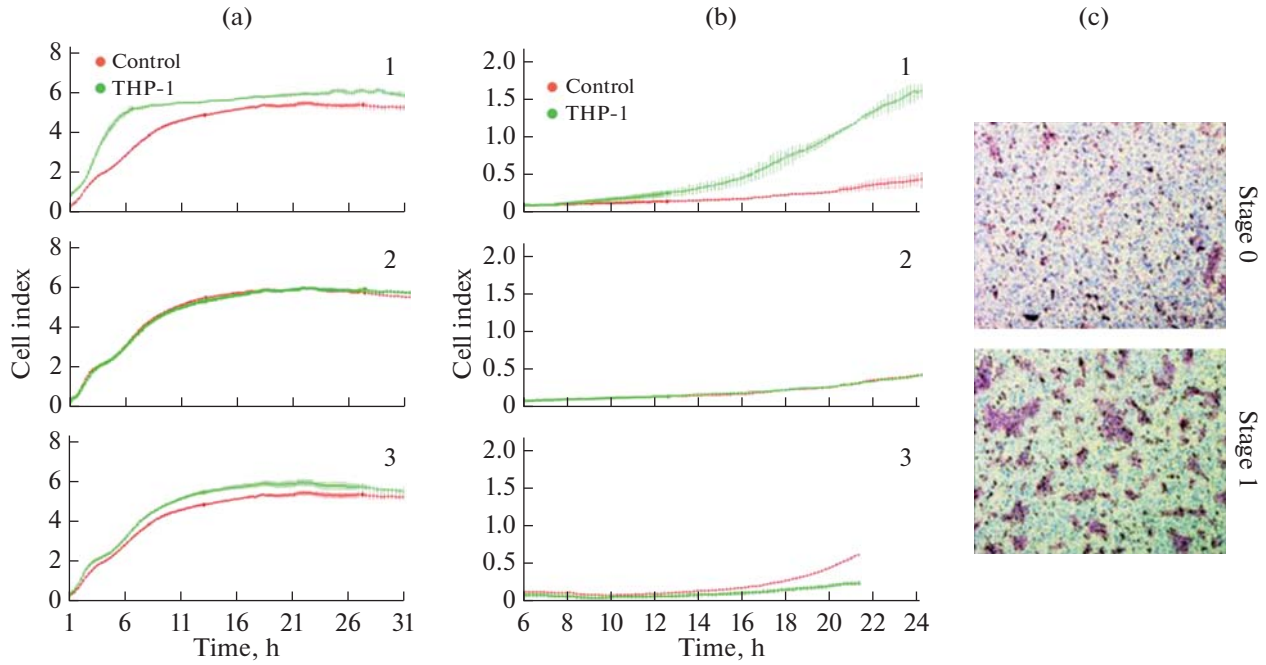

Fig. 4. Enhanced proliferation and migration of A431 cells after cocultivation with monocyte-like THP-1 cells. After cocultivation with THP-1 cells (stages 1, 2, 3), A431 cells were plated in (a) E-plates to assay proliferation or (b) CIM plates of xCELLigence equipment to analyze migration properties; (c) migration test with a Boyden chamber and cell staining with Giemsa dye.

naïve THP-1 cells with tumor cells replaced their phenotype for the protumor one, as they had increased expression of the M2 phenotype markers F4/80 and Arg-1. However, 26.1% of THP-1 cells died by apoptosis after stage 1 of cocultivation with A431 cells. The number of apoptotic cells is higher (42%) in cells with the F4/80 marker on their surface (Fig. 2). The number of apoptotic cells with F4/80 expression increased at stage 2 and reached 76% at stage 3 (Fig. 2). As a result, the total cell number with F4/80 marker expression steadily declined in the THP-1 population (Fig. 2b).

The analysis of cytokine profile in the medium of A431 and THP cocultures clarified the causes of mass macrophage death. Multiplex assay revealed that IL-1β, IL-6, IL-8, and TNF-α appeared in the culture medium at stage 1 (Fig. 3). Importantly, the cytokine profile contained TNF-α, a powerful apoptosis inducer that triggers the cell death program via the receptor TRAIL (TNF-related apoptosis-inducing ligand) (Fiorucci et al., 2005), Its level reached 120 pg/m. THP-1 cells have a high level of TRAIL expression (Wei et al., 2010; Liu et al., 2017), which makes them very sensitive to TNF-α. Supposedly, TNF-α release induces apoptosis in THP-1 cells. The apoptotic program was feasible at stage 1, 2, and even 3 when TNF-α was absent in the medium (Fig. 3). Presumably, the death of macrophages with a protumor phenotype was the cause of unaltered A431 properties during further cocultivation. Proliferative and migration properties of A431 cells associated with increased content of IL-1β, IL6, and IL8 (Fig. 3), as well as level of MMP-2 and MMP-9 (Fig. 3b), in the medium were increased only during the first contact of naïve tumor cells with macrophages (Fig. 4).

It is currently unknown whether the phenomenon described is universal and will be reproduced with other tumor cells. Taking into account that tumors are usually accompanied with chronic inflammation (Balkwill and Mantovani, 2001), which makes them a "nonhealing wound" (Dvorak, 1986), it can be suggested that tumor cells secrete cytokines that are important for the life and death of macrophages falling under influence of tumor cells. This means that TME elements, no less than tumor cells, should be a target for anticancer therapy.

ACKNOWLEDGMENTS

We are grateful to M. Prokhorova (Merck) and E. Belyaeva (Alamed) for the opportunity to use MagPix equipment and help in analysis of multiplex data.

This study was supported by the Russian Science Foundation, grant no. 14-50-00068.

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Translated by I. Fridlyanskaya