

FUNCTIONS OF TUMOR-ASSOCIATED MACROPHAGES AND MACROPHAGES RESIDING IN REMOTE ANATOMICAL NICHES IN EHRlich CARCINOMA BEARING MICE

T.V. Symchych, N.I. Fedosova, A.V. Chumak, N.L. Cheremshenko, O.M. Karaman, A.P. Burlaka,
I.M. Voyeykova, V.F. Chekhun*

*R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine, Kyiv
03022, Ukraine*

Background: The impact of growing tumor on polarization and functions of tumor-associated macrophages is well known while its influence on residential macrophages occupying different anatomical niches reminds to be elucidated. **Aim:** To study changes in polarization and functions of macrophages isolated from discrete anatomical niches in tumor-bearing mice at different stages of tumor growth. **Materials and Methods:** Ehrlich carcinoma was transplanted intramuscularly to Balb/c male mice. On days 7, 14, 21 and 28 after tumor transplantation, macrophages from tumor tissue, peritoneal cavity and spleen were isolated and analyzed. Nitric oxide production was measured by standard Griess reaction, arginase activity was determined by the measurement of urea, reactive oxygen species production was checked using NBT dye reduction assay and electron paramagnetic resonance spectroscopy, cytotoxic activity was estimated in MTT-assay. **Results:** Independently of their localization in different anatomic niches, macrophages in mice with transplanted Ehrlich carcinoma gradually lose their tumoricidal activities while arginase activity is upregulated. This indicates the shift of polarization from M1-like towards M2-like phenotype. **Conclusion:** Our findings demonstrated that growing tumor could be able to subvert functioning of macrophages at the systemic level.

Key Words: Ehrlich carcinoma, peritoneal macrophages, spleen macrophages, tumor-associated macrophages, polarization, functions.

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For a long time, macrophages were considered as the major players in anticancer defense. As a part of the natural immune response, macrophages take part in the cytotoxic elimination of cancer cells, presentation of tumor associated antigens, production of cytokines and regulation of immune reactions, etc. However, since the early 2000th the situation has changed. It appeared that except anticancer role macrophages often easily play another one — potent procancerous. Macrophages can favor tumor progression by promoting angiogenesis, invasion and metastasis and, on the other hand, by eliciting immune suppression [1]. Today the former ones are referred to as “classically activated” or M1 macrophages, while the latter are named “alternatively activated” or M2 macrophages. Therefore, the changes in macrophages polarization and as consequence their functions and role in cancer progression must be clearly understood and can be used for diagnostic and prognostic goals whereas manipulation of macrophages polarization possibly can be applied for disease treatment.

Tumor-associated macrophages (TAMs) form the most abundant and diverse part of the tumor-infiltrating leukocytes. Dependence of macrophage functions and polarization on the localization in the tumor tissue (for example, normal or hypoxic areas) or during tumor progression is quite well examined [2–4]. On the other hand, macrophages consist of very diverse populations

of tissue-resident macrophages, which differ by their functions, surface markers, pattern of response to stimuli, and the origin [5–8]. Tissue of residence strongly influences the functions and the fate of macrophages [9]. However, it is less understood whether macrophages of different anatomic zones are affected in tumor-bearing host. The aim of our research was to study changes in polarization and functions of macrophages isolated from the discrete anatomical niches in tumor-bearing mice at different stages of tumor growth.

MATERIALS AND METHODS

Animals and tumor transplantation. The study has been carried out on male Balb/c mice 2.0–2.5-month old weighting 20–21 g, bred at the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology. The use and care of experimental animals have been performed in accordance with standard international rules on biologic ethics and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes [10] and was approved by Institutional Animal Care and Use Committee. In total 32 animals were used, of which 12 were used as intact control, and 20 were transplanted with Ehrlich carcinoma cells. Ehrlich carcinoma cells ($5 \cdot 10^5$ cells/mouse) were injected i.m. into the right hind leg.

The scheme of the experiment. The mice (5 tumor-bearing and 3 intact per one observation point) were sacrificed on days 7, 14, 21 and 28 after the tumor transplantation. The macrophages of peritoneal cavity, spleen and tumor tissue were isolated and subjected to functional activity analysis. The reactive oxygen species (ROS) and nitric oxide (NO) production together with arginase (Arg),

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*Correspondence: E-mail: symchychtv@gmail.com

Abbreviations used: Arg – arginase; CTA – cytotoxic activity; CATI – cytotoxic activity index; EPR – electron paramagnetic resonance; NBT – nitroblue tetrazolium; NO – nitric oxide; ROS – reactive oxygen species; TAMs – tumor-associated macrophages.

cytotoxic and nitroblue tetrazolium (NBT) reduction activities of the macrophages were studied. Data on the tumor-bearing mice were compared with the intact mice of the same strain, sex and age (referred as the intact control).

Isolation of peritoneal macrophages. Mice were euthanized, and 3 ml of ice-cold phosphate-buffered saline supplemented with heparin (5 U/ml) were injected into abdomen. The fluid was withdrawn and abdomen was washed twice with the same volume of heparin-containing phosphate-buffered saline. The resulted cell suspension was centrifuged (550 g for 10 min), and the cell pellet was resuspended in 1 ml of 0.9% NaCl solution supplemented with 2.0% penicillin-streptomycin. These peritoneal exudate cells were counted and divided into four parts (cell count in each part depended on further technique which was planned to be performed) and cultured on 96-wells flat bottomed plates for 2 h (37 °C, 5% CO₂, 100% humidity). After that, non-adherent cells were removed, and the adherent cells were washed twice with 0.9% NaCl and taken for further investigation.

Isolation of splenic macrophages. Splenocytes were obtained from aseptically removed spleen by homogenizing with Potters homogenizer in medium 199 supplemented with 10% of bovine serum. Cell suspensions were placed on Ficoll-verografin gradient ($\rho = 1.077 \text{ kg/m}^3$) and centrifuged (550 g, 30 min). After two consecutive washings (medium 199, 550 g for 10 min), cell pellets were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and placed in the wells of 96-well flat bottomed plates for 2 h (37 °C, 5% CO₂, 100% humidity). Non-adherent cells were removed, and the adherent cells were washed twice with 0.9% NaCl and taken for further investigation.

Isolation of macrophages from tumor tissue. Tumor nodules were aseptically removed, shredded with scissors and homogenized using Potters homogenizer in medium 199 supplemented with 10% of bovine serum. After two consecutive washings (medium 199, 550 g for 10 min) cells were resuspended in medium 199, counted and $1 \cdot 10^6$ cells were placed in the wells of 96-well flat bottomed plates and cultured for 2 h (37 °C, 5% CO₂, 100% humidity). Non-adherent cells were removed, and the adherent cells were washed twice with 0.9% NaCl and taken for further investigation.

Cytotoxic activity (CTA) assay. CTA was determined by MTT-assay [11]. Ehrlich carcinoma cells were used as a target. In brief, target cells ($2 \cdot 10^4$ cell/well) in RPMI medium supplemented with 10% fetal bovine serum (all reagents from Sigma, USA) and antibiotics, were placed in a flat-bottom 96-well plates where macrophages ($4 \cdot 10^5$ cell/well) were adhered beforehand, and incubated for 18 h in a 100% humidity atmosphere with 5% CO₂ at 37 °C. Control wells contained target cells or adhered macrophages. Then 0.01 ml of MTT solution/well (5 mg/ml, Sigma, USA) was added, and incubation continued for 2 h. Then the plates were centrifuged (550 g for 15 min) and washed twice with 0.9% NaCl solution. After that, 0.12 ml of 2 M KOH and 0.14 ml of dimethyl sulfoxide (50% solution) were added into each well. Optical density was measured at $\lambda = 545 \text{ nm}$ vs $\lambda = 630 \text{ nm}$ using a mi-

croplate ELISA reader (StatFax-2100, USA). Each sample was done in triplicate.

Cytotoxic activity index (CTAI, %) was calculated by the formula:

$$CTAI = [1 - (OD_{\text{mph+tc}} - OD_{\text{mph}}) / (OD_{\text{tc}} - OD_{\text{blank}})] \cdot 100\%,$$

where OD_{mph} — optical density of wells in which only adhered macrophages were incubated; OD_{tc} — optical density of wells in which only tumor cells were incubated; $OD_{\text{mph+tc}}$ — optical density of wells in which tumor cells and macrophages were incubated; OD_{blank} — optical density of wells with the culture medium only.

Analysis of ROS production. ROS production by macrophages was checked using nitroblue tetrazolium (NBT) dye reduction test as described previously, with some modifications [12, 13]. In brief, macrophages ($1 \cdot 10^6$ cell/ml, 0.2 ml/well) were incubated with 0.02 ml/well of 0.2% NBT solution (Sigma, USA). After incubation (1 h, 5% CO₂, 37 °C), the plates were washed two times with 0.9% NaCl solution. The 2 M KOH solution (0.06 ml/well) and 50% dimethyl sulfoxide solution (0.07 ml/well) were used to dissolve diformazan granules. Optical density was measured at $\lambda = 630 \text{ nm}$ using a microplate ELISA reader (StatFax-2100, USA). Each sample was analyzed in triplicate. The results are expressed in optical units (OU).

Measurement of NO production. NO production was measured by standard Griess reaction [14]. In brief, cell suspensions ($2 \cdot 10^6$ cell/well) were placed in a volume of 200 μl in 96-well flat-bottom tissue culture plates and cultured for 24 h. Each cell culture was investigated in duplicate. At the end of the incubation period, supernatants were collected and NO production was assessed by the accumulation of nitrite (as stable metabolite of NO) by Griess reaction. An aliquot of culture supernatant (100 μl) was mixed with an equal volume of Griess reagent (Acros Organics, Belgium) and incubated for 1 h at room temperature in the dark. The reaction products were colorimetrically quantified at $\lambda = 550 \text{ nm}$. The standard curve plotted by the results of measurements of the solutions containing known concentration of NaNO₂ was used for converting the absorbance to micromolar concentrations of NO expressed in $\mu\text{M NO}_2^-$ per 10^6 cells.

Measurement of Arg activity. Arg activity was determined based on the measurement of urea [14, 15]. Macrophages were lysed by double freezing and melting. Then 50 μl of 50 mM Tris-HCl (pH 7.4) and 10 μl of 50 mM MnCl₂ were added to each sample. Samples were then heated at 56 °C for 10 min, and upon addition of 100 μl of 0.5 M L-arginine (pH 9.7) heated for further 30 min (37 °C). The reaction was stopped with 800 μl of acidic mixture (1:3:7, 96% H₂SO₄:85% H₃PO₄:H₂O). Then 40 μl of α -isonitrosopropiophenone (Sigma-Aldrich) was added to the solution, which was heated for 30 min (95 °C) and incubated for 30 min at 4 °C. Urea concentration was measured spectrophotometrically at $\lambda = 550 \text{ nm}$. Values of optical density were converted to mass of urea based on calibration curve of standard urea solution. Arg activity was calculated as described in [16]. One unit of Arg activity means the amount of the enzyme hydrolyzing 1 μM of arginine per 1 min. Results are expressed as units/ 10^6 cells.

Assessment of superoxide radical formation.

Formation of superoxide radical-anions was assessed by electron paramagnetic resonance (EPR) spectroscopy with the use of 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidine (Russia). The rate of superoxide radical-anions generation was expressed as nmol/g fresh tissue/min.

Mathematical and statistical analysis. In some cases, modulation index (MI) was calculated for better illustration of differences between the data on control and experimental groups:

$$MI = [(data_{\text{experiment}} - data_{\text{control}}) / data_{\text{control}}] \cdot 100\%,$$

where $data_{\text{experiment}}$ — results in the group of experimental tumor-bearing animals; $data_{\text{control}}$ — results in the group of intact mice.

The statistical analysis was conducted using Student's *t*-test. The difference was considered significant if $p < 0.05$; a case when $0.05 < p < 0.1$ was considered to show a tendency.

RESULTS AND DISCUSSION

To study how tumor growth influences polarization and functioning of residential macrophages localized in the remote anatomical niches of mice bearing Ehrlich carcinoma, the mice were sacrificed on days 7, 14, 21 and 28 after tumor transplantation and macrophages from spleen, peritoneum and the tumor were isolated. Functional activity of the isolated macrophages was examined.

Today, different techniques based on detection of cytokines, chemokines or surface markers are used for identification of macrophage polarization state [17] but the problem is that macrophages can manifest a so called mixed polarization type in which expression of surface markers or chemokines do not reflect the functions the macrophage is currently performing [18–20]. Moreover, expression of surface markers and cytokines rapidly changes during different time points following diverse type of stimulating factors [21] making this type of assessment not reliable. On the other hand, M1 and M2 macrophages differ in arginine pathway: M1 utilize arginine to produce NO while in M2 macrophages arginine is a precursor in ornithine synthesis. These two ways of arginine utilization are competitive [22] allowing us to distinguish between differently polarized macrophages. NO to Arg (NO/Arg) ratio may depict more clearly which way of arginine utilization is more active, thereby pointing to the type of the macrophage polarization.

As it was expected, in tumor-isolated macrophages, NO production (Fig. 1) was overwhelmed by the Arg activity (Fig. 2): NO/Arg ratio dropped from 38.4 on day 14 to 14.3 on day 28 of the tumor growth (Fig. 3). Of note, changes in NO/Arg ratio happened due to increased Arg activity, while NO production remained almost at the same level.

Independently of the source of isolation, macrophages of intact mice intensively produced NO while Arg activity was comparatively low; NO/Arg ratio was 11.8 and 10.9 in splenic and peritoneal macrophages, respectively. Transplantation of Ehrlich carcinoma induced statistically significant increase of NO production on day 7 after tumor challenge. The effect was evident in both splenic and peritoneal

macrophages (see Fig. 1). On day 14 after the tumor transplantation, production of NO by splenic and peritoneal macrophages significantly decreased compared to that on day 7 and remained at almost the same level till the end of the experiment (day 28). Even after mentioned sharp fall on day 14, production of NO by splenic macrophages was higher than that by peritoneal macrophages. This finding is in agreement with the results of Liu *et al.* [8] demonstrating that upon activation with LPS splenic macrophages produce considerably higher amounts of NO than their peritoneal counterparts do.

Arg activity was low on day 7 after tumor transplantation in both splenic and peritoneal macrophages (see Fig. 2). On day 14 and till the end of the experiment, Arg activity of the peritoneal macrophages dramatically increased and was significantly higher than that on day 7 in tumor-bearing group, in intact mice, and Arg activity of the splenic macrophages ($p < 0.05$ in all three cases). Arg activity of the splenic macrophages did not demonstrate such a radical change. It was below the intact control level till day 21 and significantly increased on day 28 though it did not exceed the intact control level.

NO/Arg ratio (see Fig. 3) in both splenic and peritoneal macrophages on day 7 after tumor transplantation was high (46.7 and 34.4 respectively). But with the tumor growth, this index in macrophages isolated from both sites was gradually falling and reached the lowest value on day 28 (13.4 for splenic and 7.2 for peritoneal macrophages). That is to say, that at early stages after tumor cell transplantation, the type of macrophage polarization was M1-like, but it turned to M2-like with tumor growth. Importantly, the changes in the macrophage polarization were observed not only in tumor tissue but at the systemic level and did not depend on the source of macrophage isolation — spleen or peritoneal cavity. Moreover, despite initial differences in functioning caused by the residential characteristics of macrophages (splenic macrophages produced more NO than peritoneal ones) the overall change of polarization was similar to both counterparts.

Then we assessed other activities of macrophages, which are known to diverge in differently polarized cells — CTA and production of ROS. In general, M1 polarized macrophages possess potent CTA towards different types of pathogens, infected, dying and transformed cells etc. Repolarization of the M1 macrophages to M2 type causes the loss of CTA [23]. In the experiment, the macrophage CTA was assessed in MTT-assay using Ehrlich carcinoma cells as a target.

CTA of the macrophages isolated from tumor tissue showed clear tendency to decline with progressive tumor growth (Fig. 4, a). As shown on Fig. 4, b, and 4, c, in the intact mice, CTA of the splenic or peritoneal macrophages did not differ significantly and were below 20.0% (19.2% and 14.7%, respectively). Tumor transplantation did not elicit rise in macrophage CTA. On day 7 and 14 after tumor transplantation, CTA of splenic or peritoneal macrophages did not differ from that of the intact mice. Progressive growth of the tumor (day 21 and 28) induced significant decrease in CTA of splenic and peritoneal macrophages compared to the intact mice level or day 14.

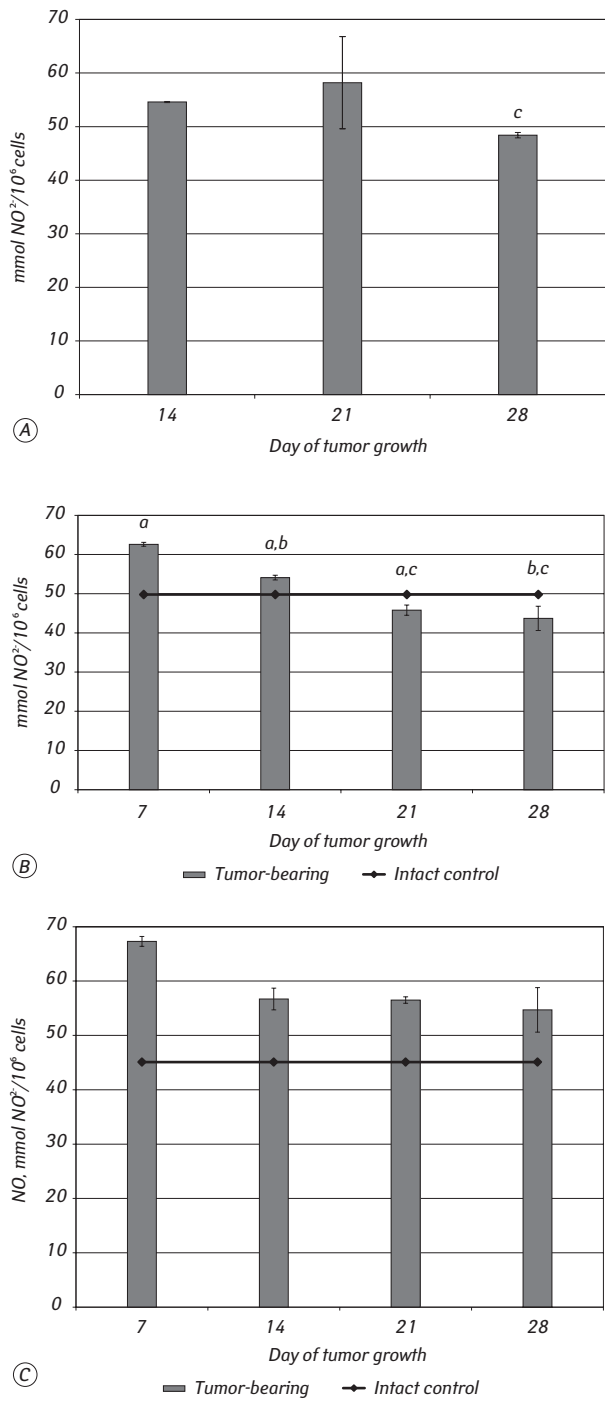


Fig. 1. Production of NO by tumor-isolated (A), peritoneal (B) and splenic (C) macrophages in Balb/c mice bearing Ehrlich carcinoma; $p < 0.05$ compared to the intact control group (a), day 7 of tumor growth (b), day 14 of tumor growth (c)

Therefore, independently on the isolation source CTA of macrophages decreased along with tumor progression. This finding goes in line with the decrease of the NO/Arg ratio evidencing on the repolarization of the macrophages during tumor growth in tumor tissue as well as in the body of the tumor-bearing host.

High production of ROS is another characteristic feature of the M1 polarized macrophages [23, 24]. Upon activation, M1 macrophages upregulate the NADPH production leading to increased ROS generation [25] which, in turn, are utilized to kill pathogens in phagosomes [26].

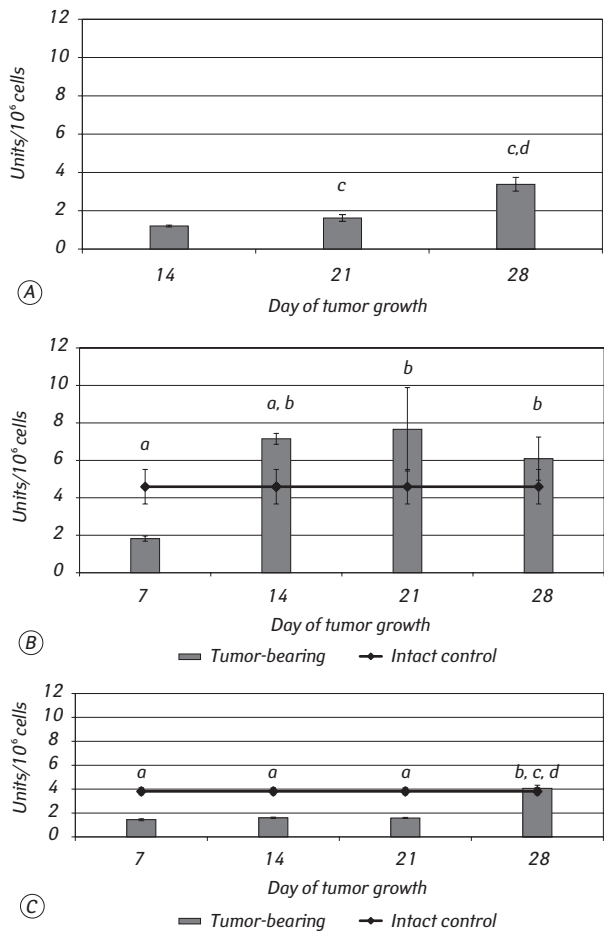


Fig. 2. Arg activity in tumor-isolated (A), peritoneal (B) and splenic (C) macrophages in Balb/c mice bearing Ehrlich carcinoma; $p < 0.05$ compared to the intact control group (a), day 7 of tumor growth (b), day 14 of tumor growth (c), day 21 of tumor growth (d)

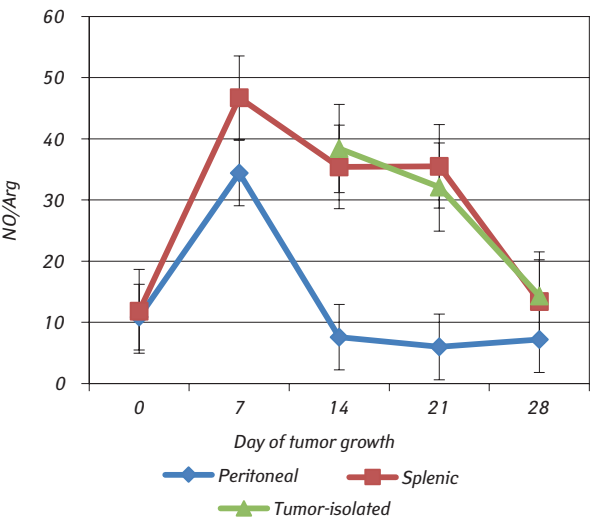


Fig. 3. Changes in NO/Arg ratio for tumor-isolated, peritoneal and splenic macrophages isolated in Balb/c mice bearing solid Ehrlich carcinoma

We used two techniques to study ROS production by macrophages — NBT reduction test and EPR spectroscopy.

The activity of macrophages assessed in NBT test is depicted in Fig. 5. Independently of the isolation source, it followed almost the same pattern: increased (splenic macrophages) or normal (peritoneal macrophages)

indices on day 7 after tumor transplantation and gradual decline during the further time points. The most evident rise in the activity was observed in splenic macrophages (on day 7, $p < 0.05$ compared to the intact control), the sharpest decrease in the activity was noted in the peritoneal macrophages (on day 28, $p < 0.05$ compared to the intact control). Therefore, the changes of the activity in NBT assay were similar to those of CTA activity and were dependent more on tumor progression rather than the macrophage isolation source.

As was detected by EPR spectroscopy, macrophages isolated from the tumor tissue intensively produced ROS on day 14 of tumor growth (Fig. 6, a). Production of ROS was by 140.0% and 79.0% higher than that produced by peritoneal and splenic macrophages of the intact mice, respectively. Nevertheless, on days 21 and 28, ROS production in intratumoral macrophages significantly dropped (compared to day 14) and was comparable with that produced by peritoneal and splenic macrophages of the tumor bearing mice at these time points.

Neither peritoneal, nor splenic macrophages produced high levels of ROS on day 7 and 14 after tumor transplantation (Fig. 6, b and 6, c). On day 21, production of ROS by macrophages isolated from peritoneum

and spleen decreased by 42% and 55% respectively compared to the intact mice level and was significantly lower compared to day 14 in the group. On day 28 ROS production by peritoneal macrophages was even lower (–70% of intact mice level) while splenic macrophages regain the ability to produce ROS (+33% as compared to that on day 21).

Monocyte-macrophage lineage represents a very heterogeneous leukocyte population widely spread across the body. These cells occupy distinct anatomical compartments and perform different functions. Predominantly tissue macrophages originate from embryonic precursors and can self-maintain by mostly local proliferation [5, 27]. TAMs are thought to originate merely of bone marrow derived blood monocytes [28, 29], which are recruited by the developing tumor. Tumor-produced substances and tumor-induced milieu subvert functions of macrophages so that they switch towards procancerous activity. Whereas the tumor affects the functions of macrophages resident in different organs distant from the primary tumor lesion is less understood. We used peritoneal and splenic macrophages as examples of different residential macrophages, which display considerable phenotypic and functional heterogeneity [5] and different pattern of response to activation stimuli [8].

It is known that functions and fate of residential macrophages are strongly influenced by the tissue of residence [9]. Even monocytes, upon entering tissue, can acquire tissue-specific transcriptomic identity of the

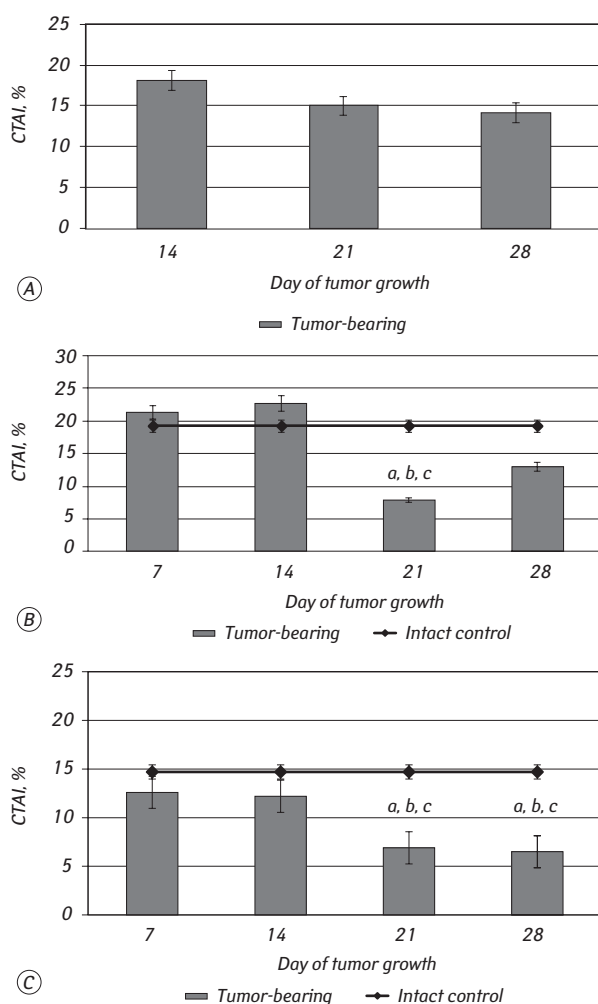


Fig. 4. Cytotoxic activity of tumor-isolated (A), peritoneal (B), and splenic (C) macrophages in Balb/c mice bearing Ehrlich carcinoma. $p < 0.05$ compared to the intact control group (a), day 7 of tumor growth (b), day 14 of tumor growth (c)

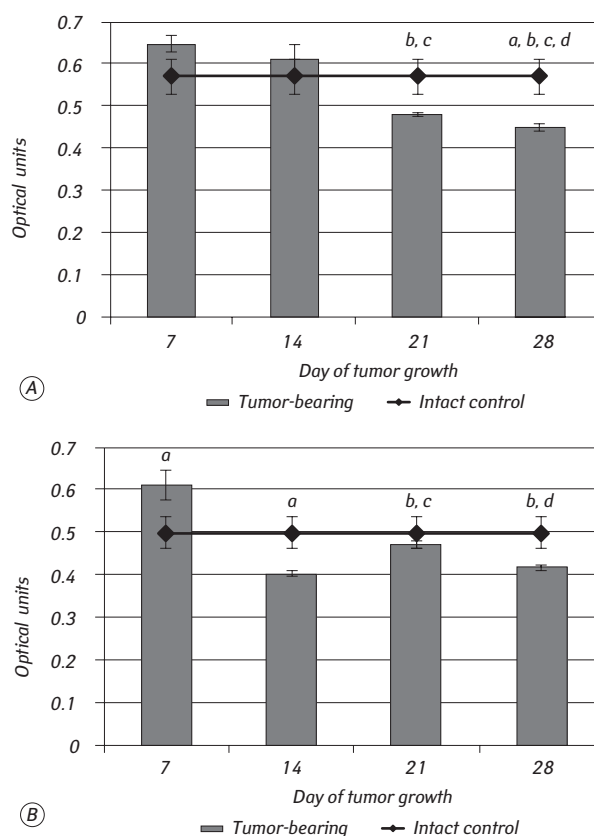


Fig. 5. ROS production by peritoneal (A), and splenic (B) macrophages in Balb/c mice bearing Ehrlich carcinoma as determined by NBT reduction assay. $p < 0.05$ compared to the intact control group (a), day 7 of tumor growth (b), day 14 of tumor growth (c), day 21 of tumor growth (d)

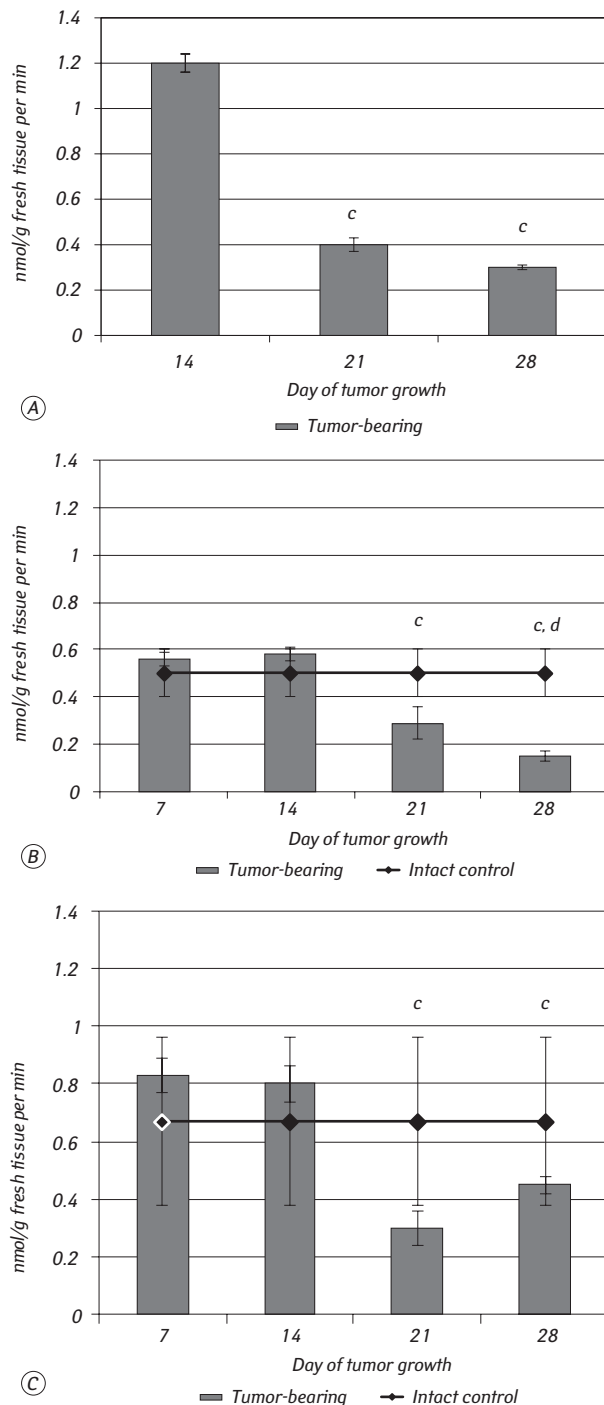


Fig. 6. ROS production by tumor-associated (A), peritoneal (B) and splenic (C) macrophages in Balb/c mice bearing Ehrlich carcinoma as revealed with EPR spectroscopy; $p < 0.05$ compared to day 14 of tumor growth (c), day 21 of tumor growth (d)

tissue-resident macrophages [30]. The results of our experiment showed that tumor influence is more potent than that imposed by the tissue of residence: independently of their anatomical niches, macrophages gradually lose their CTAs possibly acquiring protumoral ones (as can be concluded from increased Arg activity). Splenic macrophages are known as the cells expressing higher levels of MHC-II and co-stimulatory molecules [8] and being more functionally heterogeneous [5] than peritoneal ones, and they changed their functional activities comparably with their peritoneal counterparts. Moreover,

changes of macrophage activity appeared at relatively early stages of tumor growth— on day 14 after tumor transplantation — and were detectable in macrophages isolated from different niches. There is some evidence that reprogramming of macrophages to the opposing phenotype is dependent on the initial polarization state, and macrophages polarization toward M2 type (at least in *in vitro* settings) is much more easily achievable process than *vice versa* [31]. Taking into account that tumor can relatively early influence the fate of TAMs and residential macrophages, to be efficient, the interventions aimed at influencing macrophages activity should be attempted as early as possible or after primary tumor resection.

Considering that tissue macrophages appear to be nonmigratory cells [5], similar changes in functioning of peritoneal, splenic and intratumoral macrophages reflect the overall influence of the growing tumor on its host. At the same time, the factors influencing TAMs polarization are studied quite well [1]. On the other hand, splenic and peritoneal macrophages are located in distant anatomical niches; therefore it should be elucidated, which tumor-induced factors can guide their polarization, and whether these factors are the same as in the case of TAMs. Obviously, tumor influences the functions of residential macrophages through soluble factors. Some clues can be found in the experiments of DiNapoli *et al.* [32] showing that the decreased CTA of peritoneal macrophages is associated with altered NO production and *iNOS* gene expression which, in turn, can be induced by increased levels of tumor-derived GM-CSF [33, 34] and simultaneous decrease of IFN-gamma production by T lymphocytes in tumor-bearing mice [35]. PGE_2 was not involved in downregulation of macrophage CTA [33]. Further research in this field is important because growing number of data indicate that macrophages can contribute to metastases taking part in forming so called premetastatic niche [36, 37] (of note, in some circumstances Ehrlich carcinoma metastasizes into abdominal lymph nodes and spleen [38]). Therefore, neutralizing the factors undesirably influencing functions and polarization of residential macrophages can improve the results of cancer treatment via reducing the immunosuppressive influence of tumor and, possibly, slowing metastasis.

The results of our study showed that tumor growth negatively influence functioning of intratumoral macrophages as well as residential macrophages isolated from distant anatomical niches — peritoneal cavity and spleen. Independently of their isolation site, macrophages lose their tumoricidal properties (CTA, NO and ROS production) acquiring M2-like polarization type.

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