

THE EFFECT OF INTRANASALLY ADMINISTERED TLR3 AGONIST LARIFAN ON METABOLIC PROFILE OF MICROGLIAL CELLS IN RAT WITH C6 GLIOMA

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Glioma-associated microglia/macrophages (GAM) represent an attractive therapeutic target for the development of the alternative methodology in the treatment of gliomas. This study was aimed to investigate the effect of intranasally administered TLR3 agonist Larifan on microglial cell metabolic profile in rats with C6 glioma. Our results demonstrate progressive generation microglial cell population with immunosuppressive and pro-inflammatory properties in C6 glioma-bearing brain. Intranasally delivered TLR3 agonist is capable to abrogate the creation of this pro-tumoral immune infiltrates, probably, through the effect on myeloid-derived suppressor cells, and can be considered as a promising agent for glioma therapy aimed the GAM re-education.

Key words: glioma, microglia, toll-like receptor agonist, immunotherapy.

Gliomas account for more than 70% of primary brain tumors, and the vast majority of those tumors are highly malignant glioblastomas. Glioblastomas (GB) are extremely aggressive brain tumors with a very poor prognosis [1]. In spite of substantial advances that were achieved over the past several decades in the treatment of many malignant neoplasms, GB remains essentially untreatable [2]. Meaning, that most therapeutic approaches targeting tumor cells have failed. GB is heavily infiltrated with myeloid cells, mainly brain-resident microglia and peripheral phagocytes, that are collectively referred to as glioma-associated microglia/macrophages or “GAM” [3, 4]. Up to 30% of the cells in GB tissue are GAM [5]. GB cells promote metabolic shift of GAM to the immunosuppressive, proinvasive phenotype with the expression of M2 markers: arginase 1 (Arg1), interleukin 10, CD206 etc. These polarized GAM play a crucial role for GB sustained growth and invasion [6]. Unlike cancer cells, stromal cells within the tumor microenvironment including GAM are genetically stable and therefore represent an attractive therapeutic target

with minimum risk of resistance and/or tumor recurrence. There are currently two types of methodological approaches in the glioma treatment targeting GAM. First approach aims physical elimination of GAMs and abolishment their protumoral effects. There are reports concerning the successfully applied GAM ablation for the cancer treatment [7]. However, not all GAM subpopulations are protumoral, therefore complete GAM elimination may be undesirable. The second approach aims to re-educate GAM or reprogram them toward a tumour-suppressive phenotype. One can suggest that re-education of these cells, rather than ablation per se, may be an effective strategy for treating GB [8, 9]. Different substances are considered as a polarizing agents for tumor-associated phagocytes, including agonists of pattern-recognizing receptors, like CpG oligodeoxynucleotides (CpG-ODN), polyinosinic:polycytidylic acid (polyI:C), toll-like receptor (TLR) 9 ligand, TLR 7/8 agonists etc [10, 11]. Among others, TLR3 agonists are considered “safe” agonists for the use in GB immunotherapy, because as a result of their use, the tumor is generally inhibited in the absence of tu-

mor promotion [12]. TLR3 agonists up-regulate expression of both programmed death-ligand (PD-L)1 and PD-L2 on glioma cells followed by cell cycle arrest and apoptosis induction and prime the tumor microenvironment for antitumor immune response [13, 14].

There are two main routes for the drug delivery to brain tumor: direct-to-brain and systemic. However, direct-to-brain drug delivery has serious limitations due to its traumatic effect. The blood-brain barrier (BBB) is a substantial obstacle for the systemic introduction of the drugs including immunotherapeutic preparations. Intranasal route is considered as an alternative way. This route has a number of advantages: BBB ceases to be an obstacle, the method is noninvasive and is easily repeatable [15, 16]. This study was aimed to investigate the effect of intranasally delivered TLR3 agonist Larifan on microglial cell metabolic profile in rats with C6 glioma. Larifan contains TLR3 agonist - natural origin dsRNA. The preparation has an interferonogenic and immunomodulatory properties. It exhibited an antitumoral effect in the investigations with experimental tumor models [17, 18].

Materials and Methods

Animals. C6 glioma model. Study design. *In vivo* studies were performed on Wistar male rats ($n = 30, 40-50$ g). Animals were bred in the vivarium of the Educational and Scientific Centre "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv, and were maintained on a standard diet at 25 ± 1 °C. Experiments were conducted in accordance with the standards of the Convention on Bioethics of the Council of Europe's 'Europe Convention for the Protection of Vertebrate Animals' used for experimental and other scientific purposes (1997), the general ethical principles of animal experiments, approved by the First National Congress on Bioethics in Ukraine (September 2001). Animal protocol was reviewed and approved by the Taras Shevchenko National University animal welfare committee according to the Animal Welfare Act guidelines.

The C6 glioma tumor cell line was kindly provided by National Bank of Cell Lines and Transplanted Tumors of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of National Academy of Sciences of Ukraine. The cells were cultured *in vitro* in RPMI medium (Sigma, USA) supplemented with 10% fetal calf serum,

2 mM l-glutamine and 40 mg/ml gentamycin at 37 °C in humidified atmosphere with 5% CO₂. Before the surgery, rats were anesthetized (intraperitoneally) with ketamine (0.1 mg/g animal) and sedazine (0.02 mg/g animal). Tumor cells (5×10^5 in the volume of 50 µl) were transplanted intracerebrally in the left parietal zone (the anterior lobe of the left lateral ventricle) at a distance of 3 mm from sinus sagittalis to avoid intracranial bleeding, according to a stereotaxic atlas. After transplantation of tumor cells, the experimental animals were randomized by weight and distributed in three groups with ten animals per group: group 1 – intact animals, group 2 – animals with transplanted C6 glioma, group 3 – animals with C6 glioma and injected intranasally with Larifan (Larifan Ltd) at a dose of 200 µg daily for 23 days. Animal behavior was monitored daily, body weight for each rat was recorded 3-5 times a week. In a day after the cessation of Larifan introduction (on 24th day after tumor cell inoculation) animals were sacrificed. C6 glioma cell cycle analysis and microglia cell phenotype examination were conducted on 14th and 24th days after tumor cell transplantation. Metabolic characteristics (phagocytosis and oxidative metabolism) of microglia were examined at the time of experiment cessation (24th days after tumor cell transplantation).

Cell cycle assay. Cell cycle and DNA content analysis was conducted using the BD Cycletest Assay according to the manufacturer's instruction (BD Biosciences, San Jose, CA). To analyze cellular DNA content by flow cytometry, isolated from tumor tissue C6 glioma cells ($>10^6$ cells) were washed in phosphate-buffered saline (PBS) twice. After this, cells were suspended in 1 ml of propidium iodide/Triton-X 100 staining solution (20 µg/ml PI, 0.1% Triton-X 100, 0.2 mg/ml ribonuclease A (RNase A, Sigma) in PBS) and incubated 30 min at room temperature in the dark. The DNA content was then analyzed by flow cytometry (FACScan, BD Bioscience). The proliferation index (PI) was used to present cell proliferation activity $PI (\%) = S + G2 + M$.

Microglia cell isolation. Microglia cells were isolated on day 14 and day 24 of the experiment as described by Matthew G. Frank [19] with slight modifications. Rats were euthanized by i.p. injection of 200 µl pentobarbital-sodium (Narcoren, Pharmazeutischen Handelsgesellschaft), brain was rapidly extracted on ice, hippocampus was dissected and perfused using a phosphate buffered saline (PBS). Isolated tissue was gently dissociated in

ice cold PBS supplemented with 0.2% glucose for 15 min at room temperature with the use of Potter homogenizer. Homogenate was filtered through a 40 nm cell strainer (BD Biosciences Discovery) for additional tissue shredding and then was transferred to a 15 ml tube and centrifuged at 350 g for 10 min at room temperature. Homogenate was then suspended in 1 ml of 70% isotonic Percoll solution. 1 ml of 50% Percoll solution was softly layered on top of the 70% layer, and 1 ml of PBS solution was then layered on top of the 50% Percoll layer. Density gradient was centrifuged for 40 min at 1200 g. After centrifugation, the layer at the interface between the 70% and 50% Percoll phases contained highly enriched microglia was aspirated and cells were washed twice in PBS by centrifugation. Purity of isolated microglia was estimated by flow cytometry with the use of fluorescein isothiocyanate (FITC) mouse anti-rat CD11b (BD Pharmingen™) and phycoerythrin (PE) mouse anti-rat CD45 (BD Pharmingen™). The proportion of CD11b+CD45+ cells was $89.79 \pm 2.47\%$. Cell viability was determined by Trypan blue exclusion test. The proportion of viable cells was $\leq 92\%$.

ROS assay. Reactive oxygen species (ROS) levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA, Invitrogen) [20]. Carboxy-H₂DCFDA is converted to impermeable oxidized green-fluorescent form by the activity of ROS the cell. 2×10^5 microglial cells were incubated with PBS containing 10 μ M carboxy-H₂DCFDA for 30 min at 37 °C in the dark. The cells were then transferred to polystyrene tubes with cell-strainer caps (Falcon, Becton Dickinson) and analysed with flow cytometry (excitation: 488 nm, emission: 525 nm). Only living cells, gated according to scatter parameters, were used for the analysis.

Phagocytosis assay. The flow cytometry phagocytosis assay was performed as described by Cantinieaux et al. [21] with slight modifications. *Staphylococcus aureus* Cowan I cells (collection of the Department of Microbiology and Immunology of ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv) were grown on beef-extract agar and subsequently were heat inactivated and FITC labeled. The stock of FITC-labeled *S. aureus* at a concentration of 1×10^7 cells/ml in a volume of 5 μ l was added to 2×10^5 microglial cells. A tube with cells only served as a negative control. All probes were incubated at 37 °C for 30 min. At the end of the assay, phagocytosis was arrested by the addition of cold stop solution (PBS

with 0.02% EDTA and 0.04% paraformaldehyde). Fluorescence of phagocytes with ingested bacteria was determined by flow cytometry. The results were registered as phagocytosis index that representing the mean fluorescence per one phagocytic cell (ingested bacteria by one cell).

Immunofluorescence labeling. PE anti-CD80 antibodies and Alexa Fluor 647 labeled anti-CD206 antibodies (Abcam) were used to determine the mean fluorescence intensity (MFI) of CD206 and CD80 on microglial cells on 14th and 24th days of experiment. The antibodies were added (5 μ l) to the samples (50 μ l). The cells were incubated for 25 min at room temperature. Samples were then analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed using CELLQuest software (BD; Franklin Lakes, NJ, USA).

Statistical analysis. All experimental results are reported as mean \pm SD. Statistical significance of the results was determined by Student's *t*-test. Means were compared and differences were considered significant at $P < 0.05$ or less.

Results and Discussion

Intranasal Larifan delivery affect C6 glioma cell cycle. Taking into account the fact that there are no unambiguous recommendations regarding dosing and time schedule for the use of TLR agonists in cancer treatment [22, 23], we have used prolonged treatment schedule accompanied by the examination of DNA state of C6 glioma cells isolated from tumor tissue as well as by the investigation of phenotypic profile of GAM at different time points within the course of treatment. Additionally, we have characterized the effect of intranasally delivered TLR3 agonist on animal behavior and body weight. For the first 14 days of C6 glioma growth, tumor-bearing animals were characterized by lowered body weight in comparison with intact animals. Docile behavior and decreased activity were also observed. All rats treated with Larifan had active behavioral state, their body weight was moderately higher than that in their untreated counterparts until 14th day of observation (Fig. 1). The first deaths ($n = 2$) were registered in the control group on 14th day in contrast to the Larifan-treated group, where the onset of animal death ($n = 2$) was observed on day 20 of the experiment (data are not presented). At the end of the observation period (24th days after tumor cell transplantation), statistically significant differences between

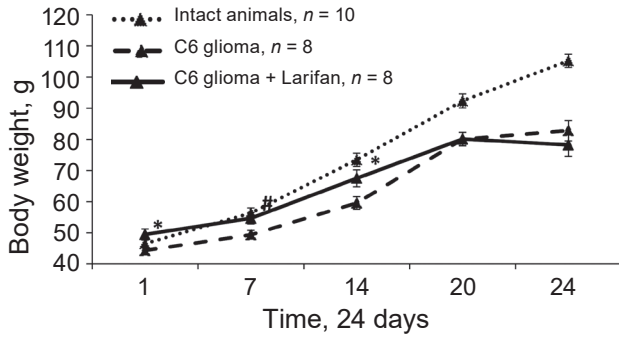


Fig. 1. Body weight of C6 glioma bearing rats treated with Larifan. 5×10^5 C6 glioma cells were intracranially implanted on day 0. Starting from day 1 to day 23, C6 glioma-bearing rats were treated i.n. with PBS (C6 glioma group) or Larifan at a dose of 200 μ g daily (C6 glioma + Larifan group). * The differences are statistically valid compared to the intact animals (*t*-test; $P < 0.05$); # the differences are statistically valid compared to C6 glioma group (*t*-test; $P < 0.05$)

body weight of animals in control tumor-bearing and treated groups has not been established (Fig. 1). All tumor-bearing animals presented instability in walking, hemiplegia, cachexia and eye conjunctival congestion which are typical for quickly growing C6 glioma [24].

Analysis of DNA state in C6 glioma cells revealed that treatment with Larifan was associated with significant decrease of proliferative fraction in tumor tissue: PI in treated animals was 3.8 times lower than that in control tumor-bearing rats at day 14, and 1.9 times lower – at 24th day (Table, Fig. 2). As TLR3 is frequently expressed by various types of malignant cells and can directly trigger tumor cell apoptosis, we can't exclude the direct antiproliferative effect of Larifan on C6 glioma cells [25].

In addition, the inhibition of C6 glioma proliferative activity can be stipulated by the effect of the drug on tumor microenvironment including GAM [26].

Phenotypic profile of microglial cells in C6-glioma bearing rats in the course of treatment with Larifan. As mentioned above, GAM, like other tumor-associated phagocytes, display M2-like functional profile, that is characterized by reduced functional maturity (decreased CD14 expression), increased expression of immunosuppressive cytokines, up-regulated arginase activity along with lowered NO production [27, 28]. According to general ideas, these cells are not efficient at antigen presentation, and express low levels of MHC a co-stimulatory molecules. In accordance with data from different research groups, they can exhibit reduced or augmented phagocytic activity along with up-regulated expression of M2 phenotypic markers such as the mannose receptor (CD206) and the hemoglobin/haptoglobin scavenger receptor (CD163) [29, 30].

In our experiments, distinct expression of key M1 and M2 phenotypic markers on GAM have been registered at different time points of the course of glioma growth (Fig. 3, A and B). Phenotypic profile of microglial cells were affected by the treatment with Larifan. Microglial surface expression level of CD206 in C6 glioma-bearing rats on day 14 after the tumor cell transplantation was 2.7 times higher than that in intact animals (Fig. 3, A). Treatment with Larifan, that was associated with substantial reduction of C6 glioma cell proliferative activity at this time point, was accompanied by the significant decrease of surface expression of this M2 marker in C6 glioma-bearing animals. By the 24th day, the difference between levels of CD206 expression in microglia of intact animals and animals with C6 glioma was less significant (Fig. 3, B). In animals treated with Larifan, expression of this M2 marker

DNA state of microglial cells in C6 glioma-bearing rats treated with Larifan

Animal group	G0/G1, %	G2/M, %	S, %	S + G2/M, %
<i>14th day after C6 glioma cell transplantation</i>				
C6 glioma, n = 8	89.82 ± 1.25	5.14 ± 0.12	5.04 ± 1.125	10.18 ± 1.245
Larifan-treated, n = 8	97.99 ± 0.69*	1.08 ± 0.28*	1.34 ± 0.45	2.70 ± 0.14*
<i>24th day after C6 glioma cell transplantation (end of experiment)</i>				
C6 glioma, n = 8	85.85 ± 0.42	6.31 ± 1.10	7.83 ± 1.52	14.14 ± 0.42
Larifan-treated, n = 8	93.50 ± 1.06*	2.40 ± 0.24	4.06 ± 1.27	7.51 ± 0.18*

* The differences are statistically valid compared to the intact animals (*t*-test; $P < 0.05$)

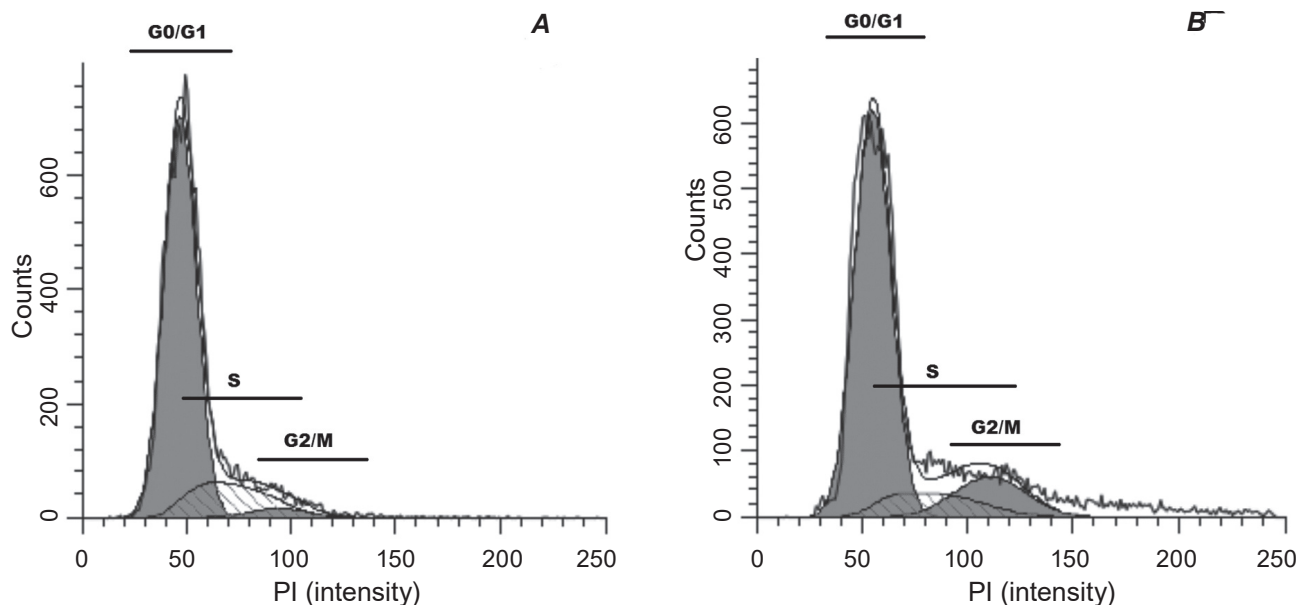


Fig. 2. Representative histogram overlay for DNA state of C6 glioma-bearing rats treated with Larifan. **A** – control tumor-bearing rats; **B** – tumor-bearing rats treated with Larifan

didn't differ significantly from that of untreated tumor-bearing rats at this time point. It was associated with lowered antiproliferative effect of the drug and was accompanied by the onset of mortality of animals. One can suggest, that at this time point there is a redistribution of cell populations in the microglia with a decrease in the fraction of the alternatively activated (M2) CD206⁺ mononuclear phagocytes.

Surface expression of co-stimulatory molecules CD80 is mainly considered as a characteristic

of M1 functional skew of activated microglial cells with tumor-suppressive properties. Overexpression of CD80 is commonly associated with down-regulation of M2 markers such as CD206 [31]. Unexpectedly, our experiments revealed significant progressive increase of surface microglial expression of CD80 in C6 glioma-bearing animals along with overexpressed CD206 (Fig. 3). Treatment with Larifan, that was associated with the retardation of C6 glioma cell proliferation, was accompanied by the

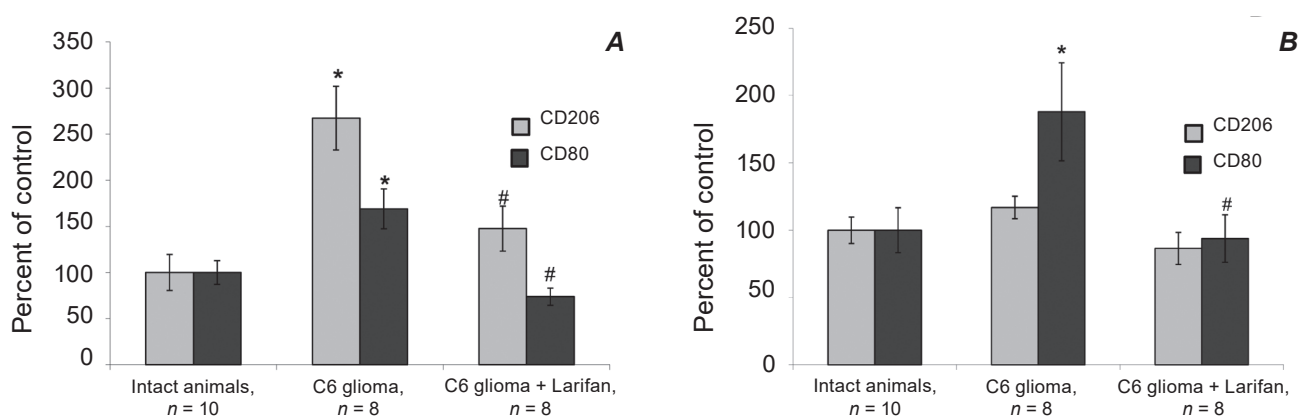


Fig. 3. Surface expression of CD206 and CD80 by microglial cells of C6 glioma-bearing rats after treatment with Larifan. Microglial cells were isolated at 14th (**A**) and 24th (**B**) days after the tumor cell inoculation, and were examined for mentioned marker expression by flow cytometry. The results are expressed as the percentage of control; microglial cells from intact animals with an MFI of 153.9 for CD206 and 639.8 for CD80 were defined as 100%. * The differences are statistically valid compared to the intact animals (t-test; $P < 0.05$); # the differences are statistically valid compared to the untreated tumor-bearing animals (t-test; $P < 0.05$)

substantial reduction of CD80 surface expression. Immune infiltrates of glioma-bearing brain is reported contain three subpopulations of CD11b+CD45+ myeloid cells: resident CD11b+CD45^{low} microglia cells, recruited CD11b+CD45^{high} macrophages and CD11b+CD45^{high}Gr1+ myeloid-derived suppressor cells (MDSC) [32, 33]. Though, just MDSC are characterized by the CD80 overexpression in tumor microenvironment, and this cell fraction rises gradually in the course of tumor growth [34]. Within this experiments, we were unable to differentiate mentioned above three subpopulation of microglial cells in C6 glioma-bearing rat brain.

However, one can assume that CD80 overexpression in analyzed microglia cells occurred at the expense of increased MDSC fraction. Further studies are warranted to confirm this assumption. Our assumption is indirectly supported by the results from animals which were treated with Larifan. Surface microglial expression of CD80 in animals from this group was significantly lower than that in untreated animals. CD80 expression is one of the markers that correlate with suppressive function of MDSC. Down-regulation or preventing CD80 expression on MDSC delay tumor growth [35]. It has been shown that TLR3 agonists provoke the conversion of tumor-associated MDSC into mature macrophages with tumor-suppressive properties and down-regulated co-stimulatory molecule expression [36, 37]. In our experiments, treatment with TLR agonists Larifan was also associated with the retardation of proliferative activity of tumor cells along with the decrease of CD80 expression level in the population of microglial cells. It provides grounds to

assume treatment with Larifan exerts negative effect on MDSC.

ROS generation and phagocytic activity of microglial cells in C6-glioma bearing rats after treatment with Larifan. ROS generation is linked to the classical (pro-inflammatory and tumor-suppressive) microglia activation [38, 39]. At the same time, ROS production is one of the key immunosuppressive mechanisms of MDSC. Increased ROS generation in MDSC is a result of up-regulated activity of NADPH oxidase in these cells, that in turn is mediated by tumor-derived factors [35, 40]. In our experiments, ROS generation was significantly elevated in C6 glioma-bearing rats in comparison with intact animals (Fig. 4, A). Treatment with Larifan resulted in down-regulation of this process. As mentioned above, systemically introduced TLR3 agonists are capable to induce the differentiation of MDSC into mature macrophages with the loss of immunosuppressive properties [36]. Such differentiation is accompanied, among other things, by a decrease in the NADPH oxidase activity and, as a consequence, by a decrease in the ROS synthesis [41]. One can suggest, that ROS decrease in microglia cells from tumor-bearing rats treated with Larifan is stipulated by MDSC inhibition.

Phagocytosis is one of the fundamental properties of all subpopulations of GAM. According to “dead cell clearance hypothesis”, pro-tumoral phenotype of tumor-associated phagocytes including GAM is characterized by the increased phagocytic activity [42]. Moreover, increased phagocytic activity is characteristic for MDSC and correlates with overexpression of PD-L1 and immunosuppressive

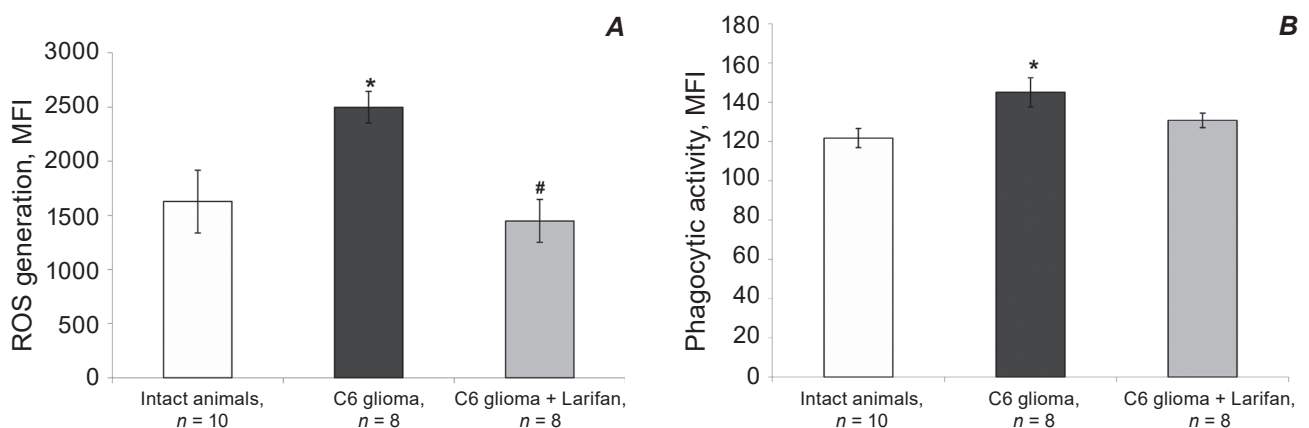


Fig. 4. ROS generation (A) and phagocytosis (B) in microglial cells of C6 glioma-bearing rats after treatment with Larifan. * The differences are statistically valid compared to the intact animals (*t*-test; $P < 0.05$); # the differences are statistically valid compared to the untreated tumor-bearing animals (*t*-test; $P < 0.05$)

properties of these cells [43]. In our experiments, phagocytic activity of C6 glioma-bearing animals was moderately higher than that in intact rats (Fig. 4, B). Treatment with Larifan resulted in slight decrease of microglia phagocytosis. Along with other results, it can indicate the overcome of immunosuppressive properties of GAM.

Our results demonstrate progressive generation microglia population with immunosuppressive and pro-inflammatory properties in C6 glioma-bearing brain as indicated by phenotypic and functional indices of these cells. We postulate that MDSC predominate in this heterogenous cell population. This is in accordance with the findings of Brandenburg et al. [33] as well as Gieryng with colleagues [44]. Intranasally delivered TLR3 agonist Larifan is capable to abrogate the creation of this pro-tumoral immune infiltrates, probably, through the effect on MDSC, and can be considered as a promising agent for GB therapy aimed the GAM re-education. Our results also indicate that short-term (not more than 14 days) course of TLR agonist is more effective than prolonged one (in our experiment – for 23 days) as positive alteration in phenotypic and functional characteristics of analysed microglial cells as well as statistically significant decrease of tumor cell PI were registered on day 14 of the experiment. The determination of most effective treatment schedule as well as the estimation of the efficacy of Larifan use in combined therapy of C6 glioma are planned in our further studies.

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All authors listed have contributed sufficiently to the project to be included as authors. All authors approved the manuscript and this submission. To the best of our knowledge, no conflict of interest, financial or other, exists.

ЕФЕКТ ІНТРАНАЗАЛЬНОГО ВВЕДЕННЯ TLR3 АГОНІСТА ЛАРИФАНУ НА МЕТАБОЛІЧНИЙ ПРОФІЛЬ МІКРОГЛІАЛЬНИХ КЛІТИН ЩУРІВ ІЗ ГЛІОМОЮ С6

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Гліомаасоційовані мікроглія/макрофаги (ГАМ) є привабливою терапевтичною мішенню для розробки альтернативного методу лікування гліом. Метою роботи було дослідження впливу інтраназального введення агоніста TLR3 препарату Ларифан на метаболічний профіль мікрогліальних клітин щурів із гліомою С6. Результати проведених нами досліджень засвідчують прогресивне формування в головному мозку в умовах розвитку гліоми популяції мікрогліальних клітин з імуносупресивними та прозапальними властивостями. Інтраназальне введення зазначеного агоніста перешкоджає формуванню імуного інфільтрату з імуносупресивними властивостями, імовірно, за рахунок впливу на міелоїдні супресорні клітини. Це дає підстави розглядати препарат Ларифан як перспективний агент для лікування гліом, спрямованого на зміну метаболічного профілю ГАМ.

Ключові слова: гліома, мікроглія, агоніст рецептора, імуноterapia.

**ЭФФЕКТ ИНТРАНАЗАЛЬНОГО
ВВЕДЕНИЯ TLR3
АГОНИСТА ЛАРИФАНА НА
МЕТАБОЛИЧЕСКИЙ ПРОФИЛЬ
МИКРОГЛИАЛЬНЫХ КЛЕТОК
КРЫС С ГЛИОМОЙ С6**

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Глиомаассоциированные микроглия/макрофаги (ГАМ) являются привлекательной терапевтической мишенью для разработки альтернативного метода лечения глиом. Целью работы было исследование влияния интраназального введения агониста TLR3 препарата Ларифан на метаболический профиль микроглиальных клеток крыс с глиомой С6. Результаты проведенных нами исследований засвидетельствовали прогрессивное формирование в головном мозге в условиях роста глиомы популяции микроглиальных клеток с иммуносупрессивными и провоспалительными свойствами. Интраназальное введение означенного агониста TLR3 препятствует формированию иммунного инфильтрата с иммуносупрессивными свойствами, вероятно, за счет влияния на миелиоидные супрессорные клетки. Это дает основания рассматривать препарат Ларифан в качестве перспективного агента для лечения глиом, направленного на изменение метаболического профиля ГАМ.

Ключевые слова: глиома, микроглия, агонист рецептора, иммунотерапия.

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