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IMMUNE MONITORING OF DENDRITIC CELL-BASED CANCER VACCINE IN GLIOBLASTOMA PATIENTS

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The purpose of the study was to define the most informative immunological criteria for assessing the immunotherapy efficacy applying dendritic cell-based cancer vaccine in glioblastoma patients. Dendritic cell-based cancer vaccine immunotherapy does not affect significantly the count of the major populations of peripheral blood cells (T-, B- and NK-cells). The most significant changes in dendritic cell-based cancer vaccine glioblastoma patients were evident in subpopulations of $CD3^+HLA^+$- and $CD38^+$-activated lymphocytes as well as suppressor cells (MDSC and T-reg ($CD4^+25^+127^{low}$)). According to ROC analysis the sufficient sensitivity and specificity were estimated for 3 biomarkers, namely the relative count of activated $CD38^+$- and $CD3^+HLA^+$-lymphocytes and T-reg cells. The threshold levels for the counts of these cells have been determined. The obtained results allow recommending these indices as the immunological criteria for assessing the efficacy of dendritic cell-based cancer vaccine in the treatment of glioblastoma.

Key words: oncology, immunotherapy, dendritic cells, immunological criteria.

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ІМУНОМОНІТОРИНГ ДЕНДРИТНО-КЛІТИННОЇ ПРОТИПУХЛИННОЇ ВАКЦИНИ У ХВОРИХ НА ГЛІОБЛАСТОМУ

Метою роботи було визначити найбільш інформативні імунологічні критерії ефективності імунотерапії на основі дендритних клітин у хворих на гліобластому. Проведення імунотерапії на основі дендритних клітин значно не впливає на зміни у кількісних показниках основних популяцій периферичної крові (Т-, В- та НК-лімфоцитів). Найбільш виражені зміни у хворих на гліобластому спостерігались у субпопуляціях активованих лімфоцитів із фенотипом CD3+HLA+ і CD38+-лімфоцитів та супресорних клітин (MDSC та Т-рег (CD4+25+127-). За допомогою ROC-аналізу встановлено, що достатню чутливість та специфічність мають 3 біомаркера: відносна кількість активованих CD38+- і CD3+HLA+-лімфоцитів та Т-рег (CD4+CD25+CD127-лімфоцити). Встановлені їх порогові значення. Одержані результати дозволяють рекомендувати дані показники для застосування в якості імунологічних критеріїв ефективності імунотерапії на основі дендритних клітин у хворих на гліобластому.

Ключові слова: онкологія, імунотерапія, дендритні клітини, імунологічні критерії.

This study is a fragment of the research project "To study the efficacy of the adjuvant immunotherapeutic and radiotherapeutic technologies in complex treatment of malignant glial brain tumors", state registration No. 0019U103900.

Glioblastoma is the commonest and most aggressive brain tumor accounting for up to 52 % of the primary brain tumors and up to 20 % of all intracranial neoplasms. The standard treatment that includes the surgical resection of tumor at the maximally possible extent followed by chemoradiotherapy and adjuvant therapy with temozolomide provides for two-year survival only in quarter of glioblastoma patients, while 46.7 % of patients with newly diagnosed glioblastoma die within less than one year after the diagnosis [1].

Immunotherapy is considered as one of the challenging treatment modalities to be hopeful for improving treatment efficacy in glioblastoma patients. The major aim of the immunotherapy is to induce the systemic immune response and inhibit immune suppression exerted by tumor growth [11]. Designing cancer vaccines is believed as the most promising field in immunotherapy development. Cancer vaccines are capable of inducing the immune response directed against both primary tumor and metastases. Since the efficacy of the first cancer vaccines was rather limited, the new concept of specific immune therapy has been developed envisaging the combination of the antigenic material with the factors enhancing specific immune response such as potent adjuvants, for example. The adjuvants of the natural origin such as dendritic cells (DC) as the major antigen-presenting cells of the immune system are undoubtedly of great interest in this context. The use of DC allows for overcoming several mechanisms that limit the effectiveness of the specific immune therapy in cancer patients [9].

Since specific immune response to the tumor-associated antigens comprises a complex of the processes with interplaying effector and regulation mechanisms, the immune monitoring of the vaccine therapy should be based on the immunological parameters that are most informative for displaying this

response. The parameters characterizing cell-mediated tumor immune response can be used for assessing clinical efficacy of the vaccine therapy since cell-mediated response is usually considered as the basic effectiveness indicator. Moreover, the current approaches for immune monitoring of cancer vaccine therapy in most cases do not take into account the peculiar characteristics of the state of immune system varying in cancers of different histogenesis. However, the lack of the prognostic criteria of the efficacy of cancer vaccines impedes the progress in attaining their maximal therapeutic effect [6]. Meanwhile, the delineation of the most specific and sensitive immunological markers is essential for developing the novel antitumor immunotherapeutics and the guidelines of their use to be translated into the clinical practice.

The purpose of the study was to define the most accurate immunological criteria for assessing the efficacy of the dendritic cell-based cancer vaccine in glioblastoma patients.

Materials and methods. Twenty patients with histologically proven glioblastoma (WHO grade IV) were included in this prospective study. At the time of the first DC-vaccination the median age of the patients was 51.8 years (range 27–70 years). 12 men and 8 women were enrolled. Surgery and postoperative radiation therapy (RT) (median total dose 60 Gy; 2 Gy per daily fraction) were performed in all cases. RT was applied with concomitant chemotherapy (Temozolomide 75 mg/m^2), followed by maintenance chemotherapy (Temozolomide 200 mg/m^2 on days 1–5 of every 28 days for up to six cycles). According to surgical reports gross total resection (GTR) and subtotal resection (STR) of the tumor were obtained in 6 and 9 cases respectively. Five patients underwent stereotactic biopsy. Patients were eligible if their ECOG performance status and Karnofsky performance status were 0–2 and 100–70 accordingly.

All patients were informed on the study and gave the informed consent for participation. All procedures performed in study were in accordance with the ethical standards adopted by the Ukrainian law (Approval decision No. 2 of 15.04.2019 by the Institutional Ethics Committee of SI Romodanov Neurosurgery Institute, NAMS of Ukraine).

DC were generated from peripheral blood monocytes cultured with growth and maturation factors (GM-CSF, IL-4, LPS, IFN-α). The lysate of tumor cells was used as the source of tumor antigens. All procedures were performed under aseptic conditions. On the Day 8 of DC culture, DC were counted in Goryaev hemocytometer. For the assessment of DC viability, the cells were stained with 0.4 % trypan blue solution. The viability of the DC obtained was not less than 95 %; with lymphocyte admixture not exceeding 20 %.

DCV was used in adjuvant schedule following chemoradiotherapy with DC concentration of (2.0–5.0) x10^6 cells per injection (intracutaneously paravertebrally) with 5 injections in total once a month.

The immunological parameters were assessed thrice: prior to therapy, at the initial steps of therapy (1st-2nd injections), and at the final steps (3rd-5th injections). As biological material for immune monitoring, a fraction of peripheral blood mononuclear cells was collected at each three steps. As a control, blood from 15 apparently healthy donors was used.

For the study of peripheral blood lymphocyte subpopulations, the expression of the following surface markers was estimated: CD3, CD19, CD4, CD8, CD16$^+$56$^+$ (for T-, B- and NK-cells); HLA-DR, CD38 (for activated lymphocytes); HLA-DR (absence), CD11b and CD33 (for myeloid-derived suppressor cells) and CD4, CD25 and CD127 (for T-reg lymphocytes).

The expression of the markers was analyzed by the direct immunofluorescence using the monoclonal antibodies conjugated with fluorescent stains FITC, PE, APC, PE-Cy5 (Becton Dickinson, USA; Caltag, USA). FACSCalibur flow cytofluorimeter (Becton Dickinson, USA) equipped with two lasers (488 and 625 nm) was used for cytometry study. The data were processed by CellQuest-PRO (USA) software.

Statistical analysis was performed using Statistica version 10 (StatSoft Inc, USA) and MedCalc 12.1 (MedCalc Software Ltd, USA). Gaussian distribution of the group was checked with Shapiro-Wilk test. Statistical analysis included Mean ± SE for Gaussian distribution and Median ± Percentiles (Q_1 and Q_3) for non-parametric data. To compare the data in the groups, we used t-test for Gaussian distribution and Mann–Whitney U-test for nonparametric ones. Prognostic significance of immunological markers was verified with the ROC-curve (Receiver Operating Characteristic curve). The H_0-hypothesis of variables equality was rejected at $p<0.05$.

Results of the study and their discussion. DCV administration was well tolerable without any accompanying side or toxic effects in the patients. The general well-being of the patients was satisfactory. Neither allergic or autoimmune disorders nor regional lymphadenopathy was evident. Hyperthermic reaction (elevation of body temperature to 38^0 C) occurred in 1–6 hours after the injection was observed in 15 % of patients. Nevertheless, this problem was eliminated by antipyretic drugs.

DCV has not been shown to affect substantially on the hematologic parameters of the peripheral blood (table 1).

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Hematological parameters in the peripheral blood of glioblastoma patients in the course of DC-based immunotherapy

Immunotherapy stage	Leukocyte count 10^9/l	Lymphocyte count, %	Monocyte count, %	Granulocyte count, %
Prior to immunotherapy	8.60±3.60	14.60±0.69	11.77±1.79	75.20±0.17
Initial stage of immunotherapy	6.27±0.23	30.37±2.55	9.29±0.21	57.01±2.74
End of immunotherapy	6.04±0.25	27.84±0.92*	9.23±0.23	64.56±1.06
Apparently healthy persons	4.0–9.0	28.0±2.01*	6.5±0.78	59.5±2.80

Note. * p<0.05 compared to the indices prior to therapy.

Prior to DCV, the count of granulocytes and monocytes in patients was significantly higher than in the apparently healthy persons (p<0.05). DCV in fact contributed to normalizing these parameters. Only lymphocyte count changed significantly upon DCV increasing 1.9-fold compared to lymphocyte count prior to the therapy (p=0.049).

Immune monitoring in the course of DCV in glioblastoma patients was one of the tasks of the present study. We demonstrated that DCV does not affect significantly the percentage of the major populations of T- and B-cells in the peripheral blood.

It should be noted that before therapy initiation, the content of $CD3^+$ T-cells and $CD4^+$ T-helpers was significantly lower compared to the indices in the apparently healthy persons, p<0.05 (fig. 1a). DCV contributed to normalizing these indices. However, the content of $CD8^+$ T-cells exceeded that in the apparently healthy persons throughout all DCV steps (fig. 1b). At the end of DCV therapy, the amount of $CD8^+$ T-cells in patients was 1.27-fold higher than in the healthy persons. In addition, we have shown that DCV was not change the distribution of B-cell population in glioblastoma patients compared to that in the healthy persons.

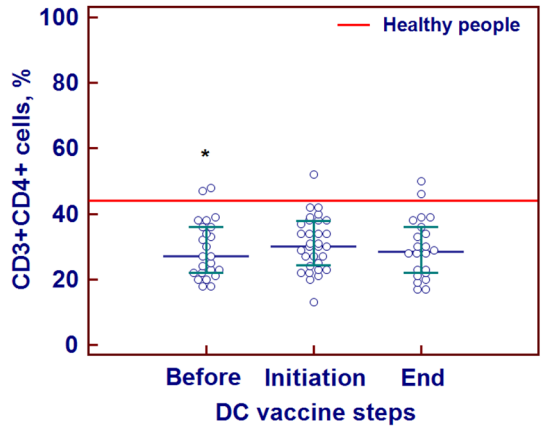

Fig. 1a. T-helper amount in the peripheral blood lymphocytes in patients with glioblastoma after DCV treatment. * – p<0.05 compared to healthy people.

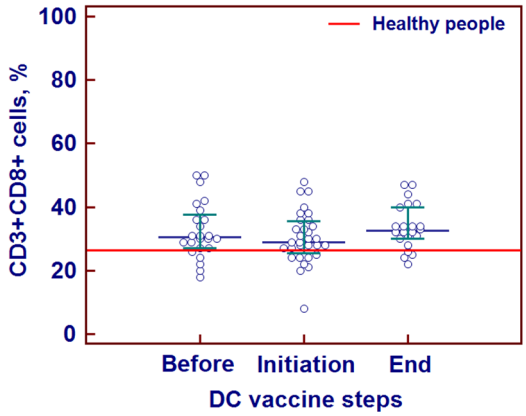

Fig. 1b. T cytotoxic cell amount in the peripheral blood lymphocytes in patients with glioblastoma after DCV treatment.

The results of studying the changes in the state of the natural component of the immunity, namely the subpopulation of the natural killer (NK) cells with $CD3^-16^+56^+$ phenotype playing the leading part in cancer immunity. Prior to DCV initiation, the proportion of NK cells in glioblastoma patients was slightly higher than in the healthy persons (17.78±1.38 % vs. 16.5±2.0 %). At the end of DCV therapy, the proportion of NK cells increased up to 19.24±1.63 %. Nevertheless, these changes were only at the trend level.

Next, we assessed the quantitative changes in the subpopulations of the activated lymphocytes (fig. 2.a). DCV therapy resulted in the significant increase of the amount of activated T-cells ($CD3^+HLA^+$ cells) compared to healthy persons (p<0.05). In the course of immunotherapy, this proportion increased further (1.42-fold compared to the value prior to DVC initiation).

The activated lymphocytes are those expressing CD38 (fig. 2b). As the transmembrane receptor, CD38 may transduce both positive and negative signals regulating proliferation and differentiation of T- and B-cells. We have shown that prior to initiation of DCV the fraction of $CD38^+$ lymphocytes in the peripheral blood of glioblastoma patients was 1.50-fold higher than in the healthy persons (60.29±4.20 % vs. 40.3±3.90 %; p<0.05). DCV therapy normalized the relative count of $CD38^+$ lymphocytes.

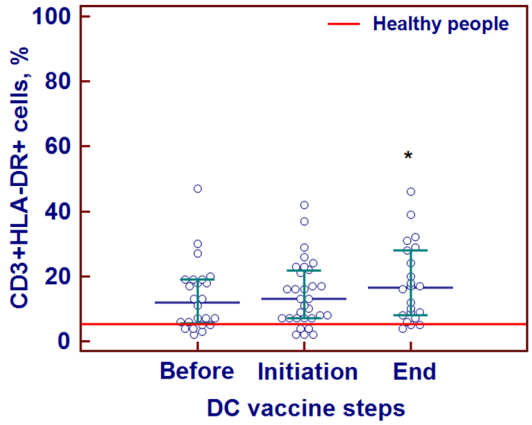

Fig. 2a. Activated T-cells amount in the peripheral blood lymphocytes in patients with glioblastoma after DCV treatment. * – p<0.05 compared to healthy people.

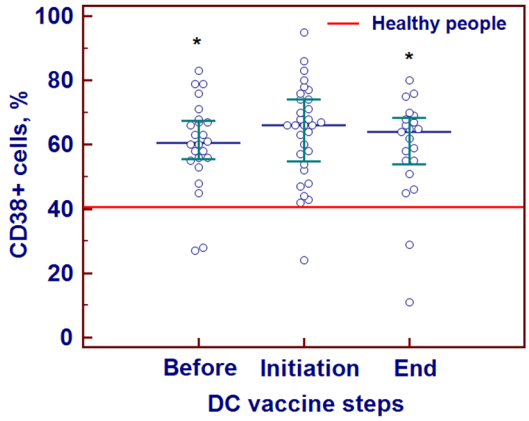

Fig. 2b. CD38+ cell amount in the peripheral blood lymphocytes in patients with glioblastoma after DCV treatment. * – p<0.05 compared to healthy people.

IL-7 receptor (CD127) is known as biomarker of Treg human lymphocytes. In the peripheral blood, T-cells possessing high suppressive activity and high FOXP3 expression may be delineated by detecting the cells expressing combination of CD4, CD25 and CD127 on their surface. We have shown that prior to DCV, the fraction of these cells was within the values 8.27±1.91 % vs. 6.0±1.5 % detected in the healthy persons (fig. 3a). DCV resulted in the significant increase of the relative count of Treg lymphocytes (15.92±4.14 %) compared to the initial values ($p \leq 0.05$).

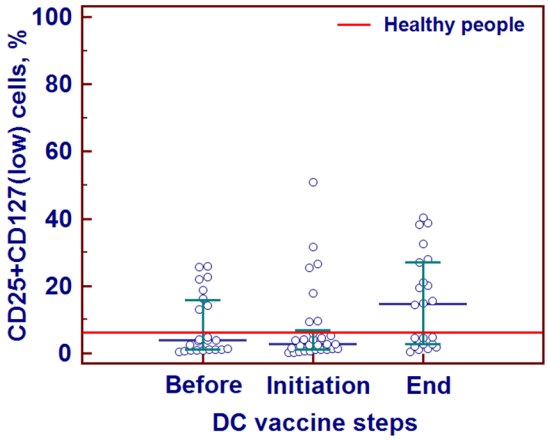

Fig. 3a. Lymphoid suppressor cell amount in the peripheral blood lymphocytes in patients with glioblastoma after DCV treatment. * – p<0.05 compared to healthy people.

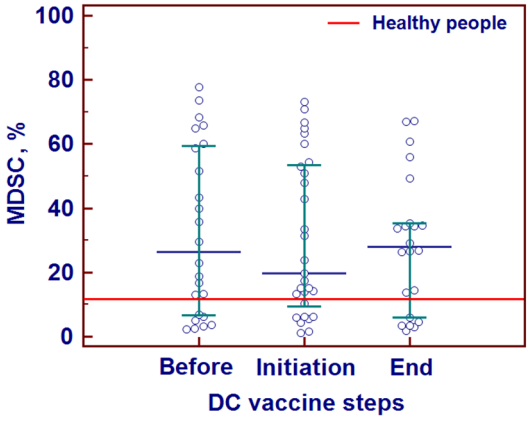

Fig. 3b. Myeloid suppressor cell amount in the peripheral blood lymphocytes in patients with glioblastoma after DCV treatment.

We have established (fig. 3b) that prior to the initiation of DCV, the relative count of the myeloid-derived suppressor cells (MDSC) with HLA-DR$^-$11b$^+$33$^+$ phenotype in the peripheral blood of glioblastoma patients was 2.84-fold higher than in the healthy persons (32.67±5.40 % vs. 11.9±2.13 %, $p \leq 0.05$). In the setting of immunotherapy, slight decrease in MDSC relative count was registered only at the trend level.

Taken together, the results of our immunological studies demonstrate that the most pronounced changes in the course of DCV in glioblastoma patients occur in the subpopulations of CD3$^+$HLA$^+$ and CD38$^+$- activated lymphocytes as well as suppressor cells – MDSC and Treg lymphocytes (CD4$^+$25$^+$127$^-$).

The association between the relative counts of the cells stated above and the survival of glioblastoma patients after DCV therapy is presented in fig. 4.

The further task of our study was to assess the suitability of these immunological parameters as the markets of the clinical effectiveness of DCV in glioblastoma patients. For this purpose, we used ROC analysis as the most precise statistical method for the assessment of the diagnostic and prognostic significance of the markers. As seen in Table 2, the following biomarkers have the sufficient sensitivity (>75 %) and specificity (>65 %): the relative count of activated CD38$^+$- and CD3$^+$HLA$^+$- lymphocytes and T-reg cells with CD4$^+$CD25$^+$CD127$^-$ phenotype. These markers demonstrate strong correlation with the overall survival of glioblastoma patients after DCV. The threshold values for these markers were determined to allow the efficacy of immunotherapy with sensitivity from 62 % to 90 %.

So, we have found out the peculiar features of the immune response of glioblastoma patients caused by specific active DC-based immunotherapy. The most specific and sensitive immunological markers for forecasting the clinical effectiveness have been defined.

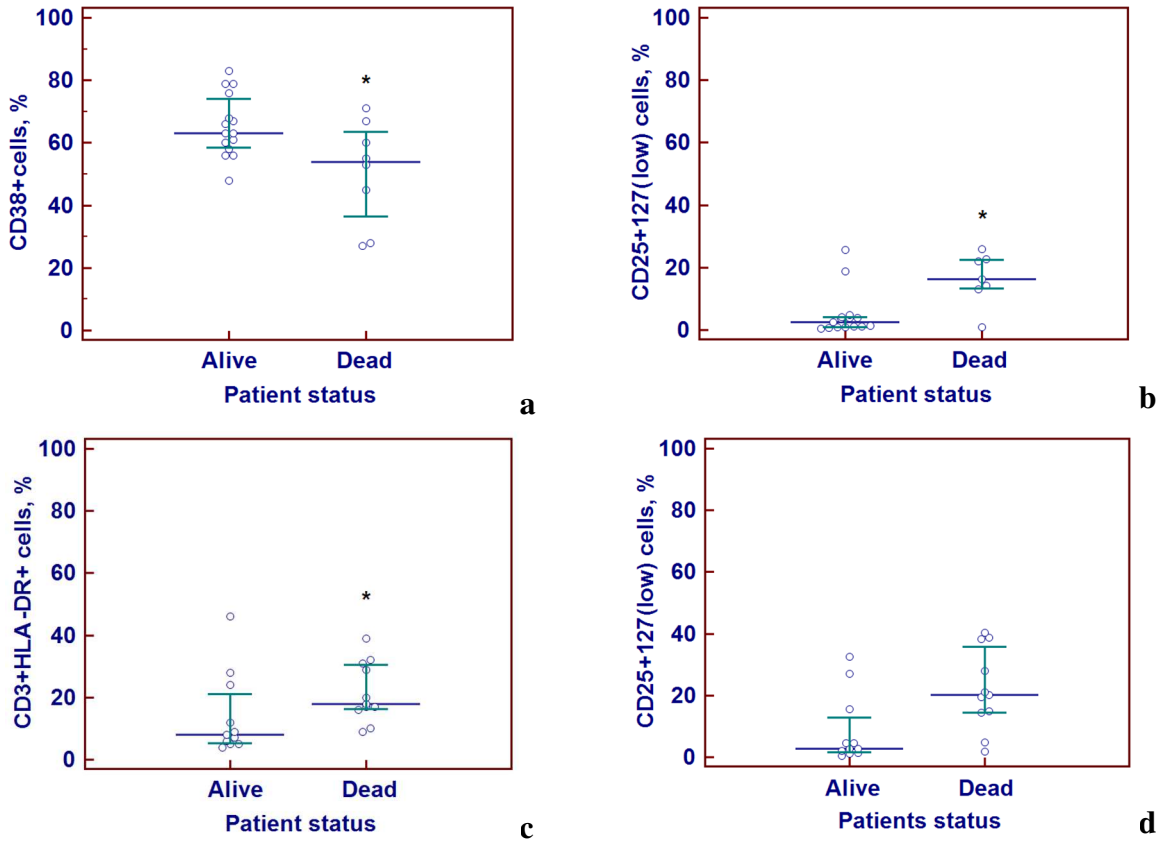

Figure 4. The distribution of activated and suppressor lymphoid cells in the peripheral blood patients under DCV treatment. (a and b – peripheral blood samples were collected before DCV treatment; c and d – peripheral blood samples were collected after 3rd, 4th and 5th DCV administration). * – p<0.05 compared to alive patients.

In spite of the numerous findings related to the immune response in the course of immunotherapy, the most important immunological criteria of such response have not been definitely stated. This could be explained in part by multifunctionality of the immune system and pleiotropic immune effects that complicates making forecasts based on single immunological parameters [5].

At present, the major task of immune monitoring consists in defining the parameters of the immune response that correlate with the clinical outcome of immunotherapy. Of great importance is also the delineation of the parameters suitable as the surrogate markers for the assessment of the clinical effectiveness of the immunotherapy. The analysis of the subpopulations of the peripheral blood lymphocytes is a basic and obligatory laboratory assay to follow the course of the immunotherapy in cancer patients. Nevertheless, it remains to be seen which of the parameters should be assessed depending on the specific nosology [4].

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ROC curve characteristics of immunological parameters in the course of DCV therapy of glioblastoma patients

№	Parameter	Step of immu-notherapy	Sensitivity Se, %	Specificity Sp, %	Optimal criterion	AUC	p
1	Relative count of $CD38^+$ cells	before	62.50	93.33	≤55.0	0.78	0.0018
2	Relative count of T-reg cells ($CD4^+CD25^+CD127^-$)	before	85.71	86.67	>4.88	0.92	0.0092
3	Relative count of $CD3^+HLA^+$ cells	after	81.82	73.93	>8	0.77	0.016
4	Relative count of T-reg cells $CD4^+$ $CD25^+$ $CD127^-$)	after	90.91	72.73	>4.55	0.80	0.0026

Our findings demonstrate that the relative count of $CD3^+$ T-cells in glioblastoma patients prior to the DCV initiation is slightly lower than in the healthy persons. These data are in line with those by H. Mostafa et al., although there is a discrepancy between their data and our findings relating to the content

of NK-cells [8]. In our study, the relative count of NK-cells increased compared to the healthy persons in contrast to the decrease observed by H. Mostafa et al.

A lot of data indicate the pivotal role of myeloid-derived suppressor cells (MDSC) in tumor development. This cell population exerts immune suppression impairing immune surveillance and disturbing antitumor cytotoxicity [10]. Our data on the significant increase of circulating MDSC in glioblastoma patients are in line with the findings published elsewhere [2]. The decrease in the relative count of MDSC in the course of immunotherapy may be useful as the immunological criterion of therapy effectiveness. To prove this, the study involving more extended cohorts is needed.

It is known that T-reg cells may affect the malignancy grade, the course of the disease and the response to treatment in many types of cancer including glioblastoma [3]. We confirmed the findings obtained by other researchers indicating that the increased count of $CD4^+CD25^+CD127^-$ T-cells is associated with the worse response to therapy and decreased overall survival of glioblastoma patients.

The necessity of including biomarkers into the standard treatment schedules is increasingly debated now [7]. Our data suggest three markers that may be used as immunological criteria of DCV effectiveness in glioblastoma patients: the relative count of $CD38^+$-lymphocytes prior to DCV (threshold≤55.0 %); the relative count of $CD3^+HLA^+$–lymphocytes after DCV (threshold>8 %); and relative count of $CD4^+CD25^+CD127$ T-reg cells prior to and after DCV (threshold>4.55 %). These criteria will be advantageous for timely assessment and individualization of the approaches for applying specific immunotherapy.

Conclusions

1. DCV does not affect significantly the major lymphocyte populations in peripheral blood (T-, B-, NK-cells) in glioblastoma patients.

2. The relative count of activated lymphocytes with $CD3^+HLA^+$ and $CD38^+$ phenotype in glioblastoma patients was significantly higher than in healthy persons ($p<0.05$).

3. The relative count of MDSC and T-reg ($CD4^+25^+127^-$ lymphocytes) in glioblastoma patients was significantly higher than in healthy persons ($p< 0.05$). DCV normalized these values.

4. ROC analysis allowed us to define three statistically significant biomarkers: the relative count of $CD38^+$-lymphocytes prior to DCV (threshold ≤55.0 %); the relative count of $CD3^+HLA^+$- lymphocytes after DCV (threshold >8 %); and relative count of $CD4^+CD25^+CD127$ T-reg cells prior to and after DCV (threshold >4.55 %).

5. The data obtained suggest using the parameters stated above as the immunological criteria for assessing the DCV efficacy in glioblastoma treatment.

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