

# Treatment with cyclophosphamide supported by various dendritic cell-based vaccines induces diversification in CD4<sup>+</sup> T cell response against MC38 colon carcinoma

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**Abstract.** The present study shows that an application of cyclophosphamide (CY) supported by dendritic cell (DC)-based vaccines affected differentiation of the activity of CD4<sup>+</sup> T cell subpopulations accompanied by an alteration in CD8<sup>+</sup> cell number. Vaccines were composed of bone marrow-derived DCs activated with tumor cell lysate (BM-DC/TAg<sup>TNF- $\alpha$</sup> ) and/or genetically modified DCs of JAWS II line (JAWS II/Neo or JAWS II/IL-2 cells). Compared to untreated or CY-treated mice, the combined treatment of MC38 colon carcinoma-bearing mice resulted in significant tumor growth inhibition associated with an increase in influx of CD4<sup>+</sup> and CD8<sup>+</sup> T cells into tumor tissue. Whereas, the division of these cell population in spleen was not observed. Depending on the nature of DC-based vaccines and number of their applications, both tumor infiltrating cells and spleen cells were able to produce various amount of IFN- $\gamma$ , IL-4 and IL-10 after mitogenic *ex vivo* stimulation. The administration of CY followed by BM-DC/TAg<sup>TNF- $\alpha$</sup>  and genetically modified JAWS II cells, increased the percentage of CD4<sup>+</sup>T-bet<sup>+</sup> and CD4<sup>+</sup>GATA3<sup>+</sup> cells and decreased the percentage of CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup> and CD4<sup>+</sup>FoxP3<sup>+</sup> lymphocytes. However, the most intensive response against tumor was noted after the ternary treatment with CY + BM-DC/TAg<sup>TNF- $\alpha$</sup>  + JAWS II/IL-2 cells. Thus, the administration of various DC-based vaccines was respon-

sible for generation of the diversified antitumor response. These findings demonstrate that the determination of the size of particular CD4<sup>+</sup> T cell subpopulations may become a prognostic factor and be the basis for future development of anticancer therapy.

## Introduction

The proper stimulation of the immune system and targeting action require the application of various forms of therapy including cytostatic drug therapy. Cyclophosphamide (CY) is a well-characterized DNA-alkylating agent, widely used in the treatment of malignancies (e.g., breast, ovarian, small cell lung cancer and leukemias). Both the tumor-eradicating and immunomodulating effects of CY are dose-dependent. At high doses, CY induces mainly a cytotoxic effect, whereas at low doses it stimulates the differentiation of effector CD4<sup>+</sup> T cells and inhibits the regulatory T (Treg) cell activity (1,2). The influence of CY on the promotion of T helper (Th)1 cell activity has been confirmed in rodent tumor models (3). This was demonstrated by an increase in the production of cytokines specific for Th1 (mainly IL-2 and IFN- $\gamma$ ) but a decrease in the secretion of IL-10 (4).

In order to improve the effectiveness of the treatment with cytostatics, attempts are made to combine chemotherapy with immunotherapy. The reasonable forms of the latter are dendritic cell (DC)-based vaccines. In some animal models and early clinical trials, the DC-based vaccines were used for stimulation of immune response and for improving the antitumor effect initiated by CY administration. Such therapy affected the inhibition of Treg cells and the increase of cellular cytotoxicity against tumor cells. Furthermore, it prolonged the survival of colorectal cancer-bearing mice as well as stimulated the production of IFN- $\gamma$  by lymphocytes derived from these animals (5,6).

It is well known that DCs influence the differentiation, migration and activation of CD4<sup>+</sup> T cells using cell-to-cell contact and cytokine production. For antitumor immunotherapy based on DCs, various strategies of antigen loading have been proposed (including whole tumor-cell lysates). It is believed that the simultaneous cytokine use can support DC maturation. Balkow and coworkers reported (7) that DC

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*Abbreviations:* BM-DCs, bone marrow-dendritic cells; MC38, murine colon carcinoma; TAg, tumor antigens; TNF- $\alpha$ , tumor necrosis factor  $\alpha$

*Key words:* CD4<sup>+</sup> T cell subpopulations, CD8<sup>+</sup> T cells, chemotherapy, cyclophosphamide, dendritic cell-based vaccine, MC38 colon carcinoma

matured by anti-CD40 monoclonal antibodies in the presence of IL-12 and IL-18 exhibited potent activity of these cells against growing tumors. Brunner *et al* (8) showed that DC stimulated with tumor antigens and TNF- $\alpha$ , expressed the MHC class II, CD80 and CD86 molecules at higher level than cells stimulated only with tumor antigens. However, complete *in vitro* maturation of DCs causes the high expression of MHC class II antigens and costimulatory molecules, but the application *in vivo* of fully matured DCs has led to decrease in DC-mediated T-cell activation (9). Thus, various levels of activation of antigen-specific T cells during the formation of antitumor response can result from diverse maturity of the DC contained in vaccines. The use of different viruses as carriers of antigenic protein genes has also been reported (10). Several lines of evidence indicate that genetically modified DC involved in cellular vaccines are capable of triggering a long-lasting tumor growth delay along with an increase in the number of cytotoxic T cells as well as cytokine-producing lymphocytes. Genetic modifications of DCs for expressing cytokine genes (e.g., interleukin 2) (IL-2) may enhance their activity (11). However, the effectiveness of the clinical protocols employing various types of DC-based vaccines is still unsatisfactory and needs further investigation. DCs are believed to stimulate naive CD4<sup>+</sup> T cells which are a key element of numerous immune mechanisms. Th1 cell subpopulation containing the IFN- $\gamma$ -producing cells supports cellular immunity; IL-4-producing cells representing the Th2 cell subset is associated with humoral immunity. The Th17 cells, secreting IL-17A and IL-17F, are responsible for pro-inflammatory activity. The Treg cells play a critical role in active suppression of immune response and are believed to be the main subpopulation of cells able to secrete IL-10.

Many experimental and clinical results confirm that the presence of CD4<sup>+</sup> T cells is required during development of antitumor response, and their infiltration into the tumor tissue can connote a good prognosis in many types of cancers. However, based on the type of tumor tissue and cytokine environment the migration and activation of different subpopulation of CD4<sup>+</sup> T cells can be observed.

There is still only limited evidence demonstrating the immune mechanisms responsible for the effect of the combined CY and DC-vaccine therapy on differentiation of T cells involved in the response against growing tumor. For this reason, the aim of the present study is to elucidate whether the various types of DC-based vaccines applied after CY administration caused diversity in CD4<sup>+</sup> T cell subpopulations directly related with the inhibition of murine MC38 colon cancer growth. This was achieved by analyzing the changes in CD4<sup>+</sup> lymphocyte infiltration into tumor tissue, ability of these cells to express T-bet, GATA3, ROR $\gamma$ t and FoxP3 transcription factors and to produce specific cytokines. The alteration in systemic response was represented by trends in splenic reactivity: cytokine secretion and diversity in transcription factor expression. The applied treatment resulted in the increase in the number of Th1 and Th2 cells followed by time-dependent activation of CD8<sup>+</sup> cells and a decrease in the number of Th17 and Treg lymphocytes.

The observed alteration in the ration of CD4<sup>+</sup> T cell subpopulations may have a great value as a prognostic factor and be the basis for future development of anticancer therapy.

## Materials and methods

**Animals.** Female C57BL/6 mice were obtained from the Center for Experimental Medicine of the Medical University of Bialystok (Bialystok, Poland). Mice were housed under specific pathogen-free conditions, and were transferred to a conventional environment two weeks before the experiment. All animal experiments were approved by the Local Ethics Committee.

**Preparation of dendritic cell-based vaccines.** Dendritic cells generated from bone marrow of healthy mice were cultured for 8 days in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Seelze, Germany) and 40 ng/ml GM-CSF (Invitrogen, Carlsbad, CA, USA) and 10 ng/ml IL-4 (ImmunoTools, Friesoythe, Germany). On the seventh day, cells were activated with MC38 tumor cell lysate (10% v/v) and stimulated with 50 ng/ml TNF- $\alpha$  (Invitrogen) (BMDC/TA $\gamma$ <sup>TNF- $\alpha$</sup> ). Dendritic cells of JAWS II cell line (CRL-11904) (obtained from ATCC, Manassas, VA, USA) transduced with retroviral vector carrying *mIL-2* gene (JAWS II/IL-2 cells) or neomycin resistance gene (JAWS II/Neo, used as a control of transduction) were maintained in RPMI-1640 and  $\alpha$ MEM (Gibco) (1:1) culture medium supplemented with 10% FBS and 5 ng/ml GM-CSF (11). BMDC/TA $\gamma$ <sup>TNF- $\alpha$</sup>  alone or with genetically modified JAWS II cells were used as antitumor vaccines.

For cell characterization, cell surface molecule expression and cytokine production were analyzed. DCs were stained with anti-CD80 (conjugated with fluorochrome APC; clone 16-10A1, hamster anti-mouse), anti-CD86 (PE-Cy7; clone GL1, rat anti-mouse), and anti-I-A<sup>b</sup> (FITC; clone 25-9-17, mouse anti-mouse) monoclonal antibodies (all from BD Biosciences, San Jose, CA, USA). The expression of molecules was measured by FACSCalibur flow cytometer (BD Biosciences) and analyzed by Flowing software 2.5.1. The concentrations of IL-2, IL-12, IL-6 and IL-10 cytokines in 48 h cell culture supernatants were measured with commercially available ELISA kits (BD Biosciences) according to the manufacturer's instructions.

**Mouse treatment schedule.** Eight- to ten-week-old C57BL/6 mice were inoculated subcutaneously (s.c.), into right flank, with MC38/0 cells ( $1 \times 10^6/0.2$  ml/mouse). After 14 days, the MC38 colon carcinoma-bearing mice were treated intraperitoneally (i.p.) with CY (Baxter, Deerfield, IL, USA) (150 mg/kg body weight). On days 17, 24 and 31, mice were administered peritumorally (p.t.) BMDC/TA $\gamma$ <sup>TNF- $\alpha$</sup> , JAWS II/IL-2, JAWS II/Neo cells ( $1.5 \times 10^6$  cells/mouse), or their combination ( $1 \times 10^6$  BMDC/TA $\gamma$ <sup>TNF- $\alpha$</sup>  +  $0.5 \times 10^6$  JAWS II cells/mouse). Twice a week tumor growth was assessed morphometrically using electronic calipers, and tumor volumes were calculated according to the formula:  $(a^2 \times b) \div 2$ , where a is the shorter and b is a longer tumor diameter. Spleen and tumor tissue were collected on day 31, 38 and 45 of the experiment. Mice were monitored and the median of tumor volume were estimated.

The values of two parameters were calculated:  $\Delta$ TRV, the difference in the median time required for the tumor to reach a volume of 1 cm<sup>3</sup> compared to the group of untreated mice and TGI, tumor growth inhibition. The TGI was used to demon-

strate the percentage of the tumor volume inhibition calculated for groups of treated mice compared to the untreated mice ( $TGI^1$ ) or to the CY-treated control ( $TGI^2$ ).

**Lymphocytes in spleen and tumor tissue.** Single cell suspension was prepared from tumor nodules or spleens. Cells were incubated with anti-CD45 (PE-Cy7, clone 30-F11) and anti-CD4 (APC, clone RM4-5) monoclonal antibodies (mAbs) or anti-CD8 (FITC, clone 53-6.7) (all rat anti-mouse and all from BD Biosciences), and analyzed by flow cytometer FACSCalibur (BD Biosciences) and Flowing software 2.5.1.

**Lymphocyte stimulation and intracellular staining.** Splenocytes or CD4<sup>+</sup> cells isolated from tumor tissue by anti-CD4 antibody-coated magnetic beads (BD IMag™), were cultured in RPMI supplemented with 10% FBS and 0.5 μg/ml concanavalin A (Con A; Sigma-Aldrich). Cell culture supernatants were used for estimation of cytokine production by stimulated cells (ELISA). For cytokine and transcription factor expression analysis, brefeldin A (10 μg/ml) and monensin (2 μM) (both from eBiosciences, San Diego, CA, USA) were added to the cell culture for the last 6 h. Cells were stained with anti-CD4 antibody (FITC, clone RM4-5), fixed and incubated with anti-mouse CD16/CD32 mAb, and stained for cytokine and/or transcription factor expression with the intracellular staining kit from eBiosciences. The following fluorochrome-conjugated mAbs were used: anti-Tbet (PE-Cy 7, clone 4B10), anti-GATA3 (PE, clone TWAJ), anti-RORγt (PE, clone AFKJS-9), anti-FoxP3 (APC or PE, clone FJK-16s), or their appropriate isotype control (all anti-human/mouse, all from eBiosciences). Flow cytometry was performed using FACSCalibur cytometer (BD Biosciences). All data were analyzed with Flowing software version 2.5.

**Cytokine production.** IFN-γ, IL-4, IL-10 (BD Biosciences) and IL-17A (eBiosciences), and production by splenocytes or CD4<sup>+</sup> lymphocytes isolated from tumor tissue was measured with commercially available ELISA kits according to the manufacturer's instructions.

**Estimation of spleen cell cytotoxicity.** Spleen cells obtained from mice after chemoimmunotherapy or from healthy mice, untreated MC38-bearing mice and CY-treated MC38-bearing mice were isolated and co-cultured (restimulated) for 4 days with mitomycin C-treated MC38 cells (mitomycin C were obtained from Sigma-Aldrich) (50 mg mitomycin C/3x10<sup>6</sup> cells/ml/30 min at 37°C) in the presence of IL-2 as described (12). The source of murine IL-2 was X63mIL-2 plasmacytoma cell supernatant used at 5% concentration, corresponding to 100 U/ml. After 4 days of restimulation the cytotoxicity of the spleen cells was tested by flow cytometry. Target MC38/0 cells were stained with DiOC<sub>18</sub> lipophilic dye (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C and then washed with PBS. After restimulation, spleen cells were incubated with labeled target cells for 4 h at 37°C at a 10:1 ratio of effector to target cells. The cells were then washed and dead cells were stained with propidium iodide (PI; Molecular Probes). Samples were analyzed using FACSCalibur flow cytometer and Flowing software 2.5.1. Percentage of cytotoxicity was determined according to formula: the percentage

of dead target MC38/0 cells previously incubated with splenocytes minus the percentage of dead MC38/0 cells cultured alone.

**Statistical analysis.** Results are presented as the median (volume of tumor nodules) or mean ± SD (all other results). Data were analyzed by the non-parametric Mann-Whitney U test and Kruskal-Wallis ANOVA by ranks (Statistica 10 software). The values of P<0.05 were considered as statistically significant.

## Results

**Dendritic cells used for the preparation of DC-based vaccines.** The present study was carried out with the use of two types of DCs: genetically modified cells of JAWS II line and bone marrow-derived DCs. For the characterization of dendritic cells, a surface antigen expression as well as cytokine production were analyzed. JAWS II dendritic cells exhibited low expression of co-stimulatory (CD80, CD86) and MHC class II molecules. JAWS II cells modified to produce IL-2 (JAWS II/IL-2) revealed slight increase in the expression of CD86 and MHC class II molecules compared to parental JAWS II cells or JAWS II/Neo cells being a control of transduction (Fig. 1), making them able to present tumor antigens only marginally. Interleukin 2 was only produced by JAWS II/IL-2 cells (for the needs of the present study, 17.3 ng/ml). Whereas, IL-6 was produced by JAWS II/Neo cells in higher amounts (18.9 ng/ml) than by unmodified JAWS II (13.08 ng/ml) cells and JAWS II/IL-2 cells (4.08 ng/ml) (data not shown). JAWS II cells, in general, turned out to be unable to produce IL-12 and IL-10. Thus, JAWS II/IL-2 cells administered into mice, acted mainly as a source of IL-2 and only to a small extent as the antigen-presenting DCs.

The bone marrow-derived DCs after *ex vivo* stimulation with tumor antigens and TNF-α (BM-DC/TAg<sup>TNF-α</sup>) were exploited for presentation of TAG. These cells exhibited higher expression of costimulatory molecules (CD80 and CD86) and MHC class II antigens compared to other groups of BM-DC (Fig. 1). The BM-DC/TAg<sup>TNF-α</sup> were able to produce IL-6 (19.1 ng/ml) and small amounts of IL-12 (78 pg/ml), but did not secrete IL-10 and IL-2 (data not shown). Although expression of surface antigens and secretion of proinflammatory cytokines increased insignificantly, the BM-DC/TAg<sup>TNF-α</sup> were likely able to activate T cells in host.

**The use of DC-based vaccines improves the antitumor CY effect.** In order to analyze the effect of the CY treatment followed by DC-based vaccines, the C57BL/6 mice bearing advanced MC38 colon carcinoma were used. Fourteen days after MC38/0 cell inoculation, when the tumors became palpable, the mice were treated with single dose of CY (150 mg/kg body weight). Starting three days later, cellular vaccines (1.5x10<sup>6</sup>/mouse) were administered three times, at weekly intervals, as is shown in Fig. 2A. Vaccines consisted of BM-DC/TAg<sup>TNF-α</sup>, JAWS II/IL-2, JAWS II/Neo cells, or their combinations (BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells, BM-DC/TAg<sup>TNF-α</sup> + JAWS II/Neo cells with cell ratio 2:1). Fig. 2 illustrates the therapeutic effect on the tumor growth delay in one of the three independent experiments (Fig. 2B).

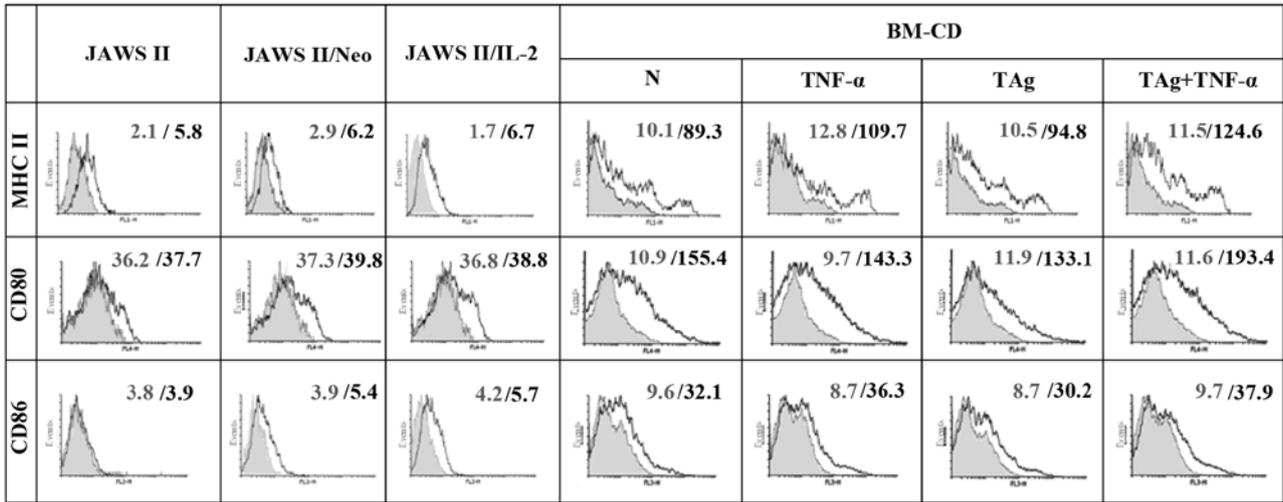


Figure 1. Phenotypic characteristics of dendritic cells. Dendritic cells of JAWS II line unmodified or transduced with neo (neomycin-resistance) or IL-2 gene as well as bone marrow-derived dendritic cells (BM-DC) unstimulated (N) or stimulated with TNF- $\alpha$ , TAg, or TNF- $\alpha$  + TAg were stained with anti-MHC class II, anti-CD80 or anti-CD86 antibodies. The image presents results from one representative experiment. The numbers correspond to MFI (mean fluorescence intensity) for the isotype control (gray) vs. the examined surface antigen (black).

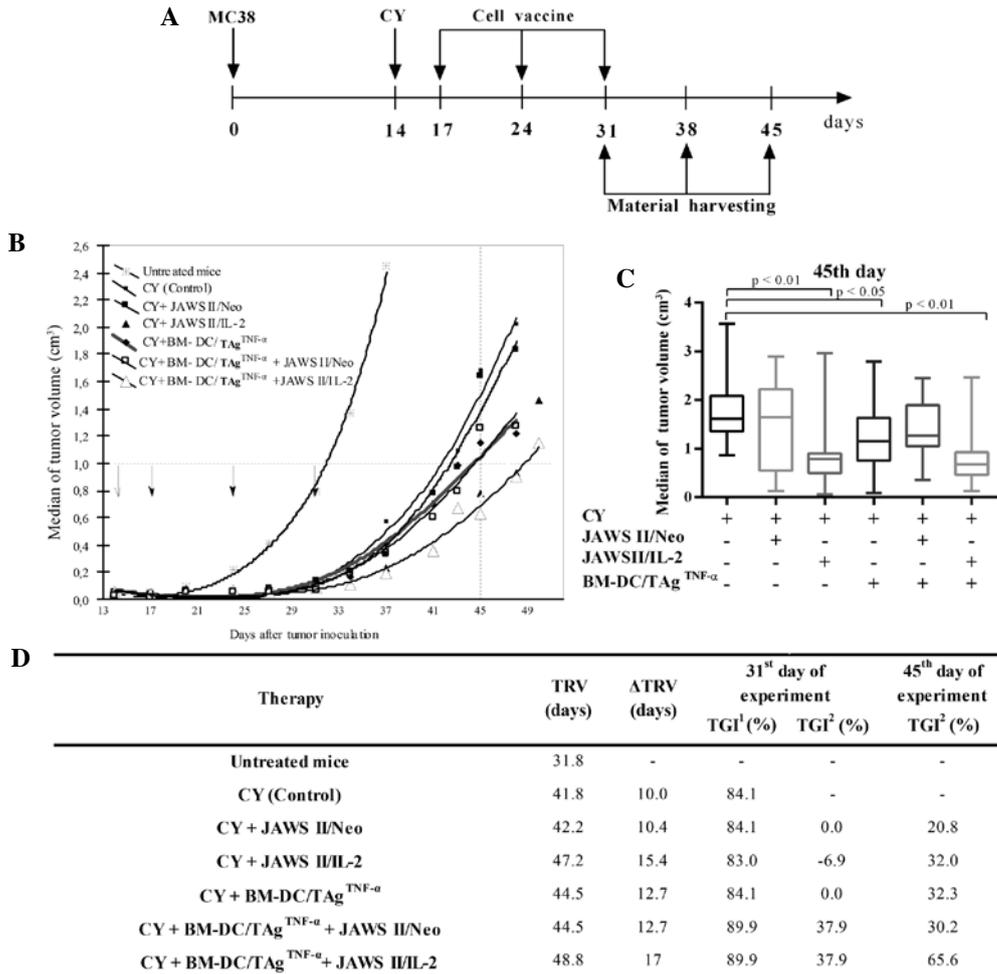


Figure 2. MC38 tumor growth in C57BL/6 mice after treatment with CY and DC-based vaccines. (A) Mice bearing advanced tumors (12-15 per group) were injected i.p. with CY on the 14th day of the experiment. Three days later, cell vaccines were given p.t. for three consecutive weeks. (B) Kinetics of tumor growth was presented as tumor volume curves (C) diversity among median of tumor volumes calculated on the 45th day. (D) In the table are presented: TRV, the median time required for the tumor to reach a volume of 1 cm<sup>3</sup> (days);  $\Delta$ TRV, difference in the TRV compared to the group of untreated mice (days); TGI, tumor growth inhibition, calculated according to the formula  $100 - (T/C \times 100)$  (%), where T represents the ratio of median tumor volume at a given time to median tumor volume at the start of treatment (14th day) for the group of treated mice and C represents the ratio of median tumor volume at a given time to median tumor volume on the 14th day for the group of untreated mice (TGI<sup>1</sup>) or control group of mice that received only CY (TGI<sup>2</sup>). The 12-15 mice/group were monitored.

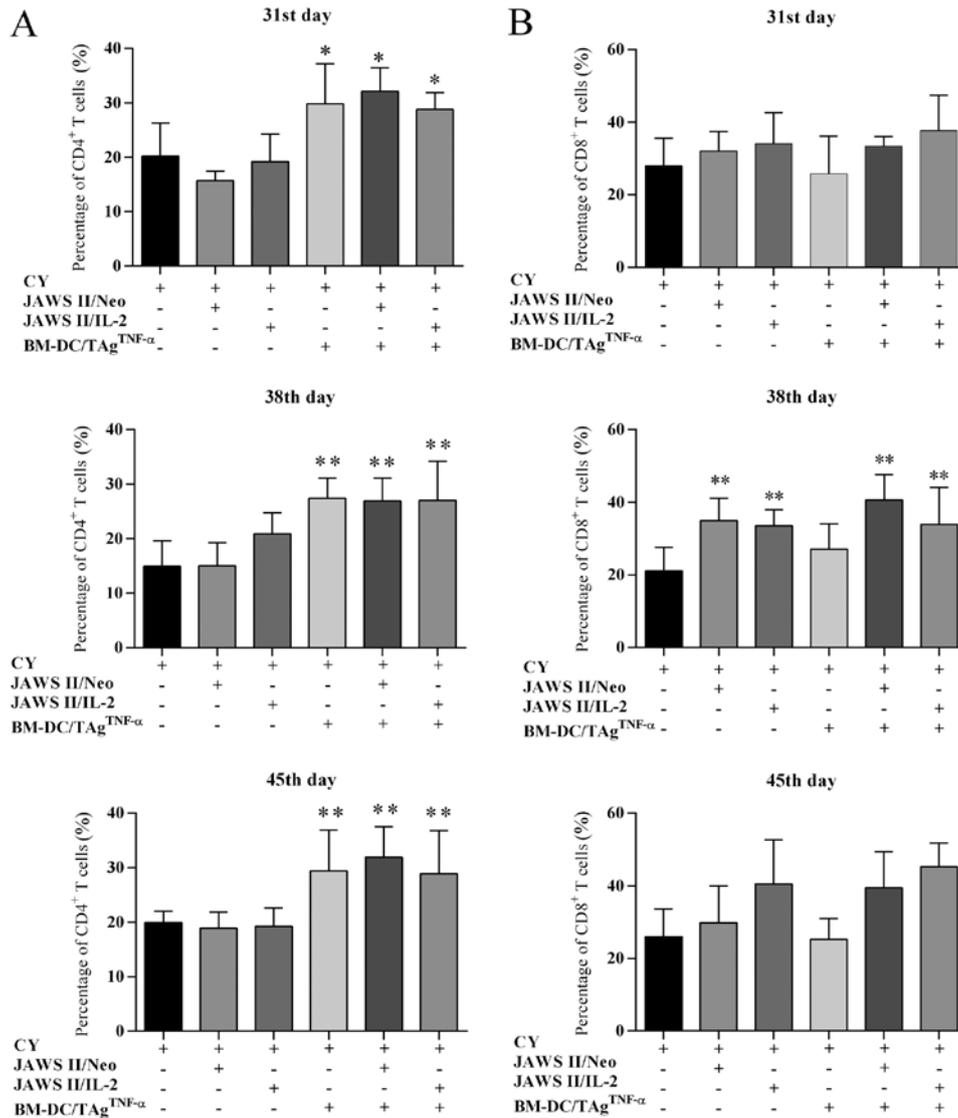


Figure 3. Tumor tissue infiltration by T lymphocytes. Tumor tissues from CY and cell vaccine-treated mice (N=3-6 per group, two independent experiments) were collected on the 31st, 38th and 45th day of the experiment. The percentage of CD4<sup>+</sup> (A) or CD8<sup>+</sup> (B) cells among leukocytes (CD45<sup>+</sup>) from tumor tissue. The suspensions of cells from tumor tissue were incubated with anti-CD45 and anti-CD4 or anti-CD8 antibodies and analyzed by flow cytometry. The percentage of CD4<sup>+</sup> or CD8<sup>+</sup> cells among CD45<sup>+</sup> cells was calculated (mean ± SD). The statistical significance (\*P<0.05 and \*\*P<0.01) vs. control (CY) by Mann-Whitney U test.

The data were completed by statistical significance of the treatment estimated on day 45 of the experiment (Fig. 2C). Using Kruskal-Wallis test as well as Mann-Whitney U test, the statistically significant differences were observed between group of mice obtaining CY and groups treated with CY + DC/TA<sub>g</sub> (P=0.034) and/or JAWS II/IL-2 (CY vs. CY + JAWS II/IL-2 at P=0.001; CY vs. CY + BM-DC/TA<sub>g</sub> + JAWS II/IL-2 at P=0.0002). However, differences between CY + JAWS II/IL-2 cells and CY + BM-DC/TA<sub>g</sub> + JAWS II/IL-2 cell treatment proved to be insignificant (P=0.78565).

The administration of CY (or CY + JAWS II/Neo cells) induced tumor growth delay reaching ~10.4 days compared to untreated group (Fig. 2D). Further increase in the ΔTRV was observed after the use of CY with BM-DC/TA<sub>g</sub><sup>TNF-α</sup> or with BM-DC/TA<sub>g</sub><sup>TNF-α</sup> + JAWS II/Neo cells (12.7 days). However, the replacement of JAWS II/Neo cells by JAWS II/IL-2 cells resulted in the increase of ΔTRV to 15.4 days, whereas, the

application of BM-DC/TA<sub>g</sub><sup>TNF-α</sup> + JAWS II/IL-2 cells elicited an increase to 17 days.

The differences in TGI illustrate the influence of DC-based vaccines on the final effect of the treatment. On the 31st day of the experiments, the slight diversification among the groups of mice was observed compared to untreated mice (TGI<sup>1</sup> ranged from 84.1 to 89.9%), whereas TGI<sup>2</sup> reached 37.9%, but only in case of the mice receiving CY + BM-DC/TA<sub>g</sub><sup>TNF-α</sup> and transduced JAWS II cells. The obtained result highlighted that ternary combinations were able to hasten the antitumor response already in its early phase but the significant effects of such therapy were found on the 45th day (14 days after the third vaccination). The differences between the medians of tumor volumes calculated on this day proved to be statistically significant, but the most intensive response against the tumor was revealed after the use of CY + BM-DC/TA<sub>g</sub><sup>TNF-α</sup> + JAWS II/IL-2 cells (TGI<sup>2</sup> amounted to 65.6%).

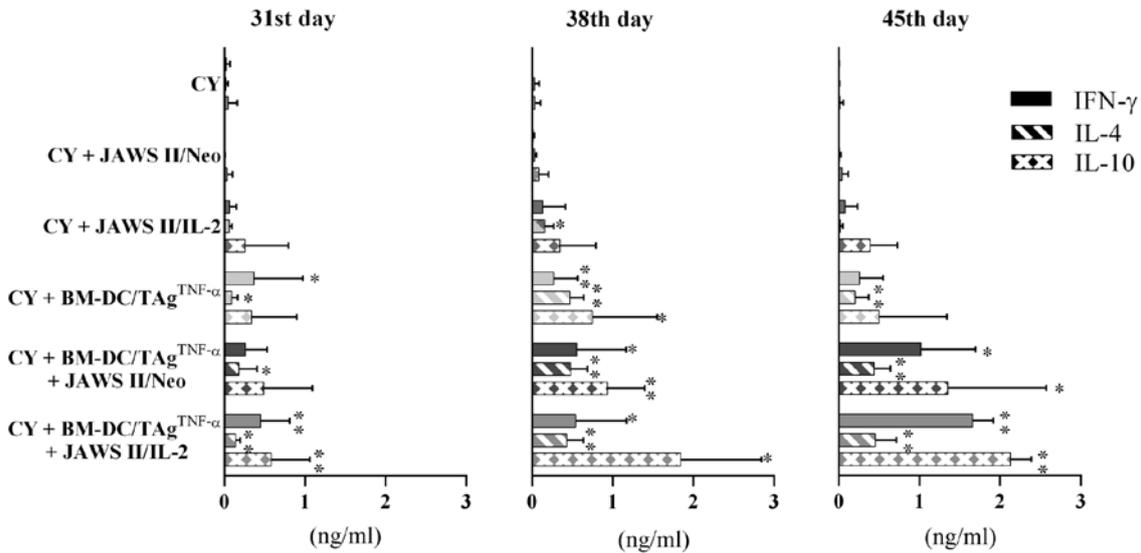


Figure 4. Cytokine production by CD4<sup>+</sup> cells isolated from tumor tissue. Tumor tissues were collected from mice (N=3-6 per group, two independent experiments). The CD4<sup>+</sup> cells were isolated from tumor tissue using anti-CD4 antibody-coated magnetic beads. Cells were cultured for 48 h with Con A. The concentrations of IFN- $\gamma$ , IL-4 and IL-10 in cell culture supernatants were measured by ELISA. Differences by mean  $\pm$  SD were estimated. The statistical significance was calculated (for details see Fig. 3).

*Changes in lymphocyte infiltration into the tumor tissue generated by combined treatment.* Taking into consideration the multidirectional activity of the T cell subpopulations participating in tumor antigen (TA<sub>g</sub>)-specific response, it was important to determine whether the combined treatment affected the changes in CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocyte infiltration into the MC38 tumor tissue. For this purpose, tumor nodules were harvested from untreated mice on the 31st day of the experiments and from mice of treated groups on the 31st, 38th and 45th day. We determined a percentage of CD4<sup>+</sup> cells among leukocytes by flow cytometry (Fig. 3A). In the case of CY-treated mice, the CD4<sup>+</sup> cell percentage among CD45<sup>+</sup> leukocytes amounted to 20.2% (the 31st day), 14.9% (the 38th day) and 19.9% (the 45th day). The use of CY + transduced JAWS II cells did not increase the percentage of CD4<sup>+</sup> T cells. Only after the replacement of JAWS II cells by BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>   $\pm$  transduced JAWS II cells the statistically significant growth in the percentage of infiltrating CD4<sup>+</sup> T cells compared to CY-treated control was noted (Fig. 3A).

Changes in the percentage of CD8<sup>+</sup> T tumor infiltrating lymphocytes were dependent on the presence of transduced JAWS II cells in DC-vaccines (Fig. 3B). Each day of the harvesting of experiments, the lowest percentage of these lymphocytes in mice treated with CY  $\pm$  BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>  was found. Whereas, the supplementation of cytotostatic treatment by transduced JAWS II cells resulted in the gradual increase of percentage of CD8<sup>+</sup> T cells. Although this process was dependent on duration of the experiments, a statistically significant increase of the CD8<sup>+</sup> T cell percentage (at  $P < 0.01$ , compared to CY-treated control) was on the 38th day. However, the most intensive growth of CD8<sup>+</sup> T cell subpopulation was observed on the 45th day of experiments after application of CY + BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>  + JAWS II/IL-2 cells (45.2% of CD45<sup>+</sup> cells).

Overall, the application of CY followed by DC-based vaccines resulted in a significant delay of MC38 tumor growth, which was accompanied by considerable changes in

CD4<sup>+</sup> and CD8<sup>+</sup> tumor infiltrating lymphocytes (TILs). The intensity of the influx of TILs and their maintenance in tumor tissue depended to a great extent on the nature of the cellular vaccines; a CD4<sup>+</sup> cell infiltration was associated with the presence of BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>  in vaccines, whereas the increase of CD8<sup>+</sup> cell influx, with the presence of JAWS II/IL-2 cells.

*Influence of DC-based vaccines on diversity of CD4<sup>+</sup> cell subpopulations infiltrating the tumor tissue.* In the following part of the investigation, we focused especially on the ability of CD4<sup>+</sup> T cell populations to trigger the antitumor response. For this purpose, after magnetic isolation of cells from tumor tissue they were stimulated with concanavalin A (Con A). Analysis of their activity was based on cytokine secretion and changes in the percentage of cells expressing transcription factors specific for main CD4<sup>+</sup> subpopulations (T-bet, GATA3, ROR $\gamma$ t and FoxP3).

Between the first and last harvesting day of the experiments, CD4<sup>+</sup> T cells isolated from mice treated with CY  $\pm$  transduced JAWS II cells produced  $< 0.13$  ng/ml IFN- $\gamma$  (Fig. 4). On day 31, the application of CY + BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>  caused a significant increase of the production which amounted to 0.37 ng/ml, but the highest amount of IFN- $\gamma$  was released by CD4<sup>+</sup> T cells derived from mice treated with CY + BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>  + JAWS II/IL-2 cells (0.45 ng/ml). In both cases, the differences in cytokine production level were statistically significant compared to CY-treated group. However, the largest alterations in the level of IFN- $\gamma$  production were found on the 45th day. Administration of CY + BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>  + JAWS II/Neo cells, caused production of IFN- $\gamma$  at a level of 1.0 ng/ml ( $P = 0.015$ ). Whereas, the use of the CY + BM-DC/TA<sub>g</sub><sup>TNF- $\alpha$</sup>  + JAWS II/IL-2 cells induced the highest IFN- $\gamma$  secretion amounting to 1.7 ng/ml ( $P = 0.004$  compared to CY-treated control).

The level of IL-4 production by CD4<sup>+</sup> T cells, likewise, was diversified in the vaccine- and time-dependent manner. CD4<sup>+</sup>

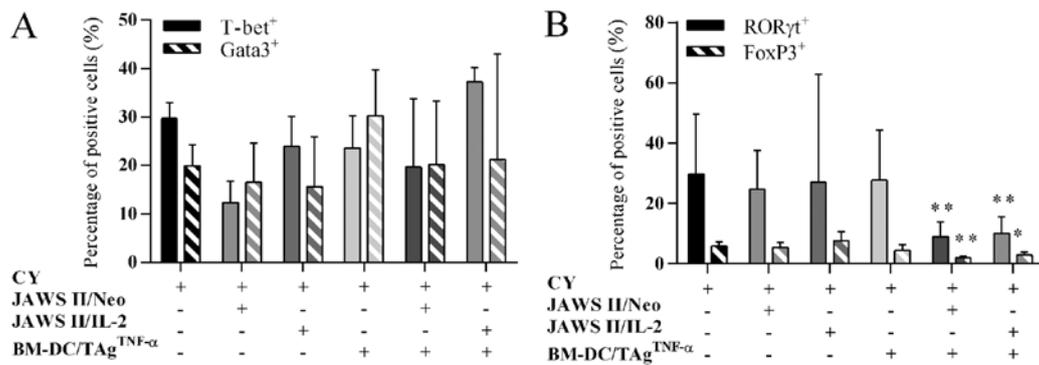


Figure 5. Activity of CD4<sup>+</sup> cells isolated from tumor tissue. On the 45th day of the experiments, tumor tissues were collected from mice (N=3-6 per group, two independent experiments). The CD4<sup>+</sup> cells were isolated from tumor tissue using anti-CD4 antibody-coated magnetic beads. Cells were cultured for 48 h with Con A and the next they were fixed and stained with anti-CD4 antibody and anti-T-bet, anti-GATA3 (A), anti-RORγt, or anti-FoxP3 (B) antibody or appropriate isotype control and analyzed by flow cytometry. The percentage of cells expressing transcription factors among CD4<sup>+</sup> cells was estimated by mean ± SD. The statistical significance was calculated (for details see Fig. 3).

T cells isolated from tumor tissue of mice treated with CY or mice receiving CY + JAWS II/Neo cells, generally produced less IL-4 than 0.03 ng/ml (Fig. 4). On the 38th day, the administration of CY + JAWS II/IL-2 cells caused a significant increase in the cytokine production. However, the treatment with CY + BM-DC/TAg<sup>TNF-α</sup> or with ternary combinations was more potent for CD4<sup>+</sup> T cell activation. Lymphocytes isolated from mice treated with CY + BM-DC/TAg<sup>TNF-α</sup> + transduced JAWS II cells on the 45th day produced a very similar amount of cytokine. The diversity in the level of IL-4 production was statistically significant (P<0.01 vs. CY-treated control). Of note, regardless of the level of cytokine production, the treatment with ternary combinations resulted in the long-lasting activity of both IFN-γ- and IL-4-producing CD4<sup>+</sup> T cells which in further perspective could affect the polarization of immune response towards Th1 or Th2.

According to the potential role of IL-10-producing T regulatory cells in modulation of immune response against growing colon cancer, we decided to determine the level of IL-10 production by CD4<sup>+</sup> T cells separated from tumor tissues. The cells originated from control mice or mice treated with CY + JAWS II/Neo cells produced <0.08 ng/ml of IL-10 (Fig. 4). On day 31, only the application of CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells caused a statistically significant increase in the IL-10 production compared to the CY-treated control (0.58 ng/ml, P=0.004). On the 38th day, the amount of IL-10 significantly increased after application of both ternary combinations and ranged from 0.9 to 1.85 ng/ml (at P<0.05 compared to CY-treated mice). On the 45th day, the application of CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells caused the highest cytokine production by CD4<sup>+</sup> TILs (2.13 ng/ml, P=0.004). Thus, the secretion of IL-10 was time-dependent, and related to the nature of cellular vaccines in a similar manner to IFN-γ and IL-4 production. It was also associated with the largest therapeutic effect.

The activity of the CD4<sup>+</sup> T lymphocytes after stimulation with Con A was confronted also with the percentage of cells expressing transcription factors specific for analyzed Th cell subpopulations (Fig. 5).

Tumor tissue from treated mice was infiltrated with growing number of TILs. The percentage of CD4<sup>+</sup>T-bet<sup>+</sup> cells on the

31st day ranged from 8.0% (CY-treated control) and 17.7% (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells), while on the 38th day reached 24.7% (CY + BM-DC/TAg<sup>TNF-α</sup>) and 32.0% (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells) (data not shown). However, the number of CD4<sup>+</sup>T-bet<sup>+</sup> cells in these groups decreased on the 45th day (Fig. 5A) and ranged between 12.3% (CY + JAWS II/Neo cells) and 23.9% (CY + JAWS II/IL-2 cells), compared to the CY-treated mice (29.7%). Only the treatment with CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells resulted in considerable increase in percentage of CD4<sup>+</sup>T-bet<sup>+</sup> lymphocytes (37.2%). As stated previously, the differences in the size of CD4<sup>+</sup>T-bet<sup>+</sup> cell subpopulation were dependent on time and the nature of vaccines.

The obtained results are essential in the light of our assumption that the BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells were involved in stimulation of Th1-type response which was accompanied by the increase in IFN-γ production to mediate the response polarization.

The second TIL-subpopulation was CD4<sup>+</sup>GATA3<sup>+</sup> cells. On the 38th day, the percentage of CD4<sup>+</sup>GATA3<sup>+</sup> cells was higher than on 31st day of experiments and increased to 20.1% (CY + BM-DC/TAg<sup>TNF-α</sup>) or 21.7% (BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells) (data not shown). On the 45th day (Fig. 5A), the size of CD4<sup>+</sup>GATA3<sup>+</sup> cell population increased to 30.2% only in the case of mice treated with CY + BM-DC/TAg<sup>TNF-α</sup> whereas the application of other types of vaccines diminished or remained at constant the percentage of CD4<sup>+</sup>GATA3<sup>+</sup> cells. The strongest effect of treatment with BM-DC/TAg<sup>TNF-α</sup> highlighted the relationship between the nature of the vaccine and CD4<sup>+</sup>GATA3<sup>+</sup> cell population differentiation.

On the 31st day of the experiments the percentage of CD4<sup>+</sup>RORγt<sup>+</sup> cells was relatively low and ranged from 9.4% (for CY + JAWS II/IL-2 group) to 15.2% (for CY-treated group). One week later (38th day) we observed ~2-fold increase in all groups (from 18.3% for CY + BM-DC/TAg<sup>TNF-α</sup>, 20.8% for CY + JAWS II/IL-2 group, to 30.8% for CY-treated group) (data not shown). The statistically significant diversity in the number of CD4<sup>+</sup>RORγt<sup>+</sup> T cells was found only on the 45th day (Fig. 5B). When mice were treated with CY, the percentage of lymphocytes amounted to 29.7%. The application of CY with transduced JAWS II cells or with BM-DC/TAg<sup>TNF-α</sup> dimin-

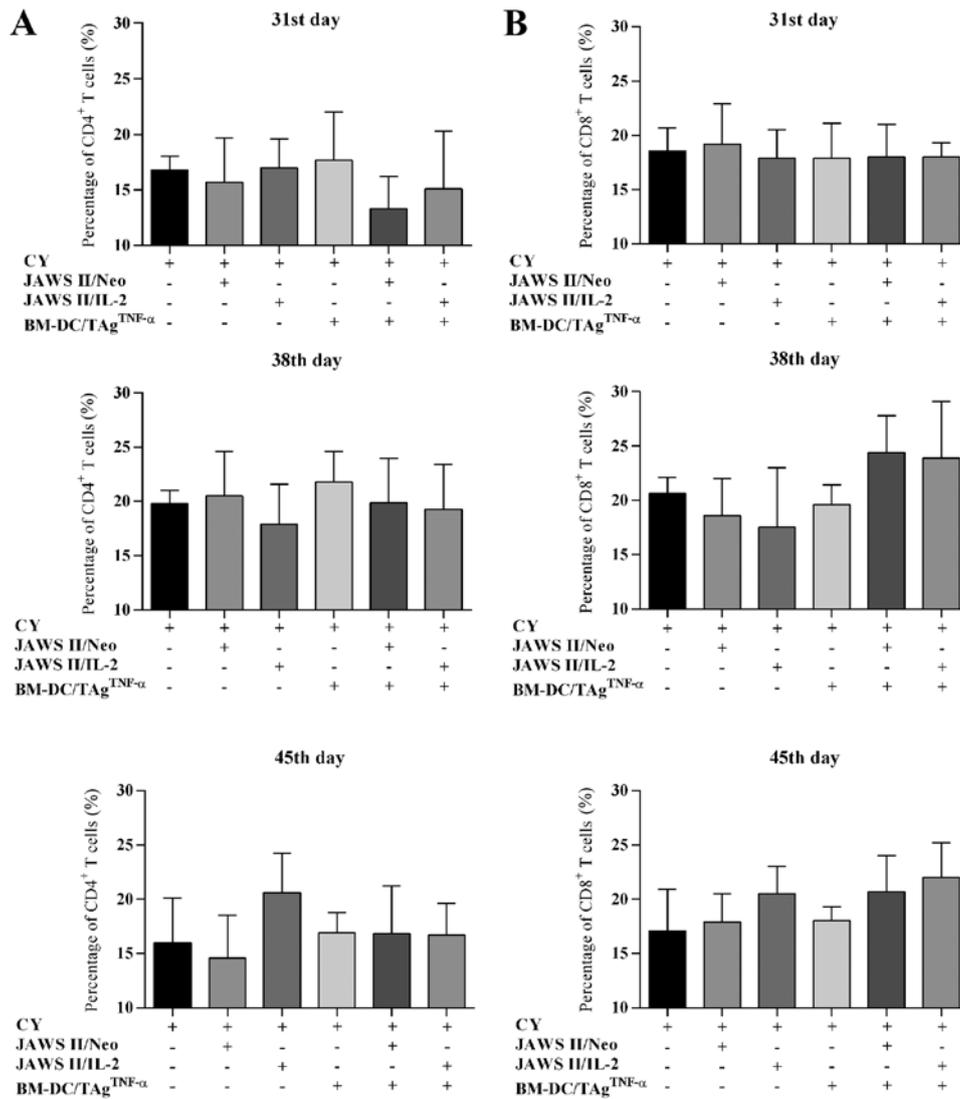


Figure 6. Percentage of T lymphocytes in the spleen. Spleen CD4<sup>+</sup> (A) or CD8<sup>+</sup> (B) cells from mice treated with CY ± DC-vaccines (N=3-6 per group, two independent experiments) were collected on the 31st, 38th and 45th day of the experiments. The cell suspensions were incubated with anti-CD4 or anti-CD8 antibodies and analyzed by flow cytometry. The percentage of CD4<sup>+</sup> or CD8<sup>+</sup> cells was not statistically significant.

ished the percentage to 24.7 or 27.7%, respectively. However, a significant decrease in percentage of CD4<sup>+</sup>RORγt<sup>+</sup> T cells was noted after the application of CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells (10.1%, P=0.004) or CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/Neo cells (9.1%, P=0.008), compared to CY-treated control. This decrease of CD4<sup>+</sup>RORγt<sup>+</sup> percentage was perhaps associated with differentiation of cells during the Th1 or Th2 polarization of immune response at least in the case of CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cell application.

CD4<sup>+</sup> T lymphocytes expressing FoxP3 transcription factor were the fourth analyzed subpopulation. On the 31st day as well as on the 38th day of the experiments in tumor tissue derived from treated mice a gradual decrease in percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> cells was noted (data not shown). Especially, when mice were administered CY + BM-DC/TAg<sup>TNF-α</sup> or CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells, a reduction in the number of TILs (3.4 and 4.1%, respectively) was observed compared to 9.5% for CY-treated control (data not shown). On the 45th day (Fig. 5B), the application of CY + BM-DC/TAg<sup>TNF-α</sup> ± transduced JAWS II cells caused statistically significant

decrease in the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> lymphocytes amounting to 2.0% (P=0.004) or 2.9% (P=0.015) compared to CY-treated control. The differences found in the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> cells turned out to be similar to those observed for CD4<sup>+</sup>RORγt<sup>+</sup> T cell subpopulation. However, considering the decrease of CD4<sup>+</sup>FoxP3<sup>+</sup> cell percentage and the increase of IL-10 production by CD4<sup>+</sup> TILs, we postulate that other subpopulations of CD4<sup>+</sup> cells than CD4<sup>+</sup>FoxP3<sup>+</sup> cells are also able to produce this cytokine during the long-lasting fight against tumor.

*Changes in systemic antitumor response generated by CY and DC-based vaccines.* Firstly, we decided to analyze the effect of the therapy on an alteration in size of two main spleen cell populations. On the 31st day the percentage of CD4<sup>+</sup> splenocytes obtained from treated mice ranged between 13.3% (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/Neo cells) and 17.7% (CY + BM-DC/TAg<sup>TNF-α</sup>) vs. 16.8% of CY-treated mice (Fig. 6A). A slight increase in the size of CD4<sup>+</sup> T cell population ranged from 17.9% (CY + JAWS II/IL-2) to 21.8%

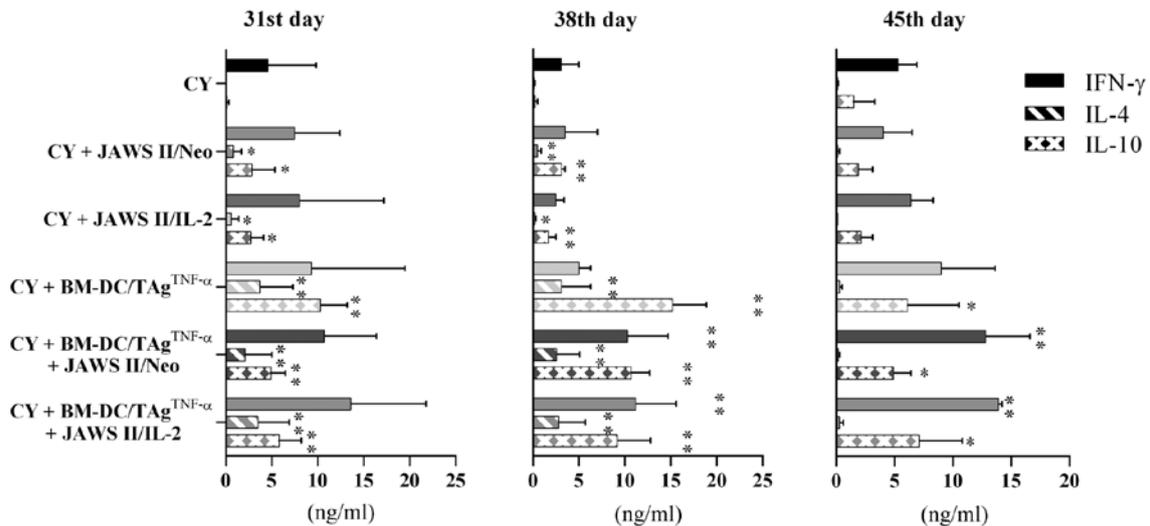


Figure 7. Cytokine production by spleen cells. On the 31st, 38th and 45th day of the experiments, spleens were obtained from CY  $\pm$  DC-based vaccine-treated mice (N=3-6 per group, two independent experiments). Splenocytes were cultured in a presence of Con A (0.5  $\mu$ g/ml). After 48 h, cell culture supernatants were collected, and the concentrations of IFN- $\gamma$ , IL-4 and IL-10 were measured by ELISA (mean  $\pm$  SD). The statistical significance was calculated (for details see Fig. 3).

Table I. Tumor-specific cytotoxic activity of splenocytes.

Therapy	Percentage of cytotoxicity $\pm$ SD (%)		
	31st day	38th day	45th day
CY (Control)	13.84 $\pm$ 6.3	24.88 $\pm$ 11.4	28.43 $\pm$ 8.4
CY + JAWS II/Neo	19.62 $\pm$ 9.2	28.03 $\pm$ 5.6	34.02 $\pm$ 13
CY + JAWS II/IL-2	14.82 $\pm$ 9.9	20.93 $\pm$ 10.9	25.50 $\pm$ 10.1
CY + BM-DC/TAg <sup>TNF-<math>\alpha</math></sup>	18.34 $\pm$ 9.4	32.94 $\pm$ 9.7	32.86 $\pm$ 7.2
CY + BM-DC/TAg <sup>TNF-<math>\alpha</math></sup> + JAWS II/Neo	23.77 $\pm$ 5.8 <sup>a</sup>	31.21 $\pm$ 5.6	36.41 $\pm$ 12.5
CY + BM-DC/TAg <sup>TNF-<math>\alpha</math></sup> + JAWS II/IL-2	19.27 $\pm$ 7.1	39.00 $\pm$ 9.9	37.56 $\pm$ 13.0

Spleen cells from mice treated with CY  $\pm$  DC-vaccines (N=3-6 per group) were collected on the 31st, 38th and 45th day and were *in vitro* restimulated in the presence of MC38/0 cells for 4 days. Activated spleen cells were next incubated with DiOC<sub>18</sub>-stained MC38/0 cells for 4 h at the effector to target cell ratio 10:1. The specific killing of target cells illustrated by PI incorporation was evaluated by flow cytometry. The percentage of dead cells (PI-positive) among the DiOC<sub>18</sub>-stained target cells represented the percentage of cytotoxicity. Cytotoxic activity of splenocytes derived from healthy mice amounted to 12.4%, of cells from untreated tumor-bearing mice obtained on the 31st day, diminished to 10.2%. The statistical significance was calculated (for details see Fig. 3).

(CY + BM-DC/TAg<sup>TNF- $\alpha$</sup> ) was observed on the 38th day. On the 45th day of experiments, in most of the treated groups the size diminished to 14.6% (CY + JAWS II/Neo cells) or 16.9% (CY + BM-DC/TAg<sup>TNF- $\alpha$</sup> ), except from mice treated with CY + JAWS II/IL-2 cells, in which the percentage of CD4<sup>+</sup> T splenocytes reached 20.6%. Thus, on consecutive harvesting days, only slight differences, independent on the nature of vaccines, could be observed. In contrast to CD4<sup>+</sup> T splenocytes, changes in CD8<sup>+</sup> cell percentage depended to a great extent on the vaccines (Fig. 6B). On the 31st day, percentage of CD8<sup>+</sup> T cell subpopulation obtained from treated mice ranged between 17.9% (CY + BM-DC/TAg<sup>TNF- $\alpha$</sup>  or CY + JAWS II/IL-2 cells) and 19.2% (CY + JAWS II/Neo cells). However, on the next harvesting day, the percentage of these splenocytes diminished after treatment with CY  $\pm$  single cell vaccines and increased when mice were treated with ternary combinations

(23.9 and 24.4%). On the 45th day of experiments, in all groups the percentage of CD8<sup>+</sup> cells was similar to that previously recorded. No statistically significant changes were found but the most visible changes were exhibited on the 38th day, this is 7 days after the third vaccination.

Taking into consideration the inhibition of tumor growth and significant differences in number and activity of CD4<sup>+</sup> TILs we decided to analyze the level of spleen cell reactivity, within the cytotoxic activity of splenocytes harvested from treated mice. On the 31st day of experiments, when CY + BM-DC/TAg<sup>TNF- $\alpha$</sup>  + JAWS II/Neo cells were applied, the cytotoxicity amounted to 23.8% (P=0.03; compared to CY-treated group) (Table I). On the 38th day of experiments, the splenocytes cytotoxicity increased considerably in all groups, and after treatment with CY + BM-DC/TAg<sup>TNF- $\alpha$</sup>  + JAWS II/IL-2 cells were on average 39% and remained similar to those

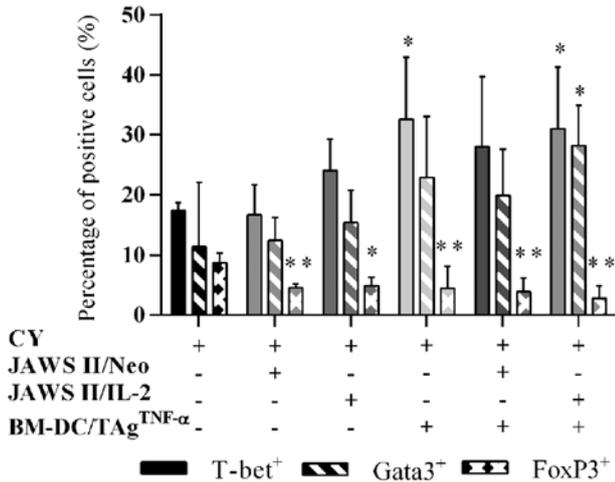


Figure 8. Activity of CD4<sup>+</sup> spleen cells. On the 45th day of the experiments, spleens were obtained from CY ± DC-based vaccine-treated mice (N=3-6 per group, two independent experiments). Splenocytes were stimulated for 48 h with Con A (0.5 μg/ml). For the last 6 h, brefeldin A (10 μg/ml) and monensin (2 μM) were added to cell culture. Stimulated spleen cells were fixed, and stained with anti-CD4 antibody and anti-T-bet, anti-GATA3, anti-FoxP3 antibody. As a control, the appropriate isotypes were used. Samples were analyzed by flow cytometry. The percentage of positive cells among CD4<sup>+</sup> splenocytes was calculated (mean ± SD). The statistical significance was calculated (for details see Fig. 3).

observed on the 45th day (37.6%). On the contrary, the use of CY + JAWS II/IL-2 cell vaccines elicited the weakest but permanently growing cytotoxicity of spleen cells. Overall, the cytotoxic activity of spleen cells gradually increased in the consecutive harvesting days, excluding therapy with CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2, which induced the highest and stable cytotoxicity on the 38th day. The data shed light on the strong relationship between kinetics of systemic antitumor reaction and the nature of the vaccines.

In order to determine the effect of the combined treatments on the spleen cell ability for cytokine production, the cells were stimulated with Con A, and concentrations of IFN-γ, IL-4, IL-17A and IL-10 in supernatants from the above cell cultures were measured. Splenocytes from healthy mice were also stimulated and only IFN-γ production was found (3.7 ng/ml).

Splenocytes from treated tumor-bearing mice elicited strong differences in IFN-γ production. On the 31st day (Fig. 7), the concentration of IFN-γ ranged from 4.6 ng/ml (CY-treated mice) to 13.6 ng/ml (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells). On the 38th day, this production ranged from 3.1 ng/ml (CY-treated mice) to 11.2 ng/ml (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells). Whereas, on the 45th day, it was 6.4 ng/ml (CY + JAWS II/IL-2 cell) to 13.9 ng/ml (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells). This increase was statistically significant (P<0.01) compared to CY-treated control (Fig. 7).

The ability of spleen cells to produce IL-4 was changed. On 31st or 38th days of the experiments we observed that the combined treatments affected the splenocytes to secrete a significantly higher IL-4 concentration than CY-treated group (Fig. 7). The statistically significant increase in the cytokine production was observed when mice received CY + BM-DC/TAg<sup>TNF-α</sup> ± transduced JAWS II cells (P<0.01).

However, the highest IL-4 concentration was noted after treatment with CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells. Spleen cells harvested on the 45th day secreted <0.3 ng/ml of IL-4.

The level of IL-10 production by splenocytes was strongly associated with the nature of the treatment of mice. Cells from CY-treated mice secreted IL-10 in a gradually growing manner (from 0.1 to 0.2 ng/ml to 1.5 ng/ml respectively, on the consecutive harvesting days). On the 38th day, the application of CY + transduced JAWS II cells caused a significant increase (vs. CY-treated group) in the IL-10 production amounting to 1.7 or 3.1 ng/ml. After treatment with ternary combinations, IL-10 production was 10.7 or 9.2 ng/ml. However, only after treatment with CY + BM-DC/TAg<sup>TNF-α</sup> they exhibited the highest secretion of cytokine (15.2 ng/ml). On the 45th day, the decrease in the production was observed. Of note, the use of CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells, regardless of harvesting day, resulted in extended IL-10 production on a moderate level.

It has to be highlighted that the concentration of IL-17A in culture supernatants of splenocytes was very low (<0.2 ng/ml) and did not differ significantly among groups and in time (data not shown).

The changes in the level of cytokine production revealed that long-lasting spleen cell activity depended on the nature of the vaccines. The highest IL-4 and IL-10 production was elicited on the 38th day, whereas the IFN-γ secretion increased up to the 45th day of the experiments.

*Influence of DC-based vaccines on diversity of CD4<sup>+</sup> cell subpopulations in spleen.* The observed changes in the cytokine secretion seemed to be associated with the stage of CD4<sup>+</sup> T lymphocyte differentiation. Therefore, the estimation of the percentage of T-bet<sup>+</sup>, GATA3<sup>+</sup>, RORγ<sup>+</sup> and FoxP3<sup>+</sup> cells among harvested splenocytes was reasonable.

On the 31st and the 38th days of the experiments, the percentage of CD4<sup>+</sup>T-bet<sup>+</sup> lymphocytes increased gradually accordingly to the type of treatment (data not shown). On the 45th day (Fig. 8), the percentage grew significantly and ranged from 17.4% (CY-treated mice) to 28.0% (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/Neo cells) and 31.0% (CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 cells). However, the highest percentage of CD4<sup>+</sup>T-bet<sup>+</sup> lymphocytes (32.6%) was induced by application of CY + BM-DC/TAg<sup>TNF-α</sup> (statistically significant vs. CY-treated control at P<0.05). The obtained results indicate that one-third of the splenocytes were able to express the T-bet transcription factor and probably to provide the high IFN-γ production.

On the 31st day of the experiments the percentage of CD4<sup>+</sup>GATA3<sup>+</sup> cells was not varied among treated groups (from 5.1% for CY-treated group, 6.5% for CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/Neo, to 8.0% for CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 group), but on the next harvesting day, it grew approximately two times after application of CY + BM-DC/TAg<sup>TNF-α</sup> ± transduced JAWS II cells (14.8% for CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/Neo group and 13.1% for CY + BM-DC/TAg<sup>TNF-α</sup> + JAWS II/IL-2 group) and was statistically significant (at P<0.01) vs. CY-treated control (data not shown). On the 45th day (Fig. 8), the use of CY alone or followed by transduced JAWS II cells resulted in a slight increase in the percentage

of splenocytes; treatment with CY + BM-DC/TA $g^{TNF-\alpha}$ , CY + BM-DC/TA $g^{TNF-\alpha}$  + JAWS II/Neo cells caused additional increase of the percentage to 22.9 or 19.9%, respectively. The increase of percentage of CD4 $^{+}$ GATA3 $^{+}$  cells to 28.2% (P=0.019 vs. CY-treated control) was a result of application of CY + BM-DC/TA $g^{TNF-\alpha}$  + JAWS II/IL-2 cells and highlighted the role of this vaccine in differentiation of CD4 $^{+}$  cells towards GATA3 $^{+}$  Th2 cells. Of note, the use of any combination of treatment did not affect the changes in the number of CD4 $^{+}$ ROR $\gamma$ t $^{+}$  spleen cells (data not shown) that was consistent with the lack of IL-17A production.

The relatively high level of IL-10 production by splenocytes, suggested the presence of the Treg cell population in spleen. To confirm this assumption, the size of CD4 $^{+}$ FoxP3 $^{+}$  splenocytes subpopulation was analyzed. On the 31st and 38th day of experiments, we noted considerable decrease in these percentages compared to CY-treated groups (data not shown). A similar process was observed on the 45th day (Fig. 8). The use of CY + BM-DC/TA $g^{TNF-\alpha}$  resulted in a decrease in the lymphocyte percentage to 4.5%, compared to 8.7% for CY-treated mice. The application of CY + BM-DC/TA $g^{TNF-\alpha}$  + JAWS II/Neo cells induced a further reduction of the lymphocyte population (3.9%) but the lowest percentage of CD4 $^{+}$ FoxP3 $^{+}$  cells was demonstrated after the application of CY + BM-DC/TA $g^{TNF-\alpha}$  + JAWS II/IL-2 cells (2.8%). These changes were statistically significant vs. CY-treated control (P<0.05).

The inverse relationship between the number of CD4 $^{+}$ GATA3 $^{+}$  cells and CD4 $^{+}$ FoxP3 $^{+}$  cells associated with application of CY + BM-DC/TA $g^{TNF-\alpha}$  + JAWS II/IL-2 cells suggested that other subpopulations of CD4 $^{+}$  cells were able to produce IL-10 during the long-lasting fight against tumor. The treatment with ternary combinations turned out to be necessary for effective stimulation of both Th1-type and Th2-type response in spleen and creation of the opportunity to reduce the percentage of Treg cells but not other CD4 $^{+}$  T cell subpopulations participating in the prolonged IL-10 production.

## Discussion

Cyclophosphamide (CY) is DNA-alkylating agent widely used in the treatment of malignant diseases. Acting in dose-dependent manner, it induces cytotoxic effect, promotes Th1 cells, and inhibits the T regulatory cell activity (1,3). In the present study, CY was administered at a single dose of 150 mg/kg body weight to reduce the volume of the MC38 tumor nodules, and to eliminate suppressor cells.

The potency of DCs for tumor growth inhibition has been confirmed both in experiments with animal tumor models and in clinical trials (13). Despite extensive research on the harnessing of DCs for immunotherapy, its effects are still unsatisfactory (14-16). DCs, exploited as therapeutic tools, are often subjected to *ex vivo* antigenic stimulation (17,18). In the present study, bone marrow-derived DC stimulation was induced by the MC38/0 cell lysate (TA $g$ ) and TNF- $\alpha$  (BM-DC/TA $g^{TNF-\alpha}$ ). After the BM-DC maturation, an increase in expression of CD80 and slight CD86 was observed. In light of the results obtained by Kuchroo and Ranger (19,20) the DCs with high expression of CD80, especially due to their capacity to produce the inflammatory cytokines, should be able to

induce differentiation of Th1 more than Th2 lymphocytes. The JAWS II cells transduced with IL-2 gene (JAWS II/IL-2) were used as element of vaccines responsible for tumor growth inhibition (11,21). As in our previous studies these cells were exploited mainly as a source of IL-2 supporting antitumor response.

Chemo-immunotherapy using CY and DC-based vaccines is considered as an effective tool in the fight against cancer, including the generation of immune memory to prevent cancer recurrence (3,5,6). In the MC38-model, we observed that such treatment affected statistically significant tumor growth inhibition (Fig. 2) which was the most spectacular on the 45th day of the experiment, 14 days after the third application of CY + BM-DC/TA $g^{TNF-\alpha}$  + JAWS II/IL-2 cells (65.6% of TGI and TRV amounted to 17 days).

Based on our earlier study and achievements presented by other authors (11,21-25) we recognized an infiltration of lymphoid cells into tumor tissue as an important prognostic factor. High lymphocyte influx was observed already on the 31st day of the experiments, especially after the application of CY + BM-DC/TA $g^{TNF-\alpha}$   $\pm$  transduced JAWS II cells (Fig. 3). Such treatment resulted in increased number of CD4 $^{+}$  and CD8 $^{+}$  T infiltrating tumor lymphocytes (TILs). These results were consistent with our previous observation in which immunotherapy with various combined cellular vaccines elicited more intense influx than vaccines containing only one type of DCs (11,21,26).

For further analysis of the relationship between nature of cellular vaccines and stimulation of lymphoid immune cells, we attempted to determine the effect of IL-2-producing and/or stimulated DCs, included in the vaccines supporting the CY-therapy, on differentiation of the main CD4 $^{+}$  T cell subpopulations and their prolonged presence in the tumor tissue. For this purpose, we estimated the production of Th-specific cytokines and the expression of corresponding transcription factors. We found changes in the number and reactivity of the Th1 (T-bet $^{+}$ ), Th2 (GATA3 $^{+}$ ), Th17 (ROR $\gamma$ t $^{+}$ ), and Treg (FoxP3 $^{+}$ ) subpopulations corresponding with a creation of antitumor response, both in tumor tissue and in the spleens. The present study showed that the level of cytokine production was time-dependent. Regardless of the level of cytokine production, the treatment with ternary combinations resulted in the long-lasting activity of both IFN- $\gamma$ - and IL-4-producing CD4 $^{+}$  TILs (Fig. 4) and in consequence would be responsible for Th1 or Th2 polarization of the immune response. These results were relevant in light of our assumption that the BM-DC/TA $g^{TNF-\alpha}$  + JAWS II/IL-2 cells contribute to stimulation of dominant Th1 cell activity. It was consistent with the observation that the treatment with ternary combinations elicited the rise of splenocyte ability to produce IFN- $\gamma$  as well as with the data obtained in other investigations both on material harvested from patients with cancers (27-29) and in experimental models (3,6,30).

Production of IL-4 and GATA3 expression are both believed to be features of Th2-type cells. The production of this cytokine by CD4 $^{+}$  TILs remained constant after the 38th day in contrast to splenocytes which were not able to produce IL-4 on the 45th day. On the last harvesting day, diversity of CD4 $^{+}$ GATA3 $^{+}$  cells, similarly to CD4 $^{+}$ T-bet $^{+}$  cells, depended on the nature of cellular vaccines. However, the strongest

CD4<sup>+</sup>GATA3<sup>+</sup> cell response in tumor tissue was elicited by the application of CY + BM-DC/Tag<sup>TNF- $\alpha$</sup> , whereas, the highest percentage of spleen-derived CD4<sup>+</sup>GATA3<sup>+</sup> cells was found after treatment with CY + BM-DC/Tag<sup>TNF- $\alpha$</sup>  + JAWS II/IL-2 cells.

The relationship of IFN- $\gamma$  and IL-10 secretion by CD4<sup>+</sup> TILs and this observed in the case of spleen cells was more complicated, due to their inverse nature (Figs. 4 and 7): CD4<sup>+</sup> TILs were able to produce more IL-10 than IFN- $\gamma$  while spleen cells produced much more IFN- $\gamma$  than IL-10. This finding suggests that duration of CD4<sup>+</sup> T lymphocyte activity could be associated with the induction of their polarization by vaccines containing BM-DC/Tag<sup>TNF- $\alpha$</sup>  and enhanced by JAWS II/IL-2 cells.

Admittedly, administration of CY is believed to reduce percentage of Treg cells, but in the present study, its effect was not observed. This could be due to the fact that cytostatic effect was short-term, especially that the time interval between single CY administration and the last day of material harvesting amounted to 31 days. Thus, gradual decrease in percentage of the CD4<sup>+</sup>FoxP3<sup>+</sup> and also CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup> T cells was strongly determined by multiplicity application and nature of vaccines not only in the tumor tissue but also in the spleen (Figs. 5 and 8). The inverse relationship between the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> cells and CD4<sup>+</sup>T-bet<sup>+</sup> or CD4<sup>+</sup>GATA3<sup>+</sup> T cells might be associated with an inhibition of differentiation of Treg cells or their ability to convert to the other subpopulations, as was suggested by Zhu and Paul (31). Additionally, in a report by Yamamoto *et al.* (29) the elimination of Treg cells in the spleen effectively induced an increase in the number of Th1 cells (producing IFN- $\gamma$ ) and reduced the number of Th2 cells (producing IL-10). In another case, the application of autologous DC-based vaccine together with low dose of IL-2 resulted in generation of response against kidney cancer associated with a decrease in the number of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and a promotion of a Th1-type response (32). Furthermore, polarization of anti-tumor response induced by administration of DC vaccines was associated with the elimination of Treg cells, and increased the cytotoxic activity of splenocytes *in vitro* and the number of IFN- $\gamma$ -secreting CD4<sup>+</sup> and CD8<sup>+</sup> cells (26,33). The cytotoxic activity of spleen cells was also revealed by us. It gradually increased in the consecutive harvesting days (Table I) but after ternary combinations the highest cytotoxicity was observed on the 38th day.

Interesting relationships were found between the expression of FoxP3 and the secretion of IL-10, both believed to be features of Treg cells. The greatest difference in IL-10 production by CD4<sup>+</sup> TILs was found on the 45th day. The growing body of evidence points out that among CD4<sup>+</sup> lymphocytes, Treg cells are not the only ones that are able to produce IL-10. For example, IL-10 can be secreted by IFN- $\gamma$ -producing cells representing T-bet<sup>+</sup> cell subpopulation (34) or by GATA3<sup>+</sup> cells (35). Shoemaker *et al.* (36) found a positive correlation between the level of IL-10 production and GATA3 expression by splenocytes. They demonstrated that splenic Th2 lymphocytes from healthy mice can produce IL-10. Even after treatment with DC-vaccines producing IL-12 the splenocytes are able to produce IL-10 (26). Taking into consideration that the percentage of CD4<sup>+</sup> T cells expressing GATA3 was higher than cells expressing FoxP3, we assume

here that in the MC38-tumor model CD4<sup>+</sup>GATA3<sup>+</sup> T cell are mainly responsible for IL-10 production and CD4<sup>+</sup>FoxP3<sup>+</sup> lymphocytes only slightly contribute to this process. The inverse relationship between the number of CD4<sup>+</sup>GATA3<sup>+</sup> cells and CD4<sup>+</sup>FoxP3<sup>+</sup> cells associated with application of CY + BM-DC/Tag<sup>TNF- $\alpha$</sup>  + JAWS II/IL-2 cells suggested that other subpopulations of CD4<sup>+</sup> cells were able to produce IL-10 during the long-lasting fight against tumor. Under a proper stimulation Th2 cells secrete IL-4 responsible for stabilization the Th2 status of CD4<sup>+</sup> T cells, lack of cytotoxic of the CD8<sup>+</sup> T cells and protection lymphocytes from apoptosis. On the contrary, IL-4 can promote tumor growth due to presence of IL-4R on surface of many types of tumor cells (37). Thus, the temporary potential of CD4<sup>+</sup> lymphocytes to produce IL-4 in our model can be associated with an increase of CD8<sup>+</sup> cell activation direct towards tumor cells. Some authors show that IL-10 exploited the generation of immunosuppression, while others demonstrate its immunostimulatory properties. This functional duality of IL-10 is substantial in the context of the regulation of tumor growth (35). Additionally, it is observed that relatively high systemic doses of IL-10, were not immunosuppressive and, when given in combination with IL-4, countered its suppressive effect.

In conclusion, the administration of CY followed by multiple application of various DC-based vaccines into MC38 colon carcinoma-bearing mice elicited MC38 tumor growth delay. This was accompanied by an increase in the number and activity of Th1 and Th2 cells and a decrease in the number of Th17 and Treg cells. The gradual changes in Th1 and Th2 cell populations of both tumor-derived CD4<sup>+</sup> lymphocytes and spleen cells were followed by the increase in the production of IFN- $\gamma$ , IL-10 and temporarily IL-4. This was also manifested by an increase in the percentage of T-bet<sup>+</sup> and GATA3<sup>+</sup> T cells, a reduction in the number of FoxP3<sup>+</sup> cells (but in late stage of immune response). Vaccines consisting of BM-DC/Tag<sup>TNF- $\alpha$</sup>  appeared to be the most effective in the stimulation of Th2-type response, while BM-DC/Tag<sup>TNF- $\alpha$</sup>  together with JAWS II/IL-2 cells were an appropriate support for the stimulation of Th1-type responses. Generally, the DC-based vaccines were able to elicit the alteration in the tumor environment and, in this way, they intensified the effect of CY.

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