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CELLULAR IMMUNE RESPONSE IN RATS WITH 1,2-DIMETHYLHYDRAZINE-INDUCED COLON CANCER AFTER TRANSPLANTATION OF PLACENTA-DERIVED MULTIPOTENT CELLS

ABSTRACT

We describe the state of the immune system at the late stage of 1,2-dimethylhydrazine (DMH)-induced colon cancer and after administration of placenta-derived multipotent cells (PDMCs). The spleen and thymus indices did not differ among the groups of intact and DMH-treated rats and were not affected by the administration of placenta-derived multipotent cells following the DMH treatment. Moreover, no difference in spontaneous or stimulated phagocytic activity of peritoneal macrophages was observed between healthy rats or the animals with DMH-induced colon cancer (with or without the administration of PDMCs). However, the proliferation of the T cells in the spleen was lower in rats with colon cancer regardless of the administration of PDMCs. Similarly, no changes were observed in the cell cycle distribution of proliferating spleen cells after stimulation by lipopolysaccharide. Our data demonstrate the absence of the active reaction by peritoneal macrophages and spleen cells to a colon cancer at mid/late stage. Additionally, the administration of PDMCs does not result in a measurable anti-tumor immune response.

KEYWORDS: *1,2-dimethylhydrazine, colon cancer, placenta-derived multipotent cells, immune response*

To date, considerable attention has been attracted to the inflammation as a major risk factor in colon cancer development [1-3]. Recent research focused on the changes of the immune system activity at different stages of cancer development. One of the novel approaches to stimulating anti-tumor immune response is placenta-derived multipotent cells (PDMCs)-based therapy that capitalizes on the immunomodulating effect of PDMCs following intravenous administration [4, 5].

The role of tumor-associated macrophages in colon cancer development has been investigated, but what extent overall macrophage activity is altered during colon cancer development remains unclear. Activated macrophages are known to produce pro-inflammatory cytokines and exert cellular cytotoxicity. However, activated macrophages recruited by the tumor can stimulate new vessel formation and tumor progression [6]. Hence, conflicting reports describe the role and involvement of tissue macrophages in colon cancer development. Illustrating the ambiguity of current understanding of macrophage system involvement in cancer pathogenesis, tumor-associated macrophages were shown to promote tumor growth, angiogenesis and metastases in orthotopic syngeneic mouse model [7], while also being able to reduce colon cancer metastasizing [6]. Little is known about the involvement of peritoneal macrophages in the late stage of colon cancer. However, activation of peritoneal macrophages at an early stage of colon and gastric cancer may inhibit tumor growth [8].

The spleen cells respond to bloodborne pathogens and play a critical role in the immune surveillance [9]. The spleen houses both the innate and adaptive compartments of the immune system, enabling an immediate innate reaction to microbial penetration, and an adaptive antigen-specific immune response [10]. However, how the spleen lymphocytes react to colon cancer development or to the transplantation of allogeneic stem cells remains mostly unknown.

Colon tumors at mid/late stages can evade recognition and elimination by the immune system [11]. The immune escape is believed to depend on both the changes of tumor cells and the defects in the immune functions [12-14]. The placenta is fundamental for maintaining fetomaternal tolerance during pregnancy, therefore it can be hypothesized that cells present in placental tissue may have immunomodulatory properties [5, 15]. Thus, PDMCs administration could help to begin the immune reaction on colon tumors. Moreover, PDMCs are allogeneic for recipient organism, the changes in activity of immune system cells are expect. PDMCs have some advantages for the clinical application: the lack of ethical restrictions, painless procedures endured by donors, abundant number of stem cells and their low immunogenicity [16, 17]. Additionally, PDMCs

are characterized of low expression of major histocompatibility complex (MHC) class II molecules, allowing these cells to be applicable in allogeneic transplantation [18].

To better understand the involvement of the immune system in the late stage of colon cancer and effects of transplanted placenta-derived multipotent cells we modeled colon cancer development by inducing tumors with 1,2-dimethylhydrazine (DMH) and analyzed cellular immune response in experimental rats.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF RAT PDMCS

All animal experiments were performed in compliance with the international principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (European Convention, Strasburg, 1986) and article 26 of the Ukraine Law «On protection of animals from cruelty» (# 3447-IV, 21.02.2006), and by following the standards of biosafety. The experimental protocol was approved by The Bioethics Committee of The Education and Scientific Centre «Institute of Biology» of Taras Shevchenko National University of Kyiv (Protocol # 8, 03.04.2014).

Pregnant female rats were sacrificed on the 21st day of pregnancy by using carbon dioxide asphyxia, and the placentas were immediately collected. Placentas from male fetuses were selected (n = 10). Rat placentas were washed in Hanks balanced salt solution supplemented with 2.5 μg/mL amphotericin B, 50 μg/mL streptomycin and 100 U/mL penicillin (*Sigma*, USA). Tiny villous tissues were dissected into pieces of approximately 1-5 mm^3 using surgical scissors and forceps and then washed in Hanks balanced salt solution. The tissue fragments were placed in cell culture dishes. To allow cell migration from the tissue onto the culture plate, tissue fragments were covered with high-glucose DMEM with 10 % FBS (*HyClone*, USA). Primary cultures were maintained using standard culture conditions, i.e., a humidified atmosphere at 37 °C (5 % CO_2). The culture medium was changed twice a week. The cells were trypsinized (0.05 % Trypsin, 0.02 % EDTA) when cell colonies reached 90 % confluency and were seeded at a density of 5•10^3 cells/cm^2.

Placental cells were directed to differentiate into osteogenic and adypogenic directions to confirm multipotency and expression of surface markers for mesenchymal stem cells was determined.

To evaluate osteogenic potential of the isolated cells, confluent third passage culture was used. For this purpose, the osteogenic medium consisted of medium supplemented with 10^{-7} M dexamethasone (*Sigma*, USA), 0.1 mM ascorbic acid 2-phosphate (*Sigma*, USA), 10 mM β-glycerophosphate (*Sigma*, USA) and 10 % FBS in high-glucose DMEM. After differentiation, the cells were stained with Alizarin Red S to detect calcium deposition.

For adipogenic differentiation the cultures were treated with adipogenic differentiation medium composed of 10^{-6} M dexamethasone (*Sigma*, USA), 60 μM indomethacin (*Sigma*, USA), 500 μM 3-isobutyl-1-methylxanthine

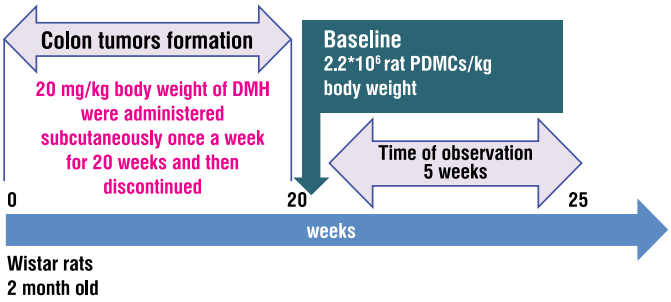

Fig. 1. Design of experiment. DMH – 1,2-dimethylhydrazine, PDMCs – placenta-derived multipotent cells.

(*Sigma*, USA), 5 μg/mL insulin (*Biochrom,* USA), and 10 % FBS in high-glucose DMEM for 21 days. Oil red O staining was performed to visualize the presence of lipid droplets. As controls were used the cultures cultured the same period of time without differentiation factors.

For the immunophenotyping the following fluorochrome-labeled monoclonal antibodies were used: PE Mouse Anti-Rat CD90/Mouse CD90.1 (Cat. No. *551401, Becton Dickinson*, USA), APC-Cy7 Mouse Anti-Rat CD45 (Cat. No. 561586, *Becton Dickinson*, USA), and CD44 antibody [OX-50] (FITC) (Cat. No. GTX76381, GeneTex, USA) according to manufacturer's instructions. For CD29 assessment, suspension of cells were washed and fixed in 2 % buffered paraformaldehyde for 20 min, then permeabilized in 0.3 % saponin for 30 min and stained with Integrin beta-1/CD29 antibody [EP1041Y], C-term rabbit monoclonal antibodies (1:50, Cat. No. GTX61413, *GeneTex*, USA). Alexa Fluor 405 conjugate Goat anti-Rabbit IgG (1:700, Cat. No. A-31556, Invitrogen, USA) was used as a secondary antibody. Phenotyping was performed with a FACSAria cell sorter (*Becton Dickinson*, USA). Obtained cultures could successfully differentiate in two mesodermal lineages and had CD90$^+$CD44$^+$CD29$^{+/low}$CD45$^-$ immunophenotype, that prove multipotency of placental-derived cultures.

IN VIVO EXPERIMENTAL DESIGN

Experiments were performed using 2-month-old male albino Wistar rats (n = 122), weighing 180-200 g, obtained from the Central Animal House of The Educational and Scientific Centre «Institute of Biology» of Taras Shevchenko National University of Kyiv. DMH (*Sigma*, USA) was dissolved in saline adjusted to pH 6.5 with 2 M NaOH immediately before use. To induce tumor development, rats were subcutaneously injected with 20 mg/kg b. wt. DMH in 0.1 mL of saline weekly for 20 weeks (n = 107) [19]. The control group (n = 10) received physiological saline (PS) only. **Figure 1** shows the DMH treatment protocol. Physiological saline or PDMCs at passage 3 were injected intravenously into the rats (n = 59) at 20th week. Ten random choose rats were sacrificed on 20th week of modelling using carbon dioxide asphyxia to perform routine histological analysis and confirm tumor formation the equivalent of stage $T_{1-2}N_{0-1}M_0$ of human colorectal cancer.

The groups are described in **table 1.** All rats were weighed every week. The thymus and spleen indices were calculated according to the

GROUP	N	DMH-INDUCED COLON CANCER	ADMINISTRATION	TIME OF SACRIFICING, WEEKS
Baseline	1	+	-	20
Intact rats+PS, 1 week	5	-	PS	21
Intact rats+PS, 5 weeks	5	-	PS	25
DMH rats+PS, 1 week	5	+	PS	21
DMH rats+PS, 5 weeks	9	+	PS	25
DMH rats+PDMCs, 1 week	5	+	PDMCs	21
DMH rats+PDMCs, 5 weeks	10	+	PDMCs	25

Table 1. Experimental groups of rats.

Notes:
DMH – 1,2-dimethylhydrazine, PS – physiological saline, PDMCs – placenta-derived multipotent cells.

following formula: thymus or spleen index = weight of thymus or spleen (mg) / body weight (g) [16].

INDUCED PROLIFERATION OF SPLEEN T AND B LYMPHOCYTES

Spleen tissue was rinsed in RPMI-1640 (*HyClone*, USA) with 100 µg/mL streptomycin and 100 U/mL penicillin, homogenized, and filtered through 100 µm filter. Spleen cells at $5 \cdot 10^6$ per mL were incubated three days at standard culture conditions in RPMI-1640 with 10 % FBS, 2 mM glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin, 25 mM 2-mercaptoethanol (*Sigma*, USA). T cells were stimulated with phytohemagglutinin (PHA) at 30 µg/mL, and B cells were stimulated with lipopolysaccharide (LPS) at 100 µg/mL (*Sigma*, USA). After that, 10^6 cells per sample were centrifuged at 300x g for 10 min, resuspended in 1mL of hypotonic lysis buffer containing 0.1 % sodium citrate, 0.1 % Triton X-100, 5 µg/mL propidium iodide (PI), and incubated for 30 min. DNA content in lymphocytes was measured by flow cytometry using FACS Calibur cytometer (*BD Biosciences*, USA). Cell cycle histograms were analyzed using Mod Fit LT 3.0 software (*Verity Software House*, USA). Stimulation index (SI) was calculated as a ratio of the number of mitogen-stimulated proliferating cells to the number of non-stimulated proliferating cells.

PERITONEAL MACROPHAGE PHAGOCYTOSIS ASSAY

Peritoneal macrophages from rats were collected in cold phosphate buffer saline (PBS). Erythrocytes were lysed in 0.9 % NH4Cl lysis buffer. FITC-labeled St. aureus cells (109/mL) were added to $2 \cdot 10^6$ macrophages and the mixtures were incubated for 30 min at +37 °C. Macrophages without St. aureus were used as a negative control. Two mL of cold PBS was added to each suspension, the cells were spun down at 300x g for 5 min and resuspended in 0.4 mL of 4 % buffered formaldehyde. Flow cytometry data was acquired by using FACS Calibur cytometer (*BD Biosciences*, USA) equipped with blue and red lasers. The data was analyzed using Cell Quest-PRO software.

STIMULATED NADPH OXIDASE ACTIVITY OF PERITONEAL MACROPHAGES

Spontaneous and induced activities of peritoneal macrophages were measured by Nitro-blue tetrazolium (NBT) reduction test. To detect spontaneous NADPH oxidase activity (SA), NBT was added to the macrophages in the wells of a 96 well plate (0.1 mL of 0.2 % NBT added to $1 \cdot 10^5$ macrophages in 0.1 mL media). To detect stimulated activity (STA), phorbol myristate acetate (PMA) at 20 nM was included in the NBT reagent solution. The cells were incubated under standard conditions (+37 °C, 5 % CO_2) for 1 h. Plates were centrifuged at 300x g for 10 min, the supernatants were gently removed and 0.2 mL 100 % ethanol was added to each well. Plates were centrifuged again, and the accumulated formazan was released by resuspending the cell pellets in 0.1 mL of 100 mM potassium hydroxide and 0.1 mL of DMSO per well. The absorbance at 540 nm was measured by using the Multiscan Spectrum photometer NanoDrop 2000 (*Thermo Scientific*, USA). The percentage of SA was calculated as (STA absorbance – SA absorbance) x 100 % / SA absorbance.

STATISTICAL ANALYSIS

Data was analyzed using the one-way ANOVA followed by Tukey's post-hoc test and presented as means ± SD. P-values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The weight of the whole body and of immune system organs in rats with DMH-induced colon cancer is not affected by PDMCs administration

The weight of intact control rats increased throughout the entire period of observation (till 25th weeks after cancer modeling), while the weight of DMH-treated rats began to decrease 16 weeks post-treatment. By the time of PDMCs administration (20 weeks post DMH treatment), the average weight of rats in the control and DMH-treated groups differed significantly (**Table 2**). Importantly, PDMCs administration did not result in the normalization of the whole body weight.

The spleen and thymus indices did not differ in intact rats, DMH-treated rats + PS and DMH-treated rats + PDMCs at 21st and 25th weeks of the experiment (**Fig. 2**), showing that neither the development of colon cancer nor the subsequent administration of PDMCs directly affected the mass of key organs in the peripheral immune system. Contrary to our observations in rats, previous work demonstrated that the spleen index in the mice with hepatocellular carcinoma was significantly greater than that in the normal group [21]. On the other hand, previous studies found that the thymus weight in the tumor-bearing group was much lower than that in the normal control group, and that the spleen performed no immunologic function with the progression of cancer [22, 23].

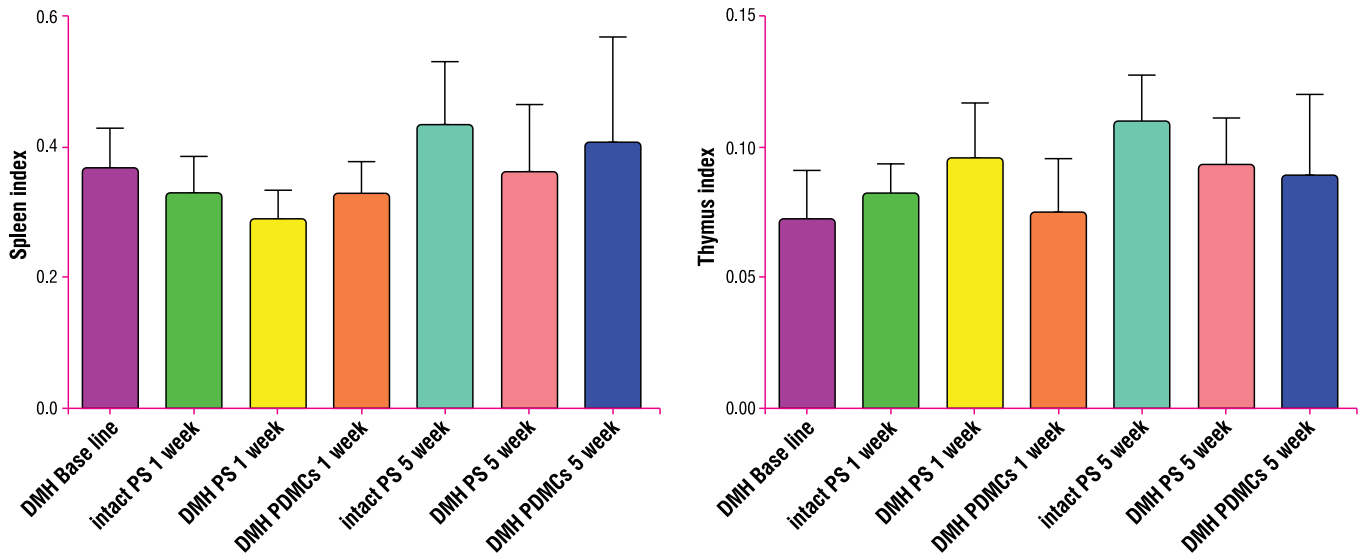

Fig. 2. Spleen and thymus indexes in different groups. DMH – 1,2-dimethylhydrazine, PS – physiological saline, PDMCs – placenta-derived multipotent cells.

PHAGOCYTIC ACTIVITY AND STIMULATED NADPH OXIDASE ACTIVITY OF PERITONEAL MACROPHAGES

Macrophage activation is required to establish control of the inflammation and progressive disease. Non-antigen-specific effector cells, which include natural killer cells, macrophages, neutrophils, and dendritic cells play an important role in antitumor protection [24, 25]. Moreover, local or systemic inhibition of the activity of aforementioned resistant effector cells has been shown to promote cancer development and metastasizing [26]. PDMCs suppress alloreactive lymphocytes proliferation in mixed lymphocytes reaction assay [15, 27-29]. Depending on the method of PDMCs isolation there was observed inhibition or promotion of a proliferative response of T lymphocytes *in vitro* [15]. Simultaneously PDMCs expressed MHC-I, but they did not express MHC-II molecules (HLA-DR), therefore they could be used in allogeneic transplantation [18, 29, 30].

Thus, we decided to examine whether the colon tumor development and PDMCs administration *in vivo* would result in the mobilization of immune cells into the peritoneal cavity. As can be seen in **Fig. 3**, there is no significant difference in spontaneous and stimulated macrophages activity in rats with colon cancer at late stages, as compared to control intact rats. Additionally, no effect is seen in five weeks after PDMCs administration in both experimental (colon cancer-bearing) and the control intact groups. Earlier work with mouse Lewis carcinoma model demonstrated that tumor development failed to affect spontaneous peritoneal macrophage phagocytic activity; however, contrary to our data, stimulated phagocytic activity of peritoneal macrophages was upregulated [31-33]. This fact may suggest that peritoneal macrophages are not involved in colon carcinogenesis at late stages and did not react on allogeneic PDMCs administration.

INDUCED PROLIFERATION OF SPLEEN T AND B LYMPHOCYTES

To evaluate the functional capabilities of spleen T and B lymphocytes, splenocytes were cultured under polyclonal activation conditions (with PHA or LPS for T and B lymphocytes respectively). Colon cancer at late stage negatively affected the proliferative status of both T and B cells. In DMH-treated rats, the proliferation activity of unstimulated lymphocytes was decreased. As can be seen in **Fig. 4, A**, the number of lymphocytes in S and G2/M phases was decreased, while the number of lymphocytes in G0/G1 phase was increased in DMH-treated rats as compared to intact control animals. PDMCs administration did not affect proliferative potential of lymphocytes. Additionally, in DMH-treated rats the level of T cell proliferation was lower (**Fig. 4, B**), which was not significantly changed following PDMCs administration. Interestingly, although the proliferative status of the T cells was lower, we did not find any changes in the levels of B cell proliferation (**Fig. 4, C**). Similar to our observations, previous study with hepatocellular carcinoma model did not find any significant differences in cell-cycle profiles of spleen lymphocytes from the tumor-bearing and the normal control animal groups. [34]. Colon cancer negative impact on T cell proliferation in rats and PDMCs administration to rats with colon cancer did not have significant effect on proliferation T or B cells and macrophages. Furthermore, we found a significant decrease of proliferation in the PHA-stimulated T cells derived from DMH-treated animals at 25 weeks post treatment as compared to the T cells derived at 20 weeks (**Fig. 4, B**). In contrast, changes in the cell cycle progression of unstimulated T cells were clearly seen in DMH-treated rats + PS at both 25 and 20 weeks post treatment. The increase of the T cell proliferative activity in the animals of parallel intact control group at 25 weeks vs. 20 weeks may reflect its dependence on age.

Table 2. Changes in average weight of rats.

GROUP	AVERAGE WEIGHT OF RATS, G					
	1ST WEEK		20TH WEEK		25TH WEEK	
	MEAN	SD	MEAN	SD	MEAN	SD
Intact rats + PS (n = 15)	139.8	30.0	354.4	10.1	387.5	12.9
DMH-rats+ PS (n = 48)	155.9	23.8	329.6*	5.7	332.1*	7.0
DMH-rats+ PDMCs (n = 59)	155.9	25.5	315.4*	6.5	313.0*	7.7

*Notes: DMH – 1,2-dimethylhydrazine, PS – physiological saline, PDMCs – placenta-derived multipotent cells. * – significant difference ($p \leq 0.05$) compare to intact rats, ANOVA with Tukey's post hoc test.*

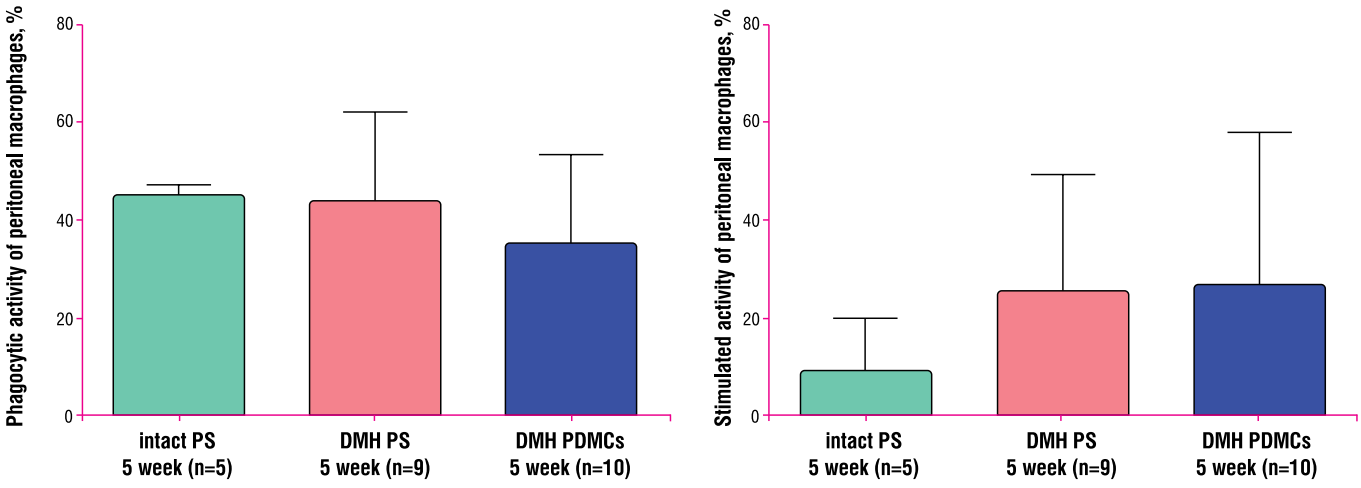

Fig. 3. Phagocytic and stimulated activity of peritoneal macrophages. DMH – 1,2-dimethylhydrazine, PS – physiological saline, PDMCs – placenta-derived multipotent cells.

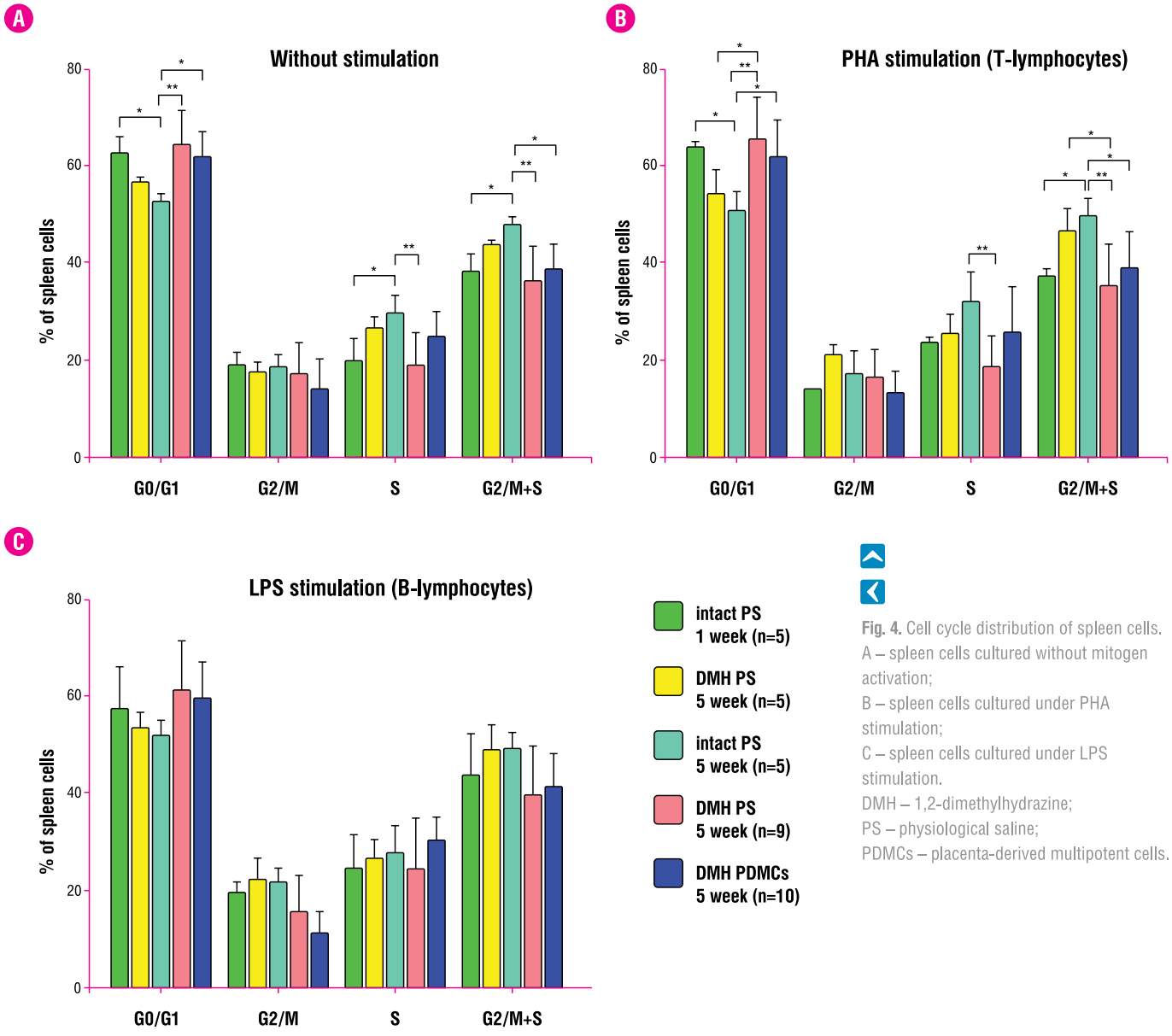

Fig. 4. Cell cycle distribution of spleen cells.
A – spleen cells cultured without mitogen activation;
B – spleen cells cultured under PHA stimulation;
C – spleen cells cultured under LPS stimulation.
DMH – 1,2-dimethylhydrazine;
PS – physiological saline;
PDMCs – placenta-derived multipotent cells.

CONCLUSION

The state of immune system at DMH-induced colon cancer at mid/late stages is altered. Spleen and thymus indices did not differ in intact, DMH-treated, or DMH-treated rats regardless of the PDMCs administration, although the weight of rats with colon cancer was decreased. Colon cancer presence with or without PDMCs administration affected neither spontaneous non stimulated phagocytic activity of peritoneal macrophages. However, proliferation of splenocytes was inhibited in the tumor-bearing group. Moreover, the cell-mediated immunity was suppressed in rats with colon cancer and was unaffected by the presence of transplanted PDMCs. Similarly, no changes were observed in the cell cycle profile of LPS-stimulated spleen B cells. This result was unexpectedly, because PDMCs in co-cultures studies inhibited the proliferation of allogeneic PHA stimulated T cells in a dose dependent manner [18], but in our studies there were no changes in lymphocytes proliferation under PDMCs administration.

Thus, PDMCs administration did not augment the functionality of the immune system at mid/late stages of colon cancer due to could not directly contact with lymphocytes in spleen. Our results can supplement knowledge about system immune reaction of the peritoneal macrophages and spleen lymphocytes at later stages of DMH-induced colorectal cancer in rats alone and with following PDMCs administration.

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