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Myeloid-derived suppressor-like cells – does their frequency change in patients with different stages of CRC?

Ćelije nalik supresorskim ćelijama mijeloidnog porekla – da li se njihov broj menja kod bolesnika u različitim stadijumima kolorektalnog karcinoma?

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Abstract

Background/Aim. Colorectal cancer (CRC) is one of the most common cancers in the population, often leading to lethal outcomes. Myeloid-derived suppressor cells (MDSCs) belong to a heterogeneous group of immature cells thought to have an immunosuppressive effect that may aid in tumor development and spreading. The aim of this study was to analyze the frequency and significance of MDSC-like cells at different stages in patients with CRC. **Methods.** Peripheral blood (PB) samples of 83 patients at different stages of the disease and 12 healthy subjects (control group) were analyzed. MDSC-like cells were identified and enumerated in the PB samples of the participants based on the immunophenotypic characteristics of the cells. **Results.** A statistically significant increase in the absolute and relative number of polymorphonuclear (PMN) MDSC (PMN-MDSC)-like cells was observed in the PB of all the patients with CRC, compared to the healthy control group ($p < 0.0001$). No significant increase was observed in monocytic MDSC (M-MDSC)-like cells when they were analyzed without CRC stage stratification ($p > 0.05$). When the relative and absolute numbers of PMN-MDSC-like cells were analyzed in relation to the stages of CRC disease (TNM classification), a statistically significant difference was observed between the control group and patients in stages III and IV of the disease ($p = 0.0005$ vs. $p = 0.0003$ and $p < 0.0001$ vs. $p < 0.0001$, respectively). There was, as well, a significant difference when the numbers of PMN-MDSC-like cells in patients in stages I and II were compared to numbers in patients in stage IV of the CRC ($p = 0.0161$ vs. $p < 0.0001$ and $p = 0.0065$ vs. $p < 0.0001$, respectively). A statistically significant difference in the relative and absolute number of M-MDSC-like cells was observed only between patients in stages II and IV of the disease ($p = 0.0014$ and $p = 0.0002$, respectively). The highest number of MDSC-like cells was observed in stage IV of the disease according to the TNM classification. A positive correlation between the presence of these cells and the number of organs affected by metastatic changes was observed ($p < 0.0001$ for the relative and absolute number of PMN-MDSC-like cells and $p = 0.003$ and $p = 0.0004$ for the relative and absolute number of M-MDSC-like cells). **Conclusion.** CRC patients had a statistically significant increase in PMN-MDSC-like cells compared to healthy controls. The increase in absolute and relative numbers of these cells mostly follows the growth and progression of CRC, while a statistically significant difference in the number of M-MDSC-like cells is observed only between stages II and IV of the disease. The absolute and relative numbers of both subtypes of MDSC-like cells significantly correlate with the number of organs affected by CRC metastases.

Key words:
colorectal neoplasms; myeloid-derived suppressor cells; neoplasm metastasis; neoplasm staging.

Apstrakt

Uvod/Cilj. Kolorektalni karcinom (KRK) je jedan od najčešćih karcinoma u populaciji, koji često dovodi i do smrtnog ishoda. Supresorske ćelije mijeloidnog porekla (SĆMP) pripadaju heterogenoj grupi nezrelih ćelija, za koje se smatra da imaju imunosupresivni efekat, koji može da pomogne razvoju i širenju tumora. Cilj rada bio je da se analizira učestalost i značaj ćelija nalik SĆMP kod bolesnika u različitim stadijumima KRK. **Metode.** Analizirani su uzorci periferne krvi (PK) 83 bolesnika u različitim stadijumima bolesti i 12 zdravih ispitanika koji su činili kontrolnu grupu. U uzorcima PK su, na osnovu imunofenotipskih obeležja, identifikovane ćelije nalik SĆMP i određen je njihov broj. **Rezultati.** Utvrđen je statistički značajan porast apsolutnog i relativnog broja ćelija nalik

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polimorfonuklearnim (PMN) SĆMP (PMN-SĆMP) u PK svih bolesnika sa KRK, u odnosu na kontrolnu grupu ($p < 0,0001$). Kada nije vršeno poređenje prema stadijumima KRK, nije uočen statistički značajan porast broja ćelija nalik monocitnim SĆMP (M-SĆMP) ($p > 0,05$). Kada su analizirane relativne i apsolutne brojnosti ćelija nalik PMN-SĆMP u odnosu na stadijume bolesti KRK (TNM klasifikacija), utvrđena je statistički značajna razlika između kontrolne grupe i bolesnika u III i IV stadijumu bolesti ($p = 0,0005$ vs. $p = 0,0003$ i $p < 0,0001$ vs. $P < 0,0001$, redom). Takođe, nađena je statistički značajna razlika brojnosti ćelija nalik PMN-SĆMP poređenjem bolesnika u I i II stadijumu bolesti, u odnosu na brojnost tih ćelija kod bolesnika u IV stadijumu KRK ($p = 0,0161$ vs. $P < 0,0001$ i $p = 0,0065$ vs. $p < 0,0001$, redom). Statistički značajna razlika u relativnom i apsolutnom broju ćelija nalik M-SĆMP uočena je samo između bolesnika u II i IV stadijumu bolesti ($p = 0,0014$ i $p = 0,0002$, redom). Najveći broj ćelija nalik SĆMP uočen je u IV stadijumu bolesti, prema TNM klasifikaciji. Uočena je pozitivna korelacija između prisustva tih ćelija i broja organa koji su zahvaćeni metastatskim promenama ($p < 0,0001$ za relativni i apsolutni broj ćelija nalik PMN-SĆMP i $p = 0,003$, $p = 0,0004$ za relativni i apsolutni broj ćelija nalik M-SĆMP). **Zaključak.** Oboleli od KRK imali su statistički značajan porast broja ćelija nalik PMN-SĆMP u odnosu na zdrave ispitanike. Porast apsolutnih i relativnih vrednosti broja ovih ćelija većim delom prati rast i napredovanje KRK, dok je statistički značajna razlika broja ćelija nalik M-SĆMP uočena samo između II i IV stadijuma bolesti. Apsolutni i relativni broj oba podtipa ćelija sličnih SĆMP značajno koreliše sa brojem organa zahvaćenih metastazama u KRK.

Ključne reči:
kolorektalne neoplazme; kostna srž, ćelije, supresorske; neoplazme, metastaze; neoplazme, određivanje stadijuma.

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the population after breast and lung cancer. CRC ranks second in mortality from malignant diseases [1]. It usually occurs sporadically and less frequently as a consequence of inflammation and hereditary diseases. Prevention of CRC, early diagnosis, as well as modern therapy, can significantly reduce the occurrence and improve the successful treatment of this tumor. The therapy for the advanced stages of the disease is still insufficient. Recently, the attention of scientists has been focused not only on malignant CRC cells but also on cells with a pronounced immunosuppressive effect, which can facilitate the progression of tumors with their presence. Important cells with such an effect are myeloid derived suppressor cells (MDSCs) that derive from the bone marrow and are also present in healthy individuals in a small percentage. The increase in the number of MDSCs is not a feature of exclusively malignant diseases, and they can be elevated in many other pathological conditions such as inflammatory diseases, trauma, graft vs. host disease, as well as in some non-pathological conditions (pregnancy, obesity, aging) [2–4]. Increased production of these cells occurs under the influence of a strong impulse, which leads to increased myelopoiesis [5]. In addition to bone marrow myelopoiesis, the increase in the number of MDSCs is aided by extramedullary hematopoiesis, as well as the plasticity of myeloid cells [6]. Similar to many other examples in practice, the identification and testing of these cells was first done on mice in the 1970s, and after several years these cells were also found in humans. MDSCs were officially named in 2007, and their phenotyping was proposed in 2016. Today, three types of these cells are known. Polymorphonuclear (PMN) MDSCs – PMN-MDSCs – are the most common MDSCs (comprising three-quarters of total MDSCs), morphologically similar to neutrophils and defined as $CD14^-CD15^+CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ or $CD11b^+CD14^-CD66b^+$ [7]. The recently discovered lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (LOX-1) as a marker of PMN-MDSCs in humans has facilitated the differentiation of these cells from neutrophils without the use of a gradient separation [8]. Monocytic MDSCs (M-MDSCs) are morphologically similar to monocytes and are defined as $CD14^+CD15^-CD11b^+CD33^+HLA\text{-}DR^-/Lin^-$ or $CD14^+CD15^+CD11b^+CD33^+HLA\text{-}DR^-/Lin^-$. There is another smaller group of early-stage MDSCs (es-MDSCs) that lacks markers for both monocyte and granulocyte populations and whose phenotype is Lin^- (CD3, CD14, CD15, CD19, CD56)/$HLA\text{-}DR^-/CD33^+$ and contain immature progenitor and precursor cells [9, 10]. In addition to phenotypic determination, the molecular and functional definition has been used to confirm MDSCs. An important feature of these cells, unlike mature neutrophils and monocytes, is immunosuppression. It primarily affects T-cells, natural killer (NK) cells, and regulatory T-cells; the mechanisms by which suppression occurs include arginine, cysteine metabolism, oxidative stress, activation and regulation of other regulatory or suppressive cells, and macrophage activity. Furthermore, a difference in the mechanism of action of PMN-MDSCs and M-MDSCs was observed [11, 12].

In our study, we tried to determine whether there is a difference in the incidence of MDSC-like cells in healthy and CRC patients, as well as whether there are statistically significant changes in the incidence of MDSC-like cells at different stages of the disease, including a subdivision of patients in stage IV according to the number of metastatic affected organs.

Methods

Patients and healthy controls

The study included 83 patients diagnosed with CRC in different stages of disease according to the last, 8th Tumor, Nodus, Metastasis/American Joint Committee of Cancer (TNM/AJCC) classification, and 12 healthy controls. The study protocol was approved by the Ethics Committee of the

Military Medical Academy (MMA) in Belgrade, Serbia (from March 10, 2016) and every patient provided a signed consent form. None of the participants underwent chemotherapy or irradiation therapy, or some other immunosuppressive therapy prior to sampling. A blood sample was taken from the patients at the Clinic of Gastroenterology and Hepatology, MMA. They were then monitored from June 2016 until January 2018. Sample processing was performed at the Institute for Medical Research, MMA.

Samples

In the study, 3 mL of venous blood was sampled from the patients with CRC and participants from the control group. Immediately after sampling, erythrocytes were removed by lysis (EDTA, NH_4Cl, $KHCO_3$) for 20 min with constant stirring. Double washing of nucleated cells in culture medium (RPMI 1640) with 5% normal human serum was then performed with subsequent centrifugation and resuspension. To separate peripheral blood mononuclear cells (PBMC) for comparative analysis, we applied LSM 1077 lymphocyte separation medium. Separation was performed by centrifugation at 1,200 × g for 20 min. The interlayer was separated and washed twice in a culture medium. The number of cells was determined manually in the Neubauer chamber and automatically on the Beckman Coulter AcT blood cell counter. The cells were then resuspended at a concentration of 1×10^6 cells per 100 µL suspension for further staining.

Immunophenotyping of cells

We used the following monoclonal human antibodies to perform cell immunophenotyping: CD15-FITC and PECy7; CD33-PE and PECy7; CD45-ECD, HLA-DR PECy5, CD14-PEC-y7, CD16-FITC and PECy7; CD11b-PE, CD10-PECy7, CD3-FITC, CD19-FITC and CD56-FITC (Beckman Coulter, USA). Stained cells were then analyzed on a Beckman Coulter FC 500 flow cytofluorimeter using CXP analytical software. Upon completing the procedure, we determined the relative and absolute number of PMN-MDSC-like cells and M-MDSC-like cells in all study participants. MDSC-like cells were phenotypically defined as $Lin^-(CD3^-/CD19^-/CD56^-)$ /$HLA\text{-}DR^{-/low}CD11b^+$ cells. PMN-MDSC and M-MDSC-like subtypes were determined based on the expression of CD14 and CD15. PMN-MDSC-like cells were defined as $CD14^-$ $CD15^+$ and M-MDSC-like cells were defined as $CD14^+CD15^-$. Further differentiation of PMN-MDSC-like cells was done using CD10 and CD16 markers. The gating strategy for the detection of MDSCs in study participants was based on the previous work by Stanojević et al. [13].

Statistical analysis

All statistical analyses were performed in GraphPad Prism 9.0.2. The Kolmogorov-Smirnov, D'Agostino-Pearson, and Shapiro-Wilk tests were used to determine whether the data followed a normal, Gaussian distribution. In our study, we used a *t*-test, Chi-square test, and Mann-Whitney *U* test.

Results

Immunophenotypic characteristics of PMN-MDSC-like cells and M-MDSC-like cells

PMN-MDSC-like cells and M-MDSC-like cell subsets were identified according to the expression of CD15 and CD14, respectively, within the $HLA\text{-}DR^{-/low}CD11b^+$ $CD33^{low}Lin^-$ population in 83 patients in different stages of CRC according to the AJCC classification and in 12 healthy controls. Detection of PMN-MDSC-like cells and M-MDSC-like cells is shown in Figures 1 and 2, respectively. Briefly, for both major MDSC-like cell subpopulations – PMN-MDSCs and M-MDSCs – the initial gate was set on CD45 (pan-leukocyte antigen) positive cells ($CD45^+$ gate, Figures 1A and 2A, respectively) in order to allow final expression of the percentages of MDSC-like cells relative to all leukocytes present in the peripheral blood sample, taking the $CD45^+$ gate as the "parental gate". In the next step, $CD45^+$ events were plotted on the HLA-DR vs. CD11b dot plot, where the $HLA\text{-}DR^{-/low}$ and $CD11b^+$ events were selected for further evaluation (Figures 1B and 2B). For detection of PMN-MDSC-like cells, the next step included the assessment of the $CD11b^+HLA\text{-}DR^{-/low}$ events for CD16 molecule expression on CD16 vs. SS log dot plot (side scatter logarithmic) where the events with lower CD16 as well as lower SS log, compared to mature granulocytes (confirmed by CD10 expression on mature granulocytes in a different test tube combination: CD16FITC/CD11bPE/CD45ECD/HLA-DR PeCy5/CD10PECy7) (Figure 1E and J), were gated and colored black for further tracking (Figure 1C). Finally, the PMN origin of $CD45^+Lin^-CD33^+CD11b^+HLA\text{-}DR^{neg/low}$ $CD16^{low}$ cells was confirmed by strong expression of CD15 on these cells (Figure 1D). The assessment of Lineage cocktail (CD3FITC/CD19FITC/CD56FITC) negativity and CD33 positivity was performed in different test tube combinations (LinFITC/CD11bPE/CD45ECD/HLA-DRPECy5/CD16PECy7 and CD16FITC/CD11bPE/CD45ECD/HLA-DRPECy5/CD33PECy7, respectively, not shown). The same gating strategy in a healthy donor sample is shown in Figure 1 F–J.

For the detection of M-MDSC-like cells, the $CD45^+CD11b^+HLA\text{-}DR^{-/low}$ events were plotted on the CD14 vs. SS log dot plot in order to confirm their monocyte origin, gated and colored black for further tracking (Figure 2C). A further distinction from PMN cells, in addition to a clearly lower SS signal on CD45 vs. SS log dot plot, was achieved by demonstration of CD15 negative measurement (Figure 2D). The overlay histogram (Figure 2E) shows clearly negative HLA-DR expression in selected monocytoid cells compared to lymphocytes, which are used as an internal test control. The assessment of Lineage cocktail (CD3FITC/CD19FITC/CD56FITC) negativity and CD33 positivity was performed in different test tube combinations based on the CD14 positive events (LinFITC/CD11bPE/CD45ECD/HLA-DRPECy5/CD14PECy7 and LinFITC/CD33PE/CD45ECD/HLA-DRPECy5/CD14 PECy7, respectively, not shown). The same gating strategy in a healthy donor sample is shown in Figure 2 F–J.

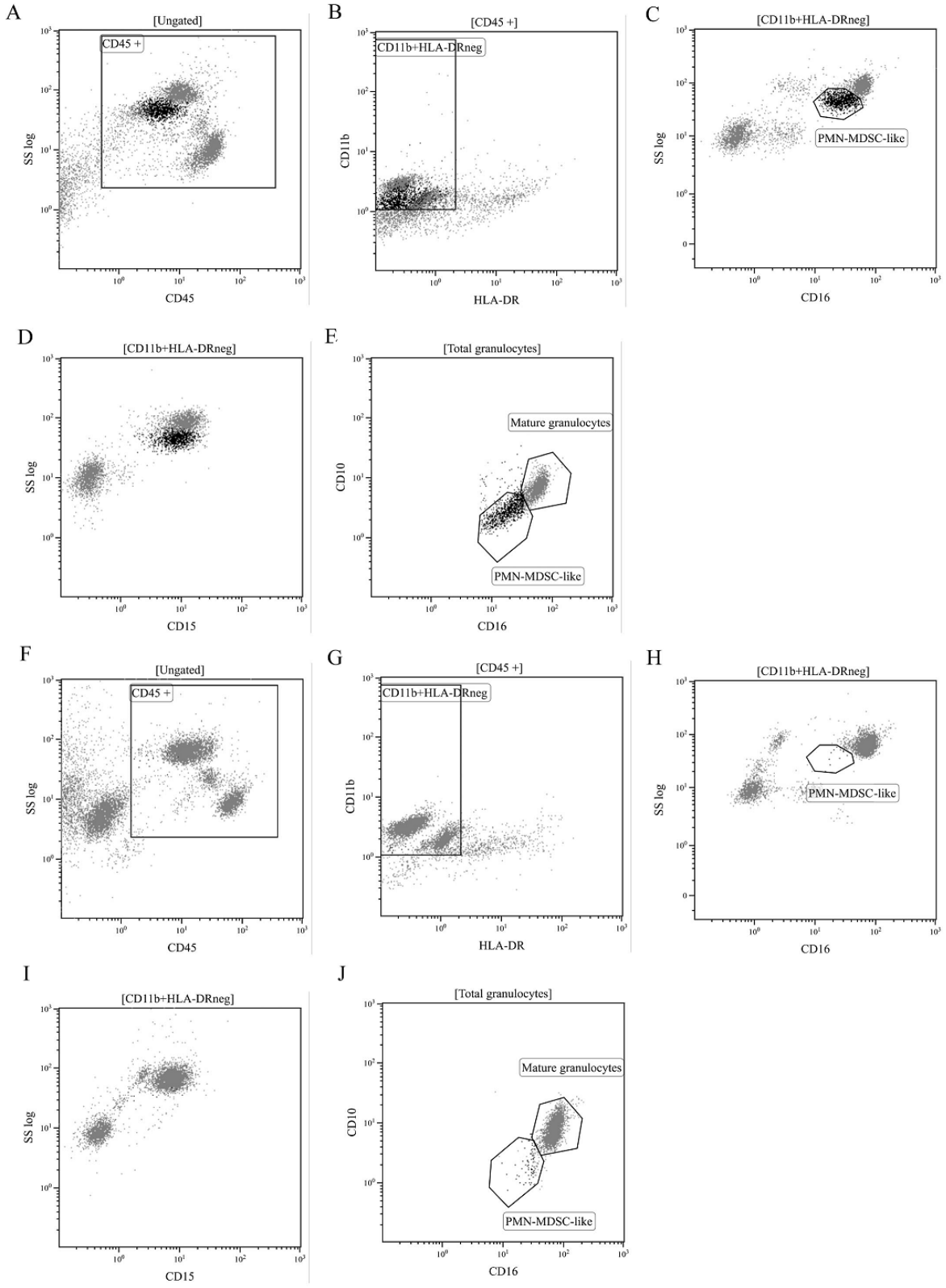

Fig. 1 – Detection of PMN-MDSC-like cells: In the representative CRC patient PB sample, the CD45 positive events are selected for analysis (A) and plotted on to HLA-DR vs. CD11b dot plot, next, $CD11b^{+}$ and $HLA\text{-}DR^{-/low}$ events (B) are further evaluated for CD16 expression, showing clearly lower signal of the examinee, as well as lower side scatter value (C); Strong expression of polymorphonuclear CD15 marker (D) and negative expression of mature granulocytes marker – CD10 (E); The healthy donor PB sample subjected to the same gating strategy, showing the substantially lower quantity of PMN-MDSC-like cells (F-J). PMN-MDSC – polymorphonuclear myeloid-derived suppressor cells; CRC – colorectal carcinoma; PB – peripheral blood.

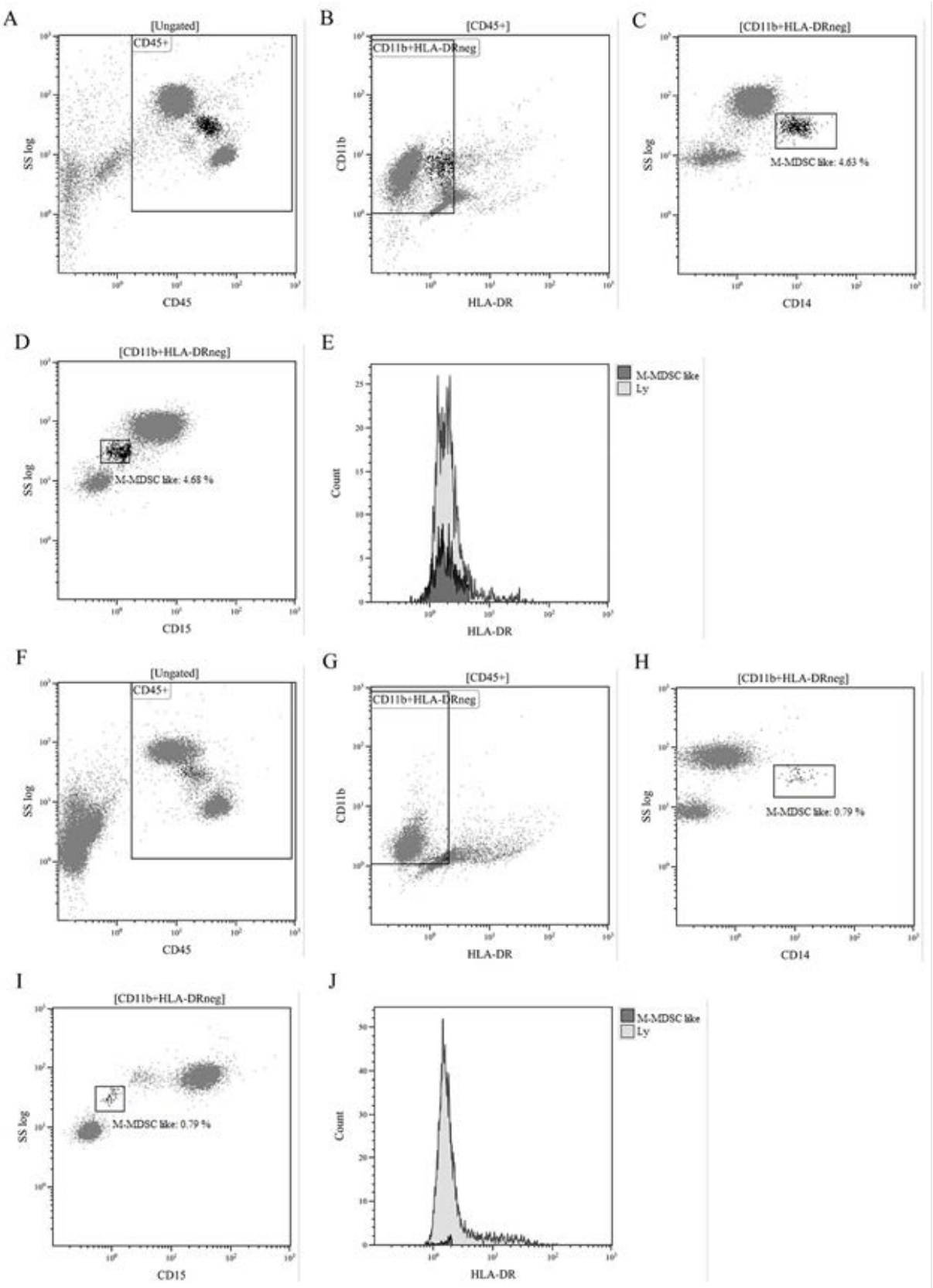

Fig. 2 – Detection of M-MDSC-like cells: In the representative CRC patient PB sample, the CD45 positive events are selected for analysis (A) on CD11b and HLA-DR expression (B); Selected CD11b^{+}/HLA-DR$^{neg/low}$ events showing positive CD14 staining (C) and negative CD15 signal (D); Overlay histogram showing HLA-DR negative signal in CD14 positive cells compared with lymphocytes as an internal control (E); The healthy donor PB sample subjected to the same gating strategy (F-J).
M-MDSC – monocytic-MDSCs. For other abbreviations, see Figure 1.

The study involved 83 patients with CRC and 12 healthy subjects who made up the control group; their descriptive statistics are presented in Table 1. It is known that CRC mainly affects a slightly older population, more often males, which is confirmed in this study as well. Namely, the average age of our patients was 63.6 years, while there was a statistically significant difference between the number of male (65.1%) and female (34.9%) patients with CRC (Chi-Square = 5.568; p = 0.018). Furthermore, despite some observed differences between our patients and the control group, statistical testing showed that there was no significant difference between these groups regarding their average age (t = 0.263, p = 0.793), average BMI (Mann-Whitney, U = 555.5, p = 0.381), or proportion of males and females (Chi-Square = 2.438; p = 0.118). The study lasted 19 months, and the patients were divided into groups depending on the stage of the disease for further testing. The lowest number of subjects belonged to stage I, according to the TNM/AJCC classification, and the highest to stage IV of the disease (Table 1).

First, we compared the percentages and absolute values of both MDSC-like cell subtypes in healthy and all diseased individuals, and complete statistical results are depicted in Figure 3. Patients with CRC had a highly significant increase in the percentage and absolute number of PMN-MDSC-like cells compared to healthy individuals (4.21 ± 4.30 vs. 1.46 ± 1.24, $p < 0.0001$ for percentage and 0.36 ± 0.59 vs. 0.09 ± 0.09, $p < 0.0001$, for absolute number, respectively). On the contrary, a statistically significant relationship was not observed in the case of M-MDSC-like cells (0.70 ± 0.33 vs. 0.39 ± 0.33, p = 0.3027 for percentage and 0.05 ± 0.06 vs. 0.03 ± 0.21, p = 0.4346 for absolute number of M-MDSC-like cells in patients with CRC and healthy persons) (Figure 3).

Table 1

Demographic and clinical characteristics of study participants

Parameters	Groups	
	CRC (n = 83)	Control (n = 12)
Age (years), mean	63.6	62.6
Gender, n (%)		
female	29 (34.9)	7 (58.3)
male	54 (65.1)	5 (41.7)
BMI, kg/m^2	26.0	24.9
CRC stage (TNM classification), n		
I	5	–
II	31	–
III	18	–
IV	29	–

TNM – Tumor Node Metastasis; CRC – colorectal carcinoma; BMI – body mass index; n – number of patients.

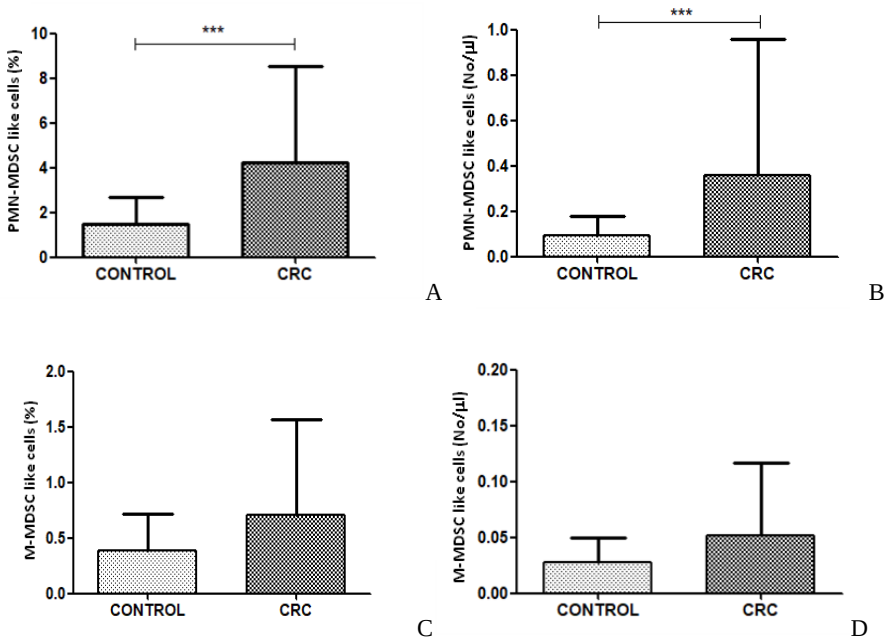

Fig. 3 – Comparison of the numbers of PMN-MDSC- like and M-MDSC-like cells between healthy subjects (control group) and CRC patients. The average percentage (A) and the absolute number (B) of PMN-MDSC-like cells; The average percentage (C) and the absolute number (D) of M-MDSC-like cells. Mann-Whitney *U* test was used (*$p < 0.0001$). Some data points are outside the axis limits and excluded for better figure presentation (number of excluded data points ≤ 3). For abbreviations, see Figures 1 and 2.**

Then, we refined our analysis by comparing the percentages and absolute numbers of PMN-MDSC-like cells and M-MDSC-like cells in patients with different clinical stages according to the TNM/AJCC classification. All participants were divided into five groups, and the first group comprised healthy individuals. The other four groups consisted of patients in different stages of CRC according to the TNM/AJCC classification, starting from the lowest (I) to the highest (IV) stage of the disease. Descriptive statistics for these groups are presented in Table 2.

After a descriptive analysis was performed and the normality of the distribution was checked, more rigorous statistical testing was completed with the Mann-Whitney *U* test, and the complete results are shown in Figure 4. Comparing the data of healthy individuals and patients in different stages of the disease according to the TNM classification, a statistically significant difference of PMN-MDSC-like cells in relative and absolute numbers was noticed between the control group and patients in stage III and IV (1.46 ± 1.2 vs. 3.72 ± 1.64, $p = 0.0032$ and 1.46 ± 1.24 vs. 6.78 ± 6.15, $p < 0.0001$ for percentage and 0.09 ± 0.09 vs. 0.32 ± 0. 29, $p = 0.0003$ and 0.09 ± 0.09 vs. 0.64 ± 0.92, $p < 0.0001$ for absolute numbers). Likewise, there was a significant difference in relative and absolute numbers of PMN-MDSC-like cells between patients in stage I and stage IV of the disease (2.02 ± 1.85 vs. 6.78 ± 6.15, $p = 0.0161$ for percentage and 0.12 ±

Table 2

Descriptive data by stages based on the latest AJCC classification

Subjects		PMN-MDSC-like cells		M-MDSC-like cells	
		%	No/μL	%	No/μL
Control group (n = 12)	mean ± SD	1.46 ± 1.24	0.09 ± 0.09	0.39 ± 0.33	0.03 ± 0.02
	median	1.01	0.06	0.29	0.03
CRC stage					
I (n = 5)	mean ± SD	2.02 ± 1.85	0.12 ± 0.10	0.55 ± 0.54	0.03 ± 0.03
	median	1.09	0.06	0.48	0.02
II (n = 31)	mean ± SD	2.45 ± 1.66	0.16 ± 0.12	0.40 ± 0.53	0.03 ± 0.05
	median	2.07	0.13	0.15	0.01
III (n = 18)	mean ± SD	3.72 ± 1.64	0.32 ± 0.29	0.61 ± 0.62	0.04 ± 0.04
	median	3.70	0.24	0.30	0.04
IV (n = 29)	mean ± SD	6.78 ± 6.15	0.64 ± 0.92	1.12 ± 1.13	0.08 ± 0.08
	median	4.32	0.36	0.63	0.05

AJCC – American Joint Committee of Cancer; n – number of subjects; SD – standard deviation; % – relative number of cells; No/μL – absolute number of cells.
For other abbreviations, see Figures 1 and 2.

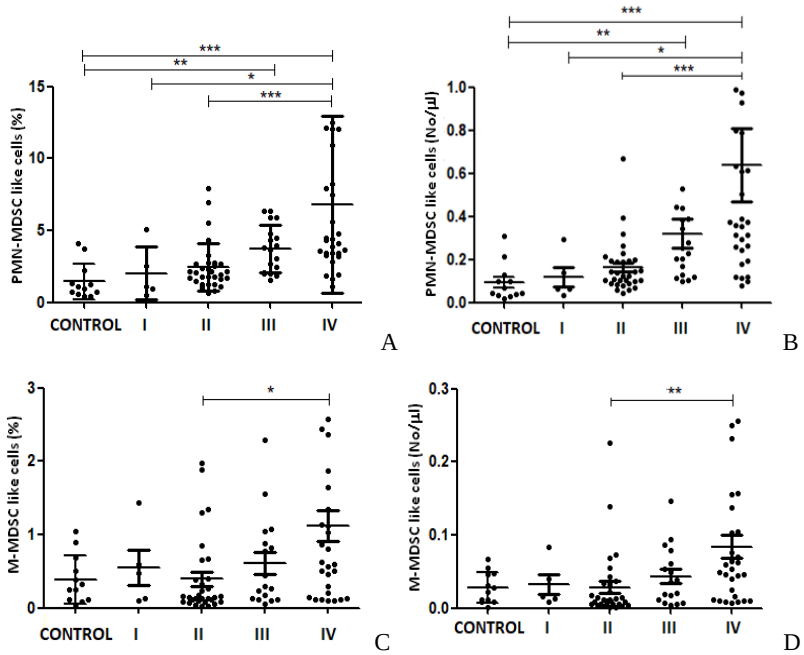

Fig. 4 – Comparison of the numbers of PMN-MDSC-like and M-MDSC-like cells between healthy subjects (control group) and patients with CRC divided into four stages of the disease. The average percentage (A) and the average absolute number (B) of PMN-MDSC-like cells; The average percentage (C) and the average absolute number (D) of M-MDSC-like cells. Mann-Whitney *U* test (*$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$). Some data points are outside the axis limits and excluded for better figure presentation (number of excluded data points ≤ 3).
For abbreviations, see Figures 1 and 2.

0.10 vs. 0.64 ± 0.92, $p = 0.0065$, for absolute numbers, respectively).

Statistical significance in M-MDSC-like cells was observed when we compared the percentages and absolute numbers of these cells between CRC patients within stage II and stage IV (0.40 ± 0.53 vs. 1.12 ± 1.13, $p = 0.0014$ for percentage and 0.03 ± 0.05 vs. 0.08 ± 0.08, $p = 0.0002$, for absolute numbers, respectively). The highest values of both MDSC-like cell subtypes were observed in patients with disseminated disease in phase IV of CRC (6.78 ± 6.15 and 0.64 ± 0.92 for the percentage and absolute number of PMN-MDSC-like cells and 1.12 ± 1.13 and 0.05 ± 0.08 for the percentage and absolute number of M-MDSC-like cells, respectively). As illustrated in Figure 4, the differences between other analyzed groups were not found to be statistically significant in our sample.

In the next step, we looked at the correlation between the percentages and absolute numbers of MDSC-like cells and the metastatic spread of the disease. For the purpose of this analysis, all patients were divided into four groups. The first group (group 0) consisted of the largest number of patients who did not have a metastatic spread of CRC. Then we made the division into three more groups depending on whether metastatic changes were observed in one organ (group 1), two organs (group 2), or three or more organs (group 3). Descriptive statistics for these groups are presented in Table 3.

Based on Spearman's correlation coefficient and statistical tests, it was observed that there was a positive correlation between the presence of MDSC-like cells and the number of organs affected by metastatic changes. This relationship is moderately strong for PNM-MDSC-like cells, while it is somewhat weaker for M-MDSC-like cells ($p < 0.0001$ for the relative and absolute number of PMN-MDSC-like cells and $p = 0.003$ and $p = 0.0004$ for the relative and absolute number of M-MDSC-like cells) (Table 4; Figure 5).

Discussion

MDSCs make up about 0.5–1% of peripheral blood neutrophils in healthy individuals [8]. A higher frequency of these cells has been observed in people with malignancies – breast cancer [14], pancreas cancer [15], lung cancer [16], but also in many inflammatory diseases such as chronic hepatitis C [17], active ulcerative colitis [18], or sepsis [19].

Patients diagnosed with CRC require further treatment. It often includes surgery, but sometimes, besides surgical treatment, depending on the stage and localization of the disease, patients also require chemotherapy and radiotherapy. The effect of chemotherapy and radiotherapy, as well as immunosuppressive drugs, on MDSCs in cancer patients, is well known [20, 21]. Therefore, none of our subjects, regardless of the stage of the disease, underwent these types of treatments prior to MDSCs sampling. Peripheral

Table 3

Descriptive data for patients with a different number of organs affected by metastases

Groups		PMN-MDSC-like cells		M-MDSC-like cells	
		%	No/μL	%	No/μL
0 (n = 54)	mean ± SD	2.82 ± 1.75	0.21 ± 0.20	0.49 ± 0.56	0.03 ± 0.04
	median	2.18	0.14	0.20	0.02
	min–max	0.51–7.93	0.03–1.35	0.02–2.29	0.00–0.23
1 (n = 12)	mean ± SD	3.99 ± 2.90	0.33 ± 0.23	1.12 ± 1.48	0.08 ± 0.11
	median	3.45	0.26	0.46	0.05
	min–max	1.09–12.13	0.12–0.93	0.10–4.35	0.01–0.31
2 (n = 13)	mean ± SD	8.53 ± 7.33	0.67 ± 0.61	1.21 ± 1.01	0.09 ± 0.08
	median	7.48	0.61	0.87	0.06
	min–max	1.86–30.14	0.10–2.43	0.12–3.15	0.01–0.26
≥ 3 (n = 4)	mean ± SD	9.99 ± 6.83	1.51 ± 2.17	0.81 ± 0.54	0.09 ± 0.06
	median	9.06	0.50	0.87	0.09
	min–max	3.42–18.42	0.27–4.76	0.14–1.34	0.01–0.16

0 – patients with CRC and without metastases; 1 – patients with CRC and metastases in only one organ; 2 – patients with CRC and metastases in two organs; 3 – patients with CRC and metastases in three or more organs. % – relative number of cells; No/μL – absolute number of cells; min-max – value range minimum to maximum.
For abbreviations, see Figures 1 and 2.

Table 4

Spearman's correlation coefficient with statistical significance testing between PMN-MDSC-like cells and M-MDSC-like cells and the number of organs affected by metastases in patients with CRC

Parameter	Metastases and PMN-MDSC-like cells %	Metastases and PMN-MDSC-like cells No/μL	Metastases and M-MDSC-like cells %	Metastases and M-MDSC-like cells No/μL
Spearman's coefficient	0.5126	0.5115	0.3234	0.3793
p-value	<0.0001	<0.0001	0.003	0.0004

% – relative number of cells; No/μL – absolute number of cells.
For abbreviations, see Figures 1 and 2.

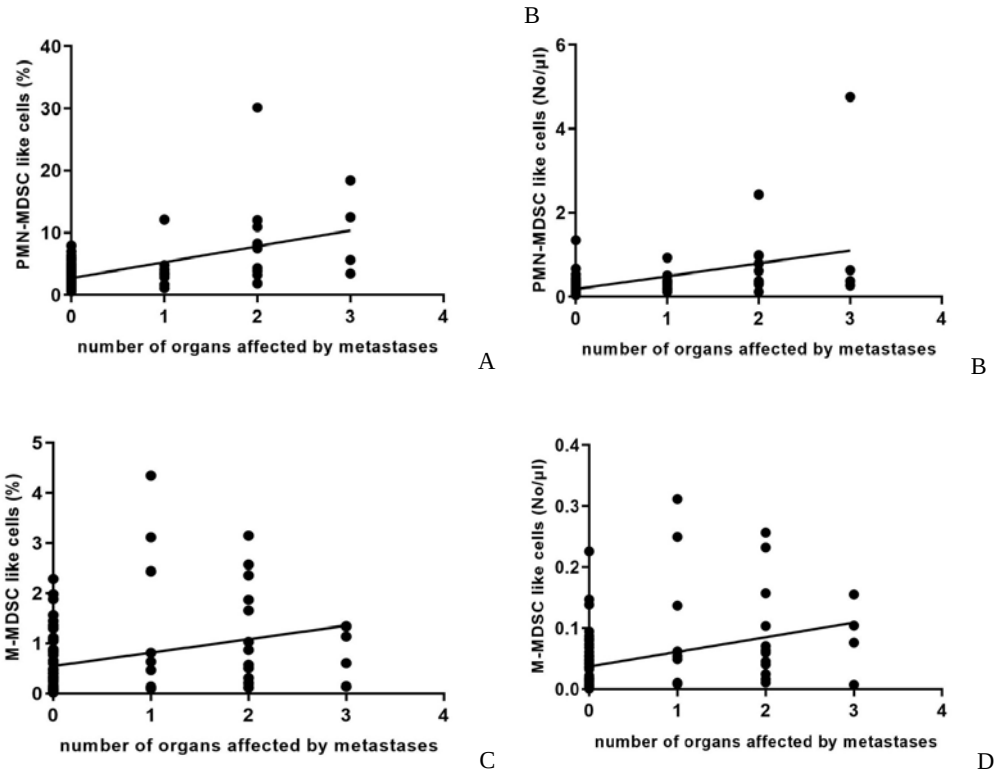

Fig. 5 – Graphic presentation of the Spearman's correlation between MDSC-like cells and the number of organs affected by metastases in patients with CRC. The relative number (A) and the absolute number (B) of PMN-MDSC-like cells; The relative number (C) and the absolute number (D) of M-MDSC-like cells. For abbreviations, see Figures 1 and 2.

blood samples were taken immediately after the diagnosis of CRC was made and before possible surgery treatment because such treatment has an impact on the frequency of MDSCs [22, 23].

First, we compared the percentages and absolute numbers of PMN-MDSC-like cells and M-MDSC-like cells in circulation in healthy individuals and patients with CRC. Our results showed a highly statistically significant increase in the percentages and absolute numbers of PMN-MDSC-like cells in CRC patients compared to the control group and are in agreement with the study by Zhang et al. [24], who also indicated an increased presence of MDSCs in patients with colon cancer compared to the healthy population. Such a conclusion was also reached in the study by Toor et al. [25]. However, when measuring the number of M-MDSC-like cells that were otherwise less present in peripheral blood compared to PMN-MDSC-like cells, no statistical significance was observed between the control group and total CRC patients. The obtained results are in agreement with the reports of other studies. Namely, data from studies by Hossaini et al. [26] indicated that, in CRC patients, the MDSCs subpopulation with the highest percentage was PMN-MDSCs. They also found that M-MDSCs were present in a smaller percentage compared to PMN-MDSCs and were not increased in CRC patients. It should be emphasized that some studies have indicated an increase in the number of both types of MDSCs in CRC, not only PMN-MDSCs [27].

We divided patients by cancer stage using TNM classification. It was assumed that the number of PMN-MDSCs and M-MDSCs would increase with disease progression. Significant differences were seen in the percentage and absolute number of circulating MDSC-like cells between healthy donors and patients with advanced stages – III and IV. Moreover, significant differences were seen between patients within stages I and IV, as well as between patients in stages II and IV. Looking at M-MDSC-like cells, patients in stage IV had a statistically significant increase in both absolute and relative cell numbers compared to those who were in stage II, according to the TNM classification. A positive correlation between the presence of MDSCs and the number of organs affected by metastatic changes clearly indicates a significant relationship between tumor burden and spread of the disease on the one hand and MDSCs accumulation on the other.

The highest values of PMN and M-MDSC-like cells were registered in stage IV of CRC in patients with advanced disease involving other organs. Observing the relationship between the stage of the disease and the presence of PMN-MDSCs, OuYang et al. [12] showed that increased presence in the peripheral blood in patients with CRC was associated with more severe clinical stages of the disease and lymph node metastasis. However, this observation is not unique to colon cancer and can be observed in other malignancies as well [13, 16]. The correlation between MDSCs

and disease progression is a consequence of the communication between these cells and cancer cells. Many mediators secreted by different tumors in a hypoxic environment lead to an increase in the number and activation of MDSCs. MDSCs also promote tumor survival and expansion through various immunosuppressive effects [28]. Malignant tumors often lead to increased myelopoiesis due to disorders in the regulation of the production of growth factors that affect hematopoiesis [29, 30]. The immunosuppressive effect of MDSCs is achieved in the tumor microenvironment and the peripheral blood, and it includes the mobilization and induction of other suppressive cells (i.e., macrophages) and altered metabolism of amino acids such as arginine and cysteine [7]. However, it should be emphasized that MDSCs also accelerate tumor progression. These effects of MDSCs take action through the process of angiogenesis and metastasis [31]. MDSCs activate growth factors such as vascular endothelial growth factor, basic fibroblast growth factor, Bombina variegata peptide 8, and platelet-derived growth factor, which are important for angiogenesis. MDSCs help cancer cells enter the circulation by releasing proteolytic enzymes – metalloproteinases, that transform vascular structures. An increase in the permeability of the vascular wall results in easier entry of cancer cells into the circulation and easier extravasation of the cells into the tissues. Even the "seeding" of malignant cells into other organs is not a random choice but is preceded by soil preparation, referred to as a premetastatic niche. MDSCs help its formation by influencing neovascularization by enabling oxygenation and nutrient supply to the future metastatic lesion [32].

It can be seen that the "cooperation" between the tumor and MDSCs is present at several levels, which may result in an increase in the number of these cells as the disease progresses. Our data confirm higher MDSC-like cells numbers in patients with advanced and metastatic disease in CRC. This fact may indicate that a possible blockade of the immunosuppressive effect of MDSCs could enhance the treatment of patients with CRC. In recent years, many different preclinical and clinical studies have tested various therapeutic approaches, such as the following: inhibition of MDSCs expansion and proliferation; differentiation of MDSCs into mature, less suppressive myeloid cells; inhibition of their immunosuppressive function and depletion of MDSCs in tumor microenvironment [33–38]. Targeting these cells as a treatment modality requires additional research.

Conclusion

Based on our results, we can conclude that the role of MDSCs is important in the process of progression and very likely in the formation of CRC. We showed for the first time significant correlations between the absolute and relative number of both subtypes of MDSC-like cells with the number of organs affected by metastases in CRC by using an original clinical protocol for identifying MDSC-like cells in total blood leukocytes. The increase in the absolute and relative numbers of MDSC-like cells registered in the advanced disease in our CRC patients, especially in patients with multiple organs affected by metastases, confirms the presumed link between the accumulation of these cells and CRC progression and may be helpful in monitoring these patients.

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