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Original Research

Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery

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ABSTRACT

Colorectal cancer (CRC) is the third most common malignancy. Its development and progression is associated with natural immunosuppression related, among others, to myeloid derived suppressor cells (MDSCs).

Overall, 54 patients in different stage of CRC, before any treatment were recruited into the study. The analysis included flow cytometry evaluation of blood MDSCs subsets, correlation their level with the tumor stage and T cell subsets. In the case of 11 patients, MDSCs level was evaluated before and 3 days after surgery, and these patients were monitored for cancer recurrence over 5 years.

The results showed that frequency of circulating MDSCs subsets is increased significantly in CRC patients, with highest level detected in most advanced tumor stages. Moreover, only monocytic MDSCs (Mo-MDSCs) positively correlate with regulatory Treg, and negatively with tumor Her2/neu specific CD8+ T cells. Circulating MDSCs, in contrast to tumor resident (mostly Mo-MDSCs), are negative for PD-L1 expression. Additionally, after surgery the blood level of Mo-MDSCs increases significantly, and this is associated with tumor recurrence during a 5-year follow-up.

In conclusion, Mo-MDSCs are pivotal players in CRC-related immunosuppression and may be associated with the risk of tumor recurrence after surgery.

Introduction

Colorectal cancer (CRC) is the third most common malignancy diagnosed worldwide and recent data documents a shift in the mean age of CRC onset from 72 to 66 years of age [1,2]. Surgery, followed by adjuvant chemotherapy, remains the main form of CRC treatment, however for patients with selected genetic profiles, immunotherapy with bevacizumab, cetuximab, and panitumumab or molecular-targeted personalized therapy might also be included [3]. Yet approximately 50% of treated patients will develop metastases during the rest of their life [4]. Cancer development is accompanied by tumor infiltration with different immune cell subsets, having both pro- and anti-tumor activities and overall affecting the tumor growth and patients' survival [5]. It was noticed that a low rate of blood lymphocyte-to-monocyte ratio in CRC may be associated with poor survival [6]. Moreover, the number of T lymphocytes infiltrating the tumor tissue may have a prognostic value in CRC, as CD4+ T cells were shown to negatively correlate with cancer stage [7]. On the other side, patients with a bigger population of cytotoxic lymphocytes tend to have more favorable outcomes relative to those with a higher proportion of suppressive Tregs, who succumb to earlier disease recurrence [8]. A significant component of the CRC microenvironment also constitutes the myeloid cells. This population is highly heterogenic and contains tumor associated macrophages (TAM), tumor associated neutrophils (TAN) and myeloid-derived suppressor cells (MDSCs) - all by the means of various mechanisms may contribute to tumor escape from the immune surveillance and can enhance cancer progression [9]. Among them, MDSCs currently turn attention in the context of their potential targeting in cancer therapy.

Abbreviations: ARG-1, arginase-1; CRC, colorectal cancer; iNOS, inducible NO synthase; FMO, fluorescence minus one; mAbs, monoclonal antibodies; MDSC, myeloid derived suppressor cells; PBMC, peripheral blood mononuclear cells; TAA, tumor-associated antigen; TAM, tumor associated macrophages; TAN, tumor associated neutrophils; Treg, T regulatory cells.

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Table 1
Clinical characterization of the patients.

		CRC	Limited[a] UICC (I+II)	Metastatic[a] UICC (III+IV)
Characteristic		$n = 54$	$n = 27$	$n = 27$
Age[b] (years)	64±13	65±11.7	63.1 ± 14.3	
Gander	Male	30	14	16
	Female	24	13	11
TNM stage[c]	I	7	7	–
	II	20	20	–
	III	16	–	16
	IV	11	–	11
Tumor site[d]	proximal	27	13	14
	distal	27	14	13
Histological grade	well/moderate	36	19	17
	poor/ undifferentiated	13	6	7
	undefined	5	2	3

[a] CRC patients with limited disease correspond to stage I and II, and those with metastatic disease correspond to stage III and IV.
[b] Mean ± SD.
[c] Staging based on TNM classification of CRC according to UICC.
[d] Tumor site was classified as proximal or distal to the splenic flexure.

MDSCs are composed of three different subpopulations, defined depending on their origin: granulocytic (PMN-MDSCs), monocytic (Mo-MDSCs) and early-stage (e-MDSCs), which are most likely progenitors of the other two [10]. Their immunosuppressive function is mainly directed on effector T cells, being driven by a complicated signal network, where inducible NO synthase (iNOS) and arginase-1 (ARG-1) play a major role [11]. While PMN-MDSCs preferentially settle the peripheral lymphoid organs and are mainly responsible for ROS production and the release of granule-derived myeloperoxidase (MPO), Mo-MDSCs persist rather in the tumor bed and show a higher expression of iNOS, being the main producers of NO [12].

Here we aimed at characterization of MDSCs role in CRC patients, particularly in respect to the level of their subsets and its correlation with the tumor recurrence after surgery during a 5-year follow-up. In CRC, several antigens could be associated with the induction of tumor-specific T cells, including carcinoembryonic antigen (CEA), mucin-1 (MUC1), guanyl cyclase C (GUCY2C, GCC), and HER-2/neu [13,14]. In this context we asked whether MDSCs correlates with HLA-A_2 HER-2/$neu_{369–377}$ specific $CD8^+$ T cells and other T cell subsets, including T reg and Th17 cells in CRC patients.

Materials and methods

Patients

Patients in different stage of CRC (TNM classification according to IUCC guidelines [15]), before any treatment were recruited into the First and Second Department of General Surgery, Jagiellonian University Medical College in Krakow. Overall, 54 patients were included in the study, however due to the several aspects investigated, the varying numbers of patients were involved in the individual phases of the study. For 11 CRC patients, examination of the blood level of MDSCs was done before and 3 days after surgery and further correlated with disease recurrence in a 5-year follow-up. In some cases, the tumor biopsy was taken in parallel. Simultaneously, 41 adult healthy blood donors were recruited as a control group. Characterization of the patients has been presented in Table 1, according to Zhang et al. [16]. All procedures were approved by the Jagiellonian University Bioethics Committee (approval no. 122.6120.128.2015 and 1072.6120.70.2018) and all subjects gave written informed consent to participate in the study.

In each case, 5–10 ml of a whole blood was drawn to EDTA-containing tubes (BD Diagnostics, Vacutainer System, San Jose, CA) and within a maximum of 2 h, the peripheral blood mononuclear cells (PBMC) were isolated by standard Pancoll (PAN-Biotech, Aidenbach, Germany) density gradient centrifugation.

MDSCs analysis

For MDSCs analysis, PBMC (app. 1×10^6 cells) were stained (20 min at 4 °C) with the set of monoclonal antibodies (mAbs) listed in Table 1 (Supplementary data). After incubation, the cells were washed twice in PBS and suspended in 0.2 ml PBS. To determine the level of non-specific antibody staining and cell autofluorescence, the fluorescence minus one (FMO) control samples were incubated in parallel. The samples were analyzed in FACSCanto flow cytometer (BD Biosciences, Immunocytometry Systems, San Jose, CA) using FACSDiva 8.1 (BD Biosciences) and FlowJo v.10 software (BD Biosciences, Franklin Lakes, NJ). The Mo-MDSCs were characterized as $LIN^-HLA\text{-}DR^{low/-}CD33^+CD11b^+CD15^-CD14^+$ cells, whereas PMN-MDSCs, as $LIN^-HLA\text{-}DR^{low/-}CD33^+CD11b^+CD15^+CD14^-$ cells, and e-MDSCs as $LIN^-HLA\text{-}DR^{low/-}CD33^+CD11b^+CD15^-CD14^-$, all presented as a percent value of nucleated cells (NC) – cells positive for staining with SYTO™41 (Invitrogen, Eugene, OR) from PBMC.

In the case of tumor biopsy material, prior to staining with monoclonal antibodies tissue was fragmented and strained using small cell strainers with a nylon mesh having 40 μm pores (BD Biosciences) and washed twice in PBS. Thereafter, the cells were stained with monoclonal antibodies and MDSCs subsets were characterized as above within $CD45^+$ cells and presented as a percent value of NC.

Detection of T regulatory (Treg) and Th17 cells

For Tregs and Th17 cells analysis, a whole blood was stained using Th17/Treg Human Phenotypic Kit (BD Pharmingen, San Diego, CA) with anti-CD4-Peridinin-Chlorophyll-Protein (PerCP), anti-IL-17A-Phycoerythrin (PE) and anti-Foxp3-Alexa Fluor (AF) 645 – conjugated mAbs, according to manufacturer's instructions. In parallel, the isotype controls were prepared to determine a non-specific binding of antibodies. Samples were analyzed in FACSCanto flow cytometer using FACSDiva software. The Treg and Th17 cells, were defined as $CD4^+Foxp3^+$ and $CD4^+IL\text{-}17A^+$ respectively, and their level was presented as percent value from $CD4^+$ population.

HLA-A_2 typing

PBMC (1×10^6/test) were incubated (20 min at 4 °C) with mouse anti-HLA-A_2-Allophycocyanin (APC)-conjugated mAb (BD Pharmingen) or respective isotype control, then fixed (BD Fixative, BD Biosciences), washed in PBS and suspended in 200 μl PBS. Samples were analyzed using a FACSCanto flow cytometer and FACSDiva software. Patients positive for HLA-A_2 expression were selected to the next stage, where $CD8^+$ T cells specific for HER-2/$neu_{369–377}$ (KIFGSLAFL) immunodominant peptide were analyzed.

Detection of $CD8^+$ T cells specific for HER-2/neu epitope

The HER-2/neu antigen specific $CD8^+$ T cells were identified using PE-labelled HLA-A_2 pentamer complex (Pro5Pentamer; ProImmune, Oxford, UK) folded around the HER-2/$neu_{369–377}$ specific epitope, as described previously [17]. As a negative control, staining with an HLA-A_2 Negative Control Pentamer (ProImmune) was used. To minimize a non-specific staining, each pentamer was tittered before the final use. Isolated PBMC were resuspended in PBS and incubated with the indicated pentamer for 10 min. at 20 °C followed by washing and staining with FITC-conjugated anti-CD8 mAB (BD Biosciences) for 20 min. at 4 °C in the dark. Cells were washed twice and fixed before analysis by flow cytometry. Data from a minimum of 50.000 lymphocytes were collected.

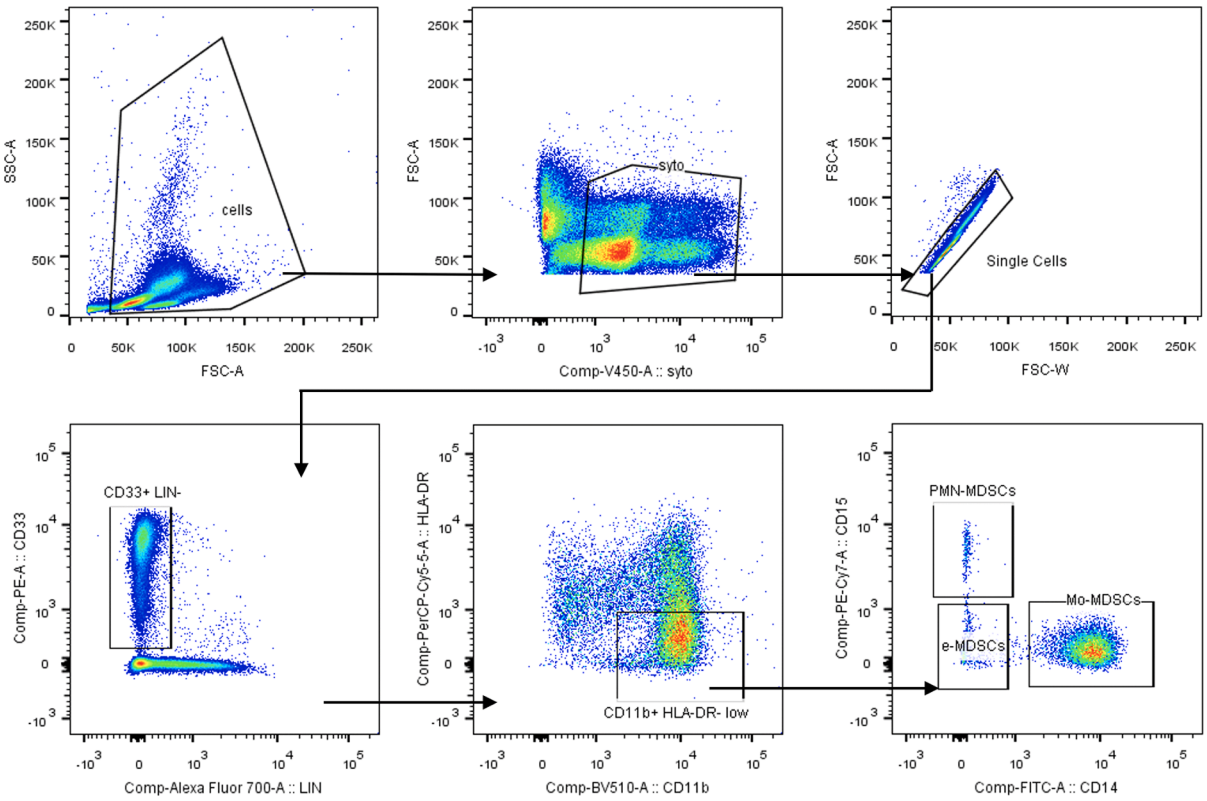

Fig. 1. Gating strategy for MDSCs population analysis.
The myeloid cells were gated as $LIN^{-}CD33^{+}$ from nucleated cells (SYTO41-positive) and single cells, then $HLA\text{-}DR^{-/low}$ $CD11b^{+}$ cells were selected and PMN-MDSCs were identified as $CD15^{+}$, Mo-MDSCs as $CD14^{+}$, whereas e-MDSCs as $CD15^{-}CD14^{-}$.

MDSCs morphology assessment

The MDSCs populations were sorted using a FACSAria II, according to the following criteria: PMN-MDSCs as $LIN^{-}HLA\text{-}DR^{low/-}CD33^{+}CD66b^{+}CD14^{-}$, Mo-MDSCs as $LIN^{-}HLA\text{-}DR^{low/-}CD33^{+}CD66b^{-}CD14^{+}$, and e-MDSCs as $LIN^{-}HLA\text{-}DR^{low/-}CD33^{+}CD66b^{-}CD14^{-}$, followed by cytospin preparations (Cytospin, Sheldon, UK) and the Wright's staining (Merck, Darmstadt, Germany). Slides were analyzed by Olympus XC50 camera (Olympus, Tokyo, Japan).

Suppression of T cell proliferation assay

The MDSCs induced suppression of T cell proliferation was analyzed by H^3-thymidine incorporation assay. In preliminary experiments, to establish the optimal ratio for the MDSCs inhibitory effect in cultures with autologous T cells, the FACS purified $CD3^{+}$ cells and MDSCs subsets were cultured in RPMI-1640 medium containing 10% (v/v) FBS at different ratios (8:1; 4:1; 2:1) (Supplementary Fig. 1) in the presence of autologous monocytes (10% of the T cell number), and stimulated with phytohemagglutinin (PHA; Sigma Aldrich, Saint Louis, MO). After 3 days of culture, cells were pulsed with H^3-thymidine (1 μCi/well; Hartmann Analytic, Braunschweig, Germany) and the ß-minus radiation was measured in a liquid scintillation counter (Beckman Coulter LS1801, Inc., Mississagua, Ontario, CA) as counts per minute (cpm). The results were calculated as mitotic index. For further tests, the ratio 2:1 (T cells: MDSCs) was selected.

$$mitotic\ index = \frac{PHA\ stimulated\ test\ culture\ [cpm]}{non\ stimulated\ control\ culture\ [cpm]}$$

iNOS expression and NO detection

Total RNA was isolated from Mo-MDSCs, and monocytes sorted by flow cytometry, using the Universal RNA Purification Kit (EURx, Gdansk, Poland), according to the manufacturer's specifications. 5 μg of total RNA was reverse transcribed to complementary DNA using NG dART RT-PCR kit (EURx). Quantitative real-time PCR was performed using SG qPCR Master Mix (2x) (EURx) and oligonucleotides complementary to transcripts of the analyzed genes using the Quant Studio 7 Real-Time PCR system (Applied Biosystems). The following oligonucleotides were used in this study:

iNOS: 5′- CAGCGGGATGACTTTCCAAG AGGCAA-GATTTGGACCTGCA-3′

Gene expression was assessed by using the $2^{-\Delta Ct}$ method. Expression level of the target genes was calculated by normalization to the reference gene - *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*). To detect NO production by MDSCs, a 4.5-diaminofluorescein-2/diacetate (DAF-2/DA, Abcam, Cambridge, UK) was used according to Strijdom et al. [18] and the cells were analyzed by flow cytometry (FACSCanto, BD Biosciences).

Statistical analysis

Statistical analysis was performed using the PRISM GraphPad 5 package (GraphPad Software Inc., San Diego, CA). Obtained data were analyzed using a T-test or one-way analysis of variance (ANOVA) with Tuckey test as a post hoc. The magnitude of the relationship between two quantitative features was evaluated using Pearson's correlation coefficient. The Kaplan–Meier curves with Log-rank analysis were used to determine the disease recurrence in two arbitrary selected patients' groups. All data are expressed as median ± interquartile range. The *p*value < 0.05 was considered statistically significant.

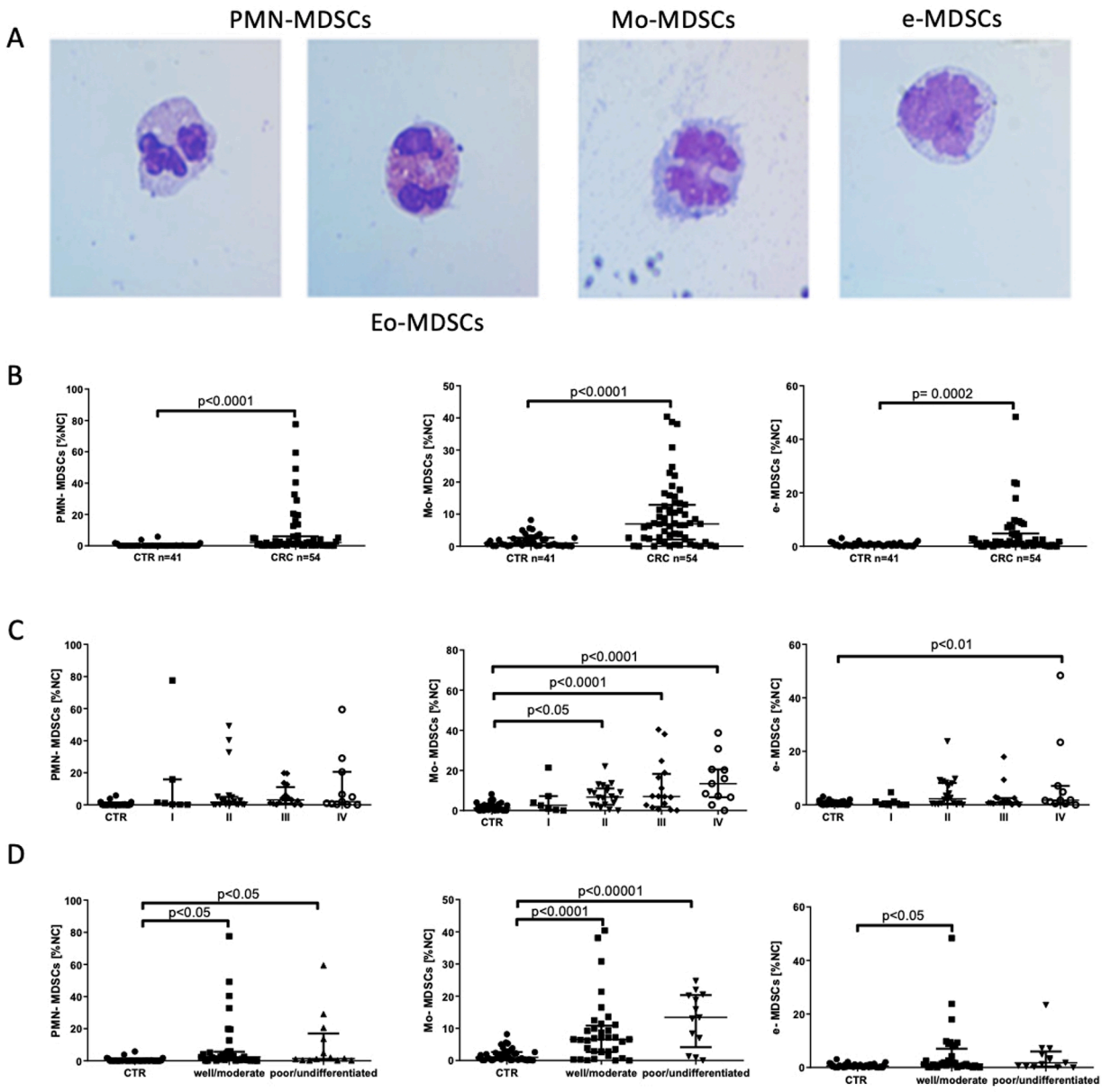

Fig. 2. MDSCs subset distribution in patients with CRC.
(A) The Wright's staining of isolated PMN-MDSCs, Mo-MDSCs and e-MDSCs. MDSCs populations were sorted out according to the following criteria: PMN-MDSCs as $LIN^{-}CD33HLA\text{-}DR^{-/low}CD66b^{+}CD14^{-}$, Mo-MDSCs as $LIN^{-}CD33^{+}HLA\text{-}DR^{-/low}CD66b^{-}CD14^{+}$, and e-MDSCs as $LIN^{-}HLA\text{-}DR^{-/low}CD33^{+}$ $CD66b^{-}CD14^{-}$ followed by cytospin preparation and the Wright's staining. Note, that within PMN-MDSCs, cells with eosinophil morphology (Eo-MDSC) were detected.
(B) Flow cytometry analysis of PMN-MDSCs, Mo-MDSCs and e-MDSCs level in CTR ($n = 41$), and patients with CRC ($n = 54$). MDSCs populations were identified by flow cytometry after gating according to cell surface marker expression, as described in A, and their levels are presented as percent values of PBMC.
(C) Frequency of PMN-MDSCs, Mo-MDSCs and e-MDSCs in relation to the stage of CRC (TNM classification, I $n = 7$; II $n = 20$; III $n = 16$; IV $n = 11$).
(D) Frequency of PMN-MDSCs, Mo-MDSCs and e-MDSCs in relation to the histological grading of CRC (well/moderate $n = 36$; poor/undifferentiated $n = 13$).
CTR - healthy donors, CRC - colorectal cancer patients, NC-nucleated cells.

Results

Peripheral blood of patients with CRC contains high level of circulating MDSCs

Populations of PMN-MDSCs, Mo-MDSCs and e-MDSCs were determined in peripheral blood of CRC patients according to the expression of previously defined cell surface markers [9]. The gating strategy for identification of MDSCs subsets by flow cytometry is presented in Fig. 1. The obtained data show that in CRC patients, the level (% value of PBMC) of circulating PMN-MDSCs, Mo-MDSCs and e-MDSCs was significantly higher than in healthy donors (Fig. 2B). These cells were further isolated by FACS and their morphology was analyzed microscopically after the Wright's staining (Fig. 2A). This analysis revealed that population of PMN-MDSCs is composed of two morphologically different subsets, including a recently described population of eosinophilic origin (Eo-MDSCs) [19]. Considering diversity in the clinical stage of the patients, we further analyzed how MDSCs subpopulations are distributed among the groups of patients with different CRC stages. In this context, higher levels of Mo-MDSCs and e-MDSCs in patients with most advanced cancer stage (IV) were observed. In the case of Mo-MDSCs, their level was also higher in stage II and III, while there was no difference in case of PMN-MDSCs (Fig. 2C).

In respect to tumor grading, the level of Mo-MDSCs and PMN-MDSCs

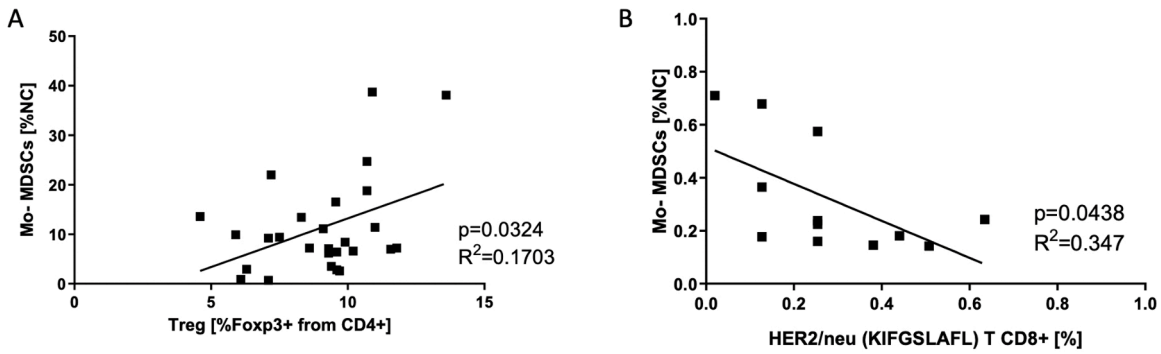

Fig. 3. The relationship of Mo-MDSCs with T cell subpopulations.
(A) Correlation of the blood level of Mo-MDSCs with Treg cells. Mo-MDSCs were identified in peripheral blood by flow cytometry, as described in "Materials and methods", and Treg cells as $CD4^+$ $Foxp3^+$. Mo-MDSCs' levels are presented as percent values of nucleated cells (NC) from PBMC while Treg levels are presented as percent of $CD4^+Foxp3^+$ lymphocytes ($n = 27$).
(B) Correlation of the blood level of Mo-MDSCs with $CD8^+$ T cells specific for Her2/neu, identified by flow cytometry. Blood level of Mo-MDSCs and HLA-A_2 HER-2/$neu_{369–377}$ specific $CD8^+$ T cells is presented as percent of PBMCs and lymphocytes, respectively ($n = 12$).

was higher in the blood of patients, regardless the histological tumor classification (Fig. 2D). On the other hand, the level of e-MDSCs was elevated only in the blood of patients with the tumor differentiation status "well/moderate" (Fig. 2D). There was no difference in the level of MDSCs between patients with or without local lymph node metastases (Supplementary Fig. 2A). However, a trend for increased Mo-MDSCs and decreased of e-MDSCs frequency in patients with large tumor was observed (Supplementary Fig. 2B).

Mo-MDSCs positively correlate with the level of Treg cells and negatively with TAA $CD8^+$ T cells in CRC patients

Although the interactions between MDSCs and T cells in cancer are well-documented, the relationship between MDSCs subpopulations and T cell subsets, including Treg in CRC is a matter of debate [20]. To shed more light on this aspect, we compared the level of MDSCs subsets with Treg, Th17 and $CD8^+$ T cells specific for HLA-A_2 HER-2/neu immunodominant epitope, considered as a tumor-associated antigen (TAA) in CRC [21]. We found that only Mo-MDSCs positively correlated with the level of Treg in this group of patients (Fig. 3A and Supplementary Fig. 3B). Furthermore, only these cells affected the level of $CD8^+$ T cells specific for HLA-A_2 HER-2/$neu_{369–377}$ (KIFGSLAFL) immunodominant epitope in CRC patients (Fig. 3B and Supplementary Fig. 3C).

MDSCs isolated from peripheral blood of CRC patients are immunosuppressive

In the next set of experiments, we examined if identified cellular subsets indeed possess suppressive activity, typical for MDSCs. This was analyzed *in vitro* by H^3-thymidine incorporation assay, evaluating the effect of MDSCs specific subsets on T-cell proliferation induced by PHA. MDSCs subsets were isolated by flow cytometry cell sorting and co-cultured for 3 days with autologous T cells, containing 10% of monocytes as accessory cells, and after pulse with H^3-thymidyne, the proliferation of lymphocyte was measured. Initially, in a co-culture setting we have established a most effective T cells to MDSCs ratio as 2:1, which was used in further experiments. By this approach we show that PMN-MDSCs, Mo-MDSCs and e-MDSCs isolated from CRC patients' blood were able to significantly inhibit T cell proliferation induced by PHA (Fig. 4A), confirming their suppressive activity.

Mo-MDSCs possess iNOS activity and differ in PD-L1 expression from the tumor-originated counterparts

Regulatory role of Mo-MDSCs is mainly associated with iNOS activity and NO production. To confirm this mechanism as operating in CRC patients, in the next step we analyzed the expression of iNOS-mRNA and NO production by Mo-MDSCs isolated from the patients' blood. The data obtained by RT-PCR have shown significantly higher iNOS-mRNA expression in Mo-MDSCs than in normal blood monocytes (Fig. 4B). In parallel, using a fluorogenic dye - DAF-2 and flow cytometry analysis we have documented a significant production of NO by Mo-MDSCs isolated from the CRC-patients' blood (Fig. 4C). In this context, we have also analyzed the expression of PD-L1 on Mo-MDSCs, as relevant in direct, cell-to-cell mediated immune suppression. The data presented in Fig. 4D show that blood Mo-MDSCs are negative, while their counterparts isolated directly from the tumor mass are highly positive for PD-L1 expression, supporting the role of Mo-MDSCs locally in the CRC tumor (Fig. 4D).

Surgical removal of colorectal cancer increases the level of circulating Mo-MDSCs which may correlate with tumor recurrence

Surgery is still a first-line therapy for CRC patients. In this context we asked, if surgical removal of the tumor may affect the level of MDSCs in patients' blood. To this end we analyzed the level of MDSCs subsets in peripheral blood of CRC patients before and 3 days after surgery. The obtained results showed that after the treatment, frequency of circulating Mo-MDSCs increases significantly (Fig. 5A) – with an average increase of 13.5 ± 11.01%. Referring to these data, we have arbitrary divided our patients into two groups – first, composed of patients whose increase in Mo-MDSCs level was above an average value (group I, $n = 4$) and the second one, with the level of increase below the average (group II, $n = 7$) (Fig. 5B). During a five-year observation period it turned out that between these two groups there is a difference in the tumor recurrence, and patients with significant increase in the level of Mo-MDSCs after surgery present much more frequent relapse of the disease (characterization of the patients in these groups is presented in Supplementary Table 2).

Discussion

Our study documents a significant increase in the level of PMN-MDSCs, Mo-MDSCs and e-MDSCs in the blood of CRC patients. In respect to PMN-MDSCs and Mo-MDSCs, this observation corroborates the previous reports [5,22], although the studies showing such a pattern solely for PMN-MDSCs also exist [12]. A correlation between CRC and e-MDSCs level has been already shown [10] but without reaching a statistical significance. Therefore, this paper is the first reporting an increased blood level of all identified MDSCs subsets in this group of patients. Moreover, we noticed that PMN-MDSCs contain, in majority, cells of neutrophil origin but eosinophilic MDSCs (Eo-MDSCs) can also

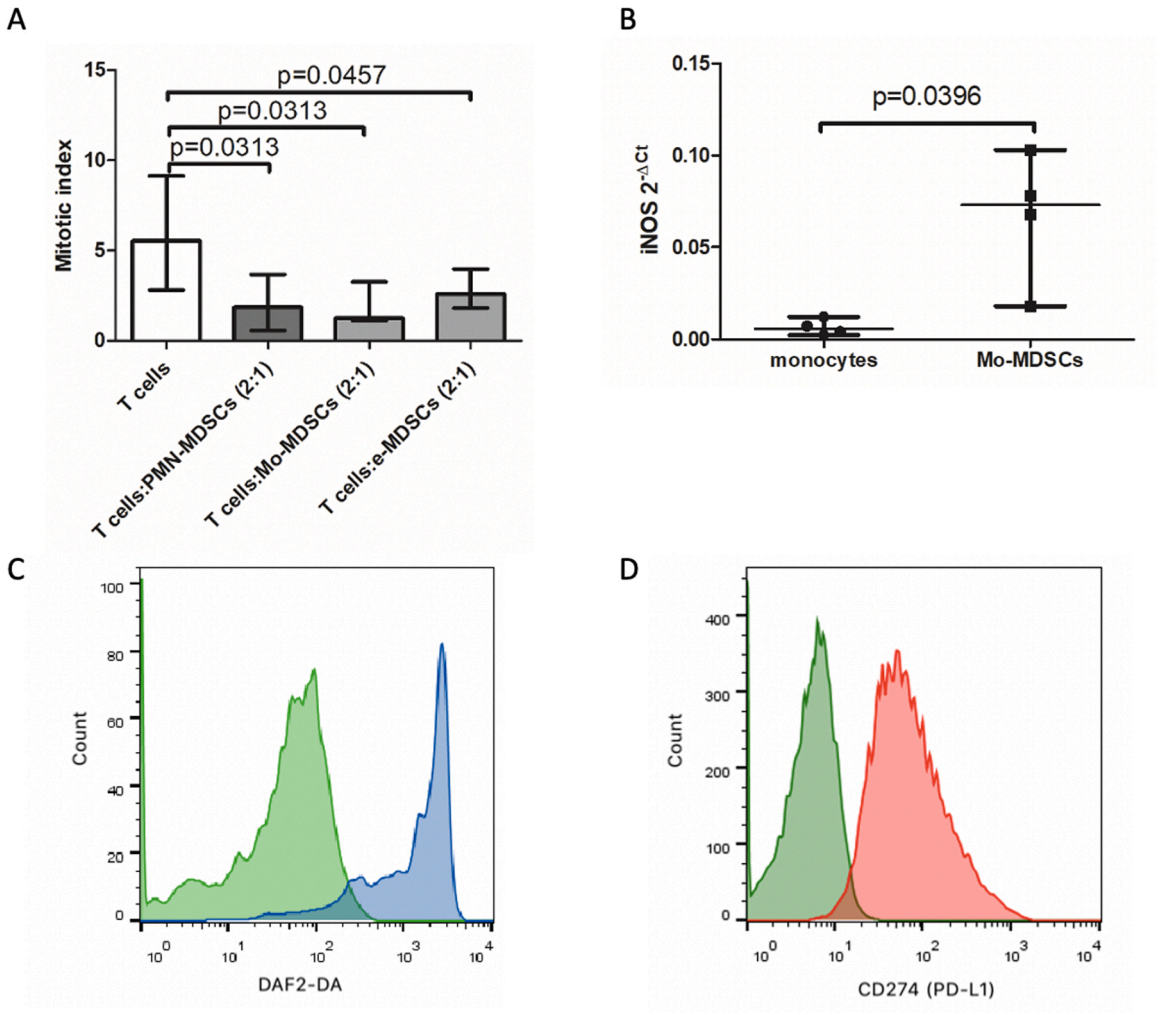

Fig. 4. Suppressive activity of MDSCs subsets. iNOS expression and NO production by Mo-MDSCs from CRC patients and expression of PD-L1 on Mo-DSCs depending on their localization

(A) MDSCs inhibit proliferation of autologous T-cells in CRC patients ($n = 6$). T cells were stimulated with PHA for 3 days in the presence of MDSCs. MDSCs subsets were sorted out by FACS as $Lin^{-}HLA\text{-}DR^{low/-}CD33^{+}CD66b^{+}CD14^{-}$; $LIN^{-}CD33^{+}HLA\text{-}DR^{low/-}CD66b^{+}CD14^{+}$; $LIN^{-}CD33^{+}HLA\text{-}DR^{low/-}CD14^{-}CD15^{-}$ cells from PBMCs. MDSCs subsets were added to the culture of FACS purified autologous $CD3^{+}$ T cells with 10% of autologous monocytes (sorted as $CD14^{+}HLA\text{-}DR^{+}$ cells). After 3 days of co-culture, T-cells were pulsed with H^{3}-thymidine for additional 6 h and β^{-} radiation was measured as cpm in a liquid scintillation counter. The index of proliferation was calculated as a ratio of PHA stimulated test culture [cpm] to non-stimulated culture [cpm].

(B) iNOS expression in monocytes and Mo-MDSCs from CRC patients detected by qRT-PCR ($n = 4$). The cells were sorted out by flow cytometry as $Lin^{-}CD33^{+}HLA\text{-}DR^{low/-}CD66b^{-}CD14^{+}$ and $CD14^{+}HLA\text{-}DR^{+}$ from PBMCs, respectively.

(C) Histogram overlay shows the fluorescence intensity of DAF2-DA corresponding to NO production by Mo-MDSCs (blue) in comparison to negative control (green). Data from one representative analysis out of four performed are presented.

(D) Histogram overlay shows the fluorescence intensity of PD-L1 (CD274) on Mo-MDSCs from blood (green) in comparison to Mo-MDSCs isolated from the tumor mass (red). Data from one representative flow cytometry analysis out of four performed are presented. Mo-MDSCs were gated as $Lin^{-}CD33^{+}HLA\text{-}DR^{low/-}CD15^{-}CD14^{+}$in PBMC and $CD45^{+}LIN^{-}HLA\text{-}DR^{low/-}CD33^{+}CD15^{-}CD14^{+}$ in the tumor tissue, respectively.

be detected. This population was described for chronic bacterial infections [19], and to the best of our knowledge, this is the first study documenting the occurrence of such cells in cancer, and specifically in CRC.

It is widely accepted that the level of circulating MDSCs increases in the late stage of cancer [23]. In our study we have confirmed this observation only for Mo-MDSCs and e-MDSCs population frequency in blood was highest in stadium IV of CRC. Considering the level of MDSCs subsets, we did not detect any significant difference neither between the groups of patients with or without local lymph node metastases, nor between the patients with large and small tumors, however a tendency suggesting that particularly Mo-MDSCs may contribute to these aspects of CRC progression has been observed.

In our work, for phenotype identification of MDSCs subsets we followed the recommendations by Bronte et al. [9], however, recently new tips for characterization of these cells were proposed [24]. In the case of PMN-MDSCs and Mo-MDSCs, our panel of markers did not differ from the latest recommendations, but in respect to e-MDSCs the use of anti-CD123 mAb to exclude basophils from analysis has been suggested [25]. In this context, our data, although did not include CD123 staining, clearly show that e-MDSCs do not resemble basophils by morphology, and possess strong suppressive activity, a key feature for MDSCs identification [25]. Regarding phenotype, it has been further indicated that PD-L1 expression on MDSCs is increased in CRC patients, suggesting that it may be a potent mediator of immunosuppression [26]. In our study MDSCs subsets isolated from the patients' blood were negative for PD-L1 expression, nevertheless they all efficiently inhibited proliferation of autologous T cells, confirming that MDSCs-mediated T cell suppression

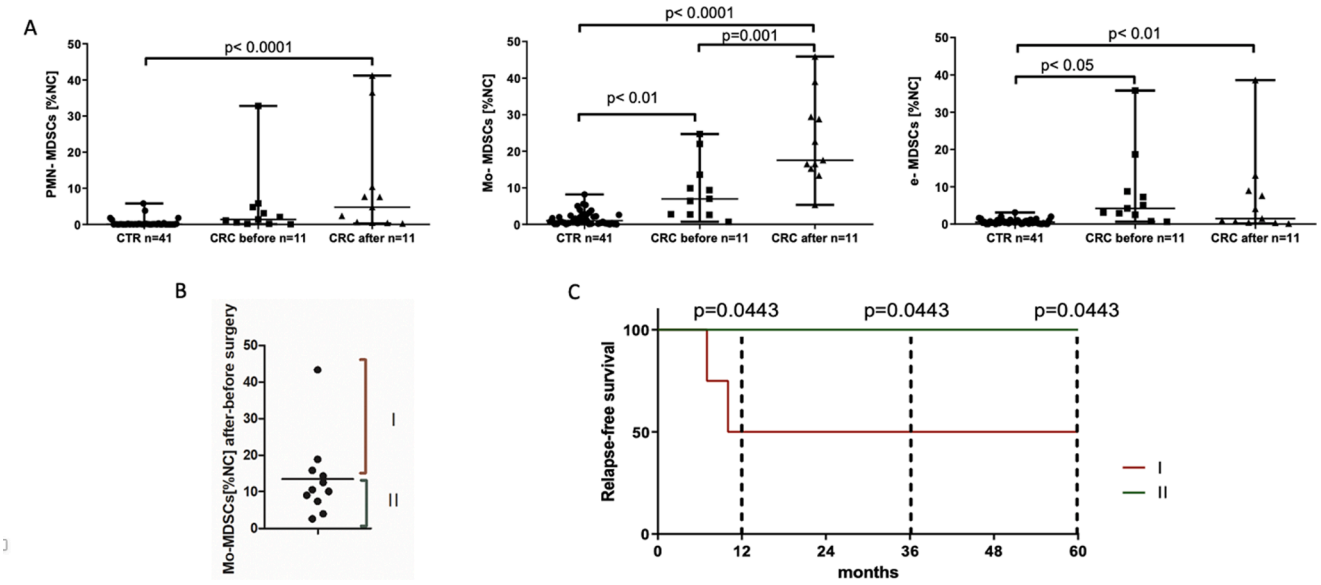

Fig. 5. MDSCs level before and after surgery treatment in relation to the CRC relapse-free time.
(A) Frequency of PMN-MDSCs, Mo-MDSCs and e-MSCSs in CRC patients' blood before and 3 days after surgical removal of the tumor. MDSCs populations were identified by flow cytometry, as described in "Materials and methods" and presented as percent of PBMC ($n = 11$).
(B) Index of the increase of Mo-MDSCs level in CRC patients' blood after surgery, calculated as the ratio of the Mo-MDSCs level after and before the treatment. Patients were divided into two groups according to the index of increase in the Mo-MDSCs level - first group (I), composed of patients whose increase in Mo-MDSCs level was above an average value ($n = 4$), and the second group (II), with the level of increase below the average value ($n = 7$).
(C) Kaplan-Meier analysis of relapse-free survival of CRC patients from group I and II during a 5-year follow-up (I $n = 4$; II $n = 7$).
CTR - healthy donors, CRC - colorectal cancer patients.

does not require the expression of PD-L1 [27], and suggesting that suppressive features of MDSCs represent a functional state of these cells. Contrary to peripheral blood, in the tumor MDSCs become more suppressive and are positive for PD-L1, specifically Mo-MDSCs, which are more prominent in this location, comparing to PMN-MDSCs [12]. This observation is in favor for PD-L1 upregulation by hypoxia, occurring in the tumor mass [28], and suggests that in the tumor, suppression is more dependent on direct cell-to cell contacts, although iNOS expression is also relevant for the suppressive nature of Mo-MDSCs [29]. In keeping, in our study Mo-MDSCs from CRC patients showed higher expression of iNOS mRNA than monocytes, and were able to produce NO, further documenting such an activity. In this context, we cannot exclude direct regulation of PD-L1 expression by iNOS activity in the tumor mass, as tumor-located MDSCs were shown to have a much higher expression of iNOS and NO production than their counterparts in peripheral lymphoid organs [30]. This would further clarify why tumor MDSCs are much more suppressive than their counterparts in the spleen or blood.

Immunosuppression in cancer is also associated with Treg [31], and increase in the Treg/Th17 ratio in cancer patients usually correlates with the disease progression [32]. Also, in our group of patients we observed a slightly increased blood Treg/Th17 ratio (Supplementary Fig. 2A), indicating a shift into the immune suppressive endotype. Considering the relationships between different populations of cells with regulatory functions, we further analyzed a potential correlation between the blood level of Treg and MDSCs. Although known for other types of cancer [33], in our study this was confirmed only in relation to Mo-MDSCs. Interestingly, there is no previous studies in the literature showing such a correlation in CRC patients. The regulatory functions of MDSCs can also be directed against tumor-antigen specific T cells [34]. In this context, we examined a correlation between Mo-MDSCs and HER-2/neu specific $CD8^+$ T cells in CRC patients. While the role of HER-2/neu overexpression/amplification in CRC is less clear than in breast or gastric cancer, and controversial expression rates, ranging from 2.7 [35] to 47.7% [36] have been published, the HER2 protein levels are correlated with clinical outcomes in CRC [37]. Our data showed an inverse correlation between the blood levels of these two cell subsets. To the best of our knowledge, this is a first such an observation in CRC patients.

Although immunotherapy or molecular-targeted personalized therapy are currently available for patients with the selected genetic profiles, surgery is still a basic form of CRC treatment. However, there is little data on the effects of surgery on the MDSCs in CRC, while the available observations are largely contradictory [14,19,38,39]. In our study, we have shown that 3 days after surgery the significant increase in the frequency of blood Mo-MDSCs occurs, and its value above the average may be relevant as a prognostic factor, correlating with the recurrence of CRC during a 5-year observation period. This was not observed for other MDSCs subsets, indicating that blood level of Mo-MDSCs after surgery should be taken into consideration for assessing the risk of CRC recurrence. Similarly, the increased level of MDSCs was noticed in patient with rectal cancer 7 days after surgery [40]. These results seem to be consistent with our data in prostate cancer patients, showing that surgery was not effective in reducing the level of circulating Mo-MDSCs, or could even induce its increase [41].

Our study has potential clinical relevance but must be interpreted with caution. Its main limitation is a small size of the group of patients with a 5-year follow-up after surgery. In this context it must be stressed that patients were qualified to this group randomly and originally the group contained 18 patients, but 7 patients had to be excluded because of a highly advanced CRC (IV stage). This aspect must be taken into consideration in future studies to obtain a more potent data, however it is worth mentioning that similarly to our results, an increased frequency of postoperative $CD14^+HLA\text{-}DR^{-/low}$ MDSCs was already correlated with an early recurrence of hepatocellular carcinoma [42].

In summary, our report indicates that in CRC patients, Mo-MDSCs play important role in cancer-related immunosuppression, and correlate with tumor size (prognostic marker in CRC) [43]. Moreover, Mo-MDSCs may be associated with tumor recurrence after surgery. Despite some limitations, our study suggests that MDSCs, and Mo-MDSCs particularly, seem potential targets for immunotherapy in CRC. In support of this view, first reports on successful therapy causing selective depletion of MDSCs in patients with advanced cancers,

including CRC, have been already released [44].

CRediT authorship contribution statement

Izabela Siemińska: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Kazimierz Węglarczyk:** Methodology. **Marta Walczak:** Methodology. **Agata Czerwińska:** Methodology. **Radosław Pach:** Methodology. **Mateusz Rubinkiewicz:** Investigation, Methodology. **Antoni Szczepanik:** Investigation, Methodology. **Maciej Siedlar:** Supervision. **Jarek Baran:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data statement

Upon reasonable request, the raw data supporting the results of this article will be made available by the authors without undue reservation.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101346.

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