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Charakterystyka fenotypowa i funkcjonalna
mieloidalnych komórek supresorowych w chorobie
nowotworowej na przykładzie raka jelita grubego
i raka prostaty - implikacje dla immunoterapii
nowotworów

Phenotype and functional characteristics of myeloid-
derived suppressor cells in solid tumors based on the
example of colorectal and prostate cancer -
implications for cancer immunotherapy

Praca doktorska

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Wykaz stosowanych skrótów

ARG1 (ang. *arginase-1*), arginaza-1

CRC (ang. *colorectal cancer*), rak jelita grubego

e-MDSC (ang. *early-stage myeloid-derived suppressor cells*), mieloidalne komórki supresorowe zatrzymane na wczesnym etapie rozwoju,

EVs (ang. *extracellular vesicles*), pęcherzyki zewnątrzkomórkowe,

Gr-MDSC (ang. *granulocyte myeloid-derived suppressor cells*), mieloidalne komórki supresorowe pochodzenia granulocytarnego

IBD (ang. *inflammatory bowel disease*), nieswoiste choroby zapalne jelit

IDO (ang. *indoloamine 2,3-dioxygenase*), 2,3-dioksygenaza indoloaminy

iNOS (ang. *inducible nitric oxide synthase*), indukowalną syntezę tlenu azotu

Mo-MDSC (ang. *monocyte myeloid-derived suppressor cells*), mieloidalne komórki supresorowe pochodzenia monocytarnego

NO (ang. *nitrogen oxide*), tlenek azotu

PBMC (ang. *peripheral blood mononuclear cell*), jednojądrzaste komórki krwi obwodowej

PC (ang. *prostate cancer*), rak prostaty

PD-L1 (ang. *programmed death ligand 1*), ligand receptora dla programowanej śmierci

PHA (ang. *phytohemagglutinin*), fitohemaglutynina

ROS (ang. *reactive oxygen species*), reaktywne formy tlenu

TAA (ang. *tumor associated antigens*), antygeny związane z nowotworem

TCR (ang. *T cell receptor*), receptor limfocyty T

Treg (ang. *T regulatory cells*), limfocyty T regulatorowych (Treg)

Rozdział 1. Wykaz publikacji będących podstawą rozprawy doktorskiej

Rozprawa doktorska stanowi cykl czterech publikacji:

1. **Siemińska I**, Rychlicka, E, Jaszczyński J, Palaczyński M, Bukowska-Strakova K, Ryś J, Dumański J, Siedlar M, Baran J. The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy. *Polish Journal of Pathology*. 2020;71(1), 46-54. doi.org/10.5114/pjp.2020.95415.

IF 0.92 MNiSW 40

2. **Siemińska I**, Baran J. Myeloid-derived suppressor cells in colorectal cancer. *Frontiers in Immunology*. 2020;7(11), 1526. doi: 10.3389/fimmu.2020.01526.

IF 5.1 MNiSW 140

3. **Siemińska I**, Węglarczyk K, Walczak M, Czerwińska A, Pach R, Rubinkiewicz M, Szczepanik A, Siedlar M, Baran J. Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery. *Translational Oncology*. 2022 17, 101346. doi.org/10.1016/j.tranon.2022.101346.

IF 4.23 MNiSW 100

4. **Siemińska I**, Baran J. Myeloid-derived suppressor cells as key players and promising therapy target in prostate cancer. *Frontiers in Oncology*. 2022 (w druku).

IF 6.24 MNiSW 100

Sumaryczny IF: 16.49 Sumaryczna punktacja MNiSW: 380

Rozdział 2. Wprowadzenie. Przegląd literatury dotyczącej tematu rozprawy

Rak jelita grubego (CRC ang. *colorectal cancer*) jest trzecim pod względem częstości występowania nowotworem na świecie. Szacuje się, że w samym 2022 roku odnotuje się 1.9 miliona nowych przypadków i 935 tysięcy zgonów z powodu CRC (1). W ciągu ostatnich lat w Polsce odnotowano 4-krotny wzrost zachorowań na CRC u mężczyzn i 3-krotny u kobiet (2). Z kolei rak prostaty (stercza) (PC ang. *prostate cancer*) jest drugim pod względem częstości nowotworem u mężczyzn i czwartym najczęściej występującym nowotworem u ludzi w ogóle. W roku 2022 szacuje się zdiagnozowanie 1.4 miliona nowych przypadków PC i 375 tys. zgonów na całym świecie (1).

W rozwoju obu nowotworów istotną rolę odgrywają zarówno predyspozycje genetyczne (3,4), jak i czynniki środowiskowe, w tym złe nawyki żywieniowe, palenie tytoniu, nadużywanie alkoholu czy brak aktywności fizycznej (5–8). Innym istotnym czynnikiem zwiększającym ryzyko rozwoju tych nowotworów jest przewlekły stan zapalny. W przypadku CRC taką zależność wykazano w odniesieniu do nieswoistych chorób zapalnych jelit (IBD ang. *inflammatory bowel disease*), gdzie przewlekły stan zapalny błony śluzowej może prowadzić do jej dysplazji i rozwoju nowotworu (9). W przypadku PC stan zapalny również związany jest z ryzykiem jego rozwoju (10), ale co istotne, wiąże się z nim nie tylko miejscowy stan zapalny w obrębie prostaty (stercza), ale także reakcja ogólnoustrojowa, w przebiegu przewlekłych chorób zapalnych, w tym astmy i alergii (11).

Na początku XX wieku zauważono, iż w przebiegu choroby nowotworowej dużą rolę odgrywa układ immunologiczny, którego komórki mogą zarówno wspomagać, jak i niestety, stymulować rozwój guza (12). Z początkiem XXI wieku w publikacjach dotyczących PC i CRC zaczęły pojawiać się doniesienia o nowej populacji komórek o właściwościach immunosupresyjnych (13,14). Późniejszy rozwój badań doprowadził do wyodrębnienia komórek mieloidalnych sprzyjających rozwojowi nowotworu i określenia ich mianem mieloidalnych komórek supresorowych (MDSC ang. *myeloid-derived suppressor cells*) (15). Obecnie wiadomo, iż komórki te stanowią heterogenną populację

i u zdrowych osób występują w krwi obwodowej tylko w niewielkim odsetku. Jednakże w trakcie różnych stanów chorobowych np. przewlekłego zapalenia, infekcji, choroby nowotworowej, itp. dochodzi do wyraźnego wzrostu ich poziomu (16). Biorąc pod uwagę ich ontogenezę, u człowieka wyróżnia się trzy subpopulacje MDSC: pochodzenia monocytarnego (Mo-MDSC), granulocytarnego (Gr-MDSC) oraz komórek prawdopodobnie zatrzymanych na wczesnym etapie rozwoju, tzw. „early-stage” (e-MDSC), fenotypowo scharakteryzowanych odpowiednio jako: $LIN^- HLA-DR^- CD11b^+ CD14^+ CD15^- CD33^+$, $LIN^- HLA-DR^- CD11b^+ CD14^- CD15^+ CD33^+$ oraz $LIN^- HLA-DR^- CD11b^+ CD14^- CD15^- CD33^+$ (17,18).

W procesie nowotworowym MDSC ułatwiają wzrost guza, promują jego unaczynienie, a nawet wspomagają tworzenie odległych przerzutów (19). Ekspansja MDSC regulowana jest przez sieć sygnałów, w której istotną rolę odgrywa stan zapalny i jego mediatory pochodzące z mikrośrodowiska guza (20). W powstawaniu MDSC wykazano istotną rolę chemokin i ich receptorów (np. CCL2/CCL12-CCR2, CXCL8-CXCR1/2), interleukin (np. IL-6, IL-10, TGF-beta), prostaglandyn (np. PGE2), czynników wzrostu (np. GM-CSF), jak również pęcherzyków zewnątrzkomórkowych, głównie pochodzenia nowotworowego (EVs ang. *extracellular vesicles*) (21-25). Wiele typów komórek, w tym komórki nowotworowe, ma zdolność uwalniania EVs i transportowania w nich m.in. funkcjonalnych białek (26), mRNA (27) czy cząsteczek niekodującego miRNA (28). Ich rola w indukcji MDSC, a tym samym wpływ na przeciwnowotworową odpowiedź układu odpornościowego jest obecnie przedmiotem licznych badań (25). Z drugiej strony, również MDSC mogą wydzielać EVs wspomagające rozwój guza (29), a samo mikrośrodowisko, oprócz generowania MDSC, może też wpływać na wzmocnienie ich właściwości immunosupresyjnych, np. dzięki silnej hipoksji (30).

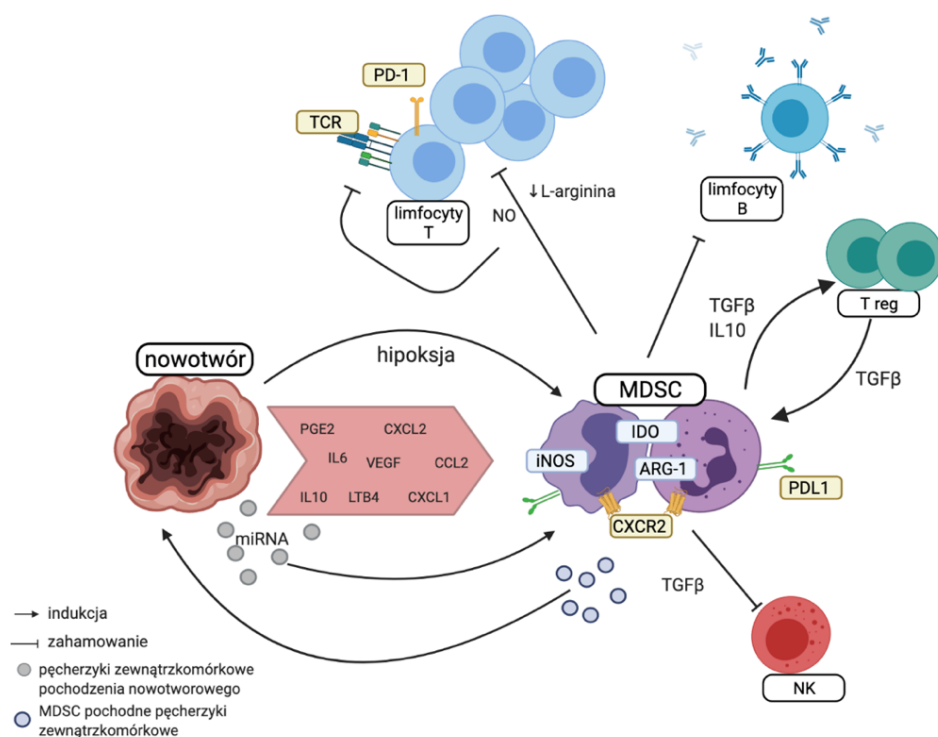
Rola MDSC w odpowiedzi na rozwijający się nowotwór została opisana w pracy przeglądowej, której jestem współautorem (31). W publikacji tej, będącej wynikiem badań międzynarodowego konsorcjum w ramach projektu COST „My-EUNITER”, opisano jak skomplikowane i dalekosiężne relacje tworzą komórki MDSC z innymi komórkami układu

odpornościowego. W tym kontekście, zarówno Mo-MDSC, jak i Gr-MDSC głównie hamują aktywność efektorowych limfocytów T, podczas gdy działanie e-MDSC nie zostało jeszcze w pełni scharakteryzowane (17,32,33). Opisane do tej pory mechanizmy immunosupresji MDSC obejmują produkcję arginazy-1 (ARG1), indukowalną syntezę tlenku azotu (iNOS), wydzielanie TGF β , IL-10, COX2, czy zubożenie środowiska w tryptofan poprzez aktywność 2,3-dioksygenazy indoloaminy (IDO) (34, 35). MDSC są również zdolne do indukcji limfocytów T regulatorowych (Treg), te zaś aktywują komórki MDSC, pozostając we wzajemnym dodatnim sprzężeniu zwrotnym (36). Limfocyty Treg i ich poziom często są również korelowane z progresją nowotworów, w tym PC i CRC (37,38). Najważniejszą jednak funkcją MDSC wydaje się ich zdolność do hamowania odpowiedzi cytotoksycznych limfocytów T, czy to poprzez obniżenie lokalnego stężenia argininy czy poprzez nitrozylację TCR (ang. *T cell receptor*) (39). Dodatkowo, MDSC dzięki ekspresji cząsteczki PD-L1 (ang. *programmed death ligand 1*) mogą np. indukować „wyczerpanie” /anergię limfocytów T w szlaku PD-L1-PD-1 (30). Nieliczne dane literaturowe wskazują, iż MDSC mogą hamować również produkcję nowotworowo swoistych przeciwciał (40), a także odpowiedź komórek NK (41). Główne mechanizmy powstawania i działania MDSC w chorobie nowotworowej przedstawiono na Rysunku 1.

Chociaż wysoki poziom MDSC zwykle kojarzony jest ze złym rokowaniem dla pacjentów onkologicznych, w tym również z CRC i PC (42-44), to mechanizmy działania MDSC w różnych nowotworach nie muszą być jednakowe. Stąd szczegółowe opisy działania MDSC w CRC i PC zawarte zostały w przeglądach literatury włączonych do cyklu.

Pomimo wspomnianej podstawowej charakterystyki fenotypowej, populacje MDSC u człowieka nie posiadają swoistego/unikalnego markera, w związku z czym, funkcjonalna analiza tych komórek nabiera kluczowego znaczenia przy ich identyfikacji (17). Ze względu na dużą wrażliwość tych komórek na czynniki zewnętrzne, takie jak temperatura czy czas izolacji, ich badanie powinno jednak spełniać wiele warunków, ograniczających ryzyko uzyskania fałszywych wyników (45,46). W przypadku PC i CRC, wiele dotychczasowych badań prowadzono jednak bez przestrzegania tych podstawowych zaleceń (17). W tym

kontekście, pogłębiona charakterystyka populacji MDSC u człowieka, prowadzona z zachowaniem opracowanych wytycznych, może pozwolić na wykorzystanie badania poziomu MDSC w krwi jako markera prognostycznego w chorobach nowotworowych, w tym w CRC i PC (47).



Rysunek 1. Powstawanie i aktywność MDSC w chorobie nowotworowej.

Rozdział 3. Cel pracy z uzasadnieniem podjętej tematyki badawczej będący odniesieniem do publikacji stanowiących rozprawę doktorską

Nadrzędne cele pracy:

- I. Ocena występowania w krwi obwodowej pacjentów z chorobą nowotworową różnych populacji MDSC oraz ich charakterystyka fenotypowa i funkcjonalna, na przykładzie CRC i PC.
- II. Mając na uwadze, iż dotychczas opisano kilka subpopulacji MDSC, istotnym było też sprawdzenie czy wszystkie z nich występują z podobną częstością w obu badanych nowotworach.
- III. Dodatkowo, ze względu na fakt, iż poziom MDSC w krwi może stanowić jeden z markerów prognostycznych w chorobie nowotworowej (wysoki kojarzony jest ze złym rokowaniem), kolejnym celem było zweryfikowanie zależności pomiędzy poziomem MDSC w krwi pacjenta, a stadium zaawansowania nowotworu. Wydawało się to istotne, zwłaszcza w odniesieniu do CRC, gdyż w literaturze światowej do tej pory nie było doniesień na ten temat.

Szczegółowe cele z uwzględnieniem poszczególnych publikacji:

1. Publikacja przeglądowa pt. „*Myeloid-derived suppressor cells in colorectal cancer*” (publikacja 2) miała na celu usystematyzowanie dotychczasowej wiedzy na temat MDSC w CRC, tak aby zagadnienia poruszane później w pracy oryginalnej nie miały charakteru odtwórczego, a wypełniały luki w dotychczasowych badaniach.
2. W tym kontekście głównym celem badań przedstawionych w publikacji oryginalnej pt. „*Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery*” była pełniejsza charakterystyka populacji MDSC w krwi obwodowej pacjentów z CRC.

Cele szczegółowe obejmowały:

- określenie zależności pomiędzy poziomem MDSC, a populacjami limfocytów T - regulatorowych i cytotoksycznych, swoistych dla wybranego antygenu związanego z nowotworem (Her2/neu), obu bardzo istotnych w odpowiedzi przeciwnowotworowej,
 - wobec rozbieżności danych na temat wpływu zabiegu chirurgicznego na poziom MDSC w krwi pacjentów z CRC, dodatkowo w badaniach podjęto próbę sprawdzenia czy po chirurgicznym usunięciu guza jako podstawowej formy leczenia CRC, poziom MDSC i ich subpopulacji ulega zmianie i czy może on korelować ze wznową choroby w okresie 5 lat od zabiegu (publikacja 3).
3. W przypadku PC, brak było w literaturze światowej danych na temat wpływu podstawowych form leczenia tego nowotworu na poziom MDSC krążących w krwi pacjentów.

Celem szczegółowym tych badań było więc:

- określenie takiego wpływu, a także ocena zależności poziomu MDSC w krwi od PSA jako powszechnego markera tej choroby - publikacja 1 pt. *„The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy”*.
4. W ostatniej pracy cyklu pt. *„Myeloid-derived suppressor cells as key players and promising therapy target in prostate cancer”* przedstawiłam usystematyzowaną wiedzę na temat mechanizmów działania MDSC w PC oraz zawarłam informacje na temat nowych potencjalnych form terapii PC, nakierowanych na eliminację i/lub zahamowanie funkcji MDSC (publikacja 4).

Rozdział 4. Realizacja celów naukowych

Zaprezentowany w publikacji 2 przegląd literatury na temat MDSC w CRC stanowił teoretyczne podstawy do podjęcia badań eksperymentalnych (publikacja 3). Natomiast publikacja przeglądowa dotycząca MDSC jako celu terapeutycznego w PC (publikacja 4) stanowi rozwinięcie tematu wpływu standardowej terapii PC na poziom MDSC w krwi pacjentów, poruszonego w ramach publikacji oryginalnej (publikacja 1).

Materialy i metody

Metodologia badań została szczegółowo opisana w sekcji „Metody” w pracach oryginalnych, wchodzących w skład rozprawy doktorskiej (publikacja 1 i 3).

Pacjenci

Badaniami objęło łącznie 97 pacjentów z następujących grup:

- 54 pacjentów ze zdiagnozowanym CRC w różnym stadium zaawansowania wg. klasyfikacji TNM Union for International Cancer Control's (UICC) oraz oceny histologicznej, leczonych w I i II Kliniki Chirurgii Ogólnej Szpitala Uniwersyteckiego w Krakowi i 41 zdrowych kontroli
- 43 pacjentów ze zdiagnozowanym PC w różnym stadium zaawansowania wg. klasyfikacji American Joint Committee on Cancer (AJCC), skali Gleasona oraz poziomu PSA, leczonych w Centrum Onkologii-Instytutu im. Marii Skłodowskiej-Curie Oddział w Krakowie i 23 zdrowych kontroli.

Szczegółową charakterystykę grup pacjentów przedstawia Tabela 1 i 2.

Na prowadzenie przedmiotowych badań wydane zostały zgody Komisji Bioetycznej Uniwersytetu Jagiellońskiego nr 122.6120.128.2015 i 1072.6120.70.2018 oraz zgoda Okręgowej Izby Lekarskiej nr 6/KBL/OIL/2014. Badaniami objęto jedynie pacjentów, którzy po przedstawieniu celu i rodzaju badań wyrazili na udział w nich swoją pisemną zgodę. Z uwagi na fakt, iż sposób pobrania materiału

(antykoagulant) i izolacji jednojądrzastych komórek krwi obwodowej (PBMC ang. *peripheral blood mononuclear cell*) różnił się u pacjentów z CRC i PC, utworzono dwie grupy kontrolne spójne w metodologicznej obróbce materiału z grupą badaną. Grupę kontrolną stanowiły osoby, u których po planowej klinicznej i laboratoryjnej diagnostyce nie rozpoznano niedoboru odporności, choroby nowotworowej czy przewlekłego stanu zapalnego.

Tabela 1. Charakterystyka pacjentów z CRC zakwalifikowanych do badań.

Pacjenci		CRC	Ograniczony ^a UICC (I+II)	Przerzutujący ^a UICC (III+IV)
Charakterystyka		n=54	n=27	n=27
wiek ^b (lata)		64±13	65±11.7	63.1±14.3
płeć	męska	30	14	16
	żeńską	24	13	11
stadium TNM ^c	I	7	7	-
	II	20	20	-
	III	16	-	16
	IV	11	-	11
Lokalizacja nowotworu ^d	proksymalna	27	13	14
	dystalna	27	14	13
Stopień histologiczny	Dobry/umiarkowany	36	19	17
	zły/nieodróżniony	13	6	7
	nieokreślony	5	2	3

^a Pacjenci z CRC z ograniczoną chorobą odpowiadają stadium I i II, a chorzy z przerzutami odpowiadają stadium III i IV.

^b Średnia ± SD

^c W oparciu o klasyfikację CRC TNM według UICC.

^d Lokalizacja guza została sklasyfikowana jako proksymalna lub dystalna względem zagięcia śledzionowego.

Tabela 2. Charakterystyka pacjentów z PC zakwalifikowanych do badań.

Pacjenci		Wszyscy	Przed leczeniem
		43	21
Poziom PSA (ng/ml)		33.67 ±35.02y ^a	30.14 ±34.44y ^a
Stage ^b	2b	11	6
	2c	2	2
	3a	7	1
	3b	17	9
	4a	5	3
	4b	2	1
Grade ^c	1 (6=3+3)	1	-
	2 (7=3+4)	14	7
	3 (7=4+3)	15	6
	4 (8=3+5;4+4)	7	4
	5 (9 – 10 = 4 + 5; 5 + 4; 5 + 5)	6	3

^a Średnia ± SD.

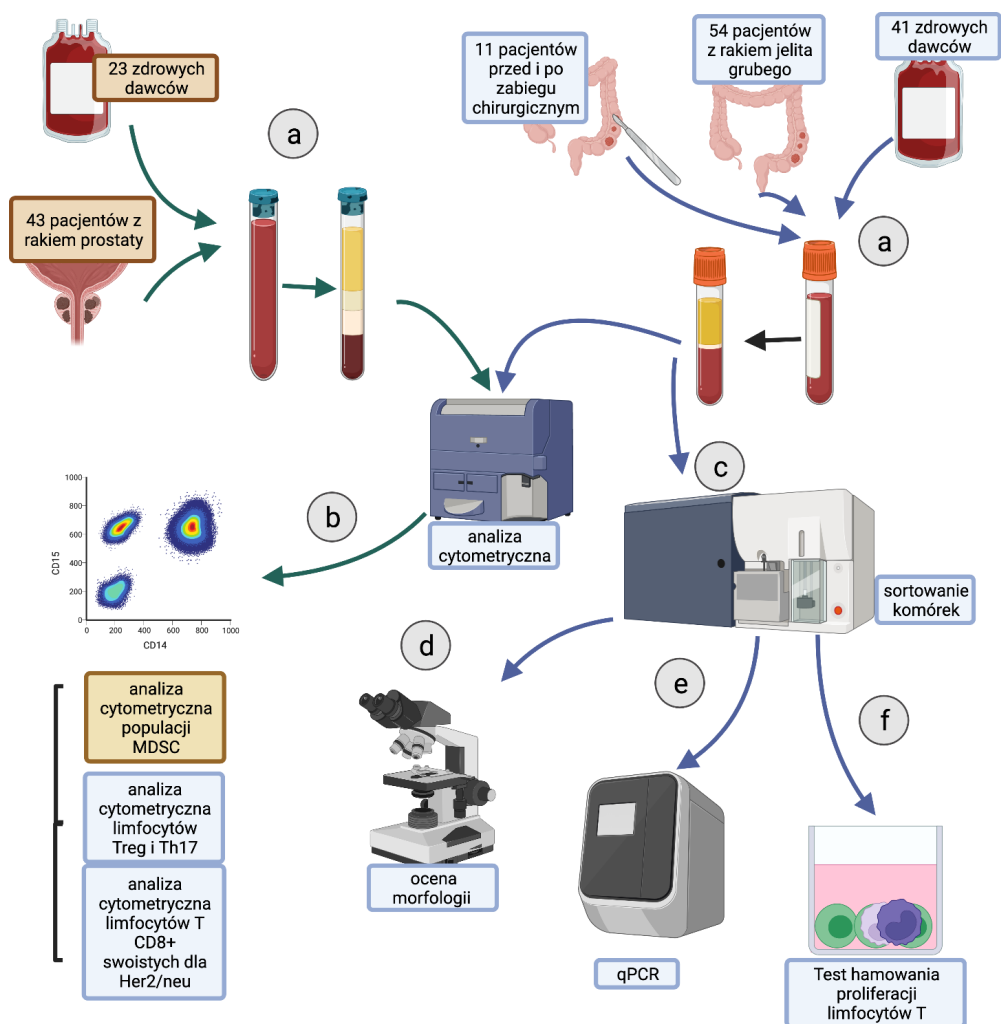
^b W oparciu o klasyfikację według AJCC

^c W oparciu o skalę Gleasona

W celu realizacji postawionych celów badawczych zastosowano:

- Izolację jednojądrzastych komórek krwi obwodowej
- Analizę cytometryczną populacji komórek MDSC oraz limfocytów Th17 i Treg i limfocytów T CD8⁺ swoistych dla antygenu Her/2neu.
- Sortowanie subpopulacji MDSC, limfocytów T oraz monocytów krwi obwodowej.
- Ocenę morfologii wyizolowanych populacji MDSC.
- Ocenę ekspresji *iNOS* oraz ocenę produkcji tlenu azotu (NO) przez Mo-MDSC.
- Test hamowania proliferacji limfocytów T.

Zastosowane metody badawcze schematycznie zestawiono na Rysunku 2.



Rysunek 2. Metody badawcze wykorzystane w pracy doktorskiej. Na niebiesko zaznaczono punkty odnoszące się do badań pacjentów z CRC, zaś na pomarańczowo punkty wspólne dla pacjentów z PC i CRC.

Wyniki

W artykule pt. „*The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy*” (publikacja 1) wykazano podwyższony poziom Gr-MDSC i Mo-MDSC w krwi obwodowej pacjentów z PC w stosunku do grupy kontrolnej, bez różnic w poziomie e-MDSC. Dodatkowo, stwierdzono pozytywną korelację pomiędzy poziomem PSA, a poziomem krążących Mo-MDSC. W kolejnym etapie badań sprawdzano, jaki jest poziom MDSC i PSA w poszczególnych stadiach zaawansowania nowotworu, wg skali Gleasona i TNM. W obu typach klasyfikacji poziom krążących w krwi Gr-MDSC i Mo-MDSC był istotnie wyższy niż w grupie kontrolnej, a w odniesieniu do PSA, wyższy względem poziomu przyjętego za wartość referencyjną. Nie wykazano natomiast istotnych różnic w wartościach tych parametrów pomiędzy poszczególnymi stadiami choroby. Ostatni etap analizy obejmował ocenę poziomu Gr-MDSC, Mo-MDSC i PSA w odniesieniu do rodzaju leczenia, jaki otrzymali pacjenci. W tym celu pacjentów podzielono na cztery grupy: przed leczeniem, po zabiegu chirurgicznym, terapii hormonalnej i terapii kombinowanej (łącznie minimum dwie z czterech form leczenia, tj. chirurgia, chemioterapia, radioterapia, leczenie hormonalne). Przeprowadzone analizy wykazały, iż w grupie pacjentów przed jakimkolwiek leczeniem obserwuje się istotnie wyższy poziom krążących Gr-MDSC, natomiast w grupach pacjentów objętych leczeniem stwierdzono, iż poziom Gr-MDSC w krwi osiągał wartości zbliżone do kontroli. W odniesieniu do Mo-MDSC, u pacjentów po leczeniu skojarzonym poziom tych komórek we krwi był porównywalny do grupy kontrolnej. W pozostałych grupach chorych (nieleczona, leczona chirurgicznie i leczona hormonalnie) stwierdzono wyraźnie wyższy poziom Mo-MDSC niż w grupie kontrolnej i grupie pacjentów otrzymujących leczenie skojarzone.

W kolejnym artykule oryginalnym pt. „*Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery*” wykazano, iż w krwi pacjentów z CRC występuje podwyższony poziom wszystkich subpopulacji MDSC, zarówno pochodzenia granulocytarnego, monocytarnego, jak i tzw. ‘early stage’. Badania morfologii tych komórek pozwoliły stwierdzić, iż wśród Gr-

MDSC występują nie tylko komórki odpowiadające neutrofilom, ale również eozynofilom. Jest to pierwsza obserwacja w literaturze światowej, wskazująca na występowanie takich komórek w krwi pacjentów z chorobą nowotworową.

W dalszych badaniach sprawdzono, czy poziom MDSC w krwi pacjentów z CRC koreluje ze stadium zaawansowania nowotworu wg klasyfikacji TNM i oceny histologicznej. Biorąc pod uwagę klasyfikację TNM, jedynie w przypadku Mo-MDSC ich poziom był znacząco wyższy w bardziej zaawansowanych stadiach choroby (II-IV stadium) w porównaniu z grupą kontrolną, ale nie stwierdzono istotnych różnic pomiędzy stadiami. Dodatkowo, niezależnie od histologicznej klasyfikacji guza, poziom Mo-MDSC i Gr-MDSC był zawsze wyższy w krwi pacjentów w stosunku do osób zdrowych. Natomiast, nie wykazano różnic w poziomach MDSC w krwi pacjentów w zależności od obecności lub braku przerzutów do lokalnych węzłów chłonnych. Zaobserwowano jednak tendencję do wzrostu poziomu Mo-MDSC i obniżenia poziomu e-MDSC u pacjentów z dużym guzem (T3/4). W kolejnym etapie określono relacje pomiędzy poziomem MDSC, a populacjami limfocytów Treg, Th17 i limfocytami T CD8⁺ swoistymi dla antygenu HER-2/neu, w CRC zaliczanego do grupy antygenów związanych z nowotworem (TAA ang. *tumor associated antigens*) (48). Przeprowadzone badania wykazały, iż jedynie poziom populacji Mo-MDSC dodatnio korelował z poziomem limfocytów Treg. Ponadto, poziom Mo-MDSC ujemnie korelował z obecnością limfocytów T CD8⁺ swoistych dla HER-2/neu. Zależności takich nie stwierdzono w odniesieniu do innych populacji MDSC.

Następnie skupiono się na ocenie aktywności poszczególnych subpopulacji MDSC u pacjentów z CRC. Aktywność tę oceniano *in vitro* w teście transformacji blastycznej z użyciem H³-tymidyny, badając wpływ poszczególnych subpopulacji MDSC na proliferację autologicznych limfocytów T indukowaną mitogenem PHA (fitohemaglutynina). Stwierdzono, iż wszystkie 3 wyizolowane z krwi pacjentów subpopulacje MDSC (Gr-MDSC, Mo-MDSC, e-MDS) wykazywały aktywność supresyjną wobec autologicznych limfocytów T, typową dla tych komórek, co potwierdziło ich właściwą selekcję.

Zgodnie z danymi literaturowymi rola regulacyjna Mo-MDSC jest związana głównie z aktywnością iNOS i produkcją tlenku azotu (NO). Aby zweryfikować działanie tego mechanizmu u pacjentów z CRC, w kolejnym etapie badań przeanalizowano produkcję NO, a także samą ekspresję *iNOS* w Mo-MDSC. Dane uzyskane za pomocą techniki PCR wykazały znacznie wyższą ekspresję *iNOS*-mRNA w Mo-MDSC niż w monocytach krwi. Równolegle, stosując fluorogenowy barwnik DAF-2 i analizę metodą cytometrii przepływowej, udokumentowano produkcję NO przez Mo-MDSC. Dodatkowo, oceniono również ekspresję cząsteczki PD-L1, istotnej w mechanizmach bezpośredniej supresji limfocytów T. Okazało się, że krążące MDSC nie wykazywały wysokiej ekspresji PD-L1. Ekspresję taką wykazano natomiast na powierzchni MDSC wyizolowanych z masy guza. I w tym przypadku, pozytywne pod względem ekspresji PD-L1 były głównie Mo-MDSC.

Ostatnim etapem była 5-cioletnia obserwacja poziomu MDSC u 11 pacjentów z CRC po chirurgicznym usunięciu guza. Zebrane w tym czasie wyniki pozwoliły stwierdzić, iż wkrótce po zabiegu (3 dni) dochodzi do istotnego wzrostu poziomu Mo-MDSC w krwi pacjentów. Odnosząc się do tych danych, arbitralnie podzielono pacjentów na dwie grupy – pierwszą składającą się z chorych, u których wzrost poziomu Mo-MDSC był powyżej wartości średniej dla całej grupy (grupa I, n = 4) i drugą, z poziomem wzrostu poniżej średniej (grupa II, n = 7). Pomimo małej liczebności obu grup stwierdzono, iż u pacjentów ze znacznym wzrostem poziomu Mo-MDSC po zabiegu chirurgicznym (grupa I), w ciągu pięcioletniego okresie obserwacji występował znacząco częstszy nawrót choroby nowotworowej, co sugeruje istotną rolę populacji Mo-MDSC w tym procesie.

Dyskusja i podsumowanie

Wyniki zgromadzone w ramach badań nad MDSC u pacjentów z PC dokumentują w ich krwi wyższy poziom komórek pochodzenia granulocytarnego (Gr-MDSC) i monocytarnego (Mo-MDSC), bez ekspansji e-MDSC, w stosunku do grupy kontrolnej. W odniesieniu do Gr-MDSC i Mo-MDSC, dotychczasowe dane literaturowe potwierdzają powyższe obserwacje (49), brak jest jednak takich danych dla e-MDSC. W tym kontekście, przedstawione wyniki należy traktować jako pionierskie. W odniesieniu do pacjentów z CRC, w ich krwi zaobserwowano podwyższony poziom wszystkich 3 subpopulacji MDSC. Wzrost poziomu Gr-MDSC i Mo-MDSC w tej grupie chorych był już opisywany (14,50), natomiast dane na temat e-MDSC były ubogie i wskazywały u chorych jedynie na tendencję wzrostową poziomu tych komórek (40). Tym samym w publikacji 3 po raz pierwszy wykazano istotny wzrost poziomu wszystkich trzech subpopulacji MDSC w krwi pacjentów z CRC. Ponadto, w obrębie subpopulacji Gr-MDSC zidentyfikowano komórki morfologicznie odpowiadające eozynofilom (Eo-MDSC). Występowanie takiej populacji stwierdzono wcześniej w trakcie przewlekłych infekcji bakteryjnych (51), ale nigdy dotąd w chorobie nowotworowej. Jest to więc pierwsze doniesienie dokumentujące występowanie Eo-MDSC w CRC.

W wielu typach nowotworów Mo-MDSC uznaje się za populację głównie zasiedlającą guz, podczas gdy Gr-MDSC mają przeważać w krwi obwodowej (52). Przyczyną takiego stanu może być fakt, iż Mo-MDSC charakteryzują się wysoką ekspresję iNOS i wytwarzają duże ilości NO, który pozostaje dłużej aktywny w środowisku niż reaktywne formy tlenu (ROS z ang. *reactive oxygen species*), produkowane głównie przez Gr-MDSC. Wskazywałoby to, iż Gr-MDSC, w przeciwieństwie do Mo-MDSC wymagają bliższego (bezpośredniego) kontaktu z komórkami docelowymi np. limfocytami T, aby zahamować ich aktywność (52,53). Z odmiennym mechanizmem działania tych dwóch subpopulacji MDSC może wiązać się nie tylko ich preferencyjna lokalizacja, ale również „moc” stosowanej przez nie immunosupresji. W tym aspekcie dane literaturowe nie są jednak jednoznaczne - jedni autorzy sugerują, iż Mo-MDSC są

bardziej supresyjne (33), podczas gdy inni za bardziej supresyjne uznają populację Gr-MDSC (54,55).

Analizy korelacji poziomu MDSC z poziomem limfocytów Treg sugerują jednak, iż populacja Mo-MDSC może odgrywać kluczową rolę w procesach immunosupresji w CRC. Limfocyty Treg i ich aktywność supresyjna jest również istotna w progresji choroby nowotworowej (65). W tym kontekście nie dziwi, iż poziom obu populacji komórek jest regulowany na drodze dodatniego sprzężenia zwrotnego (36). Taka zależność podkreślana jest w literaturze dla wielu nowotworów (57), niemniej dotychczas nie była opisana w CRC, tym bardziej w kontekście korelacji poziomu limfocytów Treg z subpopulacją Mo-MDSC. Co ciekawe, podobnych obserwacji dokonano w PC, wykazując dodatnią korelację pomiędzy poziomami obu tych populacji (58).

Ponieważ aktywność MDSC wpływa również na limfocyty T cytotoksyczne swoiste dla antygenów nowotworowych (59), postanowiono sprawdzić, czy istnieje zależność pomiędzy poziomem MDSC a populacją limfocytów T CD8⁺ swoistych dla HER-2/neu. Chociaż rola nadekspresji *HER-2* w CRC nie jest do końca jasna, obecność samego białka HER2/neu wykazuje zależność z przebiegiem klinicznym CRC (60). Również w tym przypadku korelacja, tym razem negatywna, została wykazana dla poziomów limfocytów T CD8⁺ swoistych dla HER-2/neu i subpopulacji Mo-MDSC.

Formalną podstawę do określenia komórek jako MDSC, stanowi spełnienie kryteriów fenotypowych i potwierdzenie supresyjnego charakteru badanych komórek (17). Niniejsze badania wykazały, iż wszystkie 3 subpopulacje wyizolowane z krwi pacjentów z CRC wykazują aktywność supresyjną. Najnowsze zalecenia dotyczące badań fenotypowych MDSC wskazują na dodatkowe stosowanie przeciwciał anty-CD123 jako markera przydatnego do wykluczenia z analizy bazofili, potencjalnie „kontaminujących” populację e-MDSC (61). W przedstawionej pracy, pomimo braku takiego barwienia, analiza morfologii tych komórek po ich izolacji oraz, co ważniejsze, ich silnie supresyjny charakter udokumentowany w testach funkcjonalnych, pozwoliła na odrzucenie tezy o błędnej identyfikacji e-MDSC (62).

Jedną z kluczowych cech fenotypowych MDSC, świadczącą o ich supresyjnym charakterze jest ekspresja cząsteczki PD-L1. Zwiększoną ekspresję PD-L1 wykazano już na powierzchni MDSC u pacjentów z CRC (63). W przedstawionej pracy MDSC izolowane z krwi pacjentów z CRC nie wykazywały jednak wysokiej ekspresji PD-L1, niemniej w testach funkcjonalnych wszystkie skutecznie hamowały proliferację autologicznych limfocytów T, potwierdzając, iż supresja limfocytów T nie wymaga ekspresji PD-L1 na MDSC (64). W przeciwieństwie do komórek izolowanych z krwi obwodowej, MDSC izolowane z guza wykazywały wysoką ekspresję PD-L1 i były to głównie Mo-MDSC. Obserwacja ta potwierdza dane literaturowe o predyspozycji Mo-MDSC do lokalizacji w obrębie guza (52). Współgra również z wcześniejszymi danymi na temat indukcji ekspresji PD-L1 przez hipoksję występującą w mikrośrodku guza (30).

W celu pełniejszego scharakteryzowania mechanizmów supresyjnych Mo-MDSC izolowanych z krwi pacjentów z CRC, wynikających chociażby z opisanej w literaturze ekspresji iNOS (65), w kolejnym etapie badań sprawdzono zdolność tych komórek do produkcji NO, oraz ich cytoplazmatyczną ekspresję iNOS. Otrzymane wyniki potwierdziły produkcję NO przez Mo-MDSC, jak i wyższą ekspresję *iNOS*-mRNA w porównaniu do monocytów krwi.

Przyjmuje się, iż poziom krążących MDSC wzrasta wraz ze stadium zaawansowania nowotworu (66). W publikacji oryginalnej skoncentrowanej na MDSC w PC nie stwierdzono jednak takiej zależności - o ile w przypadku PSA jego poziom był wyższy (nieistotnie) w IV stadium PC, tak poziom MDSC był podobny, a nawet nieznacznie niższy w ostatnich stadiach zaawansowania choroby. Należy jednak wziąć pod uwagę, iż w badaniu tym uczestniczyli pacjenci zarówno przed jak i po leczeniu, a leczenie może mieć tu znaczący wpływ. Patrząc na klasyfikację histologiczną w PC, również nie widać było różnic, czy istotnej przewagi w poziomie MDSC w zaawansowanych stadiach tego nowotworu. W badaniach tych skorelowano również poziom PSA z poziomem MDSC, uzyskując dodatnią korelację jedynie dla Mo-MDSC, podobnie jak w innych opublikowanych badaniach (13). Natomiast w przypadku pacjentów z CRC tendencję wzrostową można zaobserwować

w odniesieniu do Mo-MDSC i (w mniejszym stopniu) do e-MDSC. Najwyższy poziom MDSC stwierdzono u pacjentów ze złym/nieodróżnionym stopniem histologicznym i były to komórki populacji Mo-MDSC. Dodatkowo, u pacjentów z CRC zauważyć można tendencję do wzrostu poziomu Mo-MDSC w krwi wraz ze wzrostem wielkości guza.

Znając rolę MDSC w rozwoju i progresji nowotworu, nie dziwi fakt, iż oczekiwanym efektem leczenia winno być obniżenie poziomu tych komórek (49,67). Wyniki przedstawione w publikacji 1 sugerują jednak, iż rutynowe leczenie wiąże się z obniżeniem poziomu jedynie subpopulacji Gr-MDSC. Natomiast w przypadku Mo-MDSC sytuacja jest bardziej skomplikowana. Wydaje się, że skuteczne obniżenie poziomu Mo-MDSC osiąga się u pacjentów, u których zastosowano terapię skojarzoną. Natomiast u pacjentów leczonych wyłącznie chirurgicznie czy hormonalnie, poziom Mo-MDSC był znacząco wyższy i porównywalny do grupy bez leczenia. O ile w literaturze znaleźć można doniesienia o indukcji Mo-MDSC w przebiegu radioterapii (68), tak dotychczas nie prowadzono badań dotyczących MDSC u chorych z PC w kontekście różnych form leczenia. Choć publikacja ta ma charakter wstępny, a grupy pacjentów były niewielkie, jej wyniki sugerują, iż każda forma monoterapii w PC nie eliminuje krążących MDSC, zwłaszcza tych pochodzenia monocytarnego, a wręcz przeciwnie, może podnosić ich poziom. Dodatkowo według danych literaturowych pacjenci z PC, u których po leczeniu zaobserwowano podwyższony poziom Mo-MDSC w krwi, wykazywali gorsze przeżycie w stosunku do tych, u których wzrost ten nie występował (69). W takich przypadkach zastosowanie terapii dodatkowej, nakierowanej na MDSC, mogłoby polepszyć efekty leczenia PC, zwłaszcza w kontekście generowania przez te komórki oporności na leczenie (70). W tym celu potrzebne są oczywiście dalsze badania, w tym wydłużona obserwacja i analiza przeżycia pacjentów.

W przypadku CRC dane literaturowe wskazują, iż chemioterapia z użyciem gencytabiny, 5-fluorouracylu i doksorubicyny (71,72) obniża poziom krążących MDSC, w odniesieniu do zabiegu chirurgicznego brak jest natomiast jednoznacznych doniesień na ten temat (73,74–76). Wyniki uzyskane w trakcie prowadzonych badaniach wykazują jednak zbliżony

efekt jak w PC. Gdyż u pacjentów z CRC wkrótce po zabiegu (3 dni) zaobserwowano wzrost poziomu krążących Mo-MDSC. Dodatkowo u części z nich, u których wystąpił bardzo znaczący wzrost Mo-MDSC (powyżej średniej), częściej dochodziło do wznowy choroby nowotworowej w ciągu 5 lat obserwacji. Takich zależności nie stwierdzono w odniesieniu do pozostałych subpopulacji MDSC. Choć są to obserwacje prowadzone na bardzo małych liczebnie grupach, potwierdzają one wcześniejsze doniesienie pokazujące wzrost poziomu MDSC po zabiegu w raku odbytu (77). Pokrywają się one także z danymi z badań w raku piersi i płuca, gdzie po zabiegu chirurgicznym również wykazano wzrost poziomu Mo-MDSC (78,79). Również w tamtych badaniach zmiany te miały niekorzystny wpływ na rokowanie dla pacjentów (78,79). Wpływ zabiegu chirurgicznego *per se* na indukcję MDSC i progres choroby nowotworowej w CRC dobitnie pokazały badania na mysim modelu CRC, gdzie sama laparotomia wiązała się z indukcją Mo-MDSC, których poziom ujemnie korelował z przeżyciem zwierząt (75), a nawet sprzyjał powstawaniu przerzutów (76). Przedstawione w niniejszej pracy badania są pierwszymi dokumentującymi, podobnie jak w modelu mysim, iż wzrost poziomu subpopulacji Mo-MDSC po zabiegu może wiązać się ze wznową choroby nowotworowej u pacjentów z CRC.

Powyższe badania mają potencjalne znaczenie kliniczne, choć ze względu na mało liczne grupy pacjentów należy je interpretować z ostrożnością. W kontekście 5-letniej obserwacji należy podkreślić, że pierwotnie losowo zakwalifikowano 18 pacjentów, ale 7 pacjentów musiało zostać wykluczonych z powodu wysoce zaawansowanego CRC (stadium IV). Jednakże wyniki przeprowadzonych badań sugerują, iż aspekt Mo-MDSC powinien być wzięty pod uwagę przy opracowaniu przyszłych skojarzonych terapii, również nakierowanych na tę populację komórek.

Podsumowując, powyższe badania wskazują, iż zarówno u pacjentów z PC jak i CRC istotną rolę w progresji nowotworu odgrywają MDSC pochodzenia monocytarnego. W odniesieniu do PC jedynie skojarzona forma leczenia wpływała na obniżenie poziomu tej populacji w krwi pacjentów do wartości zbliżonych do grupy kontrolnej. W przypadku monoterapii PC tj. leczenia chirurgicznego lub

hormonalnego, poziom Mo-MDSC utrzymywał się na wysokim poziomie. W odniesieniu do CRC, chirurgiczne usunięcie guza prowadzi do wzrostu poziomu wszystkich subpopulacji MDSC, w tym również Mo-MDSC. Wyłącznie jednak wzrost poziomu Mo-MDSC może wiązać się ze zwiększonym ryzykiem wznowy choroby nowotworowej w ciągu 5-ciu lat. Obserwacje te sugerują, iż w obu nowotworach istotne może być leczenie wspomagające, nakierowanie na Mo-MDSC. Kontynuacją przedstawionych w mojej rozprawie badań jest aktualnie realizowany przeze mnie projekt Preludium, finansowany z Narodowego Centrum Nauki. Projekt ten ma na celu zbadanie mechanizmów generowania Mo-MDSC w CRC przez pęcherzyki zewnątrzkomórkowe pochodzenia nowotworowego, zwłaszcza w kontekście transportowanego w nich miRNA. Mam nadzieję, iż wyniki tych badań pozwolą na wytypowanie specyficznych punktów uchwytu mogących mieć wykorzystanie w immunoterapii nakierowanej na Mo-MDSC w chorobie nowotworowej.

Rozdział 5. Wnioski

1. MDSC w CRC i PC stanowią realny problem kliniczny, co potwierdzają zarówno prace oryginalne, jak i przeglądowe zawarte w cyklu publikacji.
2. MDSC pochodzenia monocytarnego (Mo-MDSC) wydają się być potencjalnym markerem prognostycznym w PC ze względu na korelację ich poziomu we krwi pacjentów z poziomem PSA.
3. U pacjentów z PC leczenie chirurgiczne i hormonalne zastosowane w formie monoterapii nie jest wystarczające do eliminacji krążących Mo-MDSC. Dopiero u pacjentów, u których zastosowano terapię skojarzoną zaobserwowano wyraźnie obniżony poziom Mo-MDSC w krwi.
4. U pacjentów z CRC Mo-MDSC odgrywają istotną rolę w immunosupresji. Ich poziom we krwi wykazuje tendencję do wzrostu wraz ze wzrostem wielkości guza i koreluje dodatnio z poziomem limfocytów T regulatorowych. Natomiast odwrotną korelację dla tego parametru stwierdzono w odniesieniu do poziomu limfocytów T cytotoksycznych, swoistych dla antygenu Her2/neu.
5. W krwi pacjentów z CRC wśród Gr-MDSC wyróżnić można nie tylko populację komórek odpowiadających morfologicznie neutrofilom, ale również komórkom pochodzenia eozynofilowego.
6. U pacjentów z CRC obecność PD-L1 na Mo-MDSC izolowanych z guza przy jej braku na Mo-MDSC krążących we krwi sugeruje, iż Mo-MDSC obecne w mikrośrodoisku nowotworu charakteryzują się silniejszą aktywnością immunosupresyjną.
7. Zabieg chirurgicznego usunięcia CRC wiąże się z szybkim wzrostem poziomu krążących Mo-MDSC (w 3 dobie po zabiegu).
8. Wzrost poziomu Mo-MDSC w krwi po zabiegu u pacjentów z CRC może stanowić marker prognostyczny wznowy nowotworu.
9. Zarówno w PC, jak i CRC MDSC, a zwłaszcza Mo-MDSC wydają się być istotnym celem immunoterapii.

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Rozdział 7. Streszczenie w języku angielskim

Prostate cancer (PC) and colorectal cancer (CRC) remain the most diagnosed malignancies worldwide. Despite genetic predispositions, a crucial role in their development plays environmental factors, including dietary habits, sedentary lifestyle, and chronic inflammation. The role of cells of the immune system in their development and progression was confirmed as well. This pro-tumorigenic response is associated with immunosuppression where the myeloid-derived suppressor cells (MDSC) are of the most pivotal players. The MDSC constitute a heterogenic population in which cells of granulocytic (Gr-MDSC), monocytic (Mo-MDSC) origin, as well as so called “early-stage” MDSC (e-MDSC) have been distinguished. Despite decades of research on MDSC and their role in various malignancies, many aspects remain unclear. Hence, this study was focused on the characterization of MDSC in PC and CRC patients, and their relation to the cancer stage and the used treatment. In the case of PC patients, we addressed the question whether commonly used therapies may affect the level of circulating MDSC. Thus, we compared the level of Gr-MDSC, Mo-MDSC and e-MDSC in the blood of patients with different clinical stages and different tumor grading scores, receiving various forms of treatment (surgery or hormonal therapy alone, and a combined therapy, including radiotherapy). Whereas in the case of CRC patients, we verified the effect of surgery on the level of MDSC and their subsets. The obtained results showed that in both cancers the Mo-MDSC seem to be most relevant. In CRC, the blood level of Mo-MDSC positively correlated with regulatory T cells, and negatively with the level of tumor antigen - Her2/neu, specific CD8⁺ T cells. Considering the effect of the treatment in PC patients, the study revealed that surgery or hormonal therapy alone did not affect the level of Mo-MDSC. In this case, only a combined therapy caused significant decrease in their level. In respect to Gr-MDSC, the level of this subset was comparable to the control group, regardless of the treatment the patients received. These results were independent of the PSA level, tumor grading and the clinical stage of the patients. In the case of CRC patients, after surgery it comes to increase of Mo-MDSC blood level, and its significant increase may be associated with tumor recurrence during a 5-year follow-up. In conclusion, Mo-MDSC seem to be pivotal players in CRC related immunosuppression and may be associated with the risk of tumor recurrence after surgery. Whereas in PC, Mo-MDSC are resistant to a single form of therapy, such as surgery or hormone therapy. Thus Mo-MDSC should be considered as a potential therapy target in the treatment of PC and CRC to enhance its anti-tumor effectiveness.

Rozdział 8. Streszczenie w języku polskim

Rak prostaty (PC) i rak jelita grubego (CRC) należą do najczęściej diagnozowanych nowotworów złośliwych na świecie. Oprócz predyspozycji genetycznych, kluczową rolę w ich rozwoju odgrywają czynniki środowiskowe tj. nawyki żywieniowe, siedzący tryb życia oraz przewlekły stan zapalny. W rozwoju obu tych nowotworów potwierdzono też udział komórek układu odpornościowego. Ta pro-nowotworowa odpowiedź związana jest z immunosupresją, w indukcji której istotną rolę odgrywają mieloidalne komórki supresorowe (MDSC). MDSC stanowią heterogenną populację, w której wyróżnia się MDSC pochodzenia granulocytarnego (Gr-MDSC), monocytarnego Mo-MDSC i tzw. „early stage” MDSC (e-MDSC). Pomimo dekad badań nad MDSC i ich rolę w różnych nowotworach, wiele aspektów pozostaje wciąż niewyjaśnionych. Niniejsze badania koncentrowały się na charakterystyce populacji MDSC w krwi pacjentów z PC i CRC, korelacji ich poziomu ze stadium zaawansowania nowotworu, poziomem limfocytów T lub markerów nowotworowych, takich jak PSA. W przypadku pacjentów z PC próbowano odpowiedzieć na pytanie, czy stosowana terapia przeciwnowotworowa wpływa na poziom populacji krążących MDSC. W tym celu porównano poziom Gr-MDSC, Mo-MDSC i e-MDSC w krwi pacjentów w różnych stadiach zaawansowania nowotworu i otrzymujących różne formy leczenia (leczenie chirurgiczne lub hormonalne, albo otrzymali leczenie skojarzone, w tym np. radioterapię). W przypadku pacjentów z CRC zweryfikowano wpływ chirurgicznego usunięcia nowotworu na poziom krążących MDSC. Uzyskane wyniki wykazały, że w obu nowotworach populacja Mo-MDSC wydaje się być najbardziej istotna. W CRC poziom Mo-MDSC dodatnio korelował z poziomem limfocytów T regulatorowych, a ujemnie z poziomem limfocytów T CD8⁺ specyficznych dla antygeny Her2/neu. Biorąc pod uwagę formy leczenia PC, okazało się, że sam zabieg chirurgiczny lub terapia hormonalna, nie wpływają istotnie na obniżenie poziomu Mo-MDSC. Dopiero terapia skojarzona, prowadziła do wyraźnego obniżenia poziomu Mo-MDSC w krwi pacjentów. W przypadku populacji Gr-MDSC, niezależnie od formy leczenia, poziom tych komórek był zbliżony do obserwowanego w grupie kontrolnej. W odniesieniu do pacjentów z CRC, wydaje się, że wkrótce po operacji dochodzi u nich do wzrostu poziomu Mo-MDSC. Wyraźny wzrost poziomu tych komórek po zabiegu może wiązać się z nawrotem nowotworu w ciągu najbliższych 5 lat. Podsumowując, Mo-MDSC wydają się odgrywać kluczową rolę w immunosupresji związanej z CRC i mogą wiązać się z ryzykiem nawrotu choroby po usunięciu guza. Podczas gdy w PC Mo-MDSC wydają się być odporne na monoterapię, np.

zabieg chirurgiczny lub terapia hormonalna. Uzyskane wyniki badań sugerują, iż populacja Mo-MDSC stanowi istotny potencjalny cel w leczeniu PC i CRC.

Rozdział 9. Załączniki

The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy.

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ORIGINAL PAPER

THE LEVEL OF MYELOID DERIVED-SUPPRESSOR CELLS IN PERIPHERAL BLOOD OF PATIENTS WITH PROSTATE CANCER AFTER VARIOUS TYPES OF THERAPY

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Prostate cancer is one of the most frequent cancers in men. Although several treatment options exist, their clinical effectiveness is still not satisfactory. One the possible reason of such situation might be the presence of myeloid-derived suppressor cells (MDSC) and their pro-tumorigenic activity. MDSC possess immunosuppressive ability and in many studies were shown to support tumor development and progression. In this study we addressed the question whether commonly used therapies of prostate cancer affect the level of MDSC populations in the patients' blood. We compared the level of granulocytic (Gr-MDSC), monocytic (Mo-MDSC) and early stage MDSC (eMDSC) in the blood of patients at different clinical stage and different tumor grading scores, who underwent either surgery or hormonal therapy alone or were given a combined treatment, including e.g. radiotherapy. The obtained results showed that the level of Gr-MDSC was significantly lower in all treated patients comparing to untreated group. On the other hand, surgery or hormonal therapy alone did not affect the level of Mo-MDSC. These results were independent of the PSA level, the tumor grading and clinical stage of the patients. In conclusion, we suggest that Mo-MDSC should be considered as a potential therapy target in the course of prostate cancer treatment to enhance its anti-tumor effectiveness.

Key words: prostate cancer (PC), myeloid-derived suppressor cells (MDSC), flow cytometry, prostate specific antigen (PSA).

Introduction

Prostate cancer (PC) is the second most frequently diagnosed cancer in men and the fifth leading cause of cancer death worldwide [1]. Economic development, increased consumption of animal fat, obesity and lack of physical activity have been considered as

modifiable risk factors of this type of cancer [2, 3]. On the other hand, many factors including improved treatment and early detection of PC, e.g. thanks to preventive programs, reduce the death rate [2]. In the early 1990s, soon after the introduction of a prostate-specific antigen (PSA) testing to the routine diagnostic procedure, the rapid growth

of incidence of prostate cancer was observed [2, 4]. Since then, due to frequent false positive results of PSA test and severe side effects of subsequent unnecessary treatment, the usefulness of routine PSA screening is a matter of debate [5, 6]. Therefore, efforts are undertaken to discover new more reliable tests, such as urine-based, that could play a major role in a primary screening [7]. In the middle of 20th century it was noticed that prostate cancer had been influenced by androgens and could be inhibited by elimination of these hormones, either through castration or neutralization of androgen activity by estrogen injections [8]. However, despite the low level of androgens, the so called castration-resistant prostate cancer still has an ability for progression. With many possibilities for prostate cancer treatment, including watchful waiting, surgery, radio-, chemo- and biological therapy [9,10], there is still a lack of satisfactory results, as tumor resistance to the applied treatment develops quite often. Recent studies indicate that the failure of prostate cancer treatment may be due to the myeloid-derived suppressor cells (MDSC), which were shown to support tumor development and recurrence [11].

MDSC compose a heterogeneous population of myeloid cells at different stage of differentiation, which regulate the immune response [12]. Nevertheless, under chronic inflammatory conditions, infections or cancer, due to the inhibition of myeloid cell differentiation the level of MDSC is elevated [13]. Numerous studies present evidence that inflammatory factors produced by tumor may induce generation of MDSC and/or increase their activity [14]. Despite high heterogeneity, MDSC are frequently distinguished as granulocytic (Gr-MDSC) and monocytic (Mo-MDSC) [12]. Recently, a population of so called early stage MDSC (eMDSC) has also been identified [15]. In humans, minimal phenotype characteristics define Gr-MDSC as CD11b+ CD14- CD15+, while Mo-MDSC as CD11b+ CD14+ HLA-DR-/lo CD15-. Population of eMDSC is recognized as CD11b+ CD14- CD15- [16]. Both Mo-MDSC and Gr-MDSC effectively inhibit T lymphocytes activity, although using different mechanisms, whereas function of eMDSC is still not clear [16, 17, 18]. Gr-MDSC preferentially express arginase - 1 (ARG1) and NADPH oxidase. Hence, Gr-MDSC show a higher production of reactive oxygen species (ROS) when compared to Mo-MDSC, which, on the other hand, comparing to Gr-MDSC display increased expression of inducible nitric oxide synthase (NOS2) and NO production [19]. MDSC could also induce T regulatory cells, which are often correlated with cancer progression [20].

Here, we asked whether commonly used therapies of prostate cancer affect the level of MDSC populations in peripheral blood of patients.

Material and methods

Patients

Patients with PC were recruited from the Maria Skłodowska-Curie Cancer Center in Krakow. 43 PC patients (treated or untreated) and 23 healthy age-matched controls (CTR) (mean age 67.64 ± 9.08 PC vs. 69.6 ± 6.31 CTR; $p = 0.062$) were enrolled into the study. As the PC group was heterogeneous in respect to the treatment options, it was further divided into four following subgroups: I – patients before treatment – 21, II – patients after a surgical removal of the tumor – 7, III – patients who received combined therapy (more than one type of treatment chemo-/radio-/ hormonal therapy/ surgery) – 9 and IV – patients with hormonal therapy alone – 6. PSA level (ng/ml), the Gleason scoring and clinical staging were performed routinely. The Gleason score assigns two most common histological patterns from 1 for highly differentiated to 5 for low differentiated cancers, with summary score ranging from 2 to 10. Further characterization of the patients' group is presented in Table I, while their classification according to the American Joint Committee on Cancer (AJCC) is presented in Table II [21]. All patients provided a signed informed consent and the study protocol was approved by the local bioethics committee of the Regional Board of Medical Doctors in Krakow (no. 6/KBL/OIL/2014).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples obtained from prostate cancer patients and healthy controls by standard density gradient centrifugation. Blood was collected into BD CPTTM mononuclear cell preparation tubes containing sodium citrate and FicollTM HypaqueTM Solution (Vacutainer System; Becton Dickinson, San Jose, CA) and the tubes were centrifuged at $1400 \times g$ for 15 min at room temperature. PBMCs were collected from the interphase, washed in PBS, resuspended in 0.5 ml of PBS and used for further analysis.

Flow cytometry analysis of Gr-MDSC, Mo-MDSC and eMDSC

For Gr-MDSC and Mo-MDSC analysis, PBMC (app. 1×10^6 cells/100 μ l) were stained with following monoclonal antibodies (mAbs): anti-LIN-AF700 (anti-CD3 clone UCTHT1, anti-CD19 clone HIB19, anti-CD56 clone B159), anti-CD33- PE (clone P67,6), anti-HLA-DR-PerCp (clone L243), anti-CD11b-BV510 (clone ICR F44), anti-CD14-FITC (M ϕ P9), anti-CD15-PE-Cy7 (clone HI98) (BD Biosciences; San Jose, CA) for 20 min in 4°C. After

Table I. Characterisation of the patients group. The patients in the stage group were qualified according to the AJCC guidelines (Table II). The grade group depends on Gleason score (summary of the two most common histological patterns of the tumor with scores ranging from 1 for highly differentiated to 5 for low differentiated cancer)

PROSTATE CANCER PATIENTS	ALL	BEFORE TREATMENT
	43	21
PSA level (ng/ml)	33.67 ± 35.02	30.14 ± 34.44
Stage groups		
2b	11	6
2c	2	2
3a	7	1
3b	17	9
4a	5	3
4b	2	1
Grade groups		
1 (6 = 3 + 3)	1	-
2 (7 = 3 + 4)	14	7
3 (7 = 4 + 3)	15	6
4 (8 = 3 + 5; 4 + 4)	7	4
5 (9 – 10 = 4 + 5; 5 + 4; 5 + 5)	6	3

incubation, cells were washed twice in PBS, suspended in 0.2 ml PBS and analyzed using FACSCanto flow cytometer (Becton Dickinson, CA, USA) and FACS-Diva software (BD). The Gr-MDSC, Mo-MDSC and eMDSC subsets were characterized as LIN- CD33+ HLA-DR- CD11b+ CD14- CD15+, LIN- CD33+ HLA-DR- CD11b+ CD14+ CD15- and LIN- CD33+ HLA-DR- CD11b+ CD14- CD15- respectively, and presented as a percent of nucleated cells (%NC) (Fig. 1). In order to determine the level of non-specific staining and cellular autofluorescence, the fluorescence minus one (FMO) control samples were prepared and analyzed in parallel.

Statistical analysis

Statistical analysis was performed using the PRISM GraphPad 5 package (GraphPad Software Inc., San Diego, CA, USA). Obtained data were analyzed using Mann Whitney test after Shapiro-Wilk normality test or one-way analysis of variance (ANOVA) with Newman-Keuls Multiple Comparison Test as post hoc test. All data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

Results

In the present study we aimed to evaluate the level of MDSC populations in the blood of prostate cancer patients, depending on the PSA level, tumor grading, clinical stage of the patients and treatment option they received. For this purpose we used flow

Table II. Clinical classification of the patients with prostate cancer according to the AJCC guidelines [21]. The TNM staging system describes the size and extent of the primary tumor (T), metastasis to the local lymph nodes (N), and distant metastasis (M); × means any result

T	N	M	PSA (NG/ML)	GRADE GROUP	STAGE GROUP
cT1a-c	N0	M0	< 10	1	I
cT2a	N0	M0	< 10	1	I
pT2	N0	M0	< 10	1	I
cT1a-c	N0	M0	≥ 10 < 20	1	IIa
cT2a	N0	M0	≥ 10 < 20	1	IIa
pT2	N0	M0	≥ 10 < 20	1	IIa
cT2b-c	N0	M0	< 20	1	IIa
T1-2	N0	M0	< 20	2	IIb
T1-2	N0	M0	< 20	3	IIc
T1-2	N0	M0	< 20	4	IIc
T1-2	N0	M0	≥ 20	1-4	IIIa
T3-4	N0	M0	×	1-4	IIIb
Tx	N0	M0	×	5	IIIc
Tx	N1	M0	×	×	IVa
Tx	Nx	M1	×	×	IVb

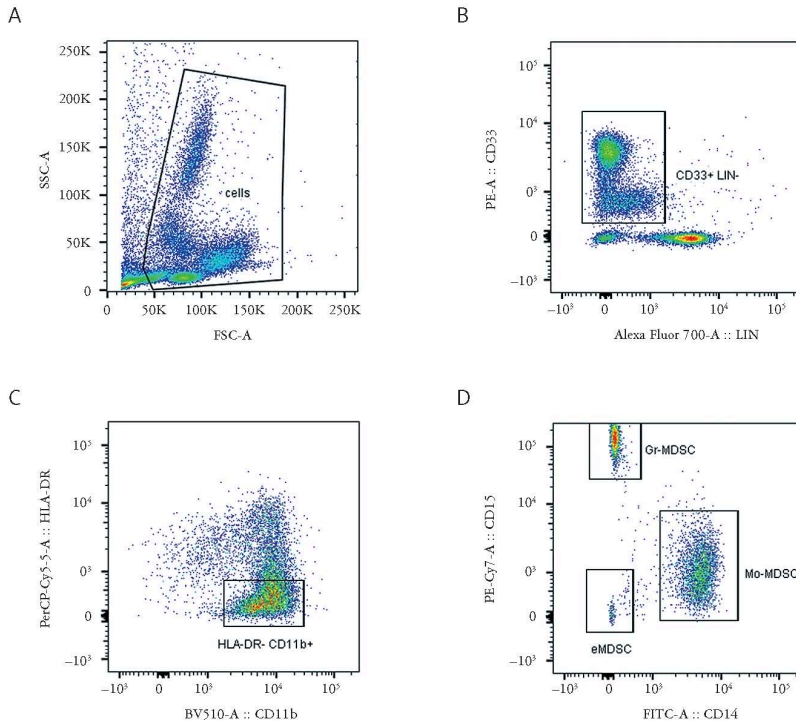


Fig. 1. Gating strategy for flow cytometry identification of Gr-MDSC, Mo-MDSC, eMDSC in peripheral blood of prostate cancer patients

cytometry analysis of peripheral blood leukocytes. The calculated mean percent value of Gr-MDSC in the whole group of PC patients was statistically higher when compared to control healthy individuals (2.53 ± 2.81 in PC vs. 0.30 ± 0.33 in CTR; $p < 0.0001$) (Fig. 2), as was the level of Mo-MDSC (10.67 ± 8.15 in PC vs. 1.17 ± 0.94 in CTR; $p < 0.0001$) (Fig. 2). In case of eMDSC, no statistically significant difference between patients and healthy controls was observed (data not shown). Although the usefulness of monitoring the PSA level is a matter of debate, it is still generally considered as a biomarker of the disease state. With this in mind, in the next step we asked if there is any correlation between the MDSC percentages and related PSA levels. We have shown such a correlation for Mo-MDSC (Pearson $r = 0.31$; $p = 0.047$) but not for Gr-MDSC (Fig. 3). In this context, it was interesting to find out whether the level of MDSC correlates with the tumor grading according to Gleason scale or clinical

staging of the patients. Therefore, considering Gleason score we divided our patients into four groups (according to the AJCC classification) and compared them with the level of Mo-MDSC and Gr-MDSC in peripheral blood, respectively. The obtained results indicated that regardless of Gleason score, the prostate cancer patients presented higher level of both Gr-MDSC and Mo-MDSC populations (Fig. 4) in comparison to healthy individuals. Moreover there was no significant differences in the level of Gr-MDSC and Mo-MDSC between the groups of patients (Fig. 4). These aspects were also analyzed in respect to the clinical stage of the patients, taking into account the PSA level, TNM classification and Gleason score (grade group) (Table II) [21]. In this case, due to the small number of patients in the groups IIb and IIc, IIIa, and IIIb, and IVa and IVb they were included to group II, III and IV, respectively. In such analysis, the level of both populations of MDSC was higher

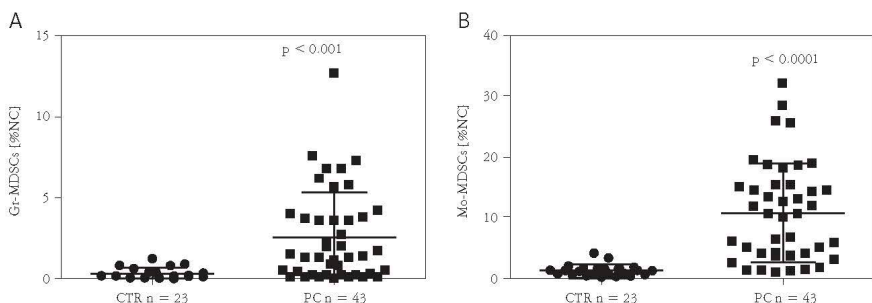


Fig. 2. Level of Gr-MDSCs (A) and Mo-MDSCs (B) in healthy control subjects and prostate cancer patients (% NC – % of nucleated cells)

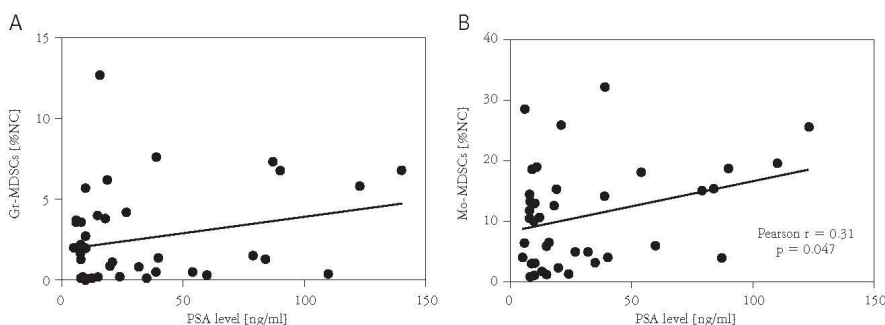


Fig. 3. Correlation between PSA level (ng/ml) and Gr-MDSC (A) and Mo-MDSC (B) percentage (% NC – % of nucleated cells)

in cancer patients in comparison to healthy controls, despite the disease stage (Fig. 5).

In the next step of analysis, a logical consequence was to evaluate the level of Gr-MDSC and Mo-MDSC in the patients' blood in respect to the mode of therapy they received. For this purpose the group of patients was subdivided into four groups, including patients before treatment, receiving surgery or hormonal therapy alone or receiving a combined therapy. The obtained data show that the mean proportion of Gr-MDSC in peripheral blood of patients was significantly higher in the group before treatment, comparing to healthy controls (3.82 ± 3.23 vs. 0.30 ± 0.33 ; $p < 0.001$) (Fig. 6), whereas the patients with formerly implemented therapy had reduced percentage of Gr-MDSC, reaching the level detected in the blood of healthy controls. In the group of patients after surgery (II group) and hormonal therapy (IV group) the results did not differ significantly (1.81 ± 1.73 vs. 1.6 ± 2.15) (Fig. 6), while in patients receiving combined therapy the level of Gr-MDSC was the lowest (0.69 ± 0.70). In relation to Mo-MDSC, their level in the group of patients before treatment

(I) and patients treated with hormonal therapy (IV group) was significantly higher than in the control group (15.40 ± 10.94 in I group, 17.37 ± 5.06 in IV group vs. 1.17 ± 0.94 CTR; $p < 0.0001$ for both) (Fig. 6) and the highest level was observed in patients after surgery (15.40 ± 10.94). Moreover, again, patients after combined therapy showed the lowest level of Mo-MDSC (5.50 ± 5.40) (Fig. 6).

When comparing the PSA level with the treatment option the patients received, we found that the patients after surgical removal of the tumor or those receiving a combined therapy presented the lowest level of PSA (14.00 ± 11.16 and 20.44 ± 17.45 , respectively), whereas the patients before any treatment had medium values of this marker (30.14 ± 34.44). On the other hand, patients after hormonal therapy presented the highest PSA level (77.00 ± 40.03 ; $p < 0.01$) (Fig. 6).

Discussion

Our study documents increased level of Gr-MDSC and Mo-MDSC in the prostate cancer patients'

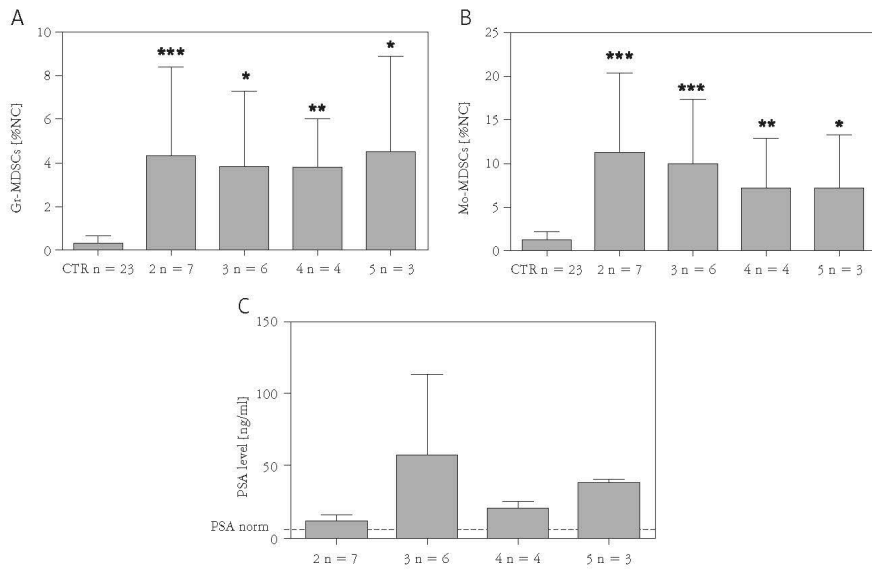


Fig. 4. Gr-MDSC (A) Mo-MDSC (B) PSA level (C) in healthy control subjects (CTR) and prostate cancer patients divided into four groups according to Gleason score. PSA normal level is indicated

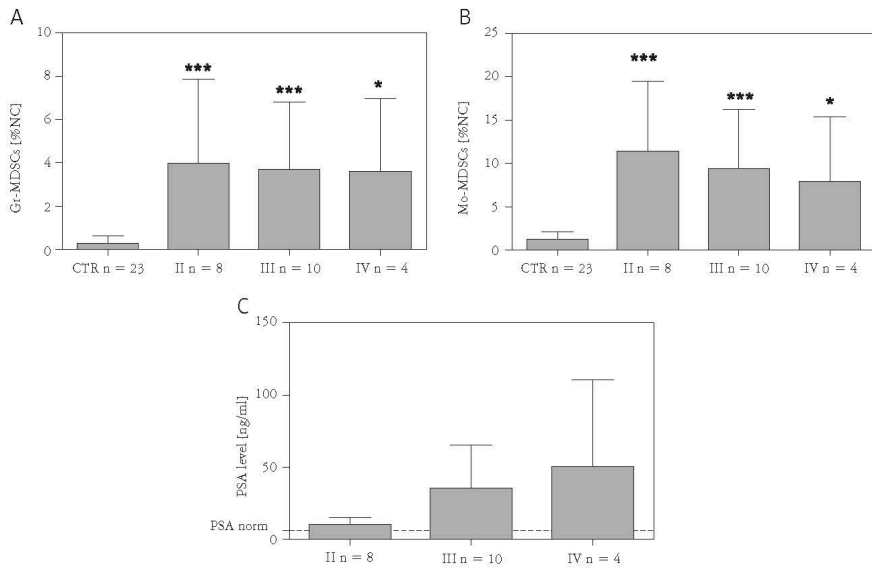


Fig. 5. Gr-MDSC (A) Mo-MDSC (B) PSA level (C) in healthy control subjects and prostate cancer patients divided into three groups according to the AJCC guidelines. PSA normal level is indicated

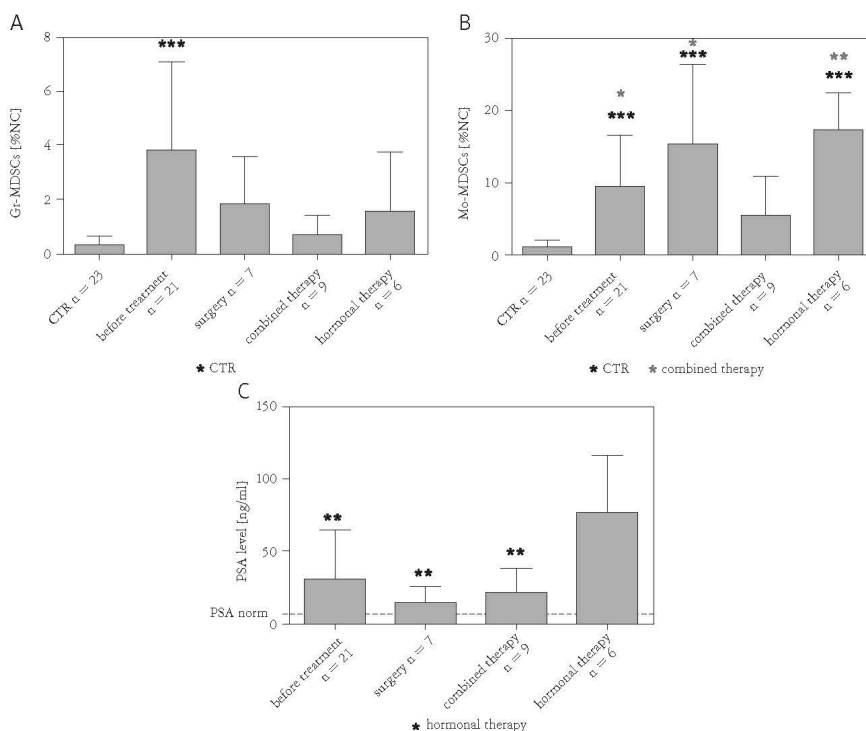


Fig. 6. Gr-MDSCs (A) Mo-MDSCs (B) PSA level (C) in healthy control subjects and prostate cancer patients divided in four groups depending on the treatment option. PSA normal level is indicated

blood, corroborating the previous reports [22, 23]. However, to the best of our knowledge the assessment of the level of MDSC populations in the blood of prostate cancer patients in respect to different forms of their treatment has not been performed so far. In our report we did not show any difference in the eMDSC level between patients and healthy individuals but data on this specific MDSC subset in prostate cancer is still missing in the literature. Although, in many types of cancer, Mo-MDSC mainly settle the tumor whereas Gr-MDSC prevail in the blood [24], in case of prostate cancer this relation is quite opposite, as higher level of Mo-MDSC than Gr-MDSC has been detected in peripheral blood [25]. This observation has been confirmed in our study. One of the first studies on MDSC in prostate cancer indicated that the level of Mo-MDSC in the blood of patients was positively correlated with the PSA level [26]. Our results corroborate this observation, however it is worth noting that the PSA level did correlate neither with the tumor grading

nor with the clinical stage of the patients. It was already shown that the level of Mo-MDSC in peripheral blood of patients with prostate cancer increases with the progression of the tumor [27]. Knowing the role of MDSC in cancer development and progression one would expect that the treatment will reduce the level of these cells [22, 23]. Our results suggest that this is a case only for Gr-MDSC. Despite the treatment option the patients received, we observed significantly lower levels of Gr-MDSC in comparison to untreated group. This however, was not true for Mo-MDSC as their level in the blood of patients post-surgical treatment or after hormonal therapy did not differ significantly from the non-treated group. These results may suggest that surgery or hormonal therapy alone have no effect on the blood level of Mo-MDSC or these forms of treatment can even induce appearance of Mo-MDSC, as it was shown already for radiotherapy [28]. The study by Koga *et al.*, demonstrated that prostate cancer patients with increased level of Mo-MDSC after the treatment had overall

worse survival [29]. Similar pattern, documenting an increased level of Mo-MDSC after therapy we observed also when analyzed blood from patients with colorectal cancer receiving surgery (manuscript in preparation). In case of prostate cancer, only patients receiving a combined treatment showed the level of Mo-MDSC comparable to this observed in a group of healthy donors and significantly lower than in a group before any treatment or receiving surgical or hormonal therapy alone. Although the others indicated a correlation between the level of MDSC and the clinical stage of prostate cancer patients [30], in our studies we did not observe such a connection. The level of Gr-MDSC or even Mo-MDSC was independent of the tumor grading and clinical stage, most likely due to the relatively small study groups. Similar pattern we observed in respect to the PSA level. In this case all patients presented the PSA level higher than the normal range, however no correlation between this parameter and the clinical stage or tumor grading was detected.

Up to now there have been no studies on the level of MDSC populations in the blood of prostate cancer patients in respect to different forms of treatment. Although, our work has a preliminary character and the groups of patients were small, it suggests that single method of treatment is not sufficient for the elimination of circulating MDSC, especially of monocyte origin. If this, however should be replaced by a combined therapy, further prolonged observations, including patients' survival analysis is required.

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The authors declare no conflict of interest.

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Myeloid-Derived Suppressor Cells in Colorectal Cancer

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Colorectal cancer (CRC) remains one of the most common malignancies diagnosed worldwide. The pathogenesis of CRC is complex and involves, among others, accumulation of genetic predispositions and epigenetic factors, dietary habits, alterations in gut microbiota, and lack of physical activity. A growing body of evidence suggests that immune cells play different roles in CRC, comprising both pro- and anti-tumorigenic functions. Immunosuppression observed during cancer development and progression is a result of the orchestration of many cell types, including myeloid-derived suppressor cells (MDSCs). MDSCs, along with other cells, stimulate tumor growth, angiogenesis, and formation of metastases. This article focuses on MDSCs in relation to their role in the initiation and progression of CRC. Possible forms of immunotherapies targeting MDSCs in CRC are also discussed.

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INTRODUCTION

Colorectal Cancer (CRC): Epidemiology and Immunity

According to the World Cancer Research Foundation, colorectal cancer (CRC) (referring to malignancy of colon, rectum, or anus) is the third most common malignancy worldwide. In 2018, more than 1.8 million new cases of CRC were diagnosed (1). About 20–25% of CRC cases are caused by genetic predispositions, including monogenic mutations in mismatched repairing genes associated with, e.g., DNA repair, the cell cycle, and apoptosis (2). Alongside inherited genetic mutations, epigenetic changes also play a significant role in CRC development (3). The remaining 75–80% of cases develop spontaneously and are related to environmental factors such as lack of physical activity, dietary habits, and smoking or alcohol abuse (4). Currently, alterations in the composition of the gut microbiome and its metabolites (playing a role in damaging local tolerance) are also considered as risk factors for CRC (5). An increased risk of CRC is often associated with chronic inflammation of the mucous membrane, which may lead to cell dysplasia, as was proven for patients with inflammatory bowel disease (IBD) (6).

The role of inflammation in CRC development was further supported by data showing that non-steroidal anti-inflammatory drugs (NSAIDs) may decrease the risk of both CRC and colon polyps, which are considered as a premalignant stage (7, 8). The tumor-infiltrating leukocytes (TILs), especially lymphocytes, contribute to the immunoscore classification, where the density of CD3⁺ and CD8⁺ T-cell infiltrate is used as a predictor of anti-tumor response and the prognostic marker in CRC (9, 10). However, further studies have shown that most of the immune cells may actually have a dual activity—anti- and pro-tumor, depending on the signals received from the tumor microenvironment. Interestingly, the so-called myeloid-derived suppressor cells (MDSCs) can switch the polarization of other cells to the status with pro-tumorigenic activity (11).

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

Already in the early 1900s, it was shown that cancer development is often accompanied by extra-medullary hematopoiesis (EMH) and neutrophilia (12). These “fresh” leukocytes were further characterized by suppressive activity and were called immature myeloid cells (ImC) or myeloid suppressor cells (MSC) (13). Eventually, in 2007, their name was changed to MDSCs (13). These cells represent a heterogeneous population of granulocytes and monocytes that rapidly expand during infection, inflammation, and cancer (14, 15). MDSCs, together with the tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs), and regulatory dendritic cells, compose the population of myeloid regulatory cells (MRC), strongly cooperating with each other during cancer development, and progression (16). Based on mouse data, the MDSC population has been divided into two subgroups: of monocyte (Mo-MDSCs), defined as $CD11b^+Ly6G^-Ly6C^{high}$, and polymorphonuclear (PMN-MDSCs), $CD11b^+Ly6G^+Ly6C^{low}$, origin (11, 17, 18). Reflecting MDSC populations already defined in mice, human MDSCs have been described as $Lin^-HLA-DR^-/lowCD11b^+CD14^-CD15^+CD33^+$ for PMN-MDSCs and $Lin^-HLA-DR^-/lowCD11b^+CD14^+CD15^-CD33^+$ for Mo-MDSCs. Very recently, a population of early-stage MDSCs (e-MDSCs) was detected and defined as $Lin^-HLA-DR^-/lowCD11b^+CD14^-CD15^-CD33^+$ (17, 19). As their name suggests, these cells possess immunosuppressive function and help cancer to escape the surveillance of the immune system and support further tumor development (17). Most studies point out that the suppressive role of MDSCs in cancer is associated with the activation of their two enzymes, namely inducible NO synthase (iNOS) and arginase-1 (ARG1) (20–22). These enzymes are responsible for metabolism of L-arginine, which is essential for the proliferation and proper functioning of T cells (23). Moreover, NO and ROS produced in these reactions are involved in the inactivation of the T-cell receptor (TCR), causing a decrease in the expression of CD3 ζ chain and inducing T-cell apoptosis (18, 19, 22).

Expansion and Activation of MDSCs in CRC

It is widely accepted that the level of circulating MDSCs increases in the late stage of cancer, correlating with disease progression and formation of metastases (15, 24–26). However, recently, Ma et al. showed that the MDSC level in circulation also increases in premalignant states, such as colon polyposis (27).

The development of MDSCs is caused by various mediators released under chronic inflammatory conditions, including the release of chemokines (11, 15, 28, 29). One of them that is particularly relevant is CCL2, which contributes to tumor growth, progression, and metastasis development in many tumors, including breast, ovarian, prostate, and CRC (30–33). Previous studies in mice showed that CRC growth could be supported by myeloid cells recruited by the CCL2-CCR2 signaling pathway (33). CCL2 caused accumulation of MDSCs and enhanced their immunosuppressive function during

colorectal carcinogenesis (34). It was also shown that the level of CCL2 increased simultaneously with the progression of CRC (humans), while the deletion of CCL2 led to the reduction of the MDSC level (mouse model) (34). Further, RNS produced by MDSCs may nitrite chemokines, e.g., CCL2 to N-CCL2, which do not attract CD8 $^+$ T cells (like unmodified CCL2 does) but instead recruit myeloid cells, e.g., monocytes (35). On the other hand, several studies documented that CXCL1 is elevated in human CRC (36–38). Further data indicated that CXCR2-positive MDSCs are recruited through CXCR2 ligands, e.g., CXCL1 and CXCL2 are essential for chronic colonic inflammation and colitis-associated tumorigenesis (39).

In addition to chemokines, an important role in the regulation of MDSC activity is attributed to other inflammatory mediators such as histamine and prostaglandins. It has been documented that histamine induces MDSC proliferation and promotes ARG1 and iNOS expression in Mo-MDSCs. At the same time, histamine inhibits the expression of ARG1 and iNOS in PMN-MDSCs, promoting the production of IL-13 and IL-4 (40). Thus, histamine may activate Mo-MDSCs and PMN-MDSCs in different ways (40, 41). Prostaglandin E2 (PGE2), on the other hand, is a strong proinflammatory mediator produced by COX-2 (42) and may activate MDSCs through STAT3 phosphorylation (43, 44). In CRC, persistent STAT3 activation is associated with tumor growth (45, 46) and activation of MDSCs (47, 48). These observations are consistent with the results showing effectiveness of COX-2 inhibitors in the reduction of the MDSC level through blocking COX-2 and subsequent inhibition of the STAT3 pathway (43, 44, 49, 50). Another arachidonic acid metabolite, leukotriene B4 (LTB4), a product of 5-lipoxygenase (5LO), acts as a chemoattractant for MDSCs, leading to their accumulation. Deficiency of 5LO is associated not only with a lowered circulation level of MDSCs but also with decreased activity of ARG1 and iNOS (51).

The tumor microenvironment stimulates MDSCs also by other factors induced by local hypoxia and low pH (52, 53). One of them is hypoxia-inducible factor (HIF). Over-expression of HIF-1 α and also HIF-2 α is associated with poor prognosis in the majority of cancers, including CRC (54). HIF-1 α is associated with increased activity of ARG1 and iNOS in MDSCs, leading to stronger inhibition of T-cell functions (55). Moreover, HIF-1 α can also enhance the suppressive nature of MDSCs by inducing expression of programmed death-ligand 1 (PD-L1) (56), a ligand for PD-1, leading to inhibition of IL-2 production and decreased proliferation of cytotoxic T cells (56, 57). Additionally, HIF-1 α , by binding to a conserved hypoxia response element in the *V-domain of Ig suppressor of T-cell activation* (VISTA) promoter, upregulates VISTA expression on MDSCs, thereby inducing their suppressive activity in the tumor microenvironment (58).

Many studies have shown that not only soluble mediators but also extracellular vesicles, e.g., exosomes secreted by tumor cells, may directly induce MDSC development and modulate their activity (59). This was demonstrated for many malignancies, including melanoma, breast, lung, and CRC (60). The role of cancer exosomes in CRC is complex, based on the type of cargo material transferred from cancer cells to the cells of the immune system, including MDSCs. This may occur through the delivery

of tumor proteins, e.g., FasL (61) and Hsp72 (62), mRNA (63), and non-coding microRNAs (miRNA) (64). The role of miRNA in CRC, in particular, has been documented recently, with an elevated level of miRNA-21 in patients' sera correlating with poor prognosis (65, 66).

MDSC Action in CRC

The suppressive function of MDSCs in CRC is mainly associated with their ability to inhibit T-cell proliferation and to stimulate Treg development (15). One of the important factors involved in interactions between T cells and MDSCs is L-arginine, an amino acid that is essential for T-cell proliferation and proper functioning. MDSCs highly express ARG1, which uses L-arginine, causing its depletion from the microenvironment (21, 22), which in turn affects T-cell functionality. Lack of L-arginine blocks T-cell proliferation and decreases expression of CD3 ζ chain and IFN γ production (67–69). Studies on CRC have shown that MDSCs impair T-cell activation through O $_2^-$ production and iNOS activity (70, 71), which can be reversed by MDSC depletion or the use of iNOS and O $_2^-$ inhibitors (72). The mechanism of ARG1- and iNOS-dependent T-cell suppression has been explained by studies showing that, under conditions where the L-arginine level is reduced due to ARG1 activity, L-arginine is preferentially used by iNOS for O $_2^-$ and NO production, while under normal conditions, where the L-arginine level is high, only NO is produced (73). After mutual reaction of NO with O $_2^-$, a strongly reactive oxidizing agent, peroxynitrite (ONOO $^-$), is formed. It can cause nitration of proteins (74, 75) as well as the induction of T-cell apoptosis through the TCR/CD3 complex tyrosine phosphorylation pathway (22, 76, 77). Recent results have also shown that MDSC level correlates with reduction in the adaptive immune response to tumor antigens, e.g., MUC-1, both by lowering the production of specific antibodies and activation of tumor-specific T cells (27).

The interactions between MDSCs and Tregs in cancer are well-documented. Mainly, the activation of Tregs by MDSCs is caused by cytokines, including IL-10 and TGF- β , where the latter is also associated with MDSC induction (78). However, the relationship between MDSCs and Tregs in CRC is questionable. Some authors indicate that MDSCs in CRC do not induce Tregs development *in vitro* (70). On the other hand, mouse MDSCs were able to induce Tregs *in vitro* and *in vivo* through the IL-10- and IFN- γ -dependent pathway (79).

In addition to the role of MDSCs in immunosuppression that is observed during tumor progression, they may also directly stimulate tumor growth and metastases, inducing, in cooperation with VEGF, angiogenesis. Furthermore, MDSCs may introduce high levels of MMP9 and pro-MMP9 into the extracellular milieu, regulating VEGF bioavailability for colorectal cancer cells (80, 81). At the initial stage of cancer, MDSCs, through TGF- β , can also induce the epithelial to mesenchymal cell transition (EMT) process, which is essential for metastases at the late stage. These cells participate in extracellular matrix degradation in order to prepare distant tissue for receiving metastatic cells (82, 83). The latest findings reveal that PMN-MDSCs also enhance CRC growth by exosomes and exosomal

protein S100A9 in the tumor microenvironment, especially under hypoxic conditions (84).

Both populations of MDSCs can effectively inhibit T-cell activity but using different mechanisms (85, 86). Some authors suggest that Mo-MDSCs are more suppressive than PMN-MDSCs (87), while others show the opposite result (88, 89). PMN-MDSCs are mainly responsible for ROS production, while Mo-MDSCs have high expression of iNOS, producing large amounts of NO, which has a longer activity than ROS. Thus, PMN-MDSCs, in contrast to Mo-MDSCs, need direct cell-to-cell contact to suppress T cells (85, 90). In this context, it has been documented that PMN-MDSCs preferentially settle the peripheral lymphoid organs, while Mo-MDSCs mainly persist in the tumor bed (85). In addition, MDSCs can also downregulate innate immune response, e.g., affecting the activity of NK cells (91). The crosstalk between MDSCs and cells in the CRC microenvironment is summarized in **Figure 1**. According to some authors, in human CRC, a major proportion of the MDSCs in peripheral blood are PMN-MDSCs (86). However, there are also studies showing an increased level of both populations (92–95). Additionally, an e-MDSC population was also detected in CRC patients (27, 96).

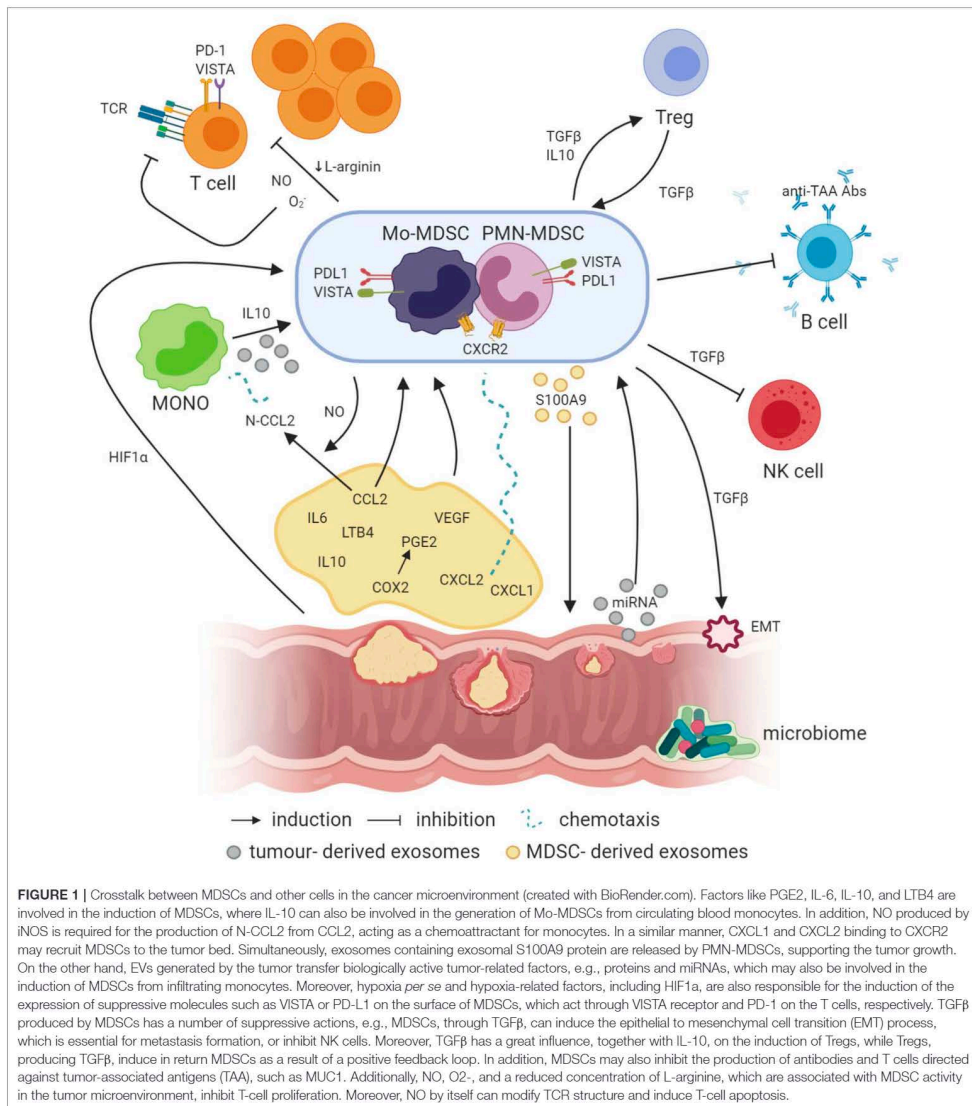
DETECTION OF MDSCs IN CRC

The composition of phenotype markers used for MDSC detection and characterization in CRC quite often differs between studies. The phenotype markers and functional characteristics of MDSCs from various studies on human CRC are presented in **Table 1**. While the majority of the authors agree that the general phenotype of MDSCs is CD11b $^+$ HLA-DR $^-$ Lin $^-$ CD33 $^+$ or functional markers, e.g., iNOS $^+$ and ARG1 $^+$, there is no consensus with respect to more specific markers such as CD14, CD15, PD-L1, or CD124 (IL-4 α R). The recent recommendations of the COST-Mye-EUNITER consortium provide the minimal phenotype characteristics necessary to identify cells as MDSCs: CD14 $^-$ CD11b $^+$ CD15 $^+$ (or CD66b $^+$) for PMN-MDSCs; CD11b $^+$ CD14 $^+$ HLA-DR $^{low/-}$ CD15 $^-$ for Mo-MDSCs, and Lin $^-$ (CD3/14/15/19/56)/HLA-DR $^-$ /CD33 $^+$ for e-MDSCs (17).

TARGETING MDSCs IN CRC

Despite the availability of chemo- and immunotherapy, surgery is still the primary method of CRC treatment. However, in a mouse model, it was shown that surgical removal of tumor mass recruits MDSCs to the peritoneal cavity and promotes tumor progression due to the surgical trauma, downregulating the CXCL4 expression. CXCL4 inhibits tumor growth and angiogenesis, which might be due to its inhibitive impact on the recruitment of MDSCs (97). In this context, it seems that MDSC-targeted therapy is urgently required for this type of cancer.

There are numerous studies concerning different small-molecule compounds that are able to inhibit the suppressive activity of MDSCs. In this section, however, the compounds with potential for CRC treatment are mainly being discussed.



One such is AT38, an inhibitor of RNS, which was used in a mouse model of CRC where it proved to effectively reduce nitration of chemokines, including CCL2. Administration of AT38 also decreased the level of iNOS and ARG1 (35). Another

example is nitroaspirine, which, in a mouse model, increased the number of tumor antigen-specific T cells and reduced both ARG1 and iNOS activity in MDSCs (98). Triterpenoids were also shown to reduce the suppressive functions of MDSCs through

TABLE 1 | The phenotype markers and functional characteristics of MDSCs as published in various studies on human CRC.

Organ/Tumor stage	Phenotype	Suppressive activity	References
Circulating/I-IV	Lin ⁻ HLA-DR ⁻ CD11b ⁺ CD33 ⁺ CD13 ⁺ CD115 ^{low} CD117 ^{low} CD124 ^{low} CD14 ⁻ CD15 ⁻ CD66b ⁻ CD34 ⁺ CD39 ⁺ CD73 ⁻ PD-L1 ^{low} PD-L2 ⁻ PD-1 ⁻	MDSCs correlate with tumor metastasis. Inhibition of CFSE-labeled autologous CD3 ⁺ T cell proliferation at 2:1 ratios with MDSCs in the absence or presence of CD3/CD28 antibody stimulation for 3 days.	(26)
CD33 ⁺ from PBMC were co-cultured with SW480/SW620 cells to induce tumor MDSCs	CD33 ⁺ CD11b ⁺ HLA-DR ⁻ , CD14 ⁺ CXCR4 ⁺ CD39 ⁺ ARG-1 ⁺ iNOS ⁺ ROS ⁺ PD-L1 ⁺ CD73 ⁻ CD117 ⁺ CD34 ⁺ CD66b ⁺ CD15 ^{weak}	Tumor-induced MDSCs promoted SW480 and SW620 cell growth in a co-culture system <i>in vitro</i> . Tumor-induced MDSCs suppressed the proliferation of PBMCs labeled with CFSE more strongly than CD33 ⁺ cells cultured in medium alone.	(70)
Circulating/tumor tissue	CD33 ⁺ CD11b ⁺ HLA-DR ⁻ CD14 ⁺ CXCR4 ⁺ CD39 ⁺ ARG-1 ⁺ iNOS ⁺ PD-L1 ⁺ ROS ⁺ CD73 ⁻ CD117 ⁺ CD34 ⁺ CD66b ⁺ CD15 ^{weak} MDSCs from tumor tissue have higher PD-L1 expression	Advanced disease stage was associated with an elevated level of circulating MDSCs; also, tumor resection reduces the level of circulating MDSCs and Tregs measured 7 days after surgery.	
Circulating/IV	CD14 ⁺ HLA-DR ^{-/low} S100A9 ^{high} iNOS ⁺	–	(71)
Circulating/tumor tissue/III IV	CD124 ⁺ CD14 ⁺ CD124 ⁺ CD15 ⁺ tumor tissue CD15 ⁺ CD14 ⁺	Mixed lymphocyte reactions in which gamma-irradiated PBMC, CD14 ⁺ , CD14 ⁻ , and PMN from CRC patients were added as stimulator to responder PBMC derived from healthy donors. These experiments showed two main subpopulations with suppressive activity present among CD14 ⁺ monocytes in one and among PMN in the other.	(93)
Colorectal tumor/III	PMN-MDSCs CD45 ⁺ Lin ⁻ HLA-DR ⁻ CD11b ⁺ CD33 ⁺ CD66b ⁺ Mo-MDSCs CD45 ⁺ Lin ⁻ HLA-DR ⁻ CD11b ⁺ CD33 ⁺ CD14 ⁺	PMN-MDSCs isolated from tumor inhibited the proliferation of activated autologous CFSE-labeled T cells and IFN- γ production in medium containing CD3 and CD28.	(94)
Circulating	CD33 ⁺ HLA-DR ⁻ CD11b ⁺ CD15 ⁺ CD33 ⁺ HLA-DR ⁻ CD11b ⁺ CD15 ⁻ CD33 ⁺ HLA-DR ^{-/low} CD14 ⁺	Upregulated plasma levels of IL-6 and IL-10, where IL-6 correlates with 15 ⁺ MDSCs and IL-10 with 15 ⁻ MDSCs. Also, CD15 ⁺ and CD15 ⁻ MDSCs correlated with reduced IFN- α responsiveness in CD4 ⁺ T cells.	(95)
Circulating/Metastasis	PMN-MDSCs CD33 ⁺ HLA-DR ^{-/low} CD15 ⁺ CD124 ⁺ PD-L1 ⁺ CD73 ⁺ CD39 ⁺ Mo-MDSCs CD33 ⁺ HLA-DR ^{-/low} CD14 ⁺ PD-L1 ⁺ CD73 ⁺ CD39 ⁺	Accumulation of PMN-MDSCs was associated with poor prognosis; also, PMN-MDSCs have higher levels of PD-L1, CD39, and CD73 expression and a stronger immunosuppressive function than Mo-MDSCs. Reduced TNF- α production and Ki67 proliferation marker of CD3 ⁺ T cells, especially by PMN-MDSCs.	(89)
Circulating/I-IV	CD33 ⁺ CD11b ⁺ HLA-DR ^{-/low} CD15 ⁻ CD14 ⁺ ARG-1 ⁺ CD33 ⁺ CD11b ⁺ HLA-DR ⁻ CD15 ⁺ CD14 ⁺ ARG-1 ⁺⁺	–	(96)
Tumor tissue/I-IV	CD33 ⁺ CD11b ⁺ HLA-DR ^{-/low} CD15 ⁻ CD14 ⁺ ARG-1 ⁺ CD33 ⁺ CD11b ⁺ HLA-DR ⁻ CD15 ⁺ CD14 ⁺ ARG-1 ⁺ CD33 ⁺ CD11b ⁺ HLA-DR ⁻ CD15 ⁻ CD14 ⁻	–	
Circulating	PMN-MDSCs CD14 ⁺ CD33 ⁺ HLA-DR ⁻ CD66b ⁺	Human MDSCs increase fatty acid uptake and expression of FAO-related enzymes, and, in mice, inhibition of FAO blocked the tolerogenic function and immunosuppressive mechanisms of MDSCs. Inhibition of CFSE-labeled CD3 ⁺ T-cell proliferation after co-culturing with MDSCs from mice in the presence of anti-CD3.	(86)
Circulating	Mo-MDSCs CD14 ⁺ HLA-DR ^{-/lo} PMN-MDSCs CD33 ⁺ CD11b ⁺ CD14 ⁻ CD15 ⁺ SSC ^{hi}	Mo-MDSC population was significantly expanded in CRC patients; the immunosuppressive capacity of these cells was evaluated in a T-cell suppression assay using a 3-way allogenic mixed leukocyte reaction (MLR).	(92)
Circulating/cancer and adenoma	Total MDSCs: CD11b ⁺ HLA-DR ^{-/low} CD33 ⁺ PMN-MDSCs: CD11b ⁺ HLA-DR ^{-/low} CD33 ⁺ CD15 ⁺ CD14 ⁻ Mo-MDSCs: CD11b ⁺ HLA-DR ^{-/low} CD33 ⁺ CD15 ⁻ CD14 ⁺ e-MDSCs: CD11b ⁺ HLA-DR ^{-/low} CD33 ⁺ CD14 ⁻ CD15 ⁻	PMN-MDSCs are the main immunosuppressive population, as depletion of CD15 ⁺ cells spares Mo-MDSCs and eliminates most of the suppression of T-cell proliferation and interferon production. MDSC levels negatively correlated with anti-MUC1 IgG levels.	(27)

downregulation of ROS and inhibition of STAT3. However, they did not exert any effects on ARG1 activity, on NO production, or on the frequency of MDSCs (99). In the human CRC, amiloride, normally used to reduce high blood pressure, can also inhibit tumor exosome formation, which has been shown to induce suppressive functions in MDSCs (62). It was also reported that H₂ blockers, e.g., cimetidine, appear to induce apoptosis of MDSCs through a Fas-FasL-dependent pathway (100).

Another therapeutic approach involves the reduction of MDSC expansion by using COX2 or PGE2 inhibitors, as PGE2 production could be associated with MDSC expansion in cancer (43). Such inhibitors, e.g., indomethacin, celecoxib, meloxicam, and acetylsalicylic acid (ASA), were able to reduce tumor growth in various tumor models, including CRC (101–103). This treatment could also modulate MDSC functions by inhibiting ARG1 expression and ROS and NO production (104, 105). ASA also reduced the level of chemokines, including CCL2, a potent chemoattractant for MDSCs (106). Another way to block MDSC accumulation is the inhibition of stem cell factor (SCF), which causes MDSC recruitment when produced in the tumor environment (107).

Another option for targeting MDSCs is inducing their differentiation. For example, curcumin used in a mouse model of CRC was able to decrease the level of PMN-MDSCs and to induce differentiation of Mo-MDSCs into cells with M1-like phenotype (108).

Another strategy for potential MDSC-targeted therapy was suggested by Condamine et al. who pointed to a shorter lifespan for MDSCs compared with neutrophils and monocytes (109). This was associated with their increased apoptosis rate in the periphery, related to high expression of *TNF-related apoptosis-induced ligand* receptors (TRAIL-Rs) due to the stress in endoplasmic reticulum (ER) occurring under pathophysiological conditions like cancer. Thus, targeting TRAIL-Rs by selective agonists can be considered as a future therapy for reducing MDSC activity and number (109).

Immunotherapy designed to target the checkpoint inhibitors of the PD-1–PD-L1 pathway is currently one of the most promising possibilities for reducing MDSC activity. Currently, four monoclonal antibodies are already approved by the FDA for the inhibition of this pathway: anti-PD-1 nivolumab and pembrolizumab, and anti-PD-L1 atezolizumab and avelumab. These inhibitors and several other checkpoint modulators are under clinical investigation for CRC treatment (110). In the clinical studies, nivolumab and pembrolizumab showed good response rates of 26 and 57%, respectively (111). Better results were obtained in the case of nivolumab combined with ipilimumab (anti-CTLA-4) (111–113). However, in the context of MDSCs, more satisfactory results were obtained where the PD-L1 inhibitor was used (56). Recently, several chemotherapeutic agents, e.g., gemcitabine, 5-fluorouracil, and doxorubicin, which are used in conventional cancer chemotherapy have been found to reduce MDSC numbers through the induction of apoptosis in tumor tissues as well as in the peripheral lymphoid organs

(114–116), and combining these agents with immunotherapy improved survival of tumor-bearing hosts. In keeping with this, Limagne et al. in their study, provided a clinical rationale for combining chemotherapy with anti-PD-1/PD-L1 antibodies for more effective reduction of the immunosuppression caused by PMN-MDSCs in metastatic CRC (89). In this context, FOLFOX (5-fluorouracil + oxaliplatin) chemotherapy was shown to act synergistically with anti-PD-1 (117).

In the context of immunotherapy, it is worth mentioning the heterogenic genetic composition of CRC, which has important therapeutic implications. The effectiveness of immunotherapy, particularly immune checkpoint inhibition therapy, such as CTLA-4 and PD-1, has been confirmed in mismatch-repair-deficient (dMMR) and microsatellite instability-high (MSI-H) (dMMR-MSI-H) tumors, while it was ineffective in mismatch-repair-proficient (pMMR) and microsatellite instability-low (MSI-L) (pMMR-MSI-L) tumors (118). This resistance for immunotherapy of MMR-MSI-L tumors results from the inability of immune cells to recognize MSI-L mutated tumor cells and thereby reduced T-cell infiltration (119). However, it was noticed that pMMR-MSI-L tumors are more extensively infiltrated by Tregs and MDSCs than dMMR-MSI-H, which may also explain the poor immune response (120). Thus, to use of MDSC-targeted therapy seems to be a beneficial opportunity to assist the effectiveness of surgery in patients with pMMR-MSI-L cancer.

CONCLUSIONS

Tumor develops a variety of mechanisms to escape from immune system surveillance, including the generation of MDSCs. There is substantial evidence that MDSCs are involved in CRC development and progression. MDSCs can be detected both in the peripheral blood and tumor tissue; however, it is not known if both or one of them are relevant for predicting the prognosis for patients in the clinic. Therefore, more in-depth investigation of the mechanisms of MDSC actions in the tumor bed is still needed. Finally, more advanced pharmacological data on specific treatments targeting MDSCs are required. This could significantly improve the effectiveness of the treatment of CRC patients, and also those with pMMR-MSI-L tumors, who respond poorly to current forms of immunotherapy.

AUTHOR CONTRIBUTIONS

IS wrote the paper. JB critically revised the paper. All authors contributed to the article and approved the submitted version.

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

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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) n = 27	Metastatic ^c UICC (III+IV) n = 27
Characteristic		n = 54		
Age ^b (years)	64±13	65±11.7	63.1 ± 14.3	
Gender				
	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), guanylate cyclase C (GUCY2C, GGC), and HER-2/neu [13,14]. In this context we asked whether MDSCs correlates with HLA-A₂ HER-2/neu₃₆₉₋₃₇₇ 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-DR^{low}/CD33⁺CD11b⁺CD15[−]CD14⁺ cells, whereas PMN-MDSCs, as LIN[−] HLA-DR^{low}/CD33⁺CD11b⁺CD15⁺CD14[−] cells, and e-MDSCs as LIN[−] HLA-DR^{low}/CD33⁺CD11b⁺CD15[−]CD14[−], all presented as a percent value of nucleated cells (NC) – cells positive for staining with SYTOTM41 (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-17A⁺ respectively, and their level was presented as percent value from CD4⁺ population.

HLA-A₂ typing

PBMC (1×10^6 /test) were incubated (20 min at 4 °C) with mouse anti-HLA-A₂-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-A₂ expression were selected to the next stage, where CD8⁺ T cells specific for HER-2/neu₃₆₉₋₃₇₇ (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-A₂ pentamer complex (Pro5Pentamer; ProImmune, Oxford, UK) folded around the HER-2/neu₃₆₉₋₃₇₇ specific epitope, as described previously [17]. As a negative control, staining with an HLA-A₂ Negative Control Pentamer (ProImmune) was used. To minimize a non-specific staining, each pentamer was titrated 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.

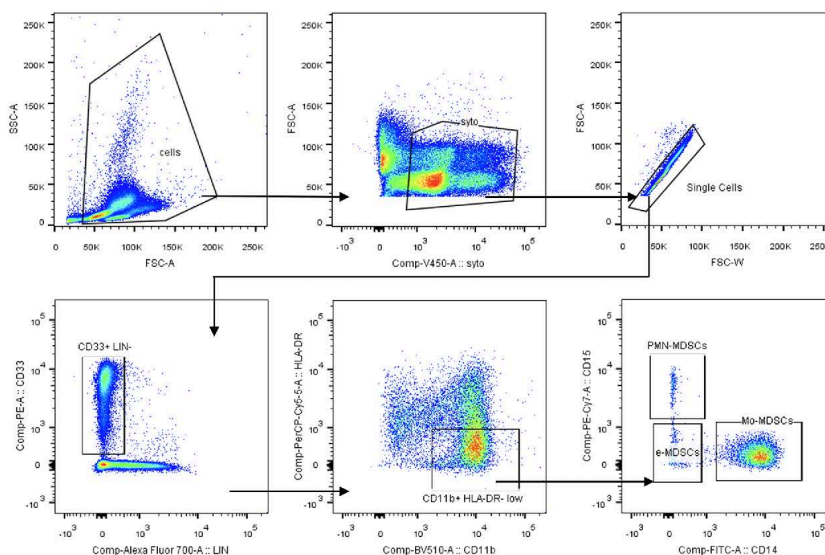


Fig. 1. Gating strategy for MDSCs population analysis.

The myeloid cells were gated as LIN⁺CD33⁺ from nucleated cells (SYTO41-positive) and single cells, then HLA-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-DR^{low}/CD33⁺CD66b⁺CD14⁺, Mo-MDSCs as LIN⁺HLA-DR^{low}/CD33⁺CD66b⁺CD14⁺, and e-MDSCs as LIN⁺HLA-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 ³H-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 CD33⁺ 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 ³H-thymidine (1 μCi/well; Hartmann Analytic, Braunschweig, Germany) and the β-minus radiation was measured in a liquid scintillation counter (Beckman Coulter LS1801, Inc., Mississauga, 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.

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

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'- CAGCGGGATGACTTTCGAAG AGGCAA-
GATTGGACCTGCA-3'

Gene expression was assessed by using the 2^{-ΔCt} 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.

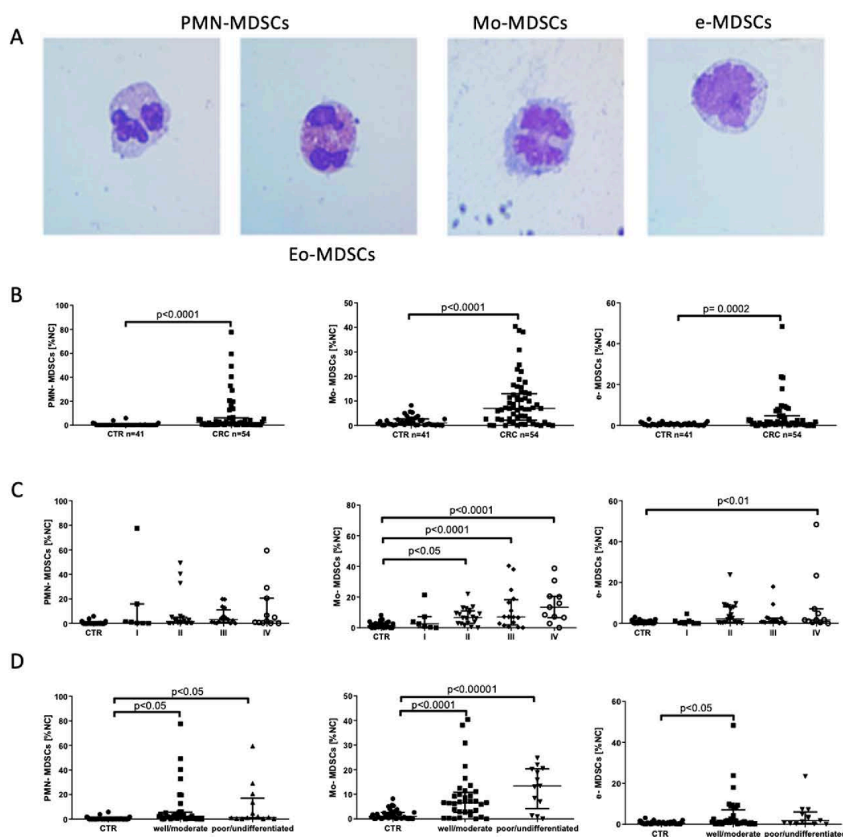


Fig. 2. MDSCs subset distribution in patients with CRC.

(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 $\text{LIN}^{-}\text{CD33}^{\text{HLA-DR}}/\text{low}\text{CD66b}^{+}\text{CD14}^{-}$, Mo-MDSCs as $\text{LIN}^{-}\text{CD33}^{+}\text{HLA-DR}}/\text{low}\text{CD66b}^{-}\text{CD14}^{-}$, and e-MDSCs as $\text{LIN}^{-}\text{HLA-DR}}/\text{low}\text{CD33}^{+}\text{CD66b}^{-}\text{CD14}^{-}$ 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

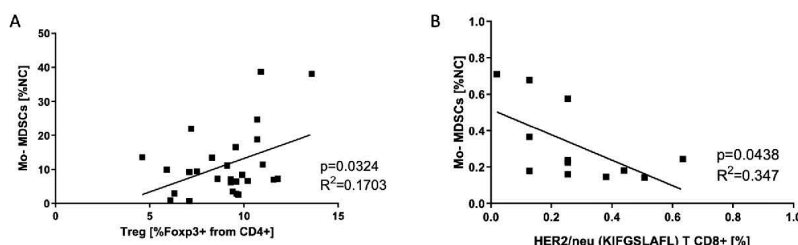


Fig. 3. The relationship of Mo-MDSCs with T cell subpopulations.

(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-A₂ HER-2/neu₃₆₉₋₃₇₇ 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-A₂ 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-A₂ HER-2/neu₃₆₉₋₃₇₇ (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 H³-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 H³-thymidine, 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 $13.5 \pm 11.01\%$. 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

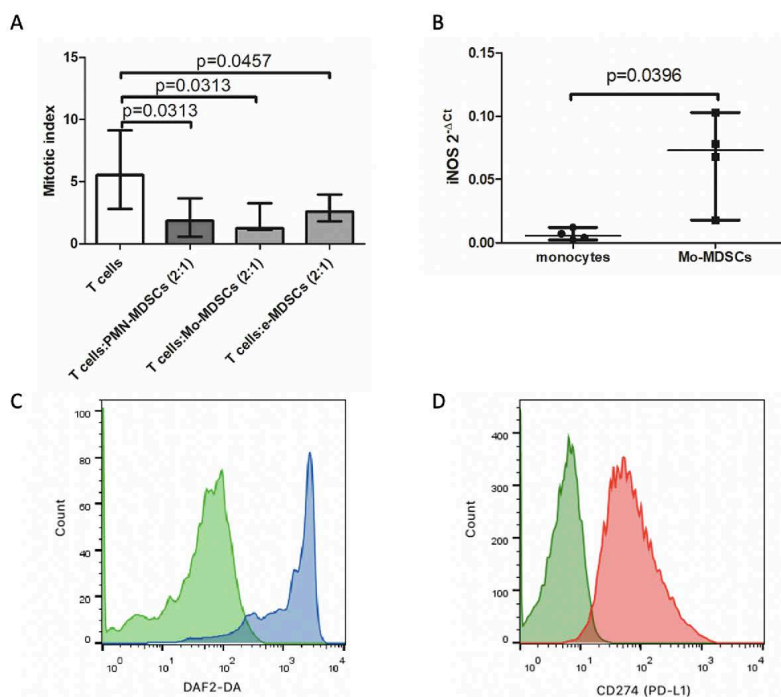


Fig. 4. Suppressive activity of MDSCs subsets. iNOS expression and NO production by Mo-MDSCs from CRC patients and expression of PD-L1 on Mo-MDSCs depending on their localization

(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-DR^{low/-}CD33⁺CD66b⁺CD14⁺; Lin⁺CD33⁺HLA-DR^{low/-}CD66b⁺CD14⁺; Lin⁺CD33⁺HLA-DR^{low/-}CD14⁺CD15⁺ cells from PBMCs. MDSCs subsets were added to the culture of FACS purified autologous CD33⁺ T cells with 10% of autologous monocytes (sorted as CD14⁺HLA-DR⁺ cells). After 3 days of co-culture, T-cells were pulsed with ³H-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-DR^{low/-}CD66b⁺CD14⁺ and CD14⁺HLA-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-DR^{low/-}CD15⁺CD14⁺ in PBMC and CD45⁺Lin⁺HLA-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

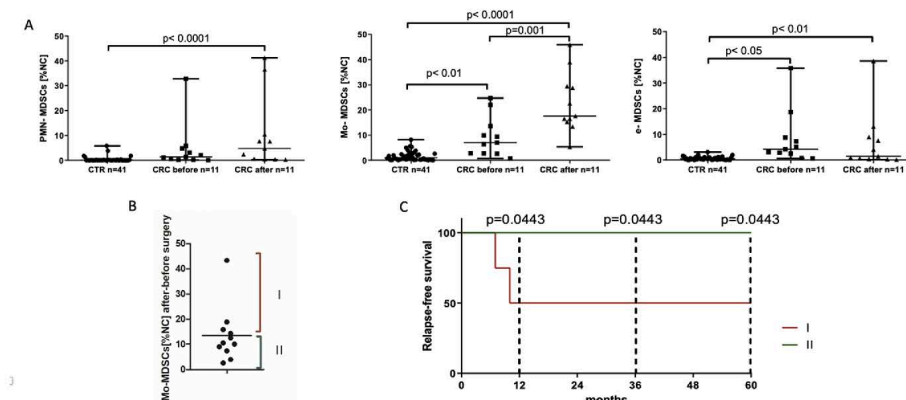


Fig. 5. MDSCs level before and after surgery treatment in relation to the CRC relapse-free time.

(A) Frequency of PMN-MDSCs, Mo-MDSCs and e-MDSCs 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-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 Czerwinska:** Methodology. **Radosław Pach:** Methodology. **Mateusz Rubinkiewicz:** Investigation, Methodology. **Antoni Szczepaniak:** 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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Graphical abstract created with BioRender.com.

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

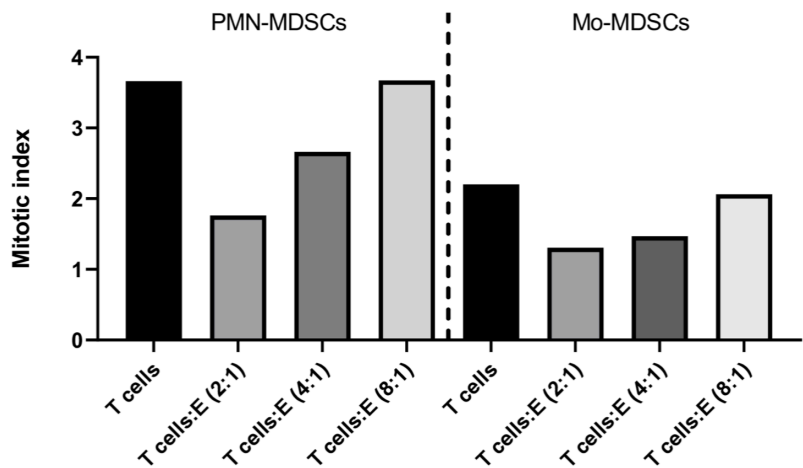
Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2022.101346](https://doi.org/10.1016/j.tranon.2022.101346).

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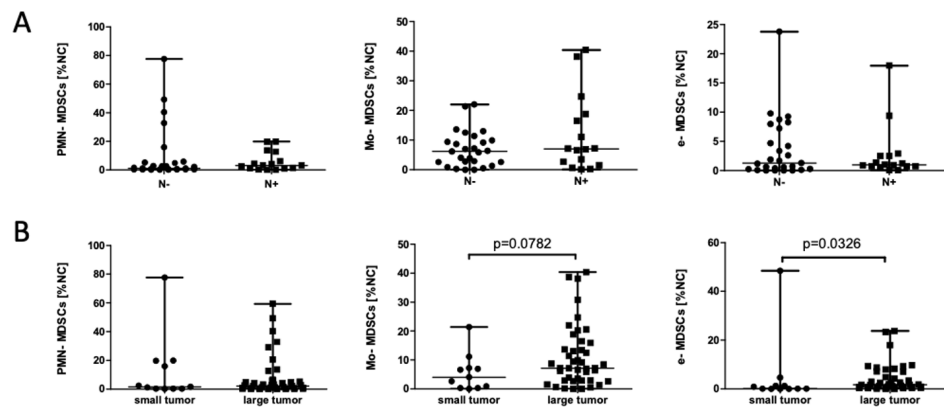
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Supplementary data

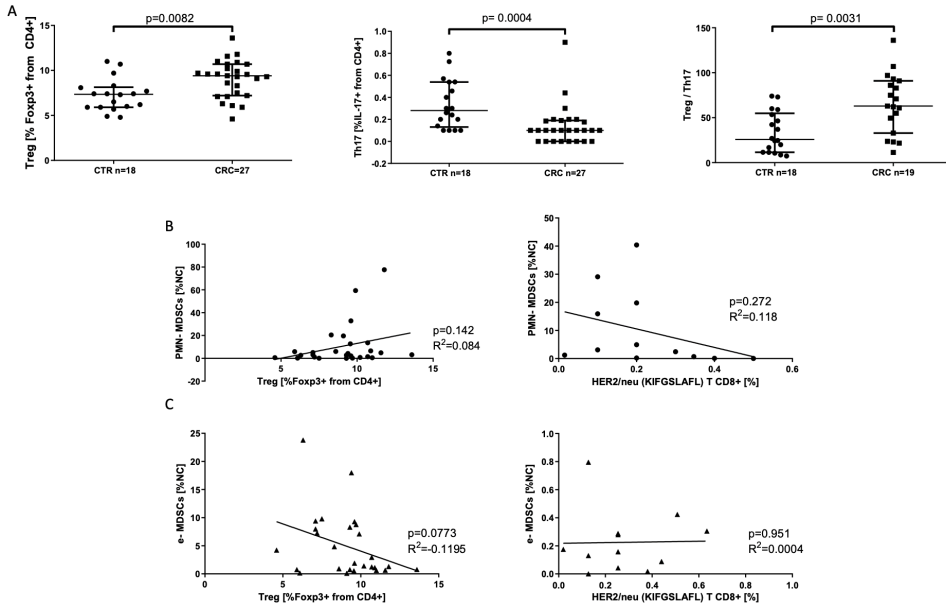


Supplementary Figure 1. Determination of optimal T cell to MDSCs (effector cells - E) ratio for proliferation assay. The subsets of MDSCs were sorted by FACS as Lin⁻HLA-DR^{low/-}CD33⁺CD66b⁺CD14⁻ (PMN-MDSCs) and LIN⁻CD33⁺HLA-DR^{low/-}CD66b⁺CD14⁺ (Mo-MDSCs) from PBMCs and added in different ratio to the cultures of FACS purified autologous CD3⁺ T cells with 10% of monocytes (sorted as CD14⁺ HLA-DR⁺ cells), stimulated with PHA. After 3 days of co-culture, T-cells were pulsed with H³-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 culture [cpm] to non-stimulated culture [cpm].



Supplementary Figure 2. MDSCs subset distribution in patients with CRC.

- (A) Level of PMN-MDSCs, Mo-MDSCs and e-MDSCs in relation to occurrence of local lymph node metastases (w/o n=27 vs. with n=16).
- (B) Level of PMN-MDSCs, Mo-MDSCs and e-MDSCs in relation to the tumor size (small = T1+T2; n=11 vs. large = T3 + T4; n=43).



Supplementary Figure 3. Correlation of Treg, Th17 and CD8⁺ T cells specific for Her2/neu frequency with the level of PMN-MDSCs and e-MDSCs in CRC patients.

- (A) Treg, Th17 and Treg/Th17 ratio in CRC and CTR. Treg and Th17 cells were analyzed by flow cytometry from lymphocytes as CD4⁺Foxp3⁺ and CD4⁺IL-17A⁺ cells, respectively.
- (B) Correlation of PMN-MDSCs and e-MDSCs level with Treg. PMN-MDSCs and e-MDSCs were identified in peripheral blood by flow cytometry, as described in “Materials and methods”. PMN-MDSCs and e-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).
- (C) Correlation of PMN-MDSCs and e-MDSCs level with CD8⁺ T cells specific for Her2/neu (n=12).

Supplementary Table 1. Monoclonal antibodies and isotype controls used in the study.

Antibody		Vendor	Clone	Fluorochrome
anti-LIN	CD3	BD Biosciences; San Jose, CA	UCHT1	AF-700 (Alexa Fluor-700)
	CD19			
	CD56			
anti-CD3		BD Biosciences	UCHT1	APC (Allophycocyanin)
anti-CD33		BD Biosciences	P67.6	PE (Phycoerythrin)
anti-HLA-DR		BD Biosciences	L243	PerCP (Peridinin-Chlorophyll-Protein)
anti-CD11b		BioLegend, San Diego, CA	ICR F44	BV510 (Brilliant Violet 510)
anti-CD14		BD Biosciences	MφP9	FITC (Fluorescein isothiocyanate)
anti-CD14		BioLegend	63D3	APC-CY7 (Allophycocyanin-Cyanine7)
anti-CD15		BioLegend	HI98	PE-CY7 (Phycoerythrin-Cyanine7)
anti-CD66b		BD Biosciences	G10F5	FITC
anti-CD274		BD Biosciences	MIH1	APC
Mouse IgG1, κ		BD Biosciences	MOPC-21	APC
Mouse IgG1, κ		BioLegend	MOP-173	APC
Mouse IgG1, κ		BD Biosciences	MOPC-21	AF-700
Mouse IgG1, κ		BD Biosciences	MOPC-21	PE
Mouse IgG2a, κ		BD Biosciences	X39	PerCP
Mouse IgG1, κ		BD Biosciences	X40	BV510
Mouse IgG2a, κ		BD Biosciences	27-35	FITC
Mouse IgG2a, κ		BD Biosciences	27-35	APC- CY7
Mouse IgG1, κ		BD Biosciences	G155-228	PE-CY7
Mouse IgM, κ		BD Biosciences	G10F5	FITC

Supplementary Table 2. Clinical characterization of the patients included in a 5-year follow-up after surgery.

Group		I	II
Characteristic		n=4	n=7
Age ^b (years)		65.2±7.9	69.1±8.7
Gender	male	2	4
	female	2	3
TNM stage ^c	I	-	-
	II	2	4
	III	2	3
	IV	-	-
Tumor site ^d	proximal	-	5
	distal	4	2
Histological grade	well/moderate	3	4
	poor/undifferentiated	1	2
	undefined	-	1

^aCRC with limited disease corresponds to stage I and II, and those with metastatic disease corresponds to stages III and IV.

^bMean ± SD.

^cStaging based on TNM classification of CRC according to UICC.

^dTumor site was classified as proximal or distal to the splenic flexure.

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Prostate cancer (PC) is the second most often diagnosed malignancy in men and one of the major causes of cancer death worldwide. Despite genetic predispositions, a crucial role in PC development plays environmental factors, including a high-fat diet, obesity, sedentary lifestyle, infections of the prostate, and exposure to chemicals or ionizing radiation. Moreover, due to a lack of, or insufficient T cell infiltration and its immunosuppressive microenvironment, PC is frequently classified as a "cold" tumor. This is related to the absence of tumor-associated antigens, lack of T cell activation and their homing into the tumor bed, and the presence of immunological cells with regulatory functions, including myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg), and tumor-associated macrophages (TAMs). All of them by a variety of means hamper anti-tumor immune response in the tumor microenvironment (TME), stimulating tumor growth and formation of metastases. Therefore, they are emerging as potential anti-cancer therapy targets. This article is focused on MDSCs concerning their function and role in the initiation and progression of PC. Clinical trials directly targeting this cell population or affecting its biological functions, thus limiting its pro-tumorigenic activity, are also presented.

Keywords: prostate cancer, myeloid-derived suppressor cells, immunosuppression, immunotherapy, Anti-tumor immune response

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Myeloid-derived suppressor cells as key players and promising therapy target in prostate cancer

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Abstract

Prostate cancer (PC) is the second most often diagnosed malignancy in men and one of the major causes of cancer death worldwide. Despite genetic predispositions, a crucial role in PC development plays environmental factors, including a high-fat diet, obesity, sedentary lifestyle, infections of the prostate and exposure to chemicals or ionizing radiation. Moreover, due to a lack of, or insufficient T cell infiltration and its immunosuppressive microenvironment, PC is frequently classified as a "cold" tumor. This is related to the absence of tumor associated antigens, lack of T cell activation and their homing into the tumor bed and the presence of immunological cells with regulatory functions, including myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg) and tumor-associated macrophages (TAMs). All of them by a variety of means hamper anti-tumor immune response in the tumor microenvironment (TME), stimulating tumor growth and formation of metastases. Therefore, they are emerging as potential anti-cancer therapy targets. This article is focused on MDSCs concerning their function and role in the initiation and progression of PC. Clinical trials directly targeting this cell population or affecting its biological functions, thus limiting its pro-tumorigenic activity, are also presented.

1 Prostate cancer – epidemiology.

PC is the most common, after lung cancer, malignancy in men - in 2020 more than 1.4 million new cases of PC were diagnosed worldwide (1,2). Advanced age, race, ethnicities such as African descent and family history are well-established risk factors of PC (3–6). Additionally, a higher incidence of PC has been associated with a diet rich in saturated animal fat and red meat, low intake of fruits/vegetables, obesity, hyperglycemia, lack of physical activity, prostate inflammation, but also exposure to chemicals or ionizing radiation (6–8). Most common genetic predispositions for PC development are related to aberrations of the *PTEN* tumor suppressor gene. Inactivation of *PTEN* by deletion or mutations is identified in ~20% of primary PC and as many as 50% of advanced castration-resistant tumors (9). The role of the immune system and prostatitis in PC development was also confirmed, indicating that inflammatory mediators may promote prostatic carcinogenesis *via* inhibition of apoptosis, promotion of cell proliferation and even loss of the tumor suppressor genes (10). Importantly, not only the local, prostate inflammation, but also systemic reaction associated with chronic inflammatory diseases, including asthma and allergies, are associated with the higher risk of PC (11).

Most of the patients develop a low-risk neoplasm (12), however, approximately 15% of men with localized PC present high-risk tumors, which will progress, metastasize, and finally will result in death (13). In men with advanced metastatic prostate cancer (mPC), hormonal - androgen deprivation therapy, is a method of choice with a good response rate. In some patients, however, the mPC will evolve to metastatic castration-resistant prostate cancer (mCRPC) (14). While a radical prostatectomy may be beneficial for patients with high-risk PC (15), only multimodal treatment, including surgery, radiation and systemic therapy gives the best chance for a

long-term progression-free outcome (13). Nowadays, immunotherapy options, including anti-PC vaccines, e.g. Sipuleucel-T (Provenge), and the use of immune checkpoint inhibitors (anti-CTLA-4 and anti-PD-1/PD-L1 monoclonal antibodies or antagonists), further improve the effectiveness of the PC treatment (16).

PC is often considered a "cold" tumor, meaning that due to the reduced or complete lack of T cell infiltration, e.g. because of the missing tumor associated antigens, lack of T cell activation and their homing into the tumor bed and local immunosuppression - it does not trigger a strong immune response. This term emphasizes the role of the immune system in PC progression (16,17). Studies indicate that regulatory T cells (Tregs) and other cell populations, namely the myeloid-derived suppressor cells (MDSCs; attracted to TME by low-grade chronic inflammatory signals) and tumor-associated macrophages (TAMs) (17) are mainly responsible for the immunosuppression observed in PC (18). Among them, MDSCs are emerging as potential therapeutic targets (19).

2 Myeloid-Derived Suppressor Cells (MDSCs) – their origin and activity

The term "myeloid-derived suppressor cells" has been used in the literature since 2007, however, the history of these cells dates back to the early 20th century, when it was shown that cancer is often accompanied by extramedullary hematopoiesis (EMH) and neutrophilia (20,21). These immature leukocytes were further characterized by their suppressive activity and called myeloid suppressor cells (MSC) (22). This term was further changed to MDSCs (22), and although current the progress in resolution techniques, including a high-dimensional single-cell analysis, have raised concerns regarding the development and activation state of MDSCs secretion (23), it is still accepted that MDSCs represent a heterogeneous population of immature myeloid cells, promptly expanding during pathological conditions, including infection, inflammation, and cancer (24). With respect to their origin, MDSCs have been divided into two main subsets – monocytic (Mo-MDSCs) and granulocytic or polymorphonuclear (PMN-MDSCs). Recently, a third population of so-called early-stage MDSCs (e-MDSCs) was also described (25). In cancer, the accumulation of MDSCs is inseparably related to the production of pro-inflammatory mediators by the tumor microenvironment (TME), which activate and drive their suppressive activity (26). The immunosuppressive mechanisms developed by MDSCs are diverse and may include arginase-1 (ARG1) and inducible nitric oxide synthase (iNOS) activity, secretion of TGFβ, IL-10, cyclooxygenase-2 (COX-2) and depletion of tryptophan by indoleamine 2,3-dioxygenase (IDO) (27). Although the immunosuppressive nature and the induction of antigen-specific T cell tolerance, is common for all the MDSCs subsets (28), they differ in the mechanism of action. In this context, Mo-MDSCs suppress T-cell response in both antigen-specific and unspecific manner, utilizing the mechanisms associated with iNOS activity and production of nitric oxide (NO) (29,30). On the other hand, PMN-MDSCs suppress immune response primarily in an antigen-specific manner, using the STAT3-mediated mechanisms of NADPH-oxidase and ARG1 activities (31). PMN-MDSCs store ARG1 in the granules and release it to the extracellular milieu, leading to the local depletion of L-arginine, affecting T-cell functionality. Both MDSCs subsets release ROS, which are essential for their immunosuppressive activity, and for retaining their undifferentiated status. Numerous studies confirmed the interplay between chronic inflammatory factors and expansion of MDSCs (24,32). A central role in the generation and functioning of MDSCs plays the transcription factor STAT3 (33–35). Various cytokines, including IL-6, IL-1β, IL-10, GM-CSF and VEGF, secreted mainly in the TME by tumor cells (26), are involved in the activation of pSTAT3. On the other side, chronic inflammation is associated with the initiation and progression of the tumor (10). In this context, chemokines and their receptors, e.g. CCL2/CCL12-CCR2, CXCL5/2/1-CXCR2, CCL3/4/5-CCR5, CCL15-CCR1, CXCL8-CXCR1/2, are relevant for a rapid progression of PC and the recruitment of MDSCs (36,37). Prostate cancer patients were shown to have higher MDSCs infiltration than those with a benign prostate hyperplasia (38). Therefore, the role of inflammation in the development and expansion of MDSCs, and hence in PC progression, is unquestionable.

3 Expansion of MDSC in PC

Studies with the use of *PTEN* KO murine PC model documented that lack of this gene was associated with upregulated inflammatory response (enhanced production of CSF-1 and IL-1β), and an extensive MDSCs tumor infiltration (39). Another mechanism involved in the recruitment of MDSCs in PC could be linked to the Hippo–

YAP signaling. This pathway, relevant for the regulation of cell proliferation and apoptosis, is often deregulated in human solid tumors and associated with enhanced cancer cell proliferation (40). In PC, the hyperactivated Hippo–YAP signaling causes the upregulation of CXCL5 in cancer cells, which promotes the MDSCs recruitment *via* CXCL5–CXCR2 axis (41,42). The recruitment of MDSCs to the tumor mass may also benefit from the tumor-related hypoxia. This is supported by the observation, that the hypoxia-targeted therapy may lead to a long-lasting decrease in the accumulation of MDSCs in the tumor (43). A significant role in the recruitment of MDSCs to PC has been also assigned to chromodomain helicase DNA-binding protein 1 (CHD1), an essential tumor suppressor (44). Its depletion was found in 29.7% of cases in African Americans, and 11.0% of European PC patients (45). It has been shown that CHD1 deficiency may recruit MDSCs *via* IL-6 dependent mechanism (46). Interestingly, a positive correlation between CHD1 and CD15 expression (a surface marker of PMN-MDSCs) in PC was also documented (46).

A growing list of evidence suggests that miRNA carried by tumor-derived extracellular vesicles (TEVs) may also play a role in the generation of MDSCs in many types of cancer (47-49). Although there is no data confirming such a role of EVs miRNA in PC, some miRNAs already shown as relevant in the induction of MDSCs in other cancers, have been also considered for PC (50).

The crosstalk between MDSCs and the TME in PC has been schematically presented in Figure 1.

4 Role of MDSCs in PC development and progression

In various cancers, the level of tumor infiltrating MDSCs has been proposed as a prognostic marker (51,52). In PC, however, such data are scarce and refer mainly to the *PTEN* mouse model (39). On the other hand, there are observations that the MDSCs blood level could be a useful parameter for monitoring the disease burden in PC, allowing to distinguish between metastatic cancer, localized PC and cancer-free men (53). Additionally, circulating MDSCs correlates well with PSA level and metastasis (33,54). The pivotal role of MDSCs in the development and progression of PC was further confirmed in randomized clinical studies showing that the increased level of MDSCs after the treatment is associated with the overall worse patients' survival (55,56). Moreover, in a mouse model of PC, the lung infiltration by MDSCs was associated with the formation of lung metastases (57). However, what type of MDSCs subpopulation is pivotal and prevalent in PC remains controversial, mainly due to the lack of reproducibility and standardization of such research. The work showing MDSCs as a negative prognostic marker in mCRPC indicates only blood Mo-MDSCs as relevant (58). Furthermore, in patients with mCRPC, a positive correlation between Mo-MDSCs and Treg cells has been described (58), suggesting a mutual positive feedback loop (59). Generally, most of the studies in PC have been focused on Mo-MDSCs rather than on PMN-MDSCs (55,58,60). Even early reports on circulating immunosuppressive cells in patients with PC were concentrated on CD14+HLA-DR^{low} monocytes (54). This may result from the fact that Mo-MDSCs are more frequent in peripheral blood than PMN-MDSCs (61,62). Another reason could be the fact, that in many studies a cryopreserved material was used (63), affecting the recovery of PMN-MDSCs (64). Recently, Wen et. al. documented infiltration of the primary prostate tumor by cells referred to as PMN-MDSCs (65), however, the markers used for their identification did not allow to distinguish them from the population of tumor-associated neutrophils (TANs) (25). In this context, the phenotype definition of circulating blood PMN-MDSCs seems more reliable but, even though, this should be further confirmed by functional tests, documenting the immunosuppressive nature of these cells (25). Studies in PC showed that Mo-MDSCs and PMN-MDSCs are transcriptomically different (61), pointing out the ARG1 as typical for PMN-MDSCs (66) and iNOS or IDO for Mo-MDSCs (58,60). Moreover, PMN-MDSCs can exert their immunosuppressive action also by the release of neutrophil elastase (NE), which was shown to stimulate the proliferation, migration, and invasion of cancer cells both *in vitro* and *in vivo* in a mouse model of PC (67,68).

It is proposed that in PC the tumor-infiltrating PMN-MDSCs express up-regulated IL-1 β and IL-23a (66). Although the IL-1 β restrained antitumor immunity was described before for other tumors (69), the secretion of IL-23 by PMN-MDSCs, so far has been documented only for PC. In this context, it was shown that IL-23 preserves the androgen receptor's (AR) functionality, enabling survival and proliferation of PC in the androgen-deprived environment. The same mechanism is postulated as a driving force in the development of castration

141 resistance (40). However, castration resistance may also be related to the secretion of IL-8 and subsequent tumor
142 infiltration by PMN-MDSCs (66).

143 5 Targeting MDSCs in PC

144 Due to a lack of, or insufficient T cell infiltration and immunosuppressive microenvironment in PC, there is a
145 need to design new therapies that could "turn up the heat on the cold immune microenvironment" (17), to
146 enhance the local anti-tumor immune response (16). Radiation *per se* has been found to activate the immune
147 response (70), however, studies using the animal models of PC revealed that radiotherapy induces a rapid
148 increase in the tumor infiltrating MDSCs (71). Our previous studies showed that surgery or hormonal therapy
149 alone did not reduce the level of circulating Mo-MDSCs in PC patients (62). In this context, in addition to the
150 standard treatment, immunotherapy (72) or dietary strategies (73) are implemented, targeting cells with
151 immunosuppressive potential, including MDSCs. One of the major challenges in targeting human MDSCs is
152 their heterogeneous nature, e.g. differences in phenotype and mechanisms of suppression. A kind of a "universal"
153 approach, covering the above aspects, may be the use of Gemtuzumab ozogamicin, a calicheamicin-conjugated
154 anti-CD33 humanized monoclonal antibody, already approved to treat a subset of patients with acute myeloid
155 leukemia, which has also been highly effective against MDSCs in many solid tumors, including PC *in vitro* (61).
156 Clinically, MDSCs may be targeted by different approaches, including e.g. inhibition of MDSCs expansion,
157 MDSCs depletion, induction of their differentiation, functional inhibition or multifactorial treatment. The
158 clinical trials concerning all these potentially therapeutic strategies in PC have been described below.

159 5.1 Inhibition of MDSCs expansion

160 Currently there are three registered clinical trials, aiming at the inhibition of MDSCs expansion in PC. As
161 mentioned, chemokines and their receptors are pivotal for the recruitment of MDSCs and rapid progression of
162 PC (36,37), therefore targeting the chemokine receptors or the use of chemokine inhibitors seems to be a
163 promising form of immunotherapy in PC (74). One of the ongoing clinical trials (NCT03177187) seems to be
164 verifying this hypothesis by using the CXCR2 antagonist (AZD5069) in combination with enzalutamide - the
165 androgen receptor's antagonist in patients with mCRPC (75). An important additional factor associated with
166 MDSCs expansion is VEGF (26), thus administration of cabozantinib (small molecule inhibitor of tyrosine
167 kinase receptor, including the VEGF pathway), followed by radical prostatectomy vs. prostatectomy alone
168 (NCT03964337) is being tested in men with high-risk PC. Moreover, cabozantinib has already shown inhibitory
169 effects on MDSCs (76). Another trial concerning dietary intervention, NCT03654638, is focused on soy bread,
170 containing isoflavones, which were shown to reduce the level of pro-inflammatory cytokines and MDSCs (77).

171 5.2 MDSCs depletion

172 MDSCs isolated from both mice and humans display elevated levels of STAT3, while inhibition of its pathway
173 resulted in enhanced antitumor activity (28,78). Circulating Mo-MDSCs maintain high levels of STAT3 until
174 they reach the tumor, where hypoxia induces its rapid downregulation, causing differentiation of MDSCs to
175 TAMs (79). STAT3 regulates the expression of the main factors of MDSCs activity, e.g. IDO, ARG1, IL-6, IL-
176 10, IL-1 β , VEGF among others, suggesting this pathway as an attractive therapeutic option (26). In this context,
177 a fungal-derived pSTAT3 inhibitor - galiellalactone, was recently assessed for its ability to prevent PC-induced
178 generation of MDSCs *in vitro* (53). In keeping, the clinical trial, NCT03709550, aiming at testing decitabine (5-
179 aza-2'-deoxycytidine), a hypomethylating agent with the ability to selectively deplete Mo-MDSCs, in mCRPC
180 patients was implemented (80).

181 5.3 Inhibition of MDSCs differentiation

182 Another therapeutic option involves a controlled differentiation of MDSCs towards the M1 anti-tumor
183 macrophages with the use of curcumin (81). This approach will be considered in the recruiting clinical trial
184 (NCT03769766). Similar approach will be used in the phase I clinical study in patients with biochemically
185 recurrent PC, testing the effectiveness of the white button mushroom (WBM) extract containing β -glucan
186 (NCT04519879). B-glucans, the most abundant carbohydrates found in yeast and mushrooms (82), may induce
187 MDSCs differentiation to antigen-presenting cells, eliminating their suppressive abilities (83). The rationale for

188 this concept was additionally grounded on the preclinical data showing that dietary WBM powder reduced not
189 only the frequency of circulating MDSCs but also decreased the level of prostate-specific antigen (PSA) (84).

190 5.4 Inhibition of MDSCs induced suppressive circuits

191 There is also a possibility to inhibit some of the MDSCs induced suppressive mechanisms, operating in PC. One
192 of such approaches is represented by a combination of abiraterone – a novel hormone therapy available for
193 CRPC (85) and Tildrakizumab (anti-IL-23 mAb) (NCT04458311), altering production of IL-23 and therefore
194 having a potential to target the MDSCs function specific for PC (41). In another clinical trial, a combination of
195 Ipatasertib (inhibitor of all three isoforms of protein kinase AKT, which blocks the PI3K/AKT signaling pathway
196 – a key driver of cancer cell growth and proliferation in PC), Atezolizumab (anti-PD-L1 monoclonal antibodies)
197 - a checkpoint inhibitor on MDSCs (86) and Docetaxel (NCT03673787), will be tested in patients with mCRPC.
198 Currently, in Europe, there is one registered clinical trial focused on blocking the MDSCs function in PC patients
199 (no. 2017-001857-14). It is testing the combination of vinorelbine - a cytostatic drug, and two checkpoint
200 inhibitors: Durvalumab and Tremelimumab, which are anti-PD-L1 and anti-CTLA-4 mAb, respectively.

201 5.5 Multifactorial intervention: inhibition of MDSCs expansion and blocking their suppressive 202 activity

203 Combinations of both, the inhibition of MDSCs expansion and blocking their suppressive activity, provide the
204 opportunity for multifactorial interventions with potential better therapeutic effectiveness. One of such trial is
205 testing the combination of STAT3 inhibitor (AZD9150), a selective CXCR2 antagonist (AZD5069) and the PD-
206 L1 inhibitor (MEDI4736) (no. 2015-002525-19), where each can inhibit either the MDSCs expansion or
207 function. Gemcitabine and RQ-00000007 (grapiprant) is an another drug combination that is being tested, where
208 gemcitabine inhibits MDSCs expansion (87), while grapiprant - an inhibitor of PGE2-receptor, reduces the
209 differentiation, expansion and suppressive activities of Mo-MDSCs (88), confirming its role in MDSCs
210 functioning (26).

211 5.6 Potential new targets

212 Despite a wide scope of the ongoing clinical research, there are other available potential therapeutic options
213 targeting MDSCs in PC. One, yet unexplored route, concerns the angiotensin-converting enzyme (ACE) –
214 angiotensin pathway, where the overexpression of ACE in monocytic cells was shown to reduce the generation
215 of MDSCs (89), while angiotensin was able to reduce the tumor malignancy in PC (90). Nowadays, in the SARS-
216 CoV-2 pandemic, this pathway, however, takes on a quite different significance. However, other forms of
217 angiotensin may impact the biological properties of PC cells by modulating inflammatory reaction, or even
218 genes, including down-regulation of HIF1a and upregulation of CDH-1 (91) expression, both associated with
219 MDSCs recruitment. Another potential approach involves estrogen, used previously in PC therapy (92). The
220 combined therapy, linking activation of estrogen receptor β (ER β) and checkpoint inhibitor - anti-PD-1 mAb,
221 diminishes MDSCs infiltration in mouse models of colorectal and breast cancer (93). Interestingly, apoptosis
222 and/or differentiation of PC cells may be promoted during the ER β activation (94). Additionally, studies
223 confirmed the benefits of ER β activation in androgen-dependent CRPC, decreasing viability of the tumor cells
224 (95). Also ARG1 is a potential therapeutic target in PC and its inactivation through STAT3 inhibition was
225 already confirmed (34). The ongoing clinical trials aiming at targeting MDSCs may be a trigger for more
226 frequent use of immunotherapy in combination with other forms of PC treatment.

227 6 Conclusion

228 Although the first observations reporting a negative role of MDSCs in antitumor responses in PC dates for the
229 beginning of the 21st century, the last decade bloomed in studies, indicating their mechanisms of action and
230 clinical relevance (96). Although several questions remain unanswered, the role of MDSCs in the development
231 and progression of PC seems unquestionable, suggesting their potential as a therapeutic target. Hence, the
232 implementation of the combination therapy, e.g. radiotherapy and immunotherapy, targeting both the tumor and
233 MDSCs in PC seems crucial. Such therapy may increase the frequency of the abscopal response, which is a

phenomenon associated with tumor shrinkage, occurring not only locally at the site of the treatment but also in other locations, where the tumor has already spread (97).

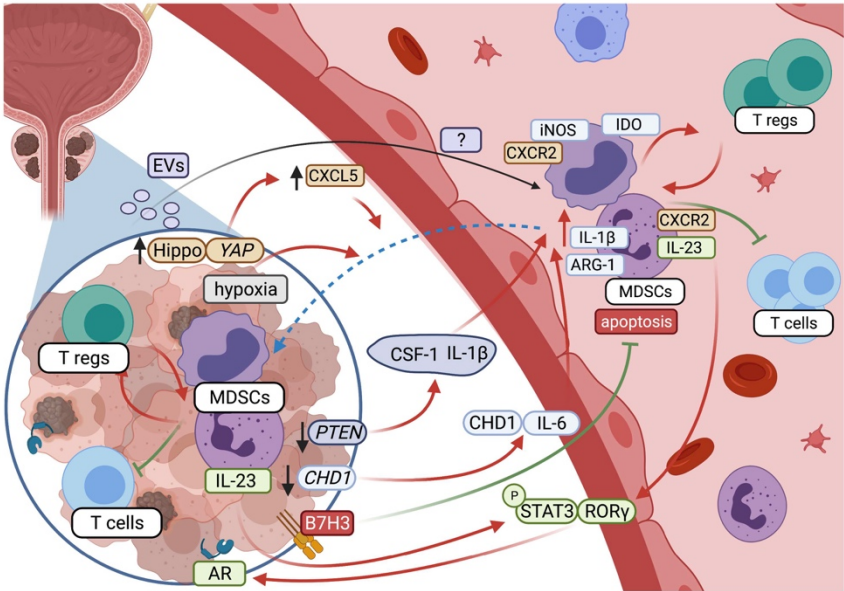


Figure 1. Crosstalk between MDSCs and tumor microenvironment in prostate cancer (created with BioRender.com).

Table 1. Clinical trials targeting MDSCs in PC patients.

Inhibition of MDSCs expansion						
No.	Title	Condition or disease	Interventions	Mechanism of action	Trial number	Status
1	Combination Study of AZD5069 and Enzalutamide. (ACE)	Metastatic Castration Resistant Prostate Cancer	CXCR2 antagonist + enzalutamide	CXCR2 antagonist may block recruitment of MDSCs to the tumor (41)	NCT03177187	Recruiting
2	Immediate Prostatectomy vs. Cabozantinib Followed by Prostatectomy in Men with High-Risk Prostate Cancer (SPARC)	Prostate Cancer Prostate Cancer Adenocarcinoma Non-Metastatic	Cabozantinib (small molecule inhibitor of tyrosine kinase receptor) + Radical Prostatectomy	Cabozantinib may reduce the tumor infiltration by MDSCs (76)	NCT03964337	Recruiting
3	Soy Bread Diet in Improving Immune Function in	Prostate Adenocarcinoma	Dietary Intervention	Soy bread isoflavones may reduce pro-	NCT03654638	Recruiting

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	Participants With Prostate Cancer			inflammatory cytokines and MDSCs level (77)		
MDSCs depletion						
4	Enzalutamide and Decitabine in Treating Patients with Metastatic Castration Resistant Prostate Cancer	Metastatic Castration Resistant Prostate Cancer	Decitabine (nucleic acid synthesis inhibitor)	Decitabine (5-aza-2'-deoxycytidine), a hypomethylating agent with the ability to selectively deplete Mo-MDSCs (80)	NCT03709550	Withdrawn
Stimulation MDSCs differentiation						
5	Trial of Curcumin to Prevent Progression of Low-risk Prostate Cancer Under Active Surveillance	Prostate Cancer	Curcumin	Curcumin may promote the differentiation of MDSCs (81)	NCT03769766	Recruiting
6	White Button Mushroom Sup for the Reduction of PSA in Patients with Biochemically Rec or Therapy Naive Fav Risk Prostate CA	Prostate Adenocarcinoma PSA Failure PSA Progression Recurrent Prostate Carcinoma Stage I Prostate Cancer stage IIA-C, III A, C Prostate Cancer	White Button Mushroom (WBM) Extract	WBM powder as a source of β -glucan may induce MDSCs differentiation to antigen-presenting cells (83) and reduce the number of circulating MDSCs (84)	NCT04519879	Recruiting
Inhibition of MDSCs induced suppressive mechanisms						
7	Abiraterone Acetate in Combination with Tildrakizumab (ACTIon)	Metastatic Castration Resistant Prostate Cancer	Abiraterone Acetate (selective inhibitor of CYP17) + Tildrakizumab (anti-IL-23)	Tildrakizumab (anti-IL-23 mAb), alters the production of IL-23 in PC, has the potential to affect castration resistance caused by MDSCs (41)	NCT04458311 / 2019-003485-40	Recruiting
8	A Trial of Ipatasertib in Combination with Atezolizumab (IceCAP)	Solid Tumor Glioblastoma Multiforme Prostate Cancer Metastatic	Ipatasertib (inhibitor of all three isoforms of protein kinase AKT) + Atezolizumab (anti-PD-L1)	Atezolizumab (anti-PD-L1 monoclonal antibodies) - a checkpoint inhibitor on MDSCs (86)	NCT03673787	Recruiting
9	A phase I/II basket trial evaluating a combination of Metronomic Oral Vinorelbine plus	Patients with locally advanced or metastatic solid tumors	Vinorelbine (cytostatic) Durvalumab (anti-PD-L1) +	Anti-PD-L1 and anti-CTLA-4 checkpoint inhibitors on MDSCs (86)	2017-001857-14	Ongoing

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	anti-PD-L1/anti-CTLA4 Immunotherapy in patients with advanced solid tumors.		Tremelimumab (anti-CTLA-4)			
Multifactorial action: inhibition of MDSCs expansion and blocking their suppressive activity						
10	A Phase 1b/2, Open-Label, Multicentre Study Assessing the Safety, Tolerability, Pharmacokinetics, and Preliminary Anti-tumor Activity of MEDI4736 in Combination with AZD9150 or AZD5069 in Patients with Advanced Solid Malignancies and Subsequently Comparing AZD9150 and AZD5069 Both as Monotherapy and in Combination with MEDI4736 as Second Line Treatment in Patients with Recurrent and/or Metastatic Squamous Cell Carcinoma of the Head and Neck.	Part A: advanced solid tumor Part B: Recurrent and/or spreading tumor of Head and Neck	MEDI4736 (Durvalumab-anti-PD-L1) + AZD9150 (Danvatirsen-STAT3 inhibitor) + AZD5069 (CXCR2 antagonist)	Combination of STAT3 inhibitor, selective CXCR2 antagonist, and PD-L1 inhibitor, where each of them has the potential to inhibit MDSCs activity (41,60,86)	2015-002525-19	Restarted or completed depending on the country
11	Phase II Trial of EP4 Receptor Antagonist in Advanced Solid Tumors	Prostate Cancer Non-Small Cell Lung Cancer Breast Cancer	Grapiprant + Gemcitabine	Gemcitabine may reduce the level of MDSCs (87), whereas grapiprant, a PGE2-receptor inhibitor, may block induction of MDSCs (88) and their suppressive effect (26)	NCT02538432	Withdrawn

7 Conflict of 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.

8 Author Contributions

IS wrote the draft version of the manuscript. JB revised and edited the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Oświadczenia autora i współautorów

Kraków, dnia 22.02.2022

mgr Izabela Siemińska
Zakład Immunologii Klinicznej
Katedry Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*: **The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy. *Polish Journal of Pathology*, 2020, 71.1** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i

opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 65% i polegał na**:

- zaplanowaniu doświadczeń
- opracowaniu koncepcji pracy
- wykonaniu badań części eksperymentalnej metodą cytometrii przepływowej
- opracowaniu, analizie oraz interpretacji wyników
- przygotowaniu manuskryptu

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje mój indywidualny wkład polegający na**:

- opracowaniu koncepcji badań,
- wykonaniu badań części eksperymentalnej metodą cytometrii przepływowej,
- opracowaniu i interpretacji wyników,
- przygotowaniu manuskryptu.

Sieminska Izabela
.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Gdańsk, dnia 07.03.2022

Dr n. med. Edyta Rychlicka-Buniowska
Międzynarodowa Agenda Badawcza
Gdański Uniwersytet Medyczny

OŚWIADCZENIE

Jako współautor pracy*: **The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy. Polish Journal of Pathology, 2020, 71.1** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 5% i polegał na**:

- izolacji jednojądrzastych komórek krwi obwodowej,

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopiśmie naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabeli Siemińskiej** polegający na**:

- opracowaniu koncepcji badań,
- wykonaniu badań części eksperymentalnej metodą cytometrii przepływową,
- opracowaniu i interpretacji wyników,
- przygotowaniu manuskryptu.

Rychlicka-Buniowska
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 21.04.2022

Dr n. med. Karolina Bukowska-Strakova
Zakład Immunologii Klinicznej
Katedry Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

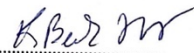
Jako współautor pracy*: **The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy. *Polish Journal of Pathology*, 2020, 71.1** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 5% i polegał na**:

- opracowaniu koncepcji metodologii cytometrii przepływowej.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- opracowaniu koncepcji badań,
- wykonaniu badań części eksperymentalnej metodą cytometrii przepływowej,
- opracowaniu i interpretacji wyników,
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(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 09.06.2022

Prof. dr hab n. med. Maciej Siedlar
Zakład Immunologii Klinicznej
Katedry Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*: **The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy. *Polish Journal of Pathology*, 2020, 71.1** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 5% i polegał na**:

- nadzorce merytorycznym manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopiśmie naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- opracowaniu koncepcji badań,
- wykonaniu badań części eksperymentalnej metodą cytometrii przepływową,
- opracowaniu i interpretacji wyników,
- przygotowaniu manuskryptu.



(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 09.06.2022

Kraków, dnia 10.06.2022

dr hab n. med. Jarosław Baran, Prof. UJ
Zakład Immunologii Klinicznej,
Katedra Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

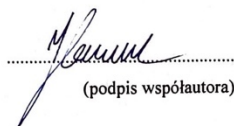
Jako współautor pracy*: **The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy. *Polish Journal of Pathology*, 2020, 71.1** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 10% i polegał na**:

- opracowaniu koncepcji badań,
- przygotowaniu manuskryptu,
- nadzorze merytorycznym manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopiśmie naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- opracowaniu koncepcji badań,
- wykonaniu badań części eksperymentalnej metodą cytometrii przepływowej,
- opracowaniu i interpretacji wyników,
- przygotowaniu manuskryptu.


(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 22.02.2022

mgr Izabela Siemińska
Zakład Immunologii Klinicznej
Katedry Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*: **Myeloid-derived suppressor cells in colorectal cancer. *Frontiers in Immunology*, 2020, 11: 1526** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 60% i polegał na**:

- przygotowanie pierwotnej wersji manuskryptu,
- opracowanie grafik.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje mój indywidualny wkład polegający na**:

- przygotowanie pierwotnej wersji manuskryptu,
- opracowanie grafik.

Siemińska Izabela.

(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 10.06.2022

dr hab n. med. Jarosław Baran, Prof. UJ
Zakład Immunologii Klinicznej
Katedra Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

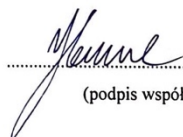
Jako współautor pracy*: **Myeloid-derived suppressor cells in colorectal cancer. *Frontiers in Immunology*, 2020, 11: 1526** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 40% i polegał na**:

- przygotowaniu ostatecznej wersji manuskryptu,
- nadzorze merytorycznym manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- przygotowanie pierwotnej wersji manuskryptu,
- opracowanie grafik.


.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 22.02.2022

mgr Izabela Siemińska
Zakład Immunologii Klinicznej
Katedry Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*: **Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery. *Translational Oncology*, 2022, 17: 101346** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 60% i polegał na**:

- opracowaniu koncepcji badania,
- wykonaniu części eksperymentalnej badania metodą cytometrii przepływowej,
- wykonanie testów proliferacyjnych,
- opracowaniu i interpretacji wyników,
- przygotowaniu manuskryptu.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje mój indywidualny wkład polegający na**:

- opracowaniu koncepcji badania,
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- opracowaniu i interpretacji wyników,
- przygotowaniu manuskryptu.

Siemińska Izabela

(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 21.04.2022

Dr hab. n. med. Mateusz Rubinkiewicz
II Katedra Chirurgii Ogólnej
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*: **Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery. *Translational Oncology*, 2022, 17: 101346** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 5% i polegał na**:

- kwalifikacja pacjentów do badania,
- pobraniu materiału (krwi obwodowej) do badania,
- koordynowaniu klinicznych aspektów pracy.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- opracowaniu koncepcji badania,
- wykonaniu części eksperymentalnej badania metodą cytometrii przepływownej,
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- opracowaniu i interpretacji wyników,
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(podpis współautora)

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**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 09.06.2022

dr hab. n. med. Antonii Szczepanik
I Katedra Chirurgii Ogólnej
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

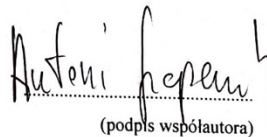
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- kwalifikacji pacjentów do badania,
- koordynowaniu klinicznych aspektów pracy,
- nadzorze merytorycznym manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopiśmie naukowych.

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(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

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Kraków, dnia 09.06.2022

Prof. dr hab n. med. Maciej Siedlar
Zakład Immunologii Klinicznej
Katedry Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*: **Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery. *Translational Oncology*, 2022, 17: 101346** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 5% i polegał na**:

-nadzorze merytorycznym manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- opracowaniu koncepcji badania,
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- opracowaniu i interpretacji wyników,
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(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 10.06.2022

dr hab n. med. Jarosław Baran, Prof. UJ
Zakład Immunologii Klinicznej
Katedra Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

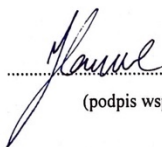
Jako współautor pracy*: **Mo-MDSCs are pivotal players in colorectal cancer and may be associated with tumor recurrence after surgery. *Translational Oncology*, 2022, 17: 101346** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 10% i polegał na**:

- opracowaniu koncepcji badań,
- przygotowaniu manuskryptu,
- nadzorze merytorycznym manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- opracowaniu koncepcji badania,
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- wykonaniu testów proliferacyjnych,
- opracowaniu i interpretacji wyników,
- przygotowaniu manuskryptu.


.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 10.06.2022

mgr Izabela Siemińska
Zakład Immunologii Klinicznej
Katedry Immunologii Klinicznej i Transplantologii
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*: **Siemińska I, Baran J. Myeloid-derived suppressor cells as key players and promising therapy target in prostate cancer. *Frontiers in Oncology*. 2022.** (w druku) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 60% i polegał na**:

- przygotowanie pierwotnej wersji manuskryptu,
- opracowanie grafik.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje mój indywidualny wkład polegający na**:

- przygotowanie pierwotnej wersji manuskryptu,
- opracowanie grafik.

Siemińska Izabela

(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 10.06.2022

dr hab n. med. Jarosław Baran, Prof. UJ
Zakład Immunologii Klinicznej
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Jako współautor pracy*: **Siemińska I, Baran J. Myeloid-derived suppressor cells as key players and promising therapy target in prostate cancer. *Frontiers in Oncology*. 2022.** (w druku) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji wynosi 40% i polegał na**:

- przygotowaniu ostatecznej wersji manuskryptu,
- nadzorze merytorycznym manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr Izabelę Siemińską** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład **mgr Izabelę Siemińską** polegający na**:

- przygotowanie pierwotnej wersji manuskryptu,
- opracowanie grafik.


(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.