Original Article

Identification and Characterization of Myeloid-Derived Suppressor Cells (MDSC) Using a Simple Approach for Conventional Flow Cytometry Analysis

Identificação e Caracterização das Células Supressoras de Linhagem Mielóide (MDSC) Usando uma Abordagem Simples para Análise por Citometria de Fluxo Convencional

Cátia F. Rodrigues[1,2,3,4*], Catarina A. Rodrigues[1,2,5,6*], Patrícia Maia[1,2,3,7*], M. Emília Sousa[3], Inês Godinho[3], Bruno M. Neves[8], Iola F. Duarte[9], Lúcio Lara Santos[1,2,10,11], Carlos Palmeira[1,2,3,10], Gabriela Martins[3]

1. Experimental Pathology and Therapeutics Group, Research Center of IPO-Porto (CI-IPOP), Portugal
2. RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO-Porto) / Porto Comprehensive Cancer Center Raquel Seruca, Porto, Portugal
3. Department of Immunology, Portuguese Oncology Institute of Porto (IPO-Porto), Porto, Portugal
4. Department of Chemistry, University of Aveiro, Aveiro, Portugal
5. Instituto de Investigação e Inovação em Saúde (i3S), University of Porto, Porto, Portugal
6. Faculty of Medicine of the University of Porto (FMUP), Porto, Portugal
7. Clinical Research Unit, Research Center of IPO Porto (CI-IPOP), Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal
8. Department of Medical Sciences and Institute of Biomedicine (iBiMED), University of Aveiro, 3Aveiro, Portugal
9. Department of Chemistry, (CICECO & LAQV/REQUIMTE), University of Aveiro, Aveiro, Portugal
10. Health School of University Fernando Pessoa, Porto, Portugal
11. Department of Surgical Oncology, Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal

* These authors equally contributed to this work.

Corresponding Author/Autor Correspondente:
Carlos Palmeira [carlospalmeira@ipoporto.min-saude.pt]
Department of Immunology, Portuguese Oncology Institute of Porto, 4200-162 Porto, Portugal

https://doi.org/10.34635/rpc.1099

Received/Recebido: 22/03/2025 **Accepted/Aceite:** 08/05/2025 **Published online/Publicado online:** 21/06/2025 **Published/Publicado:** 30/06/2025

ABSTRACT

Introduction: Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that accumulate under pathological conditions, such as cancer, and suppress immune responses through various mechanisms. Distinguishing MDSC populations remains challenging due to their shared surface markers with neutrophils and monocytes, complicating accurate identification and quantification. This study aimed to improve MDSC identification and quantification using conventional flow cytometry panels and assess their functional activity for integration into the cancer immunogram of cancer patients at the Immunology Department of IPO-Porto.

Methods: Identification and quantification of circulating MDSCs were performed by flow cytometry using an 8-color multiparametric panel.

Results: Cancer patients showed significantly higher levels of PMN-MDSCs (12-fold) and M-MDSCs (1.1-fold) than healthy donors. MDSC function was evaluated by qPCR after cell sorting (FACS), revealing increased transcriptional levels of *NOS2* and *TGFB1*, which are associated with immunosuppressive activity. Moreover, reduced zeta chain (CD247) expression in T lymphocytes and NK cells was observed, with lower mean fluorescence intensity (MFI) ratios in cancer patients, indicating impaired immune signaling.

Conclusion: This study confirmed that circulating MDSC levels are elevated in cancer patients, reinforcing their relevance in the cancer immunogram. It also identified potentially useful phenotypic and functional MDSC markers that require validation in larger sample sets. The findings contribute to refining flow cytometry analysis panels, enabling more accurate and standardized identification of MDSC populations using conventional platforms.

Keywords: Biomarkers, Tumor; Flow Cytometry; Myeloid-Derived Suppressor Cells; Neoplasms

RESUMO

Introdução: As células mieloides supressoras (MDSCs) são uma população heterogénea de células mieloides imaturas que se acumulam em condições patológicas, como o cancro, e suprimem as respostas imunitárias através de vários mecanismos. A distinção entre populações de MDSC continua a ser um desafio devido à partilha de marcadores de superfície com neutrófilos e monócitos, dificultando a sua identificação e quantificação precisas. Este estudo teve como objetivo melhorar a identificação e quantificação das MDSCs utilizando painéis de citometria de fluxo convencional e avaliar a sua atividade funcional para integração no imunograma de cancro de doentes oncológicos no Serviço de Imunologia do IPO-Porto.

Métodos: A identificação e quantificação das MDSCs circulantes foram realizadas por citometria de fluxo utilizando um painel multiparamétrico de 8 cores.

Resultados: Os doentes com cancro apresentaram níveis significativamente mais elevados de PMN-MDSCs (12 vezes) e M-MDSCs (1,1 vezes) em comparação com dadores saudáveis. A função das MDSCs foi avaliada por qPCR após a separação celular (FACS), revelando níveis de transcrição aumentados de *NOS2* e *TGFB1*, associados à atividade imunossupressora. Além disso, foi observada uma redução na expressão da cadeia zeta (CD247) em linfócitos T e células NK, com rácios de intensidade média de fluorescência (MFI) inferiores nos doentes com cancro, indicando um comprometimento na sinalização imunitária.

Conclusão: Este estudo confirmou que os níveis de MDSCs circulantes estão elevados em doentes com cancro, reforçando a sua relevância no imunograma de cancro. Também identificou uma combinação de marcadores fenotípicos e funcionais potencialmente úteis, que necessitam de validação em amostras de maior dimensão. Os resultados contribuem para o aperfeiçoamento dos painéis de análise por citometria de fluxo, permitindo uma identificação mais precisa e padronizada das populações de MDSCs em plataformas convencionais.

Palavras-chave: Biomarcadores Tumorais; Células Supressoras Mieloides; Citometria de Fluxo; Neoplasias

INTRODUCTION

Cancer encompasses a heterogeneous group of diseases characterized by the uncontrolled growth and dissemination of abnormal cells.[1] The interactions between the immune system and cancer development have been extensively studied over the years, leading to key insights into tumor biology and significant advances in cancer treatment.[2] The cancer immunogram was first described by Blank *et al*,[3] is a framework

that incorporates multiple multidimensional biomarkers influencing cancer-immune system interactions, aiming to guide the selection of the most effective cancer therapy for individual cases.[4] The cancer immunogram consists of seven parameter classes: tumor foreignness, general immune status, immune cell infiltration, absence of checkpoints, absence of soluble inhibitors, absence of inhibitory tumor metabolism, and tumor sensitivity to immune effectors.[3,5,6]

Myeloid-derived suppressor cells (MDSCs) – one of the key parameters analyzed in the cancer immunogram – are a heterogeneous population of myeloid lineage cells defined by their immature state and capacity to suppress immune responses.[7,8] Under normal conditions, hematopoietic stem cells (HSC) in the bone marrow give rise to immature myeloid cells (IMCs), which later differentiate into mature macrophages, dendritic cells (DC), or neutrophils. However, under pathological conditions such as chronic inflammation, infection, and especially cancer, abnormal myelopoiesis is triggered by persistent stimulation from growth factors, cytokines, and chemokines. This leads to the accumulation of immature myeloid cells, which acquire immunosuppressive properties in the peripheral microenvironment and are collectively termed MDSCs.[8,9]

In humans, there are three main subsets of MDSCs: polymorphonuclear MDSCs (PMN-MDSC), which are phenotypically identical to neutrophils; monocytic MDSCs (M-MDSC), which are similar to monocytes; and early-stage MDSCs (e-MDSC), a smaller population with a mixed immature phenotype.[10,11] In healthy individuals, PMN-MDSCs represent less than 1% of circulating neutrophils, but in cancer patients, they can increase to 4%–15% of total neutrophils and up to 40% in tumor-infiltrating neutrophils.[12,13] In solid tumors, M-MDSCs can rapidly differentiate into tumor-associated macrophages (TAM), suppressing immune responses and promoting tumor progression. Elevated MDSC levels in peripheral blood are positively correlated with advanced cancer stage, increased tumor burden, shorter progression-free survival, and poor response to chemotherapy.[12,14,15]

The PMN-MDSC to M-MDSC ratio is crucial, as these cells employ different mechanisms to suppress T-cell responses. M-MDSCs suppress T cell activation through antigen-specific and nonspecific mechanisms, involving increased NOS2 expression, NO production, and secretion of inhibitory cytokines like IL-10. In contrast, PMN-MDSCs primarily mediate antigen-specific suppression, inducing $CD8^+$ T cell tolerance through increased ARG1 expression and elevated ROS levels.[16-18]

PMN-MDSCs are characterized as $CD11b^+$ $CD14^-$ $CD15^+$ $CD33^+$ $HLA\text{-}DR^-$/low, M-MDSCs as $CD11b^+$ $CD14^+$ $CD15^-$ $CD33^+$ $HLA\text{-}DR^-$/low, and e-MDSCs as $Lineage^-$ (CD3, CD14, CD15, CD19, CD56) $CD33^+$ $HLA\text{-}DR^-$. However, these surface markers are not specific to MDSCs, making it difficult to distinguish them from neutrophils and monocytes.[19,20] In the past few years, lectin-type oxidized LDL receptor-1 (LOX-1) has been identified as a distinct marker for PMN-MDSCs not only in cancer but in other pathologies, as PMN-MDSCs are LOX-1 positive, while neutrophils are mostly LOX-1 negative.[21-23]

This study aims to explore the phenotypic and functional characteristics of MDSCs using conventional flow cytometry panels to improve their identification and quantification in cancer patients.

MATERIAL AND METHODS

1. PATIENTS AND SAMPLES

Peripheral whole blood (PB) samples from 31 patients with solid or hematologic tumors (22 with multiple myeloma (MM), one with MM and prostate cancer, one with diffuse large B-cell lymphoma, one with Waldenström's macroglobulinemia and seven with colon carcinoma) of the Portuguese Oncology Institute of Porto (IPO-Porto) were collected. For control, 12 samples from healthy donors were used. All samples were collected after obtaining informed consent from all individual participants and were in accordance with the ethical standards of the institutional ethics committee and with the Declaration of Helsinki.

2. FLOW CYTOMETRIC DETECTION OF MDSC

For the processing of peripheral blood, a specific lysing solution – BulkLysis™ (Cytognos) – was used according to the manufacturer's protocol. After this step, each sample was stained with an eight-color panel using the appropriate amount of the following fluorescently labeled antibodies (Table 1). An incubation step of 30 min at room temperature (RT) protected from the light was performed, and then 1X FACS™ lysing solution (BD Bioscience) was added. Another incubation of 10 min at RT protected from the light, a centrifugation at 1800 rpm for 5 min and a washing step with 2 mL of washing solution was performed. The supernatant was discarded, and the cell pellet was resuspended in 500 µL of acquisition buffer (FACSFlow™ BD Biosciences) for further data acquisition in the BD FACSCanto™ II (at least 5 million cells were acquired). If the cells were not immediately acquired, they were stored at 4°C. The data analysis was performed using Infinicyt™ 1.7 software (Cytognos).

Table 1. Comprehensive parameter panel for MDSC Characterization

Marker	Clone	Fluorochrome (s)	Vendor	µL/100µL	Purpose in the Painel
CD15	MMA	V450	BD Biosciences	5	Monocyte lineage marker, improved lymphocyte gate purity
CD45	MHCD4530	PO	Life Technologies	2.5	Pan leucocyte marker
Lineage Cocktail 2 (CD3, CD14, CD19, CD20 and CD56)	n.a	FITC	BD Biosciences	10	Exclusion of lymphocyte and NK cell populations, focusing on the analysis of the myeloid population
LOX-1	n.a	PE	BioLegend	5	Distinction marker for PMN-MDSCs
CD33	P67.6	PerCP-Cy™ 5.5	BD Biosciences	10	Myeloid cell marker; Distinction between M-MDSCs and PMN-MDSCs
HLA-DR	n.a	PC7	Beckman Coulter	1	Essential marker for M-MDSCs identification
CD11b	clone D12	APC	BD Biosciences	10	General myeloid marker
CD14	MψP9	APC-H7	BD Biosciences	5	Monocyte and M-MDSCs marker

3. ANALYSIS OF THE MRNA LEVELS OF ARG1, *IDO1*, *NOS2* AND *TGFB1*

To study the immunosuppressive mechanisms involved in MDSC activity, the two MDSC subsets were isolated by fluorescence-activated cell sorting (FACS), RNA was extracted, and the transcription levels of *ARG1*, *IDO1*, *NOS2*, and *TGFB1* were evaluated. For the FACS technique, samples of PB from a group of patients that exhibit a higher frequency of MDSCs were stained using the previously mentioned antibodies and processing protocol. Also, a PB sample from 1 healthy donor was collected to establish the settings for the acquisition of the FACS and the T cells separated by FACS by staining with CD45-PO (clone MHCD4530, Life Technologies) and CD3-APC (clone UCHT, Beckman Coulter). Samples were acquired using a BD FACSAria™ II.

The sorted cells were preserved in TripleXtractor (GRiSP) at –80ºC for further RNA extraction. The RNA extraction and purification were performed using the GRS total RNA kit – blood and cultured cells (GRiSP). After the extraction, the RNA was quantified using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific) and reversely transcribed using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Quantitative PCR (qPCR) reactions were performed using SYBR Green (BioRad) as a probe on a Bio-Rad CFX Connect device. *GAPDH* was used as the reference gene, and the results presented as the ratio of mRNA molecules of the studied genes/mRNA molecules of *GAPDH*. T cells from the healthy donor were used as a negative control. Primer sequences were designed using Beacon Designer software version 8 (Premier Biosoft International, Palo Alto, CA, USA) and thoroughly tested (Table 2).

Table 2. qPCR primers for detecting mRNA expression of interested genes

Gene Name	Primer Sequences	Reference
NOS2	Forward: TCAGTATCACAACCTCAG Reverse: TTCTGGAGACTTCTTTCC	ID NM_000625
IDO1	Forward: CCTGACTTATGAGAACAT Reverse: ATTGCCTTGAATACAGTA	ID NM_002164
ARG1	Forward: AAGAGAAGTGTCAGAGCATGAG Reverse: CTCGTGGCTGTCCCTTTG	ID NM_000045
TGFB1	Forward: GGAAACCCACAACGAAATC Reverse: GCTCTGATGTGTTGAAGAAC	ID NM_000660
GAPDH	Forward: ACAGTCAGCCGCATCTTC Reverse: GCCCAATACGACCAAATCC	ID NM_002046

4. EVALUATION OF ZETA CHAIN EXPRESSION IN T LYMPHOCYTES AND NK CELLS

This study implied the optimization of the mAb CD247-Alexa Fluor 647. For the mAb to work in its best condition, it was necessary to separate peripheral blood mononuclear cells (PBMC). The PB samples were transferred to centrifuge tubes using an aseptic technique, and an equal amount of phosphate-buffered saline 1× (PBS 1×) was added to each. The mix was gently transferred to tubes containing Lymphoprep™ (STEMCELL Technologies) and centrifuged for 20 min at room temperature (RT) at 2100 rpm. After centrifugation, the mononuclear cells layer was retained, washed twice, and stained with fluorescently labeled antibodies (Table 3). An incubation of 30 min at RT was performed, and cells were washed with a washing solution (centrifugation at 1800 rpm, 5 min). The supernatant was removed, and then the staining of cytoplasmic antigen – CD247 – was performed using the BD Cytofix/Cytoperm Kit, according to the manufacturer's protocol. Before acquisition, cells were resuspended in 500 µL of acquisition buffer (FACSFlow™), and data acquisition was performed in the BD FACSCanto™ II. If the cells were not immediately acquired, they were stored at 4°C. The data analysis was performed using Infinicyt™ 1.7 software (Cytognos).

5. STATISTICAL ANALYSIS

The statistical analysis was performed using the two-tailed unpaired *t*-test, or the Mann-Whitney U test, using the GraphPad Prism 8.0 software. Statistical significance was considered whenever $p \leq 0.05$.

RESULTS

A gating strategy was used to remove the doublets and cellular debris using FSC-A and SSC-A parameters to identify PMN-MDSCs. $CD45^-$ cells were excluded (data not shown), followed by selecting $CD15^+$ cells based on SSC-A characteristics. Neutrophils were then selected upon eosinophils exclusion based on their characteristic position based on their characteristic position in the CD45 *versus* SSC-A plot. Cells co-expressing CD33 and CD11b were then selected, and $CD14^+$ and $HLA\text{-}DR^+$ cells were excluded by applying a stringent gate on the $CD14^-$ and $HLA\text{-}DR^-$ populations in the CD14 *versus* SSC-A and HLA-DR *versus* SSC-A plots, respectively. Finally, PMN-MDSCs were identified as $CD15^+$ cells expressing LOX-1.

For the identification of M-MDSC, after excluding the doublets, cell debris, and the $CD45^-$ cells, the CD14+ cells were selected using a CD14 *vs* SSC-A plot. The cells co-expressing CD33 and CD11b were selected, and the CD15+ cells were excluded using a gate on a CD15 *vs* SSC-A plot. Finally, M-MDSC were identified as the CD14+ cells without HLA-DR expression.

The gating strategy for both PMN-MDSCs (**A**) and M-MDSCs (**B**) is shown in Fig. 1.

In the cancer patient group, PMN-MDSC percentages ranged from 0.134% to 3.136% of the total neutrophils, with a median value of 0.531%. These values correspond to a 12-fold increase compared to healthy donors, whose PMN-MDSC percentages ranged from 0.004% to 0.151%, with a median value of 0.045%.

Regarding M-MDSCs, percentages in the cancer patient group ranged from 0.144% to 8.495% of the total monocytes, with a median value of 0.931%, corresponding to a nearly 1.1-fold increase to those observed in healthy donors, where percentages ranged from 0.197% to 2.185%, with a median value of 0.845%.

Table 3. Comprehensive panel of parameters for characterizing the zeta chain (CD247) on T and NK cells

Marker	Clone	Fluorochrome (s)	Vendor	µL/100µL	Purpose in the Panel
CD27	M-T271	BV421	BD Horizon	1	Differentiation, Co-stimulation
CD45	MHCD4530	PO	Life Technologies	2,5	Pan leucocyte marker
CD45RA	ALB11	FITC	Beckman Coulter	2,5	Differentiation
CD8	B9.11	PE	Beckman Coulter	10	Identification of CD8+ T cell subsets
CD4	SK3	PerCP	BD Biosciences	10	Identification of CD4+ T cell subsets
CD56	N901 (NKH-1)	PC7	Beckman Coulter	10	NK cells marker
CD247	6B10.2	Alexa Fluor 647	BD Biosciences	10	Essential marker to study T cell activation and function
CD3	SK7	APC-H7	BD Biosciences	10	T cell lineage marker

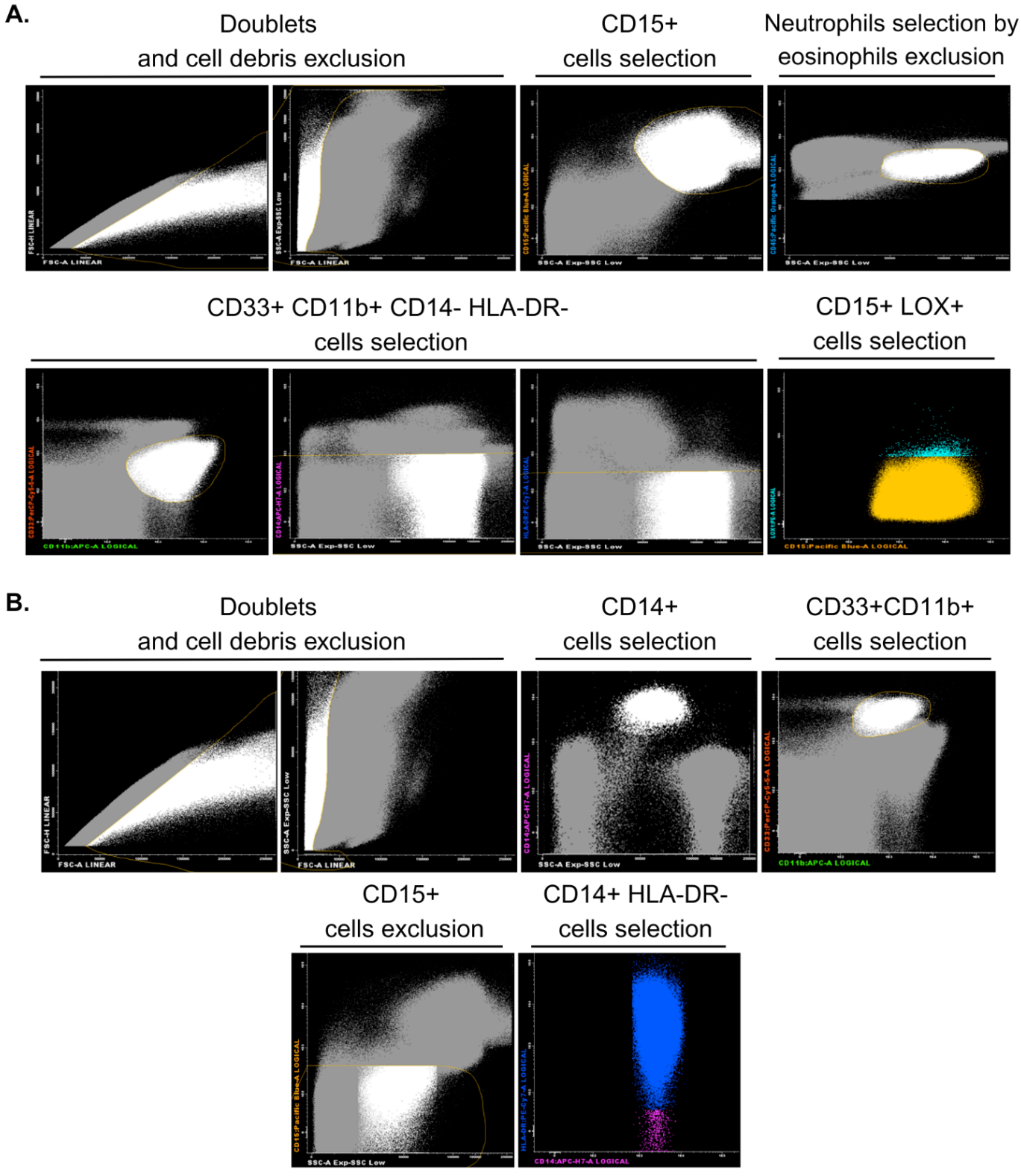

Figure 1. Gating strategy used for the identification of human PMN-MDSC (A) and M-MDSC (B) in peripheral blood samples. (A) After excluding doublets and cell debris, CD15+ cells were first selected. Neutrophils were then selected upon eosinophils exclusion based on their characteristic position on the CD45 *versus* SSC-A plot. Subsequently, cells expressing CD33+ CD11b+ CD14- HLA-DR- were selected. Finally, PMN-MDSCs were identified by selecting only those CD15+ cells that also express LOX-1, a marker specific to this population. (B) After excluding doublets and cell debris, CD14+ cells were initially selected. Cells co-expressing CD33 and CD11b were then included. CD15+ cells were excluded using a gate based on the CD15 *versus*. SSC-A plot. Finally, CD14+ cells lacking HLA-DR expression were selected, corresponding to the M-MDSC population.

The difference in PMN-MDSC percentages between cancer patients and healthy donors was statistically significant (p<0.0001). However, although a slight increase was observed for M-MDSC percentages between the two groups, it was not statistically significant (p>0.5).

The quantification of MDSC subsets in cancer patients and healthy donors and representative plots illustrating the differences observed in PMN-MDSCs and M-MDSCs between cancer patients and healthy donors are presented in Figs. 2 and 3, respectively.

Next, to evaluate the production of key immunosuppressive factors, including *NOS2*, *TGFB1*, *ARG1*, and *IDO1*, by PMN-MDSCs and M-MDSCs, their relative mRNA levels were measured using quantitative PCR (qPCR).

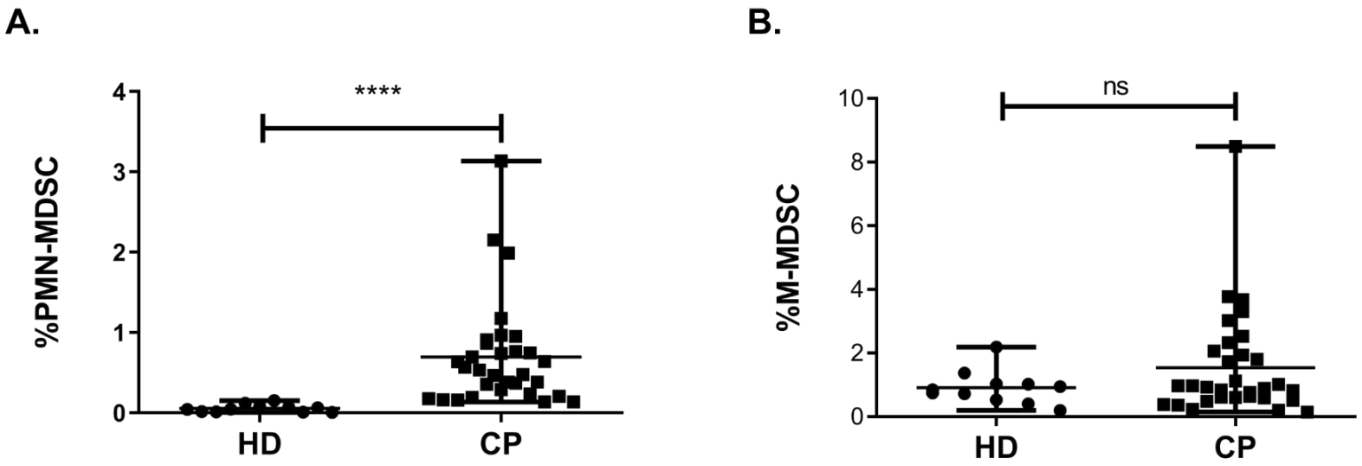

Figure 2. Quantification of MDSC subsets in peripheral blood of cancer patients and healthy donors. The percentages of PMN-MDSC (**A**) and M-MDSC (**B**) were compared between cancer patients (CP) and healthy donors (HD). The percentage of PMN-MDSC in cancer patients was approximately 12 times higher than in healthy donors (**** p<0.0001). In contrast, the percentage of M-MDSC was approximately 1.1 times higher in cancer patients compared to healthy donors, although this difference was not statistically significant (ns p>0.05).

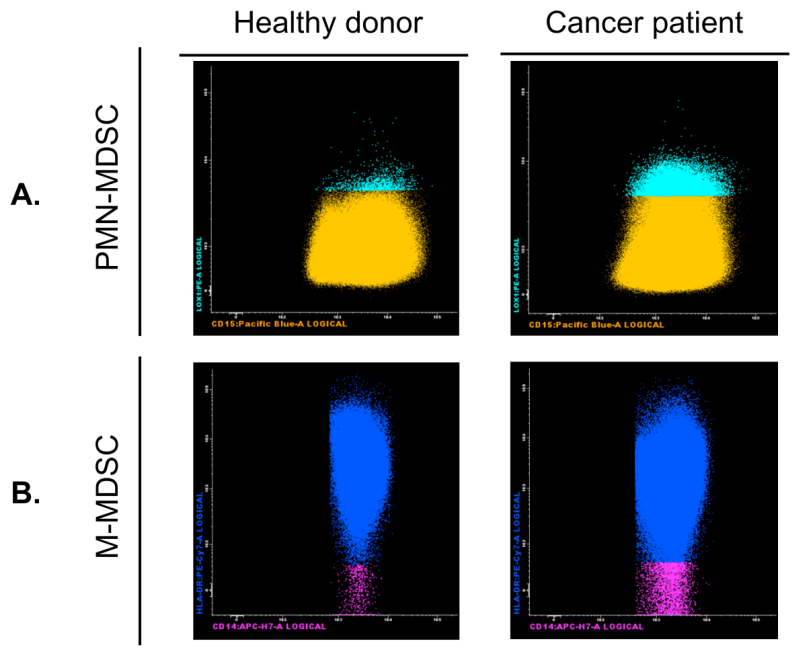

Figure 3. Representative plots showing the differences in PMN-MDSC (A) and M-MDSC (B) populations between healthy donors and cancer patients. LOX-1 *versus* CD15 (A) and HLA-DR *versus* CD14 (B) plots were used for the identification and quantification of PMN-MDSC and M-MDSC, respectively.

The detection of *NOS2* and *TGFB1* mRNA in these cells from the cancer patients group confirms their immunosuppressive activity, consistent with their known roles in promoting immune evasion and tumor progression. More specifically, *NOS2* expression was significantly higher ($p<0.05$) in PMN-MDSCs than in M-MDSCs of cancer patients (Fig- 4A). In contrast, *TGFB1* was predominantly produced by M-MDSCs (Fig. 4B).

Although it was expected that MDSCs would present increased transcription of both *ARG1* and *IDO1*, their levels were below the detection threshold of the qPCR assay used in this study. This suggests that either their transcription levels are inherently low in the analyzed samples, or post-transcriptional regulation may limit their mRNA availability.

Finally, the zeta chain (CD247) expression was analyzed by calculating the mean fluorescence intensity (MFI) ratios of CD247 expression between T cells and B cells and between NK cells and B cells. B cells were used as a negative control since these cells do not express CD247.

As shown in Fig. 5, the MFI ratios of CD247 expression between T and B cells and between NK and B cells were

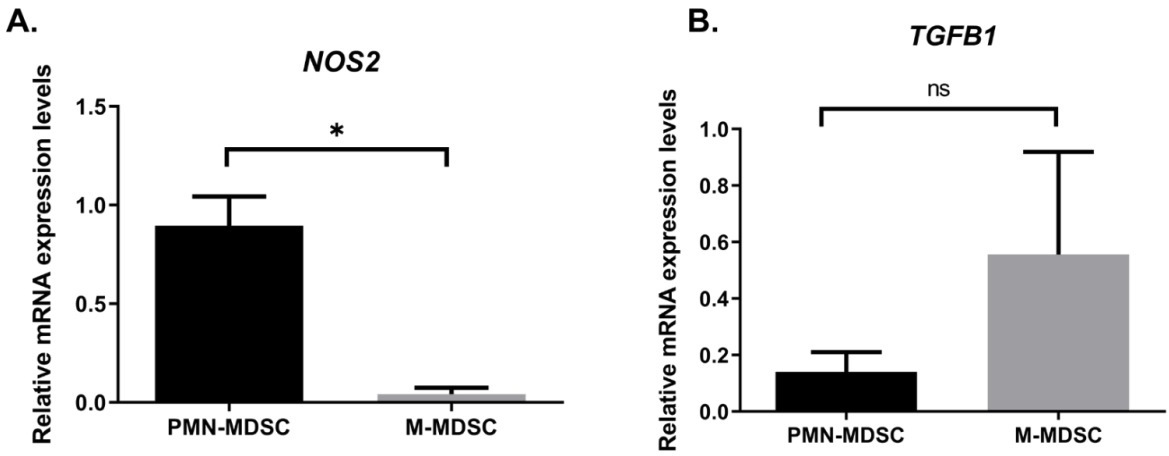

Figure 4. Relative mRNA levels of NOS2 and TGF-β. PMN-MDSC and M-MDSC were sorted from cancer patients for RNA isolation. *NOS2* mRNA was detected in both MDSC subsets, but approximately 21 times lower in M-MDSC compared to PMN-MDSC (* $p<0.05$) (**A**). For *TGF-β*, mRNA levels were about 4 times lower in PMN-MDSC compared to M-MDSC (**B**).

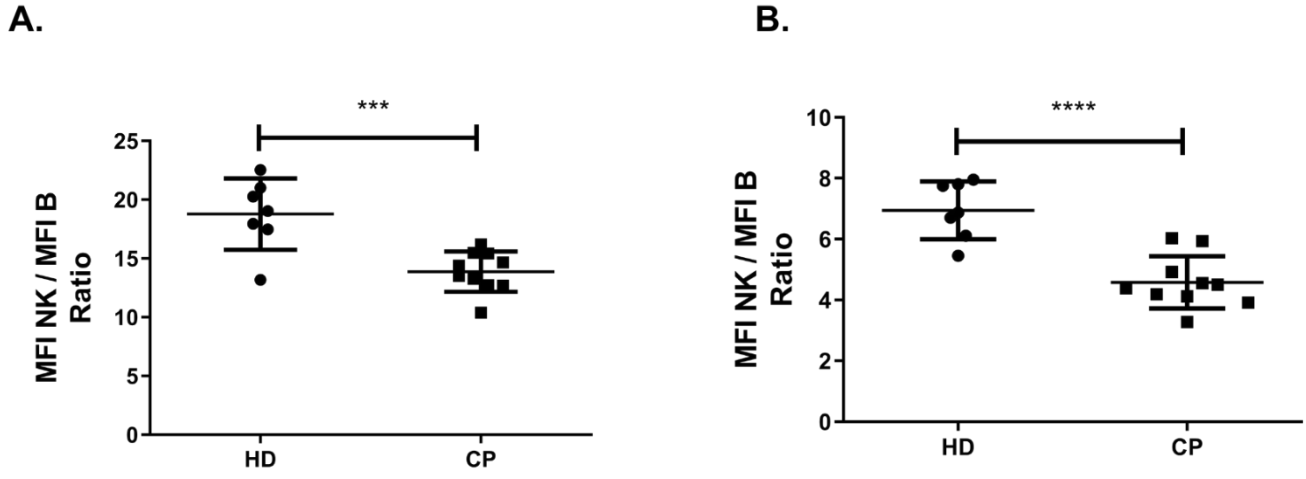

Figure 5. Comparison of mean fluorescence intensity (MFI) ratios of CD247 expression between NK and B cells (A) and between T and B cells (B) in healthy donors (HD) and cancer patients (CP). Lower MFI ratios were observed for both T lymphocytes and NK cells in cancer patients, with statistically significant differences (*** $p<0.005$ for NK cells and **** $p<0.0005$ for T cells, respectively).

significantly lower in the cancer patient group compared to the healthy donor group ($p<0.0005$ and $p<0.005$, respectively). These findings indicate a reduced zeta chain expression in T lymphocytes and NK cells from cancer patients, suggesting a potential impairment in their activation and signaling capacity.

DISCUSSION

MDSCs are a heterogeneous population of immature myeloid cells that contribute significantly to the immunosuppressive tumor microenvironment and are increased in most cancer patients.[1] They are responsible for suppressing immune responses and are correlated with a poor clinical outcome and metastatic propensity.[24,25]

In this work, the expression of the LOX-1 receptor was used as a marker to define the PMN-MDSC population, as previously described by Condamine *et al,*[26] who suggested its utility in distinguishing human neutrophils and PMN-MDSCs without a gradient. This distinction is based on PMN-MDSCs expressing LOX-1, while normal neutrophils scarcely do.[27] However, no similar marker has yet been identified for distinguishing M-MDSC from monocytes. Additionally, defining the cut-off value on the HLA-DR *versus* CD14 plot remains challenging, as a fluorescence minus one (FMO) control cannot be performed in this context.[27] Therefore, for both MDSC populations, there remains a need to expand the existing panel of markers to facilitate a more straightforward phenotypic distinction.[9]

Regarding quantification, the levels of both MDSC subsets were significantly higher in the cancer patients group than in the healthy donors. The percentage of PMN-MDSC in healthy donors had a median value of 0.045% of total neutrophils, which is aligned with the expected low percentage (< 1%). However, in the peripheral blood of cancer patients, these values are typically reported to increase to 4%-15% of total neutrophils.[28] Our cancer patients cohort's median value was 0.531%, which remains below 1% of the total neutrophils. This discrepancy could be attributed to the gating strategy used, leaning toward a stringent definition of the MDSC population. Regarding M-MDSC, higher percentages were observed in cancer patients and healthy donors relative to the PMN-MDSC subset. This could be explained by the absence of specific markers for identifying M-MDSCs, complicating their phenotypic distinction from other monocyte populations.

MDSCs are not only defined by their surface markers but also by their functional properties, particularly their ability to suppress immune responses.[9,29] As Hao ***et al***[30] described, inhibiting T cell activity remains the gold standard for evaluating MDSC function. In this study, the immunosuppressive activity of MDSCs is investigated, and the transcription of several key molecules involved in suppression mechanisms was assessed by qPCR in MDSC subpopulations isolated by FACS. The mRNA levels of *NOS2* and *TGFB1* were detected, indicating that these cells possess immunosuppressive activity. According to the literature, M-MDSC-mediated immunosuppression is primarily associated with elevated *NOS2* expression and nitric oxide (NO) production. In contrast, PMN-MDSC immunosuppression is more closely linked to increased *ARG1* expression and high levels of reactive oxygen species (ROS) and peroxynitrite (PNT).[12,30,31] Interestingly, in this study, *NOS2* mRNA levels were higher in PMN-MDSCs than in M-MDSCs, contrasting with previously reported findings.[32] Conversely, *TGFB1* was predominantly produced by M-MDSCs in the analyzed samples, while the transcription of *ARG1* and *IDO1* was not detected. These discrepancies may stem from the limited number of biological samples analyzed and the small amounts of RNA available for qPCR analysis, which may have made detecting low-abundance transcripts such as *ARG1* and *IDO1* challenging. To improve the sensitivity and accuracy of the results, it would be ideal to perform cell sorting using larger volumes of peripheral blood samples or to use Cells-to-CT™ kits, which allow for the direct measurement of relative gene transcription without the need for RNA purification before reverse transcription and qPCR run.

Another immunosuppressive mechanism of MDSCs involves their capacity to modulate the expression of the zeta chain (CD247). CD247 is a subunit of the T-cell receptor (TCR) complex, essential for its surface expression and signaling function in T lymphocytes.[33,34] Natural killer (NK) cells also express the zeta chain as a heterodimer associated with CD16.[35] Reduced levels of zeta chain can impair T and NK cell activation, proliferation, and cytokine production due to the diminished availability of tyrosine residues for phosphorylation, thereby compromising downstream signaling events such as the recruitment and activation of ZAP-70.[35,36] Flow cytometry analysis revealed a statistically significant reduction in zeta chain expression in T lymphocytes and NK cells in the cancer patient group. This reduction could be attributed to the immunosuppressive activity of MDSCs since, as demonstrated in this study, elevated levels of MDSCs were present in the cancer patient group.

CONCLUSION

Accurate and reproducible measurement of MDSC levels remains challenging due to the overlap of immunophenotypic markers shared with other myeloid cells, such as monocytes and neutrophils. There is a continuing need to expand the existing panel of markers to facilitate a more precise

and reliable phenotypic distinction of MDSCs. Therefore, improving the identification and quantification of these cells remains a key priority in the field.

The immunosuppressive capacity of MDSCs, mediated through multiple mechanisms, is a defining functional characteristic of these cells. In this study, the immunosuppressive activity of MDSCs was evaluated by assessing the increased transcription of key molecules, namely *NOS2* and *TGFB1*, as well as the reduced expression of the zeta chain in both T cells and NK cells. However, these functional assays require further optimization and validation using more biological samples to strengthen the reliability of the observed trends.

In summary, this study confirmed that circulating MDSC levels are elevated in cancer patients compared to healthy individuals, reinforcing the relevance of including MDSCs in the cancer immunogram. Additionally, this study identified and confirmed potentially useful phenotypic and functional MDSC markers that warrant validation in larger sample sets. The findings from this study could contribute to the refinement of flow cytometry analysis panels, enabling more accurate and standardized identification of MDSC populations using conventional flow cytometry platforms.

ACKNOWLEDGEMENTS / AGRADECIMENTOS

The authors acknowledge the support of the i3S Translational Cytometry (TraCy) Scientific Platform with the assistance of Catarina Meireles. The authors also acknowledge Artur Paiva's collaboration in support of flow cytometry data acquisition. Moreover, the authors wish to acknowledge the Portuguese Foundation for Science and Technology (FCT) for the PhD grant of Catarina A. Rodrigues (UI/BD/153103/2022). The research units CICECO (UIDB/50011/2020; UIDP/50011/2020, LA/P/0006/2020), LAQV-REQUIMTE (LA/P/0008/202; UIDP/50006/2020; UIDB/50006/2020), and to iBiMED (UIDB/04501/2020, DOI identifier https://doi.org/10.54499/UIDB/04501/2020 and project reference UIDP/04501/2020, DOI identifier https://doi.org/10.54499/UIDP/04501/2020) financed by national funds through the FCT/MCTES (PIDDAC) are also acknowledged.

ETHICAL DISCLOSURES

Conflicts of Interest: The authors have no conflicts of interest to declare.

Financing Support: This work has not received any contribution, grant or scholarship

Confidentiality of Data: The authors declare that they have followed the protocols of their work center on the publication of patient data.

Protection of Human and Animal Subjects: The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and those of the Code of Ethics of the World Medical Association (Declaration of Helsinki as revised in 2024).

Provenance and Peer Review: Not commissioned; externally peer-reviewed.

RESPONSABILIDADES ÉTICAS

Conflitos de Interesse: Os autores declaram a inexistência de conflitos de interesse na realização do presente trabalho.

Fontes de Financiamento: Não existiram fontes externas de financiamento para a realização deste artigo.

Confidencialidade dos Dados: Os autores declaram ter seguido os protocolos da sua instituição acerca da publicação dos dados de doentes.

Proteção de Pessoas e Animais: Os autores declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pela Comissão de Ética responsável e de acordo com a Declaração de Helsínquia revista em 2024 e da Associação Médica Mundial.

Proveniência e Revisão por Pares: Não comissionado; revisão externa por pares.

CONTRIBUTORSHIP STATEMENT

CFRs and CP: Conceptualization; methodology; data curation; investigation; validation; formal analysis; writing – original draft.

CAR and PM: Formal analysis; writing – original draft; writing – review and editing.

MES and IG: Data curation; methodology; writing – review and editing.

BMN: Conceptualization; methodology; project administration; resources; supervision; writing – review and editing.

IFD: Conceptualization; methodology; supervision; writing – review and editing.
LLS: Conceptualization; supervision; writing – review and editing.
GM: Supervision; writing – review and editing.
All authors approved the final version to be published.

DECLARAÇÃO DE CONTRIBUIÇÃO

CFRs e CP: Conceptualização; metodologia; curadoria de dados; investigação; validação; análise formal; redação – rascunho original.
CAR e PM: Análise formal; escrita – rascunho original; escrita – revisão e edição.
MES e IG: Curadoria de dados; metodologia; redação – revisão e edição.
BMN: Conceptualização; metodologia; administração do projeto; recursos; supervisão; escrita – revisão e edição.
IFD: Conceptualização; metodologia; supervisão; escrita – revisão e edição.
LLS: Conceptualização; supervisão; escrita – revisão e edição.
GM: Supervisão; redação – revisão e edição.
Todos os autores aprovaram a versão final a ser publicada.

REFERENCES

1. Brown JS, Amend SR, Austin RH, Gatenby RA, Hammarlund EU, Pienta KJ. Updating the Definition of Cancer. Mol Cancer Res. 2023;21:1142-47.
2. Gupta I, Hussein O, Sastry KS, Bougarn S, Gopinath N, Chin-Smith E, et al. Deciphering the complexities of cancer cell immune evasion: Mechanisms and therapeutic implications. Adv Cancer Biology – Metastasis. 2023;8:100107. doi: 10.1016/j.adcanc.2023.100107
3. Blank CU, Haanen JB, Ribas A, Schumacher TN. CANCER IMMUNOLOGY. The "cancer immunogram". Science. 2016;352: 658-60.
4. Mellman I, Chen DS, Powles T, Turley SJ. The cancer-immunity cycle: Indication, genotype, and immunotype. Immunity. 2023;56: 2188-205.
5. Yang K, Lu R, Mei J, Cao K, Zeng T, Hua Y, et al. The war between the immune system and the tumor – using immune biomarkers as tracers. Biomark Res. 2024;12:51. doi: 10.1186/s40364-024-00599-5.
6. Kim SI, Cassella CR, Byrne KT. Tumor burden and immunotherapy: impact on immune infiltration and therapeutic outcomes. Front Immunol. 2021;11:629722. doi: 10.3389/fimmu.2020.629722.
7. He S, Zheng L, Qi C. Myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment and their targeting in cancer therapy. Mol Cancer. 2025;24:5. doi: 10.1186/s12943-024-02208-3.
8. Liu T, Rosek A, Gonzalez De Los Santos F, Phan SH. Chapter 1 – Detection of myeloid-derived suppressor cells by flow cytometry. In: Jiménez-Cortegana C, Galluzzi L, editors. Methods in Cell Biology. . London: Academic Press; 2024. p.1-15.
9. Li K, Shi H, Zhang B, Ou X, Ma Q, Chen Y, et al. Myeloid-derived suppressor cells as immunosuppressive regulators and therapeutic targets in cancer. Signal Transduct Target Ther. 2021;6:362. doi: 10.1038/s41392-021-00670-9.
10. Glover A, Zhang Z, Shannon-Lowe C. Deciphering the roles of myeloid derived suppressor cells in viral oncogenesis. Front Immunol. 2023;14:1161848.
11. Wang S, Zhao X, Wu S, Cui D, Xu Z. Myeloid-derived suppressor cells: key immunosuppressive regulators and therapeutic targets in hematological malignancies. Biomarker Res. 2023;11:34.
12. Raskov H, Orhan A, Gaggar S, Gögenur I. Neutrophils and polymorphonuclear myeloid-derived suppressor cells: an emerging battleground in cancer therapy. Oncogenesis. 2022;11:22.
13. Groth C, Weber R, Lasser S, Özbay FG, Kurzay A, Petrova V, et al. Tumor promoting capacity of polymorphonuclear myeloid-derived suppressor cells and their neutralization. Int J Cancer. 2021;149:1628-38. doi: 10.1002/ijc.33731.
14. Davidov V, Jensen G, Mai S, Chen S-H, Pan PY. Analyzing one cell at a TIME: Analysis of myeloid cell contributions in the tumor immune microenvironment. Front Immunol.2021;11:645213. doi: 10.3389/fimmu.2020.645213.
15. De Cicco P, Ercolano G, Ianaro A. The new era of cancer immunotherapy: targeting myeloid-derived suppressor cells to overcome immune evasion. Front Immunol. 2020;11:1680. doi: 10.3389/fimmu.2020.01680.
16. Shi H, Li K, Ni Y, Liang X, Zhao X. Myeloid-derived suppressor cells: implications in the resistance of malignant tumors to T cell-based immunotherapy. Front Cell Dev Biol. 2021;9:707198. doi: 10.3389/fcell.2021.707198.
17. Gabrilovich DI. Myeloid-Derived Suppressor Cells. Cancer Immunol Res. 2017;5:3-8.
18. Ge Y, Cheng D, Jia Q, Xiong H, Zhang J. Mechanisms underlying the role of myeloid-derived suppressor cells in clinical diseases: good or bad. Immune Netw. 2021;21:e21. doi: 10.4110/in.2021.21.e21.
19. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun. 2016;7:12150. doi: 10.1038/ncomms12150.
20. Ni M, Cui J, Yang X, Ding Y, Zhao P, Hu T, et al. Dual roles of $CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}CD14^{-}$ myeloid-derived suppressor cells

with a granulocytic morphology following allogeneic hematopoietic stem cell transplantation: from inflammation promoters to immune suppressors within 90 days. Front Immunol. 2024;15:1403272. doi: 10.3389/fimmu.2024.1403272.

21. Coudereau R, Waeckel L, Cour M, Rimmele T, Pescarmona R, Fabri A, et al. Emergence of immunosuppressive LOX-1+ PMN-MDSC in septic shock and severe COVID-19 patients with acute respiratory distress syndrome. J Leukoc Biol. 2022;111:489-96. doi: 10.1002/JLB.4COVBCR0321-129R.

22. Sanchez-Pino MD, Dean MJ, Ochoa AC. Myeloid-derived suppressor cells (MDSC): When good intentions go awry. Cell Immunol. 2021;362:104302.

23. Tian X, Wang T, Zheng Q, Tao Y, Dai L, Shen H. Circulating CD15(+) LOX-1(+) PMN-MDSCs are a potential biomarker for the early diagnosis of non-small-cell lung cancer. Int J Clin Pract. 2021;75:e14317.

24. Lasser SA, Ozbay Kurt FG, Arkhypov I, Utikal J, Umansky V. Myeloid-derived suppressor cells in cancer and cancer therapy. Nat Rev Clin Oncol. 2024;21:147-64. doi: 10.1038/s41571-023-00846-y.

25. Bruderek K, Schirrmann R, Brandau S. Immunophenotyping of Circulating Myeloid-Derived Suppressor Cells (MDSC) in the Peripheral Blood of Cancer Patients. Methods Mol Biol. 2021;2236:1-7. doi: 10.1007/978-1-0716-1060-2_1.

26. Condamine T, Dominguez GA, Youn JI, Kossenkov AV, Mony S, Alicea-Torres K, et al. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. Sci Immunol. 2016;1:aaf8943. doi: 10.1126/sciimmunol.aaf8943.

27. Cassetta L, Baekkevold ES, Brandau S, Bujko A, Cassatella MA, Dorhoi A, et al. Deciphering myeloid-derived suppressor cells: isolation and markers in humans, mice and non-human primates. Cancer Immunol Immunother. 2019;68:687-97. doi: 10.1007/s00262-019-02302-2.

28. Cassetta L, Bruderek K, Skrzeczynska-Moncznik J, Osiecka O, Hu X, Rundgren IM, et al. Differential expansion of circulating human MDSC subsets in patients with cancer, infection and inflammation. J Immunother Cancer. 2020;8:e001223. doi: 10.1136/jitc-2020-001223.

29. Ostrand-Rosenberg S, Lamb TJ, Pawelec G. Here, There, and Everywhere: Myeloid-Derived Suppressor Cells in Immunology. J Immunol. 2023;210:1183-97.

30. Ren R, Xiong C, Ma R, Wang Y, Yue T, Yu J, et al. The recent progress of myeloid-derived suppressor cell and its targeted therapies in cancers. MedComm. 2023;4:e323. doi: 10.1002/mco2.323.

31. Zarobkiewicz M, Kowalska W, Chocholska S, Tomczak W, Szymańska A, Morawska I, et al. High M-MDSC Percentage as a Negative Prognostic Factor in Chronic Lymphocytic Leukaemia. Cancers. 2020;12:2614. doi: 10.3390/cancers12092614.

32. Ren W, Zhang X, Li W, Feng Q, Feng H, Tong Y, et al. Circulating and tumor-infiltrating arginase 1-expressing cells in gastric adenocarcinoma patients were mainly immature and monocytic Myeloid-derived suppressor cells. Sci Rep. 2020;10(1):8056. doi: 10.1038/s41598-020-64841-4.

33. Ye W, Zhou Y, Xu B, et al. CD247 expression is associated with differentiation and classification in ovarian cancer. Medicine. 2019;98:e18407.

34. Reuven O, Mikula I, Jr., Ashkenazi-Preiser H, Twaik N, Ben-Meir K, Meirow Y, et al. Functional assays evaluating immunosuppression mediated by myeloid-derived suppressor cells. Curr Protoc. 2022;2:e557. doi: 10.1002/cpz1.557.

35. Joshi S, Sharabi A. Targeting myeloid-derived suppressor cells to enhance natural killer cell-based immunotherapy. Pharmacol Ther. 2022;235:108114. doi: 10.1016/j.pharmthera.2022.108114.

36. Hwang JR, Byeon Y, Kim D, Park SG. Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. Exp Mol Med. 2020;52:750-61. doi: 10.1038/s12276-020-0435-8.