



Philadelphia-negative myeloproliferative neoplasms display alterations in monocyte subpopulations frequency and immunophenotype

Vitor Leonardo Bassan¹ · Gabriel Dessotti Barretto¹ · Felipe Campos de Almeida¹ · Patrícia Vianna Bonini Palma² · Larissa Sarri Binelli^{2,3} · João Paulo Lettieri da Silva^{2,3} · Caroline Fontanari¹ · Ricardo Cardoso Castro¹ · Lorena Lôbo de Figueiredo Pontes^{2,3} · Fabiani Gai Frantz¹ · Fabíola Attié de Castro¹

Received: 4 July 2022 / Accepted: 14 August 2022 / Published online: 29 September 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract

Philadelphia-negative myeloproliferative neoplasms (MPN) are clonal hematological diseases associated with driver mutations in JAK2, CALR, and MPL genes. Moreover, several evidence suggests that chronic inflammation and alterations in stromal and immune cells may contribute to MPN's pathophysiology. We evaluated the frequency and the immunophenotype of peripheral blood monocyte subpopulations in patients with polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF). Peripheral blood monocytes from PV ($n = 16$), ET ($n = 16$), and MF ($n = 15$) patients and healthy donors ($n = 10$) were isolated and submitted to immunophenotyping to determine the frequency of monocyte subpopulations and surface markers expression density. Plasma samples were used to measure the levels of soluble CD163, a biomarker of monocyte activity. PV, ET, and MF patients presented increased frequency of intermediate and non-classical monocytes and reduced frequency of classical monocytes compared to controls. Positivity for JAK2 mutation was significantly associated with the percentage of intermediate monocytes. PV, ET, and MF patients presented high-activated monocytes, evidenced by higher HLA-DR expression and increased soluble CD163 levels. The three MPN categories presented increased frequency of CD56⁺ aberrant monocytes, and PV and ET patients presented reduced frequency of CD80/86⁺ monocytes. Therefore, alterations in monocyte subpopulations frequency and surface markers expression pattern may contribute to oncoinflammation and may be associated with the pathophysiology of MPN.

Keywords Myeloproliferative neoplasms · Monocytes · Immune cells · Inflammation · Flow cytometry · Pathophysiology

✉ Vitor Leonardo Bassan
vitorbassan05@hotmail.com

Gabriel Dessotti Barretto
gabrielbarretto@usp.br

Felipe Campos de Almeida
fcampos13@gmail.com

Patrícia Vianna Bonini Palma
palmapvb@hemocentro.fmrp.usp.br

Larissa Sarri Binelli
larissabinelli@usp.br

João Paulo Lettieri da Silva
lettieri_84@yahoo.com.br

Caroline Fontanari
fontanari@usp.br

Ricardo Cardoso Castro
ricardocastro@usp.br

Lorena Lôbo de Figueiredo Pontes
lorenafgdo@usp.br

Fabiani Gai Frantz
frantz@usp.br

Fabíola Attié de Castro
castrofa@fcfrp.usp.br

- 1 Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Avenida do Café, s/nº - Vila Monte Alegre, Ribeirão Preto, São Paulo 14040-903, Brazil
- 2 Regional Blood Center of Ribeirão Preto, Rua Tenente Catão Roxo, 2501 - Vila Monte Alegre, Ribeirão Preto, São Paulo 14051-260, Brazil
- 3 Department of Medical Images, Hematology and Clinical Oncology, University Hospital of the Ribeirão Preto Medical School, University of São Paulo, Rua Tenente Catão Roxo, 3900 - Vila Monte Alegre, Ribeirão Preto, São Paulo 14015-010, Brazil

Introduction

Philadelphia-negative myeloproliferative neoplasms (MPN) are clonal hematological diseases characterized by expansion of precursor and mature myeloid cells in bone marrow and peripheral blood. The most frequent are polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF) [1, 2]. Erythrocyte hyperproliferation and high hemoglobin and hematocrit levels characterize PV, which may be accompanied by increased granulocytes count. ET and MF patients display atypical megakaryocyte proliferation: ET is characterized by increased peripheral platelet count, while MF is associated with inefficient erythropoiesis and deposition of collagen and reticulin fibers in bone marrow [3, 4].

MPN pathophysiology is associated with genetic driver mutations in the Janus Kinase 2 (JAK2), calreticulin (CALR), and thrombopoietin receptor (MPL) genes, which are gain-of-function mutations that trigger autonomous and constitutive activation of the JAK-STAT pathway. The JAK2V617F mutation is the most frequent genetic alteration in MPN. It is present in more than 95% of PV patients, while 50–70% of ET and 50–60% of MF patients harbor the JAK2 mutation. In addition, 20–30% of ET and MF patients may harbor CALR mutation and 5–10% patients may present MPL mutation [1, 2, 5].

Beyond the genetic alterations, MPN are considered as oncoinflammatory diseases due to the exacerbated inflammatory status [6–8]. Recent studies have reported increased levels of pro-inflammatory cytokines, chemokines, and growth factors in bone marrow niche and peripheral blood from PV, ET, and MF patients [6, 9, 10] and associated them with patients' clinic-laboratory parameters [9, 10]. Therefore, the pro-inflammatory profile seems to contribute to genetic and epigenetic instability, predisposition to cardiovascular events, fibrotic progression, and leukemic transformation in MPN [5, 6, 11, 12].

MPN patients may present alterations in immune cell compartments, such as decreased frequency and effector activities of natural killer cells [13], dysfunctions in lymphocyte subpopulations [14, 15], and immune response failure against pathogens [16–18]. Neutrophils, monocytes, and platelets activities have been associated with oncoinflammation and predisposition to cardiovascular events [19, 20].

Classical monocytes (CD14⁺⁺CD16⁻) correspond to 80–95% of peripheral blood monocytes in normal subjects and react against pathogens through phagocytosis, expression of scavenger receptors, high peroxidase activity, and antimicrobial mechanisms. Intermediate monocytes (CD14⁺⁺CD16⁺) corresponding to 2–10% of peripheral

blood monocytes are associated with inflammation potentiation, neoangiogenesis, lymphocytes chemoattraction, and secretion of interleukin (IL)-1 β , IL-6 and tumor necrosis factor alpha (TNF- α). Non-classical monocytes (CD14⁺CD16⁺⁺) correspond to 2–10% of peripheral blood monocytes and are related to vascular patrolling, immune surveillance, antiviral response, natural killer, and T lymphocytes modulation, and angiogenesis process [21–24].

In MPN, monocytosis has been associated with reduced life expectancy in older patients, worse prognosis in younger MF patients and the occurrence of cytogenetic and epigenetic abnormalities in PV patients [6, 25, 26]. Considering that immune cells play major roles in the modulation of inflammatory response, which is a key component in MPN pathophysiology, the present work investigated the frequency and immunophenotype of monocyte subpopulations in PV, ET, and MF patients.

Subjects, material, and methods

Demographic and clinic-laboratory features of patients and control subjects

Twenty milliliters of peripheral blood samples were collected from 16 PV patients (8 women, 8 men, median age = 62 years), 16 ET patients (8 women, 8 men, median age = 66.5 years), and 15 MF patients (5 women, 10 men, median age = 63 years) previously diagnosed according to the 2016 World Health Organization criteria. The control group consisted of 10 healthy individuals (6 women, 4 men, median age = 49 years old) from the university community.

In the PV group, 13 patients were JAK2V617F positive and three had undetermined mutational status; in the ET group, 12 patients were JAK2V617F positive, two were CALR positive, and two were triple negative; in the MF group, nine patients were JAK2V617F positive, two were CALR positive, one was triple negative, and three patients had undetermined mutational status. At the time of blood collection, eight PV patients, 11 ET patients, and nine MF patients were undergoing treatment with hydroxycarbamide (Table 1). None of the MPN patients evaluated in this study was in use of JAK inhibitors or interferon-alpha at the time of blood collection.

The Ethics Committee for Human Research from the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (FCFRP-USP) and from the University Hospital of the Ribeirão Preto Medical School (HCFMRP-USP), University of São Paulo (USP), Ribeirão Preto, Brazil approved the study protocols (#24508619.1.0000.5403 and

Table 1 Demographic and clinic-laboratory data from patients and control subjects

	CTRL	PV	ET	MF
Age (years) Median, range	49 (25–65)	62 (36–89)	66.5 (28–81)	63 (47–85)
Gender (n)	<i>n</i> = 10	<i>n</i> = 16	<i>n</i> = 16	<i>n</i> = 15
Male	4	8	8	10
Female	6	8	8	5
<i>Mutation status</i>				
JAK2V617F ⁺	–	13	12	9
CALR ⁺	–	–	2	2
Triple negative	–	–	2	1
Undetermined	–	3	–	3
<i>Treatment</i>				
Hydroxycarbamide	–	8	11	9

CTRL Controls; ET Essential thrombocythemia; MF Primary myelofibrosis; PV Polycythemia vera

Table 2 Features of the antibodies used for immunophenotyping

Antibody	Clone	Fluorochrome	Volume	Source
CD14	M5E2	APC	5µL	BD Pharmingen
CD16	3G8	PECy5	5µL	BD Pharmingen
CD45	2D1	APC-H7	5µL	BD Pharmingen
CD56	B159	FITC	5µL	BD Pharmingen
CD64	10.1	FITC	5µL	BD Pharmingen
CD80	2D10.4	PE	5µL	BD Pharmingen
CD86	IT2.2	PE	5µL	BD Pharmingen
HLA-DR	G46-6	FITC	5µL	BD Pharmingen

#24508619.1.3001.5440). All the participants signed the informed consent form.

Plasma samples

Plasma samples were obtained after centrifuging peripheral blood collected in EDTA tubes (Vacutainer®; Becton, Dickinson and Company) at 400 × *g* for 10 min at 22 °C (Eppendorf 5810R Centrifuge) and stored at – 80 °C in a freezer for further quantification of soluble CD163 (sCD163) biomarker using ELISA.

Isolation of peripheral blood monocytes

Blood samples were diluted in phosphate buffered saline (PBS) and submitted to Ficoll®-Paque (Sigma-Aldrich, San Luis, MO, USA) density gradient centrifugation protocol at 400 × *g* for 35 min at 22 °C (Eppendorf 5810R Centrifuge) to obtain peripheral blood mononuclear cells. The cells were counted in a hemocytometer and the cell concentration was adjusted to 1 × 10⁷ cells per ml for immunophenotyping.

Immunophenotyping of monocyte subpopulations

For monocyte immunophenotyping, 500 µl of mononuclear cells suspension in PBS were incubated with 5 µl of combined antibodies as follows: (i) an unlabeled tube for negative cells acquisition; (ii) a tube with anti-CD45 APC-H7, anti-CD14 APC, anti-CD16 PECy5, and anti-CD80/86 PE antibodies; (iii) a tube with anti-CD45 APC-H7, anti-CD14 APC, anti-CD16 PECy5 and anti-HLA-DR FITC antibodies; and (iv) a tube with anti-CD45 APC-H7, anti-CD14 APC, anti-CD16 PECy5, and anti-CD56 FITC antibodies (Table 2).

After antibody labeling, cells were washed once with PBS, centrifuged at 400 × *g* for 5 min, filtered, and suspended in 1 mL of PBS and analyzed in a BD FACSAria™ Fusion flow cytometer, with 10,000 events acquired.

The monocyte population was gated by forward scatter area (FSC-A) and side scatter area (SSC-A) using pseudocolor dot plots (Supplementary Fig. 1-I). Doublets were excluded applying FSC-A versus FSC-height (FSC-H) gate (Supplementary Fig. 1-II). CD14⁺ cells—total monocyte population—were selected (Supplementary Fig. 1-III). Monocyte subpopulations were defined applying CD45 versus CD16 dot plots (Supplementary Fig. 1-IV). CD16 heatmap gradient expression and CD16 median of fluorescence intensity (MFI) were used to delimit the monocyte subpopulations in classical (CD16-), intermediate (CD16+), and non-classical (CD16++) (Supplementary Fig. 1-V). The frequency of each monocyte subpopulations were analyzed by dot plots and expressed by percent of labeled cells.

The CD64, CD80/86, HLA-DR, and CD56 expression in monocytes were evaluated by histograms in each monocyte subpopulations gates and the results were expressed in median of fluorescence intensity (MFI). The data were analyzed using BDFACST™ Diva software in the flow cytometer and FlowJo™ v10 software.

Quantification of soluble CD163 in plasma

Plasma concentration of the monocyte biomarker sCD163 was quantified using the DuoSet ELISA kit (R&D Systems, Minneapolis, Minn, USA), according to the manufacturer's instructions. The concentration of sCD163 was determined by linear regression and the results were expressed in pg/mL.

Statistical analyses

The Mann–Whitney and ANOVA non-parametric statistical tests were used to compare monocyte subpopulations frequency and surface markers MFI among PV, ET, and MF patients and the control group. Statistical differences were considered significant when the p value was <0.05 . The results were expressed as mean and standard deviation. Statistical analyses were performed using the GraphPad Prism 5.0 software (Graph-Pad Software, San Diego, CA, USA).

Results

Frequency of monocyte subpopulations in MPN

Compared with the control group, (i) PV (35.68 ± 24.42 , $p < 0.0001$), ET (39.56 ± 25.32 , $p = 0.0002$) and

MF (32.34 ± 26.02 , $p < 0.0001$) patients presented decreased frequency of classical monocytes; (ii) PV and MF patients presented increased frequency of intermediate (PV = 39.00 ± 20.03 , $p = 0.0002$; MF = 31.66 ± 15.67 , $p = 0.0002$) and non-classical monocytes (PV = 27.35 ± 26.56 , $p = 0.0072$; MF = 33.53 ± 26.90 , $p = 0.0043$); and (iii) ET patients presented increased frequency of intermediate monocytes (38.53 ± 20.84 , $p = 0.0012$). PV, ET, and MF patients presented similar frequencies of monocyte subpopulations (Fig. 1).

Frequency of intermediate monocytes is associated with JAK2 mutation in MPN

The frequencies of classical, intermediate, and non-classical monocytes from PV, ET, and MF patients were analyzed according to JAK2 mutation status. JAK2V617F⁺ MPN patients presented higher frequency of intermediate monocytes than JAK2V617F⁻ patients (50.60 ± 22.82 , $p = 0.0090$) (Fig. 2). ET and MF patients positive for JAK2V617F or CALR mutations had similar frequencies of monocyte subpopulations (data not shown).

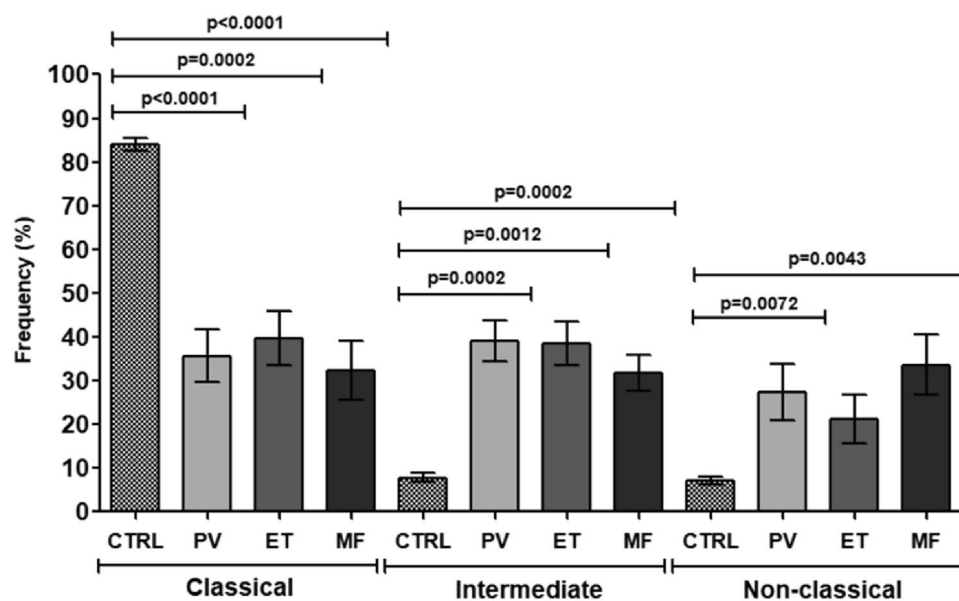
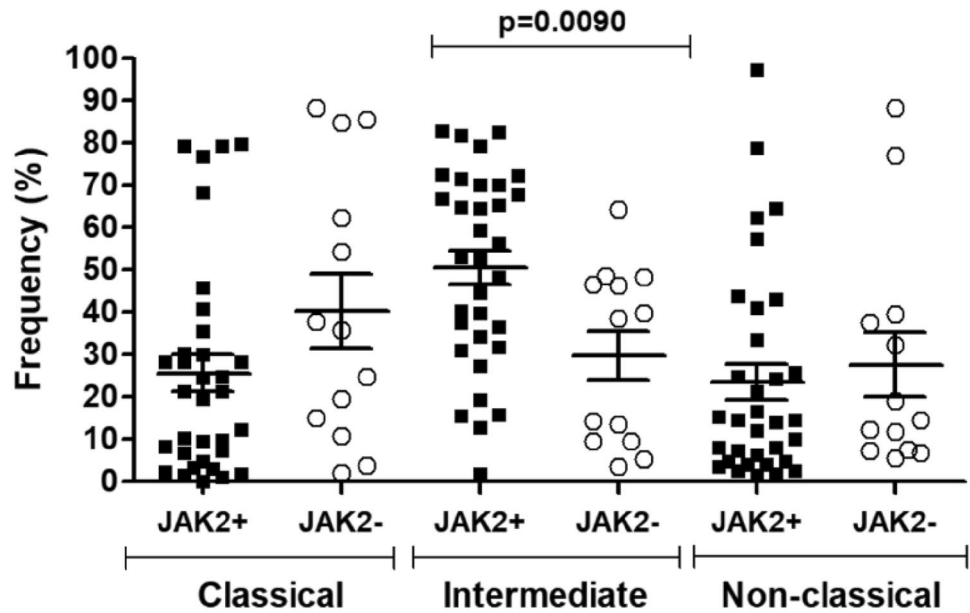


Fig. 1 Percentage of monocyte subpopulations in myeloproliferative neoplasms (MPN). Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF) patients presented lower frequency of classical monocytes ($p < 0.0001$, $p = 0.0002$, $p < 0.0001$, respectively); PV, ET, and MF presented increased intermediate monocytes frequency ($p = 0.0002$, $p = 0.0012$, $p = 0.0002$,

respectively). PV and MF patients presented increased frequency of non-classical monocytes compared to the controls ($p = 0.0072$, $p = 0.0043$, respectively). Statistical differences were calculated using ANOVA non-parametric test and considered significant when $p < 0.05$. The results were expressed in mean and standard deviation

Fig. 2 Frequency of monocyte subpopulations according to JAK2 mutation status in myeloproliferative neoplasms (MPN). The percentage of classical, intermediate, and non-classical monocytes in MPN patients was shown according to positivity for JAK2V617F mutation and plotted in the scatter plot. JAK2-positive MPN patients presented higher frequency of intermediate monocytes than JAK2-negative patients ($p=0.0090$). Statistical differences were calculated using the Mann–Whitney test and considered significant when $p < 0.05$



Surface markers distribution in monocyte subpopulations from MPN patients

The frequency of CD64⁺ monocyte subpopulations did not differ in PV, ET, and MF patients (data not shown).

In PV, there is higher frequency of CD80/86⁺ non-classical than CD80/86⁺ classical monocytes (classical = 18.81 ± 22.54 , non-classical = 37.77 ± 25.60 , $p=0.0428$) (Fig. 3A). In ET, there is more CD80/86⁺ non-classical monocytes than classical and intermediate monocytes (classical = 33.45 ± 37.25 , intermediate = 35.64 ± 30.86 , non-classical = 60.03 ± 25.82 , $p=0.0203$, and $p=0.0229$, respectively) (Fig. 3B). There were no differences in MF patients (Fig. 3C).

PV patients presented higher frequency of HLA-DR⁺ classical monocytes than HLA-DR⁺ non-classical monocytes (classical = 90.06 ± 18.64 , non-classical = 63.86 ± 35.13 , $p=0.0104$) (Fig. 3D). The frequency of HLA-DR⁺ intermediate monocytes is also higher than the frequency of HLA-DR⁺ non-classical monocytes (intermediate monocytes = 84.05 ± 23.04 , non-classical = 63.86 ± 35.13 , $p=0.0454$) (Fig. 3D). ET and MF patients did not present significant differences (Fig. 3E–F).

CD56⁺ non-classical monocytes frequency is higher than CD56⁺ classical monocytes in PV (classical = 30.23 ± 18.28 , non-classical = 51.74 ± 26.25 , $p=0.0414$) (Fig. 3G). CD56⁺ non-classical monocytes frequency is higher than CD56⁺ classical and intermediate monocytes in ET patients (classical = 33.68 ± 26.85 , intermediate = 46.84 ± 24.92 , non-classical = 69.71 ± 24.50 , $p=0.0056$, and $p=0.0240$) (Fig. 3H). No differences were seen in MF patients (Fig. 3I).

Frequency of monocytes positive for CD64, CD80/86, HLA-DR and CD56 in MPN

MPN patients and the control group had similar frequencies of CD64⁺ monocytes. Compared with the control group, PV (30.08 ± 24.59 , $p < 0.0001$) and ET (43.14 ± 33.50 , $p=0.0016$) patients exhibited reduced frequency of CD80/86⁺ monocytes, but the CD80/86 positivity in total monocytes population was unaltered in MF patients (Fig. 4A).

Compared with the control group, PV (18.81 ± 22.54 , $p=0.0013$, and 26.76 ± 20.22 , $p=0.0005$, respectively) and ET (33.45 ± 37.25 , $p=0.0406$, and 35.64 ± 30.86 , $p=0.0128$, respectively) patients exhibited lower frequencies of CD80/86⁺ classical and intermediate monocytes. MF patients presented higher frequency of CD80/86⁺ intermediate monocytes than PV (43.74 ± 36.58 , $p=0.0007$) and ET patients (60.03 ± 25.82 , $p=0.0229$). PV patients had lower frequency of CD80/86⁺ non-classical monocytes than the control group (37.77 ± 25.60 , $p=0.0194$) and ET patients (60.03 ± 25.82 , $p=0.0440$) (Fig. 4B).

PV (77.66 ± 31.65), ET (73.17 ± 30.14), and MF (82.51 ± 31.28) patients displayed increased frequency of HLA-DR⁺ monocytes when compared with controls ($p < 0.0001$) (Fig. 4A). Classical monocytes from PV (90.06 ± 18.64 , $p=0.0046$), ET (83.63 ± 19.17 , $p=0.0251$), and MF (83.22 ± 30.48 , $p=0.0229$) patients had higher HLA-DR positivity than controls. Intermediate monocytes from PV (84.05 ± 23.04 , $p=0.0026$), ET (72.79 ± 29.13 , $p=0.0185$), and MF (80.62 ± 32.34 , $p=0.0111$) presented the same profile as classical monocytes. PV (63.86 ± 18.43 , $p=0.0312$) and MF (84.01 ± 35.67 , $p=0.0262$) patients exhibited higher frequency of HLA-DR⁺ non-classical

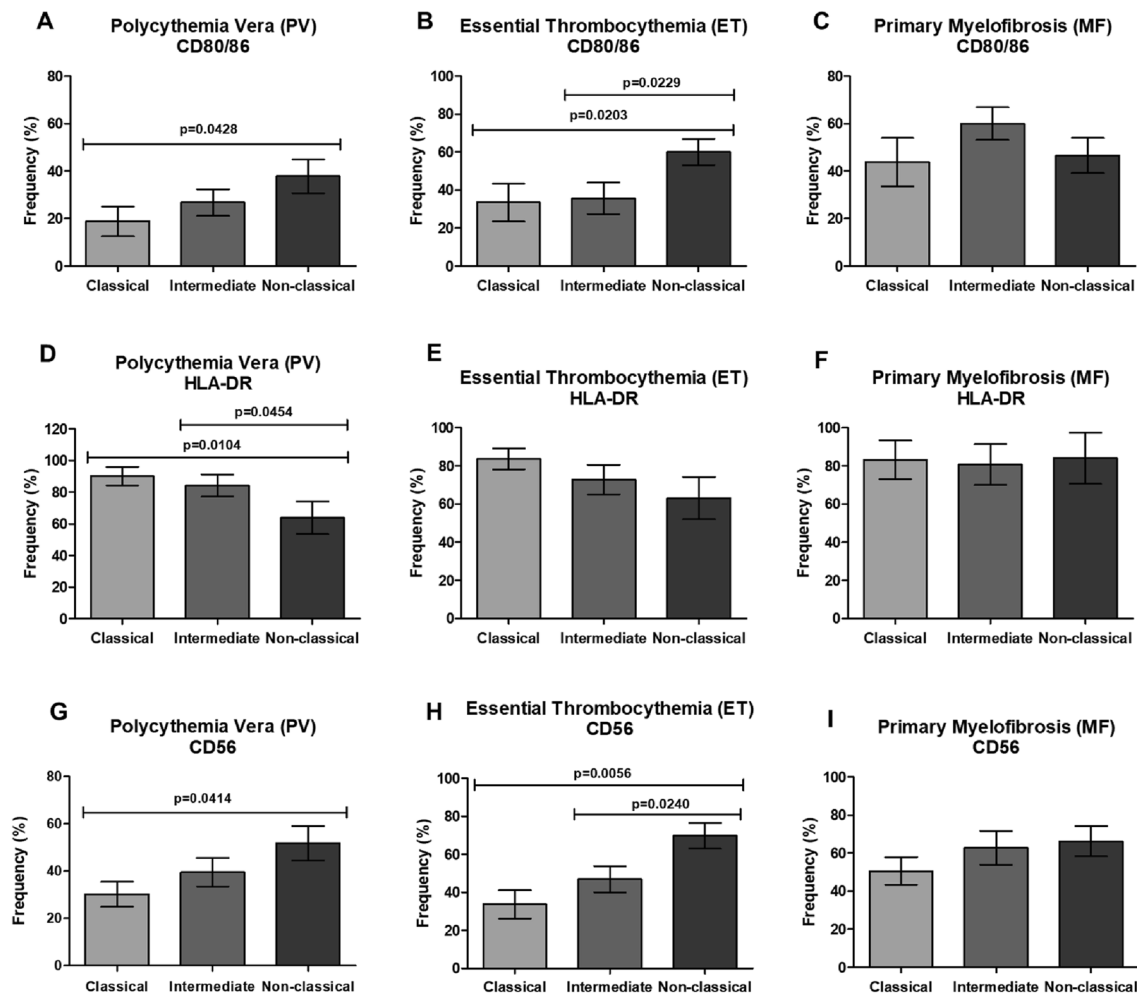


Fig. 3 Surface markers distribution in monocytes subpopulations in myeloproliferative neoplasms (MPN). The percentage of classical, intermediate, and non-classical monocytes expressing the surface markers CD80/86, HLA-DR and CD56 was calculated in patients with polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF) and control subjects (CTRL). **A–C** Per-

centage of CD80/86⁺ monocytes in PV, ET and MF. **D–F** Percentage of HLA-DR⁺ monocytes in PV, ET and MF. **G–I** Frequency of CD56⁺ monocytes in PV, ET and MF. Statistical differences were calculated using ANOVA non-parametric test and considered significant when $p < 0.05$. The results were expressed in mean and standard deviation

monocytes than controls. MF patients had higher frequency of non-classical HLA-DR⁺ monocytes than PV patients ($p = 0.0384$) (Fig. 4B).

Total monocytes from PV (40.77 ± 21.09 , $p = 0.132$), ET (50.08 ± 29.00 , $p = 0.0026$), and MF (59.58 ± 27.07 , $p < 0.0001$) patients displayed higher CD56 positivity than controls. MF patients had more CD56⁺ monocytes than PV patients ($p = 0.0038$; Fig. 4A). PV (30.23 ± 18.28 , $p = 0.0060$), ET (33.68 ± 26.85 , $p = 0.0174$), and MF (50.58 ± 25.26 , $p = 0.0005$) patients presented higher frequency of CD56⁺ classical monocytes than controls. MF patients exhibited higher frequency of CD56⁺ intermediate monocytes (62.70 ± 29.55 , $p = 0.0185$); ET (69.71 ± 24.50 , $p = 0.0140$) and MF (66.27 ± 26.19 , $p = 0.0462$) patients

presented higher frequency of CD56⁺ non-classical monocytes (Fig. 4B).

Expression density of CD64, CD80/86, HLA-DR and CD56 in MPN monocytes

MPN patients and control subjects did not significantly differ in monocytes CD64 expression. Compared with controls, PV patients expressed lower CD80/86 levels (329.81 ± 440.44 , $p = 0.0275$), while MF patients expressed similar CD80/86 levels in the total monocyte population (Fig. 5A).

PV (2900.03 ± 2527.01 , $p = 0.0025$), ET (1420.83 ± 1255.32), and MF (9355.63 ± 6921.76 , $p = 0.0033$) patients expressed more HLA-DR in the

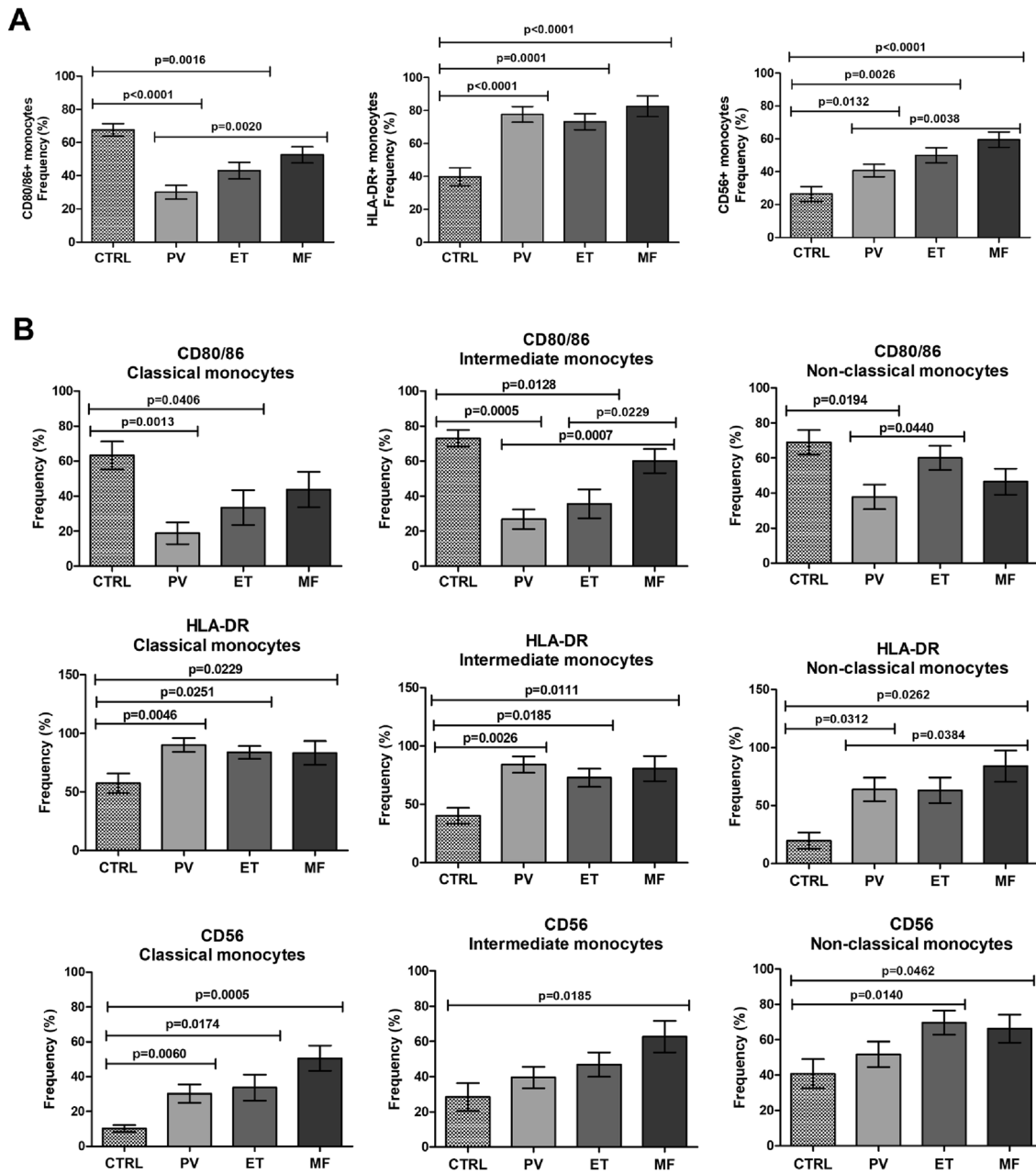


Fig. 4 Frequency of CD64, CD80/86, HLA-DR, and CD56-positive monocytes in myeloproliferative neoplasms (MPN). The percentage of monocytes positive for surface markers was analyzed in total monocyte population and its subpopulations. **A** Frequency of monocytes in the total monocyte population expressing CD64, CD80/86, HLA-DR, and CD56 in patients with polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF), and

control subjects (CTRL). **B** Frequency of monocytes in the classical, intermediate, and non-classical monocyte subpopulations expressing CD64, CD80/86, HLA-DR, and CD56 in patients with PV, ET, and MF, and CTRL. The results were expressed as mean and standard deviation. Statistical differences were determined using ANOVA non-parametric test and considered significant when $p < 0.05$

total monocytes population than control subjects (775.86 ± 616.83). MF patients expressed more HLA-DR in the total monocytes population than PV ($p = 0.0066$) and ET ($p = 0.0040$) patients (Fig. 5A). PV (2759.00 ± 2533.28 , $p = 0.0172$) and ET (1085.36 ± 912.21 , $p = 0.0109$) patient's

classical monocytes and MF patient's intermediate monocytes (9840.06 ± 7074.64 , $p = 0.0250$) presented increased HLA-DR expression than controls (Fig. 5B).

PV (208.61 ± 554.73 ; $p = 0.0132$) and ET (505.77 ± 672.22 ; $p = 0.0026$) patients' total monocytes population expressed lower CD56 levels than

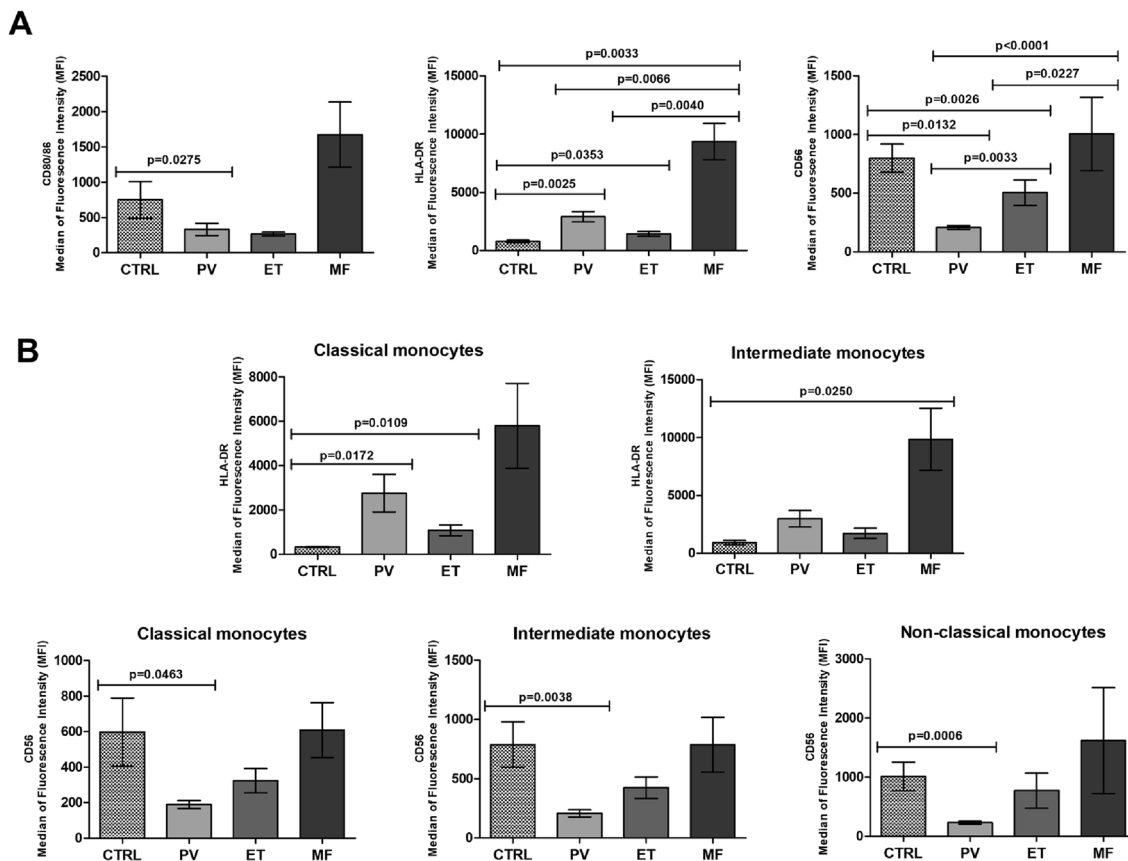


Fig. 5 Expression of CD64, CD80/86, HLA-DR, and CD56 in myeloproliferative neoplasms (MPN) monocytes. The median of fluorescence intensity (MFI) was calculated to determine surface antigen density in monocytes from control subjects (CTRL) and patients with polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF). **A** CD64, CD80/86, HLA-DR, and CD56

expression in total monocytes population. **B** CD64, CD80/86, HLA-DR, and CD56 expression in classical, intermediate, and non-classical monocyte subpopulations. The results were expressed as mean and standard deviation. Statistical differences were determined using ANOVA non-parametric test and considered significant when $p < 0.05$

controls (798.76 ± 554.73). MF patients' monocytes presented higher CD56 expression than PV and ET (1004.62 ± 1432.14 , $p = 0.02247$, and $p < 0.0001$), while ET patients' monocytes presented higher CD56 expression than PV ($p = 0.0033$) (Fig. 5A). Compared with the control group, monocyte subpopulations from ET and MF patients expressed similar levels of CD56, while classical, intermediate, and non-classical monocytes from PV patients expressed lower CD56 levels (189.36 ± 74.22 , $p = 0.0463$; 206.55 ± 100.38 , $p = 0.0038$; and 229.91 ± 86.61 , $p = 0.0006$, respectively) (Fig. 5B).

sCD163 concentration in MPN patients' plasma

Plasma sCD163 concentration was quantified in PV ($n = 13$), ET ($n = 13$), MF ($n = 14$) patients and control subjects ($n = 11$). PV ($11,454.52 \pm 4939.96$, $p = 0.0499$), ET ($11,675.92 \pm 4652.72$, $p = 0.0281$), and MF ($16,247.93 \pm 9556.81$, $p = 0.0281$) patients presented

higher sCD163 concentration than the control group (Fig. 6). The three groups of MPN patients did not differ with respect to the sCD163 plasma concentration.

Discussion

The pro-inflammatory *status* seem to contribute to MPN pathophysiology [4, 6, 8], once the isolated presence of driver mutations are insufficient to fully explain the mechanisms underlying the MPN pathogenesis, progression, and phenotypes [27]. Many authors have reported the association of oncoinflammation [6–8, 27] with genetic instability, symptomatology, endothelial dysfunction, and pro-coagulant state in MPN [9, 10, 18].

PV, ET, and MF patients, in the present study, exhibited increased frequency of intermediate and non-classical monocytes in peripheral blood. Intermediate and non-classical monocytes are cells that present mature phenotypes

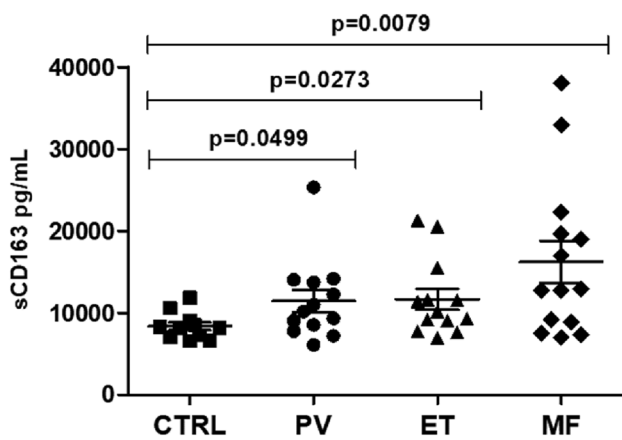


Fig. 6 Quantification of soluble CD163 (sCD163) in myeloproliferative neoplasms (MPN). The plasma concentration of the monocyte activity biomarker sCD163 in patients with polycythemia vera (PV, $n = 13$), essential thrombocythemia (ET, $n = 13$), primary myelofibrosis (MF, $n = 14$), and control subjects (CTRL, $n = 11$) was plotted in the scatter plot. The MPN patients presented higher plasma concentration of sCD163 than CTRL group (PV: $p = 0.0499$; ET: $p = 0.0273$; MF: $p = 0.0079$). Statistical differences were determined using ANOVA non-parametric test and considered significant when $p < 0.05$

compared with classical monocytes and play complex activities, including modulation of effector functions of helper lymphocytes and natural killer cells, neutrophil recruitment, intravascular homeostasis, and immune surveillance. These monocyte subpopulations are able to induce potent inflammatory responses that give support to the chronic-inflammatory milieu in neoplastic diseases [24].

Murine B-cell acute lymphoblastic leukemia model present increased frequency of non-classical and reduced frequency of classical monocytes in peripheral blood and spleen [28]. The frequency of intermediate and non-classical monocytes is augmented in acute myeloid leukemia and associated with genetic instability and worse prognosis. The increased frequency of these cells in acute myeloid leukemia is inversely correlated with TCD4⁺ lymphocytes and positively correlated with granulocytes count. This data emphasizes that CD16⁺ monocytes may modulate the effector functions of other immune cells [29].

Tie-2-expressing monocytes, with pro-angiogenic ability, and monocytes with T lymphocytes-suppressive capacity were described in chronic lymphocytic leukemia [30]. The gene signature in chronic lymphocytic leukemia is associated with increased CD16 expression and corresponds to the expansion of intermediate and non-classical monocytes. In addition, monocytes' genomic abnormalities, immunosuppressive characteristics and dysfunctions in phagocytic and costimulatory activity were associated with the leukemic cells pro-tumorigenic microenvironment [30], emphasizing the influence of the microenvironment in the phenotype and effector functions of monocytes.

Monocyte subpopulations contribute to the pathogenesis and progression of cardiovascular diseases like thrombosis—a frequent comorbidity of MPN patients, reflecting the pro-inflammatory status [31, 32].

Expansion of intermediate and non-classical monocytes occurs in bone marrow and peripheral blood from PV patients, in which high plasma levels of the pro-inflammatory cytokines IL-6, IL-8, MIP-1 α , and TNF- α secreted by monocytes correlate with disease severity and risk to leukemic transformation [33].

In our study, higher frequency of intermediate monocytes in JAK2⁺ patients strengthens the hypothesis that exacerbated inflammation linked to JAK-STAT pathway activation may contribute to pro-inflammatory monocytes differentiation [33].

The higher frequency of non-classical monocytes in MF patients when compared with PV and ET patients and the control group was a relevant finding, as it suggested that these cells participate in the mechanisms of secretion of factors associated with fibrosis. The significant increased frequency of non-classical monocytes in PV patients' peripheral blood when compared with the control group suggests the potential participation of these cells in the risk for thromboembolic events. The literature has reported the participation of non-classical monocytes in intravascular immune responses, wound healing, and regulation of vascular homeostasis [21, 34].

Here we described the altered expression of monocytes markers in PV, ET, and MF patients. PV and ET patients presented lower frequency of CD80/86-expressing monocytes than controls and MF patients. Low CD80 expression is associated with weak induction of immune surveillance and facilitation of neoplastic cells spread in patients with colorectal cancer [35]. This profile was also reported in other neoplasms, like multiple myeloma, leukemia and carcinomas, and represents one of the mechanisms of immune response evasion that leads to insufficient activation of T lymphocytes [36].

The lower frequency of CD80/86⁺ monocytes in PV and ET patients compared with healthy individuals and MF patients suggested that circulating monocytes, in these patients, present less costimulatory capacity. On the other hand, CD80/86 expression levels in MF patients' monocytes were similar to those detected in controls, pointing to differences in costimulation profile between the three diseases.

The frequency of HLA-DR⁺ monocytes was higher in PV, ET, and MF than in controls. High HLA-DR expression is related to monocytes activation and pro-inflammatory cytokines secretion [37]. In inflammatory diseases like rheumatoid arthritis and sepsis, high HLA-DR expression and low CD80/86 expression are associated with increased activation of circulating monocytes and reduced costimulatory activity, respectively [37]. PV and ET patients from the

present study exhibited this profile, which is also associated with tumor cells resistance to apoptosis in other neoplasms, such as in colon cancer [38, 39].

The fact that PV, ET, and MF patients exhibited high sCD163 plasma levels, which is a specific biomarker of monocyte activation, suggested the presence of high-activated monocytes in peripheral blood of these patients. Plasma CD163 concentration correlates to the exacerbated activity of monocytes and macrophages in bacterial sepsis, hepatitis, rheumatoid arthritis, Crohn's disease, ulcerative colitis, atherosclerotic events, and type 2 diabetes [40].

Newly diagnosed patients with multiple myeloma present increased number of extracellular vesicles carrying sCD163 in plasma and increased CD163 antigenic expression on intermediate monocytes seems to be implicated in malignant transformation of plasma cells [41]. sCD163 levels have an important prognostic value in solid and hematological neoplasms, as they correlate with patients' worse overall survival and lower progression-free survival [42].

PV, ET, and MF patients presented high frequency of CD56⁺ aberrant monocytes in peripheral blood. CD56⁺ monocytes expand during chronic-inflammatory and neoplastic conditions, but their effector functions were not fully elucidated [43]. CD56⁺ monocyte counts are increased in peripheral blood from patients with endocrine neoplasms in thyroid and adrenal glands, gastrointestinal neoplasms, leukemias, and lymphomas [44]. This subpopulation is known as "tumor-lysing monocytes", express natural killer cell markers and induce apoptosis of tumor cells when stimulated *in vitro* by IFN- α . It highlights their possible contribution to immune surveillance as lysis-promoting cytotoxic cells when stimulated [44].

CD56⁺ monocytes are expanded in patients with moderate and severe COVID-19 as compared with mild-asymptomatic patients. These cells are associated with IFN- γ and granzyme B secretion and high-activated profile; their number increase in the first 5 days of infection and they remain proliferative at the 16th day after the PCR test confirmation. CD56⁺ monocytes probably participate in the exacerbated immune response that lead to tissue and organ damage and dysfunction in COVID-19 and the frequency of these cells positively correlate with hypertension in these patients [45].

The frequency of CD56⁺ monocytes in acute myeloid leukemia correlates with worse prognosis and low patients' overall survival [46]. In MPN, CD56⁺ monocytes were recently reported in ET patients and associated with CXCL1 secretion, a chemokine that have been considered as a potential biomarker of ET evolution to MF [47].

The present work reported higher CD56 expression in MF monocytes, which indicates a possible association between CD56 expression and MPN severity, once MF is the disease category with worse prognosis.

Taken together, the results point to alterations in monocyte subpopulations frequency in MPN patients' peripheral blood, suggesting that exacerbated oncoinflammatory activity affects the monocyte compartments. JAK2V617F mutation is probably linked to the intermediate monocytes shifting. Surface markers pattern alterations and increased sCD163 levels indicates hyperactivation and possible perturbations in monocytes' effector activities. Therefore, monocytes may contribute to oncoinflammation in MPN.

Further investigations are necessary to better understand the immune response alterations in MPN in order to determine the immune profiles of PV, ET, and MF patients, helping to develop new therapeutic interventions that restore the immune cell antitumor activity and dampen the exacerbated oncoinflammation in MPN.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12032-022-01825-6>.

Author contributions VLB collected the samples, performed monocytes isolation and immunophenotyping, analyzed data, and wrote the paper. GDB, FCA, LSB, and JPL helped to collect samples, isolate monocytes, and select patients. PVBP performed samples acquisition for the immunophenotyping analysis. CF and RCC performed the ELISA assay. VLB, LLFP, FGF, and FAC designed the study, discussed the results, provided the research funding, and revised the manuscript.

Funding This study received financial support from São Paulo Research Foundation (FAPESP, grants #2019/18013-8 and #2018/19714-7), National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES, grant #88887.369858/2019-00) and Center for Cell-based Therapy (CTC, Grant #2013/08135-2). FAC is a recipient of CNPq fellowship.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

References

1. Vainchenker W, Kralovics R. Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. *Blood*. 2017;129(6):667–79. <https://doi.org/10.1182/blood-2016-10-695940>.
2. Greenfield G, McMullin MF, Mills K. Molecular pathogenesis of the myeloproliferative neoplasms. *J Hematol Oncol*. 2021;14(1):103. <https://doi.org/10.1186/s13045-021-01116-z>.
3. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–405. <https://doi.org/10.1182/blood-2016-03-643544>.
4. Spivak JL. Myeloproliferative neoplasms. *N Engl J Med*. 2017;376:2168–81. <https://doi.org/10.1056/NEJMra1406186>.
5. Agarwal A, Morrone K, Bartenstein M, et al. Bone marrow fibrosis in primary myelofibrosis: pathogenic mechanisms and the role

- of TGF- β . *Stem Cell Investig.* 2016;3:5. <https://doi.org/10.3978/j.issn.2306-9759.2016.02.03>.
6. Hasselbalch HC. Perspectives on chronic inflammation in essential thrombocythemia, polycythemia vera, and myelofibrosis: is chronic inflammation a trigger and driver of clonal evolution and development of accelerated atherosclerosis and second cancer? *Blood.* 2012;119(14):3219–25. <https://doi.org/10.1182/blood-2011-11-394775>.
 7. Gleitz HFE, Benabid A, Schneider RK. Still a burning question: the interplay between inflammation and fibrosis in myeloproliferative neoplasms. *Curr Opin Hematol.* 2021;28(5):364–71. <https://doi.org/10.1097/MOH.0000000000000669>.
 8. Fleischman AG. Inflammation as a driver of clonal evolution in myeloproliferative neoplasm. *Mediators Inflamm.* 2015. <https://doi.org/10.1155/2015/606819>.
 9. Cacemiro MC, Cominal JG, Tognon R, et al. Philadelphia-negative myeloproliferative neoplasms as disorders marked by cytokine modulation. *Hematol Transfus Cell Ther.* 2018;40(2):120–31. <https://doi.org/10.1016/j.htct.2017.12.003>.
 10. Cominal JG, Cacemiro MDC, Berzoti-Coelho MG, et al. Bone marrow soluble mediator signatures of patients with philadelphia chromosome-negative myeloproliferative neoplasms. *Front Oncol.* 2021;11:665037. <https://doi.org/10.3389/fonc.2021.665037>.
 11. Landskron G, De Le FM, Thuwajit P, et al. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res.* 2014. <https://doi.org/10.1155/2014/149185>.
 12. Desterke C, Christophe Martinaud C, Ruzehaji N, et al. Inflammation as a keystone of bone marrow stroma alterations in primary myelofibrosis. *Mediators Inflamm.* 2015. <https://doi.org/10.1155/2015/415024>.
 13. Arantes AQ, Leal CT, Silva CA, et al. Decreased activity of NK cells in myeloproliferative neoplasms. *Blood.* 2015;126(23):1637. <https://doi.org/10.1182/blood.V126.23.1637.1637>.
 14. Holmström MO, Hasselbalch HC, Andersen MH. Cancer immune therapy for philadelphia chromosome-negative chronic myeloproliferative neoplasms. *Cancers.* 2020;12(7):1763. <https://doi.org/10.3390/cancers12071763>.
 15. Cervantes F, Hernández-Boluda JC, Villamor N, et al. Assessment of peripheral blood lymphocyte subsets in idiopathic myelofibrosis. *Eur J Haematol.* 2000;65(2):104–8. <https://doi.org/10.1034/j.1600-0609.2000.90262.x>.
 16. Skov V, Thomassen M, Riley CH, et al. Gene expression profiling with principal component analysis depicts the biological continuum from essential thrombocythemia over polycythemia vera to myelofibrosis. *Exp Hematol.* 2012;40(9):771–780.e19. <https://doi.org/10.1016/j.exphem.2012.05.011>.
 17. Barone M, Catani L, Ricci F, et al. The role of circulating monocytes and JAK inhibition in the infectious-driven inflammatory response of myelofibrosis. *Oncoimmunology.* 2020;9(1):1782575. <https://doi.org/10.1080/2162402X.2020.1782575>.
 18. Strickland M, Quek L, Psaila B. The immune landscape in BCR-ABL negative myeloproliferative neoplasms: inflammation, infections and opportunities for immunotherapy. *Br J Haematol.* 2022;196(5):1149–58. <https://doi.org/10.1111/bjh.17850>.
 19. Ferrer-Marín F, Cuenca-Zamora E, Guijarro-Carrillo PJ, et al. Emerging role of neutrophils in the thrombosis of chronic myeloproliferative neoplasms. *Int J Mol Sci.* 2021;22(3):1143. <https://doi.org/10.3390/ijms22031143>.
 20. Bar-Natan M, Hoffman R. New insights into the causes of thrombotic events in patients with myeloproliferative neoplasms raise the possibility of novel therapeutic approaches. *Haematologica.* 2019;104(1):3–6. <https://doi.org/10.3324/haematol.2018.205989>.
 21. Sampath P, Moideen K, Ranganathan UD, et al. Monocyte subsets: phenotypes and function in tuberculosis infection. *Front Immunol.* 2018;9:1726. <https://doi.org/10.3389/fimmu.2018.01726>.
 22. Italiani P, Boraschi D. From monocytes to M1/M2 macrophages: phenotypical vs functional differentiation. *Front Immunol.* 2014;5:514. <https://doi.org/10.3389/fimmu.2014.00514>.
 23. Richards DM, Hettinger J, Feuerer M. Monocytes and macrophages in cancer: development and functions. *Cancer Microenviron.* 2013;6(2):179–91. <https://doi.org/10.1007/s12307-012-0123-x>.
 24. Narasimhan PB, Eggert T, Zhu YP, et al. Patrolling monocytes control NK cell expression of activating and stimulatory receptors to curtail lung metastases. *J Immunol.* 2020;204(1):192–8. <https://doi.org/10.4049/jimmunol.1900998>.
 25. Boiocchi L, Espinal-Witter R, Geyer JT, et al. Development of monocytosis in patients with primary myelofibrosis indicates an accelerated phase of the disease. *Mod Pathol.* 2013;26(2):204–12. <https://doi.org/10.1038/modpathol.2012.165>.
 26. Morsia E, Gangat N. Myeloproliferative neoplasms with monocytosis. *Curr Hematol Malig Rep.* 2022;17(1):46–51. <https://doi.org/10.1007/s11899-021-00660-2>.
 27. Masselli E, Pozzi G, Gobbi G, et al. Cytokine profiling in myeloproliferative neoplasms: overview on phenotype correlation, outcome prediction, and role of genetic variants. *Cells.* 2020;9(9):2136. <https://doi.org/10.3390/cells9092136>.
 28. Escobar G, Barbarossa L, Barbiera G, et al. Interferon gene therapy reprograms the leukemia microenvironment inducing protective immunity to multiple tumor antigens. *Nat Commun.* 2018;9(1):2896. <https://doi.org/10.1038/s41467-018-05315-0>.
 29. Jiang XQ, Zhang L, Liu H, et al. Expansion of CD14(+)CD16(+) monocytes is related to acute leukemia. *Int J Clin Exp Med.* 2015;8(8):12297–306.
 30. Maffei R, Bulgarelli J, Fiorcari S, et al. The monocytic population in chronic lymphocytic leukemia shows altered composition and deregulation of genes involved in phagocytosis and inflammation. *Haematologica.* 2013;98(7):1115–23. <https://doi.org/10.3324/haematol.2012.073080>.
 31. Wypasek E, Padjas A, Szymańska M, et al. Non-classical and intermediate monocytes in patients following venous thromboembolism: Links with inflammation. *Adv Clin Exp Med.* 2019;28(1):51–8. <https://doi.org/10.17219/acem/76262>.
 32. Urbanski K, Ludew D, Filip G, et al. CD14+CD16++ “non-classical” monocytes are associated with endothelial dysfunction in patients with coronary artery disease. *Thromb Haemost.* 2017;117(5):971–80. <https://doi.org/10.1160/TH16-08-0614>.
 33. Fowles JS, Fisher DAC, Zhou A, et al. Altered dynamics of Monocyte subpopulations and pro-inflammatory signaling pathways in polycythemia vera revealed by mass cytometry. *Blood.* 2019;134:4210.
 34. Tahir S, Steffens S. Nonclassical monocytes in cardiovascular physiology and disease. *Am J Physiol Cell Physiol.* 2021;320(5):C761–70. <https://doi.org/10.1152/ajpcell.00326.2020>.
 35. Scarpa M, Scarpa M, Castagliuolo I, et al. CD80 down-regulation is associated to aberrant DNA methylation in non-inflammatory colon carcinogenesis. *BMC Cancer.* 2016;4:388. <https://doi.org/10.1186/s12885-016-2405-z>.
 36. Mir MA. Concept of reverse costimulation and its role in diseases. In: Mir MA, editor. *Developing costimulatory molecules for immunotherapy of diseases.* New York: Elsevier; 2015. p. 45–81. <https://doi.org/10.1016/B978-0-12-802585-7.00002-9>.
 37. Skirecki T, Mikaszewska-Sokolewicz M, Hoser G, et al. The early expression of HLA-DR and CD64 myeloid markers is specifically compartmentalized in the blood and lungs of patients with septic shock. *Hind Mediat Inflamm.* 2016;2016:3074902.
 38. Chauv P, Moutet M, Faivre J, et al. Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7–1

- and B7–2 costimulatory molecules of the T-cell activation. *Lab Invest.* 1996;74(5):975–83.
39. Ugurel S, Lindemann M, Schadendorf D, et al. Altered surface expression patterns of circulating monocytes in cancer patients: impaired capacity of T-cell stimulation? *Cancer Immunol Immunother.* 2004;53:1051. <https://doi.org/10.1007/s00262-004-0565-1>.
 40. Etzerodt A, Moestrup SK. CD163 and inflammation: biological, diagnostic, and therapeutic aspects. *Antioxid Redox Signal.* 2013;18(17):2352–63. <https://doi.org/10.1089/ars.2012.4834>.
 41. Kvorning SL, Nielsen MC, Andersen NF, et al. Circulating extracellular vesicle-associated CD163 and CD206 in multiple myeloma. *Eur J Haematol.* 2020;104(5):409–19. <https://doi.org/10.1111/ejh.13371>.
 42. Qian S, Zhang H, Dai H, et al. Is sCD163 a clinical significant prognostic value in cancers? a systematic review and meta-analysis. *Front Oncol.* 2020;10:585297. <https://doi.org/10.3389/fonc.2020.585297>.
 43. Van Acker HH, Capsomidis A, Smits EL, et al. CD56 in the immune system: more than a marker for cytotoxicity? *Front Immunol.* 2017;8:892. <https://doi.org/10.3389/fimmu.2017.00892>.
 44. Papewalis C, Jacobs B, Baran AM, et al. Increased numbers of tumor-lysing monocytes in cancer patients. *Mol Cell Endocrinol.* 2011;337(1–2):52–61. <https://doi.org/10.1016/j.mce.2011.01.020>.
 45. Dutt TS, LaVergne SM, Webb TL, et al. Comprehensive immune profiling reveals CD56+ monocytes and CD31+ endothelial cells are increased in severe COVID-19 disease. *J Immunol.* 2022;208(3):685–96. <https://doi.org/10.4049/jimmunol.2100830>.
 46. Alegretti AP, Bittar CM, Bittencourt R, et al. The expression of CD56 antigen is associated with poor prognosis in patients with acute myeloid leukemia. *Rev Bras Hematol Hemoter.* 2011;33(3):202–6. <https://doi.org/10.5581/1516-8484.20110054>.
 47. Øbro NF, Grinfeld J, Belmonte M, et al. Longitudinal cytokine profiling identifies GRO- α and EGF as potential biomarkers of disease progression in essential thrombocythemia. *Hemasphere.* 2020;4(3):e371. <https://doi.org/10.1097/HS9.0000000000000371>.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.