International Immunology, Vol. 28, No. 2, pp. 87–97
doi:10.1093/intimm/dxv053
Advance Access publication 21 September 2015

A subpopulation that may correspond to granulocytic myeloid-derived suppressor cells reflects the clinical stage and progression of cutaneous melanoma

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Received 27 February 2015, *accepted* 11 September 2015

Abstract

Seventy-eight melanoma patients and 10 healthy individuals were examined. Follow-up examinations of all melanoma patients were performed regularly every three months. Myeloid-derived suppressor cells (MDSC) were defined as lineage negative ($CD3^-$, $CD19^-$, $CD56^-$), $HLA\text{-}DR^{-/low}$, $CD11b^+$ and $CD33^+$. Classification of granulocytic (GrMDSC) and monocytic (MoMDSC) subsets was based on the CD15 and CD14 expression, respectively. Unlike the MoMDSC, that were present in 60% of healthy controls and 15% of melanoma patients, the GrMDSC were present in all examined participants, and the melanoma patients were found to have statistically higher frequencies compared with healthy controls. Accordingly, we kept focused on GrMDSC frequencies in relation to the melanoma stages and course of the disease. The GrMDSC values are highest in stage IV melanoma patients, with statistical significance compared with stages IA, IB, IIA and IIB. Patients with progression had statistically higher GrMDSC counts comparing with those with stable disease ($P = 0.0079$). Patients who had progression-free interval (PFI) < 12 months showed significantly higher GrMDSC values compared with those with PFI > 12 months ($P = 0.0333$). GrMDSC showed significant negative correlation with PFI intervals ($P = 0.0095$). The GrMDSC subset was predominant in all our patients. We confirmed that GrMDSC do accumulate early in the peripheral blood of melanoma patients and their frequencies correlate narrowly with the clinical stage and the spread of the disease. The increase in GrMDSC frequencies correlates well with a progressive disease and could be considered a potential predictive biomarker of high-risk melanoma cases that are more likely to have a shorter PFI.

Keywords: immunophenotype, MDSC, progression, subset

Introduction

The presence of suppressor cells of myeloid origin in tumor-bearing mice was identified in the late 1980s (1). The interest in these cells was renewed by Bronte *et al.* who shed a new light on the suppression of the $CD8^+$ T-cell responses (2). There is sufficient evidence that myeloid-derived suppressor cells (MDSC) are present in cancer patients and that their immunosuppressive activities undoubtedly contribute to the tumor progression (3).

Immature myeloid cells with the same phenotype as MDSC are continually generated in the bone marrow of healthy individuals and they differentiate into mature myeloid cells without causing detectable immunosuppression. Neoplastic cells

condition distant sites, such as the bone marrow and spleen, by releasing soluble factors that drive the accumulation of highly immunosuppressive immature myeloid cells within the tumor as well as dendritic cells, macrophages and granulocytes with immunosuppressive function in the tumor (4).

MDSC research is ongoing in several areas: their role in tumor progression, in the prognosis of survival, in the prediction of response to therapy and finally, their use for targeted anti-cancer immunotherapy.

The immunophenotyping and counting of MDSC is a challenging task because of their heterogeneity and the absence of MDSC-specific markers. On the basis of the expression of Gr-1 and CD11b in mice, MDSC can be subdivided into at least six different subsets (5). The two most relevant subsets are granulocytic MDSC (GrMDSC) and monocytic MDSC (MoMDSC). The GrMDSC subset is recognized as $CD11b^{+}Gr\text{-}1^{high}Ly6G^{+}Ly6C^{low/int}$ and the MoMDSC as $CD11b^{+}Gr\text{-}1^{int}Ly6G\text{-}Ly6C^{high}$ (5). Similar to the mice, there are two major subtypes of MDSCs in humans, granulocytic MDSC ($CD11b^{+}CD14\text{-}CD15^{+}CD16^{low}CD33^{+}CD66b^{+}CD124^{+}$) and monocytic MDSC ($CD11b^{+}CD14^{+}CD15^{+/-}CD16^{-}CD33^{+}CD66b^{+/-}CD124^{+}$) (3).

Different MDSC subsets are detected in different cancer types; however, it is little known whether any particular subset is predominant. Various researchers are currently questioning whether the cancer type influences the subset of these cells or whether this is a result of different markers being used (3).

There are only a few studies on MDSC and clinical outcome in melanoma patients (6–8). The aim of our study was to identify the predominant subset of MDSC in melanoma patients and to evaluate if it relates to clinical course of melanoma disease. We found significant accumulation of a subpopulation of granulocytes that may correspond to granulocytic MDSC and the acronym GrMDSC in the following text refers to this subpopulation.

Methods

Patients and healthy controls

Seventy-eight melanoma patients and 10 healthy individuals as a control group were recruited during October and November in 2012 from the Clinics of Dermatovenerology and Plastic Surgery, the Military Medical Academy, Belgrade, Serbia. The patients and healthy controls consented to this research, which was approved by the local Research Ethics Committee. The experimental group of melanoma patients and control group were matched for sex and age.

Recorded parameters

The seventh edition of the American Joint Committee on Cancer (AJCC) and TNM melanoma staging and classification were used. The follow-up examinations of all melanoma patients were performed regularly every three months. Various parameters were obtained from clinical and dermoscopic examination, laboratory blood tests (complete and differential blood-cell count, general biochemical analyses, LDH and S100A protein), an ultrasound of regional lymph nodes, radiographic imaging and periodic multislice computed tomography imaging. The progression-free interval (PFI) was defined as the time from the first diagnosis to the time of tumor progression into a higher stage and was expressed in months.

Samples

Three to six milliliters of venous blood were collected from the melanoma patients and healthy controls and erythrocytes were removed using the lysing buffer (EDTA, NH_4Cl, $KHCO_3$) for 10 min with constant mixing. The remaining nucleated cells were washed out twice in the RPMI 1640 culture medium with 5% of normal human serum, centrifuged and resuspended. Separation of PBMC for the comparative analysis was performed using Lymphocyte Separation Medium LSM 1077. The separation process was performed by centrifugation at 1.200 × *g* for 20 min. The interphase layer between the plasma and the separation solution was extracted with a Pasteur pipette and washed twice in culture medium. The cell counting was done manually, in an improved Neubauer chamber, and automatically, using the Beckman Coulter ACT differ blood counter. Finally, the suspension with 1×10^6 cells per 100 μl was aliquoted in 12 × 75 mm test tubes for further immunostaining.

Neutrophil/lymphocyte ratio calculation

Complete and differential blood counts in the peripheral blood samples were done on the Beckman Coulter ACT differ blood counter and the neutrophil/lymphocyte ratio (NLR) was calculated by dividing the neutrophil count by the lymphocyte count.

Immunophenotyping of cells

The following antihuman mAbs were used in different combinations for multicolor analysis of the fresh peripheral blood samples: CD15-FITC and PECy7, CD33-PE and PECy7, CD45-ECD, HLA-DR PE/Cy5, CD14-FITC, CD16-PE, CD11b-PE, CD10-PECy7, CD3-FITC, CD19-FITC and CD56-FITC (Beckman Coulter, USA). The flow cytometry was performed using a Beckman Coulter FC 500 flow cytometer with CXP analysis software. MDSC were defined as lineage negative ($CD3^{-}$, $CD19^{-}$, $CD56^{-}$), $HLA\text{-}DR^{-/low}$, $CD11b^{+}$ and $CD33^{low}$. The MDSC were initially gated based on a CD11b versus HLA-DR dot plot. The $HLA\text{-}DR^{-/low}$ and $CD11b^{+}$ cells were further analyzed for the lineage markers (CD3, CD19 and CD56), as well as CD10, CD14, CD15, CD16 and CD45. The classification of granulocytic and monocytic subsets was based on the CD15 and CD14 expression, respectively. The GrMDSC were separated from the mature granulocyte population on the basis of CD10 negativity, lower and inhomogeneous expression of virtually all positive markers (CD11b, CD15, CD16, CD33 and CD45) and lower positioning on the CD45 versus Side Scatter (SS) dot plot. The MDSC frequency was expressed as a percentage of these cells out of all nucleated cells.

Statistical analysis

Data analysis was performed using the GraphPad Prism 5 software. Depending on the normality of data, the following tests were used: the Student's *t*-test, the Mann–Whitney test, the one-way ANOVA test and the nonparametric Kruskal–Wallis

test. To assess the strength of association between obtained values, we used the Pearson's and Spearman's correlation tests.

Results

Immunophenotype and subsets of the MDSC in melanoma patients and healthy controls

The MDSC frequencies were determined in 78 melanoma patients and 10 healthy controls (Fig. 1). The detected immunophenotype of the targeted MDSC populations is given in Table 1.

GrMDSC and MoMDSC subsets were identified according to the expression of CD15 and CD14, respectively, within the HLA-DR$^{-/low}$CD11b^{+}CD33^{low}Lin^{-} population. We found that the MoMDSC subset was present in only 60% of healthy controls and 15% of our melanoma patients (Table 2). Furthermore, MoMDSC were absent in all melanoma patients in stage IIIC and stage IV. On the contrary, we found that the GrMDSC subset was present in the peripheral blood of all melanoma patients and all healthy controls; therefore, we decided to focus on this particular subset only.

Comparative analysis of GrMDSC in lysed peripheral blood samples to GrMDSC in density-gradient-separated PBMC

In the pilot study including six melanoma patients, we performed comparative analysis of GrMDSC detected in lysed peripheral blood samples to GrMDSC obtained in the PBMC fraction separated on a density gradient. We found that HLA-DR$^{-/low}$CD10^{-}CD11b^{+}CD14^{-}CD15^{+}CD16$^{low/int}$CD33^{low}CD-45^{low}Lin^{-} cells copurified with the PBMC on density gradient and their counts correlated positively with the cells of the same phenotype found in lysed peripheral blood samples (Pearson $r = 0.9964$, $P < 0.0001$, data not shown).

Phenotypic differences between the GrMDSC and mature granulocytes

With the exception of CD10, which was negative on GrMDSC (Fig. 2A), the GrMDSC and mature granulocyte populations were positive for the same examined markers but with major differences in intensity level and homogeneity of expression. As expected by their immature state, the GrMDSC expressed low levels of CD45—common leukocyte antigen. In virtually all patients, the GrMDSC population was positioned in a region with the lowest CD45 intensity level and lower SS relative to mature granulocytes on the CD45 versus SS dot plots (Fig. 2B). Comparison of the SS average values between the GrMDSC and mature granulocytes showed significantly lower SS positioning of the GrMDSC (59.8 ± 1.3 versus 97.7 ± 3.7, $P < 0.0001$, Fig. 2C). All the positive markers on the GrMDSC population (CD11b, CD15, CD16, CD33, CD45) showed inhomogeneous and lower expression relative to mature granulocytes. Only the CD15 expression could reach the expression intensity of mature granulocytes in the substantial part of GrMDSC (Fig. 2D–H). Comparison of mean fluorescence intensity average values between GrMDSC and mature granulocytes confirmed lower expression on GrMDSC for all examined markers (Fig. 2I).

The GrMDSC frequencies correlate inversely with the frequencies of mature granulocytes without significant elevation of NLR in the peripheral blood

The GrMDSC frequencies in the peripheral blood of the melanoma patients correlated inversely with the frequencies of mature granulocytes with high statistical significance (Pearson $r = -0.5709$, $P < 0.0001$, data not shown). There was no such correlation between the GrMDSC and mature granulocytes in the peripheral blood of healthy individuals.

The average NLR in the peripheral blood of the melanoma patients was slightly higher compared with healthy controls, but statistical significance could not be reached (1.9 ± 0.2 versus 1.7 ± 0.2, $P = 0.7368$, data not shown).

The GrMDSC frequencies are elevated in the peripheral blood of melanoma patients when compared with the control group

Regardless of clinical and pathohistological stage, the GrMDSC frequencies were significantly higher in the samples of melanoma patients than in the healthy controls (8.2 ± 0.7 versus 0.9 ± 0.07, $P < 0.0001$, Fig. 3A). This significant difference was further confirmed after the classification of patients according to TNM or AJCC criteria. The patients from all TNM stages (Fig. 3B) and all AJCC stages (Fig. 4A) had significantly higher GrMDSC frequencies than healthy controls.

The GrMDSC frequencies correlate well with the clinical stage of melanoma

The GrMDSC frequencies are the highest in stage IV melanoma patients, significantly higher in stage IV than in stages IA, IB, IIA and IIB (Fig. 4B). Interestingly, the patients with melanoma stages IIC, IIIA, IIIB and IIIC, all at high risk for the development of distant metastases, showed no statistically significant differences in the GrMDSC frequencies compared to patients with melanoma stage IV.

The GrMDSC frequencies correlate well with melanoma progression

Eighteen out of 78 patients (23%) had a melanoma progression. Patients with progression had significantly higher frequencies of GrMDSC comparing to those with a stable disease (11.7 ± 2.0 versus 7.4 ± 0.6, $P = 0.0079$, Fig. 5A).

Negative correlation between GrMDSC frequencies and PFI

Patients with the progression of melanoma were classified into two groups depending on the length of time measured in months. A cut-off value of 12 months was used. The patients who had PFI < 12 months ($n = 8$) showed significantly higher GrMDSC frequencies compared to those with PFI > 12 months ($n = 10$) (15.9 ± 3.1 versus 7.9 ± 1.8, $P = 0.0333$, Fig. 5B). In addition, we found a significant negative correlation between GrMDSC frequencies and PFI intervals (Spearman $r = -0.6090$, $P = 0.0095$, Fig. 5C).

Patients with metastatic melanoma have higher frequencies of GrMDSC in their peripheral blood than patients with localized disease

Melanoma patients were divided into two groups according to the tumor stage when they were tested for MDSC.

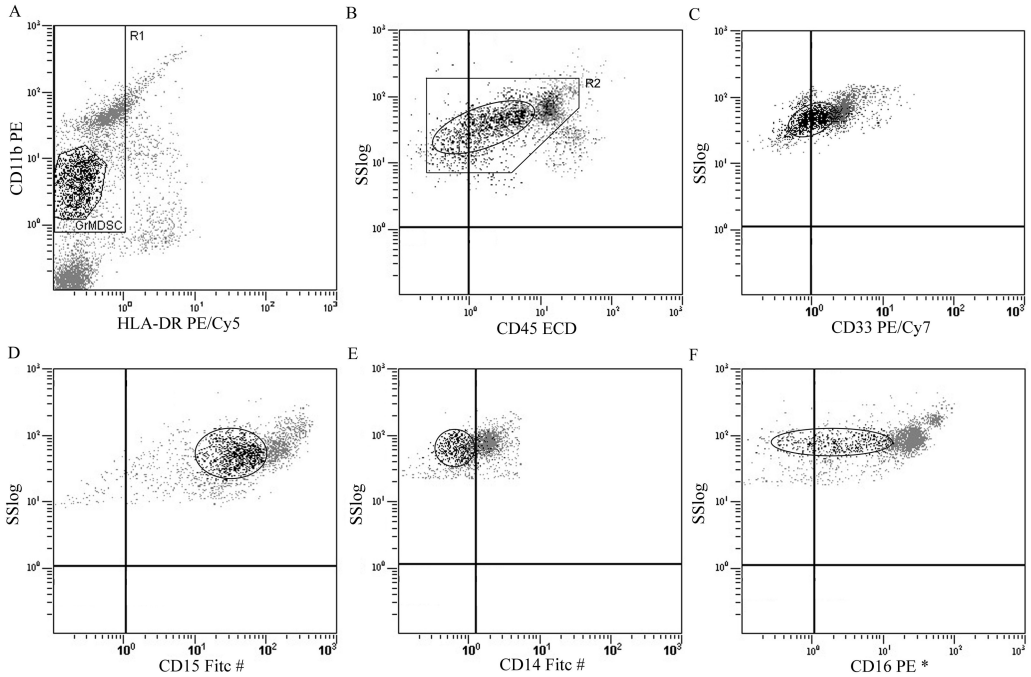

Fig. 1. Detection of GrMDSC by flow cytometry. Representative two-parameter dot plots showing identification of GrMDSC in lysed peripheral blood samples of melanoma patients (n = 78) stained for the indicated surface membrane markers. Quadrant regions were adjusted to the isotype control fluorescence. (A) On the HLA-DR versus CD11b dot plot ungated events are shown. Rectangular region R1 is plotted on HLAD$^-$/CD11b$^+$ events and represents the initial region in the detection process. The polygonal GrMDSC region within the R1 region paints the encompassed cells into black. Ellipses indicate positioning of the black-colored GrMDSC for easier tracking on the following dot plots. (B) The CD45 versus SS dot plot, gated on R1, shows CD45 positivity on black-colored cells. (C) The CD33 versus SS dot plot, gated on R1, shows black-colored cells positive, but with low expression of CD33. (D) The CD15 versus SS dot plot, gated on R1, shows strong expression of CD15 on black-colored cells. (E) The CD14 versus SS dot plot, gated on R1, shows black-colored cells negative for CD14 (#monoclonal antibodies conjugated with the same fluorochrome were examined in different tubes). (F) The CD16 versus SS dot plot shows positive, inhomogeneous expression of CD16 on black-colored cells (*the CD16 versus SS dot plot was gated on the R2 region drawn on the CD45 versus SS dot plot, in a different tube, and the black color is assigned on events on the CD45 versus SS dot plot, which was previously shown to be HLA-DR$^-$CD11b$^+$CD14$^-$CD15$^+$CD33^{low}). The black-colored population was negative for CD3, CD19 and CD56 (data not shown).

Table 1. Immunophenotype of detected MDSC populations

MDSC subset	HLA-DR	CD10	CD11b	CD14	CD15	CD16	CD33	CD45	CD3	CD19	CD56
GrMDSC	–/low	–	+	–	+	low/int	low	low	–	–	–
MoMDSC	–/low	–	+	+	–	–/low	low	low	–	–	–

Granulocytic (GrMDSC) and monocytic (MoMDSC) subsets were identified according to the expression of CD15 and CD14, respectively, within the HLA-DR$^{-/low}$CD11b$^+$CD33^{low}Lin$^-$ population.

The first group was composed of patients with localized disease in stages I and II (n = 60) and the second group was composed of patients with regional or distant metastases in stages III and IV (n = 18). We found that patients with a very advanced metastatic disease had significantly higher frequencies of GrMDSC (11.6 ± 2.0 versus 7.0 ± 0.6, P = 0.0032) compared to patients with localized disease (Fig. 6A).

The GrMDSC frequencies correlate well with the increase in the risk of developing distant metastases

We divided the patients without a distant metastatic disease according to their risk of developing distant metastases into three groups: a low-risk group with melanoma stage IA, a medium-risk group with melanoma stages IB, IIA and IIB and a high-risk group with melanoma stages IIC, IIIA, IIIB and IIIC. The fourth group comprised patients with the metastatic disease stage IV. When the first three groups of melanoma patients without distant metastasis were compared with the fourth group of the patients in stage IV, we found that melanoma patients in stage IV had significantly higher frequencies of GrMDSC in comparison with the low-risk group (15.17 ± 3.260 versus 5.678 ± 1.032, P = 0.0062) and the medium-risk group (15.17 ± 3.260 versus 7.343 ± 0.6619, P = 0.0006). The differences in the frequency of GrMDSC between stage IV melanoma patients and high-risk group were not statistically significant (Fig. 6B).

Table 2. Distribution of MDSC subsets in melanoma patients

Clinical stage	GrMDSC		MoMDSC	
	Number of patients	Mean ± SEM	Number of patients	Mean ± SEM
		%		%
IA	10	5.7 ± 1.0	1	*
IB	25	7.4 ± 0.7	4	6.5 ± 1.5
IIA	13	7.4 ± 2.0	2	3.5 ± 0.5
IIB	7	6.9 ± 0.9	2	5.5 ± 0.5
IIC	5	9.0 ± 3.2	1	*
IIIA	4	8.6 ± 4.5	1	*
IIIB	4	9.0 ± 2.2	1	*
IIIC	4	11.8 ± 6.6	0	ND
IV	6	15.2 ± 3.3	0	ND
Controls	10	1.6 ± 0.7	6	0.4 ± 0.1

Average value of detected MDSC per stage (mean ± SEM). ND, not detected

*Only one patient with detectable MoMDSC in the group.

Discussion

Since previously published data on MDSC subsets in melanoma patients have not been clearly established and have been considered almost controversial, in this clinical study conducted on 78 melanoma patients, we tried to clarify the immunoprofile of particularly engaged MDSC subtypes in melanoma patients. Although some authors reported MoMDSC to be the main subtype in melanoma patients (9–11), there is emerging evidence that GrMDSC are also present (12) and that both aforementioned subsets do co-exist in the same melanoma patient (12–14). We start our discussion emphasizing the importance of the blood sample handling during flow cytometric detection of the MDSC. As it turned out, different sample preparation methods (centrifugation on density gradient or lysis of erythrocytes) could be the important factor that influences interpretation of acquired results. On confirmation of altered density of the targeted cells, we continued our study with lysed peripheral blood samples for several reasons: (i) to prevent possible loss of the granulocytic component of MDSC; (ii) to prevent possible changes in the expression of the surface membrane markers; (iii) to compare the MDSC frequencies and phenotype with the rest of the leukocyte populations, including granulocytes; and (iv) to express the MDSC frequency as a percentage of total leukocytes.

By using fresh and lysed peripheral blood samples, we found the population of MDSC with the following immunoprofile in all 78 melanoma patients recruited in this study: HLA-DR$^{-/low}$CD10^{-}CD11b^{+}CD14^{-}CD15^{+}CD16$^{low/int}$CD33^{low}CD45^{low}Lin^{-}. This particular immunophenotype is compatible with the granulocytic subset of MDSC (15). Furthermore, in 12 out of 78 patients, we detected an additional MDSC subset with HLA-DR$^{-/low}$CD10^{-}CD11b^{+}CD14^{+}CD15^{-}CD16$^{-/low}$CD33^{low}CD45^{low}Lin^{-} immunophenotype, which is indicative of a monocytic subset (15). Our substantial finding is attributable to the GrMDSC as a consistently present subset in all our examined melanoma patients. This differs from the data published by other authors (9, 11). In our opinion, this discrepancy in the reported subsets of MDSC could be due to two reasons. The first reason could be that in previous research, the MDSC were analyzed in the density-gradient-separated PBMC fraction, which could result in a partial loss of GrMDSC. The second one could be that previous researches were performed on cryopreserved blood samples, which could lead to the loss of temperature sensitive antigens. To our knowledge, a very few researchers used fresh and lysed peripheral blood samples (16, 17). Previously published data regarding MDSC in various mouse tumor models, including the mouse melanoma model specifically designed for the investigation of MDSC, showed that the predominant subset in the mouse melanoma is the GrMDSC subset (18–20). Dumitru *et al.* presented the data on peripheral blood GrMDSC in different human cancer types—including melanoma, but did not comment on whether GrMDSC is the dominant subset (21). We found that the GrMDSCs are not only present but also the predominant subset in melanoma patients.

Interestingly, we found that 10 out of 12 patients (83%) with detectable MoMDSC were in the earlier stages of melanoma (stages I and II). Only one patient in melanoma stage IIIA and one patient in stage IIIB had detectable MoMDSC but this same subset could not be detected in the peripheral blood of patients in melanoma stages IIIC and IV. This could be due to the fact that there were fewer patients with advanced melanoma stage in our study (stages III and IV), or perhaps, due to the change of the type of MDSC subset with the progression of the disease. Taking into account MDSC plasticity seen in mice (22), it is possible that various tumor-derived factors released in different melanoma stages can cause differentiation into different subsets of MDSC (13). So far, the published data have shown high plasticity of MDSC and the capability of these cells to transdifferentiate into antigen-presenting cells or into endothelium-like cells (23, 24). The process of transdifferentiation means the change of one mature phenotype into another mature phenotype and is also known as phenotypic plasticity. The switching between a protumor and anti-tumor activity of the MDSC through the process of polarization and reprogramming of MDSCs was reported (25), but we could not find any publications regarding this direct conversion (transdifferentiation) between the GrMDSC and MoMDSC subsets. In our opinion, it would be interesting to investigate if there is a change in the predominant MDSC subset in the same patient with the progression of melanoma.

There are several mechanisms by which MDSC impair antitumor immunity in both an antigen-specific and an

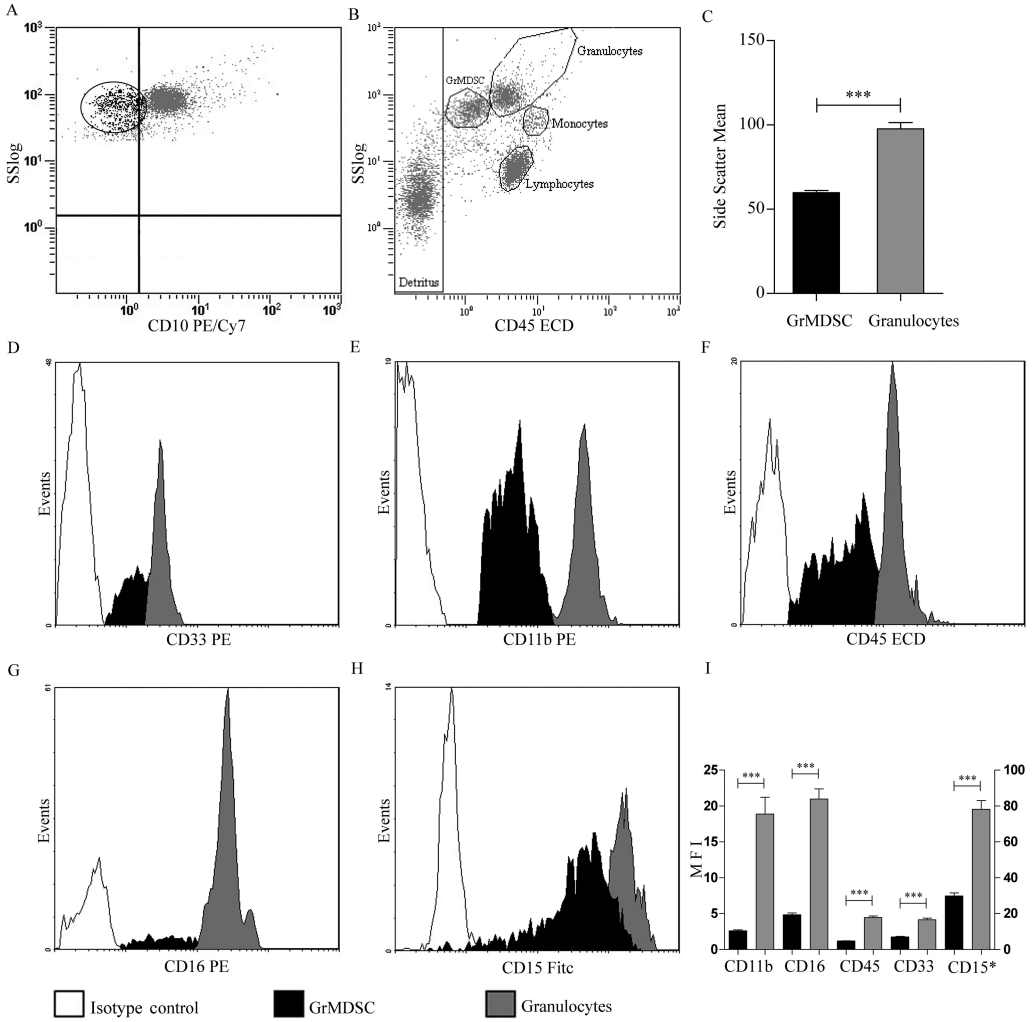

Fig. 2. Phenotypic differences between GrMDSC (black) and mature granulocytes (gray). (A) The CD10 versus SS dot plot, gated on the R1 region (see Fig. 1A), shows CD10 positive mature granulocytes and black-colored GrMDSC negative for CD10 (marked with an ellipse). (B) On the CD45 versus SS dot plot, there is a noticeably lower positioning of the GrMDSC on the SS axis, as well as lower CD45 expression relative to mature granulocytes. (C) Bar graph shows the result of the comparison of SS average values between GrMDSC (black) and mature granulocytes (gray) confirming significantly lower SS positioning of the GrMDSC compared with mature granulocytes. Mean values of 78 measurements were compared using Mann–Whitney test. (D–H) Overlay histograms of indicated markers showing different pattern and intensity of expression on GrMDSC (black) relative to mature granulocytes (gray). All the surface membrane markers found to be positive on both populations, the GrMDSC and the mature granulocytes, showed lower and inhomogeneous expression on the GrMDSC. (I) Bar graph showing results of the comparison of mean fluorescence intensity (MFI) between GrMDSC (black) and mature granulocytes (gray), confirming significantly lower expression of indicated markers on the GrMDSC population. Mean values of 78 measurements were compared using Mann–Whitney test. *The bars showing CD15 MFI are plotted on the right *y*-axis, ***$P < 0.0001$.

antigen-nonspecific manner (26–29). Some of the first studied and described are the high expression of arginase-1 (ARG1), production of reactive oxygen species (ROS) and production of nitric oxide (NO) (30). To date, the difference in the production of ROS and NO between GrMDSC and MoMDSC is well established, wherein the GrMDSC express high levels of ROS and low levels of NO, whereas the MoMDSC express low levels of ROS and high levels of NO (18, 31). Both subsets express high levels of ARG1 (18), although some authors showed that ARG1 is mainly expressed by GrMDSC (32, 33). Consequently, identification of the predominant MDSC subset in a given cancer patient could be of therapeutic importance because, by the analogy with mice models (34), different subsets of human MDSC could use different mechanisms to suppress T-cell function. Blockage of the specific mechanism, using the proper agent such as synthetic triterpenoids that reduce ROS production in MDSC, or nitro-aspirin that inhibits inducible NO synthase, could improve effectiveness of anticancer immunotherapy (35).

On the basis of the fact that we found that only the GrMDSC subset was consistently present in the peripheral blood of all our melanoma patients and all healthy controls, we therefore decided to focus our research on this particular subset. It should be emphasized that the distinction between GrMDSC and activated granulocytes is a challenging task not only for immunophenotyping but also from a functional point of view,

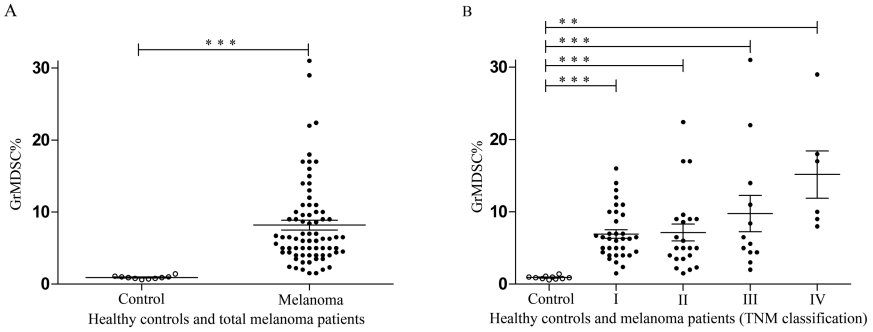

Fig. 3. Comparison of the GrMDSC frequencies between healthy controls and melanoma patients. (A) Comparison of GrMDSC frequencies between healthy controls and all melanoma patients regardless of clinical and pathohistological stage, showing significantly higher values in melanoma patients. (B) Comparison of GrMDSC frequencies between healthy controls and melanoma patients in different clinical stages classified according to the TNM classification, showing significantly higher values in all melanoma stages. GrMDSC are shown as a percentage of total leukocytes (mean ± SEM). Mann–Withney test (unpaired, two tailed, $^{**}P < 0.01$, $^{***}P < 0.001$).

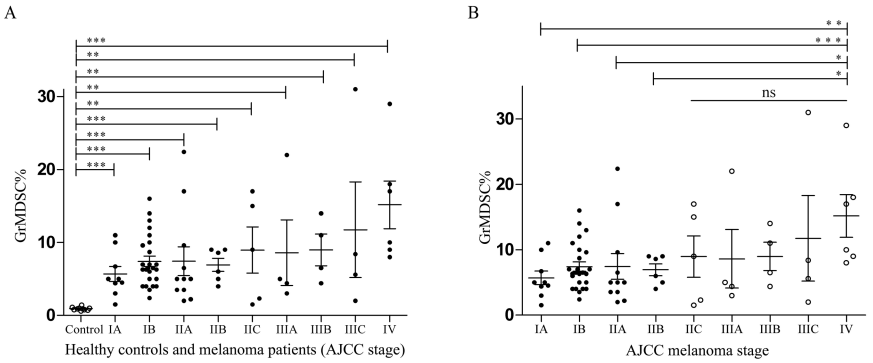

Fig. 4. (A) Comparison of GrMDSC frequencies between healthy controls and melanoma patients classified according to AJCC staging for melanoma, showing significantly higher values in all melanoma stages. (B) Comparison of GrMDSC frequencies between different AJCC melanoma stages showing the highest values in the stage IV, with statistical significance in comparison with IA, IB, IIA and IIB melanoma stages. GrMDSC frequencies are shown as a percentage of total leukocytes (mean ± SEM). Student's *t*-test (unpaired, two tailed, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant).

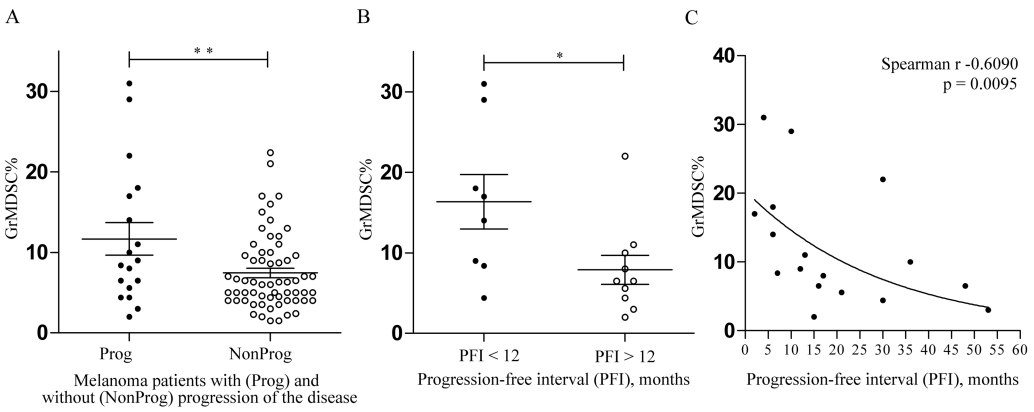

Fig. 5. (A) Comparison of GrMDSC frequencies between groups of melanoma patients with and without progression, showing significantly higher values in the group of patients with progressive melanoma disease. (B) Comparison of GrMDSC frequencies between groups of patients with progressive disease according to their PFI (cut-off value 12 months), showing significantly higher values in the group of patients with the PFI shorter than 12 months. GrMDSC frequencies are shown as a percentage of total leukocytes (mean ± SEM). Student's *t*-test (unpaired, two tailed, $^{*}P < 0.05$, $^{**}P < 0.01$). (C) Correlation of GrMDSC frequencies in the peripheral blood of melanoma patients and PFI measured in months, showing statistically significant inverse relationship (Spearman's correlation test).

since it has been shown that *in vitro* activated granulocytes from healthy donors are also capable of mediating suppressive effects on T cells (36). A precise immunophenotype of the GrMDSC referring to different cancer types—including melanoma—has still not been defined. Markers that have been predominantly used include HLA-DR, CD11b, CD14, CD15

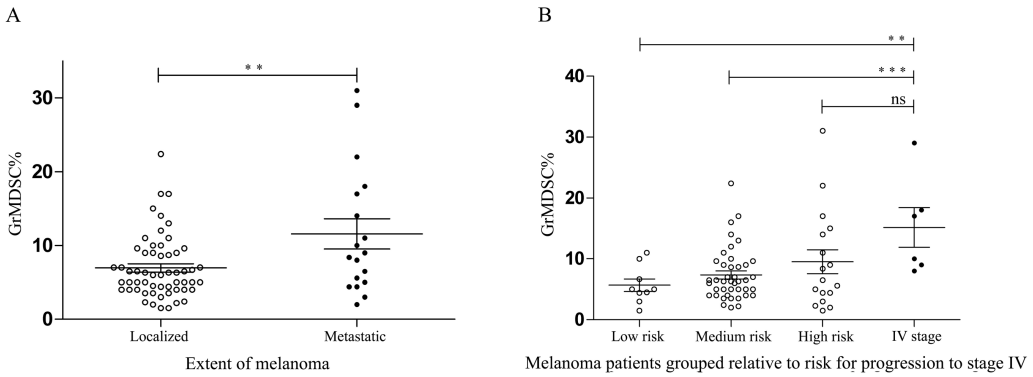

Fig. 6. (A) Comparison of GrMDSC frequencies between the patients with localized melanoma and the patients with metastatic melanoma disease, showing significantly higher values in the group of patients with metastatic disease. (B) Comparison of GrMDSC frequencies between the groups of melanoma patients with different risk for developing distant metastases and the group of patients within stage IV of melanoma, showing significantly higher values in the group of patients within stage IV in comparison with the groups with low and medium risk. The difference between high-risk group and stage IV melanoma patients was not significant. GrMDSC frequencies are shown as a percentage of total leukocytes (mean ± SEM). Student's *t*-test (unpaired, two tailed, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant).

and CD33 (5), as well as CD66b and CD16 for additional subclassification of GrMDSC (21). Accordingly, we defined our GrMDSC as $CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ cells within the $HLA\text{-}DR^{-/low}$ population. However, the presented immunophenotype also fits to the phenotype of mature granulocytes (37) and carries a risk of losing $CD11b^{-}$ MDSC from analysis (38), imposing a careful and highly critical approach to the flow cytometric enumeration of GrMDSC, with special emphasis on pattern and intensity of surface membrane marker expression. Potential significance of membrane markers expression intensity, in relation to the particular cell subsets function, has been shown in animal models. Youn *et al.* showed that the GrMDSC expressed lower levels of CD11b than mature granulocytes in different mouse tumor models—including melanoma (39). Similarly, Greifenberg *et al.* identified two subsets of neutrophils with a different CD11b expression in the spleens of healthy mice, wherein only the relatively low CD11b-expressing cells were found to be immune suppressive, exhibiting characteristics of GrMDSC (40). Our findings show lower and inhomogeneous expression of all GrMDSC positive markers—including CD11b—relative to mature granulocytes. We consider that the intensity and pattern of expression of surface membrane markers are of great importance when discussing the phenotype of GrMDSC. In this regard, the presence of mature granulocytes in lysed peripheral blood samples allows comparison between the two cell populations.

Since the expression of the CD10 molecule in neutrophilic granulocytes occurs only in mature forms (37), we used this marker to assess the maturity of the GrMDSC and for their phenotypical separation from the mature granulocytes. In addition to the lower SS positioning and inhomogeneous pattern of expression of all the positive surface membrane markers on the GrMDSC (CD11b, CD15, CD16, CD33, CD45), we propose the CD10 as one of the useful markers for discrimination between GrMDSC ($CD10^{-}$) and mature granulocytes ($CD10^{+}$). An additional value of CD10 tracking lies in the regulatory role of this molecule and its up-regulation upon activation of granulocytes by various stimuli, such as GM-CSF, TNF, C5a or formyl-methionyl-leucyl-phenylalanine (FMLP) (37, 41). Activation of granulocytes causes changes in their density leading to altered buoyancy and copurification with PBMC on density gradients (36, 42). Thus, up-regulation of CD10 on mature activated granulocytes allows their flow cytometric separation from CD10 negative GrMDSC, which also copurify with PBMC. Moreover, Sagiv *et al.* shed a new light on the plasticity of mature neutrophils showing that they can change from 'normal' high-density to low-density neutrophils and vice versa in the peripheral blood of tumor-bearing mice and human lung cancer patients (43). They showed that the high-density neutrophil fraction (HDN) was almost completely composed of mature cells, whereas the low-density fraction (LDN) contained two types of granulocytes: immature—presumably the GrMDSC, and mature granulocytes—derived from HDN fraction. This finding gives even more sense to the monitoring of CD10 expression in low-density granulocytes. In addition, Sagiv *et al.* observed that their LDNs contained two subpopulations distinct in size according to forward scatter in flow cytometric analyses. We think it would be very interesting to examine whether there are differences in the expression of CD10 between the two populations. Unlike us, they did not find differences in SS between HDNs and LDNs.

Regarding other markers that are useful for maturity assessment, our GrMDSC showed highly inhomogeneous expression of CD16 with a wide range of fluorescence intensity, revealing the presence of granulocyte precursors in different developmental stages within the single GrMDSC population. The CD16 molecule is absent from the surface of neutrophil precursors in the earliest stages of development and appears at the metamyelocyte stage with gradually increasing expression toward mature neutrophils (37). In combination with CD14 and CD15, low and inhomogeneous, but still positive expression of CD16 allowed us to distinguish our $CD14^{-}CD15^{+}CD16^{low/int}$ GrMDSC from monocytes ($CD14^{+}CD15^{-/low}$), eosinophils ($CD15^{+}CD16^{-}$) and mature neutrophils ($CD15^{+}CD16^{high}$) (37, 44).

As mentioned, our targeted population of $HLA\text{-}DR^{-/low}$ $CD10^{-}CD11b^{+}CD14^{-}CD15^{+}CD16^{low/int}CD33^{low}CD45^{low}Lin^{-}$ cells copurified with the PBMC on density gradient. Although copurification with PBMC, by itself, does not prove immunosuppressive capabilities of the GrMDSC, it is in accordance with many studies that clearly showed that granulocyte-like cells with altered density do exert immunosuppressive activities (6, 12, 36, 42). Further on, we ascertained significant

positive correlation of GrMDSC frequencies with ARG1 activity, IL-5 and NO in the sera of our melanoma patients and a very intriguing inverse correlation between CD16 expression on GrMDSC and IL-1b, IL-4, IL-5, IL13 and IL-10 (unpublished data). These findings are consistent with the immunosuppressive activities of the GrMDSC and factors that promote their accumulation (32, 42, 45–47).

There are several publications regarding the expansion of MDSC in animal models with various solid tumors and, to a lesser extent, in human patients with different solid tumors—including melanoma (9, 10, 48). Our findings confirm that there is a significant increase in the MDSC frequency, specifically GrMDSC, in the peripheral blood of melanoma patients compared with healthy individuals. Regardless of the melanoma clinical stage and classification method, TNM or AJCC, GrMDSC frequencies appeared to be significantly higher in melanoma patients, compared with healthy controls. Even the patients in stage IA with the smallest tumor burden had a significantly increased frequency of GrMDSC in their peripheral blood compared with healthy controls, indicating accumulation of these cells as an early event in melanoma development. This observation is in agreement with the study of Montero *et al.* (11), though there are studies demonstrating enlarged MDSC accumulation only in the later stages in some other solid tumors (49) We speculate that the expansion of GrMDSC is more common in the early stage of melanoma than in other cancer types because of the specific genetic background of melanoma cells and higher capacity for producing factors involved in MDSC accumulation (50).

An intriguing observation regarding GrMDSC accumulation was the disturbed myelopoiesis in our melanoma patients, wherein the GrMDSC and the mature granulocytes showed a significant inverse relationship despite the fact that the NLR did not differ significantly from the healthy controls. This finding suggests that the production of the GrMDSC happens at the expense of production of mature granulocytes. Nevertheless, Sagiv *et al.* showed that despite a large accumulation of LDGs in the peripheral blood of tumor-bearing mice, there was no significant reduction of HDNs in the bone marrow (43).

Similar to other investigators (6, 7), we also found that melanoma patients in stage IV had highest frequencies of GrMDSC in their peripheral blood. We found statistically significant differences in GrMDSC frequencies between the stage IV and stages IA, IB, IIA and IIB. On the contrary, we did not find statistically significant differences in GrMDSC frequencies between the stage IV and stages IIC, IIIA, IIIB and IIIC. Interestingly, patients with IIC, IIIA, IIIB and IIIC melanoma stages, whose GrMDSC frequencies did not differ significantly from the stage IV melanoma patients, were the patients with a high risk for developing distant metastases (51). This suggests a continuous increase in the number of GrMDSC with the progression of melanoma from the early stage IA to the stage IV. Similarly, Filipazzi *et al.* showed that there is the accumulation of MDSC in the early stage of melanoma followed by a gradual increase toward the stage IV (52). Our results show that for every subsequent higher stage of melanoma, there is a small gradual increase in the GrMDSC frequency. Interestingly, we did not find any statistically significant difference in the frequencies of GrMDSC when compared stage by stage.

Additionally, we confirmed significantly higher GrMDSC frequencies in melanoma patients with an extensive disease (stages III and IV) compared with those with localized disease (stages I and II). Diaz-Montero *et al.* found significantly higher frequencies of MDSC in the peripheral blood of patients with advanced melanoma but, unlike us, they tested patients in the stage III and stage IV as two separate groups (7).

The importance of MDSC and their role in melanoma development is well described in mouse models (21) but on the contrary, very few studies have been undertaken on the subject of melanoma in humans. Although recent studies confirmed the accumulation of MDSC in the tissue of human melanoma in the stages III and IV and highlighted the immunosuppressive function of these cells (9, 10, 52), the majority of these studies did not compare frequencies of GrMDSC separately for each stage in the same patient during the progression of their melanoma. An exception to the aforementioned is the study of Kimberly *et al.*, who examined the frequency, suppressive function and role of multiple MDSC subsets in melanoma patients depending on the stage of their disease. Among other findings, Kimberly *et al.* showed that the frequency of HLA-DR$^-$CD33$^+$CD14$^-$ MDSC correlated well with an overall survival and disease progression and that the high frequency of these cells predicts a significantly increased risk of disease progression. They proposed that these cells could be used as a potential prognostic biomarker (6). Unfortunately, Kimberly *et al.* were unable to identify the GrMDSC subset of these cells due to the loss of a CD15 antigen in the process of blood cryopreservation and CD15 is one of the crucial markers for this subset (53). One could speculate that Kimberly's HLA-DR$^-$CD33$^+$CD14$^-$ MDSC could actually be GrMDSC, which lost their surface CD15 during cryopreservation. We managed to overcome this limitation by using fresh peripheral blood samples instead of frozen and consequently, we were able to detect CD14$^-$CD15$^+$ MDSCs (GrMDSC) in all tested patients and controls. Our patients who had the progression of their melanoma had significantly higher GrMDSC frequencies compared to those with a stable disease. Moreover, we recorded the PFI for each patient with a progressive disease and then correlated PFI with the frequencies of GrMDSC in the peripheral blood. There was a statistically significant inverse correlation between the GrMDSC frequencies and the time for progression. Thus, we found that the greater the frequency of GrMDSC, the shorter the period of time to progression. In addition, we found that patients who on follow-up progressed within the first 12 months had significantly higher frequencies of GrMDSC in the peripheral blood compared with patients whose PFI was longer than 12 months.

Conclusion

Although the technique with fresh lysed blood was commonly available, it was not frequently utilized by previous researchers interested in MDSC. We spotted the advantage of the lysed fresh blood over frozen, especially for thermo-sensitive antigens like CD15, which are essential for immunophenotyping of the GrMDSC subset. A careful and critical approach to a pattern of surface marker expression and use of CD10 and

CD16 staining are of great importance in phenotypic separation between GrMDSC and mature granulocytes. The key result in this study is the finding that GrMDSC was the predominant subset of MDSC detected in melanoma patients. We confirmed that GrMDSC do accumulate early in the peripheral blood of melanoma patients and their frequencies are significantly elevated in melanoma patients compared with healthy individuals. The frequencies of these cells correlate well with the clinical stage and the spread of the disease. The increase in the GrMDSC frequency correlates well with a progression of the disease and could be considered a potential predictive biomarker of high-risk melanoma cases that are more likely to have a shorter PFI. Disturbed myelopoiesis in melanoma patients could contribute to the impaired host defense not only by production of suppressive myeloid cells but also with the decreased production of the cells of the first line of defense—mature granulocytes. Further investigations of humoral factors involved in the differentiation and expansion of the GrMDSC subset in the early stage of melanoma are required.

Funding

Ministry of Defense of the Republic of Serbia (MFVMA/3/13–15, MFVMA/24/13–15); the Ministry of Education, Science and Technological Development of the Republic of Serbia (III41018).

Acknowledgements

We are grateful to Marija Bankovic for her help in proofreading the text, to Verica Smiljanic for her technical assistance and to all colleagues in our laboratories. We kindly appreciate and we are particularly grateful to the reviewers for their suggestions and criticism, helping us make this a much better paper.

Conflict of interest statement: The authors declared no conflict of interests.

Disclosure

The results presented in the manuscript have not been published or submitted for publication anywhere else.

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