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Myeloid-derived suppressor cells in human peripheral blood: Optimized quantification in healthy donors and patients with metastatic renal cell carcinoma

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ABSTRACT

Induction of myeloid-derived suppressor cells is an important mechanism leading to tolerance against tumors. Phenotypic characterization of MDSC has been established and heterogeneous populations with monocytic or granulocytic features have been characterized. Increased levels of MDSC have been described in metastatic renal cell carcinoma and seem to correlate with an adverse outcome. As MDSC constitute only small populations in peripheral blood of cancer patients, it is highly important to achieve technically optimized conditions for quantification. Different cell preparation techniques – besides freezing and thawing – are potential sources of substantial variation. Our study was focused on an optimized quantification of MDSC in pB of healthy donors and patients with mRCC, in whom major technical sources of variation were analyzed.

Whole blood and peripheral blood mononuclear cells were used for the flow cytometric quantification of MDSC in the pB of mRCC patients and healthy donors. We compared (1) analysis in whole blood vs. PBMC after Ficoll gradient centrifugation and (2) immediate analysis after blood drawing vs. analysis one day later. Finally, in order to evaluate our optimized technical approach, pB of 15 patients with histologically confirmed mRCC under treatment with either sunitinib or sorafenib was analyzed.

No difference in the number of MDSC was observed after analysis in whole blood vs. PBMC. In contrast, the time point of analysis was a source of substantial variation (one day later vs. immediate analysis after blood drawing).

In conclusion, for optimal analysis of MDSC, immediate analysis of whole blood after blood drawing rather than one day later seems to be most appropriate under the aspect of practical feasibility and reliability. Using this method, we were able to confirm both (a) increased numbers of MDSC in patients with mRCC and (b) a decrease of MDSC under sunitinib therapy.

1. Introduction

Significant immunosuppression has been described in different cancer types including RCC [1–4]. The mechanisms underlying the suppression of antitumor immunity are diverse. However, cell-mediated interactions between the tumor and immune cells seem to play a major role. MDSC play a key role in tumor-induced immunosuppression, leading to a tumor-permissive microenvironment with evasion from host anti-tumor responses [5].

MDSC consist of myeloid cells with substantial heterogeneity concerning phenotype and maturity. Under pathological conditions such as cancer, a partial differentiation block leads to the expansion of MDSC with immunosuppressive functions [5]. Direct suppressive effects of MDSC on T cell effector functions have been described in previous studies [6–9]. Furthermore, MDSC may also promote tumor invasion, angiogenesis and metastases [10,11].

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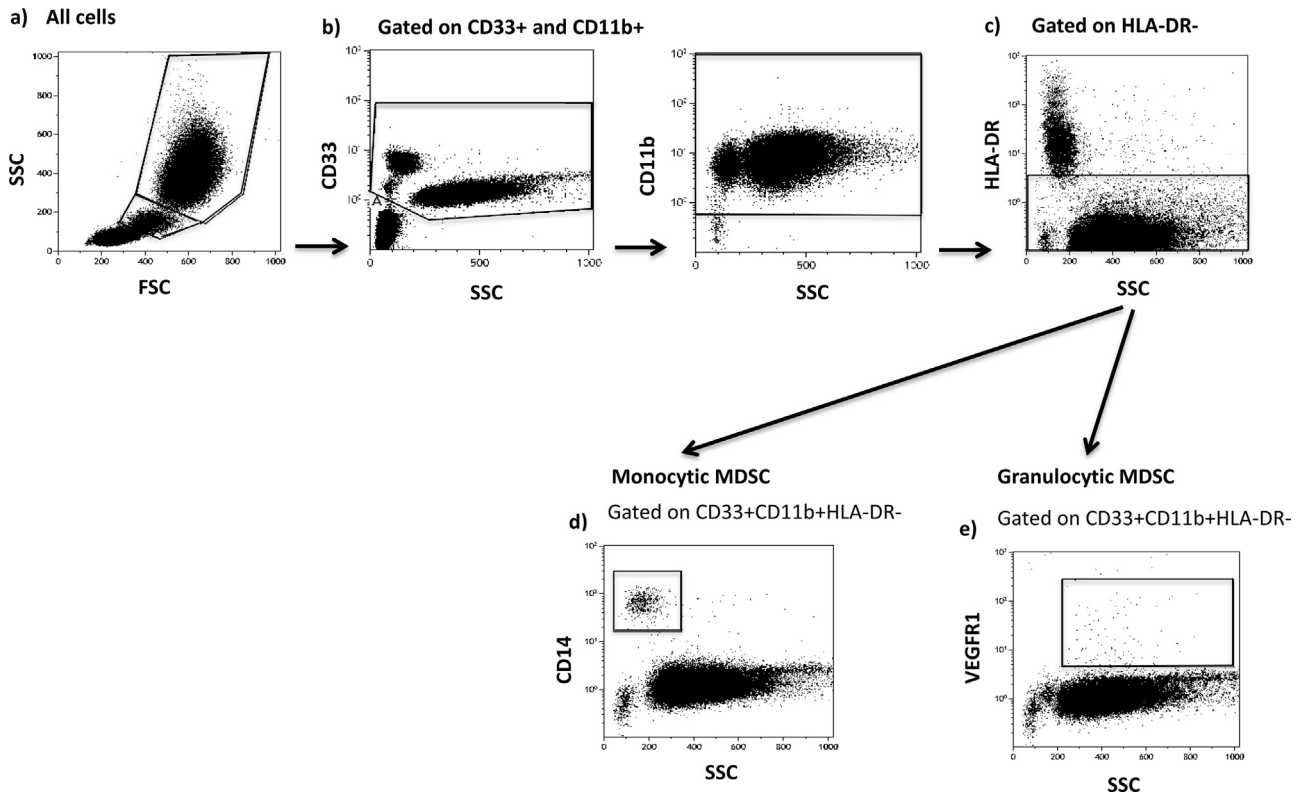

Fig. 1. Gating strategy for MDSC from whole blood.
MDSC were quantified by combining gates for (a) granulocytes/monocytes (light scatter), (b) CD33+ cells and CD11b+cells, and (c) HLA-DR-cells. Finally, granulocytic and monocytic MDSC were detected by either CD14 (d) or VEGFR expression (e).

In healthy individuals, MDSC constitute approximately 0.5% of PBMC, in cancer patients they may accumulate in pB, lymphoid tissues and tumor tissue. In RCC patients, it has been shown that the number of MDSC is increased in tumor tissue and pB [5,9,12–14].

Several groups have described MDSC in humans [5,7,9,13–16]. While different subsets have been identified, it is increasingly accepted to define MDSC by CD33+, CD11b+, HLA-DR-, with monocytic (CD14-positive) and granulocytic (CD14-negative) subsets (reviewed in [9]). In studies analyzing the frequency of MDSC in pB, different preparation methods have been used and it is not clear, to what extent these different methods have influenced final results [12,17–20].

Sunitinib and sorafenib are oral multi-kinase inhibitors, which belong to the standard treatment in mRCC and have been shown to improve progression-free survival (PFS) [21–25]. Sunitinib was able to reverse MDSC accumulation in the pB of RCC patients [12], while intratumoral MDSC do not seem to be affected by sunitinib therapy [26]. The effect of sorafenib on MDSC has only been tested in a murine liver cancer model showing a decrease of suppressive immune cell populations such as MDSC [27]. No clear correlation could be established between the sunitinib-induced reduction of MDSC and tumor burden, response to treatment, or survival. Nevertheless, it has clearly been shown that depletion of MDSC partly reverses tumor-induced immunosuppression. Therefore, MDSC may reflect immunosuppression and future immunotherapeutic strategies might include depletion of MDSC and warrant standardization of techniques for the quantification of MDSC in pB.

Therefore, this study aimed at defining standard conditions for the quantification of MDSC in pB. In particular, the impact of detection in whole blood vs. PBMC and the influence of time between blood drawing and analysis were analyzed. Subsequently, pB of mRCC patients under treatment with sunitinib or sorafenib was analyzed using our optimized approach.

2. Materials and methods

2.1. Healthy donors and RCC patients

Healthy donors were randomly selected for blood donation. Blood samples were taken from 9 healthy donors and from 15 patients before and during the 1st month of therapy. 15 Patients with histologically confirmed mRCC were treated with either sunitinib or sorafenib (see Table 1). Sunitinib-treated patients received 50 mg p.o. qd for 28 days followed by 14 days rest. Sorafenib-treated patients received 800 mg p.o. qd. The study was performed after informed consent and in accordance with local ethical guidelines.

2.2. Preparation of cells and flow cytometry

FACS analysis was performed in whole blood samples on day 0 within 2 h after blood drawing. Additionally, FACS analysis was performed in the same blood samples after preparation of PBMC by density gradient centrifugation with Lymphoprep ™ solution (Fresenius/Axis-Shield PoC AS, Oslo, Norway).

For FACS analysis, fluorochrome-labeled antibodies against CD45, CD33, CD14, CD11b, VEGFR1, HLA-DR, and isotype controls (all Becton & Dickinson, Heidelberg, Germany) were used according to the manufacturer's instructions. Additionally, 7-AAD staining was utilized to exclude dead cells. Overall, viability in our samples was always >91%. More than 90% of 7AAD-positive cells were localized outside of our routine gates for the quantification of monocytic and granulocytic MDSC. Analyses with or without a 7AAD gate did not substantially differ. Therefore, finally we did not include a live gate into our protocol. 60,000 cells were analyzed on a FACSCalibur using BD Cell Quest Pro software, version 5.2.1 (Becton & Dickinson).

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Table 1
Clinical characteristics of patients with mRCC.

	Age	Gender	MSKCC risk score	Histology	Metastasis location	Medication	Clinical response after 3 months of therapy	Overall
1	81	M	1	Clear cell	Lymph nodes, Lung	Sunitinib	SD	SD
2	56	F	1	Clear cell	Bone, Pancreas	Sunitinib	SD	CR
3	65	F	1	Clear cell	Liver, Pancreas	Sunitinib	SD	SD
4	73	F	1	Clear cell	Bone, Gluteal	Sunitinib	SD	SD
5	66	F	1	Clear cell	Lung, Lymph nodes	Sunitinib	SD	PR
6	70	M	2	Clear cell	Lung, Liver, Thyroid, Adrenal gland	Sunitinib	SD	PR
7	69	F	1	Clear cell	Lung, Pancreas	Sunitinib	SD	PR
8	66	M	2	Clear cell	Bone, Lung	Sunitinib	SD	SD
9	62	F	3	Clear cell	Lung, Liver	Sunitinib	PD	PD
10	64	M	2	Clear cell	Lung, Gluteal	Sorafenib	SD	SD
11	59	F	2	Papillary	Lung, Liver	Sorafenib	SD	SD
12	70	M	1	Clear cell	Lung, Bone, left adrenal gland	Sorafenib	SD	SD
13	42	M	1	Clear cell	Lung	Sorafenib	SD	SD
14	64	F	2	Clear cell	Liver, Pancreas, Lung	Sorafenib	SD	SD
15	49	F	1	Clear cell	Lung, cerebral	Sorafenib	PD	PD

15 patients were treated either with sunitinib or sorafenib. MSKCC = Memorial Sloan-Kettering Cancer Center risk score [27]. SD = stable disease, PR = partial remission, PD = progressive disease.

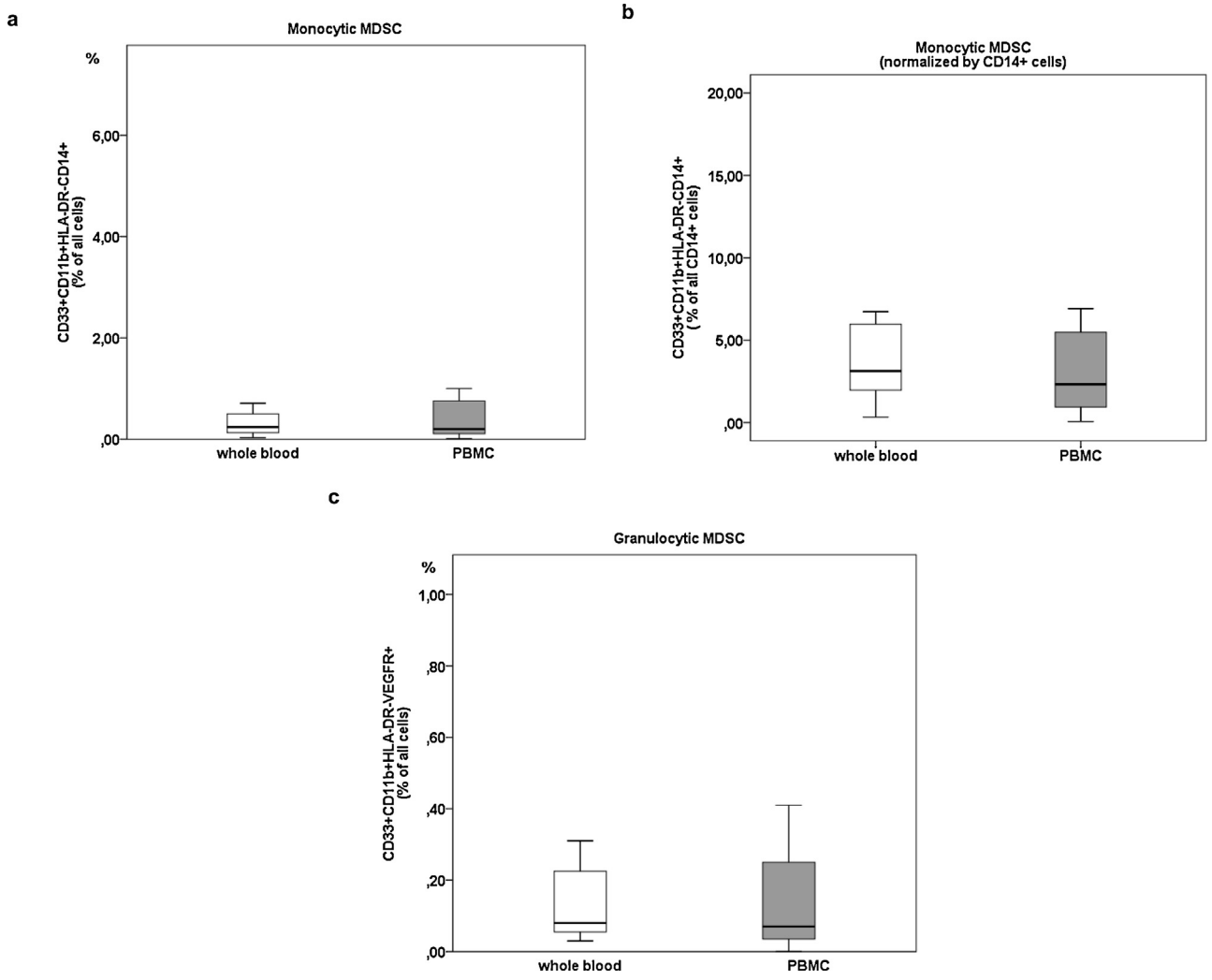

Fig. 2. Frequency of monocytic MDSC in pB after detection in whole blood or PBMC.
Analysis of monocytic MDSC in pB showed no significant difference between whole blood and PBMC, neither in relation to whole blood cell count (median in % of all cells: 0.24 vs. 0.2, $p = 1.0$) (a), nor after normalization by CD14+ cells (b) and (c) frequency of granulocytic MDSC in pB after detection in whole blood or PBMC. Analysis of granulocytic MDSC in pB showed no significant difference between whole blood and PBMC (median in % of all cells: 0.07 vs. 0.08 $p = 0.710$).

The number of MDSC was determined by measuring CD33+CD11b+HLA-DR-cells within a predefined gate according to the previously described phenotype [17,28]. In order to distinguish monocytic from granulocytic MDSC, CD33+CD11b+HLA-DR-cells were analyzed for their CD14 and VEGFR1 expression (for gating strategy see Fig. 1) [13,17,28,29]. In order to characterize the influence of detection in whole blood vs. PBMC, the same samples were simultaneously analyzed by the two different methods.

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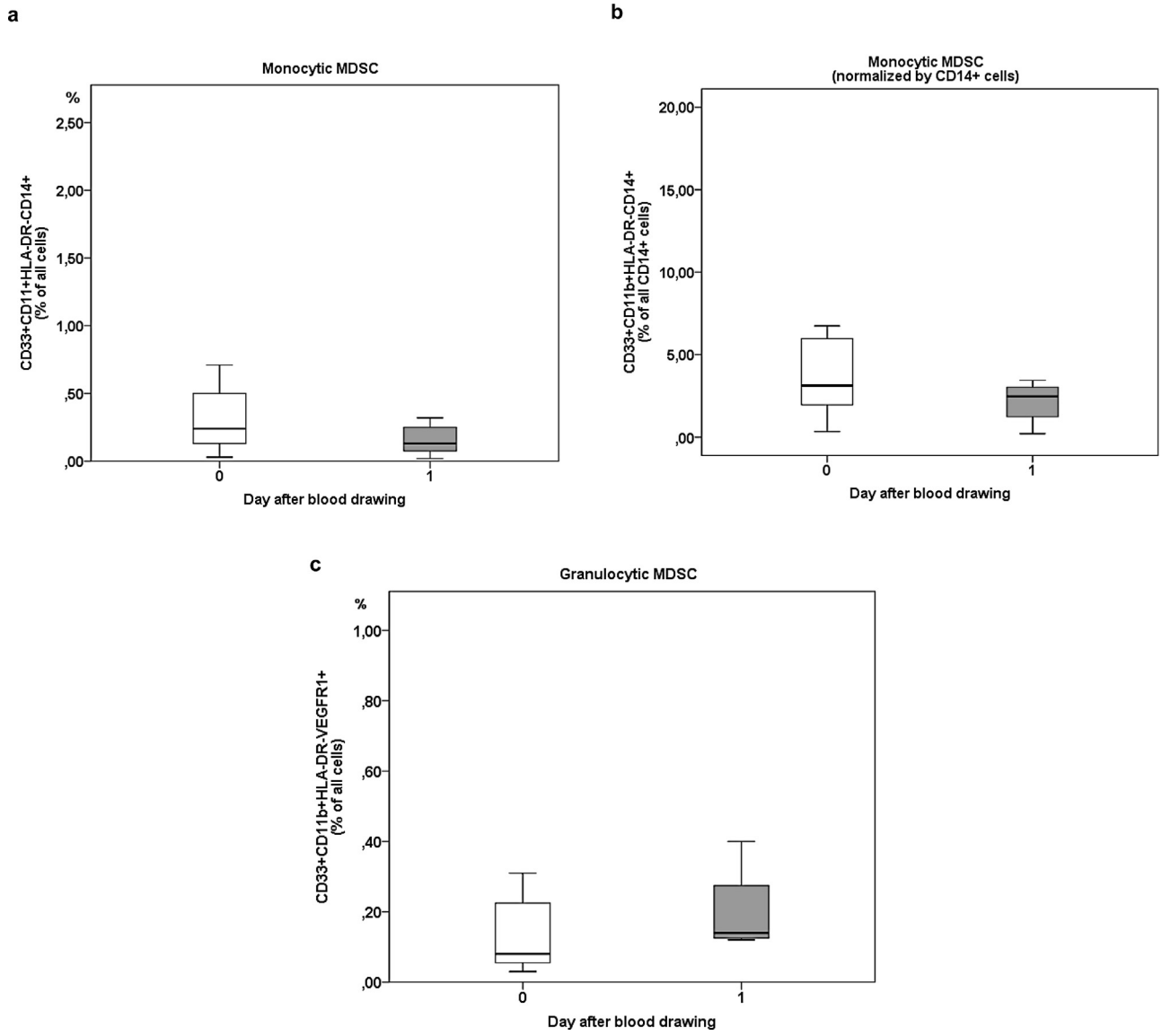

Fig. 3. Frequency of monocytic MDSC in whole blood: influence of time after blood drawing.
Analysis of monocytic MDSC on day 0 and day 1 showed no significant difference, neither in relation to whole blood cell count (median % of all cells: 0.24 vs. 0.13, $p = 0.456$) (a), nor after normalization by CD14+ cells (b) and (c) frequency of granulocytic MDSC in whole blood: influence of time after blood drawing. Analysis of granulocytic MDSC on day 0 and day 1 showed no significant difference (median % of all cells:0.08 vs. 0.14, $p = 0.259$).

Furthermore, the influence of time after blood drawing on the quantitative results was studied. In order to exclude that changes in the relative number of CD14+ MDSC are in fact caused by other cells than monocytic MDSC, results were also normalized by CD14+ cells and reported accordingly.

2.3. Statistical analysis

Statistical analysis was done descriptively. The differences in parameters between two subsets or time points were calculated and the Mann–Whitney-*U*-Test was applied in order to determine statistical significance. The data, expressed as percentages of leukocytes, PBMC or CD14+ cells, are presented as medians in box plots including upper and lower quartiles. Differences with p values <0.05 were considered to be statistically significant. Analyses were done by means of SPSS software (version 22).

3. Results

3.1. Influence of the preparation method and the time point of analysis on the frequency of MDSC

The frequency of MDSC in whole blood samples was compared to analyses in PBMC preparations with regard to total MDSC numbers and monocytic and granulocytic subsets.

Additionally, whole blood samples and samples after Ficoll gradient separation were analyzed immediately on day 0 (within 2 h after blood drawing) vs. day 1 (24 h later).

3.2. Influence of the preparation method (whole blood vs. Ficoll preparation of PBMC)

Analyses from whole blood were compared to analyses after preparation of PBMC by Ficoll gradient centrifugation. We did not find substantial quantitative variation in the number of MDSC between these two methods: both analyses of the monocytic subset

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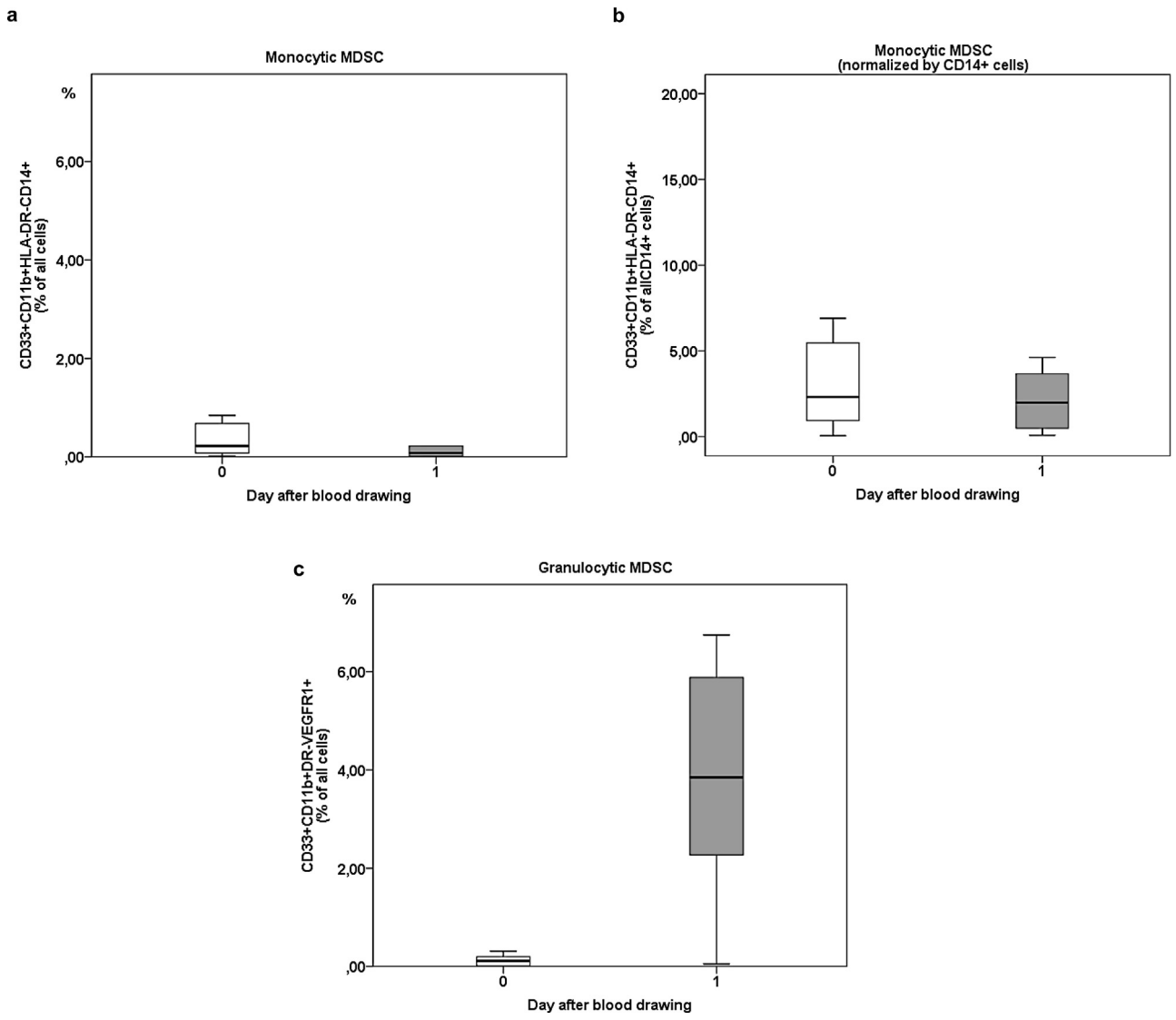

Fig. 4. Frequency of monocytic MDSC in PBMC after Ficoll preparation on day 0 vs. day 1 after blood drawing.
Analysis of monocytic MDSC in PBMC on day 0 and day 1 showed no significant difference, neither in relation to whole blood cell count (median % PBMC: 0.08 vs. 0.22, $p = 0.259$) (a), nor after normalization by CD14+ cells (b) and (c) frequency of granulocytic MDSC in PBMC after Ficoll preparation on day 0 vs. day 1 after blood drawing. Analysis of granulocytic MDSC in PBMC on day 1 (when compared to day 0) showed a significant increase of granulocytic MDSC (median % PBMC: 3.85 vs. 0.11, $p = 0.007$).

(median in % of all cells: 0.24 vs. 0.2; $p = 1.0$) and the granulocytic subset (median in % of all cells: 0.07 vs. 0.08; $p = 0.710$) gave comparable results (Fig. 2).

3.3. *Influence of the time point of the analysis (day 0 vs. day 1 after blood drawing) on the frequency of MDSC*

Whole blood samples and PBMC after Ficoll were analyzed immediately (within 2 h after blood drawing) on day 0 vs. 24 h later on day 1.

Analyses in whole blood showed that measurement on day 1 (as compared to day 0) caused a slight decrease in the relative number of monocytic MDSC (0.24 vs. 0.13 ($p = 0.456$) and consequently a slight increase of the granulocytic subset (0.08 vs. 0.14 ($p = 0.259$). However, these changes were not statistically significant (Fig. 3).

In contrast, analysis of MDSC in PBMC (day 0 or day 1) was significantly influenced by the time between blood drawing and measurement, in particular with respect to the granulocytic sub fraction: the granulocytic subset constituted 0.11% of all cells immediately after blood drawing on day 0, whereas 3.85% of all cells was measured on day 1. This difference was highly significant ($p = 0.007$) and was mainly due to an increase in VEGFR1 positive cells on day 1 as compared to day 0. In contrast, no significant change was found in the number of monocytic MDSC, when measurements on day 0 and day 1 were compared (0.22% vs. 0.08% of all cells, n.s.) (see Fig. 4).

In conclusion, granulocytic and monocytic subsets of MDSC may be quantified immediately after blood drawing both in whole blood samples and in PBMC without causing substantial variation by the detection method. In contrast, the time point of the analysis (immediately after blood drawing vs. 24 h later) seems to have strong influence on quantification. This time-dependent variation is particularly prominent, when PBMC are used for the quantification and when the granulocytic subset of MDSC is under investigation.

3.4. *MDSC in pB of mRCC patients under therapy*

The number of MDSC was analyzed in the pB of 15 mRCC patients treated with sunitinib or sorafenib. 9 patients were treated with sunitinib and 6 patients with sorafenib. 40% of the patients were

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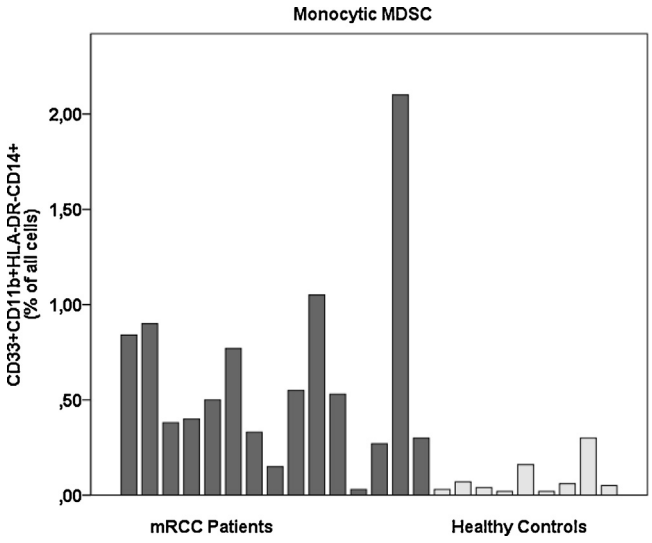

Fig. 5. Analysis of monocytic MDSC in whole blood (within 2 h after blood drawing) in healthy controls vs. mRCC patients.
15 mRCC patients and 9 healthy controls were analyzed for the frequency of MDSC. The median baseline levels of monocytic MDSC before therapy were significantly elevated as compared to healthy controls (mean in % of all cells: 0.08 vs. 0.63, $p = 0.0001$).

male, the median age was 65 years. All patients had undergone prior nephrectomy. 14 patients had clear cell carcinoma, 1 patient had a papillary carcinoma. Applying the Memorial Sloan-Kettering Cancer Center prognostic criteria [30], 15 patients were intermediate risk ($n = 8$ in sunitinib group, $n = 6$ in sorafenib group) and 1 was poor risk ($n = 1$ in the sunitinib group).

MDSC were determined from whole blood immediately (within 2 h) after blood drawing, since this method – as we have shown – seems to be less susceptible to variation. The baseline levels of MDSC before sunitinib therapy (measurement of whole blood on day 0) were significantly elevated compared to healthy controls ($p = 0.0001$, Fig. 5). Sunitinib therapy led to a significant decrease of monocytic MDSC during therapy (0.43 vs. 0.13%, $p = 0.031$, Fig. 6). Granulocytic MDSC were not significantly altered by sunitinib therapy (0,78% vs. 0.98%, $p = 0.439$, Fig. 6).

Sorafenib did not substantially alter the number of MDSC in pB (monocytic MDSC: 0.59% vs. 0.46%, $p = 0.0937$; granulocytic MDSC 0.85% vs. 0.59%, $p = 0.699$).

4. Discussion

For flow cytometric analysis of MDSC from pB different techniques of preparation have been applied and standard conditions for blood sampling and preparation of PBMC have been extensively reported in the literature [31]. However, the influence of these different preparation methods on MDSC quantification in pB is not fully understood [17–20,32]. Therefore, this study aimed to systematically analyze differences caused by (1) the time point of analysis (immediate analysis within 2 h after blood drawing vs. day 1 after blood drawing) and (2) the preparation method (whole blood vs. PBMC), both in the monocytic and the granulocytic subset of MDSC.

In our study, the analysis of MDSC from whole blood samples was feasible and consistent results were obtained. Compared to analysis from PBMC after Ficoll preparation, the whole blood method showed a lower standard deviation and was less susceptible to time-dependent changes (day 0 vs. day 1 after blood drawing). This was particularly relevant for the quantification of the granulocytic subset. However, when MDSC were analyzed immediately after blood drawing, no significant quantitative differences were found between the whole blood method and an analysis from PBMC, showing that both methods can be used to analyze fresh peripheral blood. Whether the increased standard deviation of the results obtained in PBMC is caused by cellular activation by the procedure itself cannot be determined. So far, our results confirm previous studies in the literature [19,20], which have demonstrated no relevant differences between fresh whole blood and analysis from PBMC. In addition, we could show that the time point of the analysis may be a source of substantial variation. The largely comparable results for gMDSC in whole blood and PBMC are in line with previous reports showing that MDSC separate with PBMC when centrifuged over a Ficoll gradient [17,33]. This may explain the somewhat surprising finding that the vast majority of gMDSC is not depleted by Ficoll gradient centrifugation. Since most normal granulocytes are depleted by Ficoll gradient centrifugation, we cannot completely explain this observation, but it has also been reported by several other authors [17,33,34]. As far as time-dependent changes in the number of MDSC are considered, no relevant differences were observed with the whole blood method—neither for the monocytic nor for the granulocytic subset. However, quantification of MDSC from PBMC preparations resulted in a significantly higher variation, particularly in the granulocytic subset, when the analysis was performed on the next day and not immediately after blood drawing. Increasing numbers of granulocytic MDSC on day 1 were mainly caused by a higher number of VEGFR1-positive cells on day 1. Our data do not allow to explain this change in VEGFR1 expression, however, it was obviously induced in vitro. Interestingly, upregulation of VEGFR1 on myeloid cells has been previously described under oxidative stress conditions [35]. Furthermore, McKenna et al. reported on granulocytic activation mimicking granulocytic MDSC, if blood samples were stored for more than two hours [36]. We conclude that the increased numbers of granulocytic MDSC on day 1 do not reflect the situation in vivo and are therefore a source of substantial variation. Therefore, the quantification of MDSC in fresh peripheral blood using the whole blood method seems to be most robust against variation. Consequently, the analysis of MDSC in mRCC patients under therapy was performed with this technique (within 2 h of blood drawing). In 15 mRCC patients, we could confirm the feasibility and reliability of the method and we were able to show that sorafenib does not substantially alter the number of MDSC in pB. Reduction of MDSC by sunitinib has previously been shown in mice and in mRCC patients [12]. In line with these previous studies, we also found that sunitinib treatment was able to reverse increased numbers of monocytic MDSC in mRCC patients. However, in contrast to previously published reports, we did not observe a decrease of granulocytic MDSC in our cohort of patients. A limitation of our study might be the lack of an exactly age-matched healthy control group for our mRCC patients. However, since all subjects belonged at least to a similar spectrum of age, we do not believe that this is likely to explain the profound differences between the two groups.

The influence of targeted agents such as sunitinib and sorafenib on the immune system is still poorly understood. Since their effects are not restricted to tumor cells, a possible influence on immune responses is an interesting clinical issue and may even determine therapeutic outcome in combination therapies with immunotherapeutic agents, particularly in “immunoresponsive” tumors such as RCC. The abundance of MDSC in tumor patients has been identified as a major mechanism for the induction of profound tolerance against tumors and anti-angiogenic drug resistance [5–7,37]. Therefore, the exact quantification of MDSC as a part of the immunoregulatory network will become increasingly important during therapy with kinase inhibitors and particularly for immunotherapy trials in cancer patients.

Immunological effects of kinase inhibitors in immunoresponsive tumors such as RCC might also be relevant for the design of

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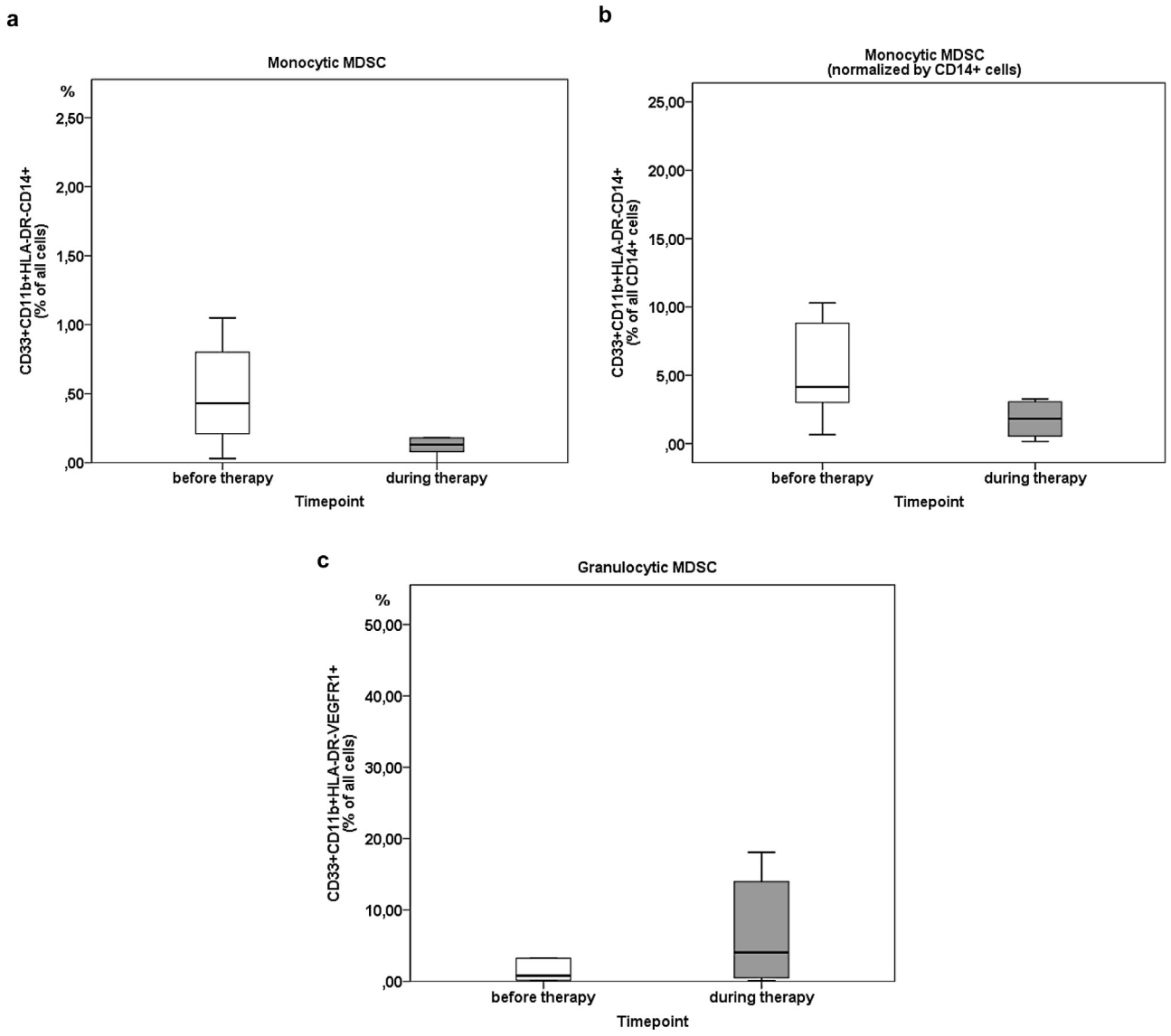

Fig. 6. Analysis of monocytic and granulocytic MDSC in mRCC patients treated with sunitinib.
Sunitinib therapy led to a significant decrease in monocytic MDSC during therapy (median% of all cells, $p = 0.031$), both in relation to whole blood cell count (a) and after normalization by CD14+ cells (b). Analyses were performed in whole blood from 9 mRCC patients within 2 h after blood drawing. (c) Granulocytic MDSC were not significantly altered by sunitinib therapy (median% of all cells, $p = 0.439$). Analyses were performed in whole blood from 9 mRCC patients within 2 h after blood drawing.

combination trials with immunologically active agents. For these trials, reliable quantification of MDSC will be an important issue.

Our present study clearly shows that MDSC quantification in whole blood or in PBMC gives comparable results as long as fresh blood samples are used. However, if there is substantial delay between blood drawing and the analysis, the whole blood method should be preferred.

Conflict of interest

The authors declare that they do not have any affiliations that would lead to a conflict of interest with respect to this work.

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