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Human Cancer Biology

See commentary by Tang and Lotze, p. 5157

Myeloid Cells Obtained from the Blood but Not from the Tumor Can Suppress T-cell Proliferation in Patients with Melanoma

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Abstract

Purpose: Myeloid-derived suppressor cells (MDSC) have emerged as an immune-regulatory cell type that is expanded in tumor-bearing mice, but less is known about their immune-suppressive role in patients with cancer.

Experimental Design: To study the importance of MDSC in patients with melanoma, we characterized the frequency, phenotype, and suppressive function of blood myeloid-derived cells and tumor-infiltrating myeloid cells in 26 freshly resected melanomas.

Results: Blood and tumor-infiltrating myeloid cells (Lin^- $CD11b^+$) could be phenotypically and morphologically classified into monocytes/macrophages, neutrophils, eosinophils, and immature myeloid cells according to marker expression ($CD14^+$, $CD14^-$ $CD15^{hi}$, $CD14^-$ $CD15^{int}$, and $CD14^-$ $CD15^-$, respectively). In contrast to the expansion of MDSC reported in tumor-bearing mice, we found no differences in the frequency and phenotype of myeloid subsets in the blood of patients with melanoma compared with healthy donors. Myeloid cells represented 12% of the live cells in the melanoma cell suspensions, and were phenotypically diverse with high tumor-to-tumor variability. Interestingly, a positive association was found between the percentage of Tregs and granulocytic cells (Lin^- $CD11b^+$ $CD14^-CD15^+$) infiltrating melanoma tumors. However, melanoma-infiltrating myeloid cells displayed impaired suppression of nonspecific T-cell proliferation compared with peripheral blood myeloid cells, in which monocytes and eosinophils were suppressive.

Conclusions: Our findings provide a first characterization of the nature and suppressive function of the melanoma myeloid infiltrate and indicate that the suppressive function of MDSC in patients with melanoma seems far less than that based on murine tumor models. *Clin Cancer Res; 18(19); 5212–23.* ©*2012 AACR.*

Introduction

Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of myeloid-derived cells which are greatly expanded in experimental models of cancer (1–3). Both monocytic ($CD11b^+$ $Ly6C^+$ $Ly6G^-$) and granulocytic ($CD11b^+$ $Ly6C^-$ $Ly6G^+$) myeloid-derived suppressor cells (MDSC) have been described in murine tumor models and their unique distinctive trait compared with cells with similar phenotype in healthy mice is their ability to suppress T-cell responses *in vitro* and *in vivo* (4–7), in part through interfering with L-arginine metabolism (8). Knowledge of MDSC has been primarily established in preclinical models and the phenotypic diversity of MDSC as well as the lack of common markers to study these cells in mice and humans has generated ambiguity in their characterization in patients with cancer. The nature, distribution, frequency, and the contribution of their suppressive phenotype in patients with cancer are highly debated. Studies in humans carried out thus far have reported an increased frequency as well as immune-suppressive activity in some of the myeloid-derived subsets present in peripheral blood of patients with cancer (9, 10). The accumulation of immature myeloid cells (IMC) Lin ($CD3/CD19/CD56/CD14/CD16)^-$ $CD33^+$ $HLA\text{-}DR^{-/low}$ in the blood correlated with the tumor burden as well as with the stage of the disease in 2 independent studies including a diverse set of cancer types (11, 12). In addition, the frequency of either monocytic ($CD11b^+$ $CD14^+$ $HLA\text{-}DR^{-/low}$) or granulocytic ($CD11b^+$ $CD15^+$) myeloid-derived cells with immune-suppressive function have been found to be increased in patients with renal cell carcinoma (13, 14), HCC (15), NSCLC (16), glioblastoma (17), gastrointestinal (18, 19), and prostate cancer (20).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Both expansion and acquisition of immune-suppressive function of myeloid-derived cells have been described in multiple experimental models of cancer. Our study evaluates the frequency, phenotype, and suppressive function of myeloid-derived cells in peripheral blood, but also in the tumor of patients with metastatic melanoma. Unlike the expansion of myeloid-derived suppressor cells (MDSC) found in tumor-bearing mice, the frequency and phenotype of the different myeloid subsets found in peripheral blood of patients with metastatic melanoma and healthy donors were similar. Furthermore, myeloid-derived cells isolated from melanoma tumors were unable to suppress T-cell proliferation compared with homologous subsets obtained from peripheral blood. Our findings provide a first characterization of the nature and suppressive function of the melanoma myeloid infiltrate and suggest that the frequency and contribution of these cells to the inhibition of T-cell proliferation in patients with metastatic melanoma seem far less than that based on murine tumor models.

MDSCs have also been shown to suppress natural killer (NK) cell function in addition to T-cell function (21). In the particular case of metastatic melanoma, both monocytic (22–24) as well as granulocytic (25) blood MDSC with immune-suppressive function have been studied independently and proposed to exert an immune-regulatory role.

The immune-suppressive activity of MDSC is highly dependent on cell-to-cell contact suggesting that their most important role in dampening tumor-specific T-cell responses is more likely to be at the tumor site, where they have been found to accumulate in murine tumor-bearing models. In addition, the ability of myeloid cells to suppress T-cell responses can be potentiated in the hypoxic tumor microenvironment through an increase in the expression of Arginase I and iNOS via signaling through hypoxia-inducible factor 1α (4, 26). MDSCs ($CD11b^+$ $Gr1^+$) from skin tumors in a spontaneous melanoma tumor model were capable of reducing T-cell proliferation to 40% relative to the absence of MDSC (100%) and more efficiently than MDSCs from bone marrow (70%; ref. 27). Moreover, the suppression exerted by MDSCs from a transplantable prostate tumor model was greater in MDSCs from the tumor site compared with MDSCs from peripheral tissues (28). Despite of recurrent evidence supporting superior *in vitro* suppressive activity of tumor-infiltrating myeloid cells in murine models (6), most human studies of MDSCs have focused on peripheral blood and bone marrow (29) and thus, the frequency and actual immune-regulatory role of human tumor-infiltrating myeloid cells is largely unexplored. A population of granulocytic myeloid cells ($CD11b^+$ $CD14^-$) infiltrating 3 human head and neck tumors was capable of suppressing nonspecific T-cell proliferation compared with the homologous population from peripheral blood (4). Ovarian carcinomas, on the other hand, recruit macrophages ($CD45^+$ $CD14^+$) that inhibit T-cell proliferation (30). The complexity of myeloid cells within human melanoma lesions remains poorly characterized with the phenotype relying on single or double marker detection by immunohistochemistry and lack of immune-suppressive functional testing (31) and therefore, the importance of this cell type in T-cell tolerance in human melanoma patients is unknown.

Our previous findings have identified regulatory elements that contribute to dampening tumor-specific T-cell responses in melanoma tumors, such as PD-1 inhibitory receptor expression in tumor-infiltrating lymphocytes (32) and increased frequency of $CD4^+$ $CD25^+$ FOXP3+ cells (33). Building on these findings, and as a result of evidence supporting the suppressive role of myeloid-derived cells, we sought to investigate the frequency, phenotype, and immune-suppressive function of the different myeloid subsets present in the blood and in tumors of patients with metastatic melanoma.

Materials and Methods

FACS analysis of blood and tumor samples

Tumor and blood specimens were collected from patients with metastatic melanoma (patient characteristics specified in Table 1), and blood samples were also collected from age and gender matched healthy donors ($n = 10$). Patients included in this study were not undergoing therapy when their samples were collected and they all had progressive disease. The method used to digest the tumor samples and to analyze the frequency and function of tumor-infiltrating myeloid cells [1-hour digest in gentleMACS Dissociator

Table 1. Patient characteristics

Variable/trait	Total (%)
Total no. patients	32 (100)
Sex	
Male	22 (69)
Female	10 (31)
Age	
11–20	1 (3)
21–30	5 (16)
31–40	3 (9)
41–50	12 (37)
51–60	7 (22)
61–70	4 (13)
Prior treatment	
Surgery	28 (88)
Chemotherapy	9 (28)
Radiotherapy	2 (6)
Immunotherapy	19 (59)
Any 2 or more	18 (56)
Any 3 or more	13 (41)

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versus overnight digest in RPMI-1640 with L-glutamine (Lonza), 1 mg/mL Collagenase type IV (Sigma-Aldrich), 30 U/mL Pulmozyme (Genentech, Inc.), and antibiotics] was chosen by comparing the yield of myeloid cells and frequency of different subsets for 6 tumor samples with the different methods (data not shown). The 1-hour digest of the minced tumor in a gentleMACS Dissociator was chosen to study the tumor-infiltrating myeloid cells, as the total frequency of myeloid cells (Lin^- $CD11b^+$) as well as the frequency of the different subsets (samples digested overnight contained less Lin^- $CD11b^+$ $HLA\text{-}DR^-$) was less impacted by this method. Briefly, tumor specimens from patients with melanoma were minced under sterile conditions into 2-mm pieces and digested during 1 hour using the gentleMACS Dissociator (Miltenyi) at 37°C. Cell suspension was filtered through a 100 μm mesh and washed twice with Hank's balanced salt solution (HBSS; Lonza). Simultaneously, matched peripheral blood samples were obtained and processed fresh. Red blood cells (RBC) were lysed with 2 to 3 cycles of hypotonic cell lysis (0.2% NaCl for 30 seconds and 1.6% NaCl), cells were resuspended in fluorescence-activated cell sorting (FACS) buffer [0.5% bovine serum albumin (BSA), 2 mmol/L EDTA], and counted. 0.5e6 live cells (trypan blue exclusion) from the tumor cell suspension and peripheral blood samples were blocked with Fc block for human cells (Miltenyi) and stained with the antibodies specified. Propidium iodide (PI) and PerCP/Cy5.5 anti-CD235ab (HIR2) antibody (BioLegend) were added to exclude dead cells and RBCs. For FACS analysis of the frequency and phenotype of myeloid cells, we excluded aggregates, dead cells, and the remaining RBCs from the tumor and peripheral blood samples and calculated the percentage of live cells (excluding aggregates, dead cells, and RBCs) expressing a given phenotype. Intracellular staining for the quantification of T-regulatory cells was conducted as previously described (33).

Antibodies and reagents

The following monoclonal antibodies (mAb) specific for human antigens and their corresponding isotype controls were purchased from BD Biosciences: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (SK7), anti-CD19 (HIB19), anti-CD20 (2H7), anti-CD56 (NCAM 16.2), anti-CD57 (NK-1), phycoerythrin (PE)-Cy7–conjugated anti-HLA-DR L243 (G46-6), allophycocyanin cell (APC)-conjugated anti-CD15 (H198), Alexa-700–conjugated anti-CD14 (M5E2), and APC-Cy7–conjugated anti-CD11b (ICRF44). APC-conjugated anti-human FOXP3 antibody (236A/E7) and its isotype were purchased from eBioscience.

Isolation of blood and tumor-infiltrating myeloid cells

Whole blood and tumor samples from the same patient were processed as described. Myeloid cells were first enriched using positive selection with CD11b beads (Miltenyi Biotec) and then counted, stained with PI and anti- Lin (CD3, CD56, CD57, CD19, CD20), CD11b, CD14, CD15, and HLA-DR antibodies, and PI-negative cells (live cells) were sorted with FACS Aria (Becton Dickinson) using a 100 μm nozzle. The purity (exceeded 90% except $CD11b^+$ $CD15^{int}$ population from peripheral blood of patient #2 which was approximately 60%) and viability of myeloid populations sorted was assessed by FACS after the sorting procedure by adding PI to a small fraction of the sorted cells. Cytospins of the cell populations isolated were stained with a modified Wright-Giemsa stain using Diff-Quick (Siemens) to assess purity as well as morphology. Representative pictures were taken under 400× magnification.

In vitro proliferation assays

Autologous CD3 cells were isolated by negative selection from a ficolled blood preparation using Pan T cell isolation kit (Miltenyi) and labeled with 10 μmol/L carboxyfluorescein diacetate succimidyl ester (CFSE; Invitrogen) for 10 minutes at 37°C. Sixty thousand CFSE-labeled cells were stimulated with a 1:1 ratio of Dynabeads HumanT-Activator CD3/CD28 (Invitrogen) in complete medium (RPMI-1640 with L-glutamine (Lonza) containing 10% human serum, antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL, and gentamicin 10 μg/mL) in absence or presence of 60,000 live myeloid cells (counted after FACS sort by trypan blue exclusion) from the subpopulations separated. Cocultures were conducted in duplicates. CFSE dilution was assayed at day 4, and the percentage inhibition of proliferation exerted by a given myeloid population was calculated as = [(sample-control)/(nonstimulated-control)] × 100, using the percentage of cells that have not diluted CFSE from the sample (cocultured with a given myeloid population), the positive control (no myeloid cells added), and the nonstimulated cells as a negative control.

Statistical analysis

P values were calculated using a 2-tailed Mann–Whitney test. $P < 0.05$ was considered statistically significant. Error bars represent the SE of the mean. Correlation of T-regulatory cell frequency and myeloid cells was conducted using the Spearman test.

Results

Frequency and phenotype of myeloid cells from peripheral blood of patients with metastatic melanoma and healthy donors are similar

Myeloid cells consist of a heterogeneous population of cells that lack expression of lineage markers (Lin: CD3, CD19, CD20, and CD56, CD57 expressed on T cells, B cells, and NK cells, respectively) and express either CD11b or CD33 common myeloid markers. Previous studies have described an enhanced frequency and immune-suppressive function in some of the peripheral blood myeloid cells in patients with cancer. Human MDSCs can be classified into monocytic ($CD14^+$ $HLA\text{-}DR^-$; ref. 23), granulocytic ($CD14^-$ $CD15^+$; ref. 25), or immature myeloid cells ($CD14^-$ $CD15^-$; ref. 11). To study all the subsets of myeloid cells present in peripheral blood of patients with melanoma, we conducted multicolor FACS of the whole blood after hypotonic lysis of RBCs from the patients with melanoma

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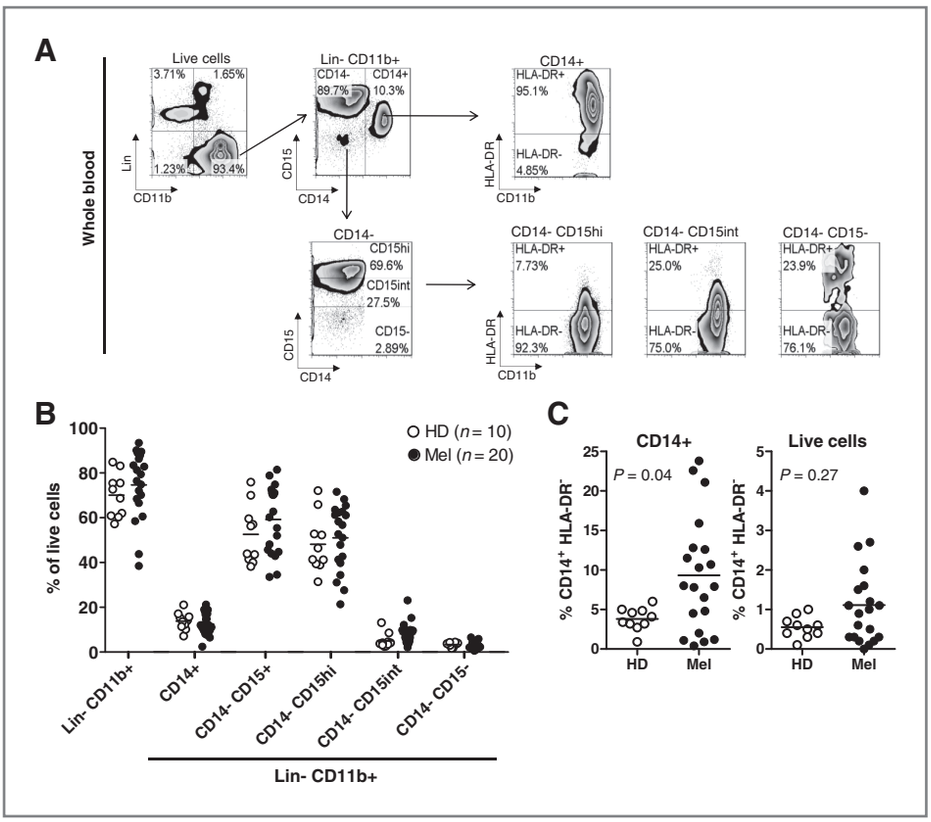

Figure 1. Characterization of myeloid cells in peripheral blood of healthy donors and melanoma patients. A, flow cytometry evaluation of expression of Lin (CD3/CD19/CD20/CD56/CD57), CD11b, CD14, and CD15 in whole blood. An example of representative dot plots after excluding aggregates, dead cells, and RBCs is shown. Gates were set based on isotype controls. Numbers represent the percentages from the parental populations gated. Names above FACS plots indicate the population gated that was analyzed. Markers analyzed are indicated in the axis of each FACS plot. The gating strategy used to analyze the samples is illustrated. B, frequency of the different phenotypes of myeloid cells in peripheral blood of healthy donors (HD; $n = 10$) and patients with melanoma (Mel; n = 20) within all the live cells. Each dot represents one patient. C, frequency of $CD14^+$ $HLA\text{-}DR^-$ cells within $CD14^+$ cells (left) and frequency of $CD14^+$ $HLA\text{-}DR^-$ within all live cells (right) in peripheral blood.

(Mel; $n = 20$) and healthy donors (HD; $n = 10$) using all the previously mentioned markers. Figure 1A shows the representative dot plots for one of the patients included in the study to illustrate the gating strategy used. In our work, we consistently observed a previously undescribed subpopulation, which expressed similar markers as neutrophils (Lin^- $CD11b^+$ $CD14^-$ $CD15^{hi}$) but lower expression of CD15 (Lin^- $CD11b^+$ $CD14^-$ $CD15^{int}$). In addition, the $CD15^{int}$ population showed a lower forward scatter, higher side scatter (not shown), and differences in expression of HLA-DR compared with the $CD15^{hi}$ population, which lead us to study it as a distinct population. Figure 1B depicts the relative frequencies of the myeloid subsets present in peripheral blood of HD compared with melanoma patients. We found no statistical difference in the frequency of myeloid cells (Lin^- $CD11b^+$) present in peripheral blood. In addition, contrary to the description in a prior report of an increase in $CD11b^+$ $CD15^+$ cells from approximately 20% in healthy donors to 80% in patients with stage III/IV metastasic melanoma (25), we found the percentage of Lin^- $CD11b^+$ $CD15^+$ to be comparable in normal donors and patients with melanoma. The percentage of Lin^- $CD11b^+$ $CD14^-$ $CD15^{int}$ population, a population that has not been previously studied, was also similar. A more detailed frequency of the different myeloid cells, including HLA-DR expression, represented as a percentage of all live cells and absolute cell counts in peripheral blood is shown in Table 2 and the frequency of each of the subsets within the myeloid cells is shown in Table 3. Although there were no differences in the percentage of $CD14^+$ cells (13.8 ± 1.2 in healthy donors vs. 12.6 ± 1.1 in patients with melanoma), the percentage of $HLA\text{-}DR^-$ cells within $CD14^+$ cells was statistically increased (Fig. 1C, left), reproducing differences in the frequency of this particular subset found in previous studies (22, 23). However, when these cells were represented as a percentage of all the live cells in peripheral blood in Fig. 1C, right, the percentage of $CD14^+$ $HLA\text{-}DR^-$ cells doubled in patients with melanoma, but this increase was not statistically significant. Finally, the percentage of live cells in peripheral blood corresponding to immature myeloid cells (Lin^- $CD11b^+$ $CD14^-$ $CD15^-$ $HLA\text{-}DR^-$) was low (2.3 ± 0.3) and was not altered in patients with melanoma (2.0 ± 0.3).

To test whether the phenotypic classification of the subsets actually corresponded to different myeloid cell types in peripheral blood, we sorted these cells by FACS according to the expression of the previously mentioned cell surface markers and characterized them morphologically using Wright-Giemsa stains on cytospins (Fig. 2A). Indeed, the Lin^- $CD11b^+$ (myeloid cells) contained morphologically distinct cell types that could be separated into monocytic cells (Lin^- $CD11b^+$ $CD14^+$) with their characteristic bean-shaped nuclei or into a subset enriched in granulocytic cells (Lin^- $CD11b^+$ $CD14^-$, most of which were $HLA\text{-}DR^-$). This group of cells could be further separated according to the expression of CD15; $CD15^{hi}$ expression corresponding to

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Table 2. Frequency and absolute numbers of myeloid subsets in peripheral blood of healthy donors and melanoma patients

	Healthy donor		Melanoma		*P*	
	%	(e5 cells/mL)	%	(e5 cells/mL)	%	(e5 cells/mL)
Lin^- $CD11b^+$	70.1 ± 3.1	(18.9 ± 3.4)	74.6 ± 3.4	(24.5 ± 5.0)	0.23	(0.68)
$CD11b^+$ $CD14^+$	13.8 ± 1.2	(3.7 ± 0.7)	12.6 ± 1.1	(4.0 ± 0.7)	0.48	(0.98)
HLA-DR^+	13.3 ± 1.2	(3.5 ± 0.7)	11.5 ± 1.1	(3.5 ± 0.5)	0.31	(0.87)
HLA-DR^-	0.6 ± 0.1	(0.2 ± 0.03)	1.1 ± 0.2	(0.5 ± 0.2)	0.27	(0.26)
$CD11b^+$ $CD14^-$ $CD15^+$	52.6 ± 4.2	(14.1 ± 2.4)	59.2 ± 3.4	(19.6 ± 4.3)	0.17	(0.49)
HLA-DR^+	0.7 ± 0.1	(0.2 ± 0.05)	1.2 ± 0.5	(0.3 ± 0.1)	0.52	(0.98)
HLA-DR^-	51.9 ± 4.2	(13.9 ± 2.4)	57.4 ± 3.5	(19.1 ± 0.4)	0.28	(0.58)
$CD11b^+$ $CD14^-$ $CD15^{hi}$	48.2 ± 4.0	(13.1 ± 2.5)	51.1 ± 3.3	(17.2 ± 4.0)	0.55	(0.65)
HLA-DR^+	0.4 ± 0.1	(0.1 ± 0.05)	0.5 ± 0.2	(0.2 ± 0.06)	0.26	(0.87)
HLA-DR^-	47.7 ± 4.1	(13.6 ± 2.5)	50.5 ± 3.2	(17.0 ± 4.0)	0.51	(0.65)
$CD11b^+$ $CD14^-$ $CD15^{in}$	5.0 ± 1.1	(1.5 ± 0.4)	8.1 ± 1.1	(2.4 ± 0.4)	0.02	(0.09)
HLA-DR^+	0.3 ± 0.1	(0.1 ± 0.04)	0.7 ± 0.3	(0.2 ± 0.08)	0.72	(0.87)
HLA-DR^-	4.7 ± 1.1	(1.4 ± 0.4)	7.4 ± 0.9	(2.2 ± 0.4)	0.02	(0.1)
$CD11b^+$ $CD14^-$ $CD15^-$	3.4 ± 0.3	(0.9 ± 0.3)	2.8 ± 0.4	(0.9 ± 0.2)	0.12	(0.72)
HLA-DR^+	1.1 ± 0.2	(0.3 ± 0.06)	0.8 ± 0.0.1	(0.2 ± 0.04)	0.05	(0.43)
HLA-DR^-	2.3 ± 0.3	(0.7 ± 0.2)	2.0 ± 0.3	(0.6 ± 0.1)	0.21	(0.94)

NOTE: Numbers represent the mean percent of live cells in peripheral blood ± SEM. Absolute counts are shown in parenthesis. *P* values were calculated using a 2-tailed Mann–Whitney test. Healthy donor $n = 10$; Melanoma patients $n = 20$.

neutrophils, $CD15^{int}$ expression to eosinophils (with their characteristic bi-lobed nuclei and eosinophilic granules), and $CD14^-$ $CD15^-$ cells, which contained a mix of immature polymorphonuclear and monocytic cells, which have not yet acquired CD15 and CD14 markers typical of more mature myeloid cells.

Table 3. Relative frequency of myeloid subsets in peripheral blood and tumors

		Melanoma			*P*
			Metastasis		
	HD blood	Blood			
	% of Lin^- $CD11b^+$	% of Lin^- $CD11b^+$	% of Lin^- $CD11b^+$	(e6 cells/g)	
$CD14^+$	19.5 ± 2.5	17.2 ± 1.6	49.8 ± 5.3	(1.3 ± 0.3)	0.495
HLA-DR^+	18.7 ± 2.4	15.7 ± 1.6	49.4 ± 5.3	(1.3 ± 0.3)	0.301
HLA-DR^-	0.8 ± 0.1	1.5 ± 0.3	0.4 ± 0.1	(0.01 ± 0.01)	0.243
$CD14^-$ $CD15^+$	69.8 ± 5.8	79.0 ± 1.8	28.9 ± 1.7	(1.2 ± 0.5)	0.202
HLA-DR^+	1.0 ± 0.2	1.5 ± 0.6	12.1 ± 3.0	(0.3 ± 0.1)	0.55
HLA-DR-	68.9 ± 5.8	76.5 ± 2.2	17.7 ± 3.8	(0.9 ± 0.4)	0.333
$CD14^-$ $CD15^{hi}$	68.6 ±3.3	67.8 ± 2.4	22.0 ± 4.1	(1.0 ± 0.4)	0.947
HLA-DR^+	0.6 ±0.1	0.7 ± 0.2	6.2 ± 1.2	(0.2 ± 0.1)	0.333
HLA-DR^-	68.0 ± 3.3	67.1 ± 2.5	15.8 ± 3.7	(0.8 ± 0.4)	0.983
$CD14^-$ $CD15^{int}$	7.8 ± 1.5	11.23 ± 1.5	8.9 ± 2.7	(0.2 ± 0.08)	0.019
HLA-DR^+	0.6 ± 0.2	0.9 ± 0.3	5.9 ± 2.1	(0.1 ± 0.04)	0.397
HLA-DR^-	7.2 ± 1.5	10.1 ± 1.3	3.0 ± 1.3	(0.1 ± 0.06)	0.068
$CD14^-$ $CD15^-$	4.7 ± 0.7	3.8 ± 0.5	18.9 ± 3.9	(0.3 ± 0.09)	0.311
HLA-DR^+	1.5 ±0.3	1.0 ± 0.2	14.1 ± 2.7	(0.3 ± 0.08)	0.123
HLA-DR^-	3.2 ± 0.6	2.7 ± 0.4	4.5 ± 1.2	(0.07 ± 0.01)	0.441

NOTE: Numbers represent the mean percent of cells within the Lin^- $CD11b^+$ gate ± SEM (HD blood n = 10; Melanoma blood n = 20; melanoma metastasis n = 26).

P values comparing the frequencies (%) of the subsets in the blood of healthy donors (HD) and melanoma patients were calculated using a two-tailed Mann-Whitney test.

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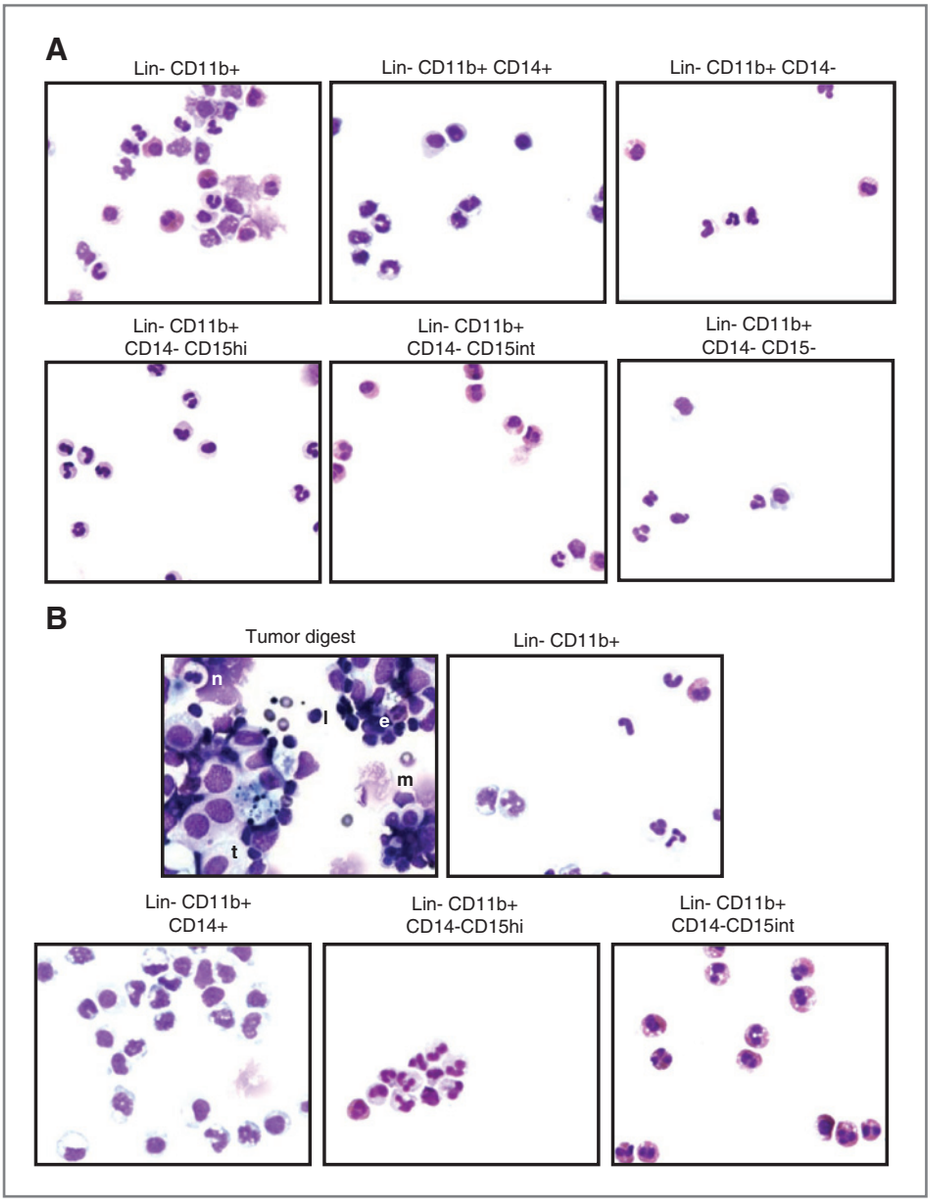

Figure 2. Morphologic traits of myeloid cells in peripheral blood and tumors of patients with melanoma. Myeloid cells were stained with PI and cell surface antibodies for Lin (CD3/CD56/CD57/CD19/CD20), CD11b, CD14, and CD15, and PI-negative cells (live cells) were sorted according to cell surface expression of Lin, CD11b, CD14, and CD15 and cytospins and Wright-Giemsa stains were conducted. Representative pictures of the populations sorted from patient #2 are shown. Cytospins of myeloid cells sorted from peripheral blood (A) and from melanoma single cells suspension (B).

Myeloid cells infiltrating metastatic melanoma lesions are phenotypically heterogeneous

The existing literature related to cancer MDSCs is largely based on murine models or myeloid cells from blood/bone marrow of patients with cancer. In an effort to study the myeloid cells in the tumor microenvironment, we assessed the expression of the same markers used to characterize blood myeloid cells (CD11b, CD14, CD15, HLA-DR) by FACS in the single cell suspensions obtained after enzymatic digestion of 26 freshly resected melanoma tumors. Cytospins and Wright-Giemsa stain of the cells isolated from one tumor digest is shown in Fig. 2B. This tumor contained morphologically diverse cells including tumor cells (t), lymphocytes (l), and myeloid cells that could be identified as macrophages (m), neutrophils (n), and eosinophils (e) that could be efficiently enriched from the single cell suspension by separating them according to expression of $CD14^{+}$, $CD14^{-}$ $CD15^{hi}$, and $CD14^{-}$ $CD15^{int}$, respectively. Figure 3A illustrates the gating strategy of one of melanoma samples studied. Myeloid cells (Lin^{-} $CD11b^{+}$ cells) were an infrequent population of cells infiltrating metastatic melanoma deposits (Fig. 3B). The mean percentage of myeloid cells in the tumors (% Lin^{-} $CD11b^{+}$ within all the live cells in the tumor after gating out aggregates, dead cells, and RBCs) was 12.0 ± 3.5, and they were less prevalent than other tumor-infiltrating leucocyte populations (Supplementary Fig. S1). Notably, the phenotype of the myeloid cells in the tumor did not resemble the phenotype of

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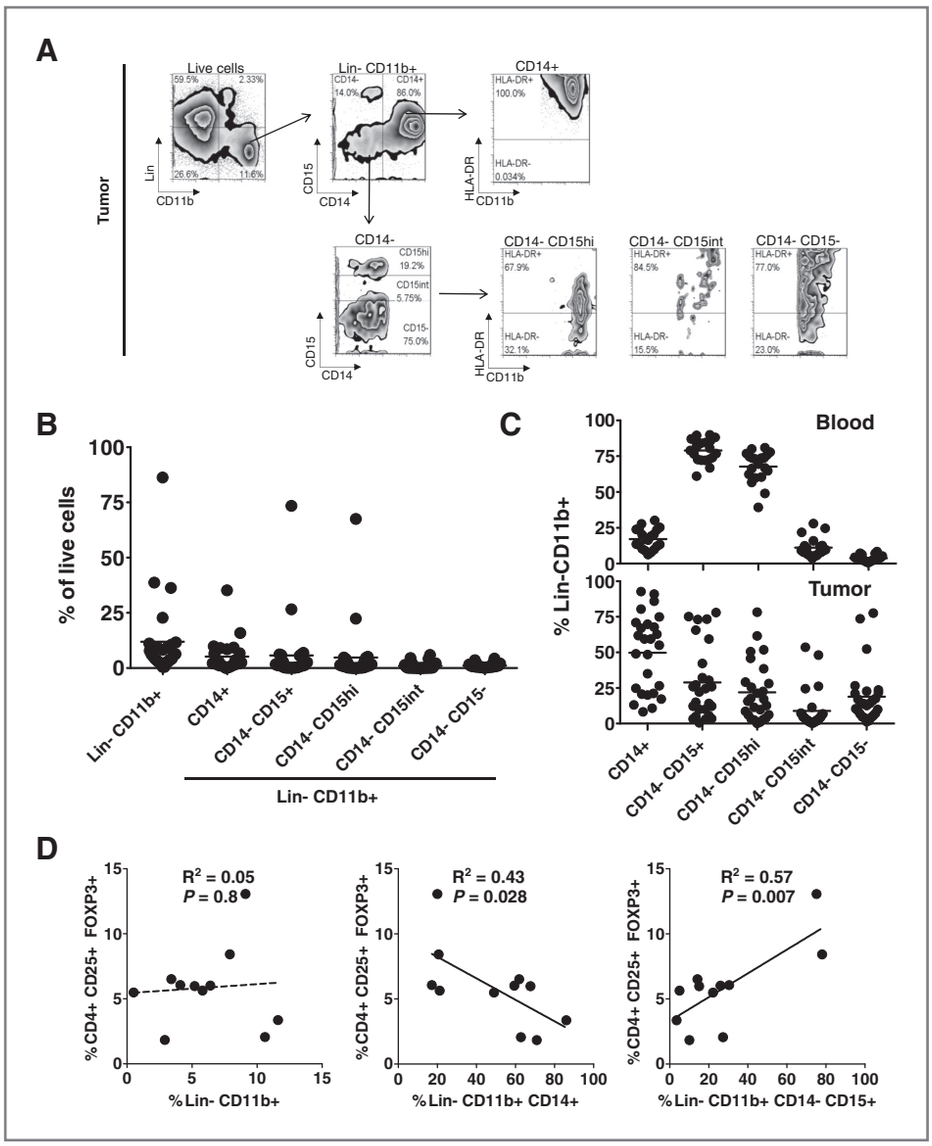

Figure 3. Characterization of myeloid cells infiltrating fresh melanoma tumors. A, flow cytometry evaluation of expression of Lin (CD3/CD19/CD20/CD56/CD57), CD11b, CD14, and CD15 in melanoma tumor cell digests. An example of representative dot plots after excluding aggregates, dead cells, and RBCs is shown. Gates were set based on isotype controls. B, frequencies of myeloid cells and subpopulations of myeloid cells infiltrating melanoma tumors ($n = 26$) represented as a percentage of all the live cells in the tumor (after excluding aggregates, dead cells, and RBCs). Each dot represents one melanoma sample. C, differences in the relative frequency of the myeloid subpopulations present in blood and tumors from patients with melanoma (Blood $n = 20$; Tumor $n = 26$). D, correlation of tumor-infiltrating myeloid cells and T-regulatory cells. For 11 melanoma tumors, both T-regulatory cell frequency (CD4+ CD25 FOXP3+) and frequency of myeloid cells as well as frequency of CD14+ and CD14- CD15+ within myeloid population were determined and correlation was assessed using a Spearman test.

peripheral blood myeloid cells (Fig. 3C and Table 3), indicating that these cells were indeed tumor-infiltrating and not just a reflection of the blood contained within tumors. Although in the blood, the relative percentage of each of the myeloid subsets studied was highly homogenous from patient to patient with a predominant neutrophil population ($CD14^-$ $CD15^{hi}$), tumor-infiltrating myeloid cells comprised a phenotypically diverse population, with high tumor-to-tumor variability. The most predominant myeloid subset (gating on myeloid cells Lin^- $CD11b^+$) in melanoma tumors was $CD14^+$ cells (49.8 ± 5.3); however, some tumors were highly enriched in granulocytic cells, most of these being neutrophil-like ($CD14^-$ $CD15^{hi}$). In addition, all the subpopulations of myeloid cells infiltrating melanoma tumors displayed higher expression of HLA-DR compared with the peripheral blood myeloid cells (Table 3), a marker that most probably reflects a more active and differentiated state. Previous mouse and human studies have suggested that some myeloid-derived cells could potentially inhibit T-cell proliferation through Treg induction (34, 35). To study whether the presence of any of the myeloid cells in the tumor was associated with an increased frequency of Tregs, we correlated the frequency of $CD4^+$ $CD25^+$ FOXP3+ cells in 11 tumors with the myeloid infiltrate. Although there was no association between the percent of myeloid cells (lin^- $CD11b^+$) and Treg cells, we found a negative association between the percent of $CD14^+$ -infiltrating myeloid cells and Tregs (Fig. 3D). Intriguingly, the percentage of intratumoral $CD15^+$ cells correlated with an increased frequency of Tregs, suggesting that perhaps granulocytic cells could inhibit T-cell proliferation indirectly through induction of other inhibitory cells.

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Melanoma-infiltrating myeloid cells display an impaired ability to inhibit *in vitro* T-cell proliferation compared with peripheral blood myeloid cells

We next evaluated the ability of myeloid cells in blood and tumor to suppress T-cell proliferation. We thus isolated by FACS the previously mentioned myeloid subsets (those that could be isolated depending on the frequency for each particular sample) from peripheral blood and from the tumor of the same patient and cocultured them with autologous CFSE-labeled $CD3^+$ cells stimulated nonspecifically with anti-CD3/CD28 beads. The CFSE dilution of T cells cocultured with blood and tumor-infiltrating myeloid cells isolated from one representative melanoma patient (Fig. 2 displays the purity and morphology of myeloid cells isolated) is shown in Fig. 4. Cumulative data assessing the suppressive activity of blood myeloid–derived subsets from 7 patients are represented in Fig. 4D and summarized in Supplementary Table S1. The whole myeloid population (Lin^- $CD11b^+$) as well as the $CD14^+$ (monocytes) and $CD14^-$ (neutrophils, eosinophils, and immature myeloid cells) populations displayed weak ability to suppress T-cell proliferation (mean inhibition of 15.4 ± 7.21%, 19.4 ± 9.94%, and 16.6 ± 9.57%, respectively). Despite this, we found that both the $CD14^+$ $HLA\text{-}DR^+$ cells and the $CD14^+$ $HLA\text{-}DR^-$ monocytic cells showed an enhanced ability to inhibit T-cell responses compared with the parental population (53.7 ± 21.3% and 59.5 ± 32.85% inhibition of proliferation, respectively). Furthermore, within the $CD14^-$ cells, the less common eosinophil population ($CD14^-$ $CD15^{int}$) showed an enhanced ability to inhibit T-cell

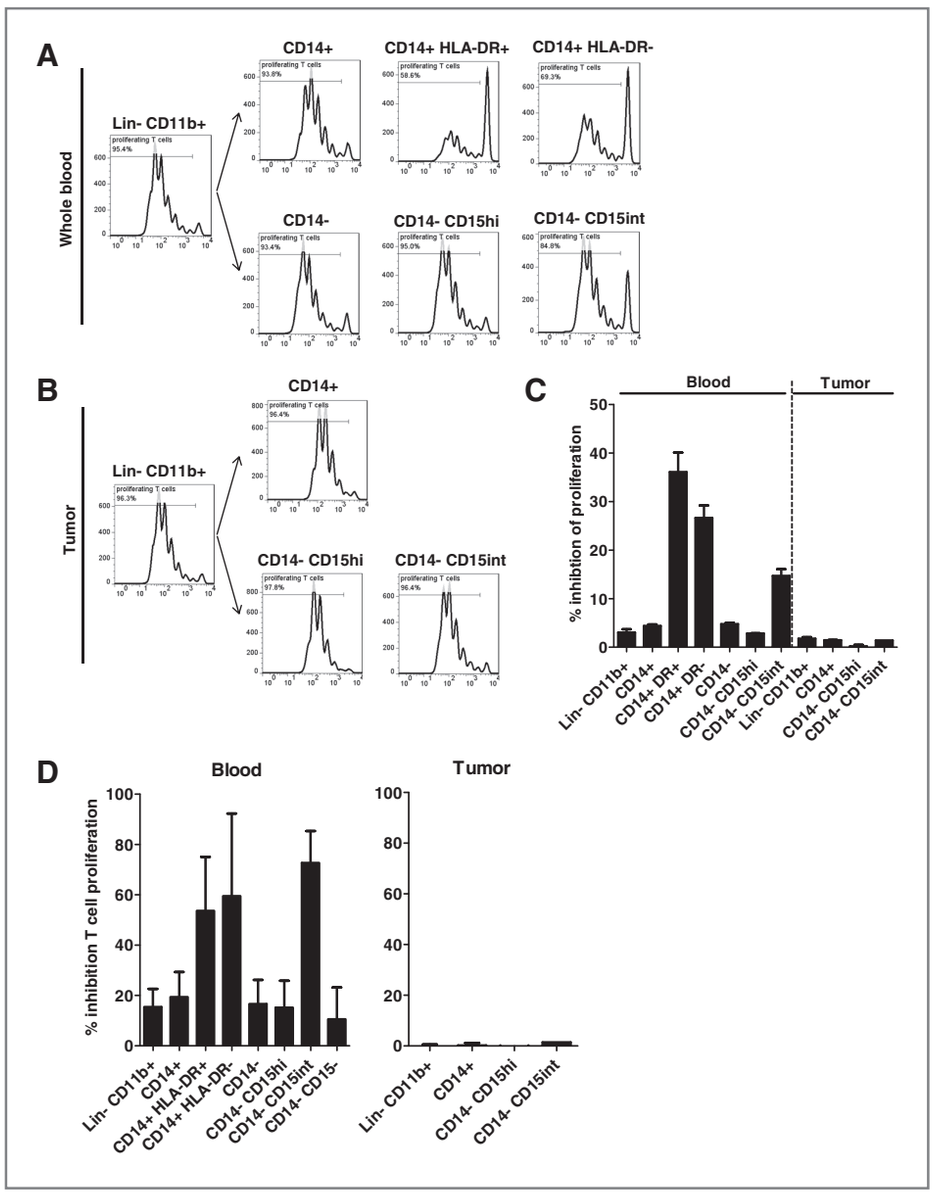

Figure 4. Melanoma-infiltrating myeloid cells are not capable of suppressing T-cell proliferation. Myeloid cells and subpopulations were isolated from blood (A) and fresh melanoma tumor digest (B) of a melanoma patient (P#2) and cocultured together with CFSE-labeled autologous CD3 cells at a 1:1 ratio in presence of anti-CD3/anti-CD28 beads. Four days later, proliferation of T cells was assessed by measuring percentage of T cells that diluted CFSE. C, percentage inhibition of T-cell proliferation exerted by blood and tumor myeloid cells. D, cumulative percentage inhibition of T-cell proliferation exerted by blood ($n = 7$) and tumor-infiltrating ($n = 6$) myeloid subsets. Average percentage inhibition of T-cell proliferation in presence of the different myeloid subsets isolated and SEM are represented.

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proliferation compared with the $CD14^-$ $CD15^{hi}$ cells, as shown by the percentage inhibition of T-cell proliferation (72.7 inhibition ± 12.69% when considering 6 independent experiments) when coculturing this subset with autologous T cells.

When we compared the suppressive activity of tumor-infiltrating myeloid cells with those in peripheral blood from the same patient, we found that these were unable to inhibit T-cell proliferation (Fig. 4C for patient#2 and Fig. 4D and Supplementary Table S2 for cumulative data from 6 tumors). The general myeloid population (Lin^- $CD11b^+$) from 6 tumors displayed a mean percentage of inhibition of T-cell proliferation of 0.1 ± 0.5. Furthermore, the $CD14^+$ population, which in the tumor consisted of macrophages characterized by high expression of HLA-DR, failed to inhibit T-cell proliferation. In fact, the mean suppressive activity of tumor-infiltrating Lin^- $CD11b^+$ $CD14^+$ cells from 4 independent tumors was 0.2 inhibition ± 0.9%. Despite the low frequency of Lin^- $CD11b^+$ $CD14^-$ $CD15^{hi}$ and $CD15^{int}$ subsets infiltrating melanoma metastasis, we succeeded in the isolation and assessment of suppressive activity of these cells from 2 and 1 tumor, respectively. These cells also failed to inhibit T-cell proliferation (−0.2% inhibition for neutrophils and 1.4% inhibition for eosinophils), in contrast to the high immune-suppressive activity of the $CD14^-$ $CD15^{int}$ population of cells found in peripheral blood. In conclusion, when tested under the same conditions myeloid cells isolated from metastatic melanoma deposits displayed a diminished ability to inhibit nonspecific T-cell proliferation compared with the suppressive function of monocytic cells and eosinophils present in peripheral blood of patients with melanoma.

Discussion

Morphologic, phenotypic, and functional heterogeneity is a hallmark of MDSCs (2). However, this heterogeneity has also generated ambiguity in the definition of MDSC. Currently, one of the biggest limitations to address the importance of these cells in human cancer patients is the lack of more specific markers defining a suppressive population within monocytic and granulocytic myeloid cells. Despite several attempts to overcome this drawback, the use of markers such as CD124 (6), CD115 (36), and CD244 (37) either remain controversial (3) or have not yet been extensively validated. Therefore, most of the characterizations of MDSCs conducted in patients with cancer have been limited to parallel the 2 main subsets described to be expanded and acquire suppressive activity in tumor-bearing mice (3). These are the monocytic MDSCs [$Ly6C^+$ $Ly6G^-$ in mice and Lin^- $CD11b^+$ $CD14^+$ $HLA\text{-}DR^-$ in human (15, 23)], granulocytic MDSCs [$Ly6C^-$ $Ly6G^+$ in mice, and Lin^- $CD11b^+$ $CD14^-$ $CD15^+$ in human (38)], and an additional population of immature myeloid cells described in patients with cancer(Lin^- $CD33^+$ $CD14^-$ $CD15^-$ $HLA\text{-}DR^-$; ref. 39). Far from narrowing down the suppressive phenotype to a specific myeloid population that differs in phenotype from a parental population, these subsets include all the myeloid cells present in peripheral blood including monocytes (Lin^- $CD11b^+$ $CD14^+$), neutrophils (Lin^- $CD11b^+$ $CD14^-$ $CD15^+$), eosinophils (Lin^- $CD11b^+$ $CD14^-$ $CD15^{int}$), immature myeloid cells, and basophils (both Lin^- $CD11b^+$ $CD14^-$ $CD15^-$). In addition, most human studies focus on only one of the MDSC subsets described (monocytic, granulocytic, or immature myeloid cells) and include patients with several types of cancer in the analysis, which gives an incomplete characterization of MDSCs and can possibly skew the results, as MDSCs are shaped according to the tumor-secreted factors, which can differ among tumor types (2, 3). In an attempt to attribute the suppressive phenotype of MDSCs, more specifically to one or several of the myeloid subsets in patients with melanoma, our work studied the frequency and function of the main existing myeloid subsets (monocytes/macrophages, neutrophils, eosinophils, immature myeloid cells) ± expression of HLA-DR, a marker that has been repeatedly used to discern suppressive from nonsuppressive monocytes. Our study combines a detailed characterization of the frequency, phenotype, and suppressive function of multiple phenotypically/morphologically distinct myeloid subsets in blood of patients with melanoma, which adds insight into the relative contribution and importance of each of these cells types to the inhibition of T-cell proliferation in patients with this cancer.

The suppressive function of monocytic cells in patients with melanoma has generally been attributed to the $CD14^+$ $HLA\text{-}DR^-$ subset, suggested to represent less mature monocytic cells that have not yet become activated. Studies regarding the suppressive activity of monocytic cells in the blood of patients with melanoma provide suggestive evidence indicating that their inhibitory function in patients with melanoma is increased compared with that of healthy donors (22, 23). Authors suggest that the increased frequency of the $CD14^+$ $HLA\text{-}DR^-$ subset in peripheral blood rather than only the modest increase in suppressive activity in this population between patients with melanoma and healthy donors could account for an enhanced tolerogenic environment in patients with cancer. We were able to detect the previously described alteration in the percentage of $HLA\text{-}DR^-$ cells within $CD14^+$ (22, 23) cells in whole blood (3.8 ± 0.5% in healthy donors and 10.5 ± 1.6% in patients with melanoma). However, when determining the frequency of $CD14^+$ $HLA\text{-}DR^-$ within all the live cells in peripheral blood (after RBC lysis), we found that there was a small yet not statistically significant increase (from 0.6 ± 0.1% to 1.1 ± 0.2%) in patients with metastatic melanoma. Our data not only highlights the small difference in the percentage of $CD14^+$ $HLA\text{-}DR^-$ cells in patients melanoma compared with healthy donors, but also, and more importantly, the small frequency of these cells in peripheral blood.

There is only one article reporting the existence of suppressive granulocytic myeloid cells in peripheral blood of patients with melanoma (25). The authors describe a 4-fold increased frequency of neutrophils in patients with melanoma (in their study characterized as $CD11b^+$ $CD15^+$ cells; 20% for healthy controls and approximately 80% in stage

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III/IV melanoma patients; ref. 25). We were not able to reproduce this remarkable difference between healthy donors and patients with melanoma. However, a letter to the editor concerning this article reported that 4 independent laboratories were unable to reproduce some of the findings in this study (40). This paper also reported that neutrophils, rather than a mixed population of granulocytes (neutrophils and eosinophils), are responsible for inhibiting antigen-specific T-cell proliferation. Our study provides evidence that the $CD11b^+$ $CD15^+$ population includes not only neutrophils ($CD11b^+$ $CD15^{hi}$) but also a less predominant eosinophil population with lower expression levels of CD15. In addition, our results show that eosinophils can display a highly suppressive function, repeatedly more potent than neutrophils. Similar to other myeloid populations, eosinophils have been associated with local immune-stimulating and immune-suppressive activities depending on the microenvironment (41, 42). Paraneoplasic blood hypereosinophilia is not common but has been reported in several nonsolid malignancies (43), and in contrast to the tumor-associated tissue eosinophilia, is usually associated with aggressiveness and poor prognosis (44). Recently, an increased frequency of immune-suppressive $CD15^+$ $CD16^-$ granulocytes in the peripheral blood of patients with terminal cancer has been associated with poor prognosis and performance status (45). The authors associate the suppressive activity of these cells to activated granulocytes, which downregulate CD16 expression upon activation. However, the $CD15^+$ $CD16^-$ phenotype is typical of eosinophils in the steady state, as these cells can be highly enriched and separated from neutrophils in peripheral blood by conducting negative selection of $CD16^+$ cells (46). Another recent work proposes CD244 as a specific marker to distinguish granulocytic MDSCs from neutrophils in tumor-bearing mice (37). Intriguingly, this marker is typically expressed in human eosinophils but not in neutrophils (47). Our results uncover a potential contribution of eosinophils to the suppressive phenotype of Lin^- $CD11b^+$ $CD15^+$ granulocytic MDSCs that has been commonly attributed to the more frequent neutrophil population.

One of the main unresolved issues is the nature and function of myeloid cells infiltrating tumor tissues. Indeed, in the case of T-regulatory cells, the slight increased percentage of Tregs in peripheral blood of patients with cancer compared with healthy donors clearly underestimates their importance in the tumor, where they accumulate to represent an important percentage of the total $CD4^+$ cells (33, 48). In contrast to T-regulatory cells, our study found that myeloid cells were an infrequent population infiltrating melanoma tumors relative to other leucocytes. The mean percentage of myeloid cells in tumor single cell suspensions was 12%. The phenotypic diversity of myeloid cells infiltrating melanoma tumors clearly contrasts with the homogeneity in frequency and phenotype of the different myeloid subsets in peripheral blood. This previously undocumented diversity of tumor-infiltrating myeloid cells adds an additional layer of complexity to the understanding of their immune-regulatory role at this particular site and suggests that the tumor microenvironment is shaped according to the tumor-specific factors secreted by each particular tumor. Contrary to the low frequency of CD14+ cells in peripheral blood, a predominance of $CD14^+$ cells was found in many tumors (Fig. 3C). However, there was high variability and some tumors displayed a high proportion of granulocytic cells or even contained some immature myeloid cells. Despite the phenotypic diversity, the Lin-$CD11b^+$ population infiltrating melanoma tumors did not suppress nonspecific T-cell proliferation. Furthermore, isolation of some of the subpopulations of myeloid cells infiltrating melanoma metastasis also did not inhibit T-cell proliferation as compared with the homologous populations from peripheral blood. Whether the inability to suppress nonspecific T-cell proliferation, as opposed to antigen-specific proliferation, reflects their real functional status in the tumor microenvironment remains an open question, as low yield of these cells have limited antigen-specific response studies. Interestingly, the most prevalent myeloid population infiltrating melanoma tumors was $CD14^+$, most likely tumor-associated macrophages, (TAM). TAMs, typically exposed to Th2 cytokines (IL-4 and IL-13), have been postulated to promote tumor progression through secretion of angiogenic factors, growth factors, proinvasive factors, and immune-suppression (49). Indeed, culture of peripheral blood $CD34^+$ progenitors cells in presence of cancer cell line supernatants *in vitro* resulted in an altered differentiation of dendritic cells ($CD14^+$ cells), as they did not efficiently express HLA-DR and other markers of mature and differentiated APCs (50, 51). According to our data, melanoma-infiltrating $CD14^+$ cells expressed high levels of HLA-DR (Fig. 3A and Table 3). Perhaps, this more differentiated phenotype, characterized by high HLA-DR expression, can account for the lack of suppressive function of these cells in melanoma tumors. The only evidence that we found supporting an indirect immune-regulatory role of tumor-infiltrating myeloid cells is the correlation between infiltrating granulocytic cells and T-regulatory cells, but functional experiments will be required to further study the relevance of this association.

Overall, our results show that both monocytic cells and eosinophils from peripheral blood of patients with melanoma are capable of inhibiting nonspecific T-cell proliferation. Despite expression of PD-1 and frequency of T-regulatory cells were increased in melanoma-infiltrating lymphocytes as previously reported by our group (data not shown; refs. 32, 33), supporting other mechanisms of immune dysfunction in the population of melanoma patients studied, we did not observe any major differences in frequency and phenotype in any of the myeloid subsets in peripheral blood of patients with melanoma compared with healthy donors. Furthermore, in preliminary experiments, we observed a similar ability to suppress T-cell proliferation with myeloid cells obtained from 2 healthy donors tested (Supplementary Fig. S2). A heterogeneous myeloid population was found infiltrating melanoma tumors, but these cells were not capable of suppressing T-cell proliferation. The lack of alterations in frequency of

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myeloid derived cells that we observed between patients with melanoma and healthy donors contrast with the expansion of these cells reported in experimental models of cancer (3, 6, 27). Possibly, the accumulation of MDSCs is less evident in melanoma, a unique tumor capable of responding to immunotherapy (52, 53). Divergence between fast-growing subcutaneously implanted tumors and the slow evolution of human tumors could also account for some differences. But perhaps, the biggest hurdle that will need to be overcome to address the importance of these cells in patients with cancer is the lack of more specific markers defining a suppressive population within monocytic and granulocytic myeloid cells. Although the presence of a minor suppressive myeloid subpopulation cannot be ruled out, our findings suggest that the frequency and contribution of these cells to the inhibition of T-cell proliferation in patients with metastatic melanoma is less important than initially thought. The suppressive function displayed by peripheral blood derived myeloid cells but not by tumor-infiltrating myeloid cells in patients with melanoma was unexpected and the immunologic relevance of the suppressive function of these circulating myeloid cells warrants further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by the authors.

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Clinical Cancer Research

Myeloid Cells Obtained from the Blood but Not from the Tumor Can Suppress T-cell Proliferation in Patients with Melanoma

Alena Gros, Simon Turcotte, John R. Wunderlich, et al.

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