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Brief Conclusive Report

Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties

Sven Brandau,[*,1] Sokratis Trellakis,* Kirsten Bruderek,* Dominik Schmaltz,[†] Gabriele Steller,[†] Motaz Elian,* Henrik Suttmann,[‡] Marcus Schenck,[§] Jürgen Welling,[||] Peter Zabel,[||] and Stephan Lang**

Departments of *Otorhinolaryngology and [§]Urology, University Hospital Essen, Essen, Germany; Departments of [†]Immunology and Cell Biology and [||]Pneumology, Research Center Borstel, Borstel, Germany; and [‡]Department of Urology, University of Lübeck, Lübeck, Germany

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ABSTRACT

In tumor-bearing mice, immunosuppressive granulocytic and monocytic MDSC have been identified. The identity and function of MDSC in cancer patients are less clear and need further characterization. We analyzed the peripheral blood of 103 patients with HNC, lung cancer, or cancers of bladder and ureter. Based on sedimentation properties in density gradients, a subset of LD-PMN was identified and analyzed. LD-PMN were expanded in the peripheral blood of cancer patients, suppressed proliferation, and IFN-γ production of polyclonally stimulated T cells and thus, qualify as human MDSC. Immunophenotyping and morphological analysis revealed the accumulation of immature PMN in the MDSC fraction. Neutrophilic MDSC showed altered surface marker expression, prolonged survival, and impaired effector functions when compared with conventional, mature PMN of regular density. MDSC displayed markedly reduced chemotaxis toward tumor-conditioned medium and lacked expression of chemokine receptors CXCR1 and CXCR2, which are normally required for PMN extravasation from the bloodstream and subsequent tissue infiltration. Collectively, our data suggest the accumulation and persistence of long-lived, immature granulocytic MDSC with T cell-suppressive function and impaired migratory properties in the peripheral blood of cancer patients. *J. Leukoc. Biol.* 89: 311–317; 2011.

Introduction

Numerous studies in the last two decades have substantiated the finding that tumors modulate and deviate the differentiation of myeloid cells. The disturbed differentiation of DCs [1], together with the presence of immature myeloid cells and $CD34^+$ progenitor cells [2], has been identified as means by which the tumor skews host anti-tumor immunity. More recently, in murine tumor models, a heterogeneous subset of so-called MDSC has been identified [3–5]. MDSC have been described as immunoregulatory cells that accumulate and expand in the blood and lymphoid organs of tumor-bearing mice [6, 7]. Murine MDSC consist of subsets of granulocytic, monocytic, and intermediate cells [8, 9]. An arsenal of mechanisms that includes but is not limited to arginase activity, production of ROS and nitrogen species, and different cytokines has been linked to the immunosuppressive activity of MDSC and to their capacity to impair T cell function [5, 10–14].

Stimulated by murine studies, investigators aimed at defining putative MDSC in human cancer patients. Recently, Rodriguez and colleagues [15] suggested that in patients with RCC, activated granulocytes exhibit features of MDSC. Other groups suggested a subset of CD14-positive cells as putative human MDSC [16]. In an earlier study, Almand and colleagues [17] found that the fraction of immature myeloid cells in their patients was mainly composed of monocytic cells and did not express the granulocytic marker CD15. Some studies have also used the myeloid progenitor marker CD33, along with HLA-DR negativity, to characterize and isolate human MDSC [17, 18]. In mice, expression of IL-4Rα on a subset of immunosuppressive, CD11b-positive monocytic cells was crucial for their suppressive activity on CD8 T cells [19]. In human cancer patients, IL-4Rα is up-regulated on monocytes and granu-

Abbreviations: APC=allophycocyanin, ATCC=American Type Culture Collection, CEA=carcinoembryonic antigen, HD-PMN=polymorphonuclear granulocytes of high density, HNC=head and neck cancer, HNSCC=head and neck squamous cell carcinoma, LD-PMN=polymorphonuclear granulocytes of low density, MDSC=myeloid-derived suppressor cells, MNC=mononuclear cells, NSCLC=non-small cell lung carcinoma, PMN=polymorphonuclear granulocytes, RCC=renal cell carcinoma, ROI=reactive oxygen intermediate, SSC=sideward scatter

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany. E-mail: sven.brandau@uk-essen.de

locytes, but the functional relevance of this finding still has to be determined [20].

Overall, current evidence suggests a complex alteration of myeloid cell differentiation and function in human cancer patients that involves polymorphonuclear and monocytic cells. To date, the majority of studies about human cancer patients investigated relatively small cohorts of patients and analyzed cell populations, which in many cases, were heterogeneous in terms of monocytic versus granulocytic, of activated versus resting, and of immature versus mature cells. Thus, two key questions, which at this stage, have not been clarified sufficiently are: first, do equivalents of murine MDSC also exist in human cancer patients, and what is their identity? Second, does altered myelopoiesis in human cancer patients result in the peripheral expansion of subsets of immature myeloid cells, and what is their cellular function? Given the substantial differences of granulocytic and monocytic MDSC in mice [6, 8, 9], it seems to be crucial to separately investigate granulocytic and monocytic cells in human patients with cancer.

We focused our attention on a subset of leukocytes with high SSC in flow cytometry that copurified with MNC in density gradients. Investigating large cohorts of cancer patients, we found that this subset was highly enriched in patients and contained different stages of immature neutrophil granulocytes. These neutrophils were suppressive on T cells and had reduced migratory properties and reduced effector cell functions. Our study provides new insights into the biology and function of MDSC in cancer patients.

MATERIALS AND METHODS

Study subjects and tumor characteristics

Tumor patients were enrolled in the respective clinical departments of the University of Lübeck (Lübeck, Germany) and the University of Duisburg-Essen (Essen, Germany) between 2004 and 2010. Detailed characteristics of patients and tumors are presented in **Table 1**. Collection of material in this explorative clinical study was approved by the ethics committees of the Medical Faculties of the Universities of Lübeck and Duisburg-Essen. Written, informed consent was obtained from all individuals before collection of samples.

Isolation of PMN from peripheral blood

Peripheral blood was drawn into 3.8% sodium citrate anticoagulant and admixed with PBS at a ratio of 1:1 v/v before separation by density gradient centrifugation (lymphocyte separation medium; PAA, Paschingen, Austria). When applying peripheral blood of cancer patients to this procedure, the interphase contains MNC and cosedimenting cells of high SSC. In the context of this manuscript, we termed these cells PMN of low density (LD-PMN).

Purification of LD-PMN

For purification of LD-PMN, we developed a multi-step isolation protocol, resulting in purity and viability of LD-PMN higher than 90% (Supplemental Materials and Methods).

Isolation of conventional PMN

For isolation of conventional PMN of regular high-density (HD-PMN), we applied a previously used protocol [21]. Purity and viability of PMN isolated by this method were higher than 98%.

TABLE 1. Patient and Tumor Characteristics

	Head and neck		Urologic		Lung	
Characteristics	*n*	%	*n*	%	*n*	%
All samples	57	100	16	100	30	100
Sex						
Male	47	83	11	69	18	60
Female	10	17	5	31	12	40
Tumor localization/type[a]						
A	24	42	14	88	2	7
B	9	16	1	6	16	53
C	12	21	1	6	11	37
D	12	21	0	0	1	3
T category[b]						
Ta	0	0	2	13	0	0
T1	4	7	0	0	1	3
T2	15	26	8	50	6	20
T3	17	30	2	13	3	10
T4	21	37	4	25	20	67
Tumor stage[b]						
0	0	0	2	13	0	0
I	3	5	0	0	0	0
II	10	18	2	13	4	13
III	10	18	9	56	15	50
IV	34	60	3	19	11	37

Blood samples were collected from patients before oncologic therapy and from healthy volunteers as control subjects. Patients were excluded from the study if they had recent radiotherapy or chemotherapy, a synchronous carcinoma in another location or a known hematological disease, or a severe concomitant systemic infectious disease. [a]Head and neck region: squamous cell carcinoma of oropharynx (A), hypopharynx (B), larynx (C), and oral cavity (D). Urological cancer of bladder and ureter: urothelial (A), squamous cell (B), adenocarcinoma (C), and others (D). NSCLC: mixed (A), squamous cell (B), adenocarcinoma (C), and others (D). [b]Determined according to the tumor node-metastasis classification system for staging malignancy. For urological tumors, the pathological stage for the rest the clinical stage is given.

LD-PMN-T cell interaction

Responder lymphocytes from HNC patients were isolated by CD3 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer's instructions. Isolated responder T cells were stained with 5 μM CFSE (Invitrogen, Carlsbad, CA, USA). Cells/mL (0.25×10^6) were stimulated with biotinylated antibodies directed against CD2, CD3, and CD28 (Miltenyi Biotec; regulatory T cell suppression inspector kit) and with anti-biotin MACSiBeads™ particles to induce proliferation. Autologous LD-PMN were added at ratios of 1:1–40:1 (T cell:PMN). CFSE fluorescence intensity was analyzed by flow cytometry after 7 days of coculture and proliferation. Supernatants of the coculture were collected, and IFN-γ was measured by ELISA (R&D Systems, Wiesbaden, Germany), according to the manufacturer's protocol.

Flow cytometry

For six-color immunophenotyping of neutrophilic MDSC, eosinophils were excluded by labeling of CD125 (IL-5Rα). Characterization of MDSC subsets was performed with the following antibodies: mouse anti-human CD125 1:50 (clone 26815, R&D Systems); rat anti-mouse IgG1 PerCP 1:20 (BD Biosciences, Heidelberg, Germany); anti-CD11b APC-Cy7 1:200 (clone macrophage-1 antigen; CR3), anti-CD16 PE-Cy7 1:200 (clone 3G8), anti-CD33 PE

1:20 (clone WM53), and anti-HLA-DR APC 1:20 (clone G46-6; all from BD Biosciences); anti-CD66b FITC 1:100 (clone 80H3, Beckman Coulter, Fullerton, CA, USA); and anti-CXCR1 PE 1:50 (clone 8F1/CXCR1), anti-CXCR2 PE 1:50 (clone 5E8/CXCR2), and anti-CXCR4 PE 1:10 (clone 12G5, all from BioLegend, München, Germany).

Apoptosis assay

Apoptosis of MDSC/PMN was determined by an Annexin V-PE apoptosis detection kit (BD Biosciences) after culture for 24 h in medium or with or without 100 ng/mL G-CSF (Chugai Pharma, Frankfurt, Germany). Cells were analyzed on a BD FACSCanto II using DIVA 6.0 software.

Cell line and HNSCC supernatant

The human hypopharyngeal carcinoma cell line FaDu (ATCC, Manassas, VA, USA) was cultured in RPMI culture medium supplemented with 10% FCS and antibiotics. We followed the guidelines provided by ATCC Technical Bulletin No. 8, 2007, to assess the quality and identity of cell lines used in our studies. To obtain supernatant, we cultured the cells at 2×10^6/mL for 24 h. Cell debris was removed by centrifugation, and the supernatant was stored at –20°C.

Chemotaxis assay

MDSC/PMN (3×10^5) were allowed to migrate through 3 μm transwells (BD Falcon) toward medium or FaDu supernatant for 3 h at 37°C. The migrated cells were counted with a Casy Model TT cell counter (Roche Innovatis, Bielefeld, Germany).

RESULTS AND DISCUSSION

Identification of immature neutrophilic MDSC in the peripheral blood of patients with different types of solid cancer

Occasional reports have indicated the existence of leukocytes with altered buoyancy and altered sedimentation properties in the peripheral blood of cancer patients applied to density gradients [22, 23]. However, the cell biological properties of those leukocyte subsets and the possible functional implications of these findings remained largely unexplored. Using conventional density gradient centrifugation to separate and isolate MNC from the peripheral blood of cancer patients and healthy donors, we investigated a subset of leukocytes with high SSC that copurified with MNC. When lineage and marker antigens specific for the different leukocyte subsets in the peripheral blood were applied, we noticed that this population expressed high amounts of CD66b antigen (**Fig. 1A**). CD66b (CEA-related cell adhesion molecule 8) is a member of the human CEA family and is uniquely expressed on human PMN [24]. Next, we determined the frequency of this CD66b-positive subset in cohorts of patients with different types of solid cancer. CD66b-positive cells with high SSC were present in a frequency of 2–10% of the MNC fraction in most patients analyzed but in some patients, also exceeded 20% (Fig. 1B). CD66b-positive cells were virtually absent in the MNC fraction of healthy donors.

To enable functional experiments, we next developed a multistep isolation protocol for $SSC^{high}CD66b^+$ cells. This isolation protocol yielded a purity of higher than 90% and a viability of higher than 98% in the majority of experiments (Supplemental Fig. 1). Earlier reports have suggested that activated granulocytes may inhibit T cell functions in cancer patients [22]. More recently, activated granulocytes have been defined as MDSC in patients with RCC [15]. As inhibition of T cell activation is a hallmark of MDSC function [4, 5], we first tested our purified $SSC^{high}CD66b^+$ cells for this function. We found that proliferation (**Fig. 2A**) and IFN-γ production (Fig. 2B) of polyclonally stimulated T cells were strongly inhibited by $SSC^{high}CD66b^+$ cells. This finding, together with the increased number of those cells in the peripheral blood of cancer patients (Fig. 1), suggested to us that $SSC^{high}CD66b^+$ cells qualify as human MDSC. Although MDSC in tumor-bearing mice are relatively well-characterized [5, 6], cellular identity and function of human MDSC are still poorly defined. A frequently used combination of markers for human MDSC includes $CD33^+/CD11b^+/HLA\text{-}DR^-$ [18, 25] and $CD14^+/HLA\text{-}DR^{low}$ [16, 26] to define monocytic MDSC that usually do not express CD114 [18]. Other studies have used $CD66b^+/CD15^+/CD11b^+/CD14^-$ for the identification of granulocytic MDSC [15, 20]. Mandruzzato and colleagues [20] reported on IL-4Rα-positive cells in monocytic and granulocytic myeloid subsets, where IL-4Rα expression correlated

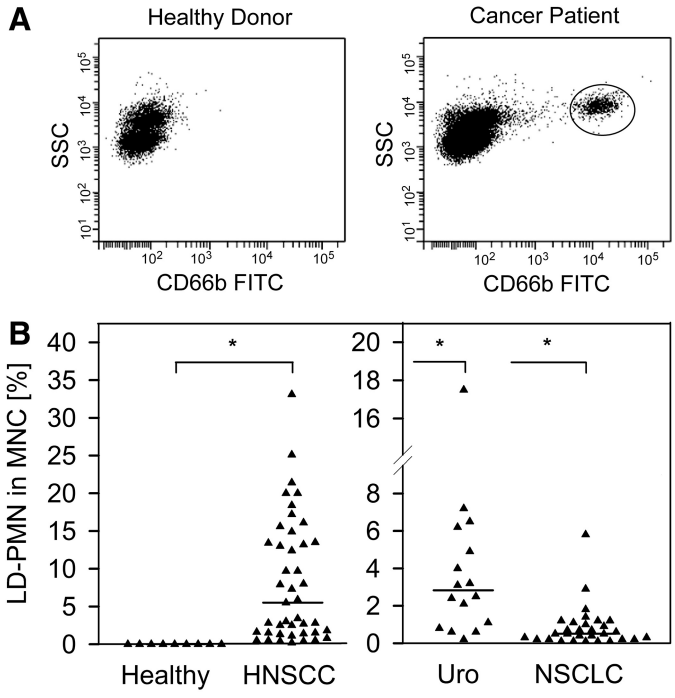

Figure 1. Identification and frequency of CD66b-positive cells in the MNC fraction isolated from the peripheral blood of cancer patients. (A) MNC were isolated from the peripheral blood of cancer patients and healthy donors by density gradient centrifugation. Anti-CD66b mAb were used to identify PMN within the MNC fraction. (B) CD66b-positive cells were detected in patients with HNSCC (n=57), cancer of the urinary tract (Uro; bladder and ureter; n=16), and NSCLC (n=30) but not in healthy donors (n=9). The median percentage of CD66b-positive cells within the MNC is indicated for each cancer (5.5% for HNSCC, 2.8% for urological cancer, and 0.5% for NSCLC). Asterisks indicate P values of tumor patients versus healthy donors (P<0.001 each; calculated by Mann-Whitney U test). We applied a significance level of 0.05 for each statistical test.

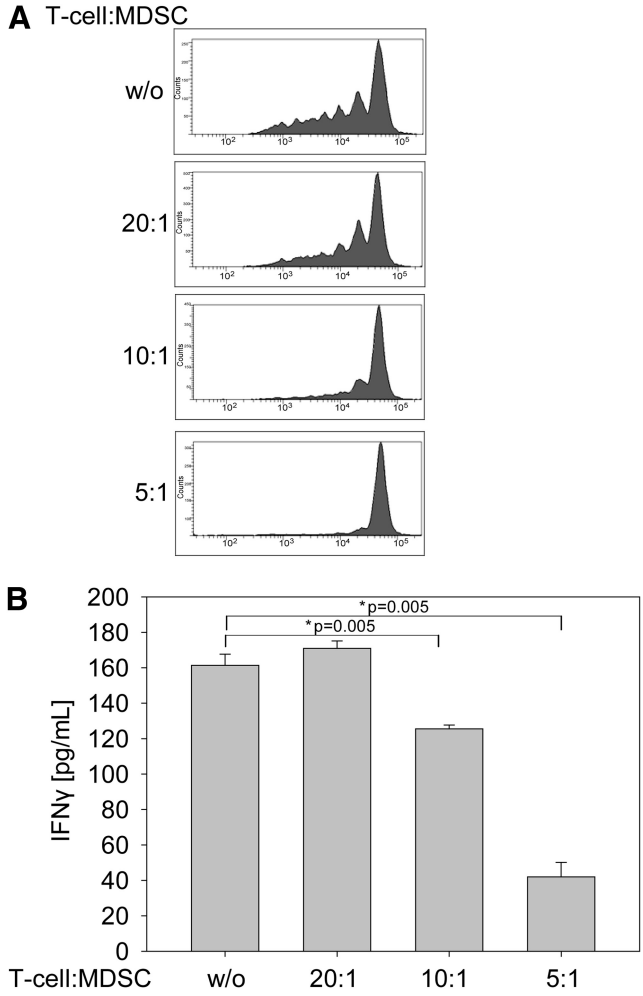

Figure 2. Neutrophilic MDSC inhibit T cell activation. (A) CFSE-labeled responder T cells from HNC patients were stimulated with α-biotin MACSiBeads™ and with biotinylated anti-CD2, -CD3, and -CD28 antibodies in the presence or absence of autologous MDSC. Lymphocyte proliferation was measured at Day 7. T cell:MDSC ratios of 20:1, 10:1, and 5:1 are shown. Data are representative for three HNC patients analyzed. w/o, Without. (B) Expression of IFN-γ in the T cell-MDSC coculture was measured by ELISA. T cell:PMN ratios of 20:1, 10:1, and 5:1 are shown. Data are presented as mean ± SD of three independent experiments. The *t* test was used for statistical analysis.

with immunosuppressive activity only in monocytic cells. It has to be noted that although useful, those markers do not unequivocally define human myeloid leukocyte subsets. For example, CD14 must be used with care as a monocyte lineage marker, as PMN also express low amounts of this antigen [27]. Similarly, MHC class II molecules may also be expressed by activated PMN [28]. To further define the identity of $SSC^{high}CD66b^{+}$ human MDSC, we investigated the nuclear morphology of those cells. Pappenheim staining on purified cells revealed the presence of different neutrophilic developmental stages, including cells with myelocytic, metamyelocytic, and band-like nuclear morphology (data not shown). Neutrophil development and differentiation are accompanied by the gradual increase and decrease of characteristic surface markers that may be used to identify the different stages of neutrophil development [29, 30]. We used six-color immunophenotyping to further define $CD66b^{+}$ MDSC (**Fig. 3**). Using CD125 (IL-5Rα), an eosinophil-specific marker [31], we detected small numbers of eosinophils in some patients. As we wanted to focus our analysis on neutrophilic MDSC, we excluded $CD125^{+}$ cells from our subsequent immunophenotyping. SSC^{high}, CD66b-positive, CD125-negative MDSC consistently expressed CD33 and were negative for HLA-DR. Within the $CD66b^{+}/CD33^{+}$ cells, two subsets of cells, $CD16^{+}$ and $CD16^{-}$, were detected (Fig. 3). In a subgroup of patients with urological cancers or HNC, we were able to perform further immunophenotyping using CD16 and CD11b as additional markers. These analyses indicated the existence of three subsets within neutrophilic MDSC. The relative frequency of $CD16^{+}/CD11b^{+}$, $CD16^{-}/CD11b^{+}$, and $CD16^{-}/CD11b^{-}$ MDSC showed a high degree of variability among different individuals, as illustrated for three representative patients in Fig. 3 (bottom panel). CD16 and CD11b are absent on promyelocytes and appear during neutrophil differentiation at the myelocyte/metamyelocyte stage. During this process, expression of CD11b precedes expression of CD16 [29]. Thus, our immunophenotyping, by using previously published markers, plus new combinations of surface antigens, enabled us to define more precisely putative neutrophilic MDSC in human cancer patients and suggested that MDSC fractions in patients contain large numbers of different developmental stages of immature neutrophils.

Despite progress in the characterization of human MDSC, the factors that direct differentiation, expansion, and activation of human MDSC remain to be explored. The transcription factor C/EBPβ has been identified as a master regulator of granulopoiesis under so-called "emergency" conditions [32]. In an elegant study, the Bronte group [33] has recently identified C/EBPβ as an important regulator of MDSC-mediated immunosuppression in cancer and defined cell culture conditions that mimic generation of human MDSC in vitro. They cultured human bone marrow cells in the presence of cytokine mixtures and identified GM-CSF and IL-6 as potent inducers of T cell-suppressive myeloid cells. Interestingly, their immunophenotyping of in vitro-generated MDSC (see Fig. 7 and Supplemental Fig. 6 in ref. [33]) reveals striking similarities with the neutrophilic MDSC identified in our patient study.

Functional properties of peripheral blood immature neutrophilic MDSC

To further define and to assess the main functional properties of MDSC, we pursued a comparative side-by-side analysis of neutrophilic MDSC and conventional, mature PMN obtained from the same donor. For these experiments, we only used preparations of high purity (>90%). First, we tested for viability and spontaneous ex vivo apoptosis of purified cells. Consistent with their immature state, we found that neutrophilic MDSC showed a reduced apoptosis during in vitro culture, when compared with conventional PMN of higher density (layered on top of the erythrocyte sediment in density gradients; termed HD-PMN; **Fig. 4**). G-CSF is a growth factor involved in the regulation of differentiation and survival of neutrophils. As expected, G-CSF enhanced the number of viable HD-PMN by

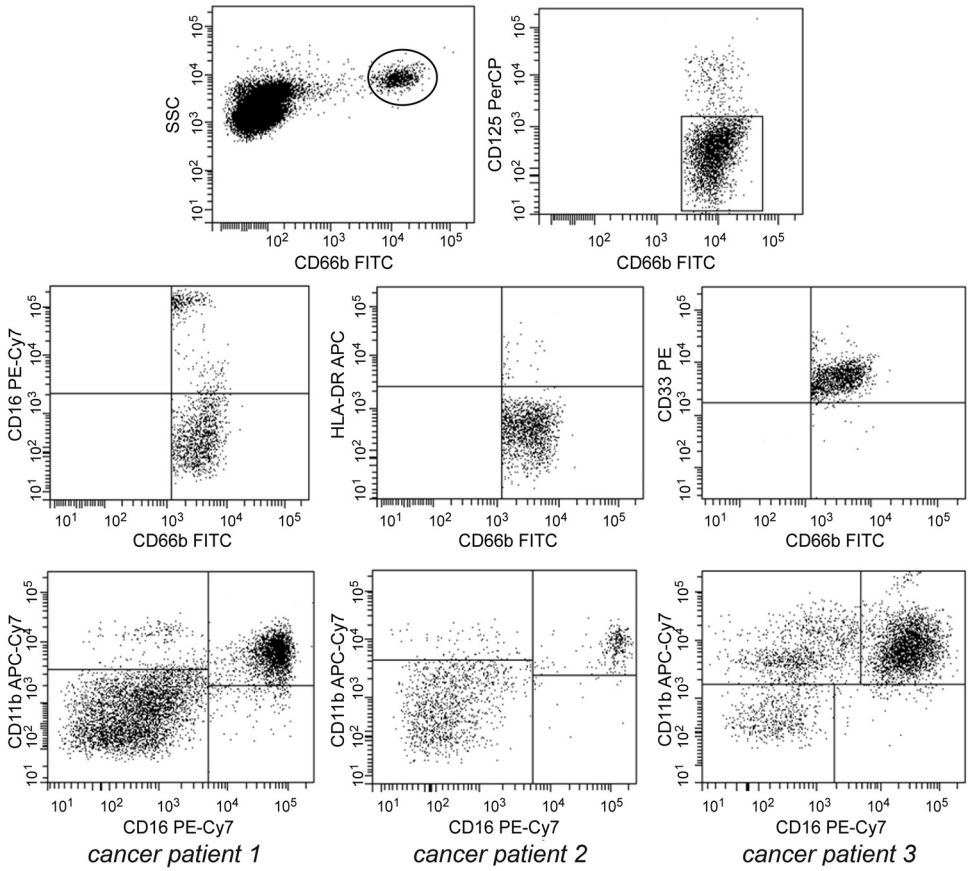

Figure 3. Neutrophilic MDSC contain large numbers of immature developmental stages. Immunophenotyping of neutrophilic MDSC was performed by six-color flow cytometry. The neutrophilic MDSC were gated as $CD66b^+/CD125^-$ (top panel) before analysis of HLA-DR, CD33 and CD16 expression (middle panel). CD16 and CD11b allowed the identification of three subsets in $CD66b^+/CD33^+/CD125^-/HLA\text{-}DR^-$ cells. Dot plots of three representative cancer patients are shown to illustrate subset variability between immature and mature developmental stages (bottom panel).

two- to threefold. The effect of G-CSF on MDSC was less pronounced and increased viability from 63% to 71%.

Production of ROI is a major effector function of PMN and has been implicated in the inhibition of T cell activity by MDSC/PMN [5]. We investigated production of ROI by MDSC during primary, fMLP-induced oxidative burst and during secondary burst induced by particulate material such as latex beads. Our results show that constitutive and induced oxidative burst activity of MDSC was strikingly lower when compared with HD-PMN (Supplemental Fig. 2). Production of cytokines is an important immunological effector function of PMN [34]. We found that LPS-induced secretion of IL-8 was reduced in neutrophilic MDSC compared with HD-PMN (data not shown). Taken together, these data suggest that consistent with their immature state, neutrophilic MDSC exhibit prolonged survival and reduced classical effector functions in comparison with mature PMN of the peripheral blood.

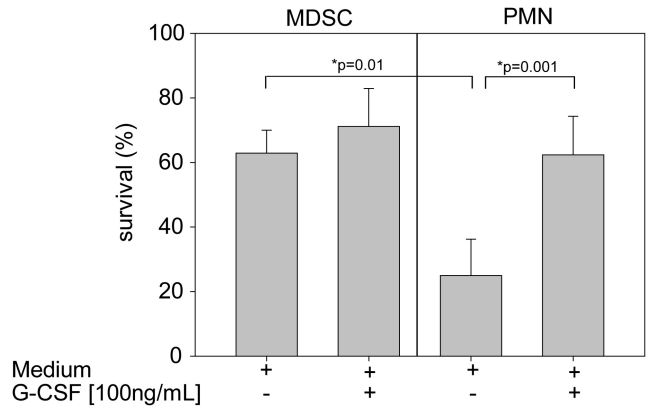

Figure 4. Reduced apoptosis of neutrophilic MDSC. The spontaneous cell death of isolated MDSC and PMN was determined by costaining with Annexin V-PE and 7-amino-actinomycin D after 24 h of culture in medium or with or without 100 ng/mL G-CSF. Data are depicted as mean ± SD of three independent experiments. The *t* test was used for statistical analysis.

It is important to note that MDSC described in this study are distinct from MDSC described by Rodriguez et al. [15] in RCC. Rodriguez et al. [15] describe activated, mature granulocytes with a segmented nuclear morphology. We focus our manuscript on a subset of immature, nonactivated PMN with reduced classical effector function. Further, Rodriguez et al. [15] found no difference in apoptosis between MDSC and conventional PMN. In our study, survival of LD-PMN/MDSC was strongly prolonged compared with conventional PMN. Instead, our MDSC seem to have similarities with immunosuppressive myeloid cells, which can be generated in vitro from human bone marrow precursors in the presence of GM-CSF and IL-6 [33]. It should be highly informative to compare cell biological functions of those in vitro-generated MDSC with our MDSC isolated from cancer patients.

As the number of MDSC was remarkably induced in some patients of our cohort, we asked whether this would correlate with increased numbers of intratumoral granulocyte counts. However, when we analyzed a subgroup of patients with HNC, where sufficient tissue material was available, we found no correlation between increased MDSC counts in the peripheral blood and CD66b counts in the tumor (data not shown). This finding prompted us to analyze the migratory properties of MDSC. When we exposed MDSC and HD-PMN to tumor cell conditioned medium in a transwell setting, we observed significantly less chemotactic activity in the MDSC fraction (**Fig. 5A**). Migration of granulocytes and other leukocytes is directed by different classes of chemokine receptors. The receptors CXCR1 and CXCR2 are of prime importance for the migration of granulocytes to sites of inflammation [35], and CXCR4 regulates neutrophil release from the bone marrow [36]. Interestingly, we observed differential expression of chemokine receptors on MDSC and HD-PMN. A significant number of MDSC strongly expressed CXCR4, and this receptor showed homogenous and low expression on HD-PMN. In contrast, CXCR1 and CXCR2 are strongly expressed on HD-PMN, and they are low or even absent on MDSC (Fig. 5B). This differential expression of CXCR1 and CXCR2 between MDSC and HD-PMN was maintained after 24 h ex vivo culture in the presence of G-CSF (Supplemental Fig. 3). CXCR4 was induced in HD-PMN under ex vivo culture conditions (data not shown). These data suggest that human neutrophilic MDSC lack expression of important chemokine receptors responsive to inflammatory chemokines. As a consequence, this would dramatically impair their capability to migrate to malignant tissue. Instead, MDSC would remain in the peripheral blood, most likely for prolonged periods of time compared with conventional PMN, as a result of their reduced rate of apoptosis. In contrast to mice, T cell-suppressive capacity of human MDSC cannot be tested in vivo. However, it is tempting to speculate that the prolonged persistence of neutrophilic MDSC in the peripheral blood (reduced apoptosis, reduced migration/extravasation) makes those cells attractive candidates for peripheral immunosuppression in cancer patients.

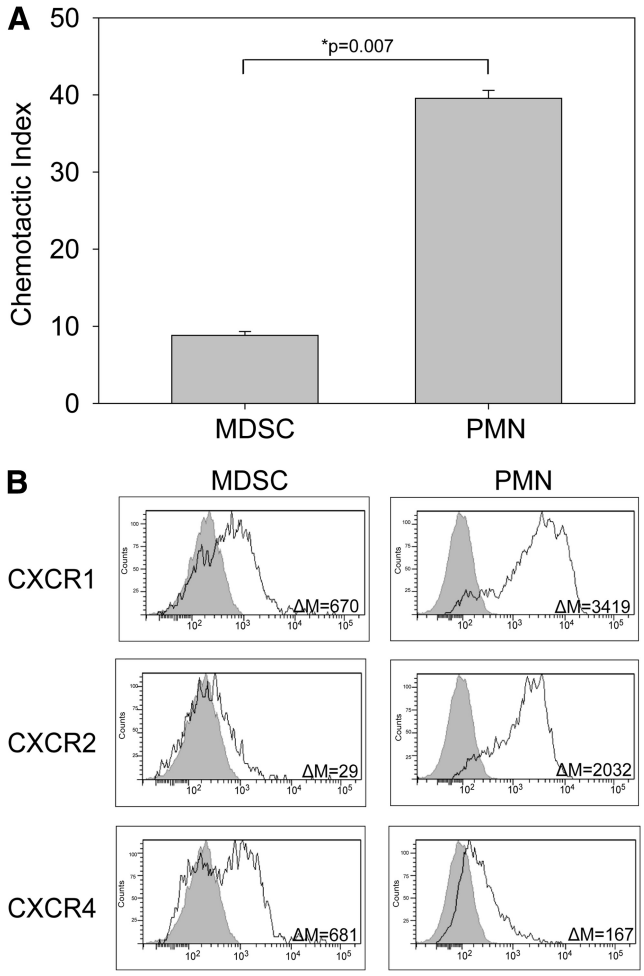

Figure 5. MDSC lack expression of CXCR1/2 and have impaired chemotactic activity. (A) Chemotaxis of MDSC and PMN toward culture supernatant of the HNC cell line FaDu was measured using 3 μm transwell inserts. Data of one representative experiment out of three are shown. The *t* test was used for statistical analysis. (B) MDSC and PMN were stained by flow cytometry for expression of CXCR1, CXCR2, and CXCR4 directly after isolation. Data are shown as overlays: isotype (gray) and surface marker (white). Representative results from one out of three independent experiments are shown.

AUTHORSHIP

S.B. conceived of the study, analyzed and interpreted data, and wrote the manuscript; S.T. collected and analyzed data and clinical material and wrote parts of the manuscript; K.B., D.S., G.S., and M.E. performed experiments and analyzed and interpreted data; H.S., M.S., and J. W. collected clinical material; P.Z. edited the manuscript; and S.L. provided clinical management and edited the manuscript.

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