Myeloid-derived suppressor cell heterogeneity and subset definition

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Myeloid derived suppressor cells (MDSCs) are defined in mice on the basis of CD11b and Gr-1 marker expression and the functional ability to inhibit T lymphocyte activation. Nevertheless the term ‘heterogeneous’ remains the first, informal feature commonly attributed to this population. It is clear that CD11b⁺Gr-1⁺ cells are part of a myeloid macropopulation, which comprises at least two subsets of polymorphonuclear and monocytic cells with different immunosuppressive properties. While recent literature shows substantial agreement on the immunoregulatory property of the monocytic MDSC subset, there is still contrasting evidence on the role of the granulocytic fraction. Moreover, this dichotomy holds true for human MDSCs. We attempt here to summarize conflicting findings in the field and provide some possible, unifying explanations.

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Introduction

The term myeloid-derived suppressor cells (MDSCs) is used to define mouse cells elicited under pathological situations, including tumor development, that express CD11b and Gr-1 antigens, do not or weakly express other markers of mature myeloid cells, upregulate genes either related to the metabolism of amino acids (such as L-arginine) or involved in production of reactive oxygen (ROS) and nitrogen species (RNS); more importantly, however, MDSCs have the potential to suppress immune responses of T lymphocytes both in vitro and in vivo [1,2]. Solving MDSC complexity could help us to understand whether these heterogeneous cells are linked by a common biological role or rather a precise immunosuppressive ‘core’ exists, bounded by auxiliary cells supporting its function or providing it with a pool of precursors for its maintenance and expansion.

On the basis of their definition, MDSCs differ from other populations of myeloid-related cells with immunoregulatory properties: tumor-associated macrophages (TAMs), for example, are not considered a subset of MDSCs, even though several findings suggest that circulating MDSCs could differentiate into Gr-1⁻ F4/80⁺ TAMs when they reach the tumor site [3–5]. MDSCs and TAMs can also cooperate in skewing immunity towards a tumor-promoting type 2 immune response by a reciprocal interplay resulting in decreased IL-12 production by macrophages and, conversely, augmented IL-10 secretion by MDSCs [6]. Moreover, another population of tumor-induced monocytes, which express the tyrosine kinase receptor Tie-2 (TEMs) and is mainly involved in promoting neovascularisation of developing tumor mass [7], seems to possess a peculiar identity, even thought it shares some features of both TAMs and MDSCs [8,9].

The elusive Gr-1 molecule

Anti-Gr-1 mAb (RB6-8C5) has long been used to stain MDSCs and allows the distinction of at least two cellular fractions according to its brightness: a Gr-1⁹⁺, mainly composed of immature and mature granulocytes, and a Gr-1⁺, comprising monocytes and other immature myeloid cells [10–13]. The use of anti-Gr-1 mAb has been criticized since it binds, though with different specificity, two molecules belonging to the Ly6 superfamily, Ly6G and Ly6C, which preferentially reside on the surface of granulocytes and monocytes, respectively [14–16]. However, despite their common use as markers, very little is known about these two molecules and their expression spans over a wide variety of cells: Ly6G was, in fact, first detected on myeloid cells, whereas Ly6C on endothelial, T lymphocytes, NK cells, monocytes and macrophages [14,17]. More recently, Ly6C expression was used as marker to distinguish various monocyte subsets and developmental stages: monocytes enter the circulation as Ly6C⁹⁺ and, under steady state haematoipoiesis, mature along with a progressive decrease of this marker; inflammatory conditions can augment monocytopoiesis producing Ly6Cmed/low cells that reach the site of inflammation and mature into exudate macrophages, whereas Ly6Chigh cells develop into mature DCs [16]. Ly6G and Ly6C ligands and functions are also poorly
examined. An interesting approach based on chimeric proteins produced by the fusion of various Ly6 family members, among which Ly6C, with murine IgM heavy chain, identified putative ligands of Ly6C on B lymphocytes [18]. Very recent evidence implicated Ly6G molecule in regulating cell proliferation and apoptosis. The well documented neutrophil depletion mediated by RB6-8C5 mAb in healthy mice and, in case of TNF-α-primed systemic inflammation, the severe cardiovascular and respiratory response to its in vivo administration were both independent from complement and related to possible Ly6G-mediated signal transduction, since some of these effects were mimicked by in vitro and in vivo Ly6G cross-linking [19]. Moreover, anti-Gr-1 mAb efficiently depleted inflammatory peritoneal Ly6CmedLy6Ghigh neutrophils along with the appearance of apoptotic cells, supporting a complement-independent pathway mediated by Ly6G and Ly6C molecules; on the contrary, bone marrow neutrophils were resistant to depletion due to the expression of anti-apoptotic Mcl-1 protein, and anti-Gr-1 mAb in this district rather promoted myelopoiesis and macrophage differentiation [20**]. In vitro the Gr-1 mAb transmitted signals via STAT-1, STAT-3, and STAT-5. These data suggest a note of caution in using Gr-1 mAb to physically separate different myeloid subsets since the extent of cross-linking might provide signals, either positive or negative that alter the functional status of the separated cells. Granulocytes, which posses the highest levels of Gr-1 and Ly6G, might be particularly sensitive to Gr-1/Ly6G cross-linking. This might explain some discrepancies about function of MDSC subsets described below.

**Mouse MDSC subset definition and isolation**

Different studies have addressed MDSC heterogeneity, and apart from dissimilarities in experimental approaches and assumptions, the common goal has been to clarify which portion of MDSC macropopulation is directly responsible for the detrimental action on antigen-activated T cells. MDSC composition is largely dependent on tumor-derived soluble factors (TDFs), the combination of which relies on tumor histotype, burden, and anatomical localization, all variables complicating the conclusive and unique phenotype identification of MDSCs [2,21].

In a model of colon carcinoma engineered to produce high levels of GM-CSF, MDSCs could be subdivided into two subsets on the basis of the expression of the α chain of the IL-4 receptor (IL-4Rα/CD124): when low doses of MDSCs were cultured together with CD8+ T lymphocytes activated by alloantigens, IL-4Rα cells, which uniformly displayed a prevalent monocyteic morphology, suppressed CD8+ T cell function, whereas IL-4Rα cells, which comprised granulocytes at various stages of differentiation including band cells, did not [11]. Almost at the same time, Huang and colleagues showed similar results by analyzing the marker CD115, the M-CSF/CSF-1 receptor. In a model of chemically induced colon carcinoma, either Gr-1+CD115+ or Gr-1+F4/80+ monocytic MDSCs isolated from bone marrow were able to induce immunosuppression in vitro to the detriment of T cells, in a manner dependent from the inducible nitric oxide synthase (NOS2); moreover, monocytic MDSCs were also able to trigger CD25+ Treg induction in vivo, contributing to the suppression of antitumor response [22]. Although these observations did point to a prevalent immunoregulatory activity within the monocytic-like cells, it was later found that IL-4Rα and CD115 expression levels in distinct MDSC subsets were strictly related to the tumor model analyzed [23,24], making impractical their use as widespread markers for MDSC isolation. Nonetheless, these works led the way to further dissection of MDSC macropopulation.

In the past two years, different groups physically separated MDSC subsets from the spleen of tumor-bearing mice on the basis of the expression of Ly6C and Ly6G molecules, thus enriching either polymorphonuclear (PMN–MDSCs) or mononuclear (MO–MDSCs) MDSCs [23,25*]. PMN–MDSCs were described as CD11b+Gr-1highLy6G+Ly6Clowint cells with high SSC endowed with immunosuppressive properties on antigen-specific CD8+ T lymphocyte proliferation. These cells expressed arginase (Arg) but suppressed antigen-specific CD8+ T cells mainly through a ROS-mediated mechanism [23], while according to another work suppression was not recovered by ROS, ARG, or NOS2 inhibitors, but required instead IFN-γ acting through a not yet identified STAT1-independent pathway [25*]. Moreover, in the latter study, PMN-MDSCs did not require MHC I direct presentation for their inhibitory functions [25*].

MO–MDSCs, on the contrary, were mainly CD11b+Gr-1intLy6G−Ly6Chigh cells with low SSC and, at least in some tumor models, expressed higher levels of F4/80, CD115, 7/4, and CCR2 compared with PMN-MDSCs, further substantiating their monocyte origin [23,25*]. MO–MDSCs suppressed antigen-specific CD8+ T lymphocyte proliferation through a Nos-mediated mechanism, as demonstrated by the use of Nos inhibitors, high nitrite content in cultures, and increased nitrotyrosine levels in co-cultured CD8+ T cells (nitrotyrosine being a hallmark of enhanced production of RNS such as peroxynitrates). Immunosuppressive activity was, at least partially, IFN-γ/STAT1-dependent [23,25*]. Similarly to what previously mentioned about TAMs, this monocytic subset was also shown to contain progenitors of strongly suppressive CD11bhighGr-1lowLy6G F4/80highMHC II+ adherent macrophages that gradually accumulated during in vitro culture, suggesting how these cells possessed suppressive potential both as immature precursors and mature cells [13,25*], as previously shown [3,26].
Novel studies, however, have re-examined the relative role of these two subsets. Although the specificity might be an issue, anti-Gr-1 mAb is still useful since it allows to distinguish several cell subsets. CD11b+ splenocytes from tumor-bearing mice can be divided, in fact, into at least three populations with different Gr-1 staining intensities, that is, Gr-1<sup>high</sup>, Gr-1<sup>int</sup>, and Gr-1<sup>low</sup> cells [24]. Gr-1<sup>high</sup> cells isolated by magnetic beads coated with anti-Ly6G mAbs were Ly6G<sup>+</sup>Ly6C<sup>low</sup>SSC<sup>int</sup> granulocytes but differed from previously described PMN-MDSCs since, in different tumor models, they exerted weak or no suppression on both antigen-specific and allo-specific CD8<sup>+</sup> T cells in vitro, at least until they did not exceed in culture the numbers found in the spleens of tumor-bearing mice [24]. More importantly, PMN-like MDSCs did not induce tolerance of antigen-primed CD8<sup>+</sup> T cells following their in vitro adoptive transfer [24]. ARG1 or NOS2 proteins were not detected in this subset, whereas Arg2 and Nos3 transcripts were overexpressed compared with Gr-1<sup>int</sup> cells. When these cells were used at very high suppressor to effector ratios, their inhibitory activity could be overcome with the use of L-NMMA and catalase, indicating that NO and ROS might have some role in their action under these conditions [24]. An indirect role for PMN-MDSCs in immunosuppression could not be ruled out since they released high amounts of IFN-γ, required by MDSCs for triggering their inhibitory function [11,24]. Gr-1<sup>int</sup>-low cells were quite variable among various tumor models in their morphology and composition, as they contained a mixture of Ly6G<sup>+</sup> and Ly6G<sup>−</sup> cells with some immature cells bearing ‘ring-shaped’ nuclei, but also expressed F4/80 and CD68 macrophage markers, upregulated ARG1 and NOS2 enzymes, and were always suppressive in vitro and, more importantly, in vivo [24]. Gr-1<sup>low</sup> cells were Ly6G<sup>−</sup>Ly6C<sup>high</sup>F4/80<sup>−</sup>SSC<sup>low</sup> resembling MO-MDSCs and displayed strong suppressive potential in vitro in some but not in all tumor models. Interestingly, Gr-1<sup>int</sup> and Gr-1<sup>low</sup> were expanded among CD11b<sup>+</sup> cells in tumor-bearing hosts mainly by tumor-released GM-CSF, while G-CSF was more directly responsible for Gr-1<sup>high</sup> cell increase [24].

There is a striking resemblance of these data with the recent findings from Greifenberg and colleagues regarding inflammation-induced MDSCs [27]. In fact, LPS + IFN-γ administration to mice expanded three populations closely related to Gr-1<sup>high</sup>, Gr-1<sup>int</sup> and Gr-1<sup>low</sup> fractions. While CD11b<sup>high</sup>/Gr-1<sup>high</sup> subset, composed of mature granulocytes, was not suppressive, CD11b<sup>+</sup>Gr-1<sup>high</sup> early myeloid cells with ring-shaped nuclei and CD11b<sup>+</sup>Gr-1<sup>low</sup>SSC<sup>low</sup> monocytes (but not CD11b<sup>+</sup>Gr-1<sup>low</sup>SSC<sup>high</sup> eosinophils) were able to inhibit antigen-specific CD8<sup>+</sup> T cell proliferation [27].

Polymorphonuclear and mononuclear MDSC subsets have also been described in tumor microenvironment. In two different tumor models, tumor-infiltrating MDSCs were more than 90% CD11b<sup>+</sup>Gr-1<sup>low</sup>F4/80<sup>−</sup>IL-4Ra<sup>+</sup>CCR2<sup>+</sup>CX<sub>3</sub>CRI<sup>+</sup> monocytes that showed markers peculiar of both M1 and M2 macrophages, with residual cells being Gr-1<sup>high</sup>F4/80<sup>low</sup> [28]. Tumor-infiltrating polymorphonuclear Gr-1<sup>high</sup> MDSCs were shown to be recruited to the tumor site by complement C5a fragment that favored both CD11b overexpression and leukocyte extravasation, while mononuclear MDSCs were less sensitive to this chemotactic activity but were primed by the C5a to increase their ROS and RNS production [29]. Interestingly, blockade of TGF-β by administration of type I TGF-β receptor kinase inhibitor lead to a marked influx of CD11b<sup>+</sup>/Gr-1<sup>high</sup> neutrophils with hypersegmented nuclei that exerted cytotoxic activity on cancer cells and promoted CD8<sup>+</sup> T cell-dependent rejection of tumor, suggesting that, as for TAMs, also neutrophils might oscillate between pro-tumoral (N2) and anti-tumoral (N1) state depending on microenvironmental signals [30].

Intratumoral distribution of MDSC subsets might also be tightly regulated. Chemokine-mediated turnover of tumor-infiltrating MDSCs, in fact, controlled the accumulation of CD11b<sup>+</sup>Gr-1<sup>high</sup>Ly6C<sup>int</sup>SSC<sup>low</sup> neutrophils near the center of the tumor whereas ARG1-expressing CD11b<sup>+</sup>Gr-1<sup>int</sup>Ly6C<sup>high</sup>SSC<sup>low</sup> macrophages were distributed throughout the tumor mass [31]. A population of tumor-infiltrating CD11b<sup>+</sup>Gr-1<sup>int</sup>SSC<sup>high</sup> eosinophils was also found in tumor mass whereas in the spleen and bone marrow of the same tumor-bearing mice, three subsets were observed: CD11b<sup>+</sup>Gr-1<sup>high</sup>Ly6C<sup>int</sup> granulocytes, CD11b<sup>+</sup>Gr-1<sup>int</sup>Ly6C<sup>low</sup> immature myeloid cells at various differentiation stages (promyelocytes, myelocytes), and CD11b<sup>+</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup> monocyctic cells. In all the analyzed compartments, monocytes/macrophages displayed higher proliferating potential than polymorphonuclear cells but no insight was provided for their direct suppressive properties [31].

Similar findings on pathologic induction of MDSC subsets also stem out from other inflammatory conditions, like mycobacteria-induced infection that was associated with the expansion of a NO-producing immunosuppressive Ly6G<sup>−</sup>Gr-1<sup>monocytic</sup> fraction and a O<sub>2</sub> – releasing Ly6G<sup>+</sup> granulocytic population with no inhibitory effect on T cell proliferation [32]; moreover, in a model of experimental autoimmune encephalomyelitis, four distinct myeloid subsets were identified that reminded those induced by LPS + IFN-γ; when CD11b<sup>+</sup> cells were then sorted on the basis of Ly6G and Ly6C markers, only CD11b<sup>+</sup>F4/80<sup>−</sup>Ly6C<sup>high</sup>Ly6G<sup>−</sup> mononuclear population demonstrated strong inhibition of both activated CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes through NO-mediated apoptosis [33].

**Human MDSCs**

The absence of a Gr-1 gene homolog in humans hampered the identification of human MDSCs but evidence
accumulated in support of both granulocytic and monocytic subset existence. PBMCs from renal cancer patients had increased arginase activity restricted to a subset of CD11b+/CD14+ /CD15+ cells with a polymorphonuclear/granulocyte morphology and markers [34]. Moreover, depletion of CD11b+ but not CD14+ cells re-established T cell proliferation, therefore suggesting that suppressor myeloid cells producing ARG1 were present in the mononuclear fraction of the blood of cancer patients. Subsequently the same group defined more precisely that MDSCs in renal cancer patients belonged to a subset of activated granulocytes, expressing CD66b and VEGFR1, able to degranulate and thus release ARG1 into plasma [35].

In partial contrast with these findings, MDSCs were recently identified in the monocyte fraction of melanoma patients by the markers CD14+/CD11b+/HLA-DRlow/neg and secretion of TGF-β, which was recognized as a mediator of the immune suppression [36]. These MDSCs were significantly expanded in metastatic melanoma patients but undetectable in healthy donors. Interestingly, this monocytic MDSC population was monitored in some patients during the course of a cancer vaccination protocol containing GM–CSF and its expansion after vaccination was significant only in the group of patients lacking immunological response, as determined by the absent recognition of the autologous tumor [36]. MDSCs with similar phenotype were also observed among PBMCs isolated from prostate cancer patients [37], and CD14+ human MDSCs susceptible to phosphodiesterase 5 inhibitors were described in multiple myeloma and head and neck cancer patients [38]. Moreover, by analyzing CD14+ monocytes in the blood of 111 hepatocellular carcinoma patients, a subpopulation of HLA-DR+ monocytes was described that significantly increased in both peripheral blood and tumor infiltrating lymphocytes of patients; these cells suppressed autologous T cell proliferation and had high arginase enzymatic activity [39]. Interestingly, these MDSCs induced an IL-10 secreting, CD4+CD25+Foxp3+ Treg population when cocultured with autologous T cells [39].

To clarify whether human MDSCs should be searched among cells carrying markers, morphology, and physical properties of either monocytes or granulocytes, we recently studied different subsets of leukocytes isolated from the whole blood of melanoma and colon cancer patients, including freshly isolated PMN that are normally lost during the freezing process and, therefore, large retrospective studies do not include this leukocyte population. We demonstrated that in the blood of the cancer patients analyzed there were two main subpopulations functionally able to suppress the immune response, that is, CD14+ monocytes and CD15+ PMNs. However, PMNs were not recovered in the mononuclear fraction (as shown in previous studies) but in the granulocyte layer. Of interest, these two immunosuppressive fractions expressed both IL4R/CD124 but the inhibitory activity was directly correlated to the percentage of IL4R/CD124 positive cells among PBMCs and not among PMNs [40*].

Despite recent description of human monocytic and granulocytic MDSCs, earlier studies did identify MDSCs in the mononuclear compartment but unveiled an immature myeloid cell (ImC) population, consisting of early stage myeloid cells, immature monocytes, and DCs functionally able to block T cell proliferation [41]. About one third of ImC were represented by immature cells of the macrophage/DC cell lineage and the remaining were cells at earlier stages of myeloid differentiation. This ‘immature signature’ is still a common finding. In renal cell carcinoma, for example, putative MDSCs can be also isolated from the blood in the fraction negative for lineage-specific and HLA-DR markers (Lin-/DR-); after enrichment of this population, characterized by the CD33 marker, the morphology was still heterogeneous, composed of both monocytic cells and granulocytic cells [42]. MDSCs were also defined as CD11b+/CD14+/CD15+ /CD33+ cells in patients with advanced non-small cell lung cancer (NSCLC), being this subset increased in peripheral blood of patients and able to reduce the expression of CD3ζ chain in co-cultured CD8+ T lymphocytes [43]. MDSCs with a partially similar phenotype (CD11b+/CD33+/CD15+) were also analyzed in NSCLC patients treated with a vaccine based on NSCLC cell lines transfected with CD80 plus MHC II genes [44]. Other important issues addressed in cancer patients are whether MDSC presence correlates with clinical cancer stage and whether their presence in the blood can be modulated by chemotherapy. An interesting paper by Monteiro and co-workers analyzed blood from 106 samples of PBMCs from solid tumor patients ranging from stage I to stage IV; moreover, in some of these patients blood was also analyzed before and after chemotherapy [45]. MDSCs were defined as Linlow+, HLA-DR+, CD33+, CD11b+ and determined on whole blood by flow cytometry. This study showed a significantly higher percentage of circulating MDSCs in cancer patients as compared to healthy donors, and demonstrated a significant increase in patients of all disease stages, with a significant trend from stage I to stage IV. Moreover, among stage IV patients those with extensive metastatic burden had the highest percentage of MDSCs. Two chemotherapy regimens were evaluated and in both cases MDSCs were increased after therapy, although with different levels of expansion [45].

**Conclusions**

Despite the heterogeneous composition of MDSCs in different organs and under inflammatory/neoplastic conditions, many data in mouse models converge on an important role for the monocytic fraction of MDSCs,
generally referred to as CD11b’Gr-1’Ly6C’ cells, in restraining the immune response in mice. However, the term ‘monocytic’ might be both misleading and erroneous since, in almost all the studies, this MDSC fraction contains an enriched proportion of cells with immature morphology. Moreover, expression of CD115 in this subset is extremely low [23,24,25,27] and CD115 is mandatory for monocytic lineage differentiation [46**]. It must be pointed out that markers used to separate distinct myeloid cell subsets under steady state haematopoiesis might not be as informative during altered haematopoiesis, as in the case of cancer-bearing hosts [47]. In fact, surface markers might be upregulated or downregulated by TDFs in different cell subsets, thus making comparison with normal counterpart improbable. Moreover, cell subsets which represent a minor percentage of myeloid cells, such as intermediate myeloid stage cells, might be released in circulation during tumor development. The phenomenon of the discharge of immature myeloid cells is clinically used as sign of underlying pathology. The term ‘left shift’ or ‘blood shift’ indicates that the neutrophils present in the blood are at a slightly earlier stage of maturation than usual. This is often seen in acute infections, and during severe infections, hypoxia, and shock, sometimes even myelocytes can appear in the blood [48]. Myeloid suppressive populations described in different cancer types might thus be ascribed to a minor percentage of common immature precursors with highly immunoregulatory potential and capable to differentiate toward either a monocytic or granulocytic MDSCs. The tumor itself may dictate the ultimate fate of the suppressive precursors, shaping the MDSCs toward a cell type or another and influence the differentiation program, which could be blocked to a certain stage or act also on terminally differentiated cells. Future studies are needed to ascertain the role of the different growth factors released by tumors on progenitor cells, to evaluate their possible role not only in MDSC expansion, but also in skewing/shaping the MDSC composition and function.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- - of outstanding interest


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This study shows, for the first time, that the activity of anti-GR-1 antibody in vivo might not depend on complement-mediated depletion of cells expressing the Ly6G molecule but rather on a complex signalling pathway activated in the target population.


This manuscript confirms the existence of suppressor cells among human both granulocytic and monocytic fractions of patients’ blood, unveils the enhanced expression of CD124 marker, and suggests that whole blood instead of ficol-purified mononuclear cells might be necessary to fully monitor changes in human MDSCs.


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Comprehensive and updated review about monocytic differentiation under normal and pathological conditions; it also attempts to place MDSCs in the context of monocytic development.