Study of myeloid cells endowed with suppressive activity is an active field of research which has particular importance in cancer, in view of the negative regulatory capacity of these cells to the host's immune response. The expansion of these cells, called myeloid-derived suppressor cells (MDSCs), has been documented in many models of tumor-bearing mice and in patients with tumors of various origin, and their presence is associated with disease progression and reduced survival. For this reason, monitoring this type of cell expansion is of clinical importance, and flow cytometry is the technique of choice for their identification. Over the years, it has been demonstrated that MDSCs comprise a group of immature myeloid cells belonging both to monocytic and granulocytic lineages, with several stages of differentiation; their occurrence depends on tumor-derived soluble factors, which guide their expansion and determine their block of differentiation. Because of their heterogeneous composition, accurate phenotyping of these cells requires a multicolor approach, so that the expansion of all MDSC subsets can be appreciated.

This review article focuses on identifying MDSCs and discusses problems associated with phenotyping circulating and tumor-associated MDSCs in humans and in mouse models. © 2014 The Authors Cytometry Part B: Clinical Cytometry Published by Wiley Periodicals, Inc.

Key terms: immune suppression; MDSC; immunophenotyping; immunology; oncology

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lopoiesis and recruitment of immature myeloid cells (1). However, it should be noted that MDSC expansion during cancer progression represents a pathological rather than a physiological event. In fact, tumor cells have been demonstrated to induce MDSC expansion by secreting tumor-derived factors (TDFs), which comprise a variety of biologically active compounds, including growth factors, cytokines and chemokines (2). The role of TDFs is to promote not only MDSC recruitment and expansion, but also to support myeloid cell development toward an immuno-suppressive phenotype, and several lines of evidence indicate that blocking differentiation in immature myeloid cells is one of the characteristics of this process.

As discussed later in this article, the differentiation step blocked in such tolerogenic cells is not clearly defined, but involves cells with monocytic and granulocytic characteristics, as well as other immature and undifferentiated cells. In each tumor, a characteristic expansion of one or more subsets of myeloid cells occurs, each of which may have various stages of differentiation, but they all share a common function, that is suppression of cells in the immune system.

THE PUZZLING QUESTION OF MDSC HETEROGENEITY: EVIDENCE FROM MOUSE STUDIES

Intensive study of mouse MDSCs started in the late 1990s, during experimental research on therapeutic anticancer vaccines. Initial observations during vaccination protocols with powerful immunogens revealed dysfunction of CD8+ cytotoxic T-lymphocytes in immuno-competent hosts (3,4). This phenomenon was accompanied by the accumulation of splenic CD11b+Gr1+ cells, deletion of which restored CD8+ T-cell functionality both in vitro and in vivo. Subsequent studies showed that these cells are endowed with great immuno-suppressive power, activated by many concurrent mechanisms (5–8). Early phenotypic characterization of murine CD11b+Gr1+ immuno-suppressive cells showed the lack of mature myeloid-associated markers, and morphologic observations indicated that MDSCs are a heterogeneous population comprising monocytes, polymorphonuclear cells, and immature myeloid cells (9). This phenotypic and functional heterogeneity prompted researchers to speculate that only a small fraction of MDSCs was endowed with immuno-suppressive activity, responsible for their qualities of immune regulation (10). During the past 20 years, intensive research has led to the discovery of several potential markers, such as CD124, CD115, CD40, and CD80, which identify a monocytic-like fraction of MDSCs accounting for most of their immune regulatory activity (11–15). However, although several laboratories have confirmed that mouse monocytic MDSCs (Mo-MDSCs) have higher suppressive activity than the granulocytic fraction (called polymorphonuclear MDSCs or PMN-MDSCs) (16–18), the above markers are not universally discriminant in all experimental models (15). For this reason, the combination of markers CD11b and Gr-1 protein isoforms (LY6C and LY6G, discussed later) still remains the most useful MDSC marker combination (1). CD49d (VLA4), a member of the integrin α-chain family of proteins, has been shown to be specifically expressed on mouse monocytic MDSCs and can be used in combination with anti-Gr-1 antibody as an alternative method to individual staining of LY6C and LY6G isoforms (19). Regarding the role of CD124 (IL4Rx) as a marker of MDSCs, while some studies showed little if any functional role for this marker (20) and a weak expression of CD124 on circulating MDSCs (21–23), others demonstrated a significant up-regulation of CD124 on circulating myeloid cells of cancer patients (24–29) and an involvement of this receptor in MDSC function (29,30) and survival (30). These different results could depend on the high plasticity of MDSCs in response to different stimuli. In fact, MDSCs can modulate the expression of surface molecules in response to the tumor-released factors present in the tumor microenvironment.

Several studies have documented the accumulation of splenic MDSCs, displaying variable intensity of the Gr-1 marker in various tumor models (17,19,31,32). This heterogeneity is the consequence of the ratio between Gr-1int Mo-MDSCs and Gr-1high PMN-MDSCs, the composition of which reflects the milieu of MDSC-recruiting soluble factors released by several types of tumors (17,33). However, the Gr-1 marker does not always distinguish unambiguously between Mo-MDSCs and PMN-MDSCs, especially in tumor tissues or bone marrow in which Gr-1 staining is a continuum of events with various degrees of brightness. Anti-Gr-1 monoclonal antibody (mAb) binds two molecules belonging to the Ly6 superfamily, Ly6G, and Ly6C, which are preferentially located on the surface of granulocytes and monocytes, respectively. For this reason, double staining with antibodies against Ly6C and Ly6G is actually preferable. According to this separation, PMN-MDSCs are thus classified as CD11b+Ly6G-Ly6Chigh cells with high side scatter (SSC), and Mo-MDSCs are generally called CD11b+Ly6G-Ly6Clow cells with low SSC (1). Three-color staining with these markers allows unambiguous detection of the two main populations of murine MDSCs within both tumor and lymphoid organs (see Fig. 1 and Supporting Information Fig. 1). It should be emphasized that a human homolog of the Gr-1 molecule has not been described so far, and human MDSCs are therefore characterized by a different combination of surface markers (see later).

Other markers expressed by mouse PMN-MDSCs are CD115 and CD244 (34), whereas Mo-MDSCs are positive for markers of inflammatory monocytes such as F4/80, CD115, and CCR2 (13). It should be noted that, from a phenotypic point of view, PMN-MDSCs, and Mo-MDSCs are almost identical to neutrophils and inflammatory monocytes, respectively, although with several important differences. The main difference is functional, since neutrophils and inflammatory monocytes lack any form of immune suppression against T-lymphocytes. Mouse PMN-MDSCs are less phagocytic than neutrophils, and express higher levels of arginase 1 (ARG1),
myeloperoxidase, and production of reactive oxygen species (ROS), which are responsible for their immuno-suppressive ability (34,35). Otherwise, Mo-MDSCs can up-regulate both ARG1 and inducible nitric oxide synthase (iNOS), both of which allow the production of peroxynitrites which cause dysfunction in CD8\(^+\) T-lymphocytes (13,36), while inflammatory monocytes do not up-regulate ARG1 and iNOS simultaneously. Mo-MDSCs also have an immature phenotype and do not differentiate into fully competent antigen-presenting cells when injected into tumor-bearing hosts (37). Immuno-suppression assays should be mandatory, to distinguish MDSCs from their myeloid counterparts found in healthy individuals (for an accurate description of how to evaluate the immunesuppressive activity of mouse MDSCs, see (38)). Although the search for truly specific markers is still intense, we believe that effort should be focused on molecules involved in the suppressive activity of MDSCs, their main characteristic. At present, the prevailing hypothesis is that MDSCs represent a pathologic, functional state of myeloid cells normally found in healthy individuals (i.e., granulocytes and monocytes) summoned by chronic inflammatory pathologies. A unique characteristic of MDSCs, which still lacks a finalistic biologic explanation, is the presence of immature myeloid precursors in both MDSC subsets (9,13,34). Interestingly, the immature fraction of mouse monocytic MDSCs can differentiate into PMN-MDSCs, but the purpose of this lineage relationship in the tumor burden is not known (39,40). We believe that this monocytic, immature cell fraction may explain most of the suppressive potential of monocytic MDSCs, but this hypothesis cannot be addressed without specific markers. An integrated approach, which includes extensive molecular analysis and cell fate mapping studies, may better define the relationship between myeloid differentiation steps and immune regulation. These powerful multidisciplinary strategies may enhance flow cytometry analysis, as we discuss below.

**HUMAN MDSCs FROM PAST TO PRESENT: THE NEED FOR INCREASINGLY COMPLEX PHENOTYPIC ANALYSIS**

The first study documenting the presence of immature cells endowed with suppressive ability in head and neck cancer tissues and regional lymph nodes was published in 1995, and the presence of CD34\(^+\) cells was correlated with the ability of tumor cells to secrete Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) (41), a cytokine which has been associated with MDSC expansion in several tumor models (4). In addition, removal of CD34\(^+\) cells from tumor infiltrates increased IL-2 secretion by intratumoral T lymphocytes, and CD34\(^+\) cells could grow into colonies in soft agar (41). Although in this article these cells are not characterized as myeloid, their similarity with mouse MDSCs in terms of immaturity, suppressive ability on T-cell function and correlation with the presence of GM-CSF make them good candidates for human homologous of mouse MDSCs. Treatment of patients with Granulocyte Colony-Stimulating factor (G-CSF) and GM-CSF for autologous stem cell transplantation causes suppressive CD14\(^+\) monocytes to induce CD4\(^+\) and CD8\(^+\) T-cell apoptosis, resulting in inhibition of T-cell function (42–45). Collectively, these studies support the concept that a myeloid population, belonging to the monocyte lineage, may become suppressive under the influence of growth factors, like GM-CSF and G-CSF.

In 2001, after the finding that dendritic cells (DCs) in the peripheral blood of cancer patients were reduced and correlated with the appearance of immature cells lacking markers of mature lymphoid and myeloid cells, such immature cells were shown to be capable of inhibiting T-cell response (46). These cells were called immature myeloid cells (ImCs), since they were characterized...
by the absence of staining of Lineage markers (CD3, CD14, CD19, CD57); in addition, ImCs were CD33<sup>+</sup>, CD11b<sup>+</sup> but lacked HLA-DR and CD15 expression. Although the term “ImCs” was used by some groups for a few years (47,48), the alternative acronym Myeloid Suppressor Cells (MSC) was also proposed. This term broadly includes cells of myeloid origin and the ability to suppress the immune response (10,49).

In the same years, another myeloid subset was identified, mainly composed of activated granulocytes with high SSC and low forward scatter (FSC), co-purifying with peripheral blood mononuclear cells (PBMCs) separated from patients with metastatic adenocarcinoma of the pancreas and colon and breast cancer, but not from healthy donors (50). Along the same line of research, myeloid cells endowed with suppressive activity were described in the peripheral blood of patients with metastatic renal cell carcinoma (51). This suppressive subset showed polymorphonuclear granulocyte morphology and expressed CD15 but not CD14; in a mouse model, these cells showed a significant increase in ARG activity and affected T-cell proliferation and CD3<sup>+</sup> expression (51,52). Further characterization of MDSCs was extended to renal cell carcinoma patients, showing that these cells were a subpopulation of activated PMNs expressing high levels of CD66b, CD11b, and VEGFR1 and low levels of CD62L and CD16 (53). The expansion of this MDSC subset has also been documented in several types of cancer patients [reviewed in (54)].

The family of MSC thus included not only immature cells but also more highly differentiated elements belonging to the monocyte and granulocyte lineages, so that the terms “MSC” and “ImC” no longer appeared to be appropriate. To define them better and avoid confusion with other abbreviations, a group of leading scientists working in the field proposed a new acronym for MDSCs and this is now the usual term (55).

The presence of a myeloid suppressive population characterized as CD14<sup>+</sup> with down-regulation of HLA class II expression (HLA-DR<sup>low/−</sup>) was found for the first time in melanoma patients treated with a vaccine containing GM-CSF as adjuvant (56). Expansion of these cells was later described in the peripheral blood of patients with melanoma, hepatocellular, renal cell and bladder carcinoma, prostate, gastrointestinal, lung, and head and neck cancer, multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and glioblastoma [reviewed in (54)].

Another marker associated with MDSCs is the α chain of the IL4 receptor (CD124), expressed on CD14<sup>+</sup> monocytes and CD15<sup>+</sup> PMNs in melanoma and colon cancer patients (24–29). This work demonstrated that both granulocytic and monocytic fractions show potential immuno-suppressive activity, but inhibition of T cell proliferation is directly correlated with the percentage of CD124/CD14 positive cells in PBMCs, but not with the percentage of CD124/CD15 positive cells in freshly purified PMNs.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme which is a key regulator of tryptophan metabolism and represents an essential pathway suppressing antitumor immunity (57). Interestingly, this enzyme is increased in CD14<sup>+</sup>/HLA-DR<sup>low/−</sup> cells in both CLL patients and patients after allogeneic hematopoietic stem cell transplantation (58,59). Remarkably, Programmed Death-Ligand 1 (PD-L1) has also been found negatively correlated with HLA-DR expression in monocytes from melanoma patients (60) and increased in the Mo-MDSCs of CLL patients (58,60). These results indicate that functional markers, such as IDO and PD-L1, may help in identifying the expansion of CD14<sup>+</sup>/HLA-DR<sup>low/−</sup>/MDSCs, since they can be used in association with surface antigens. However, an intracellular marker such as IDO not only complicates analysis but also the choice of a suitable monoclonal antibody against IDO. Lastly, the functional importance of these markers remains to be established, since mAb neutralizing PD-L1 in CLL patients does not efficiently abrogate T-cell suppression, which is instead induced by IDO inhibitor.

The subset of immature MDSCs was further characterized by a combination of the markers Lineage /HILA-DR /CD15<sup>+</sup>/CD11b<sup>+</sup> in breast cancer patients (61). This myeloid subset has been identified in other cancer types and shown to be correlated with the clinical response [reviewed in (54)]. This subset of MDSCs was also recently named promyelocytic MDSCs (62), since they share the same phenotype as MDSCs morphologically resembling promyelocytes and expanded in vitro from bone marrow progenitors with the addition of the G-CSF and GM-CSF combination (63). A critical step in phenotyping immature MDSCs is the right choice of antibodies admixed in the Lineage cocktail. Different studies in fact used different Lineage cocktails, all of which included CD3, CD19, and CD56 to exclude mature lymphocytes; in some cases, CD14 was also added to exclude monocytes and, in others, CD16 was included to eliminate granulocytes. We believe that the Lineage cocktail containing CD3, CD19, CD56, CD14, and CD16 is the cocktail of choice for staining whole blood (WB). In particular, the addition of CD16 has several advantages, as CD16<sup>+</sup> granulocytes can easily be excluded from the analysis and immature MDSCs (CD16<sup>−</sup>) can be separated from PMN-MDSCs (CD16<sup>+</sup>). We also suggest adding CD15 to the Lineage Cocktail, to avoid the possibility of confusing eosinophils, which are phenotypically CD16<sup>+</sup>CD15<sup>+</sup>, with immature Lineage-cells. However, it should be considered that CD14 and CD15 could be expressed not only by monocytes and terminally differentiated granulocytes, respectively, but also earlier in the pathway of differentiation (64). Along this line of research, we also demonstrated that promyelocytic immature MDSCs express CD15 (63). Therefore, to correctly identify the stage of MDSC differentiation, it could be useful to implement the phenotypic analysis with a morphological characterization.

To increase phenotypic heterogeneity further, the phenotype of other MDSC subsets [Lineage /HILA-DR /CD11b<sup>+</sup>(65) and CD14<sup>+</sup>/CD33<sup>+</sup>/HLA-DR (66)] have
recently been proposed, although an analysis has not been performed to find out whether these myeloid subsets overlap with others previously described.

All these considerations show that knowledge on the contribution of human MDSCs to the tumor immunosuppressive network and tumor progression emphasizes the need for thorough MDSC monitoring in cancer patients, to correlate MDSC percentages with clinical outcomes. Analysis of the path leading to MDSC definition shows that the greatest difficulties in unambiguously identifying MDSCs in cancer patients is due to the lack of specific markers. In addition, complicating this scenario, at present at least seven MDSC subsets have been identified, through gradual implementation of MDSC-associated markers stemming from efforts begun 20 years ago. Very few studies have analysed more than one subset at a time, and until now the only comprehensive survey is represented by immune monitoring by 9-color analysis of six subsets of MDSCs in a randomized, phase II clinical trial of vaccination of renal cancer patients (67). Interestingly, five out of the six MDSC subsets were significantly expanded in the blood of 68 monitored patients. The levels of two of the MDSC subsets, prior to vaccination, were significantly correlated with overall survival (OS) (67), thus highlighting the importance of monitoring the levels of these cells and, indirectly, the need to reach harmonization in the field.

In view of the frequent discrepancies, we and others decided to propose an MDSC proficiency panel for human cells, within the CIMT immuno-monitoring group. In this panel, 10 myeloid cell subsets, representing known or potential MDSC subsets, are evaluated simultaneously by 23 experienced laboratories in Europe and the US, thus representing the most extensive MDSC analysis undertaken so far. The project is ongoing and results are expected to reduce inter-laboratory differences and to reach consensus on the most debated issues.

Moreover, it should be pointed out that, at present, there is also a high variability in the literature regarding the method of evaluation of the MDSC immunosuppressive activity. For human studies, the effect of MDSCs on T cell proliferation or on cytokines' production is mainly tested. A consensus on this topic is still lacking since some groups measured proliferation with thymidine incorporation assays or with the dilution of fluorescent dyes as carboxyfluorescein succinimidyl ester (CFSE), while other studies measured the impact of MDSCs on the effector function of T cells, by testing the production of IFN-gamma by ELISA or ELISPOT. Given these premises, we believe that an effort should be made to harmonize the protocols used to test the suppressive potential of MDSCs.

**IMMUNO-PHENOTYPING OF CIRCULATING MDSCs**

Immuno-phenotyping and quantification of human circulating MDSCs are influenced by a number of parameters, apart from the problem of marker combinations. A brief summary of the most common human MDSC phenotypes reported in the literature is shown in Figure 2. The focus of this section is to indicate some of the potential sources of variability in MDSC immuno-monitoring, from blood collection to the identification and analysis of the various MDSC subsets. At least 70 reported papers describe the expansion of MDSCs in cancer patients. Most of them analyzed expansion of MDSCs in peripheral blood; tumor-infiltrating MDSCs are described only in 23 studies. The reasons for this choice are the relative ease of obtaining blood, as opposed to a biopsy from either lymph nodes or metastases, and the simpler analysis of a myeloid cell subset in blood rather than in a complex tissue, like a solid tumor, with an often variable necrotic component, severely influencing correct determination by flow cytometry. In such studies, phenotyping of circulating MDSCs was performed on either WB or isolated fractions (i.e., PBMCs, monocytes, and granulocytes). Most studies evaluated MDSCs on PBMCs, although a few used WB and a very small number identified MDSCs among purified monocytes or granulocytes.

Only one study directly compared monocytic and granulocytic MDSCs phenotyped from blood or PBMCs, and produced concordant results in the case of CD14+/HLADR-low/− MDSCs but not of CD15+/CD14+/CD11b+/CD33+ PMN-MDSCs (68). The increased frequency of monocytic MDSCs, defined as CD14+HLADR-low/−, seen in cancer patients compared with healthy donors, is consistent in WB and either fresh or frozen PBMCs, whereas the absolute number of these cells is slightly higher in the WB of patients compared with isolated PBMCs (68). Conversely, the percentage of these PMN-MDSCs is higher in WB compared with PBMCs (68). This is not unexpected, because when WB is used for phenotyping, conventional high-density PMN (HD-PMN) cannot be discriminated from PMN-MDSCs because they share expression of granulocytic markers. In the first studies describing PMN-MDSCs, these cells were defined as CD15+/CD11b+/CD66b+ which co-purified with PBMCs following density gradient centrifugation and because of their physical properties, were later on called low-density PMN (LD-PMN) (53,69). Some authors have proposed distinguishing these populations according to their differential expression of CD16, CD66b, and CD11b (53), they also showed that LD-PMN and HD-PMN have distinct functional properties and that suppressive activity is confined to LD-PMN (53,69). In a recent work (28), a population of MDSCs expanded in melanoma and colorectal cancer patients and described as CD15+/IL4Rα− was included in HD-PMN. Clearly, the choice between PBMCs and WB as the source for MDSC characterization influences results and may lead to potential bias in the evaluation of granulocytic cell subsets.

It thus turns out that blood separation is an important aspect. In this regard, Zhou et al. (70) compared various isolation procedures for human monocytes and granulocytes and found that positive selection is the procedure of choice for functional studies or when high purity is required. Of note, the Ficoll density gradient significantly reduces the surface expression of some
granulocytic markers such as CD66b and CD16 and those of functional receptors such as TLR4 and TLR2, compared with positive selection (70). Although this phenomenon was not explained, it should give rise to caution regarding the methods used for isolating the various myeloid subsets, to avoid misleading results.
Another parameter influencing MDSC phenotyping is the use of cryo-preserved samples. They are particularly suitable for multicenter clinical studies because they guarantee the possibility of performing batch analyses. Approximately, half of the reports in the literature phenotyped MDSCs from cryo-preserved PBMC samples. Five studies compared the results of MDSC values obtained with either frozen or fresh PBMCs (21,65,68,71,72). Three of these demonstrated that monocytic MDSCs, defined as CD14\(^+\)/HLA-DR\(^{low/−}\) could be efficiently detected even in frozen samples and that their frequency and absolute number were significantly correlated across various processing methods (65,68,71). However, other studies dealing with PMN-MDSCs demonstrated that the percentage of these cells is significantly reduced after thawing, due to their fragile nature (21,65,72). In view of the critical sensitivity of PMN-MDSCs to cryo-preservation, three thawing procedures were evaluated in one of these studies, and it was found that none of the protocols could avoid the loss of PMN-MDSCs after thawing (72). The same results were further confirmed in a study in which blood samples were stored at room temperature for various periods of time (up to 24 h) before staining and analysis (21). The authors showed that PMN-MDSCs with unaltered viability could only be stored for up to 6 h at room temperature before staining and analysis. Only one study investigated the effect of cryo-preservation in immature MDSCs, and the authors also concluded that such cells are sensitive to cryopreservation (65). In a survey of the literature, we found that 10 out of 25 papers estimating PMN-MDSCs and 7 out of 16 evaluating immature MDSCs used frozen PBMCs, and that only a minority of these studies included a dead cell marker to track subset viability. We believe that harmonization of the storage conditions of biological samples for immuno-phenotyping of PMN-MDSCs and immature MDSCs is essential, if these methods are to be transferred to clinical practice and comparable results obtained in multicenter trials.

It should also be noted that cryo-preservation can affect not only MDSC phenotyping but also functional assays. Kotsakis et al. (65) compared the suppressive functions of various MDSC subsets sorted from fresh blood samples of head and neck cancer patients, and showed that HLA-DR\(^−/\)CD11b\(^+\) cells suppress the proliferation of autologous CD4\(^+\) T cells. However, when functional assays were repeated on frozen PBMCs, the same cells failed to maintain their suppressive activity. Nevertheless, most studies on suppressive assays made use of fresh PBMCs, thus preventing any clear conclusions on this point.

A third aspect to be examined in MDSC phenotyping concerns the conditions in which blood is handled. Two interesting studies examined the possible bias due to sample handling in evaluating HLA-DR expression on monocytes, and this is of particular importance for CD14\(^+\)/HLA-DR\(^{low/−}\) Mo-MDSCs. The first study demonstrated that HLA-DR could be up-regulated ex vivo by a set of conditions masking the pre-existing in vivo down-regulation, and noted that blood samples, anticoagulated with either EDTA or citrate, stored on ice and analyzed within 4 h from blood withdrawal, were the best for assessing HLA-DR down-regulation without interference (73). Similar results were described in a recent study on melanoma patients undergoing Ipilimumab treatment: the expression of HLA-DR progressively increased as the interval between phlebotomy and analysis became longer, thus masking the pre-existing physiological down-regulation of this marker (74). To validate alternative methods of blood preservation, the same authors have compared standard heparinized tubes with new devices for blood collection which either contain Ficoll for immediate PBMC separation (Vacutainer® CPT\(^\text{TM}\) tubes) or are designed for blood preservation (Cyto-check\(^\text{®}\) blood collection tubes). Cyto-check\(^\text{®}\) tubes turned out to be more efficient than standard heparinized tubes in maintaining HLA-DR expression unaltered, whereas Vacutainer\(®\) CPT\(^\text{TM}\) tubes altered such expression, although it was still correlated with that obtained with Cyto-check\(®\) (74). These considerations are important for the design and management of multicenter studies, in which MDSC determination must be compared across blood samples from several different hospitals.

**CHARACTERIZATION OF MDSCs IN SOLID TUMORS**

**Mouse Tumors**

Despite considerable advances in multicolor flow cytometry and in our understanding of myeloid cell biology, phenotypic analysis of tumor-infiltrating myeloid cells still remains a problem. Major barriers are the plasticity of these cells, which respond to an elaborate microenvironment like the tumor bed, and technical difficulties during processing of tumor samples for flow cytometry.

Tumor myeloid infiltrates are phenotypically complex because several macro-populations are recruited by a plethora of locally secreted chemokines and growth factors. These macro-populations further divide into functionally distinct subsets with specific pro- or anti-tumoral tasks, depending on stimuli from the local microenvironment (75). Myeloid functional heterogeneity is of course reflected in the expression of surface markers; unfortunately, these proteins have often not been functionally linked to the populations which they identify, or else their biologic activity is simply unknown. Unambiguous definition of myeloid subsets within complex tissues such as those of tumors is therefore difficult. In addition, the assumption that specific cell surface markers univocally define myeloid populations regardless of the tissues and conditions examined, generates risks in describing new subsets without other complementary identification (76–78). Along this line of research, new approaches are expanding to implement flow cytometry data, examples being fate mapping studies, functional assays, and genome-wide analyses (79–82). The considerable potential of these experimental designs was
brilliantly demonstrated during the deciphering of the complicated nature of tumor-associated macrophages (TAMs) (82). The author showed that, although TAMs express markers of mature lymphoid DCs like CD11c and MHC-II, they actually hamper the cytotoxic activity of tumor-infiltrating T-lymphocytes. Also, surprisingly, gene-expression profiling showed that mammary carcinoma associated TAMs did not resemble alternatively activated “M2” macrophages, in contrast with normal mammary tissue macrophages. Bearing in mind that surface markers can give us only partial information about a certain cell population, some aspects of flow cytometry analysis of tumor-infiltrating MDSCs should be re-examined.

In line with analysis of lymphoid organs, MDSCs within tumor tissues can be phenotyped as CD11b<sup>+</sup>Ly6C<sup>high</sup>Ly6G<sup>-</sup> Mo-MDSCs, and CD11b<sup>+</sup>Ly6C<sup>low</sup>Ly6G<sup>-</sup> PMN-MDSCs (1). These two subsets are found in virtually all tumor tissues, regardless of the model in question, although substantial differences may occur in their percentages (see later). These cell populations can be further divided if we take into account the functional attributes of MDSCs and their lineage relationship with other myeloid cells. MDSCs usually lack the surface markers of fully mature mononuclear phagocytes like CD11c and MHC-II, in line with their poor antigen-presenting ability (4, 16, 17). The expression of F4/80, which is a marker of monocytes, macrophages, and some non-lymphoid DC subsets (79), is found only on Mo-MDSCs, but its surface expression is decreased with respect to that of TAMs. Consequently, careful evaluation of the expression levels of markers allows MDSCs to be recognized as F4/80<sub>low</sub>/negLy6C<sub>int/high</sub>Ly6G<sup>-</sup>, compared with TAMs defined as F4/80<sub>high</sub>Ly6C<sub>low/neg</sub>Ly6G<sup>-</sup> after gating on CD11b<sup>+</sup> tumor-infiltrating myeloid cells, although this definition may require accurate optimization of Ly6C and F4/80 staining (83).

Very few papers have rigorously analyzed the entire composition of tumor-infiltrating myeloid cells by flow cytometry in various tumor models (84, 85). In the work by Movahedi et al., the CD11b<sup>+</sup> myeloid infiltrate was divided into seven populations, according to Ly6C and MHC-II expressions. The Mo-MDSCs resembled a cell population described by the authors as composed of inflammatory monocytes (Ly6C<sup>high</sup>MHC-II<sup>-</sup>), whereas PMN-MDSCs were very similar to neutrophils (Ly6C<sup>high</sup>MHC-II<sup>-</sup>Ly6G<sup>-</sup>). Unfortunately, the authors did not test the suppressive ability of the two populations, and thus clear identification of such cells as tumor-infiltrating MDSCs is lacking. Interestingly, Ly6C<sup>high</sup>MHC-II<sup>-</sup> inflammatory monocytes were shown to differentiate into two populations of Ly6C<sup>low</sup>MHC-II<sup>low/high</sup>TAMs, a finding confirmed in subsequent papers (85, 86). This is reminiscent of the ability of Mo-MDSCs to differentiate into TAMs, a proposal made now nearly 10 years ago and demonstrated in several studies (87–89).

Interestingly, the two populations of TAMs can suppress the proliferation of activated T-cells, and both express the enzymes ARG1 and iNOS (84). MDSCs upregulate both enzymes while differentiating in TAMs and consequently acquire the ability to suppress CD8<sup>+</sup>T-cell responses in an antigen nonspecific manner (89). However, it should be stressed that the ability of Mo-MDSCs to differentiate in TAMs may be very difficult to analyze separately for suppressive activity. Tumor-infiltrating MDSCs spontaneously up-regulate ARG1 and iNOS when cultured in vitro for 24 h and phenotypically acquire macrophage characteristics, without requiring stimuli (13, 87). This may indicate that an intrinsic differentiation program is triggered when these cells enter the tumor microenvironment.

Apart from functional assays, cytofluorimetric detection of molecules involved in immune suppression may be helpful in assessing the immune functions of myeloid cells. Determination of ROS, ARG1, and myeloperoxidase levels can discriminate between PMN-MDSCs and normal neutrophils (15, 34), and coordinated up-regulation of both iNOS and ARG1 or staining of immunosuppressive cytokines like IL-10 and TGF-β can help to discriminate Mo-MDSCs from inflammatory monoocytes (11, 13). Although intracellular staining of these low-abundance molecules is sometimes difficult, especially in samples with a high fluorescence background like tumors, techniques such as fluorescent multilayer or enzymatic amplification staining can be very useful if carefully optimized (90, 91). Transgenic mice with fluorescent reporter genes also represent an interesting alternative (92, 93).

One of the most reliable mechanisms of MDSC immune suppression, especially in the tumor microenvironment, is the production of reactive nitrogen species (RNS), which hamper T-cell functions in several ways (35, 36, 94). MDSCs exploit RNS production and oxidative stress to perform their suppressive functions, because enzymatic inhibitors of NADPH oxidase, iNOS, and ARG1 usually restore T-cell responsiveness to near-full levels during suppression assays, regardless of the MDSC subsets or their tissue origin (1, 95). RNS production induces nitrination of both membrane surface and intracellular proteins, which mainly results in post-translational modifications of tyrosine residues (96). The resulting modified amino acid, 3-nitrotyrosine, can be recognized by specific antibodies, although their widespread reactivity with nitrated proteins limits their use in flow cytometry, especially in tissues with strong nitrative stress like tumors. However, as RNS-producing MDSCs probably exhibit discrete levels of nitration at proteomic level, the development of antibodies against specific nitrated proteins may open the way for the discovery of new functionally-related markers of MDSCs (94).

**Human Tumors**

The body of literature addressing the characterization of human MDSCs infiltrating tumor sites is less extensive than that focusing on peripheral blood, due to the technical difficulties typical of these biological samples.

Many research groups define the phenotype of tumor-infiltrating MDSCs only by the myeloid antigens CD33 and/or CD11b (89, 97–102), together with more specific
markers such as IL4Rα (97), CD66b (97), P-STAT3 (98), CD13 (97), CD34 (98), ARG1 (98), iNOS expression (89), ROS production (89), and low HLA-DR expression (97,98,102).

Other studies characterize tumor MDSCs more accurately. Many of these papers refer to monocytic MDSCs and characterize them by CD14 expression (103) together with IL4Rα (29), low or negative HLA-DR expression (27,29,103–105), presence of CD34 (27). Only two works report the expansion of granulocytic MDSCs at the tumor site (106,107) and define them as CD33+/CD11b+/CD15+ cells with negative HLA-DR expression (107). So far, immature Lin+/HLA-DR+/CD33+/CD11b+ MDSCs have been described only in melanoma patients (108).

The phenotypes of MDSCs are often defined without the use of the CD45 marker to discriminate among leukocytes and tumor cells (27,29,89,101,102,105,107,108) and only a few works have added it in flow cytometry analysis (97,99,100,103,104,106,109). In addition, only three groups evaluated the viability of cells at the tumor site: with DAPI, to stain the nuclei of viable cells (89,110), or with annexin in combination with propidium iodide to discriminate between apoptotic and necrotic cells (103).

It should be noted that many authors define these cell populations as MDSCs only on the basis of their phenotype, without results from functional assays (100,102,104–109). Although analysis of tumor samples is often difficult, because of the small amount of biological material available coupled to the low frequency of MDSCs [see (97,98)], we believe that functional assays are mandatory in identifying MDSCs, because suppressive activity on the immune response is a hallmark of their activity and also because non-suppressive myeloid populations from healthy donors are often phenotypically indistinguishable from MDSCs. A few research groups have evaluated the functional activity of MDSC populations from tumor tissue (27,29,89,97–99,103) and also identified the mechanisms of action by means of which these cells inhibit T-cell proliferation or function. The expression of ARG1 is the main mechanism by which these cells inhibit T-cell proliferation or function. The same study also identified the mechanisms of action by means of which MDSCs exert their function (29,98,106), followed by ROS production (98) and iNOS expression (89). Corzo et al. compared CD11b+/CD14+/CD33+ MDSCs in the peripheral blood and tumor tissues of cancer patients and reported that ROS are mainly produced by peripheral blood MDSCs, whereas iNOS expression is higher in tumor MDSCs (89). Another metabolic enzyme, IDO, is involved in the immuno-suppression mediated by CD33+/CD13+/CD14+/CD15+ MDSCs present in breast neoplastic tissues (97). IDO expression depends on the phosphorylation of STAT3, known to be responsible for ARG1 regulation (98), thus highlighting the role of P-STAT3 in MDSC activity. The same study compared the immuno-suppressive activity of MDSCs in peripheral blood, lymph nodes, and tumors of patients with head and neck squamous cell carcinoma, and made the interesting discovery that CD14+/HLA-DRlow/– MDSCs in lymph nodes and tumor sites had stronger suppressive activity on the proliferation of T-cells than that of MDSCs in peripheral blood (98).

The clinical importance of MDSCs at tumor sites has also been investigated (99,104,108). A negative correlation between Lin+/CD45+/CD33+ MDSC infiltration in ovarian tumor tissues and OS and disease-free survival has been shown in both primary and metastatic ovarian cancers (99). In another study, patients with pancreatic cancer were stratified into four groups according to the ratio between CD14+/CD8+ T-cell frequency, and patients with predominant CD14+/CD8low tumor infiltrates had a significantly reduced OS. As most CD14+ cells in the tumors had the phenotype of CD14+/HLA-DRlow/– MDSCs, these data provide evidence of a negative correlation between Mo-MSC expansion and patient prognosis (104). Another study evaluating the effect of Ipilimumab on the frequency of MDSCs in melanoma patients reported that a decrease in Lin+/HLA-DR+/CD33+/CD11b+ MDSCs after treatment was associated with improved progression-free survival at 1 year (108). Collectively, these results indicate that tumor-infiltrating MDSCs play an important clinical role, thus highlighting the need for deeper characterization of these cells through proper handling and analysis of the tumor district.

### MDSCs in Hematological Tumors

In MM patients, five MDSC subsets were expanded, corresponding to CD14+/HLA-DRlow/– (111,112), CD11b+/CD14+/HLA-DRlow/– (111,112), CD33+/CD15– (113), CD11b+/CD14+/CD33+/CD15+ (110), CD33+/CD11b+/CD14+/CD15+ (114). CD11b+/CD14+/CD33+ MDSCs were expanded in Chronic Myeloid Leukemia (CML) patients (115), and CD14+/HLA-DRlow/– cells in CLL (58).

The suppressive activity of MDSCs separated from MM patients has also been tested (110,112–114) and the expression of ARG1 and iNOS reported as responsible for MDSC-mediated immuno-suppression (112). The work by Noonan et al. examined the effects of taladafil treatment on MM patients, reporting reduced IL4Rα expression and ROS production on CD14+/HLA-DRlow/– cells 11 months after the beginning of treatment (112). Taladafil also reduced Arg-1 and iNOS expression in the bone marrow of MM patients leading to a decrease in tyrosine nitrosylation at this site (112). Of note, these events were associated with a dramatic increase in tumor-specific immunity of the marrow-infiltrating T-cells after 11 months of treatment, suggesting that reduced suppressive activity of MDSCs is correlated with recovery of the antitumor response (112). In MM patients, the effect of the immuno-modulatory drug lenalidomide was evaluated on MDSCs defined as CD14+/CD15+ and functionally able to inhibit both CD4+ and CD8+ T-cell proliferation in vitro. Lenalidomide-treated patients showed a significant increase in MDSCs, compared with untreated patients, and a significant positive correlation...
between MDSC levels and T central memory/T effector memory cells and Tregs (regulatory T cells) (114).

A link between MDSC frequency and prognosis has also been reported for CML; the percentage of CD11b+/CD14+/CD33- immuno-suppressive cells was higher in patients with Sokal high risk (HR) with respect to those with Sokal low risk (LR) (115).

In CLL patients, the expansion of CD14+/HLA-DRlow/-MDSCs was described by Jitschin (58). This subset suppressed T-cell proliferation in a dose-dependent manner and its levels were negatively correlated with the absolute number of circulating T lymphocytes. IDO was highly expressed in CD14+/HLA-DRlow/- MDSCs, and its blockade through the competitive inhibitor 1-MT resulted in a significant increase in T-cell proliferation, thus implying its central role in MDSC-mediated immune suppression.

TECHNICAL PROBLEMS IN ANALYZING MDSCs IN MOUSE AND HUMAN TUMOR TISSUES

There are several sources of potential bias to be considered in cytfluorimetric analysis of mouse MDSCs. The extent of their recruitment depends on tumor type, and correlates positively with both tumor size and growth time (116). Although large tumors have higher frequencies of MDSCs in lymphoid organs, the composition of single samples may be negatively influenced by the excessive presence of necrotic areas. The physical aspects of tumor growth, such as vascularization, ulceration or even invasion of the peritoneal cavity (in the case of subcutaneous injection of cancer cell lines in the flank of the body) have also been observed to affect MDSC frequency to a great extent, highlighting the bias introduced by a single operator’s ability to transplant tumor cells. MDSCs are also recruited in response to chronic infections, so animals should be housed in specific pathogen free (SPF) facilities to avoid experimental bias due to hidden pathogens.

Besides these biological considerations, there are a number of technical aspects regarding sample processing which must be carefully evaluated, especially while working with tumor samples of both human and mouse origin. Solid tissues require more handling than peripheral blood to obtain cell suspensions, and certain steps in sample processing may alter the expression of surface antigens, giving rise to misleading determinations of MDSCs. A review of the literature shows that many research groups acknowledge the need to process samples within a few hours of surgical removal and to preserve them in culture media or saline solutions, to avoid tissue degradation and antigen alteration (27,100,103,106,107,109). In mice, although CD11b, Ly6C, and Ly6G are relatively stable markers, cells should be kept in ice-cold solutions during cell manipulation, to maximize cell viability, avoid clump formation, and block antibody-induced endocytosis of specific membrane surface molecules. FcγR blocking with specific reagents is important in reducing non-specific binding of fluorochrome-conjugated antibodies. Careful optimization of staining conditions is, of course, mandatory, as already reported (117,118).

However, the most critical step in both human and mouse tissues is enzymatic digestion. Mulder et al. analyzed the effect of collagenase and two types of DNases, differing in their degree of purity, on the expression of some membrane markers on human PBMCs (119). DNase treatment of PBMCs greatly reduced the cell surface expression of CD2, CD4, CD8, CD14, and CD44 antigens and caused also a slight reduction in CD16, CD28, and CD56. This effect was reported for both types of DNases. Collagenase treatment also led to an alteration in the expression of cell-surface molecules but with a pattern differing from that of DNase because CD4, CD14, CD16, and CD56 molecules were the most sensitive (119).

Analyzing the dissociation protocols reported in the literature, we note marked variations among groups, in both procedure and enzymatic cocktail. Collagenase, hyaluronidase, and DNase are the most frequently used enzymes, although the concentrations and the times of digestion vary from one group to another (89,101,106,107,109). In some cases, these enzymes are added to other proteases (89,109) or associated with mechanical disintegration (106). Two papers reported the use of liberase alone (98) or combined with DNase (100). Some research groups simply apply mechanical sample processing (102,103), sometimes followed by centrifugation on Ficoll layer (102). To avoid antigen cleavage due to excessive sample processing, it is thus mandatory to optimize the protocol, the enzyme cocktail and the digestion time specific to each type of tumor.

The use of viability dyes is always recommended when analyzing tumor samples, and skillful exclusion of doublets and cell clusters can greatly enhance data quality. Although the combination of CD11b, Ly6C, and Ly6G is sufficient to detect the two main MDSC subsets, complementary markers and/or “dump gates” (120) allow further testing of staining quality. For example, adding the F4/80 marker should result in negative staining of PMN-MDSCs, to check whether unspecific binding of the antibody cocktail occurs: Mo-MDSCs should stain positive for F4/80 but with lower expression with respect to TAMs, thus demonstrating the high sensitivity of the staining. Another example is plotting Ly6G versus Ly6C after gating on CD11b- cells: this allows exclusion of Ly6G+Ly6Clow PMN-MDSCs, which usually have strong autofluorescence, and better identification of Ly6G+Ly6Chigh Mo-MDSCs. In this respect, once the gating strategy has been established, one very practical approach when choosing fluorochrome-marker combinations is to use two fluorochromes with low compensation requirements, to solve the problem of high autofluorescence, which is typical of tumor samples. Another problem sometimes encountered when analyzing tumor samples is the coexpression of MDSC markers by either tumor or epithelial cells. In CML patients, analysis of MDSC expansion is complicated by the fact that CML cells share many markers with MDSCs. As 95% of CD34+ cells are also positive for Philadelphia.
chromosome, CD34 antigen is used to discriminate tumoral cells, although CD34 is also expressed by 35% of MDSCs, gated as CD11b+/CD14+/CD33− cells, leading to a partial overlap of the two populations during analysis (115).

Similar data on CD15 expression have also been reported by Eruslanov et al. (109). The CD15 marker was detected not only in granulocytic myeloid cells infiltrating urothelial carcinoma but also in the CD45-negative fraction, presumably composed of epithelial cancer cells. Using the CD45 marker in tumor tissues is therefore mandatory for proper analysis of leukocytes, together with other specific antigens in the case of hematological tumors.

**FUTURE PERSPECTIVES**

Multiparametric flow cytometry is the leading technique for MDSC characterization and monitoring. Although three-color staining (CD11b/Ly6C/Ly6G) of mouse cells reveals the main MDSC subsets, a more complex panel, containing at least six markers (CD14/CD15/HLA-DR/CD33/Lin (composed of anti-CD3-14-19-56 Ab)/CD124) should be used to evaluate the human counterpart. A detailed description of staining panels suited to identify murine and human MDSCs is reported in Table 1. We believe that harmonization of MDSC phenotyping will enrich our knowledge of MDSCs, provided that high-quality datasets are generated. In this regard, we have discussed here the main technical issues which may influence MDSC identification by flow cytometry for both circulating and tumor-associated MDSCs, such as optimization of primary sample handling and specimen dissociation, and including the preparation and choice of reagents compatible with the samples to be analyzed.

Synergy between the new molecular and bioinformatics methods and efforts toward simplification and harmonization of MDSC phenotypes will enrich our knowledge in this field and enhance the use of MDSCs as both therapeutic targets and diagnostic parameters.

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**Table 1**

| Antigen | Clone | Fluorochrome |
|---------|-------|--------------|
| Staining panel for mouse MDSCs | | |
| CD11b | M1/70 | PE-Cy7 |
| LY6C | HK1.4 | Pacific Blue |
| LY6G | 1A8 | APC-Cy7 |
| F4/80 | CI:A3-1 | FITC |
| CD11c | HL3 | APC |
| I-A/I-E | M5/114.15.2 | PerCP-Cy5.5 |
| | (C57Bl/6) 2G9 (BALB/c) | |
| Staining panel for human MDSCs | | |
| CD11b | ICRF44 | Alexa-700 |
| HLA-DR | L243 | APC |
| CD15 | MAA | V450 |
| CD33 | P67.6 | PE-Cy7 |
| CD14 | M109 | APC-Cy7 |
| IL4Rα | 25463 | PE |
| Lineage cocktail | (UCHT1, M5E2, HIB19, CD3-14-19-56) | FITC | (NCAM16.2) |
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