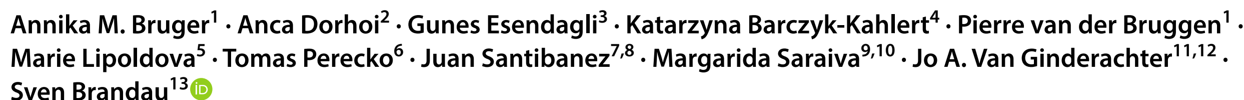
Cancer Immunology, Immunotherapy (2019) 68:631–644
https://doi.org/10.1007/s00262-018-2170-8

SYMPOSIUM-IN-WRITING PAPER

How to measure the immunosuppressive activity of MDSC: assays, problems and potential solutions



Annika M. Bruger[1] · Anca Dorhoi[2] · Gunes Esendagli[3] · Katarzyna Barczyk-Kahlert[4] · Pierre van der Bruggen[1] · Marie Lipoldova[5] · Tomas Perecko[6] · Juan Santibanez[7,8] · Margarida Saraiva[9,10] · Jo A. Van Ginderachter[11,12] · Sven Brandau[13]

Received: 22 February 2018 / Accepted: 2 May 2018 / Published online: 21 May 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of mononuclear and polymorphonuclear myeloid cells, which are present at very low numbers in healthy subjects, but can expand substantially under disease conditions. Depending on disease type and stage, MDSC comprise varying amounts of immature and mature differentiation stages of myeloid cells. Validated unique phenotypic markers for MDSC are still lacking. Therefore, the functional analysis of these cells is of central importance for their identification and characterization. Various disease-promoting and immunosuppressive functions of MDSC are reported in the literature. Among those, the capacity to modulate the activity of T cells is by far the most often used and best-established read-out system. In this review, we critically evaluate the assays available for the functional analysis of human and murine MDSC under in vitro and in vivo conditions. We also discuss critical issues and controls associated with those assays. We aim at providing suggestions and recommendations useful for the contemporary biological characterization of MDSC.

Keywords Myeloid-derived suppressor cells · T cells · Immunosuppression · Arginase · Proliferation · Mye-EUNITER

Abbreviations

ATRA	All-trans retinoic acid
Arg1, *ARG1*, ARG1	Arginase-1
BrdU	Bromodeoxyuridine
CFSE	Carboxyfluorescein succinimidyl ester
COST	European Cooperation in Science and Technology
DCFDA	2′,7′-dichlorofluorescin diacetate
EU	European Union
KO	Knock-out
M	Monocytic
MDSC	Myeloid-derived suppressor cell(s)
Nos2, *NOS2*, NOS2	(inducible) nitric oxide synthase 2
PMN	Polymorphonuclear
ROS	Reactive oxygen species

Jo A. Van Ginderachter and Sven Brandau co-senior authors.

This paper is part of a Symposium-in-Writing in *Cancer Immunology, Immunotherapy* by members of the *European Network of Investigators Triggering Exploratory Research on Myeloid Regulatory Cells* (Mye-EUNITER network), funded by the COST programme of the European Union (http://www.mye-euniter.eu).

✉ Sven Brandau
sven.brandau@uk-essen.de

Extended author information available on the last page of the article

Introduction

In the context of acute infection or inflammation, myeloid cells respond to immunogenic molecules categorized as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). A key characteristic of this response is the presence of considerably strong danger signals which decline when the infection is being cleared [1]. This acute activation of myeloid cells triggers multiple protective immune functions such as phagocytosis, cytokine secretion and activation of T cells. In the context of chronic disease and particularly in malignancies, myeloid cells are exposed to persistent signals, often of lower strength and longer duration [2]. This continuous stimulus has fundamental effects on myeloid cell differentiation and polarization [3]. A prototypic example of this is the expansion of myeloid-derived suppressor cells (MDSC) [4].

The term MDSC illustrates two important biological features of these cells. First, MDSC are derived from myeloid precursors and can consist of a mixture of different myeloid lineages such as monocytic or granulocytic cells. Second, MDSC possess capacities to suppress immune responses [5]. The latter aspect is a "sine qua non" condition for this cell type. Without such explicit and functional characterization (summarized in Table 1), myeloid cells should not be classified as MDSC [5, 6].

MDSC expansion has been recognized as an important pathophysiological principle in most types of cancer and other diseases associated with chronic inflammation. The persistence of high levels of monocytic MDSC was, for example, correlated to disease progression, poor survival and poor responses to checkpoint inhibitor therapies targeting PD-1 and CTLA-4 in patients with advanced melanoma [7, 8]. Multiple further excellent reviews (including companion reviews of this Symposium-In-Writing by Umansky et al. and Dorhoi et al.) have summarized key biological features of MDSC and their implications for disease [4, 9]. These aspects will not be repeated here.

Despite their relevance, surprisingly little attention has been paid to the careful development, standardization and harmonization of functional tests for MDSC. To address this issue, in the EU-COST Mye-EUNITER consortium (http://www.mye-euniter.eu), a working group has been formed. In this review, members of this working group summarize the current state-of-the-art and knowledge on testing the functional activity of MDSC. Since T cells are regarded as the primary targets of MDSC [10–12], this short review focuses on functional assays which assess the impact of MDSC on T cell responses. Here, we review the analysis of MDSC functions in murine and human systems, both in vitro and in vivo. We also critically assess the pros and cons as well as the pitfalls and limitations of current assays.

As stated above, immunosuppressive activity is a central and defining element of MDSC [5, 6]. When preparing this review, we performed a comprehensive analysis of the literature and surveyed research on MDSC published in the last decade. The search was performed on PubMed (US National Library of Medicine, National Institutes of Health) where "MDSC" was used as keyword. Of note, only the original articles using the term MDSC for myeloid-derived suppressor cells have been considered. Hence, approximately 850 full-text papers were retrieved and their materials and methods sections were thoroughly reviewed.

This literature survey revealed that the majority (2/3) of published studies in mouse models and humans employed selected functional assays to characterize MDSC in the respective experimental settings. However, hundreds of papers are still being published that use the term "MDSC" without reporting on the functional characterization of these cells.

With this review, we aim to provide an overview and useful guidelines and suggestions to further accelerate insights into this important field of immunology. We are aware of the fact that MDSC can and will influence other immune effector cells besides T cells. However, we consider it to be critical to first optimize T cell assays. This appears challenging enough, yet urgently needed to be able to reliably compare MDSC biology and function across laboratories and in different disease settings. Lastly, assays developed, optimized and standardized for MDSC research may also be used to test the function of other myeloid immune cells and compare their activity with MDSC.

Functional analysis of murine MDSC

Phenotype, source tissue, isolation method

Murine MDSC are phenotypically characterized as Gr-1$^{dim/+}$CD11b^{+} cells and are further subdivided into monocytic (M)- and neutrophil (PMN)-like cells based on expression of Ly6C and Ly6G, respectively [5]. These surface markers are shared with other immune cells, including bona fide phagocytes such as monocytes and neutrophils, and selected lymphocyte populations, thereby calling for careful gating strategies for flow cytometric characterization or sorting experiments. Markers, such as CD244, which have been assigned a unique expression in murine PMN-MDSC require further validation [13]. Differentiation of M-MDSC and tumor-associated macrophages (TAM) may partially be achieved based on positivity of the latter for F4/80, low expression or lack of Ly6C, abundant IRF8 and reduced endoplasmic reticulum (ER)-stress markers [5]. Biochemical features, primarily (co)expression of arginase 1 (Arg1) and inducible nitric oxide synthase (Nos2), and to a lesser extent Nadph oxidase (Nox1), indoleamine dioxygenase (Ido) and cyclooxygenase (Cox1), further support the classification as MDSC in mice. However, such enzymes may also be regulated in bona fide phagocytes. Owing to the lack of unique phenotypic markers, identification of MDSC cannot solely rely on immunophenotyping but instead requires a demonstration of their lymphocyte suppressive function.

MDSC have been isolated from diverse tissues and different disease models, with the isolation method often depending on the tissue type. The spleen is mostly processed by mechanical disruption [14], whereas lung tissue and often solid tumors are usually submitted to enzyme-based enzymatic digestion [15]. A more challenging organ is the liver, where mononuclear cell purification in Percoll gradients has been reported [16]. Bone marrow preparation mainly involves flushing of the femoral bones, and thus represents little manipulation compared to procedures for parenchymal

Table 1 Functional MDSC assays in mice and humans

Method	Mouse		Human	
	In vivo	In vitro	In vivo	In vitro
T cell proliferation				
Methods	Adoptive transfer of dye-labeled T cells In vivo bromdesoxyuridin (BrdU) pulse (2–3 h) Ki67 positivity (IHC or ex vivo flow cytometry)	Tracking dye dilution (e.g., CFSE) BrdU incorporation Ki67 positivity Thymidine incorporation	–	Tracking dye dilution (e.g., CFSE) BrdU incorporation Thymidine incorporation
Target cells	Flow cytometry analysis on CD3+CD4+and CD3+CD8+T cells, using either total cell suspensions of the organs of interest or purified from these organs	Cell suspensions (bulk splenocytes, lymphocytes—pan-T cell isolation, T cells, B cells, lymphocyte subsets—CD4+, CD8+, CD19+; thymic epithelial cells) purified from various tissues Antigen-specific/non-specific (e.g., OVA peptides, tumor/viral/bacterial peptide; polyclonal CD3/28 stimulation, mitogens) Naïve/activated T cells	–	PBMC PBMC-CD14-depleted CD3+ CD4+ CD8+
Source	Autologous T cells from diseased mice Adoptively transferred antigen-specific T cells (eg TCR transgenic T cells)	Inbred/allogenic (mostly C57BL/6 and BALB/c) Transgene antigen-specific (e.g., OT-I/II; CL4 etc.)	–	Autologous Allogenic
Cytokine release assays				
Methods	Serum levels of cytokines Cytokine reporter mice ex vivo culture of in vivo stimulated T cells, followed by supernatant collection and cytokine measurement	ELISA ELISpot ICS (flow cytometry) Bead-based arrays (luminex)	–	ELISA ELISpot
Targets	IFNγ, IL-2, IL-4, IL-6, TNFα IL-10, TGF-β	IFNγ, IL-2, IL-4, IL-6, TNFα, IL-10 TGF-β	–	IFNγ, TGF-β, IL-10, IL-2
ARG1				
Methods	Serum levels of arginine ARG1 detection via IHC in vivo use of inhibitors (eg nor-NOHA) Conditionally Arg1-deficient mice	RT-PCR Western blotting Colorimetric assay/enzyme activity/metabolite detection (urea) flow cytometry T cell proliferation assay with Arg1 inhibitors Knock-out (KO) cells (genetic evidence)	Serum levels of ARG1	RT-PCR Western blotting Colorimetric assay
IDO				
Methods	IDO detection via IHC in vivo use of inhibitors (eg 1-MT) Ido-deficient mice	RT-PCR metabolite detection (kynurenine) T cell proliferation assay with IDO inhibitors KO cells (genetic evidence)	–	RT-PCR Western blotting Immunohistochemistry T cell proliferation assay with IDO and STAT3 inhibitors

Table 1 (continued)

Method	Mouse		Human	
	In vivo	In vitro	In vivo	In vitro
NOS2				
Methods	NOS2 detection via IHC in vivo use of inhibitors (eg L-NAME) Nos2-deficient mice	RT-PCR colorimetric assays (Griess reaction) flow cytometry T cell proliferation assay with Nos2 inhibitors KO cells (genetic evidence)	–	RT-PCR Western blot Flow cytometry
Nitration/nitrosylation				
Methods	Flow cytometry: nitrosylated CD3ζ downregulation of CD3ζ	See below please	Flow cytometry: nitrosylated CD3ζ downregulation of CD3ζ	
ROS				
Methods	Flow cytometry on ex vivo cells (2′,7′ –dichlorofluorescin diacetate DCFDA) in vivo use of antioxidants Mice deficient in the NADPH complex	Flow cytometry (DCFDA) Hydrogen Peroxide/Peroxidase Assay Kit T cell proliferation assay with ROS scavengers KO cells (genetic evidence)	–	Flow cytometry (DCFDA). Destructive
Checkpoint inhibitors				
Methods	Flow cytometry on ex vivo cells Checkpoint inhibitor detection via IHC Use of checkpoint blockade inhibitors in vivo (anti-PD-1/PD-L1/CTLA-4)	PD-1/PD-L1/2 by flow cytometry Checkpoint blockade inhibitors (PD-1/CTLA-4) KO cells (genetic evidence)	–	PD-1 (by flow cytrometry) after co-incubation
Regulatory T cells				
Methods	Foxp3 detection via IHC Foxp3 reporter mice Modulation of Treg activity via conditionally deficient mice (Foxp3-cre)	% CD4+CD25+Foxp3+ Cytokine detection (IL-10, TGF-β) via ICS, bead-based array	–	% CD4+CD25+Foxp3+ after co-incubation by flow cytometry

Suppression of lytic activity of cytotoxic CD8: ^{51}Cr-release assay; granzyme B expression

Loss of cell surface CD3ζ/dissociation of TCR/CD3ζ complex: qRT-PCR; Flow cytometry; Western Blot/IP-FCM (immunoprecipitation of multiprotein complexes detected by flow cytometry), FRET (fluorescence resonance energy transfer)

Suppression NK cells: proliferation, cytokine release, perforin, granzymes

organs [17]. Tissue digestion may affect the viability of the cells and in addition may modulate the surface epitopes. Furthermore, such procedures may activate myeloid cells, e.g., upregulation of enzymes in case alarmins or pathogen-associated molecular cues are present, particularly upon extended processing time. Such factors may affect the phenotype, as well as the functional features of the murine MDSC.

MDSC can either be enriched by magnetic selection or sort purified. MDSC enrichment by magnetic selection has been achieved using commercially available MDSC kits, which allow for enrichment of $Ly6G^{+}Gr1^{high}$ and $Ly6G^{-}Gr1^{dim}$ cell subsets [15], or alternatively by positive or negative selection based on CD11b/Gr1 [14] or Ly6C/Ly6G markers. Flow cytometry sorting of murine MDSC is mainly based on a combination of CD11b/Ly6G/Ly6C cell markers. Such basic sort protocols may be refined by exclusion of lymphocytes (CD3+ and CD19+ cells) or based on pre-enrichment of $CD11b^{+}$ cells before sorting. Both commercial kits and homemade sorting protocols likely result in enrichment of cells other than MDSC, given the universality of the markers employed. As such, until unique MDSC markers are identified and validated, the expression of functional molecules and functional assays will remain crucial for the characterization of isolated cells.

Mechanisms of suppression

Murine MDSC commonly suppress T cell proliferation by the release of cytokines and through enzymatic products downstream of Nos2 and Arg1. Arginine is a non-essential amino acid that is required for T cell proliferation. NOS2 catalyzes the production of nitric oxide (NO) from L-arginine, while ARG1 catabolizes arginine into ornithine and urea. Cells expressing NOS2 or ARG1 could inhibit T cell proliferation by depleting available extracellular arginine. Additional distinct mechanisms have been reported to be responsible for the ARG1- and NOS2-mediated suppression of T cell proliferation, such as decreased expression of the CD3ζ chain, cell cycle arrest in G0/G1 phase, induction of T cell apoptosis, inhibition of MHC class II on APC, as well as reduced JAK3 and STAT5 expression in T cells [18]. A heightened production of reactive oxygen species (ROS) is unique to MDSC isolated from tumor-bearing mice and appears critical for MDSC-mediated suppression of T cell function [19]. Peroxynitrite is abundant at sites of MDSC accumulation and induces unresponsiveness in CD8+ T cells, probably subsequent to nitration/nitrosylation of the T cell receptor and the CD8 molecule [20]. Inhibitory ligands belonging to the B7 family, such as PD-L1 and PD-L2, are expressed on MDSC and further contribute to the suppression of T cells [21]. MDSC produce copious amounts of IL-10 and TGF-β and thereby promote expansion of regulatory T cells (Tregs), which in turn suppress effector T cells [22]. Cysteine sequestration by MDSC limits the amino acid's availability for T cells, and together with the downregulation of L-selectin expression on naïve T cells which decreases the T cells' tissue homing ability, represent additional immunosuppressive mechanisms of murine MDSC [11, 23]. Thus, the suppressive effects of murine MDSC are mediated by many concurrent mechanisms, which may vary depending on the disease. In addition, the outcome of MDSC-mediated suppression may also depend on the mode of T cell activation (e.g., mitogens versus immunogenic peptides).

Assays and technologies for functional MDSC analysis in vitro

Significant differences in functional assay outcomes have been reported depending on the genotype [24] and sex [25] of the animals, and for in vitro derived murine MDSC generated through different protocols [26]. Establishment of a widely accepted panel of functional assays for in vitro testing is critical for the cross-validation of results obtained in various laboratories and in distinct mouse models. Generally, in vitro flow cytometry or radiometric assays using co-cultures of MDSC with allogeneic T cells are the most frequently used tests for investigating suppressive activity. Such assays allow the monitoring of Treg expansion (e.g., CD4+Foxp3+), the proliferation of CD4+ and/or CD8+ T cells, IFN-γ release as well as T cells un-/hypo-responsiveness [26]. Identification of the suppressive mechanisms is classically performed by either measuring or inhibiting key suppressive mechanisms. As such, enzymatic activity, notably levels of metabolites downstream of ARG1 (e.g., urea measurement), NOS2 (e.g., NO measurement via the Griess reaction), ROS production (e.g., Fe 3+ -xylenol orange reaction, hydroethidine staining to detect superoxide), have been described and identified. Also, ex vivo analysis of mice deficient in those key enzymes, facilitates the identification of the mechanism of suppression of the MDSC [21].

In vivo activity of murine MDSC

In the vast majority of investigations, MDSC suppressive activity is analyzed in in vitro assays following purification from animals with ongoing pathology. Although such assays are certainly informative, the true suppressive nature of MDSC as well as ultimate proof of a physiologically relevant level of immunosuppressive activity stems from in vivo assays. To assess the T cell suppressive activity of MDSC in a living animal, essentially two approaches can be considered: (i) adoptive transfer of purified MDSC to animals that

do not harbor functional MDSC, (ii) depletion or modulation of MDSC during an ongoing pathology.

Adoptive transfer of MDSC is an elegant approach to specifically address the functionality of these cells in vivo. To this end, both purified MDSC and antigen-specific T cells (for example, TCR transgenic T cells) can be transferred intravenously into congenic naïve animals, after which the mice are immunized with the relevant antigen. A few days later, antigen-specific T cell activity is scrutinized (proliferation, cytokine production, expression of activation markers) [20]. Notably, immunization is not only required to trigger T cell activation, but MDSC may need to present the relevant peptide(s) to be suppressive. Alternatively, MDSC can be pulsed with antigenic peptide before transfer into the recipient [27]. Though elegant, this approach may be less suitable to assess the function of tissue-restricted MDSC, such as those derived from the tumor microenvironment. Tumor-associated MDSC are strongly suppressive in an antigen-independent fashion and depend on local conditions, such as hypoxia, for their function [28]. However, those microenvironmental conditions will not be recapitulated in the naïve recipient of transferred MDSC.

To draw clear-cut conclusions from MDSC depletion experiments, the depletion strategy should be as MDSC-specific as possible. This is however not trivial, considering the paucity of markers or signaling pathways that unequivocally discriminate MDSC from their non-suppressive counterparts, such as monocytes and neutrophils. Anti-Gr-1 antibodies (clone RB6-8C5) have been amply used to eliminate MDSC in mice. However, Ly6C and Ly6G (molecular targets of anti-Gr-1) are also highly expressed on classical monocytes and neutrophils, respectively [29], and depletion of target cells is transient, followed by repopulation of the animal with immature granulocytic myeloid cells [30]. The same holds true for anti-Ly6G (clone 1A8) that depletes both PMN-MDSC and neutrophils [31]. Some chemotherapeutic drugs, such as gemcitabine and especially 5-fluoroacil, were advocated as being specifically cytotoxic for MDSC [32]. However, multiple cancer cell types are also affected by these compounds, creating an uncertainty whether reduced tumor growth is due to a reduced MDSC functionality or a direct toxicity towards cancer cells. A blockade of MDSC trafficking by blocking the chemokine receptor CXCR2 [33], the use of MDSC differentiating agents such as all-trans-retinoic acid (ATRA) [34] or the application of phosphodiesterase-5 inhibitors [35] have all been shown to affect MDSC functionality in vivo. Other approaches aimed at blocking the suppressive machinery of MDSC, such as nitroaspirin [36], that diminishes the activity of NOS2 and ARG1, and the triterpenoid CDDO-Me [37], that reduced the production of ROS, have been proposed. However, it should be realized that none of these suppressive mechanisms are unique to MDSC, so the application of inhibitors in vivo may affect other cell types as well. Altogether, we can conclude that in vivo assays to test the T cell suppressive capacity of MDSC are absolutely essential, but the potential caveats of each assay should be taken into account.

Functional analysis of human MDSC

Phenotype, source tissue, isolation method

Phenotyping and isolating MDSC is much more complex and challenging in humans than it is in mice (for in-depth review of this topic compare review by Cassetta et al. in this symposium-in-writing series). First, patient samples are difficult to obtain and often only provide few (less than ten thousand) cells, making standard functional assays difficult to perform. In most cases, MDSC are isolated from peripheral blood of cancer patients [38–40]. Rarely, cancerous ascites [41], bone marrow aspirates [42], splenocytes [43], and PBMC from healthy donors for in vitro generation [44] are used to source MDSC. The density gradient which is used to separate whole blood components is required to distinguish low density PMN-MDSC from high density neutrophils in the absence of MDSC-specific markers for whole blood. While it cannot be excluded that density gradient separation induces changes in the expression of surface markers of the activation state of MDSC, no evidence of this has been reported in the literature. Second, isolation methods, immunophenotyping and gating vary largely between different research groups. This is because MDSC are heterogeneous and no relatively simple marker system mirroring the murine CD11b-Gr-1 paradigm exists in the human setting. This results in high divergence between laboratories in the phenotypic definition of MDSC subsets [45]. In response to this challenge, Bronte et al. proposed strategies to characterize defined subpopulations of MDSC phenotypically and to determine their suppressive activity in functional assays [5]. The review defines PMN-MDSC as HLA-DR$^-$CD11b$^+$CD14$^-$CD15$^+$ and M-MDSC as HLA-DR$^{-/lo}$CD11b$^+$CD14$^+$CD15$^-$. CD15 may be replaced by CD66b. CD33 can substitute for CD11b, considering that M-MDSC express high levels of CD33, while PMN-MDSC are CD33^{dim} [46]. However, common isolation practices are less uniform and more simplistic. Three basic isolation methods exist: (i) magnetic bead sorting, (ii) magnetic bead enrichment followed by flow cytometry sorting, (iii) flow cytometry sorting. Magnetic bead sorting is the least complex method and is used in one- or two-step protocols, focusing on a maximum of two markers only, while flow cytometry isolation methods allow for more complex multiparameter sorting. Single step magnetic bead isolation protocols are favored by studies that use in vitro derived MDSC and focus on CD33 positive selection or CD11b depletion

after in vitro generation from PBMC or bone marrow for 4–7 days [42, 44]. In particular, bone marrow-derived (BM-) MDSC have been generated and isolated in vitro by incubating lymphocyte-depleted bone marrow aspirate samples with G-CSF and GM-CSF for 4 days and depleting CD11b+ cells to obtain CD11b^{low}CD16^{-} immature and CD11b^{+}CD16^{+} mature BM-MDSC [42]. Single step isolation protocols from clinical samples are rare. One example is Obermajer et al. who selected all CD11b+ cells from ovarian cancer ascites by magnetic bead sorting [41]. Most human MDSC are isolated from PBMC. Here, the isolation protocols vary widely and use magnetic bead sorting only, flow cytometry sorting only or a combination of the two with different degrees of complexity. An example of a magnetic bead only isolation protocol from PBMC uses a two-step isolation protocol that involves first the depletion of HLA-DR-positive cells followed by positive selection for CD33 [47]. Similarly, simple isolations using a combination method, isolate CD14+ cells by magnetic sorting first and then HLA-DR positive and negative cells by flow cytometry. This method focuses on M-MDSC only [39]. However, these simple isolation protocols are obviously not suited for high purity isolation of MDSC subsets. Flow cytometry sorting provides the most comprehensive method of isolating MDSC and should be preferred over bead sorting. An example of a complex multiparameter panel for sorting of MDSC from PBMCs by flow cytometry includes CD11b, CD14, CD15, CD16, CD33 and HLA-DR and distinguishes between M- and PMN-MDSC [48]. Clearly, only multi-parameter isolation methods can achieve simultaneous purification of multiple well-defined MDSC subsets and should therefore be preferred.

Suppressive phenotypic characteristics

MDSC are defined by surface markers in conjunction with suppressive function [5]. Tumors expressing cyclooxygenase (COX)-1 and -2 produce high levels of prostraglandin-E2 (PGE-2) which recruits MDSC to the tumor site and induces immunosuppressive functions such as ARG1 expression in MDSC and monocytes [41, 49]. Increased ARG1 levels in patient serum have been correlated with increased numbers of MDSC in the blood [50]. *ARG1* expression in MDSC is determined in vitro through qRT-PCR, and the presence and activity of ARG1 is determined by Western blotting, and less commonly by flow cytometry, and through ARG1 activity assays that measure urea concentration colorimetrically [38, 50–52]. While qRT-PCR and Western blotting confirm the presence of *ARG1* transcription and translation in MDSC, in some instances the protein levels recorded are comparable to that in monocytes and much lower than in macrophages [51]. It is important to note that standard culture media such as RPMI contain high levels of arginine (1 mM), which exceed physiologic levels (30–125 µM). Using media low in arginine or quantifying ARG1 levels in media may be required to fully appreciate the potential contribution of arginine depletion in MDSC-mediated immunosuppression.

IDO catabolizes tryptophan through the kynurenine pathway. Similarly to arginine, the depletion of tryptophan by IDO-expressing cells limits T cell proliferation and survival [53]. Tryptophan levels in RPMI (25 µM) exceed the proliferation-limiting 2 µM. The presence of IDO is determined by qRT-PCR, Western blotting, and in rare instances immunohistochemistry [38, 41, 42]. IDO activity is not commonly assessed. One study assessed the influence of MDSC IDO activity on T cell proliferation, cytokine release and apoptosis using IDO and STAT3 inhibitors [42]. However, the MDSC used were generated from lymphocyte-depleted bone marrow, isolated via CD11b and CD16 depletion and thus may not conform with the current phenotypic consensus criteria [5, 42].

In mice, NOS2 activity results in the nitrosylation of the TCR and its consequent downregulation and internalization. While the work in mice is extensive and convincing, little data has been published in human settings. Nitrosylation was tested on the surface of CD8+ T cells from breast or head and neck cancer patients but no direct causative link to MDSC was established [20]. Testing TCR nitrosylation with specific antibodies is not commonly used to assess human T cells and their function. The expression of *NOS2* by MDSC is tested by qRT-PCR and by Western blotting [41, 51]. Liu (2010) compared the expression of NOS2 in MDSC from non-small cell lung carcinoma patients to those in macrophages and monocytes [51]. However, MDSC expressed NOS2 at similar levels to monocytes. The activity of NOS2 can be assessed by flow cytometry [47]. Unfortunately, NOS2 activity measured in this way was not compared to monocytes or macrophages. Furthermore, this method is destructive and the cells are no longer available for co-culture assays with T cells. ROS released by MDSC are measured in a similar manner and retain the same caveats [47]. Although determining the presence of phenotypic markers on MDSC that could mediate suppression is interesting, they are not sufficient to fully assess suppressive activity.

Functional analysis of human MDSC T cell interaction and suppression in vitro

The almost universal standards to assess MDSC suppressive activity are T cell proliferation and cytokine release assays [41–43, 47]. T cell proliferation assays follow a common simple principle. However, procedural details between reports vary widely. Usually, T cells are co-incubated with MDSC in a 96-well with RPMI and 10% fetal calf serum for 3–5 days (Table 2). The proliferation of T cells is assessed

through either the dilution of a tracking dye such as carboxyfluorescein succinimidyl ester (CFSE) or through tritiated thymidine incorporation. Suppression of cytokine production is assessed by ELISA using the supernatant of the co-incubation. IFNγ is the most common effector cytokine tested. Other cytokines of interest include IL-10, IL-2, and TGF-β. T cell stimulation is usually achieved through anti-CD3 and anti-CD28 antibodies. However, the type of stimulation varies between soluble, plate-bound or bead-bound antibodies. Each method has advantages and pitfalls. Soluble antibodies might not provide sufficient cross-linking of the TCR and CD28 to achieve T cell activation. Plate-bound antibodies might not provide homologous activation especially in round-bottom plates. Beads are ingested by several types of myeloid cells and therefore skew the accuracy of the observed suppression.

Large variations between studies are evident in the isolation methods of target cells and MDSC. Autologous and allogenic cells are used as target cells for suppression assays and include CD3+, CD8+, CD4+, PBMC and CD14-depleted PBMC. The method of isolation is usually magnetic bead sorting. Defined subpopulations of MDSC should be isolated after Bronte et al. for use in suppression assays [5]. Jordan et al. follows these guidelines well and uses splenocytes and blood samples from a variety of human carcinoma patients to isolate MDSC. The use of human splenocytes as a source of MDSC resulted in high yields of MDSC, but of course these cells are only available for research under special conditions. The suppression of T cell proliferation was assessed by CFSE dilution after four days of MDSC co-culture and compared to that elicited by HLA-DR + control cells. In this system, the percentage of T cells proliferating in non-suppressed control conditions was low (only 15%). CD14+ and CD15+ MDSC reduced the frequency of proliferating T cells to 12 and 10%, respectively. This was the only functional test performed [43]. However, as previously described, MDSC isolation protocols are highly variable between laboratories (Table 2).

Further suppressive effects of MDSC on T cells include the induction of Foxp3, increased PD-1 expression and downregulation of CD3ζ on T cells after co-culture with MDSC [42, 51]. All three markers by themselves are not definitive signs of T cell functional suppression. Increased PD-1 expression and TCR downregulation are known markers of T cell activation as well. The induction of regulatory T cells by MDSC is well-established in mice [22]. However, all human T cells are able to express Foxp3 if they are exposed to TGF-β. Consequently, Foxp3 expression alone does not indicate the presence of regulatory T cells in humans.

In vivo activity of human MDSC

Obtaining evidence for in vivo activity of human MDSC poses obvious challenges to the research community. As mentioned above, functional analyses are mostly performed ex vivo with MDSC freshly isolated from the peripheral blood or the pathologically affected and/or inflamed tissues [5]. On the other hand, establishing an accurate approach to monitor in situ actions of MDSC in human subjects is desirable. Currently, in conjunction with surface markers, analyses of certain immune regulatory molecules and metabolites which are directly or indirectly related to the MDSC functions (such as ARG1, NOS2, IDO, NO, ROS, prostaglandin-E2 (PGE2), PD-L1, TGF-β1, IL-10, S100A8/A9 and S100A12), are performed and presented as surrogate markers for the MDSC functionality in vivo. Biopsy, surgical excision specimens or serum samples are collected for this purpose. However, due to the limited accessibility of human tissue materials, the majority of the studies are performed with peripheral blood MDSC. Several technical drawbacks, such as small amounts of MDSC and the requirement for enzymatic degradation, may complicate or limit the assays which can be performed with MDSC from tissues [54, 55]. Moreover, variations in processing time, cell biological changes of MDSC during ex vivo manipulation, and the changes dictated by isolation procedures may influence the results.

In murine models, depletion of MDSC or adoptive transfer represent state-of-the-art assays to assess in vivo function of MDSC. Evidently, these approaches are not possible in humans because of the ethical concern. Nevertheless, a number of conventional therapeutics that are approved and conventionally used for the treatment of certain diseases has been shown to reduce MDSC levels. Low-dose chemotherapy, agents inducing myeloid maturation such as ATRA, and anti-inflammatory regimens modulate MDSC frequency and function in patients [56]. Alternatively, recombinant human G-CSF administered to healthy donors or cancer patients after myelodepletion enriches the peripheral blood hematopoietic stem cell pool which eventually leads to a considerable increase in the immature myeloid cells with regulatory actions compatible with those of MDSC [57]. However, these interventions often do not allow concluding on modulation of effector immune responses by MDSC.

An interesting alternative is the analysis of MDSC in humanized mouse models (HMM). For instance, NOD scid γ_c-deficient (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice engrafted with human CD34^{+} hematopoietic stem cells develop multi-lineage human immune cells which can serve as an efficient platform to study in vivo behavior of human immune cells [58]. In addition, MDSC-depleted PBMCs from systemic lupus erythematosus (SLE) patients, after transfer into humanized NOD/SCID mice with a SLE-like

Table 2 Variables in proliferation assays—human MDSCs

	Variable			References
Method	Tracking dye dilution (e.g., CFSE)			[38–42]
	BrdU incorporation			[36]
	Thymidine incorporation			[37]
T cells				
Source	Autologous PBMC			[37–39, 41, 42]
	Allogenic PBMC			[36, 37, 40]
Isolation	Positive magnetic bead sorting			[36, 38, 41, 42]
	Negative magnetic bead sorting			[39]
Phenotype	PBMC			[37, 40]
	CD3			[36, 38, 41, 42]
	CD8			[39]
State	Unknown/unclear			[42]
	Fresh			[37–41]
Cells/well	Unknown	[38–40]	2×10^5	[42]
	1×10^4	[37]	4×10^5	[36]
	1×10^5	[41]	5×10^5	[37]
MDSC				
Source	PBMC			[36–38]
	Splenocytes			[41]
	Derived in vitro from bone marrow cells			[40]
	Derived in vitro from healthy PBMC			[42]
	Cancer ascites			[39]
Isolation	Flow cytometry sorting			[36–38, 41]
	Magnetic bead sorting—CD11b + or CD33+			[36, 37, 39, 40, 42]
State	Fresh			[36–42]
	Frozen			None
Ratio T cells:MDSC	Unknown	[39]		
	1:4	[37]	4:1	[42]
	1:3	[36, 37]	5:1	[38]
	1:1	[36–38, 40, 41]	10:1	[38]
	2:1	[37, 42]	20:1	[38]
Stimulus				
Antibodies	Anti-CD3	Coated	1 μg/ml	[40]
	Anti-CD28	Soluble	5 μg/ml	[40]
Beads	CD3/CD28 Dynabeads $CD3^{-}CD28^{-}CD2^{-}$biotinylated beads			[36, 38, 39, 42]
Ratio (:T cells)	Unknown	[39, 42]	1:1; 1,5:1	[36]

Table 2 (continued)

	Variable			References
IL-2	100 U/ml, 500 IU/ml			[36, 42]
MLR	4×10^4 monocyte-derived DC from healthy donor, allogenic PBMC			[41]
Medium	Unknown			[38, 39, 41, 42]
	RPMI + fetal calf serum			[36, 40]
	RPMI + human serum			[37]
Days of incubation	3	[36, 37, 42]	5	[39]
	4	[39–41]	7	[38]
Tissue culture plate	Unknown	[37–39, 41]	96-well, flat	[40]
	96-well	[42]	24-well, flat	[36]
Activation controls	Medium			[42]
	Absence of MDSC			[36, 38–42]
	Patient peripheral blood-derived CD33+			[36]
	Healthy donor peripheral blood-derived CD33+			[36]
	HLA-DR + myeloid cells			[37, 41]
Suppression controls	Unknown			[36, 37, 39] or none

syndrome, mitigate the disease and provided evidence for in vivo functions of human MDSC [59]. In the future, mice encoding human cytokines (i.e., MITRG and MISTRG) could also help to better understand human MDSC functionality in vivo [58]. Non-invasive in vivo imaging technologies such as micro positron emission tomography (μPET), single-photon emission computed tomography (SPECT) and optical imaging systems that would employ functional and molecular imaging agents designed for tracing MDSC would be useful in humanized mouse models [60, 61].

Although the targeting is not always MDSC-specific, 45 clinical trials related to MDSC targeting or monitoring are registered (https://clinicaltrials.gov). We believe that these studies will help to better elucidate in vivo functions of MDSC in human diseases.

Critical issues and controls

The design of functional assays for MDSC suppression requires the careful consideration of several critical issues, most importantly the phenotyping and isolation of cells, cell viability and integrity, and finally the use of appropriate activation and suppression controls. Human MDSC in particular display heterogeneous phenotypes and require complex flow cytometry strategies for identification and isolation. Reviews that aim at standardizing MDSC phenotyping should be consulted and adopted for both phenotyping and isolation strategies. Until unique MDSC marker candidates, which correlate with function, are further validated and confirmed, at least for human PMN–MDSC, density gradient isolations remain necessary to separate MDSC from neutrophils [62, 63].

The complexity of isolation strategies raises further complications:

- *Yield*. MDSC are rare and sensitive cells. Complex multiple step isolation protocols could reduce already low yields. This greatly impacts the range of functional assays performable with one sample and reproducibility.
- *Cell viability*. MDSC, in particular PMN-MDSC are cryosensitive [64] and do not survive long ex vivo. This could impact yields and multiple day assays. Cells should be processed quickly and monitored with viability dyes.
- *Activation/Modulation*. Immature myeloid cells and neutrophils readily respond to physical stress. Cooling, centrifugation, resuspension and sorting could all activate MDSC and neutrophils in a way that does not represent their in vivo state. Gentle handling and sorting settings as well as stable temperatures should be used at all times.

The second critical issue is the use of appropriate controls. Studies frequently lack relevant controls for comparison:

- *Flow cytometric analyses*, e.g., of NOS2 or ROS presence, should be compared to monocytes and macrophages. Comparing the MDSC data to unstained controls only indicates the presence of the analyzed parameter, but not the degree of expression. For example, in qRT-PCR and Western blotting ARG1 and NOS2 expression can be compared to that in monocytes, and M1 or M2 macrophages.
- *Biological functional controls*. Most assays compare the functional activity of stimulated T cells in the absence or presence of MDSC. However, activating and suppressing biological control cells should be considered to improve reliability of such assays. Activating controls could be HLA-DR + monocytes. Suppressive controls are not reported and it is difficult to identify a reliably suppressive and comparable myeloid-derived cell type. In vitro generated immunosuppressive dendritic cells (DC-10 s) are under investigation for this role within the Mye-EUNITER network [65].
- *Target cells*. MDSC may have different effects on T cells versus other target cells. NK cells, for example, are important contributors to the elimination of malignancies. MDSC suppress NK cell and T cell functions through similar mechanisms that prominently involve the expression of ARG1 and NO. However, besides the suppression of cytokine production and proliferation, Fc receptor- and IL2-activated NK cells were also suppressed in their killing activity and intracellular signaling pathways [66–68]. Although discussing this aspect in detail is outside the scope of this review, the possible implications should not be underestimated. Similarly effector functions such as proliferation, cytotoxicity and cytokine release may not be equally affected by MDSC for a given target cell. As such the selection of the test system is a complex and probably underestimated issue in the field.

Conclusions and future perspectives

MDSC evolve as a result of pathological expansion, differentiation and functional modulation of myeloid cells. Consequently, MDSC share immunophenotypic markers with other myeloid cells such as monocytes, macrophages and granulocytes. At present, unique validated MDSC markers are not yet available. Thus, MDSC cannot be identified by immunophenotyping only and functional assays are mandatory for their appropriate characterization (see Table 1). Historically, the suppression of T cell activation emerged as the most often studied MDSC functional property. However, MDSC feature additional immune suppressive as well as disease-promoting properties. At present, it is not well-understood what regulates bona fide suppression versus other disease-promoting properties.

Despite these uncertainties, the suppression of T cell activity appears to be a key assay for defining MDSC identity and is most often measured in vitro after MDSC isolation. Currently, the functional activity of MDSC is difficult to compare between laboratories, experimental models and different cohorts of patients with disease. Careful standardization and optimization of MDSC immunophenotyping, isolation and functional testing is required to better appreciate the clinical relevance especially of human MDSC. Surrogate markers of suppression could be useful for in vivo assessment of MDSC in patient tissue and blood, but it will be important to correlate expression of these markers with target cell function and disease follow-up in future studies.

With further improvements in immunomonitoring and functional analysis, in the future, we should be able to fully appreciate the role of MDSC in experimental disease models as well as their emerging clinical relevance.

Acknowledgements We thank all members of Mye-EUNITER for contributions and discussions during the preparation of this manuscript. We also thank all members of the Esendagli laboratory at Hacettepe University Cancer Institute for their help with the quantitative and qualitative analysis of the literature.

Author contributions AMB and SB conceptualized the review. All authors contributed to the writing and editing of the review. All authors approved the final version.

Funding This work was supported by COST (European Cooperation in Science and Technology) and the COST Action BM1404 Mye-EUNITER (http://www.mye-euniter.eu). COST is part of the EU Framework Programme Horizon 2020.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Pradeu T, Cooper EL (2012) The danger theory: 20 years later. Front Immunol 3:287. https://doi.org/10.3389/fimmu.2012.00287
2. Libby P (2007) Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutr Rev 65:S140-6
3. Iqbal AJ, Fisher EA, Greaves DR (2016) Inflammation-a critical appreciation of the role of myeloid cells. Microbiol Spectr. https://doi.org/10.1128/microbiolspec.MCHD-0027-2016
4. Gabrilovich DI (2017) Myeloid-derived suppressor cells. Cancer Immunol Res 5:3–8. https://doi.org/10.1158/2326-6066.CIR-16-0297
5. Bronte V, Brandau S, Chen S-H, Colombo MP, Frey AB, Greten TF, Mandruzzato S, Murray PJ, Ochoa A, Ostrand-Rosenberg S, Rodriguez PC, Sica A, Umansky V, Vonderheide RH, Gabrilovich DI (2016) Recommendations for myeloid-derived

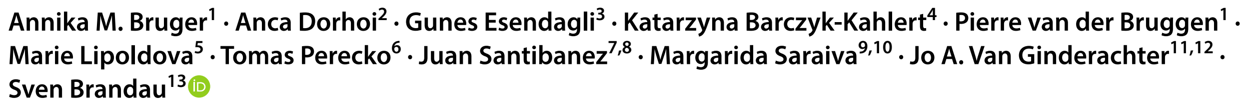
suppressor cell nomenclature and characterization standards. Nat Commun 7:12150. https://doi.org/10.1038/ncomms12150
6. Haile LA, Greten TF, Korangy F (2012) Immune suppression: the hallmark of myeloid derived suppressor cells. Immunol Invest 41:581–594. https://doi.org/10.3109/08820139.2012.680635
7. Weber J, Gibney G, Kudchadkar R, Yu B, Cheng P, Martinez AJ, Kroeger J, Richards A, McCormick L, Moberg V, Cronin H, Zhao X, Schell M, Chen YA (2016) Phase I/II Study of metastatic melanoma patients treated with nivolumab who had progressed after ipilimumab. Cancer Immunol Res 4:345–353. https://doi.org/10.1158/2326-6066.CIR-15-0193
8. de Coana YP, Wolodarski M, Poschke I, Yoshimoto Y, Yang Y, Nystrom M, Edback U, Brage SE, Lundqvist A, Masucci GV, Hansson J, Kiessling R (2017) Ipilimumab treatment decreases monocytic MDSCs and increases CD8 effector memory T cells in long-term survivors with advanced melanoma. Oncotarget 8:21539–21553. https://doi.org/10.18632/oncotarget.15368
9. Chesney JA, Mitchell RA, Yaddanapudi K (2017) Myeloid-derived suppressor cells-a new therapeutic target to overcome resistance to cancer immunotherapy. J Leukoc Biol 102:727–740. https://doi.org/10.1189/jlb.5VMR1116-458RRR
10. Monu NR, Frey AB (2012) Myeloid-derived suppressor cells and anti-tumor T cells: a complex relationship. Immunol Invest 41:595–613. https://doi.org/10.3109/08820139.2012.673191
11. Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S (2010) Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. Cancer Res 70:68–77. https://doi.org/10.1158/0008-5472.CAN-09-2587
12. Raber PL, Thevenot P, Sierra R, Wyczechowska D, Halle D, Ramirez ME, Ochoa AC, Fletcher M, Velasco C, Wilk A, Reiss K, Rodriguez PC (2014) Subpopulations of myeloid-derived suppressor cells impair T cell responses through independent nitric oxide-related pathways. Int J Cancer 134:2853–2864. https://doi.org/10.1002/ijc.28622
13. Youn J-I, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI (2012) Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. J Leukoc Biol 91:167–181. https://doi.org/10.1189/jlb.0311177
14. Liu C, Yu S, Kappes J, Wang J, Grizzle WE, Zinn KR, Zhang H-G (2007) Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. Blood 109:4336–4342. https://doi.org/10.1182/blood-2006-09-046201
15. Knaul JK, Jörg S, Oberbeck-Mueller D, Heinemann E, Scheuermann L, Brinkmann V, Mollenkopf H-J, Yeremeev V, Kaufmann SHE, Dorhoi A (2014) Lung-residing myeloid-derived suppressors display dual functionality in murine pulmonary tuberculosis. Am J Respir Crit Care Med 190:1053–1066. https://doi.org/10.1164/rccm.201405-0828OC
16. Li H, Han Y, Guo Q, Zhang M, Cao X (2009) Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. J Immunol 182:240–249
17. Rieber N, Singh A, Öz H, Carevic M, Bouzani M, Amich J, Ost M, Ye Z, Ballbach M, Schäfer I, Mezger M, Klimosch SN, Weber ANR, Handgretinger R, Krappmann S, Liese J, Engeholm M, Schüle R, Salih HR et al (2015) Pathogenic fungi regulate immunity by inducing neutrophilic myeloid-derived suppressor cells. Cell Host Microbe 17:507–514. https://doi.org/10.1016/j.chom.2015.02.007
18. Gabrilovich DI, Nagaraj S (2009) Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 9:162–174. https://doi.org/10.1038/nri2506
19. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI (2004) Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol 172:989–999
20. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J, Gabrilovich DI (2007) Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat Med 13:828–835. https://doi.org/10.1038/nm1609
21. Youn J-I, Nagaraj S, Collazo M, Gabrilovich DI (2008) Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 181:5791–5802
22. Huang B, Pan P-Y, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen S-H (2006) Gr-1+ CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res 66:1123–1131. https://doi.org/10.1158/0008-5472.CAN-05-1299
23. Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S (2009) Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells. J Immunol 183:937–944. https://doi.org/10.4049/jimmunol.0804253
24. Schmid M, Zimara N, Wege AK, Ritter U (2014) Myeloid-derived suppressor cell functionality and interaction with Leishmania major parasites differ in C57BL/6 and BALB/c mice. Eur J Immunol 44:3295–3306. https://doi.org/10.1002/eji.201344335
25. Su N, Yue Y, Xiong S (2016) Monocytic myeloid-derived suppressor cells from females, but not males, alleviate CVB3-induced myocarditis by increasing regulatory and CD4(+)IL-10(+) T cells. Sci Rep 6:22658. https://doi.org/10.1038/srep22658
26. Carretero-Iglesia L, Bouchet-Delbos L, Louvet C, Drujont L, Segovia M, Merieau E, Chiffoleau E, Josien R, Hill M, Cuturi M-C, Moreau A (2016) Comparative study of the immunoregulatory capacity of in vitro generated tolerogenic dendritic cells, suppressor macrophages, and myeloid-derived suppressor cells. Transplantation 100:2079–2089. https://doi.org/10.1097/TP.0000000000001315
27. Sierra RA, Thevenot P, Raber PL, Cui Y, Parsons C, Ochoa AC, Trillo-Tinoco J, Del Valle L, Rodriguez PC (2014) Rescue of notch-1 signaling in antigen-specific CD8+ T cells overcomes tumor-induced T-cell suppression and enhances immunotherapy in cancer. Cancer Immunol Res 2:800–811. https://doi.org/10.1158/2326-6066.CIR-14-0021
28. Corzo CA, Condamine T, Lu L, Cotter MJ, Youn J-I, Cheng P, Cho H-I, Celis E, Quiceno DG, Padhya T, McCaffrey TV, McCaffrey JC, Gabrilovich DI (2010) HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. J Exp Med 207:2439–2453. https://doi.org/10.1084/jem.20100587
29. Bronte V, Wang M, Overwijk WW, Surman DR, Pericle F, Rosenberg SA, Restifo NP (1998) Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. J Immunol 161:5313–5320
30. Moses K, Klein JC, Männ L, Klingberg A, Gunzer M, Brandau S (2016) Survival of residual neutrophils and accelerated myelopoiesis limit the efficacy of antibody-mediated depletion of Ly-6G+ cells in tumor-bearing mice. J Leukoc Biol 99:811–823. https://doi.org/10.1189/jlb.1HI0715-289R
31. Clavijo PE, Moore EC, Chen J, Davis RJ, Friedman J, Kim Y, Van Waes C, Chen Z, Allen CT (2017) Resistance to CTLA-4 checkpoint inhibition reversed through selective elimination of granulocytic myeloid cells. Oncotarget 8:55804–55820. https://doi.org/10.18632/oncotarget.18437
32. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, Martin F, Apetoh L, Rébé C, Ghiringhelli F (2010) 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res 70:3052–3061. https://doi.org/10.1158/0008-5472.CAN-09-3690
33. Highfill SL, Cui Y, Giles AJ, Smith JP, Zhang H, Morse E, Kaplan RN, Mackall CL (2014) Disruption of CXCR2-mediated MDSC

tumor trafficking enhances anti-PD1 efficacy. Sci Transl Med 6:237ra67. https://doi.org/10.1126/scitranslmed.3007974

34. Nefedova Y, Fishman M, Sherman S, Wang X, Beg AA, Gabrilovich DI (2007) Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. Cancer Res 67:11021–11028. https://doi.org/10.1158/0008-5472.CAN-07-2593
35. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I (2006) Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med 203:2691–2702. https://doi.org/10.1084/jem.20061104
36. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, Melani C, Guiducci C, Colombo MP, Iezzi M, Musiani P, Zanovello P, Bronte V (2005) Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. Proc Natl Acad Sci USA 102:4185–4190. https://doi.org/10.1073/pnas.0409783102
37. Nagaraj S, Youn J-I, Weber H, Iclozan C, Lu L, Cotter MJ, Meyer C, Becerra CR, Fishman M, Antonia S, Sporn MB, Liby KT, Rawal B, Lee J-H, Gabrilovich DI (2010) Anti-inflammatory triterpenoid blocks immune suppressive function of MDSCs and improves immune response in cancer. Clin Cancer Res 16:1812–1823. https://doi.org/10.1158/1078-0432.CCR-09-3272
38. Yu J, Du W, Yan F, Wang Y, Li H, Cao S, Yu W, Shen C, Liu J, Ren X (2013) Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. J Immunol 190:3783–3797. https://doi.org/10.4049/jimmunol.1201449
39. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Krüger C, Manns MP, Greten TF, Korangy F (2008) A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. Gastroenterology 135:234–243. https://doi.org/10.1053/j.gastro.2008.03.020
40. Brandau S, Trellakis S, Bruderek K, Schmaltz D, Steller G, Elian M, Suttmann H, Schenck M, Welling J, Zabel P, Lang S (2011) Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. J Leukoc Biol 89:311–317. https://doi.org/10.1189/jlb.0310162
41. Obermajer N, Muthuswamy R, Lesnock J, Edwards RP, Kalinski P (2011) Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. Blood 118:5498–5505. https://doi.org/10.1182/blood-2011-07-365825
42. Pinton L, Solito S, Damuzzo V, Francescato S, Pozzuoli A, Berizzi A, Mocellin S, Rossi CR, Bronte V, Mandruzzato S (2016) Activated T cells sustain myeloid-derived suppressor cell-mediated immune suppression. Oncotarget 7:1168–1184. https://doi.org/10.18632/oncotarget.6662
43. Jordan KR, Kapoor P, Spongberg E, Tobin RP, Gao D, Borges VF, McCarter MD (2017) Immunosuppressive myeloid-derived suppressor cells are increased in splenocytes from cancer patients. Cancer Immunol Immunother 66:503–513. https://doi.org/10.1007/s00262-016-1953-z
44. Lechner MG, Liebertz DJ, Epstein AL (2010) Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. J Immunol 185:2273–2284. https://doi.org/10.4049/jimmunol.1000901
45. Mandruzzato S, Brandau S, Britten CM, Bronte V, Damuzzo V, Gouttefangeas C, Maurer D, Ottensmeier C, van der Burg SH, Welters MJP, Walter S (2016) Toward harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study. Cancer Immunol Immunother 65:161–169. https://doi.org/10.1007/s00262-015-1782-5
46. Dumitru CA, Moses K, Trellakis S, Lang S, Brandau S (2012) Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology. Cancer Immunol Immunother 61:1155–1167. https://doi.org/10.1007/s00262-012-1294-5
47. Kusmartsev S, Nagaraj S, Gabrilovich DI (2005) Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. J Immunol 175:4583–4592
48. Heuvers ME, Muskens F, Bezemer K, Lambers M, Dingemans A-MC, Groen HJM, Smit EF, Hoogsteden HC, Hegmans JPJJ., Aerts JGJV. (2013) Arginase-1 mRNA expression correlates with myeloid-derived suppressor cell levels in peripheral blood of NSCLC patients. Lung Cancer 81:468–474. https://doi.org/10.1016/j.lungcan.2013.06.005
49. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, Gilbert J, Ochoa AC (2005) Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med 202:931–939. https://doi.org/10.1084/jem.20050715
50. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, Ochoa AC (2009) Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res 69:1553–1560. https://doi.org/10.1158/0008-5472.CAN-08-1921
51. Liu C-Y, Wang Y-M, Wang C-L, Feng P-H, Ko H-W, Liu Y-H, Wu Y-C, Chu Y, Chung F-T, Kuo C-H, Lee K-Y, Lin S-M, Lin H-C, Wang C-H, Yu C-T, Kuo H-P (2010) Population alterations of l-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14−/CD15+/CD33+ myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. J Cancer Res Clin Oncol 136:35–45. https://doi.org/10.1007/s00432-009-0634-0
52. Toor SM, Syed Khaja AS, El Salhat H, Bekdache O, Kanbar J, Jaloudi M, Elkord E (2016) Increased levels of circulating and tumor-infiltrating granulocytic myeloid cells in colorectal cancer patients. Front Immunol 7:560. https://doi.org/10.3389/fimmu.2016.00560
53. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL (1999) Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med 189:1363–1372
54. Toor SM, Syed Khaja AS, El Salhat H, Faour I, Kanbar J, Quadri AA, Albashir M, Elkord E (2017) Myeloid cells in circulation and tumor microenvironment of breast cancer patients. Cancer Immunol Immunother 66:753–764. https://doi.org/10.1007/s00262-017-1977-z
55. Cao LY, Chung J-S, Teshima T, Feigenbaum L, Cruz PD, Jacobe HT, Chong BF, Ariizumi K (2016) Myeloid-derived suppressor cells in psoriasis are an expanded population exhibiting diverse T-cell-suppressor mechanisms. J Invest Dermatol 136:1801–1810. https://doi.org/10.1016/j.jid.2016.02.816
56. Wesolowski R, Markowitz J, Carson WE (2013) Myeloid derived suppressor cells—a new therapeutic target in the treatment of cancer. J Immunother Cancer 1:10. https://doi.org/10.1186/2051-1426-1-10
57. Luyckx A, Schouppe E, Rutgeerts O, Lenaerts C, Fevery S, Devos T, Dierickx D, Waer M, Van Ginderachter JA, Billiau AD (2012) G-CSF stem cell mobilization in human donors induces polymorphonuclear and mononuclear myeloid-derived suppressor cells. Clin Immunol 143:83–87. https://doi.org/10.1016/j.clim.2012.01.011
58. Walsh NC, Kenney LL, Jangalwe S, Aryee K-E, Greiner DL, Brehm MA, Shultz LD (2017) Humanized mouse models of clinical disease. Annu Rev Pathol 12:187–215. https://doi.org/10.1146/annurev-pathol-052016-100332
59. Wu H, Zhen Y, Ma Z, Li H, Yu J, Xu Z-G, Wang X-Y, Yi H, Yang Y-G (2016) Arginase-1-dependent promotion of TH17

differentiation and disease progression by MDSCs in systemic lupus erythematosus. Sci Transl Med 8:331ra40. https://doi.org/10.1126/scitranslmed.aae0482
60. Liu G, Hu Y, Xiao J, Li X, Li Y, Tan H, Zhao Y, Cheng D, Shi H (2016) 99mTc-labelled anti-CD11b SPECT/CT imaging allows detection of plaque destabilization tightly linked to inflammation. Sci Rep 6:20900. https://doi.org/10.1038/srep20900
61. Eisenblaetter M, Flores-Borja F, Lee JJ, Wefers C, Smith H, Hueting R, Cooper MS, Blower PJ, Patel D, Rodriguez-Justo M, Milewicz H, Vogl T, Roth J, Tutt A, Schaeffter T, Ng T (2017) Visualization of tumor-immune interaction—target-specific imaging of S100A8/A9 reveals pre-metastatic niche establishment. Theranostics 7:2392–2401. https://doi.org/10.7150/thno.17138
62. Moses K, Brandau S (2016) Human neutrophils: their role in cancer and relation to myeloid-derived suppressor cells. Semin Immunol 28:187–196. https://doi.org/10.1016/j.smim.2016.03.018
63. Condamine T, Dominguez GA, Youn J-I, Kossenkov AV, Mony S, Alicea-Torres K, Tcyganov E, Hashimoto A, Nefedova Y, Lin C, Partlova S, Garfall A, Vogl DT, Xu X, Knight SC, Malietzis G, Lee GH, Eruslanov E, Albelda SM et al (2016) Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. Sci Immunol 1:aaf8943. https://doi.org/10.1126/sciimmunol.aaf8943
64. Trellakis S, Bruderek K, Hütte J, Elian M, Hoffmann TK, Lang S, Brandau S (2013) Granulocytic myeloid-derived suppressor cells are cryosensitive and their frequency does not correlate with serum concentrations of colony-stimulating factors in head and neck cancer. Innate Immun 19:328–336. https://doi.org/10.1177/1753425912463618
65. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, Hauben E, Roncarolo M-G (2010) Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. Blood 116:935–944. https://doi.org/10.1182/blood-2009-07-234872
66. Stiff A, Trikha P, Mundy-Bosse BL, McMichael EL, Mace TA, Benner B, Kendra K, Campbell A, Gautam S, Abood D, Landi I, Hsu V, Duggan MC, Wesolowski R, Old M, Howard JH, Yu L, Stasik N, Olencki T et al (2018) Nitric oxide production by myeloid derived suppressor cells plays a role in impairing Fc receptor-mediated natural killer cell function. Clin Cancer Res. https://doi.org/10.1158/1078-0432.CCR-17-0691
67. Mao Y, Sarhan D, Steven A, Seliger B, Kiessling R, Lundqvist A (2014) Inhibition of tumor-derived prostaglandin-e2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity. Clin Cancer Res 20:4096–4106. https://doi.org/10.1158/1078-0432.CCR-14-0635
68. Goh CC, Roggerson KM, Lee H-C, Golden-Mason L, Rosen HR, Hahn YS (2016) Hepatitis C virus-induced myeloid-derived suppressor cells suppress NK Cell IFN-γ production by altering cellular metabolism via arginase-1. J Immunol 196:2283–2292. https://doi.org/10.4049/jimmunol.1501881

Affiliations


Annika M. Bruger[1] · Anca Dorhoi[2] · Gunes Esendagli[3] · Katarzyna Barczyk-Kahlert[4] · Pierre van der Bruggen[1] · Marie Lipoldova[5] · Tomas Perecko[6] · Juan Santibanez[7,8] · Margarida Saraiva[9,10] · Jo A. Van Ginderachter[11,12] · Sven Brandau[13]

1 de Duve Institute, Université catholique de Louvain, Avenue Hippocrate 74, 1200 Brussels, Belgium

2 Institute of Immunology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany and Faculty of Mathematics and Natural Sciences, University of Greifswald, Greifswald, Germany

3 Department of Basic Oncology, Hacettepe University Cancer Institute, Ankara, Turkey

4 Institute of Immunology, University of Münster, Münster, Germany

5 Laboratory of Molecular and Cellular Immunology, Institute of Molecular Genetics AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic

6 Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dubravska cesta 9, 841 04 Bratislava, Slovak Republic

7 Molecular Oncology group, Institute for Medical Research, University of Belgrade, Belgrade, Republic of Serbia

8 Centro Integrativo de Biología y Química Aplicada (CIBQA), Universidad Bernardo O'Higgins, Santiago, Chile

9 Instituto de Investigação e Inovação em Saúde, University of Porto, Porto, Portugal

10 Instituto de Biologia Molecular e Celular, University of Porto, Porto, Portugal

11 Cellular and Molecular Immunology Lab, Vrije Universiteit Brussel, Brussels, Belgium

12 Myeloid Cell Immunology Lab, VIB Center for Inflammation Research, Brussels, Belgium

13 Research Division, Department of Otorhinolaryngology, West German Cancer Center, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany