Arch. Immunol. Ther. Exp. (2018) 66:113–123
DOI 10.1007/s00005-017-0492-4

REVIEW

Myeloid-Derived Suppressor Cells in the Tumor Microenvironment: Current Knowledge and Future Perspectives

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Received: 12 April 2017 / Accepted: 5 September 2017 / Published online: 14 October 2017

Abstract The current knowledge on tumor-infiltrating myeloid-derived suppressor cells (MDSCs) is based mainly on the extensive work performed in murine models. Data obtained for human counterparts are generated on the basis of tumor analysis from patient samples. Both sources of information led to determination of the main suppressive mechanisms used by these cell subsets in tumor-bearing hosts. As a result of the identification of protein targets responsible for MDSCs suppressive activity, different therapeutics agents have been used to eliminate/reduce their adverse effect. In the present work, we review the current knowledge on suppressive mechanisms of MDSCs and therapeutic treatments that interfere with their differentiation, expansion or activity. Based on the accumulation of new evidences supporting their importance for tumor progression and metastasis, the interest in these cell types is increasing. We revise the methods of MDSC generation/differentiation *ex vivo* that may help in overcoming problems associated with limited numbers of cells available from animals and patients for their study.

Keywords Myeloid-derived suppressor cells (MDSCs) · Immunosuppression · Myeloid regulatory cells · Tumor progression · Metastasis · Immunotherapy

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Introduction

The presence of myeloid cells with suppressive activity within tumors was first observed in the early 1960s. These were characterized as immature cells, lacking the expression of lineage markers specific for B cells, T cells, natural killer (NK) cells or macrophages. Therefore, they could not be classified and were called null cells (Duwe and Singhal 1979a, b; Slavin and Strober 1979). Nevertheless, it was evident that the accumulation of myeloid cells within tumors was a sign of poor prognosis. The initial observations on the inhibition of $CD8^+$ cytotoxic lymphocytes in immunocompetent hosts by these myeloid cells were made independently by Tsuchiya et al. (1988) and Bronte et al. (1998, 1999) while developing therapeutic anticancer vaccines. In 2007, those natural suppressive cells were finally tagged as myeloid-derived suppressor cells (MDSCs) (Gabrilovich et al. 2007).

MDSCs represent a heterologous population of immature myeloid cells which accumulate during tumor progression as a result of chronic inflammation caused by numerous soluble and exosome-bound factors produced by tumors. In murine tumor models, MDSCs were defined by the expression of CD11b and Gr1 surface markers. Further studies resulted in the distinction of monocytic M-MDSCs and granulocytic G-MDSCs subsets characterized by the expression of $CD11b^+$ $Ly6G^-$, $Ly6C^{hi}$ and $CD11b^+$ $Ly6G^+$, $Ly6C^{lo}$, respectively. While some investigators assume that these subsets arise from different differentiation pathways, or even myeloid lineages, the results of Corzo and colleagues confirmed by Lliechtenstein and colleagues demonstrated that G-MDSCs are the result of M-MDSCs maturation (Corzo et al. 2010; Liechtenstein et al. 2014).

However, human MDSCs are certainly different and not that easy to classify. In humans, Gr-1 is absent and the

division on monocytic and granulocytic subsets is based on the following expression profiles: $CD11b^+$, $CD33^+$, $CD14^+$, $CD15^-$ $HLA\text{-}DR^{-/lo}$, (M-MDSCs) and $CD11b^+$, $CD33^+$, $CD14^-$, $CD15^+$ $HLA\text{-}DR^{-/lo}$ $CD66b^+$ for human G-MDSCs, respectively. Because of the use of differing antibody panels in a wide variety of experimental systems, different research groups propose additional markers to describe human MDSC (reviewed by Elliott et al. 2017; Talmadge and Gabrilovich 2013). Murine MDSC differentiation from bone marrow (BM) precursors can be easily achieved by numerous published protocols, so their characterization is not a challenging task. For the human counterparts, the starting material is frequently peripheral blood mononuclear cells (PBMCs). This is likely the reason why the efficiency of MDSCs differentiation using most of the published protocols is still very poor. Therefore, the detailed differentiation and characterization of human MDSCs populations is still at very early stages.

Suppressive Mechanisms of MDSC

The term MDSCs represents cell populations that either create or maintain suppressive conditions in the tumor microenvironment. These cells mediate a potent suppression of the immune system through different mechanisms (summarized in Fig. 1). The best characterized of these are the depletion of essential amino acids from the extracellular medium [through the activity of arginase (ARG1), inducible nitric synthase (iNOS), and indoleamine 2,3-dioxygenase 1 (IDO)], generation of NO and ROS [through the activity of iNOs and NADPH oxidase (NOX2)] and finally production of high levels of anti-inflammatory cytokines, such as the classical cytokines transforming growth factor (TGF)-β and interleukin (IL)-10 amongst other immunosuppressive factors. Other mechanisms of T cell suppression include expression of ADAM17 and galectin 9 that affects T cell maturation and cause apoptosis, respectively.

Nutrient Depletion

L-Arginine

MDSCs express arginase 1 and nitric oxide synthase 2 (Nos2 or iNOS)—enzymes that use L-arginine as a substrate. Arginase 1 converts arginine in urea and L-ornitine while iNOS generates high levels of NO and L-citrulline. The result of their enzymatic activities is the depletion of L-arginine from the tumor microenvironment. Shortage of L-arginine blocks proliferation of T cells, downregulation of the T cell receptor (TCR) component CD3ζ expression and arrest of the cell cycle in G0 to G1 phase that is associated with an inability of T cells to up-regulate cyclin D3 and cyclin-dependent kinase 4 (Rodriguez et al. 2007). Depletion of arginine levels also affects interferon (IFN)-γ production by NK cells as recently demonstrated in a model of hepatitis C virus infection (Goh et al. 2016).

L-Cysteine

T cells are deficient in the cystathionase enzyme responsible for conversion of methionine to cysteine. In addition, they cannot import cystine and reduce it to cysteine since they lack an intact xc-transporter. As a consequence, T cells do really depend on cysteine uptake from the extracellular medium generated by macrophages and dendritic cells (DCs). MDSCs import cystine but do not export cysteine, so that they deplete in this way the levels of this amino acid, blocking T cell activation and causing their proliferative arrest (Srivastava et al. 2010).

L-Tryptophan

Another enzyme overexpressed in MDSCs is IDO. IDO is the first enzyme in the tryptophan degradation pathway. Overexpression of IDO causes accumulation of tryptophan degradation metabolites—such as kynurerine metabolites—that have a potent immunomodulatory effect (Belladonna et al. 2009). Low levels of tryptophan results in inhibition of the mTOR kinase pathway that regulates immune responses, and activation of GCN2 pathway, which acts upon amino acid deficiency (Munn et al. 2005). GCN2 kinase phosphorylates and inhibits the translation initiation factor 2α, and causes as a consequence the arrest of protein synthesis and cell growth. Additionally, the activity of IDO enhances the differentiation of regulatory T cells (Tregs) from naïve T cells as tryptophan starvation and exposure to tryptophan catabolites induce the regulatory $FOXP3^+$ phenotype (Fallarino et al. 2006). Moreover, the activity of IDO on pre-existing Tregs enhances their suppressive activity and protects them from reprogramming into T-helper cells (Baban et al. 2009; Sharma et al. 2007, 2009).

Oxidative Stress

Reactive Oxygen and Nitrogen Species

The important role of iNOS, ARG1 and NADPH oxidase (NOX2) expressed by different subsets of MDSCs regulate different elements of the T cell response. While M-MDSCs exert their function through iNOS and ARG1, G-MDSCs are characterized by NOX2 expression and generation of high reactive oxygen species (ROS) levels. These nitrogen and oxygen reactive species affect the tumor microenvironment in multiple ways. They can act directly or indirectly. NO can react with the superoxide ion (O_2^-) to form peroxynitrites

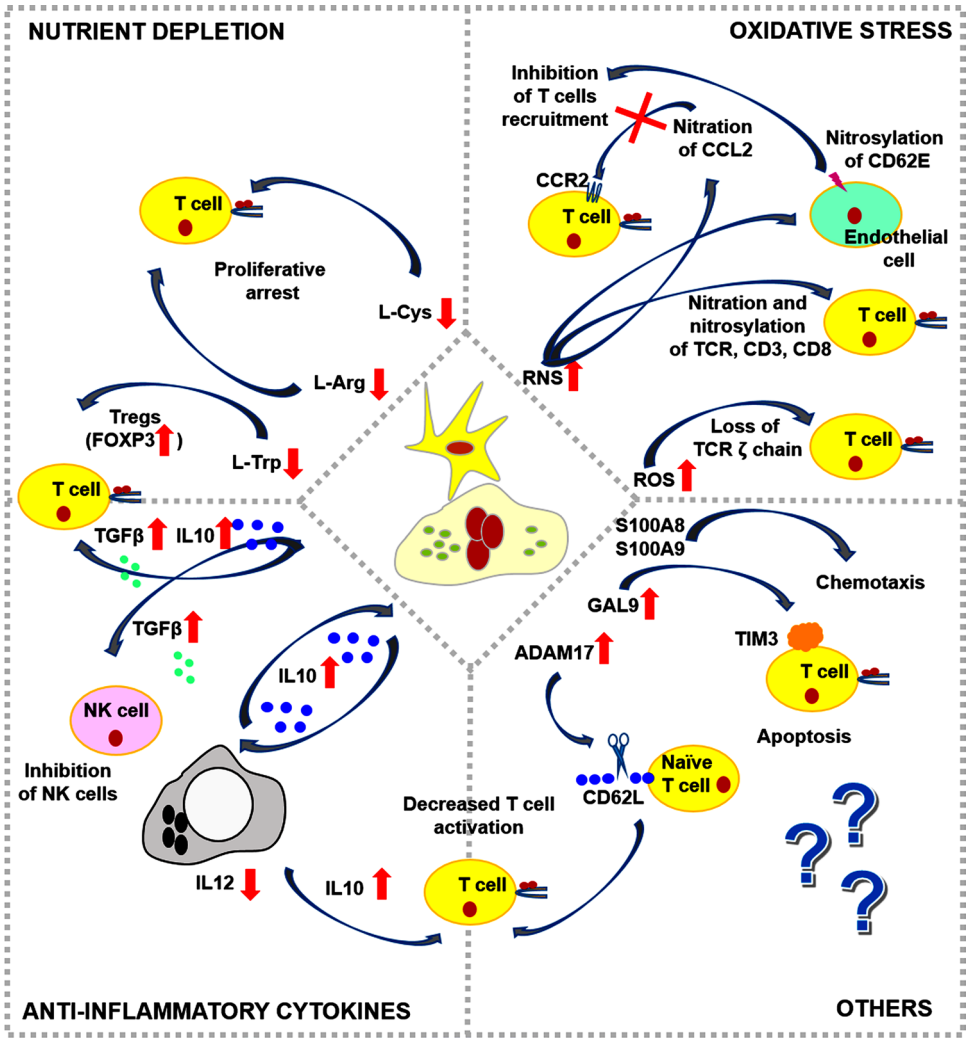

Fig. 1 Mechanisms of myeloid-derived suppressor cells (MDSCs) inhibition of effector cells of immune system. MDSCs affect the anti-tumor action of the immune system using several mechanisms. Nutrient depletion: MDSCs cause amino acid (Arg, Trp and Cys) depletion and as a result they inhibit T cell proliferation and enhance the development of FOXP3-positive regulatory T cells. Oxidative stress: Generation of reactive nitrogen species (RNS) results in nitration of CCL2 that is required for T cell recruitment to the tumor site. Nitrosylation of CD62E on the surface of endothelial cells is another mechanism that blocks attraction of T cells to the tumor site. Nitration and nitrosylation of CD3, CD8 and TCR affect effector function of T cells. Generation of high levels of ROS causes loss of TCR and as a result it again affects the effector function of T cells. Anti-inflammatory cytokines: Generation of TGF-β and IL-10 favors the establishment of Treg phenotype. TGF-β affects the generation of IFN-γ by NK cells. High levels of IL-10 induce M2 macrophage phenotype and upregulation of IL10 production by this tumor-infiltrating subset. At the same time, IL-12 production by macrophages is downmodulated. This change in the cytokine milieu disturbs activation of T cells. Other suppressive mechanisms: Among other well-characterized proteins produced by MDSCs are S100A8 and A100A9. These proteins are responsible for attraction of MDSCs into tumor sites and increase their suppressive phenotype. The proteolytic activity of ADAM17 reduces CD62L levels on the surface of T cells – CD62L is a molecule required for homing of T cells to proximal lymphatic nodes and their activation. Galectin 9 on the surface of MDSCs interacts with TIM3 on the surface of T cells resulting in their apoptosis. There are also numerous mechanisms still to be discovered, that will come to surface with the progress of investigation in the field of MDSCs

that are one of the strongest oxidants generated in the organism. The inhibitory effect of NO on MHCII expression was described by Grimm et al. (2002). In addition, it has been shown by Nagaraj et al. (2007) that the production of ROS and reactive nitrogen species by MDSC caused the nitration of TCR-tyrosines affecting $CD8^+$ T cell responsiveness. Nitration of CCl2 also affects the infiltration of T cells in tumor microenvironment (Molon et al. 2011). It is worth noting that generation of prostaglandin E2 (PGE2) that can stimulate MDSCs expansion and differentiation depends on the available pool of arachidonic acid and is affected by nitrosylation cytosolic phospholipase A2α (cPLA2α) that is responsible for cleavage of arachidonic acid from membrane phospholipids. cPLA2α increases its activity significantly

upon nitrosylation (Slomiany and Slomiany 2010). It is tempting to hypothesize that this mechanism could also be in place in the tumor microenvironment. Another example of protein activity modification by tyrosine nitration is modulation of the activities of sirtulins. Considering the importance of these proteins to different cellular processes, changes caused by oxidative stress are of high significance—review by Santos et al. (2016). The study of the characterization protein modifications by nitroso groups and their effects over cellular processes are still in its infancy due to technological limitations that prevent their systematic analysis (modification by nitrosylation and nitration are very labile).

Anti-Inflammatory Cytokines

TGF-β

Production of TGF-β has been known for a long time to have ample immunosuppressive effects on many different cell types. TGF-β influences development of the Tregs phenotype (Huang et al. 2006), as it is required for the expansion of inducible Tregs (Arce et al. 2011), as well as it suppresses IFN-γ production by NK cells (Li et al. 2009).

IL-10

Production of IL-10 have been correlated with downmodulation of IFN-γ production by $CD4^+$ cells and induced by different populations of MDSC (Mundy-Bosse et al. 2011).

Others

Galectin 9

It has been observed by Sakuishi et al. (2011) that the expression of galectin 9 by MDSC induce apoptosis of T cells by interaction with T cell immunoglobulin mucin 3.

Adam17

Another protein that influence MDSCs suppressive activity is Adam17 (disintegrin and metalloproteinase domain-containing protein 17) that cleaves L-selectin (CD62L) from the surface of T cells (Hanson et al. 2009). As a result of its activity, T cells cannot undergo homing to peripheral lymph nodes where they would be activated.

S100A8 and S100A9

S100A8 and S100A9 are proinflammatory proteins that stimulate recruitment of MDSCs to tumor site and enhance their suppressive activity (Sinha et al. 2008).

Role of MDSC in Tumor Progression and Metastasis

Quite important factor of MDSCs suppressive activity lays in activation of different signaling pathways. It has been shown by De Henau et al. (2016) that activity of PI3Kγ is responsible for unresponsiveness to immunotherapies. Authors demonstrated that use of NCT2637531-specific PI3Kγ inhibitor (currently in clinical trials) resulted in repolarization of myeloid population to less suppressive phenotype. Consequence of repolarization of myeloid population is associated with T cell infiltration and Tregs level drop. Another example of signaling molecule of great importance for protumorigenic activity of MDSC is mTOR—another element of PI3Kγ pathway. It has been shown that inhibition of mTOR1 by siRNA in the model of lysosomal acid lipase (–/–) mice resulted in repolarization of MDSC and reversion of T cell suppression (Ding et al. 2014). The same group have shown role of MDSC in tumor cell proliferation *in vitro*, and tumor growth and metastasis *in vivo* (Zhao et al. 2015). The importance of different signaling pathways and role of Ras family of GTP-binding proteins in MDSC development and tumor progression was reviewed elsewhere (Trikha and Carson 2014).

Therapeutic Strategies to Counteract the Immunosuppressive Effects of MDSC

Considering the significant overall negative effects of MDSCs on different antitumor treatments several strategies are being developed to reduce the impact of these cells in antitumor treatments. The current approaches can be divided into four groups: (i) Inducing MDSCs differentiation into mature myeloid cells; (ii) Reducing/depleting MDSCs numbers; (iii) Blocking MDSCs development; (iv) Inhibiting the immune suppressive activities of MDSCs.

(i) Inducing Differentiation of MDSC into Mature Myeloid Populations

Differentiation of MDSCs and their accumulation in tumor-bearing hosts is the result of aberrant myelopoiesis, so one of the strategies is to normalize this process. One of the first key tested agents was all-trans retinoic acid (ATRA: active metabolite of vitamin A) that differentiates MDSCs into matured myeloid cells. It was the first drug successfully tested by Almand et al. (2001) on populations isolated from blood of patients with head and neck squamous cell carcinoma (HNSCC). At the same time, Gabrilovich et al. (2001) obtained similar results in a murine tumor model. In both cases, effective differentiation of DC was achieved. A later study on the treatment of renal cell carcinoma (RCC) in combination with IL-2 showed improved DC functions and recovery effector T

cell functions (Mirza et al. 2006) The use of 25-hydroxyvitamin D3 in HNSCC patients increased HLA-DR expression, plasma IL-12 and IFN-γ levels, and improved T cell blastogenesis (Lathers et al. 2004). The combination of ATRA administration with DC vaccination in patients with extensive stage small cell lung cancer substantially improved immune responses to vaccination (Iclozan et al. 2013). Recent data on CAR therapy in a Ewing sarcoma xenograft model in combination with ATRA treatment revealed an enhancing effect of this molecule in antitumor therapy (Long et al. 2016). Treatments with taxanes, such as paclitaxel, was reported to cause maturation of MDSC into DC in murine melanoma models (Michels et al. 2012; Sevko et al. 2013). Use of docetaxel, another taxane, caused polarization of MDSC into M1-like phenotype, and activation and expansion of cocultured $CD4^+$ effector T cells in a murine mammary tumor (4T1) model (Kodumudi et al. 2010).

(ii) Reducing/Depleting Numbers of MDSC

Reducing numbers of MDSCs can be achieved by inhibition of their proliferation. This has been targeted using multiple conventional therapeutic agents including different cytotoxic molecules as: 5-fluorouracil, gemcitabine, cisplatin, monoclonal antibodies against IL-6R. It was demonstrated that 5-fluorouracil increased IFN-γ production by tumor-infiltrating $CD8^+$ T cells and stimulated T cell-dependent antitumor responses *in vivo* (Vincent et al. 2010). The use of gemcitabine reduced spleen- and tumor-infiltrating MDSC numbers without affecting effector $CD8^+$ and $CD4^+$ T cells and NK cells (Suzuki et al. 2005). Combining cisplatin with a vaccine in TC-1 tumor-bearing mice led to decreased number of MDSCs and upregulation of effector $CD8^+$ T cell activity (Tseng et al. 2008). Sumida et al. (2012) demonstrated that administration of monoclonal antibodies against IL-6R resulted in decreased accumulation of MDSCs and reduction of tumor size. The combination of this treatment with gemcitabine significantly improved the results when compared to anti-IL-6R treatment alone in a skin squamous cell carcinoma CMC-1 in murine model (Sumida et al. 2012). Analyses of multiple cytokines in plasma of patients bearing pancreatic, esophageal and gastric cancers showed that IL-13 levels were strongly associated to an increment of MDSCs accumulation (Gabitass et al. 2011). This fact was used by Hall and cols to decrease MDSCs levels by coupling IL-13 to *Pseudomonas* endotoxin leading to an extended life in a murine model of HNSCC (Hall et al. 2013). Interestingly, the use of HSP90 inhibitors as a treatment in the MCA205 sarcoma model was associated with enhanced activation of T cells and decrease of MDSCs numbers (Rao et al. 2012).

(iii) Blocking MDSC Development

Tyrosine kinase signaling is well known to be involved in the differentiation of MDSCs. Different research teams have evaluated different drugs interfering with the STAT3 signaling pathway. Inhibitors, such as curcumin derivatives that inhibit STAT3 phosphorylation have been shown to be effective in inhibiting MDSCs differentiation (Bill et al. 2010; Lin et al. 2010). Apart from curcumin derivatives, JAK2/STAT3 inhibitors, such as Icariin flavone, and its derivative 3,5,7-trihydroxy-4´-emthoxy-8-(3-hydroxy-3-methylbutyl)-flavone (Zhou et al. 2011), AZD1480 (Liechtenstein et al. 2014; Lu et al. 2012), have also been tested. Use of a sunitinib, a multityrosine kinase inhibitor, proved to be very effective at inhibiting the accumulation of MDSCs in RCC patients (Ko et al. 2009, 2010). Another molecule shown to be effective is CDDD-Me (methyl ester of 2-cyano-3, 12 dioxooleana-1,9 (11)-dien-28-oic acid) shown to be effective in reduction of ROS levels, improved T cell function and reduced tumor growth in MC38 colon carcinoma, Lewis lung carcinoma, and EL-4 thymoma mouse tumor models (Nagaraj et al. 2010). Ahmad et al. (2008) identified JAK1 and STAT3 as directs targets of CDDO-Me and have shown that complex formation between the protein targets and the compound inhibits activation of the JAK1-STAT3 pathway. Moreira and colleagues demonstrated inhibition of STAT3 by CpG-STAT3-siRNA that was rapidly internalized by Toll-like receptor 9 expressed by G-MDSCs in colorectal cancer patients (Moreira et al. 2015).

(iv) Inhibition of the Immune Suppressive Activity of MDSC

The most extensively explored area of research is inhibition of the suppressive activities of MDSCs. As mentioned before, a major element regulating MDSCs suppressive activities is NO production. Potent anti-NO effects have been shown for nitroaspirin by different groups, and the use of AT38 leads to reversion of T cell inhibition caused by CCL2 nitrosylation. It is certainly important to acknowledge that protein nitrosylation can fundamentally change the landscape of protein functions, stability and interactions. Therefore, it is highly likely that inhibition of NO synthesis strongly affects many signaling pathways in a wide variety of cells from the tumor microenvironment. Many of those mechanisms are still to be discovered as characterization of modification by NO is challenging. NO production also inhibits E-selectin expression on endothelial cells and T cell recruitment to the tumor site (Gehad et al. 2012). The inhibitor of NO synthesis N(G)-Nitro-L-Arginine Methyl Ester (L-NAME) causes downregulation of NOS production at the transcriptional level leading to reduction of NO levels (Nanni et al. 2009). Importantly, arginase 1 is another target of L-NAME. Reisser et al.

(2002) observed that L-NAME inhibited arginase activity in vitro in lysates of colon and liver cancer cells, and in vivo in nodules and liver. Other arginase 1 inhibitors that have been used are L-NOHA (N omega-hydroxy-L-arginine) and nor-NOHA (N^{ω}-hydroxy-nor-L-arginine). The use of in vitro of L-NOHA and Nor-NOHA, or injection of these inhibitors in tumor-bearing animals prevented the loss of T cell function resulting in antitumor immune responses. Inhibition of tumor growth correlated with the dose of inhibitor, and was not observed in severe combined immunodeficient mice suggesting that antitumor effect was dependent on the activity of lymphocytes (Rodriguez et al. 2004).

It was observed by independent groups that PDE5 inhibition leads to degradation of cyclic guanosine monophosphate and as a consequence inhibition of iNOS and ARG1 expression (Serafini et al. 2006). PDE5 inhibitors have been shown to diminish the suppressive activity of MDSC. Recent work by Booth et al. (2017) demonstrated that PDE-5 inhibitors (sildenafil) enhanced the lethality of pemetrexed by inhibition of heat shock proteins. Nagaraj et al. (2010) observed that the synthetic triterpenoid C-28 methyl ester of 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid (CDDO-Me; bardoxolone methyl) decreased levels of reactive oxygen species in murine models of colon carcinoma (MC38), Lewis lung carcinoma, EL-4 thymoma and in blood samples from patients of renal cell carcinoma and pancreatic cancer. It is known that CDDO-Me is a strong activator of NRF2, a transcription factor responsible for upregulation of anti-oxidant genes, such as thioredoxin, catalase, haeme oxygenase, superoxide dismutase, and NAD(P)H: quinone oxidoreductase 1. The upregulation of NRF2 expression profiles and its activities decreased intracellular ROS levels.

COX2 inhibition was described by different authors as an efficient method for blocking MDSCs expansion in different tumors. The use of celecoxib alone or in combination with a DC-based immunotherapy in a murine mesothelioma model proved to be efficient in reducing systemic MDSCs expansion (Veltman et al. 2010). Nonsteroidal anti-inflammatory drugs, such as acetylsalicylic acid or celecoxib, have been shown to be efficient in reducing tumor-infiltrating and systemic MDSCs numbers as well as increasing tumor CTL infiltration in a murine model of glioma (Fujita et al. 2011). The COX2 inhibitor SC58236 has been shown to inhibit primary tumor growth and MDSCs accumulation in a 4T1 murine mammary tumor model (Sinha et al. 2007).

As described before, IDO is another key enzyme responsible for MDSC immunosuppressive activities. Although there is currently no IDO inhibitor in clinical use, there are already undergoing trials where the IDO inhibitor Indoximod is being tested. Interestingly, its application resulted in the increased production of autoantibodies directed towards tumor antigens (Soliman et al. 2016).

Ex vivo-derived MDSCs: an essential tool for future studies. Myeloid-derived suppressor cells are one of the key populations influencing the effector activities of NK and T cells. MDSC are responsible for tumor progression, not only by blocking antitumor response of host but also abolishing the effects of radio- and chemo-therapy through the expression of ROS scavenger proteins and overexpression of enzymes involved in degradation of xenobiotics (Liechtenstein et al. 2014). Despite the significant progress in the characterization of MDSCs, there are still major gaps in the mechanisms of ex vivo MDSCs differentiation, particularly from blood of healthy individuals. Relationship between murine and human counterparts have to be clarified. It can be pursued if both murine and human MDSCs could be successfully generated *ex vivo* in high numbers. Many different protocols which at times can be too complex have been published.

Ex Vivo Models for MDSC Differentiation in Murine Models

MDSC Differentiation Medium Based on Tumor Cell-Derived Conditioning Medium

Multiple protocols describe the differentiation of MDSCs from BM in murine models using different approaches. For example, the direct use of supernatant from Lewis lung carcinoma cell cultures can differentiate BM precursors towards myeloid cells with immunosuppressive activities (Young et al. 1989). MDSCs differentiation can also be achieved using different combinations of growth factors, such as GM-CSF, G-CSF, M-CSF, and CSF, instead of conditioning media from cancer cell cultures. In fact, treatment with just GM-CSF at high doses has been shown to differentiate MDSCs from BM precursors and maintaining them alive for a relatively long period of time (Morales et al. 2010). Many of the published protocols for MDSCs differentiation are based on recombinant cytokine supplementation of supernatants from tumor cell line cultures. The major limitation of most methods lays in their low efficiency (25–30%). Nevertheless, using these methods many research groups have studied MDSCs successfully, leading to the characterization of the two MDSCs subsets in at least ten different tumor models. A different approach has been used by Liechtenstein and colleagues whereby the authors genetically modified tumor cell lines with lentivectors expressing cytokine genes to generate medium that was used to subsequent differentiation and expansion of MDSCs (Dufait et al. 2015; Liechtenstein et al. 2014). Surprisingly, this protocol was highly efficient and resulted in generation of MDSCs with characteristics equivalent to those of tumor-infiltrating subsets, characterized by high iNOS levels and TGF-β secretion. Using comparative proteomic analyses, the authors showed that these cells were characterized by a metabolic

reprogramming specific for the tumor microenvironment and an upregulated lipid metabolism. Similar observations were made in MDSC isolated from tumors of cancer patients (Hossain et al. 2015). Instead of using classical cytokines, Xiao et al. (2015) used the soluble version of MICB, a ligand of NKG2D receptor that is on the surface of NK and T cells and responsible for their activation. Medium from cultures expressing MICB supplemented with GM-CSF was an effective inducer of MDSCs (up 70%) (Xiao et al. 2015). De Veirman et al. (2015) demonstrated that multiple myeloma 5T33MMvt medium was sufficient to trigger differentiation of BM-derived precursors into MDSC with strong suppressive activity towards activated T cells. The use of tumor cell exosomes was also proven to be sufficient to initiate MDSC differentiation (Xiang et al. 2009). Elegant work of Wu et al. (2011) with CCSP-rtTA/(tetO)7-CMV-Stat3C bitransgenic mice model demonstrated that MDSC differentiation can be achieved from alveolar monocytes/macrophages isolated from wild-type mice by incubation with bronchoalveolar lavage fluid isolated from bitransgenic mice containing different cytokines. Authors tested separately or in combinations those factors [GM-CSF, IL-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α or vascular endothelial growth factor (VEGF)] and have shown their different MDSC differentiation capacities (Wu et al. 2011).

MDSC Differentiation Based on Defined Media

The use of tumor cell-derived supernatants to differentiate MDSC has its benefits as it mimics *in vivo* conditions. However, those differentiated MDSCs will differ according to differences in tumor cell lines producing distinct cytokine secretion profiles. Additionally, it might be possible that there will be variability strongly affected by the number of passages of tumor cell lines. The development of defined differentiation medium would solve those incoherencies. However, despite supplementing the medium with recombinant cytokines that permits the differentiation of MDSCs; the efficiency of the process is still very low (Escors et al. 2013).

Although GM-CSF is considered a key factor for MDSC differentiation, it is not sufficient to fully achieve MDSC differentiation. Therefore, the testing of different cytokine combinations including GM-CSF, IL-6 and G-CSF for the differentiation of BM-derived precursors revealed that GM-CSF and IL-6 or GM-CSF with G-CSF had higher differentiation activity that any of the cytokines alone. MDSC generated in those experiments were suppressing the activity of activated T cells (Marigo et al. 2010). Highfill et al. (2010) successfully differentiated MDSC using GM-CSF and G-CSF combinations, and they demonstrated that supplementation with IL-13 significantly increased levels of arginase 1. The authors showed that these differentiated MDSC inhibited graft-versus-host disease after administration into mice together with T cells without affecting beneficial graft versus host leukemia effects. Xiao et al. (2015) used purified soluble MICB to supplement medium together with GM-CSF and they showed that this combination was sufficient to drive MDSC differentiation.

Models for Ex Vivo Differentiation of Human MDSC

MDSC Differentiation Medium Based on Tumor Cell-Derived Conditioning Medium

Human MDSCs are still poorly investigated. It has to be taken into account that for murine models, the source of MDSCs precursors come from multipotent BM cells and differentiation is therefore much easier. For models of human MDSC differentiation, the source of precursors is most of the times peripheral blood monocytic cells (Lechner et al. 2010; Obermajer and Kalinski 2012). The isolation of BM precursors is an invasive procedure and not practical for these studies. Nevertheless, the differentiation efficiency is still much lower compared to murine models. In addition, MDSCs isolated from patients are still poorly characterized and it is challenging to compare *ex vivo* differentiated MDSCs with their natural counterparts. Similarly to murine differentiation models, human MDSCs can also be differentiated with the exosomal fraction of tumor cell cultures. (Valenti et al. 2006). MDSCs generated in this way were characterized by $CD14^+$ $HLA\text{-}DR^{neg}$ and low level of co-stimulatory molecules. A following study showed that these cells inhibited T cells through TGF-β secretion.

De Veirman et al. (2015) have shown that MDSCs can be differentiated with conditioning medium from a multiple myeloma cell line. Surprisingly, they proved that this specific media did not contain GM-CSF and that an alternative mechanism of differentiation relied on Mcl-1 (De Veirman et al. 2015).

MDSC Differentiation Based on Defined Media

Similarly to murine models, human BM precursors may differentiate to MDSCs with cytokine combinations, such as GM-CSF plus G-CSF or GM-CSF with IL-6. The phenotype of these cells was $CD11b^+$ $CD16^-$ and possessed strong T cell suppressive activities (Marigo et al. 2010). Using PBMCs and 15 different immune factors expressed by tumor cell lines, Lechner et al. (2010) identified IL-1β, IL-6, M-CSF, COX2, and IDO as potential MDSC differentiation factors. They tested several combination of cytokines and found that MDSC differentiated with GM-CSF plus IL-6 and GM-CSF, IL-6 and VEGF had the strongest suppressive activities towards T cells. Interestingly, treatment with GM-CSF alone or GM-CSF plus IL-1β, GM-CSF plus VEGF or GM-CSF plus TNF-α generated MDSCs with the lowest

suppressive capacities. The authors showed MDSC differentiated with a combination of GM-CSF with PGE2 were weakly immunosuppressive and that TGF-β interfered with cytokines that promoted MDSCs differentiation (Lechner et al. 2010). The work by Obermajer et al. (2011) confirmed the importance of PGE2 in human MDSCs differentiation. The authors used GM-CSF plus IL-4 in combination with PGE2 and generated MDSCs similar to those isolated from human ovarian cancer ascites (Obermajer et al. 2011). The importance of proinflammatory cytokines such as IL-6 to differentiate MDSCs with immunosuppressive capacities was demonstrated by Chen et al. (2014).

Cell Lines that Model MDSC

An attractive alternative to *ex vivo* differentiation of MDSC from precursors would be the development of immortalized MDSC-like cell lines. The major advantage of this system would be the sustained cell growth without the necessity of obtaining them from BM precursors or PBMCs. Such a cell line could be used in basic research or pharmacological screening. The approach to generate an immortal MDSC-like cell line was undertaken by Apolloni et al. (2000). The authors generated these cells by co-expression of v-raf and v-myc oncogenes by $CD11b^+$ $Gr1^+$ murine cells (Apolloni et al. 2000). A different method was used by Ding et al. (2015) who isolated a cell line from peritoneal macrophages from SV40-T transgenic mice bred with lysosomal acid lipase knock-out. Despite the lack of classical MDSC markers, these cells were characterized by strong immunosuppressive capacities (Ding et al. 2015). However, the study of their metabolic footprint showed that in contrast to primary MDSCs that use lipid metabolism as an energy source, these immortal cells upregulated the glucose metabolism (Hossain et al. 2015; Liechtenstein et al. 2014). This apparent contradiction might be explained by the lack of lysosomal lipase and adaptation of these MDSC-like cells to the subsequent decreased lipid metabolism.

However, it has to be taken into the account that these genetically modified cells will probably have different characteristics than tumor-infiltrating MDSC. Additionally, similarly to macrophage cell lines, an immortal MDSCs-like cell line will probably have different signaling pathways activated and would behave in a different way.

Conclusions

Based on the growing body of literature, it is more evident that MDSCs play a significant role in tumor growth and progression, metastasis and resistance to different therapies. Importantly, there is accumulating evidence on the crosstalk of different tumor-infiltrating populations, such as macrophages, tolerogenic DC, regulatory T and B cells as well as tumor-associated neutrophils. This intercellular collaboration strongly promotes tumor progression and metastasis. In fact, there is a growing tendency to categorize MDSC together with other tumor-infiltrating subsets as macrophages M2, immature DC and neutrophils which exert immunosuppressive activities, and define them as myeloid regulatory cells (De Vlaeminck et al. 2016; Keskinov and Shurin 2015). Considering their suppressive activities and the presence of common surface markers, this classification seems accurate. In fact, it has been shown that silencing the transcription factor STAT3, which is considered a central immune checkpoint regulator, affected the number of myeloid suppressive populations and regulatory T cells. At the same time, improvement of CD8 T cell infiltration and increase in the presentation of cancer-specific antigens was observed – reviewed by Kortylewski and Moreira (2017). However, it has to be taken into account that knowledge on the metabolism and function of these subsets within the tumor environment apart from their suppressive activity is still very limited. It could be very likely that in-depth characterization of those populations (Mass spectrometry, transcriptomics) may identify subtle differences that establish their unique role in tumor progression.

The experimental evidences from studies in murine models concentrated only on MDSC subsets clearly show that anticancer treatments including immunotherapies are far more efficient in animals that have been depleted of MDSCs. Implementation of *in vivo* studies in murine models is fairly easy as published protocols for *ex vivo* generation of MDSCs are quite straightforward and easy to implement. It has been already shown that many of the therapeutic treatments that are at present in the clinic affect MDSCs function. Nevertheless, testing of the new therapeutics in human MDSC *ex vivo* is still a challenge. In case of human MDSCs, the published protocols use different combinations of recombinant cytokines, so the resulting MDSCs differ from protocol to protocol and may not represent MDSCs found in human patients. MDSCs isolated from patients are present in samples in very low levels and as a result of this, it is challenging to characterize them in detail. Development of simple protocols for *ex vivo* generation of human MDSCs resembling tumor-infiltrating populations at high levels would be definitely a giant step accelerating systematization of information already gathered from patient-derived samples. Such methods would speed up the identification of novel potential therapeutic targets. Development of new treatments could be tested directly in cell-based high throughput screening assays based on *ex vivo*-generated MDSC.

Acknowledgements This work was partially supported by a crowdfunding project by FECYT (Spain), a CAIXA grant, a BIOEF grant (Government of the Basque Country), a FIS grant (PI14/00579) from

the Instituto de Salud Carlos III, and a grant from the Government of Navarre (BMED 033-2014), Spain. We thank SARAY foundation (Navarra, Spain) and Sandra Ibarra foundation (Spain) for their financial support. M. Gato is funded by a PhD fellowship granted by the Government of Navarre. M. Zuazo is funded by a fellowship granted by Universidad Publica de Navarra (UPNA), Spain. M. Ibáñez-Vea is funded by a Sara Borrel Fellowship. D. Escors is funded by a Miguel Servet Fellowship (CP12/03114) awarded by the Instituto de Salud Carlos III (Spain).

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