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Mini-Review

Antigen specificity of immune suppression by myeloid-derived suppressor cells

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ABSTRACT

Among the mechanisms set in motion by the tumor to escape the control of the immune system, MDSCs play a central role in inducing tolerance to a variety of antitumor effectors, including T lymphocytes. It has been demonstrated that MDSCs expand in tumor-bearing mice and in cancer patients, leading to an impairment of T cell reactivity against the tumor. However, as the presence of MDSCs is not correlated with a general immune suppression, it was advanced that a mechanism regulating the specificity of MDSC inhibition must be present. In this article, we review the literature showing that MDSCs exert their immune-suppressive function on Ag-specific T cell responses but at times, also on mitogen-activated T lymphocytes, therefore bypassing the Ag dependency. We propose that the features of MDSC-mediated immune suppression might be influenced not only by the specific microenvironment in which MDSCs expand and by the tumor characteristics but also by the levels of activation of the target lymphocytes. *J. Leukoc. Biol.* 90: 31–36; 2011.

Introduction

MDSCs are a heterogeneous collection of immature myeloid cells at different stages of differentiation that have been shown to hamper the immune response in cancer patients. It is currently believed that the origin of MDSCs (reviewed in refs. [1, 2]) is a result of a delay in the myeloid development process caused by cytokines and growth factors released within the tumor microenvironment: the immature myeloid cells fail to develop fully in the BM and are released in the circulation. After being recruited to the peripheral lymphoid organs and tumor site, MDSCs may undergo a process of activation and trigger mechanisms of suppression of T cell function through cell-surface interactions and the release of short-lived, soluble mediators [2].

In mice, MDSCs are identified as cells that simultaneously express the cell markers CD11b and Gr-1, and in cancer patients, the characterization of MDSCs is less accurate, and these regulatory cells have been defined by several authors with different combinations of myeloid markers [1]. The analysis of different subsets is beyond the purposes of this review; however, three main MDSC populations have been defined in mice and humans and will be considered here: monocytic, PMN, and immature myeloid precursors [1].

Several mechanisms of suppression exerted by MDSCs have been documented at a molecular level, but an accurate picture of the key events in MDSC suppression is not yet available. In this regard, an open issue is the specificity of suppression exerted by MDSCs, as the presence of MDSCs is not correlated with a generalized immune suppression in tumor-bearing hosts. Accordingly, two possible hypotheses have been advanced by the researchers working in this field: the Ag specificity or the unspecific but anatomically confined nature of the inhibitory function exerted by MDSCs.

The Ag specificity hypothesis requires that MDSCs take up antigens and present them to T lymphocytes, therefore realizing a strict contact between the two cells; this implies that MDSCs should exclusively target Ag-specific T cells in their inhibitory pathways; on the contrary, a nonspecific response presumes that the suppressive activity of MDSCs can be exerted on activated T cells, regardless of direct presentation of an Ag. In this review, we summarize the evidence supporting the hypotheses of the Ag-specific versus nonspecific nature of immune suppression, attempting to reconcile often conflicting results on initial triggering and modulation of MDSC activation.

Abbreviations: ARG1=arginase 1, BM=bone marrow, BM-MDSC=bone marrow-derived myeloid-derived suppressor cell, Foxp3$^+$=forkhead box p3$^+$, HCC=hepatocellular carcinoma, HNC=head and neck cancer, MDSC=myeloid-derived suppressor cell, MSC-1/2=myeloid suppressor cell 1/2, PC=prostate cancer, PDE5=phosphodiesterase-5, RCC=renal cell carcinoma, Treg=T regulatory cell, TT=tetanus toxoid

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Ag-SPECIFIC AND NONSPECIFIC IMMUNE SUPPRESSION IN TUMOR-BEARING MICE

Initial work carried out by our group to study the suppression induced in mice immunized with strong viral immunogens identified a population of myeloid cells that stained positive for the Gr-1 and CD11b antigens and was responsible for a decreased CTL activity. Interestingly, this suppression was observed not only in vitro but also in vivo, required activation of T lymphocytes by the cognate Ag, and was relatively long-lived [3].

To further investigate the nature of MDSCs, we immortalized $CD11b^+/Gr\text{-}1^+$ cells isolated from the spleen of immunosuppressed mice and generated two cell lines: MSC-1 and MSC-2; MSC-1 was constitutively suppressive and completely inhibited the function of alloreactive CTLs generated in a mixed leukocyte reaction, whereas MSC-2 acquired the ability to suppress alloreactive CTLs following pretreatment with IL-4 [4]. These two cell lines were also able to inhibit mitogen-induced proliferation, as well as peptide-specific responses [5]. However, MSC lines used at least two mechanisms, both involving the metabolism of L-arginine, to inhibit T cell function depending on the stimulus provided to activate T cells. MSC-1 and -2 restrained mitogen-induced T cell proliferation by a NOS-dependent mechanism requiring IFN-γ and direct contact with activated lymphocytes. In addition, MSC-2 were able to dampen alloreactive T lymphocyte proliferation through ARG1 [6]. We therefore suggested that under some circumstances, NOS2 and ARG1 could be active at the same time and in the same intracellular environment and also that the type of activation in T lymphocytes was the key factor triggering a specific molecular pathway responsible for the suppressive mechanisms by MDSCs [5, 6].

Consistent with our data, $Gr\text{-}1^+$ cells isolated from BM or spleen of mice bearing colon carcinomas showed an immunesuppressive activity markedly higher in comparison with the activity of the same cells derived from tumor-free BM or spleen; in this study, MDSCs strongly inhibited the proliferative response of naïve T cells activated with anti-CD3 and anti-CD28 agonistic mAb or by alloantigens [7]. The suppression exerted by these myeloid cells appeared a result of a significant production of NO, and this mechanism was activated by the secretion of IFN-γ by T cells stimulated with anti-CD3 and anti-CD28 [7]. Despite the observation that $Gr\text{-}1^+$ cells were increased in the spleen and BM of tumor-bearing mice, and depletion of $Gr1^+$ cells restored lymphocytic proliferation in vitro, in this work, the add-back experiments proving the functional activity of MDSCs were performed by isolating the suppressive subset through the fractionation of the BM cells on a Percoll density gradient, a protocol that did not allow achievement of a high purity of the myeloid fractions. In contrast, a more highly purified subset of $Gr\text{-}1^+$ cells separated by cell sorting from MethA sarcoma or C3 tumor-bearing mice did not affect Con A-inducible proliferation and IL-2 production in T cells. Instead, $Gr\text{-}1^+$ cells appeared to mediate only inhibition of Ag-specific $CD8^+$ immune responses, in vivo and in vitro, via cell-to-cell contact [8]. This study demonstrated that the machinery by which $Gr\text{-}1^+$ cells inhibited T cell proliferation in this model involved the MHC class I presentation of the Ag, as the use of a mAb-blocking MHC class I expression on the surface of $Gr\text{-}1^+$ cells abrogated the immunosuppression. In this context, NO and possibly NOS2 might be involved in $Gr\text{-}1^+$ cell-mediated inhibition of T cell responses, but its exact role did not emerge from this study [8].

Using a model of adoptive transfer of different populations isolated from tumor-bearing mice into tumor-free recipients, the same group confirmed the previous results showing that only $Gr\text{-}1^+$ cells from tumor-bearing mice, but not from tumor-free mice, abrogated Ag-specific CTL activity and that the unresponsiveness was restricted by MHC class I molecules, whereas $CD8^+$ T cells retained the ability to respond to a nonspecific stimulus, such as anti-CD3 [9]. Although the molecular mechanisms of T cell tolerance were not addressed, this work nonetheless established that $Gr\text{-}1^+$ cells were able to take up soluble proteins, process them, and present antigenic epitopes on their surface, inducing Ag-specific T cell anergy.

An interesting insight into the mechanism of suppression by which $CD11b^+Gr\text{-}1^+$ MDSCs act on $CD8^+$ T cells was proposed recently by a study showing that MDSCs, through the generation of peroxynitrite, the result of the combination of ROS and NO, can bring about the nitration of tyrosines in the TCR-CD8 complex of the T lymphocytes; as a consequence of this post-translational modification, there is an alteration in the binding of the complex with the specific peptide-MHC complex that prevents the antigen-specific response by CTLs [10]. As peroxynitrite is a short-lived molecule and cannot be active at long distances, the basis for ensuing the inhibitory machinery is the close proximity between MDSCs and the T cell, realized only by a strict cell-to-cell contact.

The need for antigen presentation by MDSCs to activate an immunosuppressive program was not confirmed in a chicken OVA-transfected EL-4 thymoma model, in which EG7 tumors were grown in WT or β2-microglobulin-deficient mice. In the latter context, the deficiency of MHC class I expression did not impair the suppression of Ag-specific proliferation exerted by mononuclear and PMN-MDSCs. Moreover, MDSCs isolated from tumor-bearing mice were able to suppress Ag-specific T cell responses by an IFN-γ-dependent mechanism but were not able to interfere with anti-CD3-induced T cell proliferation [11]. In a different tumor model, Watanabe et al. [12] demonstrated recently that $Gr\text{-}1^+$ cells isolated from fibrosarcoma MCA205 were able to inhibit $CD4^+$ and $CD8^+$ T cell activation triggered by anti-CD3 mAb. These results clearly highlight the discrepancies in the field and point to factors other than cell heterogeneity as the explanation, as discussed further below. However, it must be pointed out that the composition of MDSCs can certainly influence the immunosuppressive potency. We recently demonstrated in different tumor models that the immunosuppression mediated by MDSCs is inversely related to Gr-1 marker expression, stressing the importance to define not only the functional activity of MDSCs but also the phenotypical features of the suppressive cells [13]. One important caveat is that in many studies, the immunoregulatory function of different MDSC subsets was evaluated only in vitro and not following in vivo adoptive transfer. How in vitro data relate to in vivo immune dysfunctions induced by tumors is

not entirely known. However, our experience in mouse models suggests that the most tolerogenic cells, in vitro and in vivo, are monocytic- rather than PMN-MDSCs [1, 13, 14].

THE NATURE OF MDSC-MEDIATED IMMUNOSUPPRESSION IN CANCER PATIENTS

The characterization of the molecular mechanisms involved in human MDSC immunosuppression has been more difficult to dissect, as human studies must deal with the limitation in the supply of biological material, a prerequisite for MDSC extensive molecular and phenotypic characterization. At present, surrogate markers, predicting which patients will present an expansion of MDSCs, are not known, and consequently, acquisition of sufficient numbers of MDSCs is often a limitation for functional studies.

One of the first studies addressing the nature of human MDSCs showed that myeloid immature cells, isolated from peripheral blood of patients with advanced cancer, actively inhibited a T cell response in an IFN-γ-dependent and NO-independent manner. In particular, the addition of MDSCs not only reduced TT-specific T cell proliferation but also abrogated the production of IFN-γ by CTL exposed to the specific influenza virus-derived peptide, suggesting that MDSCs are able to interfere with an Ag-specific T cell response [15].

Subsequently, the same group analyzed the expansion and function of MDSCs in cancer patients with solid tumors enrolled in a clinical trial, potentially affecting in vivo MDSC differentiation. In this study, patients were treated with VEGF-Trap, a fusion protein of VEGFR1 and -2, coupled with the Fc portion of human IgG1, with the aim to affect the tumor vasculature and prevent tumor dissemination [16]. This study revealed that cancer patients did not show defects in anti-CD3-induced T cell proliferation, but they failed to activate a full T lymphocyte response to antigens, PHA, TT, and influenza virus. When patients characterized by a decreased or stable level of MDSCs were considered, a significant improvement of antigen- and mitogen-specific immune responses was observed after the treatment with VEGF-Trap. On the contrary, patients who showed an increase in MDSCs during the trial showed no improvement in immune responses, therefore supporting the concept that MDSCs can play a pivotal role in inhibiting immune reactivity.

Sildenafil, an inhibitor of the enzyme PDE5, was identified as an efficient drug able to block human MDSC inhibitory function, as it restored in vitro proliferation of T cells from multiple myeloma and HNC patients [17]. Specifically, in this study, the myeloid-suppressive function was attributed to $CD14^+$ cells isolated from PBMCs of cancer patients. In fact, unfractionated PBMCs were not able to respond to stimulation with anti-CD3 and anti-CD28, and lymphocyte proliferation in response to this mitogenic stimulation was achieved only after adding to culture sildenafil or the combination of N^{ω}-hydroxy-nor-arginine and L-NG-monomethyl arginine, inhibitors of ARG1 and NOS2 enzymes, respectively. The same rescue of lymphocyte proliferation was observed when PBMCs were stimulated with the same mitogens, after depleting the immune-suppressive fraction represented by the $CD14^+$ cells.

These results were confirmed by another study, in which MDSCs identified as $CD14^+/HLA\text{-}DR^{low/-}$, isolated from PC patients, inhibited autologous T lymphocyte proliferation stimulated with anti-CD3 and anti-CD28 significantly better than the $CD14^+/HLA\text{-}DR^+$ cells [18]. A cell population with the same phenotypical features was also described in patients with HCC and melanoma [19, 20]. MDSCs from HCC patients suppressed proliferation and IFN-γ secretion of CD3/CD28-stimulated, autologous PBMCs, and the depletion of MDSCs from melanoma patients induced a boost in lymphocyte proliferation in response to PHA.

MDSCs, identified as $CD11b^+/CD33^+$ cells in patients with advanced, nonsmall cell lung cancer, were found to decrease significantly T cell proliferation induced by anti-CD3 and anti-CD28 [21]. Recently, it was shown that $CD33^+/HLA\text{-}DR^+$ MDSCs from metastatic breast, colon, or PC patients exerted a high immunosuppressive function on T cells stimulated with anti-CD2, anti-CD3, and anti-CD28, compared with the same population isolated from healthy donors [22].

Analogously to mice, human MDSCs have also been described as a myeloid population with morphology and cell markers of PMN cells. In particular, PMN-MDSCs have been found often in metastatic RCC patients and were characterized recently as a subpopulation of activated PMN that express high levels of CD66b, a member of the human carcinoembryonic antigen family expressed on human PMN, contained in specific/gelatinase granules, which has been proposed as a marker of the human PMN-MDSC subset. The depletion of $CD66b^+$ cells restored the proliferation of autologous $CD8^+$ T cells activated by mitogens [23, 24]. The presence of this suppressive subset in RCC patients was accompanied by a reduction of the CD3ζ chain expression in peripheral T cells, a phenomenon that was associated with the release of ARG1 from intracellular granules of PMN-MDSCs into the microenvironment, where it depletes L-arginine and induces T cell dysfunction.

In a recent study, we evaluated the immune response of colon carcinoma and melanoma patients, and we observed that there are two main subpopulations endowed with suppressive activity circulating in the blood of cancer patients: one is present among $CD14^+$ monocytes, and another can be found among PMNs [25]. The allogeneic response of PBMCs from healthy donors was increased significantly if $CD14^+$ cells were depleted from PBMCs of cancer patients. We also observed that the $CD14^+$ population isolated from cancer patients was able to suppress not only the allogeneic response but also the anti-CD3-induced T cell proliferation, therefore demonstrating that these suppressor cells are also able to interfere with an Ag-independent response.

THE CROSSTALK AMONG MDSCs, CD4, AND Tregs

Many studies investigating the suppression exerted by MDSCs are focused toward $CD8^+$ T cells, and significantly less is known about the effect on $CD4^+$ T cells. Although earlier data indicated that MDSCs would be able to present Ags only in the

context of MHC class I and block selectively MHC class I-restricted $CD8^+$ T cell responses [8], more recent data highlighted a role of MDSCs in also suppressing $CD4^+$ T cells. MDSCs isolated from tumor-bearing mice were shown to decrease IFN-γ production, not only of tumor-specific $CD8^+$ but also of $CD4^+$ T lymphocytes in the spleen of tumor-bearing mice in vivo [22]. Accordingly, Ostrand-Rosenberg and coworkers [26] showed that MDSCs isolated from mammary, carcinoma-bearing mice were able to suppress Ag-specific $CD4^+$ and $CD8^+$ T cells and that this mechanism was not MHC-restricted. Using the same tumor model, authors clarified later that MDSCs blocked $CD4^+$ and $CD8^+$ T cell activation by limiting the extracellular pool of cysteine, which is required for T cell proliferation [27]. Moreover, very recently, it was confirmed that MDSCs are able to suppress $CD4^+$ T cells by using the OVA antigen-specific, syngeneic $CD4^+$ T cell model [28].

MDSCs and $CD4^+$ Tregs are important for the immune regulation, and in some instances, they have been found to cooperate. It was shown that a population of Gr-1^+CD115^+ MDSCs isolated from colon carcinoma-bearing mice not only suppressed $CD4^+$ splenocytes bearing a TCR specific for the antigen HA but was also able to induce the expansion of Tregs in vitro; moreover, in this report, authors provided the first evidence that the adoptively transferred Gr-1^+CD115^+ MDSCs could also induce the development of $CD25^+$ $Foxp3^+$ Tregs in vivo and that the key molecules involved in this process were IL-10 and IFN-γ. These results highlighted a possible interaction between MDSCs and Tregs to establish and maintain T cell tolerance in a tumor-bearing host [29]. The authors later examined the role of CD40 in MDSC-induced T cell tolerance, and they concluded that CD40 is critically important for MDSC-mediated Treg expansion, as the use of MDSCs derived from CD40-deficient or the blockade of CD40/CD40 ligand interaction through an anti-CD40 antibody suppressed Treg development [30]. In another study, MDSCs isolated from lymphoma-bearing mice were shown to inhibit $CD8^+$ T cells specific for the influenza HA but not HA-specific $CD4^+$ T cells; however, these MDSCs were found to mediate the expansion of HA-specific Tregs [31]. More importantly, the treatment with sildenafil, a PDE5 inhibitor, effectively reversed the immunosuppression by reducing the number of tumor-specific Tregs [31]. The relationship between MDSCs and Tregs was also investigated in a Lewis lung cancer model, in which the Fas signal was shown to promote the accumulation of MDSCs and $Foxp3^+$ Tregs within the tumor microenvironment; MDSCs appeared to be recruited to the tumor tissues earlier than Tregs to facilitate the differentiation of Tregs [32].

With regard to cancer patients, it was shown that the proliferation of $CD8^+$ T cells in PBMCs from RCC patients is inhibited more profoundly compared with $CD4^+$ T cells and that depletion of MDSCs completely restored $CD8^+$ T cell proliferation but only enhanced $CD4^+$ T cell proliferation [23]. In a different study, $CD14^+$/HLA-$DR^{low/-}$ monocytes isolated from PC patients inhibited proliferation of $CD4^+$ and $CD8^+$ T cells more than age-matched, noncancerous controls [18]. The link between MDSCs and Treg was also suggested in metastatic RCC patients: in this study, in fact, patients had elevated levels of MDSCs that were reduced after sunitinib treatment, and this decrease correlated with the decline in Tregs [33]. In another study, $CD14^+$ HLA-$DR^{low/-}$ MDSCs in HCC patients were shown to suppress tumor-specific $CD4^+$ T cell responses through induction of $CD4^+CD25^+Foxp3^+$ Tregs [19]. However, in several studies, the specific effects on $CD8^+$ or $CD4^+$ T cells were not investigated in detail, as the inhibitory activity of MDSCs was analyzed on whole T cells without discriminating between $CD4^+$ and $CD8^+$ T lymphocytes [14, 16, 20, 22, 25, 34]. Thus, on the basis of these results, we cannot exclude that MDSCs expanded in cancer patients might exert a different suppressive activity on $CD4^+$ and $CD8^+$ T cells by blocking preferentially only one of them, suppressing them at different times during tumor development, or mediating their effects indirectly (i.e., inducing Tregs to control proliferation and activation of other T lymphocytes). Certainly, further studies are needed to clarify this important issue.

IS THE MDSC-MEDIATED SUPPRESSION DRIVEN BY THE Ag SPECIFICITY OR BY THE LYMPHOCTYES' ACTIVATION?

From all of these data, it is evident that human and mouse MDSCs can play a role at times in Ag-specific responses, but in other circumstances, they are also able to suppress a CD3/CD28-mediated T lymphocyte activation, i.e., a condition in which almost 100% of cultured T lymphocytes are activated, bypassing the recognition of the Ag by the TCR in the target cells and the requirement for an Ag presentation by the same MDSCs.

To explain this apparent discrepancy in terms of Ag specificity, it was proposed recently that MDSCs may exert an immunosuppressive effect in an Ag-specific and nonspecific manner depending on their localization, the specific characteristics of the tumor, and the prevailing inflammatory microenvironment [35]. One recent study supporting this hypothesis evaluated the phenotype and function of MDSCs isolated from the spleen or the tumor sites [36]. Splenic MDSCs containinig a high level of ROS but a modest amount of NO and ARG1 were able to produce peroxynitrites and exerted their effect only via cell-to-cell contact with activated, Ag-specific T cells; on the contrary, tumor MDSCs characterized by a high level of NO and ARG1 but not of ROS were not only more potent inhibitors of antigen-specific T cell functions than spleen MDSCs but also suppressed T cells nonspecifically. In contrast to MDSCs isolated from the spleen, MDSCs in the tumor microenvironment rapidly differentiated into tumor-associated macrophages, through a process mediated by the hypoxia-inducible factor-1α. These data support the concept of the Ag-specific nature of MDSC immunosuppression in peripheral organs and at the same time, may explain the lack of systemic immune suppression in tumor-bearing mice and in cancer patients.

This hypothesis was also tested on human MDSCs, defined as $CD14^-CD11b^+CD33^+$ immature myeloid cells, by analyzing MDSCs from matched peripheral blood and tumor tissues of HNC patients. The results confirmed that tumor tissue had a significantly lower ROS amount than MDSCs in peripheral blood, and NOS2 levels in tumor MDSCs were substantially

higher than in blood MDSCs. When the authors tested the inhibitory activity exerted by MDSCs isolated from the two sites, they observed that only tumor MDSCs were able to interfere with lymphocyte activated by PHA [36].

We demonstrated recently that GM-CSF, G-CSF, and IL-6 allow a rapid generation of MDSCs from precursors present in human BM, named BM-MDSC, and that these cells share the phenotype of MDSCs isolated from cancer patients and are able to inhibit alloantigen- and mitogen-activated T cells [14]. Subsequently, we observed that the suppressive activity is fully induced in BM-MDSCs only after direct and strict contact with activated T lymphocytes, thus implying that suppressive cells need a cell membrane signal to be fully armed, a result analogous to data obtained with mouse MDSCs (unpublished results and ref. [37]).

To reconcile all of these discrepant observations, we propose that MDSCs may become fully competent in their suppressive function under two circumstances: when reaching the tumor site or in the presence of highly activated T lymphocytes. We hypothesize that beside the microenvironment, a key factor driving the triggering of the suppression mechanism of MDSCs might be the level of T cell activation. In other words, Ag is required to activate T cells, but Ag could be recognized on cells other than MDSCs. Once activated, T cells could prime MDSCs to restrain any actively proliferating T cell, while sparing nearby inactive lymphocytes. This hypothesis is in agreement with the consideration that suppressor cells physiologically fulfill their role of turning down an immune response when it reaches a critical threshold of activation to restrain a potentially dangerous and uncontrolled progression of lymphocyte activation; accordingly, suppressive cells are unable to harm resting or homeostatically proliferating lymphocytes, and the direct contact between these two cells ensures that the signals delivered by MDSCs are confined only to target cells and not to bystander cells.

It is clear, however, that this mechanism can be overwhelmed by changing the ratio between responders and suppressors: in the case of an excess of responders, it is possible to hypothesize that some lymphocytes might be spared from the "death kiss" of MDSCs and actually eliminate them, as in the case of CTLs recognizing Ag presented by MDSCs. This might explain why above a given threshold of lymphocyte excess and with potent activation signals, suppression might be limited or absent in some in vitro settings.

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