Immature myeloid-derived suppressor cells: A bridge between inflammation and cancer (Review)

CATERINA MUSOLINO[1*], ALESSANDRO ALLEGRA[1*], GOVANNI PIOGGIA[2] and SEBASTIANO GANGEMI[3*]

[1]Division of Hematology, Department of General Surgery, Pathological Anatomy and Oncology, University of Messina; [2]Institute of Clinical Physiology, IFN CNR, Messina Unit; [3]School and Division of Allergy and Clinical Immunology, Department of Clinical and Experimental Medicine, University Hospital 'G. Martino', Messina, Italy

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Abstract. Chronic inflammation is considered to be one of the hallmarks of tumor initiation and progression. Changes occurring in the microenvironment of progressing tumors resemble the process of chronic inflammation, which begins with ischemia followed by interstitial and cellular edema, appearance of immune cells, growth of blood vessels and tissue repair, and development of inflammatory infiltrates. Moreover, long-term production and accumulation of inflammatory factors lead to local and systemic immunosuppression associated with cancer progression. Of the several mechanisms described to explain this anergy, the accumulation of myeloid cells in the tumor, spleen, and peripheral blood of cancer patients has gained considerable interest. A population of suppressive $CD11b^+Gr\text{-}1^+$ cells has in fact been designated as myeloid-derived suppressor cells (MDSCs). MDSCs are a unique category of the myeloid lineage, and they induce the prevention of the development of cytotoxic T lymphocytes (CTLs) *in vitro*, and the induction of antigen-specific $CD8^+$ T-cell tolerance *in vivo*. Therapeutic approaches directed toward the manipulation of the MDSC population and their function may improve chemoimmune-enhancing therapy for advanced malignancies.

Contents

Correspondence to: Dr Alessandro Allegra, Division of Hematology, Department of General Surgery, Pathological Anatomy and Oncology, University of Messina, Messina, Italy
E-mail: aallegra@unime.it

[*]Contributed equally

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1. Inflammation and cancer

The concept that chronic inflammation facilitates malignant growth was originally proposed by Virchow in the late 1800's (1). In fact, chronic inflammation is considered to be one of the hallmarks of tumor initiation and progression. Moreover, long-term production and accumulation of inflammatory factors lead to local and systemic immunosuppression associated with cancer progression. Epidemiological studies offer strong support for this concept. For example, the risk of some organ-specific cancers, particularly colorectal cancers, is significantly higher in individuals with chronic inflammation of the target organ (2-4).

Moreover, the correlation between inflammatory mediators, and the clinical outcome of malignant patients has been frequently investigated. Inflammatory components have been shown to induce DNA damage, which contributes to genetic instability and transformed cell proliferation; to promote angiogenesis and thereby enhance tumor growth and invasiveness; and to impair myelopoiesis and hemopoiesis, which cause immune dysfunction and inhibit immune surveillance (5-7). However, the mechanisms by which inflammation mediates its effects are not well understood, and the mechanisms behind this interaction have remained elusive.

Chronic inflammation is clearly involved in shaping the tumor microenvironment and has been referred to as 'host reaction' to the tumor, although it might be more appropriate to think of it as a 'tumor promoting' reaction. Changes occurring in the microenvironment of the progressing tumor resemble the process of chronic inflammation, which begins with ischemia followed by interstitial and cellular edema, appearance of immune cells, growth of blood vessels and tissue repair, and development of inflammatory infiltrates (8).

Among the factors that determine the nature of inflammatory infiltrates found in the tumor microenvironment

is the hypoxic environment. It is created early in tumor development through activation of hypoxia-responsive genes in tumor cells (9). It favors the influx of those inflammatory cells that depend on the glycolytic pathway for survival, namely, phagocytic macrophages and granulocytes (8). These cells not only survive in the hypoxic environment but contribute to it by hyperproduction of ROS upon local activation (10). Moreover, genetic alterations in oncogenes and tumor-suppressor genes, or epigenetic changes in the tumor that modulate tumor growth and invasion into the surrounding tissue orchestrate the persistence of inflammatory infiltrates. These cellular infiltrates modulate tumor development and progression. The tumor infiltrates vary by size and composition in diverse tumor types and at different stages of tumor development. The tumor programs the cellular infiltrates to sustain a dysregulated inflammation that is hyporesponsive to the tumor. Characterization of the complex interactions among the infiltrates and tumor will aid in defining their role in tumor progression (11).

An initial goal of the inflammatory response is to destroy an invader, which in this case is the tumor. Therefore, the 'immune phase' of tumor-driven inflammation involves a recruitment and influx of antitumor effector cells to the tissue site. However, compared with cellular and humoral responses that are generated in tissues upon infections by exogenous pathogens, those mediated by the tumor are weak. This is probably because most tumor-associated antigens are considered 'self', in contrast to infections with bacteria or viruses which are viewed by the host as 'danger signals' (12).

With respect to anticancer immune responses, many studies have shown that host immune competence in both innate immunity and adaptive immunity is important for cancer prevention, cancer immunosurveillance and the control of cancer progression (13,14). Indeed, in many instances tumors can escape the host immune response (Table I). Many mechanisms of tumor escape operating in the tumor microenvironment have been proposed. Low expression of molecules on tumor cells involved in tumor target cell recognition; absence of costimulation leading to tolerization of T cells; soluble factors secreted by tumor cells inhibiting T cell response and regulatory T cells, and stromal cells may impair immune-cell responses to tumors. Furthermore, tumors can release soluble molecules such as HLA-I (sHLA-I). This, in turn, reduces T cell-mediated immune response and induces apoptosis of cytolytic effector cells such as natural killer and $CD8^+$ T lymphocytes through the engagement of HLA-I receptors such as CD8 and/or activating isoforms of the inhibitory receptor superfamily. The release of soluble ligands for activating receptors, e.g. IL-16-binding proteins and/or MHC class I-related proteins A and B, the natural ligands of NKG2D, may impair activation, effector cell-mediated recognition and cytolysis of tumor cells. Furthermore, the elimination of antitumor effector cells may be achieved by induction of apoptosis consequent to triggering elicited via activating molecules, such as receptors responsible for natural cytotoxicity, upon their binding with ligands expressed on tumor cells (15).

Immunologic anergy is a common observation in patients and rodents with cancer. This tumor-induced phenomenon may block the potential therapeutic benefit of immunotherapy. In the 1980's, a new cell population known as natural suppressor cells, distinct from T and NK cells, was described in tumor-bearing mice (16). Of the several mechanisms described to explain anergy, the accumulation of myeloid cells in the tumor, spleen, and peripheral blood of tumor-bearing mice and cancer patients has gained considerable interest (17-21).

Table I. Mechanisms of tumor escape.

Low expression of molecules involved in tumor recognition
Soluble factors inhibiting T cells
Tolerization of T cells
Apoptosis of cytolytic effector cells
Accumulation of myeloid-derived suppressor cells

Table II. Features of myeloid-derived suppressor cells.

Phenotypic characterization
$CD11b^+CD33^+CD34^+CD14^-$
$CD14^+CD11b^+HLA\text{-}DR^{low/neg}$
Ability to suppress T cells
Inhibition of NK cell activity
Inhibition of IL-2 utilization by NK cells
Development of Treg cells

In fact, recently, a population of suppressive $CD11b^+Gr\text{-}1^+$ cells has been designated as myeloid-derived suppressor cells (MDSCs) (14). MDSCs are a unique category of the myeloid lineage, and they induce the prevention of the development of cytotoxic T lymphocytes (CTL) *in vitro* (18), and induction of antigen-specific $CD8^+$ T-cell tolerance *in vivo* (16). When cultured *in vitro* in the presence of appropriate growth factors immature myeloid cells are differentiated into dendritic cells (DCs) or macrophages (22-24).

Generated in bone marrow under the influence of soluble factors produced by tumors, these cells are derived from a mixed population of myeloid cells found at different differentiation stages (25).

2. Immature myeloid-derived suppressor cells

Myeloid cells are the most abundant hematopoietic cells in the human body and have diverse functions. Mounting evidence indicates that the tumor microenvironment alters myeloid cells and the concept of MDSCs has emerged (26,27).

MDSCs are a heterogeneous population of immature myeloid cells consisting of myeloid progenitors and precursors of macrophages, granulocytes and DCs (Table II). In mice, MDSCs are identified by antibodies that detect cell surface expression of Gr1 and CD11b. Gr1 includes the macrophage and neutrophil markers Ly6C and Ly6G, respectively, while CD11b is characteristic of macrophages. More recently, MDSCs have been subdivided into different subtypes based on their expression of Ly6C and Ly6G. $CD11b^+Ly6G^+Ly6C^{low}$ cells with granulocytic-like morphology and multilobed nuclei are called granulocytic MDSCs, whereas $CD11b^+Ly6G^-Ly6C^{hi}$

cells with monocytic-like morphology are referred to as (Mo)-MDSCs (28).

In cancer patients, MDSCs are identified by surface expression of the myeloid marker CD33 and the lack of expression of markers of mature myeloid and lymphoid cells. They are typically $CD11b^+CD33^+CD34^+CD14^-$ cells that vary in CD15, CD124, CD66 and MHC class II expression, along with other markers (29). Some authors also use CD31 (also known as platelet/endothelial cell adhesion molecule 1 or ER-MP12) antibodies to further identify these cells (22). CD31 is present on myeloid precursor cells but is downregulated on mature macrophages and neutrophils.

(Mo)-MDSCs with a phenotype of $CD14^+CD11b^+HLA\text{-}DR^{low/neg}$ in melanoma patients (30) and a phenotype of $CD11b^+CD14^-CD15^+CD33^+$ in non-small cell lung cancer (NSCLC) patients (31,32) have been identified. Due to the variation in MDSC gene expression between different tumor microenvironments, it has been challenging to identify a unique set of markers for human MDSCs. Thus, along with phenotypic characterization, the functional ability of MDSCs to suppress T cells is the defining hallmark of a MDSC.

Myeloid suppressive cells have also been called 'immature myeloid cells', 'inhibitory macrophages' and 'early myeloid cells' (33). Such immature myeloid cells are present in the bone marrow and spleen of healthy mice, and differentiate into mature myeloid cells under normal conditions (23). The accumulation of $Gr\text{-}1^+CD11b^+$ cells in large numbers of tumor-bearing mice probably results from various tumor-derived factors. Release of interleukin (IL)-10 (34), transforming growth factor-β (35), IL-6 (36), vascular endothelial growth factor (37), prostanoids (38), prostaglandin E2 (39) and stromal-derived factor-α (40) by tumors has been implicated in preventing the differentiation and maturation of immunoregulatory cells and hampering the induction of antitumor immunity. These cells are also associated with immune suppression during viral infection, transplantation, UV irradiation and cyclophosphamide (CTX) treatment (6).

It has also been shown that the accumulation of MDSCs within either the tumor microenvironment or peripheral blood correlates with a poor prognosis (41,42).

3. Inflammation, cancer and immature myeloid suppressor cells

Whereas an acute inflammatory response is vital for the immediate immune defense against pathogens and for the clearance of abnormal self-cells and molecules, a chronic inflammatory response could be detrimental to the host under conditions in which the host is unable to clear the pathogen because of the developing immunosuppression. Thus, the delicate balance between inflammatory response and its extent is critical to a normal or immunosuppressed immune system.

Many studies have shown that inflammatory environments induce the production and the accumulation of MDSCs which are able to block CD4 and $CD8^-$ immune responses, and lead to cancer development. Indeed, tumor cells secrete a large variety of cytokines that allow the recruitment of MDSCs in lymphoid organs or peripheral blood and direct their differentiation into suppressor cells (23). That global inflammation controls MDSC recruitment is best illustrated by observations showing that the reduction of inflammatory potential in $IL\text{-}1R^{-/-}$ mice allows the delay of MDSC accumulation thus reducing tumor and metastatic growth (43).

The IL-1β-induced MDSCs described are effective suppressors of $CD4^+$ and $CD8^+$ T lymphocytes. The finding that IL-1β upregulates MDSC accumulation in tumor-bearing mice has led to propose the following causal relationship linking chronic inflammation with tumor progression: as tumor cells proliferate they induce an inflammatory microenvironment consisting of IL-1β and other proinflammatory mediators. The persistence of these mediators causes the accumulation and retention of MDSCs. The MDSCs, in turn, initiate and maintain an immune suppressive state that block immune surveillance, thereby facilitating the survival and proliferation of transformed cells (44).

Jiang *et al* (45) performed a complex analysis of various inflammatory factors in the peripheral blood of patients suffering from malignant melanoma of different stages. They demonstrated that levels of serum IL-1β, IFN-γ and CXCL10 were significantly increased in advanced melanoma patients. In addition, these factors were found to be associated with an increased frequency of MDSCs as compared to age- and gender-matched healthy donors. Importantly, advanced melanoma patients with signs of progression displayed markedly elevated concentrations of IL-1β and CXCL10 as compared to patients with stable disease. Moreover, an enrichment of circulating (Mo)-MDSCs was significantly correlated with a decreased progression-free survival of these patients.

Chronic inflammation associated with infectious agents, such as schistosomiasis and *Helicobacter pylori*, is also thought to predispose to malignancy, and some of these agents are associated with MDSC accumulation (46,47). Collectively, these published observations plus other reported results support the hypothesis that chronic inflammation facilitates tumor growth by inducing MDSCs that downregulate immune surveillance, thereby providing an environment favorable for tumor progression. However, other cytokines beyond IL-1 may play a significant role in the MDSCs in cancer patients.

Kohanbash *et al* (48) hypothesized a causal role for IL-4Rα and myeloid suppressor cells in glioma development. In both mouse *de novo* gliomas and human glioblastoma cases, IL-4Rα was upregulated on glioma-infiltrating myeloid cells but not in the periphery or in the normal brain. Mice genetically deficient for IL-4Rα exhibited a slower growth of glioma associated with reduced production in the glioma microenvironment of arginase, a marker of myeloid suppressor cells which is critical for their T cell inhibitory function. Supporting this result, investigations using bone marrow-derived myeloid cells showed that IL-4Rα mediates IL-13-induced production of arginase. Furthermore, glioma-derived myeloid cells suppressed T cell proliferation in an IL-4Rα-dependent manner, consistent with their identification as MDSCs. Granulocyte-macrophage colony-stimulating factor (GM-CSF) plays a central role in the induction of IL-4Rα expression on myeloid cells, and it was found that GM-CSF is upregulated in both human and mouse glioma microenvironments compared with normal brain or peripheral blood samples. Together, these findings establish a GM-CSF-induced mechanism of immunosuppression in

the glioma microenvironment via upregulation of IL-4Rα on MDSCs (48).

TNF is also a key inflammatory cytokine regulating the immune system. TNF is well known to cause inflammatory reactions such as tissue injury in autoimmune diseases mainly by activation of TNF receptor type 1 (TNFR1). Accordingly, blockade of TNF in patients with chronic inflammation and autoimmune diseases is currently being used therapeutically. However, clinical observations in patients after treatment with TNF antagonists indicated that TNF has more complex immune regulatory properties. MDSCs seem to be affected by TNF and TNFR2. TNF-dependent immune suppression was correlated with the development of functional MDSCs in a model of chronic inflammation induced by repetitive application of BCG (49). TNFR2 expression is required for MDSC accumulation during tumor growth and TNFR2 signaling is necessary and sufficient for protection of MDSCs from apoptosis (50).

Other factors may provide the link through which inflammation can contribute to cancer. Proinflammatory S100 proteins promote MDSC accumulation and suppressive activity (51,52). The S100A8 and S100A9 proteins are members of a large family of proteins that includes inflammatory molecules. Heterodimeric S100A8/A9 complexes are calcium-binding proteins that are released by neutrophils, activated monocytes, tumor cells and MDSCs. MDSCs have receptors for S100A8/A9 complexes and enhance the levels of S100A8/A9 in the tumor microenvironment through an autocrine loop. Antibody blockade of the receptors reduces the number of MDSCs in the tumors and secondary lymphoid organs of tumor-bearing mice. Mice genetically deficient for S100A9 are resistant to challenge with colon carcinoma but become susceptible if MDSCs are adoptively transferred from wild-type mice to S100A9-deficient mice. S100A8/A9 heterodimers mediate these effects through at least two mechanisms: they block the differentiation of myeloid precursors to differentiated DCs and macrophages through a STAT3-dependent mechanism, and they chemoattract MDSCs into the tumor through a NF-κB-dependent pathway (51,52).

Toll-like receptors (TLRs) play a major role in the induction of innate and adaptive immune system suppression; repetitive administration of single TLR 2, 3, 4 or 9 agonists, which do not exhibit any virulent or immune invasive properties, was sufficient to induce a bystander NK- and T-cell immunosuppression associated with ζ chain downregulation mediated by MDSCs (53). Vaknin *et al* (54) identified a 35-amino acid region within the ζ chain as being responsible for its degradation under TLR-mediated chronic inflammation. Furthermore, they provide evidence that ζ chain levels could serve as a biomarker for chronic inflammation-dependent immunosuppression.

Downregulation of TCR ζ chain expression associated with T cell dysfunction was described in various pathologies, such as advanced cancer (55-61).

The fact that pathologies that differ in their physiology and etiology show decreased ζ chain expression and exhibit T cell dysfunction suggests the existence of a common denominator linking these conditions.

Ezernitchi *et al* (62) demonstrated that under chronic inflammation, secondary lymphatic organs display various immunological milieus; ζ chain downregulation and T cell dysfunction are induced in the spleen, peripheral blood and bone marrow, but not in lymph nodes, correlating with elevated levels of $Gr1^+Mac\text{-}1^+$ MDSCs.

Sustained exposure to Ag that induces chronic inflammatory conditions differentially affects various secondary lymphatic organs; while T cells in the lymph nodes remain functional and express normal ζ chain levels, cells in the spleen, peripheral blood and bone marrow are negatively affected and reduce ζ expression levels and their function is impaired (63,64).

4. Myeloid-derived suppressor cells and cancer

Several studies have shown that the number of MDSCs is markedly increased in the peripheral blood of tumor-bearing animals and of patients with cancer, whereas the number of DCs are decreased (65). Upregulation of STAT-3 and secretion of tumor-derived cytokines, such as VEGF, TGF-β, GM-CSF, IL-10 and prostaglandin, have been shown to arrest differentiation of adenomatous polyposis coli (APC) from their myeloid progenitors and trigger accumulation of MDSCs (66,67).

Expansion of MDSCs should stem from the ability of tumors to secrete myeloid-influencing factors (68). In particular, GM-CSF recruits MDSCs into lymphoid secondary organs and suppresses antigen-specific T cells when produced by gene-modified cancer cells or when administered exogenously in tumor-bearing mice. This evidence raises the possibility that GM-CSF, a cytokine broadly used in cancer patients for its properties on bone marrow mobilization and immune functions, may promote the expansion of myeloid suppressive components, with negative consequences on tumor antigen-specific immune responses (69,70).

As many as 45% of tumors have infiltrates that are predominantly of the $GR1^+$ $CD11b^+$ immature myeloid phenotype. This has been recently reported for murine glioblastoma (71) and colon (72) cancer models (Table III).

A similar condition has been detected in other neoplastic diseases. Melanoma is considered the prototype of immunogenic tumors in humans. However, a role of the immune system in controlling disease may be claimed only in the initial phases, when the presence of T cells deeply infiltrating tumor lesions favorably impacts prognosis and reduces recurrence risk. With disease progression, immune responses start displaying functional defects and may even turn into mere indicators of tumor burden. In mouse models, these deficiencies are, at least in part, attributed to the accumulation of early differentiated myeloid cells (6).

Similarly, melanoma of the eye's uveal tract is a rare, aggressive cancer with a high mortality rate because of the development of metastatic disease, primarily in the liver, that almost invariably is refractory to therapy. Immune mechanisms have been implicated in uveal melanoma progression, and MDSCs have been identified in the tumors or blood of patients (73).

These cells are significantly and reproducibly increased in melanoma patients compared with healthy donors and are further expanded after administration of an antitumor vaccine that includes GM-CSF. Conversely, melanoma patients do not

Table III. Cancer models with increased myeloid-derived suppressor cells.

Cancer	Authors	Reference
Melanoma	Serafini *et al*	(6)
	Kusmartsev and Gabrilovich	(5)
Glioblastoma	Fujita *et al*	(71)
Colon cancer	Mundy-Bosse *et al*	(72)
Head and neck, lung and breast cancer	Almand *et al*	(79)
Pancreatic tumors	Tinder *et al*	(80)

Table IV. Mechanisms of action of myeloid-derived suppressor cells.

Arginase 1 synthesis
Nitric oxide production
Peroxynitrite production
Loss of CD3ζ signaling in T cells
Downregulation of T cell L-selectin
Inhibition of IFN-γ and NKG2D expression on NK cells
Pro-angiogenic factors
Induction of matrix metalloproteinases
Cysteine/cystine deprivation

present any of the myeloid alterations described in patients affected by other cancer histotypes, which include expansion of lineage-negative cells, myeloid CD34 precursors, and immature cells expressing both monocyte (i.e. CD11b) and granulocyte (i.e. CD15) markers (74,75).

It is therefore certain that the development of metastasis in uveal melanoma is associated with changes in immune effector and regulatory cells consistent with lessening tumor immune surveillance, and these changes are associated with changes in plasma and cellular levels of immune regulatory miRs (76).

Also similar to many human cancers, 4T1 mammary carcinoma induces a profound immune suppression, which can be partially reversed if the primary tumor is removed (77). The finding that $CD1^{-/-}$ mice, whose primary tumors are surgically removed survive indefinitely despite the presence of metastatic disease, has led to hypothesize that immune surveillance is blocked in wild-type mice. It is now clear that the immune suppression present in mice with 4T1 primary tumors is also mediated by $Gr1^{+}CD11b^{+}$ MDSCs (78).

An accumulation of phenotypic MDSCs was also found in the peripheral blood of patients with head and neck, lung and breast cancer (79). Finally, the increased proinflammatory milieu correlates with an increased percentage of myeloid suppressor cells in pancreatic tumors and tumor draining lymph nodes (80).

5. Myeloid-derived suppressor cell-induced immunosuppression mechanisms

Abundant evidence exists to indicate that tumor-specific T cells undergo inhibitory regulation and become anergic in tumor-bearing hosts (81-83). MDSCs are capable of inhibiting the T-cell proliferative responses induced by alloantigens (84), CD3 ligation (85) or various mitogens (86), and can also inhibit IL-2 utilization by NK cells as well as NK cell activity (87). Moreover, T-cell inactivation by MDSCs *in vitro* can be mediated through several mechanisms: IFN-γ-dependent nitric oxide (NO) production (88), Th2-mediated IL-4/IL-13-dependent arginase-1 (Arg-1) synthesis (18), loss of CD3ξ signaling in T cells (89) and suppression of the T-cell response through reactive oxygen species (90-92) (Table IV).

MDSC-mediated downregulation of T-cell L-selectin (CD62L) further impairs T-cell activity. CD62L is a plasma membrane molecule necessary for the homing of naive T cells to lymph nodes for activation by tumor antigens. MDSCs downregulate CD62L on naive T cells, which reduce the T cell capacity to migrate to lymph nodes (93).

MDSCs can also inhibit NK cell activity through membrane-bound TGF-β1, resulting in inhibition of IFN-γ and NKG2D expression (94). The effect shows a high efficacy since the addition *in vitro* of only 3% of MDSCs was able to completely block T-cell proliferation.

As mentioned above, MDSCs induce immunosuppression through two enzymes involved in arginine metabolism: inducible NO synthetase 2, which generates NO, and Arg-1, which depletes L-arginine, generating amino acid starvation for T-cell activation (21).

MDSCs producing high levels of Arg-1 block T-cell function by depleting L-arginine in cancer, chronic infections, and trauma patients. However, the mechanisms that induce Arg-1 in MDSCs in cancer are unknown. Using the 3LL mouse lung carcinoma, Rodriguez *et al* (95) characterized these mechanisms. Arg-1 expression was independent of T-cell-produced cytokines. Instead, tumor-derived soluble factors resistant to proteases induced and maintained Arg-1 expression in MDSCs.

Moreover, 3LL tumor cells constitutively express cyclooxygenase (COX)-1 and COX-2, and produce high levels of PGE_2. Genetic and pharmacological inhibition of COX-2, but not COX-1, blocked Arg-1 induction *in vitro* and *in vivo*. Signaling through the PGE_2 receptor E-prostanoid 4 expressed in MDSCs induced Arg-1. Furthermore, blocking Arg-1 expression using COX-2 inhibitors elicited lymphocyte-mediated antitumor responses (95).

Peripheral blood mononuclear cells from 123 patients with metastatic renal cell carcinoma (RCC), prior to treatment, were found to have a significantly increased arginase activity. These patients had markedly decreased cytokine production and expressed low levels of T-cell receptor CD3ζ chain. Cell separation studies showed that the increased arginase activity was limited to a specific subset of $CD11b^{+}$, $CD14^{-}$ and $CD15^{+}$ cells with a polymorphonuclear granulocyte morphology and markers, instead of macrophages or DCs described in mouse models. Furthermore, these patients had low levels of arginine and high levels of ornithine in plasma. Depletion of the $CD11b^{+}$ and $CD14^{-}$ myeloid suppressor cells re-established T-cell proliferation and CD3ζ chain expression (29).

How low does arginine have to be to impair T-cell function? Arginine concentrations <60 μmol/l decreased T-cell proliferation, cytokine production and CD3ζ expression *in vitro* (96). Trauma patients, who experience rapidly increased arginase activity in PBMCs, have profoundly depleted arginine to 0-50 μmol/l (normal levels range 50-150 μmol/l), resulting in T-cell anergy and loss of CD3ζ (97,98). Therefore, arginase-producing cells may decrease arginine levels in the circulation, a phenomenon that may be more profound in the tumor or lymphoid organ microenvironment where these cells are also found.

Unfortunately, replenishment of arginine alone does not seem to be a simple solution. Although arginine replenishment does reestablish CD3ζ chain expression, it may also stimulate tumor growth (24,99). Therefore, inhibition of the signals that induce Arg-1 in these cells may be an alternative approach.

However, Huang *et al* (145) proposed a novel mechanism by which tumor-induced MDSCs can suppress tumor-specific T-cell responses. MDSCs mediate the development of Treg cells, which can induce and maintain T-cell tolerance in tumor-bearing hosts. Furthermore, Treg induction and NO-dependent suppressive activity mediated by MDSCs seem to be independent pathways since iNOS-deficient MDSCs lost *in vitro* suppressive activity but could still induce the development of Tregs both *in vitro* and in tumor-bearing mice (24,100).

Moreover, it was observed that induced immunosuppression is the consequence of a chronic inflammatory response associated with a dramatic enlargement and disrupted architecture of the spleen attributable to the abnormal accumulation of MDSCs. Immunohistochemical staining of spleen sections from LPS-treated mice revealed that MDSCs tightly border and in some regions invade the T-cell zones. The observed *in vivo* intimate interaction between MDSCs and T cells in the affected spleens supports *ex vivo* data demonstrating that MDSCs confer their immunosuppressive effect on contact or close proximity with T cells. Co-incubation in the presence of a Transwell was found to abrogate the immunosuppressive effect of the MDSCs (62,101). There is recent data to suggest that these cells also contribute to tumor angiogenesis (102).

MDSCs can directly incorporate into tumor endothelium. They secret many pro-angiogenic factors as well. In addition, they play an essential role in cancer invasion and metastasis through inducing the production of matrix metalloproteinases (MMPs) and chemoattractants and creating a pre-metastatic environment (103).

Moreover, increasing evidence supports the idea that cancer stem cells (CSCs) are responsible for tumorigenesis, resistance to therapies, invasion and metastasis (103). Ye *et al* (104) hypothesized that CSCs may 'hijack' MDSCs for use as alternative niche cells, leading to the maintenance of stemness and enhanced chemotherapy and radiotherapy resistance. The countermeasure that directly targets MDSCs may be useful against angiogenesis and for preventing cancer from invasion and metastasis.

More recently, peroxynitrite has emerged as a key mediator of T-cell function suppression by MDSCs. Indeed, peroxynitrite is a product of a chemical reaction between NO and the superoxide anion, and is one of the most powerful oxidizers. It induces amino acid nitration and nitrosylation such as cysteine, methionine, tryptophan, and tyrosine. High levels of peroxynitrite have been found in areas where inflammatory cells and MDSCs accumulate. These high levels of peroxynitrite have also been associated with tumor progression in many types of cancer which have been linked to the absence of T-cell responses (105-107).

It appears that the peroxynitrite production by MDSCs during direct contact with T cells leads to TCR and CD8 molecule nitration, changing the specific binding peptide of T cells and making them intensive to specific antigen stimulation. Also, it has been shown that MDSCs are able to induce TCR/CD3ζ complex disruption through tyrosine nitrosylation/nitration, partly through NADPH oxidase 2 activity (108,109).

Finally, two studies identified a new mechanism of suppression based on modulation of local amino acid metabolism and homeostasis. This mechanism, shared with FoxP3$^+$ Tregs is called cysteine/cystine deprivation (110,111).

6. Therapeutical regulation of immature myeloid suppressor cells

Regardless of the mechanism, it has been proposed that elimination of MDSCs will likely be a valuable strategy to lessen tumor-induced immunosuppression, improve antitumor responses and enhance the effects of cancer immunotherapy (112). To date, however, there have been few practical approaches (Table V).

Salvadori *et al* (113) showed that surgical resection of large tumors led to a very rapid loss of Gr-1$^+$/CD11b$^+$ splenocytes with restoration of some antitumor immunity. Unfortunately, surgical removal of most metastatic tumors is not feasible.

Earlier experiments demonstrated that depletion of murine Gr-1$^+$ cells significantly improved CD8$^+$ T-cell immune response and allowed for eradication of tumors (114), while a more recent study demonstrated that depletion of Gr-1$^+$ myeloid cells *in vivo* prevented tumor recurrence (115).

Depletion of myeloid suppressor cells using anti-Gr-1 antibodies has been suggested. However, this also depletes all the mature granulocytes leading to severe immunosuppression. This depletion is also followed by a rapid rebound of cells (112).

Different approaches that target MDSCs are currently being explored in tumor-bearing hosts. They can be divided based on their ability to control MDSC differentiation into mature DCs and macrophages capable of APC activity, MDSC maturation from precursors, MDSC proliferation, MDSC accumulation, MDSC cytotoxicity, or MDSC function/activation (116).

One approach to therapeutic targeting of MDSCs is the use of agents that promote the differentiation of myeloid cells. Nefedova *et al* (117) demonstrated that differentiating MDSCs to DCs and macrophages by using all-*trans*-retinoic acid (ATRA) reduced MDSC numbers and augmented the responses to cancer vaccines. Mirza *et al* (118) also tested the possibility of pharmacological regulation of myeloid cell differentiation using ATRA. They observed that ATRA dramatically reduced the number of MDSCs. Moreover, ATRA significantly improved the myeloid/lymphoid DC ratio and the ability of mononuclear cells of patients to stimulate allogeneic T cells. This effect was associated with significant

Table V. Approaches to therapeutic targeting of myeloid-derived suppressor cells.

Therapy	Agent
Differentiation of myeloid cells	ATRA, IL-4
Inhibition of COX-2 enzyme activity	Celecoxib
Chemotherapeutic agents	Gemcitabine
Inhibition of IL-1	
Sunitinib	
Sildenafil	
c-kit ligand inhibition	
N-acetyl cysteine	
Polyamine-blocking therapy	

COX, cyclooxygenase; ATRA, all-*trans*-retinoic acid.

improvement in the tetanus-toxoid (T-T) specific T-cell response (119).

Selective inhibitors of COX-2 enzyme activity have shown chemopreventive activity in carcinogen-induced and transgenic rodent tumor models and clinically for colon cancer. However, the mechanisms by which COX-2 inhibitors reduce carcinogenesis remains controversial. Talmadge *et al* (119) reported that administration of the selective COX-2 inhibitor, celecoxib, significantly reduced the number of $Gr1^+CD11b^+$ MDSCs during chemoprevention of 1,2-dimethylhydrazine diHCl (1,2-DMH) induction of large intestinal tumors in Swiss mice. The 1,2-DMH induction of large intestinal tumors was associated with a 4-fold increase in MDSCs, and a decrease in splenic T-cell number and function. In addition to delaying tumor induction, reducing tumor number and increasing lymphocyte infiltration of tumors, celecoxib therapy reversed $CD4^+$ T-cell loss, decreased MDSC numbers and increased mRNA levels of nitric oxide synthase-2 (NOS-2) and arginase in the spleen (120).

Chemotherapeutic drug gemcitabine, given at a dose similar to the equivalent dose used in patients, was able to dramatically and specifically reduce the number of MDSCs found in the spleens of animals bearing large tumors with no significant reductions in $CD4^+$ and $CD8^+$ T cells, and NK, macrophages or B cells. The loss of MDSCs was accompanied by an increase in the antitumor activity of $CD8^+$ T cells and activated NK cells. Combining gemcitabine with cytokine immunogene therapy using IFN-β markedly enhanced antitumor efficacy (120).

Although it is clear that gemcitabine was able to eliminate the majority of splenic $Gr\text{-}1^+/CD11b^+$ cells in tumor-bearing animals, the mechanism by which this occurred is not known. One possibility was that gemcitabine induced a massive efflux of these cells into the blood and into other organs. Another possibility would be selective killing of the $Gr\text{-}1^+/CD11b^+$ cells. *In vitro* studies indicate that gemcitabine accelerates the death of $Gr\text{-}1^+/CD11b^+$ cells without affecting the numbers of $CD4^+$ and $CD8^+$ T cells or B cells. Using flow cytometry, it was possible to show a significantly increased rate of apoptosis in splenocytes at specific time-points after gemcitabine treatment. It is possible that gemcitabine induced differentiation of the $Gr\text{-}1^+/CD11b^+$ cells into more mature cells (121).

To note, not all chemotherapeutic agents have this action. The alkylating agent CTX has been included in various chemoimmunotherapy regimens because of its well-known immunostimulatory effects. Paradoxically, CTX can also induce suppressor cells that inhibit immune responses. However, the identity and biologic relevance of these suppressor cells are poorly defined. Jiang *et al* (124) reported that CTX treatment drives the expansion of inflammatory monocytic myeloid cells ($CD11b^+Ly6C^{hi}CCR2^{hi}$) that possess immunosuppressive activities. In mice with advanced lymphoma, adoptive transfer (AT) of tumor-specific $CD4^+$ T cells following CTX treatment (CTX+CD4 AT) provoked a robust initial antitumor immune response, but also resulted in enhanced expansion of monocytic myeloid cells. These therapy-induced monocytes inhibited long-term tumor control and allowed subsequent relapse by mediating functional tolerization of antitumor $CD4^+$ effector cells through the PD-1-PD-L1 axis. PD-1/PD-L1 blockade after CTX+CD4 AT therapy led to persistence of $CD4^+$ effector cells and durable antitumor effects. In addition to CTX, it was found that melphalan and doxorubicin can also induce (Mo)-MDSCs. These findings reveal a counter-regulatory mechanism elicited by certain chemotherapeutic agents and highlight the importance of overcoming this barrier to prevent late tumor relapse after chemoimmunotherapy (122).

It is well known that proinflammatory IL-1 promotes the development of MDSCs, and IL-1 may play a role in promoting uveal melanoma progression. Inhibiting IL-1 with IL-1Rα inhibits tumor growth *in vivo* but not *in vitro*. Tumor stroma is modified, MDSCs are reduced and M1 macrophage polarization is increased *in vivo* (123).

Interleukin-4 (IL-4), a cytokine closely associated with the differentiation of myeloid cells, was expressed locally at the tumor site with its dose and expression time tightly regulated by a Tet-Off system. Early exposure of high-dose IL-4 to the tumor stromal cells effectively prevented the generation of myeloid suppressor cells and led to a T-cell-mediated tumor rejection. However, IL-4 had no effect several days after tumor growth, when myeloid suppressor cells had been generated and T cells were tolerized. Importantly, co-inoculation of IL-4 receptor (IL-4R)-deficient tumor cells with IL-4R competent, but not IL-4R-deficient myeloid cells led to IL-4-mediated tumor regression in IL-4R-deficient mice, indicating that IL-4 acts directly on myeloid cells (124).

Several studies have evaluated the effect of the TKI sunitinib on MDSCs. The administration of sunitinib, a receptor TKI inhibitor, has been shown to reduce the frequency of MDSCs and reverse T-cell immune suppression in the peripheral blood of patients with metastatic RCC and in several murine tumor models. However, sunitinib has a variable impact in reducing MDSCs and restoring T-cell activity in the tumor microenvironment, which seems to be tumor-dependent (125,126).

Pan *et al* (127) demonstrated that the expression of c-kit ligand [stem cell factor (SCF)] by tumor cells may be important for MDSC accumulation in tumor-bearing mice, and that blocking the c-kit ligand-c-kit receptor interaction can reverse MDSC-mediated immune suppression. Mice bearing tumor cells with SCF siRNA knockdown exhibited significantly reduced MDSC expansion and restored proliferative responses of tumor-infiltrating T cells. The blockade of SCF receptor

(c-kit)-SCF interaction by anti-c-kit antibodies prevented tumor-specific T-cell anergy, Treg development and tumor angiogenesis (127).

N-acetylcysteine (NAC) has been proposed as an antitumorigenic agent due to its ability to reduce the oxidative stress that promotes genetic instability (128). NAC may have the additional benefit of facilitating T-cell activation by increasing extracellular pools of cysteine in the presence of high levels of MDSCs in cancer patients. Although NAC targets the cysteine pathway of MDSC-mediated T-cell suppression, MDSC production of arginase and NO can still maintain the suppressive effects of MDSCs. However, administration of NAC, in combination with other agents that block additional MDSC suppressive pathways (Arg-1 and NO), may be more effective at inhibiting MDSCs and facilitate the treatment of cancers (128).

Polyamine elevation in cancer, a common metabolic aberration in aggressive lesions, contributes significantly to tumor immunosuppression, and a polyamine depletion strategy can exert antitumor effects that may also promote immunity. A polyamine-blocking therapy (PBT) that combines the well-characterized ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO) with AMXT 1501, a novel inhibitor of the polyamine transport system, blocked tumor growth in immunocompetent mice but not in athymic nude mice lacking T cells. PBT had little effect on the proliferation of epithelial tumor cells, but it increased the number of apoptotic cells. Analysis of $CD45^+$ tumor immune infiltrates revealed that PBT decreased levels of $Gr\text{-}1^+CD11b^+$ myeloid suppressor cells and increased $CD3^+$ T cells. Strikingly, in a model of neoadjuvant chemotherapy, mice administered with PBT one week before surgical resection of engrafted mammary tumors exhibited resistance to subsequent tumor rechallenge (129).

Polyphenon E reduced the number of tumor-infiltrating myeloid cells, and inhibited the development of spontaneous neuroblastomas in TH-MYCN transgenic mice. In therapeutic models of neuroblastoma in A/J, but not in immunodeficient NOD/SCID mice, Polyphenon E inhibited tumor growth by acting on MDSCs and CD8 T cells. *In vitro*, Polyphenon E impaired the development and motility of MDSCs and promoted differentiation to more neutrophilic forms through the 67-kDa laminin receptor signaling and induction of GM-CSF. The proliferation of T cells infiltrating a patient metastasis was reactivated by Polyphenon E (130).

Phosphodiesterase-5 (PDE5) inhibitors (sildenafil) are agents currently in clinical use for nonmalignant conditions. Sildenafil treatment decreased the suppressive activity of MDSCs by downregulating Arg-1 and inducible NOS-2 expression. Sildenafil restored *in vitro* T cell proliferation of PBMCs from multiple myeloma patients. By reverting MDSC suppression, sildenafil enhanced intratumoral T cell infiltration and reduced tumor outgrowth *in vivo* (131).

Treatment with aminobiphosphonate was shown to reduce MDSC expansion in tumors and peripheral blood by inhibiting MMP-9 (132). Since VEGF has been shown to block DC differentiation and maturation in preclinical models, high levels of VEGF in cancer patients may induce an accumulation of immature and functionally impaired DCs contributing to tumor escape from immunosurveillance. It was hypothesized that tumor-derived VEGF might exert its inhibitory effect at the stage of immature $HLA\text{-}DR^-$MDC precursors within the MDSC fraction blocking their development into pMDCs, while simultaneously skewing their differentiation towards a newly identified population of myeloid $CD14^+HLA\text{-}DR^{neg/low}$ suppressor cells with immunosuppressive traits (132).

VEGFR/PDGFR inhibitors have demonstrated clinical efficacy as a first-line therapeutic agent in the setting of a renal cell carcinoma, via mechanisms that include the suppression of angiogenesis and inhibition of MDSC and Treg function *in vivo* (134-142).

However, the therapeutic benefits of agents that regulate MDSCs are only evident when they are combined with immune therapy and not when they are administered alone. Thus, cancer immune therapy offers an attractive therapeutic addition, delivering treatment with high specificity, low toxicity and prolonged activity.

7. Conclusion and future perspectives

Priming of the adaptive immune response occurs during the early stage of tumor growth and results in development of $CD8^+$ T cells reactive to tumors (1). Despite evident host recognition of tumor antigens, coincident with or immediately subsequent to T-cell priming, the antitumor immune response is inadequate to eliminate the tumor and is eventually dampened, thereby leading to tumor escape. Understanding how cancer growth affects the antitumor immune response and discovering how escape from antitumor immunity can be reversed are major goals in tumor immunology (143).

Tumors escape immune attack by a variety of mechanisms, including differentiation and recruitment of immunosuppressive $CD11b^+Gr\text{-}1^+$ myeloid suppressor cells into the tumor microenvironment.

However, despite numerous studies on the subject, we are still far from a full understanding of the mechanisms regulated by these cells and even on the action that these cells may have in specific conditions. New data continuously accumulate and even on their exact identification confusion and doubt exist.

Hart *et al* (144) used the chemokine receptor CX3CR1 to identify distinct populations within the monocyte, macrophage and DC lineages. They found a population that is functionally distinct from the CX3CR1-positive cellular subsets within the $CD11b^+$ cellular compartment of ascites from ovarian tumor-bearing mice. They functionally identified $CX3CR1^-$ cells as myeloid suppressor cells and as a cellular subset with pathological specificity. Importantly, the $CX3CR1^-$ cells exhibit early IL-10 production in the ovarian tumor microenvironment, which was shown to be critically tied to suppression and additional MDSC accumulation.

Huang *et al* (145) identified a more specific population within Percoll fraction 2 MDSCs that expresses the myeloid markers Gr-1, CD115 (M-CSF receptor) and F4/80, which has much stronger suppressive activity compared with the classic $Gr\text{-}1^+CD11b^+$ MDSCs. Although $Gr\text{-}1^+$ immature myeloid cells from the spleens of tumor-bearing mice have been shown to suppress the proliferation of $CD4^+$ and $CD8^+$ T cells, the sorted Gr-1 single positive cells of Percoll fraction 2 did not suppress HA-mediated proliferation of CD4.

On the other hand, Tomihara *et al* (146) showed that $CD11b^+Gr\text{-}1^+$ cells found in ascites of epithelial ovarian cancer-bearing mice at advanced stages of disease were immunostimulatory rather than being immunosuppressive. Immunostimulatory $CD11b^+Gr\text{-}1^+$ cells can strongly cross-prime, augmenting the proliferation of functional CTLs via signaling through the expression of costimulatory molecule CD80. Adoptive transfer of these immunostimulatory $CD11b^+Gr\text{-}1^+$ cells from ascites of ovarian cancer-bearing mice resulted in the significant regression of s.c. tumors even without being pulsed with exogenous tumor Ag prior to AT.

Several observations of nonsuppressive $CD11b^+Gr\text{-}1^+$ cells have actually been reported. For example, activated NKT cells have been shown to induce the conversion of MDSCs to immunogenic APCs, presumably by producing soluble factors from activated NKT cells (147). It has also been shown that $Gr\text{-}1^+CD11b^+F4/80^+$ macrophage-like cells suppressed T-cell proliferation, but that $Gr\text{-}1^+CD11b^+F4/80^-$ neutrophil-like cells were not suppressive in a tumor-bearing mouse model (148). Even suppressive $Gr\text{-}1^+CD11b^+F4/80^+$ cells induced NK cell-mediated killing in an RMA-S tumor, whereas anti-Gr-1 mAb administration resulted in enhanced tumor growth. Interestingly, recent publications have demonstrated a clear distinction between the immunologic function of $CD11b^+Gr\text{-}1^{high}$ cells and $CD11b^+Gr\text{-}1^{int/low}$ cells isolated from spleens of either naive or tumor-bearing mice (149-151). In these cases, $CD11b^+Gr\text{-}1^{high}$ cells exhibited much less immunosuppressive function compared with $CD11b^+Gr\text{-}1^{int/low}$ cells. Immunostimulatory $CD11b^+Gr\text{-}1^+$ cells generated in ID8/ascites were morphologically similar to $CD11b^+Gr\text{-}1^{high}$ cells, but only immunostimulatory $CD11b^+Gr\text{-}1^+$ cells exhibited strong immunostimulatory properties with cross-priming. Furthermore, the expression pattern of surface molecules and a shift in side scatter in flow cytometry were dissimilar. Therefore, immunostimulatory $CD11b^+Gr\text{-}1^+$ cells may be a distinct population from $CD11b^+Gr\text{-}1^{high}$ cells.

The biological significance and clinical relevance of these cells remain to be determined. Finally, the discovery of new mediators of the action of MDSCs creates new possible intervention methods to eliminate the immune anergy that accompanies cancer.

SHIP is a 145-kDa protein that possesses 5'phosphatase activity, and thus can hydrolyze the 5'phosphate on phosphatidylinositol-3,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, which are products of PI3K activity (152). SHIP is primarily expressed in hemopoietic cells, but it can also be expressed in mouse embryonic fibroblasts. The SHIP locus also encodes a stem cell-specific isoform called s-SHIP that lacks the Src homology 2 domain and is expressed by pluripotent and tissue-specific stem cells. SHIP's role in signal transduction allows it to regulate cell survival, proliferation, apoptosis, and homeostasis of certain hemopoietic cell types, as well as primitive stem cell populations. Analysis of SHIP-deficient mice revealed that this protein also has a prominent role in the immune system. Significant pathologies have been observed in $SHIP^{-/-}$ mice, including splenomegaly and an infiltration of myeloid cells into the lungs that contributes to their reduced life span (153,154).

The induction of SHIP deficiency in adult mice leads to a rapid and significant expansion of MDSCs in peripheral lymphoid tissues. Consistent with expansion of MDSCs, splenocytes and lymph node cells from adult mice with induced SHIP deficiency are significantly compromised in their ability to prime allogeneic T-cell responses. These results demonstrated that SHIP regulates homeostatic signals for these immunoregulatory cells in adult physiology (155).

Similarly, TIMP-2 is a multifunctional protein, secreted into the extracellular matrix. TIMP-2 is a negative regulator of MDSCs with important implications for the immunotherapy and/or anti-angiogenic treatment of NSCLC (156). But unexpected new fields of study seem to continually open up to researchers.

The effectiveness of attenuated *Salmonella* in inhibiting tumor growth has been demonstrated in many therapeutic models, but the precise mechanisms remain incompletely understood. Kaimala *et al* (157) showed that the antitumor capacity of *Salmonella* depends on a functional MyD88-TLR pathway and is independent of adaptive immune responses. Since MDSCs play a critical role in tumor growth, they investigated the consequences of *Salmonella* treatment on myeloid cell recruitment, phenotypic characteristics, and functional activation in spleen and tumor tissue of B16 and F1 melanoma-bearing mice. *Salmonella* treatment led to increased accumulation of splenic and intratumoral $CD11b^+Gr\text{-}1^+$ myeloid cells, exhibiting significantly increased expression of various activation markers such as MHC class II, costimulatory molecules, and Sca-1/Ly6A proteins. Gene expression analysis showed that *Salmonella* treatment induced expression of iNOS, Arg-1 and IFN-γ in the spleen, but downregulated IL-4 and TGF-β. Within the tumor, expression of iNOS, IFN-γ and S100A9 was markedly increased, but Arg-1, IL-4, TGF-β and VEGF were inhibited. Functionally, splenic $CD11b^+$ cells maintained their suppressive capacity following *Salmonella* treatment, but intratumoral myeloid cells had significantly reduced suppressive capacity. Their findings demonstrated that administration of attenuated *Salmonella* leads to phenotypic and functional maturation of intratumoral myeloid cells making them less suppressive and hence enhancing the host's antitumor immune response (157).

In conclusion, MSDCs are metabolically plastic, evidenced by their ability to differentiate under the influence of select cytokines and differentiation factors into more mature cell types both *in vitro* and *in vivo*. Significantly, forced maturation of MDSCs *in vivo* was associated with enhancement of chemotherapy efficacy, suggesting a potentially novel therapeutic strategy. Therapeutic approaches directed toward the manipulation of the MDSC population and their function may improve immune-enhancing therapy for advanced malignancies.

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