Pathways Mediating the Expansion and Immunosuppressive Activity of Myeloid-Derived Suppressor Cells and Their Relevance to Cancer Therapy

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Abstract Cancer immunotherapy has focused on inducing and expanding CTLs and improving the immune recognition of weak antigenic determinants expressed by tumors. However, few positive clinical outcomes have been reported due, in part, to tumor-associated immunologic tolerance, supporting the need for an emphasis on overcoming immunosuppression. Systemic immunosuppression is associated with abnormal myelopoiesis secondary to tumor growth, myelosuppressive therapy, and growth factor administration and subsequent expansion/mobilization of bone marrow–derived immunosuppressive cells. These myeloid-derived suppressor cells (MDSC) reduce activated T-cell number and inhibit their function by multiple mechanisms, including depletion of L-arginine by arginase-1 (ARG1) production of nitric oxide, reactive oxygen species, and reactive nitrogen oxide species by inducible nitric oxide synthase. Increased numbers of MDSCs are associated with neoplastic, inflammatory, infectious, and graft-versus-host diseases where they restrain exuberant or novel T-cell responses. In this review, we discuss critical components of MDSC-mediated suppression of T-cell function, including cellular expansion and activation-induced secretion of immunosuppressive mediators. Both components of MDSC bioactivity are amenable to pharmacologic intervention as discussed herein. We also focus on the relationship between MDSCs, tumor growth, therapeutic responses, and the mechanisms of cellular expansion, activation, and immunosuppression.

Host immunity has a critical role in regulating tumor outgrowth and patient survival; however, the expansion of tumor-specific T cells is also limited by tumor growth. Recently, there has been a resurgence of interest in myeloid-derived cells in association with their role in tumor progression and potential to limit therapeutic responses. Myeloid-derived suppressor cells (MDSC) are increased in numerous pathologic conditions, including infections, inflammatory diseases, graft-versus-host disease, traumatic stress, and neoplastic diseases (1, 2), and serve to limit T-cell responses. Tumor growth results in the expansion of this highly heterogeneous cellular population, which includes granulocytic, macrophage, dendritic cell, and early myeloid progenitors. MDSCs inhibit not only activation of T cells by anti-CD3 and superantigen, but also antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. The mechanisms of MDSC immunosuppression are diverse, including up-regulation of reactive oxygen species (ROS), nitric oxide (NO), and L-arginine metabolism, as well as immunosuppressive cytokines. MDSCs were initially described in the late 1970s when they were identified as natural suppressor cells and defined as cells without lymphocyte-lineage markers that could suppress lymphocyte response to immunogens and mitogens (3–5). MDSCs in mice are CD11b and Gr1 positive (6, 7) and also express CD31 (8), CD124, interleukin (IL)-4 receptor α-chain (9) and CD115, and macrophage-colony stimulating factor receptor (10). Human MDSC equivalents were originally described in patients with head and neck cancer (11) and more recently in the peripheral blood of renal cell carcinoma patients (12, 13). Human MDSCs have an immature phenotype, including lineage negative (Lin^-), $CD14^-$, human leukocyte antigen DR–negative ($HLA\text{-}DR^-$), $CD15^+$, $CD34^+$, $CD11b^+$, $CD33^+$, and $CD13^+$ cells. Several cytokines and transcription factors are involved in the expansion, mobilization, and activation of MDSCs as reviewed in Fig. 1.

Antigen-Presenting Cells

The binding of an antigen to an antigen-presenting cell (APC) for presentation to cognate T-cell receptors stimulates the differentiation of T cells into type 1 or type 2 subsets, depending on the cellular and cytokine milieu, which results in either T-cell activation or tolerance. Following antigen binding, signaling by the CD3ζ chain regulates the T-cell response. Thus, one mechanism of immunosuppression is the down-modulation of the CD3 chain. In type 1 versus type 2 paradigm, type 1 T cells secrete proinflammatory cytokines including IFN-γ, IL-2, IL-12, and IL-18 that promote cellular immunity. In contrast, type 2 T cells are anti-inflammatory, promoting humeral immunity and allergic responses via secretion of IL-4, IL-5, IL-10, and IL-13. The type 1 cytokines activate macrophages

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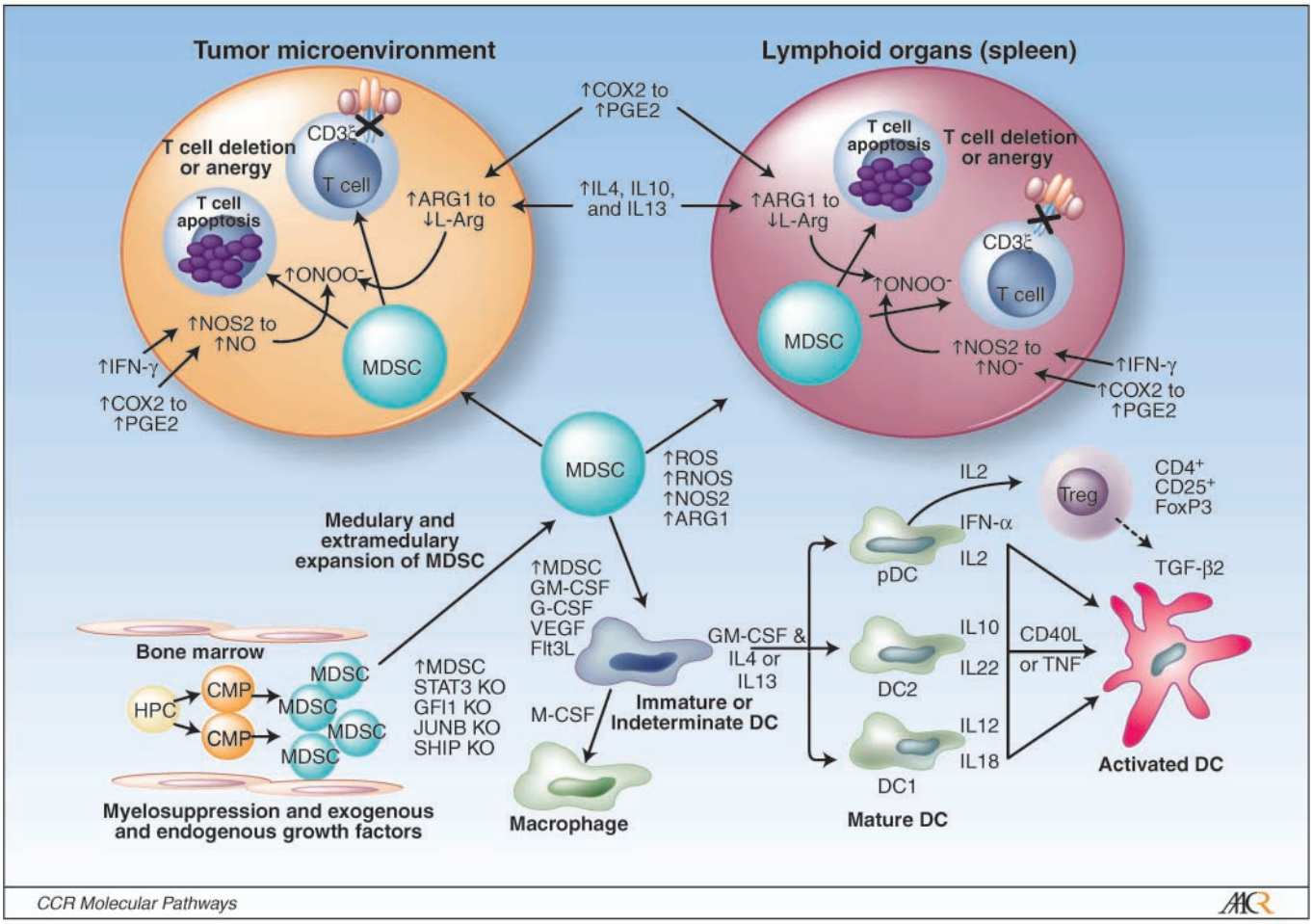

Fig. 1. Schema of MDSC expansion and activation. Hematopoietic progenitor cells (*HPC*) proliferate, differentiate, and commit to various hematopoietic lineages including committed myeloid progenitors (*CMP*). Under conditions of myelosuppression or increased levels of growth factors (*GF*), significant increases in MDSCs occur in the peripheral blood (*PB*), spleen, and tumors. During expansion, MDSCs are mobilized into the circulation, lymphoid organs, and tumor microenvironments. As a general rule in rodent models, the frequency of MDSC is higher within spleens than in the tumor microenvironment. This can result in a MDSC frequency as high as 10% to 40% of the cells within the spleen, and, with few exceptions, the MDSC frequency is <10% of the nonparenchymal cells (*NPC*) within a tumor. The expansion of MDSCs is associated with increased levels of growth factors including VEGFs. MDSC secretion of ARG1 decreases L-arginine levels, resulting in a translational blockade of the CD3ζ chain, leading to T-cell suppression. In addition, high levels of NOS2 and NO are induced by the activation of MDSCs, which result in nitrosylate cysteine residues in target proteins, and affect the production of cyclic guanosine-3′,5′-monophosphate (*GMP*). This affects IL-2 receptor signaling by blocking the phosphorylation of signal-inducing molecules coupled to IL-2 receptor and altering the stability of IL-2 mRNA. Up-regulation of both enzymes, in addition to affecting the two pathways described above, can increase the production of other reactive oxygen species (*ROS*) and reactive nitrogen oxide species (*RNOS*), including O_2, $ONOO^-$, and H_2O_2. This occurs either by nitrating tyrosine residues or controlling BCL-2 and CD95 ligand (*CD95L*) expression, resulting in T-cell apoptosis. Signaling elements that have been shown to regulate this process include signal transducer and activator of transcription (*STAT3*), GFL-1, JunB, and Src homology 2 – containing inositol phosphatase (*SHIP*). These processes of immune suppression can be regulated by differentiation of MDSC into dendritic cells (*DC*), maturation by macrophage-colony stimulating factor (*M-CSF*) into macrophages or into dendritic cells by GM-CSF and IL-4 or IL-13. This includes myeloid dendritic cells, which are immunosuppressive via the production of IL-10 and IL-21, which can be further differentiate into immune-augmenting dendritic cells secreting IL-12 and IL-18 or plasmacytoid dendritic cells (*pDC*), which have important roles in the response to viral infections via the production of IL-2 and IFN-α. These high levels of IL-2 can increase the frequency of Tregulatory cells. All of these dendritic cells can be activated to increase the expression of costimulatory molecules by a variety of cytokines including CD40 ligand (*CD40L*) and tumor necrosis factor α (*TNF-α*).

and dendritic cells, augmenting antigen processing and presentation and the secretion of reactive oxygen species and NO. In contrast, type 2 cytokines, predominant following parasitic infections, provide help to B-cell responses and attenuate type 1 and inflammatory responses. Critical to these processes are dendritic cells, which act as immune sentinels, shaping the adaptive immune response as directed by micro-environmental factors including stromal and tumor cells.

Growth Regulation of MDSCs

The consequences of extensive T-lymphocyte activation, as seen during graft-versus-host disease and septic shock, can be life threatening and are controlled, in part, by MDSC-mediated deletion of reactive lymphocytes (14). Increased levels of granulocyte-macrophage-CSF (GM-CSF) are directly associated with MDSC-dependent suppression and can be reversed with antibodies to GM-CSF (6). Mice bearing transplantable tumors, which secrete GM-CSF, have increased numbers of MDSCs and suppressed T-cell immunity (15, 16). This contrasts with the vaccine adjuvant activity of GM-CSF (17), which is associated with GM-CSF levels such that high levels expand MDSC numbers, reducing vaccine responses, whereas lower levels augment tumor immunity. Similar effects on MDSCs have been suggested with other growth factors including fms-like tyrosine kinase 3 (Flt3) ligand (18), macrophage-colony stimulating factor, and IL-3.

Vascular endothelial growth factor (VEGF) can also suppresses tumor immunity (19) via an inhibitory effect on dendritic cell differentiation (20). A correlation has been shown between plasma VEGF levels in cancer patients, a poor prognosis (21), and VEGF-induced abnormalities in dendritic cell differentiation (22), resulting in an inverse correlation between dendritic cell frequency and VEGF expression in cancer patients (23). Neutralizing VEGF antibodies not only can reverse VEGF-induced defects in dendritic cell differentiation (24), but can also improve dendritic cell differentiation in tumor-bearing mice (25, 26). VEGF has also been directly correlated with MDSC expansion such that tumor progression and multiplicity correlate with serum levels of VEGF and numbers of MDSCs in the blood and spleen (27).

Mechanism of MDSC Activation

The inducible cyclooxygenase-2 (COX2) enzyme is overexpressed in most human cancers (28), is critical to the production of prostaglandin E_2, affects tumor progression and immunosuppression (29), and stimulates arginase-1 (ARG1) and nitric oxide synthase (NOS2) secretion from MDSCs (30). Translational studies using selective COX2 inhibitors have shown chemopreventive and therapeutic activity, increased lymphocyte infiltration of tumors, increased IL-12 and IFN-γ levels, and decreased IL-10 (31) and MDSCs (22, 32). In addition, monotherapy with COX2 inhibitors can significantly increase the T-cell response to tumor vaccines, inhibit tumor growth, and prolong survival (33–35). The COX2 inhibitor Celebrex is approved by the U.S. Food and Drug Administration for chemoprevention of intestinal polyps and colon cancer (36). This therapeutic strategy has been extended to breast cancer patients who have impaired dendritic cell and T-cell function, which is correlated with COX2 and prostaglandin E_2 expression, as well as reduced T-helper 1 cells and increased T-helper 2 cytokine levels (37). In addition to stimulation of ARG1 and NOS2 levels by the COX2 metabolite prostaglandin E_2, various T-cell cytokines (38) may also have a role in T-cell regulation by MDSCs. ARG1 and NOS2 are differentially regulated by cytokines such that inflammatory cytokines up-regulate NOS2 activity and T-helper 2 cytokines up-regulate ARG1 activity. Specifically, ARG1 is up-regulated by IL-4, IL-13 (39), IL-10, transforming growth factor-β (40), and GM-CSF (41), whereas NOS2 activity is induced by IFN-γ, tumor necrosis factor α, or IL-1α (42, 43). Further, NOS2 has two domains: an NH_2-terminal oxygenase domain and a COOH-terminal reductase domain. Electrons donated by conversion of $NADP^+$ (NADPH) to NADP are transferred to the oxygenase domain, catalyzing a reaction between oxygen (O_2) and L-arginine and generating L-citrulline and NO. Under conditions of low L-arginine levels, the reductase and oxygenase domains of NOS2 transfer electrons to the cosubstrate O and produce O_2^-, which reacts with other molecules, generating reactive nitrogen oxide species and reactive oxygen species such as hydrogen peroxide (H_2O_2).

These observations show the complex interrelationship between L-arginine metabolism, immunity, and tumorigenesis (44). Tumor-infiltrating MDSCs seem to be the primary source of ARG1 activity in tumors (13, 45) and treatment with ARG1 inhibitors impairs tumor formation in immunologically intact, but not immunodeficient, mice, indicative of an immune-mediated effect (45). MDSC infiltration of tumors reduces the expression of CD3ζ chains on T cells, a finding also observed in cancer patients (13, 44) in which increased ARG1 activity is accompanied by a decreased expression of CD3ζ and diminished production of IL-2 and IFN-γ (13). Further, depletion of MDSCs restores T-cell proliferation, cytokine production, and CD3ζ expression. MDSC reduction of L-arginine by ARG1 prevents the reexpression of the CD3ζ chain after T-cell receptor signaling–induced internalization (45). The MDSC-associated loss of T-cell function seems to be critical to tumor escape from immunity, such that inhibition of ARG1 activity slows tumor growth in a dose-dependent manner (45). ARG1 activity from MDSCs leads to the depletion of L-arginine in the local tumor environment and the formation of urea, altering the translation of mRNAs through the kinase pathways. L-Arginine starvation also blocks the access of methionyl tRNA to the ribosome, impairing the initiation of translation (46). Decreased concentrations of amino acids or increased production of urea may also function in the suppression of T-cell function.

NO also has an important role in MDSC immunosuppression as has been shown with NOS inhibitors and NOS2-deficient mice. NO-mediated suppression of T-cell activation is associated with a signaling cascade downstream of IL-2 receptor binding, such that NO negatively regulates intracellular signaling proteins either directly by S-nitrosylation of cysteine residues or indirectly by activation of soluble guanylate cyclase and cyclic guanosine-3′,5′-monophosphate–dependent protein kinase (47–49). In T cells, the phosphorylation of signaling molecules, including Janus-activated kinases 1 and 3, signal transducer and activator of transcription 5, and extracellular signal–regulated kinase, and Akt (50), also has a regulatory role. Signal transducer and activator of transcription-3 activation can also affect tumor progression via an antiapoptotic effect on neoplastic cells and an alteration of immune surveillance via an inhibition of dendritic cell maturation (51, 52). Further, recent studies have shown that Janus-activated kinase 3 is oxidized and inactivated following exposure to NO. In addition, a direct proapoptotic effect on T cells exposed to high concentrations of NO is initiated by signaling through CD95, other tumor necrosis factor receptor family members, and caspase-independent pathways (53).

The relationship between ARG1 and NOS2 from MDSCs is complex because ARG1 activation limits L-arginine availability as a substrate for NOS2. However, depletion of cytosolic L-arginine in MDSCs by ARG1 affects the generation of superoxide ion (O_2^-; ref. 54). ARG1 depression of L-arginine levels switches NOS2 activity from the production of primarily NO to predominantly O_2^- (54, 55), both of which have multiple inhibitory activities for T cells. Combined activity of ARG1 and NOS2 from tumor-infiltrating MDSCs (56) and MDSCs from spleens of tumor-bearing mice (54) has been shown to have T-cell immunosuppressive activity. This indicates that coexpression of NOS2 and ARG1 provides a molecular marker of immunosuppressive MDSCs. Peroxynitrites ($ONOO^-$) are one of several reactive nitrogen oxide species that are formed as a by-product following reactions between O_2^- and NO. These highly reactive oxidizing agents damage biological targets (57) and induce posttranslational modification of proteins by nitrating tyrosine residues. At high concentrations, $ONOO^-$ can also directly control cell death by nitration of and damage to mitochondria (58). H_2O_2 is induced by the interaction of

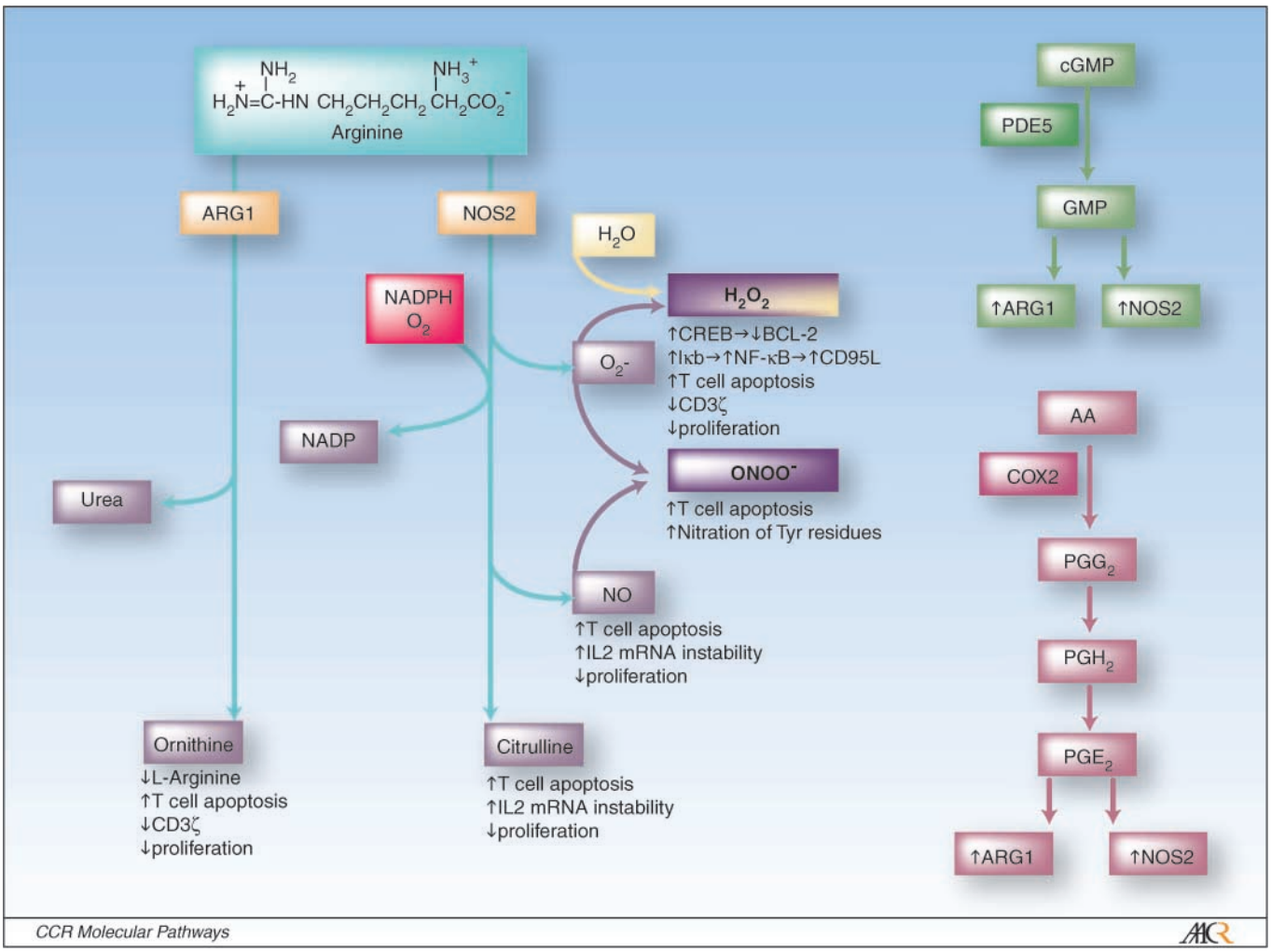

Fig. 2. Schema of ARG1 and NOS2 metabolic pathways. The bioactivities of ARG1 and NOS2 are illustrated together with the downstream pathways that are activated. In addition, relevant parallel pathways involving COX2 and phosphodiesterase type 5 (*PDE5*) are identified. It is emphasized that when L-arginine concentrations are limiting, electrons are transferred by NOS2 subunits to the cosubstrate O_2, which generates O_2^-. O_2^- from this or other reactions can combine either with NO to generate $ONOO^-$ or with H_2O to produce H_2O_2. Note that other oxidative reactions can generate O_2^- and other reactive oxygen species. Alternative mechanisms of action are also shown, including $ONOO^-$ generation as a by-product of the reaction between O_2^- and NO. The outcomes of these metabolic events and the resultant mechanisms of immune suppression are shown, including T-cell apoptosis, nitration of tyrosine, increased IL-2 mRNA instability, depressed T-cell proliferation, depression in CD3ζ chain expression, and various signaling events, including cyclic AMP-responsive element binding protein (*CREB*), nuclear factor-κB (*NF-κB*), IκB, and BCL-2. AA, arachidonic acid; MOA, mechanism of action.

O_2^- with hydrogen protons (H^+) from H_2O (59), which can reduce CD3ζ chain expression by naive T cells *in vitro*. This activity can be blocked by removing MDSCs (60) or adding oxygen radical scavengers (61). These data, together with the observation that ARG1 is expressed by MDSCs from patients with RRC (13), support the role of MDSCs in the immunosuppression of tumor-bearing patients. H_2O_2 may also have a direct role in inducing apoptosis of antigen-activated T cells by down-regulating intracellular levels of B-cell lymphoma 2 (BCL-2) and by increasing CD95 ligand expression via the nuclear factor-κB signaling pathway (62).

Clinical-Translational Advances

Regulating the bioactivity of NOS2 and ARG1 from MDSCs has therapeutic potential. ARG1 inhibitors have antitumor activity (63), although inhibition of both ARG1 and NOS2 pathways improves therapeutic activity (56). Inhibition of NOS2 has minimal effect on ARG1 immunosuppression whereas blocking ARG1 activity increases NOS2 levels, suggesting a complex relationship. COX2 inhibitors reduce both ARG1 and NOS2 levels, and anti-inflammatory drugs created by coupling conventional nonsteroidal anti-inflammatory drugs to a NO-releasing group (63) have shown significant activity, including increased number and function of tumor antigen-specific T cells, or preventive and therapeutic activity with cancer vaccines (56).

Translation studies have focused on MDSC-mediated immunosuppression by reducing MDSCs functions, including antibody-mediated depletion of MDSCs, induction of MDSC differentiation, and selective blockade of MDSC-specific, suppressive molecular pathways. Mice injected with anti-Gr1 have slowed tumor progression (64) and enhanced $CD8^+$ T-cell responsiveness, resulting in tumor rejection (64). Differentiation of MDSC into functional APC with all-*trans* retinoic acid has been shown to reduce MDSC number, with therapeutic

outcome independent of direct all-*trans* retinoic acid antitumor effect (65). Furthermore, all-*trans* retinoic acid differentiates MDSCs into mature dendritic cells, macrophages, and granulocytes, which increase responses to cancer vaccines. In contrast, 1α,25-dihydroxyvitamin D3 has an indirect action via a reduction in myelopoiesis (66). Low doses of IFN-γ and tumor necrosis factor can also reduce MDSC number by inducing cellular differentiation (67). Recently, Dr. Finke (68) showed that the administration of sunitinib, a receptor tyrosine kinase inhibitor that induces clinical responses in patients with metastatic renal cell carcinoma, also resulted in a significant diminution of MDSCs within that patient population.[1] Whether this was associated with a reduction in VEGF levels and neoangiogenesis or as an inhibition of Flt3-mediated expansion of MDSCs remains to be addressed.

The metabolism of L-arginine by ARG1 and NOS2 provides promising targets for the control of MDSC-induced immune suppression; see schema in Fig. 2. The relevance of NOS2 in the MDSC suppressive pathway is supported by studies with specific NOS2 inhibitors that showed a reversal of T-cell unresponsiveness (69), although not always sufficient to recover T-cell responsiveness (56). In one tumor model, functional CTLs could be recovered only by inhibition of both enzymes (70). Studies using aspirin derivates, which affect ARG1 and NOS2 metabolism in tumor-bearing mice, have shown an increase in tumor-specific T cells and enhanced cancer vaccine efficacy in mice immunodepressed by MDSC cells (56). In addition, a recent report using a phosphodiesterase type 5 inhibitor showed that blocking up-regulation of both ARG1 and NOS2 had significant therapeutic activity in tumor models following a reduction in MDSC numbers (71). Similarly, COX2 inhibitors reduce ARG1 and NOS2 levels, as well as MDSC numbers, in tumors and secondary immune organs in tumor-bearing mice (30, 32).

[1] J. Ko, et al., in preparation.

Summary

Despite advances in the phenotypic identity of MDSCs and insight into their mechanism of action, much remains to be learned. This includes the role of ARG1 versus NOS2, systemic versus tumor-associated activity, and the potential to overcome immune tolerance. Initial clinical studies manipulating MDSCs have been undertaken, including maturation of MDSCs with all-*trans* retinoic acid during therapeutic vaccination, manipulation of stem cell products during allogeneic transplantation to reduce graft-versus-host disease, and manipulation of MDSC activation with COX2, ARG1, and or NOS2 inhibitors. Although clinical translation has only recently been initiated, the therapeutic regulation of MDSCs provides an attractive therapeutic target not only for oncology but also for graft-versus-host disease, inflammation, and autoimmune diseases.

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