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Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives

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Summary

Patients with cancer have an impaired T-cell response that can decrease the potential therapeutic benefit of cancer vaccines and other forms of immunotherapy. L-arginine (L-Arg) is a conditionally essential amino acid that is fundamental for the function of T lymphocytes. Recent findings in tumor-bearing mice and cancer patients indicate that increased metabolism of L-Arg by myeloid derived suppressor cells (MDSCs) producing arginase I inhibits T-lymphocyte responses. Here we discuss some of the most recent concepts how MDSC expressing arginase I may regulate T-cell function in cancer and other chronic inflammatory diseases and suggest possible therapeutic interventions to overcome this inhibitory effect.

Keywords

arginase; myeloid derived suppressor cells; T-cell function

Introduction

The clinical experiments of William Coley in the 1890s and the work of Prehn and Main in the 1950s firmly demonstrated the presence of an immune response against tumor antigens that could potentially be used in the treatment of cancer. New concepts in carcinogenesis, including the viral etiology of some malignancies, the presence of mutated onco-proteins, and the over-expression of certain normal antigens, further supports the concept that an antigen-specific immune response can be generated to control tumor growth. However, two decades of clinical trials in cancer immunotherapy have also made evident that tumor cells have sophisticated mechanisms to evade the immune response. Recent findings have characterized several molecular mechanisms triggered by the tumor microenvironment resulting in the impairment of T-cell anti-tumor function. The molecular and cellular bases underlying these mechanisms are a matter of extensive research and debate, in part because of their complexities and possible overlapping pathways. However, they represent a possible key to being able to modulate the immune response for the therapeutic benefit of patients with cancer and other chronic diseases. Even though most researchers agree that the

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malignant cells trigger the events that ultimately lead to T-cell tolerance, the intermediaries in this process vary and include regulatory T cells, suppressor macrophages, and more recently myeloid derived suppressor cells (MDSCs). The latter subpopulation of immune cells has become a focus of much research recently because of the novel observations demonstrating their ability to regulate T-cell responses by controlling the availability of the amino acid L-arginine (L-Arg). The demonstration of this mechanism in tumor models, patients with cancer, and various other chronic inflammatory diseases, and the potential for therapeutic intervention has created a major interest in MDSC. Here, we discuss some of the most recent concepts of how myeloid cells metabolizing L-Arg regulate T-cell function in disease and suggest possible therapeutic applications to inhibit MDSC activity.

Alterations of the immune response in cancer

A dysfunctional immune response in cancer patients manifested by the loss of delayed type hypersensitivity to bacterial and chemical antigens was demonstrated in cancer patients several decades ago (1–4). Initial explanations included the development of 'blocking antibodies' (5), the production of suppressor factors by tumor cells (6, 7), and the generation of suppressor macrophages and dendritic cells (DCs) (8). However, the significance of these findings on the progression of the disease and its outcome were unknown. Although cancer patients generally do not develop the characteristic opportunistic infections that affect patients immunosuppressed by the use of high dose chemotherapy or corticosteroids (which ablates granulocytes and mononuclear immune cells equally), they have indeed been shown to have an impaired delayed type hypersensitivity response to bacterial and/or chemical antigens and a poor *in vitro* response to mitogens (9–12). These results suggested that cancer primarily affects T-cell responses but not the myeloid response (granulocytes). Not until the advent of immunotherapy trials in cancer in the 1980s and 1990s was the real impact of the T-cell dysfunction made apparent. Several animal tumor models and many clinical trials demonstrated that immunotherapy in mice or patients with advanced tumors failed to achieve a therapeutic response as a result of the loss of T-cell responses (reviewed in 13). In addition, several vaccine trials demonstrated the progression of tumors in spite of a robust T-cell response (14). The development of cellular and molecular models leading to T-cell anergy provided important insights to understand how cancer (and other chronic inflammatory diseases) could selectively cause T-cell dysfunction (15). This development provided the basis for the discovery of new mechanisms including the role of immunoregulatory molecules in antigen-presenting cells (APCs) such as B7.1, B7.2, B7-H1, and B7-H4 (16–20), the development of regulatory T cells (21, 22) and the generation of MDSC (23–26). Although most models are in agreement that tumor cells are the initiators of the suppressor phenomenon, they also coincide that APCs, in the form of macrophages or DCs, play a central role in directly inducing T-cell anergy or generating regulatory T cells (27, 28).

Young *et al.* (29) demonstrated that suppressor macrophages blocked T-cell responses by producing interleukin-10 (IL-10), transforming growth factor-β (TGFβ), and prostaglandin E_2 (PGE_2). Gabrilovich *et al.* (30) demonstrated that vascular endothelial growth factor (VEGF) produced by the tumor arrested the differentiation of DCs, resulting in immature myeloid cells that induce T-cell dysfunction. These immature myeloid cells were increased in patients with breast, head and neck, and lung cancer (31, 32). More recently, Mellor and Munn (33, 34) demonstrated that an impaired T-cell response can also occur as a result of the depletion of the amino acid tryptophan by plasmacytoid DCs producing indoleamine-2, 3-dioxygenase (IDO). Tryptophan starvation induced cell cycle arrest in normal T lymphocytes and sensitized activated T cells to apoptosis before cell division (35). The mature and immature APCs can play a central role in the induction of tolerance.

Signaling alterations in anergic T cells in cancer

In the 1990s, we and others showed that T cells from cancer patients and tumor-bearing mice had multiple changes in the expression of signal transduction molecules, including a decreased expression of the T-cell receptor (TCR) ζ chain (CD3ζ), a diminished tyrosine kinases $p56^{lck}$, $p59^{fyn}$, and an inability to upregulate Janus kinase-3 (Jak-3), and to translocate NFκBp65, all of which resulted in a diminished *in vitro* T-cell response (36–38). These T-cell signal transduction alterations were accompanied by a diminished ability to mobilize Ca^{++} and a decreased tyrosine phosphorylation (39), and provided a possible molecular explanation for the T-cell dysfunction reported in tumor-bearing mice and cancer patients. The initial findings in tumor-bearing mice were confirmed in patients with renal cell carcinoma, melanoma, Hodgkin's disease, ovarian cancer, colon carcinoma, and cervical cancer among others (40–42). Patients with renal cell carcinoma showed changes in CD3ζ expression in the tumor-infiltrating T cells (43), while patients with colon carcinoma showed the most significant loss of CD3ζ in the draining lymph nodes closest to the tumor (44), suggesting that the tumor microenvironment played an important role in inducing these changes. Preliminary studies also suggested an association between alterations in signal transduction and a decreased survival in cancer patients. Patients with melanoma and patients with head and neck tumors who had a decreased expression of CD3ζ had significantly shorter survival compared with patients expressing normal levels (42, 45). The absence of a mechanism to explain these changes and the apparent lack of specificity of these alterations created some initial controversy around these observations.

Mechanisms leading to a decreased CD3ζ chain in disease

Otsuji *et al.* (46) and Kono *et al.* (47, 48) were the first to demonstrate that the co-incubation of activated murine peritoneal macrophages with naive T cells induced the loss of CD3ζ chain in the latter population. This phenomenon could be blocked by the addition of oxygen radical scavengers and was therefore thought to be mediated by the release of H_2O_2 (49). A similar effect was suggested in a report by Schmielau *et al.* (36) in patients with pancreatic and breast cancer where an increase in the number of activated neutrophils and the production of H_2O_2 in peripheral blood was closely associated with a diminished expression of CD3ζ chain (50). Another mechanism suggested that the loss of CD3ζ chain was a consequence of Fas–Fas ligand (FasL)-induced T-cell apoptosis (51, 52). Still an additional mechanism was proposed by the work of Baniyash and colleagues (53), where they demonstrated that chronic stimulation of T cells by specific antigens led to the decreased expression of CD3ζ chain of the TCR and induction of anergy. However, none of these models reproduced the multiple alterations found in T cells of cancer patients.

Reports from other diseases suggested that the loss of CD3ζ was not unique to cancer. Zea *et al.* (54, 55) described that patients with lepromatous leprosy or active pulmonary tuberculosis presented similar alterations in peripheral blood T cells. More recently mice with severe trauma were found to have a loss of CD3ζ chain and T-cell function (56). Furthermore, trauma patients had a rapid depletion of L-Arg levels in serum, which was paralleled by the loss of T-cell function. Animal models confirmed this observation (57) and further demonstrated that the replenishment of L-Arg by the infusion of high doses of L-Arg in mice or in trauma patients resulted in the recovery of T-cell function and an increase in the number of $CD4^+$ cells (58–60). This finding led us to study whether regulation of the levels of L-Arg might be involved in the induction of T-cell dysfunction in patients with cancer.

L-Arg and immune response

The association of L-Arg and the immune system was initially suggested in the 1970s by reports demonstrating that the injection of L-Arg in mice undergoing extensive surgery prevented a well-described phenomenon of post-surgical thymus involution and appeared to increase the number of T cells (61). In the late 1980s, Albina and Mills (26, 62) demonstrated that L-Arg was fundamental for wound healing processes possibly by increasing the production of proline and collagen. A different but equally important association was suggested by reports showing that the rapid depletion of plasma levels of L-Arg was accompanied by a markedly decreased T-cell function in patients undergoing liver transplantation, in trauma patients, or in murine models of trauma (58–60). Furthermore, the state of anergy caused by trauma could be rapidly reversed by the enteral or parenteral supplementation of L-Arg (63).

Our initial experiments demonstrated that culturing Jurkat T cells in tissue culture medium with L-Arg levels < 50 μM resulted in the gradual loss of CD3ζ and caused a significant decrease in proliferation (64). Experiments using primary T cells (murine or human) did not show any effects of L-Arg deprivation on resting T cells. However, T cells activated in an L-Arg-free environment developed all the alterations previously described in tumor-bearing mice and cancer patients, i.e. the decreased expression of CD3ζ, an inability to upregulate Jak-3, and a decreased translocation of NFκB-p65. T cells cultured without L-Arg also failed to proliferate and did not produce interferon-γ (IFN-γ). However, these changes were selective because other functions, including the production of IL-2 and the upregulation and expression of the IL-2 receptor chains (CD25, CD122, CD132), were similar to cells cultured in medium with L-Arg (L-Arg concentration in RPMI is 1100 μM). These results suggested a potential role for L-Arg depletion as a mechanism for the induction of T-cell dysfunction.

In healthy adults, L-Arg is considered to be a non-essential amino acid, because it is synthesized endogenously from citrulline by the collaboration between the epithelial cells of the small intestine and the proximal tubules of the kidney (reviewed in 65). Normal levels of L-Arg in serum range between 50 and 150 μM. However, L-Arg is also classified as a conditional essential amino acid in certain physiological conditions involving changes in the L-Arg metabolic status, including trauma and cancer (66). L-Arg is the substrate for four enzymes, several of which exist as multiple isoforms: nitric oxide synthases (NOS1, NOS2, and NOS3), arginases (arginase I and II), arginine:glycine amidinotransferase (AGAT), and L-Arg decarboxylase (ADC) (Fig. 1). To encounter all enzymes involved in its metabolism, dietary L-Arg must be taken up by intestinal epithelial cells and must traverse the plasma membrane of all cells via the y+system of cationic amino acid transporters (CAT) (67). Once inside the cells, LArg is metabolized by NOS enzymes to produce citrulline and nitric oxide, which plays an important role in cytotoxic mechanisms and vasodilatation (68, 69). Alternatively, arginase I and arginase II metabolize L-Arg to L-ornithine and urea, the first being the precursor for the production of polyamines that are essential for cell proliferation and the second an important mechanism for detoxification of protein degradation (70). Arginase and NOS enzymes have been widely studied *in vitro* and *in vivo*; however, there is limited information about the regulation and the immunological role of ADC and AGAT. ADC converts L-Arg to agmatine, which in turn is converted to putrescine and urea by agmatinase. Mammalian ADC is highly expressed in the brain (71, 72), while AGAT is expressed in the brain and the heart (73, 74).

Arginase I and NOS2 play important roles in the immune response. The expression of arginase I and NOS2 in murine macrophages is differentially regulated by T-helper 1 (Th1) and Th2 cytokines (75, 76). Stimulation of murine macrophages with IFN-γ upregulates

NOS2 exclusively, while IL-4, IL-10, and IL-13 (77, 78) or TGFβ (79) induce arginase I. The mitochondrial isoform arginase II is not significantly modulated by Th1 or Th2 cytokines (80). Furthermore, inhibition of arginase I leads to an increased NOS2 expression and consequently promotes NO production (81). Conversely, upregulation of arginase I functionally inhibits NOS activity and contributes to the pathophysiology of several disease processes, including vascular dysfunction and asthma (82). The mechanisms of inhibition NOS2 expression by arginase I are partially understood. L-Arg depletion by arginase I blocks the induction of NOS2 expression and the subsequent NO production in macrophages through an arrest in the synthesis of NOS2 protein (83). In addition, very low levels of NOS can induce nitrosylation of cysteine residues of human arginase I, which increases the biological activity of arginase I and reduces L-Arg (84). This process in turn blocks the expression of NOS2 and the production of NO.

Activation of peritoneal macrophages with Th1 or Th2 cytokines also had different effects on the extracellular levels of L-Arg. Peritoneal macrophages stimulated with IL-4 + IL-13 increased the expression of arginase I and CAT-2B. This resulted in a dramatic increase in the uptake of extracellular L-Arg with the consequent reduction of L-Arg in the tissue culture medium. In contrast, macrophages stimulated with IFN-γ preferentially expressing NOS2 did not increase CAT-2B and did not deplete L-Arg levels from the tissue culture medium (80). In both culture conditions, macrophages constitutively expressed arginase II, suggesting that this enzyme does not deplete extracellular L-Arg. L-Arg depletion was not caused by the release of arginase I into the tissue culture medium by murine macrophages. In addition, data from arginase I and arginase II knockout animals suggested that the only L-Arg metabolizing enzyme able to modify serum levels of L-Arg is arginase I (85, 86). Macrophages producing arginase I or NOS2 were then co-cultured with activated T cells using transwells. Only macrophages producing arginase I, and not macrophages expressing NOS2, caused the loss of CD3ζ and inhibited T-cell proliferation. Furthermore, the addition of arginase inhibitors *N*-Hydroxy-nor-L-Arg (Nor-NOHA) and N-Hydroxy-L-Arg (NOHA) or exogenous L-Arg reversed the CD3ζ loss (80). Thus, arginase I expression in macrophages inhibited T-cell function by impairing the expression of CD3ζ chain of the TCR.

Molecular effects of L-Arg starvation on T cells

The initial data in Jurkat cells and in primary T cells cultured in medium lacking L-Arg showed a decrease of CD3ζ and a decrease in proliferation, which were not associated with an increase in apoptosis (64, 80, 87, 88). Both CD3ζ chain expression and cell proliferation rapidly recovered after replenishment of L-Arg and citrulline (88). We initially hypothesized that the main mechanism of T-cell dysfunction induced by L-Arg starvation was the loss of CD3ζ chain expression. However, the low expression of CD3ζ chain alone could not fully explain the almost complete inhibition of T-cell proliferation, even in T cells stimulated with phorbol myristate acetate (in the absence of L-Arg), which bypasses the TCR (unpublished data). In addition, T cells cultured in the absence of L-Arg had similar patterns of calcium flux and tyrosine phosphorylation (during the first 12 h of culture) as T cells cultured with L-Arg, and these cells were able to upregulate the expression of activation markers CD25 and CD69, suggesting that signaling through the TCR was intact during the early stages of culture without L-Arg (88). We therefore studied whether L-Arg deprivation specifically inhibited the mechanisms regulating T-cell proliferation.

Propidium iodide labeling of T cells' nuclei showed that cells cultured in the absence of L-Arg were arrested G_0–G_1 phase of the cell cycle (without the induction of apoptosis), while cells cultured with L-Arg progressed into S and G_2–M phases after 72 h of culture (89). The simple replenishment of L-Arg to physiological levels (50–150 μM) reestablished cell cycle

progression even after 96 h of culture. In mammalian cells, cyclin-dependent kinase 4 (cdk4) and cdk6 are closely associated with the D-type cyclins (cyclin D1, D2, and D3) and regulate the progression through early G_1 and later into the S phase of the cell cycle (90). This regulation requires inactivation of cyclin-D/cdk complex inhibitors (INK4, KIP) and the phosphorylation of the Rb protein family (91). Activated T cells cultured in the absence of L-Arg were unable to upregulate cyclin D3 and cdk4 mRNA and protein but not cdk6 (89). In fact, silencing of cyclin D3 in the human Jurkat T-cell line induced a similar inhibition of proliferation as that induced by L-Arg starvation. Results from knockout mice had demonstrated that cyclin D3 is essential for the maturation of T cells in the thymus (92) and suggested a potential and selective role in T-cell proliferation. In addition and as a consequence of cyclin D3 inhibition, T cells activated and cultured in the absence of L-Arg had a significant decrease in Rb phosphorylation and a markedly decreased nuclear translocation of E2F-1.

We further explored why L-Arg starvation negatively regulated the expression of cyclin D3 and cdk4 mRNA but not cdk6. Results demonstrated that L-Arg starvation induced a decrease in cyclin D3 mRNA transcriptional rate, as demonstrated by 'Run-on' experiments, as well as a decreased cyclin D3 mRNA stability. Furthermore, the L-Arg starvation impaired the translation of cyclin D3. Therefore, the expression of cyclin D3 and cdk4 in the absence of L-Arg are blocked through transcriptional, post-transcriptional, and translational mechanisms (89). What mechanism(s) may be triggered by the depletion of a non-essential amino acid to explain this apparently complex process is still a matter of ongoing research.

Amino acid deprivation in eukaryotes has been shown to activate mechanisms that inhibit translation. The accumulation of empty aminoacyl tRNAs caused by amino-acid starvation activates GCN2 kinase, which phosphorylates the translation initiation factor eIF2α (34). The phosphorylated form of eIF2α binds more tightly than usual to eIF2B, whose job is to exchange guanosine triphosphate (GTP) for guanosine diphosphate (GDP) in the eIF2 complex. When eIF2B is bound to the phosphorylated eIF2α, it is unable to exchange GDP for GTP, which inhibits the binding of eIF2 complex to methionine aminoacyl tRNA and finally leads to inhibition in translation initiation (Fig. 2-Translation off).

We first determined whether the absence of L-Arg led to the activation of GCN2 by testing eIF2α phosphorylation. A significant increase in the phosphorylation of eIF2α was observed in activated T cells cultured in the absence of L-Arg but not in cells cultured in the presence of L-Arg (89). We then tested the role of GCN2 as a central mediator of the effects on T cells induced by the absence of L-Arg, Indeed, T cells from GCN2 knockout mice did not show an arrest in cell cycle or a decreased proliferation, and they were able to upregulate the expression of cyclin D3 and cdk4 when cultured in medium without L-Arg (89). These results confirm the role of GCN2 as the sensing protein in the signaling induced by L-Arg starvation in T cells (Fig. 2). Interestingly, these results are similar to those induced by the depletion of tryptophan, an essential amino acid that when depleted by the enzyme IDO also results in the induction of a profound T-cell anergy (33, 34).

How amino acid availability decreases mRNA stability is still unclear. Some mRNA stability is increased by L-Arg starvation, which has been shown using the CAT-1 transporter where amino acid limitation increases the stability and translation of the mRNA in rat glioma cells, at a time when global protein synthesis decreases. The increased mRNA stability requires an 11 nucleotide AU-rich element within the distal 217 bases of the 3′-untranslated region (93). In addition, amino-acid starvation triggers an increased translocation of the DNA binding protein HuR from nucleus to cytoplasm which appears to increase the stability of CAT-1 mRNA (94). However, the mechanisms leading to a

diminished stability of cyclin-D3 induced by L-Arg depletion remain unclear at the present time.

Arginase expression in tumors

Several tumor lines from non-small lung carcinoma and breast carcinoma have been shown to express arginase (95–97). This expression has been thought to be a mechanism for the production of polyamines needed to sustain the rapid proliferation of tumor cells. Results from our laboratory suggested instead that arginase I was preferentially expressed in myeloid cells infiltrating tumors, which inhibited T-cell function as a possible mechanism of tumor evasion (98). Myeloid cells infiltrating murine 3LL lung carcinoma and expressing arginase I have the morphology and express the markers of mature macrophages. However, the myeloid cells found in the spleen of tumor-bearing mice (including 3LL tumors) and expressing arginase I appear to be immature myeloid cells (98). Even though there has been some variation in cell morphology and maturation markers found between different tumor models and between murine and human tumors, these myeloid cells are able to suppress T-cell responses. Recently, a panel of leading investigators in the field agreed to use the common term 'myeloid derived suppressor cells' (MDSCs) to name these arginase I-producing cells (99). MDSC commonly express the markers CD11b and GR-1 and have a similar phenotype that of alternatively activated macrophages (also known as M2 macrophages) in the mouse. MDSC are present in the bone marrow of healthy mice; however, they accumulate in the spleen and tumors in tumor-bearing mice (with a higher ability to suppress T-cell function) (100–103). Depletion of MDSC using antibodies against GR-1 induced an anti-tumor effect, which was mediated by $CD8^+$ T cells (104–106).

MDSC induce T-cell dysfunction by the depletion of extracellular L-Arg by arginase I metabolism and an increased uptake of L-Arg through the CAT-2B (98, 107, 108). Reduction of extracellular levels of L-Arg induces an arrest in the proliferation of activated T cells and blocks the re-expression of CD3ζ chain (98) (Fig. 3). The addition of arginase I inhibitors Nor-NOHA or NOHA *in vitro* or its injection into tumor-bearing mice prevented the loss of T-cell function and resulted in an immune-mediated anti-tumor response, respectively. Furthermore, tumor growth was significantly inhibited, in a dose-dependent manner, by the subcutaneous injection of the arginase inhibitor Nor-NOHA starting at the time of tumor implantation (day 0). The inhibition in tumor growth caused by Nor-NOHA, however, was lost in tumorbearing *severe combined immunodeficient* mice, strongly suggesting that the anti-tumor effect caused by the inhibition of arginase was dependent on lymphocyte function (98).

MDSC not only inhibit T-cell function by the depletion of L-Arg but have also been shown to exert a strong T-cell inhibition through cell:cell contact. These mechanisms require the co-expression of arginase I and NOS2. Cultures of MDSC and activated T cells in the presence of arginase and NOS2 inhibitors completely reestablished T-cell function (109). Under limiting amounts of L-Arg (induced by arginase I), NOS2 produces peroxinitrites ($ONOO_2$), a highly reactive oxidizing agent that nitrates proteins and induces T-cell apoptosis (110). This appears to affect the conformational flexibility of the TCR and its interaction with major histocompatibility complex (MHC) by inducing nitration of TCR proteins in $CD8^+$ cells. Thus, MDSC directly disrupt the binding of specific peptides on MHC to $CD8^+$ T cells (111).

The suppression effect induced by MDSC co-expressing arginase I and NOS2 has been related preferentially to the impairment of $CD8^+$ T-cell function (98, 102, 110, 112). MDSC inhibit the function of $CD8^+$ T cells by blocking their ability to secrete IFN-γ when stimulated with specific antigens (101, 113), which results in the induction of T-cell

apoptosis (110). This suppression is dependent on cytokines IL-13 and IFN-γ (102, 114, 115) and signaling through the signal transducer and activation of transcription 1 (STAT1) (110). In addition, it has also been suggested that MDSC can induce the expansion of regulatory T cells (116), a process that appears to be dependent on the production of IFN-γ and IL-10. Blocking of stem cell factor (SCF) signaling in MDSC significantly impairs their ability to generate regulatory T cells (117). A recent report has also suggested that MDSC suppress macrophage function by switching their phenotype toward M2 macrophages (118).

MDSC in human tumors

Although MDSC have been well studied in murine models, their role in human disease has only recently started to be understood. In contrast to murine MDSC, the human MDSC phenotype varies significantly, ranging from immature DCs (119) to activated granulocytes and expressing $CD11b^+$, $CD14^-$, $CD15^+$, $CD34^+$, $CD33^+$, and $CD13^+$ (120). A retrospective study of patients with metastatic renal cell carcinoma (RCC) demonstrated a 6–10-fold increase in arginase activity in the peripheral blood mononuclear cells (PBMCs) as compared with normal controls (120). Separation of the different subpopulations in the PBMCs of these patients demonstrated that the cells containing all arginase activity were activated granulocytes, which separated with the PBMCs when centrifuged over ficoll-hypaque. These patients also had a significantly diminished expression of the CD3ζ chain. There also was an inverse statistical correlation between arginase activity and MDSC numbers with the expression of CD3ζ chain in T cells. Finally, T-cell proliferation and IFN-γ production were re-established (*in vitro*) only after the depletion of MDSC. A similar sub-population of activated granulocytes had previously been described by Schmielau and Finn (121) in patients with pancreatic cancer, where they demonstrated a correlation between the presence of activated granulocytes and alterations in T cells such as reduced CD3ζ chain expression and decreased cytokine production (121). Clinical trials with IL-2 in patients with RCC and melanoma have also shown an association of increased numbers of granulocytes in peripheral blood with a poor response and outcome in these patients (122).

Although human MDSC also express high levels of arginase I, this expression does not appear to be upregulated by cytokines or other signals once these cells are in circulation, nor is there an apparent enhanced uptake of L-Arg as was seen in murine MDSC. Instead, arginase I stored in primary (123) or gelatinase granules (124) of MDSC is released to the microenvironment, inducing a significant decrease in L-Arg levels, which impairs T-cell function and CD3ζ chain expression (120, 125, 126). In fact, the release of arginase I in placenta appears to be an important tolerance mechanisms in pregnancy (125). High release of arginase I into the sera of RCC patients induced a decrease in plasma of L-Arg levels to < 50 μM and an increase in ornithine levels (120). In addition, the low levels of L-Arg correlated with low expression of CD3ζ chain in T lymphocytes, demonstrating that arginase I not only had a metabolic effect (L-Arg depletion) but also a negative effect on the T-cell response.

Regulation and activation of MDSC in cancer

Different cytokines have been involved in the recruitment of MDSC from the bone marrow, including VEGF and granulocyte–macrophage-CSF (GM-CSF). In fact, serum levels of VEGF directly correlated with numbers of MDSC in the blood and spleen (127) and have been associated with poor prognosis in cancer patients. Tumor-derived VEGF has been previously related with arrest in DC maturation (128, 129) through inhibition in NF-κB signaling. Increased levels of GM-CSF have also been associated with MDSC-dependent suppression, which could be reversed with antibodies to GM-CSF (103). Similar effects on MDSC have been suggested with other growth factors including Fms-like tyrosine kinase 3

(Flt3) ligand (131) and FSC (117). Treatment of MDSC with all-*trans* retinoic acid has been shown to induce their differentiation into functional APCs (130).

We used the 3LL murine lung carcinoma model to further determine what factors might be inducing the production of arginase I in the MDSC infiltrating tumors. We initially found that enriched MDSC isolated from 3LL tumors and placed in culture in regular RPMI lose arginase I expression within 24 h. However, if freshly isolated MDSC were co-cultured in transwells with 3LL cells or with 3LL supernatants, they maintained arginase I expression, suggesting that the induction of arginase I was caused by soluble factor(s) produced by tumor cells (132). Cytokines such as IL-4, IL-13, TGFβ, and others were not detected in the supernatants of the 3LL single cell suspensions. Instead, we detected a very high expression of the inducible cyclooxygenase-2 (COX-2) and an increased production of prostaglandins including PGE_2. The incubation of freshly isolated MDSC with PGE_2 maintained the expression of arginase I and induced the expression in those cells that had lost it (132). Furthermore, the addition of COX-2 inhibitors into the co-cultures of 3LL tumor cells and MDSC or the silencing COX-2 in 3LL cells completely blocked their ability to induce arginase I in MDSC (132). The effect of PGE_2 on MDSC was dependent on the expression of the E-prostanoid receptor (EP4) on MDSC and was associated with an increased cyclic adenosine 3′5′ monophosphate levels (132). Consequently, treatment of tumor-bearing mice with the COX-2 inhibitor sc-58125 decreased the expression of arginase I in MDSC infiltrating the tumor and induced an immune mediated antitumor effect (132). Similar results have been reported in mice bearing the 4T1 breast carcinoma. 4T1 tumor-bearing mice treated with the COX-2 inhibitor SC-58236 reduced the accumulation of MDSC in the spleen in an EP2-dependent manner (133). Similar results were found using the selective COX-2 inhibitor celecoxib in a model of induction of large intestinal tumors in Swiss mice by 1,2-dimethylhydrazine diHCl-(1,2-DMH) (134). Some other factors may also play a role in the induction of arginase in MDSC, including hypoxia-inducible factor 1 (HIF-1) and HIF-2 (reviewed in 135). In conclusion, although the mechanisms of induction of arginase I in MDSC have been partially identified in mice, the factors inducing the activation of MDSC in human have not been identified.

MDSC: lessons from other diseases and future applications

The advent of immunotherapy of cancer made apparent that in spite of powerful biological agents that could prime tumor-specific T cells, tumors had sophisticated mechanisms to escape the immune response. Among these is the induction of MDSC, which, through the depletion of a simple non-essential amino-acid L-Arg by the enzyme arginase I, inhibit specific signaling molecules and arrest the T-cell cycle, resulting in a state of tolerance. This phenomenon however is not unique to cancer. Trauma patients and patients with chronic infections including active pulmonary tuberculosis also have increased MDSC expressing arginase I and have an inhibition of T-cell function. This finding suggests that instead of a unique mechanism triggered by tumor cells and aimed specifically at escaping the immune response, MDSC may represent a normal process triggered by tissue damage (danger signal) with the aim of protecting the integrity of the tissues and ‘healing’ the initial injury. A demonstration of this mechanism was described in the late 1980s by Albina *et al.* (62) studying the healing of surgical wounds. They described that the tissue surrounding a surgical wound was initially infiltrated by cells expressing iNOS, which would most likely eliminate offending agents (bacteria and dead cells) contaminating the wound. This infiltration would be followed by cells expressing arginase I, which would metabolize L-Arg to ornithine and to proline, which in turn would trigger the synthesis of collagen by fibroblasts, ultimately leading to the healing of the surgical wound. In cancer or chronic infections, tissue damage would trigger a similar response with the proliferation of fibroblasts producing collagen aimed at isolating and healing the damaged tissue (i.e.

malignant growth). As a matter of fact, many tumors are surrounded by dense fibrous tissue that makes difficult its surgical excision. The major difference between the disease processes (surgical wound versus malignant tumor) would be that the surgical wound would heal, ending the role for arginase-producing MDSC. In contrast, the malignant tumor would not stop growing and destroying tissue (would not 'heal'), which would trigger instead a chronic inflammatory process mediated by MDSC that would ultimately lead to the depletion of L-Arg from the microenvironment and the development of T-cell anergy. Therefore, it is our hypothesis that tumors 'hijack' a normal healing process, making it instead a vicious cycle that results in the inhibition of a potentially protective T-cell anti-tumor response (Fig. 3). Although we are sure that this is an oversimplified version of the complex mechanisms triggered *in vivo*, it provides a model with which to understand a complex event in the development of cancer and probably design new therapeutic approaches that may interrupt this dysfunctional response.

Much has been learned about the role of MDSC in the progression of tumors in the last 5 years. Multiple approaches have been taken to block MDSC suppression using alltransretinoic acid (130), inhibiting nitric oxide function with nitro-aspirin (136), inhibiting phosphodiesterase-5 (137), and blocking arginase activity with specific arginase inhibitors such as Nor-NOHA (98). It is likely that the appropriate combination of inhibitors blocking MDSC function and stimuli protecting T cells may overcome this powerful tumor-derived mechanism that impairs the promise of cancer immunotherapy.

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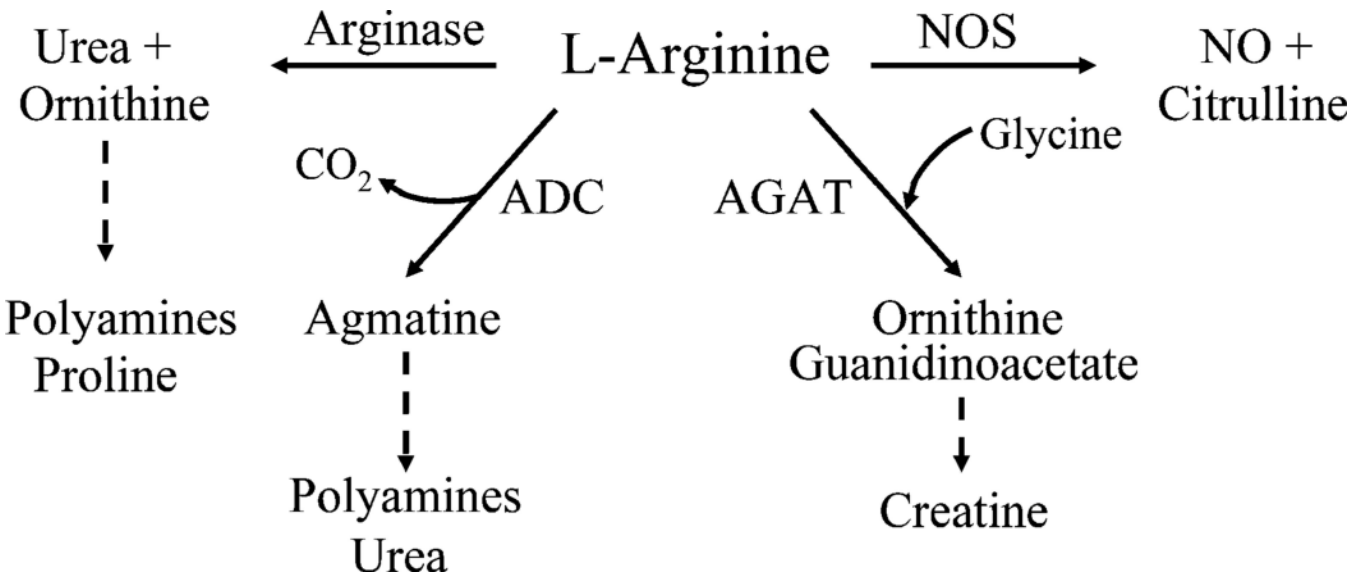

Fig. 1. L-Arg metabolism

L-Arginine can be metabolized by NOS into NO and citrulline. Alternatively, arginase (I and II) can convert L-Arg into urea and ornithine, with the latter being the substrate for the synthesis of polyamines need to sustain cell proliferation. For the sake of simplicity, emphasis is on enzymes that directly metabolize L-Arg. This diagram should not be interpreted to indicate that all of these enzymes are expressed simultaneously in any given cell type.

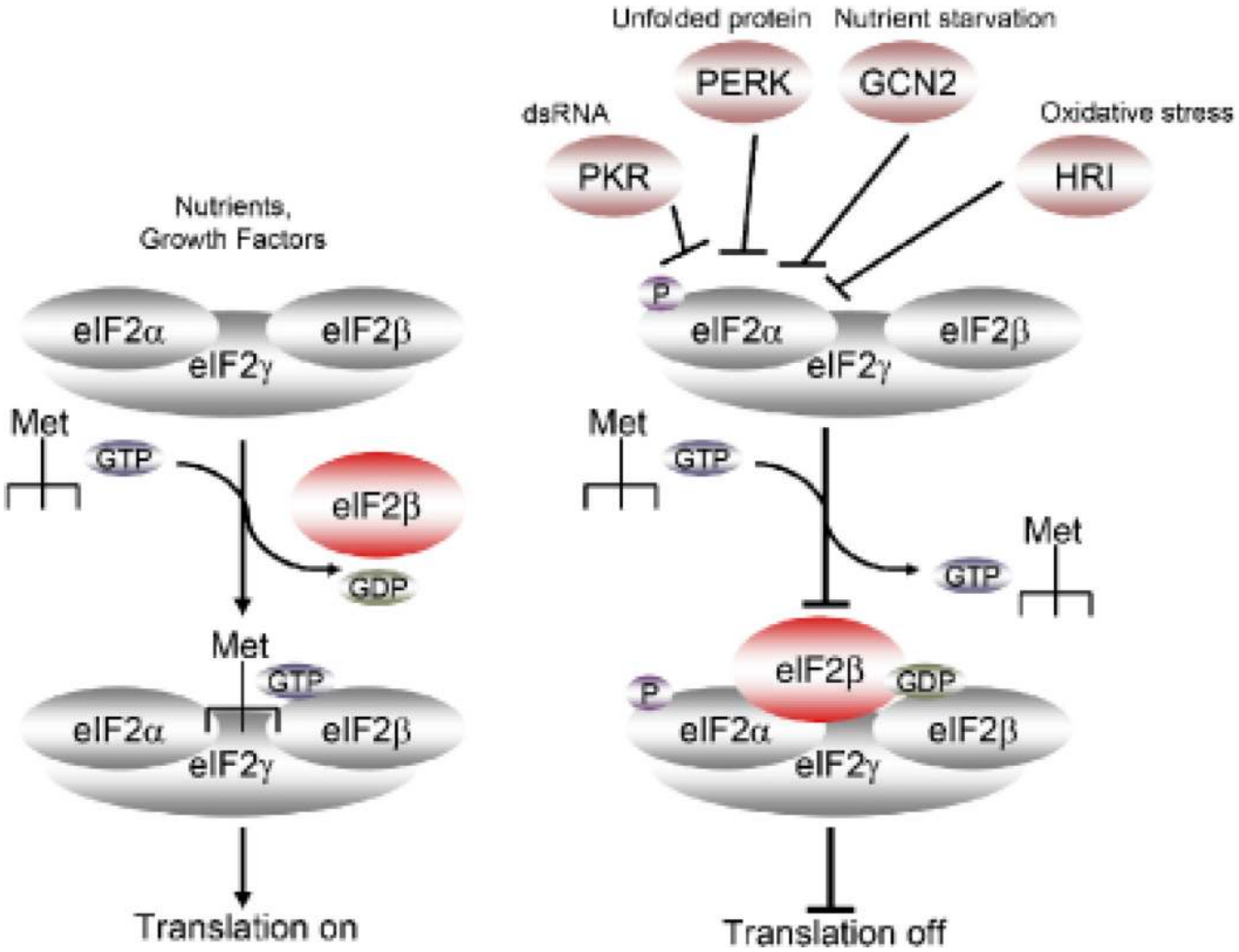

Fig. 2. Control of translation by eIF2α

Multiple signals can trigger the phosphorylation of eIF2α, including nutrient starvation (GCN2), dsRNA (PKN), unfolded proteins (PERK), and oxidative stress (HRI). Phospho-eIF2α binds to the IF2β complex, preventing its translocation and inhibiting translation.

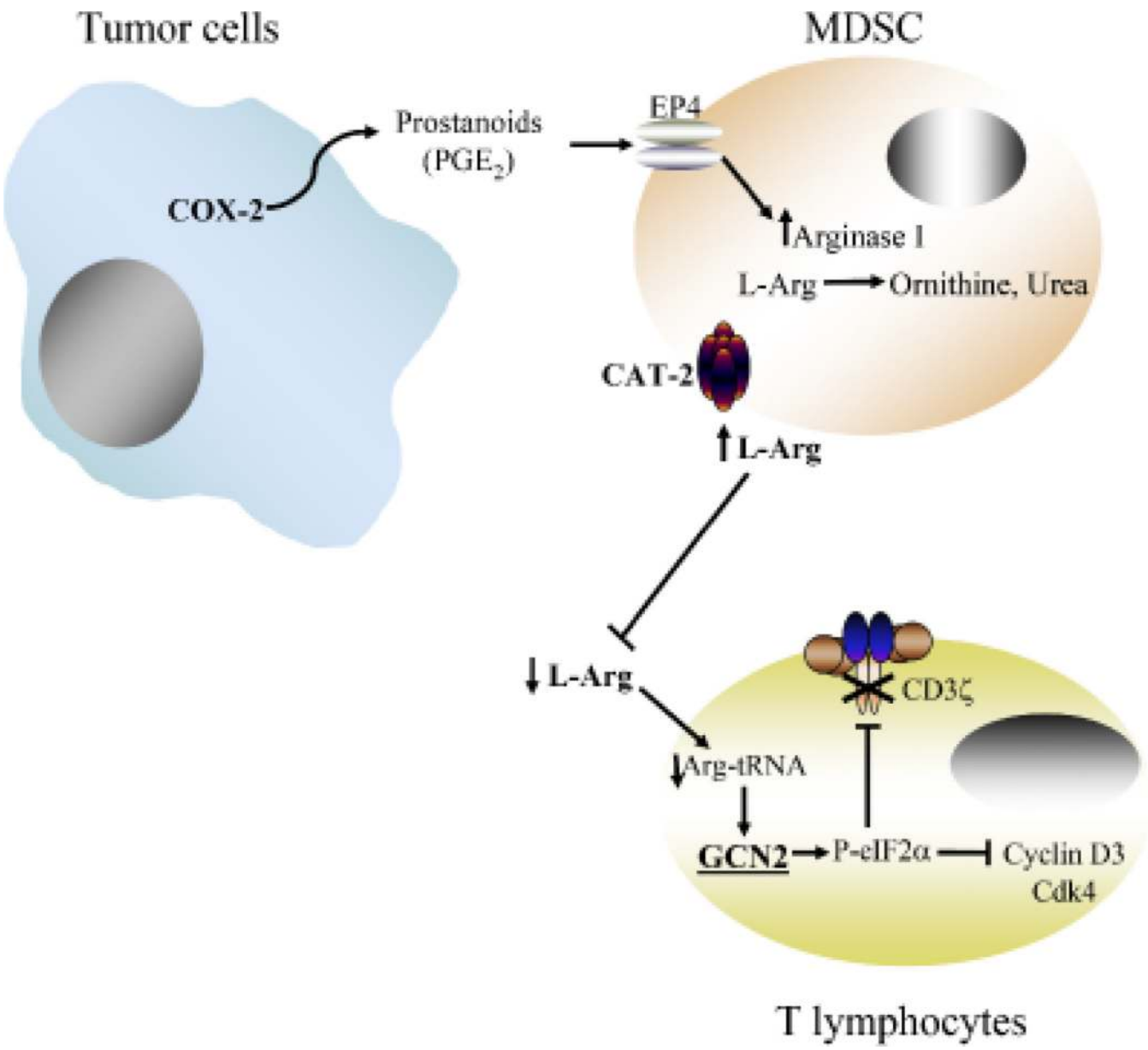

Fig. 3. T-cell dysfunction induced by arginase I

Tumor cells expressing COX-2 and releasing PGE_2 induce the expression of arginase I and CAT-2B in MDSC. This expression leads to a reduction of extracellular levels of L-Arg, which activates GCN2 and inhibits the expression of CD3ζ, cyclin D3, and cdk4 through post-transcriptional and translational mechanisms.