Arginine and Immunity[1–3]

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Abstract

For many years, dietary arginine supplementation, often combined with other substances, has been used as a mechanism to boost the immune system. Considerable controversy, however, exists as to the benefits and indications of dietary arginine due in part to a poor understanding of the role played by this amino acid in maintaining immune function. Emerging knowledge promises to clear this controversy and allow for arginine's safe use. In myeloid cells, arginine is mainly metabolized either by inducible nitric oxide (NO) synthases (iNOS) or by arginase 1, enzymes that are stimulated by T helper 1 or 2 cytokines, respectively. Thus, activation of iNOS or arginase (or both) reflects the type of inflammatory response in a specific disease process. Myeloid suppressor cells (MSC) expressing arginase have been described in trauma (in both mice and humans), intra-abdominal sepsis, certain infections, and prominently, cancer. Myeloid cells expressing arginase have been shown to accumulate in patients with cancer. Arginase 1 expression is also detected in mononuclear cells after trauma or surgery. MSC efficiently deplete arginine and generate ornithine. Through arginine depletion, MSC may control NO production and regulate other arginine-dependent biological processes. Low circulating arginine has been documented in trauma and cancer, suggesting that MSC may exert a systemic effect and cause a state of arginine deficiency. Simultaneously, T lymphocytes depend on arginine for proliferation, ζ-chain peptide and T-cell receptor complex expression, and the development of memory. T-cells cocultured with MSC exhibit the molecular and functional effects associated with arginine deficiency. Not surprisingly, T-cell abnormalities, including decreased proliferation and loss of the ζ-chain, are observed in cancer and after trauma. J. Nutr. 137: 1681S–1686S, 2007.

Arginine, first discovered over 100 y ago, is a basic amino acid naturally ingested in our diets at a rate of ~3–5 g/d (1). Arginine is particularly rich in certain food products, such as meats and nuts. Arginine, initially found to be a nonessential amino acid (2,3), was reported to be necessary for the growth of young rodents (4,5). Thus, an initial classification of arginine as a semiessential amino acid was given (6).

In the 1970s, and as a result of the simple experimental observations, it was noticed that arginine prevented thymic involution after surgery and appeared to increase the number of lymphocytes (7–9). In addition, arginine was found to be necessary for adequate wound healing (9–11). These observations were summarized in the statement that arginine was a "conditionally essential amino acid," which should be supplemented at times of physical stress such as after surgery or trauma (12,13).

In 1987, nitric oxide (NO)[4] production from arginine was identified in endothelial cells (14) and shortly after that in macrophages (15). Eventually, it was shown that inflammatory stimuli induced the expression of a specific isoform of NO synthase (iNOS) in myeloid cells and other cell types (16). There are multiple roles for iNOS in different disease processes (17). Stimuli that induce iNOS include that of T helper 1 (Th1) cytokines (IL-1, TNF-α, and IFN-γ) and endotoxin (18,19). Arginine is the sole amino acid substrate for the production of NO by all isoforms of NOS. Regulating arginine availability is a potential mechanism that can lead to the control of NO production (13,20,21). Arginase 1, an enzyme that metabolizes arginine to ornithine and urea, can actually play that role (18,19,22). Arginase 1 is induced in myeloid cells by T helper 2 (Th2) cytokines, such as IL-4 and IL-13 (23,24), and also by IL-6, IL-10, TGF-β (23), prostaglandins (PGE) (25,26), and catecholamines (23,27–29). Thus, activation of iNOS or arginase (or both) reflects the type of inflammatory response in a

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[4] Abbreviations used: ADS, arginine deficiency syndrome; CAT, cationic amino acid transporter; IED, immune-enhancing diet; iNOS, inducible nitric oxide synthase; MSC, myeloid suppressor cell; NO, nitric oxide; PGE, prostaglandin; Th1, T helper 1; Th2, T helper 2.

specific disease process (18,19). For example, sepsis is associated with a predominance of iNOS but trauma exhibits a preferential induction of arginase (29,30).

Myeloid cells expressing arginase efficiently deplete arginine from the surrounding environment (31,32). Through this mechanism, myeloid cells suppress arginine-dependent functions and are thus appropriately called myeloid suppressor cells (MSC) (33,34). MSC have been described in trauma (in mice and humans), certain infections, and prominently, in cancer (31,35–37). In humans, arginase expression is mainly observed in granulocytes, which can in turn exert a suppressive effect, suggesting a significant difference between species (38). Nevertheless, the mouse models continue to provide important knowledge as to the regulatory effect of arginase-expressing cells.

T lymphocytes depend on arginine for multiple key biological processes, including proliferation, the expression of the TCR complex and the ζ-chain peptide, and the development of memory (34,39). Soon after a traumatic stimulus, MSC invade the marginal (T-cell) zones of the spleen (31). MSC are also present in certain tumors (37,40,41). T-cells cocultured with MSC exhibit the molecular and functional effects associated with arginine deficiency. In vivo, trauma patients (42) or patients with cancer (41) exhibit T-cell abnormalities, including decreased proliferation and loss of the ζ-chain characteristic of arginine deficiency.

Arginase 1 is also pathologically released from nonimmune cells and can result in arginine deficiency. Arginine deficiency caused by the release of arginase after hepatocyte necrosis (43) or hemolysis (44) is associated with abnormally low production of NO, inappropriate vasoconstriction, poor organ perfusion, and pulmonary hypertension (43,44).

In the 1980s, some animal experiments suggested that arginine could have some beneficial effects in restoring T lymphocyte counts under conditions of stress. Pursuant to these observations, commercial diets were created to enhance immunity and hopefully prevent and/or decrease the severity of infections (45,46). The amount of arginine added was significantly higher than that of the normal dietary intake, ranging from 8 to 30 g/d. Diets containing arginine were also complemented with other substances, which were thought to have immune effects. Thus, n-3 fatty acids, nucleotides, and certain micronutrients were used along with arginine to create the so-called immune-enhancing diets (IED) (25,45,46).

Dietary strategies aimed at overcoming arginine deficiency exhibit mixed outcomes. To date >40 trials using IED have been performed in a wide variety of patient populations (45). These trials demonstrate a consistent benefit by reducing infections in patients undergoing high-risk surgery such as colon or pancreatic resection. Trauma patients also appear to benefit from these diets, but they must be started soon (ideally within 24 h) after injury. The use of IED in critically ill medical (nonsurgical) patients has failed to demonstrate any significant benefit. More concerning is the fact that in some of these studies, mixed results have been observed in septic critically ill patients, with some studies describing increased mortality whereas others report just the opposite. These uncertain results make the use of IED highly controversial in sepsis (25,44–46).

We are therefore still in the process of identifying which patient populations and/or disease processes are benefited by arginine-containing diets. The identification of states of MSC and/or the pathologic release of arginase, along with molecular biomarkers of arginine deficiency, promises to provide clues as to which patients would benefit from arginine replacement. We hope that understanding the biological role played by arginine deficiency in different disease processes will lead to its early identification and to the design of new and novel treatments.

Arginine metabolism in myeloid cells

Under resting conditions, little arginine is used by myeloid cells due to a lack of expression of high affinity cell membrane transporters. In addition, in the absence of immune stimulation, myeloid cells do not express the major arginine metabolizing enzymes, iNOS and arginase 1 (47). Thus, dietary arginine supplementation cannot enhance myeloid cell function in the absence of disease. It is only after stimulation that arginine transport into the myeloid cell is greatly increased, mainly as a result of increased expression of the high-affinity cationic amino acid transporters (CAT) (48,49). CAT are highly regulated and are coinduced with arginine-metabolizing enzymes (32,50,51). Rodriguez and coauthors (32) have reported the coinduction of the CAT, CAT2B, with arginase 1 in MSC in cancer-bearing mice.

The metabolism of arginine by iNOS or arginase 1 produces radically different biological products (22,47). iNOS generates NO in large quantities and under physiologic conditions plays an important role in killing parasites, bacteria, viruses, and cancer cells and producing vasodilatation (52). Arginase 1 generates ornithine and urea. Ornithine is a precursor of different products, including polyamines and proline, and thus may play an important role in cell proliferation (53) and wound healing (11,12). We are only beginning to understand how the expressions of iNOS and/or arginase 1 are regulated in myeloid cells (**Table 1**). Classic pro-inflammatory cytokines IL-1, TNF-α, IFN-γ, and IL-2 induce iNOS. In turn, humoral antiinflammatory cytokines IL-4, IL-10, IL-13, and TGF-β induce arginase 1 expression. Endotoxin appears to induce both iNOS and arginase 1 (12,18,19). Upon induction, iNOS exerts a regulatory effect on arginase activity through the production of hydroxy-L-arginine, an intermediate product in the generation of NO. Arginase 1 in turn regulates NO through depletion of arginine availability (22).

The observation that iNOS and arginase 1 are regulated (at least partially) by opposing stimuli allows the investigator (at least theoretically) to determine the inflammatory state generated by the disease process being studied (18,19). If iNOS expression and high NO production is observed, an inflammatory (cellular) stimulus may be predominant. In contrast, if significant arginase 1 expression is detected with little or no increase in expression of iNOS, it would appear that the condition favors a humoral response. This indeed appears to be the case in severe trauma and in sepsis, both associated with severe critical illness and organ dysfunction. In 1991, we demonstrated that there was an accumulation of NO metabolites in human plasma during sepsis (54). This coincided with increased circulating endotoxin levels and decreased systemic vascular resistance (as a measure of excessive vasodilatation). In contrast, trauma is associated with a large induction of arginase 1 expression and a concomitant decrease in circulating NO metabolites (nitrite/nitrate), although a modest induction of iNOS messenger RNA can be observed under certain conditions (31,55).

TABLE 1 Regulation of iNOS and arginase I expression

Enzyme	Cytokine stimulation	Other stimulation
iNOS	IL-1, TNF-α, IFN-α, IFN-β, IFN-γ	Endotoxine, lipopolysaharide
Arginase I	IL-4, IL-6, IL-10, IL-13, TGF-β	PGE1, PGE2, catecholamine

Because expression of arginase 1 and iNOS is reciprocally influenced by Th1 and Th2 cytokines, activation of each may be restricted to separate subsets of myeloid-derived cells, termed alternative activation (56,57). It has been shown recently, however, that both enzymes are produced in CD11b+IL-4 receptor α+MSC and that MSC produce both Th1-type and Th2-type cytokines (IFN-γ and IL-13) (58). Therefore, the designation "alternatively activated" may not be applicable or relevant in the characterization of MSC. The determination that MSC produce both arginase 1 and iNOS provides insights that can be potentially translated to the clinic (59).

To further study this phenomenon, we created a mouse model of trauma in which a mouse is subjected to an exploratory laparotomy under anesthesia and allowed to recover after surgical closure of the abdomen. Arginase and iNOS expression are then measured at different time points in immune tissues such as the spleen. Under some circumstances, endotoxin (LPS) is injected to simulate a sepsis state. Using this model of trauma, we demonstrated a significant induction of arginase 1 messenger RNA but not that of iNOS. In fact, arginase 1 activity in spleen myeloid cells appeared to be directly proportional to the severity of injury (our unpublished data). When control animals were injected with LPS, significant increase in serum nitrates is observed with only modest increased arginase 1 activity. In traumatized animals, however, there was spontaneous increased arginase 1 activity, which was further boosted in response to LPS. Nitrate levels in the serum after trauma modestly increased in response to LPS (our unpublished data). In contrast, septic mice exhibited a huge accumulation of NO metabolites. Thus, at least regarding the metabolism of arginine, sepsis and trauma appear to demonstrate a widely different behavior in the expression of the enzymes and the end biological products that accumulate. Therefore, arginine supplementation may produce widely different biological responses depending on the disease process where it is provided.

Similarly, in a mouse model of cancer, gradual increased arginase 1 activity has been detected in splenocytes starting as early as 3 d after tumor cell (MC38, colon cancer) injection (our unpublished data). Furthermore, if placed in culture, only splenocytes from tumor-bearing animals exhibit over 100-fold increase in arginase activity (our unpublished data). Increased arginase activity has also been reported by multiple investigators in animal models of cancer (37,41) and recently in humans (60,61).

Myeloid cells expressing arginase 1

Myeloid cells expressing arginase 1 are described in a growing number of disease processes, prominently in cancer, autoimmune diseases, and in graft vs. host disease (12,26,33–37). We recently demonstrated the accumulation of arginase 1-expressing myeloid cells in spleens in mice after surgical trauma (31). Myeloid cells harvested after trauma prominently exhibit CD11b and Gr-1 markers and are cataloged as immature cells. However, in mouse models of cancer, arginase 1-expressing myeloid cells may not express Gr-1 markers. In fact, in the different disease processes reported, arginase 1-expressing myeloid cells exhibited considerable heterogeneity. MSC are uniformly characterized as CD11b+ but express surface markers indicative of a mix of partially differentiated monocytes and dendritic cells (26,31,37). The exact differentiation status of MSC is difficult to establish with precision, because their function and phenotype may vary in different body compartments. Thus, other than the expression of arginase 1, phenotypical characterization is difficult due to the heterogeneity of these cell types. Under the right conditions, arginase 1-expressing myeloid cells spontaneously proliferate and differentiate. After being stimulated with selected cytokines and/or growth factors, arginase 1-expressing myeloid cells can differentiate into macrophages, dendritic cells, or even granulocytes (37). In humans, arginase 1-expressing myeloid cells have been reported in cancer and appear to be granulocytic in origin (60). We recently demonstrated that arginase 1 expression increased in mononuclear cells collected from cancer patients. In our study, arginase 1 concentrations directly correlate with the presence of circulating CD16+ cells in patients with pancreatic cancer (our unpublished data). The concentration of arginase activity inversely correlates with the absolute lymphocyte number, suggesting its importance and influence on immune functions. Interestingly, arginase activity as well as number of circulating CD16+ cells decreased significantly after surgical resection of tumor (our unpublished data). It has been shown in a mouse model that removal of the primary tumor results in normalization of the number of systemic MSC (62).

Undoubtedly, the most striking of all properties of arginase 1-expressing myeloid suppression cells is their capacity to deplete arginine from the culture media with the consequent accumulation of ornithine (31). Rapid arginine depletion is the result of increased coexpression of CAT2B that allows arginine import into cells where it is quickly metabolized by arginase 1 (32). The ornithine produced is then exported by the same CAT in exchange for another molecule of arginine (63,64). Arginine depletion is quite efficient and is observed in myeloid cells harvested from tumors and after trauma. Through arginine depletion, arginase 1-expressing cells exert a unique role, regulating at least 2 important biological processes: production of NO and T-cell function.

That NO production (by any of the 3 NOS) is proportional to the available extracellular arginine is an observation better known as the arginine paradox. Indeed, the pharmacokinetics of the NOS enzymes would suggest that under the physiologic range of arginine availability, NO generation would be independent of the concentration. This is, however, not the case, because it appears that arginine availability does determine NO production. Thus, it is not surprising that induced arginase 1 expression may limit the production of NO as it limits arginine availability. In addition, an interesting observation suggests that the presence of arginine is necessary for adequate translation of iNOS (65,66). Thus, increased arginase 1 expression could also limit the production of NO through translational control. Finally, under conditions of low arginine and activation of iNOS, superoxide is produced, which can produce highly reactive peroxynitrites, which can in turn nitrosylate susceptible amino acid side chains, especially tyrosine (67).

T lymphocyte responses and arginine

For many years, investigators have shown that arginine availability is essential for normal T-cell proliferation and function (12,32,42,53). Neither iNOS nor arginase 1 is induced in T lymphocytes, which is a marked difference between these cells and myeloid cells. As in myeloid cells, arginine utilization is minimal during resting conditions, but its uptake is increased dramatically as a result of increased CAT activity upon T-cell activation. Interestingly, maximum T-cell proliferation is achieved with culture media arginine levels of $\approx$100μmol/L with no further increase in proliferation with higher concentrations. Thus, apparently no rational basis supports the hypothesis that arginine supplementation at supraphysiologic concentrations enhances the immune system.

Several investigators including us have further investigated the importance of arginine on specific cellular and molecular functions in the T lymphocyte (31,32,42,53). Upon arginine deprivation, there is a progressive reduction (to ~25% of basal levels) in the number of T-cell receptors on the cell membrane. This is principally due to the translational regulation of the expression of the ζ-chain peptide (31,32,40), an essential component of the T-cell receptor complex. Interestingly, loss of the ζ-chain is observed in certain cancers (41) and after surgery/trauma (40), both disease processes associated with decreased T-cell function and increased arginase 1 expression.

Another effect of arginine deprivation is that of the transcriptional upregulation of argininosuccinate synthase expression. The upregulation of argininosuccinate synthase thus allows the T lymphocytes to generate endogenous arginine from citrulline even in the absence of arginine or in the presence of increased arginase 1 expression by myeloid cells (31,68,69). This observation opens therapeutic possibilities for the use of citrulline as a solution for overcoming arginine deficiency.

T lymphocytes and MSC

Considerable excitement has been generated in the last 5 y regarding the interaction between T-cells and MSC. Soon after trauma, a virtual invasion of MSC to the marginal (T-cell) zones of the spleen was observed (31). When stained with a vital dye and injected into a nontraumatized mouse, MSC once again localized to the marginal zones within the spleen (31). These observations demonstrate an obvious tropism of the MSC toward T lymphocytes and suggest that these 2 cell types are closely interacting. To study these possible interactions, several investigators, including us, using different models, such as cancer or trauma, have demonstrated that MSC inhibit T-cell proliferation, ζ-chain expression, and IL-2 production (31,32,42,53). Abnormal T-cell function caused by MSC is restored through arginine repletion or through blockage of arginase 1 function (26). Through these studies, investigators have come to the conclusion that MSC regulate T-cell function through arginine depletion, a completely novel mechanism of T-cell regulation. Thus, a new term, immunosuppression by starvation, has been coined to depict the T-cell–regulating capacity of MSC (12,34,70).

Arginine deficiency syndrome

We propose that the pathologic production of arginase 1 in MSC could cause sufficient arginine depletion, resulting in compromised T-cell function and NO production and ultimately leading to increased susceptibility to infection. Accumulating data demonstrates that this hypothesis, to our knowledge first forwarded by us (71), is indeed most possibly true. Progress in the cancer research field strongly suggests that the pathologic upregulation of arginase 1 in MSC invading the tumor cause T-cell dysfunction through arginine depletion. Similar observations suggest that arginine deficiency could also be a cause of T-cell dysfunction after surgery and trauma, increasing the susceptibility to infection.

A syndrome is defined as a "collection of signs or symptoms that characterize a specific disease or condition." Pathologic arginine deficiency indeed meets all the necessary requirements for it to be called by the name arginine deficiency syndrome (ADS). The set of signs or symptoms (biomarkers) in ADS are: a pathologic increase in arginase 1, a decrease in arginine availability, decreased NO production, abnormal T-cell function characterized by the loss of the ζ-chain, and a deleterious biological consequence (**Table 2**).

TABLE 2 Disease condition and arginine deficiency

Disease condition	Arginine level	Arginase activity	Abnormal biomarkers
Cancer (41)	↓	↑ in MSC	↓ ζ-Chain ↓ T-cell proliferation
Trauma (42)	↓	↑ in MSC	↓ ζ-Chain ↓ T-cell proliferation
Chronic infection tuberculosis (83)	↓	↑ in MSC	↓ ζ-Chain ↓ T-cell proliferation
Liver necrosis (43)	↓↓	↑ in serum	↑ Pulmonary hypertension
Hemolytic disease (44)	↓	↑ in serum	↑ Pulmonary hypertension

A growing number of diseases in humans appear to be associated with arginine deficiency and ADS and are susceptible candidates for treatment. These include certain tumors such as renal cell and prostate cancer, tuberculosis, and condition after surgery or trauma (72–75). In addition, there are a growing number of diseases where the pathologic release of intracellular arginase from erythrocytes and hepatocytes can deplete arginine (43,44). In these cases, arginase, which is constitutively expressed, is released through hepatocyte necrosis (43) or upon destruction of the erythrocyte (observed during hemolysis) (44). In these cases, MSC are not involved.

Treating ADS

Treatment for arginine deficiency should become a clinical priority if indeed it is a syndrome associated with pathologic consequences. We discuss several potential treatment strategies below.

Inhibition of development and/or activation of MSC. Inhibiting the development of MSC might be approached by targeting host- and/or tumor-derived factors that enhance myelopoiesis (e.g. granulocyte-macrophage colony-stimulating factor; GM-CSF). Alternatively, inhibition of signal transducer and activator of transcription 3 (STAT3) might be useful, because its activation in precursor cells has been shown to be essential for development of MSC. (76). Recently, it has been shown that MSC expresses IL-4 receptor α, which might be used for the targeted depletion or inactivation of MSC in vivo (58).

Blockade of arginase 1 expression and upregulation in MSC. Prevention of arginase 1 induction is possible as we learn how it is upregulated. For example, PGE E2 production by tumors appears to be central in arginase 1 induction in MSC in a mouse model of cancer. The use of *COX-2* inhibitors has been shown to prevent arginase 1 upregulation and inhibit tumor growth (26).

Pharmacologic blockade of arginase 1 function. A growing number of arginase inhibitors are being developed (77–81), although none still has reached any degree of clinical application. Of these, we have successfully used Nor-hydroxy-L-arginine to overcome the T-cell dysfunction that occurs in trauma MSC/T-cell cocultures (31).

Dietary use of arginine. The use of dietary arginine supplementation along with n-3 fatty acids has been extensively tested in over 40 studies in high-risk surgical patients, leading to a consistent and significant reduction in postoperative infections. Based on these studies, the European Society of Parenteral and Enteral Nutrition has recently published its guidelines recommending the routine use of arginine-containing diets in surgical

patients (82). Citrulline, an arginine precursor, has recently become a very interesting alternative, because it is well tolerated and may be beneficial even under conditions with high arginase 1 activity (68,69).

Dietary arginine supplementation is still largely misunderstood, probably due to a lack of knowledge of its exact role in the regulation of immune functions. Recently, investigators, including us, have demonstrated that under some circumstances, such as cancer or after trauma, there is an upregulation of myeloid cells expressing arginase 1, capable of depleting arginine necessary for normal physiologic processes such as NO production and/or T-cell function. The pathologic presence of MSC may lead to significant immunosuppression and thus to poor outcomes. Treatment of arginine deficiency involves several possible strategies, including prevention of MSC accumulation and activation, prevention of arginase 1 upregulation, blockade of arginase activity, or dietary strategies aimed at restoring arginine plasma concentrations.

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