L-arginine metabolism and its impact on host immunity against *Leishmania* infection

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Abstract Leishmaniasis is a vector-borne disease found in many countries worldwide. The causative agent of the disease, *Leishmania spp*., lives as an obligate intracellular parasite within mammalian hosts. Since tissue macrophages are major target cells for parasite replication, the outcome of infection depends largely on the activation status of these cells. L-arginine is a crucial amino acid required for both nitric oxide (NO)-mediated parasite killing and polyamine-mediated parasite replication. This review highlights the significance of L-arginine as a factor determining the outcomes of *Leishmania* infection in vitro and its influences on host immune responses in vivo. Various therapeutic approaches targeting L-arginine metabolic pathways during infections with *Leishmania* are also discussed.

Keywords L-arginine transporter · Arginase · *Leishmania* · Host immune response · Macrophages · Nitric oxide

Abbreviations
DCs   Dendritic cells
MΦs   Macrophages
NO   Nitric oxide

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iNOS   Inducible nitric oxide synthase
IFN-γ Interferon-gamma
TNF-α Tumor necrosis factor
IL   Interleukin
LPS   Lipopolysaccharide
ODC   Ornithine decarboxylase
CAT   Cationic amino acid transporter
LOHA N^{ω}-Hydroxy-L-arginine
SNAP   S-nitroso-acetyl-penicillamine

Introduction

Leishmaniasis is a vector-borne disease found in 88 countries of tropical and subtropical regions worldwide. *Leishmania* infection can give rise to a wide spectrum of clinical manifestations, ranging from self-healing skin ulcers to disfiguring mucosal lesions and fatal visceral infections. Cutaneous leishmaniasis is the most common form of infection that leads to a development of skin papules or nodules [1]. While mucocutaneous leishmaniasis is associated with damages of soft tissues of the nasal, oral mucosa and other mucocutaneous junctures of the skin, visceral leishmaniasis often causes fever, dark spots on the skin, and splenohepatomegaly. The latter form of the disease can be fatal, if not properly treated. *Leishmania* parasites exhibit a dimorphic life cycle. Flagellated motile promastigotes are transmitted into a mammalian host by an infected sand fly during its bloodmeal. Once inside host cells, promastigotes transform into aflagellated amastigotes, which can multiply inside parasitophorous vacuoles (PVs) of the infected cell and eventually burst free to spread infection in the host. While many cell types, including dendritic cells (DCs) [2], fibroblasts [3], and neutrophils [4], can be infected with *Leishmania* parasites, macrophages (MΦs) are the major target cells for parasite replication. Since MΦs are professional phagocytes capable of killing intracellular organisms, *Leishmania*'s ability to subvert MΦ antimicrobial mechanisms and persist for long-term infection within these cells represents an intriguing advantage for the parasite but a major challenge for the host to control this infection.

The fate of intracellular *Leishmania* parasites is determined by the activation status of MΦs. Fully activated MΦs can produce leishmaniacidal molecules, such as NO and oxidative mediators, and kill parasites effectively, whereas "suboptimally" and "alternatively" activated MΦs preferentially activate the arginase pathway to produce polyamines and enhance parasite replication/persistence [5, 6]. Although two distinct types of MΦ activation can lead to divergent outcomes of infection, these two pathways use L-arginine as a common substrate for their enzymatic activities. Therefore, L-arginine is situated at a crossroads between the life and death of the intracellular parasites, and its metabolism is a key determinant for infection outcome. While the roles of L-arginine have been studied extensively in cancer, trauma models (see review in [7]) and *Trypanosoma cruzi* infection [8], this review will focus on the roles of L-arginine in host immune responses against *Leishmania* infection and its biological relevance to the disease outcome.

L-arginine metabolism and transport in mammalian cells, especially in MΦs

L-arginine is involved in many metabolic pathways, including those involved in the synthesis of NO, agmatine, creatine, and urea [9]. Two major L-arginine metabolic

pathways are particularly relevant to *Leishmania* infection due to their roles in regulating MΦ effector functions (Fig. 1). L-arginine in MΦs can either be catabolized by inducible nitric oxide synthase (iNOS) to produce NO, or by arginase for polyamine synthesis, depending on the type of extracellular stimuli. When MΦs are exposed to Th1 cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), together with toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS), the expression of iNOS enzyme is upregulated, driving L-arginine metabolism toward NO production [10, 11]. In addition, several Th1–favored chemokines, including MΦ inflammatory protein-1α (MIP-1α/CCL3), MΦ chemoattractant protein-1 (MCP-1/CCL2) [12, 13], and gamma interferon-inducible protein-10 (IP-10/CXCL10) [14] can also trigger iNOS activity and promote parasite killing in MΦs. In contrast, Th2 cytokines, such as interleukin (IL)-4, IL-10, IL-13, and transforming growth factor-β(TGF-β), preferentially induce L-arginine utilization toward the production of polyamines [15, 16]. These Th2 cytokines induce expression and activity of arginase, which converts L-arginine to L-ornithine, a substrate for ornithine decarboxylase (ODC) that converts it to putrescine [17]. Since polyamines (e.g., putrescine, spermidine, and spermine) are important nutrients being directly utilized by *Leishmania spp.*, the exposure to Th2 cytokines leads to the promotion of parasite growth [15, 18].

Due to the critical roles of L-arginine pathways in determining parasite killing and proliferation, it is anticipated that these pathways are tightly regulated to permit them to
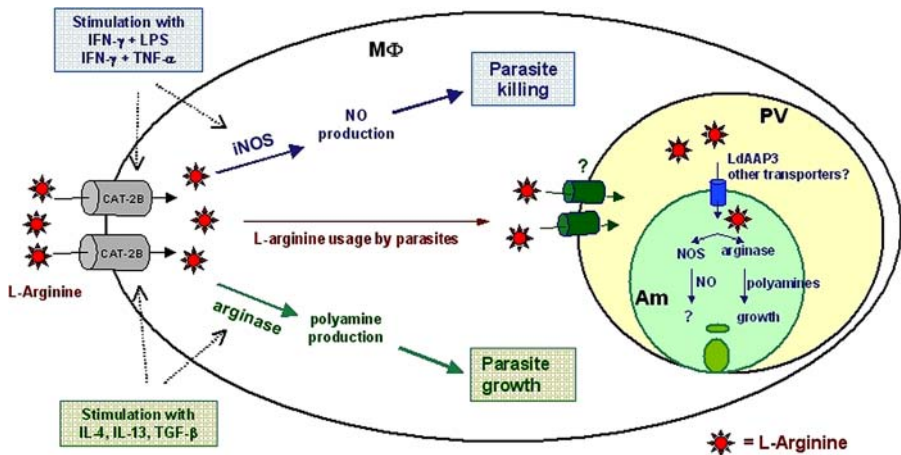

Fig. 1 L-arginine metabolic pathways and their influence on *Leishmania* infection. Classical activation of MΦs via stimulation of Th1 cytokines (IFN-γ plus LPS or IFN-γ plus TNF-α) results in an enhanced expression of CAT-2B, increased L-arginine availability and iNOS activity, ultimately leading to parasite killing. Conversely, Th2-mediated activation of MΦs (via IL-4, IL-13, and TGF-β) enhances CAT-2B expression and arginase activity, resulting in parasite growth. In suboptimally activated MΦs (primed by low concentrations of IFN-γ), CAT-2B is moderately induced, but iNOS and arginase remain relatively inactivated, allowing *Leishmania* amastigotes (Am) to take up and use available L-arginine for their proliferation. While it is unclear which molecules are responsible for the transport of L-arginine across the parasitophorous (PV) membrane, at least one permease, LdAAP3, has been identified as a transporter of L-arginine across the membrane of *L. donovani* promastigotes. Once inside the amastigotes, L-arginine can be utilized by parasite-derived NOS or arginase. While *Leishmania*-derived arginase is involved in polyamine synthesis necessary for the parasite growth, the exact role of *Leishmania*-derived NOS during infection is unclear

compete for available intracellular L-arginine. Several counter-regulatory mechanisms between the iNOS and arginase pathways have been documented. For example, N^{ω}-hydroxy-L-arginine (LOHA), an intermediate product of the iNOS pathway, is known to strongly inhibit arginase function [19]. Conversely, arginase can reduce iNOS activity by competing for L-arginine [20]. Thus, activities of iNOS and arginase can be greatly influenced by L-arginine availability. Although, L-arginine has been described as a non-essential dietary amino acid, because it can be synthesized in virtually all cell types from L-ornithine or L-citrulline, urea cycle intermediates [21], the endogenous synthesis of L-arginine is insufficient for certain cellular activities, including the production of NO via iNOS and polyamine synthesis [22]. In MΦs lacking an L-arginine transporter, NO and polyamine synthesis was reduced when these cells were stimulated with Th1 and Th2 cytokines, respectively [23, 24], suggesting that L-arginine availability is the bottle-neck control step for both iNOS and arginase. Therefore, L-arginine transport from the extra-cellular milieu is critically important for parasite growth and killing within an infected cell.

L-arginine can enter a cell via several transporters, although a Na^+-independent transport system, named system y^+ (y represents lysine, the first substrate described in this system; and $^+$ represents the positive charge amino acids) is postulated to be the main route for L-arginine entry in most cells [25]. Transporters in the y^+ system include those of the cationic amino acid transporter (CAT) family. To date, four members in the CAT family have been identified, CAT-1 to CAT-4, encoded by *Slc7A1–4* genes, respectively [26]. While CAT-1, -2, and -3 are known to transport cationic amino acids—such as L-arginine, L-ornithine, and L-lysine—the function of CAT-4 is unclear [26]. The most relevant CAT member involved in MΦ functions is CAT-2, which can be expressed in two splice variants. CAT-2A is constitutively expressed in the liver and muscle cells [27], whereas the expression level CAT-2B is inducible in MΦs and strongly unregulated by IFN-γ, IFN-γ coupling with LPS, IL-4, and GM-CSF [28–30]. In the context of *Leishmania* infection, the expression and function of CAT-2B in MΦs are critical, because CAT-2B-mediated L-arginine transport potentially affects both iNOS and the arginase pathways. When stimulated with IFN-γ, LPS, or IFN-γ coupled with LPS, MΦs from CAT-2 knockout mice displayed an impaired NO production [22]. These cells also produced significantly decreased levels of L-ornithine and spermine when stimulated with IL-4 and IL-10 [22]. Although these studies demonstrated the importance of CAT-2B in MΦ activities, there is limited information on a direct role of CAT-2B in *Leishmania* infection. Our recent study on *L. amazonensis* infection has shown that both Th1 (IFN-γ/LPS)-mediated killing and Th2 (IL-4)-mediated growth enhancement of amastigotes were suppressed in peritoneal MΦs derived from CAT-2B knockout mice [28]. Given that IFN-γ alone can enhance the growth of *L. amazonensis* amastigotes in MΦs [31], it is interesting to find that IFN-γ-mediated growth enhancement was also diminished in CAT-2B-deficient MΦs [28], suggesting that L-arginine transport via CAT-2B can significantly influence the outcome of *Leishmania* infection (Fig. 1). At present, it is still unclear whether the deletion of CAT-2B can alter the outcome of *Leishmania* infection in vivo. Since there appeared to be a variation in susceptibility to NO-mediated killing in various *Leishmania* species and developmental stages (promastigotes versus amastigotes) [32], it will be necessary to examine whether CAT-2B can influence the outcome of infection caused by different parasite species and developmental stages.

L-arginine metabolism in *Leishmania* parasites

As found in higher eukaryotes, some lower eukaryotes like *Leishmania* and *Trypanosoma* also have their own L-arginine metabolism pathways [33]. L-arginine has long been identi-fied as an essential amino acid for *Leishmania* growth. As early as the 1970s, Krassner et al. demonstrated that *Leishmania* promastigotes could not be maintained in L-arginine-free media [34, 35], suggesting mechanisms of L-arginine uptake and utilization in *Leish-mania* parasites. It is now confirmed that *L. donovani* can transport L-arginine through an amino acid permease called LdAAP3 [36]. LdAAP3 displays several distinct properties when examined in comparison to the CAT-2B found in MΦs. Unlike CAT-2B, that binds with high affinity to several basic amino acids (e.g., L-arginine, L-lysine, and L-ornithine), LdAAP3 specifically binds with high affinity to L-arginine [36]. Moreover, unlike CAT-2B that shows only 50% activity at low pH [37], LdAAP3 is highly active at pH 5.5, a physio-logical pH of *Leishmania*-containing PVs [36]. This unique ability to function at low pH makes LdAAP3 a possible key factor that can interrupt arginine metabolism in MΦs and thus outwit the host antimicrobial function (Fig. 1).

In addition to the L-arginine transporter, *Leishmania* also express other enzymes in the L-arginine metabolic pathway, including arginase and ODC. The expression of arginase in *Leishmania* was first suggested via the presence of its enzymatic activities in parasite lysates [33]. Gene-encoding arginase has been identified in *L. major* and *L. amazonensis* [38] and has been cloned from *L. mexicana* promastigotes. The characterization of *Leish-mania*-derived arginase revealed that it is a single-copy gene expressed in glycosomes [39] and is essential for the parasite survival, as indicated by the failure to maintain arginase-deficient *L. mexicana* promastigotes in vitro in the absence of supplemented L-ornithine or polyamines [39]. Interestingly, the expression of arginase in *Leishmania* can be influenced directly from a host protein. As suggested by Vendrame et al., the treatment of parasites with insulin-like growth factor-I (IGF-I) can significantly induce *Leishmania*-derived argi-nase [40]. The direct correlation between arginase expression in IGF-I-treated *Leishmania* and the increased parasite burden in infected MΦ [40] suggests that parasite-derived argi-nase plays a critical role in enhancing parasite growth in vivo. *Leishmania* parasites also express ODC, an enzyme that converts the arginase-enzymatic product, L-ornithine, to polyamine putrescine [17]. Studies in *L. donovani* revealed that, as found with arginase, ODC is also a single-copy gene vital for parasite growth [41]. Targeted gene deletion of ODC is lethal, unless polyamines (e.g., putrescine or spermidine) were supplemented [41]. Interestingly, the ODC of *Leishmania* is much more stable than that of the mammalian homologs [42]. The slow turnover rate of *Leishmania*-derived ODC makes it a prime target for anti-*Leishmania* drugs (discussed below).

MΦ-derived iNOS and NO production are known for their pivotal roles in parasite killing. It is somewhat surprising that *T. cruzi* [43] and *Leishmania* parasites [44] also have ability to produce various amounts of NO. For example, a significant amount of NO was detected in supernatants of *L. amazonensis*, *L. braziliensis*, and *L. chagasi* cultures [45]. The isolation and characterization of NOS from *L. donovani* promastigotes [44] and *L. amazonensis* axenic amastigotes and promastigotes [46] revealed that this parasite-derived enzyme closely resembles its mammalian constitutive NOS (cNOS), which requires Ca^{2+}, calmoduline, and NADPH for its activity [46]. Importantly, culture media derived from amastigotes or highly infectious metacyclics appeared to contain relatively high NOS activities [46], implying a potential correlation between *Leishmania* NOS and parasite infectivity [47]. However, the biological relevance of these findings in parasitic infections remains unclear.

The findings showing that *Leishmania*, like mammalian systems, display transporters (LdAAP3) and enzymes needed for L-arginine metabolism (arginase and NOS) highlight the common need for the parasite and host cell to compete for the same nutrients and allow us to emphasize that in a certain microenvironment (e.g., within the acidic PV), the parasite's capacity to compete for L-arginine appears to be favored. This is particularly important, given the fact that *Leishmania* parasites have additional strategies to suppress host cell activation and signaling pathways and to halt host cell growth (reviewed in [48, 49]). Therefore, L-arginine metabolic pathways in *Leishmania* may represent a novel strategy by which the parasites can compete for a limited nutrient/biological precursor with the host and evade host immune responses. Several key questions in this area remain to be addressed. How does parasite-derived arginase or iNOS compete for L-arginine? Can the parasite's L-arginine metabolic machinery interfere with the host's L-arginine metabolic pathways and affect the outcome of infection?

The influences of L-arginine and its metabolism on other host immune components

L-arginine not only serves as a crucial amino acid for MΦ activation, but also influences other arms of host immune responses, including T and B cells [50]. Although the role of L-arginine in *Leishmania*-specific T cell responses is unclear, the crucial roles of L-arginine in T-cell proliferation and activation have been demonstrated in other disease models, such as certain tumors [51] and in cases of *Helicobactor pylori* infection [52]. Using mouse tumor-associated myeloid cells (CD11b$^+$, CD16$^+$/CD32$^+$, I-A/I-E$^+$) that express high levels of arginase, Rodriquez et al. demonstrated that L-arginine depletion, due to the activation of arginase in these MΦ-like myeloid cells, led to the suppression of T-cell responses [51]. Specifically, the CD3ζ chain, the main signaling chain of the T-cell receptor, was significantly downregulated in reduced concentrations of extracellular L-arginine [51, 53]. A similar suppression was documented when extracellular L-arginine concentration was reduced in the presence of *H. pylori*-derived arginase [52]. Moreover, L-arginine starvation can arrest stimulated T cells in the G_0–G_1 phase of the cell cycle [54]. These studies suggest that the co-localization of T cells with cells displaying high arginase activities may render T cells hyporesponsive. Defective T-cell proliferation and activation are hallmarks in *L. amazonensis*-infected mice [55] and humans [56]. Since *L. amazonensis* infection induces the production of MΦ-derived IL-10 [18, 57] that can activate arginase function [15], local L-arginine depletion, together with suppressed antigen-presentation function of DCs [58], may collectively contribute to T-cell hyporesponsiveness in infected hosts.

L-arginine availability also plays a role in regulating functions of B cells. Using F/A-2^{++} mice that over expressed arginase, but reduced tissue L-arginine concentration, de Jonge et al. demonstrated that L-arginine deficiency was correlated with a dramatic reduction of B-cell maturation and a significant decrease in serum IgM, due to an impaired transition from pro- to pre-B cells [59]. The findings that oral supplementation of L-arginine enhanced antigen-specific IgA in mice immunized with tetanus toxoid further support a role for L-arginine in B-cell regulation [60]. Studies using CAT-2-deficient mice revealed that the lack of an L-arginine transporter led to an increase in DC activation in the lungs [59], suggesting that L-arginine concentrations in vivo can influence DC function. In addition, CAT-2-deficient mice displayed marked eosinophilia and neutrophilia, before and after weaning, respectively [59], suggesting that L-arginine plays a role in cellular migration. This notion was supported by a study showing that a correlation among L-arginine concentration, expression of adhesion molecules on endothelial cells, and neutrophil

trans-endothelial migration [61]. These studies collectively indicate multiple roles of L-arginine in regulating host immune functions [50].

Targeting L-arginine metabolic pathways as a therapeutic approach for the control of *Leishmania* infection

Since L-arginine metabolic pathways are crucial for parasite growth and host defense, these pathways may be explored as therapeutic approaches for the control of *Leishmania* infection. One approach is to interfere with arginase activity using a physiological inhibitor, LOHA. Treatment of bone marrow-derived MΦs containing either *L. major* or *L. infantum* promastigotes with LOHA results in a dramatic decrease in both the number of intracellular amastigotes per cell and the percentage of infected cells [19]. Interestingly, LOHA appeared to suppress both MΦ- and *Leishmania*-derived arginases [19]. Inhibition of arginase activity can also reduce the severity of leishmaniasis in vivo. For example, Kropf et al. demonstrated that administration of the synthetic arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) to *L. major*-infected BALB/c mice during infection significantly reduced arginase activity, lesion sizes, and tissue parasite burdens [62].

While inhibition of arginase is a possible means of controlling *Leishmania* infection, the stimulation of iNOS-mediated parasite killing is a more attractive approach because iNOS induction is known to be correlated with the effectiveness of anti-*Leishmania* treatment. For example, high levels of iNOS induction were observed following successful treatment of *L. donovani*-infected BALB/c mice with IFN-γ coupled with synthetic peptide containing an amino acid sequence of a cysteine protease inhibitor, cystatin [63]. Similarly, a reduced parasite burden in *L. donovani*-infected BALB/c mice treated with the phosphotyrosine phosphatase inhibitor bpV(phen) was associated with the ability of bpV(phen) to induce high levels of iNOS activity [64]. Of note, iNOS function in vivo can be enhanced when L-arginine availability is increased. Treatment of *L. donovani*-infected hamsters with an iNOS inducer, polyinosini-polycytidylic acid stabilized with polylysine and carboxy-methylcellulose (poly ICLC), was improved when L-arginine was supplemented [65], highlighting the importance of L-arginine in iNOS-mediated control of leishmaniasis.

Since *Leishmania* parasites exhibit L-arginine transporters and enzymes of L-arginine metabolism, some drugs are designed to specifically target *Leishmania*-derived ODC. Since this parasite enzyme has a lower turnover rate, when compared to mammalian homologs [42, 66], treatment of experimental leishmaniasis with ODC inhibitors, such as D, L-α-difluoromethylornithine (DFMO) [67] and 3-aminooxy-1-aminopropane (APA) [68–70], is an effective measure for controlling parasitic infection [42, 71]. In addition to the enzymes in L-arginine metabolic pathways, the L-arginine transporter may serve as a potential target for anti-leishmania drugs. Although there is no known difference in the turnover rates of *Leishmania* and host L-arginine transporters, current anti-*Leishmania* drugs, i.e., the diamidines, partially exert their antileishmanial activities by targeting *Leishmania's* L-arginine transporter. Kandpal et al. demonstrated a direct correlation between the inhibition of L-arginine uptake by *L. donovani* promastigotes and the antileishmanial activities caused by pentamidine and dibromopropamidine, a highly potent antileishmanial diamidine [72]. Treatment of *L. donovani* and *L. amazonensis* promastigotes with pentamidine also resulted in a significant decrease in the intracellular pool of L-arginine [73]. Since L-arginine is essential for the survival of intracellular parasites, a reduced concentration of intracellular L-arginine may exert a detrimental effect on these parasites.

Concluding remarks

L-arginine is an important amino acid involved in many crucial pathways in host MΦs and *Leishmania* parasites. L-arginine metabolic pathways not only participate in the regulation of iNOS-mediated parasite killing and arginase-mediated parasite growth, but also are involved in the regulation of other immune components, including T cells, B cells, DCs, and neutrophils. Although several anti-*Leishmania* reagents which target L-arginine metabolic pathways have been studied, more research in this area is still needed. Given that several *Leishmania*-specific proteins in L-arginine pathways have been characterized (i.e., LdAAP3, *Leishmania*-derived arginase, and NOS), and that more of such proteins are likely to be identified in the near future due to the completion of the *Leishmania* genome project, research focusing on disrupting functions of L-arginine metabolic pathways in *Leishmania* may provide a novel and potentially effective therapeutic approach for the control of leishmaniasis.

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