Arginase I Induction during Leishmania major Infection Mediates the Development of Disease

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In a previous work, we demonstrated that the induction of arginase I favored the replication of Leishmania inside macrophages. Now we have analyzed the differential expression of this enzyme in the mouse model of L. major infection. Ours results show that arginase I is induced in both susceptible and resistant mice during the development of the disease. However, in BALB/c-infected tissues, the induction of this protein parallels the time of infection, while in C57BL/6 mice, the enzyme is upregulated only during footpad swelling. The induction of the host arginase in both strains is mediated by the balance between interleukin-4 (IL-4) and IL-12 and opposite to nitric oxide synthase II expression. Moreover, inhibition of arginase reduces the number of parasites and delays disease outcome in BALB/c mice, while treatment with l-ornithine increases the susceptibility of C57BL/6 mice. Therefore, arginase I induction could be considered a marker of disease in leishmaniasis.

The relationship between the immune system and the evolution of infection in leishmaniasis is well studied in the experimental murine model. There has been substantial progress in understanding the immunopathogenesis of cutaneous leishmaniasis in several murine models of infection over the past 20 years (17). It is also now evident that cytokines released during the initial contact of pathogens with cells of the innate immune system play a decisive role in shaping the subsequent induced response orchestrated by T cells (21).

In the case of L. major infection in mice, genetically determined resistance and susceptibility to the infection are clearly related to the development of polarized Th1 and Th2 responses, respectively (10). The dominant Th1 response corresponds with a localized, benign, and spontaneously resolved lesion, whereas a predominant Th2 response, as the one generated by the BALB/c strain, involves a severe wide and non-spontaneously healing lesion that finally drives to the development of systemic disease (2).

Macrophages, an important host cell for Leishmania infection, induce the enzyme arginase I in response to Th2-derived cytokines as well as by other mediators (3, 4). Under these conditions, they present different subsets of innate immunity receptors and therefore have been called alternatively activated macrophages (9). Arginine hydrolysis by arginase generates urea and ornithine that, in turn, may be diverted toward polyamine synthesis or collagen synthesis.

Leishmania parasites have an unique gene coding for arginase that has been recently demonstrated to be essential for parasite survival, since arginase-deficient parasites are unable to generate polyamines, although they could obtain them from the extracellular environment. Therefore, knockouts actually become polyamine auxotrophs (16). This reinforces the hypothesis that an increase in the pool of polyamines in the host should favor the intracellular parasitic growth. Indeed, our previous results demonstrated that the in vitro growth of L. major inside macrophages was increased in the presence of arginase I induction (12). Moreover, both l-ornithine and putrescine, added to infected macrophages, were able to promote the growth of intracellular Leishmania in vitro, suggesting that arginase induction in the host was being used for polyamine generation. In fact, the intracellular parasitic growth could be controlled by inhibiting arginase (11).

It is known that parasites have evolved a great number of strategies to invade the host and escape the immune response. Recent studies (7, 24) are consistent in proposing a common mechanism by which different parasites are able to avoid nitric oxide toxicity and its derivatives, the induction of arginase by the host, which has at least two consequences: first, the depletion of l-arginine, common substrate for nitric oxide synthases (NOS IIs) that results in reduced levels of nitric oxide radicals, and second, the deviation of this amino acid toward other metabolic routes such as collagen or polyamine synthesis.

Therefore, our next aim has been to investigate the precise role of arginase in a mouse model of L. major infection in order to ascertain the contribution of the enzyme to disease susceptibility. We have found that the induction of arginase I in the host is closely correlated with susceptibility to the infection, because in susceptible BALB/c mice, its expression was directed by interleukin-4 (IL-4) induction and paralleled footpad swelling. In contrast, in resistant C57BL/6 animals, the establishment of a protective response restores the enzyme levels to baseline along with the resolution of the lesions.
Arginase activity in infected tissues (n = 5) was measured in footpad homogenates used for arginase activity.

IL-4 and IL-12 concentration in sera (n = 5) were measured by enzyme-linked immunosorbent assay (Bender MedSystems, Vienna, Austria) following the manufacturer’s instructions. The detection limits for IL-4 and IL-12p40 were 3 pg/ml and 30 pg/ml, respectively.

Immunohistochemical analysis (n = 4), two independent experiments for each mouse strain performed in duplicate, were made to detect arginase I in formalin-fixed, paraffin-embedded tissue sections. Samples were deparaffinized and rehydrated and the antibody epitopes unmasked by a 5-min incubation in PBS-0.5% Tween 20 at room temperature. Endogenous peroxidase activity was quenched by a 5-min incubation in 3% H2O2. Finally, samples were incubated with mouse anti-arginase I IgG1 (BD-Transduction laboratories, Lexington, KY) or normal mouse IgG1 (Sigma-Aldrich, Spain) as a negative control by using the MasVision kit (Anacron diagnostics, Sevilla, Spain) as directed by the manufacturer. The labeling was visualized with 0.2 mg/ml 3-amino-9 ethylcarbazole and counterstained with hematoxylin. Micrographs are representatives of more than 50 stained sections.

Arginase I and NOS II immunoblotting (n = 5) were performed in the same tissue homogenates used for arginase activity.

Equal amounts of 20 μg protein/lane were subjected to electrophoresis in 12.5% acrylamide for arginase I or 7.5% acrylamide for NOS II. The positive controls were mouse liver for arginase I and lipopolysaccharide-treated macrophages for NOS II.

After semidry electrotransfer (Bio-Rad), the nitrocellulose membrane (Amersham) was first blocked with PBS-Tween 20–1% bovine serum albumin and incubated with the anti-arginase I or anti-NOS II antibody (Transduction Lab), followed by a peroxidased secondary antibody. Signals were detected with a chemiluminescence kit (Amersham) according to the manufacturer’s directions. A rabbit polyclonal anti-actin antibody (produced against the C-terminal fragment; Sigma, Spain) was used as internal loading control following the manufacturer’s instructions.

Treatment of infected mice with nor-NOHA and l-ornithine. BALB/c mice (n = 5) were infected with 1 × 106 promastigotes and given a daily dose of 10 μg Nω-hydroxy-nor-l-arginine (nor-NOHA; Calbiochem, Spain) dissolved in PBS and injected intradermally next to the footpad. On the other hand, C57BL/6 mice (n = 5) were treated with 500 μg l-ornithine (Sigma-Aldrich, Spain) with the same protocol. In both experiments, control infected mice were treated with an identical dose of PBS.

FIG. 1. Arginase I induction correlates with footpad swelling and is opposite to NOS II induction. Mice (n = 10) were infected with 10^6 stationary-phase L. major promastigotes, and lesion size (n = 10), parasites/mg of tissue (n = 3) (A), or arginase activity/mg (n = 5) (B) were measured as described in Materials and Methods. (C) Tissue homogenates (20 μg protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-arginase I, anti-NOS II, or anti-actin as internal control. C, positive controls; L, L. major lysate. One of three independent repeats is represented. The data presented are representative of three independent experiments. Values are the means ± standard deviations of n (mice per time point). **, P < 0.01; ***, P < 0.001, versus those for day 0 by Student’s t test.

MATERIALS AND METHODS

Mice, parasites, and infections. Six- to eight-week-old BALB/c and C57BL/6 mice were from Charles River Laboratories (France) and purchased by S. L Janvier España. The Health Report of these animals certified that they were free of pathogens. Mice were kept under conventional conditions in isolation facilities during all of the experimental phase, and all procedures were approved by the institutional animal care and use committee.

Leishmania major (WHOM/IR/−/173) promastigotes were cultured at 26°C in Schneider’s medium (Sigma-Aldrich, Spain) supplemented with 20% heat-inactivated fetal calf serum. Parasites were kept in a virulent state by monthly passages in BALB/c mice. Animals were infected with a single dose of 10^6 stationary-phase promastigotes inoculated in the right hind footpad. At fixed times after infection, 10 mice per group were sacrificed in order to analyze several parameters:

Footpad swelling (n = 10) was evaluated by using a digital caliper and represented as the difference in mm between the infected and noninfected footpads. The number of parasites in the lesions (n = 3) was calculated as previously described (1) and was represented either as parasites/mg (Fig. 1) or parasites/mg of tissue. The number of parasites in the lesions (n = 3) was calculated as previously described (4). Arginase-specific activity was expressed in mU/mg of tissue.

Arginase activity/mg (n = 5) was measured as described in Materials and Methods. The detection limits for IL-4 and IL-12p40 were 3 pg/ml and 30 pg/ml, respectively.

Further description of methods related to the experiments presented in the figure.
As it can be appreciated, our results were in agreement with previously reported results (23), while arginase I was preferentially induced in BALB/c tissues. However, these data also reflect that arginase I and NOS II are coincided at fixed points after infection, especially at days 28 and 50, since the degree of induction strictly parallels the course of infection in both strains.

**RESULTS AND DISCUSSION**

Our previous in vitro studies pointed to a dependence of parasite proliferation on an efficient arginase I induction, because the inhibition of this enzyme controlled parasite proliferation inside macrophages (11). Studies made by Gobert et al. on *Trypanosoma brucei*, were the first showing of the role of macrophage’s arginase in favoring the in vivo growth of these parasites occurred (7). These findings have now been confirmed by others (22) and extended not only to *Leishmania* but also to other microorganisms, such as *Helicobacter pylori* (8).

With these premises, we wanted to confirm in our in vitro data in a murine model of *L. major* infection. In this context, we could assess two important questions. One was whether the induction of arginase could be associated with susceptibility to the infection. The second was to study arginase I regulation along the time of disease in two opposite cases: resolution of infection in C57BL/6 or progression in BALB/c mice.

**Arginase I activity correlates with footpad inflammation.** The evolution of infection in these two mice (Fig. 1A) was in agreement with previously reported results (1): BALB/c mice developed a progressive increase in footpad thickness that ended in a nonhealing phenotype. In contrast, in C57BL/6 mice, footpad swelling declined progressively after 3 weeks, consistent with a resistant phenotype. In the same experiments, we measured arginase-specific activity in footpad homogenates (Fig. 1B) and found that unexpectedly, both mouse strains showed the same degree of induction until the third week of infection. Thereafter, arginase activity in resistant mice was decreasing progressively while remaining high in BALB/c mice. In the same experiments, we measured arginase-specific activity in footpad homogenates (Fig. 1B) and found that unexpectedly, both mouse strains showed the same degree of induction until the third week of infection. Thereafter, arginase activity in resistant mice was decreasing progressively while remaining high in BALB/c mice.

**Next, we compared protein levels at fixed points postinfection.** As it can be appreciated, our results were in agreement with those of activity, because in susceptible mice, arginase I was already upregulated at day 14 and its induction increased proportionally to the time of disease, while in C57BL/6 mice, protein levels were increased only at day 28 (Fig. 1C). However, proteins from *L. major* (10^12 parasites) and BALB/c did not present a positive signal as expected, because the anti-arginase I antibody used does not cross-react with the *L. major* enzyme. Therefore, it is possible to discern between the host and parasite arginasins. In fact, the calculated sequence identity for both is only 34.23%. These data allow us to demonstrate that, although the measurement of total enzyme levels in footpads included the arginase activity of parasites, the differential activity levels found in susceptible versus resistant animals are mainly a reflection of the different patterns on arginase I induction.

One of the essential enzymes in *L. major* infection is NOS II, induced as a consequence of a Th1 predominant response and associated with resistance to this parasite (23). Arginase and NOS share arginine as substrate, and both are induced in macrophages. Thus, we compared the levels of NOS II in infected footpads at fixed points postinfection (Fig. 1C). Results showed that NOS II induction was higher in resistant mice and its expression correlated with the resolution of lesions, as previously described (23), while arginase I was preferentially induced in BALB/c tissues. However, these data also reflect that arginase I and NOS II are coincided at fixed points after infection, especially at days 28 and 50, since the degree of induction strictly parallels the course of infection in both strains.

**Immunohistochemistry of arginase I expression in infected tissues.** The immunohistochemical staining of infected footpads confirmed previous results and gave us important information about the distribution of this protein. At day 14, arginase I expression was located mainly in the mononuclear infiltrate of the dermis but also surrounding the connective tissue (Fig. 2A and D). Both mice presented similar locations; however, in susceptible mice, the higher cellular infiltrate increased the number of arginase-staining versus -resistant mice. At day 28 (Fig. 2B and E), arginase expression began to increase, together with the mononuclear infiltrate, and was found mainly inside macrophages (Fig. 2B, inset) but also in dendritic cells, polymorphonuclear cells, and fibroblasts (data not shown). Again at this point of infection, arginase was upregulated in both mice but the degree and spreading of arginase I-positive cells was significantly lower in resistant mice (Fig. 2E). The diffusion and intensity of arginase-positive staining further increased at day 42 in BALB/c animals (Fig. 2C), reaching even subhypodermal areas. At this point of infection, the histological pattern was characterized by the presence of an ulcerative process and massive necrosis, with loss of tissue structure, as can be appreciated in the negative control for arginase I (Fig. 2C, inset). In contrast, in C57BL/6 mice (Fig. 2F), where the process of tissue repair was earlier, the staining was much lower, probably restricted to areas of tissue remodeling.

These results again show the same pattern of arginase induction between BALB/c and C57BL/6 mice. In susceptible mice, the induction of the enzyme paralleled the progression of the infection (Fig. 2A to C), while in resistant mice, the maximal levels were found at day 28 (Fig. 2D to E), the time in which the values of footpad swelling (Fig. 1A) were still very high in this strain.

**The Th1/Th2 cytokine pattern explains the differences in arginase activity between BALB/c and C57BL/6-infected mice.** In order to better understand the regulation of arginase I induction, we decided to measure the concentration of IL-4 and IL-12 in the sera of infected animals. We found that both strains presented an initial burst in IL-4 levels during the first 2 weeks of infection (Fig. 3A), although it was much higher in BALB/c. In the same serum samples, IL-12 p 40 levels could not be detected until day 7 and were surprisingly similar between mice during the first 2 weeks postinfection (Fig. 3B).

The significant increase of IL-12 in C57BL/6 mice (day 14) was found immediately prior to the decrease in IL-4 concentrations. Thus, this could mark the time in which the Th1 response started to be predominant, leading to an increase in gamma interferon concentrations (10) with the consequence of NOS II induction and arginase I downregulation (13). In contrast, in BALB/c mice, the decrease in IL-12 levels (days 14 to 21) together with an increase in the second pick of IL-4, could be sufficient to establish the Th2 predominance. Under these conditions, arginase could remain fully induced. Although we have not measured the local cytokine concen-
In lymph nodes, there is substantial information about
the kinetics of IL-4 and IL-12 production in this model (17)
and it is well established that IL-12 is essential for maintaining
a Th1 response during *L. major* infection (15, 18). Therefore,
it could be possible to detect significant IL-12 concentrations in
sera during the infection, at least in C57BL/6 mice. In fact, an
elevation in IL-12 levels has also been found in the sera of
C57BL/6 mice 4 weeks after infection with *L. major* (14).

On the other hand, both susceptible and resistant mice are
able to generate an early IL-4 response (20) and it is also
possible to find IL-12 production early after infection of
BALB/c mice. Indeed, BALB/c mice treated with an anti-IL-12
antibody have been found to further increase the lesions (19).

Thus, data presented in Fig. 3 are in agreement with those
previously reported.

Moreover, by comparing the levels of IL-4 with those of
arginase activity in Fig. 1, the data show that the timing of
enhanced arginase activity in the resistant mice and the time in
which arginase begins its increase in BALB/c mice (day 21)
actually correspond to a nadir in IL-4 levels (Fig. 3A). Thus, it
might be possible that the systemic immune response does not
primarily determine the local arginase activity during the early
period of infection.

Finally, it is worth mentioning that arginase I is also trig-
gerated by IL-10 and TGF-β, both of them are induced during
*Leishmania* infection and play important roles in the develop-
ment of the disease, being important for parasite survival (5, 6). Therefore, the regulation of arginase by these cytokines deserves further investigation, since they may determine the precise cell type in which the enzyme is expressed.

Inhibition of arginase delays the disease in BALB/c mice, while treatment of resistant mice with L-ornithine increases its susceptibility to the infection. Finally, we tried to control *L. major* infection by treating susceptible mice with nor-NOHA, a competitive arginase inhibitor. Results presented in Fig. 4 show that, although we could not protect mice from developing full infection at later times, both lesion size and parasite replication were controlled during the first 3 weeks of treatment (Fig. 4A). Afterwards, and probably due to the huge levels of arginase found in tissues, parasite and host arginases could not be inhibited by the protocol used.

To try a different strategy, we treated resistant animals with 500 µg/animal/day of L-ornithine, which resulted in an increase not only in footpad swelling but also in the number of parasites/lesion (Fig. 4B), despite the predominant Th1 response confirming the avidity of parasites for this amino acid needed for polyamine generation.

Therefore, although L-ornithine treatment delayed the resolution of the lesions and thus prolonged the disease in resistant mice, treatment with the arginase inhibitor nor-NOHA could not change the phenotype of BALB/c mice. These results led us to reconsider the use of this inhibitor as a good candidate for the treatment of Leishmaniasis, at least in the visceral form of the disease, where other routes of administration may inhibit the liver isoform I, the same induced in macrophages.

In conclusion, arginase I accompanies the development of the disease in this model, being expressed mainly in the phagocytic infiltrate (macrophages and neutrophils) but also in the fibroblasts of connective tissue. All these cell types may be infected by *Leishmania*, suggesting that arginase-positive cells could be a reservoir for parasite survival. Moreover, we do not discard the possibility that the different cellular locations of arginase could imply other metabolic fates for L-arginine in host cells. One of these roles could be the generation of ornithine directed toward tissue repair, which is necessary while the infection is being controlled, as occurs in resistant animals. Therefore, we think that this work may open new perspectives for the study of arginase alternative functions in Leishmaniasis.

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**FIG. 3.** Kinetics of circulating IL-4 (A) and IL-12 (B) levels in sera of *L. major*-infected BALB/c and C57BL/6 mice. Blood samples were taken at different times of infection, and cytokine concentrations were measured by enzyme-linked immunosorbent assay as described in Materials and Methods. Data are representative of three independent experiments. Values are the means ± standard errors of the means of five mice per point. Significance values are comparing the increase in cytokine concentrations from day 2 postinfection for IL-4 or day 0 for IL-12. ***, P < 0.001.**

**FIG. 4.** Nor-NOHA delays disease in BALB/c mice, while L-ornithine increases the susceptibility to the infection in C57BL/6 mice. Animals (five per group) were injected daily with a dose of 10 µg/50 µl nor-NOHA (Fig. 4A) or 500 µg/50 µl L-ornithine (Fig. 4B) next to the footpad. Control mice (BALB/c and C57BL/6) were treated with the same amount of PBS. Parasites/lesion at indicated times are represented in numbers. One in five comparable experiments for nor-NOHA and one in two for ornithine treatments are shown. Significant values are comparing treated animals versus nontreated animals. ***, P < 0.01; ****, P < 0.001.
REFERENCES


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