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Infection with Arginase-Deficient *Leishmania major* Reveals a Parasite Number-Dependent and Cytokine-Independent Regulation of Host Cellular Arginase Activity and Disease Pathogenesis

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The balance between the products of 1-arginine metabolism in macrophages regulates the outcome of *Leishmania major* infection. 1-arginine can be oxidized by host inducible NO synthase to produce NO, which contributes to parasite killing. In contrast, 1-arginine hydrolysis by host arginase blocks NO generation and provides polyamines, which can support parasite proliferation. Additionally, *Leishmania* encode their own arginase which has considerable potential to modulate infectivity and disease pathogenesis. In this study, we compared the infectivity and impact on host cellular immune response in vitro and in vivo of wild-type (WT) *L. major* with that of a parasite arginase null mutant (*arg−*). We found that *arg−* *L. major* are impaired in their macrophage infectivity in vitro independent of host inducible NO synthase activities. As with in vitro results, the proliferation of *arg−* *L. major* in animal infections was also significantly impaired in vivo, resulting in delayed onset of lesion development, attenuated pathology, and low parasite burden. Despite this attenuated pathology, the production of cytokines by cells from the draining lymph node of mice infected with WT and *arg−* *L. major* was similar at all times tested. Interestingly, in vitro and in vivo arginase levels were significantly lower in *arg−* than in WT-infected cases and were directly correlated with parasite numbers inside infected cells. These results suggest that *Leishmania*-encoded arginase enhances disease pathogenesis by augmenting host cellular arginase activities and that contrary to previous in vitro studies, the host cytokine response does not influence host arginase activity. *The Journal of Immunology*, 2009, 183: 8068–8076.

Cutaneous leishmaniasis is a spectral disease caused by the intracellular protozoan parasite *Leishmania major*. Although the disease is endemic in tropical and subtropical countries, international tourism, migration, and military operations in endemic regions have increased the number of imported cases to low-prevalence areas, particularly in the western countries (1). After inoculation into the host by the sand fly, the promastigotes are rapidly phagocytosed by macrophages where they differentiate into amastigotes. Following phagocytosis, one of two opposing forms of macrophage activation will occur (classical or alternative), resulting in differential 1-arginine metabolism by two key enzymes: inducible NO synthase (iNOS) and arginase (2, 3). Arginase hydrolyzes 1-arginine to form urea and ornithine, which results in alternative macrophage activation (4). Ornithine is an essential substrate for the synthesis of polyamines, which are essential nutrients for growth and proliferation of cells including *Leishmania* parasites (3, 5–8). Classical macrophage activation occurs when iNOS oxidizes 1-arginine to NO in a two-step process with hydroxy-arginine and citrulline as intermediates (4). NO is a potent cytotoxin involved in clearance or inhibition of a variety of intracellular pathogens including *Leishmania* (3, 5, 7, 8). Because the two enzymes compete for the availability of arginine (4, 9), activation of one results in down-regulation of the other directly or indirectly through their intermediate products. For example, hydroxy-arginine is a powerful arginase inhibitor (3); and treatment of *L. major*-infected mice with its synthetic analog Nω-hydroxy-1-arginine (NOHA), causes a 4-fold reduction in lesion size and as much as 4 million fold reduction in parasite burden (5).

*Leishmania major*-infected mice mimic several forms of the human disease: healing and nonhealing disease occurs depending on the mouse strain. Resistant strains (e.g., C57BL/6J, CBA) produce a Th1 response to infection characterized by high levels of IFN-γ and TNF-α. These cytokines activate macrophages classically to produce NO via iNOS thereby facilitating parasite killing (6, 10). As a result, cutaneous lesions in these strains completely heal and the mice acquire life-long immunity dependent on the presence of persistent parasites. In contrast, susceptible strains (e.g., BALB/c) produce a Th2 response to infection characterized by high levels of IL-4, IL-13, and IL-10 (5, 11–14). The Th2 cytokines increase host cellular arginase activity (3, 5) resulting in alternative macrophage activation (3, 5, 6, 15) and enhanced production of polyamines for parasite growth and proliferation (3, 8).

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It is widely believed that the susceptibility of BALB/c mice to *L. major* is due to the production of high amounts of IL-4 by their CD4+ T cells, which increases macrophage arginase activity. This promotes availability of large amounts of polyamines favoring extensive parasite proliferation leading to uncontrolled pathology (16–20). Notably, *Leishmania* organisms encode an active arginase that may potentially impact on the host immune response and disease pathogenesis (6, 21). *Leishmania* arginase gene (*ARG*), which is ~50% identical with its mammalian homologue (11), is essential for parasite survival in vitro in axenic conditions because the survival and proliferation of arginase deficient parasites is dramatically compromised in the absence of exogenously supplied polyamines (6, 21). Importantly, a previous report using *L. mexicana* arginase null mutant showed that parasite-derived arginase are attenuated in vivo, due in part to increased NO production resulting from presumably increased l-arginine bioavailability for iNOS (22).

Although both Old and New World *Leishmania* cause the same spectrum of disease collectively known as cutaneous Leishmaniasis, their pathobiologies are remarkably different. For instance, the parasitophorous vacuoles of *L. major* are narrow and usually contain one parasite per vacuole whereas those of *L. mexicana* are very spacious and contain many amastigotes (23). Furthermore, whereas *L. major* causes acute and rapidly growing lesions in mice, the lesions of *L. mexicana* take a longer time to develop, are very slow in progression and do not ulcerate and the nature of host immune response is different (24). Importantly, recent reports show that the functional consequences of genetic ablation of key conserved genes such as *LPG2* are different in *L. major* and *L. mexicana* (25).

In the present study, we investigated the role of *L. major*-derived arginase in infectivity, parasite proliferation, and disease pathogenesis in vitro and in vivo. Similar to *L. mexicana arg* (22), we show that deficiency of *L. major*-derived arginase severely impaired their survival and proliferation in macrophages in vitro and in vivo, resulting in delayed disease onset, reduced pathology, and lower parasite burden. In contrast to *L. mexicana arg*, this impairment was not due to overproduction of NO in *L. major arg*-infected cells. Paradoxically, despite the dramatic differences in disease pathology, there was no significant difference in the kinetics, pattern, and quality of cytokine responses induced by WT and *arg*− parasites. Collectively, these results show that the influence of parasite-derived arginase on disease severity is not related to its modulatory effect on the host immune response. They further suggest that contrary to popular belief, the host cellular arginase activity in *L. major*-infected mice may not be regulated by cytokines as previously proposed.

**Materials and Methods**

**Mice**

Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories or Central Animal Care Services, University of Manitoba. All mouse experiments were approved by the University of Manitoba Animal Care Committee in accordance with the regulation of the Canadian Council on Animal Care.

**Parasites and infection**

All parasites were derived from the wild-type (WT) *Leishmania major* line LV39cl5 (RHO/SU/59/P). Homozygous null mutant derivatives lacking the *LPG2* Golgi GDP-mannose transporter (*lpg2−*) were described previously (26). The generation and biochemical characteristics of homozygous null mutants lacking arginase *Δarg*:HYG/Δarg::PAC, referred to as *arg−*, and its complemented line Δarg*:HYG/Δarg::PAC+/pXG-ARG (referred to as *arg−/+ARG*) are described elsewhere (21). Parasites were grown at 25°C in M199 medium (HyClone) supplemented with 10% heat-inactivated FBS, 2 μg/ml biotin, 100 μM penicillin, 100 μg/ml streptomycin, 5 μg/ml hemin, 1 μg/ml biotin, 0.1 mM adenine, 40 mM HEPES (pH 7.4), 20 mM l-glutamate, and 50 mM putrescine (for *arg−* L. major). All medium additives were purchased from Sigma-Aldrich. For infection, 7-day stationary phase promastigotes were washed three times in PBS and 5 million parasites (suspended in 50 μl PBS) were injected into the right hind footpad. After infection, the development and progression of footpad lesion was monitored weekly by measuring the diameter of infected footpad with calipers.

**Estimation of parasites burden**

At various times, mice were sacrificed and parasite burdens in the footpads of infected mice was quantified by limiting dilution analysis as previously described (27, 28).

**In vitro recall response and intracellular cytokine determination**

At sacrifice, single cell suspensions of lymph nodes draining the infection site (dLN) were cultured in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), in 24-well tissue culture plates (Falcon). Cells were stimulated with or without soluble leishmanial Ag (50 μg/ml) for 72 h and the culture supernatants were collected and stored at −20°C until assayed for cytokines by ELISA. Some wells were stimulated with PMA (50 nm), ionomycin (500 ng/ml), and BFA (10 μg/ml) for 4–6 h and used for intracellular cytokine staining (IL-4, IL-10, and IFN-γ) as previously described (29).

**Cytokine ELISAs and measurement of NO**

The levels of IL-4, IL-13, IL-10, and IFN-γ in dLN culture supernatants were determined by sandwich ELISA using Ab pairs from eBiosciences according to the manufacturer’s suggested protocols. In some experiments, IL-10 levels in culture supernatant fluids were measured by cytometric bead array using Cytomix Bead Kit (Benders, Montreal Biotech) according to manufacturer’s suggested protocols. NO levels in culture supernatants of BMDMs were measured at 24, 48, and 72 h post infection by the Griess assay as described (30). In some experiments, NO was also measured in 3-day culture supernatant fluids of lymph node cells from infected mice.

**In vitro infection of bone marrow-derived (BMDM) and peritoneal macrophages**

Bone marrow cells were isolated from the femur and tibia of mice and differentiated into BMDMs as described previously (31). In brief, after depletion of erythrocytes with ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), marrow cells were resuspended in macrophage medium (complete DMEM containing 30% L929 cell supernatant), seeded in Petri dishes at 4 × 10⁶/ml (10 ml/Petri dish) and allowed to differentiate at 37°C in CO₂ incubator. The culture medium was changed at day 3 and adherent macrophages were harvested by gentle scraping on day 7, washed, resuspended in complete medium (10⁶/ml), and used for in vitro experiments.

Peritoneal macrophage cultures were obtained by injecting 10 ml of RPMI 1640 medium (Life Technologies) into the peritoneal cavity of euthanized mice using a 21-gauge needle. The peritoneal cavity was rocked gently several times for ~3 min and the medium was aspirated into a syringe. The cell suspension was washed, counted, and resuspended at 10⁶/ml in complete medium for use in experiments.

For infection, aliquots (500 μl) of BMDMs or peritoneal cells in 5 ml polypropylene tubes were incubated with stationary phase promastigote parasites (day 7) at a ratio of 1:10 (cell: parasite). After 6 h, the free parasites were washed away with fresh medium (spun at 500 rpm for 5 min, 3 x) and infected cells were cultured in complete medium in the presence or absence of IFN-γ (100 U/ml) and LPS (10 μg/ml). At different times after infection, cytoplasm preparations were made, stained with Giemsa and infection was determined by microscopy.

**Arginase activity assay**

Arginase activity in vitro was quantified as previously described (32). One unit of enzyme activity is defined as the amount of enzyme that converts l-arginine to 1 μM urea/min. For in vivo arginase activity assay, infected feet were rinsed in chlorhexiderm and ethanol and homogenized by hand in PBS containing 2% Pen/Strep. The homogenate was first centrifuged at low speed (500 rpm for 5 min) to remove large tissue debris and the supernatant was then centrifuged again at high speed (3000 rpm for 15 min) to pellet the cells and amastigotes. Both pellets were incubated for 30 min in 2 ml lysis buffer (0.1 M Tris-HCl, 300 μM NaCl, 1 μM PMSF,
Similar results were obtained with peritoneal macrophages (C and D) were infected in polypropylene tubes with wild type (WT) or arginase deficient (arg^{-/}) L. major promastigotes (MOI 1:10; macrophage to parasite ration). After 6 h, free parasites were washed away with medium by three cycles of low speed centrifugation (see Materials and Methods) and the cells were resuspended in 500 μl of fresh medium and incubated at 37°C. At various times postinfection, slides were prepared by cytospin and stained with Giemsa. Slides were analyzed under a microscope at ×1000 magnification to estimate infection levels. Each point is the mean ± SE of three slides. Data are presented as mean ± SE and is a representative of three different experiments with similar results. *p < 0.05; **p < 0.01; ***p < 0.001.

Prostaglandin assay

Prostaglandin E_{2} was measured in lymph cell culture supernatant using a competitive ELISA kit from Cayman Chemicals by following the manufacturer’s directions. The sensitivity of the assay is 15 pg/ml.

Statistical analysis

A two-tailed Student’s t test or ANOVA was used to compare means of lesion sizes, parasites burden, and cytokine production from different groups of mice. Correlation statistics were analyzed by Pearson’s correlation at the 95% confidence interval. Significance was considered if p < 0.05.

Results

Lack of parasite-derived arginase compromises L. major proliferation inside macrophages in vitro

Previous studies using the host-specific arginase inhibitor NOHA demonstrated the dependence of L. major on exogenous (host cell-derived) polyamines for intracellular proliferation and growth in vitro (3, 5, 7, 8). Because *Leishmania* arginase is also sensitive to this inhibitor, to establish more directly the role of parasite arginase we studied an arginase *Leishmania major* null mutant (arg^{-/}) created previously by homologous gene replacement (21). We infected BMDM or peritoneal macrophages with WT, arg^{-/} or complemented (arg^{+/} ARG) L. major, and monitored infection progression by microscopy at set times postinfection. Consistently, at 6 h postinfection, the number of parasites per infected cell and the percentage of infected cells were the same regardless of whether the cells were infected with arg^{-/} or WT parasites (Fig. 1, A and B). Similar results were obtained with peritoneal macrophages (Fig. 1, C and D). Therefore, even in the absence of endogenous arginase, L. major parasites retain the ability to invade macrophages. However, beyond the initial cell invasion, subsequent survival and/or proliferation of arg^{-} parasites decreased dramatically in infected cells such that by 72 h postinfection, the percent infectivity and number of arg^{-} parasites per infected cells were reduced by as much as 50 and 60%, respectively compared with WT parasites (Fig. 1, A–D). The failure to survive and/or proliferate in infected macrophages was specific to the arg^{-} mutation, as restoration of arginase expression (arg^{-+}/ARG, “add back” parasites) restored their ability to proliferate in infected cells, to levels similar to WT parasites (Fig. 1, A and B). Taken together, these results indicate that the absence of parasite-derived arginase significantly impaired but did not completely abolish in vitro macrophage invasion by L. major.

arg^{-} L. major-infected BMDM show decreased arginase activity in vitro

It has been previously shown that increasing host cellular arginase levels enhances proliferation of L. major in vitro (3, 5, 7, 8). Because proliferation of arg^{-} L. major was impaired in infected cells, we hypothesized that the expression of parasite-derived arginase may function to increase total intracellular arginase activity leading to increased polyamine availability for the parasites. Therefore, we next quantified the effect of parasite-derived arginase on total cellular arginase activity. Fig. 2A shows the changes in arginase activity in BMDMs 24 and 144 h postinfection with WT and arg^{-} L. major. At 24 h postinfection, there was no significant difference in arginase activity between any of the groups. Differences in arginase activity became apparent from 72 h postinfection, which corresponds to the onset of significant differences in parasite proliferation in vitro (see Fig. 1). By 144 h post infection, arginase activity in WT L. major-infected cells was as much as three to four times higher than in arg^{-} L. major-infected cells, and five times greater than cells alone (Fig. 2A).

Previous reports indicate that host cellular arginase level is regulated by T cell-derived cytokines, particularly IL-4 (10, 12, 13, 17, 33). Consistent with these reports, addition of IL-4 into the cultures increased arginase levels in all treatment groups (by as much as 20–80%, Fig. 2B). However, this IL-4-induced increase in arginase levels was not sufficient to overcome the impaired cellular arginase activity in the absence of parasite-derived arginase,
Decreased arginase activity in arg
/H11002 studies suggest a direct correlation between intracellular parasite with LPS (10
alone or in combination (anti-CD40 plus IFN-
mastigotes) WT, in the right footpad with 2 million (stationary phase pro-
highly attenuated in vivo. BALB/c mice were infected
experiments with similar results. Data are presented as mean ± SE and is a representative of three to four different experiments with similar results. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

such that the total cellular arginase levels in cells infected with arg
L. major plus IL-4 were still significantly (p < 0.001) lower than those infected with WT L. major plus IL-4 (Fig. 2B). These studies suggest a direct correlation between intracellular parasite proliferation and total cellular arginase activities in vitro, which is independent of IL-4 concentration in cultures of infected cells.

Decreased arginase activity in arg
L. major-infected macrophages does not lead to increased NO production

L-arginine is the common substrate for iNOS and arginase and thus these enzymes must compete for it in vivo directly or indirectly (4, 10, 12, 34). Thus, we investigated whether the significantly lower arginase activity in arg
infected cells resulted in significantly enhanced iNOS activity, through increased availability for arginine. Therefore, we measured nitrite production (as a function of iNOS activity) by WT and arg
L. major-infected macrophages at different times after infection and after stimulation with LPS or IFN-γ plus LPS. In the absence of inducers, no nitrite was generated. LPS-induced production of NO by cells infected with WT L. major was slightly but consistently lower than that produced by uninfected cells (Fig. 2C). Surprisingly, despite the observed differences in arginase activity (Fig. 2, A and B), there was no significant difference in nitrite levels between cells infected with WT or arg
L. major at all times tested (Fig. 2C, 24 and 96 h data are shown). Interestingly and consistent with previous reports, stimulation of infected cells with CpG ODN, anti-CD40 or IFN-γ either alone or in combination (anti-CD40 plus IFN-γ) did not lead to any detectable NO production (data not shown). These results indicate that the depressed total arginase activities in cells infected with arg
L. major is not accompanied by increased iNOS activities. They further suggest that parasite-derived arginase is not responsible for the suppression of NO production in L. major-infected cells.

Lack of parasite-derived arginase compromises parasite proliferation in vivo

To discern whether the impaired proliferation of arg
parasites in vitro is representative of what happens in a real in vivo infection; we infected BALB/c mice with WT, arg
and add-back (arg
/ARG) L. major and monitored the onset and progression of lesion development over time. Although the arg
parasites were able to induce some pathology (21), our studies showed that the arg
parasites were highly attenuated in vivo (Fig. 3). Visible lesions took up to 2 wk longer to develop in these mice, and the pathology did not reach the same level of severity as seen in WT or arg
/ARG L. major infection (Fig. 3A). However, because at 7 wk the pathology in mice infected with WT parasites was such that we had to euthanize them, the long-term pathology associated with arg
L. major infection remains unclear. We quantified parasite burden in the infected footpad at different times postinfection to get a better picture of the disease process (Fig. 3B). At 7 days postinfection (before onset of lesion development), there was no significant difference in parasite titer in mice infected with WT, arg
, or arg
/ARG parasites (data not shown). However, by 2, 4, and 7
FIGURE 4. \( \text{arg}^- \) and WT *Leishmania major* induce comparable immune responses in vivo. At various times after infection, mice were sacrificed and draining lymph nodes were removed, made into single cell suspensions and cultured for 3 days in the presence of soluble *Leishmania* Ag (SLA, 50 \( \mu \)g/ml). After 72 h, the culture supernatant fluids were collected and assayed for IFN-\( \gamma \) (A), IL-4 (B), IL-10 (C), and IL-13 (D) by ELISA. Some of the cultures were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h and Brefeldin A (BFA, 10 \( \mu \)g/ml) was added in the last 2 h to enhance intracellular protein accumulation. The cells were surface stained for CD4 and intracellularly for IFN-\( \gamma \) (E), IL-4 (F), and IL-10 (G). The culture supernatant fluids were also assayed for PGE2 by ELISA using a commercially available kit (H). Data are presented as means ± SE of pools of four (A and B), three (E and F), and two (G) independent experiments, or as a representative of three (C) and two (D and H) independent experiments with similar results. *, \( p < 0.05; **, \( p < 0.01 \).

wk post infection (Fig. 3B), parasite burden in WT and \( \text{arg}^- \) ARG *L. major*-infected footpads was 2- to 3-logs higher than in \( \text{arg}^- \) *L. major*-infected mice, indicating that without endogenous arginase *L. major* cannot replicate successfully in vivo. Similar results were observed in C57B6/J mice indicating the result is not mouse strain specific (data not shown). Thus while the \( \text{arg}^- \) parasites were able to survive in mouse infections, they were highly attenuated.

Arginase deficient and WT *L. major* induce similar cytokine responses in vivo

In other knockout constructs of *L. major*, e.g., *lpg2*\(^-\) and *dhfr-ts*\(^-\), attenuated pathology and lower parasite burden are typically associated with low or altered cytokine responses in vivo (35, 36). Therefore, we investigated whether the same was true of the \( \text{arg}^- \) parasites. At different times postinfection, we sacrificed WT and \( \text{arg}^- \) *L. major*-infected mice and determined the production of cytokines (IL-4, IL-10, IL-13, and IFN-\( \gamma \)) by cells from lymph nodes draining the infection site (dLN) by ELISA or flow cytometry. Paradoxically, there was no trend toward a lower immune response by cells from \( \text{arg}^- \) infected mice over the course of infection (Fig. 4, A–D). Our results were also confirmed by intracellular cytokine analyses using flow cytometry where there were no significant differences in percentages of IL-4, IL-10, and IFN-\( \gamma \)-producing CD4\(^+\) T cells (Fig. 4, E–G). Interestingly, pooling of results from four independent experiments revealed that infection with \( \text{arg}^- \) *L. major* induced more IL-4 production than WT parasites at 7 wk postinfection (Fig. 4B). Thus, despite attenuated pathology, \( \text{arg}^- \) *L. major* induced comparable cytokine response to WT parasites.

\( \text{arg}^- \) *L. major* elicits decreased prostaglandin E\(_2\) production in vivo

In addition to cytokines, prostaglandins have also been found to increase arginase activity (16, 10). In terms of lesion pathology following *L. major* infection, the relationship between arginase and prostaglandin, particularly prostaglandin E\(_2\) (PGE\(_2\)), is relevant because arginase plays an important role in wound healing (37). Thus, in the local tissue microenvironment, prostaglandin may increase host arginase activity independently of the adaptive immune response. Therefore, we investigated PGE\(_2\) production in cell culture supernatant from the popliteal lymph nodes draining the infection site. At 3 and 14 days postinfection, there was no significant difference in PGE\(_2\) production, although there was a trend toward higher production by cells from WT *L. major*-infected mice at 14 days postinfection. By 28 days post infection there was a significant difference (\( p < 0.05 \)) in PGE\(_2\) production whereby WT infection resulted in almost 20 times more PGE\(_2\) production than \( \text{arg}^- \) infection (Fig. 4G). Because PGE\(_2\) has been shown to influence inflammatory responses (38, 39), this result is not surprising given the significantly lower inflammation in \( \text{arg}^- \) *L. major*-infected mice.

Parasite number in the infected footpad correlates with arginase activity in vivo

Previous in vivo studies have demonstrated that Th2 cytokines enhance host cellular arginase activities resulting in increased parasite proliferation and disease pathology (5, 7, 8, 10, 15). Given that the IL-4 and IL-10 responses in WT and \( \text{arg}^- \) *L. major*-infected mice were comparable or even higher (at 7 wk) in mice infected with \( \text{arg}^- \) *L. major* (Fig. 4, B and C), we hypothesized that arginase activities at lesion sites would not be different. Therefore, we determined arginase activity in footpad homogenates of infected mice. There were no significant differences in arginase levels in the footpads of mice infected with WT and \( \text{arg}^- \) *L. major* through 2 wk postinfection (Fig. 5A). In contrast, arginase activity was significantly (\( p < 0.001 \)) lower in the \( \text{arg}^- \) than WT-infected mice at 4–7 weeks postinfection, corresponding to the onset of significant differences in cutaneous lesions, and parasite numbers
Our study demonstrates that parasite-derived arginase enhances infectivity in vitro and disease pathogenesis in vivo. In the in vitro infection, parasite proliferation was significantly reduced in the absence of endogenous parasite-derived arginase but the initial ability to invade macrophages remains unaffected. Furthermore, arginase deficient parasites were unable to induce overall increases in macrophage arginase levels as wild-type parasites. Interestingly, despite the marked increase in arginase activity induced by WT L. major infection, there was no significant difference in NO production in the arg−/− infected condition, we assessed dLN cells for arginase activity ex vivo. As shown in Fig. 5E, samples from arg−/− infected mice were not greater than samples from WT infected mice. These results clearly indicate that reduced pathology in arg−/− infected mice (shown in Fig. 3) is not the result of increased parasite clearance due to a competitive advantage to metabolize L-arginine via the inducible NO synthetase pathway.

**Discussion**

Our study demonstrates that parasite-derived arginase enhances infectivity in vitro and disease pathogenesis in vivo. In the in vitro infection, parasite proliferation was significantly reduced in the absence of endogenous parasite-derived arginase but the initial ability to invade macrophages remains unaffected. Furthermore, arginase deficient parasites were unable to induce overall increases in macrophage arginase levels as wild-type parasites. Interestingly, despite the marked increase in arginase activity induced by WT L. major infection, there was no significant difference in NO production suggesting that in infected cells, the additional parasite-derived arginase plays an insignificant role in influencing the competition for L-arginine between arginase and iNOS. Furthermore, parasite number directly or indirectly influences arginase activities, modulating the outcome of L. major infection in mice.

To gain further insight into NO production in the arg−/− infected condition, we assessed dLN cells for arginase activity ex vivo. As shown in Fig. 5D (4 wk post infection), arginase activity in lymph node cells from arg−/− infected mice was significantly lower than WT infected mice. However, when supernatant from these cultures was analyzed for nitrite formation by Griess assay (Fig. 5E), samples from arg−/− infected mice were not greater than samples from WT infected mice. These results clearly indicate that reduced pathology in arg−/− infected mice (shown in Fig. 3) is not the result of increased parasite clearance due to a competitive advantage to metabolize L-arginine via the inducible NO synthetase pathway.

**FIGURE 5.** Tissue arginase levels at infected site correlate with parasite titer in vivo. BALB/c mice were infected with 2 × 10⁶ WT and arg−/− L. major and at various times (3 days and 2, 4, and 7 wk postinfection), mice were sacrificed and the infected footpads were homogenized in buffer (see Materials and Methods) and parasite burden (see Fig. 3B) and the total arginase activity was determined (A). The parasite burden was compared with in vivo arginase activity using Pearson correlation test (B, p = 0.0233; r² = 0.9539). In some experiments, additional groups of mice were infected with WT, arg−/−, and phosphoglycan-deficient (lpg2−/−) L. major and arginase activity in the infected footpad homogenates was determined at 4 wk postinfection (C). Also, culture supernatants of cells from lymph nodes draining the infection site were assessed for arginase (D, wk 4) or nitrite levels (E) as indicated in the Materials and Methods. Data are presented as means ± SE and are representative of three (A and B) and two (C–E) independent experiments with similar results. *p < 0.05; **p < 0.01; ***p < 0.001.

and PGE2 levels (see Figs. 3, A and B, and 4H). Similar results were also obtained from cells of lymph nodes draining the infection site (Fig. 5D). In contrast and consistent with our in vitro data (Fig. 2C), the production of NO in culture supernatant from lymph node cells of arg−/− infected mice was not significantly greater than that of WT infected mice (Fig. 5B). Correlation analysis between parasite titer and arginase activity showed a strong positive correlation between parasite titer and arginase levels in infected mice (r² = 0.954, p = 0.023; Fig. 5B), suggesting that parasite numbers may directly influence host arginase activity. Because arg−/− parasites lack arginase, this increase is likely to be of host origin.

To further investigate the role of parasite numbers in regulating host arginase activities, we compared arginase levels in mice infected with WT and lpg2−/− L. major. lpg2−/− L. major parasites retain arginase, but lack GDP-Golgi mannose transporter required for the synthesis and surface expression of glycoconjugates that are essential for in vitro and in vivo virulence (26, 40). As a result, infection with lpg2−/− L. major does not induce cutaneous lesion formation or any measurable cytokine response even though low levels of parasite persist at infected sites for years (27, 36). As shown in Fig. 5C and similar to arg−/− L. major, lpg2−/− parasites induced significantly (p < 0.01) less arginase levels at the site of infection than WT L. major (70% less than WT parasites). Importantly and consistent with previous reports, parasite burden was low and the cytokine response (particularly IL-4, IL-10, and IFN-γ) was very low or undetectable in lpg2−/− infected mice (data not shown). Together with our in vitro results, these results suggest that parasite number directly or indirectly influences arginase activities, modulating the outcome of L. major infection in mice.
the addition of exogenous IL-4 was not sufficient to change the trend of arg-parasites inducing significantly less arginase activity than WT parasites, a first indication that some other factor(s) (distinct from cytokines) may be more important in regulating host cellular arginase activity. Importantly, and consistent with the in vitro results, in vivo pathology and parasite burden were significantly reduced in L. major arg-infected mice. Paradoxically, despite this reduction in pathology and parasite burden, cytokine responses were not significantly different between arg and WT L. major-infected mice. Furthermore, despite similar cytokine profiles, the arg L. major-infected mice had significantly lower arginase activities than WT L. major-infected mice, strongly suggesting that cytokine profiles and arginase activity in mice infected with L. major are unrelated.

Because of their intracellular lifestyle, the nature of macrophage activation significantly impacts on the outcome of L. major infection (41). A key factor that regulates the outcome of macrophage activation is the product of L-arginine metabolism, a process that is regulated by the expression of two key enzymes: iNOS and arginase. iNOS is up-regulated by Th1 cytokines, notably IFN-γ and TNF-α, while arginase is induced by Th2 cytokines, including IL-4, IL-10, and IL-13. Previous studies suggest that in BALB/c mice infected with L. major, the overproduction of IL-4, IL-10, and IL-13 leads to enhanced arginase activities, production of polyamines, excessive parasite proliferation and uncontrolled disease pathology (3, 7, 8, 13, 16). This increased arginase activity in macrophages is believed to decrease L-arginine availability and competitively inhibit iNOS activity and NO production. In the present study, we found no relationship between arginase levels at the site of infection and the host cytokine response in the draining lymph nodes in mice infected with WT and arg-parasites. Furthermore, despite significant differences in the total arginase levels, infection with arg-parasites did not result in higher NO levels than WT infection (Fig. 5E). Thus, we speculate that impaired parasite growth in cells infected with arg L. major may be directly related to decreased polyamine availability (due to decreased arginase activity) and is unrelated to compensatory iNOS overactivity and NO production. Similar effects have been observed in mice treated with nor-NOHA, a synthetic analog of arg-OH, which blocks arginase activity in vitro and in vivo (5).

Although we found that addition of IL-4 significantly enhanced the proliferation of arg-parasites in infected macrophages, this was not enough to rescue them to the level seen in cultures containing WT parasites and rIL-4 (Fig. 2). Furthermore, despite the dramatically impaired arginase activities in vivo, the production of both Th1 and Th2 cytokines by cells from the draining lymph nodes of mice infected with arg L. major was not lower than their WT-infected counterparts (Fig. 4). Thus, the inability of arg L. major to proliferate in infected mice could not be accounted for by altered cytokine responses. Instead, we found that parasite burden (and disease pathology) directly correlated with arginase levels in vitro and in vivo. PGE2 production also follows the same trend. It is therefore tempting to hypothesize that parasite-derived arginase may function to enhance the initial survival and proliferation of L. major inside macrophages by increasing local polyamine concentration around the parasitophorous vacuoles, thereby enhancing its availability to the parasites. A testable prediction from this hypothesis is that increasing polyamine concentration in macrophage cultures will not be effective in rescuing the growth defects of arg L. major in vitro. Indeed, Gaur et al. (22) found that although additional putrescine and ornithine can increase intracellular parasite proliferation, they were unable to rescue the defect of L. mexicana arg infection. Thus, it appears that there is distinct compartmentalization of responses between what influences the cell as a whole and what influences the intracellular amastigote microenvironment.

Prostaglandin E2 (PGE2) is an important mediator of inflammation produced by a variety of immune and nonimmune cells during tissue damage (38, 39). One of the products of arginase activity is proline, which is essential for collagen deposition in wound healing. In the local tissue microenvironment, prostaglandin induction related to lesion development may increase host arginase activity independently of the adaptive immune response but directly related to parasite proliferation. In our study, we found that prostaglandin levels increased substantially in WT infection but remained relatively unchanged in arg infection (Fig. 4H). We hypothesize that the pathology associated with increasing parasite proliferation induces PGE2 in lesions and that this local PGE2 increases host arginase activity. In the arg infection, parasite burden and lesion size remain significantly lower than in WT infection over the course of the study. We believe that this lower PGE2 is insufficient to induce host arginase levels similar to those observed in WT infections. These hypotheses can be tested using the PGE2 inhibitor indomethacin with which we would expect to see a decrease in total arginase levels in WT infection. It is conceivable that additional PGE2 may not rescue the proliferation defect of arg infection just as additional polyamines did not rescue parasite proliferation in vitro.

As previously mentioned, Leishmania parasites also encode their own arginase (6). This arginase bears resemblance to both isoforms found in humans (11) but is distinct from human forms, as evidenced by an inability to detect it in western blots using a human arginase probe (5). In the absence of an exogenous source of polyamines, i.e., growth in cell-free medium, Leishmania promastigotes are completely dependent on their own arginase for proliferation. Thus, arginase knockout L. major parasites are auxotrophic for polyamines and their precursors (specifically putrescine, ornithine, or spermidine) (6). These mutant parasites can proliferate successfully if the growth medium is supplemented with polyamines, highlighting the ability of L. major organisms to scavenge polyamines. Importantly, amastigotes of L. amazonensis have also been shown to express parasite-derived arginase at the same levels as promastigotes of this strain (11), suggesting that enzyme levels and activity are not stage specific and that gene expression is maintained when inside the host cell. As with L. amazonensis, we have detected arginase activity in parasites recovered from infected footpads confirming that the gene is expressed by the amastigotes in vivo (data not shown).

The inability of arg L. mexicana to proliferate in macrophages has been attributed to overall lower arginase activity in host cells, which is in part contributed by the absence of parasite derived arginase resulting in more substrate availability for NO production by the enzyme iNOS (22). This study reported increased NO production in arg-infected macrophages over those produced by WT-infected cells at 48 h postinfection. Because this study also found no differences in total arginase levels in WT and arg-infected cells at 48 h postinfection, it is unlikely that the increased NO production observed at this time in arg-infected cells was due to enhanced iNOS activity resulting from decreased arginase activity (22). Furthermore, we did not observe any increase in NO production (over that induced by WT parasites) in the absence of parasite-derived arginase at any time during in vitro and in vivo infections (Figs. 2C and 5E). Thus, our data are consistent with our conclusion that arginase activity in infected cells or tissues does not influence iNOS activity and the impaired proliferation of arg L. major is unrelated to increased NO production. Gaur et al. (22) also found that arg L. mexicana infection induces a type one immune response based on their finding of higher IFN-γ levels and
lower IL-4 levels in culture supernatant of cells from arg⁻ infected mice at 4 wk postinfection compared with WT infection (22). In contrast, we show that the pattern, quality and quantity of cytokine response in WT and arg⁻ L. major-infected mice are similar (and showed lack of predictable dominance of type one over type two immune response or vice versa) over a period of 7 wk postinfection (Fig. 4, A–D). The L. mexicana arg⁻ study investigated the adaptive immune response only at one time point (at 4 wk) postinfection providing only a snapshot of the response whereas we undertook a systematic kinetic examination of immune response over 7 wk. In addition to differences in experimental design, it is possible that differences in the biology of New and Old world parasites may also be important. For instance, there are marked differences in the nature of interaction of L. major and L. mexicana with macrophages, particularly in the formation of parasitophorous vacuoles (23). Indeed, differences in virulence following deletion of the glycoprophosphate conjugates in Leishmania major and Leishmania mexicana have been reported (26, 42).

The metabolism of polyamines (putrescine, spermine, and spermidine) is an integral part of cellular function in all living cells (12). In mammals, the cellular polyamine content correlates with the rate of cell division, and polyamine levels are increased in cancer cells compared with normal cells (12, 15). As in mammals, polyamines are essential for the growth of all Trypanosomatidae. African trypanosomes (including T. brucei and T. congolense) encode most of the mammalian enzymes or their homologues necessary for the biosynthesis of polyamines (12). In contrast, Trypanosoma cruzi, which is closely related to Leishmania, encodes an enzyme, cruzipain, which increases host arginase levels in a cytokine-like manner aiding the uptake and interconversion of putrescine and spermidine from their environment (12, 43, 44). In addition, parasites from other families have been found to possess such arginase effectors; such as the antioxidant thioredoxin peroxidase produced by Fasciola hepatica that is shown to induce alternative activation and arginase up-regulation in peritoneal macrophages (45). The possession of a molecule capable of increasing host arginase activity is advantageous for the parasites as it provides a larger polyamine pool for optimal parasite proliferation. Our observation of a positive correlation between parasite titer and arginase activity (Fig. 5) suggest that Leishmania major may also produce such a molecule which, combined with parasite-derived arginase, ensures optimal availability of polyamines thereby facilitating efficient intracellular survival.

With respect to the exact role of L. major-derived arginase in de novo polyamine synthesis, we suggest a “take ratios for the invasion” hypothesis. The intracellular environment of macrophages is extremely hostile. During the early phase of infection, the newly transformed amastigotes require survival factors to proliferate successfully inside the cell. In the absence of parasite-derived arginase, early proliferation and establishment of L. major parasites may be impaired to such an extent that the addition of exogenous polyamines or cytokines cannot rescue their proliferation to WT levels. Thus, the ability to synthesize polyamines de novo gives L. major a survival advantage during this difficult time. Once a significant number of amastigotes are present in the host, parasite-derived products or exoantigens would be produced in sufficient quantity to sustain the infection. We hypothesize that one or more of these products is a molecule for up-regulating host arginase.

In conclusion, we have clearly shown that L. major-derived arginase plays an important role in disease pathogenesis by augmenting host arginase activities leading to efficient parasite proliferation. This effect is unrelated to enhanced iNOS activity and/or NO production. In our kinetic study, we show that adaptive immune response does not predict in vivo arginase activity or vice versa. Thus, we conclude that in mice infected with L. major, the in vivo cytokine responses may be of lesser importance in modulating host cellular arginase activity than parasite numbers. The results further suggest that parasite-derived product(s) or host inflammatory mediators like PGE₂ may play important role in modulating host arginase activities in vivo that result in effective intracellular parasite proliferation.

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Disclosures
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References
9. Goerd, S., O. Politz, K. Schledzewski, R. Birk, A. Gratchev, P. Guillot, N. Hakky, C. D. Klemke, E. Dippel, V. Kodelja, and C. E. Orfano. 1999. Alkaline arginase: a survival advantage during this difficult time. Once a significant number of amastigotes are present in the host, parasite-derived products or exoantigens would be produced in sufficient quantity to sustain the infection. We hypothesize that one or more of these products is a molecule for up-regulating host arginase.

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PARASITE NUMBERS REGULATE HOST CELLULAR ARGINASE LEVELS


