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*J Immunol* 2007; 179:8446-8453; doi: 10.4049/jimmunol.179.12.8446

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An Effect of Parasite-Encoded Arginase on the Outcome of Murine Cutaneous Leishmaniasis

Upasna Gaur,*† Sigrid C. Roberts,2‖ Rahul P. Dalvi,* Inés Corraliza, Buddy Ullman,‖ and Mary E. Wilson3§

Classical activation of macrophages infected with *Leishmania* species results in expression and activation of inducible NO synthase (iNOS) leading to intracellular parasite killing. Macrophages can contrastingly undergo alternative activation with increased arginase activity, metabolism of arginine along the polyamine pathway, and consequent parasite survival. An active role for parasite-encoded arginase in host microbialic responses has not previously been documented. To test the hypothesis that parasite-encoded arginase can influence macrophage responses to intracellular *Leishmania*, a comparative genetic approach featuring arginase-deficient mutants of *L. mexicana* lacking both alleles of the gene encoding arginase (*Δarg*), as well as wild-type and complemented *Δarg* controls (*Δarg[pArg]*) was implemented. The studies showed: 1) the absence of parasite arginase resulted in a significantly attenuated infection of mice (*p* < 0.05); 2) poorer survival of *Δarg* in mouse macrophages than controls correlated with greater NO generation; 3) the difference between *Δarg* or control intracellular survival was abrogated in iNOS-deficient macrophages, suggesting iNOS activity was responsible for increased *Δarg* killing; 4) consistently, immunohistochemistry showed enhanced nitrotyrosine modifications in tissues of mice infected with *Δarg* compared with control parasites. Furthermore, 5) in the face of decreased parasite survival, lymph node cells draining cutaneous lesions of *Δarg* parasites produced more IFN-γ and less IL-4 and IL-10 than controls. These data intimate that parasite-encoded arginase of *Leishmania mexicana* subverts macrophage microbicidal activity by diverting arginine away from iNOS. *The Journal of Immunology*, 2007, 179: 8446–8453.

*Leishmania* spp. are dimorphic parasitic protozoa that cause an array of disfiguring or fatal human diseases. The obligate intracellular amastigote stage of the parasite resides within the phagolysosome of mammalian mononuclear phagocytes including macrophages. Genetic susceptibility or resistance to *Leishmania* infection varies among different strains of mice. Resistance is almost uniformly accompanied by expansion of type 1 CD4+ (Th1) cells that produce the cytokine IFN-γ in response to parasite Ag (1–3). In contrast, the immunological response of mice susceptible to infection by different species of *Leishmania* is highly variable. Susceptibility to *Leishmania major* is mediated through expansion of a strong type 2 immune response against a predominant Ag (*Leishmania* homologue of receptors for activated C-kinase) leading to IL-4 and IL-13 expression, whereas suppression of a type 1 response without Th2 expansion is characteristic of other species (3, 4). TGF-β is a major factor suppressing the type 1 immune response during infection with *Leishmania chagasi* (5).

A remarkable feature of *Leishmania* is its ability to survive and replicate in the phagolysosome of infected mammalian macrophages, a hostile environment that is lethal to many microbes (6). Interestingly, the amino acid arginine appears to play a key role in the mechanism by which *Leishmania* survive intracellularly in the mammalian host (7). Macrophages harbor two competing pathways for arginine metabolism initiated by the enzymes inducible NO synthase (iNOS) and arginase, respectively (8, 9). The first pathway involves IFN-γ activation of NOS2 and produces its protein product iNOS. iNOS catalyzes the two-step NADPH-dependent conversion of arginine through NOHA to citrulline and NO2, the latter a potent inorganic microbicidal molecule. This INOS pathway is used by classically activated murine macrophages to kill intracellular *Leishmania* (10–12). In contrast, macrophages can be alternatively activated by other stimuli including IL-4 or IL-13 and express an arginase I activity that hydrolyzes arginine to urea and ornithine (8). Ornithine is a key intermediate in the synthesis of glutamine, proline, and polyamines in mammalian cells (13). However, in *Leishmania* promastigotes the sole role of ornithine is the production of polyamines, which are ubiquitous essential cations that play critical roles in a variety of cellular processes needed by proliferating cells (14, 15).

Previous studies have demonstrated that the balance between iNOS and arginase activities is competitively regulated by the type 1 and type 2 cytokines (16). Specifically, IFN-γ enhances expression of iNOS, whereas IL-4 induces both increased expression of

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*Abbreviations used in this paper: iNOS, inducible NO synthase; BMM, bone marrow macrophage; MOL, multiplicity of infection; MuLu, mink lung fibroblast.*
the arginase protein and increased arginase activity (17). Furthermore, just as the type 1 and type 2 cytokines are mutually inhibitory, the induction of iNOS or arginase is also regulated in a reciprocal fashion (13). iNOS is regulated both at the level of activity and gene expression as well as by substrate (arginine) availability (17). It has been demonstrated that the local availability of arginine is an important determinant of NO-mediated killing of Trypanosoma brucei (18) and that host cell NO production is regulated by the scavenging of arginine by Helicobacter pylori arginase (19).

Using arginase inhibitors, Lienstra et al. (7, 16) showed that arginase activity is necessary for the survival and growth of both L. major and Leishmania infantum in murine macrophages. However, because the host macrophage and the parasite cells each express functional arginase enzymes, it is not clear whether one or both arginases are needed for parasite survival. The generation of Leishmania mexicana gene deletion mutants deficient in parasite arginase activity enables a dissection of the relative contributions of parasite and host cell arginases to parasite survival both in vitro in the host macrophage and in vivo within the host (14). The results suggest that parasite-derived arginase presents a novel defense mechanism that enhances parasite survival through local depletion of the iNOS substrate arginine.

Materials and Methods

Mice

Female BALB/c mice (4–6 wk old) were purchased from Harlan Breeders; iNOS knockout mice in a C57BL/6 background and wild-type C57BL/6 mice were purchased from The Jackson Laboratory. Studies using mice were approved by the Animal Care and Use Committees of the University of Iowa and the Iowa City Veterans Affairs Medical Center.

Parasite culture

All genetically modified lines were derived from wild-type MNYC/BZ/62/M379 L. mexicana. The creation and characterization of the Δarg knockout and the Δarg[pArg] cell lines has been reported (14). The Δarg strain was isolated after two rounds of targeted gene replacement, whereas the Δarg[pArg] parasites possess a Δarg chromosomal background and harbor a multicopy episomal plasmid encompassing the arginase gene (14). All L. mexicana lines were cultivated in DME-L, a completely defined DMEM (14). The growth medium for the genetically manipulated L. mexicana contained the following additions: Δarg knockout parasites were supplemented with 50 μg/ml phleomycin, 50 μg/ml hygromycin, and 100 μM putrescine, and episomally complemented Δarg[pArg] parasites were supplemented with 50 μg/ml phleomycin, 50 μg/ml hygromycin, and 20 μg/ml G418. Some parasite cultures were tested for growth in the presence of no supplements, 200 μM putrescine, or 200 μM ornithine.

For limiting dilution assays to quantify parasite infections in mice, serial 4-fold dilutions of footpad lysates were cultured in 96-well plates in Schneider’s medium to which 20% FCS and 20 μg/ml gentamicin, and 20 μg/ml hygromycin, and 20 μg/ml putrescine, and episomally complemented Δarg[pArg] parasites were supplemented with 50 μg/ml phleomycin, 50 μg/ml hygromycin, and 20 μg/ml G418. Some parasite cultures were tested for growth in the presence of no supplements, 200 μM putrescine, or 200 μM ornithine.

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Cellular arginase assays

Parasite arginase activity was assayed by measuring the conversion of L-[14C(U)]arginine to [14C]urea and [14C]ornithine over 48 h (20–22). Briefly, [14C]arginine (NEN-specific activity 360 mCi/mM) at 0.25 μM was added to 96-well plates each containing 105 parasites in 200 μl of growth medium. After 48 h at 26°C, the assay was terminated by the addition of 150 μl of cell culture supernatant to 0.8 ml of a solution of 250 mM acetic acid, 100 mM urea, 10 mM arginine (pH 4.5). Remaining [14C]arginine and [14C]ornithine in supernatants was removed by binding to Dowex HCR-W2 cation-exchange resin (Sigma-Aldrich) and, after centrifugation, the [14C]urea reaction product in 0.5 ml was counted by liquid scintillation. The limits of assay detection were 0.005–2.5 nM [14C]arginine conversion to urea.

Bone marrow macrophages (BMMs)

Bone marrow cells (BMMs) obtained from BALB/c mouse femurs were cultured at 37°C, 5% CO2 in RPMI-1640; Invitrogen Life Technologies containing 20% cell culture supernatant from L929 cells (American Type Culture Collection) as a source of M-CSF. After 7–9 days, differentiatied adherent macrophages were detached from the petri dish with 2.5 mg/ml trypsin containing 1 mM EDTA (Invitrogen Life Technologies) (23). A total of 5 × 104 macrophages were then allowed to adhere to coverslips in 24-well plates and infected with promastigotes at a multiplicity of infection (MOI) of 5:1. The infection was synchronized by centrifugation (3 min, 330 × g, 4°C), and infected macrophages were incubated in 5% CO2 at 37°C. Extracellular parasites were removed by rinsing macrophages 30 min postinfection.

In another experiment, the macrophage growth medium was supplemented with increasing amounts of ornithine (A) or putrescine (B). After 48 h, triplicate coverslips were fixed, Wright-Giemsa stained, and macrophages and amastigotes were enumerated. Parasite intracellular growth was compared with that of wild-type parasites, and p values were calculated by the Student t test (paired test).

Nitrile measurements

To measure NO* generated by cellular iNOS, nitrile, which forms readily from NO* in the presence of oxygen (24), was measured using the colorimetric Griess assay (25). Briefly, 50 μl of the culture supernatants were added to 96-well plates containing 100 μl of freshly prepared Griess reagent (0.1% N-1-naphthylenediamine-HCl in water and 1% sulfanilamide in 2.5% H3PO4). After a 15-min incubation at room temperature, absorbance at 550 nm was determined on an ELISA plate reader.

Footpad model of L. mexicana infection

BALB/c mice were infected in the right hind footpad with a single injection of 1 × 107 stationary phase wild-type, Δarg, or Δarg[pArg] L. mexicana promastigotes. Footpad thickness was evaluated with a Mitutoyo digital caliper. At the termination of the experiment the foot pads were excised, and parasite loads were quantified by limiting dilution as described (26).

Lymph node cell culture and cytokine production

The lymph node draining cutaneous lesions were removed 4 wk after infection. Pooled cells from five mice in each group were seeded at 0.2 × 106 cells/well and were restimulated with 1 × 106 stationary phase wild-type, Δarg, or Δarg[pArg] L. mexicana promastigotes. After 48 h, culture supernatants were harvested, and cytokines were quantified on a Bioplex system (Bio-Rad) with a LINCOplex Mouse Cytokine Panel kit (Linco Research). In each experiment, a standard curve was run in parallel. The detection limits of the assay ranged from 0.3 to 20 pg/ml.

TGF-β biosassay

Mink lung fibroblasts (MvLu) stably expressing a luciferase construct under control of the TGF-β-responsive promoter for the plasminogen activator inhibitor were provided by D. Rifkin (New York University, New York, NY). These were used to assess bioactive and total TGF-β in culture supernatants as described (27, 28). Cells from lymph nodes of mice infected with wild-type, Δarg, or Δarg[pArg] parasites were cultivated in serum-free medium for 48 h. MvLu cells were incubated in either regular growth medium in the absence or presence of various concentrations of TGF-β for use as a standard curve (1–3000 pg/ml) or with culture supernatants. Luciferase activity was assayed with a luciferase kit from Promega. Control wells contained medium. All conditions were tested in triplicate. The detection limits of the assay were 30–3000 pg/ml.

Histopathology and immunohistochemistry

NO* can initiate covalent modification of proteins particularly on tyrosine residues. Thus, the presence of nitrosylated tyrosine is a stable marker for NO*-mediated cellular damage. Footpads from 4-wk-infected BALB/c mice were incubated overnight in 30% sucrose-PBS as a cryoprotectant, freeze dried, and embedded in Tissue-Tek OCT Compound (Sakura Finetek) in liquid nitrogen. Five- or 10-μm sections were sliced from frozen tissues, fixed in paraformaldehyde, blocked in a PBS solution containing 5% milk, and incubated with a 1/100 rabbit polyclonal anti-nitrotyrosine Ab (Upstate Biotechnology) in PBS-0.3% BSA overnight at 4°C. Sections were then incubated for 1 h in 1/200 Alexa Fluor 546-labeled goat anti-rabbit Ig followed by 5 min in TOPRO-3 nuclear stain diluted 1/3000. After rinsing and mounting with Vectashield H-1000 (Vector Laboratories), slides were examined on a Zeiss 510 laser confocal microscope (www.zeiss.com/micro) and images were captured on a laser scanning microscope (LSM) 510 version 3.2 software. Confocal optical sections were further analyzed using the LSM 5 image browser. Slides were stained with H&E.
after alcohol dehydration and a distilled water rinse and then mounted in xylene-based mounting medium.

Statistical analysis

Statistical analyses was performed using either one-way ANOVA, Kruskal-Wallis one-way ANOVA on ranks, or paired t test algorithms using Sigma Stat software (SPSS).

Results

Arginase-deficient and -complemented parasites

To verify the functional consequences of the gene replacements in Δarg parasites, arginase activity was measured in wild-type, Δarg, and Δarg[pArg] parasites. Whereas significant arginase activity was detected in both wild-type and Δarg[pArg] L. mexicana, arginase activity in Δarg parasites was essentially undetectable.

Although knockout organisms were unable to proliferate in unsupplemented growth medium, the growth of Δarg L. mexicana promastigotes (14) or amastigotes (data not shown) in axenic culture was restored by the addition of either 200 μM putrescine or 200 μM ornithine.

Decreased survival of Δarg L. mexicana in macrophages

To investigate the role of parasite-encoded arginase on tissue amastigote proliferation, the abilities of wild-type, Δarg, or Δarg[pArg] parasites to survive and maintain an infection in BMMs was analyzed. Initial parasite entry into macrophages, quantified 2 h postinfection, did not differ significantly among the wild-type, Δarg, and Δarg[pArg] strains (70.3 ± 17.6, 71.5 ± 15.8, 74.8 ± 12.8 parasites/100 BMMs, respectively). In contrast, the number of Δarg parasites that survived in BMM cultures 24 or 48 h after infection was significantly lower than those obtained for either wild-type or Δarg[pArg] parasites (Fig. 1). This was not due to significant difference in the enhanced total macrophage arginase activity in macrophages infected with mutant compared with wild-type parasites (Table I). Arginase activity due to the parasite itself was only a fraction of that observed in the total macrophage (see Table I). However, the local arginase activity influencing arginine availability in the parasitophorous vacuole surrounding the intracellular parasite could be significantly influenced by parasite enzyme activity at a level not detectable in an assay of total macrophage arginase.

To evaluate whether the decreased intracellular growth of the Δarg parasites in macrophages could be ascribed to a lack of salvageable polyamines, the growth medium of infected bone marrow-derived macrophages was supplemented with ornithine and putrescine after removal of extracellular parasites. The addition of either ornithine or putrescine increased the number of amastigotes of both wild-type and Δarg cells (Fig. 1, C and D), implying that intracellular L. mexicana amastigotes are capable of salvaging both ornithine and putrescine from the macrophage and extracellular medium. These findings confirm previous observations that the availability of increased amounts

| Table I. Arginase activity in infected macrophages, \(^a\) uninfected macrophages, \(^b\) or parasites alone\(^b\) |
|-------------------------------------------------|---------|---------|---------|---------|---------|
| Pathogen | No Infection | IL-4 | Wild Type | Δarg | Δarg[pArg] |
| Macrophages\(^a\) | 0.038 ± 0.019 | 0.905 ± 0.461 | 0.418 ± 0.248\(^b\) | 0.508 ± 0.321\(^b\) | 0.473 ± 0.096\(^b\) |
| 10\(^6\) Parasites\(^a\) | 0.0235 ± 0.0037 | 0.0002 ± 0.0001 | 0.395 ± 0.0134 |

\(^a\) Arginase activity; [\(^14\)C]arginine conversion to urea (mean ± SE nanomoles/2 × 10\(^6\) macrophages).

\(^b\) Differences are not statistically significant.

\(^c\) Arginase activity was measured in macrophages stimulated with IL-4 (20 ng/ml) and infected with no parasites or with wild-type, arginase knockout (Δarg), and complemented (Δarg[pArg]) L. mexicana promastigotes. Macrophages were infected with 10\(^6\) stationary phase promastigotes of each line. Arginase activity was measured in extracts of 2 × 10\(^5\) macrophages in medium containing [\(^14\)C]arginine as described in the text.

\(^d\) Arginase activity was measured in wild-type, arginase knockout (Δarg), and complemented (Δarg[pArg]) L. mexicana promastigotes. Data are the means of three replicate experiments, each conducted with triplicate conditions.
IFN-γ differences were observed among the three strains in the absence of exogenous addition of either putrescine or ornithine to infected parasites. Macrophages were preincubated for 24 h with 100 U recombinant murine IFN-γ/H9004 either wild-type or arg knockout, which iNOS activity can be regulated, even in the presence of the type 1 cytokine IFN-γ.

Enhanced NO production by BMMs infected with Δarg parasites

It has been reported that the intracellular concentration of arginine is rate-limiting for iNOS activity in vivo (19). We hypothesized that parasite arginase could deplete host arginine supplies that might otherwise be available to iNOS as a substrate. If this is the case, then eliminating parasite arginase should enhance the metabolic flux through iNOS in the infected host cell macrophage. To test this conjecture, the amount of cellular NO released into culture supernatants of BMMs infected with wild-type, Δarg, or Δarg[pArg] L. mexicana was assessed by measuring the amount of nitrite ion produced. Because IFN-γ is necessary for leishmanial cidal activity and transcription of iNOS (29, 30), some of the cells were preincubated for 24 h with 100 U recombinant murine IFN-γ/ml. Forty-eight hours after infection was initiated, nitrite was significantly increased in IFN-γ-primed BMMs infected with the Δarg knockout compared with IFN-γ-primed BMMs infected with either wild-type or Δarg[pArg] parasites (Fig. 2). No significant differences were observed among the three strains in the absence of IFN-γ priming. These findings support the hypothesis that parasite arginase may reduce host cell arginine pools as a mechanism by which iNOS activity can be regulated, even in the presence of the type 1 cytokine IFN-γ.

Intracellular proliferation of wild-type and Δarg parasites in the absence of host macrophage iNOS

Mice lacking both alleles of the Nos2 gene encoding iNOS are significantly more vulnerable to *Leishmania* spp. infection than wild-type mice (31). Macrophages from Nos2 knockout mice on a C57BL/6 background were exploited to further explore the hypothesis that enhanced killing of Δarg parasites by macrophages is due to increased availability of arginine and consequent increased iNOS activity. If the hypothesis is correct, the enhanced killing of Δarg parasites should be ameliorated in the absence of host cell iNOS. The intracellular survival of wild-type, Δarg, and Δarg[pArg] parasites in BMMs from iNOS-deficient mice was compared with that in BMMs from wild-type C57BL/6 mice (Fig. 3). No significant differences among entry rates of any of the parasite strains were observed between macrophages of wild-type C57BL/6 or iNOS knockout mice (data not shown; paired *t* test). Similar to data obtained with BALB/c BMMs shown in Fig. 1, the Δarg parasites did not survive as well as wild-type or Δarg[pArg] parasites in C57BL/6 macrophages. However, the absence of host cell iNOS in BMMs from Nos2 gene-deficient mice resulted in a significantly enhanced survival of Δarg parasites. Differences among the intracellular survival of wild-type, Δarg, or Δarg[pArg] parasites in iNOS knockout BMMs were statistically insignificant. These data suggest that the increased sensitivity of Δarg parasites to macrophage killing is dependent on the presence of host iNOS.

In vivo infection with control vs Δarg L. mexicana

To assess the effects of a genetic lack of parasite arginase on virulence in an animal model, BALB/c mice were infected in one hind footpad with wild-type, Δarg, or Δarg[pArg] stationary phase promastigotes (Fig. 4). Although footpad swelling was similar among the three parasite strains during the first 4 wk of infection, the lesion size in mice infected with wild-type *L. mexicana* increased more rapidly than Δarg- or Δarg[pArg]-induced lesions in the ensuing weeks (Fig. 4, left panel). Lesions from Δarg[pArg] parasites were initially smaller than those caused by wild-type parasites, but they eventually developed to the same size as the wild-type controls. In contrast, mice infected with Δarg parasites developed lesions more slowly than mice infected with the other strains. Limiting dilution assay of parasites recovered from each footpad at 4 and 16 wk postinfection suggested the smaller size lesions in mice infected with Δarg parasites could be ascribed to...
lower parasite numbers in these lesions (Fig. 4, right panel). Statistically significant differences in lesion size between footpads infected with wild-type vs Δarg parasites occurred at all time points between 8 and 17 wk postinfection with the exception of week 13 (p < 0.05).

**Immune responses to infection with wild-type, Δarg, or Δarg[pArg] parasites**

The changes in NO levels elicited by the three parasite strains reflect shifts in the microbicidal activity of the macrophage. To determine whether there was an associated adjustment in the adaptive immune response, we examined the variations in type 1, 2, and 3 cytokine levels produced by draining lymph node cells of infected animals after restimulation in vitro with live promastigote Ag (Fig. 5). The decreased lesion sizes of mice infected with Δarg organisms was correlated with significantly increased IFN-γ and decreased IL-4 levels compared with lymph node cells derived from either wild-type or Δarg[pArg] parasites. The amount of IL-10 in lymph node cells from mice infected with the null mutant was also diminished compared with the wild-type and Δarg[pArg] controls, although the differences were within the statistical margins of error.

**Lack of a detectable disease-exacerbating role for TGF-β**

Susceptibility of BALB/c mice to L. chagasi correlates with increased local production of TGF-β (28), a macrophage-deactivating cytokine that is known to induce arginase expression in mammalian macrophages (32). Due to the inverse relationship between TGF-β and IFN-γ, we investigated whether TGF-β activity might be lower in mice infected with Δarg than with wild-type or Δarg[pArg] parasites, correlating with their decreased intracellular survival. Because ELISAs detect both inactive and active TGF-β whereas bioassays are more sensitive indicators of TGF-β activity, an assay using a transfection cell line expressing luciferase under control of the TGF-β-responsive plasminogen activator inhibitor promoter was used (27).

Infection with wild-type L. mexicana did not lead to detectable bioactive TGF-β activity, although inactive TGF-β was present. Surprisingly, mice infected with Δarg L. mexicana showed significant increases in both bioactive and total TGF-β levels compared with mice infected with wild-type or add-back parasites (Fig. 6). Thus, the lower level of infection with Δarg L. mexicana cannot be ascribed to a decrease in the suppressor cytokine TGF-β. The mechanism leading to increased TGF-β during Δarg infection and whether this is a compensatory response to other changes in the local tissue cannot be discerned from these data.

Other suppressive mechanisms might be active as well within tissue. IL-10 and IL-4 were not found to increase but rather to diminish in cultured cells from mice infected with Δarg parasites (Fig. 5). Other factors that could influence the response were not measured, such as PGs. TGF-β is only one of several immunosuppressive molecules that would be expected to influence the course of disease.
**FIGURE 7.** Immunohistochemical detection of nitrosylated tyrosine in infected footpads. Enhanced nitrotyrosine modifications were detected in footpad sections of mice infected with Δarg parasites. A–C, Sections from infected animals were probed with TOPRO-3 nuclear stain and with rabbit polyclonal anti-nitrotyrosine (followed by Alexa Fluor 546-labeled goat anti-rabbit Ig) and mounted with Vectashield. The slides were examined on a Zeiss confocal. Micrographs are representative of three repeats with similar findings. D–F, H&E staining of similar section of OCT embedded footpad. Due to the small size of amastigotes, a higher magnification is shown in B compared with A. Magnified regions in H&E stains correspond to the base of the epidermis. Scale bar, 10 μm.

### Histopathology in infected animals

NO\(^{2}\) generation can lead to nitration of tyrosyl residues in proteins. These nitrotyrosines can be used as a marker of the local toxic effects of NO (33). To determine whether there is increased NO\(^{2}\) generation in vivo during infection with Δarg parasites, frozen sections were prepared from the footpads of mice infected with wild-type, Δarg, or Δarg[pArg] parasites and stained with Ab to nitrotyrosine residues (Fig. 7, A–C). There was an increase in nitrosylated tyrosine staining in the foot paws of mice infected with Δarg compared with either wild-type or Δarg[pArg] parasites. H&E staining of morphology revealed the presence of amastigotes in the dermis and epidermis from the region of increased staining (Fig. 7, D–F).

### Discussion

The manifestations of leishmaniasis result from a balance between host microbicidal and parasite defense mechanisms. *Leishmania* spp. are obligate intracellular parasites residing primarily in host macrophages. One of the most important antimicrobial effector molecules in murine macrophages is NO\(^{2}\) (9, 10, 34), and a role for NO\(^{2}\) in human macrophage microbicidal function has been documented in a few instances (29, 35, 36). NO\(^{2}\) is produced from arginine by iNOS, the product of the NOS2 gene. Arginase competes for the arginine substrate of iNOS by converting the amino acid to ornithine and liberating urea. Ornithine, in turn, is the immediate precursor for the synthesis of polyamines, essential growth factors for *Leishmania* (13). Thus, the balance between iNOS and arginase activities may determine whether the intracellular environment within the macrophage is microbicidal or supportive of *Leishmania* survival and multiplication.

The outcome of *Leishmania* infection in mice is dependent on whether the host develops an adaptive type 1 immune response resulting in production of IFN-γ and consequent microbicidal activity, or whether the type 1 response is suppressed by type 2 cytokines (e.g., IL-4) or other factors such as TGF-β (2, 3). The immune response is determined in part by the parasite species initiating infection and in part by host factors. The course of murine infection with *L. major* has been shown to be under genetically determined immunoregulatory controls that are different from those associated with *L. mexicana* infection (37–39). Whereas a majority of inbred mouse strains develop self-healing lesions when infected s.c. with *L. major*, virtually all mice develop rapidly growing large nonhealing lesions full of parasites following s.c. *L. mexicana* infection (40, 41). BALB/c mice are an exception in that both parasites cause progressive disease (3, 4, 40, 41).

There is an extensive body of literature indicating that the type 2 cytokines (IL-4, IL-9, IL-13) are associated with murine susceptibility to *L. major* infection (reviewed in Refs. 1 and 3). However, the contribution of type 2 cytokines to progressive infection with other *Leishmania* species is variable. For instance, during *L. chagasi* infection the type 1 response is inhibited by TGF-β (42), whereas type 1 immunity is inhibited by other non-IL-4 factors during infection with *L. mexicana* and *Leishmania donovani* (4, 43).

*Leishmania* promote their intracellular survival within macrophages through mechanisms that enable them to resist or inactivate reactive oxygen species generated by reactions of the NADPH oxidase and iNOS enzymes (44–46). It is becoming apparent that *Leishmania* also have mechanisms by which they can manipulate the infected host cell such that microbicidal responses are blunted or fail to develop. Examples include the ability of the parasite to suppress macrophage protein kinase C activation, MHC class II expression, and IFN-γ pathway signaling (47–49).

Previous studies have demonstrated that functional arginase is important for *Leishmania* spp. survival. The growth of both *L. major* and *L. infantum* in BALB/c macrophages is inhibited by N^ω^-hydroxy-L-arginine (called nor-NOHA or LOHA in different publications), and this effect is reversed by inhibiting iNOS with N-monomethyl-L-arginine (L-NMMA) (16). Arginase I protein levels progressively increase during lesion development in susceptible BALB/c mice, whereas a low protein level is present in self-healing resistant C57BL/6 mice infected with *L. major* (7).

Arginase inhibitors used in the above studies are active against both host and parasite arginase. During the current investigation, the specific role of leishmanial arginase as a virulence factor that acts directly on host cell was analyzed. A genetic strategy exploiting a Δarg null mutant of *L. mexicana* with wild-type and complemented Δarg controls was implemented (14). The Δarg knockout was impaired in its ability to survive in vitro in macrophages and in vivo in mice. The growth defect of the mutant in axenic...
culture was reversed by the addition of exogenous putrescine or ornithine, which provided precursors for polyamine synthesis. However, the intracellular growth defect in the macrophage was not reversed by putrescine or ornithine despite evidence that the parasites were able to scavenge these compounds when added extracellularly. Therefore, we hypothesized that the accumulation of the arginase substrate, rather than merely the deficiency of the arginase product, contributed to the defective intracellular growth.

Consistent with the above hypothesis, the Δarg elicted increased NO production by infected macrophages. Furthermore, the differences observed between Δarg and wild-type or Δarg [pArg] survival were abrogated in host macrophages that lack iNOS, suggesting that survival of Δarg was impaired at least in part because host iNOS activity was greater in these macrophages compared with macrophages infected with wild-type parasites. An increase in nitrosylated tyrosine staining in Δarg-infected mouse tissues supported this hypothesis.

NO synthesis is regulated to a great extent at the level of transcription of the NOS2 gene encoding iNOS. IFN-γ, a type 1 cytokine, up-regulates NOS2 transcription and in so doing enhances macrophage microbialic capacity (50). Not as well-recognized but equally as important, iNOS activity has been shown to be regulated at the level of substrate availability. Arginine depletion but equally as important, iNOS activity has been shown to be regulated at the level of substrate availability. Arginine depletion

Mouse infection with Δarg parasites lacking arginase developed a significantly different adaptive immune response from those infected with wild-type or arginase “add-back” controls. Ag-specific cellular responses in lymph nodes draining lesions caused by Δarg parasites were characterized by a type 1 response with increased IFN-γ and decreased IL-4 and IL-10 compared with lesions caused by wild-type or Δarg[pArg] parasites. We cannot discern from these data whether the phenotype observed in mice infected with Δarg was due to poor establishment of infection or preferential induction of a type 1 immune response, although we favor the former possibility. These data suggest that parasite arginase plays a crucial role in directing host macrophage microbialic activity through iNOS substrate depletion, and that infection with the parasite favors a type 1 response.

In contrast to our observations using Δarg L. mexicana, pharmacological inhibition of arginase did not blunt the type 2 immune response to L. major (15). The difference between the two discrepancies could be attributable to different roles of arginase between the two Leishmania sp., or to the different effects of partial vs total arginase inactivation.

Leishmania arginase plays a critical role in the synthesis of the polyamines putrescine and spermidine. The aroxotroph conferred by the Δarg null mutation in vitro can be bypassed in promastigotes by either low concentrations of putrescine, high concentrations of ornithine or spermidine, or episomal complementation (14). Even though the virulence of Δarg L. mexicana is diminished in mice and in the infected macrophage, slow proliferation of footpad lesions induced by Δarg parasites was detectable and parasites were recovered after 16 wk. This suggests that the mutant parasites were able to scavenge ornithine or polyamines from the host. The decreased virulence of Δarg compared with wild-type and Δarg [pArg] L. mexicana could reflect the combined effects of 1) enhanced iNOS activity due to increased availability of arginine, 2) lower levels of trypanothione in the absence of arginase-derived spermidine, making the parasite more vulnerable to NO-mediated toxicity; 3) enhanced development of cells producing the type 1 cytokine IFN-γ; and 4) decreased endogenous parasite polyamines (51).

Only a few studies document a role for parasite-encoded molecules in directing the host microbialic activities (49, 52). Data presented in this report suggest that parasite-encoded arginase can be added to the list of parasite-derived molecules that actively modify infection through its influence on the microbialic function of the macrophage infected with L. mexicana.

Acknowledgment

We are grateful to Melissa Miller for her technical expertise and support. We thank Jian Shao and the University of Iowa Central Microscopy facility for training and help with microscopy. We also thank Paul Reimann for help in preparation of illustrations.

Disclosures

The authors have no financial conflict of interest.

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