Arginase Is Essential for Survival of Leishmania donovani Promastigotes but Not Intracellular Amastigotes

Jan M. Boitz,a Caslin A. Gilroy,a* Tamara D. Olenyik,a,b Dustin Paradis,b Jasmine Perdeh,b Kristie Dearman,b Madison J. Davis,b Phillip A. Yates,a Yuexin Li,c Michael K. Riscoe,c,d Buddy Ullman,a Sigrid C. Robertsb

Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, Oregon, USAa; Pacific University School of Pharmacy, Hillsboro, Oregon, USA; VA Medical Center, Experimental Chemotherapy Laboratory, Portland, Oregon, USA; Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, Oregon, USAd

ABSTRACT Studies of Leishmania donovani have shown that both ornithine decarboxylase and spermidine synthase, two enzymes of the polyamine biosynthetic pathway, are critical for promastigote proliferation and required for maximum infection in mice. However, the importance of arginase (ARG), the first enzyme of the polyamine pathway in Leishmania, has not been analyzed in L. donovani. To test ARG function in intact parasites, we generated Δarg null mutants in L. donovani and evaluated their ability to proliferate in vitro and trigger infections in mice. The Δarg knockout was incapable of growth in the absence of polyamine supplementation, but the auxotrophic phenotype could be bypassed by addition of either millimolar concentrations of ornithine or micromolar concentrations of putrescine or by supplementation with either glycosomal or cytosolic versions of ARG. Spermidine supplementation of the medium did not circumvent the polyamine auxotrophy of the Δarg line. Although ARG was found to be essential for ornithine and polyamine synthesis, ornithine decarboxylase appeared to be the rate-limiting enzyme for polyamine production. Mouse infectivity studies revealed that the Δarg lesion reduced parasite burdens in livers by an order of magnitude but had little impact on the numbers of parasites recovered from spleens. Thus, ARG is essential for proliferation of promastigotes but not intracellular amastigotes. Coupled with previous studies, these data support a model in which L. donovani amastigotes readily salvage ornithine and have some access to host spermidine pools, while host putrescine appears to be unavailable for salvage by the parasite.

KEYWORDS Leishmania, arginase, polyamines

Parasites of the genus Leishmania cause a variety of devastating and often fatal diseases in humans and domestic animals. Leishmaniasis ranges from cutaneous ulcerative lesions to fatal visceralizing infections and affects an estimated 12 million people worldwide (1). Among diseases of parasitic origin, visceral leishmaniasis is the second leading cause of mortality in humans worldwide (2). The heteroxenous pathogen lives as the extracellular, flagellated promastigote within its insect vector, the phlebotomine sandfly, and resides as the intracellular, immotile amastigote within the phagolysosomes of infected macrophages and other reticuloendothelial cells of the mammalian host. Due to the absence of effective vaccines, empirical chemotherapies have offered the only avenue of defense for the treatment of leishmaniasis (3–5). Unfortunately, the current arsenal of drugs used to treat leishmaniasis is far from ideal due to the lack of target specificity and emerging drug resistance. Thus, the need to
validate new therapeutic targets and to better understand host-parasite interactions that impact these putative targets is acute.

One pathway that has been validated as an antiparasitic drug target in the evolutionarily related pathogen *Trypanosoma brucei* is that for polyamine biosynthesis (6–9). Polyamines are ubiquitous aliphatic cations that play vital roles in a variety of fundamental cellular processes, including growth, differentiation, and macromolecular synthesis (10–13). In addition to general functions, spermidine is also vital for the modification and activation of eukaryotic initiation factor 5A in parasites, as well as in higher eukaryotes (14–16). Furthermore, in a reaction unique to trypanosomatids, spermidine is conjugated with two glutathione molecules to produce trypanothione, a thiol reductant that serves to maintain the intracellular redox balance and for defense against oxidative stress (17–20). Because of the requirement for polyamines in parasites, inhibitors of polyamine pathway enzymes represent a rational paradigm for the treatment of parasitic diseases (20–25). D,L-α-difluoromethylornithine (DFMO), a suicide inhibitor of ornithine decarboxylase (ODC), the enzyme that converts ornithine to putrescine, has shown remarkable therapeutic efficacy in treating African sleeping sickness caused by *Trypanosoma brucei gambiense* (6–9). DFMO is also effective at killing other genera of protozoan parasites (26–29) and has been found to reduce *Leishmania* infections in mouse and hamster models (30–32).

The polyamine pathway in *Leishmania* consists of four enzymes: arginase (ARG), ODC, spermidine synthase (SPDSYN), and S-adenosylmethionine decarboxylase (ADOMETDC). ARG, the first and committed step in polyamine biosynthesis, converts arginine to ornithine, which is subsequently metabolized to the diamine putrescine by the catalytic action of ODC. The enzyme SPDSYN then generates spermidine via the addition of an aminopropyl group that is donated from decarboxylated S-adenosylmethionine, the product of ADOMETDC. Spermine, a prevalent polyamine of higher eukaryotes, is not made or metabolized further by *Leishmania* (33), and there is no spermine synthase (SPMSYN) homolog in the leishmanial genome (34). ODC, SPDSYN, and ADOMETDC have all been validated as essential for survival and growth of the promastigote form of *L. donovani*, as gene knockouts of each enzyme confer polyamine auxotrophy to the parasite and mutants cannot grow in the absence of appropriate polyamine supplementation (33, 35, 36).

Although ARG has not yet been validated as crucial for *L. donovani* promastigote survival, null mutants at the ARG locus have been created in *L. mexicana*, *L. major*, and *L. amazonensis* (37–39), species that are etiologic agents of cutaneous leishmaniasis. These Δarg strains are all auxotrophic for polyamines and exhibit significantly reduced infectivity levels in mice compared to wild-type parasites, although they are all still able to establish infections (38–41). These findings imply that cutaneous amastigotes are able to access ornithine and/or polyamines to some extent from the phagolysosome but demonstrate nevertheless that the parasite ARG is required for maximum infection by these *Leishmania* species. In contrast, Δodc and Δspdsyn knockouts in *L. donovani* both elicit striking diminutions in parasite loads in infected mouse organs, although the impact of the Δodc lesion on parasite burdens in both liver and spleen is much greater than that of the Δspdsyn genetic alteration (42, 43). The basis for the observed variations in infection levels among the visceralizing *L. donovani* Δodc and Δspdsyn mutants and the cutaneous *Leishmania* Δarg species is unknown.

Interestingly, the polyamine pathway of *Leishmania* is partitioned between the cytosol and the glycosome (37), a peroxisome-like microbody unique to trypanosomatids (44, 45). ARG is located in the glycosome, while ODC, SPDSYN, and ADOMETDC are cytosolic enzymes (37). This discrete cellular segregation of ARG and the downstream polyamine pathway enzymes may afford better control of polyamine biosynthesis and/or facilitate spatial partitioning of the amino acid arginine for polyamine versus protein biosynthesis. Mislocalization of ARG to the cytosol in both *L. mexicana* and *L. amazonensis* dramatically reduced parasite burdens, implying that the glycosomal milieu, although not critical for ARG function in promastigotes (37), is indispensable for proper ARG function in amastigotes (39, 46).
To address the differential impacts of Δarg lesions in cutaneous strains on infectivity with those in genes encoding downstream polyamine enzymes in *L. donovani* and to assess the importance of glycosomal targeting of ARG in both activity and infection, we created and characterized *L. donovani* Δarg strains. We found that ARG is essential for polyamine biosynthesis *de novo* and for promastigote growth in unsupplemented media. The Δarg deletion triggered a one order of magnitude reduction in parasite burdens in livers of mice after a 4-week standard infection but did not impact parasite loads in spleens. A comparison of murine infectivity in Δarg, Δodc, and Δspdy lines supports a model in which *L. donovani* amastigotes in the phagolysosome scavenge ornithine efficiently and salvage spermidine to some extent, while putrescine acquisition is basically insufficient to support amastigote maintenance.

**RESULTS**

*L. donovani* ARG. The coding and flanking sequences for the ARG locus of *L. donovani* (LdBPK_351490.1) were obtained from www.genedb.org. A multisequence alignment showed that the *L. donovani* ARG open reading frame (ORF) is 97%, 96%, and 95% identical to the predicted ARG proteins of *L. major*, *L. mexicana*, and *L. amazonensis*, respectively (see Fig. S1 in the supplemental material). Like other previously reported *Leishmania* ARG sequences (37, 39), the *L. donovani* gene encodes a C-terminal tripeptide that mediates the translocation of the protein to the glycosome. It should be noted that the ARG genes from *L. donovani* and *L. major*, both Old World *Leishmania* species, encompass an AKL C-terminal triad, while the ARG gene from *L. mexicana* and *L. amazonensis*, both New World *Leishmania* species, encode an SKL C-terminal tripeptide. Both AKL and SKL are archetypical topogenic signals for targeting proteins to the glycosome (47, 48). The *L. donovani* ARG protein is 43% and 39% identical to the two human ARG enzymes, ARGI and ARGII, respectively.

Creation and genotypic characterization of Δarg mutants and add-back cell lines. To investigate the functional role of ARG in parasite growth and infectivity, gene deletion mutants were created by double targeted gene replacement from wild-type *L. donovani*. ARG/arg heterozygotes were first generated by replacing one copy of ARG with either a HYG or PHLEO drug resistance marker, and the expected chromosomal rearrangements were confirmed by PCR. Both ARG/arg heterozygotes were then subjected to a second round of transfection to generate Δarg null mutants, and two clonal Δarg null mutant cell lines, derived from a HYG heterozygote, were selected for further analysis. In addition, add-back lines were made via insertion of the ARG coding region or a mutated arg gene lacking the C-terminal AKL glycosomal topogenic signal into the ribosomal locus of a Δarg cell line. Both PCR and Western blot analyses were employed to confirm the predicted genotypes of the mutant parasites (Fig. 1). The PCR analysis demonstrated that the ARG coding region could not be detected in the Δarg background but was present in wild-type, heterozygous, and add-back parasites (Fig. 1A). Western blot analysis with lysates harvested from either promastigotes or axenic amastigotes confirmed the absence of ARG protein from Δarg parasites and the presence of ARG protein in wild-type, heterozygous, Δarg(ARG), and Δarg[argΔAKL] cells (Fig. 1D). Somewhat surprisingly, the heterozygous parasites expressed very little ARG protein compared to the much more robust expression of ARG protein in wild-type parasites (Fig. 1D), an observation that could be ascribed to allele-specific expression differences. Immunofluorescence studies confirmed that the wild-type and Δarg[ARG] parasites expressed ARG in the glycosomes and that the Δarg[argΔAKL] cell line produced Arg protein that mislocalized to the cytosol (Fig. 2).

Nutritional assessment of the *L. donovani* Δarg mutant. Growth assays confirmed that the Δarg lesion conferred a conditionally lethal growth phenotype to *L. donovani*. Only the Δarg cell line was unable to grow in the absence of ornithine or polyamine supplementation, while wild-type, Δarg[ARG], and Δarg[argΔAKL] parasites grew at comparable rates and to similar densities in polyamine-free medium (Fig. 3A). The phenotypic consequences of the genetic lesion in Δarg parasites could be avoided by supplementation of the medium with the downstream metabolite putrescine (Fig. 3B).
3A and B), while spermidine, the penultimate polyamine in \textit{Leishmania}, did not rescue \(\Delta\text{arg}\) proliferation at concentrations of up to 1,000 \(\mu M\) (Fig. 3B). The concentrations of ornithine and putrescine that restored \(\Delta\text{arg}\) cell growth were determined by incubation of the knockouts in medium containing different ornithine or putrescine levels (Fig. 3B and 4). An initial screen indicated that optimal growth of \(\Delta\text{arg}\) parasites could be achieved at 100 \(\mu M\) putrescine, whereas supplementation of the medium with 100 \(\mu M\) ornithine had essentially no growth-stimulatory effect (Fig. 3A). Only modest growth could be achieved at 500 \(\mu M\) to 1 mM ornithine (Fig. 3B). Incubation of \(\Delta\text{arg}\) promastigotes in medium containing serial dilutions of either ornithine or putrescine revealed effective concentrations of additive that achieved 50% maximum cell densities (EC\(_{50}\)) of 750 \(\pm\) 229 \(\mu M\) for ornithine and 7.5 \(\pm\) 5 \(\mu M\) for putrescine (Table 1 and Fig. 4A). Because ornithine and putrescine incorporation by the intracellular amastigote form is ultimately key for the clinical validation of ARG as a potential therapeutic target, the requirement for ornithine and putrescine was also assessed in axenic amastigotes. The EC\(_{50}\) calculated for ornithine and putrescine were 383 \(\pm\) 147 \(\mu M\) and 6.25 \(\pm\) 3.86 \(\mu M\), respectively (Fig. 4B).

Previous studies with \textit{L. mexicana} promastigotes revealed that transport capacities for putrescine and ornithine are similar and thus cannot explain the difference in ornithine and putrescine concentrations that are required to efficiently bypass a \(\Delta\text{arg}\) lesion (37). However, ornithine levels are much higher than those for putrescine in \textit{L. mexicana} (37), suggesting that ODC is the rate-limiting step in polyamine production. To test this conjecture, we investigated whether overproduction of ODC in the \(\Delta\text{arg}\) parasites could reduce ornithine requirements for parasite proliferation by constructing a \(\Delta\text{arg}\) derivative line, the \(\Delta\text{arg[ODC]}\) strain, that overexpressed ODC. Overproduction of the ODC protein in the \(\Delta\text{arg[ODC]}\) line was confirmed by Western blotting (Fig. 5A). Growth analysis in serial dilutions of ornithine revealed that the \(\Delta\text{arg[ODC]}\) parasites indeed required smaller amounts of ornithine than the parental \(\Delta\text{arg}\) parasites (Fig. 5B).

**Effect of ARG inhibitors on promastigote proliferation.** Because ARG is essential for promastigote proliferation, the arginine analogues N\(\text{\textsuperscript{-}}\)-hydroxy-L-arginine (NOHA),...
N\textsuperscript{\textdagger} hydroxy-nor-L-arginine (nor-NOHA), S-(2-boronoethyl)-L-cysteine (BEC), and 2(S)amino-6-boronohexanoic acid (ABH), all potent inhibitors of the recombinant \textit{L. mexicana} ARG (49), were tested for their growth-inhibitory effects against \textit{L. donovani} promastigotes. EC\textsubscript{50}s of 275 \textmu M and 610 \textmu M were calculated for NOHA and nor-NOHA, respectively (data not shown). Addition of putrescine negated the observed growth inhibition caused by NOHA and nor-NOHA completely (EC\textsubscript{50} of >1,000 \textmu M). That the modest growth inhibition triggered by NOHA and nor-NOHA in \textit{L. donovani} could be reversed by putrescine supplementation indicates that ARG is the intracellular target of the two ARG enzyme inhibitors. The other two ARG inhibitors tested, ABH and BEC,
were less effective growth inhibitors. An EC$_{50}$ of $\sim 2.5$ mM was obtained for ABH, while BEC displayed no growth-inhibitory effect on promastigote growth up to 5 mM (data not shown). Interestingly, NOHA exhibited efficacy against \textit{L. major} (38) but not against \textit{L. mexicana} promastigotes (46). The relatively poor efficacy of the ARG enzyme inhibitors, at least for \textit{L. mexicana} and \textit{L. donovani}, can be ascribed to potentially poor uptake of the charged compounds (46, 49).

**Infectivity analyses.** To determine the impact of an ARG deficiency on infectivity, parasite burdens in both liver and spleen tissues were assessed in BALB/c mice inoculated with either wild-type, $\Delta$arg, $\Delta$arg\[ARG\], or $\Delta$arg\[argΔAKL\] parasites. Mice were sacrificed 4 weeks postinfection, since a time course experiment had previously determined that hepatic parasite burdens in our mouse model peaked between two and 4 weeks while splenic parasite loads were much lower but remained at steady levels in the spleen for up to 10 weeks (Fig. S2). Previous infectivity studies with $\Delta$odc and $\Delta$spdsyn \textit{L. donovani} strains were also performed 4 weeks postinoculation (42, 43). The average liver parasite loads of the five mice infected with wild-type parasites varied between $10^4$ and $10^5$ parasites/g, and the average parasite numbers of mice infected with the two independent $\Delta$arg knockout strains were an order of magnitude lower (Fig. 6A). Statistical analysis (paired $t$ test) revealed a significant difference in liver parasite numbers harvested from mice injected with either wild-type or knockout...
Parasites (P values of 0.0261 and 0.0279 for a comparison between the wild type and the Δarg mutants 1 and 2). Parasite burdens in livers obtained from mice infected with the Δarg[ΔAKL] add-back parasites were equivalent to those of mice inoculated with wild-type parasites, intimating that the infectivity deficit incurred by the Δarg lesion can be ascribed to ARG loss and not to some ancillary genetic alteration that occurred through the extended creation of the null line. Parasite loads in livers of mice infected with Δarg[ΔargΔAKL] parasites expressing the mislocalized cytosolic arg were intermediate between those from mice injected with wild-type and Δarg parasites. Only inconsequential differences in parasite numbers were observed in splenic preparations among mice infected with either wild-type, Δarg knockout, or add-back parasites (Fig. 6B).

**DISCUSSION**

To evaluate the functional role of ARG, the first and committed step in polyamine biosynthesis in *L. donovani*, a Δarg knockout was created by double targeted gene replacement and characterized with respect to its growth and infection capabilities. The Δarg lesion conferred polyamine auxotrophy to the parasite that could be rescued by supplementation of the growth medium with either ornithine or putrescine. The finding that putrescine could fully rescue the deleterious consequences of a Δarg null mutation in both promastigotes and axenic amastigotes authenticates that the sole function of ARG in *L. donovani* is to support polyamine biosynthesis, a result similar to previous findings obtained with promastigotes of Δarg null mutants of several cutaneous species of *Leishmania* (37–39). In contrast, addition of spermidine to the culture medium up to a concentration of 1 mM did not enable growth of Δarg promastigotes.

| Supplement | EC50 (μM) for Δarg mutant of |
|------------|--------------------------------|
|            | *L. donovani* | *L. mexicana* | *L. major* |
| Ornithine  | 750 ± 229     | 500**         | 1,000**    |
| Putrescine | 7.5 ± 4.95    | 2*            | 30**       |

*EC50s for *L. donovani* Δarg promastigotes were determined, as shown in Fig. 4, by incubating parasites in serial dilutions of ornithine or putrescine for 5 days. Proliferation was evaluated by assessing resazurin conversion as a measure of metabolic activity. Each value represents the means and standard deviations from three independent experiments set up in duplicate. The EC50s for *L. mexicana* Δarg and *L. major* Δarg mutants have been reported elsewhere (*, reference 37; **, reference 38).*

**FIG 5** Ornithine requirement for Δarg[ODC] promastigotes. (A) Western blot analysis was performed with cell lysates prepared from wild-type parasites, the parental Δarg line, and the Δarg[ODC] ODC overproducer strain. Parasite lysates were fractioned by SDS-PAGE and the blot probed with polyclonal antibodies against *L. donovani* ODC and an anti-tubulin antibody as a loading control. (B) Growth phenotypes of Δarg (gray squares) and Δarg[ODC] (black triangles) promastigotes were established in increasing concentrations of ornithine. Parasites were incubated at 5 × 10⁵ parasites/ml, and percent proliferation was evaluated after 5 days via the ability of parasites to convert resazurin to resorufin as assessed by fluorescence, and readings obtained with the highest supplement concentrations were equated with 100% proliferation. The experiments were set up in duplicate and repeated three times with similar results.
Because spermidine is efficiently transported into *Leishmania* (50–52), the observation that spermidine could not rescue the polyamine auxotrophy of the *L. donovani* Δarg mutant implies that putrescine is an essential metabolite for proliferation of *L. donovani* promastigotes. Coupled with previous findings that the *L. donovani* Δspdsyn mutant accumulates putrescine as a result of the genetic block but also display polyamine auxotrophy (35), our data establish for the first time that both of the polyamines found in *L. donovani*, putrescine and spermidine, are each of their own accord critical for the survival and growth of the parasite. That spermidine by itself is indispensable for parasite proliferation is unsurprising, since the polyamine is a component of two vital downstream reactions. Spermidine is a constituent of trypanothione, the thiol reductant in *Leishmania* and related trypanosomatids that replaces the role of glutathione in mammalian systems (17–20), and hypusine, an unusual amino acid that is the result of a posttranslational modification in eukaryotic translation initiation factor 5A and is essential for its proper function (14–16). In contrast to spermidine, a specific function for putrescine is unknown other than that the diamine serves as a general aliphatic polycation. Finally, the fact that supplementation of the growth medium with spermidine cannot rescue a Δarg deficiency in *L. donovani* (Fig. 3B), together with our previous observation that spermine supplementation cannot rescue a Δodc deficiency (33), proves the absence of a back-conversion pathway from spermine to spermidine and putrescine in *Leishmania* that is operative in mammalian cells (53, 54).

The basis for why higher concentrations of ornithine than putrescine are required to rescue the phenotypic consequences of a Δarg deficiency in both promastigotes and axenic amastigotes is not known. Ornithine and putrescine uptake rates into *L. mexicana* promastigotes (37) are roughly equivalent, although an analysis of ornithine and putrescine uptake into *L. donovani* promastigotes has not been undertaken. Because *L. mexicana* promastigotes contain vastly more ornithine (~130 nmol/10⁷ parasites) than putrescine (~2.5 nmol arginine/10⁷ parasites) (37), we speculate that ODC is the rate-limiting enzyme in polyamine biosynthesis, converting only small amounts of stockpiled ornithine into putrescine. Our finding that overexpression of ODC in the Δarg background reduced the levels of ornithine required to enable growth (Fig. 5)
supports this conjecture. Little is known about the regulation of polyamine synthesis in trypanosomatids, although it has long been established that mammalian cells utilize an intricate regulatory system to ensure low and stable polyamine levels (55–58). Our supposition that ODC is a rate-limiting enzyme in Leishmania together with the recent discovery of the controlled expression of prozyme, necessary for the activity of ADOMETDC, in African trypanosomes (24, 59–62) suggests that polyamine biosynthesis is also regulated in trypanosomatids.

The cellular localization of ARG does not appear to be crucial for its function in L. donovani promastigotes or axenic amastigotes, as add-back parasites expressing glycosomal or cytosolic mislocalized ARG both exhibit polyamine prototrophy (Fig. 3). Similar results have also been observed in L. mexicana and L. amazonensis promastigotes (37, 39). However, mislocalization of ARG to the cytosol in both L. mexicana and L. amazonensis, unlike L. donovani (Fig. 6), dramatically reduced parasite burdens, implying that the glycosomal milieu, although not critical for ARG function in promastigotes, is indispensable for proper ARG function in amastigotes of the two species (39, 46). Why the cellular environment appears to be important for ARG function in intracellular L. mexicana and L. amazonensis amastigotes but not extracellular parasites is unclear but could be due to limiting availability of arginine in the host cell (63).

There exists an extraordinary positional variation in the impacts that specific lesions in polyamine biosynthesis exert on L. donovani infection capacity. Among the three null L. donovani mutants deficient in polyamine biosynthesis that have been tested in mice, the Δodc deletion effectively obliterates the capacity of the parasite to trigger a mouse infection (42), the Δspdsyn lesion shows reductions of three and two orders of magnitude in parasite burdens in liver and spleen, respectively (43), and the Δarg mutation confers a statistically significant 10-fold reduction in liver parasite loads but does not compromise splenic parasite numbers (Fig. 6). All determinations of parasite loads in these experiments were performed 4 weeks postinfection, the interval in which both hepatic and splenic infections were maximal in our system (see Fig. S2 in the supplemental material). It should be noted, however, that although it has long been known that hepatic infections of visceralizing Leishmania strains are self-limiting in mice, splenic parasite numbers in murine infections tend to increase throughout month-long infections (64–68). The basis for the discrepancies in the time courses of splenic infections between our laboratory and others (64–68) is unclear and was not pursued further, because the objective was to compare the impacts of specific genetic defects in the polyamine pathway upon infection.

Although it is problematic to compare infectivity phenotypes between visceral and cutaneous strains of Leishmania, a similar statistically meaningful reduction in parasite virulence, as determined by footpad lesion size, was also observed with Δarg L. mexicana, L. major, and L. amazonensis mutants, although all three cutaneous Δarg strains retained significant infection capacity (38–41). The growth deficits of the Δodc and Δspdsyn L. donovani mutants cannot be due to a lack of polyamine transport capacity, because axenic amastigotes are able to efficiently import both putrescine and spermidine, the products of ODC and SPDSYN, respectively (50, 51). Proficient putrescine uptake is also functionally demonstrated by the observation that Δarg axenic amastigotes grew well in medium supplemented with micromolar concentrations of putrescine (Fig. 4). Most likely, the divergence in the infection capacities among the Δarg, Δodc, and Δspdsyn L. donovani mutants reflects the relative ornithine and polyamine pools within the phagolysosome of infected macrophages. It is logical to propose that the parasite has access to ample ornithine to bypass a Δarg lesion, essentially no putrescine to evade the consequences of a Δodc deficiency, and intermediate spermidine pools that enable partial bypass of a Δspdsyn mutation. It is intriguing that Δarg axenic amastigotes require much higher concentrations of ornithine than putrescine for optimal growth (Fig. 4B), thus the physiological concentrations of ornithine would have to be high in the phagolysosome to circumvent the arg deletion. The model of putrescine and relative spermidine deficiencies in the host cell is supported by the observation that macrophages rapidly convert these smaller
polyamines to spermine (69). Previous Leishmania infection models have proposed that an increased host ARGI activity in infected macrophages provides polyamines for salvage and parasite proliferation (70, 71); however, our findings imply a refined model where ornithine, rather than polyamines, is available for salvage by the parasite (Fig. 7). This hypothesis can be tested in the future by measuring nutrient concentrations in macrophages and, ideally, phagolysosomes.

MATERIALS AND METHODS

Materials, chemicals, and reagents. Resazurin, G418, hygromycin, putrescine, and ornithine were purchased from VWR International (Radnor, PA). Phleomycin was procured from Thermo Fisher Scientific (Waltham, MA). N-hydroxy-L-arginine (NOHA), N-hydroxy-nor-L-arginine (nor-NOHA), S-(2-boronoethyl)-L-cysteine (BEC), and 2(S)-amino-6-boronohexanoic acid (ABH) were bought from Enzo (Farmingdale, NY). Restriction enzymes were acquired from New England BioLabs (Ipswich, MA). The Wizard SV gel and PCR clean-up system was purchased from Promega (Madison, WI), and the DNeasy kit was obtained from Qiagen Inc. (Valencia, CA). The pCR 2.1-TOPO vector and synthetic oligonucleotides were acquired from Invitrogen Corp. (Carlsbad, CA), and the Advantage HF2 DNA polymerase mix was purchased from BD Bioscience (Palo Alto, CA). The pRP-M vector was a gift from Phillip A. Yates, Oregon Health & Science University.

Cell lines, cell culture, and assessment of growth phenotypes. All genetically manipulated parasites were derived from the wild-type LdBob strain of L. donovani (72) that was originally obtained from Stephen M. Beverley (Washington University, St. Louis, MO). Wild-type LdBob and genetically manipulated derivative strains were routinely cycled between the promastigote and axenic amastigote stages at 26°C, pH 7.4, and 37°C, pH 5.5, respectively, using previously reported cell culture conditions, in order to maintain infectivity (73, 74). Promastigotes were incubated in completely defined DME-L (75), while amastigotes were grown in an M199-based medium (73). Wild-type, Δarg, and ΔargΔAKL parasites were routinely cultured in medium with no ornithine or putrescine supplementation, while the Δarg cells were maintained in medium supplemented with 50 μg/ml hygromycin, 50 μg/ml phleomycin, and 100 μM putrescine.

Growth phenotypes were established by incubating wild-type and mutant L. donovani promastigotes or axenic amastigotes seeded in a volume of 100 μl in 96-well plates at a density of 5 × 10⁶/ml in the absence or presence of various concentrations of ornithine, putrescine, or spermidine as specified. Growth experiments with spermidine added to the medium necessitated replacement of fetal bovine serum with chicken serum to avoid polyamine oxidase-mediated toxicity (35, 76). After 5 days, 10 μl of 250 μM resazurin was added to each well to assess cell density, and plates were incubated for an

---

**FIG 7** Polyamine salvage model for promastigotes and intracellular amastigotes. (A) L. donovani promastigotes efficiently transport arginine, ornithine, and polyamines. Arginine is an essential amino acid (88, 89), while promastigotes are able to synthesize ornithine and polyamines endogenously. Putrescine and spermidine, but not ornithine, are essential for parasite growth and survival, and gene deletion mutants depend on polyamine supplementation of the media. (B) L. donovani amastigotes reside inside the phagolysosome (shaded gray) of host macrophages. Our data support a model wherein ornithine salvage is hypothesized to be efficient, while spermidine and especially putrescine salvage pools are too limited to support robust infections. Although amastigotes have putrescine and spermidine transporters (50, 51), both polyamines have been shown to be rapidly metabolized to spermine in macrophages (69) and thus are not accessible to the intracellular parasite. Spermine cannot meet the polyamine requirements of Leishmania parasites (33).
additional 4 h. Conversion of resazurin to resorufin was evaluated on a BioTek Synergy plate reader by monitoring fluorescence (excitation wavelength=579 nm; emission wavelength=584 nm). Graphs were prepared using GraphPad Prism version 4.0 or 6.0f for Mac (GraphPad Software, La Jolla, CA).

Creation of $\Delta arg$ parasites. The $\Delta arg$ knockouts were generated in the Ldb8bob background by double targeted gene replacement (77, 78). To construct drug resistance cassettes for the replacement of ARG, the 5' ARG flanking region was amplified by PCR using the following primers containing HindIII and Sall restriction sites (underlined): forward primer, GCATGCTGACTGAGCCCTGTCACCAGAGAC; reverse primer, GCGCCGGCCGATGTGCTGATCTCACTGCAAGAG. The resulting PCR product was then subcloned into the pCR 2.1-TOPO vector, excised with HindIII and Sall endonucleases, and inserted into the HindIII and Sall sites of pX63-HYG and pX63-PHLEO to generate pX63-HYG-5'ARG and pX63-PHLEO-5'ARG, respectively. The 3' flanking region was then amplified by PCR using the following primers containing SmaI and BglII sites (underlined): forward primer, GGGCCCGCGCATGTGTTCCTTCCAAG; reverse primer, GCATGGATCCGCGCATGTGTTCCTTCCAAG, and the above-noted reverse primer containing the BglII site.

The resulting PCR product was subcloned into the pCR 2.1-TOPO vector and then introduced into the BamHI/BglII sites of pX63-PHLEO-5'ARG. The correct orientations of the 5' and 3' flanking regions within the gene-targeting plasmids were verified by limited nucleotide sequencing of the insert junctions.

The plasmids pX63-HYG-Δarg and pX63-PHLEO-Δarg were digested with HindIII and BglII to liberate 6-kb and 5.5-kb linear fragments, designated X63-HYG-Δarg and X63-PHLEO-Δarg, respectively. X63-HYG-Δarg and X63-PHLEO-Δarg were purified from DNA agarose gels using the Wizard SV gel and PCR clean-up system according to the manufacturer’s protocol and then transfected into parasites using standard electroporation conditions (79). Two heterozygous cell lines, ARG/Δarg::HYG and ARG/Δarg::PHLEO, were generated first and selected in semisolid agar plates containing 50 μg/ml hygromycin or 50 μg/ml phleomycin, respectively. The genotypes of the two heterozygous cell lines were confirmed by PCR and then subjected to a second round of transfection. The ARG/Δarg::HYG heterozygotes were transfected with the PHLEO-Δarg fragment, and the ARG/Δarg::PHLEO heterozygotes were transfected with the X63-HYG-Δarg fragment. Potential homozygous Δarg::HYG/Δarg::PHLEO (Δarg) knockouts were selected in semisolid agar plates containing 50 μg/ml hygromycin, 50 μg/ml phleomycin, and 200 μM puromycin. The clones were picked and parasite cultures expanded. Derived homologous gene replacements were confirmed by PCR (Fig. 1). Two Δarg knockout clones, derived from the hygromycin-resistant heterozygotes (here designated ARG/arg), were picked for further analysis.

Complementation of Δarg cell lines. To generate a stably complemented add-back line, ARG was introduced into Δarg parasites by homologous recombination at the ribosomal locus (80). Two different constructs were generated, one containing the wild-type ARG sequence ($\Delta arg$ARG) and one containing a mutated version lacking the C-terminal AKL tripeptide ($\Delta arg$ΔAKL), an archetypal signal that targets proteins for glycosomal localization in Leishmania (47, 48, 81). The ARG coding region was amplified with the following primers, each containing an SfiI restriction site, GGCCNNNNNGGCC (underlined): forward primer GAGGCCACCTGGGCCTCATCATGGAGCACGTGCAG and reverse primer GAGGCCACCTGGGCCTCATCATGGAGCACGTGCAG. The resulting PCR product was first subcloned into the pCR 2.1-TOPO vector, excised with HindIII and SalI endonucleases, and inserted into the HindIII reverse primer, GCATGGTCGACTGACCCTGTCACCACCAGAC. The resulting PCR product was first subcloned into the pCR 2.1-TOPO vector and then inserted into the SmaI/BglII site of pX63-HYG-Δarg. Because of the presence of an SmaI site within the PHLEO coding region of pX63-PHLEO, a different cloning strategy was employed to insert the 3'-flanking region of ARG into pX63-PHLEO-Δarg. The 3'-flanking region was amplified by PCR using a forward primer containing the BamHI restriction site, GGGCCCGCGCATGTGTTCCTTCCAAG, and the above-noted reverse primer containing the BglII site. The resulting PCR product was subcloned into the pCR 2.1-TOPO vector and then inserted into the SmaI/BglII site of pX63-HYG-Δarg to generate pX63-HYG-Δarg.

ODC overproducers in the Δarg background. The construction of the ODC overexpression vector pSNBR[ODC] has been described previously (82). The Δarg[ODC] parasites were generated by transfecting Δarg parasites with the pSNBR[ODC] plasmid using standard electroporation conditions (79). Parasites harboring the episomal construct were selected with 100 μg/ml G418 in bulk culture and the correct phenotype verified by Western blot analysis.

PCR and Western blotting. Genomic DNA from the wild type, ARG/arg heterozygotes, two independent Δarg clones, and the Δarg[ARG] and Δarg[ΔAKL] add-backs was prepared for Southern blot analysis using the DNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s protocol. The following primers were utilized to amplify a 900-bp ARG coding region fragment: ATGGAGCACTGTCGACGCAGGCAG and CACGTCGGCATCTGCAAGCCG. Additional primers were designed to detect the replacement of the ARG gene with either the PHLEO or HYG drug resistance cassette. The forward primer (CCATCGTGACTGCTTATAG) was designed from sequence upstream of the deletion construct, and reverse primer were selected from the PHLEO (GAAGTCGTCCTCCACGAAGT) or HYG (CCCGCAGGACATATCCAC) coding region. The presence and size of the PCR products were determined on ethidium bromide-stained agarose gels.
Cell lysates from wild-type, \(\text{ARG}^+/\text{arg}^+, \Delta\text{arg}^+, \Delta\text{arg}^+/\text{arg}^+\text{AKL}\) cells were prepared from both promastigotes and axenic amastigotes that were in the late exponential-growth phase. Lysates were fractionated by SDS-PAGE (83) and blotted onto Immob-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA), and Western blot analysis was performed according to standard procedures (84). The membranes were probed with polyclonal antibodies raised in rabbits against the purified recombinant \(L. \text{mexicana} \text{ARG} \) protein (46) and commercially available anti-\(\alpha\)-tubulin mouse monoclonal antibody (Calbiochem, La Jolla, CA). Cell lysates were also prepared from \(\Delta\text{arg}^+/\text{ODC}\) promastigotes and Western blots probed with polyclonal antibodies raised against the purified recombinant \(L. \text{donovani} \text{ODC} \) protein (33).

**Immunofluorescence microscopy.** The immunofluorescence assay was performed on \(L. \text{donovani} \) promastigotes as described previously (81, 85, 86) using a 1:500 dilution of anti-\(\text{LdARG} \) antibody and a 1:10,000 dilution of goat anti-rabbit Oregon green-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA). Cells were mounted with VECTASHIELD antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and photographed on a Zeiss Axiovert 200 inverted microscope (Carl Zeiss Microimaging) using a 63× oil immersion lens. Photos were taken with a Zeiss AxioCam MR camera using AxioVision 4.2 software (Carl Zeiss Microimaging) and compiled using Adobe Photoshop Creative Suite 4.

**Inhibitor studies.** Wild-type promastigotes were incubated in serial dilutions of NOHA, nor-NOHA, ABT, or BEC in the presence or absence of 100 \(\mu\)M putrescine. Parasites were seeded at a density of 3 \(\times\) 10^7/ml in a volume of 100 \(\mu\)l in 96-well plates. After 5 days, parasite viability was evaluated by their ability to metabolize resazurin as described above.

**Murine infection studies.** Cohorts of five 6- to 7-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were inoculated via the lateral tail vein with 5 \(\times\) 10^5 stationary-phase promastigotes of either the wild type, two independently derived \(\Delta\text{arg} \) clones, or the \(\Delta\text{arg}^+/\text{ARG} \) or \(\Delta\text{arg}^+/\text{AKL}\) add-back. Prior to inoculation, all parasite lines were cycled between the promastigote and axenic amastigote stages. Livers and spleens were harvested 4 weeks after infection as described previously (42, 87). Single-cell suspensions from mouse organs were prepared by passage through a 70-\(\mu\)m cell strainer (BD Falcon), and parasite burdens were determined in 96-well microtiter plates using a standardized limiting-dilution assay (42, 87).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/IAI00554-16](https://doi.org/10.1128/IAI00554-16).

**TEXT S1**, PDF file, 0.6 MB.

**ACKNOWLEDGMENTS**

This investigation was supported by grant AI041622 (to B.U.) from the National Institute of Allergy and Infectious Diseases.

**REFERENCES**

1. Kedzierski L. 2011. Leishmaniasis. Hum Vaccin 7:1204–1214.
2. Bern C, Maguire JH, Alvar J. 2008. Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Negl Trop Dis 2:e313. [https://doi.org/10.1371/journal.pntd.0000313](https://doi.org/10.1371/journal.pntd.0000313).
3. Kaye P, Scott P. 2011. Leishmaniasis: complexity at the host-pathogen interface. Nat Rev Microbiol 9:604–615. [https://doi.org/10.1038/nrmicro2608](https://doi.org/10.1038/nrmicro2608).
4. Kedzierski L, Sakthianandeswaren A, Curtis JM, Andrews PC, Junk PC, Kedzierska K. 2009. Leishmaniasis: current treatment and prospects for new drugs and vaccines. Curr Med Chem 16:599–614. [https://doi.org/10.2174/09298670978458489](https://doi.org/10.2174/09298670978458489).
5. Muller R. 2007. Advances in parasitology, vol 65. Academic Press, San Diego, CA.
6. Bacchi CJ, McCann PP. 1987. Protozoon protozoa and polynamines, p 317–344. In McCann PP, Pegg AE, Sjoerdsm A (ed), Inhibition of polyamine metabolism: biological significance and basis for new therapies. Academic Press, Orlando, FL.
7. Burri C, Brun R. 2003. Effluxinohine for the treatment of human African trypanosomiasis. Parasitol Res 90(Supp 1):S49–S52.
8. Docampo R, Moreno SN. 2003. Current chemotherapy of human African trypanosomiasis. Parasitol Res 90(Supp 1):S10–S13.
9. Fairlamb AH. 2003. Chemotherapy of human African trypanosomiasis: current and future prospects. Trends Parasitol 19:488–494. [https://doi.org/10.1016/j.pt.2003.09.002](https://doi.org/10.1016/j.pt.2003.09.002).
10. Bachrach U. 2005. Naturally occurring polyamines: interaction with macromolecules. Curr Protein Peptide Sci 6:559–566. [https://doi.org/10.2174/13892030574933240](https://doi.org/10.2174/13892030574933240).
11. Janne J, Alhonen L, Keinanen TA, Pietila M, Uimari A, Pirinen E, Hyvonen MT, Jarvinen A. 2005. Animal disease models generated by genetic engineering of polyamine metabolism. J Cell Mol Med 9:379–390. [https://doi.org/10.1111/j.1582-4934.2005.tb00385.x](https://doi.org/10.1111/j.1582-4934.2005.tb00385.x).
12. Janne J, Alhonen L, Pietila M, Keinanen TA. 2004. Genetic approaches to the cellular functions of polyamines in mammals. Eur J Biochem 271:877–894. [https://doi.org/10.1111/j.1432-1033.2004.00409.x](https://doi.org/10.1111/j.1432-1033.2004.00409.x).
13. Tabor CW, Tabor H. 1984. Polyamines. Annu Rev Biochem 53:749–790. [https://doi.org/10.1146/annurev.bi.53.070184.003533](https://doi.org/10.1146/annurev.bi.53.070184.003533).
14. Chawla B, Jhingran A, Singh S, Tyagi N, Park MH, Srinivasan N, Roberts SC, Madhubala R. 2010. Identification and characterization of a novel deoeyhypusine synthase in Leishmania donovani. J Biol Chem 285:453–463. [https://doi.org/10.1074/jbc.M109.048850](https://doi.org/10.1074/jbc.M109.048850).
15. Park MH, Lee YB, Joe YA. 1997. Hypusine is essential for eukaryotic cell proliferation. Biol Signals 6:115–123. [https://doi.org/10.1159/000109117](https://doi.org/10.1159/000109117).
16. Chawla B, Kumar RR, Tyagi N, Subramanian G, Srinivasan N, Park MH, Madhubala R. 2012. A unique modification of the eukaryotic initiation factor 5A shows the presence of the complete hypusine pathway in Leishmania donovani. PLoS One 7:e33138. [https://doi.org/10.1371/journal.pone.0033138](https://doi.org/10.1371/journal.pone.0033138).
17. Fairlamb AH. 1990. Trypanothione metabolism and rational approaches to drug design. Biochem Soc Trans 18:717–720. [https://doi.org/10.1042/bst0180717](https://doi.org/10.1042/bst0180717).
18. Fairlamb AH, Cerami A. 1992. Metabolism and functions of trypanothione in the Kinetoplastida. Annu Rev Microbiol 46:695–729. [https://doi.org/10.1146/annurev.mi.46.100192.003403](https://doi.org/10.1146/annurev.mi.46.100192.003403).
19. Kraus Boitz et al. Infection and Immunity
21. Roberts SC, Jardim A, Carter NS, Heby O, Ullman B. 2001. Genetic analysis of spermidine synthase from Leishmania donovani. Mol Biochem Parasitol 113:1–11.

22. Basselin M, Lawrence F, Robert-Gero M. 1996. Pentamidine uptake in Leishmania donovani and Leishmania amazonensis promastigotes and axenic amastigotes. Biochem J 315(2):631–634. https://doi.org/10.1042/bj3150631.

23. Hasne MP, Ullman B. 2005. Identification and characterization of a polyamine permease from the protozoan parasite Leishmania major. J Biol Chem 280:20920–20924. https://doi.org/10.1074/jbc.M501092200.

24. Millner-Lemons, O’Linn-Sandovol V, Campbell K, Raiser M. 2015. Remaining mysteries of molecular biology: the role of polyamines in the cell. J Mol Biol 427:2980–2989. https://doi.org/10.1016/j.jmb.2015.06.020.

25. Perez-Leal O, Merali S. 2012. Regulation of polyamine metabolism by translational control. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.

26. Miller-Lemons L, O’Linn-Sandovol V, Campbell K, Raiser M. 2015. Remaining mysteries of molecular biology: the role of polyamines in the cell. J Mol Biol 427:2980–2989. https://doi.org/10.1016/j.jmb.2015.06.020.

27. Perez-Leal O, Merali S. 2012. Regulation of polyamine metabolism by translational control. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.

28. Shantz LM, Pegg AE. 1999. Polyamine catabolism and disease. Biochem J 342:323–338. https://doi.org/10.1042/BJ3420323.

29. Cervelli M, Amendola R, Polticelli F, Mariotti P. 2012. Spermine oxidase: ten years after. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.

30. Perez-Leal O, Merali S. 2012. Regulation of polyamine metabolism by translational control. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.

31. Miller-Lemons L, O’Linn-Sandovol V, Campbell K, Raiser M. 2015. Remaining mysteries of molecular biology: the role of polyamines in the cell. J Mol Biol 427:2980–2989. https://doi.org/10.1016/j.jmb.2015.06.020.

32. Perez-Leal O, Merali S. 2012. Regulation of polyamine metabolism by translational control. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.

33. Shantz LM, Pegg AE. 1999. Polyamine catabolism and disease. Biochem J 342:323–338. https://doi.org/10.1042/BJ3420323.

34. Cervelli M, Amendola R, Polticelli F, Mariotti P. 2012. Spermine oxidase: ten years after. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.

35. Miller-Lemons L, O’Linn-Sandovol V, Campbell K, Raiser M. 2015. Remaining mysteries of molecular biology: the role of polyamines in the cell. J Mol Biol 427:2980–2989. https://doi.org/10.1016/j.jmb.2015.06.020.

36. Perez-Leal O, Merali S. 2012. Regulation of polyamine metabolism by translational control. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.

37. Shantz LM, Pegg AE. 1999. Polyamine catabolism and disease. Biochem J 342:323–338. https://doi.org/10.1042/BJ3420323.

38. Cervelli M, Amendola R, Polticelli F, Mariotti P. 2012. Spermine oxidase: ten years after. Amino Acids 42:441–450. https://doi.org/10.1007/s00726-011-1014-2.
essential trypanosome polyamine biosynthetic enzyme by a catalytically
dead homolog. Proc Natl Acad Sci U S A 104:8275–8280. https://doi.org/
10.1073/pnas.0701111104.
60. Willert EK, Phillips MA. 2008. Regulated expression of an essential allo-
steric activator of polyamine biosynthesis in African trypanosomes. PLoS
Pathog 4:e1000183. https://doi.org/10.1371/journal.ppat.1000183.
61. Willert EK, Phillips MA. 2009. Cross-species activation of trypanosome
S-adenosylmethionine decarboxylase by the regulatory subunit protezome. Mol Biochem Parasitol 168:1–6. https://doi.org/10.1016/
j.molbiopara.2009.05.009.
62. Beswick TC, Willert EK, Phillips MA. 2006. Mechanisms of allosteric
regulation of Trypanosoma cruzi S-adenosylmethionine decarboxylase.
Biochemistry 45:7797–7807. https://doi.org/10.1021/bi603975.
63. da Silva MF, Floeter-Winter LM. 2014. Arginase in Leishmania. Subcell
Biochem 74:103–117. https://doi.org/10.1007/978-94-007-7305-9_4.
64. Engwerda CR, Ato M, Kaye PM. 2004. Macrophages, pathology and
parasite persistence in experimental visceral leishmaniasis. Trends Para-
tol 20:524–530. https://doi.org/10.1016/j.pt.2004.08.009.
65. Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, Zubiari S,
Engwerda CR. 2004. The immunopathology of experimental visceral
leishmaniasis. Immunol Rev 201:239–253. https://doi.org/10.1111/j.1600-
2896.2004.0018.x.
66. Mukherjee P, Ghosh AK, Ghose AC. 2003. Infection pattern and immune
response in the spleen and liver of BALB/c mice intracardially infected
with Leishmania donovani amastigotes. Immunol Lett 86:131–138.
https://doi.org/10.1016/S0165-2478(03)00021-X.
67. Rezai HR, Farrell J, Soulsby EL. 1980. Immunological responses of L.
donovani infection in mice and significance of T cell in resistance to
experimental leishmaniasis. Clin Exp Immunol 40:508–514.
68. Smelt SC, Engwerda CR, McCrossen M, Kaye PM. 1997. Destruction of
follicular dendritic cells during chronic visceral leishmaniasis. J Immunol
158:3812–3821.
69. Kropf P, Fuentes JM, Fahnrich E, Arpa L, Herath S, Weber V, Soler G,
Beswick TC, Willert EK, Phillips MA. 2006. Mechanisms of allosteric
regulation of Trypanosoma cruzi S-adenosylmethionine decarboxylase.
Biochemistry 45:7797–7807. https://doi.org/10.1021/bi603975.
70. Balana-Fouce R, Calvo-Alvarez E, Alvarez-Velilla R, Prada CF, Perez-
Pertejo Y, Reguera X. 2012. Role of trypanosomatid’s arginase in
promastigote viability and has an unusual tetrameric structure that
mediates the switch from proliferation to survival in Leishmania promastigotes. J Protozool 59:717–724.
https://doi.org/10.1111/j.1948-7035.2012.00341.x.
71. Robinson KA, Beverley SM. 2003. Improvements in transfection efficiency
and tests of RNA interference (RNAi) approaches in the protozoan
parasite Leishmania. Mol Biochem Parasitol 128:217–228. https://
doi.org/10.1016/S0166-6851(03)00079-3.
72. Soyas R, Tran KD, Ullman B, Yates PA. 2016. Integrating ribosomal
promoter vectors that offer a choice of constitutive expression profiles
in Leishmania donovani. Mol Biochem Parasitol 204:89–92.
73. Boitz JM, Ullman B. 2006. A conditional mutant deficient in
steric activator of polyamine biosynthesis in African trypanosomes. PLoS
Pathog 2:e20. https://doi.org/10.1371/journal.ppat.002020.
74. Debrabant A, Joshi MB, Pimenta PF, Dwyer DM. 2004. Generation of
Leishmania donovani axenic amastigotes: their growth and biological
characteristics. Int J Parasitol 34:205–217. https://doi.org/10.1016/
j.ijpara.2003.10.011.
75. Iovannisci DM, Ullman B. 1983. High efficiency plating method for
Leishmania promastigotes in semidefined or completely-defined me-
dium. J Parasitol 69:633–636. https://doi.org/10.2307/3281131.
76. Kaur K, Emmett K, McCann PP, Sjoerdsma A, Ullman B. 1986. Effects
of DL-alpha-difluoromethylornithine on Leishmania donovani promastigotes.
J Protozool 33:518–521. https://doi.org/10.1111/j.1550-
7408.1986.tb05654.x.
77. Cruz A, Coburn CM, Beverley SM. 1991. Double targeted gene replace-
ment for creating null mutants. Proc Natl Acad Sci U S A 88:7170–7174.
https://doi.org/10.1073/pnas.88.16.7170.
78. Boitz JM, Beverley SM. 1990. Gene replacement in parasitic protozoa.
Nature 348:171–173. https://doi.org/10.1038/348171a0.
79. Engwerda CR. 2004. The immunopathology of experimental visceral
leishmaniasis. Immunol Rev 201:239–253. https://doi.org/10.1111/j.1600-
2896.2004.0018.x.