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Heme Uptake Mediated by LHR1 Is Essential for *Leishmania amazonensis* Virulence

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The protozoan parasite *Leishmania amazonensis* is a heme auxotroph and must acquire this essential factor from the environment. Previous studies showed that *L. amazonensis* incorporates heme through the transmembrane protein LHR1 (*Leishmania Heme Response* 1). *LHR1*-null promastigotes were not viable, suggesting that the transporter is essential for survival. Here, we compared the growth, differentiation, and infectivity for macrophages and mice of wild-type, *LHR1*-single-knockout (*LHR1/Δlhr1*), and *LHR1*-complemented (*LHR1/Δlhr1* plus *LHR1*) *L. amazonensis* strains. *LHR1/Δlhr1* promastigotes replicated poorly in heme-deficient media and had lower intracellular heme content than wild-type parasites. *LHR1/Δlhr1* promastigotes were also less effective in reducing ferric iron to ferrous iron, a reaction mediated by the heme-containing parasite enzyme LFR1 (*Leishmania Ferric Reductase* 1). *LHR1/Δlhr1* parasites differentiated normally into aflagellated forms expressing amastigote-specific markers but were not able to replicate intracellularly after infecting macrophages. Importantly, the intracellular growth of *LHR1/Δlhr1* amastigotes was fully restored when macrophages were allowed to phagocytose red blood cells prior to infection. *LHR1/Δlhr1* parasites were also severely defective in the development of cutaneous lesions in mice. All phenotypes observed in *LHR1/Δlhr1 L. amazonensis* were rescued by expression of episomal *LHR1*. Our results reveal the importance of efficient heme uptake for *L. amazonensis* replication and vertebrate host infectivity, reinforcing the potential usefulness of LHR1 as a target for new antileishmanial drugs.

Leishmaniasis is a complex disease caused by infection with the protozoan parasite *Leishmania*. Clinical symptoms vary depending on the species, immune status of the host, and region of endemicity and range from cutaneous lesions (localized, mucosal, disseminated, and diffuse) to life-threatening visceral disease (1). The current treatment of leishmaniasis is mainly based on the parenteral administration of toxic and expensive drugs, such as pentavalent antimonials and amphotericin B (2).

The proliferative extracellular forms of *Leishmania*, procyclic promastigotes, replicate within the digestive tract in sand flies. A few days after infecting a sand fly, procyclic promastigotes differentiate into metacyclic promastigotes, the infective stages that are inoculated into the dermis of vertebrate hosts during the next blood meal. Once inside the human host, metacyclic promastigotes are engulfed by macrophages, where they transform into intracellular amastigotes and multiply within parasitophorous vacuoles (PVs) with properties of phagolysosomes (reviewed in references 3 and 4).

Heme is one of many indispensable nutrients that must be scavenged from the phagolysosomal compartment by *Leishmania* (5). In metazoa, heme biosynthesis occurs through 8 highly conserved chemical reactions, resulting in the insertion of ferrous iron into the protoporphyrin IX ring (6). However, several unicellular organisms, such as bacteria, trypanosomatids (*Leishmania* and *Trypanosoma*), and anaerobic protozoan parasites (*Giardia*, *Trichomonas*, and *Entamoeba*), lack several of the enzymes necessary for a complete heme-biosynthetic pathway (7, 8). Therefore, *in vitro* cultivation of trypanosomatids requires addition of a heme source, such as hemin, hematin, or hemoglobin. Heme not only is a crucial component of cytochromes in the respiratory chain, but also functions as an essential cofactor for hemoproteins, such as the ones involved in the biosynthesis of polyunsaturated fatty acids and sterols (8–10).

Earlier studies showed that *Leishmania donovani* promastigotes can internalize and target hemoglobin for degradation through a surface hemoglobin-binding protein (11). Heme uptake was also shown to act as a source of iron in *L. infantum* (12). However, until recently, the molecular mechanism responsible for direct heme uptake by *Leishmania* was not understood. Our group has recently clarified this issue by identifying and characterizing LHR1 (*Leishmania Heme Response* 1), a surface protein with 4 transmembrane domains that directly promotes heme uptake when expressed in *Saccharomyces cerevisiae*. *LHR1* transcripts are increased when promastigotes are cultured in the absence of heme (13), and deletion of one *LHR1* allele (the *LHR1/Δlhr1* mutant strain) significantly reduces intracellular heme pools and uptake of zinc mesoporphyrin (ZnMP). Despite several attempts to obtain an *LHR1*-null mutant by replacement of the second *LHR1* allele, it was not possible to recover viable parasites. These results suggested that *LHR1* is essential for viability in *Leishmania amazonensis* (13).

In the present work, we characterized the role of LHR1 by analyzing the biological properties of the *LHR1/Δlhr1* single-knockout strain, such as replication, differentiation, and *in vitro* and *in vivo* infectivity. Our results show that parasites lacking one copy of *LHR1* are more sensitive to heme deficiency as promastigotes and incapable of replicating as amastigotes in macrophages, an effect that is fully rescued when the endosomal pathway of macrophages is loaded with red blood cells, a rich source of heme.

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As expected, this intracellular-growth defect led to strong inhibition of lesion development in mice. Our data demonstrate for the first time that heme acquisition plays a critical role in the establishment of *L. amazonensis* infections in the mammalian host.

MATERIALS AND METHODS

Parasite cultivation. *L. amazonensis* (IFLA/BR/67/PH8) promastigotes were cultivated at 26°C in medium 199 (Gibco, Invitrogen) supplemented with 5% penicillin/streptomycin, 0.1% hemin (25 mg/ml in 50% triethanolamine), 20% heat-inactivated fetal bovine serum (FBS), 10 mM adenine (pH 7.5), and 5 mM L-glutamine. Heme-depleted medium was prepared by treating heat-inactivated FBS with 10 mM ascorbic acid for 4 h, followed by extensive dialysis in phosphate-buffered saline (PBS). Heme depletion was verified by measuring the optical absorbance at 405 nm (14). Aliquots were sterilized through a 0.2-μm filter and stored at −20°C. The single-knockout (*LHR1*/Δ*lhr1*) strain used in this study was obtained by the replacement of the region containing the *LHR1* gene with the hygromycin B phosphotransferase (HYG) gene (13). Parasites were maintained in the presence of hygromycin B (100 μg/ml). The *LHR1*-complemented (*LHR1*/Δ*lhr1* plus *LHR1*) strain was obtained after transfection of *LHR1*/Δ*lhr1* with the *LHR1*-3×FLAG episomal expression plasmid (13) and was grown in the presence of hygromycin B (100 μg/ml) and nourseothricin (100 μg/ml). Axenic amastigotes were obtained by mixing 5-day cultures (~2×10^6 to 3×10^6/ml) with equal volumes of acidic amastigote medium (M199 supplemented with 0.25% glucose, 0.5% Trypticase, and 40 mM Na succinate, pH 4.5). Culture flasks were kept for 16 h at 26°C and then transferred to a 32°C incubator for 3 days. After this period, the cultures were split at a 1:5 ratio. Axenically transformed amastigotes were kept for up to 5 days at 32°C.

Measurement of the intracellular heme concentration. The intracellular heme concentration was determined using the pyridine hemochrome method (15). Promastigotes cultivated in regular or heme-depleted M199 were collected by centrifugation, counted, washed twice with PBS, and resuspended in 1 ml of 1 mM Tris-HCl, pH 8.0. The parasites were sonicated for 2 min using a Branson digital sonifier at a 30% power setting in pulses of 5 s intercalated with 5 s of cooling. Aliquots of 840 μl were transferred to 13- by 100-mm glass tubes containing 100 μl of 1 N NaOH and vortexed for 20 s following addition of 200 μl of pyridine solution (Sigma-Aldrich) to each sample. The samples were vortexed and then transferred to a 1-ml cuvette, and a baseline absorbance spectrum was obtained on a DU 700 Series Beckman Coulter spectrophotometer. Sodium dithionite crystals (2 mg) were added to each sample, and the reduced hemochrome absorbance spectrum between 500 and 600 nm was acquired after 1 min. Heme concentrations were calculated based on the millimolar extinction coefficient of 20.7 for the difference in absorption between the spectrum peak at 557 nm and the valley at 541 nm.

Ferric reductase activity assay. Reduction of extracellular ferric iron to ferrous iron by the parasites was measured with the cell-impermeable compound potassium hexacyanoferrate [$K_3Fe(CN)_6$] as previously described (16). In brief, promastigotes from log phase were washed twice in PBS, resuspended at 4×10^6 parasites/ml in regular M199 without hemin, and incubated for 15 h at 26°C for promastigotes. The promastigotes were harvested by centrifugation and washed twice in PBS, and approximately 1×10^8 parasites were resuspended in 1 ml of Hanks balanced salt solution (HBSS) (Gibco, Invitrogen) containing 1 mM $K_3Fe(CN)_6$. Reduction of the ferric to the ferrous form of $K_3Fe(CN)_6$ was monitored by the absorbance change at 420 nm. For each time point, 50-μl samples were centrifuged at 10,000 × *g* for 5 min and read on a SpectraMax 5e plate reader (Molecular Devices). A standard curve of $K_3Fe(CN)_6$ was used to convert readings of the optical density at 420 nm (OD_{420}) into millimolar $K_3Fe(CN)_6$. Three independent assays were performed, and the data represent means ± standard errors.

Quantitative real-time PCR. Total RNA was isolated from cultures of 1×10^8 axenic *L. amazonensis* amastigotes using a Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. An on-column DNase digestion step was performed using an RNase-Free DNase Set (Qiagen) to ensure removal of any contaminating DNA. Synthesis of cDNA was performed using 1 μg total RNA and qScript cDNA Supermix (Quanta Biosciences). The levels of specific mRNA transcripts in individual samples were quantified using 2 μl of each cDNA sample for amplification with gene-specific primers in PerfeCTa SYBR green FastMix (Quanta Biosciences) according to the manufacturer's protocol, using a C1000 thermocycler fitted with a CFX96 real-time system (Bio-Rad Laboratories). The primer sequences used were as follows: *Amastin-like 1* LmxM.08.0760 forward, 5′-AGGTGTGATGTGCTGAACGACGAT-3′, and reverse, 5′-ACGGGAGCATCAGGAAGATGATGT-3′; *Amastin-like 2* LmxM.08.0770 forward, 5′-CATCTTCGTGTACGGCTTTGCGTT-3′, and reverse, 5′-TTCGGTAAGTCACCACCATGAGCA-3′; and *P27* LmxM.28.0980 forward, 5′-TTACCGCTTCCGCTATAACGTGCT-3′, and reverse, 5′-ACCACACCGTGTAGATCACGACAA-3′. Three technical and three biological replicates of each reaction were performed, and gene expression was normalized to the mRNAs encoding ubiquitin hydrolase (*UbH* LmxM.08_29.2300) as described previously (17).

Field emission scanning electron microscopy. Axenically transformed amastigotes (1×10^6/ml) were washed twice with PBS and fixed in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 24 h at 4°C. Cells were allowed to adhere to poly-L-lysine-coated coverslips. The attached cells were osmicated, dehydrated in ethanol series, critical point dried from liquid CO_2, and coated with gold in a Balzers gold sputtering system. The cells were observed under an Amray 1820D scanning electron microscope operating at 20.0 kV.

Bone marrow macrophage infection and red blood cell oxidation. C57BL/6 mouse bone marrow macrophages (BMM) were obtained as previously described (18). Approximately 4×10^5 BMM were plated on glass coverslips in RPMI medium (Gibco, Invitrogen) supplemented with 20% endotoxin-free FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 50 ng/ml human macrophage colony-stimulating factor (M-CSF) (PeproTech) and incubated for 24 h at 37°C, 5% CO_2. Red blood cells (RBC) were purified from human blood collected by finger prick using heparinized capillaries (Ram Scientific Inc.). The RBC fraction was washed 3 times in PBS, and RBC were oxidized by 4% hematocrit treatment using 5 mM ascorbate and 0.4 mM $CuSO_4$ at 37°C for 90 min (19). Then, the RBC were washed 3 times with PBS containing 0.2% EDTA to remove the Cu^{2+}. This step was followed by 3 washes with PBS. RBC were counted using a hemocytometer and stored at 4°C for up to 2 days. Infections were performed in BMM preexposed or not to 200 RBC per BMM for 2 h at 37°C, 5% CO_2. After washing the cells with PBS, axenic amastigotes were added at a ratio of 5 parasites per BMM for 1 h at 34°C. The cells were washed 3 times with PBS and incubated for 1, 24, 48, and 72 h. Coverslips were then fixed in 4% paraformaldehyde, and BMM and *Leishmania* DNA were stained with 10 mg/ml DAPI (4′,6-diamidino-2-phenylindole) for 1 h, after permeabilization with 0.1% Triton X-100 for 10 min. The number of intracellular parasites was determined by counting the total macrophages and the total intracellular parasites per microscopic field (Nikon E200 epifluorescence microscope). Counts were performed in triplicate for each period of infection.

Immunofluorescence. Infected cells fixed with 4% paraformaldehyde for 10 min were washed with ice-cold PBS 3 times and quenched with 15 mM NH_4Cl for 20 min. Coverslips were washed 3 times with PBS, incubated in blocking solution (3% bovine serum albumin [BSA] in PBS) for 1 h, and washed 3 times with PBS. Cells were permeabilized using 0.15% saponin-PBS for 15 min and then incubated with rat anti-mouse Lamp-1 monoclonal antibody (MAb) (Developmental Studies Hybridoma Bank) diluted in PBS-1% BSA-0.15% saponin for 1 h. After that period, the coverslips were incubated with anti-rat IgG Alexa Fluor 488 for 1 h. For parasite staining, coverslips were permeabilized with 0.1% Triton X-100 for 10 min and incubated with mouse polyclonal antibodies prepared against axenic amastigotes of *L. amazonensis* (20), followed by anti-mouse IgG Texas Red. All samples were incubated with 10 mg/ml DAPI for nuclear staining. Coverslips were mounted with ProLong

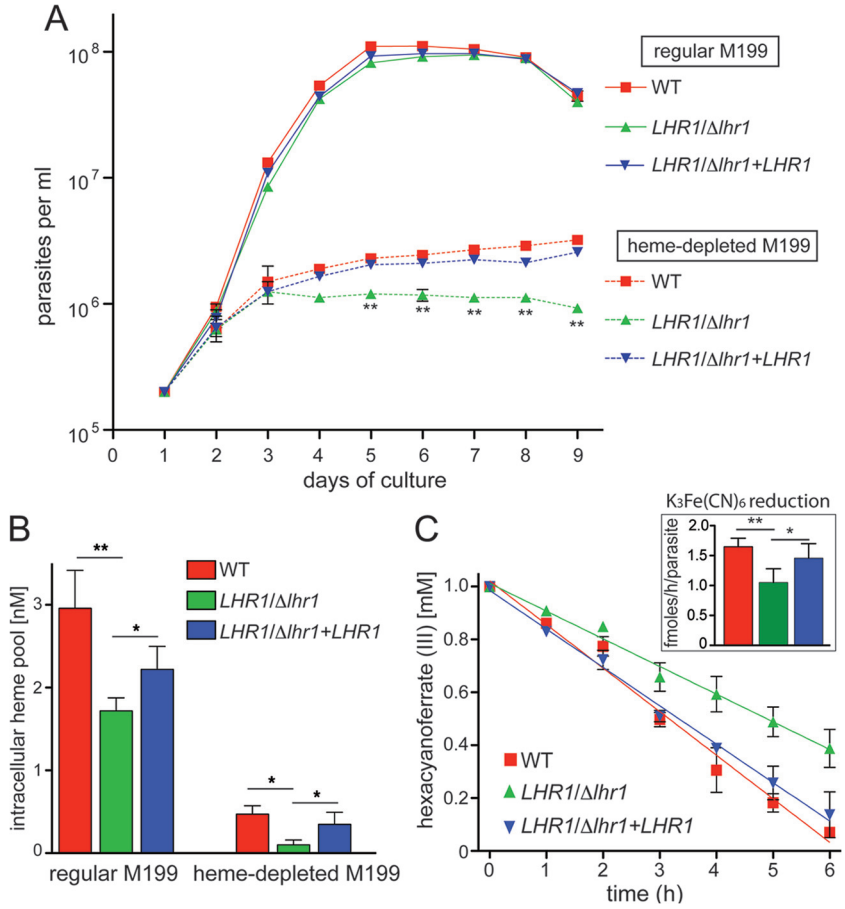

FIG 1 *LHR1/Δlhr1 L. amazonensis* promastigotes are more sensitive to heme deficiency, a phenotype that is reversed by gene complementation. (A) Growth curves obtained after cultivation of WT, *LHR1/Δlhr1*, and *LHR1/Δlhr1* plus *LHR1* promastigotes in regular (solid lines) or heme-depleted (dashed lines) M199 medium. The parasites were maintained in the presence of 100 μg/ml hygromycin B (*LHR1/Δlhr1*) or 100 μg/ml hygromycin B and 50 μg/ml nourseothricin (*LHR1/Δlhr1* plus *LHR1*). Each time point represents the mean of triplicates ± standard deviations (SD). **, $P \leq 0.006$, *LHR1/Δlhr1* versus WT (Student's *t* test). (B) Intracellular heme pool in promastigotes after 9 days of cultivation in regular or heme-depleted M199 medium. The values correspond to the means of triplicates plus SD. Regular M199: **, $P = 0.008$, *LHR1/Δlhr1* versus WT; *, $P = 0.04$, *LHR1/Δlhr1* versus *LHR1/Δlhr1* plus *LHR1*. Heme-depleted M199: *, $P = 0.02$, *LHR1/Δlhr1* versus WT; *, $P = 0.03$, *LHR1/Δlhr1* versus *LHR1/Δlhr1* plus *LHR1*. (Student's *t* test.) (C) Ferric iron reductase activity. Mid-log-phase promastigotes were transferred to M199 lacking hemin for 15 h, and ferric reductase activity was monitored for 6 h. (Inset) Ferric reductase activity expressed as the potassium hexacyanoferrate concentration/hour/parasite. The values represent the means ± standard errors of the mean (SEM) of three independent experiments. **, $P = 0.009$, *LHR1/Δlhr1* versus WT; *, $P = 0.05$, *LHR1/Δlhr1* versus *LHR1/Δlhr1* plus *LHR1* (Student's *t* test).

Gold antifade reagent (Invitrogen). Secondary antibodies were purchased from Molecular Probes. Images were acquired through a Leica SPX5 confocal microscope using a 63×/1.4 objective and processed using Volocity Suite (PerkinElmer).

Mouse infection assay. Six-week-old female C57BL/6 mice ($n = 5$ per group) were inoculated with 1×10^6 axenic amastigotes of wild-type (WT), *LHR1/Δlhr1*, or *LHR1/Δlhr1* plus *LHR1 L. amazonensis* in the left hind footpad in a volume of 50 μl PBS. Lesion progression was monitored once a week by measuring the left and right hind footpads with a caliper (Mitutoyo Corp., Japan). The parasite burden recovered from the infected tissue was determined after 9 weeks by limiting dilution (21). The Institutional Animal Care and Use Committee at the University of Maryland approved the animal study protocol.

Statistical analysis. Data were analyzed by two-tailed Student's *t* test using GraphPad Prism software. A result was considered significant at a *P* value of <0.05.

RESULTS AND DISCUSSION

Heme uptake-deficient promastigotes are more sensitive to heme deficiency. Prior studies showed that cultivation of *Leishmania amazonensis*, *L. donovani*, and *Leishmania tarentolae* promastigotes require the addition of hemin (a heme source) or hemoglobin-containing fetal bovine serum to the culture medium (22–24). We found that WT, *LHR1* single-knockout (*LHR1/Δlhr1*), and *LHR1*-complemented (*LHR1/Δlhr1* plus *LHR1*) promastigotes replicated and reached stationary phase at very similar rates in regular medium 199 (Fig. 1A) (13). However, when comparing the growth curves of these strains in promastigote medium without hemin and containing 20% heme-depleted FBS (heme-depleted M199), we observed that the cultures of *LHR1/Δlhr1* promastigotes reached stationary phase at significantly lower densities than cultures of the WT and *LHR1/Δlhr1* plus *LHR1* strains (Fig. 1A) ($P \leq 0.006$).

To verify whether the growth defect of *LHR1/Δlhr1* promastigotes was related to a smaller accumulation of intracellular heme, we quantified the total intracellular heme pool for each strain after 9 days of culture, using the pyridine hemochrome method (Fig. 1B). *LHR1/Δlhr1* promastigotes harvested from heme-replete reg-

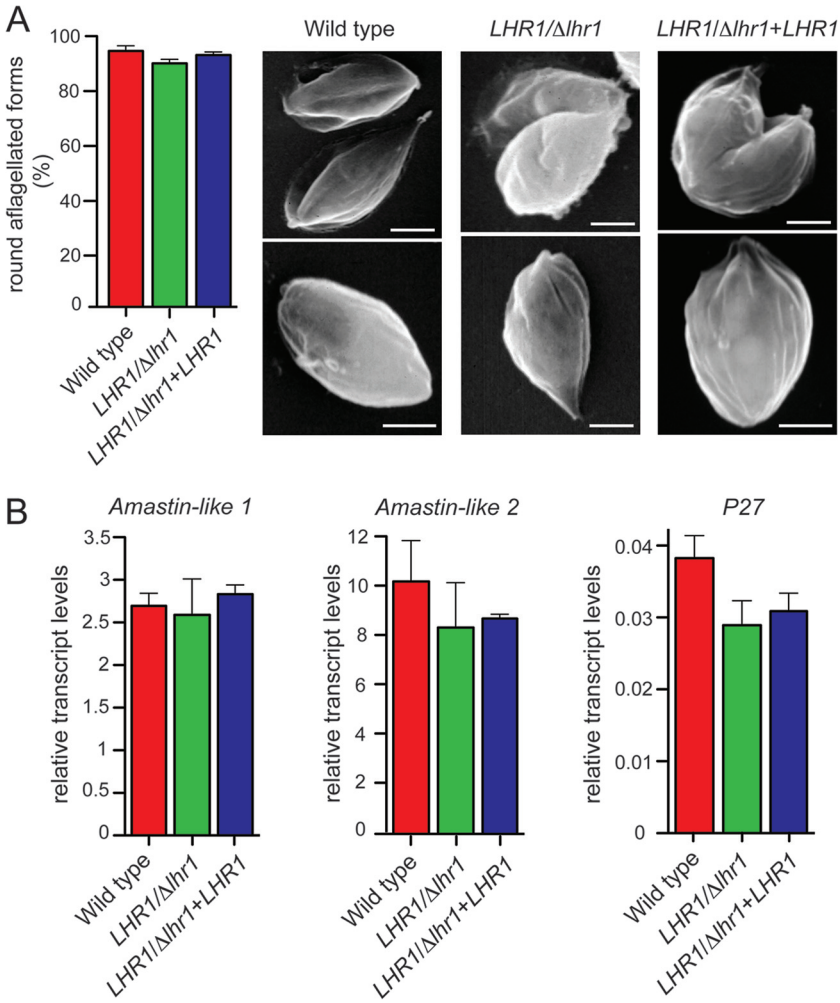

FIG 2 *LHR1* deficiency does not affect the generation of axenic amastigotes. Amastigotes of *L. amazonensis* were obtained axenically (see Materials and Methods) and cultivated for 5 days. (A) The percentage of round aflagellated forms was determined microscopically, and the morphology was analyzed by scanning electron microscopy. The values represent the means plus SD of triplicate determinations. Scale bar = 1.0 μm. (B) Transcripts of *Amastin-like 1* and *2* (LmxM.08.0760 and LmxM.08.0770) and *P27* (LmxM.28.0980) were analyzed by quantitative PCR (qPCR) and normalized to the levels of the ubiquitin hydrolase mRNA. The values represent the means and SD of triplicate determinations and are representative of three independent experiments. There were no significant differences between the *LHR1*/Δ*lhr1* versus WT or *LHR1*/Δ*lhr1* versus *LHR1*/Δ*lhr1* plus *LHR1* strains ($P > 0.05$; Student's *t* test).

ular medium showed a significant decrease in intracellular heme content compared to WT (~50% reduction) and *LHR1*/Δ*lhr1* plus *LHR1* (~25% reduction) parasites. Cultivation in heme-depleted M199 led to markedly reduced levels of intracellular heme in all strains compared to regular medium, but the levels of heme detected were significantly lower in *LHR1*/Δ*lhr1* parasites (Fig. 1B). This result emphasizes the importance of heme uptake and maintenance of an intracellular pool for the optimal *in vitro* growth of *L. amazonensis* promastigotes. The small intracellular heme pools resulting from the reduced heme availability in heme-depleted M199 (Fig. 2B) are likely responsible for the decreased growth rates of WT and *LHR1*/Δ*lhr1* plus *LHR1* strains compared to regular M199 (Fig. 1A). However, both strains were still able to reach higher densities than the *LHR1*/Δ*lhr1* single knockout (Fig. 1C), indicating that high levels of LHR1 expression allow the parasites to acquire sufficient heme from the trace amounts remaining in heme-depleted culture medium.

L. amazonensis expresses a NADPH-dependent ferric reductase, LFR1 (16). Since metalloreductases are heme-dependent enzymes (25), we measured spectrophotometrically the plasma membrane ferric reductase activity of *L. amazonensis* promastigotes as a reporter for heme availability within the parasites. Reductase activity was monitored after promastigote incubation in heme-depleted M199 for 15 h (Fig. 1C). The enzymatic activities detected for WT, *LHR1*/Δ*lhr1*, and *LHR1*/Δ*lhr1* plus *LHR1* promastigotes were 1.65 ± 0.14, 1.05 ± 0.23, and 1.46 ± 0.24 fmol of hexacyanoferrate/hour/parasite, respectively ($P = 0.009$; *LHR1*/Δ*lhr1* versus WT) (Fig. 1C, inset). These results independently demonstrate that the lack of one copy of the heme transporter LHR1 in the *LHR1*/Δ*lhr1* single-knockout strain reduces the availability of intracellular heme that is necessary for assembly of active hemoproteins. Notably, *LHR1* add-back complemented the *LHR1*/Δ*lhr1* single-knockout strain, restoring the ferric reductase activity to levels similar to those seen in WT parasites (Fig. 1C). This result emphasizes the interplay between heme and iron uptake in *L. amazonensis* that is required for parasite infectivity, since

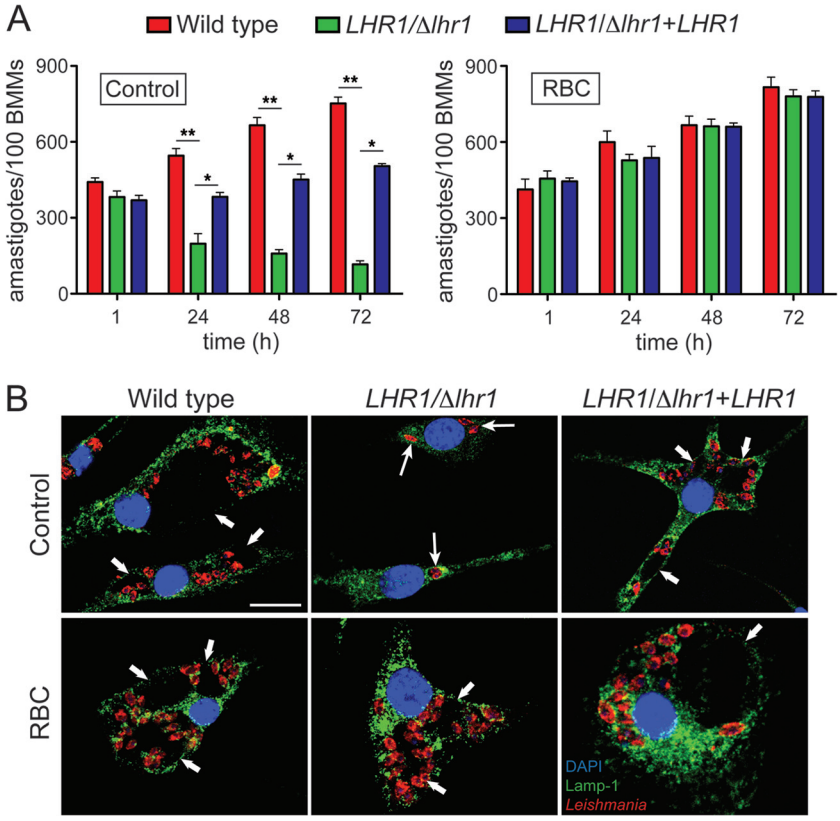

FIG 3 *LHR1*/Δ*lhr1* amastigotes are unable to proliferate in BMM. (A) BMM left untreated (Control) or preincubated with red blood cells (RBC) were infected with *L. amazonensis* axenic amastigotes (multiplicity of infection [MOI] = 5) for 1, 24, 48, and 72 h, followed by quantification of intracellular parasites. Infections using WT, *LHR1*/Δ*lhr1*, and *LHR1*/Δ*lhr1* plus *LHR1* amastigotes are shown. The values represent the means and SD of triplicates and are representative of three independent experiments. **, $P < 0.001$; *, $P < 0.01$ (Student's *t* test). (B) Immunofluorescence confocal images of BMM infected for 72 h (top row, control infection; bottom row, BMM preexposed to RBC and then infected with *L. amazonensis*). Red, anti-*Leishmania* antibodies; blue, DAPI-stained nuclei; green, lysosomes and PV membranes stained with anti-Lamp-1. The short arrows point to expanded PVs containing replicating parasites; the long arrows indicate single *LHR1*/Δ*lhr1* amastigotes in small PVs. Scale bar = 8 μm.

LFR1 is required for generating ferrous iron, the substrate for the iron transporter LIT1, which is required for intracellular replication (16).

Differentiation into infective stages is not affected by deletion of one copy of the *LHR1* gene. To test whether partial deletion of *LHR1* interferes with *Leishmania* differentiation into infective stages, we investigated the generation of metacyclic promastigotes by recovering metacyclic forms from stationary-phase cultures using the specific antibody m3A.1. This monoclonal antibody specifically agglutinates *L. amazonensis* promastigotes, yielding purified metacyclic forms in the supernatant (26). Wild-type, *LHR1*/Δ*lhr1*, and *LHR1*/Δ*lhr1* plus *LHR1* strains generated similar percentages of purified metacyclics after 6 days in culture (data not shown).

The transformation of promastigotes into amastigotes was also examined using the axenic amastigote differentiation protocol (see Materials and Methods). The percentages of round aflagellated forms for each strain were determined microscopically, and no differences were observed between wild-type, *LHR1*/Δ*lhr1*, and *LHR1*/Δ*lhr1* plus *LHR1* strains (Fig. 2A), suggesting that transformation into axenic amastigotes is also not affected by the partial *LHR1* deficiency. This conclusion was reinforced by scanning electron microscopy examinations of parasite morphology (Fig. 2A, right) and quantification of mRNA levels of three proteins expressed specifically by *L. amazonensis* amastigotes: Amastin-like 1, Amastin-like 2, and P27 (17) (Fig. 2B).

Collectively, our findings indicate that *LHR1*/Δ*lhr1* parasites are able to transform into infective amastigote stages at the same level observed for WT and add-back strains. The axenically transformed amastigotes from all strains were viable, as indicated by their redifferentiation into promastigote stages when cultivated in regular M199 at 26°C for up to 7 days (data not shown).

LHR1 single-knockout *L. amazonensis* is defective in intracellular replication in macrophages. Next, we investigated whether deletion of one *LHR1* copy affected the parasites' ability to infect and proliferate in BMM. BMM infections were performed with axenic amastigotes of each strain, and the numbers of intracellular parasites were determined after 1, 24, 48, and 72 h. As expected, wild-type amastigotes survived intracellularly and increased in numbers over time. In sharp contrast, *LHR1*/Δ*lhr1* parasites failed to replicate in BMM, and their numbers gradually decreased, indicating intracellular death. Complementation with *LHR1* in the *LHR1*/Δ*lhr1* plus *LHR1* strain fully rescued intracellular survival and partially restored the parasites' ability to replicate in BMM (Fig. 3A, Control).

Because *LHR1*-single knockout parasites contain smaller amounts of intracellular heme (13), we investigated whether delivering an excess of exogenous hemoglobin to BMM phagolyso-

somal compartments was able to rescue their intracellular growth defect. Prior studies demonstrated that phagosomes containing phagocytosed particles merge very efficiently with *L. amazonensis* PVs (27). To this end, BMM were allowed to phagocytose oxidized RBC and were subsequently infected with axenic amastigotes. Quantification of intracellular parasites over time showed that phagocytosed RBC entirely rescued the growth defect of *LHR1*/Δ*lhr1* parasites, which replicated in BMM at the same levels observed with the wild-type and *LHR1*/Δ*lhr1* plus *LHR1* strains (Fig. 3A, RBC).

Immunofluorescence experiments revealed that *L. amazonensis* PVs in BMM infected with the *LHR1* single-knockout strain were small and contained single parasites after 72 h. This phenotype was fully reversed in BMM preloaded with RBC, which showed expanded Lamp-1-positive PVs containing multiple replicating parasites, as seen for infections with wild-type and *LHR1*/Δ*lhr1* plus *LHR1* strains (Fig. 3B). These results suggest that the heme-enriched environment generated within *L. amazonensis* PVs through the phagocytosis of oxidized RBC is sufficient to sustain the growth of heme uptake-deficient parasites, possibly through alternative low-affinity mechanisms of uptake. This finding has important implications for the pathogenesis of *Leishmania* infections, since it indicates that the vigorous process of erythrophagocytosis characteristic of macrophages can confer a survival advantage on these parasites. Billions of senescent RBC are cleared daily from circulation by macrophages from the reticuloendothelial system and degraded in phagolysosomes, where breakdown of hemoglobin and cytosolic release of heme occur (28, 29). Free heme within the phagolysosome is then translocated into the macrophage cytosol by the heme transporter HRG1 (*H*eme-*R*esponsive *G*ene 1) (19). The presence of HRG1 on the phagolysosome membrane emphasizes the need for LHR1, the *Leishmania*-encoded heme transporter, to allow parasites replicating within PVs to successfully compete with the host for heme. This intracellular competition for the available heme raises intriguing questions, such as the possibility that mutations interfering with HRG1 function (19) may affect host susceptibility to *Leishmania* infections.

The intracellular-growth defect of *LHR1*/Δ*lhr1* parasites is in agreement with a study that described the inability of *L. amazonensis* axenic amastigotes to proliferate in iron-depleted medium, an effect that is reversed after addition of hemoglobin (12). The typhoid fever agent *Salmonella enterica* exploits the hemophagocytic ability of BMM to survive during chronic *in vivo* infections (30), and heme was also shown to function as a source of iron for additional bacterial pathogens residing within macrophage phagosomes, such as *Mycobacterium haemophilum* and *Listeria monocytogenes* (31, 32).

LHR1 single-knockout *L. amazonensis* is defective in cutaneous-lesion formation in mice. To determine whether deletion of one *LHR1* copy affected the *in vivo* infectivity of *L. amazonensis*, axenic amastigotes were injected into the left hind footpads of C57BL/6 mice, and the development of cutaneous lesions was followed for 9 weeks. Unlike the wild-type strain (which caused lesions of 2.7 to 3.3 mm on average during this period), *LHR1*/Δ*lhr1* parasites were not able to induce detectable cutaneous lesions. Importantly, lesion development was restored in the complemented *LHR1*/Δ*lhr1* plus *LHR1* strain, albeit at a lower level than was observed with wild-type parasites (Fig. 4A). This was not unexpected, since a lack of robust complementation has been frequently observed in transgenic *Leishmania* (33–35). Nine weeks

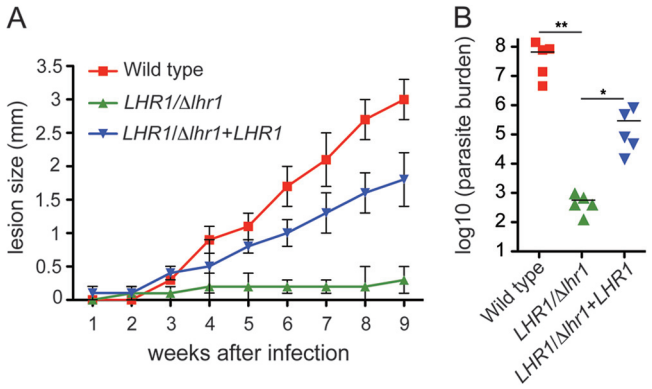

FIG 4 *LHR1*/Δ*lhr1* amastigotes are defective in cutaneous-lesion development, a phenotype reversed by gene complementation. C57BL/6 female mice were inoculated with 1×10^6 amastigotes of WT, *LHR1*/Δ*lhr1*, or *LHR1*/Δ*lhr1* plus *LHR1 L. amazonensis* in the left hind footpad. (A) Lesion progression was recorded weekly. The values represent the means ± SD of 5 mice. (B) The parasite load in the footpad tissues was quantified 9 weeks after infection ($n = 5$). **, $P = 0.007$; *, $P = 0.011$ (Student's *t* test).

after infection, the mice were sacrificed, and footpad tissue was collected for parasite quantification by limiting dilution. The parasite burden in animals infected with *LHR1*/Δ*lhr1* parasites was lower than that observed for mice infected with the wild type and the complemented strain (Fig. 4B). Thus, loss of only one *LHR1* copy is sufficient to abolish infectivity in *L. amazonensis*, indicating that a threshold level of the protein must be maintained to allow the parasites to scavenge sufficient heme while replicating within macrophage PVs.

Conclusion. In this study, we characterized in detail the biological properties of a strain of *L. amazonensis* lacking one copy of *LHR1*, which encodes the first heme transporter to be identified in trypanosomatid parasites. We found that a full dose of the protein is required for promastigote replication under heme deprivation and for assembly of active ferric iron reductase, a heme-containing protein that is required for *L. amazonensis* infectivity (16). Importantly, we also found that loss of only one *LHR1* copy renders the parasites incapable of replicating in macrophages and avirulent for mice. Several attempts to delete the second *LHR1* allele were unsuccessful, indicating that LHR1 is essential for *L. amazonensis* viability and a possible useful target for the development of novel antileishmanial drugs.

Remarkably, our results showed that delivering hemoglobin to the macrophage PV in the form of phagocytosed RBC fully restores intracellular growth of the single-knockout strain. This finding is particularly relevant for our understanding of infections caused by the visceralizing species of *Leishmania*, which cause very severe disease by replicating in spleen, liver, and bone marrow macrophages (36). These cells are actively engaged in the recycling of iron through the phagocytosis and degradation of senescent RBC and therefore have facilitated access to heme (37, 38). Human patients infected with *L. amazonensis* (39) present a broad spectrum of clinical disease, suggesting that conditions within the host, which may include variations in the interplay between parasite and host heme transporters, can influence the course of the disease. In future studies, it will be important to determine the role of LHR1-mediated heme acquisition in additional species of *Leishmania* and whether potential functional differences in the

transporter will represent an opportunity to investigate its role as a determinant of disease severity in leishmaniasis.

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