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MECHANISMS OF RESISTANCE

Lipase Precursor-Like Protein Promotes Miltefosine Tolerance in *Leishmania donovani* by Enhancing Parasite Infectivity and Eliciting Anti-inflammatory Responses in Host Macrophages

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ABSTRACT The oral drug miltefosine (MIL) was introduced in the Indian subcontinent in the year 2002 for the treatment of visceral leishmaniasis (VL). However, recent reports on its declining efficacy and increasing relapse rates pose a serious concern. An understanding of the factors contributing to MIL tolerance in *Leishmania* parasites is critical. In the present study, we assessed the role of the lipase precursor-like protein (Lip) in conferring tolerance to miltefosine by episomally overexpressing Lip in *Leishmania donovani* (LdLip^{++}). We observed a significant increase (~3-fold) in the MIL 50% inhibitory concentration (IC_{50}) at both the promastigote (3.90 ± 0.68 μM; $P < 0.05$) and intracellular amastigote (9.10 ± 0.60 μM; $P < 0.05$) stages compared to the wild-type counterpart (LdNeo) (MIL IC_{50}s of 1.49 ± 0.20 μM at the promastigote stage and 3.95 ± 0.45 μM at the amastigote stage). LdLip^{++} parasites exhibited significantly ($P < 0.05$) increased infectivity to host macrophages and increased metacyclogenesis and tolerance to MIL-induced oxidative stress. The susceptibility of LdLip^{++} to other antileishmanial drugs (sodium antimony gluconate and amphotericin B) remained unchanged. In comparison to LdNeo, the LdLip^{++} parasites elicited high host interleukin-10 (IL-10) cytokine expression levels (1.6-fold; $P < 0.05$) with reduced expression of the cytokine tumor necrosis factor alpha (TNF-α) (1.5-fold; $P < 0.05$), leading to a significantly ($P < 0.01$) increased ratio of IL-10/TNF-α. The above-described findings suggest a role of lipase precursor-like protein in conferring tolerance to the oral antileishmanial drug MIL in *L. donovani* parasites.

KEYWORDS *Leishmania donovani*, lipase precursor, miltefosine, overexpression, parasite persistence

Miltefosine (MIL), an alkylphosphocholine, was registered as the first oral drug to treat visceral leishmaniasis (VL) in the Indian subcontinent in the year 2002 (1). Although it exhibited an initial cure rate of more than 94%, reports of its declining efficacy (2) and increasing rates of relapse in the Indian subcontinent are of great concern (3). The long half-life of the drug (150 to 200 h) may cause parasites to develop resistance in the field, as evident by the quick *in vitro* induction of resistance against MIL in *Leishmania donovani* (4). A recent study reported the existence of field isolates of VL in India that were resistant to MIL (5).

Leishmania parasites are rich in ether-lipid complexes that are found mainly in the glycosylphosphatidylinositol-anchored glycolipids and glycoproteins present on the surface of the parasites (6). MIL induces apoptosis-like cell death in *Leishmania*, and inhibition of apoptotic cell death has been documented in MIL-resistant parasites (7–9). MIL plays a role in impairment of acidocalcisome function, activation of the sphingosine-dependent plasma membrane Ca^{2+} channel, and inhibition of cytochrome *c* oxidase in *L. donovani* (10, 11). *Leishmania* undergoes metabolic reconfiguration during oxidative stress to resist reactive oxygen species (ROS) (12). Fatty acids are involved in the

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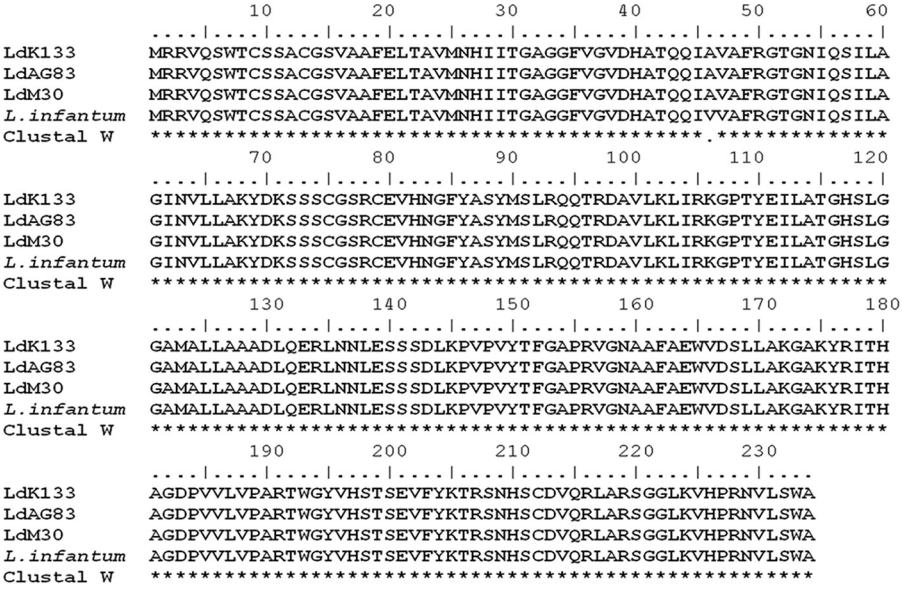

FIG 1 Clustal W amino acid sequence alignment of the lipase precursor in both wild-type *L. donovani* parasites (LdK133 and LdAG83) and *L. donovani* parasites made experimentally resistant to MIL (LdM30). There is a synonymous mutation at the 46th position in *L. donovani* in comparison with *L. infantum*.

biosynthesis of sphingolipids and ether-lipids and also serve as an important bioenergetic fuel for *Leishmania* via the beta oxidation pathway (13). Defective inward translocation of drugs due to mutations in the putative MIL transporter LdMT and its accessory protein LdRoS3 has been well explained in experimental resistant parasites (14).

A study of differential gene expression between MIL-resistant and MIL-sensitive *L. donovani* parasites revealed upregulated expression of genes associated with lipid metabolism, *viz*., lipase (LinJ.31.2540) and the lipase precursor (LinJ.31.0870), indicating the involvement of lipase-mediated free fatty acid metabolism in MIL-resistant *Leishmania* (15). Lipases are the building blocks for the synthesis of complex parasite lipids, important for membrane remodeling, and help in the acquisition of the host's resources for energy metabolism in a variety of parasitic organisms (16). MIL resistance in *Leishmania* affects lipid biochemical pathways, like fatty acid elongation, fatty acid desaturase, and C-24-alkylation of sterols. A comparative study of MIL-sensitive and -resistant *Leishmania* parasites revealed lower contents of unsaturated phospholipid alkyl chains in the MIL-resistant parasite plasma membrane, suggesting a lower fluidity of the MIL-resistant parasite membrane (17).

Here, we investigated the role of the lipase precursor (Lip) molecule in imparting increased tolerance to MIL in *L. donovani* parasites. We episomally expressed Lip in a wild-type *L. donovani* isolate and assessed the parasites overexpressing Lip (LdLip^{++}) for *in vitro* drug susceptibility, infectivity to macrophages, metacyclogenesis, tolerance to MIL-induced oxidative stress, and accumulation of MIL in the parasites. Since host immune responses are critical for jeopardizing the chemotherapeutic efficacy of antileishmanials (18), we also assessed modulation of the expression of proinflammatory and anti-inflammatory cytokines in macrophages infected with LdLip^{++} parasites.

RESULTS

Comparative sequence analysis of the lipase precursor from MIL-sensitive and MIL-resistant *L. donovani* parasites. The DNA sequence for the lipase precursor was determined for both sensitive and resistant *L. donovani* parasites. The amino acid sequence was identical and showed 99% similarity with the lipase precursor from *Leishmania infantum* (Fig. 1). A synonymous mutation was seen at 46th position, where valine was replaced by alanine.

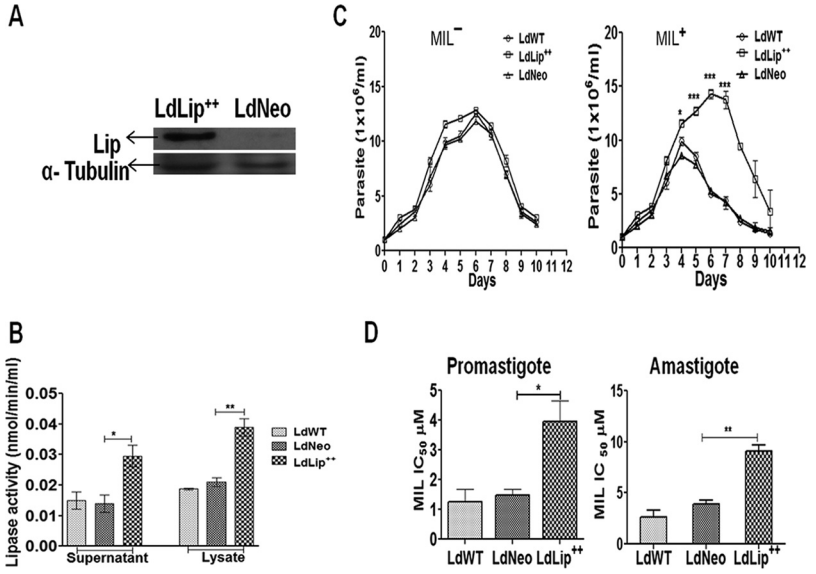

FIG 2 Western blot analysis, lipase activity, growth kinetics, and *in vitro* MIL susceptibility of LdLip^{++} parasites. (A) Whole-cell lysates from LdLip^{++} and LdNeo parasites probed with HRP-conjugated mouse anti-HA monoclonal antibody. The arrow denotes the 26-kDa LdLip^{++}::HA chimeric protein. (B) Lipase activity in culture supernatants and whole-cell lysates of *L. donovani* transfectants. Lipase activity was measured fluorometrically by using BioVision fluorometric assay kit III as described in Materials and Methods. Asterisks show levels of significance (*, $P < 0.05$; **, $P < 0.01$). (C) Growth kinetics of wild-type and transfected *L. donovani* parasites without MIL (MIL^{-}) or with MIL (MIL^{+}) pressure. Asterisks show significance (*, $P < 0.05$; ***, $P < 0.001$). (D) *In vitro* MIL susceptibility (IC_{50}) of transfected parasites in comparison with the wild type at the promastigote level following a standard resazurin assay and at the intracellular amastigote level using mouse peritoneal macrophages as described in Materials and Methods. Values represent means ± SD of data from three independent experiments. Asterisks show levels of significance (*, $P < 0.05$; **, $P < 0.01$).

LdLip^{++} parasites exhibit enhanced enzymatic activity of lipase precursor protein. Western blot results using whole-cell lysates showed that the chimeric gene construct was translated into the ~26-kDa LdLip^{++}::HA (hemagglutinin) protein in LdLip^{++} parasites (Fig. 2A). We measured the enzymatic activity of the lipase precursor-like protein in culture supernatants and cell lysates of LdLip^{++}, LdNeo, and wild-type *L. donovani* (LdWT) parasites. The activities in culture supernatants and cell lysates of LdLip^{++} parasites (0.029 mU/ml and 0.039 mU/ml) were significantly (~2-fold) higher than those of LdNeo (0.014 mU/ml and 0.021 mU/ml) or LdWT (0.015 mU/ml and 0.012 mU/ml) parasites (Fig. 2B).

LdLip^{++} parasites show enhanced proliferation in the presence of miltefosine. The growth of transfected LdLip^{++} and LdNeo parasites were monitored in comparison with LdWT parasites under MIL pressure (3 μM) at the promastigote level for a period of 10 days. In the presence of MIL, the transfected LdLip^{++} parasites showed significantly increased proliferation compared to LdNeo or LdWT parasites at day 4 ($P < 0.05$) and at days 5 to 7 ($P < 0.001$), while in the absence of MIL, the growth of LdLip^{++} parasites was comparable to that of LdNeo or LdWT parasites (Fig. 2C).

LdLip^{++} parasites display a reduction in susceptibility to miltefosine. *In vitro*, the MIL susceptibility of LdLip^{++} parasites was significantly reduced (up to 3-fold) compared to LdNeo, at both the promastigote ($P < 0.05$) and intracellular amastigote ($P < 0.01$) stages (Fig. 2D). The susceptibility of LdLip^{++} parasites to other antileishmanial drugs tested (sodium antimony gluconate [SAG] and amphotericin B [AmB]) was not significantly different in comparison with LdWT or LdNeo parasites (Table 1).

LdLip^{++} parasites do not exhibit alterations in miltefosine uptake. Liquid chromatography-mass spectrometry (LCMS) analysis revealed that levels of MIL uptake (nanograms per 10^8 promastigotes) were comparable in both LdLip^{++} (163.0 ± 2.8 ng/10^8 promastigotes) and LdWT (158.0 ± 1.4 ng/10^8 promastigotes) parasites. Overex-

TABLE 1 *In vitro* drug susceptibility of transfected parasites overexpressing the lipase precursor (LdLip^{++}) to antileishmanial drugs[a]

Isolate	Mean MIL IC_{50} (μM) ± SD		Mean AmB IC_{50} (μM) ± SD		Mean SAG IC_{50} (μg/ml) ± SD at amastigote stage
	Promastigote	Amastigote	Promastigote	Amastigote	
LdWT	1.26 ± 0.40	2.66 ± 0.67	0.78 ± 0.13	0.44 ± 0.03	4.83 ± 0.42
LdNeo	1.49 ± 0.20	3.90 ± 0.45	0.76 ± 0.16	0.42 ± 0.16	5.33 ± 0.57
LdLip^{++}	3.90 ± 0.68	9.10 ± 0.60	0.89 ± 0.11	0.49 ± 0.20	6.22 ± 0.46

[a]MIL, miltefosine; AmB, amphotericin B; SAG, sodium antimony gluconate; IC_{50}, 50% inhibitory concentration; LdWT, wild-type *L. donovani* parasites; LdNeo, mock-transfected (vector only) *L. donovani* parasites; LdLip++, transfected *L. donovani* parasites episomally expressing the lipase precursor protein.

pression of the lipase precursor protein therefore did not affect MIL uptake by *L. donovani* parasites (Fig. 3A).

Metacyclogenesis is enhanced in LdLip^{++} parasites. We observed a significantly increased percentage of the metacyclic LdLip^{++} promastigote population (38.20% ± 1.82%; $P < 0.05$) compared to LdNeo parasites (19.40% ± 1.10%), based on negative selection with peanut agglutinin (PNA) (Fig. 3B).

LdLip^{++} parasites display increased infectivity to host macrophages. LdLip^{++} parasites showed a significantly increased percent infectivity in comparison to their wild-type counterparts at both 48 h (LdLip^{++} = 76.0% ± 1.4%; LdNeo = 65.0% ± 2.8% [$P < 0.05$]) and 72 h (LdLip^{++} = 77.5% ± 2.1%; LdNeo = 63.0% ± 1.4% [$P < 0.05$]) postinfection (Fig. 3C).

LdLip^{++} parasites show increased tolerance to reactive oxygen species. The level of ROS increased with increasing MIL concentrations in LdNeo and LdWT parasites, while it remained unaltered in LdLip^{++} parasites. The ROS level was significantly lower in LdLip^{++} promastigotes at higher MIL concentrations (10 to 20 μM) than in LdNeo parasites (Fig. 4A). Similarly, we evaluated the levels of ROS without and with MIL exposure in macrophages infected with LdLip^{++}, LdNeo, or LdWT parasites. Results showed comparable levels of ROS in macrophages infected with LdLip^{++} parasites without (mean fluorescence intensity [MFI], 551.5) and with (MFI, 622.0) MIL exposure (20 μM). After MIL exposure, the level of ROS in macrophages infected with LdLip^{++}

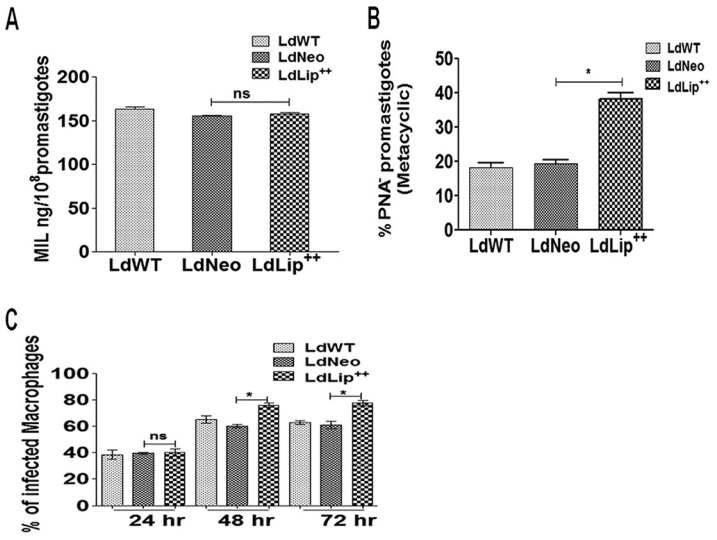

FIG 3 MIL uptake, percent metacyclogenesis, and infectivity to macrophages of transfected parasites with respect to wild-type parasites. (A) MIL uptake, estimated using LCMS, in 1×10^8 promastigotes. Data represent means ± SD of results from two independent experiments, each in triplicate. (B) Percent metacyclogenesis of the promastigote population estimated based on negative selection with peanut agglutinin (%PNA$^-$ promastigote). Values represent means ± SD of data from two independent experiments. Asterisks indicate significance (*, $P < 0.05$). (C) Mouse peritoneal macrophages infected with wild-type or transfected parasites at a 1:10 (cell/parasite) ratio. Percent infectivity was determined at 24 h, 48 h, and 72 h postinfection by counting the number of infected cells out of 100 macrophages at a ×1,000 magnification after staining with Diff-Quik. Data represent means ± SD of results from three independent experiments, each in duplicate. Asterisks indicate significance (*, $P < 0.05$). ns, not significant.

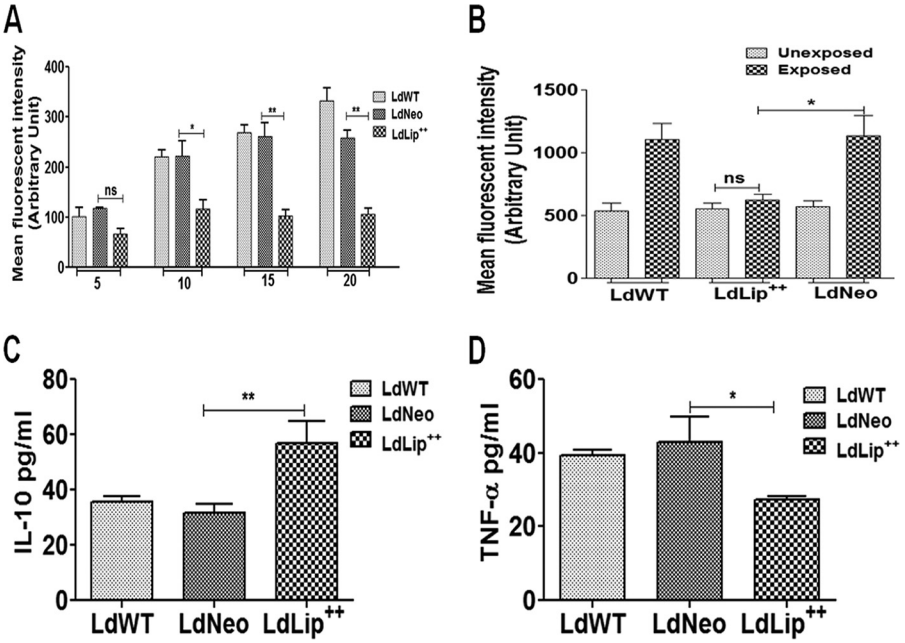

FIG 4 MIL-induced oxidative stress (ROS level) and cytokine expression in culture supernatants of infected macrophages. (A) Dose-dependent accumulation of ROS in LdWT, LdNeo, and LdLip^{++} promastigotes, measured fluorometrically at 495-nm excitation and 535-nm emission wavelengths using the cell-permeable probe H_2DCFDA (40 nM). Data represent means ± SD of results from three independent experiments, each performed in duplicate. Asterisks indicate significance (*, $P < 0.05$; **, $P < 0.01$). (B) Accumulation of ROS in macrophages infected with LdWT, LdNeo, or LdLip^{++} parasites before and after MIL exposure (20 μM), assayed fluorometrically at 495-nm excitation and 535-nm emission wavelengths using the cell-permeable probe H_2DCFDA (30 μM). Data represent means ± SD of results from three independent experiments, each in triplicate. Asterisks indicate significance (*, $P < 0.05$). (C and D) Expression of IL-10 and TNF-α in host macrophages infected with LdWT, LdNeo, or LdLip^{++} parasites. Data represent means ± SD from two separate assays, each in triplicate. Asterisks indicate significance (*, $P < 0.05$; **, $P < 0.01$).

was significantly lower than the level of ROS (MFI, 1,137.0) in macrophages infected with LdNeo parasites (Fig. 4B).

Cytokine profile of host macrophages infected with LdLip^{++}. Host macrophages prestimulated with lipopolysaccharide (LPS) (1 μg/ml) and infected with LdLip^{++} parasites showed significantly elevated expression levels of interleukin-10 (IL-10) (1.6-fold; $P < 0.05$) and reduced expression levels of tumor necrosis factor alpha (TNF-α) (1.5-fold; $P < 0.05$) compared with macrophages infected with LdNeo (Fig. 4C and D). The IL-10/TNF-α ratio in LdLip^{++} parasites (2.04) was elevated by 3.9-fold compared with that in LdNeo parasites (0.52), which may contribute to the increased persistence of LdLip^{++} parasites within host macrophages. IL-4, IL-12, and gamma interferon (IFN-γ) could not be detected, while expression levels of IL-2 were comparable in the two.

DISCUSSION

Proposed targets of MIL in *Leishmania* include disturbed ether-lipid metabolism, glycosylphosphatidylinositol anchor biosynthesis, and signal transduction as well as decreased activity of acyl transferase, an enzyme involved in lipid remodeling (6). Lipase and the lipase precursor involved in free fatty acid metabolism provide an alternate source of energy generation during stress (15). Therefore, we chose to investigate the role of the lipase precursor, which showed consistently high expression levels in all *L. donovani* isolates from cases that relapsed after MIL treatment (19). Lip encodes a putative enzyme lipase precursor that is supposedly involved in free fatty acid metabolism via the beta oxidation pathway and helps parasites to evade drug-induced oxidative stress by providing extra energy. We generated transfected *L. donovani* parasites, designated LdLip^{++}, exhibiting 2-fold-higher lipase activity and investigated LdLip^{++} parasites for various parameters, including (i) *in vitro* susceptibility to MIL, SAG,

and AmB; (ii) infectivity to macrophages; (iii) metacyclogenesis; (iv) MIL uptake; (v) tolerance to oxidative stress; and (vi) cytokine expression in LdLip^{++} parasite-infected host macrophages. Episomal expression of a gene provides an essential tool to study the phenotypic changes in parasites and to assess their fitness toward antileishmanial drugs (20). *Leishmania* parasites overexpressing histone H2A, HSP83, and P299 genes exhibited reduced susceptibility to antimony and MIL (20–22). In the present study, we observed that parasites overexpressing Lip (LdLip^{++}) showed a 3-fold reduction in MIL susceptibility compared to wild-type parasites; however, the susceptibility of LdLip^{++} to SAG and AmB remained unaltered. Possibly, lipase overexpression perturbed the membrane dynamics, leading to a reduced interaction of MIL with the ether-lipid complex, which might be responsible for reduced MIL susceptibility in LdLip^{++} parasites. We observed higher MIL 50% inhibitory concentration (IC_{50}) values at the amastigote stage than at the promastigote stage. Higher or similar MIL IC_{50} values at the amastigote stage compared to those at the promastigote stage have been observed in previous studies (23–25). A strong correlation ($r = 0.70$; $P = 0.0018$) between MIL susceptibilities of amastigotes and promastigotes was observed (24). Additionally, we have used mouse peritoneal macrophages (PEMs) for *in vitro* assays, which would have contributed to higher MIL IC_{50}s of the amastigotes, as the *in vitro* activity of miltefosine is host cell dependent, being the lowest in PEMs (26).

LdLip^{++} parasites exhibited enhanced proliferation in the presence of MIL compared with wild-type and mock-transfected parasites. Based on previous findings implicating Lip in increased virulence (16, 27), we assessed metacyclogenesis and infectivity to host macrophages of LdLip^{++} parasites. We observed that metacyclogenesis and macrophage infectivity of LdLip^{++} parasites were significantly increased. Lipase-mediated hydrolysis of triacylglycerol leads to products that compromise host immunity, thus allowing the pathogen to survive within host cell (27). Increased infectivity and increased metacyclogenesis have been associated with MIL tolerance and documented in *L. donovani* from relapse cases after MIL treatment in the Indian subcontinent (19, 28).

The investigation for MIL-induced oxidative stress tolerance in LdLip^{++} parasites revealed that ROS production was significantly reduced in LdLip^{++} promastigotes at higher MIL concentrations. Subsequent to MIL exposure in macrophages infected with LdLip^{++} parasites, the ROS level was substantially decreased, which reiterates the findings with the MIL-tolerant phenotype in our previous study (19). Likewise, the intracellular ROS level was lower in a mutant strain of *Escherichia coli* efficient in utilizing fatty acids as a carbon source than in wild-type *E. coli* (29).

Previous studies have demonstrated that increasing resistance to MIL in *L. donovani* might be associated with underdosing or underexposure of the drug to the parasites (30, 31). Differential expression of the aminophospholipid LdMT and its accessory protein LdRoS3 as well as point mutations in these genes have been linked with poor accumulation of MIL within parasites (32). Such altered expression or point mutations in LdMT-LdRoS3 could not be detected in a set of clinical isolates of *L. donovani* from relapsed cases of VL (33). The parasite isolate studied here did not exhibit any point mutation in the LdMT-LdRoS3 genes, and increased MIL tolerance in LdLip^{++} parasites could not be associated with reduced MIL accumulation (15).

The expression of the anti-inflammatory cytokine IL-10 is responsible for pathogen persistence (34). MIL has an immunostimulatory role that helps in parasite elimination from the host cell (35). Infected macrophages show low levels of proinflammatory cytokines (TNF-α, IL-12, and IFN-γ) that are significantly increased upon MIL treatment (35). IL-10 expression shows a positive correlation with parasite burden in VL (36) and plays an important role in parasite persistence and inhibition of host leishmanicidal activity (37, 38). Studies have shown that an increased ratio of IL-10/TNF-α correlates positively with *Plasmodium vivax* parasitemia (39–42). In experimental models of leishmaniasis, TNF-α plays a critical role in controlling parasite growth (43). However, in splenic aspirate cultures of *Leishmania*, neutralization of TNF-α does not affect parasite replication, although it inhibits IFN-γ production, while IL-10 levels remain unaltered

(44). LdLip^{++} parasites induced increases in IL-10 and reductions in TNF-α levels in host macrophages, leading to marked increases in the IL-10/TNF-α ratios. It has been reported that overexpression of lipase increases virulence by downregulating the Th1 immune response in *Mycobacterium tuberculosis*-infected mice (27). The data suggest that the lipase precursor molecule favors parasite persistence within host macrophages, as evident by increased proliferation and infectivity during MIL stress compared to control parasites.

In conclusion, the present findings suggest a role of the lipase precursor molecule in increasing tolerance to MIL in *L. donovani* by (i) promoting the utilization of host free fatty acids as an alternate source of energy generation; (ii) membrane remodeling that reduces the drug interaction on the parasite surface, resulting in a lower level of accumulation of ROS; and (iii) increasing parasite infectivity and proliferation within host macrophages by promoting IL-10 expression in host cells. This molecule can be a potential target to counter the parasite's defense mechanism that operates during MIL exposure.

MATERIALS AND METHODS

Parasite cultures. The *L. donovani* isolate (K133 [MHOM/IN/2000/K133]; termed LdWT here) was derived from bone marrow aspirates of a VL patient admitted to Safdarjung Hospital, New Delhi, India, under the guidelines of the ethics committee. *L. donovani* promastigotes were propagated in M199 medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 IU/ml penicillin G, and 100 μg/ml streptomycin at 25°C. Wild-type parasites were made experimentally resistant by stepwise exposure to increasing drug concentrations (MIL up to 30 μg/ml) as described previously (15).

Generation of *Leishmania* parasites with episomal expression of lipase precursor-like protein (LdLip^{++}). DNA encoding the *L. donovani* lipase precursor-like protein was amplified with gene primers containing the SpeI site and an HA tag for subcloning into the *Leishmania* expression plasmid pKSNEO using forward primer 5′-ACTAGTATGCGTCGCGTTCAGTCATGGACGTG-3′ and reverse primer 5′-ACTAGT **CTACGCGTAGTCCGGCACGTCGTACGGGTA**AGCCCCACGAAGAACATTGCGAG-3′ (the SpeI site is underlined, and the HA tag is in boldface type).

The construct (20 μg) was transfected into a wild-type *L. donovani* isolate (K133) by electroporation using a Gene pulser XCell instrument in 2-mm-gap cuvettes at 450 V and 500 mF to produce K133Lip^{++} (LdLip^{++}) parasites. The positive transfectants were selected by using G418 (50 μg/ml) as described previously (45). Parasites transfected with the empty vector pKSNEO (LdNeo) were used as a control.

Western blotting. Preparation of the parasite lysate and Western blot analysis were performed according to methods described previously (46). Lysates (100 μg) from LdLip^{++} and LdNeo parasites were transferred onto a nitrocellulose membrane following separation by SDS-PAGE. The membrane was probed with horseradish peroxidase (HRP)-conjugated monoclonal anti-HA antibody (1:4,000 dilution) produced in mouse (Sigma-Aldrich, USA). The blot was developed by using Western blot detection enhanced chemiluminescence (ECL) detection reagent (GE Healthcare, UK). The image was scanned with ChemiDoc (Bio-Rad, USA) and analyzed by using Image Lab 5.1 software (46).

Estimation of enzymatic activity of lipase precursor-like protein. The enzymatic activity of the lipase precursor-like protein was assessed fluorometrically, as described previously (16), in culture supernatants and whole-cell lysates from LdLip^{++}, LdNeo, and LdWT promastigotes using lipase activity fluorometric assay kit III (BioVision, USA), according to the manufacturer's instructions.

Briefly, 2×10^7 promastigotes (day 5) were centrifuged at 1,800 $\times$ *g* for 10 min, the supernatant was collected, and the cells were lysed in 400 μl of chilled assay buffer. Both the supernatant and cell lysate were analyzed by measuring florescence at excitation/emission (Ex/Em) wavelengths of 529/600 nm at two different time points (T_1 and T_2) of incubation.

A standard curve was generated in the range of 0 to 100 pmol, using different volumes (0 to 10 μl) of a methylresorufin solution (0.1mM), and the volume was adjusted to 100 μl/well with lipase assay buffer. Florescence was measured at Ex/Em wavelengths of 529/600 nm using a cytofluorimeter (Infinite M200; Tecan, Switzerland).

Activity was calculated by using the formula lipase activity (nmol/min/ml = mU/ml) = $[B/(T_2 - T_1) \times V] \times$ sample dilution factor, where B is the amount of methylresorufin from the standard curve and V represents the pretreated sample volume (in milliliters).

First and second readings (R_1 and R_2) were taken at 30 min (T_1) and 60 min (T_2), respectively.

Growth kinetics of LdLip^{++} parasites. A total of 1×10^6 LdWT, LdNeo, or LdLip^{++} promastigotes were inoculated in complete M199 medium without or with MIL (3 μM). Growth of LdLip^{++}, LdNeo, or LdWT parasites was monitored for a period of 10 days by counting parasite numbers using a Neubauer chamber under a microscope at a $\times$200 magnification.

***In vitro* drug susceptibility of LdLip^{++} promastigotes.** *In vitro* susceptibilities to MIL and AmB were assessed by using a resazurin-based fluorometric assay as described previously (23). Briefly, late-log-phase LdLip^{++}, LdNeo, and LdWT promastigotes were plated into a 96-well culture plate (10^5 promastigotes/well) and exposed to increasing concentrations of either MIL (0.4 μM to 390 μM) or AmB (0.027 μM to 2.157 μM) for 72 h. Plates were further incubated for 24 h after the addition of 50 μl of

resazurin (0.0125%). Viability of cells was measured fluorometrically (excitation wavelength, 550 nm; emission wavelength, 590 nm). Calculation of the half-maximal inhibitory concentration (IC_{50}) was done by sigmoidal regression analysis using Microcal Origin 6.0 software. All experiments were performed twice in quadruplicates.

***In vitro* drug susceptibility of LdLip^{++} intracellular amastigotes.** *In vitro* susceptibility to MIL, AmB, and sodium antimony gluconate (SAG) was assessed by using an intracellular amastigote model described previously (47). Briefly, primary peritoneal macrophages derived from 6-week-old female BALB/c mice were infected with 6-day-old LdLip^{++}, LdNeo, or LdWT promastigotes at a ratio of 10:1 (parasites/macrophages) in 200 μl complete RPMI 1640 medium in 8-well chambered slides and incubated for 16 h at 37°C in 5% CO_2. Infected macrophages were washed and incubated further for 48 h, with increasing concentrations of either MIL (1, 5, 10, 20, and 30 μM), AmB (0.027, 0.054, 0.108, 0.539, and 2.0 μM), or SAG (1, 5, 10, 20, 30, and 40 μg/ml). Macrophages were stained with Diff-Quik solutions and subsequently examined for intracellular amastigotes. Calculation of the IC_{50} was done by using Microcal Origin 6.0 software.

Infectivity to macrophages. The percent infectivity of LdLip^{++} parasites to macrophages was investigated by using a mouse macrophage-amastigote model as described previously (19, 48). The percent infectivity of LdLip^{++} parasites was calculated by counting the number of infected macrophages.

Metacyclogenesis in *L. donovani*. Metacyclogenesis of LdLip^{++} promastigotes was assessed according to methods described previously (19, 49). The percent metacyclic population was calculated by counting the promastigotes that did not agglutinate with peanut agglutinin (PNA) out of the total promastigote cell density.

Oxidative stress tolerance. MIL-induced oxidative stress tolerance in LdLip^{++} parasites was assessed fluorometrically by using the cell-permeable probe 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCFDA; Molecular Probes, USA) as described previously (19, 47, 50). Fluorimetric measurements were expressed as mean fluorescence intensity (MFI) units, which represent the levels of ROS.

MIL accumulation in LdLip^{++} parasites. Accumulation of MIL was investigated in log-phase promastigote cultures according to standard procedures (19, 51). Briefly, promastigotes (10^8 cells/ml), after treatment with 100 μM MIL for 90 min, were centrifuged at 1,500 $\times$ *g* for 10 min. The cell pellet was digested in 2 N HNO_3 by overnight incubation. The supernatant was collected by centrifugation at 2,000 $\times$ *g* for 15 min and later analyzed for MIL content by using liquid chromatography-mass spectrometry (LCMS).

Multiplex enzyme-linked immunosorbent assay (ELISA) for cytokine estimation. Estimation of cytokine levels was done according to methods described previously, with modifications (52). Cytokine levels in culture supernatants of mouse peritoneal macrophages prestimulated with 1 μg/ml of lipopolysaccharide (LPS) from *Escherichia coli* and then infected with LdLip^{++}, LdWT, or LdNeo parasites for 48 h were determined by using a Bio-PlexPro mouse cytokine kit (Bio-Rad, USA) according to the manufacturer's protocol. Briefly, a 50-μl cell supernatant sample was incubated with antibody-coupled beads. Immune complexes were washed and incubated with biotinylated detection antibody, followed by streptavidin-phycoerythrin treatment prior to assessing cytokine concentrations. Manufacturer-provided standards were used to prepare the standard curve for each cytokine. Proinflammatory (IL-2, IL-12, TNF-α, and IFN-γ) and anti-inflammatory (IL-4 and IL-10) cytokines were analyzed by using a multiplex array reader from the Luminex instrumentation system (Bio-Plex workstation; Bio-Rad Laboratories).

Statistical analysis. Statistical analysis of the data was carried out by using Graph Pad Prism 5 software (San Diego, CA, USA). Results are represented as means $\pm$ standard deviations (SD). The *P* value was calculated by performing Student's *t* test. *P* values of <0.05 were considered significant.

Ethics statement. The study was approved by the Institutional Animal Ethics Committee (IAEC-3/2010) of the National Institute of Pathology, New Delhi, India. Guidelines for animal care and handling protocols recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) were followed.

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We report no conflict of interest.

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