THE ROLE OF THE DIHYDROXYACETONE PHOSPHATE ACYLTRANSFERASE *Lm*DAT IN LIPOPHOSPHOGLYCAN SYNTHESIS, METACYCLOGENESIS AND AUTOPHAGY IN *LEISHMANIA MAJOR*

by

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ABSTRACT

Glycerolipids are the most abundant lipids and are important constituents of various virulence factors in the protozoan parasite *Leishmania*. The dihydroxyacetone phosphate acyltransferase *Lm*DAT catalyzes the first step of the ether, and possibly ester glycerolipid biosynthetic pathway. A *L. major* null mutant of *LmDAT* grew slowly, died rapidly during the stationary phase of growth, and more importantly, was attenuated in virulence in mice. The goal of this study was to determine the molecular basis responsible for the attenuated virulence. Western blot analysis revealed that the *Δlmdat/Δlmdat* null mutant synthesized altered versions of the virulence factor lipophosphoglycans that were not released in the media, suggesting that its lipid anchor structure was altered. The *Δlmdat/Δlmdat* strain differentiated into virulent metacyclics, but with lower efficiency compared to the wild type. Using the autophagosomal marker ATG8-GFP, the *Δlmdat/Δlmdat* line produced twice as many autophagosomes as the wild type, suggesting that it is either defective in degradation of autophagosomes or that autophagy is simply induced. In conclusion, the attenuated virulence of *Δlmdat/Δlmdat* may be explained by i) its inability to synthesize and release normal forms of lipophosphoglycan, ii) its inability to fully differentiate into virulent metacyclics, and iii) altered autophagy.

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DEDICATION

To my wonderful Mother, Father, and my Fiancé.

1. INTRODUCTION

1.1 *Leishmania* is the causative agent of human and animal diseases, leishmaniasis

Leishmania are unicellular protozoan parasites that cause significant human and animal diseases, termed leishmaniasis. At least 12 million people are affected by this disease, and 2 million new cases are reported every year, mostly in the tropic and subtropic area of the world (www.who.int). More than 20 species of *Leishmania* are pathogenic to humans or animals. There are three major forms of leishmaniasis: the cutaneous form, caused by *L. major* mainly, is characterized by local self-healing skin ulcers and lesions. The muco-cutaneous form involves the destruction of the mucosal tissues of the mouth, nose and throat, and is caused by the *L. brasiliensis* subspecies in Southern America. The visceral form is fatal if left untreated, and is caused by *L. donovani* in India and Africa. Visceral leishmaniasis is a systemic infection characterized by fever, weight loss, and swelling of the spleen and liver (www.who.int).

1.2 The parasite *Leishmania*

Leishmania is a unicellular, diploid, eukaryotic protozoa with a single flagellum. There are more than 30 species of *Leishmania*, which belongs to the order of *Kinetoplastida* of the phylum Euglenozoa. All members of the *Kinetoplastida* are parasitic, and are transmitted by an insect vector. These flagellated protozoa share an unusual mitochondrial DNA or kDNA, located at the base of the flagellum. This DNA consists of a network of mini- and maxi-circles. Maxi-circles code for mitochondrial proteins, while mini-circles code for guide RNA implicated in RNA editing. In addition,

they exhibit unusual gene expression mechanisms, such as polycistronic transcription (Johnson, Kooter et al. 1987), transplicing of precursor RNAs (Parsons, Nelson et al. 1984), and lack of obvious promoters for RNA polymerase II.

1.3 *Leishmania* life cycle

Leishmania have a complex life cycle, alternating between two hosts: an insect vector and a vertebrate host. The vector consists of a female sand fly of the genus *Phlebotomus* or *Lutzomia*, where the parasites reside within the midgut as extracellular, flagellated promastigotes. When the sand fly takes a blood meal, promastigotes are injected into the vertebrate host and are taken up specifically by macrophages, where they differentiate into oval-shaped aflagellated amastigotes, which are responsible for the pathological symptoms.

1.4 Structure and expression of lipophosphoglycan

Leishmania promastigotes are covered by a thick glycocalyx, which is mainly composed of the glycocolipid lipophosphoglycan (LPG). The salient feature of LPG is a linear chain of galactose-mannose-phosphate repeat units (phosphoglycan domain) attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor via a hexasaccharide core (Fig. 1; (McConville, Thomas-Oates et al. 1990). In the case of *L. major*, galactoses can be substituted with monosaccharide or oligosaccharide side chains, composed of β-linked galactose, glucose or arabinose. Other species of *Leishmania* carry different side chain substitutions or no substitutions at all. The phosphoglycan repeat domain terminates with a cap made of the oligosaccharide Manα1-2Man. The GPI

anchor is composed of an unusual 1-alkyl-2-*lyso*-phophatidylinositol, with a very long $C_{24:0}$ or $C_{26:0}$ fatty alcohol. Lipophosphoglycan is a promastigote specific virulence factor, and its expression is severely down-regulated in amastigotes, or even absent in some species (Turco and Descoteaux 1992).

1.5 The roles of LPG during the life cycle of *Leishmania*

1.5.1 LPG mediates Leishmania sand fly interaction

Soon after amastigotes are taken up by the sand fly, they differentiate into promastigotes and begin to express LPG. LPG plays an important role for the parasite development within the sand fly, it mediates attachment of the parasite to the midgut wall, so that they are not excreted with the digested blood meal (Pimenta, Turco et al. 1992). In the case of procyclic *L. major*, midgut lectin receptor galactin recognize the βGal terminating side chain of LPG (Sacks 2001).

LPG determines the specificity of *Leishmania* vector interaction. For example, the sand fly *Phlebotomus papatasi* can only transmit *L. major*, while *P. argentipes* can be infected with several species of *Leishmania*, such as *L. major*, *donovani*, *tarentolae*, or *amazonensis* (Pimenta, Saraiva et al. 1994; Sacks 2001).

After the digestion of the blood meal, promastigotes undergo a differentiation process termed metacyclogenesis, during which non virulent dividing procyclic promastigotes transform into non-replicating, virulent metacyclics. This differentiation is a necessary pre-adaptation for the parasite survival within the vertebrate host (discussed in section 1.4.2). *In vitro*, metacyclogenesis correlates with the stationary phase of growth (Sacks 1989).

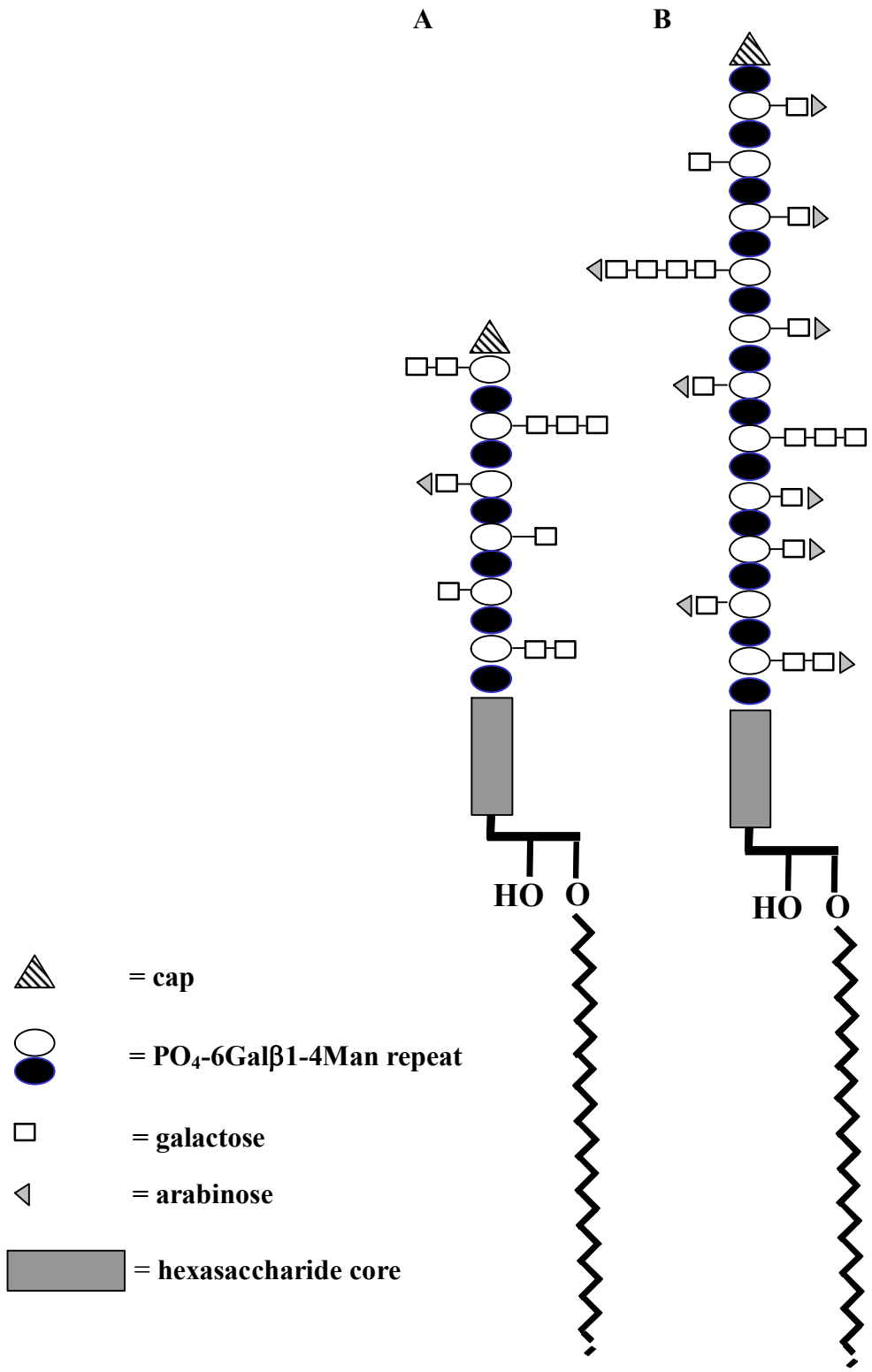

FIGURE 1: Structure of procyclic (A) and metacylic (B) forms of LPG. Metacyclic LPG displays a longer disaccharide repeat domain and higher levels of arabinosylated side chains. See text for detailed description.

One characteristic of metacyclics is the expression of structurally modified LPG. In the case of *L. major* metacyclics, LPG modifications include: i) doubling in the number of phosphoglycan repeats from 14 to 30 repeats, which leads to a thicker glycocalyx, and ii) an increase in the number of arabinose-capped side chains (McConville, Turco et al. 1992). These structural modifications mediate the release of the parasite from the midgut of the sand fly, to allow the parasite to migrate towards the mouth parts for transmission (Sacks, Brodin et al. 1990).

Other characteristics associated with metacyclogenesis include morphological changes; metacyclics are characterized by their slender body compared to the more ovoid procyclics, and an extended flagellum (Sacks 1989). Furthermore, metacyclogenesis is associated with a general down-regulation of gene expression and cessation of cell division; however, fewer genes are up-regulated in metacyclics. Such a gene is *SHERP* (small hydrophilic endoplasmic reticulum associated protein) that encodes a very hydrophilic 6.2 kDa protein (Knuepfer, Stierhof et al. 2001). SHERP localizes both to the ER and the outer mitochondrial membrane, but its function is unknown. *SHERP* belongs to a gene family, *LmcDNA16,* which contains other differentially expressed genes.

1.5.2 Roles of LPG in the vertebrate host

1.5.2.1 Resistance to complement- mediated lysis and binding to macrophage receptors

After injection into the vertebrate host, parasites must first resist lysis by the complement system. Unlike non-infective procyclic promastigotes, infective metacyclics are resistant to complement lysis. The production of longer, metacyclic LPGs, might

sterically hinder the insertion of the lytic C5b-9 complex into the plasma membrane, and thus, prevents lysis (Puentes, Da Silva et al. 1990).

LPG plays a role in parasite phagocytosis by binding directly or indirectly to macrophage surface receptors. LPG binds to complement receptor 3 (CR3) and mannose-fucose receptor on the plasma membrane of the macrophage. Indirect binding of LPG to complement receptors CR1 and CR3 is mediated by C3 component of the complement system, while indirect binding to receptor for C-reactive protein is conferred by C-reactive protein (Culley, Harris et al. 1996). CR1 and CR3-mediated phagocytosis is advantageous for the parasite, because this event prevents activation of macrophage anti-microbial defense mechanisms, such as the oxidative burst (Puentes, Sacks et al. 1988).

1.5.2.2 Protection against oxygen radicals and modulation of cytokine production

Leishmania have evolved multiple strategies to promote their survival within macrophages. LPG plays pivotal roles in protecting the parasite from toxic oxygen radicals and nitric oxide (NO) produced by the macrophage as a defense mechanism against pathogens. LPG controls the oxidative burst by two main strategies: i) by inhibiting macrophage protein kinase C activity that is linked to oxidative burst, and ii) by scavenging oxygen radicals (Chan, Fujiwara et al. 1989; Descoteaux, Matlashewski et al. 1992). LPG also inhibits the synthesis of nitric oxide (NO) by interfering with the signal transduction pathway that leads to expression of inducible nitric oxide synthase (iNOS; (Liew, Wei et al. 1997). In addition, LPG down-regulates the production of IL-12, which is pivotal in inducing Th1 lymphocyte activation (Carrera, Gazzinelli et al. 1996). Purified LPG has been shown to down-regulate IL-12 synthesis via inhibition of

the MAP kinase Erk1/2 (Feng, Goodridge et al. 1999). Piedrafita and colleagues demonstrated that the phosphoglycan repeats of LPG are implicated in this process (Liew, Wei et al. 1997; Piedrafita, Proudfoot et al. 1999).

1.6 The dihydroxyacetone phosphate acyltransferase initiates the glycerolipid biosynthetic pathway

Glycerolipids are the most abundant lipids in cell membranes, representing 70% of total lipids in *Leishmania* (Wassef, Fioretti et al. 1985). The common features of all glycerolipids are a glycerol backbone and fatty acids. There are two major classes of glycerolipids: the ester lipids, that carry an acyl group at position 1 of the glycerol backbone and represent two thirds of total glycerolipids, and the less abundant ether lipids that bear an alkyl moiety at the same position.

The initial step in ether lipid synthesis involves the acylation of dihydroxyacetone phosphate (DHAP) into 1-acyl-DHAP by a dihydroxyacetone phosphate acyltransferase (DHAPAT; Fig. 2). An alkyl dihydroxyacetone phosphate synthase then converts 1-acyl-DHAP into 1-alkyl-DHAP which is subsequently reduced by an alkyl/acyl-DHAP reductase into 1-alkyl-glycerol-3-phosphate, the precursor for all ether lipids. The intermediate 1-acyl-DHAP can also be reduced to 1-acyl-glycerol-3-phosphate by an alkyl/acyl-DHAP reductase, and serves as a precursor for ester lipid synthesis as well.

L. major expresses a single DHAPAT, *Lm*DAT, which displays 35% identity and 55% similarity to other eukaryote orthologs. Surprisingly, *Lm*DAT harbors a large N-terminal extension that is not similar to any known proteins (Zufferey and Ben Mamoun 2006). *Lm*DAT is a low-affinity DHAPAT enzyme, specific for palmitoyl-CoA as acyl-

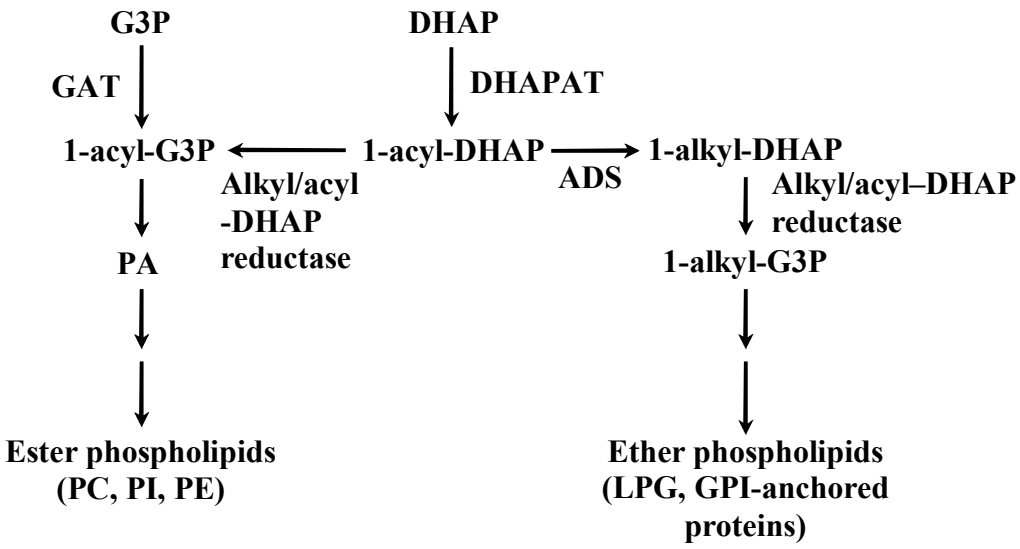

FIGURE 2: Glycerolipid biosynthetic pathways in *Leishmania*. DHAP, dihydroxyacetone phosphate; DHAPAT, DHAP acyltransferase; G3P, glycerol-3-phosphate; GAT, G3P acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; ADS, alkyl-DHAP synthase.

CoA donor. *Lm*DAT localizes to the glycosome, an organelle related to peroxisomes in *L. major* and related parasites (Opperdoes and Borst 1977; Zufferey and Ben Mamoun 2006). A null mutant of the gene *LmDAT, Δlmdat/Δlmdat*, grew twice as slowly as the wild type, reached only half of the maximal cell density of that of the wild type and complemented strain. In addition, it died rapidly soon after it entered the stationary phase of growth. More importantly, the *Δlmdat/Δlmdat* mutant was strongly attenuated for virulence *in vivo*.

1.7 The role of autophagy in differentiation and virulence

Autophagy is a process by which cytoplasmic proteins and organelles are degraded. Cytoplasmic components are engulfed by double-membrane structures termed autophagosomes, which then fuse with lysosomes, which leads to the degradation of their content. This particular process is referred to as macroautophagy or shortly autophagy. Other less investigated types of autophagy includes microautophagy, crinophagy and chaperone-mediated autophagy. Microautophagy involves the degradation of cellular components by engulfing them by the lysosomal membrane itself. Crinophagy involves incorporating secretary granules into the lysosome to be degraded, while chaperone-mediated autophagy occurs when chaperones bind to proteins to be degraded and transports them into the lysosomes. Under starvation conditions, macroautophagy is induced to degrade macromolecules that can then be recycled as a form of nutrients (Seglen and Bohley 1992). Furthermore, autophagy has been associated with cell remodeling and structural differentiation.

Autophagy is a process that is conserved among eukaryotes. *Saccharomyces cerevisiae,* has provided a great model to identify and study autophagy related genes (*ATG*). The role of ATG proteins, as well as the role of autophagy during starvation or differentiation, was also investigated in plants, as well as in mice (Mizushima, Yamamoto et al. 2004; Yoshimoto, Hanaoka et al. 2004). ATG proteins are involved in two ubiquitin-like conjugations pathways that play a role in autophagosome formation. The first conjugation system involves the linkage of ATG5 to ATG12, a reaction catalyzed by ATG7 and ATG10, which are related to E1 and E2 of ubiquitin activation. The ATG5-ATG12 conjugate then binds to ATG16 forming a complex that is localized to and necessary for the formation of autophagosome membrane (Mizushima, Sugita et al. 1998). The second system involves the cleavage and lipidation of ATG8 by conjugation to phosphatidylethanolamine, which mediates the completion of the autophagosome. The cysteine peptidase ATG4 cleaves ATG8, exposing a C-terminal glycine which is then conjugated to phosphatidylethanolamine by ATG7 and ATG3, which allows ATG8 integration into the autophagosomal membrane (Ichimura, Kirisako et al. 2000).

Recently, ATG homologs have been identified in *Leishmania* (Besteiro, Williams et al. 2006). Using GFP-ATG8 as an autophagosomal marker (Fig. 3), it has been demonstrated that *Leishmania* can undergo autophagy and occurs as a response to nutrient deprivation (Besterio *et al* 2006). Autophagy is induced in *Leishmania* when the parasite progresses from early log to stationary phase of growth, which correlates with the differentiation of procyclics into virulent metacyclic promastigotes, and when the parasite differentiates from metacyclics into the significantly smaller amastigotes

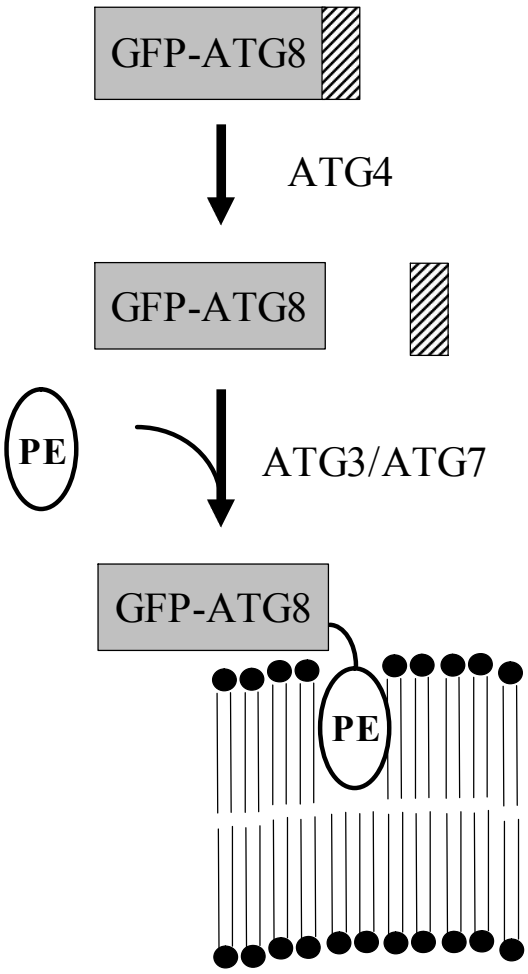

FIGURE 3: GFP-ATG8 processing during autophagy. GFP-ATG8 is normally present in the cytoplasm, as a diffuse green stain. Upon induction of autophagy, GFP-ATG8 is cleaved by ATG4. The C-terminal glycine is then conjugated to phosphatidylethanolamine (PE), which mediates GFP-ATG8 integration into the autophagosomal membrane; GFP-ATG8 would appear as green dots by fluorescence microscopy.

(Besteiro, Williams et al. 2006; Williams, Tetley et al. 2006). Furthermore, autophagy is essential for parasite differentiation and virulence, because a mutant lacking the *ATG4.2* gene, that codes for the cysteine peptidase involved in the formation of the autophagosome, displayed altered autophagy (harbored higher numbers of autophagosome-containing cells than wild type), did not differentiate into metacyclics, and was attenuated for *in vivo* virulence (Besteiro, Williams et al. 2006).

1.8 Research aims

The *Δlmdat/Δlmdat* ether lipid mutant died rapidly during the stationary phase of growth, and more importantly, displayed attenuated virulence *in vivo* (Zufferey and Ben Mamoun 2006). The goal of this work is to characterize the roles of the DHPAT enzyme *Lm*DAT in *Leishmania* biology, to gain a better understanding of how lack of *Lm*DAT negatively affects parasite virulence. The research aims are as follows:

1. Assess the ability of the null mutant *Δlmdat/Δlmadat* to synthesize and release the virulent factor LPG.
2. Investigate the ability of the *Δlmdat/Δlmadat* mutant to differentiate into virulent metacyclics based on three criteria:
   i) synthesis of arabinosylated LPGs
   ii) expression of the metacyclic gene *SHERP*
   iii) morphological changes by fractionation by Ficoll gradient centrifugation followed by quantification
3. Determine the ability of the *Δlmdat/Δlmadat* mutant to undergo autophagy using the autophagosomal marker GFP-ATG8.

2. MATERIALS AND METHODS

2.1 Parasite culture

The wild-type strain of *Leishmania major* used in this work is Friedlin V1 (MHOM/IL/80/Friedlin). Promastigotes of *L. major* were cultivated in liquid (Table 1) or semi-solid (M199-based media supplemented with 1% Noble agar) M199-supplemented media at 26ºC.

Table 1: 1x M199 supplemented media (per L)

Components	Final concentration
M199 (Sigma)	10.6 g
$NaHCO_3$	0.35 g
HEPES, pH 7.4	40 mM
Adenine	0.1 mM
Hemin	5 mg
Penicillin (Invitrogen)	100,000 units
Streptomycin (Invitrogen)	100 mg
Fetal bovine serum, heat inactivated	100 ml
Biopterin (Sigma)	2 mg

Semi-solid M199 plates were prepared as follows: one volume of warm 2x M199 supplemented medium was rapidly mixed with 2% noble agar (autoclaved and cooled down to 55°C), blasticidin and hygromycin was added at 20 and 10-25 μg/ml, respectively, as appropriate. A volume of 20 ml was poured per plate.

Cell density was monitored with a hemacytometer after cells were fixed with phosphate buffer saline (PBS: 136 mM NaCl, 2.7 mM KCl, 8.5 mM KH_2PO_4, 1 mM Na_2HPO_4, pH 7.2) supplemented with 4% paraformaldehyde (g/v).

2.2 Freezing of parasites

End log cultures (2-5x10^7 parasites/ml) were centrifuged at 2,500 rpm for 5 minutes. Supernatants were discarded. Cell pellets were resuspended in 0.5-1 ml of sterile, cold "freezing medium" (1x M199 containing 20% heat inactivated fetal bovine serum instead of 10%, and 11% dimethyl sulfoxide or glycerol; stored at 4°C), and transferred into freezing tubes (Nalgene). Tubes were frozen immediately at -75°C for at least 24 hours before being transferred into the liquid nitrogen tank (Cryo safe; -196°C) for long term storage.

2.3 Thawing of parasites

Frozen parasites were rapidly thawed by warning the tubes with the hands, transferred to a conical tube containing 5 ml of warm 1x M199 medium, and centrifuged at 2,500 rpm for 5 minutes. The supernatant was discarded. Parasites were resuspended in 5-10 ml of warm 1x M199 medium and transferred into a small tissue culture flask.

2.4 Transformation and selection of *Leishmania* parasites

Transformation of the parasites was achieved by electroporation (Ngo, Tschudi et al. 1998). Mid-log cells (5x10^6 parasite/ml) were centrifuged at 2,500 rpm for 10 min. The cell pellet was washed once with one volume of sterile cold cytomix (120 mM KCl,

0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES pH7.4, 2 mM EDTA, 5 mM $MgCl_2$, (Ngo et al (1998)) and subjected to centrifugation at 2,500 rpm for 10 min at 4°C. The supernatant was discarded, and the cell pellet was then resuspended in cytomix in 1:20 of the original volume. For each transfection, 400 µl of cells were mixed with 5-10 µg of DNA, transferred into a UV-sterilized, pre-chilled electroporation cuvette (4 mm gap). Transformation was achieved with an electroporator (BioRad) using the following setting: 1500 V and 25 µF. Cells were immediately transferred into 10 ml of pre-warmed M199-supplemented medium, and incubated at 26°C for 15-24 hrs. The culture was centrifuged at 2,000 rpm for 5 minutes, and cells were plated on selective semi-solid M199-derived plates containing blasticidin or hygromycin at 20 and 10-25 µg/ml, respectively, as appropriate.

2.5 *Leishmania* genomic DNA purification (Medina-Acosta and Cross 1993)

A volume of 5-10 ml of early stationary phase (2-4x10^7 parasite/ml) parasite culture was centrifuged at 2,500 rpm for 6 minutes. The supernatant was discarded and the cell pellet was lyzed by addition of 500 µl of TELT (2.5 M LiCl, 4% Triton X-100, 0.0625 M EDTA, 0.05 M TrisHCl, pH8). One volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed by hand-shaking for 1 min. The sample was then centrifuged at 13,000 rpm for 10 minutes at room temperature. The water (upper) phase was transferred to a new eppendorf tube, 500 µl of chloroform was added, mixed by hand-shaking for 1 min, and centrifuged at 13,000 rpm for 5 min. The upper phase was again transferred to a new eppendorf tube, 1 ml of 100% absolute ethanol was added and mixed to precipitate the DNA. After centrifugation at 13,000 rpm

for 5 minutes, the supernatant was carefully decanted and the DNA containing pellet washed with 500 µl of cold 70% ethanol. Again the sample was centrifuged for 5 minutes and the supernatant was carefully decanted. The DNA containing pellet was then dried in a vacufuge (Eppendorf) and resuspended in TE buffer (10 mM TrisHCl, pH7.5, 1 mM EDTA). Genomic DNA was stored at 4°C.

2.6 *Leishmania* total RNA purification

Cultures containing a total of 2.5-5x10^8 parasites were centrifuged at 2,500 rpm for 5 minutes. Supernatants were discarded. Cell pellets were resuspended in 1 ml of Trizol reagent (Invitrogen) and incubated at room temperature for 5 minutes. A 26 gauge needle syringe was used to shear the genomic DNA. A volume of 0.2 ml of chloroform was added, mixed by hand-shaking for 15 seconds, and incubated at room temperature for 3 minutes. The mixture was then centrifuged at 13,000 rpm for 15 minutes at 4°C. The water (upper) phase was transferred into a new eppendorf tube before 0.5 ml of isopropanol was added, mixed by hand shaking for 30 seconds, and incubated at room temperature for 10 minutes. The sample was then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the RNA containing pellet was washed with 1 ml of 75% ethanol, which was prepared using diethyl pyrocarbonate (DEPC) treated water. The sample was centrifuged again at 13,000 rpm for 5 minutes at 4°C. The ethanol was discarded, and the RNA containing pellet was dried using a vacufuge (Eppendorf). The RNA was dissolved in deionized formamide and stored at -20°C. The concentration of RNA was determined spectrophotometrically by measuring

the absorbance at a wavelength of 260 nm (Genesys 10UV from Thermo Electron Corporation).

2.7 Northern blot analysis

A 1% agarose gel was prepared by melting 1 g of agarose in 72 ml of water. This solution was then cooled down to 55°C before 10 ml of 10x MOPS (0.4 M MOPS, 0.1 M sodium acetate, 10 mM EDTA) and 18 ml of formaldehyde were added. The mixture was then poured into a gel cast. Each RNA sample was diluted to the desired concentration with formamide to which one volume of 2x sample buffer (0.08 M MOPS, 0.02 M sodium acetate, 2 mM EDTA, 13% formaldehyde, 10% glycerol, 5.4% bromophenol blue dye, 0.3 mg/ml of ethidium bromide and DEPC-treated water) was added. RNA was denatured by heating at 70°C for 10 minutes, and then immediately cooled down on ice for at least 1 minute. Each sample, containing 5 μg of RNA, was loaded onto the gel. The gel was run overnight at a constant voltage of 25 mV until the bromophenol blue dye reached the bottom one third of the gel. The gel was then washed twice with 5 volumes of purified, autoclaved water by shaking for 15 minutes. RNAs were transferred onto a nylon membrane (Roche) by the upward capillary transfer method using 10x SSC as a transfer buffer (1.5 M NaCl, 0.15 M trisodium citrate, pH7.0). After the transfer was completed, the membrane was washed with 2x SSC (0.3 M NaCl, 0.03 M trisodium citrate, pH7.0) for 5 minutes, air dried, and crosslinked by exposure to UV light for 5 minutes.

The probe used for hybridization encompasses the *SHERP* gene, which was amplified with the primers 114 (‘5-GATCCGCGCAGACCAAGATG-3’) and 115 (‘5

CAGAGAACGGCGAAGGGACTG-3') by polymerase chain reaction (PCR). The PCR was performed in a total volume of 50 µl, containing 1x buffer, 0.2 mM deoxynucleotides dCTP, dATP, dGTP and dTTP, 2.5 mM $MgCl_2$, 5 pmol of each primer, 2 µg of genomic DNA from *L. major*, and 1 unit of HiFi Taq polymerase (Invitrogen). The reaction consisted of 25 cycles of three sequential steps: denaturation at 94ºC for 20 seconds, annealing at 60ºC for 30 seconds, and elongation at 68ºC for 1 min. Before loading on an agarose gel, the amplified DNA was mixed with 5 µl of 10x DNA loading dye (70% glycerol, 200 mM EDTA, 0.25% bromophenol bule, 0.25% xylene cyanil FF). The agarose gel was made as follows: 1 g of agarose was melted in 1x TAE buffer (4.84 g Tris base, 0.11 % glacial acetic acid, 0.001 M EDTA). The mixture was allowed to cool down to approximately 60ºC and a final concentration of 0.1 µg/ml ethidium bromide was added before the gel was pored in a cast. Electrophoresis was carried out at a constant voltage of 100 mV for one hr. DNA was visualized by exposing the gel to UV light. The amplified DNA was excised and purified using a gel extraction kit of Roche Applied Science. Fifty ng of DNA was denatured by boiling for 10 min and rapidly chilled on ice for at least 2 min. The denatured DNA was labeled with ^{32}P-dCTP (MP Biologicals) by random priming using the Hi-Prime kit of Roche Applied Science. The radiolabeled probe was separated from the non-incorporated ^{32}P-dCTP nucleotides by gel filtration. The column was centrifuged for 2 min at 4,000 rpm per min. The flow-through, containing the radiolabeled probe, was used for hybridization. The radiolabeled probe was denatured as described above, before being transferred to the pre-warmed (65ºC) hybridization buffer CHURCH (10g/L bovine serum albumine, pH7.2, 1 mM EDTA, 0.2625 M sodium hydrogen phosphate buffer, 7% SDS). The hybridization was carried

out overnight at 65ºC in a glass tube with the "RNA side" of the membrane facing inward. The next day, the membrane was washed once with 2x SSC containing 0.1% SDS for 20 min at 65ºC, followed by a second wash with 0.2x SSC containing 0.1% SDS for 20 min at 65ºC. The membrane was then exposed to X-ray films.

2.8 Western blot analysis

Leishmania cell pellets were prepared from mid-log ($5x10^6$ parasites/ml), end-log/early stationary ($2\text{-}4x10^7$ parasites/ml), and three-day stationary phase cultures. Cells were centrifuged at 2,500 rpm for 6 minutes and washed once with cold PBS before being frozen at -20ºC until use. Cell pellets were lyzed with 1x protein sample buffer (2% SDS, 0.7 M β-mercaptoethanol, 10% glycerol, 62 mM TrisHCl pH6.8, 0.05% bromophenol blue) before loading on a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and separating gels contained 3.5% and 10% polyacrylamide, respectively. Electrophoresis was performed at a constant current of 37 mA per 1.5 mm thick mini-gel until the bromophenol blue dye reached the bottom of the gel (approximately 90 min). Proteins or glycolipids were transferred onto polyvinylidene fluoride (BioRad) membrane that was previously soaked with methanol before being equilibrated in transfer buffer (0.024 M Tris base, 0.2 M glycine, 20% methanol, 0.02% SDS). Transfer to the polyvinylidene fluoride membrane was performed with a semi–dry transfer apparatus (BioRad) at a constant voltage of 15 V for 1 hr 45 min. After the transfer was completed, the membrane was washed with PBST buffer (1x PBS supplemented with 0.05% Tween 20, (v/v)) for 5 minutes, then blocked with 5% dry milk in PBST for 1 hr at room temperature. The membrane was then incubated overnight at

4°C with primary antibody in 5% milk containing PBST, and subsequently washed four times for 12 minutes with PBST. The membrane was hybridyzed for 1 hr with secondary antibody at a 1:5,000 dilution in 5% dry milk containing PBST. The membrane was washed again four times as described above. Antibody complexes were revealed with a chemiluminescent substrate (Pierce) followed by development on X-ray film (Fuji).

2.9 Antibodies

The monoclonal antibody 3F12, which recognizes the arabinose-galactose disaccharide (Sacks and da Silva 1987), was used at a dilution of 1:1,000. The WIC79.3 monoclonal antibody, which recognizes the galactosylated mannose-phosphate galactose repeat of LPG (de Ibarra, Howard et al. 1982), was used at a dilution of 1:5,000.

2.10 Preparation of *Leishmania* supernatants

Mid-log cultures (cell density of 2-5x10^6 parasites/ml) were centrifuged at 2,750 rpm for 10 minutes and supernatants were discarded. Cell pellets were resuspended with 1x M199 base media (without supplements) using 1:10 of the original culture volume. After 4-5 hours of incubation at 26°C, cultures were centrifuged at 3,000 rpm for 10 minutes. Supernatants were carefully transferred into a new tube and stored at – 20°C. To visualize released LPGs, supernatants were diluted with 2x protein sample buffer, separated on a SDS-PAGE, and revealed by Western blot analysis in the presence of the monoclonal antibody WIC79.3.

2.11 Isolation of *Leishmania* metacyclic promastigotes (Spath and Beverley 2001)

A 40% Ficoll (Fisher) stock solution in water was prepared. For the assay, the stock solution was diluted with 5x M199 medium (without supplements) to obtain a final concentration of 10% Ficoll in 1x M199. All dilutions and reagents were prepared fresh. A discrete gradient was prepared as follows: 2 ml of 40% Ficoll was transferred into a 15 ml conical tube (Sarstedt), topped by 2 ml of 10% Ficoll in 1x M199. The equivalent of 10^9 stationary phase parasites was obtained by centrifuging cultures at 3,522 rpm for 10 min. The cell pellet was then resuspended in 6 ml of 1x DMEM (Dulbecco's Modified Eagle Media, Invitrogen) and 2 ml of this cell suspension was layered on top of the 10% Ficoll containing solution. The gradients were centrifuged for 20 min at 2,913 rpm. Metacyclics were recovered from the upper interphase between the 10% Ficoll layer and the cell suspension using a pasteur pipette. The cells were then centrifuged at 3,522 rpm for 10 min. The metacyclic parasites were enumerated with a hemacytometer.

2.12 Sample preparation for fluorescence microcopy

Cover slips were incubated with 1:3 diluted poly-lysine solution for 10 minutes at room temperature, before being washed five times with water. Cells derived from mid-log, early stationary and three-day stationary phase cultures were centrifuged at 2,500 rpm for 5 minutes, and washed once with one volume of PBS before being fixed in PBS supplemented with 4% (w/v) paraformaldehyde. The equivalent of $1x10^6$ parasites were applied to each poly-lysine coated cover slip by centrifugation at 800 rpm for 5 minutes. The unbound cells were discarded, and the cover slips were washed with 1 ml of PBS and

then were mounted onto slides with antifade (Invitrogen) as a mounting media. Samples were cured for 24 hours at room temperature in the dark before being sealed with nail polish.

2.13 Visualization of autophagosomes

The autofluorescence of GFP-ATG8 (the plasmid was a gift from J.C. Mottram, UK) was visualized with a fluorescence microscope (Leica). The experiment was performed in triplicates using three independent clones per strain. For each clone, 200-300 green cells were assessed for the presence or absence of autophagosomes, which were visible as distinct green dots. The number of cells containing autophagosomes was enumerated, as well as the number of autophagosomes per positive cell.

2.14 Statistical analysis

The ANOVA algorithm was used for statistical analyses to calculate P values between groups.

3. RESULTS

3.1 The *Δlmdat/Δlmdat* mutant synthesized altered forms of lipophosphoglycan

Lipophosphoglycans (LPG) are important virulence factors that are essential for parasite infectivity in both the insect vector and the vertebrate host (Handman and Goding 1985; Schlein, Schnur et al. 1990). They harbor an unusual lipid anchor that consists of a 1-alkyl-2-*lyso*-phosphatidylinositol (McConville, Turco et al. 1992). Because the *Δlmdat/Δlmdat* mutant lacks dihydroxyacetonephosphate acyltransferase activity (Zufferey and Ben Mamoun 2006) required for the production of ether lipids, we hypothesized that the null *Δlmdat/Δlmdat* mutant may not express LPG or synthesize an altered version of it. To determine whether LPG is made in the null mutant, and get some insight into its structure, whole cell extracts derived from mid-log, early stationary and three-day stationary phase cultures from wild type, *Δlmdat/Δlmdat* null mutant and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]* were subjected to Western blot analysis. LPG was visualized with the anti-LPG monoclonal antibody WIC79.3, which recognizes the galactosylated mannose-galactose-phosphate domain of LPG (de Ibarra, Howard et al. 1982). Results showed that the *Δlmdat/Δlmdat* mutant is capable of synthesizing LPG in similar levels as the wild type and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]* (Fig. 5, upper panel). However, LPG produced by the *Δlmdat/Δlmdat* mutant exhibited a slower apparent mobility on SDS-PAGE when compared to that of the wild type and complemented line (Fig. 5, upper panel). These data demonstrate that the null mutant synthesized altered forms of LPG.

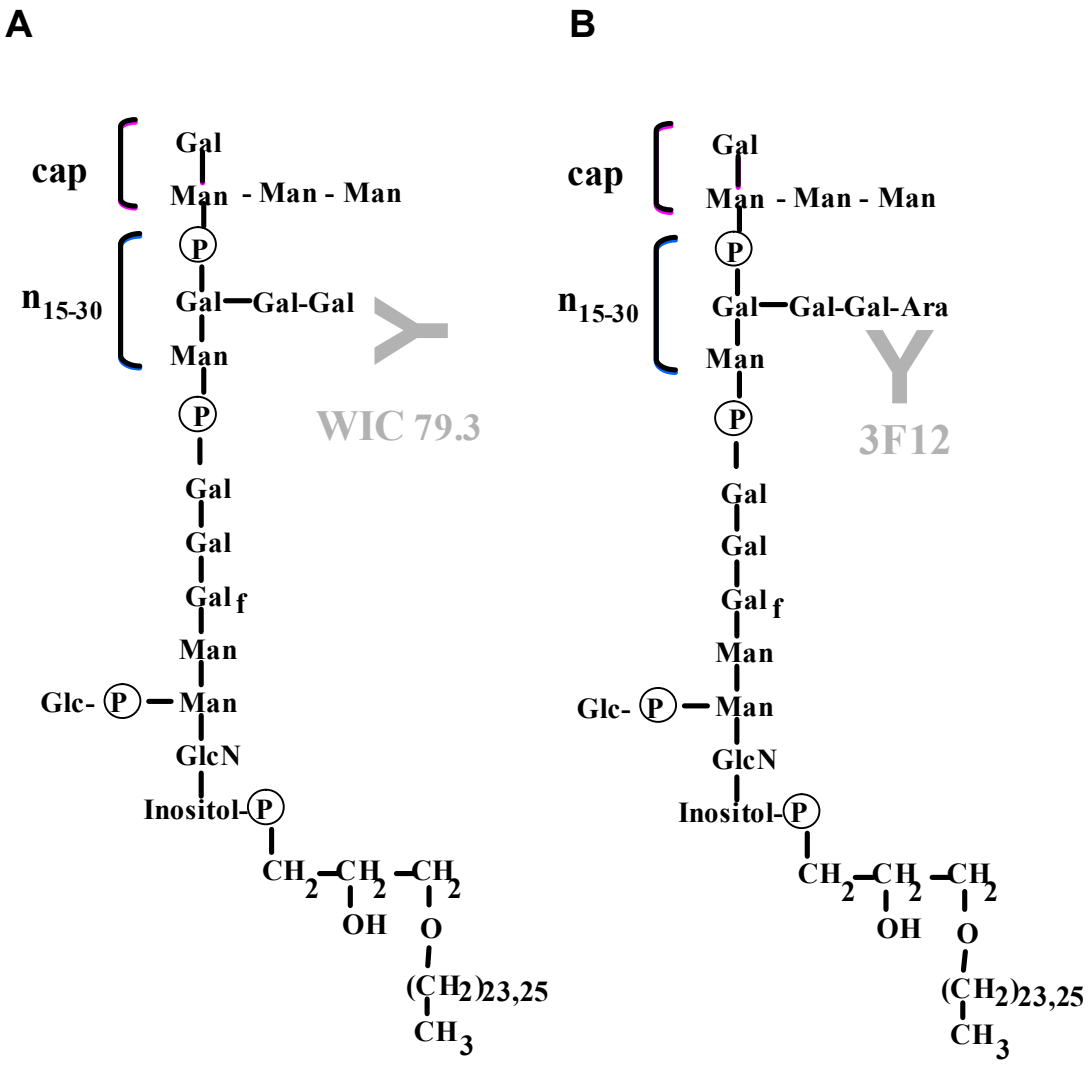

FIGURE 4: Detailed structure of LPG showing antibody specificity. (A) WIC79.3 monoclonal antibody recognizes the galactosylated mannose-phosphate galactose repeat of LPG. (B) 3F12 antibody recognizes the arabinose-galactose disaccharide.

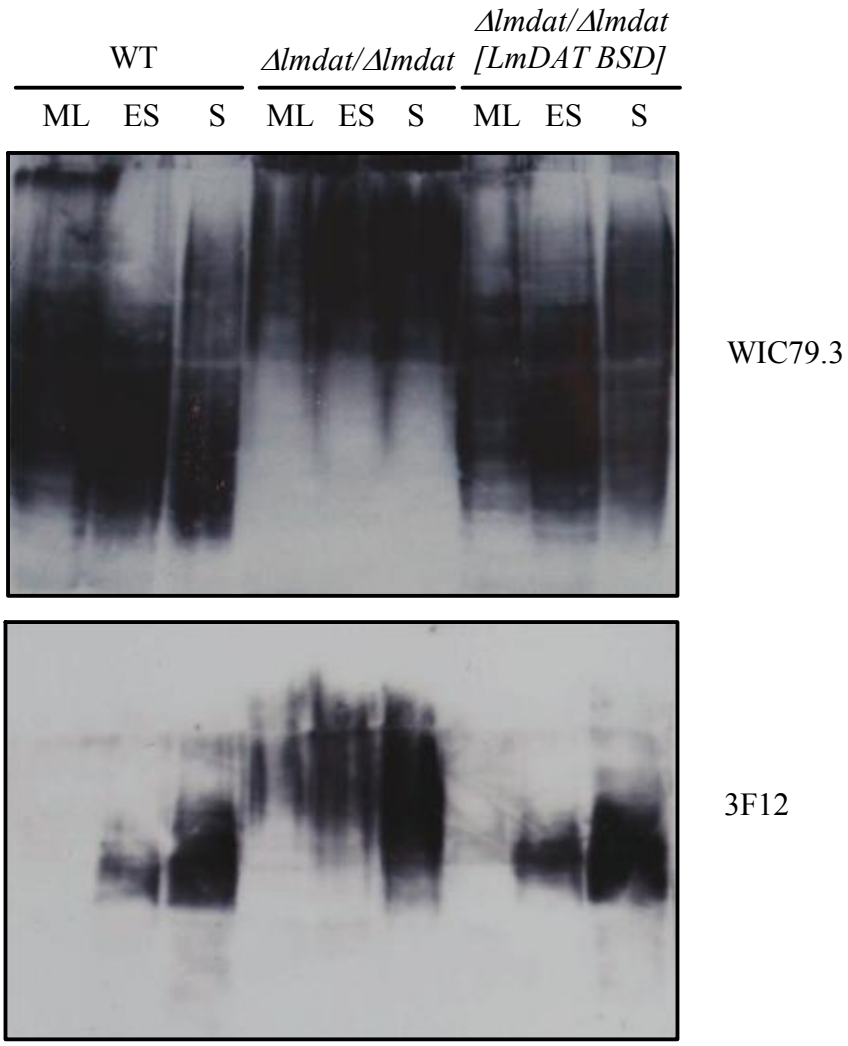

FIGURE 5: The *Δlmdat/Δlmdat* null mutant synthesized arabinosylated lipophosphoglycans. Western blot analysis of cell extracts derived from three different growth stages of wild type (WT), *Δlmdat/Δlmdat* mutant and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]*. Equivalent of $2x10^7$ cells/lane were loaded on a 10%-4% SDS-PAGE, and LPG was visualized using the WIC79.3 (upper) and 3F12 (lower) monoclonal antibody. WIC79.3 was also used here as a positive control for the lower panel. ML, mid-log phase; ES, early stationary phase; S, three-day stationary phase.

3.2 The *Δlmdat/Δlmdat* mutant was unable to release LPG in the medium

LPG is rapidly released in the medium with a half time of approximately 20 min, very likely because it is anchored to the plasma membrane by only one fatty alcohol instead of two fatty acids, or one fatty alcohol and one fatty acid (Proudfoot, Schneider et al. 1995). In contrast, protein-free glycoinositolphospholipids (GIPLs) that bear a 1-alkyl-2-acylphosphatidylinositol anchor are much slower released in the medium, with a half time of approximately 24 hrs. Because the *Δlmdat/Δlmdat* mutant is defective in the first step in the ether lipid biosynthetic pathway, we anticipate that the lipid anchor of LPG may be altered. Therefore, to determine whether the *Δlmdat/Δlmdat* mutant is able to release LPG into the medium, cell supernatants from mid-log phase promastigotes of wild type, null mutant, and complemented strain were analyzed by Western blot analysis. LPG was revealed using the anti-LPG monoclonal antibody WIC79.3. Unlike the wild type and the complemented strain, the *Δlmdat/Δlmdat* mutant was unable to release LPG into the medium, suggesting that its lipid anchor moiety is altered (Fig. 6).

3.3 The *Δlmdat/Δlmdat* mutant synthesized arabinosylated LPG

Because the *Δlmdat/Δlmdat* mutant was attenuated in virulence and died rapidly in stationary phase (Zufferey and Ben Mamoun 2006), we surmise that this phenotype is a result of the inability of the *Δlmdat/Δlmdat* promastigote to differentiate into virulent metacyclic parasites. During metacyclogenesis, which occurs *in vitro* during the stationary phase of growth, LPG of *L. major* undergoes two structural modifications: i) carbohydrate side chains become capped with arabinose residues which mediates the release of the parasite from the midgut lectins of the sand fly for transmission when the

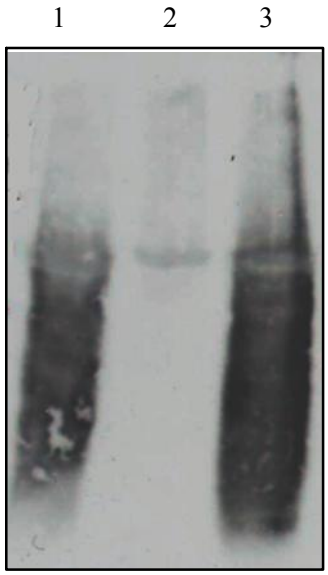

FIGURE 6: The *Δlmdat/Δlmdat* mutant was unable to release lipophosphoglycan in the medium. Western blot analysis of mid log cell supernatants derived from wild type (1), *Δlmdat/Δlmdat* mutant (2), and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]* (3). Thirty microliters of cell supernatant were loaded in each lane, and resolved on a 10%/4% SDS-PAGE. Lipophosphoglycan was revealed using the anti-LPG antibody WIC79.3.

insect takes a blood meal; and ii) its disaccharide domain is twice as long as in replicating, non virulent procyclics (McConville, Turco et al. 1992). To test whether the *Δlmdat/Δlmdat* null mutant is capable of adding arabinose residues to LPG side chains, whole cell extracts derived from mid log, early stationary and three-day stationary growth phase of wild type, mutant and complemented strain were analyzed by Western blot analysis. Arabinose capped LPGs were visualized with the monoclonal antibody 3F12, which recognizes the Ara-Gal motif (Sacks and da Silva 1987). Even though the null mutant died rapidly during the stationary phase of growth, the mutant *Δlmdat/Δlmdat* can still add arabinose moieties to LPG (Fig. 5, lower panel). As a loading control for the presence of LPG, the membrane was incubated in the presence of the LPG-specific WIC79.3 antibody (Fig. 5, upper panel).

3.4 The *Δlmdat/Δlmdat* mutant expressed normal levels of *SHERP*

SHERP is a gene that is only expressed in metacyclics (Knuepfer, Stierhof et al. 2001); hence, the expression of this gene could be used as a useful marker for metacyclics. To further test whether the *Δlmdat/Δlmdat* mutant promastigotes are capable of differentiating into virulent metacyclics, we assessed whether *SHERP* was normally expressed. Total RNAs purified from mid-log and three-day stationary phase cultures of wild type, *Δlmdat/Δlmdat* and *Δlmdat/Δlmdat [LmDAT BSD]* complemented strain were analyzed by Northern blot analysis, and hybridized with a *SHERP*-specific probe. Results showed that the *Δlmdat/Δlmdat* null mutant expressed *SHERP* at comparable levels as the wild type and *Δlmdat/Δlmdat [LmDAT BSD]* complemented strain (Fig. 7).

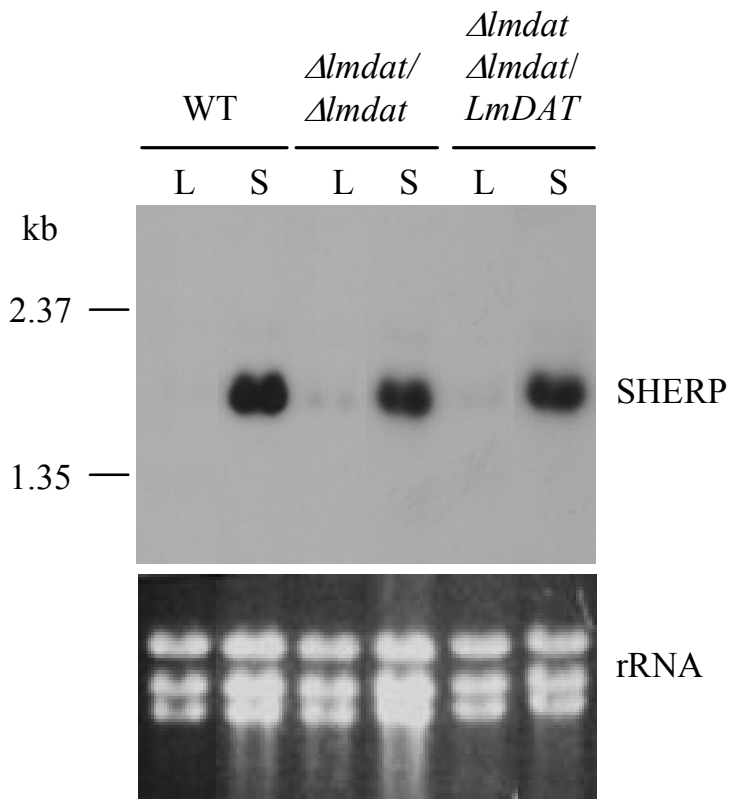

FIGURE 7: Northern blot analysis of total RNA from cells derived from two different growth stages of wild type (WT), null mutant *Δlmdat/Δlmdat,* and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]*. The membrane was hybridized with a *SHERP*-specific probe (upper panel). Ethidium bromide stained ribosomal rRNA is used as a loading control (lower panel). L, mid-log phase; S, three-day stationary phase. The ladder is shown on the left.

3.5 The *Δlmdat/Δlmdat* promastigotes differentiated into metacyclics

Because of their distinct smaller and slender morphology, metacyclics have different sedimentation properties and thus, can isolated from the rest of the cell population (procyclics) using density gradient centrifugation assays (Spath and Beverley 2001). Ficoll gradients were used to fractionate metacyclics of wild type, *Δlmdat/Δlmdat* mutant and *Δlmdat/Δlmdat [LmDAT BSD]* complemented strain from three-day stationary phase cultures. Metacyclics were recovered from the 10% Ficoll-medium interface and quantified with a hemacytometer. Results showed that approximately 4% of wild type and complemented strain cells were metacyclics, while only 2% of the *Δlmdat/Δlmdat* total cells were metacyclics (Table 2).

3.6 The *Δlmdat/Δlmdat* mutant formed autophagosomes

Alterations in autophagy have been shown to be associated with impaired procyclic-metacyclic differentiation and rapid death during the stationary phase of growth (Besteiro, Williams et al. 2006). Because the *Δlmdat/Δlmdat* mutant exhibited a similar phenotype as the autophagy mutant *Δatg4/Δatg4*, we assessed whether it displayed similar alterations in this process. The autophagosomal marker GFP-ATG8 was used to monitor autophagy (Besteiro, Williams et al. 2006). Wild-type, null mutant and complemented parasites expressing GFP-ATG8 were harvested from early log ($2x10^6$/ml), early stationary and three-day stationary growth phases. Parasites were washed, fixed, mounted on cover slips, and the autofluorescence of GFP-ATG8 was visualized with a fluorescence microscope. Cells were monitored for the presence of autophagosomes that were visible as intracellular green dots (Fig. 8). Early log cells of all

TABLE 2: Percentage of metacyclics present in three-day stationary cultures of wild type, *Δlmdat/Δlmdat* and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]*. Results were obtained from two independent experiments, and standards deviations are shown. P value for wild type (or complemented line) and null mutant pair was <0.05.

Strains	**Percentage of metacyclics (%)**
Wild type	3.79 + 0.41
Δlmdat/Δlmdat	2.00 + 0.35
Δlmdat/Δlmdat [LmDAT BSD]	3.65 + 0.21

three lines displayed an evenly distributed green stain throughout the cytoplasm, and no autophagosomes were observed (Fig. 8, upper panel). In contrast, autophagosome containing cells were observed in early stationary cultures, and their number increased slightly in three-day stationary phase parasites (Fig. 8, lower and middle panels). The wild type and the complemented strain exhibited comparable levels (12-18%) of autophagosome containing cells (Table 3). In contrast, the *Δlmdat/Δlmdat* mutant had twice as many autophagosome containing cells (25-32%) in both early stationary and three-day stationary cultures (Fig. 9; Table 3). The average number of autophagosomes within each cell was also calculated. While the wild type and complemented strain had a similar number of 1.2-1.4 autophagosomes per cell, the mutant contained an average of 1.7 autophagosomes per cell (Table 4). For all lines, no major differences were observed in numbers of autophagosomes per cell when comparing early stationary and three-day stationary phase parasites.

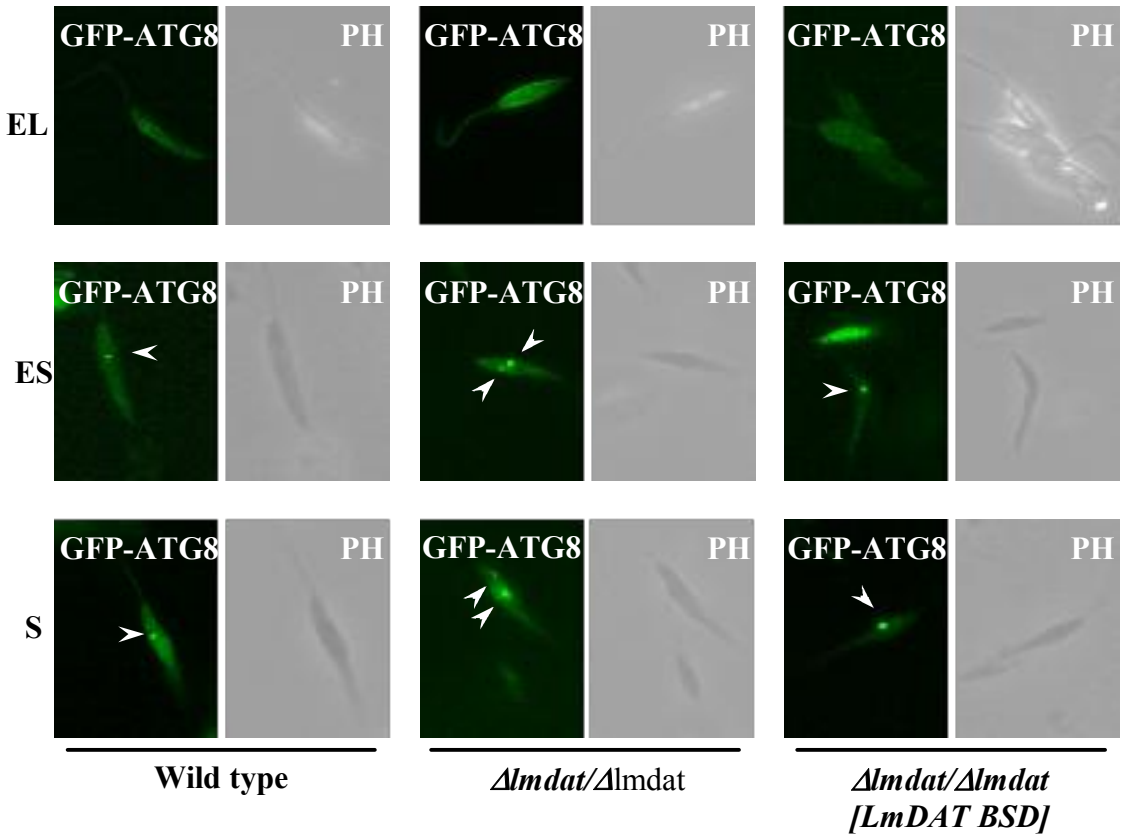

FIGURE 8: Visualization of autophagosomes. The autofluorescence of GFP-ATG8 in fixed parasites was captured by fluorescence microscopy. Slides were prepared from early log (EL), early stationary (ES), and three-day stationary phase (S) cells corresponding to wild type, *Δlmdat/Δlmdat* mutant, and *Δlmdat/Δlmdat [LmDAT BSD]* complemented strain. Autophagosomes are shown by arrowheads. PH, phase contrast.

TABLE 3: The *Δlmdat/Δlmdat* can form autophagosomes.
Autophagosome-containing cells were enumerated from early log, early stationary and three-day stationary cultures of wild type, null mutant *Δlmdat/Δlmdat,* and complemented line *Δlmdat/Δlmdat [LmDAT BSD]*. Numbers are averages obtained from three independent clones per strain. P value for wild type and null mutant or complemented line and null mutant pair was <0.05.

Strains	Early log	Early stationary	Three-day stationary
WT	< 0.5	11.6 + 4.9	16.7 + 7.6
Δlmdat/Δlmdat	< 0.5	25 + 5.1	32.3 + 4.0
Δlmdat/Δlmdat [LmDAT BSD]	< 0.5	15.2 + 4.1	18.4 + 5.7

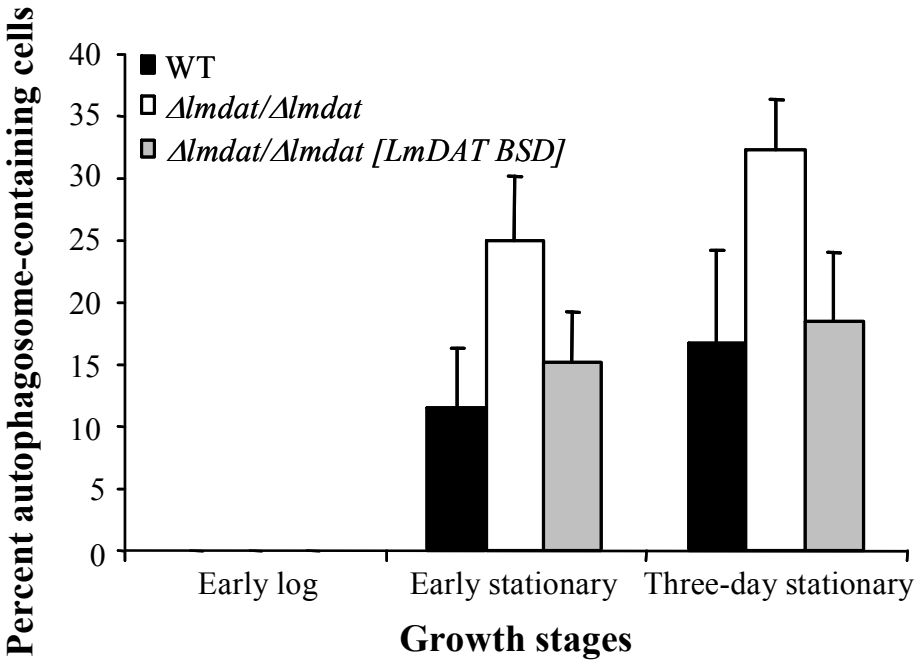

FIGURE 9: Autophagosome formation in the *Δlmdat/Δlmdat* mutant. Percentage of autophagosome-containing cells present in wild type, *Δlmdat/Δlmdat* mutant, and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]* at different growth stages. Results were obtained from three independent clones per strain. P value for wild type and null mutant or complemented line and null mutant pair was <0.05.

TABLE 4: Number of autophagosomes per cell. The number of autophagosomes per cell was enumerated in three independent clones per strain, and standard deviations are shown. P value for wild type and null mutant or complemented line and null mutant pair was <0.05.

Strains	**Early log**	**Early stationary**	**Three-day stationary**
WT	< 0.5	1.17 + 0.11	1.22 + 0.11
Δlmdat/Δlmdat	< 0.5	1.70 + 0.20	1.68 + 0.14
Δlmdat/Δlmdat [LmDAT BSD]	< 0.5	1.30 + 0.06	1.39 + 0.23

4. DISCUSSION

The goal of this study was to determine the importance of the dihydroxyacetone phosphate acyltransferase *Lm*DAT in the biosynthesis of the virulence factor LPG, in metacyclogenesis, and in autophagy of *L. major*. Because LPG is an ether lipid derivative, we first assessed whether lack of the dihydroxyacetone phosphate acyltransferase *Lm*DAT that initiates the ether lipid biosynthetic pathway affects synthesis of LPG. Results showed that LPG is expressed in the *Δlmdat/Δlmdat* null mutant at similar levels as in the wild type and complemented line. However, it was structurally altered, displaying reduced electrophoretic mobility compared to that of the wild type and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]*. This result is consistent with the phenotype of another ether lipid mutant, the *Δads1/Δads1* mutant that lacks the second enzyme of the ether lipid biosynthetic pathway, the alkyl dihydroxyacetone phosphate synthase (Zufferey, Allen et al. 2003). Similar to the *Δlmdat/Δlmdat*, the *Δads1/Δads1* null mutant synthesized LPG that displayed reduced electrophoretic mobility, which was due to a longer disaccharide repeat domain (Zufferey, Allen et al. 2003). Thus, we speculate that the phenotype observed for the *Δlmdat/Δlmdat* LPG might be due to a longer disaccharide repeat domain as well, because both mutants are defective in the same biosynthetic pathway. The reason of the deregulation of the LPG length control is not known. This may be due to overactive glycosyltransferases that build the phosphoglycan repeat domain of LPG, or to a slower transit through the secretory pathway. Extensive phosphoglycosylation of LPG is also observed during metacyclogenesis. However, the extensive phosphoglycosylation, observed in mid-log phase *Δlmdat/Δlmdat* mutant is very unlikely due to an early

differentiation into metacyclics, because the expression of metacyclic markers, such as arabinosylated LPG and SHERP, was not observed in mid log phase mutant cells.

The LPG of *L. major* is rapidly released into the medium with a half life of 20 minutes. In contrast, glycoinositolphospholipids (GIPLs), a related surface glycolipid, is released much slower into the medium with a half life of approximately 24 hours (Proudfoot, Schneider et al. 1995). This is probably due to differences in anchorage into the membrane. LPG is likely more easily released in the medium, because it is anchored to the plasma membrane by only one fatty alkyl chain, while GIPLs is slowly released due to the stronger attachment to the membrane via a 1-alkyl-2-acylphosphatidylinositol anchor. We used the release/non-release of LPG into the medium as a criterion for alteration of the GPI anchor. Our results showed that the *Δlmdat/Δlmdat* null mutant is unable to release LPG into the medium, very likely because its lipid anchor is altered. Proudfoot and colleagues demonstrated that the lipid anchor of LPG is synthesized from a glucosamine phosphatidylinositol with a 1-alkyl-2-acyl-PI structure, followed by cleavage of the fatty acyl moiety (Proudfoot, Schneider et al. 1995). However, *Leishmania* also synthesizes lower levels of glucosamine-PI precursors with a diacyl-PI structure. Thus, in absence of glucosamine-1-alkyl-2-acyl-PI precursors, *Leishmania* may use these glucosamine-diacyl-PI precursors for LPG synthesis instead. Further studies are awaited to determine the structure of the LPG anchor in the null mutant.

The inability of the *Δlmdat/Δlmdat* null mutant to release LPG might contribute to its attenuated virulence. The release of LPG by the parasite is essential for inhibition of macrophage activation by interfering directly with signal transduction pathways or other macrophage functions, such as assembly and recruitment of the NADPH oxidase to the

phagosomal membrane, that are localized in the cytoplasm of the macrophage (Denkers and Butcher 2005; Lodge, Diallo et al. 2006).

Metacyclogenesis is a differentiation process by which procyclics differentiate into virulent metacyclics, a process that occurs during the stationary phase *in vitro*. Because the *Δlmdat/Δlmdat* mutant was attenuated in virulence and was dying rapidly in stationary phase, we assessed whether the *Δlmdat/Δlmdat* mutant is capable of differentiating into metacyclics, by testing three criteria associated with metacyclogenesis. The first criterion was the ability of the *Δlmdat/Δlmdat* mutant to synthesize arabinosylated LPG; results showed that the null mutant was capable of synthesizing such molecules during the stationary phase of growth. The second characteristic was the ability of metacyclics to sediment on the 10% layer of a discrete Ficoll gradient due to their distinct short, slender morphology. Our results showed that a fraction of the stationary phase *Δlmdat/Δlmdat* cells sediment/fractionate displayed a morphology that resembles metacyclics, however the percentage of these metacyclics was less than the percentage of metacyclics isolated from wild type and complemented strain. The last criterion that we have investigated was the expression of *SHERP*, a gene that is only expressed in metacyclics. Nothern blot analysis showed that *SHERP* was expressed in the *Δlmdat/Δlmdat* mutant at comparable levels as the wild type and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]*. Altogether, these results demonstrated that the null mutant *Δlmdat/Δlmdat* was able to differentiate into metacyclics, although to a lesser efficiency. This may contribute to its attenuated virulence.

Autophagy is a process by which cytosolic proteins and organelles are degraded, and occurs in eukaryotes as a response to nutrient deprivation as well as to stress-induced differentiation (Seglen and Bohley 1992; Levine and Klionsky 2004). In *Leishmania*, autophagy is induced during the stationary phase of growth as the parasite differentiates from procyclic into metacyclic promastigote (Besteiro, Williams et al. 2006). Because autophagosome mutants exhibited similar phenotypes as the *Δlmdat/Δlmdat* mutant, slow growth and rapid death during the stationary phase of growth, we investigated the importance of *Lm*DAT in autophagy (Besteiro, Williams et al. 2006). Autophagy was monitored using the GFP tagged autophagosomal marker, GFP-ATG8. We showed that in early log cultures of wild type, *Δlmdat/Δlmdat* and *Δlmdat/Δlmdat [LmDAT BSD]* ATG8 was distributed throughout the cytosol, and no autophagosomes were observed. In contrast, autophagosomes were observed in early and three-day stationary cultures. Interestingly, early and three-day stationary cultures of the null mutant *Δlmdat/Δlmdat* displayed twice as many autophagosomes containing cells when compared to control cultures of wild type and complemented strain *Δlmdat/Δlmdat [LmDAT BSD]*. Furthermore, the *Δlmdat/Δlmdat* mutant displayed a slightly higher number of autophagosomes per cell. These results suggest that the null mutant *Δlmdat/Δlmdat* might accumulate autophagosomes because autophagosomes can not fuse with lysosomes for degradation. Studies on membrane fusion using protein-free membrane models demonstrated that membrane ether lipids facilitate/initiate membrane fusion, and that synaptic vesicles that undergo rapid and intensive membrane fusion are ether lipid rich (Gorgas, Teigler et al. 2006). This might explain not only the higher number of

autophagosomes containing cells, but also the higher number of autophagosomes per cell observed.

The fact that the null mutant *Δlmdat/Δlmdat* is defective in processing autophagosomes might, at least in part, contribute to the observed lower percentage of procyclics differentiating into metacyclics. This is consistent with published results demonstrating that a null mutant of *ATG4*, that encodes the cysteine peptidase implicated in ATG8 processing, was defective in degrading autophagosomes, and failed to differentiate into metacyclics (Besteiro, Williams et al. 2006). It is worth noting that in our study, the number of autophagosome containing cells as well as the number of autophagosomes per cell for all three cell lines was much lower than those obtained by Besteiro *et al* (2006). The differences are likely due to the use of different growth media.

Alternatively, the autophagy process might be simply induced in the *Δlmdat/Δlmdat*. Recent studies with mouse embryonic fibroblasts have shown that induced autophagy might be a mechanism by which cells can undergo programmed cell death upon exposition to specific drugs, a process known as autophagic death (Tsujimoto and Shimizu 2005). On the other hand, nutrient starvation induces autophagy as a mechanism of survival in the same cells. Thus, induced autophagy in the *Δlmdat/Δlmdat* mutant can be either a death or a survival mechanism. This could be further investigated by monitoring the growth and survival rate of the mutant in the presence or absence of autophagy inhibitors.

In conclusion, we demonstrated that the ether lipid mutant *Δlmdat/Δlmdat* was unable to produce normal forms of LPG, differentiated less efficiently into metacyclics,

and exhibited altered autophagy process, which may contribute to its attenuated virulence.

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