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Original article

# *Leishmania donovani* lipophosphoglycan inhibits phagosomal maturation via action on membrane rafts

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**Abstract**

Lipophosphoglycan (LPG), the major surface glycoconjugate on *Leishmania donovani* promastigotes, is crucial for the establishment of infection inside macrophages. LPG comprises a polymer of repeating Galβ1,4Manα-$PO_4$ attached to a lysophosphatidylinositol membrane anchor. LPG is transferred from the parasite to the host macrophage membrane during phagocytosis and induces periphagosomal F-actin accumulation correlating with an inhibition of phagosomal maturation. The biophysical properties of LPG suggest that it may be intercalated into membrane rafts of the host-cell membrane. The aim of this study was to investigate if the effects of LPG on phagosomal maturation are mediated via action on membrane rafts. We show that LPG accumulates in rafts during phagocytosis of *L. donovani* and that disruption of membrane rafts abolished the effects of LPG on periphagosomal F-actin and phagosomal maturation, indicating that LPG requires intact membrane rafts to manipulate host-cell functions. We conclude that LPG associates with membrane rafts in the host cell and exert its actions on host-cell actin and phagosomal maturation through subversion of raft function.


*Keywords:* *Leishmania*; Lipophosphoglycan; Membrane rafts; Phagosomal maturation; Actin

## 1. Introduction

The protozoan parasite *Leishmania donovani* causes visceral leishmaniasis (Kala Azar), and is transmitted to humans by infected *Phlebotomus* sand flies [1]. Its life cycle includes a flagellated, infective promastigote form primarily expressed in the gut of the sand fly, and an amastigote form, which is induced inside the macrophage phagosome in the mammalian host.

The ability of *L. donovani* to survive inside macrophages is crucial for establishment of infection [2], and depends on the action of several molecules including lipophosphoglycan (LPG) [3]. LPG is a polymer of the repeating Galβ1,4Manα-$PO_4$ unit linked to the membrane of the promastigote via a unique lipid anchor; 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol with an unusually long saturated fatty acid chain of 24–26 C [4].

LPG has several effects on macrophage functions [5], including inhibition of phagosomal maturation [6–8]. This is illustrated by the finding that wild type (WT) promastigotes block phago-lysosomal formation, whereas mutants lacking phosphoglycans, including LPG, are found in a phago-lysosomal compartment [6–8]. LPG causes accumulation of F-actin around phagosomes carrying WT *L. donovani* [8]. The periphagosomal F-actin, which could act as a physical barrier to prevent phago-lysosomal fusion, is formed through impaired dissociation of the actin regulators Cdc42 and Rac1 from the phagosomal membrane [9–11]. Upon parasite attachment, LPG is transferred from the promastigote surface to the host-cell

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plasma membrane [12]. The lipid anchor of LPG is characterized by an extended, saturated fatty acid residue [13], suggesting that it may be intercalated into host-cell detergent-resistant membranes, DRM. We have recently shown that lipoarabinomannan (LAM) from *Mycobacterium tuberculosis*, whose molecular structure is reminiscent of LPG, is inserted into the membrane rafts of the host cell [14]. From this platform, LAM is able to delay phagosomal maturation, thereby being beneficial for *M. tuberculosis* virulence. The aim of the present study was to investigate whether LPG from *L. donovani* acts in a similar manner.

## 2. Methods

### 2.1. Cells

Human monocyte-derived macrophages were isolated from heparinised donor blood as previously described [14]. The cells were differentiated in Macrophage SFM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 4 mM L-glutamine (Life Technologies). The cells were used after ten days of differentiation. Bone marrow-derived macrophages (BMM) were obtained by growing marrow cells from female BALB/c mice at 37 °C in 5% $CO_2$ in complete medium [Dulbecco's Modified Eagle Medium with glutamine (Life Technologies Inc., ON, Canada), containing 10% heat-inactivated FBS (Hyclone, Logan, UT), 10 mM HEPES pH 7.4, and antibiotics] in the presence of 15% (v/v) L929 cell-conditioned medium for seven days. BMM were made quiescent by culturing them in the absence of CSF-1 for 18 h prior to being used.

### 2.2. Cholesterol depletion

To deplete cholesterol, the medium was exchanged for fresh Macrophage SFM containing 10 mM β-cyclodextrin (βCD, Sigma Chemical Co) followed by incubation at 37 °C for 60 min. After three washes in 37 °C KRG with 2% bovine serum albumin (BSA, Boehringer-Mannheim GmBH) (KB), fresh Macrophage SFM was added. Using Amplex Red Cholesterol Assay Kit (Molecular Probes) we found that βCD extracted 60% of the cholesterol in the cells (not shown). Incubation in βCD made the cells round up, but viability was not significantly affected (not shown). Incubating the cholesterol-depleted cells in 10% AB serum over-night restored morphology and function (not shown).

### 2.3. Phagocytic prey and phagocytosis

Wild type *L. donovani* 1S promastigotes (WT) and the isogenic Galβ1,4Manα-$PO_4$-defective mutant *lpg2*$^-$KO, both expressing green fluorescent protein (GFP), were prepared as previously described [8]. The promastigotes were cultured at 26 °C in modified M199 medium with 500 μg/ml G418 (all from Gibco BRL/Life Technologies) [3]. Expression of GFP was assessed by fluorescence microscopy. The promastigotes were spun down and resuspended in the same volume of fresh growth medium 12–14 h before the experiment. Before addition to the macrophages, the promastigotes were again spun down and resuspended in fresh Macrophage SFM at 37 °C. Promastigotes in stationary phase of growth were added to the cells at a parasite-to-cell ratio of 10:1. After 20 min at 37 °C and 5% $CO_2$ (pulse), excess and unbound parasites were removed by three washes. Preparations for analysis of the distribution of LPG in the plasma membrane were fixed for 15 min at 4 °C in 2.0 % (w/v) paraformaldehyde (PFA, Sigma Chemical Co.) in KRG, and washed in PBS. For analysis of phagocytic capacity, periphagosomal F-actin and phagosomal maturation, incubation was continued at 37 °C for 30 min (chase), followed by fixation, as described above.

### 2.4. Fluorescent labelling

GM-1: cells were pre-fixed in 0.1% PFA in PBS, stained with Alexa Fluor 488-conjugated cholera toxin subunit B (CtxB) (Molecular Probes, Inc.) in PBS, washed and post-fixed for 15 min. LAMP-1 or LPG: fixed cells were incubated with PBS pH 7.6 with 2% BSA, 10% normal goat serum (Dakopatts AB) and 0.1% saponin (Sigma Chemical Co.), followed by washing and incubation with rat monoclonal antibodies against LAMP-1 (kindly provided by Dr. Sven Carlsson, Umeå University) or mouse monoclonal antibodies against LPG (CA7AE, Cedarlane Laboratories), washed and incubated with Alexa594 Fluor-conjugated goat anti-rat or anti-mouse antibodies (Molecular Probes, Inc.). Controls for unspecific labelling were made by substituting the primary antibody with purified rat or mouse IgG (not shown). F-actin: fixed cells were treated with PBS pH 7.6 with 2% BSA and 100 μg/ml lysophosphatidylcholine (Sigma Chemical Co.) and incubated with Alexa Fluor 594-conjugated phallacidin (Molecular Probes, Inc.). All cells were washed and mounted in an anti-fading medium with 20% Airvol 203 (Air Products and Chemicals, Utrecht, The Netherlands) and 4% Citifluor/Glycerol (Citifluor Ltd.) in 20 mM Tris buffer (pH 8.5) and left to set at 4 °C overnight.

### 2.5. Confocal microscopy

Confocal imaging was performed in a Sarastro 2000 microscope (Molecular Dynamics) equipped for dual activation and detection through a Nikon microscope with an ×60, NA 1.4 oil immersion objective. The 488 nm and 514 nm lines of the Argon laser were used for parallel excitation of FITC/GFP and Alexa594 Fluor. "535 nm" and "595 nm" beam splitters were employed for separation of the excitation and emission light, respectively. A 540DF30 band pass filter was employed for detection of the green signal (FITC/GFP) and an EFLP 600 long pass emission filter for the red signal (Alexa594 Fluor). This filter set-up ensured negligible red fluorescence in the green channel or vice versa.

### 2.6. Analysis of translocation of LAMP-1

Translocation of LAMP-1 to individual phagosomes was investigated in randomly selected confocal images of