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

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Original Publication:


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Running title: Lipophosphoglycan acts on membrane rafts

Key words: Leishmania, lipophosphoglycan, membrane rafts, phagosomal maturation, actin
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-PO₄ 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.
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-PO₄ 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 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% CO₂ 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 x60, 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 cholesterol-depleted or control cells after phagocytosis of WT *L. donovani* promastigotes for 20 + 30 min or 1µg/ml purified LPG for 50 min instead of the parasites. The translocation of LAMP-1 to individual phagosomes containing GFP-expressing
promastigotes was classified as either (+) or (−). Typically, 50-100 cells, from preparations done in duplicate on two or three independent occasions were analyzed under each condition.

2.7. Quantification of phagocytosis and periphagosomal F-actin

Phagocytic capacity was assessed after a 30-min chase to allow maximal internalization of the promastigotes. The number of GFP-expressing promastigotes per cell was counted in randomly scanned confocal images of samples labelled with Alexa594-phallacidin. All data were normalized against controls phagocytosing lpg2 KO promastigotes. The results were compiled from results from three independent experiments.

Periphagosomal F-actin was measured in randomly scanned confocal images of Alexa594-phallacidin-labeled samples containing GFP-expressing promastigotes as described in [8]. In short, the F-actin rim around each phagosome, i.e. periphagosomal F-actin, was manually traced in the red channel of the confocal image, the fluorescence intensity profile along the trace recorded, and the median intensity of the profile calculated. To avoid potential overlaps with cortical F-actin, only the part of the phagosome facing the cytosol was studied. The results were compiled from data obtained from samples prepared on at least three independent occasions. To compensate for possible variations in instrument performance between experimental days, all results were normalized against data from phagosomes in control cells incubated for 20 + 30 min with WT promastigotes and measured in parallel. The cells were focused in white light to avoid destruction of the fluorophores by bleaching and biased selection of certain sections of the preparations. Image analysis was performed on a Silicon Graphics OS2 workstation equipped with ImageSpace v3.2 (Molecular Dynamics).

2.8. Fractionation of cell membranes and detection of membrane components

Detergent resistant membranes were isolated as described in [14, 15] with some modifications. Briefly, adherent cells were incubated in 4°C lysis buffer, containing 1% Triton
X-100 (Merck-Schuchard), 2 μg/ml Aprotinin, 2 μg/ml Pepstatin, 2 μg/ml Leupeptin and 1 mM Pefa-block (all from Roche Diagnostics Corporation) for 30 min. Nuclei and whole cells were spun down at 500g for 10 min. The supernatant was mixed with an equal volume of 85% sucrose (w/v in lysis buffer) and transferred to an ultracentrifugation tube. A step gradient was constructed by overlaying the sucrose-sample mixture with 5 ml 30% sucrose followed by a layer of 3 ml 5% sucrose. The gradient was centrifuged for 17–19 h at 200 000g in an ultracentrifuge with a Beckman SW 41 rotor. After centrifugation, 10 fractions of 1 ml each were collected from top to bottom of the tube. The fractions were labelled 10 (top) to 1 (bottom, containing Triton X-100 soluble material). The fractions were transferred to nitrocellulose membranes by dot blot, and blocked in 5% non-fat milk in Tris-buffered saline (TBS) with 0.05 % Tween overnight at 4°C. GM-1 was detected using horse radish peroxidase (HRP)-conjugated CtxB (1:2500, Sigma Chemical Co.). Mouse monoclonal antibodies (all diluted 1:1000) were used to detect the repeating Galβ1,4Manα-PO₄ units present on LPG (CA7AE, Cedarlane Laboratories), CD44 and CD45, followed by HRP-conjugated goat anti-mouse antibodies (1:5000) (all from DAKO). The blots were analysed with a commercial enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience). Labelling was quantified from digital images of the blots using ImageJ v1.32j.

2.9. Colocalization of LPG and lipid rafts

Late stationary phase *L. donovani* promastigotes were opsonized with mouse serum [10]. Zymosan particles (Sigma-Aldrich) were washed, and coated with purified LPG, kind gift from Dr S. Turco, University of Kentucky, Lexington, KY, USA) as described [8]. For synchronized phagocytosis assays, macrophages were incubated at a particle-to-cell ratio of 10:1 for 15 min at 4°C. Excess particles were removed and phagocytosis was triggered by transferring the cells at 37°C for the indicated time points. Macrophages were next fixed,
permeabilized, and blocked as previously described [16]. Cells were incubated with the anti-LPG mouse monoclonal antibody (CA7AE) and labelled with Alexa488 Fluor-conjugated goat anti-mouse antibody (Molecular Probes, Inc.) and Alexa Fluor 594-conjugated cholera toxin subunit B (Molecular Probes, Inc). All coverslips were mounted on slides with Fluoromount-G (Southern Biotechnology Associates). Detailed analysis of protein localization on the phagosome was performed essentially as described [10] using an oil immersion Nikon Plan Apo 100 (N.A. 1.4) objective mounted on a Nikon Eclipse E800 microscope equipped with a Bio-Rad Radiance 2000 confocal imaging system (Bio-Rad).

2.10. Statistical analysis

Statistical analysis was performed using Student’s t-test. Error bars are SEM.
3. Results

3.1. LPG localizes to membrane rafts in *L. donovani*-infected macrophages

The cholera toxin subunit B (CtxB) binds to glycosphingolipids with a strong affinity for GM-1 and a lower affinity for other gangliosides [17], and can therefore be used as a marker for membrane rafts [18]. Fluorescent labelling of MDM with CtxB revealed that a substantial part of the plasma membrane contained GM-1 (not shown). Membrane extraction with cold Triton X-100 followed by density centrifugation, fractionation and dot-blot [14] showed that GM-1 was present in fractions 7 and 8 (Fig. 1A), indicative of the membrane raft fraction [19]. Another raft marker, CD44 [20], was also enriched in fractions 7 and 8 (Fig. 1A), whereas CD45, a molecule which is excluded from rafts [21], was found only in fraction 1 corresponding to the Triton X-100 soluble fraction (Fig. 1A). Membrane rafts are enriched in cholesterol [22, 23], and cholesterol-depletion by agents such as β-cyclodextrin (βCD) disrupts their structure and function [24]. The cholesterol content of MDM was reduced by approximately 60% after incubation in 10 mM βCD for 60 min at 37°C (not shown). Cholesterol-depleted macrophages remained adherent, but displayed a more rounded morphology compared to controls (not shown). Membrane fractionation showed a pronounced reduction of CD44 in rafts following incubation in βCD as well as a 50% loss of GM-1 reactivity (Fig. 1A). When MDM were infected with WT *L. donovani* promastigotes, dot-blot analysis of the membrane fractions revealed an enrichment of LPG in fractions 7 and 8 (corresponding to membrane rafts; Fig. 1B). However, the promastigote membrane itself also contains rafts rich in LPG [25], and the detergent-resistant membrane in fractions 7 and 8 could thus represent a mixture of macrophage and promastigote rafts. To assess whether the detected raft fractions originated from the macrophages and/or the parasites, we subjected the equal number of parasites as used for infections to Triton X-100 extraction followed by density gradient centrifugation and dot-blot. When comparing this sample with a preparation
of macrophages plus promastigotes, we found that LPG originating from promastigote rafts represented approximately 50% of the total amount of LPG detected in fractions 7 and 8 (not shown).

Figure 1. Dot-blot analysis of membrane fractions from human monocyte-derived macrophages (MDMs). Lysates of MDMs were applied on a sucrose gradient and fractionated by centrifugation. Ten fractions were collected and analyzed using dot-blot. To disrupt membrane rafts the MDMs were preincubated in β-cyclodextrin (βCD) before lysis. GM1 was detected with the β-subunit of cholera toxin, CD44 and CD45 were detected with mouse monoclonal antibodies. Blots from representative experiments are shown (n=3-5) in A. Arrows indicate fractions containing membrane rafts (fraction 7 and 8), characterized by the presence of GM1 and CD44 and the absence of CD45. B: Analysis of GM1 and LPG after membrane fractionation of MDMs infected with WT L. donovani promastigotes.
To further demonstrate the localization of LPG in membrane rafts, we infected macrophages with either WT promastigotes or LPG-coated zymozan. As shown in Fig. 2A, at 10 min (left panel) and 30 min (right panel) after the initiation of phagocytosis, LPG was present in the phagosome membrane and colocalized with GM-1 (arrows). We also observed that at 30 min, the distribution of GM-1 labelling in the phagosomal membrane was more uniform than at 10 min, suggesting that LPG may influence raft integrity. Similar results were obtained following the internalization of LPG-coated zymozan, where colocalization of LPG and GM1 was observed at the phagosomal membrane (Fig. 2B).

**Figure 2.** Insertion of LPG into GM1-positive lipid rafts on phagosomal membranes. A. BMM were infected with WT promastigotes for 10 min (left panel) or 30 min (right panel), fixed, and labelled for LPG (green) and GM1 (red). Colocalization analysis showed that LPG is delivered early to GM1-enriched domain on newly forming phagocytic cup (left panel). Insertion of LPG into lipid microdomains is accompanied by a loss of the punctuated distribution of GM1 in the phagosomal membrane (right panel). B. BMM were allowed to internalize LPG-coated zymozan for 10 min, fixed, and labelled for LPG (green) and GM1 (red). LPG colocalizes with GM1-enriched domain. Bar, 3 µm.
3.2. The effect of LPG on phagosomal maturation requires membrane rafts

To further investigate the importance of membrane rafts in *Leishmania* pathogenesis, we studied the effect of cholesterol extraction on phagocytosis, periphagosomal F-actin and phagosomal maturation in *L. donovani*-infected cells. Macrophages ingested lpg2KO promastigotes slightly more effectively than WT promastigotes (Fig. 3). Cholesterol-depletion reduced the capacity of macrophages to ingest *L. donovani* by 29% for lpg2KO promastigotes and by 36% (*p*<0.01) for WT promastigotes compared to non-treated cells (Fig. 3). Quantification of periphagosomal F-actin around promastigote-containing phagosomes showed that its accumulation around phagosomes carrying WT promastigotes was reduced in cholesterol-depleted cells (1.0 ± 0.08 vs. 0.62 ± 0.06, *p*<0.001; Fig. 4A). Cholesterol-depletion itself caused a slight increase in periphagosomal F-actin (0.59 ± 0.04 vs. 0.46 ± 0.03, *p*<0.01) around phagosomes containing the lpg2KO mutant (Fig. 4A). The decreased levels of F-actin around WT promastigote-containing phagosomes in cholesterol-depleted cells correlated with increased translocation of the late endosomal marker LAMP-1 to these phagosomes (Fig. 4B-E). Thus 62.5 (± 5.9, SEM) % of WT promastigote-containing phagosomes were LAMP-1 positive in cholesterol-depleted macrophages, compared to 27.0 (± 3.1, SEM) % in control cells (*p*<0.01). We found no evidence of reduced transfer of LPG to the plasma membrane of cholesterol-depleted macrophages compared to controls (not shown).
Figure 3. Phagocytic capacity of monocyte-derived macrophages (MDMs) interacting with L. donovani promastigotes. Cholesterol was extracted from the plasma membrane of MDMs using β-cyclodextrin (βCD). The cells were then infected with GFP-expressing WT or lpg2 KO promastigotes followed by fixation. The preparations were labelled with phallacidin and the average number of promastigotes per cell, in random confocal images, was assessed. Each group contains data from 124–308 cells from at least three independent experiments. Error bars indicate standard error of the mean (SEM). ** represents statistically significant differences p<0.01.
Figure 4. Periphagosomal F-actin and translocation of LAMP-1 to L. donovani phagosomes. Monocyte-derived macrophages (MDMs; A open bars, B, D) or MDMs in which cholesterol has been extracted from the plasma membrane with β-cyclodextrin (βCD; A filled bars, C, E) were allowed to interact with GFP-expressing WT or lpg2–KO promastigotes. (A) Fixed preparations were labelled with phallacidin, and examined by confocal microscopy. Periphagosomal F-actin was measured in randomly scanned confocal images. Each group contains data from 108–130 phagosomes from at least three independent experiments. Error bars indicate standard error of the mean (SEM). (B-E) Fixed cells were labelled with antibodies directed towards LAMP-1, and subjected to confocal microscopy. Images show the distribution of LAMP-1 (red channel; D-E) in MDMs containing WT promastigotes (green channel). Merged images are shown in B and C. The percentage of LAMP-1 positive phagosomes of more than 50 phagosomes for each condition and from three separate experiments was determined, and is shown in D (untreated, 27%) and E (βCD-treated, 63%). SEM is shown in brackets. Arrows indicate phagosomes. The scale bar is 10 µm. ** and ***, represent statistically significant differences, p<0.01 and p<0.001, respectively.
Discussion

The aim of this study was to determine whether LPG affects the phago-lysosomal fusion machinery in human macrophages by disturbing membrane raft function. We found that LPG is transferred to membrane rafts of the host cells during infection in a way reminiscent of *M. tuberculosis* LAM [14]. Like LAM, LPG is known to retard the phagosomal maturation process in macrophages [8], and we show here that this ability of LPG is dependent on functional membrane rafts. The consequences of LPG-insertion into rafts, ultimately resulting in retarded phagosomal maturation, still remain obscure. We know from earlier studies that LPG causes an accumulation of periphagosomal F-actin [8] which is achieved by prevention of the release of active Cdc42 and Rac1, known regulators of F-actin, from the phagosomal membrane [9, 10]. Although further investigation is required, the present data suggest that intact rafts are required for LPG to retain Cdc42 and Rac1 at the membrane, thereby preventing their inactivation. Similarly, functional membrane rafts are essential for recruitment and assembly of the NADPH oxidase complex [26]. Our data raise the possibility that association of LPG with these membrane microdomains contributes to the observed LPG-mediated inhibition of NADPH oxidase assembly at the phagosome membrane [27].

The reduced effect of LPG on periphagosomal F-actin and phagosomal maturation observed in cholesterol-depleted cells could equally well/alternatively be attributed to reduced transfer of LPG to membranes in these cells. However, this was ruled out by our control experiment showing that LPG translocated efficiently to membranes of cholesterol-depleted cells.

The observation that LPG localises to the raft fraction of MDMs during infection with WT *L. donovani* promastigotes is not surprising since there is a striking biophysical resemblance
between the LPG lipid anchor, with its unusually long, fully saturated fatty acid residue [13], and the highly saturated sphingolipids present in the target membrane [22].

Previous studies have shown that insertion of LPG into one leaflet of a lipid bilayer is sufficient to increase total membrane rigidity and inhibit membrane fusion [28, 29]. Similarly, increased membrane rigidity due to integration of LAM has been shown to reduce phagolysosomal fusion [30]. Thus, alteration of the biophysical properties of membranes carrying these microbial glycolipids may be an alternative explanation for the reduced ability of the host macrophages to accomplish phagosomal maturation upon infection.

Pucadyil et al. [31] found that cholesterol depletion resulted in reduced uptake of unopsonized WT L. donovani promastigotes while phagocytosis of opsonized promastigotes and Escherichia coli remained unaffected. This suggests that the receptor(s) responsible for internalization of unopsonized promastigotes are localized to membrane rafts, or depend on intact rafts for function. Our results showing reduced uptake of both WT and lpg2 KO promastigotes after cholesterol depletion point towards involvement of raft-associated receptors in the phagocytic process of L. donovani. However, since phagocytosis was not completely abolished in the absence of functional rafts additional uptake mechanisms may be involved.

We have previously shown that LPG prevents translocation of PKCα to the newly formed phagosomes, and that this correlates with accumulation of periphagosomal F-actin [8]. Macrophages depleted of cholesterol also fail to translocate PKCα to the membrane upon stimulation with phorbolmyristate acetate (unpublished data), but this only slightly increases the amount of F-actin around the phagosome (Fig. 4A, lpg2-KO phagosomes). Therefore, we
concluded that impaired translocation of PKCα only partially delivers an explanation for the accumulated F-actin at the *L. donovani* phagosome. A more profound effect of the interaction of LPG with rafts may be the retention of Cdc42 and Rac1 causing accumulation of F-actin [9, 10] and an inhibition of the assembly of the NADPH oxidase at the phagosome membrane [27].

In conclusion, the present study shows that during phagocytosis of *L. donovani* promastigotes, LPG partitions to membrane rafts in the phagosomal membrane. Functional membrane rafts are required for the action of LPG on host-cell actin and for inhibition of phagosomal maturation. Our results show that functional rafts of the host cell are required for LPG to exert its action and thus for *Leishmania* virulence. The transfer of glycolipids from pathogens to host cell rafts, as observed with *M. tuberculosis* [14] and *L. donovani* (present study), may represent a general mechanism for manipulation of host cell function.
4. Acknowledgements

We are grateful to Dr. Sam Turco, University of Kentucky, USA, for providing purified LPG and to Dr. Sven Carlsson, Umeå University, Sweden for providing the LAMP-1 antibody. The project was supported financially by the Swedish Medical Research Council (grants # 6251; K.E.M., # 13103; E.S.), the Swedish Research Council (grants # 621-2001-3570; K.E.M., 529-2003-5994; M.L., 2005-7046; M.L. and 521-2002-6393; B.R.), the Swedish Society for Medical Research (B.R.), the Swedish Medical Association (B.R., E.S.), Magn. Bergvalls Stiftelse (B.R.), Stiftelsen Lars Hiertas Minne (B.R., E.S.), and the Canadian Institutes of Health Research (grant # MOP-12933; A.D.). A.D. is the holder of a Canada Research Chair and was chercheur-boursier from the FRSQ. A.F.V. was partly supported by a studentship from the Fondation Armand-Frappier. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
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