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Modulation of phagolysosome biogenesis by the lipophosphoglycan of *Leishmania*

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Abstract

Promastigotes of the protozoan parasite *Leishmania* are inoculated into the mammalian host by an infected sandfly and are phagocytosed by macrophages. There, they differentiate into amastigotes, which replicate in phagolysosomes. A family of glycoconjugates, the phosphoglycans (PGs), plays an important role in the ability of promastigotes to survive the potentially microbicidal consequences of phagocytosis. Lipophosphoglycan (LPG), an abundant promastigote surface glycolipid, has received considerable attention over the past several years. Of interest for this review, lipophosphoglycan confers upon *Leishmania donovani* promastigotes the ability to inhibit phagolysosome biogenesis. This inhibition correlates with an accumulation of periphagosomal F-actin, which may potentially form a physical barrier that prevents *L. donovani* promastigote-harboring phagosomes from interacting with late endosomes and lysosomes. Thus, similar to several other pathogens, *Leishmania* promastigotes hijack the host cell's cytoskeleton early during the infection process. Here, we review this phenomenon and discuss the potential underlying mechanisms.
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Keywords: *Leishmania*; Macrophage; Phagocytosis; Virulence; Glycolipids

Introduction

The parasite Leishmania

Protozoan parasites of the genus *Leishmania* are responsible for a spectrum of diseases ranging from self-healing ulcers to fatal visceral infection in humans. Based on the symptoms and clinical manifestations caused by the various *Leishmania* species, three principle and distinct forms of human leishmaniases are delineated. Cutaneous leishmaniasis, which is caused by *Leishmania major*, *Leishmania tropica*, *Leishmania mexicana*, and various South American species and subspecies, is characterized by the presence of ulcerative lesions of the skin. The lesions are generally self-healing, although some forms may persist and disseminate. A variant form of cutaneous leishmaniasis, mucocutaneous leishmaniasis, is caused by *Leishmania braziliensis braziliensis*. This parasite has a particular tropism for the macrophages of the oronasopharyngeal region. Such tissue tropism remains a poorly understood phenomenon and may involve several factors including sensitivity of the parasite to temperature, complement, as well as permissiveness of the various macrophage populations. The most severe form of the various diseases caused by *Leishmania* is visceral leishmaniasis, also known as Kala-azar. Here, *Leishmania donovani* or *Leishmania chagasi* disseminates and infects macrophages of the liver, the spleen, and the bone marrow. This infection is chronic and may be fatal if left untreated.

Transmission of the parasite is mediated by the blood-sucking female sandfly, of either the genus *Phlebotomus* (in the Old World) or the genus *Lutzomyia* (in the New World). In the sandfly, *Leishmania* exists in the promastigote form, which is approximately 10 to 20 μm in length and 2 μm in width with a long anterior flagellum. Dividing, noninfective

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promastigotes (procyclic) are attached to the midgut epithelium and transform into a nondividing infective (metacyclic) form [1]. Metacyclic promastigotes detach from gut epithelial cells and migrate towards the anterior end of the digestive tract and are inoculated into the vertebrate host during the blood meal of the infected sandfly. There, promastigotes resist the lytic action of complement and are phagocytosed by mononuclear phagocytes. Once inside, they differentiate into nonmotile amastigotes, which are 2–5 μm in length and oval to round in shape, and proliferate within acidic and hydrolase-rich phagolysosomes [2,3]. Propagation of amastigotes to uninfected macrophages occurs when infected macrophages rupture, leading to their release into the surrounding environment. When feeding on an infected mammal, the sandfly may ingest amastigotes or amastigote-infected phagocytes. During digestion of the blood meal, amastigotes differentiate into promastigotes, which attach to the sandfly midgut epithelium, thus avoiding being excreted.

Leishmania is thus exposed to hydrolytic environments in both hosts, namely, the sandfly midgut and macrophage phagolysosomes [4]. Existence of *Leishmania* in both hosts requires the expression of stage-specific virulence determinants, including a family of related glycoconjugates, the phosphoglycans, which have in common repeating Galβ1,4Man-PO_4 units [5]. In this review, we will discuss the role of this family of glycoconjugates during the establishment of infection in mammals, with an emphasis on the macrophages and inhibition of phagosome maturation.

The phosphoglycans: a family of *Leishmania* glycoconjugates

Leishmania synthesizes a family of glycoconjugates which have in common the disaccharide-phosphate Galβ1,4Man-PO_4, which is not found in mammals. Some members of this phosphoglycan family are membrane-bound, whereas other members are secreted.

Lipophosphoglycan

Lipophosphoglycan (LPG), the major surface glycoconjugate of promastigotes [5–8], was the first member of this family of glycoconjugates to be characterized at the structural level. At approximately 5 million copies per cell, LPG covers the entire surface of the promastigote to form a dense glycocalyx. In essence, LPG consists of a polymer of repeating Galβ1,4Man-PO_4 units attached to a glycan core that is inserted into the membrane via a 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol anchor (Fig. 1). The molecule is capped by a small oligosaccharide structure which varies among species. The backbone of the repeating units is conserved, but the LPGs from several *Leishmania* species may have additional oligosaccharide chains branching off the backbone sugars [5–8]. Finally, LPGs from all species of *Leishmania* have an identical lipid anchor and glycan core.

LPG undergoes several important modifications during the life cycle that are characteristic for each *Leishmania* species. During the acquisition of virulence features in the sandfly midgut, LPG elongates, the number of repeating units doubling from about 15 in procyclic promastigotes to about 30 in metacyclic forms [5]. In addition, substitutions to the repeating units occur in several *Leishmania* species. These structural changes are required for the detachment of infectious, metacyclic promastigotes from the sandfly midgut (discussed below) [9] and contribute to the ability of metacyclic promastigote to resist complement-mediated lysis in the mammalian host [10]. In addition to stage-specific structural features, LPG levels are down-regulated in the amastigote stage by at least three orders of magnitude [11,12].

Surface or secreted phosphoglycans

In addition to LPG, repeating Galβ1,4Man-PO_4 units are present on various secreted molecules. One such molecule, the secreted extracellular phosphoglycan (PG, formerly 'excreted factor'), consists of a polymer of disaccharide-phosphate repeating units bearing LPG-like capping sugars [13]. Other molecules, which include the secreted acid phosphatase (sAP) and a secreted mucin-like proteophosphoglycan (PPG) [14–16], consist of proteins to which the repeating Galβ1,4Man-PO_4 units are *O*-linked to phosphoserine residues [14].

LPG-related glycolipids: the glycosyl-inositolphospholipids

A family of small and structurally related glycosyl-inositolphospholipids (GIPLs) is present at high levels in both promastigotes and amastigotes. GIPLs vary in sugar and lipid compositions; some GIPLs are precursors of LPG or protein GPI anchors, whereas others are distinct surface entities [17,18].

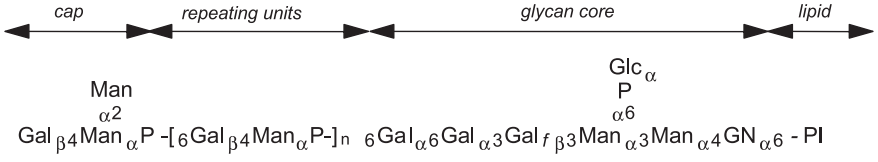

Fig. 1. Structural organization of *L. donovani* LPG.

Roles of LPG in the mammalian host

Biochemical and genetic evidence revealed that LPG is required for the interactions of promastigotes with both sand flies and mammals. In the sandfly, LPG is critical for the attachment and detachment of the promastigotes from the midgut epithelium and protects them during digestion of the blood meal. Moreover, the interspecies polymorphisms in the LPG phosphoglycan domain may account for species-specific vector competence [9]. Details on the roles of LPG in the interaction of *Leishmania* with the sandfly can be found in recent reviews [9,19].

Interaction with serum components

Following their inoculation into the mammalian host, metacyclic promastigotes are exposed to serum components. Promastigotes resist the lytic action of the complement system and use it to gain access to a safe heaven, the inside of a macrophage [20]. LPG confers resistance to the lytic action of complement in humans, but not in mice [10,21]. Binding to other serum proteins may also promote promastigote uptake by host macrophages. Mannose-terminating oligosaccharides in the cap structure of LPG bind the mannan-binding protein which acts as an activator of the complement cascade, thereby providing an additional mechanism for the formation of a C3 convertase and the subsequent formation of C3b which participates in the attachment to macrophages [22]. Uptake of *L. donovani* promastigotes by human macrophages is also enhanced by opsonization with the C-reactive protein, a major acute phase protein present in the serum during inflammation which specifically binds to the Gal(β1,4)Man(α1-PO_4) repeating units of *L. donovani* LPG [23].

Depending on the *Leishmania* species, LPG contributes to the establishment of infection inside macrophages possibly by creating conditions propitious for the promastigote-to-amastigote differentiation [8,21,24–27]. The requirement for LPG in the establishment of macrophage infection by *L. major* and *L. donovani* was evidenced by the demonstration that LPG-defective mutants are destroyed following phagocytosis and that restoration of LPG expression by genetic complementation restored their ability to survive [26,28]. In the case of *L. mexicana*, phosphoglycan-defective mutants appear as virulent as their wild-type counterparts [16,29], suggesting that, for this species, phosphoglycan synthesis is not an absolute requirement for virulence.

Interaction with the macrophage

To fully grasp the strategies by which microorganisms such as *Leishmania* manage to survive inside macrophages, one must take a closer look at the phagocytic process itself. Phagocytosis is the way by which macrophages internalize large particles [30]. It is a receptor-mediated process and is initiated by the interaction of either ligands intrinsic to the particle (in the case of nonopsonic phagocytosis) or opsonins (such as complement components and immunoglobulins) with phagocytic receptors [30,31]. The resulting vacuole, which is initially formed by the invagination of the plasma membrane, is termed a phagosome. Newly formed phagosomes undergo several transformations as they interact with the surrounding endocytic organelles, a process referred to as phagosome maturation which usually leads to the formation of phagolysosomes [30,31]. These microbicidal organelles are fully competent to process antigens and generate peptide:MHC-II complexes that are transported to the cell surface and presented to T cells [32]. Thus, phagosome maturation is a key process in the initiation of adaptive immune responses.

Recognition and attachment

Engulfment of *Leishmania* promastigotes by macrophages is initiated by the recognition of surface receptor molecules by the parasite and the host cell. Two major molecules of the parasite, LPG and gp63, are probably involved in binding, given that they interact with the complement units C3b and C3bi [33]. Indeed, the macrophage complement receptors (CRs) CR1 and CR3 (Mac-1) have been shown to mediate promastigote attachment and entry [34]. However, LPG is not necessary for binding or internalization of promastigotes, since *Leishmania* mutants defective in phosphoglycan synthesis are internalized with equal or even greater efficiency by macrophages [28,35]. Although CRs are implicated in the phagocytosis of either unopsonized or complement-opsonized promastigotes, other receptors, such as the mannose-fucose receptor (MFR), are also involved [34]. The major consequence for using such receptors is the absence of a proinflammatory responses and no oxidative burst, thus enabling promastigotes to enter macrophages silently [36]. Hence, the role of LPG and other promastigote surface molecules, along with their macrophage receptor counterparts, in *Leishmania* entry needs to be further investigated.

Evasion of proinflammatory signaling

Failure to activate macrophages during the invasion process may contribute to the successful establishment of *Leishmania* within the mammalian host [37]. In murine bone marrow-derived macrophages, attachment and internalization of *L. donovani* promastigotes do not induce the phosphorylation of the three major mitogen-activated protein kinases (MAPKs), namely, p38 MAPK, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK). In contrast, in IFN-γ-primed macrophages, *L. donovani* promastigotes activate ERK1/2 and p38 MAPK which are both required for parasite-induced

TNF-α release [38]. Since MAPKs play a central role in the regulation of innate immune responses, including the production of proinflammatory cytokines and NO, evasion of MAPK pathways activation by *L. donovani* promastigotes may therefore represent a key step in the evasion of macrophage innate functions. However, in contrast to wild-type promastigotes, LPG-defective mutants selectively induce a rapid and transient activation of ERK1 and ERK2 [38], suggesting that LPG repeating units may contribute to the silent entry of promastigotes inside naive macrophages. One possible explanation is that, in the absence of LPG, which forms a tick glycocalyx covering the entire surface of promastigotes, molecules normally buried under LPG are recognized by macrophage receptors.

Inhibition of protein kinase C activity

Manipulation of signaling pathways by pathogenic microorganisms is a widely used strategy to subvert host cell functions [39,40]. To turn off host microbicidal functions, *Leishmania* activates macrophage phosphotyrosine phosphatases and inhibits protein kinase C (PKC) activity [39,41,42]. A role for LPG in the inhibition of macrophage signaling pathways was suggested with the in vitro demonstration that LPG is a potent inhibitor of PKC activity ($K_I < 1$ μM) [43]. The 1-*O*-alkylglycerol fragment exhibits the most potent inhibitory activity, although the phosphoglycan portion also causes significant inhibition of purified PKC activity [44]. GIPLs, which represent the most abundant glycoconjugates of the amastigote stage, also display an inhibitory activity towards PKC [44]. Treatment of macrophages with purified LPG inhibits several PKC-dependent events including induction of the oxidative burst [45], LPS- and diacylglycerol-induced c-*fos* gene expression [46], and the phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) [35]. Despite the positions of LPG and PKC on opposite sides of the membrane, LPG is capable of trans-bilayer inhibition of PKC activity [47]. Moreover, while 1% or 2% LPG modestly inhibited binding of PKC to sucrose-loaded vesicles, the presence of 5% LPG completely prevented binding. Although the implications are not fully elucidated, impairment of PKC-dependent pathways by parasite-derived LPG and other glycolipids may contribute to the ability of *Leishmania* to attenuate macrophage antimicrobial functions.

Phagocytosis of Leishmania

In the early steps of phagocytosis, receptor recognition of the foreign particle leads to a localized accumulation of F-actin at the site of internalization and the beginning of an F-actin-rich phagocytic cup surrounding the phagosome [48,49]. In some cases, protruding extensions of the cytoplasm, driven by actin-derived motors, also help in enveloping the particle [50]. These early events are followed by the activation of kinases, changes in phospholipid metabolism, and membrane trafficking [48,50]. For example, type I PI 3-kinases (p85α/p110β) are involved in phosphatidylinositol 3,4,5-triphosphate (PI3,4,5P) production, whereas type III PI 3-kinases (Vps34-like) generate phosphatidylinositol 3-phosphate (PI3P) [51]. The accumulation of PI3,4,5P around the phagocytic cup and the inhibition of phagocytosis by the PI 3-kinase inhibitors wortmannin and LY294002 [52], illustrate that type I PI 3-kinases act early in the process of phagocytosis [31,51]. In contrast, type III PI 3-kinases exert a strong effect on later steps of phagosomal maturation, particularly through the action of PI3P, which serves to recruit the FYVE domain of the early endosomal proteins EEA1 and Rabenosyn-5. Hence, several components of the early endocytic compartment are involved in the maturation of the nascent phagosome.

To survive intracellularly, parasites use several strategies to perturb cellular processes [53]. For instance, *Mycobacterium tuberculosis* stalls the maturation of the phagosome and replicates in a vacuole similar to that of early endosomes [54]. In the case of *Leishmania*, in addition to using a biphasic life cycle adapted to different environments, promastigotes modulate the molecular composition of the phagosome early after its formation.

Disruption of phagosomal maturation

Usually, early endosomes interact with phagosomes within minutes of their formation, and these interactions may be facilitated by the greater accessibility of the phagosomal membrane as the F-actin-rich phagocytic cup disappears [30,55]. The small GTPase Rab5 is essential for this interaction to take place [56]. Although exactly how Rab5 is recruited to the phagosome remains to be determined, this small GTPase recognizes both types I and III PI 3-kinases in its GTP-activated form. It is required for homotypic and heterotypic endosomal fusion, through several effector proteins such as EEA1, Rabaptin-5, and Rabex-5. Although the latter two effectors are important for Rab5 action on early endosomes, their presence on phagosomes remains to be established. Nevertheless, Rab5 and EEA1 participate in the tethering of endosomes to the phagosome, since disrupting the function of either protein affects phagosome biogenesis [51,57] and impairs recruitment of lysosomal proteins such as the LAMPs.

The central role of Rab5 in endosome–phagosome interaction makes it a potentially important target for intracellular parasites in the macrophage. Indeed, when Rab5 is constitutively locked in an active form, *L. donovani* promastigotes survival is enhanced 5- to 10-fold [56]. The expression of the mutant Rab5 protein also leads to uncontrolled fusion events and the formation of giant phagosomes, an effect due to the continual fusion of endosomes with the phagosome [56].

Although several other Rab proteins associate with the phagosome, very little is known with respect to their exact function in phagocytosis. Rab7, by analogy to Rab5 for early endosomes, is involved in the interaction of the phagosome with late endosomes and in its maturation into a phagolysosome [57]. Therefore, specific Rab proteins would be involved with the sequential exchange of factors between the early or late endocytic compartments with the phagosome. This progressive transfer of proteins, through transient fusion events ("kiss-and-run") with selected organelles, ultimately leads to the mature phagolysosome [58,59].

In the case of phagosomes harboring *L. donovani* promastigotes, interactions with late endosomes and lysosomes are inhibited [60] (Fig. 2). This process is characterized by the impaired recruitment of proteins such as Rab7 and PKC-α, and a slower recruitment of others such as the LAMPs [61,62]. Using phosphoglycan-defective mutants, we showed that this inhibition of phagosome maturation is dependent on LPG repeating unit domain, for phagosomes harboring phosphoglycan-defective mutants quickly mature into Rab7- and LAMP-positive phagolysosomes [60,61]. The exact mechanism by which LPG exerts such an effect on the phagosome remains unclear; however, the insertion of LPG in cellular membranes [63] may alter their fusogenic properties [64].

Another important effect of LPG on the phagosomal membrane is the disruption of lipid rafts. Lipid rafts are cholesterol-rich specialized microdomains present at the plasma membrane, on endosomes and phagosomes. They also have a high content of glycosphingolipids and sphingomyelin. Several proteins are present in rafts, particularly those which have lipid modifications, such as GPI-linked, palmitoylated, or myristoylated proteins. Signaling molecules associated to receptor complexes are recruited to lipid rafts, underlining their role as important signaling platforms [65]. These crucial functions are potential targets for many toxins and pathogens, thus making rafts an ideal gateway to invade host cells [66]. Lipid rafts found on phagosomes are devoid of LAMP1 but enriched in flotillin-1, α and β subunits of heterotrimeric G proteins, and the proton pump V-ATPase [67]. In macrophages infected with *L. donovani* promastigotes, flotillin-rich lipid rafts are poorly recruited to phagosomes [67], possibly a consequence of the insertion of LPG in the rafts through its GPI anchor.

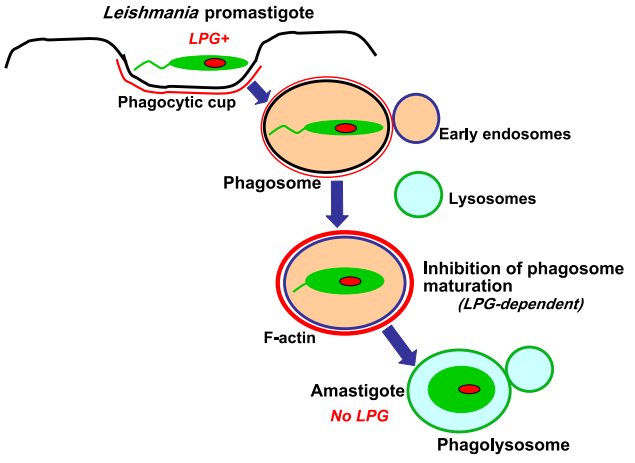

Fig. 2. Schematic diagram illustrating inhibition of phagosome maturation in a macrophage that has ingested a *L. donovani* promastigote. Although F-actin (red ring) accumulates around them, promastigote-harboring phagosomes interact with early endosomes (blue). However, these phagosomes do not interact with late endosomes or lysosomes (green) until the promastigote has fully differentiated into an amastigote.

Whereas *L. donovani* and *L. major* promastigotes inhibit phagosome maturation, this is not necessarily the case for all *Leishmania* species. A striking example is that of *Leishmania amazonensis*, of which infected macrophages display no impaired protein recruitment at their phagosomal membrane or reduced interaction with the endocytic-lysosomal pathway [68]. Furthermore, LPG-deficient *L. mexicana* promastigotes differentiate into amastigotes and proliferate as efficiently as their wild-type counterparts in mouse peritoneal macrophages [25]. Of interest, both *L. amazonensis* and *L. mexicana* amastigotes reside in large communal parasitophorous vacuoles, compared to the tight single parasite harboring phagosomes found in *L. donovani*- or *L. major*-infected macrophages [69]. It is also noteworthy that when amastigotes of either species of *Leishmania* enter macrophages, no inhibition of phagosome maturation is observed [69,70]. This corresponds to the absence/low levels of LPG present in the mammalian stage of the parasite.

F-actin, its effectors, and the Leishmania-containing phagosome

The fact that cytochalasin B, an F-actin polymerization inhibitor, blocks internalization of antibody-coated erythrocytes was one of the first observations that underlined the importance of F-actin in phagocytosis. F-actin recruitment to the phagocytic cup—in membrane ruffles and protrusions that surround nascent FcγR-driven (type I) phagosomes—is not specific to FcγR-mediated phagocytosis; F-actin also clusters around early phagosomes formed by complement-receptor (CR)-mediated internalization (type II phagocytosis). This is of interest since CR-mediated phagocytosis is otherwise mechanistically distinct from type I phagocytosis in that complement-opsonized particles sink (without the need of extending protrusions) into the plasma membrane without eliciting any inflammatory response. F-actin is also found on early phagosomes containing targets ingested through mannose-fucose (MFR) or scavenger receptor (SR)-mediated internalization. Although MFR- and SR-driven phagocytoses are mechanistically dynamic (involving F-actin-rich protrusions and ruffles, as in FcγR-mediated phagocytosis), they are similar to CR-mediated internalization in as much as they do not trigger inflammation [30,48,50]. F-actin is therefore essential for phagocytosis

and, as will be further discussed, is involved in several steps of the phagosome's formation and maturation.

Since *L. donovani* promastigotes preferentially use the complement or mannose-fucose receptors for entry into macrophages, the nascent phagosome is partially enveloped by an F-actin-rich phagocytic cup during the first steps of the internalization process. Whereas the presence of periphagosomal F-actin is normally transient, phagosomes harboring wild-type *L. donovani* promastigotes are characterized by an accumulation of F-actin [62] (Fig. 3). This may not hinder the interaction of the young phagosome with early endosomes (and Rab5 recruitment), but the accumulating F-actin may eventually form a barrier to prevent interaction of between the phagosome and late endocytic-lysosomal compartments [62]. Induction of periphagosomal F-actin accumulation has not yet been described for other intracellular microorganisms, but it is reminescent of the retention of coronin-1 (TACO) around *Mycobacterium*-containing phagosomes [71].

The mechanism by which *L. donovani* promastigotes induce the F-actin accumulation around the phagosome is not yet fully understood; however, genetic and biochemical evidence indicates that LPG is essential for this process to take place [62]. Hence, no accumulation of periphagosomal F-actin is observed during the phagocytosis of *L. donovani* LPG-defective promastigotes [62]. Furthermore, LPG-coated heat-killed yeast induces progressive accumulation of F-actin around the phagosome, whereas no F-actin is detected around "naked" heat-killed yeast-containing phagosomes [62]. Although the phenomenon of periphagosomal F-actin accumulation has not been surveyed for all *Leishmania* species, it has not been observed around *L.*

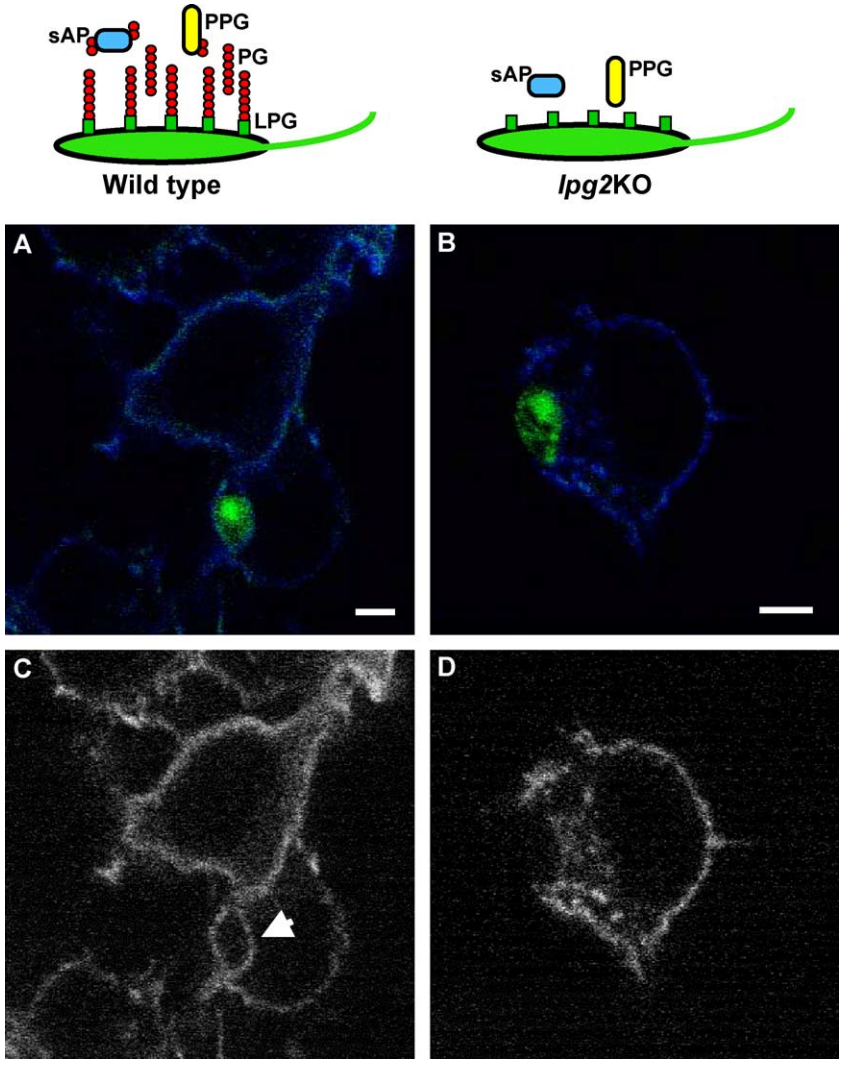

Fig. 3. LPG-dependent periphagosomal F-actin accumulation. *Leishmania* promastigotes synthesize large amounts of glycoconjugates that contain repeating phosphoglycan units (red circles). *lpg2KO* promastigotes are defective in phosphoglycan synthesis, including LPG. RAW264.7 cells were infected with either wild-type or LPG-deficient *L. donovani* promastigotes expressing GFP. Macrophages were then fixed and stained with phalloidin to detect F-actin. WT promastigotes (green in merged image A) are found in phagosomes that are surrounded by F-actin (in blue in merged image A, and arrow in C, which shows F-actin staining as white), whereas no F-actin accumulates (blue in merged image B) around LPG-deficient promastigote (green in merged image B)-containing phagosomes (see corresponding F-actin staining in D). Bar = 3 μm.

amazonensis promastigote-harboring phagosomes [68]. These observations hint to the different use of cellular F-actin by the various *Leishmania* species.

F-actin polymerization in phagocytosis is brought on by several different factors: for example, the previously mentioned PI3,4,5P and phosphatidylinositol 4,5 biphosphate (PI4,5P), both of which are synthesized early during phagosome assembly [31]. Another group of F-actin effectors are the Rho-family of small GTPases, which are involved in cell morphology and cytoskeletal remodeling besides phagocytosis [72,73]. Two members of this family, Rac1 and Cdc42, are essential for FcγR-mediated internalization (type I phagocytosis), whereas RhoA is essential for CR-mediated phagocytosis (type II phagocytosis) [74]. These small Rho-family GTPases are activated by guanine-nucleotide exchange factors (GEFs); however, no GEF has yet been clearly shown to be linked to phagocytosis. Another small GTPase important for FcγR-mediated uptake is ARF6 of the ADP-ribosylation factor (ARF) family, which is necessary for membrane protrusion and extension during phagocytosis, possibly through its interaction with the Rac1 pathway [75,76]. Signaling by the small GTPases leads to the activation and recruitment of other downstream factors early in the engulfment process.

Very little is known about the role of Rho family of GTPases in the internalization of *Leishmania*. Recently, Morehead et al. [77] reported that *L. amazonensis* amastigotes internalized by CHO cells activated the Rho and Cdc42 GTPases but not Rac-1. When these amastigotes were IgG-opsonized and taken in by Fc-receptor-expressing CHO cells, Rac-1 activation was required for the uptake of the parasites. These results are in agreement with the specific roles of RhoA and Rac-1 in types II and I phagocytosis, respectively [74]. However, Rac-1 may also participate in unopsonized target internalization [78]. Given their importance in F-actin polymerization, it will be of interest to investigate the possibility that members of the Rho-family GTPases are involved in LPG-mediated periphagosomal F-actin accumulation.

Nucleation and reorganization of F-actin at the phagocytic cup are regulated by the Arp2/3 complex, a seven subunit F-actin-interacting group of proteins that is activated by the Wiskott-Aldrich Syndrome (WASP) family of proteins [79]. Both Arp2/3 and WASP are recruited to phagosomes, where WASP may act as a link between early internalization effectors, such as Rho family GTPases and PI4,5P, and F-actin nucleating proteins such as Arp2/3 [79]. Other F-actin-interacting proteins, such as the motor protein myosin [80,81] and coronin [82], are also recruited to the phagosome. The impact of *Leishmania* promastigotes or that of LPG on these actin-associated proteins is not known.

Members of the PKC family are recruited to the phagocytic cup and to the phagosome with varying kinetics [30]. Although the substrates phosphorylated by PKC during phagocytosis have not been extensively characterized, the F-actin cross-linking protein, myristoylated alanine-rich C kinase substrate (MARCKS), is known to be one [55,83]. Recruitment of PKC family members to the phagocytic cup and the phagosome may play a role in downstream signaling events. Analysis of PKC-α distribution in macrophages infected with *L. donovani* promastigotes revealed that its recruitment to the phagosome is inhibited by LPG [62,84]. Absence of PKC-α from *L. donovani* promastigote-containing phagosomes may contribute to the accumulation of periphagosomal F-actin. This inference was suggested by the finding that phagosomes from macrophages overexpressing a dominant-negative mutant of PKC-α show F-actin accumulation and impaired recruitment of LAMP1 [84]. These observations point to an important role for PKCα in the interaction of *L. donovani* promastigotes with phagosomes and give insights on how LPG mediates periphagosomal F-actin accumulation.

Other F-actin-interacting proteins are necessary for filament cross-linking, bundling, or depolymerization, which are processes likely to contribute to formation of the phagosome as well as to its maturation and movement within the cell. Both α-actinin and cofilin associate to phagosomes, but their exact role in phagocytosis remains unclear. The focal adhesion molecules vinculin, paxilin, and talin also seem to be enriched on phagosomes: their precise role, if any, remains to be defined [48]. Many unconventional myosin motor proteins are involved in the movement of phagosomes and in the final steps of the phagocytic process. Indeed, the contraction created by the motor proteins on the organelle would help close up the vacuolar space [80]. Type III PI 3-kinase and the clathrin-coated vesicle budding proteins amphiphysin/dynamin II are also likely involved in sealing the phagosome membrane [85,86]. Finally, it is noteworthy to point out that some F-actin remains associated to the phagosome, as certain F-actin-associated proteins (α-actinin and annexins) are found on the phagosomal membrane. This could be related to the role of F-actin polymerization as a motor force for organellar movement inside the cell [48]. All these actin-associated proteins are further potential targets for *Leishmania* promastigotes. Their possible altered functions or distribution by LPG, as shown for PKCα, has yet to be investigated.

Significance of the inhibition of phagosome maturation

Various bacterial and protozoan pathogens have evolved strategies to evade destruction by phagocytosis and to live an intracellular lifestyle [87,88]. One of these strategies consists in blocking the maturation process of the phagosomes. An immediate consequence of such inhibition is the avoidance of a degradative environment. Modulation of phagosome composition by the parasite may also impair the functionality of this organelle, including the ability to process antigens. Therefore, the possibility exists that inhibition of phagosome maturation by *L. donovani* promastigotes may have an impact on antigen processing

and presentation, two important functional properties of phagosomes [32]. Future studies will address whether phagosome remodeling by *L. donovani* promastigotes [60] contributes to the ability of *Leishmania* to evade an effective adaptive immune response.

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