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Leishmania donovani lacking the Golgi GDP-Man transporter *LPG2* exhibit attenuated virulence in mammalian hosts

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ABSTRACT

Surface phosophoglycans such as lipophosphoglycan (LPG) or proteophosphoglycan (PPG) and glycosylinositol phospholipids (GIPLs) modulate essential interactions between Leishmania and mammalian macrophages. Phosphoglycan synthesis depends on the Golgi GDP-mannose transporter encoded by *LPG2*. *LPG2*-null (*lpg2*$^-$) *Leishmania major* cannot establish macrophage infections or induce acute pathology, whereas *lpg2*$^-$ *Leishmania mexicana* retain virulence. *lpg2*$^-$ *Leishmania donovani* has been reported to survive poorly in cultured macrophages but *in vivo* survival has not been explored. Herein we discovered that, similar to *lpg2*$^-$ *L. major*, *lpg2*$^-$ *L. donovani* promastigotes exhibited diminished virulence in mice, but persisted at consistently low levels. *lpg2*$^-$ *L. donovani* promastigotes could not establish infection in macrophages and could not transiently inhibit phagolysosomal fusion. Furthermore, *lpg2*$^-$ promastigotes of *L. major*, *L. donovani* and *L. mexicana* were highly susceptible to complement-mediated lysis. We conclude that phosphoglycan assembly and expression mediated by *L. donovani LPG2* are important for promastigote and amastigote virulence, unlike *L. mexicana* but similar to *L. major*.

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1. Introduction

All *Leishmania* spp. are covered by a complex glycocalyx throughout the infectious cycle, and glycoconjugates are thought to be important factors promoting their virulence (reviewed in Refs. Turco, 1990; Ferguson, 1999; Turco et al., 2001; Naderer et al., 2004). The promastigote glycocalyx is especially dense and contains high levels of phosphoglycans (PGs) comprised of polymeric [6Gal(β1,4)Man(α1-PO_4)$^-$] disaccharide phosphate-based repeating units. The main structural distinction among PGs from various *Leishmania* spp. is the side chain sugar substitutions that branch off the disaccharide-phosphate backbone. Depending on the growth phase, the abundant promastigote surface glycolipid lipophosphoglycan (LPG) contains 15–30 PG repeating units, bearing an external capping oligosaccharide and anchored to the surface by a heptasaccharide glycosylphosphatidyl inositol anchor (reviewed in Ref. Turco, 1990). Proteophosphoglycans (PPGs) comprise a family of large proteins containing Ser–Thr rich regions to which the PG repeating units are covalently linked (reviewed in Ref. Ilg, 2000). Amastigotes lack significant LPG but retain PPG expression, and both stages express high levels of smaller glycosylinositol phospholipids (GIPLs) (Elhay et al., 1988; McConville et al., 1994). The GPI anchors of LPG and PPGS show varying degrees of structural identity or similarity to those of GIPLs and GPI-anchored proteins (reviewed in refs. Turco, 1990; Ferguson et al., 1999; Turco et al., 2001; Naderer et al., 2004). Many studies have shown that purified (or synthetic) LPG, PPG and GIPLs have significant effects on parasite survival, attributed their ability to execute key steps of the infectious cycle such as suppression of host cell signaling and activation, and evasion of killing by activated complement (reviewed in Refs. Descoteaux and Turco, 1999; Naderer et al., 2004). However, one challenge to the interpretation of *in vitro* studies is that test glycoconjugate(s) are provided outside of the context of the parasite, leading to concerns about the effects of dosage and route of application (Beverley and Turco, 1998). A further challenge is the fact that these PG-containing and GPI-anchored *Leishmania* glycoconjugates show varying extents of structural relatedness, raising concerns about their cross activity during *in vitro* assays.

A second approach to glycoconjugate function has thus been to generate mutants through either forward or reverse genetic approaches (Beverley and Turco, 1998). Depending of the specific gene affected, individual or specific subsets of glycoconjugates can be defective in mutants. These living organisms permit

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assessment of glycoconjugate deficiency in the proper biological context. For example, *Leishmania major lpg1*$^-$ mutants lack the galactofuranosyl transferase activity required for synthesis of the LPG core domain but other parasite surface components remain unchanged by this mutation (Sacks et al., 2000; Spath et al., 2000, 2003a). These LPG-deficient *L. major* exhibit increased susceptibility to complement- and oxidant-mediated toxicity, and show decreased survival in macrophage infections, although they retain the ability to suppress macrophage responses leading to $NO^{\cdot}$ and IL-12 production (Spath et al., 2000, 2003a, 2003b). As expected because LPG expression is greatly down-regulated in amastigotes (Turco and Sacks, 1991; Moody et al., 1993), *lpg1*$^-$ *L. major* amastigotes are fully virulent (Spath et al., 2000).

The role of PGs, which constitute major portions of both LPG and PPGs, was first probed genetically through studies of the *lpg2*$^-$ mutants which lack the Golgi GDP-mannose transporter *LPG2* required for PG synthesis (Descoteaux et al., 1995; Ma et al., 1997; Spath et al., 2003b). Like *lpg1*$^-$ mutants, *L. major lpg2*$^-$ mutants show heightened defects in promastigote virulence, but unlike *lpg1*$^-$ mutants, they are unable to replicate as amastigotes in macrophages and/or cause acute pathology. Nonetheless, they persist indefinitely at a low level (Spath et al., 2003b) and they induce protective immunity in mice (Uzonna et al., 2004; Kebaier et al., 2006). The defect in *L. major lpg2*$^-$ amastigotes was attributed to the lack of PGs in this stage, the only known glycoconjugate synthetic defect in *lpg2*$^-$ amastigotes of *L. major*, *Leishmania donovani* and *Leishmania mexicana* (Descoteaux et al., 1995; Ilg et al., 2001; Goyard et al., 2003; Spath et al., 2003b). However, recent studies of *L. major* challenged this assumption, using a double mutant (*lpg5A*$^-$/*lpg5B*$^-$) lacking PGs through ablation of the UDP-Gal transporters encoded by *LPG5A* and *LPG5B* (Spath et al., 2004; Capul et al., 2007b, 2007a). Remarkably this mutant shows a virulence phenotype very similar to that of LPG-deficient *lpg1*$^-$ *L. major*, which affects only promastigote virulence whereas amastigotes retain full virulence (Capul et al., 2007a). Thus the loss of amastigote virulence in the *L. major lpg2*$^-$ mutant is probably not merely due to the absence of PGs. The lack of involvement of PGs in amastigote virulence agrees with results obtained with *lpg2*$^-$ *L. mexicana* which, unlike *lpg2*$^-$ *L. major,* retain amastigote virulence and induce disease despite the absence of PGs (Ilg et al., 2001).

In this work we focus on the role of *LPG2* in the virulence of *L. donovani*. Similar to *L. major, L. donovani* resides in a 'tight' parasitophorous vacuole, which differs from the 'spacious' vacuole occupied by *L. mexicana* (Castro et al., 2006). Curiously, whereas the forward genetic complementation methodology was first developed and then applied to the identification of LPG synthetic genes in LPG-deficient mutants in this species (Ryan et al., 1993; Beverley and Turco, 1998), the use of these *L. donovani* mutants for functional studies of virulence in animal infections was compromised by several factors. One was the use of heavy mutagenesis and the lack of sexual crossing to generate isogenic lines, which was solved by the use of targeted replacement methods (Spath et al., 2000). A second problem was the tendency of some *Leishmania* species and especially those causing visceral disease to rapidly lose virulence during *in vitro* culture.

Previous studies have shown that some strains of *lpg2*$^-$ *L. donovani* are unable to establish infections in macrophages *in vitro* (Lodge et al., 2006). However, the ability of these strains to survive in animal hosts has not previously been reported. In this work we make use of the observation that virulence can be maintained in *L. donovani* lines adapted for cycling between growth as promastigotes and axenic amastigotes despite extensive *in vitro* culture (Goyard et al., 2003; Debrabant et al., 2004), and generated *lpg2*$^-$ null mutants in such a strain, the LdBob line of *L. donovani* (Goyard et al., 2003). This mutant (and its complemented control line) has allowed us, for the first time, to study the role of *LPG2* in a virulent *L. donovani* background in survival in mice, and to compare the requirement with the contrasting observations reported in *L. major* and *L. mexicana*. The LdBob *lpg2*$^-$ line additionally allowed us to confirm *lpg2*$^-$ phenotypes previously described such as macrophage survival and transient inhibition of phagolysosomal fusion, and extend this to susceptibility to lysis by complement.

2. Materials and methods

2.1. Parasites

All strains studied were derivatives of the *L. donovani* strain 1S2D (MHOM/SD/62/1S-CL2D) clonal line LdBob, which were grown alternately as amastigotes and promastigotes in serum-containing medium specific for each form as described (Goyard et al., 2003). Amastigotes were cultivated at 37 °C, 5% CO_2, and promastigotes were grown at 26 °C. Parasites were converted between forms every 3 weeks.

Previously we described the homozygous *LPG2* knockout line (formally, Δ*lpg2*::HYG/Δ*lpg2*::HYG), referred to as *lpg2*$^-$ in this work, and a complemented derivative bearing an episomal *LPG2* construct (formally, Δ*lpg2::HYG*/Δ*lpg2::HYG* [pX63NEO-LPG2]), referred to as *lpg2*$^-$/+*LPG2*(e) here (Goyard et al., 2003). An integrated version of the complemented line was generated as follows: first, the *L. donovani LPG2* gene was excised by *Xho*II digestion from pXG63HYG-LPG2 [strain B1544 (Descoteaux et al., 1995)], and inserted into either the *Sma*I or *Bgl*II sites of pIR1SAT, yielding the constructs pIR1SAT-LdLPG2(a) and pIR1SAT-LdLPG2(a) (strains B5041 and B5043, respectively). These constructs were digested with *SwaI* and the targeting fragment was isolated and introduced into *L. donovani lpg2*$^-$ by electroporation (Robinson and Beverley, 2003). Clonal lines expressing the transfected construct were identified following plating on M199 media containing 5 μg/ml nourseothricin. All transfectant lines [Δ*lpg2::HYG*/Δ*lpg2::HYG* SSU:IR1SAT-Ld*LPG2*(a) or Ld*LPG2*(b), respectively] bear *LPG2* integrated into the ribosomal RNA locus, where they are stably expressed at high levels from the rRNA promoter (Robinson and Beverley, 2003). All lines showed good differentiation and similar levels of LPG expression, and are referred to here as *lpg2*$^-$/+*LPG2*(i).

2.2. Complement sensitivity

Complement sensitivity tests were performed as described (Spath et al., 2003a). Briefly, promastigotes in logarithmic or stationary phase growth were exposed to 2% fresh human serum for 30 min in the presence of propidium iodide, whose uptake by permeabilized cells was quantitated by flow cytometry.

2.3. Immunoblotting

Promastigote or amastigote proteins were separated on reducing 9% denaturing SDS polyacrylamide gels, transferred to Nytran, and blocked in 5% milk/PBS/0.01% Tween-20. Filters were incubated with the following antisera: CA7AE, specific for unsubstituted [6Gal(β1,4)Man(α1-PO_4)$^-$] phosphoglycan repeats (Goyard et al., 2003); polyclonal antiserum generated in sheep to purified *Leishmania chagasi* GP63 (1:10,000), or monoclonal antibody to α-tubulin (AB-1, 0.1 μg/ml, Oncogene, San Diego, CA) (Yao et al., 2002).

2.4. Mannan purification and analysis

Leishmania mannans were extracted by chloroform:methanol:water extraction, and fluorophore labeled. Mannans were separated by fluorophore-assisted carbohydrate electrophoresis (FACE) as described (Capul et al., 2007a).