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## Experimental Parasitology

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

Upasna Gaur<sup>a</sup>, Melissa Showalter<sup>b</sup>, Suzanne Hickerson<sup>b</sup>, Rahul Dalvi<sup>a</sup>, Salvatore J. Turco<sup>c</sup>, Mary E. Wilson<sup>a,\*,1</sup>, Stephen M. Beverley<sup>b,1</sup>

<sup>a</sup>Departments of Internal Medicine, Epidemiology and Microbiology, University of Iowa and the Veterans Affairs Medical Center, SW34-GH, 200 Hawkins Dr., Iowa City, IA 52242, USA

<sup>b</sup>Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA

<sup>c</sup>Department of Biochemistry, University of Kentucky Medical Center, College of Medicine, Lexington 40536, USA

## ARTICLE INFO

## Article history:

Received 2 December 2008

Received in revised form 3 March 2009

Accepted 6 March 2009

Available online 27 March 2009

## Keywords:

GIPL, glycosylinositol phospholipids  
LPG, lipophosphoglycan  
PPG, proteophosphoglycan  
RP-10, macrophage growth medium with RPMI and 10% FCS  
PG, phosphoglycan

## ABSTRACT

Surface phosphoglycans 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*<sup>-</sup>) *Leishmania major* cannot establish macrophage infections or induce acute pathology, whereas *lpg2*<sup>-</sup> *Leishmania mexicana* retain virulence. *lpg2*<sup>-</sup> *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*<sup>-</sup> *L. major*, *lpg2*<sup>-</sup> *L. donovani* promastigotes exhibited diminished virulence in mice, but persisted at consistently low levels. *lpg2*<sup>-</sup> *L. donovani* promastigotes could not establish infection in macrophages and could not transiently inhibit phagolysosomal fusion. Furthermore, *lpg2*<sup>-</sup> 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<sub>4</sub>)<sup>-</sup>] 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

\* Corresponding author. Fax: +1 319 384 7208.

E-mail address: [mary-wilson@uiowa.edu](mailto:mary-wilson@uiowa.edu) (M.E. Wilson).

<sup>1</sup> These authors contributed equally to the work.

assessment of glycoconjugate deficiency in the proper biological context. For example, *Leishmania major* *lpg1*<sup>-</sup> 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 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*<sup>-</sup> *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*<sup>-</sup> 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*<sup>-</sup> mutants, *L. major* *lpg2*<sup>-</sup> mutants show heightened defects in promastigote virulence, but unlike *lpg1*<sup>-</sup> 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*<sup>-</sup> amastigotes was attributed to the lack of PGs in this stage, the only known glycoconjugate synthetic defect in *lpg2*<sup>-</sup> 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*<sup>-</sup>/*lpg5B*<sup>-</sup>) 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*<sup>-</sup> *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*<sup>-</sup> 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*<sup>-</sup> *L. mexicana* which, unlike *lpg2*<sup>-</sup> *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*<sup>-</sup> *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*<sup>-</sup> 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 vir-

ulent *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*<sup>-</sup> line additionally allowed us to confirm *lpg2*<sup>-</sup> 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<sub>2</sub>, and promastigotes were grown at 26 °C. Parasites were converted between forms every 3 weeks.

Previously we described the homozygous *LPG2* knockout line (formally,  $\Delta lpg2::HYG/\Delta lpg2::HYG$ ), referred to as *lpg2*<sup>-</sup> in this work, and a complemented derivative bearing an episomal *LPG2* construct (formally,  $\Delta lpg2::HYG/\Delta lpg2::HYG$  [pX63NEO-LPG2]), referred to as *lpg2*<sup>-</sup>/+*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*I digestion from pXG63HYG-LPG2 [strain B1544 (Descoteaux et al., 1995)], and inserted into either the *Sma*I or *Bgl*III sites of pIR1SAT, yielding the constructs pIR1SAT-LdLPG2(a) and pIR1SAT-LdLPG2(b) (strains B5041 and B5043, respectively). These constructs were digested with *Swa*I and the targeting fragment was isolated and introduced into *L. donovani* *lpg2*<sup>-</sup> 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 [ $\Delta lpg2::HYG/\Delta lpg2::HYG$  SSU:IR1SAT-LdLPG2(a) or LdLPG2(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*<sup>-</sup>/+*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<sub>4</sub>)<sup>-</sup>] 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).

2.5. Animals

Female BALB/c mice (age 4–6 weeks) were purchased from Harlan Laboratories (Indianapolis, IN). Mice were infected with  $10^7$  stationary phase parasites belonging to different lines intravenously through the tail vein. At specified time points, animals were euthanized, organs were removed, and parasite loads were determined microscopically from touch preparations and organ weights as described (Stauber, 1958; Wilson et al., 1987). All animal procedures were approved by the University of Iowa and the Iowa City VA institutional animal care and use committees.

2.6. Murine and human macrophage infections

Bone marrow cells (BMMs) from BALB/c mouse femurs were cultured at 37 °C, 5% CO<sub>2</sub> in RP-10 (RPMI with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin/ml, and 50 µg of streptomycin/ml [GIBCO, Carlsbad, CA.] containing 20% L929 cell culture supernatant (American Tissue Type Collection, Manassas, VA) as a source of macrophage colony-stimulating factor. After 7–9 days, differentiated adherent macrophages were detached with 2.5 mg trypsin/ml plus 1 mM EDTA (GIBCO) (Coligan et al., 2007).

Human mononuclear cells were isolated from the peripheral blood of normal healthy donors by density sedimentation in Ficoll–Hypaque (Sigma Chemical Co., St. Louis, MO). Monocytes were separated by adherence to six-well plates (flow cytometry) for 2–3 h at 37 °C and 5% CO<sub>2</sub> in RP-10.

$5.0 \times 10^5$  macrophages were allowed to adhere to coverslips in 24-well plates and infected with opsonized promastigotes or amastigotes at a multiplicity of infection (MOI) of 5:1 as described (Rodriguez et al., 2006). The infection was synchronized by centrifugation (3 min, 330g, 4 °C) and infected macrophages were incubated in 5% CO<sub>2</sub> at 37 °C. Extracellular parasites were removed

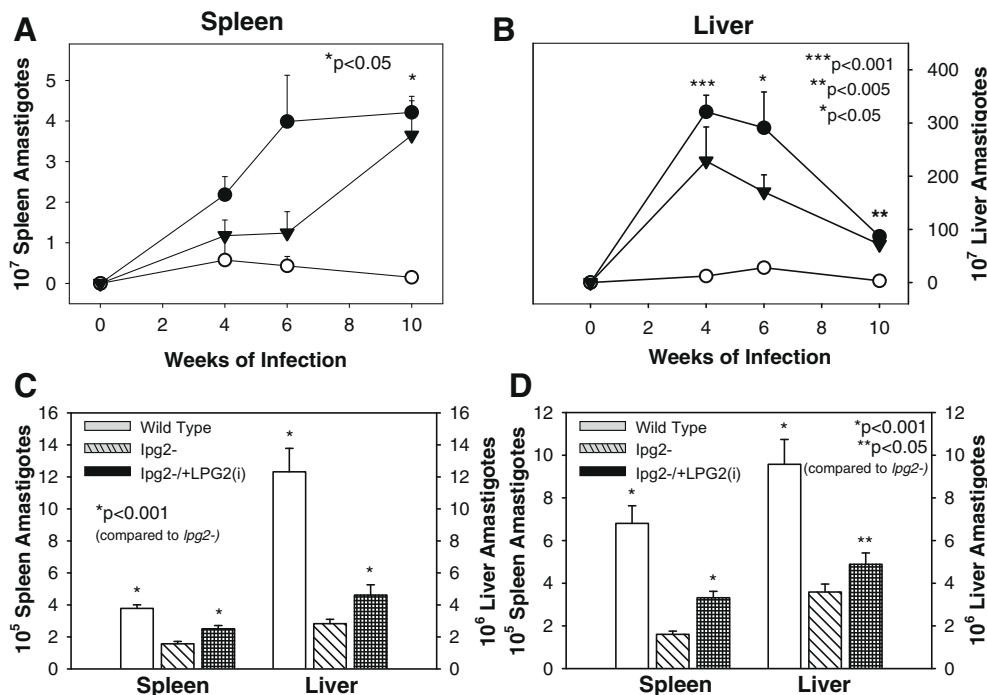
by rinsing macrophages 30 min post-infection. At each time point, coverslips were fixed and stained with Diff-Quik (Fisher Scientific).

2.7. Flow cytometry

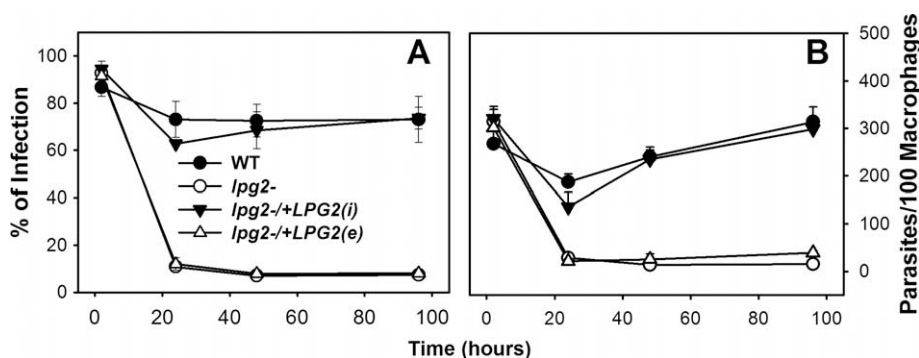
Promastigotes were incubated in 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 30 min in a 37 °C water bath, washed, and suspended at  $2 \times 10^7$ /ml in Hanks balanced salt solution as described (Chang et al., 2007). Adherent monocytes were infected with fluorescent promastigotes at a 5:1 MOI, synchronized by centrifugation, and incubated at 37 °C, 5% CO<sub>2</sub> for 15 min. Five micrometers of dihydroethidium (DHE, Molecular Probes) were added, and after an additional 10 min at 37 °C, promastigotes were removed by rinsing and phagocytes were detached in citric saline (0.135 M KCl, 0.015 M Na citrate). Cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (EMS, Hatfield, PA) and analyzed on a Becton Dickinson FACSCalibur equipped with a 488 nm argon laser (BD Biosciences, San Diego, CA). Fluorescence was monitored at 480/30 nm (CFSE) and 580/42 nm (DHE), and 10,000 events were examined. Data were analyzed using Cell Quest or Flow Jo software (BD Biosciences).

2.8. Confocal microscopy

$5 \times 10^5$  macrophages on 12 mm coverslips were infected with promastigotes stained with CFSE as described (Chang et al., 2007). Lysosomes were pre-labeled by incubation of macrophages in 0.8 µg of Mr 10,000 TRITC-conjugated dextran/ml (Molecular Probes) for 16 h, followed by a rinse and chase in RP-10 without dextran for an additional 30 min. At the time of infection the endosomal pathway was marked in positive control macrophages by addition of 10,000 Mr fluorescein-labelled dextran (Molecular Probes) at the time of “infection”. Infected or control cells were fixed in 2% paraformaldehyde (30 min), permeabilized in 0.2% Tri-



**Fig. 1.** Survival of *L. donovani lpg2*<sup>-</sup> mutants or controls in mice. (A–B) Female BALB/c mice were infected i.v. with  $10^7$  WT (●), *lpg2*<sup>-</sup> (○), or *lpg2*<sup>-</sup>/+LPG2(e) (▲) stationary phase promastigotes. Parasite loads in the spleen (panel A) or liver (panel B) were quantified microscopically. Shown is a representative of three replicate experiments; values represent the mean ± SEM for six mice at each time point. (C–D) Female BALB/c mice were infected with  $10^7$  stationary phase WT or *lpg2*<sup>-</sup> promastigotes as above, or with the same number of *lpg2*<sup>-</sup>/+LPG2(e) (panel C) or *lpg2*<sup>-</sup>/+LPG2(i) (panel D) add back organisms. After 4 weeks, mice were euthanized and parasites loads were quantified microscopically. Statistical analyses compared parasite loads in mice infected with both controls to mice infected with *lpg2*<sup>-</sup> organisms.



**Fig. 2.** Survival of *lpg2*<sup>-</sup> *L. donovani* in murine bone marrow macrophages. Macrophages were synchronously infected with opsonized promastigotes at a 5:1 MOI for 30 min before rinsing to remove extracellular parasites. At the indicated time points, infection levels were quantified by light microscopy. Values represent the mean ± SE for three experiments, each with triplicate conditions. (Panel A) Percent of macrophages infected; (Panel B) the number of parasites per 100 macrophages. Cell lines tested were WT (●), *lpg2*<sup>-</sup> (○), *lpg2*<sup>-</sup>/+LPG2(i) (▼), or *lpg2*<sup>-</sup>/+LPG2(e) (△).

ton X-100 (15 min), incubated in 50 mM glycine (15 min), and blocked in 5% non-fat dry milk/PBS (30 min). Macrophages were incubated with 50:1 goat anti-LAMP-1 (Santa Cruz) followed by 200:1 Alexa fluor 647 (blue) donkey anti-goat Ig at room temperature for 1 h. After rinsing in PBS and mounting with Vectashield H-1000 (Vector Labs, Burlingame, CA), slides were examined on a Zeiss 510 laser scanning confocal microscope, and captured using the LSM 510 version 3.2 software. Microscopic studies were performed at the University of Iowa Central Microscopy Research Facility.

### 3. Results

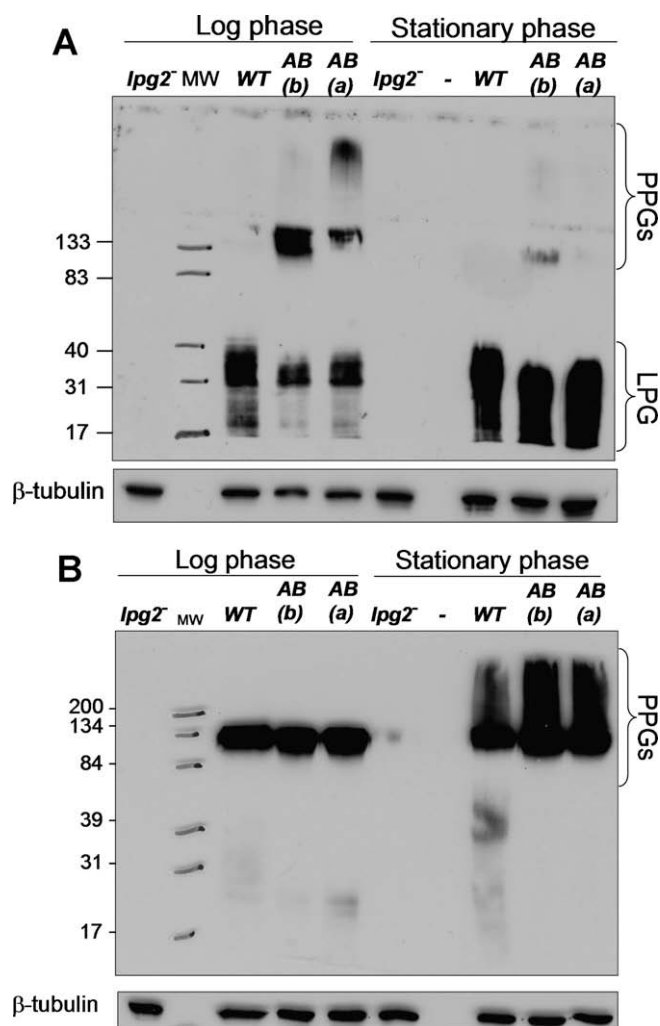
#### 3.1. In vivo defect of *lpg2*<sup>-</sup> *L. donovani* in mouse infections

Previously we reported the generation and properties of an *lpg2*<sup>-</sup> mutant in the LdBob line of *L. donovani*, able to differentiate between the promastigote and amastigote stage *in vitro* when cultured under appropriate conditions (Goyard et al., 2003), along with complemented derivatives where *LPG2* expression was restored by transfection with an episomal *LPG2* expression vector [*lpg2*<sup>-</sup>/+LPG2(e)]. BALB/c mice were infected i.v. with WT, *lpg2*<sup>-</sup>, or *lpg2*<sup>-</sup>/+LPG2(e) stationary phase *L. donovani*, and at appropriate times animals were euthanized and parasite loads were quantified by microscopy (Fig. 1). As observed previously, WT parasites multiplied most quickly in the livers (Fig. 1B), peaking at 4–6 weeks and declining thereafter, whereas infection began slowly but progressed in the spleens of infected animals (Kaye and Farrell, 2002; Wilson and Weinstock, 1996). In contrast, *lpg2*<sup>-</sup> infections showed considerably fewer parasites at all time points in both organs (Fig. 1). In the liver, *lpg2*<sup>-</sup> parasites were found at 4% of WT at the peak of the infection at week 4 (Fig. 1). Similarly, in the spleen *lpg2*<sup>-</sup> parasites were present at 3.5% of WT levels at week 10 of infection. Importantly, restoration of *LPG2* expression partially restored parasite replication in both livers and spleens [Fig. 1; *lpg2*<sup>-</sup>/+LPG2(e)], although the effect was variable in different experiments (compare Figs. 1A,B with a second experiment shown in Fig. 1C).

#### 3.2. Survival of *lpg2*<sup>-</sup> *L. donovani* in macrophages

Given the attenuation of *lpg2*<sup>-</sup> *L. donovani* replication in mouse infections, we studied its survival in murine bone marrow macrophages. In these studies, WT stationary phase *L. donovani* enter macrophages successfully and after a slight decrease in numbers after 24 h, replicated about 2-fold over the next ~72 h (Fig. 2). In contrast, while *lpg2*<sup>-</sup> *L. donovani* were taken up by macrophages at a similar level as WT (Fig. 2; 2 h time point), they were rapidly

destroyed with their numbers declining to less than 5.2% of WT by 96 h. However and unlike the mouse infections experiments, one of the control lines in which *LPG2* was restored on an extra-



**Fig. 3.** PG levels in *lpg2*<sup>-</sup> mutants and controls. Whole cell extracts from the indicated lines grown either as promastigotes (panel A) or *in vitro* amastigotes (panel B) were subjected to western blot analysis with antiserum CA7AE which detects unmodified PG repeats (Tolson et al., 1989). Blots were re-probed with 1:1000 antibody to  $\beta$ -tubulin as a loading control. The migration of LPG and PPGs are as indicated in the figure. AB(b) and AB(a) refer to *lpg2*<sup>-</sup>/+LPG2(i) parasites harvested “before” or “after” infections.

chromosomal plasmid did not restore macrophage survival, i.e. the *lpg2*<sup>-</sup>/*LPG2*(e) line was destroyed as efficiently as *lpg2*<sup>-</sup> (Fig. 2). This was similar to results obtained in another recent study in which episomal vector-based rescue of another PG deficient mutant (lacking the Golgi UDP-Gal nucleotide sugar transporters encoded by the *LPG5A* and *LPG5B* genes) was observed in mouse but not macrophage infections (Capul et al., 2007a). There we found that restoration of PG synthesis using a strong integrating *LPG5A+LPG5B* expression vector fully restored both mouse and macrophage virulence. Thus we hypothesized that the lower levels episomal *LPG2* arising from the episomal expression vectors might account for the failure to rescue. This idea meshed well with our results with *lpg2*<sup>-</sup> *L. donovani*, as previous data showed that the *lpg2*<sup>-</sup>/*LPG2*(e) line did not fully restore LPG expression back to WT levels either (Goyard et al., 2003).

We therefore generated several new transfectant restoration lines termed *lpg2*<sup>-</sup>/*LPG2*(i), where *LPG2* expression was restored using a strong rRNA-integrating expression vector (pIR1SAT; Methods). These *lpg2*<sup>-</sup>/*LPG2*(i) lines synthesized WT levels of LPG and/or PPG, in stationary phase promastigotes, tested both before (Fig. 3A) and subsequent to mouse infections (Fig. 4B), and in axenic amastigotes (Fig. 3B). Curiously, restoration of LPG synthesis was not identical to WT in level or pattern in log phase promastigotes (Fig. 3A). However, we deemed this acceptable since virulence studies were carried out with infectious stationary phase promastigotes, which appeared normal in PG expression. Mannan levels were similar in WT and *lpg2*<sup>-</sup> *L. donovani* (Fig. 4A). In agreement with previous studies in *L. major* and *L. mexicana lpg2*<sup>-</sup>, GP63 levels were similar in WT, *lpg2*<sup>-</sup> and *lpg2*<sup>-</sup>/*LPG2*(i) promastigotes (Fig. 4B).

In animal tests, the results with the *lpg2*<sup>-</sup>/*LPG2*(i) line resembled the *lpg2*<sup>-</sup>/*LPG2*(e) line in showing partial but significant rescue of the *lpg2*<sup>-</sup> phenotype (Fig. 1D). Now when tested in bone marrow macrophage infections, *lpg2*<sup>-</sup>/*LPG2*(i) infected and replicated with kinetics indistinguishable from WT, whereas *lpg2*<sup>-</sup> and the *lpg2*<sup>-</sup>/*LPG2*(e) lines entered but were rapidly destroyed as before (Fig. 2). These data established that the macrophage vir-

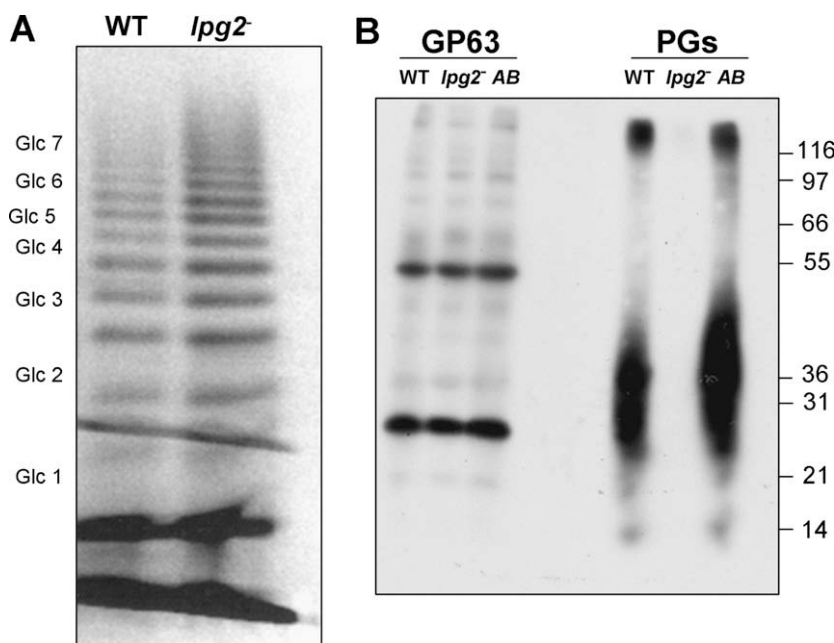
ulence defect of the *lpg2*<sup>-</sup> line was specifically attributable to the absence of *LPG2*. They also stress the importance of achieving full restoration of *LPG2* expression, in a form that is maintained in the absence of drug pressure, in the restoration lines.

### 3.3. *lpg2*<sup>-</sup> mutants of all three *Leishmania* species are susceptible to lysis by complement

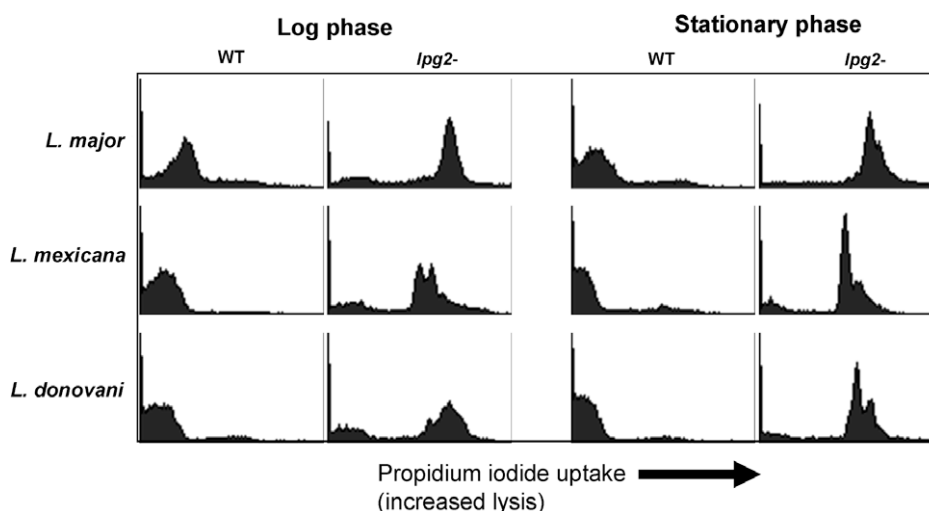
We assessed the susceptibility of logarithmic and infective stage stationary phase *lpg2*<sup>-</sup> mutants to lysis by complement (fresh human serum). Prior report of this assay indicates that lysis of *Leishmania* is mediated exclusively by complement (Spath et al., 2003a). Both growth phases of *lpg2*<sup>-</sup> *L. donovani* were susceptible to complement under these conditions, as seen by their uptake of propidium iodide in the presence but not absence of serum, whereas WT *L. donovani* were not lysed (Fig. 5, bottom panels). These results were similar to those obtained with the *lpg2*<sup>-</sup> *L. major* controls studied previously (Spath et al., 2003a) (Fig. 5, top panel). Interestingly, both growth phases of *lpg2*<sup>-</sup> but not WT *L. mexicana* promastigotes were highly susceptible to complement-mediated lysis (Fig. 5, middle panels). These data suggest that *LPG2* may similarly contribute to *L. mexicana* promastigote virulence by mediating resistance to lysis by complement in human serum, presumably through PG synthesis.

### 3.4. *L. donovani lpg2*<sup>-</sup> induces a stronger respiratory burst

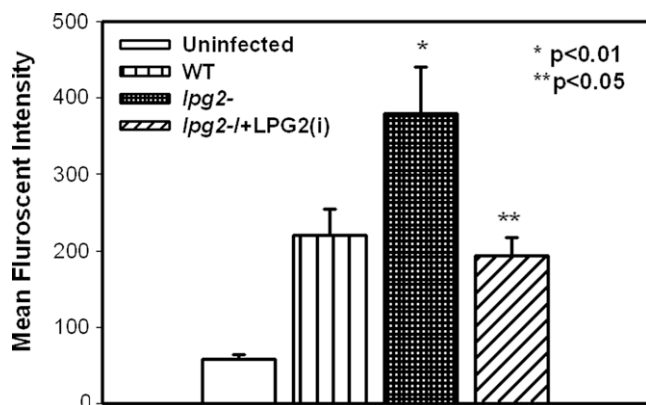
We previously developed a flow cytometry-based method for measuring mononuclear phagocyte oxidative response to *L. chagasi* promastigote phagocytosis, following oxidation of dihydroethidium, which primarily detects the products of the NADPH oxidase (Chang et al., 2007). Application of this methodology here showed that *lpg2*<sup>-</sup> *L. donovani* induced somewhat higher oxidant production than WT or *lpg2*<sup>-</sup>/*LPG2*(i) upon infection of human mononuclear phagocytes (1.7-fold; Fig. 6). These data suggest that PGs and/or other *LPG2*-dependent molecules may assist in limiting oxidant levels produced by phagocytes (Chan et al., 1989; Spath et al., 2003a).



**Fig. 4.** Glycoconjugates in WT, *lpg2*<sup>-</sup>, or *lpg2*<sup>-</sup>/*LPG2*(i) *L. donovani* promastigotes. Parasites were recovered after 12 weeks of infection and grown as promastigotes. (A) Cytoplasmic mannans from wild type and mutant *L. donovani* separated by FACE are shown. (B) Lysates from wild type (WT), *lpg2*<sup>-</sup>, or *lpg2*<sup>-</sup>/*LPG2*(i) lines were analyzed in immunoblots probed with polyclonal anti-GP63 antisera or CA7AE anti-PG monoclonal antibody.



**Fig. 5.** Complement sensitivity of *lpg2*<sup>-</sup> mutants. The complement sensitivity of WT or *lpg2*<sup>-</sup> mutants, of *L. major*, *L. mexicana*, or *L. donovani*, was measured as described in Section 2, and lysis measured by quantitation of the uptake of propidium iodide and flow cytometry. The X axis depicts propidium iodide uptake and the Y axis depicts cell numbers. Parasites were tested in logarithmic or stationary phase of growth as indicated.



**Fig. 6.** *LPG2* expression correlates with depressed respiratory response in phagocytes. Human peripheral blood monocytes were infected for 10 min with the CFSE-labeled WT, *lpg2*<sup>-</sup> or *lpg2*<sup>-</sup>/+*LPG2*(i) promastigotes, and oxidant production was detected after additional 10 min incubation with DHE (described in Section 2). Monocytes are gated based upon CD14 staining and forward-side scatter characteristics and infected monocytes were discerned by positive CFSE stain. The average  $\pm$  standard deviation mean fluorescent intensity for DHE stain in infected monocytes, calculated from three separate experiments, is shown.

### 3.5. *lpg2*<sup>-</sup> *L. donovani* promastigotes are defective in their ability to delay phagolysosomal fusion

Studies of *lpg1*<sup>-</sup> and *lpg2*<sup>-</sup> mutants of *L. major* and *L. donovani* have shown that LPG is responsible for the transient delay in the ability of infective promastigotes to delay fusion of phagosomes with lysosomes (Desjardins and Descoteaux, 1997; Dermine et al., 2000; Spath et al., 2003a, 2003b). To assess phagolysosomal fusion, we used confocal microscopy to assess the degree of overlapping parasite and lysosomal markers after phagocytosis by murine bone marrow macrophages (only the 2 hr time point is shown in Fig. 7). As previously reported, the ability to delay phagosome–lysosome fusion was greatly reduced in *lpg2*<sup>-</sup> promastigotes, but returned to WT levels following restoration of *LPG2* expression (Figs. 7 and 8).

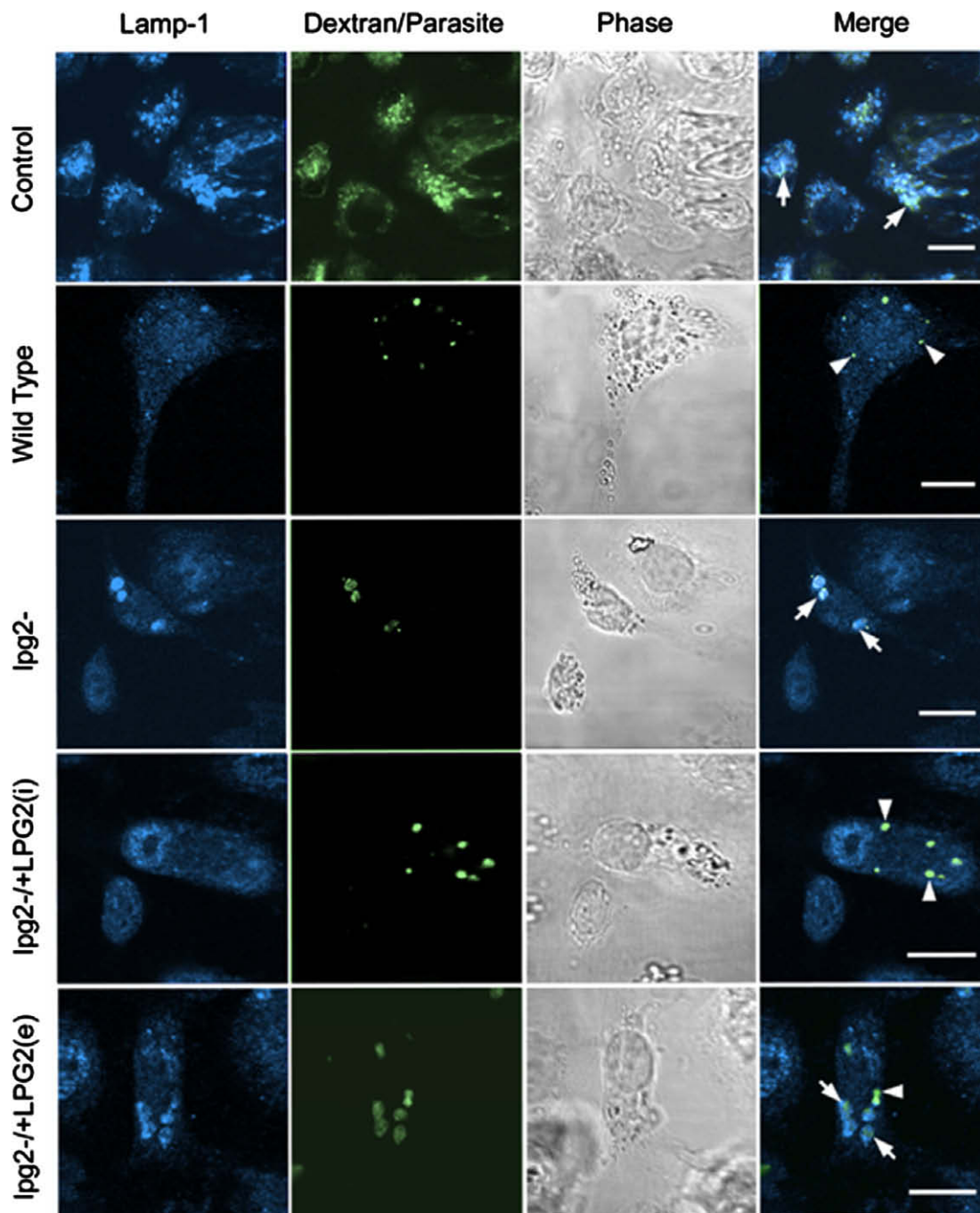
## 4. Discussion

In this study we explored the effects of *LPG2* ablation on the virulence of *L. donovani*, a parasite causing human visceral leishman-

iasis in the Old World. Using mutants on the LdBob strain background, our studies confirm earlier reports that *lpg2*<sup>-</sup> *L. donovani* are deficient in PG-containing glycoconjugates (Descoteaux et al., 1995), resulting in a defect in its ability to delay phagosome–lysosome fusion, and a failure to replicate in isolated macrophages (Lodge and Descoteaux, 2005; Lodge et al., 2006). Importantly, our work extends these observations by providing the key biological finding that *lpg2*<sup>-</sup> promastigotes induce very low levels of infection in spleens and livers of mice. These results indicate that the virulence phenotype of *lpg2*<sup>-</sup> *L. donovani* is more similar to *L. major* and unlike *L. mexicana*, and may contribute to our understanding of the mechanism of persistence.

Our studies show that the virulence defects of the *lpg2*<sup>-</sup> *L. donovani* were specific, as restoration of *LPG2* expression to the *lpg2*<sup>-</sup> mutant fully or partially restored virulence in the tests performed. Curiously, while restoration of *LPG2* expression by either episomal or integrating expression vectors yielded similar partial restoration of virulence in animal infections (Fig. 1), only the integrated *LPG2* rescue line (*lpg2*<sup>-</sup>/+*LPG2*(i)) showed restoration of macrophage survival when tested in BMMs *in vitro* (Fig. 2). This was similar to results obtained in another recent study in which episomal vector-based rescue of another PG deficient mutant (lacking the Golgi UDP-Gal nucleotide sugar transporters encoded by the *LPG5A* and *LPG5B* genes) was observed in mouse but not macrophage infections (Capul et al., 2007a). As in that study, we found that integration of *LPG2* into the ribosomal RNA locus resulted in a more complete restoration of glycoconjugate expression (Fig. 3). Thus for some reason the *in vitro* macrophage assay is more sensitive to the imperfect re-expression of *LPG2* whereas the mouse assay, in which expression is allowed over a longer period of time, allows differences between knockout and add-back to emerge. It remains to be seen the exact reason for these differences.

Our findings emphasize some of the challenges posed by virulence tests in *Leishmania*. First and as indicated above, available *Leishmania* expression vectors lead to over-expression of *LPG2* from multicopy episomes or at still higher levels following integration into the rRNA locus (Kapler et al., 1990; Misslitz et al., 2000). A similar previous study of the HSP100 gene (Hubel et al., 1997) showed that *Leishmania major* Hsp100 is required chiefly in the mammalian stage of the parasite, and that over expression can be deleterious in various ways. Perhaps *LPG2* when highly overexpressed in *L. donovani* is detrimental to virulence *in vivo*, although this phenomenon was not seen with studies of *LPG2* in *L. major*

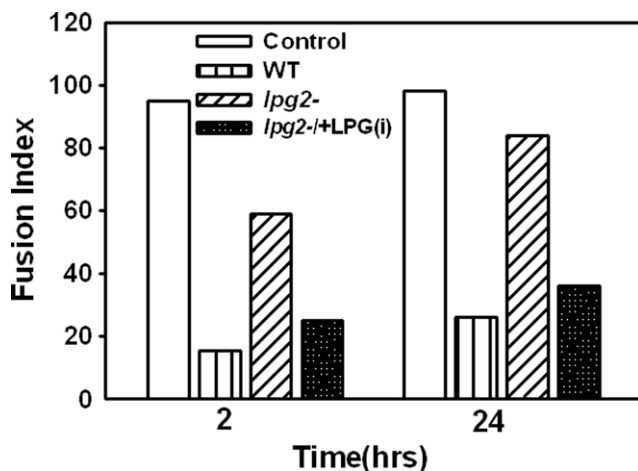


**Fig. 7.** *LPG2* expression correlates with delayed lysosomal fusion. Murine bone marrow macrophages were infected with CFSE-labeled WT, *lpg2*<sup>-</sup>, *lpg2*<sup>-</sup>/*+LPG2(e)*, or *lpg2*<sup>-</sup>/*+LPG2(i)* promastigotes. Control macrophages were pulsed at time 0 with GFP-labeled dextran which traffics to lysosomes. Two and 24 h later, samples were fixed and stained with a blue LAMP-1 marker. Shown are example micrographs from the 2 h time point. Arrows show merged blue-green signals. Arrowheads indicate green parasites that have not merged with the LAMP-1 positive marker.

(Spath et al., 2003b). Second, some variation in virulence restoration may be attributable to the well known tendency of these parasites to lose virulence during culture and/transfection (Cruz et al., 1993), rendering some transfectants less virulent for this reason rather than the intended genetic manipulation. Thus we and other investigators have adopted the operational criterion that successful restoration of virulence may nonetheless sometimes be incomplete or variable amongst different clonal transfectants, for the reasons cited above. Thus because of the tendency of *Leishmania* to lose virulence spontaneously, when examining independent clo-

nal lines, negative results are meaningless, as long as one is able to achieve a successful phenotypic rescue with at least some of them.

Given the similar biochemical consequences of *LPG2* ablation between *L. major*, *L. donovani* and *L. mexicana*, it is not immediately evident why the effects on virulence differ so greatly between *L. major*/*L. donovani* versus *L. mexicana*. These findings emphasize the well known concept that virulence determinants often play different roles in different stages of pathogens with complex life cycles. One possible explanation is that there could be as yet undetected differences in the *LPG2*-dependent glyco-repertoire between



**Fig. 8.** Quantitative measurement of the delay in lysosomal fusion. Micrographs from the experiment illustrated in Fig. 7 were classified by a blinded observer for fusion with lysosomes or lack of fusion. The “fusion index” was calculated as the ratio of parasites in vacuoles fused with lysosomes divided by total parasites  $\times 100$ . Data were derived from at least 200 macrophages for each parasite type.

these species. Whereas abundant *Leishmania* glycoconjugates have been extensively studied, potentially less abundant but functionally active molecules could remain to be discovered. Indeed, a similar explanation has been advanced to account for the fact that PG-deficient *L. major* mutants generated through ablation of the UDP-Gal transporters *LPG5A* and *LPG5B* show normal virulence as amastigotes, although they remain attenuated as promastigotes like *lpg1*<sup>-</sup> and *lpg2*<sup>-</sup> mutants (Capul et al., 2007a).

A second explanation to account for the differences between *L. major*/*L. donovani* and *L. mexicana* invokes fundamental differences in the nature of the parasitophorous vacuole inhabited by the parasites. The parasitophorous vacuole harboring intracellular *L. mexicana* is relatively ‘spacious’, and typically houses multiple organisms, whereas the parasitophorous vacuoles occupied by *L. major* and *L. donovani* are considered to be ‘tight’, with the parasite and host membranes in close proximity, and typically housing only a single organism (Castro et al., 2006). It is possible that the species specific differences in parasite survival reflect different interactions of LPG and/or other *LPG2*-dependent metabolites with these two types of vacuoles. Indeed, there are several studies which suggest that the fusogenicity of the vacuole differs between the *Leishmania* species (Courret et al., 2002). As the effects of LPG on fusion of the *L. mexicana* vacuole has been less studied than the *L. donovani* vacuole, more specific hypotheses cannot yet be drawn.

Parasitophorous vacuoles harboring virulent *L. donovani* promastigotes show a transiently decreased ability to fuse with lysosomes for a period of up to 24 h following infection. Studies of *lpg1*<sup>-</sup> *L. donovani* and *L. major* in the literature suggest this effect is mediated exclusively by LPG, according to studies with *lpg1*<sup>-</sup> mutants which specifically lack LPG but retain PPGs and other known glycoconjugates (Dermine et al., 2000; Spath et al., 2003a). As predicted, the LPG-deficient *lpg2*<sup>-</sup> *L. donovani* studied here was impaired in its ability to inhibit phagolysosomal fusion (Figs. 7 and 8). Amastigotes, in contrast to promastigotes, lack LPG and reside within highly fusogenic vacuoles (Alexander and Russell, 1992; Lang et al., 1994; Naderer et al., 2004).

The functional consequences of LPG-dependent transient phagolysosomal fusion inhibition may be multifold. Initial studies assumed the delay served merely to provide sufficient time for promastigotes to differentiate into amastigotes (Desjardins and Descoteaux, 1997). However, mechanistic studies of the *lpg1*<sup>-</sup> mutants showed a delay in fusion does not necessarily promote para-

site survival (Spath et al., 2003a). Recent studies suggest that LPG alters phagolysosomal biogenesis profoundly, one consequence of which in *L. donovani* infections is the formation of a periphagosomal barrier of F-actin which hinders the ability of the NADPH oxidase to assemble on the phagocyte membrane (Dermine et al., 2005; Lodge and Descoteaux, 2005; Lodge et al., 2006). Consistently, stationary phase WT parasite induce significant levels of oxidants, but the levels are heightened during infection with LPG-deficient mutants (Fig. 6). Transient inhibition of phagolysosome fusion presumably allows the parasite to avoid exposure to physically separate itself from toxic lysosomal contents. Inhibition may also play a role in other processes, for example in modulating early interactions of macrophages with other arms of the immune system (Lodge and Descoteaux, 2005). The summation of lower oxidant generation, residence in an unfused vacuole surrounded by an actin barrier that may prevent access of oxidants, and resistance to oxidant-mediated toxicity conferred by the LPG and PPGs of WT promastigotes (Spath et al., 2003a; Lodge et al., 2006) likely accounts for the lowered survival of *lpg2*<sup>-</sup> compared to WT *L. donovani* in host macrophages. The reciprocal relationship of LPG and the oxidative stress may have been even more remarkable if the infectious metacyclic forms of WT promastigotes had been used, since their LPGs contain approximately twice the number of the oxidant-scavenging repeating units compared to procyclic promastigote LPGs (Chan et al., 1989). There is unfortunately no method that could obtain metacyclic *lpg2*<sup>-</sup> parasites comparable to this control.

Previous studies implicated LPG as a major determinant promoting parasite resistance to lysis by complement. There are important differences between the lytic arm of the complement pathway in murine versus human hosts, with serum from the former exhibiting considerably weaker lytic activity than in other mammals (Ong and Mattes, 1989). Thus while sensitive to lysis by human complement, LPG-deficient *Leishmania* are resistant to lysis by murine complement, and no alterations in the course of *L. major* infection are seen in comparisons between C5-deficient and normal mice (Spath et al., 2003a). During the current study, we showed for the first time that as expected *lpg2*<sup>-</sup> *L. donovani* was exquisitely sensitive to complement-mediated lysis (Fig. 5). Furthermore and despite its lack of *in vivo* attenuation in mouse models, the *L. mexicana lpg2*<sup>-</sup> mutant was similarly susceptibility to lysis by human complement. Because of the differences between complement of mice and humans, it is possible that the sensitivity of *lpg2*<sup>-</sup> mutants is of little consequence in mouse infections, whereas in human hosts the complement susceptibility of *lpg2*<sup>-</sup> mutant promastigotes of all three species of *Leishmania* could result in attenuation.

In summary, our studies emphasize several important differences in the effects of LPG2 on the virulence of three *Leishmania* species, revealing an unexpected complexity and divergence in outcomes. We have focused in this work on the consequences for virulence as measured by parasite replication and/or pathology. Future studies may focus on immune responses to WT vs. *lpg2*<sup>-</sup> *L. donovani* in comparison to WT vs. *lpg2*<sup>-</sup> *L. major* (Uzonna et al., 2004; Kebaier et al., 2006). It may be of interest to perform quantitative comparisons of *L. major* and *L. donovani lpg2*<sup>-</sup> mutant infections. If the numbers of persistent *lpg2*<sup>-</sup> *L. donovani* are indeed greater than *lpg2*<sup>-</sup> *L. major* footpads (typically  $\sim 10^3$ ) as suggested in Fig. 1 ( $>10^8$  and  $>10^6$  in liver and spleen, respectively) (typically  $\sim 10^3$ ; Spath et al., 2003b, 2004), this could reflect differences in the infected cell types and/or the size or cell numbers between these organs and footpad infection sites. Alternatively, this could reflect fundamental differences between the biology of the two parasite species, manifesting *in vivo* but less apparent in macrophage infections *in vitro* (Fig. 2). These studies will have some practical value, as parasites showing ‘persistence without pathology’



such as *lpg2*<sup>-</sup> *L. major* and *L. donovani* hold promise as live attenuated vaccine lines (Spath et al., 2003b; Uzonna et al., 2004).

## Acknowledgments

This work is supported in part by NIH Grants AI045540 and AI067874 (M.E.W.), a VA Merit Review grant (M.E.W.), NIH Grant AI31078 (S.M.B. and S.J.T.), and NIH training Grants T32 AI07511 and T32 AI007260-22 (support of U.G.). We thank Tamara Barron for technical assistance and members of our laboratories for discussions. The authors are also grateful to Jian Shao at the University of Iowa Central Microscopy Facility for technical assistance.

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