The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts

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The abundant cell surface glycolipid lipophosphoglycan (LPG) was implicated in many steps of the *Leishmania* infectious cycle by biochemical tests. The presence of other abundant surface or secreted glycoconjugates sharing LPG domains, however, has led to uncertainty about the relative contribution of LPG *in vivo*. Here we used an *Leishmania major lpg1⁻* mutant, which lacks LPG alone and shows attenuated virulence, to dissect the role of LPG in the establishment of macrophage infections *in vivo*. *lpg1⁻* was highly susceptible to human complement, had lost the ability to inhibit phagolysosomal fusion transiently, and was oxidant sensitive. Studies of mouse mutants defective in relevant defense mechanisms confirmed the role of LPG in oxidant resistance but called into question the importance of transient inhibition of phagolysosomal fusion for *Leishmania* macrophage survival. Moreover, the limited lytic activity of mouse complement appears to be an ineffective pathogen defense mechanism *in vitro* and *in vivo*, unlike human hosts. In contrast, *lpg1⁻* parasites bound C3b and resisted low pH and proteases normally, entered macrophages efficiently and silently, and continued to inhibit host-signaling pathways. These studies illustrate the value of mechanistic approaches focusing on both parasite and host defense pathways in dissecting the specific biological roles of complex virulence factors such as LPG.

phosphoglycans | trypanosomatid protozoan parasite | oxidant resistance | inhibition of macrophage activation | adhesin

The protozoan parasite *Leishmania* is an intracellular pathogen that resides within an acidified phagolysosome of vertebrate host macrophages and is transmitted by biting phlebotomine sand flies. Within the phagolysosome, the parasite must resist the hydrolytic environment and avoid macrophage activation. People infected with *Leishmania* can develop diseases ranging from mild to disfiguring to fatal, depending on the species of parasite and the host factors. Current chemotherapy is inadequate, and although vaccination is thought to be feasible, no clinically effective vaccine exists.

Leishmania promastigotes are covered with a dense surface glycocalyx, composed largely of molecules attached by glycosylphosphatidylinositol (GPI) anchors (1). These GPI-anchored molecules include proteins such as the parasite surface protease gp63 and proteophosphoglycans (PPGs), as well as short GPI-anchored glycosylinositolphospholipids (GIPLs). The most abundant constituent is a large GPI-anchored phosphoglycan called lipophosphoglycan (LPG) (2–4). In all *Leishmania* species, the GPI anchor of LPG is composed of a 1-*O*-alkyl-2-lysophosphatidylinositol lipid anchor and a heptasaccharide core, to which is joined a long phosphoglycan (PG) polymer composed of 15–30 [Galβ1,4Manα1-PO₄] repeating units (substituted with other sugars in some species) and which is terminated by a capping oligosaccharide. These domains are shared with other molecules; the PG repeating units and caps occur on secreted proteins such as PPG or secreted acid phosphatase, and

the core GPI anchor domains have similarities with those present in both GIPLs and GPI-anchored proteins (1, 5, 6).

After vertebrate infection, infective metacyclic *Leishmania* must resist the action of complement, attach and enter macrophages, resist host defenses such as oxidants and hydrolytic enzymes, inhibit macrophage activation, and differentiate to the amastigote stage, which is adapted for long-term survival and replication within an acidified phagolysosome. LPG has been implicated in many steps required for establishment of macrophage infections and for survival in the insect vector (3, 4, 7–10). LPG does not play a role in the amastigote stage, however, because this parasite stage synthesizes little or no LPG and does not require LPG for virulence. However, amastigotes continue to make structurally related glycoconjugates (3, 4, 7, 9, 11).

Typically, LPG roles were studied with purified LPG and sophisticated biochemical and cellular assays. However, concerns have been raised: LPG may be applied in routes and amounts that may not be physiologically relevant, and the sharing or similarity of LPG domains to those of other parasite molecules discussed above raises the issue of specificity and the possibility of cross-activity. For example, many of the functions attributed to LPG above have been ascribed also to PPG, GIPLs, and/or GPI-anchored proteins (3, 6, 9, 12–14). Thus, teasing out the specific contributions of LPG within the complex milieu of the parasite glycocalyx remains a significant challenge.

Here we describe studies of a *Leishmania major* mutant specifically lacking LPG, which is defective in its ability to infect sand flies, mice, and macrophages (11, 15). The *lpg1⁻* mutant was obtained by targeted inactivation of a putative galacto-furanosyltransferase necessary for synthesis of the LPG glycan core; the *lpg1⁻* mutant was otherwise normal in PG, GPI-anchored proteins, GIPLs, and metacyclic gene expression (11, 16, 17). We also made use of mouse mutants defective in relevant host defenses to extend and confirm our findings on the role(s) of LPG. Overall, the studies establish a role for LPG in many but not all of the steps previously identified in macrophage invasion and survival and, in some cases, raise questions about the relevance of several aspects of the interaction of *Leishmania* with its hosts assumed previously to be important.

Materials and Methods

***Leishmania* Culture.** *L. major* LV39 clone 5 promastigotes (Rho/SU/59/P) (18) were grown in medium 199 (M199) (19). The

LPG1 mutant (*LPG1::HYG/LPG1::PAC*), referred to as *lpg1⁻*, and its *LPG1*-restored derivative (*LPG1::HYG/LPG1::PAC* [pSNBR-*LPG1*]) referred to as *lpg1⁻/+LPG1* or the *LPG1* "add-back," were grown briefly in the absence of selective agents before use (11). Metacyclics were prepared from day 4 stationary-phase cultures by an LPG-independent density-gradient centrifugation method (17).

Mouse Strains. BALB/c mice were obtained from Charles River Breeding Laboratories. C57BL/6-*Cybb^{tm1}* null mutant (*phox⁻*), C57BL/6-*Nos2^{tm1Lau}* inducible NO synthase null mutant, C57BL/6, C5-deficient B10.D2-*H2^d H2-T18^c Hc^0*/oSnJ, and C5-sufficient control B10.D2-*H2^d H2-T18^c Hc^1*/nSnJ mice were obtained from The Jackson Laboratory.

Mouse Infections. Virulence was assessed after inoculation of mouse footpads (20). Groups were injected s.c. into the footpad with 10^5 metacyclic parasites per mouse. Infections were monitored by comparing the thickness of the injected and uninjected footpads with a Vernier caliper. Parasites were enumerated in the infected tissue by a limiting-dilution assay (21).

Macrophage Infections. Infections of mouse peritoneal exudate macrophages (PEMs) were performed and relative survival was calculated by normalizing values to the initial parasite burden at 2 h postinfection (11, 17, 22). Typically 10^4 macrophages were seeded in 24-well plates and incubated with 10^5 parasites in 500 μl. For synchronous infections, parasites were incubated at 4°C for 20 min for attachment, free parasites were removed by washing, and cultures were shifted to 37°C. Macrophages were activated with 100 ng/ml lipopolysaccharide (LPS; *Escherichia coli* serotype O26:B6) and 100 units/ml IFN-γ (Sigma). All media were endotoxin-free by the Pyrotell *Limulus* amoebocyte lysate test (Associates of Cape Cod).

Opsonization. For infections, parasites were incubated for 30 min with 4% C5-deficient mouse serum in DMEM (22). For flow cytometry, parasites were incubated with 25% normal or heat-inactivated mouse serum for 20 min at room temperature (RT). C3b binding was revealed with goat anti-C3b (clone H11, 1:100 dilution; Research Diagnostics, Flanders, NJ) and Alexa Fluor 488-conjugated anti-goat IgG. IgM binding was revealed with Alexa Fluor 488-conjugated anti-mouse IgM (1:100 dilution; Molecular Probes).

Measurement of Oxidant, pH, and Protease Resistance. The xanthine–xanthine oxidase system (23, 24) was used to generate oxidants *in vitro*. Logarithmic-phase promastigotes were washed with PBS and suspended in 150 μM xanthine in PBS at 5×10^6 cells per ml, and 0.5–5 units of xanthine oxidase per ml of cells. After 1 h at RT, cells were allowed to recover for 2 h in M199, plated at various densities (10^3 to 10^4 cells) in duplicate on M199 semisolid media plates, and incubated for 7–14 days to allow colony formation. Colony numbers were normalized to controls (plating efficiencies ranged between 10% and 30%), an "EC_{50}" was calculated (typically 2 units/ml for WT), and the results were expressed as fold resistance relative to WT cells (Fig. 5B). Limiting-dilution assays were used to test the susceptibility of parasites to proteases (2.5% trypsin in DMEM for 30 min at RT), and pH effects were tested in growth assays in M199 at pH 5.5.

Phagolysosomal Fusion. PEMs were seeded in 12-well plates onto 18-mm glass coverslips (3×10^5 cells per ml) and incubated overnight (at least 12 h) in DMEM supplemented with 10% FCS and 2.5 mg/ml FITC-conjugated dextran (10 kDa, lysine fixable; Molecular Probes). Cells were washed vigorously and synchronous uptake of either zymosan (10^6 particles per ml) or parasites [multiplicity of infection (moi) of 10 for WT, or of 3 and 10 for
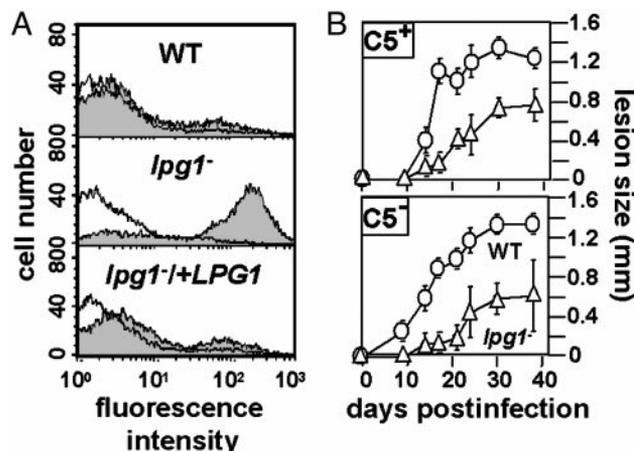
Fig. 1. *lpg1⁻* metacyclic promastigotes are sensitive to human but not mouse complement. (*A*) Flow cytometric analysis of lysis by human serum. Metacyclic WT, *lpg1⁻*, and *lpg1⁻/+LPG1* lines were incubated in the presence (shaded) or absence (open) of 2% human serum for 30 min in the presence of propidium iodide and then subjected to flow cytometry. (*B*) Infections of normal and C5-deficient mice. WT (○) or *lpg1⁻* (△) metacyclics (10^5) were inoculated into the footpad of isogenic WT (*Upper*) or C5-deficient (*Lower*) mice, and lesion formation was followed. The average and standard deviation are shown for groups of five mice; this experiment is representative of three independent tests.

lpg1⁻ low- and high-infectivity infections, respectively] was achieved as described above. Fusogenic FITC-positive phagosomes were quantified over a period of 3 h after uptake by fluorescence microscopy on paraformaldehyde-fixed preparations.

Complement Lysis. Human serum was obtained from healthy volunteers. After incubation of blood to allow clotting for 10 min at RT, serum was recovered by centrifugation for 10 min at $6,000 \times g$ at 4°C. C5-depleted human serum and human C5 were obtained from Sigma. Mouse serum was obtained by heart puncture of anesthetized animals. Washed parasites (10^6 cells in 500 μl of DMEM with 40 μg/ml propidium iodide, lacking FBS) were incubated at RT with human serum for 30 min, and fluorescence (lysis) was measured by flow cytometry.

Results

***lpg1⁻* Parasites Are Sensitive to Lysis by Human but Not Mouse Serum.** Purified metacyclic promastigotes were incubated with various amounts of fresh human serum, and the motility and integrity of the parasites were assessed. *lpg1⁻* parasites were lysed rapidly by 1% serum, whereas WT were resistant up to 10% serum (data not shown). Quantitation of lysis by flow cytometry confirmed that WT and the *LPG1* add-back controls were resistant, whereas *lpg1⁻* parasites were lysed (Fig. 1*A*). This lysis was due to complement (and not other) serum factors, because C5-deficient human serum was inactive unless C5 was added (data not shown).

In contrast, incubations with up to 60% fresh mouse serum (BALB/c or C57BL/6) failed to show detectable lysis of the *lpg1⁻* parasite (data not shown). Isogenic C57BL/10Sn WT and C5-deficient mice were infected with WT and *lpg1⁻* parasites. The *lpg1⁻* parasites showed a delay in lesion progression in the C57BL background similar to that seen in susceptible BALB/c mice (11) (Fig. 1*B*; as expected, the magnitude of the lesion was reduced in this genetically resistant strain). Second, attenuation of the *lpg1⁻* parasite did not differ in the C5-sufficient or deficient C57BL mice (Fig. 1*B*). Together these data suggest that
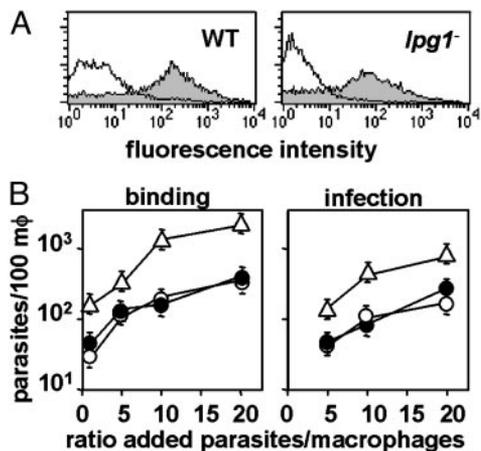
Fig. 2. *lpg1⁻* metacyclic promastigote parasites are opsonized efficiently by C3b and can invade macrophages. (*A*) Opsonization by C3b. WT (*Left*) or *lpg1⁻* (*Right*) metacyclics were treated with normal (shaded) or heat-inactivated (open) mouse serum, and deposition of C3b was quantitated by flow cytometry. Controls in which anti-C3b was omitted gave results similar to that obtained with heat-inactivated serum (data not shown). (*B*) Attachment and entry of opsonized metacyclics into macrophages (mφ). Metacyclics were opsonized with C5-deficient mouse serum and incubated with cultured PEMs (3×10^5 per well; WT, ○; *lpg1⁻*, △; and *lpg1⁻/+LPG1*, ●) at the indicated moi. For attachment (*Left*), parasites were incubated for 20 min at 4°C; for uptake (*Right*) parasites were incubated for 2 h more at 37°C. Free parasites were removed by washing with cold PBS, and cells were fixed with 3.5% paraformaldehyde, stained, and counted. The bars show the standard deviations of three replicates and are representative of at least three independent experiments.

the lytic activity of mouse complement plays little role against *Leishmania in vitro* or *in vivo*.

lpg1⁻ Parasites Enter Macrophages Normally. Parasites were incubated with normal or heat-inactivated mouse serum, labeled with anti-C3b antiserum, and analyzed by flow cytometry. Both WT and *lpg1⁻* parasites bound similar levels of C3b, which suggests that LPG is not required for opsonization (Fig. 2*A*). Similar results were obtained for IgM binding to both WT and *lpg1⁻* (data not shown). We then examined the ability of the opsonized cells to enter cultured PEMs over a 2-h period as a function of the multiplicity of infecting parasites per PEM (Fig. 2*B*). WT and the control add-back parasites entered macrophages identically, whereas *lpg1⁻* parasites showed a 4-fold increase (Fig. 2*B*). We have noticed a tendency for this and other LPG-deficient parasites in virulent *Leishmania* backgrounds to form aggregates in culture, which could account for the apparent increase. These data argue that LPG is not an essential adhesin.

lpg1⁻ Parasites Are Sensitive to Oxidants. Serum-opsonized WT and *lpg1⁻* parasites induced similar levels of O_2^- production in PEMs, ≈50% of that induced by the opsonized zymosan control (Fig. 3*A Left*). This finding suggested that neither LPG nor *L. major* down-regulate the oxidative burst during macrophage invasion. The *lpg1⁻* cells showed a significant 2-fold increase in their sensitivity to oxidants generated by the xanthine–xanthine oxidase system; the control add-backs showed similar sensitivity as WT (Fig. 3*A Right*).

To assess the significance of LPG-dependent oxidant resistance *in vivo*, PEMs were prepared from resistant C57BL/6 control and *phox⁻* mice (defective in the phagocyte oxidase system) (25), and infected by WT and *lpg1⁻* metacyclics (Fig. 3*B*). As for BALB/c PEMs (11), WT survived well and *lpg1⁻* parasites showed considerable destruction within 2 days (Fig. 3*B Left*). In contrast, the host *phox⁻* mutation was able to compen-
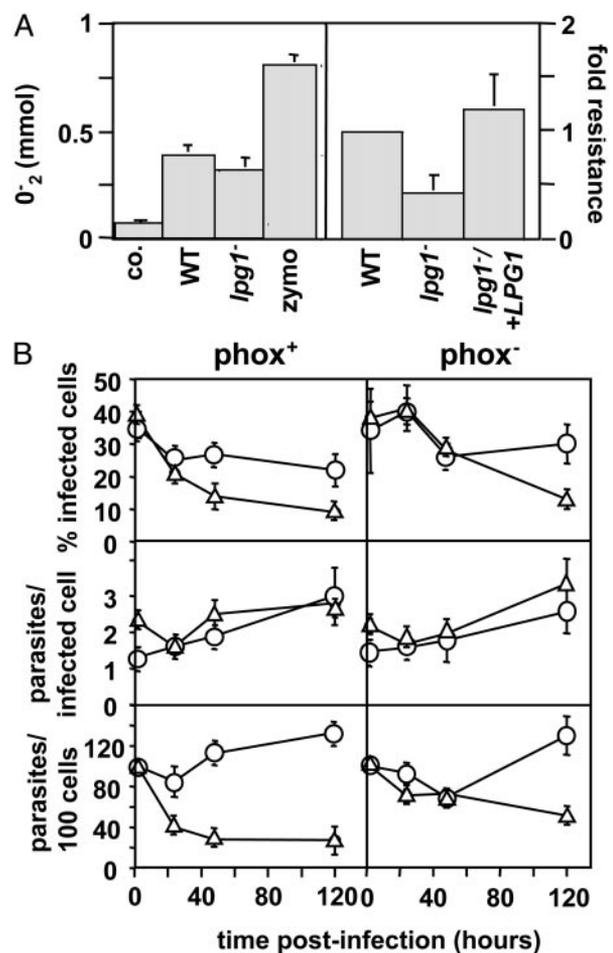

Fig. 3. *lpg1⁻* promastigotes are susceptible to oxidative stress. (A) *lpg1⁻* and WT *Leishmania* induce comparable PEM oxidative bursts (*Left*). PEMs were treated with zymosan or incubated with 4-day stationary-phase WT or *lpg1⁻* promastigotes for 90 min at 37°C, and superoxide production was determined (45). The average and standard deviation of triplicate determinations are shown, representative of two independent experiments. *lpg1⁻* parasites are more susceptible to oxidative stress (*Right*). The relative resistance of WT, *lpg1⁻*, and *lpg1⁻/+LPG1* promastigotes to oxidants generated by the xanthine–xanthine oxidase system is shown. An EC₅₀ was calculated, data were normalized to WT, and the average and standard deviation are shown. (*B*) *lpg1⁻* survives normally in infections of *phox⁻* macrophages. Infection efficiency (% infected cells, *Top*), intracellular growth (parasites per infected cell, *Middle*), and parasite survival (parasites per 100 cells, *Bottom*) of C3b-opsonized WT and *lpg1⁻* metacyclics in PEMs derived from C57BL/6 control (*phox⁺*) or isogenic oxidant-deficient (*phox⁻*) mice are shown. Three independent experiments were performed; bars show the standard deviations of one representative triplicate experiment.

sate for the LPG-dependent oxidant sensitivity, as WT and *lpg1⁻* parasites showed comparable abilities to survive in infections of *phox⁻* PEMs during the first 2 days (Fig. 3*B Right*). Thus, LPG plays an important role in oxidant resistance under physiologically relevant conditions.

Curiously, if the infection was extended to 5 days, a time at which LPG is normally absent, *lpg1⁻* survival was decreased in the *phox⁻* macrophages as well (Fig. 3*B Right*). The significance of this finding is not obvious.

Release of PGs from _L. major_ During Macrophage Invasion and Inhibition of Phagolysosomal Fusion. We performed synchronous macrophage infections of serum-opsonized metacyclic WT and *lpg1⁻* parasites, and we monitored the release of PGs with the
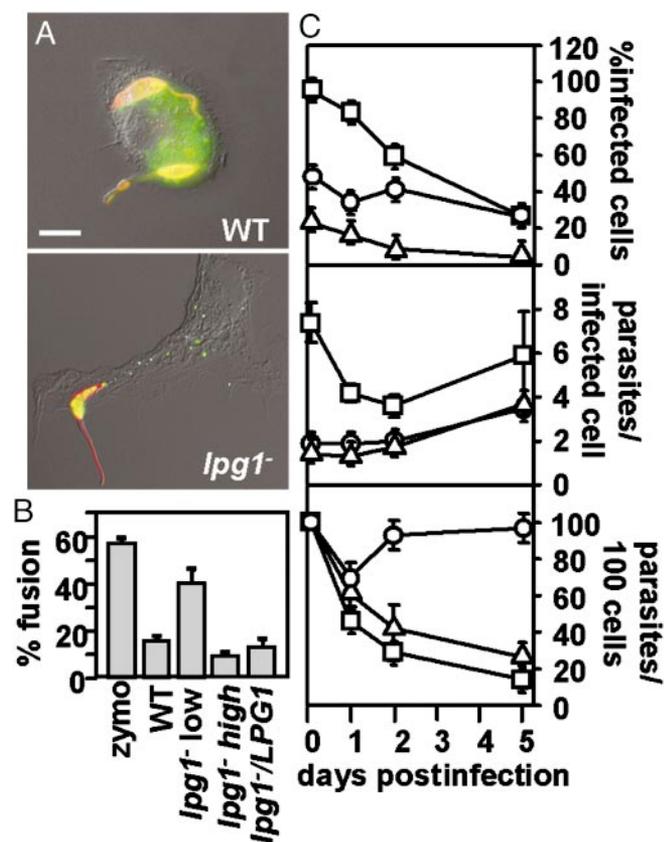
Fig. 4. Release and effects of PGs in infected macrophages. (*A*) Release of PGs. PEMs were incubated (20 min at 4°C) with serum-opsonized metacyclic WT or *lpg1⁻* promastigotes (moi of 10 or 3, respectively, to normalize for initial uptake; see Fig. 2*B*) and then incubated for up to 3 h at 37°C. Cells were fixed with 3.5% paraformaldehyde (5 min at RT), permeabilized with 100% ethanol (15 min at 4°C), incubated for 20 min at 37°C with anti-PG antibody (1:500 dilution of WIC79.3) and anti-tubulin antibody (1:2,000 dilution of anti-tubulin primary antibodies), washed with PBS, and incubated further with an FITC-conjugated goat anti-mouse IgG and Texas-red-conjugated donkey anti-rabbit IgG (each at 1:200 dilution; Jackson Immunochemicals). Exposures were 0.66 s for WT-infected macrophages and 0.25 s for *lpg1⁻*-infected macrophages (because PG levels are much higher in WT *Leishmania*). (Scale bar = 5 μm.) (*B* and *C*) *lpg1⁻* metacyclic parasites are defective in inhibition of phagolysosomal fusion and survival. Phagolysosomal fusion was quantitated by labeling of parasitophorous vacuoles with FITC-dextran, loaded previously into the lysosomal compartment (see *Materials and Methods*). The ratio of infecting *lpg1⁻* to PEMs was varied, and the effect on phagolysosomal fusion (*B*, 3 h postinfection) or parasite survival (*C*) was determined in triplicate. Purified C3-opsonized metacyclic *lpg1⁻* promastigotes were infected at an moi of 3 (low) or 10 (high); at the 2-h time point, 1.4 or 7.4 intracellular parasites per infected macrophage were obtained, respectively. The bars indicate standard deviations; one experiment is shown, which is representative of three independent experiments.

monoclonal antibody WIC 79.3 (Fig. 4*A*). Immediately after infection, PGs were confined to the parasite, but after 1 h, PG labeling was seen throughout WT-infected macrophages with evident punctate and diffuse localization (Fig. 4*A* and data not shown). Only a weaker punctate labeling pattern was evident with *lpg1⁻* parasites (Fig. 4*A*), reflecting the deposition of other less abundant PGs (such as PPGs; ref. 11) within a vesicular compartment. The kinetics of macrophage PG deposition was similar for the WT and *lpg1⁻* parasites, and by 1 h most macrophages were labeled (data not shown).

A fluorescence microscopy assay was used to measure inhibition of phagolysosomal fusion after invasion of C3-opsonized *Leishmania*. Within 3 h, most control zymosan-containing

phagosomes showed fusion with the FITC-dextran-labeled lysosomes, whereas only 20% of the phagosomes containing WT or *lpg1⁻*/+*LPG1* had fused (Fig. 4*B*). We compared the ability of *lpg1⁻* parasites to inhibit phagolysosomal fusion and survive in WT PEMs as a function of moi; in these experiments, the actual intracellular moi was determined. At low mois (1–2 parasites per infected macrophage), *lpg1⁻* parasites failed to inhibit phagosomal fusion and did not survive (Fig. 4 *B* and *C*, "*lpg1⁻* low").

Later studies, however, questioned the role of phagolysosomal fusion in parasite survival. At high mois (>6 parasites per infected macrophage), the *lpg1⁻* parasites showed inhibition of phagosomal fusion comparable to WT (Fig. 4*B*, "*lpg1⁻* high"), possibly resulting from the low levels of residual PGs in the *lpg1⁻* parasite. Despite their ability to inhibit phagosomal fusion at high mois (Fig. 4*C*), the *lpg1⁻* parasites were eliminated (Fig. 4*C*). Similarly, survival of the *lpg1⁻* parasites in *phox⁻* macrophages occurred despite the fact that these macrophages showed fusogenicity identical to that of control macrophages when infected with either WT (17% vs. 14.5% fusion for *phox⁻* vs. WT PEMs) or *lpg1⁻* (64.5% vs. 55% fusion) parasites. Last, *lpg1⁻* parasites showed no difference from WT in their resistance to low pH or treatment with proteases (data not shown).

lpg1⁻ Parasites Retain the Ability to Inhibit Macrophage Activation Pathways. Purified LPG inhibits macrophage signaling and activation *in vitro* (13, 26, 27), as revealed by monitoring key effectors such as NO and IL-12. However, infection of BALB/c PEMs by *lpg1⁻* did not lead to significant induction of either effector, despite destruction of >70% of the invading parasites (Fig. 5*A*). Addition of the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (NMMA) did not improve *lpg1⁻* survival, confirming the lack of involvement of NO in *lpg1⁻* destruction (Fig. 5*A*). That these macrophages were competent to kill *Leishmania* in an NO-dependent manner was shown in infections of PEMs previously activated by treatment with LPS + IFN-γ. These PEMs synthesized abundant NO and destroyed WT and *lpg1⁻* parasites completely, whereas inhibition of NOS activity with NMMA restored survival to that seen in nonactivated macrophages (30%; Fig. 5*A*). The lack of involvement of NO in the elimination of *lpg1⁻* parasites was supported by infections of PEMs derived from inducible NOS-knockout mice, where *lpg1⁻* parasites were destroyed also (data not shown).

We then asked whether infection by *lpg1⁻* parasites rendered infected macrophages resistant to subsequent activation. Parasites were allowed to infect PEMs for 6 h and were treated with LPS + IFN-γ for 24 h. As expected, control uninfected macrophages produced NO and IL-12, whereas macrophages infected with WT parasites showed inhibition of both NO and IL-12 production (Fig. 5*B*). Again, the *lpg1⁻* parasites resembled WT in that production of both IL-12 and NO was also inhibited despite considerably decreased survival (Fig. 5*B*). Thus, LPG is not required for the *Leishmania* parasite to enter the host cell quietly (without macrophage activation) or to inhibit the ability of macrophages to become activated by other stimuli.

Discussion

The availability of the *L. major lpg1⁻* mutant specifically defective in LPG synthesis (11, 17) allowed us to assess the contribution of LPG to specific steps implicated in parasite survival and establishment of infection in its mammalian host. In support of previous studies, LPG-deficient *L. major* were highly sensitive to human complement, more susceptible to oxidants generated by a xanthine–xanthine oxidase system, and unable to inhibit phagolysosomal fusion immediately after invasion (Figs. 1, 3, and 4). In these properties, the *in vitro* and *in vivo* results were concordant.

The contribution of these LPG-dependent functions to parasite virulence *in vivo* was assessed with the aid of mutant
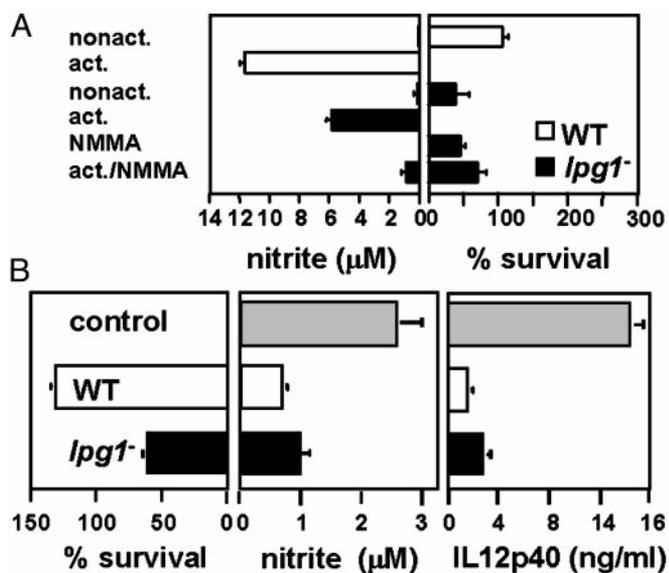
Fig. 5. Intact LPG is not required for quiet entry (without macrophage activation) or for inhibition of macrophage activation. (*A*) *lpg1⁻* promastigotes do not activate PEMs. PEMs were infected with purified C3b-opsonized WT (open bars) or *lpg1⁻* (filled bars) metacyclics. As indicated, the PEMs were incubated 12 h before infection with 100 μM NMMA, 100 ng/ml LPS plus 100 units/ml IFN-γ, or NMMA plus LPS plus IFN-γ. NO production was measured after 24 h and parasite survival was monitored; only the 5-day survival data are shown. Two independent experiments were performed; bars show standard deviations of a representative triplicate experiment. (*B*) *lpg1⁻* parasites retain the ability to inhibit PEM activation. Purified serum-opsonized WT (open bars) or *lpg1⁻* (filled bars) metacyclics were allowed to infect PEMs for 6 h, and LPS plus IFN-γ were added for another 24 h. Controls (gray bars) were mock-infected PEMs. NO-derived nitrite in culture supernatants was determined by the Griess reaction (46). IL-12 p40 levels were determined in the PEM culture supernatants by an ELISA capture method (PharMingen). Two experiments were performed; the average and standard deviation for one with triplicate samples are shown.

macrophages or mice defective in specific defense mechanisms. The increased susceptibility of the *lpg1⁻* parasite to oxidants was relatively modest, albeit comparable to that seen in other pathogens defective in several well-studied oxidant defense systems (28, 29). Interestingly, WT and *lpg1⁻* parasites induced similar levels of oxidant production in normal macrophages (Fig. 3*A*). Its importance was shown in infections of oxidative-burst-defective *phox⁻* mouse macrophages, where the LPG-deficient parasites survived as well as WT during the first 2 days when LPG is present. This finding established from both the parasite's and the host's perspective *in vivo* the importance of LPG in resistance to oxidative defenses.

A different perspective emerged from our studies showing that loss of LPG conferred susceptibility to lysis by human complement (Fig. 1; ref. 30). This result occurred in the presence of the abundant surface protease gp63, which has also been implicated in susceptibility to complement lysis in several *Leishmania* species (31, 32). Because complement resistance in *Leishmania* arises by prevention of binding of the membrane attack complex (8), these data suggest that any perturbation leading to disruption of the dense parasite glycocalyx will confer complement sensitivity.

We were surprised to find that complement lytic activity was not an effective defense mechanism against *Leishmania* in the inbred mice commonly used as virulence models. This finding arose from direct tests of mouse serum and mice genetically lacking the C5 component of complement (Fig. 2*B*). Although common inbred strains of laboratory mice were known to have

vanishingly low lytic complement activity (33, 34), the relevance to murine models of microbial pathogenesis has not been widely appreciated. Our data suggest that for *Leishmania* and perhaps other pathogens, inbred mice may not be good models for probing the role of the lytic functions of complement relevant to human infectious diseases (in contrast to their utility in studying other aspects of the complement pathway) (8).

Whereas *Leishmania* amastigotes reside in acidified, fusogenic phagosomes (35), metacyclic *L. major* promastigotes transiently inhibited phagolysosomal fusion in an LPG-dependent manner (Fig. 4*B*), as seen in *Leishmania donovani* (36). Transient fusion inhibition was thought to be required for protection while the parasite completes its differentiation to the amastigote stage within the first 2 days. Several predictions of this model, however, are not supported. First, infections of *phox⁻* macrophages restored survival of the LPG-deficient parasite nearly to that of WT in the first 2 days (when LPG is normally present), yet the *lpg1⁻* parasites resided in fusogenic phagosomes in the *phox⁻* macrophages (Fig. 3). One would not expect *phox⁻* rescue if fusion inhibition was important for survival during this time. Second, infection of macrophages with high numbers of parasites, which restored inhibition of phagolysosomal fusion by the *lpg1⁻* parasites, did not reverse parasite destruction (Fig. 4). Third, *lpg1⁻* parasites were as resistant as WT to proteases, low pH conditions, and, previously, to sand fly gut proteases *in vivo* (15). Last, in *Leishmania* infections of macrophages of *STAT1*-knockout mice, which show a defect in phagosome acidification, rescue of *lpg1⁻* survival relative to WT was not observed (G.F.S., P. Schlesinger, R. Schreiber, and S.M.B., unpublished work).

Thus, the data collectively lead to the remarkable conclusion that although transient LPG-mediated phagolysosomal fusion inhibition occurs after parasite uptake, it does not play a prominent role in the ability of *Leishmania* to establish macrophage infections. Possibly, inhibition of fusion is just an ancillary effect of LPG peripheral to its other roles in parasite virulence, as seen with amphipathic molecules with structural features resembling LPG in a variety of contexts (37). Alternatively, transient inhibition of fusion may lead to effects downstream of the "early" acidification or hydrolytic processes, as suggested by the finding that *lpg1⁻* parasites survive poorly in the *phox⁻* macrophages after 5 days, a time when LPG has largely disappeared (Fig. 3*B*). Other possibilities include steps that are unrelated to parasite survival in macrophages *in vitro* but that are important in later steps in pathogenesis in mice, such as antigen presentation (38).

Our data also did not provide support for a prominent role for LPG in several processes required for macrophage infection. One involves parasite uptake and entry; studies have shown that LPG serves as a major adhesin mediating binding and entry of *Leishmania* into macrophages, probably after opsonization by C3 (39). However, C3b deposition and uptake of the *lpg1⁻* mutant were not altered, suggesting that *Leishmania* possess an abundance of alternative C3 acceptors (Fig. 2). Similar results were obtained with IgM binding, which has also been implicated in *Leishmania* entry (40).

Interestingly, ligation of complement receptors after uptake of opsonized parasites has been suggested to mediate deactivation of macrophage signaling pathways relevant to infection, such as those leading to NO and IL-12 synthesis (8, 40). This hypothesis could explain why the *lpg1⁻* parasite retained the ability to enter macrophages without stimulation of NO or IL-12 production and to inhibit these pathways after establishment of infection (Fig. 5). However, many macrophage receptors have been implicated in *Leishmania* uptake with no clear consensus on which (if any) are specifically required for survival (41). The *lpg1⁻* mutant synthesizes a residual LPG glycan core-phosphatidylinositol anchor (11), related to the abundant heterogeneous parasite GIPLs also implicated in modulation of host cell signaling (14, 27, 42).

Possibly, the role of LPG in macrophage deactivation could be redundant with GIPLs or other parasite molecules such as PPG, which has also been implicated in parasite virulence (13, 14, 43). To resolve these questions, it will be necessary to generate other *L. major* mutants defective in individual or multiple classes of candidate molecules.

In contrast to the importance of LPG to the virulence of *L. major*, *lpg1⁻* mutants of *Leishmania mexicana*, an agent of cutaneous leishmaniasis in the New World, show very little effect on parasite virulence in infections of mice or macrophages (12). This discrepancy extends to other LPG genes (refs. 5 and 44; G.F.S., L.-F. Lye, H. Segawa, D. L. Sacks, S.J.T., and S.M.B., unpublished work). These data and studies of the host immune response suggest that different *Leishmania* species place different emphasis on the importance of canonical virulence determinants, including LPG (5). Our mechanistic studies of the defects of LPG-deficient *L. major* not only provide confirmation of its importance in this species but also will serve as a guide for future work concerning the differences among species. In this regard, limitations in the ability of murine models to assess candidate *Leishmania* virulence factors relevant to human infection (such as lysis by complement) may contribute to the perceived differences among species. Regardless, our findings firmly establish the importance of LPG in several key steps of the infectious cycle of *L. major* and emphasize the importance of mechanistic approaches involving both the host and the pathogen.

1. McConville, M. J., Mullin, K. A., Ilgoutz, S. C. & Teasdale, R. D. (2002) *Microbiol. Mol. Biol. Rev.* **66,** 122–154.
2. Beverley, S. M. & Turco, S. J. (1998) *Trends Microbiol.* **6,** 35–40.
3. Ilg, T. (2001) *Med. Microbiol. Immunol.* **190,** 13–17.
4. Ilgoutz, S. C. & McConville, M. J. (2001) *Int. J. Parasitol.* **31,** 899–908.
5. Turco, S. J., Späth, G. F. & Beverley, S. M. (2001) *Trends Parasitol.* **17,** 223–226.
6. Ilg, T., Handman, E. & Stierhof, Y. D. (1999) *Biochem. Soc. Trans.* **27,** 518–525.
7. Descoteaux, A. & Turco, S. J. (1999) *Biochim. Biophys. Acta* **1455,** 341–352.
8. Mosser, D. M. & Brittingham, A. (1997) *Parasitology* **115,** S9–S23.
9. Handman, E. (1999) *Adv. Parasitol.* **44,** 1–39.
10. Sacks, D. & Kamhawi, S. (2001) *Annu. Rev. Microbiol.* **55,** 453–483.
11. Späth, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J. & Beverley, S. M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 9258–9263.
12. Ilg, T. (2000) *EMBO J.* **19,** 1953–1962.
13. Proudfoot, L., Nikolaev, A. V., Feng, G. J., Wei, W. Q., Ferguson, M. A., Brimacombe, J. S. & Liew, F. Y. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10984–10989.
14. Proudfoot, L., O'Donnell, C. A. & Liew, F. Y. (1995) *Eur. J. Immunol.* **25,** 745–750.
15. Sacks, D. L., Modi, G., Rowton, E., Späth, G. F., Epstein, L., Turco, S. J. & Beverley, S. M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 406–411.
16. Huang, C. & Turco, S. J. (1993) *J. Biol. Chem.* **268,** 24060–24066.
17. Späth, G. F. & Beverley, S. M. (2001) *Exp. Parasitol.* **99,** 97–103.
18. Marchand, M., Daoud, S., Titus, R. G., Louis, J. & Boon, T. (1987) *Parasite Immunol. (Oxf.)* **9,** 81–92.
19. Kapler, G. M., Coburn, C. M. & Beverley, S. M. (1990) *Mol. Cell. Biol.* **10,** 1084–1094.
20. Titus, R. G., Muller, I., Kimsey, P., Cerny, A., Behin, R., Zinkernagel, R. M. & Louis, J. A. (1991) *Eur. J. Immunol.* **21,** 559–567.
21. Titus, R. G., Marchand, M., Boon, T. & Louis, J. A. (1985) *Parasite Immunol. (Oxf.)* **7,** 545–555.
22. Racoosin, E. L. & Beverley, S. M. (1997) *Exp. Parasitol.* **85,** 283–295.
23. Ager, A. (1982) *Agents Actions* **11,** Suppl., 73–81.
24. Chan, J., Fujiwara, T., Brennan, P., McNeil, M., Turco, S. J., Sibille, J. C., Snapper, M., Aisen, P. & Bloom, B. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2453–2457.
25. Pollock, J. D., Williams, D. A., Gifford, M. A., Li, L. L., Du, X., Fisherman, J., Orkin, S. H., Doerschuk, C. M. & Dinauer, M. C. (1995) *Nat. Genet.* **9,** 202–209.
26. Descoteaux, A., Turco, S. J., Sacks, D. L. & Matlashewski, G. (1991) *J. Immunol.* **146,** 2747–2753.
27. McNeely, T. B., Rosen, G., Londner, M. V. & Turco, S. J. (1989) *Biochem. J.* **259,** 601–604.
28. Tsolis, R. M., Baumler, A. J. & Heffron, F. (1995) *Infect. Immun.* **63,** 1739–1744.
29. Seyler, R. W., Jr., Olson, J. W. & Maier, R. J. (2001) *Infect. Immun.* **69,** 4034–4040.
30. Puentes, S. M., Sacks, D. L., da Silva, R. P. & Joiner, K. A. (1988) *J. Exp. Med.* **167,** 887–902.
31. Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P. & Mosser, D. M. (1995) *J. Immunol.* **155,** 3102–3011.
32. Joshi, P. B., Kelly, B. L., Kamhawi, S., Sacks, D. L. & McMaster, W. R. (2002) *Mol. Biochem. Parasitol.* **120,** 33–40.
33. Ong, G. L. & Mattes, M. J. (1989) *J. Immunol. Methods* **125,** 147–158.
34. Atkinson, J. P., McGinnis, K. & Shreffler, D. (1980) *J. Immunol. Methods* **33,** 351–368.
35. Russell, D. G., Xu, S. & Chakraborty, P. (1992) *J. Cell Sci.* **103,** 1193–1210.
36. Desjardins, M. & Descoteaux, A. (1997) *J. Exp. Med.* **185,** 2061–2068.
37. Miao, L., Stafford, A., Nir, S., Turco, S. J., Flanagan, T. D. & Epand, R. M. (1995) *Biochemistry* **34,** 4676–4783.
38. Moll, H. (2000) *Adv. Exp. Med. Biol.* **479,** 163–173.
39. Mosser, D. M. & Rosenthal, L. A. (1993) *Semin. Cell Biol.* **4,** 315–322.
40. McDowell, M. A. & Sacks, D. L. (1999) *Curr. Opin. Microbiol.* **2,** 438–443.
41. Alexander, J., Satoskar, A. R. & Russell, D. G. (1999) *J. Cell Sci.* **112,** 2993–3002.
42. Frankenburg, S., Leibovici, V., Mansbach, N., Turco, S. J. & Rosen, G. (1990) *J. Immunol.* **145,** 4284–4289.
43. Piedrafita, D., Proudfoot, L., Nikolaev, A. V., Xu, D., Sands, W., Feng, G. J., Thomas, E., Brewer, J., Ferguson, M. A., Alexander, J. & Liew, F. Y. (1999) *Eur. J. Immunol.* **29,** 235–244.
44. Ilg, T., Demar, M. & Harbecke, D. (2001) *J. Biol. Chem.* **276,** 4988–4997.
45. Johnston, R. B., Jr. (1978) *Fed. Proc.* **37,** 2759–2764.
46. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. & Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138.

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