highly conserved, but the mechanism or mechanisms determining germ cell fate are distinct, especially in mammals. Recent studies have begun to elucidate the molecular pathways by which nanos proteins maintain germ cell development through their association with Pumilio in *Drosophila* (*2*, *14*) and through a translational repression mechanism (*15*). Human NANOS1 has also recently been shown to interact with human PUMILIO-2 and these are coexpressed in spematogonia (*16*), indicating a similar function in the human testis. Advances in proteomics are likely to identify additional components of these pathways. Finally, nanos2- and nanos3-deficient mice may provide excellent model systems to evaluate the cause of sterility and infertility in humans.

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17. We thank Y. Nishimune and T. Muramatsu for TRA104 and 4C9 antibodies, respectively. Supported by Grants-in-Aid for a CREST project from Japan Science and Technology Corporation.

Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5637/1239/DC1
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2 April 2003; accepted 22 July 2003

Persistence Without Pathology in Phosphoglycan-Deficient *Leishmania major*

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***Leishmania* infections involve an acute phase of replication within macrophages, typically associated with pathology. After recovery parasites persist for long periods, which can lead to severe disease upon reactivation. Unlike the role of host factors, parasite factors affecting persistence are poorly understood. *Leishmania major* lacking phosphoglycans (*lpg2*$^-$) were unable to survive in sand flies and macrophages, but retained the ability to persist indefinitely in the mammalian host without inducing disease. The *L. major lpg2*$^-$ thus provides a platform for probing parasite factors implicated in persistence and its role in disease and immunity.**

After transmission from sand fly vectors, infective metacyclic *Leishmania* promastigotes are taken up by macrophages, where they differentiate into amastigotes adapted for survival within phagolysosomes. During the acute phase of infection *Leishmania* can induce severe pathologies in humans and animals, ranging from mild cutaneous to fatal visceral leishmaniasis. Cutaneous disease is usually controlled, with a dimunition of overt pathology accompanied by the establishment of silent and life-long persistence, which is associated with immunity (*1*–*3*). Reactivation of persistent infections underlies some of the most severe forms of leishmaniasis, including post-kala azar dermal leishmaniasis and opportunistic infections associated with human immunodeficiency virus (*4*, *5*). Persistent parasites present in asymptomatic infections may also play key roles in the transmission of leishmaniasis (*4*, *6*). Host factors that act to keep persistent parasites in check include interleukin-12 (IL-12), NO, and interferon-γ (IFN-γ), whereas those that enable parasite persistence include IL-10 (*4*, *7*, *8*). Little is known about parasite factors required for persistent infections.

The most abundant *Leishmania* surface molecules are phosphoglycans (PGs), consisting of polymers of a disaccharide phosphate repeat backbone [Galα1,4Manα1-PO_4] attached through GPI (glycosylphosphatidylinositol) anchors, as in lipophosphoglycan (LPG), or through proteins such as proteophosphoglycans (PPGs) (*9*–*11*). Together with related small glycoinositol phospholipids (GIPLs) and other GPI-anchored proteins such as gp63, PGs form a dense glycocalyx implicated in parasite survival and virulence (*10*). In *Leishmania major*, specific loss of LPG through ablation of the *LPG1* galactofuranosyl transferase results in strong defects in the ability of parasites to survive within the sand fly host and to establish infections in mammalian macrophages due to increased susceptibility to host complement and oxidative stress (*12*, *13*). However, LPG synthesis is highly down-regulated in *L. major* amastigotes and plays no role in virulence there (*12*, *14*).

Amastigotes abundantly synthesize other PGs, including PPG, which may contribute to survival (*9*). We tested this hypothesis using globally PG-deficient *L. major* parasites, obtained through deletion of the parasite Golgi guanosine diphosphate–mannose transporter LPG2 (*15*). *lpg2*$^-$ null mutants were generated by homologous replacement of the entire LPG2-coding region (*16*). They lacked all PGs, including LPG and PPG, which were restored after introduction of an episomal LPG2 gene in the *lpg2*$^-$*/+LPG2* line (Fig. 1, A and B). *lpg2*$^-$ parasites synthesized normal levels of the small GIPLs, along with the expected LPG biosynthetic "core" intermediate (Fig. 1D). Other GPI-anchored proteins such as gp63 and gp46 were present at normal levels (Fig. 1C) (*17*). Thus, except for the specific deficiency in PGs, *L. major lpg2*$^-$ was like the wild type.

In the natural sand fly vector *Phlebotomus papatasi*, *lpg2*$^-$ showed 10-fold decreased survival in the first 3 days before bloodmeal digestion and complete elimination thereafter (fig. S1) (*16*). Because *lpg1*$^-$ parasites are not compromised for early survival, these data show that PGs contribute to survival early in the sand fly, whereas LPG plays a role in parasite retention (*18*).

To study the effects of PG deficiency on mammalian infections, we used *lpg2*$^-$ parasites at the infective metacyclic stage, purified from stationary-phase cultures by an LPG-independent method (*19*). *lpg2*$^-$ para-

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sites manifested more severe defects than those previously seen in LPG-deficient *lpg1*$^-$ *L. major* (*13*). *lpg2*$^-$ was sensitive to lysis by human complement (Fig. 2A), and after uptake into macrophages, resided in fusogenic phagosomes (Fig. 2B), where they were completely destroyed (Fig. 2C). During the first 2 days of macrophage infection, the survival of *lpg2*$^-$ was comparable to that of the wild type in macrophages defective in the oxidative burst (*phox*$^-$), establishing that PGs mediate resistance to the oxidative burst in vivo (Fig. 2C). However, *lpg2*$^-$ was eliminated during later stages of both wild-type and *phox*$^-$ macrophage infections (Fig. 2C). Thus, PGs were required for both establishment and replication of *L. major* in vertebrate macrophages.

PG-deficient *lpg2*$^-$ parasites retained a number of properties of virulent *L. major*, including the ability to enter macrophages efficiently after opsonization by C3b without inducing NO or IL-12 expression (*17*), and to inhibit macrophage activation and production of NO and IL-12 in response to stimulation with IFN-γ and lipopolysaccharide (LPS) (Fig. 2D). Remarkably, this occurred even as parasites were being destroyed (Fig. 2C). Thus, whereas in vitro studies suggest that PGs are able to inhibit macrophage activation (*20*), the in vivo results with *L. major lpg2*$^-$ minimally suggest that PG inhibition of macrophage signaling is redundant with inhibition caused by other parasite molecules, and the possibility that PGs do not mediate this role in vivo must be considered.

We next studied the ability of the *lpg2*$^-$ parasite to cause infections and persist in susceptible BALB/c mice for periods from 200 to 759 days. A standard footpad infection model was used in which high numbers of parasites were inoculated, because of the extreme attentuation of the *lpg2*$^-$ parasite in macrophages (Fig. 2C) and the inability of the LV39 clone 5 wild-type parent to survive in a dermal model, which uses a smaller number of parasites (*3*, *6*, *17*). Typically, inoculation of up to 10^6 metacyclic *lpg2*$^-$ parasites yielded no pathology (Fig. 3A). Limiting-dilution assays showed that despite the lack of pathology, parasites persisted at levels of 100 to 1000 per infected mouse (Fig. 3B). Similar results were obtained at time points well beyond 200 days, including two mice at day 759 that showed no pathology but maintained 3200 to 3800 parasites at the site of infection. Thus, although PGs were essential for acute virulence and pathology, they were not required for long-term persistence.

NO is required for both innate and adaptive resistance to *Leishmania* (*21*). Thus, whereas C57BL/6 mice controlled infections, inducible nitric oxide synthase (iNOS)–deficient mice rapidly succumbed to infection with wild-type *L. major* (Fig. 3C). In contrast, *lpg2*$^-$ infections of either mouse strain failed to yield lesion formation or pathology (Fig. 3C), yet parasites persisted at similarly low levels (Fig. 3D). This result does not rule out a role for "enabling" host factors such as IL-10 or other cytokines in *lpg2*$^-$ persistence (*4*, *7*, *8*). For example, the persistence of *L. mexicana* cysteine protease mutants arose from alterations in the interactions with the host immune response (*22*, *23*). In contrast, limitation of pathology and persistence is an intrinsic property of PG-deficient *L. major* and does not require containment by the host responses. Thus, the *L. major lpg2*$^-$

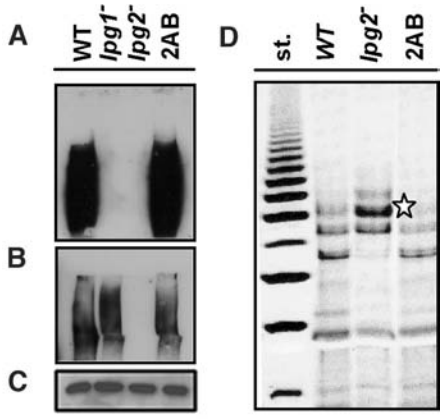

Fig. 1. Characterization of *L. major lpg2*$^-$ mutants. The lines studied were wild type (WT), LV39 clone 5; *lpg1*$^-$ and *lpg2*$^-$, homozygous knockouts of *LPG1* and *LPG2*, respectively, in LV39 clone 5 (*12*, *16*); and 2AB, *lpg2*$^-$ in which *LPG2* expression had been restored [*lpg2*$^-$/+*LPG2* (*16*)]. (**A** to **C**) Immunoblots of LPG (A) and PPG (B) with antibodies to *L. major* PG (WIC79.3) and to gp63 (#235) (C) (*16*). LPG and PPG were examined in crude extracts from log phase parasites. (**D**) GIPLs were isolated from log phase cells (*28*), subjected to nitrous acid deamination, labeled with 9-aminonaphthalene 1,3,6-trisulfate, and analyzed by fluorophore-assisted carbohydrate electrophoresis (Glyco, Novato, CA). Glucose ladder standards (st.) are indicated. The asterisk indicates the expected LPG biosynthetic intermediate seen previously in *L. donovani lpg2*$^-$ mutants [derived from Gal_2-Gal_f-(Glc-P)-Man_2-GlcN-*lyso*-alkyl-PI (*29*)].

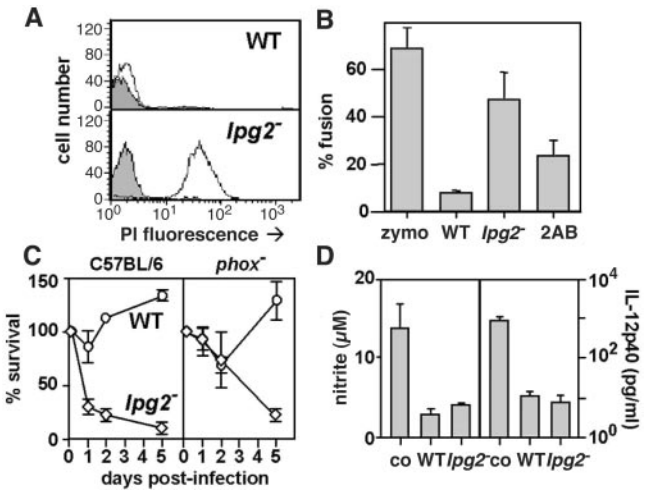

Fig. 2. *lpg2*$^-$ mutants are complement sensitive and unable to survive in macrophages, but suppress macrophage activation. (**A**) Complement lysis. A total of 10^6 purified wild-type (WT) or *lpg2*$^-$ metacyclics (*19*) were incubated with (open areas) or without (shaded areas) 2% human serum for 30 min at room temperature in Dulbecco's minimum essential medium (DMEM) containing propidium iodide (40 μg/ml). Lysis was quantified by flow cytometry. Previous studies showed that lysis was mediated by complement and was not due to other serum factors (*13*). (**B**) Transient inhibition of phagolysosomal fusion. Starch-elicited peritoneal exudate macrophages (PEM) from BALB/c mice were incubated overnight in DMEM with 10% fetal calf serum and fluorescein isothiocyanate (FITC)-conjugated dextran (2.5 mg/ml, 10 kD, lysine fixable; Molecular Probes, Eugene, OR). Phagolysosomal fusion was monitored during synchronous parasite infection as described (*19*). FITC-dextran labeled PEM were incubated with C3b-opsonized zymosan (zymo), wild-type (WT), *lpg2*$^-$, or 2AB metacyclics at 4°C for 20 min to allow binding, washed, and further incubated at 37°C. Fusion was estimated by scoring FITC-positive phagolysosomes 3 hours after infection. Results are representative of three independent triplicate experiments; error bars show standard deviations. (**C**) Macrophage infections. Infections of starch-elicited PEM from C57BL/6 (left) or isogenic oxidant-deficient (*phox*$^-$, right) mice with serum-opsonized metacyclic parasites were performed as described (*12*, *16*). Survival was calculated relative to the number of parasites present 2 hours after infection (100%). The results shown are representative of at least two experiments with each point done in triplicate; error bars indicate the standard deviations. (**D**) Wild-type (WT) and *lpg2*$^-$ parasites suppress macrophage NO and IL-12 production. BALB/c PEM were either not infected (control, "co") or were infected with metacyclic wild-type or *lpg2*$^-$ parasites as described in (B); 6 hours after infection, cultures were treated with LPS (100 ng/ml) plus IFN-γ (100 U/ml), and after a further 24-hour culture, supernatants were assayed for IL-12 (IL12p40 ELISA assay; Pharmingen, San Diego, CA) and NO production (*30*) as described (*19*). Two to four independent experiments were performed in triplicate; one representative triplicate experiment is shown, and error bars represent standard deviations.

mutation eliminates macrophage survival as well as disease pathology, yet retains persistence.

Attempts to determine the cell type harboring persistent *lpg2*$^-$ have thus far been unsuccessful, despite the use of green fluorescent protein (GFP)–tagged parasites (*17*). The data above suggest it is unlikely to be macrophages. One hypothesis for parasite persistence is the "safe haven" model, in which persistent parasites are thought to reside in an alternative cell type from which they may occasionally reactivate and go on to cause disease through propagation in macrophages (*24*, *25*) (fig. S2). This suggests an appealing model, in which wild-type *L. major* survive in macrophages through PG-dependent mechanisms, whereas both persistent wild-type and *lpg2*$^-$ survive in alternative cell types through PG-independent mechanisms (fig. S2). In other work, the genetic basis for the PG dependency of *L. major* macrophage survival was supported by the recovery of a second site revertant of *lpg2*$^-$, *lpg2*$^-$*REV*, which remained defective in the ability to become established in macrophages after metacyclic invasion, but recovered the ability to replicate in macrophages as amastigotes in the absence of PGs (*17*). In this latter respect the revertant resembles *L. mexicana*, in which PGs are not essential for macrophage survival (*26*), and it is tempting to speculate that the pathway activated in the *L. major* revertant is normally operational in *L. mexicana*.

Leishmania with intrinsic defects in acute virulence but not persistence may prove informative in efforts to develop antiparasitic vaccines, because persistent parasites have been implicated in the induction and/or maintenance of immunity in *L. major* infections (*3*, *7*). Notably, a *DHFR-TS* knockout that showed limited persistence also showed limited efficacy in vaccination tests in rodents and primates (*27*). In contrast, preliminary studies with the *lpg2*$^-$ parasite showed promising vaccination efficacy, accompanied by a novel immune response that differed from the one observed in previous vaccination studies with crude parasite antigen preparations or wild-type persistent parasites (*23*).

The properties of *L. major lpg2*$^-$ suggest that it may provide a new tool for the study of persistent *Leishmania* infections. Such a tool could be used in an experimentally tractable manner without the need for lengthy experiments and without interference arising from processes inherent in acute virulence. Parasite persistence following disease has not received extensive study owing to the complexity of the available models, which typically involve lengthy experiments and difficulties associated with low parasite numbers (*1*, *2*). Knowledge of persistence mechanisms is vital to understanding their role in human diseases arising from reactivation—for example, visceral leishmaniasis in immunosuppressed patients with acquired immunodeficiency syndrome (*5*). Our studies provide strong evidence for the existence of a class of parasite genes specifically active in enabling the survival of persistent parasites.

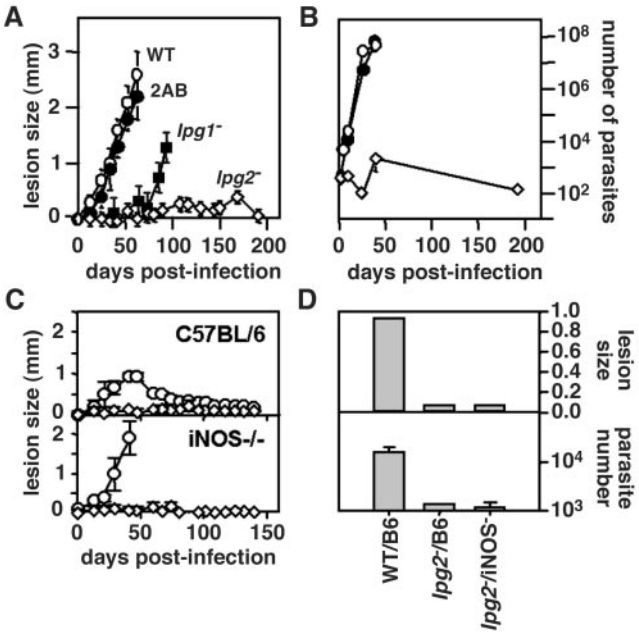

Fig. 3. *L. major lpg2*$^-$ parasites show no pathology but persist indefinitely. (**A**) Lesion formation in BALB/c mice. A total of 10^6 metacyclic WT (○), *lpg1*$^-$ (■), *lpg2*$^-$ (◇), or *lpg2*$^-$/+*LPG2* (2AB; ●) parasites were inoculated subcutaneously into the footpads of susceptible BALB/c mice, and lesion formation was monitored by measuring with a Vernier caliper the footpad thickness relative to that of the uninjected foot (*31*). (**B**) Limiting-dilution assay. Lesions of three mice were harvested at the time points indicated, pooled, and homogenized; debris was removed by brief centrifugation, and parasite numbers were determined in triplicate by limiting dilution (*32*). (**C**) *lpg2*$^-$ does not cause pathology in iNOS-deficient mice. A total of 10^6 metacyclic wild-type (○) or *lpg2*$^-$ (◇) parasites were inoculated subcutaneously into the footpads of C57BL/6 or isogenic iNOS$^-$ mice (five mice per group), and lesion formation was monitored as described in (A). (**D**) *lpg2*$^-$ persists in *iNOS*-deficient mice. The lesion size and parasite numbers at day 50 in the experiment shown in (C) were determined in triplicate as described in (B).

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33. We thank P. Scott and J. Uzona for discussions and permission to mention preliminary findings, E. Sholl for creating GFP-tagged *lpg2*$^-$ parasites, S. Hickerson for efforts to identify parasites in situ, and D. E. Dobson for reading this manuscript. Supported by NIH grant AI31078, and the Deutscher Akademischer Austauschdienst (DAAD) and Human Frontier Science Program (G.F.S.).

Supporting Online Material

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Materials and Methods
Figs. S1 and S2
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2 June 2003; accepted 18 July 2003

Supporting Online Information

1. Methods and Materials

1A. *Leishmania* strains, DNAs, culture and transfection.

Leishmania major LPG2 was isolated from a cLHYG genomic cosmid library (*S1*) using a [^{32}-P]-labeled *L. donovani LPG2* probe. A 3.8 kb *LPG2 XhoI* fragment from cosmid B2 (lab strain B3798) was inserted into the *XhoI* site of pZERO (pZERO-LMLPG2-XhoI; B3801) and its sequence determined using specific primers and an Applied Biosystems ABI-373 automated DNA sequencer (GenBank accession no. AF350492). The 1278 bp *RcaI-HpaI* fragment containing the *LPG2* open reading frame was replaced with the blunt-ended 2.8 kb *SmaI-XhoI* fragment from pX63HYG (*S2*) providing the *DHFR-TS* splice acceptor sequence and *HYG* marker to yield the targeting construct *LPG2::HYG* (strain B3867). This construct was linearized by digestion with *Xho*I; 5 μg was electroporated into *L. major* strain LV39 clone 5 (Rho/SU/59/P), and cells were plated on M199 semisolid medium containing 50 μg/ml Hygromycin B. Several clonal heterozygotes bearing a disrupted *LPG2* allele (*LPG2*/*LPG2*::*HYG*) were identified by Southern blotting using the 5' *XhoI*/*RcaI* fragment as a probe. Homozygous replacements were generated by a loss of heterozygosity protocol (*29, S3*); briefly, a heterozygote was cultured in media containing 10 to100 μg/ml hygromycin B, and plated to identify clones no longer expressing LPG as monitored by ricin agglutination. Southern blot analysis confirmed they were the desired *LPG2*::*HYG*/*LPG2*::*HYG* knockout. These were electroporated with 10 μg of pSNBR-LPG2 (B4831, obtained by inserting a 3.8 kb *Xho*I genomic fragment containing the *LPG2* ORF into the H*in*dIII/*Xba*I site of shuttle vector pSNBR) and plated on medium containing 20 μg/ml G418). Parasites from these colonies showed restoration of LPG expression. Sibling clonal lines behaved similarly and the results of

one representative null mutant line (cl.1.2), referred to as *lpg2*, and its pSNBR-LPG2 transfected derivative (cl.1.2.1), referred to as *lpg2*$^{-}$*/+LPG2* (or 2AB) are presented.

1B. Monoclonal antibodies and lectins.

Monoclonal antibody WIC79.3 recognizes *L. major* Gal-substituted [Gal-Man-P] repeating units (*S4*). Monoclonal antibody 235 recognizes the major GPI-anchored surface protease gp63 (*S5*). Polyclonal anti-gp46 antibody was from D. McMahon-Pratt (Yale University School of Medicine, New Haven, CT). Fluorescein-conjugated ricin agglutinin and PNA agglutinin were from Sigma (St.Louis, MO).

1C. Mouse strains.

BALB/c mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). The B6.129P2-Nos1 iNOS-deficient mutant, C57BL/6-*Cybb*^{tm1} oxidase cytochrome b null mutant (*phox*$^{-}$), C57BL/6 controls (*phox*$^{+}$) and complement C5-deficient B10.D2-*H2*^{d} *H2-T18*^{c} *Hc*^{0}/oSnJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

1D. Immunoblot analysis. (Fig. 1).

Crude cell extracts from logarithmic-phase cultures were subjected to Western blot analysis with WIC79.3 antibody (Fig. 1A,B) or anti-gp63 antibody 235 (Fig. 1C), at dilution of 1:1000 and 1:100 respectively. For LPG and gp63, SDS/PAGE was performed with a 10% polyacrylamide separating gel and 2.5 x 10^{6} cells (Fig. 1A,C). For PPG (Fig. 1B), SDS/PAGE was performed with a 3% polyacrylamide gel and 1 x 10^{7} cells. Reactivity with the indicated antibodies was revealed using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia).

1E. Sand fly infections.

3-5 days old female *Phlebotomus papatasi* sand flies (maintained in the Department of Entomology at the Walter Reed Army Institute of Research) were infected by feeding on heparin-treated mouse blood containing 10^6 metacyclics/ml WT, *lpg1⁻*, *lpg1⁻/+LPG1* (1AB), *lpg2⁻* and *lpg2⁻/+LPG2* (2AB) parasites (*18*). For all lines, 100% of the flies were successfully infected. For day 3 data only flies showing the presence of the blood meal were included, while for day 4 only flies showing loss (excretion) of the blood meal were included. Parasite numbers were estimated microscopically in dissected mid guts and the percent of flies showing infections deteremind; for all experiments, 8-10 flies per group were dissected and standard deviations for parasites numbers of infected flies are shown.

1F. Macrophage infections.

Infections of starch-elicited peritoneal exudate macrophages (PEM) from C57BL/6 (Fig. 2C, left panel) or isogenic oxidant-deficient (*phox⁻*, Fig. 2C, right panel) mice were performed as described (*12*). Similar results were obtained with BALB/c PEM (*17*). Metacyclic parasites were prepared from stationary phase cultures (*19*), opsonized by 30 min incubation in DMEM medium containing 4% C5 deficient mouse serum (*S6*), and allowed to invade macrophages in DMEM medium containing 0.7% BSA for 2 hours at 33°C at a multiplicity of infection of 10 and 3 parasites/PEM for wild-type (o) and *lpg2⁻* parasites (◊) respectively. Parasite numbers and morphology were monitored microscopically.

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Figure S1. Sand fly infection.

Parasite survival in *Phlebotomus papatasi* sand flies is shown at day 3, only in flies showing the presence of blood meal (open bars), and at day 4, only in flies showing loss/excretion of the blood meal (closed bars). 8-10 flies per group were dissected and standard deviations for parasites numbers of infected flies are shown. (*) indicates the presence of parasites in 100% of the dissected flies. The numbers indicate the percent of infected sand flies.

Figure S2. A model for PG dependency of pathology and persistence in WT and *lpg2⁻* *Leishmania major*.

In this model, metacyclic *Leishmania major* promastigotes enter and replicate within macrophages through a PG-dependent mechanism (heavy arrows), which is defective in the *lpg2⁻* null mutant. At some low frequency, parasites may infect an unknown cell type in which they are able to persist for long periods; this may occur directly (light arrow) or possibly, following release of amastigotes from infected macrophages (dotted arrow). The identity of the cell type harboring persistent parasites is unknown as discussed in the text. During reactivation from persistence, parasites are able to once again infect macrophages through PG-dependent

mechanism (heavy arrow). While PG-deficient *lpg2*¯ *L. major* are unable to survive and replicate in macrophages (heavy arrows), they persist in cell type X.

Fig. S1

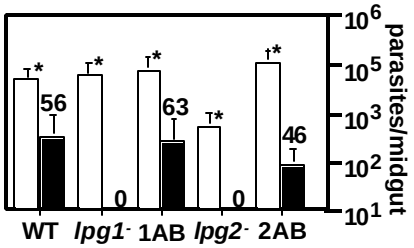

Fig. S2