

Vaccination with Phosphoglycan-Deficient *Leishmania major* Protects Highly Susceptible Mice from Virulent Challenge without Inducing a Strong Th1 Response¹

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Long-term immunity to *Leishmania* may require the continued presence of parasites, but previous attempts to create attenuated parasites that persist without causing disease have had limited success. Since *Leishmania major* mutants that lack lipophosphoglycan and other secreted phosphoglycans, termed *lpg2*⁻, persist indefinitely in infected mice without inducing any disease, we tested their ability to provide protection to virulent *L. major* challenge. In response to leishmanial Ag stimulation, cells from *lpg2*⁻-infected mice produced minimal levels of IL-4 and IL-10, as well as very low levels of IFN- γ . Nevertheless, when BALB/c mice infected with *lpg2*⁻ parasites were challenged with virulent *L. major* they were protected from disease. Thus, these findings report on attenuated parasites that may be used to induce long-term protection against leishmaniasis and indicate that the immunity induced can be maintained in the absence of a strong Th1 response. *The Journal of Immunology*, 2004, 172: 3793–3797.

Leishmaniasis is an endemic disease occurring in several continents of the world. It is estimated that >12 million people are infected and another 400 million people are at risk of infection (1). There is as yet no effective vaccination against human cutaneous leishmaniasis despite numerous studies on the mechanisms and correlates of protective immunity against *Leishmania*. Although vaccination with heat-killed *Leishmania* or recombinant proteins does not induce long-term immunity in humans (2, 3), recovery from natural infection or deliberate infection with virulent *Leishmania major* on regions of the body where resultant scarring is hidden (known as leishmanization) induce life-long protection (2, 4). Similarly, while recovery from virulent infection in mice is associated with long-term immunity, vaccination with soluble leishmanial Ag (SLA)⁴ or recombinant proteins with or without adjuvants (including IL-12) induce only short-term immunity (5). Recent reports indicate that the inability of recombinant leishmanial Ags or heat-killed *Leishmania* to maintain durable anti-*Leishmania* immunity may be related to a requirement for live parasites to maintain immunity against *L. major* (6, 7).

Several approaches have been used to generate live-attenuated organisms that can persist after infection, including long-term in vitro passage (8, 9) and targeted deletion of parasite essential and/or virulence genes (10–12). These organisms have not gained

acceptance as potential vaccine candidates because attenuated organisms may revert to virulence, and targeted deletion of essential or virulence genes results in either complete parasite destruction (11) or in mutants that induce only a delay in lesion development (10, 12). For example, a conditional auxotroph produced by targeted deletion of an essential metabolic gene, dihydrofolate reductase thymidylate synthase (*DHFR-TS*) (13, 14), induces only limited protection against virulent challenge, possibly due to their rapid elimination (11). Similarly, BALB/c mice infected with a *Leishmania mexicana* mutant lacking cysteine proteinases exhibited only a delay in lesion development when the animals were challenged with virulent parasites (12). Another *L. major* mutant, *lpg1*⁻, was generated by deleting the *LPG1* gene, which encodes a putative galactofuranosyl transferase involved in the biosynthesis of the lipophosphoglycan (LPG) core glycan (15, 16). However, although these parasites are attenuated, they still induce disease (10), precluding their use as a vaccine.

Recently we generated *L. major lpg2*⁻ mutants by targeted deletion of the *LPG2* gene (17). This gene encodes the enzyme involved in the transport of GDP mannose to the Golgi for the assembly of disaccharide-phosphate repeats of LPG and other phosphoglycan-containing molecules (18, 19). As a result, these parasites fail to synthesize LPG and other surface and secreted phosphoglycans, are highly attenuated in vitro, and upon infection of mice persist for several months without causing any overt cutaneous disease (17). Since these mutant parasites persist without causing any disease (a desirable attribute of a potential attenuated anti-*Leishmania* vaccine candidate), we investigated the host immune response following infection with *lpg2*⁻ mutants and asked whether these mutants can confer protection against virulent *L. major* challenge. In this study, we show that vaccination with *lpg2*⁻ *L. major* induces dramatic protection against virulent challenge, which suggests that *lpg2*⁻ mutants may serve as a live attenuated vaccine for leishmaniasis. Interestingly, while this immunity was associated with decreased IL-4 and IL-10 responses, no delayed-type hypersensitivity (DTH) or significant increase in IFN- γ production was observed, suggesting that *lpg2*⁻ parasites may induce a different type of memory response from that previously reported in leishmanial vaccine models.

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⁴ Abbreviations used in this paper: SLA, soluble leishmanial Ag; LPG, lipophosphoglycan; DTH, delayed-type hypersensitivity; KO, knockout; LN, lymph node; WT, wild type.

Materials and Methods

Mice

Female BALB/c, BALB SCID (Cby.Smm.CB17-Prkdc^{scid}/J), and C57BL/6-*Irfng*^{mt1ts} (GKO) mice (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-12p40 (originally from The Jackson Laboratory) knockout (KO) and STAT4 KO (originally provided by Dr. M. Kaplan, Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN), both on C57BL/6 background, were obtained from in-house breeding colonies. Mice were maintained in a specific pathogen-free environment at the School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, and the colony was screened regularly for the presence of murine pathogens.

Parasites and infections

L. major strain LV39 clone 5 promastigotes (Rho/SU/59/P; wild type (WT)), were grown in M199 medium supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete M199 medium). The homozygous *lpg2*^{-/-} mutant and parasites complemented with *LPG2*, termed *lpg2*^{-/+}*LPG2*, all derived from the WT clone, were generated as previously described and maintained in their respective selective media (17). For infection of mice, parasites were washed three times in PBS and 50 µl containing 5 × 10⁶ parasites were injected into the right hind footpad. For challenge infections, age-matched naive and mice previously infected with *lpg2*^{-/-} parasites (10 wk) were infected with 50 µl of PBS containing 2 × 10⁶ WT *L. major* in the left footpad.

For DTH responses, footpad swelling was measured in naive BALB/c mice or mice infected with *lpg2*^{-/-} parasites 72 h after challenge with *L. major*. As a positive control, C57BL/6 mice that had healed a primary infection with *L. major* were similarly tested for DTH.

Measurement of lesion size and estimation of parasite burden

Lesion size was measured with dial calipers and determined by subtracting the size of uninfected from that of infected footpad. Parasite burdens in the infected footpad and draining popliteal lymph node (LN) were estimated by limiting dilution analysis as previously described (20).

Cytokine production

At different times after infection, groups of mice were sacrificed and single-cell suspensions from spleens and draining LNs of infected mice were made. Cells (4 × 10⁶/ml) were plated in 24- or 96-well plates (1 ml or 200 µl, respectively) in complete tissue culture medium (DMEM supplemented with 10% FBS, 5 × 10⁻⁵ µM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) and stimulated with 50 µg/ml SLA as previously described (21). Cells were incubated at 37°C for 72 h and supernatants were assayed for cytokines by ELISA as previously described (21).

Proliferation assays by [³H]thymidine incorporation

LN cells were stimulated with soluble anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml) Abs or SLA (50 µg/ml). After 72 h, the cultures were pulsed with [³H]thymidine (0.5 µCi) overnight and incorporation of thymidine was assessed by a scintillation counter.

Results

lpg2^{-/-} *L. major* are highly attenuated in vivo

As previously reported (17), we found that *lpg2*^{-/-} *L. major* are highly attenuated, as BALB/c mice infected with *lpg2*^{-/-} *L. major* fail to develop any cutaneous lesions, even after 20 wk. In contrast, mice infected with WT parasites developed uncontrolled and progressive lesions and had to be euthanized after 6 wk. Mice infected with *lpg2*^{-/-} parasites complemented with the *LPG2* gene (*lpg2*^{-/+}*LPG2*) manifested a phenotype similar to those infected with WT parasites, indicating that the inability of *lpg2*^{-/-} parasites to induce lesion formation was due to a specific lack of *LPG2* gene products. However, despite the absence of a lesion, *lpg2*^{-/-} parasites were recovered from the footpad and draining LN of infected mice at 6 and 10 wk after infection (data not shown) (17).

In order for *lpg2*^{-/-} *L. major* to be considered as a vaccine, it is critical that the parasites remain attenuated in the absence of an immune response. Therefore, we infected SCID mice (which lack both B and T cells and are highly susceptible to *L. major*) with

lpg2^{-/-} parasites and monitored lesion development. As shown in Fig. 1, both BALB/c mice (Fig. 1A) and SCID mice (Fig. 1B) infected with *lpg2*^{-/-} failed to develop any disease. In contrast, *lpg1*^{-/-} parasites, which only lack the expression of LPG, induced delayed but progressive lesions in both BALB/c and SCID mice, suggesting that the absence of pathology in *lpg2*^{-/-} parasites is due to the deficiency of one of the phosphoglycans other than LPG.

SCID mice have elevated numbers of NK cells which produce IFN-γ and can therefore enhance resistance. To ensure that IFN-γ was not contributing to the failure of *lpg2*^{-/-} parasites to induce pathology, we infected IFN-γ KO, IL-12p40 KO, and STAT4 KO mice with WT and *lpg2*^{-/-} parasites and monitored the course of lesion progression. As shown in Fig. 1, C and D, no disease was evident following infection of mice lacking IFN-γ or lacking the pathway required for IFN-γ production in leishmaniasis, with *lpg2*^{-/-} parasites, even though parasites were recovered at the termination of the experiment in all of the strains. Taken together, these results indicate that host immune responses are not responsible for keeping persistent *lpg2*^{-/-} parasites in check. Rather, they suggest that *LPG2* gene-dependent molecule(s) are required for optimal parasite proliferation in vivo, although in the absence of these molecules, *lpg2*^{-/-} parasites are able to persist.

lpg2^{-/-} *L. major* induce distinct immune responses from WT parasites

To determine the influence of phosphoglycans on immunity, we measured the inflammatory response and the production of effector cytokines by cells from mice infected with WT and *lpg2*^{-/-} *L. major* at different times after infection. The number of cells isolated from the draining LNs and their proliferative potential at 3 days after infection were similar in mice infected with WT and *lpg2*^{-/-} parasites, suggesting that the absence of *LPG2* gene products does not alter the early inflammatory responses to *L. major* (Table I). However, by 4 wk of infection, the number of cells in the draining LNs of mice infected with WT and *lpg2*^{-/+}*LPG2* parasites was 10-fold greater than in mice infected with *lpg2*^{-/-} parasites (WT: 50 ± 9 × 10⁶; *lpg2*^{-/+}*LPG2*: 42 ± 6 × 10⁶; *lpg2*^{-/-}: 4 ± 0.4 × 10⁶), suggesting that although *lpg2*^{-/-} parasites persisted in BALB/c mice their stimulation of the immune system may be limited.

The cytokine responses in mice infected with WT or *lpg2*^{-/+}*LPG2* parasites for 4 wk were as expected (little or no IFN-γ and very high IL-4 and IL-10, Fig. 2, A–C). In contrast, cells from mice infected with *lpg2*^{-/-} parasites produced very low levels of IL-4,

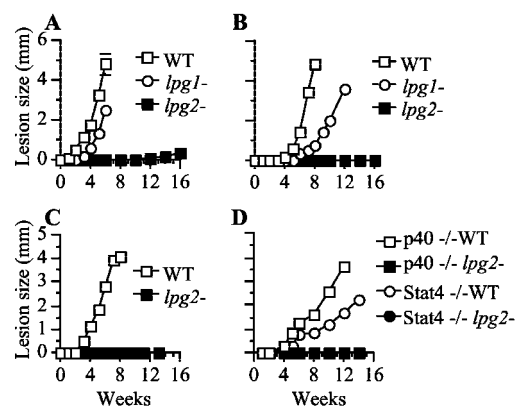


FIGURE 1. *lpg2*^{-/-} parasites differ from *lpg1*^{-/-} parasites in their capacity to induce lesions and do not induce lesions in immunocompromised mice. Course of lesion progression in BALB/c (A), SCID (B), IFN-γ KO (C), and IL-12p40 and STAT4 KO (D) mice infected with 5 × 10⁶ stationary phase promastigotes of WT, *lpg1*^{-/-}, or *lpg2*^{-/-} *L. major*.

Table 1. *lpg2*⁻ parasites induce comparable early inflammatory and proliferative responses as WT *L. major*^a

	Cells/LN ± SE (×10 ⁶)	Proliferation ± SE (×10 ³ cpm)		
		Medium	SLA	Anti-CD3
Uninfected	1.9 ± 0.2	0.2 ± 0.5	0.3 ± 0.1	57.1 ± 12.6
WT	14.6 ± 0.5	8.5 ± 1.5	16.7 ± 1.9	39.7 ± 1.2
<i>lpg2</i> ⁻	17.0 ± 3.8	25.6 ± 3.7	21.3 ± 3.7	50.2 ± 1.30
<i>lpg2</i> ^{-/+} LPG2	14.0 ± 4.0	25.8 ± 10.8	28.4 ± 4.7	65.3 ± 18.3

^a Naive (uninfected) mice or BALB/c mice infected for 3 days with WT, *lpg2*, or *lpg2*⁻/LPG2 *L. major* were sacrificed and the total number of cells in the draining popliteal LNs were enumerated. Cells were stimulated with SLA or anti-CD3/anti-CD28 for 3 days and pulsed with [³H]thymidine for 18 h, and cellular proliferation was measured by counting the amount of radioactivity incorporated by the cells.

IL-10, and IFN- γ (Fig. 2, A–C). A similar cytokine pattern was observed at 10 wk (data not shown). Furthermore, when challenged at 10 wk with virulent parasites, *lpg2*⁻-infected mice failed to exhibit a DTH response (*lpg2*⁻-infected: <0.1 mm; naive controls: <0.1 mm). The absence of DTH is notable since resistant mice, such as C57BL/6 mice that have healed a primary infection with *L. major*, exhibit a substantial DTH (0.8–1.2 mm). Thus, the immune response induced by *lpg2*⁻ parasites was not associated with a strong cell-mediated immune response. However, while the IFN- γ levels were low in *lpg2*-infected mice, the ratio of IFN- γ :IL-4 production by cells from these mice was significantly ($p < 0.01$) higher than in WT-infected mice (Fig. 2D).

Vaccination with *lpg2*⁻ parasites protects mice against virulent *L. major* challenge

To investigate whether *lpg2*⁻ parasites would provide protection to *L. major*, mice previously infected for 10 wk with *lpg2*⁻ parasites, as well as naive control mice, were challenged in the contralateral footpad with virulent *L. major*. Control mice developed progressive disease and had to be euthanized after 8 wk. In contrast, mice previously infected with *lpg2*⁻ parasites displayed a dramatic resistance, exhibiting no lesions until after 12 wk, at which time a small cutaneous lesion appeared in one mouse (Fig. 3A). Mice were sacrificed (8 wk for control (naive) and 13 wk for *lpg2*⁻ mutant infected) and the parasite burden in the footpad was estimated by limiting dilution. In agreement with lesion size, mice

previously infected with *lpg2*⁻ parasites had 4–5 logs fewer parasites than controls (Fig. 3B). Although we have not differentiated between the WT and *lpg2*⁻ parasites in this assay, our assumption is that the parasites we are measuring at the contralateral site of challenge are WT parasites. In any case, however, the results indicate that dramatic protection is obtained in *lpg2*⁻ mutant-infected BALB/c mice following challenge with virulent WT organisms.

Resistance induced by *lpg2*⁻ parasites is associated with suppression of IL-4 and IL-10 responses

Despite very weak, in some cases, undetectable immune responses in mice infected with *lpg2*⁻ parasites at the time of challenge, these mice were strongly protected against virulent *L. major* challenge. To determine whether the immune responses were enhanced after challenge infection, LN cells from control and *lpg2*⁻ mutant-infected mice were stimulated in vitro with SLA and the production of IL-4, IL-10, and IFN- γ was assessed. IFN- γ production was very low in both groups, although there was a 2- to 3-fold increase in *lpg2*⁻ mutant-infected mice (Fig. 4A). In contrast, the production of IL-4 and IL-10 was dramatically different; control mice produced ~100- and 10-fold more IL-4 and IL-10, respectively, than mice previously infected with *lpg2*⁻ parasites (Fig. 4, B and C). Thus, the protection observed in mice previously infected with *lpg2*⁻ parasites may be attributable to a more efficient activation of macrophages by IFN- γ in the complete absence and/or pronounced reduction of IL-4 and IL-10. These results suggest that what may be most important for protection is the ratio of Th1 (IFN- γ) and Th2 (IL-4, IL-10) cytokines.

Discussion

There is as yet no effective vaccination against human cutaneous leishmaniasis. Since persistent parasites have been associated with

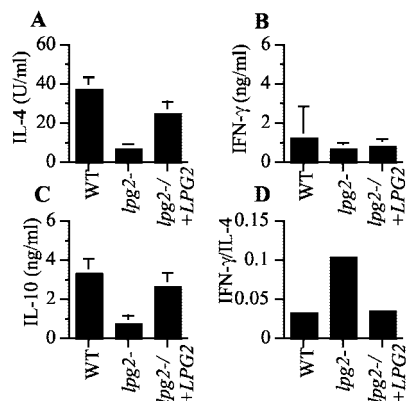


FIGURE 2. *lpg2*⁻ parasites induce minimal Th1 or Th2 immune responses. Ag-specific recall cytokine response by LN cells from mice infected for 4 wk with WT, *lpg2*⁻, or *lpg2*^{-/+} LPG2 *L. major*. Cells isolated from the draining LNs of infected mice were stimulated in vitro with SLA for 72 h and the supernatants were assayed for IL-4 (A), IFN- γ (B), and IL-10 (C) by ELISA. D, ratio of IFN- γ :IL-4 in culture supernatants of cells from infected mice. Data presented are representative of two experiments with similar results.

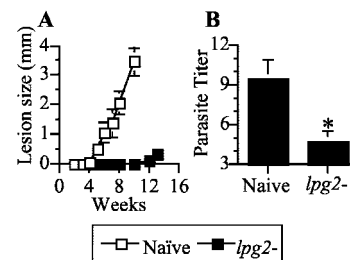


FIGURE 3. Vaccination of BALB/c mice with *lpg2*⁻ parasites confers strong resistance against virulent challenge. Naive and 10-wk *lpg2*⁻ mutant-infected mice were challenged with 2×10^6 virulent *L. major* and lesion progression was monitored (A). Mice were sacrificed at 10 (naive) or 13 (*lpg2*⁻) wk after challenge to estimate parasite burden (B). The data presented are representative of two experiments with similar results. *, $p < 0.01$.

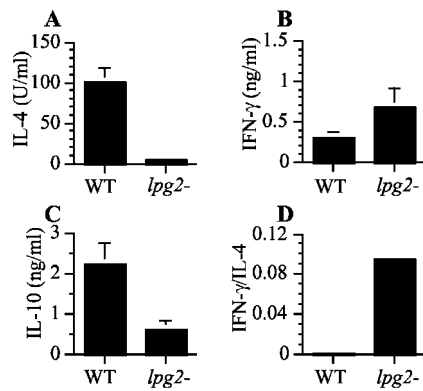


FIGURE 4. Protection induced by *lpg2*⁻ parasites is associated with slightly enhanced IFN- γ and dramatic suppression of IL-4 and IL-10 responses. At 8 (naive controls) and 13 (*lpg2*⁻-infected group) wk postchallenge, infected mice were sacrificed and mononuclear cells isolated from the draining LNs were stimulated with SLA and the production of IL-4 (A), IFN- γ (B), and IL-10 (C) was assessed by ELISA. D, Ratio of IFN- γ :IL-4 in culture supernatants of cells from infected mice.

long-term protection, the idea of using live-attenuated organisms as a vaccine against leishmaniasis has been pursued by several laboratories, but has been hampered by limited efficacy (11, 22, 23). We report here that *lpg2*⁻ parasites, which persist without causing overt cutaneous lesions (17), induce protection against virulent challenge in an experimental model, indicating that these parasites might be used as an attenuated vaccine for leishmaniasis.

Paradoxically, resistance in *lpg2*⁻-infected mice is not associated with a significant Th1 response as measured by in vitro IFN- γ production to leishmanial Ags or DTH. This raises two questions. First, how is the protection being mediated and second what are the appropriate correlates of immunity in leishmaniasis? Although the IFN- γ levels in recall responses were low, it is likely that the final effector mechanism involved in protection is the activation of macrophages by IFN- γ . Indeed, the IFN- γ :IL-4 ratio was higher in *lpg2*⁻-infected mice than in mice infected with WT parasites, suggesting the presence of an IFN- γ -dependent protective mechanism. We hypothesize that in the absence of a strong IL-4 or IL-10 response, the low levels of IFN- γ produced may be sufficient for protection. However, why the levels of IFN- γ are so low remains unclear, although characterization of the T cells that provide protection in *lpg2*⁻-infected mice should help address this issue. One possible scenario consistent with the data is that protection is mediated by a resting memory T cell population, which may be analogous to central memory T cells, that provides an expanded pool of Ag-experienced cells that can become effector T cells upon challenge (24, 25). Indeed, it has been postulated that central memory T cells can provide better protection than effector T cells (26, 27), although their role in immunity to leishmaniasis has yet to be determined.

The protection induced by the *lpg2*⁻ parasites could be similar to that seen in BALB/c mice given very low numbers of virulent *L. major* (28). However, in contrast to the resistance obtained by low-dose virulent *L. major* infection of BALB/c mice (6, 28, 29), no DTH was observed following challenge of *lpg2*⁻ mutant-infected mice, strongly suggesting that distinct mechanisms operate to maintain immunity in low-dose- and *lpg2*-induced resistance. Moreover, the dose of *lpg2*⁻ parasites used in these experiments would be expected to induce an IL-4 response in BALB/mice, which was not the case. Thus, these results suggest that *lpg2*⁻ parasites differ in their interactions with the immune system. However, our finding that the early inflammatory response in the drain-

ing LN of mice infected with WT and *lpg2*⁻ parasites is similar suggests that *LPG2*-dependent *Leishmania* phosphoglycans are not required to induce host innate responses. In contrast, the absence of particular *LPG2* gene-dependent phosphoglycans may qualitatively influence the early adaptive immune response, resulting in the ability of these parasites to induce protective immunity. For example, *Leishmania* phosphoglycans have been linked with inhibition of IL-12 production by macrophages (30). Studies are underway to determine how these molecules influence the early immune response to *L. major* in BALB/c mice.

These results also indicate that DTH and in vitro IFN- γ production in response to leishmanial Ags do not always correlate with immunity. Taken together with our finding that immunization of vervet monkeys induces both of these immune responses without inducing significant resistance to infection (31), it is clear that reliable correlates of immunity for leishmaniasis have yet to be defined. Understanding how immunologic memory is induced and maintained should aid in defining useful correlates of immunity. Indeed, further analysis of the immunity induced by *lpg2*⁻ parasites may uncover previously undefined pathways of memory T cell induction that can lead to protection. Thus, in addition to their potential use as a vaccine, these parasites will be useful for studying the mechanisms of parasite persistence and their role in the maintenance of resistance against *L. major*.

One of the arguments against the use of live-attenuated organisms for vaccine purposes is the potential of the parasites to cause disease in an immunodeficient individual. Previously, it was shown that *lpg2*⁻ *L. major* fails to induce disease in inducible NO synthase-deficient mice, which argues that the attenuation of *lpg2*⁻ parasites is independent of the immune response (17). To confirm this, we infected several additional immunodeficient mice with *lpg2*⁻ parasites, including SCID mice that lack B and T cells, as well as IFN- γ , IL-12p40, and STAT4 KO mice, and observed no disease. These results indicate that this *lpg2*⁻ line is inherently attenuated. Nevertheless, further studies to understand the nature of this attenuation will be required before these parasites can be used as a vaccine, since infection with another line of *lpg2*⁻ *L. major* eventually leads to lesion development and *lpg2*-deficient *L. mexicana* are as virulent as their WT counterparts (17, 32).

Overall, our results show that vaccination with *lpg2*⁻ *L. major* provides protection in BALB/c mice against virulent *L. major* challenge. Although additional work needs to be done with these mutants as vaccine candidates, they offer hope for the development of a live-attenuated vaccine against *L. major*. Moreover, since the protection induced by *lpg2*⁻ parasites is striking despite the absence of a strong IFN- γ response or DTH to leishmanial Ags, our studies may have revealed an as yet undescribed mechanism of maintaining immunity to *Leishmania*. How leishmanial phosphoglycans and the ability of parasites to persist contribute to that immunity can be investigated using these mutants. Thus, in addition to the potential use of *lpg2*⁻ parasites as an attenuated vaccine, these mutants will also be useful for defining new pathways of resistance, probing the role of parasite persistence in immunologic memory, and understanding how phosphoglycans modulate immune responses to *Leishmania*.

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