Binding of Leishmania infantum Lipophosphoglycan to the Midgut Is Not Sufficient To Define Vector Competence in Lutzomyia longipalpis Sand Flies

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ABSTRACT The major surface lipophosphoglycan (LPG) of Leishmania parasites is critical to vector competence in restrictive sand fly vectors in mediating Leishmania attachment to the midgut epithelium, considered essential to parasite survival and development. However, the relevance of LPG for sand flies that harbor multiple species of Leishmania remains elusive. We tested binding of Leishmania infantum wild-type (WT), LPG-defective (Δlpg1 mutants), and add-back (Δlpg1 + LPG1) lines to sand fly midguts in vitro and their survival in Lutzomyia longipalpis sand flies in vivo. Le. infantum WT parasites attached to the Lu. longipalpis midgut in vitro, with late-stage parasites binding to midguts in significantly higher numbers than were seen with early-stage promastigotes. Δlpg1 mutants did not bind to Lu. longipalpis midguts, and this was rescued in the Δlpg1 + LPG1 lines, indicating that midgut binding is mediated by LPG. When Lu. longipalpis sand flies were infected with the Le. infantum WT or Le. infantum Δlpg1 or Le. infantum Δlpg1 + LPG1 line of the BH46 or BA262 strains, the BH46 Δlpg1 mutant, but not the BA262 Δlpg1 mutant, survived and grew to numbers similar to those seen with the WT and Δlpg1 + LPG1 lines. Exposure of BH46 and BA262 Δlpg1 mutants to blood-engorged midgut extracts led to mortality of the BA262 Δlpg1 but not the BH46 Δlpg1 parasites. These findings suggest that Le. infantum LPG protects parasites on a strain-specific basis early in infection, likely against toxic components of blood digestion, but that it is not necessary to prevent Le. infantum evacuation along with the feces in the permissive vector Lu. longipalpis.

IMPORTANCE It is well established that the presence of LPG is sufficient to define vector competence of restrictive sand fly vectors with respect to Leishmania parasites. However, the permissiveness of other sand flies with respect to multiple Leishmania species suggests that other factors might define vector competence for these vectors. In this study, we investigated the underpinnings of Leishmania infantum survival and development in its natural vector, Lutzomyia longipalpis. We found that LPG-mediated midgut binding persists in late-stage parasites. This observation is of relevance for the understanding of vector-parasite molecular interactions and suggests that only a subset of infective metacyclic-stage parasites (metacyclics) lose their ability to attach to the midgut, with implications for parasite transmission dynamics. However, our data also demonstrate that LPG is not a determining factor in Leishmania infantum retention in the midgut of Lutzomyia longipalpis, a permissive vector. Rather, LPG appears to be more important in protecting some parasite strains from the toxic environment generated during blood meal digestion in the in-
sect gut. Thus, the relevance of LPG in parasite development in permissive vectors appears to be a complex issue and should be investigated on a strain-specific basis.

**KEYWORDS** LPG, *Leishmania*, parasite binding, parasite survival, sand fly, sand fly midgut, vector competence

Phlebotomine sand flies (Diptera—Psychodidae) are biological vectors of *Leishmania* parasites (Kinetoplastidae). Different species of *Leishmania* cause a spectrum of diseases collectively known as leishmaniasis. Leishmaniasis is endemic in over 88 countries, putting over 350 million people at risk of becoming infected (1). Overall, 2 million people are infected with *Leishmania* annually, resulting in between 40,000 and 90,000 patient deaths due to complications from the most dangerous form of the disease, visceral leishmaniasis (1).

In order to establish a mature transmissible infection, *Leishmania* needs to escape from the barriers imposed by the sand fly midgut (2–8). Digestive enzymes in the sand fly gut were shown to be detrimental to the transitional stages during differentiation of *Leishmania major* amastigotes to procyclic promastigotes (5, 7). This susceptibility was also attributed to toxic by-products from the digested blood for *Leishmania donovani* (9). Upon activation, the sand fly immune system is also known to be effective at reducing parasite loads by means of the Imd pathway (8). The peritrophic matrix (PM) represents another strong barrier to *Leishmania* development in the sand fly midgut (2, 3, 5, 10). *Leishmania* relies on breakdown of the PM, mediated by a sand fly-secreted chitinase, to escape its confinement (2). After this step, the parasites attach to the midgut epithelium (6, 11), and such attachment in restrictive vectors requires specific carbohydrate side chains on the surface of the parasite that bind a specific receptor on the midgut microvilli (6, 12).

Among the barriers preventing *Leishmania* development within the sand fly, midgut attachment has been suggested as the defining factor of sand fly vector competence (6, 12). The *Leishmania* surface is decorated with a rich glycocalyx (13–15), exhibiting four major types of GPI (glycosylphosphatidylinositol)-anchored glycoconjugates (16). The lipophosphoglycan (LPG) molecule is the most abundant component of the promastigote surface coat and consists of an oligosaccharide cap, a backbone of galactose-mannose-repeated units [Gal(β1,3)Man(α1)-PO₄], a conserved glycans core [Gal(α1,6)Gal(α1,3)Gal(β1,3)Glc[(α1)-PO₄]Man(α1,3)Man(α1,4)-GlcN(α1)], and a PI (1-O-alkyl-2-lyso-phosphatidylinositol) anchor (13, 14, 17). The galactose-mannose repeats exhibit different carbohydrate side chains, depending upon the *Leishmania* species, strains, and stages (16, 18). It has been demonstrated that variations in the nature of the side chain sugars decorating the LPG molecule of nonmetacyclic stages (nonmetacycles) confer specificity to interactions with vectors (16). Further, these side chains are modified to ensure release of metacyclic parasites (metacycles) from the midgut (16); in some instances, the LPG molecule conformationally prevents binding of sugars to the midgut (16, 19–21). Such studies of vector competence, mostly undertaken under laboratory conditions, sorted sand flies into two groups (16). The restrictive, or specific, vectors, such as *Phlebotomus papatasi*, *P. duboscqi*, and *P. sergenti*, are able to support the development of only one species of *Leishmania* (16). The permissive sand fly vectors, such as *Phlebotomus perniciosus*, *Phlebotomus argentipes*, and *Lutzomyia longipalpis*, are capable of supporting multiple *Leishmania* species (22–24).

The functional binding properties of *Le. major* LPG have been extensively studied (6, 12, 25, 26). It has been demonstrated that the purified nonlipidic portion of the *Le. major* LPG (PG) binds only to midgut receptors of its natural sand fly vector, *Phlebotomus papatasi*, but that the binding is restricted to the LPG from the procyclic stage (6). Whereas the PG of the nonbinding metacyclic stage is a much longer molecule and displays arabinose side chains (6), the procyclic LPG is shorter and exhibits galactose side chains (6), which are recognized by a galactose-binding lectin (PpGalec) expressed on the *P. papatasi* midgut epithelium (11). Supporting the importance of LPG in sand fly vector competence, a *Le. major* strain devoid of LPG side chains was not capable of...
developing in *P. papatasi* (12), nor was wild-type (WT) *Le. major* fed along with anti-PpGalec antibodies (11). In both cases, the infections were mostly lost once the digested blood was passed. *Le. major lpg1* knockout (KO) parasites also failed to develop in another natural vector of *Le. major*, *Phlebotomus duboscqi* (25, 26).

Although the factors defining vector competence of the sand fly *P. papatasi* for *Le. major* are well known (6), they may not govern interactions of other sand fly-*Leishmania* pairs (12, 24–29). In fact, purified LPGs from multiple *Leishmania* species bind to the midgut of the sand fly *P. argentipes* (12), a permissive vector, despite exhibiting divergent carbohydrate side chains (16). Interestingly, *Le. major* and *Le. tropica* out-compete *Le. infantum* binding to midguts of its natural vector *Lu. longipalpis* when simultaneously in contact with the epithelium (29). In addition, the *Le. major lpg1* knockout line can successfully develop in both permissive *Lu. longipalpis* (25) and permissive *P. perniciosus* (26) sand flies. Even though an LPG-independent mechanism based on a potential *Leishmania* lectin attaching to the microvilli glycocalyx has been proposed for *Leishmania* development in permissive vectors (24, 28, 30) and a candidate midgut mucin of 45 kDa that binds to *Le. major* was identified previously (30), confirmation of its function as a midgut receptor is yet to be demonstrated. Further, there is also a possibility that this phenomenon is restricted to *Le. major* development in permissive vectors and is not extendable to other *Leishmania* species naturally transmitted by such vectors. Such species-specific features have been demonstrated for the FLAG1/SMP1 flagellar protein that mediates midgut attachment of *Le. major* to *P. papatasi* but not *Le. infantum* to *Lu. longipalpis* (31, 32), and the data are further supported by apparent survival of *Le. infantum* line Δ*lpg1* in *P. perniciosus* (27) and of *Le. mexicana* line Δ*lpg1* in *Lu. longipalpis* (33).

The nature of the mechanisms defining vector competence in permissive sand fly vectors is still an open issue. The importance of LPG for midgut binding and parasite survival needs to be further analyzed, particularly as the lack of Δ*lpg1 + LPG1* add-back lines in a previous study (27) precluded a definitive conclusion about the importance of LPG in vector competence of permissive vectors. Here, we assessed midgut binding and survival of *Le. infantum* in the sand fly *Lu. longipalpis* using multiple wild-type strains as well as two Δ*lpg1* mutants and Δ*lpg1 + LPG1* add-back lines to answer the following questions. (i) Does *Le. infantum* bind to the midgut of *Lu. longipalpis*? (ii) If it does, is the *Leishmania* binding to the midgut stage specific? (iii) Is *Le. infantum* LPG necessary for parasite binding to the midgut? (iv) Is the *Le. infantum* LPG sufficient to define vector competence in the natural permissive vector? (v) Do components of the blood bolus affect *Le. infantum* survival?

**RESULTS**

*Le. infantum* binds to the midgut epithelium of *Lu. longipalpis*. In order to confirm previous results (6) and standardize the technique, we exposed *P. papatasi* midguts to *Le. major* (WR 2885) harvested from a 3-day-old culture. As expected, *Le. major* parasite bound to *P. papatasi* midguts (median 7,500 parasites/midgut; Fig. 1A). Unexpectedly, *Le. infantum* LLM-320 (MHOM/ES/92/LLM-320) harvested on day 3 failed to bind to the midguts of the sand fly *Lu. longipalpis* (median 2,700 parasites/midgut; Fig. 1B). To our surprise, *Le. infantum* parasites from an older, day 4 culture showed a greater number of parasites binding to *Lu. longipalpis* open midguts (median 17,850 parasites/midgut; Fig. 1C). In a control experiment, 4-day-old parasites did not bind intact, unopened, *Lu. longipalpis* midguts (median 1,600 parasites/midgut), pointing to the specificity of parasite binding to the sand fly midgut epithelium (Fig. 1C).

*Le. infantum* binding to the midgut epithelium of *Lu. longipalpis* is stage dependent. To validate and extend our observations, we used a different strain of *Le. infantum*, MCAN/BR/09/52 (LAB), and assessed its binding to sand fly midguts. Parasites harvested on day 3 attached to the midgut epithelium of *Lu. longipalpis* at a median of 5,250 parasites/midgut (Fig. 2A). As the LAB parasite cultures aged, the number of parasites binding the sand fly midgut increased proportionally, peaking with day 6 parasites at a median of 8,500 parasites/midgut (Fig. 2A). A greater proportion of
parasite binding to the midgut epithelium as the culture aged was also observed for the *Le. infantum* BH46 strain (see Fig. S1A to D in the supplemental material), ranging from a median of 4,200 parasites/midgut for 4-day-old culture parasites (Fig. S1A) to a median of 26,000 parasites/midgut with 6-day-old culture parasites (Fig. S1C). As the culture got older (Fig. 2B; see also Fig. S2A), the parasites began to differentiate to the infective form, the metacyclic promastigotes, increasing from 10% to 30% on day 4 to about 70% to 80% on day 7 of culture (Fig. 2C; see also Fig. S2B). As we had observed that many, if not most, of the bound parasites were at late stages of differentiation (leptomonads and metacyclic), we used a Ficoll gradient to separate early-stage parasites (ESP) from late-stage parasites (LSP) and tested if they could bind to sand fly midgut epithelium. Surprisingly, we observed that significantly more LSP than ESP bound to the sand fly midgut epithelium, for both *Le. infantum* LAB (Fig. 2D and E) and BH46 (Fig. 2F and G) strains, harvested on both day 4 and day 5.

In order to assess the abundance of LPG on the surface of BH46 WT parasites, we stained LSP and ESP Ficoll-purified parasites with a LPG backbone-specific monoclonal
Temporal and stage-specific binding of *Leishmania infantum* parasites to unfed *Lutzomyia longipalpis* midguts. (A to E) *Le. infantum* (MCAN/BR/09/52, LAB). (A) Binding of parasites harvested on different days of culture. n = 2. 3d, day 3; 4d, day 4; 5d, day 5; 6d, day 6. (B) Growth curve in culture. n = 3. (C) Metacyclic emergence on day 4, day 5, day 6, and day 7 of culture. n = 3. (D and E) Differential binding of ESP and LSP from cultures harvested on days 4 (D) and 5 (E). n = 2. (F and G) Differential binding of ESP and LSP of *Le. infantum* BH46 wild-type strain from cultures harvested on days 4 (F) and 5 (G). n = 2. ESP, early-stage parasites; LSP, late-stage parasites. Midguts were opened longitudinally along the anterior-posterior axis. *, statistically significant at P < 0.05. (H and I) Numbers of BH46 wild-type parasites stained by anti-LPG antibody in metacyclic-enriched samples (light green) and procyclic-enriched samples (dark green) for the 4-day-old (H) and 5-day-old (I) parasite cultures. FITC, fluorescein isothiocyanate.
antibody (CA7AE). Using flow cytometry, we found that LSPs exhibited 2-fold-higher and 20-fold-higher levels of antibody binding than ESP samples for 4-day-old and 5-day-old cultures, respectively (Fig. 2H and I), indicative of the increased abundance of LPG in the former. The increase in fluorescence intensity as the parasites aged (Fig. 2H and I) correlated with a greater number of parasites binding the sand fly midgut from 4-day-old and 5-day-old cultures (Fig. 2F and G). In order to further evaluate such observations, we measured LPG abundance in these parasites by confocal microscopy (Fig. 3A to H). The LPG staining that we observed was more intense in LSP (Fig. 3A to D) than in ESP (Fig. 3E to H). In comparison, 3-day-old parasite cultures stained poorly with the CA7AE antibody (Fig. 3I to L).

FIG 3 Immunostaining of LPG on the surface of different Leishmania infantum BH46 developmental stages. (A to L) Wild-type parasites were stained with green fluorescent protein (GFP)-conjugated anti-LPG CA7AE antibody. (A to H) Seven-day-old cultures were harvested, and parasites were sorted into metacyclic (A to D) and procyclic (E to H) promastigotes in a Ficoll gradient. (I to L) Staining of 3-day-old culture parasites. Bar = 5 μm. Green = LPG. Blue = DAPI (nuclear DNA). Gray pictures = DIC (differential interference contrast).

Le. infantum binding to the midgut epithelium of Lu. longipalpis is LPG dependent. In order to assess whether or not the major surface glycoconjugate of Le. infantum (LPG) was the parasite ligand attaching to the midgut epithelium of Lu. longipalpis, we carried out midgut binding assays with the Le. infantum BH46 wild-type strain (BH46 WT) as well as with both the LPG-deficient (Δlpg1) and add-back (Δlpg1/H11001 LPG1) lines. Similarly to what was observed for unfed midguts (Fig. S1), aging parasites from the BH46 WT line bound to the epithelium of midguts dissected 5 days after blood feeding with increasing efficiency, whereas parasite binding was limited in unopened midguts (Fig. 4). For both fed and unfed Lu. longipalpis midguts, binding of the BH46 Δlpg1 + LPG1 line was intermediate and the level of binding was significantly higher than that seen with the BH46 Δlpg1 mutant, which failed to bind, exhibiting less than 1,500 parasite/midgut regardless of the age of the harvested parasites or the feeding status of the midguts (Fig. 4; see also Fig. S1). Similar results were obtained with the Le. infantum BA262 Δlpg1 mutant, which also failed to bind to unfed midguts (Fig. S3). Comparatively, the BA262 WT bound with increased efficiency as the parasites aged (Fig. S3 and S4). Despite the low level of midgut binding of the BA262 Δlpg1 + LPG1 line, such parasites bound to midguts in significantly greater proportions than the BA262 Δlpg1 mutant (Fig. S3).
**Le. infantum** BH46 Δlpg1 mutants grow in the midguts of *Lu. longipalpis*. As the presence of *Le. infantum* LPG is sufficient to mediate midgut epithelium attachment *in vitro* but binding was substantially increased in LSP compared to ESP, we tested whether this ligand is necessary for *in vivo* parasite development in the midgut of the sand fly *Lu. longipalpis*. We infected *Lu. longipalpis* sand flies with the BH46 WT, BH46 Δlpg1, and BH46 Δlpg1 + LPG1 lines and followed both parasite load and infection prevalence at five time points after infection. When seeded at 5 million parasites/ml, the BH46 Δlpg1 mutant exhibited a significantly lower parasite load and a lower infection

**FIG 4** Binding of *Leishmania infantum* BH46 wild-type, Δlpg1, and Δlpg1 + LPG1 lines to *Lutzomyia longipalpis* midguts dissected 5 days after blood feeding. (A to D) Cultures of *Le. infantum* BH46 WT (wild-type), Δlpg1, and add-back (Δlpg1 + LPG1) lines were harvested on days 3 (A), 5 (B), 6 (C), and 7 (D) and incubated with midguts of sand flies 5 days after blood feeding. Midguts were opened longitudinally along the anterior-posterior axis. The WT strain was also incubated with intact (unopened) midguts. *n = 2*, *, statistically significant at *P* < 0.05.
prevalence than the BH46 WT strain or the BH46 Δlpq1 + LPG1 strain on day 3 postinfection (Fig. 5A and B), but it recovered on subsequent days, exhibiting a parasite load and infection prevalence similar to those seen with either the BH46 WT or BH46 Δlpq1 + LPG1 line (Fig. 5C to J). When the infection was started with 2 million parasites/ml, the BH46 Δlpq1 mutant displayed a lower, and yet not statistically significant, parasite load on day 3 (Fig. S5A) but higher loads than either the WT strain or the Δlpq1 + LPG1 line from day 6 onward (Fig. S5B to E).

Le. infantum BA262 Δlpq1 mutants fail to grow in the midguts of Lu. longipalpis. As the LPG side chain decorations are polymorphic among Le. infantum strains (34), we also assessed the ability of the BA262 WT strain, along with the BA262 Δlpq1 and BA262 Δlpq1 + LPG1 lines (35), to develop in Lu. longipalpis (Fig. 6). Differing from the BH46 strain, which exhibits side chains with 1 to 3 β-glucose residues, the LPG of BA262 is devoid of side chains, like most of the Le. infantum strains (34). Seeded at 5 million parasites/ml, the BA262 Δlpq1 mutant displayed lower parasite loads on days 2 and 3 (median 0 to 500 parasites/midgut) than the WT (median 3,000 to 5,000 parasites/midgut) and the Δlpq1 + LPG1 line (median 1,600 to 13,750 parasites/midgut) lines and a lower infection prevalence before the blood was passed (Fig. 6A to D). After the blood meal was passed, the BA262 Δlpq1 mutant was lost in the majority of sand flies, persisting in only a few specimens that displayed a reduced number of parasites and a decreased infection prevalence (Fig. 6E to L). The BA262 WT and Δlpq1 + LPG1 lines, on the other hand, developed well in the midguts, reaching medians of 27,000 and 35,000 parasites per midgut, respectively, and an 80% to 85% infection prevalence on day 15 postinfection (Fig. 6K and L).

Le. infantum BH46 and BA262 Δlpq1 mutants display different susceptibilities to components of the blood meal. To investigate the differences in sand fly survival rates of the BH46 and BA262 Δlpq1 mutants, we tested if resistance to by-products of blood meal digestion could be a factor that explains these differences. For this, we incubated in vitro parasites in the exponential phase of growth with extracts of midguts, collected at 24 and 48 h after blood feeding, for 4 h at 26°C, and compared them to the WT and Δlpq1 + LPG1 lines (Fig. 7). BH46 Δlpq1 mutants were not affected by the components of the digested sand fly blood meal (Fig. 7A). In contrast, the BA262 Δlpq1 mutants were severely affected by incubation with midguts collected 24 h and 48 h post-blood feeding (Fig. 7B). The WT and Δlpq1 + LPG1 lines of both strains were not affected by incubation with midgut extracts (Fig. 7).

DISCUSSION

Many studies have demonstrated that binding of Le. major to the midguts of the restrictive vectors is mediated by LPG (6, 11, 12, 24–26, 36). Incubation of P. papatasi midguts with purified PGs from procyclic Le. major parasites prevented the binding of procyclic Le. major parasites (6), and Le. major Δlpq1 parasites cannot develop in P. papatasi or P. duboscqi (25, 26) sand flies. On the other hand, the Le. major Δlpq1 mutant binds to midguts and develops well in permissive vectors, such as Lu. longipalpis (24, 25), as well as Phlebotomus arabicus (24), P. argentipes (26), and P. perniciosus (26). Based on such findings, an LPG-independent mechanism for midgut binding was proposed for the permissive vectors (28). Differences in midgut glycosylation between restrictive and permissive vectors were observed that could possibly account for nonspecific binding of parasites in the latter. It was hypothesized that a lectin on the Leishmania surface might bind to the O-linked glycans of the midgut microvilli of permissive vectors, thus allowing parasite binding (26, 28, 30). Nonetheless, such studies were carried out using Le. major and unnatural permissive vectors (24–26, 28); thus, the LPG-independent mechanism might be restricted to Le. major development in unnatural permissive vectors. Therefore, we decided to revisit the role of LPG in parasite development in natural permissive vectors, focusing on two Le. infantum strains bearing intraspecies polymorphisms in their LPGs (25). The LPG of one strain, BA262, is devoid of side chains (type I) whereas that of the BH46 strain has β-glucose sugars branching off the repeat unit backbone (type III [20]). Here, we demonstrate that Le.
FIG 5 *Lutzomyia longipalpis* midgut infection with *Leishmania infantum* BH46 wild-type, Δlpg1, and Δlpg1 + LPG1 parasites. (A to J) Upon infection with 5 million parasites per ml, parasite load and infection prevalence were assessed on days 3 (A and B), 6 (C), 9 (D), and 12 (E) post-infection. (Continued on next page)
infantum (BH46) wild-type and Δlpg1 + LPG1 lines bound to both unfed and day-5-post-feeding midgut epithelia of Lu. longipalpis in vitro. In contrast, the LPG-deficient Δlpg1 mutant failed to bind to Lu. longipalpis unfed and fed midguts. Similar results were reported for Δlpg1 mutants of Le. mexicana (MPRO/BR/72/M1845) and Le. infantum (MHOM/BR/76/M4192), which exhibited poor binding to midguts of Lu. longipalpis and P. perniciosus (27), respectively. Altogether, these data indicate that LPG mediates Leishmania binding to midguts of natural permissive vectors and suggest that the previously described LPG-independent midgut binding mechanism may be limited to Le. major binding to midguts of permissive vectors.

Similarly to the conclusions regarding Le. major binding to the midgut of its natural restrictive vector, previous reports claimed that Le. infantum and Le. donovani binding to the midgut of a naturally permissive vector was also restricted to early-stage parasites (20, 21). Nonetheless, the use of peanut agglutinin (PNA) to sort procyclic and metacyclic Le. infantum (21) and Le. donovani (20) parasites may have confounded interpretation of results. PNA has been used to purify Le. major metacyclics as the LPGs of such parasite stages are decorated with arabinose, which replaces numerous β-galactose sugars on side chains of early-stage parasites (37). In contrast, Le. infantum (21) and Le. donovani (20) LPGs display only a single β-galactose residue in the cap. Whereas it is not clear whether or not the same residue is absent in the Le. infantum metacyclic stage LPG cap (21), the Le. donovani cap bears β-galactose or mannose residues at the same proportions as procyclic parasites (20), precluding accurate purification of procyclics and metacyclics by PNA. Additionally, experiments showing the developmental differences of Le. infantum LPG (21) and Le. donovani LPG (20) found that PNA-purified metacyclic LPG was representative of only 10% of the parasites in stationary-phase cultures (20, 21). It is possible that PNA-purified metacyclic LPGs are representative of a small metacyclic subpopulation that does not bind to PNA, as the whole metacyclic population usually accounts for about 80% of late-phase cultures. Importantly, this small population of nonbinding/free-swimming Leishmania parasites likely comprises the metacyclics that are transmitted, as the parasites inoculated by sand flies represented only 0.02% to 14% of the population in a mature Leishmania infection in all sand fly species investigated to date (38–40). The observed increase in the intensity of LPG labeling as Le. infantum aged in culture, which correlated positively with the number of parasites bound to midguts, supports this hypothesis. Knowing whether stronger LPG labeling is related to an increase in the number and/or length of LPG molecules during metacyclogenesis will shed light on the mechanism of binding of the Le. infantum LPG to the receptor on the midgut microvilli.

Electron microscopy images of Le. infantum developing in Lu. longipalpis show that the parasites, previously described as long and short nectomonads (41) and now termed nectomonads and leptomonads (42), respectively, were observed attached to the posterior and anterior midguts throughout the parasite life cycle in the sand fly (41). In our experiments, early-stage parasites were not able to attach to midguts in vitro, which might be explained by a lack of bona fide nectomonad parasites in cultures expressing LPG on their surface. On the other hand, we observed strong Lu. longipalpis midgut binding by late-stage Le. infantum parasites, enriched in leptomonads and metacyclics, confirming early observations by electron microscopy that Le. infantum late-stage parasites also bind to the Lu. longipalpis midgut epithelium (41). Whether midgut binding by late-stage parasites is necessary for parasite migration to the anterior midgut (42), for genetic exchange (43, 44), and/or for preventing the pushing of metacyclic parasites to posterior midgut upon sequential blood meals (45) has yet to be determined.

Our proposed model of LPG-mediated Le. infantum attachment to the midgut of a

FIG 5 Legend (Continued)
D, 9 (E and F), 12 (G and H), and 15 (l and J) postinfection (PI), respectively. Data represent WT (wild-type), Δlpg1, and Δlpg1 + LPG1 lines. Low, 500 to 5,000 parasites/midgut; Moderate, 5,000 to 10,000 parasites/midgut; Heavy, 10,000 parasites/midgut. n = 2. *, statistically significant at P < 0.05.
FIG 6. Lutzomyia longipalpis midgut infection with Leishmania infantum BA262 wild-type, Δlpg1, and Δlpg1/H11001 parasites. (A to J) Upon infection with 5 million parasites per ml, parasite load and infection prevalence were (Continued on next page)
permissive vector complements and expands upon previous findings that demonstrated that changes in LPG during metacyclogenesis mediated parasite detachment from the midgut epithelium to be transmitted. Here, we propose that metacyclics encompass two subpopulations: one that binds to the midgut epithelium, as was observed in this study and also earlier (41), and a free-swimming one that is transmitted by a sand fly bite (20, 21). The existence of such subpopulations is also supported by polymorphisms in the LPG cap of metacyclic Le. infantum (21) and Le. donovani (20) parasites.

It was previously shown that lack of LPG in Le. infantum seems to affect the ability of this parasite to develop in the midgut of the permissive natural vector P. perniciosus (27). A closer look at the results, nonetheless, suggests that the Le. infantum Δlpg1 infection showed some recovery on day 8 postinfection, after the digested blood was passed, compared to day 4, a time point when the blood meal is still being digested.

FIG 7 Leishmania infantum survival after incubation with extracts of blood-engorged Lutzomyia longipalpis midguts in vitro. (A) BH46 WT (wild-type), Δ/− (Δlpg1), and add-back (Δlpg1 + LPG1) lines were exposed in vitro to either a PBS control (CTR) or extracts of engorged midguts dissected at 24 h or 48 h post-blood meal. (B) BA262 WT (wild-type), Δ/− (Δlpg1), and add-back (Δlpg1 + LPG1) parasites were exposed in vitro to either a PBS control (CTR) or extracts of engorged midguts dissected at 24 h or 48 h post-blood meal. n = 2. *, statistically significant at P < 0.05.
(27), as the prevalence of sand flies infected with heavy (>10,000 parasites/midgut) and moderate (5,000 to 10,000 parasites/midgut) populations increased. In order to further investigate the importance of LPG for *Le. infantum* development in *Lu. longipalpis*, we assessed survival and development of the *Le. infantum* BH46 and BA262 WT, Δ*lpg1*, and Δ*lpg1* + LPG1 lines in the naturally permissive vector *Lu. longipalpis* for 15 days. In accordance with a previous report (27), the *Le. infantum* BH46 Δ*lpg1* mutant struggled to survive at the time of blood digestion on day 3, and yet it thrived after the blood was passed, reaching parasite loads similar to those seen with the WT and add-back lines at later time points. In contrast, the BA262 Δ*lpg1* mutant not only struggled to survive up to day 3 but succumbed afterward, revealing important interstrain differences in *Le. infantum* in vivo development. These data indicate that LPG-mediated epithelium binding is not a determinant of parasite survival in the midgut for this parasite-vector pair. However, the presence of LPG does confer protection to some *Le. infantum* strains such as BA262.

Toxicity of blood components can affect the survival of *Leishmania* parasites in the sand fly midgut during digestion (9). In the present study, incubation of *Le. infantum* strains BH46 and BA262, harvested from 3-day-old cultures with extracts of engorged midguts, produced different outcomes. Whereas the BH46 and BA262 WT and Δ*lpg1* + LPG1 lines, as well as the BH46 Δ*lpg1* mutant, survived exposure to the toxic components of the digested blood, the BA262 Δ*lpg1* mutant was highly affected by midgut extracts. These results correlated well with the survival of the BH46 Δ*lpg1* mutant and the lack of further development of the BA262 *lpg1* KO line in sand flies. Together, these results indicate that neither LPG type I nor LPG type III is needed to prevent *Le. infantum* from being eliminated along with the digested blood bolus in a permissive vector, but it is likely important for early survival during the digestive period for some but not all parasite strains. The survival of the BH46 Δ*lpg1* mutant after exposure to engorged midgut extracts is intriguing; the nature of the glycosylation of the galactose-mannose backbone of other surface glycoconjugates may correlate with protection for this strain and needs to be further explored. This possibility is supported by early findings showing that *Le. major* lacking LPG survived within the bloodmeal (24, 26) whereas *Le. major* lacking LPG and proteophosphoglycans succumbed within the blood bolus (25, 26).

As stated previously by Sacks and Kamhawi (16), for permissive species such as *Lu. longipalpis*, persistence of parasites in the midgut after blood is passed is possibly due to factors other than attachment, such as a slower peristaltic movement. These observations are supported by our findings which indicate that LPG docking of parasites does occur but appears not to define vector competence for *Le. infantum* in *Lu. longipalpis*. Rather, vector competence in permissive vectors seems to be more complex, affected by strain-specific differences and involving multiple stages of parasite development in the midgut.

**MATERIALS AND METHODS**

**Sand flies, *Leishmania* strains, and parasite cultivation.** The sand flies used in this study belonged to either the *Lu. longipalpis* Jacobina colony or the *P. papatasi* Jordan colony, both maintained at the Laboratory of Malaria and Vector Research (LMVR)/NIH sand fly insectary. The different *Le. infantum* strains used in this study, including strain LAB (MCAN/BR/09/S2) (46), the BH46 wild-type strain (BH46 WT; MCAN/BR/89/BH46), the LPG-deficient BH46 Δ*lpg1* and the BH46 Δ*lpg1* + LPG1 add-back (BH46 res) strains, strain BA262 (MCAN/BR/89/BA262) (35), the LPG-deficient BA262 Δ*lpg1* and the BA262 Δ*lpg1* + LPG1 (BA262 res) strains, the RFP strain (MHOM/ES/92/LLM-320, red fluorescent protein [RFP] expressing) (47), and *Le. major* WR 2885 (RFP expressing) (38), were cultivated in Grace medium (Lonza BioWhittaker and Sigma-Aldrich) supplemented with 20% heat-inactivated fetal calf serum (FCS; Gibco, 16140071) and penicillin/streptomycin (pen/strep; 100 μg/ml) in 25-cm² flasks. For BA262, FCS was subjected to further heat inactivation at 56°C for 1 h. For all the experiments, cultures were seeded with 1 × 10⁸ parasites/ml, and parasites were grown at 26°C in a biological oxygen demand (B.O.D) chamber. For both the BH46 Δ*lpg1* and BH46 Δ*lpg1* + LPG1 add-back lines, hygromycin (50 μg/ml) and geneticin (G418, 5 μg/ml) were added to the medium. In addition, Zeocin (75 μg/ml) was added to BH46 add-back cultures. For both the BA262 Δ*lpg1* and BA262 Δ*lpg1* + LPG1 lines, hygromycin (50 μg/ml) and Geneticin (G418, 70 μg/ml) were added to the medium. In addition, Zeocin (100 μg/ml) was added to BA262 Δ*lpg1* + LPG1 cultures.
**Sorting of early-stage and late-stage parasites in Ficoll gradient.** The sorting procedure was performed as described elsewhere (48), with slight modifications. Briefly, cultures were spun down, and parasites were washed twice in phosphate-buffered saline (PBS) and resuspended in 2 ml PBS. Then, parasites were overlaid with 40% Ficoll–2 ml PBS, followed by addition of 10% Ficoll–2 ml M199 medium, and were spun at 365 × g for 10 min at room temperature. Metacyclic-enriched parasites were collected from the layer in the interface between 10% Ficoll and PBS, whereas procyclic-enriched parasites were collected after removing supernatant and resuspending the pellet. Thereafter, both parasite samples were washed twice in PBS for further experimentation.

**Parasite growth curves.** Parasite cultures were seeded with 1 × 10^6 parasites/ml as described above, and parasites were counted daily direct from the medium or diluted in PBS using Neubauer improved chambers (Incysto).

**Midgut binding assays.** Parasite cultures were spun down once and washed with PBS twice at 3,500 rpm for 15 min. Parasites were counted and diluted to 5 × 10^6 parasites/ml in PBS. The midguts of *P. papatasi* and *L. longipalpis* unfed parasites as well as *L. longipalpis* parasites 5 days after blood feeding were dissected in a drop of PBS and opened up transversally in Y shape with fine entomological pins when needed, and groups of 10 midguts were exposed to 2 × 10^6 parasites in 40 μl of PBS in a well of an electron microscopy 9-cavity Pyrex pressed plate (Fisher Scientific). The preparations were transferred to a humidified chamber, and incubation was performed for 45 min at room temperature. Afterward, midguts were passaged through fresh PBS twice and transferred individually to 1.7-ml Eppendorf tubes (Denville Scientific) with 30 μl of PBS.

**Sand fly infections.** Defibrinated naive rabbit blood (Noble Life Sciences, Gaithersburg, MD), was spun down at 2,000 rpm for 10 min, and plasma was collected and transferred to a fresh vial. Red blood cells (RBCs) were washed at least twice with PBS (until most of the free heme was removed), whereas plasma was subjected to heat inactivation at 56°C for 1 h. Parasite cultures were spun down and washed twice with PBS as described above. Then, RBCs were reconstituted with heat-inactivated plasma and seeded with either 5 × 10^6 (BH46 and BA262) or 2 × 10^6 (BH46) parasites/ml. Infectious blood was loaded into a custom-made glass feeder (Chemglass Life Sciences, CG183570), capped with a chick skin, and heated by a circulating water bath set for 37°C. Sand flies were allowed to feed for 3 h. Afterward, midguts were dissected and transferred individually to 1.7-ml Eppendorf tubes (Denville Scientific) in 50 μl of PBS.

**Midgut Leishmania load assessment.** Midguts were homogenized by the use of a cordless motor and disposable pellet mixers (Kimble). In order to assess metacyclic proportions, formalin was added to the vials at a 0.005% final concentration. Samples were diluted as necessary, and 10 μl was loaded onto Neubauer improved chambers (Incysto).

**Leishmania incubation with extracts of blood-engorged midguts.** For sand fly feeding on naive rabbit blood (Noble Life Sciences, Gaithersburg, MD), RBCs and plasma were processed as described above. Sand flies were fed on the naive (heat-inactivated) blood, and midguts were dissected at 24 and 48 h after feeding. Midguts were individually transferred to 0.2-ml tubes, frozen in dry ice, and stored at −80°C. Two batches of midguts were obtained from sand flies fed on two different days. Before incubation with parasites, midguts underwent 10 cycles of freeze-thaw (dry ice/room temperature; 5 min each cycle). Parasites were harvested from 3-day-old cultures, washed twice in 1 × PBS, and diluted to 5 × 10^6 parasites/ml in complete Grace medium. One microliter (5,000 parasites) was incubated with either the extract of a single midgut or PBS (1 μl) for 4 h at 26°C. Afterward, 20 μl of PBS was added to each sample, and parasites were counted with Neubauer improved chambers.

**Flow cytometry.** Metacyclic- and procyclic-enriched samples were sorted from both BH46 and BA262 4-day-old and 5-day-old cultures by Ficoll gradient centrifugation as described above. One million parasites of each sample/strain were washed twice in cell staining buffer (BioLegend, 420201) and resuspended in 100 μl of the LPG backbone-specific CA7AE antibody at 0.125 μl/100-μl dilution in staining buffer. Samples were incubated with primary antibody for 30 min at 4°C, washed twice with staining buffer, and then incubated with the Alexa Fluor 488 goat anti-mouse IgG secondary antibody under the same conditions. After this step, samples were fixed in 250 μl fixation buffer (BioLegend, 420801) for 20 min at 4°C. Flow cytometer experiments were performed with a MACSQuant 16 analyzer (MACS Miltenyi Biotec). For compensation, an AbC total antibody compensation bead kit (Molecular Probes, A10497) was used upon incubation with both primary and secondary antibodies, following manufacturer recommendations. Data were analyzed with FlowJo software (BD).

**Statistical analyses.** For all the data sets, the nature of the distribution was tested with the D’Agostino-Pearson normality test. Pairwise comparisons of data following a normal distribution were carried out with unpaired t tests; otherwise, the Mann-Whitney U-test was performed. In order to assess the statistical significance of infection prevalence, the chi-square test was carried out. Statistical evaluation was carried out with GraphPad Prism v.8.
**Ethics statement.** All animal experimental procedures were reviewed and approved by the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee under animal protocol LMVR4E. The NIAID Division of Intramural Research (DIR) Animal Care and Use Program complies with the Guide for the Care and Use of Laboratory Animals and with the NIH Office of Animal Care and Use and Animal Research Advisory Committee guidelines. Details of the NIH animal research guidelines can be accessed at https://oma1.od.nih.gov/manualchapters/intramural/3040-2/.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, PDF file, 0.4 MB.

**FIG S2**, PDF file, 0.4 MB.

**FIG S3**, PDF file, 0.1 MB.

**FIG S4**, PDF file, 0.4 MB.

**FIG S5**, PDF file, 0.4 MB.

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I.V.C.-A. designed and performed the experiments and analyzed the data. J.O. supported flow cytometry experiments. W.D.C. performed parasite staining. T.R.W. helped with sand fly infections. C.M. performed sand fly insectary work. R.P.S. provided data collection and analysis, decision to publish, or preparation of the manuscript.

I.V.C.-A. wrote the first draft of the manuscript. J.G.V. and S.K. edited the manuscript.

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