Leishmania major Glycosylation Mutants Require Phosphoglycans (lpg2−) but Not Lipophosphoglycan (lpg1−) for Survival in Permissive Sand Fly Vectors

Anna Svárovská1, Thomas H. Ant2, Veronika Seblová1, Lucie Jecná1, Stephen M. Beverley3, Petr Volt1*

1 Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic, 2 Keele University, Keele, Staffordshire, United Kingdom, 3 Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, United States of America

Abstract

Background: Sand fly species able to support the survival of the protozoan parasite Leishmania have been classified as permissive or specific, based upon their ability to support a wide or limited range of strains and/or species. Studies of a limited number of fly/parasite species combinations have implicated parasite surface molecules in this process and here we provide further evidence in support of this proposal. We investigated the role of lipophosphoglycan (LPG) and other phosphoglycans (PGs) in sand fly survival, using Leishmania major mutants deficient in LPG (lpg1−), and the phosphoglycan (PG)-deficient mutant lpg2−. The sand fly species used were the permissive species Phlebotomus perniciosus and P. argentinés, and the specific vector P. duboscqi, a species resistant to L. infantum development.

Principal Findings: The lpg2− mutants did not survive well in any of the three sand fly species, suggesting that phosphoglycans and/or other LPG2-dependent molecules are required for parasite development. In vitro, all three L. major lines were equally resistant to proteolytic activity of bovine trypsin, suggesting that sand fly-specific hydrolytic proteases or other factors are the reason for the early lpg2− parasite killing. The lpg1− mutants developed late-stage infections in two permissive species, P. perniciosus and P. argentinés, where their infection rates and intensities of infections were comparable to the wild type (WT) parasites. In contrast, in P. duboscqi the lpg1− mutants developed significantly worse than the WT parasites.

Conclusions: In combination with previous studies, the data establish clearly that LPG is not required for Leishmania survival in permissive species P. perniciosus and P. argentinés but plays an important role in the specific vector P. duboscqi. With regard to PGs other than LPG, the data prove the importance of LPG2-related molecules for survival of L. major in the three sand fly species tested.

Introduction

The distribution of diseases caused by the protozoan parasite Leishmania is limited by the distribution of the sand fly vectors and their capacity to support parasite development. Survival of Leishmania parasites during bloodmeal digestion and their attachment to the midgut epithelium have been identified as two critical steps determining the vector competence. Based upon experimental tests of their ability to support development of wide or limited range of Leishmania species, sand flies have been classified as permissive or specific vectors [1]. According to previous investigations, there is a close evolutionary fit between Phlebotomus papatasii and P. sergenti with Leishmania major and L. tropica respectively, as other Leishmania species survive poorly in these sand fly hosts. In contrast, other sand flies tested (P. argentinés, P. halensis, P. arabicus and Latzonyia longipalpis) were broadly permissive to the development of different Leishmania parasites. This classification is based on experimental studies and does not imply the constraints of natural transmissions (vector capacity). However, it reflects the vector competence of permissive sand flies for transmission of various parasites [for review see [1,2]].

Leishmania surface molecules have been strongly implicated in parasites survival within sand fly vectors. Leishmania promastigotes synthesise an abundance of glycoconjugates composed of polymeric units based upon a conserved Gal-Man-P phosphoglycan (PG) repeating unit for review see [3]. These include the membrane-attached glycosylphosphatidylinositol (GPI) anchored lipophosphoglycan (LPG) and proteophosphoglycan (PPG), as well as secreted forms of PPGs and secretory acid phosphatases (sAPs). PGs have been implicated in the early survival of L. donovani within the bloodfed midgut [4], presumably by conferring resistance to, or by modulating the activity of digestive enzymes. The role of these molecules in sand fly interactions has been studied by biochemical methods using purified LPG, PPG and other...
Leishmania Mutants in Sand Flies

Author Summary

Phlebotomine sand flies are small blood-feeding insects, medically important as vectors of protozoan parasites of the genus Leishmania. Sand flies species can be divided roughly into two groups, termed specific or permissive, depending on their ability to support development of one or a few strains vs. a broad spectrum of these parasites. In this study, we explored the ability of two Leishmania major glycoalyx mutants to survive within these different types of vectors. The lpg1 mutant, which specifically lacks lipophosphoglycan (LPG), was able to survive normally in two permissive species, Phlebotomus argenteus and P. perniciosus, but was only able to survive within the specific species P. duboscqi for a limited time prior to dissolution of the peritrophic matrix. Consistent with its classification as a specific sand fly vector, P. duboscqi was not able to support development of L. infantum. The lpg2 L. major mutant, which is a broader mutant and lacks all phosphoglycans including LPG and proteophosphoglycans, was unable to survive in all the three species tested. This study extends the knowledge on the role of Leishmania major surface glycoconjugates to development in three important vector species and gives supporting evidence for the existence of an LPG-independent mechanism for survival in sand flies, as well as the importance of LPG2-dependent glycoconjugates in parasite survival.

In this work we further test this hypothesis by infections of three additional sand fly species with WT and mutant L. major. In addition, we attempted to assess the importance of LPG and other LPG2-dependent molecules in protection against proteolytic attack by exposing the mutant parasitc lines to the action of bovine trypsin in vitro. Importantly, the three sand fly species used in the study are important vectors known to transmit Leishmania. Phlebotomus duboscqi is a vector of cutaneous leishmaniasis caused by L. major in sub-Saharan Africa [21,22]. It is a sister species of P. papatasi and belongs to the same subgenus. Unlike P. papatasi, some populations of P. duboscqi have been shown experimentally to support development of L. tropica [16]. Midgut glycosylation and the degree of permissivity of this species are unclear. We addressed the question of permissivity of P. duboscqi sand flies in this study by infecting them with L. infantum. The other two species used are permissive vectors transmitting parasites of L. donovani complex. Myaskova et al. [20] demonstrated that both, P. argenteus and P. perniciosus posses midgut glycoproteins with HPA (Helix pomatia agglutinin, lectin with specificity to N-acetyl-D-galactosamine)-binding epitopes. Phlebotomus argenteus is a vector of visceral anthroponotic leishmaniasis caused by Leishmania donovani in the Indian subcontinent [23]. In experimental conditions it supports development of L. donovani, L. amazonensis, L. major and L. tropica [4,19,24]. Phlebotomus perniciosus is a vector of Leishmania infantum in the western Mediterranean and in experimental conditions it supports the development of L. tropica (V.S. and P.V., unpublished results).

Materials and Methods

Parasites

Leishmania infantum MHOM/TR/2000/OG-VL and three lines of Leishmania major LV39 clone 5 (MRHO/SU/1959/Neal P) [25] were used in this work. The L. major LPG1 and LPG2 knockout mutants lpg1 and lpg2 were generated in the LV39 clone 5 background previously [7,9]. Parasites were maintained at 23°C on medium 199 supplemented with 20% foetal calf serum (Gibco) and gentamicin (50µg/ml). For the mutated lines, selection antibiotics were added to the culture medium as follows: hygromycin B (15µg/ml) for the lpg2 mutant; hygromycin (15 µg/ml) and puromycin (11µg/ml) for the lpg1 mutant. Prior to sand fly infections, parasites were washed by centrifugation and resuspended in saline solution.

Sand fly colonies

Laboratory colonies of three sand fly species were used: Phlebotomus perniciosus (originally from Spain), P. argenteus (originally from India) and P. duboscqi (originally from Senegal). Colonies were maintained in conditions described previously [26]. Adults were maintained at 26°C and fed on 50% sucrose ad libitum.

Leishmania development in sand flies

Female sand flies (5–10 days old) were fed through a chinck skin membrane with 4–5 day old promastigotes at cell density of 5×10⁶ (P. duboscqi infections with L. major) or 1×10⁸ promastigotes/ml (all other infections, including P. duboscqi with L. infantum) in heat inactivated rabbit blood (Bioveta, Ivanovice). Blood-engorged females were maintained at 26°C with access to cotton wool soaked in 50% solution of sugar in distilled water and sacrificed for microscopic examination and counting of parasites in the midgut 2 and 5 or 9 days post infection. Intensity of infection was graded as light (<100 parasites/gut), moderate (100–1000 parasites/gut) or heavy (>1000 parasites/gut) as described previously [27]. Phlebotomus perniciosus and P. duboscqi defecate between 75 and
95 hours post-feeding [28] and L. major colonized their stomodeal valve on days 7–9 post-feeding [27]. Preliminary experiments showed that Phlebotomus argentipes defecates 2–3 days post-feeding and parasites reached the stomodeal valve by day 5 already. Experiments were repeated twice. The \( \chi^2 \) test was used for comparison of infection rates (number of infected versus uninfected females) and intensities of infection (heavy, moderate, light, zero) between the WT and the mutant lines using S-PLUS 2000 programme.

Parasite susceptibility to bovine trypsin

Promastigotes of a 4-day culture were washed in medium 199 (M 199), adjusted to the concentration of \( 3 \times 10^6 \) cells/ml of M 199 and exposed to bovine trypsin (13,500 BAEE units/ml) (Sigma) alone or bovine trypsin plus 6% human haemoglobin (Sigma; one BAEE unit will produce a \( \Delta A_{253} \) of 0.001 per min at pH 7.6 at 25 °C using benzyoi-L-arginine ethyl ester (BAEE) as substrate). In control groups, parasites were cultivated in M 199 alone. After 24 hours at 23°C, parasite numbers were determined by haemocytometer counting. Assay was performed in triplicate and the experiment was repeated twice. Data were evaluated statistically by means of ANOVA test.

Detection of glycoconjugates in P. duboscqi midgut lysates

Midguts of female P. duboscqi were homogenized in Tris buffer (20mM Tris, 150mM NaCl, pH 7.6) and proteins were analyzed by SDS PAGE (10% gel, reducing conditions, 100mM NaCl, pH 7.6) and proteins were analyzed by western blotting. The nitrocellulose membrane was incubated in Tris buffer with 0.05% Tween 20 (Tris-Tw) with 5% bovine serum albumin overnight and then with biotinylated agglutinin (PSA, 2.5 μg/ml), Ricinus communis agglutinin (RCA, 0.5 μg/ml), Soybean agglutinin (SBA, 10 μg/ml). After repeated washing the blots were incubated with streptavidin peroxidase (2.5 μg/ml) in Tris-Tw and developed in 3,3′-diaminobenzidine solution. The specificity of Con A and PSA reactions were controlled by addition of 250mM methyl-mannopyranoside as an inhibitory sugar.

Results

Development of L. major in P. duboscqi

Phlebotomus duboscqi sand flies were infected with WT, \( lpg1^- \) and \( lpg2^- \) mutants of L. major in order to study the role of LPG and other PGs. On day 2 post-infection, no differences were observed between development of WT and the \( lpg1^- \) line, with both showing very high rates of infection (97% and 93% respectively), with about 75% of heavy infections (Fig. 1). The \( lpg2^- \) mutant survived less well however; their infection rate was significantly lower (74%; \( P<0.01 \)), with only 24% of heavy infections. In contrast to day 2, on day 9 post infection the \( lpg1^- \) mutant survived much more poorly than WT, with only 25% of the flies retaining parasites, as opposed to 92% for WT (\( P<0.01 \)). Moreover, the \( lpg1^- \) line produced very few mature infections colonizing the stomodeal valve in only 10% of females. Notably the \( lpg2^- \) line did not survive, as no \( lpg2^- \) parasites were found in the midguts by day 9 (Fig. 1). Previous studies have established that phenotypes arising from the \( lpg1^- \) and \( lpg2^- \) mutants in diverse settings are specific, as they are reversed by complementation with the cognate gene, and thus do not arise as a result of nonspecific culture passage or other sources [4,7,9,13]. Thus these mutant

![Figure 1. Development of L. major mutants in P. duboscqi. Phlebotomus duboscqi females were infected with Leishmania major wild type (WT) or mutants lacking LPG (lpg1^-) or all LPG2-dependent molecules (lpg2^-). Day 2 - dissection before defecation (48hours post-infection), day 9 - dissection after defecation. Infections were classified into three cathegories: heavy (more than 1000 promastigotes per gut) - black bars, moderate (100–1000) - grey bars, light (1–100) - white bars. Numbers above the bars indicate the number of dissected females. doi:10.1371/journal.pntd.0000580.g001](https://www.plosntds.org/static/image/1/f1v4.png)

![Figure 2. Development of L. major mutants in P. argenteips. Leishmania major lines tested were the same as in Fig. 1. Day 2 - dissection before defecation (48hours post-infection); day 5 - dissection after defecation. The intensity of infections was evaluated as described in Fig. 1. Numbers above the bars indicate the number of dissected P. argenteips females. doi:10.1371/journal.pntd.0000580.g002](https://www.plosntds.org/static/image/1/f2v4.png)
differences in infection intensity and rate between the $lpg2^-$ and the WT were statistically highly significant ($P<0.01$).

Day-5 dissections revealed a continuation of the trends described above during late stage infections. Females infected with WT and $lpg1^-$ mutants retained high infection rate and high parasite loads (no statistically significant differences between the lines). Also the localizations of parasites were remarkably similar; WT and $lpg1^-$ parasites reached the stomodeal valve in 46% and 48%, respectively. In contrast, $lpg2^-$ line showed a remarkable decrease in infection rate, with only 18% of positive females (highly significant difference from the WT parasites, $P<0.01$) and no colonization of the stomodeal valve.

**Development of L. major lines in P. perniciosus**

On day 2, no significant differences were observed between the three lines, all of them survived well inside the peritrophic sac producing heavy infections in about 25% of females.

On day 9, $lpg2^-$ mutants were eliminated while $lpg1^-$ mutants developed similarly to the WT parasites [Fig. 3]. WT and $lpg1^-$ lines developed mature infections colonizing the stomodeal valve with high parasite burdens in majority of females. In contrast, none of the $lpg2^-$ parasites were able to persist until day 9, suggesting that they were lost during defecation.

**P. duboscqi is refractory to L. infantum**

In order to test the degree of permissivity of $P. duboscqi$, females of this species were infected with $L. infantum$, a parasite that is not transmitted by $P. duboscqi$ in nature. Promastigotes were able to survive inside the peritrophic sac during digestion of the bloodmeal but they were not able to persist beyond defecation of the blood remnants. On day 2 post infective bloodmeal, 91% of the flies (11 of 12) were Leishmania positive while on day 8, no parasites were found in any female tested ($n = 15$) (data not shown). These results demonstrate that $P. duboscqi$ is refractory to $L. infantum$.

**P. duboscqi midgut glycosylation**

As detected by western blotting with lectins, $P. duboscqi$ midgut lysate displays molecules that bind Con A and PSA, lectins detecting terminal mannose residues of glycans [Fig. 4]. Controls with inhibitory sugar (250mM methyl-mannopyranoside) confirmed the specificity of lectin reactions (data not shown). In contrast, HPA, RCA and SBA reactions were negative indicating absence of β-galactose or N-acetyl-D-galactosamine residues in the midgut glycoproteins [Fig. 4]. The lectin binding profile is similar to that previously observed in specific sand fly vectors $P. papatasi$ and $P. sergenti$. In contrast, midgut lysates of all permissive sand fly species tested to date contain N-acetyl-D-galactosamine displaying glycoconjugates as detected by lectin affinity blotting [20].

**Effect of bovine trypsin on Leishmania**

The *in-vitro* growth of $L. major$ promastigotes of WT, $lpg1^{-}$ and $lpg2^{-}$ lines in M 199 medium was not affected either by bovine trypsin (13.500 BAEE units/ml) or by bovine trypsin plus 6% human hemoglobin.

No significant differences in numbers of viable parasites were observed between the groups in any of the three lines tested ($P = 0.84$).

**Discussion**

The classic studies of Sacks and co-workers established a paradigm for the role of LPG in the survival of $L. major$ and $L. donovani$ in sand flies, mediated through binding of LPG to the sand
In the case of *L. major* this interaction is now known to be mediated by the *P. papatasi* lectin PpGal ([15]). However in 2007 we reported the occurrence of LPG-independent sand fly survival, importantly only in sand fly species now termed ‘permissive’ as defined by their ability in experimental tests to support the development of a wide range of *Leishmania* species. In contrast, previous studies of LPG-dependency were now associated with ‘selective’ sand fly species (again defined by experimental tests as supporting the development of a narrow range of *Leishmania* species and/or isolates) ([20]). Given the implications of this new paradigm, it was important to garner additional data testing its validity by examining additional permissive and selective sand fly species, using the same well characterized LPG mutants studied previously. Additionally we have culled the literature and summarized the available data pertinent to this model (Table 1).

We found that *L. major* mutants specifically lacking LPG remain able to develop in the permissive vectors *P. perniciosus* and *P. argentipes* at levels resembling those of wild type parasites, with full midgut development and colonization of the stomodeal valve. These data suggest that in *P. perniciosus* and *P. argentipes* the LPG is required neither for parasite protection against digestive enzymes nor for midgut binding. LPG-independent development was previously reported for *L. major* in the permissive sand fly vectors *Lu. longipalpis* and *P. arabicus* ([20]), and *Le. mexicana* development in *Lu. longipalpis* [29]. These data confirm and extend the results obtained in sand fly infections with all LPG-deficient *Leishmania* carried out by various laboratories to date (Table 1). Collectively these data provide strong support for the role of LPG in specific but not permissive sand fly vectors.

Within this data set we could only identify one potential exception, involving a study of the permissive vector *P. argentipes*, where the *L. donovani* LPG-deficient mutant line known as the ‘R2D2’, also defective in LPG1 expression [30], did not survive when examined on day-5 post-infection [19]. Notably R2D2 was obtained following heavy mutagenesis and selection for LPG-deficiency, unlike the *lpg1*/*L. major* which was generated following precise gene targeting procedures [31]. It is well established in the genetics literature that mutagenesis frequently results in off-target deleterious effects. Our previous work established that the phenotypic alterations in the *lpg1* and *lpg2* arose specifically from alterations in these genes, as restoration of LPG1 and LPG2 function returned the phenotype to WT [7,9]. In contrast, R2D2

### Table 1. Requirement for lipophosphoglycan (LPG) and other phosphoglycans (PGs) during *Leishmania* development in various sand fly species.

| Vector species (colony origin) | Leishmania species | Interpretation | Mutant lines | Infection outcome | Reference                |
|-------------------------------|-------------------|----------------|--------------|------------------|-------------------------|
| *P. papatasi* (Israel)        | *L. major* (natural) | LPG required in late phase | *lpg1*<sup>-</sup> (LPG-deficient) | Low percentage of infected flies on day 5 | Sacks et al. [4]         |
| *P. papatasi* (Turkey)        | *L. major* (natural) | LPG required in late phase | *lpg1*<sup>-</sup> (LPG-deficient) | Low percentage of infected flies on day 8 | Myskova et al. [20]     |
| *P. dubosci* (Mali)           | *L. major* (natural) | LPG required in late phase | *lpg1*<sup>-</sup> (LPG-deficient) | Fewer than a 1000 parasites/gut on day 7 | Secundino et al., (submitted) |
|                               |                   | PGs required from early phase | *lpg5A<sup>-</sup>/5B<sup>-</sup> (PG-deficient) | Impaired on day 3; no parasites on day 7 |                       |
|                               | *P. dubosci* (Senegal) | LPG possibly required in late phase | *lpg1*<sup>-</sup> (LPG-deficient) | Comparable to WT on day 4, partially impaired on days 8 and 10 | Boulanger et al. [32]    |
|                               |                   | LPG2-related molecules required from early phase | *lpg2<sup>-</sup> (PG-deficient) | Impaired on day 4, no parasites on days 8 and 10 |                       |
|                               |                   |                   | *lpg1<sup>-</sup> (LPG-deficient) | Comparable to WT on day 2; impaired on day 9 | This study             |
|                               |                   | LPG2-related molecules required from early phase | *lpg2<sup>-</sup> (PG-deficient) | Impaired on day 2; no parasites on day 9 |                       |
|                               |                   |                   | *lpg1<sup>-</sup> (LPG-deficient) | High level of infection on day 7 | Myskova et al. [20]     |
|                               |                   | LPG2-related molecules required from early phase | *lpg2<sup>-</sup> (PG-deficient) | High level of infection on day 7 | Secundino et al., (submitted) |
|                               |                   |                   | *lpg1<sup>-</sup> (LPG-deficient) | Mature infections on day 7–9 | Rogers et al. [29]       |
|                               |                   | LPG-independent | *lpg1<sup>-</sup> (LPG-deficient) | High level of infection on day 7 | Myskova et al. [20]     |
| *L. longipalpis* (Brazil)     | *L. major* (unnatural) | LPG-independent | *lpg1<sup>-</sup> (LPG-deficient) | High level of infection on day 7 | Secundino et al., (submitted) |
|                               |                   |                   | *lpg1<sup>-</sup> (LPG-deficient) | Mature infections on day 7–9 | Rogers et al. [29]       |
|                               |                   | LPG-independent | *lpg2<sup>-</sup> (PG-deficient) | Impaired on day 3; no parasites on day 7 | Secundino et al., (submitted) |
| *P. arabicus* (Israel)        | *L. major* (unnatural) | LPG-independent | *lpg1<sup>-</sup> (LPG-deficient) | High level of infection on day 7 | Myskova et al. [20]     |
| *P. perniciosus* (Spain)      | *L. major* (unnatural) | LPG-independent | *lpg1<sup>-</sup> (LPG-deficient) | High level of infection on day 7 | This study             |
|                               |                   | LPG2-related molecules required from early phase | *lpg2<sup>-</sup> (PG-deficient) | Comparable to WT on day 2; no parasites on day 9 |                       |
|                               |                   |                   | *lpg1<sup>-</sup> (LPG-deficient) | High level of infection on day 5 | Thist study             |
|                               |                   | LPG2-related molecules required from early phase | *lpg2<sup>-</sup> (PG-deficient) | Impaired on day 2; low numbers of parasites on day 5 |                       |
|                               |                   |                   | *lpg1<sup>-</sup> (LPG-deficient) | Severely impaired on day 5 | Pimenta et al. [19]; Sacks et al. [4] |
|                               |                   | PGs required from early phase | *lpg2<sup>-</sup> (PG-deficient) | Impaired on day 2; no parasites on day 5 | Sacks et al. [4]         |

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failed this test, as restoration of LPG1 expression to R2D2 only weakly restored both LPG and survival in P. argentipes [4].

In contrast to the permissive vectors, the development of L. major \( lpg1 \) mutants was severely impaired in the specific vector P. duboscqi. Although the early infections were similar to those of the WT parasites, there was a substantial decrease in the \( lpg1 \) infections rate after defecation of the bloodmeal. In very few females the \( lpg1 \) mutants produced mature late stage infections. Our results extend those reported in the study by Boulanger et al. [32] performed with a small number of sand flies. Similar results with L. major \( lpg1 \) mutants in P. duboscqi were recently obtained by Secundino et al. (submitted) (Table 1). Our additional experiment confirmed that P. duboscqi is not permissive to L. infantum development and can therefore be classified as a specific vector. Moreover, lectin affinity blotting revealed that unlike P. perniciosus and P. argentipes, there are no N-acetyl-D-galactosamine-displaying epitopes in P. duboscqi midgut (Fig 4). Such glycoconjugates have been suggested as potential Leishmania ligands in the midgut of permissive vector species [20]. In conclusion, this study gives supporting evidence to the present distinction of sand flies into categories based on their susceptibility to various Leishmania species [1]. Together with the results of Myskova et al. [20] and Rogers et al. [29], our studies of L. major \( lpg1 \) development suggest the presence of a LPG-independent parasite-binding mechanism within the midgut of permissive sand flies.

Unlike LPG-deficient \( lpg1 \) mutants, PG-deficient \( lpg2 \) mutants additionally were impaired in early development in sand fly and unable to survive at all stages in all sand fly species tested. In P. argentipes and P. duboscqi parasites of this line are severely impaired as early as day 2 post infection. For P. duboscqi, these data stand in line with those of Boulanger et al. [32]. A similar finding was described for PG-deficient mutants of L. donovani in P. argentipes by Sacks et al. [4]. Moreover, Secundino et al. (submitted) have recently made similar observations in P. duboscqi originating from Mali and in Lu. longipalpis (see Table 1).

It has been hypothesized that parasite death in the pre-defecated sand fly midgut is attributable to digestive enzymes and that the phosphoglycans other than LPG confer resistance to the proteolytic attack [33], specifically phosphoglycans dependent on the activity of LPG2. While LPG2 has been suggested to affect synthesis of other glycoconjugates beyond PGs, this possibility was excluded through studies of a second PG-deficient mutant, defective due to a lack of the UDP-Gal transporters LPG3A and LPG3B by gene targeting, showing that it is also unable to survive the late stages of P. duboscqi infection (Secundino et al., submitted). Thus, it is likely that the defects in \( lpg2 \) infection of midguts described here and previously arise primarily through loss of PGs other than LPG, potentially the PPGs common to all species, or sAPs which occur in L. major albeit to lesser extents than in other Leishmania species [34].

In this work we also attempted to assess the importance of LPG2-dependent molecules by exposing parasites to the action of a proteolytic enzyme in vitro. As trypsin-like proteases were described as the most abundant digestive enzymes in both P. papatasi and Lu. longipalpis midgut after bloodfeeding [35,36], bovine pancreatic trypsin was chosen for these experiments. The bovine enzyme used shares all the conserved amino acid residues that influence the substrate specificity with sand fly midgut trypsin-like molecules. The \( lpg2 \) promastigotes lacking surface PGs did not prove to be more vulnerable to trypsin activity than the WT and \( lpg1 \) parasites whose resistance to trypsin has been previously reported [13]. These results could argue against a role of PGs in conferring resistance of promastigotes to the trypsin-like digestive enzymes in the sand fly gut. However, in light of the studies of Secundino et al (submitted), we think it more likely that bovine trypsin is not a good model for the activity and/or properties of all the proteolytic contents of the sand fly midgut. Most importantly, Secundino et al showed that inhibition of trypsin and other proteolytic activity in the midgut does in part rescue the survival of \( lpg2 \) parasites, although they do not exclude the possibility that other factors contribute to the inability of the \( lpg2 \) to survive within the bloodmeal. Sand fly immunity has been shown to play a major role in the control of bacterial and parasitic infections [32] and potentially that molecules such as antimicrobial peptides secreted to the midgut lumen could contribute to the destruction of the \( lpg2 \) mutant parasite.

In summary, this study demonstrates that an LPG-independent mechanism of attachment of Leishmania is a feature common to permissive sand fly species. It also proves the importance of LPG2-dependent molecules in the survival of L. major in various sand fly vectors. Moreover, it brings an evidence that P. duboscqi is not able to support development of L. infantum and therefore can be classified as a specific vector.

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Author Contributions
Conceived and designed the experiments: AS PV. Performed the experiments: AS THA VS LJ. Analyzed the data: AS THA VS LJ SMB PV. Contributed reagents/materials/analysis tools: SMB. Wrote the paper: AS THA SMB PV.

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