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

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Abstract

Background: Sand fly species able to support the survival of the protozoan parasite Leishmania have been classified as permissive or specific, based on 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. argentipes, 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. argentipes, 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. argentipes 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. argentipes, P. halepensis, P. arabicus and Lutzomyia 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). PPGs have been implicated in the early survival of L. donovani within the blooded 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

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 parasite 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. Myšková et al. [20] demonstrated that both, P. argentipes and P. perniciosus possess midgut glycoproteins with HPA (Helix pomatia agglutinin, lectin with specificity to N-acetyl-D-galactosamine-) binding epitopes. Phlebotomus argentipes 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/NeaP) [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. argentipes (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 thick skin membrane with 4–5 day old promastigotes at cell density of 5×10⁶ (P. duboscqi infections with L. major) or 1×10⁶ promastigotes/ml (all other infections, including P. duboscqi with L. infantum) in heat inactivated rabbit blood (Biovetra, 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 microbiological 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 $3 \times 10^5$ 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 $\Delta A_{253}^{\text{mM}}$ of 0.001 per min at pH 7.6 at 25 °C using benzoyl-L-arginine ethyl ester (BAEE) as substrate). In control groups, parasites were cultivated in M 199 alone. After 24 hours at 25°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, 10 mM protein per lane) followed by western blotting. The nitrocellulose membrane was incubated in Tris buffer with 0.05% Tween 20 (Tris-Tw) with lane) followed by western blotting. The nitrocellulose membrane was incubated in Tris buffer with 0.05% Tween 20 (Tris-Tw) with addition of 250mM methyl-mannopyranoside as an inhibitory sugar.

In contrast to day 2, on day 9 post infection the $lpg^{1+}$ 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 $lpg^{1+}$ line produced very few mature infections colonizing the stomodeal valve in only 10% of females. Notably the $lpg^{2-}$ line did not survive, as no $lpg^{2-}$ parasites were found in the midguts by day 9 (Fig. 1). Previous studies have established that phenotypes arising from the $lpg^{1+}$ and $lpg^{2-}$ 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 parasites were found in 62% of flies but with very low parasite loads (less than 100 Leishmania, except for 1 fly). The

Results

Detection of glycoconjugates in P. duboscqi midgut lysates

Phlebotomus duboscqi sand flies were infected with WT, $lpg^{1+}$ and $lpg^{2-}$ 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 $lpg^{1+}$ line, with both showing very high rates of infection (97% and 93% respectively), with about 75% of heavy infections (Fig. 1). The $lpg^{2-}$ mutant survived less well however; their infection rate was significantly lower (74%; P<0.01), with only 24% of heavy infections.

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differences in infection intensity and rate between the \( \text{lipg2}^- \) 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 \( \text{lipg1}^- \) 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 \( \text{lipg1}^- \) parasites reached the stomodeal valve in 46% and 48%, respectively. In contrast, \( \text{lipg2}^- \) 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 \( L. \text{major} \) lines in \( P. \text{perniciosus} \)

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, \( \text{lipg2}^- \) mutants were eliminated while \( \text{lipg1}^- \) mutants developed similarly to the WT parasites (Fig. 3). WT and \( \text{lipg1}^- \) lines developed mature infections colonizing the stomodeal valve with high parasite burdens in majority of females. In contrast, none of the \( \text{lipg2}^- \) parasites were able to persist until day 9, suggesting that they were lost during defecation.

\( P. \text{duboscqi} \) is refractory to \( L. \text{infantum} \)

In order to test the degree of permissivity of \( P. \text{duboscqi} \), females of this species were infected with \( L. \text{infantum} \), a parasite that is not transmitted by \( P. \text{duboscqi} \) in nature. Promastigotes were able to survive inside the peritrophic sac during digestion of the blood-meal 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 \( P. \text{duboscqi} \) is refractory to \( L. \text{infantum} \).

\( P. \text{duboscqi} \) midgut glycosylation

As detected by western blotting with lectins, \( P. \text{duboscqi} \) 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 \( \beta \)-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 \( P. \text{papatasi} \) and \( P. \text{sergenti} \). 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 \( L. \text{major} \)

The \( \text{in-vitro} \) growth of \( L. \text{major} \) promastigotes of WT, \( \text{lipg1}^- \) and \( \text{lipg2}^- \) 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 \( L. \text{major} \) and \( L. \text{donovani} \) in sand flies, mediated through binding of LPG to the sand
fly midgut [4,19]. 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 L. 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>
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<tr>
<th>Vector species</th>
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<th>Interpretation</th>
<th>Mutant lines</th>
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<tr>
<td>P. papatasi (Israel)</td>
<td>L. major (natural)</td>
<td>LPG required in late phase</td>
<td>lpg1– (LPG-deficient)</td>
<td>Low percentage of infected flies on day 5</td>
<td>Sacks et al. [4]</td>
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<tr>
<td>P. papatasi (Turkey)</td>
<td>L. major (natural)</td>
<td>LPG required in late phase</td>
<td>lpg1– (LPG-deficient)</td>
<td>Low percentage of infected flies on day 8</td>
<td>Myskova et al. [20]</td>
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<tr>
<td>P. duboscqi (Mali)</td>
<td>L. major (natural)</td>
<td>LPG required in late phase</td>
<td>lpg1– (LPG-deficient)</td>
<td>Fewer than 1000 parasites/gut on day 7</td>
<td>Secundino et al., (submitted)</td>
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<td></td>
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<td>lpgSA /SB– (PG-deficient)</td>
<td>Impaired on day 3; no parasites on day 7</td>
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<tr>
<td>P. duboscqi (Senegal)</td>
<td>L. major (natural)</td>
<td>LPG possibly required in late phase</td>
<td>lpg1– (LPG-deficient)</td>
<td>Comparable to WT on day 4, partially impaired on days 8 and 10</td>
<td>Boulanger et al. [32]</td>
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<td></td>
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<td></td>
<td>lpg2– (PG-deficient)</td>
<td>Impaired on day 4, no parasites on days 8 and 10</td>
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<td>P. duboscqi (Senegal)</td>
<td>L. major (natural)</td>
<td>LPG required in late phase</td>
<td>lpg1– (LPG-deficient)</td>
<td>Comparable to WT on day 2; impaired on day 9</td>
<td>This study</td>
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<td>lpg2– (PG-deficient)</td>
<td>Impaired on day 2; no parasites on day 9</td>
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<tr>
<td>Lu. longipalpis (Brazil)</td>
<td>L. major (unnatural)</td>
<td>LPG-independent</td>
<td>lpg1– (LPG-deficient)</td>
<td>High level of infection on day 7</td>
<td>Myskova et al. [20]</td>
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<td>lpg1– (LPG-deficient)</td>
<td>High level of infection on day 7</td>
<td>Secundino et al., (submitted)</td>
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<td>lpg2– (PG-deficient)</td>
<td>Impaired on day 3; no parasites on day 7</td>
<td>Secundino et al., (submitted)</td>
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<tr>
<td>L. mexicana (unnatural)</td>
<td>LPG-independent</td>
<td>lpg1– (LPG-deficient)</td>
<td>Mature infections on day 7–9</td>
<td>Rogers et al. [29]</td>
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<tr>
<td>P. arabicus (Israel)</td>
<td>L. major (unnatural)</td>
<td>LPG-independent</td>
<td>lpg1– (LPG-deficient)</td>
<td>High level of infection on day 7</td>
<td>Myskova et al. [20]</td>
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<tr>
<td>P. perniciosus (Spain)</td>
<td>L. major (unnatural)</td>
<td>LPG-independent</td>
<td>lpg1– (LPG-deficient)</td>
<td>High level of infection on day 9</td>
<td>This study</td>
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<td>lpg2– (PG-deficient)</td>
<td>Comparable to WT on day 2; no parasites on day 9</td>
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<tr>
<td>P. argentipes (India)</td>
<td>L. major (unnatural)</td>
<td>LPG-independent</td>
<td>lpg1– (LPG-deficient)</td>
<td>High level of infection on day 5</td>
<td>This study</td>
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<td></td>
<td></td>
<td></td>
<td>lpg2– (PG-deficient)</td>
<td>Impaired on day 2; low numbers of parasites on day 5</td>
<td></td>
</tr>
<tr>
<td>L. donovani (natural)</td>
<td>LPG-independent</td>
<td>lpg1– (LPG-deficient)</td>
<td>Severely impaired on day 5</td>
<td>Pimenta et al. [19]; Sacks et al. [4]</td>
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Table 1. Requirement for lipophosphoglycan (LPG) and other phosphoglycans (PGs) during Leishmania development in various sand fly species.
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* lpg1Δ 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 lpg1Δ infections rate after defecation of the bloodmeal. In very few females the lpg1Δ 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* lpg1Δ 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* lpg1Δ development suggest the presence of a LPG-independent parasite-binding mechanism within the midgut of permissive sand flies.

Unlike LPG-deficient lpg1Δ mutants, PG-deficient lpg2Δ 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 lpg2Δ 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 lpg2Δ promastigotes lacking surface PGs did not prove to be more vulnerable to trypsin activity than the WT and lpg1Δ 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 trypsic and other proteolytic activity in the midgut does in part rescue the survival of the lpg2Δ parasites, although they do not exclude the possibility that also other factors contribute to the inability of the lpg2Δ 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 lpg2Δ 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. Contributed reagents/materials/analysis tools: SMB. Wrote the paper: AS THA SMB PV.

References


