Leishmania major survival in selective Phlebotomus papatasi sand fly vector requires a specific SCG-encoded lipophosphoglycan galactosylation pattern

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**Leishmania major** Survival in Selective Phlebotomus papatasi Sand Fly Vector Requires a Specific SCG-Encoded Lipophosphoglycan Galactosylation Pattern

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**Abstract**

Phlebotomine sand flies that transmit the protozoan parasite *Leishmania* differ greatly in their ability to support different parasite species or strains in the laboratory: while some show considerable selectivity, others are more permissive. In “selective” sand flies, *Leishmania* binding and survival in the fly midgut typically depends upon the abundant promastigote surface adhesin lipophosphoglycan (LPG), which exhibits species- and strain-specific modifications of the dominant phosphoglycan (PG) repeat units. For the “selective” fly *Phlebotomus papatasi* (*Ppap*), side chain galactosyl-modifications (scGal) of PG repeats play key roles in parasite binding. We probed the specificity and properties of this scGal-LPG PAMP (Pathogen Associated Molecular Pattern) through studies of natural isolates exhibiting a wide range of galactosylation patterns, and of a panel of isoënic *L. major* engineered to express similar scGal-LPG diversity by transfection of SCG-encoded β1,3-galactosyltransferases with different activities. Surprisingly, both ‘poly-scGal’ and ‘null-scGal’ lines survived poorly relative to *Ppap*-sympatric *L. major* FV1 and other ‘mono-scGal’ lines. However, survival of all lines was equivalent in *P. dubosqui*, which naturally transmit *L. major* strains bearing ‘null-scGal’-LPG PAMPs. We then asked whether scGal-LPG-mediated interactions were sufficient for *Ppap* midgut survival by engineering *Leishmania donovani*, which normally express unsubstituted LPG, to express a ‘*Ppap*-optimal’ scGal-LPG PAMP. Unexpectedly, these “*L. major* FV1-cloaked” *L. donovani*-SCG lines remained unable to survive within *Ppap* flies. These studies establish that midgut survival of *L. major* in *Ppap* flies is exclusively sensitive to the scGal-LPG PAMP, requiring a specific ‘mono-scGal’ pattern. However, failure of ‘mono-scGal’ *L. donovani*-SCG lines to survive in selective *Ppap* flies suggests a requirement for an additional, as yet unidentified *L. major*-specific parasite factor(s). The interplay of the LPG PAMP and additional factor(s) with sand fly midgut receptors may determine whether a given sand fly host is “selective” or “permissive”, with important consequences to both disease transmission and the natural co-evolution of sand flies and *Leishmania*.


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**Introduction**

*Leishmania* are protozoan parasites that cause a spectrum of human diseases that range from self-healing cutaneous lesions to potentially fatal visceral forms. Leishmaniasis is re-emerging as a significant world health problem, with approximately 12 million people presently infected and 2 million new cases diagnosed each year (www.who.int/leishmaniasis/burden). The world-wide distribution of different *Leishmania* is determined by the availability of transmission-competent sand fly vectors. When a sand fly bites an infected vertebrate host, *Leishmania* amastigotes residing within macrophages and other cell types are taken up in the blood meal which is surrounded by a midgut peritrophic matrix that lasts for several days. During this time amastigotes differentiate into motile, replicating promastigote forms which reside in the extracellular lumen of the sand fly alimentary tract (rev. in [1,2,3]). Barriers to *Leishmania* development in this compartment include the chitin-containing peritrophic matrix which completely encases the blood meal, and the many hydrolytic enzymes and anti-microbial molecules secreted into the gut lumen (rev. in [2,3,4,5]). Eventually the remnants of the digested blood meal are excreted by the sand fly, and this is a crucial juncture for *Leishmania* promastigotes. In transmission-competent sand flies, parasites attach to the midgut epithelium and go on to establish a stable infection; in a transmission-refractory vector, unattached parasites are expelled when the sand fly defecates (rev. in [2,3,4]). As the sand fly prepares to feed again, promastigotes transition through several forms that culminate in infectious metacyclic parasites which express a modified surface that cannot bind to the midgut epithelium (rev. in [1,3,4,6]). Thus, a key step in *Leishmania* transmission is stage-specific midgut attachment which allows *Leishmania* development to proceed.
Author Summary

Phlebotomine sand flies are tiny blood-feeding insects that transmit Leishmania protozoan parasites, which cause diseases afflicting millions of people. The worldwide distribution of Leishmania is determined by the availability of transmission-competent vectors. In the laboratory, some vectors support many different Leishmania, while others are highly restricted. This is best exemplified by P. papatasi, which transmit only L. major despite a wide distribution in regions endemic for many Leishmania species. P. papatasi “selectivity” can be reproduced experimentally, and has been attributed to β1,3-linked galactose side chains decorating the abundant L. major surface lipophosphoglycan (LPG) adhesin, which mediate parasite attachment to the P. papatasi midgut to prevent elimination when the digested blood meal is excreted. As geographically diverse L. major display very different LPG galactosylation patterns (n=0 - 8 [Gal/side chain]), we explored the consequences of this pattern diversity to survival in P. papatasi. Using natural isolates and L. major lines engineered to express a wide range of LPG galactosylation patterns, we showed L. major survival in P. papatasi PpapJ flies was optimized by expression of highly modified ‘mono-galactosylated’ LPG and extremely sensitive to LPG side chain length. Surprisingly, L. donovani lines engineered to express a “PpapJ-optimal” LPG mono-galactosylation pattern did not survive in PpapJ flies, suggesting that additional interactions are required. These studies reveal the fine specificity of Leishmania - sand fly interactions, and the nature of species- and strain-specific parasite molecules that have co-evolved to take advantage of midgut receptors specific to available sand fly vectors.

Two distinct mechanisms for regulating Leishmania attachment to sand fly midgut epithelium have been identified to date (rev. in [1,6,7,8]). One mechanism, utilized in “selective” Phlebotomus papatasi sand flies that support the complete development of only a single Leishmania species, involves a sand fly midgut epithelium receptor that binds the parasite lipophosphoglycan (LPG) adhesin. LPG is an abundant glycolipid that covers the entire surface, including the flagellum, of all Leishmania promastigote stages [9]. The basic LPG structure is highly conserved in all Leishmania species, consisting of a glycosylphosphatidylinositol anchor to which is attached a long polymer of 10-30 phosphoglycan (PG) repeating units (6Gal,1,4-Manz1-PO4), and terminated by a small neutral oligosaccharide chain (rev. in [10,11]). The PG repeating units are often modified by strain-, species-, and developmental stage-specific modifications that have been implicated in the midgut attachment and release of several Leishmania species in their respective natural vectors [12,13,14,15]. A second, LPG-independent sand fly midgut binding mechanism was recently identified using LPG-deficient Leishmania and several “permissive” sand flies that support the development of a broad range of Leishmania species in the laboratory [2,7,8,16]. While the precise binding modality is uncertain, the involvement of vector glycans has been suggested [8,16]. Leishmania phosphoglycans and/or other LPG2-dependent molecule(s) are also required for parasite survival in “permissive” sand flies [8,17]. Thus sugars, in the form of surface glycoconjugates, are key players in the productive interactions between Leishmania and sand flies that are necessary for disease transmission. This is in agreement with the general principle that important surface interactions between many microbes and their hosts involve complex glycoconjugates binding to receptors (rev. in [18]).

In this study we focused on the interactions between Leishmania major promastigotes and Phlebotomus papatasi sand flies. Phlebotomus papatasi is a “selective” vector which, despite its wide distribution in regions endemic for transmission of several Leishmania species, transmits only Leishmania major in nature and in the laboratory (rev. in [1,2,3,4,6]). Midgut attachment is mediated by modified PG repeats bearing side chain β1,3 galactosyl residues (scGal), which form the ligand recognized by the midgut LPG receptor PpGalec identified in Jordan Valley strain P. papatasi [PpapJ] sand flies [19]. As L. major procyclics develop into infectious metacyclic forms, procyclic form LPG is shed and replaced by metacyclic form LPG, which has increased numbers of PG repeats and scGal residues masked by the addition of terminal arabinose “caps”; these modifications block binding to PpGalec receptors [19] and facilitate detachment from the midgut [19,20]. Laboratory infections established the requirement for scGal-LPG in PpapJ midgut survival: L. major mutants or Leishmania species expressing scGal-deficient LPG, or lacking LPG entirely, could not establish stable PpapJ infections [12,16,21,22,23,24] and bound poorly to isolated PpapJ midguts and recombinant PpGalec receptors in vitro [19].

Notably, geographically diverse L. major strains express very different LPG side chain galactosylation patterns [which we refer to hereafter as scGal-LPG PAMPs (Pathogen Associated Molecular Patterns)], showing a Southwest-to-Northeast cline across its range from ‘null-scGal’ to ‘poly-scGal’ LPG PAMPs [23,25,26,27]; Cardoso et al., in preparation]. For example, Senegalese strain SD procyclic LPG has such low levels of single βGal modifications that it is effectively unmodified ‘null-scGal’ LPG PAMP; [23] and this report). In contrast, Israeli strain FV1 procyclic LPG is highly modified with primarily single βGal residues (‘mono-scGal’ LPG PAMP; [27]), and Central Asian strain LV39 clone 5 (LV39c5) procyclic LPG is highly modified by long polymers of up to 8 βGal residues (‘poly-scGal’ LPG PAMP; [28,29]). Amongst these natural L. major strains, FV1 is sympatric with the “selective” P. papatasi PpapJ sand fly, while SD parasites are sympatric with P. duboscqi, a closely related sibling species of P. papatasi (rev. in [3]).

Previously, we hypothesized that different scGal-LPG PAMPs resulted from the combined activity of the seven telomeric SCG (Side Chain Galactoses) gene family members, which encode PG-side chain β1,3-galactosyltransferases. SCGs exhibit different activities, combining to varying extents ‘initiating’ activities able to attach the first βGal residue to the basic PG repeat, and ‘elongating’ activities able to add additional βGal residues to the initiated βGal side chain ([30,31]; Dobson et al., in preparation). In this work, we made use of this suite of diverse SCG activities to engineer isogenic parasites bearing defined scGal-LPG PAMPs. Using both naturally-occurring L. major strains and SD-SCG transfectant lines, we show that a specific scGal-LPG PAMP is optimal for long-term parasite survival in selective PpapJ sand flies, which preferred highly substituted scGal-LPG PAMPs bearing mono-galactosyl chains, neither “too short” nor “too long”. The “PpapJ-optimal” scGal-LPG PAMP was not sufficient, however, to enhance survival of L. donovani-SCG transfectants in PpapJ sand flies. These findings lead us to propose a two-component model for long-term Leishmania survival in “selective” PpapJ sand flies: 1) a specific scGal-LPG PAMP recognized by PpGalec midgut receptors and 2) an as yet unidentified L. major species-specific factor(s).

Methods

Leishmania strains and transfections

Leishmania major strain Friedlin V1 (FV1) is a clonal derivative of the Friedlin line (MHOM/IL/80/Friedlin), L. major strain LV39...
clone 5 (LV39c5) is a clonal derivative of the LV39 line (RHO/ SU/59/P). L. major strain SD 75.1 (SD) is a clonal derivative of the NIH/SD line (MHOM/SN/74/SD). L. donovani Sudanese strain 18-2D clone Lah/Ldh is a clonal derivative (MHOM/SD/80/18-2D), and L. mexicana strain M379 is a clonal derivative (MYNC/ BZ/62/M379). All wild type (WT) lines showed good infectivity in animal models and in their natural sand fly vectors [24,32,33,34]. Cells were grown in complete M199 medium containing 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), HEPES pH 7.4 (40.5 mM), adenosine (0.1 mM), biotin (0.001%), biotin, (2 μg/ml), and hemin (0.0005%), at 25°C as described [35]. Procyelic promastigotes were harvested from logographically growing cultures.

Promastigotes were transfected by electroporation, using a low voltage [35] or high voltage [36] protocol. Clonal lines were obtained by plating on semisolid M199 media containing the voltage [35] or high voltage [36] protocol. Clonal lines were harvested from logarithmically growing cultures.

Molecular constructs and transfectants

L. major strain FV1 was the source of all SCG genes used in this study. Episomal expression constructs used here include the cosmid vector cLHYG (strain B980; [37], pXK/NEO-SCG2 (B3900; [30]), SCG3 cosmid B3979 [30], and pXG/NEO-LMSAP1 (B3092; [34]). The integrating SCG open reading frame (ORF) constructs pRISAT-SCG1 (B5097), pRISAT-SCG3 (B5101), pRISAT-SCG4 (B5103), and pRISAT-SCG5 (B5170) were created as follows: SCG ORFs liberated by BamHI digestion of appropriate pXG/PHELO-SCG ORF constructs (Dobson et al., in preparation) were ligated into the BgII restriction site of pRISAT (B3541; [36]). Each pRISAT-SCG construct was digested with Sva1 restriction enzyme, dephosphorylated with calf alkaline phosphatase, and gel-purified to yield linear SSU::RISAT-SCG ORF targeting fragments for integration into the ribosomal RNA small subunit (SSU) locus by homologous recombination during transfection [36]. Integrated SCG transfectants are referred to by the gene name and location, i.e. SD-SSU::SCG3 has the FV1 strain SCG3 ORF (SSU::RISAT-SCG3) integrated into the SD ribosomal SSU locus.

We used three Ld-transfectant lines developed previously [30]: a ‘null-scGal’ LPG PAMP line devoid of any LPG side chain modification, transfected with the episomal cosmid vector cLHYG (Ld-vector); and two different lines exhibiting ‘mono-scGal’LPG PAMPS, one transfected with SCG3 cosmid B3979 (Ld+SCG3), and a second transfected with the SCG2 ORF expression construct pXK-SCG2 (Ld-pSCG2).

Purification and analysis of LPG

LPG was prepared from exponentially-growing promastigotes as described [38]. To assess side chain modifications, phosphorylcan repeats were depolymerized using mild acid hydrolysis, dephosphorylated using E. coli alkaline phosphatase, covalently labeled with 1-aminopyrene-3,6,8-trisulfonate, and analyzed by Dionex HPLC chromatography [14] or capillary electrophoresis [39], comparing migration distances with oligomeric glucose standards.

Analysis of secreted acid phosphatase (SAP) levels

Procyelic promastigotes (5×10⁷/ml) were grown in complete M199 medium to a final density of 1×10⁷/ml. Culture supernatants were collected and centrifuged for 10 min at 2500 rpm in a Sorvall RT7000 centrifuge to remove cells and debris. 10 microliter samples of clarified culture supernatant were electrophoresed on a non-denaturing polyacrylamide gel and the gel then stained for SAP enzyme activity using α-naphthyl acid phosphate plus Fast Garnet GBC as described [40,41]. SAP was quantitated using the AlphaImager version 5.5 gel documentation system spot densitometry program (Alpha Innotech, San Leandro, CA).

Sand fly infection and dissection

Sand fly colonies were reared at the Division of Entomology, Walter Reed Army Institute of Medical Research and at the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, NIH. The following species were used in this study: Phlebotomus papatasi from colonies originating from the Jordan Valley (Ppap), Phlebotomus duboscqi from colonies originating from Mali (Pdub), and Phlebotomus argentipes from colonies originating from India (PargN).

Female 3- to 5-day-old sand flies were fed through a chick skin membrane using a feeding device containing a mixture of heparin-treated mouse blood and logarithmic phase promastigotes, as described [24]. The concentration of promastigotes used varied depending on the experiment, from 1×10⁶ to 20×10⁶ parasites/ml. Blood-engorged sand flies were separated and maintained at 28°C with 30% sucrose (v/v). At various times after feeding, flies were anesthetized, their midguts dissected and homogenized, and the number of released midgut promastigotes counted using a hemocytometer as described [24].

Statistical analyses

Parasite numbers in the midguts of infected flies after blood meal excretion do not follow a Gaussian distribution. This is likely the result of flies within groups having either completely lost their infections or retained parasites that grow exponentially prior to the time of dissection. Therefore, data sets were compared using a nonparametric Mann Whitney test. Mann Whitney calculations were done using Prism 4 (Graphpad Software, Inc. San Diego, CA).

Results

Three natural L. major strains show varying patterns of LPG side chain galactosylation

LPGs from three geographically distinct L. major strain procyclic promastigotes were purified, subjected to mild acid hydrolysis and dephosphorylation, and isolated PG repeat structures assessed by capillary electrophoresis (Methods, Table S1). Side-chain galactosylation can be characterized by two parameters: the fraction of PG repeats that were modified, and the number of βGal residues attached. In these studies we found two general patterns of LPG side chain galactosylation: one in which little or no βGal was added; and a second in which 50–90% of the PG repeats were modified, with varying numbers of βGal residues. From these data we found it useful to calculate a single parameter for comparisons amongst lines, the “average scGal chain length”, obtained by multiplying the fraction of modified PG repeats times the average number of βGal residues added per modified repeat (Tables 1, S1).

Senegalese strain SD LPG was mostly unmodified (0.02 avg. scGal chain length), consistent with prior studies using specific antisera and lectins suggesting that SD LPG was largely unmodified [23]. In contrast, Israeli strain FV1 LPG was extensively modified with predominantly single βGal residues (0.8 avg. scGal chain length). Central Asian strain LV39c5 LPG was also highly modified, but with longer polymers of up to 8 βGal residues (3.1 avg. scGal chain length). We refer to these three prototypic LPG galactosylation patterns as ‘null-scGal’, ‘mono-
scGal', and ‘poly-scGal’ LPG PAMPs (Pathogen-Associated Molecular Patterns), respectively. The results with FV1 and LV39c5 confirmed and extended previous studies [27,28,29], and were undertaken to guard against changes in LPG side chain composition occurring during laboratory propagation, as described previously [25,42].

L. major survival in selective Phlebotomus papatasi Ppapj sand flies requires a specific scGal-LPG PAMP

Ppapj sand flies were fed on the indicated L. major-infective mouse blood and midgut infections were assessed 48 hr later, a time when parasites remain within the blood meal encased by the peritrophic membrane (Fig. 1A, “+ blood, d2”). At this time all three L. major strains showed high parasite numbers in most flies examined (>33,000 parasites/midgut), with the highest numbers observed in flies infected with the SD strain, likely reflecting the faster generation time of this strain. Thus differences in the scGal-LPG PAMPs did not affect the early survival and growth of L. major promastigotes, as expected since even LPG-deficient parasites survive normally in sand flies during this interval [16,22,24].

By day 5 post-feeding, the sand fly peritrophic matrix disintegrates and the remains of the digested blood meal are expelled. At this time there were clear differences amongst the L. major strains in their ability to survive (Fig. 1A, “no blood, d5”). In agreement with previous studies [22,24], ‘mono-scGal’ FV1 persisted in most Ppapj flies at high levels (82% flies infected, 16200±16600 parasites/midgut). In contrast, ‘poly-scGal’ LV39c5 survived poorly (33% flies infected, 3860±4840 parasites/midgut; p<0.013), as did ‘null-scGal’ SD, with the exception of two strongly-infected outliers (38% flies infected, 6660±18600 parasites/midgut; p<0.005). The poor survival of ‘null-scGal’ SD was expected, as un-galactosylated LPG cannot bind to midgut PpGalec receptors [19], resulting in unattached parasites being excreted with the digested blood meal remnants [21,24]. However, the poor Ppapj survival of ‘poly-scGal’ LV39c5 (Fig. 1A,B) [24] suggested that a specific scGal-LPG PAMP, rather than simply the presence of galactosylated LPG, controls L. major promastigote survival in Ppapj midguts following blood meal expulsion.

Generation of isogenic L. major parasites bearing a range of scGal-LPG PAMPs

Since the three L. major strains studied here show an average nucleotide sequence divergence of 0.15% [43], comparable to that amongst many L. major strains, molecular differences other than scGal-LPG PAMPs were potentially responsible for the survival differences we observed in selective Ppapj sand fly infections. To generate different scGal-LPG PAMPs in an isogenic scGal-deficient LPG background, we introduced into the SD line a series of constructs expressing members of the previously characterized SCG family of telomeric phosphoglycan-side chain-β1,3galactosyltransferases (PG-scGalt) [30,31] Critical to these studies is the fact that SCG-encoded PG-scGalt has different enzymatic properties mediating the addition of different

### Table 1. Effect of LPG galactosylation pattern on Leishmania survival in “selective” Phlebotomus papatasi Ppapj sand flies.

<table>
<thead>
<tr>
<th>Leishmania line*</th>
<th>LPG-scGal modification frequency</th>
<th>avg. scGal chain length</th>
<th>scGal-LPG PAMP</th>
<th>Ppapj survival post-blood meal expulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% infected flies</td>
</tr>
<tr>
<td>WT FV1</td>
<td>71%</td>
<td>0.8</td>
<td>mono</td>
<td>96±5</td>
</tr>
<tr>
<td>WT LV39c5</td>
<td>93%</td>
<td>3.1</td>
<td>poly</td>
<td>61±8</td>
</tr>
<tr>
<td>WT SD</td>
<td>2%</td>
<td>0.02</td>
<td>null</td>
<td>38±</td>
</tr>
<tr>
<td>SD-SSU:SCG3</td>
<td>2%</td>
<td>0.02</td>
<td>null</td>
<td>61±1</td>
</tr>
<tr>
<td>SD-SSU:SCG5</td>
<td>68%</td>
<td>0.9</td>
<td>mono</td>
<td>90±4</td>
</tr>
<tr>
<td>SD-SSU:SCG3</td>
<td>86%</td>
<td>1.3</td>
<td>mono</td>
<td>88±12</td>
</tr>
<tr>
<td>SD-SSU:SCG1</td>
<td>56%</td>
<td>1.9</td>
<td>oligo</td>
<td>46±9</td>
</tr>
<tr>
<td>SD-SSU:SCG4</td>
<td>54%</td>
<td>3.1</td>
<td>poly</td>
<td>49±7</td>
</tr>
<tr>
<td>Ld-vector</td>
<td>0%</td>
<td></td>
<td>0</td>
<td>19±19</td>
</tr>
<tr>
<td>Ld-cSCG3</td>
<td>65%</td>
<td>0.7</td>
<td>mono</td>
<td>8±</td>
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<tr>
<td>Ld-pSCG1</td>
<td>62%</td>
<td>1.1</td>
<td>mono</td>
<td>26±26</td>
</tr>
</tbody>
</table>

*The L. major (FV1, LV39c5, SD) and L. donovani (Ld) wild-type (“WT”) and transfectant lines used in experimental Ppapj laboratory infections are described in the text, with supporting data in Tables S1 to S3 and Methods.

1. LPG-scGal modification frequency 2. The percentage of modified PG repeats bearing terminal Igal side chains present in purified procyclic promastigote LPG samples.

1. LPG galactosylation patterns, or ‘scGal-LPG PAMPs’, were classified by the average length of LPG Igal side chains (column 3): ‘null’, <0.1 Igal; ‘mono’, 0.7–1.3 Igals; ‘oligo’, 1.9 Igals; ‘poly’, ≥3 Igals.

2. % infected flies is the average percentage (± SEM) of Leishmania-infected Ppapj flies post-blood meal expulsion, calculated from data in Figs. 1, 2, 4, and Tables S2, S3.

3. Most lines were examined in two independent infections, except for WT FV1 (6 infections), and WT SD and Ld-cSCG3 (1 experiment each, “*”).

4. Relative parasites/midgut is the average number (± SEM) of parasites per midgut post-blood meal expulsion calculated relative to WT FV1 = 100, using data in Figs. 1, 2, 4, and Tables S2, S3. The average of two or more independent experiments is shown, except for WT SD and Ld-cSCG3 (1 experiment each, “*”).

5. Since WT FV1 was not included in this experiment, and P. argentipes PargIN is the natural vector for Ld parasites, the number of Ld-cSCG3 promastigotes was calculated relative to Ld-vector-infected PargIN flies.

6. Ld cSCG3 survival of ‘poly-scGal’ LV39c5 (Fig. 1A,B; [24]) suggested that a specific scGal-LPG PAMP, rather than simply the presence of galactosylated LPG, controls L. major promastigote survival in Ppapj midguts following blood meal expulsion.

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numbers of scGal residues, ranging from 0 to 12 ([30,31]; Dobson et al., in preparation). Thus, SD promastigotes were transfected with different SCG constructs, using either the episomal pXG-type vector which expresses passenger ORFs at moderate levels [37], episomal cosmids identified previously bearing SCG genes [30], or the integrating pIR1SAT vector which expresses passenger ORFs at high levels following integration into the ribosomal RNA small subunit (SSU) locus [36]. LPGs were purified from SD transfectants and LPG galactosylation patterns determined as described above (Methods); from these studies we chose a key set of SD-SCG lines exhibiting a range of scGal-LPG PAMPs (Tables 1, S1) briefly summarized here.

SD transfectants bearing an integrated catalytically inactive SCG3 ORF (SSU:IR1SAT-SCG3) synthesized scGal-deficient LPG indistinguishable from the parental WT SD line (‘null-scGal’ LPG PAMP; 0.02 avg. scGal chain length). Two SD transfectants expressed ‘mono-scGal’ LPG PAMPs: SD-cscG3 (0.9 avg. scGal chain length), containing the episomal SCG3 cosmid B3979; and SD-SSU:SCG3 (1.3 avg. scGal chain length), containing an integrated SCG3 ORF (SSU::IR1SAT-SCG3). SD-SSU:SCG4 transfectants bearing an integrated SCG4 ORF (SSU::IR1SAT-SCG4) synthesized a ‘poly-scGal’ LPG PAMP (3.1 avg. scGal chain length). A novel ‘oligo-scGal’ LPG PAMP (1.9 avg. scGal chain length) was synthesized by SD-SSU:SCG1, which bears an integrated SCG1 ORF (SSU::IR1SAT-SCG1). Together these SD-transfectant scGal-LPG PAMPs spanned the natural range of L. major LPG side chain variation as well as providing new LPG galactosylation patterns for study.

To confirm that SD transfectants had not experienced a general non-specific loss of “sand fly virulence” during their generation and propagation in the laboratory, we examined their survival in two independent infections involving Phlebotomus duboscqi PduM sand flies originating from Mali (Fig. S1 A,B). P. duboscqi is a sibling species of P. papatasi, and PduM flies are able to support the full development of WT SD in the laboratory [23]. Although L. major survival in P. duboscqi is LPG-dependent, it is not strongly affected by scGal-LPG PAMPs since various ‘null-scGal’, ‘mono-scGal’, or ‘poly-scGal’ L. major strains have been shown to survive expulsion of the digested blood meal [8,17,22,44]. Female PduM sand flies were allowed to feed on the indicated L. major-infective mouse blood containing ‘null-scGal’ (WT SD, SD-SSU:SCG3), ‘mono-scGal’ (WT FV1, SD-SSU:SCG3), ‘oligo-scGal’ (SD-SSU:SCG4), or ‘poly-scGal’ (SD-SSU:SCG4) promastigotes. As expected, all PduM flies were successfully infected with high numbers of parasites at early time points (Fig. S1, “+ blood” panels). Following expulsion of the digested blood meal, PduM flies infected with all L. major lines retained high numbers of midgut parasites (Fig. S1 “no blood” panels) and each line went on to establish fully mature infections in the PduM anterior midgut by day 12 post-feeding (data not shown). These data argue against a general non-specific loss in the ability of SD-SCG transfectants to survive in the phlebotomine sand fly midgut environment.

**Effect of scGal-LPG PAMPs on SD transfectant survival in “selective” Ppap sand flies**

*Ppap* flies were allowed to feed on the indicated L. major-infective mouse blood containing SD transfectants expressing different scGal-LPG PAMPs. The results from two independent experiments are shown (Fig. 2A,B). At early times post-infection when the midgut blood meal was retained, all SD transfectants behaved similarly: 100% of *Ppap* flies were infected with high numbers of promastigotes, similar to control ‘mono-scGal’ WT FV1 infections (Fig. 2A,B, “+ blood, d2”). However, we observed clear differences in *Ppap* midgut survival amongst SD lines expressing different scGal-LPG PAMPs after the digested blood meal had passed out of the midgut (Fig. 2A,B, “no blood, d5”). First, and as expected, SD-SSU:SCG5 transgenic parasites expressing a ‘null-scGal’ LPG PAMP survived poorly following blood meal excretion, with a 81–92% decrease in mean parasite numbers relative to control FV1-infected flies ([p<0.0005], and 38–40% of* Ppap* flies having lost their infections (Fig. 2A,B, “no blood, d5”).

Second, SD transfectants expressing ‘mono-scGal’ LPG PAMPs (SD-cscG3, SD-SSU:SCG3) generally survived well post-blood meal expulsion (Fig. 2A,B, “no blood, d5”). Most flies remained infected, and mean SD-cscG3 and SD-SSU:SCG3 parasite numbers (18600±4732, 18900±4900 and 18800±3100, 13200±4800 parasites/midgut, respectively) were not significantly different from control WT FV1 infections (13500±3500, 30000±4400 parasites/midgut). By contrast, ‘mono-scGal’ SD-cscG3 survival was significantly enhanced relative to ‘null-scGal’
SD-SSU:SCG5 (2560 ± 61720, p < 0.0004 and 2420 ± 600 parasites/midgut, p < 0.003; Fig. 2A,B). Although ‘mono-scGal’ SD-SSU:SCG3 survival was also enhanced relative to ‘null-scGal’ SD-SSU:SCG5, this difference reached significance in only one experiment (p < 0.0002 and p < 0.264; Fig. 2A,B).

Third, SD-SSU:SCG1 transfectants expressing a novel ‘oligo-scGal’ LPG PAMP survived poorly. Only 37–55% of SD-SSU:SCG1 flies remained infected post-blood meal expulsion and parasite levels (4120 ± 1940, 12500 ± 6300 parasites/midgut) were significantly reduced relative to control WT FV1-infected flies (p < 0.018 and p < 0.06; Fig. 2A,B). In fact, ‘oligo-scGal’ SD-SSU:SCG1 survival was not significantly better than observed for ‘null-scGal’ SD-SSU:SCG5 (p < 0.968 and p < 0.954; Fig. 2A,B).

Lastly, and consistent with the results from natural isolates, SD-SSU:SCG4 transfectants expressing a ‘poly-scGal’ LPG PAMP survived poorly. Only 42–56% of SD-SSU:SCG4 flies remained infected and parasite levels (683 ± 213, 2267 ± 831 parasites/midgut) were significantly decreased, 92–95% relative to control WT FV1 infections (p < 0.0007 and p < 0.0001, Fig. 2A,B). Thus ‘poly-scGal’ SD-SSU:SCG4 PppapJ1 survival was not significantly better than ‘null-scGal’ SD-SSU:SCG5 parasites.

These findings are summarized in Fig. 3, showing the relationship between relative PppapJ1 survival post-blood meal expulsion and the average scGal chain length in purified procyclic promastigote LPG. Isogenic SD-SSU transfectants whose LPG closely approximates the ‘mono-scGal’ LPG PAMP of the WT FV1 line (i.e. SD-c:SCG3, SD-SSU:SCG3) clearly survived well in PppapJ1 sand flies. In contrast, isogenic SD transfectants expressing either scGal-deficient LPG (‘null-scGal’ SD-SSU:SCG5) or LPG with longer side chain polymers (‘oligo-scGal’ SD-SSU:SCG1, ‘poly-scGal’ SD-SSU:SCG4) survived poorly in PppapJ1 flies, mirroring infection outcomes with naturally-occurring L. major strains SD or LV39c5 (‘null-scGal’ or ‘poly-scGal’ LPG PAMPs, respectively). Together, these data firmly implicate the scGal-LPG PAMP causally in controlling the ability of PppapJ1 flies to support L. major midgut survival post-blood meal expulsion.

Are “PppapJ-optimal” scGal-LPG PAMPs sufficient to enhance PppapJ survival of a different Leishmania species?

The studies above established that a ‘mono-scGal’ LPG PAMP was necessary for L. major survival in selective PppapJ sand flies,
following blood meal expulsion. We next asked whether this scGal-LPG PAMP would be sufficient, by examining its effect on the PpapJ survival of a different Leishmania species. We chose L. donovani Sudanese strain 1S-2D (Ld) since these parasites possess unmodified LPG ('null-scGal' LPG PAMP; [11,45]) and have been shown to survive poorly in PpapJ sand flies [12,24]. We used three Ld-transfectant lines developed previously [30]: a 'null-scGal' line devoid of any side chain sugars (Ld-vector, 0 avg. scGal length) and two different lines exhibiting 'mono-scGal' LPG PAMPs, Ld-cSCG3 and Ld-pSCG2 (0.7 and 1.1 avg. scGal chain length, respectively; Tables 1, S1).

When PpapJ sand flies were fed on L. donovani-infective mouse blood containing ‘null-scGal’ Ld-vector or ‘mono-scGal’ Ld-cSCG3 promastigotes, all flies were successfully infected with comparably high numbers of parasites when examined at a time when the midgut blood meal was present (Fig. 4A, PpapJ ‘+ blood, d3’ panel). Thus, these parasites were able to survive well in the initial steps of sand fly infection. However, following expulsion of the blood meal at day 5 post-feeding, parasites from both of these lines were completely lost in 90% of PpapJ flies, and those flies retaining infections had very low levels of parasites (180 and 125 parasites/midgut respectively; Fig. 4A, PpapJ ‘no blood, d5’; Table S3). Thus, despite generation of the optimal highly substituted ‘mono-scGal’ LPG PAMPs, Ld-cSCG3 and Ld-pSCG2 (0.7 and 1.1 avg. scGal chain length, respectively; Tables 1, S1).

When PpapJ sand flies were fed on L. donovani-infective mouse blood containing ‘null-scGal’ Ld-vector or ‘mono-scGal’ Ld-cSCG3 promastigotes, all flies were successfully infected with comparably high numbers of parasites when examined at a time when the midgut blood meal was present (Fig. 4A, PpapJ ‘+ blood, d3’ panel). Thus, these parasites were able to survive well in the initial steps of sand fly infection. However, following expulsion of the blood meal at day 5 post-feeding, parasites from both of these lines were completely lost in 90% of PpapJ flies, and those flies retaining infections had very low levels of parasites (180 and 125 parasites/midgut respectively; Fig. 4A, PpapJ ‘no blood, d5’; Table S3). Thus, despite generation of the optimal highly substituted ‘mono-scGal’ LPG PAMP in the Ld-cSCG3 line, survival in PpapJ was not enhanced (Table 1). As a control, these Ld transfectants were fed to P. argentipes PargIN, a natural vector of Ld transmission originating from India [12,24]. Previous studies have shown that midgut survival of both L. donovani and L. major in this “permissive” sand fly species is not strongly affected by LPG galactosylation patterns [8,12,24]. Due to the limited number of PargIN flies available for analysis, a single infection time point was analyzed comparing flies without blood meal remnants in the midgut on day 5 post-feeding. In contrast to the loss of midgut infections in PpapJ flies, both Ld-vector and Ld-cSCG3 promastigotes persisted and were maintained a moderate infection intensity in most PargIN flies after the digested blood meal was expelled (88% or 78% infected flies; 11263 or 6822 parasites/midgut; Fig. 4A PargIN ‘no blood, d5’, Table S3). These data argue against a
general non-specific loss in the ability of these Ld transfectants to survive in the phlebotomine sand fly midgut environment.

In separate experiments PpopJ flies were fed on Leishmania-infected mouse blood containing ‘null-scGal’ Ld-vector, ‘mono-scGal’ Ld-pSCG2, or control ‘mono-scGal’ WT L. major FV1 promastigotes (Fig. 4B,C). As expected, most PpopJ flies were infected with high numbers of parasites prior to expulsion of the blood meal, although the numbers of Ld-vector and Ld-pSCG2 were significantly less than control WT L. major FV1 (Fig. 4B “+ blood, d3” panel). However, in PpopJ flies that had expelled their blood meal, neither Ld-vector nor Ld-pSCG2 survived (0% infected flies), whereas good survival was seen with the WT FV1 control (100% infected, 1300 parasites/midgut; Fig. 4B “no blood, d4” panel; Table S2). When PpopJ sand flies were infected with a 4-fold higher concentration of parasites to compensate for the diminished early growth of Ld transfectants compared to WT FV1, we again observed poor survival of both ‘mono-scGal’ Ld-pSCG2 and ‘null-scGal’ Ld-vector parasites after the midgut blood meal had been expelled (Fig. 4C “no blood, d5” panel), despite massive parasite loads in midguts that retained their blood meals at day 3 post-feeding (Fig. 4C “+ blood, d3” panel). Ld-vector and Ld-pSCG2 numbers were each significantly decreased relative to control WT FV1 (>90%, p<0.001), although a higher percentage of PpopJ flies remained infected (38% of Ld-vector, 52% of Ld-pSCG2, 100% of WT FV1; Fig. 4C “no blood, d5” panel). These results are consistent with early observations regarding the ability of high concentration of promastigotes in the artificial blood meal to overcome the natural resistance of P. papatasi to infection with L. donovani [12,24,46]. Together, these data suggest that while necessary for survival and transmission of L. major in “selective” PpopJ sand flies, the ‘mono-scGal’ LPG PAMP alone is not sufficient to rescue L. donovani-SCG promastigotes in PpopJ sand flies during the critical time of blood meal expulsion.

**Discussion**

In this report we have studied the ability of parasites bearing different LPG side chain galactosylation PAMPs to interact with a "selective" sand fly Leishmania host, Phlebotomus papatasi PpopJ originating from the Jordan Valley. Previous data have shown that galactosylated LPG plays a key role in mediating L. major midgut survival and binding in this sand fly species, and that binding was mediated by the PpopJ midgut LPG receptor PpGalec [12,19,21,24]. We show here first that this scGal-LPG PAMP is more complex than originally proposed, as only parasites bearing short ‘mono-scGal’ LPG PAMPs survived expulsion of the digested blood meal in infected PpopJ sand flies. This was shown using both natural L. major isolates exhibiting a wide range of scGal-LPG PAMPs (Fig. 1) and isogenic derivatives of the normally scGal-deficient L. major strain SD engineered to express different scGal-LPG PAMPs via transfection of different SGC-encoded PG-side chain-[B1,3]galactosyltransferases (Fig. 2). Thus, like the fairy-tale character Goldilocks, PpopJ sand flies show an exquisite specificity for the “just right” ‘mono-scGal’ LPG PAMP, rejecting those scGal-LPG PAMPs that are either too short (‘null-scGal’, <0.02 avg. scGal chain length) or too long (‘oligo-scGal’ and ‘poly-scGal’, >1.9 avg. scGal chain length).

Several molecular scenarios may account for the inability of L. major promastigotes expressing ‘oligo-scGal’ or ‘poly-scGal’ LPG to survive expulsion of the blood meal in PpopJ flies. Since all PpopJ-
competent lines expressed LPG containing a high percentage of PG repeats bearing a single βGal residue (50–59%, Table S1), it seems likely that the low level of mono-galactosylated PG repeats in ‘oligo-scGal’ and ‘poly-scGal’ L. major lines (6–9%, Table S1) is not sufficient to mediate binding to PpGalec midgut receptors. An alternative, non-exclusive model considers interference by modified PG repeats decorated with long chains of poly-scGal residues, which could sterically interfere with the productive binding of the mono-galactosylated PG repeats. This latter model could be probed by testing parasites bearing LPG substitutions clustered differentially along the “backbone” of polymeric PG repeats; however, methods for engineering such parasites are not yet available.

Do scGal-LPG modifications control L. major “selectivity” in all Phlebotomus papatasi?

As noted earlier, many workers have grouped sand fly species according to their ability to support in experimental infections the survival (and, in some cases, experimental transmission) of a wide versus limited range of Leishmania species [7,8,12,16], with the former group termed “permissive” sand flies and the latter termed “selective” or “restricted.” The availability of Leishmania mutants specifically defective in LPG (through the deletion of the gene encoding the LPG-specific galactofuranosyltransferase LPG1) has shown that in general, “selective” sand fly species show a strong role for LPG in midgut survival and binding, while the “permissive” sand fly species show little LPG dependency [7,8,12,16,17,24]. Our panel of engineered and natural L. major, varying greatly in scGal-LPG modification, allowed us to compare the effects seen in a “selective” sand fly, P. papatasi Ppap from the Jordan Valley, which showed a strong preference for ‘mono-scGal’ LPG PAMPs (Figs. 1–3).

Recently, we have completed studies of more than 15 L. major isolates that reveal a range in the extent of procyctic promastigote scGal-LPG modification, with a general cline proceeding from scGal-deficient ‘null-scGal’ LPG modification in West Africa to short chain ‘mono-scGal’ modification in the Middle East to long chain ‘poly-scGal’ modification in Central Asia (Cardoso et al., in preparation). Together with the findings presented here, the stage is now set for further explorations of the role of scGal-LPG PAMPs in L. major transmission in other natural settings. Since one natural P. argentipes Parg sand fly vector in this geographic range showed differing abilities to support Leishmania growth which were dependent on scGal-LPG PAMPs (Figs. 1–3), it seems likely these may play an important role and perhaps even a driving force in the evolution of parasite/vector selectivity. For example, all Israeli L. major lines whose LPG has been characterized show ‘mono-scGal’ LPG PAMPs (V121 strain, avg. 1.1 scGal length; L380 strain, avg. 0.7 scGal length; calculated from data in [25,27]) and correspondingly, the ability of a P. papatasi Ppap colony established from wild caught flies from the Jordan Valley to support L. major midgut survival is strongly dependent on this scGal-LPG PAMP. In this respect it will be interesting to examine the properties of P. papatasi sand flies from Central Asia, including potential structural diversity in their PpGalec midgut LPG receptor, as L. major from this region typically elaborate a ‘poly-scGal’ LPG PAMP similar to that of LV39c5 (Cardoso et al., in preparation). Our work demonstrating a geographical origin-based specificity between Ppap sand fly vector and L. major strains also complements the work of Elfari et al. [51] who demonstrated evidence for genetic and biological diversity in L. major strains that correlated with geographical origin and their ability to infect only sympatric animal reservoir hosts.

“PpapJ-optimal” scGal-LPG modifications are not sufficient to confer PpapJ midgut survival to L. donovani

While expression of appropriate scGal-LPG PAMPs is necessary for the survival of L. major in the PpapJ sand fly midgut, is it sufficient? We tested this by engineering the ‘mono-scGal’ LPG PAMP into a Sudanese strain of L. donovani which normally expresses a completely unmodified LPG coat [43]. We showed by biochemical analyses and agglutination tests (Table S1, [30,31]) that the engineered scGal-LPG PAMPs in L. donovani-SCG transfectants were faithful replicas of L. major ‘mono-scGal’ LPG PAMPs synthesized by natural WT L. major FV1 and engineered SD-SCG3 transfectants, all of which exhibited robust long-term survival in PpapJ laboratory infections (Tables 1, S2). However, L. donovani-SCG lines bearing a ‘mono-scGal’ LPG surface coat remained unable to survive following expulsion of the blood meal in infected PpapJ flies (Fig. 4; Tables 1, S2, S3).

We then explored several possible mechanisms that could account for the failure of L. donovani bearing an L. major FV1 LPG “surface” to survive. First was the possibility that secretion of scGal-modified acid phosphatases (SAPs, [47,48,50]) competed for LPG-dependent midgut binding and parasite survival. While SAP-deficient L. donovani are not available, reconstruction experiments in L. major FV1 promastigotes expressing high levels of PG-modified SAPs (Fig. S2, Table S4) failed to reveal any alterations in PpapJ survival. Thus, competition by L. donovani scGal-SAP is unlikely to account for the failure of Lb-SCG promastigotes to survive in PpapJ midguts. A second reason was that the engineered ‘mono-scGal’ L. donovani were unable to withstand PpapJ midgut conditions, since early killing of L. donovani promastigotes in the P. papatasi midgut has been reported [52]. In fact, in comparison to the sympatric L. major FV1 strain, the L. donovani lines showed reduced growth in the early blood fed midgut (Fig. 4B), due either to their slower generation times, and/or their greater sensitivity to midgut digestive enzymes. Nevertheless, when the differences in the concentration of parasites present prior to blood meal excretion were overcome by initiating infection with a high dose inoculum, the L. donovani lines were still largely absent in flies that had passed their blood meals (Fig. 4C). Furthermore, L. donovani transfectants were able to survive within the midgut of P. argentipes Parg sand flies (Fig. 4A). Importantly, survival in this sand fly species cannot be attributed simply to a more permissive midgut environment, as P. argentipes restricts survival of L. donovani-SCG-Ld lines which lack LPG and other PGs, evidence of a strongly hydrolytic midgut environment [8,24]. These data argue that the inability of WT or engineered L. donovani lines to survive in PpapJ sand flies is not due to an inability to withstand the midgut environment, and the timing of the loss of infection is consistent with their failure to attach to the midgut.

Additional factors may be required to mediate Leishmania - sand fly midgut interactions

Thus, while specific scGal-LPG PAMPs are necessary for L. major persistence and midgut binding during expulsion of the blood meal in PpapJ flies, the inability of L. donovani expressing the appropriate L. major scGal-LPG PAMP to survive in the same fly strain suggests most simply that this interaction, while necessary, is not sufficient for midgut attachment. This in turn would argue that an additional parasite ligand(s) must be required, one shared in the closely related L. major strains but lacking in L. donovani, which diverged from L. major >80 million years ago [53]. In this model, generation of proper scGal-LPG PAMPs in L. major SD would be sufficient to promote survival, since L. major strains would retain this second L. major-specific interaction; but insufficient in L. donovani, where the second interaction was absent due to
evolutionary divergence or loss. In contrast to L. donovani - L. major hybrids observed by Voll et al. (relative to L. infantum; [54]) is thus predicted to result from the inheritance of both L. major-specific scGal-LPG-dependent and -independent ligands.

Whether this postulated second interaction is mediated through a second species-specific receptor for LPG, or an LPG-independent ligand such as the one proposed by Myskova et al. to control midgut binding of certain Leishmania species in "permissive" sand fly vectors [8,16], is unknown. Perhaps the PpgalI mono-scGal LPG midgut receptor PpgalI collaborates with a co-receptor, similar to the interactions of certain other pattern recognition receptors such as Toll-like receptors (TLR1/2/6) with each other or with other receptors (Dectin-1, CD14, TLR4; reviewed in [55,56]).

This putative species-specific co-receptor may be especially relevant to the interaction of L. major strains with P. duboscqi sand flies. This vector, while unable to support the survival of L. major lines completely deficient in LPG biosynthesis [8,22,44], is not sensitive to differences in L. major LPG galactosylation patterns (Fig. S1) and naturally transmits L. major strains in West Africa bearing effectively "null-scGal" LPG. Nonetheless, P. duboscqi is a "selective" vector, permitting only the development of L. major in experimental infections [8], Sacks et al., unpublished). These data suggest that the few interactions between predicted P. duboscqi PpgalI midgut LPG receptors [19] and the low number of mono-galactosylated PG repeats in WT SD LPG (2%), Table S1) is sufficient to mediate parasite attachment to the PdhbM midgut epithelium, in concert with a second L. major-specific midgut binding interaction that is especially strong in this particular sand fly species. It is also possible that a scGal-independent ligand present on L. major LPG binds to the alternative receptor and is a sufficient interaction to maintain infection in the PdhbM vector. When the factor(s) controlling parasite LPG-independent binding and survival of Leishmania in "selective" and "permissive" sand fly species becomes known, it should be possible to test these hypotheses.

Supporting Information

Figure S1  Survival of natural and isogenic lines of L. major in P. duboscqi, the natural vector of L. major in West Africa, is independent of scGal-LPG PAMPs. Female P. duboscqi sand flies originating from Mali (PdhbM) were fed on the indicated L. major-infected mouse blood and the number of viable parasites per midgut determined on the indicated day post-feeding, as described in Fig. 1. SD-transfectant lines are described in the text, with additional data in Fig. 2 and Table S1. Infective mouse blood contained 5x 10⁶ (panel A) or 4x 10⁵ (panel B) parasites per ml. Results from two independent experiments are shown. Found at: doi:10.1371/journal.ppat.1001185.s001 (0.32 MB TIF)

Table S1  scGal-LPG profiles of Leishmania lines used in this study.

Table S2  P. papatasi PpgalI sand fly infection outcomes after expulsion of the digested blood meal.

Table S3  Comparative outcomes in P. patatasi PpgalI and P. argentipes PargalN infections after expulsion of the digested blood meal.

Table S4  Secreted acid phosphatase (SAP) levels in Leishmania procyclic promastigote culture medium.

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Author Contributions

Conceived and designed the experiments: DED SMB DLS. Performed the experiments: DED SK PL SJT DLS. Analyzed the data: DED SJT SMB DLS. Wrote the paper: DED SMB DLS.

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