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Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG

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Abstract

Sand flies are the exclusive vectors of the protozoan parasite *Leishmania1*, but the mechanism of transmission by fly bite has not been determined nor incorporated into experimental models of infection. In sand flies with mature *Leishmania* infections the anterior midgut is blocked by a gel of parasite origin, the promastigote secretory gel (PSG)2,3. Here, we analyse for the first time the inocula from *Leishmania mexicana* infected *Lutzomyia longipalpis* sand flies. This revealed the size of the infectious dose, the underlying mechanism of parasite delivery by regurgitation, and the novel contribution made to infection by filamentous proteophosphoglycan (fPPG), a component of PSG found to accompany the parasites during transmission. Collectively, these results have important implications for understanding the relationship between parasite and its vector, the pathology of cutaneous leishmaniasis in humans and also the development of effective vaccines and drugs. These findings emphasise that to fully understand transmission of vector-borne diseases the interaction between all three participants must be considered.

Leishmaniasis is a parasitic disease that currently infects some 12 million people worldwide, causing severe morbidity and mortality4. Infection is initiated by distinct life cycle stages, metacyclic promastigotes, that are introduced into the skin by fly bite along with sand fly saliva5-7. *Leishmania* are known to express various “virulence factors” in the sand fly, which may facilitate transmission to and infection of the mammalian host8-12. However, despite these discoveries our knowledge of parasite molecules that facilitate sand fly transmission is still limited. Furthermore, a number of key issues of transmission remain unresolved, such as the true infective dose, the mechanism of parasite delivery and the biological consequences of these upon infection. Significantly, in all *Leishmania*-vector combinations examined to date a gel-like plug, the parasite-derived PSG, blocks the anterior parts of the midgut coincident with the accumulation of metacyclic promastigotes2,3. An important structural component of PSG is fPPG, an unusual mucin-like glycoprotein unique to *Leishmania*13,14. Here we address these issues regarding transmission and reveal a novel contribution made by *L. mexicana* PSG to the infection process.

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To begin to understand the nature of the infective inoculum, the number and composition of *L. mexicana* parasites delivered during transmission was determined. A membrane feeding system was adapted to collect parasites egested by infected sand flies, revealing an average of 1086 parasites delivered per bite, highly enriched in metacyclic promastigotes (86-98%) (Table 1). The only previous investigations quantitating egested parasites have been made using microcapillary forced feeding15,16. When this method was employed on *L. mexicana*-infected sand flies an average of 105 promastigotes were collected per sand fly (n=19), significantly underestimating the size of the average infective dose egested under voluntary feeding conditions. To address the origin of the average 10^3 parasites delivered during transmission, the foregut populations in sand flies were quantified. The average foregut population in pre-fed flies was 118 promastigotes (n=10), and in post-fed flies was 53 promastigotes (n=13) per sand fly. These populations are considerably smaller than the number of promastigotes that were actually egested. Therefore, the major source of egested parasites (>90%) lies behind the pharynx, thus clearly demonstrating active regurgitation of parasites from the oesophagus and behind, and not merely inoculation of the foregut population.

Next the efficiency of sand fly transmission was investigated by comparing infections caused by single fly bite with those generated by syringe inoculation. From results above, a syringe inoculum of 10^3 metacyclic promastigotes was used to simulate natural transmission, and BALB/c and CBA/Ca mice expressed non-healing and healing phenotypes, respectively (Fig 1a, 1b). However, when individual mice were infected by single fly bites, quantitatively (BALB/c) and qualitatively (CBA/Ca) different outcomes were observed. BALB/c mice exhibited greatly accelerated lesion development, these appearing 4-5 weeks earlier and rapidly increasing in size without healing (Fig 1a), while CBA/Ca mice developed non-healing chronic lesions (Fig 1b). By analyzing *Leishmania* infection using single fly bites for the first time we provide definitive evidence that delivery by sand flies makes a contribution to *Leishmania* infection beyond simple injection of parasites.

One plausible explanation for the results described above is that sand flies egest “exacerbation factors” along with metacyclic promastigotes, which assist the establishment of the parasite in the mammalian host. Such factors could be parasite and/or sand fly derived. We exploited our method to collect metacyclic promastigotes from sand flies, and investigated whether any such exacerbation factors were co-egested along with the parasites. Egestion medium, with parasites removed, or control medium were mixed with 10^3 metacyclic promastigotes and inoculated into mice. BALB/c mice showed significantly enhanced lesion development and increased parasite burden when metacyclic promastigotes were co-inoculated with egestion medium (Fig. 1c). In CBA/Ca mice the infection was both enhanced and the outcome altered by egestion medium (Fig. 1d). The lesions quickly reached a large size, decreased slightly after peaking, but did not resolve and thus behaved like those produced by sand fly bite (Fig. 1b). Again there was a significant increase in parasite burden.

This led us to consider the identity of potential exacerbation factors. Previous work has shown that co-inoculation of parasites and sand fly salivary gland homogenate by syringe can exacerbate leishmaniasis6,7, although the interpretation of these data in the context of natural transmission has been called into question1,17,18. We confirmed, as expected, the presence of saliva in the egestion medium (Supplementary Fig. 1). We also considered an additional possibility, that PSG is egested along with metacyclic promastigotes and may itself contribute to disease exacerbation. Egestion of PSG seems likely, since we have shown here that metacyclic promastigotes are regurgitated from behind the pharynx, where PSG accumulates2,3. Immunoblotting was performed on the egestion medium of infected flies

and revealed the presence of fPPG (Fig 2a), the only component of PSG detected in egestion medium. Analysis of sand fly gut homogenates showed that fPPG accumulated as the infections developed, reaching a peak on day 7 when flies were used in transmission experiments (Fig. 2b). The composition of PSG was further investigated by SDS-PAGE and immunoblotting (Fig 2c). The main component of PSG was confirmed to be fPPG. Other parasite secreted glycans of lower molecular mass were also detected in PSG together with a limited number of minor protein components. No lipophosphoglycan (LPG; 10-50 kDa) was detected in PSG or free in the lumen of infected sand fly midguts, as predicted for a parasite surface glycoconjugate that mediates attachment to the midgut9,10 (Fig 2c; Supplementary Fig. 2). Further, sand flies were infected with various *L. mexicana* null mutants selectively deficient in specific phosphoglycans: secreted acid phosphatase (sAP)19, PPG220 and LPG21. Analysis of PSG from these flies also indicated fPPG to be the dominant component (Fig. 2c). Mutants that cannot synthesise any phosphoglycans22 (*lpg2*$^{-/-}$) were unable to synthesise PSG or survive in sand flies. These data demonstrate the existence of a second potential exacerbation factor, the parasite PSG, in addition to sand fly saliva.

Comparison of the disease enhancing properties of saliva and PSG in BALB/c mice showed that saliva did not significantly exacerbate *L. mexicana* infection with 10^3 metacyclic promastigotes (Fig 3a), and the final parasite burdens were actually lower than controls. However, in CBA/Ca mice saliva did cause moderate disease exacerbation, but these lesions also resolved with time (Fig. 3b). In contrast, PSG caused substantial disease exacerbation in both BALB/c and CBA/Ca mice (Fig. 3c, 3d), and in the latter prevented healing of the lesions, which is characteristic of those generated by sand fly bite (Fig. 1b) and co-injection with egestion medium (Fig. 1d). In both mouse strains parasite burdens in PSG co-inoculated mice were higher than in their respective controls. Since both saliva and PSG are delivered by sand flies during transmission by bite, we also examined the effect of co-inoculation of both upon infection (Fig. 3e, 3f). Intriguingly, in both BALB/c and CBA/Ca mice an intermediate course of lesion development resulted, indicating that the large disease exacerbation effect of PSG and the smaller effect of saliva, rather than acting synergistically, appeared to antagonise each other. Similarly the parasite burdens in these mice were also intermediate. The antagonism between saliva and PSG presumably reflects differences in the underlying immunological mechanisms involved. However, these results demonstrate that PSG significantly increased both the pathogenicity and survival of *L. mexicana*.

Next, we considered the identity of the active ingredient(s) in PSG. First phosphoglycans (but not saliva) were adsorbed from egestion medium by binding to DEAE-Sepharose, which consequently abrogated its exacerbatory properties (Fig. 4a; Supplementary Fig. 1). Second, when PSG was ultracentrifuged the exacerbation effect segregated with the fPPG pellet fraction (Fig. 4b, 4c; Supplementary Fig. 3a). These data are consistent with the negative charge and high molecular weight of fPPG13,14. Third, PSG was fractionated by SDS-PAGE, which showed fPPG retained by the stacking gel to be the active fraction (Fig. 4d, 4e; Supplementary Fig. 3b). Fourth, PSGs from *sap1/2*$^{-/-}$, *ppg2*$^{-/-}$ and *lpg1*$^{-/-}$ *L. mexicana* null mutants19-21 were co-inoculated with metacyclic promastigotes into CBA/Ca mice and were each found to exacerbate infections equally well as wild-type PSG (Supplementary Fig. 3d-f). By eliminating naturally occurring sAP and PPG2, or the possibility of contaminative LPG molecules within PSG, the only known molecule secreted by *L. mexicana* that possesses all of these properties, and the only molecule that we could detect in egestion medium is the fPPG component of PSG. From these data we cannot completely eliminate the existence of another bioactive component that makes a minor contribution to the exacerbation effect. However, based on the evidence we conclude that the major active ingredient of PSG is fPPG.

The protein backbone of fPPG includes a repetitive serine-rich motif that is very heavily glycosylated13,14. Therefore, lastly we investigated if disease exacerbation was associated with the peptide and/or glycan moieties of fPPG. For these experiments we used wild-type and also phosphoglycan-deficient *lpg2*$^{-/-}$ parasites22. The glycans of fPPG were removed by mild acid hydrolysis and such deglycosylated fPPG completely lost its exacerbatory properties (Fig. 4f, 4g; Supplementary Fig. 3g-i). Furthermore, a chemically synthesised *Leishmania* PG23 proved to be highly effective at exacerbating *lpg2*$^{-/-}$ *L. mexicana* infections (Fig. 4h; Supplementary Fig. 3j), whilst the serine-rich repeat motif, synthesized to mimic the backbone of *L. mexicana* fPPG13,14, did not influence infections at all (Fig. 4i). Therefore, these data show that the glycan moieties of the egested fPPG were responsible for the exacerbation of infection.

Thus a new picture of the transmission of this vector-borne disease has emerged, showing how sand flies act as vectors of leishmaniasis. Proof of regurgitation as the mechanism of natural parasite delivery led to the discovery of a new component of the infective inoculum, the PSG. This can be added to the existing role that PSG plays in creating a “blocked fly” that experiences difficulty in feeding, leading to multiple and longer feeding attempts and more opportunities for transmission2,3,24. The active ingredient in PSG that led to long-term disease exacerbation was identified as fPPG, showing the advantage of secreted phosphoglycans for transmission. Thus the current report demonstrates that *L. mexicana* uses secreted fPPG to manipulate both insect vector and mammalian host. This dual role of fPPG ensures efficient transmission and proves to be a highly beneficial survival strategy. Given the selection pressures in the evolution of infectious diseases, it is not surprising that *Leishmania* has found intriguing and novel ways to secure its own transmission.

Methods

Female *Lu. longipalpis* were infected by feeding on 2×10^6 *L. mexicana* lesion amastigotes/ml rabbit blood3. *Lutzomyia longipaplis* is not the natural vector of *L. mexicana* but has been shown to support the complete development of the parasite and transmit the infection to mice by bite3. For the generation of mutant PSG flies were infected with 6×10^6 *sap1/2*$^{-/-}$20, *ppg2*$^{-/-}$21 and *lpg1*$^{-/-}$22 amastigotes/promastigotes in their first passage, to retain their infectivity. This modification was required since these mutants did not infect flies as efficiently as wild type parasites. Flies were maintained for 7-9 days to allow mature infections to develop. To obtain egested metacyclics groups of 50 *L. mexicana*-infected flies were then offered an opportunity to feed through a chick skin on promastigote culture medium3. Individual flies that alighted on the apparatus and began feeding were observed, immediately removed from the cage when they retracted their mouthparts, and kept in a separate container. After 1 hour the feeder was withdrawn and the egestion medium processed to reveal the number of egested parasites regurgitated by sand flies in 2 ml of egestion medium, which were concentrated by centrifugation, and counted. Sand flies were dissected and morphological analysis of parasites performed3. Foregut populations were regarded as parasites located anterior of the oesophagus-pharynx junction. Microcapillary forced feeding was performed as described26.

Groups of 8-10 mice were infected either by individual sand fly bite or by subcutaneous inoculation of 20 μl containing metacyclic promastigotes ± test materials (PSG, saliva, etc.) in the upper surface of the right hind foot. Sand flies with 7 day-old infections were introduced singly into a cage with an anaesthetised mouse, screened from the fly except for its right hind leg. Flies were removed from the cage when it had taken a single feeding attempt and checked by microscopy for infection. Lesion development was monitored by measuring the swelling of the foot with Vernier calipers. At the end of experiments mice

were humanely sacrificed, their feet removed and parasite burdens determined by homogenisation and counting.

Cultured metacyclic promastigotes were generated essentially as described26 except that Grace's culture medium (Invitrogen) supplemented with 10% (v/v) foetal calf serum, adjusted to pH 5.5 was used. Such *L. mexicana* cultured metacyclic promastigotes were of equal infectivity to those egested by sand flies (Supplementary Information Fig. 4), and were used in experiments where higher numbers of parasites were required.

High molecular weight PPGs were analysed using an enlarged 4% polyacrylamide stacking gel by SDS-PAGE as described13 and immunoblotting with monoclonal antibodies AP3, LT6, LT15, WIC108.3 and anti-HF-deglycosylated fPPG13,27. Infected gut homogenates were prepared as described3, centrifuged to remove cells and debris, and the supernatant mixed with an equal volume of 2x gel sample buffer. Proteophosphoglycans were concentrated by adsorption from 1 ml volumes of egestion medium by incubation with 20% (v/v) DEAE-Sepharose (Pharmacia), beads washed in PBS (10 mM sodium phosphate, 145 mM sodium chloride, pH7.2) and proteophosphoglycans eluted into 50 μl gel sample buffer.

Salivary glands were obtained by dissection from 6-8 day old female flies in PBS on cooled slides, transferred to a clean slide, followed by puncture of individual glands with fine entomological needles in 5 μl PBS. Saliva released was centrifuged and stored at −70°C. Crude whole salivary gland homogenate or sonicate was deliberately not used to avoid contamination of saliva with salivary gland epithelial cell components1. Salivary gland homogenate was found to produce significantly more disease exacerbation than saliva (Supplementary Information Fig. 5). PSG plugs were isolated from midguts of day 7 infected flies3, and PSG separated from parasites by dispersing in PBS (5 μl/plug), followed by four rounds of centrifugation (10,000g, 5 min) and retention of supernatant, and stored at −70°C. Saliva and PSG were quantified using the BCA protein assay (Pierce). Sand flies contained on average 0·97 μg saliva and 0·86 μg PSG/fly.

Filamentous PPG was fractionated from PSG by non-reducing SDS-PAGE, followed by resuspension in culture medium. Twelve PSG plugs were separated (3 plugs × 4 lanes) and the stacking gel (fPPG fraction) was removed from and processed separately to the resolving gel (other PPGs and protein fraction). Equal volumes of gel, including a control piece of acrylamide (control fraction), were washed in PBS then homogenized in 1 ml culture medium using a Teflon coated pestle until it could be injected freely using a 21-gauge needle. After centrifugation (10,000g, 5 min) the supernatants were combined with parasites to infect mice. Mild acid hydrolysis (pH2, 60°C, 1 hour) was performed as described28. Synthetic PG was synthesized as described23 and the peptide mimicking the fPPG backbone was prepared commercially (Pepsyn Ltd, Liverpool, UK). Each were used at 1μg per infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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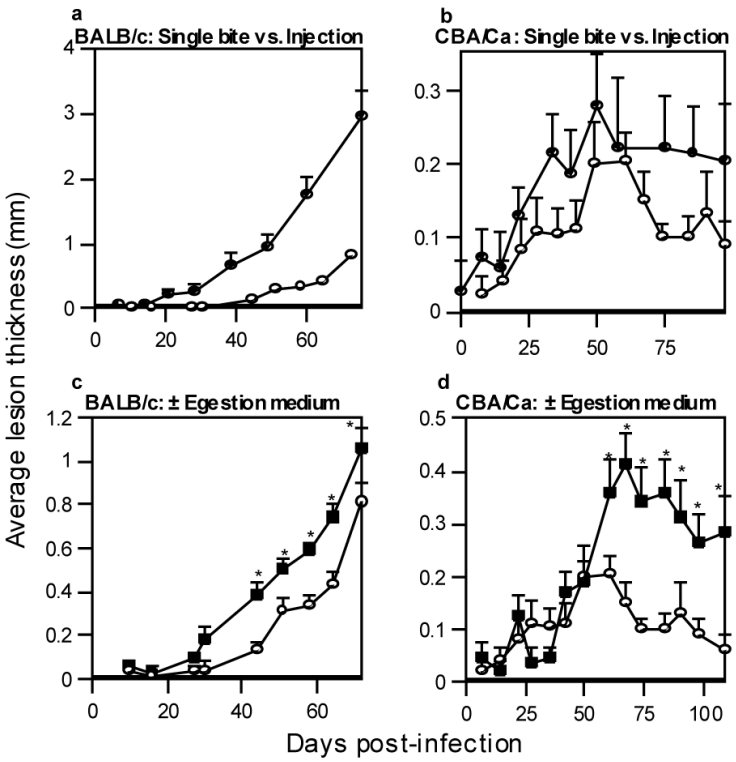

Figure 1.

Sand flies make a significant contribution to transmission and egest exacerbation factors. Comparison of lesions developing from subcutaneous syringe injections of 10^3 sand fly-egested metacyclic promastigotes (open circles) with those developing from single sand fly bites (closed circles), **a,** in BALB/c mice and **b,** in CBA/Ca mice. Data for individual fly bite in BALB/c and CBA/Ca mice were derived from 16 and 10 mice, respectively. Comparison of footpad lesions developing from injection of 10^3 sand fly-egested metacyclic promastigotes in 20 μl medium alone (control, open circles) or in 20 μl egestion medium (squares), **c,** in BALB/c mice and **d,** in CBA/Ca mice. Lesion development was monitored by subtracting the width of the uninfected foot from the contralateral, infected foot, measured with a Vernier caliper. Final parasite loads in these mice were (± s.e.m) $1{\cdot}71 \times 10^8$ (± 0·44), $2{\cdot}43 \times 10^8$ (± 0·58) and $2{\cdot}47 \times 10^8$ (± 0·65) for BALB/c mice infected by control injection, fly bite and co-inoculation with egestion medium, respectively, and $2{\cdot}0 \times 10^4$ (± 1·12), $4{\cdot}09 \times 10^6$ (± 0·62), and $5{\cdot}31 \times 10^6$ (± 0·40) for corresponding groups of CBA/Ca mice. Asterisks represent statistically significant differences using an unpaired two-tailed Student's t-test ($P < 0.05$). Error bars represent 1 s.e.m.

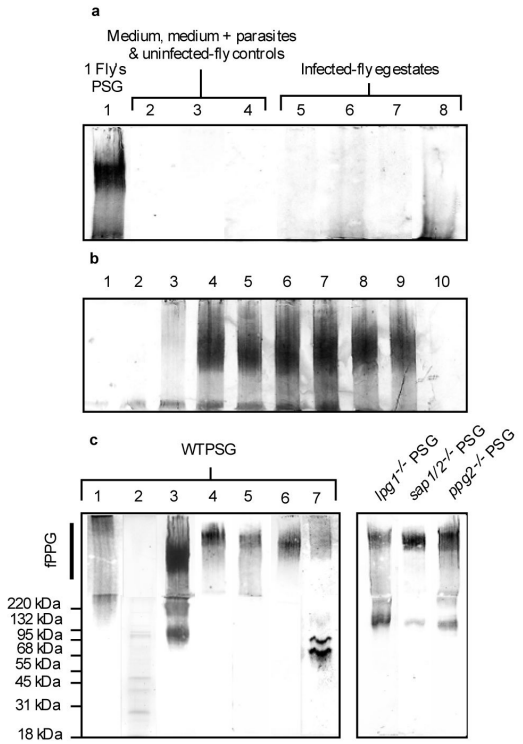

Figure 2.

The fPPG component of *Leishmania* PSG is egested by infected sand flies. **a**, Stacking gel immunoblot probed with monoclonal antibody AP327, specific for phosphoglycan terminal mannose oligosaccharide caps, of PSG (lane 1), control medium (lane 2), control medium incubated with cultured metacyclic promastigotes (lane 3), egestion medium from uninfected sand flies (lane 4), and egestion medium from infected sand flies (lanes 5-8), corresponding to the four egestion experiments in Table 1, respectively. **b**, Stacking gel immunoblot probed with AP3 of infected gut homogenates from flies sampled on day 0 (lane 1) and thereafter at daily intervals (lanes 2-9), together with a blood-fed uninfected control sample (lane 10). Each lane represents the equivalent of one sand fly. **c**, Composition of wild type PSG as revealed by Stains-All (lane 1), silver staining (lane 2), immunoblots with monoclonal antibodies raised against *Leishmania* phosphoglycans: AP3, (lane 3), LT6 (lane 4) and LT15 (lane 5), both specific for the galactose(Gal)-mannose(Man)-phosphate disaccharide repeat units, WIC108.3 (lane 6), recognizing both unsubstituted and substituted phosphosaccharides and hydroflouric acid dephosphorylated fPPG antisera (lane 7), that specifically recognizes the polypeptide backbone of fPPG13, 27. Each lane contained 1 μg of PSG, except lane 2 that contained 5 μg, and molecular mass markers are as indicated and the arrow indicated the interface between the stacking and resolving gels. Composition of PSG produced by *L. mexicana* mutants as indicated, each probed with AP3.

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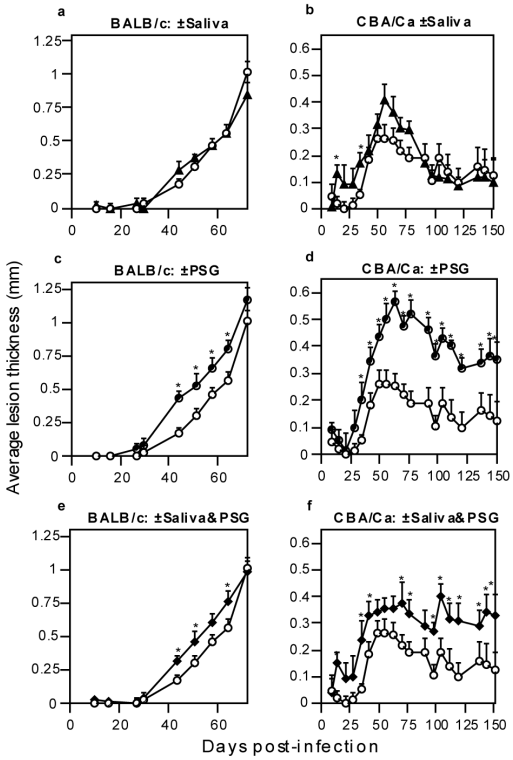

Figure 3.

PSG enhances L. mexicana disease progression more than saliva. **a, c, e,** Comparison of lesions developing from injection of 10^3 cultured metacyclic promastigotes (control, open circles), + 1 μg saliva (a, triangles), + 1 μg PSG (c, filled circles), + 1 μg saliva and 1 μg PSG (e, diamonds) in BALB/c mice. **b, d, f,** An identical set of experiments, but performed in CBA/Ca mice. Lesions were monitored as before and upon termination processed for their total parasite loads: BALB/c control 3·85 x 10^8 (± 0·49 s.e.m.); +saliva 1·81 x 10^8 (± 0·39); +PSG 4·44 x 10^8 (± 0·39); +saliva/PSG 2·69 x 10^8 (± 0·72); CBA/Ca control 4·24 x 10^5 (± 2·75); +saliva 1·13 x 10^4 (± 0·79); +PSG 1·1 x 10^6 (± 0·29); +saliva/PSG 4·09 x 10^5 (± 2·03). Asterisks represent statistically significant differences using an unpaired two-tailed t-test ($P < 0.05$). Error bars indicate 1 s.e.m.

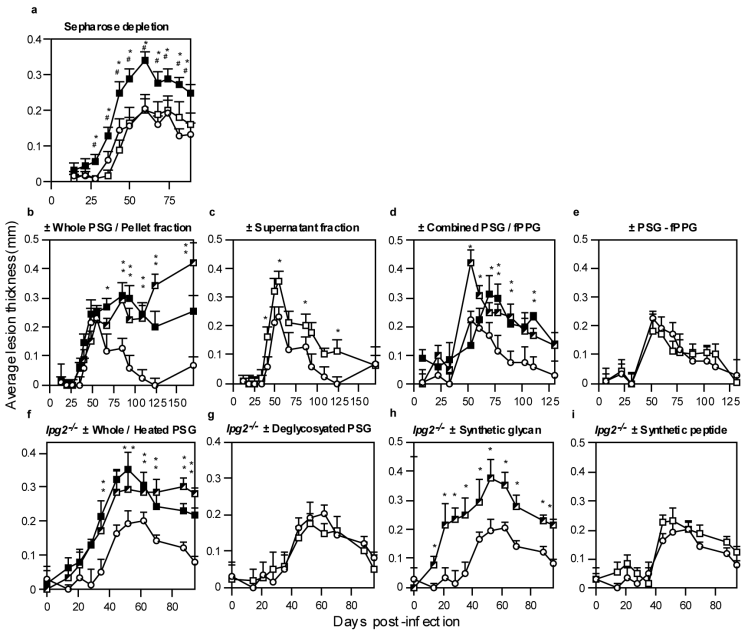

Figure 4.

The fPPG fraction of PSG is responsible for enhancement of Leishmania infectivity. a-i, Comparison of lesion development in resistant CBA/Ca mice injected with 10^3 L. mexicana metacyclic promastigotes (controls, open circles) with mice co-injected with parasites and various components of PSG. Asterisks represent statistically significant differences compared to controls, using a two-tailed t-test ($P< 0.05$). **a,** Parasites resuspended in 20 μl control medium, 20 μl egestion medium (filled squares), or 20 μl PSG-depleted egestion medium (open squares). Hash symbols indicate statistically significant differences between egestion medium and its depleted equivalent. **b,c,** After ultracentrifugation of PSG, parasites co-injected with whole PSG (filled squares), fPPG pellet (half-filled squares) (b) and supernatant fractions (open squares) (c). **d, e,** After SDS-PAGE separation, parasites co-injected with fPPG fraction (half-filled squares), all combined gel fractions (filled squares) (d) or the resolving gel components of PSG, i.e. lacking fPPG (open squares) (e). Mild acid hydrolysis (pH2, 60 0C, 1 hr)28 was used to selectively hydrolyse the acid labile phosphodiester linkages between the Gal-Man-phosphate repeat units of PSG. **f,g,** Using phosphoglycan-deficient $lpg2^{-/-}$ *L. mexicana22* , parasites co-injected with PSG (filled squares), heat-treated PSG (half-filled squares) (f),or deglycosylated PSG (open squares) (g). **h, i,** Effect of synthetic phosphoglycan23 $[Gal(\beta 1\text{-}4)Man(\alpha)\text{-}PO_3H.NH3]_{10}$ -$OH.NH_3$ (half-filled squares) (h) and synthetic peptide [APSSSSSAPSSSS] (filled squares) (i), representing the disaccharide repeat core and the protein backbone characteristic of *Leishmania* PGs and fPPG, respectively13,14. Final parasite loads reflected lesion development. Error bars indicate 1 s.e.m.

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Table 1

Egestion of metacyclic promastigotes by *Lu. longipalpis* infected with *L. mexicana*. Size of infections (± standard error)[a] were determined from a pre-feed sample of 10 flies in each experiment, and the percentage and number of metacyclic promastigotes[b] by morphological analysis. The number of promastigotes egested per fly[c] is also expressed as a percentage of the total promastigote population[a]. Similarly the number of metacyclic promastigotes egested per fly[d] is also expressed as a percentage of the total number present in the pre-feed sample[b]. Values of n for each independent experiment are the numbers of infected sand flies that delivered a single bite to the feeding apparatus.

Experiment	Size of total infection per fly[a]	Number of metacyclic promastigotes per fly[b]	Number of promastigotes egested per fly[c]	% metacyclic promastigotes in egested population	Number of metacyclic promastigotes egested per fly[d]
1 (n=9)	$6{\cdot}0 \pm 0.9 \times 10^4$	1.62×10^4 (27%)	1,015 (1.7%)	98	995 (6.1%)
2 (n=18)	$3{\cdot}6 \pm 1.77 \times 10^4$	1.26×10^4 (35%)	703 (2.0%)	91	640 (5.1%)
3 (n=13)	$4{\cdot}9 \pm 1.0 \times 10^4$	1.52×10^4 (31%)	1,376 (2.8%)	86	1183 (7.8%)
4 (n=16)	$7.5 \pm 1.02 \times 10^4$	5.15×10^4 (69%)	1,251 (1.7%)	86	1076 (2.1%)
Average	5.5×10^4	2.39×10^4 (43%)	1,086 (2.0%)	90	974 (4.1%)