**Parasites & Vectors**

RESEARCH                                                                                  Open Access

# Prostaglandin E$_2$/Leukotriene B$_4$ balance induced by *Lutzomyia longipalpis* saliva favors *Leishmania infantum* infection

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## Abstract

**Background:** Eicosanoids and sand fly saliva have a critical role in the *Leishmania* infection. Here, we evaluated the effect of *Lutzomyia longipalpis* salivary gland sonicate (SGS) on neutrophil and monocyte recruitment and activation of eicosanoid production in a murine model of inflammation.

**Methods:** C57BL/6 mice were inoculated intraperitoneally with *Lutzomyia longipalpis* SGS or *Leishmania infantum* or both, followed by analyses of cell recruitment, parasite load and eicosanoid production.

**Results:** Intraperitoneal injection of *Lutzomyia longipalpis* SGS together with *Leishmania infantum* induced an early increased parasite viability in monocytes and neutrophils. *L. longipalpis* SGS increased prostaglandin E$_2$ (PGE$_2$), but reduced leukotriene B$_4$ (LTB$_4$) production *ex vivo* in peritoneal leukocytes. In addition, the pharmacological inhibition of cyclooxygenase 2 (COX-2) with NS-398 decreased parasite viability inside macrophages during *Leishmania* infection in the presence of *L. longipalpis* SGS arguing that PGE$_2$ production is associated with diminished parasite killing.

**Conclusions:** These findings indicate that *L. longipalpis* SGS is a critical factor driving immune evasion of *Leishmania* through modulation of PGE$_2$/LTB$_4$ axis, which may represent an important mechanism on establishment of the infection.

**Keywords:** *Lutzomyia longipalpis*, *Leishmania infantum*, Saliva, Prostaglandina E$_2$, Leukotriene B$_4$

## Background

*Leishmania infantum* in America is transmitted by the bite of infected *Lutzomyia longipalpis* sand flies. Transmission of *Leishmania* sp. by hematophagous sand fly vectors occurs during blood feeding, when salivary content is inoculated with regurgitated *Leishmania* into host skin. Sand fly saliva enhances *Leishmania* infection on several experimental models [1-3] through its modulatory effects on the host immune system [4,5]. A successful blood feeding depends on the formation of a blood hemorrhagic pool [6]. In such a microenvironment there are many inflammatory cells [4], and *L. longipalpis* saliva

has been shown to enhance recruitment of different cells, including monocytes and neutrophils [7-10].

Eicosanoids display an important role during *Leishmania* infection [11-16]. In this context, there are results showing that Prostaglandin E$_2$ (PGE$_2$) production benefits parasite survival [15-18] while Leukotriene B$_4$ (LTB$_4$) is related with parasite killing by host cells [12,14,19]. In addition, sand fly saliva seems to modulate the eicosanoid production by host cells in a polarized way towards PGE$_2$ [10,11,15,20]. Maxadilan, a vasodilatory peptide present in *L. longipalpis* salivary glands, is shown to increase production of PGE$_2$ by macrophages [21]. *L. longipalpis* salivary gland sonicate (SGS) is able to modulate PGE$_2$ and LTB$_4$ release in monocytes and neutrophils recruited to the peritoneal cavity [20]. In neutrophils, SGS increases *L. infantum* infection-driven production of PGE$_2$ *in vitro* [15]. However, it remains to be addressed whether sand fly saliva

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can benefit *Leishmania* infection by control of $PGE_2/LTB_4$ axis during early steps of infection *in vivo*.

In the present study, we explore the effect of *L. longipalpis* SGS on the PGE2/LTB4 balance in the context of *L. infantum* infection *in vivo* using the peritoneal model in mice. In addition, we demonstrate that PGE2/LTB4 balance can be important for modulation of immune response elicited by SGS allowing increase in parasite viability as well as parasite burden inside leukocytes during early moments of exposure to *L. infantum*.

## Methods

### Antibodies and reagents
Schneider's insect medium, N-(1-naphthyl)-ethylenediamine and p-Aminobenzene-sulfanilamide were purchased from SIGMA (St. Louis, MO). RPMI 1640 medium and L-glutamine, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA, USA). Nutridoma-SP was from Roche (Indianapolis, In, USA). A23187 calcium ionophore was from Calbiochem Novabiochem Corp. (La Jolla, CA). NS-398, $PGE_2$ and $LTB_4$ enzyme-linked immunoassay (EIA) Kits were from Cayman Chemical (Ann Arbor, MI). Dimethylsulfoxide (DMSO) was purchased from ACROS Organics (New Jersey, NJ).

### Ethics statement
All experiments were performed in strict accordance with the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The Ethics Committee on the use of experimental animals (CEUA) of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz approved all protocols (Permit Number: 27/2008).

### Animals
Inbred male C57BL/6 mice, age 6–8 weeks, were obtained from the animal facility at Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (CPqGM-FIO-CRUZ, Bahia, Brazil). The animals were kept at a temperature of 24°C, with free access to food and water and light and dark cycles of 12 hours each.

### Parasite
*L. infantum* (MCAN/BR/89/BA262) promastigotes were cultured at 25°C in Schneider's insect medium supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stationary phase promastigotes were used in all experiments.

### Sand flies and preparation of salivary glands
Adult *Lutzomyia longipalpis* captured in Cavunge (Bahia, Brazil) were reared at the Laboratório de Imunoparasitologia/CPqGM/FIOCRUZ (Bahia, Brazil) as described previously [8]. Salivary glands were dissected

from 5- to 7-day-old *L. longipalpis* females under a stereoscopic microscope (Stemi 2000, Carls Zeiss, Jena, Germany) and stored in groups of 10 pairs in 10 μl endotoxin-free PBS at −70°C. Immediately before use, glands were sonicated (Sonifier 450, Brason, Danbury, CT) and centrifuged at $10,000 \times g$ for 4 minutes. The supernatants of salivary gland sonicate (SGS) were used for the experiments. The level of LPS contamination of SGS preparations was determined using a commercially available LAL Chromogenic Kit (QCL-1000, Lonza Bioscience) resulting in negligible levels of endotoxin in the salivary gland supernatant. All experimental procedures used SGS equivalent to 0.5 pair of salivary gland per group which possesses approximately 0.7 micrograms of proteins [22].

### Mice infection
C57BL/6 mice were submitted to intra-peritoneal (i.p.) injection with 0.1 ml of SGS (0.5 pair/cavity), 0.1 ml of *L. infantum* promastigotes from stationary phase ($3 \times 10^6$/cavity), 0.1 ml of endotoxin-free saline per cavity (negative control) or 0.1 ml of LPS (20 μg/ml; positive control-data not shown). One hour after the stimulus the total leukocytes that migrated to the peritoneal cavity was harvested by peritoneal lavage with injection of 10 ml endotoxin-free saline. Alternatively, C57BL/6 mice were previously treated with an i.p. injection of NS398 2 mg/kg or DMSO as a vehicle control. Total counts were performed on a Neubauer hemocytometer after staining with Turk's solution. Differential cell counts (200 cells total) of infected cells were carried out microscopically on cytospin preparations stained with Diff-Quick.

### Assessment of intracellular load of *L. infantum*
Intracellular load of *L. infantum* was estimated by production of proliferating extracellular motile promastigotes in Schneider medium [23]. Briefly, after 1 h of infection, peritoneal cells were centrifuged, supernatants containing non-internalized promastigotes were removed and medium was replaced by 250 μl of Schneider medium supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Infected cells were cultured at 25°C for additional 3 days. In the third day promastigotes in the cultures were counted in a Neubauer hemocytometer.

### Transmission electron microscopy
Peritoneal cells from mice infected with *L. infantum* were centrifuged (1500 rpm, 10 min) and the pellets were resuspended and fixed in a mixture of freshly prepared aldehydes (1% paraformaldehyde and 1% glutaraldehyde) in 0.1 M phosphate buffer, pH7.4 overnight at 4°C. The cells were washed in the same buffer and embedded in molten 2% agar (Merk). Agar pellets containing the cells were post-fixed in a mixture of 1% phosphate-buffered osmium
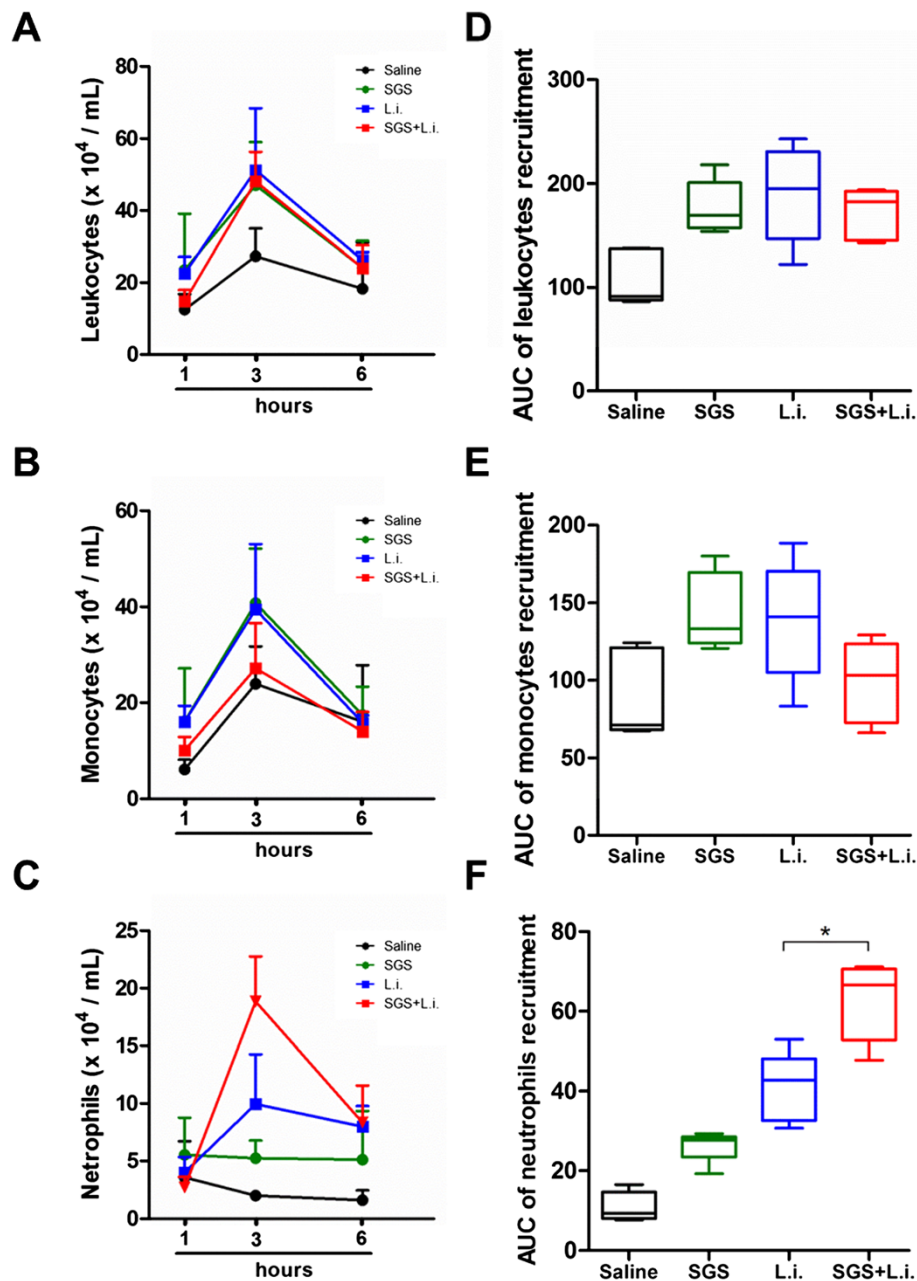
**Figure 1 Leukocyte recruitment in response to *L. longipalpis* SGS during *L. infantum* infection.** C57BL/6 mice were injected i.p. with saline (control), *L. infantum* and/or SGS as described in methods. One, 3 and 6 hours after stimulation, peritoneal cavities were washed and cells were harvested. Kinetics of total leucocytes **(A)**, monocytes **(B)** and neutrophils **(C)** were estimated using Diff-Quick-stained cytospin preparations. Area Under Curve (AUC) of the kinetic of total leukocytes **(D)**, monocytes **(E)** and neutrophils **(F)** recruitment. The data are the means and SEM from an experiment representative of three independent experiments. n = 5 per group. (*, p < 0.05 and Mann–Whitney test, two-tailed).

tetroxide and 1.5% potassium ferrocyanide (final concentration) for 1 h and processed for resin embedding (PolyBed 812, Polysciences, Warrington, PA). The section were mounted on uncoated 200-mesh copper grids and observed through a transmission electron microscope (EM 109; Zeiss, Germany). Electron micrographs were randomly taken at the magnifications of 7 000 – 30 000X

to study the entire cell profile. Viable parasites inside infected neutrophils and monocytes were enumerated by electron microscopy and presented as parasite viability index which is the ratio of percentage of viable parasite by the mean of parasites per infected cells. Parasites were considered dead according chromatin condensation and presence of cytoplasmic disorganization and vacuolization.
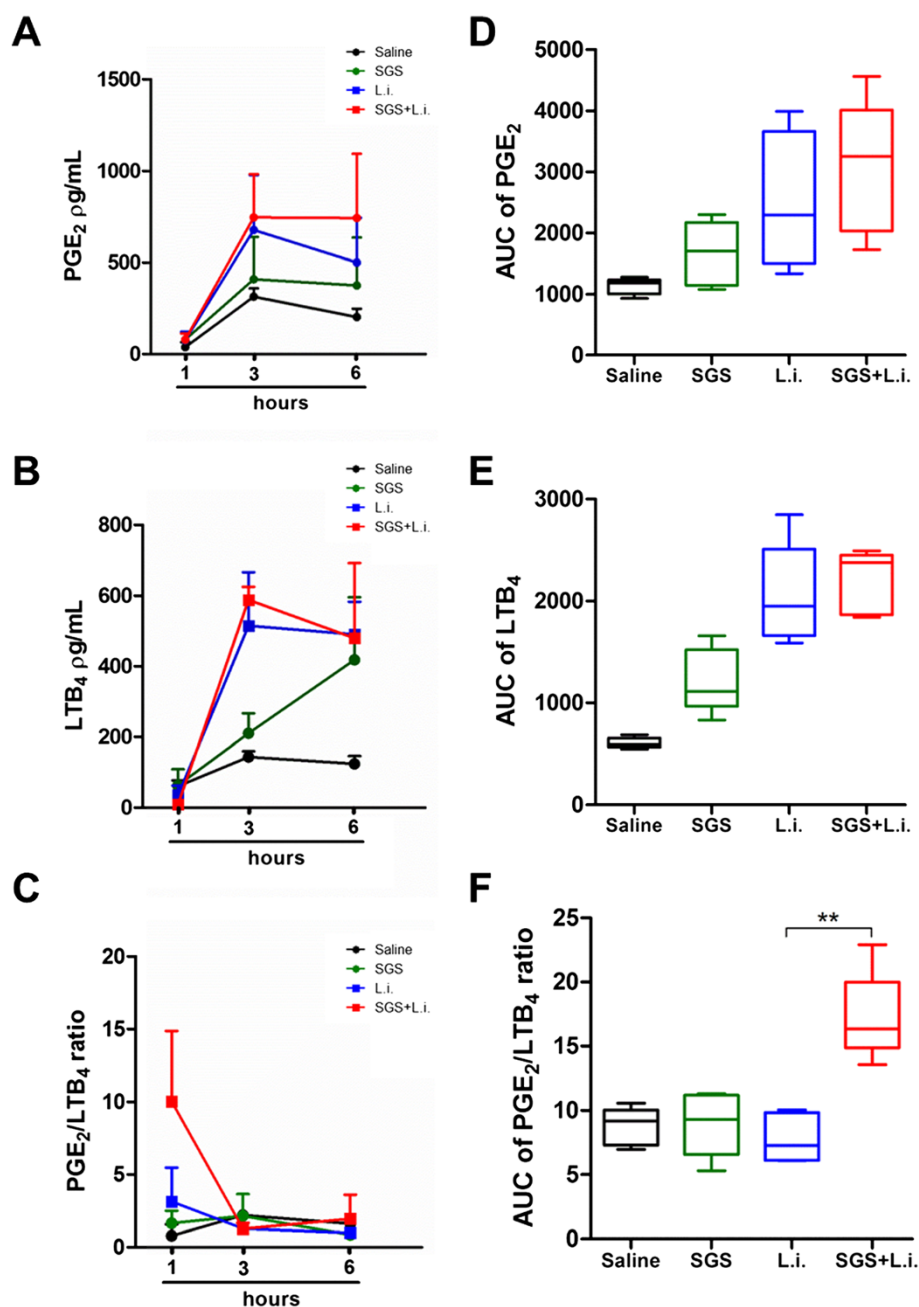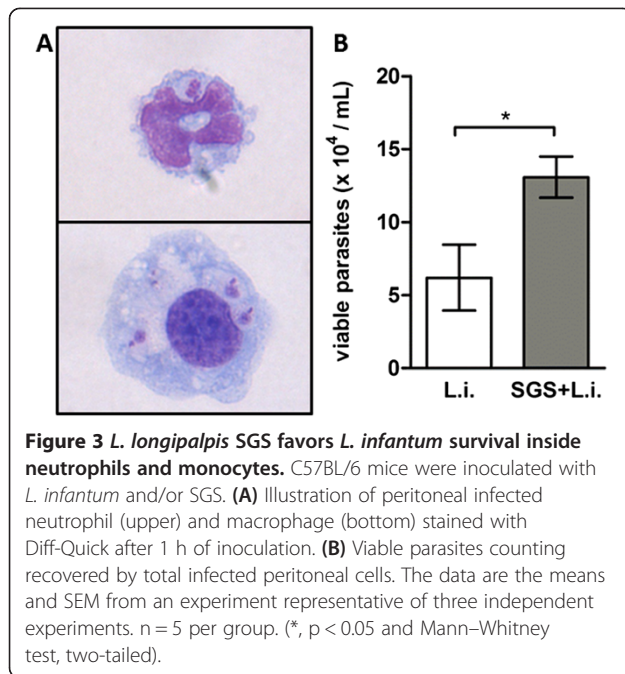
**Figure 2 PGE$_2$ and LTB$_4$ production in response to *L. longipalpis* SGS during *L. infantum* infection.** C57BL/6 mice were injected i.p. with saline (control), *L. infantum* and/or SGS. One, 3 and 6 hours after stimulation, peritoneal cavities were washed and cells were harvested. The cells were then incubated with A23187 (0.5 mM) for 15 min at 37°C to evaluate LTB$_4$ and PGE$_2$ production. The concentrations of PGE$_2$ **(A)** and LTB$_4$ **(B)** in the supernatant were measured by ELISA. **(C)** Shows the PGE$_2$/LTB$_4$ ratio. Area Under Curve (AUC) of the kinetic of PGE$_2$ **(D)**, LTB$_4$ **(E)** and PGE$_2$/LTB$_4$ ratio **(F)**. The data are the means and SEM from an experiment representative of three independent experiments. n = 5 per group. (*, p < 0.05 and Mann–Whitney test, two-tailed).

## PGE$_2$ and LTB$_4$ measurements

PGE$_2$ and LTB$_4$ levels were measured *ex vivo* from leukocytes harvested by peritoneal cavity washing with Ca$^{2+}$-Mg$^{2+}$-free HBSS. Afterwards, recovered cells (1 × 10$^6$ cells/ml) were resuspended in HBSS contained Ca$^{2+}$-Mg$^{2+}$ and then stimulated with A23187 (0.5 μM) for 15 min. Reactions were stopped on ice, and samples were centrifuged at 500 × g for 10 min at 4°C. Supernatants were collected to measure PGE$_2$ and LTB$_4$ by enzyme-linked immunoassay (EIA), according to manufacturer's instructions.
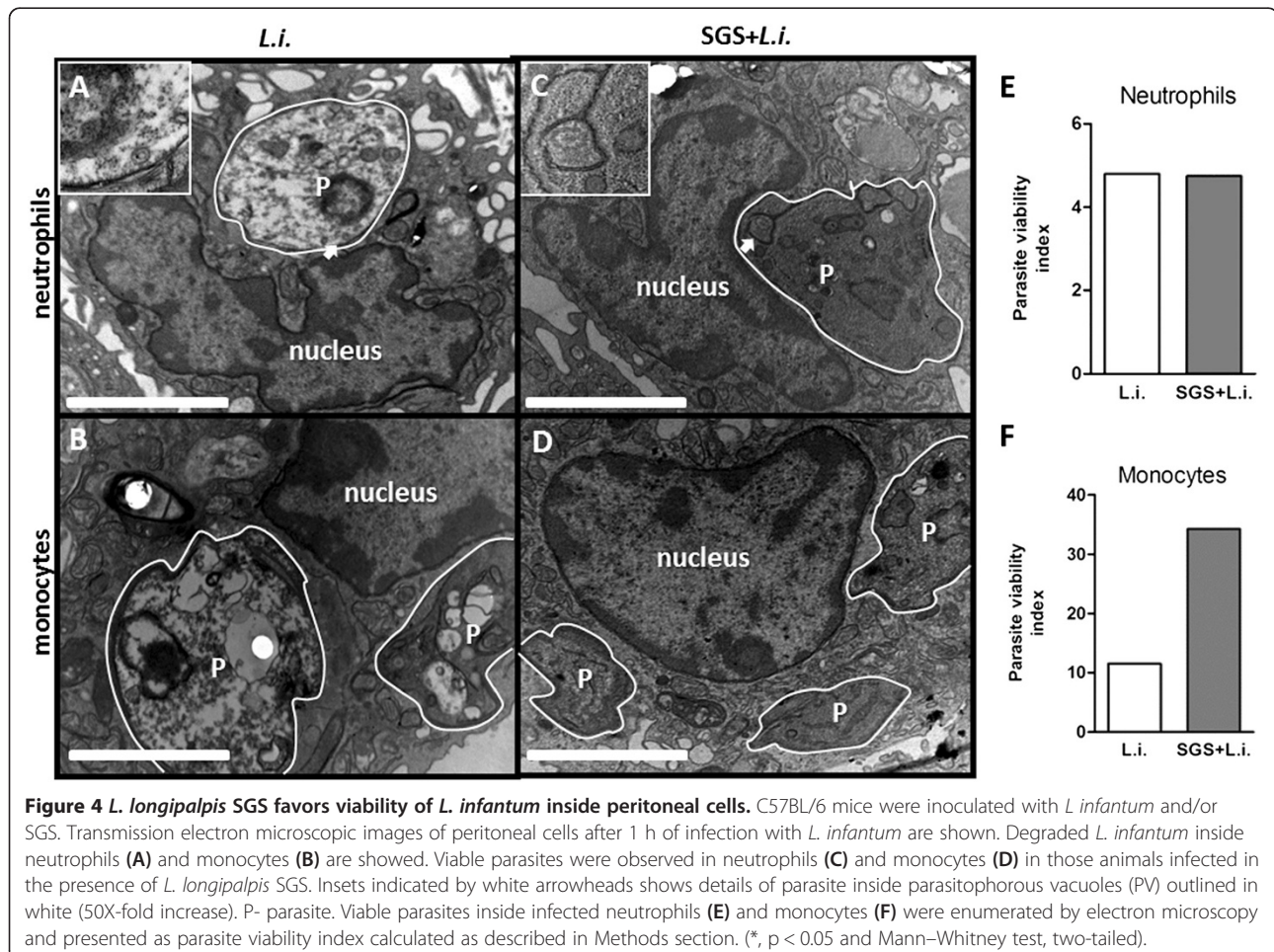
**Figure 3 *L. longipalpis* SGS favors *L. infantum* survival inside neutrophils and monocytes.** C57BL/6 mice were inoculated with *L. infantum* and/or SGS. **(A)** Illustration of peritoneal infected neutrophil (upper) and macrophage (bottom) stained with Diff-Quick after 1 h of inoculation. **(B)** Viable parasites counting recovered by total infected peritoneal cells. The data are the means and SEM from an experiment representative of three independent experiments. n = 5 per group. (*, p < 0.05 and Mann–Whitney test, two-tailed).

## Statistical analysis

The assays were performed using at least five mice per group. Each experiment was repeated at least three times. The data are presented as the mean and SEM (standard error) of representative experiments and were analyzed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Kinetic data were alternatively shown as bars graphs with area under curves calculated using GraphPad Prism 5.0 software. The comparisons between two groups were analyzed using Mann–Whitney test. The differences were considered statistically significant when p ≤ 0.05.

## Results

***L. longipalpis* SGS and *L. infantum* modulate early leucocyte recruitment and eicosanoids production *ex vivo*.**

We have previously shown that the main cell types recruited to the inoculation site by *L. longipalpis* saliva are neutrophils and monocytes [7,8,20]. Here we observed an early recruitment kinetic of total leukocytes (Figure 1A), monocytes (Figure 1B) and neutrophils (Figure 1C) in the peritoneal cavity. *L. infantum* inoculation with SGS did



**Figure 4 *L. longipalpis* SGS favors viability of *L. infantum* inside peritoneal cells.** C57BL/6 mice were inoculated with *L infantum* and/or SGS. Transmission electron microscopic images of peritoneal cells after 1 h of infection with *L. infantum* are shown. Degraded *L. infantum* inside neutrophils **(A)** and monocytes **(B)** are showed. Viable parasites were observed in neutrophils **(C)** and monocytes **(D)** in those animals infected in the presence of *L. longipalpis* SGS. Insets indicated by white arrowheads shows details of parasite inside parasitophorous vacuoles (PV) outlined in white (50X-fold increase). P- parasite. Viable parasites inside infected neutrophils **(E)** and monocytes **(F)** were enumerated by electron microscopy and presented as parasite viability index calculated as described in Methods section. (*, p < 0.05 and Mann–Whitney test, two-tailed).

not alter the number of total leukocytes (Figure 1D) and monocytes (Figure 1E), but there was a significant increase in the number of neutrophils (Figure 1F).

Different classes of eicosanoids, such as $PGE_2$ and $LTB_4$, promote both cellular recruitment and removal of inflammatory cells coordinating the initial events of inflammation [24].

We have previously demonstrated that injection of *L. longipalpis* saliva in the peritoneal cavity induced $PGE_2$ and $LTB_4$ production [20]. In the present study we investigated whether the presence of *L. infantum* alters the levels of $PGE_2$ and $LTB_4$ production induced by *L. longipalpis* saliva.

Addition of *L. longipalpis* SGS to *L. infantum* inoculation did not alter $PGE_2$ (Figure 2A,D) and $LTB_4$ (Figure 2B,E) levels significantly at the time points evaluated. Considering the fact that $PGE_2$ and $LTB_4$ exhibit antagonistic effects on inflammation and *Leishmania* infection [11,12,14-16,25], we plotted the $PGE_2/LTB_4$ ratio, following analysis of Area Under Curve (AUC) of the kinetic of these inflammatory mediators (Figure 2C,F). Injection of *L. longipalpis* salivary components plus *L. infantum* triggered high $PGE_2/LTB_4$ ratio (Figure 2F). However, this additive effect was evident only 1 hour after stimulation (Figure 2C). $PGE_2$ levels were undetectable when directly measured in the exudates (not shown).

*L. longipalpis* SGS enhances parasite viability during *L. infantum* infection *in vivo.*

Infected neutrophils and monocytes were observed in recovered cells from peritoneal cavity after 1 hour of inoculation of *L. longipalpis* SGS plus *L. infantum* (Figure 3A). The number of viable parasites from these cell cultures was increased significantly in the presence of *L. longipalpis* SGS (Figure 3B). Infected neutrophils and monocytes were not observed in later time points than 1 hour in recovered cells from the peritoneal cavity (not shown).

We have also evaluated the presence of parasites inside peritoneal neutrophils and monocytes by transmission electron microscopy (Figure 4A-D) after 1 hour post *in vivo* inoculation. Cells recovered from mice injected with *L. infantum* alone frequently presented degenerated parasite with chromatin condensation, cytoplasmic disorganization and vacuolization inside the vacuoles in neutrophils (Figure 4A) and monocytes (Figure 4B). In contrast, when *L. longipalpis* SGS was inoculated together with *Leishmania*, viable parasites were recurrently observed in both neutrophils (Figure 4C) and monocytes (Figure 4D). However, the parasite viability was increased only inside monocytes (Figure 4F), but not neutrophils (Figure 4E) as shown by analysis of electron microscopy sections (Figure 4).

Effect of *L. longipalpis* saliva on $PGE_2$ production and *L. infantum* infection *ex vivo.*

To assess if the manipulation of the eicosanoid balance driven by SGS is important to *L. infantum* infection *in vivo*, we inhibited pharmacologically the $PGE_2$ production by using a cyclooxygenase-2 (COX-2) selective inhibitor NS398. The inhibition of COX-2 decreased the number of viable parasites in peritoneal cells after *L. infantum* infection in the presence of SGS (Figure 5). Taken together, these findings suggest that the balance between $PGE_2$ and $LTB_4$ levels induced by *L. longipalpis* SGS is associated with increased *L. infantum* infection.

## Discussion

Sand fly saliva displays an important role in the first steps of *Leishmania* infection, as it induces cellular recruitment to inflammatory site, inhibits proinflammatory cytokines and deactivates dendritic cells to mobilize regulatory T cells [5]. Previous studies have shown the participation of eicosanoid in the inflammatory response triggered by sand fly saliva [9,20,21].
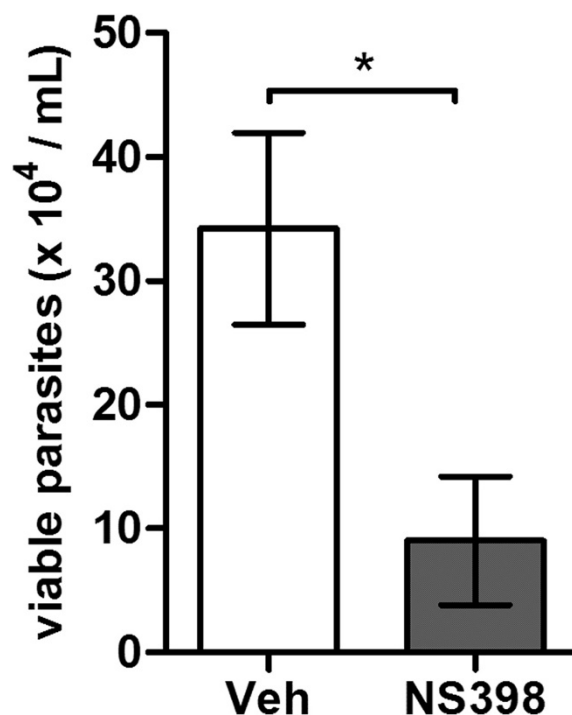


**Figure 5 Ciclooxiganese-2 inhibition affects the parasite viability *in vivo* during *L. infantum* infection in the presence of SGS.** C57BL/6 mice were treated with DMSO (vehicle – Veh) or NS398 2 mg/kg. After 1 h of treatment, mice were injected i.p. with *L. infantum* and SGS according to methods. Viable parasites counting recovered by total infected peritoneal cells after 1 h *L. infantum* or *L. infantum* plus SGS inoculation. Graph shows viable parasites counting recovered by total infected peritoneal cells. The data are the means and SEM from one experiment representative of three independent experiments. n = 5 per group. (*, p < 0.05 and Mann–Whitney test, two-tailed).

Here, we show for the first time that saliva can modulate eicosanoid profile with a balance skewed towards COX-2 driven $PGE_2$ over $LTB_4$ at early time points post *L. infantum* inoculation, possibly benefiting infection.

$PGE_2$ production favors the establishment of several pathogen infections [26]. $PGE_2$ drives *L. donovani* infection in macrophages via $PGE_2$ receptor 2 (EP2) activation [16]. In rats and mice, *Trypanosoma cruzi* infection induces $PGE_2$ by macrophages [27-29]. During *Mycobacterium bovis* infection, the increase of $PGE_2$ and TGF-β1 production by macrophages that phagocyte apoptotic neutrophils in the inflammatory site helps the establishment of the infection by this pathogen [30]. In addition, the interaction between human apoptotic neutrophils and macrophages also increases the number of parasite burden in *L. amazonensis* infection via $PGE_2$ and TGF-β1 production [31]. On the other hand, LTB4 has opposite effects on the inflammatory response. The production of $LTB_4$ is associated to the increase of pathogen killing [32,33]. In the context of *Leishmania* infection, $LTB_4$ is involved in nitric oxide production and consequently reduction of parasite burden in different cellular models of infection with *L. amazonensis* [12,14,19,25]. We have previously shown that *L. longipalpis* saliva promptly activates macrophages to produce $PGE_2$ but not $LTB_4$ *in vitro* and *ex vivo* [20]. SGS increases $PGE_2$ production by neutrophils during *L. infantum* infection [15]. Here we demonstrate that *L. longipalpis* SGS reduce the early $LTB_4$ production during *L. infantum* infection whereas it orchestrates an anti-inflammatory response by increasing $PGE_2$ production. Accordingly, in a hamster model of human VL, a role for early $PGE_2$ in *L. donovani* dissemination and impairment of NOS2 was demonstrated [34,35]. The inoculation of *L. longipalpis* SGS plus *L. infantum* increased parasite viability inside peritoneal cells. The pharmacological inhibition of COX-2 reversed the effect of SGS on enhancing parasite viability. COX-2 expression can be stimulated early dependent upon stimulus, but we do not discharge the action of NS-398 on COX-1 activity. These data strongly suggest that the presence of sand fly SGS favors an anti-inflammatory balance in the $PGE_2$/$LTB_4$ axis which could facilitate the parasite transmissibility and infection *in vivo*. Despite the lack of data about eicosanoids during later stages of *L. infantum* infection, recent publication of our group shows increased plasma levels of $PGE_2$ in patients with localized and diffuse cutaneous leishmaniasis when compared to healthy individuals [36]. Moreover the lesions of these patients present high levels of COX-2 enzyme and other enzymes associated to prostaglandin production [36], suggesting that eicosanoids balance can be important during different stages of the disease.

## Conclusion

In summary, our findings demonstrate that the eicosanoid profile induced by sand fly saliva plays an important role in the inflammatory modulation during early stages of *L. infantum* infection and point out potential implications of the PGE2/LTB4 balance in the immunopathogenesis of visceral leishmaniasis.

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