**Lutzomyia longipalpis** Saliva Triggers Lipid Body Formation and Prostaglandin E$_2$ Production in Murine Macrophages

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**Abstract**

**Background:** Sand fly saliva contains molecules that modify the host’s hemostasis and immune responses. Nevertheless, the role played by this saliva in the induction of key elements of inflammatory responses, such as lipid bodies (LB, also known as lipid droplets) and eicosanoids, has been poorly investigated. LBs are cytoplasmic organelles involved in arachidonic acid metabolism that form eicosanoids in response to inflammatory stimuli. In this study, we assessed the role of salivary gland sonicate (SGS) from *Lutzomyia* (*L.* longipalpis), a *Leishmania infantum chagasi* vector, in the induction of LBs and eicosanoid production by macrophages *in vitro* and *ex vivo*.

**Methodology/Principal Findings:** Different doses of *L. longipalpis* SGS were injected into peritoneal cavities of C57BL/6 mice. SGS induced increased macrophage and neutrophil recruitment into the peritoneal cavity at different time points. Sand fly saliva enhanced PGE$_2$ and LTBA$_4$ production by harvested peritoneal leukocytes after *ex vivo* stimulation with a calcium ionophore. At three and six hours post-injection, *L. longipalpis* SGS induced more intense LB staining in macrophages, but not in neutrophils, compared with mice injected with saline. Moreover, macrophages harvested by peritoneal lavage and stimulated with SGS *in vitro* presented a dose- and time-dependent increase in LB numbers, which was correlated with increased PGE$_2$ production. Furthermore, COX-2 and PGE-synthase co-localized within the LBs induced by *L. longipalpis* saliva. PGE$_2$ production by macrophages induced by SGS was abrogated by treatment with NS-398, a COX-2 inhibitor. Strikingly, SGS triggered ERK-1/2 and PKC-α phosphorylation, and blockage of the ERK-1/2 and PKC-α pathways inhibited the SGS effect on PGE$_2$ production by macrophages.

**Conclusion:** In sum, our results show that *L. longipalpis* saliva induces lipid body formation and PGE$_2$ production by macrophages *ex vivo* and *in vitro* via the ERK-1/2 and PKC-α signaling pathways. This study provides new insights regarding the pharmacological mechanisms whereby *L. longipalpis* saliva influences the early steps of the host’s inflammatory response.

**Citation:** Araújo-Santos T, Prates DB, Andrade BB, Nascimento DO, Clarêncio J, et al. (2010) *Lutzomyia longipalpis* Saliva Triggers Lipid Body Formation and Prostaglandin E$_2$ Production in Murine Macrophages. PLoS Negl Trop Dis 4(11): e873. doi:10.1371/journal.pntd.0000873

**Editor:** Jesus G. Valenzuela, National Institute of Allergy and Infectious Diseases, United States of America

**Received** June 29, 2010; **Accepted** October 6, 2010; **Published** November 2, 2010

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**Funding:** This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia (INCT) and Fundacao de Amparo a Pesquisa do Estado da Bahia (FAPESB). TAS, DBP, BBA, DON, PFE and ABC received fellowships from the CNPq. VMB, PTB, CIB, AB and MACSN are senior investigators from CNPq. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

To obtain a blood meal, sand flies locate blood by introducing their mouthparts into the vertebrate host’s skin, tearing tissues, lacerating capillaries and creating hemorrhagic pools upon which they feed. During this process, sand flies need to circumvent a number of the host’s homeostatic responses, such as activation of blood coagulation cascades, vasoconstriction, platelet aggregation and immune responses [1,2]. In this environment, sand flies evolved an array of potent pharmacologic components with redundant and synergistic activities that subvert the host’s physiological responses and favor the blood meal. Intense research using high-throughput analyses has been conducted to identify salivary factors and their biological activities. *Lutzomyia* (*L.* longipalpis), the main vector of visceral leishmaniasis in South America, has been extensively studied. During the inflammatory response, *L. longipalpis* saliva induces cellular recruitment, modulates both antibody production and the formation of immunocomplexes [3,4], regulates T cell activities and inhibits dendritic cells and macrophages, the latter being preferential host cells for
Leishmania [5,6]. There is also evidence that maxadilan, a L. longipalpis salivary protein with vasodilator properties, downregulates LPS-induced TNF-α and NO release through a mechanism dependent on PGE2 and IL-10 [7].

PGE2 is an eicosanoid derived from arachidonic acid (AA) metabolism by the enzyme cyclooxygenase (COX). Prostanoids and leukotrienes can be intensely produced by macrophages during inflammatory responses [8], and these mediators are implicated in cellular recruitment and activation. Among the eicosanoids, LTB4 induces neutrophil recruitment [9], whereas PGE2 and PGD2 attract mainly macrophages [10]. Previous studies used different experimental models to show that L. longipalpis saliva induces an influx of neutrophils [11] and macrophages [12], but neither the role of saliva in LTB4 and PGE2 release nor the involvement of these mediators in this process has been fully addressed.

Under inflammatory and infectious conditions, prostaglandins and others lipid mediators are mainly produced by cytoplasmic organelles called lipid bodies (LB) [13]. Intense research over the past few years has defined lipid bodies as dynamic cytoplasmic organelles. It has been demonstrated that lipid bodies compartmentalize enzymes involved in the biosynthesis, transport and catabolism of lipids, proteins involved in membrane and vesicular transport and proteins involved in cell signaling and inflammatory mediator production, including eicosanoid-forming enzymes, phospholipases and protein kinases. All of these molecules can be localized into lipid bodies in various cells under a range of activation conditions, suggesting a wide role for lipid bodies in the regulation of cellular lipid metabolism and signaling [13].

Herein, we evaluated the effect of L. longipalpis salivary gland sonicate (SGS) on the induction of LB formation as well as PGE2 and LTB4 production in vitro and ex vivo. Moreover, we explored the role of peritoneal macrophages in the production of these lipid mediators in response to L. longipalpis SGS in vitro. Finally, we found that the PGE2 production induced by L. longipalpis saliva is dependent on intracellular mechanisms involving the phosphorylation of signaling proteins such as PKC-α and ERK-1/2 and subsequent activation of COX-2.
injection and recovery of 10 mL of endotoxin-free saline. Total counts were performed on a Neubauer hemocytometer after staining with Turk’s solution. Differential cell counts (200 cells total) were carried out microscopically on cytospin preparations stained with Diff-Quick.

Lipid body staining and quantification

Cells harvested by peritoneal lavage 1, 3, 6 or 24 h after i.p. injection of 0.1 mL of L. longipalpis SGS (0.5 pair/cavity), endotoxin-free saline or LPS (20 μg/mL) were centrifuged at 400 x g and the lipid bodies within the leukocytes were stained with BODIPY 493/503 (5 μg/mL) according to Plotkowski et al. [15]. Samples were analyzed using a FACSort flow cytometer from Becton Dickinson Immunocytometry Systems (San Jose, CA) and by fluorescence microscopy.

Macrophages adhered to coverslips within 24-well plates were fixed with 3.7% formaldehyde and stained with osmium tetroxide as described previously [16]. The morphology of the fixed cells was observed, and lipid bodies were counted by light microscopy with a 100x objective lens in 50 consecutively scanned macrophages.

Resident peritoneal macrophage harvesting and treatments

For in vitro assays, macrophages were obtained by peritoneal lavage with cold RPMI 1640. Then, cells were centrifuged at 400 x g for 10 minutes. Macrophages (3 x 10^5/well) were cultured in 1 mL of RPMI 1640 medium supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in 24-well plates for 4 hours. Next, the macrophages were stimulated with different doses of L. longipalpis SGS (0.2, 0.3, 1.0, 1.5, 2.0 pairs/well). In some experiments, LPS (500 ng/well) was used as a positive control. One, 6, 24, 48 and 72 hours after stimuli, supernatants were collected and cells were fixed with 3.7% formaldehyde. For inhibitory assays, macrophages were pretreated for one hour with 1 μM NS-398, a COX-2 inhibitor; 20 nM BIS, a PKC inhibitor; or 50 μM PD98059, an ERK-1/2 inhibitor. Then, the cells were stimulated with SGS (1.5 pairs/well) or medium containing vehicle (DMSO) for 24 hours, and the supernatants were collected for eicosanoid measurement. Cell viability as assessed by trypan blue exclusion was always greater than 95% after the end of treatment.

Immunofluorescence for COX-2 and PGE-synthase

Resident peritoneal macrophages were cultured on coverslips in the presence of L. longipalpis SGS (1.5 pair/well) as described above. After 24 h, the cells were washed twice with 500 μL of HBSS γ/γ and immediately fixed with 500 μL of water-soluble EDAC (1% in HBSS γ/γ), used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins. After 15 min of incubation at room temperature (RT) with EDAC to promote both cell fixation and permeabilization, macrophages were then washed with HBSS γ/γ and incubated with 1 μM BODIPY 493/503 for 30 min. Then, the cover slips were washed with HBSS γ/γ and incubated with mouse anti-COX-2 (1:150) or anti-PGE-synthase (1:150) for 1 h at RT. MOPC 21 (IgG1) was used as a control. After further washes, cells were incubated with biotinylated goat anti-rabbit IgG secondary Ab, washed twice and incubated with avidin conjugated with PE for 30 min. The cover slips were then washed three times and mounted in Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA). The samples were observed by fluorescence microscopy and images were acquired using the software Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Western blotting analysis

Macrophages were treated or not with SGS (1.0 pair/well) for 40 min. Next, the cells were washed once with phosphate-buffered saline, homogenized in lysis buffer containing phosphatase inhibitors (10 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 0.5% v/v Nonindet-P40, 10% v/v glycerol, 1 mM DTT, 0.1 mM EDTA, 1 mM sodium orthovanadate, 25 mM NaF and 1 mM PMSF) and a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentrations were determined using the method of Lowry et al. [17] with BSA as the standard. Total proteins (20 μg) were then separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously [18] and transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TT) plus 5% BSA for 1 h before incubation overnight in the primary rabbit anti-mouse PKC-α and anti-ERK-1/2 (1:1,000) antibodies. After removal of the primary antibody and washing five times in TT, the membranes were incubated in the secondary antibody conjugated to peroxidase (1:10,000) for 1 h.

Figure 1. Leukocyte influx into the peritoneal cavity of C57BL/6 mice in response to L. longipalpis SGS. Mice were injected i.p. with endotoxin-free saline or SGS (0.5 pair/cavity). One (A), 3 (B) and 6 (C) hours after stimulation, cells were harvested by peritoneal lavage and differential leukocyte counts were performed on Diff-quick stained cytospin preparations. The data are the means and SEM from an experiment representative of three independent experiments. Groups were compared using Student’s t test at each time point. *, p<0.05 and ***, p<0.001.
doi:10.1371/journal.pntd.0000873.g001
Washed blots were then incubated with an ECL chemiluminescence kit (Amersham, UK). The membranes were discharted and immunoblotted again using primary rabbit anti-mouse phosphorylated-PKC-α and ERK-1/2 (1:1,000) antibodies according to the manufacturer’s instructions (Amersham, UK).

Quantification of the level of proteins in the western blotting membranes was determined by densitometry. Briefly, bands were scanned and processed using Adobe Photoshop 5.0 software (Adobe Systems Inc.), and arbitrary values for protein density were estimated. Ratios between phosphorylated and unphosphorylated proteins were obtained to calculate the difference between groups.

**PGE2 and LTB4 measurement**

C57BL/6 mice were inoculated i.p. with 0.1 mL of *L. longipalpis* SGS (0.5 pair/cavity), endotoxin-free saline or 0.1 mL of LPS (500 ng/mL). At 1, 3 and 6 h post-stimulus, leukocytes were harvested by peritoneal washing with HBSS/2 and 1×10⁶ cells/mL were resuspended in HBSS/2 and stimulated with A23187 (0.5 μM) for 15 min [16]. The reactions were stopped on ice, and the samples were centrifuged at 500×g for 10 min at 4°C. Supernatants from leukocytes re-stimulated *ex vivo* or those of *in vitro* assays were collected for measurement of PGE2 and LTB4 by enzyme-linked immunosassay (ELISA) according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI).

**Statistical analysis**

The *in vivo* assays were performed using at least five mice per group. Each experiment was repeated at least three times. Data are reported as the mean and standard error of representative experiments and were analyzed using GraphPad Prism 5.0 software. Disparities in leukocyte recruitment, lipid bodies and lipid mediator quantification were explored using Student’s *t* test. Means from different groups from the *in vitro* assays were compared by ANOVA followed by Bonferroni’s test or a post-test for linear trends. Differences were considered statistically significant when *p* ≤ 0.05.

**Results**

**Lipid bodies and eicosanoids in leukocytes recruited by *L. longipalpis* SGS**

To measure the leucocyte recruitment induced by SGS, we injected 100 μL of saline or SGS (0.5 pair/cavity), and 1, 3 and 6 hours after injection, we enumerated total leucocytes recruited to the peritoneal cavity. Most of the cells recruited were mononuclear cells and neutrophils (Figure 1). In this context, SGS induced mononuclear cell recruitment for 3 hours (Figure 1 A and B) and neutrophil recruitment for over 6 hours (Figure 1A–C) of stimulation when compared with the saline group. Other cell populations (eosinophils and mast cells) were not altered after SGS stimulation, and there was no variation in these numbers over time (Figure 1). The peritoneal cell population in unstimulated animals (time zero) was composed of mononuclear cells (2.985±0.027) and negligible amounts of neutrophils (0.018±0.027). At this time, macrophages are the major cells within

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**Figure 2. Kinetics of eicosanoid production in response to *L. longipalpis* SGS *ex vivo*.** C57BL/6 mice were injected i.p. with saline or SGS (0.5 pair/cavity). One, 3, 6 and 6 hours after stimulation, peritoneal cavities were washed and cells were harvested. The cells were then incubated with A23187 (0.5 μM) for 15 min at 37°C to evaluate LTB4 and PGE2 production. The concentrations of PGE2 (A) and LTB4 (B) in the supernatant were measured by ELISA. The data are the means and SEM from an experiment representative of three independent experiments. Groups were compared using Student’s *t* test at each time point. *, *p* =< 0.05.

doi:10.1371/journal.pntd.0000873.g002

**Figure 3. Lipid body formation induced by SGS *in vivo*.** C57BL/6 mice were injected i.p. with saline or SGS (0.5 pair/cavity). One, 3, 6 and 24 hours after stimulation, cells were harvested from the peritoneal cavity and stained with the neutral lipid probe BODIPY 493/503. Kinetics of LB formation in mononuclear (A) and polymorphonuclear (B) cells. Mean fluorescence intensity (MFI) histograms of mononuclear (C) and polymorphonuclear (D) cell populations at the 3-hour time point. Dotted lines indicate unstained cells, full lines indicate stained cells from the saline group (empty curves) and from the SGS-treated group (filled curves). LBs in mononuclear cells stimulated with saline (E) or SGS (F) for 3 h detected by fluorescence microscopy, nuclei stained with DAPI. Groups were compared using Student’s *t* test at each time point. *, *p* =< 0.05. MO, mononuclear; PMN, polymorphonuclear.

doi:10.1371/journal.pntd.0000873.g003
**Figure 4.** Effect of *L. longipalpis* SGS on lipid body formation in peritoneal macrophages *in vitro*. Representative image of peritoneal macrophages untreated (A) or stimulated with SGS (1.5 pair/well) (B) for 24 hours. Dose-response (C) and kinetics (D) of lipid body formation induced by SGS in peritoneal macrophages. **, *p* < 0.01 and ***, *p* < 0.001 compared with unstimulated cells.

doi:10.1371/journal.pntd.0000873.g004

**Figure 5.** COX-2 and PGE-synthase co-localize within lipid bodies induced by *L. longipalpis* SGS. Peritoneal macrophages were stimulated with SGS (1.5 pair/well) for 24 hours. BODIPY probe-labeled lipid bodies were visualized as green punctuate intra-cytoplasmic inclusions (A and D). COX-2 (B) and PGE-synthase (E) were localized with anti-COX-2 and anti- PGE-synthase antibodies, respectively. Merged images show co-localization of COX-2 (C) and PGE-synthase (F) within lipid bodies.

doi:10.1371/journal.pntd.0000873.g005
L. longipalpis SGS triggers LB biogenesis in peritoneal macrophages in vitro

To assess the role of SGS in lipid body formation in resident macrophages, we stimulated these cells with different doses of SGS (0.2–2.0 pairs/well) for different time periods (1, 6, 24, 48 and 72 hours). At 24 hours post-stimulus, SGS strongly induced LB formation compared with the untreated group (Figure 4A–D). LB formation was induced in a dose-dependent manner, and the maximum of LBs per macrophage was observed at a dose of 2.0 pairs/well (Figure 4C). Because LB formation induced by SGS (1.5 pairs/well) was more evident at 24 hours (Figure 4D), we selected this time point to perform further experiments.

L. longipalpis SGS induces macrophage PGE₂ production via the COX-2 enzyme

Prostaglandins are produced by cyclooxygenases, which occur in constitutive (COX-1) and inducible (COX-2) forms [20]. We investigated the expression and subcellular localization of COX-2 within SGS-stimulated macrophages. Immunofluorescence microscopy revealed the presence of COX-2 (Figure 5A–C) and PGE-synthase (Figure 5D–F) within LBs in macrophages stimulated with SGS.

Next, we measured PGE₂ and LTB₄ production in the supernatant of macrophage cultures. SGS induced PGE₂ production starting at 1.0 pair/well (Figure 6A), whereas LTB₄ was not detectable under any conditions (data not shown). As expected, PGE₂ production by macrophages stimulated with SGS was reduced to basal levels when the cells were pre-incubated with NS-398, a COX-2 inhibitor (Figure 6B). Thus, the PGE₂ production in peritoneal macrophages induced by SGS occurs in newly formed lipid bodies and is dependent on COX-2.

SGS induces PGE₂ production via PKC-α and ERK-1/2

Multiple pathways are involved in the signaling for PGE₂ production [13]. Recently, ERK and PKC-α were shown to be involved in COX-2 activity [21]. We observed that SGS activated both ERK (Figure 7A and C) and PKC-α phosphorylation (Figure 7B and D), but it did not alter the levels of the unphosphorylated proteins. To investigate whether these kinases are involved in the induction of PGE₂ production by SGS, we pretreated macrophages with bisindolylmaleimide I (BIS I) and PD98059, PKC-α and ERK-1/2 inhibitors, respectively (Figure 8A–B). Inhibition of both enzymes completely abrogated PGE₂ production induced by SGS (Figure 8A–B). In sum, these results suggest that PKC-α and ERK-1/2 are involved in the PGE₂ production induced by SGS.

Discussion

Sand fly saliva triggers an inflammatory response characterized by cellular influx followed by hemostatic and immune mechanism suppression. Nevertheless, the role of sand fly saliva in eicosanoid production during the early steps of the innate immune response is poorly understood. In inflammatory conditions, eicosanoids are mostly produced in cytoplasmic organelles called lipid bodies (LBs), which are formed in leukocytes and other cells involved in the inflammatory and infectious responses to several stimuli [13]. Herein, we showed that L. longipalpis saliva induces lipid body formation and PGE₂ production in peritoneal macrophages ex vivo and in vitro via kinase phosphorylation and COX-2 activation.

Previous investigations have demonstrated that sand fly saliva plays an important role in cellular recruitment in multiple experimental models [3,9,11,12], including in vivo sand fly bites [22]. Herein, we confirmed previous reports that L. longipalpis SGS induces an inflammatory infiltration composed mainly of macrophages and neutrophils. Moreover, we showed that the cellular recruitment induced by L. longipalpis saliva is concomitant with PGE₂ and LTB₄ production. In this scenario, lipid mediators
could be triggering cellular recruitment. Secretion of LTB4 by resident macrophages plays an important role in neutrophil migration [23]. In addition, lipopolysaccharides induce macrophage migration via prostaglandin D2 and prostaglandin E2 [10].

Prostaglandin E2 is an abundant eicosanoid produced by inflammatory cells, and it is known to exert anti-inflammatory and vasodilator effects. PGE2 is found in *Ixodes scapularis* saliva and is also implicated in the immunomodulatory activity of tick saliva on dendritic cell and macrophage activation [24]. Furthermore, previous studies using saliva from several *Phlebotomus* species have suggested that the anti-inflammatory properties of sand fly saliva could be attributed to PGE2 and IL-10 released by dendritic cells [9,25]. In these studies, the cellular recruitment induced by OVA stimulation was abrogated by saliva from various sand fly species [9,25], which was associated with an anti-inflammatory profile dependent on the production of IL-10, IL-4 [25] and PGE2 [9].

Intriguingly, maxadilan, a vasodilator peptide with immunomodulatory activities present in *L. longipalpis* saliva, is able to induce LPS-activated macrophages to release PGE2 via COX-1, an enzyme that is constitutively active [7]. In the present study, we showed that *L. longipalpis* SGS triggers PGE2 production in resident macrophages by an inducible pathway, since this effect was completely abrogated when the cells were incubated in the presence of NS-398, a COX-2 inhibitor. Nevertheless, whether sand fly saliva contains other molecules involved in PGE2 production or pharmacological amounts of this mediator similarly to tick saliva remains unknown.

Our study is the first to establish a direct link between *L. longipalpis* saliva, eicosanoid production and lipid body formation. Under inflammatory and infectious conditions, lipid mediators are mainly produced within LBs, which compartmentalize both the substrate and the enzymatic machinery required for eicosanoid production [13]. In this regard, the enzymes COX and 5-LO have been localized to lipid bodies in various inflammatory cells by the use of multiple techniques including fluorescence microscopy [13]. Previous studies have shown that various inflammatory and infectious stimuli are able to trigger LB formation in macrophages [13,19]. Our findings demonstrate that SGS induces LB formation in macrophages in vivo and in vitro, suggesting that *L. longipalpis* saliva acts directly on these cells, but not on neutrophils. Indeed, *L. longipalpis* SGS triggered LB formation in macrophages committed to PGE2 production via COX-2 and PGE-synthase.

Data regarding the direct effects of sand fly salivary compounds on host signaling pathways cells are scarce. The extracellular signal-regulated kinases (ERKs) and protein kinase C (PKC) are among the key enzymes implicated in signaling pathways of diverse cellular responses, including eicosanoid production. The MAP kinases ERK1 and ERK2 induce activation of cPLA2, an enzyme that hydrolyzes arachidonic acid, which is metabolized to
prostaglandin H2 by COX [13]. Previous studies have demonstrated the compartmentalization of MAP kinases and ePLA2 at arachidonate-enriched lipid bodies [20,27], as well as COX-2 and PGE-synthase [16,20,29]. Herein, it is shown for the first time that L. longipalpis SGS triggers ERK-1/2 and PKC-z phosphorylation in macrophages. Other studies have shown that COX-2 activation and PGE2 production in LPS-stimulated macrophages is dependent on the phosphorylation of protein kinases such as PKC-z [21] and ERK-1/2 [30]. We showed that the PGE2 production induced by SGS is dependent on both ERK-1/2 and PKC. This association between the activation of kinases and the metabolism of eicosanoids within lipid bodies may serve to enhance rapid eicosanoid production in response to extracellular stimuli such as sand fly saliva. Of note, in addition to their role in regulating the host response to infection by modulating inflammatory mediator production, lipid bodies may also serve as rich sources of nutrients for intracellular pathogens, thus favoring intracellular pathogen replication [31,32].

In brief, the present work provides new insights into the mechanisms involved in macrophage responses to L. longipalpis saliva, including LB formation and the signaling pathways that trigger PGE2 release. Although the roles of the newly formed LBs and PGE2 induced by sand fly saliva in the pathogenesis of leishmaniasis have not yet been addressed, several studies have shown that PGE2 is essential to the infection of macrophages [33,34] and parasite dissemination after infection [35]. The induction of PGE2 production by sand fly saliva demonstrated herein can influence the initial steps of host infection by favoring less intense macrophage activation. Our group and others have been providing strong evidence that saliva components are immunogenic and have potential as markers of exposure to sand fly vectors [36–39]. Further studies are required to determine if the immunization based on components of vector saliva interferes in eicosanoid production with consequences for the host’s immune response and the transmissibility of the parasite.

Acknowledgments

We thank Dr. Manoel Barral-Netto for critical discussion of the manuscript. We also gratefully acknowledge the technical assistance of Eduvaldo Passos, Marcos Fonseca and to Dr. Clarissa M. Maya-Monteiro and Dr. Heloiza D’Avila for intellectual contributions.

Author Contributions

Conceived and designed the experiments: TAS DBP BBA DON JC PF E CIB PTB VMB. Performed the experiments: TAS DBP BBA DON [JC PFE. Analyzed the data: TAS DBP BBA DON JC PF CIB PTB VMB. Contributed reagents/materials/analysis tools: ABC MACSN JCM PTB VMB. Wrote the paper: TAS DBP BBA PTB VMB.

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