Review Article

New Insights on the Inflammatory Role of *Lutzomyia longipalpis* Saliva in Leishmaniasis

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When an haematophagous sand fly vector insect bites a vertebrate host, it introduces its mouthparts into the skin and lacerates blood vessels, forming a hemorrhagic pool which constitutes an intricate environment of cell interactions. In this scenario, the initial performance of host, parasite, and vector "authors" will heavily influence the course of *Leishmania* infection. Recent advances in vector-parasite-host interaction have elucidated "co-authors" and "new roles" not yet described. We review here the stimulatory role of *Lutzomyia longipalpis* saliva leading to inflammation and try to connect them in an early context of *Leishmania* infection.

1. Introduction

Leishmaniasis remains a serious problem in public health, endemic in 88 countries on four continents, but most of the cases occur in underdeveloped or developing countries [1]. Visceral Leishmaniasis (VL) is a progressive infection with fatal outcome in the absence of treatment. Approximately 90% of the VL cases registered in the Americas occur in Brazil and are concentrated in the Northeast region. In the New World, *Lutzomyia longipalpis* is the principal vector of *Leishmania infantum chagasi*, the agent of American Visceral Leishmaniasis [2].

The causes related to development of distinct clinical manifestations in leishmaniasis are multifactorial and reflect the complexity at the vector-pathogen-host interface [3]. Protozoan parasites of the genus *Leishmania* are the causative agents of the disease and are transmitted to the mammalian hosts by the bite of female phlebotomine sand flies during blood repast. For blood meal obtainment, sand flies introduce their mouthparts into the skin, tearing tissues, lacerating capillaries, and creating haemorrhagic pools upon which they feed [4]. The presence of sand fly saliva in the blood pool, the environment where the parasite encounters host cells, influences the development and functions of several leukocytes. In recent years, the importance of the interaction between components of sand fly saliva and host immune mechanisms in regulating infectivity and disease progression has become clearer and suggests their consequences to disease outcome in leishmaniasis [5].

The aspects involved in immune response resulting in resistance or susceptibility widely depend on the first attempt of host's innate response to contain infection that may influence on the predominance of a pattern of future host's immune adaptive response against *Leishmania*. Many

studies have been performed to understand the mechanisms leading to protection or exacerbation of the disease however; relatively few studies have investigated the role of the sand-fly-derived salivary compounds in the innate immunity. In this paper we integrate the influence of sand fly bite with current ideas regarding the role of early steps of host inflammatory response against *Leishmania*.

2. Sand Fly Saliva: A Rich Field of Study

Sand fly vectors display a rich source of salivary biological active components to acquire blood from vertebrate hosts, a task not easy due the haemostatic, inflammatory and immune responses resultant from the bite [6]. Thus, it is not unexpected that many scientists have progressively investigated several aspects of sand fly saliva, concerning its composition and the range of mammalian response to it.

Among the New World species of sand fly which are vectors of *Leishmania*, *L. longipalpis* and its salivary gland content are the best studied. One of the first components related to *L. longipalpis* salivary gland was maxadilan [7], the most potent vasodilator peptide known and one of the two phlebotomine salivary proteins more extensively studied. Maxadilan is recognized by causing typical erythema during the feeding of *L. longipalpis* [8]. Further, it was described that maxadilan is able to modulate the inflammatory response by inhibiting cytokines such as TNF-α, by inducing IL-6 production, and by stimulating hematopoiesis [9–11]. Charlab et al. (1999) reported nine full clones and two partial cDNA clones from salivary gland from *L. longipalpis* [12]. In that work, they reported for the first time a hyaluronidase activity from sand fly saliva, an activity not yet described on phlebotomine sand flies, helping the diffusion of other pharmacological substances through the skin matrix [13]. It was also described an apyrase activity on *L. longipalpis* saliva which hydrolyses ATP and ADP to AMP, functioning as a potent antiplatelet factor [12, 14]. Interestingly, a 5′-nucleotidase activity is also present in *L. longipalpis* saliva exert vasodilator and antiplatelet aggregation role by converting AMP to adenosine [12]. One of the most abundant protein found in the *L. longipalpis* saliva is the *Yellow*-related protein [12, 13, 15, 16]. Our group has demonstrated that this family of proteins are the most recognized in sera from children living in an endemic area of visceral Leishmaniasis in Brazil [17] and by normal volunteers exposed to laboratory-reared *L. longipalpis* bites [18]. Recently, Xu et al. (2011) described the structure and function of a *yellow* protein LJM 11 [19]. In this report, the authors described that yellow proteins from *L. longipalpis* saliva act as binder of proinflammatory biogenic amines such as serotonin, histamine, and catecholamines [19]. One member of the D7 family of proteins (commonly found in dipterans saliva) is present in *L. longipalpis* [12]. The exact function of this protein in sand fly saliva is still unknown. However, its role on mosquito's saliva suggests that it could act as anticoagulant or binding biogenic amines avoiding host inflammatory events [12, 15].

Herein, we present some of the most studied proteins related to *L. longipalpis* saliva. (See [6, 15, 16, 20] for more details about this topic). Although many of them have been associated with blood-feeding, their biological functions remain undefined. Nevertheless, by modulating the host haemostatic and inflammatory response, this yet unreported sand fly salivary content remains as a research challenge, acting on host immunity to *Leishmania* during transmission and establishment of infection.

3. Immune Response to *Lutzomyia longipalpis* Saliva against *Leishmania*

There are several studies contributing to a better understanding of *L. longipalpis* saliva effects on host immunity to *Leishmania* infection. A brief exposition of these major contributions in the last 10 years is shown in Figure 1.

In mice, salivary products seem to exacerbate the infection with *Leishmania* and may, in fact, be mandatory for establishment of the parasite in vertebrate hosts. It has been shown that components of *L. longipalpis* or *Phlebotomus papatasi* salivary gland lysates mixed with *Leishmania major* resulted in substantially larger lesions compared to controls [21, 22]. Our group have shown that repeated exposure of BALB/c mice to *L. longipalpis* bites leads to local inflammatory cell infiltration comprised of neutrophils, macrophages and eosinophils [23]. Total IgG and IgG1 antibodies react predominantly with three major protein bands (45, 44, and 16 kD) from insect saliva by Western blot [23]. The injection of immune serum previously incubated with salivary gland homogenate induced an early infiltration with neutrophils and macrophages, suggesting the participation of immune complexes in triggering inflammation [23].

We have shown that in endemic areas natural exposures to noninfected sand fly bites can influence the epidemiology of the disease [17, 24]. We observed that people who presented antibodies against saliva of *L. longipalpis* also showed DTH anti-*Leishmania,* suggesting that the immune response against saliva of the vector could contribute to the induction of a protective immune response against the parasite. Recently, in a prospective study this data was reinforced by Aquino et al. (2010) evaluating 1,080 children from 2 endemic areas for VL [25]. There was a simultaneous appearance of antibodies anti-saliva and an anti-*Leishmania* DTH, or a cellular response against the parasite [25], sup-porting the idea that eliciting immunity against saliva could benefit the induction of a protective response against the parasite. The anti-sand fly antibodies can serve as epidemiological marker of vector exposure in endemic areas. In fact, we demonstrated that two salivary proteins, called LJM 17 and LJM 11, were specifically recognized by humans exposed to *L. longipalpis*, but not *Lutzomyia intermedia* [26]. We also evaluated the specificity of anti-*L. longipalpis* in a panel of 1,077 serum samples and verified that LJM 17 and LJM 11 together in an ELISA assay identified the effectiveness of these proteins for the prediction of positivity against salivary gland sonicate (SGS) [27]. In experimental model using C57BL/6 mice, immunization with LJM 11 triggered DTH response and decrease the diseased burden after *L. major* infection [19].
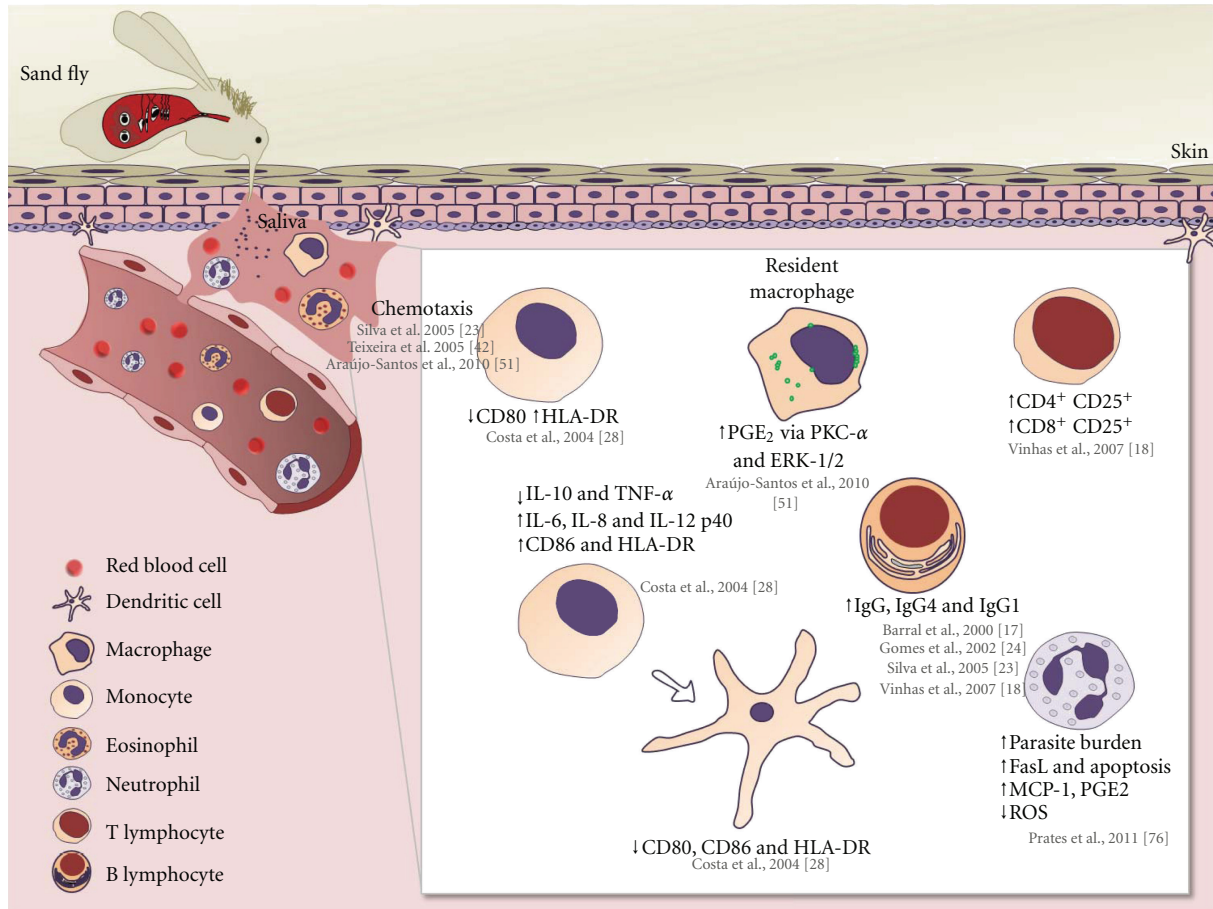
FIGURE 1: Roles of *Lutzomyia longipalpis* saliva in host immune response cell. After *L. longipalpis* saliva injection a set of events can be triggered in the host immune response. Herein, we summarized the roles of saliva on major cell populations involved in the host immune response against *Leishmania* infection.

We also characterized the immunological patterns following sand fly saliva exposure, using healthy volunteers exposed to laboratory-reared *L. longipalpis* [18]. We noticed high levels of IgG1, IgG4, and IgE antibodies anti-saliva. Furthermore, following *in vitro* stimulation with salivary gland sonicate, there was an increased frequency of CD4(+)CD25(+) and CD8(+)CD25(+) T cells as well as IFN-γ and IL-10 synthesis. Strikingly, 1 year after the first exposure, PBMC from the volunteers displayed recall IFN-γ responses that correlated with a significant reduction in infection rates using a macrophage-lymphocyte autologous culture. Together, these data suggest that human immunization against sand fly saliva is feasible and recall responses are obtained even 1 year after exposure, opening perspectives for vaccination in man [18].

Sand fly saliva also seems to exert a direct effect on human antigen presenting cells. *L. longipalpis* SGS inhibited IL-10 and TNF-α production but induced IL-6, IL-8, and IL-12p40 production by LPS-stimulated monocytes and dendritic cells [28]. Besides cytokine production, sand fly saliva also interfered with the expression of costimulatory molecules in macrophages (reduced CD80 and increased HLA-DR expression) and in monocytes (increased CD80 and

HLA-DR expression). During dendritic cell differentiation induced by CD40L, a slight reduction in CD80, CD86, HLA-DR, and CD1a expression were also observed [28].

Whereas enhancement of *Leishmania* transmission by saliva is probably due to immunomodulatory components of sand fly saliva, an explanation of the anti-*Leishmania* effect resulting from host immunization against salivary antigen is not straightforward. Immunity in this system could derive from neutralization of salivary immunomodulators such as the peptide maxadilan from *L. longipalpis* (as reviewed in [22]). Alternatively, immunity could derive from a DTH reaction at the site of the bite generated by a cellular response to salivary antigens injected by the fly [29, 30]. This particular reaction could turn the lesion and its surroundings into an inhospitable site for the establishment of *Leishmania* infection in the new host, or it could modify the environment priming the initial events of the host immune reaction to *Leishmania*.

The disease exacerbative properties of saliva, often resulting from the bioactive property of one or more of its molecules, should not be confounded with antigenic molecules in saliva that induce an adaptive immune response in the host. This acquired immunity can be either protective

or exacerbative depending on the nature and dominance of the salivary components of a vector species. Exposure to uninfected bites of the sand fly *P. papatasi* induces a strong delayed-type hypersensitivity response and IFN-γ production at the bite site that confers protection in mice challenged by *L. major*-infected flies [29]. By contrast, acquired immunity to *L. intermedia* saliva results in disease exacerbation not protection [31]. Moreover, *P. papatasi* saliva, despite its overall protective property, contains molecules that alone induce a protective (PpSP15) or exacerbative (PpSP44) im-mune response in the host [32, 33]. It is likely that *L. intermedia* saliva also contains molecules with similar profiles despite the overall exacerbative effect of total saliva.

Recently, we developed a model for visceral Leishmaniasis (VL) in hamsters, using an intradermal inoculation in the ears of 100,000 *L. chagasi* parasites together with *L. longipalpis* saliva to mimic natural transmission by sand flies [34]. Hamsters developed classical signs of VL rapidly, culminating in a fatal outcome 5-6 months postinfection. Immunization with 16 DNA plasmids coding for salivary proteins of *L. longipalpis* resulted in the identification of LJM19, a novel 11-kDa protein that protected hamsters against the fatal outcome of VL. LJM19-immunized hamsters maintained a low parasite load that correlated with an overall high IFN-γ/TGF-β ratio and inducible NOS expression in the spleen and liver up to 5 months post-infection. Importantly, a delayed-type hypersensitivity response with high expression of IFN-γ was also noted in the skin of LJM19-immunized hamsters 48 h after exposure to uninfected sand fly bites. Induction of IFN-γ at the site of bite could partly explain the protection observed in the viscera of LJM19-immunized hamsters through direct parasite killing and/or priming of anti-*Leishmania* immunity. Recently, Tavares et al. [35] showed that LJM19 was also able to protect hamsters against an infection composed by *Leishmania braziliensis* plus saliva of *L. intermedia*, the vector responsible for the transmission of this parasite in Brazil [35]. The immunization also induced a higher ratio of IFN-γ/TGF-β production in the cells from lymph nodes draining the infection site. Collin et al., (2009) immunized dogs using intradermal injections of DNA codifying salivary proteins of *L. longipalpis* (LJM17 and LJL 143), followed by injection of recombinant *Canarypox virus* containing the same genes [36]. They also observed a potential protective response against *Leishmania*, showing high concentrations of IFN-γ in PBMC stimulated with recombinant salivary proteins. Importantly, the bite of uninfected sand flies resulted in a strong DTH characterized by high amount of IFN-γ and low levels of TGF-β [36]. Together, these results point out the possibility to immunize against leishmaniasis using defined proteins of vector's saliva against *Leishmania*.

4. Early Steps of Host-Vector-*Leishmania* Interplay: Cell Recruitment Induced by Saliva

It is well established that the first steps in leishmaniasis are critical in determining the development of the disease. In order to understand this critical moment, several reports have investigated the early recruitment of cells induced by both *L. longipalpis* saliva alone or coinoculated with *L. chagasi*. Sand fly saliva is able to induce an inflammatory process in the host by recruiting different cells into the bite site. In fact, it was verified that *L. longipalpis* salivary gland lysate markedly modifies the inflammatory response to infection with *L. braziliensis* in BALB/c mice [37]. The saliva-associated lesions progressed to extensive accumulations of heavily parasitized epithelioid macrophages, with persistent neutrophilia and eosinophilia [37]. Eosinophilia has also been described in dogs intradermally inoculated with *L. longipalpis* saliva associated with *L. chagasi* promastigotes [38]. Interestingly, this inflammatory response was not observed in animals that received saliva or parasites alone [38]. The significance of this in the context of Leishmaniasis remains to be investigated. However, this phenomena is not exclusive to *L. longipalpis* saliva once eosinophils were described in the inflammatory course at the site of immunization of mice with the salivary recombinant 15-kDa protein from *P. papatasi*, the sand fly species vector of *Leishmania major* [32]. It is well established the abundant presence of eosinophils in both inflammatory site and allergic response. Activated eosinophils release lipid mediators as PAF, prostaglandins, leukotrienes, and lipoxins, as well as cytokines IL-10 and IL-8 that, in conjunct, trigger vasodilatation and leukocyte chemotaxis (reviewed in [39]). In the context of sand fly bite, this eosinophilic reaction could favor vector feeding but creates an unfriendly environment for *Leishmania* parasites.

Host cell infiltration induced by sand fly bite is the most physiologic approach to reinforce the inflammatory role of vector saliva. This event has been explored using *P. papatasi*, in which saliva-induced DTH response observed was associated to a possible fly adaptation to manipulate host immunity for the vector's own advantage [30]. Concerning *L. longipalpis* saliva, our group investigated the initial vertebrate reactions against sand fly saliva. We demonstrated that repeated exposures of BALB/c mice to *L. longipalpis* bites lead to an intense and diffuse inflammatory infiltrate characterized by neutrophils, eosinophils, and macrophages [23]. This response was observed by histological analysis of the ear dermis from exposed mice as early as 2 hours and was sustained up to 48 hours after challenge with the *L. longipalpis* salivary sonicate [23]. Moreover, the injection of immune serum previously incubated with salivary gland homogenate induced an early infiltration with neutrophils and macrophages, suggesting the participation of immune complexes in triggering inflammation [23]. An elegant and remarkable visual advance obtained by two-photon intravital imaging has recently demonstrated that the neutrophils represent the first cell population which is recruited to *Phlebotomus duboscqi* bite site [40]. Although the participation of vector salivary components had not been directly attributed to this inflammatory event by the authors, we could not discharge this possibility considering diverse data showing that saliva from different sand flies species exert chemotaxis. As neutrophils were observed on *L. longipalpis* bite site [23] the implications of its saliva on this cells will be further discussed in this paper.

In addition to *in vivo* models, cell chemotaxis induced by saliva has also been observed *in vitro*. This is of particular interest, indicating that *L. longipalpis* salivary components can act directly as inflammatory mediator. Using transwell system, Zer et al. (2001) showed the direct chemotatic effect of saliva on BALB/c peritoneal macrophages. In the same work, it was demonstrated that *L. longipalpis* saliva is able to both increase the percentage of macrophages that became infected with *Leishmania* in BALB/c and C3H/HeN mice and exacerbate the parasite load in these cells [41]. The authors discuss the possibility that, during natural transmission, saliva could reduce the promastigote exposure to the immune system by attracting host cells to the bite site and by accelerating the uptake of these parasites.

Exploring a straightforward and consistent model—the mouse air pouch—to investigate the inflammatory response induced by *L. longipalpis*, our group has described that *L. longipalpis* salivary gland sonicate was able to induce not only macrophages, but also neutrophil and eosinophil recruitment after 12 h in BALB/c [42]. The increased macrophage recruitment was linked to production of chemokine CCL2/MCP-1 and expression of its receptor CCR2 in the air pouch lining tissue. It was observed that *L. longipalpis* also synergizes with *L. chagasi* to recruit more inflammatory cells to the site of inoculation [42]. This is noteworthy because it increases the availability of "safe targets," the macrophages, for parasite evasion of the effector immune responses [43]. Interestingly, the recruitment profile observed in BALB/c was not observed in C57BL/6 mice, indicating that the same salivary components can induce diverse inflammatory effects depending on the host background [42]. However, because of limited number of cells that can be recovered on the air pouch model, some questions concerning early inflammatory events could not be investigated. Alternatively, the peritoneal cavity has been employed to this kind of study allowing the collection of high number of immigrating cells [44, 45]. In this regard, leukocyte recruitment into peritoneal cavity induced by *L. longipalpis* saliva has been evaluated in both BALB/c and C57BL/6 mouse strains [45]. In this work, significant neutrophil recruitment was observed six hours after administration of saliva, *L. major*, or saliva plus *L. major*. However, in BALB/c mice, all stimuli were able to induce more neutrophil migration than in C57BL/6 mice. Seven days later, it was observed that all stimuli were able to induce higher numbers of eosinophils and mononuclear cells in BALB/c when compared with C57BL/6 mice [45]. This study focused on the effect of saliva from *L. longipalpis* on adaptive immunity, evaluating CD4+ T lymphocyte migration and production of IL-10 and IFN-γ cytokines [45].

4.1. Inflammatory Events Triggered by L. longipalpis Saliva. Neutrophils rapidly accumulate at the inflammatory site (as reviewed in [46]) and have been described on the sand fly bite site [23, 40]. Focusing on inflammatory events triggered by *L. longipalpis* saliva using the peritoneal model, we could observe a distinct kinetic of neutrophil recruitment to the peritoneal cavity of BALB/c and C57BL/6 mice (Figure 2). A late neutrophil influx was observed in BALB/c mice (Figure 2(a)), whereas in C57BL/6 mice neutrophils were already evident in the first hours after *L. longipalpis* saliva inoculation compared to mice injected with endotoxin-free saline (Figure 2(b)).

The link between neutrophil recruitment induced by *L. longipalpis* saliva and other events which initiate and switch off the inflammatory response is an attractive field to be explored. Inflammation resolution is regulated by the release of mediators that contribute to an orchestrated sequence of events [47]. For simplicity, they result in predominance of neutrophils in the inflamed area which are later replaced by monocytes that differentiate into macrophages. During the resolution, inflammatory cells undergo apoptosis and are phagocytosed. Clearance of apoptotic cells by macrophages drives a response characterized by release of anti-inflammatory mediators [48]. Such safe removal of apoptotic cells has been implicated in exacerbation of *Leishmania* infection [49, 50]. The influence of *L. longipalpis* saliva in the time course of inflammation could be observed in cytospin preparations of the peritoneal cells from C57BL/6 mice. Neutrophils in contact with or phagocytosed by macrophages were observed at six hours (Figures 2(c) and 2(d)) and leukocyte phagocytosis by macrophages was an early event as well (Figure 2(e)). Moreover, apoptotic neutrophils were evident in C57BL/6 mice in the presence of saliva (Figure 2(f)). Therefore, components of sand fly saliva are able to both recruit and induce proapoptotic effects on neutrophils. These findings, in the scenario of anti-inflammatory clearance of apoptotic cells, add to the notion of beneficial effects of vector saliva on *Leishmania* transmission. Further work on mediators and mechanisms involved in this process is necessary.

5. Host Macrophage Response to *L. longipalpis* Saliva

Sand fly saliva displays an important role in the macrophage response by triggering the recruitment [42, 51] and suppressing the killing of parasites within macrophages [41, 52]. In this regard, *P. papatasi* saliva inhibits the NO production in macrophages treated with IFN-γ [52] and *L. longipalpis* saliva hampers *Leishmania* antigen presentation to T lymphocytes by macrophages [53] as well as upregulates the IL-10 production related with NO suppression in macrophages infected with *L. amazonensis* [54]. Moreover, pure adenosine from *P. papatasi* saliva decreases NO production in murine macrophages [55] and maxadilan peptide present in *L. longipalpis* saliva upregulates IL-6, IL-10, and TGF-β cytokine responses of LPS-activated macrophages and downregulates IL-12, TNF-α, and NO associated with *L. major* killing [56]. Despite this, few research reports cover the cellular pathways involved in sand fly saliva modulation of macrophage response. Previous study showed that maxadilan acts on PAC-1 receptor in LPS-activated macrophages and inhibits TNF-α production whereas it increases IL-6 and PGE$_2$ [11], and the authors suggest the participation of cAMP activation by maxadilan in this process.

Although the literature abounds with reports on the effects of sand fly saliva in the immune response and infection,
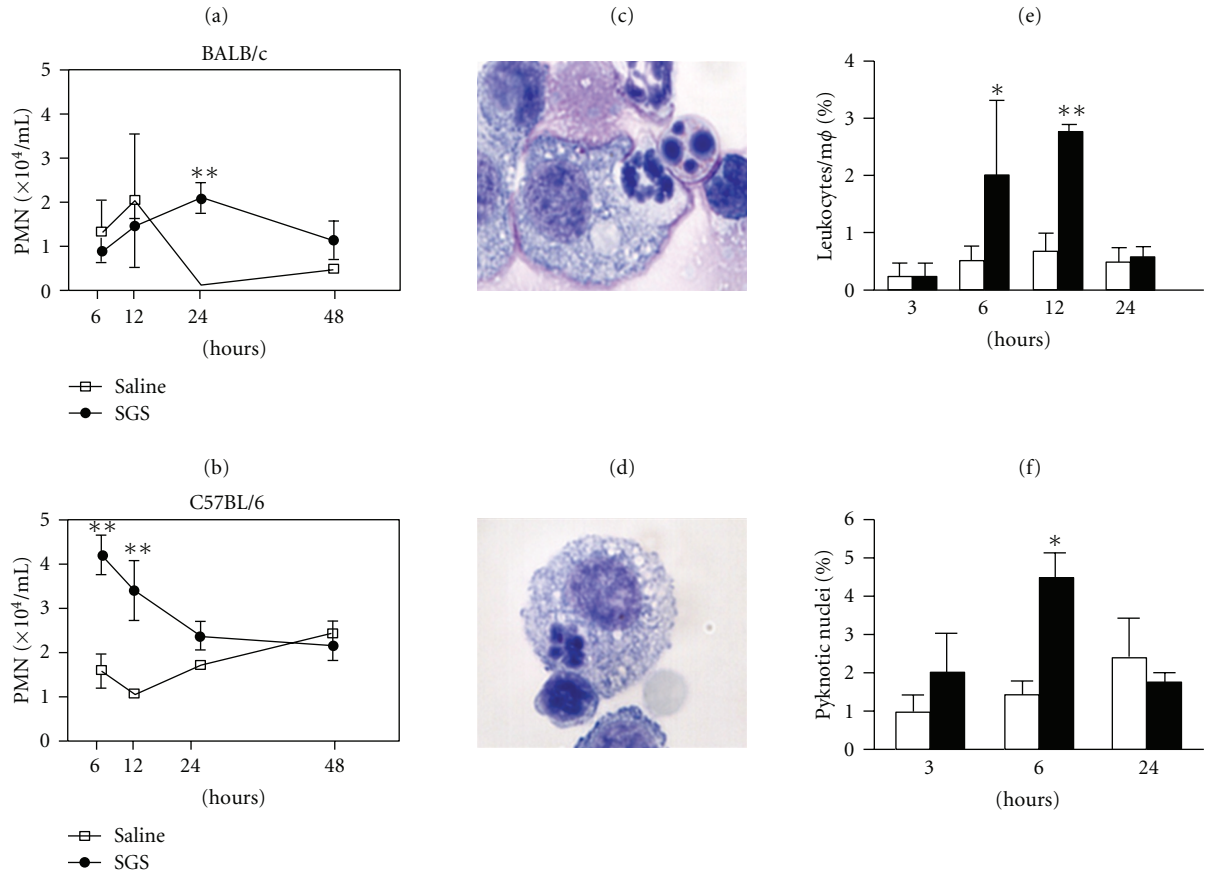
Figure 2: Neutrophil influx, apoptosis, and phagocytosis into BALB/c and C57BL/6 peritoneal cavity in response to *L. longipalpis* saliva. Mice were injected with endotoxin-free saline or *L. longipalpis* salivary gland sonicate (SGS) (0.5 pair/animal). After stimulation, peritoneal cavities were washed and differential cell counts were performed on Diff-Quik stained cytospin preparations. (a-b) Kinetics of neutrophil recruitment in BALB/c (a) and C57BL/6 (b) mice. (c-d) Representative events of C57BL/6 neutrophil phagocytosis by macrophages on Diff-Quik stained cytospin (magnification 1000x). (e-f) Phagocytosis of C57BL/6 leukocytes by macrophages (e) and neutrophil apoptosis (f) after stimulation with SGS (•) or saline (□). Data shown are from a single experiment representative of three independent experiments. Values represent means ± SEM of five mice per group. $*P < 0.05$ and $**P < 0.01$.

the effect of whole sand fly saliva on macrophages is poorly understood. Recently, we showed that *L. longipalpis* saliva activates lipid body (LB) formation in resident macrophages committed with PGE_2 production by COX-2 enzyme (Figure 3) [51]. Lipid bodies are intracellular sites related with eicosanoid production, and their formation can be triggered by activation via different intracellular pathways (as reviewed in [57]). In this context, *L. longipalpis* saliva activated ERK-1/2 and PKC phosphorylation and the inhibition of both pathways resulted in blockade of saliva-induced PGE_2 production by macrophages [51]. PGE_2 modulates the macrophage response during *Leishmania* infection in macrophages [58, 59] and is related with parasite dissemination after infection; however, the role of saliva in the PGE_2 released by macrophages during *Leishmania* infection remains to be addressed. Further studies will be necessary to clarify the importance of eicosanoids stimulated by sand fly saliva in macrophage clearance of parasites and consequently in parasite transmission after sand fly bite.

6. Neutrophils and *L. longipalpis* Saliva: A Neglected Interaction on Scenery of *Leishmania* Infection

Looking to the neutrophils as a significant host-defense cell player in both innate and adaptive response of immune system, it is surprising that few works have attempted to investigate the consequences of vector's saliva and neutrophils interaction in the pathogenesis of leishmaniasis. The reasons to encourage this special attention rise from several lines of evidence showing that neutrophils participate in *Leishmania* immunopathogenesis, by uptaking promastigote forms, producing cytokines and inflammatory mediators or interacting with macrophages enhancing infection (as reviewed in [60, 61]).

Neutrophils are considered as an initial target of *Leishmania* infection [40, 62], and they are implicated in the immunopathogenesis of murine leishmaniasis [50, 63, 64]. Moreover, significant numbers of neutrophils are present at
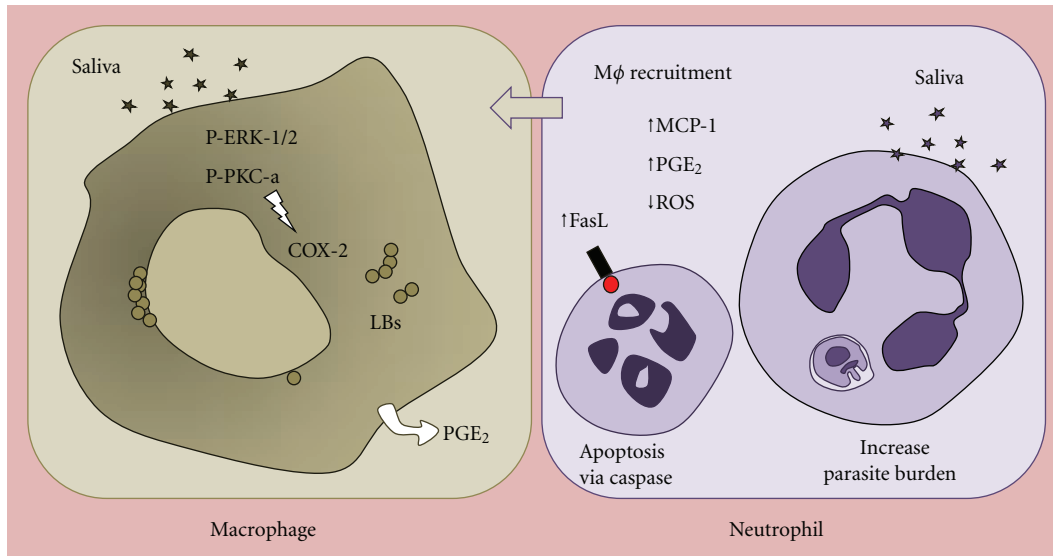
Figure 3: Effects of *Lutzomyia longipalpis* saliva on macrophage activation and neutrophil apoptosis. Macrophages and neutrophils are the first host cells to contact *Leishmania* after sand fly bite. Saliva triggers macrophages activation by lipid bodies formation committed with the PGE$_2$ production via COX-2 after phosphorilation of kinases. On the other hand, saliva induces neutrophil apoptosis by caspase and FasL activation. In addition, neutrophils activated by saliva become susceptible to *Leishmania chagasi* and release MCP-1, which is associated with macrophage recruitment. This scenario promoted by *L. longipalpis* saliva can contribute to *Leishmania* transmission in the early times of infection.

the inoculation site, lesions, and draining lymph nodes from *Leishmania*-infected mice [31, 63, 65–67]. In addition, *Leishmania* parasites undergo a silent entry into macrophages inside phagocytosed neutrophils, thus reinforcing the role of neutrophils on establishment of *Leishmania* infection [68]. *Leishmania donovani* inhibition of traffic into lysosome-derived compartments in short-lived neutrophils was suggested as a key process for the subsequent establishment of long-term parasitism [69]. On the other hand, neutrophils have also been implicated in parasite control. Phagocytosis of *L. major* by human neutrophils led to parasite killing [70]. Human neutrophils were capable to kill *L. donovani* by oxidative mechanisms [71], and, more recently, it was described the involvement of NET's (Neutrophil Extracellular Traps) on *L. amazonensis* destruction [72].

One elegant approach that reinforced the essential role for neutrophils in leishmaniasis revealed the presence of *Leishmania*-infected neutrophil on the sand fly bite site [40]. However, in that work, although the sustained neutrophil recruitment had been evident only in response to the sand fly bite, the authors did not attribute the neutrophil influx to vector salivary components. Surprisingly, besides neutrophil recruitment, there are no previous reports on further effects of sand fly saliva on neutrophil inflammatory response. Interestingly, studies performed with tick saliva disclose that the inhibition of neutrophil functions favors the initial survival of spirochetes [73–75].

Our group has recently shown the first evidence of direct effect of *L. longipalpis* salivary components on C57BL/6 mice neutrophils [76]. In summary, we described that saliva from *L. longipalpis* triggers apoptosis of inflammatory neutrophils

obtained from C57BL/6 peritoneal cavity (Figure 3). The proapoptotic effect of saliva was due to caspase activation and FasL expression on neutrophil surface. Although salivary glands from blood feeding vectors have a variety of components [76], it seems that the proapoptosis compound in *L. longipalpis* saliva is a protein. However, further work is required to elucidate which protein or proteins act in this process. Additional helpful information from this study is that preincubation of *L. longipalpis* saliva with anti-saliva antibodies abrogated neutrophil apoptosis. This allows us to propose that proapoptotic component from *L. longipalpis* saliva could be target for the host's antibodies.

Moreover, neutrophil apoptosis induced by *L. longipalpis* saliva was also increased in the presence of *L. chagasi* [76]. This is particularly interesting by reinforcing the synergistic effect of both vector component and parasite on host inflammatory response, as have been observed in cell chemotaxis [42]. Interestingly, saliva from *L. longipalpis* enhanced *L. chagasi* viability inside neutrophils. This effect was attributed to modulation of neutrophil inflammatory response [76], as treatment of neutrophils with a pan caspase inhibitor (z-VAD) and a COX-2 inhibitor (NS-398) abrogated the increased parasite burden observed. Finally, we also described a novel inflammatory function of *L. longipalpis* saliva on neutrophils, stimulating MCP-1 production, able to attract macrophages *in vitro*. Even though chemotatic activity from *L. longipalpis* saliva has been previously reported, this is the first demonstration that saliva modifies directly the neutrophil inflammatory function, inducing the release of chemotatic factors by these cells.

7. Future Directions

In this paper, we explored the new inflammatory events induced by *L. longipalpis* in the recruitment and cellular function of leukocytes, as well as the repercussion to *L. chagasi* infection. The understanding of protective mechanisms regarding the initial steps of host's response to salivary molecules that can correlate with resistance or susceptibility to *Leishmania* has been poorly explored. Further investigation should address factors that determine the success of *Leishmania* infection. Identifying new escape mechanisms used by *Leishmania* associated to the pharmacological complexity of the sand fly saliva remains a challenge. In this scenario, phylogenetic implications between vector and *Leishmania* species can result in distinct action under host cells. The insights from the inflammatory scenery approached here, as lipid body induction in macrophages and apoptotic death of neutrophils, need to be investigated during the interaction between saliva from other sand fly and *Leishmania* species. Another important point is that these inflammatory effects were detected in salivary gland extract of sand fly vector. However, recombinants proteins from *L. longipalpis* saliva that presented known immunogenic role should be tested as inducers of these inflammatory events during infection by *Leishmania* sp. The studies discussed here suggest that saliva components can act on virulence factors from parasite surface in the first steps involved the recognition, resistance to oxidative mechanisms, and modulation of inflammatory mediators' produced by host cells. However, this finding seems to be part of a "large puzzle," since they are viewed in isolation, by methodological limitations. Recent emerging imaging technologies have opened the possibility to monitor the process of *Leishmania*-host cell interaction in real time from the first moment upon sand fly bite, allowing understanding of molecular and cellular mechanisms in *Leishmania* experimental infection. These advances will enable future integrated studies that may increase understanding of immunopathogenic mechanisms induced by saliva in this intricate and fascinating interaction.

Conflict of Interests

The authors have no financial or other conflicts to declare.

Acknowledgments

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