Dual effect of *Lutzomyia longipalpis* saliva on *Leishmania braziliensis* infection is mediated by distinct saliva-induced cellular recruitment into BALB/c mice ear

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**Abstract**

**Background:** *Leishmania* parasites are transmitted to their vertebrate hosts by infected Phlebotomine sand flies during the blood meal of the flies. Sand fly saliva is known to enhance *Leishmania* spp. infection, while pre-exposure to saliva protects mice against parasitic infections. In this study, we investigated the initial inflammatory leucocyte composition induced by one or three inocula of salivary gland extract (SGE) from *Lutzomyia longipalpis* in the presence or absence of *Leishmania braziliensis*.

**Results:** We demonstrated that inoculating SGE once (SGE-1X) or three times (SGE-3X), which represented a co-inoculation or a pre-exposure to saliva, respectively, resulted in different cellular infiltrate profiles. Whereas SGE-1X led to the recruitment of all leucocytes subtypes including CD4⁺ T cells, CD4⁺CD25⁺ T cells, dendritic cells, macrophages and neutrophils, the immune cell profile in the SGE-3X group differed dramatically, as CD4⁺ T cells, CD4⁺CD25⁺ T cells, dendritic cells, macrophages and neutrophils were decreased and CD8⁺ T cells were increased. The SGE-1X group did not show differences in the ear lesion size; however, the SGE-1X group harbored a higher number of parasites. On the other hand, the SGE-3X group demonstrated a protective effect against parasitic disease, as the parasite burden was lower even in the earlier stages of the infection, a period in which the SGE-1X group presented with larger and more severe lesions. These effects were also reflected in the cytokine profiles of both groups. Whereas the SGE-1X group presented with a substantial increase in IL-10 production, the SGE-3X group showed an increase in IFN-γ production in the draining lymph nodes. Analysis of the inflammatory cell populations present within the ear lesions, the SGE-1X group showed an increase in CD4⁺FOXP3⁺ cells, whereas the CD4⁺FOXP3⁺ population was reduced in the SGE-3X group. Moreover, CD4⁺ T cells and CD8⁺ T cells producing IFN-γ were highly detected in the ears of the SGE-3X mice prior to infection. In addition, upon treatment of SGE-3X mice with anti-IFN-γ monoclonal antibody, we observed a decrease in the protective effect of SGE-3X against *L. braziliensis* infection.

**Conclusions:** These results indicate that different inocula of *Lutzomyia longipalpis* salivary gland extract can markedly modify the cellular immune response, which is reflected in the pattern of susceptibility or resistance to *Leishmania braziliensis* infection.

**Keywords:** Phlebotomines saliva, *Lutzomyia longipalpis* saliva, *Leishmania braziliensis*, Inflammatory leucocytes, Cytokines, Immunoregulation

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Background
Leishmaniasis is a vector-borne disease transmitted exclusively by sand fly bites [1], during which the host is inoculated with saliva. The saliva has been shown to downregulate the immune response allowing the establishment of successful pathogen infection [2-4]. Co-injection downregulate the immune response allowing the establishment with saliva. The saliva has been shown to downregulate the immune response allowing the establishment of successful pathogen infection [2-4]. Co-injection of Leishmania and salivary gland homogenates from either Lutzomyia longipalpis or Phlebotomus papatasi in naïve mice produces a substantial increase in lesion size and parasite burden. The increase in infectivity was associated with the capacity of the saliva to selectively inhibit antigen presentation and nitric oxide (NO) and hydrogen peroxide production thus inhibiting the ability of macrophages to kill the intracellular parasites [5,6]. Furthermore, Leishmania vector saliva inhibits the production of protective type 1 cytokines such IL-12 and IFN-γ [7-9], while enhancing the production of interleukin (IL)-10, IL-4, IL-6 and prostaglandin E (PGE)2, all of which enhance parasite survival [10-13].

Pre-exposure to saliva or bites from uninfected sand flies can lead to an increase in host resistance to Leishmania as a consequence of developing a long-term humoral immune response against the salivary components responsible for pathogen establishment [14]. However, the saliva-induced protection was associated with a delayed-type hypersensitivity (DTH) response and the upregulation of IFN-γ and IL-12 at the site of inoculation [15]. Vaccinating mice against Maxidilan (MAX), the potent salivary vasodilatador from Lutzomyia longipalpis sand fly, protected the animal from L. major infection by eliciting anti-MAX antibodies and a Th1 immune response [14]. Moreover, mice inoculated with a 15-kDa salivary protein (PpSP15) produced a strong DTH response, which even occurred in B cell knockout mice, suggesting that the cellular immune response against the saliva provided most, if not all, of the protective effect [16]. However, the mechanism responsible for the saliva-induced dual immunity observed in Leishmania infections remains unknown.

Cell recruitment is a vital event during inflammation. The cell number and cellular composition soon after an inflammatory stimulus is encountered greatly influences the future responses and the development of an adaptive immune response. Leukocyte recruitment to infected tissue is a crucial event for the control of infections such as leishmaniasis [17,18]. Furthermore, clinical leishmaniasis lesions are associated with an influx of inflammatory cells [19].

Sand fly saliva contains a mixture of pharmacologically active compounds that influence leucocyte migration. Phlebotomus duboisi saliva attracts vertebrate monocytes in vitro [20] and P. papatasi saliva attracts macrophages and enhances infections by Leishmania donovani resulting in an increased parasitic load [21]. Lutzomyia longipalpis and P. papatasi saliva recruit eosinophils and macrophages through the release of Th2 cytokines and chemokines [13,17,18]. Neutrophils are recruited to the site of Leishmania inoculation during the bite of an infected sand fly and prevent parasite surveillance via oxidoant- and protease-dependent mechanisms [22]. The co-injection of L. major with Lutzomyia longipalpis saliva increases the number of CD4+CD45RBlow T cells within the inoculation site. Undoubtedly, sand fly saliva directly influences the recruitment of leucocytes by altering the adaptive immune response. In the current study, we characterized the distinct cellular composition within BALB/c mouse ears following the inoculation of salivary gland extract (SGE) from Lutzomyia longipalpis in association with distinct patterns of resistance or susceptibility to L. braziliensis infection.

Methods
Mice
Male BALB/c mice weighing 18–22 g were housed in temperature-controlled rooms (22-25°C) with ad libitum access to water and food in the animal facility of the Department of Immunology, School of Medicine of Ribeirão Preto, University of São Paulo (Brazil). All experiments were conducted in accordance with NIH guidelines on the welfare of experimental animals, and all experiments were approved by the Ribeirão Preto School of Medicine Ethics Committee.

Salivary gland extract (SGE)
SGE was prepared from 7- to 10-day-old laboratory-bred, female Lutzomyia longipalpis as previously described [23]. Briefly, 50 pairs of salivary glands were dissected under sterile conditions in endotoxin-free PBS, placed in 50 μL of PBS and were kept at −70°C until use. Immediately before use, the glands were disrupted by sonication using a Sonifer 450 homogenizer (Branson, Danbury, Connecticut). Endotoxin levels were evaluated by using the QCL-1000(r) Chromogenic LAL Endpoint Assay kit (Lonza, Switzerland), which revealed negligible levels of endotoxin within the salivary gland supernatants.

SGE intradermal inoculation
The ear dermis of BALB/c mice was intradermally inoculated with different inoculums of SGE (SGE-1X and SGE-3X). Each inoculum consisted of 0.5 pair of SGE diluted in 10 μL of PBS /ear. SGE-1X group received one single inoculum of SGE and, other group, the mice received SGE-1X plus promastigote forms of L. braziliensis (1 × 10⁶). The protocol of immunization with saliva consisted of three inoculums of SGE, with intervals of 10 days among each ones. Alternatively, the mice received three inoculums of SGE being that, in the third one, they also received the infection with parasites. The
control group, the mice received one injection with 10 μL of PBS. Thus, the groups are: Group PBS = one injection of PBS; Group SGE-1X = one injection of SGE; Group SGE-3X = three injections of SGE; Group PBS/parasite = PBS plus parasite; Group SGE-1X/parasite = SGE-1X plus parasite; Group SGE-3X/parasite = SGE 2X + SGE-1X plus parasite.

Parasitic, intradermal infection and parasitic burden quantification

_L. braziliensis_ was cultured in Schneider (Sigma, Saint Louis, MO, USA) medium supplemented with 20% heat-inactivated fetal calf serum (Cultilab, Campinas, SP, Brazil), 4 mM NaHCO3, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Grand Island, NY, USA), and 2% v/v male human urine at 25°C. Promastigotes of _L. braziliensis_ were isolated from stationary phase cultures (5-6th day of culture), centrifuged at 1540 g at 4°C for 10 min and washed in PBS. Promastigotes of _L. braziliensis_ were isolated from stationary phase cultures (5-6th day of culture), centrifuged at 1540 x g at 4°C for 10 min and washed in PBS. The _L. braziliensis_ promastigotes (1 x 10⁶) were inoculated intradermally into the ear of mice previously inoculated with SGE (–1X or -3X) or vehicle (PBS) using a 27.5-G needle in a total volume of 10 μL. The development of lesions was monitored by measuring the diameter of the ear lesion with a vernier caliper. To quantify the parasitic burden, the dermal sheets of the infected ears were separated, deposited dermal side down, and then homogenized using a Medimachine (Becton & Dickinson Biosciences, San Diego, CA, USA) tissue grinder in a microfuge tube containing 1000 μL of supplemented Schneider medium (Sigma, Saint Louis, MO, USA) for 4 min. The tissue homogenates were serially diluted in 96-well flat-bottom microtiter plates (Corning Incorporated, NY, USA) containing biphasic medium prepared using 50 μL of NNN medium with 30% of defibrinated rabbit blood and were overlaid with 100 μL of Schneider medium (Sigma). The number of viable parasites in each tissue was determined from the highest dilution at which promastigotes could be grown after 7 days of incubation at 26°C.

Leucocyte isolation from lesions

To characterize the leucocytes within the inoculation site, the inflammatory cells were recovered as previously described [24]. Briefly, at different time points after intradermal inoculation, ears were collected and incubated at 37°C for one hour in RPMI-1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Grand Island, NY, USA) and 500 μg/ml Liberase CI (Roche, Basel, Switzerland). The tissues were processed inside Medcons using a Medimachine (both from BD Biosciences). After processing, the cells were filtered through a 50-μm filter, viability was assessed by trypan blue exclusion, and the cell concentration was determined.

Flow cytometry

The dermal inflammatory cells were gated based on their characteristic size (FSC) and granularity (SSC), and the T lymphocytes (CD4⁺CD3⁺, CD8⁺CD3⁺ and CD4⁺CD25⁺) dendritic cells (CD11c⁺CD11b⁺MHC-II⁻), macrophages (F4/80⁺CD11c⁻MHC-II⁺) and neutrophils (Gr1⁺MHC-II⁻) (BD Biosciences) were identified individually. The isotype controls used were rat IgG2b and rat IgG2a. For regulatory T cell phenotyping, CD4⁺CD25⁺ T cells were stained with anti-FoxP3 antibody conjugated to phycoeritrin (PE) (e-Biosciences). For intracellular staining, the cells were permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. For all analyses, the results were compared with the results obtained from cells stained with isotype control antibodies. Cell acquisition was performed using a FACSort flow cytometer. Data were plotted and analyzed using Cell Quest (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Cytokine release

To assess the influence of SGE on cytokine production, single-cell suspensions of the draining retromaxillary lymph nodes from the SGE-1X-, SGE-3X- or PBS-inoculated mice were prepared aseptically, diluted to a concentration of 2 x 10⁶ cells/ml, and dispensed into 48-well plates in a total volume of 500 μL of complete RPMI-1640 medium with or without 5 x 10⁵ live stationary phase _L. braziliensis_ promastigotes. Cell culture supernatants were harvested after 72 hours of culture at 37°C in 5% CO₂, and the levels of IFN-γ (BD Biosciences) and IL-10 (R&D Systems Minneapolis, MN, USA) were determined by using commercial ELISA kits, according to the manufacturer’s instructions.

In vivo depletion of IFN-γ cytokine

R46A2 hybridomas secreting rat IgG1 anti-IFN-γ were used in this study. These cells were grown as ascites in pristine (Sigma)-primed, nude-backcrossed BALB/c mice. R46A2 antibodies were purified from ascitic fluid as described elsewhere [25]. Groups of SGE-3X-inoculated BALB/c mice were given 500 μg of R46A2 mAb or normal rat IgG control antibody intraperitoneally 1 day before infection with _L. braziliensis_ (day 1) and were given 250 μg of the respective antibody every 3 days for 3 weeks thereafter.

Statistical analyses

The data are reported as the mean ± SEM and are representative of two or three independent experiments. The
means between different groups were compared by the analysis of variance (ANOVA) followed by the Tukey test for unpaired values. P<0.05 was considered to be statistically significant.

Results
Kinetics of the SGE effect in the recruitment of leucocytes to the site of inoculation in BALB/c mice
We analyzed the accumulation of leukocytes in the dermis after 0, 6, 12, 24 and 48 hours post-intradermal inoculation of *Lutzomyia longipalpis* salivary gland extract (SGE) (0.5 pair of glands/mouse) into the ears of BALB/c mice. SGE induced neutrophils (GR1⁺MHC-II⁻) cell recruitment 6 hours post-inoculation, which persisted for 48 hours. CD4⁺ T cells, CD8⁺ T cells and CD4⁺CD25⁺ T cells appeared 12 hours post-inoculation and persisted during all period analyzed. Macrophages (F4/80⁺CD11c⁻ MHC-II⁺) cell accumulation was observed 12 hours after inoculation, and dendritic cells (CD11b⁺CD11c⁻MHC-II⁺) levels did not change (Figure 1A).

To determine that the leukocyte migration is SGE-specific and not due damage inflicted by the needle injection, the kinect of leucocyte migration after similar amounts of PBS (10 μL) inoculated into ears of mice was performed. As showed, the amounts of dendritic cells, neutrophils, macrophages in PBS-inoculated mice was similar in all time points analyzed and was comparable that those recovered from naïve ears mice (Figure 1B), confirming the specificity of SGE in the leukocyte recruitment.

Inflammatory infiltrate after one or three inocula of SGE
Next, we determined whether saliva promotes or protects against leishmaniasis. First, we compared the inflammatory infiltrate after different injections of SGE. BALB/c mice received one or three intradermal ear injections of SGE, and the emigrated leucocytes were analyzed. As a control group, BALB/c mice were inoculated with PBS (time 0). Our results show that the SGE-1X group had an increased recruitment of different subtypes analyzed: CD4⁺ T cells, CD8⁺ T cells, CD4⁺CD25⁺ cells, macrophage and neutrophil (Figure 2). The leukocyte influx into the ears of mice from the SGE-3X group consisted of CD4⁺ T cells, CD4⁺CD25⁺ T cells, macrophage and neutrophil, which showed 53, 84, 71 and 53% reductions, respectively, relative to the amount of cells in the ears of mice from the SGE-1X. In contrast, the amount of CD8⁺ T cells that migrated to the ear of the SGE-3X group was 70% higher than the SGE-1X group (Figure 2B). Regarding to dendritic cells, there was no difference among all groups analyzed (Figure 2D). Therefore, pre-exposure of saliva leads to changes in the pattern of leukocyte migration to the site of inoculation.

The effect of different SGE doses on the course of *L. braziliensis* infection
Next, we evaluated whether pre-exposure to saliva interferes with the course of *L. braziliensis* infections. To this end, 1 × 10⁵ *L. braziliensis* stationary phase promastigote forms suspended in PBS or SGE were inoculated into BALB/c mice ear pretreated with PBS-2X or SGE-2X. The development of the lesion was monitored weekly by measuring the diameter of the infected ear with a vernier caliper and comparing it with the non-infected ear on the same mouse. Mice challenged with the parasite in
The presence of SGE-1X or PBS showed an increased in the lesion beginning on the 3rd week and continued to progress until the 5th week of infection (p < 0.05) (Figure 3A). After the 5th week, we observed a decrease in the ear size until the 7th week. Despite similar rates of edema in both groups (SGE-1X and PBS), mice that received SGE-1X showed higher parasite titers in the ear at the 3rd and 7th week post-infection when compared with mice inoculated with parasites in PBS (Figure 3B). Conversely, mice pretreated with saliva 2X and challenged with SGE plus parasite, referred to as SGE-3X, did not exhibit edema until the 7th week of infection. Furthermore, significantly lower numbers of parasites were detected on the 3rd and 7th week post-infection in mice that received SGE-3X when compared with mice that received parasite in SGE-1X (Figure 3B). In summary, our results are consistent with previous studies, which have shown that pre-exposure to saliva results in the protection against infection.

Furthermore, we analyzed the ability of the draining lymph node cells from SGE-1X-, SGE-3X-, or PBS-inoculated mice at the 7th week post-infection to produce IL-10 and IFN-γ in an attempt to understand the mechanism by which saliva exacerbates or protect mice against parasitic infection. Our results showed that the total lymph node cells from SGE-1X-inoculated mice produced more IL-10 after stimulation in vitro with parasitic antigen relative to mice inoculated with PBS or SGE-3X (Figure 4A). On the contrary, SGE-3X-treated mice produced significantly increased levels of IFN-γ when compared with the other groups of infected mice (Figure 4B).

The cells that migrated to the site of parasite inoculation were identified by flow cytometry. As shown in Figure 5, L. braziliensis infection induced the recruitment of T lymphocytes such as CD4+ T and CD8+ T. Likewise, both populations were detected in the ears of SGE-1X-inoculated mice. In addition, similar numbers of CD4+ T cells and CD8+ T cells producing IFN-γ ex vivo were found in both the SGE-1X and the PBS group. By comparison, the leukocyte influx was altered in the ears of SGE-3X-inoculated mice. SGE-3X markedly increased the numbers of CD4+ T and CD8+ T cells by two- to three-fold when compared with the SGE-1X group. Furthermore, the production of IFN-γ by both T lymphocyte populations was higher in the SGE-3X group.
*L. braziliensis* infection induced the migration of CD4+FOXP3+ regulatory T cells to the ear lesion (Figure 5G). However, SGE-1X treatment enhanced the number of CD4+FOXP3+ cells by three- to four-fold in the site of infection. Furthermore, in contrast with aforementioned cells, the number of CD4+FOXP3+ T cells was significantly reduced by one- to two-fold in the SGE-3X group. Our results also shown that, despite of SGE-1X presented the enhancement of neutrophil and macrophage, in the SGE-3X group both cell population was reduced. These reductions were, in average, 47% to macrophage (Figure 5H) and 48% to neutrophil (Figure 5I). These results therefore suggest that different saliva inoculums alters the inflammatory cell and cytokine composition at the site of parasite inoculation, and modulate the immune response during *L. braziliensis* infection.

The protective effect of saliva is mediated by IFN-γ release

Because SGE-3X treatment protected the mice from parasitic infection (Figure 3) and induced significant production of IFN-γ (Figure 4B) by increasing the emigration of CD4+ T cells and CD8+ T cells (Figure 5), we further investigated the impact of IFN-γ production on resistance against *L. braziliensis* infection. BALB/c mice sensitized with three treatments of saliva (SGE-3X) were depleted of IFN-γ by treatment with anti-IFN-γ mAb (R46A2 clone) and then were challenged with the parasite. As a control group, mice were also treated with a nonrelevant IgG antibody.

As shown in Figure 6A, SGE-3X mice treated with IgG control antibody developed minor edema that rapidly decreased with healing skin. Moreover, low parasitic titers were detected in this group (Figure 6B). Conversely, depletion of INF-γ reduced the protective effect
Figure 5 (See legend on next page.)
of saliva on the course of *L. braziliensis* infection leading to a significant increase in the ear lesion size (*p* < 0.05) beginning at 3rd week of infection and persisting throughout the period of analysis (Figure 6A). Importantly, mAb anti-IFN-γ treatment also resulted in an increase in parasitic load at the inoculation site.

**Discussion**

In this study, we reported that the dual effect of salivary gland extract (SGE) saliva on the course of *Leishmania* infection is characterized by distinct changes in cellular immunity due to coinoculation or pre-exposure to saliva. Defining the nature of the inflammatory leucocytes that emigrate after saliva injection may help in the understanding of *Leishmania* infection biology and, therefore, may help in the development of new vaccine approaches that effectively protect the host against parasitic infection.

Studies have reported that immunization of mice with Phlebotomine saliva confers upon the mice a protective phenotype against *Leishmania* sp., whereas parasite and saliva that is simultaneously co-injected exacerbates infection, suggesting that immune responses triggered by the Phlebotomine saliva could represent a critical step in the development of disease. In this study, we showed that SGE inoculated once (SGE-1X), representing a co-inoculation, associated with a marked recruitment of several leucocytes, and most leucocytes were of the macrophage and neutrophil lineage. Interestingly, pre-exposure to saliva (SGE inoculated three times – SGE-3X) completely changed the cellular infiltrate composition. SGE-3X itself or during infection drastically reduced the amount of neutrophils and macrophages that migrated into the inoculation site even in the absence of parasitic infection, suggesting that the blockage of neutrophils and macrophage recruitment to the inflammatory site by saliva immunization is important to induce immune protection against parasitic infection. In fact, recent studies have described that neutrophils recruited to the site of *Leishmania* infections internalize the parasite [26,27], and saliva enhances neutrophil migration to the site of infection [28]. Previous studies have also observed that parasite internalization delays the apoptosis of neutrophils and induces MIP-1β release, which recruits macrophages to the site of infection. The migrated macrophages ingest the infected apoptotic neutrophils, which stimulates the release of TGF-β and PGE₂ and downregulates macrophage activation consequently contributing to *Leishmania* infection establishment [26,27]. Together, these findings suggest that the parasites use granulocytes as “Trojan horses” to attack the macrophages [26]. In this context, the inhibition of both neutrophils and macrophages by saliva pre-exposure as described in the present investigation may represent an additional mechanism to explain the ability of Phlebotomine saliva pre-inoculation to protect mice against *Leishmania* infection.

![Figure 5](http://www.biomedcentral.com/1471-2180/13/102)

**Figure 5** Inflammatory profile during *L. braziliensis* infection after co-inoculation or pre-sensitization with saliva. BALB/c mice inoculated i.d. once (SGE-1X) or three times (SGE-3X) with *Lutzomyia longipalpis* SGE or with PBS (control) were challenged with 10⁷ *L. braziliensis* stationary phase promastigote forms. At the end of 7th week post-infection, ears were harvested, processed and inflammatory leucocytes were sorted using specific antibodies. For intracellular cytokines, the cells were in vitro re-stimulated with lived parasites. Dot plots represent the percentages of CD4⁺CD3⁺ and CD4⁺IFN-γ⁺ cells (A – left panel), CD8⁺CD3⁺ and CD8⁺IFN-γ⁺ cells (B – right panel). Total number of CD4⁺ T cells (C) and CD4⁺IFN-γ⁺ cells (D) or CD8⁺ T cells (E) and CD8⁺IFN-γ⁺ cells (F), CD4⁺FOXP3⁺ cells (G), macrophages (H) and neutrophils (I) within the ears were identified by flow cytometry. Data represent the mean ± SEM and are representative of two independent experiments (n = 4). *P < 0.05 compared with PBS. **P < 0.05 compared with the SGE-1X group.

![Figure 6](http://www.biomedcentral.com/1471-2180/13/102)

**Figure 6** Effects of *in vivo* depletion of IFN-γ on SGE-3X-inoculated mice. BALB/c mice inoculated i.d. three times (SGE-3X) with *Lutzomyia longipalpis* SGE were challenged with 10⁷ *L. braziliensis* stationary phase promastigote forms. Animals were treated with normal rat IgG or rat anti-IFN-γ. The course of infection was monitored weekly by measuring the ear lesion size with a metric caliper. In A, the lesion size was determined by the difference between the infected ear and the opposite uninfected ear in millimeters (mm) of at least five mice per group. Data represent the mean ± SEM and are representative of two independent experiments. *P < 0.05 compared with IgG control group. Ear (B) parasite burdens were determined at the 4th week post-infection via a limiting-dilution assay. The data shown are the mean ± SEM of two independent experiments, each performed with five mice per group. #P < 0.05 compared with PBS. **P < 0.05 compared with the SGE-1X group.
Stressing the relevance of our finding, we demonstrated for the first time that Phlebotomine saliva increases regulatory T cell (Treg) recruitment to the lesion site. We demonstrated that inoculation of saliva once (SGE-1X) in the absence of parasites induces the recruitment of high numbers of CD4⁺CD25⁺ cells that, although being commonly accepted phenotype of Tregs also could be related to activated cells. Accordingly, parasites co-inoculated with saliva (SGE-1X) caused an increase in the recruitment of CD4⁺Foxp3⁺ cells to the infection site, suggesting that saliva of L. longipalpis increases Tregs during the infection. Despite the fact that the parasite alone is able to induce Treg migration, saliva strengthens this migration, which maintains the persistence of the parasite in the chronic phase of infection, and suggests that the recruitment of Tregs by the saliva may contribute to the infectivity of Leishmania. In fact, increased numbers of parasites at later time points were observed in the ears of mice co-inoculated with saliva and parasite, which corresponds to the point at which the disease becomes resolved and the parasitic burden decreases in the ears of mice infected with parasite only. Previous studies have also demonstrated that during infection with L. major, the persistence of the pathogen within the skin of L. major-resistant mice is controlled by an endogenous population of Treg cells that act to suppress the immune response against L. major. Treg cells are involved in maintaining the latency status of Leishmania infections and facilitate the survival of the parasite [29]. Our group reported that CD4⁺CD25⁺ T cells present in skin lesions of patients with cutaneous leishmaniasis display phenotypic and functional characteristics of natural Treg cells [30]. Thus, Treg cells induced by saliva play an important role in modulating the immune response during Leishmania infections. Notably, CD4⁺Foxp3⁺ cells were at normal levels in the ears of mice pre-sensitized to saliva (SGE-3X), which may have contributed to the control of Leishmania growth.

Our analyses of cytokine production further support the idea that SGE affects the inflammatory cell influx. Interestingly, our data show that in vitro stimulation of draining lymph node cells from SGE-1X mice with parasitic antigens results in higher levels of IL-10, whereas the IL-10 level in SGE-3X-derived draining lymph node cells cultures remained unchanged. Whereas the production of IL-10 was unchanged in the SGE-3X mice, IFN-γ production increased in the supernatant of SGE-3X lymph node-derived cell cultures, indicating that the inhibition of IL-10 in the SGE-3X mice may have resulted in better control of Leishmania infection. In fact, the severity of disease represented by the lesion size and parasitic burden was not observed in mice pre-sensitized with saliva (SGE-3X).

IL-10 is an anti-inflammatory cytokine produced by several cell types including macrophages, neutrophils and Treg cells, and IL-10 displays diverse immunomodulatory functions [31,32]. In regard to leishmaniasis, IL-10 inhibits cytokine production by T cells (e.g., IL-2), monocytes/macrophages and dendritic cells (e.g., IL-1α and IL-1β, IL-6, IL-8, IL-12, TNF-α, and granulocyte-macrophage colony-stimulating factor) as well as the production of NO and H₂O₂ ultimately favoring parasitic survival [32,33]. The hypothesis that IL-10 induced by saliva is involved in disease progression during Leishmania infection is supported by a significant enhancement in lesion development and parasitic burden in mice that were co-inoculated with saliva and parasites. The increase in IL-10 production has been reported in treatment with other Phlebotomine saliva sources. In previous studies, we demonstrated that the saliva from the Old World species Phlebotomines P. papatasi and P. duboscqi act mainly on dendritic cells and induce the production of IL-10 by a mechanism dependent of PGE₂. In turn, PGE₂ acts in an autocrine manner to reduce the antigen-presenting ability of DCs [13]. Previous studies have also shown in vitro and in vivo examples of Lutzomyia longipalpis saliva promotes inducing IL-10 production by macrophages and T cells, which exacerbates Leishmania infection [34]. Moreover, the genetic ablation of IL-10 prevents the detrimental effect of SGE on Leishmania major and L. amazonensis infections.

The reduced ability of SGE-3X-inoculated mice to produce IL-10 may be associated with an increase in IFN-γ production. Consistently, the depletion of IFN-γ using IFN-γ-neutralizing monoclonal antibody reduced the protective profile of saliva upon Leishmania disease. Despite the significant increase in CD8⁺ T cells in the ears of mice that were pre-inoculated with saliva three times (SGE-3X), our evidence suggests that CD4⁺ T cells and CD8⁺ T cells contributed to the increased ex vivo production of IFN-γ during Leishmania infection. Although the present study is insufficient to declare the mechanism by which the immune cells mediate the control of Leishmania growth, we believe that the presence of lymphocytes producing IFN-γ at the site of inflammation may induce the direct killing of L. braziliensis by nitric oxide (NO)-dependent mechanisms. This effect could be mediated by proteins presents into saliva that are uptake by antigen-presenting cells and prime naïve CD4⁺ T cell and CD8⁺ T cells. When the mice are challenged with parasite in the presence of saliva, it triggers a rapid T cells activation and production of IFN-γ. Thus, there is a cross-reactivity of the immune response induced by salivary proteins against Leishmania braziliensis. This hypothesis has been validated in models with salivary proteins. PpSP15 protein derived from Phlebotomus papatasi provided protective immune response against L. major when the parasite was co-inoculated with P. papatasi SGE by the induction of DTH response [16]. Likewise, the
immunization of mice with proteins from *Lutzomyia longipalpis*. LJM11 and LJM19 induced the strong DTH and conferred the protective effect against different species of *Leishmania* (*L. major*, *L. infantum* and *L. braziliensis*) when the mice were challenged with parasite and SGE [35-39]. Interestingly, such responses were similar with that previously obtained using a natural sensitization with bites of uninfected sand fly [15].

Several pieces of evidence have shown that Phlebotomine saliva enhances the infectivity of many different *Leishmania* species, which can be attributed to numerous substances within the saliva that harbor pharmacological properties that induce vasodilatation, anticoagulation, anti-inflammation and immunomodulation. Thus, the active salivary constituents could serve as a prototype for the development of vaccines to control pathogen transmission. Our group is currently working on the isolation of compounds within the saliva of several blood-feeding arthropods, including Phlebotomine vectors. We recently identified adenosine (ADO) and adenosine monophosphate (AMP) as major immunomodulatory compounds present within the Old World sand fly species *Phlebotomus papatasi*, which protected mice from extreme inflammatory insults [40]. Salivary protein (SP)-15 is also present in *P. papatasi*, and SP-15 provides a protective effect against *Leishmania major* infection through an IFN-γ-dependent mechanism [16]. In the present study, neither ADO and AMP nor SP-15 is involved in the effect of SGE on *Leishmania* infection because they are not found in *Lutzomyia longipalpis* saliva. Maxadilan (MAX) is a potent vasodilator present in *L. longipalpis* saliva that exacerbates *Leishmania* sp. infection. Mice vaccinated with recombinant MAX were markedly protected from *Leishmania* infection, and this protective effect was associated with an increase in CD4+ T cells, IFN-γ and NO [14]. Presently, we cannot dismiss the possibility that the protective effect of SGE-3X could be mediated by MAX; however, several other proteins (LJM17 and LJM11 in human and LJM17, LJM11, LJJ13, LJJ23 and LJJ143 in dogs) from *L. longipalpis* saliva have been identified [41], suggesting that intensive efforts are required for the identification of salivary compounds responsible for the protective effect of sand fly saliva on leishmaniasis.

**Conclusion**

In summary, the present study provides strong evidence that different *Lutzomyia longipalpis* saliva inoculation schemes may skew the initial cellular responses, which is reflected by parasitic survival or host resistance to infection. Thus, we believe that comprehending the effects of sand fly saliva on the host immune response induced by saliva may help in the generation of new vaccine strategies that can block the effects of saliva and prevent *Leishmania* establishment in the host.

**Abbreviations**

SGE-1X: Salivary gland extract inoculated once; SGE-3X: Salivary gland extract inoculated three times.

**Competing interest**

The authors declare that they have no competing interest.

**Author contributions**

Conceived and designed the experiments: VC and JSS. Performed the experiments: VC and DLC. Analyzed the data: VC and JSS. Contributed reagents/materials/analysis tools: CIB, AMB, MB, FQC and JSS. Wrote the paper: VC and JSS. Revised the paper DLC, CIB, AMB, MB and FQC. All authors read and approved the final manuscript.

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**References**

1. Beach R, Killu G, Leeuwenburg J: Modification of sand fly biting behavior by *Leishmania* leads to increased parasite transmission. *Am J Trop Med Hyg* 1985, 34(2):276–282.

2. Ribeiro JM: Role of saliva in blood-feeding by arthropods. *Annu Rev Entomol* 1987, 32:463–478.

3. Titus RG, Ribeiro JM: The role of vector saliva in transmission of arthropod-borne disease. *Parasitol Today* 1990, 6(5):157–160.

4. Ribeiro JM: Blood-feeding arthropods: live syringes or invertebrate pharmacologists? *Infect Agents Dis* 1995, 4(3):143–152.

5. Waitumbi J, Warburg A: *Phlebotomus* papatasi saliva inhibits protein phosphatase activity and nitric oxide production by murine macrophages. * Infect Immun* 1998, 66(6):1534–1537.

6. Titus RG, Bishop JV, Mejia JS: The immunomodulatory factors of arthropod saliva and the potential for these factors to serve as vaccine targets to prevent pathogen transmission. *Parasite Immunol* 2006, 28(4):131–141.

7. Lima HC, Titus RG: Effects of sand fly vector saliva on development of cutaneous lesions and the immune response to *Leishmania braziliensis* in BALB/c mice. *Infect Immun* 1996, 64(2):542–5445.

8. Mbow ML, Bleyenberg JA, Hall LR, Titus RG: *Phlebotomus* papatasi sand fly salivary gland lysate down-regulates a Th1, but up-regulates a Th2, response in mice infected with *Leishmania major*. *J Immunol* 1998, 161(10):5571–5577.

9. Belkaid Y, Kamhawi S, Modi G, Valenzuela J, Noben-Trauth N, Rowton E, Ribeiro J, Sacks D: Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. *J Exp Med* 1998, 188(10):1941–1953.

10. Scott P, Arts D, Uzonna J, Zapf C: The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development. *Immunol Rev* 2004, 201:318–338.

11. Sacks D, Anderson C: Re-examination of the immunosuppressive mechanisms mediating non-cure of *Leishmania* infection in mice. *Immunol Rev* 2004, 201:225–238.

12. De Freitas LA, Mbow LM, Estay M, Bleyenberg JA, Titus RG: Indomethacin treatment slows disease progression and enhances a Th1 response in susceptible BALB/c mice infected with *Leishmania major*. *Parasite Immunol* 1999, 21(5):273–277.

13. Carregaro V, Valenzuela JG, Cunha TM, Veni WA Jr, Grespan R, Matsumura G, Ribeiro JM, Elnaiem DE, Silva JS, Cunha FQ: *Phlebotomine* salivas inhibit immune inflammation-induced neutrophil migration via an autocrine...
DC-derived PGE2/IL-10 sequential pathway. J Leukoc Biol 2008, 84(1):104–114.

14. Morris RV, Shoemaker CB, David JR, Lanzaro GC, Titus RG. Sandfly maxillary exudate exacerbates infection with Leishmania major and vaccinating against it protects against L. major infection. J Immunol 2001, 167(5):2525–2533.

15. Kamhawi S, Belkaid Y, Modi G, Roveton E, Sacks D. Protection against cutaneous leishmaniasis resulting from bites of unfixed sand flies. Science 2000, 290(5495):1351–1354.

16. Valenzuela JG, Belkaid Y, Garfield MK, Mendez S, Kamhawi S, Roveton ED, Sacks DL, Ribeiro JM. Toward a defined anti-Leishmania vaccine targeting vector antigens: characterization of a protective salivary glycoprotein. J Exp Med 2003, 194(3):331–342.

17. Monteiro MC, Lima HC, Souza AA, Titus RG, Romão PR, Cunha FQ. Effect of Lutzomyia longipalpis salivary gland extracts on leukocyte migration induced by Leishmania major. Am Trop Med Hyg 2007, 76(1):88–94.

18. Teixeira CR, Teixeira MJ, Gomes RB, Santos CS, Andrade BB, Raffaele-Netto L, Silva JS, Guglielmotti A, Miranda JC, Baral A, Brodskyn C, Barral-Netto M. Saliva from Lutzomyia longipalpis induces CC chemokine ligand 2 upon monocyte chemoattractant protein-1 expression and macrophage recruitment. J Immunol 2005, 175(12):8346–8353.

19. Maurer M, Dondl J, von Stebut E. What determines the success or failure of intracellular cutaneous parasitism? Lessons learned from leishmaniasis. Med Microbiol Immunol 2009, 198(3):137–146.

20. Anjili CO, Mbati PA, Mwangi RW, Githure JJ, Gblobo JO, Robert LL, Koeh K. The chemotactic effect of Phlebotomus duboscqi (Diptera: Psychodidae) salivary gland lysates to murine monocytes. Acta Trop 1995, 60:297–100.

21. Zer R, Yaroslavski I, Rosen L, Warburg A. Effect of sand fly saliva on Leishmania infection by murine peritoneal macrophages. Int J Parasitol 2001, 31(8):810–814.

22. Peters NC, Sacks DL. The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. Cell Microbiol 2009, 11(9):1200–1206.

23. Titus RG, Ribeiro JM. Salivary gland lysates from the sand fly Lutzomyia longipalpis enhance Leishmania infectivity. Science 2008, 323(5845):1306–1308.

24. Costa DL, Carregaro V, Lima-Júnior DS, Silva NM, Milanezi CM, Cardoso CR, Guadice AC, de Jesus AR, Carvalho EM, Almeida RP, Silva JS. BALB/c mice infected with antimony treatment refractory isolate of Leishmania braziliensis present severe lesions due to IL-4 production. PLoS Negl Trop Dis 2011, 5(3):e965.

25. McKinney MM, Parkinson A. A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. J Immunol Methods 1987, 106(2):271–278.

26. van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach V, Pham V, Elnaiem DE, Kamhawi S, Valenzuela JG, Andersen JF, Brodskyn C. Toward a defined anti-Leishmania vaccine targeting vector antigens: characterization of a protective salivary glycoprotein. J Exp Med 2003, 194(3):331–342.

27. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, Lawyer P, Fay MP, Germain RN, Sacks D. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmission by sand flies. Science 2008, 321(5891):970–974.

28. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. Nat Immunol 2005, 6(4):333–340.

29. Carregaro V, Roselino AM, Cavasana KA, Pereira MS, Mortara RA, Brodskyn CI, Goncalves HS, Belkaid Y, Baral Netto M, Baral A, Silva JS. CD4+ CD25+ T cells in skin lesions of patients with cutaneous leishmaniasis exhibit phenotypic and functional characteristics of natural regulatory T cells. J Infect Dis 2006, 193(9):1313–1322.

30. Sabat R. IL-10 family of cytokines. Cytokine Growth Factor Rev 2010, 21(5):315–324.

31. Moore KW, de Waal MR, Coffman RL, O’Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001, 19:693–765.

32. Ding Y, Chen D, Tarcsafalvi A, Su R, Qin L, Bromberg JS. Suppressor of cytokine signaling 1 inhibits IL-10-mediated immune responses. J Immunol 2003, 170(3):1383–1391.

33. Norsworthy NB, Sun J, Elnaiem D, Lanzaro G, Soong L. Sand fly saliva enhances Leishmania amazonesis infection by modulating interleukin-10 production. Infect Immun 2004, 72(3):1240–1247.

34. Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, Silva C, de Oliveira CI, Elnaiem DC, Kamhawi S, Valenzuela JG, Brodskyn CI. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. Proc Natl Acad Sci U S A 2008, 105(21):7845–7850.

35. Xu X, Oliveira F, Chang BW, Collin N, Gomes R, Teixeira C, Reynoso D, My Pham V, Elnaiem DC, Kamhawi S, Ribeiro JM, Valenzuela JG, Andersen JF. Structure and function of a “yellow” protein from saliva of the sand fly Lutzomyia longipalpis that confers protective immunity against Leishmania major infection. J Biol Chem 2011, 286(37):32383–32393.

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