Lutzomyia longipalpis saliva drives apoptosis and enhances parasite burden in neutrophils

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ABSTRACT

Neutrophils are considered the host's first line of defense against infections and have been implicated in the immunopathogenesis of Leishmaniasis. *Leishmania* parasites are inoculated alongside vectors' saliva, which is a rich source of pharmacologically active substances that interfere with host immune response. In the present study, we tested the hypothesis that salivary components from *Lutzomyia longipalpis,* an important vector of visceral Leishmaniasis, enhance neutrophil apoptosis. Murine inflammatory peritoneal neutrophils cultured in the presence of SGS presented increased surface expression of FasL and underwent caspase-dependent and FasL-mediated apoptosis. This proapoptosis effect of SGS on neutrophils was abrogated by pretreatment with protease as well as preincubation with antisaliva antibodies. Furthermore, in the presence of *Leishmania chagasi*, SGS also increased apoptosis on neutrophils and increased PGE_2 release and decreased ROS production by neutrophils, while enhancing parasite viability inside these cells. The increased parasite burden was abrogated by treatment with z-VAD, a pan caspase inhibitor, and NS-398, a COX-2 inhibitor. In the presence of SGS, *Leishmania*-infected neutrophils produced higher levels of MCP-1 and attracted a high number of macrophages by chemotaxis in vitro assays. Both of these events were abrogated by pretreatment of neutrophils with bindarit, an inhibitor of CCL2/MCP-1 expression. Taken together, our data support the hypothesis that vector salivary proteins trigger caspase-dependent and FasL-medi-ated apoptosis, thereby favoring *Leishmania* survival inside neutrophils, which may represent an important mechanism for the establishment of *Leishmania* infection. *J. Leukoc. Biol.* 90: 575–582; 2011.

Introduction

Neutrophils play complex roles in infection. They provide an important link between innate and adaptive immunity during parasitic infections [1, 2] but also undergo apoptosis and are ingested by macrophages, thereby triggering secretion of anti-inflammatory mediators [1, 3, 4]. At the onset of *Leishmania* infection, neutrophils establish a cross-talk with other cells in the development of an immune response [5], but the ultimate outcome is controversial, as protective [6–8] and deleterious [9–12] effects to the host have been shown.

Leishmania is transmitted by bites from sandflies looking for a blood meal. Tissue damage caused by sandfly probing [10] and sandfly saliva [13] is a potent stimulus for neutrophil recruitment, which results in a rapid migration and accumulation of neutrophils at the site of the vector's bite [10, 12, 14]. Pharmacological properties of the saliva from sandflies are diverse [15, 16], and we have shown recently that saliva from *Lutzomyia longipalpis*, the main vector of *Leishmania chagasi* in Brazil, triggers important events of the innate immune response [17]. Despite the recognition of the importance of phlebotomine saliva and neutrophils in the initial steps of leishmanial infection, the direct role of saliva on the parasite-neutrophil interplay has not been addressed.

Recent studies demonstrated the presence of *Leishmania*-infected apoptotic neutrophils at the sandfly bite site [10];

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however, a possible role of the sandfly saliva in this phenomenon remains unclear. Herein, we show an important FasL- and caspase-dependent apoptosis effect of *Lu. longipalpis* SGS upon neutrophils. In addition, the SGS-induced apoptosis favors *L. chagasi* survival inside neutrophils. These results represent the first evidence of direct effects of *Lu. longipalpis* SGS on host neutrophils and bring implications for the innate immune response to *Leishmania* infection.

MATERIALS AND METHODS

Mice and parasites

Inbred male C57BL/6 mice, aged 6–8 weeks, were obtained from the animal facility of CPqGM-FIOCRUZ (Bahia, Brazil). This study was carried out in strict accordance with the recommendations of the International Guiding Principles for Biomedical Research Involving Animals. All experimental procedures were approved and conducted according to the Brazilian Committee on the Ethics of Animal Experiments of the FIOCRUZ (Permit Number: 027/2008). *L. chagasi* (MCAN/BR/89/BA262) promastigotes were cultured at 25°C in Schneider's insect medium, supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Sandflies and preparation of salivary glands

Adult phlebotomines from a *Lu. longipalpis* colony from Cavunge (Bahia, Brazil) were reared at the Laboratório de Imunoparasitologia/CPqGM/FIOCRUZ, as described previously [16]. Salivary glands were dissected from 5- to 7-day-old *Lu. longipalpis* females under a stereoscopic microscope (Stemi 2000; Carl Zeiss, Jena, Germany) and stored in groups of 10 pairs in 10 μl endotoxin-free PBS at –70°C. Immediately before use, glands were sonicated (Sonifier 450; Brason, Danbury, CT, USA) and centrifuged at 10,000 *g* for 4 min. Supernatants of SGS were used for experiments. The level of LPS contamination of SGS preparations was determined using a commercially available *Limulus* amoebocyte lysate chromogenic kit (QCL-1000, Lonza Bioscience, Walkersville, MD, USA); negligible levels of endotoxin were found in the salivary gland supernatant. All experimental procedures used SGS in an amount equivalent to 0.5 pair of salivary glands/group, representing ~0.7 μg protein [18].

Reagents

Anti-Gr-1-FITC, anti-mouse CD178L-PE (FasL; CD95L), PE hamster IgG κ isotype control (anti-TNP), CBA mouse inflammation kit, neutralizing antibody anti-mouse FasL, and hamster IgG κ isotype control were purchased from BD Biosciences (San Jose, CA, USA). Anti-mouse Ly-6G Alexa Fluor 647 was from BioLegend (San Diego, CA, USA). Annexin-V, PI (apoptosis detection kit), and z-VAD-FMK were from R&D Systems (Minneapolis, MN, USA). NS-398 and DMSO were from Cayman Chemical (Ann Arbor, MI, USA). Proteinase K was from Gibco, Invitrogen (Grand Island, NY, USA). RPMI-1640 medium and L-glutamine, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA, USA). Schneider's insect medium and etoposide (VP-16) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nutridoma-SP was from Roche (Indianapolis, In, USA), and thioglycolate was from Difco (Detroit, MI, USA). Bindarit was from Angelini Farmaceutici (Santa Palomba-Pomezia, Rome, Italy).

Inflammatory neutrophils

Peritoneal exudate neutrophils were obtained as described previously [19]. Briefly, C57BL/6 mice were i.p.-injected with aged 3% thioglycolate solution. Seven hours after injection, peritoneal lavage was performed using 10 ml RPMI-1640 medium supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To remove adherent cells, exudate cells were incubated at 37°C in 5% CO_2 for 1 h in

250-ml flasks (Costar, Cambridge, MA, USA); cells on supernatants were then recovered and quantified in a hemocytometer by microscopy. Cell viability was >95%, as determined by trypan blue exclusion (data not shown). Nonadherent cells were stained with anti-Gr-1 and Ly-6G to assess purity and were subsequently analyzed by flow cytometry using CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA). Gr-1$^+$ Ly-6G$^+$ cells were routinely >95% pure.

Neutrophil apoptosis assay

For cell cultures, neutrophils (5×10^5/well) were cultured in 200 μl RPMI-1640 medium, supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well plates (Nunc, Denmark) in the presence of different doses of *Lu. longipalpis* SGS (0.5, 1.0, and 2.0 pairs/well). In some experiments, etoposide (20 μM) or LPS (100 ng/well) was used as a positive control. Three hours and 20 h after stimuli, neutrophil apoptosis was assessed by PS, exposed in the outer membrane leaflet through labeling with annexin-V-FITC by FACS analyses in combination with PI nuclear dye [19]. Annexin-V specificity was tested using Ca^{2+}-free buffer; binding was not observed in this case. Morphological criteria for apoptosis, such as separation of nuclear lobes and darkly stained pyknotic nuclei, were also applied for quantification purposes using cytospin preparations stained by Diff-Quick under light microscopy [19]. Neutrophils were graded as apoptotic or nonapoptotic after examination of at least 200 cells/slide. To FasL-blocked assays, neutrophils were pretreated with a neutralizing antibody specific for FasL (10 μg/mL) or an IgG isotype control (10 μg/mL) for 30 min before use. In some experiments, SGS was preincubated with sandfly antisaliva serum (0.5 salivary gland pair plus 50 μl serum preincubated for 1 h at 37°C) [20] or with proteinase K (10 mg/ml) at 65°C for 2 h and then for 5 min at 95°C for enzyme inactivation before use.

Anti-sandfly saliva serum

Hamster-derived serum was obtained as described previously [20]. Briefly, hamsters (*Mesocricetus auratus*) were exposed to bites from 5- to 7-day-old female *Lu. longipalpis*. Animals were exposed three times to 50 sandflies every 15 days. Fifteen days after the last exposure, serum was collected and tested for IgG antisaliva detection by ELISA.

Human neutrophil assay

Human blood from healthy donors was obtained from Hemocentro do Estado da Bahia (Salvador, Brazil) after donors had given written, informed consent. This study was approved by the Research Ethics Committee of FIOCRUZ-Bahia. Human neutrophils were isolated by centrifugation using PMN medium, according to the manufacturer's instructions (Robbins Scientific, Sunnyvale, CA, USA). Briefly, blood was centrifuged for 30 min at 300 *g* at room temperature. Neutrophils were collected and washed three times at room temperature by centrifugation at 200 *g*. Cells/well (10^6) were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated FBS (Hyclone, Ogden, UT, USA), 2 mM/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen) for 3, 6, and 20 h at 37°C, 5% CO_2, in the presence or absence of *Lu. longipalpis* SGS (0.5 pair/well) or etoposide (20 μM). Cells were then cytocentrifuged and stained with Diff-Quick, and pyknotic nuclei were analyzed by light microscopy.

In vitro neutrophil infection

Peritoneal neutrophils were infected in vitro with *L. chagasi* promastigotes stationary-phase at a ratio of 1:2 (neutrophil:parasites) in the presence or absence of SGS (0.5 pair/well) in RPMI-1640-supplemented medium. In some experiments, neutrophil infection was performed in the presence of etoposide (20 μM). For inhibitory assays, neutrophils were pretreated for 30 min with z-VAD-FMK (100 μM) to block caspase activation or preincubated for 1 h with NS-398 (1 μM), a COX-2 inhibitor. DMSO (vehicle) 0.4% was used as control. After 20 h, infected neutrophils were centri-

fuged, supernatants containing noninternalized promastigotes were collected, and medium was replaced by 250 μl Schneider medium, supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Infected neutrophils were cultured at 25°C for an additional 3 days. Intracellular load of *L. chagasi* was estimated by production of proliferating extracellular motile promastigotes in Schneider medium [21].

Quantification of ROS production

Intracellular ROS detection in neutrophils cultured at 5×10^5 cells/well was performed using H_2DCFDA fluorescent probe following analyses by FACS, according to the manufacturer's instructions. For investigation of ROS production, the purified neutrophil population was analyzed by forward- and side-scatter parameters following application of the H_2DCFDA-FITC probe.

Measurement of PGE$_2$ production

Supernatants from neutrophil cultures were collected 20 h after incubation with *L. chagasi* or *L. chagasi* plus SGS and cleared by centrifugation. PGE$_2$ was measured by the EIA kit from Cayman Chemical. All measurements were performed according to the manufacturer's instructions.

MCP-1/CCL2 measurement

Supernatants from neutrophil cultures were collected 20 h after incubation with RPMI medium, SGS, *L. chagasi*, or *L. chagasi* plus SGS and cleared by centrifugation. MCP-1 (CCL2) chemokine was measured using the CBA mouse inflammation kit (BD Biosciences), according to the manufacturer's instructions.

Chemotaxis assays

Neutrophils were pretreated or not with bindarit propanoic acid (Angelini Farmaceutici; 100 μM) for 30 min before incubation with medium, SGS, *L. chagasi*, or *L. chagasi* plus SGS, and supernatants were harvested. The culture supernatants were added to the bottom wells of a 96-well chemotaxis microplate ChemoTx system (Neuro Probe, Gaithersburg, MD, USA). Macrophages were obtained 4 days after i.p. injection of 1 ml 3% thioglycolate solution on C57BL/6 mice and ressuspended in RPMI-1640 medium before being added to the top wells (10^5 cells/well) and incubated for 1.5 h at 37°C under 5% CO$_2$. Following incubation, cells that migrated to the bottom wells were counted on a hemocytometer. Macrophage migration toward RPMI-1640 medium alone (radom chemotaxis) was used as a negative control and toward LPS as a positive control. The chemotaxis indexes were calculated as the ratio of the number of migrated cells toward supernatants taken from *L. chagasi*-infected or not infected neutrophils cultured in the presence or absence of SGS to the number of cells that migrated to RPMI-1640 medium alone.

Statistical analysis

The in vitro systems were performed using at least five mice/group. Each experiment was repeated at least three times. Data are reported as mean and SE of representative experiments and were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Data distribution from different groups was compared using the Kruskal-Wallis test with Dunn's multiple comparisons, and comparisons between two groups were explored using the Mann-Whitney test. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

Lu. longipalpis SGS induces neutrophil apoptosis

Different doses of *Lu. longipalpis* SGS (0.5–2.0 pairs/well) were capable of inducing apoptosis of neutrophils from C57BL/6

mice (**Fig. 1A** and **C**). Such effect was significantly higher than that observed in untreated controls (Fig. 1A and B). The occurrence of apoptosis was similar between the conditions containing diverse doses of SGS (Fig. 1A). We then decided to keep the lowest dose of SGS with biological effect in our model (0.5 pair of salivary gland/well) for further experiments.

Neutrophils exhibited markers of apoptosis up to 20 h upon incubation with SGS, such as PS exposure (Fig. 1D) and the pyknotic nuclei (Fig. 1E). At 3 h after stimulus with SGS, indicators had levels similar to those observed in unstimulated cells. Etoposide was used as a positive control to induce neutrophil apoptosis, and its effect was evident at 3 h by annexin-V detection (Fig. 1D) and 20 h by pyknotic nuclei analyses (Fig. 1E). These results confirm the proapoptotic effect of *Lu. longipalpis* SGS upon murine neutrophils.

Our further interest was to explore whether *Lu. longipalpis* SGS displays a proapoptotic effect on human neutrophils. To address this question, neutrophils obtained from healthy donors were incubated in the presence or absence of SGS or etoposide (Fig. 1F). Strikingly, 3 h after incubation, SGS induced human neutrophil apoptosis (Fig. 1F). At further times (6 and 20 h), this proapoptotic effect was no longer evident by comparison with negative control.

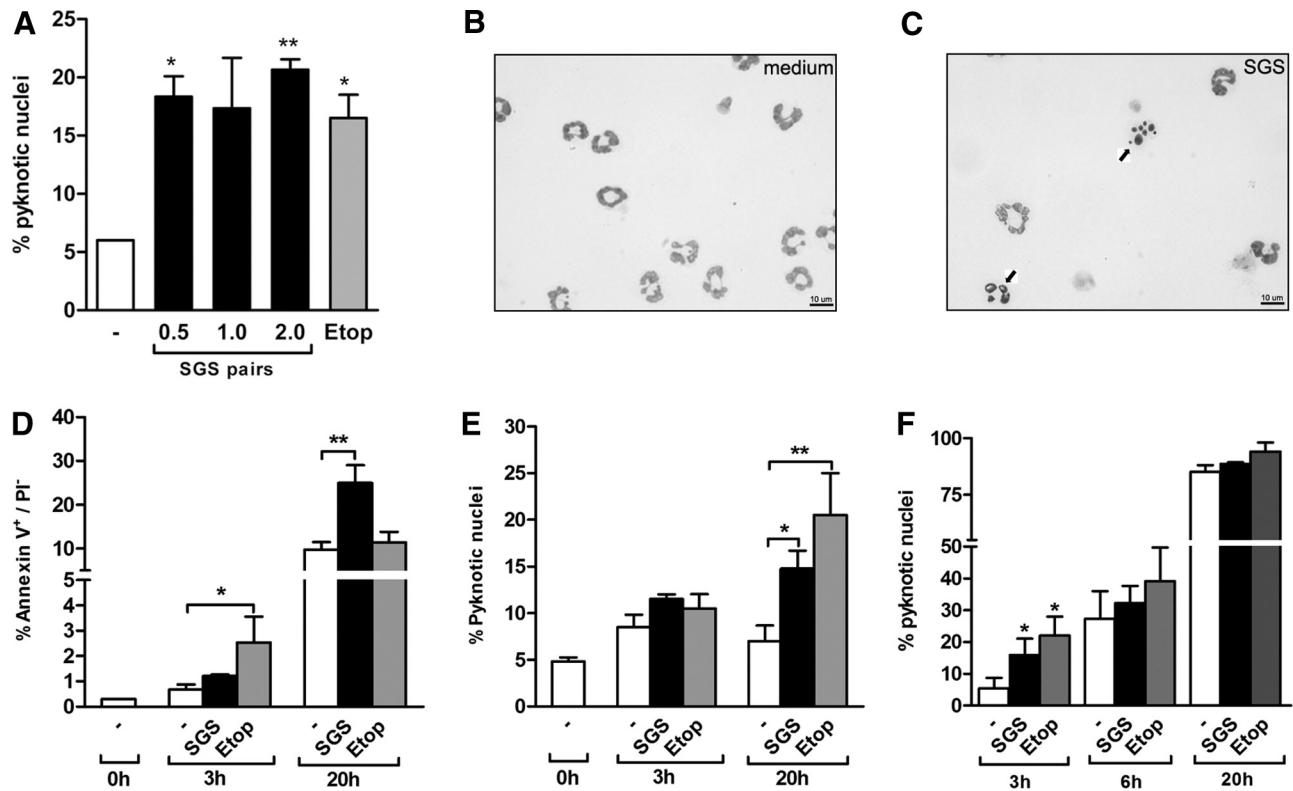## Neutrophil apoptosis induced by SGS is caspase-dependent and mediated by FasL

To evaluate the mechanisms triggered by *Lu. longipalpis* saliva to induce neutrophil apoptosis, we incubated C57BL/6 murine neutrophils with z-VAD, a pan-caspase inhibitor, for 30 min before addition of *Lu. longipalpis* SGS (**Fig. 2A**). Treatment of neutrophils with z-VAD prevented apoptosis induced by SGS, in contrast to treatment with the vehicle (DMSO) alone (Fig. 2A). Caspase activation can be induced by FasL, a molecule whose expression relates to susceptibility in *Leishmania* infection [22]. We then assessed FasL expression in neutrophils exposed to *Lu. longipalpis* SGS, which induced increased expression of FasL in neutrophils concerning intensity/cell (Fig. 2B) and also the percentage of neutrophils expressing FasL (Fig. 2C). Moreover, blockade of FasL prevented neutrophil apoptosis induced by *Lu. longiplapis* SGS (Fig. 2D). These results indicate that *Lu. longipalpis* SGS induces neutrophil apoptosis by a mechanism that involves activation of caspases and expression of FasL.

Lu. longipalpis SGS proteins induce neutrophil apoptosis

To depict initially the composition of the *Lu. longipalpis* salivary components responsible for the proapoptosis effect on neutrophils, we preincubated SGS with proteinase K before in vitro neutrophil stimulation. We observed a reduction of proapoptotic activity of SGS by incubation with proteinase K (**Fig. 3A**). This result suggests that apoptosis of neutrophils induced by *Lu. longipalpis* SGS is mediated by one or more proteic components.

Furthermore, as many evidences point out the immunogenicity of sandfly salivary proteins [13, 23, 24], we hypothesized that the

Figure 1. Effect of *Lu. longipalpis* SGS on neutrophil apoptosis. (A–E) Neutrophils from C57BL/6 mice were kept unstimulated (–) or stimulated with SGS or etoposide (Etop) 20 μM (positive control). (A) Neutrophil apoptosis induced by SGS in different doses was assessed by counting cells with pyknotic nuclei 20 h after stimulation. (B and C) Representative image of inflammatory neutrophils, unstimulated (B) or stimulated with *Lu. longipalpis* SGS (0.5 pair/well; original magnification, ×1000; C). Arrows point to neutrophil pyknotic nuclei. (D and E) Kinetic of neutrophil apoptosis in response to *Lu. longipalpis* SGS. Three hours and 20 h after stimulation, apoptosis was assessed by flow cytometry after annexin-V staining (D) and by counting cells with pyknotic nuclei (E) on Diff-Quick-stained cytospin preparations. (F) Human neutrophil apoptosis induced by SGS (0.5 pair/well). Data shown are from a single experiment that is representative of three independent experiments. *$P \leq 0.05$; **$P \leq 0.01$, compared with the unstimulated cells.

proteic component of the *Lu. longipalpis* saliva could be targets for the host's antibodies. To test this possibility, we preincubated the SGS with polled sera from hamsters pre-exposed to *Lu. longipalpis* bites. Strikingly, preincubation of SGS with specific antiserum completely abrogated induction of neutrophil apoptosis after 20 h in culture (Fig. 3B), reinforcing that components present in *Lu. longipalpis* saliva with proapoptotic activity are proteins and can be neutralized by antibodies.

Effect of *Lu. longipalpis* SGS in apoptosis and parasite burden of infected neutrophils

After determining the proapoptotic effect of *Lu. longipalpis* SGS, we evaluated whether *L. chagasi*, the parasite transmitted by this sandfly, can modify this effect in vitro. Analysis of PS exposure on inflammatory neutrophils demonstrated that *L. chagasi* was also able to induce neutrophil apoptosis (**Fig. 4A**). Moreover, this effect was exacerbated when neutrophils were coincubated with parasite and saliva (*L. chagasi* vs. *L. chagasi* plus SGS: 29.19% vs. 46.39%; Fig. 4A).

Neutrophils can act as important host cells for *Leishmania* [10, 25, 26]. As sandfly saliva exacerbates *Leishmania* infection [27], we investigated the infection of inflammatory neutrophils with *L.*

chagasi in the presence of *Lu. longipalpis* SGS in vitro. Saliva increased the viability of *L. chagasi* inside neutrophils (Fig. 4B). Infection in the presence of etoposide did not enhance parasite burden in neutrophils compared with the control cultures infected with *L. chagasi* alone (Fig. 4B). Apoptotic neutrophils displayed a high number of parasites (Fig. 4C). To investigate whether neutrophil apoptosis induced by *Lu. longipalpis* saliva affects this increase of parasite burden in vitro, we pretreated the cultures with z-VAD (Fig. 4D), which abolished the increase in *L. chagasi* replication induced by SGS (Fig. 4D). COX activation is associated with an increase of *Leishmania* infection [28]. Herein, we evaluated the role of COX-2, an inflammatory form of COX, in the increase of parasite burden triggered by SGS. NS-398, a COX-2 inhibitor, led to an inhibition of viable parasite number (Fig. 4D) when added to the neutrophil culture before infection. Moreover, PGE$_2$, a product of COX-2, favors intracellular pathogen growth, a phenomenon that could be reverted by treatment with COX-2 inhibitors [29, 30]. Indeed, our experiments show that SGS increased production of PGE$_2$ by *Leishmania*-infected neutrophils (Fig. 4E).

As ROS production is a primarily important microbicidal mechanism from neutrophils, we evaluated the effect of SGS on
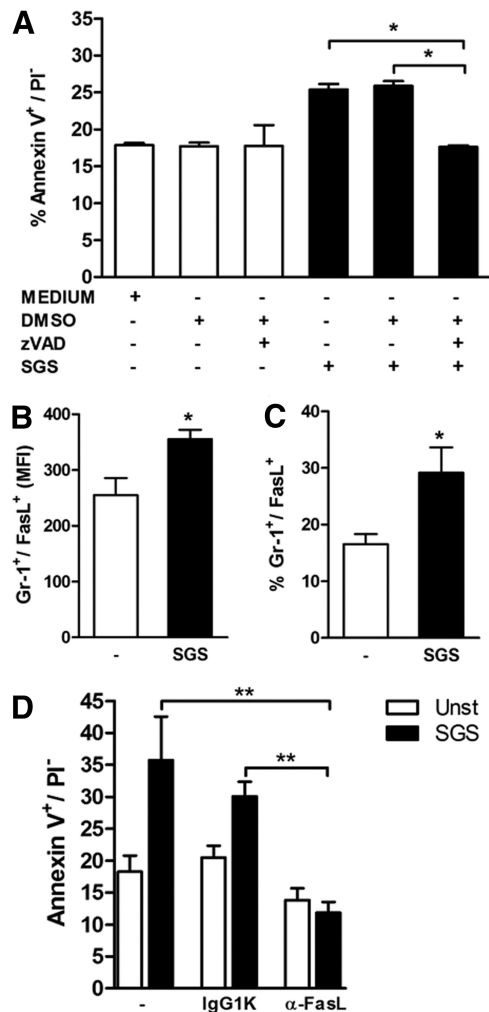
Figure 2. FasL expression and inhibition of neutrophil apoptosis by z-VAD and anti-FasL. (A) Neutrophils from C57BL/6 mice were pretreated with the pan-caspase inhibitor z-VAD (100 μM) or with vehicle (DMSO) before incubation with SGS. Twenty hours after incubation, apoptosis was assessed by annexin-V staining. (B and C) FasL expression induced by SGS on neutrophils was analyzed by flow cytometry 20 h after incubation. Results are expressed as the mean fluorescence intensity (MFI) (B) and percentage of FasL-expressing neutrophils on the Gr-1 population (C). (D) Mouse neutrophils were pretreated with neutralizing antibody specific for FasL (α-FasL; 10 μg/ml) or with IgGk1 (10 μg/ml). Apoptosis was assessed by annexin-V staining after 20 h. Data shown are from a single experiment representative of three independent experiments. –, Unstimulated (Unst) cells. *$P \leq 0.05$; **$P \leq 0.01$.

ROS production by these cells (Fig. 4E). Addition of SGS on the neutrophil cultures induced a partial reduction on ROS production 1 h after infection with *L. chagasi* (Fig. 4E). In summary, these results suggest that neutrophil apoptosis induced by *Lu. longipalpis* SGS favors *L. chagasi* infection by COX-2 activation and PGE$_2$ production, while reducing ROS generation.

## CCL2/MCP-1 released by *L. chagasi*-infected neutrophils induces macrophage recruitment

We next examined whether supernatans from neutrophils incubated with *L. chagasi* and SGS are able to induce macrophage recruitment in vitro. We found that supernatants obtained from neutrophil cultures in the presence of *L. chagasi* could attract macrophages (**Fig. 5A**) and that *Lu. longipalpis* saliva induced a synergistic effect (Fig. 5A). Analyses of the MCP-1 (CCL2) revealed that neutrophils incubated with *L. chagasi* plus SGS produced significantly higher amounts of this chemokine (Fig. 5B). To investigate whether the macrophage recruitment was a result of production of CCL2/MCP-1 induced by *L. chagasi* plus SGS, we previously treated the neutrophils with bindarit, an inhibitor of CCL2/MCP-1 synthesis, before incubation with SGS, *L. chagasi*, or both. Treatment with bindarit resulted in total reduction of macrophage chemotaxis (Fig. 5B).Taken together, these results indicate that SGS synergizes with *L. chagasi* to enhance neutrophil apoptosis, CCL2/MCP-1 production, and macrophage recruitment.

DISCUSSION

The present study provides the first evidence that salivary components from a *Leishmania* vector play a relevant and direct role on neutrophils, which in turn, influence the *L. chagasi* parasite burden. We found that *Lu. longipalpis* salivary components induced neutrophil FasL-mediated and caspase-dependent apoptosis, and this event was associated with *Leishmania* survival inside these cells.

Neutrophils are now generally considered an initial target of *Leishmania* parasites [10, 31]. Significant numbers of neutrophils are present at the parasite inoculation site, as well as in lesions and draining LNs in *Leishmania* experimentally infected mice [11, 32–35]. Moreover, *Lu. longipalpis* SGS induces accumulation of neutrophils on an air-pouch model [20]. These experimental data are reinforced by the the fact that massive dermal neutrophilic infiltrates are noted in *Lu. longipalpis* [13] and *Phlebotomus duboscqi* bite sites [10], suggesting that accumulation of this cell type may be orchestrated, at least in part, by sandfly saliva constituents. Besides neutrophil recruitment, there are no previous reports about the
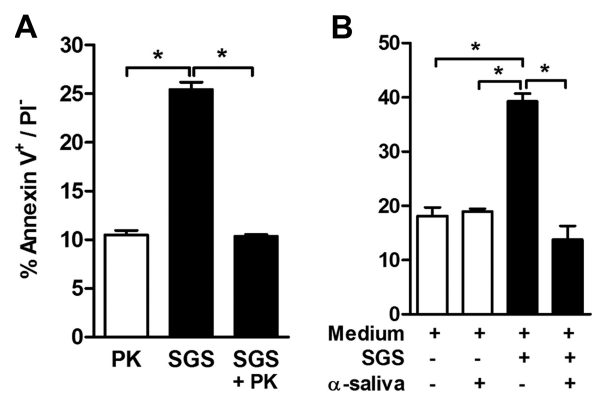
Figure 3. Inhibition of neutrophil apoptosis after *Lu. longipalpis* SGS treatment with proteinase K and α-saliva serum. Annexin-V staining from C57BL/6 mice neutrophils incubated for 20 h with SGS pretreated with proteinase K (PK; A) or with SGS preincubated for 1 h with anti-*Lu. longipalpis* saliva serum (B). Data shown are from a single experiment representative of three independent experiments. *$P \leq 0.05$.

Figure 4. Effect of *Lu. longipalpis* SGS on neutrophil apoptosis and infection.
(A) Inflammatory neutrophils from C57BL/6 mice were kept unstimulated (−) or stimulated with SGS (0.5 pair/well), *L. chagasi* (*L.c.*; 2:1) or SGS + *L. chagasi*. After 20 h, apoptosis was assessed by annexin-V staining. (B) In vitro neutrophil infection in the presence of SGS or etoposide (20 μM), followed by cultivation at 26°C and viable promastigote counts after 1, 2, and 3 days. (C) Representative image of *L. chagasi*-infected apoptotic neutrophils stimulated with *Lu. longipalpis* SGS (0.5 pair/well; original magnification, ×1000). Arrows point to infected apoptotic neutrophils. (D) Prior treatment of neutrophils with z-VAD (100 μM) and NS-398 (1 μM), followed by infection in the presence or absence of SGS. Viable promastigote counts were performed after 3 days. (E) PGE$_2$ levels of supernatants from neutrophils incubated for 20 h with *L. chagasi* and/or SGS (left side). ROS production by neutrophils cultured with *L. chagasi* for 1 h in the presence or absence of SGS
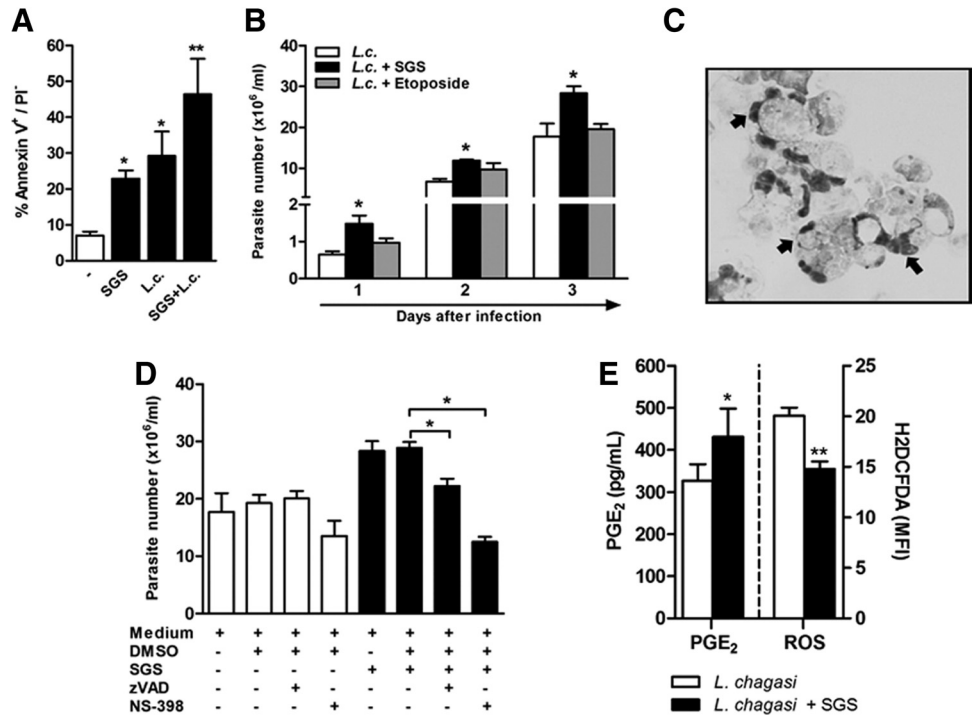
(right side). Neutrophils were incubated with H$_2$DCFDA, and ROS production was evaluated by flow cytometry. Data shown are from a single experiment representative of three independent experiments. *$P \leq 0.05$; **$P \leq 0.01$.

further effects of sandfly saliva on neutrophils. Interestingly, studies performed with tick saliva reveal that the inhibition of critical functions of neutrophils favors the initial survival of spirochetes [36–38].

Our findings on human neutrophils confirm apoptosis induction by SGS and interestingly, indicate that mice and human neutrophils have a different kinetic of spontaneous and saliva-induced apoptosis. Notably, the apoptosis of human neutrophils induced by *Lu. longipalpis* SGS also indicates that this mechanism may be important for the pathogenesis of human disease. Indeed, phagocytosis of apoptotic human neutrophils increases parasite burden in macrophages infected with *Leishmania amazonensis* [28].

It is likely that proteins from SGS trigger neutrophil apoptosis, as reincubation of *Lu. longipalpis* SGS with proteinase K abrogated its proapoptosis effect. Additionally, antisaliva serum was able of block neutrophil apoptosis. This is particularly interesting, as it reinforces the idea of a host protection mediated by the immune response against sandfly saliva, allowing for the development of an immune response against *Leishmania*. Interestingly, SGS-induced neutrophil apoptosis was associated with caspases and FasL expression. Previous studies have implicated FasL in neutrophil apoptosis [39]. Likewise, turnover of neutrophils mediated by FasL drives *Leishmania major* infection [22]. Further studies are necessary to deeply address this observation.

Our results demonstrate that SGS increases the neutrophil leishmanial burden by inducing neutrophil apoptosis, as inhibition of apoptosis by z-VAD reduced the viable parasite numbers in vitro. Indeed, treatment with z-VAD blocks lymphocyte

apoptosis and increases in vitro and in vivo resistance to *Trypanosoma cruzi* infection [30, 40]. van Zandbergen and colleagues [12] have proposed that infected apoptotic neutrophils can serve as "Trojan horses" for *Leishmania*. Alternatively, uptake of parasites egressing from dying neutrophils in an anti-inflammatory environment created by the phagocytosis of these cells, per se, could favor the infection ("Trojan rabbit" strategy) [41]. Our findings that *Lu. longipalpis* SGS could favor neutrophil apoptosis and infection by *L. chagasi* seem to give support to either of these two proposed hypotheses.

We found that neutrophil infection in the presence of SGS induced PGE$_2$ release, but was decreased in the presence of COX-2 inhibitor NS-398, indicating the participation of COX-2 products in parasite survival. Indeed, PGE$_2$, a major product from COX-2, facilitates *Leishmania* infection by deactivating macrophage microbicidal functions [19, 28–30]. Moreover, addition of exogenous PGE$_2$ to macrophage cultures induces a marked enhancement of *Leishmania* infection [19, 42]. Exposure of neutrophils to SGS caused a marked reduction of ROS production, which is a primarily important microbicidal mechanism of neutrophils. In this regard, *Lu. longipalpis* salivary proteins could be contributing to deactivation of the neutrophil inflammatory response, favoring the early steps of *Leishmania* infection. Taken together, our data suggest that the presence of sandfly SGS drives an anti-inflammatory response in *L. chagasi*-infected neutrophils by initially reducing ROS production, favoring the parasite survival. Furthermore, SGS could be triggering neutrophil deactivation through induction of apoptosis, activation of COX-2, and PGE$_2$ production by these cells. *L. major* promastigotes drive a selective fusion of azuro-
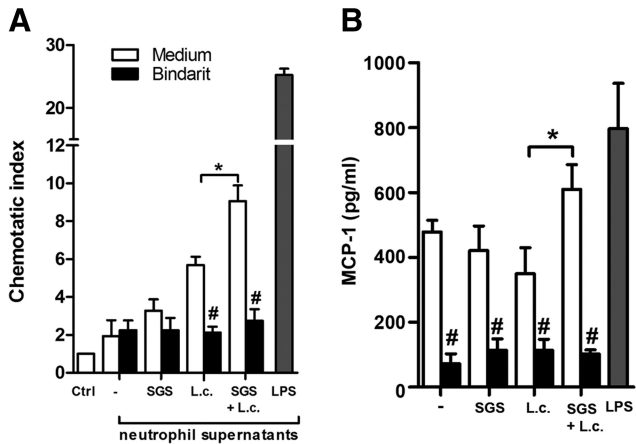
Figure 5. Macrophage recruitment and CCL2/MCP-1 release by *L. chagasi*-infected neutrophils in the presence of *Lu. longipalpis* SGS. (A) Macrophages were allowed to migrate toward supernatants from neutrophils infected or not with *L. chagasi* in the presence or absence of SGS (white bars), as described in Materials and Methods. Migration toward supernatants from bindarit-pretreated neutrophils (black bars). Following incubation, the migrated macrophages were counted, and the chemotatic index was calculated. Crtl, Negative control of radom chemotaxis. (B) CCL2/MCP-1 production (white bars) in the supernatants of neutrophil cultures after 20 h and its inhibition by bindarit pretreatment (black bars). Data are representative of two independent experiments performed in triplicate for each sample. *$P \leq$ 0.05; #$P \leq$ 0.05, compared with no bindarit-treated neutrophils.

philic granules into parasite-containing phagosomes in human neutrophils [43]. It remains to be elucidated whether, in the present system, SGS modulates neutrophil granule mobilization and contributes to early *L. chagasi* survival.

Macrophages are the preferential host cells for *Leishmania*, and the recruitment of these cells could provide safe havens for the parasite [31]. Neutrophils infected by *L. major* produce chemokines such as MIP-1β [12, 44], and sandfly SGS leads to increased expression of the macrophage chemokine MCP-1 at the site of injection [20], leading to macrophage recruitment. We have shown here that neutrophils infected with *L. chagasi* in the presence of SGS displayed higher MCP-1 production, corroborating with macrophage recruitment. This result was reinforced with the use of bindarit, an original indazolic derivative that has been shown the ability to inhibit CCL2/MCP-1 synthesis [45]. As a matter of fact, *L. chagasi*-infected neutrophil supernatants are able to recruit mouse macrophages, even though they did not induce significant MCP-1 production, which suggests that other chemotatic factors could be implicated in this event. A direct chemotactic activity of sandfly saliva has been described with several experimental models [13, 20, 46]. Herein, we also report an indirect chemotactic effect of SGS by inducing chemokine production by neutrophils.

In summary, our data demonstrate that *Lu. longipalpis* saliva orchestrates FasL- and caspase-dependent apoptosis of neutrophils. At the same time, saliva proapoptosis activity is of benefit to the parasite and may represent an important mechanism to facilitate *Leishmania* infection. These results contribute to a better understanding of the interactions be-

tween vector saliva and neutrophils in innate immunity to *Leishmania* infection.

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