EFFECT OF *LUTZOMYIA LONGIPALPIS* SALIVARY GLAND EXTRACTS ON LEUKOCYTE MIGRATION INDUCED BY *LEISHMANIA MAJOR*

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Abstract. The mechanism by which the salivary gland lysate (SGL) of *Lutzomyia longipalpis* enables *Leishmania* infection remains under investigation. One possibility is that saliva promotes cellular recruitment leading to development of skin lesions. In this study, we investigated leukocyte recruitment induced by *L. major*, *L. major* + SGL, or SGL alone into the peritoneal cavity of BALB/c mice. The administration of *L. major* with or without SGL induced neutrophil migration six hours after infection. Interestingly, after seven days, the BALB/c mice still had eosinophils and mononuclear cells in their peritoneal cavities. Flow cytometric analysis showed an increase in the CD4+ CD45RB^{low} T cell subset (effector or memory cells) compared with the CD4+ CD45RB^{high} subset (naive cells). Moreover, the co-injection of *L. major* with SGL enhanced production of interleukin-10. These results suggest that SGL can facilitate *Leishmania* infection by modulating leukocyte recruitment and Th2 cytokine production at the inflammatory focus.

INTRODUCTION

Members of the genus *Leishmania* are sand fly–transmitted protozoan parasites that cause leishmaniasis, which affects 12 million individuals at any given time with 35 million at risk of infection worldwide.[1] Phlebotomine sand flies acquire the pathogen by feeding on an infected host and transmit the parasite by regurgitating the parasite at the site of a subsequent blood meal.[2] During natural transmission, *Leishmania* promastigotes are inoculated into the vertebrate skin together with saliva. Once inoculated into the skin, the promastigotes rapidly invade macrophages at the bite site of the vector and replicate as intracellular amastigotes.[3]

The immune response to *Leishmania major* has been extensively characterized and represents one of the best-studied models in which selective activation of Th1 and Th2 cells occurs. In the resistant host (e.g., C57BL/6 mice), the Th1 response predominates, whereas, in the susceptible host (e.g., BALB/c mice) the Th2 response predominates.[3,4] The main mechanism for killing of *Leishmania* as well as other intracellular parasites in the host depends on the induction of inducible nitric oxide synthetase (NOS) with consequent production of nitric oxide. Several studies have shown that Th1 cytokines (e.g., interferon-γ [IFN-γ], tumor necrosis factor-α [TNF-α], interleukin 18 [IL-18]) induce expression of iNOS.[3–5] Conversely, Th2 cytokines (IL-14, IL-13, and IL-10) decrease the leishmanicidal activity of murine and human macrophages due to downregulation of the expression of iNOS.[6]

It has been shown that *Lutzomyia longipalpis* saliva enhances *L. major* infection in mice.[7] In the saliva of phlebotomines, a variety of anticoagulant, antiplatelet, and vasodilatory molecules are found that facilitate blood feeding. Many of these molecules are immunogenic and elicit antibody production and/or pro-inflammatory cellular responses and en-

hance the ability of pathogens to establish themselves in the host.[8,9] Different mechanisms probably contribute to this phenomenon, including inhibition of antigen presentation, reduction in nitric oxide production in *Leishmania*-infected macrophages, and enhancement of IL-4 secretion by T lymphocytes.[10–12]

Leukocytes play an important role in host defense, and their recruitment to infected tissue might be a crucial event in the control of infections such as leishmaniasis. In the present study, we investigated the influence of salivary gland lysate (SGL) of *Lu. longipalpis* on inflammatory response induced by *L. major* inoculation in resistant and susceptible animals. To this end, we evaluated leukocyte recruitment into the peritoneal cavity of BALB/c or C57BL/6 mice injected with *L. major* in the presence or absence of SGL of *Lu. longipalpis*. The peritoneal cavity was chosen as the site of infection because it is self-contained and delineated and thus is an ideal choice for determining the number and the phenotypes of immigrating leukocytes during the early phase of *L. major* infection.

METHODS

Mice. Four- to five-week-old female BALB/c and C57BL/6 mice were used in all experiments. The animals were obtained from the Animal Facility of Ribeirão Preto School of Medicine, São Paulo University, Brazil, and were maintained in sterile isolators with sterile food and water until use in the Department of Pharmacology. All experiments were conducted in accordance with National Institutes of Health guidelines regarding the welfare of experimental animals and with the approval of the Animal Ethics Committee at our Institution.

Parasite. *Leishmania major* (strain LV39) were cultured in Schneider's *Drosophila* medium supplemented with 10% fetal calf serum. Stationary-phase forms of the parasite were used in all experiments.

Sand fly salivary glands. Salivary glands from sugar-fed 3–7-day-old female *Lu. longipalpis* sand flies (Belém-Pará-Brazil) were dissected in water containing 0.1% (w/v) bovine

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serum albumin (Sigma, St. Louis, MO). To achieve complete disruption, the glands were freeze-thawed and vortexed. The osmolarity was reconstituted by adding 10× phosphate-buffered saline (PBS). Lysates were stored at -70 C until use.[7]

Infection of mice. Groups of five BALB/c or C57BL/6 mice were injected into the peritoneal cavity with *L. major* (10^5 promastigotes) or 10^5 *L. major* plus a lysate of one salivary gland of the sand fly *Lu. longipalpis*. The control groups were injected with saline or lysate of one salivary gland alone.

Determination of leukocyte migration into the peritoneal cavity. Leukocyte migration to the peritoneal cavity was evaluated at six hours and two and seven days after injections. At these time points, the animals were killed in a CO_2 chamber and cells in the peritoneal cavity were collected by injecting 3 mL of PBS containing 1 mM EDTA. Total counts were made in a cell counter (Beckman Coulter Inc., Fullerton, CA), and differential cell counts (neutrophils, eosinophils, and mononuclear cells) were made on cytocentrifuge slides (Cytospin3; Shandon Southern Products, Astmoore, United Kingdom) stained by the May-Grünwald-Giemsa method. The results are expressed as the number of leukocytes per cavity.

Determination of lymphocyte migration into the peritoneal cavity by flow cytometric analysis. Samples of 10^6 cells, obtained from the peritoneal exudate were suspended and incubated in PBS containing 2% fetal bovine serum and FcγIIR block monoclonal antibody (MAb) (CD16/CD32) for 30 minutes at 4°C to avoid nonspecific background staining. After the blocking step, cells were labeled with 1 μL of fluorescein isothiocyanate (FITC)–labeled MAb to CD4 or CD8 or phycoerythrin (PE)–labeled MAb to CD3 or CD45RB diluted in PBS-S for 30 minutes at 4°C. After labeling, the cells were washed and suspended in PBS/0.1% formaldehyde for data acquisition by fluorescence-activated cell sorting (FACS) in an FACSort (Becton Dickinson, Franklin Lakes, NJ). Erythrocytes and dead cells were excluded by their forward and side scatter, and at least 10^4 of each lymphocyte population was counted using CellQuest software (Becton Dickinson). Determination of positive and negative populations was performed based on the control staining with an irrelevant IgG isotype labeled with FITC or PE. Once determined, quadrants were rigorously maintained for all analyses.

Cytokine measurements. The concentrations of IFN-γ and IL-10 in the peritoneal exudates were determined by using an enzyme-linked immunosorbent assay (ELISA). Briefly, each well of flat-bottomed 96-well microtiter plates was coated with 100 μL of antibody specific to one of the cytokines at a dilution of 2 μg/mL of IFN-γ or 1 μg/ml of IL-10 (R&D Systems, Minneapolis, MN) in coating buffer and incubated overnight at 4°C. Plates were then washed, and nonspecific binding was blocked for 120 minutes at 37°C with 1% bovine serum. Samples (non-diluted) and standards were loaded onto plates. Recombinant murine IFN-γ and IL-10 standard curves (R&D Systems) were used to calculate the cytokine concentrations. The plates were thoroughly washed, and the appropriate biotinylated polyclonal or monoclonal anti-cytokine antibody was added. After 1 hour, the plates were washed, avidin peroxidase (diluted 1:5,000) was added to each well for 15 minutes, and the plate was thoroughly washed again. Next, the substrate (0.4 mg of *o*-phenylenediamine and 0.4 μL of H_2O_2 in 1 mL of substrate buffer) was added. The reaction was terminated with 1 M H_2SO_4 and the optical density was measured on an ELISA plate scanner (Spectra Mx 250; Molecular Devices, Menlo Park, CA) at 490 nm. The results were expressed as picograms of IFN-γ and IL-10 per milliliter of supernatant, and the optical density in samples was compared with that of standard curves.

Statistical analysis. Statistically significant differences between groups were determined by analysis of variance followed by Bonferroni's test. A *P* value < 0.05 was considered statistically significant. All experiments were done at least 2–3 times if not stated otherwise.

RESULTS

Effect of SGL from *Lu. longipalpis* upon leukocyte recruitment induced by *L. major*. The SGL of *Lu. longipalpis* promoted a marked enhancement of *L. major* lesions in the animals after 5–7 weeks of infection. This result suggested that components of SGL contained immunomodulatory activities. To test whether sand fly vector saliva interfered with leukocyte migration to infection sites, BALB/c or C57BL/6 mice were injected intraperitoneally with 10^5 *L. major*, 10^5 *L. major* plus SGL, SGL alone, or saline alone. Leukocyte migration was analyzed at six hours and two and seven days after injection. Compared with saline controls, significant neutrophil recruitment was observed six hours after administration of *L. major*, *L. major* plus SGL, or SGL alone. After this time point, neutrophil migration decreased in all groups over a 48-hour period, reaching the control levels two days after injection. However, in susceptible BALB/c mice, all stimuli were able to induce more neutrophil migration than in resistant C57BL/6 (Figure 1A and B, respectively).

Eosinophils (Figure 1C and D, susceptible and resistance mice, respectively) and mononuclear cells (Figure 1E and F, susceptible and resistance mice, respectively) showed similar time courses. Significant migrations were observed in the three experimental groups (SGL, *L .major*, and *L. major* plus SGL) two days after infection. However, after seven days, only groups inoculated with SGL (SGL alone or *L. major* plus SGL) remained statistically different compared with controls (saline alone). In susceptible BALB/c mice, all stimuli were able to induce higher numbers of eosinophils and mononuclear cells than in resistant C57BL/6.

Effect of SGL from *Lu. longipalpis* upon lymphocyte migration induced by *L. major*. To determine which lymphocyte sub-types were recruited into the peritoneal cavity, SGL was co-injected with or without 10^5 *L. major* into the peritoneal cavity of BALB/c or C57BL/6 mice. The phenotype of lymphocytes that migrated into the peritoneal cavity was analyzed by FACS at six hours and two and seven days after injection. Significant total lymphocyte (T and B lymphocytes) recruitment was observed two days after administration of *L. major*, *L. major* plus SGL, or SGL alone in BALB/c mice (Figure 2A) and C57BL/6 (Figure 2B). After this time point, only peritonea in BALB/mice injected with *L. major* plus SGL contained increased numbers of T lymphocytes (Figure 2A). In C57BL/6 mice, the numbers of these cells were increased in the animals injected with *L. major* plus SGL and SGL alone (Figure 2B). Among the T lymphocyte sub-types (CD4+ and CD8+), only CD4+ cells were increased in the peritonea of BALB/c mice seven days after injection in all experimental groups (*L. major*, *L. major* plus SGL, or SGL).
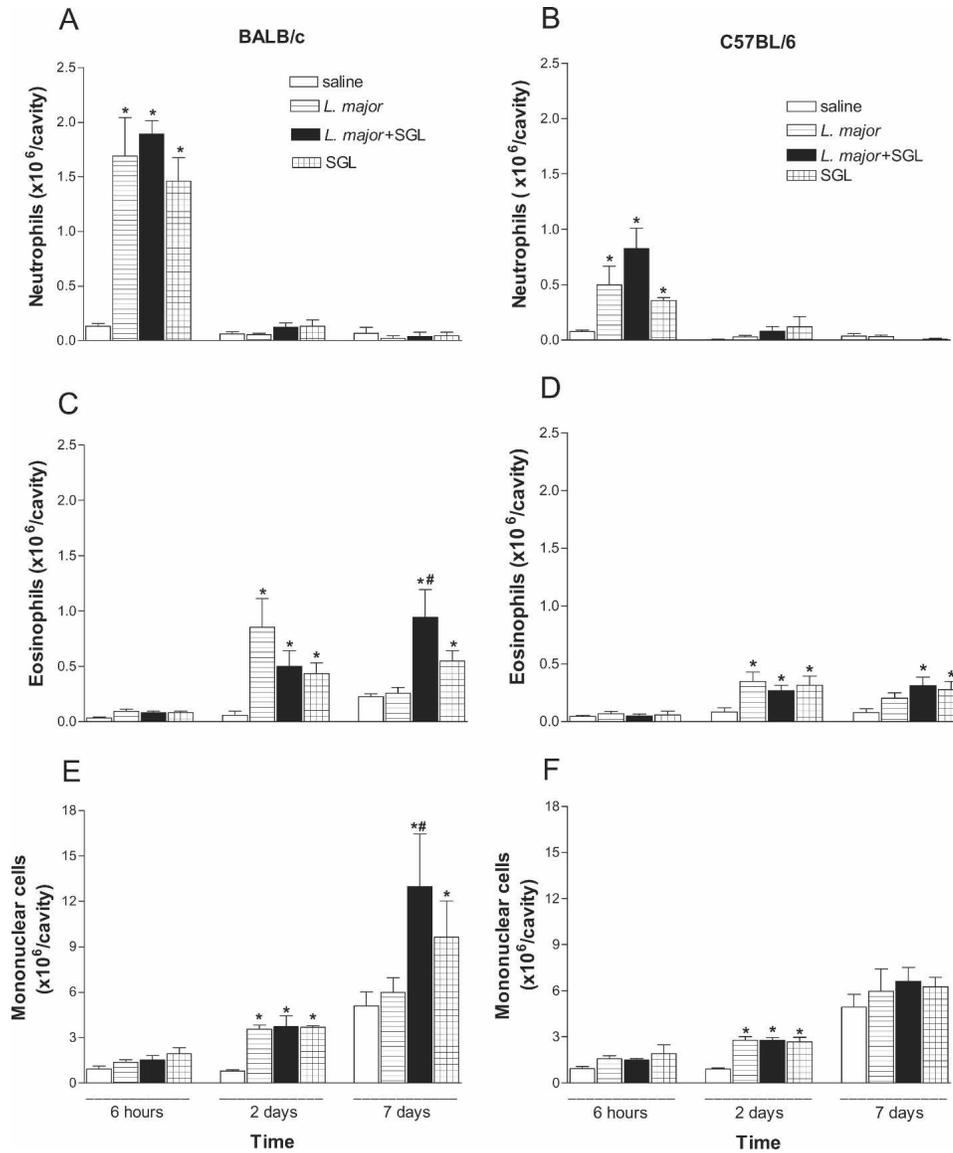
FIGURE 1.   Effect of salivary gland lysate (SGL) of *Lutzomyia longipalpis* on leukocyte migration into the peritoneal cavity of BALB/c mice (**A**, **C**, and **E**) and C57BL/6 mice (**B**, **D**, and **F**). Animals were injected intraperitoneally with saline or 10^5 *Leishmania major* promastigotes in the absence or presence of one salivary gland of *Lu. longipalpis* or one salivary gland of *Lu. longipalpis* alone. Results are representative of three experiments (n = 15) and are expressed as the mean ± SE leukocytes per cavity. *$P < 0.05$ compared with saline control group. #$P < 0.05$ compared with *L. major* group (analysis of variance followed by Bonferroni *t*-test).

No significant CD4+ or CD8+ migration was observed in C57BL/6 mice and no CD8+ migration was observed in BALB/c mice. The CD4+ migration in BALB/c induced by *L. major* plus SGL was higher than in other experimental groups (SGL or *L. major* alone; Figure 3).

Effect of SGL from *Lu. longipalpis* upon CD4+ T lymphocyte subset migration. To determine which CD4+ lymphocyte subset was recruited into the peritoneal cavity after SGL co-injection with 10^5 *L. major*, migration of CD4+ CD45RB^{high} and CD4+ CD45RB^{low} were analyzed by FACS in BALB/c and C57BL/6 mice. Migration was determined seven days after injection of *L. major*, *L. major* plus SGL, SGL, or saline alone. The CD4+ CD45RB^{low} T cells population (CD4+ T effector/memory lymphocytes) increased in the peritoneal cavities of BALB/c and C57BL/6 mice injected with *L. major* plus SGL or SGL alone. Migration was higher in BALB/c

mice than in C57BL/6 mice (Figure 4A). Moreover, the CD4+ CD45RB^{high} T cell population (CD4+ T naive lymphocytes) in the peritoneal cavities increased mainly in BALB/c mice injected with *L. major* alone or *L. major* plus SGL, although C57BL/6 mice also showed a significant increase in this CD4+ lymphocyte subset when injected with *L. major* plus SGL and SGL alone (Figure 4B).

Effect of SGL from *Lu. longipalpis* upon production of IL-10 and IFN-γ. Resistance to *L. major* infection is associated with the production of IFN-γ, and susceptibility to infection is associated with production of IL-10. Therefore, we next tested whether SGL interferes with production of these cytokines. BALB/c mice were injected with saline, *L. major*, *L. major* plus SGL, or SGL alone and the presence of IL-10 and IFN-γ in the peritoneal exudates was determined six hours and two and seven days later. A significant increase in
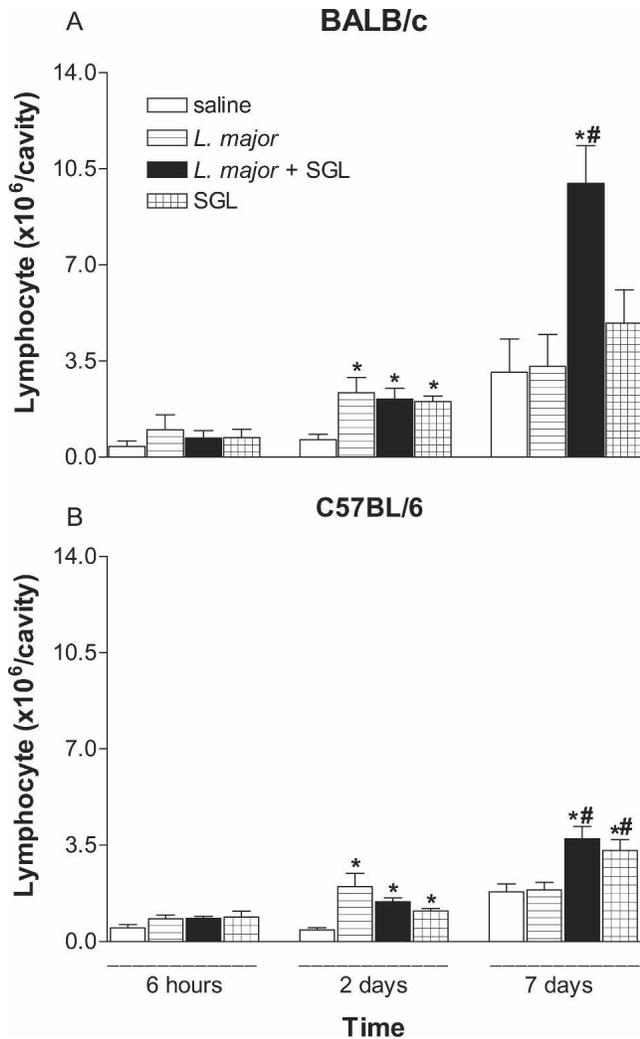
FIGURE 2. Effect of salivary gland lysate (SGL) of *Lutzomyia longipalpis* on total lymphocyte migration into the peritoneal cavity of **A**, BALB/c and **B**, C57BL/6 mice. Animals were injected intraperitoneally with saline or 10^5 *Leishmania major* promastigotes in the absence or presence of one salivary gland of *Lu. longipalpis* or one salivary gland of *Lu. longipalpis* alone. The lymphocytes were gated based on forward scatter/side scatter light scattering. Results are representative of three experiments (n = 15) and are expressed as the mean ± SE lymphocytes per cavity. *$P < 0.05$ compared with saline control group. #$P < 0.001$ compared with *L. major* group (analysis of variance followed by Bonferroni *t*-test).

FIGURE 3. Effect of salivary gland lysate (SGL) of *Lutzomyia longipalpis* on CD4$^+$ and CD8$^+$ T lymphocyte migration into the peritoneal cavity of BALB/c and C57BL/6 mice. The animals were injected intraperitoneally with saline or 10^5 *Leishmania major* (Leish) promastigotes in the absence or presence of one salivary gland of *Lu. longipalpis* or one salivary gland of *Lu. longipalpis* alone. The CD4$^+$ and CD8$^+$ T lymphocytes were stained with anti-CD4 and anti-CD8 antibodies. Results are representative of three experiments (n = 15) and are expressed as the mean ± SE lymphocytes per cavity. *$P < 0.05$ compared with saline control group. #$P < 0.001$ compared with *L. major* group (analysis of variance followed by Bonferroni *t*-test).

IL-10 concentration in the exudates of BALB/c injected with *L. major* plus SGL was observed two and seven days after injection. The injection of SGL or *L. major* alone induced significant production of IL-10 only at seven days after injection (Figure 5A). Concerning the production of IFN-γ, there were significant concentrations of this cytokine in the exudates in all times evaluated after injection of *L. major* plus SGL. Mice injected with *L. major* showed an increase in IFN-γ concentration after two days, and SGL alone induced only the production of this cytokine seven days after injection (Figure 5B).

DISCUSSION

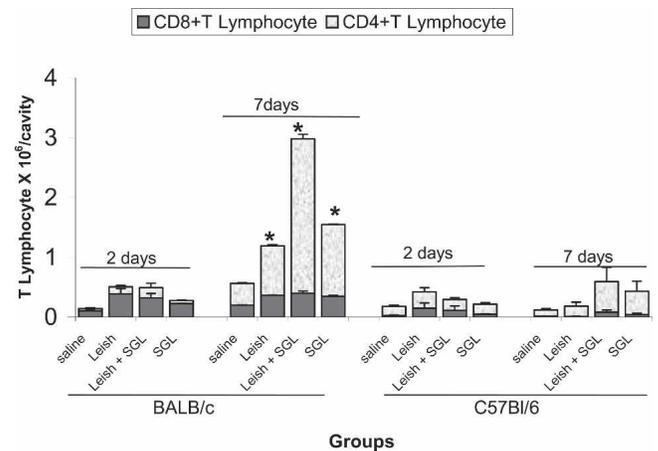Injecting *Lu. longipalpis* SGL and *L. major* into the mouse peritoneal cavity to monitor the initial phases of the parasite infection resulted in an inflammatory reaction characterized by neutrophil, eosinophil and T CD45RB+ migration. In addition, considerably more IL-10 than IFN-γ was produced. This suggests that SGL may preferentially induce type 2 cytokines and the sequential migration of neutrophils, eosinophils, and CD4+ CD45RB^{low} cells.

This study was designed to examine different inflammatory parameters mimicking the transmission of *L. major* to its host. There are few experimental models for isolating leukocytes from the inflammatory site. We therefore used the peritoneal cavity to analyze the inflammatory exudates induced by SGL. This model has been extensively used in several areas of scientific research. Recently, we analyzed the effect of SGL in ovalbumin-induced immune peritonitis.[13] However, it has not been used to evaluate the effect of SGL on infection by *L. major*, although it is well known that sand fly saliva exacerbates *Leishmania* infection.[7] Moreover, any experimental model of intradermal inoculation has demonstrated host immuno-modulation by SGL in *L. major* infection.[14]

Leishmania is an intracellular parasite that adopts several strategies for survival and replication in its host, and sand fly saliva may represent one of those strategies.[15] This effect has been demonstrated by the ability of saliva to exacerbate *Leishmania* infection.[7,16,17] Our data confirmed the results of previous studies in which *Lu. longipalpis* SGL exacerbated *L. major* lesions in susceptible mice.

The mechanism by which vector saliva contributes to *Leishmania* infection remains under investigation. Several studies demonstrated that salivary immunosuppressive components inhibit nitric oxide, hydrogen peroxide production, and antigen presentation by infected macrophages.[11] Furthermore, these components inhibit Th1 induction, enhancing the Th2-type response with an increase in IL4 and IL-10 in the infection focus.[10,12] Our results with cytokine levels confirm and expand these results.
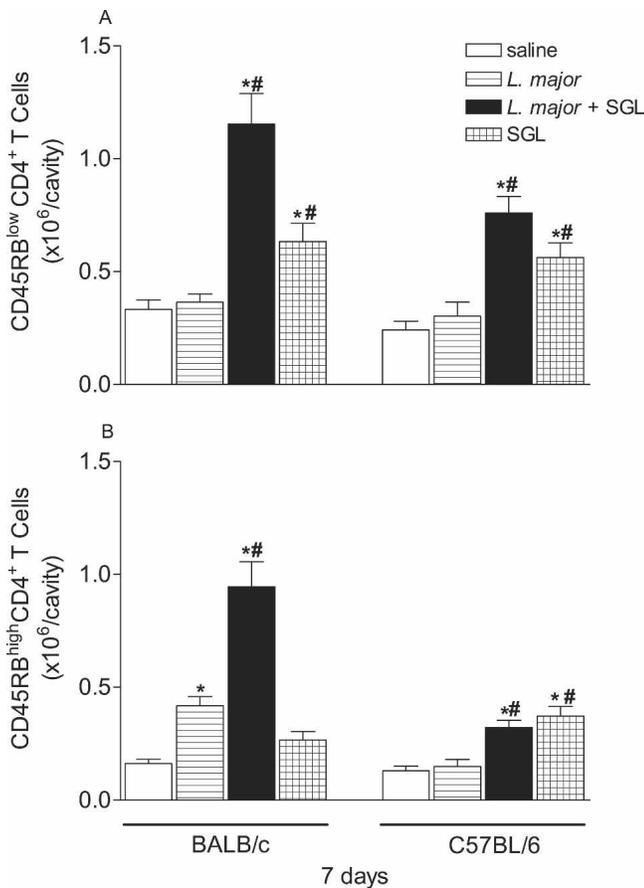
FIGURE 4. Effect of salivary gland lysate (SGL) of *Lutzomyia longipalpis* on **A**, CD45RB^{low} CD4^{+} and **B**, CD45RB^{high} CD4^{+} T lymphocyte migration into the peritoneal cavity of BALB/c and C57BL/6 mice. The animals were injected intraperitoneally with saline or 10^5 *Leishmania major* promastigotes in the absence or presence of one salivary gland of *Lu. longipalpis* or one salivary gland of *Lu. longipalpis* alone. The CD45RB^{+} CD4^{+} T lymphocytes were stained with anti-CD4 and anti-CD45RB antibodies. Results are representative of three experiments (n = 15) and are expressed as the mean ± SE lymphocytes per cavity. *P < 0.05 compared with saline control group. [#]P < 0.001 compared with *L. major* group (analysis of variance followed by Bonferroni *t*-test).

Previous studies have shown that neutrophils are the first leukocytes that appear at the subcutaneous site of *L. major* infection.[17–21] Here we demonstrated that the administration of *L. major* with or without SGL induced neutrophil migration after six hours into the peritoneal cavity of BALB/c and C57BL/6 mice. Several studies demonstrated that neutrophils could serve as host cells for *Leishmania* in the early phase of infection.[21,22] Studies reported that *Leishmania* infection extends the life span of neutrophils through inhibition of spontaneous apoptosis, and the early influx of neutrophils into the infected tissue is associated with the development of more severe disease. The depletion of neutrophils at the time of *L. major* challenge in BALB/c mice inhibited the IL-4 response and promoted partial resistance, suggesting that neutrophils might contribute to disease progression.[18,22] However, the protective role for neutrophils in leishmaniasis was shown in treatment of BALB/c and C57BL/6 mice with MAb RB68C5, an anti-polymorphonuclear leukocyte antibody, which resulted in more severe footpad lesions and an increase in the parasite load in draining lymph nodes.[19]
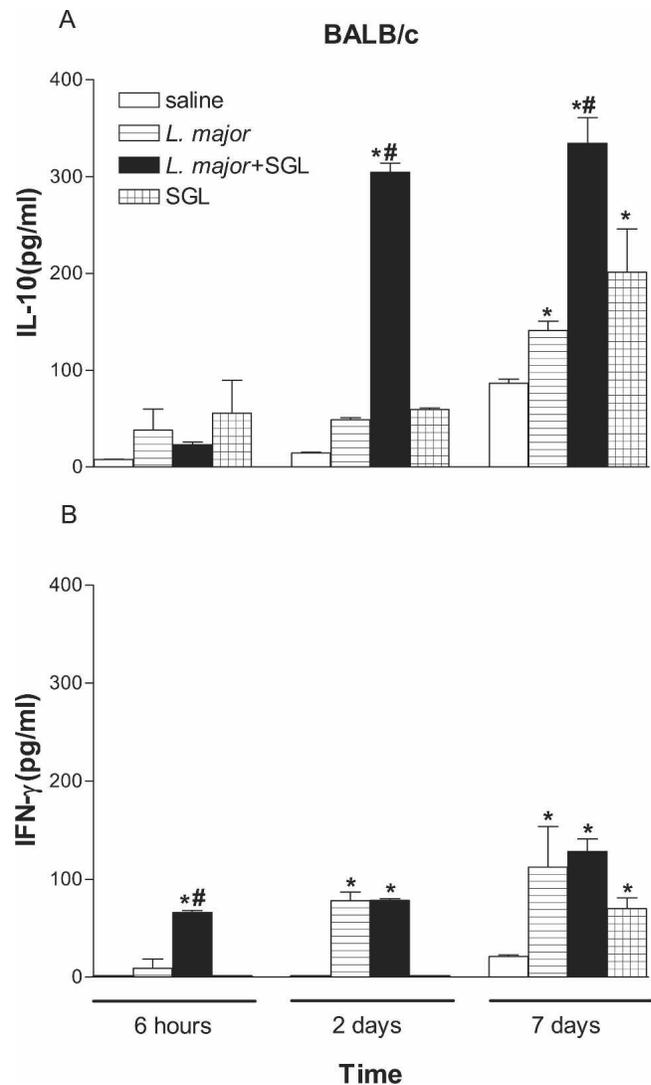

FIGURE 5. Concentration of **A**, interleukin 10 (IL-10) and **B**, interferon-γ (IFN)-γ in the peritoneal exudate of BALB/c mice challenged with saline or 10^5 *Leishmania major* promastigotes in the absence or presence of one salivary gland of *Lutzomyia longipalpis* or one salivary gland of *Lu. longipalpis* alone. Results are representative of three experiments (n = 15) and are expressed as the mean ± SE picograms per milliliter. *P < 0.05 compared with saline control group. [#]P < 0.05 compared with *L. major* group (analysis of variance followed by Bonferroni *t*-test). SGL = salivary gland lysate.

Eosinophils were present 48 hours after injection of *Leishmania* and SGL into the peritoneal cavity. These kinetics may be influenced by mast cells present at the site of injection.[23] Other studies have shown that IL-10 induces a Th2 response and eosinophil infiltration in allergy models.[24,25] Interleulin-4 has also been described as an inducer of eosinophil migration.[26] In addition, the presence of eosinophils has been associated with the initial phase of infection with *Leishmania*.[27] The experiments presented here offer further evidence that eosinophils have a role in the response to infection with *Leishmania*.

Some studies have demonstrated that CD4^{+} CD45RB^{low} T cells produce IL-4 and IL-10 and can inhibit colitis.[28,29] Therefore, one plausible explanation for our findings is that the co-injection of SGL increased IL-10 production at the

inflammatory focus by CD4+ CD45RB^{low} T cells. It has been shown previously that the early increase of IL-4 and IL-10 production is a hallmark of susceptibility to infection with *Leishmania*.[10,12] Studies demonstrated that mice co-injected with *L. amazonesis* and SGL displayed higher levels of IL-10 mRNA in the ear tissues than controls.[29,30] However, studies with IL-4-deficient mice indicated that IL-4 is not sufficient, in some cases, for susceptibility to *L. major*.[31,32] In addition, the severity of disease in IL-4-deficient mice infected by *L. major* and co-injected with SGL of *Phlebotomus papatasi* sand flies did not reduce in comparison with wild-type controls.[33] Interleukin-10 has been previously implicated in disease progression and long-term persistence of *Leishmania* in both human and experimental animal infections.[34,35] Furthermore, IL-10 and IL-4 inhibit TNF-α, IL-1β, and leukotriene B$_4$ production, downregulate major histocompatibility complex class II expression, inhibit antigen presentation by dendritic cells and monocytes/macrophages, and inhibit T lymphocyte proliferation in Th1 the immune response.[36–38]

In conclusion, our results confirm the importance of SGL in *Leishmania* infection as an inducer of a type 2 response, as well as recruitment of cells such as eosinophils and CD4+ CD45RB^{low} T cells. They also confirm the importance of early production of IL-10 for parasite success. In addition, these results lend further support to the concept that diseases transmitted by arthropods such as sand flies might be prevented by vaccinating the host against components of arthropod saliva so as to block their disease-enhancing effects.

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