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Saliva from *Lutzomyia longipalpis* Induces CC Chemokine Ligand 2/Monocyte Chemoattractant Protein-1 Expression and Macrophage Recruitment[1]

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Saliva of bloodfeeding arthropods has been incriminated in facilitating the establishment of parasite in their host. We report on the leukocyte chemoattractive effect of salivary gland homogenate (SGH) from *Lutzomyia longipalpis* on saliva-induced inflammation in an air pouch model. SGH (0.5 pair/animal) was inoculated in the air pouch formed in the back of BALB/c or C57BL/6 mice. *L. longipalpis* SGH induced a significant influx of macrophages in BALB/c but not in C57BL/6 mice. SGH-induced cell recruitment reached a peak at 12 h after inoculation and was higher than that induced by the LPS control. This differential cell recruitment in BALB/c mice was directly correlated to an increase in CCL2/MCP-1 expression in the air pouch lining tissue. In fact, treatment with bindarit, an inhibitor of CCL2/MCP-1 synthesis, and also with a specific anti-MCP-1 mAb resulted in drastic reduction of macrophage recruitment and inhibition of CCL2/MCP-1 expression in the lining tissue. CCL2/MCP-1 production was also seen in vitro when J774 murine macrophages were exposed to *L. longipalpis* SGH. The SGH effect was abrogated by preincubation with serum containing anti-SGH IgG Abs as well as in mice previously sensitized with *L. longipalpis* bites. Interestingly, the combination of SGH with *Leishmania chagasi* induced an increased recruitment of neutrophils and macrophages when compared with *L. chagasi* alone. Taken together these results suggest that SGH not only induces the recruitment of a greater number of macrophages by enhancing CCL2/MCP-1 production but also synergizes with *L. chagasi* to recruit more inflammatory cells to the site of inoculation. *The Journal of Immunology,* 2005, 175: 8346–8353.

During bloodfeeding, insect saliva is injected into the host's skin. This saliva contains a great variety of hemostatic, inflammatory, and immunomodulatory molecules. Understanding mammalian response to insect saliva is of utmost importance in several ways. Besides being related to allergy (1, 2), insect saliva is known to facilitate parasite survival (3–5). In mice, saliva from *Lutzomyia longipalpis* or *Phlebotomus papatasi* leads to larger *Leishmania major*-associated lesions than those resulting from the parasite alone (6).

The mammalian hosts do mount an anti-insect saliva response. The phlebotomine saliva enhancing effect on leishmaniasis can be abrogated by preexposure to immune sera (4, 5). Immune response against sand fly saliva is also observed in children from areas endemic for visceral leishmaniasis. Seroconversion against *L. longipalpis* saliva occurred at the same time as the host developed an anti-*Leishmania* cell-mediated immune response (7, 8).

Cell recruitment is a vital event in inflammation. Cell number and composition in the initial stages after stimuli greatly influences future responses and the development of acquired immune responses. Initial cell recruitment is also important in leishmaniasis (9, 10). Saliva from *Phlebotomus duboscqi* attracts vertebrate monocytes in vitro (11) and saliva from *P. papatasi* not only attracts macrophages but also enhances infection by *Leishmania donovani* in these cells, resulting in increased parasite load (12).

Synthesis of inflammatory mediators is a fundamental mechanism for leukocyte recruitment to an injured tissue. We thus investigated the chemotactic effect and mediators of salivary gland homogenate (SGH)[3] from *L. longipalpis* in vivo using the air pouch model because little is known about the influence of insect saliva on chemokine expression. We observed that *L. longipalpis* saliva is able to recruit a higher number of macrophages in the air pouch of BALB/c mice that correlates with the increase in CCL2/MCP-1 and CCR2 expression. Moreover, by using bindarit, a molecule that has been shown to inhibit CCL2/MCP-1 production both in vitro and in vivo (13–15) and MCP-1 neutralizing Abs, we observed a reinforced role of CCL2/MCP-1 in orchestrating the recruitment of macrophages to the BALB/c air pouch.

Materials and Methods

Mice

Inbred BALB/c and C57BL/6 mice of both sexes, 8–12 wk of age were obtained from the animal facility of Centro de Pesquisas Gonçalo Moniz-Fundação Oswaldo Cruz (CPqGM-FIOCRUZ, Bahia, Brazil). The experimental procedures were approved and conducted according to the Animal

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[3] Abbreviation used in this paper: SGH, salivary gland homogenate.

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Parasites

Leishmania chagasi (MHOM/BR00/MER/STRAIN2) promastigotes were cultured in Schneider's medium supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μl/ml streptomycin.

Air pouch and leukocyte migration

Air pouches were prepared by injecting 3 ml of air into the dorsal surface of BALB/c and C57BL/6 mice under light anesthesia. One hundred microliters of SGH (0.5 pair/animal) or SGH preincubated with anti-SGH serum (0.5 pair/animal plus 50 μl of serum preincubated for 1 h at 37°C) in endotoxin-free saline were injected into the air pouches immediately following an air injection, as previously described (16). Control mice were injected with 100 μl of endotoxin-free saline (negative control) or LPS (20 μg/ml; positive control). After 2, 4, 6, 12, and 24 h intrapouch inoculation, three to five animals per experimental group were lethally anesthetized, and the pouch washed with a total of 5 ml of endotoxin-free saline to collect leukocytes of the exudates. Lavage fluids were centrifuged at 100 $\times$ *g* for 10 min at 4°C, and pellets were resuspended in saline, stained in Turk's solution, and counted in a Neubauer hemocytometer. Differential cell counts were done microscopically on cytospin preparations stained with H&E.

Sand flies and preparation of salivary glands

L. longipalpis sand flies, 5- to 7-day-old females of the Cavunge strain, were reared at Laboratório de Imunoparasitologia (CPqGM) using a mixture of rabbit feces and rabbit ration as larval food. Sand fly colonies were maintained at 26°C. Adult male sand flies were offered a sucrose solution, and females were fed on lightly anesthetized hamsters (*Mesocricetus auratus*). Salivary glands were obtained from 5- to 7-day-old laboratory-bred *L. longipalpis* females. Salivary glands were dissected, placed in endotoxin-free PBS on ice, and stored at −70°C. Immediately before use, glands were sonicated and microfuged at 10,000 $\times$ *g* for 2 min, and the supernatant was used for the studies.

Oligonucleotide primers

Oligonucleotide primers specific for mouse chemokines and chemokine receptors were used to amplify cDNA by PCR. β-actin primers were used as a control to evaluate the expression of a housekeeping gene. The primers for chemokines, chemokine receptors, and β-actin were prepared at Invitrogen Life Technologies based on sequences published elsewhere (17, 18).

RNA isolation and cDNA preparation by reverse transcription

Total RNA was extracted from the air pouch lining tissue using TRIzol reagent (Invitrogen Life Technologies), according to manufacturer's instructions. Briefly, after cell lysis, RNA was precipitated with isopropanol, washed with 70% ethanol, and solubilized in diethylpyrocarbonate-treated water. RNA concentration and purity was determined by measuring at A_{260} and A_{280}, and samples were immediately stored at −70°C. cDNA synthesis was performed on ~1–2 μg of RNA in a total volume of 20 μl containing 2.5 μM oligo(dT)$_{12-18}$ primers, 1 mM dNTP (Invitrogen Life Technologies), 1× first-strand buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$), 20 U of RNase inhibitor, and 50 U of Superscript II reverse transcriptase (Invitrogen Life Technologies). The reaction mixture was incubated at 42°C for 50 min and stopped at 95°C for 5 min.

Chemokine and chemokine receptors mRNA detection

Expression of mRNA was analyzed by RT-PCR for the following: CCL2/MCP-1 (JE, murine homologue), CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCR2, and β-actin. A 5-μl aliquot of the cDNA obtained was amplified in a 25-μl reaction containing 1× PCR buffer (5 nmol KCl, 1 nmol Tris-HCl (pH 8.4), 1.5 nmol $MgCl_2$), 0.2 nmol each dNTP, 200 nmol each primer, and 1 U of TaqDNA polymerase (Invitrogen Life Technologies) in a PTC-100 thermal cycler (MJ Research). Reaction conditions were 30 cycles of 1 min at 94°C, 1 min at 54–55°C, and 2 min at 72°C, with a final extension step of 7 min at 72°C. For each set of primers, a negative sample (water) was run in parallel. PCR products were visualized by UV light after electrophoresis through a 6% acrylamide gel containing 0.5 μg/ml ethidium bromide. The sizes of the RT-PCR products were confirmed by comparison with a 100-bp ladder run in parallel on the same gel. The band intensity of the amplified products was analyzed using EagleSight v.3.2 software (Stratagene). The results are expressed as a ratio of expression of chemokine or chemokine receptor to β-actin expression.

J774 macrophage cell culture and stimulation

The mouse macrophage cell line J774E was maintained at 37°C in 5% CO_2 in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (HyClone Laboratories), 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin/ml (Invitrogen Life Technologies).

Cells (4×10^5 per ml) were plated in 24-well culture plates (Costar) containing 13-mm diameter glass coverslips (Novocastra Laboratories) in duplicates. After 24 h, macrophages were treated or not with LPS (50 μg/ml) or SGH (0.5 pair/well) or SGH preincubated for 1 h at 37°C with specific anti-SGH serum. Eight hours after stimulation, coverslips were removed and fixed with methanol for 5 min.

Immunohistochemical reactions

Methanol-fixed J774 cells on 13-mm diameter glass coverslips (Novocastra Laboratories) were processed for immunohistochemical assessment of chemokines. Coverslips were incubated with 10% nonfat milk for 20 min at room temperature to block nonspecific binding and to help prevent excessive drying out of the section. Primary Abs (50 μl, 1/100) of CCL2/MCP-1,

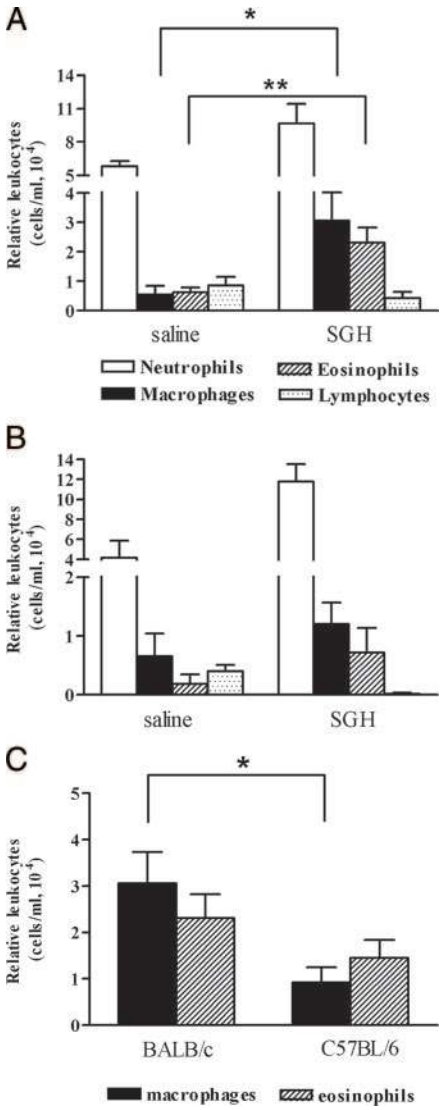

FIGURE 1. Leukocyte infiltration into BALB/c (*A*) and C57BL/6 (*B*) mice air pouch tissue. Pouches were stimulated with 0.1 ml of endotoxin-free saline, or *L. longipalpis* saliva (with SGH at 0.5 pair/animal). Twelve hours after stimulation, pouches were washed and differential cell counts performed in Turk's solution. Values represent mean ± SEM of four mice per group. *C*, Representation of difference between macrophage and eosinophil recruitment in BALB/c and C57BL/6 mice. Significant difference from value for macrophage (*, $p = 0.0390$) and eosinophils (**, $p = 0.0210$) between saline and SGH-treated groups.

CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, or CCR2 (Santa Cruz Biotechnology), diluted in PBS containing 0.01% of saponin, were added and incubated overnight at 4°C. Coverslips were washed three times with PBS containing 1% Triton X-100 (Amresco), 5 min each wash, and then secondary Ab (50 μl, 1/300 dilution of anti-rabbit or anti-goat biotin conjugate; Vector Laboratories) was added and incubated for 45 min in a moist chamber at room temperature. Coverslips were then washed three times with PBS containing 1% of Triton X-100 as previously described. Endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 20 min at room temperature, and then 50 μl of streptavidin-peroxidase (Vectastain ABC kit, mouse IgG; Vector Laboratories) was added for 30 min at room temperature. Chromogen reaction was developed with 3–3′-diaminobenzidine (DAB kit; Vector Laboratories) solution and counterstained with Mayer's hematoxylin. J774 cells stimulated with LPS were used as positive controls. Monoclonal Abs were substituted for nonimmune rabbit Igs or irrelevant mouse Abs as negative controls.

Treatments

Bindarit or 2-methyl-2-([1-phenylmethyl)-1H-indazol-3-yl]methoxy)propanoic acid (Angelini Farmaceutici) was administered at the dose of 200 mg/kg i.p. 1 h before and 7 h after air pouch induction and SGH stimulation (0.5 pair/animal). The dose of bindarit was chosen based on earlier reports showing that the molecule is a preferential inhibitor of CCL2/MCP-1 synthesis (13–15). For anti-MCP-1 mAb treatment, animals received an i.p. injection of purified neutralizing anti-MCP-1 mAb (R&D Systems) in a final volume of 200 μl (100 μg) 1 h before air pouch induction and SGH stimulation (0.5 pair/animal).

Sand fly anti-saliva serum

Hamsters (*M. auratus*) were exposed to bites from 5- to 7-day-old female *L. longipalpis* (Cavunge strain). Three animals were lightly anesthetized and exposed three times to ~50 bites every 15 days. Fifteen days after the last exposure, animals were sacrificed and serum was collected and tested for IgG anti-saliva detection by ELISA, as previously described (19).

Sensitization of mice

Groups of mice ($n = 10$) were sensitized five times by natural exposure to 15 uninfected *L. longipalpis* females in the left ear at 10-day intervals. A week after the last exposure, sera were collected to evaluate the presence of IgG anti-SGH. Mice considered positive (cutoff = 0.045) were used to induce air pouch stimulated with SGH as earlier described.

Statistical analysis

Data were reported as the mean ± SEM and were analyzed statistically by means of ANOVA or the Student t test with the level of significance at $p < 0.05$.

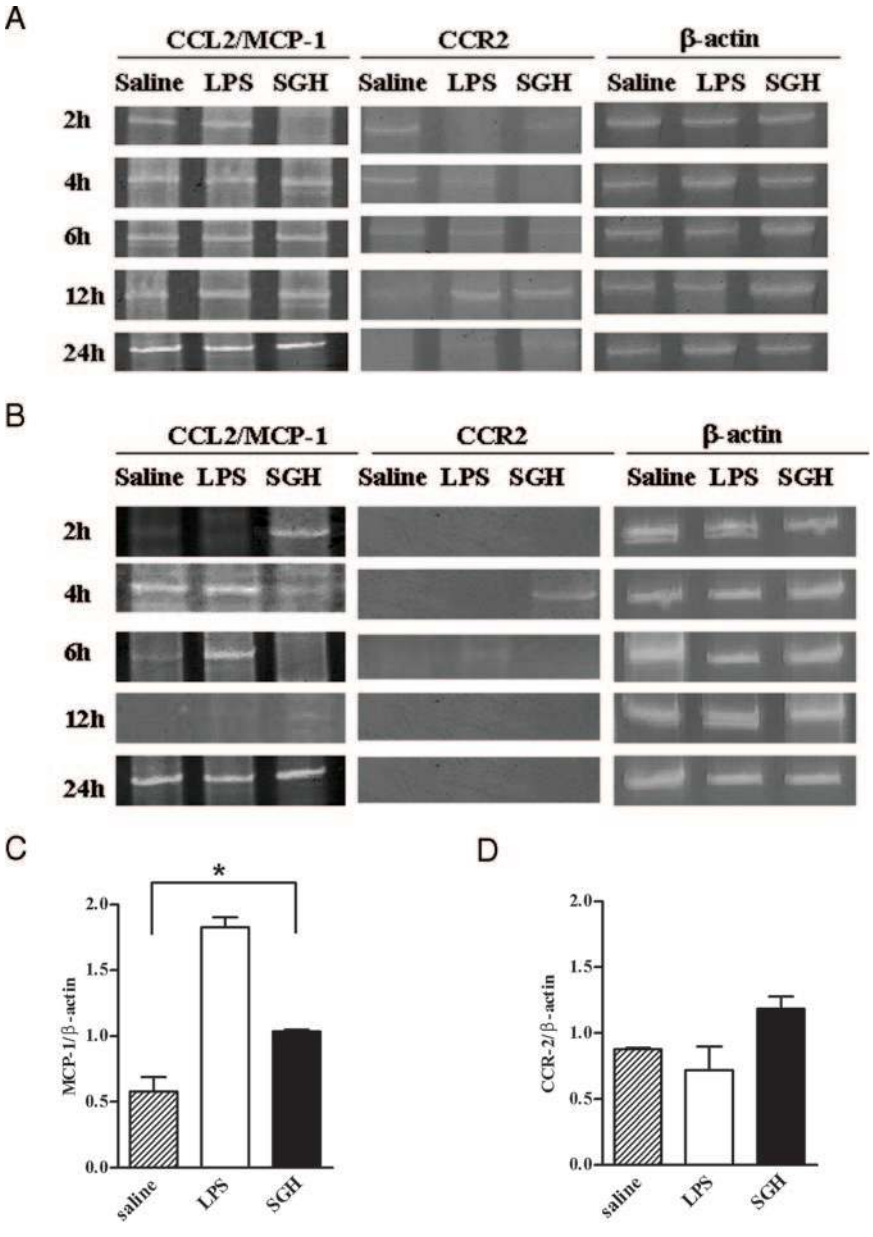

FIGURE 2. RT-PCR analysis of the expression of CCL2/MCP-1 and its receptor CCR2 from BALB/c (*A*) or C57BL/6 (*B*) air pouch lining tissue 2, 4, 6, 12, and 24 h after saline, LPS (20 μg/ml), or SGH (0.5 pair/animal) stimulation. Densitometric data of CCL2/MCP-1 (*C*) or CCR2 (*D*) expression in BALB/c mice at 12 h as the mean ± SEM of the ratio of chemokine to β-actin band from the same sample. The profiles are representative of at least two independent experiments. Significant difference (*, $p = 0.0033$) from value for negative control (saline) group and SGH-treated group.

Results

Effect of SGH on leukocyte recruitment in vivo

To investigate the inflammatory effect of *L. longipalpis* saliva we used the air pouch model previously described as an in vivo recruitment model that allows exudate analysis (20, 21). Air pouches were raised on BALB/c and C57BL/6 mice injected with SGH (0.5 pair/ml), LPS (20 μg/ml), or saline, and after 12 h the exudate was collected. Although few cells were found in the pouch exudate when endotoxin-free saline was injected, SGH from both *L. longipalpis* and LPS (data not shown) were potent recruiters for neutrophils and lymphocytes in either BALB/c or C57BL/6 mice (Fig. 1, *A* and *B*). However, only SGH induced an increase in macrophage and eosinophil recruitment in BALB/c mice, whereas this effect was not observed in C57BL/6 mice. This result was reinforced when the difference between macrophage and eosinophil recruitment in BALB/c and C57BL/6 mice was compared (Fig. 1*C*), showing that the increase was only observed in BALB/c mice after 12 h. In addition, our results describe an important inflammatory event driving macrophage chemotaxis mediated by SGH.

Expression of CCL2/MCP-1 in the air pouch lining tissue

After detecting the increase in macrophage recruitment due to SGH stimulation we decided to investigate the participation of the macrophage recruiting chemokines CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL2/MCP-1. Injection of SGH in the air pouch induced nonsignificant expression of CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES (data not shown). Of note after SGH stimulation, there was an expression of CCL2/MCP-1 at 4, 6, 12, and 24 h in the lining tissue of BALB/c, but only expression at 2 and 4 h in C57BL/6 mice (Fig. 2, *A* and *B*). The receptor for CCL2/MCP-1, CCR2, was also detected at 12 h in BALB/c and at 4 h in C57BL/6 mice. Densitometric analysis at 12 h in BALB/c mice revealed a significant increase in expression of CCL2/MCP-1 in SGH-treated mice when compared with saline control, but not as high as LPS (Fig. 2*C*), whereas the increase in CCR2 expression in SGH-treated mice was similar to the increase in saline controls (Fig. 2*D*). It is noteworthy that densitometric analysis of SGH induced CCL2/MCP-1 expression at 2 and 4 h in C57BL/6 mice (maximum expression) compared with 12 h in BALB/c mice (data not shown) showed a higher expression of CCL2/MCP-1 on BALB/c mice than in C57BL/6 mice induced by SGH. A basal CCL2/MCP-1 production in the lining tissue from the nonstimulated mice may be due to resident tissue cells acting as an early source of mediators while infiltrating cells could be the source of chemokines at a higher intensity after SGH injection (22). The higher expression of CCL2/MCP-1 only in BALB/c mice suggests the involvement of this CC chemokine in this phenomenon and may be responsible for the increased macrophage recruitment.

Production of CCL2/MCP-1 in vitro

Because we demonstrated an increased expression of CCL2/MCP-1 mRNA in the lining tissue of BALB/c, we decided to investigate whether SGH was able to induce CCL2/MCP-1 production in vitro. J774, a BALB/c macrophage cell line, was cultured in the presence of SGH. After 6 h, immunohistochemical reactions for detection of CCL2/MCP-1 were performed. SGH induced an increased production of CCL2/MCP-1 that was not seen in the untreated control (Fig. 3, *A* and *B*) and was comparable to LPS-induced CCL2/MCP-1 production (data not shown). To confirm that this effect was SGH specific, we preincubated SGH with anti-SGH serum. In this case, CCL2/MCP-1 production was completely inhibited, suggesting that the stimulatory effect induced by SGH was neutralized and again confirming the ability of SGH to induce CCL2/MCP-1 production (Fig. 3*C*).

Inhibition of CCL2/MCP-1

To investigate whether the increase of macrophage recruitment as a result of SGH stimulation was due to an increase of CCL2/MCP-1 expression, a group of BALB/c mice was previously treated with bindarit, an inhibitor of CCL2/MCP-1 synthesis, and also with anti-MCP-1 neutralizing mAb. Treatment with bindarit or anti-MCP-1 mAb treatment resulted in significant reduction of macrophage recruitment (Fig. 4, *A* and *B*). Bindarit resulted in total inhibition of CCL2/MCP-1 expression. Interestingly, anti-MCP-1 mAb treatment also led to a decrease in CCL2/MCP-1 expression evidenced by RT-PCR (Fig. 4*A*). Chemokine mRNA expression seems to be dependent on previous chemokine production and can function as a regulatory mechanism, i.e., lower concentration of protein can lead to a decrease in the message of the molecules (23, 24). Together, these results reinforce that CCL2/MCP-1 is the CC chemokine most likely responsible for the increased macrophage recruitment induced by SGH.

Inhibition of macrophage recruitment with anti-SGH serum

Because SGH effect on J774 cells was abrogated after preincubation with anti-SGH in vitro, we decided to investigate whether SGH effect could be inhibited in vivo as well. When SGH was preincubated with specific antiserum there was a reduction in macrophage recruitment. However, neutrophil, eosinophil, and lymphocyte migration remained similar to the LPS-positive control (Fig. 5). Preincubation of SGH with specific antiserum lead to a 68% abrogation of the macrophage recruitment when compared with the effect of SGH alone. Although 3.1×10^4 macrophages were recruited by SGH, only 1.0×10^4 macrophages were observed under stimulus with SGH preincubated with anti-SGH serum. No such abrogation was observed with neutrophils, eosinophils, or lymphocytes (9.68×10^4 vs 12.6×10^4 with SGH and

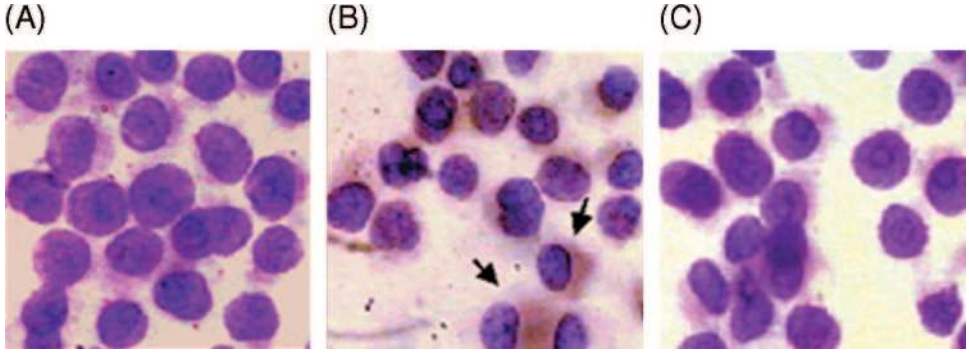

FIGURE 3. CCL2/MCP-1 production in vitro after stimulation with SGH. J774 cells were plated in 24-well culture plates containing glass coverslips. After 24 h, macrophages were untreated (*A*) or treated with SGH (0.5 pair/well) (*B*) or with SGH preincubated with anti-*L. longipalpis* SGH serum (*C*). Eight hours after stimulation coverslips were removed and immunohistochemical reaction was performed for MCP-1 detection (arrows).

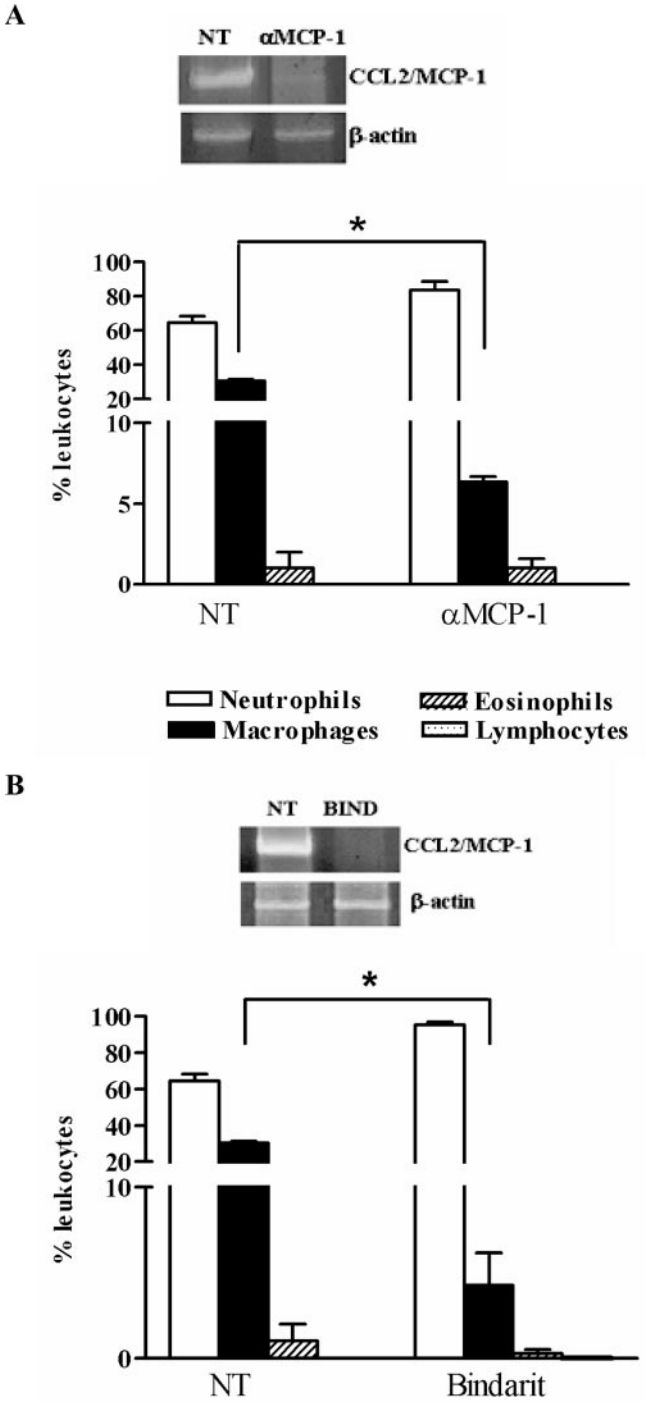

FIGURE 4. Inhibition of CCL-2/MCP-1. *A*, Leukocyte infiltration into BALB/c air pouches treated with anti-MCP-1 mAb i.p. treatment (100 μg) 1 h before SGH stimulation and RT-PCR analysis of CCL2/MCP-1 from nontreated (NT) anti-MCP-1 mAb treated (αMCP-1) BALB/c air pouch lining tissue 12 h after SGH (0.5 pair/animal) stimulation. *B*, Leukocyte infiltration into BALB/c mice air pouches treated with bindarit (200 mg/kg i.p.) 1 h before and 7 h after SGH stimulation and RT-PCR analysis of CCL2/MCP-1 from nontreated (NT) and bindarit-treated (BIND) BALB/c air pouch lining tissue 12 h after SGH (0.5 pair/animal) stimulation. Values represent mean ± SEM of four mice per group. Significant difference (*, $p = 0.0259$) from value for macrophage recruitment in control and treated groups.

SGH plus anti-SGH serum, respectively for neutrophils). Preincubation of LPS with anti-SGH serum did not result in LPS-induced inflammatory response inhibition confirming the antiserum specificity. This result suggests the presence of macrophage chemotactic components in SGH that can be neutralized by Abs present in the specific antiserum.

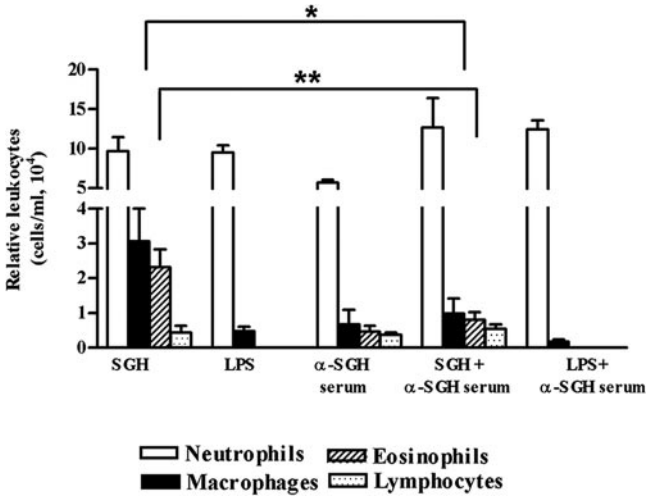

FIGURE 5. Specific inhibition of macrophage recruitment after SGH stimulation. Air pouches in BALB/c mice were stimulated with LPS (20 μg/ml), SGH (0.5 pair/animal), anti-SGH serum (α-SGH), LPS, or SGH preincubated for 1 h with anti-*L. longipalpis* SGH serum. Significant difference from value for macrophages (*, $p = 0.0364$) and eosinophils (**, $p = 0{,}0286$) between SGH and SGH + anti-SGH groups.

Inhibition of SGH macrophage recruitment by naturally induced anti-SGH Abs

Accumulating evidence points to the importance of Abs anti-saliva in protection of immunized mice (4, 5). To further understand the effect of anti-SGH serum, we decided to test whether SGH chemotatic effect could also be inhibited by Abs produced in BALB/c mice preexposed to bites from uninfected *L. longipalpis*. The chemoattractive effect induced by SGH was severely reduced in preexposed animals: macrophage recruitment was dramatically decreased (78.2% inhibition), whereas neutrophil had a smaller reduction (41.5% inhibition) (Fig. 6*A*). In addition, expression of CCL2/MCP-1 in the air pouch lining tissue of exposed mice was completely abrogated 12 h after SGH stimulation (Fig. 6*B*). This result reinforces the importance of Abs anti-saliva in SGH-mediated macrophage recruitment.

Effect of L. chagasi *and SGH on leukocyte recruitment in vivo*

After demonstrating the inflammatory effect of SGH we investigated whether it was able to modify the inflammatory response induced by *L. chagasi*, the parasite transmitted by *L. longipalpis* sand flies. Exudate cells were collected 12 h later and showed that both SGH and *L. chagasi* induced recruitment of neutrophils and macrophages in BALB/c mice (Fig. 7). However, *L. chagasi* was able to induce the recruitment of a greater number of cells to the pouch space when compared with SGH alone. The combination of *L. chagasi* with SGH resulted in an exacerbated inflammatory response indicating that SGH also has an effect on the parasite recruitment profile, which is an additive effect on *L. chagasi*-induced leukocyte recruitment.

Discussion

The data presented in this study indicate that SGH from *L. longipalpis* induces an increased macrophage recruitment into the murine air pouch. Several studies have described the use of the mouse air pouch model as a very consistent and straightforward model to investigate inflammatory response, creating the environment for

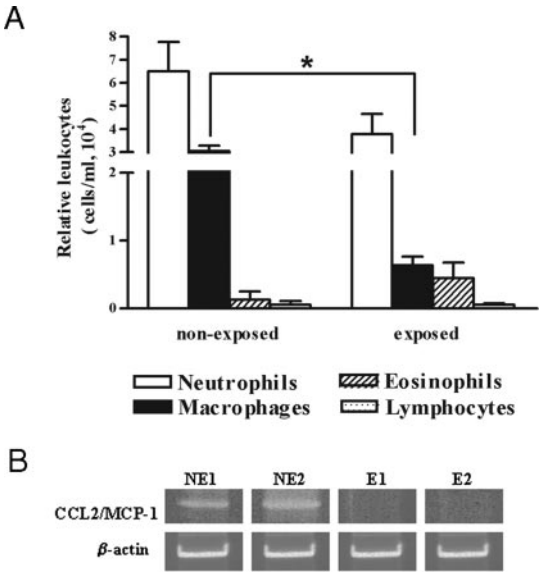

FIGURE 6. Inhibition of SGH effect in vivo in BALB/c mice ($n = 6$) sensitized with bites from *L. longipalpis*. *A*, BALB/c mice were exposed five times to *L. longipalpis* bites (10-day intervals) or nonexposed. Ten days after the last exposure, air pouches were induced and stimulated with SGH. Significant difference (*, $p = 0.0366$) from macrophage recruitment value is represented for nonexposed and exposed group. *B*, RT-PCR analysis of CCL2/MCP-1 from nonexposed (NE) and exposed (E) BALB/c air pouch lining tissue 12 h after SGH (0.5 pair/animal) stimulation. The results are representative of three experiments.

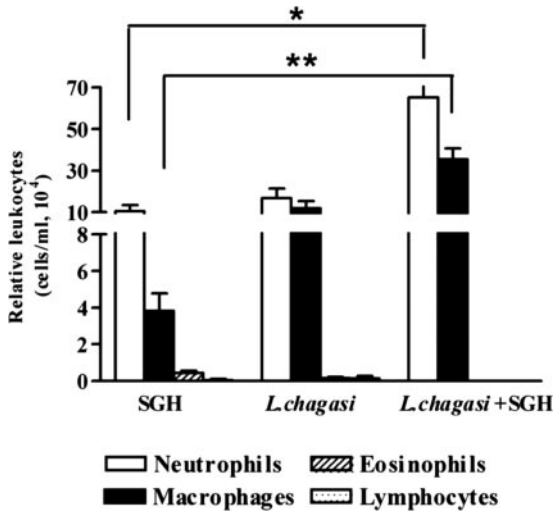

FIGURE 7. Effect of *Leishmania* on leukocyte infiltration into BALB/c mice air pouches. Pouches were stimulated with 0.1 ml of *L. longipalpis* saliva (SGH; 0.5 pair/animal), 10^7 stationary phase *L. chagasi* or *L. chagasi* + SGH for 12 h. After stimulation pouches were washed and differential cell counts performed in Turk's solution. Values represent mean ± SEM of four mice per group. Significant difference from neutrophil (*, $p = 0.0043$) and macrophage (**, $p = 0.0039$) recruitment value for SGH, *L. chagasi*, and *L. chagasi* + SGH stimulated groups is represented.

collection and phenotypic analysis of cells migrating into the pouch space (20, 21, 25). Interestingly, SGH was able to induce neutrophil, eosinophil, and macrophage recruitment after 12 h in BALB/c but not in C57BL/6 mice. Neutrophil recruitment was similar to other stimuli (LPS, for example), whereas eosinophils were increased as compared with the increase in control mice. Eosinophils have been strongly related to mosquito bites and allergies. Actually, eosinophilia was detected on dogs intradermally inoculated with saliva from *L. longipalpis* (26), in the inflammatory process at the site of immunization of mice with recombinant 15-kDa protein from *P. papatasi*, and also in cutaneous lesions in mice infected with *Leishmania braziliensis* and *L. longipalpis* saliva (27, 28). Eosinophils may participate in vasodilation favoring sand fly feeding but may alternatively be important players in creating an inhospitable environment for potential invaders transmitted with saliva (29).

Recent observations suggest that *L. longipalpis* saliva induced an intense and diffuse inflammatory infiltrate characterized by neutrophils, eosinophils, and macrophages observed up to 48 h in the ear dermis of BALB/c mice exposed to bites from uninfected sand flies (30). Of further interest was the increased macrophage recruitment induced by SGH in BALB/c but not in C57BL/6 mice. These two mouse strains differ in their susceptibility for *Leishmania*, as BALB/c mice are highly susceptible and C57BL/6 mice are partially resistant to several *Leishmania* species (31, 32). The fact that these animals, which differ in their response to *Leishmania*, also differ in their inflammatory response to sand fly saliva is noteworthy. Several studies have clearly demonstrated that salivary gland lysate inhibits NO (33), H_2O_2 production (34), and Ag presentation (35) by macrophages. The exacerbation of disease has been also associated with inhibition of the production of Th1 cytokines and with enhancement of IL-4 Th2 cytokine by saliva from *P. papatasi* (36). The capacity of macrophages to respond to Th1 activation signals against intracellular pathogens during the non-immune early phases of infection is crucial for determining whether the invading organisms proliferate or are eliminated. A lack of macrophage recruitment has also been related to failure of lesion enlargement in an immunodeficient murine model (37).

This increased macrophage recruitment was linked to CCL2/MCP-1 and its receptor CCR2 expression in the air pouch lining tissue but not other macrophage recruiting CC chemokines (CCL3/MIP-1α, CCL4/MIP-1β, or CCL5/RANTES). SGH was able to induce a strong CCL2/MCP-1 and CCR2 expression in BALB/c mice, whereas only a weak expression was observed 2 and 4 h in C57BL/6 mice. Even in these time points, the expression of this chemokine and its receptors was much weaker in C57BL/6 when compared with BALB/c mice (data not shown). Moreover, neutralization with anti-MCP-1 mAb resulted in a significant reduction of macrophages recruited and inhibition of CCL2/MCP-1 expression. This result was reinforced by using bindarit, an original indazolic derivative devoid of systemic immunosuppressive effects and of activity on arachidonic acid metabolism, which has been shown the ability to inhibit CCL2/MCP-1 synthesis both in vitro and in vivo (13–15). Bindarit-treated mice showed a significant reduction of macrophage recruitment and also inhibition of CCL2/MCP-1 expression in the lining tissue. Together, these results indicate that CCL2/MCP-1 is the CC chemokine involved in macrophage recruitment after SGH stimulation.

Because macrophages and neutrophils were the main cell populations present in the lining tissue after SGH stimulation (data not shown), we decided to investigate whether SGH was also capable of inducing CCL2/MCP-1 by J774 macrophages in vitro. SGH induced CCL2/MCP-1 production that was abrogated by specific anti-SGH serum, indicating that SGH inflammatory effect could be inhibited by specific Abs. Interestingly, saliva effect on macrophage recruitment was also specifically inhibited without affecting polymorphonuclear cell recruitment in vivo when it was preincubated with specific anti-SGH Abs or in BALB/c mice naturally exposed to uninfected *L. longipalpis* bites. This result is particularly interesting because it mimetizes the real situation in an endemic area where individuals are constantly exposed to bites from

uninfected sand flies and they develop anti-saliva immune response. In fact, in children from an endemic area the development of an anti-saliva humoral response occurred at the same time as the appearance of anti-*Leishmania* cell-mediated immunity (8). Interestingly, the decrease in macrophage recruitment was associated with a strong inhibition of CCL2/MCP-1 expression in the exposed animals, which was also detected in vitro in J774 cells. These results suggest that probably one or more immunogenic molecules present in *L. longipalpis* saliva are responsible for this effect. The importance of anti-sand fly saliva Abs has also been emphasized. *Phlebotomus argentipes* allowed to feed on hamsters previously exposed to sand fly bites, which is associated with a small amount of blood ingestion, decreased the feeding rate on animals and lowered the numbers of eggs laid, suggesting that anti-sand fly saliva Abs bind and neutralize the sand fly products (38).

We next evaluated the role of SGH in modifying the inflammatory response induced by *L. chagasi*. The parasite was able to induce a strong inflammatory response composed by neutrophils, macrophages, and eosinophils. When *L. chagasi* was associated with SGH, there was an increase in leukocyte recruitment especially on neutrophil and macrophage recruitment, indicating an additive effect of SGH. Previous work has demonstrated that *Leishmania* triggers recruitment of a mixed population of inflammatory cells, such as macrophages, eosinophils, and neutrophils, which can vary between species and strains (20, 39). The ability of SGH to modulate *L. chagasi* inflammatory response by increasing leukocyte recruitment is interesting because a potent macrophage recruitment may be related to higher susceptibility to *Leishmania* and linked to recently recruited macrophages being "safe havens" for this parasite (40).

Taken together, the results obtained in this study suggesting that SGH from *L. longipalpis* can act on inflammatory mediators leading to an increase in macrophage recruitment probably due to expression of CCL2/MCP-1 are particularly interesting. The ability of SGH to modulate initial recruitment of macrophages to the bite site might facilitate the entry of *Leishmania* into their host cells and their establishment in the hostile environment of the vertebrate skin. CCL2/MCP-1 has been associated with Th2 polarization (41) but also with *L. major* killing by human monocytes (42). Overall, understanding the role of vector saliva components is important as these molecules could be future targets for pharmacologic or vaccine interventions (43).

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Disclosures

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