

Phlebotomine salivas inhibit immune inflammation-induced neutrophil migration via an autocrine DC-derived PGE₂/IL-10 sequential pathway

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Abstract: In the present study, we investigated whether saliva from *Phlebotomus papatasi* and *Phlebotomus duboscqi* inhibited antigen-induced neutrophil migration and the mechanisms involved in these effects. The pretreatment of immunized mice with salivary gland extracts (SGE) of both phlebotomines inhibited OVA challenge-induced neutrophil migration and release of the neutrophil chemotactic mediators, MIP-1 α , TNF- α , and leukotriene B₄ (LTB₄). Furthermore, SGE treatment enhanced the production of anti-inflammatory mediators, IL-10 and PGE₂. SGE treatments failed to inhibit neutrophil migration and MIP-1 α and LTB₄ production in IL-10^{-/-} mice, also failing in mice treated with nonselective (indomethacin) or selective (rofecoxibe) cyclooxygenase (COX) inhibitors. COX inhibition resulted in diminished SGE-induced IL-10 production, and PGE₂ release triggered by SGE remained increased in IL-10^{-/-} mice, suggesting that prostanoids are acting through an IL-10-dependent mechanism. SGE treatments in vivo reduced the OVA-induced lymphoproliferation of spleen-derived cells. Further, the in vitro incubation of bone marrow-derived dendritic cells (DC) with SGE inhibited the proliferation of CD4⁺T cells from OVA-immunized mice, which was reversed by indomethacin and anti-IL-10 antibody treatments. Supporting these results, SGE induced the production of PGE₂ and IL-10 by DC, which were blocked by COX inhibition. These effects were associated with the reduction of DC-membrane expression of MHC-II and CD86 by SGE treatment. Altogether, the results showed that Phlebotomine saliva inhibits immune inflammation-induced neutrophil migration by an autocrine DC sequential production of PGE₂/IL-10, suggesting that the saliva constituents might be promising therapeutic molecules to target immune inflammatory diseases. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: antigen presentation · anti-inflammatory mediators · inflammatory diseases · cytokines · insect saliva · sand fly

INTRODUCTION

Although neutrophils have a protective role during many pathogen infections [1], they are also implicated in the pathogenesis of a number of inflammatory disorders, including rheumatoid arthritis (RA), multiple sclerosis, inflammatory bowel diseases, glomerulonephritis, immune vasculite, and psoriasis [2–5]. The deleterious role of the neutrophils in these disorders is a result of the ability of these cells to produce significant amounts of proinflammatory cytokines as well as substances such as reactive oxygen and nitrogen species, lysosomal enzymes, and metalloproteases [6, 7]. Indeed, pharmacologic strategies to limit neutrophil trafficking and/or activation have received attention as a potential treatment of autoimmune diseases.

During the inflammatory response, the migration and activation of neutrophils depend on several proinflammatory mediators [5]. In fact, TNF- α , IL-1 β , platelet-activation factor, C5a, leukotriene B₄ (LTB₄), and a variety of chemokines, such as IL-8, growth-related oncogene- α , MIP-1 α , and stromal cell-derived factor, are chemotactic mediators involved in the recruitment of neutrophils to sites of inflammation and their activation [8–10]. Previously, our group demonstrated that antigen challenge in immunized mice induces neutrophil migration that requires CD4⁺T cell-derived TNF- α . The release of TNF- α in this model depends on antigen presentation and MIP-1 α production. Moreover, TNF- α promotes neutrophil recruitment through a LTB₄-dependent mechanism [11–13].

Saliva from several species of blood-feeding arthropods arranges a formidable set of sophisticated and redundant mech-

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animals to overcome the hemostatic and inflammatory system of the vertebrate host during blood meal [14]. Vasodilators, anti-coagulants, inhibitors of platelet aggregation, and anti-inflammatory and immunomodulatory molecules are part of this salivary mixture [15, 16]. These active molecules also participate in the transmission as well as the establishment of some arthropod-borne diseases, including Leishmaniasis and Lyme disease [17, 18]. For instance, in Leishmaniasis, there is evidence that the saliva of the vectors *Lutzomyia* and *Phlebotomus* enhances the disease [19–22]. These exacerbated effects are associated with the saliva's capacity to selectively inhibit several macrophage functions, including antigen presentation, NO, and hydrogen peroxide production, thus inhibiting the ability of macrophages to kill intracellular *Leishmania major* [23–29]. Furthermore, vector saliva inhibits the production of protective type 1 cytokines such IL-12 and IFN- γ [30–32], and it enhances the production of IL-10, IL-4, IL-6, and PGE₂, all of which enhance survival of the *Leishmania* parasite [33–35].

Undoubtedly, Phlebotomine saliva contains several potent pharmacologic factors. Among those properties, identification of the anti-inflammatory and immunomodulatory moieties could be useful in the development of drugs to treat inflammatory diseases. Recently, our group demonstrated that the systemic pretreatment of mice with salivary gland extract (SGE) from the New World vector *Lutzomyia longipalpis* inhibited neutrophil migration during OVA-induced immune peritonitis. This effect was associated with inhibition of the production of the neutrophil chemoattract mediators, MIP-1 α and TNF- α [11, 36]. On the other hand, SGE treatment increased the local production of IL-10 and IL-4, which are described as anti-inflammatory cytokines in the context of immune response [36]. However, the specific site of saliva action was not addressed in the previous study. In the present study, we investigated whether salivary gland homogenates from *Phlebotomus papatasi* and *Phlebotomus duboscqi* inhibit neutrophil migration in immune inflammation as well as the mechanisms involved.

MATERIALS AND METHODS

Mice

Female BALB/c and C57BL/6 mice and mice with a targeted disruption of IL-10 (C57BL/6 IL-10^{-/-}), weighing 18–22 g, were housed in temperature-controlled rooms (22–25°C) and received water and food ad libitum in the animal facility of the Department of Pharmacology or Immunology, School of Medicine of Ribeirão Preto, University of São Paulo (Brazil).

Breeding pairs of IL-10^{-/-} were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Breeding stocks backcrossed to C57BL/6 were obtained and housed in a sterile laminar flow until experiments were conducted. The genetic status was confirmed by PCR. All experiments were conducted in accordance with National Institutes of Health (NIH) guidelines on the welfare of experimental animals and with the approval of the Ribeirão Preto School of Medicine Ethics Committee.

Sand fly SGE

Salivary glands were prepared from 7- to 10-day-old laboratory-bred females of *P. papatasi* and *P. duboscqi* from the Laboratory of Malaria and Vector Research at the NIH (Bethesda, MD, USA) as described previously [20]. Briefly, 50 pairs of salivary glands were dissected under sterile conditions in

endotoxin-free PBS, placed in 50 μ l sterile PBS buffer, and kept at -70°C until needed. Immediately before use, the glands were disrupted by sonication using a Sonifer 450 homogenizer (Branson, Danbury, CT, USA). Endotoxin levels were evaluated using the QCL-1000[®] chromogenic *Limulus* amoebocyte lysate endpoint assay kit (Lonza, Switzerland), resulting in negligible levels of endotoxin in the salivary gland supernatant.

Procedures for active sensitization with OVA

On Day 0, mice received a single s.c. injection of OVA (100 μ g) in 0.2 mL of an emulsion containing 0.1 mL PBS and 0.1 mL CFA. The mice were given booster injections of OVA in IFA on Days 7 and 14. Control mice (sham-immunized) were injected s.c. with 0.2 mL of an emulsion containing equal volumes of PBS and CFA, followed by boosters of an emulsion of PBS and IFA without OVA on Days 7 and 14. On Day 21, immunized and control animals were challenged with an i.p. injection of OVA (10 μ g) or PBS.

Leukocyte migration induced by OVA and LPS

Immunized or control (sham-immunized) mice were i.p.-challenged with PBS (0.1 mL/cavity) or OVA (10 μ g/cavity). Some naïve mice received an i.p. injection of LPS (100 ng/cavity). The total leukocytes that migrated to the peritoneal cavity were harvested by an injection of 3 mL PBS plus EDTA 1 mM at 6 h and/or 48 h post-stimulus. Total counts were performed on a cell counter, and differential cell counts (200 cells total) were carried out on cytocentrifuge slides stained with Rosenfeld. The results are presented as the number of neutrophils per cavity.

Determination of leukocyte migration into the peritoneal cavity by flow cytometry

Samples of 10⁶ cells obtained from peritoneal exudates were suspended and incubated for 30 min at 4°C in PBS containing 2% of BSA and Fc γ RI block mAb (CD16/CD32) to avoid nonspecific background staining. After the blocking step, cells were identified by characteristic size (forward-scatter) and granularity (side-scatter) combined with two-color analysis. Briefly, the lymphocytes were identified as CD19⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺; dendritic cells (DC) were identified as CD11c⁺CD11b⁺; macrophage as CD11b⁺CD11c⁻; and neutrophils as Gr1⁺ (BD Biosciences PharMingen, San Diego, CA, USA). The isotype controls used were rat IgG2b and rat IgG2a (BD Biosciences PharMingen). After staining, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry (FACScan[™] and CELLQuest[™] software, BD Biosciences PharMingen).

Effect of SGE on OVA-induced cytokine and LTB₄ production

The immunized and sham-immunized (control) animals treated with SGE or PBS were challenged (i.p.) with 10 μ g OVA. After 6 h, the animals were killed, and the peritoneal exudates harvested with 1 mL PBS containing 1 mM EDTA. Peritoneal exudates were centrifuged, and their supernatants were collected and stored at -70°C for determination of MIP-1 α , TNF- α , IL-10, and LTB₄ by ELISA and PGE₂ by radioimmunoassay (RIA).

Measurements of MIP-1 α , TNF- α , LTB₄, and IL-10 in the peritoneal exudates

The concentrations of MIP-1 α , TNF- α , LTB₄, and IL-10 in the peritoneal exudates were determined by using a double-ligand ELISA. Briefly, a flat-bottomed 96-well microtiter plate was coated with 100 μ L antibody directed to one of the above mediators at a dilution of 2 μ g/mL (MIP-1 α , TNF- α , LTB₄) or 1 μ g/mL (IL-10) in coating buffer and incubated overnight at 4°C. After washing and nonspecific-binding blockade, samples and standards were loaded into plates and incubated overnight. Subsequently, the plates were washed, and the appropriate biotinylated polyclonal anti-mediator antibody or mAb anti-mediator was added. After 1 h, the plates were washed, avidin-peroxidase (diluted 1:5000) was then added to each well for 15 min, and the plates were washed thoroughly again. Next, substrate (0.4 mg *o*-phenylenediamine and 0.4 L H₂O₂ in 1 mL substrate buffer) was added, the reaction was stopped with H₂SO₄ (1 M), and the OD was measured at 490 nm. The results were expressed as pg cytokines per mL supernatant.

Measurements of PGE₂ by RIA

Samples of peritoneal exudates of OVA-immunized and sham-immunized mice were harvested and lyophilized. A RIA kit was used to determine the PGE₂ levels, according to the manufacturer's instructions (DuPont NEN® Research Products, Boston, MA, USA).

Treatments

Mice were treated with SGE 48 h before OVA or LPS i.p. injection. Some groups also received treatments with a nonselective cyclooxygenase (COX) inhibitor (indomethacin; 5 mg/kg, diluted in 0.1 mM Tris/HCl buffer, pH 8.0) or COX-2-selective inhibitor (rofecoxib; 3 mg/kg, diluted in PBS plus Tween 80 5%) or vehicles. Drug treatments consisted of 1× during 3 days, as the first and last treatments were performed 30 min before SGE treatment or OVA challenge, respectively.

DC generation and T cell purification

DC were generated in vitro from bone marrow cells from 6- to 8-week-old wild-type BALB/c mice as described previously [37, 38]. Briefly, femurs and tibias were flushed with RPMI 1640 (Gibco-BRL Life Technologies, Grand Island, NY, USA) to release the bone marrow cells that were cultured in 24-well culture plates in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated FCS, 100 µg/ml penicillin, 100 µg/ml streptomycin, 5×10^{-5} M 2-ME (all from Sigma Chemical Co., St. Louis, MO, USA), murine GM-CSF (30 ng/ml), and IL-4 (10 ng/ml; Peprotech, Rocky Hill, NJ, USA). On Days 3 and 6, the supernatants were gently removed and replaced with the same volume of supplemented medium. On Day 9, the nonadherent cells were collected and submitted to a positive selection using anti-CD11c magnetic beads, according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA), to eliminate the residual macrophage contamination. Flow cytometric evaluation of purified DC shows that 91% of cells express CD11c^{interm} or ^{high} (marker of DC).

DC (1×10^6 /ml) in RPMI 1640 supplemented with 10% FBS were incubated with *P. papatasi* SGE (0.5 gland), IL-10 (100 µg/ml), or PGE₂ (1 µM) alone, or anti-IL-10 (α-IL-10; 10 µg/ml), indomethacin (10 µg/ml), or medium was added to the culture in the presence or absence of SGE. Briefly, these cells were incubated overnight at 37°C in 5% CO₂. In some experiments, DC were treated overnight (37°C in 5% CO₂) using the treatment described above before LPS (2 µg/ml) stimulation for 24 h. The cells were then harvested, and surface expression was characterized by flow cytometry using antibodies against MHC class-II, CD80, CD86, and CD40 conjugated to PE or FITC, as well as isotype controls.

To purify CD4⁺T cells, splenocytes from OVA-immunized mice were incubated with beads coated with antibodies against L3T4 and isolated using biomagnetic separation (Dynabeads, Dynal A.S., Oslo, Norway). The procedures were performed in accordance with the manufacturer's instructions. The isolated CD4⁺T lymphocytes (1×10^6 /ml) were cultured with or without SGE in RPMI 1640 10% FBS overnight at 37°C in 5% CO₂.

Lymphoproliferation assay

To assess the influence of SGE treatment in lymphoproliferation, OVA-immunized mice were treated in vivo with PBS or SGE from *P. papatasi* or *P. duboscqi*, and the spleens were harvested for T cell isolation for the proliferation assay. Briefly, each spleen was removed and washed twice with PBS separately. Tissues were minced, and the cells were filtered through a cell strainer and centrifuged at 500 G at 4°C for 10 min. The cell pellet was resuspended in RPMI-1640 medium to a concentration of 2.5×10^6 cells/ml, and 5×10^5 cells/well were added in 96-well microtiter plates and cultured with OVA (10 µg/ml) or medium for 72 h. Twelve hours before the termination of culture, 0.5 µCi [³H]thymidine (NEN, Boston, MA, USA) was added to each well to determine CD4⁺T proliferation. To perform in vitro culture assay, purified OVA-primed CD4⁺T cells, pretreated with or without SGE (5×10^5 cells/well of a 96-well plate), were incubated with purified DC (5×10^4 cells/well) and treated with SGE or medium. In some experiments, freshly isolated OVA-primed CD4⁺T cells were added to wells, where the DC were pretreated previously with different stimuli, as described above, to the cell ratio of 10:1. In all of the experiments, OVA (10 µg/ml) or medium were added to the culture and incubated for 72 h in a total volume of 20 µL per condition. Similar to DC, CD4⁺T lymphocytes washed twice in PBS followed the treatment before the

coculture assay to avoid the interference of drug action. Before the CD4⁺T addition, the supernatant from DC culture was harvested to measure IL-10 and PGE₂ levels by ELISA and RIA, respectively, and proliferation was measured by overnight [³H]thymidine incorporation.

Statistical analysis

Data are reported as mean ± SEM and are representative of two to four independent experiments. The results of an individual experiment were not combined, but they were analyzed individually. The means from different groups were compared by ANOVA followed by Bonferroni's *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

P. papatasi or *P. duboscqi* SGE inhibit immune peritonitis-induced neutrophil migration

In a previous study, we demonstrated that the OVA i.p. challenge in immunized mice induced a dose (3–30 µg/cavity)- and time-dependent neutrophil migration. This migration peaked 6 h after OVA challenge, decreasing thereafter and returning to the control level by the 24th hour [11]. Confirming these observations, in the present study, the i.p. administration of OVA (10 µg/cavity) in immunized mice induced a significant neutrophil migration at 6 h after challenge (**Fig. 1A**) compared with the control group (sham-immunized mice). The pretreatment of immunized mice (48 h before OVA challenge) with SGE (one salivary gland/mouse i.v., ~1 µg total protein) from *P. papatasi* or *P. duboscqi* abolished the OVA-induced neutrophil migration (**Fig. 1A**). SGE pretreatment also reduced the recruitment of other leukocyte subtypes such as B, CD4⁺T, and CD8⁺T lymphocytes and macrophages. On the other hand, SGE pretreatments induced statistically significant eosinophil migration to the peritoneal cavity (**Table 1**).

SGE inhibit the release of neutrophil chemotactic mediators

Considering that the OVA challenge-induced neutrophil migration in immunized mice is mediated by the sequential release of MIP-1α (CCL3), TNF-α, and LTB₄ [12], we analyzed the effects of SGE on the peritoneal release of these mediators. The pretreatment of immunized mice with SGE from *P. papatasi* and *P. duboscqi* inhibited OVA-induced MIP-1α (**Fig. 1B**), TNF-α (**Fig. 1C**), and LTB₄ (**Fig. 1D**) production in the peritoneal cavity.

Inhibition of OVA-induced neutrophil migration by SGE depends on sequential release of PGE₂ and IL-10

Next, we addressed whether SGE treatments inhibited neutrophil migration by inducing the production of the anti-inflammatory cytokines IL-10 or IL-4. OVA challenge (10 µg/cavity) by itself did not induce a significant release of IL-10 in PBS-pretreated mice. However, the pretreatment of OVA-immunized mice with SGE from *P. papatasi* or *P. duboscqi* significantly increased IL-10 production after OVA challenge (**Fig. 2A**). The IL-4 levels were similar in all groups analyzed (data not shown). Confirming the involvement of IL-10 in the inhibitory effects of SGE upon neutrophil migration, the SGE

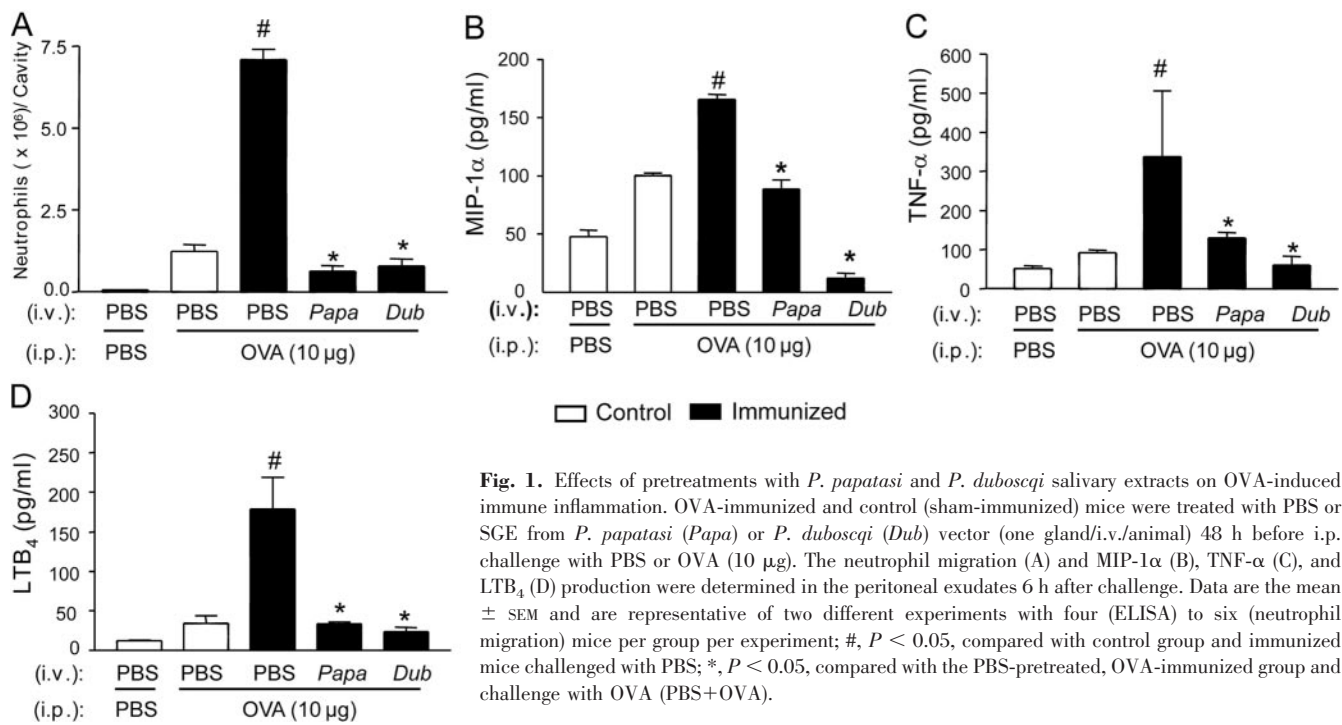


Fig. 1. Effects of pretreatments with *P. papatasi* and *P. dubosqi* salivary extracts on OVA-induced immune inflammation. OVA-immunized and control (sham-immunized) mice were treated with PBS or SGE from *P. papatasi* (*Papa*) or *P. dubosqi* (*Dub*) vector (one gland/i.v./animal) 48 h before i.p. challenge with PBS or OVA (10 μg). The neutrophil migration (A) and MIP-1α (B), TNF-α (C), and LTB₄ (D) production were determined in the peritoneal exudates 6 h after challenge. Data are the mean ± SEM and are representative of two different experiments with four (ELISA) to six (neutrophil migration) mice per group per experiment; #, *P* < 0.05, compared with control group and immunized mice challenged with PBS; *, *P* < 0.05, compared with the PBS-pretreated, OVA-immunized group and challenge with OVA (PBS+OVA).

pretreatments failed to inhibit OVA-induced neutrophil migration in immunized IL-10^{-/-} mice (Fig. 2B). Furthermore, the inhibitory effects of SGE on the peritoneal releases of MIP-1α (Fig. 2C) and LTB₄ (Fig. 2D) induced by OVA challenge were not observed in IL-10^{-/-} mice.

Taking into account previous results showing that PGE₂ is able to induce IL-10 production [39], we investigated the involvement of this eicosanoid on the SGE effects. Pretreatment of the OVA-immunized mice with SGE promoted a significant increase of OVA-induced PGE₂ production compared with PBS-pretreated mice (Fig. 3A). Moreover, the s.c. treatment of immunized mice with nonselective COX (indomethacin) or selective (rofecoxibe) COX-2 inhibitors prevented the inhibitory effects of SGE (48 h before OVA challenge) on OVA-induced, neutrophil migration (Fig. 3B), in addition to

MIP-1α (Fig. 3C) and LTB₄ (Fig. 3D) production. Further, the treatment of the immunized mice with indomethacin or rofecoxibe also prevented the increase of IL-10 production by SGE administration (Fig. 4A). On the other hand, the increase of OVA-induced PGE₂ production by *P. papatasi* SGE pretreatment was not altered in IL-10^{-/-} mice (Fig. 4B). Taken together, these results clearly demonstrate that the PGE₂/IL-10 sequential pathway is involved in the inhibitory effects of the Phlebotomine salivary extracts upon OVA-induced neutrophil migration in immunized mice.

SGE reduce antigen presentation ability of DC: autocrine role of the IL-10/PGE₂ pathway

The neutrophil migration observed during OVA-induced immune peritonitis depends on antigen presentation by APC and

TABLE 1. Effect of Sand Fly SGE on Leukocyte Migration Induced by OVA in Immunized Mice

Cells	False-immunized		OVA-immunized		
	PBS (i.v.)	PBS (i.v.)	PBS (i.v.)	OVA (i.p.)	
	OVA (i.p.)	PBS (i.p.)	PBS (i.v.)	<i>P. papatasi</i> (i.v.)	<i>P. dubosqi</i> (i.v.)
Total leukocytes	4.33 × 10 ⁶ ± 1.2	5.25 × 10 ⁶ ± 3.6	12.33 × 10 ⁶ ± 3.1 ^a	9.42 × 10 ⁶ ± 3.7 ^b	8.50 × 10 ⁶ ± 3.8 ^b
Neutrophils	12.0 ± 0.14 (29%)	0.06 ± 0.0003 (1.1%)	71 ± 0.31 (58%) ^a	0.65 ± 0.015 (6.6%) ^b	0.8 ± 0.20 (9.3%) ^b
Eosinophils	2.0 ± 0.01 (4.6%)	3.0 ± 0.02 (5.7%)	20 ± 0.1 (16.2%) ^a	57.1 ± 7.0 (60.3%) ^b	43.0 ± 6.0 (50.3%) ^b
Macrophages	1.0 ± 0.1 (2.1%)	0.4 ± 0.1 (0.7%)	3.2 ± 0.8 (2.6%) ^a	0.32 ± 0.6 (0.33%) ^b	0.6 ± 0.04 (0.72%) ^b
B cells	0.25 ± 0.02 (0.6%)	0.23 ± 0.04 (0.2%)	2.1 ± 0.1 (1.7%) ^a	0.2 ± 0.06 (0.2%) ^b	0.5 ± 0.2 (0.7%) ^b
CD4 ⁺ T	0.4 ± 0.2 (0.9%)	0.2 ± 0.09 (0.36%)	1.2 ± 0.3 (0.9%) ^a	0.2 ± 0.04 (0.2%) ^b	0.4 ± 0.08 (0.5%) ^b
CD8 ⁺ T	0.002 ± 0.0004 (0.004%)	0.003 ± 0.001 (0.006%)	0.014 ± 0.004 ^a (0.01%)	0.0052 ± 0.002 ^b (0.005%)	0.0023 ± 0.001 ^b (0.003%)

The data are mean ± SD of the number of leukocytes recovered from the peritoneal cavity of OVA-immunized and control (sham-immunized) mice treated with PBS or SGE from *P. papatasi* or *P. dubosqi* vector (one gland/i.v./animal) 48 h before i.p. challenge with PBS or OVA (10 μg). The values inside parenthesis mean the percentage of each cellular type relative to the total leukocyte number. The values are ×10⁵ per peritoneal cavities. Experimental groups included four to five animals. ^a *P* < 0.05 compared with control groups; ^b *P* < 0.05 compared with the PBS-pretreated, OVA-immunized group and challenge with OVA (PBS + OVA).

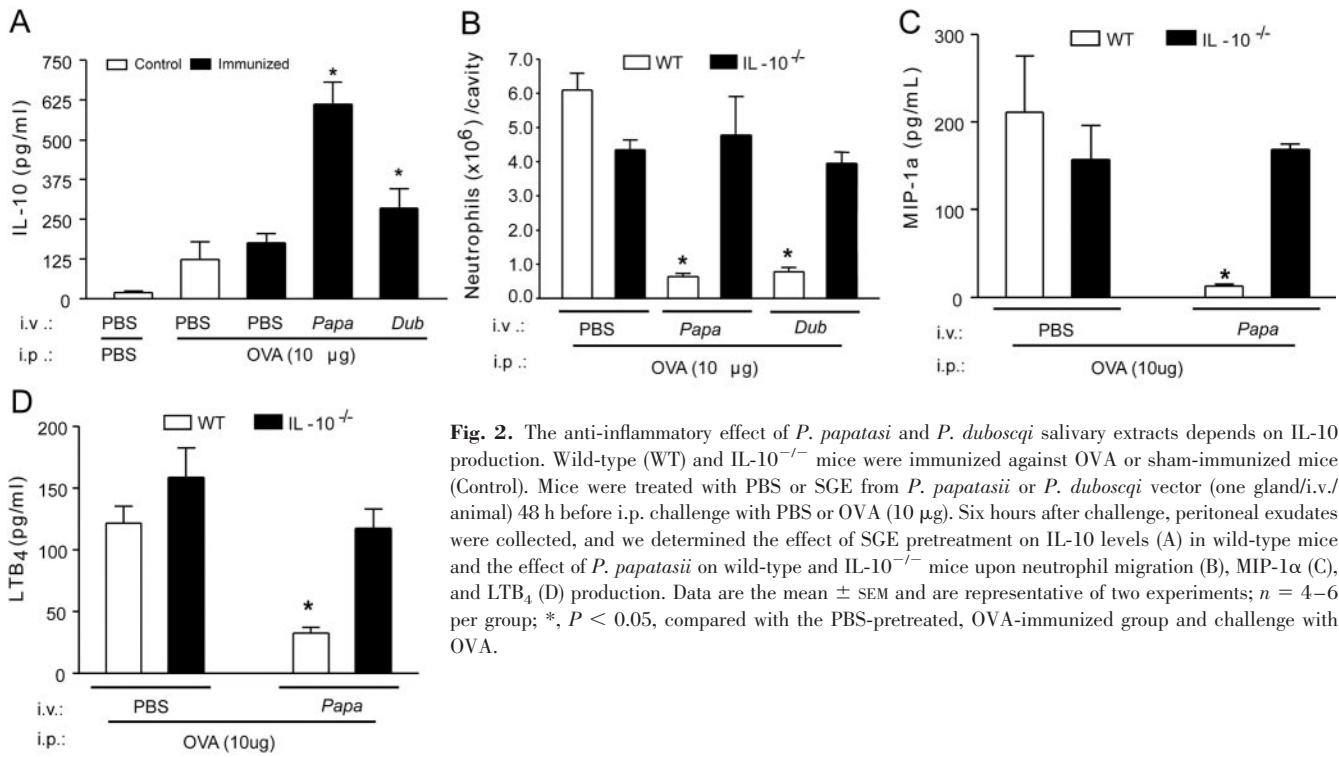


Fig. 2. The anti-inflammatory effect of *P. papatasi* and *P. duboscqi* salivary extracts depends on IL-10 production. Wild-type (WT) and IL-10^{-/-} mice were immunized against OVA or sham-immunized mice (Control). Mice were treated with PBS or SGE from *P. papatasi* or *P. duboscqi* vector (one gland/i.v./animal) 48 h before i.p. challenge with PBS or OVA (10 µg). Six hours after challenge, peritoneal exudates were collected, and we determined the effect of SGE pretreatment on IL-10 levels (A) in wild-type mice and the effect of *P. papatasi* on wild-type and IL-10^{-/-} mice upon neutrophil migration (B), MIP-1α (C), and LTB₄ (D) production. Data are the mean ± SEM and are representative of two experiments; n = 4–6 per group; *, P < 0.05, compared with the PBS-pretreated, OVA-immunized group and challenge with OVA.

consequently, CD4⁺ T cell activation [11]. In an attempt to determine the mechanism by which SGE are inhibiting OVA-induced neutrophil migration, we investigated the effects of SGE on OVA presentation by DC and an OVA-induced lymphoproliferative response. **Figure 5A** shows that spleen cells from OVA-immunized mice presented an intense lymphoproliferative response induced by OVA. There was a significant reduction in the lymphoproliferation when spleen cells were obtained from mice that had received a systemic injection (one

salivary gland/mouse) of SGE 48 h before. Considering that both SGE showed similar results concerning their anti-inflammatory activity, the *P. papatasi* SGE was used in all further experiments.

To assess which immune cell is inhibited by SGE, purified, OVA-primed CD4⁺T cells or DC obtained from bone marrow were incubated with PBS or SGE (0.5 gland/1×10⁶ cells) overnight. Afterwards, the lymphoproliferative response was assessed in the coculture of these cells. It was observed that the

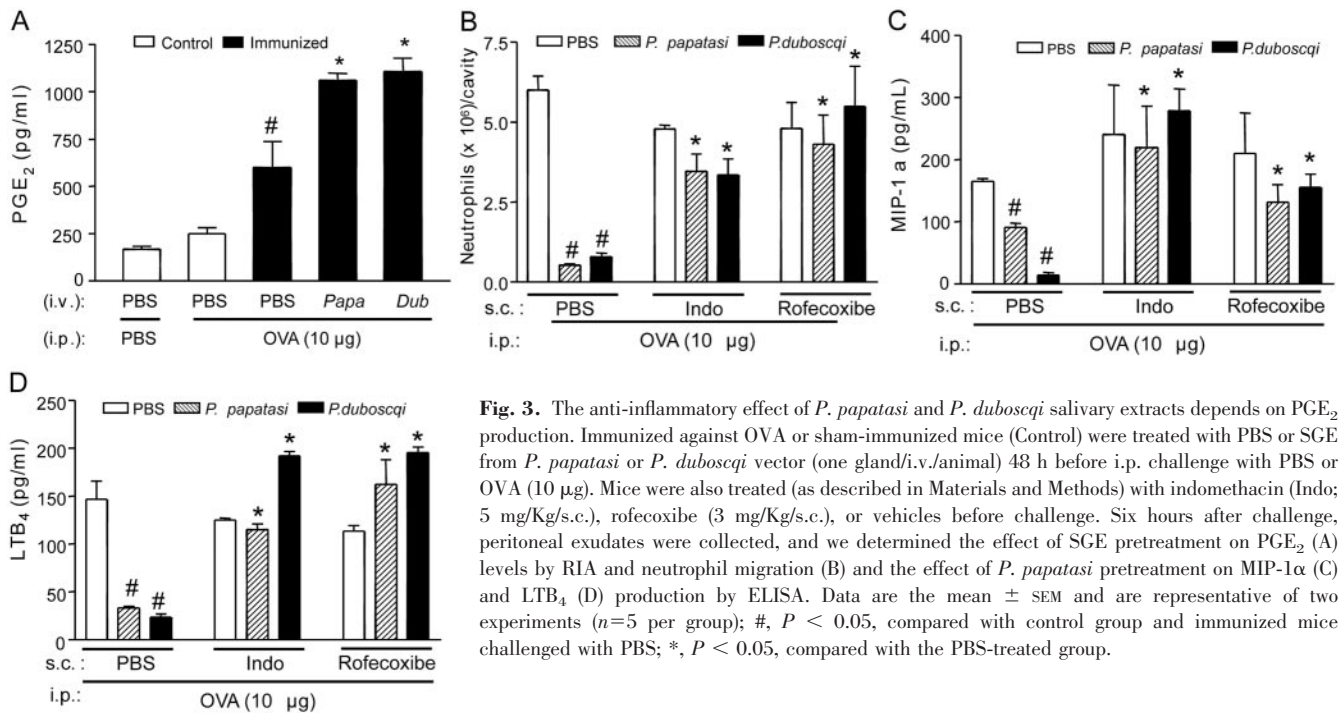
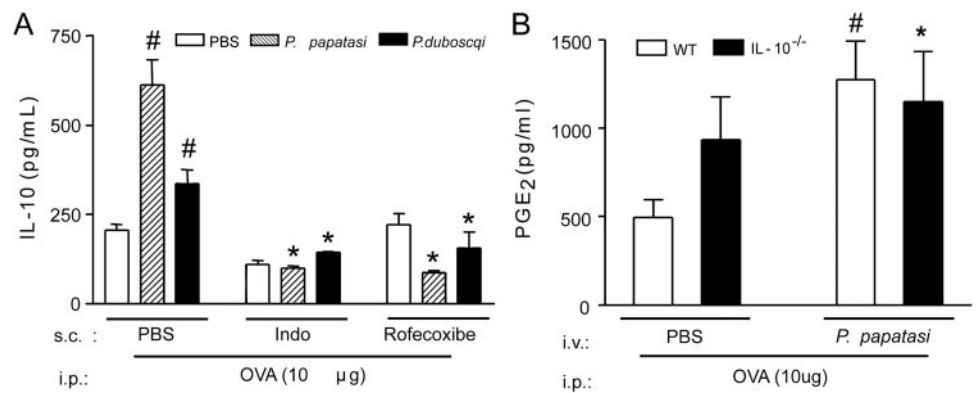


Fig. 3. The anti-inflammatory effect of *P. papatasi* and *P. duboscqi* salivary extracts depends on PGE₂ production. Immunized against OVA or sham-immunized mice (Control) were treated with PBS or SGE from *P. papatasi* or *P. duboscqi* vector (one gland/i.v./animal) 48 h before i.p. challenge with PBS or OVA (10 µg). Mice were also treated (as described in Materials and Methods) with indomethacin (Indo; 5 mg/Kg/s.c.), rofecoxibe (3 mg/Kg/s.c.), or vehicles before challenge. Six hours after challenge, peritoneal exudates were collected, and we determined the effect of SGE pretreatment on PGE₂ (A) levels by RIA and neutrophil migration (B) and the effect of *P. papatasi* pretreatment on MIP-1α (C) and LTB₄ (D) production by ELISA. Data are the mean ± SEM and are representative of two experiments (n=5 per group); #, P < 0.05, compared with control group and immunized mice challenged with PBS; *, P < 0.05, compared with the PBS-treated group.

Fig. 4. SGE induce prostanoid-dependent IL-10 production. Wild-type and IL-10^{-/-} mice were immunized against OVA or sham-immunized mice (Control). Mice were treated with PBS or SGE from *P. papatasi* or *P. dubosqi* vector (one gland/i.v./animal) 48 h before i.p. challenge with PBS or OVA (10 μg). (A) Wild-type mice were also treated with indomethacin (5 mg/Kg/s.c.), rofecoxibe (3 mg/Kg/s.c.), or vehicles before OVA challenge, and exudate samples were collected 6 h after challenge for IL-10 measurement by ELISA. (B) Peritoneal exudate samples from wild-type and IL-10^{-/-} mice were collected 2 h after OVA challenge for PGE₂ measurement by RIA. Data are the mean ± SEM and are representative of two independent experiments (n=4–5 per group); #, P < 0.05, compared with control group; *, P < 0.05, compared with the PBS-pretreated, OVA-immunized group and challenge with OVA.



lymphoproliferation was reduced when DC were incubated with SGE, whereas the incubation of OVA-CD4⁺T cells with SGE did not alter this response (Fig. 5B). These results suggest that SGE suppresses the immune response by acting preferentially on APCs.

Further, investigating the mechanism by which SGE inhibited DC function, we observed that the preincubation of DC with indomethacin or α-IL-10 prevented the inhibitory effect of SGE on the OVA-induced lymphoproliferation. These results suggest that PGs and IL-10 mediate the immunosuppressive action of SGE on APC. Corroborating this hypothesis, the treatment of DC with exogenous PGE₂ or IL-10 also inhibited

OVA-induced lymphoproliferation (Fig. 5C). Furthermore, SGE induced significant production of PGE₂ (Fig. 5D) and of IL-10 (Fig. 5E) by DC. In addition, the IL-10 production by SGE-treated DC was inhibited by incubation with indomethacin (Fig. 5E). In accordance with the previous results, IL-10 did not induce the release of PGE₂, nor did α-IL-10 prevent SGE stimulation of PGE₂ production by DC (Fig. 5E).

Inhibitory effect of SGE on DC surface molecule expression

In an attempt to determine the mechanism by which SGE inhibits the ability of DC to present antigen, we evaluated its

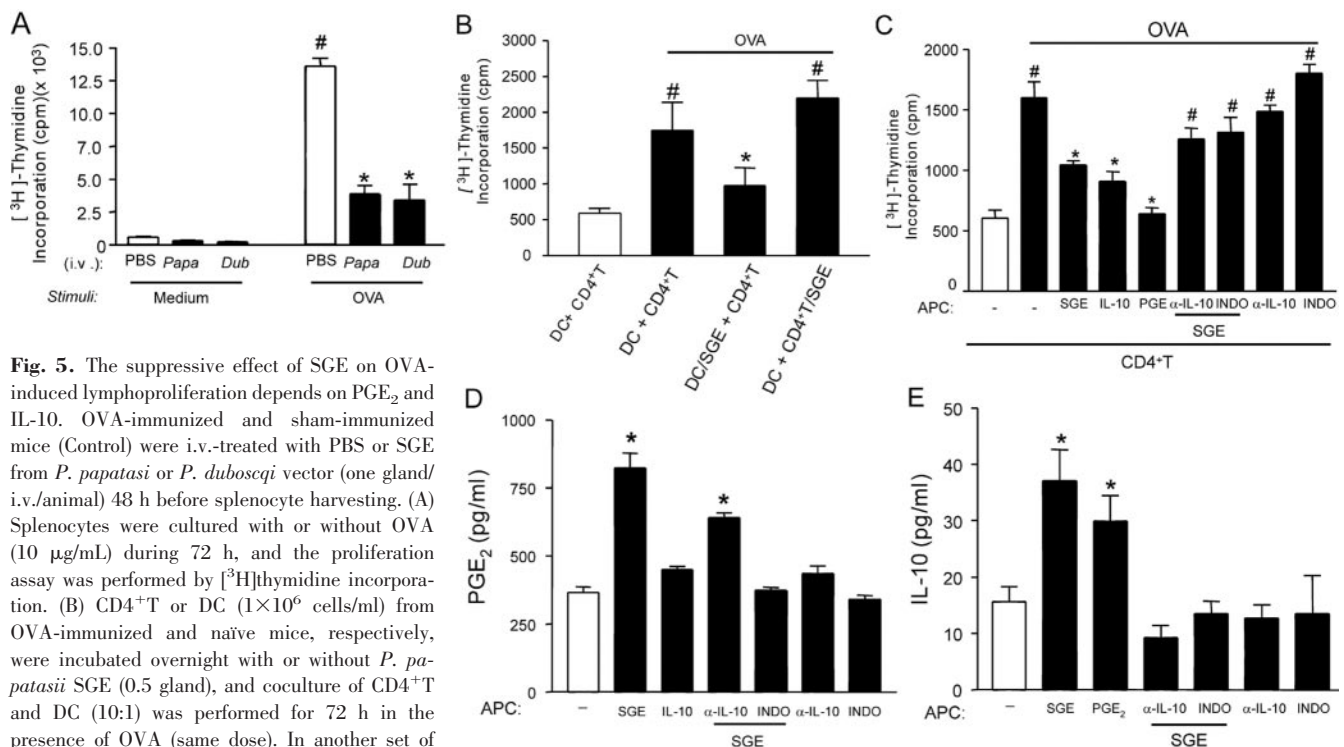


Fig. 5. The suppressive effect of SGE on OVA-induced lymphoproliferation depends on PGE₂ and IL-10. OVA-immunized and sham-immunized mice (Control) were i.v.-treated with PBS or SGE from *P. papatasi* or *P. dubosqi* vector (one gland/i.v./animal) 48 h before splenocyte harvesting. (A) Splenocytes were cultured with or without OVA (10 μg/mL) during 72 h, and the proliferation assay was performed by [³H]thymidine incorporation. (B) CD4⁺T or DC (1 × 10⁶ cells/ml) from OVA-immunized and naïve mice, respectively, were incubated overnight with or without *P. papatasi* SGE (0.5 gland), and coculture of CD4⁺T and DC (10:1) was performed for 72 h in the presence of OVA (same dose). In another set of experiments, DC (1 × 10⁶ cells) from naïve mice were incubated overnight with SGE (0.5 gland), IL-10 (100 μg/ml), PGE₂ (1 μM), antibody against IL-10 (α-IL-10; 10 μg/ml), α-IL-10 or indomethacin was also added in the presence or absence of SGE. Afterwards, CD4⁺T cells of OVA-immunized mice were added, followed by stimulation with or without OVA. The lymphoproliferation was assessed by [³H]thymidine incorporation (C), PGE₂ by RIA (D), and IL-10 by ELISA (E). The results are expressed as the mean ± SEM of at least two separate experiments made in triplicate (n=3 per group); #, P < 0.05, compared with the medium stimulus; *, P < 0.05, compared with the saliva control (PBS) and DC + CD4⁺T groups.

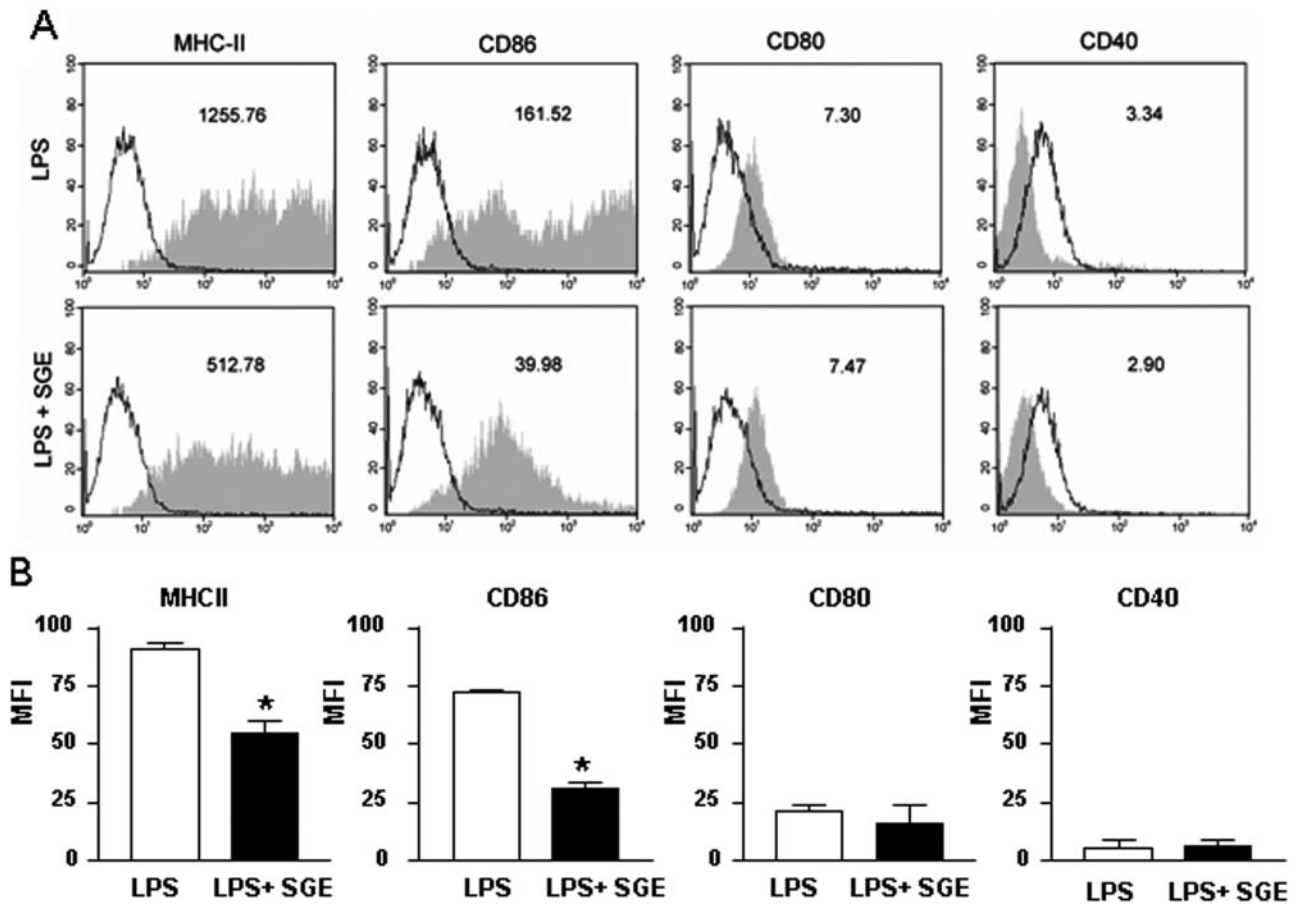


Fig. 6. SGE induces down-regulation of DC surface molecule expression. DC cells previously incubated overnight with SGE from *P. papatasi* or PBS were cultured in the presence of LPS during 24 h. Surface molecules were labeled with FITC or PE conjugated with anti-MHC class-II, anti-CD80, anti-CD86, or anti-CD40 antibodies. (A) The representative geometric mean fluorescence intensity (MFI) of DC staining from LPS or LPS + SGE cultures is shown in each box. The filled histograms represent cells labeled with the specific mAb; the empty histograms represent the same cell suspension labeled with isotypic control mAb. (B) The bars display the relative MFI obtained from one of four independent experiments made in duplicate ($n=2$); *, $P < 0.05$, compared with the LPS stimulation.

effect on expression of DC surface molecules involved in antigen presentation. The expression of MHC-II and the co-stimulatory molecule CD86 in the membranes of LPS-stimulated DC was not homogeneous. However, this finding is relatively common in the literature [40–42]. The SGE treatment inhibited the LPS-induced DC expression of these molecules by 59% and 75%, respectively (Fig. 6, A and B). On the other hand, the expression of CD80 or CD40 was not altered by SGE treatment. The low expression of these molecules in the membrane of LPS-induced DC could be a consequence of the period of DC differentiation. In fact, there is evidence in literature, including the articles that describe the methods to generate DC from bone marrow cells, where the expression of CD80 and CD40 is low in DC stimulated by LPS during 24 h [37, 40, 41]. Supporting the idea that the inhibition of neutrophil migration by SGE was associated with their ability to inhibit DC-presenting antigen, SGE from *P. papatasi* or *P. duboscqi* did not alter LPS-induced neutrophil migration to the peritoneal cavity (Fig. 7).

DISCUSSION

Several studies have reported that Phlebotomine saliva contains numerous substances with pharmacological properties

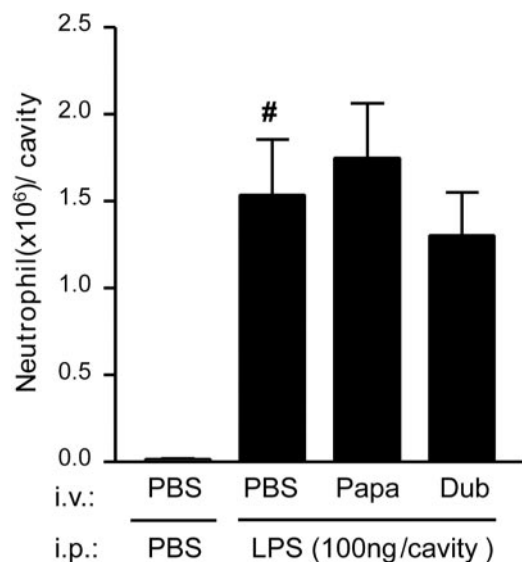


Fig. 7. Effect of SGE on LPS-induced neutrophil migration. Mice were treated with PBS or SGE from *P. papatasi* or *P. duboscqi* (one gland/i.v./animal) before i.p. injection of LPS (100 ng). The neutrophil migration was determined 6 h after the i.p. administration of LPS as described in Materials and Methods. Data are mean \pm SEM and are representative of two different experiments ($n=5$); #, $P < 0.05$, compared with PBS (vehicle of LPS) i.p. stimulus.

that include vasodilatation, anticoagulation, anti-inflammation, and immunomodulation [22, 26]. In this context, our group described previously that salivary extracts of the New World vector *L. longipalpis* present anti-inflammatory properties by inhibiting the neutrophil migration during the effector phase of a Th1-like immune response [36]. In the present study, we demonstrated that the saliva from Phlebotomines from Old World species *P. papatasi* and *P. dubosqi* inhibited the ability of DC to present antigen, and as a consequence, it reduced neutrophil migration during specific antigen-induced inflammation. The inhibitory effects of the SGE depend on sequential production of PGE₂ and IL-10 by DC, which in turn act in an autocrine manner, reducing the antigen-presenting ability of DC.

The development of an inflammatory response and consequently, neutrophil migration after OVA challenge in immunized mice result from the activation of a specific OVA-CD4⁺ T cell by APCs [11]. The activation of both cell types is responsible for the sequential release of neutrophil chemotactic mediators MIP-1 α , TNF- α , and LTB₄ at the inflammatory foci [11–13]. Therefore, the inhibition of the release of these mediators by SGE explains their ability to inhibit OVA-induced neutrophil migration. The inhibitory effect of SGE on the production of these neutrophil chemotactic mediators is dependent on the production of PGE₂ and IL-10. In fact, genetic ablation of IL-10 or pharmacologic inhibition of PG production prevent the inhibitory effect of SGE on neutrophil migration and the release of neutrophil chemotactic mediators MIP-1 α , TNF- α , and LTB₄. Moreover, the PGE₂ and IL-10 production by DC occurs in a sequential manner. It has been demonstrated consistently that IL-10 is an anti-inflammatory cytokine that plays an important role in limiting tissue injury during the specific immune inflammatory response by down-regulation of the inflammatory reactions [43]. Its anti-inflammatory activity involves the inhibition of cytokine production by T cells (e.g., IL-2), NK cells (e.g., IFN- γ), and monocyte/macrophages and DC (e.g., IL-1 α and IL-1 β , IL-6, IL-8, IL-12, TNF- α , and GM-CSF) [43, 44]. IL-10 also inhibits CC (MCP-1, MCP-5, MIP-1 α , MIP-1 β , MIP-3 α , MIP-3 β , RANTES, and macrophage-derived chemokine) and CXC chemokine (IL-8, IFN-inducible protein 10, MIP-2, and keratinocyte-derived chemokine) production by different cell types [45–48]. Although there is evidence that *P. papatasi* SGE used in the present study contains compounds that increase IL-10 levels such as adenosine/adenosine monophosphate (AMP) [49], we have demonstrated that IL-10 release depends on previous PGE₂ production by DC. Furthermore, *P. dubosqi* salivary extracts do not contain adenosine [50]. Corroborating our results, there is evidence that PGE₂ stimulates the production of IL-10 by macrophages, T cells [51–53], or bone marrow DC. It has been suggested that the molecular mechanism involved in the PGE₂-induced IL-10 production depends on the activation of EP₂/EP₄ PG receptors, which leads to an increase in the intracellular cAMP levels, ultimately responsible for IL-10 production via STAT3 [54, 55].

Our results also show that SGE treatment enhances OVA-induced eosinophil influx. Similarly, it has already been demonstrated that SGE enhances the eosinophil migration to Leishmania infection sites [56, 57]. It was not the aim of the present

study to determine the mechanism involved in SGE-induced eosinophil recruitment. However, in our experimental conditions, it could be a consequence of the increase in the production of Th2 cytokines at the inflammatory focus [58]. There is evidence in literature that SGE enhances the release of cytokines and chemokines of the Th2 pattern, such as IL-4, IL-5, IL-13, and MCP-1, which are chemotactic to eosinophils [31, 57].

Taking into account the immunomodulatory effects of PGE₂ and IL-10 released by SGE, we addressed the mechanism by which they could be inhibiting the production of neutrophil chemotactic mediators in our model. As mentioned before, the effector phase of OVA challenge-induced neutrophil migration depends on local antigen presentation by DC to CD4⁺ T cells, which in turn proliferate and release the chemotactic factor as well as TNF- α [11]. Treatment of immunized mice with SGE reduced the OVA-induced proliferation of spleen-derived T cells. This inhibitory effect of SGE was mediated by the sequential release of PGE₂ and IL-10. This conclusion is supported by the blockade of SGE inhibition of spleen T cell proliferation by indomethacin or antibody against IL-10. Moreover, PGE₂ and IL-10 mimicked the SGE inhibitory effect, and the eicosanoid effect was inhibited by antibody against IL-10. The efficient activation of CD4⁺T cells required their engagement with APC provided by interactions of TCR with MHC molecule/peptide complexes and associated costimulatory molecules, as well as with cytokines released by both cell types [59]. All of these processes can be down-modulated by IL-10 and PGE₂ as already described [60, 61]. In our system, it seems that DC are the source of SGE-stimulated PGE₂ and IL-10 production, as SGE inhibited OVA-induced CD4⁺T cell proliferation when incubated with DC but not with CD4⁺T cells. Additionally, SGE-treated DC released PGE₂ and IL-10. Thus, DC sequentially released PGE₂ and IL-10, which act in an autocrine manner, inhibiting their antigen-presentation ability.

The reduced ability of SGE-treated DC to present antigen could be associated with reduced expression of MHC-II as well as CD86 costimulatory molecules in the DC membrane. Consistently, agonists of the EP₂ and EP₄ PGE₂ receptors significantly induced the production of IL-10 by DC, which acting in an autocrine manner, decreased MHC class-II molecule expression [61–63]. The interaction of CD80 and CD86 molecules with CD28 expressed by CD4⁺T cells augments and sustains CD4⁺T cell activation [64, 65]. Likewise, the CD40–CD40 ligand interaction is also important for the maintenance of T cell immunity [66, 67]. The SGE treatment, although it did inhibit the expression of MHC-II and CD86 costimulatory molecules, affected neither CD80 nor CD40 expression.

In addition to the autocrine role of DC-derived IL-10/PGE₂, which inhibits the ability of DC to present antigen, these substances could be acting directly on CD4⁺T cells in a paracrine manner to inhibit their activation. In fact, as described in literature, PGE₂ and IL-10 induce suppression of T cell proliferation, which is related to the down-regulation of the expression of IL-2R and IL-2 production [68]. In our experimental conditions, the proliferation of CD4⁺T cells to OVA was inhibited by exogenous administration of PGE₂ in the culture but not of IL-10, suggesting that only the prostanoid is

acting in an autocrine and paracrine manner (data not shown). There is also an in vitro demonstration that the saliva of other blood-feeding arthropods, such as the tick *Ixodes scapularis*, inhibits DC maturation and function in a PGE₂-dependent mechanism. The saliva of *I. scapularis* as well as PGE₂ induces a concomitant inhibition of TNF- α and induction of IL-10 production by DC. Furthermore, although PGE₂ is a common immunomodulatory mediator among the saliva of *I. scapularis*, *P. papatasi*, and *P. duboscqi*, the mechanism seems to be different, as PGE₂ is a constituent of *I. scapularis* saliva, whereas *P. papatasi* and *P. duboscqi* saliva might contain substances that induce PGE₂ production. Another important difference is that the saliva of *I. scapularis* did not alter the expression of presentation molecules in contrast to the present data [69].

Further supporting that Phlebotomine SGE affect antigen presentation, SGE did not inhibit LPS-induced neutrophil migration, which does not depend on presentation but on direct activation of TLR with consequent production of neutrophil-chemotactic mediators. Thus, considering the novel mechanism demonstrated herein, which does not affect host defense (the response to LPS remains), we extrapolate that the anti-inflammatory effect of saliva has clinical potential, as it would inhibit only autoimmune inflammation.

Others have also demonstrated the immunosuppressive effect of Phlebotomine saliva. *L. longipalpis* SGE suppresses T cell proliferation in response to SRBCs [25], and Maxadilan, the vasodilatory peptide of *L. longipalpis*, modulates cytokine production [70–72] and T cell proliferation [73]. In the present study, Maxadilan is not involved in the SGE effects, as it is not a constituent of Phlebotomine saliva from Old World flies, *P. papatasi* or *P. duboscqi* [14, 19, 74]. In addition, in a previous experiment, we demonstrated that Maxadilan did not inhibit the OVA-induced neutrophil migration in immunized mice [36].

Recently, it was described in the literature that neutrophils recruited to the site of Leishmania infection internalize the parasite [75, 76] and that SGE enhances the neutrophil migration process [32, 56, 57]. It was also observed that the parasite internalization delays the neutrophil apoptotic death program and induces MIP-1 β release, which recruits macrophages to the infection sites. The migrated macrophages ingest the infected apoptotic neutrophils, a process that stimulates the TGF- β and PGE₂ release, which down-modulates the macrophage activation and as a consequence, contributes to Leishmania infection establishment [75, 76]. Together, these findings suggest that the parasites use the granulocytes as “Trojan horses” to invade the macrophages [75]. In this context, the inhibition of DC function by SGE described in the present investigation may also represent an additional mechanism to explain the ability of Phlebotomine saliva to exacerbate the Leishmania infection. It is important to point out that our results seem to be apparently controversial with the above-described ability of the SGE to enhance the neutrophil migration to the infection site. However, it is important to mention that the routes of SGE administration are different; whereas in the present investigation, SGE was administered systemically, in the mentioned studies, it was injected locally [32, 56, 57, 77]. It has been well demonstrated that systemic administration

of inflammatory stimuli (i.e., LPS) or of neutrophil chemotactic mediators (i.e., IL-8 and TNF- α) inhibits the neutrophil migration induced by different stimuli, whereas the local administration of the substances induces the neutrophil migration [78, 79].

To summarize, in the present study, we demonstrated a novel, anti-inflammatory mechanism in which saliva inhibits neutrophil migration during the effector phase of specific antigen-induced inflammation. These anti-inflammatory effects seem to be dependent on a sequential production of PGE₂ and IL-10, which are released by DC and act in an autocrine manner, inhibiting antigen presentation by these cells and preventing the release of neutrophil chemotactic factors with no effect on the host defense. Taking into account that the tissue damage observed in several autoimmune diseases (i.e., RA) is a consequence of the neutrophil emigration, we believe that the active constituents of the saliva could be a prototype to the development of new drugs to prevent the tissue lesion observed in such diseases. Our group is currently working on the isolation of phlebotomine salivary compounds to look for the active principles that are responsible for the anti-inflammatory activity. Thus, this will probably open perspectives for the development of novel drugs for the treatment of immune inflammatory diseases. However, these proceedings require laborious approaches concerning the reduced volume of saliva obtained.

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