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The protein LJM 111 from *Lutzomyia longipalpis* Salivary Gland Extract (SGE) accounts for the SGE-inhibitory effects upon inflammatory parameters in experimental arthritis model

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ABSTRACT

Several studies have pointed out the immunomodulatory properties of the Salivary Gland Extract (SGE) from *Lutzomyia longipalpis*. We aimed to identify the SGE component (s) responsible for its effect on ovalbumin (OVA)-induced neutrophil migration (NM) and to evaluate the effect of SGE and components in the antigen-induced arthritis (AIA) model. We tested the anti-arthritic activities of SGE and the recombinant LJM111 salivary protein (rLJM111) by measuring the mechanical hypernociception and the NM into synovial cavity. Furthermore, we measured IL-17, TNF-α and IFN-γ released by lymph nodes cells stimulated with mBSA or anti-CD3 using enzyme-linked immunosorbent assay (ELISA). Additionally, we tested the effect of SGE and rLJM111 on co-stimulatory molecules expression (MHC-II and CD-86) by flow cytometry, TNF-α and IL-10 production (ELISA) of bone marrow-derived dendritic cells (BMDCs) stimulated with LPS, chemotaxis and actin polymerization from neutrophils. Besides, the effect of SGE on CXCR2 and GRK-2 expression on neutrophils was investigated. We identified one plasmid expressing the protein LJM111 that prevented NM in OVA-challenged immunized mice. Furthermore, both SGE and rLJM111 inhibited NM and pain sensitivity in AIA and reduced IL-17, TNF-α and IFN-γ. SGE and rLJM111 also reduced MHC-II and CD-86 expression and TNF-α whereas increased IL-10 release by LPS-stimulated BMDCs. SGE, but not LJM 111, inhibited neutrophils chemotaxis and actin polymerization. Additionally, SGE reduced neutrophil CXCR2 expression and increased GRK-2. Thus, rLJM111 is partially responsible for SGE mechanisms by diminishing DC function and maturation but not chemoattraction of neutrophils.

1. Introduction

It has been shown that salivary gland extract (SGE) from *Lutzomyia longipalpis* enhances *Leishmania* infection in mice [1] not by a direct effect on the parasite virulence but rather by affecting host immune defenses [2–4]. SGE contains a variety of potent pharmacologically active substances that interfere with host homeostatic, inflammatory, and immune responses [5,6]. Several studies demonstrated that in naïve animals *Phlebotomus papatasi* and *L. longipalpis* saliva inhibit IFN-γ and enhance IL-4 and IL-10 secretion by Th1 and Th2 lymphocytes, respectively [7–9]. We previously demonstrated that *L. longipalpis* SGE inhibited OVA-induced neutrophil migration in immunized mice, apparently as a consequence of the inhibition of TNF-α and LTB_4 release [10]. However, the molecule responsible for these effects has not been identified.

At the moment, few components present in SGE of sand flies have been studied. It has been described that *L. Longipalpis* saliva contains apyrase, an anti-platelet aggregation agent [11], and adenosine deaminase, an enzyme which promotes the hydrolysis of adenosine, an immunomodulatory molecule [12,13]. Another important protein is maxadilan that besides its potent vasodilator effect, it also presents immunomodulatory effects by increasing *in vitro* secretion of IL-10 and IL-6, while inhibits TNF-α production in macrophages and reduces CD80/86 expression on murine dendritic cells [14–16]. However, maxadilan does not inhibit neutrophil migration induced by OVA in sensitized mice [10]. In recent years, Valenzuela and colleagues [17] identified and isolated the most abundant salivary proteins from the sand fly *L. Longipalpis* using high-throughoutput approaches based on massive cDNA sequencing, proteomics and bioinformatic efforts. Thus, these studies have enabled the investigation of which SGE constituents are responsible for its anti-inflammatory activities.

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Rheumatoid arthritis (RA) is an inflammatory chronic systemic autoimmune disorder, affecting mainly joints, with the crucial involvement of neutrophils, T cells, B cells, macrophages-like and fibroblasts-like synoviocytes [18,19], besides cytokines as TNF-α, IL-17 and IFN-γ [20,21]. These cytokines activate immune and structural cells in the joints, thus, promoting them to release products that lead to tissue destruction [22].

Among the different experimental arthritis models, the antigen-induced arthritis [23] model is a useful model to investigate new effective therapies, since it shows similar histopathologic and immune features to human RA [24,25]. In this way, the present study was designed to examine the effect and the mechanism of SGE and its components in experimental AIA model. In addition, the present study also aimed to identify the sand fly salivary molecule responsible for the inhibition of OVA-induced neutrophil migration and the role of this salivary molecule in the antigen-induced arthritis model.

2. Materials and methods

2.1. Animals

Sex matched BALB/c weighing 18–22 g each were housed in temperature-controlled rooms (22–25 °C) in the animal facility of the School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil, and received water and food ad libitum. The study protocols were conducted in accordance with the ethical guidelines of the School of Medicine of Ribeirao Preto, University of São Paulo (São Paulo, Brazil).

2.2. Procedures for OVA and mBSA-induced neutrophil migration

The method is original as previously described [26] and adapted [27]. On day 0, mice received a single s.c. injection of OVA (100 μg) or methylated bovine serum albumin (mBSA) (500ug) in 0.2 ml of an emulsion containing 0.1 ml of PBS and 0.1 ml of complete Freund's adjuvant (CFA). The mice were given booster injections of OVA/mBSA in incomplete Freund's adjuvant (IFA) on days 7 and 14. On day 21 immunized animals were challenged with injection of PBS (mBSA vehicle), saline (OVA vehicle), OVA (100 μg/animal; intra-peritoneally, i.p.) or mBSA (10 μg/cavity; intraarticularly, i.a.), and neutrophil migration was determined 24 h after mBSA and 4 h after OVA challenge. Groups of mice received SGE (0.3; 1 or 3 gland/10ul; i.v. route) 48 h before OVA and 24 h before mBSA-challenge. rLJM111, a protein purified from SGE, (30, 100 or 300 ng/mouse; i.v. route) was given 15 min before mBSA-challenge. Total cell counts were evaluated in a cell counter (ACT; Beckman Coulter, Miami, FL) and differential cell counts performed on cytocentrifuge slides (Cytospin 3; Shandon, Pittsburgh, PA) stained with Rosenfeld. Results were expressed as the number of neutrophils per cavity.

2.3. Articular hypernociception evaluation

The articular hypernociception (pain) of the femur–tibial joint was evaluated as previously described [28]. A nonnociceptive tip probe with area size of 4.15 mm^2 was used. An increasing perpendicular force was applied to the central area of the hind paw to induce flexion of the femur–tibial joint, followed by paw withdrawn. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until 3 subsequently consistent measurements (i.e. the variation among these measurements was less than 1 g). The results were expressed as the flexion elicited withdrawal threshold in grams (g).

2.4. Construction of DNA plasmids coding for L. longipalpis secreted salivary proteins and its treatment in immunized mice

Transcripts coding for the most abundant salivary proteins from the sand fly *L. longipalpis* were cloned into the VR2001-TOPO vector and purified as previously described [29]. Plasmids coding for LJS169, LJS238, LJL4, LJM5, LJL17, LJL35, LJL17, LJS143, LJM10, LJL13, LJM11, LJS169, LJM78, LJM111 or LJM114 and OVA-immunized mice were injected with 50 μg of these DNA plasmids (intramuscular route) in 50 μl of saline 48 h before OVA challenge. Empty plasmids were injected as control.

2.5. Expression and HPLC purification of His-tagged L. longipalpis salivary protein LJM11

Salivary recombinant protein LJM111 (rLJM111) was produced by transfecting 4000 ml FreeStyle™ 293-F cells (Invitrogen) with 500 μg of purified plasmid following the manufacturer's recommendations (Invitrogen). After 72 h, transfected cell cultures were harvested and the supernatant filtered through a 0.45 μM filter unit and concentrated to 30 ml in an AmiconR concentrator device (Millipore Corp., Bedford, MA, USA) in the presence of Buffer A (20 mM NaH2PO4, 20 mM Na2HPO4, pH 7.4 and 500 mM NaCl). A HiTrapTMChelating HP column (GE Healthcare) was charged with 5 ml of Ni2SO4 0.1 M. The concentrated recombinant protein was then added to the HiTrap Chelating HP column. The column was then connected to a Summit station HPLC system (Dionex) consisting of a P680 HPLC pump and a PDA-100 photodiode array detector. The column was equilibrated for 30 min with Buffer A at 1 ml/min. After equilibration of baseline the following gradient was used to elute the protein: minute 0–10 , 100% Buffer A; minute 10–20, 0% buffer A, 100% Buffer B (20 mM NaH2PO4, 20 mM Na2HPO4, pH 7.4, 500 mM NaCl and 50 mM imidazole); minute 20–30 100%B; minute 30–60 a gradient of 100% B to 100%C (20 mM NaH2PO4, 20 mM Na2HPO4, pH 7.4, 500 mM NaCl and 500 mM imidazole); minute 60–70 100% C. Eluted proteins were detected at 280 nm and the eluted fractions were collected every minute on a 96 well microtiter plate using a Foxy 200 fraction collector. All fractions were blotted on a nitrocellulose paper and the blot was blocked with 5% milk for 1 h and incubated for 1 hr with anti-saliva antibodies and 1 h with anti-mouse Ap conjugated secondary antibody. Positive fractions were developed with Wetern Blue stabilized substrate for alkaline phosphatase (Promega). An aliquot (5 μl) of positive fractions were run on SDS and silver stained using SilverQuest™ (Invitrogen). Imidazole was removed from the positive fractions by dialysis against PBS, pH 7.4.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-17, TNF-α and IFN-γ were quantified *in vitro* from popliteal and inguinal lymph nodes cells (5×10^5 cells/well) pre-treated or not with SGE (3 gland pairs/ml) or rLJM111 protein (2.7 μg/ml) and stimulated with mBSA (100 μg/ml) or anti-CD3 (5 μg/ml) for 36 h by ELISA. The results are expressed as picograms per milliliter.

2.7. DC generation

Dendritic cells (DC) were generated *in vitro* from bone marrow (BM) cells from 6- to 8-wk-old wild type BALB/c as previously described [30]. Briefly, femurs and tibias were flushed with RPMI-1640 (Gibco-BRL Life Technologies, Grand Island, NY) to release the BM cells that were cultured in 24-well-culture plates in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated FCS, 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 5×10^{-5} M 2-mercaptoethanol (all from Sigma), murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (30 ng/ml) and IL-4 (10 ng/ml) (Peprotech, Rocky Hill, NJ). On days 3 and 6 the supernatants were gently removed and replaced with the same volume of supplemented medium. On day 9, the non-adherent cells were collected to eliminate

the residual macrophage contamination. Flow cytometric evaluation of purified DC shows that in average 85% of cells express CD11c$^{interm\ or\ high}$ (marker of DC).

2.8. LPS-induced DC maturation

DCs (1×10^6 /ml) were incubated overnight with SGE (3 gland pairs/ml), rLJM111 (2.7 μg/ml) or medium alone (RPMI 1640, 10% FBS) at 37 °C in 5% CO_2 and then followed by stimulation with or without LPS (1 μg/ml) for 24 h. The cells were then harvested and surface expression characterized by flow cytometry using antibodies against MHC class-II and CD86 conjugated to PE or FITC, as well as isotype controls. Cytokines levels were measured into supernatant of BMDC culture by ELISA assay.

2.9. Murine neutrophil isolation

Mouse neutrophils were isolated from bone marrow by Percoll density gradient as previously described [31].

2.10. In vitro SGE or LJM 111 treatments

Neutrophils from BM of balb/c mice were treated with SGE (0.3; 1 or 3 gl/ml) or with LJM 111 protein (300, 900 or 2700 ng/ml) 30 min before MIP-2 (30 ng/ml). Chemotaxis, actin polimerazition, and CXCR2 and GRK-2 expression of neutrophils were performed as described below.

2.11. Neutrophil chemotaxis

Chemotaxis was performed using a 48-well chemotaxis chamber (Neuroprobe Inc) as previously described [31]. Neutrophils were allowed to migrate 1 h toward MIP-2 (30 ng/ml, R&D Systems) or medium alone (RPMI). After 1 h, the membrane was removed, fixed, and stained. Neutrophils that migrated through the membrane were counted under a light microscope on at least 5 randomly selected fields.

2.12. Actin polimerazition

Neutrophils were incubated with MIP-2 (30 ng/mL) at 37 °C for 5 min. Cells were fixed, permeabilized, and stained with rhodamine-phalloidin (Molecular Probes). Microscopic analysis of fluorescent images was performed using an Olympus BX40-F4 epifluorescence microscope. The mean fluorescence density was determined from a linear measurement of individual cells' fluorescence. All cells of at least 5 randomly chosen fields of each slide were analyzed.

2.13. CXCR2 and GRK-2 expression

For CXCR2 expression analysis, neutrophils were incubated with phycoerythrin-conjugated anti-CXCR2 mAb and the cells were washed, fixed, and analyzed by flow cytometry using a BD Biosciences FACSort flow cytometer. For GRK-2 expression analysis, neutrophils were incubated with rabbit anti-mouse GRK2 Ab or isotype control (Santa Cruz Biotechnology). Then Alexa-Fluor 594-conjugated goat anti-rabbit IgG Ab (Invitrogen) were added. Microscopic analysis of fluorescent images was analyzed that was described above in F-actin analysis.

2.14. Statistical analysis

Data are reported as the mean ± SEM and are representative of 2 or 3 separate experiments with an $n = 5$–6 per group in each experiment. The means of different treatments were compared by analysis of variance with Bonferroni adjustment for multiple comparisons or by Student's *t*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. The sand fly salivary protein LJM111 mimics the anti-inflammatory effects of saliva extract

L. longipalpis SGE inhibited neutrophil migration to peritoneal cavity induced by OVA in immunized mice (Fig. 1A), confirming previous data published by our group [10]. Aiming to identify the salivary proteins responsible for the anti-inflammatory effect, we tested the effect of the main sand fly secreted salivary proteins by using plasmids coding for secreted proteins. Pre-treatment of OVA-immunized mice with a plasmid coding to LJM111 protein, but not to twelve other plasmids, inhibited neutrophil migration into peritoneal cavity induced by OVA-challenge in immunized mice (Fig. 1B), indicating that LJM111 is the protein responsible for the powerful effect observed with SGE. LJM111 is a protein of 43 kDa that belongs to the yellow family of proteins identified in the saliva of sand flies and in the midgut of the mosquito *Aedes aegypti* and in the fruit fly *Drosophila*. In order to verify the activity observed on the plasmid coding for LJM111 protein, we produced the recombinant protein LJM111 (rLJM111) in mammalian cells. Both SGE and rLJM111 inhibited the neutrophil migration to articular cavity in a dose dependent manner (Fig. 2A and B, respectively)

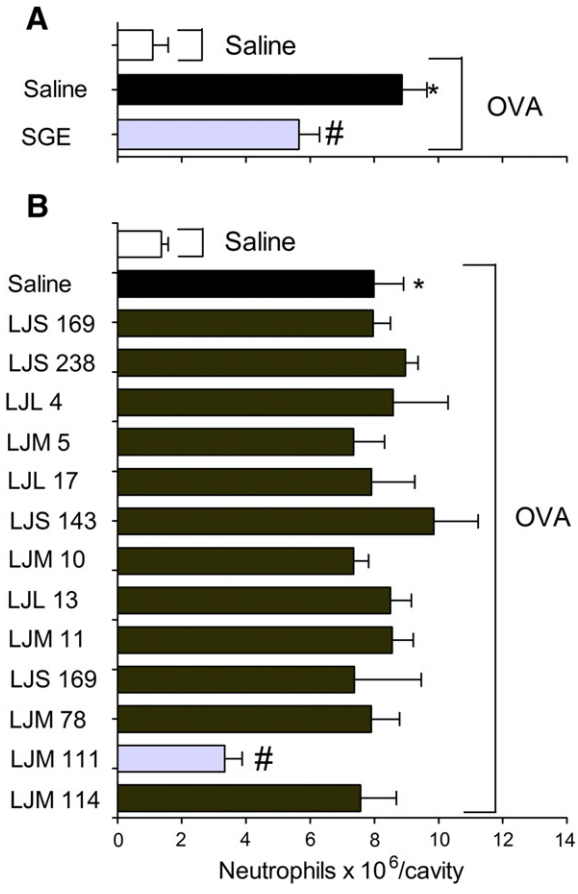

Fig. 1. SGE and plasmids codifying to LJM 111 protein inhibited neutrophil migration induced by OVA in immunized mice. (A) Immunized animals were treated with SGE (1gl/mouse) or with vehicle control 48 h before intraperitoneal (i.p.) challenge with OVA. (B) Immunized animals were treated 48 h before challenge with control plasmid (C) or plasmids codifying to indicated proteins. The neutrophil migration was determined 4 h after PBS or OVA challenge in peritoneal cavity. Values are the mean and SEM of 5 mice. * $P<0.05$ versus saline injected immunized mice, # $P<0.05$ versus immunized mice after OVA challenge, by analysis of variance with Bonferroni adjustment.

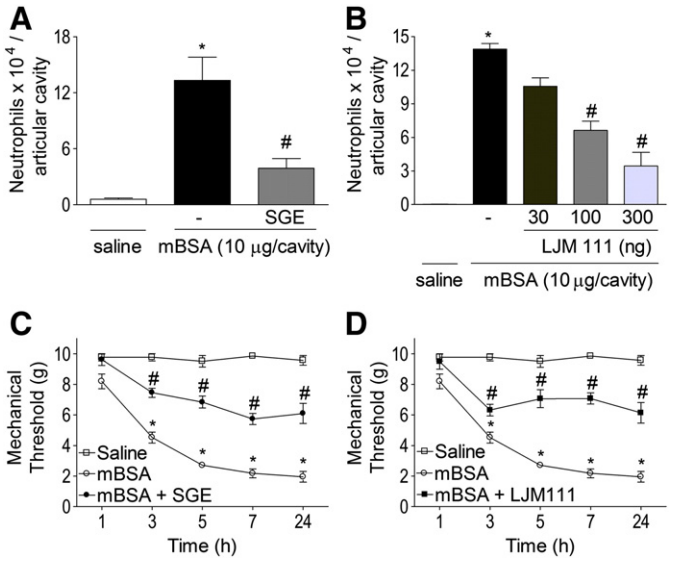

Fig. 2. SGE and LJM 111 protein reduced neutrophil migration and mechanical hypernociception on AIA model. Prior to intraarticular (i.a.) challenge with mBSA, immunized animals were treated 24 h before with 1 gl/mouse of SGE (A, C) or with recombinant LJM 111 (30, 100 or 300 ng/mouse, 15 min before, B and D). (A and B) Neutrophil migration was evaluated 24 h after saline or mBSA challenge. (C and D) Hypernociception was evaluated at the 1, 3, 5, 7 or 24 h after saline or mBSA challenge (30 μg/cavity). Values are the mean and SEM of 5 mice. * $P<0.05$ versus saline injected immunized mice, # $P<0.05$ versus immunized mice after mBSA challenge, by analysis of variance with Bonferroni adjustment.

and reduced the hypernociception (pain) induced by mBSA-challenge in immunized mice (Fig. 2C and D, respectively).

3.2. Mechanism by which SGE and rLJM111 reduce inflammatory parameters in immunized mice

Considering that pro-inflammatory cytokines are clearly involved in neutrophil recruitment and mechanical hypernociception [32,33], we investigated if SGE and rLJM111 can limit the release of pro-inflammatory cytokines in lymph node cells of immunized mice stimulated with mBSA or anti-CD3. Both SGE and rLJM111 inhibited IL-17, TNF-α and IFN-γ production by lymph nodes cells induced by mBSA *in vitro* (Fig. 3A), but did not reduce the cytokines levels from lymph nodes cells stimulated with anti-CD3 (Fig. 3B). Thereafter, we sought to investigate how SGE and rLJM111 may reduce the Ag-induced cytokine release. It was observed that both SGE and rLJM111 inhibited the expression of MHC-II and CD-86 in BMDCs stimulated by LPS (Fig. 4A). Additionaly, SGE and rLJM111 reduced TNF-α levels and increased IL-10 levels in supernatant of cultured BMDC stimulated with LPS (Fig. 4B).

3.3. SGE, but not LJM 111, inhibits the chemotactic activity of neutrophils obtained from mice

The neutrophil migration *in vivo* depends on the production of the chemotactic mediators, which stimulate the neutrophil/endotelium adhesion and neutrophil chemotaxis [32]. As shown in Fig. 5A, the pretreatment of bone marrow neutrophils from mice with SGE, but not LJM 111, reduced dose-dependently *in vitro* MIP-2-induced chemotaxis. Moreover, SGE, but not the protein LJM 111, prevented actin polymerization triggered by MIP-2 in these neutrophils (Fig. 5B). These results prompted us to investigate more extensively the mechanism by which SGE is negatively modulating the neutrophil chemotaxis.

Thus, we verified that SGE reduced neutrophil CXCR2 expression (Fig. 5C). Next we evaluated if the CXCR2 internalization was associated to increased GRK2 expression, since the G protein-coupled receptor

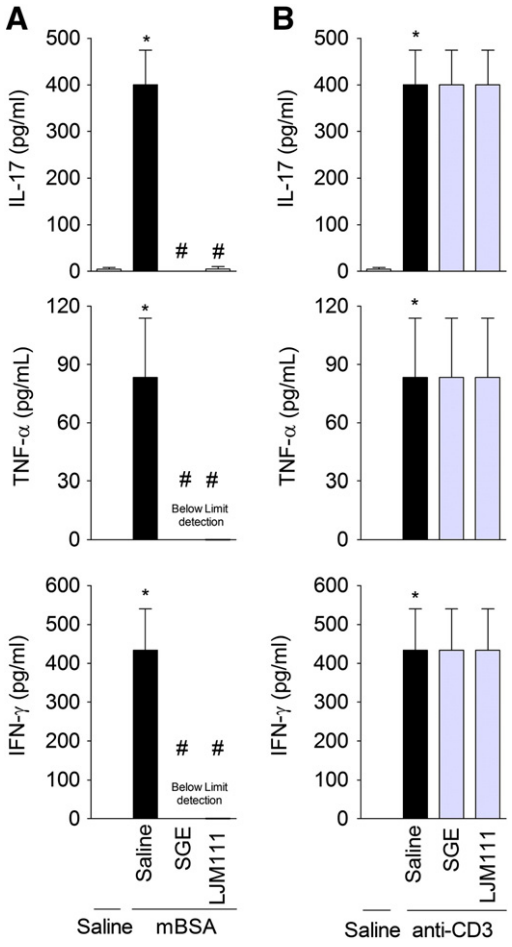

Fig. 3. SGE and LJM 111 inhibited inflammatory cytokines levels on lymph nodes cells. Concentrations *in vitro* of IL-17, TNF-α and IFN-γ in supernatant from lymph nodes cells treated or not with SGE (3 gl/ml) or protein LJM 111 (2700 ng/ml). The concentrations of cytokines were determined 36 h after **(A)** mBSA (100 μg/ml) or (**B**) anti-CD3 (10 μg/ml) stimuli. * $P<0.05$ versus cells without stimulus, # $P<0.05$ versus cells stimulated with anti-CD3 or mBSA, by analysis of variance with Bonferroni adjustment.

kinases (GRKs), regulatory molecules, are known to contribute to the GPCR internalization process [34]. As shown in Fig. 5D, the SGE enhanced neutrophil GRK2 levels. These results indicate that SGE inhibits the neutrophil chemotaxis by promoting CXCR2 internalization through the up-regulation of GRK2 expression. Besides, SGE also inhibited chemotaxis of BM neutrophils unresponsive to LPS from HeJ mice (supporting Fig. 1), thus, eliminating the possibility of LPS contamination as a cause for the observed effects.

4. Discussion

Considering the remarkable pre-clinical evidences that sand fly SGE regulates immune responses [1,8,9] and the importance of understanding the pathogenic mechanisms involved in auto-immune diseases, we investigated if sand fly SGE could have anti-inflammatory effects in an experimental model of arthritis. Our results show that SGE strongly reduces neutrophil migration and pain sensitivity in the AIA model. Importantly, this experimental models has several features observed in the actual human RA disease [25].

SGE has several componentes and accordingly we attempted to identify those responsible for the proeminent anti-inflammatory effects in experimental AIA. Valenzuela and colleagues [17] identified the most abundant secreted proteins from the salivary glands of the sand fly *Lutzomyia longipalpis*, thus, providing the experimental conditions to determine the proteins which could be promoting these

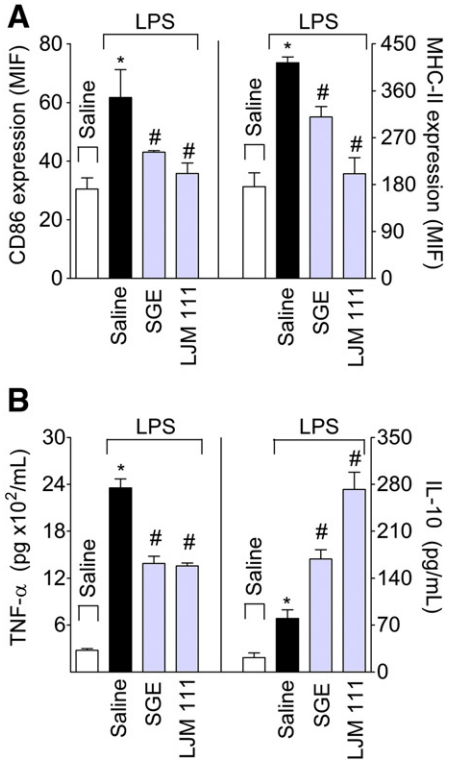

Fig. 4. SGE and LJM 111 reduced CD86, MHC-II expression and TNF-α levels and enhance IL-10 levels on dendritic cells. (A) Flow cytometry of CD86 and MHC-II expression and (B) enzyme-linked immunosorbent assay to measurement of TNF- and IL-10 levels following or not SGE (3.0 gl/ml) or LJM 111 (2700 ng/ml) treatment on dendritic cells (DC) stimulated with LPS (1 μg/ml) per 24 h. The control group (C) received only medium. * $P<0.05$ versus untreated control, # $P<0.05$ versus DC cells stimulated with LPS, by analysis of variance with Bonferroni adjustment.

SGE effects. Herein, we reported for the first time that treatment of mice with a plasmid containing a gene that codifies for the salivary protein LJM111, inhibited neutrophil migration to peritoneal cavity in OVA-challenged immunized mice. Extending such finding, the rLJM111 also reduced neutrophil recruitment and pain sensitivity in AIA. This protein is a novel protein belonging to the family of yellow-related protein of unknown function [17] and similar to *Ae. aegypti* midgut dopachrome conversion enzyme [35] and *D. melanogaster* yellow protein [36], suggesting that this protein may be altering some tryptophane metabolite. LJM111 is a protein of 43.2 kDa that belongs to the yellow family of proteins. The structure of LJM111 was recently modeled from the crystal structure of LJM11 another member of the yellow related family of proteins from sand flies. The structure of LJM111 is predicted to be of a six-bladed β-propeller fold with a single ligand binding pocket for bioamines located in the center of the propeller structure on one face of the molecule [37]. Importantly to note is that the face of the structure of LJM111 molecule is not charged as comparison to LJM11 which is positively charged. Furthermore, the isoelectric point of LJM111 is 4.7 while the one for LJM11 is 9.3. LJM11 is very antigenic while LJM111 is not [37]. It important to note that immunization with LJM111 did not produce antibodies or cellular immune response in mice [37], hamsters [38] or dogs [39]. Furthermore, dogs and humans exposed to Lutzomyia longipalpis bites did not mount an antibody response to LJM111 but mounted a very strong antibody response to LJM11 [40]. This lack of antigenicity is in agreement with the properties of LJM111 described in the current work. It is noteworthy that previous studies from our laboratory have discarded Maxadilan, a powerful vasodilatory peptide with immunomodulatory properties [14,41,42] present in SGE, as the protein responsible for these effects since, maxadilan did not reproduce the effects of SGE in inhibiting neutrophil migration in OVA-challenged immunized mice [10].

DCs play a central role in initiation of autoimmunity through their capacity to present self antigens to T cells [43–45]. This role depends on cytokine production and expression of co- and stimulatory surface molecules by these cells during antigen presentation [46,47]. SGE and rLJM111 modulated the phenotypic and functional maturation of DCs. Our results show that SGE and rLJM111 impair the complete maturation of DCs stimulated with LPS, leading to increased IL-10 production and reduced synthesis of TNF-α. This cytokine production profile was accompanied by a reduced expression of MHC-II and CD86. These results are of great interest since immature or incompletely matured DCs can prevent autoimmunity [48,49]. Previous reports have demonstrated that injections of incompletely-matured DCs are able to induce IL-10 producing $CD4^+$ T cells and to prevent EAE in mice [48]. DCs overexpressing IL-10 induce antigen-specific tolerance in experimental autoimmune myocarditis [50]. Moreover, TNF-α can improve functional maturation of DCs, selectively stimulating their capacity to induce Th1 responses, while IL-10 is widely known to be an immunosuppressive cytokine [51–53].

In line with these results, we also demonstrated that cells obtained from lymph nodes of immunized mice and treated with SGE or rLJM111 and stimulated with mBSA, but not with anti-CD3, have a remarkable reduction of IL-17, TNF-α and IFN-γ production. In fact, IL-17- and IFN-γ-secreting T cells have been associated with the induction of many autoimmune diseases [54,55] and DCs play a pivotal role in these processes [43,45]. IL-17 is directly implicated in the severity of the cartilage and bone damage in an experimental model of rheumatoid arthritis [56,57]. On the other hand, IL-17-deficient animals develop experimental autoimmune encephalomyelitis with delayed onset and diminished severity [58] and IL-17 and IFN-γ had been implicated in the development of autoimmunity. Mice deficient for the transcription factors involved in the differentiation of IFN-γ-secreting T cells, such as T-bet, are resistant to the development of EAE [59]. The molecular mechanism by which LJM111 modulates DC function and maturation is under investigation.

The excessive inflammatory responses in RA is associated with activation of G protein coupled receptors (GPCRs) by pro-inflammatory agonists as chemokines and prostaglandins [60,61]. It is well recognized that the responsiveness of these receptors is actively 'turned off' by members of the G-protein-coupled receptor kinase (GRK) [62,63]. In the context of disease, there are reports in the literature showing that in RA patients and in rats after development of adjuvant arthritis or experimental autoimmune encephalomyelitis the levels of GRK2, 3, and 6 are reduced in leukocytes from the blood or spleen [64–66]. This feature could be responsible at least in part for the outcome of these diseases since GRK induces, for instance, the internalization of CXCR2 receptors present in neutrophils, which are necessary for their chemoattraction to the inflammatory foci where neutrophils promote tissue destruction via metalloproteinases and boost cytokine production [31]. Herein, we have demonstrated that SGE provoked either CXCR2 internalization or improved GRK2 expression in neutrophils. This also leads to at least two possibilities: 1) It has been demonstrated in a monocytic human cell line that adenosine induces the internalization of adenosine A2A receptors upon binding by a mechanism related to increased GRK2 expression, which is a model of ligant binding-induced internalization. On the other hand, TNFα reduces GRK2 expression, therefore, reducing the internalization of A2A receptors [67]. Considering that SGE inhibited the production of TNFα in lymphonode cells and dendritic cells, it is also possible that SGE would inhibit a basal production of TNFα by neutrophils induced by the isolation process. It is consensus that there is some basal activation of neutrophil during isolation; and/or 2) It is possible that the SGE presents an unidentified molecule that induces GRK2 expression and G protein coupled receptor internalization.

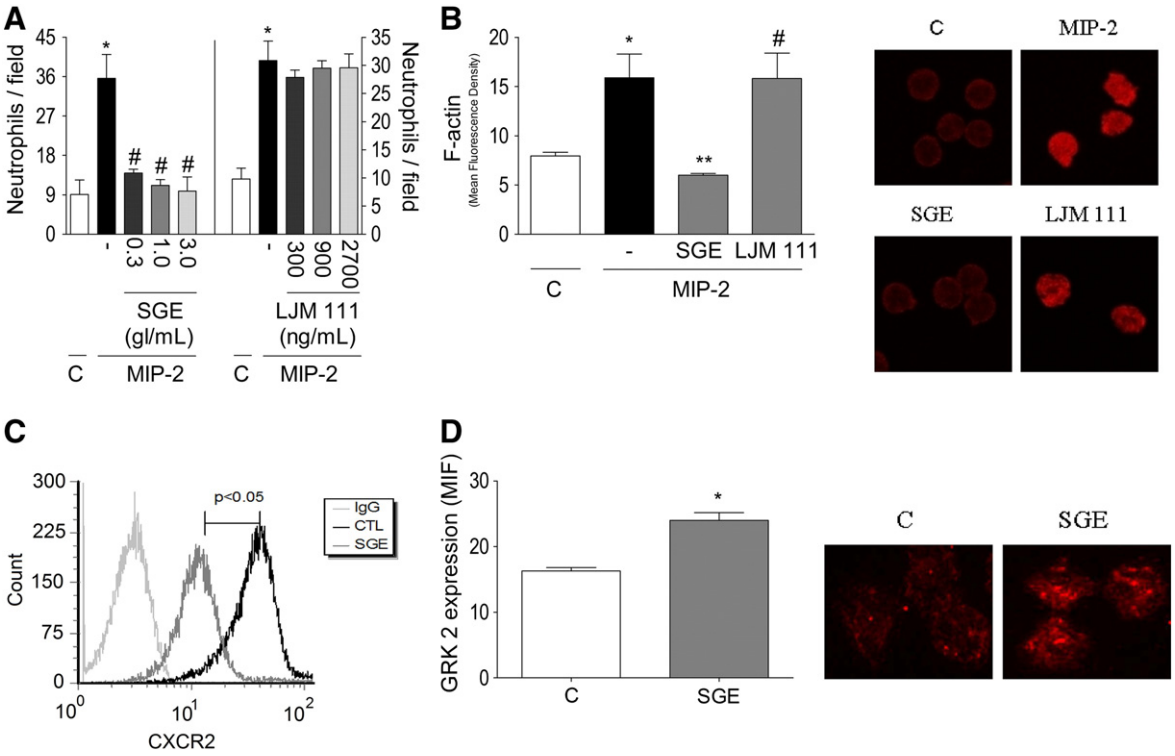

Fig. 5. The SGE, but not LJM 111 protein, inhibited *in vitro* neutrophil chemotaxis and actin polymerization, and SGE reduced CXCR2 expression and improved GRK-2 expression on neutrophils. (A) Chemotaxis of BM neutrophils to MIP-2 after 30 min SGE (0.3, 1.0 or 3.0 glands/ml) or LJM 111 (300, 900 or 2700 ng/ml) treatment. (B) F-actin polymerization of BM neutrophils to MIP-2 (30 ng/ml) after SGE (3.0 gland/ml) or LJM 111 (2700 ng/ml) treatment. Representative images (above) and quantification of F-actin polymerization by mean fluorescence density (below) are shown. (C) Flow cytometry of neutrophil CXCR2 expression and (D) immunofluorescence of GRK2 expression following SGE (3.0 glands/ml) treatment quantified by mean fluorescence intensity (MFI, below) and representative immunofluorescence microphotograph of GRK2 expression (above). $^{*}=P<0.05$ versus control group (C); $^{\#}=P<0.05$ versus group of neutrophils stimulated with MIP-2, by analysis of Student's *t*-test or one-way ANOVA where appropriate.

However, rLJM111 does not present this direct effect on neutrophils, indicating that other constituint(s) of SGE are responsible for the GRK-dependent downregulation of CXCR2 expression in neutrophil. In fact, rLJM111 inhibited the production of inflammatory mediators that are chemoattractants of neutrophils such as TNFα and IL-17. The result demonstrating that LJM111 did not affect the chemoattraction induced by MIP-2 further corroborates the suggestion that LJM111 inhibits the production of inflammatory molecules resulting in reduced in vivo neutrophil recruitment, but does not affect the effect of inflammatory molecules already produced resulting in absence of effect in the chemoattraction in vitro assay in which the active molecule is added without the need of a resident cell production. Thus, the saliva property of downregulating the inflammation justify further efforts to investigate the components responsible for this effect.

5. Conclusions

We showed that one of the mechanisms triggered by SGE and rLJM111 to inhibit the inflammatory response induced by mBSA could be by the modulation of DC function and maturation. SGE presents an additional property, which is not shared by LJM111, the inhibition of neutrophil chemotaxis a GRK-dependent downregulation of CXCR2 expression in a TLR4-independent mechanism. Therefore, LJM 111 protein, present in SGE, partially accounts for SGE anti-inflammatory activity in AIA model by modulating DC function and maturation but not acute innate inflammatory responses (neutrophil chemotaxis). This relative limitation of LJM111 mechanism compared to SGE does not reduce the importance of the finding. In fact, it increases its possible clinical relevance since in auto-immune diseases such as RA, it is desireble to inhibit auto-immune responses without affecting innate inflammatory responses necessary for host defence against infections. Furthermore, these results demonstrate the importance of distinguising the molecules present in SGE and their mechanisms.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.intimp.2012.02.004.

Competing interests

All the authors declare that they have no competing interests.

Authors' contributions

RG and FqC conceived and designed the experiments. RG, HpL, CjfO, VC, WaVJ and FS performed the experiments. RG, WaVJ, VC, JmR, JgV and FqC participated in analysis and interpretation of data. CT, JmR and JgV carried out the construction of DNA plasmids coding for proteins, expression and purification of recombinant protein and obtaining salivary gland extract of *Lutzomyia longipalpis*. RG, HpL, WaVJ, CjfO, JmR, JgV and FqC wrote the paper. All authors read and approved the final manuscript.

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