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Clinical report

Ts6 and Ts2 from *Tityus serrulatus* venom induce inflammation by mechanisms dependent on lipid mediators and cytokine production

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A B S T R A C T

Inflammatory mediators are thought to be involved in the systemic and local immune response induced by the *Tityus serrulatus* scorpion envenomation. New functional aspects of lipid mediators have recently been described. Here, we examine the unreported role of lipid mediators in cell recruitment to the peritoneal cavity after an injection with Ts2 or Ts6 toxins isolated from the *T. serrulatus* scorpion venom. In this report, we demonstrate that following a single intraperitoneal (i.p.) injection of Ts2 or Ts6 (250 μg/kg) in mice, there was an induction of leukocytosis with a predominance of neutrophils observed at 4, 24, 48 and 96 h. Moreover, total protein, leukotriene (LT)B_4, prostaglandin (PG)E_2 and pro-inflammatory cytokine levels were increased. We also observed an increase of regulatory cytokines, including interleukin (IL)-10, after the Ts2 injection. Finally, we observed that Ts2 or Ts6 injection in 5-lipoxygenase (LO) deficient mice and in wild type (WT) 129sv mice pre-treated with LTs and PGs inhibitors (MK-886 and celecoxib, respectively) a reduction the influx of leukocytes occurs in comparison to WT. The recruitment of these cells demonstrated a phenotype characteristic of neutrophils, macrophages, CD4 and CD8 lymphocytes expressing GR1+, F4/80+, CD3+/CD4+ and CD3+/CD8+, respectively. In conclusion, our data demonstrate that Ts2 and Ts6 induce inflammation by mechanisms dependent on lipid mediators and cytokine production. Ts2 may play a regulatory role whereas Ts6 exhibits pro-inflammatory activity exclusively.

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1. Introduction

Envenomation in humans is a serious public health problem that afflicts urban and rural areas throughout the world. In Brazil, recent data reveal that of a total of 13,038 accidents caused by venomous or poisonous animals, 53% of envenomation cases and 14 deaths (0.203% lethality) were caused by scorpions (Ministério da Saúde, 2011).

Tityus serrulatus venom (TsV) consists of a complex mixture of mucus, low molar mass components and neurotoxic proteins (Müller, 1993; Gwee et al., 2002; revised by Cologna et al., 2009). It is well known that *T. serrulatus* neurotoxins specifically interact with Na^+, K^+, Cl^-, and Ca^{+2} ion channels (Becerril et al., 1997). Ts2, also known as TsTX-III (Possani et al., 1991), is a neurotoxin that acts on Na^+ channels (Cestèle and Catterall, 2000; Denac

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et al., 2000). The amino acid sequences were retrieved from the Universal Protein Resource Knowledgebase (www.uniprot.org) or the Worldwide Protein Data Bank (www.pdb.org). Ts2 (Uniprot ID P68410) contains 62 amino acids, including 8 cysteine residues (Mansuelle et al., 1992). The peptide has a theoretical molar mass of 6998.0 Da and a pI of 7.70 (http://web.expasy.org/protparam/). Ts2 is a sodium channel inhibitor α-neurotoxin that inhibits the rapid inactivation of NaV1.2, NaV1.3, NaV1.5, NaV1.6 and NaV1.7, but does not affect NaV1.4, NaV1.8 or DmNaV1 (Cologna et al., 2012). The overall structure of the Ts2 model consists of three β-strands and one α-helix that are arranged in a triangular shape forming a cysteine-stabilized α-helix / β-sheet (CSαβ) motif (Cologna et al., 2012). While Ts6, also called TsTX-IV (Arantes et al., 1989), acts on K^+ channels (Coronas et al., 2003). Ts6 (Uniprot ID P59936) contains 40 amino acids, including 8 cysteine residues (Pimenta et al., 2003). The theoretical molar mass and pI of the peptide are 4514.2 Da and 8.50, respectively (http://web.expasy.org/protparam/). Ts6 is a potassium channel toxin alpha-KTx 12.1 that inhibits high conductance calcium-activated potassium channels (Novello et al., 1999) and weakly inhibits Shaker B potassium channels (Coronas et al., 2003). The structure of the peptide consists of an alpha-helix connected to a triple-stranded beta-sheet stabilized by four disulfide bonds (Oyama et al., 2005). Our group has previously demonstrated that following stimulation with Ts1, Ts2 or Ts6, the *in vitro* release of inflammatory mediators by peritoneal macrophages (J774.1) is independent of the action of these toxins on ion channels (Zoccal et al., 2011).

Several reports demonstrated that cytokines are increased after envenomation. Magalhães et al. (1999) observed an increase of interleukins (IL)-1α, IL-6, Interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the serum of patients who were stung by *T. serrulatus*. Furthermore, the increased levels of GM-CSF in severe envenomation might be involved in neutrophilia induced by *T. serrulatus* venom (Magalhães et al., 1999). The synthesis of anti-inflammatory cytokines, such as IL-10, was also observed in the plasma of patients with both moderate and severe cases of envenomation (Fukuhara et al., 2003; Petricevich, 2010). Scorpion venoms can stimulate the immune-neuroendocrine axis by inducing the release of catecholamines, corticosteroids, bradykinin (Chaudry et al., 1989; Sofer et al., 1996; Magalhães et al., 1999; Pessini et al., 2003) and eicosanoids mediators, such as prostaglandin $(PG)E_2$, lipoxin A_2 (LXA_2) and leukotriene $(LT)B_4$ (Nascimento et al., 2005; Teixeira et al., 1997).

Eicosanoids, derived from the enzymatic oxygenation of arachidonic acid (AA), are signaling molecules that control key cellular processes, including cell activation, metabolism, migration, cell proliferation and death (Funk, 2001; Yaqoob, 2003). PGE_2 and LTB_4 are AA-derived metabolites from pathways dependent on cyclooxygenase (COX) and 5-lipoxygenase (5-LO), respectively (Peters-Golden and Brock, 2000; Samuelson, 2000; Funk, 2001). These lipid mediators are involved in inflammation and several homeostatic biological functions, including vascular permeability and leukocyte influx to the bronchoalveolar fluid (Teixeira et al., 1997; Nascimento et al., 2005). PGE_2 is involved in the inflammatory response, and in the neutrophil recruitment (Fruscella et al., 2001) in mice inoculated with *T. serrulatus* scorpion venom (Pessini et al., 2006). PGE_2 is also produced after i.p. inoculation of phospholipase A_2 from the *Bothrops asper* snake venom in mice (Moreira et al., 2011). Additionally, the action of crotoxin (neurotoxin isolated from *Crotalus durissus terrificus* venom) is modulated by 5-LO-derived lipidic mediators in rats (Nogueira-Neto et al., 2008). However, there is a lack of knowledge regarding the participation of these lipid mediators in cell recruitment to the peritoneal cavity induced by *T. serrulatus* Ts2 or Ts6.

To address this question, we first demonstrated the kinetics of cell recruitment to the peritoneal cavity of mice injected with Ts2 or Ts6 isolated from the venom of scorpion *T. serrulatus*, and characterized the possible inflammatory mediators involved in cell migration. Second, we inhibited PGs and LTs synthesis by treatment with celecoxib, a COX-2 inhibitor, or MK-886, a 5-LO activation protein (FLAP) inhibitor, and characterized the cell types and cell recruitment kinetics to the peritoneal cavity of mice injected with Ts2 or Ts6.

2. Materials and methods

2.1. Toxins

Toxins Ts2 and Ts6, representing 3% and 2.5% of the total crude soluble TsV, respectively, were purified and stored at −20 °C as previously described (Arantes et al., 1989; Cologna et al., 2011, 2012). Prior to the experiments, Ts2 and Ts6 were dissolved in phosphate buffered saline (PBS) and filtered through sterilizing membranes (Spritzenfilter: 0.22 mm, TPP, Switzerland). To determine whether the purified toxins were contaminated by the endotoxin LPS, a *Limulus* Amoebocyte Lysate test (LAL) was performed according to the manufacturer's instructions (QCL-1000, Bio Whittaker, Cambrex Company, Walkersville, MD, USA).

2.2. Animals

Male 129sv mice (6–8 weeks old) were obtained from the animal facility of Faculdade de Ciências Farmacêuticas de Ribeirão Preto (FCFRP) – Universidade de São Paulo (USP). Male 5-LO deficient (5 $LO^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and raised at FCFRP-USP with their age-matched male wild type littermates (WT-background, strain 129). These mice were maintained under standard laboratory conditions. All experiments were approved and conducted in accordance with the guidelines of the University Animal Care Committee (process n^0 09.1.847.53.4).

2.3. Evaluation of leukocyte influx into the peritoneal cavity

Groups of six mice were injected i.p. with 300 μL of Ts2 or Ts6 (250 μg/kg) diluted in sterile PBS. The experiments were performed twice ($n = 12$). The controls received 300 μL of sterile PBS. After 4, 24, 48 and 96 h, the animals were euthanized in a CO_2^- chamber and 3 mL of PBS was

added into the abdominal cavity, which was gently massaged for 1 min. Peritoneal fluid was collected using a syringe with a needle inserted into the inguinal region. Total peritoneal cells were counted in Turk's solution using Neubauer chambers. Differential peritoneal leukocyte counts were performed on cytospin preparations stained with commercial kit based on the Romanowsky staining procedure (Panótico® Laborclin, Paraná, Brazil). After centrifugation ($400 \times g$, for 10 min, at 10 °C), the cell-free peritoneal fluid was stored at −80 °C.

2.4. Injection of Ts2 or Ts6 in mice and treatment with celecoxib or MK-886

Groups of six mice (129sv and 5-LO$^{-/-}$) were injected i.p. with 300 μL of Ts2 or Ts6 (250 μg/kg) diluted in PBS. Control animals received 300 μL of sterile PBS. The experiments were performed twice ($n = 12$). One group of 129sv was orally treated with celecoxib or MK-886 (5 mg/kg/0.5 mL) 1 day as well as 1 h prior to the i.p. injection with Ts2 or Ts6, and again every 24 h until the end of the experiment. After 4 and 96 h of i.p. injection, the animals were euthanized in a CO_2^- chamber, and the peritoneal fluid was collected as described above.

2.5. Quantification protein

Total proteins were quantified in the cell-free peritoneal fluid from 129sv mice injected with Ts2 or Ts6 by Coomassie protein assay reagent (Rockford, USA), according to the manufacturer's instructions.

2.6. Cytokine measurements

The cell-free peritoneal fluid obtained from 129sv mice injected with Ts2 or Ts6 was used to measure TNF-α, IL-6, IL-1β, IFN-γ, IL-10 and IL-4 by ELISA using specific antibodies (purified and biotinylated) and cytokine standards, according to the manufacturers' instructions (R & D Systems, Minneapolis, USA). Optical densities were measured at 405 nm in a microplate reader (μQuant, Biotek Instruments Inc.). For each sample, cytokine levels were obtained from a standard curve established with the appropriate recombinant cytokine (results expressed in pg/mg of total protein). Sensitivities were >10 pg/mL.

2.7. LTB_4 and PGE_2 measurements

LTB_4 and PGE_2 were quantified in the cell-free peritoneal fluid from 129sv mice injected with Ts2 or Ts6 by enzyme immunoassay (Cayman Chemical, USA). Briefly, supernatant dilutions were incubated with conjugated eicosanoid-acetylcholinesterase and antiserum in 96-well plates precoated with anti-rabbit immunoglobulin G antibodies. After incubation overnight at 4 °C, plates were washed and enzyme substrate (Ellman's reagent) was added for 60–120 min at 25 °C. Sample absorbance was determined at 420 nm in a microplate reader (μQuant, Biotek Instruments Inc.), and concentrations of eicosanoids were calculated based on the standard curve. The detection limit was approximately 13 pg/mL.

2.8. Immunophenotyping of the lavage peritoneal cells

CD3, CD4, CD8, F4/80 and GR1 expression in the cells from the peritoneal fluid recovered from 129sv mice injected with Ts2 or Ts6, with or without celecoxib or MK-886 treatment, was determined by flow cytometry immunostaining protocol using antibody conjugated with fluorochromes (BD Biosciences, NJ, USA). Specific murine IgG2a isotype controls were used to monitor non-specific binding. Stained peritoneal cells were washed with PBS containing 2% fetal bovine serum (FBS), pelleted by centrifugation at $400 \times g$ and fixed with PBS containing 1% (w/v) paraformaldehyde. A total of 30,000 events were acquired (FACSCanto™; Becton Dickinson, CA, USA) using the FACS Diva software (version 6.1.3) for data acquisition and analysis.

2.9. Statistical analyses

Data were expressed as the mean ± SEM. Statistical variations were analyzed using multi-factorial ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ts2 or Ts6 induces leukocyte influx and increase total protein into the peritoneal cavity

The response of mice to the i.p. injection of Ts2 or Ts6 was studied by evaluating the influx of leukocytes into the peritoneal cavity. Ts2 or Ts6 i.p. inoculation in mice induced an increase of total leukocyte (Fig. 1A) and neutrophil (Fig. 1B) numbers in the peritoneal cavity throughout the experimental time course (4, 24, 48 and 96 h). The mononuclear cells were increased after 4 and 96 h post Ts2 injection compared to mice inoculated with PBS. Ts6 increased mononuclear cells only after 96 h (Fig. 1C). We also determined the acute phase protein levels in the peritoneal fluid of mice injected with Ts2 or Ts6 (Fig. 2). Compared to control, the total protein levels of the peritoneal fluid peaked between 24 and 48 h and then decreased at 96 h after injection with Ts2 or Ts6 (Fig. 2). Taken together, these results demonstrated that Ts2 or Ts6 induced an inflammatory response in the peritoneal cavity, mainly during the first 24 h.

3.2. Ts2 and Ts6 induce cytokine release

Fig. 3 shows the profile of inflammatory cytokines released in the peritoneal cavity after the injection with Ts2 or Ts6. We demonstrated that Ts2 and Ts6 altered the release of specific cytokines in a time-dependent manner. Ts2 augmented the release of IL-6, IFN-γ and the regulatory cytokine IL-10 at 4 h (Fig. 3A, D and E, respectively); at 24 h, however, only IL-10 was increased (Fig. 3E). At 48 h post Ts2 injection, there was an increase in the levels of TNF-α, IFN-γ, IL-10 and IL-4 (Fig. 3B, D, E and F, respectively), while at 96 h, we only observed an increase in TNF-α, IL-1β and IL-10 (Fig. 3B, C and E, respectively). Additionally, we observed a mild difference in the cytokine profile following the Ts6 injection compared to Ts2. After 4 h, Ts6 increased the

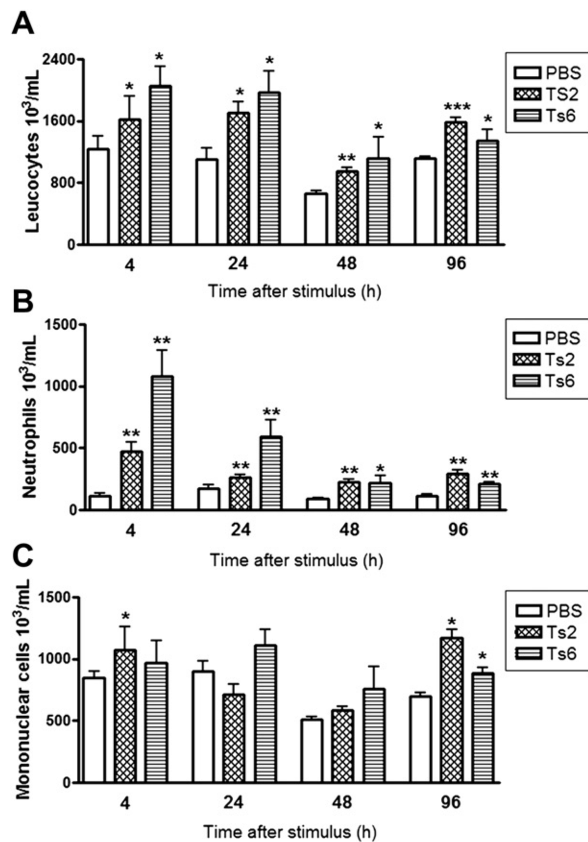

Fig. 1. Leukocyte, neutrophil and mononuclear cells recruitment induced by i.p. Ts2 or Ts6 (250 μg/kg) injection in mice. Control groups were injected with PBS. (A) Leukocyte, (B) neutrophils and (C) mononuclear cells were numbered after 4, 24, 48 and 96 h of the injection. Data are expressed as the mean ± SEM from two independent experiments ($n = 12$ mice per group). *$P < 0.05$; **$P < 0.001$ and ***$P < 0.0001$ when compared to the control PBS group.

release of IL-6, TNF-α, IL-1β and IFN-γ (Fig. 3A–D, respectively), while only the release of IFN-γ was increased at 24 h (Fig. 3D). At 48 h, TNF-α and IFN-γ levels were increased (Fig. 3B and D), while only TNF-α was increased after 96 h (Fig. 3B). For comparison, the changes in the corresponding cytokine release were also measured in a control group of mice that received PBS injection.

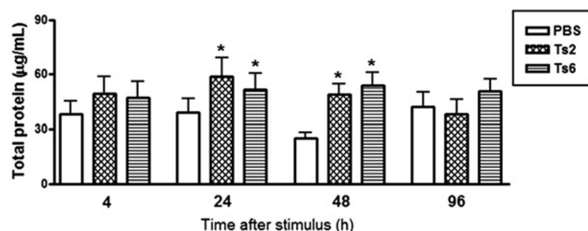

Fig. 2. Effects of intraperitoneal Ts2 or Ts6 (250 μg/kg) injections on the total protein levels in the peritoneal cavity. Control groups were injected with PBS. The total protein was determined by the Coomassie protein assay reagent after 4, 24, 48 and 96 h post injection. Data are expressed as the mean ± SEM from two independent experiments ($n = 12$ mice per group). *$P < 0.05$ when compared to PBS group.

3.3. *Ts2 or Ts6 induces the release of LTB_4 and PGE_2*

To determine whether LTB_4 and PGE_2 are produced as a result of the toxin injection, groups of mice were i.p. inoculated with Ts2 or Ts6 and the peritoneal fluid was collected after 4, 24, 48 and 96 h. Compared to controls, we observed a peak in the production of both lipid mediators 4 h and 24 h after the injection with Ts2 and Ts6, respectively (Fig. 4). Additionally, a significant increase in the LTB_4 production was observed after 24 and 48 h of Ts2 injection, followed by a decrease at 96 h, relative to control (Fig. 4A). Ts6 induced an increase in LTB_4 release throughout the experimental time course (Fig. 4B). Moreover, PGE_2 was increased in Ts2 or Ts6-dependent manner at all time points, compared to control (Fig. 4). The rate of prostaglandin-leukotriene was maintained throughout the course of the study.

3.4. *Leukocyte recruitment induced by Ts2 or Ts6 is partially dependent on the prostaglandin and leukotriene production*

To understand the role that PGs and LTs play in cell recruitment to the peritoneal cavity following Ts2 or Ts6, we treated mice concomitantly with MK-886 (FLAP inhibitor) or celecoxib (COX-2 inhibitor). Treatment of 129sv (WT) animals with MK-886 or celecoxib (5 mg/kg/day) effectively reduced the number of leukocytes at 4 and 96 h compared to the Ts2 injection, but only after 4 h compared to the Ts6 injection (Fig. 5A); neutrophils were reduced at 4 and 96 h compared to the Ts2 or Ts6 injection (Fig. 5B); mononuclear cells were reduced at 4 and 96 h compared to Ts2, but only after 4 h compared to Ts6 (Fig. 5C). The same pattern of leukocyte recruitment inhibition was observed by treating the animals with MK-886 or celecoxib. However, MK-886 was more efficient than celecoxib in inhibiting inflammatory cell recruitment in the presence of Ts6 (Fig. 5).

We also compared the WT mice (129sv) with the 5-LO$^{-/-}$ mice following the Ts2 or Ts6 injection. Compared to the WT mice that only received Ts2, we observed inhibition of the total leukocytes, neutrophils and mononuclear cells in 5-LO$^{-/-}$ mice at 4 and 96 h after Ts2 injection (Fig. 5). Ts6 inhibited leukocytes and mononuclear cells after 4 h, while neutrophils were inhibited after 4 and 96 h compared to the WT mice that received Ts6. The results demonstrated that the WT mice treated with MK-886 displayed the same behavior as the 5-LO$^{-/-}$ mice, suggesting that the Ts2 or Ts6-driven induction of leukocyte recruitment, observed primarily in neutrophils, is partially dependent on LTs.

3.5. *Immunophenotypic characterization of cell recruitment to the peritoneal cavity after Ts2 or Ts6 injection and MK-886 or celecoxib treatment*

The peritoneal cell populations, obtained after Ts2 or Ts6 injection and MK-886 or celecoxib treatment, were characterized by flow cytometry. We performed analyses using anti-GR1, F4/80, CD3, CD4 and CD8 immunoglobulins. The number of cells expressing GR1, a typical neutrophil marker, changed significantly between the

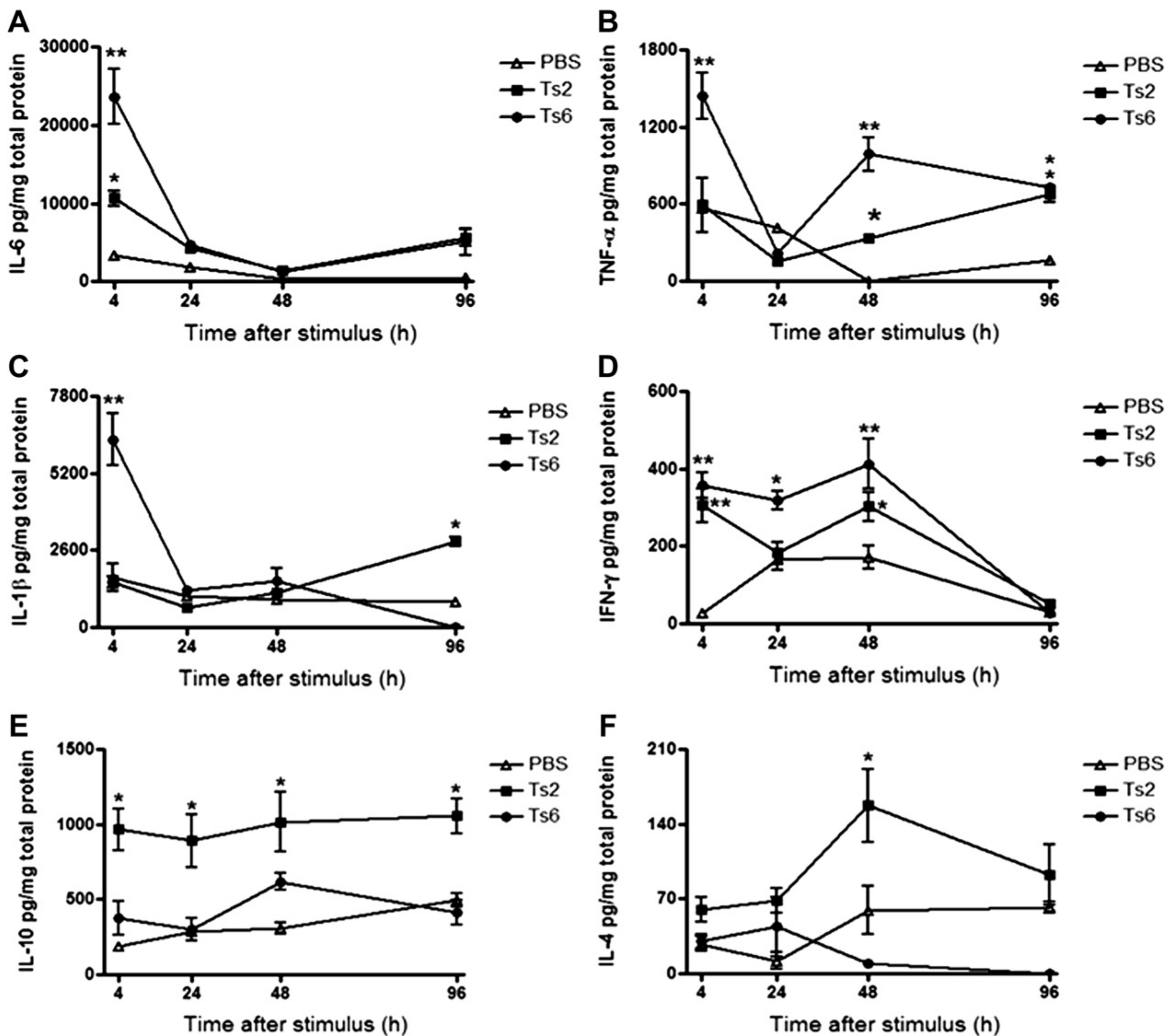

Fig. 3. Ts2 or Ts6 modulates levels of cytokines in the peritoneal cavity. (A) IL-6, (B) TNF-α, (C) IL-1β, (D) IFN-γ, (E) IL-10 and (F) IL-4 were evaluated after 4, 24, 48 and 96 h following the Ts2 or Ts6 (250 μg/kg) injection. Control groups were injected with PBS. Data are expressed as the mean ± SEM from two independent experiments ($n = 12$ mice per group). *$P < 0.05$ and **$P < 0.001$ compared to PBS group.

PBS, Ts2 or Ts6 only, and MK-886 or celecoxib treated groups. We observed an increase in GR1+ cells in the Ts2 or Ts6 only groups at 4 and 96 h. In the MK-886 or celecoxib treated groups, GR1+ cells decreased to the levels similar to the PBS group at 4 h. At 96 h, the same profile was observed (Fig. 6A). The number of F4/80 positive cells increased in the Ts2 or Ts6 group compared to PBS at 4 and 96 h, and decreased to Ts2+celecoxib and Ts2+MK-886 at 4 h compared to Ts2 and to Ts6+MK-886 group at 96 h compared to Ts6 (Fig. 6B). The same expression profile was observed in CD3+/CD4+ and CD3+/CD8 cells. Compared to PBS, CD3+/CD4+ and CD3+/CD8+ expression increased in Ts6 at 4 and 96 h and Ts2 at 96 h. CD3+/CD4+ expression decreased in Ts6+MK-886 at 4 h and Ts6+celecoxib at 96 h compared to Ts6, while Ts2+MK-886 and Ts2+celecoxib demonstrated decreased expression at 96 h compared to Ts2. CD3+/CD8+ cell number decreased following the Ts6+celecoxib and Ts6+MK-886 treatment at 4 and 96 h compared to Ts6, and Ts2+celecoxib and Ts2+MK-886 treatment at 96 h compared to Ts2 (Fig. 6C and D). These results suggest that the decreased expression of these markers can be related to the reduced number of cells recruited into the peritoneal cavity as observed in Figs. 1 and 5.

4. Discussion

Our study revealed two surprising and important new findings. First, the kinetics of cell migration induced by the active preparations permitted us to characterize a local inflammatory reaction with the gradual increase in neutrophils, inflammatory cytokines (especially in the early phase of response), and lipid mediators. Second, we demonstrated that cell recruitment is partially dependent on PGs and LTs.

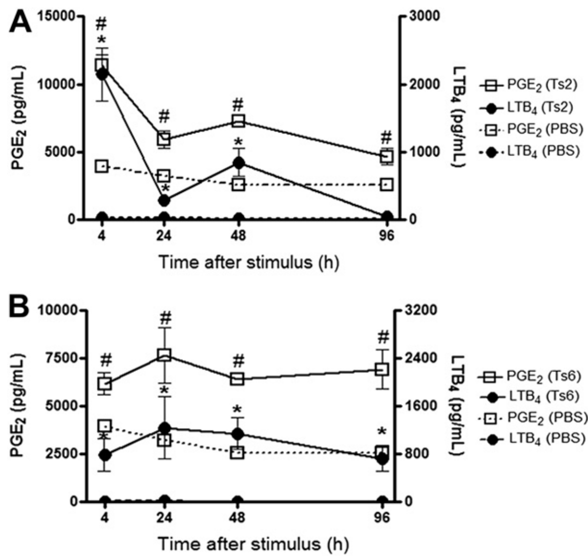

Fig. 4. Ts2 or Ts6 injection induces eicosanoid generation in the peritoneal cavity. The PGE_2 and LTB_4 release was evaluated after 4, 24, 48 and 96 h following the injection with (250 μg/kg) of Ts2 (A) or Ts6 (B). Control groups were injected with PBS. Data are expressed as the mean ± SEM from two independent experiments ($n = 12$ mice per group). $^*P < 0.001$ compared to PBS group relative to PGE_2 and $^\#P < 0.001$ compared to PBS group relative to LTB_4.

It is known that during the acute inflammatory response, depending on the stimulus, the first event is the recruitment of neutrophils, followed by the arrival of other cells, including macrophages and lymphocytes (Medzhitov, 2008). A high leukocyte count in the victims of scorpion envenomation is partially due to the action of catecholamines, released by the scorpion's venom and known to induce leukocytosis through the mobilization of marginated cells (Dàvila et al., 2002; Zeghal et al., 2000). In this study, we demonstrated that the neutrophils were the prominent cells of all cell types that migrated to the peritoneal cavity. However, we also observed an increase in the number of mononuclear cells in the later stages (at 96 h). The acute-phase response can also be characterized by an increase in total protein levels between 24 and 48 h (Fig. 2). Taken together, these results corroborate data in the literature which indicate that the total protein increase along with leukocytosis in the peritoneal cavity is a characteristic of the local inflammatory response (Petricevich, 2010).

Following the venom injection, a variety of cytokines are released and the outcome of the inflammatory response is dictated by a number of factors that include the duration of the stimulus and the balance between pro and anti-inflammatory responses (Petricevich, 2010). Increased IL-6 and TNF-α levels were observed in plasma from patients with different degrees of *T. serrulatus* envenomation, as well as in human serum and mouse macrophage supernatants (Magalhães et al., 1999; Fukuhara et al., 2003; Pessini et al., 2003; Petricevich et al., 2007). Our group demonstrated that TsV, Ts1 and Ts6 are able to stimulate macrophages to produce IL-6 and TNF-α *in vitro* (Zoccal et al., 2011). In the present study, we observed a high increase of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β and IFN-γ) in the early stage, especially in response to Ts6. Moreover, it was previously demonstrated that the regulatory cytokines IL-10 and IL-4 were increased in serum of patients envenomed with *T. serrulatus* scorpions and in experimental animals exposed to *Androctonus australis hector or Centruroides noxius* (Magalhães et al., 1999; Petricevich et al., 2007; Petricevich, 2006; Adi-Bessalem et al., 2008). IL-10 functions, in part, in key homeostatic mechanisms that control the degree and duration of the inflammatory response (Bazzoni et al., 2010). We observed an increase in the anti-inflammatory cytokine release, including IL-10 and IL-4, after Ts2 injection and primarily after 48 h. These results corroborate our previous *in vitro* results (Zoccal et al., 2011) and suggest that the venom may contain compounds with divergent activities. Here, we observed that Ts2 can induce the recruitment of neutrophils to the site of interest (Fig. 1) and also stimulate the anti-inflammatory cytokine (mainly IL-10) production *in vivo* (Fig. 3). This result corroborate partially with our previous findings which used *in vitro* stimulated peritoneal macrophages and demonstrated that Ts2 had an anti-inflammatory potential (Zoccal et al., 2011). However, it is important to take into account that the expression and production of pro or anti-inflammatory molecules by a stimulus may vary depending on the microenvironment used in the study (Bazzoni et al., 2010). Additionally, behaviors *in vivo* and *in vitro* may differ due to numerous factors, such as the presence of other resident cells, that can interfere with the inflamed site. We speculated that the neutrophils recruited by Ts2 to peritoneal cavity could be the main source of IL-10, based on the fact that these cells are present at the site of lung inflammation and function as a source of IL-10 (Zhang et al., 2009). Thus, our data suggest that Ts2 can play an important regulatory role *in vivo* due to its ability to release anti-inflammatory cytokines and recruit neutrophils to the peritoneal cavity.

During inflammation, lipid mediators such as PGs and LTs can be released in addition to cytokines. These mediators are induced after membrane disturbance that lead to increased intracellular calcium (Lewis et al., 1990; Funk, 2001). In the present work, we demonstrated through three different findings that the Ts2 or Ts6-induced recruitment of cells to peritoneal cavity is partially dependent on lipid mediators. First, we observed that Ts2 induced the production of PGE_2 and LTB_4. We suggest that the resulting cell activation that culminates in the increase of the downstream products of these pathways (LTs and PGs), and possibly in the increased phospholipase A_2 activity, a key enzyme involved in the formation of both lipid mediators. Our results demonstrate a balance between PGE_2 and LTB_4 and a corresponding increase of both lipids that could be due to the action of cytokines released at earlier time points. Unlike Ts2, Ts6 did not induce LTB_4 and PGE_2 production during the initial cell activation, but induced distinct amounts throughout the activation time course. Ts6 induced an upregulation on these mediators after 24 h and the rate of PGE_2/LTB_4 production remained constant throughout all the previous time points (Fig. 4B). Taking into consideration

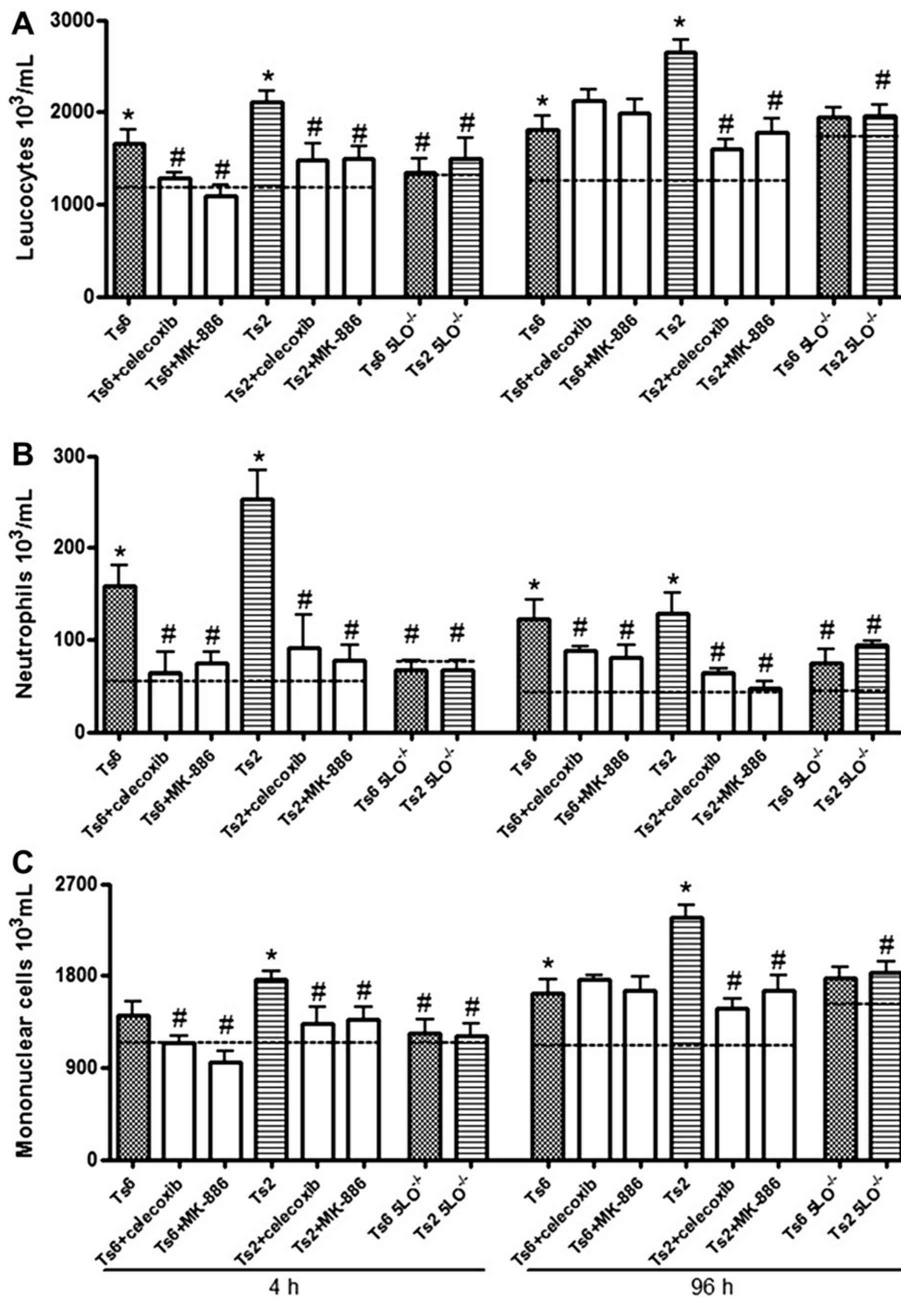

Fig. 5. Leukocyte, neutrophil and mononuclear cell recruitment increases in the peritoneal cavity after Ts2 or Ts6 injection by a mechanism partially dependent on the lipid mediators. Pre-treatment with MK-886 (5 mg/kg/0.5 mL by gavage) or celecoxib (5 mg/kg/0.5 mL by gavage) significantly reduced the number of cells in the peritoneal cavity of 129sv mice. All treatments were performed 1 day and 1 h prior to Ts2 or Ts6 injection (250 μg/kg) and every 24 h until the end of the experiment. Controls received only PBS. Data are expressed as the mean ± SEM from two independent experiments ($n = 10$ mice per group). $^{*}P < 0.001$ compared to PBS group and $^{\#}P < 0.001$ compared to 129sv mice group that received Ts2 or Ts6 injection. Leukocyte, neutrophil and mononuclear cell recruitment to the peritoneal cavity of 5-LO$^{-/-}$ (129-Alox5tm1Fun) mice is decreased compared to the strain-matched wild-type (WT) mice. Each strain was inoculated with Ts2 or Ts6 (250 μg/kg) and allowed to recover for 4 and 96 h after stimulus. Data are expressed as the mean ± SEM from two independent experiments ($n = 12$ mice per group). $^{\#}P < 0.001$ compared to 129sv mice group that received Ts2 or Ts6 injection.

our findings that revealed leukocyte recruitment following the Ts2 or Ts6 injection, we investigated the role of potent leukocyte chemoattractants known as LTs (Faccioli et al., 1991; Herschman, 1996; Medeiros et al., 1999). For this purpose, we pre-treated mice with MK-886 to inhibit LTs synthesis (Ford-Hutchinson et al., 1980) and observed reduced cell numbers after Ts2 or Ts6 injection. We also employed mice that were unable to produce LTs (5-LO$^{-/-}$) and injected them with Ts2 or Ts6. These mice demonstrated decreased cell numbers compared to WT animals. In addition, LTB_4 was increased in the peritoneal fluid of mice exposed to Ts2 or Ts6 in

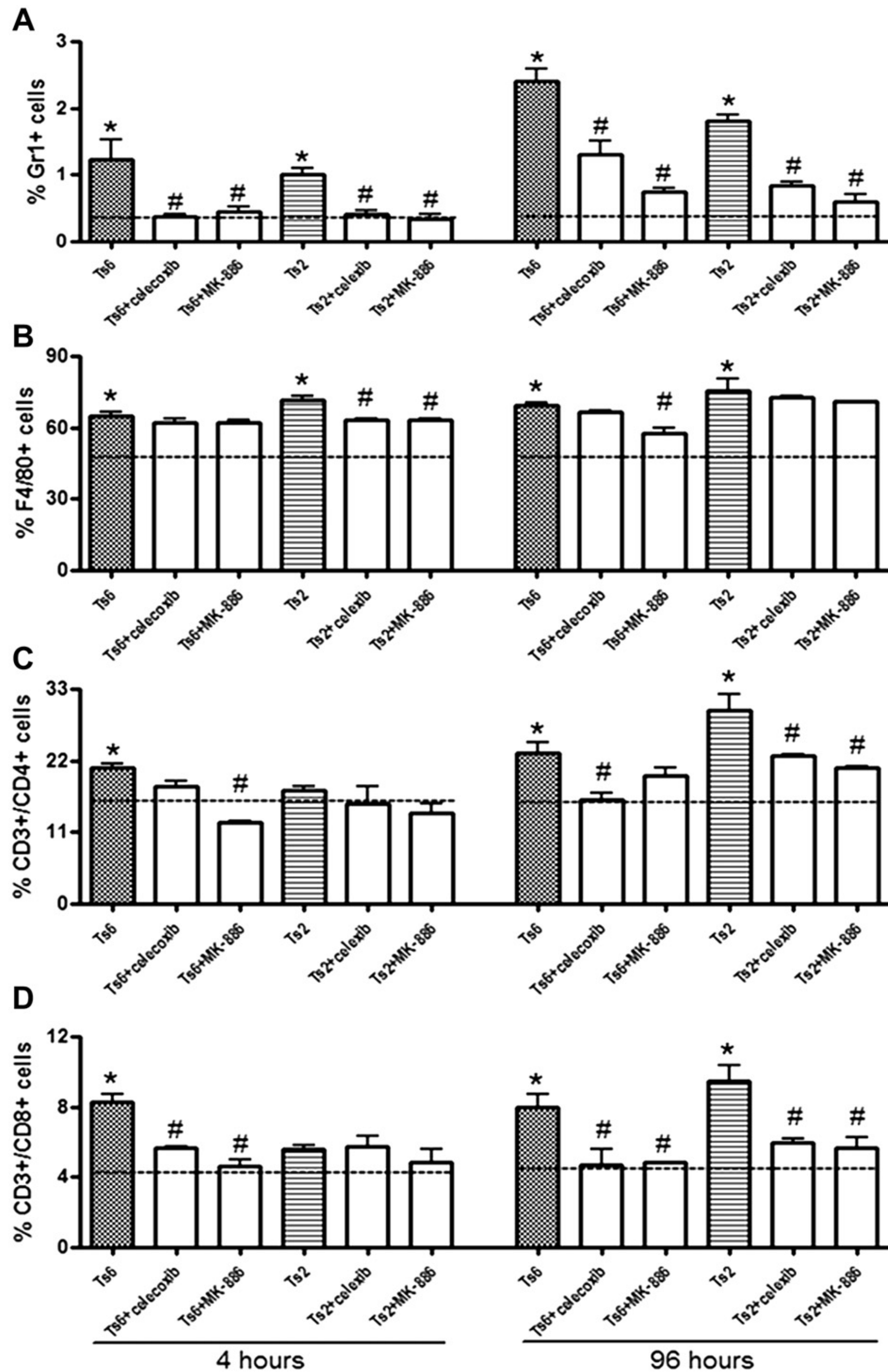

Fig. 6. Ts2 or Ts6 induced an increase of neutrophils, macrophages and lymphocytes in the peritoneal cavity. Cells were obtained from mice after the i.p. injection with PBS, Ts2 or Ts6 with or without celecoxib or MK-886 pre-treatment as described in the Material and Methods section. The neutrophil, macrophage and lymphocyte populations were analyzed for the percentage of classic markers for neutrophils, macrophages and T cells expressing an effector phenotype. (A) GR1+ for neutrophils, (B) F4/80 for macrophage, (C) CD4+ T cells and (D) CD8+ T cells. Data are expressed as the mean ± SEM from two independent experiments. $^{*}P < 0.001$ comparing PBS vs. Ts2 or Ts6; $^{\#}P < 0.005$ comparing Ts2 or Ts6 vs. celecoxib or MK-886.

comparison to mice injected with PBS (control). Taken together, these results showed that LTs, predominantly represented by LTB_4, are necessary to promote cellular migration following Ts2 or Ts6 inoculation.

Taking into consideration that prostanoids are also involved in cell recruitment, we explored the involvement of cyclooxygenase (COX)-derived PGs in the cell increase observed in our results. For that purpose, we pre-treated

mice with a COX-2 inhibitor celecoxib (Warner et al., 1999). Celecoxib-treated mice had a significantly diminished cellular migration, indicating that PGs could be involved in this process. Moreover, we also demonstrated a significant PGE_2 increase in the peritoneal fluid of mice exposed to Ts2 or Ts6 compared with the PBS control. It is known that the secretion of lipid mediators can be associated with an influx of neutrophils and an increase in inflammatory cytokines (Medeiros et al., 1999; Fernandes et al., 2007; Bagga et al., 2003). Taken together, these results demonstrated that the influx of cells to the peritoneal cavity induced by Ts2 or Ts6 is partially dependent on LTs and PGs.

Finally, we immunophenotyped the cells recruited to the peritoneal cavity after Ts2 or Ts6 injection. We observed that the cells were positive for GR1, F4/80, CD3, CD4 and CD8 markers after the Ts2 or Ts6 injection. These are the common surface markers used to characterize neutrophils (GR1), macrophages (F4/80+), CD4 (CD3+/CD4+) and CD8 (CD3+/CD8+) lymphocytes (Ramalingam et al., 2003; Pillai et al., 2009). Thus, this result reinforced the observation that neutrophils are increased in mice injected with the toxins and showed that the detected mononuclear cells are mainly macrophages and lymphocytes. As expected, after treatment with MK-886 or celecoxib, the percentage of cells expressing surface markers to GR1+ decreased. This could be due to the reduced number of cells recruited into the peritoneal cavity in relation to the recruitment induced by only Ts2 or Ts6, demonstrating that the recruitment of these cells is at least partially dependent on LTs and PGs. Moreover, our results revealed that neutrophils are the first cells recruited to the peritoneal cavity after the Ts2 or Ts6 injection. These cells together with resident cells characterize the inflammatory response by releasing inflammatory mediators, such as cytokines, LTB_4 and PGE_2, and increasing the total protein amounts. Concurrently, the same cells release anti-inflammatory cytokines, such as IL-10 and IL-4, to re-establish the homeostasis. However, the release of large amounts of inflammatory mediators overcomes the anti-inflammatory mediators. Subsequently, macrophages, CD4 and CD8 lymphocytes are recruited to re-establish the basal homeostatic state, in a mechanism partially dependent on PGs and LTs.

In conclusion, our data demonstrated that both Ts2 and Ts6 induced inflammatory response by mechanism dependent on lipid mediators and cytokines production. Moreover, the data suggested that Ts2 have a regulatory role in the inflammatory response, because it stimulated IL-10 production. Ts6 showed exclusive pro-inflammatory activity. Our results emphasize the importance of studies that aim to better understand the role of isolated toxins in envenomation. The mechanisms and the underlying signaling pathways, as well as the novel approaches for alternative treatments, might be useful in diminishing the lesions caused by *T. serrulatus* venom and will be the focus of our next work.

Ethical statement

We certify that human subjects were not used in this work.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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