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Expanding biological activities of Ts19 Frag-II toxin: Insights into IL-17 production

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ABSTRACT

Tityus serrulatus (Ts) venom is composed of a mixture of toxins presenting diverse biological functions. However, although this venom has been studied over the past three decades, omics analysis revealed that most of its toxins are not identified or their biological activities are unknown. Ts19 Frag-II is included in this group, which function is still uncertain. This study aimed to expand the biological activities of Ts19 Frag-II through *in vivo* investigation. Our results demonstrates that mice challenged with Ts19 Frag-II presented biochemical alterations, increasing serum levels of urea, ALT and β-globulin, besides decreasing γ-globulins. Moreover, this toxin was also able to induce immunological alterations, increasing NO, IL-6, TNF-α and IL-17, being considered a proinflammatory toxin. The increase of IL-17 was unprecedented regarding Ts toxins and could be a result of the overall produced-effect of cells of innate immunity cells (neutrophils, monocytes, natural killers and lymphoid tissue inducers - LTis) as well as of adaptive immunity (Th17 cells). This study expanded the biological activities of Ts19 Frag-II, suggesting that this toxin could be contributing to the Ts envenoming through alterations of biochemical parameters as well as triggering the inflammatory response.

1. Introduction

Tityus serrulatus (Ts) venom is composed of insoluble mucus, inorganic salts, nucleotides, amines, natriuretic peptide, kallikrein inhibitor, high molecular weight proteins, lipids, enzymes, free amino acids, peptides and neurotoxins (Pucca et al., 2015a). Ts compounds have been extensively studied by the scientific community, especially in recent years (Pucca et al., 2015a, 2015b, 2015c, 2015d, 2016a, 2016b; Zoccal et al., 2015; Zoccal et al., 2016), and a variety of toxins have been described (Ts1→Ts20). For a complete review of Ts compounds see (Bordon et al., 2015). Additionally, using mass spectrometry analyzes it was identified 380 different distinct masses in this venom (Pimenta et al., 2001). Thus, it is estimated that only 1% of these toxins are known (Gazarian et al., 2005; Pimenta et al., 2001). Moreover, many components already identified still have no function defined and need further studies. Ts19 Frag-II is included in this group.

Ts19 (β-KTx) was first described by Alvarenga et al. (2012) through transcriptome analysis of *T. serrulatus* gland (Alvarenga et al., 2012), although it was never been isolated so far. Nevertheless, two different fragments from Ts19 were isolated from the venom, named Ts19 Frag-I (Lima et al., 2015) and Ts19 Frag-II (Cerni et al., 2016), both produced by a Ts19 post-translational processing (post-splitting).

Ts19 Frag-II, a β-KTx toxin belonging to class (subfamily) 2, is a novel neurotoxin presenting 49 amino acid residues and a molecular mass of 5534 Da. Electrophysiological data demonstrated that this toxin is a specific blocker of voltage-gated potassium channel – Kv1.2, besides being non-cytolytic (Cerni et al., 2016). Moreover, other study showed that this fragment did not induce nociception behavior in mice (Pucca et al., 2016b). Nevertheless, it is evident that little is known about this toxin fragment. Therefore, further

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functional studies with Ts19 fragments are still needed to elucidate their role during envenoming. Based on the Ts venom effects in modulating biochemical (Correa et al., 1997; Pucca et al., 2012; Ribeiro et al., 2010) and immunological (Fialho et al., 2011; Fukuhara et al., 2003; Pessini et al., 2003, 2006; Zoccal et al., 2011) responses during envenoming, this study evaluated the ability of Ts19 Frag-II to alter biochemical parameters and modulate the immune system *in vivo*.

2. Material and methods

2.1. Ts19 Frag-II isolation

Ts venom extraction was performed using the telson electrical stimulation (12 V) method (Lowe and Farrell, 2011). After extraction, the pooled venom was desiccated and stored at −20 °C. The use of Ts venom was approved by the Genetic Patrimony Management board (CGEN/MMA), through Access and Shipment Component of Genetic Heritage for scientific research purpose (number 010214/2014-3).

Ts19 Frag-II (β-KTx class 2) was isolated as previously described (Cerni et al., 2016). Briefly, 40 mg of Ts venom (without mucus) was fractionated using Äkta Purifier UPC-10 system (GE Healthcare, Uppsala, Sweden). The unique chromatographic step was performed using a C18 column (10 mm × 250.0 mm, 5 μm particles, 300Å, Phenomenex, Torrance, Ca, USA) equilibrated with 0.1% (V/V) trifluoroacetic acid (TFA, Avantor Performance Materials Inc., Center Valley, PA, USA) at a flow rate of 5 mL/min. The samples were eluted with steps of concentration gradient from 0 to 100% of solution B (80% acetonitrile, Avantor Performance Materials Inc., Center Valley, PA, USA) in 0.1% TFA-at flow rate of 5 mL/min. The absorbance was monitored at 214 nm.

The purity of Ts19 Frag-II (fraction 25.2) was confirmed by amino terminal sequencing using a Protein Sequencer model PPSQ-33A (Shimadzu Co., Kyoto, Japan) and Edman degradation method (Edman and Begg, 1967).

2.2. In vivo experiments

2.2.1. Mice

Groups of male BALB/c mice (20–23 g, n = 5) received Ts19 Frag − II (4 or 8 μg) using subcutaneous (*s.c.*) injection in a final volume of 0.1 mL. Control groups received sterile physiological solution (0.9% W/V of NaCl − negative control) or *T. serrulatus* venom (TsV 20 μg − positive control). After 6 h of the challenge, animals were euthanized (Ketamine 60 mg/kg/Xylazine 8 mg/kg, intraperitoneally) and blood samples from the retro-orbital sinus were collected. The serum was centrifuged (1,800 × *g* for 15 min) and stored at 4° C until analysis. The use of mice are in accordance with the Ethic Principles in Animal Experimentation with the license number 13.1.41.53.4.

2.2.2. Biochemical analysis

The serum obtained as described above (2.2.1.) were used to evaluate biochemical parameters such as uric acid, creatine kinase, creatinine, glucose, lactato desidrogenase - LDH, aspartate aminotransferase - AST, alanine aminotransferase − ALT and urea. The analyses were performed using an automatic analyzer CT600i (Wiener Laboratórios, Rosario, Argentina) and their respective kits: CK-NAC UV AA, CREATININE AA, GOT (AST) UV AA, GPT (ALT) UV AA, URICOSTAT AA, UREA UV AA, GLICEMIA AA, LDH-P UV AA (Wiener Laboratórios, Rosário, Argentina).

For the serum protein electrophoresis, 0.6 μL of serum were applied in an agarose film (141001, Celmgel, CELM, São Paulo, Brazil). The electrophoresis was performed using a TRIS buffer pH 9.5 (V/V) at a voltage of 90 V for 25 min. The gel was stained with 0.2% (V/V) amido black and destained with 5% acetic acid (V/V). The densitometry of the gel was performed using a DenGo densitometer (Qualiterm, Cesário, Lange, SP, Brazil) and processed in DenGo software.

All the biochemical analyses were performed until 24 h following the blood collection.

2.2.3. Immunological analysis

The serum obtained as described above (2.2.1.) were also used for cytokines and nitric oxide (NO) analyzes. Cytokine analyzes were performed using ProcartaPlex kits - Affymetrix (eBioscience, Vienna, Austria) as specified by the manufacturer: IL-1β (EPX01A-26002-901), IL-2 (EPX01a-20601-901), IL-4 (EPX01A-20613-901), IL-5 (EPX01A-20610-901), IL-6 (EPX01A-20603-901), IL-10 (EPX01A-20614-901), IL-12p70 (EPX01A-26004-901), IL-13 (EPX01A-901), IL-17A (EPX01A-26001-901), TNF-α (EPX01A-20607-901) and IFN-γ (EPX01A-20606-901). Readings were performed on the MAGPIX®-Luminex (EMD Millipore).

NO production was evaluated according to the Griess method (Green et al., 1981). Nitrite (NO_2) is considered an indicator of NO production. The amount of nitrite in the samples was evaluated by a standard curve obtained from serial dilutions of $NaNO_2$ using absorbance at 540 nm. Quantification of nitrite was performed 48 h after collection.

3. Results

3.1. Ts19 Frag-II isolation

The Ts19 Frag-II toxin was successfully purified as previously described (Pucca et al., 2016b), which was confirmed by amino terminal sequencing (data not shown).

3.2. In vivo effects induced by Ts19 Frag-II toxin

3.2.1. Biochemical analysis

Changes in biochemical parameters induced by Ts19 Frag-II were evaluated by the determination of different analytes and enzymes in the mice serum: alanine aminotransferase (ALT), aspartate aminotransferase (AST), uric acid, creatinine, creatine kinase (CK), glucose, lactate dehydrogenase (LDH), urea and plasmatic proteins. The Ts19 Frag-II induced a significant increase of ALT (4 and 8 μg) and urea (8 μg). On the other hand, Ts venom (TsV) increased CK, urea and creatinine (Fig. 1).

The amount (%) of the serum proteins (albumin, α-1 and α-2 globulin, β-globulin and γ-globulin) was also assessed by agarose gel electrophoresis followed by gel densitometry. Administration of the Ts19 Frag-II toxin (4 and 8 μg) induced a significant increase of β-globulins (3 and 4% for 4 and 8 μg, respectively). However, it decreased the percentage of γ-globulins (4 and 8% for 4 and 8 μg, respectively). In contrast, Ts venom induced only a significant decrease in γ-globulins (5%) (Fig. 2).

3.2.2. Immunological analysis

The cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ) and oxide nitric (NO) were measured to evaluate the Ts19 Frag-II modulatory effect on the immune system. Ts19 Frag-II was administered *sc* and cytokines released systemically were evaluated in the sera of mice 6 h after injection. At 8 μg, Ts19 Frag-II induced significant increase of IL-6, TNF-α and IL-17A, while at 4 μg, Ts19 Frag-II induced an increase of TNF-α and IL-17A. Nevertheless, Ts venom (TsV, 20 μg) induced an increment of IL-6 cytokine in the sera (Fig. 3).

Administration of Ts19 Frag-II (8 μg) also induced a significant