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Contribution of endothelial cell and macrophage activation in the alterations induced by the venom of *Micrurus tener tener* in C57BL/6 mice

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ABSTRACT

An acute inflammatory response, cellular infiltrates, anemia, hemorrhage and endogenous fibrinolysis activation were previously described in C57BL/6 mice injected with *M. tener tener* venom (*Mtt*). As the endothelium and innate immunity may participate in these disturbances and due to our poor understanding of the alterations produced by these venoms when the neurotoxic component is not predominant, we evaluated the effects in an *in vitro* model. At 24 h, the release of pro-inflammatory mediators was detected in peritoneal macrophages. At different times, the release of pro-inflammatory (TNF-α, IL-6, NO and E-Selectin), pro-coagulant (vWF and TF) and pro-fibrinolytic (uPA) mediators were seen in liver sinusoidal endothelial cells (LSECs). These results suggest that *Mtt* venom activates macrophages and endothelium, thus inducing the release of mediators, such as TNF-α, that orchestrate the acute inflammatory response and the later infiltration of mononuclear cells into liver in C57BL/6 mice. In addition, endothelium activation promotes TF expression, which may in turn modulate the inflammatory and hemostatic response. These findings suggest crosstalk between inflammation and hemostasis in the alterations observed in *Micrurus* envenomation, where the neurotoxic manifestations do not predominate.

1. Introduction

Animal venoms, including snake venoms, have been widely studied, firstly to elucidate the mechanisms of action of their toxic compounds, but also due to their enormous potential as diagnostic tools and therapeutic agents (White, 2005). Venoms are deadly cocktails, which comprise a unique mixture of peptides and proteins that can disturb homeostasis and the activity of proteins, enzymes, receptors and ion channels that might alter the central and peripheral nervous systems, as well as the cardiovascular, muscular, hemostatic and immune systems (Calvete et al., 2009). For this reason, snake envenomation is a major public health problem in rural areas of tropical and subtropical countries in Africa, Asia, Oceania and the Americas (WHO, 2007).

Snake venom components may induce a systemic inflammatory response, specifically pro-inflammatory cytokine release (Petricevich, 2004; Salazar et al., 2018). In addition, active hemostatic compounds in snake venom have been identified and isolated that can decrease the coagulability of blood, induce bleeding and associated secondary effects (Sajevic et al., 2011; White, 2005). Although it is evident that components of snake venom may affect the hemostatic and inflammatory systems separately, it is not clear whether the interaction between these systems may contribute to the disturbances seen in victims.

The acute inflammatory response begins with the activation of resident immune cells including macrophages in the injured tissue, which

Abbreviations: 3FTX, 3 finger toxins; aPTT, activated partial thromboplastin time; CAM, cell adhesion molecules; CD62E, E-selectin; FBS, fetal bovine serum; FVIIa, factor VII activated; FX, factor X; FXa, factor X activated; ICAM-1, intercellular adhesion molecule 1; IL-1, interleuquin 1; IL-6, interleuquin 6; IL-8, interleuquin 8; LC_{50}, lethal concentration 50; LPS, lipopolysaccharide; LSEC, liver sinusoidal endothelial cell; *Mtt*, *Micrurus tener tener*; NO, nitric oxide; NO_2^-, nitrites; NO_3^-, nitrates; PAI-1, plasminogen activator inhibitor-1; PAR-2, protease activated receptor-2; PBS, phosphate-buffered saline; PLA_2, phospholipases A_2; PT, prothrombin time; SRB, sulphorhodamine B; SVMP, snake venom metalloproteinases; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TG, thioglycollate; Th, thrombin; TNF-α, tumor necrosis factor-α; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; VCAM-1, vascular cell adhesion protein 1; vWF, von Willebrand Factor

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release pro-inflammatory mediators such as IL-1 and TNF-α that in turn trigger the inflammatory response in the neighboring endothelial cells of post-capillary venules (Barton, 2008; Medzhitov, 2008). Likewise, in pathological conditions, a pro-inflammatory state combined with pro-thrombotic events has been demonstrated. These may produce multi-organ failure as a consequence of fibrin deposition in the micro-vasculature (Petäjä, 2011). Pro-inflammatory cytokines produce hemostatic alterations including endothelial activation, platelet activation and aggregation, thrombin generation, and suppression of the fibrinolytic system (Levi et al., 2002), but the hemostatic system may also reciprocally affect the immune system and promote inflammatory activity (Margetic, 2012; O'Brien, 2012). Hence, the study of the mechanism involved in these alterations has led to the evaluation of the crosstalk between hemostasis and inflammation, where the innate immune response plays a central role.

Micrurus envenomation is characterized by neurotoxic symptoms and may lead to death from muscle paralysis and respiratory arrest. Some reports have shown that these venoms may also contain hemorrhagic, hemostatic, hemolytic and edematogenic activities (Barros et al., 1994; Casais-e-Silva and Teixeira, 2017; Salazar et al., 2011, 2018; Tambourgi et al., 1994; Vivas et al., 2016). In the United States, only two species are responsible for all coral snake toxicity, *Micrurus fulvius fulvius* (Eastern coral snake) and *Micrurus tener tener* (Texas coral snake), and most of the information available rely on the Eastern coral snakebites. Few studies have described the clinical manifestations of patients bitten by *Micrurus tener tener* (*Mtt*), which represents 2.3% of the snakebites in Texas, which include local symptoms including pain, edema, erythema, and long-lasting paresthesia, and, in severe cases, skeletal muscle weakness and cranial nerve dysfunction (McAninch et al., 2019; Morgan et al., 2007). These effects can be attributable to the most abundant protein families shared by all species from the elapids of the New world, which are phospholipases A_2 (PLA_2) and three-finger toxins (3FTxs), although, members of other protein families, that are common but not omnipresent, including metalloproteinases, L-amino acid oxidases, Kunitz-type serine protease inhibitors, serine proteinases, and C-type lectin-like proteins can also modulate these effects (Alape-Girón et al., 1996; Bénard-Valle et al., 2014 Corrêa-Netto et al., 2011; Lomonte et al., 2016a).

In a previous article, we reported results showing that *Mtt* venom induced a pro-inflammatory response in addition to hematological and hemostatic disturbances in C57BL/6 mice that were injected intraperitoneally (i.p.) with a sub-lethal dose of this venom (Salazar et al., 2018). As the endothelium and innate immunity may participate in the disturbances seen in C57BL/6, we evaluated the inflammatory response and hemostatic alterations induced by *Mtt* crude venom in an *in vitro* model. This was done by employing cells from the same mouse strain; specifically, liver sinusoidal endothelial cell (LSEC) line and peritoneal macrophages, to extend our understanding of other mechanisms of action, besides neurotoxicity, that may be involved in the acute manifestations of *Micrurus* snake bites.

2. Material and methods

2.1. Reagents

Cadmium granules, potassium nitrate, bovine thrombin, sodium chloride, calcium chloride, lipopolysaccharide (LPS, from *Escherichia coli* serotype O26:B6), trichloroacetic acid, trizma base, sulphorhodamine B, 3′diaminobenzidine, HEPES, and other reagents were purchased from Sigma Aldrich, USA. Avidin peroxidase and streptavidin peroxidase, anti-VCAM-1, anti-ICAM-1, anti-E-Selectin antibodies were obtained from Santa Cruz Biotechnology, USA. 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Scyteck, USA. Chromogenic substrates from Chromogenix AB, Italy. Plasmin, uPA and double chain tPA standards (tcu-PA) were obtained from Sekisui, USA. Mouse IL-6 minikit and mouse TNF-α minikit, recombinant mouse TNF-α were purchased from Thermo Scientific, USA. Fetal bovine serum from Gibco, BRL, USA. vWF antibody from DAKO, USA. Murine TF standard were obtained from R&D Systems, USA.

2.2. Venoms

A pool of *Micrurus tener tener* (*Mtt*) venom was purchased from the National Natural Toxin Research Center (NNTRC) -Texas A&M University Kingsville, Kingsville, Texas, USA.

2.3. Mice

Male C57BL/6 mice, between 10 and 12 weeks of age, weighing 20–24 g, were obtained from the Instituto Venezolano de Investigaciones Científicas (IVIC) animal facility. Mice were acclimated for at least 1 week before beginning each experiment and received water and food ad libitum.

2.4. Ethical statement

All animal experiments were approved by the Bioethical Committee (COBIANIM) of IVIC (Governing Council directive #DIR1072/12). Standards and protocols for animal use and management were obtained from the Institute for Laboratory Animal Research (2011).

2.5. Cell cultures

A mouse endothelial cell line (LSEC, kindly donated by Dr. José Cardier) were grown in HAM-F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% L-glutamine, 1% pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were seeded onto 96-well plates and kept at 37 °C in a humid atmosphere with 5% CO_2. The initial number of cells per well was standardized to 20×10^3 cells.

Macrophages isolated from C57BL/6 mice peritoneal cavity were harvested 3 days after i.p. injection of 1 mL of 4% thioglycollate (TG). Mice were euthanized by CO_2 asphyxiation. A midline incision was performed, followed by 5 lavages of the abdominal cavity, using 1 mL of cold, sterile phosphate-buffered saline (PBS; pH 7.2) supplemented with 2% L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, 2% FBS, and 5 UI/mL heparin. The peritoneal fluid, containing macrophages, was collected and centrifuged at 740 *g* for 10 min at 4 °C. The supernatant was discarded, and the cell pellet resuspended in supplemented HAM-F12 medium. The cell count was determined in a Neubauer chamber using Turk solution. The cell population consisted of 90% macrophage, as determined by morphological criteria. Cells were seeded onto 96-well plates at 150×10^3 cells/well and incubated at 37 °C in a humid atmosphere with 5% CO_2. After 2 h, the plates were washed with PBS and adherent cells were maintained in a supplemented HAM-F12 medium at 37 °C in a humid atmosphere with 5% CO_2.

2.6. Cytotoxic assay

The Sulphorhodamine B (SRB) assay was used to determine cytotoxicity of *Mtt* venom over a 48 h incubation period (Vichai and Kirtikara, 2006). Briefly, cells were seeded onto 96 well plates, then incubated in the presence of a range of concentrations of *Mtt* venom (0, 12.5, 25, 50, 75 and 100 μg/mL). After 24 and 48 h, the cells were fixed with trichloroacetic acid, washed, stained for protein quantification with SRB 0.4%, washed again and the stain solubilized with 10 mM Trizma base, pH 10.5. Control wells were included to allow measurement of the original number of cells at T = 0. Plates were then read for optical density (O.D.) at 515 nm. The concentrations inducing 50% cytotoxicity (LC_{50}) after the 24 and 48 h incubation period were calculated by linear interpolation from the observed data points.

2.7. Cell activation assays

Culture cells in supplemented HAM-F12 medium were treated at 37 °C with non-cytotoxic doses of *Mtt* venom (0.5–15.0 μg/mL) or Thrombin (Th - 5 IU/mL), LPS (10 μg/mL) or TNF-α (5 ng/mL) as positive controls. At 4, 24 and 48 h, supernatant and cells were collected for the evaluation of pro-inflammatory and hemostatic parameters. LPS (10 μg/mL), as a trigger of the inflammatory response, Th (5 IU/mL), a physiologic activator of hemostasis and inflammation (Dugina et al., 2002) and TNF-α (5 ng/mL), one of the most important pro-inflammatory cytokines that promotes CAM and TF expression in various cell types (Zelová and Hošek, 2013), were chosen as cell activation controls.

2.8. Inflammatory response

2.8.1. Pro-inflammatory mediators production

At 4, 24 and 48 h post-treatment, cell supernatants were collected. Pro-inflammatory cytokines (IL-6 and TNF-α) were quantified using commercial ELISA kits (Thermo Scientific, USA). The concentrations were calculated employing a standard curve obtained with recombinant TNF-α and IL-6 (Thermo Scientific, USA) and expressed in pg/mL.

The NO concentration was evaluated indirectly using the Griess reaction after reduction of NO_3^- to NO_2^- with cadmium granules (Cortas and Wakid, 1990). Nitrite concentrations were determined from a standard curve obtained with nitrate (KNO_3) and expressed in pM.

2.8.2. Cell adhesion molecule (CAM) expression in LSEC

LSEC were seeded onto 24 well plates at 10^6 cells/plate and allow attaching for 24 h at 37 °C, in a humid atmosphere with 5% CO_2 in supplemented HAM-F12 medium. At 24 h post-treatment, the supernatant was removed, and the cells were harvested and resuspended in PBS with 1% FBS buffer solution, pH 7.2 (PBS/FSB 1% buffer). After washing in the same solution, the cells were incubated with specific antibodies conjugated with FITC or PE (anti - VCAM-1, ICAM-1 or CD62E) or the equivalent isotypic control antibody, for 30 min at 4 °C. Cells were washed once again at 400 g for 5 min at 4 °C. Finally, the cells (10^4) were analyzed by flow cytometry in a flow cytometer (FACSCalibur, Becton Dickinson, USA), and the percentage of positive cells determined using a threshold set with the isotypic controls.

2.9. Hemostatic parameters

2.9.1. Von Willebrand factor release in LSEC

The release of von Willebrand Factor (vWF) into the culture medium was measured quantitatively by Western blot. At 4 h after treatment, supernatants of LSEC were collected and subjected to SDS-polyacrylamide gel electrophoresis on 10% bis-acrylamide gels. The proteins were transferred to a nitrocellulose membrane using a transfer chamber (Bio-Rad Laboratories, USA). The membrane was blocked overnight at room temperature with a blocking solution (5% nonfat dry milk in PBS with 0.2%Tween 20, 0.2% sodium azide, pH 7.4), then incubated with the primary antibody against vWF in the same blocking solution for 6 h at room temperature. The membrane was washed twice with PBS and incubated for 2 h in blocking buffer without sodium azide with the appropriate secondary antibody conjugated to horseradish peroxidase. The membrane was washed with Tris − HCl buffer (50 mM Tris − HCl, 150 mM NaCl, pH 7.5). vWF was stained using H_2O_2 (30%) and 3-3′diaminobenzidine (0.07%). The quantification of the immunoreactive bands was assessed semi-quantitatively using the computer-assisted densitometry program ImageJ. Results were expressed as protein abundance, after being normalized to the negative control.

2.9.2. Tissue factor activity

Tissue Factor (TF) activity was measured in LSEC and peritoneal macrophages using the method of Cermak et al. (1993). Briefly, at 4 and 24 h after treatment, cells were lysed with 3 freeze-thaw and sonication cycles. TF was determined in the lysates with the coagulant activity assay. Fifty μL of platelet-poor plasma were incubated for 2 min at 37 °C in a borosilicate tube, then 50 μL of the lysate and 50 μL of a 0.025 M $CaCl_2$ solution were added and the clotting time was measured. A murine TF standard (R&D Systems, USA) was employed to quantify the final concentration of this factor produced by the cells.

Additionally, in peritoneal macrophages TF activity was also measured using the amidolytic assay described by Pereira et al. (2006). Briefly, at 4 and 24 h after treatment, supernatants were discarded and the cells were washed with HEPES saline buffer (50 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) and incubated for 30 min at room temperature with a mixture containing 5 nM FVIIa and 50 nM FX in HEPES saline buffer. The reaction was stopped with 10 mM EDTA, and TF activity was quantified indirectly with the specific substrate for FXa (S-2765, 2.7 mM). After incubation at 37 °C for 15 and 30 min, the absorbance at 405 nm was measured. A murine TF standard (R&D Systems, USA) was employed to quantify the final concentration of this factor.

2.9.3. Tissue factor expression in LSEC

LSEC were seeded onto 24 well plates at 10^6 cells/plate and allow attaching for 24 h at 37 °C, in a humid atmosphere with 5% CO_2 in supplemented HAM-F12 medium. At 24 h post-treatment, the supernatant was removed and the cells were harvested and resuspended in PBS with 1% FBS. After washing in the same buffer, the cells were incubated with the specific primary antibody for 30 min at 4 °C. The cells were then labelled with a FITC-conjugated secondary antibody for 30 min at 4 °C in darkness. Cells were washed between incubations with PBS/FSB buffer at 400 g for 5 min at 4 °C. Finally, the cells (10^4) were analyzed by flow cytometry in a flow cytometer (FACSCalibur, Becton Dickinson, USA), and the percentage of positive cells determined using a threshold set with the isotypic controls.

2.9.4. Fibrinolytic activity

The fibrinolytic activity in the supernatant and on the cell surface of peritoneal macrophages and LSEC was evaluated through the amidolytic activity using a micromethod of Guerrero and Arocha-Piñango (1992). At 4 and 24 h of treatment, cells previously washed with PBS pH 7.2, or cell supernatants (40 μL), were incubated with 10 μL of the specific substrate and recommended buffer for each substrate in a final volume of 100 μL/well. After incubation at 37 °C for 15 min, the O.D. at 405 nm was measured. Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) activities were measured with the specific substrates S-2288 (1.2 mM) and S-2444 (3.0 mM), respectively. Plasmin generation was quantified on the cell surface, after incubation with 1 μM plasminogen, the specific substrate (S-2251, 0.8 mM) and the recommended buffer. This assay was also performed adding 10 mM EDTA 30 min before the venom treatment. tPA, uPA and plasmin standard curves (Sekisui diagnostics, USA) were used to calculate the final concentration.

2.10. Statistical analysis

The data were expressed as the arithmetic means ± the standard deviation (SD) of 3 independent experiments. Student's *t*-test was employed to study possible differences between the control and experimental groups, using the GraphPad PRISM® 6.01 statistical program. Values of $p < 0.05$ and 0.01 were accepted as statistically significant for all the experiments.

3. Results

3.1. Effect of Mtt venom on LSEC and peritoneal macrophages

The SRB assay showed that the pool of *Mtt* venom used in this study

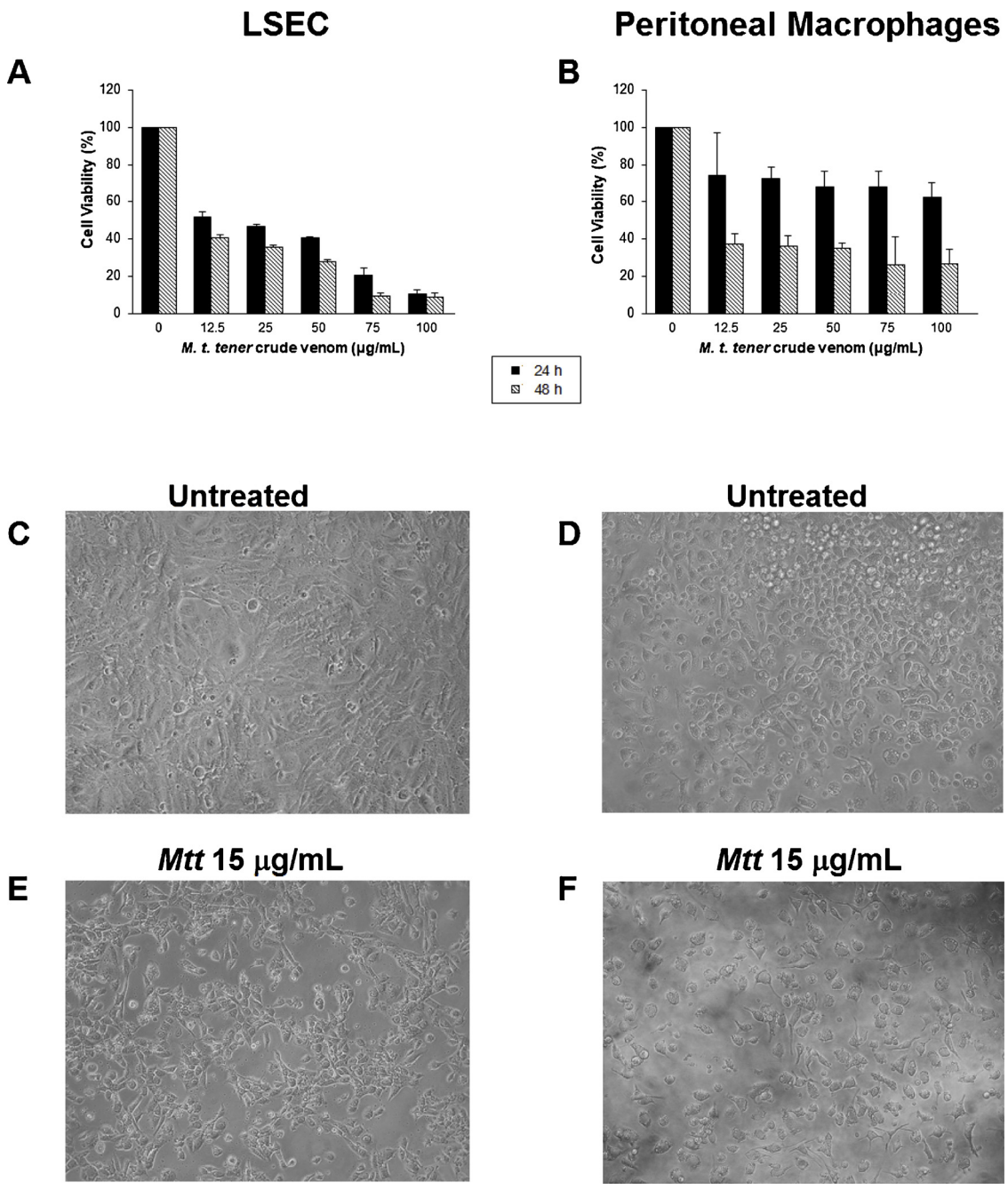

Fig. 1. Effect of *M. t. tener* crude venom on cell viability and morphology of endothelial cells and macrophages. The effect of various concentrations of *Mtt* venom (100-12.5 µg/mL) on cell viability of LSEC **(A)** and peritoneal macrophages **(B)** was measured using the SRB method after exposure of cells over a 48 h incubation period. The results are expressed as the percentage of cell viability relative to untreated cells (Concentration = 0). The results are expressed as mean ± SD (n = 3). Morphological changes in LSEC **(C, E)** and peritoneal macrophages **(D, F)** after exposure with 15 µg/mL of *Mtt* venom for 48 h. Samples were observed under light microscopy at 20x magnification.

induced a LC_{50} for LSEC of 15 and 10 µg/mL at 24 and 48 h, respectively (Fig. 1A). Meanwhile, for peritoneal macrophages, the LC_{50} values were 57 and 27 µg/mL at the same times (Fig. 1B). After 24 h, the venom produced in LSEC an increase in cytoplasmic granules along with a decrease in cell-cell contact and cell density at concentrations higher than 3.8 µg/mL (Fig. 1C and E). Meanwhile, all the concentrations tested produced macrophage activation which was evident through the morphological changes, including the appearance of vacuoles and pseudopods (Fig. 1D and F). Based on the above results, cells were treated with non-cytotoxic concentrations ranging from 0.5 to 1.9 µg/mL of *Mtt* venom. Th (5 IU/mL) did not induce any notable morphological changes in either cell type (data not shown).

3.2. Release of pro-inflammatory cytokines and NO

Our previous *in vivo* results showed a vital secretion of pro-inflammatory mediators with edema formation after venom injection. For this reason, we evaluated pro-inflammatory cytokines and NO release in endothelial cells and macrophages. At 30 min and 4 h post-treatment, no release of pro-inflammatory mediators was detected in peritoneal macrophages (data not shown). However, in LSEC, an increase in cytokine release was detected after 4 h. The higher levels of TNF-α (1509 ± 9 pg/mL) and IL-6 (151 ± 14 pg/mL) were seen in endothelial cells treated with 1.9 and 0.5 µg/mL of *Mtt* venom, respectively, compared to untreated cells that only released IL-6 (43 ± 2 pg/

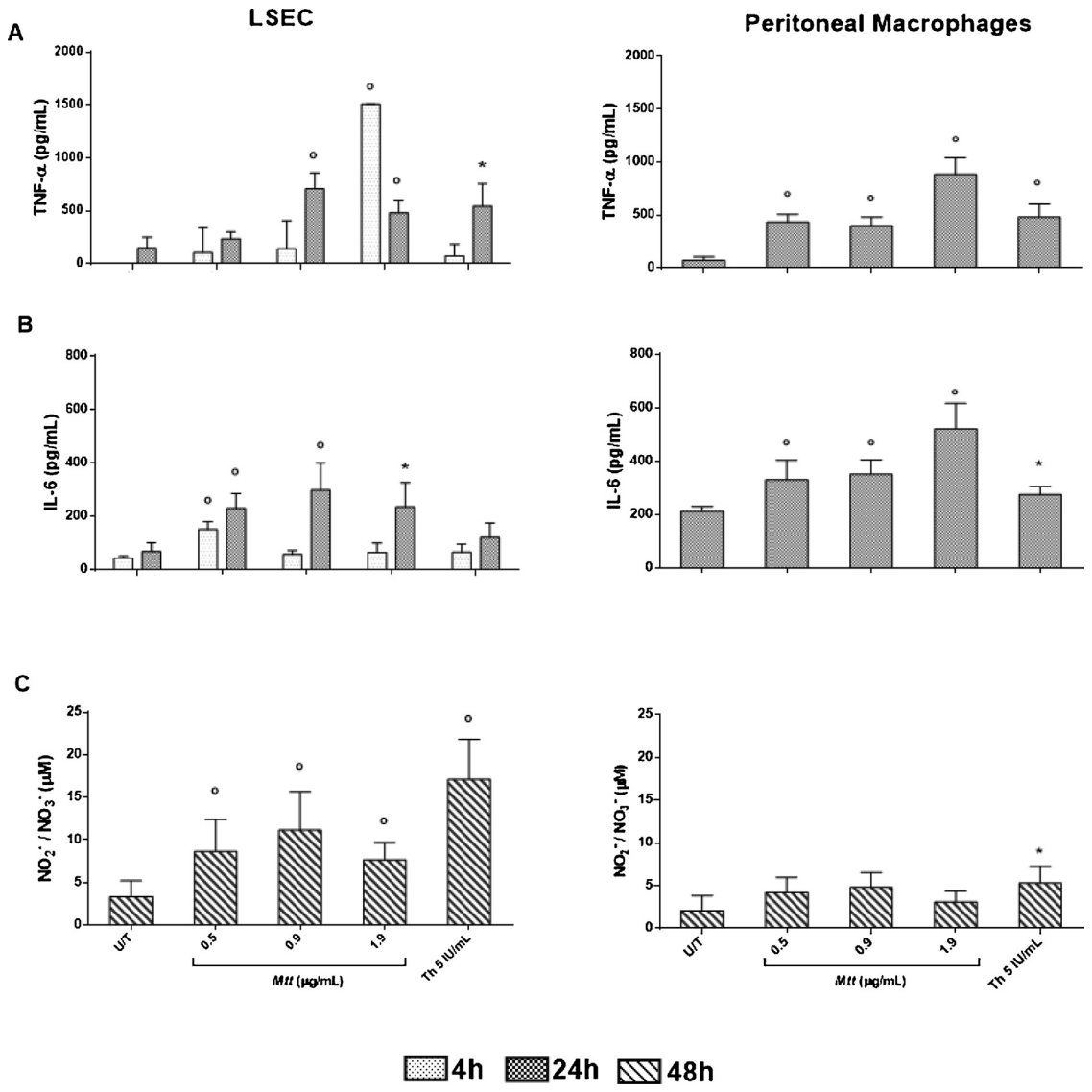

Fig. 2. Pro-inflammatory mediators release by LSEC and peritoneal macrophages. Cells were treated with *Mtt* venom (0.5–1.9 μg/mL) or Th (5 IU/mL). At 4 or 24 h, supernatants were collected and TNF-α (A) and IL-6 (B) were determined by ELISA. At 48 h, NO (C) was determined in supernatants using the Griess assay. Results represent 3 independent experiments in triplicate (mean ± SD). * $p < 0.05$ vs. untreated cells; ° $p < 0.01$ vs. untreated cells. U/T: untreated cells. Positive control: thrombin (Th).

mL) ($p < 0.01$). The venom-induced values were higher than those induced by the positive control (Fig. 2A-B).

At 24 h, TNF-α was highly secreted by both cell types, except for LSEC treated with 0.5 μg/mL of *Mtt* venom that produced similar levels than untreated endothelial cells (237 ± 26 vs. 148 ± 47 pg/mL, respectively) (Fig. 2A). In macrophages, the highest value was produced by cells treated with 1.9 μg/mL of *Mtt* venom (884 ± 71 pg/mL; $p < 0.01$), while in LSEC the maximum level of this cytokine was detected after treatment with 0.9 μg/mL of *Mtt* venom (709 ± 61 pg/mL; $p < 0.01$). IL-6 was also detected at this time (Fig. 2B), being *Mtt* venom the treatment that induced the higher release in both cell types, with values ranging 223 ± 28–299 ± 46 pg/mL for LSEC, and 331 ± 30–520 ± 39 pg/mL for macrophages ($p < 0.05$ and $p < 0.01$).

Finally, NO production was evident at latter times in both cell types (Fig. 2C). At 48 h, all treatments induced a higher release of this mediator by LSEC compared to untreated cells (3.3 ± 0.8 pM) ($p < 0.01$ and $p < 0.05$), being the highest release in cells treated with Th (17.1 ± 2.3 pM; $p < 0.01$). In contrast, only the peritoneal macrophages treated with the positive control produced a significant release of this mediator (5.3 ± 1.0 pM; $p < 0.05$).

3.3. CAM expression in LSEC

At 24 h, exposure of LSEC to the lower doses of *Mtt* venom resulted in a minor increase in E-Selectin expression (8.59 ± 0.04%), comparing to untreated cells (1.62 ± 0.26%) ($p < 0.05$), without detectable increases in ICAM-1 and VCAM-1 expression compared to untreated cells (50.17 ± 1.20 and 83.15 ± 1.55%, respectively). Endothelial cells treated with TNF-α (5 ng/mL, as positive control), showed a higher expression of ICAM-1 (94.08 ± 1.27%; $p < 0.01$) and VCAM-1 (96.27 ± 1.71%; $p < 0.05$), but there was no increase in E-Selectin expression (0.91 ± 0.14%). In LSEC treated with Th no changes were seen (Fig. 3A-C).

3.4. vWF expression in LSEC

At 4 h, LSEC treated with *Mtt* venom released vWF to the supernatant, as shown by Western blot as one band with a molecular mass weight close to 104 KDa and a remarkable band above 206 KDa, which

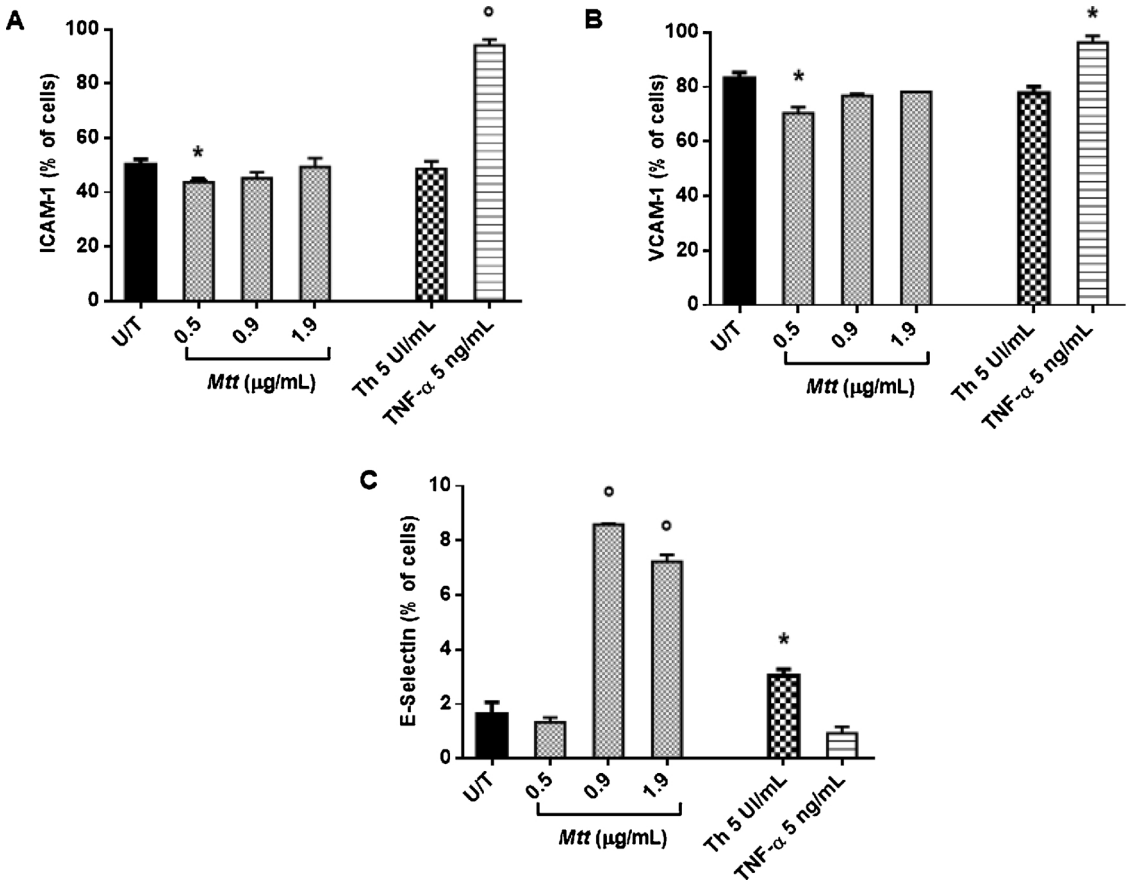

Fig. 3. Expression of CAM in LSEC treated with *Mtt* venom. Cells were treated for 24 h with several concentrations of *Mtt* venom (0.5–1.9 µg/mL). ICAM-1 (A), VCAM-1 (B) and E-Selectin (C) expression were evaluated by flow cytometry employing specific antibodies conjugated with FITC and PE. Results are expressed as the percentage of cells expressing CAM. Results of CAM expression represent 2 independent experiments in duplicate (median ± SD). The results of vWF release for one representative experiment of 6 are shown. * $p < 0.05$ vs. untreated cells; ° $p < 0.01$ vs. untreated cells. U/T: untreated cells. Positive controls: thrombin (Th) and TNF-α.

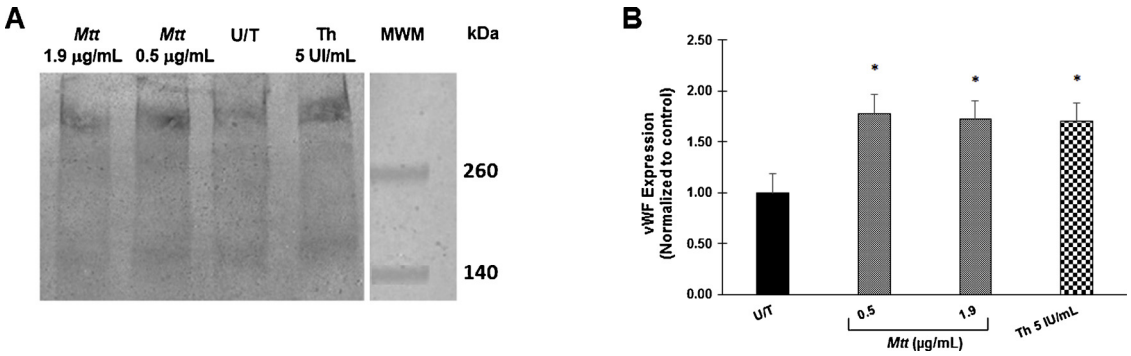

Fig. 4. Release of von Willebrand Factor in LSEC treated with *Mtt* venom. Cells were treated for 4 h with several concentrations of *Mtt* venom (0.5–1.9 µg/mL). vWF release into supernatants was evaluated by Western blotting, employing a specific primary antibody (DAKO, USA). The results of vWF release for one representative experiment of 6 are shown (A). The quantification of the immunoreactive bands was assessed semi-quantitatively using a computer-assisted densitometry program. Protein abundance was normalized to untreated cells. * $p < 0.05$ vs. untreated cells. U/T: untreated cells. Positive controls: thrombin (Th).

can be identified as subunit of mature vWF or monomers (Sadler, 1998). The densities of these bands were more intense in the cells treated with the highest concentration of *Mtt* venom (1.78) than in untreated cells (1.00) ($p < 0.05$) (Fig. 4A-B).

3.5. TF activity and expression

In order to evaluate whether *Mtt* venom may promote pro-coagulant activity, we tested TF activity using Th and TNF-α as positive controls, since these mediators are well known to induce TF expression in endothelial cells and leukocytes (Egorina et al., 2011). At 4 h, an increase in TF activity was detected in LSEC lysates after *Mtt* venom stimulation (51.1 ± 6.6–160.2 ± 21.7 pg/mL), being these results comparable to the ones induced by the positive controls (53.6 ± 11.9 and 112.1 ± 23.4 pg/mL, for Th- and TNF-α-treated cells, respectively; $p < 0.05$), and higher than untreated cells (6.4 ± 2.3 pg/mL) ($p < 0.05$). This activity decreased at 24 h, although a higher activity was registered in *Mtt* venom- and positive control-treated cells lysates, compared to the untreated cells (6.4 ± 4.1 pg/mL) (Fig. 5A).

Since snake venom metalloproteinases (SVMP) may cleave TF and

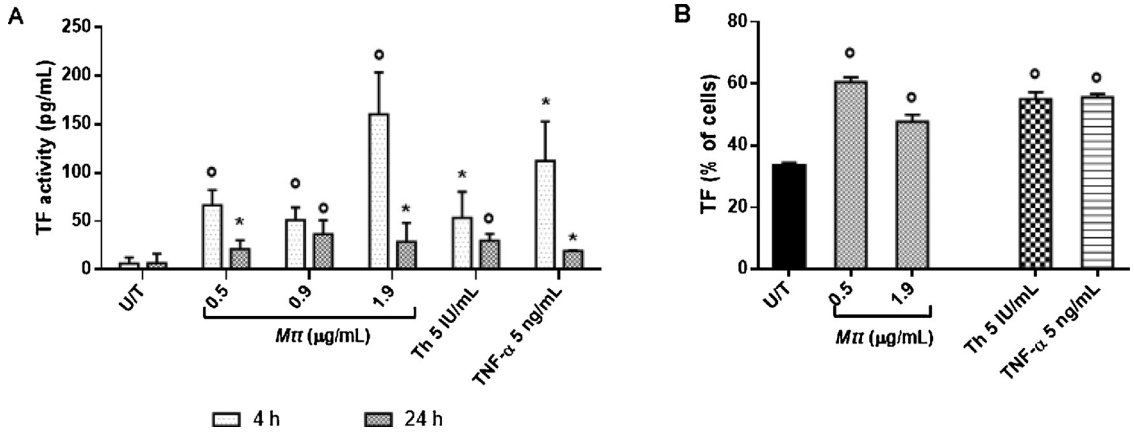

Fig. 5. Tissue Factor activity and expression in LSEC treated with *Mtt* venom. **(A)** Endothelial cells were treated with *Mtt* venom (0.5–1.9 µg/mL), Th (5 IU/mL) or TNF-α (5 ng/mL). At 4 and 24 h, cells were collected and lysed through freeze-thaw cycles to release intracellular TF, which was quantified based on its coagulant activity. The results were expressed as pg/mL. **B)** Endothelial cells were treated with *Mtt* venom (0.5 and 1.9 µg/mL), Th (5 IU/mL) or TNF-α (5 ng/mL). At 24 h, expression was evaluated by flow cytometry employing specific antibodies. Results are expressed as the percentage of cells expressing TF. Results represent 3 independent experiments in triplicate (mean ± SD). * $p < 0.05$ vs. untreated cells; ° $p < 0.01$ vs. untreated cells. U/T: untreated cells. Positive controls: thrombin (Th) and TNF-α.

promote its release to the circulation causing systemic coagulation (Yamashita et al., 2014), we evaluated TF activity in LSEC culture medium. At 4 and 24 h, *Mtt* venom-, Th- and TNF-α-treated cell supernatants did not show any activity (data not shown).

Based on the coagulant activity in LSEC, we evaluated TF expression using flow cytometry to determine if our results could be correlated with molecules already expressed, synthesized *de novo*, or stored and expressed post-activation. Fig. 5B shows that at 24 h, increased expression of TF was observed in LSEC treated with *Mtt* venom (61 ± 1 and 48 ± 1%, with 0.5 and 1.9 µg/mL of venom, respectively), Th (55 ± 1%) and TNF-α (56 ± 1%) compared to untreated cells (33 ± 1%) ($p < 0.01$).

As peritoneal macrophages could not be detached easily by conventional methods, no TF activity was detected through the coagulant assay, including cells treated with LPS as a positive control for inflammation (data not shown). In this case, TF activity was assessed through an indirect amidolytic assay. The results showed that this activity was not statistically significant at 4 h on the cell surface, compared to untreated cells (2.4 ± 0.2 pg/mL) (data not shown).

3.6. Fibrinolytic enzyme release and expression

Our previous results showed that injection of *Mtt* venom induced strong endogenous fibrinolysis activation in C57BL/6 mice. Here we show that coral venom did not enhance tPA-like activity in the supernatants of both cell types at the times tested, compared to the positive control Th (Fig. 6A). Regarding uPA-like activity (Fig. 6B), only at 4 h, an increase in this activity was detected. In LSEC, this effect was seen with 0.9 µg/mL of *Mtt* venom (27.6 ± 7.5 IU/mL; $p < 0.05$) and Th (137.6 ± 69.1 IU/mL), compared to untreated cells (5.5 ± 10.3 IU/mL). Meanwhile, in macrophages, a significant change was detected with the positive control ($p < 0.05$).

Due to the low pro-fibrinolytic-like activity seen in supernatants, we measured these activities on the cell surface, including the plasmin generation assay. Fig. 7A shows that at 4 h, tPA-like activity was increased in LSEC treated with 1.9 µg/mL of *Mtt* venom (114.5 ± 5.3 pM) ($p < 0.01$), and in both cell types after treatment with Th ($p < 0.01$ and $p < 0.05$). In addition, an increase in uPA-like activity was observed in LSEC after all treatments (Fig. 7B), and the most remarkable effect was observed with Th (1185.0 ± 68.3 IU/mL) ($p < 0.05$), while this activity was not seen with peritoneal macrophages. Plasmin generation, in the presence of plasminogen, was not seen in LSEC or macrophages, after all the treatments (Fig. 7C).

Finally, to determine whether our results concerning the fibrinolytic activity was associated with SVMP in the *Mtt* venom, we also assessed plasmin generation in LSEC in the presence of 10 mM EDTA. Fig. 7C shows that EDTA treatment led to a mild increase in plasmin generation on the cell surface in LSEC stimulated with *Mtt* venom ($p < 0.05$). No change was seen in endothelial cells treated with Th.

4. Discussion

Coral snake envenomation are relatively uncommon, however, these venoms are very potent and may produce neurotoxicity characterized by muscle paralysis and respiratory failure (Sánchez et al., 2008). The biological characterization of *Mtt* venom identified SVMP and PLA_2, as well as fibrino(geno)lytic, fibronectinolytic, gelatinolytic and hyaluronidase activities (Salazar et al., 2011). In addition, previous studies have shown that *Mtt* venom triggers an acute systemic inflammatory response within a few hours after i.p. injection into C57BL/6 mice, which may activate the hemostatic system generating a procoagulant state that could be followed by an anti-coagulated status characterized by reduced fibrinogen, prolonged coagulation tests and hemorrhage (Salazar et al., 2018). This study led us to evaluate the inflammatory response and hemostatic alterations induced by *Mtt* crude venom in an *in vitro* model employing liver sinusoidal endothelial cells and primary peritoneal macrophages from the same mouse strain, since these cells actively participate in inflammatory responses and hemostasis as part of innate immunity (Schouten et al., 2008; Verhamme and Hoylaerts, 2009).

Mtt crude venom might cause cytotoxicity in LSEC and peritoneal macrophages through PLA_2s and, although in low proportion, SVMPs present in these venoms (Bénard-Valle et al., 2014; Lomonte et al., 2016b; Rey-Suárez et al., 2011; Salazar et al., 2011). Macrophages were less susceptible than LSEC, exhibiting the typical cell activation morphology and losing viability only at high concentrations of coral venom. Macrophagic activation by elapids has only been described with *Bungarus caeruleus* venom (Bhattacharya et al., 2013). As far as we know, this is the first report of cytotoxicity and activation of peritoneal macrophages by coral snake crude venom. In addition, our results showed a higher cytotoxicity on endothelial cells compared to isolated toxins from other elapids venoms, including PLA_2s from *Micrurus lemniscatus* that were slightly cytotoxic for various cell types including HUVEC and mouse capillary endothelial cells (Casais-e-Silva et al., 2016), and 3FTxs from *Dendroaspis angusticeps*, where concentrations higher than 50 µg/mL inhibited cell viability on HUVEC (Conlon et al., 2014). This can be

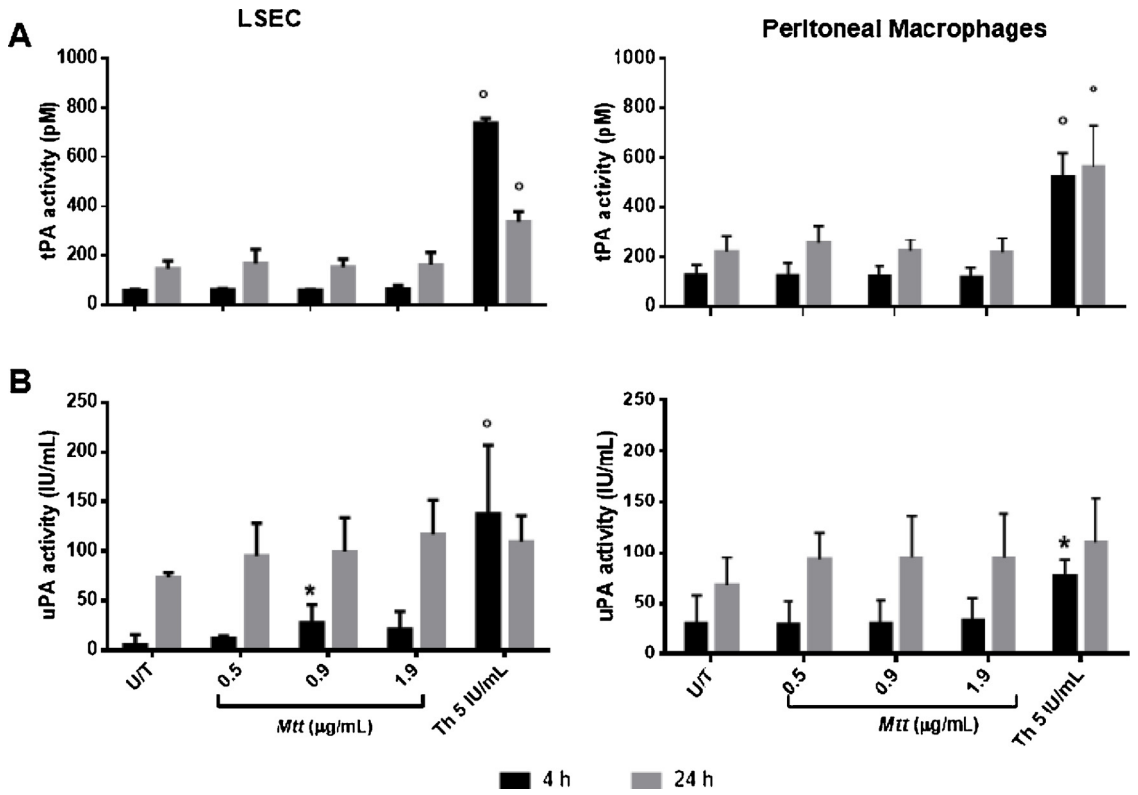

Fig. 6. Effect of *Mtt* venom on pro-fibrinolytic activity in supernatants of LSEC and peritoneal macrophages. Cells were culture for 4 and 24 h in the presence of *Mtt* venom (0.5–1.9 µg/mL), or Th (5 UI/mL) as positive control. The pro-fibrinolytic activity of supernatants was evaluated for amidolytic activity (tPA and uPA) using the specific substrates S-2288 and S-2444, respectively. Results represent 3 independent experiments in triplicate (mean ± SD). * $p < 0.05$ vs. untreated cells. U/T: untreated cells. Positive control: thrombin (Th).

due to the synergism between the toxins that comprise *Mtt* crude venom, which display higher toxicity than those from the individual venom components (Laustsen, 2016). Based on our results, non-cytotoxic concentrations of *Mtt* venom were chosen to evaluate the inflammatory and hemostatic effects.

Snake venom toxins cause local injury that leads to activation of the neighboring endothelium, release of cytokines, expression of CAM, activation and adherence of leukocytes, and their extravasation to the injury site. It is known that inflammation plays a vital role in snake venom toxicity and that some toxins directly affect cells associated with inflammation. However, the mechanisms involved in these effects are not well known, or whether they may contribute to the clinical outcome of snakebites (Lopes et al., 2012). *Mtt* venom might induce the release of pro-inflammatory mediators through different mechanisms including direct binding to specific receptors that promote gene expression. This mechanism of action has been described in the Viperidae family (Cruz et al., 2005; Furtado et al., 2014; Lee et al., 2016; Lopes et al., 2012; Schattner et al., 2005). PLA_2s and C-type lectins bind directly to membrane receptors, activating signaling cascades that lead to NF-κB activation (Chang et al., 2010; Furtado et al., 2014). In elapids, a CRiSP from *Naja atra* venom promotes CAM expression in endothelial cells through NF-κB activation (Wang et al., 2010). *Mtt* venom contains a high proportion of PLA_2s that may have high homology with other toxins of this genus, which can produce edema and immunological responses; thus, we suggest that this toxin might have a relevant role to induce the cell signaling and protein expression in LSEC and macrophages. Even though at 24 h coral venom did not promote a strong CAM in LSEC, which could be due to the lack of components typically present in the vascular milieu in our experimental conditions, e.g., basement membrane, extracellular matrix, fibroblast, and leukocytes (Lopes et al., 2012), it is important to note that in the *in vivo* model, migration of mononuclear cells to liver was seen at 72 h, so it would be necessary to evaluate CAM expression at longer periods.

Inflammation turns the endothelium to an activated pro-thrombotic profile, where it up-regulates the expression and *de novo* synthesis of molecules that favor thrombin generation including vWF and TF (O'Brien, 2012; Yau et al., 2015). At the same time, activated leukocytes express and release hemostatic factors that also modulate the innate immune response (Swystun and Liaw, 2016). In C57BL/6 mice, a pro-coagulant state was seen 24 h after *Mtt* venom inoculation, which reversed at 72 h to an anti-coagulant state (Salazar et al., 2018). Here, the hemostatic parameters were evaluated at earlier times to elucidate the mechanisms involved in the effects seen *in vivo*.

vWF is used as a marker of endothelial activation or dysfunction, its release indicates an early activation of hemostasis and its secretion has been described as constitutive, constitutive-like, or regulated, whereby agonists, including histamine and Th through PAR receptors, act as triggers of the secretion of Weibel-Palade bodies (Brott et al., 2014; Swystun and Liaw, 2016; Yau et al., 2015). At 4 h, *Mtt* venom may increase the release of vWF through an agonist-dependent mechanism, due to the similar response observed in Th treated cells, although this must be clarified since the venom could promote this effect directly.

TF is part of the nexus of coagulation and inflammation, providing a trigger for the host response to injury (Foley and Conway, 2016). In endothelial cells, *Mtt* venom might induce TF expression directly or indirectly through mediators that involve the activation of NF-κB, including damage-associated molecular patterns (DAMPs) or TNF-α (Foley and Conway, 2016; Zelová and Hošek, 2013). At 24 h, TF activity in LSEC decreased, although sustained expression of this molecule was seen, which could indicate the co-expression of the tissue factor pathway inhibitor (TFPI) that down-regulates the TF/FVIIa/FXa complex and thrombin generation (Yau et al., 2015). Regarding the results obtained in macrophages, the lack of amidolytic activity might be associated with *de novo* synthesis of TF, stored intracellularly, or

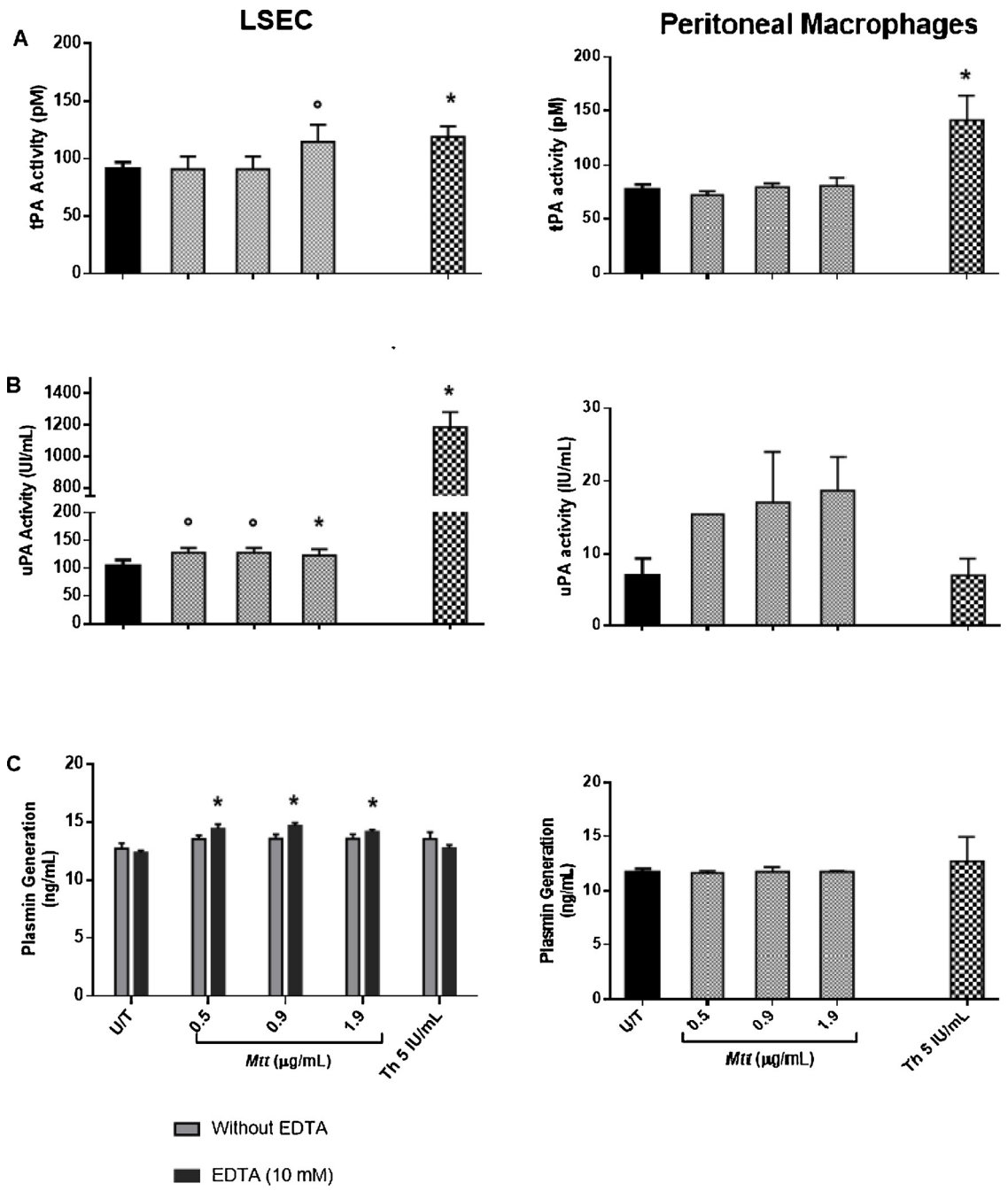

Fig. 7. Effect of *Mtt* venom on pro-fibrinolytic activity and plasmin generation in cell surface of LSEC and peritoneal macrophages. Cells were cultured for 4 h in the presence of the lower concentrations of *Mtt* venom (0.5–1.9 µg/mL) or Th (5 UI/mL) as positive control. The pro-fibrinolytic activity in cells (A–B) was evaluated through amidolytic activity (tPA and uPA) using the specific substrates S-2288 and S-2444, respectively. Plasmin generation (C) was evaluated in presence of 1 µg of plasminogen and assessed for amidolytic activity using the specific substrate S-2251. To evaluate the effect of SVMP in plasmin generation, prior the treatments, LSEC were cultured 30 min with EDTA (10 mM). Results represent 3 independent experiments in triplicate (mean ± SD). *$p < 0.05$ vs. untreated cells. U/T: untreated cells. Positive control: thrombin (Th).

expression of an encrypted form that has low pro-coagulant activity but pro-inflammatory properties when it binds to PAR-2 (Yau et al., 2015). In addition, it could also be due to TFPI expression, since this molecule has also been detected in monocytes and macrophages (Swystun and Liaw, 2016).

The fibrinolytic system degrades fibrin gradually and coordinately, favoring immune processes that facilitate wound healing and angiogenesis (Foley and Conway, 2016). In supernatants from LSEC, only a negligible pro-fibrinolytic activity was seen. The simultaneous release of pro-inflammatory mediators, such as TNF-α, may promote PAI-1 expression, thus inhibiting this activity (Levi and van der Poll, 2017; Petäjä, 2011). In addition, the low pro-fibrinolytic activity may be associated with tenerplasminin-1, a serine protease inhibitor isolated from *Mtt* venom that decreases the activity of plasmin and delays clot lysis induced by tPA (Vivas et al., 2016). Since binding to cell receptors confers protection to plasminogen activators from PAI-1 inhibition (Li et al., 2007), our next approach was evaluating pro-fibrinolytic activity on the cell surface. No uPA activity was detected in peritoneal macrophages, although the expression of its receptor (uPAR) has been widely described in these cells (Chapin and Hajjar, 2015). In contrast, we detected fibrinolytic activity on LSEC surface thus suggesting that *Mtt* venom induces an early expression of pro-fibrinolytic enzymes that are

protected once they attached to endothelial receptors. Since no significant plasmin generation was seen in this study, the pro-fibrinolytic activity mentioned above might participate in the pro-inflammatory response of LSEC. In this sense, the binding of tPA and uPA to their receptors, allow the interaction with adaptor molecules and the activation of signaling cascades that increase the inflammatory response (Foley and Conway, 2016; Lin and Hun, 2014). This binding could enhance the inflammation observed in mice and increase hemorrhage and tissue damage at 72 h, although these parameters should be measured in cells at later times.

Finally, the treatment with EDTA mildly increased plasmin generation, however, since the SVMPs represent a low proportion of *Mtt* venom, this result might not indicate an important effect in the pro-fibrinolytic activity induced by the venom. In this sense, the evaluation of plasmin generation at longer periods is necessary to elucidate how the cell activation may contribute to the effects seen *in vivo*.

It is important to mention that many of the responses seen in this study were not concentration dependent. In this sense, at 4 h, the higher activities were detected after treatment with the highest concentration of *Mtt* venom (1.9 µg/mL), while at later times, the more significant results were obtained with 0.9 µg/mL. This pattern can be attributed to the proportion of activators and inhibitors in the venom. In a previous study, the fibrinolytic activity assay revealed that high concentrations of *Mtt* venom produced an inhibition halo surrounding the degraded fibrin *in vitro* (Salazar et al., 2011). This effect may indicate that at longer periods, the higher proportion of inhibitors may suppress the activity of some toxins on cell activation pathways, causing a lower release of mediators.

In conclusion, our results suggest that endothelial cells and macrophages are involved in the alterations described in C57BL/6 mice injected with *Mtt* venom. At earlier times, these cell types expressed and released pro-inflammatory and hemostatic mediators that, along with venom toxins, might aggravate the inflammatory response and the procoagulant state seen at first in the mice model. This sustained response could then change to a pro-fibrinolytic response that may promote the hemorrhagic manifestations along with the accumulation of leukocytes into distant organs seen in the *in vivo* model. Further experiments, involving mainly TNF-α, TF, and plasmin, will address the possible crosstalk between the inflammatory response and the hemostatic alterations described in this study. As far as we know, this is the first report of the inflammatory and hemostatic effect of *Micrurus* crude venom in cultured cells.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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