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Effect of *Bothrops bilineata* snake venom on neutrophil function

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ABSTRACT

The aim of the study was to evaluate the *in vitro* effects of *Bothrops bilineata* crude venom (BbV) on isolated human neutrophil function. We proved that BbV isn't toxic towards human neutrophils. During an incubation of human neutrophils with BbV hydrogen peroxide was produced. Moreover, BbV was able to stimulate neutrophil release of proinflammatory mediators such as IL-8 and IL-6 as well as PGE_2 and NETs liberation. There is no data in the literature showing the effect of BbV on the production of IL-6 and IL-8 or NETs liberation by isolated human neutrophils. Taken together our results testify that BbV triggers relevant proinflammatory events in human neutrophils.

1. Introduction

Bothrops bilineata ((Wied-Neuwied, 1825) is an arboreal species which has a known distribution in the Amazon Forest, in some areas of the Atlantic Forests (Campbell and Lamar, 2004) and in the northeastern part of the state of Minas Gerais (Feio and Caramaschi, 2002; Bernarde et al., 2011). Recently, Carrasco et al. (2012) through morphology, phylogeny and taxonomy studies has suggested an arrangement of the *Bothrops* genus and also has recognized as sister clade synonymizing *Bothriopsis*, *Bothropoides* and *Rhinocerophis*.

It is important to note that there are few studies on the epidemiological and clinical aspects of envenomation by *B. bilineata* (Borges et al., 1999; Smalligan et al., 2004; Waldez and Vogt, 2009). And experimentally *B. bilineata* venom induces neuromuscular activity in nerve-muscle preparations isolated from vertebrates (Rodrigues-Simioni et al., 2011). In addition, *B. bilineata* venom induces a significant leukocyte accumulation at the site of tissue damage characterized by neutrophil migration (Porto et al., 2007). However, the activation state of these cells is still unclear.

Neutrophils, also named polymorphonuclear granulocytes (PMN), represent the majority of the leukocytes in peripheral blood. They have very short lifespans, spending

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only 8–12 h in circulation (Summers et al., 2010). However, various stimuli, such as cytokines and bacterial products were shown to prolong their survival (Colotta et al., 1992).

They are considered the first line of defense in the organism due to their quick migration into infected tissue thus providing an acute inflammatory response (Nathan, 2006). At the inflammation site, neutrophils perform host defense functions such as phagocytosis, release of proteolytic enzymes, generation of reactive oxygen species (ROS), and synthesis of a number of inflammatory mediators including cytokines and lipid mediators (Cassatella, 1995, 1999; Nathan, 2006; Timár et al., 2013).

In addition to these well-known neutrophil functions, the literature documents the discovery of neutrophil extracellular traps (NETs) also capable of eliminating microorganisms in the extracellular space (Brinkmann et al., 2004). These extracellular vesicles represent a form of intercellular communication carried out by lipids, proteins, and nucleic acids (Timár et al., 2013).

So, the present study aimed to evaluate the effect of *B. bilineata* venom (BbV) on the functionality of human neutrophils such as cytokine production (IL-6 and IL-8) as well as that of PGE_2, hydrogen peroxide and release of NETs.

2. Material and methods

2.1. Chemicals and reagents

MTT, RPMI-1640, L-glutamine, gentamicin, phorbol myristate acetate (PMA), Histopaque 1077, DMSO, OPD (o-1,2-phenylenediamine dihydrochloride), horseradish peroxidase and nitroblue tetrazolium (NBT) were purchased from Sigma (MO, USA). FITC anti-human CD66b was purchased from BD Pharmingen (CA, USA). DuoSet Elisa human IL-6 and DuoSet Elisa human CXCL8/IL-8 were purchased from R&D Systems (Oxon, United Kingdom). PGE_2 enzyme immunoassay kit was purchased from Cayman Chemical (MI, USA). Quant-iT™ Picogreen dsDNA was obtained from Invitrogen (CA, USA). Fetal bovine serum was obtained from Cultilab (Brazil). All salts and reagents used were obtained from Merck (Darmstadt, Germany) with low endotoxin or endotoxin-free grades.

2.2. Venom

The venom from the *B. bilineata* (BbV) snake was acquired from CEBIO-UNIR,RO. The licenses for scientific purposes are from: Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA and Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio. Numbers: 11094-2, 11094-1, 10394-1 e 15484-1.

2.3. Neutrophil isolation

Peripheral blood neutrophils were obtained from buffy coats of self-reportedly healthy donors (18–40 years), and approval for use in this study was given during the blood draw. A prior agreement from all involved was made in order to be included in the study, and the Center of Tropical Medicine Research (Rondonia, Brazil) Research Ethics Committees (number 108/2010) approved this study. Briefly after, local asepsis blood was collected in vacuum tubes containing heparin and diluted in phosphate buffered saline (PBS, 14 mM NaCl, 2 mM $NaH_2PO_4H_2O$, 7 mM $Na_2HPO_412H_2O$), pH 7.4. In order to separate the leukocytes Histopaque 1077 was added to the tubes and then the diluted blood was added carefully to the reagent. After centrifugation at 400× *g* for 30 min, the neutrophils were collected from the bottom of the tube, along with erythrocytes and transferred to another tube. Lysis of erythrocytes was performed using lysis buffer (9.98 mM $KHCO_3$, 0.1 mM Na_2EDTA). Then the solution was homogenized, incubated at −8 °C for 5 min, and centrifuged. Neutrophils were washed with PBS and an aliquot of isolated neutrophils was used for determining the total number of neutrophils in a Neubauer's chamber after cell staining (1:20, v/v) with Turk solution (violet crystal 0.2% in acetic acid 30%). The purity of the isolated cell population was determined by Panotic staining of cytospin preparations and by flow cytometry analysis with CD-66b as a granulocyte marker (FACscan). The mean purity achieved by our isolation technique was 98.5% neutrophils.

2.4. Cytotoxic assay

Neutrophils (2×10^6 cells/mL) were suspended in an RPMI culture medium, supplemented with gentamicin (100 μg/mL), L-glutamine (2 mM) and 10% fetal bovine serum. Then the cells were incubated in duplicate in 96-well plates with BbV at concentrations of 1.5, 3, 6, 12.5, 25, 50 e 100 μg/mL or RPMI (control) for 2 and 15 h, at 37 °C in a humid atmosphere (5% CO_2). Next, 10 μL of MTT (5 mg/mL) was added and incubated for 2 h. After centrifugation at 400× *g* for 5 min, the supernatant was removed and 100 μL of DMSO was added to dissolve the crystals that formed. Subsequently, the plates were kept for 18 h at room temperature. The crystals of formazam formed were evaluated in a spectrophotometer at 540 nm. The results were expressed in terms of optical density compared to the control.

2.5. Determination of hydrogen peroxide (H_2O_2) production by human neutrophils

Shortly, neutrophils (2×10^5/50 μL) were resuspended in 1.0 mL of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 0.56 mM phenol red) containing 0.05 mg/mL of horseradish peroxidase. Then the cells were incubated with BbV at 1.5, 3, 6, 12.5, 25, 50 and 100 μg/mL (experimental group), PMA (positive control group) and RPMI (negative control group) for 90 min at 37 °C in a humid atmosphere (5% CO_2). After this, the reaction was stopped by the addition of 1 M sodium hydroxide (10 μL). The absorbance was measured spectrophotometrically at 620 nm against a blank of phenol red medium. The data generated were compared to a standard curve conducted for each test. The results were expressed as μM of H_2O_2 produced.

2.6. Determination of prostaglandin E_2 (PGE_2) production by human neutrophils

PGE_2 concentration was measured in the supernatant of neutrophils (2×10^5 cells/mL) suspended in RPMI culture medium, supplemented with gentamicin (100 μg/mL), L-glutamine (2 mM) and 10% fetal bovine serum and incubated in 96-well plates with BbV at concentrations of 1.5, 3, 6, 12.5, 25, 50 e 100 μg/mL or RPMI (control) for 4 h, at 37 °C in a humid atmosphere (5% CO_2). Briefly, 100 μL aliquots of each sample were incubated with the eicosanoids conjugated with acetylcholinesterase and the specific rabbit antiserum in 96-well microtitration plates, coated with anti-rabbit IgG mouse monoclonal antibody. After the substrate's addition, the samples' absorbances were registered at 412 nm in a microplate reader, and concentrations of the eicosanoids were estimated from standard curves.

2.7. Interleukin-6 (IL-6) and Interleukin-8 (IL-8) production by human neutrophils

Neutrophils (2×10^5 cells/50 μL) were incubated with BbV at 1.5, 3, 6, 12.5, 25, 50 and 100 μg/mL (experimental group), PMA (positive control group) and RPMI (negative control group) for 4 h at 37 °C in a humid atmosphere (5% CO_2). After centrifugation the supernatant was used to determine IL-6 and IL-8 levels by specific EIA, as described by Schumaker et al (1998). Briefly, 96-well plates were coated with 100 μL of the capture monoclonal antibody anti-IL-6 or anti-IL-8 and incubated for 18 h at 37 °C. As a second a step, the plate was washed in a washer buffer (PBS/Tween20). After that, 200 μL of blocking buffer, containing 5% bovine serum albumin (BSA) in PBS/Tween20, were added to the wells and the plates were incubated for 1 h at 37 °C. Afterward, wells were washed and 50 μL of either samples or standard were dispensed on each well and the plates were incubated for 2 h at 37 °C. After this period, the plate was washed and 100 μL of the detection antibody anti-IL-6 or anti-IL-8 was added for 2 h at 37 °C. After incubation and washing, 100 μL of streptavidin-peroxidase was added, followed by incubation and addition of the substrate (100 μL/mL 3,3′,5,5′-tetramethybenzidine). Finally sulfuric acid (50 μL) was added to stop the reaction. Absorbances at 540 and 450 nm were recorded and concentrations of IL-8 and IL-6 were estimated from standard curves prepared with recombinant IL-6 or IL-8. The results were expressed as pg/mL for each cytokine.

2.8. Neutrophil extracellular traps (NETs) release

Neutrophils (2×10^5 cells/50 μL) were incubated with different concentrations of BbV (1.5, 3, 6, 12.5, 25, 50 e 100 μg/mL) or RPMI (control) or PMA (500 ng/mL, positive control) for 4 and 15 h at 37 °C in a humid atmosphere (5% CO_2). After centrifugation, the supernatant was used to determine NETs release accordingly to the procedure described in kit Quant-iT™ Picogreen dsDNA (Invitrogen). Briefly, 50 μL of samples were incubated with 100 μL of PI (Quant-iT) and 50 μL of PE buffer in a 96-well dark plate. After 15 min of incubation, absorbances at 520 nm emission and 480 nm excitation were recorded and NETs release was estimated from a standard curve. The results were represented as ng/mL of DNA.

2.9. Statistical analysis

Means and S.E.M. of all data were obtained and compared by one-way ANOVA, followed by a Tukey test with significance probability levels less than 0.05.

3. Results

3.1. BbV and its effect on human neutrophil viability

In order to investigate the effect of BbV on neutrophil function we isolated these cells using a density gradient. The purity of the isolated neutrophils obtained with the density gradient was 98.5% as determined by flow cytometry using the pan-granulocyte marker CD66b (Mannoni et al., 1982) and by Panotic staining of cytospin preparations (Inserted). We used an MTT assay to test the toxicity of BbV on isolated human neutrophils. To this end, the effect of 2 and 15 h of incubation on several concentrations of BbV was investigated. As shown in Fig. 1, incubation of BbV at all concentrations used did not affect human neutrophil viability in comparison with control cells incubated with culture medium alone at all-time intervals. This finding is evidence that BbV is not toxic to human neutrophils for these periods of time and at these concentrations.

3.2. Effect of B. bilineata venom on hydrogen peroxide production by neutrophils

To verify the ability of BbV to induce the production of hydrogen peroxide by human neutrophils, the cells were incubated with the venom in non-cytotoxic concentrations or PMA (positive control) or RPMI (negative control). As shown in Fig. 2 incubation of neutrophils at concentrations from 6.2 up to 100 μg/mL resulted in a significant increase in hydrogen peroxide production. These findings demonstrated the ability of BbV to stimulate human neutrophils to produce hydrogen peroxide.

3.3. Effect of B. bilineata venom on PGE_2 production by human neutrophils

To investigate the ability of BbV to induce the release of PGE_2 by human neutrophils, the concentration of this lipid mediator in the supernatant of neutrophils incubated with BbV (1.5, 3.1, 6.2, 12.5, 25, 50 and 100 μg/mL) or PMA (positive control; 500 ng/mL) or RPMI (negative control) was measured. Incubation of neutrophils with BbV for 4 h induced a significant increase in the basal levels of PGE_2 in the supernatant of all concentrations examined in comparison to controls (Fig. 3) suggesting that PGE_2 has a role in acute inflammation inducing the activation of neutrophils.

3.4. Effect of B. bilineata venom on IL-6 and IL-8 production by human neutrophils

To assess the ability of BbV to activate human neutrophils and stimulate pro-inflammatory cytokine release such as IL-6 and IL-8, the cells were incubated with non-cytotoxic concentrations of BbV or RPMI (control) for 4 h

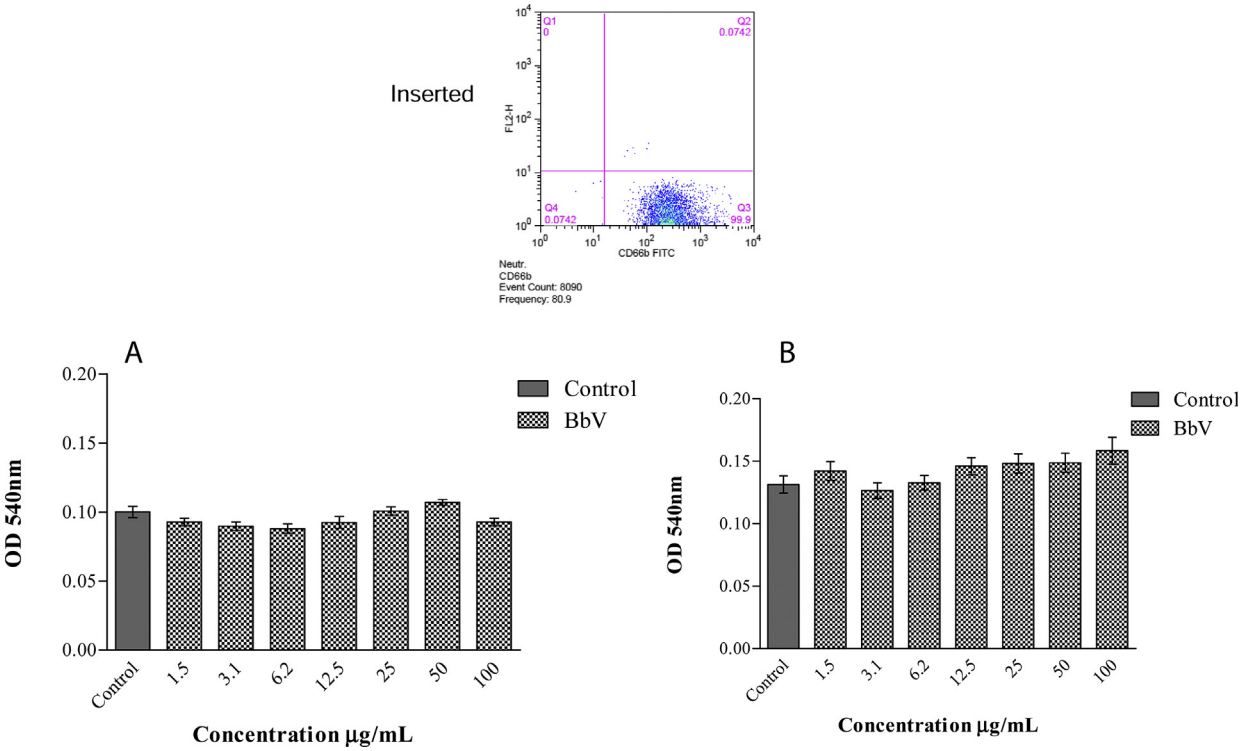

Fig. 1. Effect of *Bothrops bilineata venom* on neutrophil viability. Human neutrophils were isolated from buffy coats of healthy adult blood donors through a density gradient method and analyzed in FACScan (Inserted). 2×10^5 neutrophils were incubated for 2 h (A) or 15 h (B) with BbV (1.5, 3.1, 6.2, 12.5, 25, 50 and 100 µg/mL) or RPMI (control), at 37 °C in a humid atmosphere of 5% CO_2. Neutrophil viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Values represent the mean S.E.M. from 4 to 5 donors. $^*p < 0.05$ in comparison to control (ANOVA).

at 37 °C in a humid atmosphere of 5% CO_2. As shown in Fig. 4A, all concentrations from 6.2 up to 100 µg/mL of BbV induced a significant release of IL-6 by human neutrophils compared to control. Fig. 4B shows that after 4 h incubation of neutrophils with concentrations from 12.5 up to 100 µg/mL of BbV induced a significant release of IL-8 by human neutrophils. Our results demonstrate that BbV activated human neutrophils and induced the release of IL-6 and IL-8.

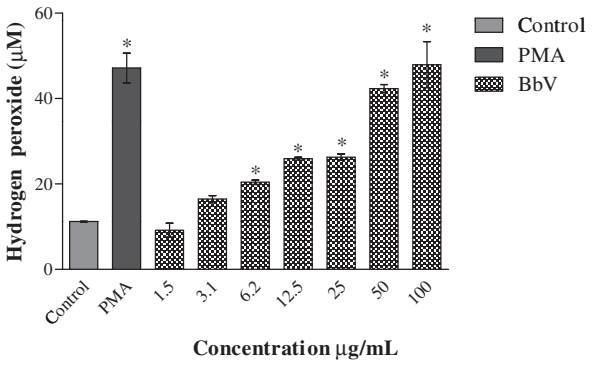

Fig. 2. Effect of *Bothrops bilineata* venom on neutrophil hydrogen peroxide production. 2×10^5 neutrophils were incubated for 90 min with BbV (1.5, 3.1, 6.2, 12.5, 25, 50 and 100 µg/mL) or PMA (positive control; 500 ng/mL) or RPMI (negative control) at 37 °C in a humid atmosphere of 5% CO_2. The absorbance was measured by spectrophotometer at 620 nm. The results were expressed as µmoles of H_2O_2 produced and represent the mean ± SEM of 4–5 donors. $^*p < 0.05$ compared to control (ANOVA).

3.5. *Effect of* B. bilineata *venom on NETs liberation by human neutrophils*

In order to investigate the ability of BbV to induce the liberation of NETs by human neutrophils, the cells were incubated with non-cytotoxic concentrations of BbV or RPMI (control) or PMA (positive control). As shown in Fig. 5A and B, 4 and 15 h of incubation of human neutrophils with different non-cytotoxic concentrations of BbV induced an increase in NETs liberation compared to the negative control (RPMI) and the positive control (PMA). These findings demonstrate the ability of BbV to stimulate human neutrophils to induce NETs liberation.

4. Discussion

The literature shows that leukocytes, and particularly neutrophils, play a critical role in skeletal muscle regeneration following myonecrosis induced by *Bothrops asper* venom (Teixeira et al., 2003). In addition, a marked inflammatory cell response with a pronounced neutrophil infiltration associated with bothropic envenomation has been reported (Gutiérrez et al., 1986; Flores et al., 1993; Farsky et al., 1997; Arruda et al., 2003; Zamunér et al., 2005; Porto et al., 2007), but the state of activation of these cells is unknown.

Besides this, it is quite possible that neutrophils – as the first cells at the site of an infection – might be able to clear a minor infection before monocytes even arrive. It therefore

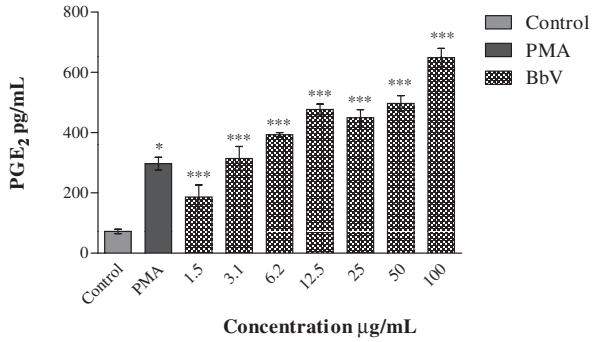

Fig. 3. Effect of *Bothrops bilineata* venom on PGE_2 production by human neutrophils. 2×10^5 neutrophils were incubated for 4 h with BbV (1.5, 3.1, 6.2, 12.5, 25, 50 and 100 µg/mL) or PMA (positive control; 500 ng/mL) or RPMI (negative control) at 37 °C in a humid atmosphere of 5% CO_2. PGE_2 concentrations were quantitated by specific EIA in supernatant collected after 4 h of incubation with BbV or PMA or RPMI. The results were expressed as µg/mL of PGE_2 produced and represent the mean ± SEM of 4–5 donors. $^*p < 0.05$ compared to control (ANOVA).

suggests the clearance of an infection by neutrophils without the classical symptoms of inflammation. Symptoms like reddening, swelling, pain and potential tissue damage are all induced by pro-inflammatory cytokines that are secreted by the later arriving monocytes (Schröder et al., 2006).

Taking this into account, we designed a study to investigate the ability of *B. bilineata* crude venom (BbV) to activate isolated human neutrophils since it has been shown that this venom causes inflammation and induces neutrophil recruitment into the peritoneal cavity of mice 4 h after its injection (Porto et al., 2007). First, the effect of BbV on human neutrophil viability was evaluated. The results showed that BbV did not affect neutrophil viability indicating its low toxicity on this cell type. The effect of BbV on human neutrophil viability was not demonstrated until now, but literature shows that *B. asper* venom decreases the viability of neutrophils isolated from mice (Moreira et al., 2009).

Since it is known that neutrophils play a central role in innate immunity and have the ability to adhere and migrate, degranulate, ingest particles by phagocytosis and release inflammatory mediators such as cytokines and reactive oxygen species (ROS) (Witko-Sarsat et al., 2000), we conducted experiments in order to verify the effect of BbV on hydrogen peroxide production. After 90 min of incubation the venom significantly stimulated human neutrophils to produce hydrogen peroxide compared to the negative control; however, there was no difference when compared with PMA (a positive control). BbV induced a significant release of hydrogen peroxide indicating that the BbV is able to stimulate neutrophils to activate the respiratory burst. In addition to our data, the literature shows that *Bothrops alternatus* venom induced the release of superoxide anion, another reactive oxygen intermediate, by mice thioglycollate-elicited macrophages (Setubal et al., 2011). Yet, the literature indicates that the injection of *B. asper* and *Bothrops jararaca* venoms in the peritoneal cavity of mice induced the production of hydrogen peroxide by peritoneal leukocytes meaning they are capable of priming leukocytes for the respiratory burst (Souza et al., 2012; Zamunér et al., 2001).

In addition to the well-known capacity of neutrophils to phagocytose and kill invading microorganisms intracellularly, they can also capture and kill pathogens extracellularly through the release of neutrophil extracellular traps (NETs). In order to understand the effect of BbV on neutrophil function, NETs liberation was assessed. Our results showed that BbV induced the liberation of NETs. However, there is no data in the literature so far showing the effect of *Bothrops* venom on NETs liberation which is the first description.

Taking this into account and to complement other studies we designed an experiment to investigate the ability of BbV to induce IL-8 release. Results showed that BbV induced the release of this chemokine. Since BbV induces IL-8 release as well as ROS production and the literature shows that cytokines and ROS induce NETs liberation (Fuchs et al., 2007; von Köckritz-Blickwede and Nizet, 2009), we suggest that IL-8 and ROS may contribute to NETs liberation induced by BbV.

To confirm our understanding of the effect of BbV on neutrophil function we decided to perform an experiment investigating the ability of BbV to induce IL-6 release. The results obtained indicate that BbV induced the release of

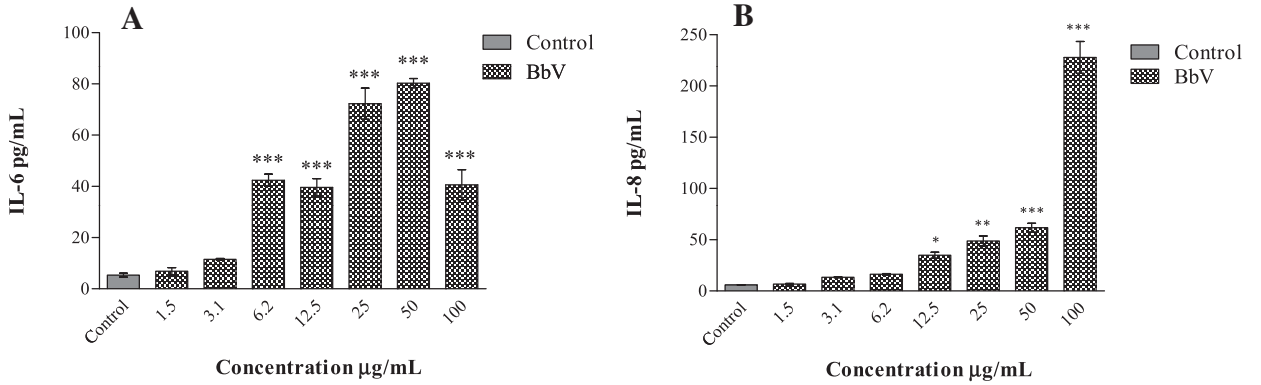

Fig. 4. Effect of *Bothrops bilineata* venom on IL-6 and IL-8 production by human neutrophils. 2×10^5 neutrophils were incubated for 4 h with BbV (1.5, 3.1, 6.2, 12.5, 25, 50 and 100 µg/mL) or RPMI (control) at 37 °C in a humid atmosphere of 5% CO_2. IL-6 (A) and IL-8 (B) concentrations were quantitated by specific EIA in supernatant collected after 4 h of incubation with BbV or RPMI. The results were expressed as µg/mL of IL-6 or IL-8 produced and represent the mean ± SEM of 4–5 donors. $^*p < 0.05$ compared to control (ANOVA).

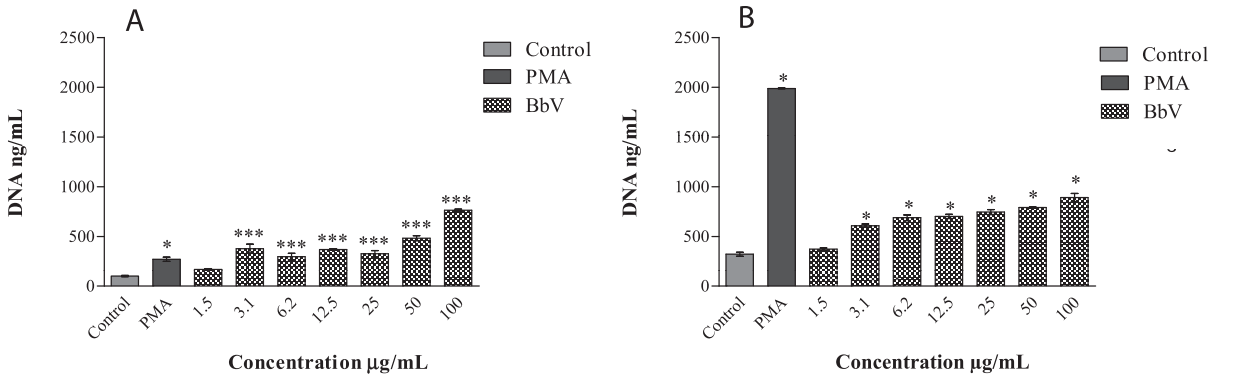

Fig. 5. Release of NETs by human neutrophils induced by BbV. Neutrophils (2×10^5) were incubated with non-cytotoxic concentrations of BbV or RPMI (control) or PMA (500 ng/mL, positive control) for 4 (A) and 15 h (B) at 37 °C in a humid atmosphere (5% CO_2). The DNA concentrations of NETs were quantified by Quant-iT™ Picogreen dsDNA kit. The results were expressed as ng/mL of DNA in the supernatant and represent the mean ± SEM of four donors. $^*p < 0.05$ compared to control (ANOVA).

this cytokine. Like IL-8 there is no data in the literature showing the effect of BbV on the production of IL-6 by isolated human neutrophils. Since BbV induces ROS production, we suggest that ROS may contribute to IL-6 release induced by BbV. Accordingly, the literature shows that intramuscular injection of *B. asper* venom induced an increase in IL-1beta and IL-6 in the muscle (Chaves et al., 2005). In addition, levels of proinflammatory cytokines IL-6 and TNF-α were significantly increased after *B. asper* venom injection (Zamunér et al., 2005). Recently, Corasolla Carregari et al. (2013) purified a new basic PLA_2 Asp-49 from *B. bilineata* that induced an increase in vascular permeability and in serum cytokine levels (IL-6, IL-1 and TNF-α) in mice.

Among the inflammatory mediators that participate in inflammatory disorders are lipid mediators. Prostaglandins are small-molecule derivatives of arachidonic acid, produced by cyclooxygenases (constitutively active COX-1 and inducible COX-2) and prostaglandin synthase. Local levels of prostaglandin E_2 (PGE_2) regulate multiple steps of inflammation and multiple functions of different immune cells (Kalinski, 2012).

Since the literature shows that IL-8 induces or enhances the expression of COX-2 (Maloney et al., 1998; Smith et al., 1996) and BbV induces IL-8, we suggest that the chemokine found in this study may contribute to signaling the induction of COX-2 expression and the release of PGE_2. Therefore we conducted experiments in order to verify the effect of BbV on PGE_2 production by human neutrophils. After 4 h of incubation the venom significantly stimulated the human neutrophils to produce PGE_2 compared to both controls. BbV induced a significant release of PGE_2 indicating that BbV is able to stimulate neutrophils to induce COX-2 expression. In addition to our data, the literature shows that *B. asper* venom induced the release of PGE_2 by mice neutrophils (Moreira et al., 2009). In this report, Moreira et al. (2009) showed that in neutrophils there is a tight correlation between the profiles of COX-2 expression and PGE_2 release, suggesting that COX-2 is a key isoform for the production of PGE_2 in these cells.

In conclusion, the data reached showed the ability of BbV to induce the activation of neutrophil function. BbV stimulates cells to produce ROS such as hydrogen peroxide. Moreover, BbV induces the release of inflammatory mediators IL-8 and IL-6, PGE_2 and induce NETs formation. It is noteworthy that this is the first description of the stimulatory effect of BbV on neutrophil function.

Authorship

J.P.Z. and S.S.S. designed the study; S.S.S., A.S.P., N.M.N. and J.S.F.B. performed the experiments; K.D.Z. provided venom; W.L.P. and O.B.C. supervised the flow cytometer studies; J.P.Z., S.S.S and A.S.P. collected and analyzed the data; L.A.C, R.G.S, J.P.Z and A.M.S. provided reagents; J.P.Z., S.S.S. and A.M.S. wrote the manuscript. All of the authors discussed the results and implications and commented on the manuscript at all stages.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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