Human neutrophils functionality under effect of an Asp49 phospholipase A₂ isolated from Bothrops atrox venom

Sulanita da S. Setúbal a, Adriana S. Pontes a, Neriane M. Nery a, Cristina M.A. Rego a, Hallison M. Santana a, Anderson M. de Lima b, Charles N. Boeno a, Mauro V. Paloschi a, Andreimar M. Soares b,c, Juliana P. Zuliani a,b,∗

a Laboratório de Imunologia Celular Aplicada à Saúde, Fundação Oswaldo Cruz (FIOCRUZ) Rondônia, Porto Velho, RO, Brazil
b Centro de Estudos de Biomoléculas Aplicadas à Saúde (CEBio), Fundação Oswaldo Cruz, (FIOCRUZ) Rondônia e Departamento de Medicina, Universidade Federal de Rondônia (UNIR), Porto Velho, RO, Brazil
c Universidade São Lucas (UnSL), Porto Velho, RO, Brazil

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ABSTRACT

Bothrops envenomation is associated with a cellular inflammatory response, characterized by pronounced neutrophil infiltration at the site of injury. Neutrophils act as the first line of defence, owing to their ability to migrate to the infected tissue, promoting an acute inflammatory response. At the site of inflammation, neutrophils perform defence functions such as phagocytosis, release of proteolytic enzymes, generation of reactive oxygen species (ROS), and synthesis of inflammatory mediators such as cytokines and lipid mediators. Neutrophils can also form neutrophil extracellular nets (NETs), webs composed of chromatin and granule proteins. This occurs after neutrophil activation and delivers high concentrations of anti-microbial molecules to the site of injury. This study evaluated the impact of BaTX-II, an Asp49 phospholipase A₂ (PLA₂) isolated from Bothrops atrox snake venom on human neutrophils in vitro. At non-toxic concentrations, BaTX-II induced hydrogen peroxide production by neutrophils, and this was reduced by wortmannin, a PI3K inhibitor. BaTX-II stimulated IL-1β, IL-8, LTB₄, myeloperoxidase (MPO), and DNA content release, consistent with NET formation. This is the first study to show the triggering of relevant pro-inflammatory events by PLA₂ Asp49 isolated from secretory venom.

1. Introduction

Phospholipases A₂ (PLA₂) are enzymes found abundantly in nature, including in mammalian fluids and tissues, as well as in high concentrations in snake, bee, and wasp venom (Harris, 1985). Although mammalian PLA₂ is non-toxic and is found in the pancreas, whereas venomous PLA₂ is highly active and toxic, they have common catalytic properties and are considerably homologous in primary, secondary, and tertiary structure (Dufton et al., 1983; Verheij et al., 1981). In addition to their role in prey digestion, venom PLA₂s have a variety of other functions, which include indirect haemolytic action, neurotoxicity, cardiotoxicity, platelet aggregation, myotoxicity, as well as anticoagulant, oedematogenic, bactericidal, and inflammatory activities (Kini and Evans, 1989; Teixeira et al., 2003).

There are two types of myotoxic PLA₂s, ‘classical’ and ‘variant’.

‘Classical’ PLA₂s contain an aspartate, at position 49 (Asp49) and catalyse, in a Ca²⁺-dependent manner, the hydrolysis of the ester linkage at the sn-2 position of glycerophospholipids. ‘Variant’ PLA₂s have no, or low, catalytic activity and contain a lysine at position 49 (Lys49) (Gutiérrez and Lomonte, 1997). Since PLA₂s present in snake venoms are homologous to these mammalian inflammatory PLA₂s, snake venom PLA₂s serve as an important tool for pharmacological studies. Neutrophils represent the largest fraction of leukocytes in the peripheral blood and are also called polymorphonuclear granulocytes (PMNs), as they have a segmented nucleus and different types of granules. These granules are classified according to their protein content, consisting of primary (azurophiles), secondary (specific), and tertiary granules (Mantovani et al., 2011). Primary granules contain myeloperoxidase (MPO), cathepsin G, defensins, elastase, and proteinase 3, among many other proteins. Secondary granules contain collagenase,
gelatinase, lactoferrin, and sialidase. Tertiary granules include gelati-
nase and β2-microglobulin (Borregaard et al., 1993; Mantovani et al., 2011).

Neutrophils have a short life span, ranging from 8 to 12 h in the
bloodstream (Summers et al., 2010). However, this life span can be
prolonged by several stimuli, such as cytokines and bacterial products
(Colotta et al., 1992). Neutrophils represent an important line of defence
for an organism, as they are attracted to infected tissue by chemokines,
promoting an acute inflammatory response (Nathan, 2006). At the site
of inflammation, neutrophils perform phagocytosis, release proteolytic
enzymes, generate reactive oxygen species (ROS), and synthesize in-
flammatory mediators such as cytokines and lipid mediators (Cassatella,
1995; Timár et al., 2013). Although neutrophils are considered an
important effector cell in the innate immune response, they also have a
function in the acquired immune response, where they are progenitor
cells of antigens, capable of priming Th1 and Th17 lymphocytes (Abi
Abdallah et al., 2011; Mantovani et al., 2011). This demonstrates the
importance of neutrophils as a link between the innate and acquired
immune responses. Neutrophils can also form neutrophil extracellular
traps (NETs), composed of webs of chromatin and granular proteins.
This occurs after neutrophil activation, providing the site of injury with
high concentrations of microbicidal molecules (Brinkmann and
Zychlinsky, 2007; Brinkmann et al., 2004).

Given these diverse functions, neutrophils are an important cell type
to study when investigating the pro-inflammatory actions of venoms and
their isolated fractions. The objective of this work was to evaluate the
effect of BaTX-II, isolated from Bothrops atrox snake venom, on isolated
human neutrophil function. We identified hydrogen peroxide produc-
tion by these neutrophils and studied the role of PI3K in this process. We
also showed release of IL-1β, IL-8, LTB₄, MPO, as well as DNA content in
response to BaTX-II.

2. Materials and methods

2.1. Venom

The venoms of Bothrops atrox specimens were from Porto Velho,
Rondônia, Brazil (SisGen authorization protocol n° AFCAB61). The
venom was extracted, pooled and lyophilized for storage biochemical
procedures.

2.2. Phospholipases A₂ isolation

2.2.1. Cation exchange chromatography

The cation exchange chromatography was performed according to
the method previously described by Andrião-Escarso et al. (2000)
with adaptations. Approximately 40 mg of B. atrox venom was suspended in
1 mL of 50 mM ammonium bicitrate buffer (NH₄HCO₃ - AMBIC) pH
8.0 and centrifuged at 3500×g for 5 min. To remove insoluble material
the venom was fractionated on a CM-Sepharose FF column (10 × 30
cm), with matrix composed of carboxymethyl (OCH₂COOH) functional
group. The column was previously equilibrated with the same buffer
used to solubilize the venom and the sample eluted under a gradient of
0–100% AMBIC 500 mM pH 8.0, in 5 columns, under a flow of 1
mL/min, in a chromatography Akta Purifier 10 (GE) system. Elution was
monitored at 280 nm and fractions were collected manually (Supple-
mental Fig. 1A).

2.2.2. Reverse phase chromatography

Reverse phase chromatography was performed according to the
method previously described by Sábel et al. (2012) with adaptations.
Fractions F7 and F9 from cation exchange chromatography were
lyophilized and solubilized in 0.1% TFA (solution A) and subjected to
high performance liquid chromatography (HPLC) in column C-18 (25
mm × 4.6 mm, Supelco), previously equilibrated with solution A and
euluted under gradient 0–70% of solution B (acetonitrile 99.9% and TFA
0.1%) in 5 column volumes, under flow of 1 mL/min, in a chromatog-
raphy Akta Purifier 10 (GE) system. Elution was monitored at 280 nm
(Supplementary Fig. 1B).

2.2.3. SDS-PAGE

Electrophoresis on 12.5% (w/v) polyacrylamide gel in the presence of
SDS (SDS-PAGE), was performed in a discontinuous pH system, in
reducing conditions, previously described by Laemmli (1970). Electro-
phoretic separation was performed at 100 V, until the bromophenol blue
reached the forehead. The gel was fixed in a 40% aqueous solution of
methanol (v/v) and acetic acid 7% (v/v) for 30 min. The protein bands
were evidenced by immersion in a solution containing Coomassie Brill-
iant Blue G-250® 0.08% (m/v), aluminum sulfate 8.0% (w/v), 1.6% o-phosphoric acid (m/v) and 20.0% (v/v) methanol for 2 h. The dye
excess was removed by immersion in a bleach solution containing 4.0%
elanol and 7.0% (v/v) acetic acid in water. Several changes of this
solution were carried out until obtaining a gel with adequate color. The
image of the gels was obtained using an Image scanner® equipment (GE
Healthcare LifeSc.) and the relative molecular mass (Mr) determined by
comparing the relative migration distances of the samples and the mo-
lecular mass standards (Supplementary Fig. 1A and 1C).

2.2.4. Phospholipase activity

The procedure was performed as described by Petrovic et al. (2001),
with modifications. For the experiment, 5 mg of 4N3OBA were diluted in
5.4 mL of acetonitrile (ACN). 0.1 mL aliquots were dried and maintained
at −20 °C. Each tube containing the 4N3OBA aliquot was diluted in 1.2
mL of sample buffer (10 mM Tris-HCl at pH 8.0, 10 mM CaCl₂ and 100
mM NaCl) and kept on ice. To determine phospholipase activity, 190 µL
of 4N3OBA reagent was combined with 10 µL of sample in triplicate. The
samples used were: B. atrox venom, a basic phospholipase Lys49
(BaTX-I) and an Asp49 (BaTX-II). After adding PL₂₅, absorbance was
determined at 425 nm using an Eon microplate spectrophotometer
(Biotek), after 30 min of incubation at 37 °C (Supplementary Fig. 1C).

2.3. Neutrophil isolation

Peripheral blood neutrophils were obtained from self-reportedly
healthy donors (18–40 years old). Informed consents were obtained
at the time of the blood draw. All participants gave informed consent
prior to their inclusion in the study, and the Brazilian IRB (Institution-
ial Review Board) of the Center of Tropical Medicine Research (CEPEM,
Rondônia, Brazil - approval number 108/2010) approved it. In brief,
according to Setúbal et al. (2013a) blood was collected in vacuum tubes
containing heparin and diluted in phosphate buffered saline (PBS, 14
mM NaCl, 2 mM NaH₂PO₄H₂O, 7mMNa₂HPO₄12H₂O), pH 7.4, after
local asepsis. For the separation of leukocytes, Histopaque 1077 was
added to the tubes and then the diluted blood was carefully added over
the reagent. After centrifugation at 400×g for 30 min, neutrophils were
collected from the bottom of the tube, along with the erythrocytes and
were transferred to another tube. Lysis of red blood cells was performed
using lysis buffer (0.15 M NH₄Cl; 0.01 M KHCO₃; 0.0001 M Na₂EDTA),
homogenized and subjected to a temperature of −8 °C for 5 min, and
then 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 anti-human
CD-66b-FITC for 30 min at a granulocyte marker analyses (Setubal
et al., 2013a) in Accuri C6 cytometer (BD, USA) (Supplementary Fig. 2).

2.4. Determination of cell viability by MTT

Thus, 2 × 10⁶ neutrophils were cultured in 96-well plates with 200
µL of RPMI supple-mented and incubated with the BaTX-II at
concentrations of 1.5, 3, 6, 12.5 and 25 μg/mL for 6 h at 37 °C in a humidified atmosphere (5% CO₂). After this time, the samples were centrifuged for 5 min at 400×g and the supernatant removed. Then 10 μL of MTT (5 mg/mL in PBS) and 90 μL of RPMI/well were added, followed by incubation at 37 °C and 5% CO₂ for 2 h. After this time, the cells were centrifuged again and the supernatant containing unreduced MTT was removed and then 100 μL of DMSO was added to solubilize the formazan crystals. The degree of reduction of MTT to formazan was quantified by the optical density (OD) measurement at 570 nm in a spectrophotometer. The results were expressed as OD.

2.5. Determination of hydrogen peroxide (H₂O₂) production by human neutrophils

The method used was described by Pick and Keisari (1980), adapted for microassay by Pick and Mizel (1981), and with modifications proposed by Russo et al. (1989). In brief, neutrophils (2 × 10⁶/50 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 an BaTX-II at several concentrations (1.5, 3, 6, 12.5 and 25 μg/mL) for 1:30 h at 37 °C in humidified atmosphere (5% CO₂). Cells were maintained simultaneously with or without stimulation by phorbol myristate acetate (PMA, 50 ng/mL). After this time, the reaction was stopped by addition of 1 N sodium hydroxide (10 μL). The absorbance was measured spectrophotometrically at 620 nm against blank constituted of phenol red medium. Generated results were compared to a standard curve conducted for each test. The results were expressed as μM of H₂O₂ produced.

To evaluate the participation of PI3K in the production of hydrogen peroxide by human neutrophils stimulated with BaTX-II, the cells were incubated with 50 μL of the Wortmannin, a PI3K inhibitor (500 nm), for 15 min prior to addition of the toxin at different concentrations, and hydrogen peroxide production was determined as previously described. The inhibitor concentration employed in this study was based on concentrations described as effective in the literature (Wortmannin - 500 ng/mL, 15 min; Ingvar et al., 1996) and did not cause adverse effects on cell viability during the assay. In the different experimental protocols, the control cells were incubated with the same concentration of vehicle used to dissolve the inhibitor.

2.6. DNA content release

Neutrophils (2 × 10⁶/100 μL) were incubated with different concentrations of BaTX-II at different concentrations (1.5, 3, 6, 12.5 and 25 μg/mL) or RPMI (negative control) or PMA (500 ng/mL, positive control) for 4 h at 37 °C in humidified atmosphere (5% CO₂) as described by Setubal et al. (2013a). After centrifugation the supernatant were used for determination of DNA content (suggesting NETs release) accordingly to 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 incubation absorbances at 520 nm emission and 480 nm excitation were recorded and DNA content release were estimated from standard curve. The results were expressed as ng/mL of DNA.

2.7. Myeloperoxidase assay

For this assay, 1 × 10⁶ neutrophils were resuspended in RPMI culture medium, plated and incubated with RPMI (negative control), LPS (1 μg/mL; positive control), PMA (500 ng/mL; positive control) or different concentrations of BaTX-II (6, 12.5 and 25 μg/mL), for 4 h at 37 °C, in a humidified atmosphere (5% CO₂) according to Pontes et al. (2016). Then, the plate was centrifuged at 400×g for 5 min and the supernatant was collected for MPO level determination. For this purpose, 50 μL of each sample was added to 100 μL of TMB (Suzuki et al., 1983). After 5 min, the reaction was interrupted with 2N sulfuric acid. MPO levels were measured with the Bio-Tek Synergy HT Multi-Detection (Winooski, VT) at absorbance wavelengths of 450 nm. Results were estimated through a standard curve prepared with recombinant MPO and expressed by ng/mL of MPO.

2.8. Interleukin-1β (IL-1β) and Interleukin-8 (IL-8) quantifications

For this assay, 2 × 10⁶ neutrophil suspensions resuspended in assay medium were plated and incubated with RPMI (negative control), PMA (500 ng/mL; positive control) or different concentrations of BaTX-II (1.5, 3, 6, 12.5 and 25 μg/mL) at 37 °C, in a humidified atmosphere (5% CO₂) for 4 h. After centrifugation, the supernatant was used for quantification of IL-1β and IL-8 levels by specific EIA, according to Pontes et al. (2014). The results were expressed in pg/mL of each cytokine.

2.9. Leukotriene B₄ (LTB₄) assay

LTB₄ concentrations were measured in the supernatant of neutrophils (2 × 10⁶ cells/mL) suspended in assay medium. Briefly, neutrophils were incubated with assay medium (negative control), PMA (500 ng/mL; positive control) or BaTX-II in different concentrations (1.5, 3, 6, 12.5 and 25 μg/mL) diluted in assay medium for 4 h at 37 °C in a humidified atmosphere (5% CO₂) according to Pontes et al. (2016). LTB₄ concentrations in the supernatant were determined by a specific enzymatic immunoassay (EIA) using a commercial kit (Cayman Chemicals, MI, USA).

2.10. Statistical analysis

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

3. Results

3.1. Effect of BaTX-II, isolated from the B. atrox snake venom, on human neutrophils viability

Neutrophil viability was assessed 6 h after incubation with different concentrations of the BaTX-II. The viability determination was performed by the MTT reduction method. The re-sults showed that the toxin at all different concentrations (1.5, 3, 6, 12.5 and 25 μg/mL) did not affect neutrophil viability in the evaluated period of time (Fig. 1).

3.2. Effect of BaTX-II on human neutrophils hydrogen peroxide production

To verify the ability of BaTX-II to induce the hydrogen peroxide production by human neutrophils, cells were incubated with the toxin with non-toxic concentrations, PMA (positive control) or RPMI (negative control). As shown in Fig. 2, incubation of neutrophils with the BaTX-II resulted in a significant increase in hydrogen peroxide production compared to the ne-gative control, at all concentrations (1.5-25 μg/mL) studied. The toxin effect at the highest concentrations was not statistically different from the positive control, PMA.

To assess the participation of PI3K in the venom Asp-49 PLA₂-stimulated hydrogen peroxide production, neutrophils were incubated with a specific inhibitor of PI3K, Wortmannin, at the concentration of 500 nM. Neutrophils were maintained for 15 min with the inhibitor and stimulated for 1:30 h with the BaTX-II. As shown in Fig. 2, when neutrophils were incubated with the BaTX-II, in the presence of Wortmannin, there was a significant decrease in the hydrogen peroxide production compared to the controls.
Fig. 1. Effect BaTX-II, isolated from the *B. atrox* snake venom on human neutrophils viability. $2 \times 10^5$ neutrophils were incubated for 6 h with BaTX-II at concentrations of 1.5, 3, 6, 12.5 and 25 μg/mL or RPMI (control) at 37 °C and 5% CO$_2$. Neutrophil viability was assessed by the reduction of MTT. The data represent the mean ±SEM of 5 independent donors.

Fig. 2. Effect of BaTX-II, isolated from *B. atrox* snake venom on human neutrophils hydrogen peroxide production. $2 \times 10^5$ neutrophils were incubated for 1:30 h with BaTX-II at concentrations (1.5, 3, 6, 12.5 and 25 μg/mL) or RPMI (negative control) or PMA (500 ng/mL; positive control) at 37 °C and 5% of CO$_2$. After incubation, the hydrogen peroxide production was determined in the presence or absence of wortmannin, a PI3K inhibitor, after reading the optical density at 620 nm. The results were expressed as μM H$_2$O$_2$. Data represent the mean ± SEM of 5 independent donors. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared to negative control. ###$P < 0.001$, ####$P < 0.0001$ compared to respective experimental without inhibitor (ANOVA).
### 3.3. Effect of BaTX-II on DNA release by human neutrophils

To assess the ability of the BaTX-II to induce the release of DNA content suggesting NETs release, neutrophils were incubated with BaTX-II in non-cytotoxic concentrations, RPMI (negative control) or PMA (positive control). As shown in Fig. 3A the toxin induced an increased in DNA release compared to the negative control after 4 h of incubation. These results evidence the ability of the toxin to activate human neutrophils and stimulate the NETs release.

### 3.4. Effect of BaTX-II on MPO release by human neutrophils

To determine the ability of the BaTX-II to induce MPO release, neutrophils were incubated with BaTX-II in non-cytotoxic concentrations, RPMI (negative control) or PMA (positive control). As shown in Fig. 3B BaTX-II induced an increase in MPO release to the supernatant compared to the negative control. These results evidenced the ability of BaTX-II to activate human neutrophils and probably causing degranulation, resulting in the MPO release.

### 3.5. Effect of BaTX-II on human neutrophils IL-1β, IL-8 and LTB4 liberation

IL-1β and IL-8 concentrations in the neutrophil supernatant was evaluated 4 h after incubation of cells with the BaTX-II (1.5, 3, 6, 12.5 and 25 μg/mL), RPMI (negative control) or LPS (positive control). Fig. 4A and B shows that in the time period studied there was a significant increase in IL-1β and IL-8 concentrations in the neutrophil supernatant after incubation with the venom BaTX-II compared to the control.

The ability of the BaTX-II to induce LTB4 production by human neutrophils was assessed by determining the concentration of this lipid mediator in the neutrophil supernatant incubated with the BaTX-II at different concentrations (1.5, 3, 6, 12.5 and 25 μg/mL), PMA (positive control; 500 ng/mL) or RPMI (negative control). Incubation of neutrophils with the BaTX-II for 4 h induced a significant increase in LTB4 levels at all concentrations used compared to the control cells (Fig. 4C) suggesting that this lipid metabolite may represent an important mediator resulting from the mechanism of action triggered by the BaTX-II on the neutrophil.

### 4. Discussion

Previous studies have demonstrated that B. atrox venom induces an increase in vascular permeability and an in vivo leukocyte influx (Barros et al., 1998; Moreira et al., 2012), consisting mainly of MNs initially and PMNs during the later stages, mediated by lipids and cytokines. This occurs directly via the action of venom-associated molecular patterns (VAMPs), activating TLR2 (Moreira et al., 2016) and MyD88 signalling (Moreira et al., 2013). Few studies have addressed the role of B. atrox PLA2 in this process (Furtado et al., 2014; Menaldo et al., 2017), and little is known about the effect of this isolated PLA2 on neutrophil function. Therefore, here we evaluated the effect of BaTX-II, isolated from B. atrox snake venom, on isolated human neutrophil function.

First, the cytotoxic activity of BaTX-II on neutrophils was evaluated. An MTT assay demonstrated that at concentrations of 1, 5, 3, 6, 12.5, and 25 μg/mL, the toxin did not alter the viability of neutrophils. Therefore, these concentrations were used in subsequent experiments. Similarly, Zuliani et al. (2005) showed that at concentrations less than...
25 μg/mL, MTX-III isolated from *B. asper* was not toxic to mice peritoneal macrophages. Other studies demonstrated that two phospholipases isolated from *B. asper* and *B. atrox* exerted toxic effects on several cell types only at high concentrations (Bultrón et al., 1993; Furtado et al., 2014; Lomonte et al., 1994; Setúbal et al., 2013b). These results show that the cytotoxic potential of BaTX-II from *B. atrox* is similar to that of the PLA₂S from snake species of the genus *Bothrops*.

Neutrophils are the first cells recruited to inflamed tissue, and after activation play a key role in innate immunity, phagocytosing and destroying microbial pathogens. This occurs through a series of fast and coordinated responses such as adhesion, migration, degranulation, and release of inflammatory mediators, proteolytic enzymes and reactive oxygen species (ROS) (Fialkow et al., 2007; Hampton et al., 1998; Witko-Sarsat et al., 2000). The increase in ROS production occurs by a
phenomenon known as the “respiratory burst” that is mediated by a NADPH oxidase complex, promoting high oxygen consumption with subsequent production of superoxide anion and hydrogen peroxide (Groemping and Ritteringer, 2005). These reactive species derived from oxygen result in microbial death and tissue damage by oxidizing a wide range of target biological molecules, including carbohydrates (Schiller et al., 1996), nucleotides, DNA (Hawkins et al., 2002), lipids (Panasenko et al., 2007; Skaff et al., 2007), and proteins (Hawkins et al., 2003). However, excessive release of hydrogen peroxide can cause tissue damage, contributing to an increase in the inflammatory reaction.

Thus, we performed experiments to verify the effect of BaTX-II on hydrogen peroxide production by human neutrophils. After 90 min of incubation, the toxin significantly stimulated neutrophils to produce hydrogen peroxide compared to a negative control; however, there was no difference when compared to PMA (a positive control). This venom PLA₂ induced the release of hydrogen peroxide, indicating that the toxin is capable of stimulating neutrophils to activate the respiratory burst. These results are in accordance with the findings of Zuliani et al. (2005), who showed that MT-II (PLA₂ Lys-49) and MT-III (PLA₂ Asp49) isolated from B. asper venom induced the release of hydrogen peroxide, by macrophages.

Phosphatidylserinol 3-kinase (PI3K) is a heterodimeric enzyme whose main product is phosphatidylserinol (3, 4, 5) triphosphate. Among other functions, it can act as a secondary modulator of ROS production in multiple cell types (Korbecki et al., 2013). Therefore, we evaluated the role of PI3K in modulating ROS production by neutrophils stimulated with BaTX-II. To determine whether the PI3K signalling pathway is involved in BaTX-II-induced ROS production by neutrophils, a PI3K inhibitor, wortmannin, was used. This is a fungal metabolite that blocks PI3K signalling (Pawis et al., 1994). When treated with wortmannin, as well as PMA (a positive control), we observed a significant decrease in the amount of hydrogen peroxide produced by neutrophils stimulated by the venom PLA₂. This suggests a role for PI3K in the signalling cascade triggered by the toxin.

In addition to the well-known microbialic capacity of neutrophils, they can also capture and kill extracellular pathogens through the release of neutrophil extracellular traps (NETs). Furthermore, ROS released by the NADPH oxidase complex can activate granular proteases and induce NET formation (Nguyen et al., 2017). ROS can cross membranes and damage nucleic acids, proteins, and cell membranes. Several enzymes located in NETs have pro-inflammatory function, such as elastase and MPO, suggesting a role for the formation of NETs in inflammation (Manfredi et al., 2018). Thus, we conducted experiments to evaluate the effect of venom BaTX-II on this important neutrophil function. Results showed that the toxin, BaTX-II, induced the release of DNA and MPO, suggesting that it induces NET formation. These observations are congruent with literature demonstrating that ROS derived from NADPH oxidase activity, as well as MPO liberation, are key signals for the generation of NETs (Rada et al., 2013). This strongly suggests a direct effect of toxin-induced ROS and MPO on the formation and release of NETs.

Further experiments were carried out to assess the capacity of the venom BaTX-II to induce the release of IL-1β, IL-8 and LTB₄ by human neutrophils. Results showed that the toxin induced the release of both IL-1β and IL-8. This is the first demonstration of the effect of PLA₂ on cytokine production by human neutrophils. Moura et al. (2014) described an increase in IL-1β concentration in bioglycocolle macrophages, which were stimulated with PLA₂ isolated from B. mcatrossensis venom. Corasolla Carregari et al. (2013) showed an increase in IL-6, IL-1, and TNF-α serum levels using a BaTX-II from B. bilineata venom. In vivo experiments, Nezam et al. (2017) showed that an acidic PLA₂ from B. atrax, BatroxPLA₂, induced IL-6, PGE₂ and LTB₄ release from mouse macrophages.

IL-1β is a pro-inflammatory cytokine produced by many cell types including macrophages, NK cells, monocytes, and neutrophils (Casserella, 1999). This cytokine has important homeostatic functions in the body, including in regulating sleep and temperature (Dinarello, 1996). However, the overproduction of IL-1β is involved in physiopathological changes which occur in inflammatory diseases (Ren and Torres, 2009).

In addition, literature suggests that the release of IL-1β is correlated with the generation of NETs by neutrophils derived from the inflammatory synovial fluid (Mitroulis et al., 2011).

As a powerful chemokine that plays a key role in innate immunity, IL-8 production could contribute to the maintenance of chemotaxis, attracting more neutrophils to the site of inflammation (Bagnoli et al., 1994). In addition, PLA₂ from B. jararacussu (Bothropsoxin-I and Bothrotoxin-II) and B. pirajai (Piratoxin-I) can induce neutrophil migration in a concentration-dependent manner (Gambero et al., 2002). However, IL-8 also contributes to tissue damage and organ failure in conditions such as acute respiratory stress syndrome, reperfusion injury, and septic shock (Feuerstein and Rabinovici, 1994). Moreover, IL-8 release, as well as ROS production, stimulates the release of NETs (Fuchs et al., 2007), suggesting that IL-8 and ROS production induced by BaTX-II could contribute to the release of NETs.

LTB₄ is a metabolite resulting from the activity of 5-lipoxygenase (5-LO) and is considered a potent lipid mediator with chemoattractant properties. It plays an essential role in the inflammatory response and is a key player in the initiation of inflammation as a lipid mediator (Henderson, 1994; Peters-Golden and Henderson, 2007). LTB₄ stimulation leads to neutrophil functional responses important to host defence, such as the secretion of lysosomal enzymes, the activation of NADPH oxidase activity, NO formation, and phagocytosis. In addition, LTB₄ stimulates the expression of the β₃-integrin (CD11b/CD18), an effect likely related to its ability to stimulate leukocyte migration and phagocytosis (Flamand et al., 2007). Our results showed that the venom PLA₂ induced a significant increase in LTB₄ release by neutrophils. This suggests that, along with IL-8, this plays a key role in neutrophil chemotaxis induced by the toxin, as has been described by Gambero et al. (2002).

Given the important role of neutrophils as front-line cells in innate immunity, as well as the importance of MPO in neutrophil functional responses, we investigated the effect of the venom PLA₂ on MPO production by this cell type. MPO, a haeme-protein, is the principal enzyme released from the neutrophil’s azurophilic granules upon neutrophil activation (Witko-Sarsat et al., 2000) when stimulated by ROS and cytokines (Arimura et al., 1993). Here, we showed that BaTX-II induced the release of IL-8, as well as the production of ROS. We also showed BaTX-II-induced MPO release, suggesting that this enzyme leads to the degradation of human neutrophils.

Taken together, our data show that the BaTX-II has the capacity to induce the activation of neutrophils (Fig. 5). The stimulation of hydrogen peroxide production involves a signalling event dependent on PI3K. In addition, BaTX-II induces the release of inflammatory mediators IL-1β, IL-8, LTB₄, MPO, and DNA, contributing to the formation of NETs. Significantly, this is the first description of the ability of BaTX-II to stimulate neutrophil functions. Our findings help contribute to a better understanding of the role of phospholipases at the site of inflammation, as well as the role of these enzymes in the inflammatory response triggered by snake venom.

**Authorship**

J.P.Z. and S.S.S. designed the study; S.S.S., A.S.P., N.M.N., H.M.S. and M.V.P. performed the experiments; A.M.S. provided venom; C.M.A. R. and C.N.B performed and supervised the flow cytometer studies; A.M. L. and A.M.S. performed the toxin isolation and characterization; J.P.Z., S.S.S and A.S.P., N.M.N., H.M.S., C.M.A.R., C.N.B, A.M.L., M.V.P. and A. M.S collected and analyzed the data; J.P.Z and A.M.S. provided reagents; J.P.Z., S.S.S and A.M.S. wrote the manuscript and M.V.P. performed the figures. All of the authors discussed the results and implications and commented on the manuscript at all stages.
Fig. 5. Proposed mechanism of action of BaTX-II, isolated from B. atrox venom on human neutrophils. The representative scheme shows BaTX-II acting on neutrophils to produce H$_2$O$_2$, IL-1β, LTB4 and IL-8. Firstly, BaTX-II interacts with the cellular membrane by an unknown mechanism, leading to PI3K stimulation and NADPH oxidase activation, culminating in H$_2$O$_2$ liberation. BaTX-II also stimulates neutrophil to secrete pro-inflammatory mediators such as cytokines IL-1β and IL-8 and the lipid mediator LTB4 and to liberate MPO and NETs. The illustration was made using the resources of “SMART - Servier Medical Art”.

Declaration of competing interest

There is no conflict of interest statement.

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