Crotoxin stimulates an *M1* activation profile in murine macrophages during *Leishmania amazonensis* infection

L. H. S. FARIAS[1,2], A. P. D. RODRIGUES[2,3], E. C. COÊLHO[1], M. F. SANTOS[4], S. C. SAMPAIO[5,6]* *and* E. O. SILVA[1,2]*

[1] *Laboratory of Parasitology and Laboratory of Structural Biology, Federal University of Para, Institute of Biological Sciences, Belém, Pará, Brazil*
[2] *National Institute of Science and Technology in Structural Biology and Bioimaging, Rio de Janeiro, Rio de Janeiro, Brazil*
[3] *Laboratory of Electron Microscopy, Department of Health Surveillance, Ministry of Health, Evandro Chagas Institute, Belém, Pará, Brazil*
[4] *Cell and Developmental Biology Department, Institute of Biomedical Sciences, University of São Paulo, São Paulo, São Paulo, Brazil*
[5] *Laboratory of Pathophysiology, Butantan Institute, São Paulo, Brazil*
[6] *Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil*

(*Received 8 November 2016; revised 28 April 2017; accepted 11 May 2017; first published online 23 June 2017*)

SUMMARY

American tegumentary leishmaniasis is caused by different species of *Leishmania*. This protozoan employs several mechanisms to subvert the microbicidal activity of macrophages and, given the limited efficacy of current therapies, the development of alternative treatments is essential. Animal venoms are known to exhibit a variety of pharmacological activities, including antiparasitic effects. Crotoxin (CTX) is the main component of *Crotalus durissus terrificus* venom, and it has several biological effects. Nevertheless, there is no report of CTX activity during macrophage – *Leishmania* interactions. Thus, the main objective of this study was to evaluate whether CTX has a role in macrophage *M1* polarization during *Leishmania* infection murine macrophages, *Leishmania amazonensis* promastigotes and *L. amazonensis*-infected macrophages were challenged with CTX. MTT [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide] toxicity assays were performed on murine macrophages, and no damage was observed in these cells. Promastigotes, however, were affected by treatment with CTX ($IC_{50} = 22{\cdot}86\ \mu g\ mL^{-1}$) as were intracellular amastigotes. Macrophages treated with CTX also demonstrated increased reactive oxygen species production. After they were infected with *Leishmania*, macrophages exhibited an increase in nitric oxide production that converged into an *M1* activation profile, as suggested by their elevated production of the cytokines interleukin-6 and tumour necrosis factor-α and changes in their morphology. CTX was able to reverse the *L. amazonensis*-mediated inhibition of macrophage immune responses and is capable of polarizing macrophages to the *M1* profile, which is associated with a better prognosis for cutaneous leishmaniasis treatment.

Key words: crotoxin, *Crotalus durissus terrificus*, murine macrophage, *L. amazonensis*.

INTRODUCTION

Leishmania, an intracellular parasite of macrophages, is responsible for over 12 million leishmaniasis cases in more than 98 countries (de Vries *et al*. 2015). Depending on the species, the protozoan can promote different clinical manifestations that are associated with cutaneous and visceral pathologies (Adade *et al*. 2014). *Leishmania (L.) amazonensis* is an important agent that induces cutaneous leishmaniasis and is responsible for the inhibition of the immune system, which promotes a high rate of parasite proliferation (Pereira and Alves, 2008).

Macrophage activation was formerly termed as simply 'activated', but is currently divided into two major profiles: classically activated, or *M1*, and alternatively activated macrophages, or M2. As regards their characteristics, *M1* macrophages are effector cells during the type 1 T helper (Th1)-type immune response and are differentiated into this profile by activation stimuli, such as the presence of interferon (INF)-γ and lipopolysaccharide (LPS) (Mosser, 2003). These *M1* cells have markers that distinguish them from other profiles, such as the high production of cytokines interleukin (IL)-12, IL-23, IL-6, tumour necrosis factor (TNF)-α and microbicidal substances such as nitric oxide (NO) and reactive oxygen species (ROS), besides morphological alterations in cell spreading and formation of cytoplasmic projections, providing the ability to react against intracellular parasites and tumour cells (Cassado *et al*. 2011; Xuan *et al*. 2015).

In contrast, M2 macrophages are activated during the Th2 lymphocyte response, being stimulated by IL-4, aiming to repair and heal tissue (Cassado

* Corresponding authors: Laboratory of Parasitology and Laboratory of Structural Biology, Federal University of Para, Institute of Biological Sciences, Belém, Pará, Brazil. E-mail: edilene@ufpa.br and Laboratory of Pathophysiology, Butantan Institute, São Paulo, Brazil. E-mail: sandra.coccuzzo@butantan.gov.br

doi:10.1017/S0031182017000944

et al. 2011). These macrophages are generally characterized by the high production of IL-10 and decreased productions of IL-12 and IL-23. M2 macrophages are characterized by producing low or no NO and ROS, characterizing a reduced microbicidal capacity (Liu *et al*. 2014; Bashir *et al*. 2016).

The drugs that are currently indicated for the treatment of leishmaniasis are of high costs and present high toxicity, and have considerable side-effects. The first line of treatment uses pentavalent antimonials, and when this approach fails in a patient, second-line drugs are employed, such as amphotericin B or pentamidine. These therapies induce adverse reactions, with the most commonly reported adverse reactions being nephrotoxicity and heart failure, respectively (McGwire and Satoskar, 2014).

Given the limited efficacy of the current drugs available to treat *Leishmania*, the development of alternative treatments is critical. Natural products have been identified as critical substances during drug discovery processes, and they are the basis for most first drugs used (Butler, 2008; Schepetkin *et al*. 2015).

Animal venoms and their purified compounds are known to exhibit a variety of pharmacological activities, including activities against pathogens *in vitro* (Quintana *et al*. 2012). In addition, snake venom proteins have been widely investigated in different areas of the life sciences. The interactions of these compounds with different cells can involve a variety of mechanisms that can result in various cellular responses, leading to the activation or blocking of physiological functions in the cell (Marcinkiewicz, 2013). The venom of the South American rattlesnake *Crotalus durissus terrificus* (*Cdt*) is reported to have various biological effects, ranging from anti-inflammatory to antitumoural activities (Sampaio *et al*. 2003; Costa *et al*. 2013). *Cdt* crude venom is composed of gyroxin, crotamine, convulxin and crotoxin (CTX), which have proteolytic, analgesic, coagulative and neurotoxic effects, respectively (Passero *et al*. 2007). CTX is the major toxic component of this venom and is composed of a non-covalent association between crotapotin, an acidic subunit, with phospholipase A_2 (PLA_2), a basic subunit.

Several studies have demonstrated the antileishmanial activity of PLA_2 derived from the venom of diverse snake species (Torres *et al*. 2010; Nunes *et al*. 2013; de Moura *et al*. 2014; Barros *et al*. 2015). Additional studies have used venoms derived from *Bothrops mattogrossensis*, *Bothrops pauloensis*, *Bothrops lutzi*, *Philodryas patagoniensis* and *Cdt*, which have also been reported to have antileishmanial activity (Passero *et al*. 2007, 2008; Peichoto *et al*. 2011; Nunes *et al*. 2013; Adade *et al*. 2014). However, few studies have studied the effects of the whole CTX molecule against *Leishmania* parasites, and most of these have demonstrated the effects of CTX that are related to the growth of the promastigote forms (Passero *et al*. 2007). There is currently no evidence of the activity of CTX, derived from *Cdt*, during macrophage–*L. amazonensis* interactions. Moreover, following on from findings showing that CTX obtained from *Cdt* can selectively modulate secretory activity in macrophages, by reinforcing the immunomodulatory actions of this toxin (Costa *et al*. 2013), we demonstrate in this study, for the first time, the immunomodulatory activity of CTX during macrophage–*L. amazonensis* interactions.

MATERIALS AND METHODS

CTX isolation

CTX (CTX-complex) was obtained from the lyophilized venom of *Cdt*, which was supplied by the Laboratory of Herpetology, Butantan Institute, São Paulo, Brazil, and maintained at −20 °C. A crude venom solution was subjected to anion-exchange chromatography as previously described in Rangel-Santos *et al*. (2004) using a Mono-Q HR 5/5 column in an fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden). The fractions (1 mL min^{-1}) were eluted using a linear gradient of NaCl (0–1 mol L^{-1} in 50 mmol L^{-1} Tris-HCl, pH 7·0). Three peaks (p1, p2 and p3) were obtained: p2 corresponded to the pure CTX fraction (approximately 60% of the crude venom), whereas peaks 1 and 3 included the other Crotalus durissus terrificus venom (CdtV) toxins. Prior to pooling, the fractions containing CTX were tested for homogeneity using non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12·5%) (Laemmli, 1970), and PLA_2 activity was assessed using a colorimetric assay with a synthetic chromogenic substrate (Lôbo de Araújo and Radvanyi, 1987).

Murine macrophage isolation

Macrophages were harvested from the peritoneal cavities of male BALB/c mice into Dulbecco's modified Eagle's medium (DMEM, pH 7·2) and incubated at 37 °C in an atmosphere containing 5% CO_2 for 1 h. Non-adherent cells were washed away with phosphate-buffered saline (PBS, pH 7·2), and the remaining macrophages were then maintained in DMEM supplemented with 10% fetal bovine serum (FBS – Gibco® Thermo Fisher Scientific) at 37 °C in 5% CO_2 for 24 h.

Murine macrophage cytotoxicity assays

Macrophages (10^6 macrophages in 1 mL of medium per well) were seeded in 24-well tissue culture plates during the adhesion step. The cells were then treated with CTX at concentrations ranging from 0·3 to 9·6 $\mu g\ mL^{-1}$ at 37 °C in 5% CO_2 for 48 h. Subsequently,

cell supernatants were collected, and the cells were washed with PBS (pH 7·2). These concentrations of CTX are the same as those used in previous studies (Sampaio *et al*. 2003, 2006*a*, *b*; Costa *et al*. 2013). The cytotoxicity of CTX was evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays in murine macrophage cultures. The cells were washed and incubated with MTT for 3 h, and the formazan crystals were then diluted in dimethyl sulfoxide. The absorbance of the resulting solution was recorded at an optical density of 570 nm, as described (Fotakis and Timbrell, 2006). As a negative control, cells were killed with methanol.

Evaluation of ROS in murine macrophages in response to CTX treatment

After treatment with CTX for 24 or 48 h, the macrophages were washed with PBS (pH 7·2) and then incubated with CellRox Green® (Life Technologies™) at a concentration of 5 μM (diluted in DMEM culture medium) for 30 min at 37 °C and 5% CO_2. After incubation, the cells were washed with PBS and mechanically harvested with cell scrapers, for flow cytometry (BD FACSCanto II) analysis. The results were evaluated using WinMDI (NIH) software to determine fluorescence intensity.

Parasites

Leishmania amazonensis promastigotes were isolated by the inoculation of lesion tissue fragments obtained from patients with localized cutaneous leishmaniasis (MHOM/BR/26361) into axenic Novy-MacNeal-Nicolle medium. Regular infections of hamsters (*Mesocricetus auratus*) were performed to maintain parasite infectivity. The parasites were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS at 27 °C.

In vitro antileishmanial assays

Antipromastigote assays. Promastigote forms were harvested at the logarithmic phase of growth, washed twice with PBS by centrifugation, counted in a Neubauer haemocytometer and seeded at a concentration adjusted to 10^6 promastigotes per well in a final volume of 1 mL in 24-well plates with RPMI medium, supplemented with 10% FBS. CTX was added to the culture at concentrations of 2·4 and 4·8 μg mL^{-1} (from a stock concentration of 1·28 mg mL^{-1}), according to the more responsive concentrations that were observed in the assays using murine macrophages. The number of parasites was counted daily for up to 96 h using a Neubauer chamber, and IC_{50} values were calculated using GraphPad Prism 6·0. Amphotericin B® (0·8 μg mL^{-1}) was used as a reference drug in analyses of parasite promastigotes.

Antiamastigote assay. Murine macrophages (5×10^5 mL^{-1}) that had been allowed to adhere to coverslips were challenged with *L. amazonensis* promastigotes at a proportion of 1:10 for 3 h at 37 °C in 5% CO_2. After incubation, the cultures were washed with PBS to remove non-adherent parasites and then treated with 2·4 and 4·8 μg mL^{-1} CTX for 24 and 48 h at 37 °C in 5% CO_2. Cells were then fixed with 4% paraformaldehyde, stained with Giemsa and mounted with Entellan® (Merck). For each sample, at least 200 cells were randomly counted using an Olympus BX41 microscope. The results are shown as phagocytic indices, which were calculated as follows: [percentage of macrophages with ingested parasites] × [mean number of intracellular parasites per macrophage].

NO production

Adherent cells were incubated with promastigotes and treated as previously described. After 24 h of treatment, the supernatants were collected, and NO production was evaluated indirectly (nitrite production) using Griess reagent, according to the methods described in Ding *et al*. (1988). At the end of the culture period, 50 μL of the supernatant was removed and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0·1% naphthalene diamine dihydrochloride, 2·5% H_3PO_4) at room temperature for 10 min. Absorbance was determined using a microplate spectrophotometer apparatus (BIO-RAD Model 450 Microplate reader) at 570 nm. The nitrite concentration was determined using sodium nitrite as the standard, and the results were expressed as μM. Control cells were activated using LPS and INF-γ at a concentration of 2 μg mL^{-1} and 0·01 ng mL^{-1}, respectively.

Morphometric analysis

We next evaluated the spreading area of macrophages that were infected with *L. amazonensis* and treated with CTX (2·4 and 4·8 μg mL^{-1}) for 24 h in an attempt to evaluate the phagocytic capacity of these cells after CTX treatment. Images of stained cells were acquired using a Carl Zeiss Axiophot A1. The area measurements and analyses were performed using ImageJ (NIH) as described (Sokol *et al*. 1987). Briefly, cell areas were measured from five random images obtained from each sample, and the mean areas were recorded.

Cytokine quantification

Cytokines present in the supernatants of *Leishmania*–macrophage interaction solutions, both treated and untreated, were quantified using cytometric bead array (CBA Th1/Th2/Th17 kit) technology, according to the manufacturer's instructions. Data were collected using a FACSCanto II flow cytometer with

FACSDiva (BD Biosciences, San Jose, CA, USA), and the results were analysed using a FCAP Array 3·0 and expressed in pg mL^{-1}, in comparison to the standard curve.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6·0. All experiments were performed with three independent experiments in triplicate. The results were tested using one-way analysis of variance with Tukey's *post hoc* tests and unpaired Student's *t*-tests. $P < 0{\cdot}05$ was considered significant.

Ethics statement

The study was carried out in strict accordance with the Brazilian animal protection law (Lei Arouca number 11·794/08) of the National Council for the Control of Animal Experimentation (CONCEA, Brazil). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Para (BIO053-12 CEPAE/ICB/UFPA).

The promastigotes of *L. amazonensis* used in this study were conceived by Evandro Chagas Institute, Pará, and its use in scientific research was approved by the institution. All samples were identified by strain number, ensuring the patients' anonymity.

RESULTS

Effect of CTX on macrophage viability and ROS production

Cells treated with CTX (0·3–9·6 $\mu g\ mL^{-1}$) for 48 h did not show signs of cytotoxicity, but at 1·2, 2·4 and 4·8 $\mu g\ mL^{-1}$, the cells demonstrated a significant increase in mitochondrial activity (Fig. 1). To evaluate ROS production, the CellRox® Green assay was performed and analysed by flow cytometry. Results demonstrated that macrophages treated with 2·4 and 4·8 $\mu g\ mL^{-1}$ CTX showed higher concentrations of ROS than the control group (1·34±0·13-fold and 1·38±0·13-fold, respectively; Fig. 2).

Evaluation of in vitro antileishmanial activity

Antipromastigote activity. The direct effect of CTX on *L. amazonensis* promastigotes was monitored for up to 96 h. Promastigotes treated with 4·8 $\mu g\ mL^{-1}$ CTX exhibited a significant decrease in their growth rate (24%, ±2·7), while those treated with 2·4 $\mu g\ mL^{-1}$ of CTX exhibited a 14·6% ±12·5 decrease in the growth of promastigotes. After 96 h of treatment, CTX was determined to have an $IC_{50} = 22{\cdot}86\ \mu g\ mL^{-1}$ (Fig. 3).

Antiamastigote activity. To analyse whether CTX stimulates *L. amazonensis* phagocytosis by

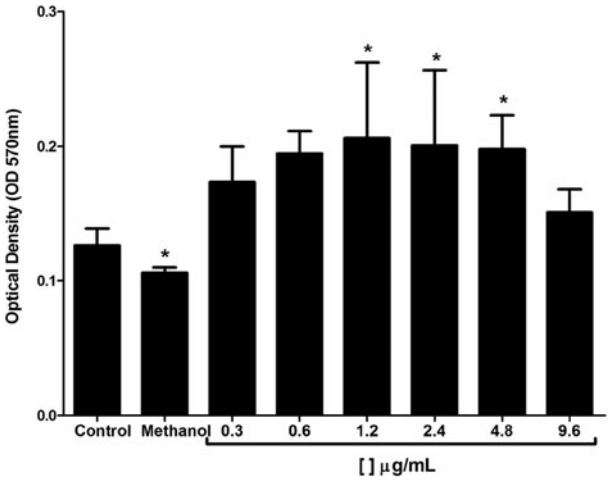

Fig. 1. Murine macrophage viability was determined using MTT assays after treatment with different concentrations of CTX for 48 h. Cells treated with 2·4 and 4·8 $\mu g\ mL^{-1}$ CTX demonstrated a significant increase in mitochondrial activity compared with the control group. Methanol was used as the negative control. $*P < 0{\cdot}05$, values are expressed as means ± S.D. and compared with the control group.

macrophages, phagocytic indices were calculated using cultured macrophages that were infected with *L. amazonensis* and treated with CTX (2·4 and 4·8 $\mu g\ mL^{-1}$) for 24 h. CTX administration resulted in an increased capacity to phagocytose promastigotes (2·85 ± 0·01-fold), compared with the untreated control group (Fig. 4A1–A3). However, to determine whether continuous treatment with CTX would eliminate the internalized parasites, treatment was performed for 48 h. Macrophages treated with CTX eliminated amastigotes once the survival index of the treated cells was reduced significantly in comparison with the untreated infected host cells (4·0 ± 2·47-fold and 5·33 ± 0·29-fold, respectively; Fig. 4B1–B3).

Morphometric analysis of macrophages infected with L. amazonensis *and treated with CTX*

After macrophages were infected with *L. amazonensis* and incubated with CTX (2·4 $\mu g\ mL^{-1}$) for 24 h, we observed a significant increase in the mean area, compared with the untreated infected group (1·21 ± 0·91-fold; Fig. 5).

CTX induces NO production in infected macrophages

NO production was also analysed in the infected macrophages because *L. amazonensis* is known to have an inhibitory effect against NO production by macrophages. In the positive control group, macrophages were incubated with LPS (1 $\mu g\ mL^{-1}$, Zhou *et al.* 2015). CTX reversed the inhibitory effect of infection after treatment with 2·4 and 4·8

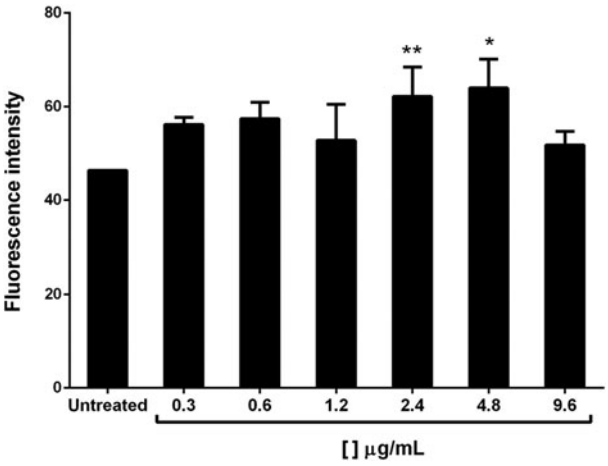

Fig. 2. A CellRox Green assay was used to analyse ROS production after macrophages were treated with different concentrations of CTX.*$P < 0{\cdot}05$; ** $P < 0{\cdot}01$, values are expressed as means ± S.D. compared with untreated group.

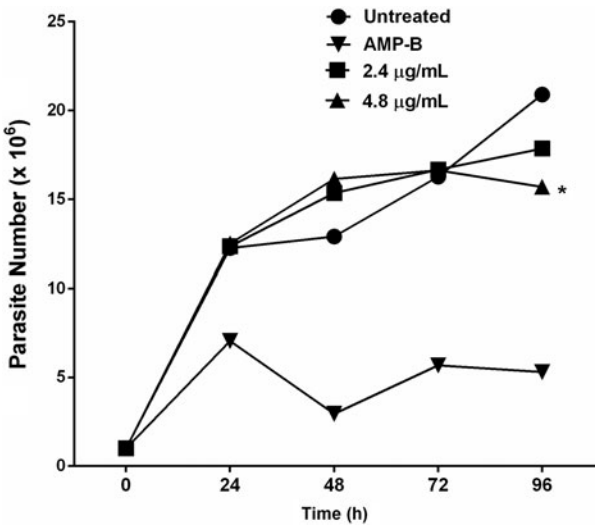

Fig. 3. Promastigote growth curve for *Leishmania amazonensis* that were treated with different concentrations of CTX for 96 h. AMP-B, amphotericin B. *$P < 0{\cdot}05$, values are compared with the untreated group.

μg mL^{-1} CTX for 24 h and increased NO production in these cells, compared with the control (1·17 ± 0·12-fold and 1·41 ± 0·23-fold, respectively; Fig. 6).

Cytokine production in infected macrophages treated with CTX

Cytokine secretion by treated macrophages that were infected with *L. amazonensis* was assessed using culture supernatants. The results obtained using flow cytometry demonstrated increased IL-6 (Fig. 7A) and TNF-α (Fig. 7B) levels in the macrophages treated with 2·4 μg mL^{-1} CTX (5·5-fold and 6·9-fold, respectively, compared with the control) and 4·8 μg mL^{-1} CTX (6·4-fold and 7·1-fold, respectively, compared with the control).

DISCUSSION

Leishmaniasis is a neglected disease, and available therapies present many disadvantages, including high toxicity and emerging drug resistance in the parasite, indicating that there is a need to develop novel compounds to treat these infections (Adade *et al*. 2014). Macrophages are crucial to the establishment and persistence of this parasitic infection (Podinovskaia and Descoteaux, 2015). Therefore, investigating new substances that show potential to improve the microbicidal and immune capacity of macrophages can be of substantial assistance in controlling the survival of the parasite. Few reports in the literature have described the use of CTX against *Leishmania* parasites or during interactions between the parasites and the host cell.

This study demonstrates that CTX did not display cytotoxicity in host cells, at the concentrations studied. Interestingly, macrophages seemed to exhibit an increase in mitochondrial activity following treatment. Based on this activity, we evaluated how CTX can modulate mitochondrial metabolism. Thus, ROS quantification was performed in host cells, and we found that CTX increased ROS production in macrophages after treatment. ROS increase oxidative stress and act as microbicidal molecules. In fact, CTX stimulated the maximal activity of hexokinase, glucose-6-phosphate dehydrogenase, citrate synthase and $^{14}CO_2$ production from [U-^{14}C]-glucose and [U-^{14}C]-glutamine in macrophages (Faiad, 2012), which led to an increase in NO or H_2O_2 production in these cells (Faiad, 2012; Costa *et al*. 2013). The importance of the activation of macrophage metabolism by other venoms during *Leishmania* infection was demonstrated in Bhattacharya *et al*. (2013), who showed that treating splenocytes with *Bungarus caeruleus* venom during a *Leishmania donovani* infection stimulated these cells to produce ROS, enabling the destruction of intracellular parasites.

One of the new findings of the present study was that CTX derived from *Cdt* displayed activity against *Leishmania* promastigotes. A significant decrease in the parasite growth rate was observed even when a low concentration of CTX was applied (IC_{50} = 22·86 μg mL^{-1}). Barros *et al*. (2015) demonstrated an IC_{50} of 52·07 μg mL^{-1} using PLA_2-derived *Cdt* against *Leishmania infantum chagasi*, and Torres *et al*. (2010) observed an IC_{50} of 86·56 μg mL^{-1} for crude venom derived from *Brothrops marajoensis*, while Muhammad *et al*. (2003) reported an IC_{50} of 0·17 μg mL^{-1} for amphotericin B. Several studies have demonstrated leishmanicidal effects by toxins derived from snakes, such as *B. caeruleus* toxin, which was

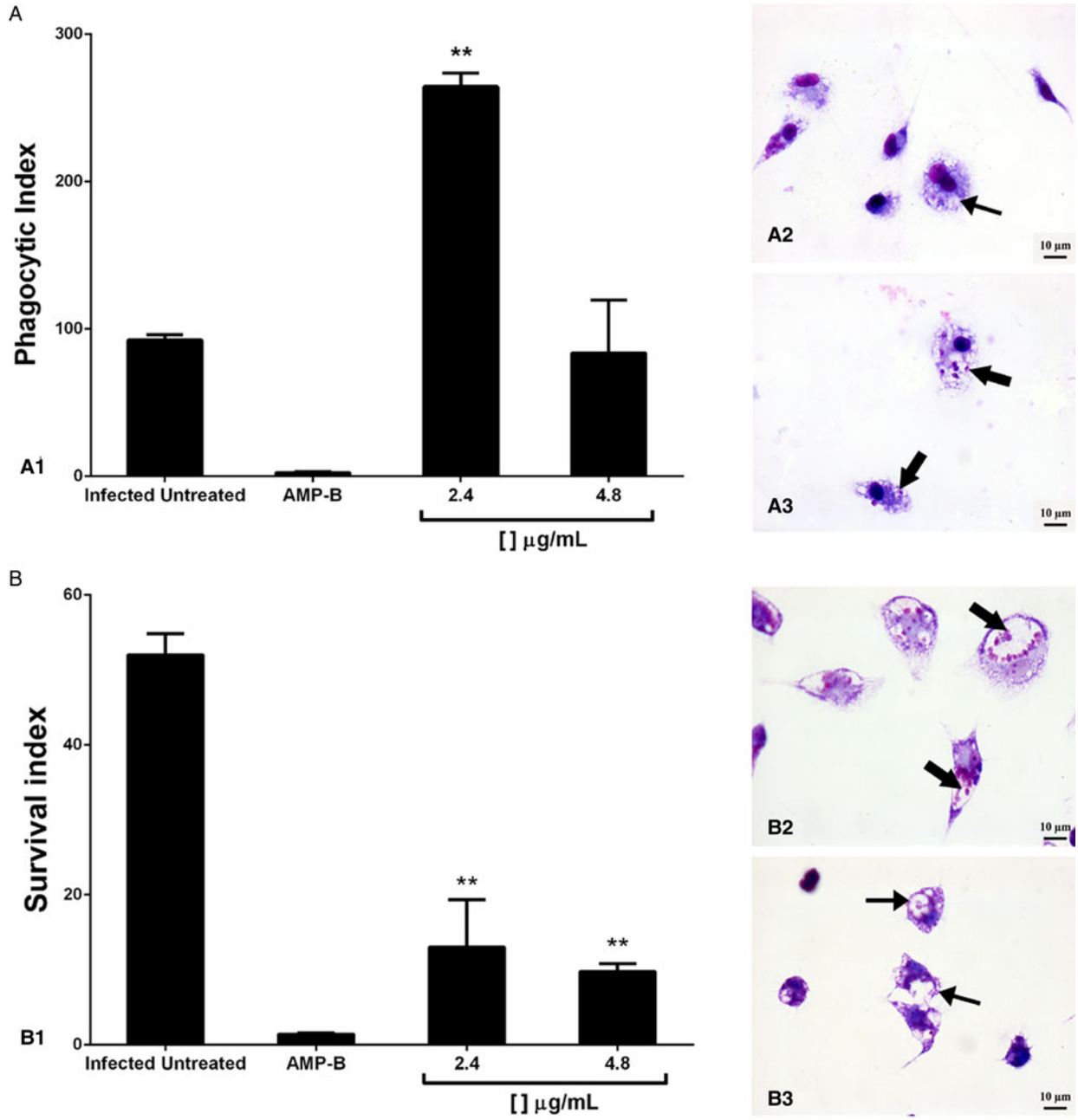

Fig. 4. Macrophages were infected with *Leishmania amazonensis* and treated with CTX for 24 h (A) and 48 h (B). (A1) The phagocytic index of host cells that were treated with CTX for 24 h. Note the significant increase in phagocytosis after treatment with 2·4 μg mL^{-1} CTX. (A2) and (A3) Giemsa staining of infected macrophages untreated (CTL) or treated with 2·4 μg mL^{-1} of CTX, respectively. Note the greater amount of amastigotes inside the macrophages (large arrows). (B1) The survival index of host cells that were treated with CTX for 48 h. (B2) and (B3) Giemsa staining of macrophages that were infected (CTL) or infected and treated with 2·4 μg mL^{-1} of CTX, respectively. $^{**}P < 0{\cdot}01$ indicates statistical significance compared with the control group; values are expressed as means ± S.D.

capable of reducing the population by up to 60% *in vitro*, and succeeded in eliminating the parasite in mice with visceral leishmaniasis (Bhattacharya *et al*. 2013). Recently, Nunes *et al*. (2013) used a fraction of *B. pauloensis* venom and observed a reduction in the growth of *L. amazonensis* promastigotes of 60–70%. These important findings provide good support for further studies of those toxins, since their activities are selective against parasites and may maintain host-cell viability.

As CTX was not toxic to host cells, and because it presented anti-promastigote activity, it was important to evaluate the activation profile that was induced by CTX during macrophage–*Leishmania* interactions. Our study demonstrated that macrophages treated with CTX were able to engulf a higher number of parasites after 24 h of treatment; moreover, CTX eliminated intracellular amastigotes after 48 h of treatment.

This stimulatory effect of CTX on phagocytic capacity differs from the inhibitory effect observed

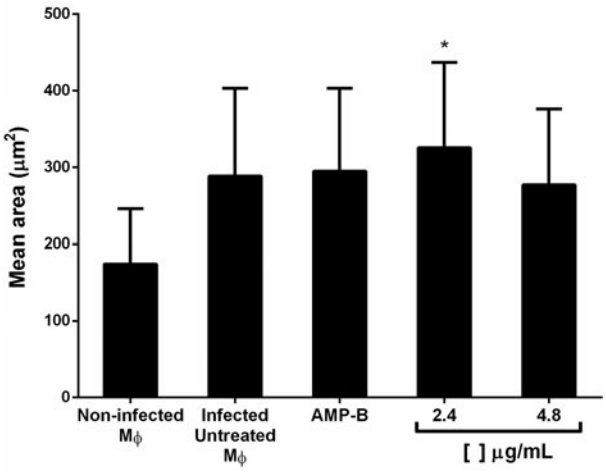

Fig. 5. Morphometric analysis demonstrating the significant increase in macrophage (Mϕ) mean areas after infected macrophages were treated with 2·4 μg mL^{-1} of CTX. *$P < 0{\cdot}05$, values are expressed as means ± S.D. and compared with the untreated group.

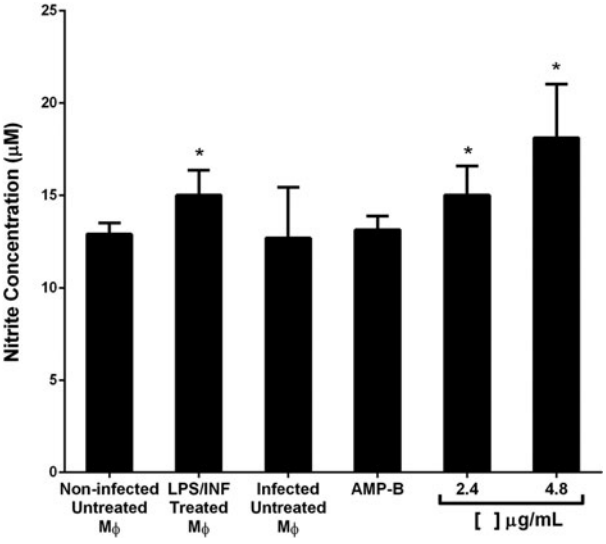

Fig. 6. Nitrite concentrations in macrophages that were infected with *Leishmania* and treated with CTX. NO production was observed indirectly by analysing nitrite concentrations and was found to be higher in the infected macrophages that were treated with CTX. Control cells were activated with LPS and INF-γ at concentrations of 2 μg mL^{-1} and 0·01 ng mL^{-1}, respectively. *$P < 0{\cdot}05$, values are expressed as means ± S.D. and compared with the untreated group.

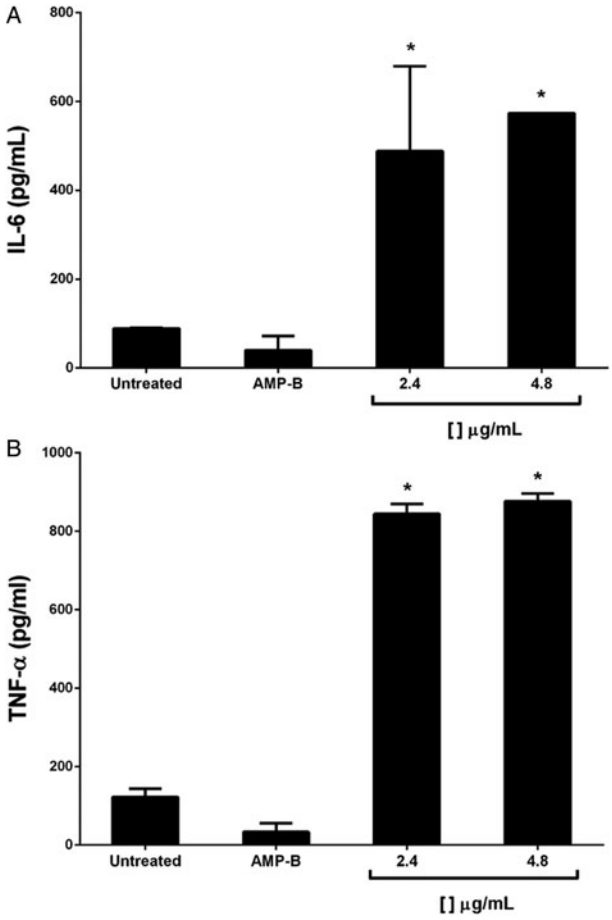

Fig. 7. Cytokine concentrations in macrophages that were infected with *Leishmania* and treated with CTX. Treated and infected macrophages produced a higher concentration of IL-6 (A) and TNF-α (B) compared with the untreated controls. *$P < 0{\cdot}05$, values are expressed as means ± S.D. and compared with the untreated group.

before (Sampaio *et al*. 2003, 2006*a*, *b*). Therefore, it is possible that incubating macrophages with parasites causes distinct signalling pathways to induce phagocytosis and the microbicidal activity that eliminates intracellular parasites. In fact, the initial interaction of the promastigote with the cells may trigger the release of intracellular survival factors by the parasite, which may subsequently modulate the phagocytic capacity of macrophages (Rotureau *et al*. 2009). In addition, it has been demonstrated that the inhibitory effect of CTX on macrophage spreading and phagocytic activities is caused by the PLA_2 subunit and that this inhibitory effect is not dependent on the type of receptor involved in the phagocytic process (Sampaio *et al*. 2005). In contrast, the present study demonstrated that, when added to the culture of macrophages infected with *Leishmania*, this toxin stimulates the phagocytic capacity of macrophages at 24 h. Costa *et al*. (2013) demonstrated that monoculture of macrophages treated with CTX present a decreased secretion of proinflammatory mediators, compatible with the anti-inflammatory profile described for this toxin. On the other hand, the secretion of proinflammatory mediators is enhanced by pretreated macrophages with CTX, when co-cultured with tumour cells, reinforcing the immunomodulatory effect described for this CTX. Therefore, we believe that the action of CTX on the phagocytic capacity of macrophages may be more associated with the

immunomodulatory activity of the CTX complex than the enzymatic activity of PLA_2. Different receptors are involved in the process of phagocytozing the parasite, including complement receptors, mannose receptors, fibronectin receptors and FCγ receptors. These receptors may be modulated according to inflammatory conditions, which may lead to the inhibition or activation of NADPH oxidase in the newly formed phagosome, which promotes promastigote clearance (Ueno and Wilson, 2012; Podinovskaia and Descoteaux, 2015). Thus, macrophage phagocytic receptors may involve a potentially distinct CTX-mediated modulatory action during their interactions with the parasite.

Studies using crude venom and toxins isolated from *Bothrops alternatus* have shown them to increase the phagocytic capacity of treated macrophages (Setubal *et al*. 2011). Interestingly, Furtado *et al*. (2014) obtained similar results when they treated J774A.1 macrophages with a *Bothrops atrox*-derived toxin. However, Macedo *et al*. (2015) studied the effect of crotamine, another toxin derived from *Cdt* venom, and did not observe any antileishmanial activity after treating infected peritoneal macrophages with up to 100 $\mu g\,mL^{-1}$ of the toxin. Thus, the higher phagocytic capacity induced by CTX in macrophages that were challenged with *Leishmania* parasites after 24 h appears to induce the destruction of the intracellular amastigotes after 48 h of treatment, and this is directly related to the development of an effective immune response. Interestingly, Costa *et al*. (2013) demonstrated that macrophages, after 24 h in medium containing CTX, demonstrated a significant increase in the secretion of lipoxin A_4 (LXA_4) and its stable analogue, 15-epi-LXA_4. However, the anti-inflammatory activity of these lipid mediators (LXs) did not compromise the ability of INF-γ-stimulated macrophages to kill intracellular parasites or to upregulate inducible nitric oxide synthase or NO synthesis, which are key antimicrobial mechanisms of macrophages. These results suggest that the LXs and their analogues are immunomodulatory rather than immunosuppressive agents (Aliberti *et al*. 2002*a*, *b*).

In fact, the antileishmanial activity induced by host cell activation is extremely important because there has been an increase in the number of parasites that are resistant to the drugs currently available to treat leishmaniasis (Fernández *et al*. 2014). Moreover, it is well documented that *L. amazonensis* can inactivate cellular immune responses, which is an important peculiarity of this protozoa infection and can cause anergic immune responses (Silveira *et al*. 2009). In the current study, during infection, we observed that treated macrophages presented larger cell areas, indicating that they have undergone morphological changes suggestive of cell activation. Furthermore, to understand how murine macrophages might eliminate *Leishmania* amastigotes, we evaluated NO production and cytokine profiles. CTX induced an increase in the production of NO, even when macrophages were challenged with *L. amazonensis*, and was able to induce macrophages to switch to a *M1* profile during *Leishmania* infection, presenting higher levels of IL-6 and TNF-α. The *M1* profile is known to be involved in cellular responsiveness, as characterized by analyses of NO and proinflammatory cytokine production, such as IL-6 and TNF-α. The *M2* profile represents the anti-inflammatory response, in which there is an increase in the production of a different set of cytokines, such as IL-10 and IL-4. This polarization profile is very beneficial to the host, and it helps to combat *Leishmania* infection and eliminate the parasite. Therefore, the data obtained in this study indicate the selective action of CTX on macrophage metabolism. Another study using crotamine isolated from *Cdt* venom also demonstrated that a higher concentration of TNF-α was observed in *Leishmania*-infected macrophages compared with the untreated and infected controls (Macedo *et al*. 2015). These data indicate that the *Cdt* venom or its isolates exhibit immunomodulatory activity.

The modulation of the macrophage response against *Leishmania*, caused by CTX, indicates a classical *M1* activation profile, which is characterized by the increased production of NO and ROS, as well as proinflammatory cytokines such as IL-6 and TNF-α, which were observed in this study (Liu *et al*. 2014; Rath *et al*. 2014; Venturin *et al*. 2016). In addition to these biochemical changes, the morphological changes, characteristic of the *M1* profile of activation, such as cell spreading and an increase in cytoplasmic projections become evident in these cells (Cassado *et al*. 2011).

To our knowledge, this report shows, for the first time, that CTX isolated from *Cdt* venom induces leishmanicidal activity against intracellular parasites and substantial activation of infected macrophages. Thus, CTX is a promising and potent antileishmanial agent and a potentially potent immunomodulatory compound that is able to induce macrophages to polarize to the *M1* type during *Leishmania* infection, leading to the elimination of intracellular parasites.

ACKNOWLEDGEMENTS

We are grateful to Instituto Evandro Chagas and Instituto Butantan.

FINANCIAL SUPPORT

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), FAPESPA (ICAAF No. 161/2014), FADESP, PROPESP-UFPA and Instituto Nacional de Biologia Estrutural e Bioimagem-INBEB (CNPq, grant number 573767/2008-4).

REFERENCES

Adade, C. M., Carvalho, A. L. O., Tomaz, M. A., Costa, T. F. R., Godinho, J. L., Melo, P. A., Lima, A. P. C. A., Rodrigues, J. C. F., Zingali, R. B. and Souto-Padrón, T. (2014). Crovirin, a snake venom cysteine-rich secretory protein (CRISP) with promising activity against trypanosomes and *Leishmania*. *PLoS Neglected Tropical Diseases* **8**, e3252.

Aliberti, J., Serhan, C. and Sher, A. (2002*a*). Parasite-induced lipoxin A4 is an endogenous regulator of IL-12 production and immunopathology in *Toxoplasma gondii* infection. *Journal of Experimental Medicine* **196**, 1253–1262.

Aliberti, J., Hieny, S., Reis e Sousa, C., Serhan, C. N. and Sher, A. (2002*b*). Lipoxin-mediated inhibition of IL-12 production by DCs: a mechanism for regulation of microbial immunity. *Nature Immunology* **3**, 76–82.

Barros, G. A. C., Pereira, A. V., Barros, L. C., Jr, A. L., Calvi, S. A., Santos, L. D., Barraviera, B. and Ferreira, R. S. (2015). *In vitro* activity of phospholipase A2 and of peptides from *Crotalus durissus terrificus* venom against amastigote and promastigote forms of *Leishmania* (*L.*) *infantum chagasi*. *Journal of Venomous Animals and Toxins Including Tropical Diseases* **21**, 48.

Bashir, S., Sharma, Y., Elahi, A. and Khan, F. (2016). Macrophage polarization: the link between inflammation and related diseases. *Inflammation Research* **65**, 1–11.

Bhattacharya, S., Ghosh, P., De, T., Gomes, A., Gomes, A. and Dungdung, S. R. (2013). *In vivo* and *in vitro* antileishmanial activity of *Bungarus caeruleus* snake venom through alteration of immunomodulatory activity. *Experimental Parasitology* **135**, 126–133.

Butler, M. S. (2008). Natural products to drugs: natural product-derived compounds in clinical trials. *Natural Product Reports* **25**, 475.

Cassado, A. D. A., de Albuquerque, J. A. T., Sardinha, L. R., Buzzo, C. D. L., Faustino, L., Nascimento, R., Ghosn, E. E. B., Lima, M. R. D., Alvarez, J. M. M. and Bortoluci, K. R. (2011). Cellular renewal and improvement of local cell effector activity in peritoneal cavity in response to infectious stimuli. *PLoS ONE* **6**, e22141.

Costa, E. S., Faiad, O. J., Landgraf, R. G., Ferreira, A. K., Brigatte, P., Curi, R., Cury, Y. and Sampaio, S. C. (2013). Involvement of formyl peptide receptors in the stimulatory effect of crotoxin on macrophages co-cultivated with tumour cells. *Toxicon* **74**, 167–178.

de Moura, A. A., Kayano, A. M., Oliveira, G. A., Setúbal, S. S., Ribeiro, J. G., Barros, N. B., Nicolete, R., Moura, L. A., Fuly, A. L., Nomizo, A., da Silva, S. L., Fernandes, C. F. C., Zuliani, J. P., Stábeli, R. G., Soares, A. M. and Calderon, L. A. (2014). Purification and biochemical characterization of three myotoxins from *Bothrops mattogrossensis* snake venom with toxicity against *Leishmania* and tumor cells. *BioMed Research International* **2014**, 1–13.

de Vries, H. J. C., Reedijk, S. H. and Schallig, H. D. F. H. (2015). Cutaneous leishmaniasis: recent developments in diagnosis and management. *American Journal of Clinical Dermatology* **16**, 99–109.

Ding, A. H., Nathan, C. F. and Stuehr, D. J. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediate from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *Journal of Immunology* **141**, 2407.

Faiad, O. J. (2012). Efeito da Crotoxina Sobre Função e o Metabolismo de Glicose e Glutamina de Macrófagos Durante a Progressão Tumoral. Master's Dissertation, USP, São Paulo. doi: 10.11606/D.42.2012.tde-18042013-085640.

Fernández, O. L., Diaz-Toro, Y., Ovalle, C., Valderrama, L., Muvdi, S., Rodríguez, I., Gomez, M. A. and Saravia, N. G. (2014). Miltefosine and antimonial drug susceptibility of *Leishmania Viannia* species and populations in regions of high transmission in Colombia. *PLoS Neglected Tropical Diseases* **8**, e2871.

Fotakis, G. and Timbrell, J. A. (2006). *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters* **160**, 171–177.

Furtado, J. L., Oliveira, G. A.., Pontes, A. S., Setúbal, S. D. S., Xavier, C. V., Lacouth-Silva, F., Lima, B. F., Zaqueo, K. D., Kayano, A. M., Calderon, L. A., Stábeli, R. G., Soares, A. M. and Zuliani, J. P. (2014). Activation of J77A.1 macrophages by three phospholipases A2 isolated from *Bothrops atrox* snake venom. *BioMed Research International* **2014**, 13.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Liu, Y.-C., Zou, X.-B., Chai, Y.-F. and Yao, Y.-M. (2014). Macrophage polarization in inflammatory diseases. *International Journal of Biological Sciences* **10**, 520–529.

Lôbo de Araújo, a. and Radvanyi, F. (1987). Determination of phospholipase A2 activity by a colorimetric assay using a pH indicator. *Toxicon* **25**, 1181–1188.

Macedo, S. R. A., de Barros, N. B., Ferreira, A. S., Moreira-Dill, L. S., Calderon, L. A., Soares, A. M. and Nicolete, R. (2015). Biodegradable microparticles containing crotamine isolated from *Crotalus durissus terrificus* display antileishmanial activity *in vitro*. *Pharmacology* **95**, 78–86.

Marcinkiewicz, C. (2013). Applications of snake venom components to modulate integrin activities in cell–matrix interactions. *International Journal of Biochemistry & Cell Biology* **45**, 1974–1986.

McGwire, B. S. and Satoskar, A. R. (2014). Leishmaniasis: clinical syndromes and treatment. *Qjm* **107**, 7–14.

Mosser, D. M. (2003). The many faces of macrophage activation. *Journal of Leukocyte Biology* **73**, 209–212.

Muhammad, I., Dunbar, D. C., Khan, S. I., Tekwani, B. L., Bedir, E., Takamatsu, S., Ferreira, D. and Walker, L. A. (2003). Antiparasitic alkaloids from *Psychotria klugii*. *Journal of Natural Products* **66**, 962–967.

Nunes, D. C. O., Figueira, M. M. N. R., Lopes, D. S., De Souza, D. L. N., Izidoro, L. F. M., Ferro, E. A. V., Souza, M. A., Rodrigues, R. S., Rodrigues, V. M. and Yoneyama, K. A. G. (2013). BnSP-7 toxin, a basic phospholipase A2 from *Bothrops pauloensis* snake venom, interferes with proliferation, ultrastructure and infectivity of *Leishmania* (*Leishmania*) *amazonensis*. *Parasitology* **140**, 844–854.

Passero, L. F. D., Tomokane, T. Y., Corbett, C. E. P., Laurenti, M. D. and Toyama, M. H. (2007). Comparative studies of the anti-leishmanial activity of three *Crotalus durissus* ssp. venoms. *Parasitology Research* **101**, 1365–1371.

Passero, L. F. D., Laurenti, M. D., Tomokane, T. Y., Corbett, C. E. P. and Toyama, M. H. (2008). The effect of phospholipase A2 from *Crotalus durissus collilineatus* on *Leishmania* (*Leishmania*) *amazonensis* infection. *Parasitology Research* **102**, 1025–1033.

Peichoto, M. E., Tavares, F. L., DeKrey, G. and Mackessy, S. P. (2011). A comparative study of the effects of venoms from five rear-fanged snake species on the growth of *Leishmania major*: identification of a protein with inhibitory activity against the parasite. *Toxicon* **58**, 28–34.

Pereira, B. A. S. and Alves, C. R. (2008). Immunological characteristics of experimental murine infection with *Leishmania* (*Leishmania*) *amazonensis*. *Veterinary Parasitology* **158**, 239–255.

Podinovskaia, M. and Descoteaux, A. (2015). *Leishmania* and the macrophage: a multifaceted interaction. *Future Microbiology* **10**, 111–129.

Quintana, J. C., Chacón, A. M., Vargas, L., Segura, C., Gutiérrez, J. M. and Alarcón, J. C. (2012). Antiplasmodial effect of the venom of *Crotalus durissus cumanensis*, crotoxin complex and Crotoxin B. *Acta Tropica* **124**, 126–132.

Rangel-Santos, A., Dos-Santos, E., Lopes-Ferreira, M., Lima, C., Cardoso, D. and Mota, I. (2004). A comparative study of biological activities of crotoxin and CB fraction of venoms from *Crotalus durissus terrificus*, *Crotalus durissus cascavella* and *Crotalus durissus collilineatus*. *Toxicon* **43**, 801–810.

Rath, M., Müller, I., Kropf, P., Closs, E. I. and Munder, M. (2014). Metabolism *via* arginase or nitric oxide synthase: two competing arginine pathways in macrophages. *Frontiers in Immunology* **5**, 1–10.

Rotureau, B., Morales, M. a., Bastin, P. and Späth, G. F. (2009). The flagellum-mitogen-activated protein kinase connection in Trypanosomatids: a key sensory role in parasite signalling and development? *Cellular Microbiology* **11**, 710–718.

Sampaio, S., Brigatte, P., Sousa-e-Silva, M. C., dos-Santos, E., Rangel-Santos, A., Curi, R. and Cury, Y. (2003). Contribution of crotoxin for the inhibitory effect of *Crotalus durissus terrificus* snake venom on macrophage function. *Toxicon* **41**, 899–907.

Sampaio, S. C., Rangel-Santos, A. C., Peres, C. M., Curi, R. and Cury, Y. (2005). Inhibitory effect of phospholipase A2 isolated from *Crotalus durissus terrificus* venom on macrophage function. *Toxicon* **45**, 671–676.

Sampaio, S. C., Santos, M. F., Costa, E. P., Rangel-Santos, A. C., Carneiro, S. M., Curi, R. and Cury, Y. (2006*a*). Crotoxin induces actin reorganization and inhibits tyrosine phosphorylation and activity of small GTPases in rat macrophages. *Toxicon* **47**, 909–919.

Sampaio, S. C., Alba-Loureiro, T. C., Brigatte, P., Landgraf, R. G., dos Santos, E. C., Curi, R. and Cury, Y. (2006*b*). Lipoxygenase-derived eicosanoids are involved in the inhibitory effect of *Crotalus durissus terrificus* venom or crotoxin on rat macrophage phagocytosis. *Toxicon* **47**, 313–321.

Schepetkin, I. A., Khlebnikov, A. I., Kirpotina, L. N. and Quinn, M. T. (2015). Antagonism of human formyl peptide receptor 1 with natural compounds and their synthetic derivatives. *International Immunopharmacology*.

Setubal, S. S., Pontes, A. S., Furtado, J. L., Kayano, A. M., Stábeli, R. G. and Zuliani, J. P. (2011). Effect of *Bothrops alternatus* snake venom on macrophage phagocytosis and superoxide production: participation of protein kinase C. *Journal of Venomous Animals and Toxins including Tropical Diseases* **17**, 430–441.

Silveira, F. T., Lainson, R., Gomes, C. M. C., Laurenti, M. D. and Corbett, C. E. P. (2009). Immunopathogenic competences of *Leishmania* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* in American cutaneous leishmaniasis. *Parasite Immunology* **31**, 423–431.

Sokol, R. J., Hudson, G., James, N. T., Frost, I. J. and Wales, J. (1987). Human macrophage development: a morphometric study. *Journal of Anatomy* **151**, 27–35.

Torres, A. F. C., Dantas, R. T., Toyama, M. H., Filho, E. D., Zara, F. J., Rodrigues de Queiroz, M. G., Pinto Nogueira, N. A., Rosa de Oliveira, M., de Oliveira Toyama, D., Monteiro, H. S. A. and Martins, A. M. C. (2010). Antibacterial and antiparasitic effects of *Bothrops marajoensis* venom and its fractions: phospholipase A2 and l-amino acid oxidase. *Toxicon* **55**, 795–804.

Ueno, N. and Wilson, M. E. (2012). Receptor-mediated phagocytosis of *Leishmania*: implications for intracellular survival. *Trends in Parasitology* **28**, 335–344.

Venturin, G. L., Chiku, V. M., Silva, K. L. O., de Almeida, B. F. M. and de Lima, V. M. F. (2016). M1 polarization and the effect of PGE_2 on TNF-α production by lymph node cells from dogs with visceral leishmaniasis. *Parasite Immunology* **38**, 698–704.

Xuan, W., Qu, Q., Zheng, B., Xiong, S. and Fan, G.-H. (2015). The chemotaxis of M1 and M2 macrophages is regulated by different chemokines. *Journal of Leukocyte Biology* **97**, 61–69.

Zhou, Y., Zhang, T., Wang, X., Wei, X., Chen, Y., Guo, L., Zhang, J. and Wang, C. (2015). Curcumin modulates macrophage polarization through the inhibition of the toll-like receptor 4 expression and its signaling pathways. *Cellular Physiology and Biochemistry* **36**, 631–641.