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Involvement of formyl peptide receptors in the stimulatory effect of crotoxin on macrophages co-cultivated with tumour cells



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ABSTRACT

Crotoxin (CTX) is the main neurotoxic component of *Crotalus durissus terrificus* snake venom. It inhibits tumour growth and modulates the function of macrophages, which are essential cells in the tumour microenvironment. The present study investigated the effect of CTX on the secretory activity of monocultured macrophages and macrophages co-cultivated with LLC-WRC 256 cells. The effect of the macrophage secretory activities on tumour cell proliferation was also evaluated. Macrophages pre-treated with CTX (0.3 µg/mL) for 2 h were co-cultivated with LLC-WRC 256 cells, and the secretory activity of the macrophages was determined after 12, 24 and 48 h. The co-cultivation of CTX-treated macrophages with the tumour cells caused a 20% reduction in tumour cell proliferation. The production of both H₂O₂ and NO was increased by 41% and 29% after 24 or 48 h of co-cultivation, respectively, compared to the values for the co-cultures of macrophages of control. The level of secreted IL-1β increased by 3.7- and 3.2-fold after 12 h and 24 h of co-cultivation, respectively. Moreover, an increased level of LXA₄ (25%) was observed after 24 h of co-cultivation, and a 2.3- and 2.1-fold increased level of 15-epi-LXA₄ was observed after 24 h and 48 h, respectively. Boc-2, a selective antagonist of formyl peptide receptors, blocked both the stimulatory effect of CTX on the macrophage secretory activity and the inhibitory effect of these cells on tumour cell proliferation. Taken together, these results indicate that CTX enhanced the secretory activity of macrophages, which may contribute to the antitumour activity of these cells, and that activation of formyl peptide receptors appears to play a major role in this effect.

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1. Introduction

Macrophages play a critical role in a host's defense against cancer. Several studies have demonstrated that when monocytes/macrophages are activated under *in vitro*

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or *in vivo* conditions, they are able to lyse tumour cells. Macrophages exist in two distinct polarisation states, as classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Mantovani et al., 1992; Gordon, 2003). In the initial stage of tumour progression, M1 macrophages release compounds that are cytotoxic to cancer cells, such as reactive nitrogen/oxygen intermediates, tumour necrosis factor α (TNF- α), IL-1 β and IL-6 (Roitt and Delves, 1992). The reactive oxygen species (ROS) that are formed during the respiratory burst of the mononuclear phagocytes have been implicated in the mechanism of killing tumour cells. In addition, ROS act as signalling molecules to induce the production of IL-1 β and the expression of inducible nitric oxide (iNOS) (Song et al., 2002). Nitric oxide (NO) has been shown to be toxic to tumour cells via mitochondrial damage, inhibition of DNA synthesis and disruption of the flux of substrates through the tricarboxylic acid cycle (Hibbs et al., 1988; Lancaster and Hibbs, 1990; Pellar et al., 1990). The production of IL-6 and TNF- α , which have a regulatory effect on tumour growth, has been implicated as one of the cytostatic/cytocidal factors in the direct anti-tumour activity of the activated macrophages (Hamilton and Adams, 1987; Lewis and McGee, 1992; Paulnock, 1992; Arinaga et al., 1992).

During tumour progression, the secretory activities of these macrophages may become altered, resulting in their being unable to lyse tumour cells (Mosmann et al., 1986; Mantovani et al., 2004, 2005; Sica et al., 2008; for review). Additionally, the lipoxygenase pathway is inhibited in macrophages upon their contact with tumour cells (Calorini et al., 2005). The inhibitory effect of tumour cells on the lipoxygenase activity of macrophages might be important for tumour progression because the lipoxygenase products, such as the lipoxins (LXs) and their analogues, are lipid mediators with anti-angiogenic and anti-tumour activities (Fierro et al., 2002; Hao et al., 2011). LXs are eicosanoids produced from arachidonic acid via the 5-lipoxygenase (5-LO) and 15-lipoxygenase (15-LO) pathways (Serhan et al., 1984) that are involved in a range of physiological and pathophysiological conditions (Serhan et al., 1995). LXA₄ and LXB₄ are the main LXs produced in mammals. The acetylation of cyclooxygenase-2 (COX-2) by aspirin (Serhan et al., 1995), or in the absence of aspirin, via S-nitrosylation of COX-2 (Birnbaum et al., 2006), or P450-derived 15R-HETE that is substrate for leucocyte 5-LO (Clària et al., 1996), lead to the transcellular biosynthesis of 15-epi-lipoxins (ATL). Released ATL, in particular the 15-epi-LXA₄ form, has more potent and longer acting effects than does the native 15S-containing LX form because it is less rapidly inactivated (Serhan et al., 1995; Serhan, 2005; for review). The native LXs and their natural analogue 15-epi-LXA₄ modulate inflammation-related signals and may play a role in regulating the genesis and development of tumours (Serhan, 2005; Li et al., 2008) and exert their effects via binding to G-protein-coupled LXA₄ receptor (ALXR, also termed FRL1) (Fiore et al., 1994; Ye and Boulay, 1997; Rabiet et al., 2007).

CTX displays an antitumour effect, reducing tumour growth both *in vivo* and *in vitro* (Newman et al., 1993; Donato et al., 1996; Cura et al., 2002; Sampaio et al., 2010 for review).

Crotoxin (CTX), the main toxic component of the venom of the South American rattlesnake *Crotalus durissus terrificus*, is a heterodimeric complex consisting of the basic and toxic phospholipase A₂ and an acidic, non-toxic, nonenzymatic component named crotapotin (Slotta and Frankel-Conrat, 1938; Bon et al., 1988). In addition to its *in vivo* anti-tumour activity, CTX, administered intramuscularly daily, inhibited the growth of Lewis lung carcinoma and MX-1 human mammary carcinomas (Newman et al., 1993; Donato et al., 1996; Cura et al., 2002). Five days of treatment with CTX significantly inhibited the growth of tumours in rat paws (Brigatte, 2005). The inhibitory effect of the toxin on tumour growth is abolished by pretreatment with Boc-2, a selective antagonist of the formyl peptide receptor (Faiad et al., 2008).

The immunomodulatory effect of *C. durissus terrificus* venom (CdtV) is retained by its major toxin, CTX, and by the isolated subunits of CTX (CA and CB) (Sampaio et al., 2010 for review). In addition, peritoneal macrophages incubated with CTX released higher LXA₄ levels than did non-treated cells (Sampaio et al., 2006b). A single dose of subcutaneously administered CTX promoted an increase of H₂O₂ release, NO production and IL-1 β and TNF- α secretion by the peritoneal macrophages obtained from Walker 256 tumour-bearing rats fourteen days later. Concomitantly, a reduction in tumour size was observed (Costa et al., 2010).

Despite the intriguing results described above, the effect of CTX on the secretory activity of peritoneal macrophages in a tumour microenvironment has not been determined. The present study investigated the following issues: 1) the effect of CTX on the secretory activity of macrophages co-cultured with LLC-WRC 256 cells, 2) the effect of CTX on tumour cell proliferation and 3) the possible involvement of formyl peptide receptors in the actions of the toxin.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 160–180 g were used in this study. All the procedures were performed in accordance with the guidelines for animal experimentation, and the Ethical Committee for the Use of Animals of the Butantan Institute approved the protocol (CEUAIB, protocol number 631/09).

2.2. Crotoxin

Lyophilised venom of *C. durissus terrificus* was obtained from the Laboratory of Herpetology, Butantan Institute, São Paulo, Brazil, and stored at –20 °C. Crude venom solution was subjected to anion-exchange chromatography as previously described by Rangel-Santos et al. (2004), using a Mono-Q HR 5/5 column in an FPLC system (Pharmacia, Uppsala, Sweden). The fractions (1 ml/min) were eluted using a linear gradient of NaCl (0–1 mol/L in 50 mM Tris–HCl, pH 7.0). Three peaks (p1, p2 and p3) were obtained: p2 corresponded to the pure CTX fraction (about 60% of the crude venom); peaks 1 and 3 included the other CdtV toxins. Prior to pooling, the fractions containing CTX were tested for homogeneity by non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis

(12.5%) (Laemmli, 1970) and the phospholipase A₂ activity was assessed by a colourimetric assay using a synthetic chromogenic substrate (Lobo-de-Araujo & Radvanyi, 1987).

2.3. Peritoneal cell preparation

The animals were euthanised in a CO₂ chamber, and the peritoneal cavity was opened. The peritoneal cavity was washed with 10 ml of cold phosphate-buffered saline (PBS), pH 7.4. After a gentle massage of the abdominal wall, the peritoneal fluid containing the resident macrophages was collected. The number of total peritoneal cells was determined using a Neubauer's chamber, and differential counts were performed on smears stained with a panchromatic dye (Rosenfeld, 1971). Samples from individual animals were used for all the measurements. The assays were always performed in duplicate.

2.4. Tumour cell culture

The cell line used was a carcinoma cell line, the LLC-WRC 256 rat Walker tumour line established by Hull (1953), which was obtained from a repository of animal cell cultures in the Dunn School of Pathology, Oxford University, UK. The cells were grown in minimum essential medium (MEM) supplemented with 10% foetal calf serum and antibiotics (penicillin 50 U/ml and streptomycin 50 µg/ml) in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For the experiments, the cells were seeded at a density of 5×10^4 cells/mL in the medium described above.

2.5. Co-culture conditions and treatment with CTX and Boc-2

The co-culture procedure that was used was based on the method described by Hauptmann et al. (1993). Co-cultures were established in 96-well tissue culture plates to determine the hydrogen peroxide (H₂O₂) liberation and nitric oxide (NO) production and to assess tumour cell proliferation. LLC-WRC 256 tumour cells (1×10^4 /100 µL per well) were allowed to adhere for 3 h, washed with PBS and incubated with fresh medium for 24 h. The adherent peritoneal macrophages (2×10^5) were pre-treated with CTX (0.3 µg/mL) for 2 h, washed, collected and transferred to a 96-well tissue culture plate containing 2×10^4 tumour cells per well. The macrophage cultures and co-cultures were maintained for 24 and 48 h at 37 °C in a 5% CO₂ humidified atmosphere. To determine the production of IL-1β, TNF-α, IL-6, LXA₄ and 15-epi-LXA₄, the adherent peritoneal macrophages (5×10^5) were pre-treated with CTX (0.3 µg/mL) for 2 h, washed, collected and transferred to a 24-well plate containing LLC-WRC 256 tumour cells (5×10^4 per well) plated in fresh medium 24 h beforehand. The macrophage cultures and co-cultures were maintained for 12, 24 and 48 h at 37 °C in a 5% CO₂ humidified atmosphere. This resulted in a macrophage:tumour ratio of 10:1. All the experiments were performed in triplicate, with macrophages from three different donors. The concentration of CTX (0.3 µg/mL) was the same as that used in previous research (Sampaio et al., 2003, 2006a,b), which did not exhibit cytotoxicity as assessed by Trypan blue exclusion and by flow cytometry for the exclusion of propidium iodide.

The involvement of the formyl peptide receptor (ALX or FPR1) in the stimulatory effect of CTX on the secretory activities of macrophages was evaluated in cells pre-treated with 100 µM of Boc-2 (butoxycarbonyl-Phe-Leu-Phe-Leu-Phe, Phoenix Pharmaceutical Inc, USA), a selective antagonist of formyl peptide receptors, for 15 min at 37 °C (Scannell et al., 2007) before incubation with CTX, as described above.

2.6. Hydrogen peroxide production

The production of H₂O₂ was measured as described by Pick et al. (1981), using phenol red. This assay is based on a horseradish peroxidase-dependent conversion of phenol red into a coloured compound by H₂O₂. A phenol red solution (PRS) containing 140 mM NaCl; 10 mM potassium-phosphate buffer, pH 7.0; 5 mM dextrose; 0.28 mM phenol red; and 8.5 U/mL of horseradish peroxidase was used for the H₂O₂ determination. A final volume of 7.4 mL was obtained using Hank's solution. After 24 h of co-culture, the supernatants were collected, and 100 µL of phenol red solution was added into each well of 96-well flat-bottomed tissue culture plates (Corning, NY), which were incubated in a humidified atmosphere at 37 °C for 1 h. Vertical row no. 1, which lacked cells, was filled with 100 µL of PRS per well. The second and third vertical rows were used to establish the H₂O₂ standard curves. These wells were filled with 100 µL of PRS, to which was added 10 µL of a H₂O₂ solution, resulting in a final concentration of H₂O₂ ranging from 5 to 40 µM. The subsequent rows contained 100 µL of PRS without (basal H₂O₂ production) or with phorbol myristate acetate (100 ng). After 60 min of incubation at 37 °C, the reaction was stopped by the addition of 10 µL of a 1 N NaOH solution. The hydrogen peroxide-dependent phenol red oxidation was spectrophotometrically measured at 620 nm using a Titertek Multiscan apparatus. The concentration of H₂O₂ was calculated from the absorbance measurements and expressed as nanomoles of H₂O₂ per 2×10^5 cells.

2.7. Nitric oxide production

To determine the nitric oxide production, nitrite was measured in the supernatants of cultures or co-cultures based on the method described by Ding et al. (1988). At the end of the culture period, 50 or 100 µL of the supernatant was removed and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, 2.5% H₃PO₄) at room temperature for 10 min. The absorbance was determined using a Titertek Multiscan apparatus at 550 nm. The nitrite concentration was determined by using sodium nitrite as the standard. Cell-free medium contained 0.2–0.3 nmol of NO₂⁻/well; this value was determined in each experiment and subtracted from that obtained with cells.

2.8. Proliferation assay

The proliferation of tumour cells was assessed using the 3-(4,5-dimethylthiazol-2-thiazyl)-2,5-diphenyl-tetrazolium bromide (MTT, Amresco[®]) assay, based on the method

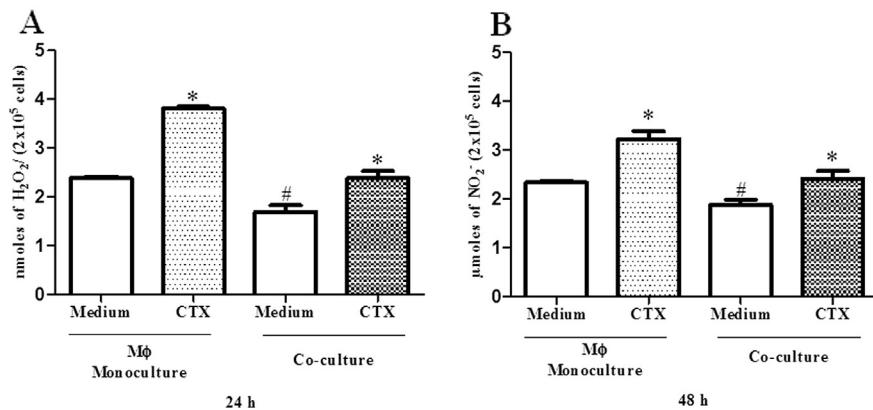


Fig. 1. Effect of CTX on Hydrogen Peroxide and Nitrite Production by Macrophages monoculture and macrophages co-cultivated with LLC-WRC 256 tumour cells. Macrophages ($M\phi$) were obtained from the peritoneal cavity of rats (resident), treated with CTX (0.3 μ g/mL) or RPMI 1640 Medium (control), at 37 °C for 2 h. After this period, the cells were cultivated in 96-well tissue culture plate or co-cultivated 96-well tissue culture plate containing tumour cell. (A) After 24 h, hydrogen peroxide production (nmoles $H_2O_2/2 \times 10^5$ cells) was determined in the presence of PMA. (B) For NO_2^- production, the results are expressed as micromoles of NO_2^- by 2×10^5 cells for 48 h. The results are expressed as means \pm SEM of values obtained from three different experiments performed with duplicate. * $P < 0.05$, significantly different from control group of co-culture. # $P < 0.05$, significantly different from control group of $M\phi$ monoculture.

described by Mossmann (1983) and Zhong et al. (2008). Cultured and co-cultured macrophages were maintained in RPMI 1640 culture medium at 37 °C in an environment of 5% CO₂ for 48 h. After this period, 30 μ L of MTT solution (5 mg/mL) was added, and the cultures were incubated for 3 h at 37 °C. During the incubation, living cells convert the tetrazolium component of the dye solution into formazan crystals. The formazan crystals were dissolved by adding 100 μ L of PBS containing 10% SDS and 0.01 N HCl and incubating the mixture for 18 h at 37 °C in 5% CO₂. The absorbance was read on a multiwell scanning spectrophotometer (ELISA reader) at 570 nm. The number of cells was estimated by comparison to a standard curve prepared using known numbers of fresh live cells added to the plates immediately before staining.

2.9. Cytokine quantification

The cytokines present in the supernatants of the cell cultures were quantified using an ELISA. Briefly, ELISA plates (Immuno Maxisorp; Nunc, NJ) were coated with mouse anti-rat monoclonal or polyclonal antibodies against IL1- β , TNF- α and IL-6 (R&D Systems, Minneapolis, MN). The plates were incubated overnight at room temperature and blocked for 1 h at room temperature on a shaker before adding the samples and standards and incubating for 2 h. Biotinylated secondary antibodies were added before 2 h of incubation, and then, peroxidase-conjugated streptavidin was added before 20 min of incubation. Tetramethyl benzidine (TMB), a peroxidase substrate, was added, and the plates were incubated in the dark for 20 min for colour development. The reaction was stopped with 2 N sulphuric acid, and the plates were read using dual wavelengths (465 and 590 nm) on a microplate reader (Spectra Max 190, Molecular Devices). The cytokine concentrations were determined by comparison to a standard curve prepared using the recombinant murine

cytokines (R&D Systems) that could be detected at 4–10 pg/mL. The cytokine concentrations were expressed as the amount of induced cytokine in picograms per 10^6 macrophages.

2.10. Extraction and quantification of eicosanoids

The production of LXA₄ and 15-epi-LXA₄ was determined from cell-free supernatants acidified with 1 N HCl to pH 3.4–3.6 and passed slowly through an octadecylsilyl silica column (C₁₈ Sep-Pak® column, Waters® Corporation, USA) that had been pre-washed with 10 ml of absolute ethanol and 10 ml of water. After activating the column with 10 ml of water, 2 ml of absolute ethanol and 2 ml of water, the eicosanoids were eluted from the column with 1 ml of water, 1 ml of ether and 2 ml of methyl formate, and the samples were dried under a stream of nitrogen. LXA₄ and 15-epi-LXA₄ concentrations were determined using an ELISA kit (Neogen Corporation, USA). The sensitivity of the assays was 2 ng/mL.

2.11. Statistical analysis

Statistical analyses of the differences between the groups were performed according to Glantz (1997) using GraphPad InStat software, version 3.01 (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance followed by Tukey's test was used for multiple comparisons (all pairs of groups) of the values from the assays using the Boc-2 antagonist. To analyse the data from the other assays, a one-way analysis of variance was used, followed by Bonferroni's test for multiple comparisons against a single control or by an unpaired Student *t*-test to compare two groups. Differences with $P < 0.05$ were considered statistically significant. The results are presented as the mean values \pm standard error of means.

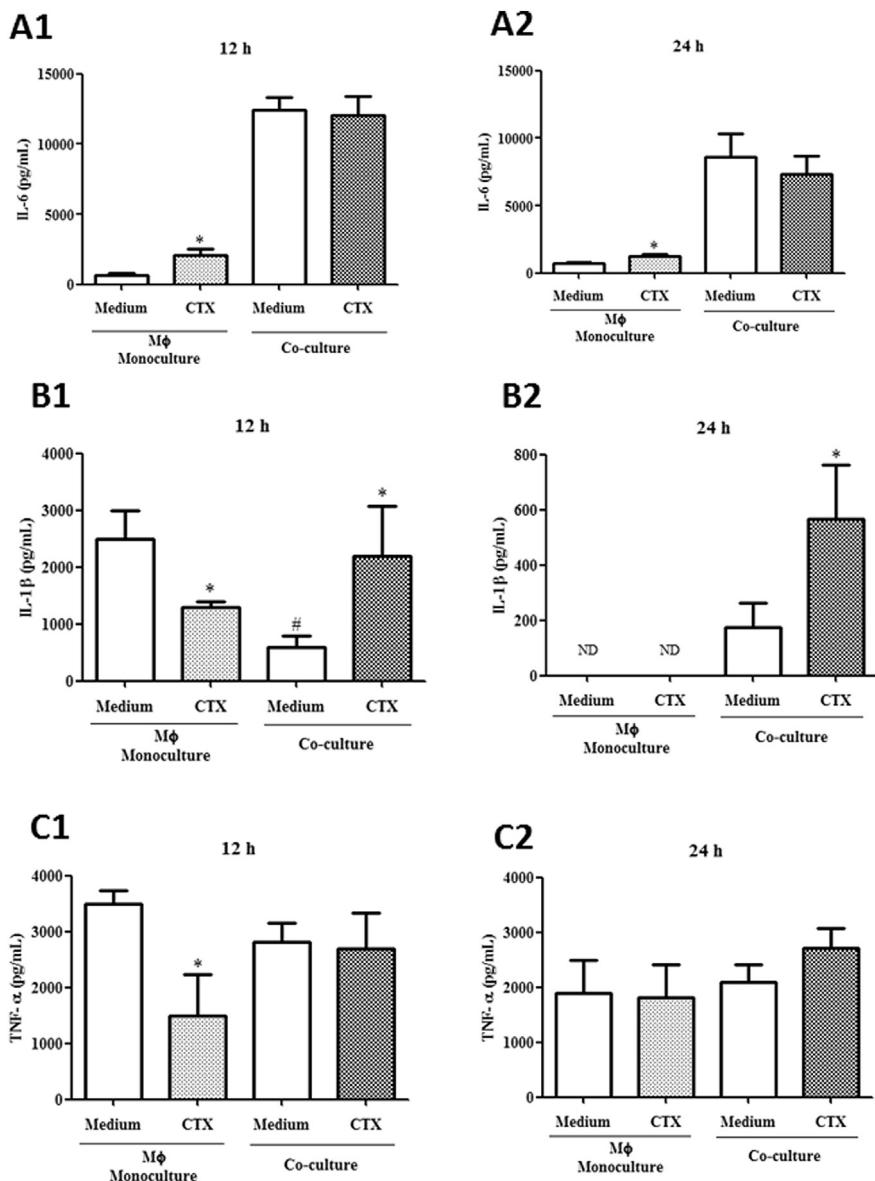


Fig. 2. Effect of CTX on Cytokine Production by Macrophages Monoculture and Macrophages Co-cultivated with LLC-WRC 256 Tumour Cells. Macrophages ($M\phi$) were obtained from the peritoneal cavity of rats (resident), treated with CTX (0.3 μ g/mL) or RPMI 1640 Medium (control), at 37 °C for 2 h. After this period, the cells were cultivated in 24-well tissue culture plate or co-cultivated 24-well tissue culture plate containing tumour cells. For determination of levels of cytokine, cell-free supernatants from monocultures and co-cultures were collected after 12 h (A1, B1 and C1) and 24 h (A2, B2 and C2) and were determined by ELISA (picograms per milliliter). IL-1 β production after 24 h $M\phi$ monoculture was not detected (ND). The results are expressed as means \pm SEM of values obtained from three different experiments performed with duplicate. * P < 0.05, significantly different from respective Control group of $M\phi$ monoculture and co-culture.

3. Results

3.1. CTX increased the hydrogen peroxide (H_2O_2) liberation and nitric oxide (NO) production of macrophage monocultures and macrophages co-cultivated with LLC-WRC 256 cells

Treatment with CTX for 2 h increased the amount of H_2O_2 liberated by the macrophage monocultures (60%) and by macrophages co-cultivated with tumour cells (41%) at

24 h of incubation (Fig. 1A). After this period, this oxygen reactive molecule was not detected in either culture. As shown in Fig. 1B, pre-treatment with CTX stimulated the NO production of macrophage monolayers (38%) and of macrophages co-cultivated with tumour cells (29%) at 48 h of incubation. The LLC-WRC 256 cell cultures produced very low levels of both reactive molecules (*data not shown*). Interestingly, the co-cultures of control macrophages with the tumour cells exhibited a marked reduction of H_2O_2

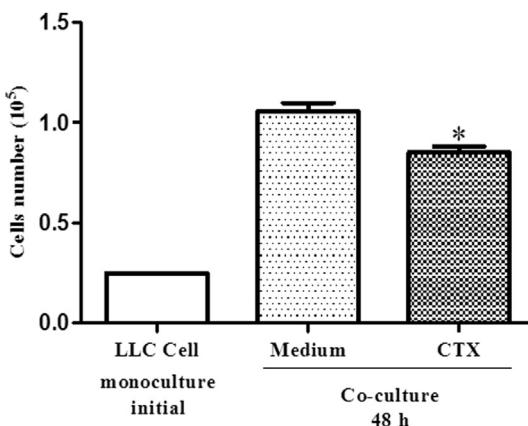


Fig. 3. Effect of Macrophages pre-treated with CTX on the proliferation of LLC-WRC 256 Tumour Cells. Macrophages ($M\phi$) were obtained from the peritoneal cavity of rats (resident), treated with CTX (0.3 μ g/mL) or RPMI 1640 Medium (control), at 37 °C for 2 h. After this period, the cells were cultivated in 96-well tissue culture plate containing tumour cell (LLC Cell), at 37 °C for 48 h. After this period, the cell number was accessed by MTT as described in Material and Methods. The results are expressed as means \pm SEM of values obtained from three different experiments performed with duplicate. * $P < 0.05$, significantly different from control group.

liberation (29%, Fig. 1A) and NO production (20%, Fig. 1B) compared to the control macrophages, suggesting that the tumour cells exerted a suppressor activity on macrophage function.

3.2. CTX stimulated the secretion of cytokines by macrophage monocultures and macrophages co-cultivated with LLC-WRC 256 cells

The secretion of cytokines by macrophages in monocultures and in co-cultures with LLC-WRC 256 cells was assessed by performing multiplexed cytokine assays on the culture supernatants. Macrophages pre-treated with CTX demonstrated increased secretion of the IL-6 cytokine (3.19-fold at 12 h, Fig. 2A1; 80% at 24 h, Fig. 2A2) but significantly decreased secretion of the IL-1 β and TNF- α cytokines at 12 h (48%, Fig. 2B1 and 57%, Fig. 2C1, respectively) when compared to the monoculture control. After 24 h, the levels of IL-1 β were undetectable (Fig. 2B2). No differences in the levels of TNF- α secreted by the two sets of macrophages were observed (Fig. 2C2). Co-culturing macrophages in the presence of tumour cells enhanced their IL-6 production, but pretreatment with CTX did not alter the level of this cytokine at the experimental time points, compared to the control group (Fig. 2A1 and A2). In contrast, increased secretion of IL-1 β (3.7-fold at 12 h, Fig. 2B1; 3.24-fold at 24 h, Fig. 2B2) was observed. Interestingly, the level of this cytokine was decreased (76%) in co-cultures at 12 h, compared to the level detected in macrophage monocultures (Fig. 2B1), suggesting a suppressive action of the tumour cells on the secretion of this mediator. Similarly, co-cultured cells secreted less TNF- α (20%) (Fig. 2C1) compared to monocultured cells. However, treatment with CTX did not affect TNF- α secretion by the macrophages co-cultured with tumour cells at 12 h or at 24 h (Fig. 2C1 and C2).

At 48 h, secreted cytokines were not detected in either the monocultures or the co-cultures (data not shown). The monocultures of LLC-WRC 256 cells secreted low levels of the cytokines analysed (data not shown).

3.3. Macrophage pre-treatment with CTX inhibited the proliferation of LLC-WRC 256 cells

Because CTX has been demonstrated to stimulate the secretory activity of macrophages and of macrophages co-cultivated with LLC-WRC 256 cells, we evaluated the effect of macrophages treated with this toxin on tumour cell proliferation. As shown in Fig. 3, the results of the MTT assay demonstrated that tumour cell proliferation was inhibited (20%) at 48 h of co-culture with macrophage pre-treated with CTX. This effect was not due to the loss of membrane integrity because the viability of the macrophages and tumour cells in monocultures was higher than 95% after 48 h, as assessed by Trypan blue exclusion.

3.4. Involvement of formyl peptide receptors in the modulatory activity of CTX on macrophages co-cultivated with LLC-WRC 256 cells

Boc-2, a selective formyl peptide receptor antagonist, abolished the stimulatory effect of pretreatment with CTX on H₂O₂ liberation and NO production by the macrophages in co-cultures (Fig. 4A and B, respectively), when compared to control co-cultures. Pretreatment with Boc-2 also abolished the increase in the level of secreted IL-1 β observed at 12 and 24 h of co-culture (Fig. 4C1 and C2) and the increase in the level of TNF- α observed at 24 h of co-culture (Fig. 4C2). Macrophages pre-treated with Boc-2 for 15 min before CTX treatment did not inhibit the proliferative activity of tumour cells in co-cultures when compared to the control co-cultures (Fig. 5). Boc-2 *per se* did not have an effect on macrophage activity. These results demonstrate the critical involvement of FPR1 in the stimulatory effect of CTX on the macrophage secretory activities and anti-proliferative function.

3.5. CTX induced eicosanoid synthesis by macrophages

Macrophage monocultures previously incubated in the presence of CTX displayed increased LXA₄ secretion (59% at 24 h, Fig. 6B). Differences in the levels of LXA₄ were not observed at 12 h (Fig. 6A) or at 48 h (Fig. 6C). In contrast, CTX enhanced the 15-epi-LXA₄ production by macrophage monocultures in all the time periods evaluated (9.3-fold at 12 h, Fig. 6D; 5.5-fold at 24 h, Fig. 6E; 2.7-fold at 48 h, Fig. 6F), compared to control monocultures. The supernatants of co-cultures of macrophages pre-incubated with CTX and LLC-WRC 256 cells produced significantly increased LXA₄ levels only at 24 h (25%, Fig. 6B). Differences in the levels of LXA₄ in the co-cultures of CTX-treated macrophages and tumour cells were not observed at 12 h (Fig. 6A) or 48 h (Fig. 6C). As shown in Fig. 6D, treatment with CTX did not affect the levels of 15-epi-LXA₄ secreted by the macrophages in co-cultures with the tumour cells. However, the 15-epi-LXA₄ levels were gradually induced over 24 h (2.3-fold, Fig. 6E) and 48 h (2.1-fold, Fig. 6F), when

compared to the controls (co-cultures with macrophages pre-incubated with culture medium and LLC-WRC 256 tumour cells). The level of 15-epi-LXA₄ in the LLC-WRC 256 cell monocultures was below the limits of sensitivity of the assay that was used (*data not shown*).

4. Discussion

In this study, an experimental model represented by macrophages cultivated together with tumour cells at a 10:1 ratio was used to evaluate the secretory activity of macrophages pre-treated with CTX growing in contact with tumour cells and the influence of this contact on tumour cell proliferation.

The data presented here demonstrate that macrophages pre-treated with CTX (0.3 µg/mL) for 2 h increased their release or secretion of effector molecules such as H₂O₂, NO and cytokines and exhibited a cytotoxic effect on tumour cells. It is important to mention that the proliferation and nitric oxide production assays were determined using

macrophages co-cultivated with three different tumour cell lines, such as, LLC-WRC 256 tumour cells, B16F10 murine melanoma cells and human breast cancer cell line MCF-7. Likewise that observed in the co-cultures with LLC-WRC 256 cells, macrophages pre-treated with CTX, inhibited proliferation of B16-F10 and MCF-7 (31% and 38%, respectively, *data not shown*). Additionally, an increase of production of NO in these co-cultures (26% and 50%, respectively, *data not shown*) was observed. Therefore, since the same effect observed regardless the tumour cell type, only the LLC WRC 256 lineage was performed in subsequent evaluation.

As shown in Fig. 1A, a marked induction of H₂O₂ liberation by CTX was observed after 24 h in both macrophage monocultures and co-cultures. After this period, liberation of H₂O₂ occurs in lower levels. Treating the macrophages with CTX resulted in an increased production of NO after 48 h of culture, as shown in Fig. 1B. Nitric oxide can modulate peroxiredoxins, an important peroxidase family that reduce H₂O₂ and other peroxides and hence, exerts an

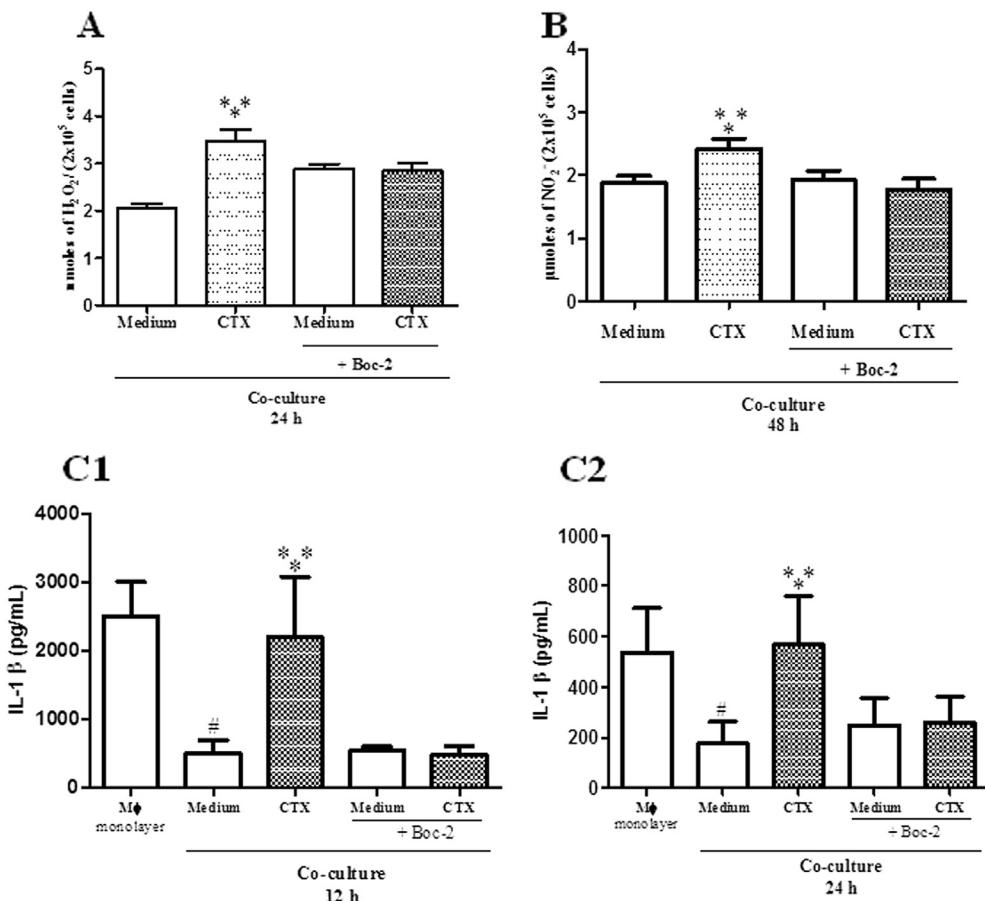


Fig. 4. Effect of Boc-2 on CTX-induced of secretory Activity of Macrophages Co-cultivated with LLC-WRC 256 Tumour Cells. Macrophages (M_φ) were obtained from the peritoneal cavity of rats (resident), incubated in the presence of Boc-2 (100 µM), for 15 min. After this period, the cells were washed with PBS and then incubated with CTX (0.3 µg/mL) or RPMI 1640 Medium (control), at 37 °C for 2 h. After this period, the cells were washed and cultivated in the tissue culture plate containing tumour cell. (A) After 24 h, hydrogen peroxide production (nmoles H₂O₂/2 × 10⁵ cells) was determined in the presence of PMA. (B) For NO₂ production, the results are expressed as micromoles of NO₂ by 2 × 10⁵ cells for 48 h. The levels of IL-1β cytokine were determined in cell-free supernatants from monocultures and co-cultures collected after 12 h (C1) and 24 h (C2) by ELISA and are expressed as picograms per milliliter. The results are expressed as means ± SEM of values obtained from three different experiments performed with duplicate. *P < 0.05, significantly different from Boc-2+CTX group. #P < 0.05, significantly different from M_φ monolayer.

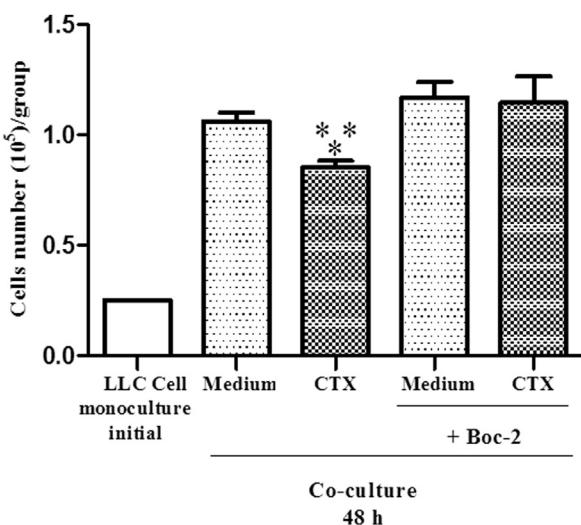


Fig. 5. Effect of Boc-2 on the anti-proliferative activity of macrophages treated with CTX on LLC-WRC 256 Tumour Cells. Macrophages ($M\phi$) were obtained from the peritoneal cavity of rats (resident), incubated in the presence of Boc-2 (100 μ M), for 15 min. After this period, the cells were washed with PBS and then incubated with CTX (0.3 μ g/mL) or RPMI 1640 Medium (control), at 37 °C for 2 h. After this period, the cells were washed and cultivated in the tissue culture plate containing tumour cell (LLC Cell), at 37 °C for 48 h. After this period, the cell number was accessed by MTT as described in Material and Methods. The results are expressed as means \pm SEM of values obtained from three different experiments performed with duplicate. * P < 0.05, significantly different from control group. ** P < 0.05, significantly different from Boc-2+CTX group.

antioxidant effect (Diet et al., 2007). This may explain, at least partially, the lower levels of amounts of H_2O_2 at 24 h in both monocultures and co-cultures.

In addition to examining the production of the oxygen and nitrogen reactive molecules, the production of cytokines was evaluated. IL-6 and TNF- α are humoral factors that are associated with the suppression of tumour cell growth (Paulnock, 1992; Arinaga et al., 1992). Enhanced production of IL-6 was observed in the co-cultures, as shown Fig. 2A1 and A2. IL-6 production is known to often precede increased levels of TNF- α and IL-1 β (Olman et al., 2004); however, IL-6 secretion was not modulated by CTX in any time period evaluated. Similarly, CTX treatment did not modulate the secretion of TNF- α , as shown in Fig. 2C1 and C2. IL-1 β production was rapidly enhanced in co-cultures of CTX-treated macrophages and tumour cells at 12 h and was maximal at 24 h (Fig. 2B1 and B2, respectively).

Moreover, monocultures of macrophages pre-treated with CTX demonstrated increased IL-6 secretion at 12 h and 24 h (Fig. 2A1 and A2) and reduced secretion of IL-1 β (Fig. 2B1 and B2) and TNF- α at 12 h, as shown in Fig. 2C1 and C2, which is compatible with the anti-inflammatory profile described for this toxin (Nunes et al., 2010; da Silva et al., 2013). The duality of the effects of CTX, depending on the model investigated, reinforces the findings reported in the literature showing that CTX accounts for the immunomodulatory action of toxin (Sampaio et al., 2010; for review). Another important observation is that the production of oxygen and nitrogen reactive molecules

and the secretion of IL-1 β were significantly decreased in control co-cultures (macrophages pre-treated with culture medium only), demonstrating that contact with tumour cells decreased the secretory capacity of the macrophages.

Macrophages release large amounts of H_2O_2 , NO and cytokines, and treatment with CTX increases their secretion; these secretory products of macrophages are known to interfere with tumour development. Our objective was to study certain properties of this phenomenon and the mechanisms involved in the anti-tumour effect of CTX.

In this regard, several studies have suggested that the tumour microenvironment decreased the ability of macrophages to kill tumour cells (Szuro-Sudol and Nathan, 1982; Ting and Hargrove, 1982; Alleva et al., 1994). This phenomenon of down-regulation of macrophage metabolism was also observed after co-culturing macrophages with tumour cells (Mitra et al., 2002). The results obtained in this study suggest that pre-treatment with CTX blocks the suppressive action of tumour cells on the secretory activity of macrophages. Studies have suggested that the appropriate activation of M1 macrophages or the conversion of M2 macrophages to M1 macrophages in tumours would be beneficial for cancer therapy (Watkins et al., 2007; Hagemann et al., 2008). Our data support this hypothesis.

Cytotoxicity against tumour cells, as well the expression of microbicidal factors, is dependent on the activation of macrophages and is closely related with the pattern of expression of several inflammatory mediators. The process of activation includes the generation of cytokines such as TNF- α , IL-6 and IL-1 β and reactive oxygen and nitrogen intermediates (Martin and Edwards, 1993; Song et al., 2002; Mantovani et al., 2004). The data presented here demonstrate that the proliferation of tumour cells cultivated in the presence of macrophages previously treated with CTX was inhibited within 48 h (Fig. 3), suggesting the up-regulation of macrophage cytotoxic activities toward the tumour cells. These results indicate that pre-treatment of peritoneal macrophages with CTX increased their metabolism and that their cytotoxic effect on tumour cells occurred through cell-cell contact. Our data are consistent with the results of Taniguchi et al. (2010), who demonstrated that cell-cell contact is critical for the cytotoxic effect of activated lung macrophages on tumour cells, because isolating these macrophages from the tumour cells using a culture insert blocks the cytotoxic effect of the macrophages on tumour cell proliferation.

Another interesting fact to consider is that the lipoxygenase pathway of murine peritoneal macrophages was affected by contact with tumour cells, resulting in the depletion of lipoxygenase products, such as LTB_4 and LXs, in the tumour microenvironment (Calorini et al., 2005). The inhibitory effect of tumour cells on the lipoxygenase activity of macrophages appears to be important for tumour progression (Calorini et al., 2005).

In this regard, studies have demonstrated that LXA₄ and its analogues effectively suppresses hepatocarcinoma *in vitro* and *in vivo*. LXs exert their biological actions by binding to specific high affinity G protein-coupled receptors, FPR2/ALX, that belong to the formyl-peptide receptor family (Chiang and Serhan, 2006; Ye et al., 2009).

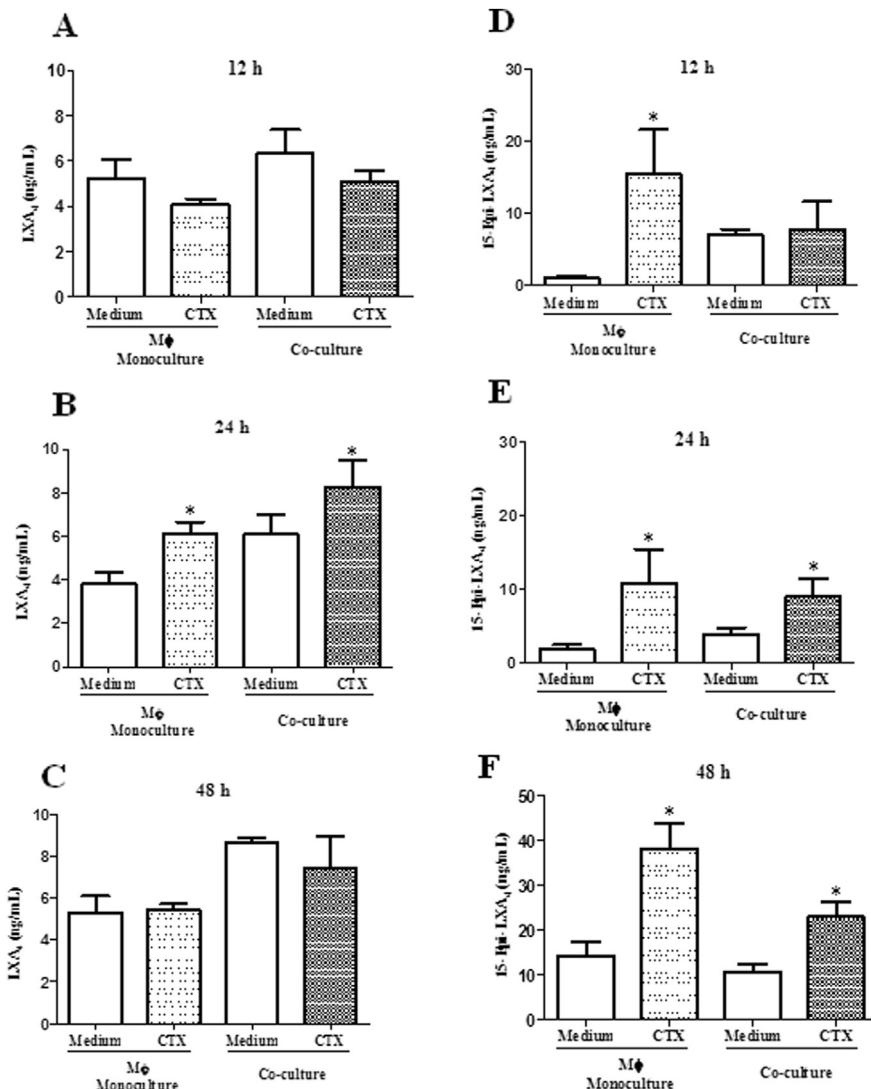


Fig. 6. Effect of CTX on induction of LXA₄ and 15-epi-LXA₄ synthesis by Macrophages Monoculture and Macrophages Co-cultivated with LLC-WRC 256 Tumour Cells. Macrophages (M_φ) were obtained from the peritoneal cavity of rats (resident), treated with CTX (0.3 µg/ml) or RPMI 1640 Medium (control), at 37 °C for 2 h. After this period, the cells were cultivated in 24-well tissue culture plate or co-cultivated 24-well tissue culture plate containing tumour cells. The levels of lipoxin A₄ (LXA₄) and 15-epi-LXA₄ (stable analogue-ATL), determined by ELISA, in cell-free supernatants from monocultures and co-cultures collected after 12 h (A, D), 24 h (B, E) and 48 h (C, F), as described in Material and Methods. The results are expressed as means ± SEM of values obtained from three different experiments performed with duplicate. *P < 0.05, significantly different from respective control group of M_φ monoculture and co-culture.

Boc-2 has been used to inhibit FPR2/ALX and FPR1, which is also a member of the FPR family (Machado et al., 2006; Stenfeldt et al., 2007). Our data demonstrate that pretreatment with Boc-2 (100 µM) abolished the stimulatory effects of CTX on the secretory activity of macrophages co-cultivated with tumour cells, as shown in Fig. 4A, B, C1 and C2. Interestingly, pretreatment with Boc-2 blocked the cytotoxic activity of CTX-treated macrophages on tumour cell proliferation (Fig. 5), suggesting that FPR are crucial for the action of this toxin.

Our previous work demonstrated that Zileuton, a 5-lipoxygenase (5-LO) inhibitor, abolished the inhibitory effect of CTX on macrophage phagocytosis (Sampaio et al.,

2006b) and the stimulatory effect of this toxin on NO production by macrophages (unpublished data), suggesting that macrophage function by CTX is regulated through the production of lipoxygenase-derived mediators. In addition, CTX induced an increase in LXA₄ production (Sampaio et al., 2006b). Macrophage effectors that mediate cellular cytotoxicity, such as cytokines and inducible nitric oxide synthase (iNOS), play critical roles in tumour progression (Keller et al., 1990). Recent insights have begun to reveal new roles for the LXs in modulating this process (Hao et al., 2011). It is important to point out that Dakin et al. (2012) showed that IL1-β induces LXA₄ release and up-regulation of FPR2/ALX expression at 24 h at least 72 h in

chronic inflammatory model. Of note, macrophages subsets are involved in this modulation (Dakin et al., 2012).

In the results presented here, CTX-treated macrophages demonstrated increased production of LXA₄ by 24 h in monocultures or in co-cultures with tumour cells (Fig. 6B). Moreover, a 2 h treatment with CTX enhanced the production of 15-epi-LXA₄ by the macrophages at 12 h, 24 h and 48 h in monocultures or in co-cultures (Fig. 6D, E and F).

LXs biosynthesis proceeds via 5-LO-mediated conversion of AA to 15-hydroxyeicosatetraenoic acid (HETE), transformed via 5-LO to LXA₄ and LXB₄ during cell-cell interactions (Spite and Serhan, 2010; for review). In the presence of aspirin, acetylated COX-2, which both prevents the generation of prostaglandins and activates the oxidation of AA to 15R-HETE (Serhan et al., 1995). This intermediate, like 15S-HETE, is transformed via 5-LO to generate epimeric lipoxins, termed aspirin-triggered or 15-epi-lipoxins (ATL), such as 15-epi-LXA₄, are more stable and more potent analogues (Parkinson, 2006). In addition, 15-epi-lipoxin biosynthesis can also be initiated by cytochrome P450 enzymes catalysed generation of 15R-HETE from AA, followed by 5-LO metabolism. This pathway may be responsible for 50% of the ATL biosynthesis in the absence of aspirin (Clària et al., 1996). Others studies demonstrated that statins promote the formation of 15-epi-LXA₄, from AA via the S-nitrosylation of COX-2 (Birnbaum et al., 2006). Similar to aspirin acetylation of COX-2, S-nitrosylated COX-2 produces 15R-HETE, both are converted by leucocyte 5-LO to form 15-epi-LXA₄ (Birnbaum et al., 2006; Spite and Serhan, 2010; for review). This may explain, in part, the significant presence of amounts of this analogue at 48 h in both monocultures and co-cultures. Again, our results indicate that CTX is able to stimulate macrophages to secrete mediators critical for tumour control, particularly by formation of 15-epi-LXA₄, and reinforce the antitumour potential of these agents.

Studies have demonstrated that differently of the other immunosuppressive agents such as glucocorticoids, LXs and their analogues (ATL) selectively regulate the secretory activity of macrophages (Aliberti et al., 2002a, 2002b; Parkinson, 2006; for review). In *in vivo* and *in vitro* studies, ATL did not compromise the ability of INF- γ -stimulated macrophages to kill intracellular parasites or to up-regulate iNOS or nitric oxide synthesis, which are key antimicrobial mechanisms of macrophages. These observations provided evidence that the LXs and their analogues are immunomodulatory rather than immunosuppressive (Aliberti et al., 2002b; Parkinson, 2006; for review). In addition, the modulation of macrophage function by immunoregulatory stimuli suggests a new immunotherapeutic strategy (Zhang et al., 2012).

In conclusion, our data demonstrate, for the first time, the ability of CTX to selectively modulate the secretory activity of macrophages co-cultured with tumour cells, which may contribute to the inhibitory effect of this toxin on tumour growth observed in *in vivo* studies, and reinforce the immunomodulatory and antitumour effects of CTX. Additionally, the activation of formyl peptide receptors, LXA₄ and the ATL receptor (ALX-R/FPRL-1) plays a major role in these effects. Therefore, the macrophage activation activity of CTX could provide new perspectives regarding

the development of substances with therapeutic properties.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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