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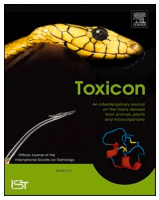

Crotoxin modulates *Encephalitozoon cuniculi*-infected macrophages toward the M1 microbicidal profile

Cristina Gabriela Nascimento de Oliveira [a], Anuska Marcelino Alvares-Saraiva [a], Elizabeth Cristina Perez [a], Sandra Coccuzzo Sampaio [b], Maria Anete Lallo [a,*]

[a] *Programa de Patologia Ambiental e Experimental da Universidade Paulista–Unip, São Paulo, Brazil*
[b] *Laboratório de Fisiopatologia, Instituto Butantan, São Paulo, Brazil*

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A B S T R A C T

Crotoxin (CTX), a bioactive extract from the snake *Crotalus durissus terrificus*, has antibacterial, antitumor, and anti-inflammatory properties. Microsporidia are opportunistic, obligate intracellular fungi that infect vertebrates and invertebrates and are highly resistant to conventional drugs. They can also subvert the microbicidal activity of M1 macrophages to an M2 profile, which is more favorable for the pathogen. Thus, in this study, we evaluated the effects of CTX on the viability of spores of the microsporidium *Encephalitozoon cuniculi*, as well as on the microbicidal activity of macrophages in vitro. *E. cuniculi* spores were treated with two concentrations of CTX (2.4 and 4.8 μg/mL) and cultivated in RK-13 cells for viability analysis. Additionally, peritoneal adherent cells (APerC), obtained from peritoneal washes of BALB/c mice, were infected with spores of *E. cuniculi* for 1 h and treated with CTX for 3 h. The profile of macrophages, cytokine production, viability of macrophages, and proliferative capacity of spores were subsequently evaluated. Treatment of *E. cuniculi* spores with CTX had no fungicidal or fungistatic effects. Compared to the macrophages in the control group, macrophages infected with *E. cuniculi* and treated with 2.4 μg/mL CTX presented an increase in the M1 profile, more necrosis, and greater production of the cytokines TNF-α and IL-6, and the spores obtained from these macrophages presented a reduction in proliferative capacity. These results indicated that CTX modulated the M1 profile of macrophages infected with *E. cuniculi*, resulting in greater production of proinflammatory cytokines and stronger microbicidal activity.

1. Introduction

Crotoxin (CTX) is the main toxin isolated from the venom of the Brazilian rattlesnake *Crotalus durissus terrificus* (*C. d. terrificus*). CTX is a heterodimeric β-neurotoxin formed by the noncovalent association of two different subunits: crotapotin (CA) and phospholipase A2 (PLA2—CB). CTX has several interesting biological effects, such as antitumor activity in vitro, in vivo, and in clinical studies (Cura et al., 2002; Sampaio et al., 2010; Reid, 2013). Several studies have also shown the modulatory function of the immune response. An immunomodulatory effect on splenic cell proliferation and cytokine secretion in vitro (Rangel Santos et al., 2004), the inhibition of phagocytic activities and the migration of resident peritoneal macrophages (Sampaio et al., 2003; Nunes et al., 2010), and the negative modulation of the intracellular signaling pathways essential for phagocytosis by peritoneal macrophages, lymphocyte recruitment, and antibody production (Silva et al., 1996; Zambelli et al., 2008) have been demonstrated.

The use of CTX at a dose of 5.0 μg/animal was found to modulate the M2 profile of macrophages in control mice and increase the M1 profile in mice with Ehrlich ascites tumors (Neves et al., 2023). In a leishmaniasis model, CTX increased the phagocytic and microbicidal activities of macrophages, which was associated with increased nitric oxide (NO) production, cell area, and proinflammatory cytokine production, characterizing the M1 profile of cellular activation (Farias et al., 2017). The survival time of mice subjected to sepsis increased after treatment with CTX; they also showed a decrease in interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels and an increase in lipoxin A4 (LXA4), prostaglandin E2 (PGE2), and IL-1β concentrations. These findings suggested that CTX can protect against sepsis-related mortality (Bretones et al., 2022).

Microsporidia are obligate intracellular fungi that infect various invertebrate and vertebrate hosts. Since the 1990s, they have emerged

* Corresponding author.
E-mail addresses: anetelallo@hotmail.com, maria.lallo@docente.unip.br (M.A. Lallo).

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as opportunistic pathogens, with the AIDS pandemic serving as an agent of enteritis. They affect immunosuppressed and/or immunodeficient patients, such as transplant recipients, patients undergoing chemotherapy, elderly individuals, and immunocompetent individuals (Han et al., 2021). The human microsporidiosis described involves 16 species, among the >1700 species identified in this group of fungi, with *Enterocytozoon bieneusi, Encephalitozoon intestinalis, Encephalitozoon cuniculi*, and *Encephalitozoon hellem* being some of the prominent species (Han and Weiss, 2017; Han et al., 2021). Intestinal and systemic infections are the most reported, and the pathogenic aspects of these infections vary according to the species involved and the quality of the immune response of the host (Moretto and Khan, 2022).

In another study, our research group induced polarization to the M1 phenotype with recombinant interferon -IFN-γ and lipopolysaccharide - LPS of mouse bone marrow (BMD) macrophages and then they were challenged with *E. cuniculi*. We demonstrated a decrease in $CD40^{+}$ expression on the surface of macrophages, which indicates a reduction in the M1 profile (Camargo Konno et al., 2024). In M2 macrophages (polarized by IL-4 treatment), *E. cuniculi* infection also resulted in a significant decrease in CD206+ cells, indicating that the pathogen also reduced the expression of molecules compatible with the M2 profile. In both conditions, *E. cuniculi* could modulate the phenotype of these cells. However, the phagocytic index of *E. cuniculi* spores was lower in M1 macrophages than in M2 macrophages (Camargo Konno et al., 2024). We also found that the pathogen favored efferocytosis and polarized the macrophages to an M2 phenotype, allowing the survival and multiplication of *E. cuniculi* within macrophages and explaining why macrophages may serve as Trojan horses in microsporidiosis (Dalboni et al., 2021). Therefore, manipulating the macrophage phenotype can facilitate the propagation and dissemination of *E. cuniculi*. Thus, in this study, we evaluated the modulatory activity of CTX on *E. cuniculi* spores and determined the effects of CTX on the modulatory activity of macrophages previously infected with *E. cuniculi* in vitro.

2. Materials and methods

2.1. Ethics statement

All the experimental procedures were performed following the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA) and were approved by the Ethics Committee for Animal Research at Paulista University under protocol number 9690131020.

2.2. Study animals

Inbred specific pathogen-free (SPF) BALB/c male and female mice (6–8 weeks old) were obtained from the animal facility at Centro de Desenvolvimento de Modelos Experimentais (CEDEME), UNIFESP, Brazil. The animals were housed in polypropylene microisolator cages under conditions of a 12-h/12-h light/dark cycle, maintained at 21 ± 2 °C and >40 % humidity, and fed standard chow and water *ad libitum*.

2.3. Encephalitozoon cuniculi spores

The spores of *E. cuniculi* (genotype I) used in this were purchased from Waterborne Inc. (New Orleans, LA, USA) and previously cultivated in a rabbit kidney cell line (RK-13, ATCC CCL-37) in RPMI medium supplemented with 10 % fetal calf serum (FCS) and gentamicin at 37 °C in 5 % CO_2. The spores were purified by centrifugation, and the cellular debris was removed with 50 % Percoll (Pharmacia) following a method described in another study (Didier et al., 1991).

2.4. Crotoxin (CTX)

Crotoxin was provided by Dr. Sandra Coccuzzo Sampaio Vessoni from the Physiopathology Laboratory of the Instituto Butantan (São Paulo, Brazil).

2.4.1. Purification of CTX

The CTX complex was purified from crude venom following the method described by Rangel-Santos et al. (2004). A 10 mg aliquot of venom was resuspended in 2 mL of Tris-HCl buffer (50 mM, pH 7.3) and centrifuged at 10,000 g for 10 min (Eppendorf ultracentrifuge) to remove insoluble material. The resulting supernatant was subjected to anion-exchange chromatography using a MONO-Q HR 5/5 column (5 mL) in an FPLC system (Fast Performance Liquid Chromatography, Pharmacia) with Tris-HCl buffer (50 mM, pH 7.3) for CTX isolation. Proteins bound to the resin were eluted using a linear gradient of 0–1 M NaCl in the same buffer. Three major peaks (Peaks I, II, and III) were obtained, with Peak II corresponding to CTX. One-milliliter fractions were collected, and protein elution was monitored by measuring absorbance at 280 nm. The fractions containing CTX were assessed for homogeneity by polyacrylamide gel electrophoresis, and phospholipase A_2 activity was evaluated using a chromogenic substrate. The fractions corresponding to purified CTX were pooled, concentrated using a Vivaspin column, and quantified by the Bradford method.

2.4.2. SDS-PAGE electrophoretic analysis for CTX purity assessment

A 12.5 % acrylamide gel was prepared and assembled in 8 × 5 cm glass plates with 1.0 mm spacers (BIO-RAD Mini Protean II System). Venom and CTX samples were diluted in sample buffer (125 mM Tris-HCl, pH 6.9, containing 2.5 % (w/v) SDS, 20 % glycerol, 1 mM PMSF, 4 mM EDTA, and 0.05 % bromophenol blue) and heated at 100 °C for 5 min. The samples were then loaded onto a 4 % stacking gel and subjected to electrophoresis at 20 mA for 2 h. Following electrophoresis, the gel was stained with Coomassie Blue. Additionally, CTX samples were tested for endotoxin (LPS) contamination using a Limulus amoebocyte lysate (LAL) immunoenzymatic assay (Cambrex/Lonza), conducted at the Quality Control Microbiology Laboratory of the Bioindustrial Center, Butantan Institute. CTX aliquots confirmed to be free of contaminants were stored at −20 °C until use.

2.5. RK-13 cell culture

The RK-13 cells (ATCC CCL 34) were cultured in RPMI medium supplemented with 10 % fetal bovine serum (FBS) and gentamicin (20 μg/mL), designated R10. The cultures were incubated at 37 °C with 5 % CO_2 at the Cell Culture Laboratory of Universidade Paulista. After complete cell confluence of the cell mat occurred in approximately two days, the medium was discarded, and the same cells were released via trypsin versene (ATV). The cells were replicated in new culture bottles, which were made available for infection with *E. cuniculi*.

2.6. Adherent peritoneal cell (APerC) cultures

APerC was obtained from the peritoneal cavities (PerC) of BALB/c mice. The PerC were washed with RPMI-1640 medium, and 0.5×10^6 cells were dispensed in each well of 24-well plates and incubated at 37 °C in 5 % CO_2 for 60 min. The nonadherent cells were discarded, and RPMI-1640 supplemented with 10 % FCS (R10) was added to the adherent fraction and incubated for 3 h before use.

2.7. Experimental design

First, *E. cuniculi* spores were incubated for 24 or 48 h with 2.4 μg/mL or 4.8 μg/mL CTX to evaluate the fungicidal or fungistatic effects of CTX on the microsporidia *E. cuniculi* (Fig. 1). The same concentrations of untreated spores and dead spores (after 20 min of autoclaving) were used as controls. After the treatment period ended, the spores were washed with PBS and incubated in RK cell culture at a ratio of one spore to each RK cell (1:1) to determine the viability of the spores based on

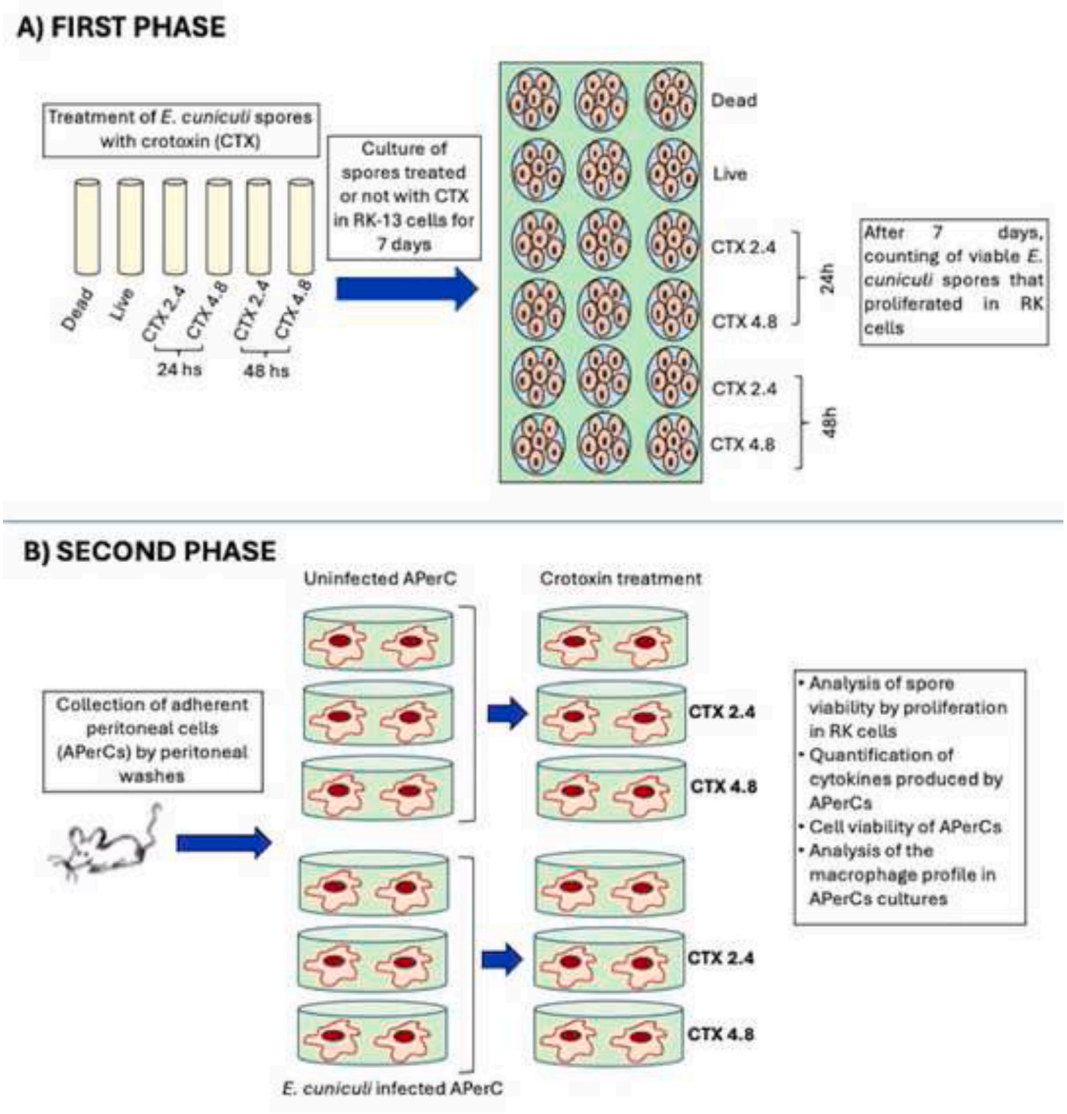

Fig. 1. Experimental design. A) First phase - *Encephalitozoon cuniculi* spores were treated with 2.4 μg/mL or 4.8 μg/mL crotoxin (CTX) for 24 h or 48 h, with live and dead controls being maintained. After treatment, the spores were infected with RK-13 cell cultures to observe their viability based on their proliferation capacity. B) In the second phase, adherent peritoneal cells (APerCs) were obtained through peritoneal lavage and cultivated on plates for 1 h. After nonadherent cells were discarded, APerCs were infected with *E. cuniculi* spores for 1 h. Finally, the infected APerCs were treated with 2.4 μg/mL or 4.8 μg/mL CTX. After 3 h, the viability of spores and APerCs, cytokine production, and the macrophage profile were assessed.

their proliferation capacity. After seven days of incubation, the spores were quantified in a Neubauer chamber. The second phase of this study involved evaluating the effects of CTX on macrophages infected with *E. cuniculi* (Fig. 2). APerC was incubated in 24-well plates as described above. Then, the supernatant was discarded, and a new medium with *E. cuniculi* spores (two spores for each macrophage, 2:1) was added. Next, 1 h after infection, the supernatant was removed, and the wells were washed again with PBS. A medium supplemented with 2.4 μg/mL or 4.8 μg/mL CTX was added. After incubation for 3 h, the cytokines released into the supernatant were quantified, the macrophage profile of the cultures and their viability were evaluated, and the viability of the spores in the medium for their proliferation in RK cells was analyzed.

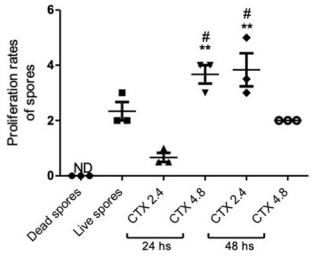

Fig. 2. Spore viability after CTX treatment was determined according to the proliferation ratio. *Encephalitozoon cuniculi* spores: nonviable spores (Dead), viable untreated spores (Live), and spores incubated with 2.4 μg/mL or 4.8 μg/mL CTX for 24 or 48 h. The proliferation ratio was determined by the difference between the number of infected spores that entered the RK cells after 6 h of culture and the number of spores produced after seven days of culture. One-way analysis of variance (ANOVA) with Tukey's post hoc test revealed a difference of $p < 0.01$** relative to Dead and $p < 0.05$# relative to CTX (2.4 μg/mL) (.

2.8. Cell viability

The viability of RK-13 cells or APerC was measured by quantifying the absolute or relative number of live or dead cells resulting from necrosis, apoptosis, or late apoptosis using the 7AAD/Annexin Kit (BD Bioscience). Following the manufacturer's guidelines, 1 μL of 7AAD and

1 μL of Annexin were added to each sample previously diluted in 100 μL of the kit buffer and incubated for 15 min in the dark at room temperature. Immediately after incubation, 100 μL of the same buffer was added, and the reading was immediately performed on a BD Accuri™ C6 flow cytometer (BD Biosciences).

2.9. Spore viability

Spores treated with CTX or recovered from cultures with APerC from the first and second phases, respectively, were cultured in RK cells to determine their viability based on their proliferative capacity. The spores were counted and inoculated in RK cells grown in 24-well plates. After 6 h of incubation, which was necessary for the internalization of the spores, the supernatant was collected, replaced with R-10 medium, and incubated for seven days. To calculate the proliferation rate, the basal number of spores (T0) was obtained from the difference between the number of inoculated spores and the number of noninternalized spores after 6 h.

After seven days, the culture supernatant was collected and stored. RK cells were released with trypsin, disrupted with 1 % Tween 20, and subjected to 20 heat shock sessions, alternating between 20 s of a hot water bath and an ice bath. The released spores were added to the supernatant, centrifuged, and diluted in 1 mL of PBS for subsequent counting in a Neubauer chamber, and the spores were considered to have proliferated within seven days (T7). The proliferation ratio was obtained by dividing the number of spores that had proliferated within seven days (T7) by the basal number of spores (T0).

2.10. Flow cytometry phenotyping of APerC

After treatment with CTX, APerCs were detached from the wells using a cell scraper in RPMI, followed by centrifugation at 2000 rpm for 5 min. The supernatant was discarded, and the cells were washed with Macs buffer, followed by incubation with an anti-CD16/CD32 antibody for 20 min in an ice bath to block Fc receptors. Next, the cells were washed with Macs buffer, and then the following monoclonal antibodies were added: mouse anti-F4/80 conjugated with allophycocyanin (APC), mouse anti-CD11b conjugated with Pacific blue (BD-Pharmingen, San Diego, CA), mouse anti-CD40 conjugated with the peridinin chlorophyll protein complex (PerCP; BD-Pharmingen, San Diego, CA), and mouse anti-CD206 conjugated with allophycocyanin (APC; BD-Pharmingen, San Diego, CA). The cells incubated with the antibodies labeled with the fluorochromes were kept refrigerated in the dark for 30 min. Finally, the cells were washed with Macs buffer and resuspended in 150 μL of PBS for flow cytometry. The data were recorded using a BD Accuri™ C6 flow cytometer (BD Biosciences).

2.11. Cytokine quantification

The cell culture supernatants were collected and stored at −80 °C to analyze the cytokines TNF-α, INF-γ, IL-6, IL-12p70, MCP-1, and IL-10 (Mouse Inflammation Kit) by flow cytometry using the cytometric bead array (CBA) method. Briefly, 20 μL of the sample (both standard curve and samples to be analyzed) was incubated with 20 μL of a mixture of capture beads and secondary antibodies conjugated to a PE fluorochrome (phycoerythrin). The samples were incubated for 2 h at room temperature in the dark. After washing, data were collected using a BD Accuri™ C6 flow cytometer (BD Biosciences). In total, 1500 events were acquired (corresponding to 300 beads per analyte to be investigated). The data were analyzed using the software for CBA (FCAP array software – BD Bioscience).

2.12. Nitric oxide (NO) production

The production of NO was measured from the amount of nitrites and nitrates present in the supernatants of the cell cultures using the indirect colorimetric method based on the Griess reaction. In a 96-well plate, 100 μL of Griess reagent (0.2 % N-1-naphthylethylenediamine and 2 % sulfanilamide in 5 % acetic acid) was added with the same volume of culture supernatant. After incubation for 10 min at room temperature in the dark, the absorbance was determined with a spectrophotometer using a 540 nm filter. To calculate the results, a sodium nitrite standard curve ranging from 0 to 100 μM was used to derive the equation of straight line. Then, the absorbance values were converted into μM of nitrite, which were used to represent nitric oxide production.

2.13. Statistical analysis

The differences between groups were determined by one-way analysis of variance (ANOVA) using the GraphPad Prism program. The values are presented as the mean of experimental replicates ±standard error. All differences were considered to be statistically significant at $p < 0.05$, with a 95 % confidence interval.

3. Results

3.1. CTX did not affect the viability of E. cuniculi spores

Crotoxin (CTX) exhibits various pharmacological activities, including antiparasitic effects; however, no study has reported the action of CTX on microsporidia. First, *E. cuniculi* spores were treated with CTX, and the fungicidal and/or fungistatic activity was analyzed based on the proliferation of the spores in RK cells. The non-proliferation of dead spores was observed in RK cell cultures, and this group was considered to be the control for loss of viability. All groups of spores treated with CTX showed proliferation and preservation of their viability relative to the dead group. Compared to the live group (live and untreated spores used as positive controls), treatment with 2.4 μg/mL CTX for 48 h and 4.8 μg/mL CTX at 24 and 48 h, resulted in a two-fold to fourfold increase in the number of spores relative to the dead group or those treated with 2.4 μg/mL CTX for 24 h (Fig. 2), suggesting the absence of fungicidal or fungistatic activity for these treatments. In contrast, the group that was treated with 2.4 μg/mL CTX for 24 h presented lower proliferation than the live spores and the other treatment groups, indicating fungistatic activity; however, at 48 h, the same concentration of CTX did not influence spore proliferation (Fig. 2).

On average, 30–35 % of uninfected or non-infected RK cells remained viable after seven days of culture. Necrosis was the predominant type of cell death in the groups infected with spores treated for 24 h with 2.4 μg/mL and 4.8 μg/mL CTX and for 48 h with 2.4 μg/mL CTX (Suppl Fig. 1). On the other hand, apoptosis and late apoptosis predominated as forms of cell death in the RK, dead, live, and CTX (4.8 μg/mL for 48 h) groups (Suppl. Fig. 1).

3.2. CTX and/or E. cuniculi modulated the macrophage profile

APerCs were challenged with *E. cuniculi* spores and then treated with 2.4 μg/mL CTX to observe the macrophage modulation profile at different time intervals (Fig. 3). After 3 h of incubation, a predominance of $CD40^{+}$ macrophages was observed in the control and CTX-treated groups compared to the infected groups (Fig. 3A); however, analysis of the $CD40^{+high}/CD206^{+high}$ ratio revealed that only the control group had a predominant M1 profile, with a ratio of 1.5 M1 macrophages for each M2 macrophage. After 6 h of incubation, the number of macrophages with the M2 profile increased in the CTX-treated group (Fig. 3B), and the M1/M2 ratio was close to zero, indicating that the macrophage profiles tended to be equal. At 12 h, M2 macrophages constituted the predominant profile in the *E. cuniculi*-infected and CTX-treated groups (Fig. 3C), although the ratio between the profiles was lower than that in the control. At 24 h (Fig. 3D), the macrophage behavior was similar to that at 6 h. Based on these results, the following experiments were performed at an incubation interval of 3 h.

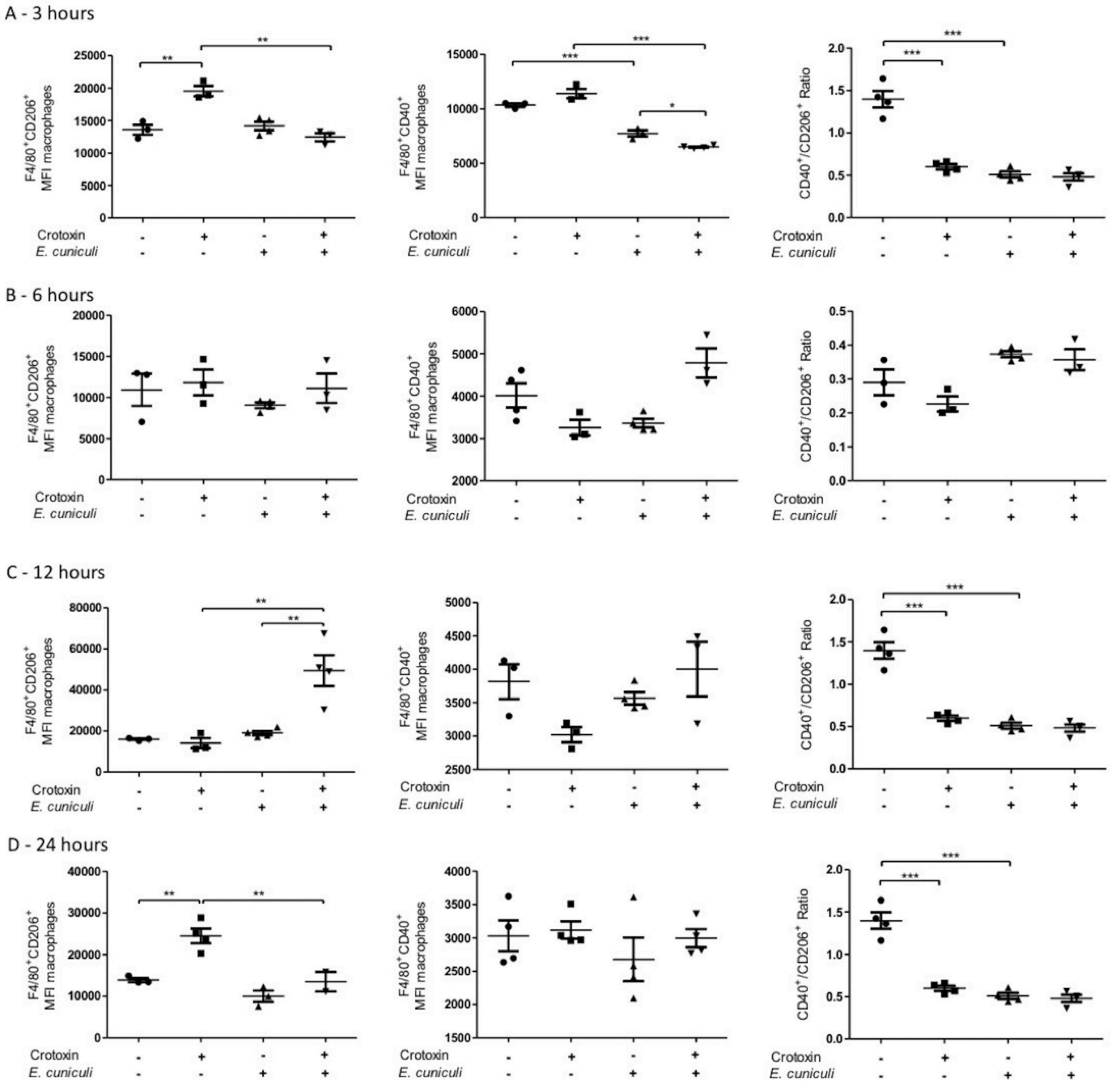

Fig. 3. Mean fluorescence intensity (MFI) of adherent peritoneal cells challenged or not challenged with *E. cuniculi* (- or +) and treated or not treated with 2.4 μg/mL CTX (- or +). A) MFI for M1 macrophages ($F4/80^{+}CD40^{+high}CD206^{+low}$), MFI for M2 macrophages ($F4/80^{+}CD40^{+low}CD206^{+high}$) and the ratio of the MFI of $CD40^{+}$/ MFI of $CD206^{+}$ after 3 h of incubation. B) MFI for $CD40^{+}$ and $CD206^{+}$ cells and the ratio of the MFI of $CD40^{+}$ to the MFI of $CD206^{+}$ cells after 6 h of incubation. C) MFI for $CD40^{+}$ and $CD206^{+}$ cells and the ratio of the MFI of $CD40^{+}$ to the MFI of $CD206^{+}$ cells after 12 h of incubation. D) MFI for $CD40^{+}$ and $CD206^{+}$ cells and the ratio of the MFI of $CD40^{+}$ cells to the MFI of $CD206^{+}$ cells after 24 h of incubation. One-way analysis of variance (ANOVA) with Tukey's post hoc test revealed $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

3.3. The concentration of 2.4 μg/mL CTX increased the microbicidal activity of macrophages

Compared to the control group or the group treated with 4.8 μg/mL CTX, the group treated with 2.4 μg/mL CTX for 3 h presented an increase in the mean fluorescence intensity (MFI) $F4/80^{+}CD40^{+high}CD206^{+low}$ or M1 profile (Fig. 4A). The number of macrophages with an M2 profile ($F4/80^{+}CD40^{+low}CD206^{+high}$) was greater in the control group (Fig. 4B); however, the ratio between the two profiles showed a greater prevalence of M1 macrophages than M2 macrophages (Fig. 4C).

Among the quantified cytokines (TNF-α, INF-γ, IL-6, IL-12p70, MCP-1, and IL-10), only TNF-α and IL-6 were detected following the instructions provided with the CBA kit used (Figs. 5D and 4E); the others were undetectable. IL-6 levels increased after treatment with 2.4 μg/mL CTX (Fig. 4E). The amount of nitrites decreased in the group treated with 4.8 μg/mL CTX, suggesting a decrease in NO production (Fig. 4F).

Compared to those in the groups infected with *E. cuniculi* or infected and treated with 4.8 μg/mL CTX, the MFI $F4/80^{+}CD40^{+high}CD206^{+low}$ or M1 profile of the macrophages in the groups infected with *E. cuniculi* or infected and treated with 4.8 μg/mL CTX increased (Fig. 5A).

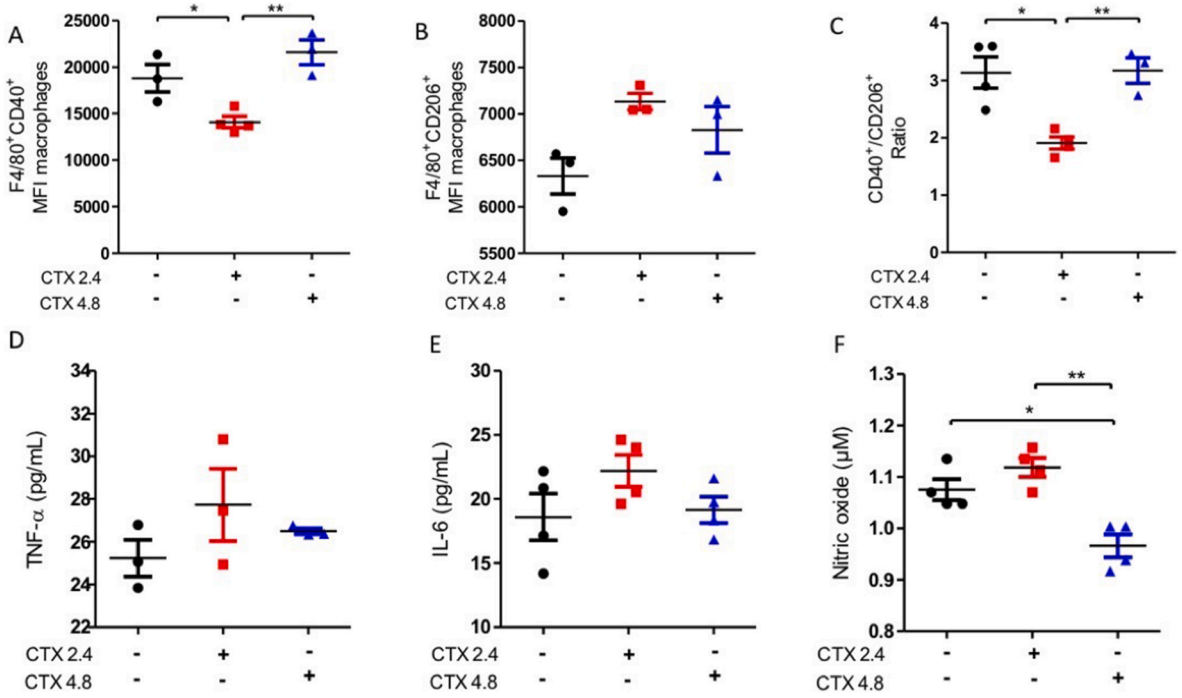

Fig. 4. Functional profile of macrophages obtained from adherent peritoneal cells treated for 3 h with CTX. MFI and production of the cytokines TNF-α and IL-6 by macrophages obtained from peritoneal cells treated (+) or not (−) with 2.4 μg/mL or 4.8 μg/mL CTX. A) MFI of F4/80^{+}CD40^{+high}CD206^{+low} macrophages. B) MFI of F4/80^{+}CD206^{+} macrophages. C) Ratio of the MFI of CD40^{+}/MFI of CD206^{+}. D) Production of TNF-α in the culture supernatant. E) Production of IL-6 in the culture supernatant. F) Measurement of nitric oxide in the culture supernatant. One-way analysis of variance (ANOVA) with Tukey's post hoc test revealed $p < 0.05$* and $p < 0.01$**.

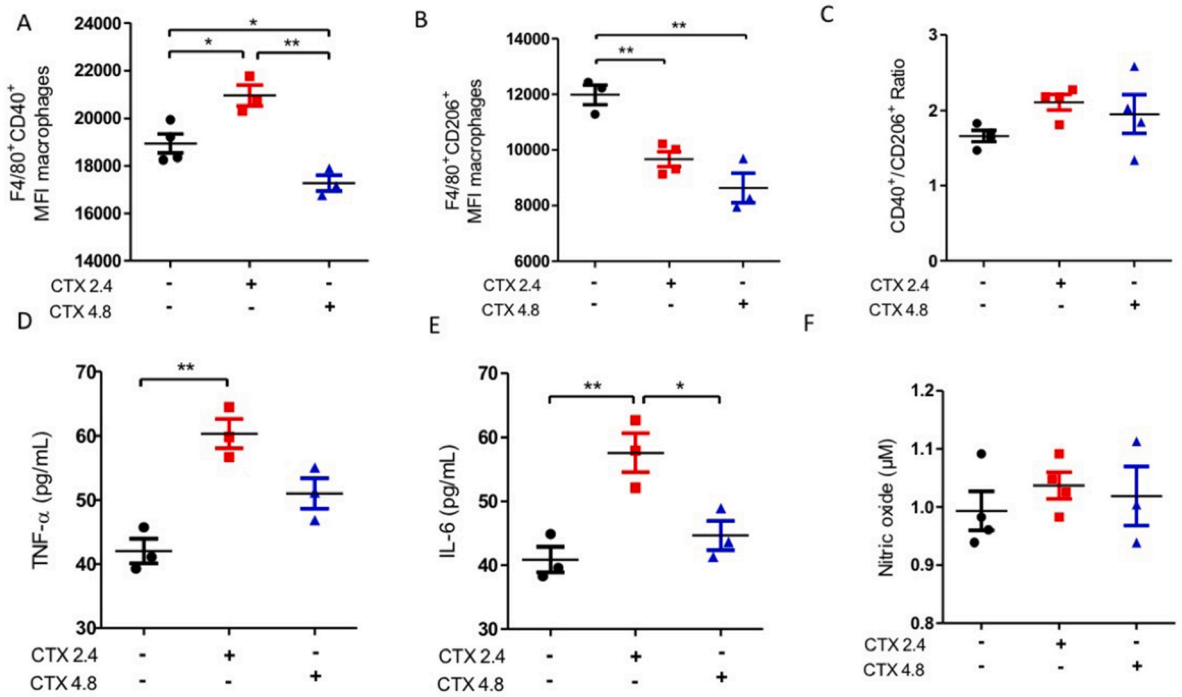

Fig. 5. Functional profile of macrophages obtained from adherent peritoneal cells upon infection with *E. cuniculi* and treatment with CTX for 3 h. MFI of macrophages obtained from adherent peritoneal cells challenged (+) or not (−) challenged with *E. cuniculi* and treated (+) or not (−) treated with 2.4 μg/mL or 4.8 μg/mL CTX. A) MFI of F4/80^{+}CD40^{+} macrophages. B) MFI of F4/80^{+}CD206^{+} macrophages. C) Ratio of the MFI of CD40^{+}/MFI of CD206^{+}. D) Production of TNF-α in the culture supernatant. E) Production of IL-6 in the culture supernatant. F) Measurement of nitrites to evaluate NO production in the culture supernatant. One-way analysis of variance (ANOVA) with Tukey's post hoc test revealed $p < 0.05$* and $p < 0.01$**.

Moreover, macrophages with an M2 profile (F4/80^{+}CD40^{+low}CD206^{+high}) were prevalent in the infected group (Fig. 5B); however, the ratio between the two profiles revealed a greater quantity of M1 macrophages than M2 macrophages (Fig. 5C). Thus, the modulation of the macrophage profile can be attributed to treatment with 2.4 μg/mL CTX. Additionally, the production of the inflammatory cytokines TNF-α and IL-6 was greater in macrophages with the M1 profile (MFI F4/80^{+}CD40^{+high}CD206^{+low}) treated with 2.4 μg/mL CTX, which confirmed the functionality of the identified profile (Fig. 5D and E). The functional behavior of macrophages infected with *E. cuniculi* and treated with 4.8 μg/mL CTX was similar to that of the untreated infected group, i.e., with low production of inflammatory cytokines (Fig. 5D and E). Nitrites concentration was low, reflecting less NO production and no differences were found between the groups (Fig. 5F).

After treatment with CTX for 3 h, spores recovered from APerC cultures were inoculated in RK cells and cultured for seven days to analyze their proliferation and viability. Following this, significantly lower proliferation was found in spores recovered from infected macrophages treated with 2.4 μg/mL CTX than in spores obtained from macrophages treated with 4.8 μg/mL CTX (Fig. 6).

3.4. CTX increased the degree of necrosis in macrophages infected with or without E. cuniculi

The viability of F4/80 macrophages present in APerC cultures after 3 h of treatment with CTX was greater than 80 % in all groups analyzed (Fig. 7A). Necrosis was the predominant type of cell death observed, with an occurrence of 5 % in uninfected macrophages or macrophages infected with *E. cuniculi* not treated with CTX. A greater percentage of necrosis was detected in macrophages treated with 4.8 μg/mL CTX than in control macrophages, both of which were uninfected (Fig. 7B). Among the infected macrophages treated with CTX, a greater percentage of necrosis occurred in the macrophages treated with 2.4 μg/mL CTX than in those treated with 4.8 μg/mL CTX and those treated with 2.4 μg/mL CTX and uninfected (Fig. 7B). No difference was found in the percentage of apoptotic cells between the groups, which presented low percentages (Fig. 7C). However, late apoptosis was greater in the control groups irrespective of whether the macrophages were infected or not; for uninfected macrophages, a difference was found between the two concentrations of CTX used, whereas, for infected macrophages, the difference was detected when the CTX concentration was 4.8 μg/mL (Fig. 7D).

4. Discussion

Microsporidia are intracellular pathogens that are highly resistant to available fungicides or fungistatics, driving research on new substances that can eliminate the pathogen and/or control its multiplication. Considering such resistance, the defense mechanism of the host against the pathogen is exceptionally important against infections by these fungi, with the adaptive immune response playing a fundamental role in the elimination of microsporidia (Aseeja et al.; Moretto and Khan, 2022). However, the innate immune response has been investigated for its primary microbicidal activity, as well as its ability to modulate the adaptive immune response and its role in the dissemination of the pathogen (Han et al., 2020).

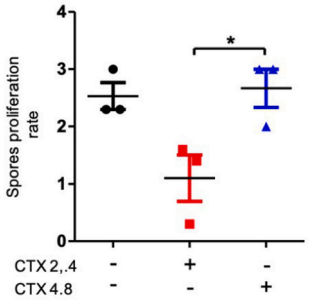

Fig. 6. Proliferation ratio of spores treated with CTX. Number of spores obtained after seven days in culture from RK cells infected with *E. cuniculi* spores and treated with 2.4 μg/mL or 4.8 μg/mL CTX for 3 h. One-way analysis of variance (ANOVA) with Tukey's post hoc test revealed $p < 0.05$*.

In this study, fungicidal and/or fungistatic effects were not observed in the treatment of spores with CTX, contrary to what was found in the treatment with the same concentrations of CTX of promastigote forms of *Leishmania amazonensis*, whose growth was reduced (Farias et al., 2017). Notably, along with the differentiated sensitivity of intracellular pathogens, *E. cuniculi* spores have a wall consisting of three layers that are extremely resistant to the environment and to the action of most disinfectants, which may partly explain the absence of fungicidal activity. Since the fungistatic effect of 2.4 μg/mL CTX for 24 h was not maintained at 48 h. De Araújo Pimenta et al. (2021) demonstrated by confocal microscopy that Crotoxin (CTX) isolated from the venom of the South American rattlesnake interacts with the cell membrane and penetrates macrophages within 24 h. A fact that could explain the fungistatic effect observed by CTX during this observation period. However, within 48 h this effect was not recognized, which could be attributed to the absence of CTX replacement in the cultures. Factors external to the cells such as pH, salt concentration or temperature variations should have little or no interference in the results. We also did not attribute the effects of CTX directly to the spores since the treatment of spores with CTX did not demonstrate interference in the viability and multiplication of *E. cuniculi* spores.

As the fungicidal or fungistatic effects of CTX on spores were absent, experiments were conducted to evaluate its activity on macrophages. Macrophages are one of the main innate cell types that exhibit plasticity; depending on the stimulus received, they can present a microbicidal (M1) or anti-inflammatory (M2) profile (Martinez and Gordon, 2014). M1 macrophages can significantly reduce *Encephalitozoon* infection (Fischer et al., 2008b), which depends on reactive nitrogen and oxygen species (Didier, 1995; Didier et al., 2010; González-Machorro et al., 2019). In this study, treatment with 2.4 μg/mL CTX without *E. cuniculi* reduced the number of M1 macrophages. However, infected macrophages treated with the same concentration of CTX presented an increase in the M1 profile, increased necrosis, and increased production of the cytokines TNF-α and IL-6, and the spores obtained from these macrophages presented a decrease in the proliferative capacity. These results indicated that this concentration increased the microbicidal profile of macrophages, as reported for *L. amazonensis* infection in peritoneal macrophages treated with CTX, which resulted in a significant increase in phagocytic capacity and elimination of intracellular parasites. *Leishmania amazonensis* also has an inhibitory effect on NO production by macrophages; in response to treatment with 2.4 μg/mL and 4.8 μg/mL CTX, NO production and the production of proinflammatory cytokines are increased, which is characteristic of the M1 profile (Farias et al., 2017). In this study, NO production probably was slightly greater in the groups treated with 2.4 μg/mL and 4.8 μg/mL CTX and infected, as indicated by nitrites amount measured.

However, the immunomodulatory activity of CTX was directly related to its concentration used, since exposing infected macrophages to 4.8 μg/mL CTX did not result in a reduction in spore proliferation. This led to the same viability as spores from untreated macrophages. Additionally, these macrophages produced fewer proinflammatory cytokines than those treated with 2.4 μg/mL CTX.

The results of this study showed that macrophages infected with *E. cuniculi* had a greater M2 profile than those uninfected or treated with 2.4 μg/mL CTX, as well as lower production of inflammatory cytokines and greater spore proliferation. In B-1 cell-deficient mice, *E. cuniculi* was found to modulate macrophages to the M2 profile, which has a lower microbicidal capacity; within this profile, polar tubule extrusion, a characteristic of spore viability, was observed (Pereira et al., 2019).

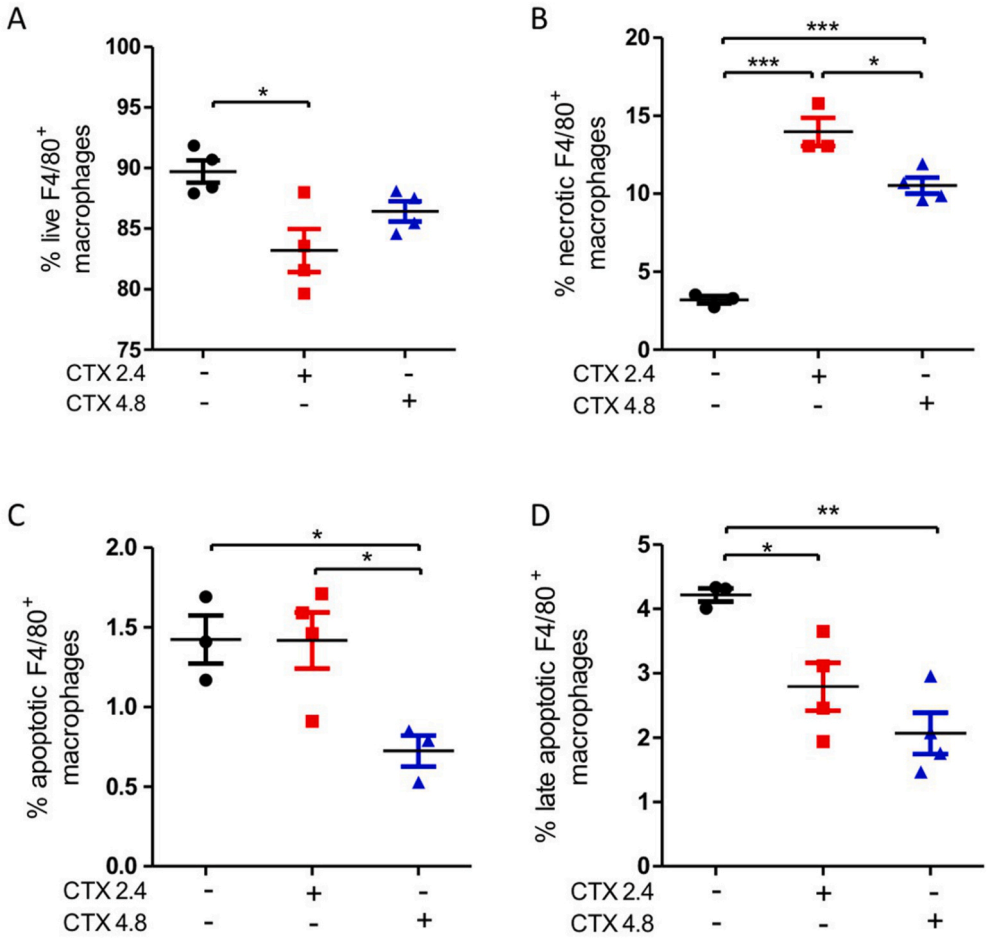

Fig. 7. Comparison of macrophage viability in response to *E. cuniculi* infection and treated (+) or not (−) treated with 2.4 μg/mL or 4.8 μg/mL CTX. A) Percentage of living macrophages. B) Percentage of dead macrophages. C) Percentage of apoptotic cells. D) Percentage of late apoptotic cells. One-way analysis of variance (ANOVA) with Tukey's post hoc test revealed $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

Under such conditions, multiplication within the macrophage results in the production of spores that can be transported as a "Trojan horse" (Mathews et al., 2009). This finding also confirmed the importance of CTX modulation for the M1 profile.

Apoptosis is a form of programmed cell death that also responds to pathogen infection and controls the immune response (Knodler and Finlay, 2001; Lüder et al., 2001; Higes et al., 2013). To proliferate for a longer duration, intracellular parasites such as microsporidia and *Toxoplasma gondii* can suppress host apoptosis (Nash et al., 1998; Higes et al., 2013; Kurze et al., 2015; Sinpoo et al., 2018; Sokolova et al., 2019). In total, 84 genes related to apoptosis were identified in the THP-1 cell line (monocytes derived from monocytic leukemia) infected with *E. cuniculi* and *Vittaforma cornea*. This showed that both can manipulate intrinsic apoptosis pathways and decrease their frequency (Sokolova et al., 2019). In contrast, in this study, a significant increase in apoptosis in RK cells infected with *E. cuniculi* was recorded, which confirmed previous findings reported by our group that demonstrated high percentages of apoptosis in macrophages from bone marrow and infected with *E. cuniculi*, especially after being preincubated with apoptotic Jurkat cells, indicating that the observed efferocytosis is a mechanism of dissemination and evasion of immunity (Dalboni et al., 2021).

In microsporidiosis, the survival of the pathogen and its dissemination depend on the host cell. The duration of the cell cycle is critical, and its metabolism induces adaptive mechanisms in the pathogen. Therefore, the suppression or activation of these genes may be associated with the type of infected cell. Thus, although apoptosis inhibition may contribute to the perpetuation of infection, apoptosis followed by efferocytosis may be an alternative strategy used by microsporidia to evade the immune response and reach other cells. In this context, the inhibition of apoptosis caused by the treatment of spores with CTX suggested that the toxin modified the survival strategies of the pathogen in the host cell, a finding that needs to be further investigated.

To summarize, exposing peritoneal macrophages infected with *E. cuniculi* to 2.4 μg/mL CTX resulted in modulation of the M1 profile, with an increase in the production of the cytokines TNF-α and IL-6 and an increase in the microbicidal activity. Treatment of *E. cuniculi* spores with CTX had no fungicidal or fungistatic effect.

In conclusion, exposure of peritoneal macrophages infected with *E. cuniculi* to 2.4 μg/mL CTX effectively modulated the M1 macrophage profile, leading to an increase in the production of pro-inflammatory cytokines TNF-α and IL-6, as well as enhanced microbicidal activity. However, CTX treatment had no fungicidal or fungistatic effects on *E. cuniculi* spores. These findings suggest that CTX may play a role in modulating immune responses without directly affecting the viability of the pathogen. Further studies are needed to explore the potential of CTX in immune modulation and its broader therapeutic applications.

CRediT authorship contribution statement

Cristina Gabriela Nascimento de Oliveira: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Anuska Marcelino Alvares-Saraiva:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis,

Data curation, Conceptualization. **Elizabeth Cristina Perez:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Sandra Coccuzzo Sampaio:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Maria Anete Lallo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics statement

All the experimental procedures were performed following the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA) and were approved by the Ethics Committee for Animal Research at Paulista University under protocol number 9690131020.

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Declaration of competing interest

The authors Cristina Gabriela Nascimento de Oliveira, Anuska Marcelino Alvares Saraiva, Elizabeth Cristina Perez, Sandra Coccuzzo Sampaio, Maria Anete Lallo declare no Competing interests in papers publication "**Crotoxin modulates *Encephalitozoon cuniculi*-infected macrophages toward the M1 microbicidal profile".**

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2025.108348.

Data availability

No data was used for the research described in the article.

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