Activation of the Kynurenine Pathway and Production of Inflammatory Cytokines by Astrocytes and Microglia Infected With *Neospora caninum*

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ABSTRACT: In the central nervous system, astrocytes and microglia contribute to homeostasis, regulating the immune response to infectious agents. *Neospora caninum* is an obligate intracellular protozoan that infects different animal species and it is encysted in their nervous tissue while triggering an immune response modulated by glia. This study aimed to evaluate the infection of primary cultures of rat glial cells by *N. caninum* through the catabolites of tryptophan, the expression of inflammatory mediators and the integrity of neural tissue. Infection with this coccidium resulted in morphological and functional changes, particularly astrogliosis and microgliosis, and increased the expression of the inflammatory mediators TNF, IL1β, IL-10, and arginase, as well as mRNA for CCL5 and CCL2, molecules involved in the CNS chemotaxis. The infection with *N. caninum* in glial cells also triggered the activation of the tryptophan pathway, characterized by increased kynurenine 2,3 monooxygenase (KMO) mRNA expression, and by the production of the excitotoxin quinolinic acid (QUIN). Moreover, glia-neuron co-cultures, when exposed to the secretome derived from *N. caninum* infected glial cells, presented greater neurons distribution and formation of neurite extensions, associated to morphological changes in astrocytes compatible with neuro-preservation. Considering that the tryptophan catabolism is associated to immune response, these findings suggest that glial activation in *N. caninum* infection should be responsible for modulating the inflammatory status in an attempt to restore the nervous system homeostasis, since excessive inflammatory response can cause irreversible damage to tissue preservation.

KEYWORDS: Kynurenine, *Neospora caninum*, neuroinflammation, glia

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Introduction

The nervous system is a complex tissue, formed by cells with specific functions and characteristics. Astrocytes, oligodendrocytes and microglia represent glia, a set of cells capable of establishing a favorable environment for the functioning of nervous tissue. These cells are responsible for maintaining homeostatic balance, in addition to supporting neurotransmission. Glial cells also participate in the response to injuries being activated by stress, including trauma and infection by restoring homeostasis, since the excessive inflammatory response can cause irreversible damage to this tissue.[1,2]

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The catabolism of tryptophan (Trp) gives rise to several active intermediates and its main route, the kynurenines pathway (KP), begins from the activation of Indolamine 2, 3 dioxygenase (IDO) resulting in important neuromodulation molecules that can contribute positively or negatively to the nervous system homeostasis.[3] In KP, 2 other enzymes stand out for producing metabolites with immunomodulatory activity: kynurenine 2,3 monooxygenase (KMO), which leads to the production of 3-hydroxi kynurenine (3-HK), and quinolinic acid (QUIN) and kynurenine 2,3 aminotransferase (KAT), which deviate the route of kynurenines for the production of kynurenic acid (KYNA). While QUIN is an agonist of NMDA receptors, KYNA is an antagonist of these receptors.[4] Similar to IDO, KMO is induced by inflammatory mediators as IL-1 β in several cell types[5] that

increase its transcription and contribute to the pathogenesis of neurological diseases. Additionally, its product, 3-HK, is responsible for the production of reactive oxygen species, causing autooxidation and degradation of the brain barrier.[6] Studies carried out by Guillemin et al[7] indicate that the metabolites of KP can induce the expression of several cytokines, chemokines, and their receptors in astrocytes. Astrocytes lack the KMO enzyme and, under physiological conditions, do not produce QUIN. Studies have shown that the induction of inflammation by LPS has no effect on KAT expression in rat brain[8] and after IFN-γ stimulation in adult human astrocytes, KAT expression is decreased.[9]

The accumulation of KP metabolites under certain pathological conditions reflects a body defense mechanism in order to avoid oxidative stress.

Neospora caninum, an apicomplexa parasite that causes neosporosis, has tropism for the nervous tissue, where it is encysted.[10] Some experimental models of *N. caninum* infection have shown that this tissue reacts to the invader through biochemical and immunological mechanisms,[11] particularly the release of IL-10 and TNF[12,13] besides the induction of neurotrophic factors[14] that allow the parasite to survive and promote a consequent neuro-preservation.[11-14] On the other hand, the infection of glial-neuron co-cultures with *N. caninum* tachyzoites after modulated by IFN-γ showed preservation of neurite outgrowth and induced the activation of indolamine 2, 3 dioxygenase (IDO), the first and rate-limiting enzyme of tryptophan catabolism through the KP, controlling the parasite proliferation.[15,16]

Moffett and Namboodiri[17] described that the Trp catabolism suppresses the immune system by reducing the supply of this amino acid avoiding the proliferation of T cells, and by promoting pro-apoptotic mechanisms. On the other hand, NAD^+, an essential cofactor in cellular metabolic reactions and one of the main products of the KP, regulates the differentiation of $CD4^+$ T cells, as well as the AMP-activated protein kinase (AMPK) pathway, and is capable of suppressing the pro-inflammatory response.[18,19] Trp catabolism is also activated by interferon gamma (IFN-γ), a pro-inflammatory cytokine with a biological significance in anti-parasitic mechanisms that does not involve oxidative stress.[20,21] The multiple actions of Trp catabolites have also been related to the triggering of several neuropsychiatric disorders and make them potentially useful as biomarkers of these pathologies.[22] The present work evaluated the infection of primary cultures of rat glial cells by *N. caninum* through the catabolism of tryptophan, the level of inflammatory mediators and the impact of the infection on the integrity of the neural tissue.

Material and Methods

Culture of N. caninum

The tachyzoites of *N. caninum* from the Nc-Bahia[23] strain were grown in cultures of VERO cells in RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS) (Gibco, Grand Island, NY) and antibiotics 100 UI/mL penicillin/streptomycin (Gibco, Grand Island, NY), according to the protocol already established.[23] Regular changes of the medium were made every 48 hours. When about 90% of the cells were destroyed by the cytopathic effect caused by the protozoan, the cells were scraped and the medium, collected and centrifuged at 4000*g* at 37°C for 10 minutes. The tachyzoites were purified in a filter unit with a 5.0-μm pore (Merck-Millipore, Darmstadt, Germany) and later used for infection of glial cell cultures.

Glial cell cultures

Wistar rats were provided by the animal facility of the Institute of Health Sciences, Federal University of Bahia (UFBA). All experiments were performed in accordance with the local Ethical Committee for Animal Use (CEUA—UFBA, Opinion nº 2841141118). Primary cultures of glial cells were obtained from the cerebral cortex of newborn Wistar rats (0-48 hours) and performed as described previously.[14] Briefly, cerebral hemispheres and meninges were removed and the tissue was mechanically dissociated into individual cells in Dulbecco's modified Eagle's medium (DMEM) with HAM F12 (1:1) (Gibco, Grand Island, NY) with the aid of a Pasteur pipette. The cells were suspended in DMEM HAM F12 medium, supplemented with 10% of FBS, sodium bicarbonate (35 mM) (Sigma Aldrich, St. Louis, U.S.A), 100 UI/mL penicillin/streptomycin, seeded at a density of 2.2×10^4 cells/cm^2 in 24-well pre-coated plates (TPP, Trasadingen, Switzerland) with 10 μg/mL poly-L-lysine (Sigma Chemical, Darmstadt, Germany) and cultured in humidified atmosphere with 5% CO_2 at 37°C. The culture medium was changed every 48 hours until the confluence of the cells, around the 15th day.

Infection of glial cell cultures with N. caninum

After confluence, glial cells were infected with *Neospora caninum* in the proportion of 1 parasite per cell (1:1) for 24 hours, according to the protocol.[14] After this period, the conditioned culture medium (cellular secretome) was collected and filtered through a 0.2-μm membrane pore and stored for further molecular analysis and treatment of glia-neuron co-cultures. The glial cells were characterized by immunocytochemistry structural markers of microglia and astrocytes.

Glia–neuron co-cultures modulated by glial cell secretome from N. caninum*–infected cultures*

For glia-neuron co-cultures, neurons were obtained from brain embryos of Wistar rats (16th-18th day of gestation). The meninges of cerebral hemispheres of the embryos were discarded and the tissue was mechanically dissociated with the aid of a Pasteur pipette. The neurons were placed on the confluent monolayer of 15-day-old glial cell cultures, in a ratio of 1:2

(neurons/glia) and at a density of 1×10^4 cells/cm^2, in a humid atmosphere with % CO_2 at 37°C. After 24 hours, the medium culture was substituted by the conditioned medium with glial cells infected by *N. caninum* (SGNc) or non-infected control glial cell cultures (SGC), for a period of 24 hours.

Immunocytochemistry and Bromodeoxyuridine cell proliferation assay

For the phenotype analysis of glia and neurons in control and *N. caninum*-infected glial cell cultures, we performed immunocytochemistry (ICQ) for the cytoskeletal proteins, glial fibrillary acidic protein (GFAP), and ionized calcium-binding adaptor molecule 1(Iba-1), respectively structural markers of astrocytes and microglia. Proliferation in glial cell cultures was determined by ICQ after 5-Bromo-2′-deoxyuridine (BrdU) incorporation associated with ICQ for the microglia marker Iba-1. Moreover, ICQ for β-tubulin III (β-tub III), a structural marker of neurons, and for GFAP was performed in glia-neuron co-cultures modulated by the secretome (SGC or SGNc).

The following primary antibodies were adopted: anti-b-Tubulin III (mouse, 1:500; BioLegend, 801202), anti-GFAP (rabbit, 1:300; DAKO, Z0334), anti-Iba-1 (rabbit, 1:200; Wako, 019-19741), and anti-BrdU (mouse, 1:200, Sigma-Aldrich, B2531). In order to determine cell proliferation, BrdU (10 μM, Sigma-Aldrich, B2531) was added to the wells since the beginning of each infection. Cells were fixed and DNA was denatured by means of the treatment with denaturing solution (2 N HCl) for 20 minutes at room temperature before ICQ. Cultures were washed with phosphate buffered saline (PBS (Sigma Aldrich) pH 7.4) and fixed in 4% paraformaldehyde (PFA) pH 7.2 diluted in PBS, at room temperature. After that, cells were permeabilized with Triton X-100 (0.5%) in PBS for 15 minutes and then washed again with PBS, 3 times for 5 minutes. Then, the non-specific binding sites were blocked by incubation with PBS containing 5% of bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO, USA) for 1 hour. The cultures were then incubated with primary antibodies diluted in PBS containing 1% of BSA, in a humidity chamber at 4°C for 12 hours. After 3 washes with PBS, cells were incubated with the secondary antibodies: goat anti-rabbit IgG Alexa Fluor 594 (1:500, Molecular Probes, Eugene, Oregon), or goat anti-mouse IgG Alexa Fluor 488, or goat anti-rabbit IgG Alexa Fluor 546 (1:500, Molecular Probes) diluted in PBS containing 1% of BSA and kept under slow agitation for 1 hour at room temperature and protected from the light. After 3 washes with PBS, cells were incubated with 5.0 μg/mL 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) for nuclear staining and mounted on slides containing 80% glycerol N-propyl gallate (Sigma-Aldrich) mounting medium. Staining was visualized and photographed under fluorescence microscopy (Leica, DFC7000). Images were captured with the Image-Pro Plus software (Media Cybernetics, Rockville, Maryland (www.mediacy.com.imageproplus). Three independent experiments were performed. Quantification was analyzed with the ImageJ 1.33u software (Wayne Rasband, National Institute of Health, Bethesda, Maryland). The quantification was performed by analyzing the total number of positive cells (per marker) divided by the total number of nuclei (DAPI positive).

Quantification of tachyzoites

For the quantification of tachyzoites in glial cell cultures, the number of tachyzoites was counted in each culture 24 hours after infection and DAPI staining as described above, under fluorescence microscopy (Leica, DFC7000). Quantification of tachyzoites was performed in 10 fields of 3 independent experiments by 2 independent investigators in a blind assay, and the results were expressed as the means of the number of tachyzoites in infected cultures.

RNA isolation, cDNA synthesis, and Quantitative PCR (qPCR)

In order to evaluate gene expression for proteins of interest, the total mRNA of glial cell cultures of *N. caninum*-infected or non-infected cells and glia-neuron co-cultures modulated by SGNc was extracted using the Trizol® reagent (Invitrogen, Life Technologies). The concentration and purity of the RNA were determined through spectrophotometry using a Kasvi nano spectrum analyzer (KASVI, São José dos Pinhais, PR, Brazil, K23-0002). RNA purity was determined using the ratio of absorbance measured at 260and 280 nm (A260/A280). Aiming to perform cDNA synthesis, a reaction was used containing 20 μL of 2.5 μg of total RNA with the Super Scriptt VILO and Master Mix (catalogue no. MAN0004286, Life Technologies). Quantitative real-time PCR was performed using TaqMan® Gene Expression Assays (Applied Biosystems, CA, USA) containing 2 primers to amplify the sequence of interest, a specific TaqMan® MGB probe and TaqMan Universal Master Mix II with UNG (catalogue# 4440038 Invitrogen, Life Technologies™, Carlsbad, CA, USA).

The marked inflammatory assays corresponding to the genes quantified in this study were TNF (Rn00563409_m1), IL-1β (Rn00580432_m1), IL-6 (Rn01410330_m1), IL-10 (Rn01525859_g1), Arginase (Rn00691090_m1), CCL-5 (Rn00579590_m1) and CCL2 (Rn00579590_m1), KMO (Rn01411937_m1), and KAT (Rn00567882_m1). Real-time PCR was performed using the Quant Studio™ 7 Flex system in real time (Applied Biosystems, CA, USA). Thermocycling conditions were performed according to the manufacturer's specifications. Reference genes (endogenous controls) were used as target α-actin (Rn00667869_s1). Data were analyzed through real-time polymerase chain reaction and based on Schmittgen and Livak,[24] using the $2^{-\Delta\Delta Ct}$ method for normalization of gene expression data in 3 independent experiments. The results represent the average of 3 independent experiments.

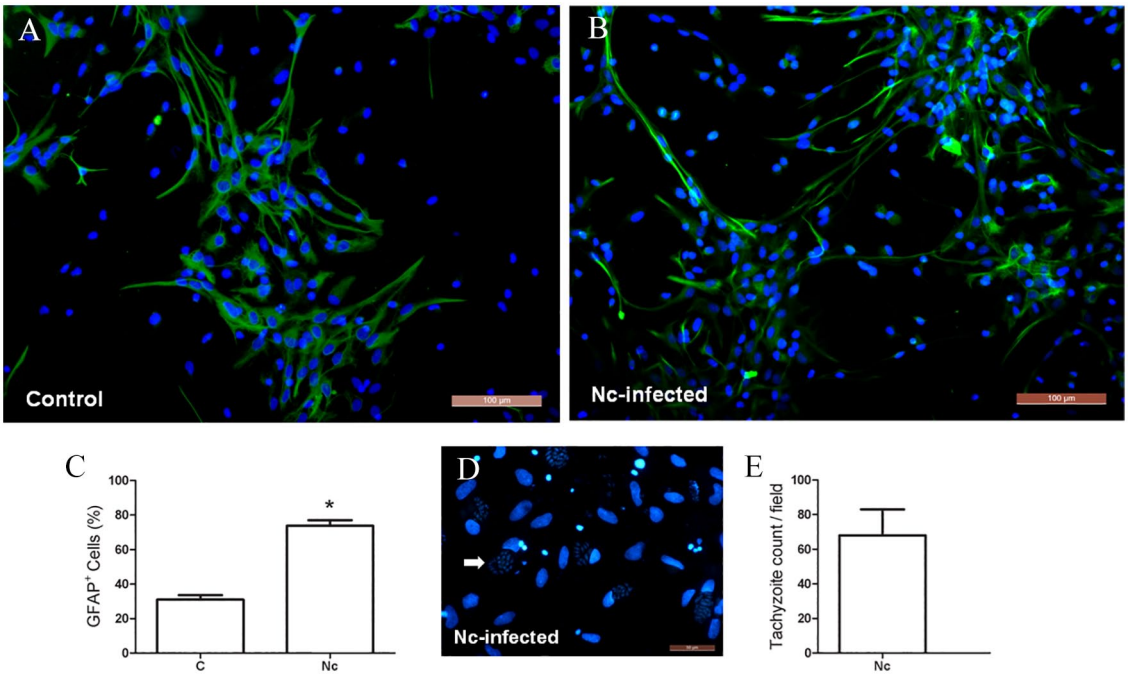

Figure 1. Effects of *N. caninum* infection in glial cell cultures: morphology and activation of astrocytes. Analysis with immunocytochemistry for GFAP (green): cultures were maintained in control conditions (A) or infected with *N. caninum* tachyzoites (Nc) (B) for 24 hours; nuclei were stained with DAPI (blue); images are representative of 3 independent experiments; objective ×20, scale bar 100 µm; (C) Percentage of GFAP$^+$ cells in control and *N. caninum*-infected cultures; groups were compared with *t*-test; statistical significance: (*) $P < .05$ in relation to control non-infected cultures. *N. caninum* tachyzoites and sites of parasitophorous vacuoles (white arrows) are evident in infected glial cell cultures (D) after DAPI staining; objective 40×, scale bar 50 µm. (E) Means of the number of tachyzoites/field in infected cultures after 24 hours.

Sample extraction and HPLC-DAD analysis of Trp metabolites

For chromatographic analysis, the samples (SGNc and SGC) were filtered through a 0.22-µm membrane, precipitated in ice pure methanol and centrifuged at 11 000*g* for 15 minutes, with 80 µL of the supernatant being analyzed. The secretome was analyzed in the High-Performance Liquid Chromatography (HPLC) system from Agilent (model infinity II 1260) coupled to a Diode array Detector (DAD). In order to elute the samples, a run took place in a reversed phase (300SB-C18) (150 mm × 4.6 mm i.d., 5 µm). The analysis was performed by gradients (methanol/acetonitrile acidified with 0.1% of formic acid) with its gradient increased linearly from 0% to 50% of acetonitrile in 8 minutes, going to 80% at 10 minutes and to 100% at 11 minutes, remaining in this condition for 12 minutes with a flow of 400 µL/minutes. The detection of the eluates by the photodiode was made between 280 and 360 nm. The samples were compared with chromatographic standard quinolinic acid, kynurenic acid, 3-hydroxykynurenine and tryptophan (Sigma–Aldrich, St. Louis, MO, USA), all diluted in water at a concentration of 500 µM.

Statistical analysis

Statistical analyses were performed with the GraphPad Prism software, version 5.0 for Windows (GraphPad Software, San Diego, California). Student's *t*-test was used for comparisons between the 2 groups. Results were expressed by means ± standard error of the mean (SEM). Values of $P < .05$ were considered as significant.

Results

N. caninum *infection alters the morphology of glial cells*

Aiming to determine the effect of *N. caninum* infection on glial cell response and reactivity, we performed immunocytochemistry (ICQ) for the glial fibrillary acidic protein (GFAP), and ionized calcium-binding adaptor molecule 1(Iba-1), respectively structural markers of astrocytes and microglia. After 15 days, glial cell cultures presented a confluent monolayer with astrocytes predominantly with polygonal morphology (Figure 1A). On the other hand, 24 hours after *N. caninum* tachyzoites, the cultures induced a significant increase in the proportion of astrocytes expressing GFAP$^+$ protein, which reached 73.8% ± 3.3% of the total of cells, compared to control cultures (31.0% ± 2.6%), associated with changes on the morphology presenting a star-like shape, with prominent extensions and centralized nucleus, characterizing astrogliosis (Figure 1B and C). The presence of *N. caninum* tachyzoites and sites of parasite proliferation was observed through DAPI staining and parasite courting reached about 68.0% ± 11.55% (Figure 1D and E).

In control glial cell cultures, Iba-1 immunostaining revealed the majority of microglia with typical branched phenotype (Figure 2A). On the other hand, Iba-1 immunostaining

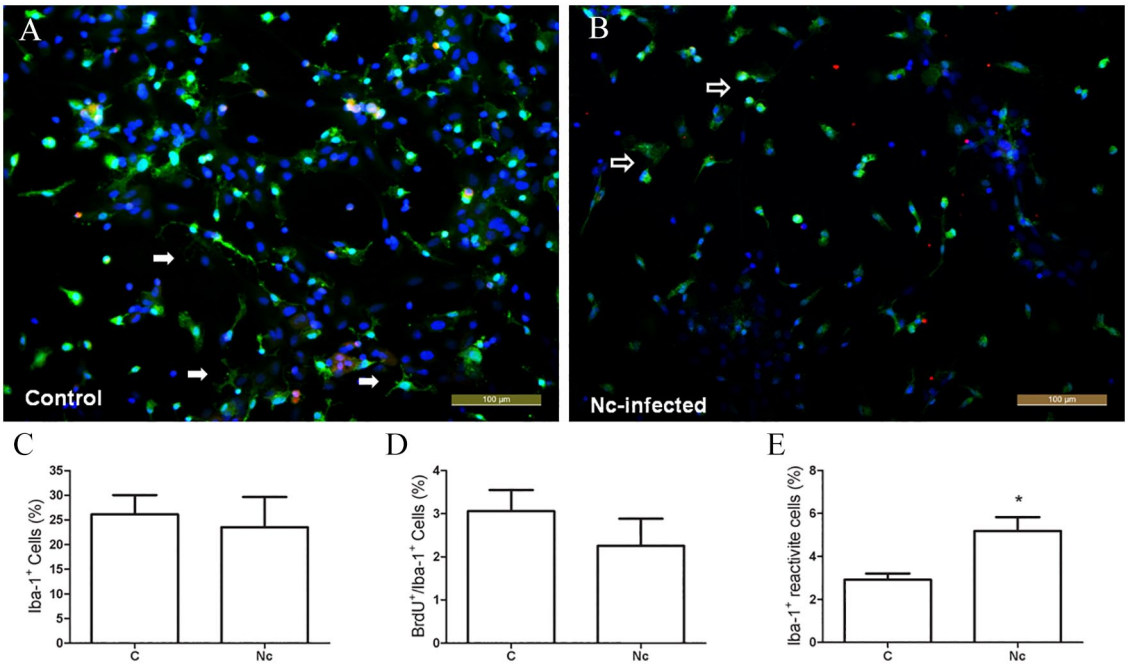

Figure 2. Effects of *N. caninum* infection in glial cell cultures: morphology and activation of microglia. Analysis with immunocytochemistry for Iba-1 (green): cultures were maintained in control conditions (C) (A) or infected with *N. caninum* tachyzoites (Nc) (B); nuclei were stained with DAPI (blue); objective ×20, scale bar 100 µm. (C) Percentage of Iba-1$^+$ cells in control and *N. caninum*-infected cultures. (D) Percentage of BrdU$^+$ in microglia cells in control and *N. caninum*-infected cultures. (E) Percentage of reactive (round or with shorter process) microglia (Iba1$^+$) in control and *N. caninum*-infected cultures; groups were compared with *t*-test; statistical significance: (*) $P < .05$ in relation to control non-infected cultures.

revealed the majority of microglia with a typical reactive phenotype in *N. caninum*-infected cultures (Figure 2B). Quantification demonstrated that the proportion of Iba-1$^+$ cells did not change in infected cultures in relation to the control cultures (Figure 2C), nor did the proportion of proliferating microglia (Iba-1$^+$/BrdU$^+$ cells) (Figure 2D). However, the proportion of reactive microglia in the *N. caninum*-infected cultures (51.8% ± 6.4%) was significantly higher to the proportion of reactive microglia in control cultures (22.5 ± 1.5) (Figure 2E).

Glial cells respond to infection by N. caninum *expressing mRNA for an inflammatory profile*

In order to evaluate the inflammatory response to infection of glial cell cultures with the parasite *Neospora caninum*, mRNA expression for inflammatory markers was measured by *RT*-qPCR. Considering inflammatory cytokines, glial cell cultures infected with tachyzoites of *N. caninum* showed after 24 hours a significant increase of about 7.8-folds in relative mRNA expression for TNF (Figure 3A) and 1.5-fold in relative mRNA expression for IL-1β (Figure 3B) compared to cell cultures under control non-infected condition. On the other hand, the levels of mRNA for the cytokine IL-6 in infected cultures were not significantly different from those observed in control cultures (Figure 3C). Otherwise, the relative expression of mRNA for the regulatory molecules IL-10 and arginase was significantly increased in *N. caninum*-infected cultures. Compared to control non-infected cultures, the relative mRNA expression for the interleukin IL-10 increased 19.8-folds in infected cultures (Figure 3D). Expression of mRNA for arginase also increased 1.5-fold in infected cultures (Figure 3E). Moreover, relative mRNA expression for chemokines CCL5 (Figure 3F) and CCL2 (Figure 3G) was also increased in about 29.3-folds and 21.5-folds in infected cultures, respectively.

Infection of glial cells with N. caninum *modulates tryptophan catabolism*

The effect of *N. caninum* infection of glial cells on the tryptophan catabolism was firstly assessed by measuring the relative expression of the 2 key enzymes involved in this pathway. An 8-fold increase in the relative expression of mRNA for KMO was observed in infected cultures, while expression of KAT, an enzyme expressed mainly by the astrocytic phenotype, was not changed after infectious stimulus (Figure 4A and B).

In order to evaluate the qualitative production of tryptophan and KP metabolites, the secretome of the control and infected cultures was analyzed by means of HPLC and the profiles of elution of Trp, QUIN, and KYNA in each condition were compared (Figure 5). The samples of non-infected cell cultures showed a chromatographic peak, like that of the Trp standard, at around 9 minutes of retention in the chromatographic column. On the other hand, the secretome from cultures infected by the parasite showed a chromatographic peak at around 13.50 minutes, consistent with the molecular pattern of the QUIN.

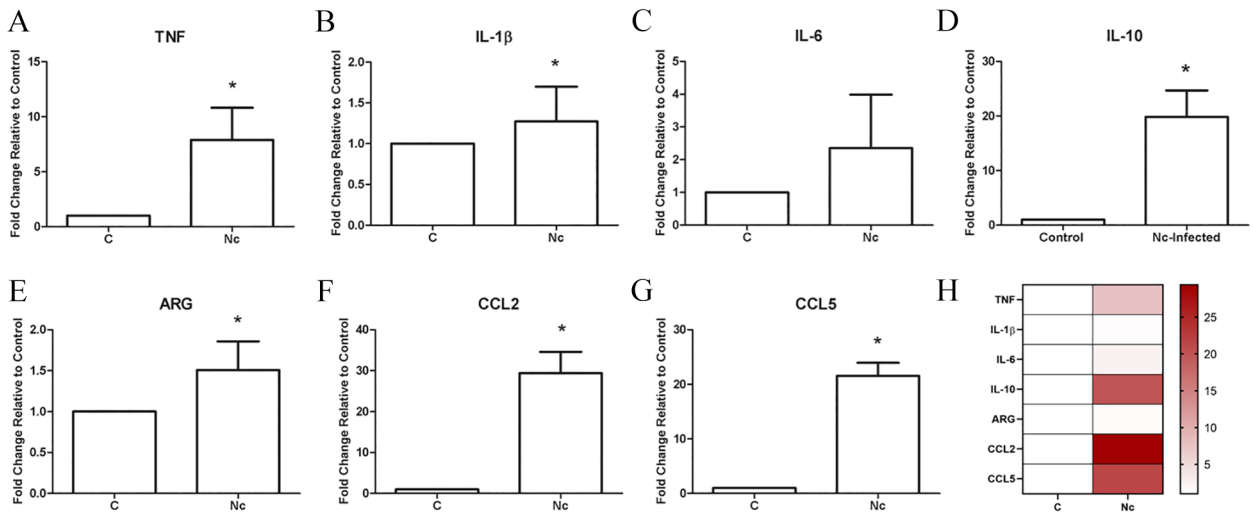

Figure 3. Effects of *N. caninum* infection in inflammatory profile of glial cells in primary cultures. Control and *N. caninum*–infected (Nc) cultures were analyzed through RT-qPCR after 24 hours. The relative mRNA expression for TNF-α (A), *IL-1*β (B), *IL-6* (C), *IL-10* (D), arginase (E), CCL5 (F), and CCL2 (G) was determined with the $2^{-\Delta\Delta Ct}$ method using β-actin for normalization of gene expression. (H) Heat map of inflammatory genes showed an important effect of *N. caninum* infection onto mRNA expression of genes involved in the control of neuro-inflammation. mRNA relative expression in both conditions was compared with the *t*-test. Data are shown as media of folds of increase in infected cultures relative to control cultures, in 3 independent experiments. Statistical significance (*) $P < .05$ compared to the control (non-infected).

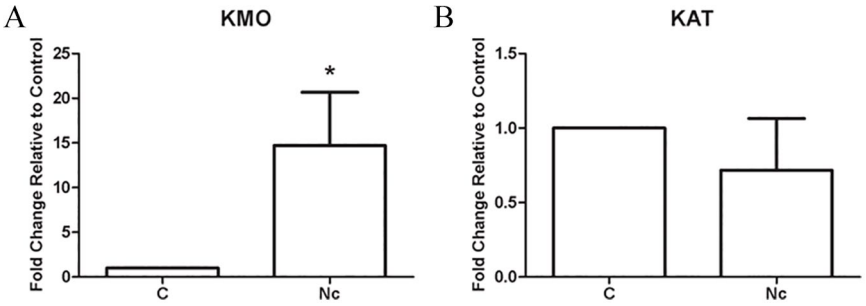

Figure 4. Effects of tryptophan catabolism of glial cells during *N. caninum*-infection. Control and *N. caninum*–infected (Nc) cultures were analyzed through RT-qPCR after 24 hours. The relative mRNA expression for T KMO (A) and for KAT (B) was determined with the $2^{-\Delta\Delta Ct}$ method using β-actin for normalization of gene expression. mRNA relative expression in both conditions was compared with *t*-test. Data are shown as media of folds of increase in infected cultures relative to control cultures, in 3 independent experiments. Statistical significance (*) $P < .05$ compared to the control (non-infected).

Neurons and glia in co-cultures modulated with the secretome derived from N. caninum*-infected glial cultures presented morphological changes compatible with neuro-preservation*

With the purpose of evaluating the effect of *N. caninum* infection of glial cells on neuronal phenotype and toxicity, glia-neuron co-cultures were exposed for a period of 24 hours to the secretome derived from infected glial cell cultures (SGNc) or derived from control non-infected cultures. Neuronal cells were analyzed through immunostaining for the specific neuron structural protein, β-III-tubulin, and for GFAP, the structural marker of astrogliosis. In the co-cultures of cells treated with SGNc (Figure 6D–F), one can observe a greater number of neurons, showing greater distribution in the culture and formation of neurite extensions, compared to the glia-neuron co-culture exposed to the secretome derived from control non-infected glial cultures (SGC) (Figure 6A–C), suggestive of neuroprotection. Moreover, the same pattern of reactive astrocyte morphology was observed in the co-culture of cells treated with SGNc compared with those observed in glial cell cultures infected with *N. caninum*.

Discussion

The response of glial cells to infection by *N. caninum* was well characterized in vitro by Pinheiro et al[12,13,25] and demonstrated that astrocytes, important cells for homeostasis in the CNS, are the target for this parasite. Studies carried out in organotypic cultures of rat brain slices showed that astrocytes are also the cells predominantly infected with *N. caninum*, acquiring reactive phenotype with filamentous and elongated morphology when immunoassayed for GFAP.[11,26] In the present work, astrocytic reactivity was observed in glial cultures infected with *N. caninum*, due to increased GFAP expression, as well as an increase in mRNA expression of TNF, IL-1β, and IL-10. The release of TNF and IL-10 in glial cells infected by *N. caninum* was already demonstrated with ELISA[12,13,25] .

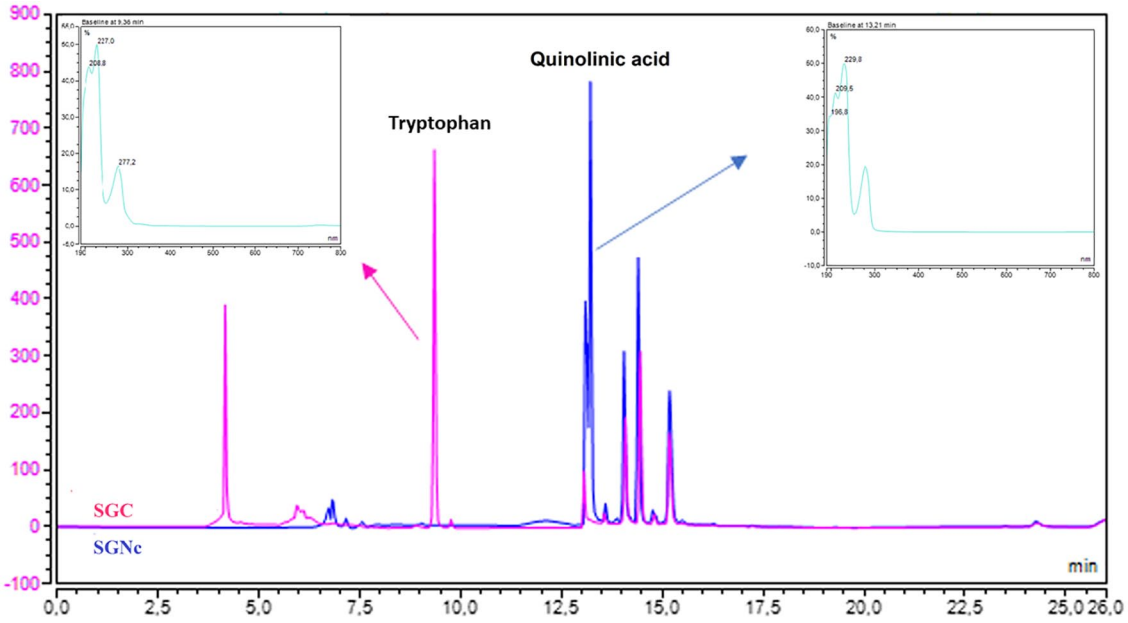

Figure 5. HPLC-DAD analysis performed with the secretome from glial cell cultures infected or not with *N. caninum* tachyzoites. The chromatogram shows the pattern of elution of control non-infected (SGC, pink) of infected cultures (SGNc, blue); the peak for tryptophan is visible at around 9 minutes and the peak for quinolinic acid is visible at 13.10 minutes of retention; the analysis was carried out with a comparison title between 3 independent experiments.

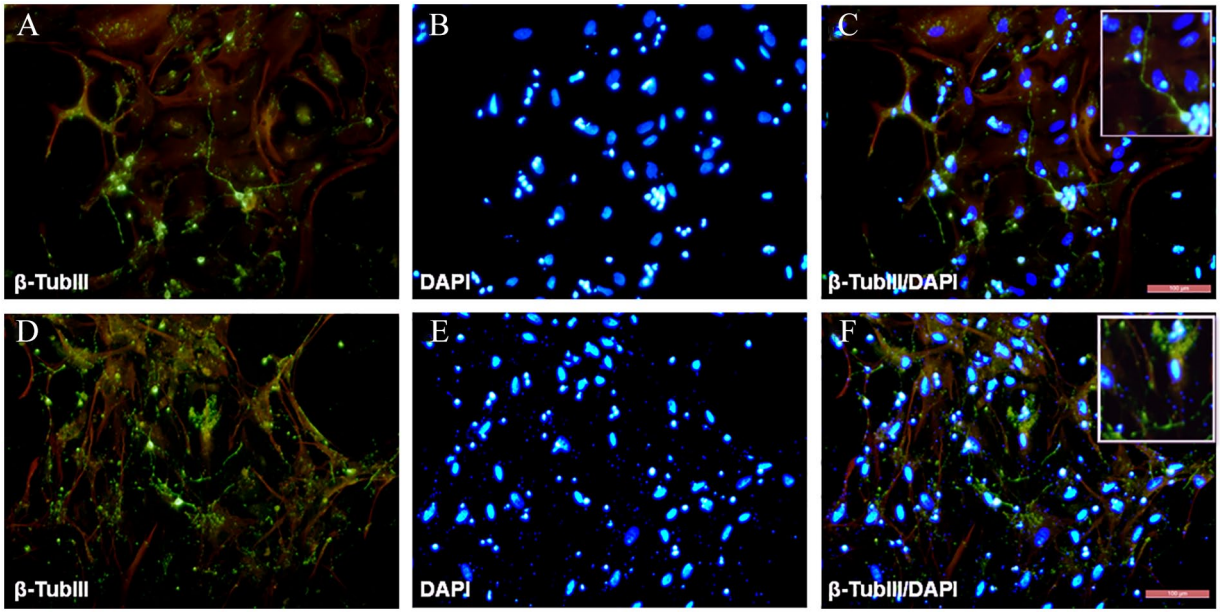

Figure 6. Morphological changes compatible with neuroprotection induced by the secretome of *N. caninum*-infected glial cell cultures. Analysis through immunocytochemistry for the proteins β-TubIII (green), a structural marker of neurons, and GFAP, a structural marker of astrocyte (red) in glia-neuron co-cultures exposed for 24 hours to the secretome derived from control non-infected glial cell cultures (A–C) or to the secretome derived from glial cell cultures infected with *N. caninum* tachyzoites (D–F); nuclei were stained with DAPI (blue); obj. ×20, scale bar 100 µm. Images are representative of 3 independent experiments.

Concerning the microglia, the infection changed them to a dimorphic phenotype, with short extension processes and a decrease in cell number. Variations in morphology and microglia density result in changes in function that can be impaired in surveillance and response to injury.[27] It was also found that the inflammatory response of these glial cells infected with tachyzoites of *N. caninum* is characterized by the modulation of arginase, as well as chemokines CCL5/RANTES and CCL2/MCP-1, which have the function of recruiting cells from the immune system and activating microglia to control the parasite infection. CCL5 also plays a role as neuromodulator, presenting protective properties in

CNS cells.[28] The combination of CCL5, CCL2, IL-10 mediators, and arginase activity in the results herein presented corroborates with the mechanisms of evasion and encystment of neurotropic parasites described by Hemphill et al[26] and with an induced tissue preservation observed in a similar model by Grangeiro et al.[14]

The microglial response to parasites is the first line of defense in the brain and the cytokines IL-1β TNF and INF-γ are responsible for the microgliosis[14,29] observed in glia-neuron co-cultures infected with *N. caninum*. In the present study, we also demonstrated microgliosis and an increase in the mRNA expression of these cytokines after *N. caninum* infection.

Recently, Jesus et al[16] demonstrated that the infection of glial cells by *N. caninum* also activated Trp catabolism and that could be an antiparasitic mechanism. The results herein described showed that infection of glial cell cultures by *N. caninum*, besides astrogliosis, also increased the expression of mRNA KMO. In physiological conditions, astrocytes do not express KMO, but in pathological situations, these cells can be stimulated to produce kynurenine in large amounts. Captured by the microglia, that activates KMO to produce QUIN.[30]

The *N. caninum* infection, inducing the production of QUIN, an agonist of NMDA glutamate receptors,[31] also modulates protein synthesis by the action of NAD^+.[3,30] Rodriguez Cetina Biefer et al[32] described a notable deviation of KP to produce NAD^+ that is associated to immune response by modulating the production of inflammatory cytokines IL-1β, TNF, and IL-6.[30] According to Tullius et al[18] and Wang et al,[19] this nucleotide also changes the pro-inflammatory profile into a resolutive response by the release of IL-10 and arginase. Our results corroborate with that and with Jesus et al,[16] who described, in a similar model of infected cell cultures, IDO1 activation favoring the control of parasitic proliferation. Activation of host antimicrobial mechanisms usually needs Trp, nitric oxide production,[9] generation of reactive oxygen species,[33] iron requirement,[34] and arginase.

Activation of Trp catabolism is an antiparasitic response of hosts to *Toxoplasma gondii*,[35] a parasite that belongs to the same family as *N. caninum*. According to Pfefferkorn et al,[36] in T.gondii infection, IFN-γ induces IDO-1 expression and activity, besides causing Trp deprivation to parasites.[20] Overexpression of IDO-1[32,37] as well as of tryptophan oxydase (TDO)[5] also inhibits the growth of *T. gondii* in vitro. This mechanism of toxoplasma growth control induced by IFN-γ seems to vary from species to species and from one cell type to another.

Once Trp is essential for both cellular and parasitic activities, our results motivated the investigation of the effect of infectious stimulus by *N. caninum* on neuron integrity. The results showed that the secretome derived from infected glial cultures preserved neurites, showing that the inflammatory response through astrogliosis and the expressive release of IL-10 bypass complications of the infectious process in order to preserve damaged tissue.

The set of these data proves that the infection of glial cells by *N. caninum* activates the KP with chemotactic deviation for microglial activation, resulting in increased expression of KMO and QUIN. Additionally, the anti-inflammatory response by astrocytosis collaborates to neuro-preservation.

Author Contributions

DSA performed all experimentation, analyzed, interpreted the data and wrote manuscript. JMPB, LSF, GAP, and MSG helped to perform cells and *N. caninum* cultures immunohistochemistry and microscope images. RC and AB contributed to HPLC and chemical analysis. VDAS, AMP, and GG revised it critically for intellectual content. GG, SLC, and MFDC supervised the study, edited, and reviewed the manuscript. All authors read and approved the final manuscript.

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