Immunopathology and Infectious Diseases

Toxoplasma gondii Prevents Neuron Degeneration by Interferon- γ -Activated Microglia in a Mechanism Involving Inhibition of Inducible Nitric Oxide Synthase and Transforming Growth Factor- β 1 Production by Infected Microglia

Claudia Rozenfeld,^{*,†} Rodrigo Martinez,[†]
Sérgio Seabra,^{*} Celso Sant'Anna,^{*}
J. Gabriel R. Gonçalves,^{*} Marcelo Bozza,[‡]
Vivaldo Moura-Neto,[†] and Wanderley De Souza^{*}

From the Laboratório de Ultraestrutura Celular Hertha Meyer,^{*}
Instituto de Biofísica Carlos Chagas Filho, the Departamento de
Anatomia,[†] Laboratório de Morfogênese Celular, Instituto de
Ciências Biomédicas, and the Departamento de Imunologia,[‡]
Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Interferon (IFN)- γ , the main cytokine responsible for immunological defense against *Toxoplasma gondii*, is essential in all infected tissues, including the central nervous system. However, IFN- γ -activated microglia may cause tissue injury through production of toxic metabolites such as nitric oxide (NO), a potent inducer of central nervous system pathologies related to inflammatory neuronal disturbances. Despite potential NO toxicity, neurodegeneration is not commonly found during chronic *T. gondii* infection. In this study, we describe decreased NO production by IFN- γ -activated microglial cells infected by *T. gondii*. This effect involved strong inhibition of iNOS expression in IFN- γ -activated, infected microglia but not in uninfected neighboring cells. The inhibition of NO production and iNOS expression were parallel with recovery of neurite outgrowth when neurons were co-cultured with *T. gondii*-infected, IFN- γ -activated microglia. In the presence of transforming growth factor (TGF)- β 1-neutralizing antibodies, the beneficial effect of the parasite on neurons was abrogated, and NO production reverted to levels similar to IFN- γ -activated uninfected co-cultures. In addition, we observed Smad-2 nuclear translocation, a hallmark of TGF- β 1 downstream signaling, in infected microglial cultures, emphasizing an autocrine effect restricted to infected cells. Together, these data may explain a

neuropreservation pattern observed during immunocompetent host infection that is dependent on *T. gondii*-triggered TGF- β 1 secretion by infected microglia. (Am J Pathol 2005, 167:1021–1031)

Although up to one third of the human population of the world is infected with the protozoan parasite *Toxoplasma gondii*¹ a pattern of an asymptomatic chronic infection is prevalent in most immunocompetent individuals² who harbor parasite cysts especially in the central nervous system (CNS).^{3,4} Considering the lifelong presence of the parasite, hosts infected with *T. gondii* must develop a powerful immune response that has to be under tight control⁵ and persistently maintained in all infected tissues.^{4,6} This continuous immune vigilance is essential to prevent reactivation of chronic diseases, which are accompanied by a high morbidity of CNS symptoms that can result in life-threatening toxoplasmic encephalitis.⁶ Cell-mediated immune mechanisms play a major role in the control of *T. gondii* infection because the parasite^{7,8} is exclusively localized intracellularly.

Interferon (IFN)- γ is the main cytokine involved with acute as well as with chronic resistance to *T. gondii* infection^{9–12} and recent studies have shown that the production and the response to IFN- γ must occur on both hematopoietic and nonhematopoietic cell lines to acquire an optimal protective host effect.^{7,13} In this way the continuous presence of IFN- γ in the CNS and its effect on

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Address reprint requests to Claudia Rozenfeld, Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, CCS, Bloco G, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21944-590, Brazil. E-mail: rozen@biof.ufrj.br.

resident CNS cells have been considered highly relevant mechanisms in keeping a host benign infection.^{7,14,15}

Microglia cells are considered the resident macrophages of the brain because they are reactive participants in immune responses in the CNS, and recently have been considered an important source of IFN- γ during *T. gondii* infection.¹⁶ Several authors have proposed that microglia play a major role in the control of infections caused by *T. gondii*^{17–19} and other protozoa, as well as being involved in pathophysiological alterations²⁰ of these infections. Microglial immune functions are induced during restricted steps of normal CNS development²¹ and in areas of neuronal infection or injury it comes out of its state of resting and becomes active producing cytokines, growth factors, complement system molecules, and reactive species of oxygen/nitrogen such as nitric oxide (NO).^{22–24}

NO results from the oxidative change of L-arginine to L-citrulline by several NO synthase isoforms (NOS).²⁵ The inducible form of NO synthase, iNOS, is strongly regulated by cytokines, with some of them acting to induce enzyme expression (IFN- γ , tumor necrosis factor- α), and others acting as inhibitory cytokines [transforming growth factor (TGF)- β , interleukin (IL)-4, IL-10, and IL-13].^{26,27} Microglial cells may up-regulate the enzyme iNOS after inflammatory stimulus, resulting in a high production level of NO.²³ This short-lived, highly reactive molecule interferes with multiple metabolic pathways required for pathogen survival but may paradoxically damage host tissue.²⁸ Neurons are extremely susceptible to the noxious effects of NO, which exerts a central role on a wide range of neurodegenerative and demyelinating diseases of the CNS such as: multiple sclerosis,^{29,30} Alzheimer's disease,³¹ Parkinson's disease,³² and the AIDS-related dementia complex.³³ Surprisingly, neuron degeneration is not commonly found during *T. gondii* infection on immunocompetent hosts although a continuous immune response accompanies the persistence of the parasite in the CNS.

The cytokine TGF- β 1 is the most abundant and best studied TGF- β isoform and it is an important component of the brain's response to injury. It is consistently increased after various forms of brain insults and in neurodegenerative diseases,³⁴ as well as being detected during the infection of microglia³⁵ by *T. gondii*. Transforming growth factor- β (TGF- β) superfamily member signals are conveyed through cell-surface serine-threonine kinase receptors to the intracellular mediators known as Smads. Activation of Smads causes their translocation from cytoplasm to the nucleus where they control gene expression,^{36,37} modulating several proteins including iNOS, which is involved with local inflammation.^{38,39}

We have recently described an *in vitro* indirect neuron-protective effect of *T. gondii* infection, dependent on inhibition of NO production by activated microglial cells, which is indirectly regulated by infected astrocytes.⁴⁰ This phenomenon was shown to be mediated by PGE₂ secretion from infected astrocytes followed by IL-10 production by IFN- γ -activated microglia.⁴⁰ Considering these data, the aim of the present study was to investigate a possible direct effect of *T. gondii* infection on

IFN- γ -activated microglia cells that could favor neuron preservation, taking into consideration that, in addition to astrocytes, microglial cells are also able to harbor parasites.¹⁷ The observations here show that the inhibitory effect of the parasite on iNOS expression by IFN- γ -activated microglia seems to be dependent on TGF- β 1 production by *T. gondii*-infected microglia, resulting in Smad-2 nucleus translocation, inhibition of NO production and neuron preservation.

Materials and Methods

Reagents and Antibodies

Chicken anti-TGF- β 1-neutralizing antibody was obtained from R&D Systems. Mouse anti-human β -tubulin III antibody, aspirin (ASA), and murine recombinant IFN- γ were purchased from Sigma. Polyclonal antibody against iNOS, polyclonal antibody against Smad-2, and polyclonal antibody against TGF- β 1 were obtained from Santa Cruz. The secondary antibodies used in this study were goat anti-rabbit fluorescein isothiocyanate-conjugated and goat anti-mouse Alexa, goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated, which were purchased from Gibco BRL, and goat anti-rabbit cy3-conjugated from Sigma.

Parasites

Tachyzoites from the virulent RH strain of *T. gondii* were maintained within the intraperitoneal passages in Swiss Webster mice and were harvested for *in vitro* studies 2 days after infection. Mice were killed by CO₂ inhalation and free tachyzoites were recovered from the peritoneal cavity after instilling 5 ml of Dulbecco's modified Eagle's medium (DMEM)/F12. The fluid obtained from infected mice was centrifuged at 200 $\times$ *g* for 7 minutes at room temperature to remove host cells and debris. The parasite-containing supernatant was collected and centrifuged at 1000 $\times$ *g* for 10 minutes. The pellet obtained was resuspended to a density of 10⁶ parasites/ml in DMEM-F12. The parasites were then used within 30 to 40 minutes, and the viability was evaluated using a dye exclusion test with trypan blue.

Serum of *T. gondii*-Infected Mice

Chronically infected mice, orally infected with Pe strain (2 to 3 months), received a boost of RH tachyzoites (10⁴) 15 days before the blood puncture and the obtaining of the serum by centrifugation. The serum of the control animal was also obtained but did not exhibit *T. gondii* staining in contrast to the serum of infected animals. In the tests using the titration specified in the paper, neither background nor nonspecific staining was observed on using the serum of infected animals.

Microglial Cultures

Murine astrocytes from BALB/c mice were cultured from the brain cortex of neonatal mice (age, between E-18 and P-0), following the procedure previously described,⁴¹ with some modifications.⁴⁰ After 14 to 15 days, microglial cells were detached from the astrocyte monolayer by shaking the culture flasks for 30 minutes. The supernatants were collected and centrifuged, and the cells were reseeded on 24-well tissue culture chamber slides (Nunc, Inc.) with 5.5-mm diameter glass coverslips, at a final concentration of 5×10^5 cells/well in 500 μ l of medium. After 40 minutes, the medium was replaced to remove nonadherent cells, and microglial cells were allowed to grow for an additional 24 to 48 hours before the experiments were started. Cells were found to be 98% microglia as judged by positive staining with isolectin b4 (peroxidase-labeled lectin from *Bandeiraea simplicifolia* BS-I, obtained from Sigma).

Microglial Infection and Activation

After washing three times in serum-free DMEM/F12, microglial cells were allowed to interact with low parasite loads (10:1 and 1:5, host cell:parasite ratio) for 2 hours. To eliminate free parasites, after this period, cultures were washed three times with DMEM/F-12. The cells were then activated with IFN- γ (500 U/ml) in a final volume of 500 U/well, for a period of 18 to 24 hours. No cell lysis was observed in this period.

Cytokine Determination

Supernatants were tested for IL-10 using a murine sandwich enzyme-linked immunosorbent assay kit (Pharmin-gen, La Jolla, CA) according to the manufacturer's instructions.

Terminal dUTP Nick-End Labeling (TUNEL) Assay

Apoptotic cells were detected by TUNEL assay using APO-BrdU TUNEL assay kit (A-23210; Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

Neuron Microglia Co-Cultures

Primary dissociated cortical neurons were prepared as previously described⁴² with some modifications. Briefly, timed pregnancy mice were sacrificed on the 17th or 18th gestational day, and embryos were removed by caesarian section. After cortex dissection as described above for glial cultures, cells were dissociated in DMEM/F-12 medium supplemented with 10% fetal bovine serum. Neurons (10^5 /well) were then plated on top of microglial monolayers, previously infected or not, 2 hours before, as described above. After 1 hour of seeding neurons, the medium was carefully replaced (500 μ l/well) and then

IFN- γ (500 U/ml) was added in the absence or in the presence of TGF- β 1-neutralizing antibodies (20 ng/ml) or ASA (100 μ mol/L) for 24 hours.

Immunocytochemistry Procedures

As previously described,⁴⁰ cultured cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100, and endogenous peroxidase activity was abolished with 3% H₂O₂. Cells were incubated with 5% bovine serum albumin (Gibco BRL) in PBS (blocking solution) and subsequently with the specified mouse anti-human β -tubulin III antibody (1:400 dilution, Sigma), diluted in blocking solution. Then, the cells were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (1:200 dilution) and peroxidase activity was revealed using a diaminobenzidine peroxidase substrate kit, which stained the cells black (Vector Laboratories). After this first step, in experiments of double staining, incubation with anti-iNOS polyclonal antibody was performed (1:100), followed by incubation with secondary horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:200 dilution). Secondary revelation was performed using the VIP (purple) substrate kit (Vector Laboratories). All antibodies were diluted in blocking solution. The preparations were dried in air and coverslips were mounted in Entellan (Merck).

Immunofluorescence Procedures

Cultured cells were fixed with 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) for 20 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Cells were incubated with 5% bovine serum albumin (Gibco BRL) plus inactivated normal mice serum (1:100) in PBS (blocking solution) for 30 minutes. Subsequently cells were incubated with anti-iNOS polyclonal antibody for 1 hour, followed by incubation with anti-rabbit fluorescein isothiocyanate-stained antibody (1:500). *T. gondii* tachyzoites were stained using serum (1:100) of chronically infected mice (Pe strain) boosted 1 week before bleeding with parasites of the same strain. After incubation for 1 hour at room temperature, a secondary goat anti-mouse rhodamine-stained antibody (1:500) was used. The same procedures, with minor changes, were used with Smad-2 immunostaining (1:50). The incubation was performed overnight at 4°C, followed by secondary anti-rabbit Cy3 stained antibody (1:5000) incubation for 1 hour. All antibodies were diluted in blocking solution. The preparations were washed several times in PBS between all steps and then coverslips were mounted in *N*-propyl-gallate solution.

Measurement of NO Production

Supernatants from microglial cells and neuron-microglia co-cultures were assayed for nitrite content, which reflects NO production, using Griess reagent (0.1% naphthylethylene diamine dihydrochloride and 1% sulfanil-

amide plus 2.5% phosphoric acid in equal volumes) as described previously.⁴³

Morphometry

Neurons stained for β -tubulin III were photographed in a Nikon microscope. Photos were scanned and neurite length was analyzed using the Sigma Scan Pro Software (Jandel Scientific). Three independent experiments were performed and at least 100 neurons were counted per sample in six or seven randomly chosen fields.

Statistical Analysis

Data were analyzed by Student's *t*-test or analysis of variance. Probability values (*P*) of 0.05 or less were considered significant.

Results

T. gondii Down-Modulates NO Production by IFN- γ -Activated-Microglia in a Parasite Load-Dependent Manner by a Mechanism Involving iNOS Inhibition

An inhibitory effect of the *T. gondii* on NO production by IFN- γ -activated microglia mediated indirectly by soluble factors released by infected astrocytes was recently described.⁴⁰ Microglia, besides astrocytes, are also a target of the parasite during CNS infection, and so the direct effect of the *T. gondii* on NO produced by microglial cells was tested. As shown in Figure 1, treatment of microglial cells with IFN- γ for 16 to 18 hours induced a strong NO production as attested by measurement of nitrite accumulation in the supernatant. A suppressive effect of the parasite on NO production by IFN- γ -activated microglia was clearly observed even in parasite loads as low as 5:1, host cell:parasite ratio (*P* < 0.05). This NO inhibitory effect was higher using increased parasite load infection (*P* < 0.01), reaching control levels, without apparent cell lysis detection. This result suggests that the inhibition of NO production was directly correlated with the parasite: host cell ratio used.

Although NO synthesis by IFN- γ -activated microglia is mainly dependent on iNOS using L-arginine as precursor, nonenzymatic production may also occur. In addition some pathogens may lead to a reduction of nitrite levels by acting as scavengers or consuming it⁴⁴ and consequently altering the bioavailability of NO. To investigate if the reduction of nitrite levels detected after *T. gondii* infection was correlated with a down-modulation of iNOS expression, immunofluorescence microscopy localization of iNOS was performed. Microglial cells in the absence of stimulation, showed undetectable basal levels of iNOS expression (Figure 1B), in contrast to IFN- γ -activated microglia where the enzyme was overexpressed (Figure 1C). This observation is in agreement with NO detection in cell supernatants (Figure 1A), strongly suggesting an enzymatic source of NO produc-

Figure 1. Inhibition of NO production by IFN- γ -activated microglia is proportional to parasite load and is mediated by down-modulation of iNOS expression during *T. gondii* infection of microglial cells. **A:** Microglial cells were allowed to interact with *T. gondii* at different parasite loads (10:1, 5:1, 1:1, 1:5), for 24 hours, in the presence of IFN- γ . After this period the supernatant was harvested and nitrite concentration was measured by Griess reaction. Negative (cont) and positive controls (IFN- γ) were maintained in the absence of the parasite. Data represent the mean SDs (error bars) of three independent experiments. **P* < 0.05 ***P* < 0.01. Microglial cells were also submitted to iNOS immunostaining under the following conditions (**B** and **E**). **B:** Control cells maintained in fresh medium expressed nondetectable amounts of iNOS. **C** and **D:** The treatment with IFN- γ (500 U/ml) induced a strong expression of iNOS by microglia (**C**), that was completely abrogated by the simultaneous infection with *T. gondii* following a host cell:parasite ratio of 1:5 (**D**). **E:** In the absence of IFN- γ , in *T. gondii* the expression of iNOS did not alter, as observed in the control. **B, C, D,** and **E** are fluorescence images; **F, G, H,** and **I** are corresponding phase contrast images.

tion after IFN- γ microglia activation. In the presence of a high *T. gondii*:host cell ratio 5:1 of infection, the expression of iNOS by IFN- γ -activated microglia was practically undetectable (Figure 1D). In the same manner, *T. gondii*-infected microglial cells in the absence of IFN- γ expressed very low levels of iNOS, similar to control (Figure 1E), suggesting that, in fact, the parasite was specifically interfering in the IFN- γ activation pathway. To investigate if this NO inhibition could result in a higher loss of microglia viability in infected cultures, trypan blue assay was applied. However, it was observed that the loss of viability induced by IFN- γ plus *T. gondii* (10%) was similar to the microglia cells activated only with IFN- γ in the absence of the parasite, despite the differences in NO detection (data not shown).

T. gondii Exerts an Autocrine Effect Inhibiting iNOS Expression by IFN- γ -Activated Infected Cells That Is Not Correlated with Microglial Apoptotic Death

The modulation of iNOS expression observed here could be a phenomenon restricted to the infected cells, or could also be affecting all cells in the culture, by a

Figure 2. iNOS expression impairment in culture is restricted to the microglia cells infected by *T. gondii*, which were not apoptotic. Microglial cells were activated with IFN- γ (500 U/ml) after the infection with *T. gondii* (host cell:parasite ratio of 1:5) for 24 hours. After this period the cells were submitted to a double immunostaining to iNOS (A) and *T. gondii* (B). On the corresponding confocal sections (C, D) *T. gondii* appears in red (rhodamine) and iNOS in green (fluorescein isothiocyanate). The inhibition of iNOS expression was not observed in the uninfected cells (arrows), as shown by this representative field (D). Microglial cells were also submitted to a double immunostaining to iNOS (E) and TUNEL assay to detect apoptotic cells (F). Through corresponding phase contrast images (G) apoptotic TUNEL-positive cells (F) can be detected as uninfected cells (arrows).

paracrine effect of the infected cells. To evaluate this, IFN- γ -activated microglia were infected using a low *T. gondii*:host cell ratio (1:5). The cultures were double stained to iNOS (green) and *T. gondii* (red) to evaluate individual iNOS expression of cells infected or not with *T. gondii*. After 18 to 24 hours a strong inhibition of iNOS expression was observed in infected cells (double staining), which however was not observed in uninfected neighboring cells on the corresponding confocal overlay sections (Figure 2; A to D). These data strongly suggest an autocrine effect of the parasite and only a slight paracrine effect on uninfected IFN- γ -activated microglia. However, a low rate of $\sim 8.71 \pm 1.04\%$ of IFN- γ -activated, infected microglia, which maintained the iNOS expression despite the presence of the parasite, was observed. In general these cells harbor parasite forms that appear to be nonreplicating and did not form rosettes (data not shown).

To investigate if the prevalent iNOS inhibition could result from an apoptotic death of infected cells, TUNEL

assay was performed in addition to iNOS immunostaining (Figure 2; E to G). In fact apoptotic microglial cells in the cultures were rarely observed and, in the few fields where they were seen (Figure 2F), these cells were iNOS-negative (Figure 2E) but uninfected, as shown with the corresponding phase contrast images (Figure 2G).

The Inhibition of iNOS Expression by T. gondii-Infected IFN- γ -Activated Microglia Leads to Neuron Protection

Neurons are particularly vulnerable to the toxic effect of high levels of nitrogen species produced by microglia activated by inflammatory cytokines such as IFN- γ .^{40,45} The next step was then to investigate if the inhibition of iNOS expression and consequent reduction of NO microglial production induced by the parasite could lead to neuron preservation. To answer this question neurons were co-cultured onto IFN- γ -activated microglia infected with *T. gondii*. The neurite outgrowth was used as a parameter of neuronal viability. Neuron microglia-uninfected co-cultures, maintained for 24 hours in fresh medium, were used as a control. In this way an increase in neurite outgrowth and a practically undetectable expression of iNOS by microglia (Figure 3, A and E) were observed. In the presence of IFN- γ (500 U/ml) a drastic impairment of neurite outgrowth was accompanied by a strong expression of iNOS by microglial cells (Figure 3, B and E). However, in the presence of IFN- γ -activated and -infected microglia, neurite outgrowth totally recovered, and the iNOS expression strongly reduced (Figure 3, C and E). No changes in neuritogenesis or iNOS expression were observed in co-cultures of *T. gondii*-infected microglia not activated by IFN- γ (Figure 3D). These results in fact suggest a correlation between iNOS inhibition by the parasite and maintenance of neuron viability. In one of the three experiments, using TUNEL assay, some rare apoptotic neurons, in co-cultures submitted to IFN- γ treatment in the absence of the parasites were observed (data not shown).

Microglial Cells Infected by T. gondii Express Intracellular TGF- β 1

Considering the TGF- β 1 deactivation effect on microglial cells^{38,39} and its production during *T. gondii* infection in several models,^{35,46} we supposed the involvement of this cytokine with the phenomenon mediated by the parasite, here described. To investigate if microglial cells infected by *T. gondii* enhance TGF- β 1 intracellular expression, immunostaining of infected and uninfected cells of this cytokine were compared in the presence or in the absence of IFN- γ (Figure 4). Aiming to correlate TGF- β 1 production with iNOS inhibition mediated by the parasite, experiments throughout short periods of 4 hours were performed because the iNOS expression by microglia appeared early in activation.⁴⁷ In the absence of the parasite and IFN- γ (Figure 4, A and E) low basal levels of TGF- β 1 expres-

Figure 3. *T. gondii*-infected microglia sustain neurite outgrowth even in the presence of IFN-γ, down-modulating iNOS expression by microglial cells. Neurons were co-cultured with *T. gondii*-infected (host cell:parasite ratio of 1:5) and uninfected microglial cells, in the presence and in the absence of IFN-γ (500 U/ml). After 24 hours the cells were submitted to a double immunostaining to iNOS (purple) and B-tub III (black), a neuronal microtubule marker. Neurite length was determined as described in Materials and Methods. **A:** In the control, neurite outgrowth showed normal development and the iNOS expression by microglia is very low. **B:** Severe impairment of neurite outgrowth was observed in co-cultures treated with IFN-γ (500 U/ml), which was paralleled by a strong expression of iNOS by microglia. **C:** The noxious effect of IFN-γ-activated microglia on neurons was completely inhibited by the presence of the parasite, with a normal neurite development and drastic inhibition of iNOS expression by microglia. **D:** In the absence of IFN-γ both neurite outgrowth and iNOS expression by microglia were similar to the control. **E:** Statistical analysis of neurite length of three independent experiments. C, control (fresh medium); Tg, *T. gondii*. **P* < 0.05. Scale bar, 100 μm.

sion were observed, which was slightly enhanced by IFN-γ (500 U/ml) treatment (Figure 4, B and F). *T. gondii*-infected cells clearly enhanced the TGF-β1 expression in the presence (Figure 4, C and G), or in the absence of IFN-γ (Figure 4, D and H). In lesser infected fields (Figure 4, D and H), infected cells had a higher expression of the cytokine (arrows) in comparison to uninfected cells (arrowhead), following the pattern of iNOS inhibition (Figure 2). These results were maintained through the 24 hours of experiments (data not shown). The production of IL-10 by infected microglia and its possible role in the iNOS inhibition was also

Figure 4. *T. gondii* triggered TGF-β1 intracellular expression in microglial cells. Microglial cells were immunostained for TGF-β1 after 6 hours in the above described conditions (**A–D**) and their respective phase contrasts (**E–H**). **A:** Control cells maintained in fresh medium exhibit a low basal level of the cytokine expression, which in the presence of IFN-γ (500 U/ml) was slightly enhanced. **B:** The *T. gondii* infection, following a host cell:parasite ratio of 1:5 in the presence of IFN-γ (500 U/ml) (**C**), or in the absence (**D**) leads to an enhancement of the intracellular expression of TGF-β1. This staining was principally detected in infected cells (arrow) in contrast to the neighboring uninfected cells (arrowhead).

investigated based on our previous data.⁴⁰ However, in the period of 24 hours of infection, we could not detect this cytokine in the culture supernatant using enzyme-linked immunosorbent assay methods (data not shown).

Figure 5. Nucleus translocation of Smad-2 protein in *T. gondii*-infected microglia. Microglial cells were immunostained for Smad-2 after 24 hours in above-described conditions following a host cell:parasite ratio of 1:5. **A:** Control cells maintained in fresh medium or IFN- γ (500 U/ml)-activated microglia (**B**) showed a cytoplasmic profile of Smad-2 localization. Differently, a nuclear concentration of Smad-2 protein was observed after *T. gondii* infection either in the presence (**C**) or in the absence of IFN- γ (500 U/ml) (**D**) by immunofluorescence staining and their respective phase contrast (**E**) and (**F**). This nuclear staining was mainly detected in infected cells (**arrow**) in contrast to the neighboring uninfected cells (**arrowhead**).

T. gondii-Infected Microglia Undergo Smad-2 Nucleus Translocation

Considering the early latent form of TGF- β 1 production,⁴⁸ the intracellular expression of this cytokine by *T. gondii*-infected microglia would not be synonymous with TGF- β 1 action and participation in the model used here. To investigate if this cytokine triggered by the parasite, in fact would be in an active form, the TGF- β 1 functional pathway through immunolocalization of Smad-2 protein (Figure 5) was analyzed. The results showed that in the absence or in the presence of IFN- γ , Smad-2 proteins were clearly located in microglial cytoplasm (Figure 5, A and B), suggesting a stage of latency (unphosphorylated form). However, in the absence or in the presence of the IFN- γ , *T. gondii* induced predominantly a nuclear localization of Smad-2 protein, mainly in infected cells (Figure 5, C and D). This change in the location profile of Smad-2 observed during microglial *T. gondii* infection is characteristic of the TGF- β 1 downstream signaling pathway. In

addition a strong Smad-2 expression was observed in parasite cytoplasm as recently described.⁴⁶ This nuclear localization of Smad-2 in IFN- γ -activated microglia during infection supports the hypothesis of the participation of TGF- β 1 in down-modulation of NO production by these cells. Further, the Smad-2 translocation mainly in infected microglia may be reflecting the up-regulation of TGF- β receptors induced by the parasite or the down modulation of inhibitory class of Smad proteins. However, a complete study of the TGF- β pathway and receptor expression during microglia infection should be performed to clear up this question. The coincident inhibition of iNOS expression and Smad-2 nucleus translocation restricted to infected microglial cell strongly suggest a correlation of this pathway. These data indicate that TGF- β 1 production by *T. gondii*-infected IFN- γ -activated microglia lead to a Smad-2 nucleus translocation followed by an iNOS inhibition, reduction of NO production, and consequently avoidance of neuron injury.

Inhibition of NO Production and Neuron Preservation Induced by T. gondii Infection of IFN- γ -Activated Microglia May Be Dependent on TGF- β 1 Production

The possible involvement of the cytokine TGF- β 1 on the effects promoted by the parasite on neurons was investigated considering that TGF- β 1 triggered by the parasite was in fact acting on microglial cells. In addition TGF- β 1 counteracted the effect of the proinflammatory cytokine IFN- γ , which has been shown to up-regulate NO synthesis⁴⁹ promoting neuronal impairment.⁴⁰ To clarify the possible effect of TGF- β 1 in the phenomenon here described, co-cultures were performed in the presence of TGF- β 1-neutralizing antibodies. As expected in the presence of IFN- γ (Figure 6, B and G) neurite outgrowth was impaired and was accompanied by high levels of NO production by neuron-microglia co-cultures (Figure 6H). The presence of infected microglia, led to a recovery of neurite outgrowth in IFN- γ -activated co-cultures (Figure 6, C and G) that was followed by a reduction of NO production (Figure 6H), however this effect was partially eliminated by 20 ng/ml of TGF- β 1-neutralizing antibodies (Figure 6; E, G, and H). The neutralization of TGF- β 1 partially abrogated the neuritogenesis-positive effect influenced by the parasite on activated microglia (Figure 6, E and G) and restored the production of NO (Figure 6H). The participation of TGF- β 1 production by IFN- γ -activated and -infected microglia in the phenomenon was evident. A possible role of PGE₂ was also suspected, based on the positive influence of TGF- β 1 over PGE₂ production by microglia⁴⁹ and considering its role in NO inhibition⁵⁰ and neuron preservation.⁴⁰ To eliminate the possible contribution of PGE₂, the co-cultures were treated with ASA (100 μ mol/L), an inhibitor of COX-1 and COX-2, which participates on limited enzymatic steps of PGE₂ production.⁵¹ However, it appeared that ASA could not abolish the down-modulation of NO production and in the absence of PGE₂, due to the ASA treatment, the

Figure 6. Sustained neurite outgrowth triggered by *T. gondii* infection of IFN- γ -activated microglia was partially abrogated by TGF- β 1-neutralizing antibody. Neurons were co-cultured with *T. gondii*-infected (host cell:parasite ratio of 1:5) and uninfected microglial cells, in the presence and in the absence of IFN- γ (500 U/ml). In addition to IFN- γ , TGF- β 1-neutralizing antibodies (20 ng/ml) or ASA (100 μ mol/L) were added to co-cultures of infected microglia (E, F). After 24 hours, morphological differences in neurons were observed by localization of β -tubulin-III immunoperoxidase, a specific marker for neuron cytoskeleton. Neurite length was determined as described in Materials and Methods. **A:** In the control, neurite outgrowth was evident, being severely reduced in co-cultures treated with IFN- γ (500 U/ml) (**B**). **C:** Infection of microglia by *T. gondii* avoided the noxious effect mediated by IFN- γ on neurons. The beneficial effect of the parasite was partially blocked by the addition of α -TGF- β 1 neutralizing antibodies (**E**) and was not affected by ASA (**F**). **D:** Microglial infection did not alter neurite outgrowth in the absence of IFN- γ . Statistical analysis of neurite length of three independent experiments (**G**) and corresponding supernatant nitrite concentration in one representative graphic (**H**) from the three independent experiments. C, control (fresh medium); Tg, *T. gondii*. * $P < 0.05$. Scale bar, 100 μ m.

neuron availability induced by *T. gondii* in neuron-microglia IFN- γ -activated co-cultures was maintained (Figure 6; F to H). No statistical differences considering neurite outgrowth and NO production were observed between control (Figure 6; A, G, and H) and co-cultures performed in the presence of infected microglia without IFN- γ (Figure 6; D, G, H).

Considering a possible effect of TGF- β 1, secreted by infected microglia, favoring directly neuron outgrowth, neurons were submitted to a treatment with conditioned mediums of infected and uninfected microglia. The experiments were performed in the absence or in the pres-

ence of NO exogenous donor sodium nitroprusside (SNP) (125 μ mol/L), which resulted in neuron outgrowth impairment as previous reported.⁴⁰ The noxious effect of SNP was partially reverted by both conditioned mediums without statistical differences. In the absence of SNP, neurons were also equally favored by both conditioned mediums (data not shown), reinforcing the hypothesis of an indirect mechanism mediated by the parasite.

Discussion

In the present study, we provided evidence that the NO production of IFN- γ -activated microglia is inhibited by direct *T. gondii* infection, which appears to favor neuron viability, in a mechanism dependent on TGF- β 1 secretion by infected microglia. This observation is in close agreement with our previous study that had shown an indirect effect of soluble immunoregulatory mediators released by *T. gondii*-infected astrocytes that appear to favor neuron viability by inhibiting NO production by IFN- γ -activated microglia.⁴⁰ It is possible that the association of these direct and indirect complementary mechanisms may ensure lower neuron damage during a chronic infection by *T. gondii*. In fact, if neurotransmitter alterations may be supposed,⁵² there is no description of an indirect inflammatory neuron loss or damage in the CNS of chronically infected immunocompetent hosts.

T. gondii is considered well adapted to the parasite life style because it is completely dependent on an intracellular environment for replication and survival. The avoidance of CNS neuron damage in intermediate hosts may be a parasite advantage, because it enhances the chances to complete its life cycle in a final host by predation.⁵³ Inhibition of NO production by IFN- γ -activated microglia indirectly through either soluble factors released by astrocytes⁴⁰ or the production of TGF- β 1 by infected microglia may prevent neuron degeneration, contributing to a harmonic host-parasite interaction.^{54,55}

The presence of different and independent pathways working together in the CNS to raise the same final effect of NO inhibition and the maintenance of neuron viability during *T. gondii* infection may reflect the undoubted importance of this phenomenon. Previously it was shown that the involvement of PGE₂ secretion by infected astrocytes, which stimulating IL-10 production by microglia, ended up in a NO down-modulation and neuron preservation.⁴⁰ The authors more recent data, however, suggest an absence of IL-10 production by *T. gondii*-infected microglia at least in the first 24 hours (data not shown) excluding its participation in the phenomenon here described. Considering the ASA inhibition of PGE₂ production by infected microglia, although ASA had not reversed the *T. gondii* effect on NO production, and the maintenance of neurite outgrowth, the participation of PGE₂ cannot be conclusively excluded, considering the methodology used.

Some evidence pointed to a possible direct effect of ASA on the inhibition of iNOS expression by microglia.⁵⁶ In this way although ASA had inhibited PGE₂ production by microglia irreversibly blocking COX-1 and COX-2, it

may bypass the absence of the prostanoid on iNOS inhibition mimicking the effect of the parasite. It has been reported that PGE₂ may constitute an important stimulus to TGF- β cytokine family production.^{57,58}

The results suggest an interference of *T. gondii* on IFN- γ -signaling cascade, as revealed by the inhibition of iNOS expression by IFN- γ -activated, infected microglia. This phenomenon is, however, observed restrictedly in infected cells, and iNOS expression was still observed on IFN- γ -activated uninfected microglia, which is in agreement with other models of peritoneal macrophage infection.^{46,59} The absence or reduced paracrine effect on neighboring cells may be explained by a possible up-regulation of TGF- β 1 receptors by infected cells. Supporting this idea, a more evident Smad-2 nucleus translocation was observed in infected microglia. These data suggest a TGF- β 1 signaling cascade acting preferentially in infected cells, which may explain a restrictive inhibition of iNOS expression by these cells. The inhibitory effect of TGF- β 1 on iNOS and NO synthesis was also shown in lipopolysaccharide-activated primary rat microglial cells³⁹ and endotoxin-activated mixed glial cell cultures.⁶⁰

The microglial production of TGF- β 1 may be stimulated by some molecules exposed on the parasite surface, such as phosphatidylserine.⁴⁶ Microglial cells are involved with the clearance of brain apoptotic cells and through this phosphatidylserine expression *T. gondii* may be recognized as a dummy apoptotic cell, in a mechanism described in distinct models as *Leishmania* infection.⁶¹ Concerning the CNS, it has already been shown that apoptotic PC12 cells exposing phosphatidylserine promote the production of anti-inflammatory molecules, as TGF- β 1 and neuroprotective molecules, by microglial cells.⁵⁴

Neurite outgrowth impairment caused by NO exogenous donor SNP was partially reverted, being equally favored by conditioned media from uninfected and infected microglia (data not shown). These data suggest that the beneficial effect of the parasite on the neurons is indirectly mediated by an anti-inflammatory effect of *T. gondii* on IFN- γ -activated microglial cells. In addition, even in the absence of this stress condition mediated by SNP, no differences between neurite outgrowths were observed in the presence of conditioned mediums from infected and uninfected microglia maintained in the presence or absence of IFN- γ . Despite a direct positive effect of *T. gondii* on neurons, an indirect effect mediated by NO inhibition by IFN- γ -activated microglia during the infection is also suggested.

An increasing number of studies reported a blockage of IFN- γ signaling cascade by the parasite in a process involving IL-12⁶² and tumor necrosis factor- α decreased levels,⁶³ inhibition of STAT-1 and down modulation of MHC expression.⁶⁴ In addition mechanisms involving inhibition of nuclear factor- κ B nucleus translocation by infected cells have been recently demonstrated^{65,66} and may be also involved in the phenomenon analyzed here. These data, however, may be regarded as immune-evasive mechanisms of the parasite, which may guarantee increased levels of *T. gondii* proliferation, host tissue

damage, and long-life parasite persistence.⁶⁷ On the other hand, the data here contribute to the idea of the blockage of some pathway triggered by IFN- γ signaling cascade in infected microglia, raising a new perspective of a host tissue preservation mechanism exerted by *T. gondii* in the CNS.

A dichotomous role for NO during acute *T. gondii* infection in mice is suggested. Recent data suggest the mechanism of parasitism control and cystogenesis being mainly dependent on IFN- γ with a minor participation of tumor necrosis factor receptor p55 and iNOS.⁶⁸ NO also appears not to be involved in the host defense activity of human fetal microglia against *T. gondii* being the late primarily dependent on the activating properties of IFN- γ , tumor necrosis factor- α , and IL-6.¹⁴ In addition, recent data has shown a complex antioxidant system in *T. gondii*, which may suggest the low vulnerability of the parasite to oxidative injury.⁶⁹ Even in models of susceptible mice, such as the C57BL/6 strain, in which some role in parasitism control is attributed to NO, it seems that a detrimental or lethal side effect occurs in the host, as demonstrated by studies with iNOS^{-/-} mice⁷⁰ partially in agreement with the data presented here.

Some data have restricted the role of NO to chronic disease control in models of susceptible strains,⁷¹ because iNOS^{-/-} animals normally survive acute infection, developing brain cysts and succumbing during the chronic stage of toxoplasmosis.⁷² Because knockout mice with a susceptible genetic background, such as C57BL/6, were used to obtain all of these data^{68,72} the role of NO in chronic disease control by resistant BALB/c strains may not follow the same pattern. The role of NO in resistance to *T. gondii* in the BALB/c (toxoplasmic encephalitis-resistant) mouse appears to be very different from that in the C57BL/6 (toxoplasmic encephalitis-susceptible) mouse. Schluter and colleagues⁷¹ demonstrated that treatment of BALB/c mice with the selective iNOS inhibitor L-NG-iminoethyl-lysine did not result in reactivation of a latent infection, although it exacerbated *T. gondii* infection in the CNS of C57BL/6 mice. Thus, NO does not appear to play a role in maintaining a latent infection in BALB/c mice. Further experiments using IFN- γ -deficient mice on a BALB/c background demonstrated that in the absence of IFN- γ , NO is still detected in the brains of *T. gondii*-infected animals, although this was insufficient to determine the resistance of these animals.⁷³ The results presented here were obtained using microglia from BALB/c mice because this resistant strain better mimics immunocompetent human infection.

The role of NO production as a mechanism of resistance against *T. gondii* infection, particularly in the brain, has also been actively debated.^{71,73} Recent data argue against a central role of NO production as an exclusive mechanism responsible for the control of *T. gondii* infection by microglia in the CNS. According to Freund and colleagues,¹⁹ an unknown NO-independent mechanism leads to inhibition of parasite growth in IFN- γ -activated microglia from both BALB/c and CBA/C mice. In humans, it is also believed that the control of *T. gondii* replication by microglia is mediated not by NO but by a decrease in the levels of infection of activated cells.¹⁴ Some authors,

however, have attributed a possible role of NO in reducing replication of *T. gondii* by murine activated microglia,⁷⁴ as observed in macrophage cells, in which NO is also considered important in triggering stage conversion.⁷⁵

As a consensus there is the persistence presence of IFN- γ in the CNS during the infection and the essential response of CNS resident cells^{7,13} to this main cytokine involved in *T. gondii* infection control.^{9,11} Neuronal death and damage have been considered to play a central role in inflammatory, autoimmune, and neurodegenerative CNS pathologies in which NO is involved. An exacerbated and persistent inflammatory immune reaction mediated by IFN- γ stimuli would lead to a noxious cellular effect on host tissue, especially in the CNS,⁷⁶ however it is not a common pattern of the infection in immunocompetent hosts.

On the basis of these facts, it can be stated that the NO down-modulation described would be pivotal in the persistence of parasites in the CNS, only favoring asymptomatic infection in the resistant host. In addition, the results here strongly indicate a potential neuroprotective and immunoregulatory role of microglial cells dependent on TGF- β 1 secretion. This observation may have therapeutic implications on inflammatory and infectious diseases of the CNS.

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