Sympathetic glial cells and macrophages develop different responses to *Trypanosoma cruzi* infection or lipopolysaccharide stimulation

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Nitric oxide (NO) participates in neuronal lesions in the digestive form of Chagas disease and the proximity of parasitised glial cells and neurons in damaged myenteric ganglia is a frequent finding. Glial cells have crucial roles in many neuropathological situations and are potential sources of NO. Here, we investigate peripheral glial cell response to *Trypanosoma cruzi* infection to clarify the role of these cells in the neuronal lesion pathogenesis of Chagas disease. We used primary glial cell cultures from superior cervical ganglion to investigate cell activation and NO production after *T. cruzi* infection or lipopolysaccharide (LPS) exposure in comparison to peritoneal macrophages. *T. cruzi* infection was greater in glial cells, despite similar levels of NO production in both cell types. Glial cells responded similarly to *T. cruzi* and LPS, but were less responsive to LPS than macrophages were. Our observations contribute to the understanding of Chagas disease pathogenesis, as based on the high susceptibility of autonomic glial cells to *T. cruzi* infection with subsequent NO production. Moreover, our findings will facilitate future research into the immune responses and activation mechanisms of peripheral glial cells, which are important for understanding the paradoxical responses of this cell type in neuronal lesions and neuroprotection.

Key words: parasite - nitric oxide - superior cervical ganglion - glial cell culture

The role of sympathetic glial cells in a variety of parasitic diseases remains unknown, despite the clear relevance of their infection and response to parasites to neuronal lesions in autonomic and enteric ganglia. Many studies have elucidated crucial roles for glial cells in neuropathological situations through the release of trophic factors, neuroprotective molecules or neurotoxic chemicals that mediate neuronal survival, function, lesion or death (Garden & Moller 2006, Giaume et al. 2007). Glial cells are in close contact with neurons in both the central and peripheral nervous systems and are believed to affect them to a greater degree than inflammatory cells and their products (Arantes et al. 2000, Almeida-Leite et al. 2007, Dewil et al. 2007, von Bernhardi 2007). In intestinal Chagas disease, parasitised glial cells and neuronal cells in close proximity to damaged myenteric ganglia is a frequent finding (Tafuri 1970, Arantes et al. 2004). Thus, it is extremely important to elucidate how glial cells are activated by and respond to pathogens, which will help to clarify the mechanisms of pathogenesis for numerous nervous system diseases.

Central glial cells such as astrocytes and microglia have been investigated in vitro and the intracellular pathways activated in response to various stimuli are well established (Jana et al. 2005, Saha & Pahan 2006). However, despite their important roles, peripheral glial cells have not been equally well investigated (Griffin & Thompson 2008, Hanani 2010).

In this study, we used a superior cervical ganglion (SCG) glial cell culture that was standardised by our group (Almeida-Leite & Arantes 2010) to study and compare how peripheral glial cells and peritoneal macrophages respond to two types of stimuli. The first stimulus was the protozoan *Trypanosoma cruzi*, which causes Chagas disease, a significant cause of morbidity and mortality that affects 7.7 million people in Latin America and caused more than 10,000 deaths in 2008 (Strasen et al. 2014). Resistance to *T. cruzi* infection is associated with the capacity of lymphocytes to generate interferon-γ (IFN-γ), which activates macrophages to produce nitric oxide (NO), the main effector molecule that controls intracellular *T. cruzi* multiplication (Gazzinelli et al. 1992, Vespa et al. 1994). NO is also related to tissue damage (Vespa et al. 1994, Chandra et al. 2002), myenteric denervation (Arantes et al. 2004), neuronal death and reduced neurite density in *T. cruzi* infection (Almeida-Leite et al. 2007).

It has been found that autonomic glial cells are susceptible to infection in human intestines (Tafuri 1970) and their parasitism and responses have been implicated in neuronal death (Tafuri 1970, Tanowitz et al. 1982). However, it is unclear whether these peripheral glial cells produce inducible NO synthase (iNOS)-derived NO and whether they exert an important role in tissue lesions and...
neuronal death, as observed in T. cruzi infection. It has been suggested that glial-derived NO contributes to the neurodegenerative process observed in the spinal cords of T. cruzi-infected interleukin (IL)-12p40 knock-out mice (Bombeiro et al. 2009). In addition to the central nervous system (CNS), the enteric nervous system is also compromised in Chagas disease; however, the autonomic glial cell production of NO has not yet been studied.

The second stimulus used in this study was bacterial lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, which induces potent NO production and immune responses (Stuehr & Marletta 1985, Ohmori & Hamilton 2001). Macrophages and resident glial cells in the CNS (astroglia and microglia) express iNOS and produce high levels of NO in response to a wide variety of pro-inflammatory and degenerative stimuli (Saha & Pahan 2006). Although the responses of these cells to these stimuli have been well characterised in vitro, the intracellular mechanisms involved are not completely understood.

Because glial cells have a crucial role in neuronal lesions and survival (Hansson & Ronnback 2003) and are able to produce NO in response to a variety of stimuli, we aimed to investigate how the response of peripheral glial cells to either T. cruzi infection or LPS differed from the response of peritoneal macrophages. Our goal was to establish the importance of peripheral glial cells as a source of NO in Chagas disease while contributing to the study of glial cell biology. Our observations contribute to the understanding of the intestinal form of Chagas disease pathogenesis based on the high susceptibility of autonomic glial cells to T. cruzi infection with subsequent NO production, ultimately leading to neuronal lesions.

**MATERIALS AND METHODS**

**SCG glial cell culture** - Pure primary cultures of SCG glial cells were prepared as previously described (Almeida-Leite & Arantes 2010). Briefly, SCGs were removed from one-four-day-old C57BL/6 wild-type (WT) mice after decapitation and enzymatically dissociated in 1X trypsin/ethylenediamine tetraacetic acid solution (Sigma Chemical Company, USA). Isolated sympathetic cells at a final concentration of 10^5 cells/well were plated in cell culture plates (96 or 24-well; Sarstedt, Germany) coated with a 1:5 dilution of Matrigel (Becton Dickinson) in sterile distilled water and cultured in 10% FBS, 100 UI/mL penicillin (Sigma-Aldrich) and 10,000 µg/mL streptomycin (Sigma-Aldrich) in DMEM (Sigma-Aldrich). After 2 h, the cultures were washed to remove the non-adherent cells, leaving a 97% pure population of adherent macrophages. The cultures were maintained at 37ºC in a humidified 5% CO_2 incubator for 24 h before interventions.

**Parasite** - The T. cruzi Y strain was used for all experiments. Trypomastigote forms were cultured and purified from the monkey kidney epithelial cell line VERO, as previously described (Braga et al. 1993). Parasites were harvested after six days in culture, centrifuged at 150 g for 10 min at room temperature (RT), counted in a Neubauer chamber, centrifuged at 450 g for 10 min at 4ºC, suspended in 10% FBS supplemented 199 medium and used to infect cultures.

**T. cruzi infection** - T. cruzi trypomastigotes were added to cultures at a parasite-to-cell ratio of 5:10:1. The cells were maintained at 37ºC in a humidified 5% CO_2 incubator for 48 h in the presence of 200 UI/mL of recombinant murine IFN-γ.

**LPS addition - Escherichia coli** LPS (L-4130; Sigma-Aldrich) was added to the cultures at a final concentration of 50 ng/mL. The cells were maintained at 37ºC in a humidified 5% CO_2 incubator for 48 h in the presence of 200 UI/mL of recombinant murine IFN-γ.

**Blockade of iNOS activity** - To define the role of NO in T. cruzi infection in glial cells in comparison to macrophages, the iNOS inhibitor aminoguanidine (AG) (300 µM, A7009; Sigma-Aldrich) was added to cultures to block iNOS activity.

**Assessment of infection** - Cultures were fixed after incubation at 48 h post-intervention (p.i.) and stained with 10% Giemsa (Doles Reagents, Brazil). The infection rate was determined by counting the intracellular amastigote forms, as previously described (Vespa et al. 1994, Silva et al. 1995).

**Immunofluorescence** - Cultured fixed cells were washed in phosphate-buffered saline (PBS), permeabilised with 0.25% Triton X-100 (Roche, Switzerland) in PBS containing 2% bovine serum albumin (BSA) (Inlab, Brazil) for 10 min at RT and incubated with 1:40 normal goat serum in PBS containing 0.25% Triton X-100 and 2% BSA for 30 min at RT to block non-specific binding sites. The primary antibodies used to characterise macrophages and glial cells were primary rat anti-mouse F4/80 (1:50; Serotec, UK) and Alexa Fluor 488 goat anti-rat IgG antibodies and primary rabbit anti-glial fibrillary acidic protein (GFAP) (1:100; Dako, Denmark) and Alexa Fluor 488 goat anti-rabbit IgG antibodies (1:400; Molecular Probes, USA), respectively. iNOS expression was detected with primary rabbit anti-iNOS (1:100; Chemicon, USA) and Alexa Fluor 546 goat anti-rabbit
IgG (1:400; Molecular Probes) antibodies. Secondary antibodies alone were used as specificity controls and uniformly resulted in very low background levels of reactivity. Nuclear staining was assessed using the fluorescent probe Hoechst (0.2 µg/mL; Molecular Probes). The cells were examined by microscopy and digital images were acquired for documentation. The fluorescence intensity was measured by the mean grey value of the binary images from two independent experiments performed in triplicate using ImageJ 1.45s [National Institutes of Health (NIH), USA].

Evaluation of NO₂ production - Culture supernatants collected at 48 h.p.i. and stored at -20°C were assayed for their nitrite concentration by mixing 0.1 mL of culture supernatant with 0.1 mL of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide plus 2.5% phosphoric acid, as previously described (Green et al. 1981). The absorbance at 540 nm was measured after 10 min and the NO₂ concentration was determined by reference to a standard curve of 1-200 µM NaNO₂.

Statistical analysis - The results were expressed as the mean ± standard error of the mean (parametric data) or as the median (non-parametric data) of at least three independent experiments. Data were analysed for statistical significance using a non-paired t test (parametric data) or a Mann-Whitney U test (non-parametric data). Probability values (p) of 0.05 or less were considered significant. All analyses were carried out using GraphPad InStat software (USA).

Ethics - All animals used in the research were acquired and cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals guidelines and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research. All animal protocols were approved by the university Ethical Committee in Animal Experimentation.

RESULTS

The glial cells in all cultures showed a flat and irregular shape, from spindle-like to polygonal and few cytoplasmic processes. Most glial cells were immunopositive for the glial cell marker GFAP (Fig. 1A). The macrophages in culture showed irregular shapes, from round to fusiform, and were immunopositive for the macrophage marker F4/80 (Fig. 1B). Comparatively, the glial cell cytoplasm was larger than the macrophage cytoplasm (Fig. 1A, B).

After 48 h of T. cruzi infection, most glial cells showed intense parasitism and swollen cytoplasm, with numerous intracellular amastigotes (Fig. 1C). Some macrophages were parasitised and a few intracellular amastigotes could be observed in the cytoplasm (Fig. 1D).

To determine the infection rate, parasitised cells in both cultures were counted. A quantitative analysis showed a higher number of infected cells in the glial cell cultures than in the macrophage cultures (Fig. 2A). However, when iNOS activity was blocked by AG, there was a significant decrease in the number of infected glial cells, with a significant increase in infected macrophages (Fig. 2A).

To calculate the degree of cell parasitism per infected cell in both cultures, the number of intracellular amastigotes was counted per infected cell of 100 cells (Fig. 2B). No statistically significant difference was observed when both cell types were compared in the presence of NO. However, cell parasitism decreased in both cell types when iNOS was blocked by AG (Fig. 2B).

The glial cells expressed iNOS and were highly infected with intracellular amastigotes (Fig. 1E). As expected, the T. cruzi-infected macrophages also showed iNOS immunopositivity (Fig. 1F). When both cultures were compared, iNOS cytoplasmic fluorescence was systematically more intense in the macrophages than in the glial cells (Fig. 1E, F). A quantitative analysis of the fluorescence intensity showed that the macrophages expressed higher iNOS immunopositivity than the glial cells (Fig. 2C).

In comparison to the controls, the T. cruzi-infected and IFN-γ-activated glial cells showed significantly higher levels of NO production at 48 h.p.i. (Fig. 3A). Similarly, treatment with IFN-γ and T. cruzi infection resulted in significant levels of NO production in comparison to all controls in macrophage cultures. Nonetheless, the macrophages were more responsive to LPS than to T. cruzi in the presence of IFN-γ (Fig. 3A, B). There was no significant difference with regard to NO production between the T. cruzi-infected and IFN-γ-activated glial cell or macrophage cultures.

LPS addition resulted in significantly higher levels of NO production in both the glial cell and macrophage cultures in comparison to the control, LPS or IFN-γ alone at 48 h.p.i. (Fig. 3B). However, the macrophages produced greater amounts of NO when compared to the glial cell cultures when both LPS and IFN-γ were present. When the T. cruzi-infected and IFN-γ-activated glial cell cultures were compared to the LPS and IFN-γ-activated glial cell cultures, there was no difference regarding NO production, regardless of the stimulus (Fig. 3) and the levels of NO production were very similar.

DISCUSSION

We used a model of primary glial cell culture from the SCG to investigate glial cell activation and iNOS-derived NO production after T. cruzi infection or LPS addition and compared this to peritoneal macrophage cultures under the same conditions. Here, we show that T. cruzi infection was greater in glial cells than in macrophages, despite similar levels of NO production. Glial cells responded similarly to both stimuli, whereas macrophages responded better to LPS than to T. cruzi infection.

Despite the close association of glial cells to neurons in the peripheral ganglia and CNS and their participation in pro and anti-inflammatory responses (Hansson & Ronnback 2003, Saha & Pahan 2006), the cellular activation pathways are not well defined for this cell type (Saha & Pahan 2006, Hanani 2010). Our group has studied SCG neurons (Almeida-Leite et al. 2007) and glial cells (Almeida-Leite & Arantes 2010) due to their participation in the neuronal degenerative changes induced by T. cruzi. This parasite has a tropism to nervous tissues in intracardiac ganglia (Machado & Ribeiro 1989) and the intramural myenteric plexus, a site where glia parasitism
has been reported, whereas neuronal infection is rarely observed (Tafuri 1970, Arantes et al. 2004). Autonomic ganglia, such as intracardiac and celiac ganglia and the enteric nervous system are preferentially affected in human and experimental Chagas disease and most likely play an important role in the disease’s physiopathology (Tafuri 1970, Machado & Ribeiro 1989, Arantes et al. 2004). The SCG is a prototype of autonomic ganglia and SCG primary neuronal cultures are widely considered a classical instrument of investigation (Blennerhassett et al. 1991). However, SCG glial cells have been less explored than neurons and have never been used for the in vitro study of *T. cruzi* infection.

Our results indicated that in vitro glial cells are susceptible to *T. cruzi* infection, as previously described in vivo (Tafuri 1970, Arantes et al. 2004). Astrocytes are very susceptible to *T. cruzi* multiplication in vitro (Troyo & Chinchilla 2003) and here we show that sympathetic glial cells are susceptible as well, which has implications for neuronal lesions in autonomic and enteric ganglia. Moreover, *T. cruzi* infection in glial cells was 1.6 times greater than in macrophages at a similar parasite to cell ratio. Despite being infected more easily, glial cells showed no morphological signs of cell degeneration or death. *T. cruzi* promotes glial cell survival through the neurotrophin-3 receptor TrkC (Weinkauf & Pereiraperrin 2009, Weinkauf et al. 2011) and it has been shown that macrophages are infected less frequently than are other cell types (Tanowitz et al. 1975), as also observed in our study. It is reasonable to suggest that glial cells have a more permissive behaviour toward *T. cruzi* infection because they have a larger cytoplasm and are non-professional phagocytes (Pilar & Landmesser 1976). Furthermore, it has been described that glial cells may favour...
parasite survival and multiplication (Troyo & Chinchilla 2003). Macrophages, in contrast, are innate immune cells and naturally resistant to intracellular infections (Tanowitz et al. 1975) and may express distinct genes that limit intracellular parasite multiplication (Crocker et al. 1987). T. cruzi invades several cell types by exploiting phagocytic or non-phagocytic mechanisms, depending on the cell involved (Caradonna & Burleigh 2011). Thus, the entry mechanism of T. cruzi into peripheral glial cells deserves further investigation, as has been performed for other cells (Burleigh & Andrews 1998, Andrade & Andrews 2004, Weinkauf et al. 2011).

One might predict that the greater infection rate in glial cells would be associated with impaired resistance and deficient NO production. Instead, the supernatant NO levels of T. cruzi-infected glial cells were similar to macrophage levels under the same conditions. However, considering that glial cells showed a higher rate of infection and produced similar levels of NO, their response appears to be less intense than that observed for macrophages. When assessing iNOS intensity by immunofluorescence, a lower quantity of expressed protein is observed. The differential regulation mechanisms of the iNOS gene in macrophages and glial cells (Gazzinelli et al. 1992, Vespa et al. 1994, Pahan et al. 1998, Saha & Pahan 2006) could explain the greater infection rate and similar NO production in glial cell cultures, in agreement with the paradoxical protective role of glial cells.

It has been shown that the parasite Toxoplasma gondii exerts an inhibitory effect on iNOS expression in microglia (Rozenfeld et al. 2005), which may also be the case for T. cruzi and SCG glial cells. Moreover, the host defence activity of human microglia against T. gondii does not depend on NO, but primarily on IFN-γ, tumour necrosis factor-α and IL-6 (Chao et al. 1992). The synthesis and secretion of such mediators, as well as leukotriene B4 (Silva et al. 1995, Talvani et al. 2002), could justify distinct infection rates in glial cells and macrophages. It is also known that T. cruzi trans-sialidase protects Schwann cells from cell death by activating the phosphatidylinositol 3-kinase/Akt pathway (Chuenkova & Pereira 2001). Hence, it is possible that our glial cells are more resistant to T. cruzi infection, showing a greater infection rate without morphological evidence of cell death and with less trypanocide activity through NO in comparison to macrophages. However, due to the proximity of glial cells to ganglionic neurons, their products of activation may have damaging effects that may be particularly harmful in both the cardiac and intestinal forms of Chagas disease, as NO has been associated with neuronal death in T. cruzi infection (Almeida-Leite et al. 2007).

Distinct roles for NO in glial cells or macrophages in the control of T. cruzi infection might be suggested by the decreased number of infected glial cells when

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**Fig. 2:** Trypanosoma cruzi infection and inducible nitric oxide synthase (iNOS) expression in glial cells and macrophages. A: percentage of T. cruzi-infected cells in glial cell or macrophage cultures. The data correspond to the mean of five independent experiments ± standard error of the mean (SEM). Asterisks mean statistically significant difference (p < 0.01, unpaired t test); B: number of amastigotes/cell in T. cruzi-infected glial cell or macrophage cultures. The data correspond to the mean of five independent experiments ± SEM (p < 0.05, unpaired t test); C: intensity of iNOS immunofluorescence in T. cruzi-infected glial cell or macrophage cultures 48 h post-intervention. The data correspond to the mean of two independent experiments in triplicate ± SEM (p < 0.05, unpaired t test); A: aminoguanidine; G: glial cell; I: interferon-γ; M: macrophage; T: T. cruzi.

**Fig. 3:** Nitric oxide (NO) production in glial cells and macrophages. Trypanosoma cruzi-infected (A) or lipopolysaccharide-activated glial cell or macrophage cultures (B). The data correspond to the median of four (A) or three (B) independent experiments in triplicate. Asterisks mean statistically significant differences to controls (medium) (p < 0.01, Mann-Whitney U test). MLI statistically different from MTI (p < 0.01, unpaired t test). G: glial cell; I: interferon-γ; L: lipopolysaccharide; M: macrophage; T: T. cruzi.
iNOS activity was blocked by AG. Although it has been shown that the absence of NO leads to higher parasitism (Arantes et al. 2004), some in vitro studies have demonstrated that the addition of AG decreased cell parasitism, as observed in our study. Other mechanisms known to control parasite replication may also be triggered and the partial inhibition of parasitism was due to cytokine production (Aliberti et al. 1999, Machado et al. 2000). Moreover, both in vivo and in vitro studies have shown that NO is not required for the control of parasitism. In addition, both in vivo and in vitro studies have shown that the partial inhibition of parasitism was due to cytokine as observed in our study. Other mechanisms known to be involved in the control of parasitism were cytokines and chemokines that control T. cruzi infection to a greater degree than macrophages. We could also assume that NO might be less effective in glial cells as a mechanism of parasite control. In contrast, the blockade of NO production reduced cell parasitism in both cell types and this may be due to distinct mechanisms of parasite uptake and replication, as previously shown (Aliberti et al. 1999).

The addition of LPS induced greater levels of nitrites in macrophage cultures compared to glial cell cultures. Indeed, it is known that T. cruzi and LPS have diverse effects in macrophages by activating different Toll-like receptors (TLRs) (Ropert et al. 2001), whereas glial cell activation pathways have not been well established (Saha & Pahan 2006). It has been shown that iNOS activation is different in astrocytes and macrophages (Pahan et al. 1998), which led us to believe that peripheral glial cells may also exhibit different levels of iNOS activation. Although macrophages showed greater NO production in LPS-primed cultures in comparison to T. cruzi-infected cultures, glial cells showed similar nitrite levels under either stimulus. Our results indicate that autonomic glial cells respond similarly to LPS and T. cruzi, which are known to activate TLR4 and TLR2, respectively, in macrophages (Hoshino et al. 1999, Ropert et al. 2001). Although the transduction signals generated by distinct TLRs are very similar, cellular responses may be quite different (Campos et al. 2001). Central glial cells respond to LPS via the activation of TLR4 (Lehnardt et al. 2003), as in macrophages (Hoshino et al. 1999); in the presence of LPS, Schwann cells express TLR4 (Cheng et al. 2007) and enteric glial cells produce NO (Cirillo et al. 2011). However, detailed information regarding TLR4 activation following iNOS induction in the presence of LPS in peripheral glial cells has not been described. Thus, it is reasonable to suggest that iNOS activation by T. cruzi and LPS in SCG glial cells involves different TLRs. Although is not known whether SCG glial cells express TLR4 or TLR2, this appears likely because they are the best characterised TLRs with regard to the recognition of microbial motifs and LPS, respectively (Hanke & Kielian 2011). Further studies of TLR expression in SCG glial cells are intended.

Our observations contribute to the understanding of Chagas disease pathogenesis, as based on the high susceptibility of autonomic glial cells to T. cruzi infection with subsequent NO production. Moreover, our findings will facilitate future research into the immune responses and activation mechanisms of peripheral glial cells, which are important for understanding the paradoxical responses of this cell type in neuronal lesions and neuroprotection.

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