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Leishmania amazonensis infection induces behavioral alterations and modulates cytokine and neurotrophin production in the murine cerebral cortex

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ABSTRACT

Neurological symptoms have been associated with *Leishmania* infection, however little is known about how the nervous system is affected in leishmaniasis. This work aimed to analyze parasitic load, production of cytokines/neurotrophins in the prefrontal cortex and behavioral changes in BALB/c mice infected with *Leishmania amazonensis*. At 2 and 4 months post-infection, infected mice showed a decrease in IFN-γ, IL-1, IL-6, IL-4, IL-10 cytokines and BDNF and NGF neurotrophins in prefrontal cortex associated with increased anxiety behavior. Parasite DNA was found in brain of all animals at 4 months post-infection, when the levels of IBA-1 (activated macrophage/microglia marker) and TNF-α was increased in the prefrontal cortex. However TNF-α returned to normal levels at 6 months post-infection suggesting a neuroprotective mechanism.

1. Introduction

Leishmaniasis is a neglected tropical disease affecting millions of people worldwide with great social impact (Desjeux, 2004). This disease is endemic in 98 countries and distributed in 5 continents. In addition, it is considered one of the six most important infectious diseases, affecting mainly the poorest people in developing countries (Alvar et al., 2012). Infections caused by *Leishmania* are characterized in three primary clinical forms: visceral, cutaneous, and mucocutaneous. The clinical form of disease is determined by species of infecting parasite, immune response and genetic background of the host (McGwire and Satoskar, 2014). *Leishmania amazonensis* is commonly associated with the cutaneous form of the disease, but it has also been found in mucocutaneous, diffuse and visceral clinical forms (Aleixo et al., 2006; de Oliveira Cardoso et al., 2010).

The host immune response infection is a determining factor in leishmaniasis and it is responsible for control or progression of disease. Classical immune response to *Leishmania*, in particular to *L. major* infection, is described by the Th1 or Th2 activation associated to resistance or susceptibility respectively (Sacks and Noben-Trauth, 2002). The development of Th1 immunity induces production of cytokines as IL-2, IFN-γ, TNF-αand IL-12 promoting activation of macrophages and intracellular parasite killing. In contrast, Th2 immune response controls Th1 functions and macrophage activation via production of IL-10 and IL-4 favors disease progression (Awasthi et al., 2004).

Mice infected by *L. amazonensis* have progressive and non-healing cutaneous lesions whereas histological examination demonstrated cellular infiltration rich in macrophages within spacious parasitophorous vacuoles, which may contain numerous parasites (Wanasen and Soong, 2008). Some reports showed that the susceptibility of mice to *L. amazonensis* was associated to lack of Th1 cells and predominance of Th2 cells (Charret et al., 2013; Soong et al., 1997). It is recognized that a susceptibility to *L. amazonensis* is dependent on IL-4 but is controversial about IL-10 production (Padigel et al., 2003). Successful macrophage activation to kill parasite consists of high nitric oxide production, however *L. amazonensis* is more resistant to the nitric oxide effects in relation to others *Leishmania* species (Gibson-Corley et al., 2014).

The leishmaniasis is not classically associated with nervous system commitment. Despite this, there are several reports of neurological manifestations in humans and animal models (Llanos-Cuentas et al., 2013; Maia et al., 2015; Petersen and Greenlee, 2011). In Africa, it is

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common patients presenting disseminated form of leishmaniasis with parasite in meninges and cerebrospinal fluid. The immunosuppression, consequence of HIV infection and immunosuppressive drugs, increases susceptibility to Central nervous system (CNS) infection by *Leishmania* parasites. Some HIV-infected patients with leishmaniasis contain parasites detected in cerebrospinal fluid (Walker et al., 2006).

Experimental studies in BALB/c mice showed that *L. amazonensis* is capable of migrating to CNS. Histological and immunohistochemical analysis of brain parenchyma from infected animals identified parasitized macrophages and polymorphonuclear infiltration. In addition, severe multifocal necrosis was found in the brain parenchyma of these animals (Abreu-Silva et al., 2003). Although the cerebral-*Leishmania* interaction study is widely lacking it is reasonable to speculate that *Leishmania* infection could influence the release of cytokines and neurotrophins. The cytokines, involved in various mechanisms of CNS responses, are responsible for a number of neurological disorders. These events may reflect cognitive and behavioral changes as well as neurodegeneration (Bilbo et al., 2012; Stolp, 2013). Thus, it is essential to study behavioral changes in diseases, especially in Leishmaniasis, a disease of chronic evolution, where neurological studies are scarce. The objective of this work was to make a temporal analysis of immunological patterns related to in the cerebral prefrontal cortex (PFC) accomplished of behavioral analyze from BALB/c mice experimentally infected with *L. amazonensis*.

2. Material and methods

2.1. Animal care

The study protocol and handling of animals were approved by the Institutional Animal Care Committee (protocol 520/2013). BALB/c mice at age of 2 months were obtained from animal-housing facilities from Universidade Federal Fluminense. Mice were kept at constant temperature (21 °C) with a light cycle of 12 h with free access to food and water.

2.2. Parasites

L. amazonensis (MHOM/BR/77LTB0016 strain) was maintained by periodic inoculation in BALB/c mice. The amastigote forms were isolated by dissociation of draining popliteal lymph node. After differentiation, promastigote forms were grown at 26 °C in Schneider's *Drosophila* medium (Sigma, USA) supplemented with 10% fetal calf serum (FCS), 100 U/mL Penicillin G potassium and 100 μg/mL streptomycin, at a pH of 7.2. Parasites were harvested from late log phase (3rd day of culture) and used to infect mice.

2.3. L. amazonensis infection and experimental design

Male 8-week-old BALB/c mice were subcutaneously infected in the left hind footpad with 10^6 promastigotes of *L. amazonensis* and sacrificed at 2, 4 and 6 months post-infection. Each experimental group (infected and non-infected) consisted of 9 animals. The infection level was observed by lesion measure and parasite quantification in footpad draining popliteal lymph node. Cutaneous lesion thickness was evaluated by measuring the diameter of footpad with a dial caliper (Mitutoyo, Yokohama, Japan). The severity of the infection was monitored by animal weight.

After the mouse was killed, the brain was removed and immediately it was performed separation of left and right hemispheres through median section by central sulcus. After separation, a tissue fragment (80 mg) was extracted from prefrontal cortex in the right hemisphere. Then, this section was divided into two parts, one used for the preparation of the sample for Western blot technique and the other for the PCR assay.

In the behavioral tests, *Leishmania* infection was performed by inoculation of 10^6 promastigotes of *L. amazonensis* in the left ear dermis of BALB/c mice group to prevent interference in the test due to the foot injury.

2.4. Immunofluorescence

Brains were collected and fixed in 10% formalin solution overnight and embedded in paraplast (Sigma, St. Louis, Missouri, USA). Sagittal sections (5-μm) were mounted on silane-coated slides (Sigma, St. Louis, Missouri, USA). Sections were blocked with phosphate-buffered saline (PBS) pH 7.4 containing 3% BSA and 1% Triton X-100 for 40 min at 20 °C. Antigen retrieval was performed by heat mediation in a citrate buffer. Immunolabeling was carried out by incubating with monoclonal anti-IBA-1 (AB_667733) (1022-5 clone) from Santa Cruz Biotechnology (California, USA) diluted 1/150 in PBS (pH 7.4) and then followed by incubation at 4 °C in a moist chamber overnight. After washing with PBS, sections were further incubated for 60 min with anti-rabbit/Alexa 488 (Molecular Probe, Oregon, USA) antibody. Imaging was performed with a Leica fluorescence microscope.

2.5. Immunoblotting for cytokine detection

Prefrontal cortex section (40 mg) from the right hemisphere to each animal was dissociated in sample buffer without bromophenol blue (62.5 mM Tris–HCl, pH 6.8, containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol by Dounce homogenizer and these material was boiled for 10 min. Total amount of protein in each sample was determined using the Bradford reagent (Bradford, 1976), with BSA as standard. After addition of bromophenol blue (0.02%), tissue homogenates (60 μg/lane) were size-fractionated on 10% SDS polyacrylamide gel and proteins transferred to PVDF membranes (GE Healthcare). These membranes were incubated with the blocking buffer TBS-T (Tris-buffered saline pH 7.6 containing 20 mM Tris–HCl, 160 mM

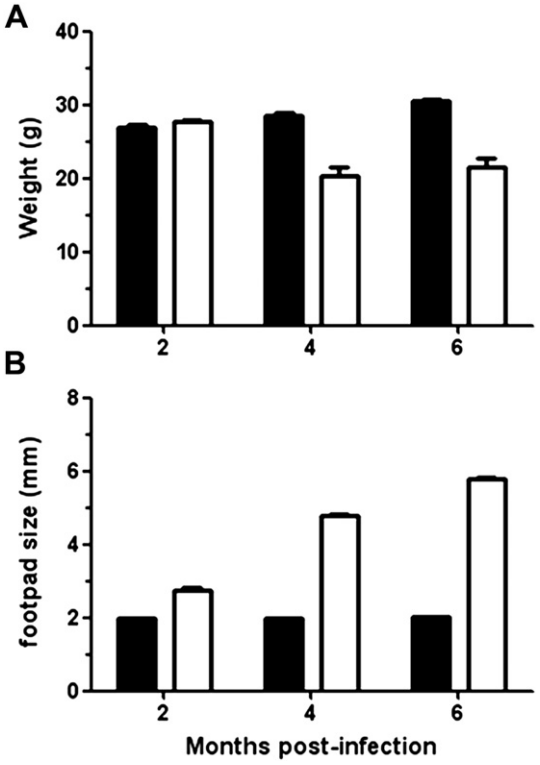

Fig. 1. Body weight (A) and primary lesion kinetic (B) in BALB/c mice subcutaneously injected with 10^6 *Leishmania amazonensis* promastigotes in footpad. Results are expressed by means ± SEM (n = 9).

NaCl, and 0.1% Tween 20) plus 5% non-fat dry milk for 2 h at room temperature. After washes in ice-cold TBS-T, membranes were incubated overnight with different primary antibodies diluted in the blocking buffer: mouse monoclonal anti-TNF-α (AB_147917) (2.1_4E10-1H11 clone) and rabbit polyclonal anti-NGF (AB_148023), BDNF (AB_148021), IL-4 (AB_147902), IL-10 (AB_147892), IL1-β (AB_147896), IL-6 (AB_147906) and IFN-γ (AB_1268232), diluted at 1:6000, both purchased from PeproTech, NJ, USA. Thereafter, membranes were washed in TBS-T and incubated for 1 h at room temperature with specific secondary antibodies diluted in blocking buffer: anti-rabbit IgG HRP-linked antibody (1:10,000), or anti-goat IgG HRP-linked antibody (1:15,000) from Biotecnology, Santa Cruz EUA. Afterwards, membranes were washed three times with TBS-T. The blots were developed using ECL-plus reagent and detected by ChemiDocL-Pix System (Loccus Biotecnologia®, SP, Brazil). The protein levels were estimated by densitometry of images using the software Scion Image (Scion Corporation, MD, USA). All data were expressed as mean ± SE, using actin protein/peroxidase (Biotecnology, Santa Cruz, EUA) as a loading control.

2.6. Polymerase chain reaction for L. amazonensis detection

DNA was extracted from 40 mg of PFC or popliteal lymph node by DNA High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. PCR was performed using primers (forward: 5′GGG GAG GGG CGT TCT GCG AA; reverse: 5′CCG CCC CTA TTT TAC ACC AAC CCC and 5′GGC CCA CTA TAT TAC ACC AAC CCC) that amplify conserved region of the minicircle molecule present in all *Leishmania* species using Rotor-gene SYBR Green PCR Kit (Qiagen®, Germany) (Fagundes et al., 2010). The amplification conditions were 30–40 cycles at 95 °C for 5 s and 60 °C for 15 s. Positive controls containing 50 ng genomic DNA of *L. braziliensis* and *L. amazonensis* and negative controls (no addition of DNA) were included in each PCR assay.

A standard curve was determined with dilutions of *L. amazonensis* DNA from *in vitro* culture containing 10^9 parasites/mL. To verify the extraction efficiency, eight serial dilutions of the DNA stock were established using nuclease-free water. Parasitic load serial dilutions of reference DNA were used to generate a standard curve with an $r^2 = 0.99659$ and reaction efficiency of 1.195. The quantity of parasite DNA in the samples was detected by comparison of the Ct (threshold cycle) values plotted on common log scale (Vitale et al., 2004). The specificity of each reaction was ascertained after the completion of the amplification protocol by performing melt curve analysis. Negative controls were performed without DNA templates. The parasite number was calculated based on the quantification of *Leishmania* DNA, considering that 1 parasite contains 88 fg of DNA, with 10% corresponding to KDNA (Fu et al., 1998).

2.7. Behavioral tests

2.7.1. Open field test

The motor activity of mice was evaluated in the open field test at distinct time of infection and the mouse was placed during 5 min into an open field arena made of wood covered with impermeable Formica (50 cm^2) and was surrounded by 60-cm high walls. The arena was divided into 25 squares (10 × 10 cm) by lines painted on the floor. Each

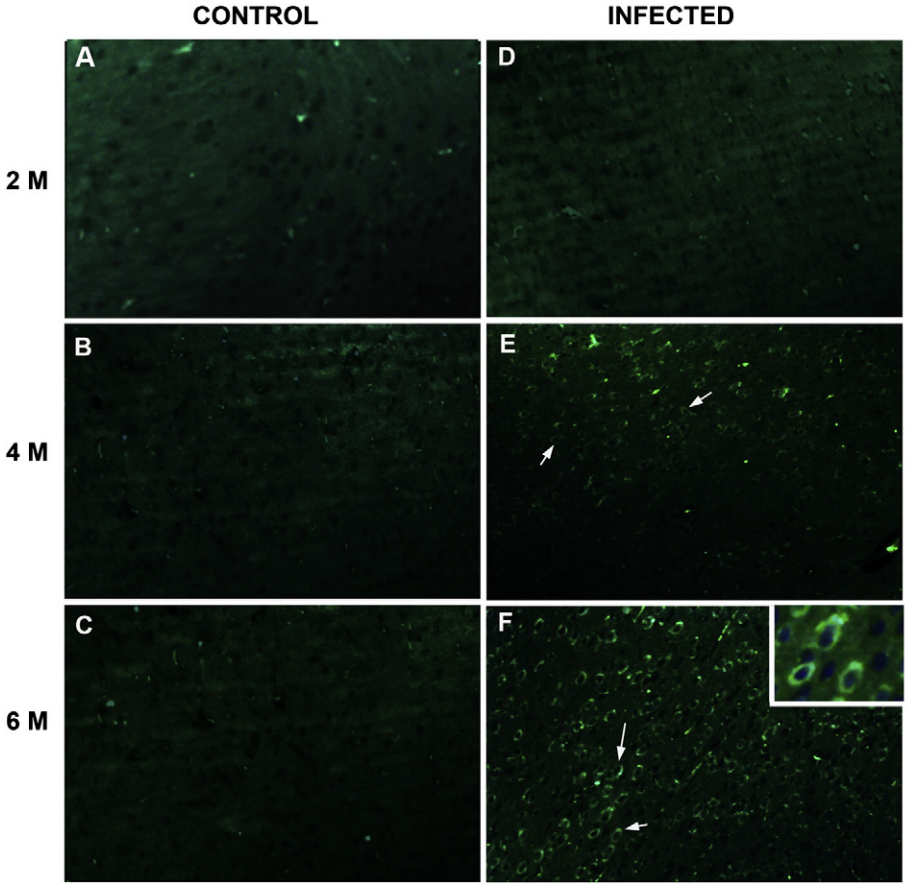

Fig. 2. BALB/c mice infected with *L. amazonensis* increase IBA-1 + microglia/macrophage in PFC. Immunolabeling for IBA-1 in PFC from control (A–C) and infected mice (D–F) at 2 (A, D), 4 (B, E) and 6 (C, F) months post infection. Higher magnification of merge IBA-1 (green) and DAPI (blue) is showed in F. M = months post infection.

mouse was placed in the center of open field and the frequency of lines crossed with the four paws (locomotion).

2.7.2. Elevated plus-maze test

The elevated plus-maze was used to evaluate anxiety-like behaviors in mice (Lister, 1987). The apparatus was made of made of wood covered with impermeable Formica. It is composed of two open arms each (18 cm long × 6 cm wide) crossed with two enclosed arms with 6 cm high walls and elevated 60 cm from the floor. Each mouse was individually placed in the central area of the maze facing an enclosed arm and observed for 5 min. The number of entries (defined by the placement of the four paws into an arm) and the time spent in open and enclosed arms were recorded. The percentage of open-arm entries and time were calculated by the number of open arm entries divided by the total number of arm entries. The number of enclosed-arm entries was used as a measure of locomotor activity.

Both behavioral experiments were conducted in a sound-attenuated room under low-intensity light and were recorded with a video camera positioned above the apparatus and monitored in an adjacent room. All apparatuses were cleaned with a 20% ethanol solution and then dried with a paper towel after each trial.

2.8. Statistical analysis

GraphPad Prism 5 (GraphPad Software Inc.) was used to calculate mean and standard deviations. One-way ANOVA and Newman-Keuls comparison test were applied to obtain statistical significance of means. Differences were considered to be statistically significant at the 0.05 level of confidence.

3. Results

3.1. Evaluation of cutaneous lesion

Clinical signs of skin lesion (edema and erythema) were observed weekly and detected after one month after infection and footpad size increased progressively all the infection period (Fig. 1A). It was observed weight loss in the animals after 4 months of infection (Fig. 1B).

3.2. Activation of microglia/macrophage in infected mice

Since microglia are normally involved in most neuroinflammations, we analyzed the expression of IBA-1, one of the most used microglia/macrophage activation markers, in the cortex of mice infected with *Leishmania* (Hoogland et al., 2015; Jeong et al., 2013). IBA-1 is expressed by monocytes and microglia, but not by neutrophils (Jeong et al., 2013). The PFC analysis showed weak activation of microglia/macrophage in *L. amazonensis* infected mice at 4 months post infection as evidenced by IBA-1 immunolabeling. However, at 6 months post-infection IBA-1 immunolabeling is very intense in PFC tissue from infected mice (Fig. 2).

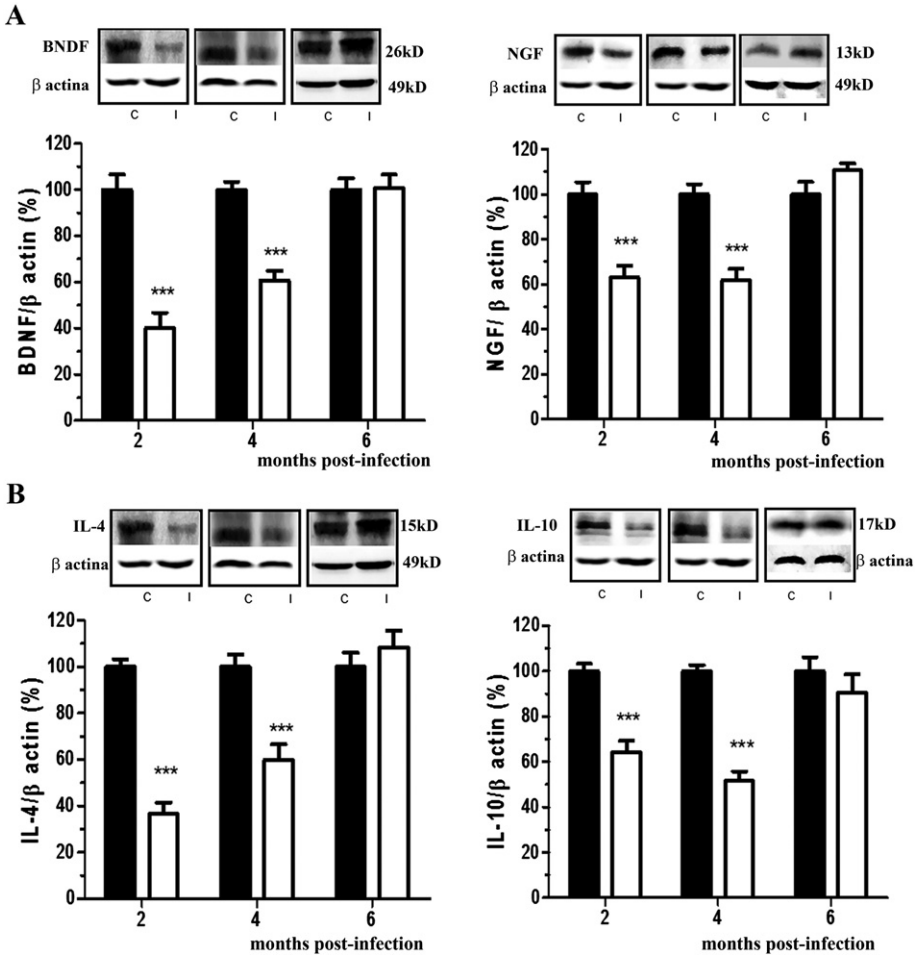

Fig. 3. Infection with *L. amazonensis* modulates neurotrophic factors and anti-inflammatory cytokine production in prefrontal cortex from BALB/c mice infected with 10^6 *L. amazonensis* promastigotes. Immunoblottings and graphs showing the relative quantification of BNDF and NGF neurotrophins is shown in A and anti-inflammatory cytokines in B. Graphs are shown as means ± SEM (n = 9) and was normalized to percent of age-matched non infected control. The differences in cytokine levels among infected and non-infected groups were evaluated by unpaired *t*-test: ***$p < 0.0001$. β-actin was used as loading control. In immunoblottings C = non infected mice and I = infected mice.

3.3. Analyses of cytokine levels in the PFC

Cytokines are a heterogeneous group of polypeptides mediators playing a fundamental role in homeostasis. Cytokines are involved in pro and anti-inflammatory responses elicited by immune cells but also can be produced and released by cells of different systems including the nervous system (Gadani et al., 2012; Vitkovic et al., 2000). Since cytokines and neurotrophins play an important role in the normal function of the nervous system, we analyzed the levels of these molecules in the PFC during different time intervals following infection of mice.

At 2 and 4 months post-infection (Fig. 3A), infected mice showed a marked decrease (59.87% and 39.39% respectively) of BDNF (Brain-derived neurotrophic factor) levels in the PFC compared to non infected mice. However, infected and non-infected control mice had a similar BDNF content in PFC at 6 months post-infection. Similarly to BDNF analysis, cortex from infected mice showed a decrease amount of NGF (Nerve growth factor) at 2 (36.90%) and 4 (38.25%) months post-infection as compared to non-infected mice (Fig. 3A). Again, NGF levels in the cortex from mice at 6 months post-infection showed no significant difference to uninfected control.

In relation to cytokine levels in PFC, *L. amazonensis* infection displayed a significant decrease in IL-4, an anti-inflammatory cytokine, at both 2 (63.34%) and 4 (40.19%) months post-infection in PFC when compared to uninfected group (Fig. 3B). At 6 months post-infection, IL-4 levels do not vary in relation to the age-matched control.

In addition, evaluation at 2 months post-infection of IL-10 amount, also an anti-inflammatory cytokine, showed a decrease of 35.65% in the levels of this cytokine compared to the control (Fig. 3B). At 4 months, IL-10 level was decreased (48.34%) in infected animals in relation to control. At 6 months post-infection, no significant difference in IL-10 levels was evidenced between infected and non-infected control animals.

Next, the levels of inflammatory cytokines IFN-γ, TNF-α, IL-1β and IL-6 were evaluated in the PFC of mice infected with *L. amazonensis* and compared to uninfected mice. We observed a decrease of 48.33% and 43.63% in the levels of IFN-γ cytokine in the infected group compared to the control at 2 and 4 months, respectively, post-infection (Fig. 4A). In the same way that the anti-inflammatory cytokines, at 6 months post-infection samples did not demonstrated changes in levels of IFN-γ cytokine compared to control.

Similarly, IL-1β analysis showed a decrease (40.67%) compared to control at 2 months post-infection (Fig. 4B). At 4 months post-infection, results showed a smaller decrease (23.25%) compared to control than at 2 months. At 6 months of infection, no significant difference was observed in IL-1β levels between control and infected animals.

As demonstrated in Fig. 4C, TNF-α showed a decrease of 61.86% compared to control at 2 months post-infection. Unlike other cytokines evaluated here, this cytokine levels increased approximately 3-fold of in relation to control at 4 months post-infection. However, TNF-α levels did not present a difference significant to age-matched non infected control at 6 months post-infection.

Our data (Fig. 4D) showed that levels of IL-6 cytokine (14.63%) decreased slightly (not significant) in the infected animals at 2 months post-infection when compared to control. Similarly, 4 months post-infection showed a decline (18.95%) of IL-6 levels in PFC from infected animals. Interestingly, at 6 months post-infection, IL-6 cytokine remained decreased (43.2%) in the PFC compared to uninfected mice, differing from all others cytokines analyzed.

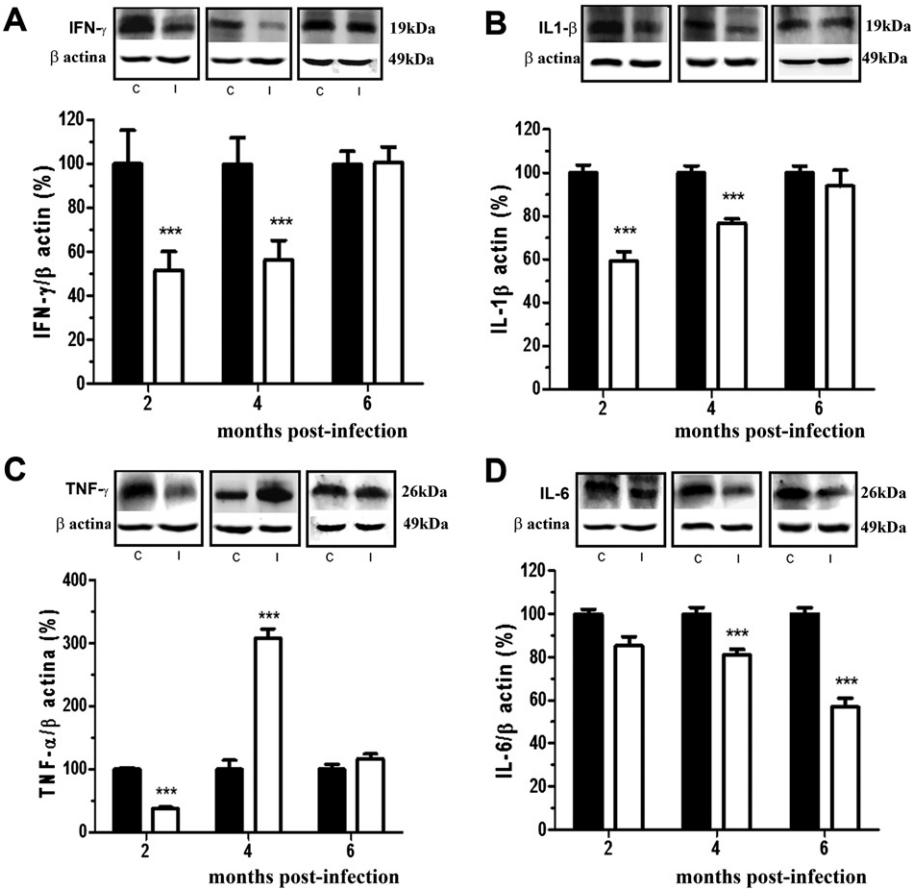

Fig. 4. Modulation of inflammatory cytokines in the PFC from mice infected with *L. amazonensis*. Graphs and immunoblottings showing relative quantification of IFN-γ (A), IL-1β (B), TNF-α (C) and IL-6 (D) in PFC from BALB/c mice infected with 10^6 *L. amazonensis* promastigotes. Graphs are shown as means ± SEM (n = 9) and was normalized to percent of age-matched non infected control. The differences in cytokine levels between infected and non infected groups were evaluated by unpaired *t*-test: ***p < 0.0001. β-actin was used as loading control. In immunoblottings C = non infected mice and I = infected mice.

Table 1
Quantification of *Leishmania* in popliteal lymph node and prefrontal cortex from Balb/c mice by qPCR.

Tissue	Months post-infection	Concentration (ng/mL)	CT cycling time	Positive animals
Lymph node	2	10,720 ± 0.420	5.60 ± 0.56	9
	4	134,633 ± 0.261	8.33 ± 0.39	9
	6	212,177 ± 5.42	7.76 ± 0.62	9
Prefrontal cortex	2	0.00015 ± 0.009	22.75 ± 2.19	3
	4	0.00528 ± 0.008	23.29 ± 0.81	9
	6	0.21220 ± 0.406	17.53 ± 0.16	9

3.4. Parasite quantification in lymph node and PFC

Number of parasites in lymph node and prefrontal cortex from each of 9 animals was estimated by qPCR assay, and they were assessed in triplicate. All lymph node samples from infected animals showed positive for *L. amazonensis*. The statistical analysis of values (Table 1) obtained for sample at different times of infection revealed a significant increase of parasite DNA concentration in lymph node at 4 months post-infection when compared with 2 months ($p < 0.05$). At 6 months parasite DNA increased compared to 2 and 4 months ($p < 0.001$) post-infection.

Finally, 3 animals were positive for *Leishmania* DNA at 2 months post-infection in PFC, but with very low parasite load. However, at 4 months post-infection, all animals were positive but parasite load was yet low load and without significant increase when compared to 2 months. At 6 months post-infection it can be observed a significant difference in relation to 2 or 4 months ($p < 0.05$). In this moment, parasite load showed intensely increased in both lymph node and PFC, but the parasite load in PFC was small when compared to lymph nodes (Fig. 5; Table 1).

3.5. Evaluation of locomotion and anxiety-like behavior in infected mice

The open field test was performed to measure locomotor activity of infected animals with *Leishmania amazonensis*. At 2, 4 and 6 months post infection, it was not observed significative difference in distance traveled at 5 min in the open field apparatus between infected and control groups (Fig. 6A). However, in the elevated plus-maze test, *Leishmania*-infected mice had a reduced percentage of time ($p < 0.05$) spent (Fig. 6B) and numbers of entries (Fig. 6C) in open arms at 2 and 4 months after infection when compared to non-infected mice, indicating an anxiety-related behavior in infected group. There was no difference in the number of entries into the enclosed arms in all the infected and control groups (Fig. 6D).

4. Discussion

Leishmania amazonensis is widely known as responsible for different clinical forms of cutaneous leishmaniasis, however it is a specie capable of disseminating to organs causing visceral form (Barral et al., 1991). Previously, in the study by Abreu-Silva et al. was shown that BALB/c is greatly susceptible to *L. amazonensis* and it presents infected macrophages in the cerebral parenchyma at 8 months after infection (Abreu-Silva et al., 2003). In the present study, parasite DNA was found in cerebral cortex at 4 months post-infection in BALB/c mice, concomitant with the increased TNF production. Besides of that, we observed a significant downmodulation of neurotrophins and inflammatory/anti-inflammatory cytokines in the PFC from infected animals at 2 and 4 months post-infection when compared to control. The cytokine and neurotrophin downmodulation in PFC was accompanied by anxiety-like traits in infected mice. However, at 6 months post-infection, microglial cells showed activated (IBA-1 +) in PFC and it was evidenced an increase of cytokine levels compared to 4 weeks, getting back close to normal levels.

At 2 months post-infection, we observed cytokine and neurotrophin downmodulation in the prefrontal cortex. Cytokine reduction in the cortex of infected animals persisted up to 4 months, except to TNF-α. Recently it was shown that stress has a differential effect in microglia, activating or inhibiting the production of cytokines (Smith et al., 2016). It is possible that the stress caused by peripheral infection can be influencing cytokine production by the microglial in infected mice. In addition, stress is associated with decreased BDNF and NGF neurotrophins in the PFC (Filho et al., 2015).

Behavioral tests showed anxiety-like traits in the *L. amazonensis* infected mice at 2 and 4 months post-infected, when cytokines and neurotrophins were decreased in the PFC. The imbalance in the release of cytokines has been associated with behavioral changes in the course of several infections (de Miranda et al., 2011; Mahmoudvand et al., 2015; Vilar-Pereira et al., 2015). In other protozoal infections, such as malaria and Chagas disease, it has been reported increased anxiety behavior (Campos et al., 2015; Vilar-Pereira et al., 2015). Considered one of the most important cytokines in the CNS, IL-6 directly participates

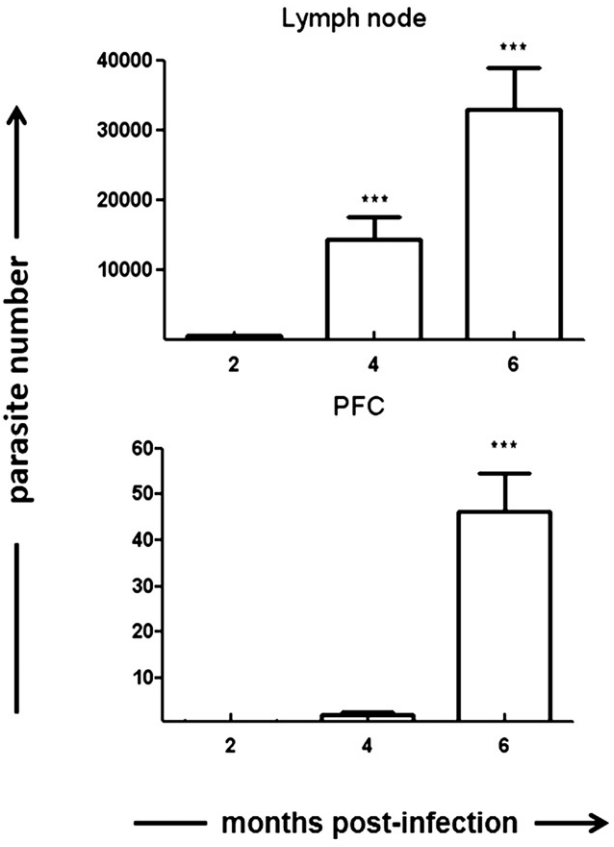

Fig. 5. Number of *Leishmania* in the popliteal lymph node and prefrontal cortex of infected animals. The number of parasites was calculated based on the quantification of *Leishmania* DNA in 40 mg of tissue at 2, 4 and 6 months post infection by qPCR reaction. All experiments were performed in triplicate and each point corresponds to the average of the animals (n = 9). PFC = prefrontal cortex. The differences were evaluated by unpaired *t*-test: ***$p < 0.0001$.

in processes of learning, memory and low levels of this cytokine are correlated with anxiety profile (Erta et al., 2015). IL-6-deficient mice showed lower levels of exploration of the open arms of the elevated plus maze than control animals showing higher level of anxiety (Armario et al., 1998; Butterweck et al., 2003). Besides, Campos and collaborators showed that lack of IFN-γ in mice increased anxiety-like and depressive-like behaviors associated with downregulation of the NGF expression in the prefrontal cortex (Campos et al., 2014). Then, negative modulation of these cytokines in the CNS during leishmaniasis may be involved in the anxious profile of the infected mice.

Changes in BDNF levels also have been associated with the development of symptoms of anxiety (Janke et al., 2015). Neurotrophins and their receptors are highly expressed during CNS development, being responsible for the regulation of proliferation, migration, differentiation, survival, apoptosis and synaptic plasticity (Ohira and Hayashi, 2009; Wang and Jin, 2014). BDNF is crucial for learning, memory consolidation, and behavioral modulation in cognitive processes. Moreover, low levels of this cytokine are associated with Parkinson's disease and Huntington's disease (Autry and Monteggia, 2012; Baquet et al., 2005; Cowansage et al., 2010) indicating an important role for this neurotrophin in the development as well as in adult life.

Linares and collaborators showed a progressive decrease in BDNF levels in different brain regions such as: thalamus, hypothalamus, cerebellum, brainstem and cortex in murine cerebral malaria (Linares et al., 2013). Behavioral tests showed memory deficits in these infected animals, suggesting a relationship between this disorder and the low levels of BDNF (Comim et al., 2012).

Not only BDNF levels are decreased in PFC from infected animals. Also NGF and IL-4 levels are altered, presenting a similar time dependent effect observed for BDNF. NGF also belongs to neurotrophin family and plays an important role in repair and functional recovery in injured nerves by promoting proliferation and differentiation of neuronal cells (Sebben et al., 2011). IL-4 is involved in neurogenesis, oligodendrogenesis process (Ponomarev et al., 2007) and regulates BDNF production (de Araujo-Martins et al., 2013). In addition, collaborating with our hypothesis, IL-4 KO mice display anxiety-like behavior with increased social exploration (Moon et al., 2015).

At 4 months post-infection, all animals showed parasite DNA in prefrontal cortex coinciding with the start of microglia activation and a significant increase of TNF-α. Previous studies showed *in vitro* and *in vivo* microglia infection by *L. amazonensis* (Abreu-Silva et al., 2003; Ramos et al., 2014). Besides, Deininger and collaborators showed that activated microglial cells produce cytokines and signalling molecules in cerebral malaria (Deininger et al., 2002). Lapara and Kelly (2010) showed that murine macrophages stimulated with LPS and infected with *L. amazonensis* increases TNF-α production in relation to uninfected macrophage, while down regulate TH2 cytokines (Lapara and Kelly, 2010). Otherwise, Gasparotto et al. found no significant increase in TNF levels in lysates of PFC from mice infected with *L. amazonensis* at 4 months post-infection, but they used different parasite inoculum and methodology (Gasparotto et al., 2015). TNF-α at high levels in the CNS induces changes in the hematoencephalic barrier mediated by the activation of metalloproteinases and may facilitate the migration of parasites into the CNS (Cardoso et al., 2010, Schwerk et al., 2010). Despite of increased levels of TNF-α, other inflammatory and anti-inflammatory cytokines remained decreased at 4 months post-infection. In agreement with our results, Gasparotto and collaborators also found significantly IFN-γ decreased, but IL-6 and IL-1 levels were similar to the uninfected control (Gasparotto et al., 2015).

At 6 months, parasite load and cytokine production (except TNF-α and IL-6) was increased in cortex when compared to 4 months post-infection. However, although there was increased cytokine production, not exceed the level found in normal mice. Possibly this is due to low parasite levels in the prefrontal cortex of infected animals and it would be important to analyze cytokines in the prefrontal cortex at 8 months post-infection. Important to note that normalization of cytokines IL-1, IFN-γ, IL-4 and IL-10 and neurotrophins NGF and BDNF was accompanied by normalization of behavioral tests.

Microglia is the main component of neuroinflammation, which can act to protect or damage brain cells in pathological situation

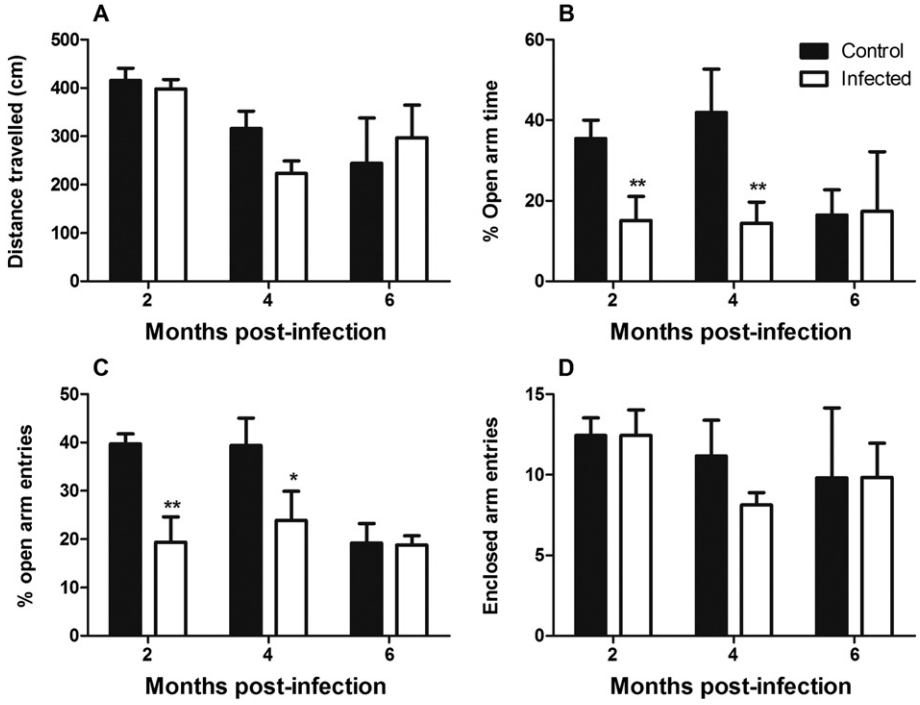

Fig. 6. Locomotion and anxiety-like behaviors in BALB/c mice infected with *Leishmania amazonensis*. BALB/c mice infected with 10^6 *L. amazonensis* promastigotes and submitted to behavior tests at 2, 4 and 6 months post-infection. In graph (A) shows distance traveled in the open field task in 5 min. To elevated plus-maze test the mice were placed in the central platform and observed for 5 min. The percentual of time spent (B) and entries (C) in the open arms were recorded. In graph (D) represents percentage of entries in enclosed arms. Results are expressed by means ± SEM (n = 9). The among infected and non-infected groups were evaluated by unpaired *t*-test: *p < 0.05, **p < 0.001).

(Hernandez-Rabaza et al., 2016). The term microglia activation is a simplification of different states of activation. As for macrophage, a dichotomy has been proposed for microglia activation: M1 (classic) or M2 (alternative) (Cherry et al., 2014). The balance between classically and alternatively activated microglial phenotypes influences neuroinflammation progression. M1 microglia produce pro-inflammatory cytokines and are neurotoxic, while M2 have a role in remodeling and repair (Hoogland et al., 2015). At 6 months post-infection with *L. amazonensis*, it was evidenced microglia activation by morphology and IBA-1 intense immunolabeling in PFC, however it was detected low inflammatory cytokine production suggesting alternative activation, M2.

The chronic inflammation can induce disastrous consequences in the CNS therefore the microglial response is tightly regulated to prevent deleterious effects to neighbouring neurons. Similarly to peripheral tissues, acetylcholine appears to have a key role in controlling neuroinflammation. In the brain, α7 nAChR is expressed in neurons and non-neuronal cells, including astrocytes, microglia, oligodendrocyte precursor cells and endothelial cells (Egea et al., 2015). The anti-inflammatory cholinergic pathway is a neuroinflammation control mechanism to prevent neurotoxic effects of microglia over activation (van Gool et al., 2010). At 6 months post-infection, infected mice had decreased levels of TNF-α and IL-6 in the prefrontal cortex compared to 4 months post-infection, suggesting that regulatory mechanisms drive to control the microglia activation.

Inflammation in the brain is a complex process involving several cell types such as microglial cells, astrocytes, blood inflammatory cells and neurons, which protecting the brain and preventing secondary injury (Jeong et al., 2013). The cytokine network is modulated to protect to infections and to maintain the homeostasis. In conclusion, this study showed that during experimental infection by *Leishmania amazonensis* there is a modulation of cytokines and neurotrophins in the PFC associated anxiety-related behavior and that even after increasing the parasitic load in the PFC, cytokine levels are controlled possibly as a neuroprotective mechanism.

Conflict of interest

All authors that participate in this study declare that have nothing to disclose regarding competing interests or funding from industry with respect to this manuscript.

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