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Inducible Nitric Oxide Synthase is required for parasite restriction and inflammatory modulation during *Neospora caninum* infection

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ABSTRACT

Neospora caninum infection is an important cause of neuromuscular disease in dogs and abortion in cattle, leading to significant economic losses in beef and dairy industries. The protective immunity against apicomplexan parasites, specifically *Toxoplasma gondii and N. caninum*, is typically achieved by inducing an IL-12-driven Th1 immune response. IL-12 stimulates IFN-γ production, which activates Inducible Nitric Oxide Synthase (iNOS) and promotes consequent Nitric Oxide (NO) synthesis, classically described as one of the main effector mechanisms for parasite elimination. Here, we aimed to evaluate the role played by iNOS during *N. caninum* infection. Our results show that *N. caninum* infection in C57BL/6 wild type (WT) mice induce NO production *in vivo* and *in vitro*. In agreement, iNOS deficient mice, as well as WT mice treated with iNOS inhibitor aminoguanidine, succumbed during acute infection with a dose lethal to 50 % of the WT mice, and presented significant increase in parasite load when submitted to sub-lethal infection protocols. Interestingly, the lack of control of parasite proliferation observed in iNOS$^{-/-}$ mice was associated with notable CNS inflammation and increased production of the main systemic proinflammatory cytokines (IL-12, IFN-γ, IL-6, TNF and IL-17A). Taken together, our findings show that iNOS plays an important role in restricting *N. caninum* replication, while also modulates the inflammatory process induced by the infection.

1. Introduction

Neospora caninum is an apicomplexan parasite that shares many morphological and biological features with *Toxoplasma gondii*. This protozoan causes neuromuscular disease in dogs and is considered the major cause of abortion in cattle, leading to significant economic losses in beef and dairy industries worldwide (Dubey and Schares, 2011; Reichel et al., 2013). There are three known infectious stages of *N. caninum*: fast replicating tachyzoites, bradyzoites contained inside tissue cysts, and sporozoites within oocysts. Those parasite forms infect canids and a wide range of intermediate hosts through transplacental infection, as well as oral exposure to meat bearing tissue cysts or drink water – and irrigated vegetables – contaminated with oocysts (Goodswen et al., 2013; Marugan-Hernandez, 2017). In the initial phase of infection, tachyzoites invade multiple organs, causing tissue lesions due to its rapid proliferation and induction of strong inflammatory responses (Hemphill et al., 2013; Mineo et al., 2010b). Like many other apicomplexan parasites, in order to survive, *N. caninum* has developed the ability to respond to changes in their living environment, through stage transformation. The activation of the host immune response induce differentiation of tachyzoites into slowly proliferating bradyzoites, which form cysts in neural and skeletal tissues, surviving for many years in immunocompetent hosts. However, disruption in physiological immune surveillance, caused by immunosuppression, pregnancy, or other conditions may result in reactivation of tissue cysts into rapidly dividing tachyzoites (Innes et al., 2002).

Data from *in vitro* and *in vivo* immunological studies indicate that innate immunity plays an important role in protection and also in the pathogenesis of protozoan infections. The first line of host immune defense against apicomplexan parasites is provided by epithelial cells, which act as a physical barrier. Beyond this basic function, the interaction between innate immune receptors and parasite molecules trigger intracellular signaling pathways that result in a Th1-biased cytokine/chemokine inflammatory response. These molecules act on immune

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cells including macrophages, polymorphonuclear leukocytes and dendritic cells, recruiting those to the site of infection to assist resident cells. Through recognition of pathogen-associated molecular pattern (PAMPs) from the protozoa, innate cells act - through its germline-encoded pattern recognition receptors (PRRs) – to restrict pathogen growth (da Silva et al., 2017; Davoli-Ferreira et al., 2016). IL-12 produced by macrophages and DCs during the acute phase of the infection stimulate the production of IFN-γ by natural killer (NK) cells and compromise CD4 + T cells into a type 1 T-helper (Th1) profile. A Th1-skewed immune response induce a wide array of effector mechanisms that are essential for the control of *N. caninum* and *T. gondii* infections, and production of nitric oxide (NO) is one of the important players in this process (Gazzinelli et al., 1993; Nishikawa et al., 2001; Tanaka et al., 2000; Yarovinsky, 2014).

NO is a cell signaling molecule involved in vascular regulation, neurotransmission, host protection against infection and/or immunosuppression, and its availability is dependent on the family of nitric oxide synthase enzymes (NOS). In mammals, three different genes encode distinct NOS isoforms: Constitutive nNOS (neuronal, NOS1) and eNOS (endothelial, NOS3), both dependent of the Ca^{2+}/calmodulin system, which produce small amounts of NO; and iNOS (inducible, NOS2), mainly expressed in innate immune cells, which yields high NO production in a Ca^{2+} independent manner during an extended period of time, playing a crucial role during many inflammatory and infectious processes (Bogdan, 2001; MacMicking et al., 1997; Stuehr, 1997).

Although work by others have shown the participation of NO in growth restriction and stage conversion of *N. caninum* (Jesus et al., 2019; Pinheiro et al., 2010; Vonlaufen et al., 2002), the actual role of iNOS during the infection by this protozoan has not been properly dissected to date. In that sense, we here present results showing that this enzyme is crucial for resistance against *N. caninum* infection in mice, promoting restriction of parasite replication and modulation of the host immune responses.

2. Materials and methods

2.1. Ethics statement, mice and experimental design

All experiments involving mice were performed in strict accordance with the rules issued by the Brazilian National Council for Control of Animal Experimentation (CONCEA) and the protocols were approved by the Ethics Commission on Animal Use (CEUA) from the Federal University of Uberlândia (UFU) – Protocol 109/16. Six- to 8-week-old, wild-type (WT) C57BL/6 background mice, along with genetically deficient littermates for iNOS (iNOS$^{-/-}$), were bred and maintained at the institutions' animal facilities (REBIR/UFU), with food and water *ad libitum*. Each experimental group was composed by 5–8 mice, which were infected by intraperitoneal inoculation (i.p.) of freshly lysed *N. caninum* tachyzoites. To assess survival rates, WT and iNOS$^{-/-}$ mice were infected with a lethal dose for 50 % of the WT mice (LD_{50}, 2×10^7 tachyzoites), and observed daily for weight loss and mortality, up to 30 dpi. A sublethal dose (1×10^6) of tachyzoites was used in experiments aimed for cytokine measurements and tissue analysis during different time points of the infection.

2.2. Host cell and parasite maintenance

N. caninum tachyzoites (Nc-1 isolate) were maintained by continuous passages in HeLa cells (ATCC CCL-2), grown in RPMI-1640 media supplemented with 10 % heat inactivated fetal calf serum (FCS), 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 250 of amphotericin B (Thermo Scientific, USA), at 37 °C and 5 % CO_2. The tachyzoites used in all infections were harvested after an approximate 70 % lysis of the cell monolayer, as previously described (Mineo et al., 2010a).

2.3. Bone marrow derived macrophages (BMDMs)

BMDMs were generated from C57BL/6 WT mice, as previously described (Marim et al., 2010). Briefly, mice were euthanized and its femurs were collected and flushed to extrude the bone marrows. Cells were seeded in petri dishes (Falcon, USA) and incubated at 37 °C in 5 % CO_2, for 7 days, in RPMI-1640 medium, supplemented with 15 mM HEPES, 1 mM L-glutamine, supplemented with 20 % FCS and 30 % cell-conditioned medium obtained from the supernatant of confluent L929 cells (LCCM), as the source of granulocyte/macrophage colony stimulating factor. For NO measurements, BMDMs were plated in 96 well culture plates (1×10^6 cells/ml), 18 h before each experiment.

2.4. In vivo and in vitro measurement of Nitric Oxide

The concentration of nitric oxide was indirectly measured in peritoneal exudate samples from infected C57BL/6 mice, using the Total Nitric Oxide and Nitrate/Nitrite Assay (R&D Systems, USA), following the manufacturer's recommendations. We also evaluated the production of NO in supernatants of Bone marrow-derived macrophages (BMDMs), from C57BL/6 WT mice infected with *N. caninum*, using the conventional Griess method. The Griess Reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 − 570 nm (Miles et al., 1996).

2.5. Histological analysis

CNS samples of WT and iNOS$^{-/-}$ mice were obtained after 30 dpi, fixed in phosphate-buffered formaldehyde solution (4 %) and posteriorly embedded in paraffin, sectioned (5 μm), mounted on glass slides and subjected to standard staining procedure in hematoxylin-eosin (H& E). The images were obtained with an automated inverted microscope (FSX100, Olympus, Japan).

2.6. Quantification of the parasite burden

The parasite burden in peritoneal cells and CNS was determined by qPCR, as previously described elsewhere (Collantes-Fernandez et al., 2002; Miranda et al., 2019). Briefly, the genomic DNA was extracted from normalized number of cells and CNS mass (50 mg), and further adjusted to 40 ng/μl estimated by 260/280 ratio (NanoDrop 1000, Thermo). The samples were amplified for the repeated Nc-5 genomic sequence (sense 5′-GCTGAACACCGTATGTCGTAAA-3′; antisense 5′-AGAGGAATGCCACATAGAAGC -3′). The assays were composed of master mix buffer (GoTaq qPCR Master Mix, Promega, USA), along with 10 pMol of each oligo along with 50 ng of the DNA templates. Parasite burden was estimated through the extrapolation of the number of Nc5 copies in the samples (three technical replicates per sample), relative to the amplification of murine *gapdh* gene (sense 5′-CTCGTCCCGTAGAC AAAATGG-3′; antisense 5′- AATCTCCACTTTGCCACTGCA -3′). This approach corrects possible discrepancies in sample extraction and also adjusts the results for the presence of potential PCR-inhibiting compounds in each DNA sample analyzed (Collantes-Fernandez et al., 2002). The amplification was carried out in a real time thermal cycler (StepOnePlus, Thermo).

2.7. Cytokines quantification

IL-12p40 and IFN-γ levels were measured in the peritoneal exudate obtained from infected WT and iNOS$^{-/-}$ mice by commercial ELISA kits (BD), following the manufacturer's instructions. The assays were read at 450 nm in a dedicated plate reader (M2e, Molecular Devices, USA). IL-6, TNF and IL-17A concentrations were evaluated using cytometric bead arrays (CBA, BD) and analyzed using a flow cytometer (FACSCanto II, BD) and dedicated software (FCAP Array, BD). Cytokine concentrations were calculated from standard curves of murine recombinant cytokines. Detection limits for each cytokine: IL-6 (1.4 pg/ml), TNF (0.9 pg/ml), IL-17A (0.8 pg/ml). IL-12p40 (3.9 pg/ml), IFN-γ (0.762 pg/ml).

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, USA). Data were expressed as mean ± SD or SEM, and were compared using *t*-test, for the direct comparison of two datasets. Additionally, One-way ANOVA followed by Bonferroni post hoc test were applied to compare the results of three or more time-points. Survival curves were compared using Kaplan–Meier survival analysis through log-rank Mantel–Cox test. Differences were considered statistically significant when: P-Values < 0.05 (*); < 0.01 (**); < 0.001 (***).

3. Results

3.1. N. caninum infection triggers NO production

NO acts as an omnipresent intercellular messenger that governs a broad spectrum of biological processes, including the elimination of many intracellular pathogens. Therefore, we first measured NO production induced by *N. caninum* infection in a mouse model. For such, WT C57BL/6 mice were infected with a sublethal dose (1×10^6) of *N. caninum* tachyzoites and peritoneal fluid samples were collected on days 0, 7, 15, 30 post-infection (dpi) for the analysis of nitrite/nitrate concentration, sub-products of NO degradation. As shown in Fig. 1A, nitrite/nitrate concentrations were considerably higher after tachyzoite inoculation, with peak production in the seventh day of infection ($p < 0.05$). As iNOS is mainly expressed in innate immune cells, we sought to determine whether macrophages were able to produce NO after infection with *N. caninum* tachyzoites *in vitro*. For that purpose, naïve C57BL/6 WT bone marrow derived macrophages (BMDMs) were infected with tachyzoites of *N. caninum* (MOI 2) and NO concentration was measured after 24, 48 and 72 h. Our experiments show that detectable amounts of NO were measured after 48 h, with a peak at 72 h of infection (Fig. 1B, $p < 0.01$), confirming innate immune production of the mediator.

3.2. iNOS-derived NO is crucial for mice survival during acute infection with N. caninum

Next, we examined whether iNOS impacts host resistance against *N. caninum* infection. For that purpose, we infected WT and iNOS deficient (iNOS$^{-/-}$) C57BL/6 mice with a lethal dose of *N. caninum* tachyzoites to 50 % of the mice (LD$_{50}$), which were monitored daily for body weight and survival. As shown in Fig. 2A, iNOS$^{-/-}$ mice were susceptible to the challenge with *N. caninum* tachyzoites, since none survived more than 12 days of infection, while 50 % (4 mice) of the WT mouse group survived the 30-day experiment ($p < 0.05$). iNOS$^{-/-}$ mice also experienced a tendency of a more severe reduction in body weight if compared to WT mice (Fig. 2B). As mice from both groups lost mean 1 g of weight in the first 24 h of infection, WT mice recovered approximately half of that loss in the subsequent days, while iNOS$^{-/-}$ mice doubled the loss after 48 h (≅ 2 g/mice), not recovering until the group started to succumb to the infection. In order to confirm that the higher mortality of iNOS$^{-/-}$ mice was a consequence of deficiency in NO production, groups of WT mice were infected with the same LD$_{50}$ and treated or not with aminoguanidine (AG, 100 mg/kg), a broad range nitric oxide synthase inhibitor, and survival rate was monitored for 30 days. We observed that infected mice treated with AG were more susceptible to the infection ($p < 0.05$) in a pattern with notable similarities to the group of mice genetically depleted of iNOS, as the whole group had to be euthanized before 10 dpi (Fig. 2C). On the other hand, 60 % of the infected control group (untreated) survived the infectious challenge, while 100 % of the drug control group (uninfected, only AG) survived the 30-day experiment ($p < 0.05$). These results demonstrate that iNOS-derived NO plays an important role during acute infection with *N. caninum*, conferring resistance to the infected hosts.

3.3. iNOS deficiency results in increased parasitism and brain injury

As NO restricts tachyzoite proliferation through the interference in the availability of essential metabolites for parasite growth (Bogdan et al., 2000; Spekker et al., 2009), we next aimed to observe other phenomena associated with the higher mortality rate of iNOS$^{-/-}$ mice. With that intent, we first evaluated the acute parasite burden of these mice at the initial site of the inoculation, during a sublethal (1×10^6 tachyzoites/mice) *N. caninum* infection. qPCR analysis of peritoneal exudate cells showed that iNOS$^{-/-}$ mice presented significantly higher parasite load if compared to its control littermates on the second day of infection (Fig. 3A, $p < 0.001$). However, both groups of mice were able to control the acute parasite burden at that site over time, since a substantial reduction in parasite load was observed on the seventh day of infection.

In the acute period of *N. caninum* infection, tachyzoites invade different host cells and quickly spread throughout the body. However, chronic phase is marked by latent stages of the parasite lodged mainly the in the host's central nervous system (CNS). Since iNOS$^{-/-}$ mice lacked proper control of acute parasite replication, we then speculated whether this phenotype would also result in an increased parasite migration to the CNS of these mice. For that, WT and iNOS$^{-/-}$ mice were infected with a sublethal dose of *N. caninum* tachyzoites, and brain tissues were measured for the concentration of parasite genomic DNA

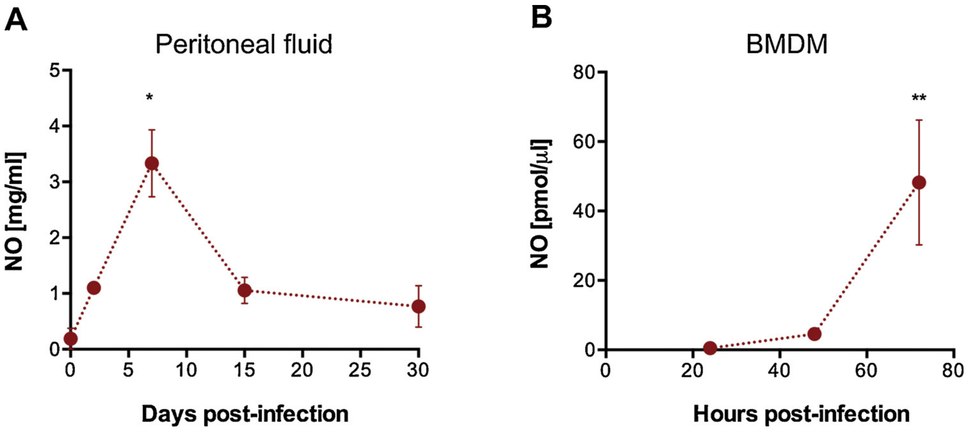

Fig. 1. *N. caninum* infection induces the production of nitric oxide (NO). (A) To determine the involvement of NO in *N. caninum* infection, C57BL/6 WT mice were infected with 1×10^6 tachyzoites and nitrite/nitrate concentration was measured in peritoneal fluid by Griess method. (B) BMDMs (1×10^6 cells/ml) were infected with tachyzoites (MOI = 2) and nitrite concentration was determined in cell culture supernatant, also by Griess, at 24, 48 and 72 h post-infection. Results, expressed as mean ± SD, are representative of two independent experiments. Asterisks indicate statistical significance level - (*) $p < 0.05$; (**) $p < 0.01$ [ANOVA followed by Bonferroni multiple comparison post-test].

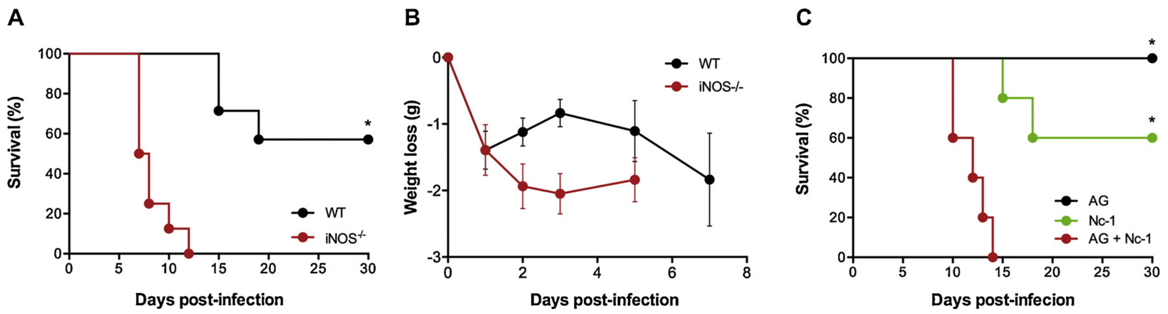

Fig. 2. iNOS is required for mice resistance against *N. caninum* infection *in vivo*. WT and iNOS$^{-/-}$ mice were infected with *N. caninum* (LD$_{50}$, 2 × 10^7 tachyzoites) and monitored daily for survival (A) and weight loss (B, mean ± SEM), through 30 dpi. (C) WT mice were treated daily with aminoguanidine [AG, 100 mg/kg] and challenged with *N. caninum* (LD$_{50}$) to evaluate survival rates, along with infection and treatment controls. Results are representative of three independent experiments. (*) indicate $p < 0.05$ statistical significance [Kaplan–Meier survival analysis through log-rank Mantel–Cox test].

on days 7, 15 and 30 after infection. As it may be observed in Fig. 3B, the concentration of parasite DNA in the brain tissues of iNOS$^{-/-}$ mice was already detectable in the 7th day of infection, increasing subsequently in the other two time points analyzed. In comparison, parasite genomic DNA was only detected in brain samples of WT mice at the 30th day of infection, with a significantly lower concentration than that observed in the CNS of iNOS$^{-/-}$ mice ($p < 0.001$).

High parasite load is usually associated with tissue damage and clinical manifestation of the disease. Therefore, we then aimed to check the inflammatory profile of the CNS samples obtained from both groups (WT and iNOS$^{-/-}$ mice), after 30 days of a sublethal infection. Compared to the WT, infected iNOS$^{-/-}$ mice presented notable sites of mononuclear cellular infiltration in the parenchyma, glial nodules and meninges, characterizing severe inflammatory lesions (Fig. 3C). The escalation of those lesions - due to the increased parasite inoculum used in the other experimental dataset - may be associated with the subsequent death of the INOS$^{-/-}$ mice, reinforcing the role of iNOS in host resistance against *N. caninum*.

3.4. *Uncontrolled parasite replication lead to a robust production of inflammatory mediators by infected iNOS$^{-/-}$ mice*

Due to the inability of iNOS$^{-/-}$ mice to properly control parasite replication, we next aimed to check whether these mice were able to produce cytokines known to trigger inflammatory responses after the infection. With that intent, we measured the production of IL-12, IFN-γ, IL-6, TNF and IL-17A, in the peritoneal fluids or serum samples of WT and iNOS$^{-/-}$ mice after 2, 7, and 15 dpi with sublethal doses of *N. caninum* tachyzoites. Surprisingly, all cytokines were shown to be partially upregulated in samples obtained from iNOS$^{-/-}$ mice, if compared to its WT littermates. As it may be seen in Fig. 4A, iNOS$^{-/-}$ mice significantly produced more IL-12 in the peritoneal fluid in all analyzed time-points ($p < 0.0001$); a phenotype partially mimicked by its close biological partner, IFN-γ (Fig. 4B, $p < 0.05$). Also, serum levels of IL-6 were higher in iNOS$^{-/-}$ mice at 2 dpi (Fig. 4C, $p < 0.0001$), TNF at 7 dpi (Fig. 4D, $p < 0.005$) and IL-17 at 15 dpi (Fig. 4E, $p < 0.05$), if compared to the infected WT mice. Together, these results indicate that,

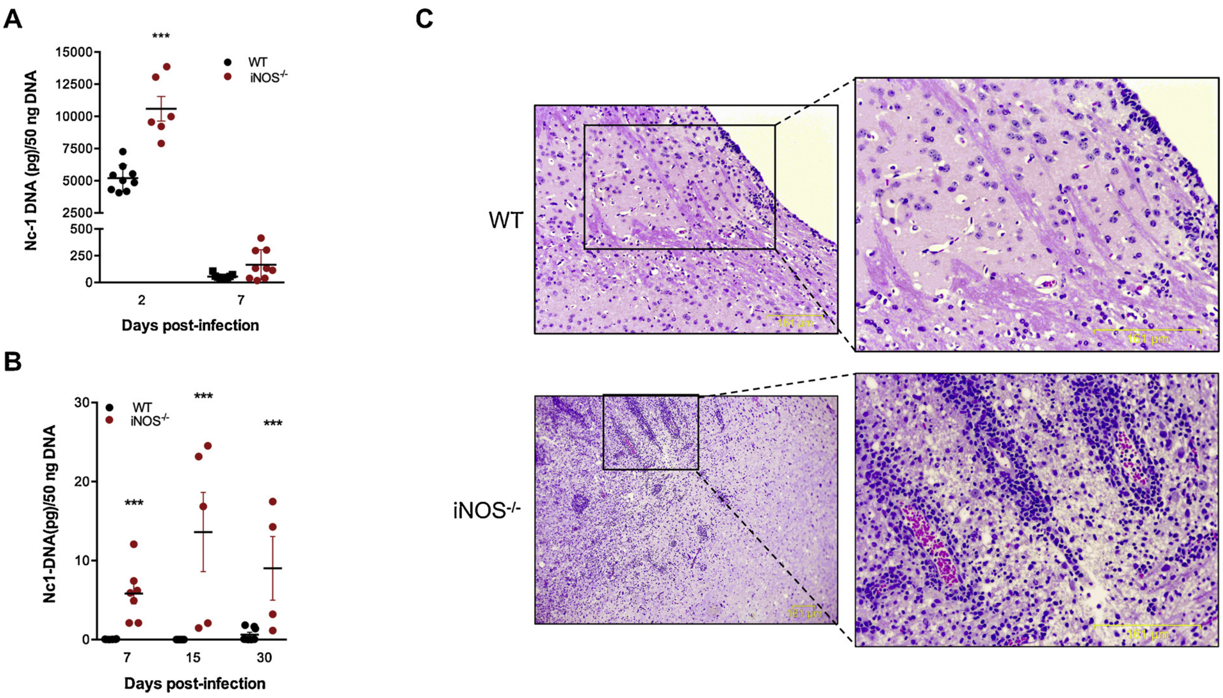

Fig. 3. iNOS is essential for the control of *N. caninum* replication and tissue homeostasis. Parasite burden was quantified by qPCR in peritoneal cells (A) and brain tissue (B) from WT and iNOS$^{-/-}$ mice, experimentally infected with a sub-lethal dose (1 × 10^6) of *N. caninum* tachyzoites. Results are expressed as picograms of parasite DNA per 50 ng of host DNA, extrapolated from the quantitative detection of the *Nc5* region normalized by the host's *gapdh* gene (C) Representative images of histopathological changes in the central nervous system of WT and iNOS$^{-/-}$ mice at 30 dpi. Results are representative of two independent experiments. (***) indicate statistical significance ($p < 0.001$) [ANOVA followed by Bonferroni multiple comparison post-test].

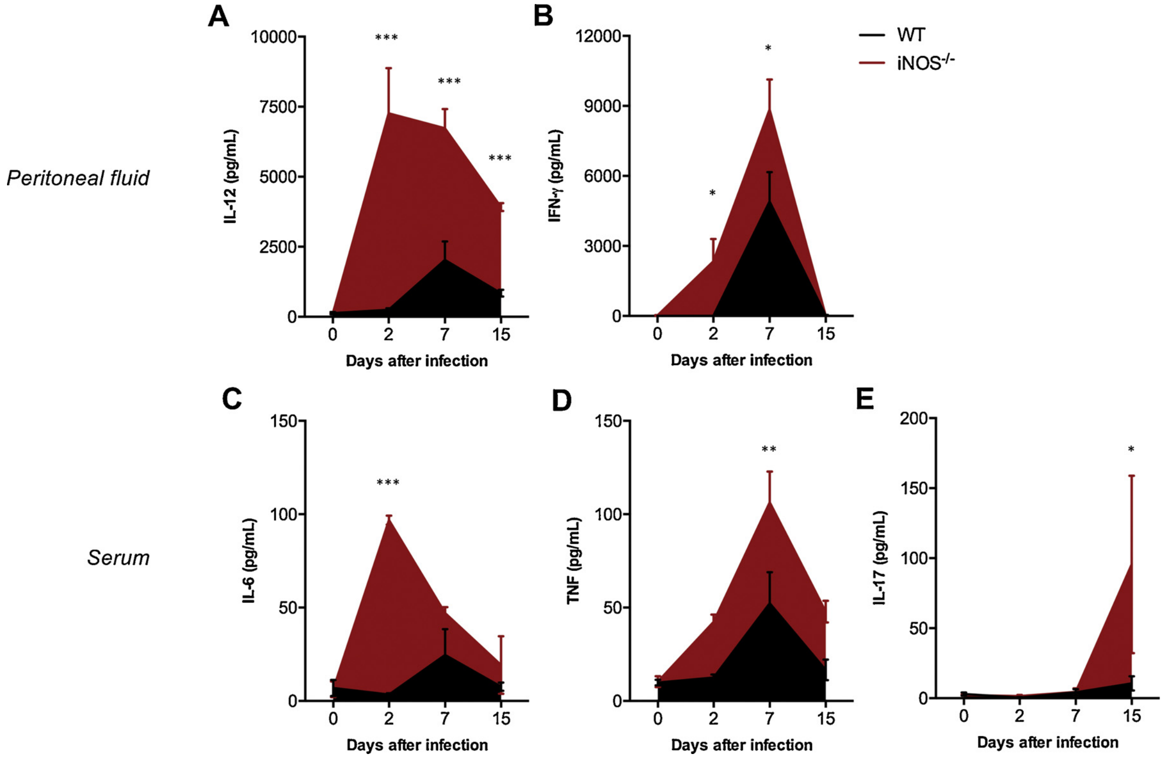

Fig. 4. iNOS$^{-/-}$ mice present systemic exacerbated production of pro-inflammatory cytokines. WT and iNOS$^{-/-}$ mice were infected with sublethal doses (1×10^6) of *N. caninum* tachyzoites and levels of IL-12 (A) and IFN-γ (B) were quantified in the peritoneal fluid. Additionally, other inflammatory mediators - IL-6 (C), TNF (D) and IL-17A (E) - were measured in serum samples of both groups of mice. Results, expressed as mean ± SEM, are representative of at least seven independent experiments (for A and B) and two independent experiments (for C–E). Asterisks indicate statistical significance level - (*) $p < 0.05$; (**) $p < 0.01$; $p < 0.001$ (***) [Student's *t*-test and ANOVA followed by Bonferroni multiple comparison post-test].

in the absence of iNOS, mice lack proper regulation of pro-inflammatory cytokine production.

4. Discussion

Protozoan pathogens of the phylum Apicomplexa are one of the major threats to human and animal health around the world (Guerra et al., 2006; Mueller et al., 2016). Despite the great economic impact of neosporosis within cattle industries worldwide, there are no vaccines or treatments currently available, and the amount of research involving this pathogen in the literature is still limited (Marugan-Hernandez, 2017; Reichel et al., 2013). A better understanding of signaling pathways and immunological mechanisms triggered by the interaction between *N. caninum* and its host cells is essential for development of effective therapeutic strategies against neosporosis. Our research group has dedicated its efforts in the last decade to help elucidate and functionally characterize pathways involved in the recognition, signaling and elimination of *N. caninum* (da Silva et al., 2017; Davoli-Ferreira et al., 2016; Mineo et al., 2009, 2010a; Mineo et al., 2010b; Miranda et al., 2019; Mota et al., 2016). Here, our goal was to evaluate the role of iNOS during *N. caninum* infection.

Many studies have shown that nitric oxide derived from iNOS acts in numerous biological processes including vasodilation, neurotransmission and host defense against pathogens of different classes (Bogdan, 2001; Bogdan et al., 2000; Calabrese et al., 2007). As expected, we show that *in vivo* and *in vitro* infections with *N. caninum* in mice triggers the production of NO. In addition, we found that in the absence of iNOS, mice present higher mortality and morbidity rates. These results are in agreement with data demonstrating that NO is an essential component of host defenses against various pathogens including viruses, bacteria and parasites (Alderton et al., 2001; Bogdan et al., 2000). Mice lacking the iNOS enzyme have been shown to display increased susceptibility to infection with *Leishmania major* (Wei et al., 1995), *Listeria monocytogenes* (MacMicking et al., 1995), *Salmonella typhimurium* (Mastroeni et al., 2000), *Paracoccidioides brasiliensis* (Livonesi et al., 2009) and chronic infection with *T. gondii* (Scharton-Kersten et al., 1997).

In an attempt to understand the mechanisms involved in this higher susceptibility of iNOS$^{-/-}$ mice, we first evaluated the parasite burden of these animals, followed by analysis of histopathological changes in the CNS and production of proinflammatory cytokines. We observed that iNOS$^{-/-}$ mice presented higher parasite DNA concentration during the acute and chronic infection, associated with higher cellular infiltration and brain inflammation. Data from *T. gondii* studies show that mortality of iNOS$^{-/-}$ mice is associated with defective control of parasite growth in the CNS, allied to a defective ability of macrophages to kill the parasite *in vitro* and *ex vivo* (Scharton-Kersten et al., 1997; Woods et al., 2013). Our data strongly suggest that iNOS is part of one of the main effector mechanisms involved in the restriction of *N. caninum* replication. In the absence of this effector pathway, mice lose their ability to efficiently contain the parasite at the site of the infection - which quickly spreads to the CNS - and where it causes uncontrolled tissue damage and consequent death.

Inflammation is a biological host defense response to harmful stimuli, such as infectious agents, tissue ischemia, autoimmune responses and toxic compounds. However, despite its great importance in the maintenance of homeostasis, when exacerbated, inflammation is a common cause of tissue damage, disease and death (Chen et al., 2018; Medzhitov, 2010). Cytokines regulate important aspects of the immune response, including the intensity and duration of inflammation. Our results show that iNOS$^{-/-}$ mice produced significantly higher levels of proinflammatory cytokines during the course of the infection that, associated with notable inflammatory lesions in the brain of these mice, demonstrate that the absence of iNOS induced an exacerbated inflammatory process, most likely due to excessive antigen availability and immune cell hyperactivation. Studies with *T. gondii* show that - although the production of proinflammatory cytokines, especially IL-12 and IFN-γ, are required for resistance to *T. gondii* (Gazzinelli et al., 1994; Scharton-Kersten et al., 1996; Suzuki et al., 1988) - excessive levels of these cytokine are lethal (Gavrilescu and Denkers, 2001; Mordue et al., 2001; Suzuki et al., 2000). Several studies have shown that iNOS expression can modulate inflammatory cytokine production. Giordano et al (Giordano et al., 2011) and Xiong et al. (Xiong et al., 2004) reported that proinflammatory cytokines (including TNF, IL-6 and IL-12) were upregulated in iNOS$^{-/-}$ mice and cells. iNOS deficient mice also are more susceptible to the development of inflammatory diseases such as EAE (Bogdan, 1998; Niedbala et al., 2011).

5. Conclusion

In conclusion, we here show that iNOS is an essential molecule for the efficient restriction of *N. caninum* replication and the regulation of the production of immune mediators, conferring mice resistance against the infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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