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# *Toxoplasma gondii*: The role of IFN-gamma, TNFRp55 and iNOS in inflammatory changes during infection

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In order to examine the role of IFN-γ, TNFRp55 and iNOS in inflammatory reaction during toxoplasmosis, IFN-$\gamma^{-/-}$, TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice were experimentally infected with *Toxoplasma gondii* ME-49 strain. The organs of the mice were evaluated for histology and immunohistochemistry in detection of tissue parasitism and iNOS positive cells. IFN-$\gamma^{-/-}$ mice presented mild inflammation in peripheral organs associated with a high parasitism and mortality in the acute phase of infection. In contrast, the peripheral organs of WT, TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice, presented a significant inflammatory reaction and low tissue parasitism in the same period of infection. The inflammatory lesions and tissue parasitism were increased and more severe in the Central Nervous System (CNS) of TNFRp55$^{-/-}$ and iNOS$^{-/-}$ with a progression of infection, when compared to WT mice. In these knockout animals, the inflammatory changes were associated with low levels or no expression of iNOS in TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice, respectively.

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## 1. Introduction

*Toxoplasma gondii* infection in most adult animals and humans is asymptomatic because of effective protective immunity (Frenkel, 1988). In immunocompetent individuals, infection with the parasite causes little or no overt signs of disease in its hosts, but in situations of immunodeficiency, or during congenital infection, *T. gondii* may emerge as a serious infection, which if untreated can lead to host death (Denkers and Gazzinelli, 1998; Denkers, 1999). Tachyzoites are the principal pathogenic stage in toxoplasmosis. If immunity is not acquired in a timely fashion, tachyzoites continue to multiply, destroying an excessive number of cells and producing lesions in several organs, with pneumonia and encephalitis being the prominent causes of illness and death (Frenkel, 1988). In immunocompromised individuals, in particular those with AIDS, the rapid multiplication of tachyzoites in the central nervous system (CNS) on reactivation of infection results in toxoplasmic encephalitis (TE) (Kasper and Buzoni-Gatel, 1998).

Virtually all mouse strains develop a strong Th1 immune response to *T. gondii*, regardless of whether they present resistant or susceptible MHC haplotypes (Gazzinelli et al., 1991, 1992). In murine models, CD4$^+$ and CD8$^+$ T cells have both important roles in resistance to *T. gondii*, which is at least in part mediated by cytokines (Suzuki and Remington, 1988; Gazzinelli et al., 1991). In vivo studies indicate that IFN-γ is a major cytokine, which mediates resistance against *T. gondii* infection (Suzuki et al., 1988). *In vitro* experiments have shown a crucial role for both IFN-γ and TNF-α in the induction of reactive nitrogen intermediates (RNI) and microbicidal activity displayed by murine macrophages against tachyzoites (Adams et al., 1990; Sibley et al., 1991; Langermans et al., 1992). Additionally, infection of murine monocyte-derived or peritoneal macrophages by *T. gondii* can induce a significant reduction in NO production (Seabra et al., 2002). Experiments with neutralization of endogenous TNF-α during chronic toxoplasmosis show reactivation of chronic infection and a lethal exacerbation of the disease (Gazzinelli et al., 1993). In addition, TNF receptor p55 and p75 (TNFRp55$^{-/-}$ and p75$^{-/-}$), and TNFRp55 (TNFRp55$^{-/-}$) deficient mice develop a lethal TE (Deckert-Schluter et al., 1998; Yap et al., 1998; Silva et al., 2002a,b). Other experiments with inhibitors of inducible nitric oxide synthase (iNOS) or the iNOS$^{-/-}$ mice have demonstrated that RNI production is important to control chronic infection, since these animals develop necrotizing encephalitis within 3 to 4 wk post-infection (Hayashi et al., 1996; Scharton-kersten et al., 1997; Silva et al., 2002a,b).

Pro-inflammatory cytokines at infection site are important to the recruitment and activation of leukocytes, which mediate local host defenses (Echtenacher et al., 1990; Eskandari et al., 1992). TNF-α and IFN-γ synergistically activate expression of chemokine genes, which play a crucial role in the chemo-attraction of

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leukocytes during inflammatory response (Sallusto et al., 1999; Lee et al., 2000). On the other hand, RNI have been shown to inhibit lymphocyte proliferation during acute phase of infection with *T. gondii* (Candolfi et al., 1995; Hayashi et al., 1996).

In the present study, we comparatively examined the role of IFN-$\gamma$, TNFRp55 and iNOS in the inflammatory responses in peripheral organs and in the CNS during the entire period of evolution of the infection with *T. gondii*. We confirmed that IFN-$\gamma$ is essential to induce inflammatory response and control of the parasite load during early stages of infection. On the other hand, the absence of iNOS in iNOS$^{-/-}$ mice and low iNOS expression combined with the absence of TNFRp55 in TNFRp55$^{-/-}$ mice is associated with an intense inflammatory immune response that is detrimental to the host who is thus unable to control *T. gondii* replication.

## 2. Materials and methods

### 2.1. Experimental animals

IFN-$\gamma^{-/-}$ and iNOS$^{-/-}$ mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME). The TNFRp55$^{-/-}$ matrices were kindly provided by Dr. Klaus Pfeffer (Technical Institute of Hygiene, University of Munich, Munich, Germany) (Pfeffer et al., 1993). The wild type (WT) and knockout mice, both on a C57BL/6 background, were bred as homozygotes and kept in the Laboratory of Nutrition and Gnotobiology from the Department of Biochemistry and Immunology, Biological Sciences Institute, UFMG. Mice were housed in pathogen-free conditions. All of the animals used for the experiments were female at age of 8–12 weeks. Animal experiments were conducted according to institutional guidelines for animal ethics and was approved by the Ethics Commission of Ethics in Animal Research (CETEA) with the protocol number 037/2006.

### 2.2. Infectious organisms

The low-virulent ME-49 strain of *T. gondii* was used to infect animals in this experiment. Cysts were harvested from the brains of C57BL/6 mice that had been inoculated with approximately 10 cysts, by the intraperitoneal (i.p.) route, 1 month prior. For experimental infections, knockout and WT mice received 10 ME-49 cysts in a volume of 0.1 ml by the i.p. route.

### 2.3. Experimental procedure and tissue processing

Groups of 3 mice were injected with anesthetics Ketamine (Syntec Brasil Ltda, SP, Brazil)/Xylazine (Schering-Plough Coopers, SP, Brazil) by i.p. route and were killed by cervical dislocation on different days post- infection (p.i.) as follow: IFN-$\gamma^{-/-}$ (5, and 8), iNOS$^{-/-}$ (5, 8, 14, and 21), TNFRp55$^{-/-}$ (5, 8, 14, 21, and 25) and WT (5, 8, 14, 21, 25 and 30). The brain, spinal cord, lung, liver, spleen, kidney, and heart tissue samples were collected, fixed in 10% buffered formalin and processed routinely for paraffin embedding and sectioning. Cervical, thoracic, lumbar and sacral regions of the spinal cord were examined. Tissue sections with 4 μm thickness (40 μm distance between sections) of each organ from each mouse were obtained in microtome and mounted in slides for histopathological and immunohistochemical studies. Tissue sections were stained with Haematoxylin and Eosin and they were observed under light microscope. In addition, the tissue sections were stained by immunoperoxidase method for identification and quantification of tissue parasitism and iNOS positive cells.

### 2.4. Immunohistochemistry

Tissue parasitism was quantified by immunohistochemistry as described previously (Welter et al., 2007; Benevides et al., 2008). For immunohistochemistry, deparaffinized sections were submitted to antigenic unmasking in a microwave oven. The sections were incubated for 30 min at 37 °C in 2% unlabeled sheep serum to reduce nonspecific binding. Then incubated in polyclonal rabbit antibody against whole parasites of ME-49 strain of *T. gondii* (total antigen parasite) (produced by our Laboratory by immunizing rabbit with soluble antigen of ME-49 strain of *T. gondii*) or rabbit anti-iNOS peptide (Santa Cruz Biotechnology, Inc., CA, USA), at 4 °C overnight. Secondary biotinylated antibodies were sheep anti-rabbit antibodies. The sensitivity was improved with the avidin–biotin technique (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, CA, USA). The reaction was visualized by incubating the section with 3,3′-diaminobenzidine tetrahydrochloride (Amresco, Solon, OH, USA) for 5 min. Control slides were incubated in the unlabeled rabbit serum. The slides were studied with an Olympus microscope and the images were captured in a digital camera.

The tissue parasitism detected by immunohistochemistry was scored as previously described (Gazzinelli et al., 1993) by counting the total number of cyst like structures and parasitophorous vacuoles in 40 microscopic fields per histological section using a 40× objective (40 μm distance between sections). Six sections were analyzed for each animal. Parasitophorous vacuoles were graded on an arbitrary scale as follow: 1+, for detection of one lesion containing few parasitophorous vacuoles in three to five histological sections; 2+, one or more lesions containing parasitophorous vacuoles in two sections; 3+, one or more lesions containing parasitophorous vacuoles per section; 4+, one or more lesions containing parasitophorous vacuoles per section and at least one extensive lesion containing abundant parasitophorous vacuoles; 5+, more than one extensive lesion containing abundant parasitophorous vacuoles per section; 6+parasitophorous vacuoles widespread for the all section. The iNOS+ cells were analyzed by counting of the number of positive cells per sagittal section of the CNS.

### 2.5. Histological and morphometric analysis

In order to score the inflammatory infiltration in cardiac tissue, the quantification was performed in six noncontiguous sections (40 μm distance between them) in 25 fields using a 20× objective in a blind manner by two researchers. An inflammatory infiltrate was considered when we detected 30 leukocytes or more in each inflammatory focus.

In the lung the inflammatory score was done as previously described (Bernardes et al., 2006) being analyzed to the inflammatory cell infiltration within the alveolar walls. In the liver, the inflammatory foci scattered in the parenchyma were quantified. In these peripheral organs, the inflammatory changes were examined in two noncontiguous sections (40 μm distance between them) from each mouse in 40 microscopic fields. For the inflammatory score in the CNS, the total numbers of focal or diffuse inflammatory foci were counted in a sagittal section and the cuffing of blood vessels and inflammatory cells infiltration in the meninges were analyzed. All of the analysis was done using a 40× objective in a blind manner by two researchers. The inflammatory score was represented as arbitrary units: 0–2, mild; 2–4, moderate; 4–6, severe; and above 6, very severe.

Histological changes and tissue parasitism were consistent among individual mice in the same group and between sections from the same organ of each mouse.

### 2.6. Statistical analysis

Statistical determinations of the difference of tissue parasitism between means of experimental groups were performed using an unpaired, two-tailed Student's *t*-test. Difference of histological

analysis among groups of animals, were compared by using Mann–Whitney U test. Statistical analysis and graphs were performed using GraphPad prism version 4.0 (GraphPad Software, San Diego, USA). Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Disease progression and tissue parasitism in WT, TNFRp55$^{-/-}$, iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice infected with T. gondii

The tissue parasitism in the liver, lungs and spleen was observed from 5 days p.i. and was more evident in the majority of these organs on day 8 p.i. (Table 1). The IFN-$\gamma^{-/-}$ mice presented a progressive disease and on day 8 p.i. the animals had ruffled fur and demonstrated severe depression with great weight loss and serious weakness, culminating with death. These animals presented ascites and tachyzoites in peritoneal exudates and simultaneously an extreme parasitism in peripheral organs (Table 1). Conversely, WT, TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice were apparently healthy in the acute phase of infection. In accordance with clinical signs, the animals presented relatively low numbers of parasites in peripheral tissues (Table 1) compared to IFN-$\gamma^{-/-}$ mice.

We verified the parasite distribution in the spinal cord in addition to the brain, in order to verify the role of IFN-γ, TNFRp55 and iNOS in this important site of *T. gondii* multiplication. The presence of parasite was more easily observed in the spinal cord on day 14 p.i., and different regions of the spinal cord presented similar parasite load. Parasitophorous vacuoles and cyst like structures were first detected in the brain on day 8 p.i. and the tissue parasitism was proportionally similar between the brain and spinal cord at each time point. The tissue presented a gradual increase of parasitophorous vacuoles and cyst like structures in the brain and spinal cord from TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice (Table 1). Closer to the time of death, TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice were severely sick presenting weakness, weight loss, piloerection and paresis, with the rear limbs being more strongly affected. In contrast, the WT presented less tissue parasitism in the CNS than that observed in TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice.

### 3.2. Histological changes in the peripheral organs of WT, TNFRp55$^{-/-}$, iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice infected with T. gondii

In acute phase of infection, the liver, spleen and lung were the most affected organs. The liver presented lesions as early as 5 days p.i., which was characterized mainly by mononuclear inflammatory foci scattered by parenchyma and portal areas (Fig. 1). The WT presented inflammatory lesions in the liver significantly higher than those observed in TNFRp55$^{-/-}$, iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice at this period of infection (Mann–Whitney $U = 0.000$; $P = 0.0022$) (Fig. 2A). Despite the low number of parasites on day 8 p.i., we observed increasing inflammatory changes (Figs. 1 and 2A) and necrosis foci in the liver from TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice. On day 8 of infection the inflammatory reaction was significantly greater in the liver from WT (Fig. 1A) and TNFRp55$^{-/-}$ (Fig. 1B) compared to iNOS$^{-/-}$ (Fig. 1C) and IFN-$\gamma^{-/-}$ mice (Fig. 1D) (Mann–Whitney $U = 0.000$; $P = 0.0095$). However, IFN-$\gamma^{-/-}$ showed less inflammatory alterations compared to iNOS$^{-/-}$ mice (Mann–Whitney $U = 0.000$; $P = 0.0095$) (Fig. 2A) and also presented necrosis foci in the organ. From day 14 p.i. the lesions presented a gradual decrease in the liver from TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice coinciding with the clearance of *T. gondii* from the organ (Table 1 and Fig. 2A).

Likewise, the pulmonary lesions were observed on day 5 p.i. coinciding with the presence of parasitophorous vacuoles in the lung (Table 1). The principal lesion observed in all animals was an intense chronic interstitial pneumonitis. This lesion was constituted of inflammatory exudates of mononucleated cells within the alveolar walls and the interstitial tissues, enlarging the pulmonary septum (Fig. 1E, F). Most of the cellular exudates were diffuse, but in some areas it was in focus. The inflammatory lesions were observed during the entire survival period in the organ of WT *T. gondii* infected mice (Fig. 2B). On day 8 of infection, the lung of IFN-$\gamma^{-/-}$ and iNOS$^{-/-}$ presented less severe inflammatory lesions (Fig. 1G,

**Table 1**
Parasitophorous vacuoles score and cyst like structures in the peripheral organs and CNS from TNFRp55$^{-/-}$, iNOS$^{-/-}$, IFN-$\gamma^{-/-}$ and WT mice infected with ME-49 strain of *T. gondii*.

| Days post-infection | Mouse strain | Parasitophorous vacuoles score[a]/number of cyst like structures (parentheses)[b] | | | | |
|---|---|---|---|---|---|---|
| | | Liver | Lung | Spleen | Heart | CNS |
| 5 | C57BL/6 | 3+ | 1+ | 1+ (0.7 ± 0.6) | – | – |
| | TNFRp55$^{-/-}$ | 1+ | 2+ | 1+ (0.3 ± 0.6) | – | – |
| | iNOS$^{-/-}$ | 3+ (0.3 ± 0.6) | 1+ | 3+ (1.3 ± 0.6) | – | – |
| | IFN-$\gamma^{-/-}$ | 3+ (1.0 ± 1.0) | 1+ | 1+ (0.3 ± 0.6) | – | – |
| 8 | C57BL/6 | – | 2+ (2.0 ± 0) | –(1.3 ± 1.2) | – | 2+ (2.3 ± 1.5) |
| | TNFRp55$^{-/-}$ | – | 3+ (8.3 ± 3.5) | –(0.3 ± 0.6) | –(0.3 ± 0.6) | 3+ (2.7 ± 1.5) |
| | iNOS$^{-/-}$ | 2+ (0.3 ± 0.6) | 4+ (5.3 ± 7.6) | 2+ (2.0 ± 1.7) | 2+ (0.3 ± 0.6) | 2+ (5.3 ± 3.5) |
| | IFN-$\gamma^{-/-}$ | 6+ (24.3 ± 1.2)* | 6+ (8.0 ± 2.0) | 6+ (19.7 ± 7.1)* | 3+ (4.0 ± 3.5) * | – |
| 14 | C57BL/6 | – | 2+ (3.0 ± 1.0) | – | – | 3+ (39.7 ± 23.1) |
| | TNFRp55$^{-/-}$ | – | 2+ (0.3 ± 0.6) | – | – | 3+ (42.0 ± 32.0) |
| | iNOS$^{-/-}$ | – | 2+ (3.0 ± 3.0) | 2+ (0.3 ± 0.6) | –(0.3 ± ± 0.6) | 4+ (72.3 ± 25.5) |
| 21 | C57BL/6 | – | – | –(1.0 ± 1.7) | – | 1+ (25.7 ± 8.1) |
| | TNFRp55$^{-/-}$ | – | –(1.3 ± 1.5) | – | –(1.0 ± 1.0) | 4+ (117.7 ± 84.1) |
| | iNOS$^{-/-}$ | – | 1+ (11.0 ± 8.9) | 1+ | – | 4+ (159.7 ± 18.7)** |
| 25 | C57BL/6 | – | – | – | – | 1+ (30.0 ± 2.8) |
| | TNFRp55$^{-/-}$ | – | 1+ (0.3 ± ± 0.6) | –(0.3 ± 0.6) | – | 5+ (154.0 ± 85.5)** |
| 30 | C57BL/6 | – | – | – | – | 3+ (41.0 ± 11.3) |

Similar results were obtained in two experiments.
*Significantly different from values obtained from WT, TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice ($p < 0.01$).
**Significantly different from values obtained from TNFRp55$^{-/-}$ and WT mice ($p < 0.001$).
[a] The parasitophorous vacuoles score was obtained from assays performed in 40 microscopic fields per histological section on 6 sections using a 40× objective (40 μm distance between sections) from three mice/group. The score was measured as described in Materials and Methods.
[b] The results are the means and standard deviations of number of cyst like structures on 40 microscopic fields from each of 6 sections (40 μm distance between sections) per mouse from three mice/group.

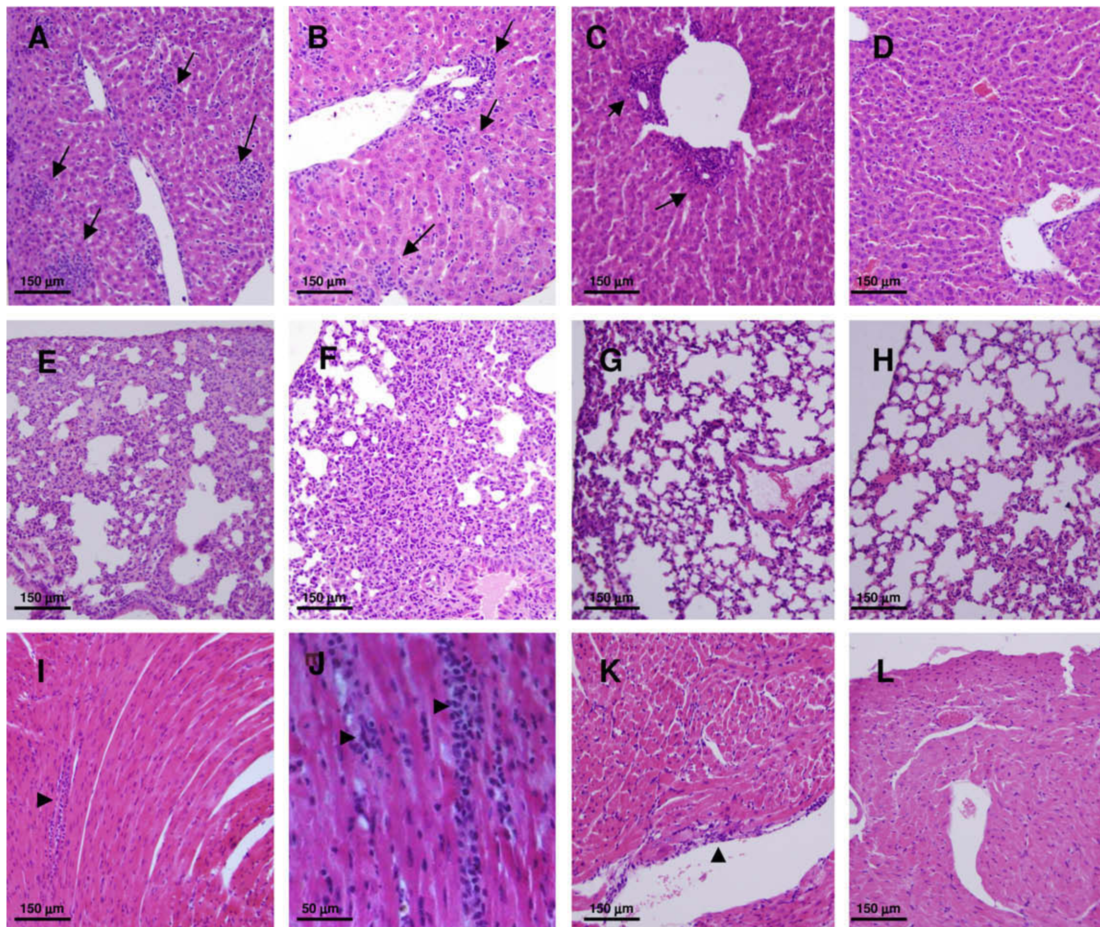


**Fig. 1.** Histopathological findings in the liver, lung and heart of WT, TNFRp55$^{-/-}$, iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice infected with ME-49 strain of *T. gondii*. (A) Hepatic inflammatory foci in the liver of WT, (B) TNFRp55$^{-/-}$, (C) iNOS$^{-/-}$, and (D) IFN-$\gamma^{-/-}$ mice on day 8 p.i., (E) pulmonary septum from WT, (F) TNFRp55$^{-/-}$, (G) iNOS$^{-/-}$ and (H) IFN-$\gamma^{-/-}$ mice on day 8 p.i, (I) inflammatory infiltrates in tissue heart from WT, (J) TNFRp55$^{-/-}$, (K) iNOS$^{-/-}$ on day 21 p.i. and (L) IFN-$\gamma^{-/-}$ mice on day 8 p.i. (H&E staining).

H) when compared to TNFRp55$^{-/-}$ and WT mice (Mann–Whitney U = 0.000; $P$ = 0.0022) (Fig. 2B). On day 14, after parasite inoculation, the pulmonary inflammatory changes were elevated in TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice. By day 21 p.i., TNFRp55$^{-/-}$ and WT presented a decrease of inflammatory lesions in the organ, in contrast to iNOS$^{-/-}$ mice which presented an intense inflammatory reaction in this period of infection (Mann–Whitney $U$ = 4.000; $P$ = 0.0260; related to WT and $U$ = 0.000; $P$ = 0.0022; related to TNFRp55$^{-/-}$ mice) (Fig. 2B). Furthermore, we observed a higher number of parasites in the tissue of iNOS$^{-/-}$ compared to TNFRp55$^{-/-}$ and WT mice. Thus, IFN-$\gamma^{-/-}$ mice showed a slight pulmonary inflammatory reaction and the lesions were delayed and persistent in the organ from iNOS$^{-/-}$ when compared to TNFRp55$^{-/-}$ and WT mice.

The spleen was affected by *T. gondii* from day 5 p.i., when we observed typical germinal centers in the white pulp (data not shown) in TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice. An intense parasitism of this organ was detected in IFN-$\gamma^{-/-}$ mice (Table 1), however, at the same time germinal centers were not discernible in the white pulp. Necrosis foci were also observed in the spleen of some *T. gondii* infected IFN-$\gamma^{-/-}$ and iNOS$^{-/-}$ mice on day 8 p.i. (data not shown).

In accordance with the low parasitism in the heart of TNFRp55$^{-/-}$, iNOS$^{-/-}$and WT mice (Table 1), the cardiac lesions were scarce and were characterized by small mononucleated inflammatory infiltrates (Fig. 1I, J, K and 2C). On day 14 p.i., the iNOS$^{-/-}$ presented more lesions in the cardiac tissue compared to TNFRp55$^{-/-}$ and WT mice (Mann–Whitney $U$ = 4.000; $P$ = 0.0260) (Fig. 2C). No inflammatory lesion was seen in the heart of IFN-$\gamma^{-/-}$ mice (Fig. 1L) despite having the highest tissue parasitism (Table 1).

### 3.3. Histological changes in the CNS from WT, TNFRp55$^{-/-}$, iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice infected with T. gondii

From day 8 p.i. *T. gondii* was observed in the brain in TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice coinciding with the observation of inflammatory changes in the brain and spinal cord. In accordance with low parasitism in the brain and spinal cord no inflammatory lesion was observed in the CNS from IFN-$\gamma^{-/-}$ mice. In general, the lesions in TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice were characterized by mononucleated cell infiltrates, glial nodules, vascular cuffing by lymphocytes and focal mononucleated cell infiltrates in the meninges (Fig. 3A). Diffuse infiltrates of mononuclear cells were also found. The lesions in the spinal cord were similar to those observed in the brain (Fig. 3A–D) contributing to neurological signs. The inflammatory changes were more frequently observed in the gray matter compared to white matter in the spinal cord (Fig. 3D). The inflammatory lesions were increased in the CNS from TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice with the progression of infection (Fig. 4A). TNFRp55$^{-/-}$ and iNOS$^{-/-}$ showed more severe inflammatory changes compared to WT mice on day 14 (Mann–Whitney $U$ = 0.000; $P$ = 0.0022) and 21 (Mann–Whitney $U$ = 0.000; $P$ = 0.0020, related to difference between TNFRp55$^{-/-}$ and WT mice; and Mann–Whitney $U$ = 0.000; $P$ = 0.0044, related to difference between iNOS$^{-/-}$ and WT mice)

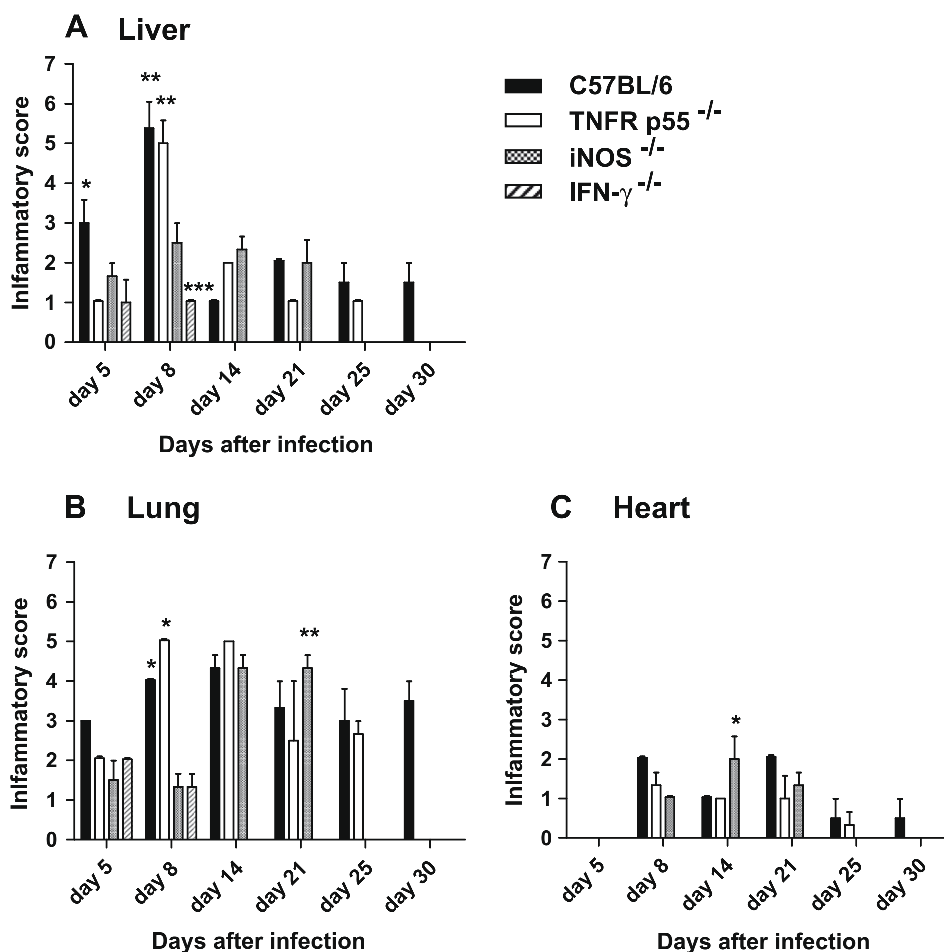


**Fig. 2.** Inflammatory score in the liver, lung and heart of WT, TNFRp55$^{-/-}$, iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice infected with ME-49 strain of *T. gondii*. The data were obtained by analyzing 40 microscopic fields per section on six sections using a 40× objective from each mouse and from three mice per group. (A) Liver, *Significantly different from values obtained from TNFRp55$^{-/-}$, iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice (Mann–Whitney $U = 0.000$; $P = 0.0022$); **Significantly different from values obtained from iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice (Mann–Whitney $U = 0.000$; $P = 0.0095$); ***Significantly different from values obtained from iNOS$^{-/-}$ mice (Mann–Whitney $U = 0.000$; $P = 0.0095$); (B) lung, *Significantly different from values obtained from iNOS$^{-/-}$ and IFN-$\gamma^{-/-}$ mice (Mann–Whitney U = 0.000; $P = 0.0022$); **Significantly different from values obtained from WT and TNFRp55$^{-/-}$ mice (Mann–Whitney U = 4.000; $P = 0.0260$; related to WT and $U = 0.000$; $P = 0.0022$; related to TNFRp55$^{-/-}$ mice); (C) heart, *Significantly different from values obtained from WT and TNFRp55$^{-/-}$ mice (Mann–Whitney $U = 4.000$; $P = 0.0260$).

p.i. (Fig. 4A). At the end of survival period we observed polymorphonucleated cells and large areas of parenchymal necrosis in the CNS from iNOS$^{-/-}$ and TNFRp55$^{-/-}$ mice culminating in the death of the majority of animals on days 21 and 25, respectively. Parasitophorous vacuoles were abundant in or close to necrotic areas. On day 21 p.i. the inflammatory lesions in the CNS, despite not being statistically significant, were more pronounced in iNOS$^{-/-}$ compared to TNFRp55$^{-/-}$ mice. In addition, iNOS$^{-/-}$ present tissue parasitism was statistically higher in the CNS when compared to TNFRp55$^{-/-}$ and WT mice, coinciding with the mortality of the animals. Less severe lesions were observed in the CNS from WT mice at 25–30 days p.i. and no necrosis focus was observed.

### *3.4. The impaired iNOS activation in the CNS from iNOS$^{-/-}$ and TNFRp55$^{-/-}$ mice contributes to major susceptibility of the knockout mice to T. gondii infection*

Immunohistochemistry analysis revealed that in the CNS of WT mice a high number of iNOS$^{+}$ cells were observed on day 25 p.i.. In contrast, the number of iNOS$^{+}$ cells in the CNS of TNFRp55$^{-/-}$ was smaller than that found in the WT mice (Fig. 3E, F and Fig. 4B) (Mann–Whitney U = 0.000; $P = 0.0095$). Thus, the smaller number and the absence of iNOS$^{+}$ cells in TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice, respectively, could have contributed to a poor control of parasite in the CNS of both knockout mice.

## 4. Discussion

We performed studies to analyze the balance between protective and pathogenic inflammatory response caused by *T. gondii* infection in TNFRp55$^{-/-}$, iNOS$^{-/-}$, IFN-$\gamma^{-/-}$ and WT mice. The parasite induces a highly polarized Th1 type immune response (Gazzinelli et al., 1991, 1992), and an appropriate immunoregulation is necessary to control this response and avoid immunopathology (Gazzinelli et al., 1996; Neyer et al., 1997; Suzuki et al., 2000). From the organs studied, the liver, lung and spleens had the most remarkable histological changes in acute infection.

We and others have previously demonstrated that mice with impaired IFN-$\gamma$ function succumb to acute infection (day 7–10 p.i.) with *T. gondii*, whereas with iNOS$^{-/-}$ (day 21–30 p.i.) and TNFRp55$^{-/-}$ (day 25–40 p.i.) mice death occurs later, when the chronic phase is establishing (Deckert-Schluter et al., 1996, 1998; Scharton-Kersten et al., 1996, 1997; Silva et al., 2002a,b). Consistent with the high susceptibility to *T. gondii* infection, the IFN-$\gamma^{-/-}$ mice presented an intense parasitism on day 8 p.i. Curiously, this intense tissue parasitism was not accompanied by intense inflam-

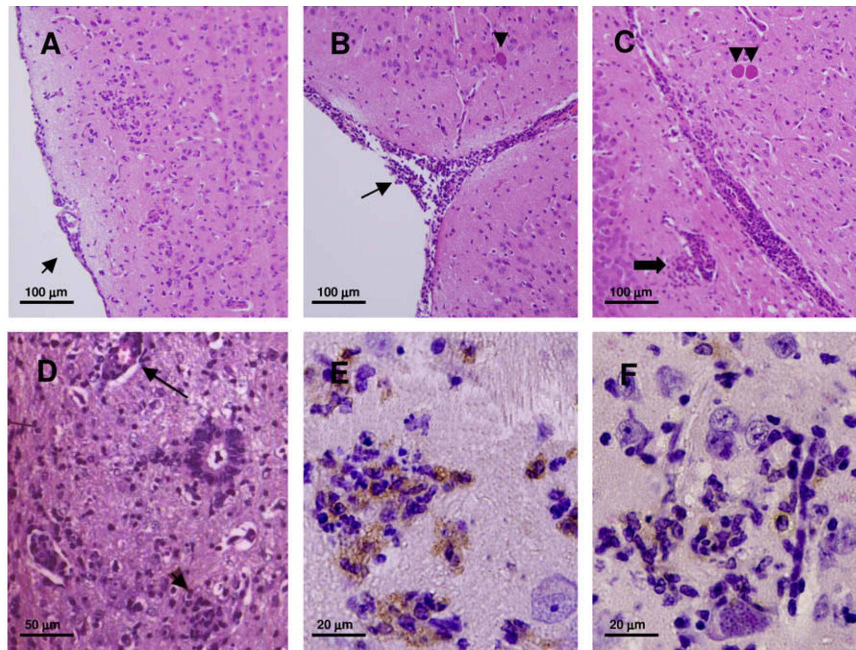


**Fig. 3.** Histological changes and iNOS staining in the CNS from iNOS$^{-/-}$, TNFRp55$^{-/-}$, and WT mice on day 21 of infection with *T. gondii*. (A) Presence of mononucleated inflammatory cells infiltrates in the meninges (meningitis) (arrow) in the brain of WT mice. (B) Meningitis (arrow) and cyst like structures (arrowhead) in the brain of TNFRp55$^{-/-}$ mice. (C) Microglial nodule (large arrow), vascular cuffing and cyst like structures (arrowheads) in the brain of iNOS$^{-/-}$ mice. (D) Presence of vascular cuffing (arrow), glial nodule (arrowhead) in the spinal cord of *T. gondii* infected iNOS$^{-/-}$ mice. (E) Large number of cells expressing iNOS in the brain of WT mice and (F) fewer cells expressing iNOS in the brain of TNFRp55$^{-/-}$ mice, respectively.

mation in the peripheral organs, when comparing to TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice. Thus, the recruitment of inflammatory cells, one of the most important immune mechanisms induced by IFN-γ (Lee et al., 2000), is involved in the control of parasite multiplication. IFN-γR$^{-/-}$ also presented an intense parasitism in peripheral organs, predominantly in the small intestine and liver when orally infected with DX strain of *T. gondii* (Deckert-Schluter et al., 1996). In those experiments the animals died by a necrotizing hepatitis, and in contrast to our studies they found prominent inflammatory infiltrates in the organ. The different results can be the response to the lineage of mouse, the different route of infection or the different strain of the parasite used in those experiments.

In contrast to IFN$^{-}$γ$^{-/-}$, TNFRp55$^{-/-}$, iNOS$^{-/-}$ and WT mice presented a lower number of parasites and a significant inflammation in the peripheral organs. The inflammatory cells or mechanisms induced by IFN-γ that are at least in part independent of TNFRp55 or iNOS, or both were able to decrease parasite proliferation in the liver, spleen and heart from TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice. It was observed that the inflammatory changes were higher in the liver and lung from WT and TNFRp55$^{-/-}$ compared to iNOS$^{-/-}$ mice on day 8 p.i., indicating that in the absence of iNOS the inflammatory infiltrates in these organs is lower or delayed. This observation is consistent with previous reports in iNOS$^{-/-}$ mice orally infected with *T. gondii*, where the knockout mice were more resistant to acute infection compared to WT mice and presented little evidence of inflammatory changes in the liver and small intestine (Khan et al., 1997). However, these previous experiments did not examine the pulmonary tissue. In contrast to WT and TNFRp55$^{-/-}$, iNOS$^{-/-}$ mice presented a persistent interstitial pneumonia contributing to the major susceptibility of this lineage of mouse compared to WT and TNFRp55$^{-/-}$ mice at the beginning of chronic stage of infection. Thus, NO has a paradoxical effect on the host immune system in that it has been implicated as a critical factor involved in the suppression of T cell responses (Mills, 1991). In addition, our experiments demonstrated that the presence of iNOS is involved in the control of inflammatory changes in the lung. On the other hand, this immune mediator is involved in degenerative changes in the liver and bowel in oral infection (Khan et al., 1997).

In the CNS from TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice, we observed through histological studies, a marked parasitism as well as inflammatory lesions from day 14 p.i., with maximum changes on days 21 and 25 to iNOS$^{-/-}$ and TNFRp55$^{-/-}$ mice, respectively. Similar to previous studies (Khan et al., 1996; Deckert-Schluter et al., 1998; Yap et al., 1998), the brain of iNOS$^{-/-}$ and TNFRp55$^{-/-}$ mice had large, loose foci of encephalitis, with extensive polymorphonuclear neutrophil infiltration and necrosis. Despite the protective effects of neutrophils in *T. gondii* infection (Bennouna et al., 2003; Bliss et al., 2001; Denkers et al., 2004), they are not sufficient to control acute toxoplasmosis in the gut (Dunay et al., 2008). In addition, neutrophils are known to degrade tissues by releasing matrix metalloproteinase (Hasty et al., 1990). Thus, in our experimental model, the presence of neutrophils in iNOS$^{-/-}$ and TNFRp55$^{-/-}$ mice is not efficient enough to control the parasite. The spinal cord of knockout and WT mice was also examined during *T. gondii* infection, showing inflammatory lesions and tissue parasitism similar to those from the brain, indicating that the whole CNS is affected by the parasite. The iNOS$^{-/-}$ was more susceptible to infection than TNFRp55$^{-/-}$ mice. The major susceptibility of iNOS$^{-/-}$ mice must be associated with a persistent interstitial pneumonia and necrotizing encephalitis in the CNS on day 21 p.i. Similar to pulmonary lesions, the CNS of iNOS$^{-/-}$ mice presented intense and increased inflammatory changes, indicating that RNI might be involved in the suppressive mechanism of the lymphocyte proliferation. As previously shown, in addition to being an important mechanism of parasite control, the production of high levels of RNI has been associated with suppression of lymphocyte proliferation in normal mice (Candolfi et al., 1995; Khan et al., 1995). This regulatory mechanism may work through the induction of apoptosis in a variety of cell types, including T cell clones (Williams et al., 1998; Dalton et al., 2000). Previous experiments have shown that, RNI produced by Th1 cells activated by specific

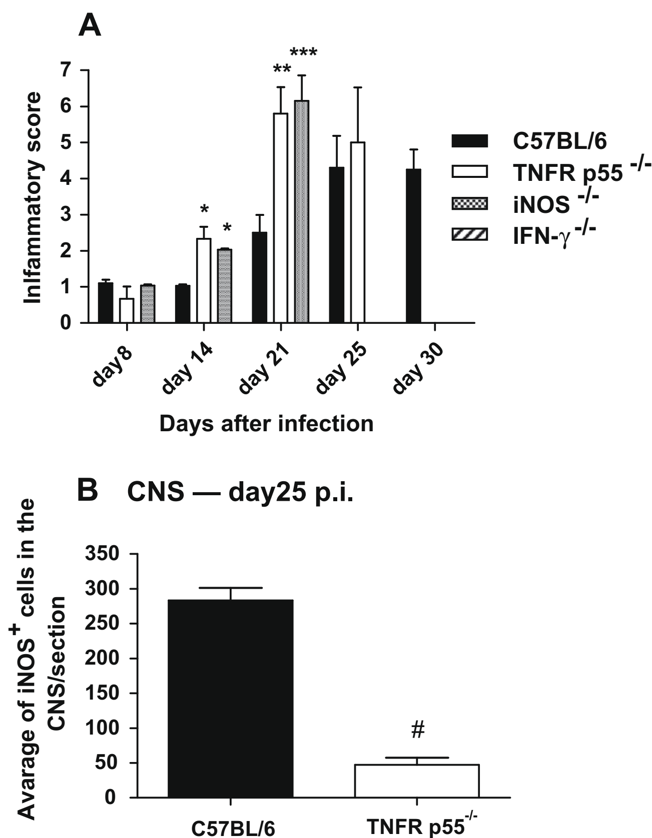


**Fig. 4.** Inflammatory score in the CNS of WT, TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice and number of iNOS$^{+}$ cells in the CNS of WT and TNFRp55$^{-/-}$ mice infected with *T. gondii*. (A) The data were obtained per sagittal section, in two sections per mouse using a 40× objective and three mice were used on each group. *Significantly different from values obtained from WT mice (Mann–Whitney $U = 0.000$; $P = 0.0022$); **Significantly different from values obtained from WT mice (Mann–Whitney U = 0.000; $P = 0.0020$); ***Significantly different from values obtained from WT mice (Mann–Whitney $U = 0.000$; $P = 0.0044$). (B) The data were obtained by counting the number of iNOS + cells per histological section using a 40× objective. # Significantly different from values obtained from WT mice (Mann–Whitney $U = 0.000$; $P = 0.0095$).

antigens or Con A can inhibit the secretion of IL-2 and IFN-γ, exerting a self-regulatory effect on Th1 cells (Taylor-Robinson et al., 1994). In agreement, iNOS$^{-/-}$ mice developed a significantly stronger Th1 immune response than WT or heterozygous mice infected with *Leishmania major* (Wei et al., 1995). Additionally, a significant amount of evidence indicates that RNI decrease expression of adhesion molecules such as VCAM-1, E-selectin, and P-selectin (Gauthier et al., 1994; DeCaterina et al., 1995; Khan et al., 1996) and RNI can modulate leukocyte recruitment and accumulation (Gaboury et al., 1996; Hickey et al., 1997; Benjamim et al., 2002). Similarly, in TNFRp55$^{-/-}$ mice the decreased iNOS expression observed in our studies might be associated with the accumulation of inflammatory cells that are producing less NO, as well as severity of the lesions and necrotic focus in the CNS in this lineage of mice.

It was previously shown that inflammatory monocytes (Gr1$^{+}$F4/80$^{+}$) expressing iNOS are able to protect against lethal oral toxoplasmosis (Dunay et al., 2008). In the present investigation, low or no iNOS expression contributed to uncontrolled parasite multiplication that in addition to inflammatory and necrotic lesions in the CNS induced the mortality of TNFRp55$^{-/-}$ and iNOS$^{-/-}$ mice. In contrast to TNFRp55$^{-/-}$ and iNOS$^{-/-}$, WT mice were more resistant to infection and presented an important, but moderate inflammatory infiltration in the organs. In normal mice, the ability of *T. gondii* to induce pro- and anti-inflammatory cytokines simultaneously is likely to be an indication of the requirement to strike a balance between controlling parasite growth and avoiding cytokine toxicity (Denkers, 1999). For other parasites this is also the case, because the absence of anti-inflammatory cytokine, IL-10, induced earlier mortality in infection with *Plasmodium chabaudi* or *Trypanosoma cruzi*, most likely due to a lethal inflammatory response rather than a fulminating parasitemia (Linke et al., 1996; Hunter et al., 1997).

Taken together, our results indicate that the inflammatory reaction induced by pro-inflammatory cytokines during *T. gondii* infection is crucial to control the parasite; however, a critical immunoregulation is required to prevent host immunopathology.

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