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Inhibition of Inducible Nitric Oxide Synthase Exacerbates Chronic Cerebral Toxoplasmosis in Toxoplasma gondii-Susceptible C57BL/6 Mice But Does Not Reactivate the Latent Disease in T. gondii-Resistant BALB/c Mice

Dirk Schlüter,* Martina Deckert-Schlüter,† Elke Lorenz,‡ Timothy Meyer,* Martin Röllinghoff, ‡ and Christian Bogdan 2‡

Infection of C57BL/6 mice with Toxoplasma gondii leads to progressive and ultimately fatal chronic Toxoplasma encephalitis (TE). Genetic deletion or inhibition of inducible nitric oxide synthase (iNOS) from the beginning of infection increased the number of T. gondii cysts in the brain and markedly reduced the time-to-death in this mouse strain. In the present study, we addressed whether iNOS also contributes to the control of intracerebral parasites in a clinically stable latent infection that develops in T. gondii-resistant BALB/c mice after resolution of the acute phase of TE. iNOS was expressed in the inflammatory cerebral infiltrates of latently infected BALB/c mice, but the number of iNOS+ cells was significantly lower than in the brains of chronically infected T. gondii-susceptible C57BL/6 mice. In BALB/c mice with latent TE (>30 days of infection), treatment with the iNOS inhibitors L-Nω-iminoethyl-lysine or L-nitroarginine-methylester for ≥40 days did not result in an increase of the intracerebral parasitic load and a reactivation of the disease, despite the presence of iNOS-suppressive inhibitor levels in the brain. However, L-nitroarginine-methylester treatment had remarkably toxic effects and induced a severe wasting syndrome with high mortality.

In contrast to BALB/c mice, L-Nω-iminoethyl-lysine treatment rapidly exacerbated the already established chronic TE of C57BL/6 mice. Thus, the containment of latent toxoplasms in T. gondii-resistant BALB/c mice is independent of iNOS, whereas the temporary control of intracerebral parasites in T. gondii-susceptible C57BL/6 mice with chronic TE requires iNOS activity. The Journal of Immunology, 1999, 162: 3512–3518.

Toxoplasma gondii is an obligate intracellular parasite that is able to persist in the central nervous system (CNS)1 of humans. The asymptomatic persistence of T. gondii is guaranteed by the immune system, which effectively prevents reactivation of the parasite. The central importance of the immune system for the control of latent T. gondii is illustrated in immunodeficient patients, in particular in AIDS patients, who are prone to a lethal reactivation of the dormant parasites in the brain.

The factors of the immune system that prevent reactivation of T. gondii have only been partially identified. Studies in experimental murine toxoplasmosis have revealed that T cells play a critical role, because simultaneous depletion of CD4+ and CD8+ T cells leads to reactivation of chronic Toxoplasma encephalitis (TE) (1). The major protective mechanism of CD4+ and CD8+ T cells is the production of IFN-γ, and neutralization of this cytokine also results in a lethal reactivation of chronic TE (2, 3). Moreover, neutralization of TNF-α, which is produced by CD4+ and CD8+ T cells, macrophages/microglial cells, and astrocytes in chronic TE, exacerbates cerebral toxoplasmosis (4, 5).

At present, it is unclear as to how IFN-γ and TNF-α control intracerebrally persisting toxoplasms. Because in vitro studies have demonstrated that IFN-γ induces a TNF-α-dependent nitric oxide (NO)-mediated toxoplasmastatic activity in murine macrophages (6, 7), it was hypothesized that IFN-γ and TNF-α also exert their protective activity in toxoplasmosis in part via the induction of NO. In fact, experiments in anti-TNF-α-treated mice, IFN-γR-deficient mice, or TNF receptor type 1 and type 1/2-deficient mice have shown that these mice die of an impaired parasite control that, apart from one study, was associated with a reduced expression of inducible nitric oxide synthase (iNOS, NOS2) (4, 8–10). In addition, studies directly addressing the role of NO in murine acute toxoplasmosis have revealed that an inhibition of iNOS with aminoguanidine beginning at the day of infection leads to an increased intracerebral parasitic load (11). Furthermore, iNOS-deficient mice had impaired parasite control and developed rapidly fatal acute cerebral toxoplasmosis (12, 13). In addition to this direct antiparasitic effect, NO exerts an important immunoregulatory role in acute toxoplasmosis by suppressing the cytokine production and proliferation of T cells (11, 14).

It is important to emphasize that all of the aforementioned studies on the role of iNOS in cerebral toxoplasmosis have been performed in T. gondii-susceptible mouse strains (i.e., C57BL/6 or 129Sv × C57BL/6 mice), which inevitably develop progressive...
and ultimately fatal TE due to a genetically determined insufficient intracerebral immune response (15, 16). As the mice were lacking the iNOS gene or were treated with nonspecific iNOS inhibitor from the very beginning of the infection (11–13), these studies did not directly analyze the function of iNOS/NO in chronic (i.e., already established) cerebral toxoplasmosis. In fact, in susceptible mice, inhibition of iNOS from the start of infection might indirectly influence the course of the subsequent encephalitis, because it prevents the inflammation and necrosis of the small bowel otherwise occurring in these mice after oral infection (12, 17). Experiments on the role of iNOS/NO in latent cerebral toxoplasmosis of *T. gondii*-resistant mouse strains (e.g., BALB/c mice) have never been reported.

Therefore, we performed a comparative analysis on the functional role of iNOS/NO in established latent vs progressive cerebral toxoplasmosis using *T. gondii*-resistant BALB/c mice and *T. gondii*-susceptible C57BL/6 mice, respectively. In both strains, cerebral toxoplasmosis was characterized by intracerebral expression of iNOS protein. Inhibition of iNOS activity resulted in a rapid and lethal exacerbation of TE in C57BL/6 mice. In contrast, parasite control of BALB/c mice remained completely unaffected. These findings argue for a critical role of NO in the control of intracerebral parasites in *T. gondii*-susceptible mice, whereas *T. gondii*-resistant strains effectively contain intracerebral toxoplasmas in the absence of iNOS activity.

### Materials and Methods

#### Mice

Female 6–8-wk-old BALB/c (H-2d) and C57BL/6 (H-2b) mice were obtained from Harlan Winkelmann (Borchen, Germany). The animals were kept on a normal rodent diet under conventional conditions throughout the experiments.

#### Parasites

Parasites were harvested from the brains of NMRI mice that had been chronically infected with the low-virulent DX strain of *T. gondii*. Brain tissue of these animals was dispersed in 0.9% NaCl. The final concentration of the infectious agent was adjusted to a dose of 10 cysts/0.5 ml, and this dosage was administered orally to the mice by gavage.

#### NO synthase (NOS) inhibitors

L-N6-iminoethyl-lysine (L-NIL), a potent selective inhibitor of iNOS (18, 19), was either synthesized at Searle/Monsanto (St. Louis, MO) or purchased from Alexis (Laufelfingen, Switzerland). Both L-nitroarginine-methyl ester (L-NAME), an inhibitor of all NOS isoforms (18, 20), and the inactive analogue L-nitroarginine-methyl ester (L-NAME) were obtained from Alexis. Either L-NAME or water was used as a control for L-NIL, because sufficient amounts of an inactive D analogue of L-NIL are not yet commercially available. The stereospecificity of L-NIL has been demonstrated previously (18, 19).

#### In vivo treatment of chronically infected mice

Genetically resistant BALB/c and susceptible C57BL/6 mice (four to six mice per group) were treated with NOS inhibitors on or after day 30 postinfection. L-NIL, L-NAME, and L-NAME were added to the drinking water (adjusted to pH 2.7 to prevent bacterial overgrowth) at a concentration of 5–10 mM and were freshly provided every other day. Survival rates, body weight, and food and water uptake were recorded.

#### Histopathology

At the indicated days postinfection, animals were intracardially perfused with 0.9% NaCl to remove the blood from brain vessels. The brain was dissected, embedded in Tissue Tek OCT compound (Miles Scientific, Napperville, IL), snap-frozen in isopentane (Fluka, Neu-Ulm, Germany), pre-cooled on dry ice, and stored at −80°C.

*T. gondii* Ag and iNOS were detected on 5–7-µm frozen sections by incubation with rabbit anti-*T. gondii* antiserum (Biogenex, Duiven, The Netherlands) and rabbit anti-iNOS peptide-antiserum (obtained from Dianova, Hamburg, Germany, or kindly provided by Drs. C. Nathan and Q.-W. Xie, Cornell University Medical College, New York, NY), respectively, followed by peroxidase-labeled goat anti-rabbit IgG (Dianova). CD4+ T cells, CD8+ T cells, and B220+ B cells were stained on frozen sections with an indirect peroxidase protocol employing rat anti-CD4 (clone G.K 1.5), rat anti-CD8 (clone 2.43), and rat anti-B220 (clone RA3-3A1/61), respectively, as primary Abs. Thereafter, sections were incubated with peroxidase-labeled sheep anti-rat IgG. F4/80+ macrophages and microglia were detected by incubation with rat anti-F4/80 (clone F4/80), followed by biotinylated mouse anti-rat IgG F(ab)2', fragments and peroxidase-labeled streptavidin complex (Dakopatts, Hamburg, Germany). Peroxidase reaction products were visualized using 3,3'-diaminobenzidine hydrochloride (Sigma, Deisenhofen, Germany) and H2O2 as cosubstrates. Negative controls included omission of the primary Ab, incubation with an irrelevant isotype-matched control Ab, or competition of the staining by the peptide used for immunization. Sections were slightly counterstained in part with hemalum (Merck, Darmstadt, Germany).

#### Preparation of serum, cerebrospinal fluid (CSF), and brain homogenates from NOS inhibitor-treated mice

Blood was obtained from anesthetized mice by puncture of the retroorbital plexus with Pasteur pipets. Serum was prepared from clotted blood by centrifugation. Clear CSF (~5–8 µl/mouse) was obtained from mice after intracardial perfusion with 0.9% NaCl by puncturing the cisterna cerebellomedullaris with a fine glass capillary and was mixed with an equal volume of sterile distilled water to reduce losses. The perfused, blood-free brains (brain volume was ~1 ml) were homogenized in sterile distilled water (1.25 ml per brain) and centrifuged (36,000 × g, 30 min), and the supernatant (~1 ml per brain) was sterile-filtered (0.22 µm). Serum, CSF, and brain homogenates were stored at −70°C before being subjected to the NOS inhibitor analysis.

#### Determination of biologically active NOS inhibitor levels in serum, CSF, and brain homogenates

Determination of NO synthase (NOS) inhibitor levels was performed in serum, CSF, and brain homogenates. Serum samples and brain homogenates were initially filtered through a 10,000 m.w. cutoff centrifuge filter (Centrifuge, Millipore, Bedford, MA) to remove remaining particles from the centrifugation step (not performed for CSF samples because of lack of volume). The endogenous arginine in the samples was converted to urea by treatment with arginase (100 U/ml, 1 h at 37°C; Sigma, St. Louis, MO), and the enzyme (with a molecular mass of ~130 kDa) was subsequently removed by filtration (10,000 m.w. cutoff filter). Samples and standards of the respective water-soluble inhibitors (l-NIL and l-NAME, with molecular masses of 223.5 and 269.7 Da, respectively) were diluted appropriately and assayed in a NOS enzyme reaction wherein NOS activity was determined by monitoring the conversion of l-[^2H]arginine to l-[^2H]citrulline. The enzyme assays were performed with DEAEP partially purified recombinant human iNOS as detailed elsewhere (21), except that a final concentration of 2.5 µM l-arginine was used. The ability of treated samples to inhibit NOS enzyme activity was compared with a standard curve from the assay, in which defined amounts of each enzyme inhibitor were added to the enzyme reaction. The quantity of inhibitor present in the original sample was calculated from this standard curve.

#### Statistics

Statistical evaluations of differences in mortality, in intracerebral parasitic load, in the size of cysts, and in the number of iNOS® inflammatory foci between the various experimental groups were determined by the Student t test. p < 0.05 was accepted as significant. For a quantitative evaluation of the intracerebral parasitic load and the size of the *T. gondii* cysts, sections (10–15/cerebrum) were cut from various regions of the brain and immunohistochemically stained for *T. gondii* Ag. The number of *Toxoplasma* cysts per section was counted in ≥100 high-power fields (×400) and is shown as mean ± SEM. The size of the *T. gondii* cysts was determined from the same sections using an ocular with a micrometer scale bar, and the mean size ± SEM is shown. For the number of iNOS® inflammatory foci (mean ± SEM), tissue sections from various regions of the brains of five animals per group were analyzed.

#### Results

Differential kinetics of the parasitic load and expression of iNOS in the brains of *T. gondii*-resistant BALB/c mice and *T. gondii*-susceptible C57BL/6 mice

Oral infection of BALB/c mice with low-virulent *Toxoplasma* cysts induced chronic cerebral toxoplasmosis (Fig. 1a). A quantitation of the intracerebral parasitic load revealed that the number...
of cysts was continuously declining during the course of disease (Table I). Histopathologically, Toxoplasma cysts were frequently and closely surrounded by a few iNOS\(^{+}\) cells (Fig. 1b), but iNOS\(^{-}\) cysts were also found (data not shown).

Compared with BALB/c mice, the number of Toxoplasma cysts was much higher in the brains of C57BL/6 mice (Fig. 1c) and continuously increased during the course of disease (Table I). At day 60 postinfection, the amount of Toxoplasma cysts in C57BL/6 mice exceeded that of BALB/c mice by \(\geq 40\)-fold \((p < 0.001)\). At the same timepoint, the cysts (as determined by T. gondii immunostaining) were more than twofold larger in the brains of C57BL/6 mice as compared with BALB/c mice (Table I). Immunohistochemical staining for iNOS regularly revealed numerous iNOS\(^{+}\) leukocytes in close vicinity to T. gondii cysts (Fig. 1d). Compared with BALB/c mice, the number of iNOS\(^{+}\) cells surrounding an individual cyst was higher (Fig. 1, b vs d), and the extent of iNOS\(^{+}\) inflammatory infiltrates was increased 10-fold at day 30 postinfection (mean number \((\pm\) SEM) of iNOS\(^{+}\) inflammatory foci per brain section was 36 \(\pm\) 3 for C57BL/6 mice and 3 \(\pm\) 1 for BALB/c mice; \(p < 0.001\)).

These findings illustrate that BALB/c mice effectively reduce the amount of intracerebral Toxoplasma cysts and develop latent TE, whereas C57BL/6 mice develop progressive TE. Expression of iNOS was observed in both mouse strains and correlated with the number of intracerebral Toxoplasma cysts in the respective mouse strain.

**Oral uptake of NOS inhibitors \(\text{l-NIL and l-NAME}\) leads to iNOS-suppressive inhibitor levels in serum, CSF, and brain tissue of T. gondii-infected mice**

To analyze the functional role of iNOS/NO in chronic TE, it is a prerequisite that the NOS inhibitors gain access to the CNS. Therefore, we determined the concentration of biologically active \(\text{l-NIL}\) and \(\text{l-NAME}\) in the serum, the CSF, and the brains of chronically infected BALB/c mice, which had received drinking water supplemented with either of the two inhibitors for 12 consecutive days, using an assay based on the suppression of the activity of recombinant iNOS. \(\text{l-NIL}\) is a potent and nontoxic inhibitor with a 30-fold selectivity for the inducible form of NOS, whereas \(\text{l-NAME}\) suppresses all isoforms of NOS with a preference for the constitutive neuronal and endothelial enzymes (18–20). As shown in Table II, the concentration of \(\text{l-NIL}\) in the brain reached \(~50\%\)

![FIGURE 1. Histopathological findings in BALB/c and C57BL/6 mice at day 30 after T. gondii infection.](http://www.jimmunol.org/)

**Table I. Kinetics of intracerebral parasitic load and cyst size in T. gondii-resistant BALB/c and T. gondii-susceptible C57BL/6 mice**

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Days Postinfection</th>
<th>Number of cysts(\text{mean} \pm \text{SEM})</th>
<th>Size of cysts(\text{mean} \mu\text{M} \pm \text{SEM})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
<td>Day 30</td>
<td>Day 60</td>
</tr>
<tr>
<td>BALB/c</td>
<td>6.3 (\pm) 2.3</td>
<td>3.3 (\pm) 0.9</td>
<td>1.8 (\pm) 0.5</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>21.1 (\pm) 2.6</td>
<td>21.6 (\pm) 4.6</td>
<td>70.0 (\pm) 9.6</td>
</tr>
</tbody>
</table>

\(a\) The number of Toxoplasma cysts was determined in 100 high-power fields/section. A total of 10–15 sections from various regions of the brain were counted, and data represent the mean \((\pm\) SEM) number of cysts/section from four mice per group.

\(b\) Significantly different from the respective BALB/c values \((p < 0.001)\).

\(c\) The exact number of cysts could not be determined, as cysts were partially confluent in some high-power fields.

\(d\) Diameter of T. gondii immunoreactive areas.

\(e\) Significantly different from the respective BALB/c values \((p < 0.01)\).
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Table II. Oral treatment with l-NIL and l-NAME leads to iNOS-suppressive inhibitor levels in serum, CSF, and brain tissue homogenate of T. gondii-infected BALB/c mice

<table>
<thead>
<tr>
<th>Oral Treatment</th>
<th>Serum</th>
<th>CSF</th>
<th>Brain homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
<tr>
<td>l-NIL (10 mM)</td>
<td>45.6 ± 6.34</td>
<td>27 ± 11.5</td>
<td>23 ± 6.3</td>
</tr>
<tr>
<td>l-NAME (10 mM)</td>
<td>75.6 ± 13.3</td>
<td>+d</td>
<td>+d</td>
</tr>
</tbody>
</table>

a At day 29 postinfection, treatment with l-NIL (10 mM), l-NAME (10 mM), or water (control) was initiated and performed for 12 days.

b NOS inhibitor was not detected (detection limit of the assay was 5 μM).

c iNOS inhibitor was not detected (detection limit of the assay was 5 μM).

d Mean ± SD of three serum pools from two or three individual mice, respectively.

e Mean ± SD of three individual determinations with supernatants from brain homogenates derived from seven individual mice.

of the respective serum levels, thus exceeding the reported concentration required for a 50% inhibition (IC50) of iNOS in vitro (0.4–3 μM) by a factor of 10–80 (18). Although the concentration of l-NAME in the brain homogenates was similar (23 ± 6.3 μM), its known IC50 against iNOS is considerably higher (~20 μM), whereas it potently inhibits neuronal NOS (IC50 = 0.5 μM) (18). Low concentrations of both inhibitors were also found in the 1/1 prediluted CSF.

These findings illustrate that treating mice orally with l-NIL and l-NAME leads to iNOS-suppressive inhibitor levels in the CNS of T. gondii-infected mice.

Inhibition of iNOS does not lead to reactivation of latent TE in BALB/c mice

To test the hypothesis that iNOS is involved in parasite control in chronically infected BALB/c mice, these mice were treated orally with the iNOS inhibitor l-NIL. l-NIL treatment (10 mM in the drinking water) was started at various timepoints during chronic TE (i.e., at days 30 and 70 postinfection) and performed for ≤40 days. l-NIL treatment did not cause mortality, reduced food and water uptake, weight loss, or any clinically overt disease in BALB/c mice (Fig. 2). In addition, l-NIL-treated mice had slightly more necrotic foci, whereas immunohistostaining showed no significant differences in the distribution and quantity of intracerebral CD4+ T cells, CD8+ T due to the inhibition of constitutive neuronal and endothelial NOS. Thus, in accordance with previous results obtained in Leishmania major-infected mice (19, 22), this compound is not suitable for exploring the function of iNOS in experimental in vivo models.

Inhibition of iNOS exacerbates chronic TE in T. gondii-susceptible C57BL/6 mice

To analyze the function of iNOS in the chronic progressive toxoplasmosis of T. gondii-susceptible mice, T. gondii-infected C57BL/6 mice were treated with l-NIL. Inhibition of iNOS induced a rapidly fatal course of disease, and all l-NIL-treated C57BL/6 mice succumbed to the infection within 14 days (Fig. 2). Clinically, an exacerbation of TE in l-NIL-treated C57BL/6 mice was characterized by a severe wasting syndrome, and these mice showed prominent weight loss compared with control mice (Table IV). Histopathology of terminally ill l-NIL-treated mice showed that these mice had a significantly increased amount of intracerebral cysts compared with control treated mice (Table IV). In addition, l-NIL-treated mice had slightly more necrotic foci, whereas immunohistostaining showed no significant differences in the distribution and quantity of intracerebral CD4+ T cells, CD8+ T
cells, B cells, and macrophages between L-NIL- and control-treated mice (data not shown).

These results illustrate that an inhibition of iNOS leads to a rapidly fatal exacerbation of progressive TE in C57BL/6 mice.

Discussion

This study shows that in chronic TE of BALB/c mice, low levels of iNOS are expressed in the vicinity of Toxoplasma cysts. However, biologically active intracerebral levels of NOS inhibitors were not able to cause a reactivation of latent TE. Thus, once an effective intracerebral immune response is established in T. gondii-resistant mice, lack of iNOS activity is efficiently compensated for with respect to parasite control. In contrast, an inhibition of iNOS in T. gondii-susceptible C57BL/6 mice resulted in an early loss of parasite control and a rapid lethal exacerbation of chronic progressive TE, demonstrating the important role of NO in these mice.

These findings extend previous in vitro studies on the role of NO for the control of intracellular toxoplasmosis. NO inhibits the growth of T. gondii in IFN-γ-stimulated human astrocytes as well as murine macrophages and microglia (6, 7, 23, 24). However, in several other human cell types, including fibroblasts, glioblastoma cells, macrophages, and retinal pigment epithelial cells, indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan degradation inhibits the growth of T. gondii (25–29). With respect to murine cells, a decisive role for IDO in the control of T. gondii has not been described thus far. However, there is evidence for a cross-regulation between NO and IDO. Mouse cells, when expressing high levels of NO, show a drastically reduced activity of IDO (30, 31). In contrast, IDO levels increase under conditions with low NO concentrations; therefore, in chronically infected BALB/c mice, which express low amounts of iNOS, IDO-mediated tryptophan starvation may become clinically relevant and contribute to the control of latent toxoplasmosis. An alternative pathway that might also participate in the control of persisting toxoplasmosis is the production of oxygen intermediates by activated macrophages (32, 33).

The observation that the control of latent intracerebral toxoplasmosis is independent of iNOS in T. gondii-resistant mice contrasts with findings in other experimental infectious diseases with persisting pathogens. In murine leishmaniasis, resistant mouse control Leishmania persisting in lymph nodes via iNOS, and inhibition of iNOS employing exactly the same protocol as in the present study caused clinical recrudescence of the disease (22). In addition, in latent murine tuberculosis, NO controls the infectious organism in the lung, and inhibition of iNOS with L-NIL or amino-guanidine reactivated the disease (34, 35). These divergent findings on the role of NO for the control of persisting pathogens may be explained by pathogen-specific features or by an influence of organ-specific factors on the ensuing immune response.

What makes NO so important in chronic TE of T. gondii-susceptible C57BL/6 mice? Previous studies have shown that T. gondii-susceptible mice succumb to chronic progressive TE due to an insufficient intracerebral immune response; this response is characterized by significantly lower levels of intracerebral IFN-γ and TNF-α as compared with BALB/c mice (36–38). An analysis of the host genes responsible for the impaired intracerebral immune response of C57BL/6 mice has revealed that genes within the MHC complex as well as genes outside of the MHC are involved (16, 36, 39). The MHC class I Lp molecule, which is expressed in BALB/c but not in C57BL/6 mice, confers protection against T. gondii (40, 41). The importance of MHC class I-restricted CD8+ T cell responses in chronic TE is further supported by the observations that CD8+ T cells determine the number of intracerebral Toxoplasma cysts (39) and that T. gondii-susceptible C57BL/6 mice have reduced numbers of intracerebral CD8+ T cells compared with T. gondii-resistant mice (36). In contrast, C57BL/6 mice have increased numbers of intracerebral macrophages (38), which have been identified as the cellular source for iNOS in TE (5). Thus, the increased production of iNOS in C57BL/6 mice that was observed in the present study most likely reflects a compensatory mechanism for the insufficient intracerebral T cell response in this strain. Inhibition of this compensatory NO production results in a rapid multiplication of the parasite and in death of the animals.

Previous studies on the function of NO in acute murine toxoplasmosis of C57BL/6 mice also revealed that either inhibition of iNOS with aminoguanidine or infection of iNOS-deficient mice resulted in an increased parasite burden of the brain and of peripheral organs (11–13). Interestingly, these studies showed that, although the number of parasites was initially increased in peripheral organs of T. gondii-susceptible mice, the protective role of NO in these organs seemed to be less important than in the brain.

Table III. Treatment with L-NIL does not lead to an increased parasite load in the brains of BALB/c mice

| Days Postinfection | Treatment Group | Days of Treatment | Number of Cysts | Weight
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td>30</td>
<td>Control (2)</td>
<td>0</td>
<td>13.1 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>Control (1)</td>
<td>33</td>
<td>3.8 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>L-NIL (1)</td>
<td>33</td>
<td>2.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>Control (2)</td>
<td>40</td>
<td>2.4 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>L-NIL (2)</td>
<td>40</td>
<td>4.9 ± 1.8</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>70</td>
<td>Control (2)</td>
<td>0</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>Control (1)</td>
<td>33</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>L-NIL (1)</td>
<td>33</td>
<td>3.6 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>L-NAME (1)</td>
<td>33</td>
<td>3.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>L-NAME (2)</td>
<td>33</td>
<td>0.9 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>Control (2)</td>
<td>35</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>L-NIL (2)</td>
<td>35</td>
<td>1.8 ± 1.6</td>
</tr>
</tbody>
</table>
| | 105 | L-NAME (2) | 35 | 1.3 ± 1.6 | 17.9 ± 1.4

* The number of mice analyzed at a given timepoint in each treatment group (consisting of six mice) is shown in parentheses. Additional control and inhibitor-treated mice were analyzed at day 54 postinfection (Expt. 1) and day 96 postinfection (Expt. 2); these mice yielded similar results and are not shown for the sake of clarity. The total number of mice analyzed by immunohistology was eight in the L-NIL and control groups and four in the L-NAME and L-NAME groups.

* The number of cysts was determined in 100 high-power fields/section. A total of 10 sections from various regions of the brain were counted, and data were expressed as the mean ± standard error of the mean.

* The mean ± standard error of the mean is shown.

* The animals were weighed individually, and the mean ± SEM is shown.

* Significantly different from the respective control values (Student’s t test for unpaired samples; p < 0.01).

Table IV. Inhibition of iNOS leads to increased intracerebral parasitic load in chronically infected C57BL/6 mice

| Days Postinfection | Treatment Group | Days of Treatment | Number of Cysts | Weight
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Expt. 1</td>
<td>35</td>
<td>Control (2)</td>
<td>0</td>
<td>29.0 ± 7</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>Control (2)</td>
<td>9</td>
<td>31.0 ± 9</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>L-NIL (2)</td>
<td>9</td>
<td>63.5 ± 12</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>35</td>
<td>Control (2)</td>
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<td>18.5 ± 8</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>Control (3)</td>
<td>12</td>
<td>22.0 ± 6</td>
</tr>
</tbody>
</table>
| | 47 | L-NIL (3) | 12 | 41.5 ± 7d | 12.4 ± 0.5

* The number of mice analyzed immunohistologically in each treatment group (six to eight mice) is shown in parentheses.

* The number of cysts was determined in 100 high-power fields/section. A total of 10 sections from various regions of the brain were counted, and data were expressed as the mean ± SEM number of cysts.

* The animals were weighed individually, and the mean ± SEM is shown.

* Significantly different from the respective control values (p < 0.0001).
because *T. gondii* could be cleared efficiently from peripheral organs despite the absence of iNOS activity, whereas parasite growth was not restricted in the brain. With respect to resistant BALB/c mice, the role of NO in acute toxoplasmosis has not been evaluated. It cannot be excluded that NO also contributes to parasite control in these mice before the onset of an efficient intracerebral immune response.

In acute murine toxoplasmosis, the function of NO is not solely confined to a direct antiparasitic effect. Within the first 14 days of *T. gondii*-infection, NO inhibits the proliferation of splenic T cells, which may contribute to the escape of the parasite from the host immune response (11, 14). Thus, NO has two effects in acute toxoplasmosis, namely a direct antiparasitic effect and an additional immunoregulatory function, which might also indirectly influence parasite control. A recent study of murine leishmaniasis further illustrates that iNOS not only influences the Ag-specific T cell response but also regulates the innate immune response by inducing IFN-γ production and NK cell responses (42). In our study on the role of iNOS in chronic TE, inhibition of iNOS was initiated when a parasite-specific intracerebral immune response had already been established. A careful histopathological analysis of brain tissue from *l-NIL*-treated and control BALB/c mice as well as C57BL/6 mice demonstrated that there were no differences in the number, composition, and topographical distribution of inflammatory leukocytes, including CD4+ cells, CD8+ T cells, B cells, and macrophages. Therefore, in contrast to acute toxoplasmosis, there is no evidence for a decisive immunoregulatory function of iNOS in chronic TE.

In addition to its antiparasitic and immunoregulatory functions, NO also has immunopathological effects. In acute toxoplasmosis of *T. gondii*-susceptible mouse strains, NO induces tissue necrosis in the gut and liver after peroral infection (12). Moreover, it has also been suggested that NO might exert a neurotoxic effect in CNS disorders. This assumption is primarily based on the observations that in vitro NO has a remarkable capacity to kill neurons (43, 44), that iNOS is detectable in some neurodegenerative diseases (e.g., Alzheimer’s disease) (45), and that the expression of iNOS correlates inversely with the outcome of the disease and leads to neuronal loss in several infectious CNS disorders (46–49). From these findings, the question arises as to whether the increased iNOS expression of *T. gondii*-infected C57BL/6 mice plays an immunopathological role and is, at least in part, responsible for the aggravated course of disease in these animals. However, TE of *T. gondii*-susceptible mice is not characterized by neuronal loss, indicating that the continuous increase of intracerebral toxoplasms is the major driving force of progressive TE. In addition, the lethal reactivation of TE by the inhibition of iNOS illustrates that the antiparasitic activity is clearly the predominant effect of NO in chronic TE of susceptible mice.

In conclusion, we demonstrate that iNOS is expressed in the brains of mice chronically infected with *T. gondii*. Inhibition of iNOS leads to a rapid lethal exacerbation of TE in susceptible mice but not in *T. gondii*-resistant mice. Thus, in contrast to previous assumptions, control of intracerebrally persisting *Toxoplasma gondii* in a truly latent phase of the infection does not require iNOS activity. Considering the tightly regulated and limited expression of iNOS in human diseases (50), this observation suggests that latent toxoplasmosis in immunocompetent humans could also be controlled in an iNOS/NO-independent manner.

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References


