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Crucial Role of TNF Receptor Type 1 (p55), But Not of TNF Receptor Type 2 (p75), in Murine Toxoplasmosis

Martina Deckert-Schützer, Horst Bluethmann, Andrea Rang, Herbert Hof, and Dirk Schützer

TNF-α exerts its biologic activity through two distinct receptors, TNF receptor type 1 (TNFR1, p55) and TNF receptor type 2 (TNFR2, p75). To analyze their function in toxoplasmosis, we orally infected mice genetically deficient for TNFR1 (TNFR1<sup>0/0</sup>), TNFR2 (TNFR2<sup>0/0</sup>), or both TNF receptors (TNFR1<sup>0/0</sup>/2<sup>0/0</sup>), as well as wild-type (wt) mice with a low-virulent strain of Toxoplasma gondii. TNFR1<sup>0/0</sup> and TNFR1<sup>0/0</sup>/2<sup>0/0</sup> mice succumbed to toxoplasmosis within 17 and 27 days, respectively, whereas TNFR2<sup>0/0</sup> and wt mice were equally resistant to acute toxoplasmosis. Histopathology attributed death of TNFR1<sup>0/0</sup>/2<sup>0/0</sup> and TNFR1<sup>0/0</sup> mice to a fulminating necrotizing encephalitis. In addition, pneumonia contributed to the fatal outcome. The poor prognosis of TNFR1<sup>0/0</sup>/2<sup>0/0</sup> and TNFR1<sup>0/0</sup> mice was reflected by a significantly increased parasitic load in the brain and lung as compared with TNFR2<sup>0/0</sup> and wt mice. Immunohistochemistry demonstrated a remarkable reduction of inducible nitric oxide synthase protein in brain and lung of TNFR1<sup>0/0</sup>/2<sup>0/0</sup> and TNFR1<sup>0/0</sup> as compared with TNFR2<sup>0/0</sup> and wt mice. Reverse-transcribed PCR showed that in contrast to TNFR2<sup>0/0</sup> and wt mice, TNFR1<sup>0/0</sup> mice were unable to up-regulate inducible nitric oxide synthase mRNA transcripts in the course of infection, whereas intracerebral levels of IFN-γ, TNF-α, and IL-1β mRNA transcripts, recruitment of immune cells to the brain, and the amount of apoptotic cells in inflammatory foci did not differ significantly among the various experimental groups. These results illustrate that in Toxoplasma encephalitis, TNF-α-mediated immune responses are of crucial importance and that signaling through TNFR1, but not TNFR2, provides the stimulus required for the induction of protective nitric oxide.

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3 Abbreviations used in this paper: CNS, central nervous system; TE, Toxoplasma encephalitis; IFN-γR, interferon-γ receptor; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TNFR, tumor necrosis factor receptor; wt, wild type; RT, reverse transcription; LCA, leukocyte common Ag; GFAP, glial fibrillary acid protein; PE, phycoerythrin; p.i., postinfection; HPRT, hydroyxphosphoribosyltransferase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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immune reactions to infectious organisms (23, 24). All of these aspects may play a significant role in the immune response to *T. gondii*, and it still remains to be clarified which TNFR(s) mediates the protective effects of TNF-α in toxoplasmosis.

In the present study, we took advantage of the recent development of TNFRI (TNFR1<sup>100</sup>)- and TNFRII (TNFR2<sup>100</sup>)-, as well as TNFRI/1 (TNFR1<sup>200</sup>)-deficient mice (23–25), which allow the analysis of the role of TNF-α as well as its receptors under exactly defined genetic conditions. Our experiments show that TNF-α-mediated immune responses are of critical importance in toxoplasmosis and that signaling through TNFRI, but not through TNFRII, is necessary to mediate protection in this parasitic infection.

**Materials and Methods**

**Animals**

Female 129/Sv × C57BL/6 TNFR<sup>100</sup>, TNFR<sup>200</sup>, TNFR1<sup>200</sup>, and 129/Sv × C57BL/6 wild-type (wt) mice at the age of 8 to 16 wk were used for the experiments. Female C57BL/6 mice (6–8 wk old) were obtained from Harlan-Winkelmann (Borchern, Germany). The animals were kept in an isolation facility before and throughout the studies.

**Infectious organisms**

Parasites were harvested from the brains of mice chronically infected with a low-virulent strain of *T. gondii* (DX strain). Brain tissue of these animals was dispersed in PBS. The final concentration of the infectious agents was adjusted to a dose of 10 cysts/0.5 ml, which was administered orally to the mice.

**Analysis of the role of TNF-α**

Institute, Palo Alto, CA). Rat anti-mouse TNF-α (clone MP6-XT22), CD4-FITC, CD8-FITC, and CD45R (B220)-FITC were purchased from PharMingen (Hamburg, Germany). A polyclonal rabbit anti-*T. gondii* antiserum was obtained from Biogenex (Duiven, The Netherlands). Polyclonal rabbit anti-mouse iNOS, alkaline phosphatase-conjugated donkey anti-rabbit IgG F(ab<sup>9</sup>)<sub>2</sub> fragments, peroxidase-conjugated goat anti-rabbit IgG F(ab<sup>9</sup>)<sub>2</sub> fragments, Texas Red-conjugated goat anti-rabbit IgF(ab<sup>9</sup>)<sub>2</sub> fragments, and biotinylated mouse serum-preadsorbed mouse anti-rat IgG F(ab<sup>9</sup>)<sub>2</sub> fragments were from Dianova (Hamburg, Germany). FITC-conjugated F4/80 was purchased from Serotec-Camom (Wiesbaden, Germany). Peroxidase-linked sheep anti-rat IgF(ab<sup>9</sup>)<sub>2</sub> fragments were obtained from Amersham-Buchler (Braunschweig, Germany), and peroxidase-linked streptavidin-biotin complex and alkaline phosphatase-linked streptavidin-biotin complex were purchased from Dakopatts (Hamburg, Germany). FITC-conjugated avidin and rabbit anti-cow gial fibrillary acid protein (GFAP) were from Sigma (St. Louis, MO). Mouse serum protein-adsorbed PE-conjugated goat anti-rat IgG and avidin PE/Cy5 were obtained from Southern Biotechnology Associates-Biozol (Freising, Germany).

**Immunohistochemistry**

Immunohistochemistry was performed on acetone-fixed 10-μm cryostat sections according to a protocol previously described in detail (2). In brief, for demonstration of CD4, CD8, CD45 (LCA), CD45R (B220), Ly-6-G, and TNF-α, as well as for visualization of *T. gondii* in iNOS, an indirect method using peroxidase-conjugated sheep anti-rat IgF(ab<sup>9</sup>)<sub>2</sub> fragments or peroxidase-conjugated goat anti-rabbit F(ab<sup>9</sup>)<sub>2</sub> fragments, respectively, as secondary Abs was used. The avidin-biotin complex technique was employed for demonstration of CD54 (ICAM-1) and VCAM. In addition, *T. gondii* Ag was demonstrated in paraffin sections in a peroxidase-anti-peroxidase protocol, as described previously (2). The peroxidase reaction products were visualized using 3,3′-diaminobenzidine (Sigma) and H<sub>2</sub>O<sub>2</sub>. Sections were, in part, lightly counterstained with hemalum (Merck).

To demonstrate TNF-α with GFAP on cryostat sections, a double-labeling immunofluorescence technique was employed. The incubation steps were 1) rat anti-mouse TNF-α, 2) biotinylated mouse anti-rat IgG F(ab<sup>9</sup>)<sub>2</sub> fragments, 3) FITC-conjugated avidin, 4) rabbit anti-cow GFAP antiserum, and 5) Texas Red-conjugated goat anti-rabbit IgG F(ab<sup>9</sup>)<sub>2</sub> fragments.

To control for nonspecific reactions, incubations either with irrelevant species-specific IgG Abs instead of the primary Ab or with omission of the primary Ab were performed.

Apopotosis was assessed on 1% glutaraldehyde-fixed cryostat sections by use of a terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) kit (Boehringer, Mannheim, Germany) according to the manufacturer’s instructions.

**Flow cytometry of brain-derived leukocytes**

Brain-derived leukocytes were analyzed by double or triple immunofluorescence staining followed by flow cytometry. Murine macrophages and microglial cells were identified by staining with CD45-biotin and F4/80-FITC followed by avidin-PE/Cy5. As reported previously (26), macrophages and microglial cells were differentiated by their different expression of CD45 (LCA). Microglial cells were F4/80<sup>CD45<sup>low</sup></sup>, whereas macrophages were F4/80<sup>CD45<sup>high</sup></sup>. For identification of B lymphocytes, double staining with CD45R (B220)-FITC and CD45 (LCA)-biotin followed by avidin-PE/Cy5 was employed. CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were identified by staining with rat anti-mouse CD4-FITC and CD8-PE, respectively, followed by goat anti-rat IgG F(ab<sup>9</sup>)<sub>2</sub> fragments. Granulocytes were identified by triple staining with rat anti-mouse Ly6-G and goat anti-rat PE followed by F4/80-FITC, CD45-biotin, and avidin-PE/Cy5. Granulocytes were defined as Ly6G<sup>F4/80<sup>CD45<sup>high</sup></sup></sup> and CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were identified by staining with rat anti-mouse CD4-FITC and CD8-PE, respectively, followed by goat anti-rat IgG F(ab<sup>9</sup>)<sub>2</sub> fragments. Control staining was performed with fluorochrome-labeled or unlabeled isotype-matched control Abs. For flow cytometry, a FACScan (Becton Dickinson, Heidelberg, Germany) was used.

**Detection of cytokine mRNA by RT-PCR**

IFN-γ, TNF-α, iNOS, IL-18, and hydroxyphosphoribosyltransferase (HPRT) mRNA transcripts were analyzed in brain tissue homogenates according to a protocol previously described in detail (27). Primer sequences and oligonucleotide probes for IFN-γ, TNF-α, and HPRT were identical with those described before. iNOS mRNA transcripts were analyzed by use of the following pair of primers: 5′-TCA CGC TTG GGT CTT GTT CAC T-3′ (sense), 5′-TGT TCT CTO GCT CCT CTG GTC A-3′ (antisense) as described by Stenger et al. (28), and as the oligonucleotide probe, 5′-TGA CCC TAA GAG TCA CCA AA-3′ was employed.

In brief, mRNA was extracted from the brains of infected and *T. gondii*-infected TNFR1<sup>100</sup>, TNFR1<sup>200</sup>, and TNFR2<sup>200</sup>, and wt mice by use of an mRNA extraction kit (Pharmacia). After RT of mRNA using the Superscript RT kit (Life Technologies), PCR reactions were conducted in a volume of 50 μL. PCR reaction conditions were optimized for each set of primers to ensure that amplification occurred in the linear range. PCR products were subjected to electrophoresis through an agarose gel, and the
DNA was transferred to a nylon membrane (Boehringer). Blots were hybridized using specific oligonucleotide probes, which were 3'-end labeled with digoxigenin by use of a DIG Oligonucleotide 3'-End Labeling Kit (Boehringer). A DIG Luminescent Detection Kit (Boehringer) was used to visualize the hybridization products.

Western blot analysis
For the analysis of iNOS production by Western blot, brain tissue of each experimental group was lysed in ice-cold lysis buffer (10 mM NaH2PO4, pH 8.0, 140 mM NaCl, 3 mM MgCl2, 0.5% IGEPAL, 1 μM DTT, 17.4 μg PMSF/ml, 100 μg aprotinin/ml; all reagents from Sigma).

In addition to brain tissues, IFN-γ and LPS-stimulated resident peritoneal cells were used as a positive control as described by Green et al. (29). In brief, peritoneal cells were isolated from a C57BL/6 mouse and incubated at a concentration of 5 × 106 cells in 500 μl in DMEM with 5% FCS (Life Technologies) in snap-cap tubes (Becton Dickinson). For the induction of iNOS, cells were treated with 10 U IFN-γ/ml (R&D Systems, Wiesbaden, Germany) and 2 ng LPS/ml (Sigma) for 48 h. Negative controls remained unstimulated. For Western blot analysis, cells of six snap-cap tubes were pooled after 48 h of incubation, and the cellular proteins were isolated as described for brain tissues.

The protein content of brain tissue and peritoneal cell lysates was determined by the Bradford assay (Bio-Rad, Munich, Germany). For Western blot analysis, 20 μg of protein was separated on 7.5% reducing SDS-PAGE and transferred in 25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol to a 0.2 μm-pore size nitrocellulose membrane (Bio-Rad). Detection of iNOS on nitrocellulose membrane was performed according to a protocol published recently by Bogdan et al. (30). In brief, membranes were blocked with 1% BSA in TTBS (25 mM Tris, pH 7.5, and 150 mM NaCl with 0.2% Tween-20) for 1 h and, thereafter, incubated with rabbit anti-mouse iNOS diluted 1:2000 in TTBS with 1% BSA at 4°C overnight. After intensive washing with Tris buffer, the membranes were incubated with alkaline phosphatase-conjugated donkey anti-rabbit IgG (Fab′)2 fragments (Di-anova) at a dilution of 1:10,000 in TTBS for 45 min at RT. After washing in Tris, the membranes were equilibrated in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM MgCl2). Thereafter, they were developed in a solution of nitroblue tetrazolium (330 μg/ml, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (165 μg/ml, Sigma).

Specificity controls included either incubation with irrelevant rabbit anti-mouse Ab instead of the primary Ab or omission of the primary Ab.

Statistical evaluation
For statistical evaluation of the parasitic load of brain and lung, the number of parasites was determined on anti-T. gondii-immunostained sections in TNFR1/2−/−, TNFR1−/−, TNFR2−/−, and wt mice. High power fields (n = 100–400) were analyzed per section in four animals from each group, and the statistical significance of the differences was evaluated by using the Wilcoxon test. Differences in the mortality rates among TNFR1/2−/−, TNFR1−/−, TNFR2−/−, and wt mice were analyzed using the χ2 test. A P value of <0.05 was accepted as significant.

Results
Mortality rates
After oral infection with 10 T. gondii cysts of the low-virulent DX strain, all mice developed toxoplasmosis. There were significant differences in the susceptibility to the parasite among the various groups of mice as reflected by the mortality rates (Fig. 1). Inactivation of both TNFRs rendered mice highly susceptible to T. gondii, and all TNFR1/2−/− mice died of acute toxoplasmosis up to day 17 p.i. In addition, TNFR1−/− mice also exhibited a significantly increased susceptibility to the infectious organism, and death occurred up to day 27 p.i. In contrast, TNFR2−/− mice were as resistant as wt animals, and TNFR2−/− mice only in rare cases succumbed to acute toxoplasmosis (Fig. 1). TNFR2−/− mice and wt mice developed a chronic toxoplasmosis and survived for at least 3 mo.

Histopathology
To assess the cause of death in TNFR1/2−/− and TNFR1−/− animals, a detailed histopathologic study was performed. TNFR1/2−/−, TNFR1−/−, TNFR2−/−, and wt mice developed acute toxoplasmosis with the same pattern of organs involved. Brain and lung were most severely affected, and major differences among TNFR1/2−/−, TNFR1−/−, TNFR2−/−, and wt animals were detected in these organs. In contrast, in all groups, liver, heart, and small and large intestine were only mildly diseased, and pathologic findings in these organs regressed up to day 25 p.i.

Histopathology attributed death of TNFR1/2−/− mice to a progressive encephalitis, which was characterized by unrestricted intracerebral multiplication of T. gondii. Interestingly, at day 10 p.i., these animals had also developed a ventriculitis with large numbers of parasites in the lumen and the wall of the lateral ventricle, which is normally not infected by T. gondii except in cases of prenatal toxoplasmosis (31), as well as in the periventricular brain parenchyma (Fig. 2g). In addition, a severe pneumonia contributed to the fatal outcome of TNFR1/2−/− mice. In these animals, the parasitic load of the brain and the lung significantly exceeded the number of parasites in all other experimental groups (Table I).

TNFR1−/− mice were also unable to control the parasite in the CNS. However, as compared with TNFR1/2−/− mice, multiplication of the parasite in the brain was significantly lower at day 10 p.i. (Table I). As observed for TNFR1/2−/− mice, the parasite also infected the lateral ventricle (Fig. 2c). Up to day 25 p.i., the parasite replicated massively in the brain (Table I) and caused widespread, large areas of tissue necrosis with trophozoites as well as T. gondii cysts located at the border (Fig. 2d). Furthermore, TNFR1−/− mice developed toxoplasmic pneumonia, which was characterized by numerous toxoplasms in association with large numbers of inflammatory leukocytes in the alveolar spaces and lung tissue. At day 10 p.i., many of the alveoli were occluded by a serous exudate. However, in contrast to the brain, pneumonia was regressive from day 10 to 25 p.i., and the amount of parasites declined significantly during this period (Table I).

The increased resistance of TNFR2−/− mice to T. gondii was reflected by a significantly lower parasitic load in brain and lung as compared with TNFR1/2−/− and TNFR1−/− mice (Table I). Although exceptional TNFR2−/− mice died of acute pneumonia up to day 17 p.i., the vast majority of TNFR2−/− mice were able to significantly reduce the amount of T. gondii in the lung from day 10 to 25 p.i. and to effectively control the parasite in the brain. In contrast to TNFR1/2−/− and TNFR1−/− mice, TNFR2−/− mice did not differ significantly from wt mice in the number of parasites in
FIGURE 2. Histopathology of T. gondii distribution in the brain. a, wt mouse, day 10 p.i. Only a few T. gondii positive cells are detectable in the frontal cortex. b, wt mouse, day 25 p.i. A single T. gondii cyst resides in the subpial layer of the frontal cortex adjacent to small inflammatory infiltrates in the melanin-pigmented leptomeninges (arrow). c, TNFR1<sup>-/-</sup> mouse, day 10 p.i. Numerous parasites have infected the lateral ventricle. T. gondii Ag is located in the lumen and the walls of the lateral ventricle. The parasite has also invaded the periventricular parenchyma (arrowheads). d, TNFR1<sup>-/-</sup> mouse, day
Table I. Parasitic load in the brain and lung

<table>
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<th>10 Days p.i.</th>
<th>25 Days p.i.</th>
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<td></td>
<td>Brain</td>
<td>Lung</td>
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<tr>
<td>WT</td>
<td>2 ± 0.6a</td>
<td>218 ± 52</td>
</tr>
<tr>
<td>TNFR1/20/0</td>
<td>92 ± 21c</td>
<td>654 ± 71c</td>
</tr>
<tr>
<td>TNFR10/0</td>
<td>39 ± 15d</td>
<td>440 ± 101d</td>
</tr>
<tr>
<td>TNFR20/0</td>
<td>9 ± 4.7</td>
<td>338 ± 68</td>
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The amount of parasites was determined in 100 to 400 high power fields of anti-T. gondii-stained sections. The mean value in 100 high power fields of four animals per experimental group ± SD is shown.

From 10 days to day 25 p.i. the parasitic load declined significantly in the lung of wt (p < 0.025), TNFR10/0 (p < 0.025), and TNFR20/0 (p < 0.005) mice.

At day 10 p.i. the parasitic load in brain and lung of TNFR1/20/0 significantly exceeded those of wt (p < 0.005 for brain and lung), TNFR10/0 (p < 0.05 for brain and lung), and of TNFR20/0 (p < 0.005 for brain and lung) mice.

At day 10 p.i. the parasitic load in the brain of TNFR10/0 mice significantly exceeded those of WT and TNFR20/0 mice (p < 0.025 for both mouse strains).

At day 10 p.i. the parasitic load in the lung of TNFR10/0 mice was significantly increased as compared to wt mice (p < 0.05).

At day 10 to 25 p.i. the parasitic load significantly increased in the brains of TNFR10/0 mice (p < 0.005). At day 25 p.i. the parasitic load in the brains of TNFR10/0 mice significantly exceeded those of wt and TNFR20/0 mice (p < 0.005 for both strains).

25 days p.i. the parasitic load in the lung of TNFR10/0 mice was significantly elevated as compared to wt and TNFR20/0 mice (p < 0.005 for both mouse strains).

Deceased.

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Production of intracerebral cytokines in normal and T. gondii-infected TNFR1/20/0, TNFR10/0, TNFR20/0, and wt mice

In uninfected TNFR1/20/0, TNFR10/0, TNFR20/0, and wt mice, low levels of TNF-α and IL-1β mRNA transcripts were occasionally observed. In contrast, IFN-γ and iNOS mRNA transcripts were not detectable (Fig. 4).

In TE, recruitment of immune cells to the brain was paralleled by an increase of IFN-γ mRNA levels in all groups (Fig. 4). In addition, in all experimental groups, intracerebral IL-1β and TNF-α mRNA transcripts increased in response to T. gondii infection (Fig. 4). An analysis of at least three mice per group revealed small variations in the expression of IL-1β and TNF-α mRNA transcripts within each group, and there were no significant differences among the various experimental groups. Since TNF-α expression is also regulated posttranslationally, additional studies were performed at the protein level. Immunohistochemistry confirmed elevated TNF-α production in TE and revealed numerous TNF-α+ leukocytes within inflammatory foci, as well as TNF-α+ astrocytes without obvious differences in their number and topographical distribution in the various experimental groups (data not shown).

In contrast, differences were observed in the expression of iNOS among TNFR1/20/0, TNFR10/0, TNFR20/0, and wt mice. Immunohistochemistry revealed that in the brains of wt and TNFR20/0 mice, iNOS+ leukocytes were already present in significant numbers at day 10 p.i., with a further strong increase toward day 25 p.i. (Fig. 5a and e). In these animals, iNOS+ cells were intimately associated with T. gondii (Fig. 5b). In contrast to wt and TNFR20/0 mice, T. gondii-associated leukocytes in TNFR1/20/0 and TNFR10/0 mice did not express, or only exceptionally single leukocytes expressed, iNOS, and large numbers of infectious organisms were devoid of accompanying iNOS+ cells (Fig. 5g). In addition, the increase in the number of iNOS+ cells, which was observed in wt and TNFR20/0 animals, was absent in TNFR10/0 mice at day 25 p.i. (Fig. 5d).

25 p.i. Huge amounts of T. gondii are present in the brain of a terminally ill TNFR10/0 mouse. The parasite has caused a large area of necrosis, which contains numerous trophozoites and cellular debris. The border of the necrosis is decorated by several T. gondii cysts (arrow), e, TNFR20/0, day 10 p.i. Only single T. gondii Ag-positive cells are present in the temporal cortex. Both the topography and the amount of parasites are comparable to wt mice at the same stage of infection. f, TNFR20/0, day 25 p.i. Parasites are detectable as single cysts or as small groups of cysts in the brain. These findings also parallel those of wt mice in the same phase of infection. In contrast to TNFR10/0 mice, necrosis is absent. g, TNFR1/20/0 mice, day 10 p.i. A prominent ventriculitis has developed. Compared with TNFR1/20/0 in the same stage of the disease, infection of the lateral ventricle is more severe, as indicated by an increased amount of T. gondii Ag in the ventricular lumen and walls. T. gondii Ag is located in epithelial cells of the choroid plexus, which is in part necrotic (arrowhead). Ependymal cells are also infected (small arrow). Furthermore, the parasite has already spread to the periventricular rostral basal ganglia, where small foci of necrosis have developed (large arrow), a–g, Anti-T. gondii immunostaining (brown) with polyclonal antibody anti-T. gondii antiserum and slight counterstaining with hemalum (blue); magnification, ×125. Control stainings (incubation with irrelevant polyclonal rabbit antiserum, with omission of the primary Ab) confirmed the specificity of the immunohistochemical reactions.
number of iNOS

1

TNFR20/0 and wt mice (data not shown), which had the highest brain lysates by Western blot analysis. This was even observed in all strains of infected mice was too low to detect iNOS protein in signals of iNOS mRNA transcripts at day 10 p.i., and, more importantly, TNFR100 mice failed to increase intracerebral iNOS mRNA transcripts up to day 25 p.i. (Fig. 4).

With respect to intracerebral iNOS production in the various experimental groups, it is of note that we made the same histopathologic observation with respect to the expression of iNOS in the lung: In contrast to wt and TNFR200 mice, which exhibited abundant iNOS+ leukocytes, iNOS expression on pulmonary leukocytes was significantly reduced or even absent in TNFR1/200 and TNFR100 mice (data not shown).

Discussion

The present study demonstrates that TNF-α-mediated immune responses play a crucial role in murine toxoplasmosis and, furthermore, that the protective effects of TNF-α are mediated through TNFR1, but not TNFR2. The observation that TNFR1/200 mice succumbed significantly earlier to the infection with a dramatically increased parasitic load of brain and lung as compared with TNFR100 mice provides evidence that in the absence of TNFR1, a part of the function of this receptor is taken over by TNFR2. This is in keeping with the observation that soluble TNF preferentially signals through TNFR1 and much less through TNFR2 (32). It also argues that membrane-bound TNF, the prime activating signal for TNFR2 (33), is not crucially involved in anti- T. gondii defense.

Histopathology revealed that in all experimental groups, the same pattern of organs was affected by T. gondii, with the brain being the most strongly diseased organ followed in severity by the lung. Thus, although TNFR1/200 and TNFR100 mice have a disturbed intestinal lymphatic architecture (17, 34) at the entry site of the parasite into the host, they were able to effectively clear T. gondii from this organ. Interestingly, IFN-γR-deficient mice on a 129SvEv background, which are at least as resistant to T. gondii as are mice on the 129/Sv × C57BL/6 background used in this study, failed to control T. gondii already in the intestine (10). Consequently, IFN-γR-deficient mice died of necrotizing hepatitis and intestinal lymphadenitis within 10 days. The remarkable differences in the survival times between IFN-γR-deficient and TNFR1/200 mice strongly indicate that in murine toxoplasmosis, IFN-γ-mediated immune responses are crucial for the control of the infection just from an early phase of infection and TNF-induced processes at a later stage in the course of infection.

TNFR100 mice had a significantly increased parasitic load in the lung and brain as compared with TNFR200 and wt mice at days 10 and 25 p.i. However, in contrast to the brain, TNFR100 mice achieved a significant reduction of the parasitic burden in the lung from day 10 to 25 p.i. These observations indicate that systemic immune responses differ significantly from intracerebral immune reactions and that TNFR1 is especially important in the brain, where the parasite persists. The CNS differs immunologically from other organs in several aspects. In toxoplasmosis, it seems particularly important that T. gondii infects neurons (35). Thus, the well-documented cytotoxic activity of CD8+ T cells against T. gondii (36, 37) is fully active in systemic organs, where all cell populations express MHC class I molecules, but ineffective in the brain. These findings indicate that in the brain, noncytotoxic immune reactions are even more important than in other organs. This may explain why, in our study, TNFR100 mice, which have a normal cytotoxic activity of CD8+ T cells (23), could compensate their genetic defect by other immune mechanisms in peripheral organs but not in the brain.

An analysis of the effect of TNFR deficiency on the ensuing immune response detected a significantly reduced production of iNOS mRNA and protein in the brains of TNFR1/200 and TNFR100 mice. The recent observation of Scharton-Kersten et al.
(13) that iNOS-deficient mice succumbed to a progressive TE within 4 wk strongly indicates that in our study, the impaired iNOS production of TNFR1/2<sup>−/−</sup> and TNFR1<sup>−/−</sup> mice significantly contributed to the poor outcome of these mice. In addition, it has been shown that neutralization of NO in acute murine toxoplasmosis leads to an exacerbation of TE (14). Interestingly, we observed a reduced iNOS production in the brain as well as in the lung of TNFR1/2<sup>−/−</sup> and TNFR1<sup>−/−</sup> mice, which indicates that the increased parasitic load of the lung also results from an insufficient production of NO. However, the significant reduction of the parasitic load in the lung further argues for a significant role of other immune mechanisms independent of TNFR1 and iNOS in systemic toxoplasmosis (38).

Previous studies have established that in TE, resident brain parenchymal cell populations including astrocytes and microglial cells are involved in the antiparasitic immune response (9, 39). Interestingly, in vitro, neither unstimulated nor IFN-γ- or TNF-α-stimulated murine astrocytes are able to control the growth of *T. gondii* (40). In addition, recent studies in our laboratory revealed that IFN-γ- and/or TNF-α-stimulated murine cerebellar granule neurons cannot control the growth of *T. gondii* and, furthermore, that constitutive NO synthase expression of neurons is insufficient to inhibit the growth of the parasite (D. Schlüter, unpublished observations). In contrast, several in vitro studies have shown that IFN-γ-stimulated microglial cells and macrophages exert a toxoplasmastatic activity, which is dependent on TNF-α-induced NO production (41). Furthermore, a strong IFN-γ- and TNF-α-independent (Ref. 12 and this study) iNOS production is observed in murine toxoplasmosis. In addition, we recently identified macrophages/microglial cells as iNOS-producing cell types in murine TE (9). From these data, the question arises as to how NO produced by cells of the macrophage lineage can control a parasite persisting in an NO-negative cell type. Recently, Stenger and coworkers described the control of *Leishmania* persisting in iNOS-negative cells in the lymph nodes by neighboring iNOS-expressing cells (42, 43). This mechanism may also be operative in murine TE. In line with this assumption is the development of a fatal TE in iNOS-deficient mice and an exacerbation of TE in mice with neutralized NO (13, 14).

Interestingly, studies of TNFR1<sup>−/−</sup> mice have also revealed a crucial role of this receptor in listeriosis and tuberculosis (23, 24, 44). As observed in our study, in murine tuberculosis TNFR1 deficiency was linked to a reduced production of NO in the early stage of the infection (44). In contrast, *Leishmania*-infected TNFR1<sup>−/−</sup> mice exhibited a normal production of NO, although parasite clearance was delayed (45). The divergent findings on the relative role of TNFR1 in NO induction among the various studies may be explained by different IFN-γ levels at the site of infection (44, 45). Additionally, TNFR1<sup>−/−</sup> macrophages, infected with *Leishmania major*, were shown to compensate for the lack of TNF/TNFRI signaling and to effectively induce NO synthesis in the presence of the parasite (45). It is noteworthy that *T. gondii*-infected normal macrophages stimulated with suboptimal doses of IFN-γ require TNF-α for an effective induction of NO as well as an inhibition of proliferation of *T. gondii* (41). Furthermore, neutralization of TNF-α in chronic murine TE resulted in a reduced production of NO and an increased proliferation of *T. gondii* leading to death in the mice (12). These findings indicate that in murine TE, endogenous TNF-α, which signals preferentially through TNFR1 (32), acts in synergy with limited concentrations of IFN-γ for an optimal NO production and parasite control.

TNF-α and TNFR are also involved in a variety of immune reactions that are independent of NO production. Studies in TNFR1<sup>−/−</sup> and TNFR2<sup>−/−</sup> mice have revealed that in cerebral malaria, TNFR2 mediates the immunopathologic hyperinduction of ICAM-1 on cerebral blood vessel endothelium leading to occlusion of cerebral blood vessels by sequestering leukocytes (21). In our model, none of the TNFR-mutant mice showed an increased expression of ICAM-1 or VCAM on cerebral blood vessels as compared with wt mice. This difference between cerebral malaria and TE is explained by the observation that in murine TE, IFN-γ is the major mediator inducing an up-regulation of cell adhesion molecules at the blood-brain barrier (M. Deckert-Schlüter, unpublished observation), whereas in cerebral malaria, TNFR2-mediated immunopathologic up-regulation of ICAM-1 is of key importance (21). Moreover, in our study, the recruitment of immune cells to the brain as well as the rate of apoptosis of intracerebral immune cells were as normal in TNFR-mutant mice as in wt mice. Thus, the number of intracerebral immune cells, especially of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which are of major importance in toxoplasmosis (2, 7, 46), was equal in the various experimental groups. Furthermore, the normal rate of apoptosis in TNFR-mutant mice indicates that CD95 (Fas) and not TNFR regulates apoptosis in TE (19). Furthermore, intracerebral production of cytokines with a documented anti-*T. gondii* activity, i.e., IFN-γ, TNF-α, and IL-1β (7, 12, 15, 47, 48), did not differ between TNFR-mutant and wt mice.

Although these data point to the reduced iNOS production as a crucial factor responsible for the high susceptibility of TNFR1/2<sup>−/−</sup> and TNFR1<sup>−/−</sup> mice, other yet undefined factors may also contribute to the insufficient control of *T. gondii* in these strains. In particular, the in vivo regulation of intraneuronal and astrocytic anti-
CRUCIAL ROLE OF TNFR1, BUT NOT OF TNFR2, IN TOXOPLASMOSIS

WT

10 days p.i.

25 days p.i.

TNFR1<sup>−/−</sup>

TNFR2<sup>−/−</sup>

TNFR1/2<sup>−/−</sup>
T. gondii immune responses by IFN-γ and TNF-α is still unknown, and one may speculate that TNFR1 deficiency directly impairs the immune response of neurons and astrocytes to T. gondii. In this context, it is of note that in TNFR1-/- and TNFR1+/- mice, intracerebral cysts developed regularly, indicating that in these animals the immunologic control of cysts, but not the induction and formation of cysts, is impaired.

In conclusion, the present study demonstrates an essential function of TNF-α- and TNFR1-mediated immune responses in murine toxoplasmosis, which cannot be compensated for by other immune mechanisms. The strong inverse correlation of susceptibility and iNOS levels strongly suggests a critical role of TNFR1-mediated iNOS and NO production in murine toxoplasmosis.

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References


