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

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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<sub>0/0</sub>/TNFR2<sup>0/0</sup>), as well as wild-type (wt) mice with a low-virulent strain of Toxoplasma gondii. TNFR1<sub>0/0</sub> and TNFR2<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<sub>0/0</sub> and TNFR2<sup>0/0</sup> mice to a fulminating necrotizing encephalitis. In addition, pneumonia contributed to the fatal outcome. The poor prognosis of TNFR1<sub>0/0</sub> and TNFR1<sub>0/0</sub> 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<sub>0/0</sub> and TNFR1<sub>0/0</sub> 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<sub>0/0</sub> 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.

Toxoplasma gondii is an obligate intracellular parasite that may cause severe infections in humans, in particular in immunocompromised patients. The parasite has a remarkably high affinity for the central nervous system (CNS),<sup>3</sup> and Toxoplasma encephalitis (TE) is a common opportunistic infection of the CNS in AIDS patients (1). The present knowledge on the immune response to T. gondii is largely based on experimental studies in mice. In these animals, the course of infection closely mimics that of human toxoplasmosis. Following oral application of T. gondii, i.e., the natural route of infection, mice develop an acute generalized toxoplasmosis followed by a chronic stage, when the disease is confined to the brain (2). Immunity to T. gondii is T cell mediated, and in addition to CD4<sup>+</sup> and CD8<sup>+</sup> T cells, macrophages and NK cells play an important role (3–6).

Numerous experimental studies have underlined the key importance of cytokine production for an effective antiparasitic immune response. In particular, IFN-γ, which is produced by NK cells as well as CD4<sup>+</sup> and CD8<sup>+</sup> T cells in murine toxoplasmosis (7–9), has gained special attention. The fundamental role of IFN-γ in toxoplasmosis is illustrated in IFN-γ-deficient mice, which rapidly succumb to fulminant acute toxoplasmosis (10, 11). In addition, neutralization of IFN-γ results in a lethal reactivation of chronic TE (7). There is increasing evidence that IFN-γ mediates its protection in part through the induction of other protective effector molecules. In both IFN-γ- and IFN-γR-deficient mice, which as well as in mice with chronic TE, which received an IFN-γ-neutralizing Ab, the lethal course of disease was characterized by a dramatic reduction of TNF-α and inducible nitric oxide synthase (iNOS) production (10–12).

The importance of iNOS for the antiparasitic immune response in vivo has recently been demonstrated in iNOS-deficient mice, which succumbed to toxoplasmosis within 4 wk (13). In addition, neutralization of nitric oxide (NO) in acute murine toxoplasmosis results in an exacerbation of TE (14). With respect to TNF-α, which is produced by CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as macrophages/microglial cells in murine TE (9), it has also been shown that its application in acute toxoplasmosis has a protective effect (15), whereas neutralization of this cytokine in chronic TE results in a lethal exacerbation of the disease (12). However, the precise biologic role of TNF-α in toxoplasmosis has not yet been defined.

TNF-α exerts its biologic activity via two receptors, the TNF receptor type 1 (TNFR1, p55) and the TNF receptor type 2 (TNFR2, p75), which are expressed in virtually all cell types (16). These two receptors have been implicated in a variety of biologic functions, including the development of lymphatic organs (17), mediation of apoptosis (18–20), regulation of cell adhesion molecule expression (21), and activation of glial cells (22), as well as
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 TNFR1 (TNFR1<sup>1/100</sup>)- and TNFR2 (TNFR2<sup>1/200</sup>), as well as TNFR1/2 (TNFR1<sup>2/300</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 TNFR1, but not through TNFR2, is necessary to mediate protection in this parasitic infection.

Materials and Methods

Animals

Female 129/Sv × C57BL/6 TNFR<sup>1/100</sup>, TNFR<sup>2/200</sup>, TNFR1<sup>1/200</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 (Borchen, Germany). The animals were kept in an isolation facility throughout and for the study. Infection

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

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, and TNF-α, as well as for visualization of T. gondii and iNOS, an indirect method using peroxidase-conjugated sheep anti-rat IgG F(ab′)<sub>2</sub> fragments or peroxidase-conjugated goat anti-rabbit IgG F(ab′)<sub>2</sub> fragments, and biotinylated mouse serum-preadsorbed mouse anti-rat IgG F(ab′)<sub>2</sub> fragments were from Dinova (Hamburg, Germany). FITC-conjugated F4/80 was purchased from Serotec-Cam (Wiesbaden, Germany). Peroxidase-linked sheep anti-rat IgG Fab′<sub>1</sub> fragments, were obtained from Amersham-Buchler (Braunschweig, Germany), and peroxidase-linked streptavidin-biotin complex and alkaline phosphatase-phosphate complex were purchased from Dakopatts (Hamburg, Germany). FITC-conjugated avidin and rabbit anti-cow glial fibrillary acidic protein (GFAP) were from Sigma (St. Louis, MO). Mouse serum protein-absorbed PE-conjugated goat anti-rat IgG and avidin PE/Cy5 were obtained from Southern Biotechnology Associates-Biozol (Freising, Germany).

Detection of cytokine mRNA by RT-PCR

The following anti-mouse mAb-producing hybridomas were obtained from the American Type Culture Collection (Rockville, MD) and kept under standard hybridoma conditions in DMEM with 10% FCS (Life Technologies).

For transfection (RT)-PCR determination of cytokine mRNA and Western blot analysis, brain tissues were snap-frozen and stored at −80°C until use for immunohistochemistry.

For immunohistochemistry on paraffin sections and conventional histology, mice were perfused intracardially with 0.9% saline while they were under deep anesthesia (Janssen, Neuss, Germany) anesthesia. Uninfected mice of all strains served as controls.

For immunohistochemistry on paraffin sections, liver, spleen, small and large intestine, heart, lung, and brain were dissected. Blocks of tissue were mounted on thick filter paper with Tissue Tek OTC compound (Miles Scientific, Naperville, IL), snap-frozen in isopentane (Fluka, Neu-Ulm, Germany) on dry ice, and stored at −80°C until use for immunohistochemistry.

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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.

Apoptosis 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 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>lo</sup></sup>, whereas macrophages were F4/80<sup>CD45<sup>hi</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> B lymphocytes were identified by staining with rat anti-mouse CD45R (B220)-FITC and CD45 (LCA)-biotin (clone 30-F11.7.19, Pharmingen). The peroxidase reaction was controlled by staining with irrelevant species-specific IgG Abs instead of the primary Ab or with omission of the primary Ab.

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-1β and hydroxyurea phosphoribosyltransferase (HRPT) 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 HRPT were identical with those described before. iNOS mRNA transcripts were analyzed by use of the following pair of primers: 5′-TCA CGC TGT GGT CCT GTT CAC T′-3′ (sense), 5′-TGT TCT CTG CCT CCT GTG 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>1/200</sup>, TNFR<sup>2/200</sup>, and wt mice by use of an mRNA extraction kit (Pharmacia). After RT of mRNA using the SuperScrip 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 tissue.

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 (F(ab′)₂), fragments (Dianova) 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 (>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. A significance level of p < 0.00001 for both strains, respectively. The mortality rate of 10 mice from each group was determined, and the results from one of two experiments, which yielded similar results, are shown.

**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. At day 17 p.i., TNFR1/2°/° and 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 mice 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 uncontrolled 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 3430 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).
brain and lung (Table I) or in the histopathology of toxoplasmic pneumonia and encephalitis. Both TNRF2<sup>0/0</sup> and wt mice had only single T. gondii Ag-positive cells in the brain at day 10 p.i. (Fig. 2, a and e). At day 25 p.i., either single cysts or small groups of cysts were scattered throughout the brain (Fig. 2, b and f).

Cellular immune reactions in the CNS

In the normal brain of TNRF1<sup>2/00</sup>, TNRF1<sup>0/0</sup>, TNRF2<sup>0/0</sup>, and wt mice, only low numbers of CD4<sup>+</sup>, CD8<sup>+</sup> T cells, B cells, macrophages, and granulocytes were present, as revealed by flow cytometry and immunohistochemistry. Infection with T. gondii induced an increase of these populations of immune cells in the brain up to 10 day p.i., with a further prominent increase up to day 25 p.i. (Fig. 3).

In parallel to the increased parasitic load, TNRF1<sup>2/00</sup> mice had 3- to 4-fold elevated numbers of intracerebral CD4<sup>+</sup> and CD8<sup>+</sup> T cells as compared with TNRF1<sup>0/0</sup>, TNRF2<sup>0/0</sup>, and wt animals at day 10 p.i. (Fig. 3). The amount of the various populations of intracerebral immune cells did not differ significantly among TNRF1<sup>0/0</sup>, TNRF2<sup>0/0</sup>, and wt mice at days 10 and 25 p.i. These data show that mutation of neither TNRF1 nor TNRF2 interferes with the recruitment of immune cells to the T. gondii-infected brain. These findings are extended by in situ immunohistochemical analysis of cell adhesion molecule expression. In all experimental groups, ICAM-1 and VCAM were strongly up-regulated on endothelial cells of cerebral blood vessels. In addition, macrophages and CD4<sup>+</sup> and CD8<sup>+</sup> T cells were recruited efficiently to infectious organisms in the brain parenchyma in every strain. Thus, in all experimental groups, the formation of inflammatory infiltrates was unimpaired.

Since TNFRs have also been shown to be involved in programmed cell death (18), we studied apoptosis in the brains of the various groups by the TUNEL technique. Whereas in the brains of all uninfected animals apoptotic cells were not present, T. gondii-infected mice from all experimental groups exhibited a similar small number of apoptotic cells in leptomeningeal infiltrates, in perivascular cuffs, and in T. gondii-associated infiltrates. Thus, these studies demonstrate that in murine TE, apoptosis of intracerebral immune cells is independent of TNRF1 and TNRF2 expression (data not shown).

Production of intracerebral cytokines in normal and T. gondii-infected TNRF1<sup>2/00</sup>, TNRF1<sup>0/0</sup>, TNRF2<sup>0/0</sup>, and wt mice

In uninfected TNRF1<sup>2/00</sup>, TNRF1<sup>0/0</sup>, TNRF2<sup>0/0</sup>, 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<sup>+</sup> leukocytes within inflammatory foci, as well as TNF-α<sup>+</sup> 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 TNRF1<sup>2/00</sup>, TNRF1<sup>0/0</sup>, TNRF2<sup>0/0</sup>, and wt mice. Immunohistochemistry revealed that in the brains of wt and TNRF2<sup>0/0</sup> mice, iNOS<sup>+</sup> leukocytes were already present in significant numbers at day 10 p.i., with a further strong increase toward day 25 p.i. (Fig. 5, a and e). In these animals, iNOS<sup>+</sup> cells were intimately associated with T. gondii (Fig. 5b). In contrast to wt and TNRF2<sup>0/0</sup> mice, T. gondii-associated leukocytes in TNRF1<sup>2/00</sup> and TNRF1<sup>0/0</sup> mice did not express, or only exceptionally single leukocytes expressed, iNOS, and large numbers of infectious organisms were devoid of accompanying iNOS<sup>+</sup> cells (Fig. 5g). In addition, the increase in the number of iNOS<sup>+</sup> cells, which was observed in wt and TNRF2<sup>0/0</sup> animals, was absent in TNRF1<sup>0/0</sup> mice at day 25 p.i. (Fig. 5d).

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Table I. Parasitic load in the brain and lung

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<tr>
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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 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>218 ± 52&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>TNRF1&lt;sup&gt;2/00&lt;/sup&gt;</td>
<td>92 ± 2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>654 ± 71&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>TNRF1&lt;sup&gt;0/0&lt;/sup&gt;</td>
<td>39 ± 15&lt;sup&gt;g&lt;/sup&gt;</td>
<td>440 ± 101&lt;sup&gt;h&lt;/sup&gt;</td>
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<tr>
<td>TNRF2&lt;sup&gt;0/0&lt;/sup&gt;</td>
<td>9 ± 4.7&lt;sup&gt;k&lt;/sup&gt;</td>
<td>338 ± 68&lt;sup&gt;l&lt;/sup&gt;</td>
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<sup>a</sup> 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.

<sup>b</sup> From day 10 to day 25 p.i. the parasitic load declined significantly in the lung of wt (p < 0.025), TNRF1<sup>0/0</sup> (p < 0.025), and TNRF2<sup>0/0</sup> (p < 0.005) mice.

<sup>c</sup> At day 10 p.i. the parasitic load in brain and lung of TNRF1<sup>2/00</sup> significantly exceeded those of wt (p < 0.005 for brain and lung), TNRF1<sup>0/0</sup> (p < 0.05 for brain and lung), and of TNRF2<sup>0/0</sup> (p < 0.005 for brain and lung) mice.

<sup>d</sup> At day 10 p.i. the parasitic load in the brain of TNRF1<sup>0/0</sup> mice significantly exceeded those of WT and TNRF2<sup>0/0</sup> mice (p < 0.025 for both mouse strains).

<sup>e</sup> At day 10 p.i. the parasitic load in the lung of TNRF1<sup>0/0</sup> mice was significantly increased as compared to wt mice (p < 0.05).

<sup>f</sup> From day 10 to 25 p.i. the parasitic load significantly increased in the brains of TNRF1<sup>0/0</sup> mice (p < 0.005). At day 25 p.i. the parasitic load in the brains of TNRF1<sup>0/0</sup> mice significantly exceeded those of wt and TNRF2<sup>0/0</sup> mice (p < 0.005 for both strains).

<sup>g</sup> 25 days p.i. the parasitic load in the lung of TNRF1<sup>0/0</sup> mice was significantly elevated as compared to wt and TNRF2<sup>0/0</sup> mice (p < 0.005 for both mouse strains).

<sup>h</sup> Deceased.

25 p.i. Huge amounts of T. gondii are present in the brain of a terminally ill TNRF1<sup>0/0</sup> 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, TNRF2<sup>0/0</sup>, 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, TNRF2<sup>0/0</sup>, 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 TNRF1<sup>0/0</sup> mice, necrosis is absent. g, TNRF2<sup>0/0</sup> mice, day 10 p.i. A prominent ventriculitis has developed. Compared with TNRF1<sup>0/0</sup> 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 rabbit 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 signals of iNOS mRNA transcripts at day 10 p.i., and, more importantly, TNFR10/0 mice had a prominent increase in intracerebral iNOS mRNA levels. In contrast to wt and TNFR20/0 mice, which exhibited abundant iNOS+ leukocytes, iNOS expression on pulmonary leukocytes was significantly reduced or even absent in TNFR1/20/0 and TNFR10/0 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 TNFR1/20/0 mice succumbed significantly earlier to the infection with a dramatically increased parasitic load of brain and lung as compared with TNFR10/0 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 TNFR1/20/0 and TNFR10/0 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 129/SvEv 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 a necrotizing hepatitis and intestinal lymphadenitis within 10 days. The remarkable differences in the survival times between IFN-γR-deficient and TNFR1/20/0 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.

TNFR10/0 mice had a significantly increased parasitic load in the lung and brain as compared with TNFR20/0 and wt mice at days 10 and 25 p.i. However, in contrast to the brain, TNFR10/0 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, which fail to express MHC class I Ags (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, TNFR10/0 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 TNFR1/20/0 and TNFR10/0 mice. The recent observation of Scharton-Kersten et al.

In addition to immunohistochemistry, Western blots were performed to analyze iNOS protein production. In contrast to cultivated peritoneal cells from C57BL/6 mice, which served as positive controls, the number of iNOS+ immune cells in the brains of all strains of infected mice was too low to detect iNOS protein in brain lysates by Western blot analysis. This was even observed in TNFR20/0 and wt mice (data not shown), which had the highest number of iNOS+ cells as revealed by immunohistochemistry.

Moreover, iNOS mRNA transcripts were analyzed. RT-PCR of brain tissue homogenates performed within the linear range of amplification detected very low levels of iNOS mRNA transcripts in wt and TNFR20/0 mice at day 10 p.i. Up to day 25 p.i., these strains had a prominent increase in intracerebral iNOS mRNA levels. In contrast, only single TNFR10/0 and TNFR1/20/0 mice had faint signals of iNOS mRNA transcripts at day 10 p.i., and, more importantly, TNFR10/0 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 TNFR20/0 mice, which exhibited abundant iNOS+ leukocytes, iNOS expression on pulmonary leukocytes was significantly reduced or even absent in TNFR1/20/0 and TNFR10/0 mice (data not shown).

FIGURE 3. Flow cytometric analysis of the phenotypic composition of intracerebral inflammatory infiltrates. Uninfected mice of all mouse strains had only a very low number of immune cells in the brain. \( T. \ gondii \) infection induced a significant increase in CD4+ and CD8+ T cells. B cells, macrophages, and granulocytes in the brain of all experimental groups. There were no significant differences in the phenotypic composition of the inflammatory infiltrates among the various experimental groups except for day 10 p.i., when the number of CD4+ and CD8+ T cells in TNFR1/20/0 was 3- to 4-fold elevated over TNFR10/0, TNFR20/0, and wt mice. Data represent the mean value of three animals per group ± SD. Data from one of two experiments are shown.
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>0/0</sup> and TNFR<sup>10/0</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 decreased iNOS production in the brain as well as in the lung of TNFR1/2<sup>0/0</sup> and TNFR<sup>10/0</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 lungs of TNFR<sup>10/0</sup> mice from day 10 to day 25 p.i. despite the persistent low production of iNOS 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-dependent (Ref. 12 and this study) iNOS production is observed in murine toxoplasmamosis. 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 TNFR<sup>10/0</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 TNFR<sup>10/0</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, TNFR<sup>10/0</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 TNFR<sup>10/0</sup> and TNFR<sup>20/0</sup> mice have revealed that in cerebral malaria, TNFR2 mediates the immunopathologic hyperinduction of ICAM-1 on cerebral blood vessel endothelia 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>0/0</sup> and TNFR<sup>10/0</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

10 days p.i.  
WT

25 days p.i.  
TNFR1^{−/−}

TNFR2^{−/−}

TNFR1/2^{−/−}
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 TNFR1-2/0 and TNFR1-0/0 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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2. Scharton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, and Daniela Sampels for expert technical assistance, and we are grateful to Wolfgang Nettekoven for help with the photographs.

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