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# TNF Is Important for Pathogen Control and Limits Brain Damage in Murine Cerebral Listeriosis[1]

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**Cerebral listeriosis is a life-threatening disease. However, little is known about the bacterial virulence factors responsible for the severe course of disease and the factors of the immune system contributing to the control of *Listeria monocytogenes* (LM) or even to the damage of the brain. To analyze the importance of the *actA* gene of LM, which mediates cell-to-cell spread of intracellular LM, the function of TNF in murine cerebral listeriosis was studied. C57BL/6 mice survived an intracerebral (i.c.) infection with *actA*-deficient LM, but succumbed to infection with wild-type (WT) LM. Upon infection with *actA*-deficient LM, macrophages and microglial cells rapidly, and later LM-specific CD4 and CD8 T cells, produced TNF. In contrast to WT mice, TNF-deficient animals succumbed to the infection within 4 days due to failure of control of LM. Histology identified a more severe meningoencephalitis, brain edema, and neuronal damage, but a reduced inducible NO synthase expression in TNF-deficient mice. Reciprocal bone marrow chimeras between WT and TNF-deficient mice revealed that hematogenously derived TNF was essential for survival, whereas TNF produced by brain-resident cells was less important. Death of TNF-deficient mice could be prevented by LM-specific T cells induced by an active immunization before i.c. infection. However, brain pathology and inflammation of immunized TNF-deficient mice were still more severe. In conclusion, these findings identify a crucial role of TNF for the i.c. control of LM and survival of cerebral listeriosis, whereas TNF was not responsible for the destruction of brain tissue.** *The Journal of Immunology,* **2006, 177: 3972–3982.**

*Listeria monocytogenes* (LM)[7] is a Gram-positive, facultative intracellular rod which may cause severe meningitis, encephalitis, and brain abscess. Patients at risk for the development of a cerebral listeriosis, which has a lethality of 30% despite antibiotic treatment, are immunocompromised, elderly, and very young individuals (1).

Several virulence factors of LM contribute to its successful intracellular lifestyle. One of these factors is the actin polymerase A (ActA), which enables intracellular LM to propel through the cytoplasm of the host cell by polymerization of host cell actin and to induce the formation of pseudopods, which extend from the infected cell to neighboring cells, triggering the uptake into uninfected cells (2–4). In the absence of ActA-mediated direct cell-to-cell spread, LM are highly attenuated (5).

Most of our knowledge on immune responses in listeriosis is based on studies in the murine model of systemic listeriosis (6). In this model, cells of the innate immune system and T cells, especially CD8 T cells, control and eradicate LM from infected tissues. Because *actA*-deficient (Δ*actA*) LM also induce a strong CD4 and CD8 T cell response (5, 7), transgenic attenuated LM expressing foreign T cell Ags are considered to be attractive and safe live vaccines in humans (8). One cytokine crucial for both control and survival of systemic listeriosis is TNF (9–11). TNF is rapidly produced after systemic infection with LM by cells of the innate immune system including macrophages, granulocytes, and dendritic cells (9, 12, 13). Infection of $TNF^{-/-}$ or $TNFR1^{-/-}$ mice with low numbers of wild-type (WT) LM results in a massive multiplication of the pathogen and rapidly fatal disease (14–16). In addition, LM-specific CD4 and CD8 T cells also produce TNF (17). However, TNF production of CD8 T cells is dispensable for protection in systemic listeriosis induced by low and moderate doses of WT LM, but not in high dose infection (13, 18, 19).

Importantly, primary systemic infection with high numbers of attenuated Δ*actA* LM induces a nonlethal infection in $TNF^{-/-}$ mice (18). However, it is not known whether attenuated LM also display such a low virulence in cerebral listeriosis nor whether TNF is dispensable for the control of these attenuated LM in the brain. In fact, recent cases of cerebral listeriosis in patients with systemic anti-TNF treatment indicate that the function of TNF in cerebral listeriosis may be even more critical than in systemic listeriosis (20–22). The role of TNF in cerebral listeriosis may not be restricted to pathogen control, because TNF can also damage the highly vulnerable brain by disturbance of the blood-brain-barrier,

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[7] Abbreviations used in this paper: LM, *Listeria monocytogenes*; ActA, actin polymerase A; WT, wild type; i.c., intracerebral; C-SF, cerebrospinal fluid; p.i., postinfection; LLO, listeriolysin O; iNOS, inducible NO synthase.



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edema formation, recruitment of increased numbers of inflammatory cells, and induction of neuronal cell death (23–26).

Experimental cerebral listeriosis induced by intracerebral (i.c.) inoculation of WT LM inevitably causes death of mice within 5 days before the onset of a LM-specific T cell response (27). The disease is characterized by a strong multiplication of LM in choroid plexus epithelial cells, ependymal cells, macrophages, microglia, and some neurons resulting in a prominent meningitis, ventriculitis, and encephalitis, and mice finally succumb to a necrotizing brain stem encephalitis (28). In addition, massive brain edema and neuronal apoptosis develop, which are reduced by intrathecally produced IL-10 (29), and may be induced or aggravated by i.c. TNF production. An active systemic immunization before i.c. challenge infection induces LM-specific CD4 and CD8 T cells, prevents death in 60% of mice and dramatically reduces pathology, especially in the brain parenchyma (27, 30).

To analyze whether ActA is an important bacterial virulence factor in cerebral listeriosis and whether TNF plays a protective or detrimental role in this disease, WT and $TNF^{-/-}$ mice were i.c. infected with WT and/or Δ*actA* LM. These experiments revealed that *actA* contributes to the virulence of LM in the brain, but that TNF is absolutely required to control multiplication and i.c. spread of attenuated Δ*actA* LM as well as for survival. In addition, the development of brain edema and neuronal damage were TNF independent, but correlated to a more widespread i.c. distribution of LM and a more intense inflammatory reaction.

## Materials and Methods

### *Animals*

Age- and sex-matched C57BL/6 WT, obtained from Harlan-Winkelmann, and $TNF^{-/-}$ mice, prepared on a C57BL/6 background (31), were used. All animals were kept under conventional conditions in an isolation facility throughout the experiments. Experiments were approved and supervised by local governmental institutions.

### *Bacteria and infection*

WT LM (serovar 1/2a, EGD, SLCC 5835), rOVA-expressing LM (LM-OVA) (32), Δ*acta* LM (33) were used. A Δ*acta* LM-OVA strain was constructed by introducing the OVA expression cassette (34) into Δ*acta* LM (33) using double crossover homologous recombination as described previously (35). LM strains were grown in tryptose soy broth, and aliquots of log-phase cultures were stored at −80°C. For each experiment, the respective strain of LM was thawed from the stock solution and diluted appropriately in sterile pyrogen-free PBS (pH 7.4). Anesthetized mice were infected i.c. with either $6 \times 10^2$ Δ*actA* LM, $6 \times 10^2$ Δ*actA* LM-OVA or $1 \times 10^2$ WT LM, respectively, into the right caudate nucleus as described before (28). For immunization, mice were infected i.p. with $1 \times 10^6$ Δ*actA* LM or $1 \times 10^5$ LM-OVA 28 days before i.c. challenge infection. For each experiment, the bacterial dose used for infection was controlled by plating an inoculum on tryptose soy agar and counting colonies after incubation at 37°C for 48 h.

### *Histopathology*

For immunohistochemistry on frozen sections, mice were perfused intracardially with 0.9% NaCl in methoxyflurane anesthesia. Brains of three to six animals per group were dissected and blocks were mounted on thick filter paper with Tissue-Tek OTC Compound (Miles Scientific), snap-frozen in isopentane precooled on dry ice, and stored at −80°C. Immunohistochemistry was performed on frozen sections as described previously (36). In brief, sections were stained by an indirect immunoperoxidase protocol using rat anti-mouse CD45 (clone M1/9.3.4.HL.2), CD4 (clone G.K.1.5.), CD8 (clone 2.43), and Ly6-G (clone RB6-8C5, all clones obtained from American Type Culture Collection (ATCC)) as primary Abs and peroxidase-linked sheep anti-rat IgG $F(ab')_2$ (Amersham Biosciences) as secondary Ab. In addition, the avidin-biotin complex technique using rat anti-mouse F4/80 (clone F4/80; ATCC) as primary Ab, biotinylated mouse serum-preadsorbed mouse anti-rat IgG $F(ab')_2$ (Dianova) as secondary Ab and streptavidin-biotin complex (DakoCytomation) was used. For histology on paraffin sections, anesthetized mice were perfused with 4% paraformaldehyde in PBS, brains were removed and fixed with 4% paraformaldehyde for 24 h. Paraffin sections (4 μm) were stained with hemalum & eosin, cresyl violet, Luxol fast blue, Gram, and periodic acid Schiff stain. LM was demonstrated immunohistochemically by incubating deparaffinized sections with a polyclonal rabbit anti-LM antiserum (Difco) followed by peroxidase-labeled goat anti-rabbit IgG $F(ab')_2$ (Dianova). Brain edema was histologically analyzed on immunostained frozen sections by visualization of Ig deposits in brain tissue. Sections were stained with goat anti-mouse IgG and goat anti-mouse IgM (Vector Laboratories) followed by biotin-labeled anti-goat Abs and the Vectastain Elite ABC kit (Vector Laboratories). Peroxidase reaction products were visualized using 3,3′-diaminobenzidine and $H_2O_2$ as cosubstrate. Sections were lightly counterstained with hemalum. For an evaluation and grading of histopathological alterations in specimens stained with H&E, cresyl violet, and Gram solution as well as in immunohistochemically stained sections various regions of the brain including the basal ganglia, the cortex and white matter, the hippocampus, the ventricular system at the levels of the lateral, third, and fourth ventricle, the subarachnoid space at the level of the forebrain, the cerebellar cisterns, and the brain stem were evaluated.

### *Determination of TNF in cerebrospinal fluid (C-SF)*

C-SF (~7 μl/mouse) was obtained from mice after intracardial perfusion with 0.9% NaCl by puncturing the cisterna cerebellomedullaris with a fine glass capillary as described previously (37). The C-SF of five mice per experimental group was pooled and mixed with an equal volume of sterile distilled 0.1 M PBS to reduce losses. C-SF was stored at −80°C before being analyzed in a commercially available mouse TNF-ELISA (Quantikine M kit; R&D Systems), which was used as recommended by the manufacturer.

### *Colony-forming units*

At the indicated time points postinfection (p.i.), brains were dissected from sacrificed mice, and homogenized separately with sterile tissue grinders. Ten-fold serial dilutions of the homogenates were plated on tryptose-soy agar. Bacterial colonies were counted microscopically after incubation at 37°C for 48 h.

### *Isolation of cerebral leukocytes*

At the indicated days p.i., animals were anesthetized with Metofane (Janssen) and intracardially perfused with 0.9% NaCl to remove contaminating intravascular leukocytes from the brain. Thereafter, brains were dissected, minced through a 100-μm cell strainer, and leukocytes were separated by Percoll gradient centrifugation (Amersham Biosciences) as described previously (38).

### *Flow cytometry analysis*

Isolated cerebral leukocytes were analyzed by double or triple immunofluorescence staining followed by flow cytometry. All Abs were rat anti-mouse Abs from BD Biosciences. To block unspecific binding of Abs to FcRs, i.c. leukocytes were first incubated with anti-CD16/32 (clone 2.4G2) at 4°C for 10 min. Subsequently, the cells were stained with a mixture of fluorochrome-labeled Abs at 4°C for 20 min. CD4 and CD8 T cells were detected by staining with anti-CD4-PE (clone RM4-5) and anti-CD8-FITC (clone 53-6.7). Microglia, macrophages, and granulocytes were identified by staining with anti-Gr-1-PE (clone RB6-8C5), anti-CD11b-FITC (clone M1/70), and anti-CD45-CyChrome (clone 30-F11). Microglia are $CD11b^+CD45^{low}$, macrophages are $CD11b^+CD45^{high}$, granulocytes are $CD11b^+CD45^{high}Gr1^{high}$ (39). Control staining was performed with isotype-matched control Abs. Flow cytometry was performed on a FACScan (BD Biosciences), and the data were analyzed with WinMDI or CellQuest software.

### *Intracellular TNF staining*

Cerebral leukocytes were first incubated with anti-CD16/32 followed by extracellular staining with anti-CD4-FITC and anti-CD8-FITC, respectively, or a combination of anti-Mac-1-FITC and anti-CD45-CyChrome. Thereafter, cells were washed twice in 0.1 M PBS, fixed with Cytofix/Cytoperm solution (BD Biosciences), and stained intracellularly with anti-TNF-PE (clone MP6-XT22; BD Biosciences). The final washing step was performed with Perm/Wash solution (BD Biosciences) in 0.1 M PBS. To analyze TNF production of *Listeria*-specific CD4 and CD8 T cells from mice infected i.c. with Δ*actA* LM-OVA, isolated cerebral leukocytes were stimulated with $OVA_{257–267}$ (CD8 specific, $10^{-8}$ M) or listeriolysin O $(LLO)_{190–201}$ (CD4 specific, $10^{-6}$ M) peptide in the presence of GolgiPlug (1 μl/ml, Cytofix/Cytoperm kit; BD Biosciences) at 37°C for 6 h before TNF staining and flow cytometric analysis.

### *ELISPOT assay*

The frequency of i.c. LM-specific CD4 and CD8 T cells was determined by an IFN-γ specific ELISPOT as described previously (29). In brief, isolated i.c. leukocytes ($1 \times 10^5$/well, $1 \times 10^4$/well, and $1 \times 10^3$/well) were added to 96-well ELISPOT plates coated with rat anti-mouse IFN-γ (BioSource International) in triplicate. Intracerebral leukocytes were coincubated with spleen cells from noninfected WT C57BL/6 mice ($2 \times 10^5$/well), which were either preloaded with $LLO_{190-201}$ ($10^{-6}$ M) or $OVA_{257-264}$ ($10^{-8}$ M) peptide. Controls included coincubation of i.c. leukocytes with spleen cells (APC) without peptide loading. All ELISPOT plates were incubated overnight and developed with biotin-labeled rat anti-mouse IFN-γ, peroxidase-conjugated streptavidin, and aminoethylcarbazole dye solution (Sigma-Aldrich). Spots were counted microscopically, and the frequency of Ag-specific cells was calculated from triplicate wells as the number of spots per leukocytes seeded.

### *Bone marrow chimeras*

Bone marrow chimeras were generated as described previously (39, 40). In brief, WT and $TNF^{-/-}$ mice were irradiated with 1000 rad and were i.v. reconstituted with $1–2 \times 10^7$ bone marrow cells isolated from the tibia and femur of $TNF^{-/-}$ or WT mice. Ten weeks after bone marrow transplantation, $TNF^{-/-} \rightarrow$WT, WT→$TNF^{-/-}$, and WT→WT chimeras as well as normal WT and $TNF^{-/-}$ control mice were i.c. infected with Δ*actA* LM. Survival of chimeras was monitored.

### *T cell depletion*

For depletion of T cells, mice were treated simultaneously with rat anti-mouse CD4 (clone GK1.5) and rat anti-mouse CD8 (clone 2.43) Abs. Abs were purified from tissue culture supernatants by protein G chromatography, adjusted to a concentration of 2.5 mg/ml in 0.1 M PBS, sterile filtered, and stored at −80°C until used. Anti-CD4 and anti-CD8 Abs were injected i.p. at a concentration of 0.5 mg/ml per mouse. Control mice were treated with 1.0 mg of rat IgG (Sigma-Aldrich). Ab treatment was started 3 days before i.c. infection with LM, and Abs were injected daily for three consecutive days. Thereafter, Abs were injected every third day. Efficacy of CD4 and CD8 T cell depletion was >95%, respectively, as controlled by flow cytometry.

### *Statistics*

For statistical evaluation of the experimental data, the WINKS software (Texasoft) was used. Survival analysis was performed with the Mantel-Haenszel log-rank test. The Student *t* test and Wilcoxon rank sum test were used to analyze differences in CFU and cell numbers between WT and TNF-deficient mice. Values of $p < 0.05$ were accepted as significant.

## Results

### *Intracerebral infection with ΔactA LM induces a nonlethal cerebral listeriosis*

To analyze whether *actA* is an important virulence factor of LM in cerebral listeriosis, WT mice were infected i.c. with WT LM or Δ*actA* LM. Whereas all mice infected with WT LM succumbed until day 5 p.i., 100% of mice infected with Δ*actA* LM survived the infection (Fig. 1*A*). In addition, i.c. CFU were significantly reduced in mice infected with Δ*actA* LM as compared with mice with WT LM infection (Fig. 1*B*), and mice cleared Δ*actA* LM from the brain until day 14 p.i. In addition to the Δ*actA* LM strain, i.c. infection with Δ*actA* LM-OVA, which harbor a strong H-$2K^b$-restricted CD8 epitope ($OVA_{257-264}$), induced a nonlethal cerebral listeriosis with a significantly lower bacterial load as compared with WT LM (data not shown). Because the virulence of Δ*actA* LM and Δ*actA* LM-OVA strains was comparable, they were used both in subsequent experiments as indicated.

### *Kinetics and cellular sources of TNF production in cerebral listeriosis*

To determine the kinetics and cellular sources of TNF in cerebral listeriosis, nonimmunized and immunized WT mice were i.c. infected with Δ*actA* LM, and TNF was measured in the C-SF by ELISA and in i.c. leukocytes by flow cytometry.

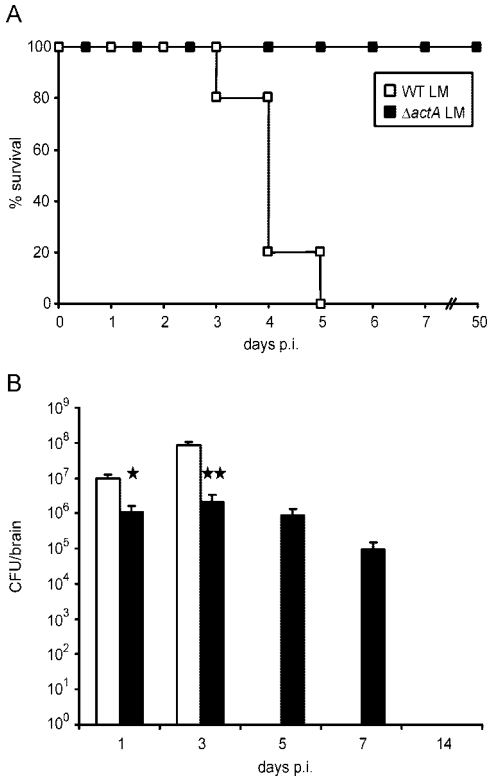


**FIGURE 1.** Survival and CFU of mice following i.c. infection with WT and Δ*actA* LM. *A*, After i.c. infection with WT LM, all mice succumb within 5 days to the infection, whereas 100% of mice survive i.c. infection with Δ*actA* LM. Survival was monitored for eight mice per experimental group. *B*, CFU were determined from three to five mice per experimental group and time point, and data represent the mean + SD. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

In the C-SF of nonimmunized mice, TNF levels peaked at day 1 p.i. and gradually declined until day 14 p.i. (Fig. 2*A*). In immunized animals, TNF levels in the C-SF were lower as compared with nonimmunized mice. Although there was a peak at day 1 p.i., TNF levels declined more rapidly and were already negative at day 5 p.i. (Fig. 2*A*).

In normal nonimmunized animals, a few microglial cells and macrophages produced TNF (Fig. 2*B*). Both cell populations strongly up-regulated their TNF production at day 1 p.i. with a further increase toward day 3 p.i. Thereafter, TNF production of microglia and macrophages gradually declined until day 50 p.i. In nonimmunized mice, only a small number of LM-specific CD4 and CD8 T cells produced TNF after restimulation with $LLO_{191-201}$ and $OVA_{257-264}$ peptides, respectively (Fig. 2*B*). The peak of LM-specific TNF-producing CD4 and CD8 T cells was at days 7 and 14 p.i.

In immunized animals, similar kinetics of TNF production by microglia and macrophages were observed with a peak at day 3 p.i. Immunization resulted in a dramatic increase in the number of LM-specific CD4 and CD8 T cells producing TNF after stimulation with their respective peptides. Numbers of TNF-producing LM-specific CD4 and CD8 T cells reached a maximum at day 7 for CD4 T cells and at day 14 for CD8 T cells. At these time points, the percentages of TNF-producing CD4 and CD8 T cells were increased as compared with microglia and macrophages. Whereas the number of TNF-producing CD4 T cells decreased rapidly beyond day 7 p.i., TNF-producing CD8 T cells had a much slower decline. At day 50 p.i., small numbers of LM-specific TNF-producing CD4 and CD8 T cells were still present in the CNS. A

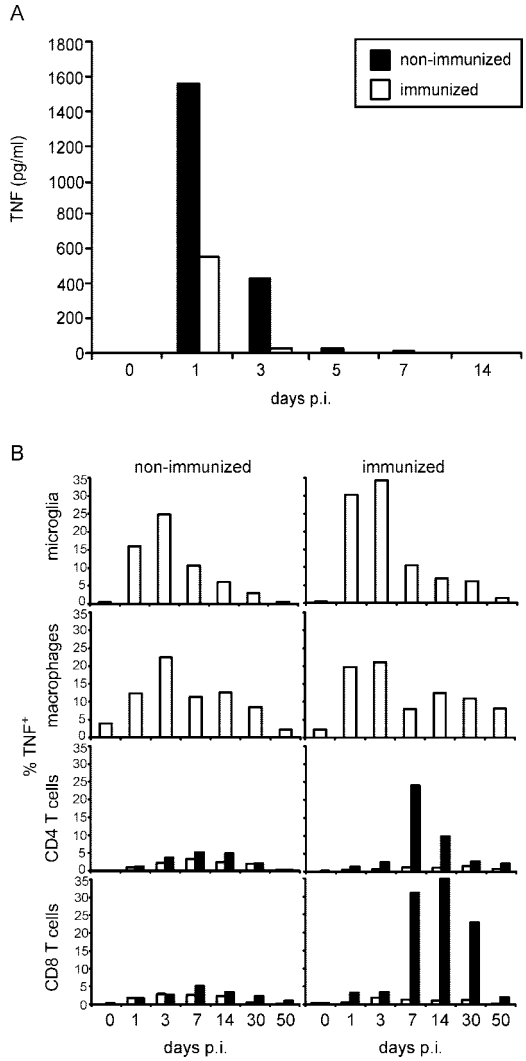


**FIGURE 2.** Kinetics of TNF production in the C-SF and i.c. TNF-producing cells. *A*, Nonimmunized mice and mice i.p. immunized 28 days before with LM-OVA were i.c. infected with Δ*actA* LM-OVA, and the C-SF was collected at the indicated time points after infection. C-SF of five mice per experimental group was pooled and TNF was determined by ELISA. *B*, Nonimmunized and immunized mice were i.c. infected with Δ*actA* LM-OVA. At the indicated time points p.i., i.c. leukocytes were isolated from three to six mice per experimental group and pooled microglia, macrophages, CD4, and CD8 T cells were stained for intracellular TNF. For CD4 and CD8 T cells, data without (□) or with (■) $LLO_{190-201}$ and $OVA_{257-267}$, respectively, peptide restimulation are shown.

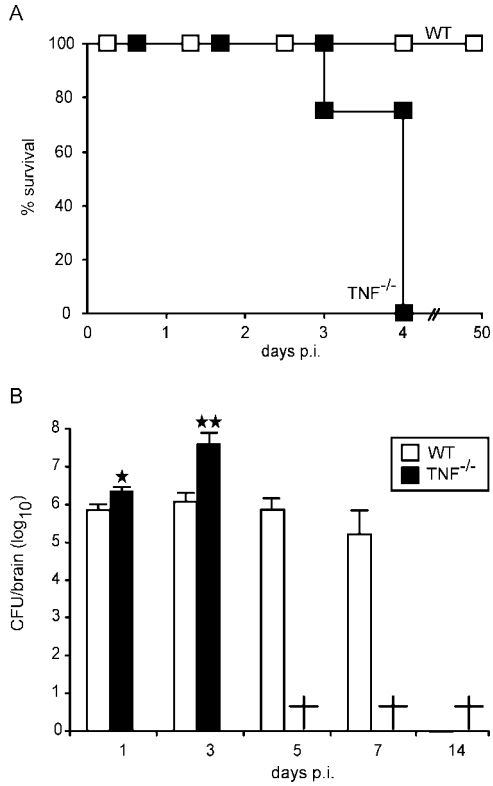


**FIGURE 3.** Survival and CFU of WT and $TNF^{-/-}$ mice following infection i.c. with Δ*actA* LM. *A*, After i.c. infection with Δ*actA* LM, all $TNF^{-/-}$ mice succumb within 4 days to the infection, whereas 100% of WT mice survive the infection. Survival was monitored for eight mice per experimental group; $p < 0.05$ for $TNF^{-/-}$ vs WT mice. *B*, CFU were determined from three to five mice per experimental group at the indicated time points p.i., and data represent the mean + SD. Mice were perfused with 0.9% NaCl before isolation of brain tissue. *, $p < 0.05$ and **, $p < 0.005$ for $TNF^{-/-}$ vs WT mice at the respective days p.i.

spontaneous TNF production, i.e., without restimulation by peptides, of CD4 and CD8 T cells was not observed.

In conclusion, these experiments illustrate that the kinetics of TNF production differ in the various anatomic compartments of the CNS and between leukocyte subpopulations in cerebral listeriosis. In addition, an active immunization before i.c. infection strongly regulated frequencies of LM-specific CD4 and CD8 T with the capacity to produce TNF.

### *TNF-deficient mice rapidly succumb to cerebral listeriosis*

To analyze the functional importance of TNF in cerebral listeriosis, WT and $TNF^{-/-}$ mice were infected i.c. with Δ*actA* LM. Whereas all $TNF^{-/-}$ succumbed to the infection between days 3 and 4 p.i., 100% of WT mice survived (Fig. 3*A*). A determination of the number of i.c. LM revealed that $TNF^{-/-}$ mice failed to control the multiplication of LM, whereas WT mice efficiently restricted the increase of LM and cleared the pathogen from the brain up to day 14 p.i. (Fig. 3*B*). These findings suggest that $TNF^{-/-}$ mice succumb to a LM-induced pathology.

### *Widespread i.c. distribution of LM and increased inflammation in $TNF^{-/-}$ mice*

To analyze the impact of TNF on the distribution of LM in the brain and the ensuing inflammatory reaction, a detailed immunohistochemical analysis was performed (summarized in Table I). CNS listeriosis developed more rapidly in $TNF^{-/-}$ mice. As early as day 1 p.i., LM had spread to the meninges and the ventricular system of $TNF^{-/-}$ mice, already invading the periventricular parenchyma, whereas only few bacteria spread to the meninges and the lumen of the lateral and fourth ventricle of WT mice without evidence for invasion of the periventricular parenchyma. Inflammatory infiltrates mainly consisted of $RB6^+$ granulocytes and some $F4/80^+$ macrophages in both strains of mice without significant differences in the total number of i.c. leukocytes ($0.53 \times 10^6$ and $0.48 \times 10^6$ leukocytes/brain for $TNF^{-/-}$ and WT mice, respectively), as determined by flow cytometry.

At day 3 p.i., CNS listeriosis had progressed in both strains. Meningitis and ventriculitis were much more severe in $TNF^{-/-}$ mice as compared with WT animals (Fig. 4, *a–f*). In addition to large numbers of LM in the enlarged lateral and fourth ventricle in $TNF^{-/-}$ mice, a widespread destruction of the ependyma allowed bacterial invasion of large areas of the brain tissue resulting in a

Table I. *Neuropathological grading of cerebral listeriosis in TNF$^{-/-}$ and WT mice*

| Immunization | Day p.i. | Mouse Strain[a] | Meningitis[b] | Encephalitis[c] | Ventriculitis[d] | Edema[e] |
|---|---|---|---|---|---|---|
| − | 1 | WT | + | − | + | + |
| | | TNF$^{-/-}$ | ++ | + | ++ | ++ |
| − | 3 | WT | + | + | ++ | ++ |
| | | TNF$^{-/-}$ | ++ | +++ | +++ | +++ |
| + | 1 | WT | + | − | + | + |
| | | TNF$^{-/-}$ | + | + | ++ | ++ |
| + | 3 | WT | + | + | ++ | + |
| | | TNF$^{-/-}$ | + | ++ | +++ | ++ |
| + | 5 | WT | + | +[f] | ++[f] | + |
| | | TNF$^{-/-}$ | + | ++ | ++ | ++ |

[a] Three mice were analyzed per group.
[b] No neuropathological alterations in the meninges, −; small foci of bacteria and inflammatory infiltrates, +; thick and widespread layers of bacteria and inflammatory cells, ++; ubiquitous meningeal inflammation, +++.
[c] No neuropathological alterations in the brain parenchyma, −; small foci of bacteria and inflammatory infiltrates, +; many bacteria and diffuse inflammatory infiltrates, ++; ubiquitously distributed and large numbers of bacteria and inflammatory infiltrates, +++.
[d] No neuropathological alterations in the ventricular system, −; single small foci of bacteria and inflammatory infiltrates, +; large numbers of bacteria and inflammatory infiltrates, enlargement of the ventricular system, focal destruction of the ependyma, ++; huge numbers of bacteria and inflammatory infiltrates, widespread destruction of the ependyma, +++.
[e] No neuropathological alterations, −; single blood vessels with IgG deposition, +; more widespread expression of perivascular IgG, focal expression of perivascular IgM, ++; ubiquitous expression of perivascular IgG and IgM, IgG and IgM deposition not confined to blood vessels, +++.
[f] Granuloma formation.

necrotizing encephalitis (Fig. 4, *c* and *e*). In contrast, destruction of the ependyma and bacterial invasion of the periventricular brain parenchyma was much less severe in WT mice (Fig. 4, *d* and *f*), and inflammatory cells located predominantly in the ventricular system and the periventricular brain tissue in WT animals (Fig. 4, *d* and *f*). Correspondingly, flow cytometry revealed that TNF$^{-/-}$ mice harbored i.c. leukocytes 2-fold increased in number as compared with WT animals ($0.98 \times 10^6$ and $0.47 \times 10^6$ for TNF$^{-/-}$ and WT mice, respectively). In both strains, RB6$^+$ granulocytes, F4/80$^+$ macrophages, activated microglia as well as some CD4 and CD8 T cells formed the inflammatory infiltrates, a finding which was confirmed by flow cytometry (data not shown). Beginning at day 5 p.i., when TNF$^{-/-}$ had already succumbed to the disease, cerebral listeriosis regressed in WT animals and was resolved at day 21 p.i.

### *Reduced inducible NO synthase (iNOS) expression in TNF$^{-/-}$ mice*

Because NO plays some protective role in systemic listeriosis (13, 41, 42) and may cause neuronal damage (43), iNOS expression was analyzed. iNOS was not expressed at day 1 p.i. and only on few inflammatory cells of TNF$^{-/-}$ mice at day 3 p.i. (Fig. 4*g*). In contrast, many cells within in the ventricular system and the periventricular brain tissue expressed iNOS protein in WT animals at days 1 and 3 p.i. (Fig. 4*h*). In addition, at day 3 p.i., iNOS protein expression of microglial cells was much more pronounced in WT animals as compared with TNF$^{-/-}$ mice (Fig. 4*h*), in which iNOS protein was only weakly expressed on a few microglial cells. At day 7 p.i., many leukocytes in perivascular cuffs and microglial cells still expressed iNOS protein in WT mice. Thereafter, iNOS expression of WT inflammatory leukocytes regressed.

### *Increased brain edema and neuronal damage in TNF$^{-/-}$ mice*

To determine the impact of TNF on the development of brain edema and neuronal damage in cerebral listeriosis, a histological analysis was conducted. Cerebral listeriosis led to much more pronounced brain edema in TNF$^{-/-}$ mice, which was detectable as early as day 1 p.i. (Fig. 5, *a* and *b*; Table I). At day 3 p.i., TNF$^{-/-}$ mice exhibited a strong deposition of IgG ubiquitously around cerebral blood vessels, most prominent in the choroid plexus, the periventricular brain parenchyma, and the hippocampus. Perivascular IgM expression was also more widespread distributed in TNF$^{-/-}$ mice (Fig. 5*c*). In contrast, perivascular IgG and IgM deposits were only rarely detected in WT mice (Fig. 5*d*).

Neuronal damage was much more pronounced in TNF$^{-/-}$ mice. Many neurons in the CA1 segment of the hippocampus, some neurons in the dentate fascia and cerebellar Purkinje cells of TNF$^{-/-}$ mice were shrunken and exhibited an increased nuclear acidophilia with a smudged nucleus (Fig. 5, *e* and *g*). These findings were only rarely observed in WT animals (Fig. 5, *f* and *h*).

### *TNF production of hematogenous cells, and to a lesser extent of brain resident cells, is required for survival of cerebral listeriosis*

Because hematogenous, bone marrow-derived cells are sensitive to irradiation while brain-resident cells including microglia are resistant (40), reciprocal bone marrow chimeras between WT and TNF$^{-/-}$ mice precisely answer the question of whether TNF produced by either brain-resident or hematogenous cells is of importance for the control of i.c. LM.

Ten weeks after irradiation, mice were i.c. infected with Δ*actA* LM, and survival was monitored up to day 21 p.i. Up to day 3 p.i., all nonirradiated TNF$^{-/-}$ control mice died (Fig. 6). All TNF$^{-/-}$ →WT chimeras also succumbed rapidly to the infection within 6 days. In contrast, 64% of WT→TNF$^{-/-}$ chimeras survived the infection. In addition, all WT→WT chimeras and, as expected, all nonirradiated WT control mice survived the infection.

Thus, hematogenously derived TNF is absolutely essential for survival of cerebral listeriosis, whereas brain-derived TNF plays a less important role, but contributes to an optimal control of i.c. LM resulting in 100% survival of the infection.

### *An active immunization before i.c. infection induces protective CD4 and CD8 T cells rescuing TNF$^{-/-}$ mice from lethal cerebral listeriosis*

In accordance with data published previously (18), TNF$^{-/-}$ and WT mice survived an i.p. infection with $1 \times 10^6$ Δ*actA* LM. More important, 80% of immunized TNF$^{-/-}$ and 100% of immunized WT survived an i.c. challenge infection with Δ*actA* LM (Fig. 7*A*), and both immunized TNF$^{-/-}$ and WT mice effectively restricted multiplication of LM in the brain without significant differences between the two experimental groups (Fig. 7*b*).

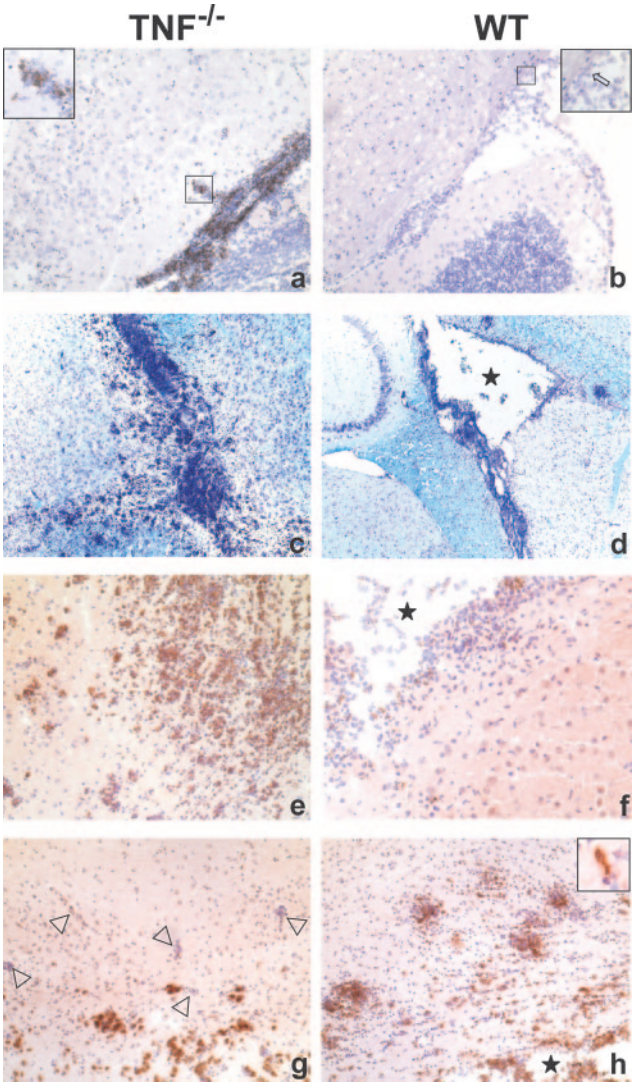


**FIGURE 4.** CNS listeriosis in nonimmunized TNF$^{-/-}$ (*a*, *c*, *e*, and *g*) and WT (*b*, *d*, *f*, and *h*) mice at day 3 p.i. *a* and *b*, Meningitis is more severe in a TNF$^{-/-}$ mouse (*a*) as compared with a WT mouse (*b*). While in a TNF$^{-/-}$ mouse (*a*), many bacteria cover the markedly inflamed meninges and, from here, have also invaded the adjacent cortex (*inset* in *a*), only single bacteria have homed to the meninges at the level of the cerebellum in a WT animal (*inset* in *b*, arrow). Anti-*L. monocytogenes* immunostaining, slight counterstaining with hemalum; original magnification, ×100; *insets*, ×400. *c–f*, Ventriculitis is more severe in a TNF$^{-/-}$ mouse, in which the wall of the lateral ventricle is largely destroyed (*c*), allowing invasion of the adjacent brain parenchyma by large numbers of bacteria (*e*). In contrast, inflammatory infiltrates (*d*) and bacteria (*f*) are largely confined to the lateral ventricle (∗) with preservation of the ependymal lining of the ventricular wall in a WT mouse. *c* and *d*, Cresyl violet and Luxol fast blue staining; original magnification, ×50. *e* and *f*, Anti-*L. monocytogenes* immunostaining, slight counterstaining with hemalum; original magnification, ×100 (*e*) and ×200 (*f*). *g* and *h*, iNOS protein expression is markedly reduced on i.c. leukocytes of a TNF$^{-/-}$ mouse (*g*) as compared with a WT animal (*h*). In the TNF$^{-/-}$ mouse, many leukocytes in inflammatory infiltrates do not express iNOS (arrowheads). In the WT mouse, numerous leukocytes in the lumen (∗) and wall of the lateral ventricle as well as in the basal ganglia and also microglial cells (*inset* in *h*) express iNOS protein. Anti-iNOS immunostaining, slight counterstaining with hemalum; original magnification, ×100; *inset* (*h*), ×400.

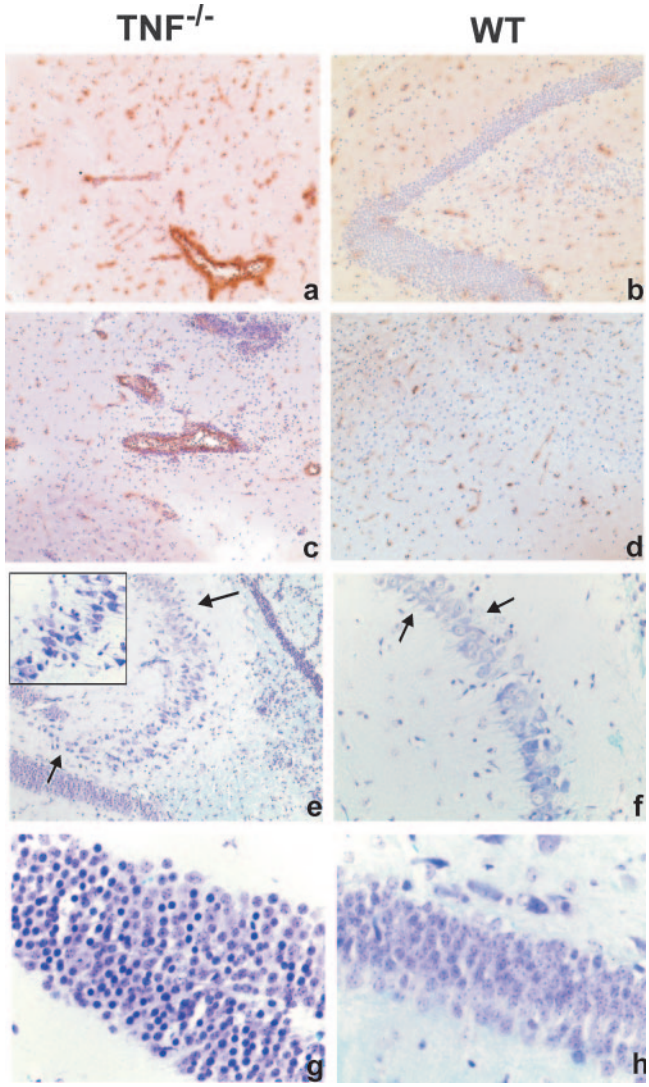


**FIGURE 5.** Increased CNS pathology in TNF$^{-/-}$ (*a*, *c*, *e*, and *g*) as compared with WT (*b*, *d*, *f*, and *h*) mice. *a* and *b*, At day 1 p.i., perivascular IgG deposition is prominent around numerous cerebral blood vessels in a TNF$^{-/-}$ mouse (*a*), however, weak in a WT animal (*b*). Anti-IgG immunostaining, slight counterstaining with hemalum; original magnification, ×100. *c* and *d*, At day 3 p.i., perivascular IgM deposition is marked and widespread in a TNF$^{-/-}$ mouse (*c*), but rarely and only weakly present in a WT mouse (*d*). Anti-IgM immunostaining, slight counterstaining with hemalum; original magnification, ×100. *e–h*, In a TNF$^{-/-}$ mouse, neuronal damage in the hippocampus (*e*) is most prominent in the CA1 segment (arrows in *e*) and the dentate fascia (*g*) with an increased nuclear acidophilia and neuronal shrinkage (*inset* in *e*). In contrast, only single neurons are shrunken in the CA1 segment (arrows, *f*) and the dentate fascia is normal (*h*) in a WT mouse. Cresyl violet and Luxol fast blue staining; original magnification, ×100 (*e*), ×400 (*f–h*, *inset* in *e*).

A depletion of CD4 and CD8 T cells in immunized TNF$^{-/-}$ and WT mice before i.c. challenge infection resulted in a strong increase of CFU in both strains of mice at day 7 p.i. (Fig. 8*A*). At this time point, immunized T cell-depleted TNF$^{-/-}$ mice were critically ill and harbored significantly more LM in the brain than immunized T cell-depleted WT animals. At day 7 p.i., nonimmunized control TNF$^{-/-}$ animals had already succumbed to the infection and nonimmunized WT mice had similar CFU as compared with immunized T cell-depleted WT mice.

To determine the impact of TNF on i.c. LM-specific CD4 and CD8 T cells, the frequency of i.c. IFN-γ-producing LM-specific CD4 and CD8 T cells was determined (Fig. 8*B*). In immunized WT and TNF$^{-/-}$ mice, peak numbers of LM-specific CD4 and CD8 T cells were synchronously detected at day 7 p.i., but numbers of

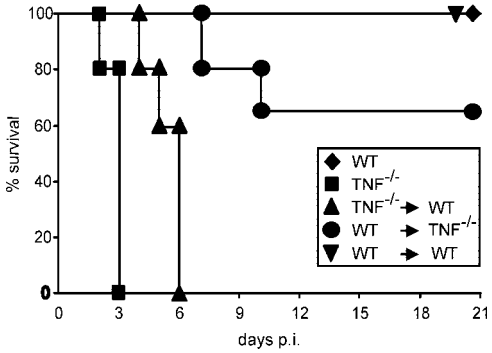


**FIGURE 6.** Survival of reciprocal WT and TNF$^{-/-}$ bone marrow chimeras following i.c. infection with Δ*actA* LM. Ten weeks after bone marrow transplantation, chimeras and control TNF$^{-/-}$ and WT mice were i.c. infected with Δ*actA* LM. Survival was monitored for five to eight mice per experimental group; $p < 0.01$ for TNF$^{-/-}$ vs WT mice and TNF$^{-/-}$ → WT chimeras vs WT mice; $p > 0.05$ for WT → TNF$^{-/-}$ chimeras vs WT mice and WT → WT chimeras vs WT mice.

LM-specific CD4 and CD8 T cells were significantly increased in TNF$^{-/-}$ mice. Thereafter, numbers of LM-specific CD4 and CD8 T cells gradually declined in both strains of mice, but numbers were still increased in TNF$^{-/-}$ mice at days 15 and 30 p.i. At day 50 p.i., equal numbers of LM-specific CD4 and CD8 T cells persisted in the brains of WT and TNF$^{-/-}$ mice.

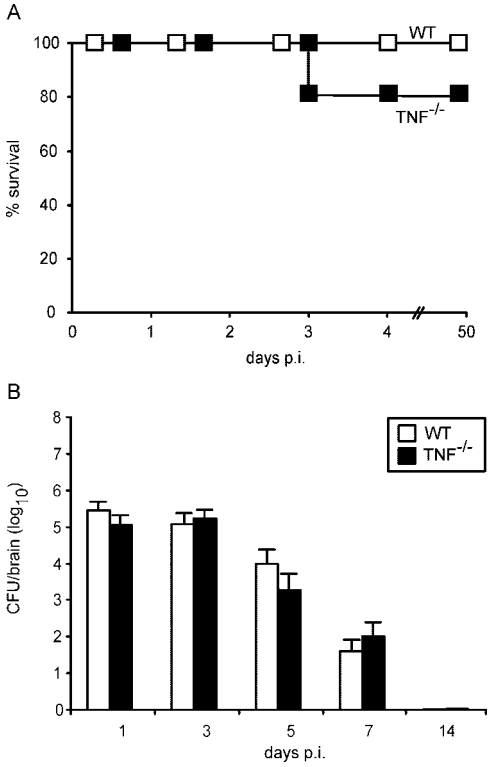


**FIGURE 7.** Survival and CFU of immunized WT and TNF$^{-/-}$ mice following infection i.c. with Δ*actA* LM. *A*, Twenty-eight days after systemic immunization with Δ*actA* LM, WT and TNF$^{-/-}$ mice were i.c. infected with Δ*actA* LM. Eighty percent of immunized TNF$^{-/-}$ mice and 100% of immunized WT survived the infection. Survival was monitored for eight mice per experimental group; $p > 0.05$ for TNF$^{-/-}$ vs WT mice. *B*, CFU were determined from three to five mice per experimental group at the indicated time points p.i., and data represent the mean. Mice were perfused with 0.9% NaCl before isolation of brain tissue.

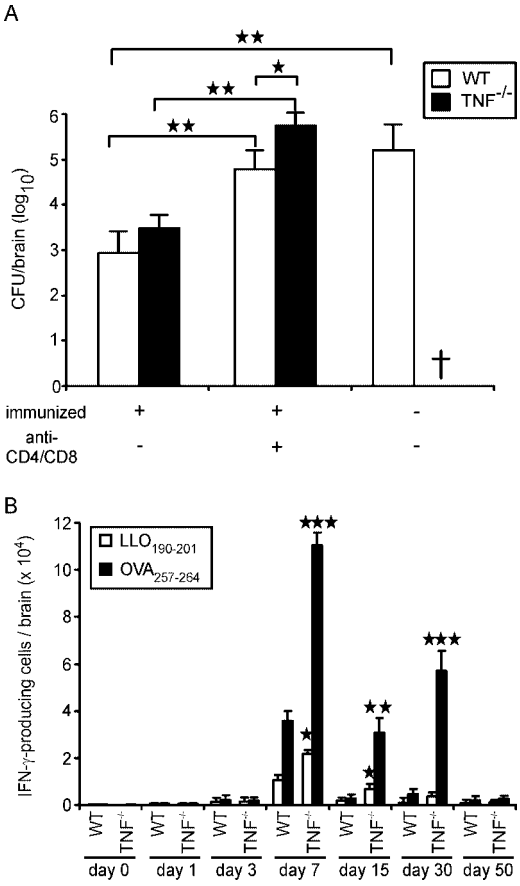


**FIGURE 8.** Influence of T cell depletion and immunization on i.c. CFU and frequencies of LM-specific CD4 and CD8 T cells in WT and TNF$^{-/-}$ mice following i.c. infection with Δ*actA* LM-OVA. *A*, After immunization with LM-OVA, WT and TNF$^{-/-}$ mice were depleted of CD4 and CD8 T cells or treated with control Abs before i.c. infection with Δ*actA* LM-OVA. CFU were determined from five mice per experimental group at day 7 p.i., and data represent the mean + SD. *, $p < 0.05$ and **, $p < 0.01$ for the respective groups, which are linked by a line. *B*, The frequency of i.c. LM-specific $LLO_{190-201}$-CD4 and $OVA_{257-267}$-CD8 T cells was determined by an IFN-γ ELISPOT. The absolute number of IFN-γ-producing $LLO_{190-201}$-CD4 and $OVA_{257-267}$-CD8 T cells is shown. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.005$ for TNF$^{-/-}$ vs WT mice at the respective days p.i. In *a* and *b*, leukocytes of three to six mice per time point were analyzed and the mean + SD is shown.

Collectively, these findings illustrate that an active immunization before i.c. challenge infection induces a TNF-independent, T cell-mediated protection from cerebral listeriosis and that in the absence of TNF numbers of i.c. LM-specific CD4 and CD8 T cells were increased.

### *Immunized TNF$^{-/-}$ mice exhibit more widespread distribution of LM, increased inflammatory reaction, and more pronounced brain damage*

In both strains of mice, immunization significantly reduced disease severity, however, CNS inflammation, edema formation, and neuronal damage were more severe in TNF$^{-/-}$ mice (Table I). At day 1 p.i., LM and leukocytic infiltrates were mainly confined to the ventricular system in both strains of mice. At day 3 p.i., the ventricular system was markedly enlarged in TNF$^{-/-}$ mice and bacteria had infiltrated the periventricular brain parenchyma. At day 5 p.i., leukocytic infiltrates, being composed of large numbers of RB6$^{+}$ granulocytes, F4/80$^{+}$ macrophages as well as CD4 and CD8 T cells, diffusely infiltrated the brain parenchyma of TNF$^{-/-}$

mice, whereas WT animals had restricted LM and infiltrates to the ventricular system. At day 7 p.i., WT mice had developed granulomas residing in the ventricular system and the periventricular brain tissue, whereas TNF$^{-/-}$ mice still showed a diffuse encephalitis and ventriculitis (Fig. 9, *a* and *b*). Beyond day 7 p.i., meningoencephalitis gradually recovered in both strains of mice with small leukocytic infiltrates persisting in the enlarged ventricular system.

Expression of iNOS protein was most prominent at day 3 p.i., when iNOS was expressed on leukocytes residing predominantly within or around the lateral ventricle in both strains of mice, however, at lower number in TNF$^{-/-}$ mice (Fig. 9, *c* and *d*). In WT mice, many hippocampal and periventricular microglial cells expressed iNOS protein, whereas microglia of TNF$^{-/-}$ mice was only exceptionally iNOS$^{+}$ (Fig. 9, *c* and *d*).

In addition, numerous blood vessels in the ventricular system, the hippocampus, the basal ganglia, and the meninges were IgM and IgG immunoreactive in TNF$^{-/-}$ mice resolving as late as day 14 p.i. (Fig. 9*e*). In contrast, WT mice exhibited only very minor edema with only single IgM$^{+}$ and IgG$^{+}$ vessels in the choroid plexus and the basal ganglia regressing until day 7 p.i. (Fig. 9*f*).

Neuronal damage was only exceptionally detectable in hippocampal and cerebellar neurons of WT mice, whereas hippocampal neurons of TNF$^{-/-}$ mice showed a marked eosinophilia at day 7 p.i. Remarkably, despite regressing CNS inflammation and bacterial clearance beyond day 7 p.i., TNF$^{-/-}$ mice still showed evidence of eosinophilic degeneration of neurons in the hippocampus, the dentate fascia, the dentate nucleus of the cerebellum, and of Purkinje cells as late as day 21 p.i. (Fig. 9*g*). At this time point, fields of neuronal loss indicative of neuronal apoptosis or necrosis were much smaller in WT animals (Fig. 9*h*).

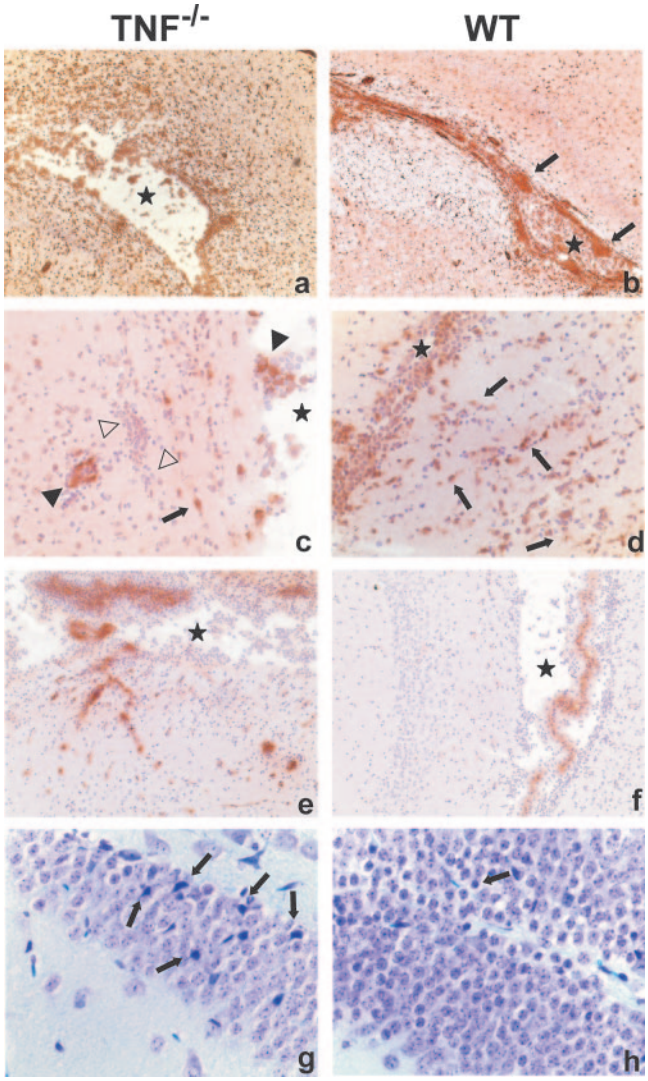


**FIGURE 9.** CNS listeriosis in immunized TNF$^{-/-}$ (*a*, *c*, *e*, and *g*) and WT (*b*, *d*, *f*, and *h*) mice. *a* and *b*, At day 7 p.i., meningoencephalitis is diffuse in the brain of a TNF$^{-/-}$ mouse (*a*) with large numbers of leukocytes in the lateral ventricle (∗) and scattered throughout the brain parenchyma. In contrast, at day 7 p.i., a granulomatous CNS listeriosis has developed in a WT mouse (*b*). Inflammation is mostly confined to the ventricular system (∗). Arrows point to granulomas within the lumen and wall of the lateral ventricle (∗). Anti-CD45 immunostaining, slight counterstaining with hemalum; original magnification, ×50. *c* and *d*, At day 3 p.i., in TNF$^{-/-}$ mouse (*c*), only some leukocytes in inflammatory infiltrates in the lateral ventricle and the adjacent brain tissue (filled arrowheads) and only exceptional microglial cells (arrow) express iNOS protein, whereas many leukocytic infiltrates are iNOS negative (open arrowheads). In contrast, in a WT mouse (*d*), many intraventricular (∗) and periventricular leukocytes as well as many microglial cells (arrows) in the periventricular parenchyma express iNOS protein at day 3 p.i. Anti-iNOS immunostaining, slight counterstaining with hemalum; original magnification, ×200. *e* and *f*, At day 3 p.i., there is a prominent perivascular IgM deposition in a TNF$^{-/-}$ mouse (*e*), which is observed in the choroid plexus of the lateral ventricle (∗), but is absent from cerebral blood vessels in the brain parenchyma in a WT mouse (*f*). Anti-IgM immunostaining, slight counterstaining with hemalum; original magnification, ×100. *g* and *h*, At day 21 p.i., shrunken cells with condensed nucleus indicative of apoptosis (arrows in *g*) are present in the dentate fascia in a TNF$^{-/-}$ mouse (*g*), but only exceptionally detectable in a WT mouse (*h*). Cresyl violet and Luxol fast blue staining; original magnification, ×400.

## Discussion

This study demonstrates, first, that ActA is an important virulence factor of LM in cerebral listeriosis, second, that hematogenously derived TNF is essential for survival of cerebral listeriosis, third, that an active immunization before i.c. infection with Δ*actA* LM prevents death of TNF$^{-/-}$ mice, and, fourth, that brain edema formation and neuronal cell death are TNF independent in cerebral listeriosis.

WT mice survived an i.c. infection with Δ*actA* LM, whereas all WT mice i.c. infected with WT LM succumbed within 5 days p.i.; this observation clearly illustrates the importance of ActA as virulence factor of LM in cerebral listeriosis. Compared with LM lacking phosphatidylcholine-specific phospholipase C, which is also involved in cell-to-cell spread (44, 45), and to LM lacking internalin A and B, which plays a role in the invasion of extracellular LM into several cell lines (46), Δ*actA* LM were clearly much more attenuated, because both phosphatidylcholine-specific phospholipase C- and internalin A/B-deficient LM induce an inevitably lethal primary murine cerebral listeriosis (47). However, it should be stressed that i.c. infection of WT mice with as few as 600 Δ*actA* LM resulted in a strong multiplication of Δ*actA* LM in the CNS with bacterial counts as high as $1 \times 10^6$ within 1 day p.i. In addition, a significant reduction of the i.c. bacterial load occurred as late as day 7 p.i. and was T cell dependent. These findings illustrate that Δ*actA* LM are not completely attenuated, but still possess a pathogenic potential in cerebral listeriosis of WT animals. These findings are in contrast to systemic infection with Δ*actA* LM. In WT mice, systemic infection with high amounts of Δ*actA* LM results only in a marginal multiplication of these bacteria in the infected liver and spleen (5) indicating that cell-to-cell spread is more important in systemic listeriosis

and that the immune response is more effective against LM in peripheral organs than in the CNS.

Distribution of LM in the CNS occurs also independent of direct cell-to-cell spread, because Δ*actA* LM migrated from the site of inoculation, i.e., the rostral basal ganglia, to the meninges, the ventricular system, including the lateral and fourth ventricles and the choroid plexus, as well as to the ependyma and the periventricular brain tissue. This pattern of distribution of Δ*actA* LM is similar to WT LM (28) with the only exception that Δ*actA* LM do not reach the brain stem parenchyma. The absence of a necrotizing brain stem encephalitis is the major factor responsible for survival of cerebral listeriosis after i.c. infection with Δ*actA* LM. The ActA-independent spread of LM may be caused be several mechanisms including spread within macrophages, which naturally reside in the leptomeninges and the choroid plexus, or extracellular spread taking advantage of the natural circulation of the C-SF in the ventricular system.

The pathogenic potential of Δ*actA* LM is even more dramatically illustrated in $TNF^{-/-}$ mice which succumbed to a primary cerebral listeriosis within 4 days p.i., i.e., before the onset of a LM-specific T cell response (6, 48). Thus, a low number of Δ*actA* LM invading the CNS is sufficient to cause death in immunocompromised hosts. This is in marked contrast to systemic murine listeriosis, because both WT and $TNF^{-/-}$ mice survive a systemic infection with at least $1 \times 10^6$ Δ*actA* LM (Ref. 18, and this study). Death of $TNF^{-/-}$ mice from primary cerebral listeriosis was due to a severe necrotizing meningoencephalitis affecting the ventricular system, the meninges, and widespread areas of the brain parenchyma including the periventricular tissue, the basal ganglia, the hippocampus, the cerebral cortex, and even the brain stem. Obviously, in the absence of TNF, the innate immune response was not able to control multiplication of Δ*actA* LM and to prevent the infection of the vital brain stem.

One of the factors which might contribute to the defective control of i.c. LM in $TNF^{-/-}$ mice is the reduced expression of iNOS. NO plays some protective role in systemic listeriosis (41, 42), and, therefore, a reduced iNOS expression in $TNF^{-/-}$ mice might result in an impaired control of LM. However, other factors including reactive oxygen intermediates and the small GTPase LRG-47 also contribute to control of LM (49, 50) in peripheral organs, and the relative role of NO and these other factors for the control of i.c. LM remains to be determined.

An active immunization before i.c. infection prevented death of 80% of $TNF^{-/-}$ mice, and immunized $TNF^{-/-}$ mice restricted the multiplication of LM as efficiently as WT mice. In both $TNF^{-/-}$ and WT mice, the improved control of i.c. Δ*actA* LM was T cell mediated, which is in agreement with previous studies illustrating that both CD8 and CD4 T cells are required for the protective action of a systemic immunization against cerebral listeriosis (27). The exact protective mechanisms of T cells have not been identified in cerebral listeriosis and may include the production of IFN-γ (6). However, even in immunized $TNF^{-/-}$ mice the inflammatory reaction was more widespread including higher numbers of inflammatory leukocytes, especially LM-specific CD4 and CD8 T cells, which were recruited to the CNS independent of TNF. LM-specific CD4 and CD8 T cells persisted for much longer times in the brain of $TNF^{-/-}$ mice despite equal numbers of i.c. LM and an identical kinetic of clearance of LM from the brain. The reason for the prolonged persistence of LM-specific T cells is yet unresolved, but a reduced apoptosis of LM-specific T cells in the absence of TNF might play a role.

The absence of LM-associated i.c. granulomas in $TNF^{-/-}$ mice also clearly illustrates that cerebral listeriosis of $TNF^{-/-}$ mice was less focused as compared with immunized WT mice. Such a granuloma inducing function of TNF has also been described in other infectious disease including LM hepatitis and is described here for the first time for cerebral listeriosis (51, 52). The importance of TNF-dependent granuloma formation for the control of LM and mycobacteria has recently been highlighted by Ehlers (21), speculating that the increased frequency of cerebral listeriosis in patients treated with anti-TNF Abs, i.e., infliximab, may be caused by the granuloma-disintegrating action of the anti-TNF Abs.

The survival rates of reciprocal $TNF^{-/-}$ and WT bone marrow chimeras i.c. infected with LM illustrate that TNF produced by hematogenous cells is essential for survival, whereas TNF derived from brain-resident cells is not absolutely essential, but contributes to an optimal survival rate. Moreover, the rapid death of nonimmunized $TNF^{-/-}$ mice before the onset of a LM-specific T cell response and the TNF-independent T cell-mediated survival of immunized $TNF^{-/-}$ mice indicate that TNF-producing cells of the innate immune system, in particular macrophages, are indispensable for the restriction of multiplication and spread of LM before T cells ultimately eliminate the bacteria from the brain. In fact, TNF was rapidly produced after i.c. infection of both nonimmunized and immunized WT mice. In extension of data by Frei and colleagues (53), flow cytometry identified macrophages, which reside both in the inflammatory meningeal and intraventricular infiltrates with direct contact to the C-SF as well as in the inflammatory infiltrates in the brain parenchyma, as a major source of TNF at days 1 and 3 p.i. In addition, microglia, the brain-resident macrophage population in the brain parenchyma, produced TNF with peak numbers of TNF-producing microglial cells at day 3 p.i., which coincides with the infection of the periventricular brain tissue by Δ*actA* LM. These findings indicate that the topographical distribution of LM strongly influences the kinetics and TNF-producing cell populations in cerebral listeriosis. Beyond day 7 p.i., LM-specific CD4 and CD8 T cells were the major TNF-producing cell types. However, these T cells produced TNF only after stimulation with their respective specific peptides, but not spontaneously, which is consistent with a rapid on/off cycling of cytokine production by LM-specific T cells (17, 54). The limited and focused TNF production by LM-specific T cells may be one important reason why TNF does not induce immunopathology in cerebral listeriosis. In conclusion, the concept that hematogenously derived TNF-producing innate immune cells are indispensable for the control of Δ*actA* LM is largely in agreement with findings in systemic listeriosis following WT LM infection, in which 1) $TNF^{-/-}$ and $TNFR1^{-/-}$ mice also succumb to the disease before the onset of a LM-specific T cell response (14–16), 2) the eradication of LM is also T cell mediated (6, 55), and 3) TNF production of T cells is dispensable for their protective action (18).

The present study clearly illustrates that TNF is not responsible for neuronal cell death in the hippocampus, the cortex, and cerebellum as well as for brain edema development in murine cerebral listeriosis. In fact, in the absence of TNF, these pathological changes were even more severe indicating that an effective control of LM in the brain and a restriction of the inflammatory reaction reduces damage of neurons and of the blood-brain-barrier. The factors inducing these pathological changes have not yet been defined. Potentially, the LM-derived highly potent pore-forming toxin listeriolysin may cause death of neurons and endothelial cells as already suggested and, importantly, has been clearly demonstrated for hepatocytes and lymphocytes (56, 57). This might even hold true despite identical CFUs in immunized $TNF^{-/-}$ and WT mice, because distribution of LM was more widespread in $TNF^{-/-}$ mice, which may facilitate interaction of listeriolysin with neurons and endothelial cells in various regions of the brain. Such a neurotoxic activity of a bacterial toxin has already been shown for

pneumolysin from *Streptococcus pneumoniae* (58, 59). Moreover, the more widespread and intense inflammatory reaction in both nonimmunized and immunized $TNF^{-/-}$ mice as compared with WT mice may contribute to development of neuronal cell death and brain edema formation. Because $TNF^{-/-}$ mice expressed less iNOS protein than WT animals, it is unlikely that excessive NO, which is potentially toxic for neurons and endothelial cells (43), contributes to the damage of neurons and blood vessel endothelial cells. Thus, other factors including an overproduction of excitatory amino acids, e.g., glutamate, may also contribute to damage of the brain (60), and it remains to be identified whether a single or several synergistic factors are responsible for neuronal cell death and brain edema formation.

In conclusion, the present study illustrates that the pathogenic potential of attenuated LM is significantly increased in the highly vulnerable CNS as compared with peripheral organs and that an appropriate immune response is even more critical in cerebral listeriosis, which has a strong impact on the use of attenuated LM as vaccines in humans as well as on therapeutic strategies in cerebral listeriosis. Therefore, a further dissection of the factors critical for the control of LM in the CNS as opposed to those factors contributing to death and neurological sequelae of surviving patients remains a major challenge.

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## Disclosures

The authors have no financial conflict of interest.

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