Rapidly Fatal Leishmaniasis in Resistant C57BL/6 Mice Lacking TNF

Patricia Wilhelm, Uwe Ritter, Stefanie Labbow, Norbert Donhauser, Martin Röllinghoff, Christian Bogdan and Heinrich Körner

*J Immunol* 2001; 166:4012-4019; doi: 10.4049/jimmunol.166.6.4012
http://www.jimmunol.org/content/166/6/4012

References
This article cites 30 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/166/6/4012.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Rapidly Fatal Leishmaniasis in Resistant C57BL/6 Mice Lacking TNF

Patricia Wilhelm,*† Uwe Ritter,*† Stefanie Labbow,*† Norbert Donhauser,∗ Martin Röllinghoff,* Christian Bogdan,* and Heinrich Körner2∗†

The resolution of infections with the protozoan parasite Leishmania major in mice requires a Th1 response that is closely associated with the expression of IL-12, IFN-γ, and inducible NO synthase. Previous Ab neutralization studies or the use of mice deficient for both TNF receptors suggested that TNF plays only a limited role in the control of parasite replication in vivo. In this study we demonstrate that resistant C57BL/6 (B6.WT) mice locally infected with L. major rapidly succumb to progressive visceral leishmaniasis after deletion of the TNF gene by homologous recombination. A reduction of the parasite inoculum to 3000 promastigotes did not prevent the fatal outcome of the disease. An influence of the altered morphology of secondary lymphoid organs in C57BL/6-TNF−−/− mice on the course of disease could be excluded by the generation of reciprocal bone marrow chimeras. Although infected B6.TNF−−/− mice mounted an L. major-specific IFN-γ response and expressed IL-12, the onset of the immune reaction was delayed. After in vitro stimulation, B6.TNF−−/− inflammatory macrophages released 10-fold less NO in response to IFN-γ than B6.WT cells. However, in the presence of a costimulus, e.g., L. major infection or LPS, the production of NO by B6.WT and B6.TNF−−/− macrophages was comparable. In vivo, inducible NO synthase protein was readily detectable in skin lesions and draining lymph nodes of B6.TNF−−/− mice, but its expression was more disperse and less focal in the absence of TNF. These are the first data to demonstrate that TNF is essential for the in vivo control of L. major. The Journal of Immunology, 2001, 166: 4012–4019.

The intracellular protozoan parasite Leishmania is able to infect a variety of mammalian hosts, including humans and mice, and to cause different manifestations of disease ranging from cutaneous to visceral forms. In the mouse model of L. major infection, the control of parasitic replication depends strongly on the genetic background of the infected mouse strain (1, 2). It has been demonstrated that the first step of a protective immune response is confinement of the parasite to the site of infection and the draining lymph node (LN). This is the result of a rapid expression of IFN-αβ, the induction of inducible NO synthase (iNOS) in macrophages and the up-regulation of IFN-γ within 24 h of infection (3, 4). The subsequent steps of protective immunity are based on induction and expansion of IFN-γ-producing CD4+ Th1 cells, which in concert with TNF activate macrophages to exert NO-mediated leishmanicidal activity (5–8). In the resistant C57BL/6 (B6.WT) mouse strain, healing of the local lesion and lifelong control of L. major replication are dependent on a permanent presence of iNOS activity in the tissue. Blocking of this enzyme results in a rapid reactivation of the disease (9).

In the mouse model of leishmaniasis, the proinflammatory cytokine TNF has been examined extensively because of its potential effector function. Treatment with TNF resulted in a reduction of lesion size and parasitic burden (7, 10, 11). Application of neutralizing anti-TNF Abs led to a transient aggravation of the disease (8, 10–12). In a transgenic model, B6.WT mice that constantly expressed a neutralizing TNF receptor 1 (p55; TNFR1)-IgG fusion protein developed a serious, nonhealing lesion at the site of infection (13). Finally, genetically targeted mice negative for TNFR1 or both TNFR1 and -2 were able to clear the parasite, although they continued to exhibit a permanent swelling at the site of infection. These mice developed a Th1 response and expressed IFN-γ and iNOS (14–16). TNFR2 was not involved in the in vivo control of the parasite (15). These findings suggest that TNF acts as a mere cofactor in the development of protective, anti-leishmanial immunity and is dispensable for the ultimate control of the infection.

In the present study we have analyzed the anti-leishmanial immune response of B6.WT mice lacking the TNF gene. Unexpectedly, these mice developed a visceral form of leishmaniasis. The parasite disseminated rapidly, and the majority of mice died 6–9 wk after infection, although the local lesion never reached the size seen in the highly susceptible BALB/c mouse strain. Fatal disease also occurred after low dose infections as well as in radiation bone marrow chimeras lacking TNF in hemopoietically derived cells. Analysis of the Ag-specific immune response revealed a delay of the induction of Th1 cells by 1 wk and demonstrated substantial deficiencies in the IgG response. In vitro, the expression of iNOS by thioglycolate-elicited peritoneal macrophages after stimulation with IFN-γ was reduced in the absence of endogenous TNF, but could be induced to wild-type levels after cosimulation with LPS and was also detectable in vivo.

Materials and Methods

Mice

Inbred B6.WT, and BALB/c mice were purchased from Charles River (Sulzfeld, Germany). The CD45-congenic strain B6.WT-Ly5.1 was obtained from Dr. Heinrich Körner, Nikolaus Sulzfeld, Germany. The CD45-congenic strain B6.WT-Ly5.1 was obtained from Dr. Heinrich Körner, Nikolaus Sulzfeld, Germany.

Copyright © 2001 by The American Association of Immunologists
from Dirk Schlüter (Universität Heidelberg/Mannheim, Mannheim, Germany), B6.TNF−/− mice were generated on a genetically pure C57BL/6 background as previously described (17) and were obtained from the Centenary Institute of Cancer Medicine and Cell Biology (Sydney, Australia). The B6.TNF−/− mice were screened by PCR using primers flanking the excised region of the TNF gene (5′ sense, GGC TCC AGC TGA CTA AAC ATC CTT C; 3′ antisense, ACC ACT AGT TGG TTG TCT TTG AGA T). The conditions of the PCR were 4 min at 94°C and 35 cycles of 94°C (1 min), 60°C (1 min), and 72°C (1 min). All mice were kept at the Institut für Klinische Mikrobiologie, Immunologie, und Hygiene (Erlangen, Germany). Mice 8–16 wk of age were used in all experiments, and experiments were performed according to the animal experimental ethics committee guidelines of the University of Erlangen-Nürnberg.

**Generation of radiation bone marrow chimeras**

Bone marrow cells were flushed with cold PBS/0.1% BSA from the long bones (femur and tibia) of matched donor mice. A single-cell suspension was prepared by gently passing the cells through 70-μm pore size cell strainer (Becton Dickinson, Franklin Lakes, NJ). The cells were washed and counted. Recipient animals were irradiated with a dose of 5.5 Gy gamma radiation on day 0. Bone marrow cells (2 × 10^7 cells/recipients) were injected i.v. on day 0. As a means to track the level of engraftment, the reconstitution was performed reciprocally between C57BL/6 and TNF-negative mice (both B6.Ly5.2) and B6.WT-Ly5.1 mice. Four weeks after engraftment peripheral blood was drawn from recipients. After RBC lysis lymphocytes were analyzed by flow cytometry for the presence or the absence of the C57BL/6 or TNF-negative congenic marker as previously described (18). The origin of the Abs used for the analysis was also detailed previously (18). Engraftment was considered to be sufficient when >80% of peripheral leukocytes exhibited the CD45 allele of the donor type.

**L. major parasites and the preparation of L. major Ag**

*L. major* promastigotes (MHOM/IL/81/FEBNI) were propagated in vitro in blood agar cultures as previously described (19). The virulence of the isolate was maintained by monthly passage in BALB/c mice. For the preparation of *L. major* parasites, stationary phase promastigotes were harvested, washed four times, and resuspended in PBS. The parasites were used for infection or subjected to four cycles of rapid freezing and thawing to prepare *L. major* Ag.

**L. major infection and evaluation of the systemic course of disease**

Mice were infected s.c. in one or both hind footpads with 3 × 10^7 stationary phase promastigotes/footpad of the third to seventh in vitro passage in a final volume of 50 μl. The increase in lesion size was monitored twice weekly by measuring the footpad thickness with a metric caliper (Kroeplin Schnelltaster, Schlechttern, Germany). When one footpad was infected, the increase in footpad thickness (percentage) was determined by the formula: thickness of infected footpad/mean thickness of noninfected footpads × 100. When both footpads were infected, the increase in footpad thickness (percentage) was determined by the formula: thickness of infected footpad/mean thickness of all footpads in the experimental group before infection × 100. Infection in one or both hind footpads did not change the course or outcome of the disease. The number of viable parasites in draining LN and spleen was estimated using limiting dilution analysis by applying Poisson statistics and the χ^2 minimization method as described previously (20).

**Serum isotype detection**

IgG1 and IgG2a serum Abs specific for *L. major* were detected by sandwich ELISA. For quantitation of different isotypes, reverse-96-well microtiter plates (Dynex Technologies, Denkendorf, Germany) were coated with 4 × 10^5 lyzed *L. major* promastigotes/well at 4°C overnight. The following steps alternated with washing of the plates with PBS/0.05% Tween 20. Microtiter plates (Dynex Technologies, Denkendorf, Germany) plus 10% FCS (Sigma). Cells were stimulated with lysed *L. major* promastigote Ag at a ratio of five parasites per cell or with Con A (final concentration, 5 μg/ml). Control wells contained medium without additives. The assay was performed in quadruplicate when possible. After 72 h of incubation, 100 μl of supernatants were removed and replaced by medium without additives. For the final 17 h of culture, cells were pulsed with [3H]thymidine (0.5 μCi/well; Amersham, Aylesbury, U.K.). Cells were harvested onto filter strips using a semiautomated cell harvester, and incorporation of radioactivity was measured in a scintillation counter.

 Supernatants of proliferation assays were stored at −70°C until the determination of IL-4 or IFN-γ concentrations. The cytokines were detected by capture ELISA. The following pairs of mAb were used: biotinylated BV4D11 and purified 11B11 in the IL-4 ELISA, and biotinylated XM12 and purified RA-6A2 in the IFN-γ ELISA (PharMingen). Round-bottom 96-well microtiter plates (Dynex Technologies) were coated with an anti-IFN-γ or an anti-IL-4 capture Ab at 4°C overnight. The following incubation steps were performed in a humidified chamber and alternated with washing of the plates three to five times using PBS/0.05% Tween 20. Supernatants were diluted 1/5 for IL-4 or 1/20 and 1/200 for IFN-γ detection and incubated with the capture Abs for 2 h at 37°C. Lymphokines were detected with biotinylated detection Abs and visualized with a streptavidin/alkaline phosphatase complex (Dako, Copenhagen, Denmark) followed by p-nitrophenyl-phosphate substrate. The plates were analyzed in a plate reader (Dynex Technologies) at 405 nm.

**Delayed-type hypersensitivity (DTH) reaction**

Three weeks after *L. major* infection *L. major* promastigote Ag (corresponding to 2 × 10^7 parasites) was injected in a volume of 20 μl in the footpad of the foreleg. As a control, 20 μl of PBS was injected in the opposite foreleg. The footpad thickness was measured with a metric caliper (Kroeplin Schnelltaster) after 24, 48, and 72 h.

**RT-PCR**

Total RNA was extracted from snap-frozen tissue or cell pellets with RNeasy columns (Qiagen, Germany) following the manufacturer’s protocol. RT of 1 μg RNA was performed using Moloney murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany). Specific products were amplified with 1 Taq polymerase (Promega). Both reactions followed a standard protocol. The PCR products were size fractioned by electrophoresis on 1% agarose gel containing ethidium bromide. The following primers were used: IFN-γ forward, 5′-TGA ACG GAC CAC ACT GCA TCT TGG-3′; IFN-γ reverse, 5′-CGA TTC CTT TTC CGC TTC CTG AG-3′; IL-4 forward, 5′-ATG GGT GTC AAT CCC CAC CAG GTA A-3′; IL-4 reverse, 5′-CGA GAT AGC CCG CAG GAT CAT GAT CAT-3′; IL-12 (p40) forward, 5′-GAC CCT GCC CAT TGA ACT GCC GGC-3′; IL-12 (p70) reverse, 5′-GAC CCT GCC CAT TGA ACT GCC GGC-3′.

**Macrophages**

Thioglycollate-elicited peritoneal macrophages were prepared from the peritoneal cavity of B6.WT or B6.TNF−/− mice 4 days after i.p. injection of 3 ml of 4% Brewer’s thioglycollate broth (Difco, Detroit, MI). RPMI 1640 medium with 2.5% FCS (Sigma) and supplemented as described above was used for tissue culture. The macrophages were seeded into 24-well plates (1 × 10^6 cells/well in 500 μl) or into eight-well LabTek chamber slides (Percarnox; Nunc, Wiesbaden, Germany) and cultured at 37°C. After 90–120 min, nonadherent cells were washed off, and subsequently macrophages were further incubated in fresh medium, stimulated with cytokines, and infected with *L. major* parasites as indicated.

**Determination of nitrite**

As an indirect measurement for the production of NO, culture supernatants were analyzed for their content of nitrite (NO2−) using the Griess reaction as previously described (20).
**Immunohistochemistry**

Tissue blocks from footpad lesions or LN were embedded in optimal cutting temperature compound (Diatec, Hallstadt/Bamberg, Germany) and stored at -70°C. Tissue sections (5-6 μm) were cut, thawed onto slides, coated with Pro-Marker (Services Sciences, Munich, Germany), air-dried, fixed in acetone (for 10 min at -20°C), and washed briefly in PBS/0.05% Tween 20. Nonspecific binding sites were blocked for 30 min with PBS/1% BSA/20% FCS. Rabbit antisera specific for L major or a mouse C-terminal iNOS peptide have both been described previously (9). Immunoperoxidase staining (with 3-amino-9-ethyl-carbazole as a substrate) and hematoxylin counterstaining were performed as described previously (9).

**Results**

**Course of L. major infection in TNF-deficient mice**

B6.WT, BALB/c, and B6.TNF-/- mice were infected with 3 × 10^6 L. major promastigotes, and the course of infection was monitored. At the site of infection, resistant B6.WT mice developed a small skin lesion, but were able to resolve the lesion and control the infection (Fig. 1A). Susceptible BALB/c mice, in contrast, developed ulcerating skin lesions that progressed without healing (Fig. 1A). In this mouse strain tissue necrosis developed soon after infection. B6.TNF-/- mice showed a moderate increase in lesion size, and ulceration was not observed. After 6 wk the local skin lesion started to resolve, and the tissue scarred (Fig. 1A). However, the disease progressed systemically, and the majority of mice died 6–9 wk after infection (Table I). On day 42 after infection, spleen and liver of B6.TNF-/- mice were substantially enlarged (spleen: B6.WT mice, 150 ± 30 mg (n = 5); B6.TNF-/- mice, 360 ± 50 mg (n = 4); BALB/c, 250 ± 10 mg (n = 3); liver: B6.WT mice, 1580 ± 50 mg (n = 5); B6.TNF-/- mice, 2030 ± 390 mg (n = 4); BALB/c, 1240 ± 60 mg (n = 3)).

An independent set of infection experiments was performed using various doses of L. major (3 × 10^6, 3 × 10^5, 3 × 10^4, and 3 × 10^3 parasites; n = 3). An inoculum of 3000 parasites was sufficient to cause fatal disease in B6.TNF-/- mice (Table I and Fig. 1B). BALB/c mice infected with the same dose showed a more pronounced footpad swelling than B6.TNF-/- mice (data not shown), but were able to control the infection and survived (Table I).

**Course of L. major infection in reciprocal bone marrow chimeras**

Reciprocal chimeras (B6.TNF-/-→B6.WT-Ly5.1, B6.WT-Ly5.1→B6.TNF-/-) were generated using a lethal irradiation protocol and a subsequent transfer of bone marrow. B6.WT-Ly5.1→B6.WT chimeras served as a control. Four weeks after bone marrow transfer flow cytometric analysis of a CD45 congenic marker was used to examine the efficacy of engraftment. At the time of the infection ~80% of recirculation leukocytes (αβ TCR+, B220+, Mac-1+) were of the donor type. Eight weeks after reconstitution the three types of chimeric mice were infected with 3 × 10^6 parasites in one hind footpad. The increase in lesion size in B6.WT-Ly5.1→B6.WT and B6.WT-Ly5.1→B6.TNF-/- chimeras after infection mirrored the lesion size in B6.WT (compare Figs. 1A and 2). In contrast, B6.TNF-/-→B6.WT-Ly5.1 displayed a more pronounced increase similar to B6.TNF-/- mice (compare Figs. 1A and 2). Only the B6.TNF-/-→B6.WT-Ly5.1 chimeras died after 6–10 wk of infection.

**Development of parasitic burden in TNF-deficient mice**

Limiting dilution assays were performed with draining popliteal LN to determine the number of viable L. major parasites in these lymphoid organs closest to the site of infection. On day 14 postinfection (p.i.), the parasitic burden in B6.WT mice was comparable to that in B6.TNF-/- mice (Fig. 3A). The number of parasites in the highly susceptible BALB/c strain was at this time point 10 times higher (Fig. 3A). On day 42 p.i., the number of parasites in B6.WT LN was 65-fold lower than that in B6.TNF-/- LN (Fig. 3A). BALB/c LN were suppurated and could not be analyzed.

Next, we examined the parasite burden in a visceral organ, the spleen. On day 14 p.i., neither B6.WT nor BALB/c nor B6.TNF-/- mice exhibited L. major titers greater than 1 parasite/100,000 splenocytes (Fig. 3B). On day 42 p.i., the number of parasites detectable in the spleens of B6.TNF-/- mice had increased remarkably. The titer of L. major in B6.TNF-/- mice exceeded the parasitic burden of B6.WT mice by >4 orders of magnitude (35,000-fold) and that of BALB/c mice by 1 order of magnitude (Fig. 3B).

On day 35 p.i. the parasitic burden of the bone marrow was analyzed. Also in the bone marrow of the TNF-negative mice, the number of L. major parasites exceeded the burden in B6.WT controls by a factor of 500 and was increased 16-fold compared with that in susceptible BALB/c mice (data not shown).

**Analysis of the cellular and humoral immune responses in TNF-deficient mice**

Protective immunity against L. major depends on the genetically determined ability to mount a Th1-type immune response (1, 2). Therefore, we isolated and analyzed cells from draining LN at various time points after infection. The relative proportions of T and B lymphocyte populations in draining LN were analyzed by
Liferative response to L. major

The presence of IFN-γ was assessed in B6.WT and B6.TNF−/− mice. The absence of exogenous Ag (Fig. 4A). On day 7 p.i., a marked L. major-specific proliferation was present in B6.WT mice, whereas in B6.TNF−/− mice only a limited proliferative response to L. major could be detected (Fig. 4A). Based on analysis of the cellularity of the draining LN, differences in total cell numbers did not explain the limited proliferative response of B6.TNF−/− LN cells to L. major Ag on day 7 after infection. After 18 days of infection, LN cells from B6.TNF−/− mice showed a stronger response to Ag than B6.WT LN cells (Fig. 4A). At this point the titers of parasites in the LN (mean >500 viable parasites/1000 cells) were so high that LN cells proliferated in the absence of exogenous Ag (Fig. 4A).

A protective Th1-type immune response is characterized by high levels of IFN-γ and IL-12 and low levels of IL-4. Therefore, we analyzed B6.WT and B6.TNF−/− popliteal LN and spleen tissue for the presence of IFN-γ, IL-12 (p40), and IL-4 mRNA by RT-PCR before the infection occurred (day 0) and 14 days after infection (day 14). At these time points these cytokines were expressed at equivalent levels in B6.WT and B6.TNF−/− mice (data not shown). To study the expression of IFN-γ and IL-4 by ELISA on a protein level, LN cells were isolated and stimulated with L. major Ag. B6.WT LN cells produced a significant amount of IFN-γ after antigenic stimulation. Interestingly, the expression of IFN-γ was substantially higher in B6.TNF−/− mice than in B6.WT mice (Fig. 4B). IL-4, as protagonist of the Th2 response, could not be detected in either supernatant (data not shown).

Protective immunity to L. major is independent of the presence of Ag-specific Abs (21). However, Ab production reflects the ability of the host to respond to Ag efficiently. Moreover, proportional distribution of serum isotypes is supposed to mirror a bias toward either a Th1 or Th2 response, whereby IgG2a marks a Th1 response, and IgG1 is preferentially expressed in Th2-type responses. Therefore, we investigated the level of Ag-specific Abs of the IgG1 and IgG2a isotypes on days 28 and 42 after infection. In B6.WT mice, we detected at these time points no preference of either isotype, but a substantial production of both IgG isotypes (Fig. 4C). Interestingly, in B6.TNF−/− mice the production of Ag-specific IgG1 was below the detection level at both time points, whereas IgG2a could be detected on day 42 p.i., however, at a 10-fold reduced concentration compared with B6.WT mice (Fig. 4C).

Table 1. Lethality after infection of B6.WT, BALB/c, and B6.TNF−/− mice with different numbers of L. major

<table>
<thead>
<tr>
<th>B6.WT</th>
<th>BALB/c</th>
<th>B6.TNF−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 × 10^6 L. major</td>
<td>3 × 10^7 L. major</td>
<td>3 × 10^8 L. major</td>
</tr>
<tr>
<td>0/9</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>6/6</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>11/11</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* The infected mice were checked twice weekly for their general health status and were sacrificed if they showed clinical signs of disease or died during the period of observation (12 wk).

** The lethality data after infection with 3 × 10^6 L. major are taken from a total of three independent infection experiments.

FACS analysis and were found to be stable throughout the early phase of infection (days 0–21 p.i.; data not shown). The determination of the total number of lymphocytes in the draining LN showed a reduction in B6.TNF−/− mice on day 3 after infection. However, this retardation of cell influx to the local LN in B6.TNF−/− mice was transient and had stopped on day 4 (data not shown). Additionally, the proliferative response of T cells to L. major Ag of infected B6.WT and B6.TNF−/− mice was studied. On day 7 p.i., a marked L. major-specific proliferation was present in B6.WT mice, whereas in B6.TNF−/− mice only a limited proliferative response to L. major Ag could be detected (Fig. 4A). Based on analysis of the cellularity of the draining LN, differences in total cell numbers did not explain the limited proliferative response of B6.TNF−/− LN cells to L. major Ag on day 7 after infection. After 18 days of infection, LN cells from B6.TNF−/− mice showed a stronger response to Ag than B6.WT LN cells (Fig. 4A). At this point the titers of parasites in the LN (mean >500 viable parasites/1000 cells) were so high that LN cells proliferated in the absence of exogenous Ag (Fig. 4A).

A protective Th1-type immune response is characterized by high levels of IFN-γ and IL-12 and low levels of IL-4. Therefore, we analyzed B6.WT and B6.TNF−/− popliteal LN and spleen tissue for the presence of IFN-γ, IL-12 (p40), and IL-4 mRNA by RT-PCR before the infection occurred (day 0) and 14 days after infection (day 14). At these time points these cytokines were expressed at equivalent levels in B6.WT and B6.TNF−/− mice (data not shown). To study the expression of IFN-γ and IL-4 by ELISA on a protein level, LN cells were isolated and stimulated with L. major Ag. B6.WT LN cells produced a significant amount of IFN-γ after antigenic stimulation. Interestingly, the expression of IFN-γ was substantially higher in B6.TNF−/− mice than in B6.WT mice (Fig. 4B). IL-4, as protagonist of the Th2 response, could not be detected in either supernatant (data not shown).

Protective immunity to L. major is independent of the presence of Ag-specific Abs (21). However, Ab production reflects the ability of the host to respond to Ag efficiently. Moreover, proportional distribution of serum isotypes is supposed to mirror a bias toward either a Th1 or Th2 response, whereby IgG2a marks a Th1 response, and IgG1 is preferentially expressed in Th2-type responses. Therefore, we investigated the level of Ag-specific Abs of the IgG1 and IgG2a isotypes on days 28 and 42 after infection. In B6.WT mice, we detected at these time points no preference of either isotype, but a substantial production of both IgG isotypes (Fig. 4C). Interestingly, in B6.TNF−/− mice the production of Ag-specific IgG1 was below the detection level at both time points, whereas IgG2a could be detected on day 42 p.i., however, at a 10-fold reduced concentration compared with B6.WT mice (Fig. 4C).

FIGURE 2. Course of L. major infection in B6.TNF−/−→B6.WT-Ly5.1 and B6.WT-Ly5.1→B6.TNF−/− radiation bone marrow chimeras. B6.TNF−/−→B6.WT-Ly5.1 (●: n = 8) and B6.WT-Ly5.1→B6.TNF−/− mice (■: n = 8) were infected with 3 × 10^6 L. major promastigotes. B6.WT-Ly5.1→B6.WT mice (○: n = 4) served as controls. The relative increase in footpad thickness is shown as the mean ± SEM. The curve of the B6.TNF−/−→B6.WT-Ly5.1 lesion size ends after the death of two animals 7 wk after infection. The experimental infection of the six remaining mice was terminated between weeks 8 and 9 when the animals became moribund. Neither control animals (B6.WT-Ly5.1→B6.WT) nor B6.WT-Ly5.1→B6.TNF−/− mice showed clinical signs of disease or died during the period of observation (12 wk).

FIGURE 3. Parasitic burden in draining LN and spleen in B6.WT, BALB/c, and B6.TNF−/− mice. The numbers of viable parasites in the draining LN (A) and the spleen (B) of B6.WT, BALB/c, and B6.TNF−/− mice were determined on day 14 p.i. (□) and day 42 p.i. (▲) by limiting dilution analysis. The mean parasitic burden in the organs of four to six mice is shown. One triangle represents one animal. One of three experiments is shown.
Delayed-type hypersensitivity

The presence of Ag-specific T cells is required to mount a DTH response. To determine the ability of B6.TNF$^{−/−}$ mice to mount such a response, we injected L. major Ag in one footpad of L. major-infected B6.WT, B6.TNF$^{−/−}$, and BALB/c mice. After 48 h the swelling of the footpads in B6.TNF$^{−/−}$ and BALB/c mice was significantly reduced compared with that in B6.WT mice. However, B6.TNF$^{−/−}$ mice showed a stronger reaction than BALB/c mice (Fig. 5).

Expression level and localization of iNOS in TNF-deficient mice

A major antimicrobial effector mechanism of the innate immune system is the IFN-γ- and TNF-dependent up-regulation of iNOS and the subsequent production of large amounts of NO (22). Thioglycolate-elicited peritoneal exudate macrophages from B6.WT and B6.TNF$^{−/−}$ mice were stimulated with IFN-γ and LPS, respectively (Fig. 6), and the accumulation of NO in the supernatant was determined. Upon stimulation with IFN-γ alone B6.WT macrophages showed a strong production of NO (34.6 ± 11.1 μM NO; mean ± SEM of four experiments; Fig. 6A), whereas NO levels generated by TNF-deficient macrophages were low (13.3 ± 9.5 μM NO; n = 4; Fig. 6A). Stimulation of TNF-negative cells with LPS or LPS plus IFN-γ resulted in an activation comparable to that in B6.WT macrophages (Fig. 6B). Thus, IFN-γ-induced, but not LPS- or LPS- plus IFN-γ-induced, production of NO is dependent on endogenous TNF.

Histological analysis of the iNOS expression of infected footpad tissue and the draining LN was performed to determine alterations in iNOS expression level and localization of iNOS-positive cells in the absence of TNF expression. On day 27 p.i., the skin lesion in B6.WT mice showed a stronger, less diffuse iNOS expression than that in B6.TNF$^{−/−}$ mice (Fig. 7, A and B). Later in the course of the infection (day 40 p.i.), the draining popliteal LN in B6.WT mice showed a weaker expression than that in B6.TNF$^{−/−}$ mice (Fig. 7, C and D).
mice exhibited a defined lymphoid microarchitecture and distinct focal expression of iNOS in the paracortical T cell area adjacent to B cell follicles. In contrast, in the LN of B6.TNF−/− mice, lymphoid structures were replaced by connective tissue. This can be interpreted as an indication for an ongoing inflammatory process with tissue destruction and subsequent scarring. The overall production of iNOS was strong, but instead of large iNOS-positive foci, much smaller iNOS-positive clusters or single iNOS-positive cells prevailed (Fig. 7, C and D).

Discussion
The clinical form of leishmaniasis, cutaneous or visceral, that develops after infection with *L. major* depends on the genetic background of the host. This makes it possible to examine mechanisms leading to an effective immune response. The infection of the resistant strain C57BL/6 that lacks the gene for TNF with *L. major* led to a visceral form of the disease and resulted in the rapid death of the animals. This outcome was unexpected and was in contrast to the results of earlier studies using mice negative for TNFR1 and -2 (15). The unaltered course of infection even after a 1000-fold reduction of the number of parasites in the inoculum revealed a high susceptibility to the pathogen in the absence of TNF. This was not due to the altered morphology of the secondary lymphoid organs (loss of B cell follicle and absence of germinal center reaction) as could be shown by infection experiments in reciprocal radiation bone marrow chimeras. The delay of 1–2 wk in the adaptive immune response of B6.TNF−/− mice suggested a complex deficiency in cell-cell interaction and priming of lymphocytes. Nevertheless, the predominant effector molecule of the innate immune response, NO, was produced in the absence of TNF.

The course of infection observed in B6.TNF−/− mice was different from the form of leishmaniasis observed in the susceptible BALB/c strain. In B6.TNF−/− mice, there was neither ulceration of the lesions nor suppuration of infected tissues or draining LN. Most importantly, B6.TNF−/− mice displayed a higher susceptibility to the pathogen, which was illustrated by a markedly shorter period of survival than that in infected BALB/c mice. The role of TNF in leishmaniasis has been analyzed in a number of previous studies using application of TNF or neutralizing anti-TNF Abs or transgenic models. Ab treatment resulted in a transient aggravation of the course of disease (7, 8, 10, 12), whereas application of therapeutic doses of TNF led to an amelioration of symptoms in susceptible mice (7, 10, 11). Unexpectedly, mice that were negative for both TNFRs, which according to our knowledge should lack any TNF-signaling, did not succumb to the parasite and were able to control the infection, but developed a nonhealing inflammatory lesion (15). The fatal course of infection in the B6.TNF−/− mice suggests that TNF can signal through a third receptor and that this signaling is necessary to confer protection in leishmaniasis. In this context it is worth mentioning that a recent report by Hayder et al. shows that a ligand other than TNF or lymphotoxin can bind to TNFR1 and -2 (23). Taken together, our data are compatible with the view that the one ligand-two receptors model might not be entirely sufficient to describe the more complex situation in vivo. Alternatively, the difference in disease outcome between TNF- and TNFR1/2-negative mice could also be the result of the use of different *L. major* strains. A comparison of the two strains will answer this question.

TNF-negative mice display changes in the microarchitecture of secondary lymphoid organs such as the absence of distinct B cell

FIGURE 7. Histologic analysis of the iNOS expression. The expression of iNOS was analyzed in footpad lesion (day 27 p.i.; A and B) and popliteal LN (day 40 p.i.; C and D) of B6.WT (A and C) and B6.TNF−/− mice (B and D) infected with *L. major*. Sections were stained with immunoperoxidase and counterstained with hematoxylin (×400).
folicles and are not able to generate germinal centers after antigenic challenge (17, 24). These alterations are relatively minor, but could influence the efficiency of the anti- \textit{L. major} immune response. To investigate this, radiation bone marrow chimeras (18) were infected with \textit{L. major}. This experiment indicates that the sole factor necessary for survival is the ability of hemopoietic cells to produce TNF. TNF-positive bone marrow cells are able to mount a protective immune response in a TNF-negative stromal environment. This also shows that TNF produced by nonhemopoietic cells such as keratinocytes in the skin cannot substitute for leukocytic TNF. Therefore, we conclude that the effects of TNF on hemopoietic cells, rather than those on the formation of the correct lymphoid microarchitecture, are critical for the control of \textit{L. major}.

Our experiments showed that the clinical course of leishmaniasis in B6.TNF^{-/-} mice correlated with the parasitic burden of the host organism. The uncontrolled replication of parasites in the spleen of B6.TNF^{-/-} mice demonstrated that as yet undefined mechanisms, which normally control the replication of \textit{L. major}, are TNF dependent and cannot be compensated for by other control mechanisms. In the mouse model of \textit{Mycobacterium tuberculosis} infection, TNF-negative mice were not able to form granulomas (25). This deficiency had already been revealed by treatment of infected mice with anti-TNF Abs (26, 27) and was attributed to a role of TNF in the priming of the immune response and leukocyte migration in general (28, 29). An insufficiency in T cell priming or migration to the site of infection or the lymphoid tissues due to the lack of TNF should impair the development of a specific cellular or humoral response to \textit{L. major}. Indeed, we were able to demonstrate that B6.TNF^{-/-} mice displayed a delay in generating a T cell response. Nevertheless, LN cells from B6.TNF^{-/-} mice produced a substantial amount of IFN-\gamma, which indicates a Th1-type response. This is consistent with results obtained in studies using TNFR1-negative mice, which also showed an elevated level of IFN-\gamma after \textit{L. major} infection (14). Therefore, the T cell response in B6.TNF^{-/-} mice was of the expected type, but it was delayed by 1–2 wk. We can exclude that the limitations of B6.TNF^{-/-} mice in starting the immune response are due to deficiencies at the level of signal transduction pathways (30). Thus, we suggest that the delay in generating specific T and B cells implies a paucity of cell-cell communication or cell migration. Moreover, infected B6.TNF^{-/-} mice experience a major deficiency in the DTH reaction to \textit{L. major} Ag. After 48 h, at the peak of the reaction, B6.WT mice showed a marked increase in footpad thickness at the injected site. Compared with the DTH response of B6.WT mice, B6.TNF^{-/-} mice only reacted at low level (35% of the B6.WT swelling 48 h after infection; Fig. 5). This is in contrast to the outcome of similar experiments in the \textit{M. tuberculosis} model (25), where the same strain of TNF-negative mice displayed a normal DTH response. This is another strong indication for a defective T cell priming in B6.TNF^{-/-} mice in the model of experimental leishmaniasis.

NO has been described as an important effector molecule of an innate and adaptive response to \textit{L. major}. Recently, the cascade of events that results in the expression of iNOS and the production of NO has in part been defined (31). An infection of macrophages with \textit{L. major} causes the production of IFN-\gamma. These cytokines are needed as a necessary second signal and induce in concert with \textit{L. major} a rapid expression of iNOS. The enzyme is readily detectable in the skin within 24 h after infection (4). This leads to an up-regulation of IFN-\gamma production of NK cells, and in a positive feedback loop to an increased production of iNOS. TNF has been shown to synergize with IFN-\gamma for the activation of macrophages (21). Furthermore, neutralization of TNF in the presence of IFN-\gamma or the stimulation of B6.TNF^{-/-} macrophages with IFN-\gamma alone demonstrated a strong dependency of the production of NO on the presence of endogenous TNF and have proven the importance of this cytokine (5–7, 31). In this study we could demonstrate that in vivo B6.TNF^{-/-} mice, like TNFR^{-/-} mice (14, 15), do not need TNF signaling for the expression of iNOS during the infection.

In conclusion, we have determined an unexpected role of TNF in the control of leishmaniasis. Abolishing the signaling through both TNFR and 2 can be compensated, whereas absence of the ligand is fatal. Our investigations have not yet revealed which defense mechanism has failed or is absent. However, we found a retarded proliferative response of T cells, a delayed or missing switch to Ag-specific, T cell-dependent serum Igs, and an impaired formation of iNOS-positive cell clusters. These results together with the virtual absence of a DTH reaction point to a deficit in cell recruitment and priming of the immune response that is currently under investigation.

References


