Immunity, Vol. 22, 93–104, January, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.immuni.2004.11.016

# Distinct and Nonredundant In Vivo Functions of TNF Produced by T Cells and Macrophages/Neutrophils: Protective and Deleterious Effects

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## Summary

**Tumor necrosis factor (TNF, TNFα) is implicated in various pathophysiological processes and can be either protective, as in host defense, or deleterious, as in autoimmunity or toxic shock. To uncover the in vivo functions of TNF produced by different cell types, we generated mice with TNF ablation targeted to various leukocyte subsets. Systemic TNF in response to lipopolysaccharide was produced mainly by macrophages and neutrophils. This source of TNF was indispensable for resistance to an intracellular pathogen, *Listeria*, whereas T-cell-derived TNF was important for protection against high bacterial load. Additionally, both T-cell-derived TNF and macrophage-derived TNF had critical and nonredundant functions in the promotion of autoimmune hepatitis. Our data suggest that T-cell-specific TNF ablation may provide a therapeutic advantage over systemic blockade.**

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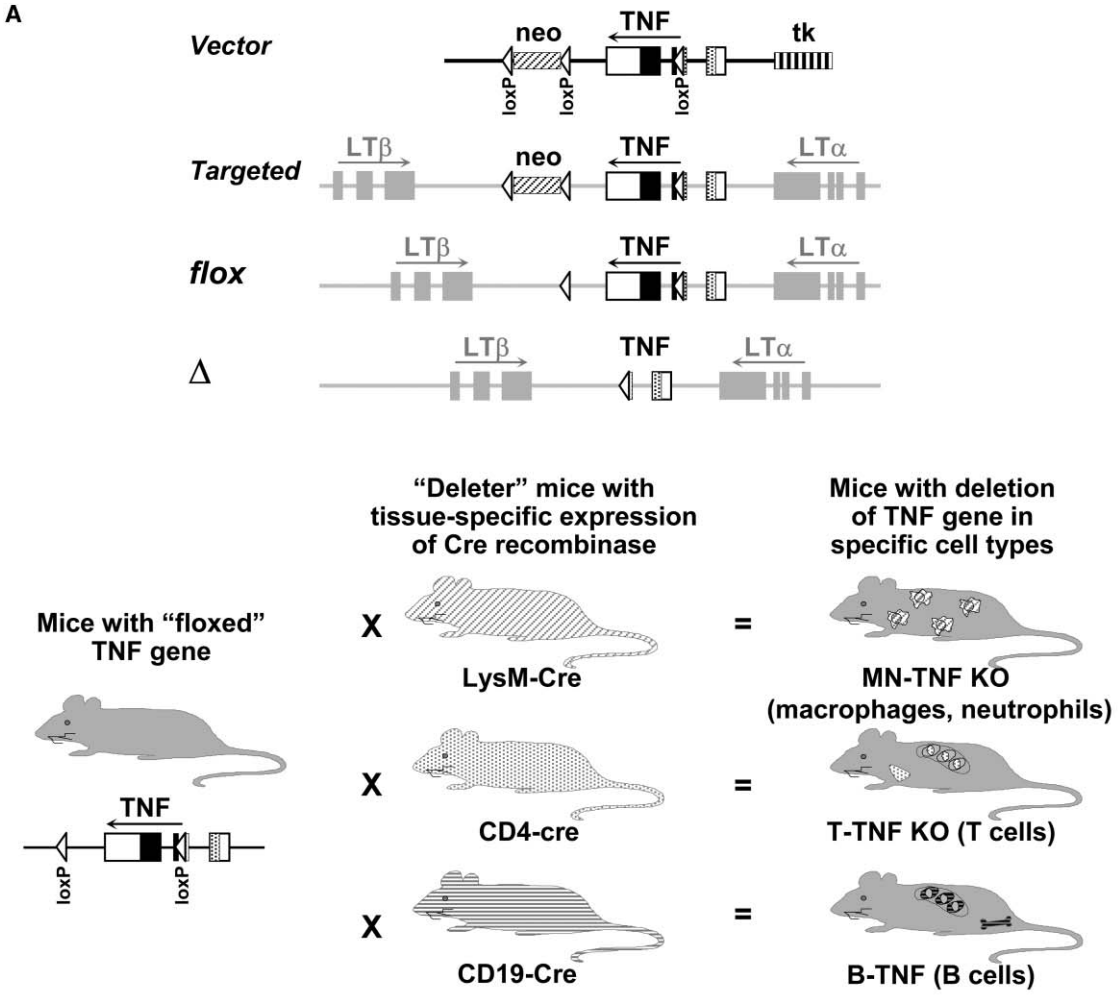
## Introduction

Tumor necrosis factor (TNF, also called TNFα) was discovered due to its potent antitumor activity in animal models (Carswell et al., 1975) and is a pleiotropic cytokine critical for cell trafficking, inflammation, maintenance of lymphoid organ structure, and host defense against various pathogens, such as *Listeria* and *M. tuberculosis* (Pfeffer et al., 1993; Rothe et al., 1993; Pasparakis et al., 1996; Flynn et al., 1995). For TNF to play a beneficial role in the resistance to pathogens, it must be produced at the right place, at the right time, and in appropriate concentrations. Deregulated TNF production can be deleterious and has been clearly associated with several autoimmune and inflammatory diseases, such as rheumatoid arthritis (RA) (Feldmann et al., 1995), septic shock (Beutler and Cerami, 1989), and inflammatory bowel disease (IBD) (Kassiotis and Kollias, 2001; Kollias et al., 1999). Recently, TNF blockers have provided important advances in control of inflammation associated with RA (Feldmann, 2002), Crohn's disease (Papadakis and Targan, 2000), and psoriasis (Baeten et al., 2003). However, systemic inhibition of TNF production may result in unwanted side effects due to compromised host defense functions, as exemplified by reported rare reactivation of tuberculosis in RA patients treated with TNF blockers (Mohan et al., 2003). Since in vivo TNF is produced by many cell types (Giroir et al., 1992; Sedgwick et al., 2000), cell-type-restricted TNF production may be one of the mechanisms by which the tightly regulated balance between beneficial and deleterious functions is maintained. In order to directly test the hypothesis of distinct physiological roles played by TNF produced by different cell types in vivo, we generated mice with highly efficient cell-lineage-restricted inactivation of TNF. In particular, we deleted the TNF gene in macrophages/neutrophils (MN-TNF KO mice) or lymphocytes (T-TNF KO and B-TNF KO mice). Our data point to unique and nonredundant roles of both macrophage/neutrophil and T-cell-derived TNF in host resistance to an intracellular pathogen (*Listeria*) and in the development of autoimmune liver injury. These findings provide a rationale for cell-type-restricted TNF blockade strategies.

## Results

### Selective and Efficient Ablation of TNF in Macrophages/Neutrophils and Lymphocytes

To inactivate TNF selectively in distinct cell types of the immune system, we utilized the Cre/loxP recombination


A
Vector
neo
TNF
tk
loxP
loxP
loxP
Targeted
LTβ
neo
TNF
LTα
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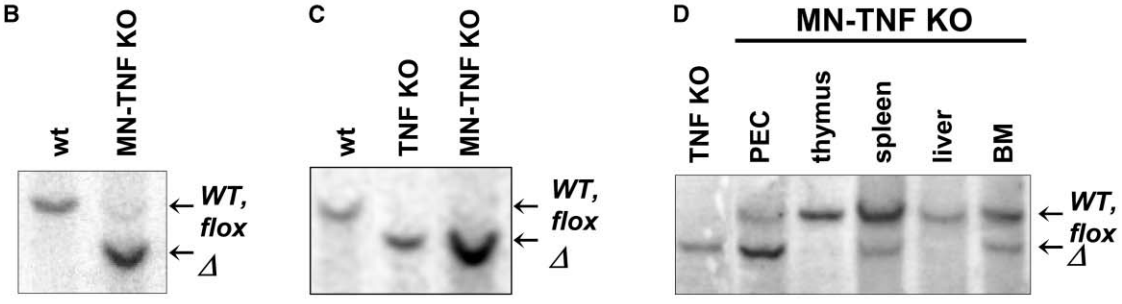
Δ
LTβ
TNF
LTα
Mice with "floxed" TNF gene
"Deleter" mice with tissue-specific expression of Cre recombinase
Mice with deletion of TNF gene in specific cell types
X
LysM-Cre
=
MN-TNF KO (macrophages, neutrophils)
X
CD4-cre
=
T-TNF KO (T cells)
X
CD19-Cre
=
B-TNF (B cells)


B
wt
MN-TNF KO
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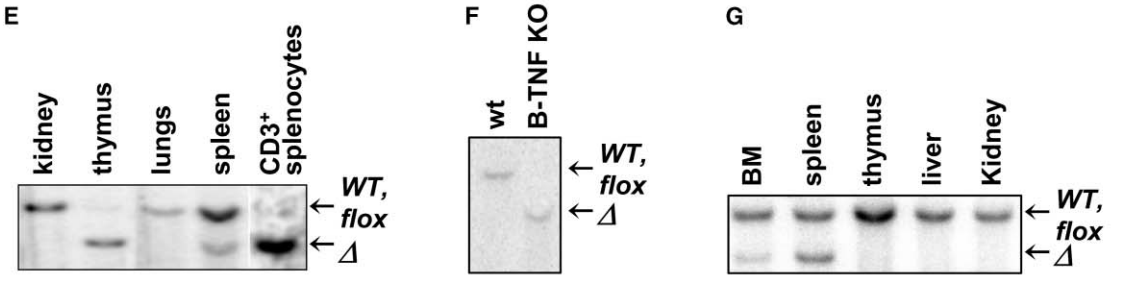
E
kidney
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G
BM
spleen
thymus
liver
Kidney
WT,
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system and a knockin/transgenic approach. We first generated the knockin $TNF^{flox/flox}$ mice (Figure 1A) in which the TNF gene was modified by the insertion of two synthetic loxP sites flanking the critical coding region and placed in such a way as to leave the TNF gene fully functional. We rigorously verified that the insertion of the loxP sites into the TNF gene did not change the wild-type phenotype of $TNF^{flox/flox}$ mice (for details, see Figure 1A and Supplemental Figure S4 at http://www.immunity.com/cgi/content/full/22/1/93/DC1/). However, in these mice the TNF gene was tagged for subsequent deletion by Cre recombinase expressed under cell-lineage-specific or inducible promoters.

In order to generate mice with specific deletion of the TNF gene in macrophages and granulocytes, we crossed TNF floxed mice with knockin LysMcre mice bearing Cre recombinase under the control of the lysozyme M promoter (Clausen et al., 1999; Faust et al., 2000). For the experiments, we used mice with the genotype $tnf^{flox/flox}$ $lysM^{Cre/wt}$, which were named MN-TNF KO mice (Figure 1A). The extent of TNF gene deletion was almost complete in neutrophils and macrophages (>98%), whereas none was detected in liver and thymus (Figures 1B–1D). TNF mRNA in bone-marrow-derived macrophages (BMDM) was barely detectable by Northern analysis after lipopolysaccharide (LPS) stimulation, in contrast to high levels of TNF transcripts in cells from control mice (Figure 2A). Virtually no TNF protein was detected in supernatants of neutrophils from MN-TNF KO mice stimulated with LPS (Figure 2B). Production of TNF by peritoneal-elicited cells (PEC) from MN-TNF KO mice stimulated in vitro by LPS was significantly reduced (Figure 2C). Finally, intracellular cytokine staining of $CD11b^+$ PEC treated with LPS revealed a dramatic reduction in TNF levels in cells from MN-TNF KO mice in comparison to wild-type (WT) (Figure 2D) or complete absence of TNF production by $F4/80^+$ cells (Figure 2E). $CD11b^+CD11c^+$ myeloid DC from peritoneal cavity were still able to produce TNF, despite marked deletion of the TNF gene in this particular subset of DC (Clausen et al., 1999). Bone-marrow-derived dendritic cells (BMDC), which are also of monocyte origin, showed partial deletion of the TNF gene (see Supplemental Figure S2). Therefore, MN-TNF KO mice may serve as a model with a remarkably complete ablation of TNF in macrophages and neutrophils.

We next generated T-TNF KO mice using CD4-Cre transgenic mice (Lee et al., 2001). Both Southern analysis and intracellular cytokine staining revealed high efficiency and specificity of TNF ablation in T lymphocytes (Figures 1E and 2F). In particular, T-TNF KO mice were characterized by almost complete deletion of the gene in thymus. Purified splenic T cells exhibited >98% of deletion, and intracellular staining of TNF revealed that $CD3^+$ splenocytes from T-TNF KO mice activated with PMA/ionomycin failed to produce any detectable TNF. On the other hand, BMDC from T-TNF KO mice produced normal amounts of TNF in response to LPS (Figure 2G).

To generate B-cell-specific TNF KO mice, we utilized CD19-Cre deleter mice (Rickert et al., 1997), which were previously used to inactivate the neighboring LTβ gene with a high efficiency and specificity (Tumanov et al., 2002). B-TNF KO mice showed approximately 99% TNF gene deletion in B cells but no detectable deletion in either macrophages, thymus, kidney, or liver (Figures 1F, 1G, and 2F).

Finally, we verified that complete loxP-mediated deletion of the TNF gene did not affect the expression of LTα or LTβ in spleen and on activated splenocytes (Supplemental Figure S4B). Splenic structure in complete TNF KO mice was reminiscent of that in the conventional TNF KO mice (Pasparakis et al., 1996, 2000; Korner et al., 1997) and was clearly distinct from LTβ KO mice (Supplemental Figure S4A).

### Deleterious TNF Can Be Produced by Various Cell Types, Depending on the Toxicity Model

Bacterial products induce innate immunity by engaging Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997). TNF production is an important downstream target of TLR signaling, in particular through TLR4 (Poltorak et al., 1998). As such, TNF was reported to be a critical mediator of septic shock in humans and of LPS/D-Gal-induced acute systemic toxicity in mice (Tracey et al., 1987).

Importantly, serum TNF levels were dramatically reduced in MN-TNF KO mice injected with LPS (although still clearly exceeding the levels in TNF KO mice), while they did not differ from the wild-type levels in T-TNF KO and B-TNF KO mice (Figure 3A). Additionally, quantification of soluble TNF protein by ELISA in supernatants of BMDM stimulated with various pathogen-derived compounds confirmed that TNF production was decreased at least 100-fold in macrophages from MN-TNF KO mice (Figure 3B).

Figure 1. Generation of a Panel of Mice with Cell-Type-Specific TNF Ablation

(A) Targeting strategy and breeding scheme. $TNF^{flox/flox}$ mice were crossed with appropriate Cre-bearing deleter in order to obtain mice with cell-type-specific TNF deficiencies.

(B) Deletion of the TNF gene in BMDM. DNA was prepared from adherent 10-day BMDM cultures from MN-TNF KO mice and subjected to Southern analysis (represents one of two independent experiments).

(C) Deletion of the TNF gene in neutrophils. DNA was prepared from FACS-sorted $CD11b^+$ $GR1^{high}$ cells from thioglycollate-elicited peritoneal cells (PEC) from MN-TNF KO mice and subjected to Southern analysis.

(D) Multiple tissue analysis of TNF deletion in MN-TNF KO mice. Genomic DNA from various tissues was prepared and subjected to Southern analysis. PEC were induced by injection of 3% thioglycollate medium i.p. 36 hr before cell collection (represents one of two independent experiments).

(E) T-cell-specific deletion of TNF. Genomic DNA from different tissues and sorted $CD3^+$ splenocytes from T-TNF KO mice was purified and subjected to Southern analysis (represents one of two independent experiments).

(F and G) B-cell-specific inactivation of TNF. Genomic DNA samples from sorted splenic B cells (F) or from various tissues (G) of B-TNF KO mice were purified and subjected to Southern analysis.

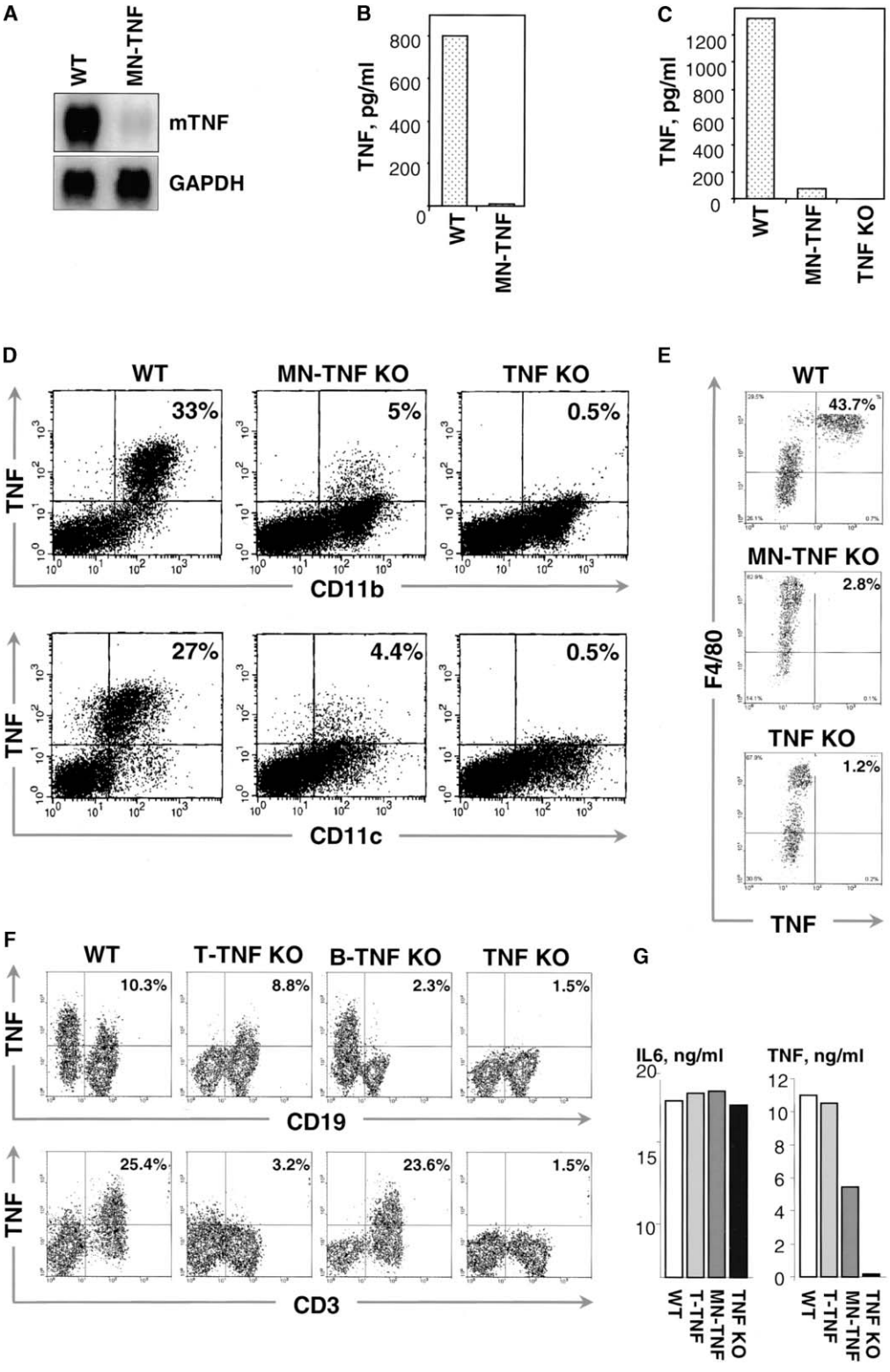


Figure 2. Efficiency of TNF Ablation in MN-TNF KO and T-TNF KO Mice

(A) TNF mRNA expression in BMDM from MN-TNF KO mice. BMDM were activated by LPS (100 ng/ml) for 1.5 hr, collected, and lysed for RNA preparation.

(B) TNF production by LPS-activated neutrophils. FACS-sorted $CD11b^{+}$ $GR1^{high}$ cells from PEC of MN-TNF KO mice were activated by LPS (100 ng/ml) for 4 hr.

(C) TNF production by LPS-stimulated PEC. PEC of MN-TNF KO mice were activated by LPS (100 ng/ml) for 6 hr. In (B) and (C), TNF was measured in supernatants by ELISA (represents one of two independent experiments).

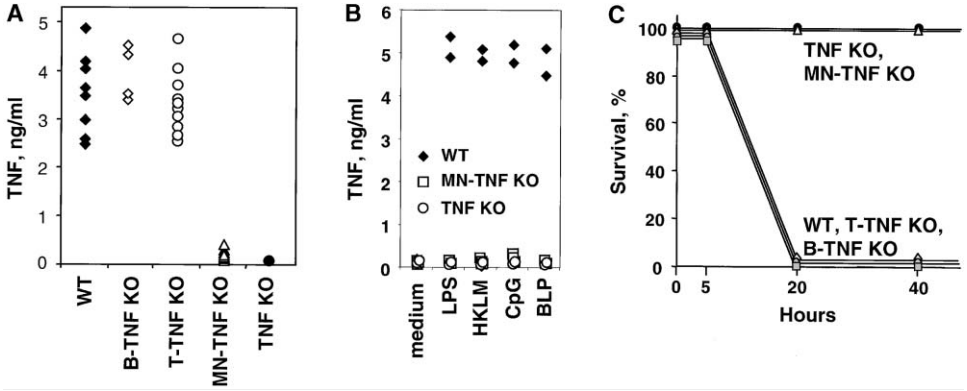


Figure 3. Macrophages and Neutrophils Are the Main Source of Systemic TNF after LPS Challenge

(A) Systemic TNF production induced by LPS. Mice were injected intraperitoneally with 100 μg of LPS, and sera were collected 1.5 hr after injection. TNF in sera was measured by ELISA (represents one of two independent experiments).

(B) TNF production by BMDM of MN-TNF KO mice. Adherent cells from 10 day cultures ($10^6$ cells/ml) were activated for 24 hr in 96-well plates with LPS (100 ng/ml), bacterial lipoprotein (BLP) (500 ng/ml), CpG (1 ng/ml), HKLM (moi 100:1), or incubated without stimulation in the presence of IFNγ (50 ng/ml). TNF production was assessed by ELISA.

(C) Deletion of TNF in macrophages and neutrophils rescues mice from LPS/D-Gal toxicity. Mice were injected intraperitoneally with 10 μg LPS and 20 mg D-Gal, monitored for survival, and sacrificed when moribund (represents one of three independent experiments).

Since both TNF- and TNFR1-deficient mice are completely resistant to LPS/D-Gal lethal toxicity (Pfeffer et al., 1993; Rothe et al., 1993; Pasparakis et al., 1996; Marino et al., 1997), we asked whether the loss of TNF production by macrophages/neutrophils was sufficient to protect mice from the septic shock. Remarkably, MN-TNF KO mice were completely resistant to this type of lethal challenge (Figure 3C). On the contrary, mice with lymphocyte-restricted TNF deficiency died with the same kinetics as the wild-type controls (Figure 3C). Even lower doses of LPS (1 μg) in combination with D-Gal were still lethal for T-TNF KO, B-TNF KO, and control mice, whereas MN-TNF KO mice remained fully resistant (data not shown). Therefore, for the LPS/D-Gal-induced toxic shock, the deleterious role of TNF is solely due to its production by neutrophils and macrophages.

In another model of toxic shock induced by superantigen from gram-positive bacteria (*S. aureus* enterotoxin B [SEB]) in the presence of D-Gal, T-TNF KO and MN-TNF KO mice showed a level of protection almost identical to that of TNF KO mice, while B-TNF KO mice remained as sensitive as wild-type mice (Supplemental Figure S6). These results implied nonredundant critical functions of both T-cell-derived TNF and macrophage/neutrophil-derived TNF in the development of superantigen-induced shock. Collectively, the data demonstrate that the source of deleterious TNF during the development of systemic toxicity can be attributed to distinct types of leukocytes, and it also depends on the nature of the pathogenic stimulus that triggers the response.

### TNF from Macrophages and Neutrophils Is Critical for Protection against *Listeria*, but at High Doses T-TNF Becomes Important

Host defense is one of the complex physiological functions in which the beneficial and protective role of TNF produced by hematopoietic cells has been clearly demonstrated using mice deficient in either TNF or in its main receptor, TNFR1 (Pfeffer et al., 1993; Rothe et al., 1993; Pasparakis et al., 1996; Marino et al., 1997). Nevertheless, the cellular sources of protective TNF are incompletely defined. In order to evaluate the relative contribution of macrophages/neutrophils versus lymphocytes in TNF production in response to intracellular bacterial infection, we used a *Listeria monocytogenes* model. Inactivation of the TNF gene only in macrophages and neutrophils was sufficient for almost complete loss of resistance against *Listeria*, similar to that observed in TNF KO (Figure 4). Indeed, MN-TNF KO mice developed uncontrolled infection, leading to death within 4 days (Figure 4A) and accompanied by a 3–4 log increase in bacterial load, both in the liver and the spleen (Figures 4B and 4C). Under the same conditions, WT and B-TNF KO mice could successfully control the infection (Figures 4C and 4D). While WT mice displayed almost normal liver histology with only small inflammatory foci, TNF KO and MN-TNF KO mice developed large, confluent inflammatory necrotic areas with many Mac-1- and Gr1-positive cells, which appeared less abundant in T-TNF KO mice and rare in WT mice (Figure 4E). Importantly, induction of the chemokine MCP-1 was reduced in spleens of MN-TNF KO mice, although not to the extent

(D and E) Intracellular TNF staining of LPS-stimulated PEC. PEC were elicited from MN-TNF KO mice 48 hr (D) or 80 hr (E) after thioglycollate injection. Cells were activated by LPS (100 ng/ml) for 5 hr and stained for intracellular TNF and for CD11b, CD11c (D), or F4/80 (E) surface markers.

(F) Intracellular labeling for TNF. Splenocytes were activated by 100 ng/ml PMA and 1 μg/ml of ionomycin for 5 hr, and intracellular labeling for TNF and surface markers was performed.

(G) ELISA quantification of IL6 and TNF in the supernatants of BMDC of T-TNF KO and MN-TNF KO mice. BMDC were stimulated by LPS in the presence of 10 ng/ml IFN γ for 24 hr.

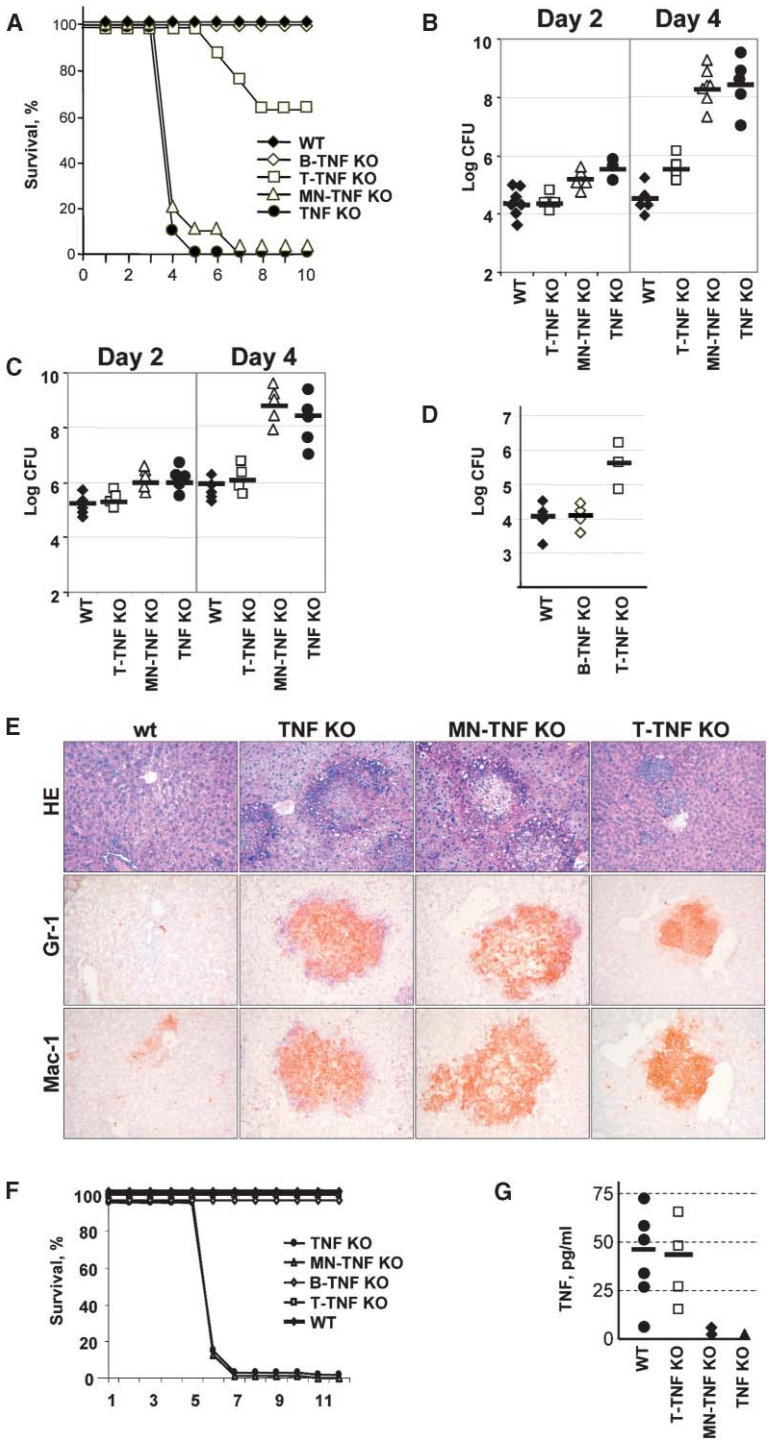


Figure 4. Role of T-Cell-Derived TNF and Macrophage/Neutrophil TNF in the Resistance against *Listeria*

(A) Mice were injected intravenously with $5 \times 10^4$ cfu (colony-forming units) of live *Listeria*, monitored for survival daily, and sacrificed when moribund (represents one of three independent experiments, 5–10 mice per group).
(B–D) Bacterial loads in the liver (B, D) and spleen (C) of infected mice. Mice were injected intravenously with $5 \times 10^4$ cfu of live *Listeria* and sacrificed on day 2, 4, and 7 (D). Livers and spleens were aseptically removed and homogenized in sterile PBS with 0.05% of Tween 20. Serial dilutions were plated into the brain-heart infusion agar and colony-forming units were calculated after 24 hr of growth at 37°C (represents one of two independent experiments).
(E) Histological examination of liver. Mice were injected intravenously with $5 \times 10^4$ cfu of live *Listeria* and sacrificed on day 3. Livers were excised and embedded into the O.C.T. compound for the preparation of frozen sections or fixed in 10% neutral buffered formalin. Upper panel, hematoxilin/eosin staining of formalin-fixed paraffin-embedded liver tissue; lower panel, serial frozen liver sections stained with CD11b and GR1. Original magnification, 100×.
(F) Mice were injected intravenously with $5 \times 10^3$ cfu of live *Listeria*, monitored for survival daily, and sacrificed when moribund (represents one of two independent experiments, 5–10 mice per group).
(G) TNF levels in serum of infected mice. After high dose of *Listeria* injection ($5 \times 10^4$ cfu i.v.), mice were bled on day 2 and serum TNF levels were determined by ELISA.

observed in TNF KO mice (Supplemental Figure S3). MCP-1 and its receptor CCR2 are important for resistance against *Listeria* (Kurihara et al., 1997; Serbina et al., 2003). Although myeloid DC displayed partial deletion of the TNF gene, they were able to produce normal amounts of IL-6, bactericidal nitric oxide, and only slightly decreased amounts of TNF in vitro (Figure 2G and data not shown), implying that the DC-mediated innate immune component was not severely affected in MN-TNF KO mice. Serum TNF levels on day 2 of infection could not be detected in MN-TNF KO mice, but TNF was clearly present in both T-TNF KO and WT mice (Figure 4G).

Interestingly, T-TNF KO mice exhibited increased sensitivity to *Listeria* at high doses of infection too, although the effects were modest in comparison with MN-TNF KO mice (Figure 4A). Specifically, T-TNF KO mice showed increased mortality, occurring on days 5–8 post infection. Bacterial loads in infected organs at the earliest stages in T-TNF KO mice were comparable with those

in WT mice but elevated on day 4 or 6 (Figures 4B–4D). However, all surviving T-TNF KO mice were able to clear *Listeria*, as no bacteria were detected in organs of mice on day 14 (data not shown). This result implied that T-cell-derived TNF possesses a distinct function in host defense: protection against high bacterial load, which cannot be provided solely by innate mechanisms. However, low-to-moderate doses of infection could be fully controlled in T-TNF KO mice, in contrast to MN-TNF KO or TNF KO mice (Figure 4F and data not shown). Therefore, the dominant role in production of protective TNF during *Listeria* infection is played by macrophages and neutrophils, while TNF-producing T cells may constitute the second line of defense.

Interestingly, generation of T cell response and of T cell memory per se appeared TNF independent, since MN-TNF KO, T-TNF KO, and even TNF KO mice immunized with ActA-deficient *Listeria* (Kocks et al., 1992) were all able to control and survive secondary challenge with virulent *Listeria* (Supplemental Figure S5A).

### Both T Cells and Macrophages/Neutrophils Produce TNF that Promotes a Model Autoimmune Disease

Despite its beneficial role in providing resistance against various pathogens, TNF is involved in the development of inflammatory and autoimmune conditions, such as RA, brain malaria, encephalomyelitis, and hepatitis. Earlier studies suggested that most of the TNF in the experimental model of autoimmune liver injury, induced by Concanavalin A (ConA) (Tiegs et al., 1992), was produced by resident liver macrophages (Kuppfer cells) (Schumann et al., 2000). However, in addition to TNF, Kuppfer cells can also produce iNOS and several chemokines, which are known to be critical for the development of hepatitis (Sass et al., 2001). Moreover, development of autoimmune liver injury also requires T cells that are the primary targets for ConA action.

In order to unveil the sources of TNF during the initiation and development of liver injury, we evaluated ConA-induced autoimmune hepatitis in a panel of conditional TNF KO mice (Figure 1). Strikingly, T-TNF KO mice showed increased resistance to the liver injury comparable to that of complete TNF KO mice, implying a critical role of T cells as TNF producers in this model of liver toxicity (Figures 5A–5C). Inactivation of TNF in macrophages and neutrophils in MN-TNF KO mice also resulted in a similar protection (Figures 5A–5C).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) liver enzyme activities measured in sera correlated with the extent of liver injury and were greatly elevated in WT mice or B-TNF KO mice, while in T-TNF KO, MN-TNF KO, and TNF KO mice these levels remained comparatively low (Figures 5B and 5C). Additionally, extensive liver necrosis was observed in WT and B-TNF KO, whereas in T-TNF KO, MN-TNF KO, and TNF KO only infiltrating cells but no necrosis or tissue destruction areas could be detected (Figure 5D). TNF serum levels correlated well with biochemical and histological parameters: grossly elevated in both wild-type and B-TNF KO mice and significantly decreased in both T-TNF KO and MN-TNF KO mice (Figure 5E). Thus, ablation of TNF in either T cells or macrophages/neutrophils is sufficient to provide significant protection against experimental autoimmune hepatitis.

## Discussion

For many years, TNF was viewed as a soluble proinflammatory cytokine produced by leukocytes of the innate immunity arm, such as macrophages and neutrophils (Giroir et al., 1992). However, TNF is expressed in both soluble and membrane-bound forms by various cell types with distinct homing and migratory properties (Sedgwick et al., 2000). Thus, different cell subsets are likely to be responsible for systemic versus local TNF production as well as for TNF-mediated responses in different physiological conditions. In this study, we employed a novel panel of mice with highly specific and efficient deletion of TNF gene in several types of leukocytes, including cells of both the innate arm of the immune response, such as macrophages and neutrophils, and of its adaptive arm, T and B lymphocytes. Using these tools, we determined the source of TNF production critical for host defense and for the development of several TNF-dependent pathologies. Our findings clearly demonstrate that both macrophage/neutrophil-derived and T-cell-derived TNF play distinct biological roles that can be either protective or deleterious. TNF from these two sources cannot substitute for each other, and they cannot be substituted by other cellular sources of TNF.

Macrophages and neutrophils are the main source of systemic TNF in vivo in response to LPS during gram-negative sepsis (Figure 3). However, TNF produced both by T cells and by macrophages/neutrophils in response to SEB, a toxin derived from gram-positive bacteria, was responsible for toxicity in SEB/D-Gal shock. In SEB-induced systemic toxicity, T cells are responsible for the induction of disease, whereas the non-T cell compartment (presumably, macrophages) is linked to disease susceptibility and to organ failure (Anderson and Tary-Lehmann, 2001). Ablation of transcriptional factor NFATp, which drives TNF expression specifically in T cells, is sufficient for significant protection against SEB/D-Gal shock (Tsytsykova and Goldfeld, 2000). Thus, both T cells (believed to be the primary TNF producers in this case) and macrophages make TNF that plays nonredundant critical roles in pathophysiological consequences of exposure to a superantigen.

From the response to bacterial products, we extended our studies to a model of intracellular infection. Resistance to listeriosis is a complex reaction by the host mediated by various cell types: macrophages, neutrophils, DC, NK cells, and T cells (Edelson and Unanue, 2000; Kaufmann, 1993; Unanue, 1997). These leukocytes produce proinflammatory cytokines such as TNF and IFN$\gamma$, which are absolutely critical for protection (Pasparakis et al., 1996; Pfeffer et al., 1993; Harty and Bevan, 1995). We show here that macrophages and neutrophils are critical TNF producers required for the resistance against the intracellular bacterial pathogen *Listeria*. These cells of the innate immunity arm represent the first line of host defense against intracellular pathogens. They produce large amounts of TNF (Figures 3A, 3B, and 4G), contributing to both the initial activation of innate immune response and to the recruitment of DC and inflammatory cells to the sites of infection. In the absence of TNF from macrophages and neutrophils, expression levels of key chemokine MCP-1, which attracts

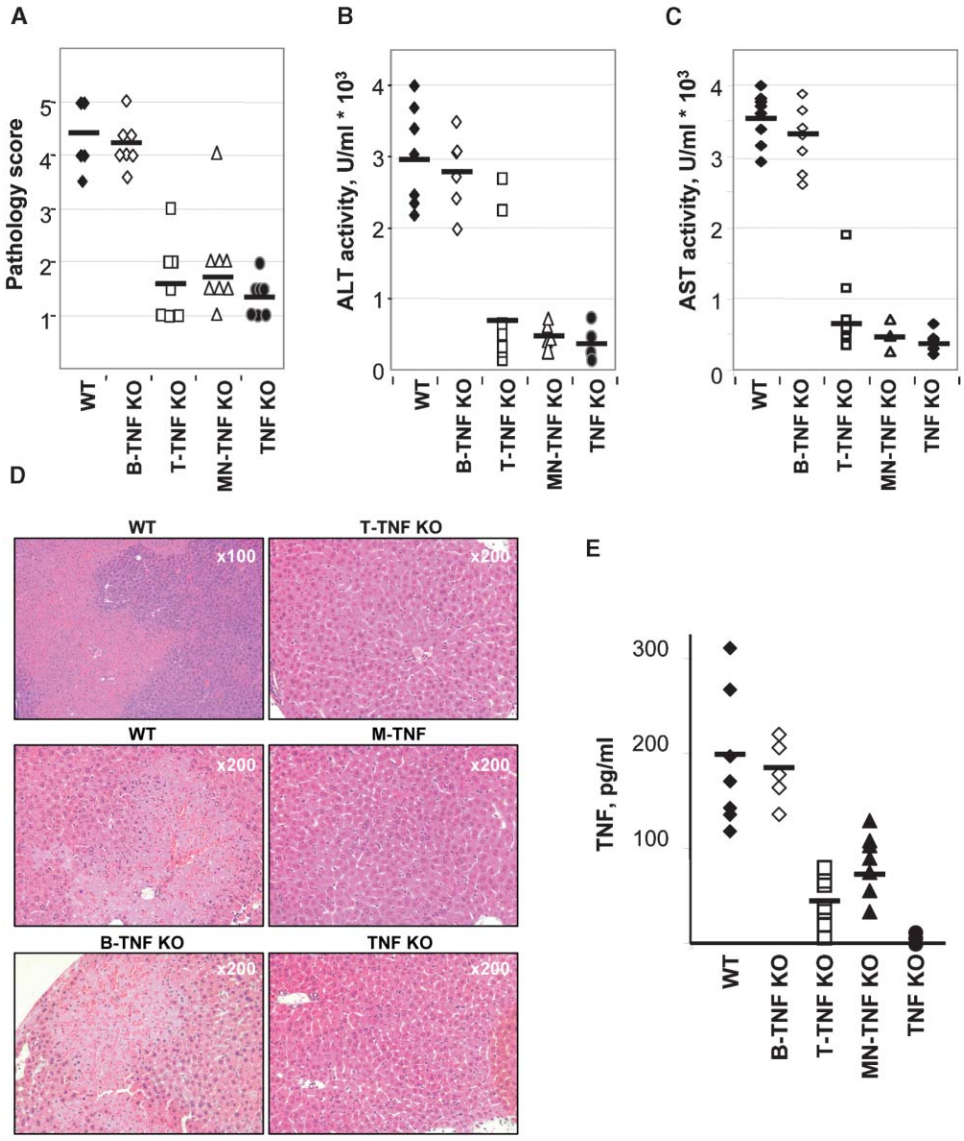


Figure 5. T-Cell- and Macrophage-Derived TNF Promote Autoimmune ConA-Induced Hepatitis

(A) ConA-induced fulminant liver pathology. Mice were injected intravenously with 15 mg/kg body of ConA. Ten hours later, mice were sacrificed and the extent of hepatitis was analyzed by a pathologist using a semiquantitative scale of clinical scores as follows: 1–2, normal liver; 3, mild hepatitis; 4, pronounced hepatitis; 5, severe hepatitis (represents one of two independent experiments).

(B and C) Alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) activity after ConA injection. Mice were injected intravenously with 15 mg/kg body of ConA. Sera were collected 10 hr after the injection, and ALT (B) or AST (C) activities were analyzed using an enzymatic colorimetric kit (represents one of two independent experiments).

(D) Histological examination of liver. Mice were injected intravenously with 15 mg/kg of ConA and sacrificed after 10 hr. Livers were excised and fixed in 10% neutral buffered formalin, followed by staining of 5 μm thick sections with hematoxilin/eosin and visualization with a photomicroscope. Original magnification: 100× or 200×.

(E) Serum levels of TNF after ConA administration. Serum samples were taken 2 hr after ConA injection (15 mg/kg) and analyzed by ELISA.

monocytes and DC to the infected organs, are significantly reduced (Supplemental Figure S3).

However, despite the apparent lack of contribution from T cells to systemic TNF levels in response to bacteria, T-TNF KO mice exhibited increased sensitivity to high dose of *Listeria* infection, implying that T-cell-derived TNF is important for controlling the infection at high bacterial loads. In T-TNF KO mice, bacteria numbers in infected organs at earliest stages of infection were comparable with those in WT mice but became elevated on day 4 or 6 (Figures 4B–4D), especially in mice succumbing to infection (data not shown). At lower doses, T-TNF KO mice were able to control the infection without increased mortality (Figure 4F), implying the predominant and sufficient role of macrophage/neutrophil-derived TNF in protection. Previous studies utilizing SCID (Bhardwaj et al., 1998) and T-cell-deficient mice (DiTirro et al., 1998) showed that T cells play a pivotal role in producing sterilizing immunity primarily by several distinct mechanisms: direct lysis of infected cells by

Table 1. Role of TNF Produced by Distinct Types of Leukocytes in Various Pathophysiological Models

| | TNF-Producing Cells | | | |
|---|---|---|---|---|
| TNF-Dependent Feature | Macrophages | Neutrophils | T Cells | B Cells |
| Resistance to intracellular pathogen | +++ | +++ | + | no role |
| Septic (LPS-induced) shock | −−− | −−− | no role | no role |
| Superantigen shock | −− | no role? | −−− | no role |
| Autoimmune hepatitis | −−− | no role? | −−− | no role |
| Lymphoid organ structure | no role | no role | ++ | +++ |

Protective (+) and detrimental (−) effects of TNF produced by various types of leukocytes in host defense against intracellular pathogens, maintenance of lymphoid organs, toxic shock conditions, and autoimmune hepatitis. Our data suggest that blockade of T-cell-derived TNF only may neutralize the destructive and toxic TNF in autoimmunity, with only minimal effects on host defense, lymphoid organ structure and antibody production.

CD8+ T cells and by production of IFN$\gamma$ (Harty and Bevan, 1995). Both $CD4^+$ and $CD8^+$ antigen-specific T cells are known to produce large amounts of TNF involved in direct killing as well as in activation of inflammatory response. For example, lung inflammation and injury in transgenic mice was recently shown to be induced by TNF exclusively expressed by $CD8^+$ T cells without any appreciable contribution from macrophages (Xu et al., 2004). Mice lacking $\alpha\beta$-T cells rapidly succumb to high-dose *Listeria* infection within 5 days (DiTirro et al., 1998), while SCID mice are able to control low-dose *Listeria* infection but fail to clear it (Bhardwaj et al., 1998).

There are at least two possible explanations for why T-cell-derived TNF may play a unique role in a high-dose infection model. First, it is conceivable that at least some TNF provided by T cells is retained on the membrane of T cells and acts via direct cell-to-cell contact (Kinkhabwala et al., 1990; Grell et al., 1995), i.e., upon antigen recognition. Indeed, mice bearing a noncleavable form of TNF showed a partial protection against mycobacteria (Olleros et al., 2002) or *Listeria* (Pasparakis et al., 1996). Membrane-bound TNF apparently cannot be substituted by TNF from other sources and can activate downstream chemokines differently from the soluble TNF (Ruuls et al., 2001). Alternatively, activated T cells secrete TNF, which nevertheless remains in tight contact with target cell inside the immunological synapse, without further diffusion. Finally, in the course of infection, TNF production by innate immune cells may be downregulated, thus leaving TNF produced by T cells indispensable for the further recruitment and activation of inflammatory cells. Additional studies are needed to shed more light on the mechanisms of T-cell-derived TNF function in the resistance to intracellular pathogen. One might expect that the role of TNF from T cells is even more pronounced in long-term infections, as was recently shown for mycobacteria (Saunders et al., 2004).

TNF has been clearly implicated in the pathogenesis of several autoimmune diseases, such as autoimmune diabetes, RA, psoriasis, and encephalomyelitis, often causing deleterious effects. In humans, continuous TNF blockade by injectable TNF inhibitors is the widely accepted treatment of autoimmune disease, such as RA (Feldmann, 2002) and psoriasis. How efficiently TNF produced by each individual subset of leukocytes in vivo can be blocked during these treatments remains to be determined.

In an experimental model of autoimmune liver disease, ConA-induced hepatitis, TNF is a critical mediator of inflammation and liver destruction (Leist et al., 1996). Liver macrophages, T cells, NK cells, and neutrophils were found to be important for the development of hepatitis onset (Muhlen et al., 2004; Schumann et al., 2000; Tiegs et al., 1992; Bonder et al., 2004), with membrane-bound TNF playing a particularly critical role (Maeda et al., 2003). We found that the deletion of the TNF gene either exclusively in T cells or exclusively in macrophages/neutrophils could lead to protection against ConA-induced liver toxicity (Figure 5). This finding leads to the conclusion that both TNF-producing cell populations contribute to liver damage. Since the depletion of Kuppfer macrophages protects mice from fulminant hepatitis and diminishes TNF production (Schumann et al., 2000), it is likely that the macrophage-derived TNF is critical for ConA-induced pathology. However, the contribution from neutrophil-derived TNF cannot be excluded (Bonder et al., 2004). Since ConA primarily targets T cells and not macrophages, our findings strongly argue that, in addition to its possible direct hepatotoxicity, T-cell-derived TNF participates in activation of macrophages. TNF can also induce upregulation of adhesion molecules, which is necessary for the recruitment of immune cells to the liver and for the development of hepatitis. Cooperation between T cells and liver macrophages during cocultivation in vitro resulted in the increased production of cytokines, particularly TNF, compared to what each of these cell types produced alone (Gantner et al., 1996). Interestingly, deregulation of TNF expression either in T cells or in macrophages in Crohn-like disease was also sufficient to induce organ inflammation and development of disease onset (Kontoyiannis et al., 2002).

Overall, this study has dissected some of the functions of TNF produced by individual types of leukocytes in vivo (Table 1). Our data demonstrate the distinct and nonredundant functions of TNF produced by macrophage/neutrophils and by T cells in autoimmune hepatitis, superantigen-induced shock, and resistance against intracellular pathogens (*Listeria*). Interestingly, no role in toxicity, host defense, or autoimmunity has been attributed so far to B-cell-derived TNF, although it was clearly implicated in the maintenance of the secondary lymphoid organs' structure (Endres et al., 1999; Wang et al., 2001; Tumanov et al., 2004). A panel of mice with targeted cell-type-specific inactivation of TNF will serve in the future for studying the role of TNF in the development of

various autoimmune disorders, including inflammatory bowel disease and arthritis.

Thus, individual subsets of immune cells can produce both "good" TNF, protecting against intracellular pathogens, and "bad" TNF, contributing to autoimmune destruction of tissues. Nevertheless, our data strongly imply that if blockade of TNF production is restricted to T cells, it may neutralize deleterious effects associated with autoimmune disease while still retaining most of protective functions necessary for host defense (Table 1). Given that the kinetics and absolute levels of TNF production are controlled differently in macrophages, T cells, and other cell types (both at transcriptional and posttranscriptional levels), it is likely that future therapeutic strategies using these differences to inhibit TNF made by T cells may find more beneficial clinical applications compared to systemic TNF blockade.

## Experimental Procedures

### Generation of TNF "Floxed" Mice

In order to generate mice for conditional TNF inactivation, correctly targeted ES cell clone #6537 (see Supplemental Experimental Procedures for details) was transiently transfected with the pIC-Cre vector. Nine neomycin-sensitive clones that have lost the neo-cassette but retained intact TNF gene were selected by PCR and Southern analysis. Several of these clones were injected into C57/Bl6 blastocysts, chimeric mice were backcrossed to C57BL/6, and germline transmission was detected by PCR using KO41 (5′-TGAGT CTGTCTTAACTAACC) and KO42 (5′-CCCTTCATTCTCAAGGCACA) primers and Southern analysis. Progeny with germline transmission were intercrossed to obtain homozygous $TNF^{flox/flox}$ mice (see Supplemental Experimental Procedures for details). TNF "floxed" mice were then crossed to cell-type-specific Cre deleters (Mlys1-Cre, CD19-Cre, or CD4-Cre), and F2 mice were further backcrossed to C57BL/6 mice at least four times. For all experiments except splenic histology, both C57BL/6 and littermate TNF floxed mice were used as wild-type controls.

### Challenges

For LPS challenge, mice were injected intraperitoneally with indicated amounts of LPS (*E. coli*-serovar 055:B5 Sigma, St Louis, MO) in sterile PBS, and sera were collected 1.5 hr after challenge. For LPS/D-Gal-induced shock or LPS septic shock, mice were injected i.p. with indicated amounts of LPS in combination with D-Gal (Sigma, St. Louis, MO) 20 mg/mouse or alone when indicated. Mice were monitored hourly and sacrificed when moribund. *S. aureus* enterotoxin B (Sigma) was injected i.v. with 20 mg of D-Gal. To induce ConA-mediated hepatitis, freshly prepared Concanavalin A (Sigma, St. Louis, MO) in sterile saline was injected i.v. (15 mg/kg body weight) as described (Tiegs et al., 1992).

### Bacteria and Infection

*Listeria monocytogenes* EGD Sv1/2a, LO 28, and ActA-deficient mutant (Kocks et al., 1992) were used for the infection. After passage through a host, the stock of bacteria ($10^9$ cfu [colony-forming units]/ml) was prepared. Aliquots were kept at −80°C in PBS containing 20% glycerol and thawed once before the experiment. Mice were injected either i.v. (tail vein) or i.p. with the indicated amounts of bacteria in sterile PBS, monitored twice a day, and sacrificed when moribund. Heat-killed *Listeria monocytogenes* (HKLM) were obtained by heating the regular stock of *Listeria* at 56°C for 2 hr.

### Cultures of Bone-Marrow-Derived Macrophages and DC

BMDM were produced as described (Muller et al., 1996). After 10 days of incubation, adherent cells were taken for the stimulation or lysed in proteinase K buffer for subsequent DNA isolation and Southern analysis. BMDC were produced as described (Abe et al., 2003).

To assess the cytokine production, BMDC and BMDM ($10^6$ cells/ml) were stimulated in 96-well plates with LPS (100 ng/ml; Sigma), bacterial lipoprotein (BLP) (500 ng/ml), CpG (1 ng/ml), or HKLM (moi 100:1).

### RNA Analysis

RNA from infected organs was prepared using Tri-Reagent (Sigma), then treated with DNase I (Promega) and reversely transcribed using ImProm-II Reverse Transcription System (Promega). For Taqman real-time PCR analysis of MCP-1, the following oligonucleotides were used: forward primer 5′-CTTCTGGGCCTGCTGTTCA, reverse primer 5′-CCAGCCTACTCATTGGGATCA′, probe 5′-FAM CTCAGC CAGATGCAGTTAACGCCCC-TAMRA.

### Immunohistochemistry

Immunohistochemistry was performed on frozen sections of 7 μm thickness, fixed in acetone as previously described (Tumanov et al., 2002). See Supplemental Experimental Procedures for the list of primary rat anti-mouse monoclonal antibodies used. For routine histology, tissues were fixed in 10% neutral formalin, embedded into paraffin, and 5 μm sections were prepared and stained with hematoxilin and eosin.

### Flow Cytometry and Intracellular Cytokine Staining

Intracellular cytokine staining using IC kit (eBioscience) and surface marker staining was performed according to the manufacturer's recommendations (see Supplemental Experimental Procedures).

## Acknowledgments

We thank M. Anver, E. Southon, M. Abshari, V. Yeremeev, M. Drutskaya, J. Goya, M. Lagarkova, E. Koltsova, and R. Kazaryan for their valuable assistance at different stages of this study and T. Stull for expert assistance with animal breeding. We are grateful to C. Wilson and H. Harowitz for CD4-Cre mice, R. Rickert for CD19-Cre mice, H. Gu for reagents and advice, and Y.-X. Fu for the gift of LTßR-Ig. We thank the personnel of Laboratory Animal Breeding Center, Branch of Schemyakin and Ovchinnikov Institute of Bioorganic Chemistry for expert help with mouse breeding and production. Finally, we are indebted to K. Rajewsky, W. Murphy, J. Keller, V. Quesniaux, F. Brombacher, and A. Apt for helpful discussions and to A. Chervonsky, R. Schreiber, S. Vogel, A. Rudensky, S. Durum, V. Yeremeev, R. Medzhitov, T. Sayers, J. Ashwell, and J. Oppenheim for critical comments on the manuscript.

This project has been funded in part by MCB grants from the Russian Academy of Sciences, Russian Foundation for Basic Research (grants 03-04-49097-a and 03-04-49083-a), and with US Federal funds from the National Cancer Institute, National Institutes of Health under Contract NO1-CO-12400. S.I.G. was supported by INTAS grant YSF 2002-0235. S.A.N. is International Research Scholar of the Howard Hughes Medical Institute.

The contents of this publication do not necessarily reflect the view or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.



Received: July 25, 2004
Revised: November 6, 2004
Accepted: November 24, 2004
Published: January 25, 2005

## References

Abe, K., Yarovinsky, F.O., Murakami, T., Shakhov, A.N., Tumanov, A.V., Ito, D., Drutskaya, L.N., Pfeffer, K., Kuprash, D.V., Komschlies, K.L., and Nedospasov, S.A. (2003). Distinct contributions of TNF and LT cytokines to the development of dendritic cells in vitro and their recruitment in vivo. Blood *101*, 1477–1483.

Anderson, M.R., and Tary-Lehmann, M. (2001). Staphylococcal enterotoxin-B-induced lethal shock in mice is T-cell-dependent, but

disease susceptibility is defined by the non-T-cell compartment. Clin. Immunol. *98*, 85–94.

Baeten, D., Kruithof, E., Van den Bosch, F., Van den Bossche, N., Herssens, A., Mielants, H., De Keyser, F., and Veys, E.M. (2003). Systematic safety follow up in a cohort of 107 patients with spondyloarthropathy treated with infliximab: a new perspective on the role of host defence in the pathogenesis of the disease? Ann. Rheum. Dis. *62*, 829–834.

Beutler, B., and Cerami, A. (1989). The biology of cachectin/TNF—a primary mediator of the host response. Annu. Rev. Immunol. *7*, 625–655.

Bhardwaj, V., Kanagawa, O., Swanson, P.E., and Unanue, E.R. (1998). Chronic Listeria infection in SCID mice: requirements for the carrier state and the dual role of T cells in transferring protection or suppression. J. Immunol. *160*, 376–384.

Bonder, C.S., Ajuebor, M.N., Zbytnuik, L.D., Kubes, P., and Swain, M.G. (2004). Essential role for neutrophil recruitment to the liver in concanavalin A-induced hepatitis. J. Immunol. *172*, 45–53.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA *72*, 3666–3670.

Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Forster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. *8*, 265–277.

DiTirro, J., Rhoades, E.R., Roberts, A.D., Burke, J.M., Mukasa, A., Cooper, A.M., Frank, A.A., Born, W.K., and Orme, I.M. (1998). Disruption of the cellular inflammatory response to Listeria monocytogenes infection in mice with disruptions in targeted genes. Infect. Immun. *66*, 2284–2289.

Edelson, B.T., and Unanue, E.R. (2000). Immunity to Listeria infection. Curr. Opin. Immunol. *12*, 425–431.

Endres, R., Alimzhanov, M.B., Plitz, T., Futterer, A., Kosco-Vilbois, M.H., Nedospasov, S.A., Rajewsky, K., and Pfeffer, K. (1999). Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. J. Exp. Med. *189*, 159–168.

Faust, N., Varas, F., Kelly, L.M., Heck, S., and Graf, T. (2000). Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. Blood *96*, 719–726.

Feldmann, M. (2002). Development of anti-TNF therapy for rheumatoid arthritis. Nat. Rev. Immunol. *2*, 364–371.

Feldmann, M., Brennan, F.M., Elliott, M.J., Williams, R.O., and Maini, R.N. (1995). TNF alpha is an effective therapeutic target for rheumatoid arthritis. Ann. N Y Acad. Sci. *766*, 272–278.

Flynn, J.L., Goldstein, M.M., Chan, J., Triebold, K.J., Pfeffer, K., Lowenstein, C.J., Schreiber, R., Mak, T.W., and Bloom, B.R. (1995). Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity *2*, 561–572.

Gantner, F., Leist, M., Kusters, S., Vogt, K., Volk, H.D., and Tiegs, G. (1996). T cell stimulus-induced crosstalk between lymphocytes and liver macrophages results in augmented cytokine release. Exp. Cell Res. *229*, 137–146.

Giroir, B.P., Johnson, J.H., Brown, T., Allen, G.L., and Beutler, B. (1992). The tissue distribution of tumor necrosis factor biosynthesis during endotoxemia. J. Clin. Invest. *90*, 693–698.

Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., and Pfizenmaier, K. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell *83*, 793–802.

Harty, J.T., and Bevan, M.J. (1995). Specific immunity to Listeria monocytogenes in the absence of IFN gamma. Immunity *3*, 109–117.

Kassiotis, G., and Kollias, G. (2001). Uncoupling the proinflammatory from the immunosuppressive properties of tumor necrosis factor (TNF) at the p55 TNF receptor level: implications for pathogenesis and therapy of autoimmune demyelination. J. Exp. Med. *193*, 427–434.

Kaufmann, S.H. (1993). Immunity to intracellular bacteria. Annu. Rev. Immunol. *11*, 129–163.

Kinkhabwala, M., Sehajpal, P., Skolnik, E., Smith, D., Sharma, V.K., Vlassara, H., Cerami, A., and Suthanthiran, M. (1990). A novel addition to the T cell repertory. Cell surface expression of tumor necrosis factor/cachectin by activated normal human T cells. J. Exp. Med. *171*, 941–946.

Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H., and Cossart, P. (1992). L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell *68*, 521–531.

Kollias, G., Douni, E., Kassiotis, G., and Kontoyiannis, D. (1999). On the role of tumor necrosis factor and receptors in models of multiorgan failure, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. Immunol. Rev. *169*, 175–194.

Kontoyiannis, D., Boulougouris, G., Manoloukos, M., Armaka, M., Apostolaki, M., Pizarro, T., Kotlyarov, A., Forster, I., Flavell, R., Gaestel, M., et al. (2002). Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. J. Exp. Med. *196*, 1563–1574.

Korner, H., Cook, M., Riminton, D.S., Lemckert, F.A., Hoek, R.M., Ledermann, B., Kontgen, F., Fazekas de St Groth, B., and Sedgwick, J.D. (1997). Distinct roles for lymphotoxin-alpha and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue. Eur. J. Immunol. *27*, 2600–2609.

Kurihara, T., Warr, G., Loy, J., and Bravo, R. (1997). Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. J. Exp. Med. *186*, 1757–1762.

Lee, P.P., Fitzpatrick, D.R., Beard, C., Jessup, H.K., Lehar, S., Makar, K.W., Perez-Melgosa, M., Sweetser, M.T., Schlissel, M.S., Nguyen, S., et al. (2001). A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity *15*, 763–774.

Leist, M., Gantner, F., Kunstle, G., Bohlinger, I., Tiegs, G., Bluethmann, H., and Wendel, A. (1996). The 55-kD tumor necrosis factor receptor and CD95 independently signal murine hepatocyte apoptosis and subsequent liver failure. Mol. Med. *2*, 109–124.

Maeda, S., Chang, L., Li, Z.W., Luo, J.L., Leffert, H., and Karin, M. (2003). Ikk$\beta$ is required for prevention of apoptosis mediated by cell-bound but not by circulating TNFalpha. Immunity *19*, 725–737.

Marino, M.W., Dunn, A., Grail, D., Inglese, M., Noguchi, Y., Richards, E., Jungbluth, A., Wada, H., Moore, M., Williamson, B., et al. (1997). Characterization of tumor necrosis factor-deficient mice. Proc. Natl. Acad. Sci. USA *94*, 8093–8098.

Medzhitov, R., and Janeway, C.A.J. (1997). Innate immunity: the virtues of a nonclonal system of recognition. Cell *91*, 295–298.

Mohan, A.K., Cote, T.R., Siegel, J.N., and Braun, M.M. (2003). Infectious complications of biologic treatments of rheumatoid arthritis. Curr. Opin. Rheumatol. *15*, 179–184.

Muhlen, K.A., Schumann, J., Wittke, F., Stenger, S., van Rooijen, N., Van Kaer, L., and Tiegs, G. (2004). NK cells, but not NKT cells, are involved in Pseudomonas aeruginosa exotoxin A-induced hepatotoxicity in mice. J. Immunol. *172*, 3034–3041.

Muller, M., Eugster, H.P., Le Hir, M., Shakhov, A., di Padova, F., Maurer, C., Quesniaux, V.F., and Ryffel, B. (1996). Correction or transfer of immunodeficiency due to TNF-LT alpha deletion by bone marrow transplantation. Mol. Med. *2*, 247–255.

Olleros, M.L., Guler, R., Corazza, N., Vesin, D., Eugster, H.P., Marchal, G., Chavarot, P., Mueller, C., and Garcia, I. (2002). Transmembrane TNF induces an efficient cell-mediated immunity and resistance to Mycobacterium bovis bacillus Calmette-Guerin infection in the absence of secreted TNF and lymphotoxin-alpha. J. Immunol. *168*, 3394–3401.

Papadakis, K.A., and Targan, S.R. (2000). Tumor necrosis factor: biology and therapeutic inhibitors. Gastroenterology *119*, 1148–1157.

Pasparakis, M., Alexopoulou, L., Episkopou, V., and Kollias, G. (1996). Immune and inflammatory responses in TNF alpha-deficient

mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J. Exp. Med. *184*, 1397–1411.

Pasparakis, M., Kousteni, S., Peschon, J., and Kollias, G. (2000). Tumor necrosis factor and the p55TNF receptor are required for optimal development of the marginal sinus and for migration of follicular dendritic cell precursors into splenic follicles. Cell. Immunol. *201*, 33–41.

Pfeffer, K., Matsuyama, T., Kundig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Kronke, M., and Mak, T.W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457–467.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Rickert, R.C., Roes, J., and Rajewsky, K. (1997). B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res. *25*, 1317–1318.

Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. (1993). Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature *364*, 798–802.

Ruuls, S.R., Hoek, R.M., Ngo, V.N., McNeil, T., Lucian, L.A., Janatpour, M.J., Korner, H., Scheerens, H., Hessel, E.M., Cyster, J.G., et al. (2001). Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. Immunity *15*, 533–543.

Sass, G., Koerber, K., Bang, R., Guehring, H., and Tiegs, G. (2001). Inducible nitric oxide synthase is critical for immune-mediated liver injury in mice. J. Clin. Invest. *107*, 439–447.

Saunders, B.M., Briscoe, H., and Britton, W.J. (2004). T cell-derived tumour necrosis factor is essential, but not sufficient, for protection against Mycobacterium tuberculosis infection. Clin. Exp. Immunol. *137*, 279–287.

Schumann, J., Wolf, D., Pahl, A., Brune, K., Papadopoulos, T., van Rooijen, N., and Tiegs, G. (2000). Importance of Kupffer cells for T-cell-dependent liver injury in mice. Am. J. Pathol. *157*, 1671–1683.

Sedgwick, J.D., Riminton, D.S., Cyster, J.G., and Korner, H. (2000). Tumor necrosis factor: a master-regulator of leukocyte movement. Immunol. Today *21*, 110–113.

Serbina, N.V., Kuziel, W., Flavell, R., Akira, S., Rollins, B., and Pamer, E.G. (2003). Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection. Immunity *19*, 891–901.

Tiegs, G., Hentschel, J., and Wendel, A. (1992). A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J. Clin. Invest. *90*, 196–203.

Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F., and Cerami, A. (1987). Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature *330*, 662–664.

Tsytsykova, A.V., and Goldfeld, A.E. (2000). Nuclear factor of activated T cells transcription factor NFATp controls superantigen-induced lethal shock. J. Exp. Med. *192*, 581–586.

Tumanov, A.V., Kuprash, D.V., Lagarkova, M.A., Grivennikov, S.I., Abe, K., Shakhov, A.N., Drutskaya, L.N., Stewart, C., Chervonsky, A.V., and Nedospasov, S.A. (2002). Distinct role of surface lymphotoxin expressed by B cells in the organization of secondary lymphoid tissues. Immunity *17*, 239–250.

Tumanov, A.V., Kuprash, D.V., Mach, J.A., Nedospasov, S.A., and Chervonsky, A.V. (2004). Lymphotoxin and TNF produced by B cells are dispensable for maintenance of the follicle-associated epithelium but are required for development of lymphoid follicles in the Peyer's patches. J. Immunol. *173*, 86–91.

Unanue, E.R. (1997). Inter-relationship among macrophages, natural killer cells and neutrophils in early stages of Listeria resistance. Curr. Opin. Immunol. *9*, 35–43.

Wang, Y., Wang, J., Sun, Y., Wu, Q., and Fu, Y.X. (2001). Complementary effects of TNF and lymphotoxin on the formation of germinal center and follicular dendritic cells. J. Immunol. *166*, 330–337.

Xu, L., Yoon, H., Zhao, M.Q., Liu, J., Ramana, C.V., and Enelow, R.I. (2004). Cutting edge: pulmonary immunopathology mediated by antigen-specific expression of TNF-alpha by antiviral CD8(+) T cells. J. Immunol. *173*, 721–725.