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Divergent effects of tumor necrosis factor-alpha and lymphotoxin-alpha on lethal endotoxemia and infection with live Salmonella typhimurium in mice

Abstract

During septic shock with Gram-negative microorganisms, mortality is determined by two independent factors: high concentrations of circulating proinflammatory cytokines and multiplication of the microorganisms in the organs of the host. We studied the role of endogenous tumor necrosis factor-

alpha (TNF) and lymphotoxin-alpha (LT) in the pathogenesis of lethal endotoxemia and infection with viable Salmonella typhimurium. Compared to wild-type control mice, TNF-/-LT-/- knock-out mice were more resistant (100% versus 25% mortality) to a lethal challenge with LPS, due to a significantly decreased production of the proinflammatory cytokines TNF, IL-1alpha and IL-1beta. In contrast, TNF-/-LT-/- mice were highly susceptible to infection with viable S. typhimurium as compared to wild-type mice (100% versus 0% mortality), and this was accompanied by a 100-fold greater bacterial load in their organs. The effect of endogenous TNF and LT during infection was mediated by a defective recruitment of neutrophils at the site of infection, as well as a reduced intracellular killing of S. typhimurium by these cells. These results show that TNF and LT have crucial, yet opposite effects on lethal endotoxemia induced by S. typhimurium LPS and on the infection of mice with live Salmonella microorganisms, and suggest caution when extrapolating results obtained in the lethal endotoxemia model to bacteremia in patients.

Full text

INTRODUCTION

Typhoid fever, a systemic disease caused by *Salmonella typhi* or *S. paratyphi*, is still one of the major health problems throughout the world, particularly in developing countries. The host defense mechanisms against *Salmonella* species, an intracellular pathogen, are complex and involve both innate and adaptive immunity. Mononuclear phagocytes play a central role in the control of *Salmonella* infections, after activation of these cells through a T-helper 1 type immune response, involving a network of cytokines, including tumor necrosis factor (TNF)-alpha, interleukin (IL)-2, IL-12, IL-18, and interferon (IFN)-gamma [1]. However, at the early stages of infection, before recruitment and activation of mononuclear phagocytes has occurred, granulocytes are critical for anti-*Salmonella* defense [2].

Tumor necrosis factor-alpha (TNF) and lymphotoxin-alpha (LT) are members of the TNF family of cytokines [3]. Although they have different structures, both TNF and LT bind to the same TNF receptors, induce overlapping intracellular pathways, and lead to similar biological effects [3]. On the one hand, these cytokines are considered to be potentially deleterious mediators of the inflammatory responses that occur during sepsis [4], and anti-TNF antibodies

protect against lethal endotoxemia [5] and intravenous Gram-negative infections in experimental models [6]. On the other hand, endogenous TNF production is required for the normal immune response against intracellular microorganisms, as has been demonstrated in various experimental models [7-12]. TNF also has important roles in the host defense against systemic *Salmonella* infection. It has been shown that *S. typhimurium* is able to induce the production of TNF both *in vitro* and *in vivo* [13-15], and neutralization of endogenous TNF by blocking antibodies has deleterious effects on the course of experimental *S. typhimurium* infection in mice [13, 16, 17]. However, the exact mechanism of TNF-mediated protection in salmonellosis is not clear. The objective of the present study was to investigate the role of endogenous TNF and LT in two models of sepsis: the lethal LPS challenge, leading to overwhelming cytokine release and septic shock, and experimental infection with viable *S. typhimurium*, in which bacterial dissemination and growth leads to organ damage. These models were investigated in mice lacking the genes for both TNF and LT ($TNF^{-/-}LT^{-/-}$).

MATERIALS AND METHODS

Mice

Both $TNF^{-/-}LT^{-/-}$ and $TNF^{+/+}LT^{+/+}$ mice have a C57Bl/6J/129sv genetic background. Female homozygous $TNF^{-/-}LT^{-/-}$ mice and $TNF^{+/+}LT^{+/+}$ control mice (20-25 g, 6-8 weeks old) were produced as previously described [18]. Briefly, the fragment +733 to +5,839 of the TNF/LT locus was replaced by a neo cassette (pMCI NeopA) in 129/sv mice. This deletion encompasses all of the LT gene and ends at the TNF exon 4 at codon 13. Mice homozygous for the deletion and wild-type littermates were derived from crosses between C57Bl/6 and 129sv mice [18]. Animals were fed standard laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*, and were housed under specific pathogen-free conditions.

Endotoxemia model

Groups of $TNF^{-/-}LT^{-/-}$ and $TNF^{+/+}LT^{+/+}$ mice were injected i.p. with 1.5 mg/mouse of LPS from *S. typhimurium* (Sigma Chemical Co., St. Louis, MO, USA). Survival was assessed over one week, in groups of 10 mice.

Salmonella typhimurium *infection*

A serum-resistant strain of *S. typhimurium* (phage type 510) described earlier [19] was grown to stationary phase culture by overnight incubation at 37° C in nutrient broth (BHI Oxoid). This strain has an LD50 of < 100 cfu in a reference lty S C57Bl/6 mouse strain [19]. Mice were injected intraperitoneally (ip) with 50 cfu of *S. typhimurium* in 100 muL of pyrogen-free saline. Survival was assessed twice daily for 28 days. A subgroup of animals (5 mice per strain per time point) was killed by cervical dislocation under ether anesthesia on days 1 and 3 of infection. Blood was collected on EDTA for plasma cytokine concentration measurements. For the assessment of the outgrowth of the microorganisms in the liver, spleen, and bone marrow, the organs were aseptically removed, weighed and homogenized in sterile saline using a tissue grinder. The number of viable *S. typhimurium* cells in the organs was determined by plating serial dilutions on blood agar plates. Colony forming units were counted after overnight incubation at 37° C. The results were expressed as the log cfu per gram of tissue.

Recruitment of neutrophils

To investigate the recruitment of PMN at the site of the *Salmonella* infection, groups of 5 $TNF^{-/-}LT^{-/-}$ mice and wild-type littermates were injected i.p. with 10^7 cfu *S. typhimurium*. After 0, 2, 4, and 24 hours, peritoneal cells were collected in sterile saline containing 0.38% sodium citrate, and the total cell number was counted in a hemacytometer. The percentage and the absolute numbers of neutrophils were determined in Giemsa-stained cytocentrifuge preparations.

Phagocytosis and killing of S. typhimurium

To obtain peritoneal exudate neutrophils, groups of 5 $TNF^{-/-}LT^{-/-}$ mice and wild-type littermates were injected i.p. with 1 ml of 10% proteose peptone, and after 4 hours the exudate cells were collected in separate sterile tubes as previously described [20]. Four hours after injection of proteose peptone, the peritoneal cell population consisted of 86-97% PMN, 3-12% macrophages, and 1-5% lymphocytes. After centrifugation and washing, the cells were resuspended at 1 x 10^6 cells/ml in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, and 100 mug/ml gentamicin (culture medium) in 1.5 mL polypropylene tubes. An equal volume containing 1 x 10^7 cfu of *S. typhimurium* was added to the cell suspension (effector to target ratio 1:10), and incubated at 37° C under continous rotation.

To assess phagocytosis after 15 min of incubation, the samples were centrifuged (1,500 x g, 5 min). The supernatant containing the uningested microorganisms was discarded, and the cells were washed twice in culture medium. Lysis of the phagocytes was performed in water containing 0.01% bovine serum albumin (Sigma Chemical Co, St. Louis, MO, USA). Serial dilutions of the suspension containing the intracellular organisms were plated on blood agar plates, and the *S. typhimurium* cfu were counted after 24 hours of incubation. To assess intracellular killing of *S. typhimurium* by PMN, the cell suspension obtained after the phagocytosis step as described above, was reincubated in culture medium at 37° C under continous rotation for an additional 60 min. After lysis of the phagocytes, *S. typhimurium* cfu were enumerated after 24 hours of culture on blood agar plates.

Superoxide production

PMN from $TNF^{-/-}LT^{-/-}$ and normal mice were obtained 4 hours after i.p. injection of 1 ml of 10% proteose peptone, and cells were collected as described above. Luminol-enhanced chemiluminescence of proteose-peptone-elicited PMN was measured on a Victor 1420 counter (Wallac, Turku, Finland) at 20° C, using white, 96-well microplates (Costar Corporation, Cambridge, MA, USA), as previously described [10]. Each well contained 2×10^5 cells, 50 muM luminol, 4.5 U/ml horseradish peroxidase and 50 ng/ml phorbol 12-myristate 13-acetate (PMA) in 200 mul of Hanks balanced salt solution without phenol red (Gibco Life Technologies, Paisley, Scotland), supplemented with 0.25% human albumin (Behringwerke, Marburg, Germany). Reactions were started by adding PMA. Each experiment was performed in duplicate. Hrp was added to the system in order to overcome peroxidase deficiency extracellularly [10]. The chemiluminescence was expressed as the total amount of superoxide produced during the assay period by integrating the area under the curve (in mV.s) per PMN.

Ex vivo *cytokine production*

Uninfected $TNF^{-/-}LT^{-/-}$ mice and wild-type controls were sacrificed, and resident peritoneal macrophages were harvested by injecting 4 ml of sterile PBS containing 0.38% sodium citrate [21]. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100 mug/ml gentamicin and 2% fresh mouse plasma (culture medium). Cells

were cultured in 96-well microtiter plates (Costar) at 10^5 cells/well, in a final volume of 200 mul. The cells were stimulated with either 1 ng/ml lipopolysaccharide (LPS, *E. coli* serotype O55:B5 or *S. typhimurium*, Sigma), or heat-killed (1 hour, 100° C) *S. typhimurium* 10^7 cfu/ml. After 24 hours of incubation at 37° C, the plates were centrifuged (500 g, 10 min), and the supernatant was collected and stored at - 80° C until cytokine assays were performed. To assess the cell-associated cytokines, 200 mul of culture medium was added to the remaining cells, and the membranes were disrupted by three freeze-thaw cycles. The samples were stored at - 80° C until measurements.

Cytokine assays

IL-1alpha, IL-1beta and TNF-alpha were determined by specific radioimmunoassays (detection limit 20 pg/ml), as previously described [10]. IL-6 was measured using a commercial ELISA (CLB, Amsterdam, The Netherlands; detection limit 16 pg/ml), as was IFN-gamma (Biosource, Camarillo, CA; detection limit 15 pg/ml), according to the guidelines of the manufacturer.

Statistical analysis

The differences between groups were analyzed by the Mann-Whitney U test, and where appropriate by the Kruskal-Wallis ANOVA test. Survival curves were analyzed by the Kaplan-Meyer log-rank test. The level of significance between groups was set at $p < 0.05$.

RESULTS

Survival of $TNF^{-/-}LT^{-/-}$ mice during endotoxemia

To assess the role of TNF in mediating LPS toxicity, mice deficient in TNF and LT, and wild-type controls were injected with *S. typhimurium* LPS. Death occurred during the first 5 days after LPS challenge, with no further deaths thereafter.

Injection of LPS into $TNF^{+/+}LT^{+/+}$ control mice resulted in 100% mortality, whereas only 25% mortality was observed in $TNF^{-/-}LT^{-/-}$ mice ($p < 0.05$). Thus, $TNF^{-/-}LT^{-/-}$ mice were significantly more resistant to *S. typhimurium* LPS, underscoring the deleterious role of TNF and/or LT in overwhelming endotoxemia.

Cytokine production by peritoneal macrophages in vitro

To test whether these differences in survival were due to a different cytokine production of mononuclear cells in the two mouse strains, resident peritoneal macrophages from $TNF^{-/-}LT^{-/-}$ and $TNF^{+/+}LT^{+/+}$ mice were stimulated with 1 ng/ml LPS of either *E. coli* serotype O55:B5 or *S. typhimurium*, or with 10^7 cfu heat-killed *S. typhimurium in vitro* for 24 hours. Macrophages obtained from $TNF^{-/-}LT^{-/-}$ mice did not produce TNF upon stimulation with LPS or heat-killed *Salmonella*, and moreover they synthesized significantly lower amounts of IL-1alpha, IL-1beta and IL-6 than macrophages from $TNF^{+/+}LT^{+/+}$ animals did upon stimulation with heat-killed *S. typhimurium* (Figure 1). Similarly, significantly reduced stimulation of IL-1alpha, IL-1beta and IL-6 was found upon stimulation of $TNF^{-/-}LT^{-/-}$ macrophages with LPS from either *E. coli* or *S. typhimurium* (data not shown). This is in agreement with earlier observations with stimulation with LPS or whole microorganisms *in vitro* [10].

Salmonella typhimurium *infection of $TNF^{-/-}LT^{-/-}$ and $TNF^{+/+}LT^{+/+}$ mice*

In contrast to lethal endotoxemia in which overwhelming production of TNF is responsible for lethality, TNF is assumed to be required for containment of the microorganisms during infection with live bacteria. To test whether this is also the case during *S. typhimurium* infection, $TNF^{-/-}LT^{-/-}$ and $TNF^{+/+}LT^{+/+}$mice were injected with a potentially lethal amount of viable *S. typhimurium* bacteria. After injection of 50 *S. typhimurium* cfu, all $TNF^{-/-}LT^{-/-}$ mice died between day 2 and day 5 of infection, whereas 100% of control mice survived beyond day 28 ($p < 0.01$; Figure 2).

The increased lethality in the $TNF^{-/-}LT^{-/-}$ mice was probably due to the load of *S. typhimurium* in their organs, since the numbers of cfu on day 1 and 3 post-infection were significantly higher in the liver, the spleen, and the bone marrow of $TNF^{-/-}LT^{-/-}$ mice than in those of $TNF^{+/+}LT^{+/+}$ mice ($p < 0.05$; Figure 3).

Circulating cytokines

Circulating concentrations of TNF-alpha, IL-alpha, IL-1beta, IL-6 and IFN-gamma were determined on day 1 and day 3 of infection. TNF concentrations were undetectable in the circulation of $TNF^{-/-}LT^{-/-}$ mice, and were 114 ± 183 pg/ml (mean ± SD, day 1) and 171 ± 206 pg/ml (day 3) in the $TNF^{+/+}LT^{+/+}$ mice. IL-1alpha

and IL-1beta were below the detection limit at all time points. Peak IL-6 blood concentrations were observed 1 day after the infection, with levels significantly higher in $TNF^{-/-}LT^{-/-}$ (26.4 ± 32.7 ng/ml) than in $TNF^{+/+}LT^{+/+}$ mice (3.1 ± 5.9 ng/ml, $p < 0.01$). On day 3, the circulating IL-6 concentrations had decreased considerably, but still tended to be higher in $TNF^{-/-}LT^{-/-}$ (3.4 ± 1.4 ng/ml) than in $TNF^{+/+}LT^{+/+}$ mice (2.9 ± 4.0 ng/ml, $p > 0.05$). IFN-gamma concentrations were undetectable on day 1. On day 3, circulating IFN-gamma tended to be higher in the $TNF^{-/-}LT^{-/-}$ (150 ± 92 ng/ml) than in $TNF^{+/+}LT^{+/+}$ mice (62 ± 88 ng/ml, $p > 0.05$).

Recruitment of PMN

To investigate the recruitment of neutrophils at the site of a *S. typhimurium* infection, groups of $TNF^{-/-}LT^{-/-}$ and $TNF^{+/+}LT^{+/+}$ mice were injected i.p. with 10^7 cfu of *S. typhimurium*, and exudate peritoneal neutrophils were harvested and counted 0, 4, 8, and 24 hours later. There was significantly less infiltration of neutrophils in the peritoneal cavity of $TNF^{-/-}LT^{-/-}$ than in that of $TNF^{+/+}LT^{+/+}$ mice. Four hours after injection of *S. typhimurium* cfu, the number of neutrophils peaked in $TNF^{+/+}LT^{+/+}$ mice ($4.7 \pm 3.6 \times 10^6$/ml). In $TNF^{-/-}LT^{-/-}$, the peak number of neutrophils was $0.8 \pm 0.6 \times 10^6$/ml ($p < 0.05$) after 4 hours. At 24 hours after injection of the inflammatory stimulus, the neutrophil infiltrate had disappeared in both mouse strains. Superoxide production by PMN from $TNF^{-/-}LT^{-/-}$ mice was similar to that by PMN from $TNF^{+/+}LT^{+/+}$ control mice (0.15 ± 0.04 *versus* 0.17 ± 0.07 mV.s/PMN, $p > 0.05$).

Phagocytosis and killing of S. typhimurium *by PMN*

The capacity of PMN to phagocytose and kill *S. typhimurium in vitro* was assessed using peritoneal exudate PMN elicited with proteose peptone. Phagocytosis of *S. typhimurium* by exudate PMN from $TNF^{-/-}LT^{-/-}$ mice was similar to that of $TNF^{+/+}LT^{+/+}$ control mice (9.4 ± 3.2% *versus* 8.2 ± 2.5%, $p > 0.05$). In contrast, PMN from $TNF^{+/+}LT^{+/+}$ control mice had killed all ingested *S. typhimurium* after 30 min of incubation, whereas the killing by PMN from $TNF^{-/-}LT^{-/-}$ mice was only 51 ± 33% ($p < 0.05$).

DISCUSSION

In the present study we show that TNF and LT have crucial, yet opposite effects on lethal endotoxemia induced by *S. typhimurium* LPS and on the infection of

mice with live *Salmonella* microorganisms. While $TNF^{-/-}LT^{-/-}$ mice were significantly protected against endotoxic shock induced by *S. typhimurium* LPS, they readily succumbed to an infection with very low numbers of viable *S. typhimurium* cfu, whereas the $TNF^{+/+}LT^{+/+}$ control C57Bl/6J/129sv mice resisted the infection. These data show that the pathogenesis of the systemic inflammatory response induced by LPS and that of bacteremia with live microorganisms are fundamentally different, and that TNF can have divergent effects in these clinical syndromes.

Lethal endotoxemia in mice has been extensively used as an experimental model of human septic shock. From the seminal studies of Beutler, it has become clear that TNF plays a central role in the pathogenesis of endotoxic shock, as anti-TNF antibodies protected mice against a lethal challenge with LPS. We and others have expanded these findings by showing a decreased susceptibility of $TNF^{-/-}$ $LT^{-/-}$ mice to a lethal challenge of *E. coli* LPS [20, 22]. In agreement with these data, in the present study we have shown that $TNF^{-/-}LT^{-/-}$ mice are also more resistant to *S. typhimurium* LPS. The lethality during endotoxic shock is mediated by overwhelming activation of the proinflammatory cytokine production, especially TNF and IL-1, leading to damage to the vascular endothelium, capillary leakage, hypotension, organ failure and ultimately, death. The $TNF^{-/-}LT^{-/-}$ mice are protected against these effects not only by the absence of TNF and LT, but also because of the subsequent defective production of other proinflammatory cytokines such as IL-1alpha and IL-1beta (Figure 1).

In contrast to the overwhelming endotoxemia models in which the "lethal cytokinemia" is responsible for the death of animals, bacterial invasion and outgrowth in the host organs is responsible for lethality in infection with live microorganisms. During infection, proinflammatory cytokines play an important role in the activation of the host defense for the recognition and elimination of the microorganisms, as has been shown for IFN-gamma [23], IL-12 [24], and IL-18 [24]. $TNF^{-/-}LT^{-/-}$ mice died earlier than control $TNF^{+/+}LT^{+/+}$ mice when challenged with live *S. typhimurium*, and this was caused by the increased burden of microorganisms in the organs of the deficient animals, both at days 1 and 3 of infection. All mice lacking TNF and LT died between day 2 and day 5 of infection with 50 cfu of *S. typhimurium*, whereas control mice with the capacity to produce TNF and LT survived. As expected, the circulating concentrations of TNF were undetectable in the knock-out mice. In contrast, the production of the antiinflammatory cytokine IL-6 was higher in $TNF^{-/-}LT^{-/-}$ mice compared to wild

type control mice both on days 1 and 3 of infection. This is probably due to the extensive outgrowth of *Salmonella* in the organs of the deficient mice, leading to a secondary cytokine response.

These findings are not surprising, since endogenous TNF has been shown to be crucial for host defense against a variety of live microorganisms, including *Mycobacterium* spp. [8], *Listeria monocytogenes* [7, 11], *Toxoplasma gondii* [11], and *Candida albicans* [10, 12]. In previous studies on experimental *S. typhimurium* infection in mice, employing neutralizing anti-TNF antibodies, the role of TNF was found to be protective. Inhibition of endogenous TNF during *Salmonella* infection worsened the outcome [25], whereas administration of recombinant TNF resulted in better survival [26, 27]. The exact mechanisms through which TNF mediates protection against *S. typhimurium* infection have only been partly elucidated. In the early phase of infection, the defense against *Salmonella* employs neutrophil recruitment, which both prevents extracellular colonization of the liver microvasculature by *S. typhimurium* [2] and induces bacterial killing through oxidative mechanisms [25, 28]. Later during infection, after approximately 5 to 7 days, macrophage accumulation, granuloma formation and NO-mediated killing is thought to be pivotal for bacterial elimination [28]. In our experiments, we have observed an early effect of the absence of TNF/LT on the outgrowth of *Salmonella* in the organs of knock-out mice. This early effect of TNF/LT is explained by a defective recruitment of neutrophils to the site of infection, as well as a reduced capacity to kill ingested *S. typhimurium*, despite normal phagocytic activity and normal synthesis of oxygen radicals. Together, these effects lead to extensive bacterial growth in the organs of the knock-out mice. Although NO-mediated mechanisms are exerted later during the infection, an additional effect of PMN/macrophage-derived NO on the effects of endogenous TNF/LT cannot be excluded.

CONCLUSION

In conclusion, $TNF^{-/-}LT^{-/-}$ mice are less susceptible to lethal endotoxemia induced by *S. typhimurium* LPS, and this is due to a strongly reduced production of all proinflammatory cytokines tested. In contrast, the $TNF^{-/-}LT^{-/-}$ mice die earlier than wild-type control mice when challenged with live *Salmonella*. The effect of endogenous TNF/LT were already apparent in the early phase of infection, and this appears to be due to a defective migration of neutrophils at the site of infection and their reduced bactericidal capacity. These data strongly

suggest caution when extrapolating results obtained in the lethal endotoxemia model to the situation found in humans with typhoid fever or related diseases.

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Figures

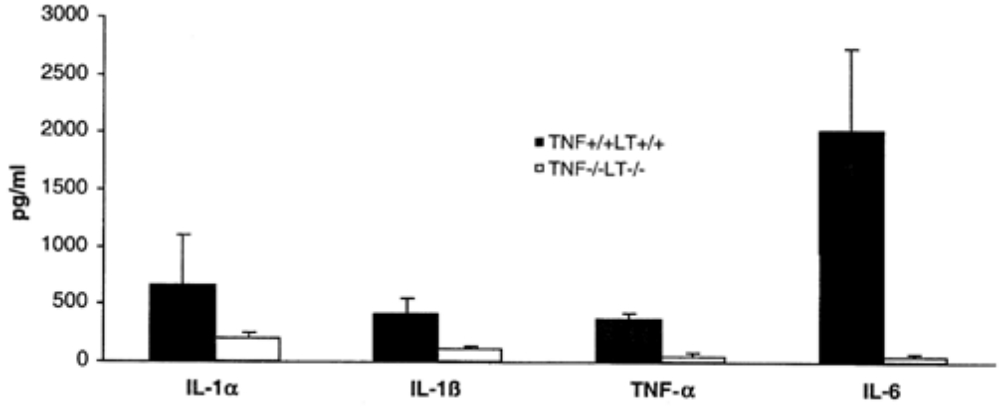


Figure 1
Production of cytokines *in vitro* by resident peritoneal macrophages of $TNF^{+/+}LT^{+/+}$ (solid bars) and $TNF^{-/-}LT^{-/-}$ mice (open bars) upon stimulation with 10^7 cfu/ml of heat-killed *S. typhimurium*. Data represent the mean ± SD of 5 mice. At all points shown, the difference between $TNF^{+/+}LT^{+/+}$ mice and $TNF^{-/-}LT^{-/-}$ mice was statistically significant ($p < 0.05$).

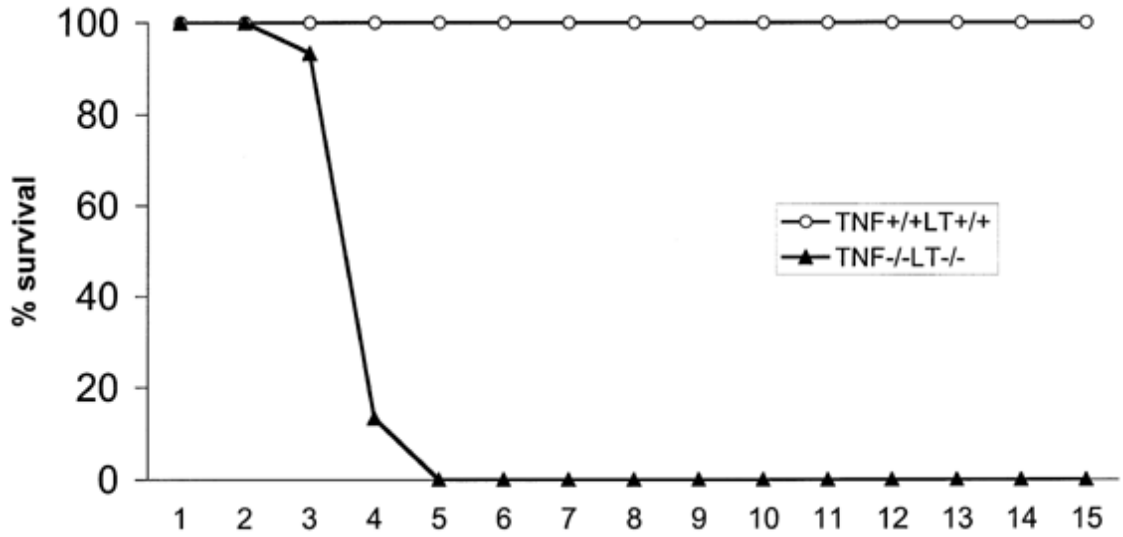


Figure 2
Survival of $TNF^{+/+}LT^{+/+}$ mice and $TNF^{-/-}LT^{-/-}$ mice after after i.p injection of 50 cfu of *S. typhimurium* in 14 animals per group.

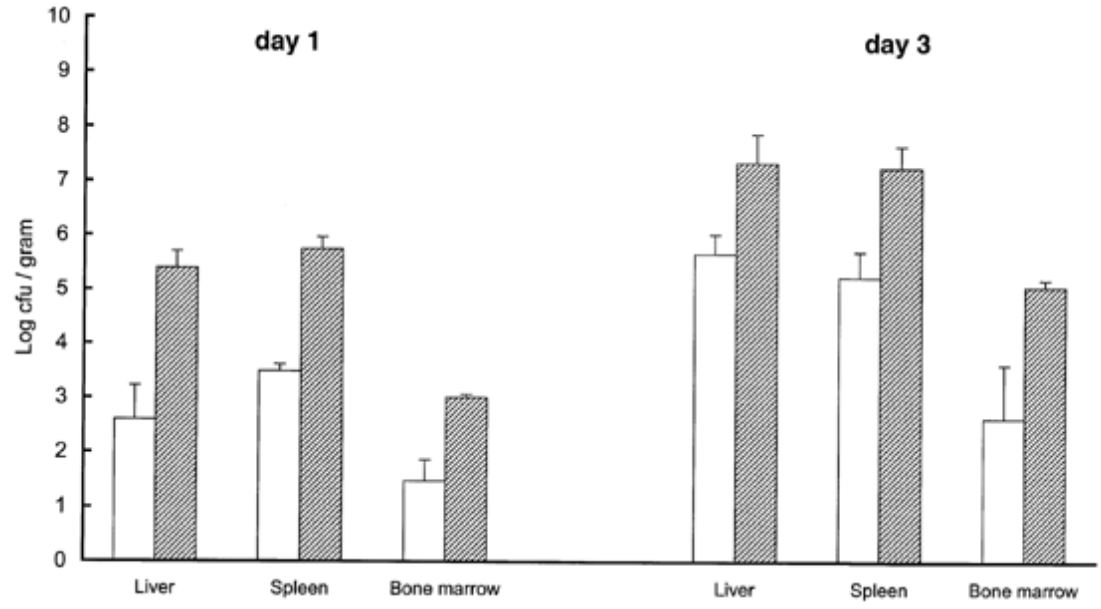


Figure 3
Growth of *S. typhimurium* in the organs of $TNF^{+/+}LT^{+/+}$ (open bars) and $TNF^{-/-}LT^{-/-}$ mice (hatched bars) on the day 1 and day 3 after i.p injection of 50 cfu of *S. typhimurium*. Data represent the mean ± SD of 5 mice. At all points shown, the difference between $TNF^{+/+}LT^{+/+}$ mice

and $TNF^{-/-}LT^{-/-}$ mice was statistically significant ($p < 0.05$).

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IL-10, IL-19, IL-20, IL-22, mda-7, AK155.

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NK cells, dendritic cells, cell-to-cell interaction, activation, proliferation.

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TNF, shock, acute phase, inflammation, alpha2-macroglobulin.

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tumor necrosis factor, mRNA stability, lipopolysaccharide, tristetraprolin.

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asthma, cerebrospinal fluid, juvenile idiopathic arthritis, sputum, synovial fluid, THP-1 cells.

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tumor necrosis factor, lymphotoxin, salmonella typhimunium, knock-out mice.

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CSF-1, tumour progression, metastasis, de-differentiation.

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J. Benjamin Dame, Nasser Chegini, Robert D. Christensen, Sandra E. Juul
IL-1beta, TNF-alpha, GM-CSF, neuron, mRNA, protein.

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