

A Mediator Role For Metallothionein in Tumor Necrosis Factor-induced Lethal Shock

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Abstract

Tumor necrosis factor (TNF) is a proinflammatory cytokine, which is centrally involved in several inflammatory disorders. Administration of TNF leads to a potentially lethal systemic inflammatory response syndrome (SIRS). We observed that (a) mice lacking functional genes for metallothionein 1 and 2 (MT-null) were protected compared with wild-type controls ($P = 0.0078$), and (b) mice overexpressing MT-1 (MT-TG) were more sensitized for the lethal effect of TNF than control mice ($P = 0.0003$), indicating a mediating role for MT in TNF induced SIRS. As MT is involved in the body zinc homeostasis, we tested whether zinc-deprivation or -supplementation alters the response to TNF. Although zinc-depletion strongly sensitized ($P = 0.036$), and pretreatment with zinc sulfate (ZnSO_4) conferred protection against the deleterious effects of TNF ($P < 0.0002$), it was also found that the protection provided by zinc is independent of MT. Our observation that hsp70 is strongly induced in jejunum after ZnSO_4 treatment, suggests a contribution of hsp70 in the protection against TNF. In addition, ZnSO_4 cotreatment allowed complete regression of inoculated tumors with TNF and interferon γ , leading to a significantly better survival ($P = 0.0045$).

Key words: systemic inflammatory response syndrome • zinc • acute-phase reaction • interferon • leptin

Introduction

During systemic bacterial infection animals achieve an internal homeostasis by balancing the activities of proinflammatory and antiinflammatory pathways. TNF is considered to be a major early mediator in the systemic inflammatory response syndrome (SIRS)* observed during Gram-negative sepsis (1). Intravenous injection of TNF in wild-type (wt) mice causes acute weight loss, metabolic derangement, hypotension, and at very high doses, shock, bowel necrosis, hepatitis, and death as a result of induction of SIRS (2). The toxicity and lethality induced by TNF is largely dependent on the presence and absence of endogenous sensitizing or protective factors: e.g., we showed previously that IL-12 or a glucocorticoid antagonist drastically sensitizes for the lethal effects of TNF (3, 4), while inversely, acute phase proteins protect (5–7). Recently, we also demonstrated the involvement of leptin in the endogenous protective mech-

anisms (8). In our attempts to elucidate the mechanisms by which leptin confers this protection we observed that leptin was a potent inducer of metallothionein (MT) both in vitro and in vivo (9).

The latter are members of a family of heavy-metal-binding proteins that have been highly conserved during evolution and are ubiquitously found in the animal kingdom. MTs have remarkable biochemical properties and are characterized by their low molecular weight (6–7 kD), high cysteine content, lack of aromatic amino acids, and the presence of 7–12 metal atoms per molecule. Zinc coordination includes four protein ligands (metal mercaptides) closely arranged in the linear sequence of the protein, leading to a unique structural motif known as zinc cluster of metallothionein. In mammals, MTs predominantly bind zinc, but it can easily be displaced by copper, cadmium, and traces of other metals. Besides by metal displacement, zinc is also released from MTs through oxidation of the multiple cysteine residues. MTs have been postulated to function in the Zn and Cu homeostasis, to participate in the inflammatory stress response and to be involved in the detoxification of heavy metals and scavenging of free radicals (10).

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*Abbreviations used in this paper: ALT, alanine aminotransferase; SAP, serum amyloid P; SIRS, systemic inflammatory response syndrome; wt, wild-type.

Of the four mouse MT-genes, the MT-1 and MT-2 genes are expressed in almost all organs in any stage of development. These genes are constitutively expressed in the liver and are highly induced during inflammation, and by metals and glucocorticoids (10). Injection of a variety of cytokines like TNF, IL-1, IL-6, leptin, and IFN- γ induces hepatic MT gene expression in vivo (9, 11, 12).

The functions of MT-1 and MT-2 have been examined in mice by inactivating both genes and also by overexpressing MT-1 gene. MT-null mice are phenotypically normal, but are more sensitive to cadmium and oxidant toxicity, and showed reduced zinc-uptake capabilities, indicating that the zinc homeostasis in these animals is disturbed (13). Similarly, MT-1 overexpression has no obvious influence on the phenotype, except that they were found to be more resistant to zinc depletion when fed on a zinc-free diet (14).

The aim of this study was to explore the role of MT in TNF-induced lethal shock using wt, MT-null mice, and MT-1 transgenic mice. A secondary objective was to investigate the role of zinc in TNF-induced inflammation and the potential use of zinc in TNF-based antitumor therapy.

Materials and Methods

Animals. Specific pathogen-free female 129S7/SvEvBrd-Mt1^{tm1Bri} Mt2^{tm1Bri} (hereafter referred to as MT-null mice) and their control strain 129S3/SvImJ were obtained from The Jackson Laboratory. C57BL/6J-TgN(Mt1)174Bri (hereafter referred to as MT-TG mice) and control strain C57BL/6J were also obtained from The Jackson Laboratory. Mice were used at the age of 9–10 wk. C57BL/6J mice, obtained from Iffa-Credo were used in the antitumor and zinc supplementation experiments at the age of 8–12 wk. All animals were housed in a temperature-controlled environment with 12 h light/dark cycles and received water and food ad libitum. The genotypes of the different strains were regularly monitored by DNA analysis of tail-tip samples as described by The Jackson Laboratory. Rectal body temperatures were measured using an electronic thermometer (model 2001; Comark Electronics). All experiments were performed according to the European Guidelines on Animal Care and Use.

Reagents. *Salmonella abortus equi* LPS, ZnSO₄ and D-(+)-galactosamine (GalN) were purchased from Sigma-Aldrich. Recombinant murine TNF (mTNF) and recombinant murine IFN- γ were produced in and purified from *Escherichia coli* in our laboratory. TNF had a specific activity of 1.0×10^9 IU/mg and contained <0.02 ng endotoxin/mg protein, as assessed by a chromogenic Limulus ameocyte lysate assay (Coatest; Chromogenix). IFN- γ had a specific activity of 1.10^8 IU/mg. TNF and IFN- γ were diluted in pyrogen-free PBS before injection. Intravenous injections had a volume of 0.2 ml and intraperitoneal injections were in 0.5 ml. Antibodies for detection of serum amyloid P (SAP) were purchased from Calbiochem. An anti-rabbit antibody, alkaline phosphatase-coupled and alkaline phosphatase-coupled streptavidin were from Sigma-Aldrich.

Measurement of IL-6, NO_x, SAP, Alpha-1-Acid Glycoprotein, Alanine Aminotransferase, and Apoptosis. Blood was collected via cardiac puncture, allowed to clot at 37°C, and subsequently centrifuged to obtain serum. IL-6 was determined as described previously using the 7TD1 cell line in a hexosaminidase colorimetric assay (15). As an estimate of NO concentration in serum, the stable NO metabolites nitrate and nitrite were measured together

(NO_x) as described (16). SAP was measured by a sandwich ELISA, also as described previously (17). Alpha-1-acid glycoprotein (AGP) was measured by ELISA using a rat anti-mouse AGP monoclonal antibody (raised by C. Libert) and a rabbit polyclonal antibody directed against mouse AGP (18). Serum alanine aminotransferase (ALT) was measured using a colorimetric test from Sigma-Aldrich. Evaluation of apoptosis in hepatocytes was done as follows: livers were perfused with 50 mM phosphate/120 mM NaCl/10 mM EDTA pH 7.4 buffer, excised, and transferred to ice-cold buffer (10 ml/g liver). The livers were homogenized using a douncer and DNA was prepared from 500 μ l of liver homogenate. The proteins were extracted with phenol/chloroform/isoamyl alcohol (50/49/1) and with chloroform/isoamyl alcohol (50/50). DNA was precipitated overnight with ammonium acetate and ethanol at -20°C. DNA was dissolved in Tris/EDTA and RNA was removed by RNase treatment. Plates were then coated with an anti-histone (H2B) antibody and the cytosolic fraction of the homogenized liver was used as an antigen source. After incubation, a biotinylated secondary antibody, specific for the nucleosome subparticles of histones H2A, H2B, and DNA (19), was added, followed by detection using an alkaline phosphatase-conjugated streptavidine (Sanvertech) and substrate (Sigma-Aldrich). Samples from untreated mice gave no signal in the ELISA. The activity was expressed as U/ml, one unit being the activity giving half maximal OD (405 nm).

Experimental Procedures. ZnSO₄ was dissolved in distilled water at a concentration of 5 or 25 mM. Mice were treated with ZnSO₄ or distilled water for 7 d. The B16BL6 melanoma subline, selected from a spontaneous melanoma B16F10 line by I. Hart and syngeneic with C57BL/6 mice, was a generous gift from Dr. M. Mareel, Ghent University, Ghent, Belgium (by courtesy of Dr. I. Fidler, University of Texas, M.D. Anderson Cancer Center, Houston, TX). Cells were expanded in vitro in DMEM containing 10% FCS, L-glutamine, sodium pyruvate, nonessential amino acids, MEM vitamins, penicillin, and streptomycin. At day 0 (the day of inoculation), cells were detached from the culture flask by trypsin/EDTA treatment and washed three times with serum-free medium and three times with pyrogen-free PBS. 500,000 tumor cells were inoculated by subcutaneous injection in 0.1 ml in the right thigh of mice that were shaved the day before. The tumor size index (TSI) was obtained with the following formula: largest diameter of the tumor \times perpendicular diameter \times 0.4. Treatment of tumor-bearing mice started at day 10 after inoculation. mTNF (10 μ g/mouse) and IFN- γ (5,000 IU/mouse) were injected simultaneously and paralesionally in a volume of 0.1 ml. Control groups received no treatment.

In the experiments assessing the effect of TNF toxicity on different strains, mice were challenged at 9 a.m. with different doses (ranging from 5 to 25 μ g) of mTNF intravenously. Survival was monitored up to 96 h. Mice that survived were still alive several weeks after the TNF treatment.

Statistical Analysis. Survival curves (Kaplan-Meier plots) were compared using a Log Rank- χ^2 test, final outcomes using a χ^2 test and means with SD using a Student-*t* test. * means $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$.

Tissue Sections and Staining. The jejunum was dissected and cut longitudinally and fixed in 10% formalin in PBS and embedded paraffin. 5- μ m sections were cut longitudinally and stained with hematoxylin and eosin.

Hsp-70 Immunoblot. Jejunum tissue was quickly removed after killing, immediately homogenized in 1 ml phosphate-buffered saline (PBS) and centrifuged to remove undissolved material. Total protein content was quantitated using the Bradford method.

3 μg of each sample was separated on a 7.5% SDS-PAGE and transferred to nitrocellulose. The membranes were blocked with 1% gelatine blocking solution (PBS, 0.1% Tween20, 1% gelatine). Next, blots were exposed to a mouse anti-hsp70 biotin-conjugated monoclonal antibody (StressGen Biotechnologies) and peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories), respectively. After washing, blots were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.) and exposed to a standard autoradiographic film. Efficiency of protein transfer on the membrane was checked by staining with Ponceau S.

Results

Altered Sensitivity to TNF-induced Toxicity in MT-null and MT-TG Mice. To investigate the role of MT in TNF-induced lethal shock, both MT-null mice and their appropriate controls (wt) were challenged with 10 μg mTNF/mouse intravenously. All mice showed apparent signs of distress such as apathy, fur ruffling, conjunctivitis, and diarrhea. These symptoms were more prominent in the control group. The dose of 10 μg mTNF caused lethality in 100% of the wt mice within 48 h after injection, whereas in the MT-null group 47% survived (** $P = 0.0078$) (Fig. 1 A). Administration of a lower dose mTNF (5 μg) was lethal in 47% of wt mice and 3% of the MT-null mice (** $P = 0.0026$; data not shown). Therefore, we conclude that targeted disruption of the MT-1 and MT-2 gene significantly increases the survival from TNF-induced shock.

To determine whether overexpression of MT influences sensitivity to TNF induced lethal shock, MT-1 transgenic mice and controls were treated with 7.5 μg mTNF. Symptoms of distress were present in both groups but were more pronounced in MT-TG mice. Moreover, we observed a survival rate of 78% in the control group whereas only 25% of the MT-TG animals survived (** $P = 0.0003$; Fig. 1 B). These data were reproduced several times and are in line with the former observation of decreased sensitivity of MT-null mice.

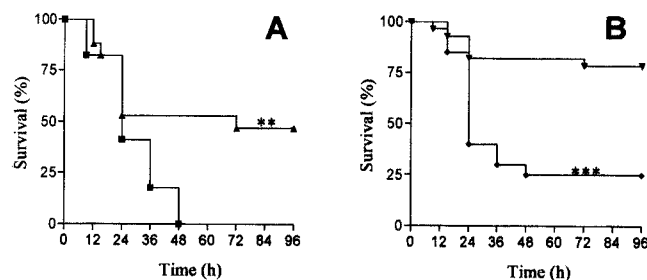


Figure 1. Sensitivity of MT-null and MT-TG mice to mTNF-induced lethality. (A) Mice (wt, MT-null) were challenged with 10 μg mTNF intravenously. The percentage survival was plotted as a function of time (hours after challenge); $n = 18$ (wt) and 18 (MT-null). ■, wt; ▲, MT-null. ** $P = 0.0078$ (wt versus MT-null). The result is a cumulative sum of two independent experiments. (B) Mice (wt, MT-TG) were challenged with 7.5 μg mTNF intravenously. The percentage survival was plotted as a function of time (hours after challenge); $n = 28$ (wt) and 20 (MT-TG). ▼, wt; ◆, MT-TG. ** $P = 0.0003$ (wt versus MT-TG). The result is a cumulative sum of three independent experiments.

The change of a number of metabolic parameters after challenge with TNF was studied in wt and MT-knockout mice. To investigate TNF-induced gene-expression, IL-6 and iNOS-produced NO_x were measured, respectively, 2 and 12 h after challenge with 7.5 μg mTNF and acute phase proteins were measured 24 h after 1 μg mTNF. As is clear from Table I, IL-6, NO_x , and acute phase proteins were equally well induced in both groups of mice.

Recently, Kimura et al. described that MT-deficient mice were more sensitive for LPS in GalN-sensitized mice (20). We could confirm these data as, compared with controls, MT-knockouts all died from a sublethal dose of 0.1 μg LPS in combination with 20 mg GalN (see Table I). However, MT-knockout mice were equally sensitive as controls in a TNF/GalN model, as judged by final lethality, drop in body temperature, appearance of liver ALT in the serum, and liver apoptosis (Table I).

Influence of Zinc Supplementation or Deprivation on TNF-induced Lethality. As MT has been shown to play a prominent role in the inflammation-mediated tissue Zn redistribution (21), we examined whether alteration of zinc metabolism could influence the sensitivity to the toxic effects of TNF. In a first experiment we tested whether zinc supplementation could influence the response to TNF-induced toxicity. Therefore, wt mice were pretreated for 1 wk with 5 or 25 mM ZnSO_4 in the drinking water and were challenged with 25 μg mTNF. Control mice, which

Table I. Changes of Metabolic Parameters upon Injection of Wild-Type and MT-knockout Mice with TNF, TNF/GalN, or LPS/GalN

Challenge ^a	Parameter ^b	wt	MT-KO	<i>P</i> value
TNF	IL-6 (ng/ml)	110 ± 15	88 ± 21	0.060 (N.S.)
	NO_x (μM)	604 ± 44	538 ± 83	0.116 (N.S.)
	SAP (μM)	206 ± 50	195 ± 25	0.672 (N.S.)
	α -1-AGP	1,990 ± 470	1,960 ± 640	0.940 (N.S.)
TNF/GalN	Lethality	5/5	5/5	1.000 (N.S.)
	Body temp ($^{\circ}\text{C}$)	29.7 ± 1.6	32.1 ± 1.7	0.051 (N.S.)
	ALT (IU/liter)	7,259 ± 7,203	5,779 ± 3,487	0.690 (N.S.)
	APO (U/ml)	244 ± 56	272 ± 45	0.465 (N.S.)
LPS/GalN	Lethality	4/7	5/5	0.045 (*)

N.S., statistically not significant.

^aTNF was injected intravenously at 7.5 $\mu\text{g}/\text{mouse}$ (IL-6 and NO_x) or 1 $\mu\text{g}/\text{mouse}$ (SAP and α -1-AGP); 0.5 μg TNF was injected intraperitoneally together with 20 mg GalN; 0.1 μg LPS was injected intraperitoneally together with 20 mg GalN.

^bMeasured in serum, 2 h after challenge (IL-6), 12 h after challenge (NO_x) or 24 h after challenge (SAP, α -1-AGP); body temperatures (Body temp), ALT, and apoptosis measured 6 h after challenge and lethality 24 h after challenge (no further deaths occurred).

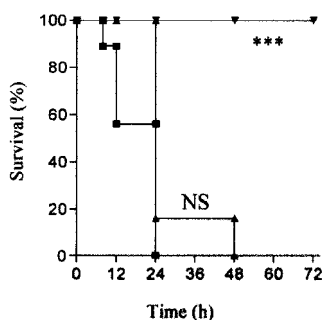


Figure 2. Protective effect of ZnSO₄ on TNF-induced lethality. Mice were treated with H₂O ($n = 18$), 5 mM ZnSO₄ ($n = 6$), or 25 mM ZnSO₄ ($n = 18$) for a period of 7 d, and were then challenged intravenously with 25 µg mTNF. The percentage survival was plotted as a function of time (hours after challenge). ■, H₂O; ▲, 5 mM; ▼, 25 mM ZnSO₄. *** $P < 0.0001$.

had received normal distilled water during 1 wk, all died within 24 h after TNF administration. 5 mM ZnSO₄ conferred no protection, but 25 mM ZnSO₄ lead to 100% protection (** $P < 0.0001$; Fig. 2). Measurement of body temperature and serum IL-6 concentrations, respectively, 6 and 8 h after TNF injection, confirmed that the inflammatory response syndrome was attenuated by ZnSO₄ pretreatment (data not shown). Furthermore, 6 h after TNF-challenge, the jejunum of control mice displayed significant loss of goblet cells, inflammatory cell infiltration, erosion, and necrosis of the epithelium (Fig. 3 A). No tissue injury could be demonstrated in the jejunum of the 25 mM ZnSO₄ treated group (Fig. 3 B). We conclude that pre-

treatment with ZnSO₄ completely desensitizes wt mice to TNF toxicity.

It is well known from literature that ZnSO₄ treatment in vivo results in hsp70 induction. To study the correlation between zinc-induced protection and hsp70 induction, mice were treated with water, 5 mM, or 25 mM ZnSO₄ and after 1 wk, hsp70 induction in the jejunum was studied by Western blot analysis. Preliminary data had shown that hsp70 was strongly induced in the jejunum. It was found (Fig. 4) that although there was detectable induction of hsp70 after 5 mM ZnSO₄ treatment, a clearly more pronounced induction was obtained after treatment with 25 mM ZnSO₄.

Because zinc distribution is disturbed in MT-null mice we also tested whether zinc supplementation or zinc deprivation could influence the protection against TNF induced toxicity in these mutant mice. It was described before that 25 mM ZnSO₄ in the drinking water is not toxic to MT-null mice (22). In a first experiment, we observed that, in analogy with wt mice, MT-null mice could be perfectly protected by adding 25 mM ZnSO₄ in the drinking water (** $P = 0.0002$; Fig. 5 A). In a second experiment wt mice (129S3/SvImJ) as well as MT-null mice were kept on a zinc deficient diet for 3 wk or on a normal control diet. Next, mice were challenged with 250 µg/kg mTNF intra-

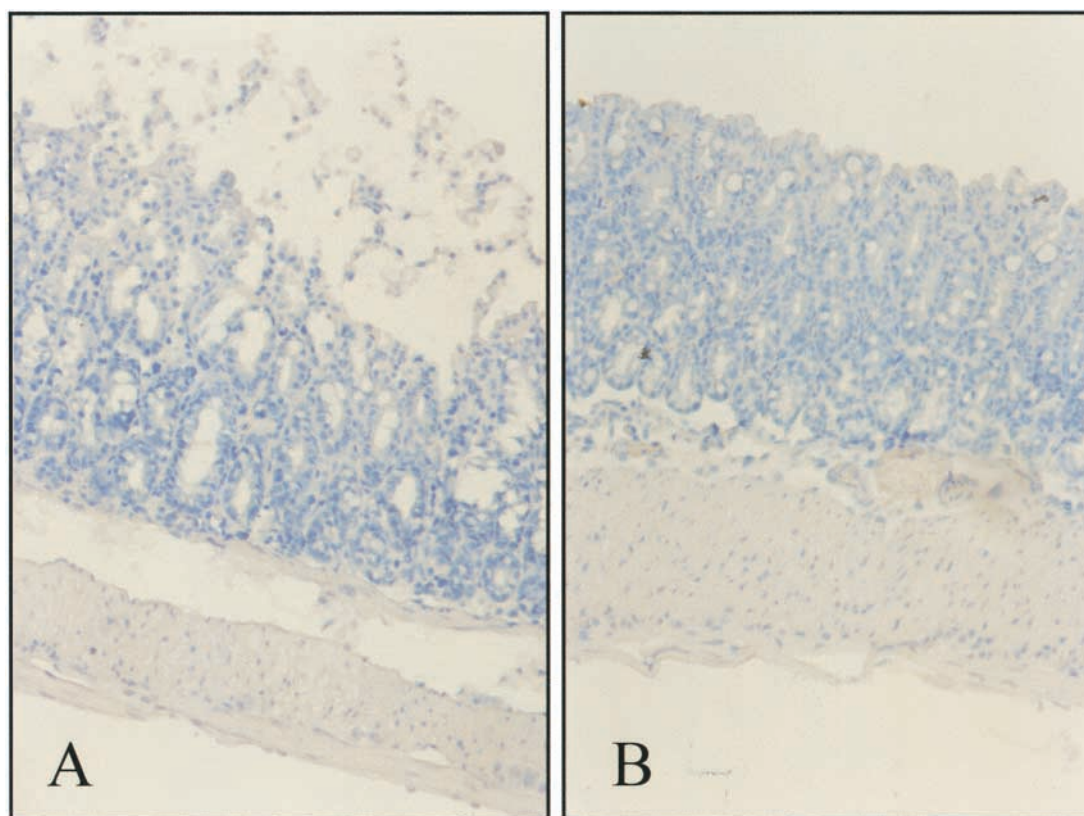


Figure 3. Protective effect of ZnSO₄ on TNF-induced bowel necrosis. Mice were treated with H₂O (A) or 25 mM ZnSO₄ (B) for a period of 7 d, and were then challenged intravenously with 25 µg TNF. 6 h after challenge, mice were killed and the bowels fixed in 10% formalin. Tissue sections were made and stained with eosin/hematoxylin.

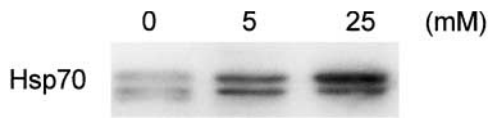


Figure 4. Western blot analysis of hsp 70 protein expression in jejunum tissue after pretreatment with ZnSO₄. Animals were treated for 1 wk in the drinking water either with 0 mM, 5 mM, or 25 mM ZnSO₄ as indicated on top.

venously. In both the wt mice and the MT-null mice survival was higher in the normal fed group as opposed to the zinc-deprived mice ($*P = 0.048$ and $*P = 0.036$ respectively; Fig. 5 B). These data illustrate that loss of MT-1 and MT-2 leads to protection to TNF, also in the absence of zinc and that zinc-deprivation leads to sensitization in both wt and MT-null mice. Administration of a dose of 500 $\mu\text{g}/\text{kg}$ of mTNF to MT-null mice leads to a survival rate of 57% in the normal fed group, whereas no animals survived in the zinc-deprived group ($***P < 0.0001$), confirming that zinc deprivation increases the susceptibility of MT-null mice to TNF toxicity (Fig. 5 C). Though MT-null mice are more sensitive to zinc deprivation (23), we observed that zinc deprived MT-null mice are still more resistant than zinc deprived wt mice ($***P < 0.0001$; Fig. 5 B).

Influence of Zinc Supplementation on Antitumor Activities of TNF. C57BL6/J mice were inoculated with B16BL6 syngeneic tumor cells on day 0. From day 3 until the end of the experiment, mice received water with or without addition of 25 mM ZnSO₄. On day 10, mice were left untreated or were subjected to a 10-d treatment of daily injection with 10 μg mTNF in combination with 5,000 IU mIFN- γ . Tumor size index (TSI) was measured daily. After 10 d of treatment all surviving mice were killed. ZnSO₄ did not inhibit the growth of the B16BL6 tumors; neither did it affect the antitumor effect of TNF/IFN- γ (Fig. 6). Untreated tumor-bearing mice were all alive on day 20. After 10 d of treatment with TNF/IFN- γ , the number of mice that had succumbed was 8/12 in the control group

and 2/13 in the ZnSO₄ group ($**P = 0.0045$). From these observations, we conclude that coadministration of ZnSO₄ offers significantly better survival prognosis in TNF/IFN- γ antitumor therapy without impairing the antitumor effect.

Discussion

TNF, which was originally discovered based on its property to destroy tumors in mice (24), is involved in the pathogenesis of many inflammatory diseases. Administration of TNF leads to a widespread SIRS, which is established by a complex network of cytokines, hormones, and low-molecular weight mediators induced by TNF (25). One of the cytokines that is induced by TNF is leptin, a hormone secreted by adipocytes which modulates food intake and energy metabolism (26, 27). Recently, we showed that leptin has protective properties against the toxicity exerted by TNF (8). Using a Representational Difference Analysis-strategy in PC12 cells stably expressing the leptin receptor, we were able to identify a set of leptin-induced genes (9). One of these genes encoded MT-2, which was of particular interest since we were able to demonstrate modulation of MT mRNA and protein levels by leptin both in vitro (PC12 cells) and in vivo (in wt and ob/ob mice). Moreover, several publications indicate that MT has a protective role against oxidative damage (11, 28–31) and that oxidative stress is implicated in the mechanism of TNF-induced cell death (32). To address the question whether MT could also protect against the toxic effects of TNF, we studied the lethal response in MT-null and MT-TG mice by challenging them with TNF intravenously. To our surprise, survival was significantly higher in MT-null mice than in wt control mice, whereas MT-TG mice exhibited an increased sensitivity in comparison with the appropriate control population. This observation claims a crucial role for MT in mediating the harmful effects of TNF to the host, which is in marked contrast with a potentially protective effect of MT in SIRS (10). Indeed, it

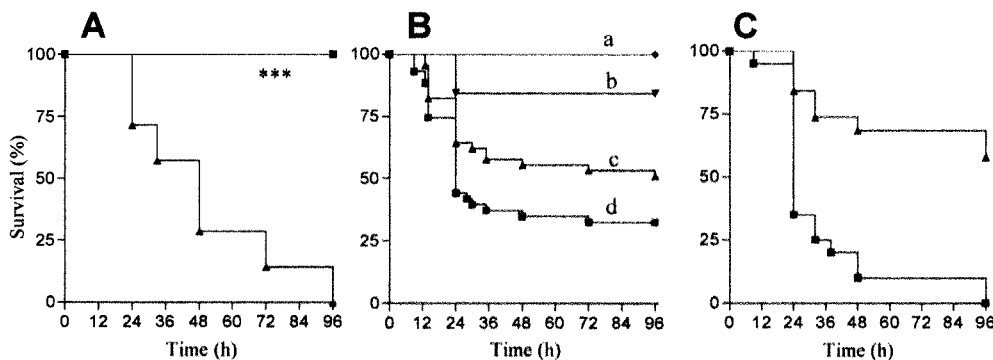


Figure 5. (A) Protective effect of ZnSO₄ on TNF-induced lethality in MT-null mice. Mice were treated with H₂O ($n = 7$) or 25 mM ZnSO₄ ($n = 6$) for a period of 7 d, and were then challenged intravenously with 12.5 μg mTNF. ▲, H₂O; ■, 25 mM ZnSO₄. $***P < 0.0002$. (B) Sensitivity of normal fed MT-null, normal fed wt mice, zinc-deprived MT-null, and zinc-deprived wt mice to mTNF-induced lethality. Mice were challenged with 250 $\mu\text{g}/\text{kg}$ mTNF intravenously.

The percentage survival was plotted as a function of time (hours after challenge); $n = 23$ (MT-null, normal diet), $n = 45$ (wt, normal diet), $n = 32$ (MT-null, zinc deficient diet), $n = 43$ (wt, zinc deficient diet); ◆, MT-null, normal diet; ▲, wt, normal diet; ▼, MT-null zinc deficient diet; ■, wt, zinc deficient diet. The result is a cumulative sum of three independent experiments. Survival curves were significantly different between a and b: $*P = 0.048$, c and d: $*P = 0.036$, a–c: $***P < 0.0001$, b–d: $***P < 0.0001$. (C) Sensitivity of normal fed MT-null and zinc deprived MT-null mice to mTNF-induced lethality. Mice were challenged with 500 $\mu\text{g}/\text{kg}$ mTNF intravenously. The percentage survival was plotted as a function of time (hours after challenge); ▲, MT-null, normal diet ($n = 19$); ■, MT-null, zinc deficient diet ($n = 20$), $P < 0.0001$.

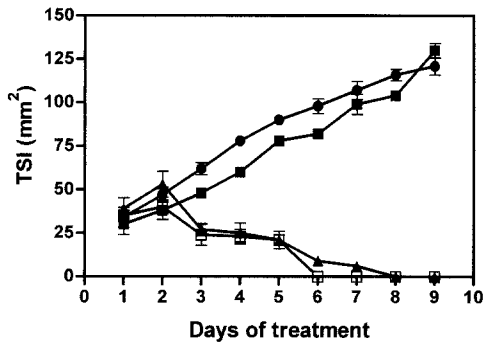


Figure 6. Influence of ZnSO₄ treatment on tumor growth and TNF/IFN- γ induced antitumor activity. Tumor cells were inoculated subcutaneous and tumors were allowed to grow for a period of 10 d. Mice were either pretreated with H₂O or with 25 mM ZnSO₄ from day 3 after inoculation. 10 d after inoculation, mice were treated daily with TNF/IFN- γ or were not treated at all. ■, H₂O, untreated ($n = 8$); ●, ZnSO₄, untreated ($n = 8$); ▲, H₂O, TNF/IFN- γ -treated ($n = 12$); □, ZnSO₄, TNF/IFN- γ -treated ($n = 13$).

was recently shown that MT-deficient mice were more sensitive in a model of LPS induced lethal shock in GalN-sensitized mice (20). We found a similar sensitization in this model but not in the TNF/GalN model, which may be related to the scavenging of reactive oxygen species, involved in the induction of TNF by LPS (33). It was found by these authors that antioxidantia such as allopurinol, or detoxifying enzymes such as superoxide dismutase and catalase protected against LPS/GalN but not against TNF/GalN. The loss of antioxidant MT activity in the TNF model is therefore clearly irrelevant.

As loss of MT leads to slightly increased NF- κ B activity in cells (34) we studied the nuclear factor (NF)- κ B and p38 dependent gene-induction by TNF in wt as well as MT knockout mice and found no difference whatsoever. These data illustrate that the resistance of MT-deficient mice to TNF is independent from NF- κ B-mediated gene expression. We also followed induction of acute phase proteins and found no difference between control and MT-null mice. This is in agreement with the data of Rofe et al. who found no changes in fibrinogen increase after LPS treatment (35).

To exclude that the effect we observed is merely a secondary effect of an altered zinc distribution in MT-null mice, we repeated the TNF challenge experiments in zinc limiting conditions. It has been shown before that MT-null mice have higher serum zinc and lower hepatic zinc level in comparison with the normal wt mice. Moreover, LPS administration leads to hypozincemia and increased hepatic zinc levels in wt mice within a few hours, while plasma zinc and hepatic zinc concentrations remain unchanged in MT-null mice (35). It was also demonstrated that zinc deprivation leads to low plasma zinc concentrations and low liver zinc concentrations in wt and MT-null mice, with bottom levels that are the same in both genotypes (23). Here, we show that zinc withdrawal clearly increased the sensitivity of both MT-null and wt mice to TNF toxicity.

Moreover, we found that zinc deprived MT-null mice are still more protected in comparison with the zinc-deprived wt mice, which argues strongly for a zinc-independent role for MT in mediating the toxic effects of TNF.

In this study we also provide evidence that the survival rate after TNF challenge is significantly enhanced in ZnSO₄ pretreated animals. Previously, Klosterhalfen and coworkers obtained similar data in a porcine model with LPS, which is a potent inducer of TNF. They showed that pretreatment with Zn²⁺ led to lower plasma peak levels of proinflammatory mediators like IL-1, IL-6, thromboxane-B2, and TNF after LPS administration, as opposed to the untreated group (36). Likewise, intraperitoneal administration of zinc chloride decreased liver damage and increased survival of rats against LPS (37). Although these data strongly suggest that protection provided by ZnSO₄ against LPS and TNF is based on the same mechanism, it is not fully understood.

Several metal responsive genes may play a role in the zinc mediated protection against TNF. One of the candidate genes is the hsp70 gene, which is induced in vivo by heavy metals (36). Overexpression of hsp70 protects against TNF-induced damage in several cell types (38, 39). Furthermore, rat hsp70-transgenic mice are protected in a model of ischemic heart failure, a disease in which TNF is a central mediator (40, 41). Using Western blot analysis, we found indeed induction of hsp70 in the jejunum after treatment of the mice with ZnSO₄. Metal ions are also potent inducers of MT-1 and MT-2, a phenomenon linked to their role in heavy metal detoxification (42). The decreased sensitivity of MT-null mice and the increased sensitivity of MT-TG mice to TNF, is at odds with a role for MT in the zinc-induced protection against TNF toxicity. Moreover, MT-null mice pretreated with ZnSO₄ are more protected than untreated MT-null mice, indicating that the protection provided by ZnSO₄ is established by an MT-independent mechanism.

Further studies will be necessary to reveal the mechanisms by which ZnSO₄ exerts its protective effects and to determine the precise contribution of hsp70 induction. The advantageous properties of ZnSO₄ may not be limited to the protection against TNF systemic response. ZnSO₄ treatment may also provide protection in other pathological conditions in which TNF plays a mediating, detrimental role, such as rheumatoid arthritis, infectious disease, and inflammatory bowel disease (1). Furthermore, our survival data suggest that ZnSO₄-cotreatment could have beneficial effects in cancer therapy. As systemic treatment with TNF leads to major side effects like hypotension, liver toxicity, and bowel necrosis, TNF therapy is currently limited to isolated organ perfusion settings (e.g., of limbs). It can be expected that if one succeeds in reducing the concomitant side effects, wider regional and even systemic application of TNF in cancer treatment may be reconsidered.

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