INFECTION AND IMMUNITY, June 1997, p. 2006–2010 Vol. 65, No. 6
0019-9567/97/$04.00+0

Generation of a Mouse Tumor Necrosis Factor Mutant with Antiperitonitis and Desensitization Activities Comparable to Those of the Wild Type but with Reduced Systemic Toxicity

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Received 18 November 1996/Returned for modification 30 January 1997/Accepted 5 March 1997

In this study, we investigated whether the recently identified lectin-like domain of tumor necrosis factor (TNF) is implicated in its biological activities on mammalian cells. To this end, a mouse TNF (mTNF) triple mutant, T104A-E106A-E109A mTNF (referred to hereafter as triple mTNF), lacking the lectin-like affinity of mTNF for specific oligosaccharides, was compared with the wild-type molecule for various TNF effects in vitro and in vivo. The triple mTNF displayed a 50-fold-reduced TNF receptor 2 (TNFR2)-mediated bioactivity but only a 5-fold-reduced TNFR1-mediated bioactivity in vitro. The specific activity of the triple mutant on L929 fibrosarcoma cells was slightly reduced compared with that of the wild type. We subsequently assessed the systemic toxicity of triple versus wild-type mTNF, since TNFR2 is partially implicated in this activity. The triple mTNF had a significantly reduced toxicity compared with that of wild-type mTNF in vivo. Moreover, we compared the effects of the triple and the wild-type mTNFs in TNFR1-mediated phenomena, such as (i) induction of tolerance towards a lethal mTNF dose and (ii) protective activity in cecal ligation and puncture-induced septic peritonitis. No significant differences between the mutant and wild-type forms were observed. In conclusion, these results indicate that triple mTNF, lacking TNF's lectin-like binding capacity, has reduced systemic toxicity but retains the tolerance-inducing and peritonitis-protective activities of wild-type mTNF.

The pleiotropic cytokine tumor necrosis factor (TNF), produced mainly by activated macrophages, is an important mediator of host defense in infections. Indeed, endogenous wild-type (wt) mouse TNF (mTNF) was shown to be essential for clearance of *Legionella pneumophila* from the lungs (3) and for survival of cecal ligation and puncture (CLP)-induced septic peritonitis in mice (5). Furthermore, mice genetically deficient for TNF receptor type 1 (TNFR1; p55), but not TNFR2 (p75), were shown to be highly sensitive to *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Candida albicans* infections (7, 14, 17, 18).

Unfortunately, the promising protective activity of TNF cannot be used therapeutically due to the high level of systemic toxicity of TNF. Human TNF (hTNF), which cannot interact with TNFR2 (11), has a profoundly reduced toxicity in mice (4), and TNFR2-deficient (*Tnfr2°*) mice are resistant to normally lethal doses of mTNF (6), although to a lesser extent than *Tnfr1°* mice (23). Approaches undertaken to reduce the systemic toxicity of TNF aimed at inhibiting the TNFR2-mediated pathway. An elegant approach to reduce the toxicity of TNF consisted of the generation of a TNFR1 agonist of hTNF (24). Unfortunately, the human TNFR1-specific mutants also induced acute hypotension, tachycardia, and organ dysfunction in the baboon, to the same extent as the wt molecule. It is not clear whether the mutant itself or the endogenously produced TNF was responsible for these early hemodynamic changes (25). An alternative approach in the mouse system made use of TNF-induced desensitization to a subsequent lethal TNF dose (26). Interestingly, this approach, which was suggested to block the TNFR2-activated pathway, induced a desensitization to systemic toxicity but not to antitumor activity of TNF (20, 21). However, the doses used to induce tolerance in healthy animals are systemically toxic for mice suffering from tumor loading or bacterial infections. Therefore, in view of these toxicity problems of impaired animals during tolerance and the possible induction of endogenous TNF by TNFR1-specific mutants, the search for other candidate TNF mutants that would not have these side effects remains interesting.

Apart from its interaction with its two receptors, TNF also has a lectin binding capacity for specific oligosaccharides, such as chitobiose and branched trimannoses (9). This lectin-like domain of TNF was recently identified and found to be situated in the tip region in the three-dimensional representation of the mTNF trimer (12). This lectin-like region is not present in lymphotoxin and is both functionally and spatially separated from the protein-protein interaction sites with the receptors. Thus, mutants of mTNF lacking its lectin-like feature but not its tumoricidal activity were able to be generated (12).

In this study, a lectin-deficient triple mutant, T104A-E106A-E109A mTNF (triple mTNF), in which the three amino acids critical for its lectin-like activity were replaced by alanines, was characterized in terms of TNFR1- and TNFR2-mediated effects in vitro and in vivo.

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MATERIALS AND METHODS

Mice. Male C57BL/6 mice and male NMRI mice 8 to 10 weeks of age were purchased from IFFA-CREDO, Les Oncins, France. *Tnfr2°* mice (6) were kindly provided by H. Bluethmann, F. Hoffmann-La Roche, Basel, Switzerland.

Cells. CT6, an interleukin-2-dependent murine cytotoxic T-cell line, was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), antibiotics, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (15). L929 fibrosarcoma cells were cultured in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and glutamine. Brain microvascular endothelial cells (MVEC) from *Tnfr2*° B6 × 129 mice (6) were isolated as described elsewhere (19) and were identified by immunostaining for factor VIII-related antigen, presence of tight junctions in electron microscopy (EM), γ-glutamyl-transpeptidase activity, and TNF-induced ELAM-1 upregulation. Cell purity was higher than 95%. The cells were passaged up to four times and were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% FCS, antibiotics, and endothelial cell growth supplement (100 μg/ml).

Biochemicals. wt mTNF, T104A mTNF, and P105A mTNF were produced and purified as described previously (12). The triple mTNF mutant was produced and purified by using a similar protocol, but a supplementary Cu^{2+}-IDA Sepharose FF metal affinity chromatography was performed. The purity of all mutants was higher than 98%, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions (Coomassie and silver staining) and by Western blot (immunoblot) analysis using rabbit-anti *Escherichia coli* and rabbit anti-mTNF polyclonal antibodies. Lipopolysaccharide (LPS) contamination of all wt or mutant mTNF preparations was lower than 0.03 endotoxin units (EU)/μg.

Specific activities of TNF mutants. The specific activities of the TNF mutants on L929 fibrosarcoma cells were determined by titration according to the standard procedure (8).

TNF cytotoxicity assay on MVEC. An ethidium homodimer 1 incorporation assay was used to screen for lysis of endothelial cells. Ethidium homodimer 1 (Molecular Probes, Leiden, The Netherlands) is a high-affinity red fluorescent DNA dye that is internalized only through altered cell membranes. This assay has recently been shown to be more sensitive than the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide] incorporation assay in screening for TNF cytotoxicity (10). Briefly, monolayers of MVEC (3×10^5 cells/ml) in DMEM without phenol red (quencher of ethidium homodimer 1), supplemented with 10% FCS and antibiotics, were treated for 18 h with various concentrations of mTNF (Innogenetics NV, Ghent, Belgium; specific activity, 2×10^8 IU/ml), in combination with actinomycin D (1 μg/ml). Subsequently, the MVEC were treated for 30 min at 37°C with 8 μM ethidium homodimer 1 solution. As controls, cells pretreated for 30 min at room temperature with 33% ethanol (positive control) and cells incubated in medium (negative control) were included. The fluorescence signal was then read with 530 nm as the excitation wavelength and 620 nm as the emission wavelength in a CytoFluor II fluorescence multiwell plate reader (PerSeptive Biosystems, Framingham, Mass.), after which the percentage of dying cells was calculated.

CT6 proliferation assay. Induction of growth on CT6 cells was determined as described previously (15). Briefly, 4×10^5 cells/well were incubated with wt or mutant mTNF. After incubation for 24 h, the assay plates were pulsed with 0.5 μCi of [^{3}H]thymidine deoxyribose per well and further incubated for 4 to 6 h. The cells were then harvested, and incorporated label was measured.

Binding assay. Soluble (extracellular) fragments of murine TNFR1 or TNFR2 (mBp55 or mBp75, respectively) were produced with the baculovirus expression system in Sf9 insect cells and partially purified (W. Declercq, Laboratory of Molecular Biology, Vlaams Interuniversitair Instituut voor Biotechnologie, University of Ghent). Microtiter plates (Nunc-Immuno BreakApart Module) were coated with mBp55 (0.66 μg/ml) or mBp75 (1 μg/ml) overnight at room temperature. Blocking was done with phosphate-buffered saline (PBS)–2% bovine serum albumin. The microtiter plates were then incubated with 10 to 20 ng of mTNF per ml, labeled with ^{125}I as described previously (24), in the presence of different concentrations of wt or mutant TNF. After incubation overnight at 4°C, the wells were washed with PBS-Triton X-100, and bound radioactivity was measured in a γ-counter.

Half-life of triple mTNF in sera. In order to assess the half-life of triple versus wt mTNF, 8-week-old C57BL/6 mice were injected intravenously (i.v.) with 1 μg of wt or triple mTNF. Subsequently, the mice were bled 30 s or 5, 10, 15, 20, or 30 min after injection (three mice per time point). The remaining levels of wt mTNF or triple mTNF in serum, with the corresponding molecule used as a standard, were then assessed with an INNOBASICS mTNF enzyme-linked immunosorbent assay kit (Innogenetics), having a detection limit of 12 pg/ml and detecting both receptor-bound and free mTNF.

Systemic toxicity. In two consecutive experiments, 8- to 10-week-old C57BL/6 mice (10 mice per group) were each injected with 7.5, 15, or 30 μg of wt mTNF or triple mTNF dissolved in sterile DMEM or with DMEM as a negative control. Subsequently, the survival of the animals was monitored every 2 h.

Desensitization experiment. In two consecutive experiments, 8- to 10-week-old C57BL/6 mice (eight mice per group) were pretreated intraperitoneally (i.p.) twice daily on three consecutive days with 2 μg of either wt mTNF or triple mTNF, dissolved in PBS (PBS injections were given as a negative control), prior to i.v. injection with 15 μg of wt mTNF per mouse. Both during the pretreatment period and after the challenge, the general condition as well as the survival of the animals was checked every 2 h.

CLP. Male NMRI mice (weight, 35 to 40 g) were injected i.p. with 600 ng of triple mTNF or wt mTNF, each dissolved in 0.5 ml of sterile PBS, or with sterile PBS only (seven mice per group). Two hours later, these mice were anesthetized by i.p. injection of 75 mg of ketamine hydrochloride (Ketanest; Parke, Davis & Company, Munich, Germany) per kg of body weight and 16 mg of xylazine hydrochloride (Rompun; Bayer AG, Leverkusen, Germany) per kg in sterile PBS. The abdominal skin of the mice was dampened with 96% ethanol, and a 0.7-cm midline incision was made. The cecum was exteriorized and, if required, filled with feces by milking stool back from the ascending colon. The distal 80% of the total cecum length was ligated and punctured twice with a 0.9- by 40-mm needle. Gentle pressure was applied on the ligated cecum to exteriorize a small amount of feces. The cecum was then returned to the peritoneal cavity, and the incision was closed with clamps. The mice were observed for 2 weeks (5, 27).

Statistical analysis. Survival curves were analyzed by using the Kaplan Meier test in the Windows SPSS program.

TABLE 1. Relative binding competition capacities and receptor-specific bioactivities as well as specific activities of mutants of mTNF[a]

Type of mTNF	R1 binding[b]	R1 activity[c]	R2 binding[d]	R2 activity[e]	Sp act[f]
wt	100	100	100	100	2×10^8
Triple	20	20	10	2	5×10^7
T104A	45	20	95	2	4×10^7
P105A	45	100	100	30	2×10^8

[a] Values are means (four samples per group) and, except for specific activity, are expressed as percentages. In all cases, the standard deviation was <8%.

[b] As assessed by a competition binding assay between ^{125}I-wt mTNF and unlabeled mutants for binding to solid-phase-bound soluble mouse TNFR1.

[c] As measured by means of actinomycin D-combined cytolytic activity on brain MVEC isolated from *Tnfr2*° mice (6).

[d] As assessed by a solid-phase competitive binding assay between ^{125}I-wt mTNF and unlabeled mutants for binding to solid-phase-bound soluble mouse TNFR2.

[e] As measured by the proliferation-inducing capacity of the mutants for the CT6 T-cell line.

[f] Expressed in international units per milligram of protein, assessed as the actinomycin D-combined cytolytic activity on L929 cells (8).

RESULTS

Interaction of triple mTNF with TNFR1 and TNFR2. Using an assay of competition binding between radiolabeled wt mTNF and the TNF mutants (triple mTNF, T104A mTNF, and P105A mTNF), we assessed the relative affinity of each of the mutants for the soluble mTNFR1 and mTNFR2 compared to that of wt mTNF. As shown in Table 1, the triple mTNF mutant had a 5-fold-lower relative affinity for the soluble TNFR1 and a 10-fold-lower relative affinity for the soluble TNFR2 than the wt TNF. A lectin-deficient T104A mTNF mutant and a control P105A mTNF mutant had twofold-reduced TNFR1 affinity and equal TNFR2 affinity compared to that of the wt mTNF. The specific activities of wt mTNF and the mutants were assessed in a TNFR1-specific bioassay, i.e., the actinomycin D-combined cytolytic activity towards brain MVEC isolated from *Tnfr2*° mice (11a). As such, the cytotoxic activities of the lectin activity-deficient triple and T104A mTNF mutants were found to be fivefold lower than the wt mTNF activity, whereas P105A mTNF had activity comparable to that of the wt. When the effects of triple mTNF and T104A mTNF on CT6 proliferation were assessed, i.e., in a TNFR2-mediated bioassay, both mutants had 50-fold-decreased activity compared to that of wt mTNF, whereas the control P105A mutant had only 3-fold-reduced bioactivity. The latter result is in contrast with the binding data, where the lectin-deficient mutants had 10-fold-reduced (triple mTNF) or equal (T104A mTNF) binding activity for the soluble TNFR2 compared to that of wt mTNF. Taken together, these experiments indicated that mutants of mTNF lacking its lectin-like activity have a significantly reduced TNFR2-mediated bioactivity but retain a substantial TNFR1-mediated bioactivity compared to that of the wt mTNF molecule.

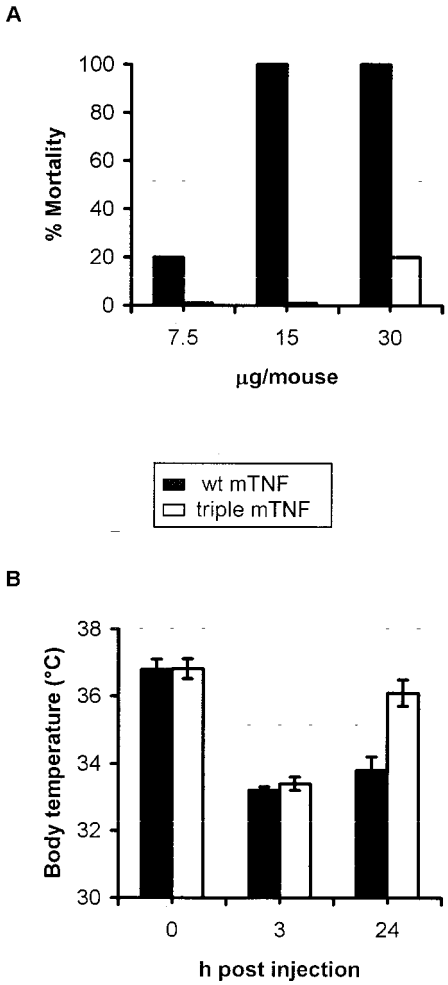

FIG. 1. (A) Survival curves for C57BL/6 mice injected i.v. with 7.5, 15, or 30 μg of triple mTNF versus wt mTNF per mouse (10 mice per group). (B) Body temperature evolution in C57BL/6 mice injected with 7.5 μg of triple mTNF versus wt mTNF (10 mice per group; values are expressed as means ± standard deviations).

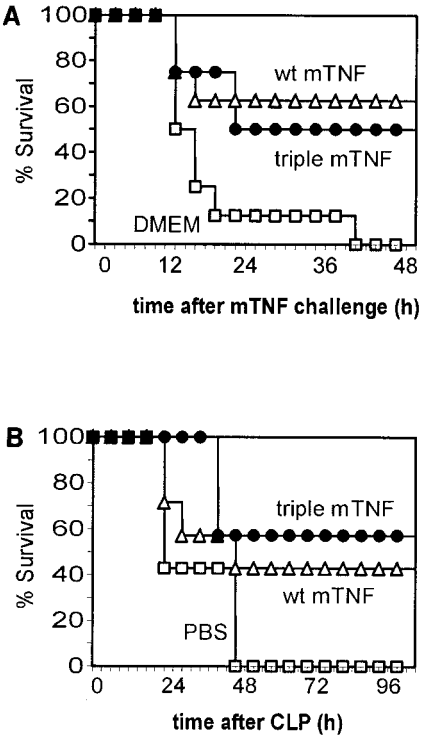

FIG. 2. (A) Comparison of capacities for desensitization towards a lethal wt mTNF challenge between triple mTNF and wt mTNF. C57BL/6 mice were treated i.p. twice daily on three consecutive days with 2 μg of either triple mTNF or wt mTNF per mouse before challenge with a lethal dose of wt mTNF (15 μg per mouse i.v.; eight mice per group; for triple versus wt mTNF, $P > 0.783$; for triple mTNF versus DMEM, $P < 0.014$). (B) Comparison of protective capacities of triple mTNF versus wt mTNF against CLP-induced septic peritonitis in NMRI mice. Mice (seven per group) were treated with 600 ng of triple mTNF, wt mTNF, or PBS i.p. 2 h before CLP (triple versus wt, $P > 0.41$; triple versus PBS, $P < 0.0003$).

Specific activities of the mTNF mutants. We compared the specific activities of triple, T104A, and P105A, and wt mTNFs in combination with actinomycin D on L929 fibrosarcoma cells. As shown in Table 1, triple mTNF and T104A mTNF had four- to fivefold-reduced specific activities compared to that of the wt molecule, corresponding to their reduced TNFR1-mediated bioactivity, whereas P105A had specific activity equal to that of wt TNF.

Systemic toxicity of triple mTNF. Since TNFR2 has been implicated in the systemic toxicity of mTNF (4, 6), we selected the lectin-deficient mutant of mTNF with the lowest TNFR2 affinity and bioactivity, i.e., the triple mTNF, and in two consecutive experiments compared the doses required for 100% mortality of C57BL/6 mice with the required dose of wt mTNF. As shown in Fig. 1A, triple mTNF had significantly reduced toxicity in C57BL/6 mice compared to that of wt mTNF. Indeed, whereas wt mTNF caused 100% mortality at a dose of 15 μg per mouse, 30 μg of triple mTNF caused only 20% mortality. Interestingly, whereas both triple and wt mTNFs induced hypothermia at 3 h postinjection, this effect persisted for at least 24 h after injection in the case of wt mTNF, whereas at this time point the temperature of mice injected with triple mTNF had returned to normal (Fig. 1B). It should be noted here that the half-life of triple mTNF, 10 ± 2.5 min (mean ± standard deviation), was not significantly different from that of wt mTNF, 11 ± 2 min.

In the following experiments, we investigated whether triple mTNF had retained the potentially useful TNFR1-mediated effects of wt mTNF in vivo, such as desensitization to a lethal mTNF dose and protection in septic peritonitis.

Desensitization capacity of triple mTNF. We pretreated C57BL/6 mice i.p. twice daily on three consecutive days with 2 μg of either wt mTNF, triple mTNF, or DMEM as a negative control per mouse. Subsequently, they were challenged with a normally lethal dose of 15 μg of mTNF i.v. per mouse. As shown in Fig. 2A, triple mTNF was as efficient as wt mTNF in inducing desensitization to the challenging wt mTNF dose, whereas all of the DMEM-pretreated mice died. These results thus indicated that triple mTNF was as efficient as wt mTNF in inducing desensitization towards a lethal mTNF challenge.

Protective capacity of the triple mutant in CLP-induced septic peritonitis. Endogenous TNF is essential for the survival of mice after CLP-induced peritonitis (5). Moreover, the observation that TNFR1-deficient (*Tnfr1°*) mice, but not *Tnfr2°* mice, are more sensitive for lethality after CLP than control mice (4a) indicated that TNFR1 is implicated in this protective activity of mTNF. In this experiment, we tested whether an i.p. injection 2 h before the onset of CLP of either wt mTNF or triple mTNF could equally confer resistance to lethal CLP-

induced peritonitis. We first identified the minimal concentration of wt mTNF giving maximal protection in CLP, and as such we selected a dose of 600 ng per mouse (data not shown). As shown in Fig. 2B, upon pretreatment of the animals with 600 ng per mouse, triple mTNF was as efficient as the wt mTNF in protecting the mice from CLP-induced lethality.

DISCUSSION

In this study, we have shown that a lectin-deficient mutant of mTNF, triple mTNF, had a significantly reduced TNFR2-signaling capacity in vitro compared to that of the wt mTNF. On the other hand, the TNFR1-mediated bioactivity of triple mTNF in vitro, such as the actinomycin D-combined cytolytic activity in brain MVEC isolated from *Tnfr2*° mice, was only slightly reduced. These results indicate that the triple mutant of mTNF has significantly reduced TNFR2-mediated bioactivity but maintained TNFR1-mediated bioactivity in vitro. Recently, lymphotoxin α (LT-α), which lacks the lectin domain of TNF, was shown to have a greatly diminished capacity, compared to that of TNF, to mediate induction of differentiation of human myeloblastic leukemia ML-1 cells (22). The affinity of human LT-α for TNFR2 was reduced only threefold compared to the affinity of hTNF, but the TNFR2-mediated signaling activity of LT-α in these cells was dramatically weakened. These results suggest that LT-α has a significantly reduced ability to signal through TNFR2, without significant loss of affinity for this receptor type. Collectively, these observations as well as ours suggest the involvement of the lectin-like domain of TNF in TNFR2 signaling.

Both TNFR1 and TNFR2 have been implicated in the systemic toxicity of mTNF, but only TNFR1 has been shown to be involved in its CLP-protective and desensitizing activities. The systemic toxicity of triple mTNF was significantly lower than that of wt mTNF, in spite of its comparable half-life in vivo. An elegant approach to reduce the toxicity of TNF can be achieved by desensitizing mice to lethal doses of TNF through pretreatment with low doses of the cytokine (26). This tolerance is mediated by TNFR1 and was suggested to result from an inhibition of the TNFR2-mediated pathway (21). During the 3-day pretreatment, the wt-mTNF-treated mice showed signs of toxicity, such as ruffled fur, trembling, and diarrhea, whereas the triple-mTNF-pretreated mice did not. However, the subsequent levels of protection from a lethal dose of wt mTNF were the same for the two groups. Hence, triple mTNF appears to exert a beneficial tolerance effect similar to that of wt mTNF.

We tested whether triple mTNF, which exhibited a significantly lower systemic toxicity than wt mTNF, could exert an equal protective activity in an animal model of septic peritonitis, i.e., CLP induced. In this model, anti-TNF treatment has been shown to aggravate CLP-induced lethality, indicating that endogenous TNF has a protective effect during the infection (5). This was confirmed by i.p. injection of hTNF, leading to significant protection of rats after CLP (1). Because hTNF does not react with rat TNFR2, this result at the same time indicated that TNFR1 is mediating the protective effect of TNF. Moreover, TNF has been shown recently not to be the cause of mortality after CLP (13, 16), contrary to its cardinal role in LPS-induced lethality (2). These results thus indicate that TNF is required for protection in polymicrobial sepsis, whereas it has a deleterious effect in LPS-induced shock. This hypothesis opens perspectives for therapeutic application of TNF in septic peritonitis. The high percentage of protection after CLP (50 to 60%) that we were able to induce by injecting mice with low doses of wt mTNF (600 ng/ml) confirms the possible interest of TNF as a potent protective agent. However, in view of the high systemic toxicity of wt mTNF, it is important to generate TNF mutants that combine a similar protective effect in CLP-induced septic peritonitis with a reduced toxicity. By comparing protective doses of wt mTNF with triple mTNF in CLP, we found that triple mTNF was as efficient as wt mTNF in protecting mice from lethality. The tolerance to a lethal TNF dose, as well as the protective effect in CLP, equal in triple-mTNF- and wt-mTNF-treated mice, is mediated mainly by TNFR1. Therefore, this suggests that the reduced systemic toxicity of triple mTNF compared to that of the wt molecule is attributable not to its fourfold-decreased TNFR1-mediated bioactivity in vitro but, rather, to the significantly reduced TNFR2-signaling capacity of triple mTNF.

In conclusion, our results indicate that triple mTNF, a mutant of wt mTNF that lacks its lectin-like activity, possibly has therapeutic advantages over wt mTNF, since it exerts tolerance and protective activities equal to those of wt mTNF in vivo, while it displays a reduced systemic toxicity.

ACKNOWLEDGMENTS

We acknowledge Wim Declercq for the generation of the soluble mouse TNF receptors, Paul Ameloot and Peter Vandenabeele for help with the binding competition experiments, and Maureen Van den Hemel and Christine Giroud for their excellent technical assistance.

This project was supported by the "Ligue Genevoise contre le cancer" (R.L. and G.E.G.) and by the Flemisk Action Programme for Stimulation of Biotechnology (VLAB; P.D.B.).

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Editor: R. E. McCallum