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Single-Chain TNF, a TNF Derivative with Enhanced Stability and Antitumoral Activity

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The inflammatory and proapoptotic cytokine TNF possesses a compelling potential as an antitumoral therapeutic agent. Possible target cells include the malignant cells themselves, the tumor vasculature, or the immune system. As the clinical use of TNF is limited by systemic toxicity, targeting strategies using TNF-based fusion proteins are currently used. A major obstacle, however, is that homotrimeric TNF ligands are prone to activity loss due to dissociation into their monomers. In this study, we report the construction of single-chain TNF molecule, a TNF mutant consisting of three TNF monomers fused by short peptide linkers. In comparison to wild-type TNF, single-chain TNF was found to possess increased stability in vitro and in vivo, displayed reduced systemic toxicity yet slightly enhanced antitumoral activity in mouse models. Creation of single-chain variants is a new approach for improvement of functional activity of therapeutics based on TNF family ligands. The Journal of Immunology, 2008, 180: 8176–8183.

The pleiotropic cytokine TNF is a prominent proinflammatory mediator associated with the pathophysiology of several acute and chronic diseases. Depending on the cell type and environment, TNF can exert opposing effects, such as immune stimulation vs immune suppression or growth stimulation vs induction of apoptosis (1, 2). Therapeutically, TNF is a potent antitumoral agent inducing both direct tumor cell apoptosis and collapse of tumor neovasculature, the latter resulting in widespread induction of hemorrhagic necrosis allowing improved penetration of anticancer agents in the tumor. In this sense, TNF can be seen as a potent bifunctional anticancer therapeutic. However, due to its high systemic toxicity, clinical use of exogenous TNF as a therapeutic strategy is currently restricted to local treatments, e.g., isolated limb perfusion and hepatic perfusion regimens.

Based on structural conformational studies, the bioactive form of TNF is shown to be a compact, noncovalently linked homotrimer (3, 4). This structure agrees with the binding of TNF to its receptors, through the insertion of the receptor into the grooves located between the individual TNF molecules of the active homotrimer. The classical soluble cytokine TNF is processed from its bioactive type II transmembrane form by the metalloprotease TNF converting enzyme. To date, two TNF receptors have been defined TNFR1 (CD120a; p55/p60) and TNFR2 (CD120b; p75/p80). Whereas TNFR1 is equally well activated by both the soluble and membrane-bound form, TNFR2 is efficiently activated only by the membrane bound form of TNF (5). This differential responsiveness of the two receptors is independent of their respective distinct signaling capacities (6) and partially overlapping signaling pathways. TNFR2 is best characterized for its transcription factor NF-κB activating activity, which requires direct interaction with the adapter protein TNF receptor associated factor 2 leading to proinflammatory and anti-apoptotic responses. In contrast, TNFR1 activates sequentially two distinct signaling pathways. Firstly, TNFR1 forms a prosurvival signaling complex at the plasma membrane recruiting and activating IkB kinases, which lead to the activation of NF-κB. A pro-death complex initiating the apoptotic program is formed in a second step and requires the internalization of the TNF/TNFR1 complex, which leads to the recruitment of Fas-associated protein with death domain and caspase-8 (7, 8).

Following in vivo administration, TNF demonstrates rapid clearance from the circulation, resulting in a requirement for repeated high-dose exposure of the agent to be used in clinical application. At low concentrations, TNF is well known to dissociate into its monomers (9, 10). Although principally reversible, the dissociation of TNF is likely to favor rapid systemic clearance and enhanced denaturation. One approach evaluated to circumvent this problem was conjugation of TNF to polyethylene glycol resulting in both an improved plasma half-life and therapeutic potency of TNF (11). Another strategy used to improve antitumor efficacy and decrease systemic toxicity of TNF is the development of tumor-targeted TNF-fusion proteins. As targeting component, typically an Ab derivative against a tumor-associated Ag is used. Several studies have successfully detailed the creation of these rTNF proteins, strongly supporting the potential and viability of this approach (12–14). The main disadvantages and limitations for these
TNF-fusion protein approaches are the obligation to create TNF trimers for activity, and a potential agonistic Ab response due to oligomerization.

In this study, we have developed a strategy to address both these concerns, TNF dissociation into monomers and synthesis of complex and bulky trimeric TNF fusion proteins. We have created a single-chain derivative of TNF (scTNF)7 through fusion of the three individual TNF monomers using two dipeptide linkers. This mono-chain derivative of TNF was shown to demonstrate both enhanced pharmacokinetics and reduced toxicity in vivo, relative to the trimeric TNF. In addition, scTNF demonstrated antitumor activity either comparable to, or slightly greater than trimeric TNF. Therefore, scTNF and single-chain variants of other TNF ligand family members are potent and viable agents with great potential for the design and development of clinically TNF-fusion proteins.

Materials and Methods

Molecular dynamics simulation studies

A model structure of scTNF was generated from its crystal structure (pdb code 1tnf). Two linker sequences and terminal residues not resolved in the crystal structure were modeled adopting a random conformation. Two linker sequences were included, each connecting the C-terminal residue of a TNF monomer with the N-terminal residue of the adjacent TNF monomer. After 4000 steps of energy minimization, a molecular dynamics (MD) simulation was used to relax the linker sequence from its original conformation. Minimization and MD simulation runs were performed using the program NAMD (15) and the CHARMM27 (16) force field. The simulation was performed in vacuum at 300 and the total simulation time was 350 picoseconds using a time step of 1 femtosecond. The backbone atoms of the TNF structure were constrained using a harmonic potential with a spring constant of 0.5 kcal/mol Å2 to maintain the overall TNF structure. The linker sequence and an additional 25 amino acids before and after the linker were free to move. Electrostatic interactions were evaluated using a cut-off radius of 15 Å and a switching function. System preparation and visualization was done using the program VMD (17).

Plasmids and PCRs

The pQe9-scTNF expression construct for human wild-type scTNF was generated by insertion of linker sequences, encoding three or four repetitions of the GGGS motif, ([GGGS]3; scTNFL1] and ([GGGS]4; scTNFL2], respectively, with a BamHI site C-terminal of two TNF modules. In addition undesirable STOP and START codons were mutagenized using standard PCR and pEE-His-TNF as template DNA. The first module was provided with a N-terminal His-tag. The generation of the expression constructs of the TNFR-selective TNF mutants is described elsewhere (18). The TNFR2-selective scTNF D143N/A145R and the TNFR1-selective scTNF R32W/S86T with substitutions in all three subunits were generated by insertion of the linker sequence encoding three repetitions of the GGGS motif and following the cloning strategy of wild-type scTNF. As templates pQe9-HisTNF D143N/A145R and pQe9-HisTNF R32W/S86T were used in the PCR. All PCR amplified sequences were verified by sequencing.

Expression and purification of scTNF

A total of 20 liters each of pQe-scTNF1 or pQe-scTNF2 transformed Escherichia coli XL1 blue were grown in a fermenter and induced with 1 mM isopropyl β-D-thiogalactoside for 4 h at 30°C. Cells were harvested and lysed in buffer A (50 mM Na2HPO4 (pH 8.0), 300 mM NaCl, 10 mM imidazol, 0.05% (v/v) Tween 20, and 1 mM PMSF) using a high-pressure homogenizer (Emulsi Flex-C5; Avestin) and a French Press (10000 p.s.i). Clarified lysates were applied to an affinity column (HiTrap; Amersham Biosciences) and protein fractions were eluted using lysis buffer containing 250 mM imidazol. Positive fractions were pooled, dialyzed against binding buffer (20 mM Tris-Cl (pH 8.5) and 10 mM NaCl) and applied to an anion exchange column (Q-Sepharose beads; Amersham Biosciences). After washing with binding buffer, protein fractions were eluted with 20 mM Tris-Cl (pH 8.5) and 400 mM NaCl. Positive fractions were pooled and purified once more using a Q-Sepharose column and eluted with 20 mM Tris-Cl (pH 8.5) and 200 mM NaCl. LPS was removed using EndoTrap blue (10 (Proفس AG) and verified by Limulus test (Cambrex), scTNF D143N/A145R and scTNF R32W/S86T were produced likewise to wild-type scTNF except that the production volume was 1 liter and the bacteria pellets were washed in 3 ml ice-cold PBS before lysis in lysis buffer B (50 mM Na2HPO4 (pH 8.0), 300 mM NaCl, 1 mM PMSF, and 1 mg/ml lysozyme) and sonication on ice. After pelleting the cell debris at 12,000 × g for 15 min at 4°C, the supernatant was applied to an affinity column (HiTrap; Amersham Biosciences) as described for wild-type scTNF. The elution of the scTNF mutants from the anion exchange column (Q-Sepharose beads; Amersham Biosciences) was achieved by 20 mM Tris-Cl (pH 9.0) and a salt gradient of 0–500 mM NaCl. A second affinity column as for wild-type scTNF was not applied. All scTNF variants were dialyzed against PBS at 4°C and the concentration was determined by several methods (OD280 nm, BCA, and silver gel).

Silver gel analysis, Western blotting, and reagents

Recombinant human TNF (2 × 10^10 U/mg) was provided by Knoll. TNF and its derivatives were denatured in Laemmli buffer, resolved by SDS-PAGE (50 ng/ml), and stained by silver staining (Amersham Biosciences) or assessed by Western Blotting using TNF-specific Abs (1 μg/ml T1; Ref. 19) and visualized by chemiluminescence (Super Signal; Pierce). Chemical crosslinking was achieved by incubation of TNF (derivatives) with BS3 (Pierce) for 30 min in PBS at room temperature and stopped by the addition of Tris-Cl (pH 7.5) (50 mM final; 15 min at room temperature). IκBα was detected using IκBα-specific Abs (rabbit anti-IκBα; BD Pharmingen).

EMSAs of NF-κB activation and JNK assay

Kym-1 cells (5 × 10^6 cells/well) were seeded in 6-well plates and grown overnight. Where indicated, cells were stimulated with 10 ng/ml TNF, scTNF1, or scTNF2 for 30 and 60 min at 37°C. Nuclear extracts were prepared as described (6), and samples were adjusted for identical protein levels. In brief, 10 μg extracted protein was incubated with 2 mg/ml poly(deoxynosine-deoxyctydine) in binding buffer (10 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM MgCl2, 1 mM DTT, and 10% (v/v) glycerol) for 15 min at room temperature in a final volume of 50 μl. A total of 30,000 cpm [32P]ATP-end-labeled and annealed NF-κB-specific oligonucleotides (5'-AGTTGAGGGGACTTTCCCAGGC-3') were added and incubated for an additional 15 min at room temperature before analysis on a nondenaturing PAGE. JNK assays were basically performed as described before (6). Following stimulation, cells (1 × 10^6 cells/well) were lysed and proteins were extracted from isolated nuclei. JNK activity was analyzed after immunoprecipitation of JNK using JNK-specific antiserum (C-17; Santa Cruz Bio-technology; 0.6 μg/assay) followed by a kinase assay using GST-c-Jun (5–89) (0.5 μg/assay) and 2 μCi/assay of [32P]ATP as a substrate.

Cell culture and cell death assays

Immortalized mouse fibroblasts generated from TNFR1/TNFFR2 double knockout mice and stably transfected with human TNFR2-Fas (MF T1; Ref. 8177) and actinomycin D (2 μg/ml; L929 cells) were added 30 min before the addition of TNF (derivatives). Next day, the cells were stained with crystal violet (20% methanol and 0.5% crystal violet) and OD at 550 nm was determined with an ELISA plate reader as described (6). The stability of the various forms of TNF was determined by incubating various TNF forms (3 ng/ml in RPMI 1640 with 5% FCS) at 37°C. Bisactivity was determined by cytotoxicity assays with Kym-1 cells followed by crystal violet staining.

Equilibrium binding studies

Equilibrium binding studies were performed as described (20). In brief, TNF was labeled with 125I by the chloramine-T method. Kym-1 cells (5 × 10^5 cells) were resuspended in PFA (PBS, 2% FCS, and 0.002% sodium azide) and binding of TNF to TNFR1 or TNFR2 was blocked by pretreatment of Kym-1 cells with the mAb H398 (30 μg/ml, TNFR1) or a TNFR2-specific rabbit serum (1/100, TNFR2) for 1 h on ice. Cells were incubated with increasing concentrations of 125I-TNF (0.25–83 ng/ml) or 125I-scTNF, (0–28 ng/ml) for 2 h on ice. Non-specific binding was determined in monolayers of a 200,000 cell number of unlabeled TNF. Cell-bound radioactivity of TNF was determined after centrifugation of the cells through a phthalate oil mixture. For calculations of the binding affinities a molecular mass of 51 kDa for wild-type TNF and 54 kDa for scTNF was used.
FIGURE 1. Construction and characterization of scTNF-L1 and -L2. A, Schematic representation of scTNF. Three single TNF modules, 1 (plus N-terminal His-tag), 2, and 3, were fused by Gly-Ser linkers containing three or four repetitions of GGGS (scTNF-L1 and scTNF-L2, respectively). B, 3D structure of scTNF-L2, as derived from molecular simulation studies. TNF (blue) shown in cartoon representation was taken from x-ray crystal structure. The backbone atoms of two linker sequences (yellow and orange) and the N- and C-termini of TNF (green and red, respectively) are shown in van der Waals representation. (C) Western Blot and (D) silver gel analyses of purified scTNF under reducing and nonreducing conditions; scTNF-L1 and scTNF-L2, were loaded onto a SDS-PAGE in the presence or absence of 5% 2-ME. For comparison, 500 ng of wild-type human TNF was used. TNF and its variants were detected by Western blot analysis. Molecular mass markers (M).

Pharmacokinetics

Animal care and all experiments performed were in accordance with federal guidelines and have been approved by university and state authorities. Female BALB/c mice (8–10 wk old; n = 3) were injected i.v. with 20 μg TNF or scTNF, each in 100 μl PBS. Blood (50 μl) was taken after 2 min (t = 0) up to 180 min. Blood plasma was generated by centrifugation for 15 min at 2000 g at 4°C and the respective samples were pooled before determination of TNF concentrations by ELISA (BD Biosciences).

Antitumoral and liver toxicity studies

The antitumoral effect of TNF was evaluated in two different model systems. First, CFS-1 cells were implanted intradermally on the back (1.6 × 10^6 cells/mouse) of 6–8-wk-old female C3H/HeN mice and analyzed in a CFS-1 methylcholanthrene-induced fibrosarcoma model (21). At day 12 and tumor sizes of ~5–6 mm in diameter, 10 μg TNF (derivatives) in PBS, or PBS alone, was injected i.p. Tumor size was measured macroscopically each day. Mice were sacrificed at day 6 and tumors were removed for histology. Excised tumors were fixed and embedded in paraffin. Equatorial cross sections (4 μm) were stained with H&E and examined microscopically for necrosis (22). At least 5 sections/tumor were examined for tumor necrosis. In the second model, 6–8-wk-old female mice (n = 5–9; nu/nu, B&K Universal) were implanted s.c. with 2–3 × 10^4 fragments of HCT116 tumor. When the tumor volume had reached ~32 mm^3, TNF (derivatives) (0.2 mg/kg) was administered. Tumor size was measured daily over 9–15 days. Tumor volume was determined using the formula: (a×b×c)/2, where a is the smaller and b the larger dimension of the tumor. In vivo liver injury was induced in male C3H/HeN mice (14–16 wk old) by coadministration of TNF or scTNF-L2 (1 μg/mouse) together with 15 mg D-galactosamine (23). Mice were observed over 24 h.

Results

High affinity binding of scTNF to both TNFR

The crystal structure of human TNF reveals the N- and C-termini being relatively close to one another (3). To prevent dissociation of TNF at low concentration (9, 10), we covalently fused the three monomers to yield scTNF (Fig. 1A). Linkers comprising three or four repeats of the sequence GGGS, L1 = [GGGS]_3, and L2 = [GGGS]_4, were fused to the TNF monomers resulting in scTNF-L1 and scTNF-L2, and an N-terminal Histidin-tag (Fig. 1A) was added. Fig. 1B was derived from a simulation study of MD of the scTNF-L2 molecule, illustrating position and length of the two linker sequences as viewed from the side (left) and along the threefold axis (right) of the TNF trimer.

scTNF-L1 and scTNF-L2 were expressed in E. coli and purified to homogeneity (Fig. 1C). Both variants showed a molecular mass of ~50 kDa by SDS-PAGE and 54 kDa by MALDI-TOF spectroscopic analyses (not shown) matching the calculated molecular mass of 54.2 kDa and 54.7 kDa for scTNF-L1 and scTNF-L2, respectively (Fig. 1, C and D).

scTNF was compared with soluble TNF performing equilibrium binding studies on the human rhabdomyosarcoma cell line Kym-1 that endogenously expresses both TNF receptors. To allow ligand binding to a single receptor species only, the second receptor was blocked by pretreatment of the cells with TNF receptor-specific Abs. As expected, similar maximum binding capacities of 13 pM for TNF and 15 pM for scTNF-L1 toward TNFR1, and of 56 pM for TNF and 62 pM for scTNF-L1 toward TNFR2 were determined (Fig. 2). However, scTNF showed an enhanced binding affinity (K_d) of 43 pM as compared with 264 pM for TNF when binding to TNFR1 (Fig. 2, B and A). Similar results were obtained for TNFR2 binding (scTNF = 32 pM and TNF = 193 pM) (Fig. 2, D and C). Additional experiments performed with TNFR1/TNFR2 negative murine embryonal fibroblasts stably expressing TNFR-Fas chimeric receptors, that bind TNF but display Fas-specific signaling (6) revealed similar results (data not shown).

In vitro bioactivity of the scTNF variants

To address the bioactivity of the scTNF variants, we first analyzed the activation of the transcription factor NF-κB and JNK signaling pathways using Kym-1 cells. Performing kinetic analyses we found comparable bioactivities for both scTNF variants and wild-type TNF in both NF-κB electromobility shift assays and IκBα degradation assays under saturating concentrations (Fig. 3, A and B). JNK kinase assays using GST-jun as substrate also demonstrated similar bioactivities for all three reagents (Fig. 3C). We next determined bioactivities on various cell lines using established cytotoxicity assay systems. In the mouse fibrosarcoma line L929, again comparable values for TNF and the scTNF variants were found (ID50_{scTNF-L1} = 10 pg/ml; ID50_{scTNF-L2} = 20 pg/ml; and ID50_{TNF} = 20 pg/ml) (Fig. 4B). Similar cytotoxic activities for the three reagents were also observed in Kym-1 cells (not shown). Induction of cytotoxicity by scTNF and TNF could be
completely blocked with TNF-specific Abs (not shown) demonstrating specificity. Finally, the two scTNF variants were compared with TNF regarding their capability to activate NF-κB in an electromobility shift assay analyzing the activation kinetics at a dose of only 10 pM in HeLa cells. Also these experiments revealed a comparable bioactivity of all three reagents used (data not shown).

The introduction of peptide linkers could also favor the formation of higher oligomers. To investigate this, TNF and scTNF variants were treated with the bivalent chemical crosslinker BS3. Western blot analyses revealed an efficient crosslinking of wild-type TNF, serving as a positive control, as observed by dimer and trimer formation even at low BS3 concentrations. However, no higher molecular aggregates were detectable for scTNFL1 or scTNFL2, even at high BS3 concentrations (Fig. 4A). These results could be confirmed investigating TNFR-Fas chimeric receptors (6). Whereas TNF and the scTNF variants showed a similar cytotoxicity on TNFR1-Fas cells (Fig. 4C), TNFR2-Fas chimeras were fully unresponsive toward both reagents (Fig. 4D). Only in combination with the TNFR2-specific Ab mAb 80M2, known to additionally crosslink ligand/receptor complexes, thus mimicking the action of the membrane bound form of TNF (5), a strong apoptotic response could be induced by both agents (Fig. 4E). Accordingly, similar to TNF, scTNF on its own did not mediate enhanced receptor crosslinking, further arguing against the formation of higher

**FIGURE 2.** Equilibrium binding studies of scTNF and wild-type TNF. Kym-1 cells were preincubated with anti-TNFR2 Abs (M80 serum, 1/100) (A and B) or anti-TNFR1 Abs (H398; 30 μg/ml) (C and D), followed by incubation with 125I-TNF (0–83 ng/ml) (A and C) or 125I-scTNFL1 (0–28 ng/ml) (B and D) on ice. Soluble and cell-bound 125I-TNF and 125I-scTNF were separated. Binding curves, 95% confidence limits (thin lines), and Scatchard plot analyses (inset) are shown. The maximum TNF binding capacities (B_{max}) and K_d values are given. One representative set of four experiments is shown.

**FIGURE 3.** Activation of NF-κB and JNK by scTNF variants. Kym-1 cells were treated with 10 ng/ml TNF, scTNFL1, or scTNFL2 for the indicated times. Cell lysates were prepared directly before (−) or after the indicated time points of stimulation. The negative control (neg. c) corresponds to control bacterial lysate that was purified likewise the scTNF variants. A, Proteins were extracted from nuclei and the presence of NF-κB was investigated by gel shift analysis. The positive control (pos. c) corresponds to HeLa cells stimulated with TNF (100 ng/ml) for 30 min. B, Whole cell lysates were prepared and the degradation of I kB was analyzed by Western blot using I kB-specific Abs. C, JNK activity was analyzed by immunocomplex kinase assay with GST-c-Jun (5-89) as a substrate. NF-κB (A), I kB (B), and GST-c-Jun (C) are indicated by arrows.
oligomers. In parallel, in the presence of mAb 80M2, scTNF efficiently induced the recruitment of the adaptor protein TNF receptor associated factor 2 to TNFR2 (data not shown), as observed by live imaging confocal microscopy using TNFR2-positive HeLa cells (6).

By the exchange of only two amino acids in TNF, mutants can be generated that show high selectivity for TNFR1 (TNF R32W/S86T) or TNFR2 (TNF D143N/A145R) (24). We designed and produced the respective scTNF variants (scTNF R32W/S86T and scTNF D143N/A145R). As expected, a marked receptor selectivity is observed.

**FIGURE 4.** Investigation of multimerization and bioactivity of scTNF. A, TNF (left) and scTNF variants (right) (500 ng each) were incubated in the presence or absence of BS3 (0–500 µM) and applied to TNF-specific Western blot analysis. The positions of TNF monomers, dimers, and trimers are indicated by arrows. B, The signaling capacity of scTNF compares to that of wild-type TNF. L929 cells were treated with wild-type TNF, scTNF R32W/S86T, and scTNF D143N/A145R (0–10 ng/ml) in the presence of 2 µg/ml actinomycin D. Cell viability was determined by crystal violet staining.

**FIGURE 5.** Characterization of single-chain TNFR-selective mutants scTNF R32W/S86T and scTNF D143N/A145R. MF TNFR1-Fas (A), MF TNFR2-Fas (B and C), or Kym-1 (D) cells were treated as indicated with wild-type TNF, scTNF R32W/S86T (TNFR1-selective), and scTNF D143N/A145R (TNFR2-selective) (0–10 ng/ml), respectively. For costimulation, cells were preincubated with the mAb 80M2 (0.5 µg/ml) before TNF and scTNF treatment. The control lysate corresponds to control bacterial lysate that was purified likewise the scTNF variants. Cell viability was determined by crystal violet staining.
of these molecules could be demonstrated (Fig. 5, A, B, and C), indicating that the introduction of peptide linkers had not affected its TNF receptor-specific interaction sites. Again, no evidence for higher molecular aggregates for the TNFR2 selective scTNF D143N/A145R was obtained, as this molecule showed no bioactivity on both TNFR2-Fas expressing fibroblasts (Fig. 5B) and Kym-1 cells (Fig. 5D). In contrast, the presence of TNFR2-specific Abs mAb 80M2 resulted in a strong cytotoxic activity of scTNF D143N/A145R on TNFR2-Fas-positive cells (Fig. 5C), being in full agreement to the results obtained with wild type scTNF (Fig. 4, D and E).

**scTNF is highly stable compared with TNF**

Soluble wild-type TNF was estimated to dissociate into monomers with a $K_d$ value of $0.2 \text{ nM}$ (25) and free monomers are likely to undergo rapid denaturation (9). To directly investigate the stability of the scTNF derivatives in vitro, they were incubated in cell culture medium at $37^\circ\text{C}$ for various time periods. scTNF L2 retained its full bioactivity over a period of 70 days, whereas wild-type TNF had lost $>90\%$ of its activity after only 7 days (Fig. 6A). Similar data were obtained for scTNF L4 and receptor selective scTNF variants (not shown). Assays using freshly isolated human serum also revealed a largely increased stability of the scTNF variants (not shown). If dissociation of TNF into monomers would favor the removal of TNF in vivo, differential pharmacokinetics of scTNF in comparison to wild-type TNF could be expected. To investigate this, single-protein doses of $20 \mu\text{g}$ were injected i.v. into mice and the serum TNF concentration determined periodically. Whereas the TNF concentration in the plasma had decreased to $10\%$ of its initial value after 53 min, the same reduction in scTNF L1-treated mice was observed not until 110 min after injection, (Fig. 6B) demonstrating that scTNF is more efficiently retained in the bloodstream.

**FIGURE 6.** In vitro and in vivo stability of scTNF variants. A, scTNF L1, scTNF L2, and TNF were freshly titrated (day 0) or as indicated incubated at 3 ng/ml for 7 (d 7) and 70 (d 70) days at $37^\circ\text{C}$ before titration on Kym-1 cells. Cell viability was determined by crystal violet staining the next day. B, Pharmacokinetics of TNF and scTNF L1 after i.v. injection in mice ($n = 3$ each). The concentration of TNF in serum was quantified by ELISA.

**FIGURE 7.** Liver toxicity and antitumoral effect of TNF and scTNF L2 in mice. A, Male C3H/HeN mice ($n = 10$) received TNF and scTNF L2 (1 $\mu\text{g/mouse}$) together with 15 mg D-galactosamine. Mice were inspected over a period of 24 h. There was no further toxicity observable after the first 12 h. (B and C) Antitumoral effect of scTNF on fibrosarcomas in C3H/HeN mice. At day 12 after tumor inoculation, TNF or scTNF L2 (10 $\mu\text{g/mouse}$; $n = 7$) were i.p. injected. At day 6 after treatment, tumors were inspected by microscopy. The arrows indicate necrotic areas in the tumor. D, Effect of scTNF on tumor volume. Tumors were inoculated in female nu/nu mice and TNF and scTNF L1 (0.2 mg/kg) were injected i.v. as daily doses over 4 days. Tumor size was determined each day. Each data value represents the mean ± SD ($n = 5–9$).
Antitumoral activity of scTNF

Next, we evaluated the in vivo bioactivity of scTNF. First, systemic toxicity was determined in a model of liver injury using D-galactosamine-sensitized C3H/HeN mice (23). At a dose of 1 μg/animal, 70% of the mice treated with wild-type human TNF died within 12 h, compared with only 20% of the scTNF-treated animals (Fig. 7A). Accordingly, scTNF showed a significantly lower toxicity in vivo, which was unexpected in light of the enhanced in vivo half-life of scTNF. We next investigated the antitumoral efficacy of scTNF in a Meth-A-induced solid tumor mouse model (26). The hemorrhagic necrotic area of a central tumor section was inspected 6 days after a single i.p. injection of wild-type TNF or scTNF into tumor-bearing mice. A total of 43% of TNF- and 70% of scTNF-treated mice showed macroscopically clearly discernible surface necrosis on their tumors (Fig. 7, B and C). Central hemorrhagic necrosis in greater than 10% of the section area could be observed in all tumors from scTNF-treated mice but only in one case of a TNF-treated animal. The observed enhanced antitumoral effect of scTNF might be caused by its increased retention period in the bloodstream (Fig. 6B) and is in accordance with its increased molecular integrity and thus functional stability as determined in vitro. In a second mouse tumor model using nu/nu mice, the relative tumor volume of an implanted section of a HCT116 tumor grown over 9–15 days was determined. No statistically significant improved antitumoral activity of scTNF compared with TNF given as daily doses over 4 days could be detected (Fig. 7D), although there was evidence from the first tumor model that the antitumoral activity of scTNF was superior.

Discussion

Soluble TNF was estimated to dissociate into monomers with a \( K_d \) value of \( \sim 0.2 \) nM (25). Once dissociated the free monomers are likely to undergo rapid denaturation. To circumvent this, we have fused three TNF monomers with two short peptide linkers to produce new TNF variants, called scTNF. Overall, scTNF retains full bioactivity when compared with the wild-type molecule, but in addition possesses some interesting new features.

First, scTNF-based molecules showed an enhanced binding affinity for TNFR1 and TNFR2 in comparison to wild-type TNF in equilibrium binding studies performed at \( 0^\circ \text{C} \) (Fig. 2). In addition, the mono-chain variants displayed an enormously enhanced stability when incubated at different concentrations in culture media with or without serum (Fig. 6A and data not shown). These properties of scTNF might be expected as a direct consequence of the covalent stabilization of the bioactive TNF homotrimer. More puzzling are our findings that the observed higher affinities for both receptors were not translated into higher bioactivities in vitro. We explicitly investigated this point in a number of different assay systems (Figs. 3 and 4) including experiments performed particularly at very low TNF concentrations, where dissociation of wild-type TNF would be expected to play a major role (Fig. 4B and data not shown). Furthermore, we took care to circumvent misinterpretation caused by inaccurate protein quantifications by using three different protein quantification methods (see Materials and Methods). Overall, there was no evidence for significant different bioactivities of wild-type vs scTNF variants. To produce highly immunogenic TNF variants, Nielsen et al. (27) inserted tetanus toxoid-derived T cell epitopes between the TNF monomers, thereby also covalently stabilizing the TNF molecule. These authors state that their TNF variants have receptor binding affinities comparable to wild-type TNF at \( 20^\circ \text{C} \), but possess enhanced bioactivity at very low concentrations.

However, when comparing affinities derived from equilibrium binding studies with dose-response curves in bioassays, care is advised. Our studies have clearly demonstrated that affinity values derived from equilibrium binding studies performed at \( 0^\circ \text{C} \) (Ref. 20 and Fig. 2) differ from those obtained at \( 37^\circ \text{C} \) (20). Accordingly, affinity values obtained from binding experiments performed at \( 0^\circ \text{C} \) cannot be directly applied to estimate dose responses under cell culture conditions. In the case of TNF/TNFFR binding, an obvious reason for this discrepancy is the complexity of underlying molecular interactions. Both TNF receptors have been shown to possess a homophilic interaction domain, called pre-ligand binding assembly domain, in their extracellular membrane distal parts (28). Pre-ligand binding assembly domain-mediated homomultimer formation of unligated receptors is believed to be essential for high affinity TNF binding and signaling (28). Moreover, there is compelling evidence that efficient signal initiation requires the formation of ligand/receptor complexes comprising more than a single (homotrimeric) ligand molecule and three receptors (6, 29). In summary, we hypothesize that formation of TNF signaling complexes is governed by multiple molecular interactions working in a cooperative manner. This could result in a remarkable insensitivity in the strength of signal initiation when the affinity of a single molecular interaction has been changed.

Regarding the pattern of intracellular signal induction we found, as expected, no differences between wild-type TNF and the scTNF derivatives. Comparable induction of typical intracellular signaling pathways, such as the induction of apoptosis (Fig. 4B), activation of the NF-κB pathway (Fig. 3, A and B), and activation of the JNK (Fig. 3C) have been demonstrated. Moreover, scTNF displayed the same inefficiency to activate TNFR2 in the absence of additional crosslinkers, as has been described for soluble wild-type TNF (Fig. 4, D and E). This provides biological evidence that both scTNF variants do not form intermolecular higher aggregates due to their linker sequences, but exist as single molecules confirming the biochemical crosslinking data (Fig. 4A).

In vivo, scTNF\(_{1,1} \) displayed \( \sim 2\)-fold reduced clearance rate (Fig. 6B), suggesting a slower kidney clearance because of the covalent stabilization of the molecule as a 54 kDa protein, not capable to reversibly dissociate. In two different mouse tumor models, scTNF\(_{1,1} \) possessed an antitumoral activity at least as potent as wild-type TNF. The superior activity of scTNF observed in the MethA model (Fig. 7, C and B) might be readily explained by the enhanced half-life of this molecule in the bloodstream of the animals (Fig. 6B) and is in accordance with its increased molecular integrity and thus functional stability as determined in vitro (Fig. 6A). An enhanced antitumoral effect could not be observed in a second model system analyzing tumor shrinkage. Although a clear tendency toward enhanced tumor regression in scTNF-treated mice compared with wild-type TNF-treated mice was observed, this effect turned out not to be statistically significant (Fig. 7D). There are no obvious reasons for the discrepancy between the two tumor models, although these models differ in the mouse strain and tumor material used.

Unexpectedly, in a model of liver injury in D-galactosamine-treated C3H/HeN mice, the toxic activity of scTNF was largely reduced (Fig. 7A). It is, therefore, tempting to speculate that reversible dissociation of TNF affects tissue distribution finally leading to enhanced toxic effects. Strong differences in the systemic toxicity in mice have also been described for human and mouse TNF, an effect which is not related to the fact that human TNF does not bind mouse TNFR2 (30). A possible explanation could be a differential interaction of scTNF and wild-type TNF with shedded extracellular TNF receptor domains in body fluids, potentially serving as inhibitors and/or storage pools. Accordingly, scTNF
interaction would be more stable because TNF dissociation into monomers, leading to a destruction of the TNF receptor binding site, is not possible. This interpretation is in agreement with our data where scTNF shows an enhanced affinity in initial binding steps, as determined in equilibrium binding studies at 0°C (Fig. 2).

In summary, the strategy to covalently link the three TNF monomers resulted in enhanced stability in vitro, prolonged in vivo availability, yet decreased liver toxicity, paralleled by an antitumor activity at least equal to wild-type TNF. This improvement could be exploitable for other members of the TNF superfamily. Indeed, we have preliminary data that this principle of converting a noncovalently associated homotrimeric ligand into a single-chain molecule of superior functional activities can also be applied for TRAIL (data not shown). Moreover, the use of sc-variants of TNF ligand family members should have advantages particularly for the construction of fusion proteins allowing targeting of stabilized cytokines to a particular tissue by, for instance, a single-chain Ab fragment. Especially TNF as an antitumor agent retained only recently once again great attention (31, 32), underlining the potential of TNF-based therapeutics.

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Disclosures
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