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Structure-Activity Profiles of Ab-Derived TNF Fusion Proteins

Stefan Bauer,* Nicole Adrian,† Eliane Fischer,* Sascha Kleber,* Frank Stenner,* Andreas Wadle,* Natalie Fadle,‡ Andy Zoellner,‡ Rita Bernhardt,‡ Alexander Knuth,* Lloyd J. Old,§ and Christoph Renner*

TNF application in humans is limited by severe side effects, including life-threatening symptoms of shock. Therefore, TNF can be successfully applied as a tumor therapeutic reagent only under conditions that prevent its systemic action. To overcome this limitation, genetic fusion of TNF to tumor-selective Abs is a favored strategy to increase site-specific cytokine targeting. Because wild-type TNF displays its bioactivity as noncovalently linked homotrimer, the challenge is to define structural requirements for a TNF-based immunokine format with optimized structure-activity profiles. We compared toxicity and efficacy of a dimerized CH2/CH3 truncated IgG1-TNF fusion protein and a single-chain variable fragment-coupled TNF monomer recognizing fibroblast-activating protein. The former construct preserves its dimeric structure stabilized by the natural disulfide bond IgG1 hinge region, while the latterimerizes under native conditions. Analysis of complex formation of wild-type TNF and of both fusion proteins with TNFR type 1 (TNF-R1) using surface plasmon resonance correlated well with in vitro and in vivo toxicity data. There is strong evidence that TNF subunits in a trimeric state display similar toxicity profiles despite genetic fusion to single-chain variable fragment domains. However, LD₅₀ of either immunodeficient BALB/c nu/nu or immunocompetent BALB/c mice was significantly decreased following administration of TNF in the formation of IgG1-derived dimeric fusion protein. Reduction of unspecific peripheral complexation of TNF-R1 resulted in higher anticancer potency by immunotargeting of fibroblast-activating protein-expressing xenografts. The broader therapeutic window of the IgG1-derived TNF fusion protein favors the dimeric TNF-immunokine format for systemic TNF-based tumor immunotherapy. The Journal of Immunology, 2006, 177: 2423–2430.

TNF is a pleiotropic cytokine with a wide variety of biological activities and immunomodulatory properties (1). The soluble form of the cytokine occurs as a trimer of three identical 17-kDa subunits. Because its systemic administration has been shown to mediate regression of murine (2) and xenotransplanted human tumors (3, 4), TNF has attracted attention as a potent antitumor agent. However, the systemic use of the cytokine for cancer therapy in humans is restricted by its very short circulatory half-life (5) and its dose-limiting toxicity (6, 7). Like other cytokines, TNF rapidly binds to its ubiquitously expressed receptor type 1 (TNFRSF1A (TNFR superfamily, member 1A; p60; TNF-R1)) in various normal tissues after i.v. application. Thus, TNF exerts multiple systemic side effects, such as activation of coagulation and inflammatory cascades, before achieving a therapeutic dose at the tumor site (8). However, TNF-mediated antitumor activity depends more on triggering indirect tumoricidal mechanisms than on direct induction of death signaling pathways in malignant cells themselves. Therefore, local exposure of high dose TNF is an obligate prerequisite to induce sufficient strength in TNF-R1 triggering with subsequent recruitment of cellular and humoral effector mechanisms for successful tumor immunotherapy (9–11). Until recently, isolated limb perfusion was the only accepted clinical method to obtain high cytokine concentrations in more localized areas (12, 13). To gain high antitumor activity by local enrichment of TNF, many investigations aimed at decreasing its unwanted toxicity to facilitate systemic application at therapeutic doses needed. Most strategies focused on Ab-directed targeting of TNF to a tumor-associated Ag by genetically engineered fusion proteins (14). Because wild-type TNF exerts its bioactivity as a homotrimeric protein, structural prerequisites for assembly of monomeric subunits were usually met by designing single-chain derived immunocytokines (15–17). However, trimerized TNF fusion proteins carry a fully active TNF molecule with high affinity to TNF-R1 and capability of signaling before the target Ag expressed by the tumor cell has been reached. As a result, similar levels of TNF-R1-mediated cytotoxicity are observed, compared with wild-type soluble TNF (18, 19). We have previously described the targeted bioactivity of TNF by an Ab-derived fusion protein, based on an IgG1-format. This fusion protein consists of a humanized anti-fibroblast-activating protein (FAP)3 Ab and human (hu)TNF replacing the IgG1 CH2/CH3 Fc domain. In contrast with trimerized wild-type TNF, this construct preserved its IgG1-derived dimeric structure with the TNF molecule forced to form a dimer. Leaving the 3-fold symmetry of TNF resulted in a significantly reduced TNF-R1-mediated toxicity in vitro and in vivo, compared with trimerized TNF at equimolar concentrations (20). The results

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3 Abbreviations used in this paper: FAP, fibroblast-activating protein; hu, human; scFv, single-chain variable fragment; S, sensitive; R, resistant; rhs, recombinant human; HC, H chain; LC, L chain; RT, room temperature.
of these studies prompted further investigations on the structural requirements for an optimal TNF-immunokine format. Therefore, we have analyzed the structure-activity relationship of trimerized (single-chain variable fragment (scFv) format) and dimerized (truncated IgG1 format) Ab-TNF conjugates. Bioactivity profiles, including TNF-R1-mediated dose-limiting toxicity and therapeutic potency, differ significantly between rTNF-conjugated Ab formats when analyzed in vitro and in vivo with a clear disadvantage for the trimeric scFv-TNF construct.

Materials and Methods

Cell lines and reagents

RPMI 1640 (supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and glutamine (0.3 mg/ml), all obtained from Invitrogen Life Technologies), was used as a standard medium. HT1080 FAP-transfected (HT1080 FAP) and mock-transfected cells (HT1080 par) were described previously (20). WEHI-164-sensitive (S) (TNF-S) and WEHI-164-resistant (R) (TNF-R) were cultured in standard medium as described (16). Recombinant huTNF (rhuTNF) and soluble TNF-R1 were purchased from Genzyme. Infiximab was obtained from Centocor (USA) and etanercept from Wyeth-Ayerst Pharmaceuticals. Marine anti-idiotypic anti-FAP was provided by Prof. A.M. Scott (Heidelberg, Victoria, Australia).

Preparation of FAP-reactive IgG1- and scFv-TNF fusion proteins

The pREN eukaryotic expression vector system with either human constant protein expression. The generation of the anti-FAP IgG1-derived TNF fusion protein has been described previously in detail (20). In brief, a modified pREN HC vector containing the inserted huTNF cDNA fragment in frame after the hinge instead of CH2 and CH3 region was used for the assembly of HC fusion proteins. The vector coding for single-chain TNF immunokines also was generated by modifying pREN HC. The variable anti-FAP HC was linked to the variable anti-FAP LC sequence by a short peptide sequence, and the final anti-FAP scFv Ab replaced the constant IgG1 HC region (BumHi and Ncol) in the pREN HC-TNF construct. In accordance with published data, a 45-bp sequence coding for 15aa (G,S) was inserted between the scFv and rhuTNF (19, 21). The TNF cDNA fragment was followed by a His6-tag (Ncol and XhoI) to allow for standardized purification (22). All constructs were sequenced on both strands.

Expression, purification, and biochemical characterization of TNF fusion proteins

Stable transfected Chinese hamster ovary cell lines were established by electroporation using a GenPulser (Bio-Rad). In brief, 400 μl of cells (1 x 10^6/ml) was mixed with 10 μg of circular DNA, electroporated (270V, 975 μF), and plated out in a volume of 150 ml of medium. Ge18 selection (0.5 mg/ml; Invitrogen Life Technologies) commenced after 2 days, methotrexate (5 nM; Lederle laboratories) was added on day 7 and increased to 100 nM over 2 wk. Stable cell lines producing high level of Abs were expanded, weaned off FCS, and transferred into a Technomouse system (Bio-Rad) for large-scale production. The supernatant was dialysed against PBS (pH 7.2) overnight (4°C), and IgG1-derived immunocytokine was purified (Ion Metal Affinity Chromatography on Zn2+/H9262-F), and plated out in a volume of 150 ml of medium. G418 selection (0.5 μg/ml, 1 h, RT), biotinylated rabbit-anti-mouse serum (1/2000; 1 h, RT) and surface plasmon resonance signal after immobilization of soluble huTNF-R1 generated 740 response units. The coupling procedure was completed by injection of 50 μl of 1 M ethanolamine hydrochloride solution (pH 8.5) to block any remaining free ester groups. The binding analysis was conducted by injecting different TNF-equivalent concentrations of either one immunocytokine or commercially available soluble rhuTNF (Genzyme) in the range of 0.1 to 10 μg/ml with a flow rate of 10 μl/min, and the refractive index changes were recorded. All protein-containing solutions were prepared in Bioace HS-BP buffer (0.01 M HEPES buffer (pH 7.4), 0.15 M NaCl, with 0.005% Surfactant P20). Five microliters of a 25 μM NaOH solution was injected to remove still-bound scFv-TNF or IgG1-TNF. The regeneration performance was evaluated by analyzing the baseline response and changes in the binding capacity after up to 30 binding cycles with 10 μg/ml scFv-TNF or IgG1-TNF, respectively. The observed changes were <10%, indicating a good regeneration performance (data not shown). Analysis of the binding curves and determination of Kd values were done using evaluation software (version 3.1; Biacore).

Optical biosensor measurements

Binding analysis

Flow cytometry was performed as described previously (20). In brief, 1 x 10^6 FAP- or mock-transfected cells were incubated with purified constructs using indicated specificity and concentration (30 min, 4°C). Cells were washed twice with PBS, incubated with rabbit anti-TNF (dilution 1/1000, 30 min, 4°C, DakoCytomation), and, finally, the complex was visualized by adding PE-conjugated goat-anti-rabbit serum (dilution 1/100, DakoCytomation). Ten thousand cells of each sample were counted and analyzed.

Binding of TNF fusion proteins also was assessed by ELISA. Ninety-six-well flat-bottom microtiter plates (Maxisorp Immuno; Nunc) were coated (overnight, 4°C) with infiximab (1 μg/ml) in 50 μl of coating buffer per well. Plates were blocked with 1.5% gelatin in PBS, and the indicated reagents solved in PBS were added in serial dilutions (1 h, room temperature (RT)). After incubation with murine anti-idiotypic anti-FAP Ab (1 μg/ml, 1 h, RT), biotinylated rabbit-anti-mouse serum (1/2000, 1 h, RT) and HRP (1/50,000, 15 min, RT; Boehringer Mannheim) were added. Plates were developed by addition of 0-phenylenediamine substrate (Sigma-Aldrich). Reaction was stopped with 3 M HCl, and plates were read on a fluorometer (model 1420, Victor 2; Wallac) at 490 nm.

Cytotoxicity assay

Cell death was determined using the EZ4U method (Biomedica Medizinprodukte) according to the manufacturer’s instructions. In brief, TNF-S WEHI-164-S or TNF-R-WEHI-164-R cells (5 x 10^4) were cultured in 96-well plates in the presence of the indicated amount of Abs or huTNF. The viability of the target cells was measured after 48 h using the EZ4U assay (Biomedica Medizinprodukte). After conversion of the tetrazolium compound to its red formazan derivative, the absorbance was measured at 492 nm, with 620 nm as a reference by a plate-reading fluorometer (model 1420, Victor 2; Wallac). Absorbance of untreated cells defined 100% viability.

Tumor model and treatment protocols

Adult male and female BALB/c nu/nu and immunocompetent BALB/c mice (6–8 wk; Charles River Laboratories) were used for toxicity and efficacy assessment. To determine TNF-induced mortality, two different administration regimens were used. First, three groups of immunocompetent BALB/c mice (10 animals per group) received repeated injections of 30 μg of rhuTNF or the equivalent doses of IgG1-TNF or scFv-TNF, respectively, at 3-h intervals, with a maximum of three i.v. injections. In a second trial, three groups of 10 BALB/c nu/nu mice were challenged in the beginning with a single i.v. administration of 30 μg of rhuTNF or the corresponding doses of IgG1-TNF or scFv-TNF, respectively. Further doses were administered using a dose-escalation regimen. Four groups of five BALB/c nu/nu mice were challenged with single TNF-equivalent doses of IgG1-TNF or scFv-TNF, respectively, at 200, 100, 50, or 0 μg/kg i.p. (50, 100, 50, or 200 μl) and monitored for death. Each control group received an equal volume of PBS alone. In this experiment, mortality was assessed 24 h after challenge. For efficacy analysis, tumors were engrafted by s.c. injection of 2 x 10^6 FAP-transfected HT1080 cells in the right flank of

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BALB/c nu/nu mice. Thirty animals were randomly divided into six experimental groups (five mice per group), and treatment was initiated when solid tumors had reached ~3 mm³. Treatment groups received 20 μg of TNF-equivalent doses (suspended in 150 μl of PBS) of anti-FAP IgG1-TNF (100 μg in total), scFv-TNF (33 μg in total), or rhuTNF (20 μg in total) or the corresponding dose of parental IgG1 (80 μg) in combination with rhuTNF (20 μg in total; suspended in 150 μl of PBS) or single-chain anti-FAP Abs (67 μg) in combination with rhuTNF (20 μg in total; suspended in 150 μl of PBS) i.v. administered by tail-vein injection every 3 days, for five consecutive injections. Control mice received the same volume of PBS alone. A second treatment regimen based on the results of the dose escalation protocol described above. FAP-expressing solid tumors were established in the flank of 15 BALB/c nu/nu mice, and animals were randomly divided into three groups of five mice. Again, treatment started when tumors reached ~3 mm³, and groups received 100 μg of TNF-equivalent dose of IgG1-TNF (400 μg in total, suspended in 400 μl of PBS) or the parental IgG1 at equal total dose and volume. Control mice received 400 μl of PBS. Mice were monitored daily to measure weight loss. Tumor size was assessed on treatment days. Animals were sacrificed when tumor volume reached >1 cm³. Comparison of tumor growth between different treatment groups was performed with t test (Microsoft Excel software). All studies were done under an existing animal ethics approval as required by federal law.

Results

Generation and characterization of anti-FAP IgG1-TNF and anti-FAP scFv-TNF

The CH2 and CH3 domains of the huIgG1-Fc region were replaced by the huTNF molecule, resulting in the formation of a dimeric Ab-derived molecule. Dimerization was stabilized by the natural disulfide bond hinge region and noncovalently assembled TNF-molecules. To generate the single-chain-based proteins, the cDNA of huTNF was fused to a 45-bp linker following the 3' end of scFv-domains (Fig. 1A). The constructs were cloned into the pREN mammalian expression vector system to transfet Chinese hamster ovary cells by electroporation. Fusion proteins were purified from the conditioned medium as described in Materials and Methods with a final yield of ~10 mg/L culture. Immunocytokines were analyzed by gel electrophoresis and Western blotting. The recombinant HC (anti-FAP-TNF) and LC products run under reducing conditions at 47 and 28 kDa, whereas the scFv-derived TNF-fusion protein migrates as a monomer of 45 kDa. Under non-reducing conditions, the IgG1-TNF appeared with the size of ~150 kDa, suggesting the dimerization of the IgG1-derived protein. ScFv-TNF showed again the expected size of a monomer (Fig. 1B).

Binding assays

The immunoreactivity of both anti-FAP TNF fusion proteins was assessed by flow cytometry. At construct, concentrations ranging from 10 ng/ml to 10 μg/ml comparable binding properties were observed without significant differences in Ag recognition. Anti-FAP moieties were demonstrated on FAP- and mock-transfected
HT1080 cells using an anti-huTNF mAb (Fig. 3 A and B). The correct biochemical assembly of TNF moiety was analyzed by ELISA, demonstrating proper binding to a chimeric anti-huTNF mAb (infliximab). To prove the integrity of the entire constructs, antigenic recognition of both fusion proteins was visualized using murine anti-idiotypic anti-FAP mAb (Fig. 3C). Furthermore, the interaction of both immunocytokines with TNF-R1 was characterized in a direct in vitro binding assay, compared with that of rhuTNF. Recombinant soluble TNF-R1 was covalently bound to the surface of a CM5 chip, after which different solutions containing anti-FAP IgG1-TNF, anti-FAP scFv-TNF, or rhuTNF in TNF-equimolar concentrations were passed over the receptor-coated surfaces. The resulting sensograms clearly showed that rhuTNF associates much faster to immobilized TNF-R1 than both immunocytokines. In contrast, both fusion proteins bind more stably to the monomeric receptor, because their dissociation is slower, compared with rhuTNF (Fig. 4). However, the $K_d$ values for the different complexes indicate that binding of trimeric scFv-coupled

**FIGURE 3.** Immunoreactivity. A, Flow cytometry analysis revealed specific binding of TNF-immunokines scFv-TNF (dotted line) and IgG1-TNF (thick line) to FAP-transfected HT1080 cells. Recognition of cell surface expressed FAP was detected via the TNF part of the constructs using rabbit anti-huTNF serum and visualized by PE-conjugated goat anti-rabbit serum. Uncoupled parental anti-FAP IgG1 (○) was used as negative control. No binding was observed when reagents were incubated with mock-transfected HT1080 cells (data not shown). B, Flow cytometry analysis was performed in triplicate. Both immunokines scFv-TNF (▲) and IgG1-TNF (■) showed equal binding moieties and concentration-dependent increase in fluorescence intensity. Uncoupled parental anti-FAP IgG1 (□) and rhuTNF (○) were used as negative control. C, Binding of both constructs (scFv-TNF (▲), IgG1-TNF (■)), uncoupled parental anti-FAP IgG1 (□), and rhuTNF (○) also was assessed by sandwich ELISA. Plates were coated with anti-TNF mAb (infliximab), and binding of immunokines was detected by murine anti-idiotypic anti-FAP Ab.

**FIGURE 4.** Binding curves. A and B, Complexation of immobilized soluble TNF-R1 by rhuTNF homotrimer, scFv-TNF trimer (▲), or IgG1-TNF dimer (■) was measured by surface plasmon resonance signal detection. Reagents were injected at a concentration of 1 μg/ml. C, Binding curves were normalized, and maximal response unit (RU) value of all curves was set to 100 to obtain a good comparison of the influence of the different multimeric states of TNF-subunits on receptor binding moieties. Curves were obtained by injecting a 10-μg/ml solution of either rhuTNF, scFv-TNF, or IgG1-TNF.
TNF fusion protein to TNF-R1 behaves like rhuTNF and is 10-fold more stable than the interaction between dimerized IgG1-TNF and TNF-R1 (Table I).

**Cytotoxicity in vitro**

We performed an cytotoxicity assay to evaluate whether this increase in receptor-ligand complex-stability of TNF-R1 and homotrimerized scFv-TNF was due to an increased TNF-R1-mediated cytotoxicity, compared with the dimeric immunocytokine. The cytotoxic capacity of both TNF-derived effector proteins was determined by coincubation with TNF-S or TNF-R WEHI-164 cells at TNF-equivalent concentrations. As expected, the WEHI-164-R clone was partially resistant to cell death mediated either by rhuTNF or both TNF-fusion proteins (data not shown). Incubation of soluble effectors with WEHI-164-S cells resulted in TNF-dependent cell lysis. However, the TNF-mediated cytotoxicity was significantly enhanced by scFv-TNF trimer, compared with IgG1-TNF dimer \((p = 0.02)\). Furthermore, similar levels of cell death were induced by trimerized fusion protein (scFv-TNF) and rhuTNF (Fig. 5A). The fact that parental anti-FAP IgG1 or anti-FAP scFv Abs could not induce measurable cell death and that lysis of WEHI-164-S cells could be inhibited by coincubation of all soluble effectors with etanercept (Fig. 5B) underscores the specific TNF-dependency of the cytotoxic effects described.

**Toxicity and therapeutic efficacy in vivo**

Due to the fast clearance of huTNF in mice, injection of high doses leads to high peak levels but short exposure time. In consequence, rhuTNF only induces death in immunocompetent mice at very high doses of \(500 \mu g\) per mouse. To circumvent limited duration and strength of TNF receptor triggering, exposure time to the TNF portion of the constructs was enhanced by repetitive injections \((23)\). Lethality of immunocompetent BALB/c mice was tested during repeated i.v. application of rhuTNF, scFv-TNF, or IgG1-TNF, respectively. These were administered every 3 h at TNF-equivalent doses of 30 \(\mu g\), and application was scheduled for one to three injections. The rate of death was similar between the groups receiving either homotrimeric rhuTNF or trimerized scFv-TNF, because all animals died after the second application. Those BALB/c mice treated with IgG1-TNF could be challenged by a third injection before LD\(_{50}\) was reached (Table II). Due to increased TNF-mediated toxicity in BALB/c nu/nu mice and taking into account the common use of this strain as test organism for xenografted tumor models, a complementary experiment was performed. After establishment of xenotransplanted fibrosarcomas, tumor-bearing animals received rhuTNF, scFv-TNF, or IgG1-TNF, respectively, at TNF-equivalent doses of 30 \(\mu g\) per mouse, because the calculated LD\(_{50}\) value for BALB/c nu/nu mice is 1.2 \(\mu g\) for murine TNF and 20 \(\mu g\) for huTNF \((24)\). Again, increased toxicity resulted from the administration of rhuTNF and scFv-TNF, because animals from both treatment groups died after the challenge. In contrast, mice receiving equal amounts of TNF dosage by IgG1-TNF did not die (Table III). Further toxicity experiments were performed using dose escalation. The study started with an initial TNF-equivalent dose of 50 \(\mu g\) of IgG1-TNF. Next, dose levels were defined by the addition of another TNF-equivalent dose of 50 \(\mu g\) of IgG1-TNF, and gradual application depended on toxicity. Using this regimen, groups of five mice were challenged by TNF-equivalent doses of 50, 100, 150, and at least 200 \(\mu g\) of IgG1-TNF. Equal volumes of PBS were administered to control mice. The first dose level was well tolerated without any signs of toxicity. Mice showed signs of stress at TNF-equivalent doses of 100 \(\mu g\) and with more intensity at 150 \(\mu g\), but all animals completed treatment without lethal side effects. The study was stopped at the dose level of 200 \(\mu g\) because administration caused death of three animals. Control groups were not affected at all.

Comparison of the activity profiles of different formatted TNF-based therapeutics was completed by demonstrating their antitumor efficacy. Xenografting of FAP-positive fibrosarcomas (HT1080-FAP\(^{+}\)) in BALB/c nu/nu mice describes an appropriate

### Table I. Binding parameters for TNF-R1 and wild-type TNF or Ab fused TNF-subunits in a dimeric or trimeric state

<table>
<thead>
<tr>
<th>TNF-R1 Reaction Partners</th>
<th>(k_{on} \text{ [M}^{-1} \text{s}^{-1})</th>
<th>(k_{off} \text{ [s}^{-1})</th>
<th>(K_d \text{ [M])</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhuTNF</td>
<td>2.24*10^4</td>
<td>4.14*10^{-4}</td>
<td>1.85*10^{-5}</td>
</tr>
<tr>
<td>scFv-TNF</td>
<td>6397</td>
<td>9.09*10^{-6}</td>
<td>1.42*10^{-5}</td>
</tr>
<tr>
<td>IgG1-TNF</td>
<td>782.4</td>
<td>1.97*10^{-3}</td>
<td>2.52*10^{-5}</td>
</tr>
</tbody>
</table>

\(\Delta \) Data were determined using the Biacore evaluation software version 3.1. Averaged interaction curves consisting of three independent measurements for different concentrations of wild-type TNF, IgG1-TNF and scFv-TNF with EDC/NHS-coupled TNF-R1. The values shown correspond to the formation of a 1:1 complex between the interaction partners. The standard deviation for all displayed values is \(\pm 20\%\) of the displayed value.
PBS alone did not result in measurable anti-tumor response. Again, no case of death was seen at this dose the first day after treatment and slight but insignificant weight loss 0.003). However, these animals showed reduced activity during /H9262 dose of 20
and rhuTNF resulted in identical lethality (150 µg in total; ), IgG1-TNF (33 µg in total, ) or the corresponding dose of parental IgG1 (80 µg, ) in combination with rhuTNF (20 µg in total, ) led to significant tumor growth delay, compared with parental IgG1 (450 µg in total, ) or PBS alone (400 µl in total; data not shown) (p = 0.003). Clinical monitoring showed slight weight loss without reaching significance.

Discussion
The clinical use of TNF is limited to local treatment schedules like isolated limb or hepatic perfusion. Those administration regimen excluding systemic delivery of TNF induce high remission rates in patients with sarcoma or melanoma of the extremities and with unresectable cancers confined to the liver (26, 27). Various targeting strategies have been developed to overcome therapy limiting toxicity arising from nonspecific TNF release (28, 29). Approaches using fusion proteins of TNF and tumor-selective single-chain Ab fragments (scFv-TNF) have shown encouraging results. Unfortunately, most trials dealing with scFv-TNF fusion proteins for tumor immunotargeting have not included in vivo comparison of cytotoxic profiles resulting from i.v. application of wild-type TNF and scFv-TNF. Therefore, it is impossible to define and compare precisely the activity profile of the recombinant fusion proteins (16, 19, 21, 30). Furthermore, most in vivo data were generated on TNF-S tumors, but those trials are irrelevant with regard to the clinical situation, because low-dose administration of TNF is already sufficient to demonstrate antitumor activity in these animal models. The challenge is to prevent targeting-independent action of the coupled TNF-domains, even when applied at therapeutically relevant doses. We designed a fusion protein on the concept of an

tumor model to characterize therapeutic activity of FAP-recover-
ing effectors (20, 25). Treatment started when solid fibrosarcomas reached ~3 mm³. Immunotherapy was performed by repeated injections of rhuTNF or rTNF-based fusion proteins in sublethal TNF-equivalent doses of 20 µg every 3 days for five consecutive injections. Control groups received equivalent doses of parental Abs in combination with either rhuTNF or PBS alone. Tumor growth was not influenced by treatment with nonfused anti-FAP IgG1 or anti-FAP scFv in combination with rhuTNF, nor by repeated injections of unbound rhuTNF alone, compared with PBS-treated mice. However, significant differences in tumor growth delay arised in the dependence of TNF exposure through IgG1 dimer or through scFv-trimer format. Application of scFv-TNF was less effective than treatment with IgG1-TNF. ScFv-TNF-treated animals reached predefined end of study resulting from tumor volume ~7 days earlier (~day 24). Fibrosarcomas of mice treated with IgG1-TNF exceeded 1 cm³ at ~day 31 (Fig. 6). To ensure FAP-Ag dependency of TNF-based immunotherapy, we performed a further trial on xenotransplanted parental HT1080 cells. Repeating the treatment schedule on mock-transfected (FAP-deficient) fibrosarcomas failed to induce any therapeutic effects (data not shown). As the toxicity profile of IgG1-TNF allowed for application of escalated TNF-equivalent doses with acceptable clinical signs of stress, we performed a corresponding therapeutic trial. FAP-expressing xenografts were established as described above, and mice were randomly assigned to treatment groups of five. Treatment started when tumors reached ~3 mm³. IgG1-TNF was injected every 3 days at a TNF-equivalent dose of 100 µg for five times. Treatment at this dose level significantly increased therapeutic efficacy (p = 0.003). However, these animals showed reduced activity during the first day after treatment and slight but insignificant weight loss (data not shown). Again, no case of death was seen at this dose level. Treatment of control groups receiving parental IgG1 Ab or PBS alone did not result in measurable anti-tumor response.

![Table II. Comparison of lethality induced by repeated administrations of wild-type TNF and TNF-based immunokines in BALB/c mice](image)

<table>
<thead>
<tr>
<th>Time Points</th>
<th>rhuTNF</th>
<th>scFv-TNF</th>
<th>IgG1-TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 0 h</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>at 0 and 3 h</td>
<td>10/10</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>at 0, 3, and 6 h</td>
<td>n.d.</td>
<td>n.d.</td>
<td>7/10b</td>
</tr>
</tbody>
</table>

* Immunocompetent BALB/c mice were challenged to i.v.-application of 30 µg of wild-type TNF or equivalent doses of TNF-fusion proteins at indicated time points. There was no difference in lethality between the groups receiving TNF in trimeric state.

* Animals treated with dimeric IgG1-based TNF-immunokine could be challenged to further application (p < 0.001).

![FIGURE 6. Therapeutic effects of TNF-based immunocytokines on growth pattern of FAP-expressing xenografts in BALB/c nu/nu mice. A. Animals were randomly assigned to different treatment groups, and i.v. administration of effectors started at day 9 every 3 days for five consecutive injections. Mice received 20 µg of TNF-equivalent doses (resuspended in 150 µl PBS) of anti-FAP IgG1-TNF (100 µg in total; ), scFv-TNF (33 µg in total, ) or the corresponding dose of parental IgG1 (80 µg, ) in combination with rhuTNF (20 µg in total, ) led to significant tumor growth delay, compared with parental IgG1 (450 µg in total, ) or PBS alone (400 µl in total; data not shown) (p = 0.003). Clinical monitoring showed slight weight loss without reaching significance.](image)
toxicity analysis by repeated administrations of TNF-containing rhuTNF administration than non-tumor-bearing animals (3). For these reasons, TNF-mediated lethality was evaluated by two different mouse strains, because BALB/c mice have a shorter blood circulation than murine TNF (23). Second, TNF sensitivity varies between different mouse strains, because BALB/c mice are more sensitive than immunocompetent counterparts (24). Therefore, we performed a second experiment in BALB/c nu/nu mice with increased TNF sensitivity as a result of immunodeficiency and xenografted tumors. As expected, all animals died after a single injection of 30 μg of trimerized huTNF (20, 24). There was no difference in lethality arising from i.v. administration of free huTNF or single-chain fused scFv-TNF, while the application of the dimeric IgG1-based fusion protein was tolerated without lethal side effects at this dose level. Concurrent dose escalation of dimeric IgG1-TNF finally defined 150 μg as its maximal tolerated TNF-equivalent dose. As a consequence of the trimeric-TNF related toxicity, we had to reduce the dose level and proved the anticancer efficacy of immunocytoxines at TNF-equivalent doses of 20 μg per injection. As prerequisite for a valid evaluation of dose-dependent therapeutic efficacy, the equivalent recognition of the targeted FAP-Ag was shown against the background of different Ab formats for both immunocytoxines (Fig. 3). Furthermore, the solid growth of FAP-expressing xenotransplants in BALB/c nu/nu mice provides an appropriate test system, because FAP expression of transfected HT1080 is stable over weeks, and exposure of tumor cells to TNF does not activate apoptotic pathways. Therefore, in accordance with the clinical situation, the anticancer activities induced by TNF-based constructs will rely on indirect tumoricidal mechanisms (20, 36). Administration of adjusted TNF-equivalent doses allowed repetitive treatment of animals resulting in measurable tumor growth delay. Although the maximum tolerated dose of anti-FAP IgG1-TNF was not reached, bioactivity of the stabilized dimeric fusion protein displayed enhanced therapeutic efficacy, compared with noncovalently trimerized scFv-TNF, unbound homotrimeric rhuTNF, or parental Abs in combination with soluble rhuTNF, respectively. Further dose escalation of IgG1-TNF increased its therapeutic efficacy, and repetitive administration led to significant tumor growth delay with tolerable clinical side effects. Our results suggest that unspecific peripheral interaction with TNF-R1, together with affected stability of trimeric-TNF proteins, above all at low concentrations (37, 38), is capable not only to increase unwanted systemic toxicity, but also to decrease the targeted antitumor activity. In the past, TNF-prodrug strategies were initiated to circumvent unspecific TNF-R1 complexation with trimeric scFv-TNF, while the application of the dimeric IgG1-based fusion protein was tolerated without lethal side effects at this dose level. Concurrent dose escalation of dimeric IgG1-TNF finally defined 150 μg as its maximal tolerated TNF-equivalent dose. As a consequence of the trimeric-TNF related toxicity, we had to reduce the dose level and proved the anticancer efficacy of immunocytoxines at TNF-equivalent doses of 20 μg per injection. As prerequisite for a valid evaluation of dose-dependent therapeutic efficacy, the equivalent recognition of the targeted FAP-Ag was shown against the background of different Ab formats for both immunocytoxines (Fig. 3). Furthermore, the solid growth of FAP-expressing xenotransplants in BALB/c nu/nu mice provides an appropriate test system, because FAP expression of transfected HT1080 is stable over weeks, and exposure of tumor cells to TNF does not activate apoptotic pathways. Therefore, in accordance with the clinical situation, the anticancer activities induced by TNF-based constructs will rely on indirect tumoricidal mechanisms (20, 36). Administration of adjusted TNF-equivalent doses allowed repetitive treatment of animals resulting in measurable tumor growth delay. Although the maximum tolerated dose of anti-FAP IgG1-TNF was not reached, bioactivity of the stabilized dimeric fusion protein displayed enhanced therapeutic efficacy, compared with noncovalently trimerized scFv-TNF, unbound homotrimeric rhuTNF, or parental Abs in combination with soluble rhuTNF, respectively. Further dose escalation of IgG1-TNF increased its therapeutic efficacy, and repetitive administration led to significant tumor growth delay with tolerable clinical side effects. Our results suggest that unspecific peripheral interaction with TNF-R1, together with affected stability of trimeric-TNF proteins, above all at low concentrations (37, 38), is capable not only to increase unwanted systemic toxicity, but also to decrease the targeted antitumor activity. In the past, TNF-prodrug strategies were initiated to circumvent unspecific TNF-R1 complexation with trimeric scFv-TNF fusion proteins. Some of these multistep approaches relying on Cu²⁺-dextran or biotin conjugates were tested in animal models despite their minor relevance with regard to the clinical situation (29, 31, 39, 40).

In conclusion, we have shown that differences in bioactivity profiles of TNF-based immunokines are related to their recombinant protein structure defining the multimeric state of TNF subunits. Binding analysis, in vitro, and in vivo toxicity data favor the dimeric IgG1-Ab-TNF format for further investigations to optimize therapeutic efficacy and warrant its clinical application.
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Disclosures
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References


