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

J Immunol 2006; 177:2423-2430; doi: 10.4049/jimmunol.177.4.2423
http://www.jimmunol.org/content/177/4/2423

References
This article cites 40 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/177/4/2423.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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. Compared to 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 latter trimerizes 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 had been shown to mediate regression of murine (2) and xenotransplanted human tumors (3, 4), TNF has attracted attention as a potent antimetastasis 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 (TNF 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 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

Copyright © 2006 by The American Association of Immunologists, Inc.
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, weaned off FCS, and transferred into a Technomouse system (Bio- Australia).

**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 glucose (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. Inflliximab was obtained from Centocor 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 IgG1 H chain (HC) or κ L chain (LC) sequences was used for recombinant 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 (BanH1 and NcoI) in the pREN HC-TNF construct. In accordance with published data, a 45 bp-sequence coding for 15aa (G,S)4 was inserted between the scFv and rhuTNF (19, 21). The TNF cDNA fragment was followed by a His-tag (NcoI 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 GenPulsar (Bio-Rad). In brief, 400 μl of cells (1 x 10⁶/ml) was mixed with 10 μg of circular DNA, electroporated (270V, 975 μF), and plated out in a volume of 150 ml of medium. G418 selection (0.5 mg/ml; Invitrogen Life Technologies) commenced after 2 days, methotrexate (5 mM; Lederle Laboratories) was added on day 7 and increased to 100 mM over 2 wk. Stable cell lines producing high level of Abs were expanded, weaned off FCS, and transferred into a Technomouse system (Bio-labs) for large-scale production. The supernatant was dialysed against PBS (pH 7.2 overnight) (4°C), and IgG1-derived immunocytokine was purified as described recently (20). The single-chain TNF construct was purified from the supernatant via its C-terminal His-tag by affinity chromatography (Ion Metal Affinity Chromatography on Zn⁺²-loaded Hi-Trap columns; Pharmacia). Possible endotoxin contamination of the final products was excluded by Limulus amebocyte assay (QCL 1000; BioWhittaker). The molecular mass determination of native immunocytokines was determined with a Superdex 200 prep gradient HiLoad 16/60 column using low and high gel filtration chromatography calibration kits (Amerham Biosciences).

The elution volumes were extracted from the chromatogram, and a calibration curve of calculated Kav values vs log m.w. was generated. The m.w. of both TNF-based fusion proteins was obtained from the calibration curve. SDS-PAGE analysis was performed as described before (20), and protein bands were visualized by staining with Coomassie brilliant blue (Sigma-Aldrich). Proteins were transferred to polyvinylidene difluoride membrane (Millipore) for Western blot analysis, and membranes were blocked with 10% nonfat dry milk before incubation with rabbit-anti-huTNF (1/1000), followed by goat-anti-rabbit IgG conjugated to HRP (1/3000; Bio-Rad), and developed by chemiluminescence (ECL-kit; Amerham Biosciences).

**Optical biosensor measurements**

Binding analysis of immunocytokines to soluble rhuTNF-R1 was performed by using a Biacore 3000 system. Monomerized TNF-R1 (Genzyme) was immobilized to the carboxymethylated dextran matrix of a CM5 sensor chip. To activate the carboxyl groups on the sensor chip, a solution containing 0.2 M N-ethyl-N-(3-dimethylaminopropyl)carbodiimide and 0.1 M N-hydroxysuccinimide was injected onto the chip with a flow rate of 5 μl/min. Then, 50 μl of 10 mM sodium acetate buffer (pH 4.0) as such or containing 25 μg/ml soluble huTNF-R1 was passed over one flow cell. 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 Biacore HBS-EP 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).

**Binding analysis**

Flow cytometry was performed as described previously (20). In brief, 1 x 10⁵ 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 Immununo; Nunc) were coated (overnight, 4°C) with inflliximab (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 O-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 10⁴) were cultured in 96-wells 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 rhTNF 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 rhTNF 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 at 50, 100, 160, or 200 μg, respectively.

Control groups received equal volumes 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 10⁶ FAP-transfected HT1080 cells in the right flank of
The results of the treatment with anti-FAP IgG1-TNF and anti-FAP scFv-TNF were evaluated in a model of FAP-expressing solid tumors in BALB/c nu/nu mice. Thirty animals were randomized 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 (suspending 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 huLgG1-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 transfected 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). Furthermore, the eluted fusion proteins were analyzed by size-exclusion fast protein liquid chromatography, and gel filtration profiles revealed dominant peaks for both TNF-constructs, with retention volumes corresponding to the apparent molecular mass of 149 kDa for IgG1-derived dimers. Migration at ~148 kDa for scFv-TNF immunokine confirmed its homotrimerization under native conditions (Fig. 2).

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. 3A 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 (H11002) 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 (A), or IgG1-TNF dimer (B) was measured by surface plasmon resonance signal detection. Reagents were injected at a concentration of $1\mu g/ml$. 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 $10\mu g/ml$ solution of either rhuTNF, scFv-TNF, or IgG1-TNF.
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( \times 10^4)</td>
<td>4.14( \times 10^{-4})</td>
<td>1.85( \times 10^{-9})</td>
</tr>
<tr>
<td>scFv-TNF</td>
<td>6397</td>
<td>9.09( \times 10^{-6})</td>
<td>1.42( \times 10^{-9})</td>
</tr>
<tr>
<td>IgG1-TNF</td>
<td>782.4</td>
<td>1.97( \times 10^{-5})</td>
<td>2.52( \times 10^{-5})</td>
</tr>
</tbody>
</table>

* 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.

TNF fusion protein to TNF-R1 behaves like rhuTNF and is more than 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 \(\approx 500 \mu g\) per mouse. To circumvent limited duration and strength of TNFR triggering, exposure time to the TNF portion of the constructs was enhanced by repetitive injections (23). Lethality of immunocompetent BALB/c mice 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 many 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, an 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
PBS alone did not result in measurable anti-tumor response.

level. Treatment of control groups receiving parental IgG1 Ab or 0.003). However, these animals showed reduced activity during the treatment schedule on mock-transfected (FAP-deficient) fibrosarcomas. Repeating the 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, ▲), rhuTNF (20 μg in total, ○), or the corresponding dose of parental IgG1 (80 μg, □ in combination with rhuTNF (20 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 regimens 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.a

<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>

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

Table III. Lethality induced by a single i.v. application of effectors in immunodeficient and tumor-bearing BALB/c nu/nu micea

<table>
<thead>
<tr>
<th>TNF-Equivalent dose</th>
<th>rhuTNF</th>
<th>scFv-TNF</th>
<th>IgG1-TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μg</td>
<td>1/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>50 μg</td>
<td>10/10</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>200 μg</td>
<td>nd</td>
<td>nd</td>
<td>3/5</td>
</tr>
</tbody>
</table>

a Administration regimens was performed to evaluate lethality following i.v. application of different formatted TNF-conjugates, compared with wild-type TNF.
b Application of IgG1-TNF was well tolerated, while toxic profiles of scFv-TNF and rhuTNF resulted in identical lethality (p > 0.001). The sublethal TNF-equivalent dose of 20 μg was used in later trials in order to compare anticancer activities of dimeric IgG1- and trimeric scFv-based immunokines.

c The maximal tolerated TNF-equivalent dose after i.v. injection of IgG1-TNF was 150 μg. Further dose escalation at 200 μg caused signs of severe toxicity and mortality.

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 PBS alone (400 μl in total; data not shown) (p = 0.003). Clinical monitoring showed slight weight loss without reaching significance.
IgG1 Ab resulting in a dimeric TNF formation. This construct has demonstrated its Ag-restricted therapeutic activity after i.v. administration with tolerable systemic toxicity (20). In consequence, a direct comparison of both immunocytokine formats and wild-type TNF under conditions of a relevant preclinical model is needed to define the optimal structure-activity relationship to establish a TNF-based immunotherapeutic principle. We produced both immunocytokines under identical conditions and yielded high expression levels of intact fusion proteins. Structure analysis confirmed expected dimerization of the IgG1-derived format, while trimerization of scFv-TNF-monomers was seen only under native conditions, because reduced stability of trim erized TNF single-chain formats has already been reported (15). To circumvent affected trimerization, TNF single-chain molecules were designed with intrinsic trimerization capability using covalent linkage via a coiled-coil trimerization domain derived from tenascin (31). Until now, in vivo reports about this construct are still missing, and enhanced immunogenicity resulting from the increased complexity of artificial recombinant fusion proteins is of great concern (32).

To further analyze the biological importance of the multimeric state of TNF-subunits, we first investigated the complex formation of immobilized monomeric TNF-R1 by IgG1-TNF dimer, scFv-TNF trim er, or wild-type TNF trim er, respectively, using surface plasmon resonance. The data given in Table I show the differences in interaction. Binding of trim erized wild-type TNF was characterized by a fast on-rate and displayed a 35-fold higher rate than trimerized scFv-TNF. However, the single-chain-coupled TNF-trimer displayed an ~10-fold increased association velocity to TNF-R1 than dimerized IgG1-TNF and proved to form the strongest complex with TNF-R1. As a consequence, the $K_d$ value for the complex with IgG1-TNF dimer was ~10-fold higher than the $K_d$ for the complex with trim erized scFv-TNF. Thus, the scFv-TNF/TFN R1 complex was much more stable than the IgG1-TNF/TFN R1 complex. To the best of our knowledge, there are no published data on the use of surface plasmon resonance to analyze complex formation of TNFR with Ab-fused TNF-molecules. Therefore, we cannot directly correlate our results with data from the literature. However, there was a strong positive correlation between binding and bioactivity profiles for the TNF domain containing reaction partners. The standard in vitro assay that depends on TNF-R1-mediated cytotoxicity in murine WEHI-164 cells confirmed these results. LC$_{50}$ of scFv-TNF was ~10-fold lower than that of rhuTNF, but nearly 10-fold higher than that of IgG1-TNF.

Signaling through TNF-R1 also will trigger cytotoxic side effects in vivo, because experiments with TNF-R1 or TNF-R2 knockout mice indicated that TNF-R1 is the main receptor governing TNF-induced symptoms of shock and, finally, lethality (33, 34). Furthermore, huTNF displays a selective species specificity in mice and interacts only with murine TNF-R1 and not murine TNF-R2 (35). Therefore, comparison of LD$_{50}$ values after i.v. application of rhuTNF and the TNF-based constructs in mice can undoubtedly serve as a preclinical model to evaluate unwanted systemic toxicity. However, activity of rhuTNF in mice is influenced not only by selective triggering of murine TNF-R1, but also by additional factors. First, huTNF is cleared much faster from the circulation than murine TNF (23). Second, TNF sensitivity varies between different mouse strains, because BALB/c nu/nu mice are 2.5 times more sensitive than immunocompetent counterparts (24). Third, tumor-bearing mice are more susceptible to toxic effects of rhuTNF administration than non-tumor-bearing animals (3). For these reasons, TNF-mediated lethality was evaluated by two different administration regimens. As a start, we performed in vivo toxicity analysis by repeated administrations of TNF-containing effectors, because huTNF stays only for a limited period of time in the blood circulation of immunocompetent BALB/c mice as a result of rapid clearance (23). Two consecutive applications after a 3-h time interval defined LD$_{100}$ at a cumulative dose of 60 µg of rhuTNF per mouse. In contrast with the identical LD$_{100}$ of rhuTNF and scFv-TNF, a further application of dimerized IgG1-TNF was needed to even reach LD$_{50}$ at a cumulative TNF-equivalent dose of 90 µg per mouse. These data clearly demonstrate the dramatically enhanced toxicity of trim erized scFv-TNF, compared with the dimerized TNF-immunokine. Considering these differences in TNF-R1 triggering, one may conclude that not only unwanted systemic toxic effects, but also therapeutic efficacy, might be induced at lower TNF-equivalent doses of i.v.-administered scFv-TNF, compared with IgG1-TNF. 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 trim erized huTNF (20, 24). There was no difference in lethality arising from i.v. administration of free rhuTNF 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 trim eric-TNF related toxicity, we had to reduce the dose level and proved the antican cer efficacy of immunocytokines 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 immunocytokines (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 antican cer 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 trim erized scFv-TNF, unbound homotrimeric huTNF, 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 trim eric-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 trim eric scFv-TNF proteins. Some of these multistep approaches relying on Cu$^{2+}$-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.
Acknowledgments
We thank Anja Reich and Nicole Jordan for excellent technical assistance. We thank the Department of Clinical and Experimental Surgery (Universität des Saarlandes, Homburg/Saar, Germany) for housing and handling of animals.

Disclosures
The authors have no financial conflict of interest.

References