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Transglutaminase-catalyzed covalent multimerization of camelidae anti-human TNF single domain antibodies improves neutralizing activity

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ABSTRACT

Tumor necrosis factor (TNF) plays an important role in chronic inflammatory disorders, such as Rheumatoid Arthritis and Crohn's disease. Recently, monoclonal Camelidae variable heavy-chain domain-only antibodies (V_HH) were developed to antagonize the action of human TNF (hTNF). Here, we show that hTNF–V_HH does not interfere with hTNF trimerization, but competes with hTNF for hTNF-receptor binding. Moreover, we describe posttranslational dimerization and multimerization of hTNF–V_HH molecules *in vitro* catalyzed by microbial transglutaminases (MTG). The ribonuclease S-tag-peptide was shown to act as a peptidyl substrate in covalent protein cross-linking reactions catalyzed by MTG from *Streptomyces mobaraensis*. The S-tag sequence was C-terminally fused to the hTNF–V_HH and the fusion protein was expressed and purified from *Escherichia coli* culture supernatants. hTNF–V_HH-S-tag fusion proteins were efficiently dimerized and multimerized by MTG whereas hTNF–V_HH was not susceptible to protein cross-linking. Cell cytotoxicity assays, using hTNF as apoptosis inducing cytokine, revealed that dimerized and multimerized hTNF–V_HH proteins were much more active than the monomeric hTNF–V_HH. We hypothesize that improved inhibition by dimeric and multimeric single chain hTNF–V_HH proteins is caused by avidity effects.

1. Introduction

TNF is an important regulator of immune cell proliferation, survival, differentiation, and apoptosis (Hehlgans and Pfeffer, 2005). As a consequence, TNF plays a pivotal role in acute and chronic inflammatory conditions, such as sepsis (Beutler et al., 1985), rheumatoid arthritis (Maini et al., 1995) and Crohn's disease (Van Dullemen et al., 1995). hTNF is present as a trimeric transmembrane protein and can be cleaved by ADAM metalloproteinases into a soluble form (sTNF) (Kriegler et al., 1988; Black et al., 1997). hTNF binds to cell-surface TNF receptor 1 (TNFR1, p55) or TNFR2 (p75) (Smith et al., 1994). These receptors undergo ligand-induced multimerization and associate with signaling molecules such as TNF receptor-associated death domain (TRADD) protein and TNF receptor-associated factors (TRAFs) which in turn initiate several downstream intracellular cascades (Heyninck and Beyaert, 2001).

Today, three anti-TNF drugs, engineered on the basis of monoclonal antibodies, infliximab (Remicade™), adalimumab (Humira™), and certolizumab pegol (CIMZIA™, CDP870), were approved for treatment of human diseases, such as rheumatoid arthritis and Crohn's disease (Abbott Laboratories 2007; Sandborn et al., 2007). Additionally, engineered soluble TNFR2, etanercept (Enbrel™), is approved for the treatment of rheumatoid arthritis and psoriasis (Ducharme and Weinberg, 2008). Infliximab is a chimeric monoclonal antibody composed of a human IgG1 constant region and a murine variable region. Adalimumab is a completely humanized monoclonal antibody. Certolizumab pegol is a pegylated, humanized monoclonal anti-TNF Fab′ (fragment, antigen binding) fragment. Etanercept consists of two extracellular domains of the human TNFR2 (p75) fused to the Fc fragment of a human IgG1. In contrast to anti-TNF antibodies, which can bind monomeric and trimeric TNF, etanercept binds only trimeric TNF, at a ratio of one trimer per one etanercept dimer. Since each monoclonal antibody molecule contains two TNF binding sites, they have the potential by bridging interactions among multiple TNF trimers to form large immune complexes (Scallon et al., 2002).

Camelidae heavy-chain antibodies might provide an alternative molecular principle to generate drugs for TNF-dependent diseases. Camelidae produce a fraction of functional immunoglobulins that

Abbreviations: hTNF, human tumor necrosis factor; V_HH, heavy-chain only antibodies; MTG, microbial transglutaminases; PARP, poly(ADP-ribose) polymerase; ScFv, single chain Fv; CDR3, complementary-determining region 3.

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are devoid of light chains (Hamers-Casterman et al., 1993). The heavy-chain antibodies bind the antigen with only a single variable heavy-chain domain (V_HH). The V_HH domain can be separately expressed and is about 10 times smaller than a conventional IgG molecule. The small size of the V_HH domain allowed production in microorganisms (Arbabi Ghahroudi et al., 1997). In contrast to conventional antibody fragments, V_HH domains are highly resistant to high temperatures and their thermal unfolding is reversible (Arbabi Ghahroudi et al., 1997). Importantly and perhaps surprisingly, V_HH and conventional antibodies are comparable in terms of specificity and affinity for their antigens, with dissociation constants (K_D) in the nanomolar range (Lauwereys et al., 1998).

Monoclonal V_HH antibodies were developed to bind GFP (Rothbauer et al., 2008), to antagonize EGFR (Roovers et al., 2007) or to inhibit the formation amyloid fibrils from human lysozyme (Dumoulin et al., 2003), and to prevent the formation of mature amyloid fibrils by stabilizing Aβ protofibrils, which is a common biochemical characteristic that occurs in Alzheimer's disease (Habicht et al., 2007).

Recently, monoclonal Camelidae heavy-chain antibodies were developed to antagonize murine and human TNF (Coppieters et al., 2006). Anti-murine TNF–V_HH domains were shown to block the development of collagen-induced arthritis (Coppieters et al., 2006). Moreover bivalent molecules consisting of two anti-TNF–V_HH domains connected by a flexible linker were shown to be much more potent than the monovalent molecules (Coppieters et al., 2006).

As alternative, we decided to posttranslationally cross-link V_HH proteins *in vitro*, catalyzed by transglutaminase. Transglutaminases specifically catalyze the acyl-transfer reaction between the γ-carboxyamide group of a Gln residue (acyl donor) and the ε-amino group of a Lys residue (acyl acceptor) of proteins and peptides, which can be adopted to cross-link target proteins via an ε-(γ-glutamyl)lysine bridge (Folk, 1983; Wold, 1985). Interestingly the cross-linked proteins, which are often of high molecular mass, are highly resistant to mechanical challenge and proteolytic degradation (Griffin et al., 2002). Products of transglutaminases are found in a variety of tissues and processes where such properties are relevant, including skin, hair, blood clotting and wound healing (Griffin et al., 2002). Microbial transglutaminases (MTG) from *Streptomyces mobaraensis* do not require Ca^{2+} for activity (Ando et al., 1989; Kanaji et al., 1993). These enzymes can be easily produced and have found several applications as biocatalysts in the food, cosmetic and textile industry (Griffin et al., 2002). Recently, MTG was used for biotinylation of monoclonal antibodies (Josten et al., 2000), and a poly(ethyleneglycol)-modification of human interleukin 2 (Sato et al., 2001). The ribonuclease S-tag-peptide was shown to work as a good peptidyl substrate in protein cross-linking reactions catalyzed by microbial transglutaminase (MTG) from *S. mobaraensis* exemplified with S-tagged enhanced green fluorescent protein (EGFP) as a model protein (Kamiya et al., 2003).

By using hTNF and the soluble TNF-antagonist etanercept, we show that hTNF–V_HH does not interfere with hTNF trimerization, but competes with the hTNF receptor p75 for binding to hTNF trimers. Moreover, the posttranslational dimerization and multimerization of S-tagged hTNF–V_HH proteins by MTG from *S. mobaraensis* led to highly potent bivalent and multivalent hTNF–V_HH molecules with improved inhibitory profiles.

2. Materials and methods

2.1. Cells and reagents

L929 cells and HEK293 cells were from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were grown in DMEM high glucose culture medium (PAA Laboratories, Marburg, Germany) supplemented with 10% fetal calf serum, penicillin (60 mg/l) and streptomycin (100 mg/l) at 37 °C with 5% CO_2 in a water saturated atmosphere. Recombinant hTNF was a gift from Daniela N. Maennel, Dept. Immunology, University of Regensburg, Germany. Etanercept (Enbrel™) was from Wyeth Pharma (Münster, Germany). Anti-c-myc antibodies were purchased from Cell Signaling, New England Biolabs (Schwalbach, Germany). Anti-PARP antibodies were from Cell signaling (Beverly, MA, USA). MTG transglutaminase was purchased from N-zyme (Darmstadt, Germany). All restriction enzymes were obtained from Fermentas (St. Leon-Rot, Germany).

2.2. Cloning of hTNF–V_HH into Escherichia coli expression plasmids

The cDNA coding for anti-hTNF–V_HH was synthesized by Geneart (Munich, Germany) (Coppieters et al., 2006). The pCRScript-anti-hTNF–V_HH plasmid was digested with NcoI and NotI and the purified cDNA coding for anti-hTNF–V_HH was cloned into the *E. coli* expression plasmid pet23a(+)-pelB-IL-6-c-myc-his (unpublished results). The resulting plasmid was named pet23a(+)-pelB-anti-TNF–V_HH-c-myc-his. The cDNA coding for the ribonuclease S-tag-peptide was synthesized by Geneart (Munich, Germany). The sequence was amplified by PCR using primers containing NotI endonuclease restriction sites (5′primer: 5′-3′: CTGAGCGCGGCCGCACTCGAGGTCGAC and 3′primer: 5′-3′: CTGAGCGGCCGCGGAGCCGGAGTCCATGTG) and digested with NotI. The purified PCR product was subcloned into pet23a(+)-pelB-anti-TNF–V_HH-c-myc-his digested with NotI. The resulting plasmid was named pet23a(+)-pelB-anti-TNF–V_HH-S-tag-c-myc-his.

2.3. Expression and purification of hTNF–V_HH and hTNF–V_HH-S-tag in E. coli

The plasmids pet23(+)-pelB-anti-TNF–V_HH-c-myc-his and pet23(+)-pelB-anti-TNF–V_HH-S-tag-c-myc-his were transformed into *E. coli* BL21 pLysS and protein expression was achieved by growing bacteria in LB-Media for 18 h at 30 °C after induction with 1 mM isopropyl-β-D-thiogalactopyranosid at an OD_{600} of 0.6–0.8. Cells were collected by centrifugation and the cleared anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag-protein-containing supernatants, respectively, were supplemented with an equal volume of phosphate buffer (500 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.4). 1 ml Ni-NTA agarose was added per 1 l supernatant and stirred at 4 °C for 2 h and then loaded on a column. Proteins were eluted with 500 mM imidazole in phosphate buffer.

2.4. Size exclusion chromatography

Recombinant anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins were further purified on a calibrated HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) using 10 mM sodium phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.5) or phosphate buffer with 150 mM NaCl as the mobile phase with a constant flow-rate of 1 ml/min. The column was calibrated with the low molecular mass standard, containing Blue Dextran 2000 (2000 kDa), bovine serum albumin (67 kDa; ±10%), ovalbumin (43 kDa; ±15%) and chymotrypsinogen A (25 kDa; ±25%) (Amersham Biosciences, Freiburg, Germany). Fractions of 2.5 ml were collected, analyzed by SDS-PAGE, pooled and concentrated.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Microtitre plates (Greiner Microlon, Solingen, Germany) were coated with recombinant hTNFα (10 μg/ml) in PBS and incu-

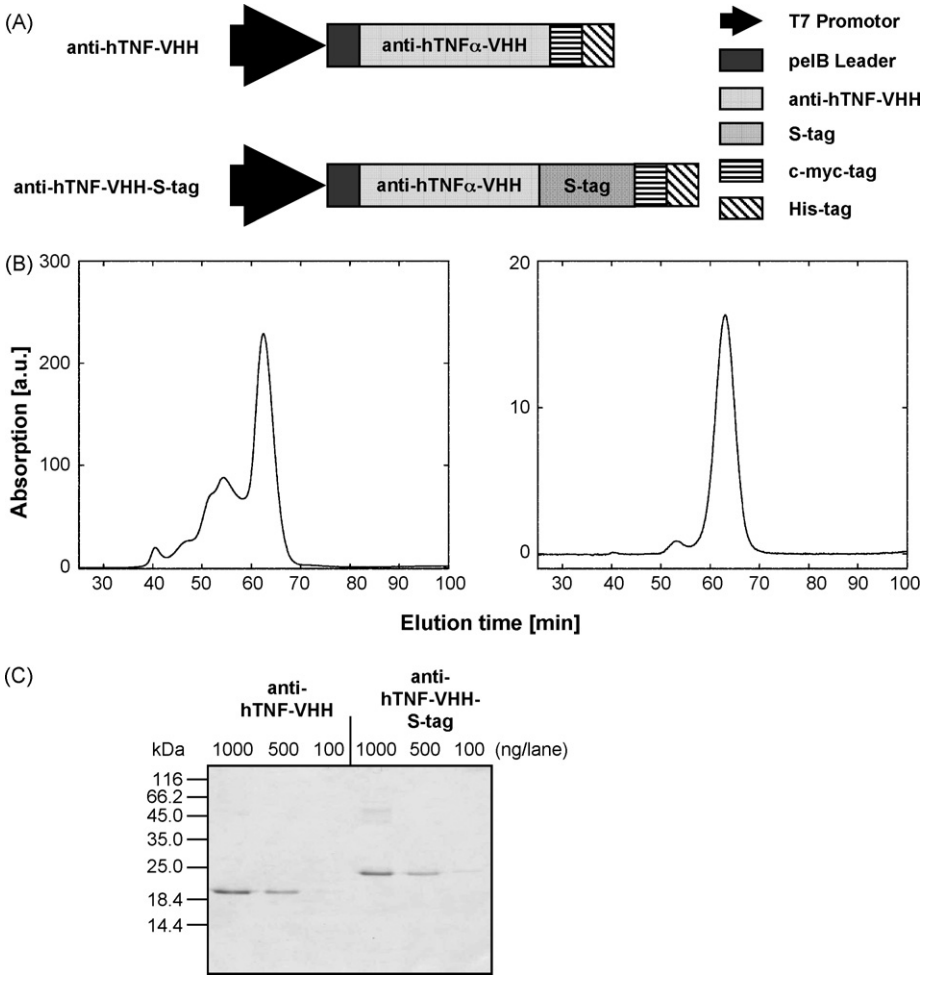

Fig. 1. Expression and purification of anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag. (A) Schematic illustration of expression constructs coding for anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag. (B) Size exclusion chromatography of anti-hTNF–V_HH-S-tag (2 ml) after purification of the protein via NTA-agarose on a calibrated HiLoad 16/60 Superdex 75 prep grade column using a 10 mM sodium phosphate buffer, pH 7.5 as the mobile phase with a constant flow-rate of 1.0 ml/min (left panel). Subsequently the fractions with monomeric anti-hTNF–V_HH-S-tag proteins were pooled, concentrated and again analyzed by size exclusion chromatography (right panel). (C) Defined amounts of anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag were separated on a 15% SDS polyacrylamide gel and visualized by coomassie staining.

bated overnight at room temperature. After blocking with 3% bovine serum albumin in PBS for 2 h, 25 ng/ml anti-hTNF–V_HH in 1% BSA PBS-T (+0.05% Tween-20) was added for 1 h. Thereafter, the V_HH–anti-hTNF solution was replaced by indicated Enbrel concentrations, which were incubated for another 1 h at RT. V_HH–anti-hTNF bound to the plate was visualized by anti-c-myc antibodies followed by anti-mouse IgG horseradish peroxidase diluted in PBS/1% BSA. The enzymatic reaction was performed with soluble peroxidase substrate (BM blue POD from Roche, Mannheim, Germany) at RT for 10 min and stopped with 1 M H_2SO_4 (50 μl/well). The absorbance was measured at 450 nm on a SLT Rainbow plate reader from Tecan (Maennedorf, Switzerland).

2.6. Immunoblotting and enhanced chemiluminescence (ECL) detection

For Western blotting L929 cells were washed once with PBS after removing the medium and lysed directly in 2× Laemmli buffer. Protein concentration was determined by BCA protein assay (Pierce Biotechnology, Rockford, USA) and 10 μg of protein was subjected to SDS-PAGE samples after heating for 5 min at 95 °C, and transferred to a polyvinylidendifluoride membrane (Hybond-P from Amersham Biosciences, Freiburg, Germany) by a semi-dry electro-blotting procedure. The membrane was blocked in a solution of TBS (10 mM Tris, pH 8, 150 mM NaCl) supplemented with 0.02% Tween-20 and 6% skimmed milk powder, and probed overnight with the anti-PARP antibody (1:2000) from Cell signaling (Beverly, MA) at 4 °C, followed by incubating with horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected using a chemiluminescence kit (ECL plus Western Blotting Detection System, Amersham Biosciences, Freiburg, Germany) following the manufacturer's instructions.

2.7. Cross-linking of anti-hTNF–V_HH-S-tag by MTG

The purified anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins were diluted to a concentration of 50 μg/ml and supplemented with MTG as indicated (0.25 U/ml to 0.015 U/ml) and incubated for 30 h at 4 °C in the 10 mM sodium phosphate reaction buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.5). For mass production of cross-linked anti-hTNF–V_HH-S-tag proteins, 500 μg/ml anti-hTNF–V_HH-S-tag protein was supplemented with MTG (0.01–0.02 U/ml) and incubated for 15 h at 4 °C. The sample was subjected to size exclusion chromatography.

2.8. L929-cytotoxicity assays with hTNF

L929 cells were seeded onto a 96-well plates at a density of 10,000 cells/well and cultured for 24 h. Thereafter the cells were incubated for additional 24 h with or without

100 ng/ml hTNF and with or without indicated concentrations of anti-hTNF–V_HH-variants. The CellTiter-Blue Cell Viability Assay (Promega, Mannheim, Germany) was used to quantify the cellular proliferation following the manufacturer's instructions and measured on a Lambda Fluoro 320 Fluorimeter (ex-filter 530/25, em-filter 590/35, sensitivity 75, Software KC4). The maximal fluorescence (560/590) values varied between 30,000 and 80,000 depending on the incubation time for fluorescence development. All samples were measured in triplicates.

2.9. NFκB-activation in HEK293 cells

Activation of the transcription factor NF-κB in HEK293 cells was quantified using a dual-luciferase reporter gene assay (Promega, Madison, WI) according to the manufacturerĩs instructions. Cells seeded on 96-well plates were transfected with 15 ng/well of pNF-κB_Luc plasmid (Stratagene, La Jolla, CA, USA) in combination with 5 ng/well of pRL-TK (Promega). Cells were stimulated with 10 ng/ml hTNF for 6 h or left untreated. Cell lysates were analyzed on a Tecan Genios Pro microplate luminometer (Tecan Trading AG, Switzerland). All samples were measured in duplicates. The results for NF-κB driven firefly luciferase activity were normalized using the reference plasmid and expressed as relative light units (RLU).

3. Results

3.1. Expression and purification of anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins from E. coli

A cDNA coding for anti-hTNF–V_HH was subcloned into the *E. coli* expression plasmid pet23a(+). Here, the anti-hTNF–V_HH was N-terminally fused to a sequence coding for a pelB leader for periplasmatic secretion and C-terminally fused to a sequence coding for the c-myc tag for immunochemical detection and the his-tag for purification via NTA-affinity chromatography (Fig. 1A). S-tagged EGFP could be specifically cross-linked in a MTG-dependent manner (Kamiya et al., 2003). Since our aim was to posttranslationally generate bivalent and multivalent anti-hTNF–V_HH antibodies, we fused the anti-hTNF–V_HH cDNA with a cDNA coding for a short linker peptide and the S-tag peptide for transglutaminase mediated protein cross-linking, the c-myc-tag and his-tag coding sequences were again placed at the very N-terminus (Fig. 1A and supplementary Fig. 1A and 1B). The plasmids were transformed into *E. coli* strain BL21 pLysS and protein expression was induced by IPTG. Recombinant anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins from *E. coli* supernatants were readily visible as faint bands in coomassie-stained SDS-PAGE gels (data not shown). Recombinant anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins were purified by NTA-affinity chromatography and size exclusion chromatography. Interestingly, recombinant anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins did not run as a single peak on the size exclusion chromatography, indicating that although most of the protein was produced as monomers, there was also a minor fraction of dimers and multimers (Fig. 1B, left panel, data not shown for anti-hTNF–V_HH). To analyze whether the monomeric fraction is stable, monomeric anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins were again analyzed by size exclusion chromatography, which led to a single elution peak (Fig. 1B, right panel, data not shown for anti-hTNF–V_HH). We concluded that the pure recombinant anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins were in a homogenous, monomeric conformation. The overall yields of pure recombinant anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag were approximately 10 mg/l. The purity of the recombinant hTNF–V_HH and hTNF–V_HH-S-tag was confirmed on coomassie-stained SDS-PAGE gels (Fig. 1C).

3.2. Anti-hTNF–V_HH binds to trimeric hTNF and competes with Enbrel for binding to trimeric hTNF

We next used size exclusion chromatography to analyze whether anti-hTNF–V_HH binds to trimeric hTNF (Fig. 2A and B). First, recombinant hTNF and anti-hTNF–V_HH-S-tag proteins were separately analyzed by size exclusion chromatography (estimated from size exclusion: 34.1 kDa, expected: 20.9 kDa). However, it has to be noted that anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag seemed to have higher molecular weights on the size exclusion chromatography and on coomassie-stained gels as calculated from amino acid composition, even though the mass was correct as determined by mass spectroscopy (data not shown). Recombinant hTNF (monomer: 17.3 kDa) eluted as di- or trimeric complex as estimated from the column calibration (estimated from size exclusion: 40.4 kDa, expected: dimeric 34.6 kDa/trimeric 51.9 kDa), whereas recombinant anti-hTNF–V_HH-S-tag was eluted as a monomer. Secondly, recombinant hTNF (final concentration: 525 μg/ml) and anti-hTNF–V_HH-S-tag (final concentration: 175 μg/ml) were mixed at 3.7:1 monomeric molar ratio, which reflects an almost 1:1 molar ratio for trimeric hTNF vs. monomeric anti-hTNF–V_HH-S-tag. Before size exclusion chromatography, the proteins were pre-incubated for 30 min at 4 °C min to allow binding of anti-hTNF–V_HH-S-tag to hTNF. Pre-incubation of hTNF and anti-hTNF–V_HH-S-tag resulted in an almost complete shift of the trimeric hTNF to a molecular weight of about 80 kDa (estimated from size exclusion: 82.5 kDa, expected: trimeric hTNF 3× 17.3 kDa + 1× anti-hTNF–V_HH-S-tag 20.9 kDa = 72.8 kDa) indicating that hTNF trimers were not destroyed by binding to anti-hTNF–V_HH-S-tag.

We then performed a competition ELISA to analyze whether the neutralizing activity of anti-hTNF–V_HH-S-tag is based on the inhibition of TNF-receptor binding to the trimeric hTNF. Therefore we coated recombinant hTNF on an ELISA plate and subsequently incubated the wells with constant amounts of the anti-hTNF–V_HH-S-tag. Thereafter unbound anti-hTNF–V_HH-S-tag was washed away and replaced by indicated concentrations of Enbrel, a soluble hTNFR2 receptor antagonist which is dimerized via a Fc fusion (Ducharme and Weinberg, 2008). It was previously shown that Enbrel exclusively binds to trimeric hTNF (Scallon et al., 2002). Binding of anti-hTNF–V_HH-S-tag proteins was detected with anti-c-myc-specific monoclonal antibodies and a secondary peroxidase-connected antibody directed against the Fc-portion of the anti-c-myc-tag antibody. In the absence of Enbrel, the anti-hTNF–V_HH-S-tag bound to immobilized hTNF (Fig. 2C). Anti-hTNF–V_HH-S-tag bound to hTNF was replaced by Enbrel in a dose-dependent manner, indicating that anti-hTNF–V_HH-S-tag and Enbrel competed for binding to trimeric TNF.

3.3. Cross-linking of anti-hTNF–V_HH-S-tag proteins by microbial transglutaminase and isolation of dimeric and multimeric anti-hTNF–V_HH-S-tag proteins

To identify optimal conditions to cross-link anti-hTNF–V_HH proteins, 50 μg/ml recombinant anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins were incubated in reaction buffer with increasing amounts of microbial transglutaminase (MTG) from *S. mobaraensis*, as schematically illustrated in Fig. 3A and described in Section 2. As depicted in Fig. 3B, recombinant anti-hTNF–V_HH proteins were not or only to a small extent cross-linked by MTG, indicating that anti-hTNF–V_HH was not a substrate for MTG cross-linking. However, S-tagged recombinant anti-hTNF–V_HH proteins were efficiently cross-linked by MTG into dimeric and multimeric products over a wide range of MTG concentrations (0.25 U/ml to 0.015 U/ml) without a decrease of cross-linking end-products. Importantly, more than 50% of the monomeric recombinant anti-hTNF–V_HH-

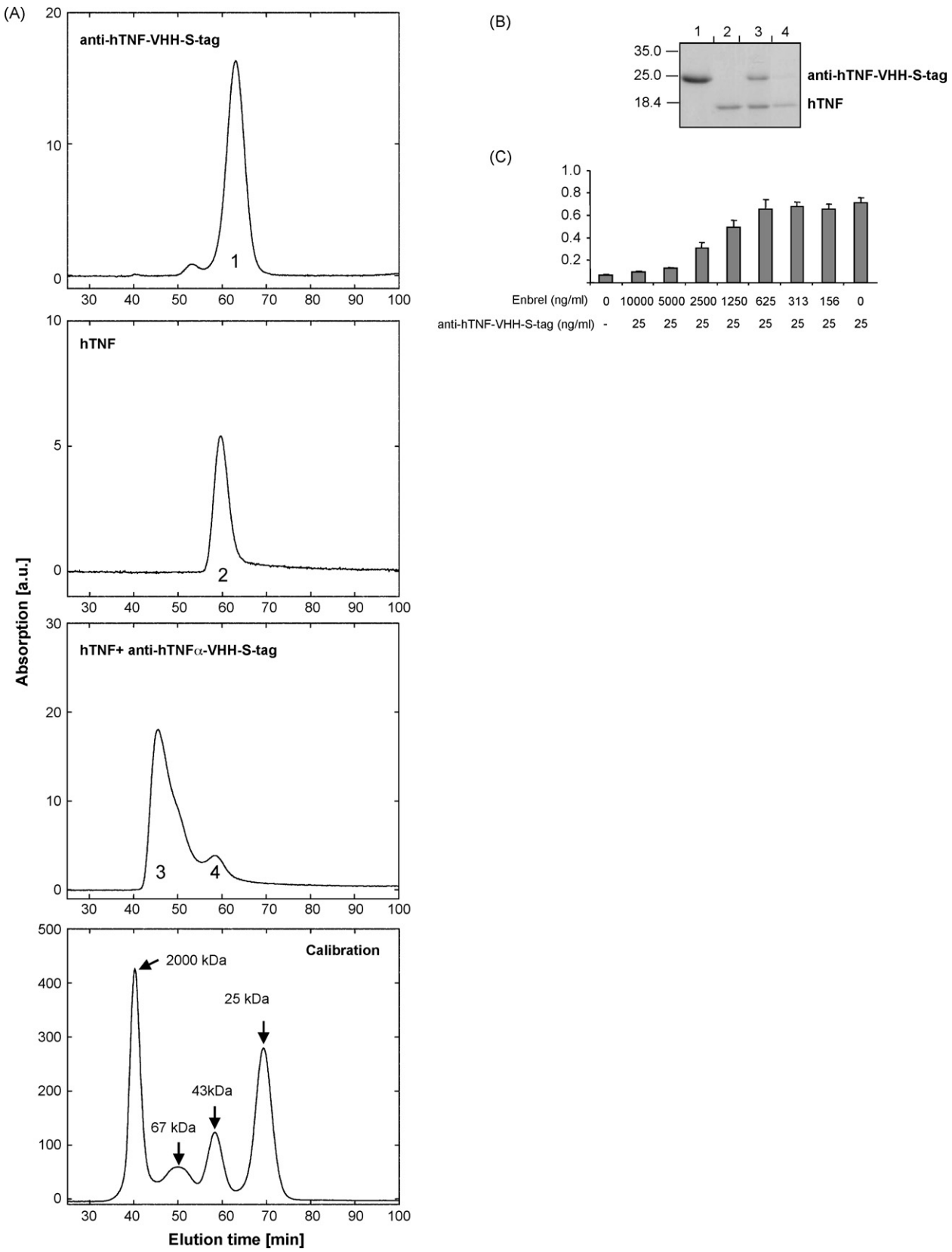

Fig. 2. Characterization of the binding properties of anti-hTNF–V_HH-S-tag to hTNF. (A) Size exclusion chromatography of anti-hTNF–V_HH-S-tag, hTNF or a combination of anti-hTNF–V_HH-S-tag and hTNF on a HiLoad 16/60 Superdex 75 prep grade column using a 10 mM sodium phosphate buffer at pH 7.5, 150 mM NaCl as the mobile phase with a constant flow-rate of 1.0 ml/min. The column was calibrated with the Blue Dextran 2000 (2000 kDa), bovine serum albumine (67 kDa), ovalbumine (43 kDa) and chymotrypsinogen A (25 kDa). (B) Fractions 1–4 indicated in (A) were separated on a 15% SDS polyacrylamide gel and visualized by coomassie staining. (C) Enbrel can compete with anti-hTNF–V_HH-S-tag for the binding of hTNF. hTNF (10 ng/ml) was coated on ELISA plates, subsequently the wells were incubated with constant amounts anti-hTNF–V_HH-S-tag (25 ng/ml) and increasing amounts of Enbrel (from 156 ng/ml to 1000 ng/ml). The residual binding of the c-myc tagged anti-hTNF–V_HH-S-tag to hTNF was analyzed.

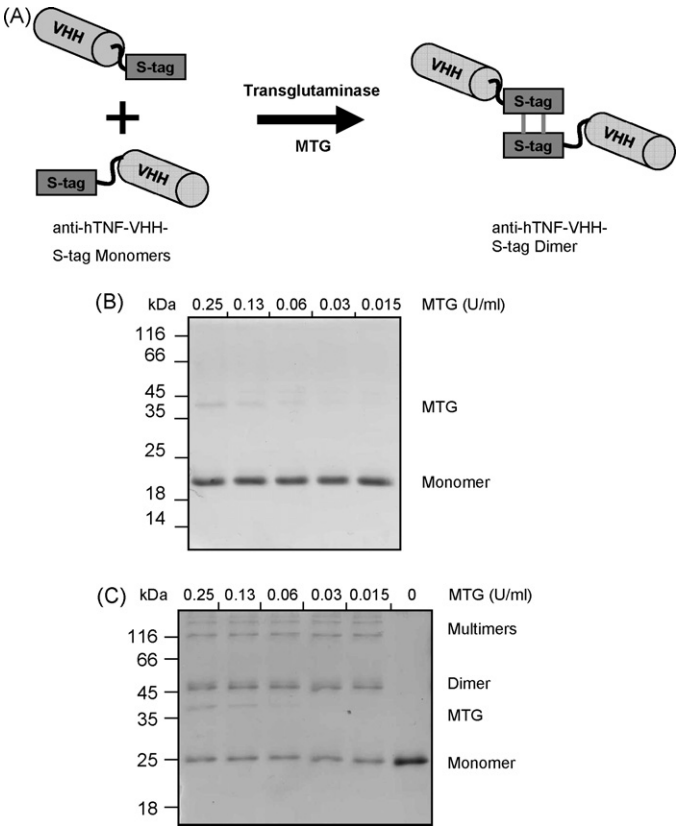

Fig. 3. Cross-linking of anti-hTNF–V_HH-S-tag by MTG. (A) Schematic illustration of MTG mediated protein cross-linking. (B) Constant amounts of anti-hTNF–V_HH (50 μg/ml) were incubated for 30 h at 4 °C with decreasing amounts of recombinant MTG (U/ml). Monomeric and cross-linking products were separated on a 15% SDS polyacrylamide gel and visualized by coomassie staining. (C) Constant amounts of anti-hTNF–V_HH-S-tag (50 μg/ml) were incubated for 30 h at 4 °C with decreasing amounts of recombinant MTG (U/ml). Monomeric and cross-linking products were separated on a 15% SDS polyacrylamide gel and visualized by coomassie staining.

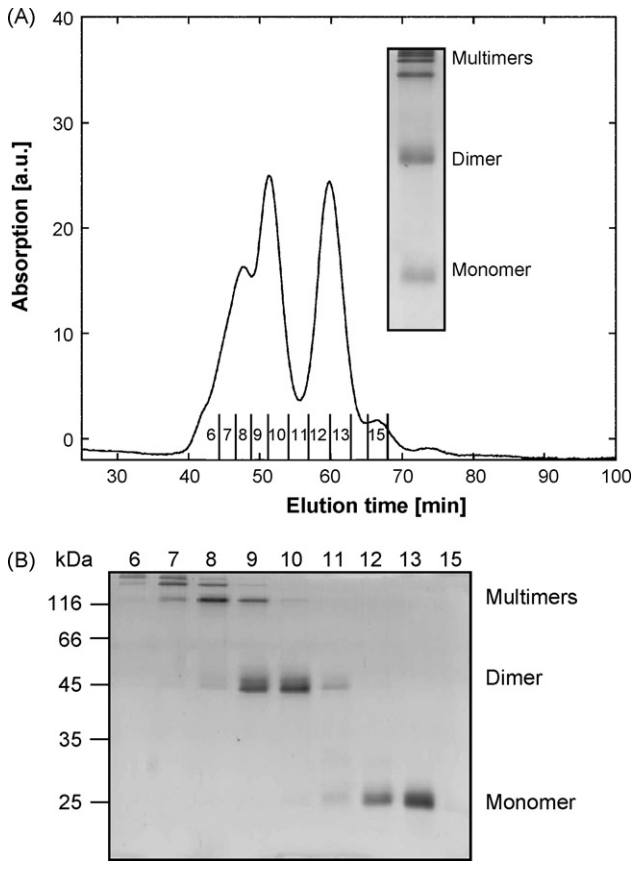

Fig. 4. Quantitative separation of anti-hTNF–V_HH-S-tag monomers, dimers and multimers by size exclusion chromatography. (A) Size exclusion chromatography of anti-hTNF–V_HH-S-tag monomer, dimers and multimers on a HiLoad 16/60 Superdex 75 prep grade column using a 10 mM sodium phosphate buffer at pH 7.5 as the mobile phase with a constant flow-rate of 1.0 ml/min. (B) Fractions 6–13 and 15 as indicated in (A) were separated on a 15% SDS polyacrylamide gel and visualized by coomassie staining.

S-tag proteins were cross-linked into di- or multimers by MTG (Fig. 3C).

The mono-, di- and multimeric anti-hTNF–V_HH-S-tag proteins were separated from each other in a single run by preparative size exclusion chromatography (Fig. 4A). The samples 6–13 and 15 from this size exclusion chromatography were analyzed by coomassie-staining of SDS-PAGE gels (Fig. 4B), and indicated that the fractions 6–8 mainly contained multimeric, fraction 10 dimeric and fractions 12–13 monomeric anti-hTNF–V_HH-S-tag proteins. The fractions 6–8 and 10 were concentrated and used for subsequent activity bioassays.

3.4. Bivalent and multivalent anti-hTNF–V_HH-S-tag dimers and multimers possess markedly higher biological activity than monovalent anti-hTNF–V_HH-S-tag monomers

L929 cells undergo apoptosis in the presence of hTNF (Schmid et al., 1986). We treated L929 cells with hTNF to estimate the inhibitory capacity of monomeric, dimeric and multimeric anti-hTNF–V_HH proteins. Initially, we compared the activity of anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag, to exclude that the N-terminal addition of the S-tag peptide interferes with anti-hTNF–V_HH activity. As depicted in Fig. 5A, anti-hTNF–V_HH and anti-hTNF–V_HH-S-tag proteins were able to inhibit hTNF-induced cell death of L929 in a dose-dependent manner. Importantly, the inhibitory profile of both proteins was identical, indicating that the N-terminal S-tag does not interfere with antigen binding.

We then compared monomeric and dimeric anti-hTNF–V_HH-S-tag proteins in the L929 cytotoxicity assay (Fig. 5B). At the molar level, referring to binding sites/molecule, the bivalent anti-hTNF–V_HH-S-tag dimers (IC_{50} = 0.19) were 5-fold more active than the monovalent anti-hTNF–V_HH-S-tag monomer (IC_{50} = 0.93), indicating that this dimerization strategy leads to fully active bivalent antibodies with improved activity as described for single-chain anti-TNF–V_HH proteins (Coppieters et al., 2006). Apoptosis induction in L929 cells was visualized by the disappearance of poly(ADP-ribose) polymerase (PARP) (Fig. 5C). Degradation of PARP could be detected in cells treated with 100 ng/ml hTNF and in cells treated with 100 ng/ml hTNF and 100 ng/ml anti-hTNF–V_HH monomer, which was in line with the results from the cytotoxicity analysis shown in Fig. 5B. On the other hand, no PARP degradation was detected in cells treated with 100 ng/ml hTNF and 100 ng/ml anti-hTNF–V_HH dimer, indicating that these cells did not undergo apoptosis, supporting the finding that the dimeric anti-hTNF–V_HH protein had improved inhibitory capacity. At higher concentrations of anti-hTNF–V_HH (1000 ng/ml) both, monomeric and dimeric anti-hTNF–V_HH, proteins were able to inhibit PARP degradation and apoptosis of L929 cells. Additionally, activation of the transcription factor NF-κB in HEK293 cells was determined using a dual-luciferase reporter gene assay. Cells were stimulated with 10 ng/ml hTNF for 6 h or left untreated in the presence of increasing amounts of monomeric and dimeric anti-hTNF–V_HH. Again we showed, that anti-hTNF–V_HH-S-tag dimers had improved inhibitory activity on

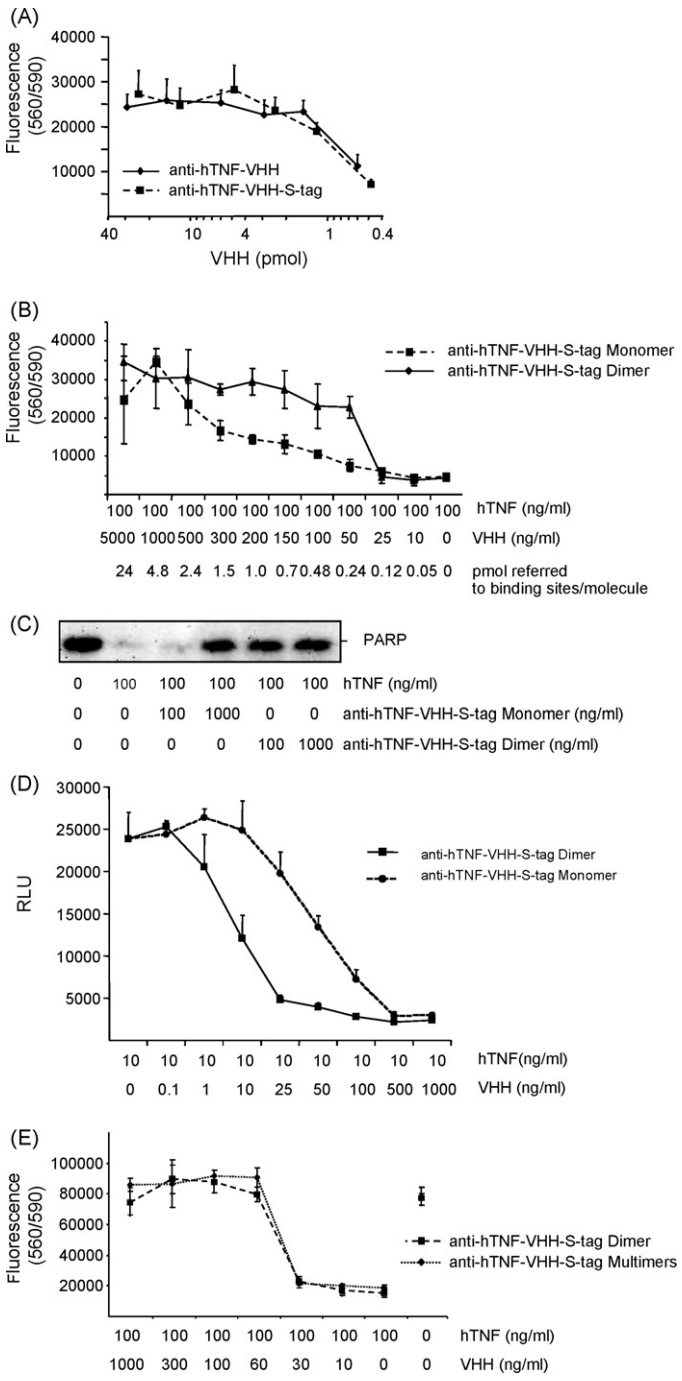

Fig. 5. Anti-hTNF–V_HH-S-tag dimers and multimers have improved inhibitory activity on hTNF-mediated cytotoxicity in comparison to monomeric anti-hTNF–V_HH. (A) L929 cells were incubated with constant amounts of hTNF (100 ng/ml, 0.2 pmol) and increasing amounts of hTNF–V_HH and hTNF–V_HH-S-tag (5000 ng/ml; 2500 ng/ml; 1000 ng/ml; 500 ng/ml; 250 ng/ml; 100 ng/ml). (B) L929 cells were incubated with constant amounts of hTNF (100 ng/ml) and increasing amounts of hTNF–V_HH-S-tag monomers and dimers. (C) L929 cells were incubated with constant amounts of hTNF (100 ng/ml) and increasing amounts of hTNF–V_HH-S-tag monomers (100 ng/ml, 1000 ng/ml) and dimers (100 ng/ml, 1000 ng/ml). The cells were harvested after 24 h of stimulation and Western blot analysis of PARP degradation was performed. (D) NFκB-activation in HEK293 cells. Activation of the transcription factor NF-κB in HEK293 cells was determined using a dual-luciferase reporter gene assay. Cells were stimulated with 10 ng/ml hTNF and the indicated hTNF–V_HH concentrations for 6 h or left untreated. (E) L929 cells were incubated with constant amounts of hTNF (100 ng/ml) and increasing amounts of hTNF–V_HH-S-tag dimers and multimers. (A, B and E) Cytotoxicity of hTNF on L929 cells was quantified as indicated in Section 2.

hTNF-mediated NFκB-activation than monomeric anti-hTNF–V_HH-S-tag (Fig. 5D).

Finally, when we compared dimeric with multimeric anti-hTNF–V_HH-S-tag proteins in the L929 cytotoxicity assay, we observed that the multivalent anti-hTNF–V_HH-S-tag multimers were as potent as the bivalent anti-hTNF–V_HH-S-tag dimers in inhibition TNF-induced cellular apoptosis. Our results indicated, that the generation of multivalent high molecular weight antibody structures by MTG catalyzed cross-linking resulted in fully biological active molecules with high potential to form complexes facilitated by the observed avidity effects (Fig. 5E).

4. Discussion

Single V_H domain antibodies with full antigen binding properties offer several advantages in comparison to conventional IgG's or single chain antibodies, including better tissue penetration due to their small size and lighter tendency for aggregation and/or proteolysis caused by linker sequences normally used in single chain Fv's (scFv's) (Hamers-Casterman et al., 1993). Removing of the V_L domain from conventional svFc's exposes a large hydrophobic surface of the V_H to the solvent. As a result, isolated V_H molecules become "sticky" and are difficult to produce in a soluble form in *E. coli* (Muyldermans, 2001). In the early 90s a unique type of antibodies without any light chain was discovered in the serum of camelides (Hamers-Casterman et al., 1993). These unusual heavy chains bind their antigen by a specific V_HH domain lacking any interface for V_L binding, facilitating both good solubility and producibility in *E. coli*. Phage display libraries using such single domain antibody genes were constructed and successfully used for the development of various anti-V_HH antibodies (for review see Muyldermans, 2001). Surprisingly, the V_HH antibody fragments can recognize unique conformational epitopes because of the dominant involvement of its long complementary-determining region 3 (CDR3). The efficacy of antigen binding can be improved by di- or multimerization of antibody fragments, which is based on higher avidity (Pluckthun and Pack, 1997). Bispecific and bivalent V_HH antibodies were constructed by conventional genetic engineering using linker peptides connecting two V_HH domains in a single protein (Conrath et al., 2001). However, dimerization can be achieved in several other ways, e.g. chemical cross-linking, fusion of heterologous or homologous self-assembling peptides or protein domains, such as leucine zippers, helix–loop–helix motif, streptavidin and the tetramerization domain of human p53 and transglutaminases (Kamiya et al., 2003; Pluckthun and Pack, 1997; Conrath et al., 2001; Pack et al., 1995; Arndt et al., 2000; Casey et al., 1996). Oligomerization does not necessarily result in a dramatic increase in avidity of the higher ordered complex, because the independent antigen binding sites of the complex must be sterically positioned correctly to bind to separate antigen molecules simultaneously. A second aspect of multimerization is the increase in molecular mass, which could also be achieved by PEGylation (Kubetzko et al., 2006), resulting in longer serum persistence of molecules in the circulation, because they cannot be filtered into the kidney glomeruli (Behr et al., 1998). On the other hand, there is an inverse correlation between the molecular mass of these molecules and their ability to penetrate into the target tissue (Graff and Wittrup, 2003).

Here, we described the functional characterization of di- and multimerized anti-hTNF–V_HH antibodies by transglutaminase-mediated covalent protein cross-linking. In our approach, the anti-hTNF–V_HH was expressed as an S-tag fusion protein with a high yield in *E. coli*. Previously, the S-tag peptide from the ribonuclease of the cow *Bos taurus* was shown to be a good peptidyl substrate in protein cross-linking reactions catalyzed by microbial transglutaminase (MTG) from *S. mobaraensis* (Kamiya et al., 2003). Trimeric

hTNF induces signal transduction via binding to trimerized receptor complexes (Smith et al., 1994). We showed that the anti-hTNF–V_HH bound to trimeric hTNF and prevented the interaction of hTNF with its receptors. The employed molarity of hTNF refers to the trimeric molecule which is based on data from size exclusion chromatography, showing that one mole of anti-hTNF–V_HH is able to almost completely shift one mole of trimeric TNF to a higher molecular state, indicating that only one anti-hTNF–V_HH binding site is accessible per trimeric hTNF and that the binding of one anti-hTNF–V_HH antibody is sufficient to neutralize one trimeric hTNF molecule. The IC_{50} of monomeric anti-hTNF–V_HH protein in L929 cytotoxicity assays was 141 ng/ml with 100 ng/ml hTNF. At the molar level, 0.85 pmol anti-hTNF–V_HH was needed to inhibit the activity of 0.2 pmol trimeric hTNF by 50%, which reflected a 4.25-fold molar excess of anti-hTNF–V_HH for inhibition by 50%. The S-tagged anti-hTNF–V_HH behaved similar with 0.93 pmol (194 ng/ml) for IC_{50}. On the other hand, only 0.094 pmol (0.19 pmol referring to binding sites per dimeric V_HH antibody) of the dimerized S-tagged anti-hTNF–V_HH was needed to inhibit the activity of 0.2 pmol trimeric hTNF by 50%. Here, the molarity of the dimeric anti-hTNF–V_HH refers to antigen binding sites per molecule, to allow a good comparison of the monomeric and dimeric anti-hTNF–V_HH antibodies. This means that one dimeric anti-hTNF–V_HH has to be viewed as two independent molecules, because of the presence of two antigen binding sites in the dimeric anti-hTNF–V_HH vs. one binding site in the monomeric anti-hTNF–V_HH antibody. Interestingly, the IC_{50} of the oligomeric anti-hTNF–V_HH molecules was identical to the IC_{50} of the dimeric anti-hTNF–V_HH. In summary, di- and oligomerized anti-hTNF–V_HH proteins were 5-fold more active than the monomeric anti-hTNF–V_HH. The IC_{50} of the dimeric anti-hTNF–V_HH was almost equimolar, indicating that only one dimeric anti-hTNF–V_HH molecule was needed to inhibit the biological activity of one/two trimeric hTNF by 50%. Since it is not possible to inhibit more TNF molecules than antibody binding sites, the activity of this dimeric molecule is at the upper limit and could not be further improved. Coppieters et al. (2006) showed that the dimeric single chain anti-TNF–V_HH is up to 500-fold more active than the monomeric V_HH, but the IC_{50} values were also in the lower picomolar range, therefore the activity of our dimeric molecule seemed to be comparable to the previously described one. We concluded that the improved neutralizing activity of the multimerized anti-hTNF–V_HH was based on avidity, which describes the combined synergistic strength of multiple bond interactions. Moreover the multimerized anti-hTNF–V_HH variants might also be able to efficiently connect several of hTNF trimers to form higher molecular weight V_HH antibody/TNF-complexes. Therefore, our results clearly showed that the protein cross-linking dimerization did not cause loss but improved antigen binding of anti-hTNF–V_HH. However, the cross-linked sample contained not only the expected dimeric anti-hTNF–V_HH proteins but also higher oligomeric complexes. The oligomeric products are the result of covalent addition of anti-hTNF–V_HH-S-tag proteins to a specific site of dimeric anti-hTNF–V_HH-S-tag conjugates. However, even the oligomeric anti-hTNF–V_HH-S-tag molecules were as active as the dimeric anti-hTNF–V_HH-S-tag molecules, indicating that oligomerization did not interfere with the functionality of the multimeric anti-hTNF–V_HH-S-tag complexes. Our results indicate that MTG catalyzed site-specific cross-linking under mild conditions may be extensively applicable to the conjugation of V_HH antibodies without negative effects on their affinity. Analysis of *in vivo* neutralizing activity of TNF with these reagents will depend on availability of models depending on pathogenic human TNF and should be done in the future.

In summary, we showed that the combination of genetic engineering and enzymatic posttranslational modification is a feasible strategy for site-specific conjugation of V_HH antibodies and might be suitable to overcome current limitations in both genetic and chemical manipulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2009.04.002.

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