

Analysis of receptor-ligand interactions with new single chain TNF constructs

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I hereby assure that I performed this work independently without further help or other materials than stated.

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Abbreviations

°C	degree celsius
µg	microgram
µl	microliter
µm	micrometer (10 ⁻⁶ m)
µM	micromolar (10 ⁻⁶ M)
Å	Ångstrom (0.1 nm)
aa	amino acids
AIDS	acquired immune deficiency syndrome
Amp	ampicillin
APS	ammonium persulfate
BS ³	bis-(sulfosuccinimidyl)-suberate
BSA	bovine serum albumin
cFLIP	cellular FLICE inhibitory protein
Ci	Curie
c-IAP	inhibitor of apoptosis protein c
cpm	counts per minute
CRD	cysteine rich domain
cysTNF	cysteine TNF
ddH ₂ O	bidistilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EC ₅₀	effective concentration 50%
ECMV	encephalomyocarditis virus
EDTA	ethylene di-amine tetra acetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
EPO-R	erythropoietin receptor
FACS	fluorescence activated cell sorting
FADD	fas-associating protein with a death domain
Fas	FS-7 cell-associated surface antigen
FasL	FS-7 cell-associated surface antigen ligand
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
GITRL	human glucocorticoid-induced TNF receptor ligand
h	hour
HEK cells	human embryonal kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG	immunoglobulin G
IgM	immunoglobulin M
IMAC	immobilized metal affinity chromatography
IκB	inhibitor of κB
JNK	c-Jun N-terminal kinase
K _d	saturation affinity constant

kDa	kilo Dalton
KOD	<i>Thermococcus kodakaraensis</i>
k_{off}	dissociation constant
l	liter
LB	Luria Bertani
LPS	lipopolysaccharide
LT α	lymphotoxin alpha
M	molar
mA	milliampere
mAb	monoclonal antibody
MAP-kinase	mitogen activated protein kinase
MD	molecular dynamics
MF	mouse embryonic fibroblasts
mg	milligram
min	minute
ml	milliliter
mM	millimolar (10^{-3} M)
mRNA	messenger RNA
mTNF	membrane bound TNF
Mw	molecular mass
n. s.	not stimulated
NF- κ B	nuclear factor-kappa B
nm	nanometer (10^{-9} m)
nM	nanomolar (10^{-9} M)
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide electrophoresis
PARP	poly (ADP-ribose) polymerase
PBA	PBS with BSA and azide
PBS	phosphate buffered saline
PBS-T	PBS with Tween-20
PCR	polymerase chain reaction
Pfu	<i>Pyrococcus furiosus</i>
PLAD	pre-ligand binding assembly domain
pM	picomolar (10^{-12} M)
PMSF	phenylmethylsulfonyl flouride
Poly DI:DC	poly (deoxyinosinic-deoxycytidylic) acid
Rel	reticuloendotheliosis oncogene
RIA	radioimmunosorbent assay
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
scTNF	single chain TNF
SDS	sodium dodecyl sulfate
sec	seconds
sTNF	soluble TNF
TACE	TNF α converting enzyme
TAE	tris-actetate-EDTA
TBE	tris-boric-EDTA
TEMED	N,N,N',N'-tetramethylethyl-diamine
TNF	tumour necrosis factor

TNFR1 (p55/60, CD120a)	TNF receptor 1
TNFR2 (p75/80, CD120b)	TNF receptor 2
TRADD	TNFR1 associated death domain
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TNF-related apoptosis-inducing ligand receptor
Tris	tris-(hydroxymethyl)-amino-methane
u	units
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
WT	wild type
XIAP	X-linked inhibitor of apoptosis protein
β -ME	beta-mercaptoethanol

Summary

Different models for the binding of the ligand tumour necrosis factor (TNF) to its receptors can be imagined. In a first model the homotrimeric ligand merges three single receptor molecules upon binding. Thereby their intracellular parts are brought into proximity, which enables signal initiation. Based on the discovery of a preassociation of the TNF receptors prior to ligand binding, a distinct model was proposed, in which the TNF trimer binds at once to several preassociated receptor molecules. A conformational change in the receptor molecules could then bring the intracellular receptor parts in proximity thereby initiating signalling.

In the TNF derivative single chain TNF (scTNF) the three TNF monomers are linked by two Glycine-Serine linkers. The observed reversible dissociation of the TNF homotrimer in its monomers is thereby prevented. Furthermore it is possible to mutate single receptor binding sites in the trimer thereby generating TNF trimers with only one or only two active binding sites. With these molecules it is possible to draw conclusions on the binding mode of TNF. If TNF binds to several preaggregated receptor molecules at once, one would expect a strong reduction in affinity in saturation binding studies at 0°C. If TNF binds only to one single receptor or to only one receptor of a preformed aggregate, the omission of single binding sites should have no strong effects.

Using this approach it was possible to show in this work that TNF receptor 2 (TNFR2) must exist in a preaggregated state prior to ligand binding, most likely as a receptor homotrimer. For TNF receptor 1 (TNFR1) the existence of preformed dimers can be assumed, because inactivation of one single binding site shows no significant effects on binding. Only when a second receptor binding site of scTNF was impaired, affinity was reduced. By using another mutation influencing receptor binding also with a scTNF with two impaired binding sites no effect on binding affinity was achieved. However, this could be the case because of a comparably weak impact of the used mutation on receptor binding in general. Alternatively, an aggregation of single receptor molecules or of receptor dimers/trimers by the ligand can be discussed.

Hence the model of binding to already preaggregated receptors could be clearly proven, at least for TNFR2. Furthermore it was shown that there exist clear differences in ligand interaction between the two TNF receptors. This might be caused by the structural differences between the receptors (differences in stem- and transmembrane domains) and/or a localisation in different areas of the cell membrane (membrane microdomains).

The partially detectable bioactivity of TNF trimers with only one binding site displaying full binding properties further gives a hint for the existence of higher aggregates of several ligand molecules, their bound receptors and intracellular signalling molecules. A signal initiation between two adjacent receptor molecules each bound to another TNF trimer could explain the observed residual activity.

Zusammenfassung

Für die Bindung des Tumor Nekrose Faktors an seine Rezeptoren sind unterschiedliche Modelle vorstellbar. Zum einen ist es naheliegend, dass der als Homotrimer vorliegende Ligand drei einzelne Rezeptormoleküle zusammenführt und dadurch die intrazellulären Rezeptorbereiche angenähert werden, was letztendlich die Signalinitiierung ermöglicht. Ausgehend von der Entdeckung, dass die TNF-Rezeptoren schon in Abwesenheit des Liganden voraggregiert auf der Membran vorliegen, wurde jedoch ein neues Modell vorgeschlagen, bei dem das TNF Trimer zugleich mehrere voraggregierte Rezeptormoleküle bindet. Eine Konformationsänderung in den Rezeptormolekülen könnte dann zur Signalinitiierung führen.

In dem TNF Derivat single chain TNF (scTNF) sind die drei TNF Monomere über zwei Glycin-Serin Linker kovalent verknüpft. Die sonst beobachtbare reversible Dissoziation des TNF-Homotrimers in seine Monomere kann dadurch verhindert werden. Zudem ist es möglich einzelne Rezeptorbindestellen im TNF-Trimer zu mutieren und dadurch TNF-Derivate mit nur einer oder nur zwei voll bindungsfähigen Bindestellen herzustellen. Mit Hilfe dieser Moleküle ist es möglich Rückschlüsse auf die Art und Weise, mit der TNF an seine Rezeptoren bindet, zu ziehen. Bei einer gleichzeitigen Bindung an mehrere Rezeptoren würde in Gleichgewichtsbindungsstudien bei 0°C ein starker Abfall der Affinität zu erwarten sein. Bei der Bindung an einen einzelnen Rezeptor oder an ein Molekül eines Rezeptoraggregats, sollte ein Wegfall einzelner Bindungsstellen keinen großen Einfluß haben. Mit diesem experimentellen Ansatz war es in vorliegender Arbeit möglich zu zeigen, dass der TNF Rezeptor 2 (TNFR2) voraggregiert vorliegen muß, sehr wahrscheinlich als Homotrimer. Für TNFR1 wird aufgrund der erhaltenen Ergebnisse ein Vorliegen von Dimeren vermutet, da das Inaktivieren einer Bindungsstelle keinen Effekt zeigte. Erst das Mutieren einer weiteren Bindungsstelle führte zu einer Affinitätsabnahme. Bei der Verwendung einer weiteren, die Bindung beeinflussenden Mutation wurde jedoch auch mit einem scTNF mit zwei beeinflussten Bindungsstellen auf TNFR1 nur ein minimaler Effekt auf die Bindungsfähigkeit erzielt. Dies könnte man sich jedoch mit einer im Vergleich eher schwachen Auswirkung der verwendeten Mutation auf die Rezeptoraffinität erklären. Alternativ kann eine Aggregation von einzelnen Rezeptormolekülen oder von Rezeptor Dimeren oder Trimeren durch den Liganden diskutiert werden.

Somit konnte das Model der Bindung an schon voraggregierte Rezeptoren zumindest für TNFR2 eindeutig belegt werden. Es konnte aber auch gezeigt werden, dass deutliche

Unterschiede zwischen beiden Rezeptoren vorhanden sind. Dies mag in der unterschiedlichen Struktur der Rezeptoren begründet sein (Unterschiede in Stiel- und Transmembrandomäne) und/oder in einer Lokalisation in verschiedenen Bereichen der Zellmembran (Mikrodomänen).

Die teilweise noch nachweisbare Bioaktivität eines TNF Trimers mit nur einer voll bindefähigen Rezeptorbindestelle ist zudem ein Hinweis auf die Existenz größerer Aggregate bestehend aus mehreren Liganden, ihren gebundenen Rezeptoren und intrazellulären Signalproteinen. Eine Signalinitiation durch zwei benachbarte Rezeptormoleküle jeweils gebunden an ein anderes TNF-Trimer könnte die vorhandene Restaktivität erklären.

1 Introduction

1.1 *Tumour necrosis factor (TNF)*

In multicellular organisms there are a multitude of processes with a need of a controlled death of cells. That starts in embryonic development with the formation of organs and complex multicellular tissues and continues in the adult organism, assuring normal cell homeostasis as well as the accurate function of the immune system. Without the suicide of self reactive T-cells autoimmune diseases would emerge and without the possibility of killing infected cells the organism would be impaired in its defence against infectious agents. The major underlying mechanism for these forms of programmed cell death is called apoptosis (from the Greek word for “falling away”, describing the dropping of leaves in fall). In contrast to the death of cells by necrosis, where the cell membrane ruptures and the cell fluid is dispersed in the surrounding tissue thereby leading to inflammation, the apoptotic cell dies by an energy depending process leading to chromatin destruction, membrane blebbing and a subsequent dissection of the cell in membrane enclosed vesicles. These apoptotic bodies can be engulfed easily by macrophages.

Events leading to apoptosis can be subdivided into intrinsic ones, triggered for example by DNA damage, leading to cytochrome c release from the mitochondria into the cytoplasm, and extrinsic ones, activated by death ligands binding to their corresponding receptors at the cell surface. The tumour necrosis factor (TNF) was the first death ligand discovered. Astonishingly it was not found because of its apoptotic effects, but because of its ability of leading to necrosis of solid tumours. At the end of the 19th century, a correlation was established between bacterial infection and a reduction in tumour mass in cancer patients. The surgeon William Coley believed in a causal relationship and tried to treat patients with bacteria derived preparations. Although some tumour regression could be observed, the side effects were too severe for further therapies (Coley, 1896). Later it was shown that the tumour mass reduction was induced by a single protein in the serum of the host, TNF, named after the hemorrhagic necrosis of tumours observed in experiments with mice (Carswell et al., 1975). Nowadays it is known that the effects of TNF on the tumours are not caused by its direct interaction with the tumour cells, but are rather an effect on tumour vasculature leading to necrosis of the tumour.

In 1984-1985 TNF was biochemically characterized and its molecular cloning was successful (Pennica et al., 1985). Thereby it was revealed that TNF had been already known as Cachectin (Beutler et al., 1985), an until that time not isolated factor playing a role in wasting

(cachexia) and gram negative sepsis. In the following years many other death ligands and their corresponding receptors were discovered, leading to the definition of two families of proteins, the TNF- and TNF-receptor-related superfamilies with TNF as the founding member (Aggarwal, 2003; Locksley et al., 2001; Branschaedel et al., 2007).

1.2 Cellular effects of TNF

Human TNF is a 26 kDa type II transmembrane protein of 157 amino acids, which is not glycosylated and contains only one disulphide bridge. It is proteolytically cleaved at the C-terminal extracellular region by a protease called TACE (TNF α converting enzyme, Black et al., 1997), giving rise to its soluble form called soluble TNF (sTNF, 17 kDa). sTNF as well as the membrane bound form (mTNF) are able to stimulate cells, mTNF acting in a juxtacrine manner on neighbouring cells, whereas sTNF can also act on the TNF producing cell itself (autocrine).

There are two known receptors for TNF, TNF-receptor 1 (TNFR1) and TNF-receptor 2 (TNFR2). TNFR1 can be activated by sTNF as well as by mTNF, whereas TNFR2 only after ligation with mTNF induces intracellular signals (Grell et al., 1995). There exist further receptors in the TNFR-superfamily which need their respective membrane bound ligand to become activated, like TRAILR2 or Fas (Wajant et al., 2001; Schneider et al., 1998). One reason for the differences of the TNFRs regarding their demand for the different ligand forms could be the distinct dissociation rates (k_{off}) observed between sTNF and TNFR1 and sTNF and TNFR2 at 37°C. From TNFR1 sTNF dissociates very slowly ($k_{\text{off}} = 0,021 \text{ min}^{-1}$), from TNFR2 sTNF dissociates very fast ($k_{\text{off}} = 0,631 \text{ min}^{-1}$). Accordingly, it has been argued that the binding of sTNF to TNFR2 might be too short to lead to an efficient signal complex formation, i.e. activation of the receptor (Grell et al., 1998).

Upon binding of sTNF or mTNF to TNFR1, two fates are possible for a cell. Either it dies by apoptosis, or it survives by processes counteracting the apoptotic signals. In the intracellular part of the molecule, TNFR1 contains a domain called death domain which can lead to the activation of caspases. Caspases are zymogens, only upon proteolysis they become active, then capable to act proteolytically on other proteins themselves (Riedl and Shi, 2004). They can be classified into initiator caspases and effector caspases, the latter ones becoming activated by the initiator caspases and finally cleaving essential cellular proteins which will lead to the break down of the cell. The counteracting antiapoptotic signals which are caused by receptor binding mainly derive from the activation of the transcription factor NF- κ B. It

belongs to the Rel family of transcription factors (Gilmore and Ip, 2005). Rel proteins bind DNA as hetero- or homodimers and can either activate or repress transcription. The most prominent NF- κ B member is a heterodimer consisting of the proteins p50 and RelA (also called p65) and usually activates transcription. Like all Rel proteins it is held in an inactive state in the cytoplasm by the interaction with an inhibitor called I- κ B (Inhibitor of NF- κ B).

Via interaction with the death domain of TNFR1 a complex of proteins forms upon ligand binding. An important protein of this complex is TRAF2. TRAF (TNFR-associated factor) proteins are used by a range of TNFR-family members to elicit antiapoptotic signals and are also commonly used by the pattern recognition receptors of the Toll-like receptor family (Wajant et al., 2001). TRAF2 passes on the signal to kinases of the MAP-kinase-cascade leading to the activation of the I- κ B kinase (IKK) complex. The latter phosphorylates two serine residues on I- κ B thereby marking it for ubiquitination which leads to proteosomal degradation of the inhibitor. NF- κ B is thereby liberated and after further activation through various posttranslational modifications translocates into the nucleus to induce the transcription of a wide range of antiapoptotic and inflammatory proteins (Hayden and Gosh, 2008).

In the meantime, TNFR1 gets internalized and the protein complex at the death domain changes its composition (Schneider-Brachert et al., 2004; Micheau and Tschopp, 2003). The protein TRADD (TNF receptor adaptor protein with a death domain) can now bind to the death domain of the receptor. Associated with TRADD is FADD (Fas associated death domain protein) which recruits a procaspase-8 dimer. The binding to FADD promotes the autoproteolytical processing of the initiator procaspase, which is then able to activate effector caspases (Salvesen and Dixit, 1999; Boatright et al., 2003). To accomplish apoptosis, the effector caspases cleave a wide range of substrates. They act on negative regulators of apoptosis as well as on proteins involved in cytoskeleton regulation, and also directly disassemble cell structures like the nuclear lamina (Thornberry and Lazebnik, 1998).

Antiapoptotic mechanisms try to interrupt the apoptotic processes. An example for an antiapoptotic protein family is the IAP family (inhibitor of apoptosis proteins). Their transcription is, like the transcription of many other antiapoptotic proteins, regulated by NF- κ B. One prominent member of the family is XIAP, for which it has been shown that it can directly inhibit caspase-3 by binding to it with its BIR (baculoviral IAP repeat-domain 2; Riedl et al., 2001; Eckelman et al., 2006). Another example for an antiapoptotic protein is the cellular FLICE inhibitory protein (cFLIP). NF- κ B signals induce the expression of cFLIP (Micheau et al., 2001). One of the splice variants of cFLIP resembles a caspase 8 molecule

without possessing an enzymatic activity (cFLIP_s). Accordingly, cFLIP_s can act as dominant negative inhibitor through its death effector domain and can thereby prevent apoptosis (reviewed in Budd et al., 2006).

TNFR2 contains no death domain but a TRAF2 binding site, therefore its ligation leads to NF- κ B activation resulting in antiapoptotic signals and inflammation (Rothe et al., 1994). When both TNFRs are coexpressed in a cell, especially prolonged stimulation of TNFR2 can enhance apoptosis evoked by TNFR1. One mechanism is the induction of TNF production in the cell, which can then lead to apoptosis via TNFR1 in an autocrine manner (Grell et al., 1999). Alternatively, or in combination, activation of TNFR2 results in a sequestration of anti-apoptotic proteins which are no more available for TNFR1 leading to an enhanced apoptotic signalling of this receptor (Weiss et al., 1998). The latter mechanism is further amplified by the fact that binding of TNF to TNFR2 leads to ubiquitination and finally to degradation of TRAF2 by c-IAP1, potentiating TNF-induced apoptosis (Li et al., 2002).

1.3 Medical applications and impact in disease

TNF is a highly conserved protein being present in a wide range of organisms. Therefore it is not surprising that it fulfils some important functions, which lie mainly in immune defence. If macrophages are not able to get rid of pathogens alone, they secrete, among other cytokines, TNF. TNF causes blood vessels to expand and the blood flow is slowing down. The cells of the endothelium are induced to express adhesion molecules and cells of the immune system accumulate near the focus of inflammation. Besides Macrophages also cytotoxic T-cells and Natural Killer cells secrete TNF to kill infected cells by apoptosis.

However, being such a highly potent immune-stimulatory agent, TNF can also cause severe problems if production and/or actions are not appropriately regulated. Raised plasma concentrations of TNF can be found as a symptom of diverse diseases, like AIDS, rheumatoid arthritis or cerebral malaria to name only a few (Aggarwal, 2003). For some diseases, like rheumatoid arthritis, neutralizing TNF-specific antibodies demonstrate the high impact of TNF, because treatment leads to a clear improvement of the patients conditions (Nash and Florin, 2005). Consistent with its role in immune defence a possible side effect of this therapy can be the reoccurrence of a latent tuberculosis infection.

As Carswell and colleagues could show that TNF can lead to necrosis of tumours (Carswell et al., 1975), it was handled as the new hope in the treatment of cancer. But soon it became clear that the side effects of systemic TNF administration are too severe, especially at

concentrations which would show beneficial effects on the tumour. Some success was achieved with the technique of isolated limb or isolated liver perfusion, where TNF is only locally administered by supplying a part of the body through an external blood circulation. Therefore it is possible to use higher concentrations without significant side effects. Common is a combination treatment together with a chemotherapeutic drug like melphalan, which leads to synergistic antitumoural effects. Because of the actions of TNF on the tumour vasculature leading to vessel permeability the drug is well distributed throughout the tumour (Horsssen et al., 2006). Unfortunately this technique is applicable only for a few types of tumours. Only 1-2% of all malignant neoplasms in adults are soft tissue sarcomas, the main type of cancer where isolated limb perfusion is useful (Schlag and Tunn, 2007). New strategies try to abolish the systemic impacts by activating the TNF ligand only at its site of action. This can be achieved for example with fusion proteins of TNF with TNFR1, where TNF is held in an inactive state by binding to the receptor part (Wajant et al., 2004). An attached tumour specific antibody targets this construct to the tumour, where tumour specific proteases activate TNF by cutting the protein resulting in the loss of the inhibitory receptor part.

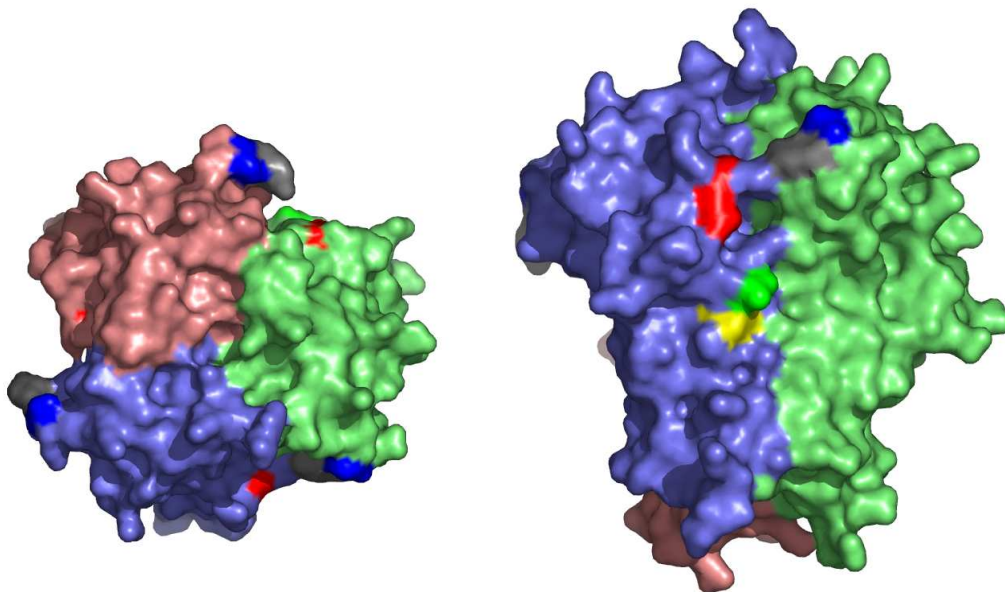


Figure 1: TNF forms a homotrimer. Shown is the structure of the TNF trimer from above (left) and from the side (right) with respect of the threefold axis. The three TNF molecules are shown in slate, lime and salmon. Depicted are amino acids crucial for receptor binding. Their mutation can lead to receptor selective binding or to no binding at all. Blue: S86, red: R32, yellow: D143, green: A145, grey: Y87. The figure was generated by using the pdb-file 1tnf (<http://www.rcsb.org>) and the program PyMOL (<http://www.pymol.org>).

1.4 Structure of TNF and its receptors

The crystal structure of sTNF shows that it forms tightly packed homotrimers (Eck et al., 1988), where the monomers are in contact via their long axes forming a bell-shaped trimer (Figure 1). There is no crystal structure of TNF bound to one of the receptors available, only one of Lymphotoxin alpha (LT- α), a closely related protein with similar structure, bound to soluble TNFR1 (Banner et al., 1993). This structure reveals that the clefts between the monomers in the ligand-trimer are connecting with the receptor, so that each trimer is able to bind to 3 receptor molecules. There were amino acids in the clefts of the TNF trimer identified which are crucial for receptor binding, and mutation of distinct amino acids in this region can also lead to a preferred binding to only one of the receptors (Loetscher et al., 1993). TNFR1 and TNFR2 both contain 4 so called cysteine-rich domains (CRDs), characteristic domains of the TNFR-family, containing six cysteine residues distributed within a stretch of about 40 amino acids. For both receptors CRD2 and CRD3 were determined to be the main interaction sites with the ligand (Banner et al., 1993; Fu et al., 1995). The cysteine rich domains can be further divided into modular units with characteristic consensus sequences and tertiary fold, common also in other members of the TNFR-family (Naismith and Sprang, 1998). In TNFR1 three types of these units are arranged: A1, B2 and C2. The letter stands for the type of unit and the numeral for the number of disulphide bridges in the unit. The CRD1 to 3 of TNFR1 each comprise one A1 and one B2 unit, CRD4 an A1 and a C2 unit. TNFR2 is predicted to contain an A1 and a B2 unit in CRD1 and CRD2, an A2 and B1 unit in CRD3 and an A1 and B1 unit in CRD4. Furthermore the receptors differ in the region between CRD4 and the putative transmembrane part of the receptors, called stem region. Whereas in TNFR1 this region comprises 14 amino acids it is in TNFR2 with 55 amino acids quite long.

Several reports showed that for signalling a complex of one ligand and 3 receptors is not sufficient and in various experimental systems microscopically visible receptor clusters have been described (Ko et al., 1999; Krippner-Heidenreich et al., 2002). This is not only true for TNF and its receptors but has also been shown for Fas and FasL (Holler et al., 2003; Siegel et al., 2004). However, until now it is unclear how this clustering is achieved. One possible explanation for this clustering are multimerized intracellular signalling components. For the adapter protein FADD as well as for caspase 8 it was shown that they exist as dimers and it was proposed that they link different receptor trimers thereby leading to higher aggregate formation (Sandu et al., 2006). Another or additional possibility is that the receptors become concentrated in special membrane compartments enriched in sphingolipids and cholesterol,

called membrane microdomains or lipid rafts (Brown and London, 2000). Localization of proteins into these domains is associated with formation of detergent resistant aggregates. For the TNF receptor family member Fas a lipid raft localisation has been shown recently by two independent groups (Feig et al., 2007 and Chakrabandhu et al., 2007). It involves palmitoylation of a membrane proximal cysteine residue of the receptor. However, earlier studies in other cell lines were not able to determine a raft localisation for Fas (Legler et al., 2003). Based on the mechanism of Fas signalling cells have been subdivided into “type I” and “type II” cells (Scaffidi et al., 1998). It seems that in type I cells Fas resides at least partially in rafts, whereas in type II cells it is excluded (Muppidi and Siegel, 2004). For TNFR1 a raft localisation has been described (Legler et al., 2003), but a palmitoylation was not reported yet. Disruption of rafts was found to sensitize cells to TNFR1-induced cell death, implying that raft localization is needed for NF- κ B induction.

In the year 2000 it was shown for some members of the TNF receptor family that they are already in a multimerized state prior to ligand binding. The CRD1 of TNFR1 and TNFR2 was shown to contain a domain responsible for this association called PLAD (pre-ligand binding assembly domain; Chan et al., 2000). Using chemical crosslinker these receptor complexes were determined to be trimers. Other data from a similar approach implicate that the receptors form mainly homodimers on the plasma membrane (Branschaedel, 2007). Because of the converging results on the number of molecules which assemble, the stoichiometry of the receptor oligomers needs to be further defined. The finding of receptor dimers is in accordance with available crystal structures, where two extracellular domains from TNFR1 form complexes in the absence of the ligand (Naismith et al., 1995). Two different structures were found at a pH value of 8.0. In the first one the receptors are arranged in a parallel form interacting via their CRD1 but also via their CRD4. The ligand binding sites are freely accessible in this homodimer. In the second structure the two receptor molecules are arranged in an antiparallel manner. In this conformation the cytoplasmic domains of the two receptors would be separated from each other more than 100 Å. In addition the ligand binding of the receptors is hidden, implying that this conformation would represent a non signalling state, if it exists at the cell surface.

The existence of preformed receptor complexes can explain why until now no heteromers of human TNFR1 and TNFR2 after TNF binding have been found (Moosmayer et al., 1994). As the PLAD-mediated TNF receptor interaction is receptor specific, a ligand mediated receptor heteromerization is hampered. Fas, also a member of the TNFR superfamily, was found to be oligomerized on the cell in the absence of ligand, too (Siegel et al., 2000). This nicely

explains the dominant interference of mutated Fas receptors with Fas-mediated apoptosis in patients with ALPS (autoimmune lymphoproliferative syndrome; Sneller et al., 1997). Some of the observed receptor mutants are not able to bind FasL anymore, but they could still act in a dominant negative manner by hetero-oligomerization with wildtyp receptors via their intact PLAD (Chan, 2000). A similar mechanism has been described to occur within the TRAIL receptor system. Here, TRAILR3 and TRAILR4, incapable to induce intracellular apoptotic signals on their own, can form heteromeres with TRAILR1 and TRAILR2, because PLAD-binding within TRAIL-receptors is not receptor specific (Clancy et al., 2005).

1.5 Single chain TNF

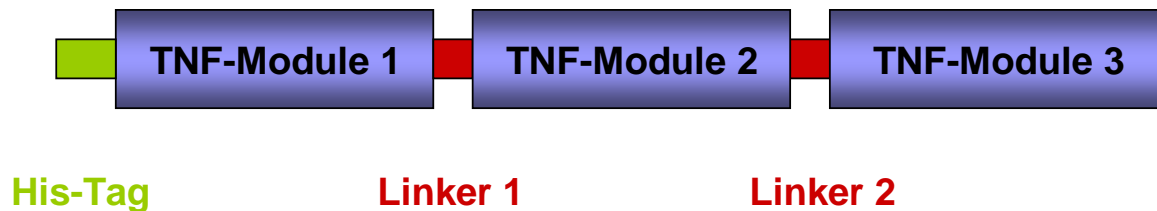


Figure 2: Schematic representation of scTNF. 3 TNF modules (blue) are linked by two Glycine-Serine Linkers (red). At the N-terminus a His-Tag (green) was added for purification.

In several publications it was reported that the TNF trimer can dissociate into its monomers (Poiesi et al., 1993; Ameloot et al., 2001). Although this dissociation is in principle reversible, dissociated TNF monomers are likely prone to hydrophobic interactions leading to activity loss. Dissociation is concentration dependent, is significant at picomolar concentrations and has been proposed to contribute to the fine regulation of TNF activity (Corti et al., 1992). Interestingly, with Suramin a substance was found which affects TNF activity by destroying its quaternary structure (Alzani et al., 1993).

Single chain TNF (scTNF) is an artificial TNF derivative, where three monomers are linked by two polypeptide linkers thereby preventing a loss of activity occurring through dissociation of the trimer (Figure 2, Krippner-Heidenreich et al., 2008). Sterically this linking is quite easy to accomplish, because all N and C termini of the three TNF monomers are in close proximity towards each other in the native trimeric TNF structure (Eck et al., 1988). Two different linker types with different lengths have been used, both of them consisting of the amino acids glycine and serine. The protein was produced in *E. coli* and purified by affinity and ion exchange chromatography. It was shown that scTNF has a higher stability in vivo and in vitro

compared to sTNF. Despite of the linkage, the scTNF molecule formed no higher protein complexes, which was confirmed by chemical crosslinking. Its bioactivity was similar to sTNF, as examined in cytotoxicity studies, but also by looking for its ability to activate NF- κ B and JNK. Surprisingly scTNF shows a higher binding affinity for both TNFR1 and TNFR2 when compared to sTNF. This higher affinity is not displayed in the bioactivity studies, probably because the binding affinities had been measured at 0°C. At this temperature events after binding which contribute to activity like the assembling into higher receptor complexes are not taking place. In two tested mouse models scTNF showed a reduced systemic toxicity but an equal or even slightly higher antitumoural activity. Because of the higher stability and the reduced toxicity in combination with the prominent antitumoural activity scTNF is an interesting molecule for the design of new TNF-based therapeutics. The concept of stabilising the ligand trimers by linker peptides has been transferred to other members of the TNF family. Up to now scTRAIL has been successfully produced and is now tested in animal models in new tumour targeted therapeutic concepts.

In therapeutic approaches it can be necessary to activate only one of the two TNF receptors, because of unwanted effects of the other receptor member. Moreover, a TNF derivative capable to activate only one of the two receptor types present on a cell is an interesting tool to answer questions regarding TNF signalling. By using mutations at the receptor binding sites (Loetscher et al., 1993; Figure 1) scTNFs selective for TNFR1 and TNFR2 have been generated (Kuehnle, 2005). Unfortunately, production in *E. coli* was hampered by the fact that the majority of the expressed receptor selective protein remained insoluble, a fact that was already observed with the wildtyp scTNF. Therefore, the yields of correctly folded protein were low. But nevertheless the produced proteins showed a bioactivity and stability comparable to wildtype scTNF, combined with the expected receptor selectivity.

1.6 Aim of the work

The aim of this work was to examine the first binding steps of TNF to its receptors. By using scTNF molecules allowing binding only to one or only to two receptors the stoichiometry of ligand and receptors should be determined. Therefore, new scTNF variants had to be constructed by using receptor selective mutations. Moreover, a new expression system had to be established to achieve adequate protein yields to enable the performance of equilibrium binding studies.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Consumables	
γ - ³² P-ATP	Hartmann Analytic GmbH, Braunschweig
Acrylamide (Rotiphorese Gel 30)	Carl Roth GmbH &Co., Karlsruhe
Agar	Carl Roth GmbH &Co., Karlsruhe
Agarose	Carl Roth GmbH &Co., Karlsruhe
Ammonium persulfate (APS)	Carl Roth GmbH &Co., Karlsruhe
Ampicillin	Sigma-Aldrich, München
Blotting Paper, 3MM	Whatman, Schleicher Schuell, Dassel
Bovine Serum Albumin (BSA)	Sigma-Aldrich, München
Bradford reagent	BioRad, Hercules, USA
Bromphenol blue	Serva, Heidelberg
Cell culture flasks and dishes	Greiner, Frickenhausen
Crystal violet	Merck, Darmstadt
Di-butyl phtalate	Sigma-Aldrich, München
Dimethylsulfoxide (DMSO)	Carl Roth GmbH &Co., Karlsruhe
Di-octyl phtalate	Sigma-Aldrich, München
DNA standard	Invitrogen, Carlsbad, USA
Dithiothreitol (DTT)	Carl Roth GmbH &Co., Karlsruhe
Ethylene glycol tetraacetic acid (EGTA)	Carl Roth GmbH &Co., Karlsruhe
Eosine	Merck, Darmstadt
Ethanol	VWR, Darmstadt
Ethylene di-amine tetra-acetic acid (EDTA)	Sigma-Aldrich, München
Glycerol	Carl Roth GmbH &Co., Karlsruhe
Glycine	Carl Roth GmbH &Co., Karlsruhe
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Carl Roth GmbH &Co., Karlsruhe
Imidazole	Merck-Schuchardt, Hohenbrunn
Iodine-125 (Na ¹²⁵ I)	Hartmann Analytic GmbH, Braunschweig
KCl	Carl Roth GmbH &Co., Karlsruhe
Methanol	Carl Roth GmbH &Co., Karlsruhe
MgCl ₂	Carl Roth GmbH &Co., Karlsruhe
Milk powder, non-fat	Applichem, Darmstadt
Thiozolyblue tetrazolium bromide (MTT)	Sigma-Aldrich, München
Nitrocellulose	Whatman, Schleicher Schuell, Dassel
PD 10 column	GE Healthcare, Uppsala, Sweden
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich, München
Poly DI:DC	GE Healthcare, Uppsala, Sweden
Probe Quant G50 microcolumns	GE Healthcare, Uppsala, Sweden
Protein marker, prestained	NEB, Ipswich, USA
Puromycin	Calbiochem, San Francisco, USA
RIA vials	Sarstedt AG, Nürnberg
RNase A	Applichem, Darmstadt
Scatchard centrifugation tubes (Micro tube 0.3 ml)	Sarstedt AG, Nürnberg

Sodium dodecyl sulfate (SDS)	Carl Roth GmbH &Co., Karlsruhe
Sodium azide	Sigma-Aldrich, München
Sodiumdisulfite	Merck, Darmstadt
Sodium iodide	Merck, Darmstadt
Tetramethylethyldiamine (TEMED)	Carl Roth GmbH &Co., Karlsruhe
Tris-(hydroxymethyl)-aminomethane (Tris)	Carl Roth GmbH &Co., Karlsruhe
Trypton	Carl Roth GmbH &Co., Karlsruhe
Tween-20	Carl Roth GmbH &Co., Karlsruhe
Xylene cyanol	Serva, Heidelberg
Yeast Extract	Carl Roth GmbH &Co., Karlsruhe
β -Mercaptoethanol (β -ME)	Sigma-Aldrich, München

2.1.2 Cell lines

MF TNFR1-Fas	Murine embryonic fibroblasts from TNFR1/TNFR2 knock-out mice stably expressing human TNFR1-Fas F. Tübing, University Stuttgart (Krippner et al., 2002)
MF TNFR2-Fas	Murine embryonic fibroblasts from TNFR1/TNFR2 knock-out mice stably expressing human TNFR2-Fas F. Tübing, IZI, University Stuttgart (Krippner et al., 2002)
MF TNFR2	Murine embryonic fibroblasts from TNFR1/TNFR2 knock-out mice stably expressing human TNFR2 R. Fischer, IZI, University Stuttgart
MF TNFR1 dd _{mut}	Murine embryonic fibroblasts from TNFR1/TNFR2 knock-out mice stably expressing human TNFR1 with mutated death domain (R347A) S. Wöhrle, IZI, University Stuttgart
Kym-1	Human myosarcoma cell line M. Seguchi, University Tokyo, Japan
HeLa	Human cervix carcinoma cell line American Type Culture Collection (ATCC), Rockville, USA

2.1.3 Cell culture reagents

10x Trypsin EDTA	Invitrogen, Carlsbad, USA
DMEM, RPMI, OptiMEM	Invitrogen, Carlsbad, USA
Eosine solution	6 mM Eosine, 10% FCS, 90% PBS, 0.02% NaN ₃
FCS	Fetal calf serum, PAA, Pasching
FCS + DMSO	90% (v/v) FCS, 10% (v/v) DMSO
HEK transfection and expression medium	OptiMEM

HEK293 culture medium	DMEM + 10% FCS
HEK293 selection medium	DMEM + 10% FCS + 4 µg/ml puromycin
HeLa culture medium	RPMI 1640 + 5% FCS
Kym-1 culture medium	RPMI 1640 + 10% FCS
LB _{Amp} agar plates	LB _{Amp} -medium + 1.5% (w/v) agar
LB _{Amp} medium	LB-medium + 100µg/ml ampicillin
LB-medium	0.5% (w/v) yeast extract, 1% (w/v) trypton, 1% (w/v) NaCl, pH 7.0
MF culture medium	RPMI 1640 + 5% FCS
MF selection medium	RPMI 1640 + 5% FCS + 2.5 µg/ml puromycin
SOC ⁺⁺	2% trypton (w/v), 0.5% yeast extract (w/v), 10 mM NaCl, 2.5 mM KCl, 20 mM glucose, 10 mM MgCl ₂ , pH 7.0

2.1.4 Solutions

10x TBE (EMSA)	1.3 M Tris, 0.4 M boric acid, 1.3 mM EDTA
4x SDS-sample buffer	40 mM Tris/HCl, pH 8.0, 0.4 mM EDTA, 140 mM SDS, 4.4 M glycerine, 0.4 % (w/v) bromphenol blue
5x binding buffer (EMSA)	500 mM KCl, 50 mM Tris/HCl pH 7.4, 25 mM MgCl ₂ , 5 mM DTT, 50% (v/v) glycerine
6x DNA-sample buffer	1 mM EDTA, 50% (v/v) glycerine, 0.025 % (w/v) bromphenol blue, 0.025 % xylene cyanol
Binding buffer (IMAC)	50 mM NaPi, 20 mM imidazol, 300 mM NaCl, pH 8
Buffer A (EMSA)	10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT(fresh), PMSF (fresh)
Buffer C (EMSA)	20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (fresh), 1 mM PMSF (fresh)
Buffer for primary antibodies	PBS + 0.025% (v/v) BSA + 0.02% (v/v) NaN ₃
Crystal violet solution	0.5% (w/v) crystal violet, 20% (v/v) methanol
Elution buffer (IMAC)	50 mM NaPi, 500 mM imidazol, 300 mM NaCl, pH 8.3
Lysis buffer	200 mM NaOH, 1% SDS
PBA (FACS)	PBS + 0.05% (v/v) BSA + 0.02% (v/v) NaN ₃
PBS	2.67 mM KCl, 1.47 mM KH ₂ HPO ₄ , 137.9 mM NaCl, 8.06 mM Na ₂ HPO ₄ , pH 7.2
PBS-B	PBS + 5% (m/v) BSA
PBS-Tween	PBS + 0.05% Tween-20
PFA (Scatchard)	PBS + 2% FCS + 0.02% (v/v) NaN ₃
Precipitation buffer	2.8 M KAc, pH 5,1
Reaction buffer for radioactive labelling	400 mM NaPi, pH 7.4
Resuspension buffer	50 mM Tris, 50 mM EDTA, 100 µg/ml RNase A, pH 8
Sample buffer for EMSA	50 mM EDTA, 80 % (v/v) glycerine, 0.01 % (w/v) bromphenol blue, 0.01 % xylene cyanol

Scatchard oil mixture (density 1.014)	53% dibutyl phthalate, 47% dioctyl phthalate (v/v)
SDS-PAGE running buffer	50 mM Tris/HCl, 380 mM glycine, 4 mM SDS, pH 8.3
Stacking gel SDS buffer (4x)	375 mM Tris/HCl, pH 8.8, 3.75 mM SDS
Separation gel SDS buffer (4x)	125 mM Tris/HCl, pH 6.8, 3.75 mM SDS
TAE-buffer for DNA gels	40 mM Tris, pH 8.3, 0.11% (v/v) acetate, 50 mM EDTA
Transfer buffer for western blotting	192 mM glycine, 25 mM Tris, 20% (v/v) methanol, pH 8.3

2.1.5 Antibodies	
anti-huTNF (mouse, clone T1)	IZI, University Stuttgart
anti-PARP (mouse, clone 7D3-6)	BD Biosciences, Franklin Lakes, USA
anti-huTNFR1 (mouse, clone H398, FACS)	IZI, University Stuttgart
anti-huTNFR2 (mouse, clone 80M2, cytotoxicity assay)	IZI, University Stuttgart
anti-huTNFR2 (mouse, clone MR2-1, FACS)	Hycult Biotechnology, Uden, The Netherlands
anti mouse, IgM + IgG, H+L chain, FITC labelled (goat, FACS)	Dianova, Hamburg
Anti-huTNF (mouse, clone 5N, cytotoxicity assay)	Hycult Biotechnology, Uden, The Netherlands
anti mouse, IgM + IgG, H+L chain, HRP labelled (goat, Western blot)	Dianova, Hamburg
Anti-Tubulin, (mouse, clone DM1A)	Thermo Fisher Scientific, Waltham, USA

2.1.6 Kits	
Lipofectamine TM 2000 Reagent	Invitrogen, Karlsruhe, USA
Protein silver staining kit	GE Healthcare, Uppsala, Sweden
Super Signal West Dura ECL substrate	Pierce, Rockford, USA
PureLink TM HiPure Plasmid DNA Purification Kit for Mini and Midi preparation of plasmid DNA	Invitrogen, Karlsruhe, USA
NucleoSpin Extract II for PCR clean up and gel extraction	Machery-Nagel, Düren

2.1.7 Primers	
Module 1 reverse (TNF-nonstop-AfeI-HindIII-rev)	atcgattaagcttcccgggcagcgctatgataccgaagtaacc tgaccagattc
Module 3 forward (TNF-AfeI-BamHI-for)	ggctggttagcgctggaaagggatcctcttctctgacccgtct gacaaccg
Module 3 reverse (TNF-Stop-XbaI-HindIII-rev)	gggggggaagctttctagatcatcacagagcgatgataccgaa gtaaac

Module 2 forward (TNF-linker- EcoRI - AfeI -for)	ggctggg gaattcagcgct gggtggaggtggtcctcttcgta ccccgtctgacaaaccg
Module 2 reverse (TNF-nonstop-linker- BamHI -rev)	ggaaatt ggatcct caccaccagagcgatgataccgaagtaa acctg
Y87Q Mutation forward	ctcgtatcgctgttcc agc agaccaaagtaa
Y87Q Mutation reverse	gttactttggtctg ctg ggaaacagcgatacgag
Module 1 forward (Pichia forward) EcoRI , EcoRV , XhoI	cgcac gaattcgatatctcg agaagagaatgagaggatcgcat caccatcacc

2.2 Methods

2.2.1 Molecular biology

Enzymes for DNA dephosphorylation, fill-in reactions and DNA digestions were utilized as recommended by the supplier (NEB, Ipswich, USA).

For PCR (Polymerase chain reaction), a DNA polymerase with proof reading functions was used (Pfu from Promega, Madison, USA or KOD from Novagen, San Diego, USA).

2.2.2 Site directed mutagenesis

For inserting a point mutation into the TNF gene, a PCR with reverse complementary primers containing the mutation was performed. The unmutated template DNA was subsequently eliminated by using the restriction enzyme *DpnI*. This enzyme digests only the methylated template DNA, not the unmethylated PCR product.

The following PCR reaction mixture was prepared:

Plasmid DNA	50 ng
KOD Polymerase	2 μ l
25 mM MgSO ₄	2 μ l
8 mM dNTPs	5 μ l
10x KOD buffer	5 μ l
Forward primer	2 μ l
Reverse primer	2 μ l
Sterile ddH ₂ O	Add 50 μ l

The reaction was performed in a PCR cycler (Mastercycler gradient, Eppendorf) as shown in Table 1.

Table 1: PCR program for site directed mutagenesis

Step	Temp.	Time
1. Denaturation	95°C	5 min
2. Denaturation	95°C	30 sec
3. Annealing	60°C	1 min
4. Extension	72°C	2 min
5. Go to Step 2 and repeat 20x		
6. Extension	72°C	10 min
7. Hold	4°C	

2.2.3 Transformation of *E. coli*

50 µl of commercially available chemically competent cells (Subcloning Efficiency DH5α Competent Cells, Invitrogen, Carlsbad, USA) were thawed on ice and incubated for 30 min with the chosen DNA. A heat shock was performed at 37°C in a water bath for 20 sec. The cells were cooled down on ice for 2 minutes. 500 µl SOC⁺⁺ medium was added, followed by an incubation for 1 h at 37°C and plating on LB agar plates containing 100 µg/ml ampicillin. For complex cloning procedures, cells with higher competence (Library Efficiency DH5α Competent Cells, Invitrogen, Carlsbad, USA) were used in the same way, except that the heat shock was executed at 42°C for 45 sec.

2.2.4 Plasmid isolation from *E. coli*

For propagation of plasmid DNA in medium scale, single clones derived from transformations were used to inoculate 50 ml of LB_{Amp} medium and grown over night at 37°C and 180 rpm (Multitron, Infors-HT, Bottmingen, Switzerland). Plasmids were extracted using commercial kits (PureLinkTM HiPure Plasmid DNA Purification, Invitrogen, Carlsbad, USA). Purity and concentration of the DNA were determined by UV-absorption measured photometrically in the nanodrop device (ND-1000, Thermo Fisher Scientific, Waltham, USA).

Plasmid isolations at a smaller scale were performed to check a range of clones for their correctness by restriction digestion. Therefore, homemade solutions were used. Single clones were inoculated in 2 ml LB_{Amp} medium and grown over night at 37°C and 180 rpm. The next day the cultures were centrifuged at 16.000 g for 5 minutes and pellets were resuspended in 150 µl resuspension buffer. 150 µl lysis buffer was added and after 5 minutes 150 µl

neutralisation buffer. The emerged protein precipitate was segregated by centrifugating at 16000 g for 5 min. The supernatant was treated with 1 ml of 100% ethanol and was incubated at -20°C for 30 min. Precipitated DNA was obtained by centrifugating for 5 minutes at 4°C and 16000 g. The DNA was air-dried and resolved in 20 µl TE buffer or ddH₂O.

2.2.5 Construction of expression vectors for different scTNF variants

All mutations which were used to confer receptor selectivity or to prevent receptor binding in general, were described in a publication by the group of W. Lesslauer from Basel (Loetscher et al., 1993).

For generation of the different scTNF mutants, PCR reactions with the appropriate primers and plasmids were performed. As template for the 3 TNF modules of scTNF WT served plasmid **pQE9 His-TNF** (Nr. 178). For TNF-modules with R1 selective mutations, plasmid **pQE9 HisTNF R32W S86T** (Nr. 179) was used, and for TNF-modules with R2 selective mutations **pQE9 HisTNF D143N A145R** (Nr. 180). To generate TNF modules containing only mutation R32W or the mutations D143N A145R together with mutation S86T, the section between the restriction sites *StuI* and *PstI* was exchanged between vectors Nr. 178 and Nr. 179 (see TNF DNA sequence in the appendix). Plasmid Nr. 179 thereby loses mutation S86T and plasmid 180 receives it. The resulting plasmids served as templates for PCR reactions generating the corresponding TNF modules for the scTNFs with mixed selectivities (scTNF R112 and R221). The mutation Y87Q for scTNF Y87Q 1x and Y87Q 2x was obtained by site directed mutagenesis (see below).

The used primers are listed in 2.1.7. The primers for module 2 contain the linkers (amino acids GGGGS) of the scTNF. The DNA sequence of the linkers differ: the codon usage of the second linker (in primer **module 2 rev**) was changed in a way that a *BamHI* site was generated. Primer **module 2 for** inserts a silent mutation in the coding sequence of TNF in front of the first linker leading to an *Eco47III* restriction site. The *Eco47III* site and the *BamHI* site are used to insert TNF module 2 between module 1 and 3.

The single modules were combined in one vector like shown in Figure 3. The arrangement of the differently mutated modules in the scTNF variants is shown in Table 2 and plasmid carts can be found in the appendix.

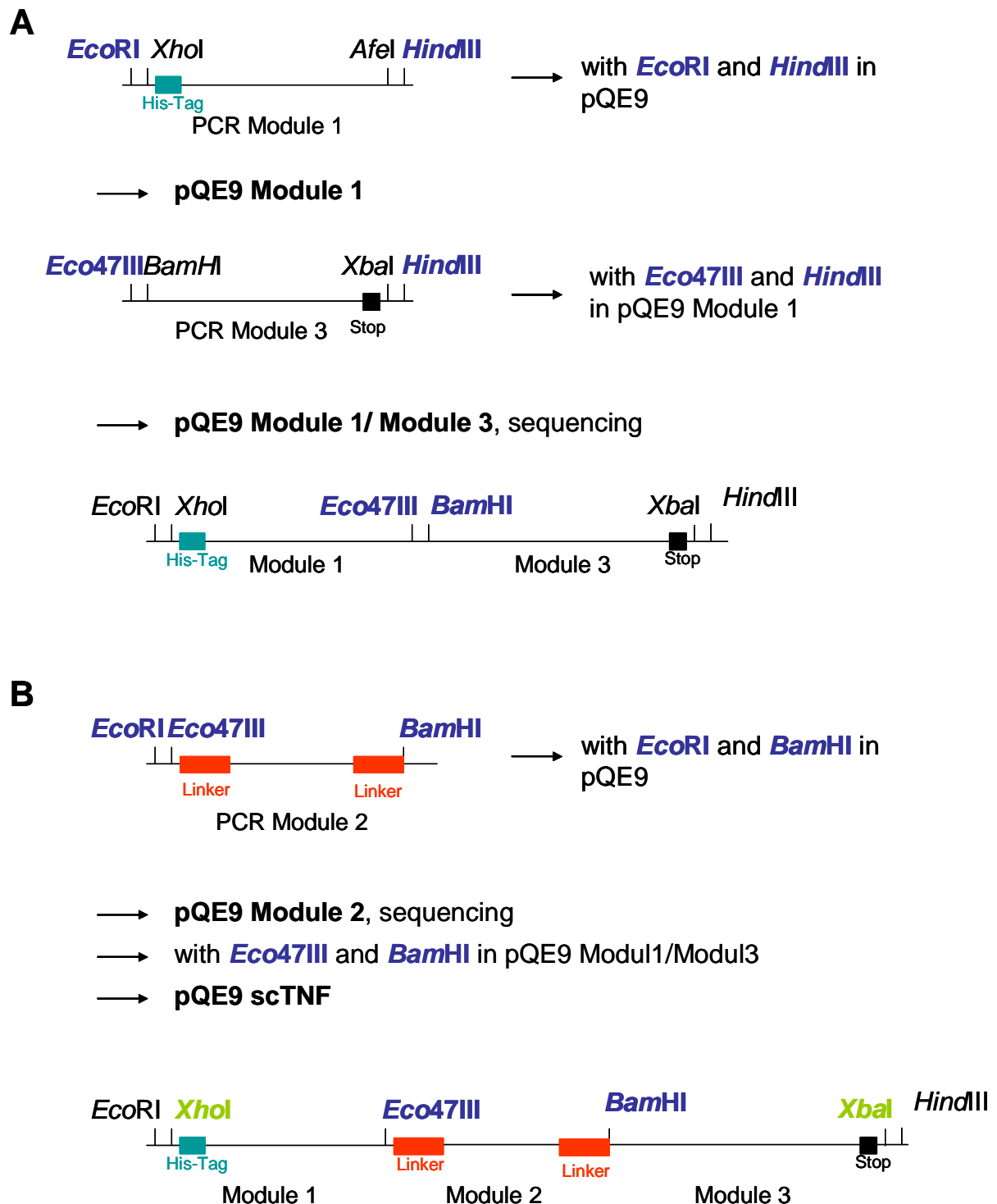


Figure 3: General cloning procedure for the new scTNF molecules. **A:** TNF module 1 and module 3 were combined in one vector by the integrated *Eco47III* and *HindIII* sites. Module 1 contains a His-Tag (dark green) for purification, module 3 the Stop codon (black). **B:** By using the restriction sites *Eco47III* and *BamHI*, TNF module 3 was added. It contains both linkers. By the light green colored restriction sites *XhoI* and *XbaI*, the scTNF gene was cloned in frame behind a signal sequence for secretion.

For generation of **pQE9 scTNF Y87Q 1x**, site directed mutagenesis was performed with vector **pQE9 module 2 TNF WT** and primers **Y87Q Mutation forward /Y87Q Mutation reverse**. The obtained mutated module 2 (codon TAC is replaced by codon CAG) was cloned into **pQE9 module 1/module 3** like shown in Figure 3B. For generation of **pQE9 scTNF Y87Q 2x**, site directed mutagenesis was performed with vector **pQE9 module 1 TNF WT** and the same primer pair. The resulting module 1 was exchanged against the corresponding wildtype module in **pQE9 scTNF Y87Q 1x**.

The coding sequences of the scTNF constructs assembled in pQE9 were subcloned into vector **pCR3 leader Fas prodrug** (Group of Jeanette Gerspach, IZI, University Stuttgart) in frame of the IgG leader sequence for protein secretion via *XhoI/XbaI*, thereby replacing the gene for the Fas prodrug. From the resulting vectors the scTNF coding sequences were cut out together with the leader sequence by digestion with *XbaI* and *HindIII*. By filling in the sticky ends of the digested DNA the scTNF gene together with the leader sequence could be cloned into the expression vector **pIRESpuro3** (linearised with *EcoRV*; Clontech, Mountain View, USA).

2.2.6 Cell culture

Mammalian cells were cultivated in a CO₂ incubator (Sheldon Manufacturing Inc., Cornelius, USA) at 37°C, 96% air humidity and 5% CO₂. For harvesting, adherent cells were washed with PBS and detached with 10% Trypsin-EDTA. The cells were centrifuged (450 g, 5 min) and resuspended in medium or the appropriate buffer. For determination of the cell concentration, the cells were stained with eosine solution and unstained cells were counted in a Neubauer counting chamber (Marienfeld, Lauda-Koenigshofen).

Table 2: Arrangement of TNF modules in the scTNF molecules

scTNF	Module 1	Module 2	Module 3
<i>WT</i>	TNF WT	TNF WT	TNF WT
<i>3R1</i>	R32W S86T	R32W S86T	R32W S86T
<i>3R2</i>	D143N A145R	D143N A145R	D143N A145R
<i>R112</i>	R32W	R32W S86T	S86T D143N A145R
<i>R221</i>	R32W	S86T D143N A145R	D143N A145R
<i>Y87Q 1x</i>	TNF WT	Y87Q	TNF WT
<i>Y87Q 2x</i>	Y87Q	Y87Q	TNF WT

2.2.7 Transfection of HEK cells

HEK293 cells were seeded over night in 6-well plates (1.4×10^6 cells/well) in DMEM + 10% FCS. On the next day medium was exchanged for OptiMEM (2 ml) and cells were transfected with 4 μ g of a scTNF pIRES construct using 5 μ l Lipofectamine 2000 (Invitrogen, Carlsbad, USA). On the next morning medium was replaced by fresh OptiMEM. Cells were incubated for 2 days, then the supernatants were tested by western blotting.

For generation of stable cell lines, cells were transferred into 10 ml cell culture flasks in DMEM + 10% FCS the day after transfection. After 2 days 4 μ g/ml Puromycin was added for selection.

2.2.8 Limited dilution

For generation of cell clones, 100 cells/well of a pool of stable cells were seeded in the first row of a 96 well plate. Cells were stepwise two fold diluted towards the end of the plate. In the following weeks plates were controlled for individual cell clones. If they were grown dense enough, these cells were first transferred to 12 well plates, then to 10 ml cell culture flasks. Protein expression was checked by western blotting.

2.2.9 Expression of different scTNF variants

Individual cell clones which produced high amounts of protein and which showed no degradation products on western blots were chosen for production. The cells were expanded in 550 ml culture flasks (4 bottles per production) with 30 ml DMEM + 10% FCS. When a density of about 90% was achieved, the medium was exchanged against 25 ml Opti-MEM per flask. Cells were incubated for 2 days, then the supernatant was collected and the cells were incubated for another 2 days with fresh medium. The supernatants (at total 200 ml per production) were centrifuged for 5 minutes at 3320 g and stored at 4°C until purification.

2.2.10 Purification of different scTNF variants by IMAC (immobilised metal affinity chromatography)

For purification HisTrapTM FF columns (GE Healthcare, Uppsala, Sweden) were used. The column was prepared as recommended by the manufacturer. For applying supernatants and buffers, a peristaltic pump at a flow rate of 1 ml/min was used (P1, GE Healthcare, Uppsala, Sweden). Prior to loading on the column, 20 ml of 200 mM imidazol was added to 180 ml

supernatant to prevent unspecific binding and to adjust the pH value to 8.0. After loading, the column was washed three times with 5 ml binding buffer, elution buffer was applied and 10 fractions of each 500 µl were collected. Fractions were tested by SDS-PAGE and silver staining and fractions containing the protein of interest were pooled and dialysed over night against PBS. Buffer was exchanged, and dialysis was continued for additional 4 hours on the following day. Dialysed protein was sterile filtrated (Acrodisc® Syringe filter with 0,2 µm HT Tuffryn® membrane, Pall corporation, Ann Arbor, USA).

2.2.11 Determination of protein concentration

Concentration and purity of the dialysed protein was checked by loading different amounts together with sTNF of known concentration (Knoll AG, Ludwigshafen) on a SDS-gel. The gel was silver stained, and concentration of the protein estimated by comparison with the serial dilutions of sTNF. Furthermore, concentration was determined by Bradford staining (Protein Assay, BioRad, Hercules, USA) with BSA as standard according to the manufacturers' instructions and by measuring the absorbance at 280 nm in the nanodrop device (ND-1000, Thermo Fisher Scientific, Waltham, USA).

2.2.12 Cytotoxicity assays

To test the cytotoxic activity of the different scTNF constructs MF TNFR1-Fas, MF TNFR2-Fas cells or Kym-1 cells were used. 7000 cells/well (for Kym-1 10.000 cells per well) were seeded in 96-well flat-bottom plates at the evening before stimulation. On the next day they were treated with different concentrations of protein. Where indicated, MF TNFR2-Fas cells were preincubated for 30 min with 1 µg/ml 80M2 antibody (own production) prior to stimulation. After 6 h, the cells were stained with crystal violet (20% methanol, 0.5% crystal violet) for 10 min. The plates were washed gently with water and air dried. Stained cells were dissolved in 50 µl methanol/well. The absorbance was measured at 550 nm with an ELISA reader (Infinite M200, Tecan, Männedorf, Switzerland).

2.2.13 Immuno-staining and flow cytometry

Harvested cells (5×10^5) were washed once with PBA and incubated with 5 µg/ml of TNFR1 specific antibody (H398, IZI, University Stuttgart) or 2 µg/ml of TNFR2 specific antibody (MR2-1, HyCult Biotechnology, Uden, The Netherlands) on ice for one hour. After washing

with PBA, cells were incubated on ice with FITC labelled goat anti-mouse antibody (goat anti-mouse FITC stained, IgM + IgG, H+L chain, Dianova, Hamburg) for one hour, washed again and analyzed by flow cytometry (Cytomics FC 500 CXP, Beckman Coulter, Fullerton, USA). As a control, cells were treated with PBA instead of the primary antibody.

2.2.14 SDS-PAGE

Proteins were separated by discontinuing Tris/glycin SDS-PAGE using a vertical gel electrophoresis chamber (Phase, Luebeck). Depending on the proteins loaded, running gels contained 7.5 to 15% of acrylamide. Stacking gels contained 4.5% acrylamide. For silver- and coomassie staining SDS-gels of 1 mm thickness were prepared, for western blotting gels of 1.5 mm thickness. The samples were boiled in 4x SDS sample buffer containing 20% β -ME for 5 min at 95°C. The gels were run for 1 h 45 min at 35 mA (1.5 mm) or at 25 mA (1 mm). Gels were subjected to western blotting (2.2.15) or were stained according to the instructions of the manufacturer of the silver staining kit (GE Healthcare, Uppsala, Sweden) or the coomassie stain (Imperial Protein Stain, Thermo Fisher Scientific, Waltham, USA).

2.2.15 Western blotting

Samples were separated by SDS-PAGE and transferred on nitrocellulose membrane (Whatman Schleicher Schuell, Dassel, Germany) for 90 min at 1.5 mA/cm² by using a semi-dry blotting chamber (Phase, Luebeck, Germany). For blocking, the membrane was incubated for 1 h in blocking buffer (PBS, 5% (w/v) non fat milk powder, 0.05% (v/v) Tween-20). Incubation with the primary antibody was carried out over night at 4°C. Secondary antibodies (horseradish peroxidase coupled) were added for 1 h on the next day. As chemiluminescence substrates, the Super Signal West Pico- or Super Signal West Dura-kit (Thermo Fisher Scientific, Waltham, USA) were used. After blocking and each antibody incubation the membrane was washed 3 times for 10 min with washing buffer (PBS, 0.05% Tween-20).

2.2.16 Chemical crosslinking

1.5 μ g of purified scTNF R221 or sTNF in 20 μ l PBS were incubated for 30 minutes on ice with the freshly resolved crosslinking reagent bis-(sulfosuccinimidyl)-suberate (BS³, Thermo Fisher Scientific, Waltham, USA). The used concentrations of the crosslinker ranged from 0 to 500 μ M. Addition of 10 nM Tris/HCl (pH 7) stopped the reaction. The samples were boiled

in 4x SDS sample buffer containing 20% β -ME prior to SDS-PAGE (OWL water cooled electrophoresis chamber, Thermo Fisher Scientific, Waltham, USA) and western blotting with an anti-TNF antibody (T1, IZI production).

2.2.17 Radioactive labelling of scTNF proteins and saturation binding experiments

Proteins were labelled with ^{125}I by the chloramine-T method. Therefore, 10 μg protein in 100 μl 400 mM NaPi buffer (pH 7.4) was added to 10 μl Na ^{125}I (1 mCi) and 10 μl of chloramine-T (0.5 mg/ml in ddH₂O). After one minute the reaction was stopped by incubating with 10 μl sodiumbissulfite (10 mg/ml in ddH₂O) for 3 minutes, and by subsequent addition of 60 μl of NaI (0.6 M). The radioactively labelled proteins were isolated from free radioactive iodine by gel filtration on a prepacked column (PD10, GE Healthcare, Uppsala, Sweden) equilibrated with PBS. The protein was eluted in 10 steps of 1 ml PBS-B. Fractions containing the labelled protein (usually fractions 2, 3 and 4) were pooled and filled up to a volume of 8 ml with PBS-B, leading to a protein concentration of 1 $\mu\text{g}/\text{ml}$ (assuming a protein loss of 20%).

The bioactivity of the radioactive proteins was measured by cytotoxicity assays on Kym-1, MF TNFR1-Fas or MF TNFR2-Fas cells compared to the corresponding unlabelled proteins. Cells were stimulated as described in 2.2.12., and analysis was carried out by adding 7.5 μl of MTT (5 mg/ml) per well for 2 h. Cells were lysed by adding 45 μl of MTT lysis buffer. Plates were incubated for several hours or over night in darkness before being measured at 540 nm in an ELISA reader (SPECTRAMax 340PC, Molecular Devices Corp., Sunnyvale, USA).

For saturation binding experiments, labelled protein was added in a range of concentrations to cells suspended in PFA (50,000 cells/sample). The reaction was stopped after 2 h incubation on ice by centrifugation of the cell suspension for 20 sec in a microcentrifuge (16000 g) through 150 μl phthalate oil mixture (density 1.014). Bound protein was determined by measuring the radioactivity of the cell pellets in a γ -counter (LB211, Berthold, Bad Wildbad). To determine nonspecific binding (NSB) cell bound radioactive protein was measured in the presence of a 100 fold excess of unlabelled sTNF. By using the program Prism (GraphPad Software Inc., San Diego, USA) NSB was subtracted and a one-site binding hyperbola was fitted to the data points.

2.2.18 Elektromobility shift assay (EMSA)

For EMSA 7×10^5 cells/well were seeded in 6 well plates and stimulated for 30 min the following day. The cells were washed on ice with 1 ml PBS, scraped off in 200 μ l buffer A and incubated for 15 min. 12.5 μ l 10% NP-40-solution was added, the samples were shaken for 2 min at 4°C, incubated 1 min at RT and centrifuged 5 min at 16000 g. The supernatant was discarded. 50 μ l buffer C was added to the pellets, they were shaken for 20 min at 4°C and centrifuged for 5 min (16000 g, 4°C). The protein concentration was determined by using the Bradford assay (Protein Assay, BioRad, Hercules, USA).

As probe 32 P-ATP end labelled NF- κ B-specific oligos (5'-AGTTGAGGGGACTTTCCCAGG-3') were used. For labelling of the oligos 10 pmol of each oligo was added to 40 u of T4 DNA Polynucleotidkinase (NEB, Ipswich, USA) and 0.05 mCi of γ - 32 P-ATP in a total volume of 50 μ l and incubated one hour at 37°C and 10 min at 65°C. Both oligos were combined and purified by Probe Quant G50-Microcolumns (GE Healthcare, Uppsala, Sweden) according to the instructions of the manufacturer.

10 μ g of the protein extracts were incubated for 20 min at RT with 4 μ g pDI:DC in binding buffer. The samples were incubated with the labelled oligos (50,000 cpm per sample, diluted in 5 μ l binding buffer) for 10 min. 4 μ l DNA sample buffer was added and the samples were loaded onto a native 6% PAA-gel. The gel was allowed to run for 90 min at 22 mA in 0.25 x TBE. The gel was vacuum dried for 2 hours (Model 583, BioRad, Hercules, USA). A screen was applied over night onto the dry gel and was scanned the next day (Molecular Probes Storm 860 Phosphoreader, Invitrogen, Carlsbad, USA). Analysis was performed with the software Image Quant v 5.0.

3 Results

3.1 Construction of different scTNF variants

The aim of this work is to get more knowledge about the stoichiometry of TNF when binding to its receptors. So far it is totally unclear what molecular events are measured when binding studies with TNF at 4°C are performed. Is it the event of a TNF trimer binding to one receptor molecule or to receptor multimers? In 2000 it was published by the group of Lenardo that TNFRs exist in a preaggregated state on the cell surface prior to ligand binding, but there exists some discordance about the number of receptor molecules incorporated in these aggregates. While in the original publication from 2000 (Chan et al., 2000) receptor trimers were postulated, findings from M. Branschaedel point at dimer formation (Branschaedel, 2007). To shed light onto this question, TNF trimers with only two or only one functional binding site have been generated and examined for their affinity and bioactivity towards TNFR1 and TNFR2. Therefore, mutations were used which confer receptor selectivity to the TNF molecule or which prevent binding at all (Figure 1, Loetscher et al., 1993). All these mutations are located within the clefts between the individual TNF monomers of the trimer, thereby influencing the binding of TNF to its receptors. To be able to mutate specific binding sites of the TNF trimer, the format of scTNF had to be used, where 3 TNF monomers are linked by two glycine-serine linkers. A reversible dissociation of the trimer is thereby prevented, thus conserving the mutated binding sites. Mutation of arginine 32 to a tryptophane (R32W) and mutation of serine 86 to a threonine (S86T) result in TNFR1 selectivity, whereas mutation of aspartic acid 143 to an asparagine (D143N) and mutation of alanine 145 to an arginine (A145R) result in TNFR2 selectivity. The affinity of a TNF containing mutations R32W and S86T towards TNFR2 is reported to be more than 6666 times lower compared to sTNF WT (Loetscher et al, 1993). If mutations A145R and D143N are used, the affinity towards TNFR1 is reported to be more than 2500 lower.

The mutations were combined as shown in Figure 4, resulting in a scTNF with two binding sites for TNFR1 and one binding site for TNFR2 (scTNF R112) and the vice versa protein (scTNF R221). On cells which express only TNFR1, scTNF R112 contains two functional binding sites and scTNF R221 only one. If cells are used which express only TNFR2, scTNF R221 contains two functional binding sites and scTNF R112 contains only one.

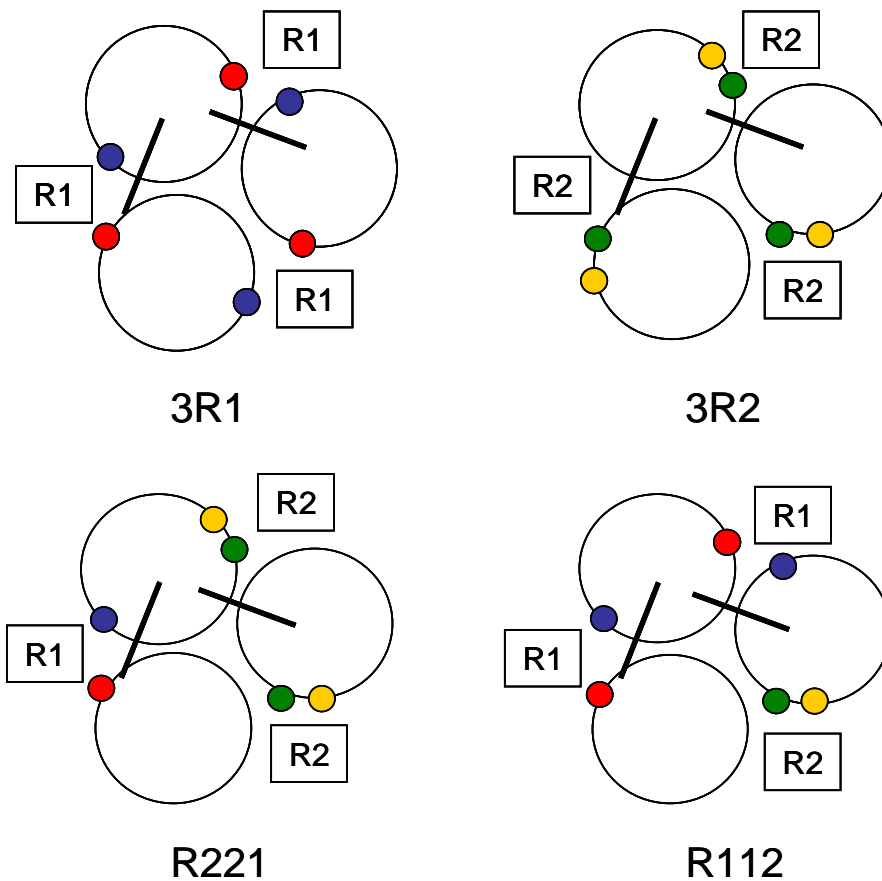


Figure 4: scTNF variants with receptor selective mutations. The TNF trimer is shown from the top. The linkers are depicted as black bars, the mutated amino acids as coloured circles and rectangles show the resulting receptor selectivity. Blue: S86T, red: R32W, yellow: D143N, green: A145R. R1: receptor 1 selectivity, R2: receptor 2 selectivity.

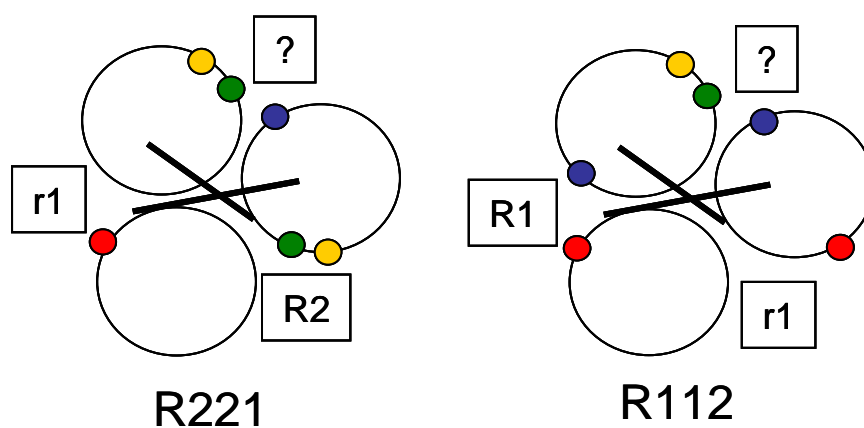


Figure 5: Wrong folding of scTNF R221 and R112. The TNF trimer is shown from the top. The linkers are depicted as black bars, the mutated aminoacids as colored circles and rectangles show the resulting receptor selectivity. Blue: S86T, red: R32W, yellow: D143N, green: A145R. R1: receptor 1 selectivity, R2: receptor 2 selectivity, r1: weak receptor 1 selectivity, ?: effects on binding not known.

All mutations lie at the clefts between the TNF monomers. The two TNFR1 selective mutations are arranged on different sites of the cleft, whereas both TNFR2 selective mutations reside on one side. Hence the resulting scTNF molecules R112 and R221 consist of three TNF monomers, all differing in their mutations (Figure 4). Accordingly, the spatial assembly of the mutations, i.e. the combination of three TNF modules differing in their mutations, has consequences. If the TNF trimer does not assemble in the correct way (here called clockwise, see Figure 4), the selectivities of the binding sites will change, as depicted in the counter clockwise arrangement in Figure 5.

One can still find two TNFR1 selective binding sites on the scTNF R112 molecule and one selective binding site on scTNF R221 in case of counter clockwise assembly (Figure 5). Namely, with a binding site containing the mutation R32W alone a good TNFR1 binding is obtained as well. One binding site contains in both proteins a combination of the two TNFR2 selective mutations D143N and A145R together with the TNFR1 selective mutation S86T. This combination of mutations was never tested, but since the TNFR2 selective mutations clearly lead to a strong loss of binding affinity towards TNFR1 (>2500, Loetscher et al., 1993), and the TNFR1 mutation already effects binding to a smaller extent on its own (see below), one might expect no TNFR1 binding.

scTNF R221 contains only one completely TNFR2 selective binding site and scTNF R112 contains none. Mutation R32W alone is reported to result in an at least 500 fold affinity loss towards TNFR2 (Loetscher et al., 1993), which would allow only weak binding to TNFR2 at this binding site. The binding site containing the TNFR2 selective mutations together with the mutation S86T would allow only weak binding as well: The TNFR1 selective mutation S86T is reported to evoke a 2300 fold loss in affinity for TNFR2 (Loetscher et al., 1993), making a strong binding to TNFR2 rather unlikely.

In consequence, formation of the TNF trimer in the wrong orientation would probably have no effect on the number of binding sites able to bind to TNFR1. In contrast, for TNFR2 one would more or less gain a molecule with only one active binding site and one molecule where no strong binding would be possible at any site.

The peptide linker bridged C- and N-termini have different distances when a scTNF molecule is arranged in a clockwise or a counter clockwise arrangement. As this distance is shorter in the clockwise arrangement, a linker short enough should prevent the wrong assembly. To determine the correct linker length, the distances between the C- and the N-termini in the crystal structure were measured. For the correct clockwise assembly measuring the distances between adjacent N- and C-terminus results in a mean distance of 14 Å, whereas in the

opposite direction the mean distance between N- and C-terminus is 21 Å. It was further determined that a linker consisting of 4 glycine and 1 serine residue should be long enough for the correct assembly of the molecule, but too short for the wrong, i.e. counter clockwise assembly. In contrast to these newly designed scTNF molecules, the classical scTNF molecules described in Krippner-Heidenreich et al., 2008, contain linkers with 3 glycines and 1 serine repeated 3 or 4 times, providing considerably longer linkers.

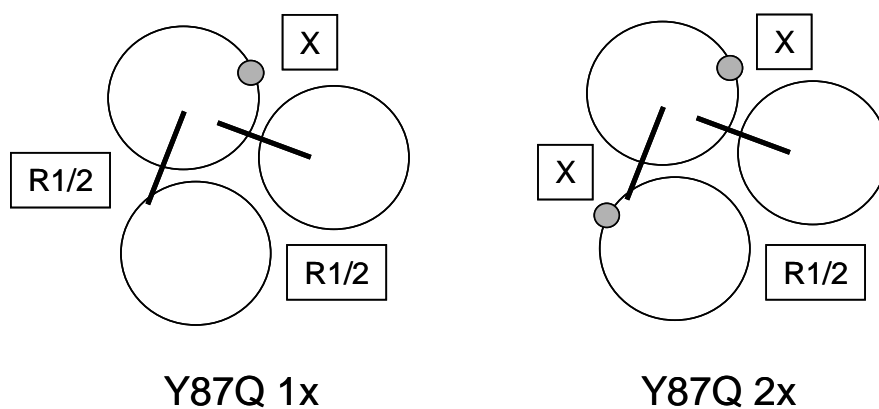


Figure 6: scTNF variants containing the nonbinding mutation Y87Q. The TNF trimer is shown from the top. The linkers are depicted as black bars, the mutated amino acids as circles and rectangles show the resulting binding properties. Grey: mutation Y87Q, R1/R2: binding to receptor 1 and to receptor 2, X: no binding.

In a molecular dynamics (MD) simulation performed by Dr. Andrew Aird (3. Physikalisches Institut, University Stuttgart) of the crystal structure of TNF (Eck et al., 1988) containing the shorter linkers no tensions in the molecule were observable, so that it was assumed that the protein would be not affected in its function by the shorter linkage.

As control molecules the completely TNFR1 selective scTNF (3R1) and the TNFR2 selective scTNF (3R2) were also constructed, using the shorter linkers (Figure 4). The mutations used for conferring receptor selectivity not only result in a strongly reduced affinity towards the receptor where binding should be abolished, but also to some affinity loss towards the receptor where binding should be retained. The affinity of a R32W S86T mutated sTNF towards TNFR1 is reported to be 2.2 times weaker, the affinity of the D143N A145R mutated sTNF is 6.7 times weaker as compared to sTNF WT when binding TNFR2 (Loetscher et al., 1993). It is therefore important to compare the affinities of scTNF R112 and R221 with the completely TNFR1 selective and TNFR2 selective scTNFs, and not with the affinity of a scTNF WT.

Mutation of tyrosine 87 to glutamine (Y87Q) results in a TNF molecule which is reported to bind very weak to TNFR1 and to TNFR2. In a solid phase radioligand binding assay, lysate of bacteria expressing this TNF mutant did not compete with radioactive labelled wildtype TNF for binding to TNFR1 or TNFR2 at the lowest dilution tested (Loetscher et al., 1993). By constructing a scTNF derivative with one mutated TNF module (scTNF Y87Q 1x) and one with two mutated modules (scTNF Y87Q 2x), the results from the scTNFs with the receptor selective mutations could be compared (Figure 6). Both scTNF molecules contain also the short GGGGS-linker. To possess the relevant control protein, scTNF WT with the GGGGS-linker was constructed.

3.2 scTNF production

Because in *Escherichia coli* the majority of the produced scTNF protein appeared in inclusion bodies, the new scTNF variants were produced in a different system. First, the methylotrophic yeast *Pichia pastoris* was used as an expression system. The advantage of this system is the directed integration of the gene of interest into the yeast genome, the inducible expression and the low costs. Although the proteins were produced at sufficiently high concentrations in this system, there were high amounts of degradation products the formation of which could not be prevented, e.g. by using protease inhibitors (data not shown).

In a second approach, HEK293 cells were used in combination with the pIRESpuro3 vector. This vector contains the internal ribosomal entry site (IRES) of the encephalomyocarditis virus (ECMV), which allows the translation of two open reading frames (ORF) from one mRNA. Behind the IRES the gene for puromycin resistance is placed, thereby coupling transgene with resistance gene transcription. After selection nearly all surviving clones will therefore express the transgene. By using an IgG-signal sequence, the protein should become secreted into the cell supernatant.

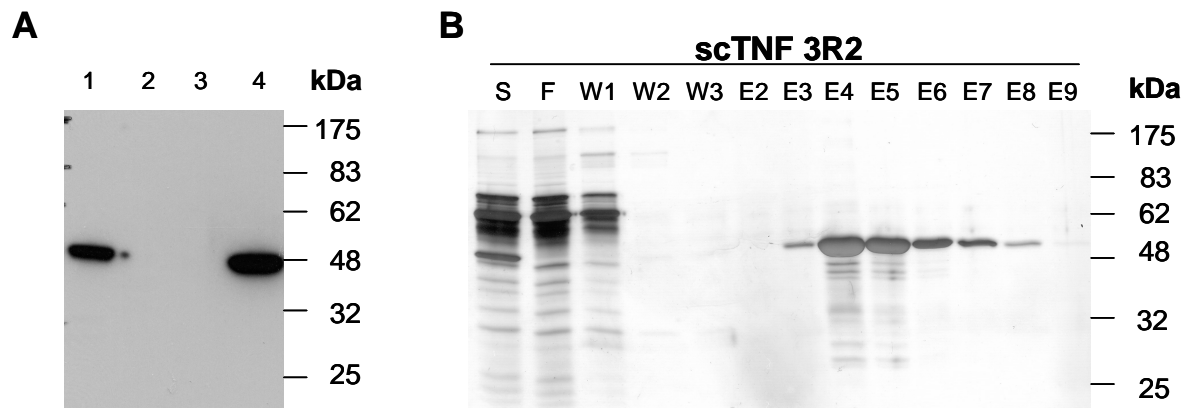


Figure 7: Transfection and Purification of scTNF derivatives. A: Western blot (10% SDS-PAGE, anti-TNF antibody T1, 1 μ g/ml) of supernatants derived from cells two days after transfection. Lane 1: 200 ng purified scTNF expressed in *E. coli*, 2: untransfected cells, 3: cells transfected with pIRES puro 3, 4: cells transfected with pIRES puro 3 Y87Q 2x. **B:** Purification of scTNF 3R2 by IMAC. The fractions from the Ni- Sepharose column were analysed by silver staining (10 % SDS-PAGE). Shown are the loaded supernatant (S), the flow through (F), the wash fractions (W1-W3) and the elution fractions (E2-E9).

Figure 7A shows the results of a transfection with the vector for scTNF Y87Q 2x expression. Expressed protein was detected by western blotting with an antibody specific for TNF. As negative controls supernatants of untransfected cells and of cells transfected with the empty expression vector pIRES puro 3 were loaded. As a positive control, 200 ng of the classical scTNF WT produced in *E. coli* (Krippner-Heidenreich et al., 2008) was used. A protein with a molecular mass of approximately 50 kDa was detected in the lane of the cells transfected with pIRES puro 3 scTNF Y87Q 2x, corresponding to the size of the loaded control scTNF protein. No protein was detected when the empty vector was transfected or when supernatant of untransfected cells was loaded. The concentration of scTNF protein in supernatants of transiently transfected cells was estimated to 1 mg/l. To obtain higher yields of protein, transfected cells were stabilized by adding 4 μ g/ml puromycin. Single clones were isolated by limited dilution and clones producing a satisfying amount of protein (highest concentration achieved: 8 mg/l) combined with a low percentage of degradation products were chosen for large scale production resulting in 200 ml of supernatant.

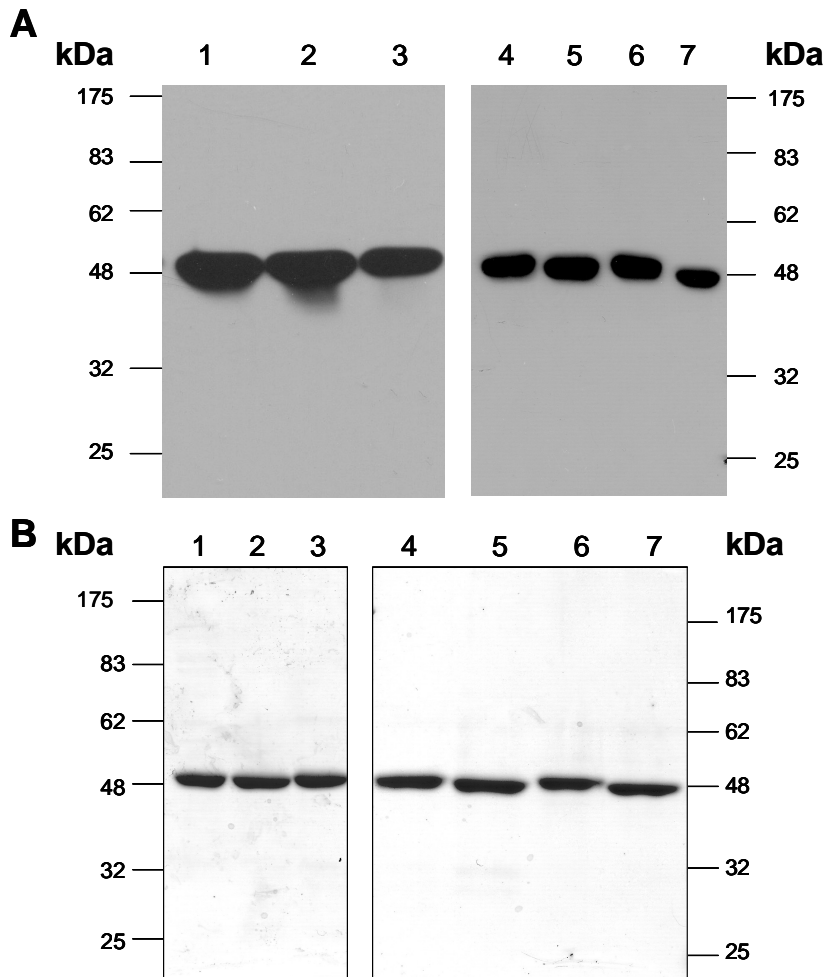


Figure 8: Overview of the produced proteins. **A:** 1 μ g of each protein was subjected to SDS-PAGE (1-3: 12.5%, 4-7: 10%) and western blotting with anti-TNF antibody (T1, 1 μ g/ml). **B:** 1 μ g of each protein was subjected to SDS-PAGE (10%), subsequently the gel was stained with coomassie. 1: scTNF Y87Q 1x, 2: scTNF Y87Q 2x, 3: scTNF WT, 4: scTNF 3R1, 5: scTNF 3R2, 6: scTNF R112, 7: scTNF R221.

The proteins were purified from the supernatants by IMAC (Immobilized metal affinity chromatography, Figure 7B) via the N-terminal His-Tag of the proteins. The majority of the protein eluted in fractions 4 and 5 and only minor amounts in fractions 6 and 7. The concentrations of the purified scTNF proteins were determined, and equal amounts were analysed by coomassie staining and by western blotting (Figure 8). All proteins showed a high purity and a molecular mass of about 50 kDa, corresponding to a TNF trimer. No additional protein bands were recognized by an antibody specific for TNF under these conditions.

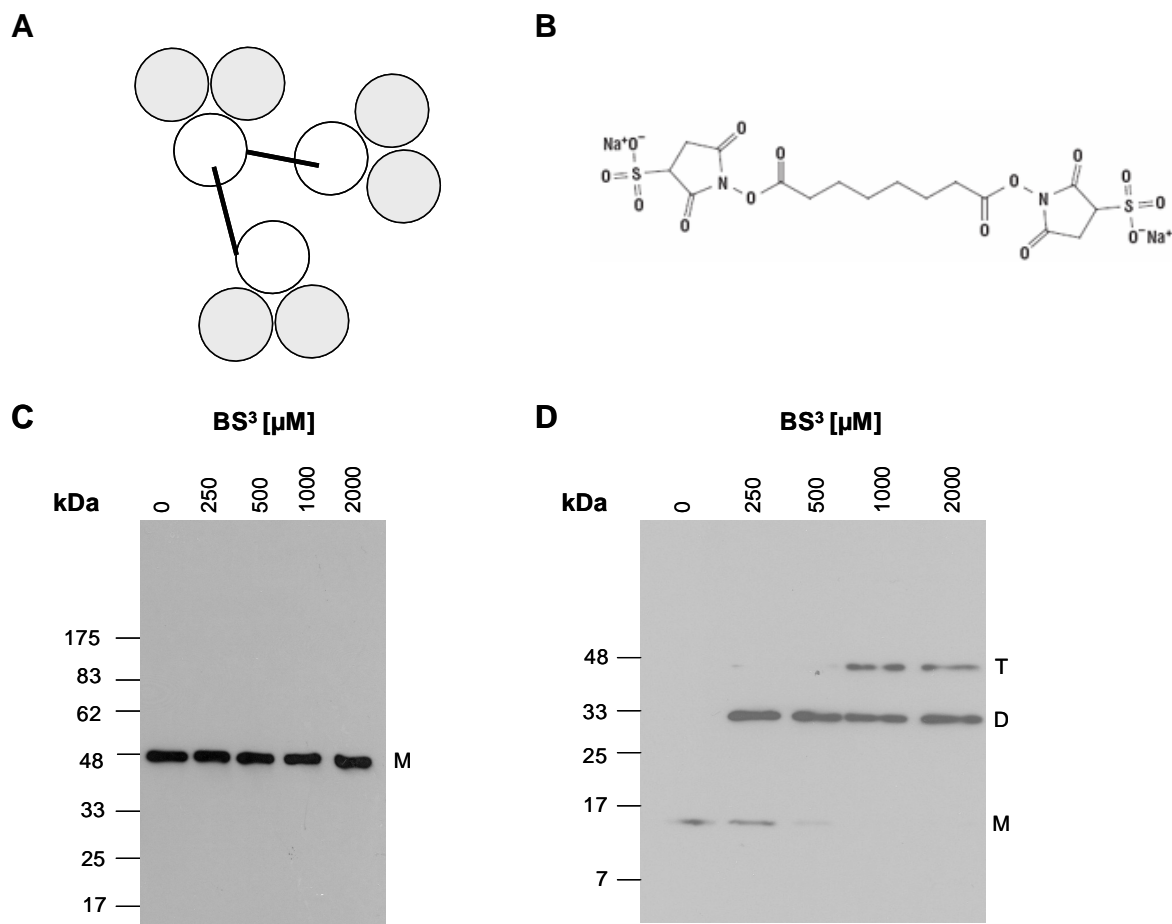


Figure 9: scTNF molecules with shorter linkers show no aggregate formation. Linkage of the TNF monomers could result in formation of higher protein aggregates by formation of intermolecular contacts (A). By treatment with the chemical crosslinker BS³ (B) these aggregates can be detected. With its 11.2 Å long linker separating two reactive ester groups it can covalently stabilize protein interactions, making them visible on SDS-PAGE. Representative for all new scTNF constructs containing GGGGS-linkers 1.5 µg of scTNF R221 per sample (C) or sTNF as a control (D) were incubated for 30 minutes on ice with the indicated BS³ concentrations. Samples were boiled with 4x SDS sample buffer containing 20% β-ME and were subjected to SDS-PAGE (C: 10% running gel, D: 15% running gel). Western blotting with a TNF specific antibody was performed (T1, 1 µg/ml). M: Monomer, D: Dimer, T: Trimer.

By combining three TNF monomers in one polypeptide, it might be possible that not the three TNF monomers of one molecule, but rather the monomers of different scTNF molecules interact (Figure 9A). Thereby large protein aggregates would build up. With the long linkers of the classical scTNFs no such aggregates were detected in an assay with the homobifunctional, noncleavable and water soluble crosslinker BS³ (Krippner-Heidenreich et al., 2008). This substance connects lysine residues of proteins located at a distance up to 11.2 Å to each other by its two ester groups (Figure 9B) and thereby makes protein interactions visible in SDS-PAGE. It was important to carry out this experiment also with the new scTNF molecules presented in this work, because the linker length had been changed. Provided the new linkers might be too short to allow a homotrimerization of the TNF monomers to form

one stable molecule, aggregate formation would be feasible. Furthermore, in TNF trimers formed by intermolecular assembly of scTNF molecules as depicted in Figure 9A, the designated selectivity and the amount of functional binding sites would not be retained. Exemplarily for all newly produced scTNF variants, scTNF R221 was incubated with different amounts of BS³. sTNF WT was used as a positive control and was efficiently crosslinked: The TNF dimer (34 kDa) and the trimer (51 kDa) are detectable in the western blot (Figure 9C). With the scTNF only the band of the monomeric protein at around 50 kDa is visible, no higher bands of crosslinked protein were detectable (Figure 9D). Therefore, it can be excluded that a significant percentage of the protein exists in an aggregated form.

3.3 *Bioactivity determinations*

3.3.1 **scTNFs with receptor selective mutations**

The scTNF variants with receptor selective mutations were tested in different assay systems to assess their bioactivity. For testing the ability of the proteins to activate caspases and thereby their ability to exert apoptosis, the cleavage of the protein PARP (Poly (ADP-ribose) polymerase) upon stimulation with different scTNF constructs was examined. PARP is a DNA-binding protein, which recognizes DNA strand breaks and assists in their repair. It is cleaved by active effector caspases resulting in a loss of its normal function, and this processing serves as a marker for the occurrence of apoptosis (Duriez and Sha, 1997). Kym-1 cells, a human rhabdomyosarcoma cell line expressing TNFR1 and TNFR2, were stimulated for 6 hours with 50 ng/ml of the different scTNF constructs and with sTNF as a control. Cells were scraped off, lysed and subjected to SDS-PAGE and western blotting. For all scTNF constructs, except scTNF 3R2, the processed 89 kDa band of PARP together with the band of the intact protein of 116 kDa could be detected in a western blot with an anti-PARP antibody (Figure 10). The samples stimulated with the completely R2 selective scTNF showed no processing, as well as the untreated control, implying that no caspase activation and thereby no apoptosis had occurred. Since with soluble TNF or soluble scTNF-derivatives only TNFR1 can be activated (Grell et al., 1995), this result was expected and demonstrates the selectivity of the D143N and A145R mutations. Already with the presence of a single binding site specific for TNFR1, however, significant PARP processing takes place, visible in the presence of the 89 kDa fragment in the scTNF R221-treated sample. Upon treatment with scTNF R112 the stimulated cells show already a level of PARP processing comparable to the fully active scTNF 3R1 protein. Clearly, a loss of one or two binding sites in the TNF trimer does not result in a complete loss of the ability to induce apoptotic signals, but rather leads to a reduction in signal intensity only.

To check if the scTNF proteins are also able to activate the nonapoptotic pathways of TNFR1, HeLa cells, which express only TNFR1, were stimulated with 100 ng/ml of the respective scTNF constructs for 30 minutes. Nuclear extracts were prepared and were subjected to an EMSA analysis to examine NF- κ B activity (Figure 11). The activity in cells stimulated with scTNF 3R2 is significantly lower in comparison to cells stimulated with the other TNF constructs and is comparable to unstimulated cells. A gradual, but not significant decline in the NF- κ B signal is also visible with decreasing amounts of binding sites for TNFR1 (Figure 11B). These data demonstrate that not only for apoptosis induction but also for the activation

of NF- κ B it holds true that a loss of one or two binding sites of a TNF trimer does not lead to a complete abrogation of signalling, but rather to a reduction in signal intensity.

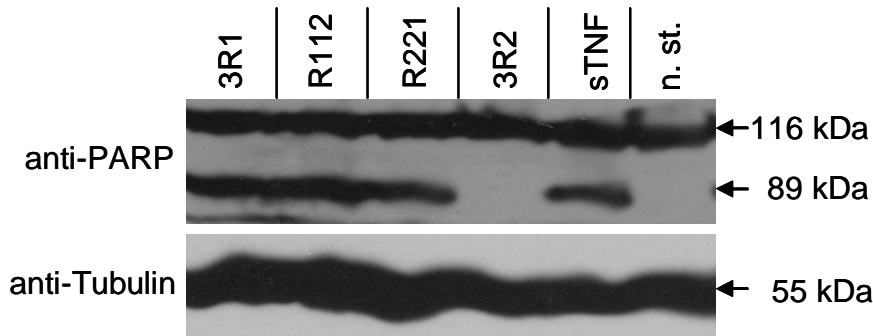


Figure 10: PARP processing in cells stimulated with scTNF derivatives containing receptor selective mutations. Kym-1 cells were stimulated for 6 h with 50 ng/ml of the indicated scTNFs, sTNF as positive control or were left untreated (n. st.). Cells were scraped off, 4x SDS sample buffer containing 20% β -ME was added, samples were boiled for 5 minutes at 95°C and for complete cell lysis sonificated 5 times. Samples were analysed by SDS-PAGE (7.5%), and western blotting with a PARP specific antibody (upper panel) was performed. As a loading control tubulin was used (lower panel). Therefore the blot was stripped and reprobred with an antibody recognizing tubulin.

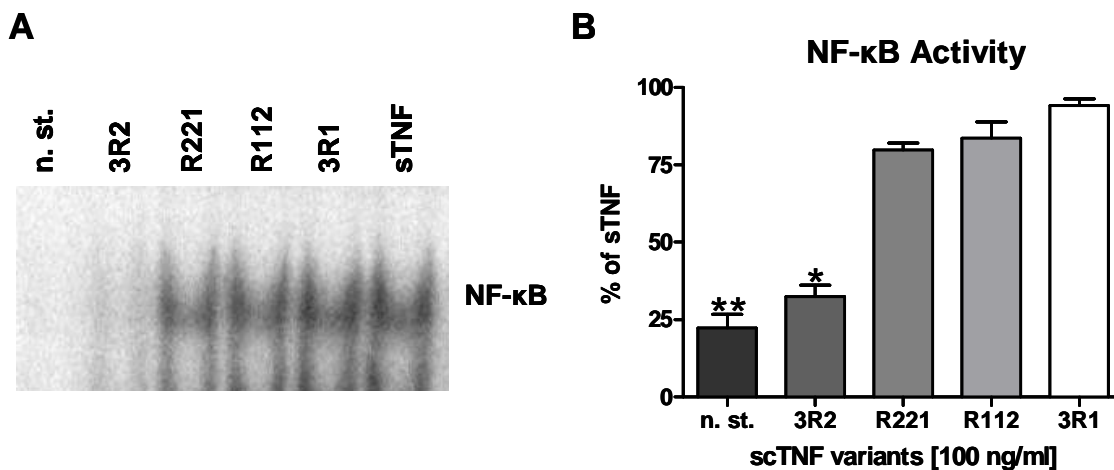


Figure 11: Activation of the nonapoptotic pathway of TNFR1. **A:** HeLa cells were stimulated for 30 min with 100 ng/ml of the respective scTNF variant, with sTNF as positive control or were left untreated (n. st.). Nucleic extracts were prepared and samples were subjected to an electrophoretic mobility shift assay (EMSA) for determination of NF- κ B activity. **B:** Mean band intensities of 3 independent experiments are displayed as percentage of the mean intensity obtained with sTNF stimulation (not shown). **: $P > 0.1$ (compared to 3R2), *: $P < 0.001$ (compared to R221).

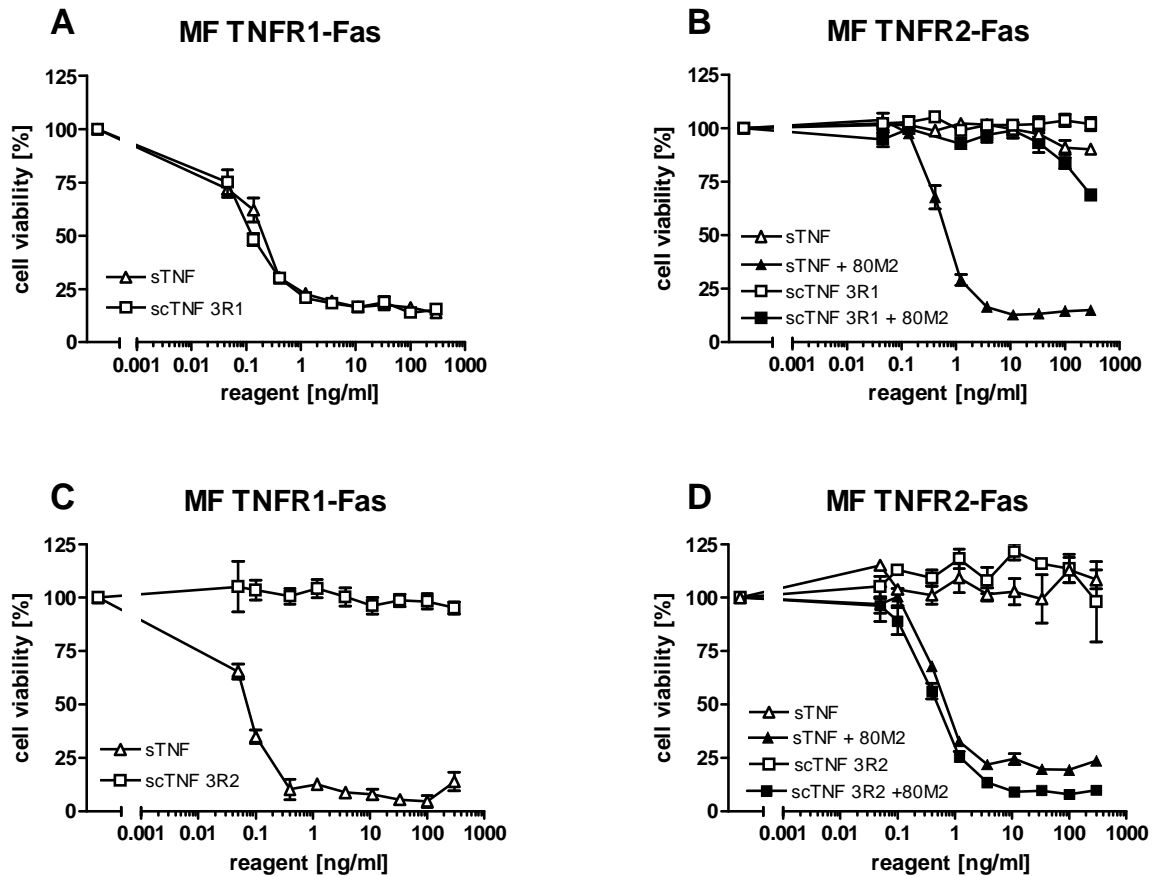


Figure 12: scTNF derivatives 3R1 and 3R2 show the expected receptor selectivity. For the depicted cytotoxicity assays cells were incubated with serial dilutions of different scTNF constructs or sTNF for 6 h or were left untreated. Living cells were quantified by crystal violet staining. Cell viability is displayed as percentage of untreated cells. **A:** scTNF 3R1 and sTNF on MF TNFR1-Fas, **B:** scTNF 3R1 and sTNF with and without the anti-TNFR2 antibody 80M2 on MF TNFR2-Fas, **C:** scTNF 3R2 and sTNF on MF TNFR1-Fas, **D:** scTNF 3R2 and sTNF with and without 80M2 on MF TNFR2-Fas cells. Data points represent mean values out of triplicates.

To examine the loss in signal intensity triggered by scTNF R112 and R221 over a large range of ligand concentrations, cytotoxicity assays were performed. To this, embryonic mouse fibroblasts from TNFR1/TNFR2 knockout mice transfected with the human TNFR1-Fas or the TNFR2-Fas receptor constructs (Krippner-Heidenreich et al., 2002) were used. In these chimeric receptors the intracellular part of the respective TNF receptor is exchanged against the intracellular signalling part of the TNF receptor family member Fas/CD95. The intracellular domain of the Fas receptor contains a death domain leading to a strong and immediate apoptotic signal upon receptor activation, but only to a weak activation of NF- κ B. Because the intracellular parts of the two TNF receptor constructs are identical, both cell types react to TNF stimulation with apoptosis. Furthermore, using these chimeric receptors it is possible to directly compare the signal strengths of the two TNF receptors to a certain

degree. However, the differential properties of the TNFRs regarding their responsiveness to soluble TNF is retained in the chimaeras. TNFR1-Fas is activated by membrane bound TNF as well as by soluble TNF, TNFR2-Fas can only be efficiently activated upon membrane TNF binding, but not with soluble TNF. For mimicking mTNF, soluble TNF in combination with the TNFR2 specific antibody 80M2 can be used (Grell et al., 1995).

For the cytotoxicity assays, the cells were stimulated with different concentrations of the scTNF derivatives and with sTNF as a positive control for 6 hours. The TNFR2-Fas cells were preincubated with 1 $\mu\text{g/ml}$ of the antibody 80M2 for 30 minutes prior to stimulation to mimic mTNF action in combination with sTNF or the scTNF derivatives. Dead cells were washed away and remaining cells were stained with crystal violet.

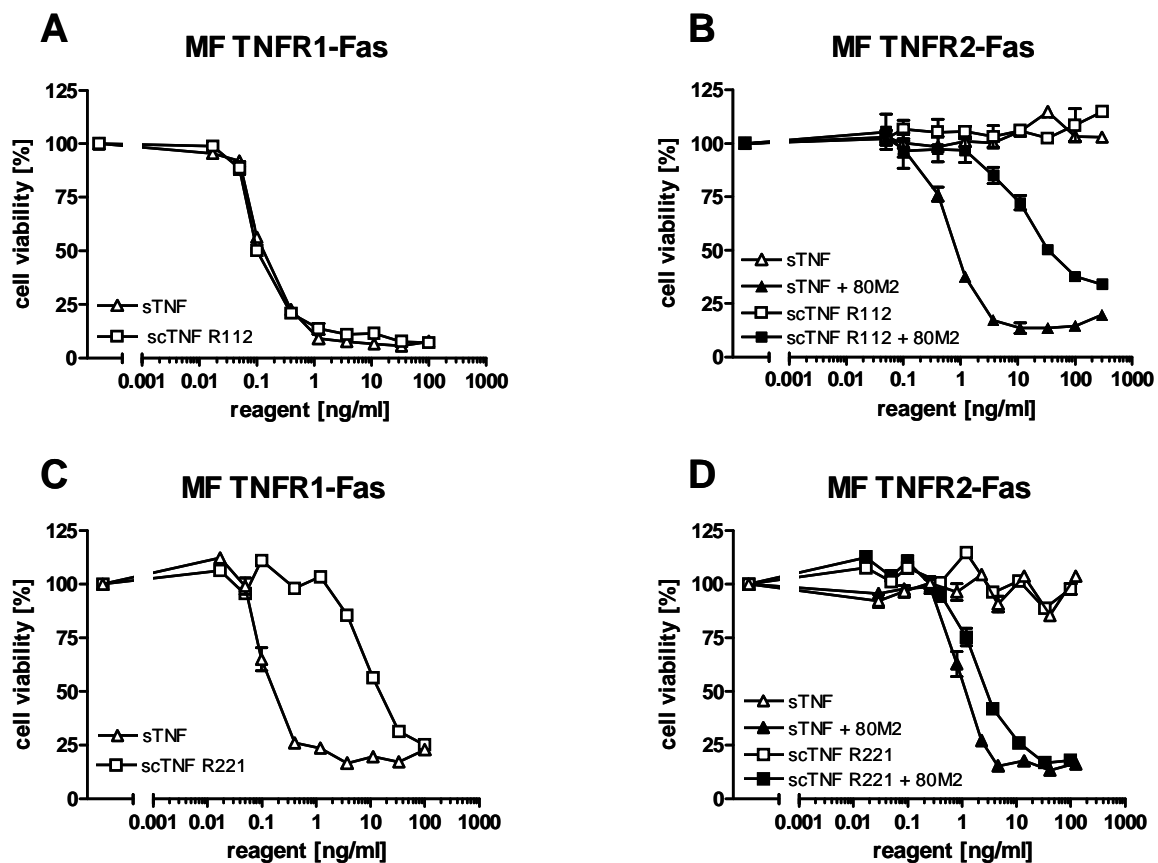


Figure 13: The loss of two functional binding sites results in a strong reduction of bioactivity. For the shown cytotoxicity assays cells were incubated with serial dilutions of different scTNF constructs or sTNF for 6 h or were left untreated. Living cells were quantified by crystal violet staining. Cell viability is displayed as percentage of untreated cells. **A:** scTNF R112 and sTNF MF TNFR1-Fas, **B:** scTNF R112 and sTNF with and without the anti-TNFR2 antibody 80M2 on MF TNFR2-Fas, **C:** scTNF R221 and sTNF on MF TNFR1-Fas, **D:** scTNF R221 and sTNF with and without 80M2 on MF TNFR2-Fas cells. Data points represent mean values out of triplicates.

As a control, cells were incubated with purified supernatant from cells transfected only with the empty expression vector. No dead cells could be observed by this treatment (data not shown). Cell death obtained by sTNF or the scTNF molecules could be blocked by adding an antagonistic antibody specific for TNF (data not shown).

The scTNF 3R1 shows a cytotoxic activity comparable to TNF on TNFR1-Fas cells, but no activity on TNFR2-Fas cells could be detected at the concentrations tested (Figure 12). As expected, scTNF 3R2 shows no activity on TNFR1-Fas cells, but TNFR2-Fas cells die upon treatment in a similar manner as if treated with sTNF. There is no activity observable when scTNF 3R2 is used without the antibody 80M2 on TNFR2-Fas cells. These results correspond to those obtained with the classical receptor selective scTNFs (Krippner-Heidenreich et al., 2008), confirming that the exchange of the linkers did not affect the activity of the proteins and their receptor selectivity.

scTNF R112 is similarly active as sTNF on TNFR1-Fas cells, but this molecule could induce apoptosis on TNFR2-Fas cells only when used at much higher concentrations than sTNF (Figure 13). In contrast, scTNF R221 shows a clear activity loss when compared to sTNF and acting on TNFR1-Fas cells, whereas it is only slightly weaker cytotoxic on TNFR2-Fas cells. Further evidence for the validity of the obtained results with scTNF R112 and scTNF R221 was obtained from experiments where partially purified proteins produced in the yeast *Pichia pastoris* had been used and similar results were obtained (data not shown).

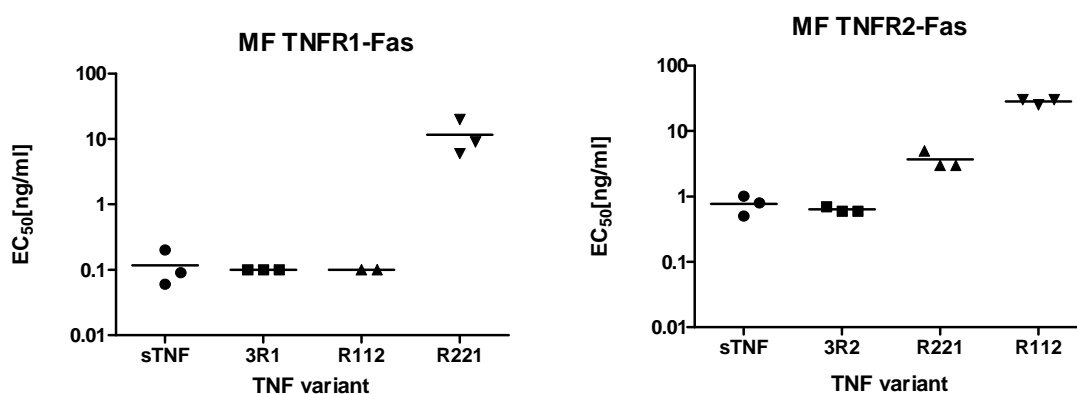


Figure 14: Overview of EC₅₀ values of scTNF variants with receptor selective mutations. Depicted are the obtained EC₅₀ values of different scTNF variants and sTNF derived from cytotoxicity assays with TNFR1-Fas and TNFR2-Fas cells and the resulting mean values.

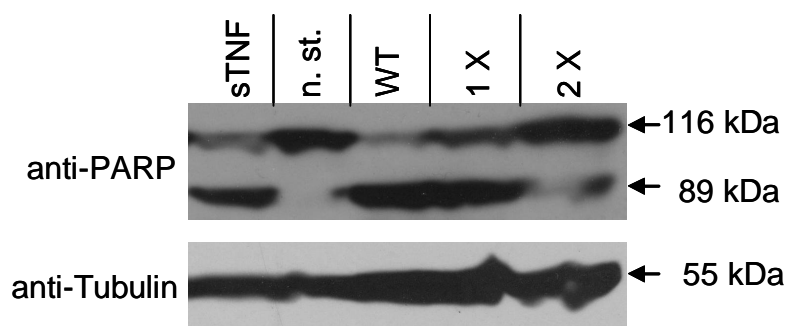


Figure 15: PARP processing in cells stimulated with scTNF variants containing the Y87Q mutation. Kym-1 cells were stimulated for 6 h with 50 ng/ml of the indicated scTNFs, sTNF as positive control or were left untreated (n. st.). Cells were scraped off, 4x SDS sample buffer containing 20% β -ME was added, samples were boiled for 5 minutes at 95°C and for complete cell lysis sonicated 5 times. Samples were analysed by SDS-PAGE (7.5%) and Western blotting with an anti-PARP antibody (upper panel) was performed. As a loading control tubulin was used (lower panel). Therefore the blot was stripped and reprobated with an anti-tubulin antibody. 1x: scTNF Y87Q 1x, 2X: scTNF Y87Q 2x, WT: scTNF WT.

For comparison of the proteins, the EC 50 values (the concentration where 50% of the cells die) of several cytotoxicity experiments were determined. Figure 14 gives an overview of the results (corresponding data can be found in Table 3). If one compares the EC 50 values of the proteins with respect to the number of binding sites for one particular receptor, it becomes obvious that a loss of a single binding site results in only a weak (TNFR2) or even in no reduction (TNFR1) of bioactivity, whereas the loss of two receptor binding sites causes a high reduction (TNFR1 and TNFR2).

3.3.2 scTNFs with mutation Y87Q

The scTNF variants containing the mutation Y87Q and the scTNF WT as a control were tested on Kym-1 cells in a PARP assay to investigate their ability for induction of apoptosis (Figure 15). The processing of the PARP protein when scTNF WT is used, is comparable to the processing in the sTNF sample. Using scTNF Y87Q 1x, a slightly reduced PARP processing is visible as indicated by the weaker band of the unprocessed protein. Using scTNF Y87Q 2x as a stimulator, an even stronger reduction in processing is apparent. Accordingly, the reduction in the number of receptor binding sites also leads to a reduction in apoptosis development in Kym-1 cells, similar to the data obtained with the receptor selective scTNF mutants, but not to a complete activity loss.

The reduction in bioactivity was also examined in cytotoxicity assays using the mouse fibroblasts expressing the TNFR-Fas chimeras (Figure 16). In some difference to the results of the PARP cleavage assay system with the Kym-1 cells, scTNFs Y87Q 1x and Y87Q 2x

show only a slightly reduced bioactivity on TNFR1-Fas cells as compared to sTNF. In contrast, when acting on TNFR2-Fas cells, scTNF Y87Q 1x is around 10 times less active as compared to sTNF. Moreover, scTNF Y87Q 1x is capable to induce apoptosis only in a subset of all cells, whereas about 40% of the cells survive. A flow cytometry analysis revealed that TNFR2 expression was rather inhomogenous between the cells (data not shown). Probably the resistant cells express the receptor only in low numbers. The scTNF Y87Q 2x shows no activity on the TNFR2-Fas cells at the concentrations tested (highest concentration tested: 400 ng/ml).

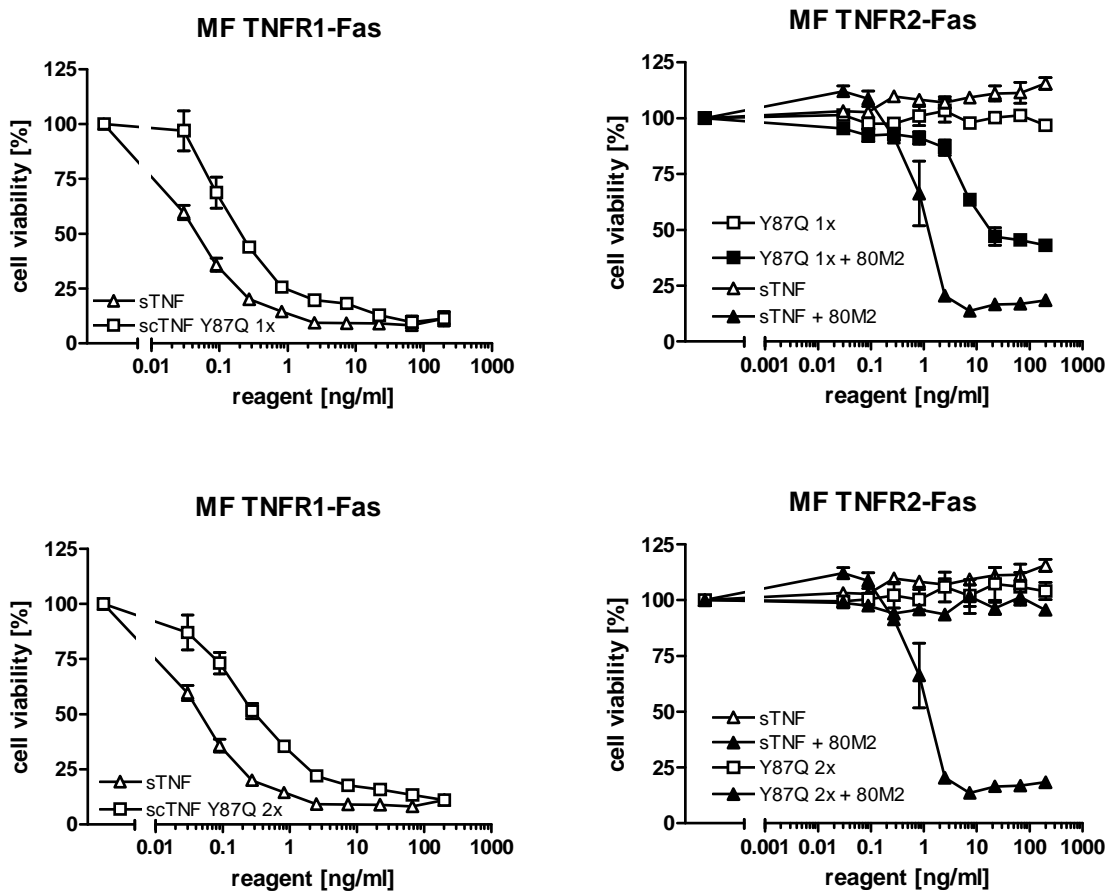


Figure 16: Y87Q scTNF derivatives show a strong bioactivity on TNFR1-Fas cells but a weak one on TNFR2-Fas cells. For the shown cytotoxicity assays cells were incubated with serial dilutions of different scTNF constructs or sTNF for 6 h or were left untreated. Living cells were quantified by crystal violet staining. Cell viability is displayed as percentage of untreated cells. **A:** scTNF Y87Q 1x and sTNF on MF TNFR1-Fas, **B:** scTNF Y87Q 1x and sTNF with and without the anti-TNFR2 antibody 80M2 on MF TNFR2-Fas, **C:** scTNF Y87Q 2x and sTNF on MF TNFR1-Fas, **D:** scTNF Y87Q 2x and sTNF with and without 80M2 on MF TNFR2-Fas cells. Data points represent mean values out of triplicates.

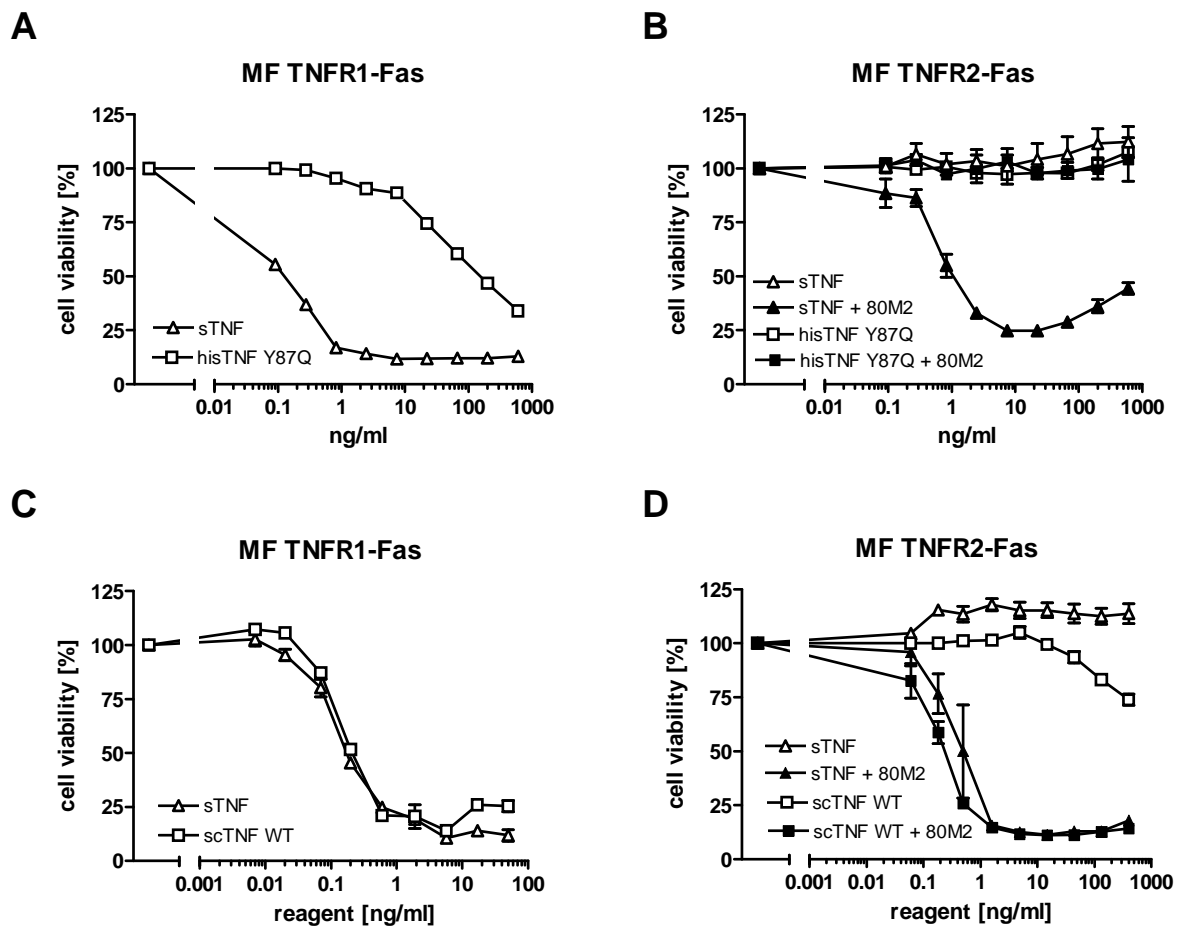


Figure 17: Bioactivity of hisTNF Y87Q and scTNF WT compared to sTNF. For the shown cytotoxicity assays cells were incubated with serial dilutions of sTNF or TNF derivatives for 6 h or were left untreated. Living cells were quantified by crystal violet staining. Cell viability is displayed as percentage of untreated cells. **A:** hisTNF Y87Q and sTNF on MF TNFR1-Fas, **B:** hisTNF Y87Q and sTNF with and without the anti-TNFR2 antibody 80M2 on mouse fibroblasts TNFR2-Fas, **C:** scTNF WT and sTNF on MF TNFR1-Fas, **D:** scTNF WT and sTNF with and without 80M2 on MF TNFR2-Fas cells. Data points represent mean values out of triplicates.

scTNF WT showed on TNFR1-Fas cells as well as on TNFR2-Fas cells an activity comparable to sTNF (Figure 17, C and D).

As a control, cytotoxicity assays were performed with the protein hisTNF Y87Q, representing a sTNF molecule containing the mutation Y87Q and a His tag for purification (produced and purified by Jessica Tepperink, IZI, University Stuttgart). Trimers of this protein contain the mutation at every binding site and should therefore display a weak activity on TNFR1 as well as on TNFR2. Consistent with these expectations, on TNFR2-Fas cells no activity could be detected at the concentrations tested (Figure 17B, highest concentration tested: 600 ng/ml). On TNFR1-Fas cells a more than 1000 times lower activity was observed (Figure 17A).

The determination of the EC_{50} values of several experiments and their comparison confirms the findings: On TNFR1-Fas cells the loss of one or two binding sites results only in a weak reduction of activity, whereas a prominent reduction is visible on TNFR2-Fas cells (Figure 18, corresponding data in Table 3).

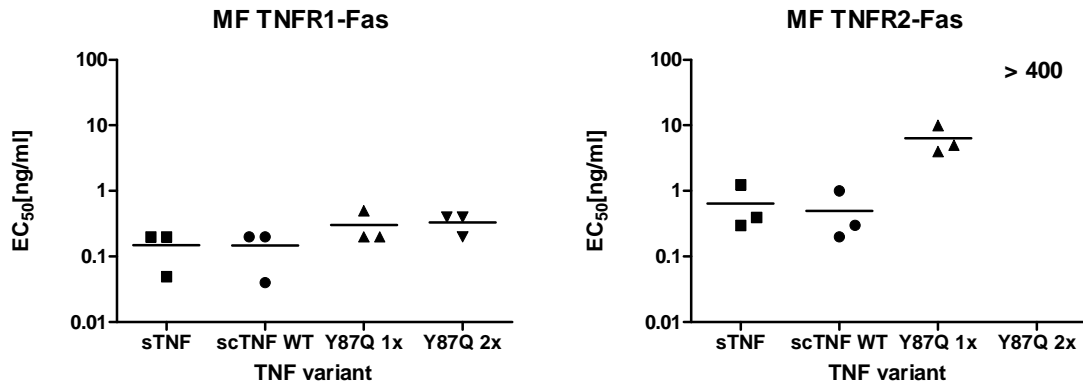


Figure 18: Overview of EC_{50} values of scTNF variants with Y87Q mutations. Depicted are the obtained EC_{50} values of different scTNF variants and sTNF derived from cytotoxicity assays and the resulting mean values. For scTNF Y87Q 2x no activity on TNFR2-Fas could be detected at the highest concentration applied (400 ng/ml).

3.4 Equilibrium binding studies

3.4.1 scTNF derivatives with receptor selective mutations

To perform binding studies on cells using the different scTNF mutants, these were labelled with radioactive iodine (^{125}I) by the chloramine T method. Chloramine T is a strong oxidant producing elementary ^{125}I from Na^{125}I , which then disproportionates resulting in the formation of iodine cations capable to react with tyrosine groups of the respective protein. As

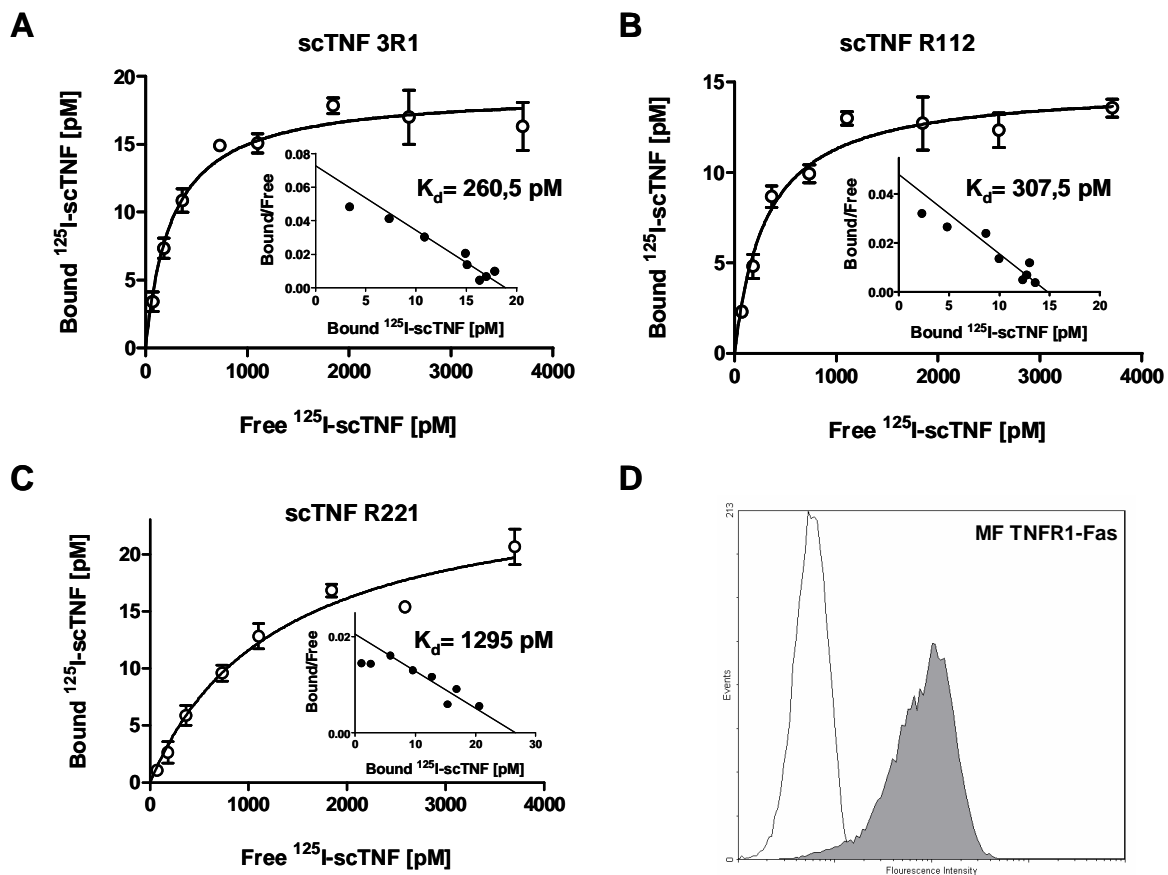


Figure 19: Equilibrium binding studies with scTNF variants containing receptor selective mutations on MF TNFR1-Fas cells. A-C: Iodinated scTNF constructs (A: scTNF 3R1, B: scTNF R112, C: scTNF R221) were incubated at different concentrations for 2 h on ice with TNFR1-Fas cells (50,000 cells per sample). Cells were separated from unbound protein by centrifugation through phthalate oil and cell bound radioactivity was measured. Free radioactive protein was plotted against bound, a one site binding hyperbola was fitted through the data points and K_d -values were determined by using the program Graphpad Prism. Data are also displayed as Scatchard plots (insets under the curves). **D**: Used cells were immuno stained with an anti-TNFR1 antibody (H398, filled histogram) or only with anti-mouse FITC-conjugated secondary antibody (D, open histogram) and analysed by flow cytometry.

the labelling procedure can result in an activity loss of the protein, labelled scTNFs were compared to their unlabelled counterparts performing cytotoxicity assays. All iodinated proteins used here, showed only a very moderate activity loss displaying more than 75% of the bioactivity of the unlabelled molecules (data not shown).

In all experiments equilibrium binding studies at 0°C were performed. Different concentrations of the labelled proteins were incubated for two hours on ice with the cells which should be examined. Unbound protein was separated by centrifugation through a phthalate oil mixture, and cell bound radioactivity was quantified. Nonspecific binding was determined in the presence of a 100-fold excess of the respective non-iodinated ligand and was subtracted. Through the obtained data points a one site binding hyperbola was fitted, and the K_d and the B_{max} values were determined.

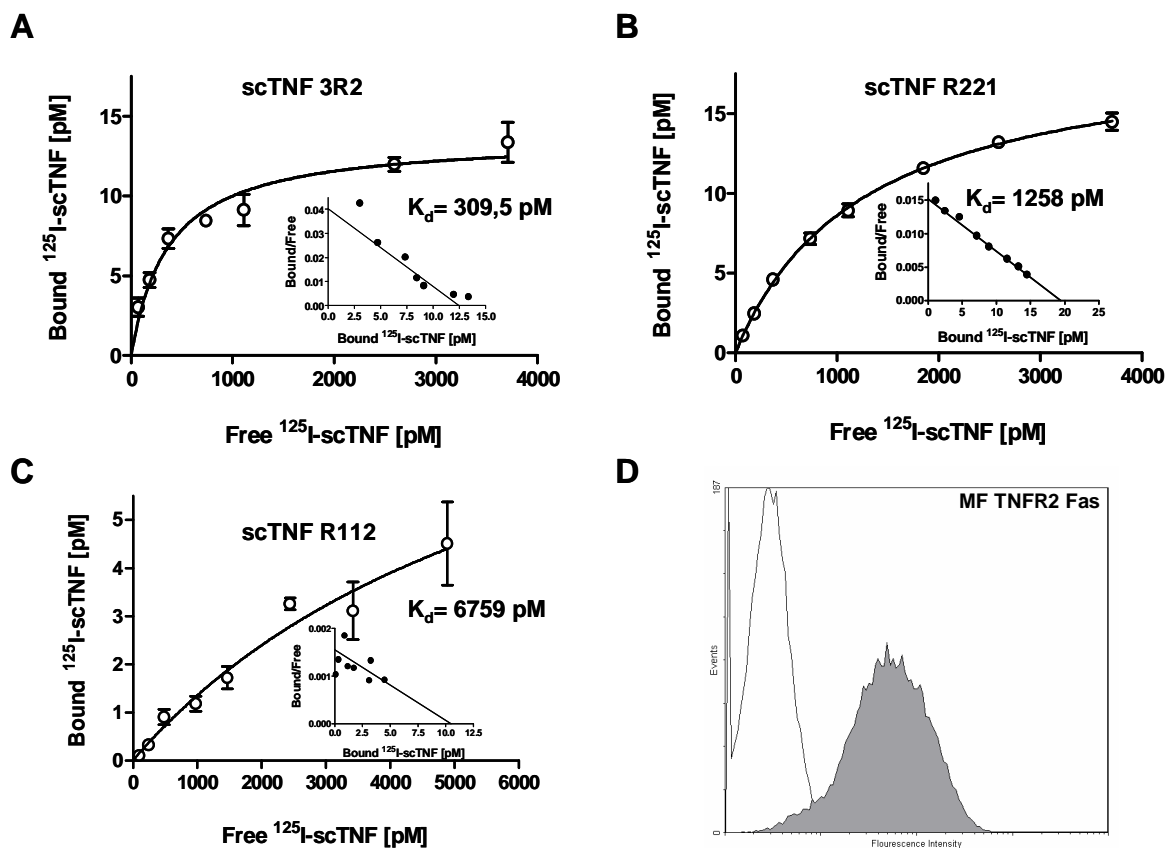


Figure 20: Equilibrium binding studies with scTNF variants containing receptor selective mutations on MF TNFR2-Fas cells. A-C: Iodinated scTNF constructs (A: scTNF 3R2, B: scTNF R112, C: scTNF R221) were incubated at various concentrations for 2 h on ice with TNFR2-Fas cells (50,000 cells per sample). Cells were separated from unbound protein by centrifugation through phthalate oil and cell bound radioactivity was measured. Free radioactive protein was plotted against bound, a one site binding hyperbola was fitted through the data points and K_d -values were determined by using the program Graphpad Prism. Data are also displayed as Scatchard plots (insets under the curves). **D:** Used cells were immuno-stained with an anti-TNFR2 antibody (MR2-1, filled histogram) or only with anti-mouse FITC-conjugated secondary antibody (open histogram) and analysed by flow cytometry.

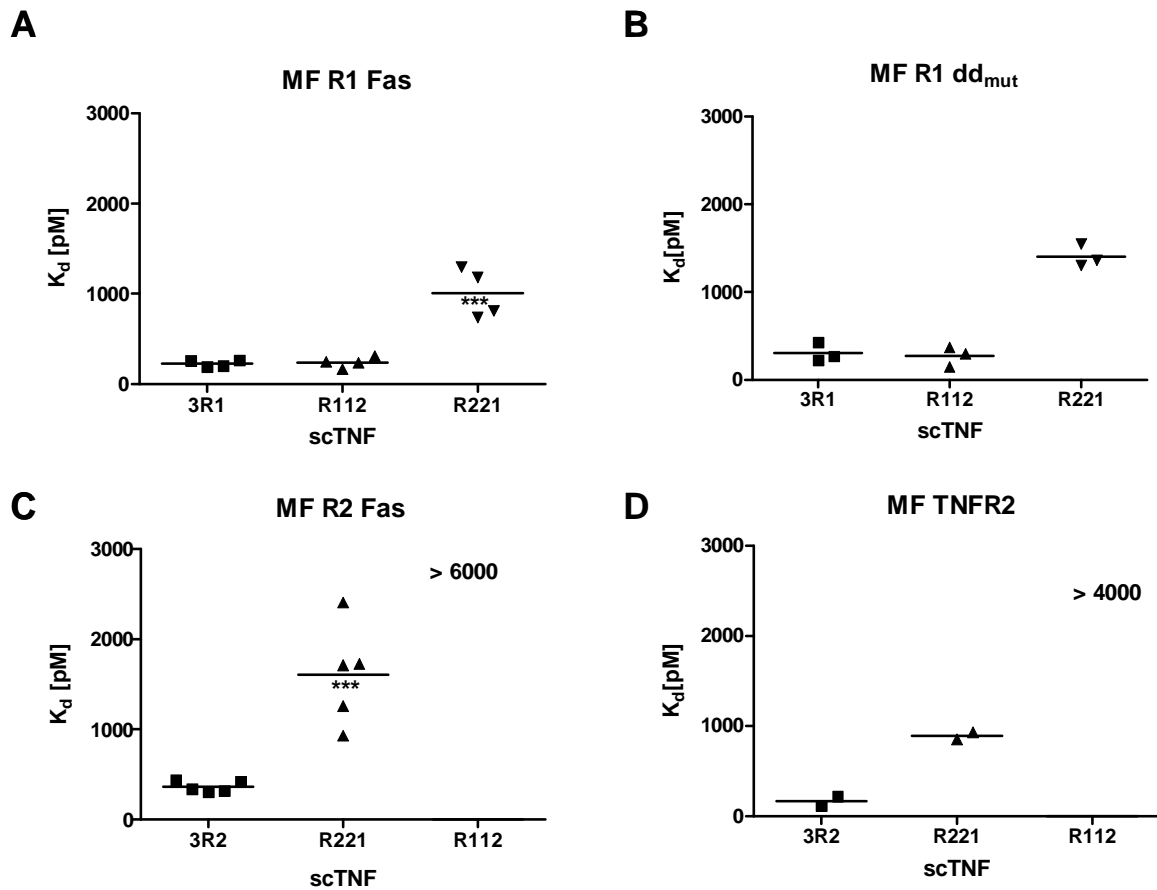


Figure 21: Overview of K_D -values of scTNF variants with receptor selective mutations. Depicted are the K_D -values derived from equilibrium binding studies and the resulting mean values. A: MF TNFR1-Fas, B: MF TNFR1 dd_{mut}, C: MF TNFR2-Fas, D: MF TNFR2. ***: $P < 0.002$.

Figure 19 and Figure 20 show typical results from performed experiments, using MF TNFR-Fas cells. In parallel the data is also displayed in Scatchard plots (small insets under the curves), because this type of a graph shows nicely the fitting of the measured data to a one site binding hyperbola, and if the data points sufficiently cover the whole range of the binding curve. By flow cytometry it was assured that the cells express the receptor in high amounts and that the percentage of cells negative for receptor expression is low (Figure 19D and Figure 20D).

scTNF 3R1 shows the expected high affinity for TNFR1 with a K_D -value of 260.5 pM (Figure 19A), scTNF R112 reveals a K_D -value of 307.5 pM, thus showing a similar affinity (Figure 19B). scTNF R221 binds with a K_D -value of 1295 pM around 4 times weaker as compared to the other two proteins (Figure 19C). On MF TNFR2-Fas cells, binding studies with the fully active protein scTNF 3R2 result in a K_D -value value of 310 pM (Figure 20A), scTNF binds with a K_D -value of 1258 pM 4 times weaker (Figure 20B). A strong decrease in affinity is visible when scTNF R112 is used, a K_D -value of only 6759 pM was determined (Figure 20C). The Scatchard plot of the latter experiment shows that the concentrations used were far from

saturation, although concentrations up to 5 nM were used. Having affinities in this range the detection limit of the assay was reached, indicated also by the fact that only in 2 out of 5 experiments a reasonable number for the K_d -value could be obtained using scTNF R112. No specific binding at all could be observed with scTNF 3R1 on TNFR2-Fas cells or with scTNF 3R2 on TNFR1-Fas cells (data not shown).

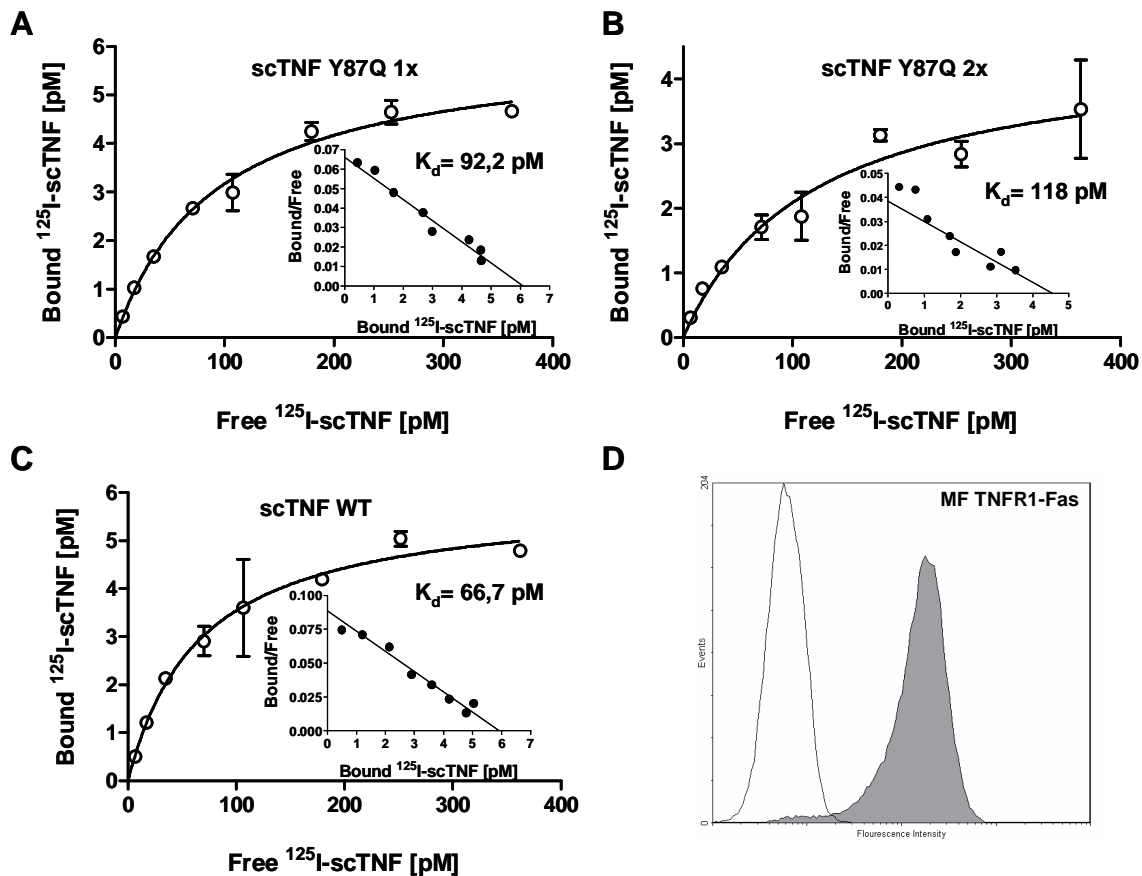


Figure 22: Equilibrium binding studies with scTNF variants containing the Y87Q mutation on MF TNFR1-Fas. A-C: Iodinated scTNF constructs (A: scTNF Y87Q 1x, B: scTNF Y87Q 2x, C: scTNF WT) were incubated at different concentrations for 2 h on ice with TNFR1-Fas cells (50,000 cells per sample). Cells were separated from unbound protein by centrifugation through phthalate oil and cell bound radioactivity was measured. Free radioactive protein was plotted against bound, a one site binding hyperbola was fitted through the data points and K_d -values were determined by using the program Graphpad Prism. Data were also displayed as Scatchard plots (insets under the curves). **D:** Used cells were immuno-stained with an anti-TNFR1 antibody (H398, filled histogram) or only with an anti-mouse FITC-conjugated secondary antibody (D, open histogram) and analysed by flow cytometry.

An overview of all performed experiments shows that on TNFR1 the loss of one binding site obtained through the chosen mutations has hardly any effect on affinity, whereas the loss of two receptor binding sites results in a significant decrease in affinity (Figure 21A, corresponding data in Table 3). On TNFR2 already the loss of a single binding site results in a significant loss of affinity, the loss of another TNF binding site results in an even stronger reduction (Figure 21C). In this case the affinity was so low that it could not be determined easily under the conditions of the assay.

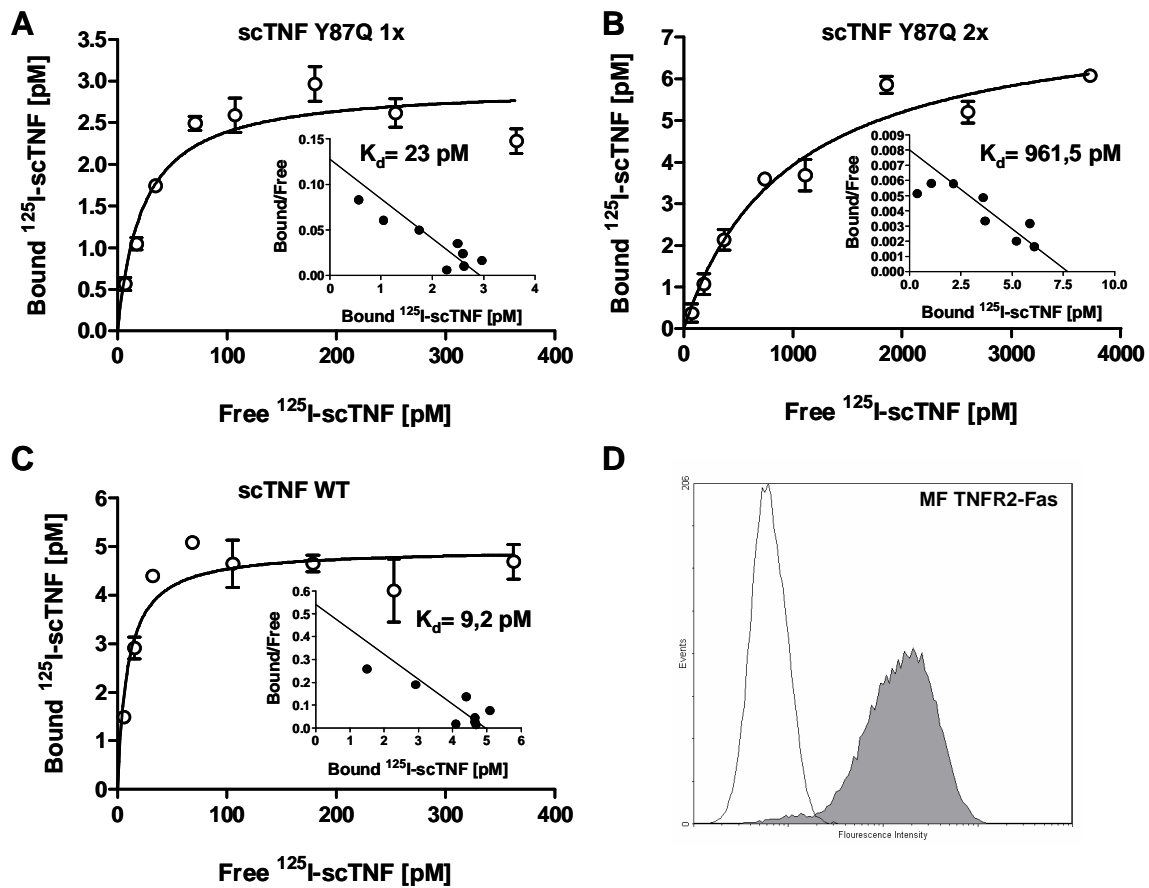


Figure 23: Equilibrium binding studies with scTNF variants containing the Y87Q mutation on MF TNFR2-Fas cells. A-C: Iodinated scTNF constructs (A: scTNF Y87Q 1x, B: scTNF Y87Q 2x, C: scTNF WT) were incubated at different concentrations for 2 h on ice with TNFR2-Fas cells (50,000 cells per sample). Cells were separated from unbound protein by centrifugation through phthalate oil and cell bound protein was measured. Free radioactive protein was plotted against bound, a one site binding hyperbola was fitted through the data points and K_d -values were determined by using the program Graphpad Prism. Data are also displayed as Scatchard plots (insets under the curves). **D:** Used cells were immuno-stained with an anti-TNFR2 antibody (MR2-1, filled histogram) or only with an anti-mouse FITC-conjugated secondary antibody (D, open histogram) and analysed by flow cytometry.

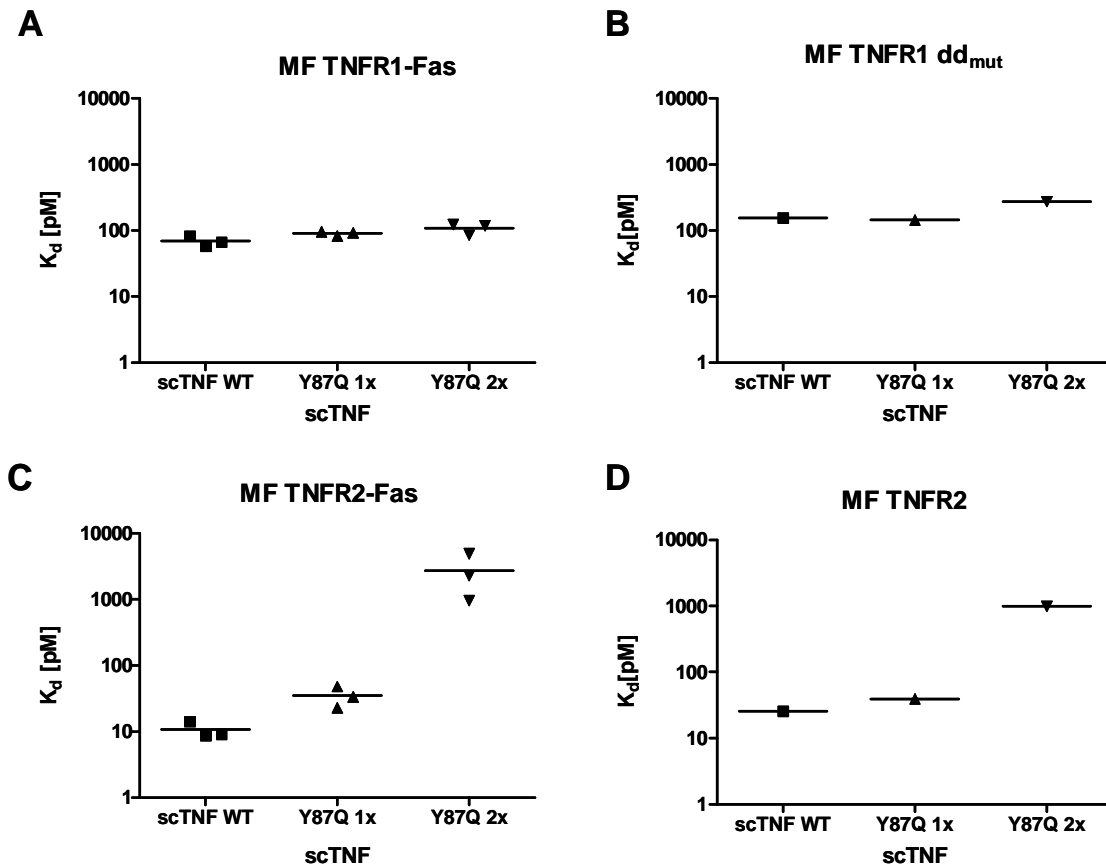


Figure 24: Overview of K_d -values of scTNF variants with Y87Q mutations. Depicted are the K_d -values derived from several binding studies and the resulting mean values. A: MF TNFR1-Fas, B: MF TNFR1 dd_{mut} , C: MF TNFR2-Fas, D: MF TNFR2.

The results reported here could be verified by using different cell lines: mouse fibroblasts expressing the wildtype TNFR2 (MF TNFR2, Figure 21D) and mouse fibroblasts expressing TNFR1 with a mutated death domain (MF TNFR1 dd_{mut} , Figure 21B). These data rule out that the intracellular Fas signalling part had an influence on the obtained results.

3.4.2 scTNFs with mutation Y87Q

The affinities of the scTNFs containing various numbers of the Y87Q mutation were compared to that of scTNF WT. For the classical scTNF molecules with the longer linkers it was reported that scTNF molecules display a higher affinity for both TNF receptors as compared to sTNF (Krippner-Heidenreich et al., 2008). This could be confirmed here, using the produced scTNF WT containing the shorter linkers. On TNFR1-Fas an affinity of 66.7 pM was measured (Figure 22C) and on TNFR2 an affinity of 9.2 pM (Figure 23C, affinity of sTNF on TNFR1: 300-600 pM, on TNFR2: 70-200 pM, taken from Grell et al., 1998).

The scTNF mutants containing two (scTNF Y87Q 1x) or only one (scTNF Y87Q 2x) active

binding site per TNF trimer show affinities very similar to scTNF WT on TNFR1-Fas cells: 92.2 pM and 118 pM (Figure 22A and B). On TNFR2-Fas a gradual decline in affinity is visible with an increasing number of mutated binding sites: a K_d -value of 23 pM for scTNF Y87Q 1x and a value of 962 pM for scTNF Y87Q 2x was determined (Figure 23A and B). In average of all performed experiments (Figure 24, corresponding data presented in Table 3) there was a clear tendency that on TNFR1 no significant affinity loss is detectable, whereas on TNFR2 a very pronounced affinity loss can be verified (Figure 24A and C). These results obtained with TNFR-Fas chimeras could be underlined by experiments using MF TNFR2 (Figure 24D) and MF TNFR1 dd_{mut} (Figure 24B), ruling out major effects caused by the intracellular Fas part of the TNFR-Fas chimeras.

Table 3: Overview of data obtained in binding studies and cytotoxicity assays. Values depicted in bold represent mean values of the data presented above.

TNFR1-Fas				TNFR2-Fas			
	1 site	2 sites	3 sites	1 site	2 sites	3 sites	
	R221	R112	3R1	R112	R221	3R2	
k_D [pM]	1295.00	307.50	261.00	6759.00	1258.00	309.50	
	1180.00	245.90	258.60	n.d.	1728.00	424.10	
	737.00	162.90	187.90	n.d.	2405.00	322.40	
	808.20	234.90	198.90	7966.00	932.00	438.50	
				n.d.	1710.00	340.70	
	1005.05	237.80	226.60	> 7000	1580.75	373.63	
B_{max} [pM]	26.59	14.78	18.88	1.60	19.45	12.45	
	23.28	15.64	18.08	n.d.	11.40	7.94	
	1.61	8.22	11.44	n.d.	10.46	4.71	
	6.76	6.71	7.20	6.46	9.92	7.10	
				n.d.	13.65	6.83	
EC_{50} [ng/ml]	6.00	0.10	0.10	30.00	3.00	0.60	
	20.00	0.10	0.10	25.00	3.00	0.70	
	9.00		0.10	30.00	5.00	0.60	
	11.67	0.10	0.10	28.33	3.67	0.63	
	Y87Q 2x	Y87Q 1x	scTNF WT	Y87Q 2x	Y87Q 1x	scTNF WT	
k_D [pM]	123.40	92.18	82.56	2300.00	48.12	14.33	
	118.00	95.65	66.68	4907.00	33.83	8.68	
	84.70	82.30	58.20	961.50	22.99	9.15	
	108.70	90.04	69.15	2722.83	34.98	10.72	
B_{max} [pM]	6.57	9.39	9.81	8.97	2.65	4.52	
	4.55	6.10	5.91	19.57	3.27	4.34	
	7.42	9.97	10.50	7.70	2.94	4.95	
EC_{50} [ng/ml]	0.50	0.40	0.04	> 400	4.00	0.20	
	0.20	0.20	0.20	> 400	10.00	0.30	
	0.20	0.40	0.20	> 400	5.00	1.00	
	0.30	0.33	0.15	> 400	6.33	0.50	

TNFR1 dd_{mut}				TNFR2			
	1 site	2 sites	3 sites	1 site	2 sites	3 sites	
	R221	R112	3R1	R112	R221	3R2	
k_D [pM]	1303.00	372.10	222.60	n.d.	931.10	218.40	
	1548.00	297.30	269.10	4486.00	850.00	112.90	
	1360.00	145.90	428.90				
	1403.67	271.77	306.87	> 4486	890.55	165.65	
B_{max} [pM]	26.59	14.78	18.88	n.d.	29.70	16.77	
	35.77	28.91	21.79	14.61	22.08	15.08	
	34.72	13.59	24.23				
	Y87Q 2x	Y87Q 1x	scTNF WT	Y87Q 2x	Y87Q 1x	scTNF WT	
k_D [pM]	271.50	143.80	154.60	988.30	39.30	25.57	
	B_{max} [pM]	18.37	18.65	23.17	38.92	16.63	22.69

4 Discussion

4.1 Attributes of the used TNF mutations

The exchange of single amino acids in the TNF molecule can have different effects depending on the location of the amino acid and its participation in receptor molecule interactions. Also the type of amino acid which is used as replacement is of importance. Mutations of residues located at the hydrophobic interfaces between the monomers of the TNF trimer were shown to affect bioactivity by interfering with subunit trimerization, whereas mutations at the clefts between the monomers often show an effect on bioactivity by influencing receptor binding (Zhang et al., 1992). As all mutations used in the scTNF variants described herein are located at the clefts, no negative effects on TNF trimerization are expected.

The extent of influence on receptor binding of mutations at the binding sites may vary between the receptors, leading to receptor selective mutations. It was shown for the TNFR1 selective mutation R31D that the selectivity is caused by the disability of TNFR2 to interact with the aspartic acid replacing arginine 31 (Reed et al., 1997). In the TNF WT, arginine 31 is predicted to form two ionic interactions with the glutamic acid residue 56 of TNFR1 and with

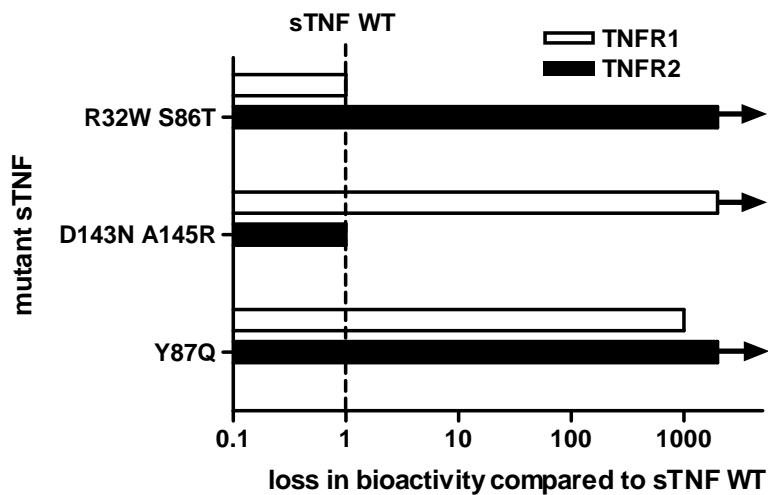


Figure 25: Scheme showing the influence of the used mutations on sTNF bioactivity. The x-axis shows the extent of reduction in bioactivity obtained compared to sTNF WT. The activity of sTNF WT is set as 1. Arrows indicate that that no activity has been observed with the concentrations which were used for the assays and so the exact extent of reduction is not known (>1000).

the corresponding glutamic acid residue 57 of TNFR2. Whereas the mutated TNF is predicted to partially substitute the lost interactions with TNFR1 by a polar interaction of the aspartic acid with serine 59, no such interaction is possible with TNFR2. This might be the cause for the strong effect of the mutation on TNFR2 binding, whereas TNFR1 binding is only moderately affected. For the mutations used in the present work similar reasons for receptor selectivity can be expected. All used receptor selective mutations moderately affect the binding to the receptor to which binding should be retained.

The effects of some mutations on the bioactivity of sTNF is depicted in Figure 25. The combination of mutation R32W and S86T in sTNF results in a bioactivity comparable to sTNF WT on TNFR1, whereas on TNFR2 a strong reduction in bioactivity is detectable. The mutations D143N and A145R result in TNFR2 selectivity. On TNFR2 the mutated TNF is as active as sTNF WT, whereas on TNFR1 bioactivity is impaired more than 1000 fold¹. In contrast, a sTNF molecule with mutation Y87Q shows a low bioactivity on both receptors. But there is a weak selectivity for TNFR1 detectable: an activity could be determined in cytotoxicity studies, although 1000 times lower than the activity of sTNF WT (see also Figure 17).

4.2 General considerations regarding the new scTNF molecules

In scTNF molecules three TNF molecules are linked by two glycine-serine linkers. Thereby the TNF trimer is covalently stabilized, making the reversible dissociation into its monomers impossible (Krippner-Heidenreich et al., 2008 and Poiesi et al., 1993). Because of this, the single chain format is perfect to study the stoichiometry between TNF and its receptors. Individual binding sites in the TNF trimer can be mutated, resulting in molecules which can only bind effectively to one or two receptor molecules instead of an intact trimer being capable to bind three receptor chains or a completely mutated TNF trimer lacking any binding capability. In this work it was possible to generate these molecules and to study the resulting differences in binding activity and bioactivity when acting on the two TNF receptors.

Two different approaches were used. The first approach involves the introduction of mutations known to result in TNF molecules which selectively bind only TNFR1 or TNFR2. In a second approach mutations were introduced reported to abrogate binding to both TNFR1 and TNFR2. The first approach results in scTNF molecules consisting of three differently mutated TNF monomers as a consequence of the spatial orientation of the mutations within the binding clefts of the TNF trimer. Based on these facts the desired design of functional

¹ Experiences with mutated sTNF molecules in the group of P. Scheurich

binding sites is only retained provided a defined orientation of the monomers to each other is retained when forming the trimer. For these reasons the linkers used to connect the monomers had to be shortened as compared to the linkers used in all previous scTNF constructs (Krippner et al., 2008) to enforce the correct orientation. Importantly, it could be shown that the introduction of these shorter GGGGS-linkers has no effect on the functionality of the proteins. In advance of the production of these molecules, scTNF WT containing the short linkers was computer simulated addressing the question if any tensions in the molecule would occur. The positive results from these simulation studies were underlined by the experimental results obtained with the respective proteins. Using the bifunctional crosslinking agent BS³ no indication for the formation of higher protein aggregates was obtained (Figure 9). Higher aggregate formation is likely to occur when the used linker peptides would be too short to allow proper hydrophobic interaction of the monomers to fold to the native trimer. By means of the crosslinker BS³ in the case of a single chain derivative of the TNF ligand family member FasL indeed aggregates could be detected (Haag, 2005), indicating the suitability of this method. In addition, the scTNF derivatives containing the new short linkers did not show any bioactivity on TNFR2-Fas positive cells, further arguing against the formation of protein aggregates. Rather, and similar to wildtype sTNF, the scTNF molecules display no bioactivity on TNFR2 in the absence of the TNFR2 specific antibody 80M2, a combination which mimics mTNF action. Provided the scTNF mutants would be present in an aggregated state, some stimulatory activity for TNFR2 would be expected, which was in fact observed with the scFasL derivative when acting on Fas positive cells (Haag, 2005). Furthermore, a TNF variant differing from wildtype sTNF only in an additional N-terminal cysteine residue (cysTNF, Bryde et al., 2005) was shown to possess some bioactivity on TNFR2 due to the formation of oligomers, most likely hexamers.

The shorter linkers also had no impact on the general bioactivity of the scTNF molecules. If one compares scTNF WT, scTNF 3R1 and scTNF 3R2, all containing the shorter linkers, with their long linker counterparts as published in Krippner-Heidenreich et al., 2008, they show the same behaviour. In cytotoxicity assays scTNF WT is as active as sTNF, but in binding studies it shows a higher affinity towards both receptors when compared to sTNF (K_d values of 69.1 pM on TNFR1 and 10.7 pM on TNFR2²). An enhanced affinity in a similar range was already reported for the scTNF WT molecules containing the longer linkers (43.4 pM on TNFR1 and 31.5 pM on TNFR2 for the scTNF WT containing the longer linkers; Krippner-Heidenreich et

² All K_d - and EC_{50} -values cited in chapter 4 are mean values of several experiments as presented in Table 3

al., 2008³). scTNF 3R1 and scTNF 3R2 show their expected receptor selectivity both at the level of their bioactivities as well as in binding studies. The affinity of scTNF 3R1 (226.6 pM) is 3 times weaker compared to the affinity of scTNF WT on TNFR1, the affinity of scTNF 3R2 (373.6 pM) around 30 times weaker than the affinity of scTNF WT on TNFR2. This loss in affinity is expected, because the mutated scTNFs have a very low affinity ($< 10^{-3}$ of the original value) for the receptors to which they should not bind anymore, but the mutations at the same time induce some affinity loss towards the other receptor, where binding should be retained. Furthermore, as reported before (Loetscher et al., 1993), the TNFR2 selective mutations result in a higher affinity loss than the TNFR1 selective mutations.

Regarding the scTNF derivatives with the receptor selective mutations one has to keep in mind that folding in the wrong orientation cannot be formally excluded. The first 5 N-terminal amino acids of sTNF are not resolved in the crystal structure from which the required linker length was derived. Therefore, it is not possible to fully exclude that the scTNF might still have a possibility to arrange also counter clockwise, resulting in proteins displaying not the desired amount of TNFR2 selective binding sites (see Figure 5 and chapter 3.1 for details).

An alternative excluding the possibilities of wrong trimeric arrangement of the monomers was a TNF derivative carrying only a single amino acid substitution in each monomer. The tyrosine residue 87 of TNF was shown repeatedly to be important for receptor 1 binding as well as for receptor 2 binding (Loetscher et al., 1994; Steed et al., 2003), hence it was of interest to confirm the results obtained with the first approach by using two scTNF molecules, one where a single monomer is mutated (Y87Q 1x) and one where two of the monomers had been mutated (Y87Q 2x). Importantly, because there is only a single amino acid exchange per TNF module necessary in this approach, the amount of active binding sites is retained regardless of the orientation in which the three TNF modules are arranged to each other.

4.3 Models for the initial steps in TNF/TNFR binding

Basic questions leading to the project described here came from observations made in binding studies with TNFR2 at 0°C (Loehden, 1995). When iodinated sTNF was used, about 30,000 binding sites per cell were determined using the cell line Kym-1. According to flow cytometry analyses, these binding sites should be mainly (about 90%) mediated by TNFR2. When the iodinated TNFR2-specific antibody 80M2 was used, 70,000 to 100,000 binding

³ Measured on Kym-1 cells with the help of antagonistic TNFR specific antibodies

sites per cell were observed. Although a TNF trimer possesses 3 binding sites and the antibody (mouse IgG2a) only two, the antibody seems to bind to more receptor molecules. This result could be explained by an aggregation of receptors on the cell surface: Several antibody molecules can bind to one receptor multimer, whereas only one TNF trimer can bind. Actually, a TNF receptor aggregation has been described in 2000 for TNFR1 and TNFR2 (Chan et al., 2000), but also for some additional TNFR-family proteins (Fas and TRAIL, Siegel et al., 2000 and Clancy et al., 2005).

As a model for binding of TNF to its preaggregated receptors it was proposed that the ligand slides into this aggregate, thereby abrogating the receptor contacts (pre-ligand assembly model, Figure 26 B; Chan, 2000). The receptor contacts are mediated by a not yet clearly defined domain in the CRD1 of the receptors, called PLAD (pre-ligand binding assembly domain). After these first binding steps it is assumed that higher receptor clusters are formed, containing the ligand, the receptors and the intracellular signalling molecules (Krippner-Heidenreich et al., 2002 and Branschaedel, 2007). Probably the released PLAD might also participate in these secondary interactions. Figure 26D shows two possible arrangements of receptor dimer or trimers and their bound ligand molecules. In such receptor-ligand lattices, binding and activation of intracellular signalling molecules probably takes place not between receptor molecules bound to one TNF trimer, but between receptors bound to adjacent ones. An earlier model for receptor binding, which came up after the publication of the crystal structure of lymphotoxin alpha (LT α) bound to TNFR1 (Banner et al., 1993), involves an aggregation of three monomeric receptors by the TNF trimer (ligand-induced trimerization model, Figure 26 A and E). The intracellular parts of the bound receptors are thereby brought into proximity and signalling can occur.

In this project the consequences on affinity and bioactivity of TNF trimers with one or two mutated binding sites have been examined in equilibrium binding studies at 0°C with the aim to gain more information about the binding stoichiometry in the very first binding steps of TNFR1 or TNFR2 interaction. At a temperature of 0°C the cell membrane is rather rigid and should not allow large movements of the membrane receptors, thereby conserving their respective preassociated or individual state. In the ligand induced trimerization model only a binding to one receptor might be expected under these conditions (Figure 26A), because diffusion of the receptors in the membrane is prevented. Furthermore, one would obtain no major differences in affinity when the TNF trimer contains a reduced number of active binding sites. On the other hand, if the trimer binds to several receptors in a preassociated state, like proposed in the pre-ligand assembly model (Figure 26B), one would estimate a

significant binding affinity reduction when using molecules with a reduced number of functional receptor binding sites.

A third way of binding is feasible where the receptors are again preaggregated, but the TNF ligand binds only to one of these receptors (Figure 26C). This would require that TNF has a direct access to the binding sites of these receptor molecules, whereas in the pre-ligand assembly model the binding sites are buried inside the receptor aggregate, i.e. not directly

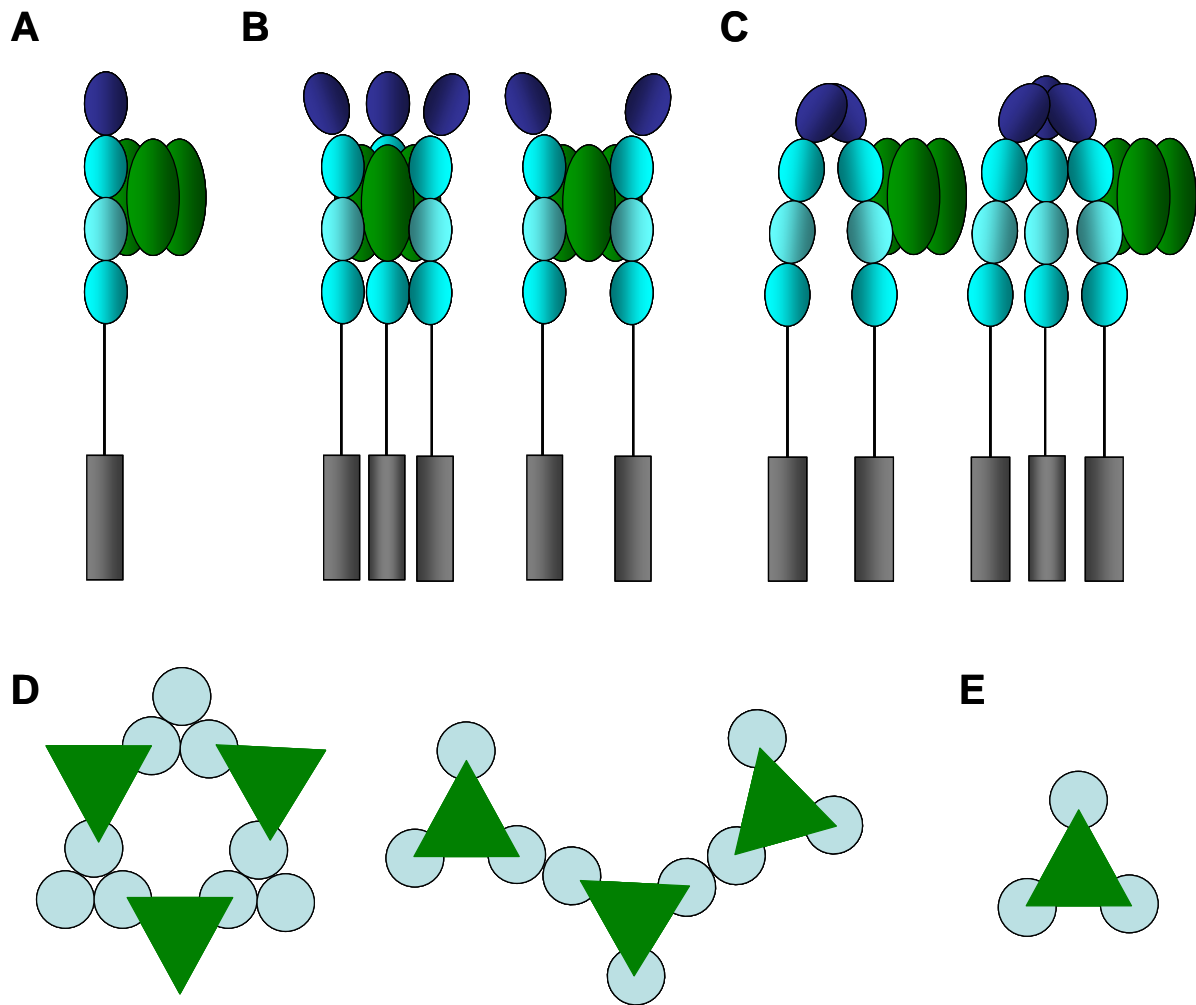


Figure 26: Different models of initial steps in TNF-receptor binding. **A:** Ligand-induced trimerization. Receptor molecules are in a monomeric state prior to ligand binding and are aggregated by ligand binding. At 0°C only binding to single receptors would be possible because of membrane rigidity. **B:** Pre-ligand assembly. Receptor molecules are preaggregated as trimers or dimers prior to ligand binding, likely by PLAD/PLAD interaction via CRD1. The ligand binding sites face inwards. The TNF trimer slides into the dimer/trimer thereby abrogating PLAD binding. TNF binds 2 or 3 preassociated receptors at 0°C. **C:** Receptors are preaggregated as dimers or trimers prior to ligand binding with the ligand binding sites facing outwards. TNF binds only to one receptor at 0°C. **D:** Models of higher receptor ligand aggregates formed after ligand binding (seen from above). Left: receptor trimers, right: receptor dimers (Branschaedel, 2007). **E:** Aggregation of only three receptor molecules as proposed by the ligand-induced trimerization model. Representatively shown in A-C is TNFR1 with the CRD1 containing the PLAD in dark blue, CRD2-4 in cyan and the intracellular death domain in grey. The TNF trimer is shown in green. In E and F the TNF trimer is shown as a green triangle and the receptor molecules as light blue circles.

accessible. Higher aggregates of ligand and receptor molecules would then form by binding of two further receptor dimers or trimers (Figure 26D). At 0°C this diffusion dependent step is prevented. One would therefore expect no strong reduction in affinity with this model at 0°C, when using molecules with a reduced number of functional receptor binding sites.

4.4 Initial steps in TNFR1 binding

4.4.1 Properties of TNFR1

As mentioned above, for the preassociation of the receptors in the cell membrane the CRD1 is important, assuming that it contains or represents a domain called PLAD. In TNFR1 the interaction between the PLAD of the individual receptors seems to be stronger compared to TNFR2. By performing crosslinking studies a dimeric preaggregation as well as a trimeric preaggregation were postulated for TNFR1 (Chan et al., 2000; Branschaedel et al., 2007).

TNFR1 was found to be associated with membrane microdomains. These domains are believed to represent islets in the membrane differing in their lipid composition. Domains containing a high proportion of sphingolipids and cholesterol are often referred to as lipid rafts (Brown and London, 2000), related microdomains containing also caveolin proteins are called caveolae (Parton and Simons, 2007). The latter ones form also grape like structures or tubules and are important for internalization processes. For both kinds of membrane domains it was shown that they are places where components of signalling complexes are gathered and where signalling takes place (Xavier et al., 1999, Gonzalez et al., 2004, Bucci et al., 2000). It was shown in U937 cells that TNFR1 is constitutively localized in caveolae-like domains and that depletion of cholesterol and thereby destroying of rafts/caveolae abolishes apoptosis induction upon TNF stimulation (Ko et al., 1999). In fluorescence correlation spectroscopy (FCS) studies it was shown that TNFR1 might be at least in parts constitutively localized to microdomains (Gerken et al., submitted), because a contingent of the receptor molecules displayed a low diffusion constant and depletion of cholesterol resulted in an enhancement of diffusion velocity.

4.4.2 Discussing the pre-ligand assembly model

In the approach using scTNF molecules carrying receptor selective mutations, no difference in affinity could be detected between a fully active TNF trimer and one containing only two active receptor binding sites (K_d of 226.6 pM for scTNF 3R1 and 237.8 pM for scTNF R112).

Only when a second binding site was mutated, a significant effect was visible, observable by a K_d value 4 times higher (K_d of 1005.5 pM for scTNF R221). A very similar pattern was also obtained when the bioactivity of these molecules on TNFR1 was examined. A more than 100 fold decrease in bioactivity was found when only one active binding site was left (EC_{50} rises from 0.1 ng/ml with both scTNF 3R1 and R112 to 11.67 ng/ml when using scTNF R221).

These results could be easily interpreted in accordance with chemical crosslinking data from M. Branschaedel in 2007 that the receptors are present at the cell surface in dimers and binding would take place in a pre-ligand assembly manner as depicted in Figure 26B. In this model the reduction of active binding sites per TNF trimer from three to two is not expected to have a high impact on affinity. At the level of bioactivity determinations it would be argued that in a postulated ligand-receptor lattice formed after binding of ligands (Krippner et al., 2002; Figure 26D) some defect interaction sites do not have a high impact on signalling. As described above, activation of intracellular signalling components could take place not between those receptors bound to one TNF trimer, but rather between receptors bound to adjacent trimers. Actually there are reports existing, supporting the theory of a signalling activation between adjacent receptor-complexes in the Fas system (Carrington et al., 2006, Sandu et al., 2006, Holler et al., 2003).

4.4.3 Discussing binding to single receptors

The second approach with the Y87Q mutated scTNF molecules Y87Q 1x and Y87Q 2x was performed to verify the results obtained with the scTNF variants containing the receptor selective mutations. On TNFR1-Fas cells the effects of these two molecules concerning binding affinity and bioactivity were only marginal (scTNF WT K_d of 69.2 pM drops to 90.0 pM and 108.7 pM for the mutants, EC_{50} drops from 0.15 ng/ml to 0.30 ng/ml). One reasonable explanation of these results could be that the mutation used here shows a weak TNFR1 selectivity. When sTNF containing this mutation was generated (resulting in a completely mutated TNF trimer) and was tested on TNFR1-Fas cells, it showed only an about 1000 fold lower bioactivity as compared to wildtype sTNF (Figure 25). For comparison, the completely TNFR2 selective scTNF 3R2 mutant revealed no bioactivity at all at the same concentrations. This implies that there is still some significant residual binding affinity for TNFR1 of the mutated sites left, which could make it difficult to detect differences between Y87Q 1x and Y87Q 2x in comparison to scTNF WT. On Kym-1 cells a clear attenuating effect was detectable in a PARP assay (Figure 15, low processing with Y87Q 2x) as well as in

cytotoxicity assays (data not shown), implying a higher effect of the mutated molecules on this cell line.

If the small difference in affinity observed between the Y87Q-mutated scTNF variants and the scTNF WT molecule represents the actual situation on the cell, this would hint to receptors which are not preaggregated (Figure 26A) or that the ligand binds only to one receptor of a preformed aggregate (Figure 26C).

In light of the results obtained with cross-linking studies showing a preassociation of TNFR1 the later model would perhaps be more likely. The short, probably not that flexible stem of TNFR1 and the higher affinity of the PLAD-interaction as compared to the PLAD of TNFR2 (Chan et al., 2000 and Branschaedel, 2007) could prevent binding as proposed by the pre-ligand assembly model (Figure 26B). Aggregation of two receptors would not only fit to the results obtained by M. Branschaedel, but would also correspond to the parallel dimeric crystal structure observed for TNFR1 without ligand (Naismith et al., 1995). In this structure the areas for ligand binding are easily accessible, which is crucial if binding happens as proposed in Figure 26C. Furthermore, formation of higher ligand/receptor complexes can be easily explained in this model by diffusion of the receptors in the membrane.

If binding takes place as proposed in the ligand induced trimerization model (Figure 26A), why do the scTNF molecules containing the mutated binding site show also a high bioactivity? In this binding model receptor clustering after binding is solely a ligand driven process which should be highly impaired when not all sites are able to bind a receptor. In fact, localization in membrane microdomains might play a role. The single TNFR1 molecules might already exist in these membrane compartments in locally high concentrations. Binding of TNF molecules containing only one active binding site might then be sufficient for signalling: Through a conformational change in the bound receptor its cytoplasmatic part could come into contact to the corresponding part of an adjacent unbound receptor, making signalling possible.

The observed bioactivities of mutated scTNF derivatives on Kym-1 cells could be caused by the lower amount of TNFR1 molecules present in these cells. The density of receptors in the microdomains might be too low to allow an effective activation by ligand trimers containing only one active binding site. Ligand dependent aggregation might be especially important under the conditions of low receptor expression and these conditions do also better represent the natural situation. An influence of the amount of expressed receptors on the easiness of signal initiation is also visible by the fact that it is not possible to generate cells expressing

high amounts of the wildtype form of TNFR1. Spontaneous activation of the receptors occurs and the cells suffer from spontaneous apoptosis induction (Boldin et al., 1995).

4.5 Initial steps in TNFR2 binding

4.5.1 Properties of TNFR2

In contrast to TNFR1, TNFR2 needs an aggregated ligand, apparent by its need of mTNF to become active, whereas TNFR1 can also become activated by sTNF (Grell et al., 1995). For TNFR2 no accumulation in membrane microdomains like rafts or caveolae was reported. Indeed, in FCS studies it could be shown that TNFR2 diffuses rather fast in the membrane in the absence of ligand (Gerken et al., submitted). However, when a mTNF analogue was added, the diffusion constant was reduced, consistent with an aggregation e.g. into microdomains. The rather weak fluorescence signal of the ligated receptors implies the formation of only small receptor oligomers after ligand binding, so the decrease in diffusion is unlikely to be mediated by formation of large receptor clusters. Depletion of cholesterol did not show an effect on the TNFR2 behaviour after ligand binding, diffusion did not become faster again. Lipid rafts or caveolae as places of aggregation are therefore unlikely, but other cholesterol independent microdomains could be involved. This implies further that TNFR1 and TNFR2 reside in different membrane compartments on the cell surface, because TNFR1 clearly shows a higher diffusion rate when cholesterol was removed from the membrane, indicating a localization in lipid rafts or caveolae.

Like TNFR1, TNFR2 was found to exist preaggregated on the cell membrane either as dimer (Branschaedel, 2007) or trimer (Chan et al., 2000). The affinity of the PLAD domain of TNFR2 was shown to be rather weak compared to the one of TNFR1⁴. Interestingly, the transmembrane domain of TNFR2 contains one, maybe two putative glycine-rich self-association motifs (GxxxG, Senes et al., 2004), which could be an indication that not only the CRD1 domain but also the transmembrane domain may contribute to receptor aggregation. Experiments where the transmembrane domain of TNFR2-Fas was exchanged against the transmembrane region of TNFR1 showed an effect of the transmembrane domain on the behaviour of TNFR2 towards sTNF: mouse fibroblasts expressing this TNFR2-(TM)_{TNFR1}-Fas chimaera are partially responsive to sTNF showing the induction of apoptosis, having thereby reduced their dependence on ligand mediated receptor crosslinking (Holeiter, 2005). This effect became even stronger when additionally the stem region was exchanged between the

⁴ A. Krippner-Heidenreich and P. Scheurich, unpublished results

receptors (TNFR2-(S/TM)_{TNFR1}-Fas; Messerschmidt, 2006). The corresponding TNFR1-Fas molecule containing stem- and transmembrane region of TNFR2 (TNFR1-(S/TM)_{TNFR2}-Fas) on the other hand lost the ability to react on sTNF binding with apoptosis to 80%. The stem regions of the two receptors are different in their length, in TNFR2 it consist of about 4 times more amino acids than in TNFR1. Interestingly, when the stem region in the TNFR1-(S/TM)_{TNFR2}-Fas exchange mutant is shortened, the receptor acts again similar to a normal TNFR1-Fas⁵.

4.5.2 Overview of the obtained results

In the first approach with the molecules carrying receptor selective mutations a gradual decline in affinity with lower numbers of active binding sites could be detected. For scTNF R221 an affinity 4 times lower than that of scTNF 3R2 was measured (K_d value of 373.6 pM for 3R2 and 1580.7 pM for R221). For the scTNF R112 molecule, containing only one active binding site for TNFR2, it was difficult to determine the K_d -value at all, probably because it was too low to be easily detectable by the assay used here. A clear reduction in affinity could be determined, however, using the Y87Q mutated molecules. scTNF WT shows a high affinity (10.7 pM) for TNFR2 and in contrast to the first approach it was easily possible to measure the affinity of scTNF Y87Q 2x, which contains only one active binding site: binding was more than 200 times weaker (2722.8 pM). The additional active binding site in Y87Q 1x results in a K_d value only 3 times weaker (35 pM).

In both cases the affinity reduction is clearly reflected in the bioactivity assays. The Y87Q mutation seems to have a higher impact than the receptor selective mutations. Using the Y87Q 1x mutant only 50% of the cells died at maximum concentration with an EC_{50} value 13 times higher compared to scTNF WT, with scTNF Y87Q 2x no activity could be detected at all, even at the highest ligand concentrations used (400 ng/ml). In contrast, the bioactivity of scTNF R221 is only 6 times weaker than the activity of scTNF 3R2 and for scTNF R112 a 45 fold lower affinity was observed. The fact that not the whole cell population died when treated with Y87Q 1x TNF could probably be explained by a toxic effect only on those cells which express high numbers of receptors. In cells with lower receptor numbers the threshold of active caspases necessary to switch the cell into death is not reached with the scTNF containing only two active binding sites even at saturating concentrations.

⁵ A. Krippner-Heidenreich and A. Zappe, unpublished results

A wrong folding of the scTNF molecules containing the receptor selective mutations results in molecules containing not the desired amount of non-functional binding sites (see Figure 5 and chapter 3.1 for details). The results obtained with the proteins containing the Y87Q mutation confirmed the results obtained with scTNF variants containing the receptor selective mutations. Therefore it can be ruled out that a large percentage of this protein exists in the wrong formation.

4.5.3 Discussing the pre-ligand assembly model

The affinity reduction detectable in both approaches clearly demonstrates that TNF binds at once to several TNFR2 molecules, present on the cell surface in a multimeric form. This pre-ligand assembly mode of action (Figure 26B) might be favoured by the weaker affinity of the PLAD-mediated interactions (Chan et al., 2000 and Branschaedel, 2007), the long flexible stem region of TNFR2 and a putative interaction site in the transmembrane region. As described above (4.5.1), the stem and the transmembrane region were found to be crucial for defining the responsiveness towards sTNF. They might also be a cause for a differential binding mode between the TNF receptors. TNF clearly binds TNFR2 like proposed in the pre-ligand assembly model, whereas it might bind TNFR1 like proposed in Figure 26C.

In the pre-ligand assembly model TNF abrogates the PLAD binding between the receptors. How can one explain then the occurrence of high aggregates after ligand binding in microscopy studies? It could be that these aggregates are formed by a secondary interaction of the receptors, probably also by the PLAD. Another possibility could be that the observed aggregates are formed because of the action of the membrane form of the ligand. The membrane bound ligand might exist in patches on the cell surface, thereby resulting in a clustering of receptors on the neighbouring cell followed by efficient signalling. Recently the formation of higher ligand aggregates was shown for the soluble form of the TNF-family protein human glucocorticoid-induced TNF receptor ligand (GITRL) by crosslinking studies and in the crystal structure (Zhou et al., 2008). Consistent with its inactivity on TNFR2, for sTNF no higher aggregates than trimers can be found. Therefore, for mTNF only interactions in its cytoplasmic or transmembrane domain or in the stem region, which links the transmembrane domain with the part comprising sTNF, can be considered. Interestingly, mTNF was reported to be palmitoylated (Utsumi et al., 2001). This kind of modification was shown to be important for localization of proteins in membrane microdomains (Zhang et al., 1998; Feig et al., 2007). Therefore, a concentration of mTNF in these domains can be considered as well, thereby resulting in the observed aggregates of bound receptors. The

antibody 80M2 might mimick this by linkage of the receptor trimers, thereby allowing activation by sTNF.

4.5.4 Are there preaggregated dimers or trimers ?

The reduction in affinity with each additional mutated binding site could be an indication for a trimerization of the receptor. This assumption is in variance to the results obtained by crosslinking studies performed with the same cell line (MF TNFR2-Fas), where predominantly dimers were found (Branschaedel, 2007). However, purified receptor protein showed trimerization and even higher aggregates after crosslinking (Branschaedel, 2007). Moreover, the group of Michael Lenardo identified trimers using a different cross-linking reagent and a different cell line (Chan et al., 2000), but the reagent they used is not stable under reducing conditions and under non-reducing conditions size determination of proteins in SDS-gels might be imprecise. A protein which is reported to exist as a non-covalently linked dimer and which is activated by a ligand induced conformational change is the Erythropoietin receptor (EpoR; Livnah et al., 1999; Remy et al., 1999). Heteromeric receptors consisting of the extracellular part of the Erythropoietin receptor, and the transmembrane and intracellular part of TNFR2 were functional, but triggering of this receptor was less efficient compared to the normal TNFR2 (Declercq et al., 1995). Therefore the results with the TNFR2-chimaeras could be a hint that the TNFR2 might rather exist as a trimer. On the other hand it could be possible that the chimeric receptor is impaired in its signal intensity because of other reasons, e.g. the assembly of the intracellular signalling complex might be affected. If the affinity reduction observed with the scTNF variants containing mutated binding sites could be indeed interpreted by the existence of preformed receptor trimers has to be confirmed by other experiments which are able to elucidate the aggregation status of the receptor.

4.6 Mutated TNF derivatives in literature

TNF mutations have been used in several attempts to generate drugs to ameliorate diverse diseases on which TNF has a negative impact. In 2003 mutated TNF variants were shown to act in a dominant negative way by forming heteromeres with intact TNF molecules (Steed et al., 2003). Apart from the TNFR2 selective mutation A145R used also in the project described here, Tyrosine 87 was mutated to a Histidine, a mutation resulting in weak binding to both receptors. When added in 20 fold excess to native sTNF the authors could show that

the variants were able to inhibit caspase activation to a large extent and to a smaller extent also the activation of the NF- κ B pathway. They were also able to show that administration of these proteins ameliorates arthritis in a mouse model. In a sandwich ELISA it could be shown that the mutated variants form heteromeres with sTNF WT and it was concluded that this was the reason for the observed antagonistic effects. At a 20 fold excess of the mutant sTNF molecules the complete sTNF WT present was shown to be incorporated in heteromers, mainly consisting of two mutant sTNF monomers and one sTNF WT monomer. In addition also homotrimeric sTNF mutant was present.

If one tries to compare these published results with those presented here, one has to take into consideration that different assays were used to assess bioactivity and that different TNF mutants have been used. Whereas in the scTNF molecules the amount of mutated binding sites is defined, in the dominant negative TNF molecules sTNF WT must be present and the proportion of heteromers formed is concentration dependent. Nevertheless the results with the dominant negative TNF molecules fit quite nicely to the results obtained with the scTNF molecules containing the receptor selective mutations and thereby to the model of pre-ligand assembly with receptor dimers. scTNF R112 displays a very weak activity of only about 1% of the activity of sTNF WT on TNFR1 (100 times lower EC_{50} compared to sTNF), consistent with the fact that most of the inactive heteromers observed in the work of Steed and colleagues consist of two inactive and one active TNF monomer. The high bioactivity observed with the Y87Q mutated scTNF molecules is at variance with these data. However, the kind of mutations chosen and their combination has a high impact on the strength of bioactivity loss as shown in this work as well as with the dominant negative TNF molecules. The scTNF molecules containing the receptor selective mutations showed a higher loss in TNFR1 bioactivity as compared to the Y87Q mutants, whereas on TNFR2 the Y87Q mutations have a higher impact than the receptor selective ones. In Steed et al. (2003) the TNFR2 selective mutation A145N alone showed only a reduction of caspase activity of 50 %, whereas in combination with mutation Y87H 80% was inhibited when TNF WT together with 20 fold more mutant TNF was added to the cells.

Another approach where TNF mutations were used and where also 3 TNF molecules were connected by two linkers was published in 2004 (Nielsen et al., 2004). In contrast to the scTNF presented in this work and to the scTNF molecules published recently (Krippner-Heidenreich et al., 2008), the aim of this project was to design a human therapeutic vaccine for induction of antibody responses against TNF in the patient. Therefore, a foreign immunodominant T helper cell epitope had been inserted into the protein to circumvent T cell

tolerance. To not disturb the structure of the TNF trimer, the epitopes were inserted into glycine linkers connecting the three TNF monomers. Because TNF is highly toxic when injected, different mutations were inserted to prevent bioactivity. The TNFR2 selective mutations D143N and A145R and also a mutation of Y87 to a serine reported to reduce binding to both receptors were used in different settings. Trimers containing mutation A145R in all 3 monomers displayed no cytotoxic activity as well as trimers containing mutation Y87S in all 3 monomers. Interestingly, a molecule containing mutation A145R in only one of the monomers (TNF_T-1x145) showed no decrease in bioactivity, data which clearly resemble the results reported here with the scTNF Y87Q 1x and scTNF R112. Also the binding of TNF_T-1x145 to TNFR1 was comparable to that of the corresponding WT protein, which could also be observed with the scTNF proteins in this work, arguing strongly for either the binding to single receptors or preformed receptor dimers of TNFR1.

4.7 Outlook

4.7.1 Existence of heteromeric receptors

In a variety of cells both TNF-receptors are coexpressed, raising the question if a formation of heteromers of TNFR1 and TNFR2 might take place after addition of ligand. Clearly, in the absence of ligand no PLAD-mediated heteromers exist, due to the selectivity of PLAD/PLAD interaction (Chan et al., 2000). Until now ligand induced heteromers have not been described in human cells (Moosmayer et al., 1994), only in the mouse system it was reported that they might exist (Pinckard et al., 1997). Formation of TNFR1/TNFR2 complexes could be prevented by localization of the receptors in different membrane microdomains and additionally it might not be favoured by the selectivity of the pre-ligand binding assembly domains. Furthermore the receptors differ in their overall structure. TNFR2 contains a longer stem region than TNFR1, thereby the interaction site of TNFR2 with TNF (CRD2 and CRD3) would probably be located further away from the membrane in comparison to the one in TNFR1. Although the bound receptors do not interact with each other when TNF is bound, as shown in the crystal structure of LTalpha bound to TNFR1 (Banner et al., 1993), this might impede interactions for the TNF trimer with both kinds of receptors at once. Anyhow, the scTNF variants containing mixed receptor selectivities (scTNF R112 and R221) would be ideal tools to look for heteromer formation on cells coexpressing both receptors, like for example Kym-1 cells. If the receptors are not preaggregated at all and aggregation is ligand-induced, the trimers with selectivities for

TNFR1 and TNFR2 might capture both receptors if they are not separated in different membrane microdomains. Experiments to look for these heteromers would involve immunoprecipitations alone, or also in combination with crosslinking to stabilize the ligand receptor complexes. These were already shown to be detectable in western blots after cross linking with BS³ (Branschaedel, 2007).

4.7.2 Higher cluster formation

TNFR2 seems to strongly depend on ligand induced aggregation for signalling, as confirmed by the high impact of the scTNF molecules containing less active binding sites. After stimulation with mTNF analogs of cells expressing TNFR2 on the cell surface receptor clusters form, which are visible in fluorescence microscopy (Krippner-Heidenreich et al., 2002). If the scTNF molecules exert a weaker activity because of their lower ability to crosslink the receptors, one would expect to find a reduced receptor cluster formation or less prominent clusters on the cell surface in microscopy. MF TNFR2-Fas cells can be transfected with FADD-EGFP and the TNFR2-Fas molecules can be visualized by using Alexa 546-labelled 80M2 antibody. Treatment with sTNF or the different scTNF molecules then induces formation of signalling complexes, visible by colocalization of the red labelled receptors with the green labelled FADD.

4.7.3 Investigating the influence of stem- and membrane region

The stem and the membrane regions of the receptors seem to have an important effect on the responsiveness towards sTNF. The TNFR2-Fas receptor in which the transmembrane and stalk region has been exchanged against the corresponding regions of TNFR1 can be well activated by sTNF (Messerschmidt, 2006). One could imagine that with this an exchange in localization on the cell surface could be involved. This could be visible in FCS studies, but should probably also have an effect on the response towards the scTNF molecules containing inactive binding sites. With a localisation like TNFR1 and no necessity of a crosslinking ligand the effect of the mutated scTNF molecules on activity should become less prominent.

4.7.4 Determination of the binding kinetics

In this work equilibrium binding studies at 0°C were performed. The assumptions are that the receptors are hold in their aggregation status at this temperature, that no secondary signalling

steps like receptor internalization take place and that an equilibrium between free and bound ligand is build up. Apart from this technique it is also possible to measure how fast the ligand binds to the receptor and how fast it dissociates at 37°C. These assays, called on-off kinetics, showed that sTNF binds fast to TNFR1 but stays bound for a long time ($t_{1/2}$ = 33.2 min), whereas on TNFR2 sTNF binds also fast, but also dissociates rapidly ($t_{1/2}$ = 1.1 min, Grell et al., 1998). These differences between TNFR1 and TNFR2 were taken as an argument to explain the different responsiveness of the two receptors towards sTNF. However, mouse fibroblasts expressing TNFR2-S1-Fas cells are partially responsive to sTNF, but still show only a short binding half live of 1.8 minutes.⁶ This implicates that the response to sTNF is not regulated solely at the level of receptor binding, but rather the membrane location might be important. Nevertheless it would be also interesting to see what impact a reduced amount of functional binding sites of the TNF trimer has on the binding kinetics. Probably one can find a faster dissociation from TNFR2 with less active binding sites being the cause for the lower affinity observed at 0°C.

4.8 Concluding Remarks

The purpose of the present project was to examine the first binding steps of TNF with the intention to find out in which kind of conformation the receptors are bound: monomers or already receptor aggregates.

For TNFR2 strong evidence is presented using two independent approaches that gradual mutation of individual binding sites in the TNF trimer results in a gradual loss in affinity. This is consistent with binding of the TNF trimer to several receptors at once, which exist in a preaggregated state before ligand binding (pre-ligand assembly model, Figure 26B). A binding to single receptors can be ruled out. The decline in affinity with each additionally mutated binding site strongly suggests a binding to three preassociated receptors, although this is in variance to results obtained in cross-linking studies using the very same cell line (Branschaedel, 2007), where only receptor dimers have been observed. mTNF, which might represent a crosslinked ligand, either by its membrane location alone or by a so far not discovered formation of higher aggregates, could lead to higher complexes of the receptor trimers. Their formation might be crucial, because signalling initiation could have to take place between two receptors bound by two adjacent TNF trimers.

For TNFR1 the two approaches revealed no consistent results. When a set of receptor selective mutations was used to construct scTNF molecules where one or two binding sites

⁶ A. Krippner-Heidenreich, unpublished results

were unable to bind TNFR1, only the molecule with two affected sites revealed a significant affinity reduction. These data again rule out that the ligands bind to monomeric receptors. They further indicate binding to preformed receptor dimers (Figure 26B). Results by two other groups, which used mutated TNF molecules support this interpretation (Steed et al., 2003 and Nielsen et al., 2004). Furthermore, in cross-linking studies dimeric receptors could be found with the same cell line as used here (Branschaedel, 2007). When the Y87Q mutation was used, the affinities and bioactivities of scTNF Y87Q 1x and scTNF Y87Q 2x were both very similar to the wildtype control, probably because of a residual affinity and thereby bioactivity of the fully mutated protein on TNFR1, indicating a moderate effect of the Y87Q mutation on TNFR1 binding only. Nevertheless, if these similar affinities represent the actual situation on the cell, they might speak against binding of several receptors at once, but rather for binding to monomeric receptors (Figure 26A). Binding of the receptors to the remaining binding sites would then take place in a diffusion dependent manner. The high bioactivity of the TNF trimers containing less active binding sites could probably be explained by a concentration of the receptors in membrane compartments, where already one active binding site might be enough for signal initiation. More likely than an existence of single receptors would be the binding to only one receptor of a preformed receptor aggregate (Figure 26C), because TNFR1 aggregates were independently found several times (Chan et al., 2000 and Branschaedel, 2007). Further receptor dimers might then bind to the remaining binding sites of the TNF trimer.

Concerning bioactivity, the question was interesting if a TNF trimer with only one or two active binding sites is still able to elucidate a signal in the cell. It was clearly shown, that this is possible for both receptors when high enough concentrations were used. One could imagine an intracellular signalling activation not between receptors bound by one TNF trimer, but rather an activation between receptors activated by two distinct trimers.

The scTNF molecules mutated in single binding sites to abolish binding, have clearly shown their usefulness for answering questions concerning TNF binding and signalling initiation. There are also further experiments imaginable, for example the examination of the binding kinetics, which could be helpful in specifying the picture obtained so far.

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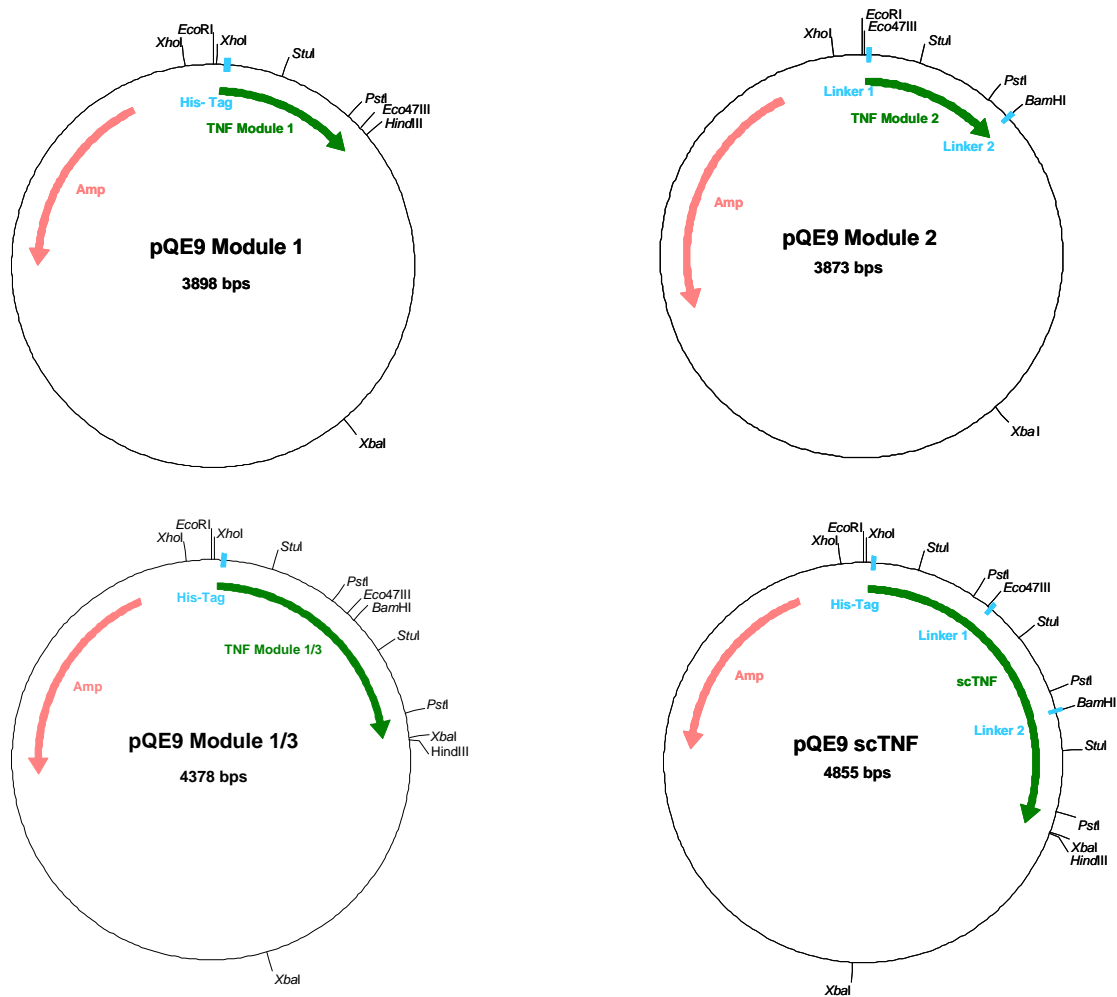
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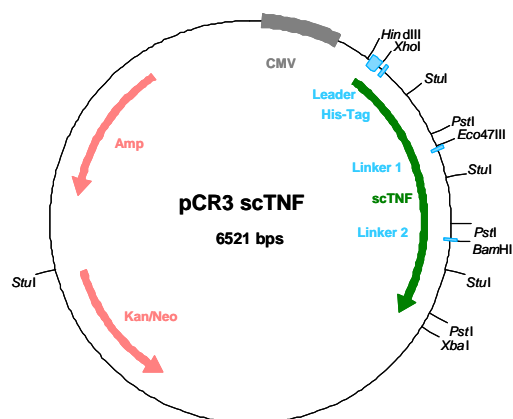
6 Appendix

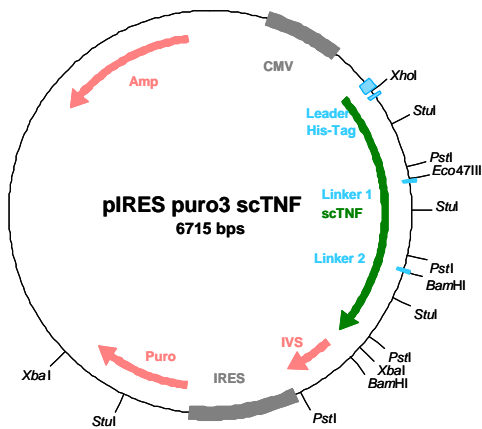
6.1 Plasmid Maps

Plasmids for the assembly of the scTNFs:



Plasmid for addition of the leader signal:



Plasmid for expression:**6.2 Sequences**

A: DNA sequence of the TNF on which the scTNFs are based upon (His-TNF in pQE9, Nr. 178)

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ACA.GAA.TTC.ATT.AAA.GAG.GAG.AAA.TTA.ACT.ATG.AGA.GGA.T
      EcoRI
CG.CAT.CAC.CAT.CAC.CAT.CAC.GGA.TCA.GCG.TCG.TCT.TCT.T
      His-Tag
CT.CGT.ACC.CCG.TCT.GAC.AAA.CCG.GTT.GCT.CAC.GTT.GTT.G
CA.AAC.CCG.CAG.GCT.GAA.GGT.CAA.CTG.CAA.TGG.CTG.AAC.C
GT.CGT.GCT.AAC.GCT.CTG.CTG.GCT.AAC.GGT.GTT.GAA.CTG.C
      R32
GT.GAC.AAC.CAG.CTG.GTT.GTT.CCG.TCT.GAA.GGC.CTG.TAC.C
      StuI
TG.ATC.TAC.TCC.CAG.GTT.CTG.TTC.AAA.GGC.CAG.GGC.TGC.C
CG.TCC.ACC.CAC.GTT.CTG.CTG.ACC.CAC.ACC.ATC.TCT.CGT.AT
C.GCT.GTT.TCC.TAC.CAG.ACC.AAA.GTA.AAC.CTG.CTG.TCT.GCA
      S86 Y87
.ATC.AAA.TCT.CCG.TGC.CAG.CGT.GAA.ACC.CCG.GAA.GGT.GCT.
GAA.GCT.AAA.CCG.TGG.TAC.GAA.CCG.ATC.TAC.CTG.GGT.GGC.
GTT.TTT.CAA.CTG.GAG.AAA.GGT.GAC.CGT.CTG.TCT.GCA.GAA.A
      PstI
TT.AAC.CGT.CCG.GAC.TAC.CTG.GAC.TTC.GCA.GAA.TCT.GGT.C
      D143 D145
AG.GTT.TAC.TTC.GGT.ATC.ATC.GCT.CTG.TGA.TGA.TAT.CTA.AC
      Stop
T.AAG.CTT.AAT.TAG.CT
      HindIII

```

Red: His-Tag for purification

Grey: Restriction sites

Start and end of the gene are indicated by arrows.

Coloured codons were mutated in this work:

R32 → TGG (W)

S86 → ACC (T)

D143 → AAC (N)

A145 → CGA (R)

Y87 → CAG(Q)

B: Amino acid sequence of scTNF WT (Linker GGGGS)

MDWTWRVFCLLA VAPGAHSLEKRMGRS **HHHHHH**GSASSSRTPSDKPVAHVVA
 NPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGGQCPSTHV
 LLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLS
 AEINRPDYLDFAESGQVYFGIIALGGGGSSSRTPSDKPVAHVVANPQAEGQLQWLN
 RANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGGQCPSTHVLLTHTISRIAVSYQT
 KVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAES
 GQVYFGIIALGGGGSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELR
 DNQLVVPSEGLYLIYSQVLFKGGQCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQR
 ETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

Grey: leader sequence for secretion

Red: His-Tag for purification

Green: Amino acids where mutation results in TNFR1 selectivity

Blue: Amino acids where mutation results in TNFR2 selectivity

Orange: Amino acids where mutation results in total inactivity

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Moleküle

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