Characterisation of TNF receptor-2 mediated signal initiation and transduction

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I hereby confirm that I performed the present work independently without further help or other materials than stated.

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For my parents
Helga and Gerd Bryde
and
for my siblings
Nikolaus and Dunja

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Abbreviations

°C	degree Celsius
µF	microfarad
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
32WCysHisTNF	R32W/S86T-cysteine/histidine-TNF, TNFR1-selective
143NCysHisTNF	D143N7A145R-cysteine/histidine-TNF, TNFR2-selective
A, ala	alanine
aa	amino acid
ab	antibody
ADAM	a disintegrin and metalloproteinase
amp	ampiciline
APS	ammoniumpersulfate
APS	3-aminopropyltriethoxysilane
ATP	adenosine triphosphate
BSA	bovine serum albumine
CD	cluster of differentiation
CIAP	calf intestinal alkaline phosphatase
cIAP	cellular inhibitor of apoptosis
cm	centimetre
cpm	counts per minute
CRD	cysteine rich domain
C-terminal	carboxy-terminal
cys	cysteine
D, asp	aspartate
DcR1	decoy receptor 1 (=TRAIL-R3, TRID)
DcR2	decoy receptor 2 (=TRAIL-R4)
DD	death domain
DISC	death-inducing signaling complex
DMSO	dimethylsulfoxide

DNA	desoxyribo nucleic acid
DR3	death receptor 3 (=Apo3, TRAMP, WSL-1,LARD)
DR4	death receptor 4 (=TRAIL-R1)
DR5	death receptor 5 (=TRAIL-R2, TRICK2, KILLER)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
CFP	enhanced cyan fluorescent protein
GFP	enhanced green fluorescent protein
EGTA	ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid
ELISA	enzyme linked immunoassay
FADD	Fas-associating death domain protein (=MORT1)
FITC	fluoresceinisothiocyanate
glu	glutamate
gly	glycine
hiFCS	heat-inactiviated fetal calf serum
h	hour
his	histidine
hu	human
I, ile	isoleucine
IAP	inhibitor of apoptosis protein
IgG	immunoglobuline G
IKK	IκB-kinase
IκB	inhibitor of κB
IPTG	isopropyl-β-thio-galactopyranoside
JNK	c-Jun N-terminal kinase (=SAPK, stress activated protein kinase)
K_d	dissociation constant
kDa	kilo-dalton
LB	Luria Bertani
LD_{50}	lethal dosis 50 (50% dead cells in a cytotoxicity assay)
LT	lymphotoxin
LTβ-receptor	lymphotoxin-β-receptor
mA	milliampere
memTNF/sTNF	membrane bound/soluble TNF
min	minute

ml	millilitre
mm	millimetre
mM	millimolar
N, asn	asparagine
NFκB	nuclear factor κB
ng	nanogram
nm	nanometre
N-terminal	amino-terminal
OD	optical density
o.n.	over night
PAGE	polyacrylamide-gel-electrophoresis
PBA	PBS with potassium azide
PBS	phosphate buffered saline
PEG	polyethylenglycol
PFA	parafomaldehyde
PLAD	pre-ligand binding assembly domain
PMSF	phenylmethylsulfonylfluoride
R, arg	arginine
RIP	receptor interacting protein
rpm	revolutions per minute
s	second
S, ser	serine
SDS	sodium dodecyl sulfate
SD	standard deviation
SODD	silencer of death domain
sulfoSMCC	succinimidyl-4-(N-maleimidomethyl) cylclohexane-1-carboxylate
T, thr	threonine
TACE	TNFα-converting-enzyme
TEMED	N,N,N´,N´-tetramethylethyldiamine
TNF	tumour necrosis factor
TNFR1 (-2)	tumour necrosis factor receptor 1 (-2)
TRADD	TNF-R1-associated death domain protein
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand (=Apo2L)

TRAIL-R1	TRAIL receptor 1 (=DR4)
TRAIL-R2	TRAIL receptor 2 (=DR5, TRICK2, KILLER)
TRAIL-R3	TRAIL receptor 3 (=DcR1, TRID)
TRAIL-R4	TRAIL receptor 4 (=DcR2)
TRANCE	TNF-related activation-induced cytokine (=RANK-L)
TWEAK	TNF related, weak ability to induce apoptosis
U	unit
W, trp	tryptophane
wt	wild type
wtCysHisTNF	wild type Cysteine-Histidine-TNF
Y, tyr	tyrosine
zVAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-Keton

Summary

In this work, silica based micro-particles covalently coated with TNF were generated as a new tool to convert soluble into an equivalent of membrane bound TNF. This tool was employed to investigate the molecular events leading to TNFR2 mediated NFκB-signalling.

For the covalent coupling of TNF to silica beads, a TNF-mutant with a Cysteine and a Histidine tag (CysHisTNF) fused to its N-terminus was used. The particles were activated with sulfoSMCC, allowing the formation of a covalent C-S-thioether between the maleimide group of sulfoSMCC and the SH-group of the free Cysteine being presented by wild type CysHisTNF. Immortalised mouse fibroblasts lacking wild type TNFR1 and TNFR2 but stably over-expressing a TNFR2/Fas receptor-chimera (composed of the extracellular and membrane spanning domains of TNFR2 and the cytosolic domain of Fas/CD95) were employed as a test system to confirm the membrane bound TNF (memTNF)-like activity of TNF-labelled beads. These cells undergo apoptosis within an hour after stimulation with memTNF but are completely resistant to activation with soluble TNF (sTNF). CysHisTNF coated beads were shown to provide a memTNF-like derivative which was able to fully stimulate wild type TNFR2 or the TNFR2/Fas chimera. To allow TNFR1 or TNFR2 selective stimulation in the presence of both receptors, CysHisTNF-mutants binding selectively to one or the other TNF-receptor were employed.

Control experiments with wildtype TNF revealed that a significant amount of the cytokine can bind to the beads by adsorption. Particles labelled with CysHisTNF, however, showed a higher bioactivity as compared to beads which had been treated with wildtype soluble TNF only.

Investigations using confocal microscopy revealed that an average 6 TNF-coated beads of 1 µm diameter or one respective particle of 10 µm diameter were sufficient to trigger apoptosis in a single TNFR2-Fas expressing mouse fibroblast. This revealed an about 20 fold higher bioactivity as compared to the best so far available memTNF mimicking agent that was generated by sTNF (derivatives) stabilised at the receptor with 80M2, a non-agonistic TNFR2-specific monoclonal mouse antibody.

TNFR1/Fas and TNFR2/Fas receptor-chimera were used to distinguish between the receptor-specific activity of sTNF or memTNF-equivalents on TNFR1 or TNFR2. Both receptor-chimera were demonstrated to activate the Fas-signal transduction pathway upon stimulation with memTNF-analoga by recruitment of FADD and/or Caspase-8 to the cytosolic portion of the receptor constructs, observed by confocal microscopy. However, only TNFR1/Fas could be stimulated with soluble TNF whereas TNFR2/Fas was fully resistant to sTNF action. Similar data were obtained after stimulation of wild type TNFR2 with a TNFR2-selective TNF-mutein being stabilised to the receptor with 80M2 or with a wtCysHisTNF-coated bead. Whereas these memTNF derivatives lead to the recruitment of TRAF2 to the TRAF-binding domain of the receptor, sTNF triggered no TRAF2-recruitment (Krippner-Heidenreich *et al.*, 2002).

In a HeLa cell overexpressing a full length wild type TNFR2-GFP construct, which was locally stimulated with a wtCysHisTNF-coated bead of 10 µm diameter, clustering of TNFR2-molecules to the bead-cell contact site as well as lateral diffusion of molecules from the direct vicinity to the bead could be observed by confocal microscopy. However, no indication of a lateral signal progression that would have generated the activation of non-stimulated receptor molecules was found.

Selective activation of TNFR2-molecules led to their internalisation via a dynamin-1-dependent mechanism. Internalised TNFR2 was found partially associated with caveolae and in late endosomes. Internalisation of TNF-coated beads of 1 µm diameter could be shown to occur independently of interaction of the beads with TNFR2 and was not inhibited by a dominant negative dynamin-1 mutant, suggesting a mechanism of internalisation different of that operative for TNFR2.

A TNFR2-receptor-construct devoid of its cytosolic domain, which was replaced by GFP, served to reveal that internalisation but not the clustering of TNFR2 is dependent of its cytosolic domain.

TNFR2 mediated NFκB-translocation, activated by the stimulation of TNFR2 with memTNF-analogues, was shown to occur within 30 minutes. Of the three investigated NFκB-molecules p65, p50 and c-Rel, p65 was found to be the NFκB-protein mainly being translocated into the nucleus after stimulation of both TNFR1 as well as TNFR2. IKKγ/NEMO could be demonstrated to be as essential for the release of NFκB from IκBα via TNFR2-signalling as is known for TNFR1. RIP, however, played no detectable role in TNFR2-mediated NFκB-translocation. Finally, TNFR2-mediated recruitment of TRAF2 was demonstrated to occur independently of TNFR2-internalisation.

Zusammenfassung

Mehrere Mitglieder der Tumor-Nekrose-Faktor-Rezeptor Familie zeigen unterschiedliche molekulare Reaktionen auf die Stimulierung mit entweder der membrangebundenen Form oder der löslichen Form ihres Liganden. So ist für TNFR1 bekannt, dass er sowohl lösliches als auch membrangebundenes TNF binden kann und zur Weiterleitung von Signalen angeregt wird. Im Gegensatz dazu kann TNFR2 zwar beide TNF-Formen binden, aber nur die Interaktion mit Membran-TNF führt zur vollständigen Aktivierung der TNFR2-vermittelten Signaltransduktion.

Um die molekularen Ereignisse, die durch die Stimulation des TNFR2 mit Membran-TNF verursacht werden, angemessen zu untersuchen, wurden in dieser Arbeit TNF-gekoppelte Mikropartikel auf Silica-Basis eingesetzt, welche die besondere Bioaktivität von Membran-TNF besitzen.

Um eine kovalente Bindung von TNF an die Silica-Partikel zu ermöglichen, wurde eine TNF-Mutante verwendet, die ein Cystein und einen Histidin-*tag* an ihrem N-Terminus aufweist. Die Oberfläche der Partikel wurde mit SulfoSMCC aktiviert und erlaubte so die Ausbildung eines kovalenten C-S-Thioethers zwischen der Maleimid-Gruppe des SulfoSMCC und der SH-Gruppe des freien Cysteinrestes. In einem Test-System aus immortalisierten Mausfibroblasten, die weder TNFR1 noch TNFR2 exprimieren, dafür jedoch mit einer TNFR2/Fas-Rezeptorchimäre (bestehend aus dem extrazellulären und dem membrandurchspannenen Teil des TNFR2 und der zytosolischen Domäne des Fas-Rezeptors) stabil transfiziert waren, wurde die Membran-TNF-Aktivität der TNF-präsentierenden Partikel untersucht. TNFR2/Fas exprimierende Mausfibroblasten können nicht durch lösliches TNF in Apoptose gebracht werden, Membran-TNF dagegen induziert Apoptose innerhalb einer Stunde nach Stimulation der Zellen. Sowohl die TNFR2/Fas-Chimäre als auch wildtyp-TNFR2 wurden durch das auf Partikeln gebundene TNF angeregt, d.h. partikulär immobilisiertes TNF wirkt als memTNF. In weiteren Versuchen wurden für die selektive Stimulation des TNFR1 bzw. des TNFR2 TNF-Muteine verwendet, die selektiv nur an jeweils einen oder den anderen Rezeptor binden.

In Kontrollversuchen mit löslichem wildtyp-TNF wurde nachgewiesen, dass die Partikel TNF auch durch Adsorption binden.

Untersuchungen mit Hilfe der konfokalen Mikroskopie zeigten, dass 6 wtCysHisTNF-Partikel von 1 µm Größe oder ein einziges entsprechendes Partikel von

10 µm Größe ausreichten, um Apoptose in einem TNFR2/Fas-Mausfibroblasten auszulösen. Daraus errechnet sich eine etwa 20-fach höhere Bioaktivität im Vergleich zu dem bisher verwendeten memTNF-Analog, das aus TNF(-Muteinen) besteht, welches mit Hilfe des nicht agonistischen TNFR2-spezifischen Antikörpers 80M2 am TNFR2 stabilisiert wird.

Für die Untersuchung der Rezeptor-spezifischen Wirkung von löslichem TNF bzw. der memTNF-Analoga wurden TNFR1/Fas und TNFR2/Fas Rezeptor-Chimären eingesetzt. Beide induzieren die Signalweiterleitung durch ihre zytosolische Fas-Domäne, die im Konfokalmikroskop auch durch die Rekrutierung von FADD und/oder Caspase-8 an die jeweilige Rezeptor-Chimäre beobachtet werden konnte. TNFR1/Fas konnte mit beiden TNF-Formen, der löslichen und der memTNF imitierenden, aktiviert werden, während TNFR2/Fas vollkommen resistent gegen eine Stimulation mit löslichem TNF war. Entsprechende Daten konnten an wildtyp-TNFR2-Molekülen reproduziert werden. Erst die Stabilisierung von TNF am Rezeptor mit Hilfe des nicht-agonistischen Antikörpers 80M2 oder der Kontakt mit TNF-gekoppelten Partikeln führte zur Rekrutierung von TRAF2 an den Rezeptor, wogegen lösliches TNF keine Reaktion auslöste (Krippner-Heidenreich *et al.*, 2002).

Ein einziges 10 µm großes, mit wtCysHisTNF überzogenes Partikel wurde eingesetzt, um eine HeLa-Zelle, die ein TNFR2-GFP Konstrukt überexprimierte, lokal zu stimulieren. Mit Hilfe eines Konfokalmikroskops konnte die Akkumulierung von Rezeptormolekülen an der Kontaktstelle zwischen Partikel und Zelle sowie die Diffusion links und rechts direkt benachbarter Molekülen in die Kontaktstelle beobachtet werden. Hinweise auf eine laterale Signal-Übertragung, die zur Aktivierung von unstimulierten Rezeptoren geführt hätte, wie sie für den Epidermal Growth Factor-Rezeptor gezeigt wurden (Reynolds *et al.*, 2003; Verveer *et al.*, 2000), wurden jedoch nicht gefunden.

Die Internalisierung von TNFR2-Molekülen und ihre Lokalisierung in Lysosomen konnte ebenso gezeigt werden wie ihre Abhängigkeit von Dynamin. In einem vorläufigen Experiment im konfokalen Mikroskop wurde eine teilweise Kolokalisierung von internalisiertem TNFR2 mit Caveolin-1 ebenso wie mit späten Endosomen beobachtet. Diese Ergebnisse sind ein Hinweis auf einen Caveolin- und Dynamin-abhängigen Internalisierungsweg des Rezeptors. wtCysHisTNF-überzogene Partikel von 1 µm Durchmesser wurden ebenfalls internalisiert und in Lysosomen lokalisiert. Dieser Vorgang war unabhängig von der Interaktion der TNF-Partikel mit TNFR2 und konnte

nicht durch Expression einer dominant-negativen Dynamin-1 Mutante in den behandelten Zellen inhibiert werden. Dies weist auf eine Internalisierung durch einen Mechanismus hin, der sich von dem für die TNFR2-Internalisierung wirksamen unterscheidet.

Mit Hilfe eines TNFR2-Konstrukts, dessen zytosolische Domäne durch ein GFP-Molekül ersetzt wurde, konnte gezeigt werden, dass die Akkumulation des TNFR2 in Clustern im Gegensatz zur Internalisierung des Rezeptors unabhängig von der zytosolischen Domäne erfolgte.

Die TNFR2-vermittelte NFκB-Translokation in den Zellkern, wie sie nach der Stimulierung des TNFR2 mit Membran-TNF-Äquivalenten erfolgt, war 30 Minuten nach der Aktivierung am stärksten. Von drei untersuchten NFκB-Proteinen p65, p50 und c-Rel, machte p65 den Hauptanteil des translozierten NFκBs aus, sowohl in der TNFR1- als auch in der TNFR2-vermittelten Signalübertragung. IKKγ/NEMO, die regulatorische Untereinheit des IKK-Komlexes, wurde für die Phosphorylierung des Inhibitors von κB (IκB) und die Freisetzung von NFκB durch TNFR2 als ebenso essentiell befunden wie bereits für TNFR1 gezeigt wurde. Dagegen konnte keine Beteiligung von RIP während der TNFR2-vermittelten NFκB-Translokation festgestellt werden. Beobachtungen von TRAF2-Rekrutierung an den TNFR2 in Zellen, die eine dominant-negative Dynamin-1-Mutante exprimierten lieferten Hinweise, dass der TNFR2-vermittelte NFκB-Signalweg unabhängig von der Internalisierung des Rezeptors erfolgt.

1 Introduction

1.1 The tumour necrosis factor superfamily

The steadily growing TNF-superfamily is composed of about twenty polypeptides which display structural similarities (Aggarwal, 2003; Locksley *et al.*, 2001). A survey of the best defined members of the TNF-superfamily and their corresponding receptors is given in table 1. Tumour necrosis factor alpha (TNF), the namesake of this family of cytokines, was identified and characterised as a serum-factor that causes necrosis of certain tumours in mice (Carswell *et al.*, 1975). Most members of the TNF-superfamily are expressed in the immune system, where they play a major role in the regulation of proliferation and in the protective function of pathogen sensitive cells. TNF itself is known to possess characteristics that allow the modulation of the immune response as well as pro-inflammatory and pathophysiological activities. Bacterial infections, e.g., cause the activation of macrophages and monocytes mainly mediated by TNF, especially after stimulation with lipopolysaccharides (Higuchi *et al.*, 1990). In endothelia, TNF participates in the regulation of the expression of surface antigens like human leukocyte antigens (HLA) and adhesion molecules (intercellular adhesion molecule (ICAM), endothelial leukocyte adhesion molecule (ELAM), vascular cell adhesion molecule (VCAM)), facilitating the adhesion of neutrophile granulocytes to vascular walls (Adolf and Fruhbeis, 1992). Furthermore, TNF induces the production of several cytokines such as interleukin-1 (IL-1), IL-6, IL-8, interferon-γ (IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF) and also TNF itself (Grell and Scheurich, 1997; Vassalli, 1992).

Thymocyte and B-cell proliferation have been shown to depend on TNF (Becker *et al.*, 1990; Tartaglia *et al.*, 1993b) as well as the induction of apoptosis in certain cells. Some tumour cell lines are sensible towards apoptosis induction by TNF, however, typically protein synthesis has to be inhibited. This requirement of protein synthesis inhibition is caused by anti-apoptotic proteins which are constitutively expressed and/or induced by TNF stimulation. They all act downstream of the activated TNF-receptor to inhibit different steps in the apoptotic signal transduction. Some of these proteins with anti-apoptotic functions are A20 (Lademann *et al.*, 2001; Opipari, Jr. *et al.*, 1992; Zhang *et al.*, 2000), c-FLIP (Krueger *et al.*, 2001; Yeh *et al.*, 2000), cIAPs 1 and 2 (Roy *et al.*,

1997; Salvesen and Duckett, 2002), XIAP (Shiozaki *et al.*, 2003; Srinivasula *et al.*, 2001; Suzuki *et al.*, 2001), and TRAF1 and -2 (Dempsey *et al.*, 2003; Wajant *et al.*, 1999).

Considering the pleiotropic nature of TNF-induced signalling in the immune system, it is not surprising to find a disregulation of TNF-expression at the core of many acute diseases as well as autoimmune diseases. TNF was identified as the main mediator of cachexia, a general reduction in vitality and strength of body and mind resulting from a debilitating chronic disease (Sharma and Anker, 2002). In patients with bacterial meningitis, sepsis, cerebral malaria, fulminant hepatitis or AIDS, enhanced TNF-levels can be found in the sera. Autoimmune diseases like rheumatoid arthritis, morbus crohn (in both diseases, high levels of membrane bound TNF are found), multiple sclerosis and systemic lupus erythematosus are thought to be caused by a defective apoptosis induction (Grell and Scheurich, 1997).

All members of the TNF ligand family, except LTα, are expressed as type II transmembrane proteins. They may exist as a membrane bound “pro” as well as a processed soluble “mature” form. Cleavage is performed by metalloproteases of the ADAM (a disintegrin and metalloproteinase)-family (Idriss and Naismith, 2000). Trimers of memTNF are already formed in the cytosol (Tang *et al.*, 1996) and migrate to the cell membrane where they can be processed by TACE (TNFa-converting enzyme) to release soluble TNF-trimers (Black *et al.*, 1997; Moss *et al.*, 1997). Both ligand forms are bioactive only as self-assembled and stable, but non-covalently linked trimers (Fesik, 2000). Differences in bioactivity between the membrane bound and the soluble ligand form have been shown for TNF (Grell *et al.*, 1995), TRAIL (Muhlenbeck *et al.*, 2000; Wajant *et al.*, 2001), and Fas/CD95 (Schneider *et al.*, 1998). The soluble, mature form of TNF is able to bind to both TNFR1 and TNFR2, but only TNFR1 is fully activated. In contrast, membrane bound TNF has been shown to activate TNFR1 as well as TNFR2 effectively (Grell *et al.*, 1995; Grell *et al.*, 1998b; Krippner-Heidenreich *et al.*, 2002). Different amino acids as well as overlapping residues of the TNF molecules are responsible for the binding to TNFR1 or TNFR2, respectively. Amino acids that are essential for the binding of TNF to TNFR1 have been identified. These are R(arginine)32, S(serine)86 and I(isoleucine)97 whereas TNFR2 selectively interacts with D(aspartate)143. Residues essential for binding of TNF to both receptors are Y(tyrosine)87 and A(alanine)145 (Steed *et al.*, 2003; Vandenabeele *et al.*, 1995). All these amino acids interact with CRDs 2 and 3 of the two respective receptors (see below).

Table 1: Cytokines of the TNF-superfamily and their receptors

Cytokine	Receptor
TNFα	TNFR1, TNFR2
TNFβ	TNFR1, TNFR2
CD95L/FasL	CD95/Fas, DcR3
LTα	LTβR, TNFR1, TNFR2, HveA
LTβ	LTβR
TRAIL	DR4/TRAILR1, DR5/TRAILR2, DcR1, DcR2
CD27L	CD27
CD30L	CD30
CD40L	CD40
VEGI	DR3, DcR3
EDA-A1, EDA-A2	EDAR, XEDAR
NGF	NGFR
RANKL	OPG, RANK
TWEAK	FN14
LIGHT	LTβR, DcR3, HveA
4-1BBL	4-1BB
OX40L	OX40
APRIL	TACI, BCMA
BAFF	TACI, BCMA, BAFFR
GITRL	GITR

1.2 The tumour necrosis factor receptor superfamily

The corresponding receptors for the TNF ligand family were gathered into one family based on structural homologies (table 1). In contrast to the ligands, receptors of the TNFR family are type I transmembrane proteins. They are characterised by 1 to 6 cysteine rich domains (CRDs) within their extracellular sections. A conserved domain was identified within CRD1 of TNFR1, TNFR2 and Fas, that allows the formation of receptor dimers and/or trimers independently of interaction with their respective ligands

(Chan *et al.*, 2000;Siegel *et al.*, 2000). This pre-ligand binding assembly domain (PLAD) differs from the domain responsible for ligand binding (CRDs 2 and 3), but is also essential for TNF binding as has been shown in PLAD deletion mutants of TNFR1 and TNFR2. Receptor chimera, whose PLADs had been exchanged, revealed that only homomeric interactions between the extra-cellular domains of receptors can take place. The autoaggregation of PLAD-expressing receptor molecules appears to be a common trait for the members of the TNFR-family.

TNFR1 is constitutively expressed in many tissues and cell types, whereas TNFR2-expression is highly regulated by several factors (Adolf *et al.*, 1994). The functionality of TNFR2 was long obscured by the fact that most signalling responses upon TNF-stimulation were mediated by TNFR1. The discovery that TNFR2 is fully activated only by membrane bound TNF, and the generation of tools to mimic memTNF allowed a closer scrutiny of TNFR2-mediated signalling. Cellular pathways activated by TNFR2 are the proliferation of thymocytes and T-cells (Grell *et al.*, 1998a; Tartaglia *et al.*, 1991; Tartaglia *et al.*, 1993b), the production of GM-CSF (Vandenabeele *et al.*, 1992), the activation of NFκB (Rothe *et al.*, 1995), the activation of JNK (Jupp *et al.*, 2001), but also in some cells the induction of apoptosis (Grell *et al.*, 1993; Grell *et al.*, 1994; Medvedev *et al.*, 1994; Zheng *et al.*, 1995). Beside a similar affinity of TNF towards TNFR1 and TNFR2, the stability of TNF-TNFR-complexes are crucial for the differential activation of TNFR1 by sTNF and memTNF and of TNFR2 by only memTNF (Grell *et al.*, 1998b; Krippner-Heidenreich *et al.*, 2002).

The intracellular signal transduction after the stimulation of TNFR-family members with their respective ligands occurs with the aid of adaptor molecules. These cooperate via protein-protein interaction motives located in the cytosolic domains of the TNFR-family members. Two subclasses of receptors regarding their intracellular domains have been defined: the death domain (DD) containing or death receptors and the non-death domain receptors. The death receptors possess homologous sequences of about 70 amino acids which form six anti-parallel α-helices with a highly conserved topology (Aravind *et al.*, 1999; Lahm *et al.*, 2003). Electrostatic interactions between the death domains of the receptor and their respective cytosolic adaptor proteins are responsible for the cytotoxic effects of the death receptors, amongst them those of TNFR1 (Tartaglia *et al.*, 1993a) and Fas. The DDs of Fas and TNFR1 were shown to auto-aggregate, a trait that leads to the induction of apoptosis upon over-expression of one of these receptors (Boldin *et al.*, 1995; Song *et al.*, 1994). Cytoplasmatic proteins which posses a

DD are, amongst others, TRADD (Hsu *et al.*, 1996b;Schwandner *et al.*, 1998), FADD (Berglund *et al.*, 2000;Schwandner *et al.*, 1998), and RIP (receptor interacting protein) (Kelliher *et al.*, 1998). The recruitment of TRAF-proteins to TNFR1 occurs only indirectly via TRADD as the adaptor molecule providing a platform for this event. In contrast to the death receptors, TRAF-binding receptors contain several amino acid motifs responsible for the binding of TRAF-molecules. For the interaction of TNFR2 with TRAF2, the sequence SxEE was found to be essential (Boucher *et al.*, 1997; Rothe *et al.*, 1994).

1.3 TNFR1 signal-transduction pathways

The initial event of TNFR1 mediated signal transduction is the trimerisation of the receptor, induced by the ligation with TNF-trimers (Smith *et al.*, 1994). Auto-aggregation of the TNFR1-DDs is inhibited by SODD (silencer of death domain), a protein that thus prevents spontaneous activation of signalling cascades by binding to the DD of TNFR1 or Fas (Jiang *et al.*, 1999). Upon stimulation, the TNFR1-SODD-complex disintegrates and the death domain of TNFR1 becomes available for interactions with other adaptor proteins. In the following chapter, TNFR1-signalling pathways relevant for this work are discussed. Figure 1 presents an overview of these pathways.

1.3.1 TNFR1 mediated apoptosis

Engagement of TNFR1 triggers the DD-mediated recruitment of TRADD, a protein that serves as a platform for the recruitment of several other proteins to the receptor. The formation of a complex of the DD-containing ser/thr kinase RIP (Chen and Goeddel, 2002;Hsu *et al.*, 1996a), TRAF2 (Hsu *et al.*, 1996b) and FADD (Zhang *et al.*, 1998), together with TRADD and TNFR1 was established as the bifurcation point between TNFR1 mediated NFκB-activation and the induction of apoptosis. The TRADD-RIP-TRAF2 complex is known to be essential for the activation of NFκB-signalling pathways (Baud and Karin, 2001) whereas the interaction between TRADD and FADD allows the recruitment and activation of caspase-8 (Hsu *et al.*, 1996b). This model has recently been challenged by the discovery that neither FADD nor caspase-8 could be found in the complex formed at the cell membrane upon TNFR1 stimulation (Harper *et al.*, 2003).

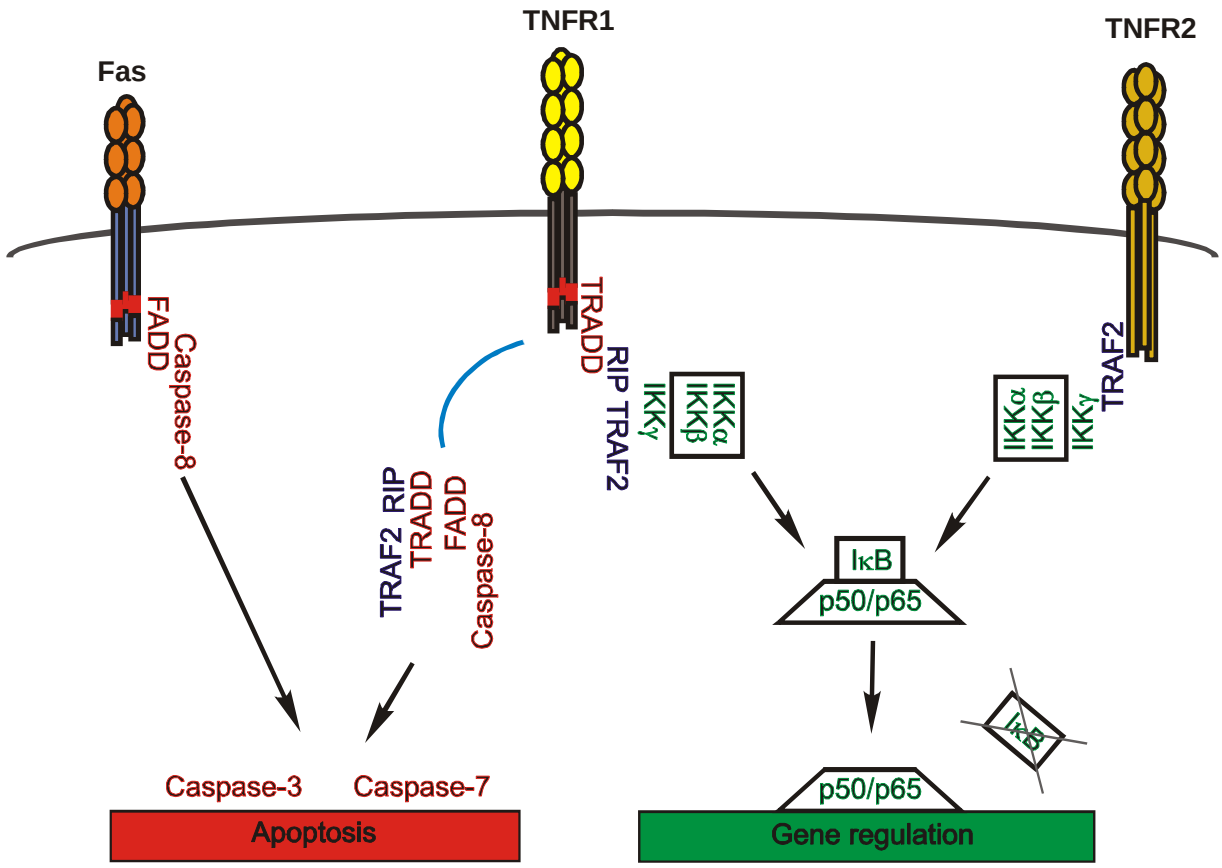


Figure 1: **Selected signal transduction pathways of Fas, TNFR1 and TNFR2**

Micheau and Tschopp (2003) proposed a new model for the TNFR1 mediated apoptosis. After stimulation with TNF, an initial complex (complex I) that contains TNFR1, TRADD, TRAF2, and RIP, and possibly other known or yet unidentified proteins, is rapidly assembled in lipid rafts. This complex triggers the activation of NFκB, as will be discussed later on. Formation of complex I is transient and a large portion of TRADD, TRAF2 and RIP dissociate within an hour from TNFR1. After dissociation, the DD of TRADD becomes available for interaction with FADD, thus leading to the binding and activation of caspase-8 in the cytosol (Micheau and Tschopp, 2003). Caspase-8 is a member of a family of cystein containing, aspartate directed proteases. These enzymes are highly specific and cleave their substrates after the amino acid aspartate. Caspases are expressed as pro-enzymes. Upon induction of apoptosis, the pro-forms are cleaved into a large subunit of about 20 kDa and a small subunit of about 10 kDa. These subunits aggregate to heterotetramers, containing two small and two large subunits. Caspases may be divided into two subfamilies: the initiator caspases and the effector

caspases. TNFR1-mediated activation of apoptosis depends on a caspase cascade instigated by the initiator caspase-8 or initiator caspase-10 (Wang *et al.*, 2001). After cleavage and activation through the recruitment to TRADD-FADD, caspase-8 or caspase-10 itself activates downstream effector caspases-3 and -7 (Shi, 2002). These events are characteristic for type I cells. In type II cells, caspase-8 activation results in the cleavage on Bid into tBid, thus initiating the intrinsic apoptotic pathway involving mitochondria. The effector caspases then cleave a variety of defined substrates like poly(ADP)-Ribose-Polymerase (PARP), ICAD (inhibitor of caspase-activated DNase), gelsolin and lamine (Fischer *et al.*, 2003) and others. The proteolysis of effector-caspase substrates is responsible for the characteristic morphological changes observed in apoptotic cells and for programmed cell death.

1.3.2 NFκB-activation by TNFR1-signalling

As mentioned in chapter 1.3.1, the activation of TNFR1 triggers the recruitment of TRADD to its death domain, followed by the DD-containing ser/thr-kinase RIP and TRAF2. TRAF2 binds to TRADD via its c-terminal TRAF-domain (Hsu *et al.*, 1996b; Takeuchi *et al.*, 1996) and recruits the IKK-complex to TNFR1 by interaction with IKKβ (Devin *et al.*, 2001). RIP, on the other hand, supports the recruitment of the IKK-complex by interaction with IKKγ/NEMO and activates the complex via its intermediary domain (Devin *et al.*, 2000; Kelliher *et al.*, 1998; Zhang *et al.*, 2000). Another report suggests that RIP activates the IKK-complex only indirectly since it binds to the mitogen activated protein kinase kinase kinase MEKK3, which serves as a linker between TRAF2/RIP and IKK and activates the latter (Yang *et al.*, 2001). The IKK-heterotrimer is composed of two catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ/NEMO (Mercurio *et al.*, 1997; Rothwarf *et al.*, 1998). IKKβ dephosphorylates the inhibitor of κB alpha (IκBα) during TNF-mediated NFκB activation whereas IKKα is dispensable during this process in most cell types (Chu *et al.*, 1999; Delhase *et al.*, 1999; Hu *et*. *al.*, 1999; Li *et al.*, 1999b) Phosphorylation of IκBα, which retains NFκB dimers (most commonly p50/p65) in an inactive state in the cytosol by masking their nuclear location sequence, leads to its poly-ubiquitination and degradation by the 26S proteasome. Subsequently, NFκB dimers translocate into the nucleus, bind DNA and activate gene transcription (Ghosh and Karin, 2002).

The NFκB-inducing pathway described above is the classical of two known ways of NFκB-activation. The alternative pathway is known to be activated by LTβR-, BAFFR- or CD40-induced signalling. These non death domain containing members of the TNFR family activate the formation of an IKKα dimer, which targets p100 for phosphorylation and processing into p52 (Coope *et al.*, 2002; Dejardin *et al.*, 2002; Senftleben *et al.*, 2001). The canonical pathway is mostly operative during infection and inflammatory processes, whereas the alternative NFκB-activation is required for B cell maturation and the formation of secondary lymphoid organs (Senftleben *et al.*, 2001).

1.4 TNFR2 signal-transduction pathways

TNFR2-signalling pathways are less intense investigated as those of TNFR1, since selective stimulation of TNFR2 was difficult to obtain. Therefore, the overall pathways initiated by activation of TNFR2 are quite well known, but the molecular mechanisms underlying these pathways are poorly understood. TNFR2 is known to trimerise upon ligation with memTNF trimers, similar to TNFR1 and other members of the TNFR-family. Upon stimulation, TRAF2 is recruited to the TRAF-binding site of its cytosolic domain in a 3:3 stochiometry (Stroud and Wells, 2004). As mentioned in chapter 1.2, TNFR2 is responsible for the proliferation of thymocytes and T-cells, the production of GM-CSF, the initiation of NFκB, the activation of JNK and the induction of apoptosis. The NFκB-activation pathway, being relevant for this work, is shown in fig. 1.

TRAF2-recruitment upon stimulation of TNFR2 with memTNF leads to the activation of the IKK-complex. As described in chapter1.3.2 for TNFR1, the IKK-heterotrimer then triggers phosphorylation of IκBα, which is poly-ubiquitinated and degraded by the 26S proteasome. The thus released NFκB dimers translocate into the nucleus, where they bind DNA and initiate gene transcription (Ghosh and Karin, 2002).

1.5 Fas mediated induction of apoptosis

Fas is the best studied member of the death receptor sub-family. As has been shown for other members of the TNFR-superfamily with their respective ligands, Fas forms trimers and higher order oligomers upon triggering with FasL (Kischkel *et al.*, 1995). This oligomerisation is followed by the direct recruitment of FADD to the DD of Fas, without

an intermediary adapter molecule as has been shown for TNFR1. FADD interacts with the initiator caspase-8 to form the death-inducing signalling complex (DISC) (Kischkel *et al.*, 1995). Similar to TNFR1 and TNFR2, Fas has been shown to form pre-assembled complexes which interact via their PLAD domains (Siegel *et al.*, 2000). Fas triggers apoptosis via the classical caspase cascade, involving the initiator caspase-8 as well as the effector caspases-3 and -7. However, according to the cell type, two different pathways of apoptosis induction were shown (Scaffidi *et al.*, 1998). In type I cells, caspase-8 is recruited to the DISC in large quantities, allowing the direct activation of caspase-3 (Stennicke *et al.*, 1998). DISC formation in type II cells is inefficient, generating only very small quantities of active caspase-8 at the cell membrane. These are not able to activate caspase-3 directly but can cleave the BH3-only protein Bid (Li *et al.*, 1998). This leads to the apoptogenic activation of mitochondria, involving caspases-9, -3 and -7. The apoptotic pathway triggered by Fas in type I cells is shown in fig. 1.

1.6 Stimulation of TNFR2 mediated signal transduction: a choice of tools designed to mimic memTNF

To investigate TNFR2 mediated signal transduction, various tools were created and employed. One of the first attempts was the generation of an agonistic monoclonal antibody specific for TNFR2, among these a monoclonal antibody named MR2-1 by the manufacturer. However, experience showed that this antibody exerts only a very weak effect and is therefore able to trigger only limited activation of TNFR2. Moreover the activity of MR2-1 was associated with a weak tendency of MR2-1 to autoaggregate. TNFR2-activation was negligible since MR2-1 was not able to cross-link the receptor molecules, an essential condition for TNFR2-mediated signalling.

Cultivation of adherent TNFR2-expressing cells together with an adherent cell line that expresses a mutated form of membrane bound TNF is another possibility to activate TNFR2. $CHO_{TNF\Delta 1\text{-}12}$ cells (Grell *et al.*, 1995) express a form of TNF that cannot be processed into soluble TNF by cleavage through TACE. Co-cultures of cells, though, may be complicated, since side effects generated by cell-cell interactions (e.g. reverse signalling; for review, see Eissner *et al.*, 2004) or by differences in the preferred settings for the cultivation of each cell line may falsify the results. Furthermore, kinetics of signalling events are extremely difficult to follow using this method, since co-cultured

cells need time to adhere to their substrate in order to avoid death by anoikis. Thus primary signalling events might be disturbed by the adhering process or falsified by cells that were not able to fully adhere.

Grell et al. (1995) established a tool to mimic membrane bound TNF which allows not only the direct comparison between sTNF and its memTNF equivalent, but which also permits the observation of signal transduction events at very early time points by microscopy. 80M2, a monoclonal antibody specific for TNFR2, has been shown to cross-link TNFR2-molecules and to stabilise the binding of TNF to the receptor (Grell *et al.*, 1995). This TNFR2-specific antibody exerts, apart from its already mentioned function, neither agonistic nor antagonistic activities, thus representing a neutral tool to stain TNFR2 for fluorescence based microscopy or FACS-analysis. To refine the TNFR2 selective stimulation, TNFR1 and TNFR2 specific mutants of TNF (R32W/S86T-TNF and D143N/A145R-TNF, respectively) were generated, allowing stimulation of only one receptor in the presence of the other receptor molecule. The TNFR2-selective mutant was shown to exercise only a weak stimulation of its corresponding receptor which could be strongly enhanced by the pre-stimulation of the cells with 80M2 (Krippner-Heidenreich *et al.*, 2002).

To construct TNF-derived mutants with membrane bound TNF-like characteristics, a His-tag and a free cysteine residue were fused to wild type TNF. Both allow the formation of higher order aggregates, leading to a comparably weak memTNF functionality of the TNF-constructs. Experience showed that to achieve full stimulation of TNFR2, wild type CysHisTNF as well as D143N/A145R CysHisTNF had to be stabilised with 80M2 at the receptor.

1.7 Soluble TNF coupled to micro-beads mimics membrane-bound TNF

Microbeads are a tool widely used to bind biological targets from biological samples, as has been published for cell sorting (Bildirici and Rickwood, 2001; Kruger *et al.*, 2000) or protein enrichment and purification (Hurst *et al.*, 1999). In this work, micro- and nanobeads were employed as a new tool to convert sTNF into a memTNF eqivalent.

The group of Günter Tovar (Fraunhofer Institute for Interfacial Engineering and Biotechnology, Nobelstr. 12, 70569 Stuttgart, Germany) has developed silica particles

that are modified to allow covalent binding of biomolecules onto their surface (Schiestel *et al*., 2004). Beads exposing streptavidin on their surface to allow coating of the

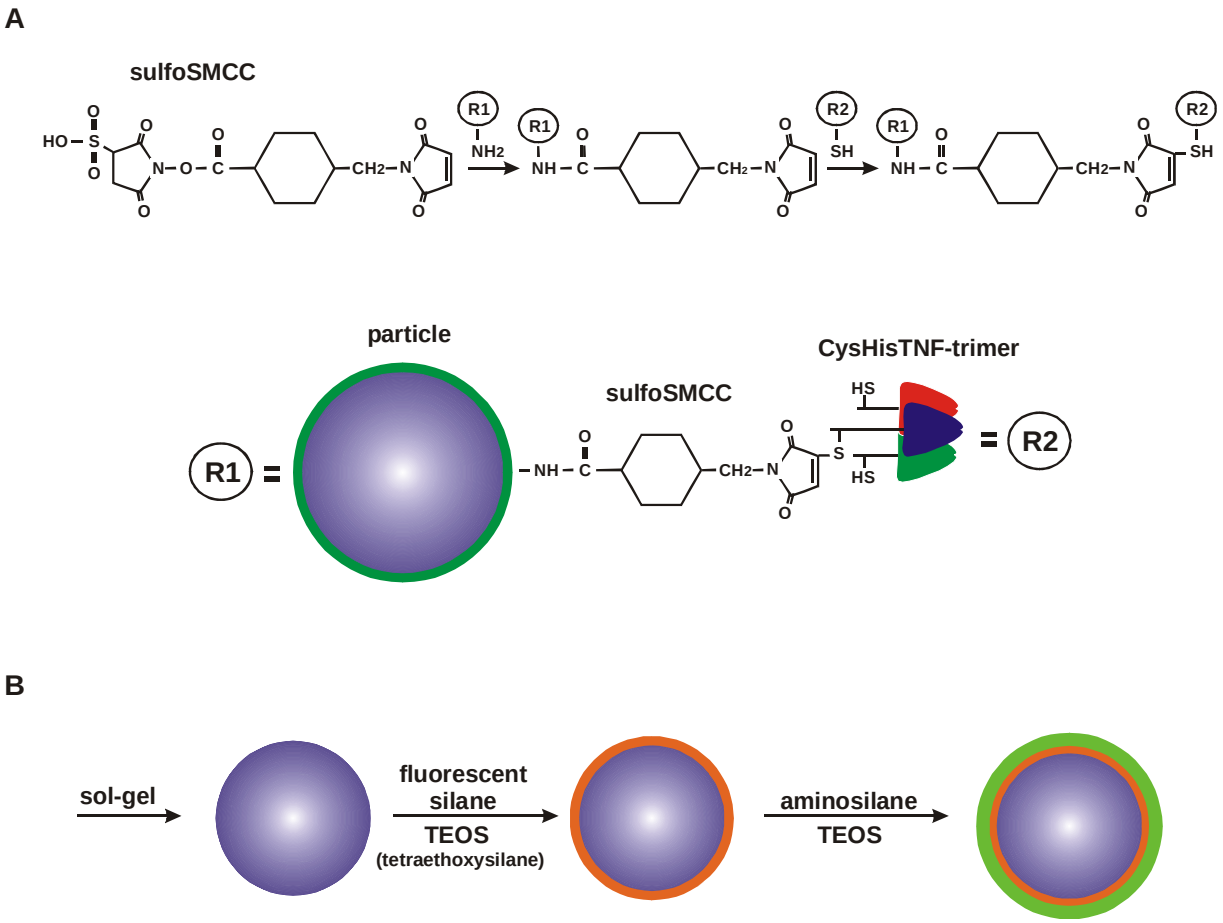


Figure 2: **The architecture of TNF-silica-particles**. **A.** Beads activated with sulfoSMCC are able to form covalent C-S-thioether bonds with TNF that exposes a free cysteine residue on its N-terminus. **B.** To beads with a silica core, red fluorescent Alexa-Fluor-568-dye is polymerised prior to modifying the surface. The aminosilane coat allows the activation of the bead with sulfoSMCC (compare with TNFR1 = bead in A).

particles with biotin-conjugated proteins were shown to represent a valuable tool for spatially restricted receptor-selective stimulation of cells (Reynolds *et al.*, 2003; Verveer *et al.*, 2000). The beads manufactured by Schiestel et al. consist of a silica core that was modified with APS (3-aminopropyltriethoxysilane) and sulfoSMCC (Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate) to allow the formation of covalent C-S-thioethers between the maleimide group of sulfoSMCC and the SH-group of a free

cysteine residue presented by the target protein (fig. 2a, Thomas Schiestel and Nadja Reinhardt, personal communication). To enable the target protein TNF to bind covalently to the silica-particles, wild type TNF that has a cysteine fused to its N-terminus was employed. Fluorescing beads were manufactured by Schiestel et al. with the red fluorescent dye Alexa-Fluor-568. This dye was polymerised onto the silica core of the beads and covered with APS and sulfoSMCC (fig. 2b).

1.8 Aim of this work

Recent publications have shown that TNFR2 signal transduction is mainly triggered by membrane bound TNF (Grell *et al.*, 1995; Krippner-Heidenreich *et al.*, 2002). In this work, silica particles activated with sulfoSMCC to allow covalent binding of TNF were established as a new tool to convert soluble TNF into a membrane bound TNF-equivalent. These beads were characterised regarding their binding capacity of sTNF vesus CysHisTNF, the manner in which TNF is bound and their bioactivity compared with soluble memTNF equivalents. Beads labelled with wild type or TNFR2 selective TNF-derivatives were used to stimulate TNFR2, thus allowing the investigation of several aspects of TNFR2-signalling. Receptor clustering and -internalisation was studied, as well as the role of RIP and IKKγ/NEMO during NFκB-activation.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Alexa-Fluor-546 protein labelling kit	Molecular Probes, Eugene Oregon, USA
Ampicillin (Amp)	Sigma, Steinheim
Bio-Rad Protein Assay	Bio-Rad, München
Blotting-paper Whatman 3MM	Chr Whatman International, England
Bromphenolblue	Serva, Heidelberg
BSA, sulfhydryl modified	Serva, Heidelberg
Cell culture flasks and dishes	Greiner, Frickenhausen
Cell culture glass bottom dishes for microscopy	Mattek Corporation, USA
Crystal violet	Merck, Darmstadt
Cycloheximide	Sigma, Steinheim
DNA standards (1 kb, 100 bp)	Gibco BRLR Life Technol. GmbH Karlsruhe
Eosine	Merck, Darmstadt
Imidazole	Amersham, Braunschweig
FCS	Biochrom, Berlin
Fluoromount-G TM	Southern Biotechnology Associates, Birmingham, USA contributor germany: Biozol Eching
Lysotracker	Molecular Probes, Eugene Oregon, USA
Milk powder, low fat	Heirler, Radolfzell
NFκB specific oligonucleotide	Gibco BRLR Life Technology Karlsruhe
Nitrocellulose	Pall Life Sciences, USA
NP-40	Sigma, Steinheim

Phenylmethylsulfonylfluoride (PMSF)	Sigma, Steinheim
Poly[dI:dC]	Amersham, Braunschweig
Prestained protein marker	New England Biolabs, Frankfurt
Protease-inhibitors (cocktail tablets)	Boehringer, Mannheim
Puromycin A	Calbiochem, San Francisco, USA
RPMI 1640	Seromed, Berlin
TEMED	Sigma-Aldrich Chemie GmbH Steinheim
Tetracycline hydroxide	Calbiochem, San Francisco, USA
Tween-20	Merck, Darmstadt
Whatman paper (3mm)	Whatman International, Great Britain
Trypsin/EDTA-solution	Seromed, Berlin
zVAD-fmk	Bachem, Heidelberg
β-Mercaptoethanol	Sigma, Steinheim
γ-^{32}P-ATP	Amersham, Braunschweig

Recombinant human TNF with a specific activity of 2 x10^7 U/mg was a friendly gift from I.-M. von Broen, Knoll AG, Ludwigshafen.

All other reagents were from Sigma, Deisenhofen, or Carl Roth GmbH & Co., Karlsruhe (2-propanol, acetate, acetic acid, aceton, acrylamide (RotiphoreseR Gel 30), agar, agarose, ammoniumpersulfate (APS), bovine serum albumine (BSA), calcium chloride, dimethylsulfoxide (DMSO), di-sodium-hydrogenphosphate (Na_2HPO_4), dithiothreitol (DTT), EGTA, ethanol, ethidiumbromide, ethylene-diaminetetraacetic acid (EDTA), glycerine, glycin, HEPES, hydro-chloride (HCl), potassium-chloride, magnesium-dichloride ($MgCl_2$), methanol, potassium acetate, sodium acetate, sodium azide (NaN_3), sodium chloride (NaCl), sodium-di-hydrogenphosphate (NaH_2PO_4), sodium docecyl sulfate (SDS), sodium hydroxide (NaOH), sodium phosphate (Na_3PO_4), Tris).

2.1.2 Enzymes

T4 DNA Ligase	MBI Fermentas GmbH, St. Leon-Rot
T4 DNA Ligase-buffer	MBI Fermentas GmbH, St. Leon-Rot

T4 Polynuccleotide kinase	New England Biolabs, Frankfurt
T4 Polynucleotide kinase buffer	New England Biolabs, Frankfurt
Calf intestinal alkaline phosphatase (CIAP)	Fermentas, St.Leon-Rot

Restriction enzymes and corresponding buffers were from New England Biolabs, Frankfurt

2.1.3 Culture media

RPMI/FCS (cell culture)	95% (v/v) RPMI 1640, 5% (v/v) FCS
Trypsin/EDTA (in PBS)	0.5 mg/ml Trypsin, 0.2 mg/ml EDTA
FCS/DMSO	90% (v/v) FCS, 10% (v/v) DMSO
LB-Culture medium (+Amp)	0.5% (w/v) yeast extract, 1% (w/v) Bacto-Trypton, 1% (w/v) NaCl, (100 µg/ml Ampicillin)
LB-Amp-Platten	0.5% (w/v) yeast extract, 1% (w/v), Bacto-Trypton, 1% (w/v), NaCl, 1.2% (w/v) Agarose, 100 µg/ml Ampicillin

2.1.4 Buffer and solutions

PBS	20 mM $Na_2HPO_4 \times 2\ H_2O$, 20 mM $NaH_2PO_4 \times H_2O$, pH 7.2
PBS-T	0.05% (v/v) Tween-20 in PBS, pH 7.2
PBS-T/B/A	0.05% (v/v) Tween-20, 0.025% (v/v) BSA, 0.02% (v/v) NaN_3 in PBS, pH 7.2
PBA	0.025% (v/v) BSA, 0.02% (v/v) NaN_3 in PBS, pH 7.2
4 × 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) Bromphenolblue, 20% (v/v) β-Mercaptoethanol (add before use)
SDS-PAGE electrophoresis buffer	50 mM Tris-HCl, pH 8.3, 380 mM Glycine, 4 mM SDS
4 x separating gel buffer	375 mM Tris-HCl, pH 8.8, 3.75 mM SDS

4 x stacking gel buffer	125 mM Tris-HCl, pH 6.8, 0.75 mM SDS
Blotting buffer	192 mM Glycin, 25 mM Tris, 20% (v/v) Methanol
5 × EMSA binding buffer	500 mM KCl, 50 mM, pH 7.4 Tris-HCl, 25 mM MgCl, 5 mM DTT, 50% (v/v) Glycerine
EMSA buffer A (hypotonic)	10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT (add before use), 1 mM PMSF (add before use)
EMSA buffer C	20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (add before use), 1 mM PMSF (add before use)
EMSA electrophoresis buffer	25% (v/v) TBE
10 x TBE	1.3 M Tris-HCl, 0.4 M Boric acid, 1.3 M EDTA
Crystal violet buffer	0.5% (w/v) Crystal violet, 20% (v/v) Methanol
6 × DNA sample buffer	0.25% (w/v) Bromphenolblue, 0.25% (w/v) Xylencyanol, 30% (v/v) Glycerine
TAE-buffer (agarose electrophoresis)	40 mM Tris, pH 8.3, 0.11% (v/v) Acetate, 50 mM EDTA
TE-buffer (DNA-storage)	10 mM Tris, pH 7.5, 1 mM EDTA
Resuspension-buffer (DNA mini-preparation)	500 mM Tris, pH 8.0, 500 mM EDTA, 100 µg/ml RNase
Lysis-buffer (DNA mini-preparation)	10 M NaOH, 10% (w/v) SDS
Equilibration buffer (DNA mini-preparation)	2.8 M potassium-acetate, pH 5.1
8 × Phosphate buffer, pH 7.4 (purification his-tagged proteins)	80 mM Na_2HPO_4 × 2 H_2O, 80 mM NaH_2PO_4 × H_2O, 4 M NaCl
Starting-buffer (purification his-tagged proteins)	12.5% (v/v) 8 × phosphate buffer, 10 mM imidazole, 500 mM NaCl
Washing-buffer (purification his-tagged proteins)	12.5% (v/v) 8 × phosphate buffer, 30 mM imidazole, 500 mM NaCl
Elution-buffer (purification his-tagged proteins)	12.5% (v/v) 8 × phosphate buffer, 500 mM imidazole, 500 mM NaCl

Binding-buffer (antibody purification)	3.3 M NaCl, 50 mM Tris, pH 8.9
Washing buffer 1 (antibody purification)	3.3 M NaCl, 5 mM Tris, pH 8.9
Washing buffer 2 (antibody purification)	150 mM NaCl, 10 mM NaPOi, pH 7. 2 (titration of 1 M NaH_2PO_4 against 1 M Na_2HPO_4 until pH 7.2)
Elution-buffer (antibody purification)	100 mM NaCitrate, pH 4.5
Regeneration-buffer (antibody purification)	100 mM NaCitrate, pH 3

2.1.5 Antibodies

Mouse anti-huTNFR2 monoclonal antibody MR2-1 (flow cytometry)	W. Buurmann, University of Limburg
Mouse anti-hu TNFR2 monoclonal antibody 80M2	IZI, University of Stuttgart
Mouse anti-hu TNFR2 monoclonal antibody 80M2 Alexa-546 conjugated (confocal microscopy)	IZI, University of Stuttgart
Mouse ant-human HLA class I monoclonal antibody (confocal microscopy)	Sigma Aldrich, Steinheim
Mouse anti-hu HLA class I monoclonal antibody FITC conjugated (confocal microscopy)	Sigma Aldrich, Steinheim
Rabbit anti-hu IκBα polyclonal antibody (Western blot)	Becton Dickinson Heidelberg
Mouse anti-hu vinkulin monoclonal antibody (Western blot)	Sigma Aldrich, Steinheim
Mouse anti-hu RelA/p65	Santa Cruz
Mouse anti-hu p50	Santa Cruz
Mouse anti-hu c-Rel	Santa Cruz
Goat anti-mouse-IgG, FITC-conjugated (flow cytometry)	Dianova
Goat anti-mouse-IgG HRP-conjugated (Western blot)	Dianova
Donkey anti-rabbit-IgG HPR-conjugated (Western blot)	Amersham

2.1.6 Vector constructs and expression vectors

pEGFP-N1, an eukaryotic expression vector, was from Clontech, Heidelberg.
pBSTNFR2 + Kpn1 was generated by Sylvia Willi, University of Stuttgart.
TNFR2-pEGFP, TRAF2-pEGFP, FADD-pEGFP, FasL-pEYFP were a friendly gift from Harald Wajant, University of Würzburg.
Endosomal marker-pECFP, Golgi apparatus-pECFP, ER-pECFP, Nucleus-pECFP was from Clontech, Heidelberg.
Caveolin-1-pECFP was a friendly gift from Lukas Pelkmans, ETH Zürich

2.1.7 Cell lines

HeLa, an epithelial cell line from cervix carcinoma, was purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA.
HeLa80, a TNFR2 over-expressing HeLa-derived cell line, was established by T. Weiß at the Institute of Cell Biology and Immunology, University of Stuttgart.
HeLadynK44A, a cell line which expresses a dominant negative form of dynamin-1 controlled by a tet on/off promoter, was a friendly gift from Dr. C. Roepstorff, Strucutral Cell Biology Unit, Panum Institute, University of Copenhagen, Danmark. This cell line, when cultured in RPMI/FCS 5% with 1 µg/ml tetracycline, expressed normal dynamin. Upon tetracycline deprivation, the cells switched to the expression of dominant negative dynamin which was fully expressed after 24 hours.
The human rhabdomyosarcoma cell line Kym-1 was a friendly gift of M. Sekiguchi (University of Tokyo, Japan).
Double knockout mouse fibroblasts deficient for TNFR1 and TNFR2 were a friendly gift from Daniela Männel, University of Regensburg.
Based on TNFR1/TNFR2 double knockout mouse fibroblasts, Fabian Tübing, University of Stuttgart, generated mouse fibroblasts expressing a TNFR1/Fas or a TNFR2/Fas receptor chimera. TNFR1/Fas or TNFR2/Fas consist of the extra-cellular and membrane spanning domain of TNFR1 or -2, respectively, fused to the cytosolic domain (including death domain) of Fas.

2.2 Methods

2.2.1 Cultivation of eukaryotic cells

All indicated cell lines were cultivated in RPMI 1640 culture medium with 5% heat-inactivated fetal calf serum at 37°C and 96% humidity with an addition of 5% CO_2 to the atmosphere.
Cell lines transfected with a plasmid pEFTNFR2 containing a kpn1 digestion site (pEFTNFR2 + kpn1) were selected with an addition of 2 µg/ml puromycin to the culture.
Adherent cell lines were harvested by incubation with trypsin (0.025%) and EDTA (10 mM). After sedimentation at 453 x g in an Eppendorf centrifuge 5810 R for 5 minutes at room temperature, the supernatant was discarded and cells were resuspended in culture medium or buffer.
The vitality and number of cells was determined with a solution of 0.4% eosin, 0.9% NaCl, 0.05% NaN_3 using a hematocytometer.

2.2.2 Transfection of eukaryotic cell lines

The respective HeLa and mouse fibroblast cell lines were harvested and resuspended in culture medium at a density of 1.25 x 10^6 cells/ml. 800 µl cell suspension were mixed with 4 to 10 µg of the desired plasmid in a sterile 4 mm electroporation cuvette (Peqlab). Electroporation was performed in an Easyject Plus 70–1010 electroporator (Equibio, Peqlab Biotechnologie GmbH, Erlangen) with a single pulse of 250 V at 1800 µF and infinite resistance. Jurkat cell lines were electroporated at a density of 4 x 10^6 cells/ml with 10 µg DNA. Instantly after electroporation, cells were seeded in 2 ml pre-warmed culture medium into a 3.5 cm glass bottom dish and cultured over night at 37°C and 5% CO_2.

2.2.3 Generation of stable cell lines: JurkatwtTNFR2, JurkatRIP-/-TNFR2, JurkatNemo-/-TNFR2

10 x 10^6 cells/ml culture medium were transfected with 30 µg of a pEFTNFR2 + kpn1 plasmid preparation with a single pulse of 250 V at 1800 µF. Immediately after

electroporation, the cells were seeded in 10 ml pre-warmed culture medium and left over night at 37°C and 5% CO_2. After 24 h, the culture medium was changed and puromycin at a final concentration of 2 µg/ml was added. Cells integrated the vector into their genome and were positively selected by addition of 1-5 µg/ml puromycin. After one to two weeks, cells were sorted using FACS (flow activated cell sorting, FACstar, Beckton Dickinson) and continually kept in RPMI 5% with 2 µg/ml puromycin. To achieve stably expressing cells, the sorting steps were performed three times.

2.2.4 Cytotoxicity assay

According to cell-size and proliferation-time, 10^6 (mouse fibroblasts), 1.2 x 10^6 (Kym 1) or 1.5 x 10^6 (HeLa) cells/well were seeded into a 96-well-plate and grown over night at 37°C and 5% CO_2. Substances that had to be tested were added at fixed concentrations or in serial dilutions. After approximately 18 h of incubation, the supernatant was discarded and cells were washed 3 times with PBS. Viable cells were stained with Crystal Violet for 15 min at room temperature to differentiate between living and dead cells (0.5% Crystal Violet, 20% Methanol). The staining solution was carefully washed away beneath running tap water and the plates were air-dried. The Crystal Violet was dissolved in 100 µl of Methanol and absorption was measured in a Spectra Max 340PC Elisa Reader (Molecular Devices).

2.2.5 FACS-analysis and cellsorting

Fluorescence activated cell sorting (FACS) was used to analyse the cell surface expression of proteins (receptors). The amount of receptors was measured in an Epics XL-MCL (Coulter Immunotech). Cellsorting was performed in a FACStar (Beckton Dickinson). For both methods, 10^6 or more cells were harvested, washed in ice-cold sterile PBA (phosphate buffered saline with 0.5% BSA) and incubated for 1 h with 5 µg/ml primary antibody in PBA at 4°C. After washing once with PBA, cells were stained with 1:200 diluted, fluorescence-labelled secondary antibody for 30 min at 4°C and washed again with PBA. Cells were resuspended in 300 µl or 4 ml PBA for protein-analysis or cellsorting, respectively.

10,000 to 30,000 cells, which were sorted for the creation of stable cell lines, were collected in culture medium and seeded in a small volume of RPMI with 5% FCS and an antibiotic for selection in a 24-well- or 6-well plate.

2.2.6 SDS-PAGE and non-reducing-PAGE

Proteins were separated according to their molecular weight in a discontinuing SDS-polyacrylamide gel electorphoresis (PAGE) system using a vertical gel electrophoresis chamber from Phase, Luebeck. Running gels containing 10% or 12.5% of polyacrylamide, and stacking gels containing 4.5% of polyacrylamide were used. Samples were mixed with Laemmli buffer and heated for 5 min at 95°C. Samples for non-reducing PAGEs were heated in Laemmli sample buffer without SDS prior to applying onto the gel. Gels were run at 40 mA for approximately 1 h 15 min. Samples with a protein contents of 50 µg -100 µg of protein were loaded. The standard used was composed of

MRP-β-galactosidase	175 kDa
MRP-paramyosin	83 kDa
Glutamic dehydrogenase	62 kDa
Aldolase	48 kDa
Triosephosphate isomerase	33 kDa
β-Lactoglobulin A	25 kDa
Lysozyme	17 kDa
Aprotinin	7 kDa

2.2.7 Immunoblotting/Western blot analysis

Protein transfer from SDS- and non-reducing PAGEs onto nitrocellulose membranes (Pall life sciences, USA) was performed in a horizontal semi-dry blotting chamber (Phase, Luebeck) with 1,5 mA/cm^2 gel for 1 h. A buffer containing 3% fat-reduced milkpowder in PBS/0.05% Tween was used to block non-specific binding sites. The membrane was washed several times with PBS/T and incubated with 1 µg/ml primary antibody in PBS/T/B/A over night at 4°C. After several washing steps with PBS/T, the membrane was incubated with a secondary horseradish peroxidase-conjugated antibody

diluted 1:20,000 in PBS-T, for 1 h at room temperature. Having washed the membrane several times with PBS-T, the blot was developed with the Super Signal Kit® (Pierce, USA).

2.2.8 Electro Mobility Shift Assay (EMSA), detection of transcription factor NFκB-translocation into the nucleus

According to cell type, cells were seeded at a density of 10^6 (HeLa) or 10^6/ml (Jurkat) in 6-cm-petri-dishes and incubated over night at 37°C and 5% CO_2. After stimulation at different time points or with different concentrations of TNF, cells were harvested in ice-cold PBS and pelleted for 5 min at 500 x g (Eppendorf) and 4°C. The pellets were washed once with PBS, resuspended in 100 µl ice cold buffer A (buffer for cytoplasmic lysates) and incubated for 15 min on ice. 6.3 µl of detergent (10% NP-40, Sigma, Deisenhofen) were added and the samples were incubated at 4°C for 2 min in a Thermomixer compact (Eppendorf) to set the nuclei free. The nuclei were centrifuged at 13,000 rpm (4°C, 1 min), the supernatants discarded and the pellets were resuspended in 25 µl of ice-cold buffer C (generation of nuclear lysates). After shaking for 20 min at 4°C and centrifugation (5 min, 13,000 rpm, 4°C), the supernatants were separated from the pellets and the protein concentration of the nuclear extracts was determined with a Bradford protein assay.

NFκB specific Oligonucleotides (Oligonucleotide 1: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', Oligonucleotide 2: 5'-GCC TGG GAA AGT CCC CTC AAC T-3') were radioactively labelled at 5' with [γ-^{32}P]ATP in a T4-polynucleotide kinase reaction. 10 pmol of oligonucleotide 1 and 2 each were mixed with 40 Units T4-polynucleotide kinase, 10 x T4-polynucleotide kinase buffer, 50 µCi [γ-^{32}P]ATP (10 mCi/ml), deionised water and incubated for 1 h at 37°C. The reaction was stopped by incubation for 10 min at 95°C and the oligonucleotides were allowed to cool slowly at room temperature. Unbound [γ-^{32}P]ATP was removed by purification of the oligo-nucleotides with ProbeQuant G50-Microcolumns according to the accompanying protocol. The radioactive labelling was measured in a β-scintillation counter (Canberra-Packard, Dreieich) with 1 µl of oligo-nucleotide.

10 µg protein from the nuclear extracts were prepared each for analysis with 2 µl 2 mg/ml poly[dI:dC] (blocking of unspecific binding sites), 5 x binding buffer and

deionised water in a final volume of 20 µl. For supershift experiments, 10 µg protein from each sample were incubated for 20 min at room temperature with 1 µg/ml anti-p65, anti-p50, anti-cRel, antibodies and deionised water prior to adding poly[dI:dC], 5 x binding buffer.

After incubation for 20 min at room temperature, 1 µl radioactively labelled oligo-nucleotide (approximately 200,000 cpm) was added and the samples were incubated for 10 min at room temperature. The specific binding of the doubly stranded oligo-nucleotide to NFκB was controlled by either adding a surplus of 100 x cold oligo-nucleotide to a positive control or a superhift was performed. Samples were mixed with 5 µl 6 x DNA sample buffer and charged onto a 1.5 mm 6% native polyacrylamide gel (6% acrylamide/polyacrylamide, 0.25 x TBE, 0.075% APS, TEMED). The gel was run at 25 mA for 1 h 20 min, dried in a BioRad Gel dryer (Model 583) and read with a Storm 860 Phosphoreader (Molecular Probes). Analysis was performed with ImageQuant v5.0.

2.2.9 Labelling of TNF-beads

wtCysHisTNF or its derivatives were pre-treated with 10 mM DTT over night at 4°C to reduce CysHisTNF-multimers. DTT was removed using Dialysis-membranes with a cut-off of 10 kDa against three changes of PBS during 4 hours, 4°C. Prior to use, the TNFs were sterilised by filtration through a syringe filter (cut-off 0.25 µm).

Silica-beads were activated with sulfoSMCC prior to labelling with CysHisTNFs. Upon delivery in a NaPOi-buffer (similar to PBS), the beads were sonified shortly to dissociate beads-clusters from the suspension. 50 µg or 25 µg CysHisTNF were added to 1 ml semi-sterile suspension of 1 mg beads of a diameter of 10 µm, 1 µm or 100 nm, respectively. During the incubation for 1 h at room temperature, the 2-ml-Eppendorf tubes containing the beads/TNF suspension were constantly rolled. Control beads were prepared adding β-mercaptoethanol at a final concentration of 200 mM to a 1 mg beads/ml phosphate buffer suspension in order to saturate free coupling groups. Labelled beads were centrifuged in a pre-cooled Eppendorf centrifuge 5403 at 14,000 rpm for 10 min at 4°C. The supernatant with surplus CysHisTNF was discarded and the beads were washed twice with sterile RPMI/5% FCS, the pellets being re-suspended by sonification. The final beads-preparation was re-suspended in 200 ml culture medium and stored at 4°C, protected from light. Bioactivity was measured in a cytotoxicity assay as described in chapter 2.2.4.

2.2.10 Indirect determination of the amount of TNF coupled to silica-beads

TNF and beads, both prepared as described in chapter 2.2.10, were incubated on a roller for 1 h at room temperature. Since TNF has a high affinity to adhere to glass and plastic surfaces, the same amount of TNF used for coupling was added to 1 ml NaPOi-buffer without beads and treated like the samples to determine loss of TNF. TNF treated for coating beads, supernatants after incubation with or without beads and washing step supernatants were collected in RPMI/FCS 5% to prevent further adhering of TNF to Eppendorf tube walls. The content of TNF in the collected solutions was measured in a sandwich ELISA from Beckton Dickinson, Heidelberg.

Calculations:

The initial amount of prepared TNF used for labelling (concentration 1) was measured to control TNF loss caused by dialysis and sterile filtration. The concentrations determined were compared with the initially known stock solution concentrations and corrected, if necessary.

TNF contents in the supernatants after incubation with beads (concentration 2), without beads (concentration 3) and after 2 washing steps (concentrations 4 and 5) were determined. Concentrations 2, 4 and 5 were subtracted from concentration 3. The result was assumed to represent the amount of TNF being coupled to 1 mg beads.

Quantities of coupled TNF were expressed as percentage of the corrected values of initially used TNF.

2.2.11 Protein labelling with Alexa-Fluor-staining

An Alexa Fluor staining kit from Molecular Probes (Oregon, USA) was used. Proteins were prepared and treated according to protocol.

2.2.12 Immuno-labelled fixed specimens for confocal laser scanning microscopy

12 mm cover slips were sterilised for 2 min at the highest energy level in a microwave. 10^5 MfR2Fas cells were seeded immediately after electroporation onto the sterile cover

slips and cultivated over night at 37°C and 5% CO_2 in culture medium. TNFR2 was pre-stained for 30 min with at least 2 μg/ml 80M2 labelled with Alexa-Fluor-546 at 37°C and 5% CO_2 or left unstained. The pre-stained cells were stimulated with 100 ng/ml sTNF, unstained cells with 1 μg/ml sTNF labelled with Alexa-Fluor-546 for 30 min at 37°C. After aspiring the culture medium carefully, the cells were washed once with ice cold PBS to stop the reaction and fixed with 3.5% para-formaldehyde for 15 min at room temperature. Prior to mounting the cover slips upside down in fluoromount-G™, they were washed 6 x with PBS and dried by tapping their edges slightly on an absorbent towel. The specimens were stored at 4°C protected from light until further examination.

2.2.13 Life imaging at the confocal laser scanning microscope

3 x 10^5 cells (MfR1Fas, MfR2Fas, HeLa) or 2 x 10^6 cells (Jurkat) were seeded immediately after electroporation into 3.5 cm coated glass bottom dishes (P35G-0-14-C, Mattek Corporation, USA) and cultured over night at 37°C and 5% CO_2 in culture medium. The cells were pre-stained with fluorophore labelled antibodies and/or stimulated with different TNF-preparations. Images of one frame were taken with a TCS SL confocal laser scanning microscope from Leica. During analysis, the cells were kept at 37°C and 5% CO_2 in a microscope adapted mini-chamber. The fluorophores were excited according to their excitation wavelength with an Ar/Kr laser, a green Kr laser or a Ne/He laser. GFP- and FITC-fluorophores were excited at 488 nm; CFP and YFP at 458 nm and 514 nm, respectively; red fluorescing compounds like Alexa-Fluor-546 labelled proteins, lysotracker and beads were excited at 534 nm and α-mouse-633 was excited at 633 nm. Analysis of the obtained data and imaging was performed with the Leica confocal software and the Adobe photoshop v8.0.

2.2.14 Spatially restricted stimulation of TNFR2

HeLa cells transiently ove-expressing a TNFR2-GFP fusion protein were prepared according to chapter 2.2.13. 20 µg wild type CysHisTNF-coated silica beads of 10 µm diameter and stained with Alexa-Fluor-568 were added to the dish. Using an Eppendorff microinjection device with a microinjection needle as a manipulation device, the beads were pushed or pulled toward a TNFR2-GFP expressing cell showing fluorescence of culture medium intensity. Images of the stimulated cell were taken every 11 seconds

over a period of five minutes. Fluorescence intensity of the GPF-fusion protein TNFR2 was measured with Leica software directly at the contact site between cell and bead, to the left and right of the bead, in the cytosol and in the Golgi apparatus.

2.2.15 Generation of pEGFP-TNFR2exTM

TNFR2 without its cytosolic domain (=TNFR2exTM) was excised from a pBSTNFR2-plasmid which contains a digestion site for the enzyme kpn1 eleven amino acids from the TNFR2 transmembrane domain. 5 U/µg DNA of restriction enzymes Acc65I, a Kpn1 isoschizomer, and Xho1 were incubated with a corresponding buffer and DNA for 2 h at 37°C in a final volume of 40 µl. pEGFP was de-phosphorylated after 1 hour incubation with 1 U CIAP (calf intestine alkaline phosphatase) to prevent re-ligation of the vector. DNA fragments were separated in a 2% agarose-gel electrophoresis and eluted with a NucleoSpin extraction kit.

DNA-fragment TNFR2exTM was ligated into the pEGFP-vector with 400 units of T4 DNA ligase, T4 DNA ligase buffer and a 1:4 vector/insert mix in a final volume of 10 µl at 16°C over night. Successful cloning was investigated by restriction analysis with DraIII, cutting in the insert and in the vector. Correct expression and functionality of the construct in cells were controlled by flow cytometry and in confocal live imaging with anti-TNFR2-antibodies.

2.2.16 Antibody purification

Protein-A Sepharose (Pharmacia, volume 10 ml) was warmed up to room temperature prior to use. Culture supernatant of an 80M2-hybridoma cell line was prepared for purification with solid NaCl to obtain a final concentration of 3.3 M and Tris (final concentration 50 mM) and adjusted to pH 8.9. To remove non-dissolved salt debris, the solution was filtered through a 0.45 µm filter. After equilibration with 5 – 10 volumes of binding buffer, the supernatant was loaded onto the column with a flow rate of 0,4 ml/min. The resin was washed until the phenolred staining from the culture medium disappeared, then 3 – 5 volumes of wash buffer 1 and 5 volumes of wash buffer 2 were applied. The antibody was eluted at a flow rate of 1 ml/min with a 0.1M NaCitrate buffer pH 4.5. Six 5 ml fractions were collected by hand and neutralised each with 1 ml 1M Tris pH 8.0. Generally, fractions 3 and 4 contained the bulk of eluted antibody. Possibly

remaining protein was further eluted with 5 column volumes of 0.1 M NaCitrate pH 3.0. Prior to pooling the fractions, the protein content of each was determined at 280 nm in an Eppendorf BioPhotometer. The eluate was dialysed over night against PBS in dialysis bags with a cut off of 10 kDa. If necessary, the antibody solution was concentrated using Centricon-tubes (Centriprep30, Amicon) with a cut-off of 30 kDa, which were centrifuged at 4°C and 4000 rpm for 30 – 60 min. After filtration through a 0.2 µm sterile filter, the protein concentration was determined with a BCA protein detection kit from Pierce, Illinois, USA. Bioactivity was determined in a cytotoxicity assay in mouse fibroblasts-TNFR2/Fas by cistimulation with 1 ng/ml human sTNF. Purity of the antibody and confirmation of the measured protein content was controlled in a 7.5% Coomassie-SDS-PAGE, with human IgG with a known concentration as reference.

2.2.17 CysHisTNF-preparation

100 ml XL1-blue Ca^{2+} competent E. coli suspension were transformed with 1 mg of plasmid-preparation (pQE0-32WCysHisTNF, pQE9-143NCysHisTNF or pQE9-wtCysHisTNF, respectively) for 5 min on ice, seeded onto LB_{Amp} agar plates and grown over night at 37°C. A 100 ml pre-culture was prepared in LB_{Amp} culture medium and grown over night. 1 l LBAmp was mixed with 100 ml pre-culture, grown for 90 min at 37°C and induced with 1mM IPTG for 6 h at 30°C. The bacteria culture was centrifuged with 5000 rpm for 10 min at 4°C in a Beckmann GPKR centrifuge and washed with 3 ml PBS/g pellet. The pellet was resuspended in 3 – 6 ml PBS/g pellet and sonified on ice with 4 x 10 impulses. After centrifugation with 12,000 x g at 4°C for 10 min (Rotor JA20, Eppendorf), supernatant 1 was filtered with a 0.2 µm sterile filter to remove cell debris and the protein content was determined at 280 nm. The pellet was, after sonification, rolled in PBS/2% Triton X-100 for 15 min at 4°C. Supernatant 2 was checked for inclusion bodies in a 15% SDS-PAGE.

A 1 ml Hi-Trap column from Pharmacia was washed with 5 volumes of deionised H_2O and loaded with 1 ml 0.1 M $NiSO_4$. After equilibration with 10 volumes of start-buffer, sn 1 was loaded at a flow rate of 1 ml/min. The column was washed with 10 ml wash buffer and the CysHisTNF eluted in 8 fractions with 8 ml elution buffer. Protein containing fractions were detected at 280 nm and pooled. The TNF-solution was dialysed over night against PBS, filtered sterile (0.2 µm filter) and the protein concentration was determined with a BCA detection kit. The bioactivity and receptor

specificity was controlled in cytotoxicity assays with Kym1, MfR1Fas and MfR2Fas with sTNF and sTNF + 80M2 as positive controls. Protein content and the purity of the TNF-preparation was controlled with 15% Coomassie-SDS-PAGE, with HSA free human TNF as reference.

3 Results

3.1 Generation of a tool for optimised stimulation of TNFR2

TNFR2 binds both forms of its ligand, sTNF and memTNF, however, complexes formed with sTNF disintegrate rapidly leading to a very weak activation of the receptor. In contrast, complexes of TNFR2-memTNF are believed to be very stable and they induce a strong response. Investigation of the TNFR2 mediated signal transduction is obstructed by a lack of adequate tools to mimic a membrane–like form of TNF. TNF preparations used as memTNF-equivalents typically consist of artificially crosslinked sTNF derivatives. Krippner-Heidenreich et al., for example, fused the soluble form of TNF with a cysteine/histidine tag. Cysteines form disulphide bonds and create covalent dimers that associate to higher order TNF-oligomers, most likely hexamers. These TNF-constructs possess only a limited memTNF-like signal capability (Krippner-Heidenreich *et al.*, 2002). Another strategy to mimic bioactive memTNF is the pre-incubation of cells with 80M2, a monoclonal TNFR2-specific antibody which cross-links receptor molecules and stabilises the binding of TNF to TNFR2 (Grell *et al.*, 1995; Krippner-Heidenreich *et al.*, 2002). This process delays the dissociation of sTNF from the receptor and allows its full activation. In this work, next to the described methods, TNF was immobilised on silica micro-particles, to effectively mimic more naturally the membrane bound form of this ligand.

3.1.1 Optimised coupling of TNF on silica-beads

In a first set of experiments, a standard protocol was established to optimise the bioactivity of beads coupled with TNF. Bioactivity of TNF-beads was tested on cells that were unresposive to sTNF but sensitive for memTNF. In addition, apoptosis-inducing qualities of TNF coupled beads were investigated along with storage abilities. Furthermore, an indirect approach was performed to determine the amount of TNF being coupled to 1 mg particles.

3.1.1.1 TNF-preparation and receptor selectivity of coated beads

Silica beads activated with sulfoSMCC expose maleimide groups on their surface. For covalent binding on the particles, a wild type TNF containing a His tag for purification purposes and a cysteine residue on its N-terminus was used. Cysteine is capable to

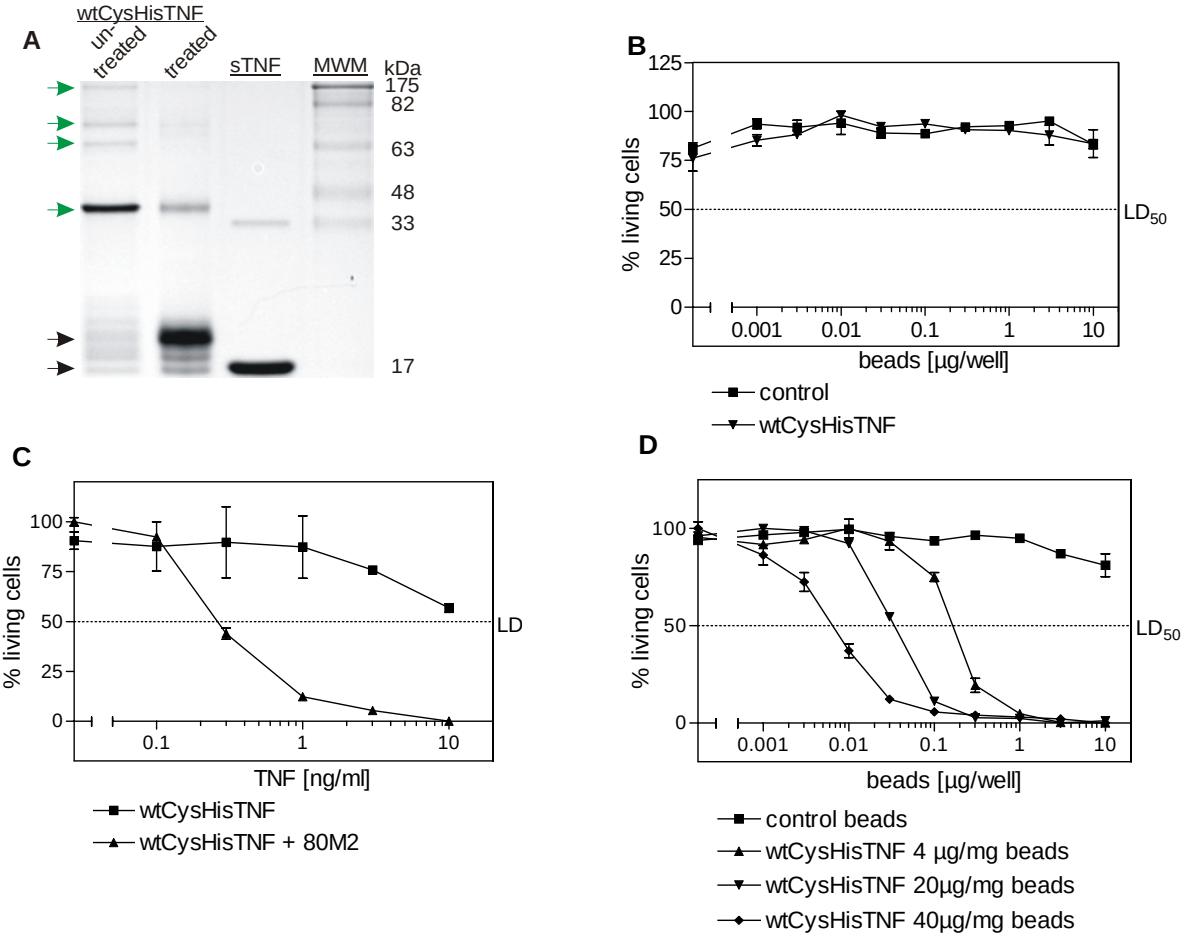


Figure 3: **Soluble wtCysHisTNF and TNF-labelled beads are TNF-receptor specific. A.** and **B.** wtCysHisTNF was incubated over night with 10 mM DTT and dialysed for 3 h. **A.** 5 µg protein per lane were analysed in a 15% non-reducing SDS-PAGE. lane 1: wtCysHisTNF without DTT-treatment, lane 2: wtCysHisTNF treated with DTT and dialysed, lane 3: sTNF, lane 4: molecular weight marker. Green arrows indicate dimeric wtCysHisTNF or higher order wtCysHisTNF-complexes. Black arrows point towards monomeric wtCysHisTNF or sTNF-monomers. **B.** DTT-treated wtCysHisTNF was coupled onto 10 µm-beads. TNF-coated particles and uncoated beads as a negative control were used to stimulate TNFR1/TNFR2 double knockout mouse fibroblasts. Cell viability was determined in a standard cytotoxicity assay. **C.** TNFR2/Fas mouse fibroblasts were stimulated with soluble wtCysHisTNF alone or after pre-incubation with 80M2 (1µg/ml). **D.** Decreasing amounts of wtCysHisTNF were used for coupling onto particles. Bioactivity of the various preparations was determined in a cytotoxicity assay on Kym1 cells. Cytotoxic effects in **B, C** and **D** were quantified with crystal violet staining. Results are expressed as the average of triple values ± SD.

form a C-S-thioether with the maleimide group of sulfoSMCC. Wild type CysHisTNF (wtCysHisTNF), treated with DTT over night to reduce disulphide bonds, was dialysed against PBS to remove DTT, which would otherwise interfere with the coupling reaction. Monomeric sTNF has a molecular weight of 17 kDa. In contrast, wtCysHisTNF-monomers are detected at a molecular weight of 19 kDa due to the addition of one cysteine and six histidines on its N-terminus. After the treatment of wtCysHisTNF with DTT to reduce disulphide bonds formed between the cysteines of two wtCysHisTNF-molecules (38 kDa), monomeric wtCysHisTNF was accordingly detected in a 15% non-reducing SDS-PAGE with a molecular weight of 19 kDa (fig. 3a). Activated particles were labelled with wtCysHisTNF according to the protocol (A. Hammer, unpublished data) and their specificity for TNF-receptors was tested. The beads showed no cytotoxic activity on TNFR1 and TNFR2 double knockout mouse fibroblasts (fig. 3b).

To test the bioactivity and receptor specificity or TNF-coated beads, mouse fibroblasts expressing a TNFR2/Fas receptor-chimera were used (Krippner-Heidenreich *et al.*, 2002). These cells are resistant to sTNF but rapidly develop apoptosis upon memTNF treatment. Prior to stimulation with monomeric wtCysHisTNF, TNFR2/Fas mouse fibroblasts were pre-incubated with or without 80M2. Cells stimulated with this memTNF equivalent, 80M2 plus wtCysHisTNF, became apoptotic with a LD_{50} of 0.15 ng/ml, whereas cells stimulated with CysHisTNF alone died only at much higher concentrations (fig. 3c and data not shown).

Furthermore, a dose response in the bioactivity of the beads was observed when declining amounts of TNF had been taken for coupling on activated silica-beads (fig. 3d). Since receptor-selectivity was not of interest in this experiment, Kym1, a cell line very sensitive to TNF-mediated apoptosis, was used to detect differences between particles coated with diminishing amounts of TNF.

3.1.1.2 Storage conditions and binding capacity of silica-beads

Stock solutions of TNF were usually prepared either in RPMI/FCS 5% for low concentrations or in PBS for concentrations higher than 10 µg/ml. These solutions were stable for several months when stored at 4°C. 10 µm-wtCysHisTNF-coated beads were stored at a concentration of 5 µg/µl in RPMI/FCS 5% at 4°C. Five weeks after preparation and frequent use, the bioactivity of the particles was analysed in a

cytotoxicity assay using the TNF-sensitive cell line Kym 1 (fig. 4). No significant loss of TNF-bioactivity could be observed.

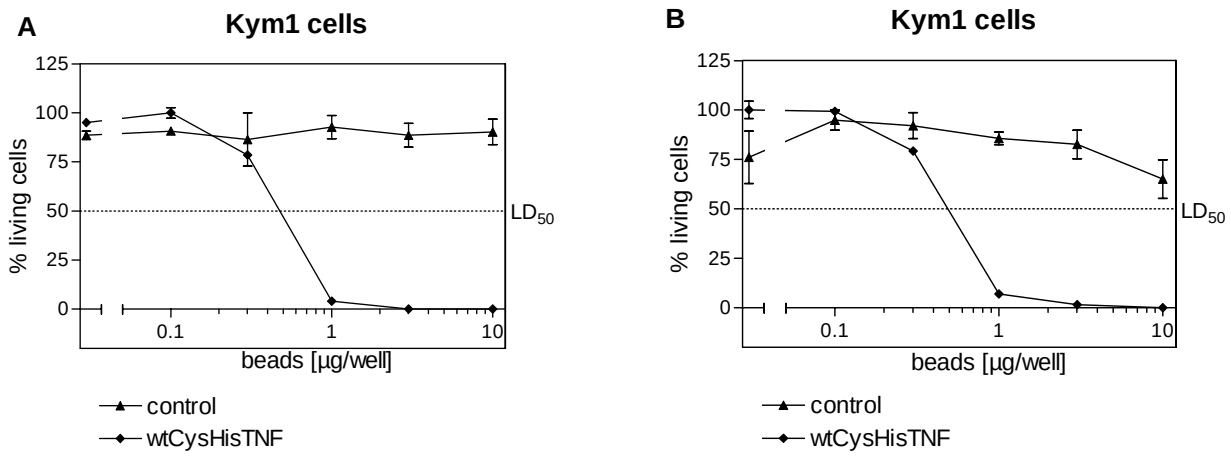


Figure 4: **The bioactivity of TNF-coated particle remains stable upon storage at 4°C.** wtCysHisTNF coupled beads were stored for five weeks at a concentration of 5µg particles/µl FCS-containing culture medium. Subsequently they were washed with RPMI/FCS 5% and, after addition of fresh culture medium, tested on TNFR2/Fas mouse fibroblasts in a standard cytotoxicity assay. **A.** Freshly prepared particles., **B.** Beads after storage for 5 weeks at 4°C in RPMI/FCS 5%. Results are expressed as the mean value of duplicates.

The amount of TNF which had been coupled onto the beads could be determined only indirectly since no direct standard was available for comparison. An initial input of 50 µg or 25 µg TNF was treated with DTT over night, dialysed and sterilised by filtration. The quantity of TNF actually used for coating was determined by an ELISA-assay to detect loss of TNF during these procedures. sTNF or wtCysHisTNF were incubated with and without particles. TNF-concentrations in the supernatants were measured and subtracted from each other (calculation see chapter 2.2.10). Data from four experiments for wtCysHisTNF coupled to 1 µm- and 10 µm-particles, three experiments for wtCysHisTNF-coated 100 nm-particles, two experiments for sTNF-beads of 10 µm and 1 µm and three experiments for sTNF-beads of 100 nm were analysed and are presented as average ± standard deviation (table 2). The amount of TNF bound to the beads was calculated in % of the input TNF-quantity. Since only minimal traces of TNFs in the supernatants of two washing steps were detected, these data were omitted (data not shown). Similar absolute quantities in a range from about 2 to 7 µg of wtCysHisTNF or of sTNF were found to bind to 1 mg beads, regardless of the size (6th row). However, during the preparation of the ligand samples for the final coupling step much more

CysHisTNF (about 70%) was lost as compared to wild type sTNF (about 20%; 4th row). Accordingly, when the amount of TNF bound to beads is calculated as the percentage of the TNF input (7th row), increasing quantities of TNF correlate with a reduced size of the beads. Percentages of sTNF bound exclusively by adsorption were found to be in a range from 11% to 33%, whereas percentages of wtCysHisTNF, attached to the beads by adsorption as well as covalent bonds, ranged from 39% to 57%.

	wtCysHisTNF (adsorption mediated and covalent binding)			sTNF (binding through adsorption)		
Beads size	10 µm	1 µm	100 nm	10 µm	1 µm	100 nm
TNF-input prior to treatment with DTT, dialysis and filtration **[µg/mg beads]**	50	25	25	50	25	25
TNF-input for coupling **[µg/mg beads]**	14.5 ± 2.7	7.2 ± 1.4	7.7 ± 1.3	41.3 ± 2.2	20.7 ± 1.1	20.7 ± 1.1
TNF bound to reaction tube walls after incubation without beads **[µg/tube]**	8.0 ± 2,6	3.8 ± 1.7	4.4 ± 1.6	27.1 ± 4.1	11.6 ± 4.3	11.6 ± 4.3
TNF bound to beads after incubation (indirect data) **[µg/mg beads]**	5,5 ± 0.6	3.2 ± 1.5	4.4 ± 1.5	0.93 7.75	3.91 2	6.7 ± 2.9
Ratio of bound TNF/TNF input	38.6%	43.1%	57.1%	11.0%	14.6%	33.0%

Table 2: **Indirect determination of the quantity of TNF coupled on beads**. An initial input of TNF was treated with 10 µM DTT overnight, dialysed against three changes of PBS and filtered sterilely (3rd row). Beads of 10 µm, 1 µm and 100 nm diameter were coated with pre-treated wtCysHisTNF or sTNF, respectively (4th row). TNF loss due to adhesion to reaction tube walls during the coupling was measured by incubation of wtCysHisTNF or sTNF with particle free buffer (5th row). TNF quantities in the supernatants of samples incubated with or without beads were determined in a sandwich ELISA. The difference between the TNF concentration measured in beads free samples and beads-containing samples was assumed to be the amount of TNF coupled to 1 mg of particles (6th row). Total amounts of TNF being coupled to beads were calculated as percentage of the input of TNF used for the coating procedure (7th row).

This method to determine the amount of TNF coupled to beads provides no direct data. Furthermore, the bioactivity of each bound molecule is unknown. TNF molecules on

beads and beads themselves might only partly interact with cells due to steric reasons. Therefore, for most experiments, comparison of particle-samples with each other and not the amount TNF coated on particles used in each experiment was performed to consider the quantity of beads as a reference point.

3.1.2 Covalent versus adsorptive labelling of silica-beads

wtCysHisTNF was found not only to form covalent bonds with the maleimide groups on micro-particles but to bind also by adsorption. This must be concluded from experiments with sTNF, which contains no free Cysteine-SH groups and is not able to form covalent S-C-thioether bonds. However, sTNF was also shown to bind to the beads surface and create bioactive beads. sTNF-coated particles were able to activate TNFR2 in TNFR2/Fas-mouse fibroblasts, less effectively, however, than wtCysHisTNF-beads (fig. 5). wtCysHisTNF-beads triggered apoptosis with an LD_{50} of 0.15 µg beads/well while the LD_{50} of sTNF-coated beads was in the range of 1 µg beads/well.

To investigate differences between covalent and non-covalent binding of TNF molecules to the particles and to discover possible positive or negative effects on their bioactivity, different methods to inhibit adsorptive binding were used.

1. Beads were pre-treated with sulfhydryl-BSA. SH-groups of free cysteines had been inactivated by sulfhydryl-reactive groups of this BSA-preparation and thus the formation of disulphide bonds was inhibited. In the case of silica particles activated with sulfoSMCC, sulfhydryl-BSA should serve to coat the adsorptive portion of the beads, leaving the maleimide groups free to form covalent bonds with free SH-groups of wtCysHisTNF. However, pre-treatment with 5% sulfhydryl-BSA prior to labelling did not significantly inhibit adsorptive binding of sTNF to the beads (data not shown).

2. PBS supplied with detergents was used to treat the beads for different lengths of time to break up non-covalent interactions between TNF and the bead surface. Again, however, no effects on sTNF adsorption could be observed (data no shown).

3. Different surface modifications were prepared by our partners to investigate the adsorptive behaviour of sTNF and wtCysHisTNF to silica-particles. APS (3-aminopropyl-trimethoxy-silan) is the basis for coating micro-particles with sulfoSMCC. To test whether APS was responsible for the adsorption of TNF to the beads, these were provided with a

PEG (poly-ethylen-glycol)-surface imposed on APS. PEG provides an inert, hydrophilic surface apart from free NH-groups necessary for the modification with sulfoSMCC. APS- or PEG-beads, either chemically activated or not activated with sulfoSMCC, were incubated with sTNF and wtCysHisTNF. But again no significant changes in their reactivity on cells could be detected in a cytotoxicity assay (data not shown).

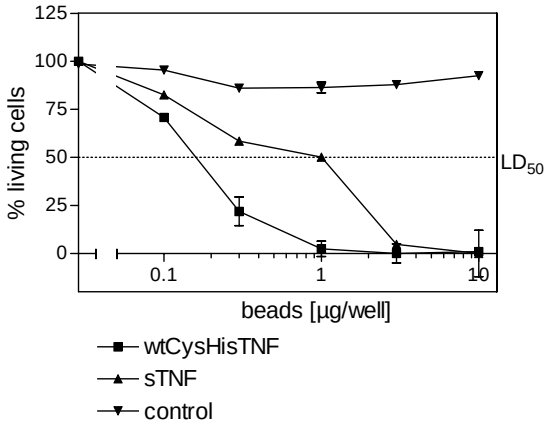


Figure 5: **wtCysHisTNF binds covalently and by adsorption, sTNF binds only by adsorption to beads.** wtCysHisTNF and sTNF were pre-treated equally with DTT over night, dialysed and incubated with beads. Labelled beads were tested on TNFR2/Fas mouse fibroblasts in a cytotoxicity assay. Cell viability was quantified using crystal violet staining and expressed as the average of triple values ± SD.

3.1.3 Receptor-selective TNF-preparations

TNFR1 is activated equally well by sTNF and memTNF. In contrast, efficient TNFR2-activation mainly relies on memTNF. To allow the selective stimulation of only one type of receptor, CysHisTNF-mutants binding selectively to TNFR1 or to TNFR2 were used. In the TNFR1-specific mutant, arginine(R)32 and serine(S)86 are exchanged for a trytophane(W) and a threonine(T), respectively. TNFR2 specific TNF has replaced the amino acids aspartate(D)143 and alaine(A)145 with asparagine(N) and arginine(R), respectively. The binding selectivity of these two CysHisTNF-mutants and their bioactivity was verified in a cytotoxicity assay using TNFR1 and TNFR2 double knockout mouse fibroblasts expressing either TNFR1/Fas or TNFR2/Fas (figs. 6a and b). As expected, the beads labelled with the CysHisTNF-mutants show a comparable receptor-selectivity in the induction of apoptosis on these cell lines (figs. 6c and d).

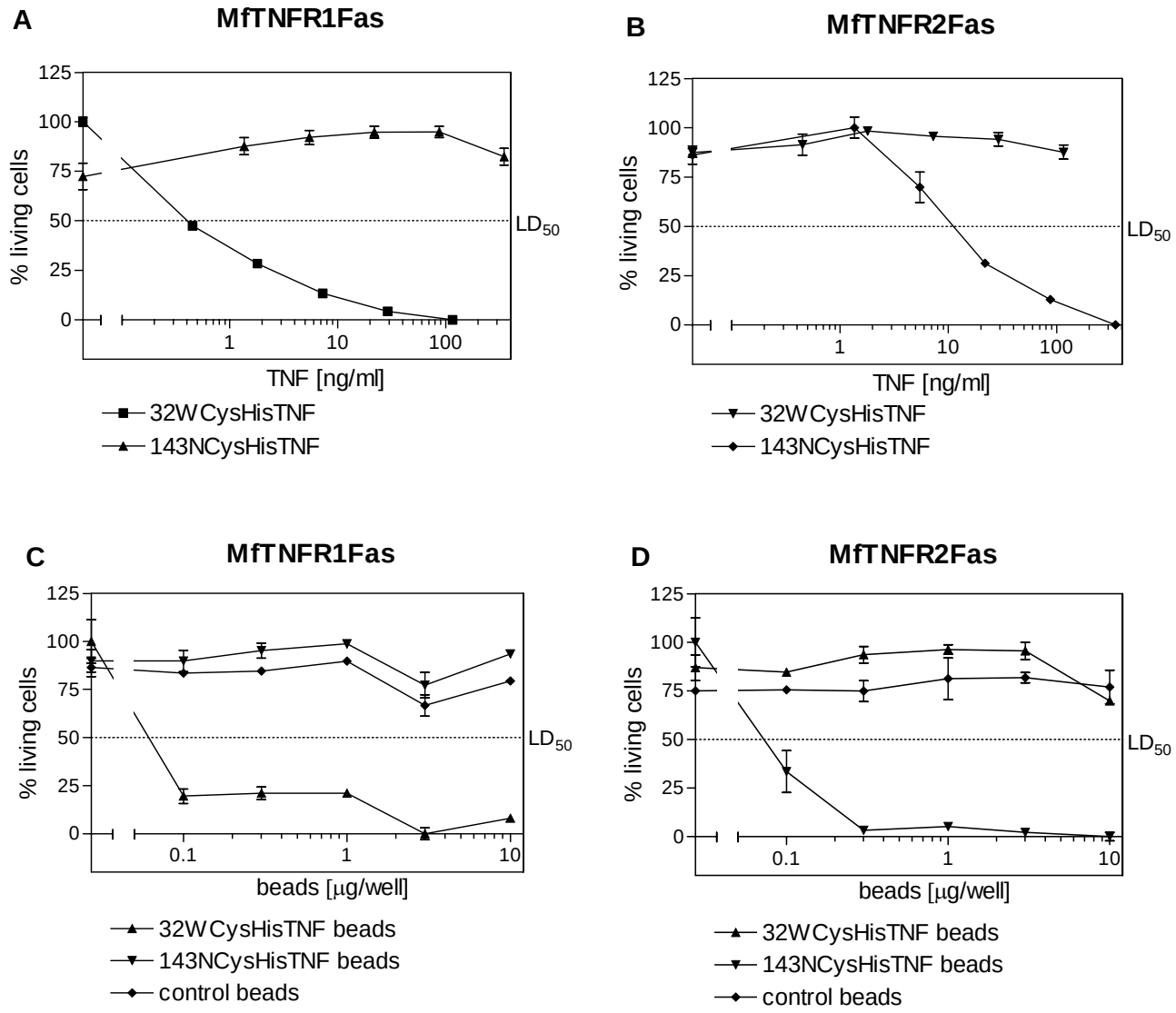


Figure 6: **Receptor specificity of TNF-mutants and beads coated with CysHisTNF-mutants. A.** and **B.** 32WCysHisTNF (TNFR1 selective) and 143NCysHisTNF (TNFR2 selective) were tested on TNFR1/Fas (**A**) and TNFR2/Fas (**B**) mouse fibroblasts. 10 000 cells/well in a 96 well plate were grown over night. The following day, cells were stimulated with TNF-mutants at 37°C over night. **C.** and **D.** 32WCysHisTNF and 143NCysHisTNF were pre-treated with DTT, dialysed and coupled onto beads. Bioactivity and receptor-specificity were determined on TNFR1/Fas (**C**) and TNFR2/Fas (**D**) mouse fibroblasts. Cell viability was quantified using crystal violet staining and expressed as average of double values.

3.1.4 Bioactivity of CysHisTNF-labelled beads

When TNF coated beads were used in a standard cytotoxicity assay, no absolute comparison with sTNF in solution could be made, as the average chance for a single bead to have an effective contact with a target cell is unknown. Therefore absolute information about the potency of the beads can be only obtained from data at the single cell level. Such experiments were performed using wtCysHisTNF-beads as an example.

3.1.4.1 10 µm-beads polymerised with Alexa-Fluor-568 as a microscopic tool

To mimic a cell expressing memTNF, but having an otherwise inert surface, wtCysHisTNF-particles of 10 µm diameter labelled with Alexa-Fluor-568-dye were used for confocal live cell imaging. TNFR2/Fas mouse fibroblasts were stimulated with 100 µg of wtCysHisTNF-beads and the specimen was searched for cells being in contact with one or more beads. In fig. 7, one TNFR2/Fas mouse fibroblast over-expressing human FADD-GFP is shown to undergo apoptosis after stimulation with three 10µm-wtCysHisTNF-beads. FADD-recruitment (see white arrow) was observed. Cell shrinking, the first physiological and optically visible sign of cell death, could already be observed after 28 min. Blebbing and fragmentation of the cell into smaller vesicles followed within additional 2 minutes.

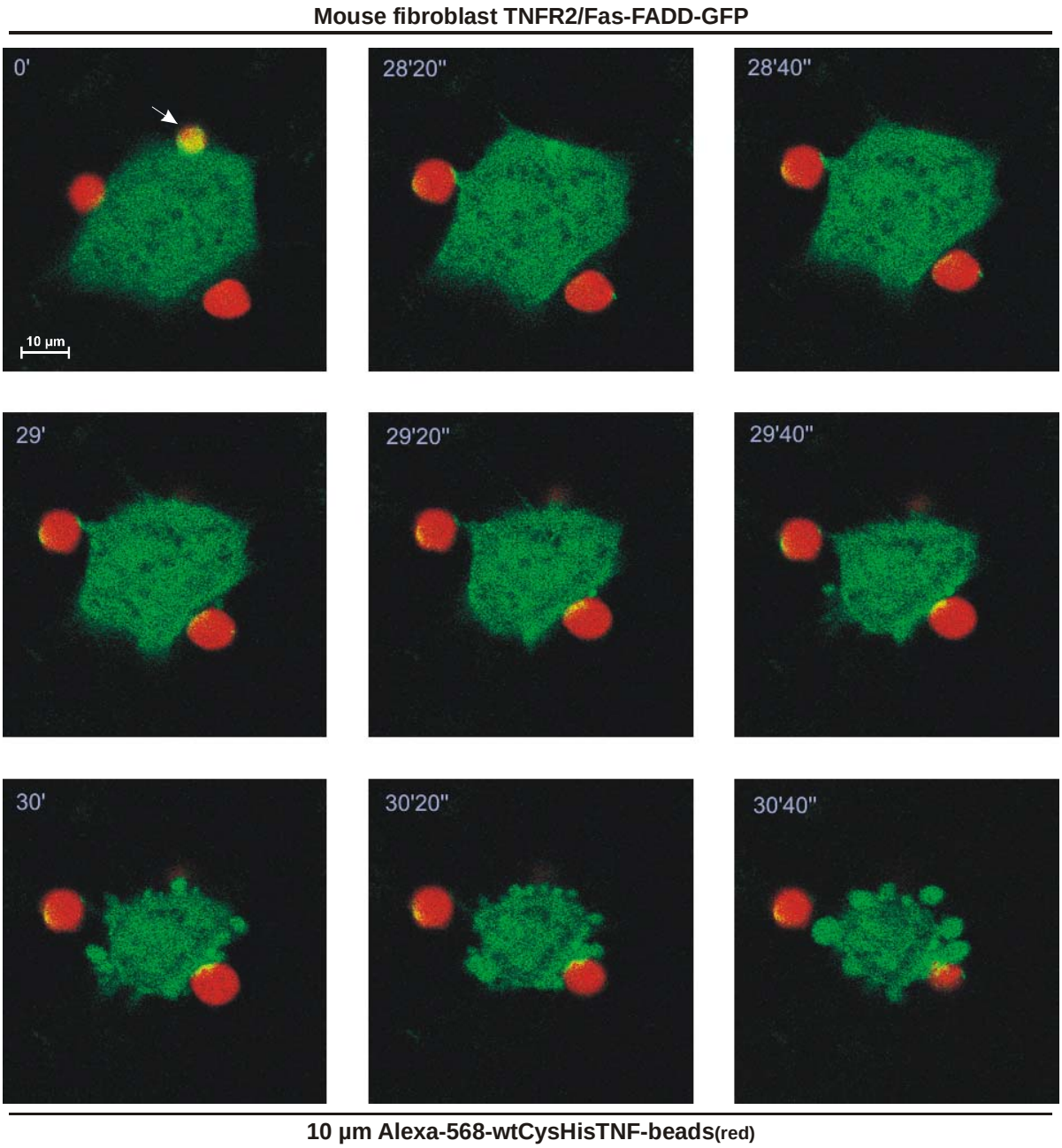


Figure 7: **Induction of apoptosis- in TNFR2/Fas mouse fibroblasts.** Alexa-Fluor-568 stained beads of 10 µm diameter were coated with 50 µg wtCysHisTNF/mg beads. 10^6 TNFR2/Fas mouse fibroblasts were transfected with 6 µg human pEGFP-FADD and grown over night in glass bottom culture dishes. 100 µg of 10 µm-wtCysHisTNF-beads were added to the cells and the induction of apoptosis was analysed using a confocal laser scanning microscope. Images were taken every 10 sec for a time period of 40 min. The white arrow indicates FADD-recruitment at the time when observation was started.

3.1.4.2 wtCysHisTNF-beads: A few particles are sufficient to trigger apoptosis

Standard cytotoxicity-assays revealed a LD_{50} of 0.15 µg wtCysHisTNF-beads/well (fig. 5). 1 mg beads of 100 nm diameter contain approximately 9.55 x 10^{11} particles.

	cell #	beads in contact	SD
	1	4	
	2	7	
	3	7	
	4	12	
	5	4	
	6	4	
	7	3	
	8	3	
	9	5	
	10	4	
	11	5	
	12	4	
	13	7	
	14	4	
	15	5	
	16	5	
	17	5	
	18	7	
	19	6	
	20	11	
	21	4	
	22	9	
	23	3	
	24	13	
	25	13	
	26	4	
	27	12	
average		6,3	3,2

	dish #	total amount of cells	apoptotic cells	% apoptotic cells
	1	158	108	
	2	172	123	
	3	121	48	
	4	78	62	
	5	101	70	
	6	94	71	
sum		724	482	66,6

Table 3: **Few beads are able to induce apoptosis.** 10^6 TNFR2/Fas mouse fibroblasts were transfected with 6-mg human pEGFP-FADD and grown over night in glass bottom culture dishes. Alexa-Fluor-568-beads of 1 µm diameter were coated with wtCysHisTNF. 25 µg particles were added to the cells and apoptosis was observed over 30 min. Images were taken and used as endpoint-material for analysis. The quantity of beads having killed one cell was then counted (left panel) and the ratio of apoptotic cells to the total amount of cells was determined (right panel).

Accordingly, 1.4 x 10^8 particles induced the LD_{50} response killing half of 20,000 cells. Confocal microscopy was performed to determine the minimal amount of beads capable to induce apoptosis in a single cell. Since single beads of 100 nm diameter cannot be resolved by light microscopy, particles of 1 µm diameter were used. TNFR2/Fas mouse fibroblasts expressing human FADD-GFP were treated with 25 µg of red fluorescent 1 µm-beads coated with wtCysHisTNF. After an incubation time of 30 min under permanent observation, beads remaining in contact with single apoptotic cells were counted and 27 cells from 9 different sample preparations were analysed. Approximately 6 x 100 cells from 6 different dishes were counted to calculate the ratio of apoptotic cells to the total cell population. Within these parameters, approximately 66% of the cell population were apoptotic. An average of 6 beads was sufficient to kill a single cell (table 3).

3.2 Signal complex formation of TNFR/Fas-receptor chimera

In this study, TNFR/Fas-chimera expressed in TNFR1 and TNFR2 double knockout mouse fibroblasts were used to distinguish receptor-specific stimulation events. The chimeras were derived from the extra-cellular and membrane spanning domains of the two TNF-receptors and the intracellular domain of Fas. Thus, TNFR2, mainly initiating survival signals, was converted into a death domain containing receptor, being able to induce apoptosis upon stimulation with memTNF.

3.2.1 TNFR1/Fas expressing mouse fibroblasts

Ligand induced oligomerisation of Fas leads to the formation of the DISC (death inducing signalling complex). This complex develops by recruitment of FADD to the intracellular death domain of Fas, leading to the activation of caspase-8 and initiation of the apoptotic cascade.

TNFR1/Fas mouse fibroblasts were transiently transfected with human FADD-pEGFP which appeared in the cells as a homogenous cytoplasmic staining. These cells have been recently shown to die upon stimulation with sTNF as well as memTNF (Krippner-Heidenreich *et al.*, 2002). After pre-incubation with Alexa-546-labelled sTNF on ice, cells were monitored by confocal microscopy. Initially, a weak receptor staining associated with the cell membrane could be observed. After 15 min of incubation with

sTNF at 37°C, receptors began to cluster and recruitment of FADD-GFP into receptor-complexes was detected (figs. 8a and b). To investigate whether membrane bound TNFR1-selective TNF has the same effect on TNFR1 as sTNF, beads labelled with 32WCysHisTNF (receptor 1 selective TNF mutant) and stained with Alexa-Fluor-

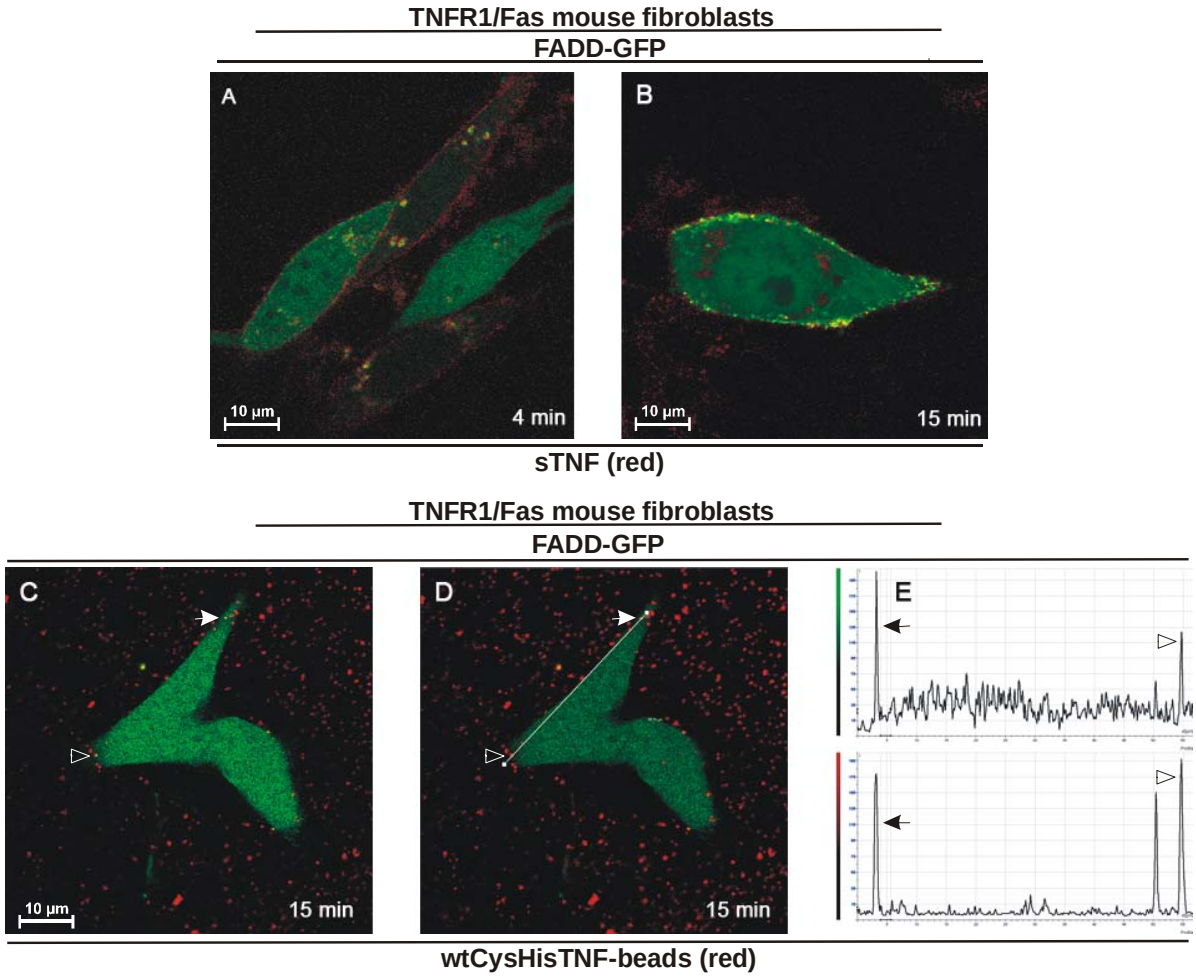


Figure 8: **FADD-recruitment in TNFR1/Fas mouse fibroblasts**. 10^6 TNFR1/Fas mouse fibroblasts were transiently transfected with 6 µg human FADD-pEGFP and grown over night in glass bottom culture dishes. **A.** and **B.** Cells were treated with Alexa-546-labelled sTNF (1.7 µg/ml) for 10 min on ice, washed and transferred to an incubation chamber at 37°C for observation in a confocal microscope. **C.** and **D.** 50 µg Alexa-Fluor-568-stained beads of 1 µm diameter coated with 1,25 µg wtCysHisTNF were added to TNFR1/Fas mouse fibroblasts. FADD recruitment was analysed in a confocal laser scanning microscope. **D.** Fluorescence intensity was measured in the Leica software accompanying the confocal microscope. **E.** Peaks indicating co-localisation of two signals are marked with open and filled arrows.

568 were used to stimulate TNFR1/Fas mouse fibroblasts transiently expressing FADD-GFP. Figures 8c and d demonstrate the recruitment of FADD-GFP molecules to the

contact site with TNF.beads already 15 min after beads addition. Figure 8d shows the analysis of fluorescence intensities.
A line of interest, indicated by the white bar crossing the cell, was defined, spanning two beads co-localising with FADD-GFP-clusters recruited to the cell membrane and the non-clustered FADD-GFP as a reference. The fluorescence intensities measured along the indicated line in the green (upper panel) and red (lower panel) channels are demonstrated in fig. 8e. Superposition of peaks in the graphs indicate co-localisation between FADD-GFP-clusters (green) and TNF-coated beads (red).

3.2.2 TNFR2/Fas expressing mouse fibroblasts

To determine whether signal transduction in TNFR2/Fas mouse fibroblasts occurred similar to that demonstrated in TNFR1/Fas mouse fibroblasts, TNFR2/Fas cells were transiently transfected with human FADD-pEGFP. The TNFR2/Fas receptor chimera was stimulated with Alexa-Fluor-546 laballed sTNF or with a memTNF equivalent. Treatment of the cells with sTNF did not provide significant clustering of receptors on the cell surface, nor was any recruitment of FADD-GFP to the receptor observed (fig. 9a). A memTNF agent, composed of Alexa-Fluor-546 labelled antibody 80M2 (which cross-links TNFR2 and stabilises the sTNF/TNFR2-complex) and sTNF, induced rapid formation of receptor clusters. FADD-GFP was recruited to the cell membrane within 2 to 8 minutes and co-localised with TNFR2/Fas clusters (fig. 9) (Krippner-Heidenreich *et al.*, 2002). In a further experiment, FADD-GFP over-expressing cells were incubated with wtCysHisTNF-coated beads (stained with Alexa-Fluor-546) Also in this group (fig. 9c), a recruitment of FADD-GFP to the contact site with the beads was observed. Similar results were obtained in TNFR2/Fas expressing cells transiently transfected with a plasmid coding for human Caspase-8(C360S)-GFP protein, a catalytically inactive caspase-8, and a murine FADD expression construct. To inhibit apoptosis, 20 µM z-VAD-fmk, a caspase inhibitor, was added to the culture medium. Again, treatment with sTNF alone did not generate receptor clustering or recruitment of caspase-8 to the membrane (data not shown). In contrast, the memTNF-like stimulus 80M2-Alexa-Fluor-546 plus sTNF caused of recruitment caspase-8-GFP to the receptor-clusters (fig. 9d).

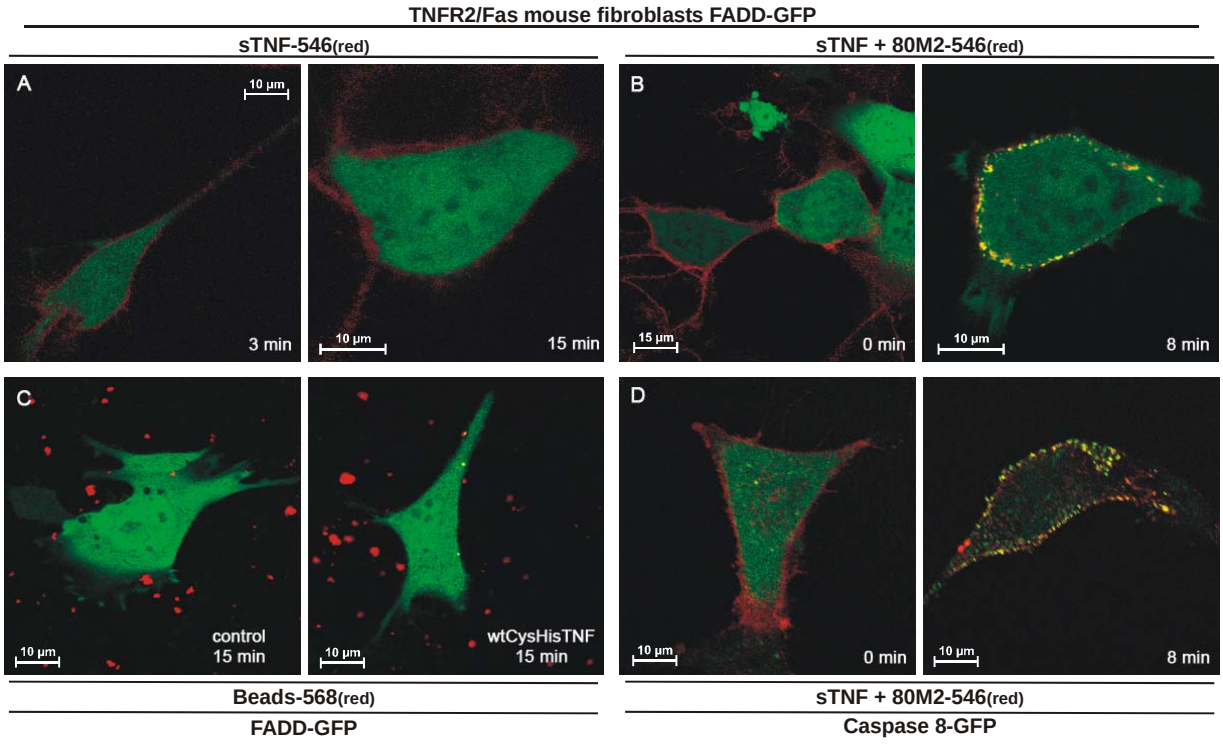


Figure 9: **Signal transduction in TNFR2/Fas mouse fibroblasts**. **A.-C.** 10^6 Cells were transiently transfected with 6 µg human FADD-pEGFP or **D.** with 6µg human caspase-8(C360S)-pEGFP plus a plasmid coding for murine FADD protein. Cells were grown over night in glass bottom culture dishes, those expressing caspase-8-GFP in 20 µM zVAD.fmk. After pre-incubation with sTNF-Alexa-Fluor-546 (1,7 µg/ml; **A**) or 80M2-Alexa-Fluor-546 (4 µg/ml; **B** and **D**) for 4 min on ice, the cells were washed with culture medium. Subsequently, 50 ng/ml unlabelled sTNF was added to the cells pre-treated with 80M2-Alexa-Fluor-546. In **C**, cells were stimulated with 1,25 µg/ml wtCysHisTNF-coated, Alexa-Fluor-568 stained beads of 1 µm diameter. FADD-recruitment was examined by live cell imaging (**A-C)**, or in fixed cells (**D**).

3.2.3 Wild type TNFR2 signal complex formation

Since sTNF has been shown in chapter 3.2.2 to activate TNFR2/Fas signal transduction only when converted in the presence of 80M2 into a memTNF equivalent, the induction of wild type TNFR2 with sTNF or memTNF-equivalent preparations was investigated. HeLa cells stably over-expressing TNFR2 (HeLa80) were transiently transfected with human TRAF2-pEGFP, which was predominantly expressed in the cytosol. Addition of sTNF-Alexa-Fluor-546 to the cells generated a red cell surface staining, but neither receptor clustering nor TRAF2 recruitment to the receptor could be observed at 37°C (fig. 10a). In contrast, cells pre-treated with 80M2-Alexa-Fluor-546 and stimulated with the TNFR2-specific mutant 143NCysHisTNF showed rapid receptor clustering and

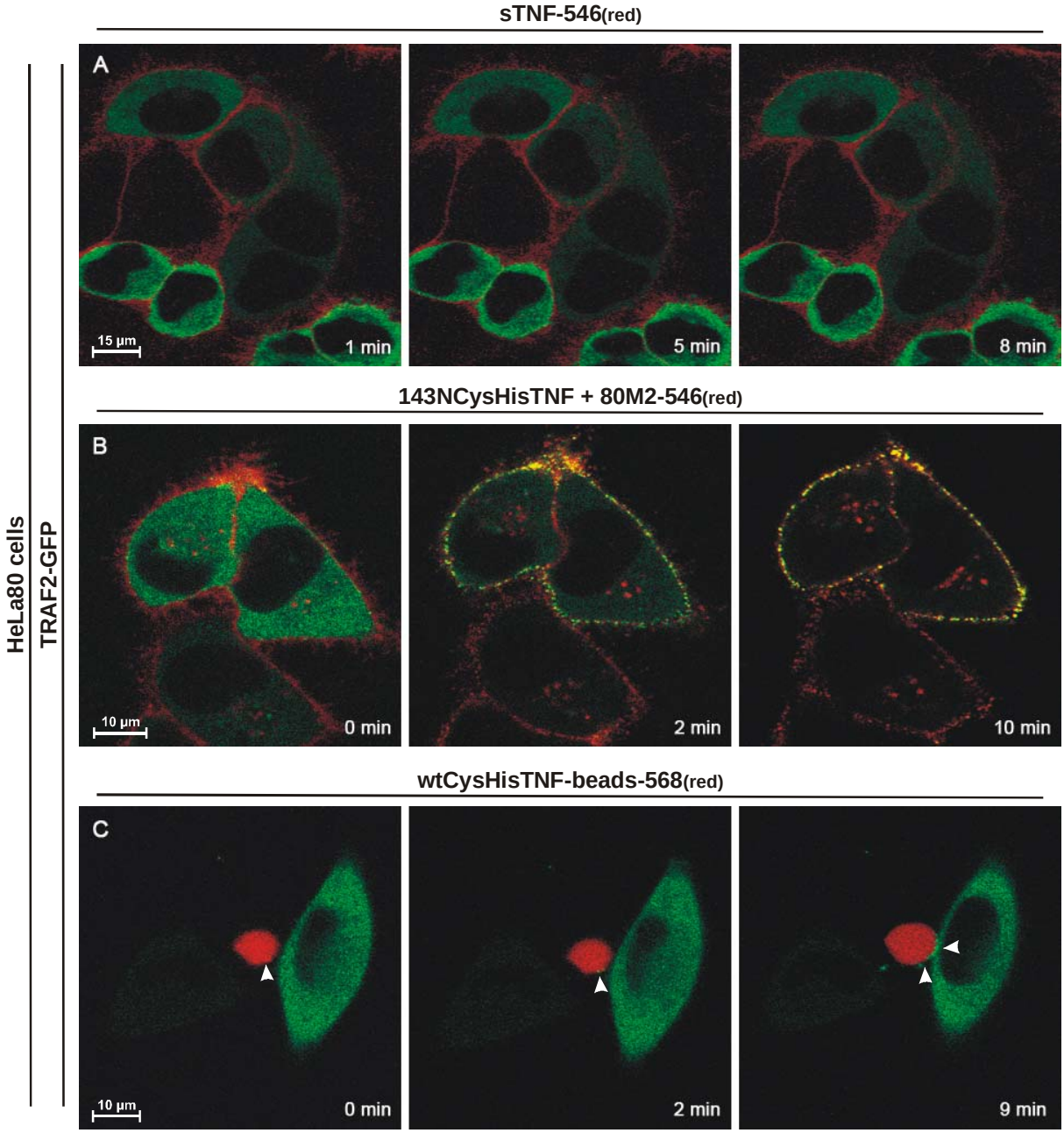


Figure 10: **TRAF2-recruitment in HeLa80 cells**. 10^6 HeLa80 cells (HeLa cells expressing TNFR2) were transiently transfected with 6 µg plasmid coding for human TRAF2-GFP and grown over night. **A.** HeLa80 cells were incubated with Alexa-Fluor-546-labelled sTNF (1,7 µg/ml) on ice, then washed with culture medium **B.** Prior to stimulation with 300 ng/ml unlabelled 143NCysHisTNF (TNFR2-specific TNF-mutant), the cells were incubated with 2 µg/ml 80M2-Alexa-Fluor-546 for 5 min at room temperature and washed with culture medium. **C.** Cells were treated with 100 µg Alexa-Fluor-568-stained beads of 10 µm diameter, coated with 5 µg wtCysHisTNF. **A.-C.** The pre-treated cells were transferred to an incubation chamber of 37°C.TRAF2-recruitment was monitored in a confocal laser scanning microscope and images were taken a the indicated time points.

recruitment of TRAF2 to the receptor complexes, visible as co-localisation in fig. 10b. Wild type CysHisTNF, immobilised on Alexa-Fluor-568 stained beads, was equally well able to induce TRAF2-recruitment to the contact site with a coated bead (fig. 10c).

3.3 Lateral diffusion of receptors and internalisation of ligand-receptor-complexes

Within the TNFR-superfamily, Fas is the best characterised member of the death receptors. Fas has been shown to cluster in lipid rafts upon stimulation with FasL (Grassme *et al.*, 2001). Contradictory findings of Algeciras-Schimnich et al. reject the importance of Fas-recruitment to lipid-rafts. However, these authors claim that ligand induced formation of receptor micro-aggregates is essential for signal transduction. Actin driven receptor-internalisation could be shown but was excluded as an event necessary to permit Fas-signalling (Algeciras-Schimnich *et al.*, 2002).

TNFR1, a close relative of Fas, has also been demonstrated to cluster in lipid rafts (Cottin *et al.*, 2002; Legler *et al.*, 2003). However, in contrast to Fas, internalisation of TNFR1 upon activation with TNF has been shown to play an essential role in pathways such as stimulation of acidic sphingomyelinase (ASMase), JNK-activation and apoptosis (Schneider-Brachert *et al.*, 2004; Schutze *et al.*, 1999).

In chapter 3.2 it has been shown that stimulation of the TNFR2/Fas receptor chimera with memTNF was followed by receptor clustering and the recruitment of signalling proteins to the DISC. The treatment of wild type TNFR2 with memTNF triggered similar events. Since ligand induced receptor-clustering appears to be a general characteristic for the signal-transduction induced by members of the TNF-receptor-superfamily, the behaviour of wild type TNFR2 at the cell membrane upon memTNF treatment was further characterised. Lateral signal propagation (Reynolds *et al.*, 2003) between stimulated and non-stimulated receptors, clustering and internalisation events were examined.

3.3.1 Lateral diffusion of TNFR2 in HeLa cells

The existence of a carrier-coupled equivalent of the membrane form of TNF allows to stimulate a single cell selectively on a locally restricted part of its cell surface, a process that is very likely to occur *in vivo.* To observe the cellular response to such a spatially directed stimulus, HeLa cells were transiently transfected with a plasmid coding for full length human TNFR2-GFP-protein (HeLa80-GFP). These cells showed a typical expression pattern for membrane proteins: TNFR2-GFP was visible in the peri-nuclear area, most likely within the Golgi apparatus, and associated with the cell membrane. The cells were treated with wtCysHisTNF-beads stained with Alexa-Fluor-568 of 10 µm diameter. The dish was searched for cells that were not in direct contact with any of the beads. A nearby lying bead was then brought in contact with the aid of an Eppendorff microinjection device, thus stimulating the cell timely controlled at one predetermined point of the membrane (fig. 11a). Subsequently, the stimulated cell was monitored for 5 minutes in 11 second-frames. The development of receptor-clusters at the cell-bead contact site appeared within few seconds, then clusters increased in size for about the following 3½ minutes. The area neighbouring the contact region in contrast lost fluorescence, i.e. it became depleted in TNFR2-GFP-molecules. Further away from the bead, neither receptor clustering events nor a change in fluorescence intensity could be observed. The interaction between wtCysHisTNF coupled onto a 10 µm-bead and TNFR2-molecules on the cell surface proved to be very strong. Mechanical separation of the bead from the cell was only possible with force and lead to the destruction of the cell (fig. 11b, compare with fig. 11a). Unlabelled control-beads did not bind to the cells and therefore did not induce any changes in the TNFR2 distribution (data not shown). Fluorescence intensity at the interaction site between bead and cell, the areas in the direct vicinity at the left and right of the bead, in the cytosol and in the Golgi apparatus was analysed as a function of time (fig. 11c). Statistic examination of seven different experiments showed that the fluorescence at the contact point increased, whereas the directly adjacent regions lost intensity in fluorescence. No changes could be observed in the cytosol or in the Golgi apparatus (fig. 11d). These results indicate a lateral diffusion of non-stimulated TNFR2-molecules in close proximity to the cell-bead contact site.

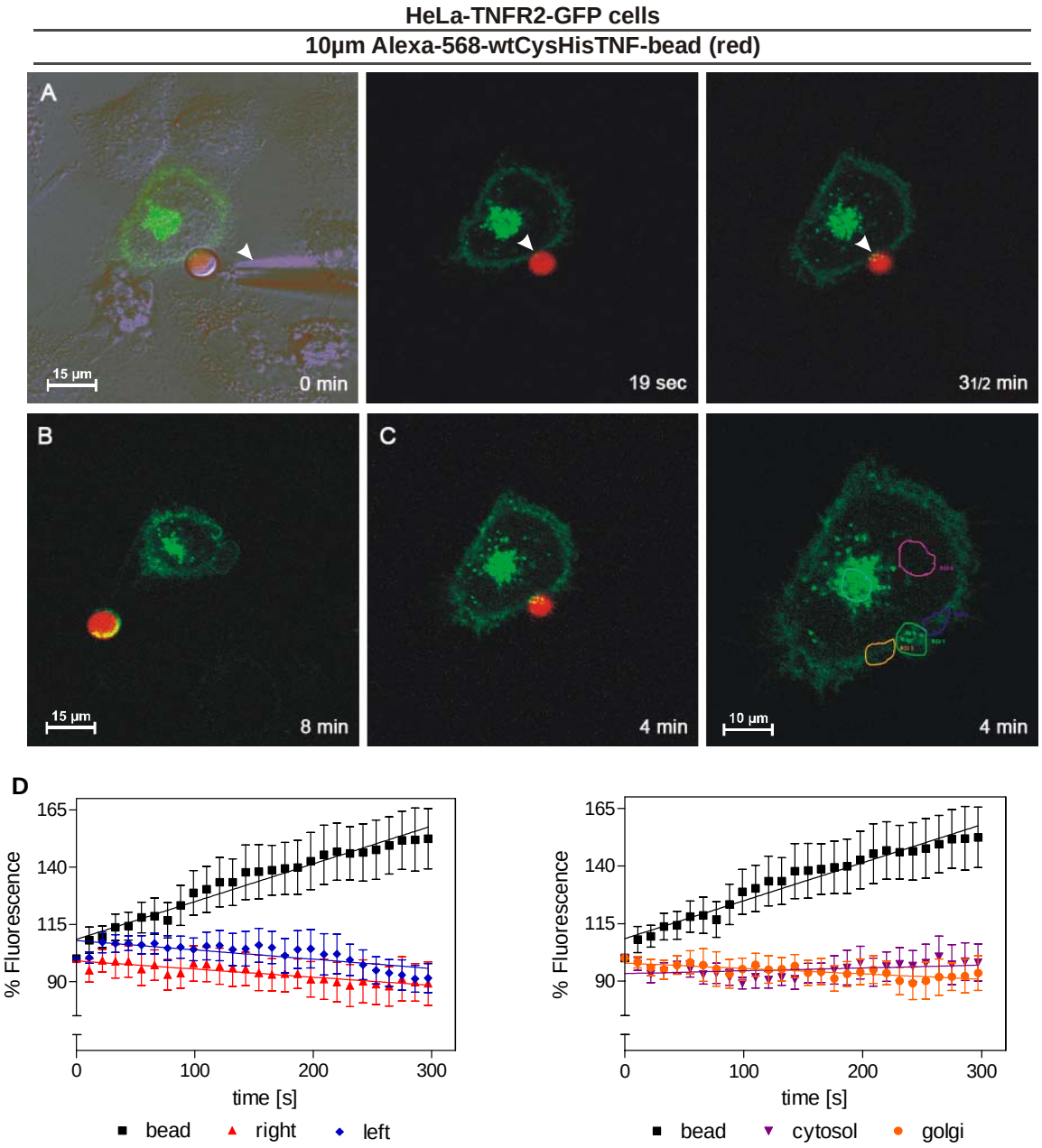
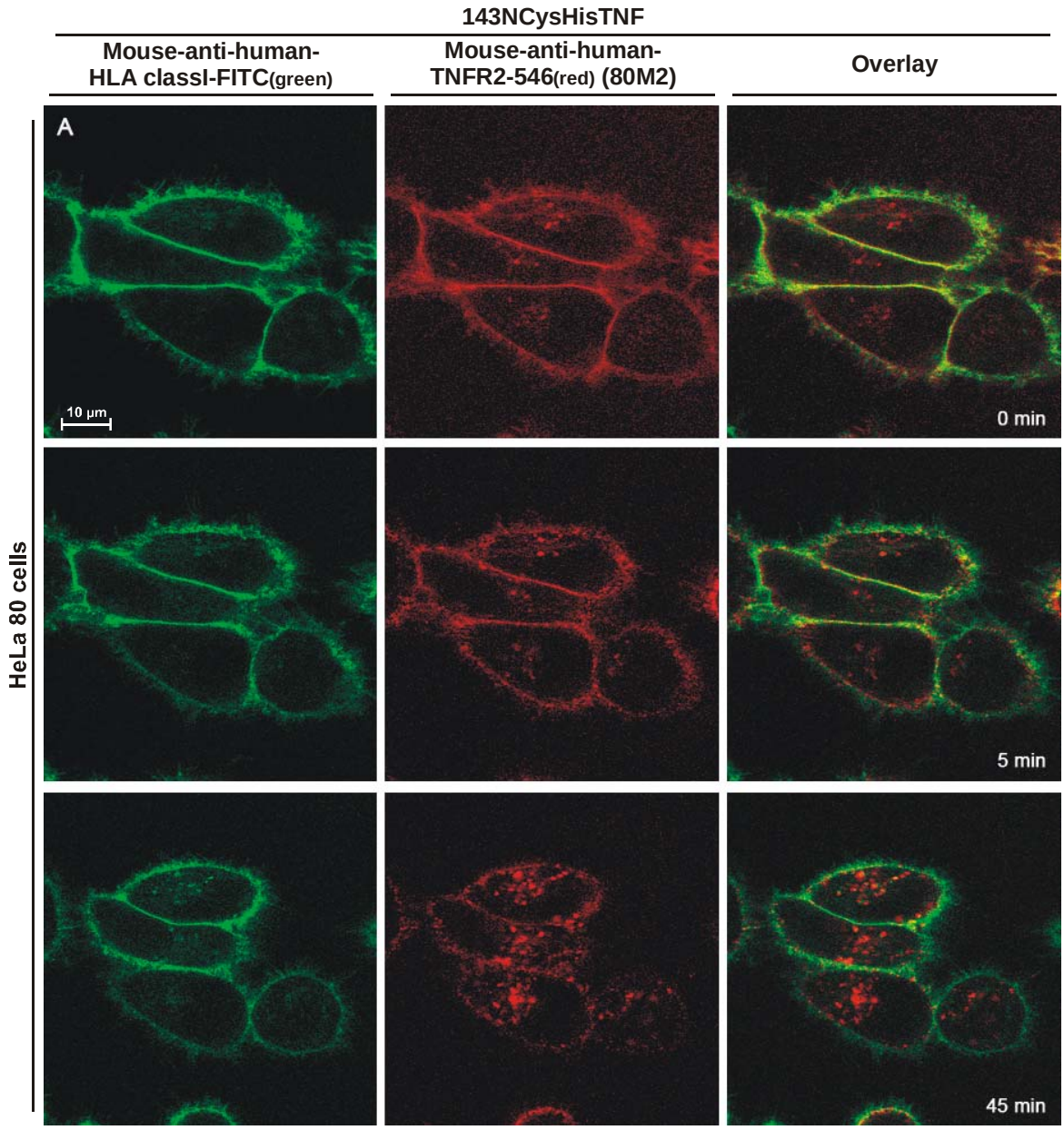


Figure 11: **Analysis of lateral diffusion in HeLa-TNFR2-GFP cells**. 10^6 cells were transiently transfected with 6µg plasmid coding for human TNFR2-GFP and grown over night. Alexa-Fluor-568 stained beads of 10 µm diameter were coated with 50 µg wtCysHisTNF/mg beads. **A.** 100 µg wtCysHisTNF-beads were added to the cells. A cell not in contact with beads was selected and stimulated with a particle pushed to the HeLa cell with a Femtojet microinjection device (Eppendorff). TNFR2-clustering and binding to the bead was monitored for 5 min with images taken every 11 sec. **B.** Particle and cell were separated with the Femtojet microinjection device. **C.** Fluorescence intensity at the contact site between cell and bead, on the left and the right hand side of the particle, in the cytosol and in the Golgi apparatus was measured by defining regions of interest being analysed in the Leica software. Measurement was performed for every image taken during 5 min. **D.** Data derived from 7 cells were statistically analysed for fluorescence intensity as described in **C**.

3.3.2 Internalisation of TNFR2 into HeLa80 cells

TNFR1 and Fas have been shown to cluster and to internalise after stimulation with their respective ligands. In the previous 2 chapters, TNFR2 has been shown to cluster upon spatially restricted stimulation with a wtCysHisTNF-labelled 10 µm-bead, but also upon stimulation with sTNF in the presence of the cross-linking antibody 80M2. To examine the events after clustering, HeLa80 cells were stained with a FITC-coupled human-HLA class I-specific antibody as a visible marker of the cell membrane. HLA class I showed a homogenous distribution in the cell membrane. Pre-incubation with 80M2-Alexa-Fluor-546 resulted in a likewise homogenous membrane staining, displayed as co-localisation with HLA class I (fig. 12a). Then the cells were stimulated with the TNFR2-specific mutant 143NCysHisTNF. Receptor-clustering started within 5 minutes after stimulation. Internalisation was complete after 45 minutes, as illustrated in fig. 12a showing the receptor localised inside the cell, whereas the human HLA class I antibody still remained membrane associated.

Localisation of TNFR2 was investigated in endosomes and Caveolae. HeLa80 cells were transiently transfected with a plasmid coding either for endosomal marker-CFP or Caveolin1-CFP and pre-incubated with 80M2-Alexa-Fluor-546. The cells were stimulated with 143NCysHisTNF and internalisation of TNFR2 was monitored for 60 minutes in the confocal microscope. Co-localisation of TNFR2 with endosomes as well as with Caveolae could be observed as soon as 20 minutes (data not shown) and was complete after 60 minutes, visible as white dots (figs. 12b and c).


143NCysHisTNF
Mouse-anti-human-HLA classI-FITC(green)
Mouse-anti-human-TNFR2-546(red) (80M2)
Overlay
HeLa 80 cells
A
10 µm
0 min
5 min
45 min

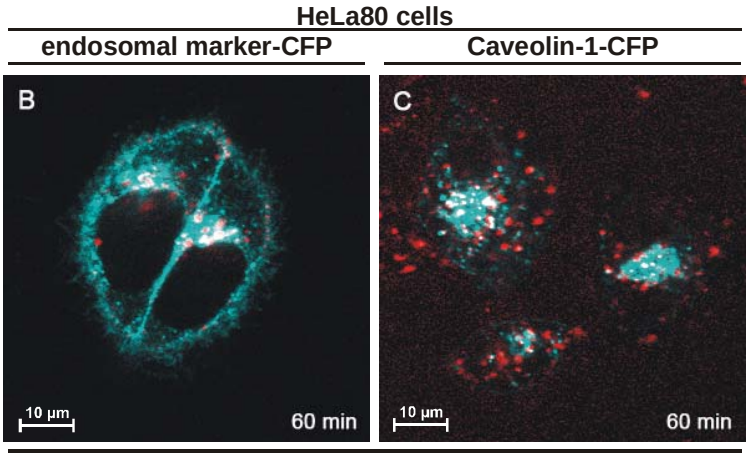

HeLa80 cells
endosomal marker-CFP
Caveolin-1-CFP
B
C
10 µm
60 min
10 µm
60 min
80M2-546(red) + 143NCysHisTNF

Figure 12. **TNFR2-clustering and internalisation in HeLa80 cells**. **A.** HeLa80 cells were pre-incubated with a FITC-coupled monoclonal anti-human-HLA class I antibody (10 µl, 1 test, green) to stain the cell membrane and with 80M2-Alexa-Fluor-546 (2 µg/ml, red) for 5 min at 37°C and washed with culture medium. 300 ng/ml of unlabelled 143NCysHisTNF (TNFR2-specific) was added and receptor-clustering and internalisation were monitored for 45 min using a confocal laser scanning microscope, with images taken every 5 min. **B.** 10^6 **C.** HeLa cells were transiently transfected with 6 µg Caveolin-1-pECFP and grown over night. Cells were treated as described in **A** and **B**. Internalisation was monitored for 45 min – 60 min after addition of 300 ng/ml 143NCysHisTNF.

3.3.3 Internalisation and localisation of wtCysHisTNF-beads

Since TNFR2 was shown to internalise within less than one hour after stimulation, the next question was whether and how wtCysHisTNF-coated beads of an adequate size would internalise when interacting with TNF-receptor. Alexa-Fluor-568 stained beads of 1 µm diameter were coated with wtCysHisTNF. HeLa80 cells were stained with a FITC-coupled α-human-HLA class I-antibody to visualize the cell membrane prior to stimulation with 100 µg wtCysHisTNF-beads. The cells were monitored for 16 hours using a confocal microscope. Internalisation was visible approximately 5 hours after addition of the beads to the HeLa80 cells (data not shown). To explore whether the internalisation of the particles was receptor-specific and/or dependent on the cell type, further experiments were performed with Kym1 cells, a line expressing high numbers of TNFR2. Either wtCysHisTNF- or uncoated (control) beads, labelled with Alexa-Fluor-568, were added over night to Kym1 cells that were cultivated with zVAD.fmk to prevent apoptosis. The cells were then stained with a FITC-coupled α-human-HLA class I-antibody and examined by confocal microscopy. Since TNF-coated particles as well as control beads could be found inside the cells in about equal amounts (fig. 13a and b), internalisation was neither dependent on interaction between the immobilised TNF and TNF-receptors, nor on the cell type. The localisation of internalised beads was again investigated using endosomal and lysosomal markers. HeLa80 cells were transiently transfected with a plasmid coding for endosomal marker-CFP, treated with wtCysHisTNF-particles or with control-beads, each Alexa-568-stained, and analysed for co-localisation. Both types of particles could be found in the cells, but no endosomal co-localisation was visible (data not shown). 1 µm FITC-stained beads could be co-localised with red fluorescing lysotracker in HeLa80 cells, visible as yellow dots (figs. 13c and d), independent whether TNF coated (fig. 13c) or control beads (fig. 13d) had been used.

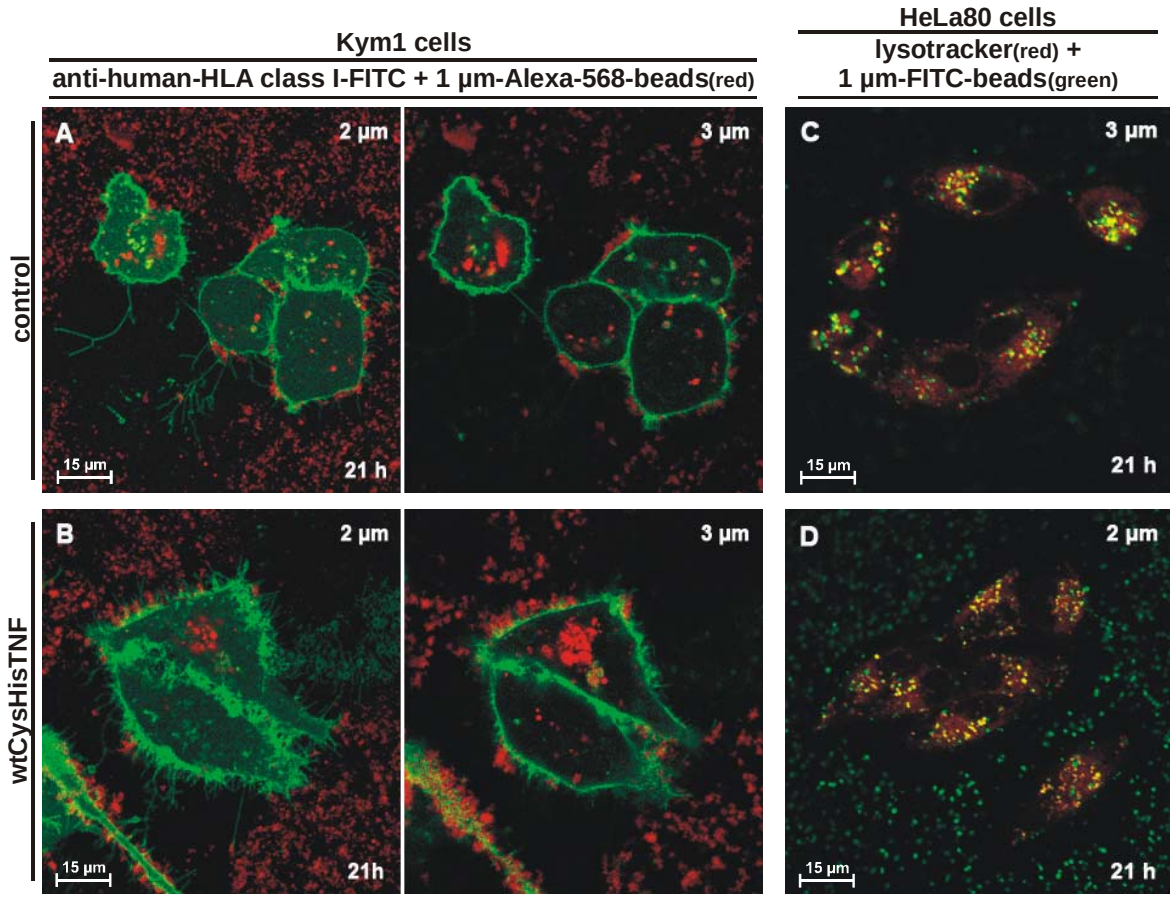


Figure 13: **Internalisation and localisation of beads in different cell lines**. Beads stained with FITC or with Alexa-Fluor-568 of 1 µm diameter were coated with 25µg wtCysHisTNF/mg beads. **A.** and **B.** 1 mg Alexa-568-beads (red) uncoated (**A**) or labelled (**B**) with wtCysHisTNF were added to Kym1 cells and incubated over night at 37°C. The cells were then stained with a FITC-coupled monoclonal anti-human-HLA class I antibody (green), washed with culture medium and analysed in a confocal microscope. **C.** and **D.** 1 mg FITC-beads unlabelled (**C**) or coated with wtCysHisTNF (**D**) were added to HeLa80 cells and left over night at 37°C. After incubation with lysotracker (red) and washing with culture medium, the cells were analysed by confocal laser scanning microscopy.

3.3.4 TNFR2-internalisation is dynamin-dependent

Several pathways of internalisation are known, depending on the type and size of the cargo (Conner and Schmid, 2003). Particles like virus and bacteria are taken up by cells through phagocytosis, whereas proteins and particles of less than 1 µm in size are internalised by pinocytosis, the formation of vesicles out of invaginations of the cell

membrane. Many endocytotic vesicle formation mechanisms require dynamin. This GTPase self-assembles into a “collar” at the necks of deep invaginations and cuts

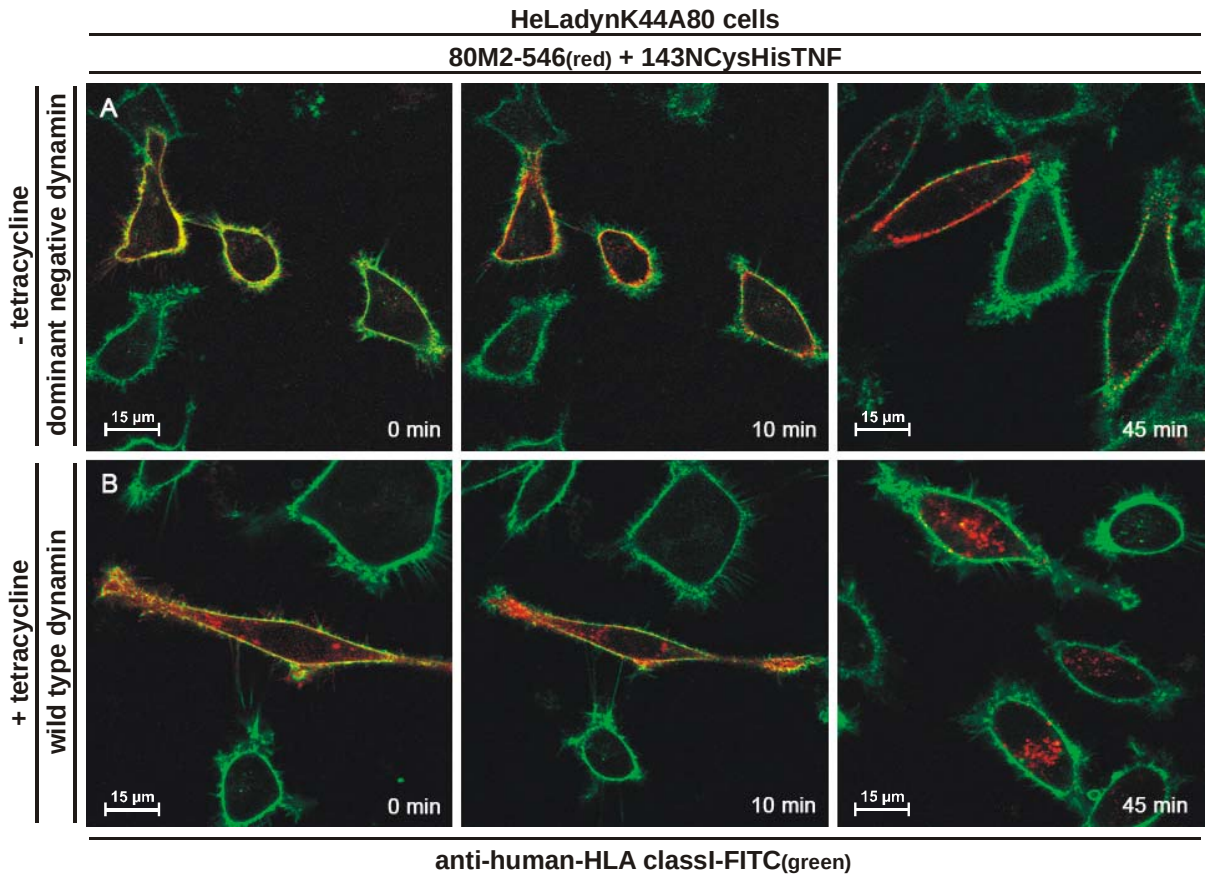


Figure 14: **Dynamin-dependent TNFR2-internalisation**. 10^6 HeLadynK44A cells, which express a dominant negative dynamin mutant controlled by a tetracycline-regulated promoter, were transiently transfected with 6 µg plasmid coding for human TNFR2. After 48 h of cultivation without or with tetracycline, the cells expressed dominant negative (**A**) or wild type (**B**) dynamin, respectively. Prior to stimulation with 300 ng/ml 143NCysHisTNF, the cells had been stained with FITC-coupled anti-human-HLA class I-antibody (10 µl, 1 test; green) and with 2 µg/ml 80M2-Alexa-Fluor-546 (red), and then washed with culture medium. TNFR2-internalisation was monitored by live cell imaging.

vesicles off from the cell membrane. In order to investigate the dynamin dependence of TNFR2-internalisation, HeLa cells expressing a dominant negative form of dynamin (DN-dynamin) regulated by a tetracycline-promoter were cultured in culture medium with 1 µg/ml tetracycline (HeLadynK44A). The cells were transiently transfected with human TNFR2 and grown for 48 hours with or without tetracycline, to activate expression of DN-dynamin. Prior to TNFR2-specific stimulation with 143NCysHisTNF, HeLadynK44A cells were stained with a FITC-coupled anti-human-HLA class I-antibody and pre-incubated with 80M2-Alexa-Fluor-546 (anti-human-TNFR2-antibody). Internalisation was

monitored for 45 minutes. Cells expressing the dominant negative form of dynamin showed a homogenous TNFR2-distribution on the cell membrane, evident as co-localisation with HLA class I as cell surface marker (fig. 14a). Within 5 minutes after stimulation, the receptor molecules clustered. Invaginations could be observed by the formation of a red ring surrounded by green fluorescent HLA class I at the cell surface. However, no internalisation was detected (fig. 14a). HeLadynK44A cells transiently expressing TNFR2, which had been cultivated with tetracycline and therefore expressed wild type dynamin, showed receptor clustering and internalisation similar to HeLa80 (chapter 3.3.2) when treated identically (fig. 14b).

1 µm-beads have been shown to internalise into different cell lines when incubated over night. HeLadynK44A cells were transiently transfected with TNFR2 and grown for 48 hours without or with tetracycline. Both cell lines, expressing DN-dynamin or wild type dynamin respectively, ingested particles when stimulated with TNF-beads over night (data not shown). These results suggest that TNFR2 is internalised by a mechanism dependent on functional dynamin, whereas beads are taken up by a dynamin-independent pathway.

3.3.5 TNFR2-internalisation depends on the receptor's cytosolic domain

Cottin et al. (2002) have demonstrated that the recruitment of activated TNFR1 to lipid rafts depends on its death domain (Cottin *et al.*, 2002). Others (Schutze *et al.*, 1999) have discussed the necessity of stimulated TNFR1 being recruited to lipid rafts in order to internalise. Internalisation of activated TNFR2 might also depend on its cytosolic domain. To investigate this question, a TNFR2-construct lacking its cytosolic domain (amino acids 301-461) was created. A GFP-molecule was fused to the potential transmembrane domain of TNFR2 (fig. 15a). The resulting hybrid construct TNFR2exTMGFP was transiently over-expressed in HeLa cells. After pre-stimulation with 80M2-Alexa-Fluor-546, which co-localised as expected completely with the receptor-construct (fig. 15b), the cells were treated with 143NCysHisTNF. Receptor clustering and internalisation was monitored as described before during 60 minutes. Although clustering occurred normally, no internalisation was observed (fig. 15b, compare with fig. 12a).

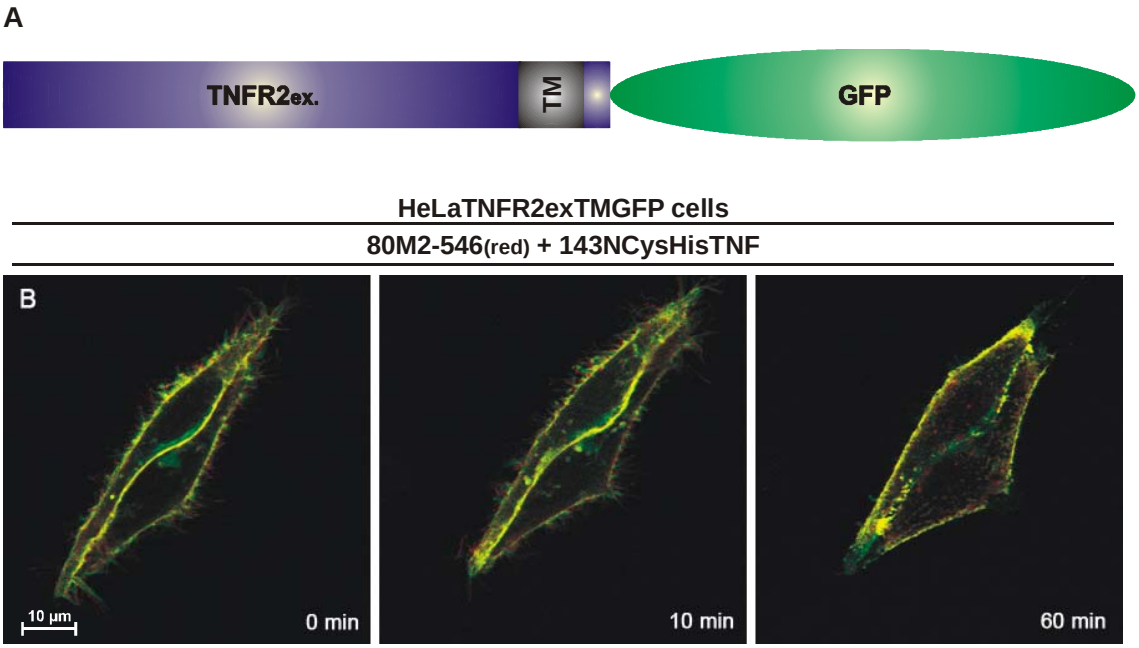


Figure 15: **Expression and clustering of TNFR2exTMGFP in HeLa cells**. **A.** Schematic representation of TNFR2exTMGFP. GFP was fused to the C-terminus of the potential transmembrane region of human TNFR2. **B.** 10^6 HeLa cells were transiently transfected with 6 µg plasmid coding for TNFR2exTMGFP and grown over night in glass bottom culture dishes. Cells were then pre-incubated with 80M2-Alexa-Fluor-546 (2 µg/ml; red) and washed with culture medium. Receptor-clustering and internalisation was observed for 60 min by live cell imaging after stimulation with 300 ng/ml of unlabelled 143NCysHisTNF.

Similar results had been obtained in TNFR2/Fas expressing mouse fibroblasts when pre-treated with 80M2 and activated with sTNF or 143NCysHisTNF (data not shown). These data indicate that internalisation, but not clustering, of the TNFR2 depends on its cytosolic domain.

3.4 TNFR2 mediated signal transduction

TNFR1-activation and signalling is by far better characterised as compared to the signalling pathways induced by TNFR2. Only in the past decade, after the realization that TNFR2 can be fully activated only by memTNF (Grell *et al.*, 1995; Krippner-Heidenreich *et al.*, 2002), its signalling pathways have become scrutinised more closely. Some of the proteins participating in the TNFR1 signal transduction are also crucial for TNFR2, like TRAF2, IκB and the IKK-complex for NFκB-activation. However, for the NFκB-activation mediated by TNFR1, RIP is indispensable, whereas its role in TNFR2-

driven NFκB-activation is not defined. In the following set of experiments, some proteins well known to participate in TNFR1 mediated signal transduction were investigated for their relevance in TNFR2-signalling.

3.4.1 NFκB-translocation after TNFR2 stimulation

HeLa cells express approximately 3000 molecules TNFR1, sufficient for a strong activation of NFκB, as well as for the induction of apoptosis in the presence of a transcription/translation inhibitor. HeLa cells express extremely low numbers of TNFR2, if any, and therefore HeLa80, a cell line stably expressing TNFR2 has been employed.

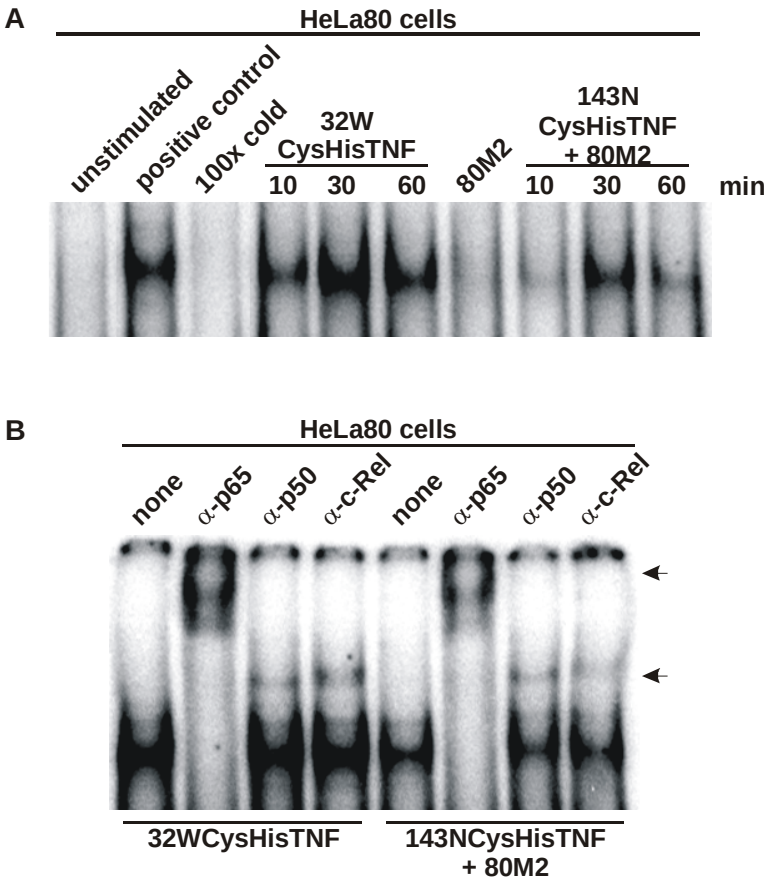


Figure 16: **NFκB-translocation in HeLa80 cells after stimulation with TNFR1- or TNFR2-selective TNF-mutants**. **A.** HeLa80 cells were stimulated with 30 ng/ml 32WCysHisTNF (TNFR1-stimulation) or with 300 ng/ml of 143NCysHisTNF + 2 µg/ml 80M2 (TNFR2-stimulation). Lysates from isolated nuclei were prepared directly after the indicated time points of TNF-stimulation and the NFκB-translocation was investigated by gel shift analysis. **B.** Lysates with the strongest response after TNF-treatment (30 minutes) were incubated with antibodies specific for p65, p50 and c-Rel. These lysates were then analysed in an electro mobility super shift assay. Arrows indicate super shifts.

HeLa80 cells express about 30,000 molecules of TNFR2 per cell at the cell surface, i.e. an about ten fold excess in comparison to TNFR1. An aliquot of cells grown over night was pre-incubated with 80M2 prior to specific TNFR2 activation. After selective receptor stimulation with either 32WCysHisTNF or 143NCysHisTNF (the 80M2 pre-treated group) at different times, activation of NFκB was analysed in an electro mobility shift assay. After stimulation of each of the two TNF receptors the cells showed similar time kinetics, with full NFκB-activation after 30 minutes. TNFR1, however, generated a much stronger signal as compared to TNFR2 (fig. 16a). The samples with maximum NFκB activations after 30 minutes of stimulation were used for super shift experiments to investigate the type of NFκB-proteins involved in the activation events. It was observed (fig. 16b) that the protein predominantly translocated into the nucleus was p65, although small amounts of p50 and c-Rel could be also detected.

3.4.2 IKKγ/NEMO is essential for NFκB-activation by TNFR2

Two main pathways leading to the activation of NFκB are known. The canonical pathway depends on IKKβ and IKKγ/NEMO interaction to phosphorylate IκBα, leading to translocation mainly of p65 and p50 heterodimers into the nucleus. The second "alternative" pathway, recently investigated in more detail, follows e.g. NIK-mediated IKKα-activation and is independent of IKKβ and NEMO. This pathway results in the processing of p100 into p52 and subsequent translocation of p52/RelB into the nucleus (Bonizzi and Karin, 2004). The data obtained in chapter 3.4.1 strongly suggest that the NFκB-pathway initiated by both TNFR1 and TNFR2 follows the canonical IKKβ-dependent pathway. To further confirm this hypothesis, wild type and NEMO deficient Jurkat cells were stably transfected with TNFR2. Wild type cells and NEMO deficient cells without or expressing TNFR2 were stimulated at different times with TNFR1-specific 32WCysHisTNF or with 143NCysHisTNF + 80M2 to exclusively stimulate one type of the TNF receptors. Lysates of the cells were analysed in a western blot for IκBα-degradation with monoclonal IκBα-antibody as an indicator for IKKß-mediated activation of NFκB. Vinkulin was determined as a loading control. The results demonstrate that both receptors show the same degradation pattern after selective stimulation. In wild type Jurkat cells, as soon as 15 minutes after stimulation diminishing amounts of IκBα could be detected after selective TNFR1 stimulation. As expected, the wild type Jurkat

cells, negative for TNFR2, were not responsive to a TNFR2-selective stimulus. NEMO deficient TNFR2 negative Jurkat cells were unresponsive to either receptor stimulus (fig. 17). Jurkat cells stably expressing TNFR2 showed the same IκBα-degradation-pattern after exclusive TNFR1 or TNFR2 stimulation. In both cases, the receptor selective stimulation induced significant I-κBα degradation, indicating an IKKβ-driven activation of NFκB. In contrast, the NEMO-deficient Jurkat cells were unresponsive. No IkB-degradation was observed after selective stimulation of TNFR1 or TNFR2. These results support an essential role of NEMO not only in TNFR1 mediated signals, but also in TNFR2 signalling. In addition, these experiments confirm that TNFR2-stimulation also activates the classical NFκB pathways in Jurkat cells (fig. 17).

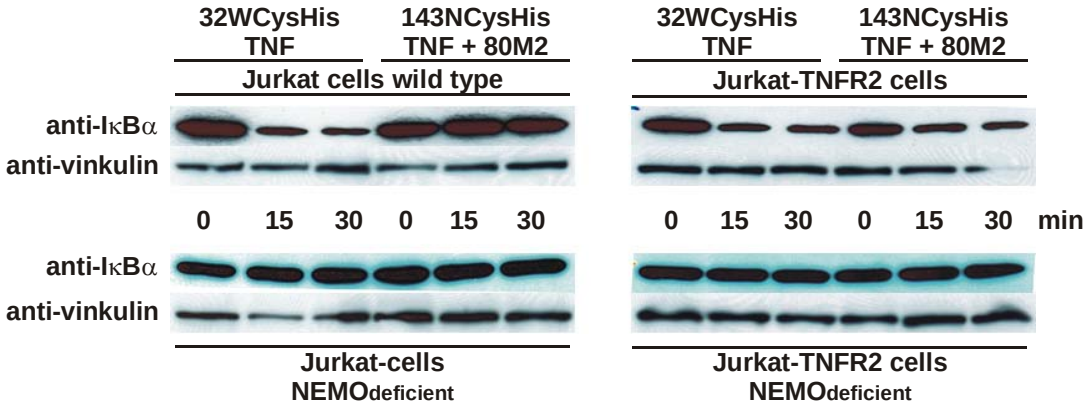


Figure 17: **IκBα-degradation in IKKγ/NEMO deficient Jurkat cells**. Jurkat cells (wt or NEMO deficient) were stably transfected with human TNFR2. Wild type or NEMO deficient cells, without or with TNFR2, were treated with receptor-specific TNF-mutants. TNFR1 was activated with 100 ng/ml 32WCysHisTNF and TNFR2 was stimulated with 300 ng/ml 143NCysHisTNF + 2 µg/ml 80M2 for the indicated time points. IκBα-degradation was detected with a monoclonal IκBα-antibody in a western blot. Vinkulin was identified as a loading control.

3.4.3 The role of RIP in TNFR2 signalling

RIP is essential in TNFR1-mediated NFκB-activation (Devin *et al.*, 2000; Pimentel-Muinos and Seed, 1999). To investigate the role of RIP in the NFκB-pathway initiated by TNFR2, four different Jurkat cell lines were used: wild type cells expressing TNFR1 but not TNFR2, TNFR2 transfectants, RIP-deficient Jurkat cells as well as their TNFR2 stably over-expressing counterparts. Wild type or RIP-deficient Jurkat cells were incubated with TNFR1-specific 32WCysHisTNF, TNFR2-overexpressing cells were

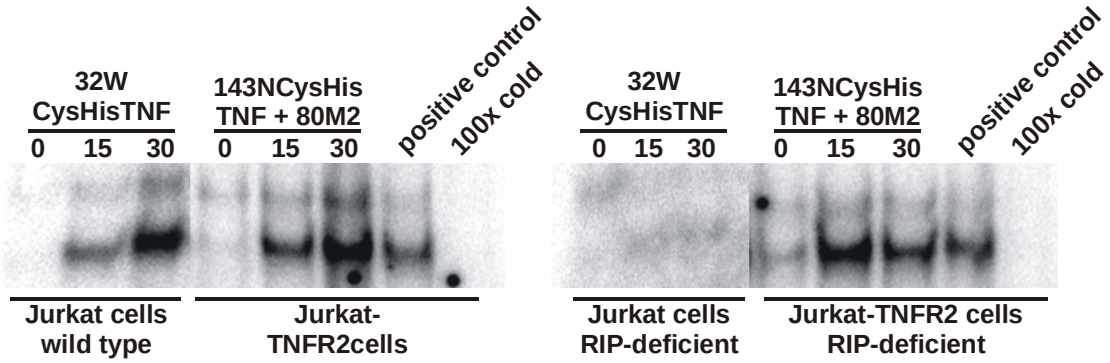


Figure 18: **NFκB-translocation in RIP-deficient Jurkat cells after selective stimulation of TNF receptors**. Jurkat cells, wild type or RIP-deficient, were stably transfected with human TNFR2. Wild type and RIP-deficient cells, without or expressing TNFR2, were stimulated with receptor specific TNF-mutants. TNFR1 was activated with 100 ng/ml 32WCysHisTNF, TNFR2 with 300 ng/ml 143NCysHisTNF + 2 µg/ml 80M2 for the indicated time points. Lysates from isolated nuclei were analysed in an electro mobility shift assay.

stimulated with 143NCysHisTNF + 80M2 for different times. Samples were analysed in an electro mobility shift assay. As expected, NFκB was translocated in wild type Jurkat cells from the cytosol into the nucleus within 15 minutes after stimulation of TNFR1. RIP-deficient, TNFR2 negative cells were not able to activate NFκB in response to a TNFR1-stimulus. Both TNFR2-containing Jurkat cell lines, however, were capable to translocate NFκB to the nucleus after selective stimulation of TNFR2 (fig. 18). These data demonstrate that RIP is dispensable for the TNFR2-mediated NFκB-activation.

3.4.4 TRAF2-recruitment is independent of internalisation

In chapter 3.3.5, the requirement of the cytosolic domain for TNFR2 for internalisation was demonstrated. TNFR2/Fas as well as TNFR2exTMGFP constructs were not able to internalise within 45 minutes as had been shown for wild type TNFR2 (figs. 12 and 15). Moreover, a dominant negative dynamin mutant inhibited TNFR2 internalisation when over expressed in HeLa cells (fig. 14). On the other side, a TNFR2/Fas chimera, although not able to internalise, had been shown to recruit FADD-GFP upon stimulation with 143NCysHisTNF + 80M2. To confirm that the signal transduction of wild type TNFR2 is similarly unaffected upon inhibition of receptor internalisation by expression of dominant negative dynamin, HeLadynK44A cells were transiently co-transfected with human TNFR2 and human TRAF2-GFP. These cells were then grown in culture medium

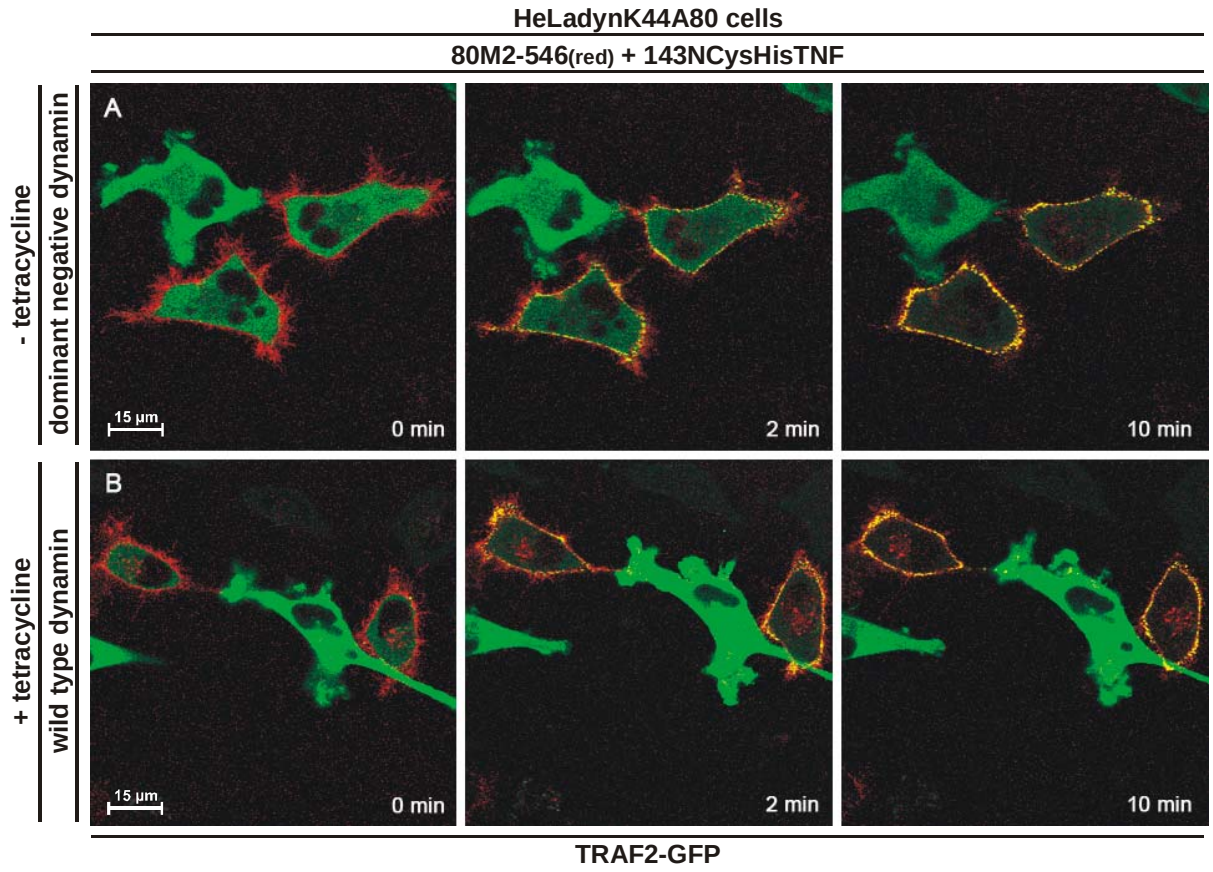


Figure 19: **Dynamin-independent TRAF2-recruitment in HeLa cells**. 10^6 HeLadynK44A cells, which express a tetracycline controlled dominant negative form of dynamin, were transiently co-transfected with 3µg human TRAF2-GFP and 3 µg human TNFR2. Cells were grown for 48 hours without (**A**) or with (**B**) tetracycline to allow expression of dominant negative or wild type dynamin. After incubation with 2 µg/ml 80M2-Alexa-Fluor-546 (red) and washing with culture medium, the cells were stimulated with 300 ng/ml 143NCysHisTNF. TRAF2-recruitment was monitored for 30 min with a confocal laser scanning microscope.

with or without tetracycline to suppress or allow expression of the dominant negative dynamin. For subsequent analyses in a confocal microscope, the specimens were pre-incubated with red fluorescent 80M2-Alexa-Fluor-546 and stimulated with 143NCysHisTNF. Receptor-clustering and TRAF2-recruitment followed the same pattern already shown in chapter 3.2.3 (figs. 19a and b, compare with fig. 10b). The receptor molecules started to cluster within few minutes after treatment with TNFR2-specific TNF, followed instantly by the recruitment of TRAF2-GFP to the membrane, visible as yellow dots of co-localisation. These data, together with those obtained in chapter 3.2.2 using TNFR2/Fas expressing mouse fibroblasts indicate that initial events in TNFR2-signalling are independent of receptor internalisation.

4 Discussion

Most of the members of the TNF-ligand superfamily exist in two bioactive forms: a membrane bound form and its soluble counterpart, processed from the membrane bound TNF by metalloproteases and/or produced by alternative splicing. Both soluble and membrane bound TNF are able to bind and stimulate TNFR1. In contrast, TNFR2 is able to bind sTNF as well as memTNF, but only memTNF is capable to produce a strong activation of TNFR2 (Grell *et al.*, 1995). Generally, the investigation of TNFR2 signalling pathways is obstructed by a lack of adequate memTNF-mimicking tools. In this work, some aspects of TNFR2 signal transduction were investigated using newly designed TNF-coated beads in comparison to the classical agonistic agent, consisting of the combination of soluble TNF and a TNFR2-specific antibody (Grell *et al.*, 1995).

TNF immobilised on silica beads – a new tool for TNFR stimulation. Silica particles of various sizes provided from the group of Günter Tovar, Fraunhofer Institute for Interfacial Engineering and Biotechnology, were coated with wtCysHisTNF, exposing free SH-groups in their Cysteine-residues to generate a memTNF equivalent (fig. 3). A standard protocol was established for optimal labelling, with excess quantities of TNF being coupled onto beads at pH 7.2 during 1 hour at room temperature to obtain maximal bioactivity.

Coating of beads with CysHisTNFs as well as with sTNF converted the particles into a tool possessing the characteristics of membrane bound TNF (fig. 5). However, wtCysHisTNF labelled beads were about 10 times more bioactive than sTNF-beads. This could be explained by two different mechanisms that are responsible for the binding of TNF to particles. One part of either wtCysHisTNF or sTNF is adsorbed due to hydrophobic and non-covalent interactions between beads and TNF. TNF, like Fibronectin, is known to strongly adhere to surfaces, amongst them glass and plastics (Grunze M., 2002). In addition to this unspecific binding, CysHisTNF is able to form covalent C-S-thioether bonds between its free Cys-residues and the maleimide group of sulfoSMCC, which was used to activate the beads for coating. This reaction allows the directed attachment of CysHisTNF to the particles, whereas the adsorbed TNF molecules might bind in any spatial order.

The directed presentation of TNF on the surface of the beads, with its N-terminus forming a covalent bound with sulfoSMCC and its C-terminus distal to the bead, permits formation of biologically active trimers with neighbouring bound molecules or with freely diffusing molecules from the supernatant (fig. 20). Adsorbed TNF molecules, in contrast, might be attached to the beads in a randomised manner, either in a way that allows the formation of active trimers or with most of their surface interacting with the beads-surface, which would obstruct formation of trimers with adjacent molecules. Thus, a portion of the adsorbed TNF is probably not bioactive.

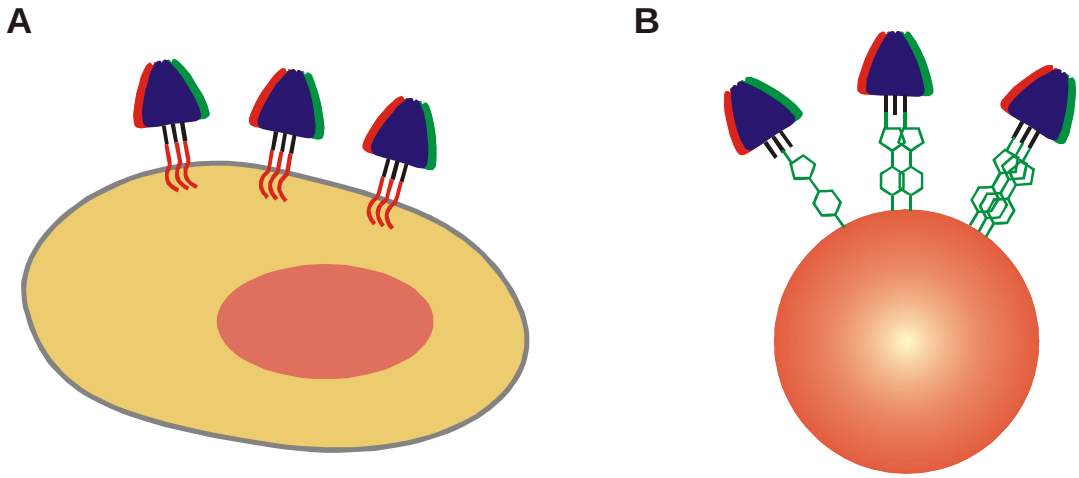


Figure 20: **TNF-exposure on cells and on beads. A.** A cell presenting membrane TNF. TNF trimers are formed in the cytosol of the cell and translocated to the cell membrane as type II transmembrane proteins. **B.** wtCysHisTNF covalently bound to a micro-particle. A Cysteine-residue fused to the N-terminus of soluble TNF forms a C-S-thioether with a maleimide-group on the beads-surface. Trimer formation may occur with freely diffusing, non-bound TNF-molecules or with molecules attached in the direct vicinity.

The amounts of TNF that had been calculated to be bound on the beads after labelling revealed similar absolute quantities for sTNF and wtCysHisTNF (see table 2). However, during the preparation of the ligand samples for the final coupling step much more CysHisTNF was lost as compared to wild type TNF (table 2). This might indicate that CysHisTNF has a higher tendency to unspecifically adhere to surfaces in general as compared to wild type TNF. On the other hand, as in the final coupling step the available TNF concentrations were quite different (table 2), unspecific binding might be also different, as it is assumed to be proportional to the respective TNF concentration. In any case, the differential behaviour of the two TNF species that have been compared in

these experiments (summarized in table 2) might complicate (half)quantitative estimations even more.

When the amount of TNF bound to beads was calculated as percentage of the TNF input, we found increasing quantities of TNF with reduced size of the beads. These data suggest that the quantity of TNF used to coat the beads is sufficient to saturate all maleimide-groups. This can be concluded from the input values in the final coupling step, ranging from 7.2 to 41.3 μg ligand per mg beads, but resulting in near to constant amounts of coupled material (4.4 and 6.7 μg in these two respective groups; see table 2). Taken together, these data suggest that the higher bioactivity of wtCysHisTNF-coated particles compared to sTNF-labelled beads might be caused by covalent, directed coupling of wtCysHisTNF-molecules.

To further assess the directed and covalent binding of wtCysHisTNF onto particles, several methods to suppress the adsorptive effect of the beads were employed. Poly-ethylen-glycol (PEG) was chosen as a hydrophilic, protein repulsive surface superimposed onto positively charged 3-amino-propyl-trimethoxy-silan (APS), which surrounds the silica core of the beads. Another approach was to coat particles with sulfhydryl-BSA, a protein whose SH-groups of free cysteines had been inactivated, prior to the addition of wtCysHisTNF to the beads. The third approach involved prolonged washing of the beads after the coupling reaction with buffers supplied with various strong detergents. These methods failed to suppress the adsorption of TNF to beads, since beads treated in the described manners maintained their bioactivity without significant loss.

One reason for this might be electrostatic interactions of the positively charged APS-coat covering the beads with negatively charged TNF. The labelling reaction is performed at pH 7.2 to allow the formation of covalent C-S-thioethers. At this pH value, TNF has a slightly negative charge, since its isoelectric point is known to be around 6. However, pH-values for the optimal reaction between the maleimide groups on the beads surface and the Cysteine residues of wtCysHisTNF range narrowly between pH 7.0 and 7.4. This does not allow the labelling of beads in a slightly acidic environment in order to provide TNF molecules without charge, since the formation of covalent bonds would be hindered. Another reason for the very strong adsorption of TNF to beads might be the rather small size of TNF-molecules. These may be able to enter the porous outer layers of the beads superficially and stay firmly embedded due to the generally strong adsorption.

Another method to avoid unspecific adsorption of TNF to beads would be to change the basic material to generate particles. Application of the particles in patients would indeed require a biodegradable substance, so the employment of poly lactic acid (PLA) as material for the generation of beads was proposed. Preliminary experiments in fact have demonstrated that adsorption of TNF to lactic acid derived beads is neglectable (Ingo Grunwald, personal communication).

The adsorption of TNF to silica particles was firm enough to allow the storage of beads without significant loss of bioactivity over several weeks (fig. 4). However, some TNF could be detected in the supernatant of stored beads (data not shown). Dissociation and re-adsorption of TNF from and to beads might take place and/or the disintegration and formation of trimers, until some equilibrium might be reached. The latter process might be counteracted by the creation of a TNF-molecule that is formed of three TNF-monomers being fused to each other. Successful design of this single-chain-TNF (scTNF) and preliminary experiments with beads labelled with scCysTNF have been performed. However, these beads showed no enhanced bioactivity compared with particles coated with monomeric wtCysHisTNF and also displayed significant adsorptive binding (data not shown).

After the coupling of TNF, the beads showed bioactivity on different TNF sensitive cell lines, however, the specificity of TNF-coated beads had to be verified. It was possible that the binding of TNF to beads might generate particles with some unspecific toxicity or TNF might be damaged during the labelling process and toxic degradation products also from the used chemical agents may arise. Such processes could lead to the induction of apoptosis or necrosis by the mechanisms different from TNF receptor stimulation. To address the question of the selectivity, TNF-coated beads were tested on the parental immortalised mouse fibroblasts, deficient in TNFR1 and TNFR2 (fig. 3b). These cells showed no cytotoxic response, indicating that TNFR1 and/or TNFR2 are the receptors that are specifically stimulated by the beads.

The finding that TNF-coated beads are TNF-receptor specific was strengthened further by the use of TNF-mutants binding either to TNFR1 or TNFR2. On mouse fibroblasts stably expressing one of the receptor chimeras TNFR1/Fas or TNFR2/Fas, R32W/S86T-CysHisTNF and D143N/A145R-CysHisTNF were shown to possess a considerable selectivity (figs. 6a-d). This was not necessarily expected, because it had been reasoned by us before that the strong receptor selectivity of the soluble TNF muteins might be reduced or even vanished by avidity mechanisms when the cytokines

had been coupled to the beads. As expected, on the other side, the TNFR2 selective mutein 143NCysHisTNF, similar to wild type TNF, was highly active on TNFR2 (TNFR/Fas chimera), when coupled to the beads.

The LD_{50} of wtCysHisTNF coated beads was found to be in the range of 0.15 µg beads/well in a standard cytotoxicity assay (fig. 5), i.e, 1.4 x 10^8 particles induced apoptosis in half of 20,000 cells. This method to assess the bioactivity of the beads is inadequate, however, since the particles, unable to become distributed by diffusion, might be only statistically come into contact with the target cells. For this reason, the memTNF-bioactivity of TNF-coated beads was investigated exactly using a single cell assay system. TNFR2/Fas expressing mouse fibroblasts, which had been shown to undergo apoptosis within 45 min, were treated with small amounts of beads of 1 µm and 10 µm diameter, respectively, and monitored by confocal laser scanning microscopy. Apoptosis could be induced by only 6 1 µm-beads or a single 10 µm-bead (see table 3, fig. 7). Assuming that the percentage of coverage of the bead surface by the cell is similar for 1µm and 100 nm-beads, 6 of the 1 µm-beads would then be equivalent to 600 particles of 100 nm diameter. Accordingly, 600 beads of 100 nm size should represent the minimum to kill a cell in a cytotoxicity assay system. An about 20 fold higher number was necessary to obtain the LD_{50}, which was roughly equivalent to the 66% cytotoxicity in the statistically evaluated experiment using the 1 µm-beads. These data indicate that in a typical cytotoxicity assay using TNF-coupled beads, only about 10% of the particles had contact to cells to exert their cytotoxic effects.

The LD_{50} value of soluble wtCysHisTNF tested in a cytotoxicity assay on the same cell line pre-incubated with 80M2 was found to be at 0.3 ng/ml (fig. 3c). We have shown that the dissociation constant (K_d value) at 37°C for sTNF stabilised by 80M2 on TNFR2 is 1.7 x 10^{-11} M (Scheurich, unpublished data). The LD_{50} value of soluble wtCysHisTNF + 80M2 therefore is 1/3 of the K_d value, indicating that about 6750 of 45,000 receptors expressed on TNFR2/Fas mouse fibroblasts are occupied by TNF molecules. Since the cell undergoes apoptosis very rapidly, the receptors are engaged only once during this process. In contrast, 6 beads of 1 µm diameter contain approximately 200,000 wtCysHisTNF molecules localised on their surface. Of these beads, only 1/3 of their surface was estimated to be in direct contact with the cell, i.e. capable to engage receptors. One bead has a surface of about 3.1 μm^2 while the surface of the average mouse fibroblast amounts to approximately 1250 μm^2. Therefore,

the contact area between six beads and one cell covers about 6.3 μm^2 or 0.5% of the cell surface. Assuming that the 45,000 receptor molecules expressed by the cell are statistically distributed in the membrane, approximately 225 receptor molecules would be assembled at the contact sites between beads and the cell to interact with TNF-molecules presented on the beads surface. Lateral diffusion and activation of unstimulated TNFR2 molecules (that will be discussed in detail later on) occurs only from the direct vicinity to the contact site between beads and cell (fig. 11). This event is estimated to trigger the activation of less than three fold more molecules, i.e. at most 675 receptors. The by far larger part of the receptor molecules remains unsitmulated.Thus, the bioactivity of wtCysHisTNF immobilised on beads is about ten fold enhanced compared to the so far most efficient memTNF analogue, sTNF or wtCysHisTNF in the presence of the antibody 80M2.

Cells from various human tissues have been shown to express between 300 and a few thousand endogenous TNF receptors. These amounts are in good accordance with our calculations estimating that a few hundred receptor molecules can be sufficient to induce apoptosis when engaged by a membrane TNF analogue.

Signal transduction in TNFR/Fas chimera. Mouse fibroblasts expressing TNFR1/Fas and TNFR2/Fas designed by Krippner-Heidenreich et al. (2002) were used to assess the bioactivity and TNFR-selectivity of silica-particles compared with soluble memTNF equivalents. Krippner-Heidenreich et al. demonstrated that the responses of TNFR1 and TNFR2 could be transferred to the Fas-signalling system, thus allowing an extra-cellular differential stimulation which resulted in identical intracellular signal transduction (Krippner-Heidenreich *et al.*, 2002). Thus the receptor chimeras allowed investigation of differences between TNF-receptors selectively stimulated with soluble or membrane bound TNF, but unaffected by distinct cytosolic domains. The conversion of TNFR2 into an apoptosis-inducing receptor by fusing its extra-cellular and membrane spanning domains with the cytosolic domain of Fas and its stable expression in TNFR1/TNFR2 double knockout mouse fibroblasts resulted in a cell line that succumbed to apoptotic signals within less than an hour. This permitted rapid live cell imaging assays with the confocal microscope without an additional treatment of the cells with e.g. CHX to inhibit protein-synthesis, as would have been necessary in assays with other cell lines.

In figs. 8a and b, recruitment of FADD to the TNFR1/Fas chimera was shown upon stimulation of the mouse fibroblasts with sTNF. The same result was achieved

when cells were stimulated with wtCysHisTNF-coated beads (figs. 8d-e), however, the FADD recruitment was spatially restricted to the contact site between cell and bead. These data suggest, moreover, that the intracellular signal transduction pathway known to be characteristic for Fas is not disturbed by the superimposition of a different extracellular portion of receptor, and that TNF bound to particles is able to exert a normal response from cells upon receptor-stimulation. A crucial event during signal transduction mediated by members of the TNFR-superfamily is the clustering of receptor molecules. Since the activation of Fas or TNFR1 as well as TNFR2 depends on this event, the superimposition of one receptor-domain onto another should not interfere with clustering.

Signal transduction in TNFR2/Fas mouse fibroblasts showed a different pattern. Whereas sTNF was not able to induce signal transduction, the memTNF equivalent sTNF stabilised with 80M2 at the receptor stimulated recruitment of both FADD and caspase-8, the protein activated downstream of FADD. Again wtCysHis labelled beads were able to elicit the same reaction as sTNF + 80M2, clearly indicating that the beads convert soluble wtCysHisTNF into an efficient membrane TNF analogue, capable to activate TNFR2. The mechanism which allows activation of TNFR2 by memTNF, proposed by Krippner-Heidenreich et al. (2002), was prolonged and stabilised contact between TNF and TNFR2. The half-life of ligand/receptor complexes was shown to be enhanced about 10 fold by stabilising TNF with 80M2 at the receptor. The conversion of sTNF into a memTNF analogue upon binding wtCysHisTNF onto beads might be caused by the same mechanism. TNF coupled to beads is immobilised and not longer able to diffuse freely. Contact of the TNF-loaded bead-surface with a TNFR2-expressing cell therefore results in a stable, prolonged contact between ligand and receptor.

The TNFR1/Fas or TNFR2/Fas expressing mouse fibroblasts served further to investigate possible cross reactivity of beads coupled with TNF-receptor-selective mutants. In fact, the data obtained indicate that 32WCysHisTNF shows indeed slight cross reactivity on TNFR2 at high concentrations, when coupled to beads or added to cells after they had been pre-incubated with 80M2 (Sylvia Willi, personal communication). However, significant cross reactivity occurred at concentrations higher than generally employed for receptor-selective stimulation. Moreover, when 32WCysHisTNF is used in experiments for selective TNFR1 stimulation, this will be typically not performed in the presence of the TNFR2-specific antibody 80M2.

Accordingly, this cross-reactivity must not be taken into account in the very most experiments.

Selective activation of the TNFR2/Fas chimera could be successfully achieved with wtCysHisTNF coated beads. In addition, the effect of sTNF- and memTNF-derivatives on wild type TNFR2 were investigated. As published by Grell et al. (1995), wild type TNFR2 cannot be fully activated by sTNF, whereas pre-treatment of cells with the TNFR2-specific antibody 80M2 prior to stimulation with sTNF or the TNFR2-selective mutant 143NCysHisTNF (Grell et al. 1995) is capable to do so. Stimulation of HeLa80 cells with wtCysHisTNF labelled beads resulted in the formation of receptor clusters and recruitment of TRAF2 to the membrane (see fig. 10). These results suggest that TRAF2-recruitment was not based on TRAF2-overexpression, but was in fact caused by the memTNF derivative employed here, in contrast to wild type sTNF. Fotin-Mleczek (2002) could demonstrate that TRAF2 is recruited to receptor-ligand complexes formed between TNFR2-expressing and memTNF-expressing cells. These complexes containing memTNF-TNFR2-TRAF2 did not internalise and were stable for several hours (Fotin-Mleczek, 2002). However, the stability and inability of these complexes to internalise might constitute side-effects caused by interactions between the two different cells. In experiments with wtCysHis-labelled beads of 10 µm diameter (see fig. 10c), which present an otherwise inert surface, the data discussed above could be verified, indicating that exclusive ligand-receptor interaction was responsible for the formation of stable memTNF-TNFR2-TRAF2-complexes.

Receptor-clustering and internalisation of ligand-receptor-complexes. An interesting feature of activation of some receptors is the lateral signal propagation upon ligand binding, as has been demonstrated for the EGF-receptor (Reynolds *et al.*, 2003; Verveer *et al.*, 2000). Lateral signal propagation was investigated with the aid of epidermal growth factor (EGF) covalently attached to beads. Upon focal stimulation of EGFR with these beads, lateral phosphorylation of non-initiated receptors over the entire cell could be observed, resulting in full activation of all receptors. To address the question whether a similar mechanism would exist for TNFR2, we focally stimulated a single HeLa cell over-expressing full length TNFR2-GFP with one wtCysHisTNF-labelled 10 µm-bead (fig. 11). A rapid accumulation of receptor-clusters at the bead-cell contact site could be observed. This was accompanied by a loss of fluorescence intensity of the

cell membrane surrounding the bead, indicating that the regions adjacent to the contact site lost their receptor molecules, likely by diffusion. This observation suggests that during the stimulation lateral diffusion of non-stimulated receptor molecules in the direct vicinity of the particle towards the bead took place. Regions more distant to the area of bead-cell-interactions showed neither clustering nor a loss in fluorescence intensity. TRAF2-recruitment, in a focally stimulated HeLa80 cell over-expressing TRAF2-GFP, as well as FADD-recruitment in TNFR2/Fas mouse fibroblasts stimulated with few wtCysHisTNF-beads of 1 µm diameter, was strictly restricted to the interaction site between bead and cell (figs. 9 and 10c). The same results were obtained in cells which expressed TRAF2-GFP only very weakly, indicating that the spatially restricted recruitment of TRAF2 to the area where TNFR2-molecules were activated by beads-bound wtCysHisTNF was not dependent on over-expression. Taken together, these findings indicate furthermore that unstimulated receptor molecules could not be activated through lateral signal propagation as has been described for the EGFR by Reynolds et al. (2003).

Receptor oligomerisation and complex formation upon stimulation with a mono- or multimeric ligand seems to be a common trait in multi-cellular organisms (Stroud and Wells, 2004). Several members of the TNF-receptor superfamily have been shown to form higher order aggregates upon initiation with their respective ligands, amongst them Fas (Algeciras-Schimnich *et al.*, 2002; Grassme *et al.*, 2001) and TNFR1 (Cottin *et al.*, 2002; Ko *et al.*, 1999; Legler *et al.*, 2003). For both receptors, internalisation has been shown to be a part of the normal signal transduction events after stimulation with their respective ligands (Algeciras-Schimnich *et al.*, 2002; Higuchi and Aggarwal, 1994; Schneider-Brachert *et al.*, 2004; Schutze *et al.*, 1999). Algeciras-Schimnich et al. proposed that the internalisation of activated Fas was important to down-regulate apoptotic signals and to protect neighbouring cells or macrophages from harmful contact with FasL. Higuchi and Aggarwal claimed the same mechanism for down-modulation of TNFR1 after stimulation, whereas TNFR2 was down-regulated by shedding through cleavage by TACE (Higuchi and Aggarwal, 1994; Solomon *et al.*, 1999). In contrast, Schütze et al. defined the internalisation of TNFR1 as a necessary step to trigger stimulation of acidic sphingomyelinase (ASMase), activation of JNK and apoptosis. In this work, internalisation of TNFR2 could be shown within 45 min after stimulation with memTNF equivalents (fig. 12). Di Guglielmo et al. were able to detect a small quantity of

internalised TGFβ-receptors in early endosomes after the receptor had internalised partially into clathrin coated vesicles. For this receptor, two different ways of internalisation have been shown, the other one being dependent on caveolae. Further investigations of this group indicated that the portion of TGFβ-receptor being internalised by coated vesicles did not enter late endosomes/early lysosomes but underwent recycling back to the membrane (Di Guglielmo *et al.*, 2003). TNFR2 could be detected partially in late endosomes as well as in caveolae (figs. 12b and c). Whether this is an indication that the two-way mechanism of endocytosis operative for the TGFβ-receptor, which regulates distinct destinations for the internalised molecules, holds also true for TNFR2 would have to be determined in future experiments.

Dynamin, a protein crucial for various endocytotic pathways, is known to oligomerise, forming a “collar” around the necks of deep invaginations of varied composition. Clathrin coated vesicles as well as caveolae and a form of clathrin and caveolin independent endocytosis process are dynamin dependent. Endocytosis of TNFR2 was inhibited by a dominant negative form of dynamin-1 (fig. 14), indicating that either caveolae or both caveolae and clathrin coated vesicles were involved. These data are supported by the time-frame established for TNFR2-internalisation. While clathrin mediated endocytosis, being crucial for synaptic function and synaptic vesicle recycling, is known to be a rapid event, caveolae have been shown to internalise very slowly (Conner and Schmid, 2003). TNFR2 shows complete endocytosis after approximately 45 min, a result that favours a caveolae-driven mechanism. However, since early endosomes form and transform very rapidly, the use of early endosomal markers might shed light on the question whether TNFR2 is also internalised in clathrin coated vesicles. These findings, together with data showing that TNFR2 stained with 80M2-Alexa-Fluor-546 clusters and internalises upon stimulation with sTNF, resulting in a more intense fluorescence, and the recruitment of TRAF2 to the beads-cell-contact site of a HeLa80 cell locally stimulated with a wtCysHisTNF-labelled bead, are in contrast to the findings of Higuchi and Aggarwal, stating that TNFR2 is down-regulated by shedding (Higuchi and Aggarwal, 1994). Furthermore, stimulation of TNFR2 with memTNF presented by a different cell line (Fotin-Mleczek, 2002), or with wtCysHisTNF bound to a bead of 10 µm diameter as shown in this study, lead to the formation of stable ligand-receptor clusters which showed no internalisation at all. Moreover, the complex formation between TNF and TNFR2 was strong enough to cause destruction of the cell

when the wtCysHisTNF-coated bead was separated by micromanipulation from its target cell (fig. 11).

Evidence has been presented that TNFR1 recruitment into lipid rafts and clustering depends on its death domain (Cottin *et al.*, 2002). Mutants of TNFR2, which had the cytosolic domain of TNFR2 replaced by either GFP or the cytosolic domain of Fas, could be induced to cluster upon stimulation with a memTNF equivalent. Internalisation, however, was completely lacking in comparison to wild type receptors (fig. 15b and data not shown). This indicates that the cytosolic domain of TNFR2 is indispensable for endocytosis of this receptor.

Having demonstrated the dynamin dependence of wild type TNFR2-internalisation after stimulation with the soluble memTNF equivalent 143NCysHisTNF + 80M2, the next step was to investigate whether beads of an adequate size would be internalised in a TNFR2-dependent manner. For this purpose, different cell lines were treated with wtCysHisTNF-labelled beads of 1µm diameter and the localisation of the beads was followed (fig. 13). Similar to wild type TNFR2 in HeLa80 cells, the beads apparently internalised into Kym1 cells as well as into HeLa80 cells. They could, however, not be localised in late endosomes labelled with CFP-fused RhoB. In contrast, localisation of beads in lysosomes could be shown approximately 16 h after stimulation of the HeLa80 cells with beads, independent of their coating. This indicates that internalisation was independent on the interaction of the beads with TNFR2 and on the cell type thus following a different pathway as compared to TNFR2. Internalisation of beads into TNFR2 negative HeLadynK44A cells, expressing a dominant negative mutant of dynamin-1, occurred similarly, indicating a dynamin-independent pathway of endocytosis. Of several known entryways into cells, most of the pinocytotic mechanisms can be discarded, since they are specific for cargo sizes of 120 nm and less. However, macropinocytosis and phagocytosis are two possible endocytotic mechanisms which are able to take up particles with a size of 1 µm and more (Conner and Schmid, 2003). Phagocytosis is a highly regulated process involving cell-surface receptors and signalling cascades mediated by Rho-family GTPases and can be inhibited by dominant negative dynamin-2. Macropinocytosis, on the other hand, is believed to play a role in the down-regulation of activated signalling molecules and is completely dynamin independent. Future experiments will have to be performed to determine which way of endocytosis is used for internalisation of beads into HeLa80 and Kym1 cells.

TNFR2 mediated signal transduction. The signalling pathways initiated by TNFR1, the death domain positive member of the two TNF-receptors, have been studied extensively (Wajant *et al.*, 2003). The investigation of TNFR2 mediated signal transduction, however, has long been obstructed by a lack of tools to stimulate this receptor adequately. In recent years, upon discovery that TNFR2 is specifically stimulated by the membrane bound form of TNF, several signal transduction pathways have been established for TNFR2, amongst them activation of JNK (Jupp *et al.*, 2001), NFκB-activation (Rothe *et al.*, 1995), production of GM-CSF (Vandenabeele *et al.*, 1992), but also the induction of apoptosis in some cellular systems (Bigda *et al.*, 1994; Grell *et al.*, 1993; Grell *et al.*, 1994; Heller *et al.*, 1992; Medvedev *et al.*, 1994; Zheng *et al.*, 1995). Molecular crosstalk between the two TNF-receptors has been clearly established in a recent publication (Fotin-Mleczek *et al.*, 2002), therefore it is not surprising to find that both receptors have some proteins in common that play a role in their respective signal transduction pathways. TRAF2 is not only one of the essential components for TNFR1 mediated NFκB-activation (Devin *et al.*, 2000; Devin *et al.*, 2001), it is moreover a bifurcation point in TNFR1-signalling towards apoptosis or cell survival that is regulated by the order of stimulation of the two TNF receptors (Fotin-Mleczek *et al.*, 2002). Exclusive stimulation of TNFR2 leads to the direct recruitment of TRAF2 to its cytosolic TRAF2-binding domain and the activation of NFκB, although this event is generally less potent than NFκB-activation induced by TNFR1. Two major and different ways of NFκB-activation are currently discussed. The canonical pathway depends on IKKβ and IKKγ as essential components of an IKKα-IKKβ-IKKγ/NEMO hetero-trimeric complex for the activation of NFκB in response to pro-inflammatory stimuli and for the inhibition of apoptosis (Chu *et al.*, 1999; Li *et al.*, 1999a; Li *et al.*, 1999b). The activated IKK-complex catalyses the phosphorylation of IκBs, leading to ubiquitination, thus releasing most commonly a p50/p65 dimer which translocates into the nucleus. Recently, an alternative, slowly acting pathway has been established, which strictly depends on IKKα to target p100 for phosphorylation and processing into active p52 (Coope *et al.*, 2002; Dejardin *et al.*, 2002; Senftleben and Karin, 2002). As non death domain containing members of the TNF receptor superfamily, like CD40 and the LTß receptor, do activate this alternative pathway, it was of great interest to investigate TNFR2 in this respect. Receptor selective stimulation of HeLa cells stably over-expressing TNFR2 showed a similar kinetics of NFκB-translocation into the nucleus with full activation after 30 min for TNFR1 as well as

TNFR2 stimulation (fig. 16). Of the five known NFκB-subunits, RelA/p65, c-Rel and p50 were investigated regarding their translocation into the nucleus. p65 was found to be translocated predominantly, together with very small amounts of p50 and c-Rel. These data indicate that both TNFR1 and TNFR2 activate the canonical, IKKβ-IKKγ/NEMO dependent NFκB-pathway. Further confirmation was found in experiments employing IKKγ/NEMO deficient Jurkat cells, stably over-expressing TNFR2. NFκB-translocation into the nucleus was completely inhibited in these cells, regardless whether TNFR1 or TNFR2 had been selectively stimulated (fig. 17). These results are in good accordance with a recent publication regarding the NFκB-activation in primary cortical neurons (Marchetti *et al.*, 2004). Also in these cells TNFR1 as well as TNFR2 were found to activate the translocation of p65/p50 heterodimers into the nucleus. The respective time kinetics of NFκB-activation differed, however, dramatically. Whereas TNFR1 elicited a rapid and transient NFκB-translocation into the nucleus, stimulation of TNFR2 led to a prolonged, protein kinase B/Akt phosphorylation dependent response. The difference in the NFκB-activation patterns between primary cortical neurons and HeLa80 cells might be explained with the role of TNFR2 as a TNFR1 antagonist with neuroprotecive function in mouse brains, whereas in somatic cell lines, TNFR2 acts as a regulator of TNFR1 mediated signal transduction (Chan and Lenardo, 2000; Fotin-Mleczek *et al.*, 2002; Li *et al.*, 2002). However, both sets of experimental settings support the findings that engagement of TNFR1 or TNFR2 does not result in the activation of the alternative pathway.

Essential components of the NFκB-pathway mediated by TNFR1 are TRAF2 as well as RIP. Whereas TRAF2 recruits the IKK components to the activated TNFR1 by interaction with IKKβ, RIP supports recruitment of the IKK complex by interaction with IKKγ/NEMO, thus playing a role in the activation of the IKK complex via its intermediary domain (Devin *et al.*, 2000; Kelliher *et al.*, 1998; Zhang *et al.*, 2000). Another protein interacting with IKKγ/NEMO is A20, which is a negative regulator of TNFR1-mediated NFκB-activation, supposedly by several mechanisms which include the phosphorylation of the IKKs (Delhase *et al.*, 1999; Zhang *et al.*, 2000). Since NFκB is known to similarly depend on TRAF2 when activated by TNFR2 (Rothe *et al.*, 1995) it is tempting to speculate that RIP may also participate in this signalling pathway. However, experiments with Jurkat cells deficient in RIP and stably over-expressing TNFR2

revealed a RIP-independent NFκB-translocation after TNFR2-selective stimulation with the memTNF-equivalent 143NCysHisTNF + 80M2 (fig. 18). This supports other data which demonstrated that TNFR2 was able to activate NFκB independently of RIP (Pimentel-Muinos and Seed, 1999). Nevertheless, RIP was proposed to have a different function regarding TNFR2-signalling. In contrast to activated TNFR1, where RIP mediates cell survival signals, RIP interaction with TNFR2 appears either to provoke caspase dependent cell death in Jurkat cells (Pimentel-Muinos and Seed, 1999) or to enhance caspase dependent programmed necrosis mediated by TNFR1 (Chan *et al.*, 2003). A similar mechanism was proposed for a Fas-mediated, caspase-independent necrosis using RIP as an effector molecule (Holler *et al.*, 2000). In this scenario, the kinase domain of RIP is indispensable for signal transduction.

Since TNFR2 mediated NFκB-activation seems to depend on TRAF2 but not on RIP, the mechanism employed to activate the IKK-complex remains unclear. TRAF2 alone might be responsible for its recruitment and activation or another, as yet unknown mechanism may participate in the recruitment or activation of the IKK complex. Whether this unknown player would have to contain a kinase activity is debatable, since RIP-mediated NFκB-activation upon TNFR1-stimulation does not depend on its kinase domain, but rather the intermediary domain. Moreover, IKK-phosphorylation by A20 appears to regulate NFκB-activation negatively (Delhase *et al.*, 1999; Zhang *et al.*, 2000). Taken together, these data indicate a differential role for RIP and TRAF2 participating in the signal transduction of the two distinct TNF receptors. The differences in function can be ascribed to the presence of several domains applying for different tasks as is the case for RIP, or to the differential activation of the protein by distinct signals, as happens with TRAF2.

Another common trait between TNFR1 and TNFR2 with different outcome is the receptor internalisation. Inhibition of the internalisation of activated TNFR1 abolished JNK-activation and apoptosis (Schutze *et al.*, 1999). These findings were refined by further experiments indicating that FADD and Caspase-8 are not recruited to the initial, membrane-proximal TNFR1-complex (Harper *et al.*, 2003; Micheau and Tschopp, 2003). On the contrary, upon recruitment of TRADD to TNFR1 and TRAF2 and RIP to TRADD, a complex formed by TRADD-RIP-TRAF2 was shown to dissociate from TNFR1 and to form a cytosolic secondary complex with FADD and Caspase-8. In contrast to these findings, inhibition of TNFR2-internalisation by a dominant negative dynamin-mutant had

apparently no impact on TRAF2-recruitment (fig. 19). These data concur with the observation that receptor molecules, co-localising with TRAF2, remained localized at the membrane for a prolonged time, although TNFR2 internalised within 45 minutes when it had been treated with the TNFR2 selective memTNF equivalent 143NCysHisTNF + 80M2.

sTNF and memTNF may elicit signal transduction by differential molecular mechanisms. Complexes formed between memTNF and TNFR2 are not able to internalise, as shown by Fotin-Mleczek (2002). Nevertheless, signal transduction is operative in these complexes, as has been demonstrated by recruitment of TRAF2 and FADD to stimulated TNFR2 and TNFR2/Fas chimera, respectively. In mice free of secreted TNF but expressing a memTNF variant that cannot be processed by TACE into sTNF (memTNF$^{\Delta/\Delta}$ mice), memTNF was shown to participate in many signalling processes. However, memTNF alone was not sufficient to drive inflammatory processes. In these mice, clinical sings were significantly delayed and reduced when compared to EAE-infected wild type mice (Ruuls *et al.*, 2001). This implies different roles for sTNF and memTNF: whereas sTNF is more important for the execution of inflammatory responses, memTNF plays a role in the development of secondary lymphoid organs and chemokine expression.

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