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A molecular genetic analysis of TNFR1 regulation in SPRET/Ei and generation of new TNFR1 inhibitory tools

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Thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR IN SCIENCES – BIOTECHNOLOGY

Promotor: Prof. Dr. Claude Libert

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Summary

Tumor necrosis factor (TNF) is a powerful pro-inflammatory cytokine that plays a key role in several inflammatory diseases, such as sepsis and autoimmune diseases. TNFR1, the main receptor for TNF, is involved in inducing and maintaining inflammation, cell proliferation, cell survival and apoptosis, while TNFR2 functions in the development of regulatory immune cells and initiates healing after inflammation. Although our knowledge of TNF biology is not yet complete, TNF inhibitors are successfully being used to treat patients suffering from these diseases. A significant proportion of the patients however do not respond to anti-TNF agents and long-term use of these drugs can cause side-effects, such as opportunistic infections and the development of additional autoimmune diseases or lymphomas. These side-effects and the lack of effect in sepsis patients are thought to be caused by inhibition of stimulation of the immunomodulatory TNFR2. More specific targeting of the pathological actions of TNF by selectively blocking TNFR1 signaling while leaving TNFR2 signaling intact may therefore be a better therapy for TNF-induced chronic diseases.

That TNFR1 is an interesting drug target was recently shown by TNFR1+/- mice which are totally resistant against TNF-induced inflammation and by using specific TNFR1 blocking antibodies. In the search for new therapeutic targets for TNF-mediated diseases, the poorly understood regulation of the TNFR1 coding gene, *Tnfrsf1a*, should be investigated. SPRET/Ei, an inbred mouse strain derived from *Mus spretus*, is extremely resistant to TNF-induced inflammation and this trait is genetically linked to a locus on proximal chromosome 2 and a locus on distal chromosome 6, harboring the *Tnfrsf1a* gene. However, despite the sequence variations in the SPRET/Ei T*nfrsf1a* gene, this TNFR1 is fully functional. Since the resistance to TNF was found to strongly depend on the TNFR1 levels, we studied whether the resistance of SPRET/Ei is attributable to a quantitative effect at the TNFR1 locus.

In the present thesis, we show that SPRET/Ei mice have a normal mRNA level of TNFR1, but display a very low expression of the TNFR1 protein, suggesting that the post-transcriptional regulation of TNFR1 in SPRET/Ei may be differential from susceptible strains such as C57BL/6. We could genetically link this quantitative trait to the same chromosomal loci as were found for TNF resistance, indicating that TNF resistance and TNFR1 expression in SPRET/Ei are indeed correlated. Since B.S^{chr6} and B.S^{chr2} congenic mice were not resistant to TNF and had equal TNFR1 levels as C57BL/6 mice, this relation proved to point to an *in trans* regulation of the TNFR1 protein expression. In the locus on proximal chromosome 2, we found miR-511, being a predicted TNFR1-regulating microRNA. This miR was found to be significantly higher expressed in SPRET/Ei and confirmed to be a genuine regulator of TNFR1 as seen *in vitro* as well as in mice. In mice, transient, liver-specific overexpression of miR-511 down-regulates TNFR1 and protects against TNF-induced lethality, while anti miR-511 up-regulates TNFR1 and sensitizes for TNF. We demonstrate specificity for TNFR1 and suggest therapeutic potential in endotoxemia and hepatitis. Since also (BxS)F1 mice were sensitized by this anti miR delivery, these data may suggest that part of the extremely robust TNF resistance of SPRET/Ei mice may depend on miR-511.

Moreover, we show that the expression of miR-511 is induced by glucocorticoids (GCs). SPRET/Ei mice were previously found to express high corticosterone levels as well as high levels of the glucocorticoid receptor (GR), which lead to high basal expression of GR-induced genes. We prove that the TNF resistance of SPRET/Ei mice is completely depending on the overactive HPA axis of these mice since irreversible blocking of the GR sensitizes SPRET/Ei mice for TNF, completely wiping out the differences in TNF response between SPRET/Ei and C57BL/6 mice. Removal of the adrenal glands led to a marked decrease of miR-511 and to an increase of TNFR1 protein expression, while treatment with Dexamethasone led to an induction of miR-511 and reduction of TNFR1, also in the serum, suggesting a mechanism independent of shedding. We therefore hypothesize that GCs protect against TNF partly via induction of miR-511 and down-regulation of TNFR1.

Furthermore, we developed a new generation of TNFR1 blockers. We generated a nanobody against human TNFR1 which shows strong binding affinity to the extracellular part of TNFR1, thereby competing with the ligand. *In vitro*, the nanobody inhibits TNF-induced NF-kB activation to the same extends as a commercially available monoclonal antibody. This nanobody will be further characterized *in vivo* to prove its therapeutic potential against TNFR1-mediated diseases. Nanobodies might have many advantages compared to monoclonal antibodies. They are encoded by single genes, which allow the design of modular proteins by combining them with each other or with other molecules, and they are extremely stable, which allows passage through the gastrointestinal tract. This offers the potential of oral administration to treat inflammatory bowel diseases, colon cancer or other disorders of the gut.

Samenvatting

Tumor necrosis factor (TNF) is een krachtig pro-inflammatoir cytokine dat een belangrijke rol speelt bij vele ontstekingsziekten zoals sepsis en auto-immuunziekten. TNFR1, de voornaamste receptor voor TNF is vooral betrokken bij het induceren en onderhouden van de ontsteking, bij cel-proliferatie, -overleving en apoptosis, terwijl TNFR2 belangrijk is in de ontwikkeling van regulerende immuuncellen en de initiatie van genezing na ontsteking. Hoewel onze kennis van de TNF biologie nog niet volledig is, worden TNF-remmers reeds met succes gebruikt bij patiënten die lijden aan deze ziekten. Een belangrijk deel van de patiënten reageert echter niet op deze anti-TNF middelen en langdurig gebruik ervan kan ook bijwerkingen veroorzaken zoals opportunistische infecties en de ontwikkeling van bijkomende auto-immuunziekten of lymfomen. Deze bijwerkingen en het gebrek aan effect bij patiënten met sepsis wordt waarschijnlijk veroorzaakt door remming van de immuunregulerende werking via TNFR2. Meer specifieke targeting van de pathologische werking van TNF door het selectief blokkeren van TNFR1 signalisatie, terwijl de TNFR2 signalisatie ongemoeid blijft, lijkt daarom een betere therapie voor TNF-geïnduceerde chronische ziekten.

Dat TNFR1 een interessante drug target is, werd onlangs aangetoond met TNFR1+/- muizen die volledig resistent zijn tegen TNF-geïnduceerde ontsteking en met specifieke TNFR1 blokkerende antilichamen. In de zoektocht naar nieuwe therapeutische doelwitten voor TNF-gemedieerde ziekten is het belangrijk om de tot nu toe onvoldoende gekende regulatie van het TNFR1 coderende gen, *Tnfrsf1a*, te onderzoeken. SPRET/Ei, een inteelt muis stam afgeleid van *Mus spretus*, is zeer resistent tegen TNF-geïnduceerde inflammatie en deze eigenschap is genetisch gelinkt met een locus gelegen op proximaal chromosoom 2 en een locus gelegen op distaal chromosoom 6, waar het *Tnfrsf1a* gen gelokaliseerd is. Ondanks de variaties in de SPRET/Ei sequentie van het *Tnfrsf1a* gen, is deze TNFR1 volledig functioneel. Aangezien de resistentie tegen TNF echter sterk afhankelijk blijkt van de TNFR1 niveaus, gingen we na of de resistentie van SPRET/Ei afhankelijk is van een kwantitatief effect in de TNFR1 locus.
In deze thesis tonen we aan dat SPRET/Ei muizen een normale mRNA level hebben van TNFR1, maar een zeer lage expressie van het TNFR1 eiwit, wat suggereert dat de post-transcriptionele regulatie van TNFR1 in SPRET/Ei anders verloopt dan in gevoelige stammen zoals C57BL/6. We konden dit kwantitatief kenmerk genetisch linken met dezelfde chromosomale loci als eerder gevonden voor TNF resistentie, wat aangeeft dat TNF resistentie en TNFR1 expressie in SPRET/Ei inderdaad gecorreleerd zijn. Aangezien B.S^{chr2} en B.S^{chr6} congene muizen niet resistent waren tegen TNF en gelijke TNFR1 levels vertoonden als C57BL/6 muizen, bleek deze correlatie te wijzen op een *in trans* regulatie van de TNFR1 eiwitexpressie. In de locus op proximaal chromosoom 2 vonden we miR-511, een voorspelde TNFR1-regulerende microRNA. Deze miR bleek aanzienlijk hoger geëxpresseerd in SPRET/Ei muizen en een echte regulator van TNFR1, zowel *in vitro* als in muizen. In muizen zorgt transiënte, lever-specifieke overexpressie van miR-511 voor neerregulatie van TNFR1 en

bescherming tegen TNF-geïnduceerde lethaliteit, terwijl anti miR-511 zorgt voor op-regulatie van TNFR1 en sensitisatie voor TNF. We tonen verder de specificiteit aan voor TNFR1 en suggereren therapeutisch potentieel van deze miR in endotoxemie en hepatitis. Aangezien ook (BxS)F1 muizen gesensitiseerd worden door deze anti miR toediening, kan het zijn dat de uiterst robuuste TNF resistentie van SPRET / Ei muizen deels te wijten is aan de werking van miR-511.

Bovendien tonen we aan dat de expressie van miR-511 wordt geïnduceerd door glucocorticoïden (GCn). In SPRET/Ei muizen werd eerder gevonden dat ze hoge corticosteron levels hebben, alsook hoge levels van de glucocorticoid receptor (GR), wat leidt tot een hoge basale expressie van GR-geïnduceerde genen. We bewijzen dat de TNF weerstand van SPRET/Ei muizen volledig afhankelijk is van de overactieve HPA-as in deze muizen aangezien een onomkeerbare blokkering van de GR SPRET/Ei muizen sensitiseert voor TNF, waarbij de verschillen in TNF respons tussen SPRET/Ei en C57BL/6 muizen volledig verdwijnen. Het operatief verwijderen van de bijnieren leidde tot een duidelijke vermindering van miR-511 en een stijging van de TNFR1 eiwitexpressie, terwijl behandeling met dexamethason leidde tot een inductie van miR-511 en een vermindering van TNFR1, ook in het serum, wat duidelijk maakt dat dit mechanisme onafhankelijk is van shedding door TACE. Onze hypothese is dan ook dat de bescherming van GCn tegen TNF deels te wijten is aan de inductie van miR-511 en de daaropvolgende neerregulatie van TNFR1.

Verder ontwikkelden we een nieuwe generatie van TNFR1 blokkers. We genereerden een nanobody tegen humaan TNFR1 met een sterke bindingsaffiniteit voor het extracellulaire deel van TNFR1 waardoor concurrentie optreedt met het ligand. *In vitro*, zorgt het nanobody voor blokkering van TNF-geïnduceerde NF-kB activatie in dezelfde mate als een commercieel verkrijgbaar monoklonaal antilichaam. Dit nanobody zal verder gekarakteriseerd worden *in vivo* om het therapeutisch potentieel tegen TNFR1-gemedieerde ziekten aan te tonen. Nanobodies kunnen veel voordelen hebben ten opzichte van monoklonale antilichamen. Ze worden gecodeerd door één enkel gen, waardoor het ontwerpen van modulaire eiwitten mogelijk is door ze te combineren met elkaar of met andere moleculen. Daarenboven zijn ze zeer stabiel, wat de transit door het maagdarmkanaal mogelijk maakt. Hierdoor kunnen ze oraal worden toediening om inflammatoire darmziekten, colonkanker of andere darmaandoeningen te behandelen.

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List of abbreviations

A	AA	amino acids
	Ab	antibody
	ACD	allergic contact hyper dermatitis
	ADAM17	a desintegrin and metalloproteinase 17
	ADP	adenosine diphosphate
	Adx	adrenalectomized mice
	AF	atrial fibrillation
	Ag	antigen
	Ago2	argonaute 2
	AIF	apoptosis-inducing factor
	ALT	alanine aminotransferase
	AMI	acute myocardial infarction
	ANOVA	analysis of variation
	AP-1	activator protein 1
	APC	antigen presenting cell
	ARE	AU-rich element
	ARTS	amino peptidase regulator of TNFR1 shedding
	ASO	antisense oligo
	AST	aspartate aminotransferase
	ATP	adenosine triphosphate
B	B	C57BL/6
	BA	binding affinity
	BAL	broncheo-alveolar lavage
	BBB	blood brain barrier
	BD	Behcet's disease
	Bcl-2	B-cell CLL lymphoma 2
	bFGF	basic fibroblast growth factor
	BMP	bone morphogenic protein
	bp	base pairs
	BSA	bovine serum albumin
	BZLF1	Epstein-Barr virus immediate-early protein
C	cAMP	cyclic adenosine monophosphate
	CASP	colon ascendens stent peritonitis
	CCV	clathrin-coated vesicle
	CD	Crohn's disease
	CDK	cyclin-dependent kinase
	cDNA	complement DNA
	CDR	complementarity-determining region
	CDS	coding DNA sequence
	C/EBPα	CCAAT/enhancer-binding protein α
	C.elegans	*Caenorhabditis elegans*
	c-FLIP	cellular FLICE inhibitory protein
	CH	constant heavy chain

	ChIP	chromatin immunoprecipitation
	CHS	contact hypersensitivity
	CIA	collagen-induced arthritis
	cIAP	cellular inhibitor of apoptosis
	CIM	composite interval mapping
	CK	creatine kinase
	CL	constant light chain
	CLP	cecal ligation and puncture
	cM	centi Morgan
	CMV	cytomegalovirus
	CNS	central nervous system
	conA	concanavalin A
	COX-2	cyclo-oxygenase 2
	CRD	cysteine rich domain
	CRP	C-reactive protein
	Crry	CRI-related y
	CSS	chromosome substitution strain
	CTR	control
	CXCL16	chemokine (C-X-C motif) ligand 16
	CYLD	cylindromatosis
D	DAMP	damage-associated molecule
	DAPK	death-associated protein kinase
	DC	dendritic cell
	DD	death domain
	DEX	dexamethasone
	DISC	death-inducing signaling complex
	DMEM	Dulbecco's modified Eagle medium
	DMSO	dimethylsulfoxide
	DNA	deoxyribonucleic acid
	DN-TNF	dominant-negative TNF
	DR	death receptor
	DSS	dextran sodium sulphate
E	EAE	experimental autoimmune encephalomyelitis
	EBV	Epstein-Barr virus
	EC	endothelial cell
	ECD	extracellular domain
	E.coli	*Escherichia coli*
	eIF	early translation initiation factor
	ELISA	enzyme-linked immunosorbent assay
	EM	expectation-maximization
	ENCODE	encyclopedia of DNA elements
	ER	endoplasmic reticulum
	ERK	extracellular signal-regulated kinase
	eTNFR1	exosome-associated TNFR1
	eQTL	expression QTL

F	Fab	antigen binding fragment
	FADD	Fas-associated protein with death domain
	Fc	constant fragment
	FCS	fetal calf serum
	FoxP3	forkhead box P3
	Fv	variable fragment
G	Gadd45b	growth arrest and DNA damage inducible β
	GalN	galactosamine
	GAS	IFNγ activated site
	GC	glucocorticoid
	Gilz	GC-leucin zipper
	GM-CSF	granulocyte macrophage colony stimulating factor
	GPCR	G protein-coupled receptor
	GR	GC receptor
	GRE	GR response element
	GRHB	GR half binding site
	GSK	GlaxoSmithKline
H	H3	histone 3
	HBS	HEPES buffered saline
	HCC	hepatocellular carcinoma
	HCEC	human conjunctival epithelial cell
	HD	Huntington's disease
	HDAC	histone deacetylase
	HEK	human embryonic kidney cell
	HEPES	hydroxyethyl piperazineethanesulfonic acid
	HMM	hidden Markov model
	hnRNPK	heterogeneous nuclear ribonucleoprotein K
	HOIL	heme-oxidized IRP ubiquitin ligase
	HOIP	HOIL-1 interacting protein
	HPA	Hypothalamus-pituitary gland-adrenal gland
	HPV	human papilloma virus
	HR	hart rate
	HRP	horse radish peroxidase
	HSV	herpes simplex virus
	Ht	hematocrit
	hTNFR1	human TNFR1
	HUVECs	human vascular epithelial cell
I	IBD	inflammatory bowel disease
	IC	inhibitory capacity
	ICAM	intercellular adhesion molecule
	ICD	intracellular domain
	IFN	interferon
	Ig	immunoglobin

	I-kB	inhibitor of kB
	IKK	I-kB kinase complex
	IL	interleukin
	iNOS	inducible NO synthase
	ip	intraperitoneal
	IPA	invasive pulmonary aspergillosis
	IPTG	isopropyl β-D-1-thiogalactopyranoside
	IRES	internal ribosome entry site
	IRF	IFN regulatory factor
	IRP	iron-responsive element-binding factor
	IU	international units
	iv	intravenous
J	JNK	c-Jun terminal kinase
K	K48	lysine 48
	KC	keratinocyte cytokine
	Kd	dissociation constant
	kDa	kilo Dalton
	KO	knock out
L	LDH	lactate dehydrogenase
	LDLchol	low density lipoprotein cholesterol
	LDT	low dose tolerance
	LMP	latent membrane protein
	LOD	logarithm of odds
	LPL	lipoprotein lipase
	LPS	lipopolysaccharide
	LRS	likelihood ratio statistics
	LT-α	lymphotoxin α
	LUBAC	linear ubiquitin chain assembly complex
M	mAb	monoclonal antibody
	MAPK	mitogen-activated protein kinase
	Mb	mega base
	MCAO	middle cerebral artery occlusion
	MCP-1	monocyte chemo attractant protein 1
	MEKK	MAPK kinase kinase
	MHC	major histocompatibility complex
	MIP	macrophage inflammatory protein
	miR	microRNA
	MK2	MAP-activated protein kinase 2
	MKK	MAPK kinase
	MMP13	matrix metalloproteinase 13
	MOG	myelin oligodendrocyte glycoprotein
	MRC-1	mannose receptor C type 1
	mRNA	messenger RNA

	MS	multiple sclerosis
	mTNFR1	mouse TNFR1
	MVB	multivesicular bodies
	MW	molecular weight
	MX1	myxovirus resistance 1
N	NA	nitroamilide
	NaCl	sodium chloride
	nb	nanobody
	NEMO	NF-kB essential modulator
	NFAT	nuclear factor of activated T-cells
	NF-kB	nuclear factor kappa B
	NGAL	neutrophil gelatinase-associated lipocalin
	nM	nano molar
	NO	nitric oxide
	NP4	neutrophil proteinase 4
	NSD	N-SMASE activation domain
	nt	nucleotides
O	O_3	ozone
	OD	optical density
	ORF	open reading frame
	OVA	ovalbumin
P	pA	poly A tail
	PAF	platelet activating factor
	PAGE	polyacrylamide gel electrophoresis
	PAMP	pathogen-associated molecular pattern
	PARP	poly (ADP-ribose) polymerase
	PBS	phosphate buffered saline
	PCR	polymerase chain reaction
	PDGF	platelet-derived growth factor
	PDI	protein disulfide isomerase
	PEC	pulmonary endothelial cell
	PEG	polyethylene glycol
	PGE2	prostaglandin E2
	PI3K	phosphatidylinositide 3 kinase
	pI	isoelectric point
	PITA	probability of interaction by target accessibility
	P.pastoris	*Pichia pastoris*
	PMA	phorbol myristate acetate
	pol	polymerase
Q	Q-PCR	quantitative PCR
	QTL	quantitative trait loci
R	RA	rheumatoid arthritis

	RBQ	retinoblastoma binding protein
	RFLP	restriction fragment length polymorphism
	RIP	receptor interacting protein
	RISC	RNA-induced silencing complex
	RNA	ribonucleic acid
	RNAi	RNA interference
	ROS	reactive oxygen species
	RR	respiratory rate
	RT	room temperature
	RT-PCR	reverse transcriptase PCR
	RTS1	
S	S	SPRET/Ei
	SDS	sodium dodecyl sulphate
	S.E.	standard error
	SEAP	secreted alkali phosphatase
	SF	synovial fluid
	sGC	soluble guanylate cyclase
	SGK	serum and glucocorticoid-inducible kinase
	SHANK	SH3/ankyrin domain gene
	SHARPIN	SHANK-associated RH domain interacting protein
	shRNA	short hairpin RNA
	SIM	simple interval mapping
	SIRS	systemic inflammatory response syndrome
	siRNA	small interfering RNA
	SLE	systemic lupus erythematosus
	snRNA	small nuclear RNA
	snoRNA	small nucleolar RNA
	SNP	single nucleotide polymorphism
	SOD	superoxide dismutase
	SODD	silencer of death domains
	SSCP	single strand conformation polymorphism
	SSLP	single sequence length polymorphism
	STAT	signal transducer and activator of transcription
	sTNF	soluble TNF
	SV-80	simian virus 80
T	TAB	TAK1-binding protein
	TACE	TNF-α converting enzym
	TAK1	TGFβ-activated kinase 1
	TAT	tyrosine amino transferase
	tBid	truncated Bid
	Tchol	total cholesterol count
	TG	triglycerids
	Th	T helper cell
	TLR	toll-like receptor
	TMD	trans membrane domain

	tmTNF	trans membrane TNF
	TNBS	trinitrobenzene sulfonate
	TNF	tumor necrosis factor
	TNFR1	TNF receptor 1
	TNFRSF1A	TNF receptor superfamily 1A
	TPA	12-O-tetradecanoylphorbol-13-acetate
	TPA	tissue plasminogen activator
	TRADD	TNFR-associated death domain
	TRAF	TNFR-associated factor
	TRAIL	TNF related apoptosis inducing ligand
	TRAPS	TNF receptor-associated periodic syndrome
	TRBP	HIV-1 TAR RNA binding protein
	tRNA	transfer RNA
	Treg	regulatory T-cell
	TSS	transcription start site
	TTP	tristetrapoline
U	UC	ulcerative colitis
	UTR	untranslated region
	UV	ultraviolet
V	VCAM	vascular cell adhesion molecule
	VEGF	vascular endothelial growth factor
	VH	variable heavy chain
	VHH	heavy chain antibody
	VL	variable light chain
	VNS	vagal nerve stimulation
W	WBC	white blood cell
	WT	wild type
X	XIAP	X-linked inhibitor of apoptosis protein

CHAPTER I

Introduction

1. Tumor Necrosis Factor

1.1. The discovery of TNF

The history of TNF can be traced back more than a century ago when William B. Coley discovered that cancer patients could be cured by having a bacterial infection and created a mixture of killed bacteria, since then known as Coley's Toxins. In 1893, he treated a boy, having a massive abdominal tumor, with consecutive injections of Coley's toxins into the tumor. The toxins produced the symptoms of an infectious disease, but not the disease itself. Seven months later, the remains of the tumor were hardly detectable. (1) Later it was shown that the active component of these toxins was lipopolysaccharide (LPS) from gram-negative bacteria and that serum from LPS-treated mice could induce tumor regression. In 1975, the serum factor that was secreted by the infection and that was able to mimic the LPS effect, was called tumor necrosis factor or TNF by Lloyd Old. (2) In 1984 and 1985, two different TNFs were identified. One protein of 25 kDa, was named lymphotoxin-α (LT-α) and the other cytotoxic factor of approximately 17 kDa was named human TNF-α. When determining the amino acid sequence to isolate the full-length cDNAs of the two proteins, it was seen that they had 50 % homology and the name of LTα was changed to TNF-β. (3-5) But even though they bind to common high-affinity cell surface receptors present on most cell types, both molecules are distinct. Whereas TNF-α is mainly produced by macrophages, TNF-β is mainly produced by lymphocytes. (6)

Meanwhile, Anthony Cerami and his colleagues investigated Trypanosoma infections in cattle. One of the main problems is that the animals lose rapidly a considerable amount of muscle and fat, leading to cachexia. They developed an anti-trypanosomal compound of which a single injection could completely cure mice and rats of the parasites. But when giving this compound to infected cows, they went immediately into shock and died. A good explanation was that these cows were overreacting to the infection, producing a mediator that was responsible for the cachexia. (7) Later on, Cerami and co-workers could show that the supernatant of macrophages that had been exposed to LPS or other molecules from infective organisms contained a mediator sharing many activities that had been associated with cachexia and anemia. (8-10) In 1981, a patent was submitted describing the biological activities of this mediator, named cachectin. Elevated levels of cachectin can lead to a number of human diseases including shock, cachexia and rheumatoid arthritis. When obtaining the amino acid sequence of isolated mouse cachectin, it was found to be similar to that of the recently cloned human TNF. (11) This came as a big surprise because several years before, Cerami had exchanged material with Lloyd Old's group to see if TNF had LPL suppressive activities or cachectin had anti-tumor activity and both assays were negative. Nevertheless, Cerami was able to show that mouse cachectin and mouse TNF were identical. (12) So, the hope that TNF would be a specific anti-tumor agent that could safely be given to patients was taken away by the pro-inflammatory activities associated with cachectin.

1.2. The TNF superfamily

The tumor necrosis factor (TNF) superfamily consists of 19 ligands and 29 receptors that all have pro-inflammatory activities, partly by activation of the transcription factor NF-kB. (Figure 1) Several members also have proliferative activities on hematopoietic cells through activation of different mitogen-activated protein kinases or play a role in apoptosis. (13) And some members have been reported to play a role in morphogenetic changes and differentiation. Most of the TNF superfamily members have both beneficial and harmful effects. (14) TNF itself, for example, has been linked with physiologic proliferation and differentiation of B-cells under steady-state conditions, but also with a wide variety of diseases, including cancer, cardiovascular -, neurological -, pulmonary -, autoimmune - and metabolic disorders.

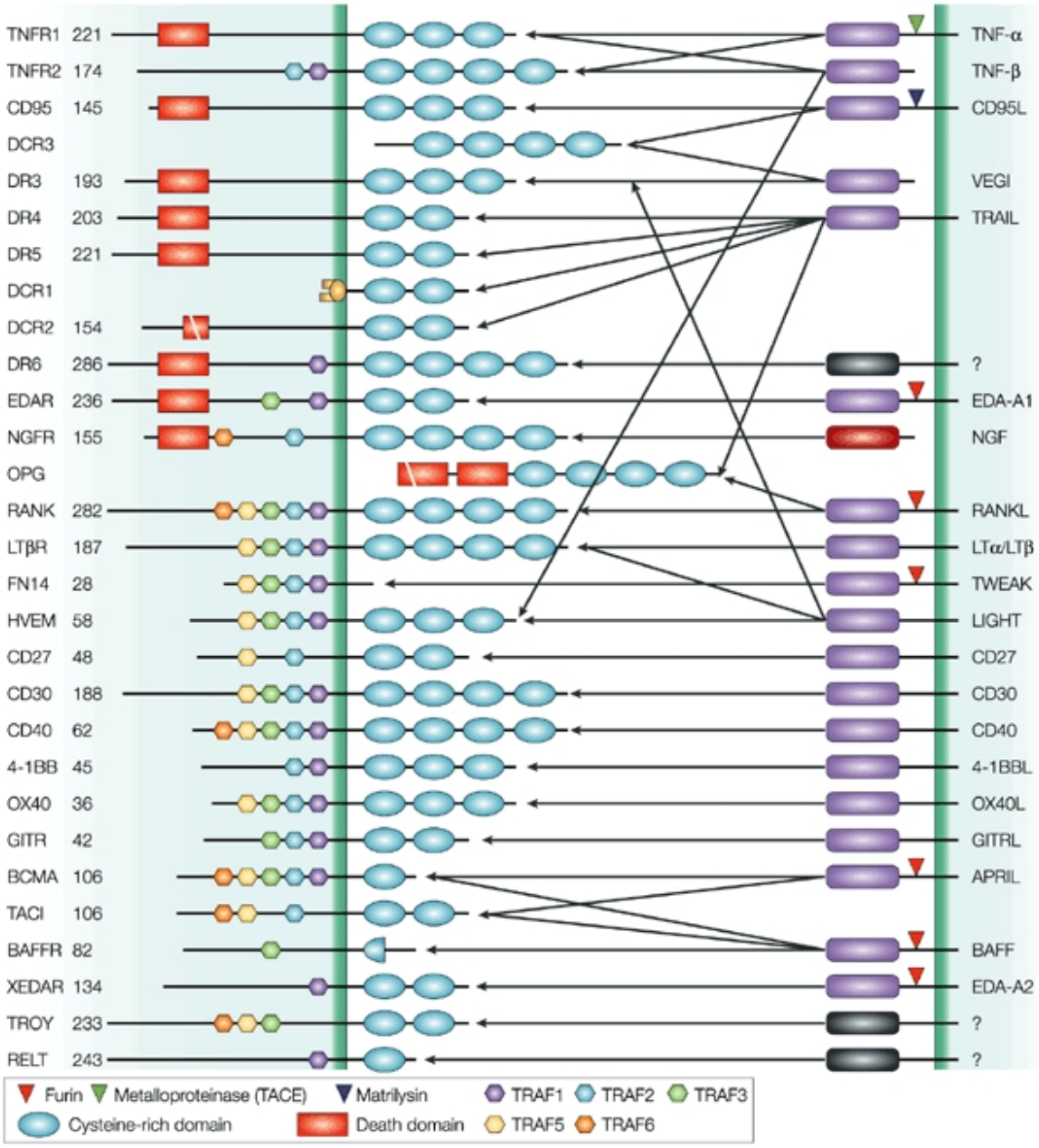

Figure 1. A diagrammatic representation of the ligands of the TNF superfamily and their receptors. (14)

1.3. Gene and protein structure

The human TNF gene (*TNFSF2*) is located on chromosome 6 (6p21.3), is 2.77 kb in size and consists of four exons. The mouse TNF gene (*tnfsf2*, *tnfsf1a*) is located on chromosome 17 (18.59 cM), is 2.607 kb in size and also has four exons. Both genes are present in a gene cluster with LTα and LTβ in the MHC locus. (15)

TNF is a type II transmembrane glycoprotein consisting of three monomers with a typical β-jellyroll structure. Each subunit consists of two packed β-sheets of five antiparallel β-strands with N-terminal three additional β-strands. In the amino acid chain the first 76 amino acids form a highly conserved hydrophobic sequence which is an anchor for precursor polypeptides in the membrane. This form of immature protein, transmembrane pro-TNF, has a molecular mass of 26kDa and is proteolytically cut mainly by the metalloprotease TNFα converting enzyme (TACE or ADAM17) to a 17kDa active unit. (16) Also other proteases, such as ADAM10 (17), MMP-7 (18) and MMP13 (19), have been shown to cut pro-TNF and lead to soluble TNF. Soluble TNF exists as a homotrimer of a molecular mass of 52kDa. Based on high resolution crystals, the protein structure of TNF has been described in great detail. (Figure 2) The shape of the TNF homotrimer has the appearance of a triangular cone or bell in which the three subunits are arranged edge to face. (20) The receptor binding sites of TNF are located in the lower half of the triangular cone in the groove between two subunits. (21)

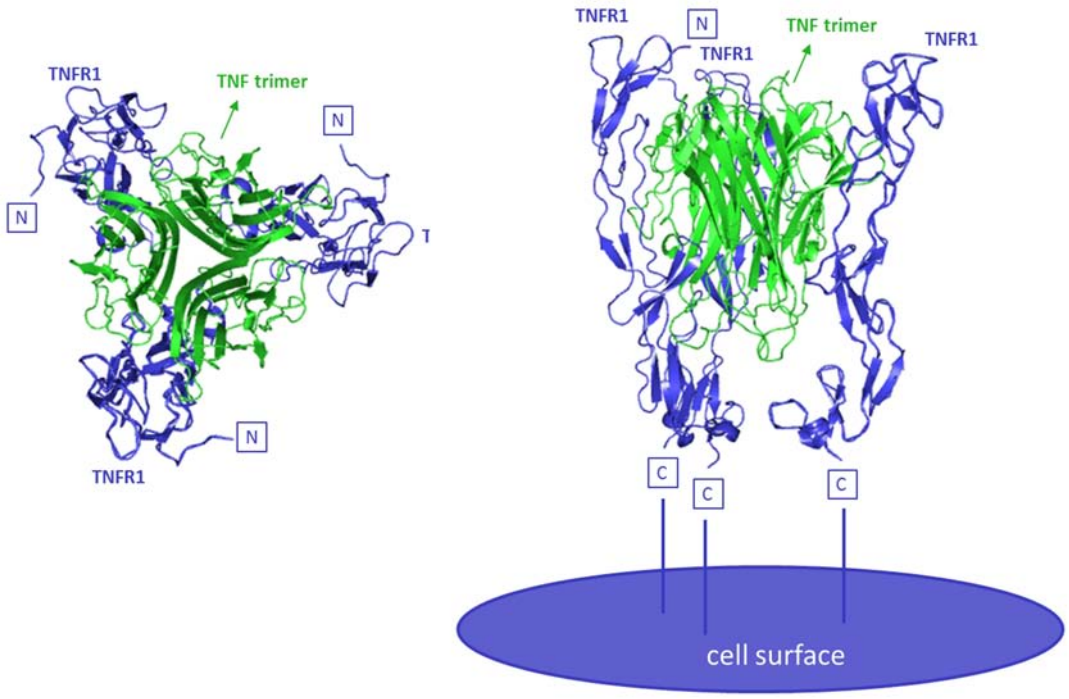

Figure 2. Crystal structure of tumor necrosis factor (PDB 1TNF in green) and binding to its receptor TNFR1 (PDB1 EXT in blue). Top view at the left, side view at the right. N=aminoterminus, C=carboxyterminus. (22, 23)

TNF binds with a high affinity to two type I transmembrane receptors, i.e. TNFR1, which is activated by both sTNF and tmTNF, and TNFR2, which is activated mainly by tmTNF. But most of the biological activities of TNF are initiated via binding to TNFR1. (24)

1.4. Transcriptional and post-transcriptional regulation

TNF is regulated by multiple and complex regulatory mechanisms controlling gene transcription, mRNA turnover and translation of the protein.

At the transcriptional level, expression of TNF is highly regulated by a network of transcription factors, co-regulators and chromatin modifications. The promoter, the third intron and an enhancer located immediately after the gene, contain binding sites for transcription factors such as NF-kB, nuclear factor of activated T-cells (NFAT), Ets, interferon-regulating factor and CCAAT/enhancer-binding protein (C/EBPα). Co-regulators without DNA-binding properties modulate gene transcription in other ways including DNA methylation or covalent modification of histons like phosphorylation and ubiquitination thereby influencing the chromatin conformation and promoting the assembly of a transactivating complex. (25-27)

Transcripts of cytokine encoding mRNAs are generally short-lived because they contain RNA destabilizing elements that contribute to a stringent regulation of cytokine production. The best known are AU-rich elements (AREs) which appear in the 3'UTR and control the stability and initiation of translation of TNF. (28) (Figure 3) The AREs are bound by tristetraprolin (TTP), a zinc finger that associates with different RNA degradation enzymes. TTP itself is induced by TNF creating a negative feedback loop. (29) Mice lacking the TNF ARE region as well as TTP KO mice have a chronic excess of circulating TNF leading to inflammatory arthritis and Crohn's-like inflammatory bowel disease. (30, 31) Translational control is dependent on MAP kinases for the phosphorylation of TTP by MK2. Phospho-TPP is unable to recruit RNA degradation enzymes like deadenylases. (32) Down-regulation of TNF to end an inflammatory reaction is mediated by the anti-inflammatory mediators IL4, IL10 and glucocorticoids. Immunization of mice with anti-IL10 resulted in a sustained increase in plasma TNF levels. (33) And inactivation of TNF is mediated by binding of soluble TNF receptors which are responsible for the clearance of TNF. (34)

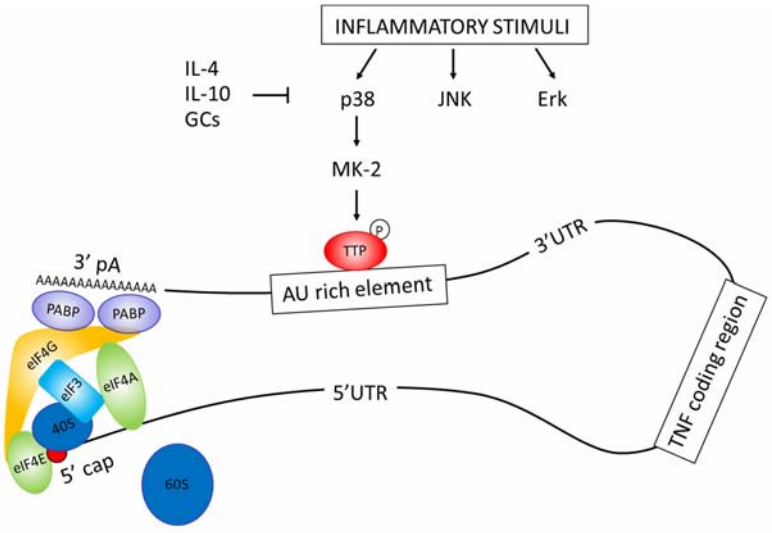

Figure 3. Posttranscriptional control of the ARE containing TNF mRNA. PABP: polyA binding protein, eIF: eukaryotic initiation factors, 40S and 60S: ribosome. For detailed description, see text above.

1.5. TNF-induced signaling

Binding of TNF to TNFR1 results in trimerization of the receptors and clustering of the death domains (DD). Subsequently, an adapter molecule called TRADD (TNFR associated death domain) binds via interactions between the DDs of TRADD and TNFR1 leading to NF-kB and AP-1 activation. TNFR1 stimulation can also lead to programmed cell death such as apoptosis and necroptosis. TNFR2, lacking a DD, can directly associate with activated TRAF2 leading to NF-kB and AP-1 activation. TNFR2 cannot directly initiate apoptosis but depletion of TRAFs and cIAPs can sensitize cells for TNFR1-induced cell death. (35) (Figure 4)

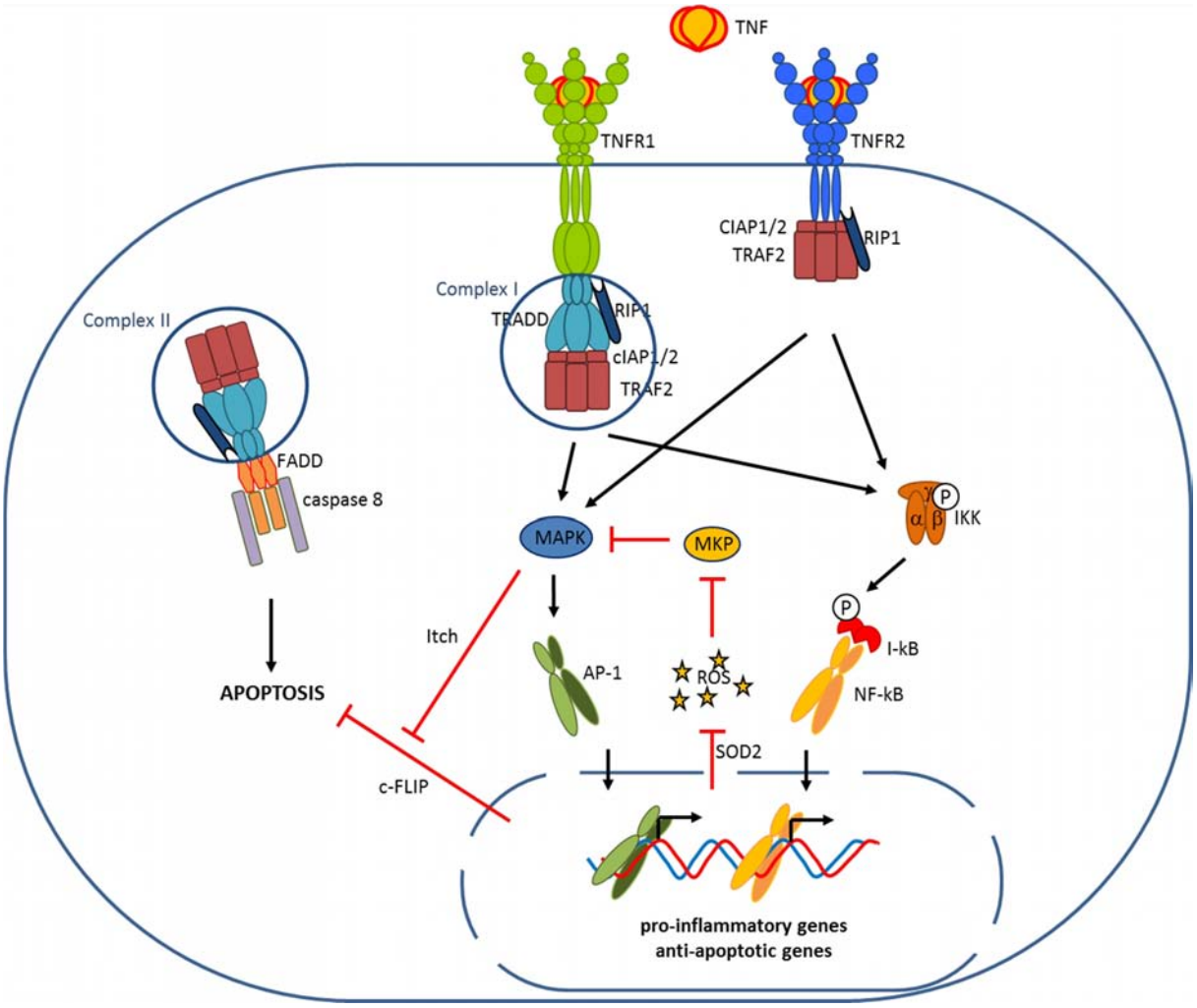

Figure 4. TNF-induced signaling. For detailed description, see further in the text.

NF-kB activation. TRADD acts as a platform adapter that has the ability to recruit TNFR associated factor 2 or 5 (TRAF2/5), cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) and receptor interacting protein 1 (RIP1) forming membrane-bound complex I. (37) (Figure 5) This allows cIAP to K63-ubiquinate RIP1 and TRAF2/5. Subsequent binding of TAB2/3 and TAK1 leads to activation of the inhibitor of kB (I-kB) kinase complex (IKK) by phosphorylation. (36) Linear ubiquitination of IKKγ or NEMO by the LUBAC complex consisting of Heme-oxidized IRP1 ubiquitin ligase (HOIL-1), HOIL-1 interacting protein (HOIP) and SHANK-

associated RH domain interactor (SHARPIN) leads to stabilization of the IKK complex. (37) Phosphorylation of I-kB by IKK ensures I-kB K48-ubiquitination which signals for degradation by the proteasome. Activation of NF-kB leads to translocation to the nucleus where it activates the expression of many pro-inflammatory and anti-apoptotic genes. However, the duration of NF-kB activation is limited by a couple of feedback mechanisms, like the induction of I-kB, CYLD and A20. CYLD, a protease that specifically cleaves K63-ubiquitin chains, de-ubiquinates TRAF2, thereby inhibiting the recruitment of TAB/TAK and activation of IKK. (38) And A20 deactivates RIP1 by removing the K63-ubiquitin chain and adding a K48-ubiquitin chain. (39)

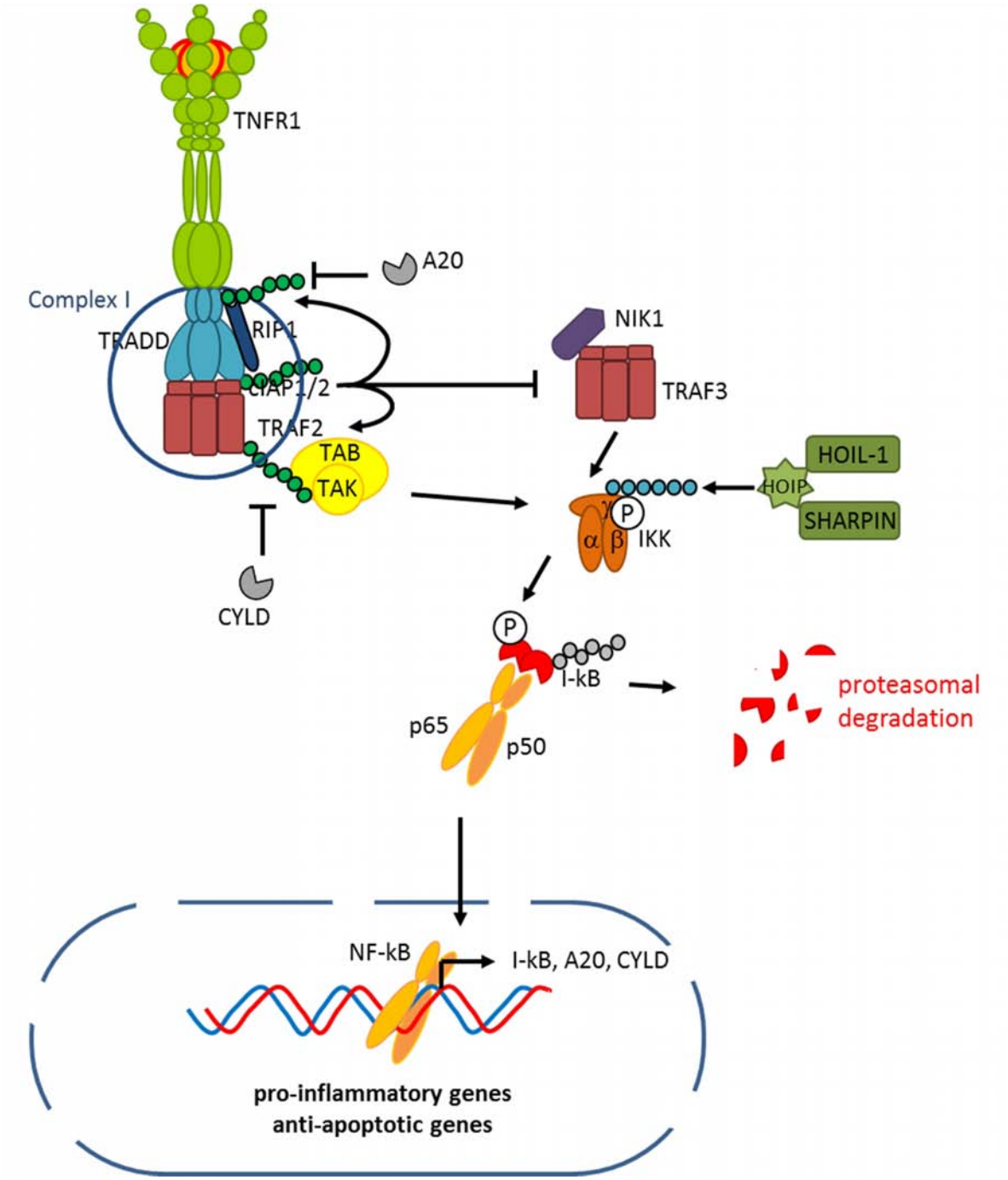

Figure 5. TNF-induced NF-kB activation. For detailed description, see text above.

AP-1 activation. Activation of AP-1 involves a cascade of phosphorylation mediated by the mitogen activated protein (MAP) kinases. (Figure 6) Activation starts with phosphorylation of MAPK kinase kinases like MEKK1 (MAP/Erk kinase kinase 1), followed by phosphorylation of MAPK kinases MKK3/6 and MKK4/7. These are responsible for the activation of c-Jun N-terminal kinases JNK1, 2 and 3 and p38, leading to activation and nuclear translocation of c-Fos and c-Jun. (40)

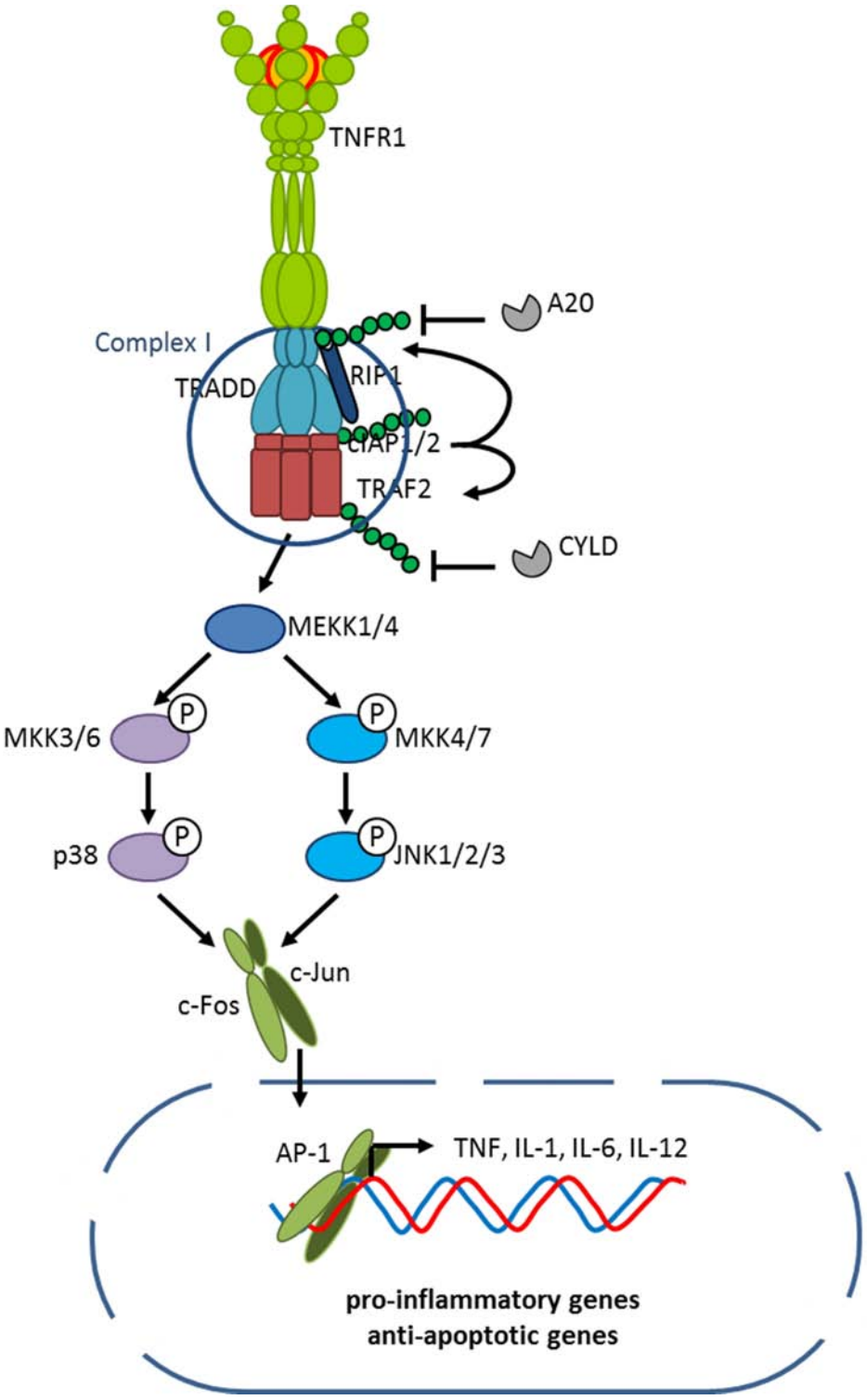

Figure 6. TNF-induced AP-1 activation. For detailed description, see text above.

Hence, complex I stimulates pathways leading to activation of NF-kB and AP-1 and induction of pro-inflammatory and anti-apoptotic proteins. (Figure 4) c-FLICE inhibitory protein (c-FLIP), a caspase-8 homolog lacking the enzymatic activity domain, can form dominant negative dimers with caspase 8, an essential molecule in the apoptotic pathway. Moreover, these c-FLIP-caspase 8 dimers are able to cleave RIP3 resulting in an inhibition of necroptosis. (41) However, JNK activation induces also pro-apoptotic proteins such as Itch, which can mediate c-FLIP degradation. (42) cIAP1 and 2 inhibit FADD-RIP1 interaction by K63-ubiquitination of RIP1 and inhibit caspase 8 activation. XIAP is able to inhibit caspase 3 and 7 activity. (43) TRAF1 enhances the interaction between cIAPs and TRAF2. Bcl-2 and Bcl-XL ensure the mitochondrial integrity. (44) NF-kB also induces the expression of proteins that inhibit the AP-1 activation like Gadd45b inhibiting MKK7. (45) And sustained AP-1 activation is prevented by MAPK phosphatases like glucocorticoid-induced MKP-1. (Figure 4) TNF-induced reactive oxygen species (ROS) are controlled by the induction of superoxide dismutase (SOD2) by NF-kB and oxidation of MKP-1 is suppressed. (46) TNF-induced gene induction often requires the activation of more than one pathway. E-selectin expression for example is dependent on the activation of NF-kB as well as AP-1. (47) Transactivation of NF-kB by p38-induced phosphorylation of histon H3 in the promoter of some inflammatory genes leads to better accessibility for NF-kB to the promoter. (48)

Apoptosis. Upon endocytosis of complex I, TRADD dissociates from TNFR1 and associates with Fas-associated protein with death domain (FADD) forming intracellular complex II or death inducing signaling complex (DISC). (Figure 7)

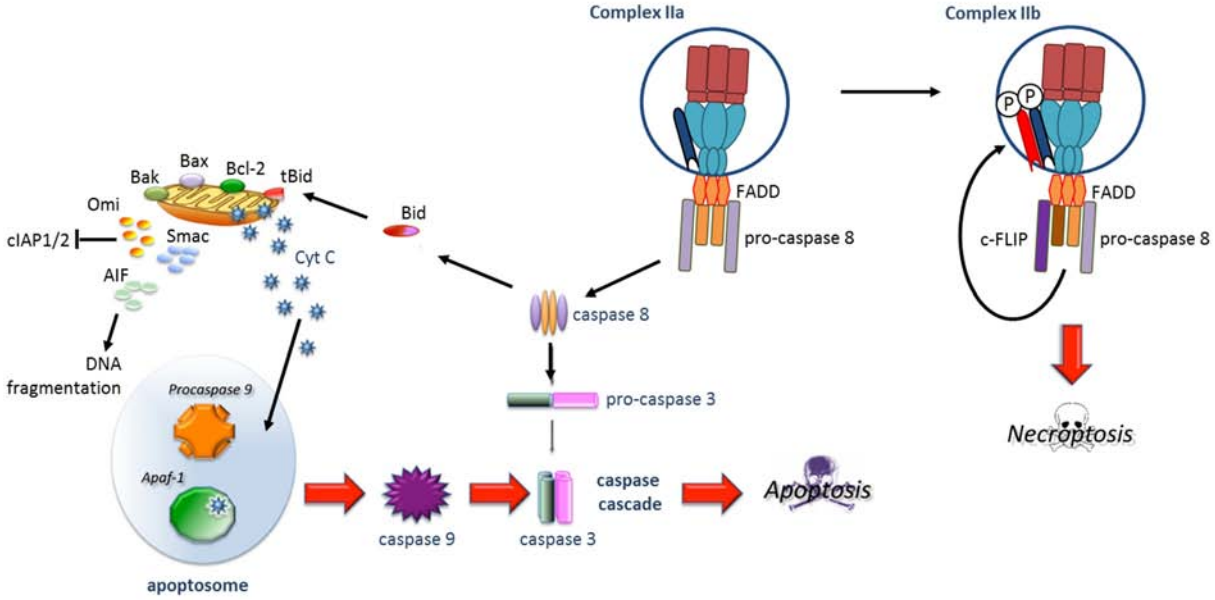

Figure 7. TNF-induced apoptosis and necroptosis, figure adapted from (49). For detailed description, see text.

De-ubiquitination of RIP1 by A20 or CYLD leads to the recruitment and autocatalytic cleavage of pro-caspase 8. Active caspase 8 can directly activate the downstream executioner

caspases 3 and 7 (the extrinsic pathway) or it can cleave Bid to tBid wich translocates to the mitochondria and induces permeabilisation of the membrane (the intrinsic pathway). Secreted cytochrome c interacts with the apoptosome resulting in activated caspase 9. Smac/diablo and Omi secretion inhibits the cIAP family and the apoptosis inducing factor (AIF) causes DNA fragmentation and chromatin condensation in the nucleus. The executioner caspases are able to cleave many substrates resulting in apoptosis. (49) In most cases, apoptosis doesn't induce an immune response. However, the death of tumor cells can release DAMPs such as HSPs, HMGB1, ATP and IFNs that can induce an inflammatory response with an antitumor effect. (50) See Figure 7.

Necroptosis. When caspase 8 is inhibited by c-FLIP and RIP-3 becomes induced, RIP1 and RIP3 become phosphorylated by auto phosphorylation or cross phosphorylation, leading to regulated necrosis or necroptosis. Necroptosis causes rapid plasma membrane permeabilization with release of reactive oxygen species (ROS) and exposure of damage-associated molecular patterns (DAMPs) strongly stimulating the immune system. (51) See Figure 7.

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2. TNFR1, the major receptor of TNF

2.1. The biological significance of TNFR1

TNF signals through two distinct receptors, TNFR1 and TNFR2. These receptors initiate diverse important effects, including proliferation, differentiation, migration, inflammation and cell death. (1) The pro-inflammatory and pathogen clearing activities of TNF are mediated mainly through activation of TNFR1, which is a strong activator of NF-kB, while TNFR2 may be more responsible for suppression of inflammation. (2)

Several groups generated TNFR1 deficient mice by gene targeting. (3-5) Studies on these mice have contributed a lot to understanding the role of this receptor in the biological activities of TNF. It is clear that TNF has a key role in immunity and immunomodulation as well as pro-inflammatory and antitumor activities. Resistance against bacterial infection, e.g. *Listeria monocytogenes*, is mediated by TNFR1: mice deficient in TNFR1 are extremely sensitive to *L. monocytogenes* and other gram-positive bacteria, including *Yersinia enterocolitica* (6, 7), *Pseudomonas aeruginosa* (8), *Legionella pneumophila* (9) and *Burkholderia pseudomallei* (10), and also to other pathogens, such as viruses, e.g. influenza virus (11) and vaccinia virus (12). However, injection of TNF in mice leads to systemic inflammation, which, depending on the dose, can be lethal. Studies on TNFR1 knockout (KO) mice have shown that lethality, which involves inflammation (13) and necroptosis (14), is mediated entirely by TNFR1. Chronically overexpressed TNF leads to arthritis and inflammatory bowel diseases, both of which depend on TNFR1. (15) TNF-induced antitumor effects are also mediated entirely by TNFR1 on host-derived neo-vascular endothelial cells, which grow into the tumor. (16)

Interestingly, TNFR1+/- mice, which express 50% TNFR1 on cells, were also found to be completely resistant to TNF-induced lethal inflammation. These data, together with others, such as those showing that mice expressing higher levels of cell-bound TNFR1 develop spontaneous inflammation, suggest that TNFR1 is a potential drug target, and that minor TNFR1 regulation might have substantial physiological and pathological effects. Hence, selective inhibition of TNFR1 signaling, like anti-TNF therapy, may inhibit detrimental inflammation but leave beneficial TNFR2 signaling untouched. (17, 18) For instance, in humans, mutations affecting TNFR1 have been linked with the development of the TNFR1-associated periodic syndromes (TRAPS), which are characterized by recurrent fever attacks and localized inflammation. (19)

Although TNF receptors form homotrimers upon activation by TNF but no heterotrimers are assembled, the TNFR1/TNFR2 protein ratio has been found to be important for the TNF response. TNF affects NF-kB activation predominantly through TNFR1, whereas TNFR2 activates transcription poorly. (20) Nevertheless, TNFR2 stimulation can result in competition for TRAF2 and cIAPs or even degradation of these molecules by the proteasome by induction of (auto)ubiquitination, and thereby inhibition of TNFR1-induced NF-kB transcription. (21) Reduction of NF-kB activation promotes apoptosis in certain cell types due to diminished production of anti-apoptotic factors. (22, 23) Hence, largely unmodulated

TNFR1 expression coupled to changeable TNFR2 levels alters the TNFR1/TNFR2 ratio and controls the response of the cell to TNF stimulation. (24)
On the other hand, the kinetics of TNF binding to TNFR2 suggests a mechanism by which TNFR2 might increase the apparent rate of TNF binding to TNFR1. TNFR2 has a higher affinity and longer TNF-binding half-life than TNFR1, and by a so-called ligand-passing mechanism it associates with TNF, increasing its concentration near TNFR1 receptors, and makes it available for activating TNFR1. (25) Clearly, the outcome of TNFR1 and TNFR2 signaling is complicated and may depend on the cell type and activation state.

2.2. Gene and protein structure of TNFR1

The human TNFR1 gene (*TNFRSF1A*) is located on chromosome 12 (12p13.2) in reversed orientation, is 13.361 kb in size and consists of nine exons. The mouse TNFR1 gene (*tnfrsf1a*) is located on chromosome 6 (59.32 cM), is 12.761 kb in size and has ten exons.
The protein of mouse TNFR1 has a length of 454 amino acids (AA) composed of an extracellular domain (ECD) of 212 AA, a helical transmembrane domain (TMD) of 23 AA, and an intracellular domain (ICD) of 219 AA. (Figure 1)

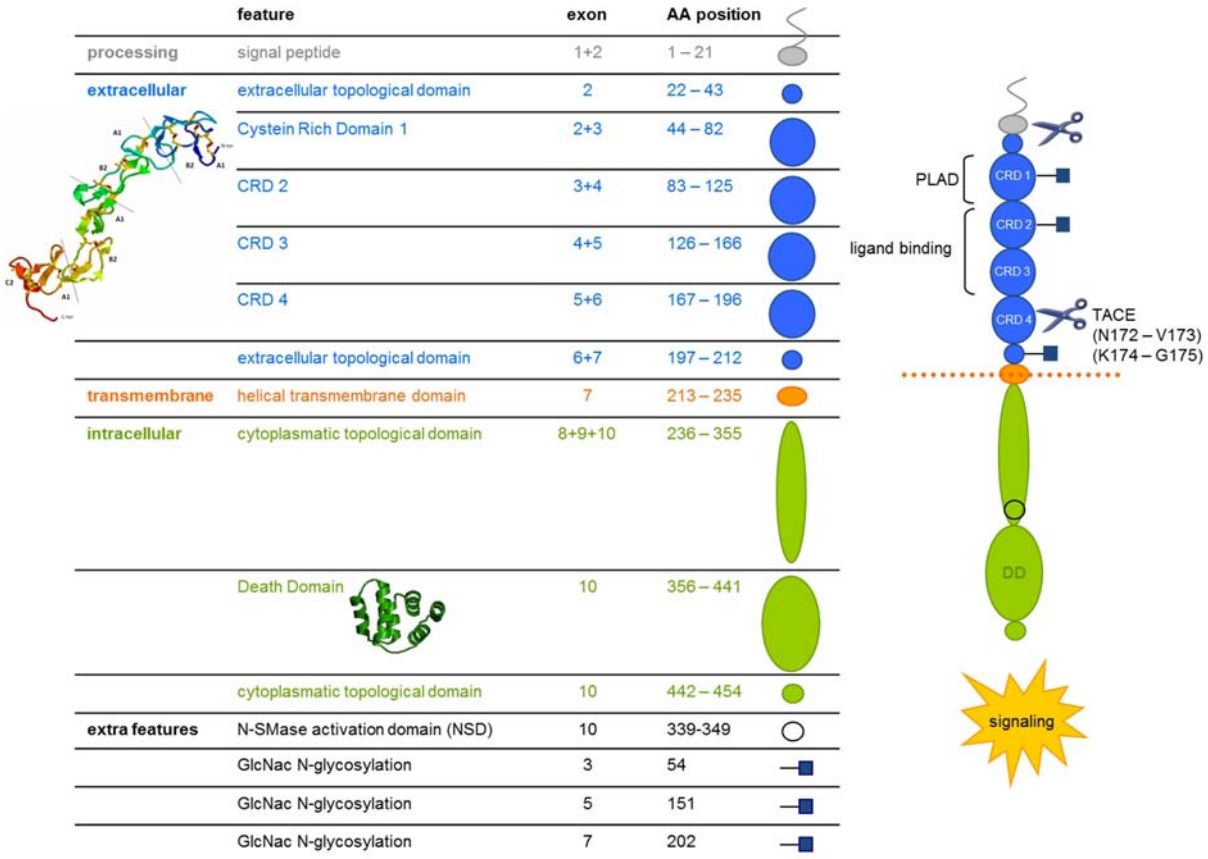

Figure 1. Representation of TNFR1 (UniProtKB P25118). For detailed description see text.

The extracellular regions of TNFR1 and TNFR2 are structurally highly homologous. The first 21 AA function as a signal peptide that interacts with the signal recognition particle and directs the ribosome to the endoplasmic reticulum, where co-translational insertion takes

place. The N-terminal ECD contains two extracellular topological domains (AA 22–43 and AA 197–212) and four cysteine rich domains (CRD) at AA 44–82, AA 83-125, AA 126-166 and AA 167-196. Each domain contains six cysteines. Transmembrane TNFR1 is also a substrate of TACE. The major TNFR1 cleavage site is the spacer region close to the transmembrane domain between Asn172 and Val173, and the minor site is between Lys174 and Gly175. (26) In contrast, there is no homology in the intracellular region between TNFR1 and TNFR2, indicating that these receptors activate distinct signaling pathways. TNFR1 contains a cytoplasmic death domain (DD), a hemophilic protein–protein interaction region of 86 AA (356–441) required for TNF induced apoptosis, and an N-SMASE activation domain (NSD) spanning an 11-AA motif N-terminal to the DD. See figure 1.

2.3. Transcriptional and posttranscriptional regulation of TNFR1

2.3.1. Transcriptional regulation of TNFR1 expression

Despite the critical role that TNFR1 plays in TNF-mediated signaling, little is known about the regulation of its promoter. It has been suggested that the TNFR1 promoter is constitutively active, like the promoters of 'housekeeping' genes, but at low levels and on nearly all nucleated cell types. (24) (27) On the other hand, TNFR2 expression is inducible and it is expressed exclusively by immune cells, endothelial cells and some neuronal populations. (28)

Promotor analysis. The 5' flanking region of TNFR1 was scanned for the presence of sequence motifs that have been associated with regulation of gene transcription. (29) According to the UCSC genome browser, the transcription start site (TSS) is located 107 bp upstream of the putative TSS that was described by Takao and Jacob. A putative **TATA box** (TTAAATT), the core promoter sequence, is now located between +63 and +69 upstream of the TSS, which presumes that it may not be a true TATA box. Two **GC-rich elements** are present between -44 and +3 and between +5 and +41. These elements have been shown to possess enhancer activity in many eukaryotic genes. (29) The TNFR1 promoter contains a functionally important **binding site** for CCAAT/enhancer binding protein **(C/EBP),** which contributes to the constitutive activity of the promoter. C/EBP transcription factors play essential roles in regulating different cellular processes, including differentiation, energy metabolism and inflammation. Both C/EBPα and C/EBPβ bind to a sequence located between +5 and +12. (29, 30) There are two copies of the consensus **binding site for AP-1**, a transcription factor that regulates gene expression in response to different stimuli, including cytokines, growth factors, stress, and bacterial and viral infections. (29) Also four potential binding sites for the **AP-2** family of transcription factors (AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε) have been found. The general functions of this family are stimulation of proliferation and suppression of differentiation during embryonic development. (31) An **NF-kB binding site** in the TNFR1 promoter between -489 and -498 activate the expression of TNFR1. In mammary-specific β-lactoglobulin $Cre^{+}/Ikk2^{flfl}$ mice, NF-kB DNA-binding activity is diminished by 50%, contributing to reduced expression of TNFR1 mRNA levels and abrogation of TNF-induced apoptosis. (32) A consensus sequence related to the **IFNγ activated site (GAS)** of signal

transducer and activator of transcription (**STAT-1**) factors has been found between -235 and -243 of the mouse TNFR1 promoter. In oligodendrocytes, IFNγ indeed induces TNFR1 transcription via activation and binding of STAT-1 homodimers to the GAS site in the TNFR1 promoter. (33) (34) (Figure 2 and Table 1)

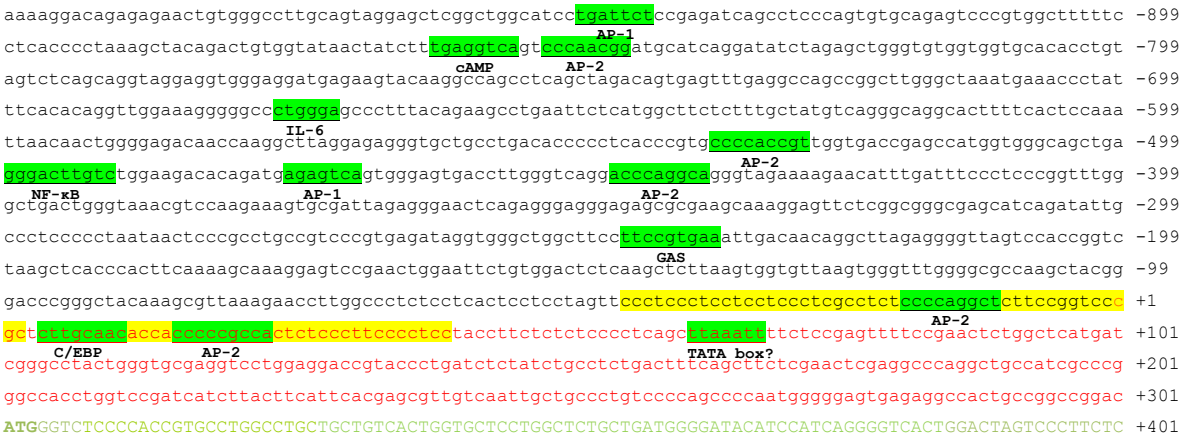

Figure 2. Promoter of mouse *Tnfrsf1a* (NM011609). The 5'UTR is shown in red, the TSS in a box and the CDS in green. Transcriptional regulatory sequences are marked in green and the GC rich element in yellow.

transcriptional element	consensus sequence	actual sequence	position
TATA box?	TATAWAW	TtaAAtT	+63/+69
C/EBP binding site	RTTGCGYAAY	cTTGCaac	+5/+12
GC rich element			-44/+3
			+5/+41
AP-1 binding site	TGASTCA	TGAtTCt	-941/-947
		aGAGTCA	-467/-473
AP-2 binding site	GCCNNNGGC	cCCAACGGa	-843/-850
		cCCCACcGt	-527/-535
		aCCCAGGca	-436/-444
		cCCCAGGct	-11/-19
		cCCCCGcca	+17/+25
NF-κB binding site	GGGACTTTCC	GGGACTTgtC	-489/-498
IL-6 responsive element	CTGGGA	CTGGGA	-669/-674
cAMP responsive element	TGACGTCA	TGAgGTCA	-853/-860
IFNγ activated site	TTCNNNGAA	TTCCGTGAA	-235/-243

Table 1. Transcriptional regulatory sequences in the promoter of *Tnfrsf1a*. (Y=C/T, R=A/G, M=A/C, K=G/T, W=A/T, S=G/C)

Factors affecting TNFR1 expression. Gene transcription is also controlled by dynamic acetylation and deacetylation of histone proteins, which alters chromatine structure and affects transcription factor access to the DNA. **HDAC5** (Histone deacetylase 5) overexpression has been shown to inhibit tumor cell growth and induce spontaneous apoptosis by altering gene expression, including a four-fold up-regulation of TNFR1. (35) Transcriptional up-regulation of TNFR1 by **NF-kB** activation has been observed in membrane-bound TNF-mediated cell-cell contact between T-cells and monocytes. (36) Moreover, **IL18** and IL18Rα up-regulate the expression of both TNFR1 and TNFR2 on the surface of

monocytes by activating the NF-kB pathway. Neutralizing mAb to T-cell membrane-bound TNF decreased the enhancement of TNF and IL1β production by IL18 on cell contact, suggesting that IL18 up-regulates TNF receptor expression and thereby stimulates cross-talk between monocytes and activated T-cells. (37) Also **Vitamin D3** enhances TNF-induced NF-kB activation and TNF-induced apoptosis in TNF sensitive MCF-7 cells. This can be explained by up-regulation of surface expression of TNFR1. The Vitamin D effect, however, changes the balance between death-inhibiting and death-promoting signals in favor of increased apoptosis. (38)

On the other hand, **IL3 and GM-CSF** can down-regulate both mRNA and surface expression of TNFR1 and TNFR2. This inhibits TNF-induced differentiation of osteoclasts from hematopoietic precursors of the monocyte lineage, which also give rise to macrophages or dendritic cells. (39) **IL10** induces down-regulation of membrane TNFR1 and TNFR2 on monocytes and reduces the pro-inflammatory potential of TNF in three ways: down-regulating membrane TNF receptor expression, increasing production of soluble TNF receptor, and inhibiting TNF release. This suggests that IL-10 may be useful in the treatment of diseases involving overexpression of TNF. (40) However, overexpression of **STAT3** completely inhibited IL10-induced suppression of TNF receptor expression. (41)

2.3.2. Post-transcriptional regulation of TNFR1 expression

A key aspect of the regulation of eukaryotic gene expression is cytoplasmic control of mRNA translation and degradation. (42) Regulation at the translational level can provide a quick response to stimuli because it does not involve the upstream processes of gene expression. Furthermore, translational regulation is usually reversible, as it is often mediated through reversible protein modifications such as the phosphorylation of translation initiation factors.

Decreased stability of TNFR1 mRNA during differentiation of human monocytes is associated with changes in the formation of RNA-protein complexes: a decrease of 66–87 % in the level of TNFR1 mRNA of these cells was noticed upon TPA-induced differentiation. This decrease is partial and thus appears to be one of several mechanisms that lead to the complete loss of TNFR1 from the surface of those cells. In contrast, in the fibroblast cell line SV-80, TPA did not induce alterations in the pattern of RNA–protein complexes, nor did it affect the level of TNFR1 mRNA or cell surface protein expression, indicating that the response to TPA is cell-type specific. Phorbol esters such as TPA activate protein kinase C and can modulate gene expression by affecting transcription. But there is also evidence that TPA affects gene regulation post-transcriptionally by altering the stability of mRNAs. To detect proteins that bind to the TNFR1 mRNA, electrophoretic mobility shift was assayed on radiolabeled full-length RNA transcribed *in vitro*. Incubation of the probe with cytoplasmic extracts from untreated U-937 cells resulted in formation of one major complex (complex A) and one minor complex with lower electrophoretic mobility (complex B). A substantially different pattern was observed for protein extracts from TPA-treated U-937 cells. Formation of complex A was decreased while that of complex B was markedly increased. In addition, complexes of intermediate mobility appeared. The nucleotide sequence involved in the

formation of the RNA–protein complexes A and B and intermediate complexes is located in exon 2 of the TNFR1 mRNA. It appears that an 18-nt region (caccctcaaaataattc), essential for all complexes formed, is on its own sufficient for formation of complex A, whereas additional 5' adjacent nucleotides are required for formation of complex B and intermediate complexes. The identities of the proteins are not known. TNFR1 mRNA does not contain AREs, but the RNAs for GM-CSF, TNF, and c-myc, which do contain AREs in their 3' UTR, efficiently competed with the TNFR1 RNA in the formation of complex B. Competition was weaker in the formation of complex A. (43, 44) Detection of the RNA binding proteins would be a big step forward in understanding the regulation of TNFR1 mRNA.

2.3.3. Post-translational regulation of TNFR1 expression

Storage. After translation in the ER, TNFR1 is localized predominantly in the **Golgi apparatus** while TNFR2 is directed to the plasma membrane. The intracellular death domain is required for retention of TNFR1 in the Golgi apparatus: deletion of the entire TNFR1 intracellular domain or the C-terminal DD allowed expression of the receptor on the plasma membrane. However, addition of the DD to the C-terminus of TNFR2 did not lead to Golgi-retention. (45) Deletions in the cytoplasmic tail demonstrated that the C-terminal sequence of 23 amino acids is required for this targeting. This sequence is mostly outside the death domain and contains an acid patch and a dileucine motif. Interaction of the sequence with membrane traffic adaptor proteins may play an important role in controlling the cell's response to TNF, because binding of signaling adaptor proteins has been demonstrated for TNFR1 on the plasma membrane but not for Golgi-localized TNFR1. (46) The Golgi pool of TNFR1 is used to replenish cell surface TNFR1 receptors. (Figure 4)

Shedding. TNFR1 can be released from the cell surface by a proteolytic process named ectodomain shedding. (Figure 4) Both soluble TNFR1 and TNFR2 are present in blood and urine of naive mice and are produced by monocytes and macrophages that can release sTNFR spontaneously. (47) Like TNF, TNFR1 and TNFR2 are cleaved by TNF-α converting enzyme (TACE), also known as 'a disintegrin and metalloproteinase 17' (ADAM-17). (48) Proteolytic cleavage of TNFR1 or TNFR2 generates antagonistic soluble receptors that regulate TNF bioactivity by feedback. (49) TACE may therefore be either pro- or anti-inflammatory, depending on whether it acts on an effector cell (e.g. releasing ligand from macrophage) or target cell (e.g. releasing receptor from endothelial cell). (50) However, low levels of sTNFR may sometimes stabilize the activity of TNF and provide a reservoir of TNF. (51) In humans, mutations affecting the shedding of TNFR1 have been linked with the development of the TNFR1-associated periodic syndromes (TRAPS), which are characterized by recurrent fever attacks and localized inflammation. Similarly, knock-in mice expressing a mutant non-sheddable TNFR1 are very sensitive to TNF-induced inflammation and develop several auto-immune diseases, such as spontaneous hepatitis, enhanced susceptibility to endotoxic shock, exacerbated TNF-dependent arthritis, and experimental autoimmune encephalomyelitis.

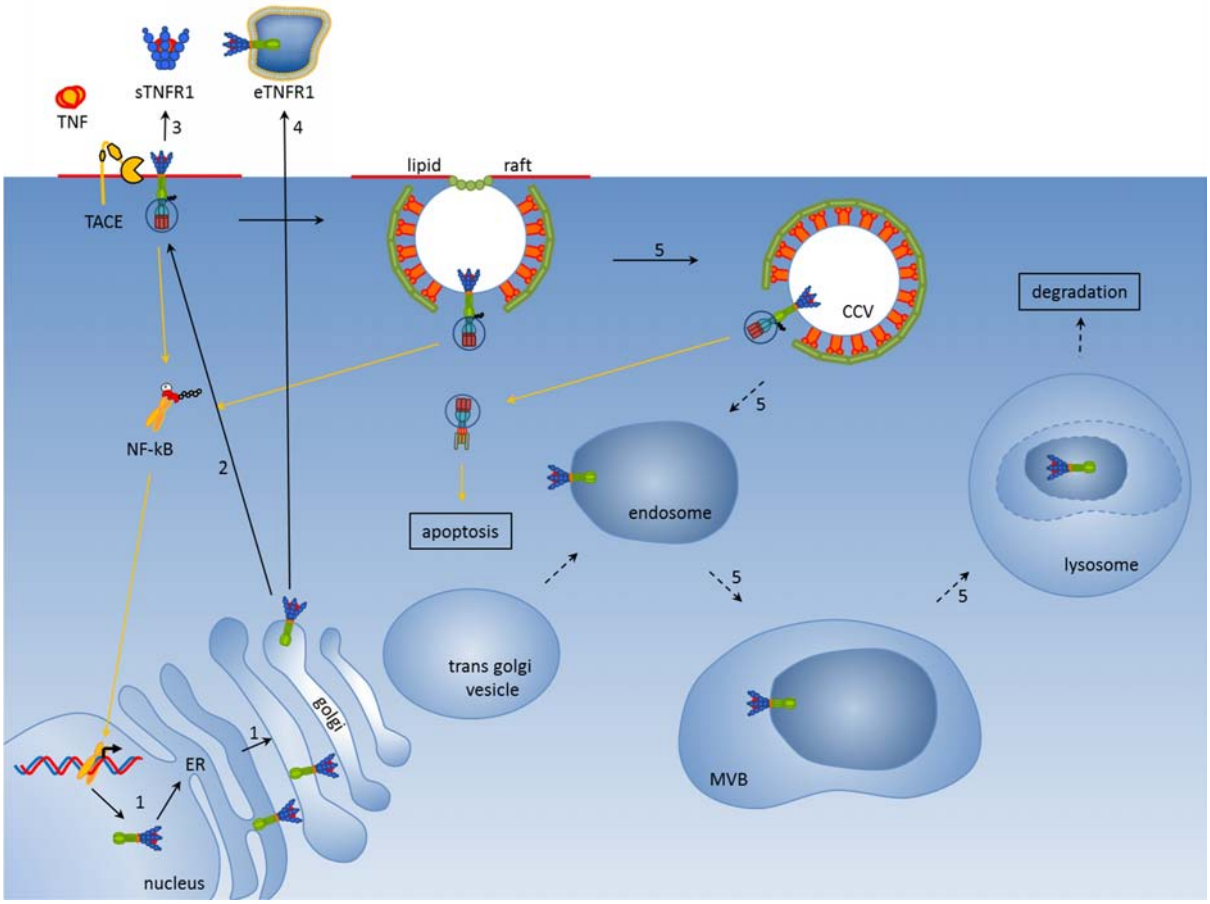

Figure 4. Compartmentalization of TNFR1 signaling. Black arrows indicate transport of TNFR1, 1=translation and storage, 2=membrane expression, 3=shedding, 4=exocytosis, 5=endocytosis. Yellow arrows indicate signaling pathways. MVB=multivesicularbodies, CCV=clathrin-coated vesicles. For detailed description, see text.

Basal expression of TACE is observed in all vasculature cell types, including endothelial cells, vascular smooth muscle cells, fibroblasts and leukocytes. TACE-mediated shedding is enhanced by stimulation with pro-inflammatory cytokines (TNF, IFNγ), TLR ligands (LPS), growth factors (PDGF, VEGF), GPCR ligands (thrombin) or oxidative stress (ROS), and thus involved in many regulatory pathways. Transcriptional regulation, maturation by furin, trafficking from storage pools, intracellular phosphorylation, changes in cellular distribution within membrane lipid rafts, interaction with adapter molecules (Tetraspanins) and conformational changes of the protease finally lead to enhanced substrate cleavage. (52) TACE is expressed in intracellular compartments and on the cell surface. Removal of its pro-domain by a furin protease probably occurs within the Golgi apparatus. (53) Distribution of TACE and its substrates within lipid rafts affects shedding because mature ADAM17 is associated with cholesterol-rich lipid rafts, and depletion of rafts induces shedding of TNF, TNFR1 and TNFR2. (54, 55) A p38- and ERK-dependent phosphorylation of TACE at Thr735 has been demonstrated (56), and ERK inactivation induced by arecoline (a muscarinic acetylcholine receptor agonist) suppressed the production of mature TACE. (57) However, analysis of TACE truncations and chimeric constructs revealed that the protease requires the transmembrane domain but not the intracellular domain for up-regulation of shedding, suggesting that intracellular phosphorylation is not necessary for regulating TACE activity.

(58) TACE is kept in a less active closed conformation by extracellular protein disulfide isomerases (PDI). (59) PMA-induced generation of reactive oxygen species (ROS) changes the redox state, leading to inactivation of PDI and thereby allowing the protease to adopt an active open conformation. (60)
The mechanism of TNFR1 shedding might also involve interactions with regulatory ectoproteins. A direct relationship exists between the level of the type II integral membrane protein **ARTS-1** (aminopeptidase regulator of TNFR1 shedding) and the degree of TNFR1 shedding. ARTS-1 overexpression increases TNFR1 shedding and decreases membrane-associated TNFR1, while expression of antisense ARTS-1 mRNA decreases membrane-associated ARTS-1 and TNFR1 shedding but increases membrane-associated TNFR1. ARTS-1 neither bound to TNFR2 nor altered its shedding, indicating its specificity for TNFR1. Findings suggest that ARTS-1 does not possess TNFR1 sheddase activity, which indicates that it is a multifunctional ectoprotein capable of binding to and promoting TNFR1 shedding. (61)

Exocytosis. TNFR1 shedding is a consequence of proteolytic cleavage of the 28-kDa ectodomain by a receptor sheddase, such as TACE. But in human vascular epithelial cells (HUVECs) and BAL fluid, the predominant form of sTNFR1 is a full-length 55-kDa protein (eTNFR1) associated with **exosome-like vesicles**. (Figure 4) Exosomes are small membrane-enclosed vesicles of 30–100 nm released from the cell by exocytic fusion with the plasma membrane. These intracellular TNFR1-containing vesicles enable constitutive release of eTNFR1 into the extracellular compartment. This is an alternative pathway for generating soluble cytokine receptors independently of proteolytic cleavage of the receptor ectodomain. (62) It has been reported that histamine induces redistribution of TNFR1 from the Golgi to vesicles and subsequently into the medium as soluble receptors, with a consequent decrease in cell surface TNFR1 (49). The precise significance of such remarkable full-length TNFR1 shedding is still unclear.

Ligand-induced translocation. Upon binding of TNF, TNFR1 translocates to cholesterol- and sphingolipid-enriched membrane microdomains named **lipid rafts** (Figure 4), where it associates with RIP and the adaptor proteins TRADD and TRAF2 to form signaling complex I. This complex triggers pathways leading to induction of pro-inflammatory and anti-apoptotic proteins. Activation of p42, a member of the MAP kinase family, leads to TNFR1 phosphorylation at a consensus MAPK site in its cytoplasmic domain. Phosphorylation of TNFR1 alters its subcellular localization, resulting in changes in its signaling properties. (63) Moreover, in lipid rafts TNFR1 and RIP1 get ubiquitinated, which leads to their degradation via the proteasome pathway. (64) Disruption of lipid rafts, by depleting cholesterol, not only abolishes ubiquitination but also totally blocks TNF-induced NF-kB activation, leading to apoptosis induction which indicates that the translocation of NF-kB to lipid rafts is essential for its activation. (65)

Endocytosis. Formation of complex I is transient because most of TRADD, RIP1 and TRAF2 dissociate from TNFR1 within an hour, when TNFR1 starts to undergo endocytosis.

Treatment of U937 cells with TNF led to maximal down-modulation of the TNF receptors within 30 minutes. These cells express both types of TNFRs, but the amount of TNFR2 is more than double that of TNFR1. Ligand-induced down-modulation of TNF receptors is caused by TNFR1 internalization and TNFR2 shedding, and the signaling for both is mediated through TNFR1. (66) The liberated DD of TRADD now binds to FADD, resulting in caspase 8 recruitment and complex II formation. Complex II initiates apoptosis (Figure 4), provided that NF-kB signaling has terminated. (65) Endocytosis or TNFR1 internalization and intracellular trafficking play an important role in selection of the signaling pathway: either internalization-independent (pro-inflammatory complex I) or internalization-dependent (pro-apoptotic complex II). Apoptosis is totally blocked by preventing internalization with monodansylcadaverine (MDC), an inhibitor of transglutaminase, a membrane-bound enzyme that actively participates in internalization of various receptor systems. (67) The endosome should therefore be recognized as a signaling organelle involved in selectively transmitting death signals from TNFR1. (68) Internalization proceeds through the classical receptor-mediated endocytosis pathway, i.e. via clathrin-coated vesicles and endosomes that fuse and enter multivesicular bodies (MVB) before accumulating in lysosomes. (69) (Figure 4) After apoptosis, TNFR1 protein is eliminated in a caspase-dependent manner. *In vitro*, the cytoplasmic tail of TNFR1 is susceptible to cleavage by the downstream executioner caspase 7, the only caspase capable of cleaving TNFR1. (70) Identification of the cleavage site revealed an EXE motif instead of the classic EXD motif. Homologous sequence alignments showed that the EXE motif is conserved in rat and pig but not in mouse and cow, which have 3–4 missing amino acids at this site. (70)

TNF thus causes the down-modulation of its own receptor by internalization which might function as a negative feedback mechanism as it will desensitize cells for further TNF stimulation until the membrane TNFR levels have recovered.

2.4. Dysregulation of TNFR1 expression

2.4.1. TNFR1 genetic variants

Missense mutations. Systemic autoinflammatory diseases are genetic disorders characterized by seemingly spontaneous inflammation without major involvement of the adaptive immune system. Among them is the TNF receptor-associated periodic syndrome (**TRAPS**), which is caused by missense mutations in the TNFRSF1A gene and characterized by periodic high fevers, rash, abdominal pain, chest pain, conjunctivitis, arthralgia and myalgia. More than 60 mutations have been identified in TNFR1, all of them in the extracellular domain. (http://fmf.igh.cnrs.fr/ISSAID/infevers) (71) TRAPS mutations occur predominantly in CRD1 and CRD2 and many of them involve intramolecular disulfide bonds. Others occur at residues predicted to have an effect on the secondary structure or at residues involved in hydrogen bonds between loops of the receptor. The absence of large deletions suggests that synthesis of the mutant protein is important for pathogenesis. It has been hypothesized that TRAPS pathology is driven by defective receptor shedding resulting in reduced serum levels

of soluble TNFR1. This hypothesis was based on observations that some TRAPS patient cells are resistant to PMA-induced shedding and that serum from TRAPS patients contained reduced levels of circulating TNFR1. However, the shedding defect was found to vary, especially between the types of cells studied, and did not always occur. (72) Molecular modeling indicates that nine TNFR1 mutants are unable to bind TNF and normal TNFR1 (H22Y, C30S, C30R, C33G, C34S, T50M, C52F, C88R, R92P), whereas R92Q and P46L can. Due to misfolding, they accumulate in the endoplasmic reticulum (ER) instead of localizing to the cell surface, and are most likely degraded by the proteasome. (73) But accumulation of mutant TNFR1 in the ER may also trigger the ER stress response, which can directly or indirectly lead to inflammation or block TNF-induced apoptosis. Interestingly, neutrophils and dermal fibroblasts from TRAPS patients with several different mutations have reduced apoptosis but produce the proinflammatory cytokines IL-6 and IL-8 normally when exposed to TNF. (74, 75) Failure of activated cells to undergo apoptosis in TRAPS could lead to accumulation of pro-inflammatory cytokines. However, TRAPS mutations might not all act by the same mechanism. (76, 77) Glucocorticoids are effective in decreasing the severity and duration of the fever attacks, although their efficacy fades with time. (78) The soluble TNF receptor Etanercept, the IL-1β receptor antagonist Anakinra and the IL-6 receptor antagonist Tocilizumab are effective in some patients, but treatment with Infliximab (anti-TNF) exacerbated the disease in some cases. (79)

Two rare single amino acid mutations in TNFRSF1A have been identified as low-penetrance risk factors for TRAPS. The R92Q substitution is carried by ~2% of North American and Irish populations, and the P46L mutation is present in 9% of African populations. TRAPS patients with these polymorphisms have a milder syndrome with almost no incidence of amyloidosis. (19) But the R92Q mutation has also been linked with other diseases associated with inflammation, such as **rheumatoid arthritis** and **atherosclerosis**. (80, 81) Clinical observations have identified some **multiple sclerosis** (MS) patients carrying the R92Q mutation and exhibiting additional TRAPS symptoms. The co-existence of MS and TRAPS could be mediated by this mutation. The R92Q mutation acts like a genetic risk factor for MS and other inflammatory diseases, including TRAPS. Nevertheless, this mutation does not appear to be a severity marker, modifying neither the progression of MS nor its response to therapy. But an alteration in TNF/TNFR1 signaling may increase pro-inflammatory signals. (82)

Single nucleotide polymorphisms (SNPs) in the TNFRSF1A gene may also influence the innate immune response against **invasive pulmonary aspergillosis** (IPA), an increasingly common opportunistic fungal infection that usually occurs in immunocompromised patients. In this infection, TNF acting through TNFR1 plays a pivotal role in immune regulation and host immune responses. Three SNPs were genotyped in 275 individuals (144 immunocompromised hematological patients with high-risk of developing IPA and 131 healthy controls): A383C and G609T in the 5’ UTR, and A36G in exon 1. The last two are associated with IPA susceptibility. The role of tnfrsf1a SNPs is also supported by significantly lower TNFR1 mRNA levels in IPA compared to IPA-resistant patients and by a strong

correlation between the –609 SNP and TNFR1 expression levels. This SNP might play a critical role in the promoter binding affinity of IRF-8, a transcription factor involved in the TNFR1-mediated activation of NF-kB. TNFR1 polymorphisms may influence the risk of IPA disease and might be useful for risk analysis. (83) The A36G mutation is also significantly associated with lower hemoglobin levels, causing iron deficiency anemia in patients with early or established rheumatoid arthritis. The frequency of anemia is higher in GG homozygous patients. (84, 85)

Alternative splicing. Two disease-associated isoforms produced by alternative splicing were recently described.

The **TNFR1-Δ6** splicing pattern is caused by a variation (rs1800693) in the exon 6/intron 6 border region that alters exon 6 splicing. The frame shift caused by skipping of exon 6 results in a protein lacking the intracellular and transmembrane domains and part of the extracellular domain. This TNFR1 splice form is associated with multiple sclerosis (MS) but not with other autoimmune conditions such as rheumatoid arthritis, psoriasis or Crohn's disease. The MS risk allele directs the expression of a soluble TNFR1 form that can block TNF. Importantly, TNF-blocking drugs can promote onset or exacerbation of MS, but they are very effective for autoimmune diseases that are not associated with rs1800693. This indicates that the clinical experience with these drugs corroborates the disease association of rs1800693, and that the MS-associated TNFR1 variant mimics the effect of TNF-blocking drugs. (86)

The **TNFR1-Δ2** splice variant is regulated by three variations in the promoter affecting the phenotype of TRAPS. They occur in the promoter, exon 1 and intron 4 (rs4149570, rs767455, rs1800692 respectively) of the *TNFRSF1A* gene. In patients with TRAPS, rs1800692 T/T homozygotes are exceedingly rare. In vitro alternative splicing and transcriptional activity assays showed that exon 2 skipping increased with the T-A-T haplotype at rs4149570–rs767455–rs1800692 as compared with the G-G-C haplotype. Furthermore, transcriptional activity increased with the T-T haplotype compared with the G-C haplotype at rs4149570–rs1800692. These results suggest that regulation of TNFR1-Δ2 expression may occur via a coupling mechanism between TNFRSF1A transcription and exon 2 splicing with a combined functional effect of the promoter, exon 1 and intron 4. Whereas TNFR1 is ubiquitously expressed, TNFR1-Δ2 is predominantly expressed in human PBLs, brain, heart, kidney, skeletal muscle, small intestine and spinal cord, but not in liver or lung. These results suggest that expression of these two *TNFRSF1A* transcripts is regulated differentially, and that TNFR1-Δ2 is expressed tissue-specifically. (87)

2.4.2. TNFR1 as a biomarker

Because TNF is a key immune system modulator and has far-reaching effects, excessive signaling can cause significant damage. A delicate balance exists between beneficial immune stimulation and pathogenesis. Several autoimmune and inflammatory diseases have been associated with the effects of dysregulated TNF activation. (88) Therefore, understanding

the regulation of TNF and its receptors is essential for elucidating how TNF can either prevent or induce various diseases.

Disease	Biomarker	Tissue	Effect	Ref.
Rheumatoid arthritis	sTNFR1 protein up	synovial membrane		(89-91)
Systemic Lupus Erythematosus	TNFR1 protein up	B-cells, T-cells	cellular activation	(92)
Lupus Nephritis	TNFR1 protein up	urine, brain	apoptosis	(93, 94)
Huntington's Disease	TNFR1 mRNA down	skeletal muscle		(95-98)
Behcet's Disease	sTNFR1 protein up	serum, synovial fluid		(99-102)
Ocular allergic inflammation	TNFR1 protein up	HCECs	I-CAM expression	(103)
Allergic skin inflammation	TNFR1 protein up	effector T-cells		(104)
Asthma	TNFR1 protein ip	endothelial cells	remodeling	(105-108)
Hepatocellular carcinoma	sTNFR1 protein up	serum, ascetic fluid		(109, 110)
Hepatocellular carcinoma	TNFR1 mRNA down	liver		(111)
Aging	TNFR1 protein up	lymphocytes	apoptosis	(112)
Acute Myocardial Infarct	TNFR1 protein up	cardiomyocytes, ECs	angiogenesis down	(113-115)
Chronic heart failure	sTNFR1 protein up	serum		(116)
Chronic atrial fibrillation	sTNFR1 protein up	serum	remodeling	(117)
Ischemic cerebrovascular diseases	sTNFR1 protein up	serum	proliferation	(118, 119)
Lung epithelium injury	sTNFR1 protein up	BALF	neutralize TNF	(120)
LPS	TNFR1 mRNA up	macrophages		(121)
Salmonella typhimurium	TNFR1 mRNA up	macrophages		(122)

Table 3. The regulation of TNFR1 in several TNFR1-mediated diseases. For detailed description, see text.

In **rheumatoid arthritis** (RA) patients, not only TNF is over-expressed in synovial fluids and the synovial membrane (123, 124), but also the TNF receptor expression is up-regulated, especially in synovial membrane areas adjacent to erosions, and increased concentrations of the shed TNF receptors appear to correlate with disease activity. (89-91)

Patients with **systemic lupus erythematosus** (SLE), a multiorgan inflammatory autoimmune disease, have altered expression of TNF-related signaling molecules, suggesting that imbalance of TNF signaling favors cellular activation rather than apoptosis. These patients have increased levels of TNFR1, TNFR2 and TRAF2 and decreased levels of RIP1 on various naive and memory B-cell and T-cell subsets. However, the levels of these molecules are not correlated with their RNA expression or with serum TNF levels in peripheral whole blood. (92)

MRL/lpr, NZM2410 and B6.Sle1.lpr mice develop spontaneous **lupus nephritis**, an inflammation of the kidney caused by SLE. In these mice, VCAM-1, P-selectin, TNFR1 and CXCL16 are enriched in the urine compared with the serum, especially at the peak of disease. All four molecules are also elevated in the urine of patients with lupus nephritis and correlate well with proteinuria and SLE disease activity index scores. These molecules may therefore be useful for diagnosis of lupus nephritis. (93) Whether the increased urinary

TNFR1 levels reflect increased shedding and are relevant to disease progression remains unclear. In contrast, deposition of complement proteins in the brain of MRL/*lpr* mice indicates complement activation causing release of inflammatory mediators that can induce apoptosis. TNFR1, iNOS and ICAM-1 are up-regulated in brains of lupus mice. Crry (CR1-related y), a C3 convertase inhibitor prevents the increased expression of these inflammatory molecules. This clearly demonstrates that apoptosis is complement dependent in lupus brains and that complement inhibition is a therapeutic possibility. (94)

Huntington's disease (HD) is a genetic neurodegenerative disorder caused by expansion of a glutamine-encoding CAG repeat. It affects muscle coordination and results in cognitive regression and psychiatric problems. A mouse model representing the earlier stages of human HD has been made by inserting CAG repeats into the mouse *huntingtin* gene ($Hdh^{CAG(150)}$ knock-in mice). (95) Another mouse model, expressing exon 1 of the human *huntingtin* gene (R6/2 transgenic mice), mimics the later stages or the rare juvenile form of human HD, which is generally more severe than adult-onset HD. (96) The same gene expression changes have been seen in both mouse models and in muscle biopsies from HD patients. In addition to metabolic changes (97), there were also HD specific changes, e.g. decreased gene expression of *Tnfrsf1a*. The complete HD signature may be a useful biomarker that reflects disease progression. (98)

Behcet's disease (BD) is a chronic, multisystemic, immunoinflammatory vasculitis that often presents with mucous membrane ulcers, ocular lesions and arthritis. Based on the systemic levels of IL10 and IL12, it was suggested that BD might be a Th1-driven disease. But patients with active BD also have increased levels of plasma sTNFR1 and sTNFR2, especially when arthritis is present. The concentrations of both receptors were three times higher in synovial fluid (SF) of arthritic joints than in the corresponding plasma. (99) TNF appears to be important in initiating the disease, as indicated by the effects of blocking TNF. Infliximab is also a promising treatment for uveitis associated with the disease, and Etanercept may be useful for patients with mainly skin and mucosal symptoms. (100-102, 125)

Up-regulation of the intercellular adhesion molecule (ICAM)-1 on human conjunctival epithelial cells (HCECs) is an important feature of **ocular allergic inflammation**. TNF in the supernatant from IgE-activated human conjunctival mast cells up-regulates the expression of TNFR1 on HCECs, resulting in a stronger TNF-mediated response, including up-regulation of ICAM-1. So, up-regulation of TNFR1 expression results in enhanced ICAM-1 expression in response to TNF stimulation. This demonstrates that targeting TNFR1 expression may be more effective than targeting TNF for treatment of ocular inflammation. (103)

The development of **allergic skin inflammation** can be prevented by repeated low-dose exposure to contact allergens, with the help of TNF and its receptors. In mice, suppressor T-cells can activate killer DCs in skin-draining lymph nodes upon contact with low dose allergens. Killer DCs then induce TNFR2-driven apoptosis in allergen-specific effector T-cells, preventing the development of **contact hypersensitivity** (CHS), which is used as a mouse model for human allergic contact dermatitis (ACD). Mice deficient in TNF, TNFR1 or TNFR2 show reduced CHS responses due to impaired activation and migration of antigen-presenting

cells (APC) during the induction phase and diminished recruitment of inflammatory immune cells to the skin during the elicitation phase. In allergen-specific immune responses, TNF may act both as an activator (resulting in CHS) and a suppressor of the immune system (resulting in tolerance), depending on the form of TNF involved (tmTNF vs sTNF), the receptor engaged (TNFR1 vs TNFR2), and the immune cell population targeted. (104)

Symptomatic **asthma** exacerbation is associated with increased BAL levels of TNF and increased airway remodeling. Airway remodeling in asthma is characterized by subepithelial fibrosis, increased extracellular matrix deposition, smooth muscle hyperplasia/hypertrophy and mucus metaplasia. TNF contributes not only to airway remodeling but also to remodeling in other diseases, such as proliferative retinopathy (105, 126), cardiac remodeling (106, 127), and remodeling of blood vessels and lymphatics in the lung (107). TNFR1/2 deficient mice in a model of chronic ovalbumin (OVA) induced airway remodeling show reduced recruitment of eosinophils expressing TGF-β1 and reduced remodeling. (108)

Hepatocellular carcinoma (HCC) is the fifth most common neoplasia in the world and the first cause of death by cancer in some regions. sTNFR1 levels in the serum and ascitic fluid of patients with HCC are significantly higher than in controls, and they correlate positively with total bilirubin and alpha fetoprotein in the peripheral blood. This reflects an abnormal immune status of HCC patients and can help to predict the progression of the tumor. (109) Moreover, the disruption of death receptor (DR)-dependent cell signaling is linked to poor survival in patients with HCC. Because there is intense crosstalk among DR and cell survival pathways in cancer cells, specific targeting of DR pathways is a potential therapeutic strategy for HCC treatment. (110) On the other hand, several genetic alterations of the TNF-TNFR superfamily in HCC were detected by sequencing HCC DNA samples. In particular, the TNFR1 promoter –329G/T polymorphism was strongly associated with primary HCC, where the T allele resulted in the repression of TNFR1 expression. Therefore, our results suggest that the TNFR1 329G/T polymorphism may play an important role in the development of HCC. (111)

Aging is characterized by increased susceptibility of T-cells to TNF-induced apoptosis due to increased constitutive expression of TNFR1 and TRADD and decreased expression of TNFR2 and TRAF-2. Moreover, there is an early and increased activation of caspase 8 and caspase 3, confirming that increased TNF-induced apoptosis may play a role in T-cell deficiency associated with human aging. (112, 128)

Development of artery diseases is associated with age-related impairment of angiogenesis. Because TNFR1 is known to mediate the cytotoxic effects of TNF, whereas TNFR2 is mostly involved in the protective effects, TNF signaling via its receptors has diverse effects on neovascularization, repair and regeneration in adult tissue after **acute myocardial infarction** (AMI). Intact signaling through both TNFR1 and TNFR2 assures sufficient NF-kB activation, followed by transcriptional activation of *VEGFA*, *FGFB* and other pro-angiogenic genes. But age-associated decrease of TNFR2, coupled with post-ischemic increase in systemic TNF, favors apoptosis in adult cardiomyocytes and ECs due to reduced NF-kB activation, leading to inhibition of angiogenesis. Decreased TNFR2 expression in adult tissue also stimulates pro-apoptotic signaling through TNFR1 by the release of vacant TRADD. (113) But signaling

via both TNFR1 and TNFR2 is necessary to prevent reperfusion injury after AMI during late preconditioning. Modulation of TNFR1 and/or TNFR2 expression at different stages of AMI may have important implications for prevention of myocardial injury and enhancement of myocardial repair and regeneration. (114) AMI induction in rats caused low blood pressure, increased leakage of lactate dehydrogenase (LDH) and creatine kinase (CK), larger infarcts, increased TNF levels, and increased TNFR1/TNFR2 ratio. (115)

Also in patients with **chronic heart failure**, TNF and TNFR1 are increased as an expression of inflammation, and they are strongly correlated with other prognostic factors, such as anemia and low lipid fractions (TChol, LDLchol, TG and Ht). (116)

Inflammation is also involved in the early stage of **chronic atrial fibrillation** (AF) and may lead to electrical and structural remodeling in atrial tissue. The fibrillating atria cause calcium accumulation within the atrial myocytes, inducing apoptosis. Dead atrial myocytes are replaced by fibrous scar tissue, which contributes to the electrical conduction abnormality. TNF elevation in serum and atrium tissue is strongly associated with AF, and sTNFR1 - as the main receptor for TNF in AF - rises accordingly in the serum. (117, 129)

Peripheral blood markers of inflammation have been associated with incidence and recurrence of **atherosclerosis and cardiovascular disease** and can also be used for prognosis after **ischemic stroke**. High TNF levels are associated with myocardial ischemia and with subclinical atherosclerosis. However, it has been suggested that the sTNFR1 level may be a better marker of atherosclerosis and vascular defects than TNF. TNFR1 is emerging as an important marker of poor outcome after both stroke and myocardial infarction, and associations exist between TNFR1 and mortality. (130)

Furthermore, it has been shown that patients with **acute ischemic cerebrovascular diseases** also have higher plasma levels of sTNFR1, neutrophil gelatinase-associated lipocalin (NGAL) and neutrophil proteinase 4 (NP4), which are markers of leukocyte activation. (118, 131) Likewise, animal studies have shown that TNFR1 is associated with decreased neuronal proliferation after stroke and that deletion of TNFR1 enhances neuroblast formation and recovery. These results provide evidence that TNFR1 is a negative regulator of stroke-induced progenitor proliferation; the proliferative response after stroke might be promoted by blocking TNFR1 signaling. (119)

Ozone (O_3) is an air pollutant that causes **lung epithelium injury** leading to inflammation. The inflammatory response to acute ozone exposure includes the production of numerous cytokines and chemokines, resulting in influx of neutrophils. Exposure chamber studies on adult humans point to a reduced response to acute O_3 with increasing age. Genetic linkage studies on both mice and humans have shown that a locus encompassing TNF plays a role in responses to O_3. In mice, age-related differences in the inflammatory response to acute O_3 exposure vary with TNFR1 expression. In WT mice, sTNFR1 in the BAL fluid is substantially higher at the age of 39 weeks than at 7 weeks. Consequently, the influx of BAL neutrophils and BAL concentrations of MCP-1, KC, and MIP-2 are lower at the age of 39 weeks. But in TNFR1 KO mice, the inflammatory responses in older mice are intact. The increase in sTNFR1 can neutralize TNF in the lung and protect older mice against O_3-induced inflammation. (120)

Bacterial **LPS** plays a major role in the early response of macrophages to bacterial infection. It causes significant changes in the expression of many genes encoding chemokines, cell surface receptors, signaling molecules and transcriptional activators. Many of these up-regulated genes encode effectors with well characterized pro-inflammatory or direct antimicrobial properties. LPS also elevates the gene expression of receptors such as TNFR1 and CD40, which allow macrophages to communicate with other cells of the immune system. (132, 133) However, up-regulation of TNFR1 occurs only in hematopoietic cells and cancer cell lines. On pulmonary endothelial cells (PECs), TNFR1 surface expression decreased substantially two hours after LPS administration, but it started to recover another 2 h later and reached ∼80% of baseline levels at 24 h after LPS. This loss of membrane bound TNFR1 on endothelial cells may limit ongoing TNF stimulation. (121)
Also *Salmonella typhimurium* **infection** of RAW 264.7 macrophages alters the expression level of many genes due to recognition of bacterial products such as LPS, as well as by activation of IFNγ as a host cell response. An overlapping spectrum of genes is expressed in response to *S. typhimurium* and to purified LPS from *S. typhimurium*. The gene expression profile is further altered by activation with IFNγ, indicating that host cell responses depend on the activation state of the cell. IFNγ is released by NK- and T-cells following *S. typhimurium* infection as a stimulator of macrophage gene expression that makes them respond more rapidly and effectively against invading pathogens. Elevated mRNA levels of signaling molecules involved in cell death or the response to IFNγ were also observed. These include the apoptosis-associated genes caspase 1, TNFR1, Fas and TRAIL, and the IFNγ-induced IFN regulatory factor 1 (IRF-1). (122)

2.4.3. Viral TNFR1 interference

TNF acting via TNFR1 is considered an important anti-viral agent, often acting synergistically with IFNγ. (134) However, growing evidence has shown that both DNA and RNA viruses can interfere with the TNFR1 pathway and thereby escape the host immune response. Viral proteins can either affect TNFR1 availability by acting upon transcription, translation, trafficking or shedding of TNFR1, or modulate TNFR1 activity by acting on internalization or signaling of TNFR1.

Epstein-Barr virus (EBV) immediate-early protein BZLF1 prevents TNF-induced activation of target genes and TNF-induced apoptosis by down-regulation of TNFR1 during the EBV lytic replication cycle. Thus, EBV has developed a mechanism for evading TNF-induced antiviral effects during lytic reactivation or primary infection. (135) The **hepatitis C virus** (HCV) core protein is not only a component of viral nucleocapsids but also a multifunctional protein influencing multiple cellular processes. In HCV core protein-activated Hep191 cells, transcriptional profiling identified decreased expression of TNFR1. Since RT-PCR confirmed that TNFR1 is down-regulated and that TNF-induced DNA fragmentation is suppressed in these cells, expression of HCV core protein at physiological levels might inhibit apoptotic cell death of HCV-infected cells. (135)

Virus	Viral protein	Interference	Mechanism	Ref.
Epstein-Barr virus	BZLF1	TNFR1 transcription	Reduction of TNFR1 promoter activity	(135)
Hepatitis C virus	?	TNFR1 transcription	Inhibition of TNFR1 mRNA expression	(136)
Herpes simplex virus	UL41	TNFR1 translation	Degradation of TNFR1 mRNA and inhibition of apoptosis	(137, 138)
Cytomegalovirus	Immediate early (IE) viral products	TNFR1 Golgi-trafficking	Down-regulation of TNFR1 surface expression	(139, 140)
Poliovirus	3A	TNFR1 Golgi-trafficking	Down-regulation of TNFR1 surface expression	(141)
Respiratory syncytial virus	?	TNFR1 shedding	IL10 production and increased production of soluble TNF receptor	(142, 143)
Adenovirus	E3-14.7 K	TNFR1 internalization	Inhibition of apoptosis and increased NF-kB signaling	(144)
Myxoma virus	secreted M-T2 (pseudo sTNFR)	TNFR1 signaling	Inhibition of TNF-induced cytotoxicity in T-cells	(145, 146)
Myxoma virus	intracellular M-T2	TNFR1 signaling	Inhibition of apoptosis (T-cells)	(145)
Orthopoxviruses	CrmB,C,D,E (pseudo sTNFR)	TNFR1 signaling	Blocking TNF function	(147)
Epstein-Barr virus	LMP-1 (pseudo TNFR)	TNFR1 signaling	Ligand independent NF-kB activation (infected B-cells)	(148-154)
Human herpes virus 6B	U20	TNFR1 signaling	Inhibition of TNFR1 signaling and apoptosis	(155)
Herpesvirus saimiri	STP (pseudo TNFR)	TNFR1 signaling	Ligand independent NF-kB activation via association with TRAFs (infected T-cells)	(156)
human papilloma virus 16	E6	TNFR1 signaling	TNFR1 binding with inhibition of TRADD binding and DISC formation leading to reduced TNF-induced apoptosis	(157)
Respiratory syncytial virus	RSV-G protein (pseudo TNFR1)	TNFR1 signaling	Inhibition of TNF-induced apoptosis by I-kB proteolysis	(158)
Hepatitis C virus	HCV core protein	TNFR1 signaling	TNFR1 DD binding with inhibition of TRADD binding, facilitating FADD binding and leading to increased TNF-induced apoptosis (HepG2, Hela)	(159, 160)
Hepatitis C virus	HCV core protein	?	Increasing NF-kB nuclear retention and DNA binding and increasing I-kB degradation leading to reduced apoptosis	(161, 162)
Hepatitis C virus	HCV core protein	?	Inhibition of TNF-induced apoptosis (MCF7)	(163)
Tanapox virus	glycopeptide	?	Inhibition of TNF-induced NF-kB activation leading to reduced cell adhesion molecules	(164)
Parvovirus	?	?	Activation of caspase 3 and down-regulation of c-*myc* leading to increased apoptosis	(165, 166)

Table 4. Viral mechanisms of escape from the host response. For detailed description, see text.

Herpes simplex virus 1 (HSV) uses many strategies to inactivate host functions that are harmful to its replication and dissemination, including taking advantage of the short half-life of TNFR1. Steady-state levels of TNFR1 require continuous renewal by translation of its mRNA, and the HSV viral protein UL41 prevents this constant replenishment by degrading the TNFR1 mRNA. (137)

Cytomegalovirus (CMV) is known to target the cell cycle, cellular transcription and immunoregulation to optimize the cellular environment for viral DNA replication. CMV infection also prevents external signaling to the cell by reducing the cell surface expression of TNFR1. Viral early gene products may be responsible for interfering with TNFR1 trafficking through the Golgi apparatus to the cell surface. So, upon infection CMV isolates the cell from host-mediated signals, forcing it to respond only to virus-specific signals. (139, 140) After an extended period of continuous virus production, CMV infection terminates by a caspase-independent cell fragmentation process, initiated by the mitochondrial serine protease HtrA2/Omi and controlled by the viral mitochondria-localized inhibitor of apoptosis (vMIA). (167)

Poliovirus also triggers host defensive reactions by activating intrinsic (intracellular) and extrinsic (receptor-mediated) apoptotic pathways. Poliovirus nonstructural protein 2A is an inhibitor of cellular translation enhancing the sensitivity to TNFR1-induced apoptosis. On the other hand, poliovirus nonstructural protein 3A neutralizes the pro-apoptotic activity of 2A by eliminating TNFR1 from the cell surface. Consequently, poliovirus infection dramatically decreases TNF receptor abundance on the surfaces of infected cells as early as four hours post-infection. Poliovirus-mediated resistance to TNF is caused by protein 3A interfering with protein trafficking through the endoplasmic reticulum and Golgi: the effect of protein 3A on TNF signaling can be imitated by brefeldin A. (141)

Alveolar macrophages and respiratory epithelial cells **infected with respiratory syncytial virus** (RSV) suppress the production of early inflammatory cytokines such as TNF by producing the Th2 cytokine IL10, which is an ineffective response to the virus. (142) Moreover, the soluble form of TNFR1, but not TNFR2, was secreted from A549 cells in a time- and RSV dose-dependent way. As the secretion of soluble TNFR1 blocks TNF responses, increased shedding might be another counteraction against the immune response. (143)

The **adenovirus** protein E3-14.7 K inhibits TNF-induced apoptosis, which is initiated by recruitment of TRADD, FADD and caspase 8 to the death domain of TNFR1 to assemble the death-inducing signaling complex (DISC). Analysis of purified magnetically labeled TNFR1 complexes from mouse and human cells stably transduced with E3-14.7K revealed that prevention of TNF-induced TNFR1 internalization resulted in inhibition of the DISC formation. In contrast, E3-14.7K did not affect TNF-induced NF-kB activation via recruitment of RIP-1 and TRAF-2. Inhibition of endocytosis by E3-14.7K is due to a failure in the coordinated temporal and spatial assembly of essential components of the endocytic machinery, such as Rab5 and dynamin 2, at the site of the activated TNFR1. This is another mechanism by which Adenoviruses escape the host immune response. (144)

Many poxviruses encode several immunomodulatory proteins, such as homologs of host cytokine receptors, also referred to as viroreceptors. These receptors mimic host function by binding to host cytokines, allowing the virus to circumvent the immune defense. The T2 protein of **myxoma virus** (M-T2) is a pseudo TNF receptor that has two distinct activities. The secreted dimeric M-T2 glycoprotein binds TNF with high affinity, inhibiting direct TNF-mediated cytolysis of infected cells and other secondary immune responses dependent on

TNF. However, intracellular M-T2 prevents myxoma-infected T-cells from undergoing apoptosis, supporting viral replication. (145, 146) The cytokine response modifier (Crm)-like pseudo TNF receptors, including CrmB, CrmC, CrmD and CrmE and a putative fifth member from cowpox virus that closely resembles CD30, have been identified in several **orthopoxviruses**. These viral TNF receptors resemble secreted versions of the extracellular domains of their counterpart cellular receptors and form functional oligomers that bind and block TNF. (147)

The latent membrane protein 1 (LMP1) of **Epstein-Barr virus** (EBV) contributes to the immortalizing activity of EBV in primary human B lymphocytes. (148) LMP1 is targeted to the plasma membrane of infected cells as a constitutive pseudo TNF receptor that activates NF-kB through two independent domains in its cytoplasmic tail. One site is similar to TNFR2 interacting with TRAF1 and TRAF2 and the second site is similar to TNFR1 associating with TRADD. As LMP1 acts independently of the ligand, it replaces the T-cell-derived activation to maintain unlimited B-cell proliferation. LMP1-mediated signaling through the TRAF system plays a role in the pathogenesis of EBV-infected lymphomas that emerge in immunosuppressed patients. (149) Furthermore, the activity of the TNFR1 promoter is dramatically decreased by the EBV protein BZLF1, helping the virus to oppose the anti-viral effects of TNF. (135) Infection by **human herpesvirus 6B** (HHV-6B) blocks caspase 3 and 8 activation and I-kB phosphorylation, indicating inhibition of both the inflammatory and apoptotic signaling pathways. The viral pseudo TNF receptor U20 was shown to localize to the cell membrane, and siRNA knockdown of U20 showed that the protein is necessary for HHV-6B-mediated inhibition of TNFR signaling during infection. (155) The STP pseudo TNF receptors of the **herpesvirus saimiri** (HVS) are stably associated with TRAF1, 2, or 3. Mutational analysis revealed that STP-C488 induces NF-kB activation that correlates with its ability to associate with TRAFs. Thus, TRAF/STP association might be involved in immortalization of T lymphocytes following HVS infection. (156) High-risk strains of **human papillomavirus** (HPV), such as HPV16, cause human cervical carcinoma. The E6 protein of HPV16 mediates the rapid degradation of p53. But transfection of HPV16 E6 into the TNF-sensitive LM cell line protects the cells from TNF-induced apoptosis independently of p53. Caspase-3 and -8 activation is significantly reduced in E6-expressing cells, indicating that E6 acts early in the TNF apoptotic pathway. In fact, E6 binds directly to TNFR1. E6 requires the same TNFR1 C-terminal part for binding as does TRADD, and TNFR1/TRADD interactions are decreased in the presence of E6. HPV E6 binding to TNFR1 interferes with formation of the DISC and thus with transduction of pro-apoptotic signals. HPV, like several other viruses, can evade the TNF-mediated host immune response. (168) Moreover, the central conserved region of the attachment protein G of **respiratory syncytial virus** (RSV) shows structural homology with the fourth subdomain of TNFR1. Although the functions of both protein domains are unknown, the structural similarity of the two protein domains suggests that the cysteine noose of RSV-G may interfere with the antiviral and apoptotic effect mediated by TNF. (158)

The **hepatitis C virus** (HCV) core protein is not only a component of viral nucleocapsids but also a multifunctional protein. The core protein binds to the cytoplasmic domain of TNFR1, namely the death domain (DD). The presence of core protein does not alter the level of TNFR1 mRNA or expression of TNFR1 on the cell surface, but it enhances TNF-induced apoptosis. (159) The findings suggest that the HCV core protein sensitizes cells to TNF-induced apoptosis due to its interaction with the cytoplasmic tail of TNFR1, thereby inhibiting TRADD binding but facilitating FADD recruitment to TNFR1. (160) By enhancing TNF-mediated apoptosis, the HCV core protein may provide a selective advantage for HCV replication by enabling evasion of host antiviral defense mechanisms. In contrast, HCV core protein was shown to inhibit TNF-induced apoptosis via NF-kB activation. The expression of the core protein enhances nuclear retention and DNA binding activity of NF-kB and TNF-triggered I-kB degradation. This ability of HCV core protein to inhibit TNF-mediated apoptotic signaling may contribute to the chronically activated, persistent state of HCV-infected cells. (161) (162) The controversy among these reports may be attributed to differences in cell types, conditions of core protein expression, and protocols of TNF stimulation. (169, 170)

Tanapoxvirus (TPV)-infected cells secrete an early 38-kDa glycopeptide that selectively inhibits TNF-induced NF-kB activation and transcriptional activation of cell-adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 on the surface of endothelial cells. (164)

Parvovirus H-1 infection leads to activation of caspase 3, leading to morphologic changes characteristic of apoptosis and resembling the effects of TNF treatment. This effect is also observed when U937 cells are infected with a recombinant H-1 virus that expresses the nonstructural proteins but in which the capsid genes are replaced by a reporter gene, indicating that the induction of apoptosis can be assigned to the cytotoxic nonstructural proteins in this system. Furthermore, the c-Myc protein, which is over-expressed in the monocytoid cell line U937, is rapidly down-regulated during parvovirus infection, consistent with a possible role of c-Myc in mediating the apoptotic cell death induced by H-1 virus infection. Interestingly several clones derived from the U937 cell line and selected for their resistance to H-1 virus fail to decrease c-Myc expression upon treatment with differentiation agents, and they also resist the induction of cell death after TNF treatment. (165, 166)

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3. Inflammation

3.1. Acute inflammation

Inflammation is a local response to tissue damage caused by an infection (bacteria, viruses, or fungi), allergens or physical stimuli (heat, ionizing radiation, UV radiation, etc.), but can also be the result of an auto-immune response of the body, such as in rheumatoid arthritis. The classical symptoms of inflammation are redness and heat due to vasodilatation, swelling or edema, pain and organ dysfunction. (1) A superficial infection can be defeated by physical barriers including epithelial tight junctions, neutrophils, anti-bacterial peptides such as defensins, complement components, mannose binding lectin and IgMs. (2-4) But the induced innate immune response relies on the recognition of bacterial components or pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors including toll-like receptors such as TLR-2, TLR-4 and TLR-8. Exposure to PAMPs triggers the signaling cascades leading to transcription of a set of genes that are associated with inflammation as well as immunity.

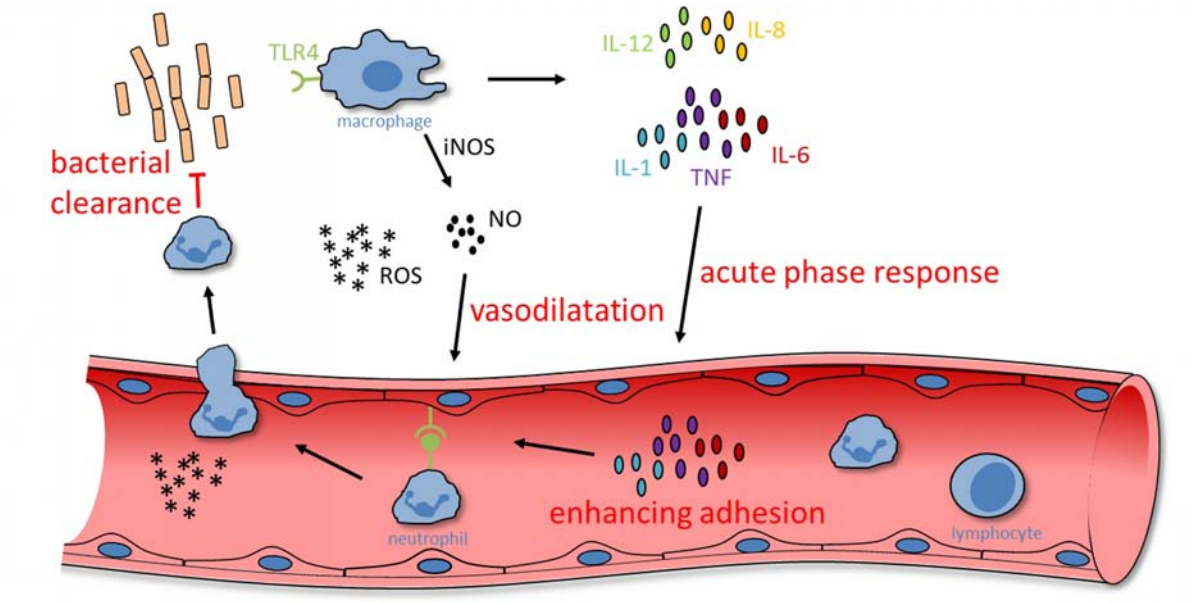

Figure 1. Acute bacterial inflammation. For detailed description, see text.

Several pathways lead to NF-kB-induced gene transcription resulting in the release of important pro-inflammatory cytokines and chemokines, such as TNF, IL1, IL6, IL12 and IL8. (Figure 1) These cytokines and chemokines, produced by activated macrophages, but also other cells, result in a controlled local immune response to the infection and clearance of the invading pathogen. (5) Moreover, the TLR-4 pathway activates signals that direct the synthesis of pro-inflammatory lipid mediators (PAF, leukotrienes, prostaglandins and thromboxanes), ROS, type 1 interferons and iNOS, which leads to increased NO production. (6) NO-induced local vasodilatation causes slowing of the blood flow, allowing tethering of neutrophils to the vessel wall while locally produced TNF and IL1 activate the vascular endothelium, enhancing adhesion molecule expression. Neutrophil transmigration is

accompanied by a significant amount of intravascular fluid, partly explaining the peripheral tissue edema, and contributes to successful clearance of bacteria. (7) TNF, IL1 and IL6 coordinate the initiation of the acute phase response, resulting in transcriptional up-regulation of many proteins, mainly in the liver, including C-reactive protein and serum amyloid A, which may play a role in antibacterial immunity, proteases and protease inhibitors, coagulation and complement components and transport proteins. (8) Beside the inflammatory response, the host produces compensating anti-inflammatory mediators such as glucocorticoids, IL4 and IL10 and soluble cytokine receptors, including soluble TNF receptors that dampen and terminate the inflammatory response. Without the induced innate immune response, the host would almost certainly succumb to a severe infection.

3.2. Systemic inflammation – sepsis

If the immune system fails to remove the pathogens from the local invasion site, an uncontrolled immune response can arise. When bacteria enter the bloodstream, the infection becomes systemic and the subsequent systemic inflammatory response is called sepsis. Severe sepsis occurs when one or more organs lose their function by poor circulation and in the worst case patients go into septic shock from a drop in blood pressure due to systemic vasodilatation. Trauma or burns can also trigger an excessive inflammatory response and shock. When no infection is involved, the situation is called systemic inflammatory response syndrome (SIRS). (9) SIRS is diagnosed when the patient suffers from more than one of the following criteria: hyperthermia or hypothermia, tachycardia, tachypnea, leukocytosis or leukopenia. (Figure 2)

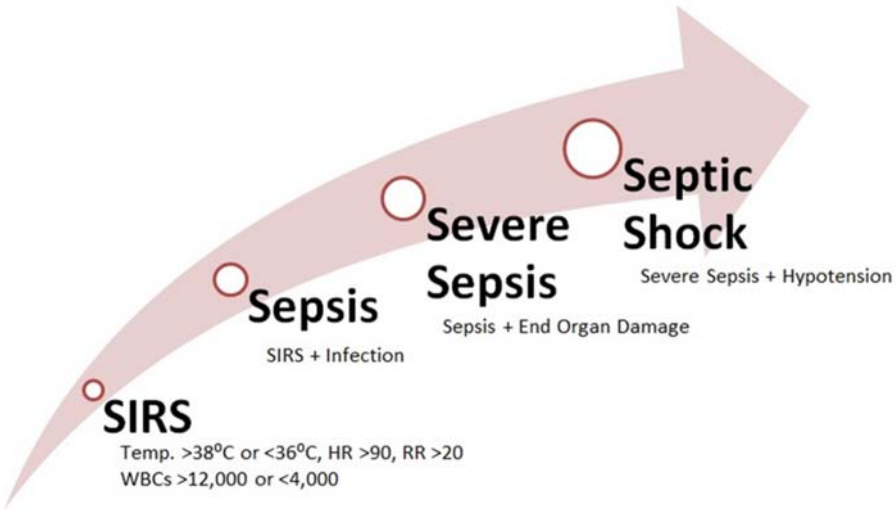

Figure 2. The relationship between SIRS, sepsis, severe sepsis and septic shock. (http://loverockmd.com) HR: heart rate, RR: respiratory rate, WBCs: white blood cells. For detailed description, see text.

The key signals initiating SIRS are cytokines and upon cellular damage, alarmins (e.g. ATP, high-mobility group box 1 and DNA) are released amplifying the induction of cytokines. The endogenous alarmins and the exogenous PAMPs together form the larger family of damage-associated molecular patterns (DAMPs). (10) In SIRS, inflammation dominates over anti-inflammatory pathways, coagulation dominates over fibrinolysis and homeostasis is lost. The

overproduction of inflammatory mediators contributes to the massive activation and recruitment of different cell types, as well as to the excessive activation of the coagulation pathways leading to an overall intravascular coagulation. This, together with NO-mediated cardiovascular anomalies, will inevitably cause tissue damage and subsequent organ failure and death.

3.3. Mouse models

Frequently used experimental models of sepsis include systemic administration of bacteria or bacterial products such as LPS, but also cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP). These models mimic the pathophysiology of human sepsis and scientists are investigating molecules, produced by immune cells during sepsis, of which the blockade might be protective.

Systemic administration of **LPS** shows that the living bacteria and their actions are not necessary to induce the host response. Injection of LPS (endotoxins) in mice results in many clinical symptoms of SIRS and sepsis, namely endotoxemia, including an increase in pro-inflammatory cytokines in the serum. In patients with sepsis, cytokine levels are elevated in relation to the severity of the disease. However, those peaks are lower and they appear later than after LPS injection. These differences, together with the failure of therapies like blocking of LPS with an antibody, suggest that the LPS model is not an accurate reflection of sepsis in patients. But nevertheless, the LPS model has an important function in the study of the underlying molecular mechanisms of SIRS and sepsis. (11)

The idea behind the **CASP** model is that intestinal leakage (e.g. after abdominal surgery) leads to bacterial invasion of the peritoneum. A stent is inserted into the ascending colon, resulting in organ failure such as acute lung injury, renal- and bone marrow cell dysfunction. (12-14) The use of stents of different sizes can modify mortality rates. Pro-inflammatory mediators such as IL1 and IFNγ are thought to play an important role in this model, but survival in CASP seems to be TNF-independent in contrast to the CLP model. (15, 16) Although pathophysiological changes after CASP appear to be similar to those in human sepsis, the nature of the model limits its use. Stenting the colon ascendens creates a constant leakage of bowel content, mimicking the situation of insufficient healing after bowel surgery, which is often leading to sepsis.

Similar to CASP, an abdominal infectious focus leads to a polymicrobial infection of the peritoneum in the **CLP** model. But unlike CASP, cecum ligation is performed immediately below the ileocecal valve followed by a single transient perforation of the cecum through needle puncture. Subsequently, mice develop typical symptoms of sepsis and usually die. (17) The main pro-inflammatory cytokines IL6 and TNF have been shown to increase following CLP and high levels of IL6 strongly correlate with survival, similar to what is seen in human sepsis. (18, 19) Blockade of IL6 results in increased survival, however TNF inhibition by administration of anti-TNF antibodies or using TNFR1-deficient mice, increased the mortality. (20, 21) A drawback is that the outcome after CLP is strongly associated with several factors during the procedure. These include the percentage of cecum that is ligated

and thus the amount of necrosis that is induced, the amount of microbial influx in the peritoneum which is dependent on the size of the needle, the number of punctures and the pressure exerted on the cecum, variability in surgical procedures like the size of the incisions made in the skin and abdominal muscle and the use of supportive therapy such as fluid resuscitation and antibiotic treatment. (22) However, due to its clinical relevance, the CLP model has become the most widely used model for experimental sepsis and may be considered as the 'gold standard', provided that it is performed with high consistency and reproducibility.

Because **TNF** is released systematically during the early phase of inflammation, SIRS can be induced by systemic administration of recombinant TNF. Injection of TNF in mice induces weight loss, hypotension, bowel and renal necrosis and eventually death. Although the TNF-induced lethal shock is caused by different mechanisms, at high doses, the effects of TNF are indistinguishable from the effects of LPS. (23) TNF induces the expression of adhesion molecules on the endothelium and leukocytes resulting in adherence and extravasation. Furthermore, TNF induces enhanced production of NO, whose concentration is inversely proportional to the blood pressure. Subsequent soluble guanylate cyclase (sGC) activation mediates vasodilatation by vascular smooth muscle relaxation. While the inhibition of NO exacerbates toxicity, inhibitors of sGC activation protected against TNF-induced lethality. (24) TNF also induces the production of cytokines (IL6 and IL1) and many secondary inflammatory mediators like leukotrienes, prostaglandins, platelet activating factor. (25)

3.4. Epidemiology and therapy

Sepsis is a major clinical challenge, afflicting around 18 million people worldwide each year. The most dangerous stages, severe sepsis and septic shock, have a combined mortality rate of 30 to 40 % making them the leading cause of death in intensive care unit (ICU) patients. (26) Several risk factors, including higher age, male gender, lower socioeconomic status, alcohol abuse, pre-existing comorbid conditions, underlying immune deficiency and the sites of infection, can significantly increase mortality in sepsis patients. (27) Over the last three decades, the incidence of sepsis has increased due to aging of the population, a rising number of invasive medical procedures, the use of immunosuppressive and chemotherapeutic agents, the emergence of the acquired immunodeficiency syndrome (AIDS) and occurrence of multiresistant bacteria. (28) Fortunately, the mortality rate have been dropping in recent years, thanks to improvements in supportive care. (29) Nevertheless, sepsis still kills around one in four affected patients because critical care specialists lack specific drug therapies for the treatment of sepsis.

The only approved antisepsis drug, recombinant activated protein C (Xigris), was withdrawn from the market because it failed in a post-marketing phase III clinical trial proving no better than a placebo at preventing death in people with high risk of septic shock. (30) Eritoran, a Toll-like receptor (TLR) 4 antagonist that prevents endotoxin signaling, also failed to show benefit in clinical trials. The failure of clinical trials can be attributed to multiple problems. One of them is that current mouse models poorly mimic human inflammatory diseases and

cannot predict results in clinical trials. (31, 32) Another problem is that the timeframe of clinical trials is often not large enough and that the participants are not stratified enough regarding, for instance, the cause or the susceptibility to sepsis. Moreover, the difficulty of designing effective sepsis drugs revolves around the complex, and not fully understood, pathophysiology of sepsis. (33)

Drugs that are currently under development target diverse step in the disease progression. Toramyxin, an antibiotic-coated hemoperfusion filter, targets pathogens, lowering the 28-day mortality rate from 53 % to 32 % when added to standard care. AB103, which mimics part of the T-cell co-receptor CD28, blocks bacterial superantigens or toxins. Other drugs, including ART-123 or recombinant thrombomodulin, an anticoagulant, and the immunomodulatory talactoferrin alfa, aim to dampen the innate immune and inflammatory response of the host. A final set of drugs, including interleukin modulators, immunoadjuvants and other ways of controlling T-and B-cell function are starting to prove importance by basic research. (34) Only by a better understanding of the fundamental processes involved in sepsis, we will be able to define novel targets for new therapies.

Meanwhile, the treatment for sepsis remains mostly supportive. Control of infection, e.g. by use of antibiotics, is not only meant to eliminate the ongoing infection, but also to prevent new infections. Support of impaired organ function consists of fluid resuscitation and the use of vasopressors to normalize blood pressure, mechanical ventilation for respiratory insufficiency and kidney dialysis for kidney failure. (35)

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4. TNF in inflammation

4.1. The role of TNF and its receptors

Studies of TNF-, TNFR1- and TNFR2-deficient mice have confirmed some important functions of TNF in immunity to intracellular and extracellular pathogens, tumor immunity and autoimmunity. (Figure 1)

Innate immunity. The absence of TNF sensitizes mice to bacterial infections such as *Listeria monocytogenes* or *Mycobacterium tuberculosis*, indicating that endogenous TNF is a critical component of the antibacterial host defense. TNF is required for the activation of immune cells such as lymphocytes and neutrophils. It induces the expression of adhesion molecules and the permeability of the endothelium resulting in adherence and extravasation. TNFR1 appears to be the key receptor for these immune functions because the sensitivity observed in TNF-deficient mice also occur in TNFR1 deficient mice and not in TNFR2 deficient mice. (2-5) The differentiation and function of IFNγ producing effector T-cells is found to be normal in TNFR1 deficient mice, but the bacterial sensitivity is associated with defective neutrophil recruitment and reduced expression of the critical adhesion molecules, P- and E-selectin, by endothelial cells. Thus, direct crosstalk between locally produced TNF and TNFR1-expressing endothelial cells is essential for neutrophil recruitment to the site of inflammation. (1) On the contrary, elimination of choriomeningitis virus-infected lymphocytes, which strictly depends on the functioning of $CD8^+$ Tc-cells, is completely unaffected in TNFR1 deficient mice. (2) This indicates that TNFR1 signaling is not needed for direct T-cell mediated immune functions, but only crucial when, in addition to T-cells, macrophages and/or neutrophils are involved. This is of great relevance for understanding the mode of action of anti-TNF therapy for autoimmune diseases in humans. Whereas neutrophil recruitment is beneficial in the clearance of bacterial infections, it is harmful in autoimmune diseases. Therefore, blocking TNF with anti-TNF mAbs or soluble TNF receptors is highly effective in diseases that critically involve neutrophil recruitment, including rheumatoid arthritis, psoriasis or inflammatory bowel disease while no consistent effects have been reported for neutrophil-independent inflammation like the chronic phase of multiple sclerosis. (3, 4) This central role of TNF in neutrophil recruitment is underlined by reports showing that concomitant infections with bacteria or mycobacteria may be more severe and difficult to cure in patients treated with TNF-blocking agents. Specific inhibition of sTNF-TNFR1 can be more effective while tmTNF alone is sufficient to retain a certain level of immunity against pathogens. It has been shown that the specific TNFR1 inhibitor R1antTNF can ameliorate disease severity in collagen-induced arthritis as effectively as the total TNF blocker Etanercept, without interfering with hepatic viral clearing. (5) tmTNF alone was able to resolve infection with Leishmania major in transgenic mice expressing an uncleavable TNF mutant. tmTNF was also sufficient to partially protect against acute Mycobacterium tuberculosis and Listeria monocytogenes infection. (6, 7) While full immunity required sTNF as well, the presence of tmTNF alone was clearly superior to the total absence of TNF in these models.

Adaptive immunity. TNF deficient mice completely lack primary B-cell follicles, well-organized follicular dendritic cell networks and germinal centers in the spleen leading to a dysregulated humoral immune response against antigens. Complementation of TNF by transgenes can reconstitute these defects, demonstrating a physiological role for TNF in regulating the development and organization of splenic follicular architecture and in the maturation of B-cells. (8)

Anti-tumor activity. TNF is also involved in the elimination of malignant cells which might explain the slightly increased risk of malignancy in anti-TNF treated patients. (9) TNF, in combination with IFNγ, is able to efficiently regress tumors. (10) Unfortunately, initial clinical trials showed that systemic administration of TNF leads to unacceptable side effects due to the strong pro-inflammatory nature of TNF. Therefore, the use of TNF/IFNγ is currently limited to locoregional treatments such as isolated limb perfusion (ILP). Angiograms following ILP showed selective elimination of tumor-associated blood vessels, indicating that the tumor endothelium is the main target of TNF/IFNγ therapy. (11) The antitumor effect of TNF/IFNγ is shown to be an indirect effect, involving the destruction of the tumor neovasculature by targeting the integrin αVβ3, an adhesion receptor that plays a key role in tumor angiogenesis. This results in detachment of the endothelial cells from the extracellular matrix leading to anoikis of the endothelial cells, irreversible injury of the tumor vasculature and arrest of tumor circulation. (12) Furthermore, both TNF-TNFR1 signaling on APCs and TNF-TNFR2 signaling on T-cells seem to be required for effective priming, proliferation and recruitment of tumor-specific T-cells. (13) Moreover, TNFR2-dependent signaling leads to production of nitric oxide, which might also result in anti-tumoral effects. (14)

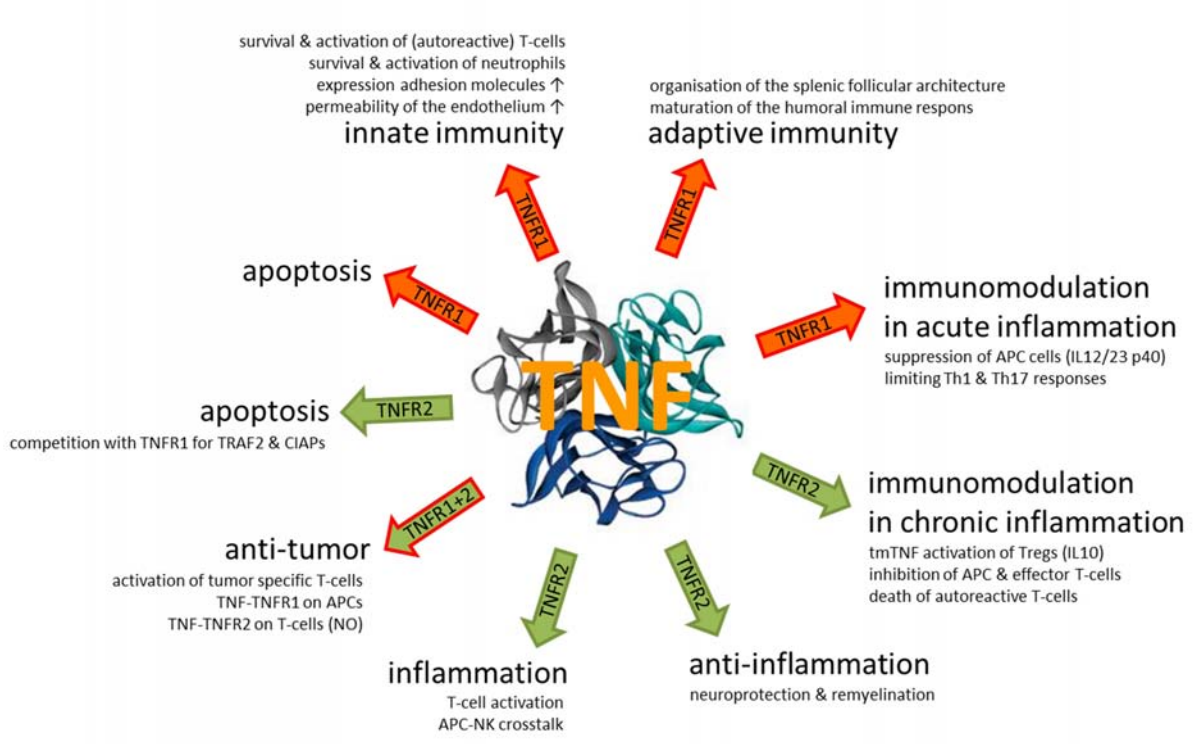

Figure 1. Distinct functions of TNF, mediated by TNFR1 or TNFR2. For detailed description, see text.

Immunomodulation in acute inflammation. The function of TNF in rapid immune responses to foreign pathogens or tumor cells is crucial, providing effector cells to act as a first line of defense or sustained effector responses of the adaptive immune system. But TNF deficient mice showed that TNF also plays a role in the recovery phase by regulating and limiting the inflammatory response. TNF deficient mice injected with heat-killed *Corynebacterium parvum* died 6 to 8 weeks after the challenge, at a time when the inflammatory lesions in wild type mice had resolved. This delayed but progressive inflammatory process characterized by high levels of IL12/IL23 p40 and IFNγ, reveals the two faces of TNF: pro-inflammatory in the initial phase of infection and an anti-inflammatory/immunomodulatory function after the infectious or toxic agent has been localized and controlled. (15) Similar results were obtained in a model of lung infection with both live and heat-killed mycobacteria, in which the animals died quickly from respiratory failure by an uncontrolled Th1 immune response with overproduction of IFNγ and IL12. Early reconstitution with TNF or depletion of $CD4^+$ and $CD8^+$ T-cells decreased IFNγ levels and prolonged the survival of the TNF deficient mice. (16) The suppression of Th1 and Th17 effector cells was shown to be mediated by TNFR1 signaling. TNFR1 deficient mice developed a more severe *Yersinia enterocolitica*-induced reactive arthritis with higher levels of IFNγ and IL17 in arthritic joints, spleen and lymph nodes and a higher number of Th1 and Th17 cells compared with wild type mice. A mechanistic analysis revealed the involvement of IL12/IL23 p40 in the generation of increased IFNγ and IL17 production under TNFR1 deficiency indicating that TNF, by interacting with TNFR1, reduces Th1 and Th17 responses by inhibiting IL12/IL23 p40 production. (17) This TNFR1-mediated immunomodulatory action appears to be a regulatory feedback mechanism limited to APCs and macrophages as the number of these cells correspondingly decreased. (18) (Figure 2)

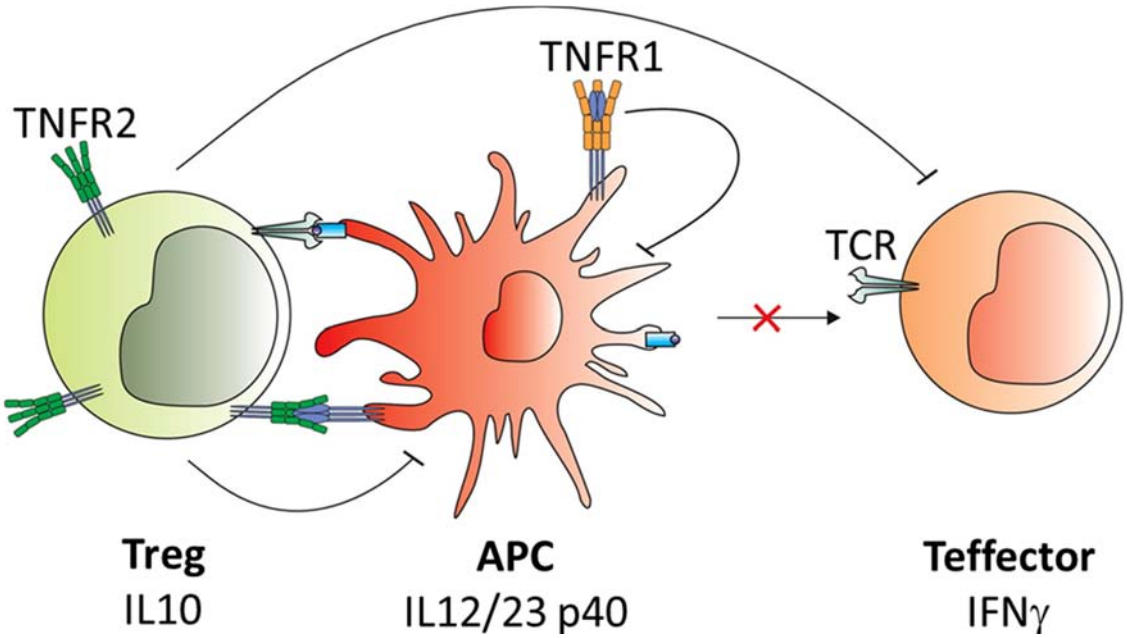

Figure 2. TNF-mediated immunomodulation mediated by TNFR1 or TNFR2. (19) In acute inflammation, sTNF/TNFR1 suppresses T effector cells by inhibiting IL12/23 p40 production of APCs. In chronic inflammation, tmTNF/TNFR2 suppresses self-reactive APCs and T effector cells by promoting the expansion and activity of Tregs. For detailed description, see text.

TNF and TNF receptor deficient mice also fail to develop immune suppression after CLP. During sepsis, TNF and activation of the TNF receptors are crucially involved in generating a suppressed immune status in the host, leading to reduced immune defenses for subsequent secondary bacterial infections. TNF thus causes the ensuing sepsis-induced immunoparalysis. (20)

Immunomodulation in chronic inflammation. Initial TNF signaling is commonly modulated by regulatory feedback mechanisms which serve to inhibit potentially harmful responses, but chronic TNF-signaling impairs I-kB resynthesis, leading to persistent activation of NF-kB and the expression of genes that promote cell survival and effector responses. (21) An inappropriate expansion of self-reactive lymphocyte populations at sites of inflammation would clearly be detrimental. However, TNF also has important immunomodulatory functions especially after prolonged TNF stimulation. (22) TNF indirectly suppresses T-cell function by promoting the expansion and activity of regulatory T-cells (Tregs) by tmTNF-TNFR2 interactions. tmTNF activates TNFR2-expressing $CD4^{+}FoxP3^{+}$ Tregs resulting in their proliferation and increased suppressive activity. (23) In turn, Tregs check for self-tolerance and autoimmunity by inhibiting self-reactive APCs and effector T-cells. (24) (Figure 2)
Inhibition of Treg functions has been correlated with decreased induction of tolerance to self-antigens, which leads to increased risk for autoimmune diseases. (25) Total blockade of TNF, including tmTNF, may compromise the functions of Tregs, which may explain the onset of new autoimmune diseases seen in patients treated with TNF blockers. (26) For example, TNF neutralization can lead to worsening or induction of psoriasis by enhancing Th17 activity and reducing the expansion of Tregs. The lack of T-cell suppression by Tregs may further enhance the expansion of Th17 cells. IL22, produced by these Th17 cells, activates keratinocytes to release antimicrobial peptides that recruit more immune cells to the site of skin inflammation and cause tissue damage. (27) TNF blockade has also been shown to expand Th1 and Th17 cells in a collagen-induced arthritis (CIA) model. However, these T-cells accumulate in the lymph nodes and not in the joints, providing an explanation for the paradox that anti-TNF therapy ameliorates arthritis despite increasing numbers of pathogenic T-cells. (28)
TNF not only has an important role in the onset of autoimmune-mediated demyelination of the central nervous system but also suppressive effects were shown in experimental autoimmune encephalomyelitis (EAE). In immunized TNF deficient mice, self-reactive T-cells to myelin could proliferate in the spleen for at least 10 weeks post-immunization while they already lose this capacity 3 weeks post-immunization in wild type mice. This abnormally prolonged self-reactivity and worsening of the disease from 5 weeks following immunization in TNF deficiency indicate that TNF is protective in the later phase of chronic EAE. (29, 30) These findings may explain the failure in clinical trials with anti-TNF in MS patients showing enhanced clinical symptoms. (31) In TNFR1 deficient mice, anti-myelin reactivity regresses normally while TNFR1R2 double deficient mice were unable to suppress late autoimmune reactivity, indicating that the immunosuppressive functions of TNF may be exerted by TNFR2. Even though the clinical course of EAE is suppressed at both the pro-inflammatory

and the autoimmune phases in TNFR1 deficient mice, TNFR1 is clearly an important target for therapy. (30) Interestingly, treatment of EAE with a TNFR1 specific inhibitor was effective and could reduce the number of Th1 and Th17 cells in lymph nodes and spleen, suggesting that TNFR2 mediated activation of Tregs remained intact. (32)

In some autoimmune diseases including type I diabetes, tmTNF-TNFR2 interactions play a key role in TNF-induced immunomodulation by selectively killing self-reactive T-cells to insulin. (33) In contrast, activated TNFR2 deficient $CD8^+$ T-cells are highly resistant to activation-induced cell death and express high levels of phosphorylated I-kB. Neutralizing TNF in these cells increased their susceptibility again, indicating that TNFR1 functions as a survival receptor promoting I-kB phosphorylation and NF-kB activation in these self-reactive T-cells. (34)

4.2. TNF-induced chronic diseases

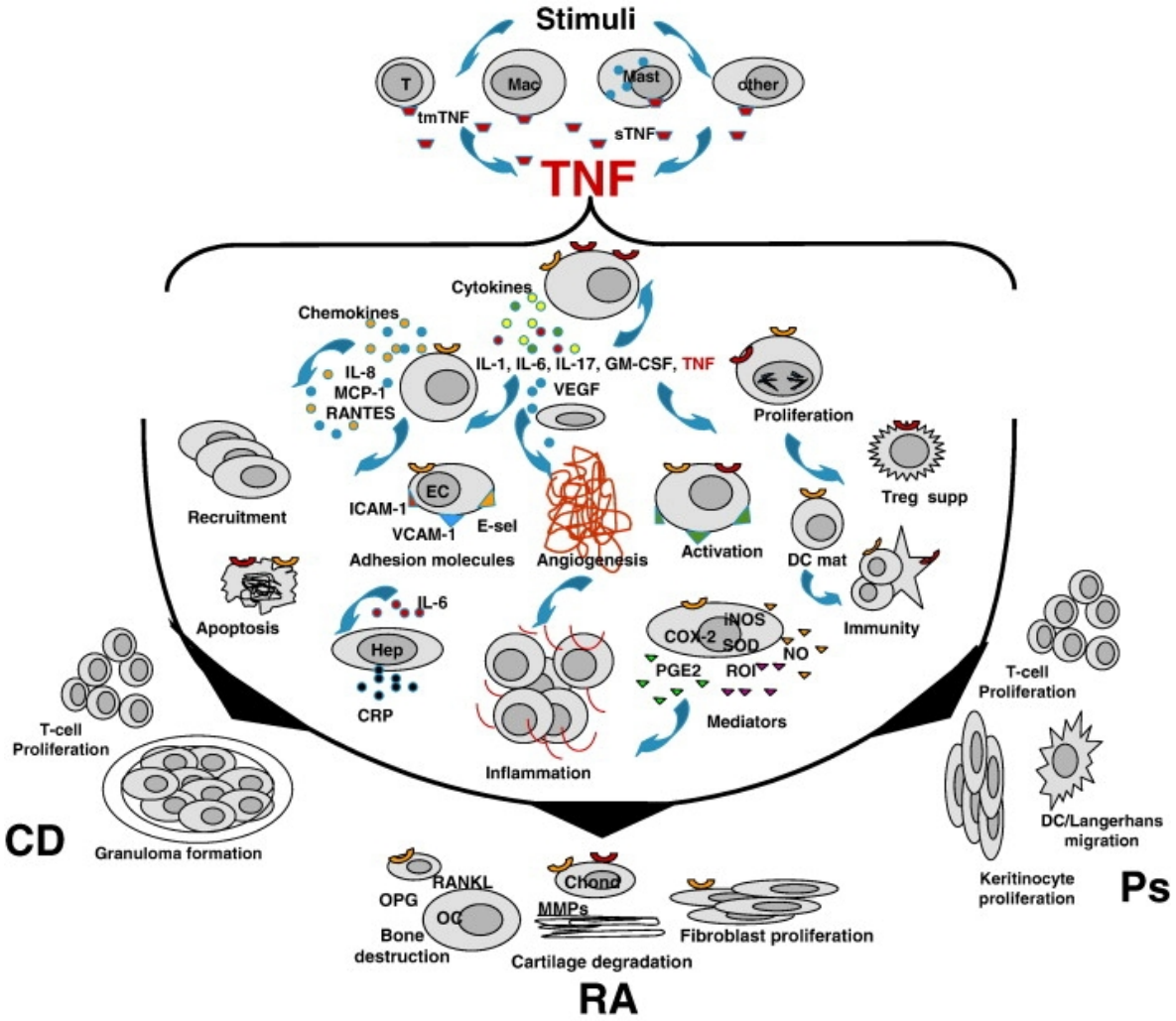

Figure 3. The pathophysiology of TNF-mediated chronic diseases. (35) In the pathophysiology of rheumatoid arthritis (RA), Crohn's disease (CD) and psoriasis (Ps), TNF is produced at high concentrations by a variety of cell types, induced by endogenous or exogenous stimuli. The cellular responses mediated by TNF that are common to RA, CD and Ps, such as those that modulate cell recruitment, cell proliferation, cell death and immune regulation, are shown in the enclosed area in the center of the diagram. Responses restricted to a particular disease, such as granuloma formation in CD, matrix degradation and osteoclastogenesis in RA or keratinocyte proliferation in Ps, are shown outside of the enclosed area. For detailed description, see text.

Defective immune cells can induce destruction of endogenous proteins, cells and tissues, which leads to the development of autoimmune diseases including rheumatoid arthritis, Crohn's disease, multiple sclerosis, type 1 diabetes and psoriasis. Some activities of TNF, such as modulation of cell recruitment, cell proliferation, cell death and immune regulation, are common to a variety of diseases. Other biological activities of TNF are more restricted to certain diseases, such as matrix degradation and osteoclastogenesis in RA or granuloma formation in Crohn's disease. Such disease-specific symptoms may be related to the different cell types in the target tissues or be unique mechanisms underlying the pathogenesis of the particular diseases. (35) See figure 3.

Rheumatoid arthritis. In rheumatoid arthritis (RA), the synovial membrane lining the joint space becomes inflamed as a result of increased vascularization and infiltration of leukocytes, resulting in hyper-proliferation of synovial tissue and progressive erosion of cartilage and bone. (36) (Figure 3) Synovial fluid and tissue from patients with RA contains TNF and other pro-inflammatory cytokines such as IL1, regardless of the duration of the disease. Activated macrophages are the primary source of TNF in inflamed synovial tissue and both the number of macrophages and the expression of TNF correlate with clinical scores for knee pain. (37) The hypothesis that TNF drives much of the pathophysiology in a rheumatic joint is supported by studies of TNF overexpression. Genetic deletion of the ARE in the 3'UTR of the TNF gene ($TNF^{\Delta ARE}$ mice) led to moderate over-expression of TNF in the synovial fibroblasts and resulted in the spontaneous development of chronic inflammatory polyarthritis. (38, 39) Furthermore, transgenic mice expressing a TACE-resistant tmTNF mutant also developed spontaneous arthritis indicating that tmTNF by itself is sufficient to mediate arthritis. (40, 41) Using TNF receptor deficient mice, it was shown that the development of tmTNF-mediated arthritis requires the presence of TNFR1 but is significantly delayed in the absence of TNFR2, suggesting a positive cooperation between the two receptors. Moreover, transgenic mice expressing a mutated nonsheddable TNFR1 ($TNFR1^{\Delta ns}$) exhibit exacerbated TNF-dependent arthritis. (42) These results indicate that blocking the activities of both sTNF and tmTNF may be required to effectively neutralize the pathogenic potential of this cytokine in arthritis.

Inflammatory bowel diseases. Inflammatory bowel diseases (IBD) are inflammatory pathologies of the colon and the small intestine with Crohn's disease (CD) and ulcerative colitis (UC) as the most common types. IBD is characterized by shortened villi, marked inflammatory infiltration and the formation of granulomas in the submucosa. (Figure 3) UC is mostly restricted to the colon, while CD can affect the whole gastrointestinal tract. The intestinal mucosa of patients with CD and UC contains elevated TNF mRNA and protein levels predominantly in the mast cells, monocytes, macrophages and T-cells. (43, 44) $TNF^{\Delta ARE}$ mice also develop a Crohn's-like inflammatory ileitis, confirming that TNF drives the pathology of Crohn's disease in humans. Interestingly, mature T- and B-cells play a major role in the development of TNF-induced inflammatory bowel disease, while they don't in the chronic inflammatory arthritis in these mice (39) Furthermore, in a mouse TNBS-induced

colitis model, a colonic inflammation similar to CD in humans was induced in TNF transgenic mice. Conversely, no significant TNBS-induced colitis could be induced in TNF deficient mice. (45) In DSS-induced chronic colitis TNFR1 deficiency led to increased mortality compared with TNFR2 deficiency, but both TNFR1 and TNFR2 deficiency led to exacerbated signs of colitis. (46)

Psoriasis. Psoriasis is a chronic autoimmune disease that affects the skin. The most common form, *psoriasis vulgaris*, is often marked by red or white scaly patches appearing mainly on the outer side of elbows and knees. The scales are a result of hyper-proliferation of the epidermis with premature maturation of keratinocytes and incomplete cornification with retention of nuclei in the stratum corneum (parakeratosis). (Figure 3) The disorder is a recurring condition ranging in severity from minor localized patches to complete body coverage. Psoriasis can also cause inflammation of the joints, which is known as psoriatic arthritis. (47) The incidence of psoriasis is caused by a combination of genetic factors and the exposure to specific triggers such as stress, skin injury, medication and infection. Genetic polymorphisms including *TNF* A-308G, show a particular pattern of association with psoriasis. (48) Skin lesions of patients with psoriasis contain elevated TNF levels localized to dermal macrophages, epidermal keratinocytes and intra-epidermal Langerhans cells. TNFR1 expression is associated with epidermal keratinocytes, a network of upper dermal dendritic cells and upper dermal blood vessels, whereas TNFR2 expression is associated with upper dermal blood vessels and perivascular infiltrating cells. (49, 50) TNF is mainly involved in the maintenance of the inflammatory state of the disease, leading to keratinocyte activation and proliferation and production of anti-microbial peptides and chemokines. The hypothesis that TNF and TNFR expression are functionally linked to disease pathology has been definitively validated by the clinical success with TNF antagonists in psoriasis. However, TNF also has anti-inflammatory properties that might explain the sporadic occurrence of psoriasis as a side effect of anti-TNF therapy. (51)

Multiple Sclerosis. Multiple sclerosis (MS) is the most common chronic autoimmune demyelinating disease of the central nervous system (CNS). The loss of myelin producing oligodendrocytes and neurons is directly associated with the clinical outcome of the disease, including loss of sensation, optic neuritis and progressive paralysis. (52) Pro-inflammatory cytokines, including TNF, and chemokines are produced by both infiltrating immune cells and resident CNS glial cells, leading to inflammation, demyelination and axonal damage. (53) (Figure 4) Transgenic mice constitutively expressing either mouse or human TNF develop clinical symptoms resembling MS, a phenotype that could be reversed by administration of anti-TNF antibodies. (54) Much knowledge concerning MS pathogenesis has resulted from studies of its animal model, experimental autoimmune encephalomyelitis (EAE). (55) Although TNF is required for normal initiation of EAE, TNF deficient mice still develop EAE and reach a clinical disease severity similar to wild type mice. (56) However, TNFR1 deficient mice were totally resistant to EAE, exhibiting reduced proliferative responses and Th1 cytokine production. In contrast, TNFR2 deficient mice exhibited exacerbated EAE, enhanced

Th1 cytokine production, and enhanced $CD4^+$ and $F4/80^+$ CNS infiltration. (57) Taken together, although TNFR1 signaling is detrimental in the initiation phase of EAE, TNF has immunosuppressive activities in the chronic progression of EAE, by signaling through TNFR2. Moreover, transgenic mice exclusively expressing tmTNF ($TNF^{tm/tm}$) showed protection against both the acute and chronic phase of EAE by retaining the autoimmune suppressive properties of TNF. (58) hence, selective blockade of sTNF/TNFR1 may be beneficial in the treatment of MS.

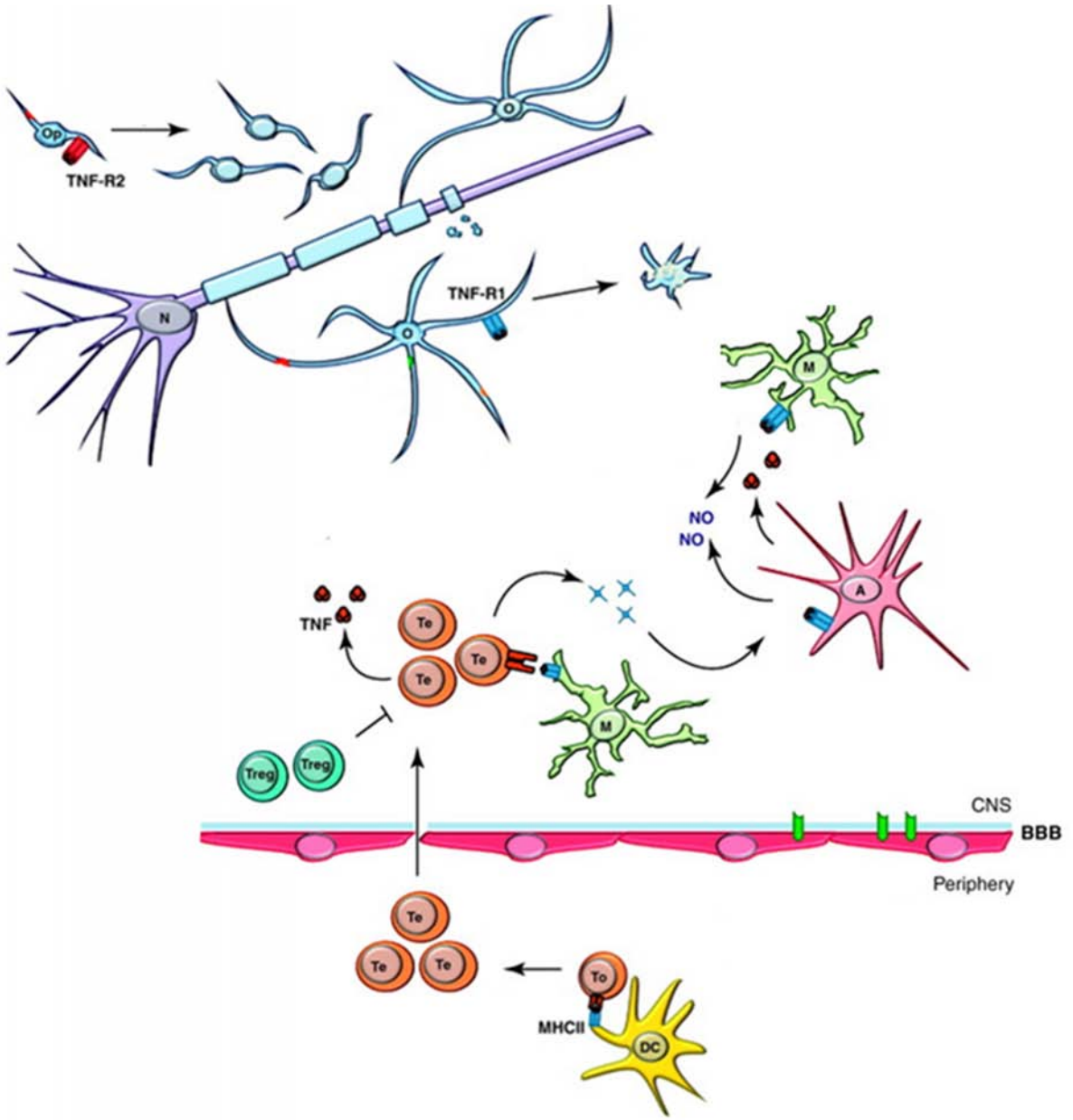

Figure 4. The pathophysiology of multiple sclerosis. (59) Antigen-presenting cells, such as dendritic cells (DCs), present an encephalogenic antigen to T-cells (To), causing a clonal expansion of encephalogenic T-cells (Te), which migrate through a disrupted blood-brain barrier (BBB). In the central nervous system (CNS), Te cells become activated when they re-encounter antigens presented by resident macrophages and microglia (M). These activated Te cells produce several inflammatory chemokines and cytokines, including TNF, which can activate astrocytes (A) and microglia (M), resulting in an excessive production of nitric oxide (NO) causing oligodendroglial and neuronal (N) damage. (60) In addition, TNF promotes oligodendrocyte precursor (Op) cell survival and proliferation through TNFR2. (61)

4.3. TNF-inhibiting therapeutics against chronic diseases

The pathophysiology of TNF as seen in chronic inflammation and autoimmune diseases suggests that the pro-inflammatory activities are mediated by TNFR1 while the immunomodulating and disease-suppressive functions are more dependent on TNFR2. Hence, inhibition of all biological active TNF is clearly not advised. Here, I will give an overview of the currently available TNF-inhibiting therapeutics already in use in the clinic, and some of the new generation drugs based on TNF biology. (Table1)

therapeutics	brand name	target	phase
Infliximab	Remicade	hTNF	in the clinic
Adalimumab	Humira	hTNF	in the clinic
Golimumab	Simponi	hTNF	in the clinic
Etanercept	Enbrel	hTNF	in the clinic
Certolizumab Pegol	Cimzia	hTNF	in the clinic
DN-sTNF A145R/Y87H	XPro 1595	soluble hTNF	in phase I clinical trials
Atrosab		hTNFR1	in preclinical trials
m5R16		hTNFR1	in preclinical trials

Table 1. Overview of TNF-inhibiting therapeutics used in the clinic or under development. For detailed description, see text.

Anti-TNF therapy. Current therapy includes five different anti-TNF agents for clinical use. (Figure 5) A chimeric mouse/human monoclonal antibody (Infliximab), two fully human monoclonal antibodies (Adalimumab and Golimumab), a fusion protein of the extracellular part of two TNFR2 molecules coupled to the Fc region of IgG1 (Etanercept) and a PEGylated Fab' fragment lacking the Fc region (Certolizumab).

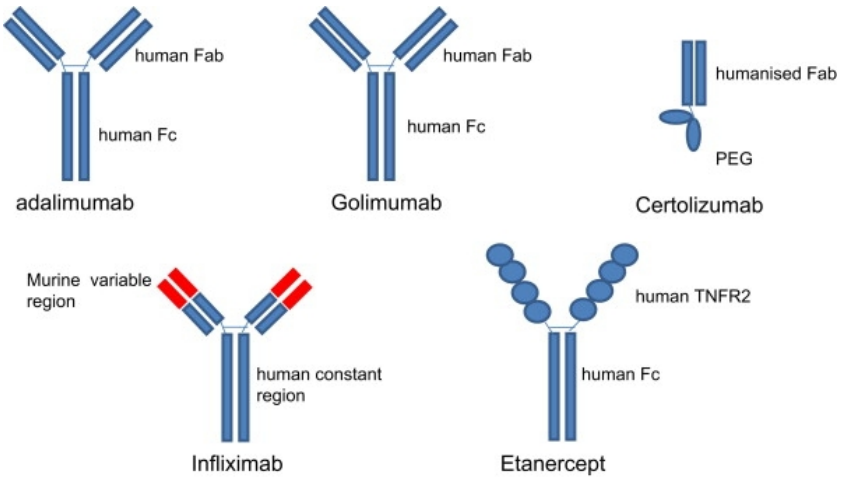

Figure 5. Schematic diagram of the structures of the five anti-TNF agents. (62)

These drugs not only differ in composition, they also differ in mechanism of inhibition, pharmacokinetics and route of application, complicating comparative evaluation of patients treated with different TNF blockers. Nevertheless, some interesting differences could be found. (35) All of the above mentioned drugs bind and inhibit sTNF with comparable

efficiency, but bind with different kinetics to tmTNF, which can influence the relative efficacy of the therapy. (63) This may be one of the reasons why Etanercept, which is a less potent inhibitor of tmTNF, is less effective in the treatment of IBD and other chronic granulomatous diseases in which macrophages that are unable to eliminate pathogens form granulomas. (64) On the other hand, the use of Etanercept is associated with a lower incidence of infections compared to the other TNF blockers, suggesting that the weaker inhibition of tmTNF might allow better functioning of the immune system. (65) Rheumatoid arthritis benefits from the approved anti-TNF therapies when co-administered with other immunosuppressive therapies, probably because it is associated with excessive levels of TNF. (66, 67) However, although anti-TNF therapy can reduce the disease-induced inflammation, it cannot reverse the underlying mechanisms of autoimmunity, sometimes leading to the development of additional autoimmune diseases such as lupus, type 1 diabetes, uveitis, multiple sclerosis, psoriasis, as well as lymphoma and leukemia**.** Some of these diseases are shared with the underlying disease for which the TNF blockers are given, making management of these conditions challenging. For example, anti-TNF therapy is used for the treatment of psoriasis, but psoriatic lesions are sometimes observed in patients receiving therapy. Similarly, arthralgia and arthritis can be observed in patients with rheumatologic diseases receiving anti-TNF agents. (68) Moreover, the U.S. Food and Drug Administration (http://www.fda.gov) has received reports of patients developing opportunistic bacterial and fungal infections while taking TNF blockers.

TNFR2 agonists. Several *in vitro* and *in vivo* studies have shown that TNFR2 agonists are associated with pancreas regeneration, heart protection, remyelination, survival of neurons and stem cell proliferation. TNFR2 agonists as a therapy for autoimmune diseases has been suggested and successfully tested in type 1 diabetes. (33) Thanks to the limited cellular distribution of its receptor, a TNFR2 agonist may exhibit less systemic toxicity than TNF. Additionally, because the agonist is given at low doses and in intervals, side effects are minimized. However, in a study performed in baboons, some adverse effects of a specific TNFR2 agonist were observed, including enhanced T-cell proliferation and a mild, transient inflammation with fever by infiltration of mononuclear cells upon intradermal administration of the agonist. (69)

sTNF-TNFR1 inhibition. Specific inhibition of sTNF, without inhibition of tmTNF signaling can be obtained using dominant-negative sTNF variants (DN-TNF) like A145R/Y87H which specifically interact with sTNF in the formation of inactive heterotrimers and thereby inhibit its binding to TNF receptors. In this way, the interactions between tmTNF-TNFR1 and tmTNF-TNFR2 can freely continue. A145R/Y87H was proven to reduce joint swelling in a collagen-induced arthritis model after PEGylation to improve *in vivo* stability. (70) This and other DN-TNFs demonstrated potent activity against inflammation in multiple models of autoimmune and neuroinflammatory disease including rheumatoid arthritis, asthma, Parkinson's, Alzheimer's and Crohn's disease without suppressing immunity to infection. (71-74)

Another strategy is the selective inhibition of TNFR1 while leaving TNFR2 signaling fully functional. For TNFR1 inhibition a variety of compounds have already been generated, including monoclonal antibodies and derivatives (Atrosab, 55R170, m5R16, DAb), antagonistic TNF variants (R1antTNF), RNAi and antisense oligos.

Atrosab is a humanized mouse anti-human TNFR1 monoclonal antibody converted into an IgG1 molecule containing a modified Fc region. The epitope was mapped to the N-terminal region (AA 1-70) comprising the first cysteine-rich domain (CRD1) and the A1 sub-domain of CRD2 thereby overlapping the PLAD domain and partially overlapping the TNF binding site. *In vitro*, Atrosab inhibited typical TNF-mediated responses such as apoptosis and NF-kB-dependent gene expression like IL6 and IL8. These findings should be further analyzed in relevant disease models in non-human primates or hTNFR1 KI mice. (75)

55R170 is a hamster anti-mouse TNFR1 monoclonal antibody from R&D that is able to neutralize TNF-induced cytotoxicity in L929 mouse fibroblasts and to inhibit LPS-triggered TNF-induced hepatocyte apoptosis in a murine non-alcoholic steatohepatitis model. (76) Moreover, this antibody protected mice against acute TNF-induced lethality in a dose-responsive way and protected B16BL6 tumor-bearing mice against toxicity of TNF/IFNγ therapy without loss of the antitumor effect, while mice treated with a control antibody showed no significant difference compared with PBS-treated animals. (77) This antibody was shown to be specific for TNFR1 as it protected wild type mice but not TNFR1 deficient mice against LPS (unpublished results of Filip Van Hauwermeiren).

M5R16 is an anti-human TNFR1 antibody generated by UCB. This PEGylated F(ab)' fragment is able to block TNF-induced lethality in hTNFR1 KI mice, allowing safe TNF/IFNγ anti-tumor therapy. (77)

A domain antibody (dAb), comprising the variable domains of either the heavy or light chain, against TNFR1 from GSK was successfully used in a ventilator-induced lung injury (VILI) model where TNF is up-regulated in the alveolar space early in the disease. Studies in TNFR1 - and TNFR2 deficient mice indicate that TNFR1 signaling is promoting while TNFR2 signaling is preventing pulmonary edema. The TNFR1-dAb substantially reduced neutrophil recruitment, expression of ICAM-1 on alveolar macrophages and IL6 and MCP1 levels in lavage fluid, while an anti-TNF antibody was ineffective. (78)

R1antTNF is a TNFR1-selective antagonistic human sTNF variant, containing mutations A84S, V85T, S86T, Y87H, Q88N, and T89Q. Its affinity for TNFR1 is almost similar to that of wild type TNF, but neutralizes TNFR1-mediated hepatic injury in TNF/GalN, CCl4 and conA hepatitis models in BALB/c mice. Pharmacokinetic parameters were higher than those of wild type TNF, indicating very fast binding and x-ray crystallography suggested that the mutation Y87H changed the binding mode from a hydrophobic to an electrostatic interaction, which may explain why R1antTNF behaves as an antagonist. (79, 80) PEGylated R1antTNF clearly improved the incidence and the clinical score of CIA showing greater therapeutic effect than Etanercept, without interfering with hepatic viral clearing. And the use of PEG-R1antTNF in EAE significantly improved the clinical score and demyelination in

the initial phase of EAE with considerable suppression of Th1 and Th17 responses in the spleen, lymph nodes and spinal cord. (5, 81)
Alternatively, RNAi with an adenoviral vector containing a shRNA against TNFR1 showed a threefold reduction of TNFR1 mRNA resulting in reduced pathology and IFNγ (Th1), IL4 (Th2) and IL17 (Th17) production in models of rheumatoid arthritis. (82)
In addition, antisense oligos (ASO) against TNFR1 resulted in 59% inhibition of liver TNFR1. In radiation-induced liver damage, induction of serum AST/ALT and liver apoptosis could be reduced by pretreating mice with these ASOs. (83) Furthermore, these TNFR1 ASOs are successfully used to prevent the toxicity of TNF/IFNγ treatment in several mouse tumor models. (84)
These results suggest that sTNF-TNFR1 inhibition is a better approach for the treatment of TNF-induced chronic diseases. It reduces the pro-inflammatory action of TNF while maintaining most beneficial TNF functions, including resistance to infection, prevention of additional autoimmune diseases and anti-tumor activity. It allows more effective treatment of diseases in which tmTNF-TNFR2 signaling is beneficial like multiple sclerosis, in which treatment with TNF blockers exacerbates the disease. However, it might not be suited for diseases in which tmTNF-TNFR2 signaling contributes to the pathology like Crohn's disease and other chronic granulomatous diseases.

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5. Genetic mapping in mice

5.1. *Mus spretus*

One of the advantages of working with mice is the availability of inbred strains, for example, C57BL/6. An inbred strain is created by intercrossing individuals of consecutive generations for at least 20 generations, but preferentially more than 60 generations. This process leads to mice that are genetically homogeneous and homozygous at all loci. During the first decades of the 20th century, a set of inbred strains (DBA/2, BALB/c, C57BL/6, C57BL/10, 129/Sv) was developed from mice obtained from mouse fanciers such as Miss Abbie Lathrop. (1) When the genome of these strains was first analyzed during the 1980s, it became clear that they had a common origin, which implies little interstrain polymorphisms and that they all were undefined genomic mixtures of two or more subspecies. (Figure 1) Indeed, the group of the house mouse (*Mus musculus*) consists of four subspecies that all contributed to the genome of the classical inbred lines. So, the classical laboratory mice do not represent a particular animal that exists in nature. (2)

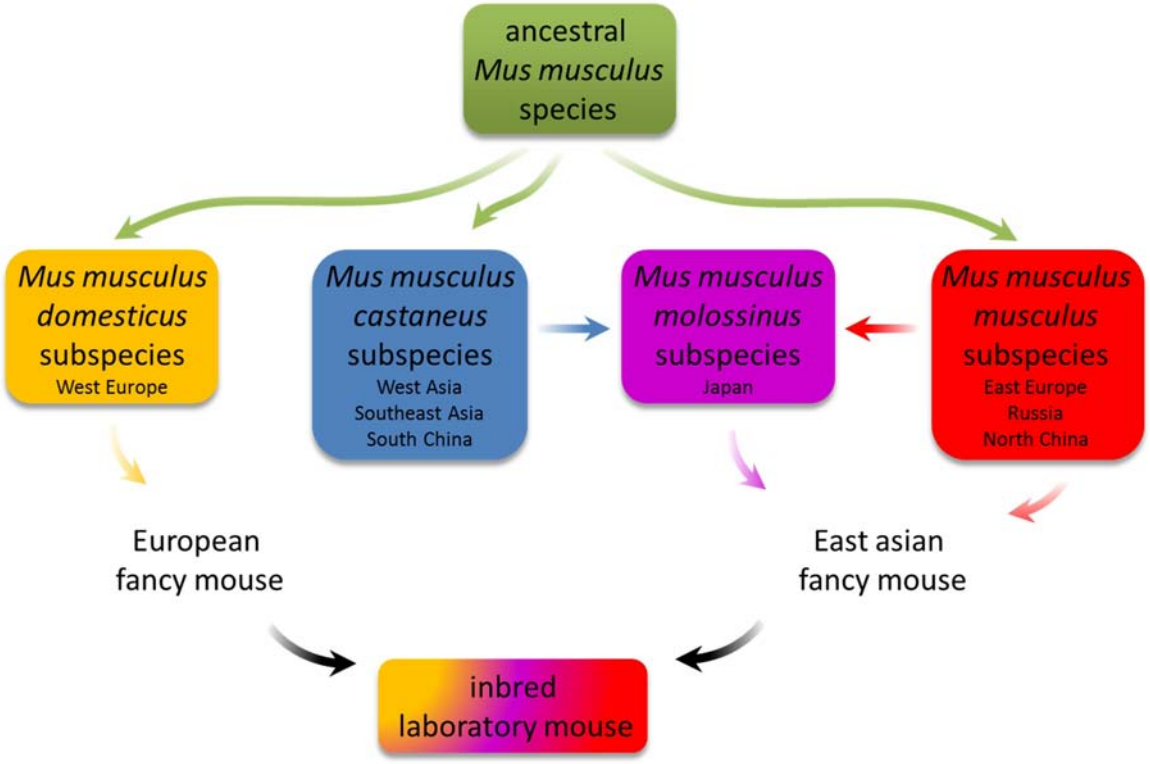

Figure 1. Origin of inbred laboratory mice. The genome of inbred strains is a mosaic derived from European (*domesticus*) and Asian (*castaneus* and *musculus*) subspecies. Figure adapted from (2).

The problem of low interstrain polymorphism could be overcome with the development of new inbred strains derived from a pair of animals captured from a single well-defined wild population. Over the last several decades, more than 450 inbred strains have been developed from animals representing each of the major subspecies in the house mouse group as well as more distant species that still form fertile hybrid females with *Mus musculus*, such as *Mus spretus*. (Figure 2) The high level of genetic variation between wild-

derived and common inbred mice makes them valuable tools for evolution and systematics research and makes progeny from crosses between them especially useful for genetic mapping.

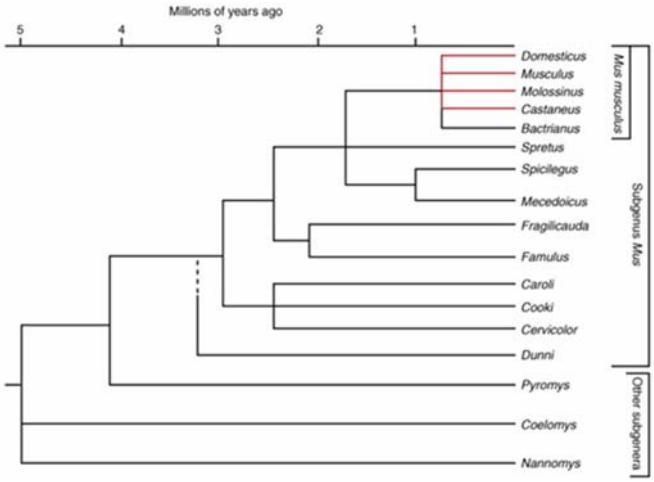

Figure 2. Evolutionary tree of the genus *Mus*. The subspecies at the origin of the classical laboratory strains are indicated in red. (3)

The wild-derived inbred strain SPRET/Ei was created in 1988 by Eva Eicher from individuals of *Mus spretus*, the western Mediterranean short-tailed mouse. Other strains derived from the *Mus spretus* group are SEG/Pas and STF/Pas by the Pasteur institute in Paris. *Mus spretus* and *Mus musculus* have diverged from each other around 1.5 million years ago which has led to a large number of genetic polymorphisms and would allow mapping to the sub cM level of resolution provided members of these two species could be crossed. SPRET/Ei mice have an agouti coat and a white belly. (Figure 3) They are very poor breeders with small litters and have a typical wild behavior. The (BxS)F1 hybrid males are sterile (Haldane's law) what makes it impossible to generate an intercross between F1 females and males, but F1 females are fertile and hence can be backcrossed to either parental. However, SPRET/Ei mice present a lot of useful features. They are extremely resistant to inflammation and all pathological changes induced by TNF (4) and LPS (5-7). And they are resistant to several cancers like skin cancer, lung cancer and thymic lymphomas (8-10) and to several viruses (7, 11).

Figure 3. SPRET/Ei (*Mus spretus*) and C57BL/6 (*Mus musculus*) can both be purchased from The Jackson Laboratory.

Any inbred strain derived from *Mus spretus* exhibits, on average, one single nucleotide polymorphism (SNP) in every 80-100 base pairs compared with any of the classical laboratory strains. This high density of polymorphisms is a major advantage when

quantitative traits are mapped, because every animal with a relevant phenotype can be genotyped for a very large number of markers. (3)

5.2. Linkage and recombination

Gregor Mendel stated in his first law of segregation that each diploid individual has two alleles of every gene, one from each parent. If the two alleles are identical, the individual is called homozygous for the gene or trait. If the two alleles contain different information, the individual is called heterozygous. For sexual reproduction, haploid gametes are formed in a process called meiosis. Prior to meiosis the chromosomes are duplicated by DNA replication, creating two exact copies or sister chromatids, attached at the centromere. They typically exchange DNA by homologous recombination leading to crossover between the maternal and paternal chromosomes. Then, the recombinant chromosomes get separated and alleles are divided into different haploid gametes. Alleles can reunite by fertilization of the gametes. The offspring then receives its own pair of alleles for each trait. Which of the two alleles is dominant determines how the offspring expresses that trait. In his second law of independent assortment, Mendel states that separate genes are passed independently of each other from parents to offspring. For two genes (a gene with alleles a and A, and a gene with alleles b and B), the possibility that a gamete will be ab, AB, aB or Ab is 25 %. However, this is only true for genes that are not linked to each other. When genes are located on the same chromosome, they are linked and will be transmitted at a frequency other than 25 %. (Figure 4)

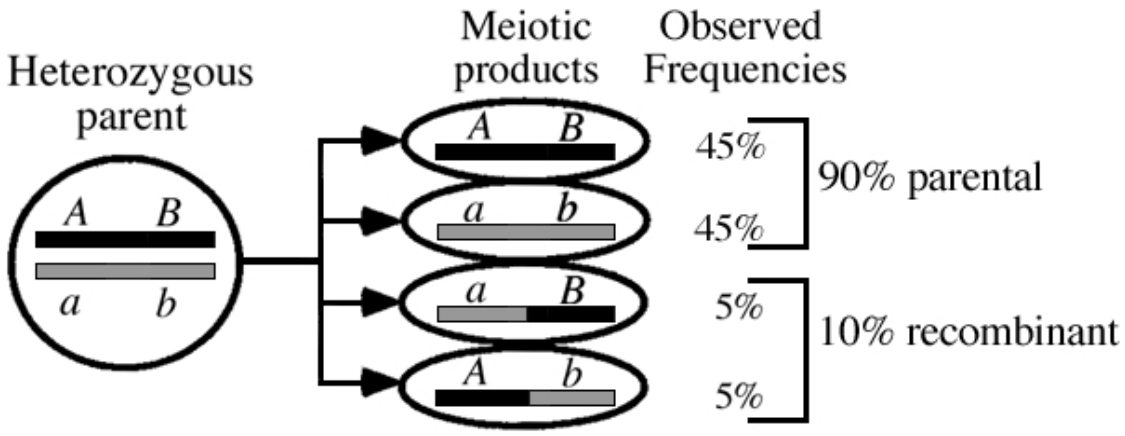

Figure 4. Non-independent assortment of genes A and B on linked loci. The parental combination of alleles will be transmitted at a higher frequency. (1)

In case of extreme linkage, when the genes are very close to each other, only the parental combination of alleles will be transmitted, each at a frequency of 50 %. But when recombination or crossover occurs, the recombination frequency is related to the genetic distance between the two genes. Genetic distance is measured in cM (1 cM is about 2 Mb in the mouse) and defined as the distance between two loci recombining with a frequency of 1 %. When double or even numbers of crossovers occur, the new combination will not be different from the original one and cannot be detected. As a consequence, the observed

recombination frequency will be less than the actual frequency. Also one recombination event on a certain location can influence the initiation of other events. This process is called interference and can restrict the resolution of a linkage map. The resolution can be calculated with the formula 100:N, where N is the number of meiotic events and in practice, the number of offspring mice used in a back- or intercross. For example, in an analysis of 200 meiotic events (200 backcross offspring or 100 intercross offspring), the average distance between crossovers will be 0,5 cM. (1)

5.3. Genetic markers

Markers used for QTL analysis (see further) need to be polymorphic, easy to identify and easy to genotype rapidly in large numbers of individuals. Several types of markers are used, including restriction fragment length polymorphisms (RFLP), single strand conformation polymorphisms (SSCP), single nucleotide polymorphisms (SNP) and simple sequence length polymorphisms (SSLP) or microsatellites.

Microsatellites are repeats of one to four nucleotides, with the most common being CA-repeats. The length can vary between 80 and 270 bp and can be polymorphic between different strains. About 50 % of the microsatellites are polymorphic between the *Mus musculus* strains, while the percentage can be as high as 84 % between C57BL/6 and wild-derived inbred strains such as SPRET/Ei. (12) Microsatellites have the advantage that they don't follow particular genes of interest, but rather provide loci that are distributed at uniform distances along each chromosome in the genome. These loci can be used as framework maps to rapidly position any QTL of interest. The flanking sequences of microsatellites are quite conserved, making it possible to design PCR primers for amplification to determine the length in different mouse strains. (Figure 5)

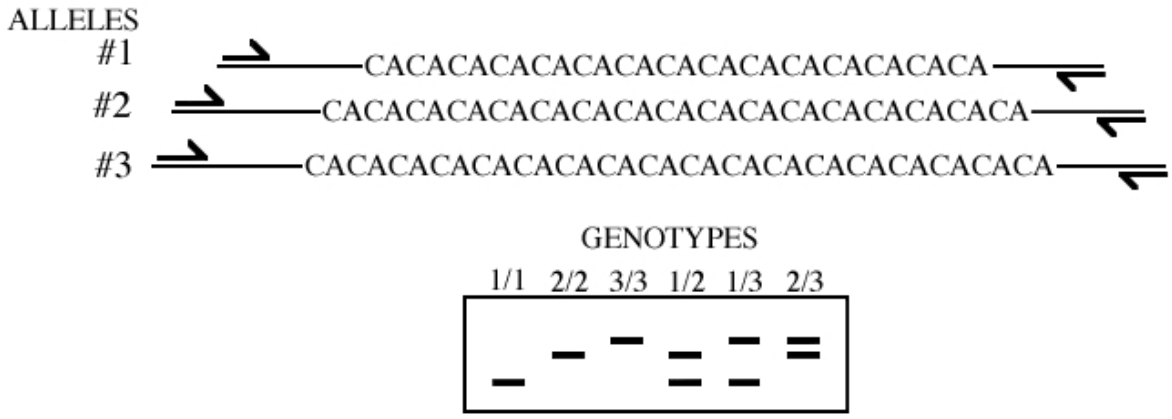

Figure 5. Genotyping of microsatellites. Three different alleles can be detected by PCR and the length of the fragments is visualized by gel electrophoresis. (1)

The number of genetic markers needed for an initial whole genome screen can be determined by the 'swept radius'. This is the distance (in cM) around a locus where linkage can be observed between a marker and this locus with 95 % confidence. The swept radius is related to the number of progeny and can be calculated by determining the recombination

fraction. For example, with 72 backcross samples, the swept radius is 20 cM, with 94 samples it becomes 24 cM. So, depending on the length of the chromosomes, for a normal sized backcross population, two or three markers are needed per chromosome. Each intercross sample is equivalent to two backcross samples meaning that a swept radius of 20 cM can be obtained with 36 samples.

5.4. Quantitative trait loci

To analyze the relationship between phenotype and genotype, there are two pathways that can be followed. One pathway (reverse genetics) starts with the mutation of a gene, the function of which is not understood and studies this mutation in various experiments to uncover the gene function. The other pathway (forward genetics) is based on the observation of an interesting phenotype and link the phenotype to a responsible gene via genetic markers. With these markers it is possible to clone the region that must contain the responsible gene and identify the gene. However, most phenotypes or diseases are complex traits caused by the interaction of multiple genes at more than one locus. Often genes work together in an additive or epistatic way. Visible traits such as skin color as well as hidden traits such as blood pressure vary over a continuous range of phenotypes and therefore are called quantitative or continuous traits. Continuous variation can also be due to non-genetic factors including environmental influences and chance.
The term quantitative trait locus or QTL is used to define a locus that affects a complex trait in a strictly quantitative way. But qualitative traits can often be considered as quantitative traits when reaching a certain threshold such as survival. Expression quantitative trait loci (eQTLs) are genomic loci that regulate expression levels of mRNAs or proteins. These expression levels are mostly regulated by a single gene with a specific chromosomal location. eQTLs that map to the approximate location of their gene-of-origin are called cis eQTLs. In contrast, trans eQTLs map to a location far from their gene-of-origin, often on a different chromosome. (13) Some cis eQTLs are detected in many tissue types while most of the trans eQTLs are tissue-dependent. (14) The best way to analyze (e)QTLs of complex traits is to generate a backcross or an intercross for mapping at the sub-chromosomal level. As described further, afterwards, the QTLs can be isolated using consomic or congenic mice. When the location of the QTL is confirmed, an additional cross can be done for fine mapping.

5.5. Crosses

The first step is an **outcross** of two homozygous inbred strains, preferably a classical lab strain (e.g. C57BL/6) and a wild-derived strain (e.g. SPRET/Ei), one of the strains having an interesting quantitative trait that differs significantly in expression from the other strain. The resulting F1 offspring, in this example (BxS)F1, will be heterozygous for all loci. The phenotype of F1 hybrids between classical lab strains is generally intermediate between the two parental strains. But when a lab strain is crossed with a wild inbred strain, the F1 hybrid is mostly identical to the phenotype of the wild-derived parent, indicating dominance of the wild-derived allele. (3)

In a **backcross** strategy, the F1 offspring are crossed back to one of the parental strains, e.g. the wildtype lab strain (B) generating N2 progeny. (Figure 6) The advantage of a backcross is that each N2 offspring can be viewed as representing an isolated meiotic event. The N2 mice are unique and can be homozygous BB (50 %) or heterozygous BS (50 %) for each locus. Those mice that still have the phenotype of interest will be heterozygous for the QTL. By determining the amount of mice having the phenotype, the amount of loci involved can be predicted. The phenotype will be monogenic when 50 % of the N2 mice show the phenotype, when the amount is lower, the trait will be complex. For a monogenic trait, the QTL can be narrowed down by typing more markers in the critical region and by analyzing more mice.

In an **intercross** strategy, the F1 offspring are intercrossed generating F2 progeny. (Figure 6) At a certain locus in the genome, 25 % of the F2 mice will be homozygous BB, 25 % will be SS and 50 % will be heterozygous BS. An important advantage over the backcross is that an intercross can be used to map QTLs defined by recessive phenotypes that interfere with viability or fertility. Both heterozygous parents will be normal and homozygous F2 offspring can be recovered at any pre- or postnatal stage. Another advantage is that meiosis causes recombination in both parents, leading to twice as much recombination events per mouse. However, this makes the obtained data also more complex because it is impossible to determine which allele, at each heterozygous locus, came from which parent.

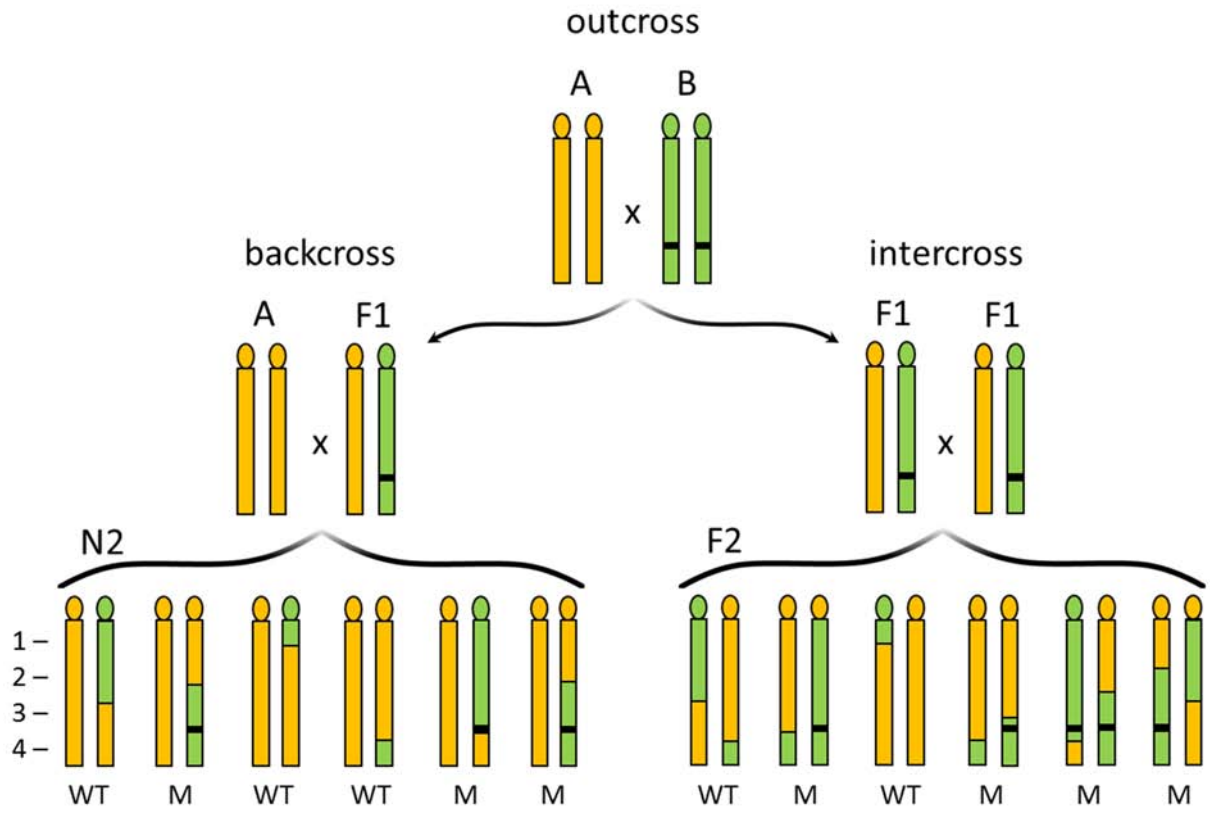

Figure 6. Generation of a backcross (left) and an intercross (right). Segregation of a monogenic trait, M=mutant, WT=wildtype, ▬=mutation.

N2 progeny	1	2	3	4	5	6
marker 1	H	A	H	A	H	A
marker 2	H	A	A	A	H	A
mutation	-	+	-	-	+	+
marker 3	A	H	A	A	H	H
marker 4	A	H	A	H	A	H

F2 progeny	1	2	3	4	5	6
marker 1	H	H	H	A	H	H
marker 2	H	H	A	A	H	B
mutation	-	+	-	+	+	+
marker 3	A	H	A	A	B	H
marker 4	H	B	A	B	H	H

Table 1. Segregation and mapping of a monogenic trait in a backcross (left) and an intercross(right). H=heterozygous, A=strain A, B=strain B.

Consomic mice or chromosome substitutions strains (CSS) are homozygous inbred mice that harbor one chromosome derived from one of the parental strains (the donor strain), while all the other chromosomes are derived from the acceptor strain. (Figure 7) They are generated by repeated backcrosses to the acceptor strain, with selection for the relevant chromosome at each generation. At each backcross generation, 50 % of the donor genome is lost. After 10 generations, the genetic background should be essentially that of the acceptor strain except for the selected chromosome.

Congenic mice are similar to consomic strains, but instead of a whole chromosome, only a part is introduced from the donor strain into the acceptor strain. The rapid elimination of heterozygosity occurs only in regions of the genome that are not linked to the donor allele. The expected length (in cM) of the differential chromosomal segment can be calculated as $[200*(1-2^{-N})/N]$ where N is the generation number. For N higher than 5, this can be simplified to [200/N]. After 10 generations, there will be a 20 cM region of the chromosome including the locus derived from the donor strain.

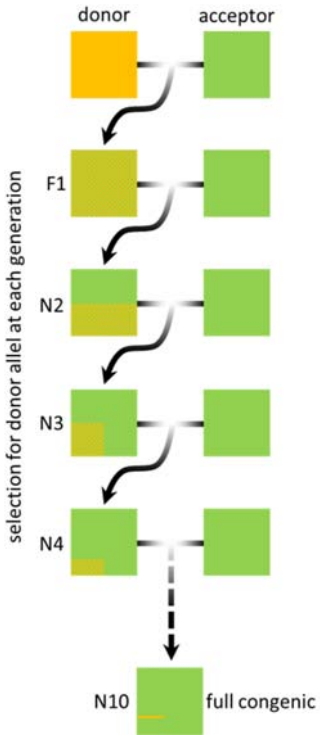

Figure 7. Generation of congenic mice. Elimination of heterozygosity. Figure adapted from (1).

5.6. QTL mapping

Markers that are genetically linked to a QTL influencing the trait of interest will segregate more frequently with trait values, whereas unlinked markers will not show significant association with the phenotype.

A simple method for QTL mapping is the **'single locus association test'** to evaluate the association between the genotype of each locus and a trait value. In a backcross, one may use a t-test to compare the averages of the two marker genotype groups. For other types of crosses like intercross, where there are more than two possible genotypes, one uses a more general form of ANOVA. The weaknesses of this method is that the QTL location is indicated only by looking at which markers give the greatest differences between genotype group averages and that the apparent QTL effect at a marker will be smaller than the true QTL effect as a result of recombination between the marker and the QTL.

Lander and Botstein developed **'simple interval mapping (SIM)'**, which is used for estimating the position of a QTL within two markers. The likelihood of a single hypothetical QTL is evaluated, one at a time, at each location in the genome. The maximum likelihood of both QTL position and QTL effect can be calculated using a genetic map of all phenotypes and marker genotypes. The results of these test van be expressed by giving a LRS or LOD score. The **'likelihood ratio statistics (LRS)'** is a value given to each association and follows a χ^2 distribution. The **'logarithm of odds (LOD)'** score compares the probability of obtaining the test data if the two loci are indeed linked, to the probability of obtaining the same data purely by chance. For example, a LOD score of 4 means that there is 10^4 times more chance that there is a QTL on a given location than that the QTL is not present. The correlation between LRS and LOD scores is a factor 4.6 (LRS = LOD x 4.6). (15)

For complex trait analysis, one can use **'composite interval mapping (CIM)'** which takes into account additional marker loci that have been shown to be associated with the trait.

But to analyze data from large crosses, we use a specific computer program called **'R/qtl'** (16). R/qtl is a freely available add-on package for the statistical software R. It includes functions for estimating genetic maps, identifying genotyping errors, and performing single-QTL and two-dimensional, two-QTL genome scans by multiple methods. The data are loaded as a comma-delimited (.csv) file of all genotypes, phenotypes and other necessary information. It is possible to summarize and plot various features of the project like the proportion of missing genotypes and a histogram of the phenotype. The function 'scanone' is used to perform a single QTL genome scan using a standard model assuming normal distribution. The maximum likelihood is determined using the expectation–maximization (EM) algorithm. (15) Scanone can also be used to perform a permutation test which yields a genome wide LOD significance threshold. In addition, you can estimate genome wide adjusted p-values for the QTLs and obtain a report of all chromosomes exceeding a certain significance level, with the corresponding LOD threshold.

A **'permutation test analysis'** is a method where the phenotypic data are randomly distributed among the progeny, while the genetic map is kept intact. This breaks the relationship between the phenotypic data and the genotypes of the markers. This procedure

is repeated multiple times (e.g. 1000 or 10000 times) and for each permutated dataset, the maximal LOD is recorded at regular intervals throughout the genome. Values at appropriate percentages of this empirical distribution are used as a threshold value above which the observed LOD is significant. The suggestive level is the one at which on average one false positive is found per genome scan.

5.7. References

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6. microRNA

6.1. Introduction

In 1993, Victor Ambros and colleagues discovered that the lin-4 gene, known to control the timing of *C. elegans* larval development, does not code for a protein but produces a pair of small RNAs. One RNA of approximately 61 nt was predicted to fold into a stem-loop and to be the precursor of a shorter one of 22 nt in length. They also noticed that these lin-4 RNAs had antisense complementarity to multiple sites in the 3' UTR of the lin-14 gene, previously proposed to be repressed by the lin-4 gene product. (1) Gary Ruvkun and colleagues could show that the regulation of lin-14 by lin-4 reduces the amount of LIN-14 protein without noticeable changes in levels of lin-14 mRNA. The lin-4 RNA is recognized as the founding member of an abundant class of tiny regulatory RNAs called microRNAs (miRNAs, miRs). (2) Over the past decade, miRNAs have emerged as important regulators of translation. Hundreds of these molecules and their possible targets have been discovered in the genomes of plants and animals. Bioinformatics analyses suggest that up to 30% of the human genes might be regulated by microRNAs. (3, 4)

6.2. Genomics

Most miRNA genes are located in regions of the genome quite distant from protein-coding genes, implying that they derive from independent transcription units. But about a quarter of the human miRNA genes are located in the introns of pre-mRNAs, preferentially in the same orientation, suggesting that most of these miRNAs are expressed after splicing of the host mRNA. This coordinated expression of a miRNA and a protein-coding mRNA could explain the conserved relationship between miRNAs and host mRNAs. (5) A striking example of this conservation is mir-7, found in the intron of heterogeneous nuclear ribonucleoprotein K (hnRNPK) in both insects and mammals. (6)

Many miRNAs have intriguing expression profiles, e.g. lin-4 which has a stage-specific expression in development. Other interesting examples are miR-1, which is particularly found in the mammalian heart (7), miR-122 in the liver (8), miR-223 in granulocytes and macrophages of mouse bone marrow (9) and the mir-290/mir-295 cluster, which is expressed in mouse embryonic stem cells but not in differentiated cells (10). These and many other results suggest that every animal cell type at each developmental stage might have a distinct miRNA expression profile. Also the level of miRNA expression can differ a lot. Some miRNAs are expressed at very high levels, including miR-2, miR-52 and miR-58 which are each present as more than 50000 molecules per adult worm cell. Whether this high expression is attributable to very potent transcription or to slow degradation, is not yet known. Other miRNAs are expressed at much lower levels, including miR-124 which is present in the adult worm as about 800 molecules per cell. (11) This lower level, though still higher than that of the typical mRNA (estimated to average ~100 molecules per cell), might be due to low expression in many cells or high expression in just a few cells. The finding that

mouse miR-124 is nearly exclusively expressed in the mouse brain supports the latter explanation. (7)

Nearly all miRNAs are conserved in closely related animals, such as human and mouse, or *Caenorhabditis elegans* and *Caenorhabditis briggsae*. Many are also conserved more broadly among the animal lineages, e.g. more than a third of the miRNAs in *C. elegans* have homologs among the human miRNAs. (5)

6.3. Biogenesis

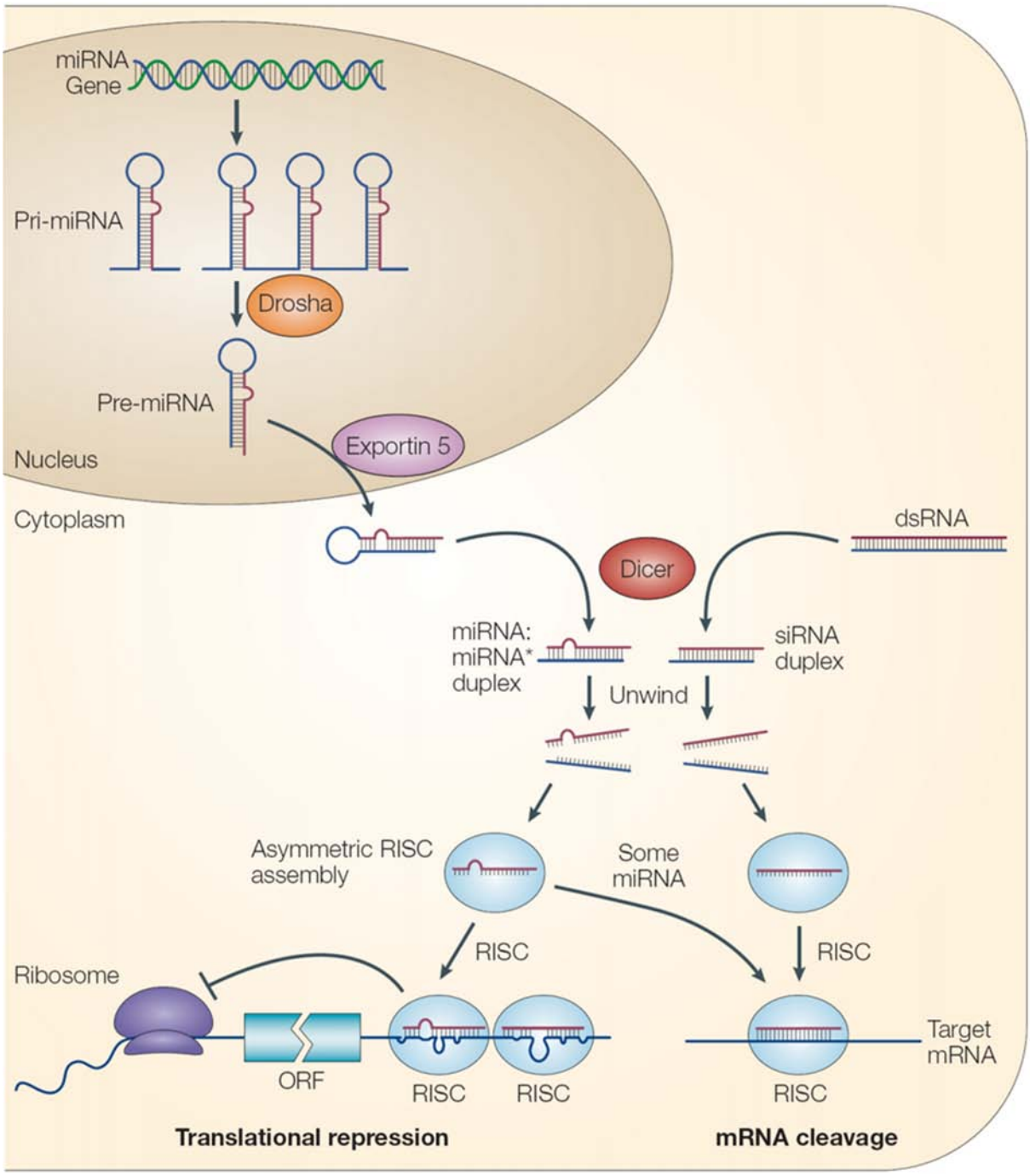

Figure 1. Biogenesis of microRNA. Transcription, maturation and function. (16) For detailed description of the different steps, see text.

The generation of miRNAs is a multistage process (Figure 1). For transcription of **pri-miRNA**s, there are two candidates, namely RNA polymerases II and III. Pol II produces mRNAs, small nucleolar RNAs (snoRNAs) and four of the small nuclear RNAs (snRNAs) of the spliceosome, while pol III produces some of the shorter noncoding RNAs, including tRNAs, 5S ribosomal RNA and the U6 snRNA. The miRNAs processed from the introns of protein-coding genes are certainly transcribed by pol II and many observations provide evidence that most of the other miRNAs might also be pol II products. (12) Most of the pri-miRNAs do not contain a 5' m^7G cap or poly A tail. (13)

The first step in maturation of mammalian miRNAs is the nuclear cleavage of the pri-miRNA transcript. This delivers a 60-70 nt hairpin, known as the miRNA precursor or the **pre-miRNA**. (13) This processing is performed by the RNase III endonuclease **Drosha**, which cleaves both strands of the stem at sites near the base, resulting in a 5' phosphate and two nucleotides 3' overhang. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of splicing and debranching, thereby bypassing the Drosha step. (14)

The pre-miRNA is actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Exportin 5.

The nuclear cut by Drosha defines one end of the mature miRNA, the other end is processed in the cytoplasm by the enzyme **Dicer**. (14) The RNase III endonuclease Dicer recognizes the double-stranded part of the pre-miRNA, possibly with particular affinity for a 5' phosphate and 3' overhang, and cuts both strands at about two helical turns away from the base. This cleavage removes the terminal loop leaving an siRNA-like imperfect duplex consisting of a 22 nt mature miRNA and a similar-sized complementary fragment, the miRNA*. These miRNA* sequences are found in much lower amounts than the mature miRNAs, indicating that the miRNA:miRNA* duplex is much less stable than the mature miRNA single strand.

One strand of the miRNA:miRNA* duplex (the guide strand) is incorporated together with Argonaute 2 (Ago2) into the RNA-induced silencing complex (**RISC**) whereas the other strand is released and degraded. The strand that enters the RISC is nearly always the one whose 5' end is less tightly paired. For a few miRNAs, both strands of the duplex accumulate suggesting that both can enter the RISC and be functional. (15)

6.4. Mechanism and function

MiRNAs can direct the RISC to down-regulate gene expression by two post-transcriptional mechanisms, e.g. mRNA cleavage or translational repression, depending on the target. Once incorporated into the RISC, the miRNA will specify cleavage if the mRNA has near perfect complementarity to the miRNA or repress translation if the mRNA has wobbled complementarity. (16) When a miRNA guides cleavage, the cut is at precisely the same site as seen for siRNA guided cleavage, i.e. between the nucleotides pairing to residues 10 and 11 of the miRNA. After cleavage of the mRNA, the miRNA remains intact and can recognize and cleave additional mRNAs. (17)

The most stringent requirement for translational repression is a contiguous and perfect base pairing of the miRNA nucleotides 2 to 8, representing the seed region. (Figure 2) Other

requirements are that there has to be a bulge in the center of the miRNA–mRNA duplex to prevent AGO-mediated cleavage of the mRNA and that there must be reasonable 3’ complementarity to the miRNA to stabilize the interaction especially when matching in the seed region is suboptimal. (18)

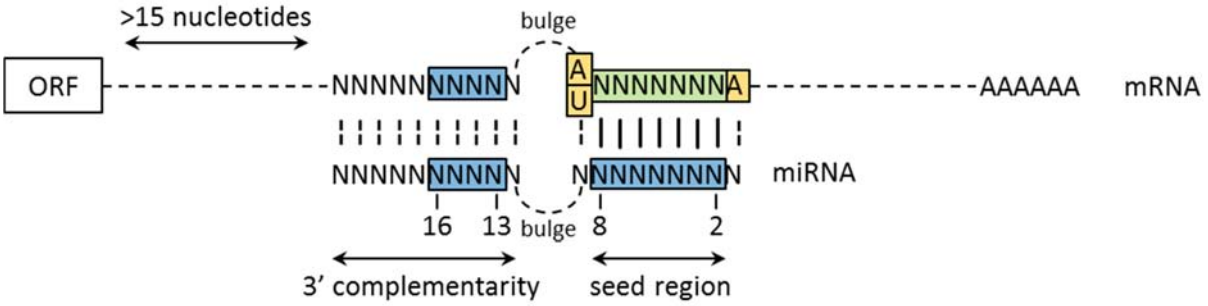

Figure 2. Principles of miRNA:mRNA interactions. (18) For detailed description, see text above.

With a few exceptions, miRNA-binding sites in animal mRNAs are located in the 3' UTR and usually present in multiple copies, which is required for effective repression of translation. In analogy to other biological regulatory systems such as transcriptional regulation, the cooperative action of multiple RISCs seems to provide the most efficient translational repression. (19) And the prediction of multiple miRNA complementary sites in most of the targets suggests that when the list of proved miRNA:mRNA regulatory interactions will become longer, control by cooperative action will be seen more common.

Translational repression can occur by targeting the initiation or the elongation of translation, or both. miRNA-induced repression of initiation of translation is dependent on the 5’ m^7G cap structure and the 3’ poly A tail of target mRNAs. The central domain of AGO contains limited sequence homology to the cap binding region of the initiation factor eIF4E allowing AGO to compete with eIF4E binding and prevent translation of capped mRNAs. (20) Deadenylation of the mRNAs prevents eIF4G mediated circularization from the 5’ m^7G cap to the 3’ poly A tail. Widespread miRNA-mediated deadenylation of mRNAs occurs during zebrafish embryogenesis. miR-430 causes the removal of hundreds of maternal mRNAs by inducing their deadenylation and subsequent degradation at the onset of zygotic transcription. (21) Interestingly, some miR-430 targets, such as nanos1 and tudor-like tdrd7 mRNAs, are repressed by miR-430 in somatic but not germ cells, indicating that target repression or destabilization can be tissue or cell specific. (22) An alternative mechanism of miRNA-induced repression of initiation of translation is binding of the AGO2-Dicer-TRBP complex to another translation initiation factor, eIF6, preventing 60S joining to the 40S ribosomal subunit. (23) But targeting of initiation is likely not the only mechanism by which miRNAs induce translational repression. Observations that IRES-mediated translation is also sensitive to miRNAs and the cosedimentation of miRNAs and AGO proteins with ribosomes did propose a drop-off model in which miRNAs make ribosomes prone to premature termination of translation. There are numerous miRNAs, like miR-1 and miR-133, that repress their target genes at the translational level, with no or only a minimal effect on mRNA degradation. (24, 25)

miRNAs and endogenous siRNAs have a partially shared biogenesis and cannot be distinguished by either their chemical composition or mechanism of action. However, important distinctions can be made in regard to their origin, evolutionary conservation and the types of genes that they silence. miRNAs are processed from transcripts that form RNA hairpin structures, while siRNAs are processed from long RNA duplexes or extended hairpins. One single miRNA:miRNA* duplex is generated from each pre-miRNA, while a multitude of siRNA duplexes are generated from each siRNA precursor molecule, leading to many different siRNAs accumulating from both strands of this extended dsRNA. miRNA sequences are generally conserved in related organisms, while endogenous siRNA sequences are rarely conserved and miRNAs direct the silencing of genes that are unrelated to the loci that encode the miRNAs themselves, while siRNAs direct self-silencing of the mRNA they are derived from. These differences make it possible to distinguish newly discovered miRNAs and endogenous siRNAs. (26)

6.5. Identification and validation of miRNA targets

In contrast to the accumulation of validated miRNA sequences, there is a lack of experimental evidence for identified miRNA targets.

Independent groups have designed computational algorithms to predict miRNA target genes. Several of these programs provide target predictions based on miRNA sequence complementarity to target sites with emphasis on perfect base-pairing in the seed region and sequence conservation e.g. TargetScan (27), PicTar (28) and TargetRank (29). Other target predictions are based on calculations of mRNA secondary structure and energetically favorable hybridization between microRNA and target mRNA, e.g. RNAhybrid (30), STarMir (31), MicroInspector (32), PITA (33), and RNA22(34). Some prediction programs combine all three requirements, e.g. MicroCosm (35), miRanda (36) and DIANA-microT (37). In addition, a few programs make a comparative analysis of several prediction programs based on algorithms focusing on different aforementioned requirements, e.g. miRWalk (38) and miRGen (39).

Functional assays. The validation of regulation of putative target genes by certain miRNAs can be done by functional assays. Most commonly used are luciferase reporter constructs containing the target 3' UTR with the putative binding site downstream of the reporter coding region. These constructs are used to transfect cells expressing the relevant miRNA or along with miRNA mimetics. Decreased luciferase activity in reporter assays indicates miRNA activity on the 3’ UTR of the target gene. (40)

Gain-of-function studies. A complementary approach for *in vitro* or *in vivo* validation of miRNA function are gain-of-function studies using miRNA mimetics, followed by quantitative analyses of target mRNA and protein levels. miRNA mimetics imitating endogenous miRNAs can be delivered in the form of synthetic pre-miR miRNA precursor molecules (41) or as pre-miR encoding vectors (42). The systemic delivery of miRNAs into mice by *intravenous* injection is mostly limited to the liver tissue. (43) Several groups have circumvented this limitation by *ex vivo* gene transfer into hematopoietic stem cells or fetal liver cells and

reconstitution into recipient mice. (9, 44) Tissue-specific overexpression of a miRNA *in vivo* can also be achieved by generation of transgenic mice. This approach was used to study the function of miR-1 in cardiogenesis. (45) Studies that are based solely on overexpression of miRNAs must be interpreted with caution. Overexpression of miRNAs could target genes that would otherwise not be affected in a physiologic context, because of either low expression levels of the endogenous miRNA or spatial differences between the miRNA and its target. Therefore, results that are based on induced expression of miRNAs should ideally be confirmed by loss-of-function experiments. (40)

Loss-of-function studies. In loss-of-function studies, the miRNA can be silenced using antisense oligonucleotides (46), antagomirs (47), locked nucleic acid (LNA) antimiR (48), or vector-based transcripts called miRNA sponges, containing multiple miRNA binding sites that absorb miRNAs and prevent binding to their targets (49).

Antisense oligonucleotides (ASOs) are single-stranded RNAs complementary to the targeted miRNA that carry complete phosphorothioate backbones and 2'-O-methoxyethyl modifications. Intraperitoneal injection of ASOs selective to miR-122, a highly abundant miRNA in the liver, could markedly reduce endogenous miR-122 levels in the liver after a 4 week treatment period. (46)

Antagomirs are cholesterol-conjugated ASOs stabilized with a partial phosphorothioate backbone and 2'-O-methyl modifications. Intravenous injection of antagomir-122 showed a similar reduction of miR-122 levels in the liver as seen with miR-122 ASOs and antagomir-16 could silence the ubiquitously expressed miR-16 in most tissues, including liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenals for up to 3 weeks. Unfortunately, antagomirs are not able to cross the blood-brain barrier nor the blood-placental barrier. (47)

Locked nucleic acids (LNA) are RNA analogs in which the ribose ring is locked in the ideal conformation for Watson-Crick binding. As a result, LNA oligonucleotides exhibit unprecedented thermal stability when hybridized to a complementary DNA or RNA strand.

Since LNA antimiRs consist of a mixture of LNA and RNA, it is possible to optimize the sensitivity and specificity of miRNA binding by varying the LNA content. A single intravenous injection on three consecutive days of a 16 nt, unconjugated LNA antimiR-122 with complete phosphorothioate backbone leads to specific, dose-dependent silencing of miR-122 resulting in lower cholesterol plasma levels.

Genetic silencing of miRNAs can be achieved by conditional silencing of *Dicer1*, leading to deficiency of all mature RNAs. Full *Dicer1* deficient mice die at day E7.5, indicating that miRNAs have an important functional role in development. To study the contribution of individual miRNAs, the expression of specific miRNAs can be restored in full *Dicer1* deficient mice using miRNA mimetics. Specific miRNA deficient mice or transgenic mice with mutated miRNA target sites in protein encoding genes can be used to confirm the role of the miRNA-target interaction for a miRNA-associated phenotype.

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CHAPTER II

Aims

Relevance of this PhD project

Tumor necrosis factor (TNF) is a powerful pro-inflammatory cytokine that plays a key role in the development of septic shock and is involved in several inflammatory and autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD) and multiple sclerosis (MS). (1) The main receptor for TNF, TNFR1, is involved in inducing and maintaining inflammation, cell proliferation, cell survival and apoptosis. (2) In addition, TNFR2 functions in the development of regulatory immune cells and initiates healing after inflammation under certain conditions. (3) Despite the fact that our knowledge of TNF biology is not yet complete, TNF inhibitors are successfully being used to treat patients with autoimmune disorders. (4) However, long-term use of these agents can cause significant side-effects, such as opportunistic infections (5) and the development of additional autoimmune diseases or lymphomas. (6) More specific targeting of the pathological actions of TNF could be possible by specifically blocking TNFR1 signaling while leaving TNFR2 signaling intact. (2) At least, development of novel, effective drugs for the treatment of many inflammatory diseases requires a better understanding of the regulation of the pro-inflammatory activities of TNF.

Previous findings that form the basis of this PhD project

Jan Staelens *et al.* studied the sensitivity of several mouse strains to TNF-induced lethal inflammatory shock and found that SPRET/Ei, an inbred strain derived from *Mus spretus*, is extremely resistant. SPRET/Ei mice are completely protected against all pathological changes induced by very high doses of TNF (up to 1000 μg per mouse, while the LD_{100} for C57BL/6 reference mice is around 30 μg) and the resistance is a dominant trait because (BxS)F1 mice are as resistant as SPRET/Ei mice. Using an interspecific backcross between (BxS)F1 and C57BL/6, they found that loci conferring resistance to TNF-induced lethality are found on proximal chromosome 2 and distal chromosome 6. Although the critical region on chromosome 6 was, at that stage of the research, still several tens of cM wide, the *tnfrsf1a* gene, encoding for the major TNF receptor TNFR1, was found in this region, suggesting that a sequence variation in this gene is associated with the resistance of SPRET/Ei mice. (7) Compared to C57BL/6, they found 12 amino acid variations in the TNFR1 protein of SPRET/Ei. However it was shown that the binding of TNF to TNFR1 is not altered in SPRET/Ei and that TNFR1 is still functional in SPRET/Ei.

In mice deficient for TNFR1, TNF-induced lethal inflammation is absent. Recently, Filip Van Hauwermeiren *et al.* published that TNFR1+/- mice which express only half of the maximal levels of TNFR1 in all organs, also show total resistance against TNF-induced lethality. In these mice, TNF induces NF-kB-dependent genes half maximally and there is a reduced degradation of I-kB. In contrast, the TNFR1 levels are sufficient for induction of apoptosis and resistance to *Listeria monocytogenes* infection. Moreover, since TNF-induced lethality depends on gene induction, while antitumor effect on apoptosis, TNFR1+/- mice show full antitumor activity without any toxicity. Furthermore, they could down-regulate TNFR1 expression in the liver with TNFR1 antisense oligonucleotides, and in this way protect mice

against acute TNF-induced lethality, without reduction of the antitumor effect, and block TNFR1 using antibodies and obtaining similar results as observed in the TNFR1+/- mice. (8) In both studies, I have been involved.

Aims of this PhD project

Inspired by the resistance of both SPRET/Ei and TNFR1+/- mice, we asked the initial question whether the TNF resistance of SPRET/Ei can be linked to lower TNFR1 levels.

- Is the TNF resistance of SPRET/Ei mice linked to lower TNFR1 levels?

We studied whether the resistance of SPRET/Ei is linked to a quantitative effect at the TNFR1 locus by determining TNFR1 mRNA and protein levels of C57BL/6 and SPRET/Ei in several organs.

The positive answer to this question leaded to several new questions arising from obtained results.

- Which loci are linked to the TNFR1 protein expression level?

Since we found that SPRET/Ei and (BxS)F1 mice show a similar lower TNFR1 protein expression level as TNFR1+/- mice while the mRNA expression level remains normal in these organs, we searched for loci that are linked to the lower TNFR1 protein level. We generated a new interspecific backcross between (BxS)F1 and C57BL/6. The N2 progeny were genotyped using microsatellite markers equally distributed over the genome and the phenotype, the TNFR1 expression level, was determined using ELISA on liver lysates.

- Which genes can we find in the loci that are linked to the TNFR1 protein level?

Since we found that the TNFR1 expression level in SPRET/Ei might be regulated post-transcriptionally, we focused on microRNAs. These noncoding RNAs have emerged as key posttranscriptional regulators of gene expression, involved in diverse physiological and pathological processes. We performed a microRNA profiling on naive livers of C57BL/6 and SPRET/Ei to look for differential expression of microRNAs. Conversely, using prediction programs we looked for microRNAs that are predicted to repress the translation of TNFR1. MicroRNAs that are predicted by several programs and that are located in the loci conferring lower TNFR1 protein level, were studied in more detail by functional analysis both *in vitro* and *in vivo*.

- Are the TNF resistance and lower TNFR1 protein level of SPRET/Ei regulated by the glucocorticoid receptor?

Since SPRET/Ei mice were found to express higher levels of the glucocorticoid receptor (GR) than C57BL/6 mice, reflecting in increased GR activity and mediating resistance to LPS-induced lethal inflammation (9), we examined whether the GR also plays a role in the TNF resistance of SPRET/Ei using a GR antagonist. In addition, we searched for glucocorticoid response elements and investigated the influence of the GR on the expression of TNFR1-

repressing microRNAs and consequently on the TNFR1 level using the GR agonist dexamethasone and adrenalectomized mice.

In addition to elucidating the TNF resistance of SPRET/Ei and the TNFR1 regulation, we want to develop potentially better therapeutics against TNF-mediated diseases.

- Can we develop a new generation of TNFR1 blockers?

Since TNFR1+/- and SPRET/Ei mice both have a reduced TNFR1 expression level and consequently are resistant against TNF-induced lethality, we are interested in ways to down-regulate TNFR1 *in vivo* and thereby induce protection. We generated nanobodies as an alternative to regular antibodies. Nanobodies are made from naturally occurring heavy chain antibodies from *Camelidae*. Nanobodies have a high specificity and affinity similar to conventional antibodies, but have some extra interesting advantages. They are easily produced and manipulated in bacteria or yeast and are very soluble due to hydrophilic amino acid residues. They have a longer shelf-life thanks to their stability to heat, pH and proteases. But what makes them really special is the ability to recognize and bind hidden epitopes thanks to their small size (15 kDa). After *in vitro* characterization, these nanobodies will be tested in inflammatory models such as TNF-induced lethality and LPS-induced endotoxemia. We hope to develop TNFR1-inhibiting nanobodies that can be used as therapeutic agents for the treatment of several auto-immune diseases by specifically blocking the TNFR1-induced inflammation.

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CHAPTER III

Results

MicroRNA-511 causes resistance to TNF by regulating TNFR1 expression

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Abstract. Tumor necrosis factor (TNF) is a powerful pro-inflammatory cytokine that plays a key role in the development of septic shock and is involved in several inflammatory and autoimmune diseases such as rheumatoid arthritis, inflammatory bowel diseases (IBD) and multiple sclerosis (MS). Development of novel, more effective drugs for the treatment of inflammatory diseases requires a better understanding of the regulation of the pro-inflammatory activities of the TNF. The mouse strain SPRET/Ei, previously reported as being extremely resistant to TNF-induced lethal inflammatory shock relative to the TNF-sensitive C57BL/6 mice, expresses significantly reduced protein amounts of the major TNF receptor TNFR1, compared to C57BL/6, while the mRNA levels are normal. This trait was found to be linked to a locus on proximal chromosome 2 and another on distal chromosome 6, the major loci that previously had been linked to the TNF resistance phenotype. This identical linkage demonstrates the coupling of TNFR1 levels to TNF resistance. B.S^{chr6} congenic mice, harboring the distal part of chromosome 6 of SPRET/Ei, including the TNFR1 encoding gene *Tnfrsf1a,* in a C57BL/6 background, display no reduction in TNFR1 protein levels and no TNF resistance. Therefore we presume that the down-regulation of TNFR1 is regulated in *trans*. MicroRNA-511 (miR-511), encoded on proximal chromosome 2, was found to be higher expressed in SPRET/Ei mice and to suppress TNFR1 protein expression *in vitro* as well as *in vivo*, after injection in C57BL/6 mice. Moreover, miR-511 could protect C57BL/6 mice against TNF-induced lethality while anti miR-511 up-regulates TNFR1 protein and sensitizes mice for TNF. We suggest that miR-511 suppresses TNFR1 expression, contributing to the resistance of SPRET/Ei against TNF-induced inflammation.

Contributions

Leen Puimège performed all the experiments in this chapter with the exceptions below.
Filip Van Hauwermeiren initiated the project and assisted in the experiments.
Sofie Lodens performed the luciferase reporter assays.
Jan Staelens generated the (BxS)N2 backcross for TNF resistance of SPRET/Ei.
Claude Libert supervised the design and performance of the experiments.

1.1. Introduction

Tumor Necrosis Factor (TNF) is a powerful cytokine that plays a key role in immunity and inflammation. (1) The diverse signaling cascades initiated by TNF lead to a range of cellular responses, including proliferation, differentiation, migration and cell death, as well as to inflammation. (2) TNF is synthesized as a transmembrane protein (tmTNF) that is subsequently cleaved by tumor necrosis factor-α-converting enzyme (TACE) to release the mature, soluble form (sTNF). (3) TNF remains usually undetectable in healthy individuals, but elevated levels can be found during inflammation, infection, injury and following exposure to various environmental challenges. (4) Dysregulation of TNF expression or signaling plays a role in the pathology of many auto-immune diseases, such as rheumatoid arthritis, inflammatory bowel disease (IBD) and multiple sclerosis. (5) TNF acts by binding to two different receptors: TNFR1 is activated by both sTNF and tmTNF, and TNFR2 is activated mainly by tmTNF. (6) In general, TNFR1 is involved in inducing and maintaining inflammation, cell proliferation, cell survival and apoptosis. (7) In addition, TNFR2 mainly functions in the development of regulatory immune cells and initiates healing after inflammation. (8) Although our understanding of TNF biology is not complete, TNF inhibitors are being used successfully worldwide to treat patients afflicted with autoimmune disorders. (9) Although these agents target TNF, they differ in their clinical efficacy. Etanercept, for example, is presumed to be less potent in inhibiting tmTNF and therefore ineffective against IBD in contrast to infliximab and adalimumab. (10) Such observations suggest that neutralization of sTNF might not be the only or best mechanism of action. Moreover, long-term use of these agents can cause significant side-effects, such as the increased incidence of infection (11) and the development of certain autoimmune diseases, such as psoriasis. (12) These side-effects are thought to be caused by inhibition of the immunomodulatory stimulation of TNFR2. More specific targeting of pathological actions could be possible by specifically blocking TNFR1 signaling while leaving TNFR2 signaling intact. (7)

While the TNFR1 signaling pathway is studied in great detail, very little is known about the transcriptional regulation of the TNFR1-encoding gene *Tnfrsf1a*. The absence of canonical TATA and CCAAT boxes and the high GC content have been associated with the promoters of housekeeping genes. (13) On the other hand, the TNFR1 protein has been shown to be regulated by post-transcriptional modifications. One mode of regulation is shedding of TNFR1 by TACE/ADAM17. (14) TNFR1 shedding and the resulting acute decrease in the number of receptor molecules on the cell surface is believed to transiently desensitize cells to TNF. (15) In addition, the pool of soluble TNFR1 could also function as physiological attenuators of TNF activity by competing for the ligand with the cell surface receptors. (16) In humans, mutations affecting TNFR1 shedding have been linked with the development of TRAPS (TNF receptor-associated periodic syndromes). (17) These disorders are characterized by recurrent fevers and localized inflammation. Additionally, mice expressing a non-sheddable TNFR1 have higher levels of TNFR1 in most tissues and are therefore extremely sensitive to LPS- and TNF-induced inflammation. (15) All these findings suggest that the quantity of TNFR1 is strictly regulated and that it plays an important role in determining

sensitivity to TNF. In our search for factors that determine the response to TNF, we had studied the sensitivity of several inbred strains of mice and found two strains that are considerably hyporesponsive to TNF-induced lethal inflammatory shock, namely, DBA/2 and SPRET/Ei mice. (18, 19) SPRET/Ei is an inbred strain derived from *Mus spretus*, which is believed to have diverged from *Mus musculus* about two million years ago. (20) Hence, SPRET/Ei mice display a large genetic diversity with *Mus musculus* strains such as C57BL/6, which allows high resolution genetic mapping. Genetic linkage analysis has shown that the TNF resistance of SPRET/Ei mice is strongly linked to two protective loci, namely one on proximal chromosome 2 and another one on distal chromosome 6. (19)

Since the TNFR1-encoding gene *Tnfrsf1a* is located on distal mouse chromosome 6 (125.3 Mb–60.55 cM), we studied whether the genetic link between TNF resistance and distal chromosome 6 has any molecular relation to the *Tnfrsf1a* gene. In this paper, we describe that such a correlation indeed exists. TNFR1 protein levels in TNF resistant SPRET/Ei mice are significantly down-regulated, presumably in *trans*, relative to C57BL/6 mice. Since miR-511, a predicted TNFR1-regulating miRNA, that is located on proximal chromosome 2 and is expressed more strongly in SPRET/Ei than in C57BL/6, leads to functional down-regulation of TNFR1 and causes resistance to TNF, we believe that this miR is partly responsible for the lower TNFR1 expression levels and TNF resistance of SPRET/Ei. This hypothesis is confirmed based on miR and anti miR studies. Our data also identify miR-511 as a potential therapeutic molecule to treat TNFR1 mediated diseases.

1.2. Results

1.2.1 TNFR1 expression is reduced in SPRET/Ei mice

We previously showed that, compared to C57BL/6 mice, SPRET/Ei mice are solidly resistant to lethal inflammatory shock induced by TNF, an activity known to be mediated by TNFR1. (21) The LD_{100} of a single *i.p.* injection of TNF is >1000 µg in SPRET/Ei mice and ~30 µg in C57BL/6 mice. This resistance was previously shown to be linked to proximal chromosome 2 and distal chromosome 6. The *Tnfrsf1a* gene, encoding TNFR1 and located at 60.55 cM/125.3 Mb, is the most likely candidate resistance gene at the chromosome 6 locus. (19) Since TNFR1+/- mice, expressing only half of the maximal levels of TNFR1 in all organs (Figure 1), show total resistance against TNF-induced lethality (21), we analyzed the expression level of *Tnfrsf1a* mRNA and its protein product in liver, kidney, lung and spleen of SPRET/Ei and C57BL/6 mice.

We found that SPRET/Ei mice express significantly less TNFR1 protein than C57BL/6 mice in all four tested tissues. Moreover, (BxS)F1 mice have similar TNFR1 protein levels as SPRET/Ei, indicating that the lower TNFR1 expression in SPRET/Ei is a dominant trait. Furthermore, the concentration of soluble TNFR1 in serum was threefold lower in SPRET/Ei mice, suggesting that the differences measured in organs are not caused by increased TNFR1 shedding by TACE. (Figure 1)

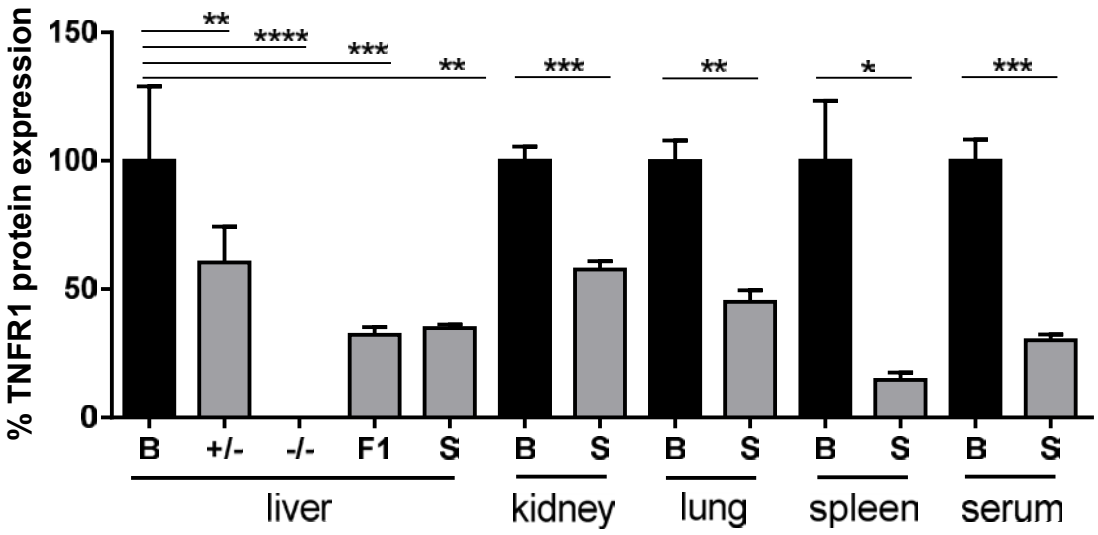

Figure 1. TNFR1 protein levels in different samples. TNFR1 protein levels were measured by ELISA and compared to C57BL/6 (n=13, set as 100 % for all organs): TNFR1+/- (n=6), p=0,059; TNFR1 -/- (n=6) , p<0,0001; (BxS)F1 (n=5), p=0,0001; SPRET/Ei liver (n=5), p=0.0040; SPRET/Ei kidney (n=4), p=0.0002; SPRET/Ei lung (n=4), p=0.0029; SPRET/Ei spleen (n=4), p=0.0109; SPRET/Ei serum (n=4), p=0.0002.

However, the low TNFR1 protein levels are not correlated with TNFR1 mRNA levels, since we found equal levels of TNFR1 mRNA in liver, kidney, lung and spleen of both SPRET/Ei and C57BL/6 mice. The primers used for the TNFR1 Q-PCR contained no SNPs between C57BL/6 and SPRET/Ei indicating that they could bind equally well to both genome transcripts. Moreover, these results could be confirmed by equal Affymetrix microarray TNFR1 mRNA expression in hepatocytes of SPRET/Ei and C57BL/6. (Figure 2)

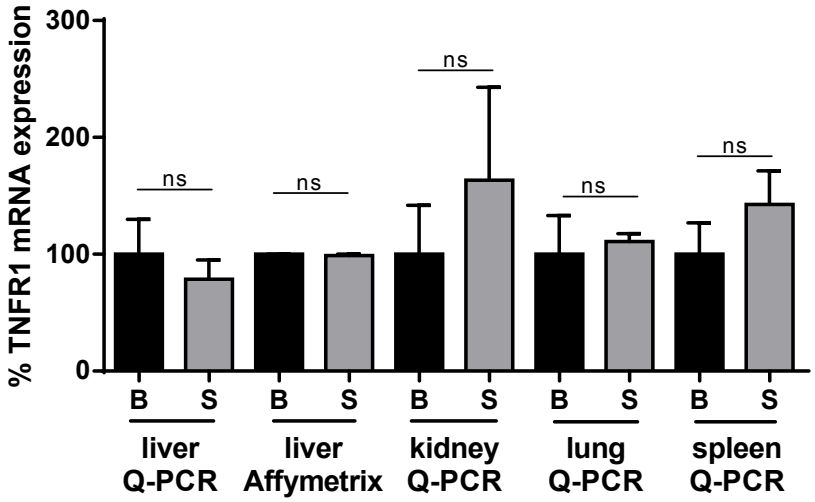

Figure 2. TNFR1 mRNA levels in C57BL/6 (n=3) and SPRET/Ei (n=3). TNFR1 mRNA levels were measured by Q-PCR (liver, p=0.5651; kidney, p= 0.5191; lung, p= 0.7229; spleen, p= 0.3399). Affymetrix microarray data in hepatocytes (p=0,3845).

We conclude that SPRET/Ei mice express similar levels of TNFR1 mRNA as C57BL/6 mice but express 2-5 fold less TNFR1 protein. It is unlikely that TACE plays a role in this difference, but

a variety of other factors could cause this dilemma, including translation blockade by microRNAs (miRNAs). (22)

1.2.2 Low TNFR1 expression and TNF resistance of SPRET/Ei are linked to the same chromosomes

To identify the genetic regions responsible for the low level of TNFR1 protein in SPRET/Ei mice, we generated 214 BSB mice by backcrossing (C57BL/6 x SPRET/Ei) female mice with C57BL/6 males. Livers were excised and TNFR1 levels were measured by ELISA. Genomic DNA was prepared and microsatellite mapping was performed by using polymorphic markers covering the entire genome of all BSB mice.

Linkage analysis using Map Manager QTX (23) software revealed linkage to a locus on chromosome 2, highly significant linkage to a locus on chromosome 6 and negative linkage to chromosome 14. (Table 1 and Figure 3)

locus	position	LRS	%	p value	CI	effect
D2Mit359	5	6.0	3	0.01420	89	-56.52
D2Mit89	32	6.0	3	0.01451	90	-55.60
D6Mit132	40	6.7	3	0.00983	81	-58.57
D6Mit104	45	8.7	4	0.00321	62	-66.68
D6Mit288	51	6.5	3	0.01061	82	-57.94
D6Mit194	61	16.0	8	0.00006	34	-89.77
D6Mit113	70	19.4	9	0.00001	29	-98.48
D6Mit254	60	12.3	6	0.00045	44	-78.67
D13Mit35	75	4.8	2	0.02778	111	50.17
D14Mit50	4	9.0	4	0.00268	60	68.99
D16Mit88	10	5.6	3	0.1817	96	-53.84
D18Mit186	45	4.2	2	0.03980	127	-47.69

Table 1. Microsatellite markers showing linkage to the TNFR1 protein level in the liver of 214 BSB backcross mice. The position is expressed in cM, % stands for the observed variance attributable to the locus, CI is the 95 % confidence interval according to Darvasi (24) and the additive effect is negative when the locus positively contributes to the phenotype.

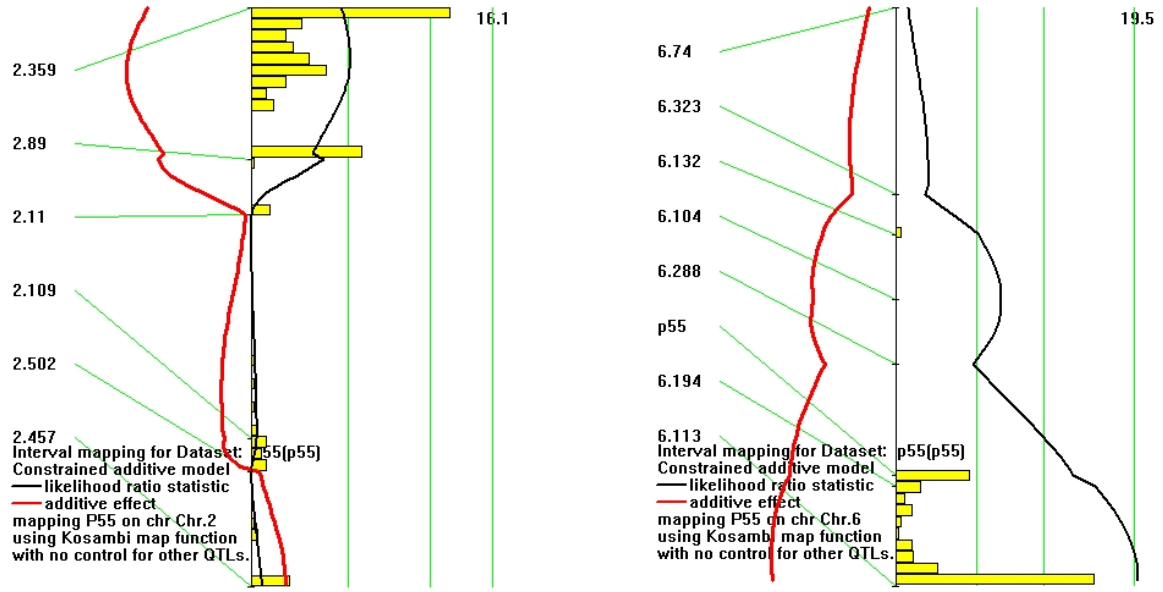

Figure 3. QTL mapping of TNFR1 protein level in the liver of 214 BSB backcross mice. The LRS scores show a significant QTL on chromosome 2 (LRS = 6.0) and chromosome 6 (LRS = 19.4). Green lines denote thresholds of significance (suggestive = 6.2, significant = 11.8, highly significant = 18.2) for 10000 permutations.

A QTL analysis using R/QTL (25) confirmed the loci on proximal chromosome 2 (1–38 cM) and on distal chromosome 6 (40–70 cM) (Figure 4) being the same chromosomal regions that previously had been linked with TNF resistance. (19)

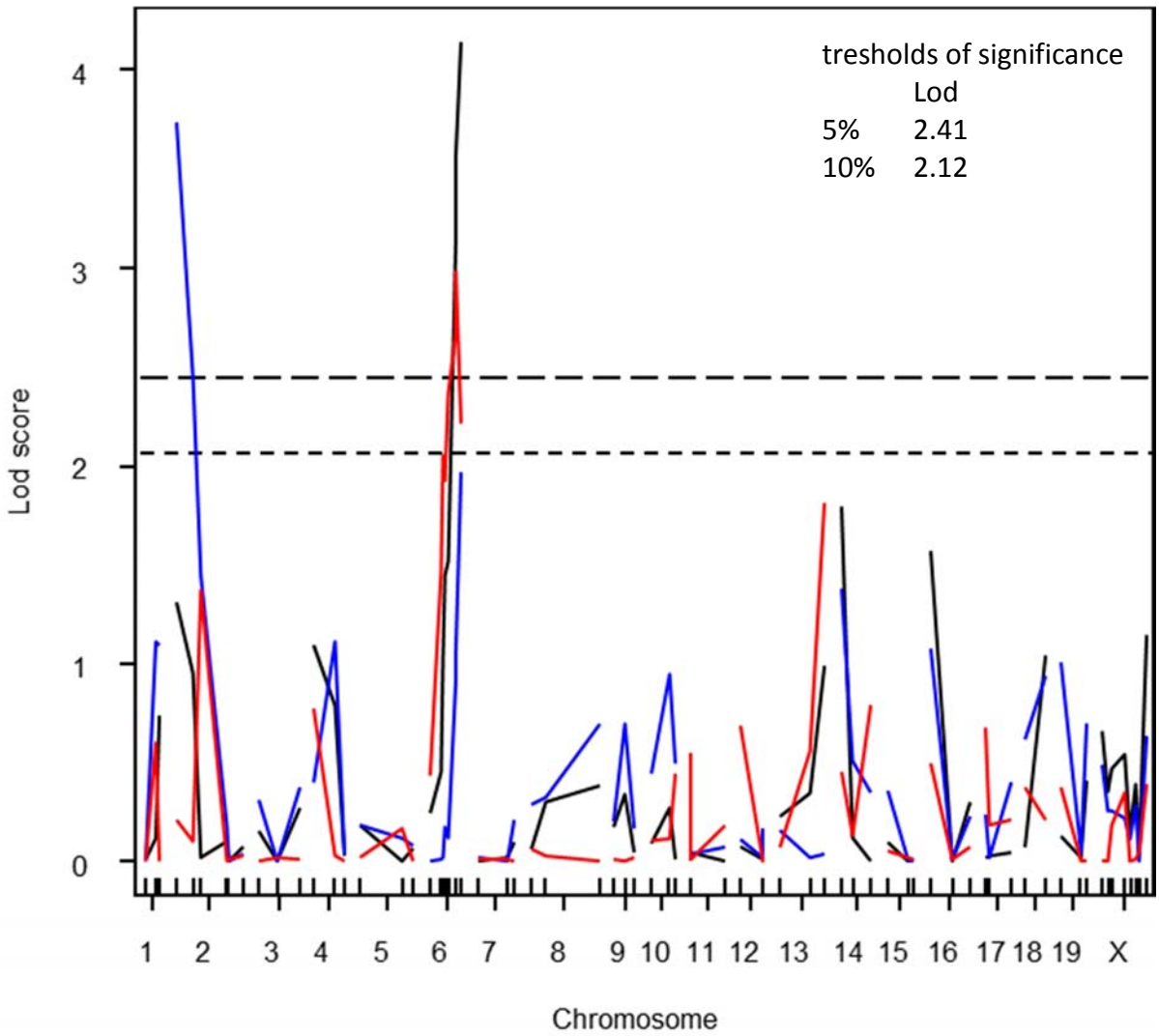

Figure 4. QTL mapping of TNFR1 protein level in the liver of 214 BSB backcross mice. The Lod scores show a significant QTL on chromosome 2 (Lod = 3.7460) and chromosome 6 (Lod = 4.1519). Dashed lines denote thresholds of significance (p=0.1 and p=0.05, 10000 permutation). Males in blue, females in red, full population in black.

The similar linkage pattern of TNF resistance and TNFR1 protein expression is quite remarkable. (Figure 5) Moreover, SPRET/Ei and TNFR1 +/- mice both having half-maximal TNFR1 protein expression levels show total resistance against TNF-induced lethality. (21) Together, these data provide evidence that the two phenotypes are causally related.

Since the Lod scores of chromosome 6 for both TNF resistance and TNFR1 expression are higher than those of chromosome 2 and the gene encoding TNFR1 (*Tnfrsf1a*) is located in

the linkage region on distal chromosome 6, we suggest that not only the TNFR1 expression but also the resistance to TNF can be associated mainly with TNFR1 regulation.

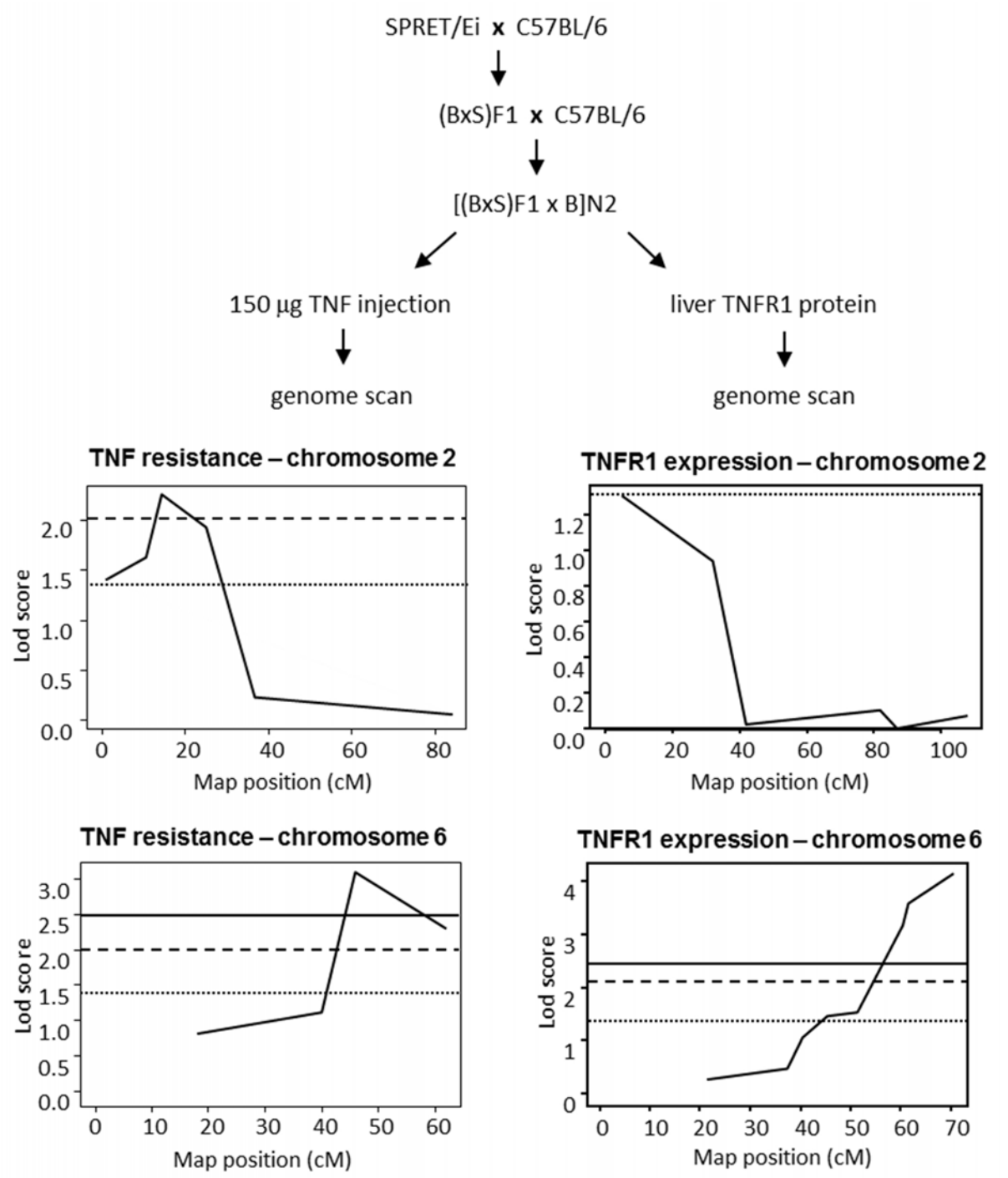

Figure 5. QTL mapping of survival (n=178, left) and TNFR1 protein level in the liver (n=214) of BSB backcross mice. The LOD scores show a QTL on chromosome 2 (15 cM, LOD=2.2454, 5 cM, LOD=1.3098) and chromosome 6 (46 cM, LOD=3.1056, 70 cM, LOD=4.1519) for both phenotypes. For TNF resistance, the 5 % significance threshold LOD score is 2.50, the 10 % threshold is 2.00 and the suggestive level is 1.4. For TNFR1 protein levels, the 5 % significance threshold LOD score is 2.41, the 10 % threshold is 2.12 and the suggestive threshold is 1.34. Horizontal lines in the figures represent highly significant (full line), significant (stripes) and suggestive (dots) LOD values.

1.2.3 Down-regulation of TNFR1 in SPRET/Ei mice is regulated in *trans*

To study how the SPRET/Ei *Tnfrsf1a* region on distal chromosome 6 participates in the regulation of *Tnfrsf1a* and its role in TNF resistance, we generated B.S^{chr6} congenic mice harboring the distal part of the SPRET/Ei chromosome 6 (40-70 cM) in a C57BL/6 background by backcrossing to the host strain for nine generations, followed by intercrossing and selecting congenic mice homozygous for the distal part of chr6, including *Tnfrsf1a* (D6Mit254), of C57BL/6 or SPRET/Ei or heterozygous for the distal part. (Figure 6)

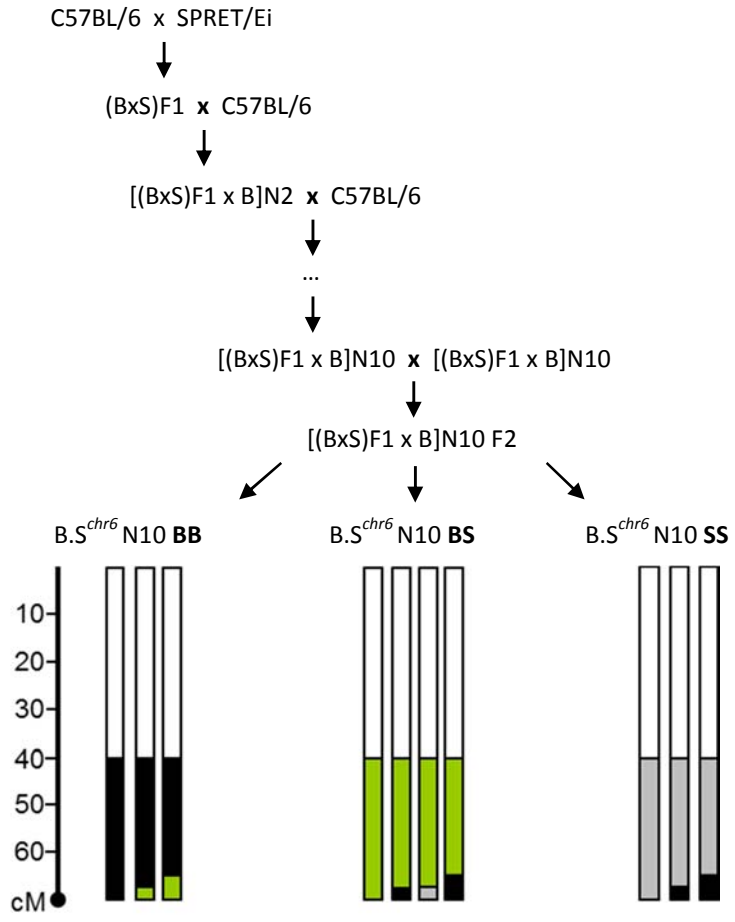

Figure 6. Breeding scheme of B.S^{chr6} congenic mice. Black represents C57BL/6, grey represents SPRET/Ei and green represents heterozygous (BxS).

These congenic mice were challenged with 20 μg of TNF and survival was recorded. B.S^{chr6} N10 SS mice were significantly sensitized to TNF in comparison with full SPRET/Ei mice, however doing better than full C57BL/6 mice. (Figure 6) This result indicates that, in a background of C57BL/6, the SPRET/Ei TNFR1 is not sufficient to achieve total resistance to TNF. On the other hand we can conclude that the SPRET/Ei TNFR1, when isolated in a C57BL/6 mouse is fully biologically active. Furthermore, liver TNFR1 protein levels were equal in the three congenic groups (Figure 7), indicating that the specific regulation of TNFR1 protein levels in SPRET/Ei does not only depend on the distal chromosome 6 region *in cis*.

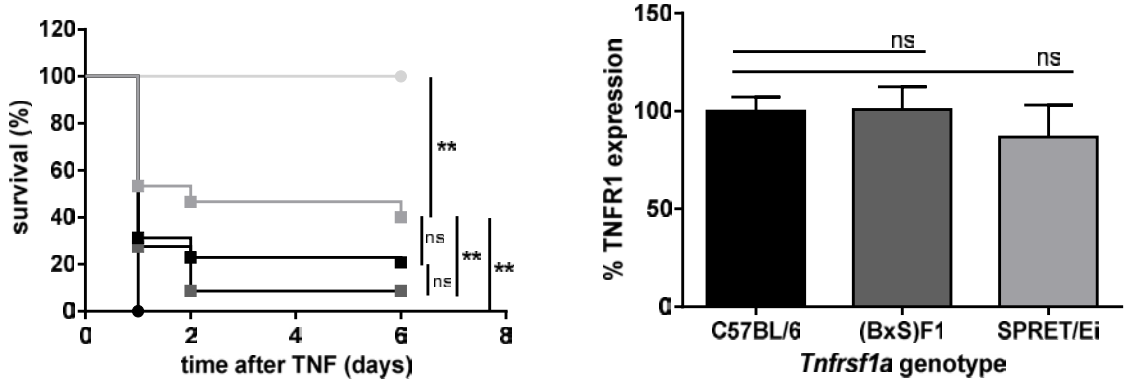

Figure 7. TNF lethality and TNFR1 protein levels in B.S^{Tnfrsf1a} congenic mice. (left) Congenic mice with a *Tnfrsf1a* gene of C57BL/6 (■, n=48), (BxS)F1 (■, n=60) or SPRET/Ei (■, n=14) and parental C57BL/6 (●, n=19) and SPRET/Ei (●, n=16) controls were given 20 μg of TNF. (right) TNFR1 protein levels were measured by ELISA in the liver of the surviving congenic mice with a *Tnfrsf1a* gene of C57BL/6 (n=10, set as 100%), (BxS)F1 (n=7) or SPRET/Ei (n=5). The three groups express equal protein levels of TNFR1 (resp. p=0.3984 and p=0.9537).

These data illustrate that the SPRET/Ei TNFR1 gene can function normally in a C57BL/6 background and that the low TNFR1 protein level in SPRET/Ei mice is genetically linked to distal chromosome 6 but is not regulated in *cis*. Our data also confirm the relation between TNFR1 protein levels and TNF sensitivity and that normalization of TNFR1 levels to the level of C57BL/6 restores TNF sensitivity. Clearly, the observed down-regulation of TNFR1 protein expression in SPRET/Ei mice is regulated in *trans*.

1.2.4 SPRET/Ei mice display increased miR-511 and miR-680-1 expression

A key aspect of the regulation of eukaryotic gene expression is the cytoplasmic control of mRNA synthesis and degradation. (26) Over the past decade, miRNAs have emerged as important regulators of translation. Hundreds of these molecules and their possible targets have been discovered in the genomes of plants and animals (27). Strikingly, bioinformatics analyses suggest that up to 30% of human genes might be regulated by miRNAs. (28)
SPRET/Ei have low TNFR1 protein levels but normal mRNA levels. Additionally, we showed that these low TNFR1 protein levels and the regulation of TNFR1 levels are linked in *trans*. Consequently, we investigated whether miRNAs might be involved in the resistance of SPRET/Ei.
We searched for miRNAs that can have *Tnfrsf1a* as a target gene. Using several miRNA target prediction programs based on algorithms focusing on different features such as (seed) complementarity, conservation and thermodynamics, we were able to make a comparative analysis. (Table 2)
We found two miRNAs on chromosome 2, including miR-296, which is located at the very end of the chromosome (174 Mb/97,88 cM). However miR-511 is located on 14 Mb/10.48 cM on chromosome 2, i.e. within the critical linkage region, and was predicted by six prediction programs to have an influence on the expression of Tnfrsf1a. Furthermore, miR-680-1, located on 130 Mb/63,44 cM on chromosome 6, about 5 Mb beyond the Tnfrsf1a gene, is predicted by eight programs. Other predicted microRNAs may be very important in

the regulation of TNFR1, however in the context of this thesis, we focused on miRNAs that may be responsible for lower TNFR1 levels in SPRET/Ei, based on the linkage results.

	chr	microCosm	miRanda	miRGen	NBmiRTar	TargetScan	miRDB	DIANA-microT	microInspector	miRWalk	PICTAR	PITA	RNA22	RNAhybrid	sum
mmu-mir-10a	11	0	0	1	0	0	0	0	0	0	0	0	0	0	1
mmu-mir-29a/b1	6	1	1	1	0	1	0	1	0	1	1	1	0	0	8
mmu-mir-29b2/c	1	1	1	1	0	1	0	1	0	1	1	1	0	0	8
mmu-mir-125a	17	1	0	1	1	1	0	1	1	1	0	1	0	0	8
mmu-mir-125b	9	1	0	1	0	1	0	1	0	1	0	1	0	0	6
mmu-mir-128-1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
mmu-miR-138-1	9	0	0	1	0	0	0	0	0	0	0	0	0	0	1
mmu-mir-142	11	0	1	0	0	0	0	0	0	0	0	0	0	0	1
mmu-mir-146a	11	0	0	0	1	0	0	0	0	0	0	0	0	0	1
mmu-mir-149	1	0	0	1	0	1	0	1	1	1	0	1	0	0	6
mmu-mi-181c	8	0	1	0	1	0	0	0	0	0	0	0	0	0	2
mmu-mi-203*	12	1	0	0	0	0	0	0	1	0	0	0	0	0	2
mmu-mir-211	7	1	0	1	0	0	0	1	1	0	0	0	0	0	4
mmu-mir-296	2	0	0	0	0	1	0	0	1	0	0	0	0	0	2
mmu-mir-335	6	0	0	0	0	0	1	0	0	0	0	1	0	0	2
mmu-mir-351	X	1	0	1	0	1	0	1	1	1	0	1	0	0	7
mmu-mir-511	2	1	1	0	0	1	0	0	1	1	0	1	0	0	6
mmu-miR-592	6	1	0	1	0	0	0	0	0	0	0	0	0	0	2
mmu-mir-680-1	6	1	1	1	0	1	0	1	1	1	0	1	0	0	8
mmu-mir-763	10	1	1	1	0	1	0	1	0	1	0	1	0	0	7

Table 2. miRNA target prediction for *Tnfrsf1a*. 1/green indicates predicted, 0/red indicates not predicted.

Meanwhile, we performed a microRNA profiling for 500 microRNAs on naive livers of C57BL/6 and SPRET/Ei to look for differential expression of microRNAs. We found an extreme difference in expression of miR-592 in SPRET/Ei compared to C57BL/6 which will be studied in detail by other members of our research group. In the context of this thesis, we focused on miR-511 and miR-680, which are also higher expressed in SPRET/Ei. (Figure 8)

MiR-511 is found in intron 5 of the gene encoding mannose receptor C type 1 (*Mrc1*). Recently, it was shown that the expression of miR-511 correlates with that of Mrc1 mRNA, supporting the notion that this intronic miRNA is regulated by the same promotor as its host gene. (29) Unfortunately, miR-511-5p, the mature form that is predicted to repress *Tnfrsf1a*, has a much lower expression profile than miR-511-3p which makes it difficult to detect.

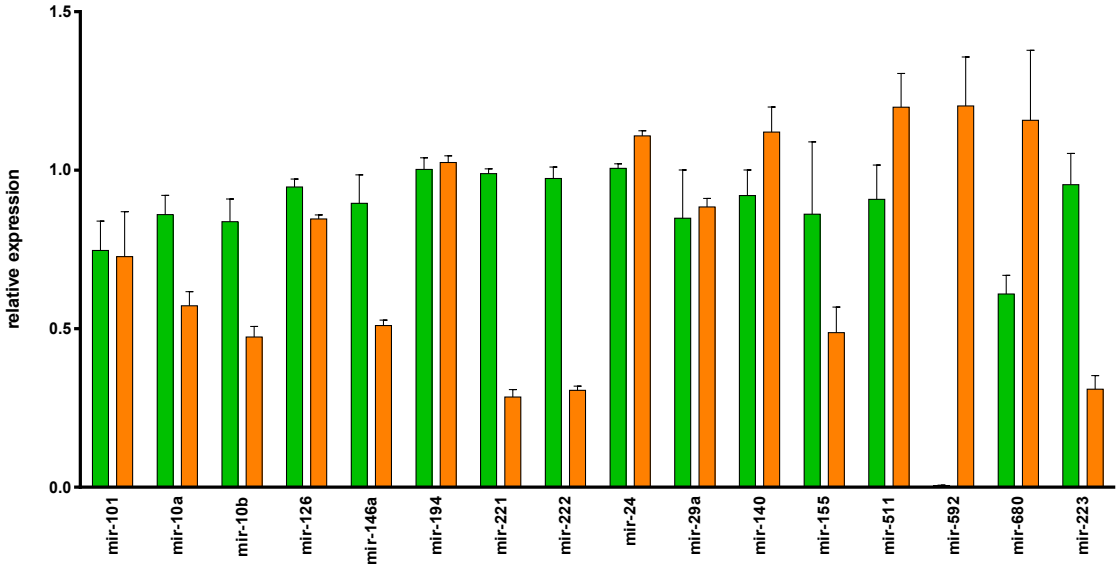

Figure 8. miRNA profiling in naive livers of C57BL/6 (n=5, green) and SPRET/Ei (n=5, orange).

According to Genevestigator and BioGPS, Mrc-1 is higher expressed in the spleen than in the liver, what makes the spleen a more interesting organ for detection. Indeed, we found that both miR-511 and Mrc1 mRNA expression levels measured in the spleen were significantly higher in SPRET/Ei mice than in C57BL/6 mice. (Figure 9)

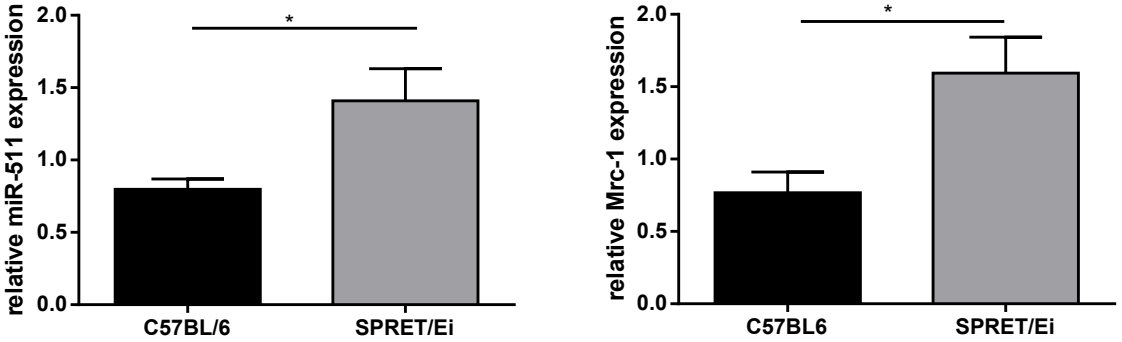

Figure 9. miR-511 and Mrc-1 mRNA levels in C57BL/6 (n=5) and SPRET/Ei (n=5). (left) miR-511 mRNA levels were measured by Q-PCR in the spleen. SPRET/Ei mice express higher miR-511 levels compared to C57BL/6 (p=0.0300). (right) Mrc-1 mRNA levels were measured by Q-PCR in the spleen. SPRET/Ei mice express higher miR-511 levels compared to C57BL/6 (p=0.0275).

MiR-680-1 is located intergenic and two SNPs were found in the pri-miR-680-1 between SPRET/Ei and C57BL/6. (Figure 10)

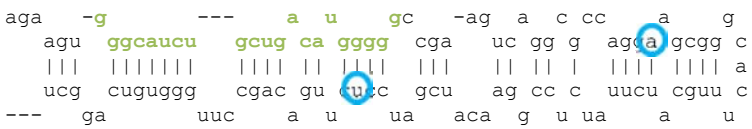

Figure 10. Stem-loop sequence of mmu-miR-680-1. Mature sequence indicated in green, SNPs between SPRET/Ei and C57BL/6 circled in blue.

1.2.5 miR-511 regulates TNFR1 protein levels

1.2.5.1 *in vitro* analysis in hepatocytes

To investigate whether miR-511 or miR-680-1 can regulate TNFR1 expression, we transfected pre-miR-511 precursor molecules or control pre-miRs with a random sequence in primary hepatocytes and 24 h later we studied the effect on TNFR1 protein levels by ELISA. miR-511 could significantly reduce the TNFR1 protein expression in both C57BL/6 and SPRET/Ei MEFs, albeit not dramatically. Whereas miR-680 transfection didn't change the TNFR1 level. (Figure 11)

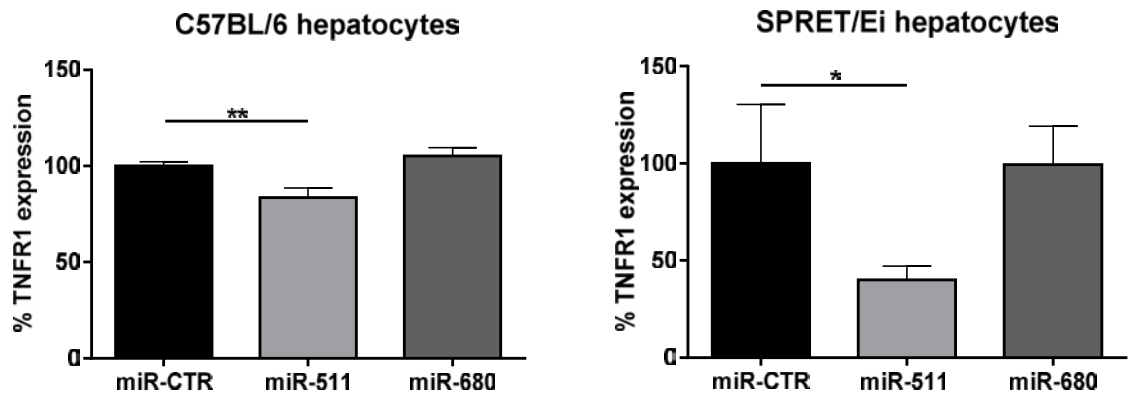

Figure 11. TNFR1 protein levels in C57BL/6 hepatocytes (n=12) and SPRET/Ei MEFs (n=4) after transfection with miRs or siRNA. TNFR1 protein levels were measured by ELISA. Transfection of miR-511 decreases the TNFR1 protein level both in C57BL/6 (p=0.0037) and in SPRET/Ei MEFs (p=0.0748), while miR-680 doesn't induce any reduction of TNFR1.

1.2.5.2 *in vitro* analysis in fibroblasts

To further demonstrate that miR-511 regulates TNFR1 expression, we transfected pre-miR-511 precursor molecules or control pre-miRs with a random sequence in mouse embryonic fibroblasts (MEFs) and 24 h later we studied the effect on cell-bound TNFR1 protein levels by ELISA. miR-511 reduced the TNFR1 protein expression to roughly 80 % in both C57BL/6 and SPRET/Ei MEFs. (Figure 12)

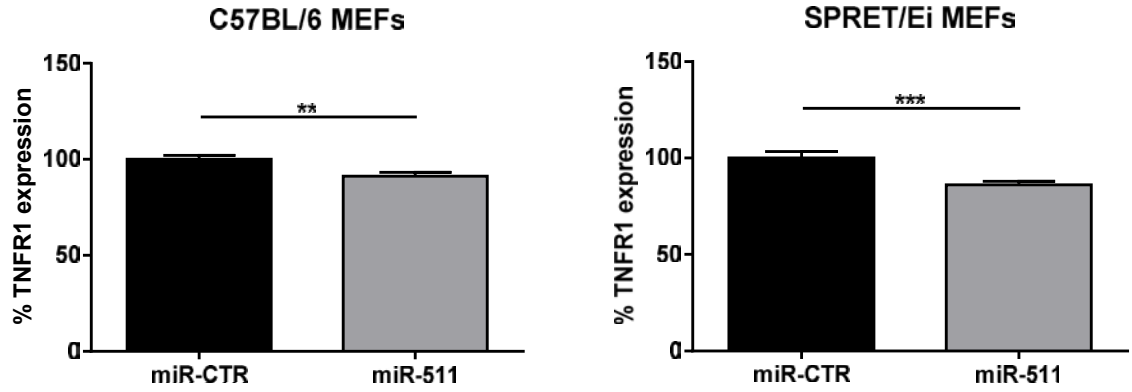

Figure 12. TNFR1 protein levels in C57BL/6 MEFs (n=18) and SPRET/Ei MEFs (n=24) after transfection with miR-511 or miR-CTR. TNFR1 protein levels were measured by ELISA. Transfection of miR-511 decreases the TNFR1 protein level both in C57BL/6 (p=0.0048) and in SPRET/Ei MEFs (p=0.0005).

Moreover, inhibition of endogenous miR-511 with an anti miR (i-mmu-miR-511-5p miRCURY LNA™ microRNA inhibitor) led to significant up-regulation of cellular TNFR1 protein expression 24 h after transfection compared to a mismatch control anti miR, in both C57BL/6 and SPRET/Ei MEFs (Figure 13).

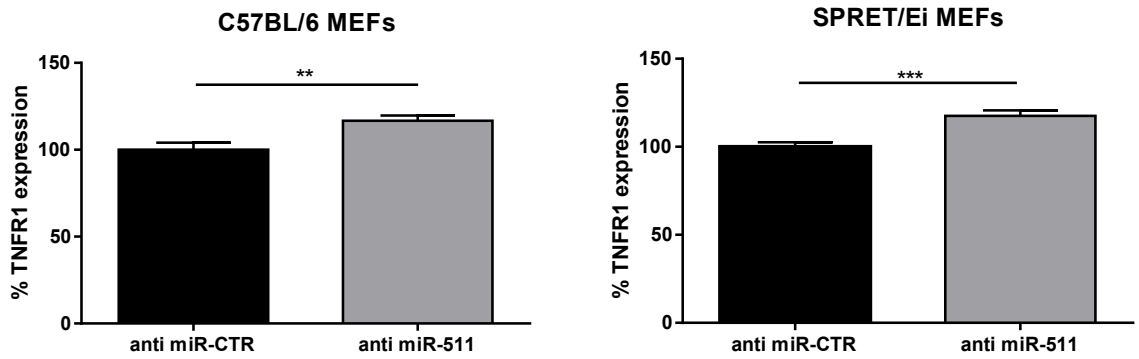

Figure 13. TNFR1 protein levels in C57BL/6 MEFs (n=18) and SPRET/Ei MEFs (n=24) after transfection with anti miR-511 or anti miR-CTR. TNFR1 protein levels were measured by ELISA. Transfection of anti miR-511 increases the TNFR1 protein level both in C57BL/6 ($p=0.0021$) and in SPRET/Ei MEFs ($p<0.0001$).

Combined, these results support the hypothesis that miR-511 is an important regulator of TNFR1 protein expression. Moreover, they further indicate that the genetic linkage between TNF resistance and TNFR1 expression level on the one hand and proximal chromosome 2 and distal chromosome 6 on the other hand may point to the miR-511 and *Tnfrsf1a* genes. Indeed, although no differences were found in the miR-511 sequence between the two mouse strains, miR-511 was clearly up-regulated in SPRET/Ei mice following the up-regulation of the *Mrc1* gene.

When studying the predicted miR-511 target sequences in the 3'UTR of the *Tnfrsf1a* gene of C57BL/6 and SPRET/Ei mice, we found two single nucleotide polymorphisms (SNPs) that can alter the binding of miR-511, in one of the two predicted 3'UTR target sequences. (Figure 14) However, our data suggest that the small sequence variation in this seed sequence does not influence the effect of miR-511 on TNFR1 regulation.

Figure 14. miR-511 binding sites in the 3'UTR of the *Tnfrsf1a* gene of C57BL/6 (B) and SPRET/Ei (S). The single nucleotide polymorphisms (SNPs) of SPRET/Ei are shown in red and two miR-511 target sequences in bold.

We cloned the 3'UTR of the *Tnfrsf1a* gene of both C57BL/6 and SPRET/Ei into the psiCHECK-2 luciferase reporter vector (Promega) and co-transfected each of them with pre-miR-511 precursor molecules or control pre-miRs in HEK-293T cells. 48 h later, we studied the effect on luciferase expression with a dual luciferase assay. Co-transfection with miR-511 significantly reduced the Renilla luciferase activity of the psiCHECK-*Tnfrsf1a*-3'UTR vector of both C57BL/6 and SPRET/Ei (Figure 15), illustrating that miR-511 regulates both B and S *Tnfrsf1a* 3'UTR sequences.

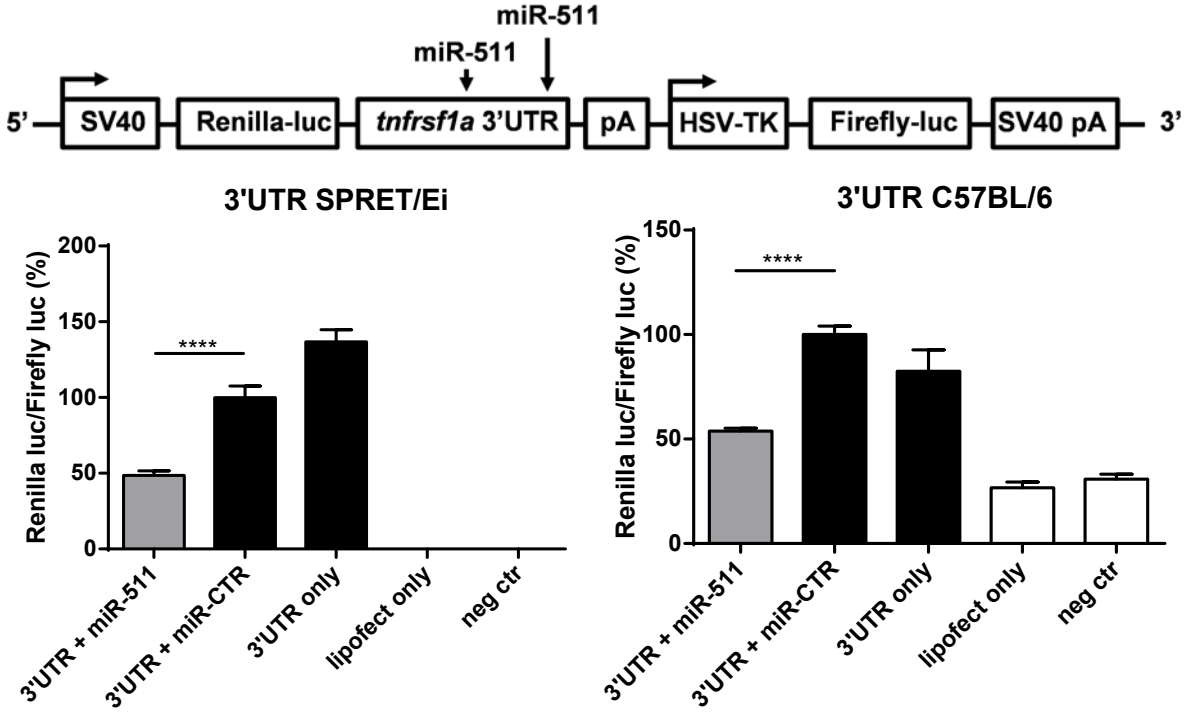

Figure 15. Presentation of the essential elements in the psiCHECK-2 vector. Inhibition of Renilla luciferase activity of the psiCHECK-*Tnfrsf1a*-3'UTR of C57BL/6 and SPRET/Ei reporter plasmid by miR-511 in HEK-293T cells. n=8, p<0,0001.

1.2.5.3 *in vivo* analysis

To examine whether increased expression of miR-511 can down-regulate TNFR1 and induce TNF resistance *in vivo*, we injected a precursor miRNA expression plasmid for mmu-miR-511 and a scrambled control plasmid in C57BL/6 and SPRET/Ei mice by high pressure tail vein injection. This technique of *in vivo* plasmid delivery leads to transient and efficient transfection, predominantly of the liver. (30) After 24 h, miR-511 significantly reduced the TNFR1 protein levels in the livers of both strains of mice relative to the control plasmid (Figure 16), moreover further demonstrating that the sequence variation in the 3'UTR between SPRET/Ei and C57BL/6 has no influence on the functioning of this miR and that the miR-511 indeed regulates TNFR1 protein levels *in vivo*.

Inhibition of endogenous miR-511 by the anti miR (i-mmu-miR-511-5p miRCURY LNA™ microRNA inhibitor) led to significant up-regulation of TNFR1 protein expression 24 h after injection compared to the mismatch control anti miR, in both C57BL/6 and (BxS)F1 mice

(Figure 17). Combined, these data support the hypothesis that miR-511 is an important regulator of TNFR1 protein expression.

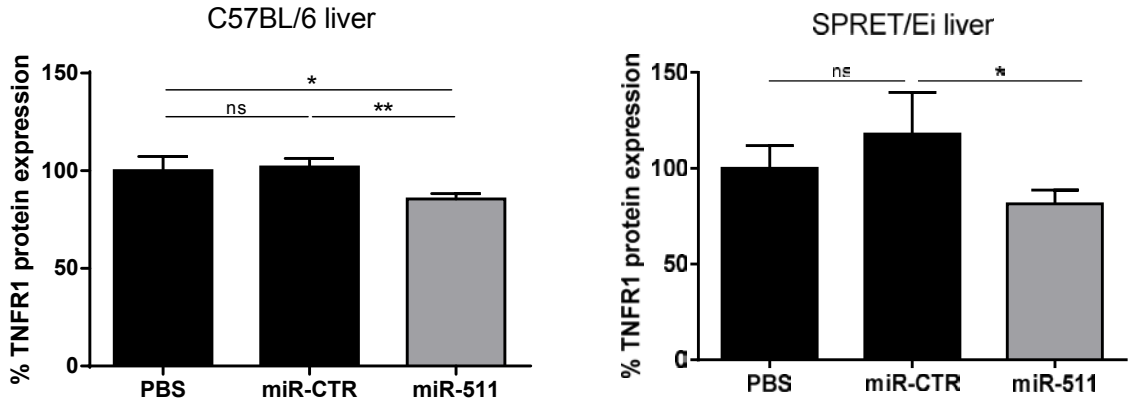

Figure 16. TNFR1 protein levels in C57BL/6 mice (n=19) and SPRET/Ei mice (n=3 vs n=6) after transfection with PBS, miR-511 or miR-CTR. TNFR1 protein levels were measured by ELISA in the liver. Transfection of miR-511 decreases the TNFR1 protein level both in C57BL/6 (p=0.0035) and in SPRET/Ei mice (p=0.0390).

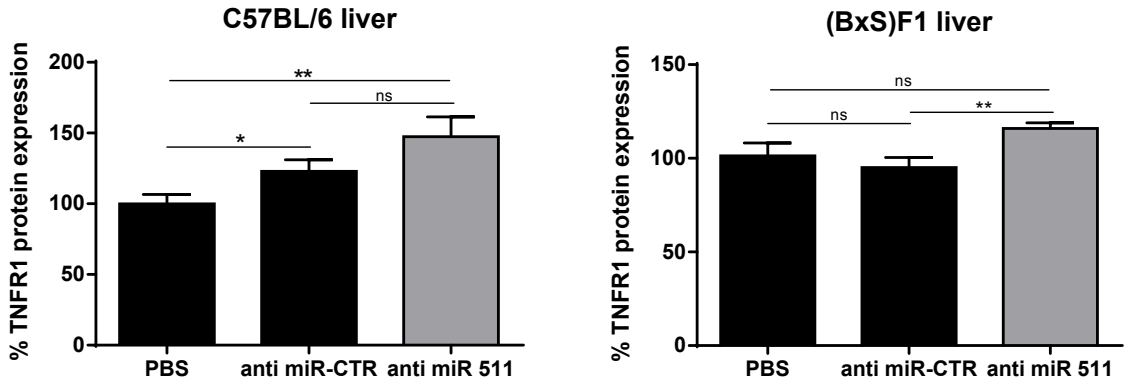

Figure 17. TNFR1 protein levels in C57BL/6 mice (n=19) and (BxS)F1 mice (n=3 vs n=6) after transfection with PBS, anti miR-511 or anti miR-CTR. TNFR1 protein levels were measured by ELISA in the liver. Transfection of anti miR-511 increases the TNFR1 protein level both in C57BL/6 (p=0.0067) and in (BxS)F1 mice (p=0.0044).

1.2.6 miR-511 causes resistance to TNF

miR-511 induced significant resistance in C57BL/6 mice against the inflammation caused by an LD_{50} dose of 25 μg mTNF (Figure 18). While anti miR-511 induced higher susceptibility in C57BL/6 mice against the inflammation caused by a dose of 20 μg mTNF (Figure 19). These data suggest that miR-511 can regulate TNFR1 protein levels and thereby influence the TNF response *in vivo*.

In addition, anti miR-511 treatment induced also higher susceptibility in (BxS)F1 mice for the inflammation caused by a high dose of 500 μg mTNF (Figure 20). Blocking miR-511 thus attenuates the resistance of SPRET/Ei and (BxS)F1.

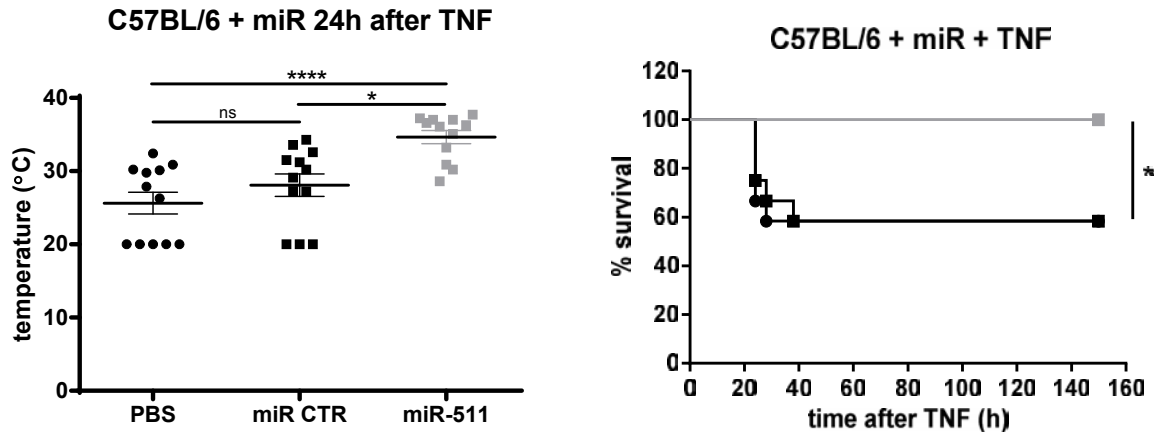

Figure 18. C57BL/6 mice 24 hours after injection of 25 μg TNF (24 h after miR-511 or miR-CTR transfection). Mice pretreated with miR-511 (■, n=12) have a significantly higher bodytemperature compared to mice pretreated with miR-CTR (■, n=12). (p=0.0013) All mice pretreated with miR-511 survived, while 5/12 of the mice pretreated with miR-CTR died. (p=0.0353)

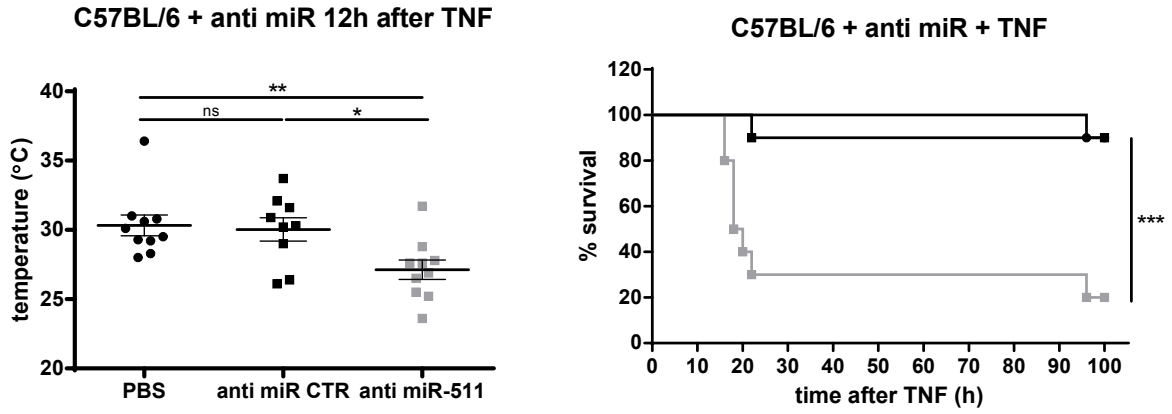

Figure 19. C57BL/6 mice 12 hours after injection of 20 μg TNF (24 h after anti miR-511 or anti miR-CTR transfection). Mice pretreated with anti miR-511 (■, n=10) have a significantly lower bodytemperature compared to mice pretreated with anti miR-CTR (■, n=10). (p=0.0156) 80 % mice pretreated with anti miR-511 (■, n=10) died, while only 10% of the mice pretreated with anti miR-CTR (■, n=10) died. (p=0.0001)

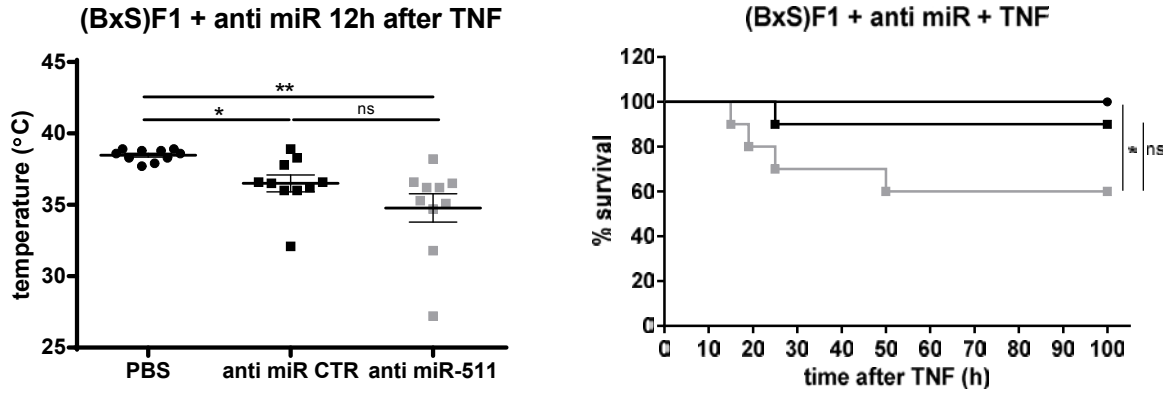

Figure 20. (BxS)F1 mice 12 hours after injection of 500 μg TNF (24 h after anti miR-511 or anti miR-CTR transfection). Mice pretreated with anti miR-511 (■, n=10) have a lower bodytemperature compared to mice pretreated with anti miR-CTR (■, n=10). (p=0.1526) 40 % mice pretreated with anti miR-511 (■, n=10) died, while only 10% of the mice pretreated with anti miR-CTR (■, n=10) died. (p=0.1223)

1.2.7 miR-511 mediates resistance to TNF by specifically regulating TNFR1 expression

To study the specificity for TNFR1 in the anti-inflammatory effect of miR-511, we investigated whether miR-511 can protect mice against LPS-induced endotoxemia, a model in which TNF is centrally involved. (31) 24h following hydrodynamic tail vein injection of miR-511, C57BL/6 mice were indeed significantly protected against an LD_{50} of LPS, *i.e*. 200 μg/mouse (Figure 21A). Because miR-511 was recently described as a regulator of the LPS receptor TLR4, under certain conditions (32), we studied whether the protection provided by miR-511 against LPS is mediated by specific TNFR1 regulation. To do so, we studied the protective effect of miR-511 injection against an LD_{50} of LPS in TNFR1 KO mice, which lack the *Tnfrsf1a* gene, i.e. 500 μg/mouse (Figure 21C). As shown in Figure 21B, miR-511 did not protect against LPS in the absence of TNFR1, suggesting that miR-511 protects against LPS in WT animals by specifically down-regulating TNFR1.

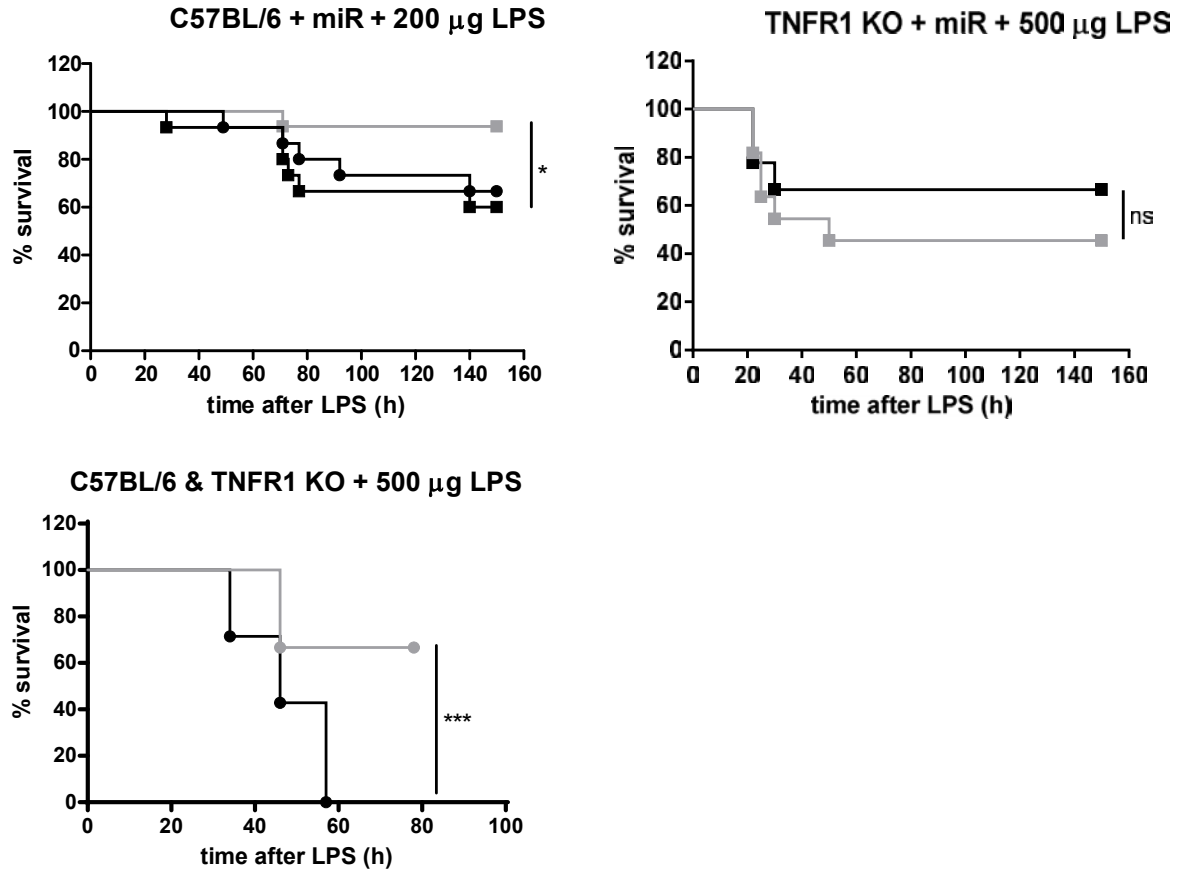

Figure 21. Survival of C57BL/6 mice and TNFR1 KO mice injected with 200 μg or 500 μg LPS, respectively, 24 h after miR-511 or miR-CTR transfection. A. C57BL/6 mice pretreated with miR-511 (■, n=16) were significantly (p=0.0278) protected against LPS compared to miR-CTR pretreated mice (■, n=15). B. No difference in survival (p=0.4066) was found between TNFR1 KO mice pretreated with miR-511 (■, n=11) or with miR-CTR (■, n=9). C. Response of WT (●, n=7) and TNFR1 KO mice (●, n=6) to 500 μg LPS, p=0,0001.

1.2.8 miR-511 causes resistance to conA-induced hepatitis

Because the used technique of *in vivo* plasmid delivery leads to transient and efficient transfection, predominantly of the liver, (30) we further investigated the therapeutic potential of miR-511 in a liver specific TNFR1-induced disease model. Concanavalin A (conA)-induced hepatitis is a cell-mediated immunoinflammatory disease similar to human autoimmune hepatitis. This disease can be induced in mice giving a single *intravenous* injection of 18 mg/kg conA causing activation of T-cells and leading to the secretion of TNF which binds to TNFR1 on macrophages. The following infiltration of the liver with neutrophils and macrophages leads to apoptosis and necrosis of the hepatocytes characterized by a markedly increased serum level of alanine transaminase (ALT) 8 to 24h after injection. (33, 34) First, we injected TNFR1 KO mice with conA to confirm the TNFR1 dependence of this model. Indeed, TNFR1 KO mice injected with 360 μg conA per 20 g *iv* showed significant resistance to conA-induced hepatitis compared to C57BL/6 WT mice. (Figure 22)

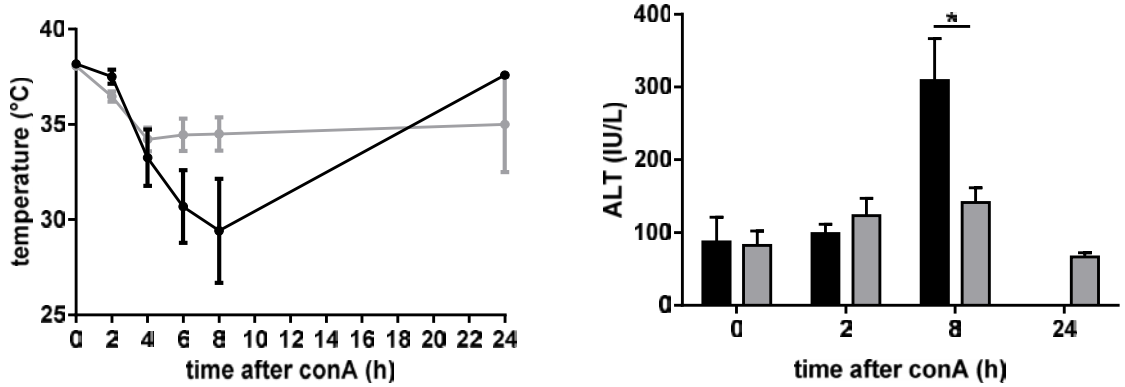

Figure 22. TNFR1 KO and TNFR1 WT mice after injection of 360 μg conA. TNFR1 KO mice are (●, n=12) have a significantly higher bodytemperature, p=0.0255, and a significantly lower serum ALT induction compared to, p=0.0255 compared to TNFR1 WT mice (●, n=12).

Then, we injected C57BL/6 mice with 360 μg conA per 20g, 24h following hydrodynamic tail vein injection of miR-511, miR-CTR or PBS. miR-511 induced significant resistance to conA-induced hepatitis as seen in a regular bodytemperature 8h after conA and almost no induction of serum ALT levels. (Figure 23) There was a normal induction of TNF levels (Figure 24), demonstrating that conA effectively induced inflammation, but that the miR-511-mediated TNFR1 repression influences the TNF response. conA-induced hepatitis thus proved to be a suitable model for studying the effects of miR-511 on TNFR1.

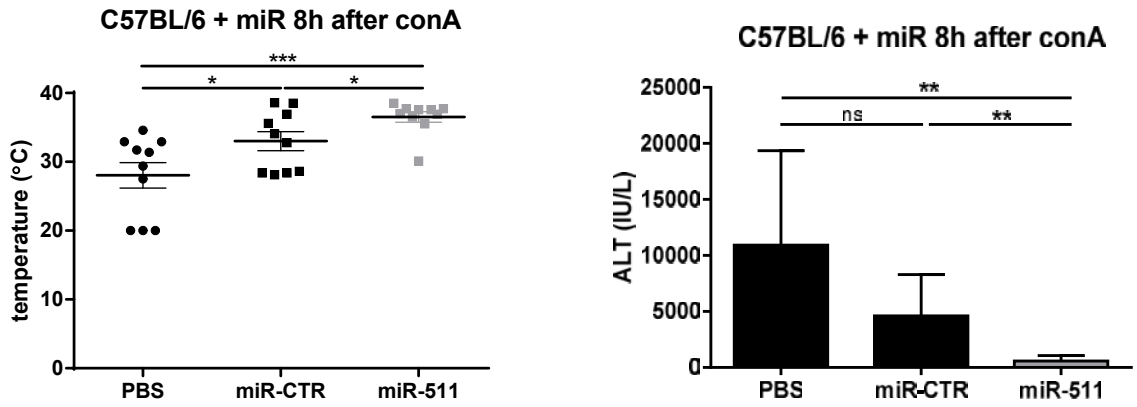

Figure 23. C57BL/6 mice 8 hours after injection of 360 μg conA (24 h after miR-511 or miR-CTR transfection). Mice pretreated with miR-511 (■, n=10) have a significantly higher bodytemperature compared to mice pretreated with miR-CTR (■, n=10), p=0.0384, and a significantly lower ALT serum level compared to miR-CTR mice, p=0.0032.

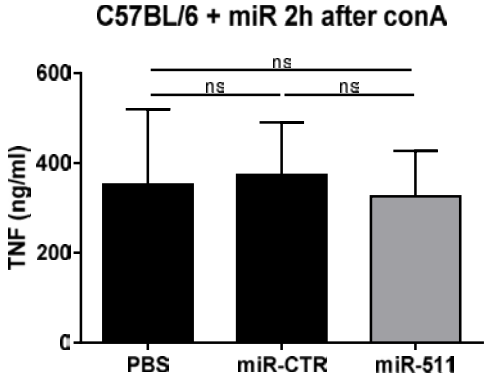

Figure 24. C57BL/6 mice 2 hours after injection of 360 μg conA (24 h after miR-511 or miR-CTR transfection). Serum TNF levels of miR-511 or miR-CTR pretreated mice are similar.

1.3. Conclusion

TNFR1 is the most important receptor in TNF-induced lethality, and TNFR1 KO mice resist most TNF effects, such as induction of cell death, inflammation and tumor necrosis. (35) As TNFR2 has immunoregulatory functions, TNFR1 could be a more specific and therefore a safer drug target than TNF itself. (7) However, not much is known about the regulation of TNFR1 expression on the level of translation.

Here, we show that the remarkable resistance of SPRET/Ei mice to TNF is linked to proximal chromosome 2 and distal chromosome 6. SPRET/Ei mice also have low levels of TNFR1 protein, a trait linked to the same chromosomal regions. Based on chromosomal location, differential miR profiling and *in silico* prediction we selected miR-511 as one of the candidates mediating these two phenotypes. Functional studies on cells and on mice revealed that miR-511 is indeed an important regulator of TNFR1 protein expression. Moreover, administration of miR-511 can protect mice against TNF-mediated inflammation by down-regulation of TNFR1.

We hypothesize that higher levels of miR-511 in SPRET/Ei compared to C57BL/6 are responsible for lower TNFR1 protein levels in SPRET/Ei which mediates resistance to TNF.

1.4. Material and methods

Mice. C57BL6/J mice were purchased from Janvier-Europe. TNFR1 KO mice generated by Dr. M. Rothe (35) were a kind gift from Dr. H. Bleuthmann. SPRET/Ei mice were obtained from The Jackson Laboratory and bred in our facility. (C57BL/6 x SPRET/Ei) F1 mice were generated by crossing C57BL/6J female mice with SPRET/Ei male mice. B.S^{Tnfrsf1a} congenic mice were generated by backcrossing (BxS)F1 mice with the host strain, C57BL6/J, and then repeatedly backcrossing to the host strain and screening the progeny for a nonrecombined SPRET/Ei donor locus of interest (*Tnfrsf1a*) in each generation. (36) B.S^{Tnfrsf1a} congenic mice heterozygous for the S *Tnfrsf1a* allele were intercrossed at the N9 generation, and mice homozygous for the B *Tnfrsf1a* allele as well as mice homozygous for the S *Tnfrsf1a* allele were identified by typing for the polymorphic marker D6Mit254, which is a sequence-tagged site (STS) for *Tnfrsf1a*. All mice were kept in individually ventilated cages under a constant dark-light cycle in a conventional animal house and received food and water *ad libitum*. The mice were used at the age of 8–12 weeks. Animal experiments were approved by the institutional ethics committee for animal welfare of the Faculty of Sciences, Ghent University, Belgium.

Injections. Recombinant mouse TNF (specific activity of 1.66×10^9 IU/mg) was expressed in *Escherichia coli* and purified in our laboratory. The preparation contained less than 6 EU/ml of endotoxin as determined by a *Limulus* amoebocyte lysate assay. Lipopolysaccharide (LPS) from *Salmonella abortus equii* and concanavalin A (conA) were purchased from Sigma. Mice were injected *intraperitoneally* with TNF or LPS in 0.3 ml of pyrogen-free phosphate-buffered saline (PBS) or *intravenously* with conA in 0.2 ml PBS.
A precursor miRNA expression clone for mmu-miR-511 (GAUACCCACCAUGCCUUUUGCUCUGCACUCAGUAAAUAAUAAUUUGUGAAUGUGUAGCAAAA GACAGGAUGGGGAUCCA), cloned in the pEZX-MR04 vector was purchased from GeneCopoeia, as well as a scrambled control clone. Ten micrograms of ultrapure high quality plasmid DNA was dissolved in PBS and injected in a volume of 2 ml in the tail vein under high pressure. This technique guarantees hepatocyte-specific uptake and expression of the plasmid. (30) Rectal body temperatures were measured with an electronic thermometer from Comark.

mTNFR1 ELISA. Liver, kidney, lung and spleen of C57BL/6, SPRET/Ei and B.S^{Tnfrsf1a} mice were excised at the age of 8 weeks or 24 h after miR/anti miR transfection and snap-frozen in liquid nitrogen. Samples were homogenized in PBS containing 0.5% CHAPS and complete protease inhibitor cocktail tablets from Roche. Homogenates were centrifuged for 30 min at 20,000 g and 4°C, after which the supernatant was collected and stored at –80°C. Blood was collected by retro orbital bleeding and allowed to clot for 1 h at 37°C. Serum was prepared and stored at −20°C. Protein concentration was determined by the Bradford method (BioRad) and 500 μg was used to perform an ELISA specific for TNFR1 using the mouse sTNF

I/TNFRSF1A duoset ELISA from R&D Systems. The levels were normalized to the levels of C57BL/6 or control samples, which were set as 100%.

Q-PCR. Liver, kidney, lung and spleen samples were stored in RNA later® from Ambion. Samples were homogenized and RNA was extracted using an RNeasy mini kit from Qiagen. RNA concentration was measured with the Nanodrop1000 from ThermoScientific and 500 ng RNA was used to prepare cDNA with iScript from Bio-Rad. Q-PCR was performed using the SYBR Green master mix and the Light cycler 480 from Roche with the following primers: 5'-CCGGGAGAAGAGGGATAGCTT-3' and 5'-TCGGACAGTCACTCACCAAGT-3' for mTNFR1, 5'-GCTGAATCCCAGAAATTCCGC-3' and 5'-ATCACAGGCATACAGGGTGAC-3' for Mrc-1, 5'-TGAAGCAGGCATCTGAGGG-3' and 5'-CGAAGGTGGAAGAGTGGGAG-3' for Gapdh, and 5'-CCTGCTGCTCTCAAGGTT-3' and 5'-TGGCTGTCACTGCCTG GTACTT-3' for Rpl13a. The best performing housekeeping genes were determined with geNorm (37). No SNPs between C57BL/6 and SPRET/Ei were included in the primers (not shown). Q-PCR for miR-511 was done using specific MultiScribe™ cDNA synthesis and TaqMan® Pri-miRNA Assays for mmu-miR-511 and three stable miRs (mmu-miR-194, mmu-miR-24 and mmu-miR-29a) from Applied Biosystems. All values shown are relative expression values normalized to the geometric mean of the selected housekeeping genes.

Quantitative Trait Loci (QTL) mapping. To map the loci responsible for low TNFR1 protein levels in SPRET/Ei mice, an interspecies backcross between female (C57BL/6 x SPRET/Ei) F1 mice and male C57BL/6 mice was set up and N2 backcross mice were generated. Tail biopsies were collected at weaning from 214 N2 mice and high quality genomic DNA was prepared by standard phenol-chloroform extraction. A genome scan on 100 ng DNA was performed with 72 microsatellite markers (Table 3). Primer sequences from the Massachusetts Institute of Technology (MIT) were obtained at www.informatics.jax.org. Coverage of the genome was estimated by taking the position of the marker loci on the Mouse Genome Database genetic map obtained from The Jackson Laboratory and applying a swept radius of 20 Cm. (38) Primer sequences from the Massachusetts Institute of Technology (MIT) were obtained at www.informatics.jax.org.
Coverage of the genome was estimated by taking the position of the marker loci on the Mouse Genome Database genetic map obtained from The Jackson Laboratory and applying a swept radius of 20 Cm. (38) Livers from the 214 N2 backcross mice were excised at the age of 8 weeks and snap-frozen in liquid nitrogen. Total protein was isolated and 500 μg was used to measure TNFR1 levels by ELISA. After the first screening, the density of markers was increased on chromosomes 2 and 6, which were shown to be linked to the trait. Linkage analysis was performed using the R/qtl software version 1.12-26 running under R 2.9.1. (39) Significance thresholds of LOD scores were estimated by 10,000 permutations of experimental data. The protein level was analyzed using a normal model by the EM algorithm. (40)

D1Mit373	D2Mit359	D3Mit21	D4Mit172	D5Mit352	D6Mit74	D7Mit112	D8Mit223	D9Mit25	D10Mit221
D1Mit24	D2Mit89	D3Mit298	D4Mit152	D5Mit95	D6Mit323	D7Mit222	D8Mit302	D9Mit155	D10Mit309
D1Mit356	D2Mit11	D3Mit114	D4Mit146	D5Mit122	D6Mit132	D7Mit291	D8Mit88	D9Mit24	D10Mit102
	D2Mit109				D6Mit104				
	D2Mit502				D6Mit288				
	D2Mit457				D6Mit254				
					D6Mit194				
					D6Mit113				

D11Mit94	D12Mit147	D13Mit300	D14Mit50	D15Mit267	D16Mit88	D17Mit83	D18Mit119	D19Mit41	DXMit53
D11Mit51	D12Mit102	D13Mit145	D14Mit122	D15Mit29	D16Mit139	D17Mit177	D18Mit186	D19Mit83	DXMit50
D11Mit182	D12Mit27	D13Mit35	D14Mit205	D15Mit34	D16Mit106	D17Mit155		D19Mit137	DXMit126
									DXMit114
									DXMit116
									DXMit130
									DXMit34
									DXMit184

Table 3. Microsatellite markers used in the genome scan for TNFR1 expression in SPRET/Ei mice.

Primary hepatocytes. The isolation of primary hepatocytes was performed by Laurent Dollé and relied on a two-step procedure, including in situ perfusion/digestion of the liver and purification based on cell density. Hepatocytes were isolated from C57BL/6 and SPRET/Ei mice. Subsequently, liver digest medium supplemented with 0.025 % collagenase P (Roche Diagnostics) was applied for enzymatic digestion of the tissue at 37°C. After digestion, the tissue was manually disrupted and filtered into medium containing 0.025 % collagenase P and Dnase I (Roche Diagnostics). The resulting cell suspension was filtered through a sieve and centrifuged two times at 50 g for 2 min. The pellet was resuspended with 24 mL of DMEM with 10% FCS. Hepatocyte enrichment relied on centrifugation at 750 g for 20 min through a three-layer discontinuous Percoll gradient (Amersham Biosciences). (41) An aliquot of the cell preparation was separated for cell count and viability analysis (light microscopy and trypan blue exclusion test). One million of hepatocytes per well were placed on rat tail collagen-1-coated well plates in hepatocyte basal medium with 10% FCS. After 4 h, the medium was changed to a medium without FCS, and hepatocytes were used immediately for further investigations.

Mouse Embryonic Fibroblasts (MEFs). MEFs were isolated from embryos 18 days postcoitum. They were cultured in DMEM medium (supplemented with 10% FCS, penicillin, streptomycin, sodium pyruvate and L-glutamine) and seeded in culture flasks. Cells were trypsinized and seeded in 24-well plates at 100,000 cells per well.

Transfections. Pre-miR™ miRNA Precursors for mmu-miR-511 (GAUACCCACCAUGCCUUUUGCUCUGCACUCAGUAAAUAAUAAUUUGUGAAUGUGUAGCAAAA GACAGGAUGGGGAUCCA), mmu-miR-680-1 (AGAAGUGGGCAUCUGCUGACAUGGGGGCCGA AGUCAGGCGCCAGGAAGCGGGCACUUUGCAUCUUAUCUCCGGAACAUCGAUCCUCUUGACAGC CUUGGGUGUCAGGCU) and a negative control were purchased from Ambion. LNA-enhanced miRCURY i-mmu-miR-511-5p (GAGTGCAGAGCAAAAGGCA) and i-miR-511-5pMMControl

were purchased from Exiqon. 50 μM of the molecules were transfected using Lipofectamine RNAiMAX from Invitrogen and 24 h later the cells were lysed with PBS containing 0.5% CHAPS and complete protease inhibitor cocktail tablets from Roche. In ELISA for TNFR1, 75 μg of total protein was used.

Luciferase reporter assay. The DNA coding for the *Tnfrsf1a* 3'UTR of C57BL/6 and SPRET/Ei were ordered at Genscript. The 514 bp sequence of C57BL/6 and the 508 bp sequence of SPRET/Ei were digested out of the vector pUC57 with SgfI and NotI and ligated in the psiCHECK-2 vector (Promega). The constructs were transformed into MC1061 cells and screened for the presence of a correct sequence with the following PCR primers, CAGATGAAATGGGTAAGTAC and AAACCCTAACCACCGCTTAA. The 3'UTR reporter plasmids were co-transfected with pre-miR precursor molecules in HEK-293T cells using Lipofectamin 2000 (Invitrogen). Cells were harvested 48 hours post transfection, washed with PBS and lysed in passive lysis buffer (PLB). Firefly luciferase activity is measured in Optiplate-96 F plates (Perkin Elmer) by adding Luciferase Assay Reagent II (Promega) to generate a luminescent signal. This is signal is quantified using the GLOMAX 96 microplate luminometer (Promega). Afterwards, this reaction is quenched and the Renilla luciferase reaction is initiated by adding Stop & Glo® Reagent (Promega) to the same wells.

Determination of TNF and ALT in the serum. Blood was collected by retro orbital bleeding and allowed to clot for 1 h at 37°C. Serum was prepared and stored at −20°C. Serum ALT determination was performed in the clinical Biology laboratory of the Ghent University Hospital. TNF bio-activity was measured with an MTT cell death assay using the sensitive L929 cell line. (42)

Identification of *Tnfrsf1a* targeting microRNAs. We searched for miRNAs that can have *Tnfrsf1a* as a target gene with the following miRNA target prediction programs : MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), miRanda (http://www.microrna.org/microrna/home.do), miRGen (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi), NBmiRTar (http://wotan.wistar.upenn.edu/NBmiRTar/index.php), TargetScan (http://www.targetscan.org), miRDB (http://mirdb.org/miRDB/), DIANAmicroT (http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi/), microInspector (http://bioinfo.uni-plovdiv.bg/microinspector), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/predictedmirnagene.html), PicTar (http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_mouse.cgi), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html).

Statistical analysis. Survival curves (Kaplan-Meyer plots) were compared by a log-rank test and final outcomes were compared by a chi-square test. Data are expressed as the means ± S.E. Statistical significance of differences between groups was evaluated with Student's *t*

tests with 95% confidence intervals and with one-way or two-way analysis of variance. Error bars in the figures represent the mean ± S.E. *, **, and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

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MicroRNA-511 mediated TNFR1 repression is regulated by Glucocorticoids and Glucocorticoid Receptor activity

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Abstract. TNF resistance of the mouse strain SPRET/Ei is found to be related to higher expression of microRNA-511 and thereby reduced expression of the major TNF receptor TNFR1. SPRET/Ei mice were previously reported to express higher levels of the glucocorticoid receptor, reflecting in increased GR activity. Here we report that blocking of the GR with RU486 sensitizes SPRET/Ei mice against TNF, indicating that GR activity is essential for the extreme resistance of SPRET/Ei mice against TNF-induced lethality. Moreover, Mrc-1, the gene in which miRNA-511 is located, was found to be a GR-inducible gene and administration of dexamethasone to C57BL/6 mice caused down-regulation of TNFR1 protein levels. Our data suggest that high GR levels in SPRET/Ei mice induce the expression of Mrc-1 and miR-511, resulting in lower TNFR1 protein levels and consequential resistance to TNF-induced lethality.

Contributions

Leen Puimège performed all the experiments in this chapter with the exceptions below.
Sofie Lodens performed the Q-PCRs for all the experiments.
Lien Dejager performed the RU486 sensitization experiment in C57BL/6 and SPRET/Ei.
Steven Timmermans performed the *in silico* analysis of the Mrc-1 promoter.
Filip Van Hauwermeiren and Claude Libert supervised the design and performance of the experiments.

2.1. Introduction

Inbred strains derived from mice obtained from the wild are often more resistant to carcinogens and to several types of pathogens compared with the commonly used inbred strains like C57BL/6. More specifically, *Mus spretus* derived SPRET/Ei mice are extremely resistant to inflammation induced by cytokines or bacterial products and to viruses. (1-3) Because of these phenotypic differences, *Mus spretus* mice have often been used for the identification of genetic factors underlying these complex traits.

The resistance of SPRET/Ei against influenza A virus proved to be a simple trait, linked to the myxovirus resistance 1 (*Mx1*) gene, but there is also evidence that the sequence variation of Mx1 in SPRET/Ei has led to a more active Mx1 form than the one found in C57BL/6 mice. (3) The LPS resistance of SPRET/Ei was shown to be mediated by GC-leucin zipper (Gilz), encoded by the *Tsc22d3* gene on the distal region of the X chromosome. (4) Compared with C57BL/6, SPRET/Ei mice were found to express higher GR levels, reflecting in increased GR activity. GR is the receptor of glucocorticoids such as corticosterone in rodents, cortisol in humans or synthetics such as dexamethasone, all of which have strong anti-inflammatory effects via GR. (5) GR functions as a dimeric transcription factor, binding to specific elements on the DNA, called GRE-elements, thereby increasing transcription of these GRE-element containing genes. (6) The higher GR levels of SPRET/Ei were found to be linked to the *Nr3c1* gene, encoding for the GR itself, on chromosome 18. (7) The Gilz gene *Tsc22d3* was shown to have a higher expression in SPRET/Ei and to contain a GR responsive element (GRE). ChIP analysis of liver lysates confirmed that the GR binds to a GRE element in the promoter of *Tsc22d3* of both C57BL/6 and SPRET/Ei mice. (4) Hence, the Gilz-mediated LPS resistance is regulated by higher GR induction in SPRET/Ei.

The resistance of SPRET/Ei against TNF-induced lethality has been shown to be linked to two protective loci, one on proximal chromosome 2 and one on distal chromosome 6. (1) Since the TNFR1-encoding gene *Tnfrsf1a* is located on distal chromosome 6, we studied whether the genetic link between TNF resistance and distal chromosome 6 has any molecular relation to the *Tnfrsf1a* gene. In chapter 1 of the results, we describe that such a correlation indeed exists. SPRET/Ei mice express significantly reduced protein levels of the major TNF receptor TNFR1, while the mRNA levels appear to be normal. This trait was found to be linked to similar chromosomal parts that were previously linked to the TNF resistance phenotype, demonstrating that the lower TNFR1 levels and TNF resistance are likely coupled phenotypes. MicroRNA miR-511, which is intronic of the mannose receptor C type 1 (*Mrc-1*) gene on proximal chromosome 2, is higher expressed in SPRET/Ei than in C57BL/6 and was found to regulate TNFR1 translation leading to a functional down-regulation of TNFR1 and causing resistance to TNF. Therefore we believe that miR-511 is partly responsible for the lower TNFR1 expression levels and TNF resistance of SPRET/Ei.

To examine whether, in analogy with the GILZ story, the higher GR induction in SPRET/Ei also plays a role in the miR-511 mediated TNF resistance of SPRET/Ei mice, we investigated the influence of the GR on the expression of Mrc-1, miR-511 and thereby on TNFR1.

2.2. Results

2.2.1. GR antagonist sensitizes SPRET/Ei against TNF-induced lethality

Glucocorticoids (GCs) are important anti-inflammatory mediators that prevent an excessive immune response. They are produced by the adrenal gland upon stimulation of the HPA (hypothalamus-pituitary gland-adrenal gland) axis and act through the glucocorticoid receptor (GR) by which they can inhibit TNF-induced lethal inflammation. (8) Administration of synthetic GCs such as dexamethasone (DEX) can protect mice from lethality to TNF, while administration of the GR antagonist RU486 or adrenalectomy strongly sensitize mice to TNF. (9) Moreover, SPRET/Ei mice were found to have higher endogenous GC and GR expression levels compared to C57BL/6 mice. (7)

To investigate whether glucocorticoids play a role in the TNF resistance of SPRET/Ei mice, we treated C57BL/6 and SPRET/Ei mice with the GR antagonist RU486. The lowest lethal dose of TNF in RU486 sensitized mice was proven to be 5 μg. We found that irreversible blocking of the GR with RU486, sensitizes SPRET/Ei mice for TNF, entirely wiping out the differences in TNF response between SPRET/Ei and C57BL/6 mice. (Figure 1) These results indicate that the GC-GR action is essential for the extreme resistance of SPRET/Ei mice against TNF-induced lethality.

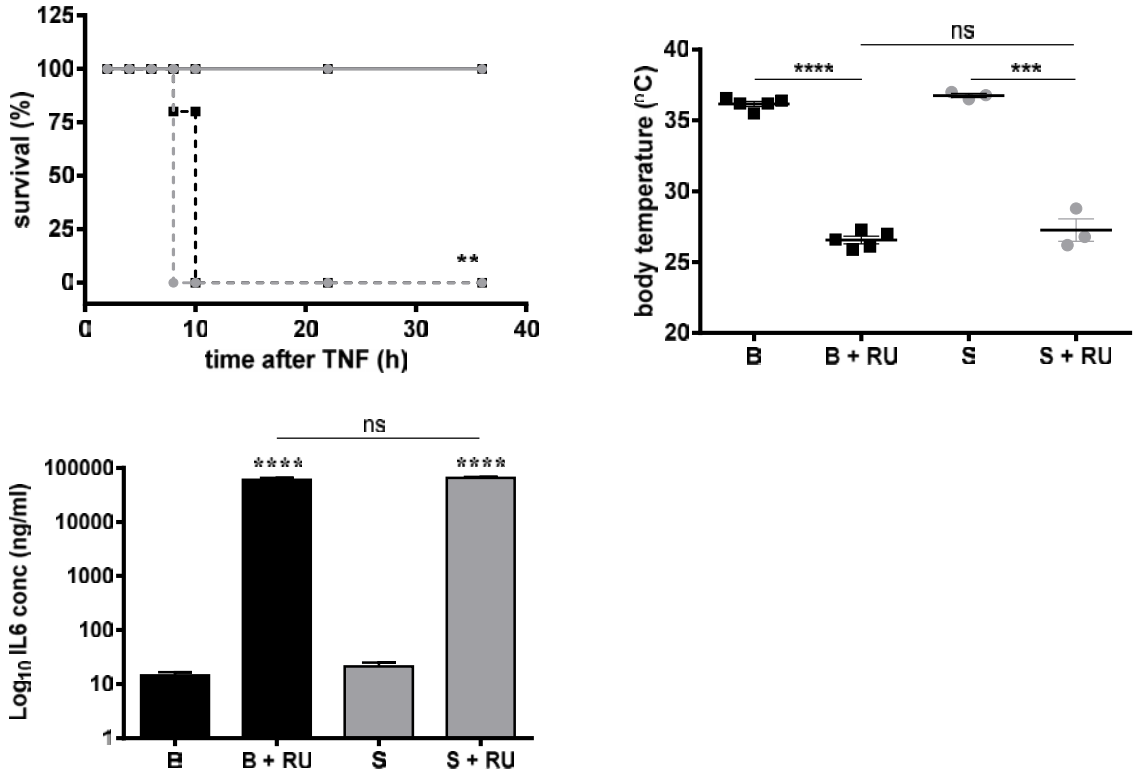

Figure 1. Survival of C57BL/6 (B) and SPRET/Ei (S) mice after injection of 5 μg TNF (30 min after RU486 treatment). A. Both C57BL/6 (■, n=5) and SPRET/Ei (●, n=3) are sensitized to 5 μg TNF after treatment with 5 mg RU486 (dotted lines), compared to DMSO (full lines) pretreated control mice. B. Both C57BL/6 and SPRET/Ei pretreated with RU have a significantly lower body temperature 6 h after TNF (resp. $p<0.0001$ and $p=0.0003$). C. Both C57BL/6 and SPRET/Ei pretreated with RU show significantly higher IL-6 levels 6 h after TNF ($p<0.0001$).

2.2.2. Glucocorticoid responsive elements

GCs have strong anti-inflammatory properties, mainly by inhibiting the production of pro-inflammatory cytokines via transrepression mechanisms, i.e. protein-protein interactions between the GR and transcription factors like NF-kB and AP-1. (10) Upon steroid ligand binding, the GR translocates to the nucleus where it can perform distinct functions. Besides transrepression, the GR can also induce transcription by transactivation. It either acts as a homodimeric transcription factor that binds to the glucocorticoid response elements (GREs) in promoter regions of GC-inducible genes or as a monomeric protein that co-operates with other transcription factors to induce transcription. (6)

Based on functional studies, we have shown that miR-511 is responsible for *in vivo* down-regulation of TNFR1 in mice. Hence, we suggest that miR-511 suppresses TNFR1 expression in SPRET/Ei mice leading to resistance against TNF-induced inflammation. MiR-511 is found in intron 5 of the gene encoding mannose receptor C type 1 (*Mrc1*) and it was suggested that miR-511 is regulated by the same promoter as the gene. (11) Indeed, we found that Mrc1 and miR-511 show a similar up-regulation in the spleen of SPRET/Ei mice compared to C57BL/6 mice.

To investigate whether Mrc-1 expression could be regulated by the GR, we searched for GRE elements in the promoter of mouse Mrc-1. The consensus GRE sequence is an inverted imperfect hexameric palindrome separated by a spacer of 3 bp (5'-AGAACAnnnTGTTCT-3'). However, the sequence of the GRE may vary among different promoters. (12) Using ConTra, we found two GRE elements in the 5' region at -1167 to -1152 and at -599 and -584. Moreover, we found a GR half binding site at positions -20 to -2, just before the transcription start site. These findings suggest that the GR is able to bind to the promoter of Mrc-1 and induce transcription. However, we don't have physical evidence of GR binding based on ChIP-Seq or classical ChIP analysis, nor on ENCODE data.

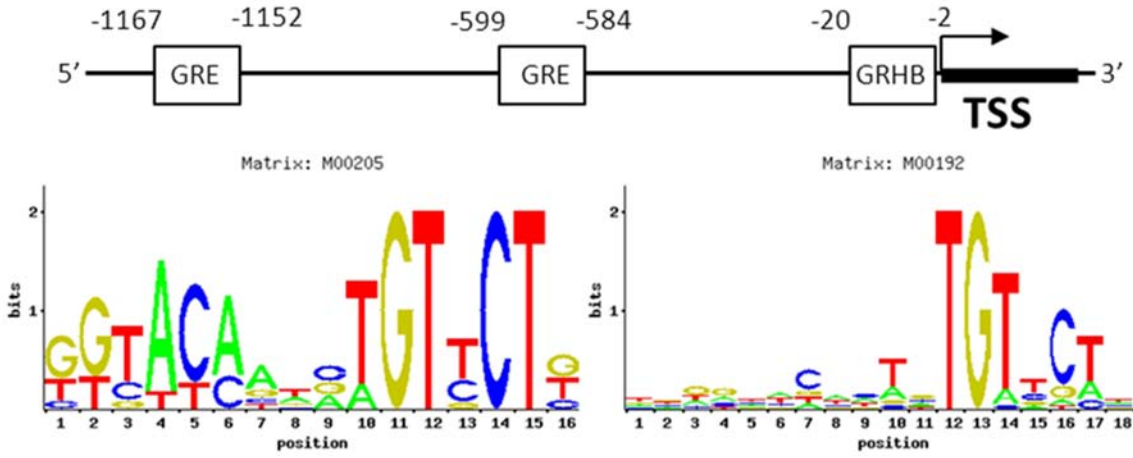

Figure 2. Two GRE elements (ConTra matrix M00205) in the 5' region of the Mrc-1 gene at -1167 to -1152, and -599 to -584. One GR half binding site (ConTra matrix M00192) in the 5' region of the Mrc-1 gene at -20 to -2.

2.2.3. Dexamethasone induces Mrc-1 and miR-511 expression in mice

It is known that the GR, through direct GRE binding, induces the gene expression of liver enzymes involved in gluconeogenesis, like tyrosine aminotransferase (TAT), serum and glucocorticoid-inducible kinase (SGK) and many anti-inflammatory proteins like MAPK phosphatase 1 (MKP-1) and GC-leucin zipper (Gilz). The *Tsc22d3* gene, encoding for Gilz, is considered to be a prototype of GC-inducible genes and is often used as a readout product of the GC-induced signaling cascade. (12)

We administered rapidexon, a soluble form of dexamethasone, to C57BL/6 mice to study possible up-regulation of Mrc-1 and miR-511 expression. Beside the induction of known GRE genes, we could see a significant induction of Mrc-1 in the liver 2h after 500 μg of DEX and in the spleen 6h after 50 μg of DEX. (Figure 3 and 4) This induction confirms the suggestion that the GR is able to bind to the promoter of Mrc-1 and induce transcription of both Mrc-1 and miR-511, though only a ChIP analysis is able to undoubtedly validate this binding.

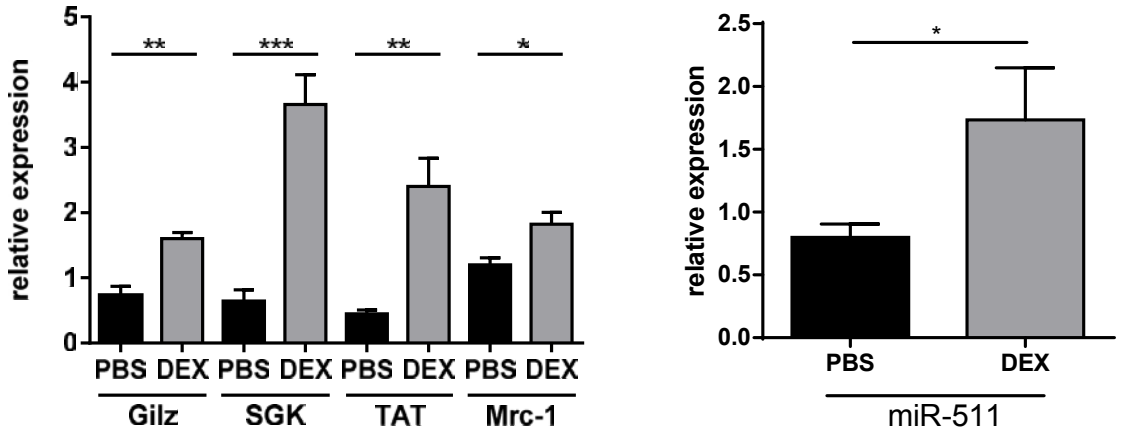

Figure 3. Relative mRNA expression in C57BL/6 liver (n=5) 6h after injection of 50 μg rapidexon. Significant up-regulation of Gilz (p=0.018), SGK (p=0.0003), TAT (p=0.0015) and Mrc-1 (p=0.001) and miR-511 (p=0.0180).

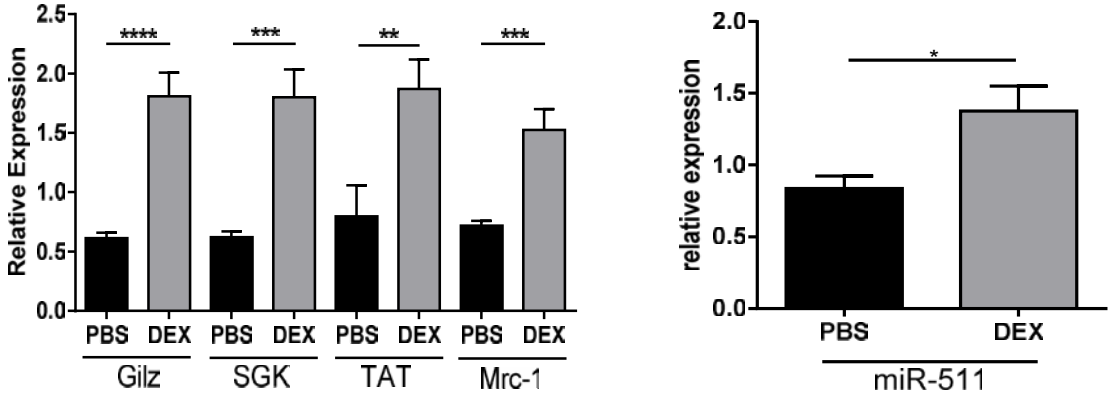

Figure 4. Relative mRNA expression in C57BL/6 spleen (n=5) 6h after injection of 50 μg rapidexon. Significant up-regulation of Gilz (p<0.0001), SGK (p=0.0001) and Mrc-1 (p=0.0008) and miR-511 (p=0.0125).

2.2.4. Adrenalectomy reduces Mrc-1 and miR-511 expression in mice

Glucocorticoids are produced by the adrenal glands and upon removal of these glands by adrenalectomy the expression of GR-induced genes declines. In addition, we also found a significant down-regulation of Mrc-1 and miR-511 in adrenalectomized mice (Figure 5) once more confirming that the GC-GR action induces transcription of Mrc-1.

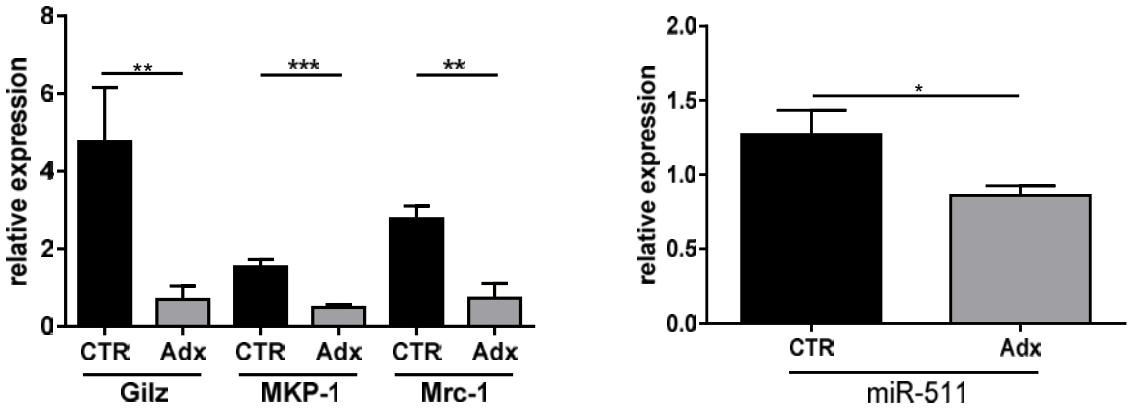

Figure 5. Relative mRNA expression in C57BL/6 mice (n=5) and adrenalectomized mice (n=6). Left, significant down-regulation of Gilz (p=0.0088), MKP-1 (p=0.0003) and Mrc-1 (p=0.0034). Right, significant down-regulation of miR-511 (p=0.0357).

2.2.5. TNFR1 expression is regulated by GR activity

Knowing that SPRET/Ei mice have a higher GR expression compared to C57BL/6 mice and that the GR can induce Mrc-1 expression, we hypothesize that the higher level of miR-511 in SPRET/Ei is also caused by GR induction. As miR-511 might be responsible for suppression of TNFR1 in SPRET/Ei mice, leading to resistance against TNF-induced inflammation, we expect to see down-regulation of TNFR1 after administration of rapidexon. Indeed, in mice 6h after injection with 50 μg rapidexon, we saw a significant down-regulation of TNFR1 in the liver and the spleen as well as in the serum, which excludes the role of shedding by TACE (Figure 6). So, we suggest that administration of dexamethasone to C57BL/6 mice causes down-regulation of TNFR1 protein levels by inducing Mrc-1 and miR-511.

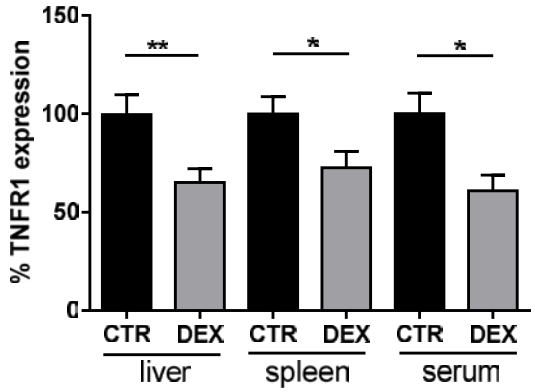

Figure 6. TNFR1 protein levels in C57BL/6 (n=5) 6h after injection of 50 μg rapidexon. Significant down-regulation of TNFR1 in the liver (p=0.0079), spleen (p=0.0296) and serum (p=0.0256).

In addition, we also found a significant up-regulation of TNFR1 in the liver and spleen of adrenalectomized mice as well as in the serum (Figure 7).

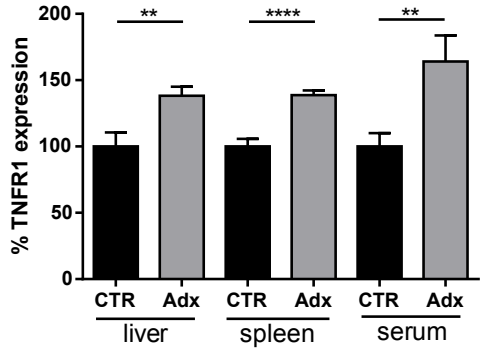

Figure 7. TNFR1 protein levels in C57BL/6 (n=10) and adrenalectomized mice (n=10). Significant up-regulation of TNFR1 in the liver (p=0.0072), spleen (p<0.0001) and serum (p=0.0077).

2.3. Conclusion

In the previous chapter of results, we describe that SPRET/Ei have higher Mrc-1 and miR-511 levels leading to translational repression of TNFR1 and TNF resistance of SPRET/Ei. SPRET/Ei mice were found to express higher GR levels, reflecting in increased GR activity.(7)
Here, we demonstrated that blocking of the GR with RU486 sensitizes SPRET/Ei mice against TNF, indicating that GR activity is essential for the extreme resistance of SPRET/Ei mice against TNF-induced lethality. Moreover, we found that Mrc-1 is a GR-inducible gene and that administration of dexamethasone to C57BL/6 mice causes down-regulation of TNFR1 protein levels. Our hypothesis is that high GR levels in SPRET/Ei mice induce the expression of Mrc-1 and miR-511, resulting in lower TNFR1 protein levels and thereby mediating resistance to TNF-induced lethality. (Figure 8)

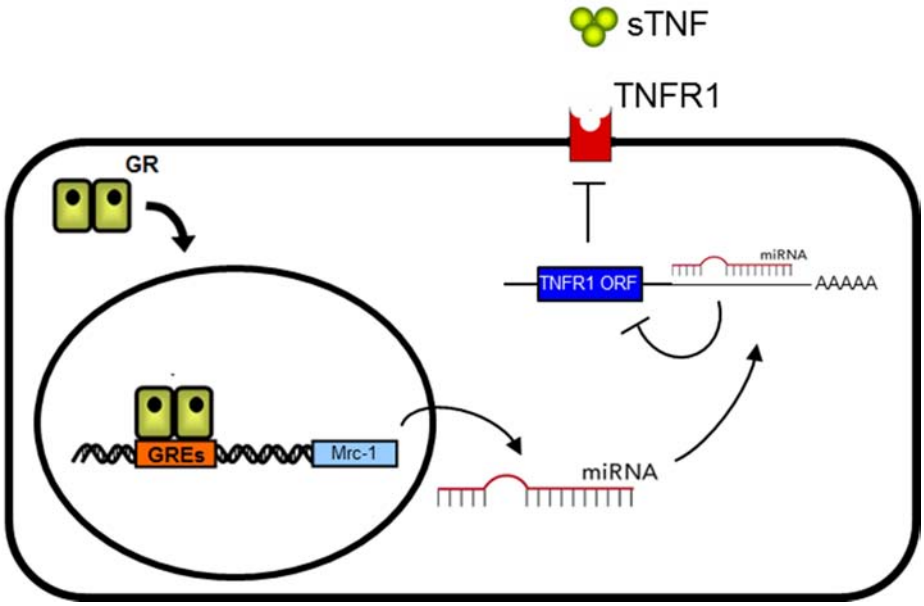

Figure 8. Hypothesis on the involvement of the GR in TNFR1 down-regulation. MicroRNA-511 mediated TNFR1 repression is regulated by GR activity.

2.4. Material and methods

Mice. Normal and adrenalectomized C57BL/6J mice were purchased from Janvier-Europe. SPRET/Ei mice were obtained from The Jackson Laboratory and bred in our facility. All mice were kept in individually ventilated cages under a constant dark-light cycle in a conventional animal house and received food and water *ad libitum*. The drinking water of Adx mice was supplemented with 0.9% NaCl. Animal experiments were approved by the institutional ethics committee for animal welfare of the Faculty of Sciences, Ghent University, Belgium.

Injections. Recombinant mouse TNF (specific activity of $1.66x10^9$ IU/mg) was expressed in *Escherichia coli* and purified in our laboratory. The preparation contained less than 6 EU/ml of endotoxin as determined by a *Limulus* amoebocyte lysate assay. Mice were injected intraperitoneally with TNF in 0.3 ml of pyrogen-free phosphate-buffered saline (PBS). RU486 (Mifepristone), a GR and progesterone receptor antagonist, was purchased from Sigma. Mice were injected intraperitoneally with 5 mg of RU486 in 0.05 ml of DMSO. Rapidexon (DEX, a synthetic GR ligand) was purchased from Medini. Mice were intraperitoneally injected with 50 or 500 mg rapidexon in 0.3 ml of pyrogen-free phosphate-buffered saline (PBS). Rectal body temperatures were measured with an electronic thermometer from Comark. Serum was prepared and stored at −20°C and serum IL-6 was determined with a 7TD1 bioassay. (13)

Q-PCR. Liver and spleen samples were stored in RNA later® from Ambion. Samples were homogenized and RNA was extracted using an RNeasy mini kit from Qiagen. RNA concentration was measured with the Nanodrop1000 from ThermoScientific and 500 ng RNA was used to prepare cDNA with iScript from Bio-Rad. Q-PCR was performed using the SYBR Green master mix and the Light cycler 480 from Roche with the following primers: 5'-CCAGTGTGCTCCAGAAAGTGTAAG-3' and 5'-AGAAGCTCATTTGGCTCAATCTC-3' for Gilz, 5'-GAGATCGTGTTAGCTCCAAAGC-3' and 5'-CTGTGATCAGGCATAGCACACT-3' for SGK, 5'-TGCTGGATGTTCGCGTCAATA-3' and 5'-CGGCTTCACCTTCATGTTGTC-3' for TAT, 5'-GTTGTTGGATTGTCGCTCCTT-3' and 5'-TTGGGCACGATATGCCCAG-3' for MKP-1, 5'-GCTGAATCCCAGAAATTCCGC-3' and 5'-ATCACAGGCATACAGGGTGAC-3' for Mrc-1, 5'-TGAAGCAGGCATCTGAGGG-3' and 5'-CGAAGGTGGAAGAGTGGGAG-3' for Gapdh and 5'-CCTGCTGCTCTCAAGGTT-3' and 5'-TGGCTGTCACTGCCTGGTACTT-3' for Rpl13a. The best performing housekeeping genes were determined with geNorm (14). All values shown are relative expression values normalized to the geometric mean of the selected housekeeping genes.

mTNFR1 ELISA. Livers of C57BL/6 mice were excised 2h or 6h after rapidexon injection and snap-frozen in liquid nitrogen. Samples were homogenized in PBS containing 0.5% CHAPS and complete protease inhibitor cocktail tablets from Roche. Homogenates were centrifuged for 30 min at 20,000 g and 4°C, after which the supernatant was collected and stored at -80°C. Protein concentration was determined by the Bradford method (BioRad) and 200 μg

was used to perform an ELISA specific for TNFR1 using the mouse sTNF RI/TNFRSF1A duoset ELISA from R&D Systems.

Statistical analysis. Survival curves (Kaplan-Meyer plots) were compared by a log-rank test and final outcomes were compared by a chi-square test. Data are expressed as the means ± S.E. Statistical significance of differences between groups was evaluated with Student's *t* tests with 95% confidence intervals and with one-way or two-way analysis of variance. Error bars in the figures represent the mean ± S.E. *, **, and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Acknowledgements. We thank Joke Vanden Berghe for technical assistance.

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Generation and characterization of TNFR1 binding and inhibiting nanobodies

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Abstract. Tumor necrosis factor (TNF) is an essential mediator in numerous inflammatory and autoimmune diseases. TNF signals through two distinct receptors, TNFR1 and TNFR2, where primarily TNFR1 is involved in the pathological actions of TNF. Currently, therapeutic interventions are based on anti-TNF agents which block signaling through both receptors. However, more specific targeting of TNFR1 without interfering with the immunomodulating functions of TNF via TNFR2, is suggested to be more appropriate for the treatment of TNF-induced chronic diseases. We have generated a nanobody against human TNFR1 that has many advantages compared to monoclonal antibodies. Our TNFR1 inhibitor is a trimeric molecule consisting of two TNFR1 targeting nanobodies coupled to a nanobody against albumin. This nanobody shows strong binding affinity to the extracellular part of TNFR1, thereby competing with the ligand. In vitro, the nanobody inhibits TNF-induced NF-kB activation to the same extent as the commercial available monoclonal antibody MAB225. These findings open the way to further characterization of the *in vivo* therapeutic potential against TNFR1-mediated diseases.

Contributions

Leen Puimège performed all the experiments in this chapter with the exceptions below.
Filip Van Hauwermeiren assisted in the experiments.
Sophie Steeland assisted with the pharmacokinetic study.
Claude Libert supervised the design and performance of the experiments.

3.1. Introduction

The TNF signaling pathway is a valuable target in the therapy of autoimmune diseases and anti-TNF treatment is used for the treatment of rheumatoid arthritis, inflammatory bowel disease and psoriasis. TNF blockers have been very successful at inhibiting the inflammation induced damage. However, some side effects have been reported, e.g. increased risk of infection and malignancy and the onset of new auto-immune diseases. Some of these effects are caused by unwanted inhibition of beneficial TNF signaling such as resistance to infection and recovery of affected tissues. More specific targeting of pathological TNF signaling might lead to a broader applicability and improved safety. Specificity can be obtained by targeting TNFR1 while leaving TNFR2 signaling untouched. Several TNFR1 inhibitors including monoclonal antibodies (Atrosab), antagonistic TNF mutants (R1antTNF), RNAi and antisense oligos have been successfully used to down-regulate TNFR1 and reduce pathology *in vivo*. (1-4)

Mammal antibodies typically consist of two heavy and two light polypeptide chains connected by disulfide bonds. The antigen binding site is located at the N-terminal end of the antigen binding fragment (Fab) which is composed of a constant (Fc) and a variable domain (Fv) from each heavy (VH) and light (VL) chain. (20) (Figure 1)

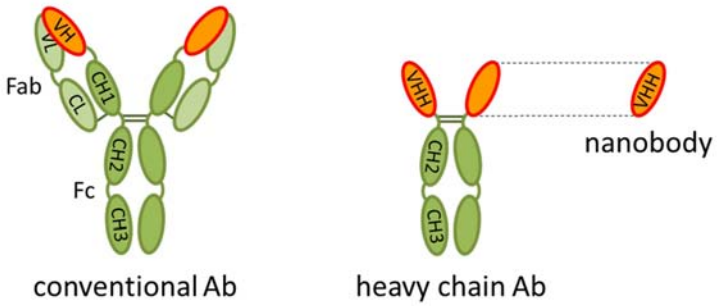

Figure 1. Schematic diagram of the structures of conventional and heavy chain antibodies and a nanobody. Adapted from (5).

Camelids (Camelidae), in addition to conventional antibodies, have a unique type of antibodies missing the light chain. These so-called heavy chain antibodies contain only a single variable domain (VHH) and two constant domains (CH2 and CH3), but still have a functional antigen binding site. (6) (Figure 1) By proteolytic cleavage it is possible to isolate the VHH domain harboring the full antigen-binding capacity of the original heavy chain antibody. These VHH domains with their unique structural and functional properties form the basis of a new generation of therapeutic antibodies called nanobodies. We believe that the nanobody technology is a good platform to develop TNFR1 specific inhibitors because nanobodies combine the advantages of conventional antibodies with important features of small molecules. Like conventional antibodies, nanobodies show high target specificity and affinity and low immunogenicity. And, like small molecules, they can easily reach hidden epitopes or receptor cavities. Nanobodies are highly soluble and extremely stable allowing passage through the gastrointestinal tract. (7) This offers the potential of oral administration

in the form of pills to treat inflammatory bowel diseases, colon cancer and other disorders of the gut. Furthermore, nanobodies are cheap and easy to produce in almost all prokaryotic and eukaryotic hosts including bacteria and yeast. (8, 9) They are encoded by single genes, which allow the design of modular proteins by combining nanobody building blocks with each other or with other molecules. (10) Nanobodies generally display a short serum half-life, but they can be generated to target abundant serum proteins, like albumin, having an inherently long serum half-life. (11) Fusion of such nanobodies to therapeutic compounds like TNFR1 targeting nanobodies provides a way to extend the serum half-life of the therapeutic. Using this technology, we wish to develop a new generation of TNF signaling blockers that will cause fewer side effects, yet still maintain high efficacy in the treatment of immune diseases.

3.2. Results

3.2.1 Generation of human TNFR1 nanobodies

TNFR1 nanobodies were generated in collaboration with the VIB Nanobody Service Facility by immunization of an alpaca by six consecutive injections of 130 μg recombinant human TNFR1. On day 37, anti-coagulated blood was collected for the analysis of the immune response and the isolation of peripheral blood lymphocytes. The mRNA coding for heavy chain antibodies was isolated from the lymphocytes and amplified by RT-PCR. The nanobody gene fragments were cloned into the phage display vector pHEN4 and a gene library containing several millions of clones was produced. Screening was done by multiple rounds of panning with recombinant human TNFR1 to identify antigen binding clones. (12) Eight TNFR1 specific nanobodies were selected and isolated. (Figure 2) Nanobodies nb19, nb20, nb96, nb107 and nb9 are very similar and their sequences suggest that they are from clonally-related B cells resulting from somatic hypermutation or from the same B cell but diversified due to PCR error during library construction. Nanobodies nb23, nb 70 and nb 22 differ more and most likely belong to distinct families. Therefore, the 8 different nanobodies are thought to belong to 4 different groups.

A

```
                    *        20        *        40        *        60        *        80        *
4TNFR19  : QVQLQESGGGVVQPGGSLTLSCTRTGLTPSTGAVGWYRQAPGKKCELVSYITIPSGRTTYTDSVKGRFAISRDKAKNTVFLQMNSLKPEDTALYYCG :  97
4TNFR20  : QVQLQESGGGVVQPGGSLTLSCTRTGLTASTNAVGWYRQAPGKKCELVSYITIPSGRTTYADSVKGRFAISRDKAKNTVFLQMNSLKPEDTALYYCG :  97
3TNFR96  : QVQLQESGGGVVQPGGSLTLSCTRTGFTASTNAVGWYRQGPGKKCEWVSYMTIPSGRTTYADAVKGRFAMSRDKAKSTVFLQMNSLKPEDTAVYYCG :  97
3TNFR107 : QVQLQESGGGVVQPGGSLTLSCTRTGFTASTNAVGWYRQGPGKKCEWVSYMTIPSGRTTYADAVKGRFAMSRDKAKSTVLLQMNSLKPEDTAVYYCG :  97
4TNFR9   : QVQLQESGGGVVQPGGSLTLSCTRTGFTASTNAVGWYRQAPGKKCELVSYITIPSGRTTYADAVKGRFAISRDKAKNTVFLQMNSLKPEDTAVYYCG :  97
3TNFR23  : QVQLQESGGGLVQPGGSLRLSCAASGRTFSYYTMAWFRQAPGKEREFVASISWSGGITYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCA :  97
3TNFR70  : QVQLQESGGGLVQAGGPLRLSCAASVRTFSSYAMGWFRQAPGKEREFVAAISWSGGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCA :  97
4TNFR22  : QVQLQESGGGLVQSGGSLRLSCRASGFTFSHYPMSWYRQAPGKKRELVAGI-YTDGSTNYADSVKGRFTISRDNAKNTVYLQMSSLKPEDTAVYYCR :  96

           100        *       120
4TNFR19  : DVPYSTIQAMCTDDGPWGQGTQVTVSS
4TNFR20  : DVPYSTIQAMCTDDGPWGQGTQVTVSS
3TNFR96  : DVPFSTLPAMCTNDGPWGQGTQVTVSS
3TNFR107 : DVPFSTLPAMCTNDGPWGQGTQVTVSS
4TNFR9   : DVPFSTLPAMCTNDGPWGQGTQVTVSS
3TNFR23  : AR----KEPGSAYFGSWGQGTQVTVSS
3TNFR70  : VL----HEDLYEYD-YWGQGTQVTVSS
4TNFR22  : R---------SMVSVYWGQGTQVTVSS
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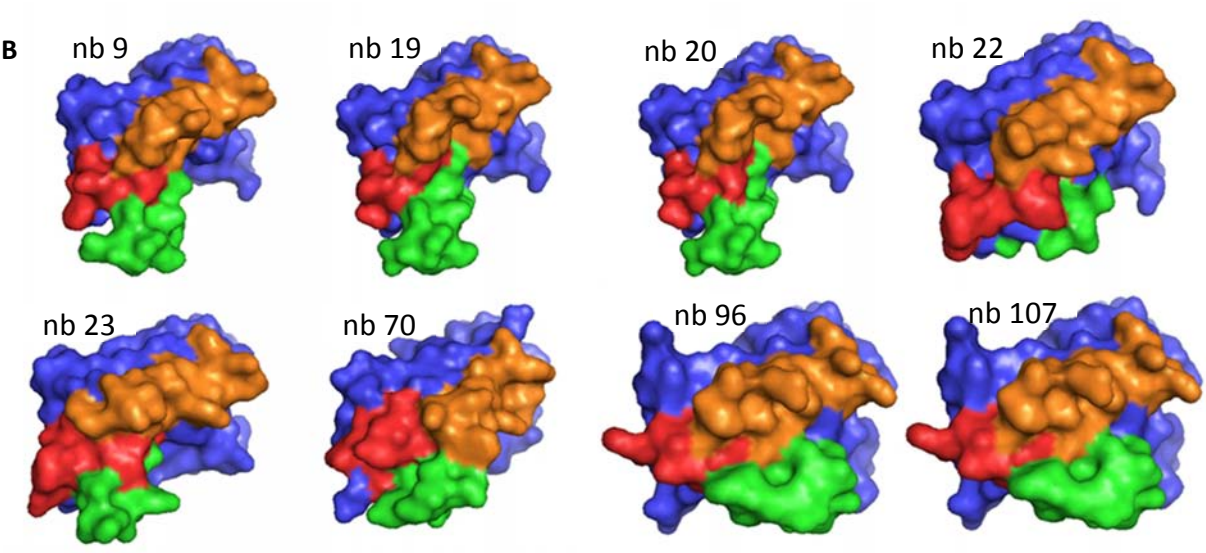


Figure 2. Primary and tertiary structure of the TNFR1 specific nanobodies. A. Alignment of the amino acid sequences of the TNFR1 nanobodies. The complementary determining regions (CDRs) are shown in boxes. Amino acids in red indicate the differences between nanobodies compared to the sequence of nb 19. B. Molecular modeling of the TNFR1 nanobodies using SWISS MODEL with 3P0G as a template. CDR1, CDR2 and CDR3 are shown in red, orange and green, respectively.

3.2.2 Expression and purification of human TNFR1 nanobodies

The eight TNFR1 nanobodies were successfully recloned into the pHEN6c vector, which introduces an N-terminal pelB leader sequence which directs the nanobody to the periplasmic space and a C-terminal His_6-tag for detection and purification, and transformed into WK6 cells. These cells will produce soluble nanobodies upon induction with IPTG. The expressed nanobody proteins, all having the expected size of about 15 kDa (table 1), were extracted from the periplasmic space and visualised by Coomassie staining on SDS-PAGE. (Figure 3) The same proteins were detected by Western blot using a specific anti-His-tag antibody. (Figure 4) For initial candidate selection studies, the nanobodies were purified with a His GraviTrap column followed by dialysis against PBS. For further *in vitro* and *in vivo* studies, the nanobodies were purified in collaboration with the VIB Protein Service Facility by Nickel affinity chromatography, followed by ion exchange and gel filtration. The yield of the pure TNFR1 nanobodies nb 9, nb 19, nb 22, nb 70, nb 96 and nb 107 was 17.5 mg, 28.25 mg, 17.75 mg, 2 mg, 25.75 mg and 46 mg respectively per liter culture. Nanobody nb70 has a very low yield, even when the bacteria are grown in large quantities in a fermentor (25.77 mg per 50 l culture).

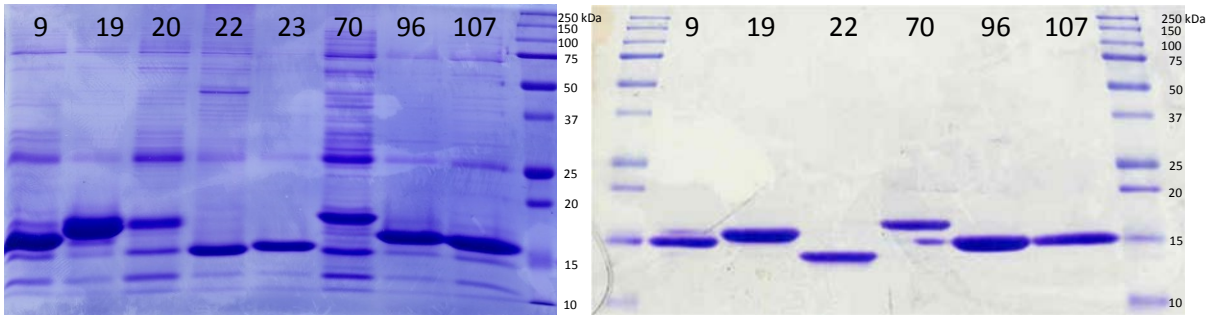
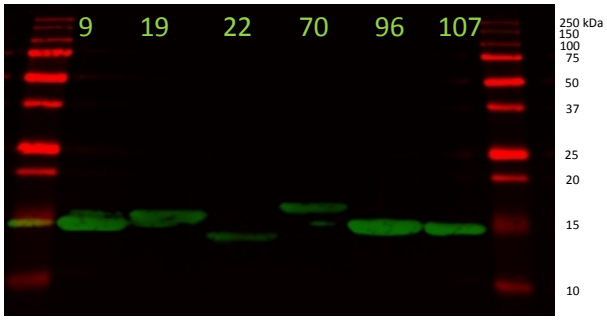


Figure 3. Analysis of nanobody purity by Coomassie staining after PAGE. Left, nanobody purity after purification with His GraviTrap columns and subsequent dialysis against PBS. Right, nanobody purity after extensive purification by the VIB Protein Service Facility.



nb	MW	pI
nb 9	14.2	8.75
nb 19	14.2	8.38
nb 22	13.6	9.39
nb 70	14.2	6.64
nb 96	14.3	8.75
nb 107	14.2	8.41

Figure 4. (left) Western blot analysis of nanobody expression. Nanobody proteins were detected with a specific anti-His-tag antibody.

Table 1. (right) Nanobody properties. Theoretical MW and pI are computed using the ExPASy bioinformatics software.

3.2.3 Binding capacity of the human TNFR1 nanobodies

The binding capacity of the nanobodies can easily be assessed by TNFR1 detection using an ELISA assay. Recombinant human TNFR1 was immobilized on a 96 well plate and incubated with a serial dilution of the nanobodies. Nanobodies nb96 and nb107 have the highest affinities followed by nb70. Nanobodies nb22, nb19 and nb9 show a very low affinity comparable to the control nanobody raised against an irrelevant protein, namely β-lactamase. (Figure 5) The commercially available hTNFR1 Ab is not detected with the anti-His secondary Ab, what gives a biased view with a higher plateau. It is likely that nanobodies specific for human TNFR1 will cross react with mouse TNFR1 because these proteins show a 83 % amino acid identity. Cross reactivity with mouse TNFR1 would allow us to use the nanobodies in many TNF-induced mouse models. Unfortunately, none of the nanobodies was able to bind to recombinant mouse TNFR1. The nanobodies were not only species specific but also receptor specific as there was no cross reactivity with human TNFR2.

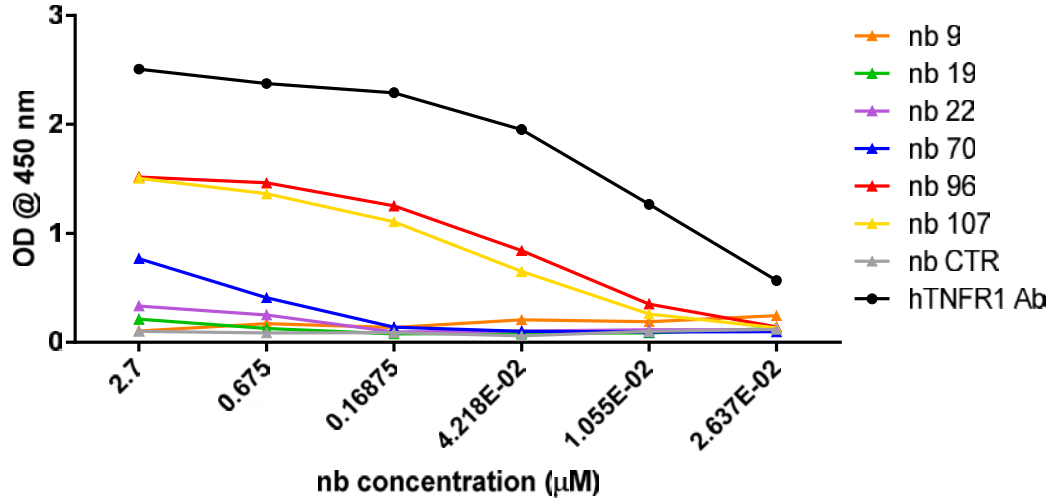


Figure 5. Binding capacity of TNFR1 nanobodies. The nanobody-TNFR1 binding was determined by anti-His and rat-anti-mouse HRP coupled IgG.

3.2.4 Inhibitory capacity of the human TNFR1 nanobodies

A nanobody capable of inhibiting TNFR1 would be a valuable tool in the therapy of autoimmune diseases in humans. To investigate the inhibitory capacity, we used HEK-2-Blue cells. These cells are stably transfected with an NF-kB/AP-1 inducible SEAP reporter gene. Upon stimulation with human TNF, the NF-kB/AP-1 promotor induces the transcription of SEAP, which will turn the substrate blue.

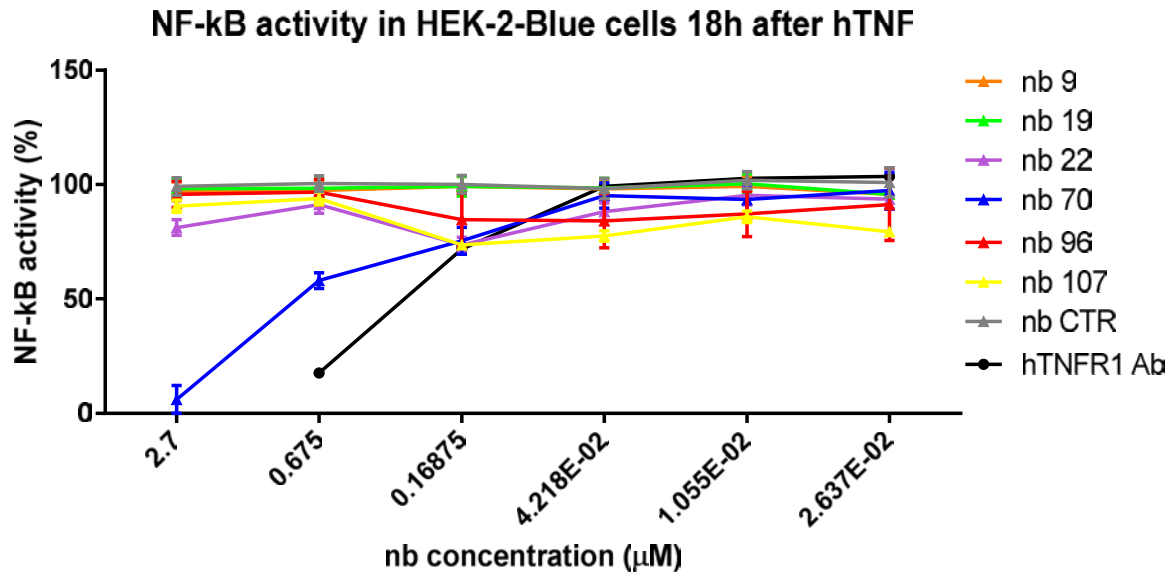


Figure 6. Inhibitory capacity of TNFR1 nanobodies. The TNFR1 inhibition was determined by NF-kB activity after TNF stimulation.

Pre-treatment with nanobodies can cause competition between TNF and the nanobodies for binding to TNFR1. A serial dilution of the nanobodies was added to the cells 30 minutes before TNF stimulation. Nanobody nb70 is the only nanobody that can inhibit the TNF-induced NF-kB activity. The inhibition is very strong and comparable to the hTNFR1 antibody. (Figure 6)

We conclude that nanobody nb70 is a potent inhibitor of TNF-induced NF-kB activation with a moderate binding affinity for hTNFR1. In addition, nb96 and nb107 have strong binding affinities to recombinant hTNFR1.

3.2.5 Docking of hTNFR1 nanobody nb70 to hTNFR1

To get an idea of the interaction of nanobody nb70 with hTNFR1, we performed a docking prediction using pyDockWEB in collaboration with Paco Hulpiau (IRC BioIT core facility). We got several possible confirmations of hTNFR1-nb70 binding of which the most probable are shown in Figure 7. In contrast to conventional antibodies, in which the antigen binding site is often a flat surface, a cavity or a groove, the CDR3 region of nanobodies possess the extraordinary capacity to form long fingerlike extensions that can easily reach hidden epitopes or receptor cavities. (13) In confirmation 1, nb70 binds with its CDR3 region to hTNFR1, but the paratope is most likely situated too close to the cell surface. In confirmation

2, nb70 binds with its CDR2 region into a cavity of hTNFR1. However to my opinion, confirmation 3 appears to be the most likely interaction. Here, nb70 binds with its CDR3 region into the cavity of hTNFR1, thereby seemingly blocking the binding of hTNF to the receptor. However, the real interaction should be deduced from crystallographic studies.

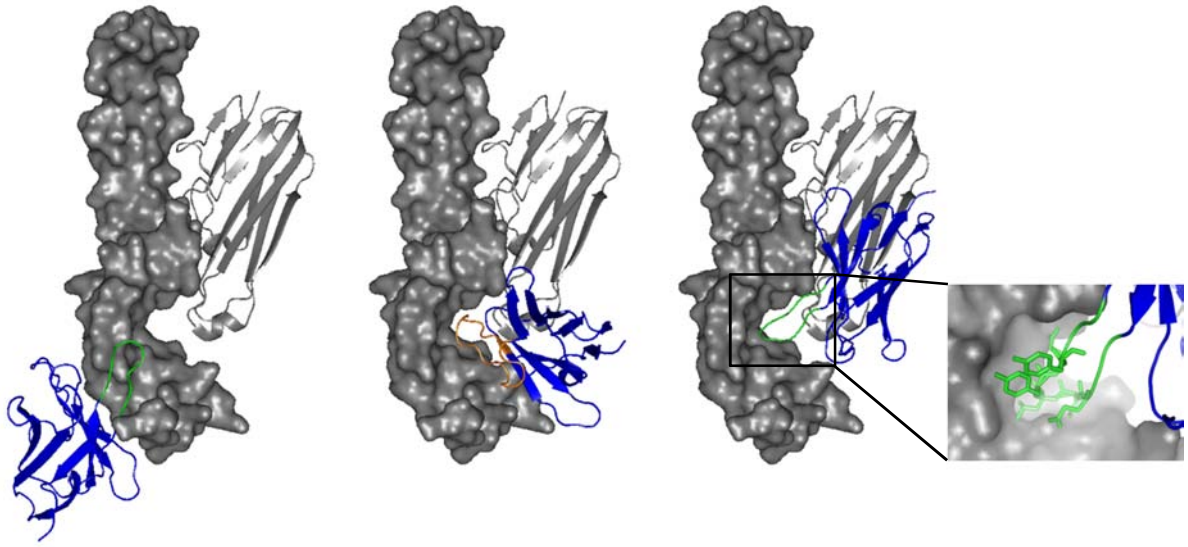
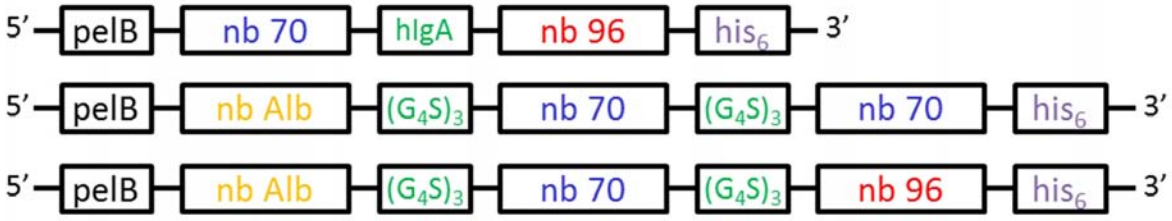


Figure 7. Docking prediction of hTNFR1 (PDB1EXT) with nb70 (based on PDB3P0G) using pyDockWEB. (14, 15) Representation of the three most probable conformations and close-up of conformation 3. hTNFR1-hTNF binding is shown in gray, nb70 is shown in blue with CDR2 in orange and CDR3 in green.

3.2.6 Generation of multimeric hTNFR1 nanobodies

In order to use the nanobodies *in vivo*, we made multimeric nanobodies to improve the half-life. By combining the inhibitory nb70 with the best binding nb96 and a nanobody against human albumin we wanted to improve the pharmacokinetics and -dynamics as well as the inhibitory capacity. The different nanobodies were coupled to each other using a cloning strategy introducing a linker. Several linkers can be used, e.g. the human IgA1 hinge (Ser-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Thr-Pro-Pro), the llama IgG2C hinge (Ala-His-His-Ser-Glu-Asp-Pro-Ser-Ser-Lys-Ala-Pro-Lys-Ala-Pro-Met-Ala) or the $(G_4S)_3$ linker (Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser). We generated a dimeric nb70-96 coupled with the rigid human IgA1 linker and trimeric nbAlb-70-96 and nbAlb-70-70, both coupled with the flexible $(G_4S)_3$ linker. In order to make the cloning feasible, we had to add a few extra amino acids, shown in black (Figure 8). These amino acids might alter the configuration of the protein and consequently the binding capacity.



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                                *         20        *         40        *         60        *         80        *
TNFR1 nbAlb-70-96 : LLLAAQPAMAQVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEWVSGISSLGDSTLYADSVKGRFTISRDNAKNTLY : 90
TNFR1 nbAlb-70-70 : LLLAAQPAMAQVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEWVSGISSLGDSTLYADSVKGRFTISRDNAKNTLY : 90
                          100       *         120       *         140       *         160       *         180
TNFR 70+96        : ----------------------------------------------------------MAQVQLQESGGGLVQAGGPLRLSCAASVRTFSS : 97
TNFR1 nbAlb-70-96 : LQMNSLKPEDTAVYYCTIGGSLNPGGQGTQVTVSSGGGGSGGGGSGGGGSQVQAQPAMAQVQLQESGGGLVQAGGPLRLSCAASVRTFSS : 180
TNFR1 nbAlb-70-70 : LQMNSLKPEDTAVYYCTIGGSLNPGGQGTQVTVSSGGGGSGGGGSGGGGSQVQAQPAMAQVQLQESGGGLVQAGGPLRLSCAASVRTFSS : 180
                                *         200       *         220       *         240       *         260       *
TNFR 70+96        : YAMGWFRQAPGKEREFVAAISWSGGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAVLHEDLYEYDYWGQGTQVTVSSSP : 193
TNFR1 nbAlb-70-96 : YAMGWFRQAPGKEREFVAAISWSGGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAVLHEDLYEYDYWGQGTQVTVSSGG : 270
TNFR1 nbAlb-70-70 : YAMGWFRQAPGKEREFVAAISWSGGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAVLHEDLYEYDYWGQGTQVTVSSGG : 270
                          280       *         300       *         320       *         340       *
TNFR 70+96        : STPPTPSPSTPPASQVQLQESGGGVVQPGGSLTLSCTRTGFTASTNAVGWYRQGPGKKCEWVSYMTIPSGRTTYADAVKGRFAMSRDKAK : 19
TNFR1 nbAlb-70-96 : GGSGGGGSGGGGS-QVQLQESGGGVVQPGGSLTLSCTRTGFTASTNAVGWYRQGPGKKCEWVSYMTIPSGRTTYADAVKGRFAMSRDKAK : 359
TNFR1 nbAlb-70-70 : GGSGGGGSGGGGS-QVQLQESGGGLVQAGGPLRLSCAASVRTFSSYAMGWFRQAPGKEREFVAAISWSGGSTYYADSVKGRFTISRDNAK : 359
                                *         380       *         400       *
TNFR 70+96        : STVFLQMNSLKPEDTAVYYCGDVPFSTLPAMCTNDGPWGQGTQVTVSSHHHHHH
TNFR1 nbAlb-70-96 : STVFLQMNSLKPEDTAVYYCGDVPFSTLPAMCTNDGPWGQGTQVTVSSHHHHHH
TNFR1 nbAlb-70-70 : NTVYLQMNSLKPEDTAVYYCAVLHEDLYEYDYWGQGTQVTVSS-----HHHHHH
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Figure 8. Alignment of the amino acid sequences of multimeric TNFR1 nanobodies. Nanobody nbAlb is shown in yellow, nb70 in blue, nb96 in red, the linker in green, the his_6-tag in purple and extra AAs in black.

3.2.7 Expression and purification of multimeric hTNFR1 nanobodies

These multimeric nanobodies were expressed in *E.coli* and purified with high purity by the VIB Protein Service Facility. However, the yield of nb70-96, nbAlb-70-96 and nbAlb-70-70 (3.5 mg/l, 0.026 mg/l and 0.0044 mg/l, respectively) was much lower than that of the single nanobodies, probably due to the sequence of nb70. Therefore we decided to move to the yeast *Pichia pastoris* for the production of the trimeric nanobodies. For this purpose we had to reclone the genes into the pAOXZalfaHC vector, which introduces an N-terminal alpha factor secretion signal which allows expression in the yeast culture supernatant. In *Pichia*, the yield was substantially higher than in *E.coli*, namely 7.17 mg/l and 0.43 mg/l for nbAlb-70-96 and nbAlb-70-70 respectively. The purified nanobody proteins were analysed by Coomassie staining on SDS-PAGE (Figure 9) and Western blot using a specific anti-His-tag antibody.

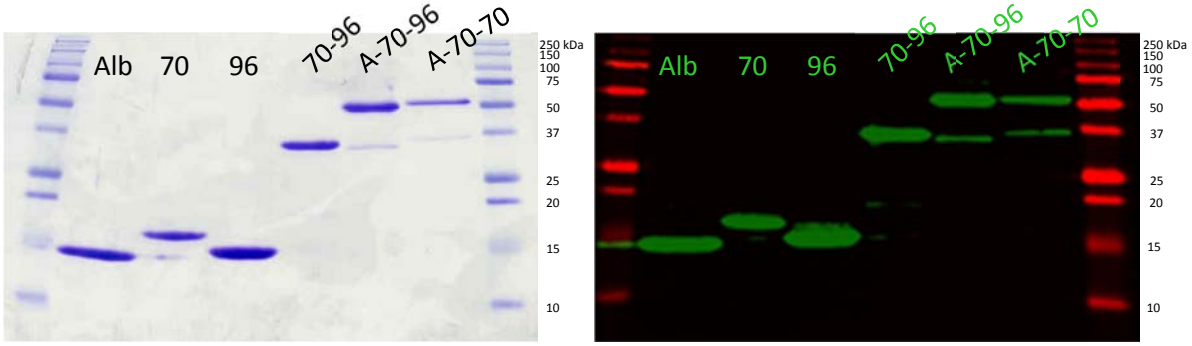

Figure 9. Analysis of nanobodies by Coomassie staining (left) and Western blot (right) after extensive purification by the VIB Protein Service Facility.

3.2.8 Binding capacity of multimeric hTNFR1 nanobodies

The binding capacity of nb70-96 (K_D 0.0556 nM) is much better than that of the individual nanobodies (K_D 8.91 nM and 4.95 nM respectively). However, the trimeric nanobodies show an intermediate binding capacity (K_D 0.110 nM and 0.263 nM), presumably due to the addition of the nanobody against albumin. (Figure 10A) The trimeric nanobodies show

sufficiently high (cross-reactive) binding to mouse serum albumin (Figure 10B), what makes them particularly suitable for *in vivo* use.

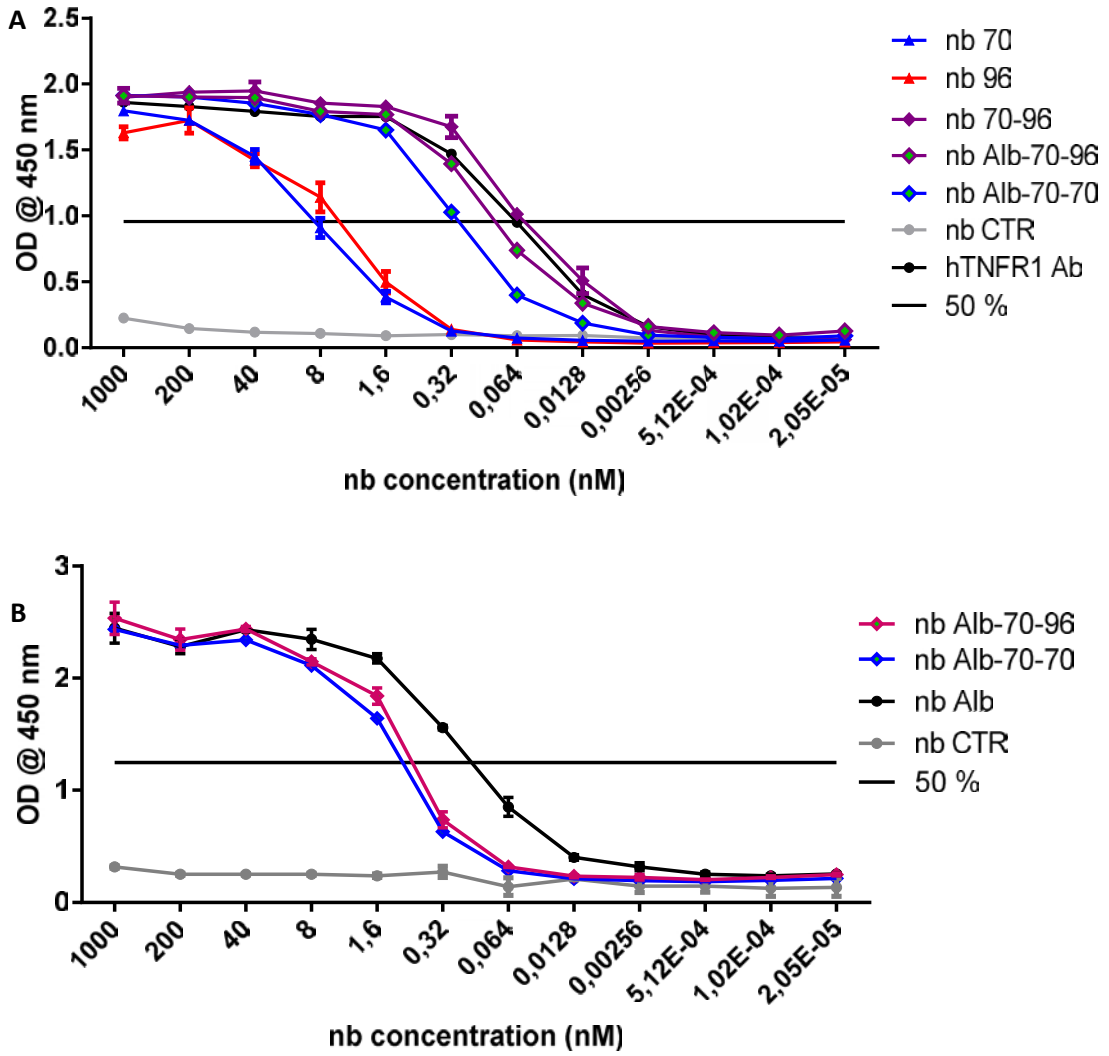

Figure 10. Binding capacity of TNFR1 nanobodies. The nanobody-TNFR1 binding (A) and the nanobody-albumin binding (B) were determined by ELISA to hTNFR1 and to mouse albumin and resolved by anti-His and rat-anti-mouse HRP coupled IgG.

3.2.9 Pharmacodynamics of multimeric hTNFR1 nanobodies

Next to the ELISA assay, we used the surface plasmon resonance technique of a Biacore system for the determination of the binding capacities of the nanobodies in collaboration with Nick Devoogth (VUB). This technique provides a more sensitive and accurate analysis of the K_D. Recombinant human TNFR1 was coupled to a CM5 sensor chip and a serial dilution of the nanobodies was run over the chip. The K_D value can be detemined by calculating K_{on}/K_{off} where K_{on} is based on the association rate of the nanobody to the chip while K_{off} is the dissociation rate at which the nanobody is released when the nanobody flow was changed to running buffer. All tested nanobodies showed dose-dependent binding curves for all dilutions and the calculated K_D values have the same proportions as determined with ELISA. (Table 2) The affinity of nb96 is clearly better than nb70, but both of them have a relatively

fast dissociation rate. The dimeric nanobody nb70-96 and trimeric nbAlb-70-96 and nbAlb-70-70 are disscociating a little slower, reflecting in a better K_D. (Figure 11)

In contrast to the flat plateau of equilibrium after a steep incline for nb96, the plateau of nb70 is not entirely flat, but rises a little. This can point to a two state model in which the conformation of the nanobody changes after binding to TNFR1. Calculating the K_D according to a 1:1 model gives a value of 38.71 nM while a two state model gives a value of 50.59 nM, which is presumably a more accurate result.

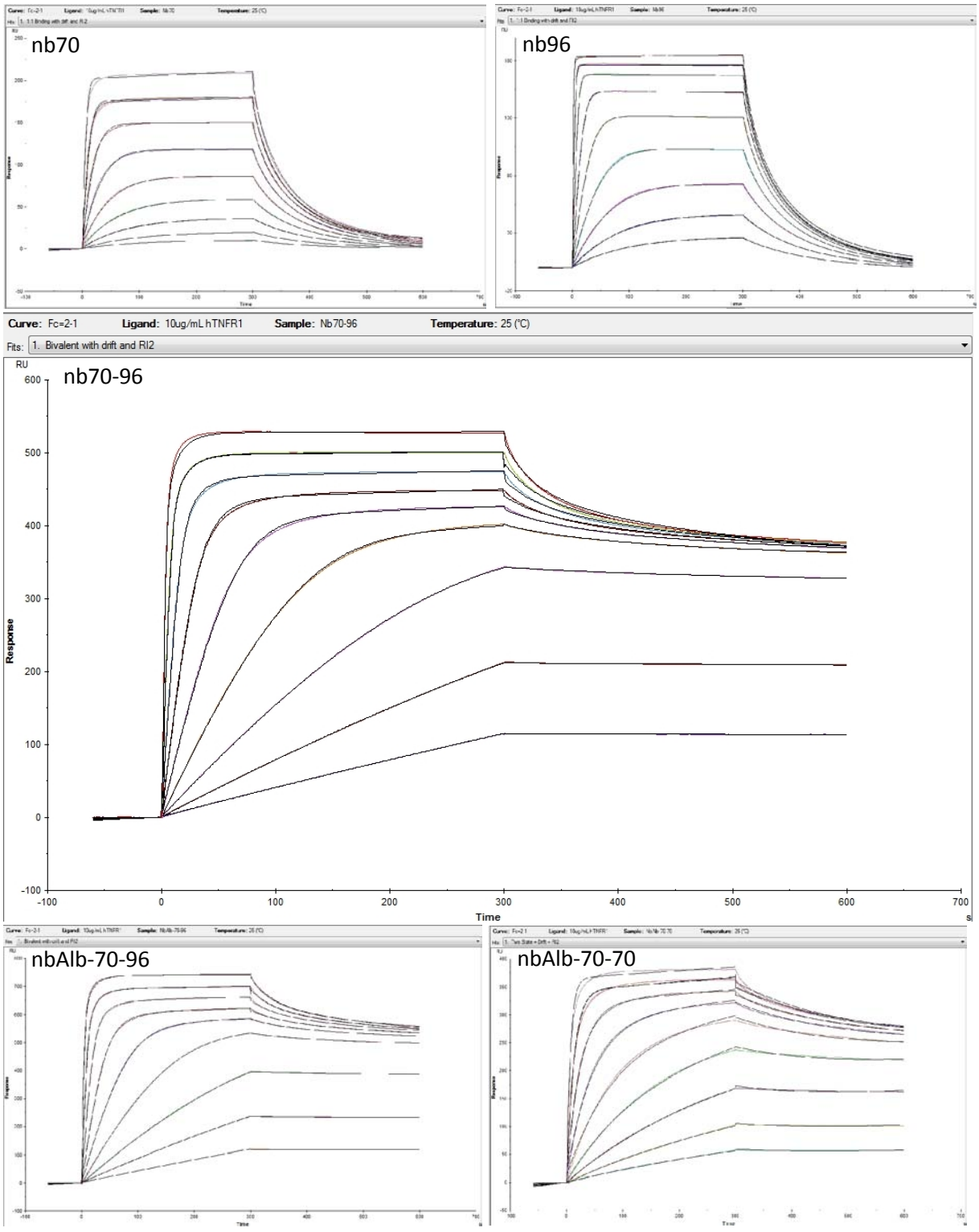

Figure 11. Biacore analysis of the interaction between human TNFR1 and nanobodies. The response is expressed in resonance units (RU). Several dilutions of the separate nanobodies were run over a sensorchip immobilized with hTNFR1. At t=0s, the nanobodies were sent over the chip, allowing determination of the K_{on} rate. The flow was stopped at t=300s and switched back to running buffer. The K_{off} rate could be determined as the rate at which the nanobody is released. The K_D can be calculated as $K_{on/Koff}$.

Next to the determination of binding affinity, the surface plasmon resonance technique can also be used to perform competition experiments. We found that nb70 and nb96 do not compete with each other for binding to hTNFR1, indicating that they recognize and bind two distinct epitopes and explaining the higher affinity of the combined nanobody nb70-96. (Figure 12)

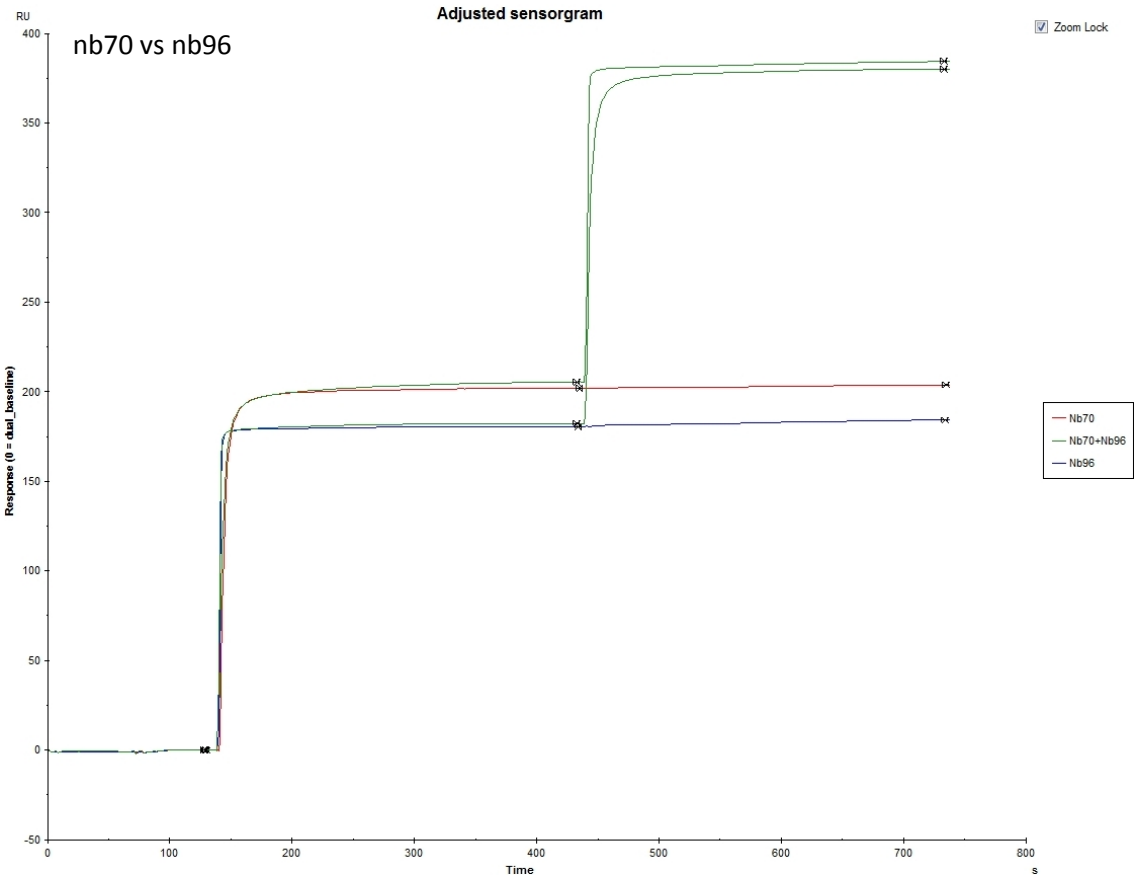

Figure 12. Competition of nanobody nb70 with nanobody nb96. nb70 (red) and nb96 (blue) were run separately or 450 s later simultaneously (green) over the chip. The additional gain in RU indicates that the two nanobodies do not interfere with each other when binding to hTNFR1.

On the other hand, we found that nb70 goes in competition with hTNF by inhibiting the binding to its receptor. The inhibition is even more pronounced in the dimeric and trimeric nanobodies. However, the competition is most apparent when the nanobodies are given before hTNF, in a prophylactic setting. When hTNF is first bound to the receptor, the nanobodies are less able to go in competition with it. (Figure 13)

The sensorgram of nanobody nbAlb-70-96 strongly resembles the one of a monoclonal antibody against TNFR1 (R&D MAB225, result not shown), indicating that it has equivalent inhibitory qualities.

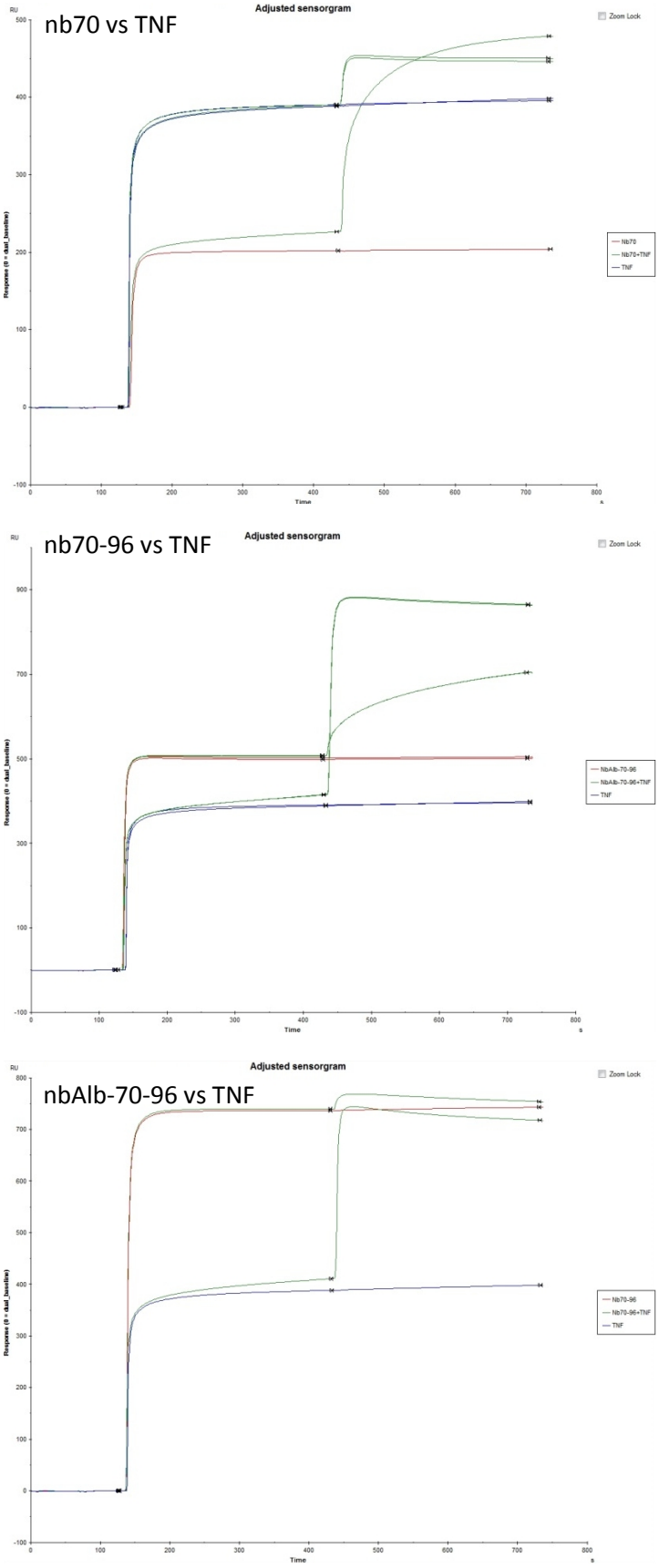

Figure 13. Competition of nanobodies with TNF. The nanbody (red) and TNF (blue) were run separately or 450 s later simultaneously (green) over the chip.

3.2.10 Inhibitory capacity of multimeric hTNFR1 nanobodies

The additional amino acids, the N-glycosylation and the combination with an Albumin nanobody might also influence the TNFR1 inhibition. The inhibitory capacity of the dimeric nb70-96 (IC_{50} 0.926 μM) is slightly better than that of nanobody nb70 (IC_{50} 1.34 μM). However, the trimeric nanobody nbAlb-70-96 is still a lot better with an IC_{50} of 0.395 μM. (Figure 14)

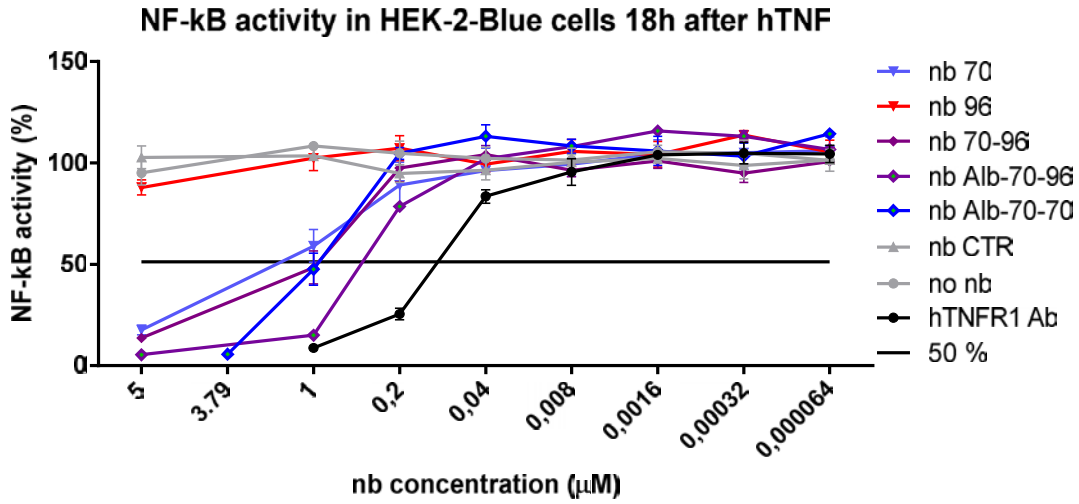

Figure 14. Inhibitory capacity of TNFR1 nanobodies. The TNFR1 inhibition was determined by NF-kB activity after TNF stimulation.

We conclude that the transition from *E.coli* to *P.pastoris* did not influence the binding and inhibitory capacities of the trimeric nanobodies. The dimeric nanobody nb70-96 has a very strong binding affinity to recombinant hTNFR1, but also the trimeric nanobodies show high binding affinities. The trimeric nanobody nbAlb-70-96 is the best inhibitor of TNF-induced NF-kB activation with high binding capacity to hTNFR1 and Albumine. This nanobody will be further investigated *in vitro*, but also *in vivo*.

nb/Ab	MW	pI	K_D Biacore	IC_{50}
nb 70	14.2	6.64	50.59 nM	1.34 μM
nb 96	14.3	8.75	11.16 nM	-
nb 70-96	28.9	8.16	3 nM	0.926 μM
nb Alb-70-96	43.1	8.35	5 nM	0.395 μM
nb Alb-70-70	43.0	8.41	9.368 nM	0.926 μM
hTNFR1 Ab	160.0	NA	NA	0.096 μM

Table 2. Nanobody properties. Theoretical MW and pI are computed using the ExPASy bioinformatics software.

3.2.11 Pharmacokinetics of hTNFR1 nanobody nbAlb-70-96

To be able to use the TNFR1 nanobodies as *in vivo* therapeutic agents, we need to know how they will be affected after administration. Drugs injected *iv* are removed from the plasma through the mechanisms of absorption and distribution to tissues as well as elimination. The

resulting decrease in plasma concentration follows a biphasic pattern. The initial phase is primarily attributed to distribution from the central circulation to the peripheral tissues, which reaches a pseudo equilibrium of drug concentration. The following phase represents a gradual decrease attributed to drug metabolism and excretion. (16) We injected 100 μg nanobody nbAlb-70-96 *iv* in 8 C57BL/6 mice and followed the elimination by taking 100 μl blood by eye puncture at several time-points. The concentration of the nanobody was determined by ELISA. Serum was added to 96 well plates with immobilized recombinant human TNFR1 and detected with anti-His and rat-anti-mouse HRP coupled IgG. We see a distribution phase until 8h after injection followed by elimination with a half-life $T_{1/2}$ of more or less 24h. (Figure 15)

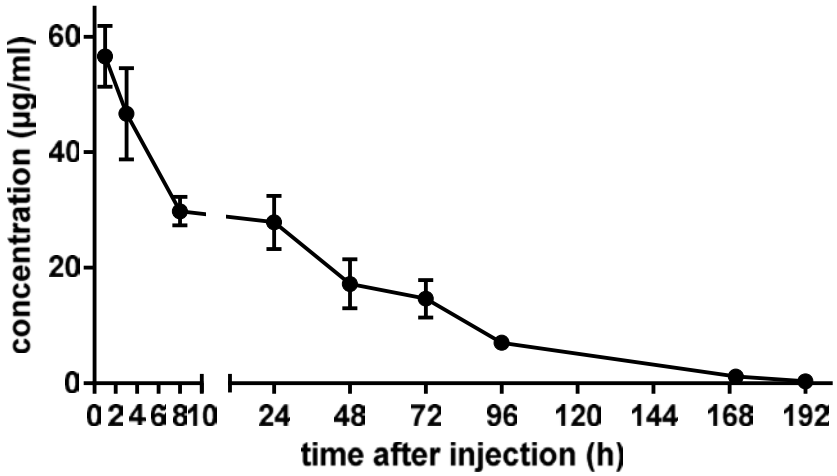

Figure 15. Pharmacokinetics of trimeric hTNFR1 nanobody nbAlb-70-96. Nanobody concentration in the serum of 8 C57BL/6 mice after *iv* injection of 100 μg.

3.3. Conclusion

We have generated a functional human TNFR1 specific nanobody. This trimeric nanobody consists of one nanobody against TNFR1 (nb96) which is shown to strongly bind to TNFR1, another nanobody against TNFR1 (nb70) which has lower affinity but strongly inhibits TNFR1 signaling, and one nanobody against serum albumin. This format was chosen to prolongue half-life and increased binding and inhibition due to bivalency. TNFR1-selective inhibition by nanobody nbAlb-70-96 results in blocking of the inflammatory signaling pathway of TNFR1, shown *in vitro* by inhibition of NF-kB induction. A pharmacokinetic study revealed that the first 8h after injection, the nanobody is distributed to the tissues, followed by elimination with a half-life $T_{1/2}$ of more or less 24h. Dosing and injection schedules can be adjusted to reach a steady state level of the nanobody in serum or tissue that is adequate for optimal neutralization of TNFR1. Further *in vivo* experiments should be performed in hTNFR1 KI mice or other humanized models. We want to test the nanobody in inflammatory models such as TNF-induced lethality and LPS-induced endotoxemia. Later, nbAlb-70-96 may be tested as a therapeutic agent especially for those diseases against which currently marketed anti-TNF therapeutics have failed or even exacerbate disease progression, including multiple sclerosis.

3.4. Material and methods

Generation of human TNFR1 nanobodies. Human TNFR1 nanobodies were generated with the help of the VIB Nanobody Service Facility. An alpaca was injected subcutaneously on days 0, 7, 14, 21, 28, 35 with about 130 μg of recombinant human soluble TNFR1 (Peprotech) per injection. On day 37, anti-coagulated blood was collected for the analysis of the immune response and for the preparation of lymphocytes. Total RNA from peripheral blood lymphocytes was used as a template for first strand cDNA synthesis. Using this cDNA, the VHH encoding sequences were amplified by PCR, digested with PstI and NotI, and cloned into the PstI and NotI sites of the phagemid vector pHEN4. Four consecutive rounds of panning were performed on solid-phase coated human TNFR1 (10 μg/well). Totally, 332 individual colonies were randomly selected and analyzed by ELISA for the presence of antigen specific VHHs in their periplasmic extracts. Sequencing of VHH genes from 34 positive colonies identified 8 different nanobodies originating from 4 clonally-unrelated B-cells. The clonally-related nanobodies recognize the same epitope but might differ in characteristics such as affinity, stability, etc. The specificities of these nanobodies were again confirmed by ELISA in an independent experiment using soluble nanobodies.

Production of human TNFR1 nanobodies. The 8 different nanobody encoding sequences were amplified by PCR, digested with PstI and BstEII, and cloned into the PstI and BstEII sites of the expression vector pHEN6c. The plasmids were transformed into WK6 cells and screened for the presence of a correct nanobody sequence. The positive colonies were grown for expression of the nanobodies after addition of 1 mM IPTG to the medium. For the initial experiments, the nanobodies were purified using His GraviTrap colums (GE Healthcare) followed by an overnight dialysis against PBS to remove buffer, salts and imidazole with a Slide-A-Lyzer cassette (Thermo Scientific) with 3500 kDa molecular weigth cut-off. For further *in vitro* and *in vivo* studies, the nanobodies were purified by Nickel Sepharose affinity chromatography, followed by ion exchange and gel filtration with the help of the VIB Protein Service Facility.

Coomassie staining and Western blot. Nanobody proteins (5 μg) were separated with 15 % SDS-PAGE after adding loading dye with β-mercaptoethanol and 5 min incubation at 95 °C. The Precision Plus Protein All Blue standard (Biorad) was loaded as a molecular weight marker. For Coomassie, the gel was colored with Coomassie brilliant blue from and destained in a solution of 30 % methanol and 7 % acetic acid. For Western blot, the gel was transferred to a nitrocellulose membrane (0.2 μm) by semi-dry blotting at 0.8 mA/cm^2 gel for 2 h. The membrane was blocked with 2 % BSA in TBS overnight at 4 °C. The next day, the membrane was incubated for 1 h at RT with 1/1000 mouse anti-His tag Ab (Abd Serotec) and washed with Tween-20 containing TBS. Subsequently, the membrane was incubated with 1/10000 anti-mouse IRDye 800 (Westburg) and washed TBS-Tween, TBS and distilled water. Visualisation was done at a wavelength of 800 nm with the Odyssey Infrared imaging system (Li-Cor Biosciences).

ELISA binding assay. A flat bottom 96 well (Nunc MaxiSorp) was coated with 100 ng recombinant human TNFR1 (Peprotech, 310-07) and incubated overnight at 4 °C. The next day, the plate was blocked with 1 % BSA for 2 h at RT and washed with PBS. The nanobodies were added at a final concentration of 1 ng/μl and 1/3 diluted. The monoclonal antibody MAB225 against human TNFR1 (R&D systems) was used as a positive control and a nanobody against β-lactamase was used as a negative control. After one hour of incubation, the plate was washed twice with Tween-20 washing buffer and incubated with 1/1000 mouse anti-His antibody (AbD Serotec). One hour later, the plate was washed and incubated with 1/2000 anti-mouse IgG-HRP (GE Healthcare). After washing, substrate was added for 30 minutes and the reaction was stopped with 1M H2SO4. The OD was read at 490 nm with a reference wavelength of 595 nm.

TNFR1 inhibition assay. HEK-2-Blue cells (Invivogen) were seeded at 20000 cells/ml DMEM detection medium in 96 well plates and incubated for 24 h at 37 °C. The next day, a ¼ nanobody dilution starting from 1 mg/ml was added to the cells and incubated for 30 min at 37 °C. Then, 100 IU/ml hTNF was added and after overnight incubation at 37 °C, the OD was measured at 655 nm.

Cloning multivalent TNFR1 nanobodies. The DNA coding for the first nanobody was amplified by PCR using primers that introduce a C-terminal linkerchain. This PCR fragment and the vector containing the second (and third) nanobody were digested with the appropriate restriction enzymes and ligated. The plasmids were transformed into WK6 cells and screened for the presence of a correct sequence. For recloning to pAOXZalfaHC the nanobody gene in pHEN6c was amplified by PCR and digested with XhoI and HindIII. The pAOXZalfa vector was once digested with XhoI and XmaI and once with HindIII and XmaI and both fragments were ligated with the digested nanobody gene in a three point ligation.

Biacore T200 affinity determination. Recombinant human TNFR1 (Peprotech 310-07, 10 μg/ml in 10 mM sodium acetate pH4) was covalently attached via amino coupling to a sensor chip CM5, causing a signal increase of 581 RU. Nanobodies (500 nM and a ½ serial dilution) were perfused over the chip in HBS running buffer (20 mM HEPES pH7.4, 115 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.4 mM K_2HPO_4) at a flow rate of 30 μl/min for 300 s after which a plateau phase was reached and further increase in signal of about 100 RU. This was followed by a dissociation step with running buffer (30 μl/min, 600 s). Regeneration was achieved by using a 25 MM NaOH/500 mM $NaCl_2$ buffer (30 μl/min, 30 s) after which the chip was washed with running buffer (30 μl/min, 300 s) for stabilization. The specificity of the signal was corrected for non-specific binding measured in a non-coupled control channel. Association and dissociation rate constants were calculated by curve fitting, using the Biacore evaluation software and assuming an 1:1 model.

Biacore T200 epitope mapping. The same chip with coupled human TNFR1 as above was used for the epitope mapping experiment. In a first step, a single nanobody was perfused over the chip in running buffer (1 μM, 5 μl/min, 240 s). In a second step, a combination of

the first single nanobody together with a second single nanobody (1 μM) was perfused over the chip for another 240 s. The chip was then regenerated by perfusion with 25 MM NaOH/500 mM NaCl2 (2 x 30 μl/min, 30 s) and washed for 300 s with running buffer (30 μl/min).

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CHAPTER IV

Discussion

1. Introduction

Inflammation is a normal response to tissue damage caused by an infection or irritation and leads to the release of pro-inflammatory cytokines, chemokines and other molecules by a variety of cell types. This results in a controlled local inflammatory and immune response and elimination of the initial stimulus. (1) However, if the immune system fails to remove pathogens from the local invasion site and these pathogens start entering the bloodstream, an uncontrolled systemic immune response (SIRS) can arise. SIRS triggers a widespread systemic inflammation, which leads to blood clots and impaired blood flow damaging organs by depriving them of nutrients and oxygen. In severe cases, one or more organs lose their function, blood pressure drops, the heart weakens and the patient goes into septic shock. Once this happens, multiple organs quickly fail and the patient can die. Every year, severe sepsis strikes about 18 million people worldwide of which 20 to 30 % die. (2) Sepsis is a major challenge in the intensive care unit, being one of the leading causes of death. A healthy and balanced immune system is therefore essential to defend our body against inflammation and infections.

Unfortunately, the immune system sometimes wrongly identifies endogenous proteins and tissues as 'foreign' and tries to destroy them. This leads to the development of autoimmune diseases such as rheumatoid arthritis, Crohn's disease, multiple sclerosis, type 1 diabetes and psoriasis, all of them causing severe symptoms with high costs for therapy. Collectively, these diseases affect up to 23.5 million Americans according to the National Institutes of Health (NIH), and the prevalence is rising.

Tumor necrosis factor (TNF), a powerful pro-inflammatory cytokine, is a central mediator in the pathology of SIRS and plays a key role in several inflammatory and autoimmune diseases. (3, 4) The diverse signaling cascades initiated by TNF lead to a range of cellular responses, including proliferation, differentiation, migration and cell death, as well as to inflammation. (5) TNF blocking agents such as Infliximab, Adalimumab, Golimumab, Etanercept and Certolizumab, therefore have gained a solid position in the top 10 of bestselling drugs worldwide. Etanercept and Adalimumab, for example, are successfully used to treat patients with rheumatoid arthritis for more than 10 years. (6, 7) Meanwhile, the indication field is extended by other forms of arthritis, psoriasis and inflammatory bowel diseases, illustrating the importance of TNF in inflammation and the need for anti-TNF therapy. (8) However, long-term use of these agents can cause significant side-effects, such as opportunistic infections (9) and the development of additional autoimmune diseases including lupus, type 1 diabetes, uveitis, multiple sclerosis, psoriasis, as well as lymphoma and leukemia. (10, 11) The pathophysiology of TNF as seen in chronic inflammation and autoimmune diseases and the problems encountered using anti-TNF agents suggest that the actions of TNF diverge on the level of the receptor. TNFR1, the main receptor for TNF, is primarily involved in inducing and maintaining inflammation, cell proliferation, cell survival and apoptosis (12) while the immunomodulating and disease-suppressive functions of TNF are more dependent on TNFR2. (13) Hence, more specific targeting of the pathological actions of TNF by selectively blocking TNFR1 is advised for the treatment of TNF-induced

chronic diseases. (12) In experimental autoimmune encephalomyelitis (EAE), for example, TNFR1 deficient mice are resistant, exhibiting reduced Th1 proliferation and cytokine production. (14) In contrast, TNF deficient and TNFR1R2 double deficient mice are unable to suppress the late autoimmune reactivity and show an abnormal prolongation and worsening of the disease, indicating that the immunosuppressive functions of TNFR2 are protective in the later phase of chronic EAE. (15) Therefore, treatment of EAE with a TNFR1 specific inhibitor is effective, showing a normal regression of anti-myelin reactivity and leaving TNFR2 mediated activation of Tregs intact (16), while clinical trials with anti-TNF failed in multiple sclerosis patients acquiring enhanced clinical symptoms. (17) This idea formed the basis of one part of my PhD.

The development of novel, more effective drugs for the treatment of inflammatory diseases requires, in the first place, a better understanding of the regulation of this receptor. Despite extensive knowledge on the TNFR1 signaling pathway, little is known about the regulation of TNFR1 expression, modifications, localization and processing. While it has been generally assumed that TNFR1 expression is constitutive and not inducible, the TNFR1 protein has been shown to be regulated by a number of post-transcriptional modifications. One mode of regulation is shedding of TNFR1 by TACE/ADAM17. (18) Inflammation-induced activation of TACE, resulting in an acute decrease in the number of receptor molecules on the cell surface, is believed to transiently desensitize cells to TNF. (19) In addition, the pool of soluble TNFR1 could also function as physiological attenuators of TNF activity by competing for the ligand with the cell surface receptors. (20) In humans, mutations affecting TNFR1 shedding have been linked with the development of TRAPS (TNF receptor-associated periodic syndromes). (21) These disorders are characterized by recurrent fevers and localized inflammation. Moreover, mice expressing a non-sheddable TNFR1 have higher levels of TNFR1 in most tissues and are therefore extremely sensitive to TNF-induced inflammation. (19) These findings suggest that the quantity of TNFR1 is strictly regulated and that it plays an important role in determining sensitivity to TNF.

We previously showed that SPRET/Ei mice, an inbred strain derived from *Mus spretus*, are highly resistant to the lethal effects of TNF and we believe that these mice could be useful to gain more insight in the TNF-induced pathology, in TNFR1 regulation and to discover new therapeutic possibilities. (22) The great genetic diversity between *Mus spretus* and *Mus musculus* has already been useful in the identification of genes that contribute to other phenotypes of SPRET/Ei such as resistance to LPS (23, 24), *Salmonella typhimurium* (25) and Influenza A (26). The resistance to TNF was shown to be linked to a locus on proximal chromosome 2 and another on distal chromosome 6. Since the TNFR1-encoding gene *Tnfrsf1a* is located on distal mouse chromosome 6 (125.3 Mb-60.55 cM), we believed that this trait might be associated with the regulation of TNFR1, and this idea formed the basis of another part of this PhD.

2. Results

2.1 TNFR1 expression is reduced in SPRET/Ei mice

Tnfrsf1a has long been an obvious candidate gene. Sequencing of the gene in SPRET/Ei and C57BL/6 revealed 12 amino acid variations of which 6 are totally unique for SPRET/Ei. However, no difference in TNF-binding efficiency was found and B.S^{chr6} consomic mice demonstrated that the TNFR1 of SPRET/Ei in C57BL/6 mice is still able to induce lethality. Inspired by the complete resistance of TNFR1+/- mice against TNF-induced lethality, we studied whether the TNF resistance of SPRET/Ei is linked to a quantitative effect at the TNFR1 locus. Indeed, we found that SPRET/Ei mice show greatly reduced TNFR1 protein levels, comparable to those of TNFR1+/- mice. Moreover, (BxS)F1 mice express similarly low TNFR1 protein levels as SPRET/Ei, indicating that the lower TNFR1 expression in SPRET/Ei is a dominant trait. Furthermore, the concentration of soluble TNFR1 in the serum was threefold lower in SPRET/Ei mice, suggesting that the differences measured in organs are not caused by increased TNFR1 shedding by TACE. Interestingly, the low TNFR1 protein levels are not correlated with TNFR1 mRNA levels, since we found equal TNFR1 mRNA levels in both SPRET/Ei and C57BL/6 mice. This suggests that the differential TNFR1 regulation in SPRET/Ei is attributable to post-transcriptional effects.

The ELISA used for determination of the TNFR1 protein levels makes us of a rat anti mouse TNFR1 capture Ab (R&D MAB425) and a biotinylated goat anti mouse TNFR1 detection Ab (R&D BAF425). Both antibodies are raised against the extracellular part of mouse TNFR1 (AA 22-212). In this region, four missense mutations between SPRET/Ei and C57BL/6 are located. This means that we cannot guarantee that the binding to TNFR1 of SPRET/Ei happens equally well as to TNFR1 of C57BL/6. To confirm the lower TNFR1 protein level of SPRET/Ei, we should use additional methods preferably without the use of antibodies. Unfortunately, our attempts to perform FACS analysis on primary macrophages and fibroblasts using biotinylated hTNF and slot blot analysis on liver extracts using distinct antibodies, gave inconclusive results probably due to the very low expression of TNFR1 in naive mice. Nevertheless, we assume that the antibodies do bind equally well to the TNFR1 of both strains since B.S^{chr6} congenic mice, expressing the SPRET/Ei receptor in a background of C57BL/6 show a normal TNFR1 expression level as in C57BL/6.

2.2 TNF resistance and lower TNFR1 expression in SPRET/Ei are causally related

To identify the genetic regions responsible for the low TNFR1 protein level in SPRET/Ei mice, we generated an interspecies backcross between (BxS)F1 and C57BL/6. The TNFR1 protein level was measured in the liver of 214 BSB offspring mice and a genomic scan was performed using polymorphic markers covering the entire genome. A QTL analysis using R/QTL software (27) revealed genetic linkage to a locus on proximal chromosome 2 (1-38 cM) and to a locus on distal chromosome 6 (40-70 cM). When the BSB population was separated into males and females, the linkage to chromosome 2 was found to be highly significant in males while the linkage to chromosome 6 was highly significant in females as well as in the total population.

Hypotheses about the underlying reason for this gender difference are difficult to make. However, the group of Xavier Montagutelli (Institut Pasteur Paris) reported a similar case of two QTLs that were only found in females and a third QTL that was present in both sexes. Further analysis using congenic mice harboring one of the QTLs or combinations of them in a highly susceptible C57BL/6 background revealed that the first QTL in females might be a spurious linkage, while the other two QTLs are both genuine QTLs controlling resistance to *Yersinia pestis* in both males and females of SEG/Pas. (28, 29) Hence, it is possible that QTLs are not detected in one of the gender-specific subpopulations, while QTLs with a highly significant Lod score are most likely true QTLs even if they are only found in one of the subpopulations.

Interestingly, the QTLs that we found for TNFR1 protein expression are similar to the ones that had been linked to TNF resistance in SPRET/Ei. (22) Moreover, SPRET/Ei and TNFR1+/- mice, both having half-maximal TNFR1 protein expression levels, show total resistance against TNF-induced lethality (30), suggesting that the two phenotypes are causally related. Since the gene encoding TNFR1 (*Tnfrsf1a*) is located in the QTL on distal chromosome 6 and the Lod scores of chromosome 6 for both TNF resistance and TNFR1 expression are higher than those of chromosome 2, we suggest that both traits are associated mainly with TNFR1 regulation.

2.3 Down-regulation of TNFR1 in SPRET/Ei mice is regulated *in trans*

Congenic mice are used to confirm and further characterize QTLs identified by linkage analysis. We therefore tried to develop a congenic strain harboring the proximal part of SPRET/Ei chromosome 2 and another strain harboring the distal part of SPRET/Ei chromosome 6 in a genomic background of C57BL/6. Unfortunately, the backcrosses to obtain B.S^{chr2} congenic mice failed due to breeding problems and at that time, the chromosome 2 of B.S^{chr6} congenic offspring was already fully C57BL/6. However, B.S^{chr2} congenic mice harboring the SPRET/Ei locus on chromosome 2 (2.23-71.17 cM) in a C57BL/6 background, had been generated in the past by Jan Staelens and offspring from the successive generations had been injected with a TNF dose ranging from 20 to 25 μg. In none of these experiments a correlation was found between the genotype and TNF lethality.

We could generate B.S^{chr6} congenic mice by selecting offspring that were homozygous C57BL/6 or SPRET/Ei for distal chr6, including a microsatellite marker intronic of *Tnfrsf1a* (D6Mit254). B.S^{chr6} SS mice showed a TNF survival rate approximately half that of SPRET/Ei mice, indicating that the SPRET/Ei TNFR1 region alone (in a C57BL/6 background) is also not sufficient to achieve resistance to TNF. Furthermore, liver TNFR1 protein levels were equal in B.S^{chr6}BB, BS and SS mice pointing to an *in trans* regulation of the TNFR1 protein expression in SPRET/Ei mice.

Although ideally, we should have generated bicongenic mice harboring both putative SPRET/Ei-derived resistance alleles in a susceptible C57BL/6 background, we can conclude that neither the QTL on chromosome 2 nor the QTL on chromosome 6 alone is able to restore complete TNF resistance when isolated in a C57BL/6 background. Moreover, our

data confirm the relation between TNFR1 protein expression and TNF resistance, both presumably regulated *in trans*. As an alternative for the bicongenic mice, we will inject B.S^{chr6} congenic mice with miR-511 or dexamethasone to study the potential synergistic effect of higher miR-511 levels and the distal part of chromosome 6 of SPRET/Ei isolated in a background of C57BL/6.

2.4 Identification of candidate genes

The identification of the genes underlying the effects on the TNFR1 regulation ideally requires a combination of approaches, including the genetic fine mapping of each putative QTL in bicongenic, congenic and subcongenic strains and a whole-genome expression study to detect differentially expressed genes in SPRET/Ei.

However, since we found a most remarkable correlation between TNF resistance and the TNFR1 protein expression, both being associated with the post-transcriptional regulation of TNFR1 *in trans*, we focused on microRNAs (miRNAs) that may have an influence on TNFR1. Moreover, the role of TACE in this story was ruled out since the concentration of TNFR1 in the serum was as low as in the organs, in SPRET/Ei compared to C57BL/6. miRNAs have emerged as key post-transcriptional regulators of gene expression, involved in diverse physiological and pathological processes. (31)

On the one hand, we searched for miRNAs that are predicted to have *Tnfrsf1a* as a target gene. Using several programs based on different algorithms, we were able to make a comparative analysis. On the other hand, we performed a microRNA expression profiling of 541 miRs on naive livers of C57BL/6 and SPRET/Ei. Combining the list of predicted miRNAs with the list of differential miRNAs, we made a shortlist of the 20 most interesting miRNAs that will be studied in detail. Beside functional analyses, we would like to identify their target sequences in *Tnfrsf1a* and other possible target genes, determine their conservation in the human genome and search for literature and ENCODE data regarding these miRNAs.

However, in the context of this thesis, we focused on miR-511 and miR-680, two predicted, differentially expressed miRNAs that are located within the regions that are linked with TNF resistance and TNFR1 protein expression.

miR-511 is located on 14 Mb-10.48 cM of chromosome 2, in intron 5 of the gene encoding mannose receptor C type 1 (*Mrc-1*). Mrc-1 is a lectin or carbohydrate binding protein that is primarily present on the surface of macrophages and dendritic cells. It recognizes and binds mannose-rich glycans on the surface of potentially pathogenic viruses, bacteria and fungi, and its activation triggers endocytosis and phagocytosis of the pathogen via the complement system. (32) Depending on the ligand, mannose receptors may also induce signaling leading to MAP kinase and NF-kB activation or may act indirectly by modulating TLRs. For example, MIMP, the functional domain of surface layer proteins of probiotic *Lactobacillus* species binds to intestinal epithelial cells via Mrc-1 and prevents the adherence and invasion of pathogens such as *E.coli.* (33) MIMP reduces the intestinal permeability via the p38 signaling pathway through a mechanism that requires Mrc-1. (34)

Recently, it was shown that the expression of miR-511 correlates with that of Mrc-1 mRNA, supporting the notion that this intronic miRNA is regulated by the same promoter as its host gene. (35) Indeed, we found that both miR-511 and Mrc-1 mRNA expression levels were significantly higher in SPRET/Ei mice than in C57BL/6 mice.
miR-680-1 is located intergenic on 130 Mb-63.44 cM of chromosome 6, about 4 cM beyond the *Tnfrsf1a* gene.

2.5 microRNA-511 regulates TNFR1 protein expression

To investigate whether miR-511 or miR-680-1 are really able to regulate TNFR1 expression, we performed several functional tests *in vitro* and *in vivo*.
The miRNAs were introduced into primary hepatocytes and fibroblasts by means of transfection with miRNA precursor molecules and at several time points after transfection, we looked at the cell-bound TNFR1 protein expression. We found that miR-511 overexpression could reduce TNFR1 expression, and that the effect was most pronounced after 24 h. One of the two predicted miR-511 target sequences in the 3'UTR of *Tnfrsf1a* contains two SNPs in SPRET/Ei compared to C57BL/6. However, miR-511 reduced TNFR1 expression in both C57BL/6 as in SPRET/Ei, indicating that it binds and recognizes the target sequence in both genomes. miR-680 however, was not able to alter the TNFR1 expression, at least not in these experimental conditions. To further demonstrate that miR-511 regulates TNFR1 expression, we blocked the endogenous miRNA by means of an anti miR specifically binding to miR-511. This inhibition led to a significant induction of cellular TNFR1 protein expression likewise in C57BL/6 and SPRET/Ei. The pri-miR-511 sequence indeed contains neither SNPs nor INDELs in SPRET/Ei compared to C57BL/6.
To confirm the target sequence of miR-511, we cloned the 3'UTR of the *Tnfrsf1a* gene of both C57BL/6 and SPRET/Ei into the psiCHECK-2 luciferase reporter vector. Co-transfection of each of these plasmids with pre-miR-511 precursor molecules in HEK-293T cells significantly reduced the Renilla luciferase activity, further illustrating that miR-511 regulates both C57BL/6 and SPRET/Ei *Tnfrsf1a* target sequences. Now, it will be essential to modify the 3'UTR sequence in the reporter assay, to identify which of the predicted sequences are true functional binding sites, since only the polymorphic target sequence might explain the genetic link to chromosome 6. Unfortunately, miR-511 is not yet included in the StarBase collection of CLIP-Seq data. Performing CLIP-Seq, via the Ago-protein, would also identify specific miRNA-mRNA interactions.
To examine whether higher expression of miR-511 can also down-regulate TNFR1 *in vivo*, as we believe is happening in SPRET/Ei mice, we injected a precursor miR-511 expression plasmid in C57BL/6 and SPRET/Ei mice by hydrodynamic tail vein injection. This technique of *in vivo* plasmid delivery leads to an efficient transient transfection, predominantly of the hepatocytes. (36) Indeed, we observed a significant reduction of TNFR1 protein expression in the liver of both mouse strains, demonstrating that miR-511 regulates TNFR1 protein expression *in vivo*. Moreover, inhibition of endogenous miR-511 by hydrodynamic tail vein injection of anti miRs, led to an up-regulation of TNFR1 expression. Future experiments

should reveal whether miRs and anti miRs can also be taken up and exert their function in other organs such as spleen, lung and the intestine after hydrodynamic tail vein injection. Alternatively, our group is evaluating other modes of administration such as *in vivo* electroporation with promising results so far in the brain and the quadriceps of mice.
Together, these results show that miR-511 regulates TNFR1 protein expression. In the future, we should further investigate other possible targets of miR-511, in particular genes that are involved in inflammation.

2.6 microRNA-511 mediates resistance to TNF-induced inflammation

Although the effect of miR-511 on endogenous TNFR1 is only about 20 %, it is clearly sufficient to induce a marked protection to TNF-induced inflammation. In addition, blocking endogenous miR-511 in the liver and consequential elevation of TNFR1 protein expression leads to higher susceptibility against TNF in C57BL/6 as well as in (BxS)F1 mice. These results are in accordance with experiments performed in collaboration with George Kollias where a 30 % reduction of TNFR1 expression led to a robust protection, but seem to be in conflict with the results obtained with Alfp-Cre *Tnfrsf1a*$^{fl/fl}$ conditional reactivation mutants and conditional knock-out mice. (30) Villin-Cre *Tnfrsf1a*$^{fl/fl}$ mice with *Tnfrsf1a* deficiency in intestinal epithelial cells significantly resisted TNF-induced toxicity, suggesting a crucial role for the intestine in TNF-induced inflammation possibly due to influx of gut bacteria or bacterial agents in the system. (37, 38) However, mice with hepatocyte-specific *Tnfrsf1a* deficiency did display a mild protection against TNF-induced toxicity, in comparison with controls, suggesting that the liver cannot be simply ruled out as an important organ in inflammation.
Our data demonstrate that miR-511 is a genuine regulator of TNFR1 protein expression and is able to affect the TNF response *in vivo*. Moreover, they further indicate that the genetic link between TNF resistance and TNFR1 expression level on the one hand and proximal chromosome 2 and distal chromosome 6 on the other hand may point to the *miR-511* and *Tnfrsf1a* genes. Although no genetic difference is found in miR-511 between C57BL/6 and SPRET/Ei, miR-511 is clearly up-regulated in SPRET/Ei mice following the up-regulation of the *Mrc-1* gene. The linkage to chromosome 2 might point to a genetic difference in the regulation of miR-511, possibly in the GRE sequences (see next paragraph).
Since miR-511 is also described as a regulator of the LPS receptor TLR4, under certain conditions (39), we demonstrated that the effect of miR-511 in our results is mediated by specific TNFR1 regulation. Indeed, the protection of miR-511 pretreated mice against LPS-induced endotoxemia, a model in which TNF is centrally involved (40), was only true in C57BL/6 and not in TNFR1 deficient mice.
For therapeutic applications, until now, we are limited to liver specific TNFR1-mediated disease models such as concanavalin A (conA)-induced hepatitis. This cell-mediated immunoinflammatory disease model for human autoimmune hepatitis can be induced by a single intravenous injection of conA. Activation of T-cells causes secretion of TNF which binds to TNFR1 on macrophages. The following infiltration of the liver with neutrophils and

macrophages leads to apoptosis and necrosis of the hepatocytes. (41, 42) miR-511 could induce significant protection to conA-induced hepatitis as seen in a regular bodytemperature an almost no induction of serum ALT levels. A normal induction of TNF levels however demonstrated that conA effectively induced inflammation, but that the miR-511 mediated TNFR1 repression determined the TNF response.

2.7 microRNA-511-mediated TNFR1 repression is regulated by GR activity.

Compared with C57BL/6, SPRET/Ei mice were found to express higher levels of the glucocorticoid receptor (GR), reflected in increased GR activity. (24) Glucocorticoids (GC) such as corticosterone in rodents, cortisol in humans or synthetics such as dexamethasone, have strong anti-inflammatory properties, which is thought to be mainly by inhibiting the production of pro-inflammatory cytokines via transrepression mechanisms, i.e. protein-protein interactions between the GR and transcription factors such as NF-kB and AP-1. (43) Upon binding of glucocorticoids, the GR translocates to the nucleus where it can perform distinct functions. Besides transrepression, the GR can also induce transcription by transactivation. It either acts as a homodimeric transcription factor that binds to the glucocorticoid response elements (GREs) in promoter regions of GC-inducible genes or as a monomeric protein that co-operates with other transcription factors to induce transcription. (44) Our research group have recently found that the dimeric GR is much more important in protecting against TNF toxicity than the monomeric form, thereby confirming that GR-induced gene expression is essential. (45)

To investigate whether glucocorticoids play a role in the (miR-511 regulated) TNF resistance of SPRET/Ei mice, we treated C57BL/6 and SPRET/Ei mice with the GR antagonist RU486. We found that irreversible blocking of the GR with RU486, sensitizes SPRET/Ei mice for TNF, entirely wiping out the differences in TNF response between SPRET/Ei and C57BL/6 mice. These results indicate that the GC-GR action is essential for the extreme resistance of SPRET/Ei mice against TNF-induced lethality.

The consensus GRE sequence for transactivation is an inverted imperfect hexameric palindrome separated by a spacer of 3 bp (5'-AGAACAnnnTGTTCT-3'). (46) Using ConTra, we found two GRE elements in the 5' UTR of *Mrc-1* at position -1167 to -1152 and at -599 to -584 relative to the transcription start site (TSS). Moreover, we found a GR half binding site at positions -20 to -2, just before the TSS. These findings suggest that the GR might be able to bind to the promoter of *Mrc-1* and induce transcription. Furthermore, after administration of rapidexon, a soluble form of dexamethasone, to C57BL/6 mice we could see a significant induction of Mrc-1 and miR-511 gene expression by qPCR in the liver and in the spleen. Since glucocorticoids are produced by the adrenal glands, the expression of GR-inducible genes declines upon removal of these glands by adrenalectomy. Indeed, we found a significant down-regulation of Mrc-1 and miR-511 in adrenalectomized mice. Although we don't have physical evidence yet, e.g. based on ChIP-seq analysis, of GR binding to Mrc-1, or a promoter analysis with an Mrc-1 promoter construct, our results strongly suggest that the GR is up-regulated in SPRET/Ei and able to bind to the promoter and induce transcription of Mrc-1

and miR-511. Besides, the administration of dexamethasone to C57BL/6 mice causes down-regulation of TNFR1 protein expression, while adrenalectomy causes up-regulation.
Since we found no genetic link between TNF resistance or TNFR1 levels and the GR locus on chromosome 18, we suggest that these phenotypes do depend on the GR, but are linked with a GRE gene that executes the anti-inflammatory effect and is located on proximal chromosome 2, namely the *Mrc-1/miR-511* locus. Therefore, it will be important to study the expression profile of miR-511 in naive conditions and during inflammation.
Hence, knowing that SPRET/Ei mice have a higher GR expression compared to C57BL/6 mice and that the GR can induce Mrc-1 expression, we suggest that the higher level of miR-511 in SPRET/Ei is caused by GR induction. Since miR-511 is found to mediate suppression of TNFR1 in SPRET/Ei mice leading to protection against TNF-induced inflammation, we hypothesize that the resistance of SPRET/Ei to TNF-induced lethality is mediated by GR-induced over-expression of Mrc-1 and miR-511, resulting in lower TNFR1 expression. (Figure 1) Since blocking of the GR is far more effective than the administration of miR-511, other GR-mediated mechanisms will certainly play a role. However, the modest repression of TNFR1 by miR-511 can provide a significant improvement of the survival. Moreover, miR-511 and anti miR-511 were delivered specifically in the liver. Therefore, it will be very interesting to study the effect in full miR-511 deficient and overexpressing mice.
If our hypothesis is confirmed, we will have a strong point to show that GCs, via GR dimerization, cause anti-inflammatory actions partly via induction of miRNAs, which will be an entirely novel mechanism.

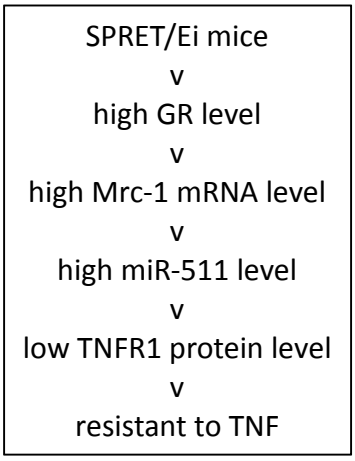

Figure 1. Representation of our hypothesis.

2.8 Generation and characterization of TNFR1 binding and inhibiting nanobodies

The TNF signaling pathway is a valuable target in the therapy of autoimmune diseases. Anti-TNF treatment has shown to be successful in the inhibition of inflammation-induced damage in rheumatoid arthritis, inflammatory bowel diseases and psoriasis. However, a number of side effects have been reported, e.g. increased risk of infection and malignancy and the onset of additional auto-immune diseases. Some of these effects are caused by the unwanted inhibition of beneficial TNF signaling such as resistance to infection and recovery

of the affected tissues, activities that are mainly dependent on TNFR2. Hence, more specific targeting of the pathological TNF/TNFR1 signaling might lead to a broader applicability and improved safety. Specificity can be obtained by targeting TNFR1 while leaving the TNFR2 signaling untouched. Several TNFR1 inhibitors including domain antibodies from GSK and antagonistic TNF mutants (R1antTNF) have already proven to have a similar therapeutic effect as anti-TNF blockers in the treatment of immune diseases such as EAE, CIA and ventilator-induced lung injury, however causing fewer side effects. (16, 47, 48)

Since we want to further explore the potential of TNFR1 as a therapeutic target and develop even better tools for its inhibition, we decided to generate nanobodies against TNFR1. We believe that the nanobody technology is a good platform to develop TNFR1 specific inhibitors because nanobodies combine the advantages of conventional antibodies with important features of small molecules. Like conventional antibodies, nanobodies show high target specificity and affinity and low immunogenicity. However, like small molecules, they can easily reach hidden epitopes or receptor cavities. Furthermore, nanobodies are extremely stable, highly soluble and easy to produce in almost all prokaryotic and eukaryotic hosts including bacteria and yeast. Moreover, they are encoded by single genes, which allow the design of modular proteins by combining nanobodies with each other or with other molecules. The TNFR1 nanobodies can, for instance, be coupled to MMP-8 inhibiting nanobodies. MMP-8 inhibition mediates resistance to sepsis mainly through effects in the brain, while for resistance by TNFR1, inhibition in the intestine and the liver are of great importance. By coupling the nanobodies, we may be able to induce an even more pronounced resistance as was already seen in TNFR1/MMP-8 double deficient mice. Another possibility is the coupling of TNFR1 nanobodies to TACE or to a TACE nanobody (Ablynx) to induce specific cleavage of TNFR1. By generating nanobodies, we will have unlimited possibilities.

The TNFR1 nanobodies were generated by immunization of an alpaca with recombinant human TNFR1. After intensive screening, two TNFR1 specific nanobodies were selected. Nanobody nb70 has a good binding affinity of about 50 nM and is the only tested nanobody that inhibits TNFR1 signaling with a strong inhibitory capacity of 1.34 μM. Unfortunately, nb70 has a very low production yield, which is probably related to its sequence. Nanobody nb96 has an even better binding affinity of 10 nM, but is not able to inhibit TNFR1 signaling. Both nanobodies are specifically recognizing human TNFR1 as there was no cross reactivity with human TNFR2. Unfortunately and despite high similarity, none of the nanobodies cross react with mouse TNFR1, limiting their *in vivo* characterization to experiments in hTNFR1 KI mice or other humanized models, however making them valuable tools in the therapy against auto-immune diseases in humans.

In order to use the nanobodies *in vivo*, we made multimeric nanobodies to improve the half-life. By combining the inhibitory nb70 with the best binding nb96 and a nanobody against human albumin we wanted to improve the pharmacokinetics and -dynamics as well as the inhibitory capacity. We generated a dimeric nb70-96 coupled with a rigid human IgA1 linker and trimeric nbAlb-70-96 and nbAlb-70-70, both coupled with a flexible $(G_4S)_3$ linker. Again,

the yield of production in *E.coli* was very low, probably due to the presence of nb70. Therefore we decided to switch to the yeast *Pichia pastoris* for the production of the trimeric nanobodies, which led to a substantially higher yield. The introduction of a few extra amino acids for the cloning into a yeast expression vector did not influence the binding capacity nor the inhibitory capacity.

The binding capacity of nb70-96 (3 nM) is much better than that of the single domain nanobodies. The trimeric nanobodies show an intermediate binding capacity (5 nM and 9 nM), presumably due to the addition of a nanobody against albumin. Importantly, the trimeric nanobodies show sufficiently high (cross-reactive) binding to mouse serum albumin, what makes them particularly suitable for *in vivo* use. The inhibitory capacity of the dimeric nb70-96, i.e. 0.926 μM, is slightly better than that of nanobody nb70. However, the trimeric nanobody nbAlb-70-96 is even better with an IC_{50} of 0.395 μM. Epitope mapping revealed that nb70 and nb96 recognize two distinct epitopes which might explain the higher affinity and thereby a better inhibitory capacity of the combined nanobody nb70-96.

A Biacore competition assay showed that the inhibitory nb70 competes with hTNF by blocking its binding to TNFR1. The competition is even more pronounced in the multimeric nanobodies especially when the nanobodies are given before hTNF, in a prophylactic setup. A docking prediction confirmed that nb70 is able to hinder the binding of TNF to its receptor by competing for the same region on TNFR1. Nb70 can bind with its protruding CDR3 loop into the cavity formed by CRD3 and CRD4 and thereby cover part of the ligand binding domain of TNFR1 with the rest of its structure. The exact confirmation of the hTNFR1-nb70 binding however needs to be deduced from crystallographic studies.

Based on the obtained results, we can conclude that the trimeric nanobody nbAlb-70-96 is the best inhibitor of TNF-induced NF-kB activation with a high binding capacity to hTNFR1 and mouse albumin. This nanobody will be further investigated *in vivo*. A preliminary pharmacokinetic study revealed that upon intravenous injection, this nanobody is absorbed from the plasma and distributed to the tissues until 8h after injection, followed by a gradual elimination. The estimated half-life of more or less 24 h is equal to other multimeric nanobodies such as the trimeric αEGFR-αEGFR-αAlb nanobody. (49) Furthermore, the similar pharmacokinetics of αEGFR-αEGFR-αAlb and mouse serum albumin itself indicates that the albumin nanobody ensures a very strong *in vivo* association to albumin. (50) Moreover, since the albumin nanobody also shows high affinity for human serum albumin, and human serum albumin was shown to have a blood half-life of about 20 days (51), we might expect that the trimeric nanobody nbAlb-70-96 will show a much longer half-life in humans.

3. Future perspectives

miR-511 might be a potential therapeutic molecule to treat TNFR1-mediated diseases. However, since each miRNA potentially targets hundreds of mRNAs and regulates different signaling pathways, it will be important to fully characterize its mechanism and all cellular targets to avoid negative outcomes. To date only few examples of therapeutic applications

are known. Miravirsen, an LNA-modified anti miR-122, provides a prolonged dose-dependent antiviral activity in clinical trials with patients having chronic HCV infection. (52) So far, no serious adverse effect of miR-122 inhibition has been observed in chimpanzees (53) or in the phase II clinical trials. However, long term studies will need to ensure that over-expressed or blocked miRNAs do not cause any harmful side-effects. To further investigate the (patho) physiologic role of miR-511, we will generate miR-511 deficient mice and transgenic miR-511 over-expressing mice.

miR-511 deficient ES cells from the EUCOMM consortium will be injected, in collaboration with Dr. Tino Hochepied, into C57BL/6 blastocysts resulting in chimeric mice, in which some cells in each tissue are derived from the ES cell lineage. Since the ES cells are heterozygous for the miR-511 deletion, roughly half of the offspring will carry the modification. Crossing the chimeras with C57BL/6 mice and subsequent intercrosses may result homozygous miR-511 deficient mice. These mice will be extensively characterized in terms of TNFR1 expression, resistance to TNF-induced inflammatory models and potential spontaneous pathologies.

miR-511 transgenic mice will be generated in close collaboration with Prof. Thierry Vandendriessche en Prof. Marinee Chuah who developed a fast and effective system based on the Piggy-Back transposon. Both transient as well as germline transmission can be induced by the introduction of two plasmids. One plasmid contains the Piggy-Back transposon (Tp), in which the miR-511 construct is flanked by Tp inverted repeats. The other plasmid contains the hyperactive Piggy-Back transposase (Tpase) that will provide highly efficient integration of Tp. Since we will deliver the plasmids by hydrodynamic injection, we expect the integration and over-expression of miR-511 predominantly in hepatocytes where it will remain for several months. In addition, we will generate permanent transgenic mice by injecting the plasmids directly into the testis, followed by an electroporation of the testis. Also these mice will be extensively characterized in terms of TNFR1 expression, resistance to TNF-induced inflammatory models and potential spontaneous pathologies.

TNFR1-selective inhibition by nanobody nbAlb-70-96 resulted in blocking of the inflammatory signaling pathway of TNFR1, shown *in vitro* by inhibition of TNF-induced NF-kB activation. The *in vitro* characterization will soon be supplemented with expression data of NF-kB and AP-1-induced genes such as IL6, IL8, TNF, A20 and IKKα. For future *in vivo* experiments, we are negotiating with the company Biomedcode for the use of hTNFR1KI mice to test the nanobody in inflammatory models such as TNF-induced lethality and LPS-induced endotoxemia. In these experiments, the properties of the nanobody will be compared with a trimeric anti-βlactamase control nanobody, e.g. nbAlb-BCll10-BCll10, and the anti-TNF agent Etanercept. Later, nbAlb-70-96 may be tested as a therapeutic agent especially for those diseases against which currently marketed anti-TNF therapeutics have failed or even exacerbate disease progression, including multiple sclerosis (EAE) and rheumatoid arthritis (in ΔARExhTNFR1KI mice). Meanwhile, we will develop our own hTNFR1KI mice by injecting a hTNFR1 containing BAC-clone into C57BL/6 zygotes. The resulting transgenic mice will have a random integration of hTNFR1 and will be crossed with

mTNFR1 deficient mouse. These 'knock in' mice will be used to further explore the therapeutic niche of the nanobody in several TNFR1-mediated models such as CLP-induced polymicrobial sepsis and kidney ischemia and reperfusion. In addition, we have contacts with Prof. Geert Leroux-Roels to use transgenic uPA-SCID mice. These mice suffer from a transgene-induced liver disease and are transplanted early after birth with primary human hepatocytes, resulting in mice with a partially humanized liver. (54) We will test whether nbAlb-70-96 is able to protect against TNF/Galactosamin-induced liver inflammation in these mice by determination of induction of human ALT levels and expression of NF-kB and AP-1-inducable genes. In a later phase, we would like to study whether gut-specific delivery of the nanobody by means of *Lactococcus lactis* is useful in a model of IBD such as ΔARE-induced ileitis. This should be done in collaboration with the company Actogenix.

Since the hTNFR1 nanobodies did not cross react with mouse TNFR1, we decided to perform a new panning with recombinant mouse TNFR1 on the existing clones of the hTNFR1 nanobody gene library. Unfortunately, the recombinant mouse TNFR1 solution that we used contained an excess of BSA as a carrier protein, resulting in the selection of nanobodies against BSA instead of nanobodies against mTNFR1. This can be explained by the fact that the alpacas are reused several times for the immunization with multiple antigens. Some of these antigens might have had BSA as a carrier protein as well, or one of the antigens might have been BSA itself. Therefore, we had to perform a new panning, which is currently in progress.

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CHAPTER V

Discussie

1. Inleiding

Inflammatie of een ontsteking is een normale reactie op weefselschade veroorzaakt door een infectie of irritatie en leidt tot het vrijkomen van pro-inflammatoire cytokines, chemokines en andere moleculen door verschillende cel types. Dit resulteert in een gecontroleerde lokale ontstekingsreactie en immuunrespons gevolgd door eliminatie van de initiële stimulus. (1) Wanneer het immuunsysteem er echter niet in slaagt om de schadelijke stoffen op te ruimen en deze pathogenen de bloedbaan binnendringen, kan een ongecontroleerde systemische immuunrespons (SIRS) ontstaan. SIRS veroorzaakt een algemene systemische ontsteking met onder meer bloeddrukdaling, koorts en een versnelde hartslag als gevolg. Bloedklonters en een verminderde doorbloeding van de organen brengen de toevoer en opname van zuurstof in het gedrang waardoor organen beschadigd kunnen raken. In ernstige gevallen verliezen een of meerdere organen hun functie, verzwakt het hart en gaat de patiënt in septische shock. Zodra dit gebeurt kunnen vele organen tegelijk bezwijken met een grote kans op overlijden van de patiënt. Jaarlijks worden wereldwijd ongeveer 18 miljoen patiënten getroffen door sepsis, waarvan 30 tot 40 % sterven. (2) Sepsis vormt, als een van de belangrijkste doodsoorzaken, een groot probleem op de afdeling intensieve zorgen. Een gezond en evenwichtig immuunsysteem is dus essentieel om ons lichaam te verdedigen tegen ontstekingen en infecties.

Helaas herkent het immuunsysteem soms ten onrechte lichaamseigen eiwitten en weefsels als 'vreemd' en probeert deze te vernietigen. Dit leidt tot de ontwikkeling van auto-immuunziekten zoals reumatoïde artritis, de ziekte van Crohn, multiple sclerose, type 1 diabetes en psoriasis, allemaal ernstige, slepende ziekten met hoge kosten voor therapie. Volgens het nationaal gezondheidsinstituut NIH treffen deze ziekten samen tot 23,5 miljoen Amerikanen en de prevalentie stijgt nog.

Tumor necrose factor (TNF), een krachtig pro-inflammatoir cytokine, is een centrale factor in de pathologie van SIRS en speelt een belangrijke rol in verschillende ontstekingsziekten en auto-immuunziekten. (3, 4) De diverse signaaltransductiecascades die geïnitieerd worden door TNF leiden tot een waaier van reacties waaronder proliferatie, differentiatie, migratie en celdood en ook inflammatie. (5) TNF-remmers zoals Infliximab, Adalimumab, Golimumab, Etanercept en Certolizumab hebben daardoor een stevige positie verworven in de top 10 van best verkochte geneesmiddelen wereldwijd. Etanercept en Adalimumab bijvoorbeeld, worden reeds meer dan 10 jaar met succes gebruikt in de behandeling van reumatoïde artritis. (6, 7) Ondertussen zijn de indicaties reeds uitgebreid met andere vormen van artritis, psoriasis en inflammatoire darmziekten. (8) Dit illustreert duidelijk het belang van TNF in inflammatie en de noodzaak van anti-TNF therapie. Langdurig gebruik van deze middelen kan echter ernstige bijwerkingen veroorzaken zoals opportunistische infecties (9) en de ontwikkeling van nieuwe auto-immuunziekten zoals lupus, type 1 diabetes, uveïtis, multiple sclerose en psoriasis, maar ook lymfeklierkanker en leukemie. (10, 11) De pathofysiologie van TNF zoals in chronische ontstekingen en auto-immuunziekten, en de problemen met anti-TNF middelen wijzen er op dat de diverse acties van TNF verschillen naar gelang de betrokken receptor. TNFR1, de belangrijkste receptor voor TNF, is voornamelijk

verantwoordelijk voor het induceren en onderhouden van inflammatie, proliferatie, overleving en apoptose (12), terwijl de immunomodulerende en ziekte-onderdrukkende functies van TNF meer afhankelijk zijn van TNFR2. (13) Daarom is, voor de behandeling van TNF-geïnduceerde chronische ziekten, een meer specifieke inhibitie aanbevolen van de pathologische werking van TNF door het selectief blokkeren van TNFR1. (12) In experimentele autoimmuun encefalomyelitis (EAE), bijvoorbeeld, zijn TNFR1 deficiënte muizen beschermd, wat zichtbaar is in verminderde Th1 proliferatie en cytokineproductie. (14) TNF en TNFR1R2 deficiënte muizen daarentegen zijn niet in staat zijn om de late auto-immune reactiviteit te onderdrukken en vertonen een abnormale verlenging en verergering van de ziekte. Dit toont aan dat de immunosuppressieve functies van TNFR2 beschermend werken in de latere fase van chronische EAE. (15) Behandeling van EAE met TNFR1 specifieke remmers is bijgevolg zeer effectief. Hierbij zien we een normale regressie van de anti-myeline reactiviteit met behoud van de TNFR2 gemedieerde activatie van Tregs. (16) Klinische trials in multiple sclerosis patiënten met anti-TNF daarentegen, faalden omwille van een versterking van de klinische symptomen. (17) Dit idee vormt de basis van een eerste deel van mijn doctoraat.

De ontwikkeling van nieuwe, meer doeltreffende geneesmiddelen voor de behandeling van inflammatoire ziekten vereist in de eerste plaats een beter begrip van de regulatie van deze receptor. Ondanks de uitgebreide kennis over de TNFR1 signalisatie, is er weinig geweten over de regulatie van TNFR1 expressie, modificatie, lokalisatie en processing. Terwijl algemeen wordt aangenomen dat de expressie van TNFR1 vrij constant is en niet induceerbaar, werd reeds aangetoond dat de TNFR1 eiwitexpressie toch afhankelijk is van een aantal post-transcriptionele modificaties waaronder het verknippen van membraangebonden TNFR1 door TACE/ADAM17. (18) Inflammatie-geïnduceerde activatie van TACE resulteert in een plotse afname van het aantal receptoren op het celoppervlak waardoor cellen tijdelijk ongevoelig zijn voor TNF. (19) Bovendien kunnen de vrijgekomen oplosbare TNFR1 moleculen ook fungeren als fysiologische remmers van de TNF activiteit door competitie voor het ligand met celgebonden receptoren. (20) Bij de mens zijn mutaties die de TNFR1 shedding beïnvloeden verantwoordelijk voor de ontwikkeling van TRAPS (TNF receptor-geassocieerde periodieke syndromen). (21) Deze aandoeningen worden gekenmerkt door steeds terugkerende koortsaanvallen die gepaard gaan met lokale inflammatie. Daarenboven zijn muizen die non-sheddable TNFR1 expresseren, en dus hogere TNFR1 levels hebben in de meeste weefsels, buitengewoon gevoelig voor TNF-geïnduceerde inflammatie. (19) Deze bevindingen suggereren dat de hoeveelheid TNFR1 strikt gereguleerd is en dat deze hoeveelheid van groot belang is voor de TNF gevoeligheid.

Eerder toonden we aan dat SPRET/Ei muizen, een inteelt stam afgeleid van *Mus spretus*, zeer goed bestand zijn tegen de dodelijke effecten van TNF en we denken dat deze muizen ons kunnen helpen om meer inzicht te krijgen in de TNF-geïnduceerde pathologie, in de regulatie van TNFR1 en in het ontdekken van nieuwe therapeutische mogelijkheden. (22) De grote genetische diversiteit tussen *Mus spretus* en *Mus musculus* heeft reeds bijgedragen tot de identificatie van genen die van belang zijn bij andere kenmerken van SPRET/Ei zoals

resistentie tegen LPS (23, 24), Salmonella typhimurium (25) en Influenza A (26). De resistentie tegen TNF werd reeds eerder gekoppeld aan een locus op proximaal chromosoom 2 en een andere op distaal chromosoom 6. Aangezien het gen dat codeert voor TNFR1 gelegen is in de distale regio van muis chromosoom 6 (125,3 Mb - 60.55 cM), waren we van mening dat de TNF resistentie kan worden geassocieerd met de regulatie van TNFR1, en dit idee vormde de basis van een tweede deel van dit doctoraat.

2. Resultaten

2.1 TNFR1 expressie is lager in SPRET/Ei muizen

Tnfrsf1a was reeds lange tijd een voor de hand liggend kandidaat gen. Sequentie bepaling van het gen in SPRET/Ei en C57BL/6 onthulde 12 aminozuurvariaties waarvan 6 volkomen uniek voor SPRET/Ei. Er kon echter geen verschil aangetoond worden in TNF-bindingsefficiëntie en B.S^{chr6} consome muizen toonden aan dat de TNFR1 van SPRET/Ei in C57BL/6 muizen nog steeds letaliteit kan veroorzaken. Geïnspireerd door de volledige resistentie van TNFR1+/- muizen tegen TNF-geïnduceerde letaliteit, onderzochten we of de TNF resistentie van SPRET/Ei gekoppeld is aan een kwantitatief effect in de TNFR1 locus. We vonden inderdaad dat SPRET/Ei muizen sterk verlaagde TNFR1 eiwitniveaus vertonen, vergelijkbaar met die van TNFR1+/- muizen. Bovendien hebben (BxS)F1 muizen een even laag TNFR1 eiwitniveau als SPRET/Ei, wat aangeeft dat de lagere TNFR1 expressie in SPRET/Ei een dominante eigenschap is. De concentratie van oplosbaar TNFR1 in het serum was eveneens drie keer lager in SPRET/Ei muizen, wat verondersteld dat de gemeten verschillen in organen niet worden veroorzaakt door een verhoogde TNFR1 shedding door TACE. De lage TNFR1 eiwitniveaus zijn echter niet gecorreleerd met lage TNFR1 mRNA niveaus. We vonden gelijke TNFR1 mRNA niveaus in zowel SPRET/Ei en C57BL/6 muizen. Dit suggereert dat het verschillende TNFR1 regulatie in SPRET/Ei te wijten is aan post-transcriptionele effecten.

De TNFR1 eiwitniveaus werden bepaald met behulp van een ELISA waarbij gebruik gemaakt wordt van een rat anti-muis TNFR1 ‘capture’ antilichaam (R&D MAb425) en een gebiotinyleerd geit anti-muis TNFR1 detectie antilichaam (R&D BAF425). Beide antilichamen werden opgewekt tegen het extracellulaire deel van muis TNFR1 (AA 22-212). In deze regio zijn echter vier missense mutaties tussen SPRET/Ei en C57BL/6 gelegen. Dit betekent dat we niet kunnen garanderen dat de binding aan TNFR1 van SPRET/Ei even goed gebeurt als aan TNFR1 van C57BL/6. Om het lagere TNFR1 eiwitniveau van SPRET/Ei te bevestigen, zouden we aanvullende methoden moeten toepassen, bij voorkeur zonder het gebruik van antilichamen. Jammergenoeg gaven onze pogingen om een FACS analyse uit te voeren op primaire macrofagen en fibroblasten met gebiotinyleerd hTNF en een slot blot analyse van lever extracten met verschillende antilichamen geen uitsluitsel, waarschijnlijk omwille van de zeer lage expressie van TNFR1 in naïeve muizen. Toch nemen we aan dat de antilichamen even goed de TNFR1 van beide stammen kunnen binden aangezien B.S^{chr6}congene muizen,

die de SPRET/Ei receptor expresseren in een achtergrond van C57BL/6, een normaal TNFR1 expressieniveau vertonen zoals in C57BL/6.

2.2 TNF resistentie en lagere TNFR1 expressie in SPRET/Ei zijn oorzakelijk verbonden

Om de genetische regio's die verantwoordelijk zijn voor de lage TNFR1 eiwitlevel in SPRET/Ei muizen te identificeren, genereerden we een interspecies backcross tussen (BxS)F1 en C57BL/6. De TNFR1 eiwitlevels werden gemeten in de lever van 214 BSB muizen en er werd een genomische scan uitgevoerd met behulp van merkers verspreid over het gehele genoom. Een QTL analyse met R/QTL software (27) onthulde een genetische link met een locus op proximaal chromosoom 2 (1-38 cM) en een locus op distaal chromosoom 6 (40-70 cM). Wanneer de BSB populatie werd gescheiden in mannetjes en vrouwtjes, werd gevonden dat de link met chromosoom 2 sterk significant was bij de mannetjes terwijl de link met chromosoom 6 sterk significant was bij de vrouwtjes en in de totale populatie. Het is niet makkelijk om veronderstellingen te maken over de onderliggende reden van dit sekseverschil. Echter, de groep van Xavier Montagutelli (Institut Pasteur in Parijs) rapporteerde een soortgelijk geval met twee QTLs die alleen werden gevonden bij vrouwtjes en een derde QTL die aanwezig was bij beide geslachten. Verdere analyse met behulp congene muizen, die een van de QTLs of combinaties ervan dragen in een achtergrond van C57BL/6, onthulde dat de eerste QTL bij vrouwtjes een onechte link bleek, terwijl de andere twee QTLs echte QTLs waren die verantwoordelijk zijn voor de resistentie tegen *Yersinia pestis* bij zowel mannetje als vrouwtjes van SEG/Pas. (28, 29) Het is dus mogelijk dat QTLs niet gedetecteerd worden in een van de specifieke subpopulaties, terwijl QTLs met een hoog significante Lod score, waarschijnlijk echte QTLs zijn zelfs al komen ze slechts voor in een van de subpopulaties.
De QTLs die we vonden voor TNFR1 eiwitexpressie in SPRET/Ei zijn analoog met degene die waren gelinkt met de TNF resistentie van SPRET/Ei. (22) Bovendien vertonen SPRET/Ei en TNFR1+/- muizen, beide met half-maximale TNFR1 eiwit expressie niveaus, een sterke resistentie tegen TNF-geïnduceerde letaliteit. (30) Dit suggereert dat de twee fenotypes oorzakelijk verbonden zijn. Aangezien het gen dat codeert TNFR1 (*Tnfrsf1a*) in de QTL op distaal chromosoom 6 ligt en de Lod scores van chromosoom 6 voor zowel TNF resistentie als TNFR1 expressie hoger zijn dan die van chromosoom 2, veronderstellen we dat beide eigenschappen geassocieerd zijn met TNFR1 regulatie.

2.3 Neer-regulatie van TNFR1 in SPRET/Ei muizen is gereguleerd *in trans*

Congene muizen worden gebruikt om QTLs, die geïdentificeerd werden door linkage analyse, te bevestigen en verder te karakteriseren. Daarom probeerden we een congene lijn te genereren die het proximale deel van SPRET/Ei chromosoom 2 bezaten en een lijn met het distale gedeelte van SPRET/Ei chromosoom 6 in een genomische achtergrond van C57BL/6. Helaas slaagden we er niet in om B.S^{QTLchr2} congene muizen te maken door problemen met de kweek en intussen was het chromosoom 2 van de B.S^{QTLchr6} congene muizen reeds volledig C57BL/6. In het verleden werden er echter reeds B.S^{QTLchr2} congene muizen gemaakt

door Jan Staelens die de SPRET/Ei locus op chromosoom 2 (2,23-71,17 cM) bezaten in een C57BL/6 achtergrond en nakomelingen van opeenvolgende generaties werden geïnjecteerd met een dosis TNF van 20-25 μg. In geen van deze experimenten werd een correlatie gevonden tussen het genotype en TNF letaliteit.
We slaagden er wel in om B.S^{Tnfrsf1a} congene muizen te genereren door het selecteren B.S^{QTLchr6} nakomelingen die homozygoot C57BL/6 of SPRET/Ei waren voor een microsatelliet merker (D6Mit254) gelegen in een intron van *Tnfrsf1a*. B.S^{Tnfrsf1a} SS muizen vertoonden na TNF injectie een overlevingskans van ongeveer 50 % in vergelijking met SPRET/Ei muizen, wat aangeeft dat de SPRET/Ei TNFR1 regio (in een C57BL/6 achtergrond) alleen ook niet voldoende is voor resistentie tegen TNF. Daarnaast waren de TNFR1 eiwit niveaus in de lever van B.S^{Tnfrsf1a} BB, BS en SS muizen gelijk, wat kan wijzen op een *in trans* regulatie van de TNFR1 eiwitexpressie in SPRET/Ei muizen.
Hoewel we idealiter dubbel congene muizen hadden moeten maken die beide SPRET/Ei resistentie allelen bevatten in een achtergrond van C57BL/6, kunnen we toch concluderen dat noch de QTL op chromosoom 2, noch de QTL op chromosoom 6 alleen volledige resistentie kunnen bereiken tegen TNF wanneer ze geïsoleerd zijn in een C57BL/6 achtergrond. Bovendien bevestigen onze gegevens de relatie tussen TNFR1 eiwitexpressie en TNF resistentie die beide waarschijnlijk *in trans* worden gereguleerd. Als een alternatief voor de bicongene muizen, zullen we B.S^{chr6} congene muizen miR-511 of dexamethasone toedienen om het potentieel synergistisch effect na te gaan van hoge miR-511 levels en het distaal deel van SPRET/Ei chromosome 6 in een achtergrond van C57BL/6.

2.4 Identificatie van kandidaat genen

De identificatie van genen die verantwoordelijk zijn voor de effecten op de TNFR1 regulatie vereist idealiter een combinatie van benaderingen, waaronder het genetische fijn mappen van elke mogelijke QTL in congene, dubbelcongene en subcongene stammen en een genoomwijde expressiestudie om differentieel tot expressie gebrachte genen op te sporen in SPRET/Ei.
Aangezien we echter een zeer opmerkelijke correlatie vonden tussen TNF resistentie en de TNFR1 eiwitexpressie, beide geassocieerd met de post-transcriptionele regulatie van TNFR1 *in trans*, hebben we ons gericht op microRNAs (miRNAs) die een invloed kunnen hebben op TNFR1. Bovendien werd de rol van TACE in dit verhaal uitsloten omdat de concentratie van TNFR1 in het serum even laag was als in de organen, in SPRET/Ei ten opzichte van C57BL/6. miRNAs hebben zich ontpopt zeer belangrijke post-transcriptionele regulatoren van genexpressie en zijn betrokken bij diverse fysiologische en pathologische processen. (31)
Enerzijds zochten we naar miRNAs waarvan voorspeld wordt dat ze *Tnfrsf1a* als doelwitgen hebben. Met behulp van verschillende programma's op basis van differentiële algoritmes, waren we in staat om een vergelijkende analyse te maken. Anderzijds deden we een microRNA expressie profiling van 541 miRs op naïeve levers van C57BL/6 en SPRET/Ei. Door de lijst met voorspelde miRNAs te combineren met de lijst van differentiële miRNAs, maakten we een shortlist van de 20 meest interessante miRNAs die in detail bestudeerd

zullen worden. Naast functionele analyses willen we ook de bindingssequenties identificeren in *Tnfrsf1a* en andere mogelijke doelwit genen, de conservatie bepalen in het menselijk genoom en zoeken naar literatuur- en ENCODE gegevens met betrekking tot deze miRNAs.
In het kader van deze thesis hebben we ons gericht op miR-511 en miR-680, twee voorspelde, differentieel geëxpresseerde miRNAs die zijn gelegen in de regio's die gelinkt zijn met TNF resistentie en TNFR1 eiwitexpressie.
miR-511 is gelegen op 14 Mb-10.48 cM van chromosoom 2, in intron 5 van het gen dat codeert voor mannose receptor C type 1 (*Mrc-1*). Mrc-1 is een lectine of koolhydraatbindend eiwit dat voornamelijk voorkomt op het oppervlak van macrofagen en dendritische cellen. Het herkent en bindt mannose-rijke glycanen op het oppervlak van potentieel pathogene virussen, bacteriën en schimmels en de activering ervan stimuleert endocytose en fagocytose van de pathogenen door het complementsysteem. (32) Afhankelijk van het ligand kunnen mannose-receptoren ook signalisatie induceren die leidt tot MAP-kinase en NF-kB activering of indirect werken door het beinvloeden van TLRs. MIMP, het functionele domein van oppervlakte eiwitten van probiotische *Lactobacillus* species, bijvoorbeeld, bindt via Mrc-1 aan darmepitheelcellen en voorkomt de vasthechting en invasie van pathogenen zoals *E.coli*. (33) MIMP vermindert de intestinale permeabiliteit door p38 signalisatie via een mechanisme dat Mrc-1 vereist. (34)
Onlangs werd aangetoond dat de expressie van miR-511 correleert met die van het Mrc-1 mRNA, waarbij het idee wordt ondersteund dat een intronisch miRNA wordt gereguleerd door dezelfde promotor als het gastheergen. (35) We vonden inderdaad dat zowel de miR-511 als Mrc-1 mRNA niveaus significant hoger waren in SPRET/Ei muizen dan in C57BL/6 muizen.
miR-680-1 ligt intergenisch op 130 Mb-63,44 cM van chromosoom 6, ongeveer 4 cM voorbij het *Tnfrsf1a* gen.

2.5 microRNA-511 reguleert TNFR1 eiwitexpressie

Om na te gaan of miR-511 of miR-680-1 in staat zijn om TNFR1 expressie te beïnvloeden, voerden we een aantal functionele tests uit *in vitro* en *in vivo*.
De miRNAs werden door middel van transfectie met miRNA precursor moleculen binnengebracht in primaire hepatocyten en fibroblasten en op verschillende tijdstippen na transfectie keken we naar de cel gebonden TNFR1 eiwitexpressie. We vonden dat miR-511 de TNFR1 expressie duidelijk deed dalen en dit het effect was het meest uitgesproken op 24 uur na transfectie. Een van de twee voorspelde miR-511 doelsequenties in de 3'UTR van *Tnfrsf1a* bevat twee SNPs in SPRET/Ei ten opzichte van C57BL/6. miR-511 verlaagt echter de TNFR1 expressie in zowel C57BL/6 als in SPRET/Ei, wat aangeeft dat het doelsequentie in beide genomen kan herkennen en binden. miR-680-1 was niet in staat de TNFR1 expressie te veranderen, althans niet in deze experimentele omstandigheden. Om verder aan te tonen dat miR-511 de TNFR1 expressie reguleert, blokkeerden we de endogene miRNA door middel van een anti miR die specifiek bindt aan miR-511. Deze inhibitie leidde tot een significante inductie van cellulaire TNFR1 eiwitexpressie eveneens in zowel C57BL/6 als

SPRET/Ei. De pri-miR-511 sequentie bevat inderdaad geen SNPs noch INDELs in SPRET/Ei in vergelijking met C57BL/6.
Om de doelsequentie van miR-511 te bevestigen kloneerden we de 3'UTR van het *Tnfrsf1a* gen van zowel C57BL/6 als SPRET/Ei in de psiCHECK-2 luciferase reporter vector. Co-transfectie van elk van deze plasmiden met pre-miR-511 precursor moleculen in HEK-293T cellen verminderde de Renilla luciferase activiteit. Dit illustreert verder dat miR-511 de *Tnfrsf1a* doelsequenties beïnvloedt in C57BL/6 en SPRET/Ei. Om te weten welke van de voorspelde doelsequenties werkelijk functioneel zijn, kunnen we nu mutaties aanbrengen in de 3'UTR sequentie in de reporter assay. Dit is belangrijk aangezien enkel de polymorfe doelsequentie de genetische link met chromosoom 6 kan verklaren. miR-511 is jammer genoeg nog niet aanwezig in de StarBase collectie van CLIP-Seq data. Een CLIP-Seq analyse zou ook specifieke miRNA-mRNA interacties kunnen identificeren.
Om te onderzoeken of over-expressie van miR-511 ook *in vivo* TNFR1 kan neer-reguleren, injecteerden we een precursor miR-511 expressieplasmide in C57BL/6 en SPRET/Ei muizen door middel van hydrodynamische staartader injectie. Deze techniek leidt tot een efficiënte, transiënte transfectie, voornamelijk van de hepatocyten. (36) We zagen inderdaad een significante daling van de TNFR1 eiwitexpressie in de lever van beide muizenstammen, waaruit blijkt dat miR-511 de TNFR1 eiwitexpressie *in vivo* kan beïnvloeden. Bovendien leidde inhibitie van endogene miR-511 door hydrodynamische staartader injectie van anti miRs tot een op-regulatie van de TNFR1 expressie. Uit toekomstige experimenten zal moeten blijken of miRs en anti miRs ook kunnen worden opgenomen door andere organen zoals milt, long en darm na hydrodynamische injectie en ook daar hun functie kunnen uitoefenen. Als alternatief onderzoekt onze groep ook andere toedieningswijzen zoals *in vivo* elektroporatie, met reeds veelbelovende resultaten in de hersenen en de quadriceps van muizen.
Deze resultaten tonen aan dat miR-511 de TNFR1 eiwitexpressie beïnvloedt. In de toekomst moeten we verder zoeken naar andere mogelijke doelwitten van miR-511, in het bijzonder genen die betrokken zijn bij inflammatie.

2.6 microRNA-511 medieert resistentie tegen TNF-geïnduceerde inflammatie

Hoewel het effect van miR-511 op endogeen TNFR1 slechts ongeveer 20 % bedraagt, blijkt dit toch voldoende groot om bescherming te induceren tegen TNF-geïnduceerde inflammatie. Bovendien resulteert het blokkeren van endogene miR-511 in de lever en de daaruit voortvloeiende verhoging van TNFR1 eiwitexpressie tot hogere TNF gevoeligheid in C57BL/6 en in (BxS)F1 muizen. Deze resultaten zijn overeenstemming met experimenten uitgevoerd in samenwerking met George Kollias, waarbij een 30 % reductie van TNFR1 expressie leidde tot een robuuste bescherming, maar lijken in strijd te zijn met de resultaten verkregen met Alfp-Cre *Tnfrsf1a* $^{fl/fl}$ conditionele reactiveringsmutanten en conditionele knock-out muizen. (30) Villin-Cre *Tnfrsf1a* $^{fl/fl}$ muizen met *Tnfrsf1a* deficiëntie in darmepitheelcellen zijn significant beschermd tegen TNF-geïnduceerde toxiciteit, hetgeen een belangrijke rol voor de darm suggereert in TNF-geïnduceerde inflammatie waarschijnlijk

door de influx van darmbacteriën of bacteriële producten. (37, 38) Muizen met hepatocyt specifieke *Tnfrsf1a* deficiëntie vertonen evenwel een lichte bescherming tegen TNF-geïnduceerde toxiciteit in vergelijking met controles, wat suggereert dat de lever zeker niet kan worden uitgesloten als belangrijk orgaan in inflamatie.
Onze resultaten tonen aan dat miR-511 een echte regulator is van TNFR1 eiwitexpressie en in staat is om de TNF respons te beïnvloeden *in vivo*. Bovendien tonen ze verder aan dat de genetische link tussen TNF resistentie en TNFR1 expressie enerzijds en proximaal chromosoom 2 en distaal chromosoom 6 anderzijds kan wijzen op de *miR-511* en *Tnfrsf1a* genen. Hoewel geen genetisch verschil te vinden is in de miR-511 sequentie tussen C57BL/6 en SPRET/Ei, is miR-511 duidelijk op-gereguleerd in SPRET/Ei muizen volgend op de op-regulatie van het *Mrc-1* gen. De link met chromosoom 2 kan daarom misschien wijzen op een genetisch verschil in de regulatie van miR-511, eventueel in de GRE sequenties (zie volgende paragraaf).
Aangezien miR-511 ook beschreven wordt als een regulator van de LPS receptor TLR4, onder bepaalde voorwaarden (39), hebben we aangetoond dat het effect van miR-511 in onze resultaten wordt gemedieerd door specifieke TNFR1 respressie. De bescherming van miR-511 voorbehandelde muizen tegen LPS-geïnduceerde endotoxemie, een model waarin TNF duidelijk betrokken is (40), was enkel te zien in C57BL/6 en niet in TNFR1 deficiënte muizen.
Voor therapeutische toepassingen zijn we momenteel nog beperkt tot lever specifieke TNFR1-gemedieerde ziektemodellen zoals concanavaline A (conA)-geïnduceerde hepatitis. Dit cel-gemedieerd immunoinflammatoir ziektemodel voor humane auto-immune hepatitis kan worden geïnduceerd door een eenmalige intraveneuze injectie van conA. Activatie van T-cellen veroorzaakt secretie van TNF dat bindt aan TNFR1 op macrofagen. De daaropvolgende infiltratie van neutrofielen en macrofagen in de lever leidt tot apoptose en necrose van de hepatocyten. (41, 42) miR-511 kon significante bescherming induceren tegen conA-geïnduceerde hepatitis zoals te zien in een normale lichaamstemperatuur en zo goed als geen inductie van serum ALT levels. Een normale inductie van TNF levels toonde aan dat conA effectief inflammatie induceerde, maar dat de miR-511 gemedieerde repressie van TNFR1 de TNF respons bepaalde.

2.7 microRNA-511-gemedieerde TNFR1 repressie wordt gereguleerd door GR activiteit.

In vergelijking met C57BL/6, vertonen SPRET/Ei muizen hogere levels van de glucocorticoïd receptor (GR) en daardoor verhoogde GR activiteit. (24) Glucocorticoïden (GC) zoals corticosteron bij knaagdieren, cortisol bij mensen of synthetische stoffen zoals dexamethason, hebben sterke anti-inflammatoire eigenschappen, die vooral worden veroorzaakt door het remmen van de productie van pro-inflammatoire cytokines via transrepressie mechanismen, bijvoorbeeld eiwit-eiwit interacties tussen de GR en transcriptiefactoren zoals NF-kB en AP-1. (43) Na binding van glucocorticoïden, zal de GR zich verplaatsen naar de kern waar hij verschillende functies kan uitvoeren. Naast transrepressie , kan de GR ook transcriptie induceren door transactivering. De GR treedt op als homodimere transcriptiefactor die bindt aan de glucocorticoïd respons elementen (GREs)

in promotorgebieden van GC-induceerbare genen of als een monomeer eiwit dat samenwerkt met andere transcriptiefactoren om transcriptie te induceren. (44) Onze onderzoeksgroep heeft recent gevonden dat de dimere GR veel belangrijker is dan de monomere vorm in het beschermen tegen TNF toxiciteit en dat de GR-geïnduceerde genexpressie hierin essentieel is. (45)

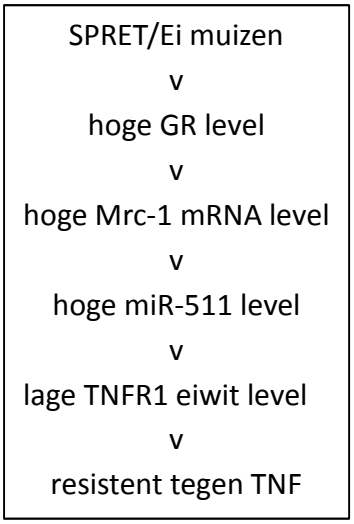
Om na te gaan of glucocorticoïden een rol spelen in de (miR-511 gemedieerde) TNF resistentie van SPRET/Ei muizen, behandelden we C57BL/6 en SPRET/Ei muizen met de GR antagonist RU486. We vonden dat een onomkeerbare blokkering van de GR met RU486 SPRET/Ei muizen gevoelig maakt voor TNF waardoor de verschillen in TNF respons tussen SPRET/Ei en C57BL/6 muizen volledig verdwijnen. Deze resultaten geven aan dat de GC-GR actie essentieel is voor de extreme resistentie van SPRET/Ei muizen tegen TNF-geïnduceerde letaliteit.

De consensus GRE sequentie voor transactivatie is een omgekeerd, onvolmaakt hexameer palindroom, gescheiden door een spacer van 3 bp (5'-AGAACAnnnTGTTCT-3‘). (46) Met behulp van ConTra vonden we twee GRE elementen in de 5' UTR van Mrc-1 op positie -1167 tot -1152 en -599 tot -584 ten opzichte van de transcriptie startplaats (TSS). Bovendien vonden we ook een GR half bindingsplaats op positie -20 tot -2, net voor de TSS. Deze vindingen suggereren dat de GR zou kunnen binden op de promotor van Mrc-1 en zo transcriptie induceren. Verder zagen we na toediening van rapidexon, een oplosbare vorm van dexamethasone, in C57BL/6 muizen een significante inductie van Mrc-1 genexpressie met behulp van qPCR in de lever en in de milt. Aangezien glucocorticoïden worden geproduceerd door de bijnieren daalt de expressie van GR-induceerbare genen na verwijdering van deze klieren door adrenalectomie. We zagen inderdaad een significante neer-regulatie van Mrc-1 en miR-511 in geadrenalectomiseerde muizen. Hoewel we voorlopig nog geen fysiek bewijs hebben, bijvoorbeeld gebaseerd op ChIP-seq analyse van GR binding GR op Mrc-1 of een promotor analyse via een Mrc-1 promotor construct, suggereren onze resultaten dat de GR is opgereguleerd in SPRET/Ei en kan binden op de promotor van Mrc-1 en zo de transcriptie van Mrc-1 and miR-511 kan induceren. Dexamethasone toediening aan C57BL/6 muizen veroorzaakt namelijk ook een neer-regulatie van TNFR1 eiwitexpressie terwijl adrenalectomie een op-regulatie van TNFR1 veroorzaakt.

Aangezien we geen genetische link vonden tussen TNF resistentie of TNFR1 levels en de GR locus op chromosoom 18, veronderstellen we dat deze fenotypes wel afhangen van de GR, maar gelinkt zijn met een GRE gen dat het anti-inflammatoir effect uitvoert en gelegen is op proximaal chromosoom 2, namelijk de *Mrc-1/miR-511* locus. Het is dus van belang om het expressieprofiel van miR-511 te onderzoeken in zowel naïeve omstandigheden als tijdens inflammatie.

Dus, aangezien SPRET/Ei muizen een hogere GR expressie vertonen vergeleken met C57BL/6 muizen en de GR Mrc-1 expressie kan induceren, suggereren we dat de hogere level van miR-511 in SPRET/Ei wordt veroorzaakt door GR inductie. Omdat miR-511 zorgt voor repressie van TNFR1 in SPRET/Ei muizen wat leidt tot bescherming tegen TNF-geïnduceerde

inflammatie, veronderstellen we dat deze resistentie van SPRET/Ei tegen TNF-geïnduceerde letaliteit wordt gemedieerd door GR-geïnduceerde over-expressie van Mrc-1 en miR-511, wat resulteert in een lagere TNFR1 expressie. (Figuur 1) Aangezien het blokkeren van de GR veel effectiever is dan de toediening van miR-511, zullen zeker ook nog andere GR gemedieerde mechanismen een rol spelen. De bescheiden repressive van TNFR1 door miR-511 kun echter toch een significante bescherming bieden. Daarenboven worden miR-511 en anti miR-511 specifiek afgeleverd in de lever. Daarom zal het zeer interessant zijn om het effect te bestuderen in volledig miR-511 deficiente en overexpresserende muizen.
Als onze hypothese bevestigd wordt, zullen we een sterk punt hebben om aan te tonen dat de anti-inflammatoire acties van GCs, via GR dimerisatie, deels via inductie van miRNAs verlopen, hetgeen een geheel nieuw mechanisme is.



Figuur 1. Voorstelling van onze hypothese.

2.8 Aanmaak en karakterisering van TNFR1 bindende en inhiberende nanobodies

De TNF signalisatie is een belangrijk doelwit in de behandeling van auto-immuunziekten. Anti-TNF behandeling werd reeds met succes toegepast voor het tegengaan van inflammatie-geïnduceerde schade in reumatoïde artritis, inflammatoire darmziekten en psoriasis. Helaas werden een aantal bijwerkingen gerapporteerd waaronder een verhoogd risico op infecties en tumoren en het ontstaan van bijkomende auto-immuunziekten. Sommige van deze effecten worden veroorzaakt door de ongewenste inhibitie van gunstige TNF signalisatie zoals resistentie tegen infecties en het herstel van aangetaste weefsels, acties die voornamelijk afhankelijk zijn van TNFR2. Vandaar dat een meer specifieke inhibitie van de pathologische TNF/TNFR1 signalisatie kan leiden tot een bredere toepasbaarheid en verbeterde veiligheid. Deze specificiteit kan verkregen worden door inhibitie van TNFR1 terwijl de TNFR2 signalisatie ongeremd blijft doorgaan. Verschillende TNFR1 blokkers, waaronder domein antilichamen van GSK en antagonistische TNF mutanten (R1antTNF), hebben reeds bewezen dat ze eenzelfde therapeutisch effect bereiken als TNF-blokkers bij de behandeling van auto-immuunziekten zoals EAE, CIA en ventilator-geïnduceerde longschade, maar met minder bijwerkingen. (16, 47, 48)

Aangezien we de mogelijkheden van TNFR1 als therapeutisch doelwit verder willen onderzoeken en nog betere inhibitoren willen ontwikkelen, hebben we besloten om nanobodies te genereren tegen TNFR1. We denken dat de nanobody-technologie een goed platform is om TNFR1 specifieke inhibitors te ontwikkelen omdat nanobodies de voordelen van conventionele antilichamen combineren met belangrijke kenmerken van kleine moleculen. Zoals bij conventionele antilichamen, vertonen nanobodies een hoge specificiteit en affiniteit en een lage immunogeniciteit. Daarenboven kunnen ze, als kleine moleculen, gemakkelijk verborgen epitopen of receptorholtes bereiken. Verder zijn nanobodies uiterst stabiel, sterk oplosbaar en eenvoudig te produceren in bijna alle prokaryotische en eukaryotische gastheren zoals bacteriën en gisten. Bovendien worden ze gecodeerd door een enkel gen, wat het ontwerpen van modulaire eiwitten mogelijk maakt door nanobodies te combineren met elkaar of met andere moleculen. De TNFR1 nanobodies kunnen, bijvoorbeeld, gekoppeld worden aan MMP-8 inhiberende nanobodies. MMP-8 inhibitie medieert resistentie tegen sepsis voornamelijk door effecten in de hersenen, terwijl voor resistentie door TNFR1, inhibitie in de darm en de lever belangrijk zijn. Door de 2 nanobodies te koppelen, zouden we in staat kunnen zijn om een meer uitgesproken resistentie te bekomen zoals reeds gezien werd in TNFR1/MMP-8 dubbel deficiënte muizen. Een andere mogelijkheid is het koppelen van TNFR1 nanobodies aan TACE of een TACE nanobody (Ablynx) om specifieke shedding van TNFR1 te bekomen. Door nanobodies te genereren, creëren we onbeperkte mogelijkheden.

De TNFR1 Nanobodies werden gegenereerd door immunisatie van een alpaca met recombinant humaan TNFR1. Na een intensieve screening werden twee TNFR1 specifieke nanobodies geselecteerd. Nanobody nb70 heeft een goede bindingsaffiniteit van ongeveer 50 nM en is het enige geteste nanobody dat TNFR1 signalisatie inhibeert met een capaciteit van 1,34 μM. Helaas geeft nb70 een zeer lage opbrengst die waarschijnlijk gerelateerd is met de sequentie. Nanobody nb96 heeft een nog betere bindingsaffiniteit van 10 nM, maar kan de TNFR1 signalisatie niet blokkeren. Beide nanobodies zijn herkennen specifiek de humane TNFR1, gezien er geen kruisreactiviteit is met de humane TNFR2. Helaas, en ondanks de hoge gelijkenis, binden geen van de nanobodies met muis TNFR1, wat hun *in vivo* karakterisering zal beperken tot experimenten in hTNFR1 KI muizen of andere gehumaniseerde modellen, maar waardoor ze wel waardevol zijn als therapie tegen auto-immune ziekten bij mensen.

Om de nanobodies in vivo te kunnen gebruiken, maakten we multimere nanobodies om de halfwaardetijd te verbeteren. Door het inhiberend nb70 te koppelen aan het best bindende nb96 en een nanobody tegen humaan albumine, wilden we de farmacokinetiek en -dynamiek verbeteren alsook de inhiberende capaciteit. We maakten een dimeer nb70-96 gekoppeld door een starre hIgA1 linker en een trimeer nbAlb-70-96 en nbAlb-70-70, beide met een soepele $(G_4S)_3$ linker. De opbrengst van de productie in E.coli was echter zeer laag, waarschijnlijk door de aanwezigheid van nb70. Daarom besloten we om over te schakelen naar de gist *Pichia pastoris* voor de productie van trimere nanobodies, wat leidde tot een aanzienlijk hogere opbrengst. De introductie van enkele extra aminozuren voor de klonering

in een gist-expressievector veranderde gelukkig niets aan de bindingscapaciteit of de inhiberende capaciteit.
Het bindingscapaciteit van nb70-96 (3 nM) is veel beter dan deze van de enkelvoudige nanobodies en de trimere nanobodies tonen een intermediaire bindingscapaciteit (5 nM en 9 nM), vermoedelijk als gevolg van de toevoeging van een nanobody tegen albumine. Belangrijk is ook dat de trimere nanobodies voldoende hoge (cross-reactieve) binding vertonen aan muis serum albumine, wat ze bijzonder geschikt maakt voor *in vivo* gebruik. Het inhiberend vermogen van de dimere nb70-96, namelijk 0,926 uM, is iets beter dan dit van nanobody nb70, maar dit van het trimere nanobody nbAlb-70-96 is zelfs nog beter met een IC50 van 0,395 μM. Uit epitoop mapping bleek dat nb70 en nb96 twee verschillende epitopen herkennen, wat de hogere affiniteit en daardoor de betere inhibitie van het gecombineerde nanobody nb70-96 kan verklaren.
Een Biacore competitie assay toonde dat het inhiberende nb70 in competitie gaat met hTNF door het blokkeren van de binding ervan aan TNFR1. De competitie is zelfs nog meer uitgesproken in de multimere nanobodies, vooral wanneer de nanobodies vooraf worden gegeven, in een profylactische setup. Een *in silico* voorspelling van de bindingsplaats bevestigt dat nb70 in staat is de binding van TNF aan zijn receptor te verhinderen door competitie voor dezelfde regio van TNFR1. nb70 kan binden met een uitstulpende CDR3-lus in de holte die gevormd wordt door CRD3 en CRD4 van TNFR1, en daardoor een deel van het ligandbindend domein van TNFR1 bedekken met de rest van het nanobody. De exacte conformatie van de hTNFR1-nb70 binding zou echter bepaald moeten worden aan de hand van een kristallografische studie.
Op basis van de verkregen resultaten kunnen we concluderen dat het trimeer nanobody nbAlb-70-96 de beste inhibitor is van TNF-geïnduceerde NF-kB activatie, met een hoge bindingsaffiniteit voor hTNFR1 en muis albumine. Dit nanobody zal verder gekarakteriseerd worden *in vivo*. Een voorlopige farmacokinetische studie toonde reeds dat bij intraveneuze injectie, dit nanobody geabsorbeerd wordt uit het plasma en gedistribueerd wordt naar de weefsels tot 8 uur na injectie, gevolgd door een geleidelijke eliminatie. De geschatte halfwaardetijd van min of meer 24 uur is gelijk aan deze van andere multimere nanobodies zoals het trimere αEGFR-αEGFR-αAlb nanobody. (49) Bovendien geeft de vergelijkbare farmacokinetiek, van αEGFR-αEGFR-αAlb en muis serum albumine zelf, aan dat het albumine nanobody zorgt voor een zeer sterke *in vivo* binding aan albumine. (50) Aangezien het albumine nanobody ook een hoge affiniteit vertoont voor humaan serum albumine en humaan serum albumine een bloed halfwaardetijd heeft van ongeveer 20 dagen (51), mogen we verwachten dat het trimeer nanobody nbAlb-70-96 een veel langere halfwaardetijd zal hebben in de mens.

3. Toekomstperspectieven

miR-511 is een potentiële therapeutische molecule om TNFR1-gemedieerde ziekten te behandelen. Aangezien elke miRNA mogelijk honderden mRNAs kan beïnvloeden en daardoor de regulatie van verschillende signalisatie wegen, zal het echter belangrijk zijn om

het mechanisme en de cellulaire targets van miR-511 volledig te karakteriseren om negatieve gevolgen te vermijden. Momenteel zijn slechts enkele voorbeelden van therapeutische toepassingen van miRs bekend. Miravirsen, een LNA-gemodificeerde anti miR-122, biedt een langdurige dosis-afhankelijke antivirale activiteit in klinische studies met patiënten met een chronische HCV-infectie. (52) Tot nu toe werden er geen ernstige negatieve gevolgen van miR-122 inhibitie waargenomen bij chimpansees (53) of in fase II klinische studies. Maar uit lange termijn studies zal moeten blijken of de over-expressie of inhibitie van miRNAs geen schadelijke bijwerkingen veroorzaakt. Om de (patho) fysiologische rol van miR-511 verder te onderzoeken, zullen we miR-511 deficiënte en transgene miR-511 overexpresserende muizen genereren.

miR-511 deficiënte ES-cellen van het EUCOMM consortium zullen geïnjecteerd worden, in samenwerking met Dr. Tino Hochepied, in C57BL/6 blastocysten wat zal resulteren in chimere muizen waarbij sommige cellen in elk orgaan afkomstig zijn van de ES cellijn. Aangezien de ES-cellen heterozygoot zijn voor de miR-511 deletie, zal ongeveer de helft van de nakomelingen de modificatie dragen. Kruisen van de chimeren met C57BL/6 muizen en daaropvolgende intercrossen kunnen resulteren in homozygote miR-511 deficiënte muizen. Deze muizen zullen uitgebreid worden gekarakteriseerd wat betreft hun TNFR1 expressie, hun resistentie tegen TNF-geïnduceerde inflammatie modellen en potentiële spontane pathologieën.

miR-511 transgene muizen zullen worden gegenereerd in nauwe samenwerking met prof. Thierry Vandendriessche en prof. Marinee Chuah, die een snel en doeltreffend systeem op basis van de Piggy-Back transposon hebben ontwikkeld. Zowel tijdelijke als blijvende transmissie kan geïnduceerd worden door de introductie van twee plasmiden. Een plasmide bevat het Piggy-Back transposon (Tp), waarin de miR-511 sequentie geflankeerd wordt door Tp inverted repeats. Het andere plasmide bevat het hyperactieve Piggy-Back transposase gen (TPase) dat voor een zeer efficiënte integratie van Tp zal zorgen. Aangezien we de plasmiden zullen binnenbrengen door hydrodynamische injectie, verwachten we dat de integratie en over-expressie van miR-511 voornamelijk zal plaatsvinden in levercellen, waar het een aantal maanden actief zal blijven. Daarnaast zullen we permanente transgene muizen gegenereren door rechtstreekse injectie van de plasmiden in de testis, gevolgd door elektroporatie van de testis. Ook deze muizen zullen uitgebreid worden gekarakteriseerd wat betreft hun TNFR1 expressie, hun resistentie tegen TNF-geïnduceerde inflammatie modellen en potentiële spontane pathologieën.

TNFR1-selectieve inhibitie door nanobody nbAlb-70-96 resulteerde in het blokkeren van de inflammatoire signalisatie van TNFR1, wat *in vitro* aangetoond werd door inhibitie van TNF-geïnduceerde NF-kB activatie. De *in vitro* karakterisering zal binnenkort worden aangevuld met expressie gegevens van NF-kB en AP-1-geïnduceerde genen zoals IL6, IL8, TNF, A20 en IKKα. Voor toekomstige *in vivo* experimenten zijn we in onderhandeling met het bedrijf Biomedcode voor het gebruik van hTNFR1KI muizen om het nanobody te testen in inflammatie modellen zoals TNF-geïnduceerde letaliteit en LPS-geïnduceerde endotoxemie. In deze experimenten zullen de eigenschappen van het nanobody worden vergeleken met

een trimeer anti-βlactamase controle nanobody, namelijk nbAlb-BCll10-BCll10, en de TNF-blokker Etanercept. Later kan nbAlb-70-96 dan getest worden als een therapeutisch geneesmiddel, in het bijzonder voor ziekten waartegen de huidige anti-TNF geneesmiddelen falen of het ziekteproces zelfs verergeren, zoals multiple sclerose (EAE) en reumatoïde artritis (in ΔARExhTNFR1KI muizen). Ondertussen zullen we ook onze eigen hTNFR1KI muizen ontwikkelen door het injecteren van een hTNFR1 bevattende BAC kloon in C57BL/6 zygotes. De resulterende transgene muizen zullen een willekeurige integratie van hTNFR1 vertonen en worden gekruist met mTNFR1 deficiënte muizen. Deze 'knock-in' muizen zullen gebruikt worden om de therapeutische niche van het nanobody verder te onderzoeken in verscheidene TNFR1-gemedieerde modellen zoals CLP-geïnduceerde polymicrobiële sepsis en nier ischemie en reperfusie. Daarnaast hebben we contacten met prof. Geert Leroux-Roels om transgene uPA-SCID muizen te gebruiken. Deze muizen lijden aan een transgen-geïnduceerde leverziekte en worden reeds vroeg na de geboorte getransplanteerd met primaire humane hepatocyten, resulterend in muizen met een gedeeltelijk gehumaniseerde lever. (54) We zullen testen of nbAlb-70-96 in staat is om te beschermen tegen TNF/Galactosamine-geïnduceerde leverontsteking in deze muizen door bepaling van de inductie van humane ALT levels en de expressie van NF-kB en AP-1-induceerbare genen. In een latere fase willen we ook onderzoeken of darm-specifieke aflevering van het nanobody door middel van *Lactococcus lactis* nuttig kan zijn in een model van IBD zoals ΔARE-geïnduceerde ileïtis. Dit zal gebeuren in samenwerking met het bedrijf Actogenix.
Aangezien de hTNFR1 nanobodies niet binden met muis TNFR1, hadden we besloten om een nieuwe panning uit te voeren met recombinant muis TNFR1 op de bestaande klonen van de hTNFR1 nanobody gen bibliotheek. Helaas bevatte de recombinante muis TNFR1 oplossing die we gebruikten, een overmaat aan BSA als carriëreiwit, met als gevolg de selectie van nanobodies tegen BSA in plaats van tegen mTNFR1. Dit kan worden verklaard door het feit dat de alpaca's verschillende keren worden hergebruikt voor de immunisatie met meerdere antigenen. Sommige van deze antigenen kunnen BSA hebben als carriëreiwit of een van de antigenen kan zelfs BSA zelf zijn. Daarom waren we genoodzaakt om een nieuwe panning te starten met carriervrij recombinant mTNFR1. Deze panning is momenteel aan de gang.

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CHAPTER VI

Addenda

Curriculum vitae

1. Personal data

name	Leen Puimège
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born	Lokeren, 11 October 1974
nationality	Belgian

2. Education

2004 – 2009	Master in Sciences: Biotechnology University of Ghent - Dresden University of Technology
Master thesis:	'HIF en MMPs in LPS geïnduceerde endotoxische shock: is er een verband?'
1996 – 1999	Graduate 'Farmaceutische en Biologische Technieken' KaHo Sint Lieven, Ghent
Graduate thesis:	'Mappen van microsatellieten rond de TNFp locus en studie van de respons van neutrofielen-elastase-deficiënte muizen.'

3. Professional experience

2009 – 2013	PhD in Sciences: Biotechnology VIB – University of Ghent
PhD thesis:	'A molecular genetic analysis of TNFR1 regulation in SPRET/Ei and generation of new TNFR1 inhibitory tools.'
1999 – 2009	Technician in the Mouse Genetics in Inflammation Unit Inflammation Research Center (VIB – UGent)

4. Skills

in vivo mouse experiments, DNA/RNA/protein isolation, PCR, RT-PCR, Q-PCR, genotyping, protein purification, western blot, zymography, ELISA, NO-assay, MPO-assay, histology, *in situ* hybridization, immunohistochemistry, macro-array and cloning

5. Courses

2005	LightCycler 480 training course (Roche Vilvoorde)
2006	Workshop Ensembl (DMBR UGent)
2006 – 2007	Proefdierkunde BCLAS cat. B (Erasmus Hogeschool Brussel – VUB)
2008	Ensembl training (DMBR – UGent)
2009	EHBO training (idewe)
2009	Workshop Nanobody imaging and therapy (VUB)
2009 – 2010	Laboratory Animal Science part I and II (UGent)
2010	EHBO training (idewe)

2010	Essential statistics course (BITS VIB)
2010	TATAA Biocenter qPCR training course (Selected Biosciences Dublin)
2010	Genevestigator training (BITS VIB)
2011	Nanobody and protein production training (VRTC VIB)
2011	Ingenuity Pathway analysis training (BITS VIB)
2011	EHBO training (idewe)
2011	Advanced Academic English: Writing skills (UCT UGent)
2011	Introduction to R course (MRC SGDP Summer School London)
2012	Workshop CLC Main Workbench (DMBR UGent)
2012	EHBO training (idewe)
2012	Workshop Impact and Research Communication Skills (UGent)
2013	Presentation Skills (Principiae)

6. National Conferences

2008	Annual BIS meeting (Liège)
2008	FWO meeting 'targets in inflammatory & autoimmune diseases'(Leuven)
2009	VIB science club cancer
2010	IUAP meeting WP4&5 (Liège)
2011	VIBes in Biosciences (Leuven)
2011	IUAP-VI-18 meeting (Ghent)
2011	Annual Meeting of EMDS (Brussels)
2011	VIB symposium 'Closer to the future' (Ghent)
2011	Annual BIS meeting (Hasselt)
2012	FWO meeting 'Signaling in inflammation and immunity' (Ghent)
2012	PhD symposium on Cytokines (Hasselt) oral presentation
2012	VIB seminar 2012 (Blankenberge) poster
2013	VIBes in Biosciences (Ghent)
2013	IUAP-VII-32 meeting (Ghent)
2013	VIB seminar 2013 (Blankenberge) oral presentation

7. International Conferences

2009	2nd Conference on Models for Immunological Disease (Athens – Greece)
2009	12th International TNF conference (Madrid – Spain) poster
2010	7th RNAi & miRNA – epigenetics (Dublin – Ireland) poster
2011	11th Introduction to R summer school (London – UK) poster
2011	13th International TNF conference (Hyogo – Japan) poster
2012	EMBO workshop on non-coding RNA (Ascona – Switzerland) poster

8. Student guidance

2009-2010	Hanne Crombez, Michiel Vandecasteele, Nele Vandersteen – bachelor 3
2009-2010	Adriaan Sticker – master 1
2010-2011	Filip Liebner – Erasmus student

2010-2011	Annelyn Wallaert – master 1
2011-2012	Elyn Hollevoet – professional bachelor
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9. References

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10. Relevant publications

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Puimège L., Van Hauwermeiren F., Lodens S., Vandenbroucke R.E., Dejager L., Staelens J., Van Roy M., Libert C. MicroRNA-511 is induced by glucocorticoids and protects against TNF via TNFR1 down-regulation. Manuscript in preparation.

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11. Additional information

languages Dutch: native language
English: good
French: moderate
German: moderate
Spanish: moderate

Manuscript in preparation

MicroRNA-511 is induced by glucocorticoids and protects against TNF via TNFR1 down-regulation.

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Acute inflammation is essential during host defense, but results in lethal conditions, such as shock, when dysregulated. TNF is essential during inflammatory shock and a well-recognized drug target. We have found that the mouse strain SPRET/Ei, which displays extreme and dominant resistance against TNF-induced shock, displays low expression of TNF receptor 1 protein (TNFR1) but normal mRNA, a trait genetically linked to the major TNFR1 coding gene Tnfrsf1a and a locus harboring the predicted TNFR1-regulating miRNA-511miR-511. This miRNA is a genuine TNFR1 regulator in cell systems. In mice, overexpression of miR-511 down-regulates TNFR1 and protects against TNF, while anti-miR-511 up-regulates TNFR1 and sensitizes for TNF. We show specificity for TNFR1 and therapeutic potential in endotoxemia and hepatitis. Furthermore, this miR is induced by glucocorticoids, suggesting a newly recognized mechanism by which glucocorticoids protect against acute inflammation.

Acute systemic inflammation is a central hallmark of many severe conditions, such as sepsis, severe burns, hemorrhage, ischemia/reperfusion and others. Although this inflammation serves to remove invading microorganisms and to restore homeostasis, an unbalanced regulation of the response is often fatal. This systemic inflammatory response syndrome (SIRS) is coordinated by a limited number of powerful pro-inflammatory cytokines. Tumor necrosis factor (TNF) is such a cytokine. It has pro-inflammatory activities, activates expression of many genes involved in cytokine production, white blood cell infiltration, blood pressure reduction and coagulation, but also induces cell death and wound healing and is involved in antibacterial immunity. Since TNF is such a central mediator in SIRS, injection of TNF in mammals, which leads to severe systemic inflammation and which is lethal with microgram doses, is considered an interesting model system. The acute inflammation induced by TNF is mediated by several secondary cytokines, such as interleukin-1 (IL-1), IL-17 and interferons, inhibition of which leads to some protection against TNF. TNF binds on two receptors, TNFR1 and TNFR2, the former of which is constitutively expressed on most cells, while the latter is inducible and has a more restricted expression pattern. The major activities of TNF (induction of inflammation and cell death) are mediated by TNFR1, while TNF's immune-regulatory effects e.g. on regulatory T cells and DC subtypes, are mediated by TNFR2. Because anti-TNF therapy, which is applied in inflammatory bowel diseases, rheumatoid arthritis and psoriasis, limits both TNFR1 and TNFR2 signals, part of the side-effects (e.g. appearance of psoriatic-like lesions) of this therapy may be prevented when TNFR1 is selectively inhibited. That TNFR1 is an interesting drug target was recently recognized by our studies that showed that the limited expression of TNFR1 of 50 % in TNFR1+/- mice led to virtually unlimited protection against TNF-induced SIRS and that these data were reproduced using specific TNFR1 blocking antibodies. In the search for new therapeutic targets for SIRS and other TNF-mediated diseases, the poorly understood regulation of the TNFR1 coding gene (Tnfrsf1a), located on distal chromosome 6 in the mouse genome should be investigated.

Glucocorticoids (GCs) are well known anti-inflammatory molecules, the expression of which is strongly activated during inflammation, as a negative-feedback loop, by the hypothalamic-pituitary (HPA) axis. GCs exert their function by binding to the ubiquitously expressed GC receptor, GR. Synthetic GCs such as dexamethasone have proven very effective in numerous inflammatory diseases, also in sepsis, and protect well against TNF-induced SIRS. The mechanism of action of GCs remains unclear, but our recent work strongly suggests that the formation of GC-stimulated GR dimers, followed by gene induction is essential, suggesting that GC/GR-induced genes are executing anti-inflammatory actions.

We have previously shown that the mouse strain SPRET/Ei displays an extreme and dominant

resistance against TNF-induced SIRS, and that this trait is linked to a locus on proximal chromosome 2 and another one on distal chromosome 6, the latter locus containing the Tnfrsf1a gene. We here report that despite the sequence variations in the SPRET/Ei Tnfrsf1a gene, this TNFR1 is fully functional, but that the expression of the TNFR1 in SPRET/Ei is significantly lower than in control C57BL6 mice, but only at the level of the protein, not the mRNA. Also this trait is linked to these chromosomes, suggesting strong correlation between low TNFR1 and TNF resistance. The locus on chromosome 2 contains miR-511, which we here confirm as a genuine TNFR1 regulator, and which is significantly up-regulated in SPRET/Ei mice. We show that therapeutic delivery of miR-511 to mice down-regulates TNFR1 protein and protects against TNF, but also against endotoxemic shock in a TNFR1 dependent way and against lethal hepatitis. We show that the TNF resistance of SPRET/Ei mice is completely depending on the overactive HPA axis of these mice and that the expression of miR-511 is induced by dexamethasone. We therefore hypothesize that GCs protect against TNF partly via induction of miR-511 and down-regulation of TNFR1.

RESULTS

SPRET/Ei mice have a low expression of a functional TNFR1.

We previously showed that, compared to C57BL/6 (B) mice, SPRET/Ei (S) mice are extremely resistant to lethal inflammatory shock induced by TNF. The LD100 of a single injection of TNF is >1000 μg in S mice and ~20 μg in B mice (Fig. 1A). This resistance is linked to proximal chromosome 2 (0-40 cM with a peak at 15 cM) and distal chromosome 6 (40-70 cM with a peak at 46 cM). With this resolution of mapping, the Tnfrsf1a gene, located at 60.55 cM and encoding TNFR1, is a most likely candidate resistance gene at the chromosome 6 locus. Sequence analysis showed that the TNFR1 protein of S has 10 AA changes compared to TNFR1 of B mice. By repetitive backcrossing the S genome into a B background for 10 generations, selecting for the S TNFR1 locus and distal part of chr6 using polymorphic markers located in and around the Tnfrsf1a locus, followed by intercrossing, we generated 99.9 % B congenic mice, harboring about 40 cM of distal chr6 with either two copies B TNFR1, two copies S TNFR1 or a copy of both (Fig. 1B). None of these mice showed any TNF resistance when injected with 20 μg TNF, suggesting that the S TNFR1 protein is functional (Fig. 1A).

Low protein expression and normal mRNA.

To further investigate the genetic link between TNF resistance and the distal chr6 locus, and because we had found that TNFR1+/- mice, expressing 50 % TNFR1, are completely resistant to TNF, we measured TNFR1 protein in several tissues of B and S and found that S mice express significantly less TNFR1 protein. In liver, we found that this trait is dominant and in spleen the levels were lowest. We also found reduced soluble TNFR1 in the serum of S mice, ruling out that the reduced tissue levels are the result of extreme shedding of soluble TNFR1. Furthermore, there was no difference in TNFR1 protein level in the three populations of congenic mice, suggesting that the TNFR1 ELISA detected B and S TNFR1 equally well, that the lack of protection of the congenic mice was reflected in normal TNFR1 levels and that the regulation of TNFR1 proteins happens in trans (Fig. 2A). Measurement of TNFR1 mRNA levels in B and S tissues, by QPCR with conserved primers, however, revealed no differences (Fig. 2B). To further prove the relation between TNF resistance and TNFR1 protein levels in S mice, we performed a new BSB genetic backcross, generating 214 N2 mice, in which we measured liver TNFR1 protein and performed genotyping using polymorphic markers. A clear link was found between low TNFR1 protein levels and proximal chr2 (0-40 cM) and distal chr6 (40-70 cM) (Fig. 2C), i.e. exactly the same subchromosomal regions that were linked with TNF resistance ($p=0.01$ that both traits are linked to the same chromosomes). The fact that the gene encoding TNFR1 (Tnfrsf1a) is located on distal chromosome 6 suggests that both traits are associated mainly with TNFR1 regulation.

MiRNA-511 is predicted and higher expressed in SPRET/Ei.

The significant in trans regulated reduction of TNFR1 protein, but not mRNA, could be based on a miRNA-based regulation. Based on the linkage data, we hypothesized that a miR on proximal chr2 might be involved. We have run 11 different prediction programs and found a group of 20 miRs that have the Tnfrsf1a gene as a target (Supplemental Fig. 2). Two miRs on chr2 were found, namely miR-296 (predicted only once and located on 97.9 cM) and miR-511, which was predicted by 6 programs and is located at 10.5 cM. MiR-511 is located in intron 5 of the Mrc1 gene, and was found to be strictly co-expressed by this gene. We measured miR-511 and Mrc-1 expression by

QPCR in livers and spleens of naïve B and S mice, and found significantly higher expression of both RNAs in both organs of S mice (Fig. 3A). By analysis of the sequence of the mature miR-511 in B and S we found no differences while a comparison of the 3'-UTR of the Tnfrsf1a gene in S revealed two miR-511 target sequences, one of which being identical with B and the other one showing two nucleotide differences that would lead to less complementarity with the miR-511 sequence (Fig. 3B).

MiRNA-511 is a genuine TNFR1 regulator and has therapeutic potential.

To study whether miR-511 is a genuine Tnfrsf1a regulator at the 3'UTR level in both B and S, we generated a reporter system, by cloning in the psiCHECK vector, the 3'UTRs controlling expression of a Renilla Luciferase gene in HEK293T cells. We found that transfection of premiR-511 significantly reduced luciferase expression, 48 h later, in both the B and S reporters (Fig. 4A). Similarly, when B and S MEF cultures were transfected with miR-511 and control miRs, TNFR1 protein levels measured in cell lysates 24 h later were significantly reduced in both B and S MEFs (Fig. 4B). Similar results were obtained using primary hepatocytes cultures (not shown). Furthermore, LNA-locked anti miRs specifically inhibiting miR-511 led to significant up-regulation of TNFR1 in both B and S MEFs, 24 h after transfection (Fig. 4C). We then evaluated whether miR-511 is able to regulate TNFR1 and response to TNF in vivo. Using a system of liver-specific plasmid delivery, based on hydrodynamic injection, pre-miR-511 under control of the CMV promoter was delivered to mice and 24 h later TNFR1 protein were measured or mice were injected with 20 μg TNF and hypothermia and lethal shock were observed. We found that miR-511 led to a 20 % reduction in TNFR1 protein expression, and that this was associated with a significant resistance to TNF-induced lethality and hypothermia (Fig 4D). To study the therapeutic potential of miR-511 in mice, as well as its specificity, we investigated whether miR-511 can protect mice against LPS-induced endotoxemia, a model in which TNF is centrally involved (1). Following hydrodynamic tail vein injection of miR-511, B mice were indeed significantly protected against an LD50 of LPS, i.e. 200 μg/mouse (Figure 4E). Because miR-511 was recently described as a regulator of TLR4, the LPS receptor (2), we studied whether the protection provided by miR-511 against LPS is mediated by specific TNFR1 regulation. measured by lethal response to a low dose of 5 μg To do so, we studied the protective effect of miR-511 against an LD50 of LPS in TNFR1 KO mice, i.e. 500 μg /mouse. As shown in Figure 4E, miR-511 did not protect against LPS in the absence of TNFR1, suggesting that miR-511 protects against LPS by down-regulating TNFR1. Finally, the therapeutic potential of miR-511 was further studied in a model of acute, TNF mediated hepatitis. 24 h after hydrodynamic pretreatment of mice with PBS, control miR or miR-511, mice were injected with Concanavalin A (ConA), leading to TNF release, cell death (ALT production) and hypothermia. Despite some minor effects of the control miRs, we found very significant protective effects of miR-511 against ALT release and hypothermia, while the amount of TNF produced remained similar (Fig. 4F). These results suggest that TNFR1 reduction by miR-511 has powerful therapeutic potential.

SPRET TNF resistance is dependent on miR-511 and on HPA axis.

To explore further whether miR-511 is linked with TNFR1 expression and TNF response, we delivered LNA-locked anti miRs by hydrodynamic injection to B mouse livers and studied TNFR1 levels and TNF response 24 h later. MiR-511-specific anti miR led to an increased expression of TNFR1 in livers of about 50 %, and to a very significant sensitization of B mice to TNF as measured both in lethal response as in hypothermia. Similar experiments were performed in (BxS)F1 mice (not in S mice for practical constraints) and resulted in a modest up-regulation of TNFR1 of 20 %, specifically by the miR-511 specific anti miR, and significant sensitization to TNF-induced hypothermia and lethality. These data suggest that inhibiting miR-511 leads to the loss of at least some fraction of the TNF resistance of these mice.

We then addressed the question about the regulation of miR-511 in mice. We have previously shown that S mice have an extremely overactive HPA axis, that they produce very high basal levels of corticosterone (CS), express high levels of the CS receptor (the glucocorticoid receptor, GR) and hence high basal levels of numerous GR-inducible (GRE-element containing) genes. We investigated whether the resistance to TNF of S mice depends on the HPA axis by pretreating mice with RU486, an irreversible inhibitor of the GR. We found that the spectacular TNF resistance of S mice was completely annihilated when GR was blocked by RU486, and that under such conditions, S and B mice become equally sensitive, as TNF, hypothermia and release of the inflammatory

mediator IL-6 (Fig. 5C). Furthermore, RU486 treatment of S mice indeed led to significantly increased expression of TNFR1 in liver and spleen (Fig. 5C).These data suggest that TNF resistance of S mice depends on GR biological activity. Since we found previously that protection of GR against TNF is depending on GR dimerization and induction of GRE-element containing genes, we next investigated whether the mouse Mrc-1 gene contains GRE elements in its promoter using the ConTra matrix and found two GRE elements and one GR half binding site (Fig. 5D).

MiR511 and TNFR1 are GC regulated.

To further support the dependency of Mrc-1 expression, miR-511 expression and TNFR1 regulation and hence sensitivity to TNF inflammation on GCs/GR, we performed adrenalectomy (Adx) to remove the adrenals as the major source of GCs in mice. We confirm that Adx significantly sensitizes mice for TNF-induced inflammation (Fig. 6A) (3) and found that Adx decreased the expression of several validated GRE-genes (Tsc22d3, encoding GILZ and Dusp1 encoding MKP1) as well as Mrc-1 and miR-511 in the spleen (Fig. 6A) and liver, as well as strongly increased the expression of TNFR1 in these organs (Fig. 6A).

Finally, synthetic GCs, such as dexamethasone (DEX) are very abundantly used as anti-inflammatory drugs, although there is still uncertainty about the mechanisms of action. As shown before, a low dose of 50 □ g DEX protects against TNF-induced lethal SIRS (Fig. 6B), and both in liver (Fig. 6B) and spleen (Fig. 6C) significantly induces expression of several GRE-genes, but also Mrc-1 and miR-511. Expression of TNFR1 mRNA remains unchanged. TNFR1 protein expression decreases significantly in both organs, as well as in the serum, suggesting that increased shedding of sTNFR1 is not involved in this response (Fig. 6D).

DISCUSSION

TNF is a central mediator in several inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease and psoriasis. Although our understanding of TNF biology is not complete, TNF inhibitors are being used successfully in patients suffering from these diseases (4). Significant groups of patients however do not respond to anti-TNF biological drugs, and long-term use of these agents can cause side-effects, such as the increased incidence of infection (5) and the development of certain autoimmune diseases, such as psoriasis (6). These side-effects are thought to be caused by inhibition of the immunomodulatory stimulation of TNFR2. More specific targeting of pathological actions could be possible by specifically blocking TNFR1 signaling while leaving TNFR2 signaling intact (7). Similarly, the lack of effect of anti-TNF drugs in sepsis may be based on the inhibition of TNFR2 signaling and thus also for sepsis, TNFR1 inhibition might be and interesting option.

Very little is known about the regulation of the TNFR1 coding gene Tnfrsf1a. The absence of canonical TATA and CCAAT boxes and the high GC content have been associated with the promoters of housekeeping genes (8). However, functional C/EBP (9) and NF-κB (10) binding sites are found. Moreover, IL-10 (11), IL-3 and GM-CSF (12) can down-regulate both mRNA and surface expression of TNFR1. The best described regulation of TNFR1 is at the post-transcriptional level, namely the shedding of TNFR1 by TACE/ADAM17 (13). TNFR1 shedding and the resultant acute decrease in the number of receptor molecules on the cell surface might serve to transiently desensitize cells to TNF action (14). In addition, the pool of soluble TNFR1 generated by shedding could function as physiological attenuators of TNF activity by competing for the ligand with the cell surface receptors (15). In humans, mutations affecting TNFR1 shedding have been linked with the development of TRAPS (TNF receptor-associated periodic syndromes) (16). These disorders are characterized by recurrent fever and localized inflammation. Additionally, mice expressing a non-sheddable TNFR1 have higher levels of TNFR1 in most tissues and are therefore extremely sensitive to TNF-induced and TNF-mediated inflammation (14), while mice only expressing 50 % TNFR1 show an extreme resistance to TNF-induced lethal inflammation. These findings suggest that understanding the mechanisms of TNFR1 regulation will be important to determine sensitivity to TNF and develop novel TNFR1 therapies.

Glucocorticoids (GCs) are well-known anti-inflammatory drugs which work by binding and activating the GR, a nuclear receptor. Despite long-term use of GCs often leads to side effects and large groups of patients show GC unresponsiveness, they are widely used (over 80 million prescriptions per year in the USA). The dominant action mechanism of GCs in SIRS has been shown to be depending on GR dimerization, followed by DNA binding on GRE elements and gene induction. Several GRE-genes with possible targets have been discovered in the genomes

anti-inflammatory functions have been described, such as those coding for GILZ and MKP-1.

The mouse strain SPRET/Ei is derived from the species Mus spretus, which has diverged some 1.5 million years ago from the house mouse, Mus musculus. The resulting genetic polymorphisms have also resulted in phenotypic differences that are relatively easy to map by linkage analysis. The SPRET/Ei (S) genome contains a SNP about every 100 bps compared to standard Mus musculus strains such as C57BL/6J mice. S mice have previously been found to be extremely resistant to TNF-induced SIRS, a trait that was found to be dominant and linked to proximal chr2 and distal chr6.

Because the Tnfrsf1a gene is centrally located in the critical region on chr6, we investigated the basis of the genetic linkage of TNF resistance to this gene. We here describe that S mice have 10 amino acids changes in TNFR1 compared to B, but that the S TNFR1 receptor is fully functional when introduced in a B background. The TNFR1 protein, however, is 2-5 fold lower expressed in S than B mice, in serum and in all organs tested. In previous work we have shown that low TNFR1 expression (50 % in TNFR1+/- mice) leads to extreme resistance to TNF-induced SIRS in a non-linear gene-dosage way. To find evidence that low TNFR1 protein expression in S mice is linked to TNF resistance, we performed a genetic linkage experiment and found that proximal chr2 and distal chr6 are linked not only to TNF resistance but also to TNFR1 levels, suggesting that the latter is responsible for the former. For TNFR1 levels, the LOD scores of chr6 were far beyond highly significant, while the chr2 LOD score reached only suggestive level. With the poor breeding performance of interspecies crosses such as those with B and S mice, only small backcross populations were possible, leading to relatively big critical regions of several tens of cM. Furthermore, chr6 consomic mice could be generated while chr2 consomics were not possible because of sterility problems, which occur frequently in interspecies crosses. The studies with the consomic mice led us to conclude that the regulation of the TNFR1 locus on chr6 was not depending on the locus on chr6 itself, but potentially on the chr2 locus and since the low expression of TNFR1 in S was only observed on the protein level and not the mRNA level, a regulation by miRs was an interesting hypothesis.

A key aspect of the regulation of eukaryotic gene expression is the cytoplasmic control of mRNA translation and degradation (17). Over the past decade, miRs have emerged as important regulators of translation. Hundreds of such molecules and their of plants and animals (18). Some miRs have validated anti-inflammatory effects, such as miR-155 and miR-9. Strikingly, bioinformatics analyses suggest that up to 30 % of human genes might be regulated by miRs (19). Most miRs bind in 3'UTRs of regulated mRNAs and lead to faster degradation of mRNA via initiation of poly(A) tail shortening, followed by mRNA decay, decapping and arrest of translation initiation, but many miRs can leave the mRNA levels intact and block translation only. An example of the latter is miR-579 (20). We found that miR-511 was recurrently predicted as TNFR1 regulating and that this miR is the only TNFR1 predicted regulator located right in the critical region on chr2 and is significantly stronger expressed in S than in B mice. MiR-511 is located in an intron of the Mrc-1 gene and since recently, it was suggested that, like most intronic miRNAs, miR-511 is regulated by the same promoter as the host gene (21), we indeed found higher Mrc-1 mRNA in S than B livers and spleens. We also found evidence that miR-511 is indeed regulating the TNFR1 3'UTR in a reporter system, and that transfection of this miR down-regulates TNFR1 protein in B and S fibroblasts and that anti miRs have the inverse effect. Interestingly, in vivo delivery of miR-511 down-regulated TNFR1 in livers of mice and protected the animals not only against TNF-SIRS, but also against endotoxemia (in a TNFR1-dependent way) and a model of lethal hepatitis. The experimental delivery system of the miRs and anti miRs targets only the livers of mice, and hence the effect of liver-specific regulation of TNFR1 on the lethal response to TNF is quite interesting. These data suggest that the development of miR-511 into a therapeutic tool may have future therapeutic value. Also, anti miRs inhibiting miR-511, delivered specifically in the liver of mice increased TNFR1 expression and sensitized them for TNF-induced SIRS. Since also (BxS)F1 mice were sensitized by this anti miR delivery, these data may suggest that part of the extremely robust TNF resistance of S mice may indeed depend on miR-511. Unfortunately, such experiments were not possible with S mice because of practical constrains. Final proof that miR-511 is responsible for part of the TNF resistance and low TNFR1 protein levels of S mice should come from genetic disruption of this miR-511 in the S genome, but genetic manipulation of S is still impossible, despite our efforts.

The genetic link of TNF resistance and low TNFR1 levels to chr6 may be explained by the minimal change in miR-511 target sequence in the Tnfrsf1a 3'UTR, but

how to explain the linkage to the miR-511 locus if the sequence of miR-511 appears to be equal in B and S mice? We have found previously that S mice have an overactive HPA axis, the axis stimulated by stress, and leading to the release of ACTH from the pituitary and to corticosterone (CS) release from adrenals. As a negative feed-back, the increased CS production normally leads to down-regulated GR levels and to inhibition of the HPA axis at the level of the hippocampus. In S mice however high CS levels are associated with high GR levels, which lead to high basal levels of expression of GR-induced genes. GR can homodimerize and act as a true transcription factor, binding on GRE-like elements. The canonical GRE element is an element resembling AGAACA(N)3TGTTCT. Such elements are abundant and can be located close to transcription start sites of genes, but also many kilobases away. Typical GRE-genes are genes involved in gluconeogenesis (SGK, PEPCK, TAT) or can have anti-inflammatory effects (GILZ, MKP-1). Interestingly, inhibition of GR by the irreversible inhibitor RU486 led to sensitization of both B and S mice to TNF, but also to complete loss of any difference in TNF-response between B and S, which suggests that the normally observed TNF resistance of S mice completely depends on the GR and the overactive HPA axis. Since we found no genetic link between TNF resistance of TNFR1 levels and the GR locus on chr18, we believe that our data suggest that these phenotypes do depend on GR, but are linked with a GRE gene that executes/mediates the anti-inflammatory effect, and is located on proximal chr2, namely the Mrc-1/miR-511 locus. We indeed found that treatment of S mice with RU486 led to decreased Mrc-1 and miR-511 expression and increased TNFR1 protein levels, that removal of adrenals in B mice led to a marked reduction in expression of basal levels of Mrc-1 and miR-511 and to an increase of TNFR1 levels and that DEX (a synthetic GC) injection in mice led to induction of these genes and reduction in TNFR1, also in the serum (suggesting a mechanism independent of shedding). Mrc-1 is also known as the macrophage mannose receptor and is strongly expressed by these cells, but also in the liver and in the spleen (genevestigator/bioGPS) and has recently been shown to be induced by GCs in macrophages and to be involved in macrophage polarization (22).

A new regulatory network relevant in TNF biology is appearing from our studies. We show that miR-511 is strongly induced by GCs. GCs have been shown to induce miRs in several cell systems. We show that this DEX-induced miR-511 is a true TNFR1 modulator. Since minimal reductions of TNFR1 have very significant effects on TNF-sensitivity, we believe that at least part of the anti-inflammatory effects of GCs may be mediated by induction of this miR, working at the level of TNFR1 expression. Whether this anti-inflammatory strategy is a widespread mechanism used by GCs, and thus this mechanism is applied at other inflammatory mediators and targets will have to be studied in the future.

MATERIALS AND METHODS

Mice

C57BL/6J (B) mice were purchased from Janvier-Europe. SPRET/Ei (S) mice were obtained from The Jackson Laboratory and bred in our facility. (C57BL/6 x SPRET/Ei) F1 mice were generated by crossing C57BL/6J female mice with SPRET/Ei male mice. B.S.chr6 consomic mice were generated by backcrossing (BxS)F1 mice with the host strain, C57BL6/J, and then repeatedly backcrossing to the host strain and screening the progeny for a nonrecombined S donor locus of interest (Tnfrsf1a) in each generation (23). B.S.chr6 consomic mice heterozygous for the S Tnfrsf1a allele were intercrossed at the N10 generation, and resulting N10F2 mice homozygous for the B Tnfrsf1a allele as well as mice homozygous for the S Tnfrsf1a allele and heterozygotes were identified by typing for the polymorphic marker D6Mit254, which is a sequence-tagged Site (STS) for Tnfrsf1a. TNFR1-/- mice generated by Dr. M. Rothe (24) were a kind gift from Dr. H. Bleuthmann. TNFR1 +/- mice were generated by crossing TNFR1-/- mice with C57BL/6J mice. Adrenalectomized C57BL/6J mice were purchased from Janvier-Europe, and received 0.9 % NaCl in their drinking water. All mice were kept in individually ventilated cages under a constant dark-light cycle in a conventional animal house and received food and water ad libitum. The mice were used at the age of 8–12 weeks. Animal experiments were approved by the institutional ethics committee for animal welfare of the Faculty of Sciences, Ghent University, Belgium. Genome sequences of SPRET/Ei were retrieved from the Sanger laboratories.

Compounds, injections and measurements.

Recombinant mouse TNF (specific activity of 1.66x109 IU/mg) was expressed in Escherichia coli and purified in our laboratory. The preparation contained less than 6 EU/ml of endotoxin as

determined by a Limulus amoebocyte lysate assay. Lipopolysaccharide (LPS) from Salmonella abortus equii was purchased from Sigma. RU486 and ConA were purchased from Sigma. Dexamethasone was bought as a ready-to-inject solution called Rapidexon from Medini N.V. Mice were injected intraperitoneally with TNF or LPS in 0.3 ml of pyrogen-free phosphate-buffered saline (PBS). Lethality was recorded on a regular basis until no further deaths occurred. Rectal body temperatures were measured with an electronic thermometer from Comark. Concanavalin A (360 µg per mouse) was injected intravenously in a volume of 200 µl. Two hours later, 100 µl of blood was withdrawn from the retro orbital plexus and TNF measured in the serum using a specific bioassay, and again six hours later, another blood sample was taken, serum prepared and alanine aminotransferase (ALT) measured. Serum ALT determination was performed in the Clinical Biology Laboratory of the Ghent University Hospital using a Hitachi kit and apparatus. Serum IL-6 was measured using a specific bioassay. RU486 was dissolved in DMSO and 5 mg was injected in 50 µl DMSO per mouse. DMSO was used as solvent control. Rapidexon was injected i.p. at a dose of 50 µg /mouse. On one occasion, for the induction of Mrc-1 in B mice, 500 µg was injected.

TNFR1 3'UTR reporter construct and luciferase reporter assay

The DNA coding for the Tnfrsf1a 3'UTR of B and S were ordered at Genscript. The 514 bp sequence of B and the 508 bp sequence of S were digested out of the vector pUC57 with SgfI and NotI and ligated in the psiCHECK-2 vector (Promega). The constructs were transformed into MC1061 cells and screened for the presence of a correct sequence with the following PCR primers, CAGATGAAATGGGTAAGTAC and AAACCCTAACCACCGCTTAA. The 3'UTR reporter plasmids were co-transfected with pre-miR precursor molecules in HEK-293T cells using Lipofectamin 2000 (Invitrogen). Cells were harvested 48 hours post transfection, washed with PBS and lysed in passive lysis buffer (PLB). Firefly luciferase activity is measured in Optiplate-96 F plates (Perkin Elmer) by adding Luciferase Assay Reagent II (Promega) to generate a luminescent signal. This signal is quantified using the GLOMAX 96 microplate luminometer (Promega). Afterwards, this reaction is quenched and the Renilla luciferase reaction is initiated by adding Stop & Glo® Reagent (Promega) to the same wells.

Hydrodynamic injections in mice

A precursor miRNA expression clone for mmu-miR-511 (GAUACCCACCAUGCCUUUUGCUCUGCA CUCAGUAAAUAAUAAUUUGUGAAUGUGU UAGCAAAAGACAGGAUGGGGAUCCA), cloned in the pEZX-MR04 vector was purchased from GeneCopoeia, as well as a scrambled control clone. LNA-enhanced miRCURY i-mmu-miR-511-5p (GAGTGCAGAGCAAAAGGCA) and i-miR-511-5pMMControl were purchased from Exiqon. Ten micrograms of plasmid DNA was dissolved in PBS and injected in a volume of 2 ml in the tail vein under high pressure. This technique guarantees hepatocyte-specific uptake and transient expression of the plasmid (25).

mTNFR1 ELISA

Liver, kidney, lung and spleen of mice were excised and snap-frozen in liquid nitrogen. Samples were homogenized in PBS containing 0.5 % CHAPS and complete protease inhibitor cocktail tablets from Roche. Homogenates were centrifuged for 30 min at 20,000 g and 4 °C, after which the supernatant was collected and stored at –80 °C. Blood was collected by retro orbital bleeding and allowed to clot for 1 h at 37 °C. Serum was prepared and stored at −20 °C. Protein concentration was determined by the Bradford method (BioRad) and 500 µg was used to perform an ELISA specific for TNFR1 using the mouse sTNF RI/TNFRSF1A duoset ELISA from R&D Systems. The levels were normalized to the levels of C57BL/6 or control samples, which were set as 100 %.

Q-PCR

Liver, kidney, lung and spleen samples were stored in RNA later® from Ambion. Samples were homogenized and RNA was extracted using an RNeasy mini kit from Qiagen. RNA concentration was measured with the Nanodrop1000 from ThermoScientific and 500 ng RNA was used to prepare cDNA with iScript from Bio-Rad. Q-PCR was performed using the SYBR Green master mix and the Light cycler 480 from Roche with the following primers: 5′-CCGGGAGAAGAGGGATAGCTT-3′ and 5′-TCGGACAGTCACTCACCAAGT-3′ for mTNFR1, 5′-GCTGAATCCCAGAAATTCCGC-3′ and 5′-ATCACAGGCATACAGGGTGAC-3′ for Mrc-1, 53′ and 5′-TCGGACAGTCACTCACCAAGT-3′ for mTNFR1, 5′-GCTGAATCCCAGAAATTCGilz, 5z, 5 5 5z, 5 , 5 CGGACAGTCA and 5and 5 , 5 CGGACAGTCACTCACCA for CGC-3 and 5and 5 , 5 CGGACAGTCACTCAC for Mrc-1, 5′-TGAAGCAGGCATCTGAGGG-3′ and 5′-

CGAAGGTGGAAGAGTGGGAG-3′ for Gapdh, and 5′-CCTGCTGCTCTCAAGGTT-3′ and 5′-TGGCTGTCACTGCCTGGTACTT-3′ for Rpl13a. The best performing housekeeping genes were determined with geNorm (26). No SNPs between C57BL/6 and SPRET/Ei were included in the primers (not shown). Q-PCR for miR-511 was done using specific MultiScribeTM cDNA synthesis and TaqMan® Pri-miRNA Assays for mmu-miR-511 and three stable miRs (mmu-miR-194, mmu-miR-24 and mmu-miR-29a from Applied Biosystems. All values shown are relative expression values normalized to the geometric mean of the selected housekeeping genes.

Quantitative Trait Loci (QTL) mapping

To map the loci responsible for low TNFR1 protein levels in S mice, an interspecies backcross between female (C57BL/6 x SPRET/Ei) F1 mice and male C57BL/6 mice was set up and N2 backcross mice were generated. Tail biopsies were collected at weaning from 214 N2 mice and high quality genomic DNA was prepared by standard phenol-chloroform extraction. A genome scan on 100 ng DNA was performed with 72 microsatellite markers. Primer sequences from the Massachusetts Institute of Technology (MIT) were retrieved from www.informatics.jax.org. Coverage of the genome was estimated by taking the position of the marker loci on the Mouse Genome Database genetic map obtained from The Jackson Laboratory and applying a swept radius of 20 cM (27). Livers from the 214 N2 backcross mice were excised at the age of 8 weeks and snap-frozen in liquid nitrogen. Total protein was isolated and 500 □ g was used to measure TNFR1 levels by ELISA. After the first screening, the density of markers was increased on chromosomes 2 and 6, which were shown to be linked to the trait. Linkage analysis was performed using the R/qtl software version 1.12-26 running under R 2.9.1 (28). Significance thresholds of LOD scores were estimated by 10,000 permutations of experimental data. For TNF resistance, the 5 % significance threshold LOD score is 2.50, the 10 % threshold is 2 and the suggestive level is 1.4. For TNFR1 protein levels, the 5 % significance threshold LOD score is 2.41, the 10 % threshold is 2.12, the suggestive threshold is 1.34. The protein level was analyzed using a normal model by the EM algorithm in R/qtl (29).

Mouse Embryonic Fibroblast (MEF) studies

MEFs were isolated from embryos 18 days postcoitum. They were cultured in DMEM medium (supplemented with 10 % FCS, penicillin, streptomycin, sodium pyruvate and L-glutamine) and seeded in culture flasks. Cells were trypsinized and seeded in 24-well plates at 100,000 cells per well. Pre-miR™ miRNA Precursors for mmu-mir-511 (GAUACCCACCAUGCCUUUUGCUCUGCACUCA GUAAAUAAUAAUUUGUGAAUGUGUAGCAAA AGACAGGAUGGGGAUCCA) and a negative control were purchased from Ambion. LNA-enhanced miRCURYi-mmu-miR-511-5p (GAGTGCAGAGCAAAAGGCA) and i-miR-511-5pMMControl were purchased from Exiqon. 50 μM of the molecules were transfected using Lipofectamine RNAiMAX from Invitrogen and 24 h later the cells were lysed with PBS containing 0.5 % CHAPS and complete protease inhibitor cocktail tablets from Roche. TNFR1 was measured using the TNFR1 ELISA

ConTra analysis of Mrc-1 promoter

Analysis of the 2000 bp upstream Mrc-1 promoter for GRE sequences across species was conducted using the ConTra online software (30), with the highest stringency of core=1.00 and similarity matrix=0.95 and a method to minimize false positives with TRANSFAC matrices.

Statistical analysis

Survival curves (Kaplan-Meyer plots) were compared by a log-rank test and final outcomes were compared by a chi-square test. Data are expressed as the means ± S.E. Statistical significance of differences between groups was evaluated with Student's t tests with 95 % confidence intervals and with one-way or two-way analysis of variance. Error bars in the figures represent the mean ± S.E. *, **, *** and **** represent $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p<0.0001$ respectively.

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FIGURE LEGENDS

Figure 1. Lethal response of different genotypes to increasing doses of TNF and generation of consomic mice. A: mice were injected with three different doses of TNF and lethality was observed for 96 hours. Numbers of deaths/total are displayed. B. Breeding scheme for the generation of B.S.chr6 consomic mice for the distal part of chr6. After intercrossing at the N10 generation, mice were obtained that had the last 40 cM of chr6 in a B homozygous way (black), BS heterozygous (green) or S homozygous (grey). The location of the Tnfrsf1a gene is indicated. In the three different groups, some contaminating BB, BS or SS genome was present at the very end of the chromosome (around 70 cM). Statistical differences in survival are calculated compared to the B group or the B.S.chr6 BB group.

Figure 2. Reduced TNFR1 protein levels in S mice and genetic linkage study of this trait. A: TNFR1 protein levels in serum and tissues of different mouse groups (n = 4-13). TNFR1 protein levels were measured by ELISA and compared to B, the levels of which were set as 100 %. Statistical significance of the differences with B was studied by a Students-t-test. B: TNFR1 mRNA levels measured by q-PCR in organs of B (n=3) and SPRET/Ei (n=4). Affymetrix microarray data in hepatocytes (Affy Mo Gene 1.0 ST Array). C: backcross experiments in mice to determine genetic linkage between TNF resistance (left) and TNFR1 liver protein levels (right). QTL mapping of lethal response (n=178) was based on the data previously obtained and discussed by Staelens et al., but re-analyzed in the current study using R/qtl software. TNFR1 protein level in the liver of N2 mice (n=214). Both for TNF resistance and TNFR1 levels, the LOD scores show a QTL on chromosome 2 (TNF resistance 15 cM, LOD=2.2454 and TNFR1 levels 5 cM, LOD=1.3098) and chromosome 6 (TNF resistance 46 cM, LOD=3.1056 and TNFR1 levels 70 cM, LOD=4.1519). Horizontal lines in the figures represent suggestive (dots), significant (stripes) and highly significant (full line) LOD values.

Figure 3. miR-511 levels, sequence and target sequences in B and S mice. A: miR-511 and Mrc-1 mRNA levels in liver (left panel) and spleen (right panel) in B (n=5) and S (n=5) mice. mRNA levels were measured by Q-PCR and levels detected in B mice expressed as 100 %. Significances of the differences were studied by a Students t-test. B: 3'UTR of the tnfrsf1a gene of B mice. The single nucleotide polymorphisms (SNPs) found in the sequence of S mice are shown in red. MiR-511 is predicted by the program MicroSNiPer (http://cbdb.nimh.nih.gov/microsniper/) to have two target sequences, shown in bold and underlined. The 3' most target sequence contains two nucleotide differences in S compared to B (in red), leading to different base-pairing with miR-511 as shown in the lower panel.

Figure 4: miR-511 is a genuine TNFR1 regulating miR and has therapeutic potential. A: Inhibition of Rluc activity of the psiCHECK-tnfrsf1a-3'UTR reporter plasmid with B sequence (left panel) and S sequence (right panel) by miR-511 transfection in HEK-293T cells. (n=8). B: TNFR1 protein levels in B MEF cultures (n=18) and S MEF cultures (n=24) 24 h after transfection with miR-511 or miR-CTR. TNFR1 protein levels were measured in cell lysates by ELISA. C: TNFR1 protein levels in B MEF cultures (n=18) and S MEF cultures (n=18) 24 h after transfection with anti miR-511 or anti miR-CTR. TNFR1 protein levels were measured in cell

lysates by ELISA. D: In vivo effect 24 h after hydrodynamic injection of B mice with plasmids expressing miR-511 (n=19), miR-CTR (n=19) or PBS (n=8). Liver TNFR1 protein levels (left panel) were measured by ELISA. 24 h after plasmid injection mice were injected with 25 μg TNF. All mice pretreated with miR-511 (■, grey, n=12) survived, while 50 % of the mice pretreated with miR-CTR (n=12) or PBS (n=12) succumbed to the TNF injection (middle panel). 24 h after injection of TNF, body temperatures of mice pretreated with miR-511 were significantly higher than those of the miR-CTR and PBS groups (right panel) (all groups n=10). E: Survival of B mice and TNFR1-/- mice injected with 200 μg or 500 μg LPS, respectively, 24 h after miR-511 or miR-CTR hydrodynamic plasmid injection. B mice pretreated with miR-511 (■, grey, n=16) were significantly protected against LPS compared to miR-CTR pretreated mice (■, n=15) or PBS pretreated mice (n=15) (left panel). No difference in survival was found between TNFR1 -/- mice pretreated with miR-511 (■, grey, n=11) or with miR-CTR (■, n=9) (middle panel). In a control experiment, TNFR1 -/- mice (grey, n=6) were significantly protected against 500 μg LPS compared to TNFR+/+ mice (n=7) (right panel). F: Effect of hydrodynamic injection of plasmids expressing miR-511, miR-CTR or PBS on ConA-induced hepatitis in B mice (all groups n=10). 360 μg ConA was injected 24 h after plasmids and 8 h later mice were analyzed. Liver damage (ALT levels, left panel) in serum. Body temperatures of mice 8 h after ConA injection (middle panel). TNF levels (right panel) 2 h after injection of ConA revealed similar concentrations of TNF in the three groups.

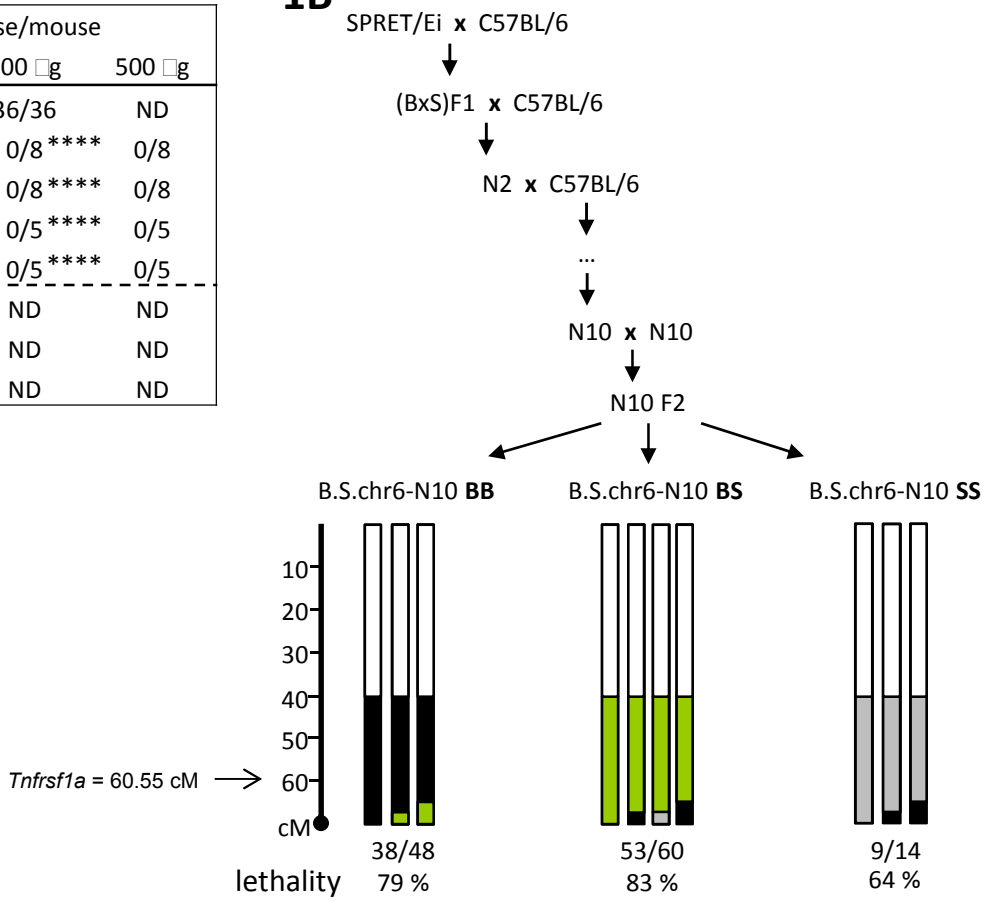
Figure 5. Inhibition of miR-511 and of GR sensitize for TNF-induced SIRS. A: In vivo effect 24 h after hydrodynamic injection of B mice with plasmids expressing anti miR-511 (n=10), anti miR-CTR (n=10) or PBS (n=10). TNFR1 protein levels were measured 24 h after hydrodynamic injection by ELISA in the liver (left panel). Survival of B mice injected with 20 μg TNF, 24 h after hydrodynamic injections (middle panel). Mice pretreated with Antago miR-511 (■, grey, n=10) were significantly sensitized for TNF-induced SIRS compared to control groups and had the strongest drop in body temperatures (right panel) 12 h after injection of 25 μg TNF. B: In vivo effect 24 h after hydrodynamic injection of (BxS)F1 mice with plasmids expressing anti miR-511 (n=9), anti miR-CTR (n=10) or PBS (n=10). TNFR1 protein levels were measured 24 h after hydrodynamic injection by ELISA in the liver (left panel). Survival of (BxS)F1 mice injected with 500 μg TNF, 24 h after hydrodynamic injections (middle panel). Mice pretreated with anti miR-511 (■, grey, n=9) were significantly sensitized for TNF compared to the PBS control group and showed the strongest drop in body temperatures (right panel) of all groups. C: Inhibition of TNF resistance of S mice by RU486. B and S mice (all groups n=5) were injected with 5 μg TNF (= LD100 in RU486 pretreated mice from preliminary experiments), 30 min after injection of RU486 or DMSO control. Both B (black dashed line) and S mice (grey dashed line) pretreated with RU were significantly sensitized for TNF-induced lethality compared to DMSO/TNF controls (full lines) (left panel) and for TNF-induced hypothermia measured 6 h after injection of 5 μg TNF. IL-6 levels 6 h after injection of TNF were equally high in RU486 sensitized B as s mice. RU486 injection in S mice (n=4) led to significantly increased TNFR1 protein expression 6 hours later, in liver and spleen compared to DMSO vehicle treated S mice (n=4). D. Two GRE elements (ConTra matrix M00205) were identified in the 5' region of the Mrc-1 gene at -1167 to -1152 and -599 to -584 relative to the TSS. One GR half binding site (ConTra matrix M00192) was found at -20 to -2 relative to the TSS.

Figure 6. MiR-511 expression depends on GCs. A: Effect of adrenalectomy (Adx) on relative mRNA expression in the spleen of B mice (n=5) and Adx mice (n=6). Left panel shows significant down-regulation of Tsc22d3 (encoding GILZ), Dusp-1 (encoding MKP-1), Mrc-1 and miR-511 in Adx mice. Middle panel displays significant up-regulation of the protein level of TNFR1 in the liver the spleen and the serum of Adx mice compared to control mice (all groups n=10). Right panel, significant protection of B mice against 30 μg TNF-induced lethal SIRS by 50 μg DEX pretreatment (-30 min, grey) compared to PBS pretreatment (250 ul, -30 min, black) (both groups n=13). B: Relative mRNA expression in the liver of B mice 6 h after injection of PBS (n=10) or DEX i.p. (n=10). Left panel, significant up-regulation of Tsc22d3, Sgk, Tat, Mrc-1 and miR-511. C: Relative mRNA expression in the spleen of B mice 6 h after injection of PBS (n=10) or DEX i.p. (n=10). Left panel, significant up-regulation of Tsc22d3, Sgk, Tat, Mrc-1 and miR-511. No effect of DEX on TNFR1 mRNA levels. D. Significant down-regulation of the protein level of TNFR1 in the liver (n=20), the spleen (n=20) and the serum (n=5) by 50 μg DEX compared to PBS.

1A

Mice	TNF dose/mouse		
	20 □g	100 □g	500 □g
C57BL/6	9/10	36/36	ND
Spret/Ei	0/10 ***	0/8 ****	0/8
(BxS)F1	0/10 ***	0/8 ****	0/8
TNFR1 -/-	0/6 ***	0/5 ****	0/5
TNFR1 +/-	0/8 ***	0/5 ****	0/5
B.S.chr6 BB	38/48	ND	ND
B.S.chr6 BS	53/60 ns	ND	ND
B.S.chr6 SS	9/14 ns	ND	ND

1B



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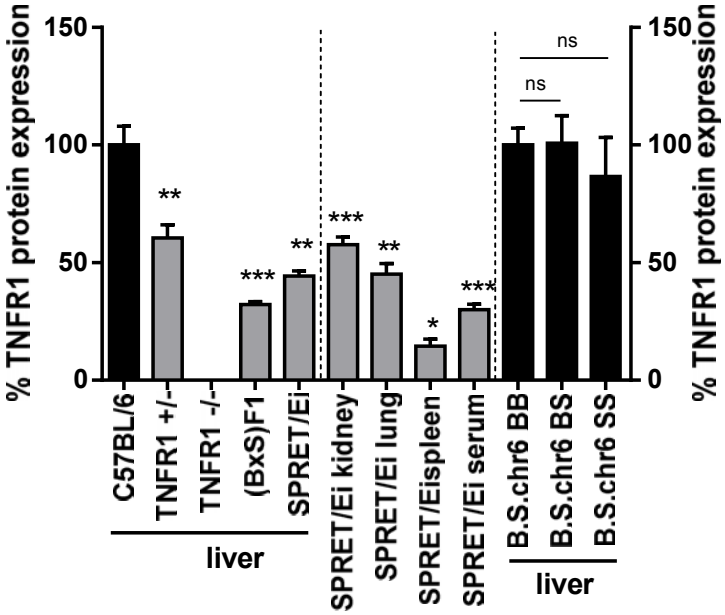

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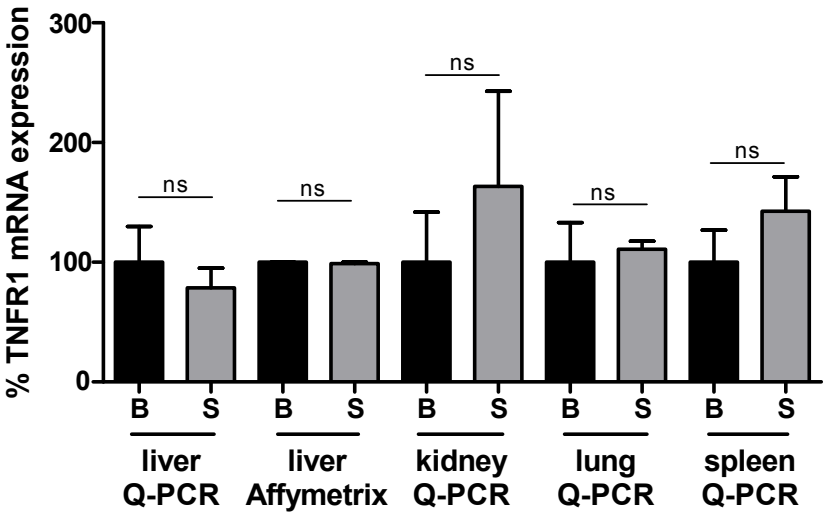
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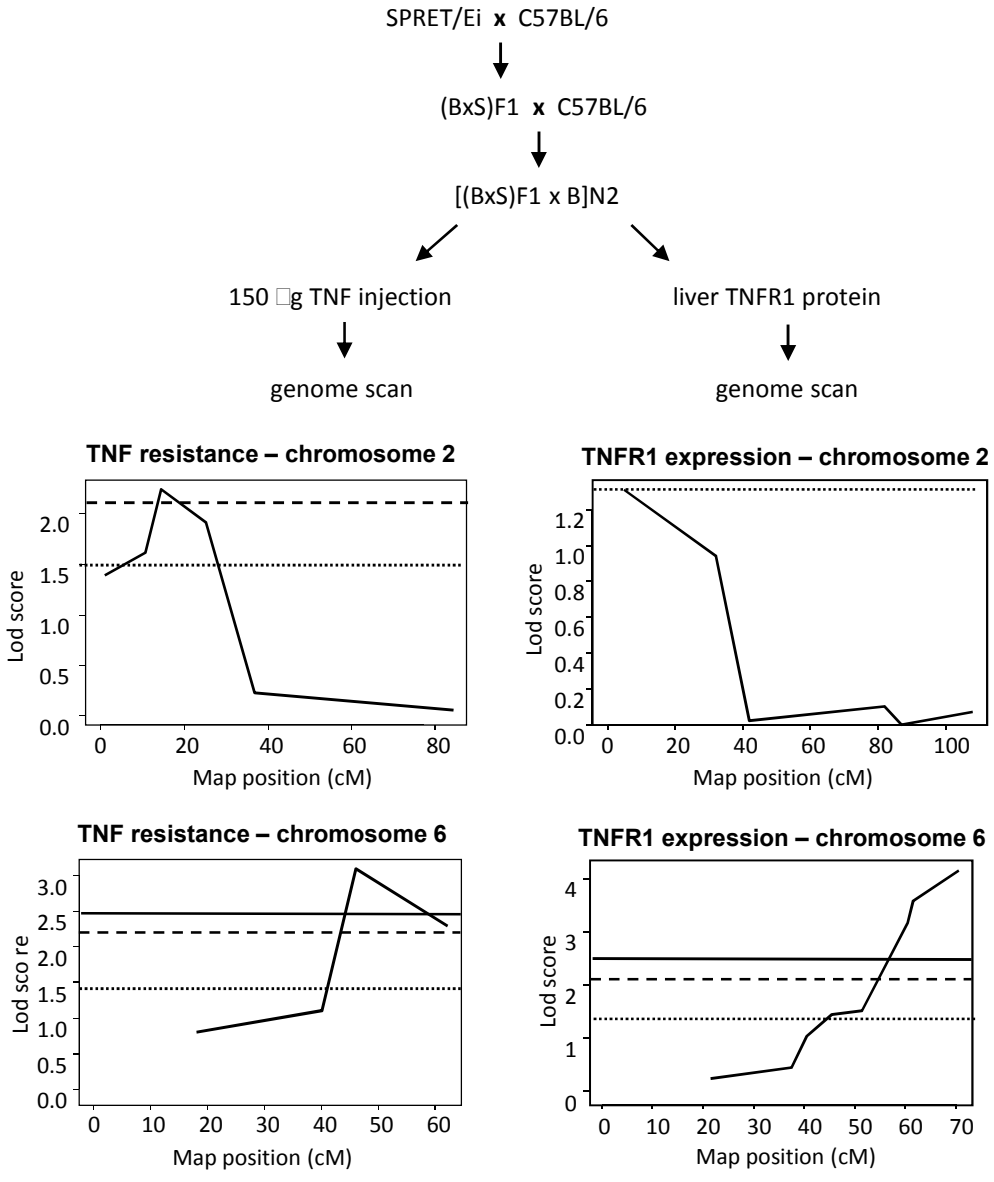
kidney Q-PCR
lung Q-PCR
spleen Q-PCR

2C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TNF resistance – chromosome 2
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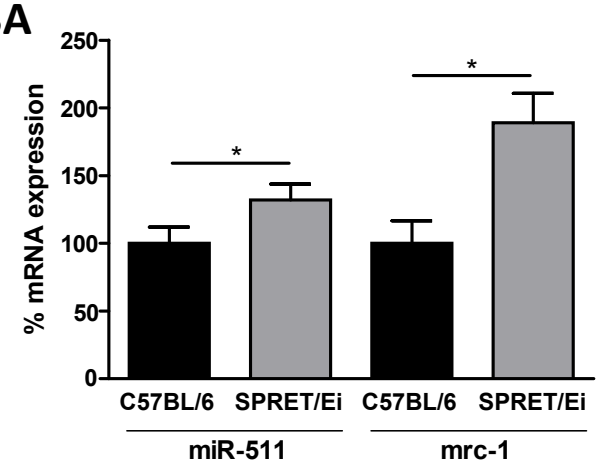
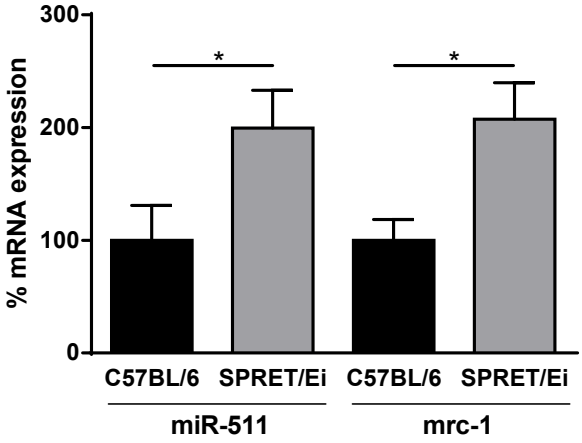
3A





3B

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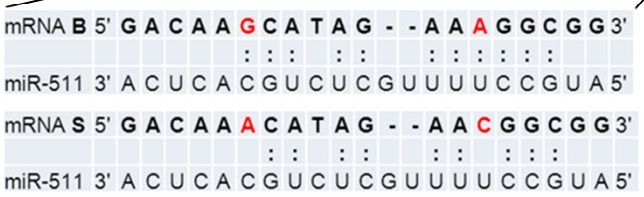
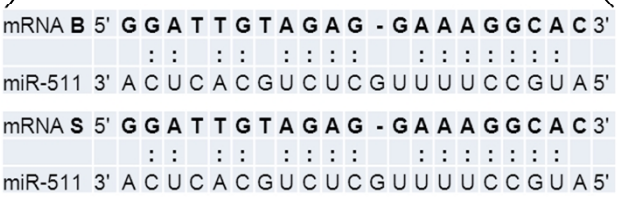
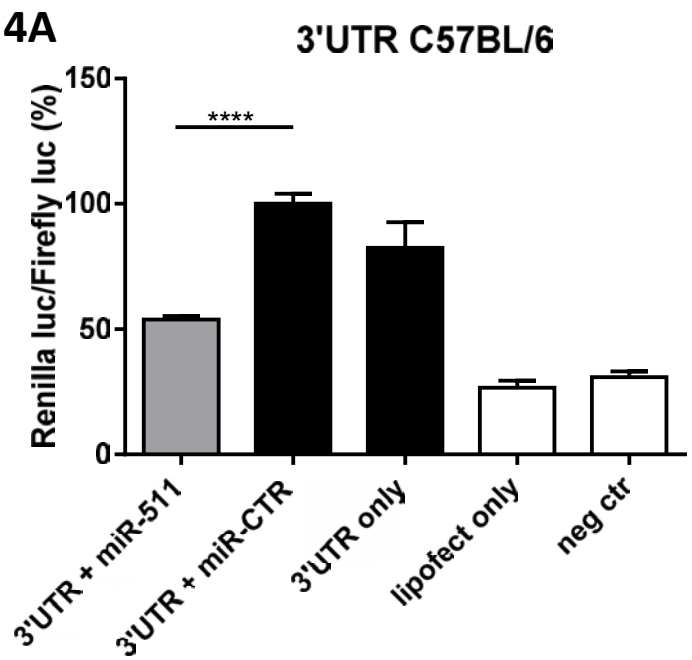
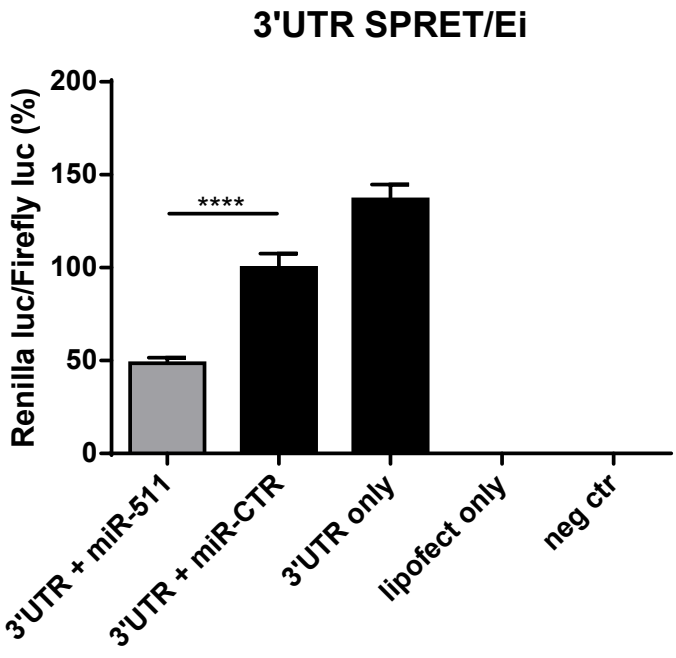
atatcacactaatggatgaactgtgtaaggtaag**gacaag**c**atagaaa**g**gcg**gggtctccagctgga**gc**ctcgactcttgtaaatacactaaac
gtctaaaaatgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa3’



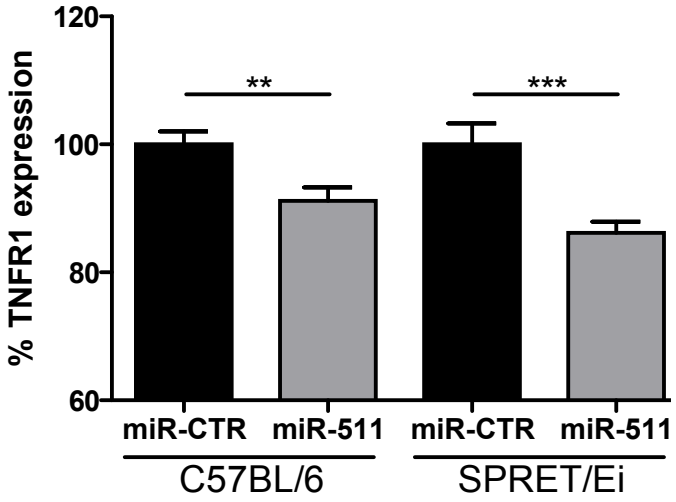


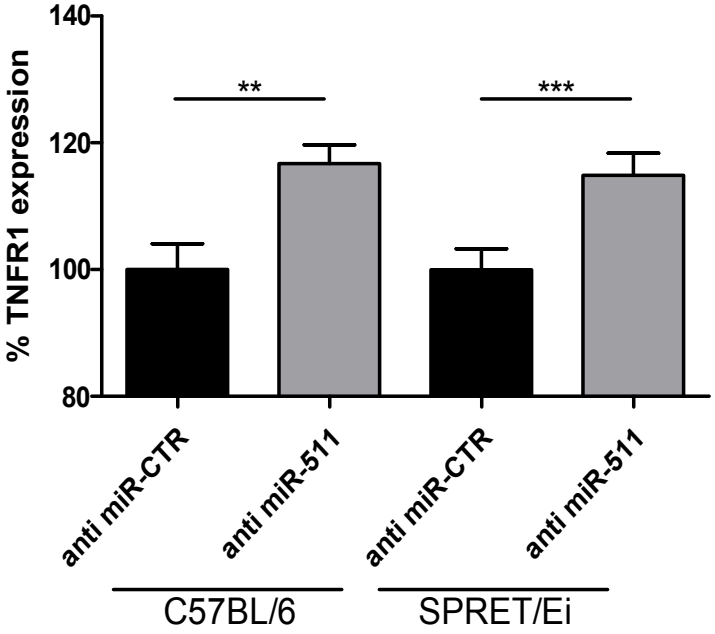
4A



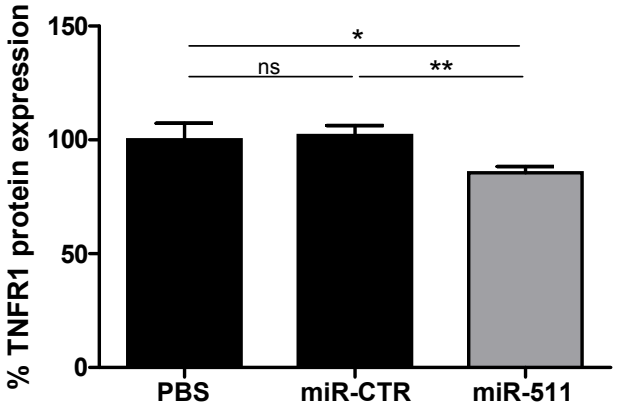


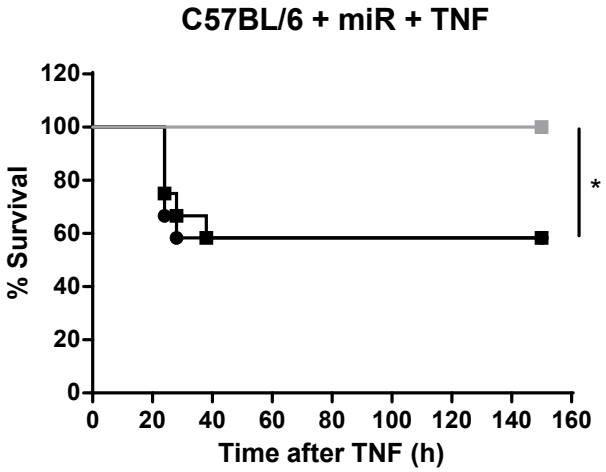
4B

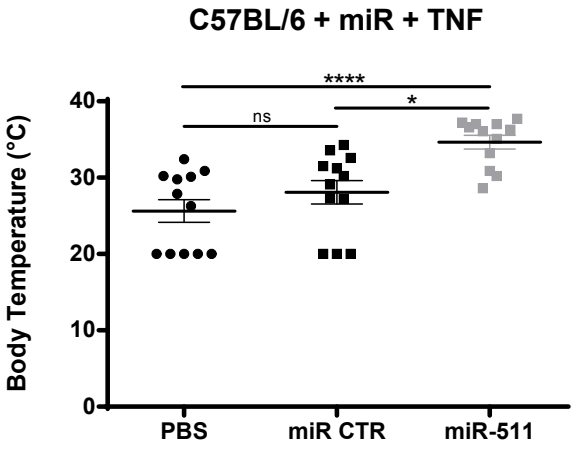

% TNFR1 expression
120
100
80
60
**
***
miR-CTR
miR-511
miR-CTR
miR-511
C57BL/6
SPRET/Ei


% TNFR1 expression
140
120
100
80
**
***
anti miR-CTR
anti miR-511
anti miR-CTR
anti miR-511
C57BL/6
SPRET/Ei

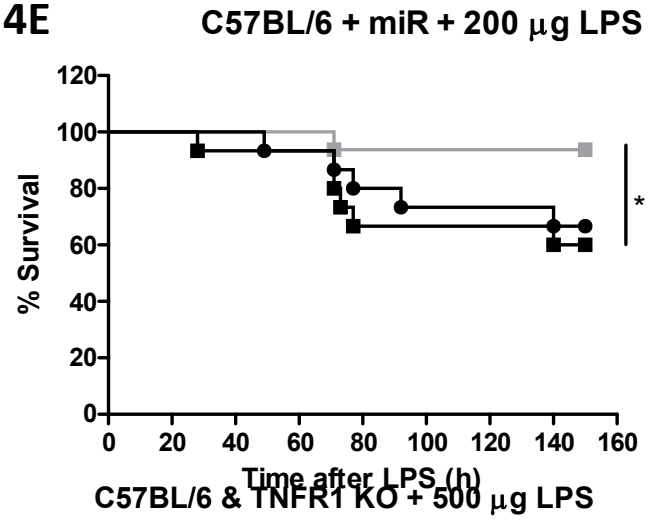
4D

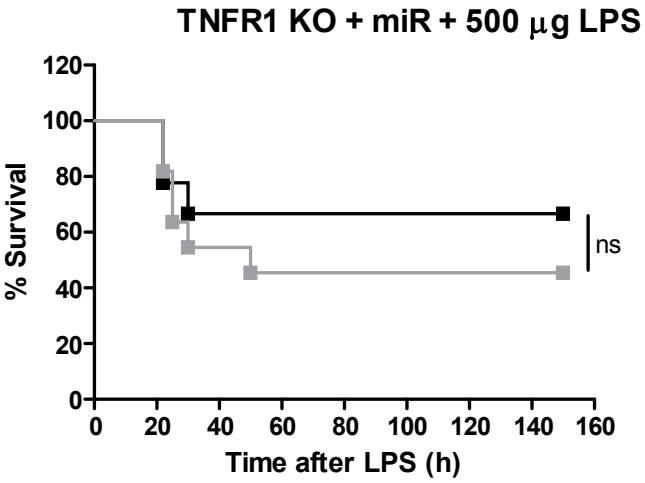

% TNFR1 protein expression
150
100
50
0
*
ns
**
PBS
miR-CTR
miR-511

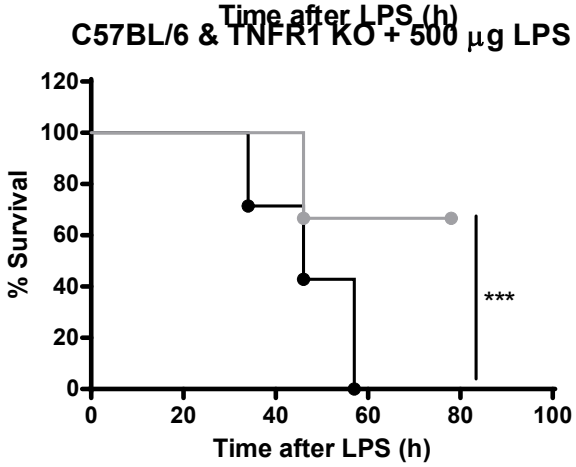

C57BL/6 + miR + TNF
% Survival
120
100
80
60
40
20
0
*
0
20
40
60
80
100
120
140
160
Time after TNF (h)


C57BL/6 + miR + TNF
Body Temperature (°C)
40
30
20
10
0
****
*
ns
PBS
miR CTR
miR-511

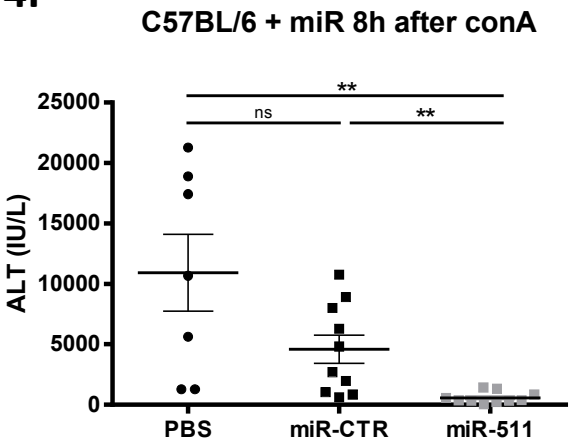
4E

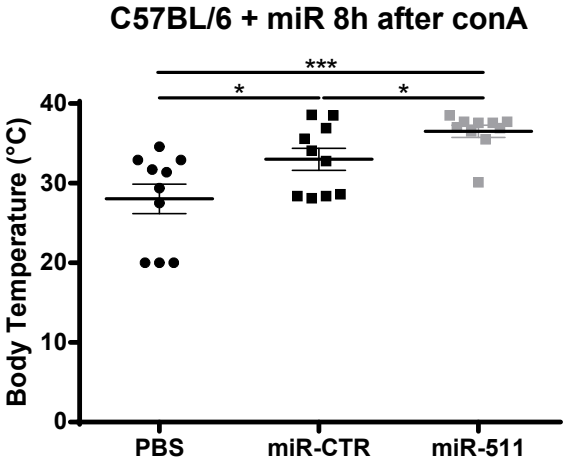

C57BL/6 + miR + 200 μg LPS
% Survival
Time after LPS (h)
*

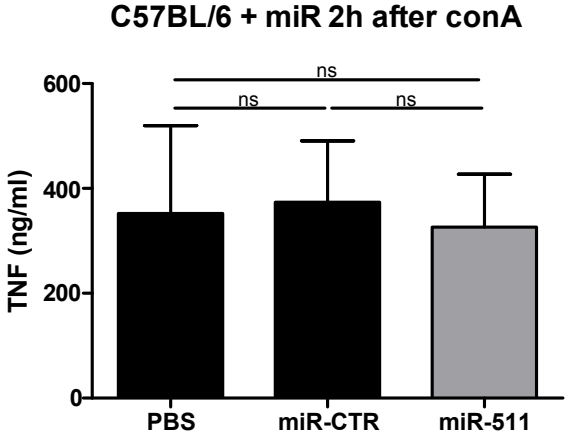

TNFR1 KO + miR + 500 μg LPS
% Survival
Time after LPS (h)
ns


C57BL/6 & TNFR1 KO + 500 μg LPS
% Survival
Time after LPS (h)
***

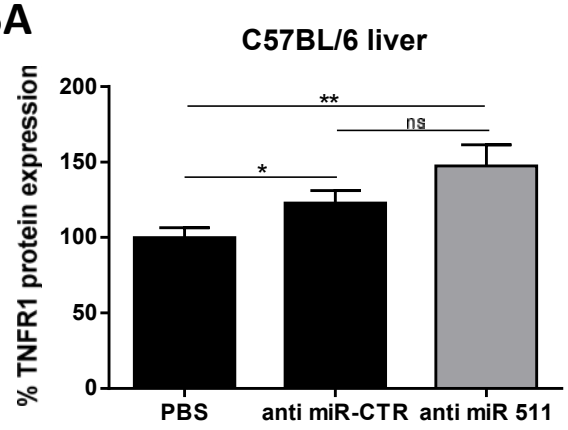
4F

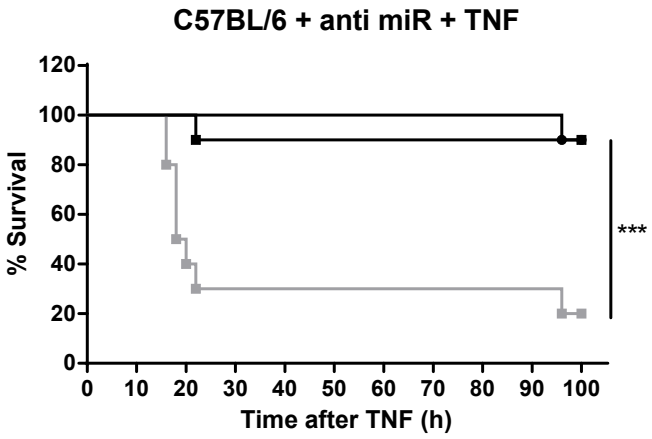

C57BL/6 + miR 8h after conA
ALT (IU/L)
**
ns
**
PBS
miR-CTR
miR-511

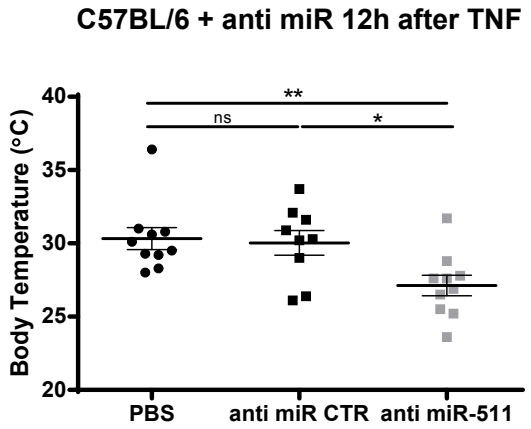

C57BL/6 + miR 8h after conA
Body Temperature (°C)
***
*
*
PBS
miR-CTR
miR-511


C57BL/6 + miR 2h after conA
TNF (ng/ml)
ns
ns
ns
PBS
miR-CTR
miR-511

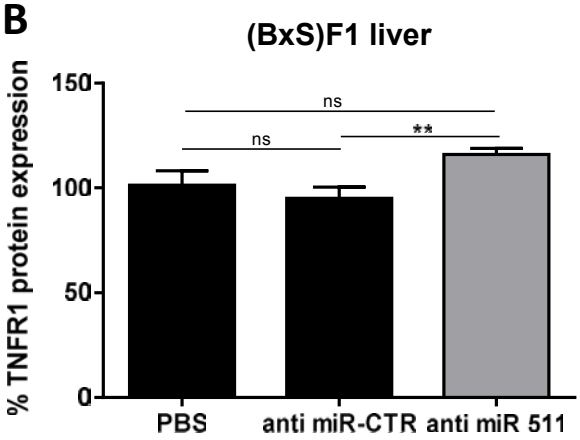
5A

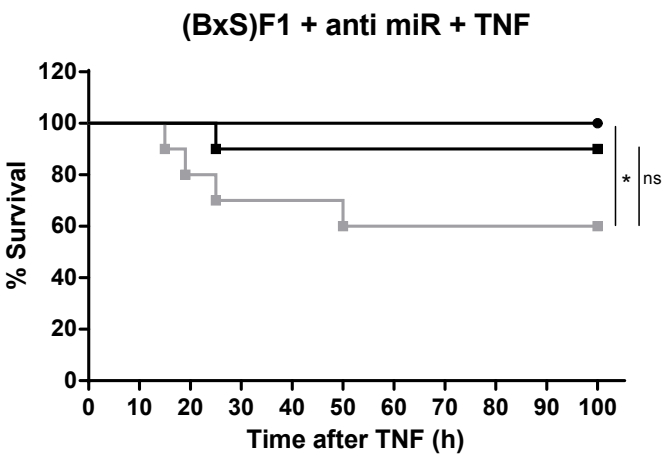

C57BL/6 liver
% TNFR1 protein expression
200
150
100
50
0
**
ns
*
PBS
anti miR-CTR
anti miR 511

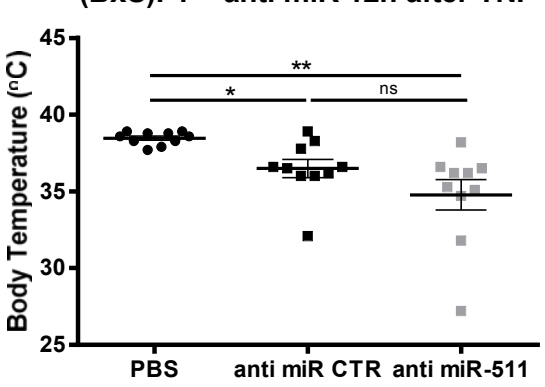

C57BL/6 + anti miR + TNF
% Survival
120
100
80
60
40
20
0
***
0 10 20 30 40 50 60 70 80 90 100
Time after TNF (h)


C57BL/6 + anti miR 12h after TNF
Body Temperature (°C)
40
35
30
25
20
**
ns
*
PBS
anti miR CTR
anti miR-511

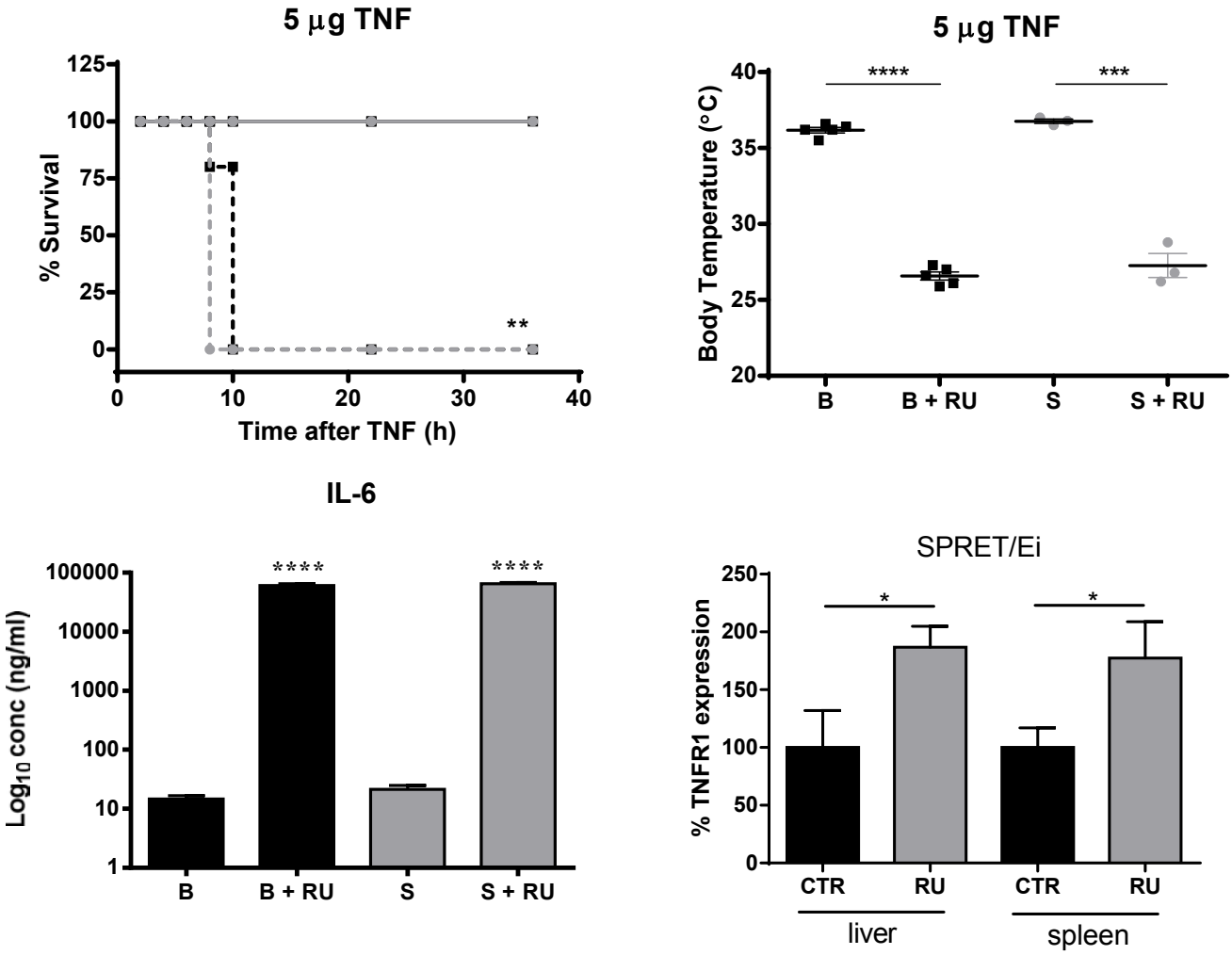
5B


(BxS)F1 liver
% TNFR1 protein expression
150
100
50
0
ns
ns
**
PBS
anti miR-CTR
anti miR 511

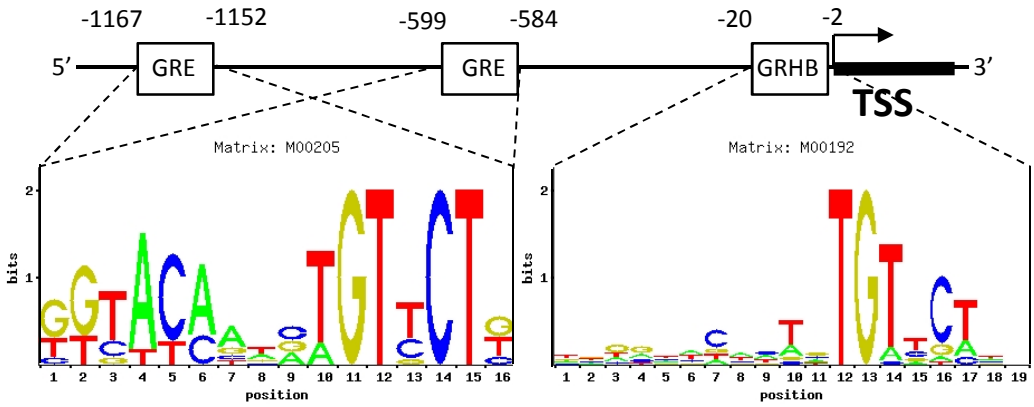

(BxS)F1 + anti miR + TNF
% Survival
120
100
80
60
40
20
0
*
ns
0 10 20 30 40 50 60 70 80 90 100
Time after TNF (h)


(BxS)F1 + anti miR 12h after TNF
Body Temperature (°C)
45
40
35
30
25
**
*
ns
PBS
anti miR CTR
anti miR-511

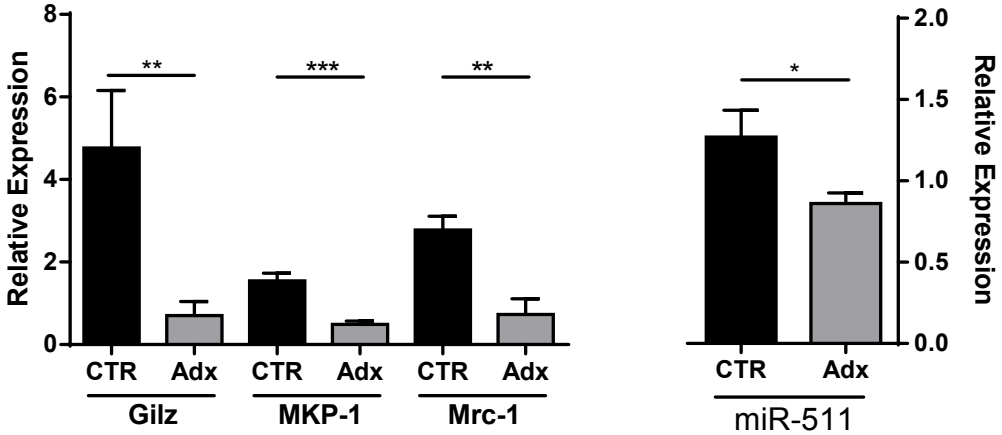
5C

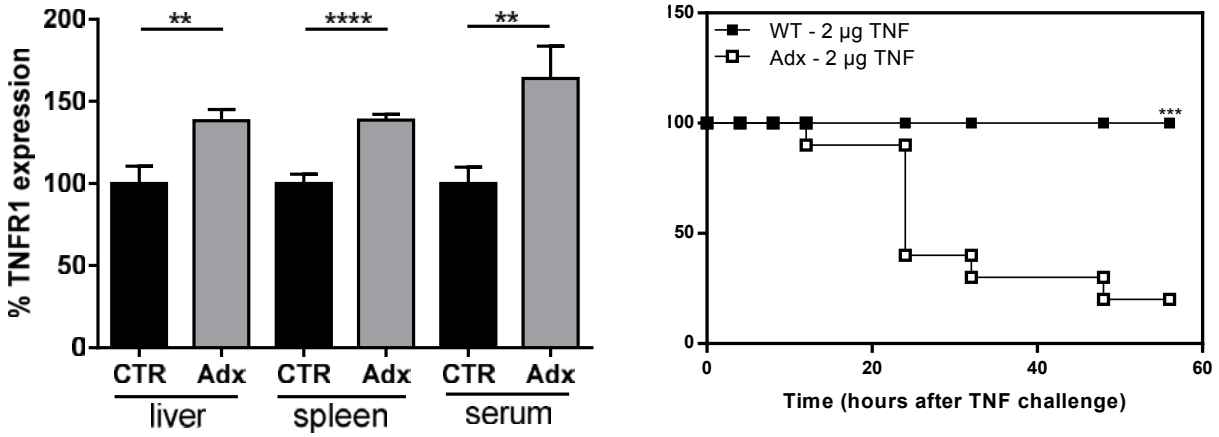

5 µg TNF
% Survival
Time after TNF (h)
**
5 µg TNF
Body Temperature (°C)
****
***
B
B + RU
S
S + RU
IL-6
Log10 conc (ng/ml)
****
****
B
B + RU
S
S + RU
SPRET/Ei
% TNFR1 expression
*
*
CTR
RU
CTR
RU
liver
spleen

5D

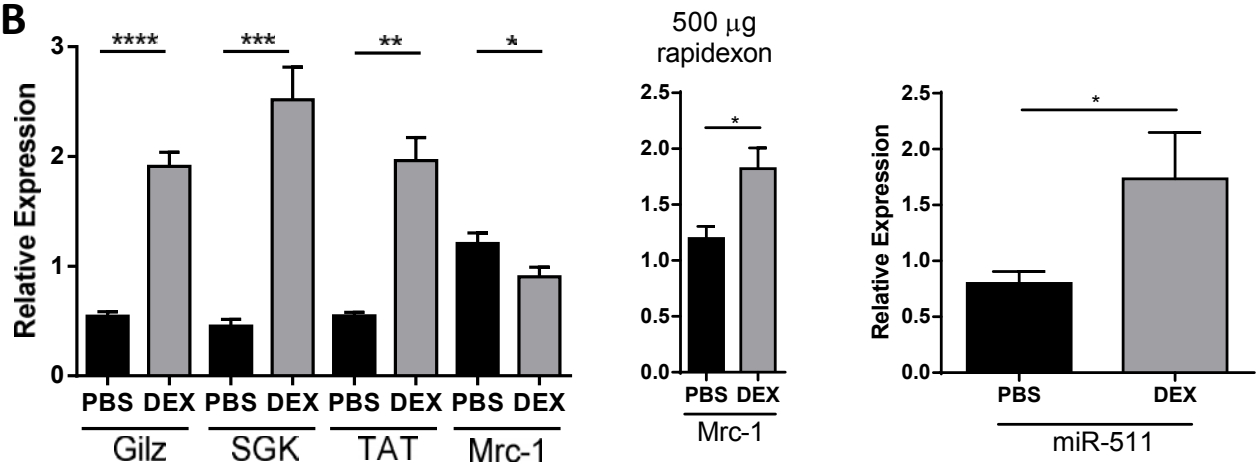

-1167
-1152
-599
-584
-20
-2
5'
GRE
GRE
GRHB
3'
TSS
Matrix: M00205
Matrix: M00192
bits
position

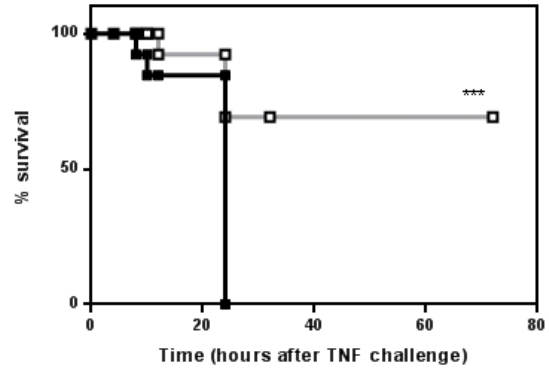
6A


Relative Expression
8
6
4
2
0
**
***
**
CTR
Adx
CTR
Adx
CTR
Adx
Gilz
MKP-1
Mrc-1
2.0
1.5
1.0
0.5
0.0
*
Relative Expression
CTR
Adx
miR-511

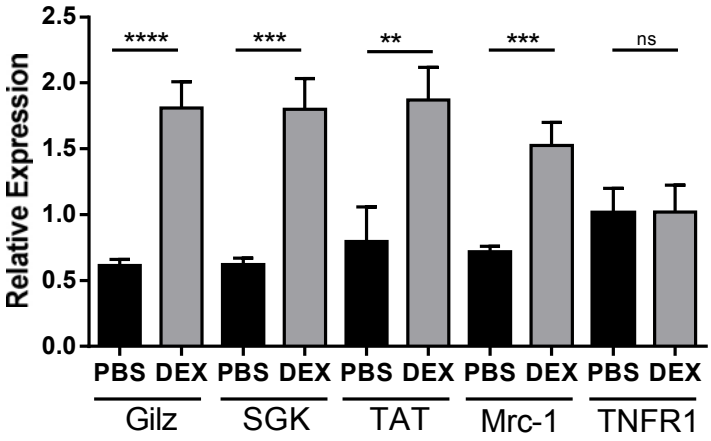

% TNFR1 expression
200
150
100
50
0
**
****
**
CTR
Adx
CTR
Adx
CTR
Adx
liver
spleen
serum
150
100
50
0
WT - 2 µg TNF
Adx - 2 µg TNF
***
0
20
40
60
Time (hours after TNF challenge)

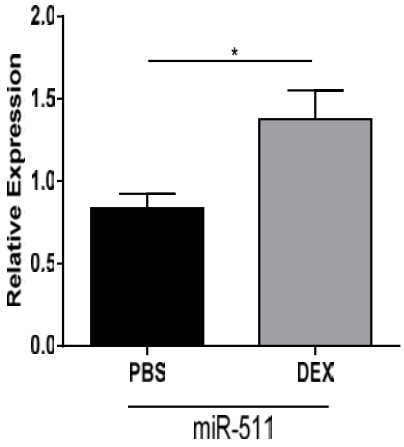
6B


Relative Expression
3
2
1
0
****
***
**
*
PBS
DEX
PBS
DEX
PBS
DEX
PBS
DEX
Gilz
SGK
TAT
Mrc-1
500 µg
rapidexon
2.5
2.0
1.5
1.0
0.5
0.0
*
PBS
DEX
Mrc-1
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
PBS
DEX
miR-511

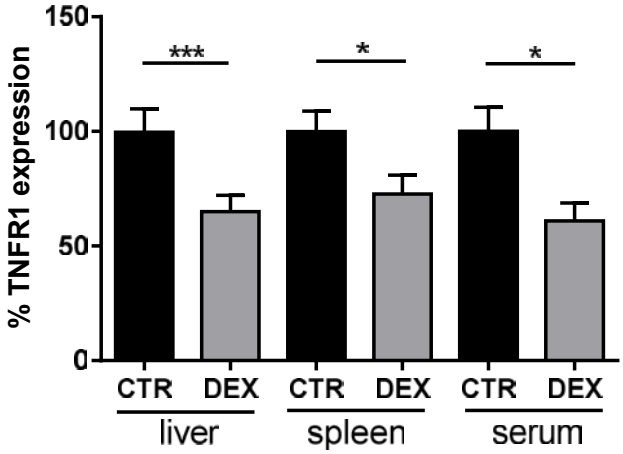

100
50
0
% survival
***
0
20
40
60
80
Time (hours after TNF challenge)

6C


Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
****
***
**
***
ns
PBS DEX PBS DEX PBS DEX PBS DEX PBS DEX
Gilz
SGK
TAT
Mrc-1
TNFR1


Relative Expression
2.0
1.5
1.0
0.5
0.0
*
PBS
DEX
miR-511

6D


% TNFR1 expression
150
100
50
0
***
*
*
CTR DEX CTR DEX CTR DEX
liver
spleen
serum

Review in preparation

REGULATION AND DYSREGULATION OF TUMOR NECROSIS FACTOR RECEPTOR 1

Leen Puimège[1,2] Filip Van Hauwermeiren[1,2] and Claude Libert[1,2]

1 Department for Molecular Biomedical Research, VIB, Ghent, Belgium
2 Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

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The cytokine TNF is an essential regulator of the immune system. Dysregulation of its expression or signaling plays a role in the pathology of many auto-immune diseases, such as rheumatoid arthritis, inflammatory bowel disease (IBD) and multiple sclerosis and several TNF-blocking agents have proven successful in the treatment of such diseases. Development of novel, safer or more effective drugs requires a deeper understanding of the regulation of the pro-inflammatory activities of TNF and its receptors. The ubiquitously expressed TNFR1 is responsible for most TNF effects, such as induction of cell death, inflammation and tumor regression, while TNFR2 has a limited expression pattern and often performs immune-regulatory functions. Despite extensive knowledge of TNFR1 signaling, the regulation of TNFR1 expression, its modifications, localization and processing are less clear and the data are scattered. Here we review the current knowledge of TNFR1 regulation and discuss the impact this has on the host.

TNFR1, THE MAJOR RECEPTOR OF TNF

TNF is a type II transmembrane glycoprotein consisting of three monomers with a typical β-jellyroll structure. Each subunit consists of two packed β-sheets of five antiparallel β-strands with three additional β-strands in the N-terminal. (Figure 1) The first 76 amino acids form a highly conserved hydrophobic sequence that anchors precursor polypeptides in the membrane. This immature protein (transmembrane pro-TNF) has a molecular mass of 26 kDa and is proteolytically cleaved, mainly by the metalloprotease TNFα converting enzyme (TACE or ADAM17), to a 17-kDa active unit. (1) Also other proteases, such as ADAM10 (2), MMP7 (3) and MMP13 (4), have been shown to cut pro-TNF and generate soluble TNF. Soluble TNF is a homotrimer with a molecular mass of 52 kDa. The **TNF** protein structure and its interaction with TNFR1 have been described in great detail from high resolution crystals. The homotrimer looks like a triangular cone or bell in which the three subunits are arranged edge to face. (5) The receptor binding sites of TNF are located in the lower half of the triangular cone, in the groove between two subunits. (6)

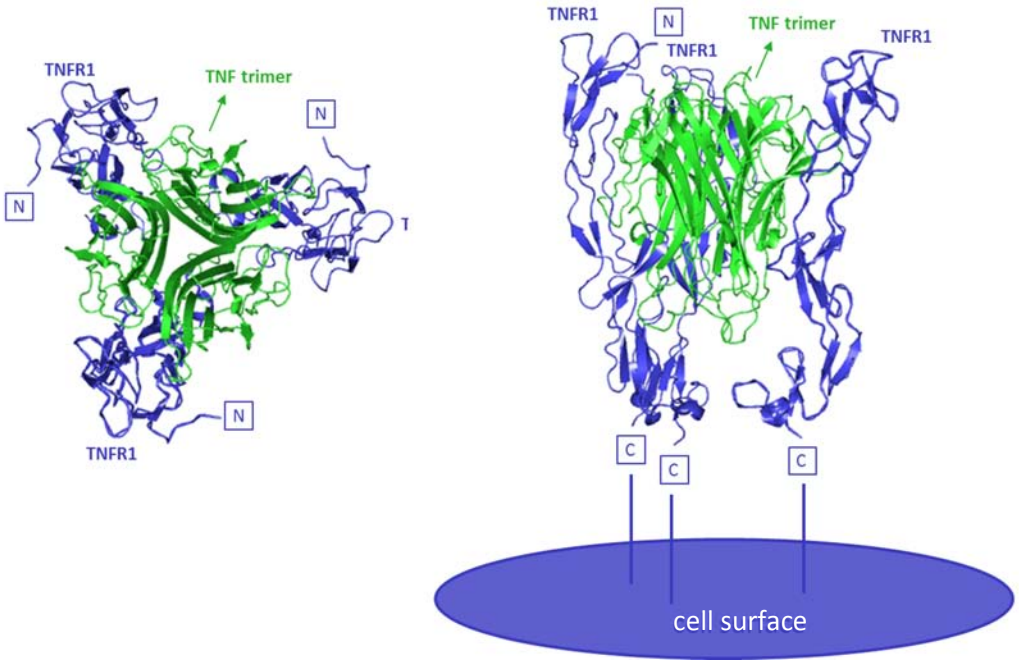

Figure 1. Crystal structure of TNF (PDB 1TNF in green) and binding to its receptor TNFR1 (PDB 1EXT in blue). Top view is on the left and side view on the right. (7, 8)

TNF binds with high affinity to two type I transmembrane receptors: TNFR1, which is activated by both sTNF and tmTNF, and TNFR2, which is activated mainly by tmTNF. Most of the biological activities of TNF are initiated by binding to TNFR1. (9)

Mouse **TNFR1** has a length of 454 amino acids (AA), of which the 21 N-terminal AAs are a signaling peptide, composed of an extracellular domain (ECD) of 212 AA, a helical transmembrane domain (TMD) of 23 AA, and an intracellular domain (ICD) of 219 AA. (Figure 2) The extracellular regions of TNFR1 and TNFR2 are structurally highly homologous. The N-terminal ECD contains two extracellular topological domains (AA 22–43 and AA 197–212) and four cysteine rich domains (CRD) at AA 44–82, AA 83-125, AA 126-166 and AA 167-196, each of which contains six cysteines. Transmembrane TNFR1 is also a substrate of TACE. The major TNFR1 cleavage site is the spacer region close to the transmembrane domain between Asn172 and Val173, and the minor site is between Lys174 and Gly175. (10) In contrast, there is no homology in the intracellular region between TNFR1 and TNFR2, indicating that these receptors activate distinct signaling pathways. TNFR1 contains a cytoplasmic death domain (DD) which is a homophilic protein–protein interaction region of 86 AA (356–441) required for TNF-induced apoptosis, and an N-SMASE activation domain (NSD) spanning an 11-AA motif N-terminal to the DD.

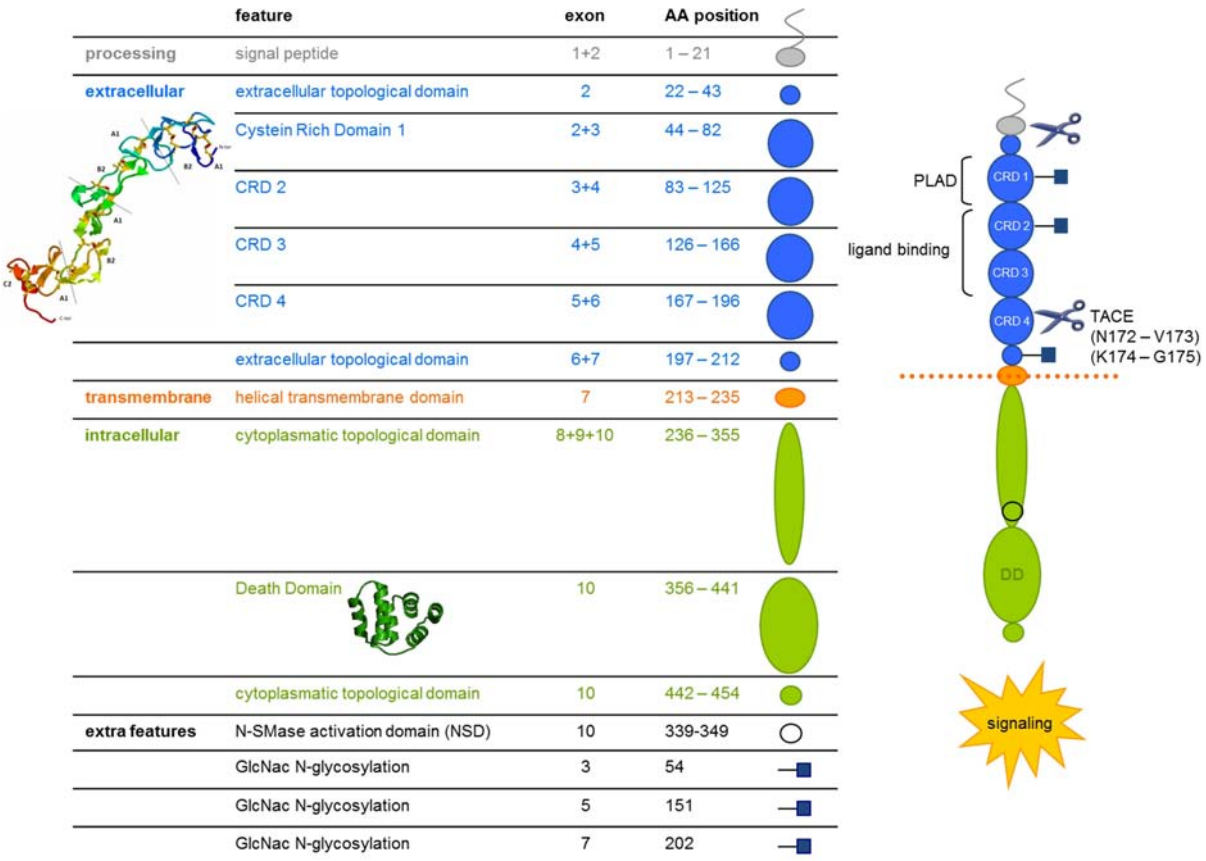

Figure 2. Representation of TNFR1 (UniProtKB P25118). For detailed description see text.

The pre-ligand assembly domain (PLAD) is a homophilic protein–protein interaction motif located in CRD1 and plays an important role in both TNFR1 and TNFR2 signaling pathways by assisting in the assembly of the receptor complex required for TNF binding. It has been proposed that PLAD-mediated homomultimer formation stabilizes CRD2 in a conformation necessary for high affinity ligand binding. (11) In autoimmune diseases, this PLAD region might serve as a target to prevent TNF signaling. A polypeptide that can mimic the binding of PLAD to TNFR1 can be an attractive alternative to the current therapeutics against TNFR1-mediated diseases. (16)

Binding of TNF to TNFR1 results in trimerization of the pre-existing receptor complexes and clustering of the intracellular death domains. Subsequently, the adapter molecule TRADD binds by interacting with

DDs of TRADD and TNFR1. (Figure 3) TRADD acts as a platform adapter that can recruit TNFR associated factor 2 or 5 (TRAF2/5), cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) and receptor interacting protein 1 (RIP1) to form the membrane-bound **complex I**. (12) This allows cIAP to K63-ubiquinate RIP1 and TRAF2/5, leading to activation of the inhibitor of κB (I-κB) kinase complex (IKK). (13) Additionally, linear ubiquitination of IKKγ or NEMO by the LUBAC complex stabilizes the IKK complex. (14) Phosphorylation of I-κB by IKK ensures I-κB K48-ubiquitination, which signals for degradation by the proteasome and consequent activation of NF-κB and its translocation to the nucleus. Activation of AP-1 involves a phosphorylation cascade mediated by the mitogen activated protein (MAP) kinases. These kinases are responsible for activating c-Jun N-terminal kinases JNK1, 2 and 3 and p38, leading to activation and nuclear translocation of c-Fos and c-Jun. (12) Hence, complex I stimulates pathways leading to activation of NF-κB and AP-1 and induction of pro-inflammatory and anti-apoptotic genes. One such anti-apoptotic gene encodes c-FLICE inhibitory protein (c-FLIP), which is a specific inhibitor of caspase 8 (an essential molecule in the apoptotic pathway). (12) However, prolonged JNK activation can also induce pro-apoptotic proteins such as Itch, which can mediate c-FLIP degradation. (15) However, the duration of NF-κB activation is limited by several feedback mechanisms, such as the induction of I-κB, CYLD and A20. CYLD, a protease that specifically cleaves K63-ubiquitin chains, de-ubiquinates TRAF2, thereby inhibiting the recruitment of TAB/TAK and activation of IKK (17), and A20 deactivates RIP1 by removing the K63-ubiquitin chain and adding a K48-ubiquitin chain. (18) Sustained MAPK activation is prevented by MAPK phosphatases such as glucocorticoid-induced MKP-1. However, TNF-induced reactive oxygen species (ROS) can disturb some of the feedback systems, for example by oxidizing MKP-1. (19)

Upon endocytosis of complex I, TRADD dissociates from TNFR1 and associates with Fas-associated protein with death domain (FADD) to form the intracellular located **complex II**. De-ubiquitination of RIP1 leads to recruitment and autocatalytic cleavage of pro-caspase 8, thereby initiating apoptosis. (20) However, in conditions where caspase 8 is inhibited, RIP3 can be recruited and RIP1 and RIP3 become phosphorylated by auto phosphorylation or cross phosphorylation, leading to necroptosis, a regulated form of necrosis. Necroptosis causes rapid plasma membrane permeabilization with release of reactive oxygen species (ROS) and exposure of damage-associated molecular patterns (DAMPs), which provide strong stimulation of the immune system. (21)

TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL REGULATION OF TNFR1

Transcriptional regulation of TNFR1 expression

Despite the critical role that TNFR1 plays in TNF-mediated signaling, little is known about the regulation of its promoter. It has been suggested that the TNFR1 promoter is constitutively active, like the promoters of "housekeeping" genes, but at low levels and on nearly all nucleated cell types. (22) (23). On the other hand, TNFR2 expression is inducible and it is expressed exclusively by immune cells, endothelial cells and some neuronal populations (24).

Promoter analysis

The 5' flanking region of TNFR1 was scanned for the presence of sequence motifs that have been associated with regulation of gene transcription. (25) According to the UCSC genome browser, the transcription start site (TSS) is located 107 bp upstream of the putative TSS that was described by Takao and Jacob. A putative **TATA box** (TTAAATT), the core promoter sequence, is now located between +63 and +69 downstream of the TSS, which presumes that it may not be a true TATA box. Two **GC-rich elements** are present between –44 and +3 and between +5 and +4. These elements have been shown to possess enhancer activity in many eukaryotic genes. (25)

The TNFR1 promoter contains a functionally important **binding site** for CCAAT/enhancer binding protein **(C/EBP),** which contributes to the constitutive activity of the promoter. C/EBP transcription factors play essential roles in regulating different cellular processes, including differentiation, energy metabolism, and inflammation. Both C/EBPα and C/EBPβ bind to a sequence located between +5 and +12. (25, 28)

There are two copies of the consensus **binding site for AP-1**, a transcription factor that regulates gene expression in response to different stimuli, including cytokines, growth factors, stress, and bacterial and viral infections. (25)

Also four potential binding sites for the **AP-2** family of transcription factors (AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε) have been found. The general functions of this family are stimulation of proliferation and suppression of differentiation during embryonic

development. (26)

An **NF-κB binding site** in the TNFR1 promoter between –489 and –498 activates the expression of TNFR1. In mammary-specific β-lactoglobulin $Cre^{+}/Ikk2^{fl/fl}$ mice, NF-κB DNA-binding activity is diminished by 50%, contributing to reduced expression of TNFR1 mRNA levels and abrogation of TNF-induced apoptosis. (27)

A consensus sequence related to the **IFNγ activated site (GAS)** of signal transducer and activator of transcription (**STAT-1**) factors has been found between –235 and –243 of the mouse TNFR1 promoter. In oligodendrocytes, IFNγ indeed induces TNFR1 transcription via activation and binding of STAT-1 homodimers to the GAS site in the TNFR1 promoter. (29) (30) (Figure 4 and Table 1)

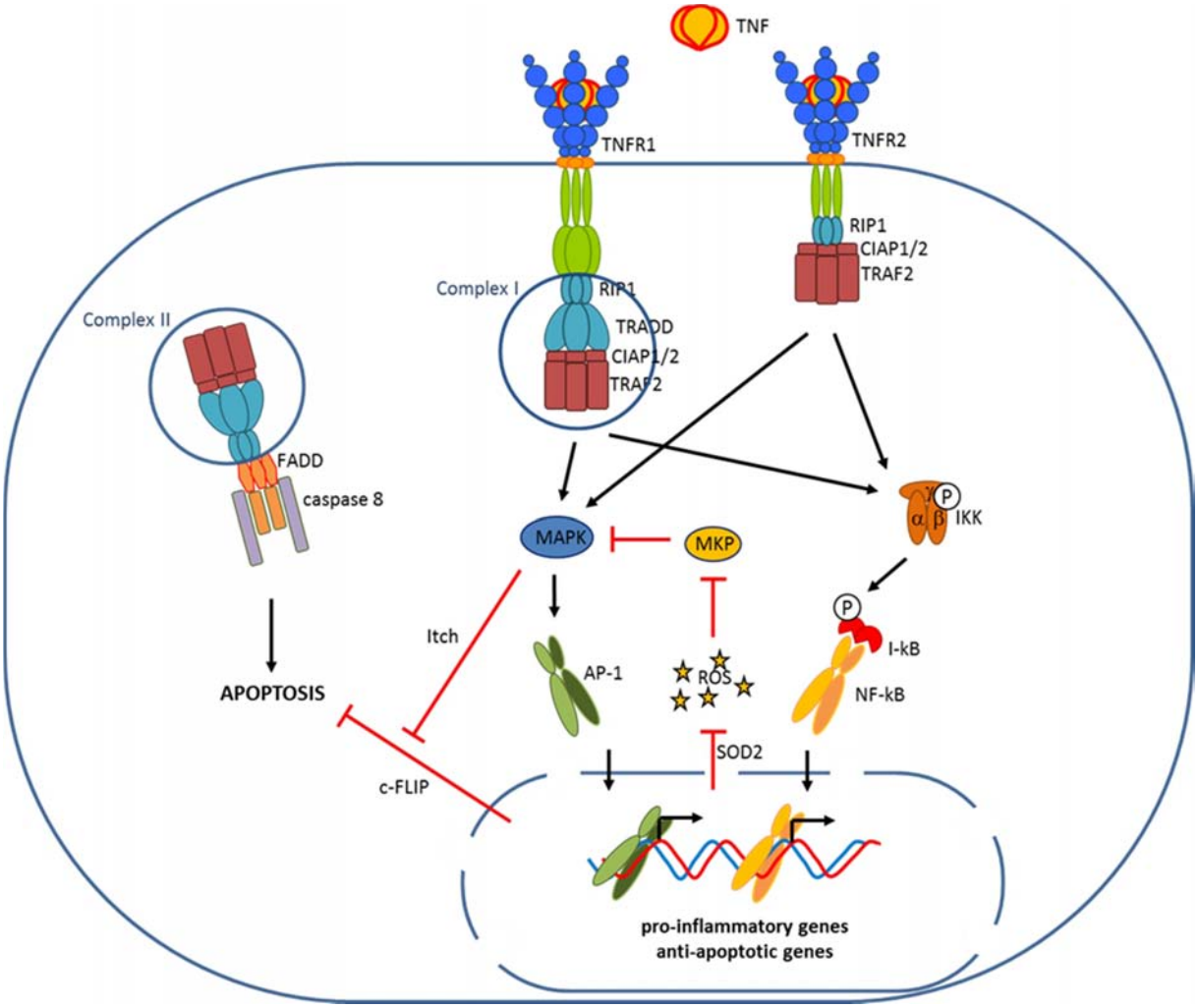

Figure 3. TNFR1 pathway. Binding of TNF to TNFR1 triggers recruitment of TRADD, TRAF2, CIAP1/2 and RIP1, forming complex I. Activation of the IKK complex, leads to proteolytic degradation of I-κB proteins and subsequent activation of NF-κB which is then translocated to the nucleus to activate transcription of target genes. Upon endocytosis of complex I, TRADD dissociates from TNFR1 and associates with FADD and caspase 8 to form intracellular complex II. Caspase 8 becomes activated and initiates apoptosis. For detailed description, see text.

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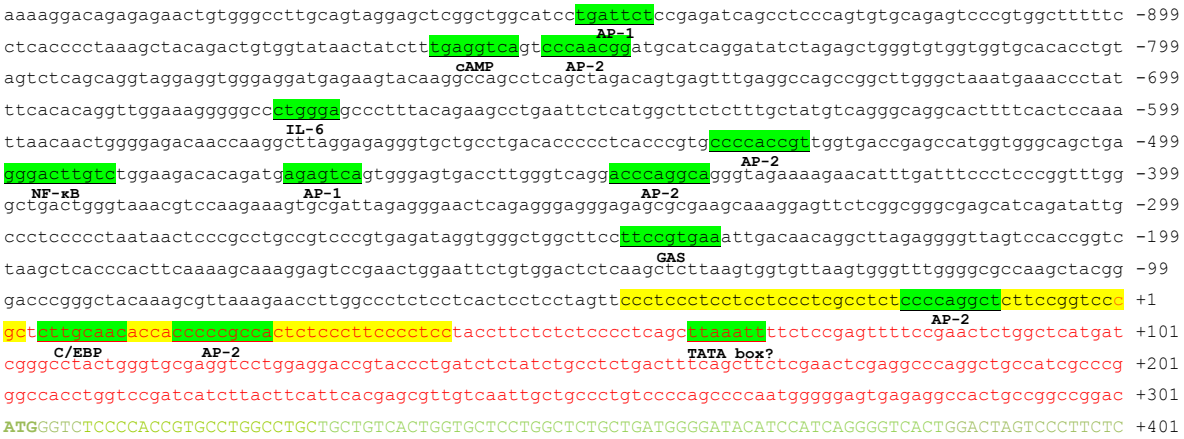

Figure 4. Promoter of mouse *Tnfrsf1a* (NM011609). The 5'UTR is shown in red and the CDS in green. Transcriptional regulatory sequences are marked in green and the GC rich elements in yellow.

transcriptional element	consensus sequence	actual sequence	position
TATA box?	TATAWAW	TtaAAtT	+63/+69
C/EBP binding site	RTTGCGYAAY	cTTGCaac	+5/+12
GC rich element			-44/+3
			+5/+41
AP-1 binding site	TGASTCA	TGAtTCt	-941/-947
		aGAGTCA	-467/-473
AP-2 binding site	GCCNNNGGC	cCCAACGGa	-843/-850
		cCCCACcGt	-527/-535
		aCCCAGGca	-436/-444
		cCCCAGGct	-11/-19
		cCCCCGcca	+17/+25
NF-κB binding site	GGGACTTTCC	GGGACTTgtC	-489/-498
IL-6 responsive element	CTGGGA	CTGGGA	-669/-674
cAMP responsive element	TGACGTCA	TGAgGTCA	-853/-860
IFNγ activated site	TTCNNNGAA	TTCCGTGAA	-235/-243

Table 1. Transcriptional regulatory sequences in the promoter of *Tnfrsf1a*. Lower case indicates deviations from the consensus. (Y=C/T, R=A/G, M=A/C, K=G/T, W=A/T, S=G/C)

Factors affecting TNFR1 expression

Gene transcription is controlled by dynamic acetylation and deacetylation of histone proteins (and other proteins), which alters chromatin structure and affects transcription factor access to the DNA. **HDAC5** (Histone deacetylase 5) overexpression has been shown to inhibit tumor cell growth and induce spontaneous apoptosis by altering gene expression, including a four-fold up-regulation of TNFR1. (31)

Transcriptional up-regulation of TNFR1 by **NF-κB** activation has been observed in membrane-bound TNF-mediated cell–cell contact between T-cells and monocytes. (32) Moreover, **IL18** and IL18Rα up-regulate the expression of both TNFR1 and TNFR2 on TNF decreased the enhancement of TNF and IL1β production by IL18 on cell contact, suggesting that IL18 up-regulates TNF receptor expression and thereby stimulates cross-talk between monocytes and activated T-cells. (36) Also **Vitamin D3** enhances TNF-induced NF-κB activation and TNF-induced apoptosis in TNF sensitive MCF-7 cells. This can be explained by up-regulation of surface expression of TNFR1. The Vitamin D effect, however, changes the balance between death-inhibiting and death-promoting signals in favor of increased apoptosis. (37)

On the other hand, **IL3 and GM-CSF** can down-regulate both mRNA and surface expression of TNFR1 and TNFR2. This inhibits TNF-induced differentiation

the surface of monocytes by activating the NF-κB pathway. Neutralizing mAb to T-cell membrane-bound or dendritic cells. (33) **IL10** induces down-regulation of membrane TNFR1 and TNFR2 on monocytes and reduces the pro-inflammatory potential of TNF in three ways: down-regulating membrane TNF receptor expression, increasing production of soluble TNF receptor, and inhibiting TNF release. This suggests that IL-10 may be useful in the treatment of diseases involving overexpression of TNF. (34) However, overexpression of **STAT3** completely inhibited IL10-induced suppression of TNF receptor expression. (35)

Transcription factor prediction

The interaction of transcription factors with transcription factor binding sites (TFBSs) is the primary mechanism in the regulation of transcription. Since TFBSs are short DNA sites of 6-15 bp, *in silico* predictions simply based on their primary sequence are less reliable. We searched for TFs that can target *Tnfrsf1a* in both mouse and human by using a new online TFBS prediction program named PhysBinder. In addition to sequence data, this program makes use of biophysical properties of protein-DNA complexes such as the flexibility of the DNA. (38) In our *in silico* TFBS predictions, we further took into account the conservation among vertebrates, DNase hypersensitivity that predicts open chromatin clusters and markers that are found near regulatory elements (H3K4Me1) and promoters (H3K4Me3) based on ENCODE data using the UCSC human genome browser. Applying a stringent threshold, we found seven specific TFBSs for human *TNFRSF1A*, five TFBS specific for mouse *Tnfrsf1a* and 25 TFBSs that are common to mouse and human (Table 2).

	MAFK	SP4	SREBF1	STAT1	RAD21	ELK4	Mycn	EGR1	REST	SP2	USF2	NANOG	CTCF	ELK1	ETS1	FOS	GABPA	GATA1	GATA2	KLF4	MAFF	MEF2C	RELA	SPI1	RUNX3	STAT3	TEAD4	ZEB1	BACH1	ELF1	ESSRA	IRF3	JUNB	GTF3C2	Essrb	Tfcp2l1	Zfx
hg19_human *TNFRSF1A*	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
mm9_mouse *Tnfrsf1a*	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 2. TF binding site prediction for *Tnfrsf1a*. 1/green indicates predicted, 0/red indicates not predicted.

Figure 5. Genomic structure of the TNFR1-coding gene.

Post-transcriptional regulation of TNFR1 expression

A key aspect of the regulation of eukaryotic gene expression is cytoplasmic control of mRNA stability, translation and degradation. (39) Regulation at the translational level can provide a quick response to stimuli because it does not involve the upstream processes of gene expression. Furthermore, translational regulation is usually reversible, as it is often mediated through reversible protein modifications such as the phosphorylation of translation initiation factors.

RNA binding proteins

Decreased stability of TNFR1 mRNA during differentiation of human monocytes is associated with changes in the formation of RNA–protein complexes: a decrease of 66–87 % in the level of TNFR1 mRNA of these cells was noticed upon phorbol esters such as TPA-induced differentiation. This decrease is partial and thus appears to be only one of several mechanisms that lead to the complete loss of TNFR1 from the surface of those cells. In contrast, in the fibroblast cell line SV-80, TPA did not induce alterations in the pattern of RNA–protein complexes, nor did it affect the level of TNFR1 mRNA or cell surface protein expression, indicating that the response to TPA is cell-type specific. TPA activates protein kinase C and can modulate gene expression by affecting transcription, but there is also evidence that TPA affects gene regulation post-transcriptionally by altering the stability of mRNAs. To detect proteins that bind to the TNFR1 mRNA, electrophoretic mobility shift was assayed on radiolabeled full-length RNA transcribed *in vitro*. Incubation of the probe with cytoplasmic extracts from untreated U-937 cells resulted in formation of one major complex (complex A) and one minor complex with lower electrophoretic mobility (complex B). A substantially different pattern was observed for protein extracts from TPA-treated U-937 cells. Formation of complex A was decreased while that of complex B was markedly increased. In addition, complexes of intermediate mobility appeared.

The nucleotide sequence involved in the formation of the RNA–protein complexes A and B and intermediate complexes is located in exon 2 of the TNFR1 mRNA. It appears that an 18-nt region (caccctcaaaataattc), essential for all complexes formed, is on its own sufficient for formation of complex A, whereas additional 5' adjacent nucleotides are required for formation of complex B and intermediate complexes. The identities of the proteins are not yet known. TNFR1 mRNA does not contain RNA destabilizing AU rich elements (AREs), but the RNAs for GM-CSF, TNF, and c-myc, which do contain AREs in their 3' UTR, efficiently compete with the TNFR1 RNA in the formation of complex B. Competition was weaker in the formation of complex A. (40, 41) Detection of the RNA binding proteins would be a big step forward in understanding the regulation of TNFR1 mRNA stability.

microRNAs

Over the past decade, microRNAs (miRNAs) have emerged as important regulators of translation and mRNA stability. MiRNAs can down-regulate gene expression by two post-transcriptional mechanisms, mRNA cleavage or translational repression, depending on the target. When a miRNA guides cleavage, the cut is at precisely the same site as in siRNA-guided cleavage, i.e. between the nucleotides pairing to residues 10 and 11 of the miRNA. After cleavage of the mRNA, the miRNA remains intact and can recognize and cleave other mRNA copies. (42)

Hundreds of microRNA molecules and their possible targets have been discovered in the genomes of plants and animals (43). Strikingly, bioinformatics analyses suggest that up to 30% of human genes might be regulated by miRNAs (44). We searched for miRNAs that can target *Tnfrsf1a* by using the following miRNA target prediction programs: MicroCosm, miRanda, miRGen, NBmiRTar, TargetScan, miRDB, DIANAmicroT, microInspector, miRWalk, PicTar, PITA, RNA22 and RNAhybrid. By using several programs based on algorithms focusing on different features such as (seed) complementarity, conservation and thermodynamics, we made a comparative analysis. (Table 3)

	microCosm	miRanda	miRGen	NBmiRTar	TargetScan	miRDB	DIANA-microT	microInspector	miRWalk	PICTAR	PITA	RNA22	RNAhybrid	sum
mmu-mir-29a/b1	1	1	1	0	1	0	1	0	1	1	1	0	0	**8**
mmu-mir-29b2/c	1	1	1	0	1	0	1	0	1	1	1	0	0	**8**
mmu-mir-125a	1	0	1	1	1	0	1	1	1	0	1	0	0	**8**
mmu-mir-680-1	1	1	1	0	1	0	1	1	1	0	1	0	0	**8**
mmu-mir-351	1	0	1	0	1	0	1	1	1	0	1	0	0	**7**
mmu-mir-763	1	1	1	0	1	0	1	0	1	0	1	0	0	**7**
mmu-mir-125b	1	0	1	0	1	0	1	0	1	0	1	0	0	**6**
mmu-mir-149	0	0	1	0	1	0	1	1	1	0	1	0	0	**6**
mmu-mir-511	1	1	0	0	1	0	0	1	1	0	1	0	0	**6**

Table 3. miRNA target prediction for Tnfrsf1a. 1/green indicates predicted, 0/red indicates not predicted.

Post-translational regulation of TNFR1 expression

Storage

After translation in the endoplasmic reticulum, TNFR1 is localized predominantly in the **Golgi apparatus** while TNFR2 is directed immediately to the plasma membrane. The intracellular death domain is required for retention of TNFR1 in the Golgi apparatus: deletion of the entire TNFR1 intracellular domain or the C-terminal DD allowed expression of the receptor on the plasma membrane. However, addition of a DD to the C-terminus of TNFR2 did not lead to Golgi-retention. (49) Deletions in the cytoplasmic tail demonstrated that a C-terminal sequence of 23 amino acids is required for targeting to the *trans*-Golgi network. This sequence is partly outside the death domain and contains an acidic cluster. Interaction of this sequence with membrane traffic adaptor proteins may play an important role in controlling the cell's

response to TNF. (45) The Golgi pool of TNFR1 is believed to serve to replenish cell surface TNFR1 receptors. (Figure 6)

Shedding

TNFR1 can be released from the cell surface by a proteolytic process named ectodomain shedding. (Figure 6) Like TNF, TNFR1 and TNFR2 are cleaved by TNF-α converting enzyme (TACE). (1) Proteolytic cleavage of TNFR1 or TNFR2 reduces their surface expression thereby desensitizing cells to TNF actions. Moreover, the generation of antagonistic soluble receptors can regulate TNF bioactivity by preventing its binding to membrane receptors. (46) TACE may therefore exert either pro- or anti-inflammatory, depending on whether it acts on an effector cell (e.g. releasing ligand from macrophage) or target cell (e.g. releasing receptor from endothelial cell). (47) However, low levels of sTNFR may stabilize the activity of TNF and provide a reservoir of TNF. (48) In humans, mutations affecting the shedding of TNFR1 have been linked with the development of the TNFR1-associated periodic syndromes (TRAPS), which are characterized by recurrent fever attacks and localized inflammation. Similarly, knock-in mice expressing a mutant non-sheddable TNFR1 are very sensitive to TNF-induced inflammation and develop several auto-immune-like diseases, such as spontaneous hepatitis, enhanced susceptibility to endotoxic shock, exacerbated TNF-dependent arthritis, and experimental autoimmune encephalomyelitis. (50) Basal expression of TACE is observed in all vasculature cell types, including endothelial cells, vascular smooth muscle cells, fibroblasts, and leukocytes. TACE-mediated shedding is enhanced by stimulation with pro-inflammatory cytokines (TNF, IFNγ), TLR ligands (LPS), growth factors (PDGF, VEGF), GPCR ligands (thrombin) or oxidative stress (ROS), and thus involved in many regulatory pathways. Transcriptional regulation, maturation by furin, trafficking from storage pools, intracellular phosphorylation, changes in cellular distribution within membrane lipid rafts, interaction with adapter molecules (Tetraspanins) and conformational changes

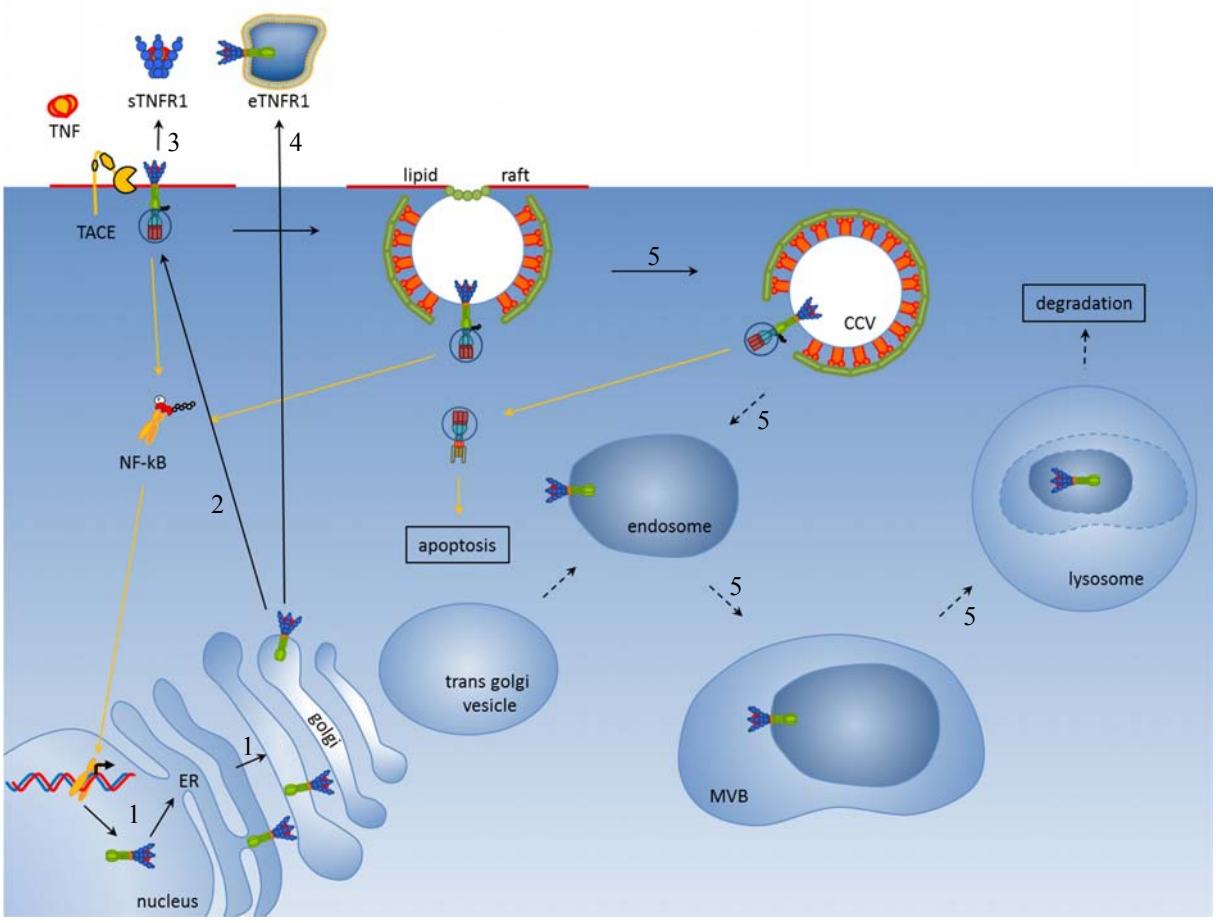

Figure 6. Compartmentalization of TNFR1 signaling. Black arrows indicate transport of TNFR1, 1=translation and storage, 2=membrane expression, 3=shedding, 4=exocytosis, 5=endocytosis. Yellow arrows indicate signaling pathways. For detailed description, see text.

of the protease all lead to enhanced substrate cleavage by TACE. (51) TACE is expressed in intracellular compartments and on the cell surface. Removal of its pro-domain by a furin protease probably occurs within the Golgi apparatus. (52) Distribution of TACE and its substrates within lipid rafts affects shedding because mature TACE is associated with cholesterol-rich lipid rafts, and depletion of rafts induces shedding of TNF, TNFR1 and TNFR2. (53, 54) A p38- and ERK-dependent phosphorylation of TACE at Thr735 has been demonstrated (55), and ERK inactivation induced by arecoline (a muscarinic acetylcholine receptor agonist) suppressed the production of mature TACE. (56) However, analysis of TACE truncations and chimeric constructs revealed that the protease requires the transmembrane domain but not the intracellular domain for up-regulation of shedding, suggesting that intracellular phosphorylation is not necessary for regulating TACE activity. (57) TACE is kept in a less active closed conformation by extracellular protein disulfide isomerases (PDI). (58) PMA-induced generation of reactive oxygen species (ROS) changes the redox state, leading to inactivation of PDI and thereby allowing the protease to adopt an active open conformation. (59)

The mechanism of TNFR1 shedding might also involve interactions with regulatory ectoproteins. A direct relationship exists between the level of the type II integral membrane protein **ARTS-1** (aminopeptidase regulator of TNFR1 shedding) and the degree of TNFR1 shedding. ARTS-1 overexpression increases TNFR1 shedding and decreases membrane-associated TNFR1, while expression of antisense ARTS-1 mRNA decreases membrane-associated ARTS-1 and TNFR1 shedding but increases membrane-associated TNFR1. ARTS-1 neither bound to TNFR2 nor altered its shedding, indicating its specificity for TNFR1. Findings suggest that ARTS-1 does not possess TNFR1 sheddase activity, which indicates that it is a multifunctional ectoprotein capable of binding to and promoting TNFR1 shedding. (60)

Exocytosis

TNFR1 shedding is a consequence of proteolytic cleavage of the 28-kDa ectodomain by a receptor sheddase, such as TACE. But in human vascular epithelial cells (HUVECs) and bronchoalveolar lavage (BAL) fluid, the predominant form of sTNFR1 is the full-length 55-kDa protein (eTNFR1) associated with **exosome-like vesicles**. (Figure 6) Exosomes are small membrane-enclosed vesicles of 30–100 nm released from the cell by exocytic fusion with the plasma membrane. These intracellular TNFR1-containing vesicles enable constitutive release of eTNFR1 into the extracellular compartment. This is an alternative pathway for generating soluble cytokine receptors independently of proteolytic cleavage of the receptor ectodomain. (64) It has been reported that histamine induces redistribution of TNFR1 from the Golgi to vesicles and subsequently into the medium as soluble receptors, with a consequent decrease in cell surface TNFR1 (46). The precise significance of such remarkable full-length TNFR1 shedding is still unclear.

Ligand-induced translocation

Upon binding of TNF, TNFR1 translocates to cholesterol- and sphingolipid-enriched membrane microdomains named **lipid rafts**, where it associates with RIP1 and the adaptor proteins TRADD and TRAF2 to form signaling complex I. (Figure 3) This complex triggers pathways leading to induction of pro-inflammatory and anti-apoptotic proteins. Activation of p42, a member of the MAP kinase family, leads to TNFR1 phosphorylation at a consensus MAPK site in its cytoplasmic domain. Phosphorylation of TNFR1 alters its subcellular localization, resulting in changes in its signaling properties. (65) Moreover, in lipid rafts TNFR1 and RIP1 become ubiquitinated, which leads to their degradation via the proteasome pathway. (66) Disruption of lipid rafts, by depleting cholesterol, not only abolishes ubiquitination but also totally blocks TNF-induced NF-κB activation, leading to a switch to apoptosis induction which indicates that the translocation of NF-κB to lipid rafts is essential for its activation. (67)

Endocytosis

Formation of complex I is transient because most of TRADD, RIP1 and TRAF2 dissociate from TNFR1 within an hour, when TNFR1 starts to undergo endocytosis. (Figure 6) Treatment of U937 cells with TNF led to maximal down-modulation of the TNF receptors within 30 minutes. These cells express both types of TNFRs, but the amount of TNFR2 is more than double that of TNFR1. Ligand-induced down-modulation of TNF receptors is caused by TNFR1 internalization and TNFR2 shedding, and the signaling for both is mediated through TNFR1. (68) The liberated DD of TRADD now binds to FADD, resulting in caspase 8 recruitment and complex II formation. Complex II initiates apoptosis, provided that NF-κB signaling has terminated. (67)

Endocytosis or TNFR1 internalization and intracellular

trafficking play an important role in selection of the signaling pathway: either internalization-independent (pro-inflammatory complex I) or internalization-dependent (pro-apoptotic complex II). Apoptosis is totally blocked by preventing internalization with monodansylcadaverine (MDC), an inhibitor of transglutaminase, a membrane-bound enzyme that actively participates in internalization of various receptor systems. (61) The endosome should therefore be recognized as a signaling organelle involved in selectively transmitting death signals from TNFR1. (62) Internalization proceeds through the classical receptor-mediated endocytosis pathway, i.e. via clathrin-coated vesicles and endosomes that fuse and enter multivesicular bodies (MVB) before accumulating in lysosomes. (63) After apoptosis, TNFR1 protein can be eliminated in a caspase-dependent manner. *In vitro*, the cytoplasmic tail of hTNFR1 is susceptible to cleavage by the downstream executioner caspase 7, the only caspase capable of cleaving TNFR1. Identification of the cleavage site revealed an EXE motif instead of the classic EXD motif. Homologous sequence alignments showed that the EXE motif is conserved in rat and pig but not in mouse and cow, which have 3–4 missing amino acids at this site. (69)

TNF thus causes the down-modulation of its own receptor by internalization which might function as a negative feedback mechanism as it will desensitize cells for further TNF stimulation until the membrane TNFR levels have recovered.

DYSREGULATION OF TNFR1 EXPRESSION

TNFR1 genetic variants

Missense mutations

Systemic auto-inflammatory diseases are genetic disorders characterized by seemingly spontaneous inflammation without major involvement of the adaptive immune system. Among them is the TNF receptor-associated periodic syndrome (**TRAPS**), which is caused by missense mutations in the TNFRSF1A gene and characterized by periodic high fevers, rash, abdominal pain, chest pain, conjunctivitis, arthralgia and myalgia. More than 100 mutations have been identified in TNFR1, all of them in the extracellular domain. (http://fmf.igh.cnrs.fr/ISSAID/infevers) (70) TRAPS mutations occur predominantly in CRD1 and CRD2 and many of them involve intramolecular disulfide bonds. Others occur at residues predicted to have an effect on the secondary structure or at residues involved in hydrogen bonds between loops of the receptor. The absence of large deletions suggests that synthesis of the mutant protein is important for pathogenesis. It has been hypothesized that TRAPS pathology is driven by defective receptor shedding resulting in reduced serum levels of soluble TNFR1. This hypothesis was based on observations that cells of some TRAPS patient are resistant to PMA-induced shedding and that serum from TRAPS patients contained reduced levels of circulating TNFR1. However, the shedding defect was not always present and varied depending on the studied cell types. (71) Molecular modeling indicates that nine TNFR1 mutants are unable to bind TNF and normal TNFR1 (H22Y, C30S, C30R, C33G, C34S, T50M, C52F, C88R, R92P), whereas R92Q and P46L can. Due to misfolding, they accumulate in the endoplasmic reticulum (ER) instead of localizing to the cell surface, and are most likely degraded by the proteasome. (79) But accumulation of mutant TNFR1 in the ER may also trigger the ER stress response, which can directly or indirectly lead to inflammation or block TNF-induced apoptosis. Interestingly, neutrophils and dermal fibroblasts from TRAPS patients with several different mutations have reduced apoptosis but produce the proinflammatory cytokines IL-6 and IL-8 normally when exposed to TNF. (80, 81) Failure of activated cells to undergo apoptosis in TRAPS could lead to accumulation of pro-inflammatory cytokines. However, TRAPS mutations might not all act by the same mechanism. (82, 83) Glucocorticoids are effective in decreasing the severity and duration of the fever attacks, although their efficacy fades with time. (84) The soluble TNF receptor Etanercept, the IL-1β receptor antagonist Anakinra and the IL-6 receptor antagonist Tocilizumab are effective in some patients. However, since no controlled clinical trials have been performed for this rare syndrome, there is currently no single recommended treatment. (85)

Two rare single amino acid mutations in *TNFRSF1A* have been identified as low-penetrance risk factors for TRAPS. The R92Q substitution is carried by ~2% of North American and Irish populations, and the P46L mutation is present in 9% of African populations. TRAPS patients with these polymorphisms have a milder syndrome with almost no incidence of amyloidosis. (86) But the R92Q mutation has also been linked with other diseases associated with inflammation, such as **rheumatoid arthritis** and **atherosclerosis**. (87, 88) Clinical observations have

identified some **multiple sclerosis** (MS) patients carrying the R92Q mutation and exhibiting additional TRAPS symptoms. The co-existence of MS and TRAPS could be mediated by this mutation. The R92Q mutation acts like a genetic risk factor for MS and other inflammatory diseases, including TRAPS. Nevertheless, this mutation does not appear to be a severity marker, modifying neither the progression of MS nor its response to therapy. But an alteration in TNF/TNFR1 signaling may increase pro-inflammatory signals. (72)

Single nucleotide polymorphisms (SNPs) in the *TNFRSF1A* gene may also influence the innate immune response against **invasive pulmonary aspergillosis** (IPA), an increasingly common opportunistic fungal infection that usually occurs in immunocompromised patients. (73) In this infection, TNF acting through TNFR1 plays a pivotal role in immune regulation and host immune responses. Three SNPs were genotyped in 275 individuals (52% immunocompromised hematological patients with high-risk of developing IPA and 48% healthy controls): A383C and G609T in the 5' UTR, and A36G in exon 1. The last two are associated with IPA susceptibility. The role of *TNFRSF1A* SNPs is also supported by significantly lower TNFR1 mRNA levels in IPA compared to IPA-resistant patients and by a strong correlation between the –609 SNP and TNFR1 expression levels. TNFR1 polymorphisms may influence the risk of IPA disease and might be useful for risk analysis. (74) The A36G mutation is also significantly associated with lower hemoglobin levels, causing iron deficiency anemia in patients with early or established rheumatoid arthritis. The frequency of anemia is higher in GG homozygous patients. (75, 76)

The pathologies induced by mutations in TNFR1 further emphasize the important role of TNFR1 in autoimmune diseases and immunity. Furthermore, they indicate that TNFR1 targeting might be a better alternative to current TNF inhibition therapies since Etanercept and Infliximab fail in certain TRAPS family with T50M, C30S and R92Q mutations. (77, 78)

Alternative splicing

Two disease-associated isoforms produced by alternative splicing were recently described.

The **TNFR1-Δ6** splicing pattern is caused by a variation (rs1800693) in the exon 6/intron 6 border region that alters exon 6 splicing. The frame shift caused by skipping of exon 6 results in a protein lacking the intracellular and transmembrane domains and part of the extracellular domain. This TNFR1 splice form is associated with multiple sclerosis (MS) but not with other autoimmune conditions such as rheumatoid arthritis, psoriasis or Crohn's disease. The MS risk allele induces the expression of a soluble TNFR1 form that can block TNF. Importantly, TNF-blocking drugs can promote onset or exacerbation of MS, but they are very effective for autoimmune diseases that are not associated with rs1800693. This indicates that the clinical experience with these drugs corroborates the disease association of rs1800693, and that the MS-associated TNFR1 variant mimics the effect of TNF-blocking drugs. (89)

The **TNFR1-Δ2** splice variant is regulated by three variations affecting the phenotype of TRAPS. They occur in the promoter, exon 1 and intron 4 (rs4149570, rs767455, rs1800692 respectively) of the *TNFRSF1A* gene. Exon 2 alternative splicing increased with the T-A-T haplotype at rs4149570–rs767455–rs1800692 as compared with the G-G-C haplotype and transcriptional activity increased with the T-T haplotype compared with the G-C haplotype, suggesting that regulation of TNFR1-Δ2 expression may occur via a coupling mechanism between transcription and splicing. Whereas TNFR1 is ubiquitously expressed, TNFR1-Δ2 is expressed tissue-specifically in human PBLs, brain, heart, kidney, skeletal muscle, small intestine and spinal cord, but not in liver or lung. (90)

TNFR1-mediated diseases

Because TNF is a key immune system modulator and has broad effects, excessive signaling can cause significant damage. A delicate balance exists between beneficial immune stimulation and pathogenesis. Several autoimmune and inflammatory diseases have been associated with the effects of dysregulated TNF activation. (9) Therefore, understanding the regulation of TNF and its receptors is essential for elucidating how TNF can either prevent or induce various diseases. (Table 4)

Patients with **systemic lupus erythematosus** (SLE), a multi-organ inflammatory autoimmune disease, have altered expression of TNF-related signaling molecules, suggesting that imbalance of TNF signaling favors cellular activation rather than apoptosis. SLE patients have increased levels of TNFR1, TNFR2 and TRAF2 and decreased levels of RIP1 on various naive and memory B-cell and T-cell subsets as compared to controls. However, the levels of these molecules are not correlated with their RNA expression or with serum TNF levels in peripheral whole blood. (91)

Disease	Biomarker	Tissue	Effect	Ref.
Systemic Lupus Erythematosus	TNFR1 protein up	B-cells, T-cells	cellular activation	(91)
Ocular allergic inflammation	TNFR1 protein up	HCECs	I-CAM expression	(92)
Aging	TNFR1 protein up	lymphocytes	apoptosis	(93)
Acute Myocardial Infarct	TNFR1 protein up	cardiomyocytes, ECs	angiogenesis down	(94-96)
Ischemic cerebrovascular diseases	sTNFR1 protein up	serum	proliferation	(97, 98)
Lung epithelium injury	sTNFR1 protein up	BALF	neutralize TNF	(99)
Lupus Nephritis	TNFR1 protein up	urine	?	(100, 101)
Behcet's Disease	sTNFR1 protein up	serum, synovial fluid	?	(102-105)
Huntington's Disease	TNFR1 mRNA down	skeletal muscle	?	(106-109)
Hepatocellular carcinoma	sTNFR1 protein up TNFR1 mRNA down	serum, ascetic fluid liver	?	(110, 111) (112)

Table 4. The regulation of TNFR1 in several TNFR1-mediated diseases. For detailed description, see text.

Up-regulation of the intercellular adhesion molecule (ICAM)-1 on human conjunctival epithelial cells (HCECs) is an important feature of **ocular allergic inflammation**. TNF in the supernatant from IgE-activated human conjunctival mast cells up-regulates the expression of TNFR1 on HCECs, resulting in a stronger TNF-mediated response, including up-regulation of ICAM-1. So, up-regulation of TNFR1 expression results in enhanced ICAM-1 expression in response to TNF stimulation. This demonstrates that targeting TNFR1 expression may be more effective than targeting TNF for treatment of ocular inflammation. (92)

Aging is characterized by increased susceptibility of T-cells to TNF-induced apoptosis due to increased constitutive expression of TNFR1 and TRADD and decreased expression of TNFR2 and TRAF-2. Moreover, there is an increased activation of caspase 8 and caspase 3, confirming that increased TNF-induced apoptosis may play a role in T-cell deficiency associated with human aging. (93, 113)

Development of artery diseases is associated with age-related impairment of angiogenesis. Because TNFR1 is known to mediate the cytotoxic effects of TNF, whereas TNFR2 is mostly involved in the protective effects, TNF signaling via its receptors has diverse effects on neovascularization, repair and regeneration in adult tissue after **acute myocardial infarction** (AMI). Intact signaling through both TNFR1 and TNFR2 assures sufficient NF-κB activation, followed by transcriptional activation of *VEGFA*, *FGFB* and other pro-angiogenic genes. But age-associated decrease of TNFR2, coupled with post-ischemic increase in systemic TNF, favors apoptosis in adult cardiomyocytes and ECs due to reduced NF-κB activation, leading to inhibition of angiogenesis. Decreased TNFR2 expression in adult tissue also stimulates pro-apoptotic signaling through TNFR1 by the release of vacant TRADD. (94) But signaling via both TNFR1 and TNFR2 is necessary to prevent reperfusion injury after AMI during late preconditioning. So, total blocking of TNF is not advised in AMI patients. Modulation of TNFR1 and/or TNFR2 expression at different stages of AMI may have important implications for prevention of myocardial injury and enhancement of myocardial repair and regeneration. (95)

Furthermore, it has been shown that patients with **acute ischemic cerebrovascular diseases** also have higher plasma levels of sTNFR1. (97, 116) Likewise, animal studies have shown that TNFR1 is associated with decreased neuronal proliferation after stroke and that deletion of TNFR1 enhances neuroblast formation and recovery. These results provide evidence that TNFR1 is a negative regulator of stroke-induced progenitor proliferation; the proliferative response after stroke might be promoted by blocking TNFR1 signaling. (98)

Ozone (O_3) is an air pollutant that causes **lung epithelium injury** leading to inflammation. The inflammatory response to acute ozone exposure includes the production of numerous cytokines and chemokines, resulting in influx of neutrophils. Genetic linkage studies on both mice and humans have shown that a locus encompassing the *TNF* gene plays a role in responses to O_3. In mice, age-related differences in the

inflammatory response to acute O_3 exposure vary with TNFR1 expression. In fact, it has been shown that sTNFR1 increases during disease in the BAL fluid, and that this sTNFR1 neutralizes TNF in the lung and so protects against O_3-induced inflammation. (99)

Other diseases may also be associated with differences in TNFR1 levels, however the effects are less clear. (Tabel 4) In mice and patients with **lupus nephritis**, an inflammation of the kidney caused by SLE, TNFR1 is found to be enriched in the urine especially at the peak of disease. Whether the increased urinary TNFR1 levels reflect increased shedding and are relevant to disease progression remains unclear. (114) Patients with active **Behcet's disease** (BD), a chronic, multi-organ immunoinflammatory vasculitis that often presents with mucous membrane ulcers, ocular lesions and arthritis, have increased levels of plasma sTNFR1 and sTNFR2, especially when arthritis is present. (102) TNF appears to be important in initiating Behcet's disease, as indicated by the effects of blocking TNF. Infliximab is a promising treatment for uveitis associated with the disease and Etanercept is useful for patients with mainly skin and mucosal symptoms. (103-105, 115) Since the level of TNFR1 correlates well with disease activity index scores of these diseases, the increase may be useful for diagnosis of Behcet's and lupus.

In **Huntington's disease** (HD), a neurodegenerative disorder caused by expansion of a glutamine-encoding CAG repeat which affects muscle coordination and results in cognitive regression and psychiatric problems, the opposite is observed. Mouse models representing the earlier stages of human HD ($Hdh^{CAG(150)}$ knock-in mice) or the later stages or the rare, but more severe, juvenile form of human HD (R6/2 transgenic mice) (106, 107) and muscle biopsies from HD patients, showed decreased *Tnfrsf1a* gene expression. (109) Studies with TNFR1 deficient mice could be very useful to confirm these observations.

Hepatocellular carcinoma (HCC) is the fifth most common neoplasia in the world and the first cause of death by cancer in some regions. sTNFR1 levels in the serum and ascitic fluid of patients with HCC are significantly higher than in controls, and they correlate positively with total bilirubin and alpha fetoprotein in the peripheral blood. This reflects an abnormal immune status of HCC patients and can help to predict the progression of the tumor. (110) Moreover, the disruption of death receptor-dependent cell signaling is linked to poor survival in patients with HCC. (111) On the other hand, several genetic alterations of the TNF-TNFR superfamily in HCC were detected by sequencing HCC DNA samples. In particular, the TNFR1 promoter –329G/T polymorphism was strongly associated with primary HCC, where the T allele resulted in the repression of TNFR1 expression. Therefore, these results suggest that the TNFR1 329G/T polymorphism may play an important role in the development of HCC. (112)

Viral TNFR1 interference

TNF acting via TNFR1 is considered an important anti-viral agent, often acting synergistically with IFNγ. (117) However, growing evidence has shown that both DNA and RNA viruses can interfere with the TNFR1 pathway and thereby escape the host immune response. In infected cells, viral proteins can either affect TNFR1 availability by acting upon transcription, translation, trafficking or shedding of TNFR1, or modulate TNFR1 activity by acting on internalization or signaling of TNFR1. **Epstein-Barr virus** (EBV) immediate-early protein BZLF1 prevents TNF-induced activation of target genes and TNF-induced apoptosis by down-regulation of TNFR1 during the EBV lytic replication cycle. Thus, EBV has developed a mechanism for evading TNF-induced antiviral effects during lytic reactivation or primary infection. (118) The **hepatitis C virus** (HCV) core protein is besides a component of viral nucleocapsids also a multifunctional protein influencing multiple cellular processes. In HCV core protein-activated Hep191 cells, transcriptional profiling identified decreased expression of TNFR1. Since RT-PCR confirmed that TNFR1 is down-regulated and that TNF-induced DNA fragmentation is suppressed in these cells, expression of HCV core protein at physiological levels might inhibit apoptotic cell death of HCV-infected cells. (118)

Herpes simplex virus 1 (HSV) uses many strategies to inactivate host functions that are harmful to its replication and dissemination, including taking advantage of the short half-life of TNFR1. Steady-state levels of TNFR1 require continuous renewal by translation of its mRNA, and the HSV viral protein UL41 prevents this constant replenishment by degrading the TNFR1 mRNA. (119)

Cytomegalovirus (CMV) is known to target the cell cycle, cellular transcription and immunoregulation to optimize the cellular environment for viral DNA replication. CMV infection also prevents external signaling to the cell by reducing the cell surface expression of TNFR1. Viral early gene products may be responsible for interfering with TNFR1 trafficking through the Golgi apparatus to the cell surface. So,

Virus	Viral protein	Interference	Mechanism	Ref.
Epstein-Barr virus	BZLF1	TNFR1 transcription	Reduction of TNFR1 promoter activity	(118)
Hepatitis C virus	?	TNFR1 transcription	Inhibition of TNFR1 mRNA expression	(120)
Herpes simplex virus	UL41	TNFR1 translation	Degradation of TNFR1 mRNA and inhibition of apoptosis	(119, 121)
Cytomegalovirus	Immediate early (IE) viral products	TNFR1 Golgi-trafficking	Down-regulation of TNFR1 surface expression	(122, 123)
Poliovirus	3A	TNFR1 Golgi-trafficking	Down-regulation of TNFR1 surface expression	(124)
Respiratory syncytial virus	?	TNFR1 shedding	IL10 production and increased production of soluble TNF receptor	(125, 126)
Adenovirus	E3-14.7K	TNFR1 internalization	Inhibition of apoptosis and increased NF-κB signaling	(127)
Myxoma virus	secreted M-T2 (pseudo sTNFR)	TNFR1 signaling	Inhibition of TNF-induced cytotoxicity in T-cells	(128, 129)
Myxoma virus	intracellular M-T2	TNFR1 signaling	Inhibition of apoptosis (T-cells)	(128)
Orthopoxviruses	CrmB,C,D,E (pseudo sTNFR)	TNFR1 signaling	Blocking TNF function	(130)
Epstein-Barr virus	LMP-1 (pseudo TNFR)	TNFR1 signaling	Ligand independent NF-κB activation (infected B-cells)	(131-137)
Human herpes virus 6B	U20	TNFR1 signaling	Inhibition of TNFR1 signaling and apoptosis	(138)
Herpesvirus saimiri	STP (pseudo TNFR)	TNFR1 signaling	Ligand independent NF-κB activation via association with TRAFs (infected T-cells)	(139)
human papilloma virus 16	E6	TNFR1 signaling	TNFR1 binding with inhibition of TRADD binding and DISC formation leading to reduced TNF-induced apoptosis	(140)
Respiratory syncytial virus	RSV-G protein (pseudo TNFR1)	TNFR1 signaling	Inhibition of TNF-induced apoptosis by I-κB proteolysis	(141)
Hepatitis C virus	HCV core protein	TNFR1 signaling	TNFR1 DD binding with inhibition of TRADD binding, facilitating FADD binding and leading to increased TNF-induced apoptosis (HepG2, Hela)	(142, 143)
Hepatitis C virus	HCV core protein	?	Increasing NF-κB nuclear retention and DNA binding and increasing I-κB degradation leading to reduced apoptosis	(144, 145)
Hepatitis C virus	HCV core protein	?	Inhibition of TNF-induced apoptosis (MCF7)	(146)
Tanapox virus	glycopeptide	?	Inhibition of TNF-induced NF-κB activation leading to reduced cell adhesion molecules	(147)
Parvovirus	?	?	Activation of caspase 3 and down-regulation of c-*myc* leading to increased apoptosis	(148, 149)

Table 5. Viral mechanisms of escape from the host response. For detailed description, see text.

upon infection CMV isolates the cell from host-mediated signals, forcing it to respond only to virus-specific signals. (122, 123)

Poliovirus also triggers host defensive reactions by activating intrinsic (intracellular) and extrinsic (receptor-mediated) apoptotic pathways. Poliovirus nonstructural protein 2A is an inhibitor of cellular translation enhancing the sensitivity to TNFR1-induced apoptosis. On the other hand, poliovirus nonstructural protein 3A neutralizes the pro-apoptotic

activity of 2A by eliminating TNFR1 from the cell surface. Consequently, poliovirus infection dramatically decreases TNF receptor abundance on the surfaces of infected cells as early as four hours post-infection. Poliovirus-mediated resistance to TNF is caused by protein 3A interfering with protein trafficking through the endoplasmic reticulum and Golgi: the effect of protein 3A on TNF signaling can be imitated by brefeldin A. (124)

Alveolar macrophages and respiratory epithelial cells infected with **respiratory syncytial virus** (RSV) suppress the production of early inflammatory cytokines such as TNF by producing IL10, resulting in an ineffective response to the virus. (125) Moreover, the soluble form of TNFR1, but not TNFR2, was secreted from these cells in a time- and RSV dose-dependent way. As the secretion of soluble TNFR1 blocks TNF responses, increased shedding might be another counteraction against the immune response. (126)

The **adenovirus** protein E3-14.7K inhibits TNF-induced apoptosis by preventing TNF-induced TNFR1 internalization, which results in inhibition of the DISC formation. In contrast, E3-14.7K did not affect TNF-induced NF-κB activation via recruitment of RIP-1 and TRAF-2. Inhibition of endocytosis by E3-14.7K is due to a failure in the coordinated temporal and spatial assembly of essential components of the endocytic machinery, such as Rab5 and dynamin2, at the site of the activated TNFR1. This is another mechanism by which Adenoviruses escape the host immune response. (127)

Many poxviruses encode several immunomodulatory proteins, such as homologs of host cytokine receptors, also referred to as viroreceptors. These receptors mimic host function by binding to host cytokines, allowing the virus to circumvent the immune defense. The T2 protein of **myxoma virus** (M-T2) is a pseudo TNF receptor that has two distinct activities. The secreted dimeric M-T2 glycoprotein binds TNF with high affinity, inhibiting direct TNF-mediated cytolysis of infected cells and other secondary immune responses dependent on TNF. However, intracellular M-T2 prevents myxoma-infected T-cells from undergoing apoptosis, supporting viral replication. (128, 129) The cytokine response modifier (Crm)-like pseudo TNF receptors, including CrmB, CrmC, CrmD and CrmE and a putative fifth member from cowpox virus that closely resembles CD30, have been identified in several **orthopoxviruses**. These viral TNF receptors resemble secreted versions of the extracellular domains of their counterpart cellular receptors and form functional oligomers that bind and block TNF. (130)

The latent membrane protein 1 (LMP1) of **Epstein-Barr virus** (EBV) contributes to the immortalizing activity of EBV in primary human B lymphocytes. (131) LMP1 is targeted to the plasma membrane of infected cells as a constitutive pseudo TNF receptor that activates NF-κB through two independent domains in its cytoplasmic tail. One site is similar to TNFR2 interacting with TRAF1 and TRAF2 and the second site is similar to TNFR1 associating with TRADD. As LMP1 acts independently of the ligand, it replaces the T-cell-derived activation to maintain unlimited B-cell proliferation. LMP1-mediated signaling through the TRAF system plays a role in the pathogenesis of EBV-infected lymphomas that emerge in immunosuppressed patients. (132) Furthermore, the activity of the TNFR1 promoter is dramatically decreased by the EBV protein BZLF1, helping the virus to oppose the anti-viral effects of TNF. (118) Infection by **human herpesvirus 6B** (HHV-6B) blocks caspase 3 and 8 activation and I-κB phosphorylation, indicating inhibition of both the inflammatory and apoptotic signaling pathways. The viral pseudo TNF receptor U20 was shown to localize to the cell membrane, and siRNA knockdown of U20 showed that the protein is necessary for HHV-6B-mediated inhibition of TNFR signaling during infection. (138) The STP pseudo TNF receptors of the **herpesvirus saimiri** (HVS) are stably associated with TRAF1, 2, or 3. Mutational analysis revealed that STP-C488 induces NF-κB activation that correlates with its ability to associate with TRAFs. Thus, TRAF/STP association might be involved in immortalization of T lymphocytes following HVS infection. (139) High-risk strains of **human papillomavirus** (HPV) such as HPV16 cause human cervical carcinoma. The E6 protein of HPV16 mediates the rapid degradation of p53. But transfection of HPV16 E6 into the TNF-sensitive LM cell line protects the cells from TNF-induced apoptosis independently of p53. Caspase-3 and -8 activation is significantly reduced in E6-expressing cells, indicating that E6 acts early in the TNF apoptotic pathway. In fact, E6 binds directly to TNFR1. E6 requires the same TNFR1 C-terminal part for binding as does TRADD, and TNFR1/TRADD interactions are decreased in the presence of E6. HPV E6 binding to TNFR1 interferes with formation of the DISC and thus with transduction of pro-apoptotic signals. HPV, like several other viruses, can evade the TNF-mediated host immune response. (150) Moreover, the central conserved

region of the attachment protein G of **respiratory syncytial virus** (RSV) shows structural homology with the fourth subdomain of TNFR1. Although the functions of both protein domains are unknown, the structural similarity of the two protein domains suggests that the cysteine noose of RSV-G may interfere with the antiviral and apoptotic effect mediated by TNF. (141)

The **hepatitis C virus** (HCV) core protein binds to the cytoplasmic domain of TNFR1, namely the death domain (DD) thereby inhibiting TRADD binding but facilitating FADD recruitment to TNFR1. (142) (143) By sensitizing cells to TNF-mediated apoptosis, the HCV core protein may provide a selective advantage for HCV replication by enabling evasion of host antiviral defense mechanisms. In contrast, HCV core protein was shown to inhibit TNF-induced apoptosis via NF-κB activation. The expression of the core protein enhances nuclear retention and DNA binding activity of NF-κB and TNF-triggered I-κB degradation. This ability of HCV core protein to inhibit TNF-mediated apoptotic signaling may contribute to the chronically activated, persistent state of HCV-infected cells. (144) (145) The controversy among these reports may be attributed to differences in cell types, conditions of core protein expression, and protocols of TNF stimulation. (151, 152)

Tanapoxvirus (TPV)-infected cells secrete an early 38-kDa glycopeptide that selectively inhibits TNF-induced NF-κB activation and transcriptional activation of cell-adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 on the surface of endothelial cells. (147) **Parvovirus** H-1 infection leads to activation of caspase 3, leading to morphologic changes characteristic of apoptosis and resembling the effects of TNF treatment. This effect is also observed when U937 cells are infected with a recombinant H-1 virus that expresses the nonstructural proteins but in which the capsid genes are replaced by a reporter gene, indicating that the induction of apoptosis can be assigned to the cytotoxic nonstructural proteins in this system. Furthermore, the c-Myc protein, which is over-expressed in the monocytoid cell line U937, is rapidly down-regulated during parvovirus infection, consistent with a possible role of c-Myc in mediating the apoptotic cell death induced by H-1 virus infection. Interestingly several clones derived from the U937 cell line and selected for their resistance to H-1 virus fail to decrease c-Myc expression upon treatment with differentiation agents, and they also resist the induction of cell death after TNF treatment. (148, 149)

THE BIOLOGICAL SIGNIFICANCE OF TNFR1

TNF signals through two distinct receptors, TNFR1 and TNFR2. These receptors initiate diverse important effects, including proliferation, differentiation, migration, inflammation and cell death. (153) The pro-inflammatory and pathogen-clearing activities of TNF are mediated mainly through activation of TNFR1, which is a strong activator of NF-κB, while TNFR2 may be more responsible for suppression of inflammation. (154)

Several groups generated TNFR1 deficient mice by gene targeting. (155-157) Studies on these mice have contributed a lot to understanding the role of this receptor in the biological activities of TNF. It is clear that TNF has a key role in immunity and immunomodulation as well as pro-inflammatory and antitumor activities. Resistance against bacterial infection, e.g. *Listeria monocytogenes*, is mediated by TNFR1: mice deficient in TNFR1 are extremely sensitive to *L. monocytogenes* and other gram-positive bacteria, including *Yersinia enterocolitica* (158, 159), *Pseudomonas aeruginosa* (160), *Legionella pneumophila* (161) and *Burkholderia pseudomallei* (162), and also to other pathogens, such as viruses, e.g. influenza virus (167) and vaccinia virus (168). However, injection of TNF in mice leads to systemic inflammation, which, depending on the dose, can be lethal. Studies on TNFR1 knockout (KO) mice have shown that lethality, which involves inflammation (169) and necroptosis (21), is mediated entirely by TNFR1. Chronically overexpressed TNF leads to arthritis and inflammatory bowel diseases, both of which depend on TNFR1. (170) TNF-induced antitumor effects are also mediated entirely by TNFR1 on host-derived neo-vascular endothelial cells, which grow into the tumor and are important for sustaining tumor growth and survival. (171)

Anti-TNF therapy is effective for treatment of autoimmune diseases such as rheumatoid arthritis and inflammatory bowel diseases, indicating that the TNF signaling pathway is an appropriate target. (172, 173) Nevertheless, long-term TNF blockade can cause substantial side-effects, such as opportunistic infections (174) and development of additional autoimmune diseases, including lupus, type 1 diabetes, uveitis, multiple sclerosis and psoriasis, as well as lymphoma and leukemia. (175, 176) Interestingly,

TNFR1 +/- mice, which express 50% TNFR1 on cells, were also found to be completely resistant to TNF-induced lethal inflammation over a huge dose range. (163) These data, together with others, such as those showing that mice expressing higher levels of cell-bound TNFR1 develop spontaneous inflammation (50), suggest that TNFR1 is a potential drug target, and that minor TNFR1 regulation might have substantial physiological and pathological effects. Furthermore, in humans, mutations affecting TNFR1 have been linked with the development of the TNFR1-associated periodic syndromes (TRAPS), which are characterized by recurrent fever attacks and localized inflammation. (86) Hence, selective inhibition of TNFR1 signaling might be a better alternative for the treatment of TNF/TNFR1-mediated autoimmune diseases because it would reduce the pro-inflammatory actions of TNFR1 without impairing the immunosuppressive properties of TNFR2. (164, 165) A variety of compounds have already been generated, including monoclonal antibodies and derivatives (Atrosab, 55R170, m5R16, Domain Ab), antagonistic TNF variants (R1AntTNF), RNAi and antisense oligo's (ASOs), which are all able to inhibit TNF-mediated NF-κB gene expression and apoptosis. Many of these inhibitors have already proven to block TNF-induced lethality, allowing safe anti-tumor therapy (163, 166) while others have shown to be protective in mouse models of several TNF-induced chronic disease, such as CIA, EAE, RA, VILI, CCL4-, conA- and TNF/GalN-hepatitis. (177-184)

TNF receptors form homotrimers upon activation by TNF but no heterotrimers are assembled and the TNFR1/TNFR2 protein ratio has been found to be important for the TNF response. TNF affects NF-κB activation predominantly through TNFR1, whereas TNFR2 activates transcription poorly. (185) Nevertheless, TNFR2 stimulation can result in competition for or TRAF2 and cIAPs and thereby inhibit TNFR1-induced NF-κB transcription. Reduction of NF-κB activation promotes apoptosis in certain cell types due to diminished production of anti-apoptotic factors. (186, 187) Hence, constant TNFR1 expression coupled to changeable TNFR2 levels alters the TNFR1/TNFR2 ratio and controls the response of the cell to TNF stimulation. (22) On the other hand, the kinetics of TNF binding to TNFR2 suggests a mechanism by which TNFR2 might increase the apparent rate of TNF binding to TNFR1. TNFR2 has a higher affinity and longer TNF-binding half-life than TNFR1. By a so-called ligand-passing mechanism TNFR2 is thought to associate with TNF, increasing its concentration near TNFR1 receptors, and making TNF available for activating TNFR1. (188) Clearly, the outcome of TNFR1 and TNFR2 signaling is complex and may also depend on the cell type and the activation status of the cell.

CONCLUSION

TNFR1 mediates the signaling of most cellular effects of TNF. It is involved in numerous physiological and pathological functions at different levels and in different cell types. However, despite extensive knowledge of its signaling pathway, the precise TNF/TNFR1 activation mechanism remains unclear (189). Moreover, there is still little knowledge about the transcriptional and translational regulation of TNFR1 expression. While the general assumption is that TNFR1 is constitutively expressed and regulated as a housekeeping gene, it is clearly sensitive to certain stimuli. Predictions of several transcription factor binding sites and microRNA target sequences suggest that there are many unknown regulators. TNFR1 availability and localization are regulated by shedding (1), exosome formation (64) and internalization (63). Evidence for the importance of these mechanisms can be found in the many viral proteins that interfere with TNFR1 signaling (128) and in inflammatory diseases that are attributable to abnormal or defective TNFR1 signaling (86).

The discovery of the PLAD and PLAD-mediated ligand-independent receptor assembly, for example, is an example of a novel possibility of preventing TNFR1 signaling. (11, 16) Further expansion of our knowledge on TNFR1 regulation might allow the development of new therapeutic strategies that more effectively target the pathogenic TNF signaling.

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