THE FUNCTIONAL CHARACTERISATION OF TUMOUR NECROSIS FACTOR RECEPTOR SUPERFAMILY 1A (TNFRSF1A) MUTATIONS THAT CAUSE SYSTEMIC INFLAMMATION

STEFAN SIEBERT

Section of Infection and Immunity
Wales College of Medicine
Cardiff University

Supervisor: Dr Paul Brennan

A dissertation submitted to the Wales College of Medicine, Cardiff University in candidature for the degree of Doctor of Philosophy

September 2005

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my parents, my wife Michelle and our sons Rowan and Julian

ACKNOWLEDGEMENTS

I am deeply indebted to the index patient for her participation and generous contribution to this project. Much of the work would not have been possible without this. I hope that the ongoing research into TRAPS yields tangible therapeutic benefits for her and others with this condition.

I would like to thank my supervisor Dr Paul Brennan for his excellent guidance, support and supervision throughout this project. I would also like to thank the following individuals who were all crucial to this work: Professor Bryan Williams for his encouragement, support and perseverance which were critical to getting this project both started and completed; Nick Amos whose encouragement, assistance and technical expertise were invaluable and Dr Ceri Fielding for his assistance in the early phases of this work and for his enthusiasm throughout.

I would like to thank Professor Martin Rowe for his encouragement and advice. I thank all the members of the Infection and Immunity department, particularly our research group, for their support and friendship, and for making my time in the department so enjoyable and fruitful.

I am grateful for the unrestricted educational grant from Schering-Plough, and the Cardiff and Vale NHS Small Grant Scheme that helped fund this work.

Finally, a special thank you to my wife Michelle and our two wonderful sons, who were both born while this work was in progress. This completed work would not have been possible without Michelle's unwavering support and encouragement, and all their many sacrifices.

SUMMARY

This work describes a study into the effects of clinically relevant TNF receptor superfamily 1A (TNFRSF1A) mutations associated with the TNF receptor-associated periodic syndrome (TRAPS). TRAPS is a dominantly inherited autoinflammatory disorder characterised by episodes of systemic inflammation.

The first part focuses on a novel TNFRSF1A mutation (C43S) from a patient with TRAPS. A primary dermal fibroblast line was established from the patient and used to study the functional effects of this TRAPS mutation. Experiments revealed that the C43S TNFRSF1A mutation resulted in reduced activation of the transcription factors NF-κB and AP-1 in fibroblasts, while production of the pro-inflammatory cytokines IL-6 and IL-8 was maintained at relatively normal levels. TNFα-induced apoptosis was also reduced in fibroblasts and peripheral blood mononuclear cells from this patient with TRAPS. TNFRSF1A shedding from neutrophils was normal.

The work was extended by generating and expressing plasmids for four recombinant TNFRSF1A TRAPS mutants, including C43S, in B-cell lines by transient transfection. All four recombinant TNFRSF1A mutants resulted in reduced NF-κB activation, suggesting that reduced TNFRSF1A signalling may be general feature of TRAPS. The four TRAPS mutants all displayed reduced surface expression of TNFRSF1A, with the receptor predominantly localised intracellularly. The signalling and expression studies also suggest that there may be subtle differences between the various TRAPS mutants.

In conclusion, the TRAPS mutations studied alter TNFRSF1A expression and localisation, and reduce TNFRSF1A-mediated signalling, revealing new insights into TNFRSF1A.

PAPERS/ PRESENTATIONS RESULTING FROM THIS WORK

Part of this work was previously presented at the following meetings:

18th Annual Postgraduate Research Day, 2003
University of Wales College of Medicine
Poster Presentation – 3rd Prize

19th Annual Postgraduate Research Day, 2004
Wales College of Medicine, Cardiff University
Oral presentation – 1st Prize

American College of Rheumatology
Annual Scientific Meeting 2004
San Antonio, USA
Poster presentation

Part of this work has previously been published (Appendix III):

Siebert, S., Amos, N., Fielding, C. A., Wang, E. C., Aksentijevich, I., Williams, B. D. and Brennan, P. (2005) Reduced tumor necrosis factor signaling in primary human fibroblasts containing a tumor necrosis factor receptor superfamily 1A mutant. *Arthritis Rheum,* **52,** 1287-92.

Siebert, S., Fielding, C.A., Williams, B.D. and Brennan, P. (2005) Mutation of the extracellular domain of tumour necrosis factor receptor 1 causes reduced NF-κB activation due to decreased surface expression. *FEBS Lett,* **579,** 5193-8.

ABBREVIATIONS USED

aa	Amino acids
ADAM	A disintegrin and metalloproteinase
AIP1	ASK1-interacting protein 1
ALPS	Autoimmune lymphoproliferative syndrome
AP	Alkaline phosphatase
AP-1	Activating protein-1
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulphate
ARE	AU-rich elements
ARTS-1	Aminopeptidase regulator of TNFRSF1A shedding 1
ASC	Apoptosis-associated speck-like protein containing a CARD
ASK1	Apoptosis signal-related kinase 1
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
bp	Basepairs
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
cDNA	Complementary DNA
CHX	Cycloheximide
cIAP	Cellular inhibitor of apoptosis
CINCA	Chronic infantile neurological cutaneous and articular syndrome
CIP	Calf intestinal alkaline phosphatase
CRD	Cysteine rich domain
CRE	cAMP responsive element
CRP	C-reactive protein
DD	Death domain
DISC	Death inducing signal complex
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECD	Extracellular domain

EDTA	Ethylenediaminetetra acetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbent assay
Emp	Empty vector
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
FADD	Fas-associated protein with death domain
FAN	Factor associated with neutral-sphingomyelinase activation
FCAS	Familial cold autoinflammatory syndrome
FCS	Foetal calf serum
FHF	Familial Hibernian fever
FITC	Fluorescein isothiocyanate
FLIP	FLICE inhibitory protein
FMF	Familial Mediterranean fever
FPF	Familial periodic fever
GCK	Germinal centre kinase
GFP	Green fluorescent protein
Grb2	Growth receptor bound protein 2
GSB	Gel sample buffer
HBSS	Hank's Balanced Salt Solution
HIDS	Hyperimmunoglobulinaemia D and periodic fever syndrome
HRP	Horseradish peroxidase
IκB	Inhibitor of NF-κB
ICD	Intracellular domain
IF	Immunofluorescence
IKK	IκB kinase
IL-	Interleukin-
JNK	Jun-N-terminal kinase
kb	Kilobasepairs
Kd	Dissociation constant
kDa	Kilo Daltons

EDTA	Ethylenediaminetetra acetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbent assay
Emp	Empty vector
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
FADD	Fas-associated protein with death domain
FAN	Factor associated with neutral-sphingomyelinase activation
FCAS	Familial cold autoinflammatory syndrome
FCS	Foetal calf serum
FHF	Familial Hibernian fever
FITC	Fluorescein isothiocyanate
FLIP	FLICE inhibitory protein
FMF	Familial Mediterranean fever
FPF	Familial periodic fever
GCK	Germinal centre kinase
GFP	Green fluorescent protein
Grb2	Growth receptor bound protein 2
GSB	Gel sample buffer
HBSS	Hank's Balanced Salt Solution
HIDS	Hyperimmunoglobulinaemia D and periodic fever syndrome
HRP	Horseradish peroxidase
IκB	Inhibitor of NF-κB
ICD	Intracellular domain
IF	Immunofluorescence
IKK	IκB kinase
IL-	Interleukin-
JNK	Jun-N-terminal kinase
kb	Kilobasepairs
Kd	Dissociation constant
kDa	Kilo Daltons

LAR	Luciferase assay reagent
LB	Luria broth
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal antibody
MADD	MAPK activating death domain
MAPK	Mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MAP4K	MAPK kinase kinase kinase
mfi	Mean fluorescence intensity
MHC	Major histocompatability complex
MIM	Mendelian inheritance in man
MLK	Mixed lineage kinases
mRNA	Messenger ribonucleic acid
mTNFα	Membrane-integrated TNFα
MWS	Muckle-Wells syndrome
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor-κB
NIK	NF-κB-inducing kinase
NLS	Nuclear localisation sequence
NRS	Normal rabbit serum
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PLB	Passive lysis buffer
PFA	Paraformaldehyde
PI	Propidium iodide
PLAD	Pre-ligand-binding assembly domain
PMA	Phorbol-myristate-acetate

PMSF	Phenylmethanesulphonylfluoride
Poly(A)	Polyadenylation
RA	Rheumatoid arthritis
RHD	Rel-homology domain
RIP1	Receptor interacting protein-1
SAA	Serum amyloid A
SAPK	Stress-activated protein kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SH3	Src homology-3
SNP	Single-nucleotide polymorphisms
SODD	Silencer of death domains
SP	Signal peptide
TACE	TNF-alpha converting enzyme
TAE	Tris-acetic acid buffered EDTA
TBE	Tris-Boric acid buffered EDTA
TE	Tris-EDTA
TEMED	NNN'N- tetramethylethylenediamine
TGF	Transforming growth factor
TMD	Transmembrane domain
TNF	Tumour necrosis factor
TNFα	TNF-alpha
TNFβ	TNF-beta
TNFR	TNF receptor
TNFRSF	TNF receptor superfamily
TRADD	TNF receptor 1-associated death domain protein
TRAF	TNF receptor associated factor
TRAPS	TNF-receptor-associated periodic syndrome
TRID	TNF-receptor 1 internalisation domain
UV	Ultraviolet
WT	Wild-type

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CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Tumour necrosis factor alpha (TNFα)

1.1.1 Background to TNFα

Tumour necrosis factor alpha (TNFα) is a potent cytokine, with pleiotropic activities in immune regulation and host defence, as well as potentially hazardous pro-inflammatory and cytotoxic functions, and has been the subject of extensive investigation over the past 30 years. In the 19th century it was first reported that tumours in humans occasionally regressed after bacterial infection (Bruns, 1868), leading to the use of bacterial extracts to treat human tumours, with some success (Coley, 1891). In 1943, lipopolysaccharide (LPS) was isolated from bacterial extracts and shown to be responsible for the tumour regression (Shear and Turner, 1943). It was subsequently shown that the effects of LPS on tumour regression are not direct, but are mediated through a factor in the serum initially termed tumour necrotizing factor (O'Malley *et al.*, 1962), and later renamed tumour necrosis factor (TNF) (Carswell *et al.*, 1975).

Macrophages were identified as the major cellular source of TNF (Carswell *et al.*, 1975) although early studies were limited by the crudeness of the preparations. The similarity with lymphotoxin (LT), a protein produced by lymphocytes that kills tumour cells (Granger and Williams, 1968), further complicated the situation. It was not until 1984 that human LT was purified to homogeneity from a B lymphoblastoid cell line (Aggarwal *et al.*, 1984) and its structure determined by protein sequence methods (Aggarwal *et al.*, 1985a). Neutralization of LT activity by antibodies to LT led to the isolation of TNF from a human myeloid cell line (Aggarwal *et al.*, 1985b). The determination of the amino acid sequence of TNF indicated that the two proteins were homologous, with 30% amino acid identity (Aggarwal *et al.*, 1985b). The cDNAs of LT and of TNF were subsequently cloned (Gray *et al.*, 1984; Pennica *et al.*, 1984). The binding of TNF to the receptor for LT, and its displacement by LT, further confirmed the

functional homology between the two proteins (Aggarwal *et al.*, 1985c). These similarities led to the renaming of TNF and LT to TNFα and TNFβ respectively. It eventually became clear that these two were members of a gene superfamily, now known as the TNF superfamily. Modern molecular techniques have shown that the receptors for these TNF superfamily proteins also constitute a TNF Receptor (TNFR) superfamily (Figure 1.1). To date, at least 16 TNF ligand superfamily members mediate their cellular response through at least 29 TNFR superfamily members. While most ligands bind to a single receptor, others bind to more than one. The reasons for this apparent redundancy remain unclear.

1.1.2 The TNFα gene

As TNFα is expressed transiently, its release requires transcription, protein synthesis and protein processing. The TNFα gene is a single copy gene found next to the TNFβ gene on the short arm of human chromosome 6 (Spriggs *et al.*, 1992). The gene is closely linked with the major histocompatability (MHC) genes on chromosome 6. The TNFα gene is around 3 kb long and consists of four exons interrupted by three introns. Exons I and II contain almost entirely leader peptide sequence, while over 80% of the mature TNFα sequence is encoded in the fourth exon. The 5' flanking region of the TNFα gene contains several CRE and κB binding sites, in addition to a MHC class II-like "Y box", which are involved in the transcriptional regulation of TNFα by the transcription factors AP-1 and NF-κB (Shakhov *et al.*, 1990; Yao *et al.*, 1997). Numerous polymorphisms of the TNFα promoter have been described, but their functional significance and association with disease remain controversial in light of conflicting results (Allen, 1999; Bayley *et al.*, 2004).

1.1.3 Translational regulation of TNFα

TNFα is also regulated at a translational level. The mRNAs of many inflammatory proteins are potentially unstable, and may be stabilised by the p38 mitogen-activated protein kinase (MAPK) pathway (Winzen *et al.*, 1999). Control of mRNA stability is believed to enable rapid changes in mRNA levels and provides a mechanism for prompt

termination of protein production (Kracht and Saklatvala, 2002). mRNAs are protected from degradation by poly(A) tails. TNFα mRNA contains destabilising AU-rich elements (AREs), consisting of 6 AUUUA motifs, in the 3' untranslated region (Brook *et al.*, 2000), which direct poly(A) shortening. Deletion of TNFα AREs in mice is associated with overexpression of TNFα and a diffuse inflammatory phenotype (Kontoyiannis *et al.*, 1999). A number of ARE-binding proteins have been identified. Overexpression of HuR stabilised an mRNA reporter containing TNFα AREs (Dean *et al.*, 2001). Tristetraprolin overexpression destabilised a TNFα ARE reporter (Lai *et al.*, 1999) while tristetraprolin deficient mice developed a severe inflammatory syndrome associated with over-production of TNFα due to increased stability of TNFα mRNA (Carballo *et al.*, 1998).

It has been shown that TNFα mRNA is stabilised by the p38 MAPK pathway (Brook *et al.*, 2000), while p38 MAPK inhibitors inhibited TNFα production to a greater extent than the level of TNFα mRNA (Young *et al.*, 1993; Prichett *et al.*, 1995). The p38 MAPK pathway may control both mRNA translation and stability, with the extent of each varying with cell type (Rutault *et al.*, 2001; Kracht and Saklatvala, 2002). The exact mechanism by which p38 MAPK stabilises mRNA is unknown, while the relationship between translational control and mRNA stability is not fully understood (Rutault *et al.*, 2001).

1.1.4 TNFα structure

TNFα exists in a membrane-integrated (mTNFα) and a soluble form (Watts *et al.*, 1997). Unless otherwise stated, TNFα refers to the soluble form. Human TNFα is expressed as a precursor molecule of a 157 amino acid, preceded by a 76 amino acid leader sequence. The presequence is highly conserved and appears to serve to anchor the precursor polypeptide in the membrane (Vilcek and Lee, 1991). mTNFα has a molecular mass of 26 kDa and is proteolytically cleaved by metalloproteinases to yield a 17 kDa soluble form (Gearing *et al.*, 1994; Black *et al.*, 1997). Tumour necrosis factor-alpha converting enzyme (TACE/ ADAM 17) appears to be the major sheddase for TNFα

(Black *et al.*, 1997; Moss *et al.*, 1997). The cleaved, mature form of TNFα lacks any methionine residues and exists in solution as a homotrimer (Smith and Baglioni, 1987).

TNFα binds to two receptors, TNFRSF1A and TNFRSF1B (Section 1.2). Soluble TNFα ligands have to trimerise to be able to activate TNF receptors (Eck and Sprang, 1989). Whether membrane-bound TNFα ligands also have to form aggregates for receptor activation is not entirely clear, although crosslinking studies have suggested that mTNFα also possess this homotrimeric structure (Tang *et al.*, 1996). The receptor binding site is formed by the groove between two neighbouring ligand monomers, and the ligand trimers therefore have three potential receptor binding sites (Figure 1.2). The crystal structure for TNFα was solved almost simultaneously by two research groups (Eck and Sprang, 1989; Jones *et al.*, 1989) and was subsequently described to a very high resolution. Both forms of TNFα are active as self-assembling noncovalent trimers, arranged like a triangular cone, such that each molecule has contact with the other two. Each monomer consists of two packed β-pleated sheets, each of eight, anti-parallel β-strands arranged as compact β-jellyroll sandwiches with an N-terminal insertion that contains three additional β-strands (Eck and Sprang, 1989; Idriss and Naismith, 2000). The outer sheet is rich in hydrophilic residues, while the monomers interact at the hydrophobic interfaces of the inner sheet (Fesik, 2000). The TNF superfamily members have 25-30% amino acid similarity, which is largely confined to these internal aromatic residues responsible for trimer assembly (Idriss and Naismith, 2000). In contrast, the external surfaces of the ligands show little sequence similarity, which accounts for the receptor selectivity. However, certain ligands and receptors in the TNF/TNFR superfamilies can bind more than one partner with high affinity. The reason for this cross utilization of ligands or receptors is unclear (Aggarwal, 2003), although it may play a role in enhancing regulatory flexibility and complexity (Idriss and Naismith, 2000) and suggests the signalling pathways are highly integrative (Ware, 2003).

1.1.5 TNFα expression

TNFα is expressed in a diverse range of cells, and is produced mostly by macrophages and monocytes, but also by T-lymphocytes, B-lymphocytes, NK cells, neutrophils, endothelial cells and fibroblasts, as well as a number of tumour cell lines (Munker and Koeffler, 1987; Vilcek and Lee, 1991). Many of TNFα's activities are species-independent, although some are species specific (Fiers, 1991).

1.1.6 Reverse signalling through mTNFα

Reverse signalling of membrane-integrated ligands is a common phenomenon in the TNF superfamily (eg CD40L, FasL, OX40L, CD30L) (Eissner *et al.*, 2004). It is now clear that mTNFα also possess receptor-like qualities, rendering it capable of reverse signalling. Not only could mTNFα provide a co-stimulatory signal for B cell activation (Aversa *et al.*, 1993), but it also induced E-selectin expression on activated T cells in the reverse direction (Harashima *et al.*, 2001). While initial experiments used anti-TNFα antibodies to trigger mTNFα, this can also occur with TNFR positive cells (Harashima *et al.*, 2001; Kirchner *et al.*, 2004b) and high concentrations of soluble TNFRSF1A (Waetzig *et al.*, 2005).

Reverse signalling by mTNFα in monocytes results in resistance to the effects of LPS (Eissner *et al.*, 2000), while binding of mTNFα by anti-TNFα antibodies can induce apoptosis in monocytes, T cells and neutrophils (Lugering *et al.*, 2001; ten Hove *et al.*, 2002; Waetzig *et al.*, 2003). It appears from these studies that anti-TNFα antibodies selectively kill activated immune cells at sites of inflammation, but not resting or circulating cells. This selectivity is presumed to be due to higher levels of mTNFα on activated cells (Kinkhabwala *et al.*, 1990; Aversa *et al.*, 1993; Van den Brande *et al.*, 2003). In addition, mTNFα has been found to be expressed on virally infected cells (Macchia *et al.*, 1993).

The signalling pathways in mTNFα reverse signalling are gradually being determined. The cytoplasmic domain of mTNFα contains a casein kinase 1

phosphorylation site whose phosphorylation is crucial for mTNFα-mediated calcium signalling (Watts *et al.*, 1999). LPS resistance due to mTNFα signalling in monocytic cells is mediated by the MAPK/ERK pathway (Kirchner *et al.*, 2004a). The apoptosis mediated by mTNFα is independent of Fas (CD95) (Lugering *et al.*, 2001). Stimulation of monocytes with soluble TNFRSF1A induces apoptosis through mTNFα signalling that is dependent on transforming growth factor (TGF)-β1 (Waetzig *et al.*, 2005). The enhanced TGF-β1 secretion activates MAPK/ERK pathways to induce apoptosis. However, TGF-β1 alone is not cytotoxic to these cells, suggesting that the soluble TNFRSF1A-mediated mTNFα signalling sensitised the cells to TGF-β1-induced apoptosis (Waetzig *et al.*, 2005). It remains to be elucidated whether the same signal transduction pathways are involved in T lymphocytes.

The biological importance of mTNFα signalling remains to be fully determined but lessons learned from the use of the anti-TNFα agents suggests this system may play an important role in therapeutic strategies involving TNFα modulation (Section 1.1.7). The molecular mechanisms of mTNFα reverse signalling are complex and the effects appear to be cell specific (Eissner *et al.*, 2000; Harashima *et al.*, 2001; Lugering *et al.*, 2001), with differential effects in different T cell subpopulations (Vudattu *et al.*, 2005).

1.1.7 TNFα in inflammatory diseases and anti-TNFα therapy

The concept that disequilibrium between pro- and anti-inflammatory cytokines was associated with a variety of diseases, led to intensive attempts to inhibit pro-inflammatory cytokines in these conditions. This has led to the establishment of anti-TNFα therapy as accepted treatment for rheumatoid arthritis (RA) (Feldmann and Maini, 2001). Irrespective of the stage of the disease, many pro-inflammatory cytokines, including TNFα, are expressed in RA synovium (Buchan *et al.*, 1988). It was subsequently shown that cultures of RA synovium spontaneously produced cytokines in a continuous manner (Brennan *et al.*, 1989a). The critical role of TNFα in the regulation of the inflammatory process was established by the demonstration that neutralising anti-TNFα antibodies inhibited IL-1 production (Brennan *et al.*, 1989b). Together these studies indicated that

TNFα was a rational therapeutic target in RA. Multicentre clinical studies subsequently demonstrated the efficacy of targeting TNFα by monoclonal antibodies, including infliximab (Maini *et al.*, 1999), adalimumab (Weinblatt *et al.*, 2003), or by the soluble p75TNFR:Fc fusion protein etanercept (Moreland *et al.*, 1997). These agents have been approved for use in the treatment of RA in Europe and the USA, where they are in widespread use. The anti-TNFα agents have also been found to be effective in a variety of other diseases, including Crohn's disease, psoriasis and ankylosing spondylitis. The major complication with the anti-TNFα agents has been the development of serious infectious diseases (reviewed in Ellerin *et al.*, 2003).

Valuable insights into TNFα signalling have emerged from the clinical responses, treatment failures and adverse events of the anti-TNFα agents in different diseases. While in RA, both the chimeric monoclonal anti-TNFα antibody infliximab and the soluble TNFR fusion protein etanercept led to a comparable clinical response (Feldmann and Maini, 2001), in Crohn's disease, only infliximab was effective (Targan *et al.*, 1997; Sandborn *et al.*, 2001). Both of these agents neutralise soluble TNFα effectively, suggesting that the mechanism of action of these two agents differs (Van den Brande *et al.*, 2003). It has subsequently been shown that the anti-TNFα antibodies, but not etanercept, bind to and induce apoptosis in activated T cells (Van den Brande *et al.*, 2003; Shen *et al.*, 2005). The evidence is accumulating that this infliximab-induced apoptosis of activated immune cells occurs through mTNFα reverse signalling (Mitoma *et al.*, 2005). However, the effects of infliximab appear to be target cell specific as infliximab down-regulates apoptosis of epithelial cell in Crohn's disease (Zeissig *et al.*, 2004) and bone marrow erythroid cells in rheumatoid arthritis (Papadaki *et al.*, 2002). The effects of the anti-TNFα agents are therefore not simply due to neutralisation of cytokines, and these insights are helping to inform further research into inflammation associated with TNFα.

1.2. TNF Receptors (TNFR)

TNFα binds to two cognate receptors, of molecular weight 55 kDa (TNFRSF1A, TNFR1, p55TNFR, CD120a) and 75 kDa (TNFRSF1B, TNFR2, p75TNFR, CD120b) (Hohmann *et al.*, 1989; Brockhaus *et al.*, 1990). The cloned cDNAs of these two receptors were reported by a variety of independent groups in 1990 (Gray *et al.*, 1990; Heller *et al.*, 1990; Kohno *et al.*, 1990; Loetscher *et al.*, 1990; Nophar *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990).

1.2.1 TNFRSF1A and TNFRSF1B genes

TNFRSF1A is encoded by a single-copy gene situated on chromosome 12p13.2 and is composed of 10 exons (Fuchs *et al.*, 1992). The structure of the TNFRSF1A gene excludes alternative splicing as cause for the generation of soluble TNFRSF1A (Fuchs *et al.*, 1992). TNFRSF1B is situated on chromosome 1p36.3-p36.2 and also contains 10 exons (Beltinger *et al.*, 1996). The overall gene structure of TNFRSF1A and TNFRSF1B is similar.

1.2.2 TNFRSF1A and TNFRSF1B structure

In common with the other TNFR superfamily members, TNFRSF1A and TNFRSF1B are both type 1 transmembrane proteins. While diverse in their primary structure, the TNFR receptor superfamily is characterised by the presence of repeated cysteine-rich extracellular sequence homology. The cysteine rich domains (CRDs) are 40 amino acid pseudorepeats that are typically defined by three intrachain disulphides generated by six highly conserved cysteine residues (Banner *et al.*, 1993; Bazan, 1993; Smith *et al.*, 1994). The disulphide bonds form a structural scaffold that contains short regions of variable amino acid sequence and length. These variable regions confer specificity for the appropriate ligand. This combination of structural conservation and sequence variability allows the members of the TNFR superfamily to recognise their cognate ligand with specificity. This recognition is enhanced by the inherent flexibility of the receptor molecule, which optimises the receptor's interaction with the ligand through a series of hinging movements (Naismith and Sprang, 1998; Idriss and Naismith, 2000).

The X-ray structure of TNFRSF1A in complex with TNFβ provided a breakthrough in understanding how TNF functions (Banner *et al.*, 1993). The TNF trimer was seen to bind three receptor molecules, one at each of the three TNF monomer-monomer interfaces (Figure 1.2). TNFRSF1A structure was subsequently visualised on its own (Naismith *et al.*, 1995), followed by the description of the high resolution structure of the entire extracellular domain of the receptor (Naismith *et al.*, 1996). TNFRSF1A is a single polypeptide consisting of extracellular, transmembrane and intracellular regions (Figure 1.3). The extracellular region contains four CRD, arranged end-to-end in a linear fashion and designated 1-4, starting from the N-terminus (Figure 1.4). Whereas the earlier studies did not allow adequate visualisation of the 4th CRD, the high resolution structure revealed this CRD to have a different structure to the first three CRDs (Naismith *et al.*, 1996). Each CRD contains three cysteine-cysteine disulphide bonds. The extracellular region contains the motifs involved in ligand binding (Section1.2.4) and pre-ligand-binding assembly (Section 1.2.5). Proteolytic cleavage of the extracellular region occurs in a short "spacer" segment between the extracellular and transmembrane regions (Section 1.2.8).

TNFRSF1A and TNFRSF1B share 28% homology, mostly in their extracellular regions. Their intracellular sequences are largely unrelated with almost no homology (Dembic *et al.*, 1990). Removal of the intracytoplasmic regions of the TNFRs abolishes signalling (Brakebusch *et al.*, 1992), indicating that this region is involved in signal transduction. However, the intracellular regions of both receptors lack intrinsic enzymatic activity and rely on cytosolic factors to transduce their activity. TNFRSF1A contains a protein-protein interaction motif of approximately 80 amino acid residues towards its carboxyl terminal, termed the death domain (DD) (Tartaglia *et al.*, 1993a), which is not present in TNFRSF1B. Both receptors also contain phosphorylation sites in their intracellular regions (Darnay *et al.*, 1994; Beyaert *et al.*, 1995; Van Linden *et al.*, 2000).

1.2.3 TNFR ligand binding and affinity

TNFRSF1A and TNFRSF1B can bind both TNFα and LTα. It appears that while both receptors can bind soluble and membrane-integrated forms of TNFα, TNFRSF1A interacts preferentially with the soluble TNFα homotrimer while activation of

TNFRSF1B is more efficiently induced by mTNFα (Grell *et al.*, 1995). Most *in vitro* laboratory research is conducted using soluble TNFα as stimulus, so these studies may underestimate the physiological role of TNFRSF1B (Grell *et al.*, 1995; MacEwan, 2002). It has also become clear that mTNFα has receptor-like qualities and is capable of reverse signalling, which may be important in local signalling through cell-to-cell contact (Section 1.1.6). Interestingly, while anti-TNFα antibodies (Mitoma *et al.*, 2005) and soluble TNFRSF1A (Waetzig *et al.*, 2005) induced apoptosis by mTNF reverse signalling, the soluble TNFRSF1A fusion protein etanercept did not (Van den Brande *et al.*, 2003). The extent to which cell-to-cell contact via membrane TNFα contributes to TNF-induced signalling *in vivo* remains unclear.

Cultured cells can respond to low picomolar or even fentomolar concentrations of TNFα (Coffman *et al.*, 1988; Meager *et al.*, 1993; Chan and Aggarwal, 1994; Khabar *et al.*, 1995), suggesting high affinity binding of TNFα to its receptors. While initial studies suggested that the TNFRSF1B may be the high affinity receptor for TNFα, there was marked variation in the reported dissociation constants (Kd) for TNFα binding to TNFRs, ranging from approximately 100 to 500 pM. TNFRSF1B binds and releases TNFα many fold faster than TNFRSF1A, indicating the TNFα-TNFRSF1B complex is kinetically less stable (Tartaglia *et al.*, 1993b; Evans *et al.*, 1994). "Ligand passing" was proposed as a mechanism whereby the rapid kinetics of TNFα-TNFRSF1B association and dissociation would serve to increase the local concentration of TNFα at the cell surface, thereby enhancing TNFRSF1A signalling (Tartaglia *et al.*, 1993b). However, comparable cellular sensitivities for TNFα could also be demonstrated with cells that only express TNFRSF1A (Hohmann *et al.*, 1990). Furthermore, the activity of TNFRSF1A is not impaired in TNFRSF1B knockout mice (Peschon *et al.*, 1998). It was subsequently demonstrated that when the binding affinities are measured at 37°C, TNFRSF1A is in fact the high affinity receptor for soluble TNFα (TNFRSF1A Kd = 19 pM; TNFRSF1B Kd= 420 pM) (Grell *et al.*, 1998). The authors suggest that the different stability of the ligand-receptor complexes under physiological conditions is an important parameter in determining TNFR signalling capacity.

1.2.4 Ligand binding region of TNFRSF1A

Both soluble and membrane forms of TNFRSF1A are able to bind TNFα and TNFβ (LT). The crystal structure of three soluble TNFRSF1A molecules in complex with a TNFβ trimer demonstrated direct involvement of the 2nd and 3rd CRD of TNFRSF1A in the interface with the ligand (Banner *et al.*, 1993). The interface appears to split into two almost separate contact surfaces, between amino acid 77 and amino acid 114 of TNFRSF1A. The involvement of the 2nd and 3rd CRD of TNFRSF1A in TNFα ligand binding was supported by deletion mapping (Chen *et al.*, 1995a). Deletion of the 1st CRD of soluble TNFRSF1A also resulted in loss of TNFα binding (Marsters *et al.*, 1992; Corcoran *et al.*, 1994), while deletion of the 4th CRD did not alter TNFα binding significantly (Corcoran *et al.*, 1994; Chen *et al.*, 1995a).

1.2.5 Pre-ligand-binding assembly domain (PLAD)

The crystal structure of TNFβ in complex with the TNFRSF1A extracellular domain revealed no contact between the individual receptor chains (Banner *et al.*, 1993). From this it was inferred that the TNFα ligand trimer recruited or cross-linked three receptor monomers into the final 3:3 ligand-receptor complex. This concept was challenged by subsequent crystallographic studies demonstrating dimerization of unligated TNFR, suggesting the receptor could cluster in the absence of ligand (Naismith *et al.*, 1995). A conserved domain in the 1st CRD (amino acids 1-54) of the extracellular region of TNFRSF1A and TNFRSF1B that mediates ligand-independent assembly of receptor trimers was subsequently identified (Chan *et al.*, 2000a). This pre-ligand-binding assembly domain (PLAD) is physically distinct from the ligand-binding domain (2nd and 3rd CRD), and explains the previously reported absence of TNFα ligand binding as a result of deletion of the 1st CRD of TNFRSF1A (Marsters *et al.*, 1992; Corcoran *et al.*, 1994). Mutations within the PLAD or removal of the PLAD rendered the receptor incapable of binding ligand (Chan *et al.*, 2000a). Therefore, the PLAD is required to pre-complex receptors that bind TNFα and mediate signalling. TNF receptors therefore seem to function as preformed complexes rather than as individual receptor subunits that oligomerize after ligand binding (Aggarwal, 2003).

1.2.6 TNFR Expression

TNFRSF1A is widely expressed on most cell types, with the exception of erythrocytes and platelets (Munker and Koeffler, 1987). The expression of TNFRSF1B is more restricted and confined mainly to immune and endothelial cells (Aggarwal, 2003). While there is marked variation in receptor densities, there appears to be no correlation between the number of receptors expressed on cells and the magnitude of the TNF-induced response (Tsujimoto *et al.*, 1985; Yoshie *et al.*, 1986; Munker and Koeffler, 1987; Beyaert and Fiers, 1994). Normal human lymphocytes express approximately 270 TNFRs per cell (Hober *et al.*, 1994) and it is known that significant biological effects of TNFα can occur at low levels of receptor occupancy (Munker and Koeffler, 1987). However, not all cell types that express TNFRs respond to TNFα ligand (Sugarman *et al.*, 1985; Munker and Koeffler, 1987), suggesting that the expression of TNFRs is necessary but not sufficient for the biological effects of TNFα (Yoshie *et al.*, 1986).

1.2.7 TNFR localisation and internalisation

TNFRSF1A localization may differ in divergent cell types. While TNFRSF1A has been shown to be localised to both plasma membrane lipid rafts and the trans-Golgi complex in most cells (Cottin *et al.*, 2002), in endothelial cells the receptor is principally localized to the trans-Golgi network and is only poorly expressed at the plasma membrane (Bradley *et al.*, 1995; Gaeta *et al.*, 2000). Little is known about how TNFRSF1A trafficks from the cytoplasm to the cell surface, although there is evidence that the TNFRSF1A stored in the trans-Golgi apparatus serves as a reservoir of receptor for translocation to the cell surface (Wang *et al.*, 2003). The intracytoplasmic sequences of TNFRSF1A appear to influence the expression and localisation of the receptor (Hsu and Chao, 1993). Removal of the intracytoplasmic region of the receptor results in significantly increased, uniform surface expression of TNFRSF1A (Cottin *et al.*, 2002; Fielding *et al.*, 2004). A region of the TNFRSF1A death domain (DD) (aa 405-412) has been shown to be responsible for lipid raft localisation (Cottin *et al.*, 2002). Interestingly, this region of the TNFRSF1A DD is also involved in the localisation to the trans-Golgi complex (aa 404-426), and contains a putative acidic cluster (aa 406-410) and a dileucine motif (aa 424-425) (Gaeta *et al.*, 2000; Storey *et al.*, 2002), sequences known to affect

protein localisation. These divergent findings may suggest that the sequences necessary for the spatial localisation of TNFRSF1A to different cell compartments differ in divergent cell types, and therefore, may further contribute to the observed heterogeneity of TNFα signalling in different cell populations.

Pairwise deletion of the six alpha-helices that compromise the DD also disrupted lipid raft localisation of TNFRSF1A (Cottin *et al.*, 2002), suggesting that an appropriately folded and functionally active DD is required for both TNFRSF1A-mediated apoptosis and localisation of the receptor to lipid rafts. It was subsequently shown that lipid rafts are essential for NF-κB signalling by TNFRSF1A (Legler *et al.*, 2003). We have also shown that a region of the intracytoplasmic C-terminus of TNFRSF1A between aa 218-324 negatively regulates TNFRSF1A cell surface expression (Fielding *et al.*, 2004). This region is known to contain a number of phosphorylation sites (Van Linden *et al.*, 2000) which, once phosphorylated, can relocalise TNFRSF1A to tubular structures located within the endoplasmic reticulum (Cottin *et al.*, 1999). This region also contains a sequence that interacts with the SH3 domain of the adapter protein growth receptor bound protein 2 (Grb2) (Hildt and Oess, 1999). The interaction of Grb2 with another receptor, epidermal growth factor receptor, has been shown to play a role in its internalisation by clarithrin-coated pits (Yamazaki *et al.*, 2002; Jiang *et al.*, 2003).

Compartmentalisation of TNFRSF1A signalling is one of the mechanisms whereby TNFα-induced signalling can be regulated. Although TNFRSF1A is predominantly localized to the trans-Golgi complex in endothelial cells, and the adapter protein TNF receptor 1-associated death domain protein (TRADD) also localizes to the Golgi region, TNFα-induced TRADD binding to TNFRSF1A has been shown to occur only at the cell membrane (Jones *et al.*, 1999). In contrast, it appears that selected apoptotic pathways require internalisation of TNFRSF1A (Higuchi and Aggarwal, 1994; Schutze *et al.*, 1999). A TNF-receptor 1 internalization domain (TRID) required for receptor endocytosis has subsequently been identified (Schneider-Brachert *et al.*, 2004). The authors show that while the adapter proteins receptor interacting protein-1 (RIP1) and TNF receptor associated factor-2 (TRAF2) were recruited to the receptor at the level of the plasma

membrane, the aggregation of TRADD, Fas-associated protein with death domain (FADD) and caspase-8 is critically dependent on receptor endocytosis. TNFα-induced signalling may proceed via the sequential formation of two distinct complexes located in different cellular compartments (Micheau and Tschopp, 2003) (see Section 1.3.2.3). It has recently been shown that caveolae may participate in TNFRSF1A internalization and some TNFRSF1A-mediated signalling (D'Alessio *et al.*, 2005).

Taken together, these results suggest that TNFRSF1A expression and localisation play an important role in restricting TNFRSF1A function and controlling the response of cells to TNFα. While the role of the intracytoplasmic region, particularly the DD, of TNFRSF1A in localization of the receptor is established, it has also recently been shown that mutations altering the conformation of the extracellular domain can affect receptor expression (Schweickhardt *et al.*, 2003).

1.2.8 Shedding of TNFRSF1A

The TNFRs are initially synthesised as membrane-anchored proteins, which can subsequently be proteolytically cleaved, forming soluble receptors capable of binding TNFα (Nophar *et al.*, 1990; Porteu and Nathan, 1990; Loetscher *et al.*, 1991). Soluble TNFRs are constitutively released into the circulation (Pinckard *et al.*, 1997) and their levels increase after stimulation with TNFα (Lantz *et al.*, 1990; Jansen *et al.*, 1995). Soluble TNFR levels have also been seen to increase in the course of various diseases (Diez-Ruiz *et al.*, 1995).

Soluble TNFRs are also rapidly produced in cell culture systems following stimulation with TNF (Lantz *et al.*, 1990), LPS (Leeuwenberg *et al.*, 1994a), and PMA (Aggarwal and Eessalu, 1987; Brakebusch *et al.*, 1992; Mullberg *et al.*, 1995), or after T cell (Leeuwenberg *et al.*, 1994b) and neutrophil activation (Porteu and Nathan, 1990). Shedding results in an acute decrease in the number of cell surface receptors and may therefore transiently desensitize the cell to TNFα (Wang *et al.*, 2003). The soluble receptors retain their high affinity binding for TNFα but do not directly mediate any biological effects. Therefore, the pool of soluble TNFR produced competes with the

membrane receptors for TNF ligand, thereby limiting the bioavailability of TNFα (Loetscher *et al.*, 1991; Higuchi and Aggarwal, 1992; Van Zee *et al.*, 1992). This concept is supported by the excellent clinical results obtained using soluble TNF receptor (etanercept) to treat RA (Section 1.1.6). It has also been postulated that the soluble TNFRs can stabilize circulating soluble TNFα and therefore function to enhance the TNFα effect (Aderka *et al.*, 1992). The authors postulate that soluble TNFRs may serve a buffering function, attenuating TNFα activity when TNFα levels are elevated and reconstituting TNFα when levels have declined (Aderka *et al.*, 1992).

TNFRSF1A shedding has been shown to be dependent solely on the sequence properties in the extracellular region close to the transmembrane regions of the receptor (Gullberg *et al.*, 1992; Brakebusch *et al.*, 1994). It has been postulated that TACE (ADAM 17) is also the metalloprotease responsible for cleaving TNFR (Dri *et al.*, 2000; Reddy *et al.*, 2000; Peiretti *et al.*, 2005). In addition, TNFR shedding appears to be regulated by aminopeptidase regulator of TNFRSF1A shedding (ARTS-1) which binds to an extracellular region of the receptor and facilitates receptor cleavage (Cui *et al.*, 2002).

Since my work has been in progress, the *in vivo* significance of TNFRSF1A shedding has been shown using knock-in mice expressing a mutated non-sheddable TNFRSF1A (Xanthoulea *et al.*, 2004). This mutation does not interfere with the signalling capacity of the receptor but completely abolishes proteolytic cleavage of the receptor. Heterozygous expression of the mutant receptor also resulted in marked reduction of receptor shedding, suggesting the mutation has a dominant effect (Xanthoulea *et al.*, 2004). While this mutation renders the mice more resistant to intracellular bacterial infections, they develop spontaneous hepatitis, and the mutation exacerbates TNF-dependent arthritis and experimental autoimmune encephalomyelitis (a mouse model of human multiple sclerosis). These results suggest that TNFRSF1A shedding constitutes an important mechanism for the regulation of TNFα activity *in vivo*, possibly by altering the thresholds of immune activation.

1.2.9 Lessons from TNFR-deficient mice

The deletion of the genes for TNFRSF1A and TNFRSF1B produced distinct effects in mice. Mice deficient in TNFRSF1A are susceptible to infection by *Listeria monocytogenes* but are resistant to endotoxin-induced shock (Pfeffer *et al.*, 1993) and TNFα-mediated cytotoxicity (Rothe *et al.*, 1993). Mice deficient in TNFRSF1A were subsequently shown to have impaired organization of secondary lymphoid organ structure and function (Pasparakis *et al.*, 2000) and are susceptible to infection with various microorganisms (Steinshamn *et al.*, 1996; Castanos-Velez *et al.*, 1998; Deckert-Schluter *et al.*, 1998; O'Brien *et al.*, 1999).

TNFRSF1B deleted mice also display decreased sensitivity to TNFα (Erickson *et al.*, 1994). The absence of TNFRSF1B did not impair the activity of TNFRSF1A in inflammatory models (Peschon *et al.*, 1998), although TNFRSF1B appears to have a critical role in organ inflammation, independent of TNFα or TNFRSF1A (Douni and Kollias, 1998; Peschon *et al.*, 1998).

Mice lacking both receptor genes have the sum of the phenotypic effects, and have lymph nodes and Peyer's patches (Peschon *et al.*, 1998). However, deletion of both the TNFα and LTα genes, which removes the only ligands known to interact with the two TNFRs, does not yield the same phenotype as deletion of both receptors. Mice lacking TNFα and LTα have no lymph nodes or Peyer's patches and no splenic white pulp (Eugster *et al.*, 1996; Korner *et al.*, 1997). The reason for this discrepancy is that LTα can form heterotrimers with LTβ, and bind the LTβreceptor, which is required for lymph node development (Rennert *et al.*, 1996).

1.3 TNFR-mediated signalling

TNFα-induced ligation of its cognate receptors mediates a variety of signalling pathways resulting in cell differentiation, proliferation, expression of inflammatory genes and apoptosis (MacEwan, 2002; Aggarwal, 2003; Dempsey *et al.*, 2003). The proteins involved in TNFR signalling were initially identified predominantly using the yeast two-

hybrid system for protein-protein interactions, together with overexpression and dominant-negative approaches. However, subsequent gene-deletion studies have occasionally provided contradictory findings, suggesting that results using the former techniques may sometimes be misleading. In addition, several TNFRSF1A-mediated effects appear to be cell type and context specific. The literature on these signalling pathways is therefore complex and often controversial. The focus here is on the major TNFα-induced signalling pathways and those most relevant to the subsequent experimental work.

Whereas some reports indicated that TNFRSF1A mediates apoptosis and TNFRSF1B mediates proliferation, others suggested that the two receptors transduce their signals cooperatively (Weiss *et al.*, 1998; Mukhopadhyay *et al.*, 2001). It has subsequently been shown that the two receptors mediate distinct but overlapping cellular responses (Erikstein *et al.*, 1991; Tartaglia *et al.*, 1991; Kalthoff *et al.*, 1993; Kalb *et al.*, 1996; Mukhopadhyay *et al.*, 2001; McFarlane *et al.*, 2002). The TNFRSF1A:TNFRSF1B ratio has been found to important in the way a cell predetermines its response to TNFα (Medvedev *et al.*, 1996; Declercq *et al.*, 1998; Baxter *et al.*, 1999).

1.3.1 Membrane proximal events in TNFR-mediated signalling

Upon TNFα binding, TNFRSF1A translocates to cholesterol and glycosphingolipid-enriched membrane microdomains, known as lipid rafts (Cottin *et al.*, 1999). This localization is necessary for the initiation of signalling pathways, including those leading to TNFα-induced NF-κB activation (Legler *et al.*, 2003) and apoptosis (Ko *et al.*, 1999).

Schematics of the three major TNFR-mediated signalling pathways relevant to this work are shown in Figures 1.5-1.7. Ligand independent signalling by TNFRSF1A is prevented by the constitutive association of silencer of death domains (SODD) with the DD of TNFRSF1A, thereby preventing the binding of TRADD (Jiang *et al.*, 1999). TNFα-binding to the receptor releases SODD from TNFRSF1A, permitting interaction with signalling adapter proteins. TNFRSF1A primarily associates, via its DD, with the

adapter TRADD (Hsu *et al.*, 1995). This association only occurs at the cell membrane (Jones *et al.*, 1999). TRADD in turn serves as a platform to recruit at least three additional mediators: TNF receptor associated factor-2 (TRAF2) (Hsu *et al.*, 1996), receptor interacting protein 1 (RIP1) (Stanger *et al.*, 1995) and Fas-associated protein with death domain (FADD) (Chinnaiyan *et al.*, 1995). Signalling strength induced by a given ligand/TNFR interaction appears to be regulated at the level of adapter protein recruitment (Krippner-Heidenreich *et al.*, 2002).

1.3.2 Downstream events in TNFR-signalling

TNFα-induced activation of TNFRSF1A leads to the activation of two major transcription factors, nuclear factor-kappa B (NF-κB) and activating protein-1 (AP-1), which are involved inflammatory responses and regulate apoptosis (Baeuerle and Henkel, 1994; Karin, 1995; Barnes and Karin, 1997). NF-κB is regulated primarily by phosphorylation of inhibitory proteins, the inhibitors of NF-κB (IκBs) (Section 1.3.2.1), while the activity of AP-1 is regulated primarily by members of the mitogen-activated protein kinase (MAPK) family (Sections 1.3.2.2). In contrast, the TNFα-induced association of TRADD with the DD of FADD initiates a caspase cascade resulting in apoptosis (Section 1.3.2.3). Unlike TNFRSF1, TNFRSF1B does not contain a DD motif and cannot recruit TRADD but recruits TRAF2, and TRAF1, directly (Rothe *et al.*, 1994) leading to NF-κB and AP-1 activation (Rothe *et al.*, 1995).

1.3.2.1 TNFα-induced activation of NF-κB

A schematic of TNFα-induced NF-κB activation is shown in Figure 1.5. The ubiquitously expressed NF-κB family of transcription factors contains five members in mammals: p50/p105, p52/p100, p65/RelA, RelB and c-Rel (reviewed in Ghosh and Karin, 2002). These five proteins share a conserved Rel-homology domain (RHD), of approximately 300 amino acids, which contains a nuclear localisation sequence (NLS) and is involved in dimerization, interaction with the inhibitory IκB proteins and sequence-specific DNA binding. RelA, RelB and c-Rel, but not p50 or p52, also contain transactivation domains required for the recruitment of transcriptional machinery (Chen

and Ghosh, 1999). The NF-κB family members combine as a variety of homo- and heterodimers.

Two major signalling pathways lead to the activation of NF-κB (Bonizzi and Karin, 2004). The classical pathway of NF-κB activation is activated by a variety of pro-inflammatory cytokines, including TNFα (Beg *et al.*, 1993), and pathogen-associated molecular patterns (such as those mediated through Toll-like receptors). The classical NF-κB pathway most commonly affects the p50-RelA NF-κB dimer and appears to be essential for innate immunity (Bonizzi and Karin, 2004). The more recently described alternative pathway is activated in response to engagement of various TNFR superfamily members, such as LTβR and CD40 but not TNFRSF1A. This pathway results in phosphorylation of the p100 precursor, causing its inducible processing to p52, which in turn results in the nuclear translocation of p52-RelB dimers (Senftleben *et al.*, 2001). The alternative pathway of NF-κB activation appears to play a central role in the development and maintenance of secondary lymphoid organs (Bonizzi and Karin, 2004).

Ligand induced aggregation of TNFRSF1A allows binding of TRADD to the DD of the receptor which in turn recruits members of the TNF receptor associated factors (TRAF) family which are involved in NF-κB activation. TRAF2 was initially shown to be a critical mediator of NF-κB (Rothe *et al.*, 1995). However, mice deficient in either TRAF2 (Yeh *et al.*, 1997) or TRAF5 (Nakano *et al.*, 1999) have intact TNFα-induced activation of NF-κB, while TRAF2/5 double knockout cells have substantially reduced NF-κB activation (Tada *et al.*, 2001). Therefore, TRAF2 and TRAF5 appear to play a redundant role in TNFα signalling to NF-κB, although the nature of this role remains to be established.

Another TRADD-recruited protein, RIP1 is also essential for TNFα-induced NF-κB activation (Ting *et al.*, 1996) although its kinase activity is dispensable for NF-κB activation (Lee *et al.*, 2004). It appears that while TRAF2 directly recruits IκB kinase (IKK), RIP1 stabilises this complex, resulting in the activation of IKK (Devin *et al.*,

2000). While it was previously thought that NF-κB-inducing kinase (NIK) was essential for TNFα-induced NF-κB activation (Malinin *et al.*, 1997), NIK was subsequently shown to be involved in the alternative NF-κB activation pathway only and not in NF-κB activation by TNFα (Yin *et al.*, 2001).

IKK activation is required to allow NF-κB to translocate to the nucleus. In most cells, inactive NF-κB is retained in the cytoplasm in complex with a member of the IκB family (Baeuerle and Baltimore, 1988; Beg *et al.*, 1992). The IκB family includes IκBα, IκBβ, Bcl-3, IκBε and IκBγ, which are characterised by the presence of five to seven ankyrin repeats that mediate binding to the RHD of NF-κB and interfere with its NLS function. The functional characteristics of IκBα, IκBβ and IκBε are primarily the result of temporal differences in their degradation and synthesis, attributable to differential transcriptional regulation of their promoters (Hoffmann *et al.*, 2002). Activated IKK mediates the specific phosphorylation of IκB (Brockman *et al.*, 1995; Brown *et al.*, 1995). This phosphorylation of IκB results in its ubiquitination and subsequent degradation by the 26S proteosome, allowing NF-κB to translocate to the nucleus (Palombella *et al.*, 1994; Chen *et al.*, 1995b). Degradation of IκB is a key step in the regulation of NF-κB is therefore tightly regulated. IKK has been shown to contain two kinase subunits, IKKα and IKKβ, and a regulatory subunit called NF-κB essential modifier (NEMO/IKKγ) (Karin and Ben-Neriah, 2000). While the classical NF-κB activation pathway involves predominantly IKKβ- and IKKγ-dependent phosphorylation of specific IκB residues, the alternative pathway is strictly dependent on the IKKα subunit (Senftleben *et al.*, 2001).

Following degradation of IκB, the released NF-κB translocates to the nucleus where it is able to bind to promoter and enhancer regions with the consensus sequence 5'-GGGRNNYYCC-3'. The transactivation domain on RelA, RelB and c-Rel promote transcription by facilitating the recruitment of coactivators and the displacement of repressors. NF-κB regulates the expression of many crucial genes involved in the immune system, including TNFα, but also regulates genes involved in embryonic and tissue development (Ghosh and Karin, 2002).

In addition to the tightly regulated IκB degradation, NF-κB is also tightly controlled at several other levels, thereby limiting the duration and intensity of NF-κB activation. Regulatory mechanisms include the rapid induction of IκBα by NF-κB (Brown *et al.*, 1993), the auto-phosphorylation of IKKβ (Delhase *et al.*, 1999) and phosphorylation of NF-κB by protein kinases (Schmitz *et al.*, 2004). Altered NF-κB regulation has been shown to be involved in the pathogenesis of a variety of chronic inflammatory disorders (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001).

1.3.2.2 TNFα-mediated activation of AP-1

A schematic of TNFα-induced AP-1 activation is shown in Figure 1.6. AP-1 is a sequence-specific transcriptional activator composed of members of the c-Jun, Fos or ATF families (reviewed in Karin *et al.*, 1997). These DNA binding proteins form a variety of homo- and heterodimers that bind to common DNA sites and activate transcription. Different AP-1 factors regulate different target genes and thus execute different biological functions (Karin *et al.*, 1997). Activation of the transcription factor AP-1 is regulated by members of the MAPK family, which enter the nucleus upon stimulation to phosphorylate DNA-bound transcription factors (Karin, 1995; Whitmarsh and Davis, 1996).

Functional MAPK cascades are three or four-tiered serine/threonine kinase modules. MAPK kinase kinase kinases (MAP4Ks) phosphorylate and activate MAPK kinase kinases (MAP3Ks), which in turn phosphorylate and activate MAPK kinases (MAP2Ks), which ultimately phosphorylate and activate the MAPKs. The MAPK pathways most relevant to TNFα signalling are the Jun-N-terminal kinase (JNK)/ stress-activated protein kinase (SAPK) and the p38 MAP kinase pathways (Han *et al.*, 1994; Kyriakis *et al.*, 1994). Both the JNKs and p38 MAPKs are rapidly and potently activated in response to TNFα through MAPK cascades (Derijard *et al.*, 1995; Lin *et al.*, 1995). In some cells, TNFα also stimulates another MAPK pathway, extracellular signal-regulated kinase (ERK), which may contribute to AP-1 activity (Westwick *et al.*, 1994).

Ligand-induced activation of TNFRSF1A results in recruitment of TRADD, which in turn recruits TRAF2, whereas TRAF2 is recruited directly by TNFRSF1B. TRAF2 is a critical mediator of JNK and p38 MAPK activation (Natoli *et al.*, 1997; Reinhard *et al.*, 1997). While TRAF2-deficient mice are still able to activate NF-κB, TNFα-induced activation of JNK is defective (Lee *et al.*, 1997). TRAF-mediated activation of JNK appears to depend on ligand-induced oligomerization of TRAF2 (Baud *et al.*, 1999) and the subcellular localisation of different TRAF molecules (Dadgostar and Cheng, 2000).

TNFα activates the MAP4K, germinal centre kinase (GCK/ MAP4K2), which interacts with both TRAF2 and MAP3K1 (Yuasa *et al.*, 1998; Chadee *et al.*, 2002), and activates the JNK pathway (Pombo *et al.*, 1995; Yuasa *et al.*, 1998). GCK may also recruit mixed lineage kinases (MLKs) to activate the JNK pathway (Whitmarsh *et al.*, 1998; Chadee *et al.*, 2002). The TRADD-recruited protein, RIP1 also interacts with TRAF2 and can activate both the JNK and p38 MAPK pathways (Yuasa *et al.*, 1998; Devin *et al.*, 2003). These results suggest that GCK and RIP1 are proximal components in redundant, bifurcating mechanisms for TRAF2-mediated JNK activation, while RIP1 is the dominant effector for TRAF2 activation of p38 MAPK.

Multiple MAP3Ks have been shown to act downstream of TRAFs to activate JNK and p38 MAPK by serine/threonine kinase cascades. It is often difficult to determine the relative importance of each of these kinases and it is possible that significant redundancy exists between these pathways. MAP3Ks upstream of the JNKs and p38 MAPKs are highly divergent and several MAP3Ks can activate multiple MAPK pathways (Kyriakis and Avruch, 1996; Xu *et al.*, 1996; Ichijo *et al.*, 1997; Takekawa *et al.*, 1997). These MAP3Ks include apoptosis signal-related kinase 1 (ASK1/ MAP3K5) and MAP3K1 (MEKK1). ASK1 is essential for JNK activation by TRAF2 (Ichijo *et al.*, 1997; Nishitoh *et al.*, 1998), and a Ras-GTPase-activating protein, ASK1-interacting protein 1 (AIP1) has been identified which plays an important role in regulating TNFα-induced ASK1 activation (Zhang *et al.*, 2003). TNFα has also been shown to activate MAP3K1 (Baud *et al.*, 1999; Xia *et al.*, 2000).

JNK is activated by at least two MAP2K; namely MAP2K4 (MKK4) and MAP2K7 (MKK7) (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Tournier *et al.*, 1997). Likewise, p38 MAPK is activated by at least two MAP2K; namely MAP2K3 (MKK3) and MAP2K6 (MKK6) (Derijard *et al.*, 1995; Raingeaud *et al.*, 1996).

Ultimately, the JNK and p38 MAPK signalling pathway result in activation of the transcription factor AP-1, which is involved in many cellular responses including proliferation, transformation and death (reviewed in Shaulian and Karin, 2002). In addition, p38 MAPK signalling pathways control the mRNA stability of several inflammatory cytokines, including TNFα (Section 1.1.3).

1.3.2.3 TNFα-induced apoptosis

One of the earliest effects described in association with TNFα was the induction of cell death, although this was not subsequently found to be the case for all cell types (Rosenblum and Donato, 1989). TNFRSF1A is the major receptor involved in mediating TNFα-induced cell death, which it may be able to do independently of TNFRSF1B (Leist *et al.*, 1995). Overexpression of the DD of TNFRSF1A is sufficient for death induction (Boldin *et al.*, 1995a), and deletion or mutation of the DD prevents TNFα-mediated apoptosis (Brakebusch *et al.*, 1992; Tartaglia *et al.*, 1993a). Unlike TNFRSF1A, TNFRSF1B does not contain a DD and its role in apoptosis is less clearly understood. However, in some cell types, it appears that both receptors cooperate to achieve optimal TNFα-induced apoptosis (Murray *et al.*, 1997; Declercq *et al.*, 1998; Chan and Lenardo, 2000b).

A schematic of TNFα-induced apoptosis is shown in Figure 1.7. FADD was initially identified as a protein that associates directly with the Fas receptor (Chinnaiyan *et al.*, 1995; Boldin *et al.*, 1995b) and has subsequently been reported to be the universal adapter used by the death receptor subfamily of the TNFR superfamily in signalling for apoptosis. TNFRSF1A recruits FADD indirectly via TRADD, through homophilic DD interactions (Hsu *et al.*, 1996). The TNFRSF1A DD sequences necessary for TNFα-induced apoptosis have been shown to be indistinguishable from those required for

TRADD binding (Telliez *et al.*, 2000). Besides a DD, FADD contains a motif called the death effector domain (DED), which interacts with the DED of the initiator caspase-8 (also called FLICE/MACH) (Boldin *et al.*, 1996). There is genetic evidence that both FADD and capase-8 are important for TNFRSF1A-mediated apoptosis (Varfolomeev *et al.*, 1998; Yeh *et al.*, 1998). However, it had not been possible to demonstrate a physical association between TNFRSF1A and either FADD or caspase-8 (Harper *et al.*, 2003; Micheau and Tschopp, 2003). This discrepancy has subsequently been explained by the fact that TNFα-induced signalling may proceed via the sequential formation of two distinct complexes located in different cellular compartments (Micheau and Tschopp, 2003). The authors suggest that following stimulation, the originally formed complex (complex I, which includes TRADD, TRAF2 and RIP) at the plasma membrane dissociates from TNFRSF1A and forms a different complex in the cytosol (complex II), which then recruits the apoptotic machinery. It has been hypothesised that the dissociation of complex I from the receptor may be due to ubiquitination or other post-translational modification (Barnhart and Peter, 2003). The formation of complex II, termed death inducing signal complex (DISC), involves two mutually exclusive proteins, the pro-apoptotic caspase-10 and the caspase-8-like molecule FLICE inhibitory protein (FLIP) (Micheau and Tschopp, 2003). Caspase-10 has been previously been shown to be involved in TNFα-mediated apoptosis (Vincenz and Dixit, 1997; Wang *et al.*, 2001), while FLIP inhibits FADD/caspase-8 mediated apoptosis (Irmler *et al.*, 1997).

TNFα-induced activation of the initiator caspase-8, via TRADD, FADD and caspase-10, results in the activation of a downstream caspase cascade. Caspases are a group of at least 14 different cysteine proteases which orchestrate induction of most types of apoptosis. Caspases are synthesised as inactive proenzymes that must be activated by proteolytic cleavage after specific aspartic acid residues (Salvesen and Dixit, 1999). Beside caspases-8 and -10, evidence suggests a crucial role for caspases-9 and -3 in TNFα-induced apoptosis. TNFα-activated caspase-8 is able to activate the executioner caspase-3 (Stennicke *et al.*, 1998). While the role of caspases in TNFα-induced apoptosis is proven, the exact nature of individual caspases is not fully clear. Much of the evidence of caspase functions has arisen from the overexpression of caspases or the use of

pharmacological inhibitors of caspases, whose claimed specificity does not always match up to their actual inhibitions at the concentrations used experimentally (Schotte *et al.*, 1999). It appears that different cell types may activate different caspase cascades (Zheng *et al.*, 1999), with the propagation of a caspase cascade depending on which caspases the cell expresses, the relative concentrations of each caspase and the kinetic efficiency of individual transactivation reactions (Wolf and Green, 1999).

FADD-caspase-8 is the major, but not only pro-apoptotic pathway engaged by TNFα. Besides being activated directly by caspase-8 via the extrinsic pathway, TNFα-mediated caspase-3 activation may also occur through the intrinsic pathway by the release of mitochondrial components which bind the adapter apoptotic protease activating factor 1 (Apaf-1) and lead to caspase-9 activation, which in turn activates caspase-3 (Li *et al.*, 1997; Ito *et al.*, 1999). In most cell types, this intrinsic mitochondrial pathway is subordinate to the dominant TNFRSF1A-mediated extrinsic pathway of caspase-8-mediated caspase-3 activation (Kuida *et al.*, 1998). Reactive oxygen intermediates, ceramide, phospholipases and serine proteases have all been implicated in TNFα-induced apoptosis (reviewed in Rath and Aggarwal, 1999).

The end of the apoptotic process is characterised by a common degradation phase with well described and remarkably consistent morphological features (Wallach *et al.*, 1997). The biochemical basis of these events is still unclear although caspases also seem to play an important role in this. The caspase substrates that may be involved have recently been reviewed (Fischer *et al.*, 2003).

Exactly how TNFα induces apoptosis under physiological conditions is still not clearly understood. TNFα-induced apoptosis is both cell type and cell context dependent. TNFα-induced apoptosis is potentiated by the addition of protein synthesis inhibitors, such as actinomycin and cycloheximide. This relationship is so well established that standard bioassays of TNFα-mediated apoptosis include the addition of a protein synthesis inhibitor (Polunovsky *et al.*, 1994). This suggests that the pro-apoptotic effectors needed for TNFα to mediate apoptosis pre-exist and that the synthesis of new

proteins suppresses TNFα-induced apoptosis. These latter proteins include TRAF1, Bcl-2, (cellular inhibitor of apoptosis) cIAP-1, c-IAP-2, FLIP and A20 zinc finger protein (reviewed in Rath and Aggarwal, 1999; Muppidi *et al.*, 2004). The expression of several anti-apoptotic genes is induced by NF-κB.

The finding that apoptotic TNFRSF1A signalling proceeds by the sequential formation of two distinct complexes suggests a further mechanism whereby cells can regulate between TNFα-induced proliferative and apoptotic signals (Micheau and Tschopp, 2003). Since the first complex can activate survival signals, via the activation of NF-κB, and influence the second complex, by the induction of NF-κB-induced anti-apoptotic genes, this mechanism provides a checkpoint to control the execution of apoptosis. In the absence of NF-κB signalling, FLIP is not produced and the pro-apoptotic caspase-10 is able to interact with caspase-8 in the second complex, resulting in apoptosis. This model is consistent with the finding that while TNFα activates NF-κB in most cell types, it rarely induces apoptosis. It also helps explain why protein synthesis inhibitors (such as cycloheximide) sensitize cells to TNFα-induced apoptosis (Polunovsky *et al.*, 1994; Aggarwal, 2003).

The precise role of JNK in apoptosis remains controversial since it has been reported to have conflicting results depending on cell type. However, it does now appear that JNK may play a positive role in TNFα-induced apoptosis (Deng *et al.*, 2003). In addition to activating anti-apoptotic genes, NF-κB can suppress apoptosis by inhibiting sustained activation of JNK, which is associated with apoptosis (Tang *et al.*, 2001). Crosstalk between the NF-κB, JNK and apoptotic pathways appears to be crucial for the outcome of a TNFα-induced signal (reviewed in Muppidi *et al.*, 2004; Varfolomeev and Ashkenazi, 2004).

1.3.2.4 Other TNFRSF1A mediated signals

The C-terminus of TNFRSF1A also contains sequences which bind the factor associated with neutral-sphingomyelinase activation (FAN) (Adam-Klages *et al.*, 1996)

and the adapter protein, growth receptor bound protein 2 (Grb2) (Hildt and Oess, 1999). The FAN binding sequence is required for activation of neutral sphingomyelinase (Adam-Klages *et al.*, 1996), suggesting that FAN regulates TNFα-induced ceramide production. The FAN and Grb2-interacting sequences are required for activation of the MAP3K, c-Raf-1 (Hildt and Oess, 1999).

TNFα also appears to alter the redox potential of cells and there is now considerable evidence linking TNFα-induced signalling to reactive oxygen intermediates, which may play a role in both pro- and anti-apoptotic signals (Janssen-Heininger *et al.*, 2000; Garg and Aggarwal, 2002; Shakibaei *et al.*, 2005). A number of other molecules, including sentrin and MAPK activating death domain (MADD), have been found to interact with the intracellular region of the TNFRs, either directly or indirectly, although their functions are often still unknown (reviewed in Aggarwal, 2000; MacEwan, 2002).

1.3.3 TNFα-induced IL-6 and IL-8 expression

TNFα is a potent inducer of a number of cytokines, including the pro-inflammatory cytokine IL-6 (Vandevoorde *et al.*, 1992) and the chemokine IL-8 (Matsushima and Oppenheim, 1989; Kasahara *et al.*, 1991). The promoters of both IL-6 and IL-8 contain functional AP-1 and NF-κB sites (Dendorfer *et al.*, 1994; Roger *et al.*, 1998). NF-κB has been shown to be the main TNFα-induced transcriptional activator for IL-6 gene induction (Shimizu *et al.*, 1990; Zhang *et al.*, 1990). However, activation of NF-κB alone is not sufficient for IL-6 gene expression in response to TNFα (Patestos *et al.*, 1993), and inhibition of the p38 MAPK pathway blocks TNFα-mediated expression of IL-6 (Beyaert *et al.*, 1996). It has subsequently been confirmed that NF-κB is essential for TNFα-induced IL-6 activation, while TNFα-activated p38 and ERK MAPK pathways contribute to transcriptional activation by modulating the transactivation capacity of NF-κB (Vanden Berghe *et al.*, 1998). TNFα-induced activation of IL-8 also appears to be mediated by the NF-κB (Jobin *et al.*, 1999) and p38 MAPK pathways (Suzuki *et al.*, 2000).

In addition to controlling NF-κB transactivation at a transcriptional level, the p38 MAPK pathway is also crucial for the stabilization of inflammatory mRNAs (Section 1.1.3). Like TNFα, the mRNAs of both IL-6 and IL-8 contain destabilising AU-rich elements that have been shown to be stabilised by the p38 MAPK pathway (Miyazawa *et al.*, 1998; Winzen *et al.*, 1999).

1. 4 Hereditary periodic fever syndromes

The hereditary periodic fevers form part of the autoinflammatory disorders. This group of disorders is characterised by recurrent episodes of seemingly unprovoked systemic inflammation. The term "autoinflammatory" is used to signify that high titre autoantibodies or antigen-specific T cells are not a feature of these conditions, unlike autoimmune disorders (McDermott *et al.*, 1999a). This relatively new term encompasses a number of diverse disorders, in addition to the hereditary periodic fevers (Galon *et al.*, 2000).

The hereditary periodic fever syndromes consist of a group of diseases with permanent genetic defects that present with intermittent clinical symptoms. These conditions are characterised by recurrent episodes of fever lasting from a few days to a few weeks, separated by symptom-free intervals of variable duration. While the fever and inflammatory attacks are intermittent, there is often no true periodicity (i.e. regularity) and their frequency can vary considerably (Grateau, 2004). The disorders can broadly be subdivided according to their pattern of inheritance: familial Mediterranean fever (FMF) and hyperimmunoglobulinaemia D and periodic fever syndrome (HIDS) are autosomal recessive, while Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS) and TNF-receptor-associated periodic syndrome (TRAPS) are autosomal dominant. The salient features and genetic defects of these conditions are described below and compared in Table 1.1. The underlying mechanisms appear to be specific for each of these diseases. Accurate diagnosis, which now relies on combined clinical and genetic data, is important because of the specific therapeutic management of each of these syndromes. Therefore, while these disorders are rare, the advances in their

genetic underpinnings offers the opportunity of gaining new insights into the function of the affected proteins and understanding mechanisms mediating inflammation that may be applicable to a range of more common diseases.

1.4.1 Familial Mediterranean fever (FMF)

FMF (MIM249100) is the most prevalent hereditary periodic fever disorder, affecting more than 10 000 patients worldwide (Drenth and van der Meer, 2001). The name FMF reflects the fact that it predominantly affects people of Mediterranean descent, particularly individuals of Turkish, Armenian, Jewish, Italian and Arab ancestry (Ben-Chetrit and Levy, 1998). Attacks generally start before the age of 20 (Ben-Chetrit and Levy, 1998) and are characterised by short (1-4 days) attacks of fever and serositis (Sohar *et al.*, 1967). Abdominal pain, as a result of peritonitis, and non-erosive monoarthritis are common features. Erysipelas-like skin lesions, which are considered to be a specific clinical finding, occur less commonly (Majeed *et al.*, 1990). The major complication of FMF is amyloidosis. Regular administration of colchicine prevents both acute attacks of FMF and the development of amyloidosis in most patients (Zemer *et al.*, 1986). The diagnosis is based on clinical manifestations, ethnicity, family history and response to colchicine. The gene responsible for FMF, designated *MEFV*, is on chromosome 16 (16p13) and was cloned in 1997 (The French FMF Consortium, 1997; The International FMF Consortium, 1997). *MEFV* encodes a protein called pyrin (or marenostrin), which contains a PYRIN domain that is shared by a number of proteins involved in apoptosis and inflammation (Kastner and O'Shea, 2001; Pawlowski *et al.*, 2001). Molecular testing can therefore be used as a confirmatory test for FMF. While at least 89 mutations in the *MEFV* gene have been reported on the INFEVERS registry (http://fmf.igh.cnrs.fr/infevers/) (Sarrauste de Menthiere *et al.*, 2003), five mutations account for more than 85% of all cases of FMF (Grateau, 2004).

1.4.2 Hyperimmunoglobulinaemia D and periodic fever syndrome (HIDS)

HIDS (MIM260920) was recognised as a separate entity in 1984 (van der Meer *et al.*, 1984) and is characterised by recurrent attacks of fever that usually start in the first year of life in patients of Western European origin. Episodes last 4-6 days and are

associated with lymphadenopathy, skin lesions, arthralgia and raised serum IgD levels (Drenth *et al.*, 1994). Amyloidosis has not been described. The gene for HIDS, designated *MVK*, is on chromosome 12 (12q24) and encodes mevalonate kinase (Drenth *et al.*, 1999; Houten *et al.*, 1999). The activity of mevalonate kinase, a key enzyme in the cholesterol biosynthetic pathway, is reduced in patients with HIDS. It is not yet known how a deficiency of this enzyme is linked to inflammatory periodic fever.

1.4.3 Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS) and chronic infantile neurological cutaneous and articular syndrome (CINCA)

MWS (MIM191900), FCAS (MIM120100) and CINCA (MIM607115) were previously described as distinct clinical entities but have subsequently been found to reflect a spectrum of diseases that are associated with mutations in the gene *CIAS1* (Hoffman *et al.*, 2001). *CIAS1* is on chromosome 1 (1q44) and encodes the protein cryopyrin which, like pyrin, contains an N-terminal PYRIN domain. There appears to be a relative phenotype/genotype correlation, suggesting that mutations have different effects on cryopyrin function or expression (Neven *et al.*, 2004). The acute febrile attacks usually start in infancy and are accompanied by an urticaria-like rash and joint involvement. The disorder may occasionally be complicated by central neurological involvement and multiorgan amyloidosis.

1.4.4 Tumour necrosis factor receptor-associated periodic syndrome (TRAPS)

TRAPS (MIM142680) was first described in 1982 in a large family of Irish ancestry, and thus called familial Hibernian fever (FHF) (Williamson *et al.*, 1982). The autosomal dominant pattern of inheritance, prolonged attacks, and response to corticosteroids but not colchicine differentiated this disorder from the more common FMF (McDermott *et al.*, 1997). Cases were subsequently reported in a variety of other ethnic groups (McDermott, 1999b). Following the identification of mutations in the TNFRSF1A gene on chromosome 12 (12p13), TRAPS was proposed as a unifying, pathogenically descriptive term (McDermott *et al.*, 1999a).

1.4.4.1 Clinical aspects of TRAPS

Patients with TRAPS generally have prolonged episodes (> 1week) of fever with serositis, skin rash, muscle pain, ocular inflammation and arthritis, although there is considerable heterogeneity in the clinical picture of TRAPS (Dode *et al.*, 2002; Aganna *et al.*, 2003). The characteristic centrifugally-migrating localised myalgia, often accompanied by an overlying erythematous rash, has been shown to be due to monocytic fasciits, and not actual myositis as previously thought (Hull *et al.*, 2002a). The skin rash in TRAPS is characterised by a perivascular dermal infiltrate of lymphocytes and monocytes (Toro *et al.*, 2000). In general, the symptoms tend to decrease in both frequency and severity with increasing age.

During a febrile attack, patients with TRAPS generally have a neutrophilia in addition to non-specific elevation of acute phase markers, such as C-reactive protein (CRP) and serum amyloid A (SAA) (McDermott *et al.*, 1999a). Most patients with TRAPS have low serum levels of soluble TNFRSF1A compared to normal controls (McDermott *et al.*, 1999a), although the levels can vary substantially. Levels can be normal during an acute attack, and normal or elevated in patients with renal impairment (Aganna *et al.*, 2003) as soluble TNFRSF1A is cleared by the kidneys (Bemelmans *et al.*, 1994; Ward and McLeish, 1996). The increase in soluble TNFRSF1A during a febrile attack of TRAPS, resulting in pseudonormalisation of the levels, is significantly less than the large increase in soluble TNFRSF1A seen in patients with active RA or systemic lupus erythematosus (McDermott *et al.*, 1999a). Soluble TNFRSF1A levels are normal in FMF (Gang *et al.*, 1999) and MWS (Aganna *et al.*, 2003).

The prognosis for patients with TRAPS is determined mainly by the presence or absence of amyloidosis (McDermott *et al.*, 1997). Amyloidosis is caused by the tissue deposition of a product of the acute phase reactant SAA, which is produced by hepatocytes in response to TNFα and IL-6 (Yamada, 1999). Amyloidosis develops in approximately 14% of patients with TRAPS (Aksentijevich *et al.*, 2001) and may be more common with cysteine mutations than non-cysteine mutations (Aksentijevich *et al.*, 2001), although this is not exclusive (Jadoul *et al.*, 2001; Dode *et al.*, 2002; Aganna *et al.*,

2003). It has been suggested that the development of amyloidosis in TRAPS may depend on other modifier genes (Dode *et al.*, 2002; Kallinich *et al.*, 2004).

Treatment of patients with TRAPS has traditionally involved nonsteroidal anti-inflammatory drugs and moderate doses of oral corticosteroids, whose prolonged use is associated with significant toxicity (McDermott *et al.*, 1997). Since the identification of the association with TNFRSF1A mutations, inhibition of TNFα signalling has been tried as a therapeutic approach. Treatment of patients with etanercept, a soluble p75TNFR:Fc fusion protein, has been demonstrated to decrease the frequency, duration and severity of attacks (Galon *et al.*, 2000; Hull *et al.*, 2002b). However, as in patients with RA, the response to etanercept is often partial (Drewe *et al.*, 2003), and the long-term effect on amyloidosis remains to be determined, as treatment with etanercept does not normalise the acute phase response (Hull *et al.*, 2002c; Drewe *et al.*, 2003; Arostegui *et al.*, 2005).

1.4.4.2 TNFRSF1A mutations associated with TRAPS

Two independent genome-wide linkage studies performed on several families of Irish/Scottish descent with familial Hibernian fever (FHF) mapped the susceptibility locus to the short arm of chromosome 12 (McDermott *et al.*, 1998; Mulley *et al.*, 1998). The human transcript maps (Deloukas *et al.*, 1998) suggested a number of potential candidate genes, including the gene for TNFRSF1A. TNFRSF1A was seen as a particularly attractive candidate in light of altered soluble TNFRSF1A levels observed in the serum of FHF patients, and the central role of TNFRSF1A in inflammation. Genomic sequencing of the coding region and splice junctions of the TNFRSF1A revealed six different single nucleotide substitutions in 40 of 43 symptomatic family members with FHF (McDermott *et al.*, 1999a). None of these mutations were found in control chromosomes screened by genomic sequencing. The affected members all tested negative for a panel of 19 known FMF mutations. In light of the diverse ethnic distribution of patients, the authors proposed the descriptive acronym TRAPS (TNF receptor-associated periodic syndrome) to describe the autosomal dominant periodic fevers, including FHF and FPF, associated with TNFRSF1A mutations (McDermott *et al.*, 1999a).

So far, at least 46 TRAPS-associated TNFRSF1A mutations have been reported on the INFEVERS registry of hereditary periodic fevers (http://fmf.igh.cnrs.fr/infevers/) (Sarrauste de Menthiere *et al.*, 2003), of which 43 are located in exons 2-4 of the TNFRSF1A gene. Virtually all of the mutations are substitutions, with 23 involving substitutions of cysteine residues. Apart from the possible increase in amyloidosis associated with cysteine mutations (Section 1.4.4.1), the type of TNFRSF1A mutation does not correlate with clinical differences seen in individual patients with TRAPS (Dode *et al.*, 2002; Aganna *et al.*, 2003).

The numbering of the amino acids for TNFRSF1A mutations has not followed the general rule of starting with the translation initiator methionine. Virtually all publications in the literature relating to TRAPS have started numbering after the leader sequence (i.e. starting with the leucine at residue 30). This latter system of numbering is used throughout this study to allow comparison with published reports (e.g. C72S is called C43S). The situation in the literature is further complicated by the use of the "correct" nomenclature at the DNA level (e.g. C43S is a nucleotide 215 G →C transition).

The status of three of the TNFRSF1A mutations, i.e. R92Q, P46L and T61I, remains to be completely established. The R92Q and P46L mutations have both been found to occur in approximately 1% of Caucasian and African American controls respectively (Aksentijevich *et al.*, 2001), and the T61I mutation occurred in 1-4% of Japanese controls (Horiuchi *et al.*, 2004; Ida *et al.*, 2004). While this may reflect low penetrance, the P46L allele frequency was found to be approximately 10% in West African populations, suggesting that the P46L variant may be a polymorphism (Tchernitchko *et al.*, 2005). It has been suggested that the R92Q variant may influence the susceptibility to inflammation in early arthritis (Aksentijevich *et al.*, 2001; Hull *et al.*, 2002b) and the non-specific inflammatory response in a variety of other disease processes, such as juvenile idiopathic arthritis (Aganna *et al.*, 2004), atherosclerosis (Poirier *et al.*, 2004) and vasculitis (Lamprecht *et al.*, 2004). The T61I mutation was also found in 2-8% of Japanese patients with systemic lupus erythematosus but was not detected in patients with RA (Horiuchi *et al.*, 2004; Ida *et al.*, 2004).

In addition to the TRAPS-associated mutations, several TNFRSF1A single-nucleotide polymorphisms (SNPs) have been reported. These SNPs were found in the promoter region, in exon 1 (silent substitution), and in introns 2, 4, 6, 7 and 8 (Pitts *et al.*, 1998a; Pitts *et al.*, 1998b; Weinshenker *et al.*, 1999; Bazzoni *et al.*, 2000). Since genome-wide screens in RA have suggested that the TNFRSF1A and TNFRSF1B loci are susceptibility loci (Cornelis *et al.*, 1998; Shiozawa *et al.*, 1998; Jawaheer *et al.*, 2001), there has been an intensive search for associations between SNPs in these genes and RA. While several studies have failed to demonstrate any association, a putative susceptibility TNFRSF1B genotype (Barton *et al.*, 2001; Dieude *et al.*, 2002) and a protective TNFRSF1A genotype (Dieude *et al.*, 2004) have both been reported in familial, but not sporadic, RA.

In their seminal description of TNFRSF1A mutations, McDermott *et al* (1999a) demonstrate that the C52F TRAPS mutation results in defective TNFRSF1A shedding from leucocytes in response to phorbol-myristate-acetate (PMA). The authors hypothesize that this decreased TNFRSF1A cleavage may reduce the amount of soluble TNFRSF1A available for neutralisation of TNFα (see Section 1.2.8), thus resulting in the inflammatory TRAPS phenotype. Although TRAPS-associated TNFRSF1A mutants have been described in proximity to the transmembrane region (Kriegel *et al.*, 2003), most mutations are distant from the site of receptor cleavage (Gullberg *et al.*, 1992; Brakebusch *et al.*, 1994), so the effects on shedding are likely to be indirect. However, not all TRAPS-related TNFRSF1A mutations result in defective receptor shedding by leucocytes (Aksentijevich *et al.*, 2001; Aganna *et al.*, 2003). The presence or absence of a receptor shedding defect appears to have no bearing on the severity of the clinical symptoms or the development of amyloidosis (Aganna *et al.*, 2003). Moreover, in some families with autosomal dominant periodic fevers without any TNFRSF1A mutations, there is a defect in receptor shedding from leucocytes (Aganna *et al.*, 2003). Recently it has also been shown that defective receptor shedding varies between cell types bearing the same mutation (Huggins *et al.*, 2004). TNFRSF1A shedding was impaired from dermal fibroblasts, but not leucocytes, from C33Y TRAPS patients. Taken together, this data suggests that the differences in shedding are not purely a function of TNFRSF1A

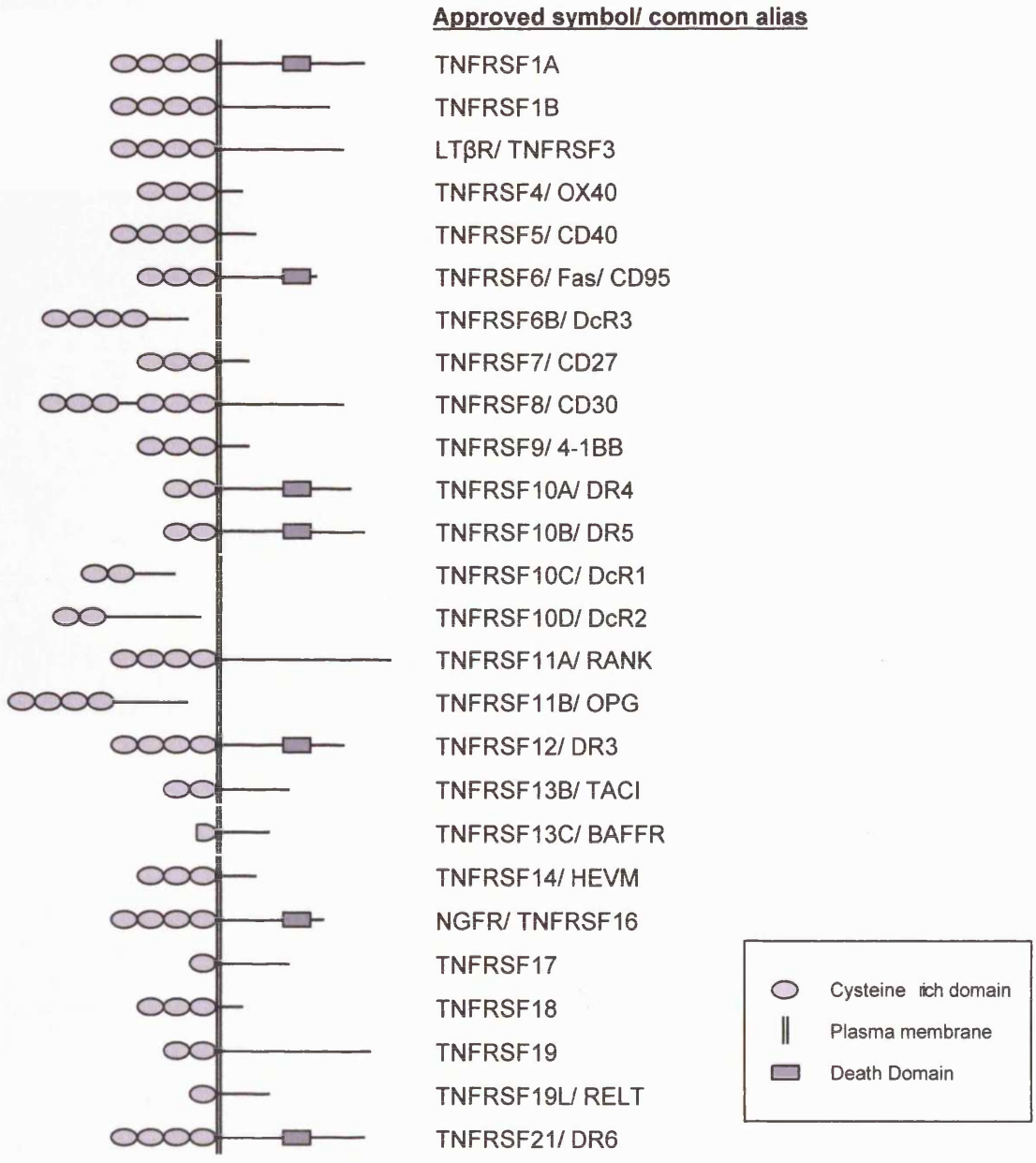
structure, but are also influenced by other genetic and/or cellular factors. Defective TNFRSF1A shedding alone cannot therefore account for the pathophysiological mechanism underlying TRAPS for all mutations. Thus, the mechanisms by which the TNFRSF1A mutations result in the inflammatory phenotype of TRAPS are not known.

1.5 Experimental outline and aims of thesis

When this project was initiated, no analysis of signalling in TRAPS patients or by TRAPS mutants had been described. We hypothesised that a constitutive or enhanced TNFRSF1A signal, perhaps through NF-κB activation, could be responsible for the TRAPS phenotype. To test this hypothesis, we took a two pronged approach:

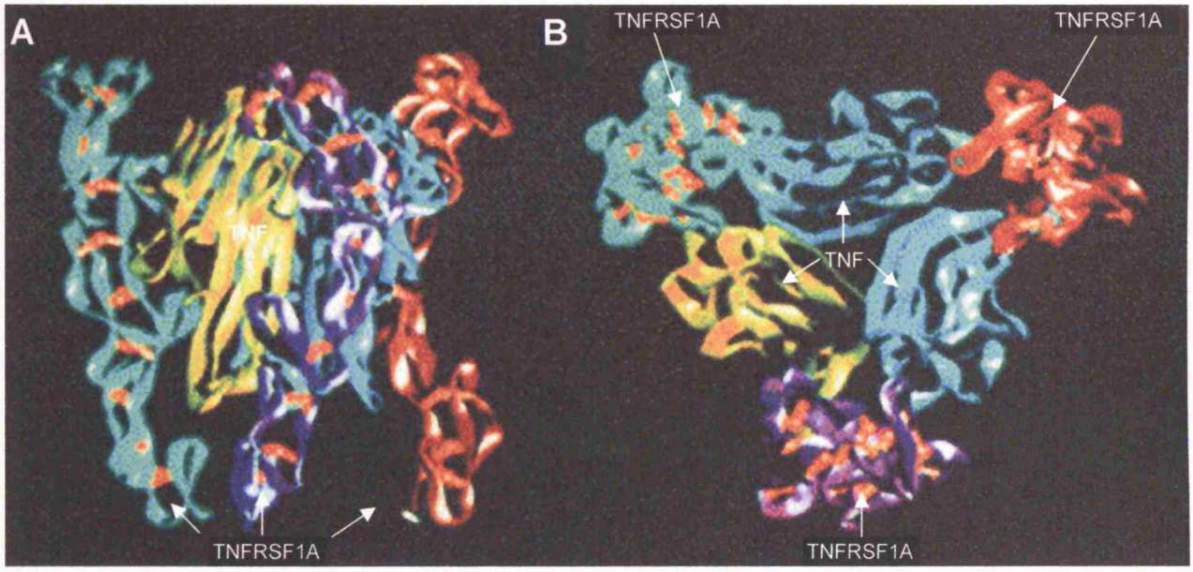
1. Analysis of TNFRSF1A signalling in a patient-derived cell line.
   This involved taking tissue from a patient with TRAPS and from healthy volunteers following the relevant ethical approval. This was followed by analysis of TNFα-induced NF-κB activation, apoptosis and cytokine production.
2. Analysis of the signalling of recombinant TNFRSF1A mutants.
   I generated four TRAPS mutants by site directed mutagenesis. These were analysed for activation of NF-κB, for surface and intracellular expression and for subcellular localisation.

Figure 1.1
Diagrammatic representation of TNFR superfamily members



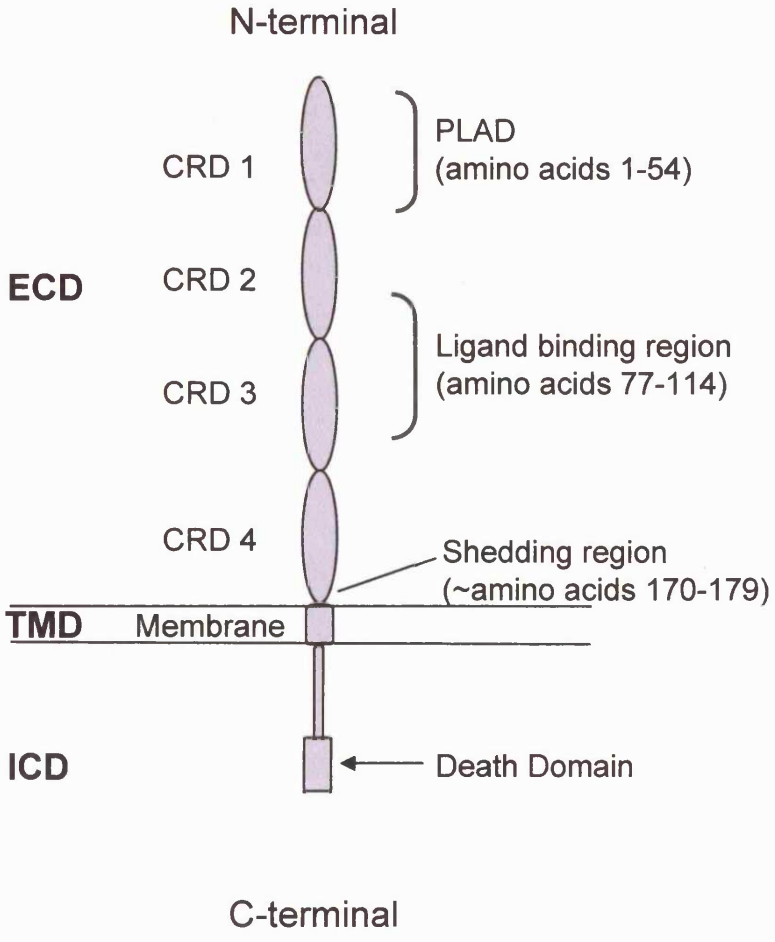
Diagrammatic representation of the TNFR superfamily members indicating the extracellular cysteine-rich domains and intracellular death domains. Approved symbols and common aliases are shown. TNFRSF = TNF receptor superfamily; LTβR = lymphotoxin beta receptor; DcR = decoy receptor; DR = death receptor; RANK = receptor activator of NFκB; OPG = osteoprotegerin; TACI = transmembrane activator and cyclophylin ligand interactor; BAFFR = B-cell activating factor receptor; HEVM = herpes-virus entry mediator; NGFR = nerve growth-factor receptor; RELT = receptor expressed in lymphoid tissue.

Figure 1.2
Structure of TNF/ TNFRSF1A interaction



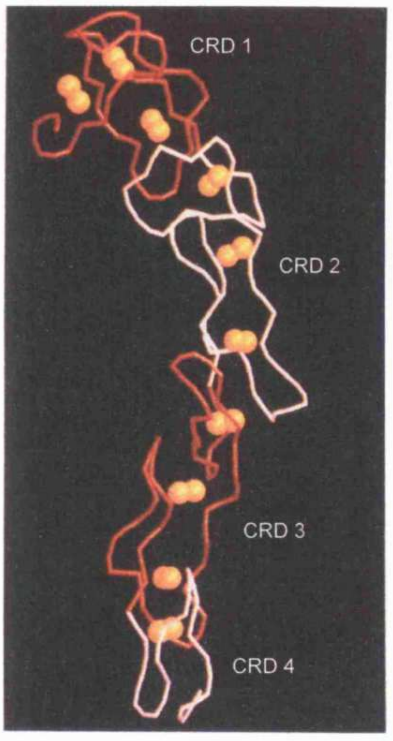
TNFRSF1A extracellular domain complexed with lymphotoxin-α (LTα) is shown from **(A)** "side" and **(B)** "top" views. The disulphide bonds of the TNFRSF1A extracellular domains (light green, blue and crimson) are highlighted in red. The ligand chains (dark green and yellow-brown) form a trimer, which binds three TNFRSF1A receptors. Text labels are shown to aid identification. Adapted from Banner *et al* (1993).

Figure 1.3
Schematic of the TNFRSF1A monomer



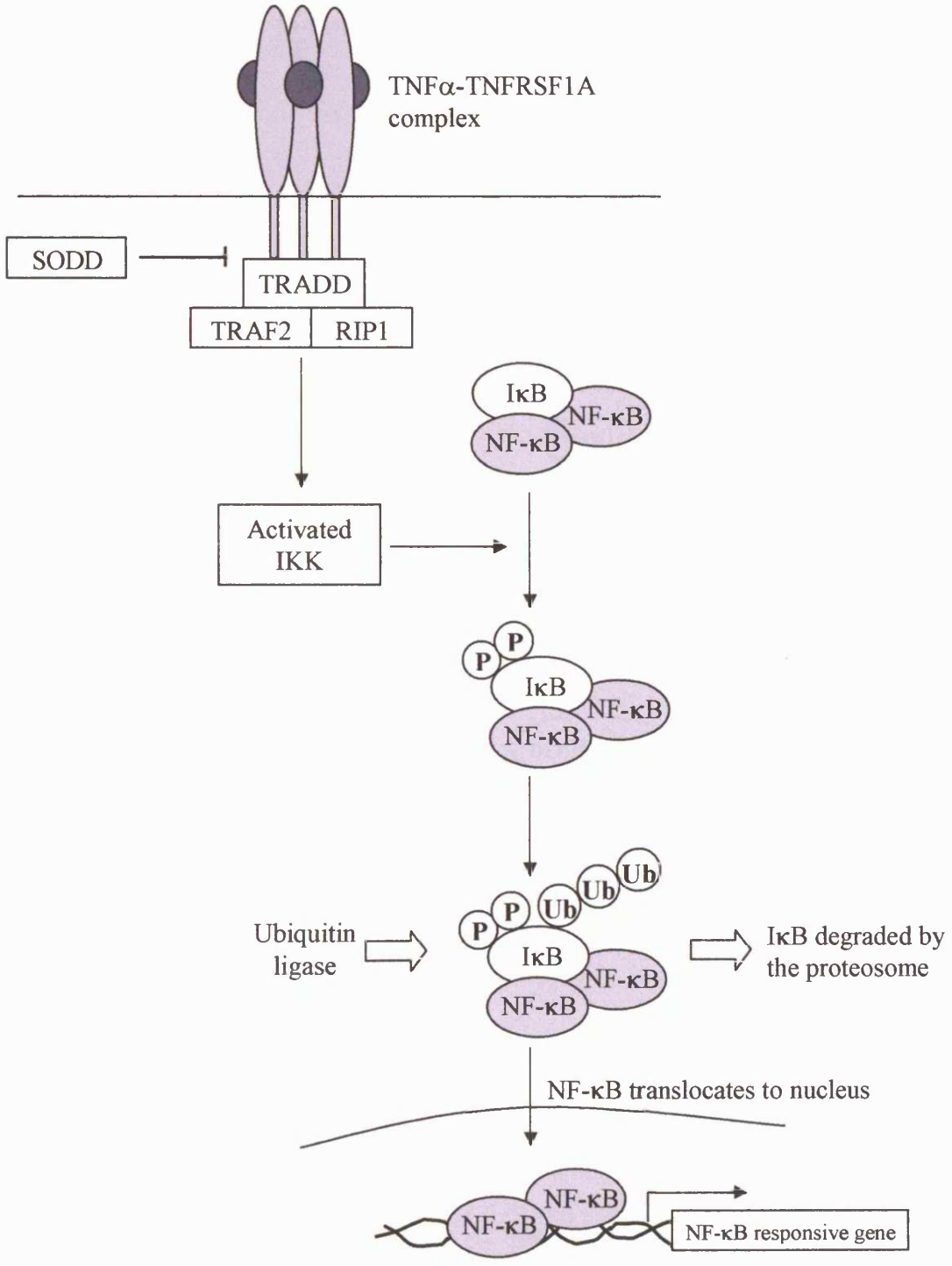
A schematic of TNFRSF1A depicting the major structural regions (labels on left) and important functional regions (labels on right). ECD = extracellular domain; TMD = transmembrane domain; ICD = intracellular domain; CRD 1-4 = cysteine-rich domains 1-4 of ECD; PLAD = Pre-ligand-binding assembly domain

Figure 1.4
Structure of the TNFRSF1A extracellular domain



The extracellular domain of TNFRSF1A contains four cysteine rich domains (CRD), arranged end-to-end in a linear fashion, and designated 1-4, starting from the N-terminus. The CRDs are characterised by three intrachain disulphide bonds which form a structural scaffold. The sulphur atoms of the disulphide bonds are shown as space-filling yellow spheres. Only the first disulphide bond of CRD 4 is shown. Figure adapted from Naismith *et al* (1995).

Figure 1.5 Schematic of TNFRSF1A-mediated NF-κB activation

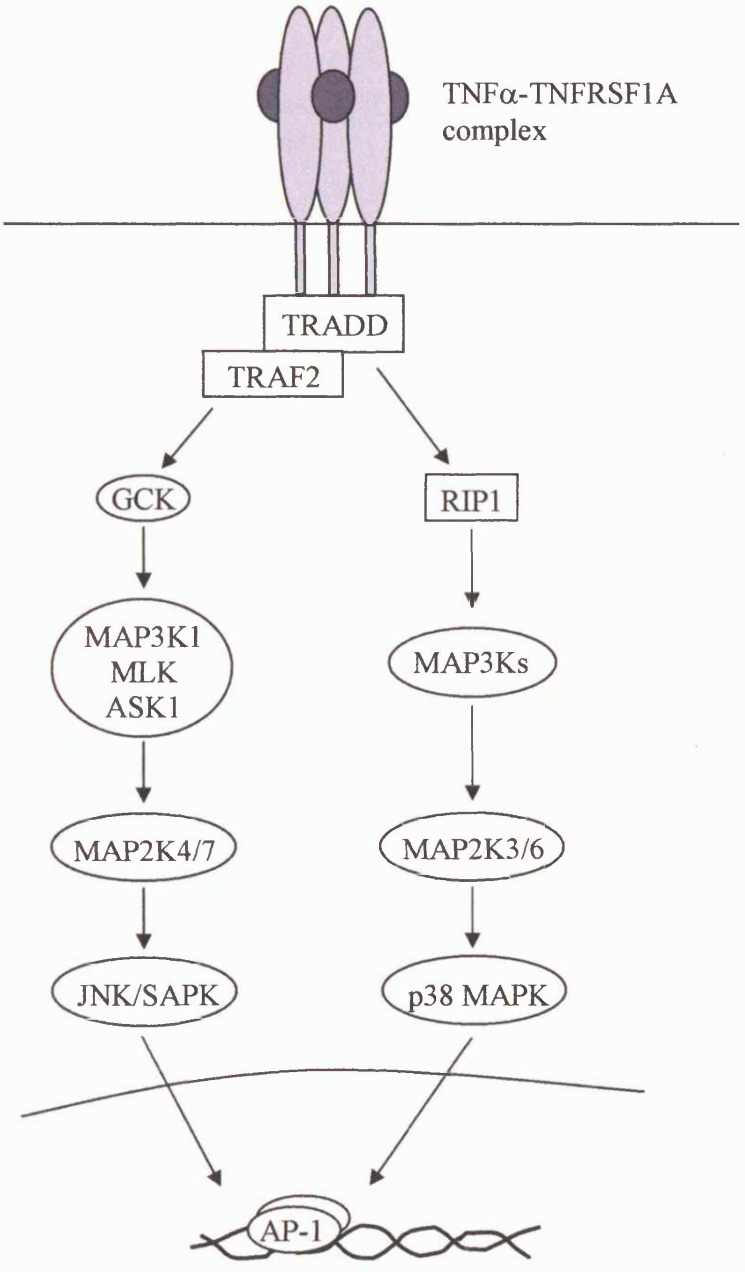


SODD = Silencer of death domains
TRADD = TNF receptor 1-associated death domain
TRAF2 = TNF receptor associated factor 2
RIP1 = Receptor interacting protein 1

NF-κB = Nuclear factor-κB
IκB = Inhibitor of NF-κB
IKK = IκB kinase

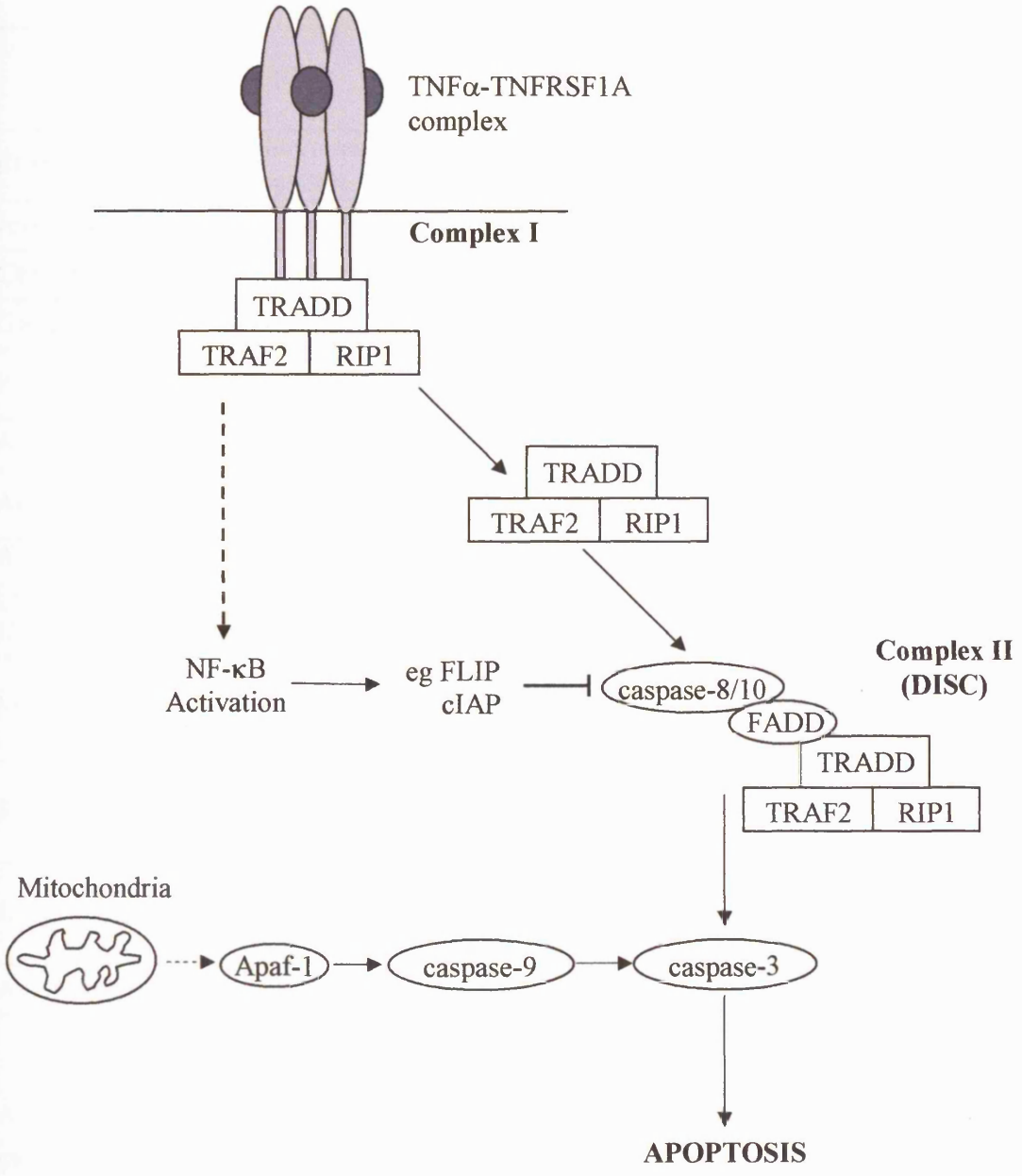
(P) = phosphate group
(Ub) = ubiquitin

Figure 1.6 Schematic of TNFRFS1A-mediated MAPK signalling



TRADD = TNF receptor 1-associated death domain
TRAF2 = TNF receptor associated factor 2
RIP1 = Receptor interacting protein 1
GCK = Germinal centre kinase
ASK1 = Apoptosis signal-regulating kinase 1
MLK = Mixed lineage kinases
MAPK = Mitogen activated protein kinase
MAP2K = MAPK kinase
MAP3K = MAPK kinase kinase
JNK = Jun-N-terminal kinase
SAPK = Stress-activated protein kinase
AP-1 = Activating protein-1

Figure 1.7 Schematic of TNFRSF1A-mediated apoptosis



TRADD = TNF receptor 1-associated death domain
TRAF2 = TNF receptor associated factor 2
RIP1 = Receptor interacting protein 1
NF-κB = Nuclear factor-kappaB

DISC = Death inducing signal complex
FADD = Fas-associated protein with death domain
FLIP = FLICE inhibitory protein
cIAP = Cellular inhibitor of apoptosis
Apaf-1 = Apoptotic protease activating factor-1

Table 1.1 Distinctive features of the four main hereditary periodic fevers

	FMF	HIDS	MWS/ FCAS	TRAPS
Ethnic group	Mediterranean descent	Western European	Various	Diverse
Inheritance	Recessive	Recessive	Dominant	Dominant
Chromosome	16p13	12q24	1q44	12p13
Gene	*MEFV*	*MVK*	*CIAS1*	*TNFRSF1A*
Protein	Pyrin (marenostrin)	Mevalonate kinase	Cryopyrin	TNFRSF1A
Age of onset	<20	Childhood	Childhood/neonatal	Variable
Attack duration	1-4 days	3-7 days	Variable	Often >1 week
Abdominal pain	Very common	Very common	Rare	Common
Chest pain	Yes	Unusual	No	Yes
Musculoskeletal	Monoarthritis	Arthralgia (polyarticular)	Arthritis	Localised myalgia, arthralgia
Rash	Rare, erysipeloid	Very common maculopapular	Urticaria, erythema	Common, migratory erythema
Other features	Pericarditis, scrotal attacks, splenomegaly	Cervical lymphnodes, headaches	Neurological signs, cold sensitivity, conjunctivitis	Periorbital oedema, conjunctivitis
Amyloidosis	Yes	Not reported	Yes	Yes

Abbreviations

FMF = familial Mediterranean fever; HIDS = Hyperimmunoglobulinaemia D and periodic fever syndrome; MWS = Muckle-Wells syndrome; FCAS = familial cold autoinflammatory syndrome; TRAPS = TNF-receptor-associated periodic syndrome

CHAPTER 2

MATERIALS AND METHODS

2.1 Molecular Biology

2.1.1 Media and Buffers

Preparation of media and buffers was as described below. Sterilisation was carried out by autoclaving for 40 minutes at 15 psi, 121°C where indicated.

SOC Medium

SOC medium was used in the transformation of competent cells.

SOC medium was prepared to a final concentration of 0.5% yeast extract (Oxoid), 2% tryptone (Oxoid), 8.5 mM NaCl (Fischer) and 2.5 mM KCl (Fischer) in distilled water, before sterilization. Once the medium had cooled, 20 mM glucose (Fischer) was added. SOC was stored at 4°C. Just before use, 10 mM sterile $MgCl_2$ was added.

Luria-Bertani Medium (LB)

LB medium was prepared to a final concentration of 0.5% yeast extract (Oxoid), 1% tryptone (Oxoid) and 0.17 M NaCl in distilled water, before sterilisation. If required for selective LB medium, antibiotics were added prior to use once the LB medium had cooled sufficiently. LB was stored at 4°C.

Luria-Bertani Agar (LB agar)

15 g bactoagar (Oxoid) was added to 1 litre of LB, before sterilisation. Antibiotics for selective LB agar plates were added when the agar had cooled sufficiently, immediately prior to pouring the agar. LB agar was stored at room temperature. LB agar plates were stored at 4°C.

Antibiotics

Ampicillin (Sigma) was used as antibiotic selection for transformed bacteria. Stock solutions were prepared at 1000x the working concentration of 50 μg/ml, in sterile distilled water and sterilised by filtration through a 0.2 μM filter (Gelman Sciences). Aliquots were stored at
-20°C and thawed just prior to use.

Terrific Broth

Terrific broth was used in the large scale preparation of plasmid DNA, using caesium chloride gradients. Terrific broth was prepared as a base solution with a final concentration of 2.4% yeast extract (Oxoid), 1.2% tryptone (Oxoid) and 0.4% glycerol (Fischer) in distilled water. A potassium phosphate solution was prepared in a separate flask to a final concentration of 0.17 M KH_2PO_4 (Sigma) and 0.72 M K_2HPO_4 (Sigma) in distilled water. The solutions were sterilized separately by autoclaving. Once cooled, 100 ml potassium phosphate solution was added to 900 ml base solution. Antibiotics for selective Terrific Broth were added to cooled medium immediately prior to use.

0.5 M EDTA pH 8

186.1 g of EDTA (Sigma) was dissolved in 800 ml distilled water and adjusted to pH 8 by the addition of sodium hydroxide pellets (approximately 20 g) (Fischer). The final volume was made up to 1 litre and sterilized by autoclaving.

Tris-EDTA (TE)

TE was used as a buffer to dissolve or dilute DNA. It consisted of 10 mM Tris-HCl pH 8 and
1 mM EDTA pH 8 and was sterilized by autoclaving.

6x DNA loading buffer

6x DNA loading buffer was prepared to the following recipe: 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), 30% glycerol (v/v) and 50 mM EDTA pH 8. DNA loading buffer was stored at 4°C.

Tris-acetic acid buffered EDTA (TAE)

TAE was used as an electrophoresis buffer. It was prepared as a 50x stock solution using 24.2 g Tris-base (USB), 57.1 ml glacial acetic acid (Fischer) and 100 ml of 0.5 M EDTA pH 8, made up to 1 litre with distilled water.

Phenol liquefied, washed in Tris buffer (Fischer)

Phenol was stored at 4°C.

Phenol: Chloroform: Isoamyl alcohol (25:24:1) (Sigma)

Phenol: chloroform: isoamyl alcohol (25:24:1) was stored at 4°C.

2.1.2 Bacterial strains

One Shot TOP10 chemically competent *E. coli* (Invitrogen) were used to in the experiments to generate recombinant plasmids. TOP10 *E. coli* have the following genotype: F{*lac*I^q, Tn*10*(Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G. TOP10 *E. coli* were stored at -80°C, and single-use aliquots were gently thawed on ice immediately prior to use.

2.1.3 Submerged horizontal agarose gel electrophoresis

Agarose gels were used to separate DNA fragments. Gels were prepared by dissolving electrophoresis grade agarose (Gibco BRL) in 1x TAE by heating in a microwave oven. 1% agarose (w/v) gels were cast and run in submerged horizontal electrophoresis tank apparatus (Flowgen). A 50 ml volume of gel was necessary for one gel (dimensions 10 cm x 10 cm). Samples were mixed with 6x DNA loading buffer and loaded into an agarose gel submerged in 1x TAE, along with Generuler 1 kB DNA ladder (MBI Fermentas). Hyperladder I DNA ladder (Bioline) was used when quantification of DNA was required. Gels were run at 100 V using a Powerpac 300 power supply (BioRad). Gels were stained with an ethidium bromide solution (0.5 μg/ml) and visualized using the gel documentation system (BioRad).

2.1.4 Restriction endonuclease digestion

Restriction enzymes and reaction buffers (New England Biolabs) were stored at -20°C and kept on ice during use. Restriction enzyme digestions for cloning were performed in the required reaction volumes (20-50 µl), containing 10% final volume of appropriate 10x reaction buffer, BSA (if required), and the appropriate Units of restriction enzyme, according to the manufacturer's instructions. All reactions were incubated for 2 hours at 37°C. Restriction enzyme digestions were analysed by submerged horizontal agarose gel electrophoresis.

2.1.5 Gel Extraction

Gel extraction of the TNFRSF1A inserts was performed using the QIAquick Gel Extraction kit (Qiagen), according to the manufacturer's instructions.

The digested DNA fragments were run on submerged horizontal agarose gel electrophoresis (Section 2.1.3) and excised from the agarose gel with a clean scalpel, keeping the size of the surrounding agarose to a minimum. The gel slices were weighed in a colourless tube and 3 volumes of Buffer QG added to 1 volume of gel (100 mg ~ 100 µl). This was incubated at 50°C for 10 minutes (or until the gel slice completely dissolved), with vortexing every 2-3 minutes during the incubation. The mixture was inspected to ensure that the pH indicator included in Buffer QG was yellow (pH ≤7.5). 1 gel volume of isopropanol was added to the sample and mixed. The sample mixture was loaded onto a QIAquick spin column, centrifuged at 13000 rpm for 1 minute in a microcentrifuge and the flow-through discarded. 0.5 ml Buffer QG was added to the column, centrifuged for 1 minute and the flow-through discarded. The DNA was eluted (Section 2.1.8) and used in the ligation reactions.

2.1.6 Alkaline phosphatase treatment of vector

Alkaline phosphatase catalyses the removal removes the free 5' phosphatase groups from the vector and prevents self-ligation of complementary overhangs produced by restriction digestion (Section 2.1.4), thereby decreasing the vector background in cloning. The insert used in the ligation was not treated with alkaline phosphatase as this would

inhibit the ligation reaction. The restriction enzyme digest of the vector was suspended in 1x reaction buffer, to which was added calf intestinal alkaline phosphatase (CIP, 0.5 units/μg vector DNA) (New England Biolabs). The reaction was incubated for 1 hour at 37°C. The CIP treated vector was then purified by PCR purification (QIAquick PCR Purification kit, Section 2.1.7).

2.1.7 PCR purification

Purification of the restriction digested, CIP-treated pcDNA3.1/myc-His vector was performed using the QIAquick PCR Purification kit (Qiagen), according to the manufacturer's instructions.

5 volumes Buffer PB was added to 1 volume of the restriction digested, CIP-treated pcDNA3.1/myc-His vector. The solution was added to a QIAquick spin column, centrifuged at 13000 rpm for 1 minute in a microcentrifuge and the flow-through discarded. The DNA was eluted (Section 2.1.8) and used in the ligation reactions.

2.1.8 DNA elution from spin columns

The column was washed twice with 0.75 ml Buffer PE (with 80% ethanol (v/v)) and the flow-through discarded each time. Residual wash buffer was removed by centrifugation at 13000 rpm for an additional minute. The DNA was eluted from the column with pre-warmed 50 μl Buffer EB (10 mM Tris-Cl pH 8.5) and centrifugation at 13000 rpm for 1 minute into a fresh 1.5 ml Eppendorf tube.

2.1.9 Ligation of DNA

The ligation reaction contained 1 μl of 10x T4 DNA ligase buffer, 400 U T4 DNA ligase (New England Biolabs), *Bam HI/Xba I*-digested, CIP-treated pcDNA3.1/myc-His vector and *Bam HI/Xba I*-digested TNFRSF1A insert in sterile distilled water to a final volume of 10 μl. Reactions contained insert: vector ratios of either 1:1 or 3:1. Control reactions, which contained vector or insert only or no T4 DNA ligase, were prepared in parallel. The ligation and control reactions were incubated at 16°C for 24 hours. The ligation products were used to transform competent bacteria (Section 2.1.10).

2.1.10 Transformation of competent cells

50 µl of One Shot TOP 10 chemically competent *E. coli* (Invitrogen) were incubated on ice with 2-3 µl of ligation reaction for 30 minutes. The competent bacteria were "heat-pulsed" by incubation in a 42°C water bath for exactly 45 seconds and then ice for 2 minutes. The cells were then incubated in 500 µl of pre-warmed (37°C) SOC medium at 37°C for 1 hour at 250 rpm in a shaking incubator. The transformation reactions were plated onto selective LB agar plates (Ampicillin, Section 2.1.1), which were inverted and incubated overnight at 37°C.

2.1.11 Growth of starter cultures

A single colony of bacteria from a streaked selective LB agar plate (Section 2.1.10) was used to inoculate 3 ml of LB, and incubated overnight at 37°C in a shaking incubator. This was repeated for a number of colonies on an LB agar plate.

2.1.12 Glycerol stocks of plasmid DNA

850 µl of an overnight starter culture (Section 2.1.11) and 150 µl autoclaved glycerol (Fischer) were added to a cryoprecipitation tube and stored at -80°C.

2.1.13 PCR Sequencing

DNA sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkins-Elmer Applied Biosystems), according to the manufacturer's instructions.

DNA sequencing reactions contained 200-500 ng DNA template, 4 µl Big Dye Ready Reaction Mix, 3.2 pmole oligonucleotide primer, and distilled water to a final volume of 10 µl, and overlayed with mineral oil. The primers used to obtain the complete overlapping sequence of the TNFRSF1A coding region were: T7 Promoter forward, TNFRSF1A 325 reverse, TNFRSF1A 310 forward and pcDNA3.1/BGH reverse (Table 2.1). The following cycling conditions were used: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes (25 cycles) in an OMN-E Thermal Cycler (Hybaid).

The mineral oil was removed prior to precipitation. The sequencing reactions were precipitated by the addition of 4 µl 3 M sodium acetate pH 4.6, 200 µl 100% ethanol and 10 µl distilled water, incubation at room temperature for 15 minutes and centrifuging at 13000 rpm for 30 minutes in a microcentrifuge. The precipitate was washed five times in 250 µl 70% ethanol (v/v). The reactions were dried on a vacuum drier and mixed with loading buffer. Reactions were separated on a 4% polyacrylamide gel run at 169 kV and analysed on an ABI Prism 377 DNA sequencer. The drying of sequencing reactions and their analysis on the DNA sequencer were performed by Joyce Hoy and Barry Francis at the WCM Central Biotechnology Services DNA Sequencing Facility. Sequencing results were analysed using the Lasergene Navigator (DNAStar Inc) software.

Table 2.1 Primers sequences used in sequencing

Primer Name	Primer Sequence (5' to 3')
T7 Promoter Forward	TAATACGACTCACTATAGGG
TNFRSF1A 325 Reverse	ATCAGTGTCTAGGCTCTG
TNFRSF1A 310 Forward	GGAAGAACCAGTACCGGC
pcDNA3.1/BGH Reverse	TAGAAGGCACAGTCGAGG

2.1.14 Small scale preparation of plasmid DNA

Small scale preparations of plasmid DNA were performed using the QIAprep Spin Miniprep kit (Qiagen), according to the manufacturer's instructions. The procedure is based on a modified alkaline lysis method (Birnboim and Doly, 1979) followed by adsorption of DNA onto silica in the presence of high salt (Vogelstein and Gillespie, 1979).

1.5 ml of an overnight starter culture (Section 2.1.11) was centrifuged at 1300 rpm for 5 minutes in a microcentrifuge and the culture medium aspirated completely. The bacterial pellet was completely resuspended in 250 µl Buffer P1, with RNAase A. 250 µl Buffer P2 was added and the suspension mixed by gently inverting the tube 4-6 times.

350 µl Buffer N3 was added and the suspension mixed by inverting 4-6 times. The cloudy solution was centrifuged for 10 minutes at 13000 rpm in a microcentrifuge, yielding a white pellet. The supernatant was decanted into the to the QIAprep spin column. The column was centrifuged for 30 seconds and the flow-through discarded. The column was washed once with 0.5 ml Buffer PB and the DNA eluted (Section 2.1.8). If required, the DNA was stored at -20°C.

2.1.15 Medium scale preparation of plasmid DNA

Medium scale preparations of plasmid DNA were performed using Midi and Maxi prep kits (Qiagen), according to the manufacturer's instructions. In the method described below, numbers in brackets represent volumes and times relating to the Maxi prep kit. Other volumes and times refer to the Midi prep kit.

A starter culture (Section 2.1.11) diluted 1 in 1000 into 25 ml (100 ml) selective LB medium was incubated for 12-16 hours at 37°C in a shaking incubator overnight. The bacteria were harvested by centrifugation and completely resuspended in 4 ml (10 ml) Buffer P1 (containing final concentration of 100 µl/ml RNAase A). 4 ml (10 ml) Buffer P2 was added and the suspension gently mixed by inverting 4-6 times. After incubation at room temperature for 5 minutes, 4 ml (10 ml) chilled Buffer P3 was added and the suspension gently mixed by inverting 4-6 times. The mixture was incubated on ice for 15 minutes (20 minutes). The white precipitate was pelleted by centrifugation at 13000 rpm for 30 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was filtered to remove any further precipitates. The filtered supernatant was loaded onto a QIAGEN-tip 100 (500) column pre-equilibrated with 4 ml (10 ml) Buffer QBT and the flow-through discarded. The column was washed twice with 10 ml (30 ml) Buffer QC and the flow-through discarded. The DNA was eluted from the column with 5 ml (15 ml) Buffer QF. The DNA was now precipitated by the addition of 3.5 ml (10.5 ml, equivalent to 0.7 volumes) isopropanol (Fischer) to the solution and centrifugation at 11000 rpm for 30 minutes at 4°C in a Sorvall SS-34 rotor. The pellet was carefully washed with 2 ml (5 ml) 70% ethanol and centrifuged at 11000 rpm for 10 minutes. The supernatant was decanted and the DNA pellet air-dried. The DNA was then re-dissolved in a suitable volume of TE

buffer and a 1 in 100 dilution was quantified on a UV spectrophotometer at 260 nm (50 μg/ml DNA solution has an A_{260nm} of 1).

2.1.16 Large scale preparation of plasmid DNA, purified by caesium chloride gradients

2.1.16.1 Large scale preparation of plasmid DNA

A starter culture (Section 2.1.11) diluted 1 in 1000 into 250 ml selective Terrific Broth was incubated for 12-16 hours at 37°C in a shaking incubator overnight. The bacteria were pelleted by centrifugation to 5000 rpm for 8 minutes at 4°C in a Beckman JA-10 rotor. The culture medium was discarded and 1 ml of 1% lysozyme from chicken egg white (w/v) (Sigma), freshly prepared in Tris-HCl pH 8, was added directly onto the pellet. The pellet was resuspended completely in 4 ml Solution I (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8) by pipetting up and down. 10 ml freshly prepared Solution II (0.2 M NaOH, 1% SDS (w/v)) was added to the bacterial suspension and incubated at room temperature for 5 minutes. 7.5 ml Solution III (3 M potassium acetate (w/v), 11.5% glacial acetic acid (v/v)) was added and incubated for 5 minutes on ice. The white precipitate was pelleted by centrifugation at 14000 rpm for 30 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was decanted into a fresh tube and 13.5 ml isopropanol added. The solution was mixed and stored on ice. The DNA was precipitated by centrifugation at 13000 rpm for 30 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was discarded and the pellet washed with 3 ml of 70% ethanol (v/v). The pellet was dried briefly, 4 ml of $T_{50}.E_{10}$ (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8) added accurately and 4 μl RNAase A (10 mg/ml) added. The solution was left on a shaker for at least 1 hour, until the pellet had dissolved. A volume of the dissolved DNA solution (3.95 g by weight) was transferred into a fresh tube, containing 4.1 g of caesium chloride (Roche). The tube was shaken to dissolve the caesium chloride. 150 μl ethidium bromide (10 mg/ml, Sigma) was added to the tube and mixed by inverting several times. The mixture was incubated on ice for 5 minutes and centrifuged at 12000 rpm for 10 minutes at 4°C. Small Beckman tubes (Beckman, 13x48 mm, 362185) were filled with the DNA/ caesium chloride/ ethidium bromide mixture, paired according to weight to balance the centrifuge, and then sealed according to the manufacturer's instructions. The tubes were centrifuged in a Beckman NVT90 near-vertical rotor (maximum 90000 rpm) at 45000

rpm, without the brake, at 22°C for 16 hours. The lower band visible by eye (or UV light if required) after centrifugation was harvested into a fresh tube by piercing the top of the tube with one needle and just below the lower band with a second needle to elute this band. Distilled water was added to the tube up to 5 g in weight, followed by 5 ml isopropanol, and the contents mixed well. If separate phases were visible, equal volumes of water and isopropanol were added until miscible. The tubes were placed on ice and centrifuged at 12000 rpm for 25 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was discarded and the pellet washed with 2 ml of 70% ethanol. The pellet was dried and dissolved in 400 µl TE pH 8.

2.1.16.2 Phenol extraction of DNA

The solution was transferred into a sterile 1.5 ml Eppendorf tube and an equal volume of phenol (Fischer) added, and mixed vigorously until cloudy. The solution was centrifuged at 13000 rpm for 2 minutes in a microcentrifuge to separate the aqueous and organic phases. The upper aqueous phase was transferred to a fresh 1.5 ml Eppendorf tube. An equal volume of phenol: chloroform was added, the phases mixed and separated by centrifugation at 13000 rpm for 2 minutes. The upper aqueous phase was transferred to a fresh 1.5 ml Eppendorf. An equal volume of chloroform (Fischer) was added, the phases mixed and separated by centrifugation at 13000 rpm for 2 minutes. The aqueous phase was again transferred to a fresh tube and heated at 60°C for a few minutes to remove any remaining chloroform.

2.1.16.3 Ethanol precipitation of DNA

A 1/10 volume of 3M sodium acetate pH 5.2 (Sigma) and a 2.5x volume of 100% ethanol AR quality (Fischer) were added to the sample and mixed. The solution was then placed at -20°C for at least 1 hour and the DNA pelleted by centrifugation at 13000 rpm for 30 minutes in a microcentrifuge. The supernatant was removed and the pellet washed in 70% ethanol. The pellet was dried and dissolved in an appropriate volume of TE buffer and a 1 in 100 dilution quantified on a spectrophotometer at 260 nm as before.

2.1.17 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions.

2.1.17.1 Primer design

Two complimentary oligonucleotides containing the desired mutation, flanked by ~10-15 bases of unmodified TNFRSF1A sequence on either side, were individually designed and synthesized (MWG-Biotech). The primers underwent HPSF (High Purity Salt Free) purification at source and were diluted to 5 μg/μl in TE pH 8, and stored at -20°C. The primer sequences used to generate the TRAPS mutants by site-directed mutagenesis are shown in Table 2.2.

Table 2.2 Primer sequences used for site-directed mutagenesis

Mutation	Primer Sequence (5' to 3')
C30R	CGATTTGCCGTACCAAGTGCCAC
C43S	CTTGTACAATGACTCTCCAGGCCCGGGGC
T50M	CCGGGGCAGGATATGGACTGCAGGGAG
C52F	GGCAGGATACGGACTTCAGGGAGTGTGAGAG

2.1.17.2 PCR Reactions for site-directed mutagenesis

The sample reactions contained 1/10 volume 10x reaction buffer, 50 ng dsDNA template (WT TNFRSF1A pcDNA3.1/myc-His), 125 ng of each complimentary oligonucleotide primer, 1 μl dNTP mix and distilled water to a final reaction volume of 50 μl. 1 μl *PfuTurbo* DNA polymerase (2.5 U/μl) was then added to the reaction. The reaction was performed in a Primus Thermal Cycler (MWG-Biotech) with hot-top assembly. The following cycling parameters were used: 95°C for 30 seconds (1 cycle); 95°C for 30 seconds, 55°C for 1 minute and 68°C for 2 minutes/kb of plasmid length (16 cycles). Following temperature cycling, the reaction was placed on ice for 2 minutes.

2.1.17.3 Restriction endonuclease digestion of parental (non-mutated) dsDNA

1 µl *Dpn I* (10 U/µl) was added directly to each amplification reaction, gently mixed by pipetting up and down, and centrifuged for 1 minute in a microcentrifuge. The reaction was incubated at 37°C for 1 hour.

2.1.17.4 Transformation into Supercompetent cells

The XL1-Blue Supercompetent cells were stored at -80°C, and gently thawed on ice just prior to use. 50 µl of the supercompetent cells was transferred to a pre-chilled Falcon 2059 polypropylene tube (BD Bioscience) and 5µl of the *Dpn I*-treated DNA added. The transformation reactions were mixed by gentle swirling and incubated on ice for 30 minutes. The reaction was "heat pulsed" at 42°C for 45 seconds and placed on ice for 2 minutes. The cells were then incubated in 500 µl of pre-warmed (37°C) SOC medium at 37°C for 1 hour at 250 rpm in a shaking incubator. The transformation reactions were plated onto selective LB agar plates (Ampicillin, Section 2.1.1), which were inverted and incubated overnight at 37°C. Starter cultures were grown (Section 2.1.11), and small (Section 2.1.14) and large (Section 2.1.16) scale preparation of plasmid DNA were performed. The cloned TNFRSF1A genes were then sequenced, as described in Section 2.1.13, to give the complete overlapping nucleotide sequences of the entire TNFRSF1A coding region and ensure that only the desired mutation had been introduced. Maps for the recombinant TNFRFS1A vectors are shown in Appendix I.

2.2 Tissue Culture

2.2.1 Tissue culture media and supplements

RPMI-1640 without glutamine (Gibco BRL)

RPMI-1640 was stored at 4°C.

Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL.)

DMEM was stored at 4°C

Foetal Calf Serum (FCS) (Gibco BRL, Batch no. 40Q9021F)

FCS was stored in aliquots at -20°C

Glutamine 200mM (Gibco BRL)

Glutamine was stored in aliquots at -20°C and used as 100x stock

Penicillin/Streptomycin 5000 U/ml and 5000 µg/ml (Gibco BRL)

Penicillin/streptomycin were stored in aliquots at -20°C and used as 100x stock

2.2.2 Tissue culture sterile solutions

Phosphate buffered saline (PBS)

50 PBS tablets (Oxoid) were dissolved in 5 litres of distilled water. 500 ml aliquots were sterilised by autoclaving and stored at room temperature.

Histopaque-1077 (Sigma)

Histopaque was used for the isolation of peripheral blood mononuclear cells (PBMCs) from whole blood. Histopaque was stored at 4°C.

Hank's Balanced Salt Solution (HBSS), without phenol red (Gibco BRL)

HBSS was used in the isolation of PBMCs from whole blood. HBSS was stored at 4°C.

Heparin (CP Pharmaceuticals)

Heparin was reconstituted to a concentration of 1000 IU/ml with PBS immediately prior to use.

Trypsin-EDTA (Gibco BRL)

Trypsin was used to suspend the adherent fibroblasts. Trypsin stocks were stored at -20°C and working stocks were stored at 4°C.

2.2.3 Reagents used to stimulate cells

Recombinant human tumour necrosis factor-α (TNFα) (Calbiochem)

TNFα was reconstituted to a concentration of 10 μg/ml with sterile PBS containing 0.1% BSA. Single-use aliquots of 10-50 μl were stored at -80°C.

Recombinant human Interleukin-1 (IL-1) (Calbiochem)

IL-1 was reconstituted to a concentration of 2 μg/ml with sterile PBS containing 0.1% BSA. Single-use aliquots of 10-50 μl were stored at -80°C.

Phorbol-myristate-acetate (PMA) (Sigma)

PMA was used to induce TNFRSF1A shedding from neutrophils. PMA was reconstituted to a concentration of 10 ng/ml.

Cycloheximide (CHX) (Sigma)

Cycloheximide was used in the apoptosis and cell viability assays. Cycloheximide was reconstituted to a concentration of 50 mg/ml in DMSO and stored at 4°C.

2.2.4 Isolation of peripheral blood mononuclear cells (PBMCs) from blood

Permission was obtained from the local ethics committee to obtain extra blood from the patient when routine monitoring blood tests were performed in the clinic. Written consent was obtained from the patient. Blood was also taken from age- and sex-matched healthy volunteers in the laboratory. Heparin (CP Pharmaceuticals) was added at 100 IU/10 ml of blood. The blood was diluted with an equal volume of sterile HBSS, and the blood components separated on a Histopaque gradient. A mixture of 2 volumes blood: HBSS was carefully placed on top of 1 volume Histopaque-1077 in a fresh tube and centrifuged in a Megafuge1.0R (Heraeus), without the brake, at 1500 rpm for 30 minutes at room temperature. The "buffy coat" interface was carefully transferred to a fresh tube, which was topped up with approximately 3 volumes of HBSS and centrifuged at 1200 rpm for 10 minutes at room temperature. The supernatant was discarded, the pellet resuspended in HBSS and centrifuged at 1200 rpm for 10 minutes. The HBSS wash was

repeated and the pellet resuspended in RPMI-1640 with 10% FCS. PBMCs were used immediately for Annexin V apoptosis assays (Section 2.9.4).

2.2.5 Isolation of neutrophils from blood

For the TNFRSF1A shedding experiments, neutrophils were isolated from whole blood using dextrose sedimentation followed by gradient centrifugation using Ficoll-hypaque (Boyum, 1968). Briefly, fresh heparinised blood was mixed with 6% dextran (Fischer) in PBS and then left for 1 hour at room temperature. The resultant leucocyte-enriched plasma components were separated by gradient centrifugation using Histopaque. The supernatant was discarded and the remaining red cells lysed by the addition of 1 ml of ice cold sterile water for 60 seconds. The neutrophils were then immediately resuspended in 20 ml HBSS, washed twice in HBSS, resuspended in RPMI-1640 with 10% FCS and used immediately for the TNFRSF1A shedding experiments (Section 2.4). Viability by trypan dye exclusion was greater than 95%.

2.2.6 Generation and maintenance of primary dermal fibroblast lines

Permission was obtained from the local ethics committee for skin biopsies to be performed on the patient with TRAPS, as well as on healthy age- and sex-matched volunteers. Written, informed consent was obtained from all participants (see Appendix II). Primary skin fibroblast lines were established using an adapted method of a previously described protocol (Borysiewicz *et al.*, 1983).

1% lignocaine was injected intradermally to anaesthetize and raise the biopsy area. A small biopsy (~2mm^3) was taken using forceps and a scalpel. This was cut into 8 small fragments with a scalpel, placed into 35mm surface-modified tissue culture dishes (Primaria Easy Grip, Becton Dickinson) and covered with a glass coverslip. Fibroblasts were then cultured in DMEM containing 20% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Media was changed at weekly intervals. When cells were confluent, they were transferred to tissue culture flasks and maintained in pre-warmed DMEM containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37°C in a humidified atmosphere containing 5% CO2. Once established,

the cells were resuspended by the addition of trypsin, and up to 66% of the cell culture was removed and replaced with fresh pre-warmed growth medium once a week. Early passage fibroblasts were stored in liquid nitrogen for later use. All subsequent experiments were performed comparing early passage (passage numbers 5-10) fibroblasts of equal passage number. All stimulations of fibroblast were performed at 60-80% confluence.

2.2.7 Maintenance of lymphoid cell lines

The cell lines used for the transient transfection experiments are of lymphoid origin. The Eli-BL (Rowe *et al.*, 1987) and DG75 (Ben-Bassat *et al.*, 1977) B-cell Burkitt's lymphoma cell lines were grown in RPMI-1640 medium, supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Up to 90% of the culture was removed twice weekly and replaced with fresh pre-warmed growth medium.

2.3 Genetic mutational analysis

Blood samples from the patient were sent to Dr I. Aksentijevich (Genetics Section, Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, USA), who performed genetic mutational analysis of the samples. Briefly, DNA was extracted from the patient's whole blood and PCR amplifications of exons and flanking intronic sequences were performed as described previously (McDermott *et al.*, 1999a). PCR products were then sequenced by dye primer chemistry (Amersham Biosciences) on an ABI 377 automated sequencer.

2.4 Shedding experiments

Neutrophils were separated from fresh whole blood by dextrose sedimentation followed by gradient centrifugation using Ficoll-hypaque (Section 2.2.5). Neutrophils were either left unstimulated or stimulated with 10 ng/ml of PMA (phorbol-myristate-acetate). Surface expression of TNFRSF1A was analysed by flow cytometry (Section

2.11.2.1) and soluble TNFRSF1A levels in the supernatant were analysed by ELISA (Section 2.8.2).

2.5 Subcellular fractionation for nuclear and cytosolic extracts

2.5.1 Reagents and buffers

Buffer A

Buffer A was prepared to a final concentration of 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$ and 10 mM KCl in distilled water. Buffer A was stored at 4°C.

Buffer C

Buffer C was prepared to a final concentration of 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0,2 mM EDTA pH 8 and 25% glycerol (v/v) in distilled water. Buffer C was stored at 4°C.

Buffer D

Buffer D was prepared to a final concentration of 8 mM HEPES pH 7.9, 0.5 mM DTT, 25 mM KCl, 0.1 mM EDTA pH 8 and 8% glycerol (v/v) in distilled water. Buffer D was stored at 4°C.

Phenylmethanesulphonylfluoride (PMSF) (Sigma)

The protease inhibitor PMSF was prepared to a final concentration of 50 mM in equal volumes of ethanol: propan-2-ol. PMSF was stored at 4°C.

Phosphatase Inhibitor Cocktails I & II (Sigma)

2.5.2 Preparation of nuclear and cytosolic extracts

Early passages (passage numbers 5-10) of fibroblasts were seeded at 3 x 10^5 cells per 60 mm tissue culture dish (Greiner). The following day (approximately 70-80% confluence), the culture medium was replaced and the cells were either mock stimulated with growth medium or stimulated with TNFα (10 ng/ml) for 1 hour. Stimulation was

terminated by removal of DMEM and the addition of ice-cold PBS. Cells were harvested, using a cell scraper. All buffers were supplemented with a protease inhibitor (PMSF) and phosphatase inhibitor cocktails I and II just prior to use. Harvested cells were centrifuged at 13000 rpm for 1 minute and resuspended in 1 ml Buffer A. Cells were centrifuged at 13000 rpm in a microcentrifuge and the supernatant discarded. The pellet was resuspended in 30 µl Buffer A containing 0.1% Nonidet-P40, incubated on ice for 5 minutes and centrifuged at 13000 rpm for 5 minutes. The supernatant, representing the cytosolic extract, was removed and stored at – 20°C. The pellet was resuspended in 10 µl Buffer C, incubated on ice for 30 minutes, with regular agitation, and centrifuged at 13000 rpm for 5 minutes. The supernatant, representing the nuclear extract, was added to 25 µl of Buffer D and either used immediately or stored at –20°C. The amount of protein in the extracts was quantified prior to use.

2.5.3 Protein determination assay

The protein in the nuclear and cytosolic extracts was quantified using an adaptation of the method of Bradford (Bradford, 1976). Doubling dilutions of a 1 mg/ml BSA solution were prepared in duplicate in a flat-bottomed 96 well microplate to produce a protein standard range. Appropriate amounts of cytosolic and nuclear extracts (1-5 µl) were added in duplicate to the microplate. 200 µl of a 1/5 solution of Protein Assay Reagent (BioRad) was added to the samples and standards, and incubated at room temperature for 5 minutes. Samples were spectrophotometrically measured in a microplate reader using a wavelength of 570 nm. Protein concentrations of the cell extracts was determined by plotting on a standard curve of protein concentration.

2.6 Electrophoretic Mobility Shift Assays (EMSA)

2.6.1 Reagents and buffers

NF-κB oligonucleotide probe (Promega)

AP-1 oligonucleotide probe (Promega)

T4 polynucleotide kinase and 10x kinase buffer (Promega)

Redivue [γ-^{32}P] ATP (Amersham Biosciences)

Acrylamide solution (BD Biosciences)

Ready-mixed 40% stock containing acrylamide and bis-acrylamide at a ratio of 37.5:1 was stored at 4°C.

Tris-Boric acid buffered EDTA (TBE)

TBE was used as electrophoresis buffer. It was prepared as a 5x stock solution using 54 g Tris base (USB), 27.5 g boric acid (Sigma), 20 ml 0.5 M EDTA pH 8 made up to 1 litre. The 5x stock solution was stored in glass bottles at room temperature and any batches that developed a precipitate were discarded. TBE was used at a working strength of 0.5x.

Binding Buffer

4% glycerol, 1 mM EDTA, 5 mM DTT, 10 mM Tris pH 7.5, 100 μM NaCl, 0.1 mg/ml nuclease-free BSA (Sigma). Binding buffer was stored at -20°C, and kept for no longer than 1 month.

Ammonium persulphate (APS) (Fischer)

NNN'N- tetramethylethylenediamine (TEMED) (Fischer)

6x DNA loading dye (MBI Fermentas)

Poly(dI-dC)·Poly(dI-dC) (Amersham Biosciences)

Made up to 1 μg/μl and stored in 200 μl aliquots at -20°C.

X-omat LS Kodak film 18 x 24 cm (Amersham Biosciences)

Hypercassette with HyperScreen (Amersham Biosciences)

2.6.2 End-labelling of oligonucleotide probes

4.375 pmol of the appropriate oligonucleotides (Promega) was incubated with 25 U T4 polynucleotide kinase, 1x kinase buffer and 9.25 MBq Redivue [γ-^{32}P] ATP at 37°C for 2.5 hours. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA pH 8. One volume of phenol: chloroform was added and the solution centrifuged at 13000 rpm for 1 minute in a microcentrifuge to separate the aqueous and organic phases. The upper, aqueous phase was removed into a fresh tube. A 1/25 volume of 5M NaCl and 2 volumes of 100% ethanol AR quality (Fischer) were added to the sample and mixed. The solution was left at -20°C for at least 1 hour and the DNA pelleted by centrifugation at 13000 rpm for 10 minutes in a microcentrifuge. The supernatant was removed and the pellet was resuspended in 50 µl of TE buffer.

2.6.3 EMSA

A 4% polyacrylamide gel was made using 5 ml 40% acrylamide, 0.1 g APS, 15 µl TEMED, 10 ml 5x TBE and 35 ml distilled water. This solution was pipetted into a Hoefer Sturdier SE400 vertical gel unit, a comb inserted and allowed to set for at least 45 minutes. Equal amounts of the nuclear extracts (Section 2.5.2) were incubated with ^{32}P-labelled oligonucleotides containing the consensus sequence for either NF-κB or AP-1 (Promega). This reaction was carried out in binding buffer and poly(dI-dC)·poly(dI-dC) at room temperature for 30 minutes. The reaction was stopped by the addition of 6x DNA loading dye. The samples were separated on 4% polyacrylamide gel that was run in 0.5x TBE at 180 V for 1.5 hours. The gel was then vacuum-dried onto Whatman filter paper at 80°C for 2 hours. The gel was then exposed to autoradiograph film at -80°C overnight. Analysis of gels for densitometry was performed using Scion Image (Scion Corporation).

2.6.4 Supershift EMSA

For the NF-κB supershift assays, the nuclear extracts were first incubated on ice for 30 minutes with antibodies to the various subunits of NF-κB (kindly provided by Prof. NR Rice, Center for Cancer Research, National Cancer Institute at Frederick, MD, USA (Rice *et al.*, 1992; Koski *et al.*, 2001) (Table 2.3), prior to the binding reaction. The remainder of the process was as described above for the EMSAs.

Table 2.3 Antibodies used for NF-κB supershift assays

NF-κB subunit Antibody	Region of NF-κB recognised by antibody	Antisera names
p50	N-terminal	1141
p52	N-terminal	1267
RelA	N-terminal	1207
RelB	C-terminal	1319
cRel	Internal region	1136

2.7 SDS-PAGE and Immunoblotting

2.7.1 Reagents and buffers

2x GSB (Gel Sample Buffer)

100 mM Tris-HCl pH 6.8, 20% glycerol, 0.2 M DTT, 4% SDS, 0.02% bromophenol blue.

Aliquots of 2x GSB were stored at -20°C.

Acrylamide solution (BD Bioscience)

Ready-mixed 40% stock containing acrylamide and bis-acrylamide at a ratio of 37.5:1 was stored at 4°C.

Ammonium persulphate (APS) (Fischer)

A 15% of APS (w/v) was prepared in diluted water.

Aliquots of 15% APS were stored at – 20°C.

Resolving gel buffer

A 4x solution was prepared using 1.5 M Tris-HCl pH 8.8, 0.24% TEMED (v/v), 0.4% SDS (w/v). This 4x stock solution was stored for up to one month at 4°C

Stacking gel buffer

A 2x solution was prepared using 0.25 M Tris-HCl pH 6.8, 0.12% TEMED (v/v), 0.4% SDS (w/v). This 2x stock solution was stored for up to one month at 4°C

Electrophoresis running buffer

A 10x solution was prepared 0.25 M Tris-base, 1.92 M glycine (Fischer), 1% SDS. The running buffer was at pH 8.3 and not adjusted with HCl. This 10x stock solution was stored at room temperature.

Transfer buffer

25 mM Tris-base, 192 mM glycine, 20% analysis-grade methanol (Fischer). The transfer buffer was stored at room temperature.

10x Phosphate-buffered saline (PBS)

50 PBS tablets (Oxoid) per 500 ml distilled water. 10x PBS was stored at room temperature.

PBS-Tween (PBS with Tween-20)

0.2% Tween-20 (v/v) in 1x PBS. PBS-Tween was stored at room temperature.

Blocking buffer

0.2% I-block (w/v) (Tropix), 0.1% Tween-20 (v/v), 0.02% NaN_2 (v/v) in PBS.
500 ml of distilled water was heated in a microwave until boiling and 5 PBS tablets added. 1 g I-block purified casein was dissolved in the hot 1x PBS solution by stirring on a magnetic mixer. After the I-block had dissolved and the solution had cooled, 500 µl of Tween-20 and 500 µl of 20% sodium azide were added. The blocking buffer was stored for up to one month at 4°C.

Alkaline phosphatase (AP) assay buffer

A 10x solution was prepared using 1 M diethanolamine pH 9.5, 10 mM $MgCl_2$. 10x AP assay buffer was stored for up to one month at 4°C.

CPD-Star development reagent; ready to use (Tropix)

CPD-Star is an alkaline phosphatase substrate for use in chemiluminescent detection protocols. The chemical name for CPD-Star is: Disodium 2-chloro-5-(4-methoxyspiro{-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.1]decan}-4-yl-1-phenyl phosphate. CPD-Star was stored at 4°C.

MESNA stripping buffer

62.5 mM Tris-HCl pH 6.8, 2% SDS (w/v), 50 mM sodium 2-mercaptoethansulfonate (MESNA) (Sigma). MESNA stripping buffer kept for up to one month at 4°C.

SDS wash buffer

62.5 mM Tris-HCl pH 6.8, 2% SDS (w/v). SDS wash buffer was stored at room temperature.

Polyethylene lay flat film 204 mm (Jencons)

Hybond-P PVDF membrane (Amersham Biosciences)

Pre-stained molecular weight markers (Sigma)

X-omat LS Kodak film 18 x 24 cm (Amersham Biosciences)

2.7.2 SDS-PAGE

Volumes stated here are sufficient to pour two polyacrylamide gels in the mini-gel apparatus used. 8% SDS-PAGE resolving gels were made up with 2.8 ml resolving gel buffer, 2.25 ml acrylamide (40%), 44 µl 15% APS and 6.1 ml distilled water, and pipetted into mini protean gel apparatus (BioRad). The resolving gel was overlayed with water-saturated butan-2-ol, and allowed to set for at least 45 minutes.

The butan-2-ol layer was poured off and the surface of the resolving gel washed with distilled water. Excess water was removed with paper tissues. The stacking gel was

made up with 3.9 ml stacking gel buffer, 0.8 ml acrylamide solution, 78 μl 15% APS, 3.1 ml distilled water and 8 μl TEMED. The stacking gel was poured over the set resolving gel, a comb inserted, and left to set for at least 20 minutes. Gels were run at 180 V for 45 minutes.

2.7.3 Blotting of polyacrylamide gels

Gels were blotted onto Hybond-P PVDF membranes, which had been pre-soaked in methanol and equilibrated in transfer buffer. The gel was placed onto the PVDF membrane between two pieces of Whatman filter paper soaked in transfer buffer, in a blotting cassette. Blotting was carried out at 100 V for 1 hour in mini-transblot apparatus, cooled with an ice bucket. The blots were then washed three times in PBS-Tween.

2.7.4 Immunostaining of western blotted filters

The blots were blocked with 15 ml blocking buffer overnight on a rocker at 4°C in a sealed polyethylene bag. The blocking buffer was discarded and replaced with 12.5 ml of primary antibody diluted in blocking buffer (see Table 2.4), and incubated on a rocker for at least 1 hour at room temperature. The immunoblot was washed three times, for 10 minutes each, with PBS-Tween, and then incubated with 12.5 ml AP-conjugated secondary antibody, diluted 1/ 10,000 in blocking buffer for 1 hour at room temperature. The blot was washed three times in PBS-Tween and once with 1x AP assay buffer, for 10 minutes each at room temperature. Excess AP assay buffer was drained off and the blot incubated with CPD-Star for 20 minutes, before removal of the excess liquid and exposure to autoradiograph film through a polyethylene envelope.

2.8 Enzyme linked immunosorbent assays (ELISA)

TNFRSF1A DuoSet (R&D Systems), IL-6 and IL-8 CytoSet (Biosource) ELISA kits were used according to the manufacturers' instructions.

2.8.1 Reagents and buffers

Phosphate-Buffered Saline (PBS)

5 PBS tablets (Oxoid) per 500 ml of distilled water. PBS was stored at room temperature.

PBS-Tween (PBS with Tween-20) Wash buffer

0.1% Tween-20 (Sigma) in PBS. PBS-Tween was stored at room temperature.

Coating buffer

Coating buffer was prepared immediately prior to use and made up to the required concentration in PBS.

Blocking Solution

The blocking solutions for the two ELISA kits were made up with different amounts of BSA, as per manufacturer's instructions. 1% BSA (w/v) in PBS (DuoSet). 0.5% BSA (w/v) in PBS (CytoSet).

Standard diluent/ Assay buffer

Different standard diluents/ assay buffers were used for the two ELISA kits, as per manufacturer's instructions. 1% BSA in PBS (DuoSet). 0.5% BSA in PBS-Tween (CytoSet). The appropriate dilution of streptavidin/ HRP was added to the assay buffer prior to use.

TMB (tetramethyl benzidine) Substrate

Stop solution

12.5% H_2SO_4 (Sigma)

Standard dilutions

Doubling dilutions of the standard were prepared in growth medium with 10% FCS prior to use.

2.8.2 TNFRSF1A DuoSet ELISA (R&D Systems)

The supernatants of neutrophils stimulated with PMA (Section 2.4) were removed and stored at -70°C until used. The samples were only thawed once and then discarded.

ELISA microplates (Nunc) were incubated with 100 µl/well of coating buffer, containing 4 µg/ml coating antibody, overnight at 4°C. The supernatant was decanted, the plate washed four times with PBS-Tween wash buffer and incubated with 300 µl/well of blocking solution (1% BSA (w/v) in PBS) for 1 hour at room temperature. The plate was washed four times with PBS-Tween wash buffer, 100 µl/well of sample or standard dilutions, in RPMI-1640 containing 10% FCS, added and incubated on a rocker for 2 hours at room temperature. The plate was washed four times with PBS-Tween, 100 µl/well of biotinylated detection antibody, diluted to 0.2 µg/ml in 1% BSA in PBS, added and incubated for 2 hours at room temperature. The plate was washed four times with PBS-Tween. Streptavidin-HRP conjugate was diluted 1/200 with assay buffer, then 100 µl/well added to the microplate and incubated on a rocker for 30 minutes at room temperature. The plate was washed four times with PBS-Tween and developed with 100 µl/well TMB substrate for 10-30 minutes. The reaction was stopped by the addition of 100 µl of stop solution and spectrophotometrically measured in a microplate reader using a wavelength of 450 nm. Sample concentrations were calculated by plotting the sample optical densities on a standard curve of concentration.

2.8.3 IL-6 and IL-8 Cytoset ELISA (Biosource)

Dermal fibroblasts were seeded at 5 x 10^4 cells/well in a 48 well plate. The following day (approximately 70-80% confluence), the culture medium was replaced and the cells were either mock stimulated with growth medium or stimulated with TNFα (10 ng/ml). After 24 hours incubation, the supernatants were removed and stored at -70°C until used. The samples were only thawed once and then discarded.

ELISA microplates were incubated with 100 µl/well of coating buffer, containing 1 µg/ml coating antibody, overnight at 4°C. The supernatant was decanted and the plate

incubated with 300 µl/well of blocking solution (0.5% BSA (w/v) in PBS) for 2 hours at room temperature. The supernatant was discarded and the plate washed four times with PBS-Tween wash buffer, 100 µl/well of sample or standard dilutions added and incubated with 50 µl/well of the appropriate biotinylated detection antibody, diluted 1/1250 with assay buffer, on a rocker for 2 hours at room temperature. The plate was washed four times with PBS-Tween. 100 µl/well of Streptavidin-HRP conjugate, diluted with assay buffer to the appropriate concentration for the specific assay (1/4000 for IL-6, 1/8000 for IL-8), was added to the microplate and incubated on a rocker for 30 minutes at room temperature. The plate was washed four times with PBS-Tween and developed with 100 µl/well TMB substrate for 10-30 minutes. The reaction was stopped by the addition of 100 µl of stop solution and spectrophotometrically measured in a microplate reader using a wavelength of 450 nm. Sample concentrations were calculated by plotting the sample optical densities on a standard curve of concentration.

2.9 Assays of cell viability

2.9.1 Cell viability by light microscopy

Toluidine Blue Stain (VWR International)

Toluidine blue stain was made to 1% sodium borate petra (w/v) and 1% toluidine blue (v/v) in distilled water. The solution was then passed through a 0.2 µM filter and stored at room temperature.

Early passages (passage numbers 5-10) of fibroblasts were seeded at 3 x 10^4 cells in 1 ml growth medium in a 24 well tissue culture plate. The following day, the culture medium was replaced and the cells were stimulated with various doses of TNFα and cycloheximide (CHX, 50 µg/ml), either alone or in combination, under usual conditions of growth. After 24 hours incubation, the culture medium was removed and filtered 1% toluidine blue stain was added for 1 minute. Cells were then washed in situ with PBS and distilled water. Images were acquired using an Olympus BX41 microscope (4x objective) fitted with an Olympus C-03030 digital camera.

2.9.2 Alamar blue viability assays

2.9.2.1 Alamar blue assay of fibroblasts

Early passages (passage numbers 5-10) of fibroblasts were seeded at 2 x 10^4 cells in 200 µl growth medium in a 96 well flat bottom microplate. The following day, the culture medium was replaced and the cells were stimulated with various doses of TNFα and CHX (50 µg/ml), either alone or in combination, under usual conditions of growth. 24 hours later, the growth medium was replaced with 200 µl of 10% alamar blue reagent (Biosource) in growth medium. After 4 hours incubation at 37°C, readings were made on a FLUOstar Optima (BMG Laboratories) using the fluorescence configuration. Fluorescence was monitored with excitation wavelength at 530-560 nm and emission wavelength at 590 nm.

2.9.2.2 Alamar blue assay of transfected lymphoid cell lines

Cells were transfected as described in Section 2.10.2 and then left in growth medium overnight. The following day 2 x 10^4 transfected cells were resuspended in fresh growth medium and placed in a 96 well flat bottom microplate. Cells were then stimulated TNFα and CHX, and alamar blue assay performed as described for the fibroblasts (Section 2.9.2.1).

2.9.3 Caspase-3 assays

Caspase-3 activity of the fibroblasts was analysed using the Caspase-3 Colorimetric Assay (R&D Systems), according to the manufacturer's instructions.

5 x 10^5 fibroblasts were seeded in 80cm^2 tissue culture flasks (Greiner). The following day, the culture medium was replaced and the cells were stimulated with TNFα (10 ng/ml) and CHX (50 µg/ml), either alone or in combination, under usual conditions of growth. At the times indicated, stimulation was terminated by the addition of ice-cold PBS. The suspension, containing non-adherent cells was removed. The remaining adherent cells were removed by the addition of trypsin. Unless otherwise indicated, the following steps were all performed on ice where possible. Both adherent and non-adherent cells were pelleted together by centrifugation at 2000 rpm for 10 minutes in a

microcentrifuge. Cold lysis buffer was added at 25 µl per 1 x 10^6 cells and incubated for 10 minutes on ice. Cell lysate was centrifuged at 10000 rpm in a microcentrifuge. The supernatant (50 µl) was transferred to a 96 well flat bottom microplate. 10 µl fresh 1 M DTT was added to 1 ml 2x reaction buffer just before use. 50 µl reaction buffer was then added to the cell lysate together with 5 µl Caspase-3 substrate (DEVD-pNA). The plate was incubated for 1 hour at 37°C and then spectrophotometrically measured in a microplate reader using a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the colour reaction. Experimental controls included non-induced cells, samples without cell lysate (substrate only), samples without substrate and as positive control, recombinant caspase-3 enzyme (R&D Systems) was added.

2.9.4 Annexin V apoptosis assays

Apoptosis of PBMCs was analysed using the TACS Annexin V-FITC kit (R&D Systems), according to the manufacturer's instructions. Annexin V Incubation Reagent was prepared, with or without propidium iodide (PI), just prior to use, and contained 1 µl Annexin V-FITC, 10 µl 10x binding buffer, 10 µl PI (50 µg/ml) (optional) and distilled water to a total volume of 100 µl. This reagent was kept on ice in the dark, and used within 1 hour.

PBMCs were isolated from whole blood using a Ficoll gradient (Section 2.2.4). 2 x 10^6 PBMCs were then placed in 2 ml RPMI-1640 with 10% FCS in a 6 well plate. Cells were either left untreated, or stimulated with TNFα (10 ng/ml) and CHX (50 µg/ml), either alone or in combination. Apoptosis assays were performed after 2 and 6 hours stimulation. A cell scraper was used to remove any adherent monocytes and all cells were then transferred to a fresh tube. Cells were pelleted by centrifugation at 1500 rpm for 10 minutes in a Megafuge1.0R (Heraeus). Cells were washed in cold PBS and pelleted by centrifugation as above. Cell pellet was gently resuspended in the pre-mixed Annexin V-FITC incubation reagent at a concentration of 1 x 10^6 cells/100 µl and incubated in the dark at room temperature for 15 minutes. 400 µl of 1x binding buffer was then added and

the samples analysed immediately by flow cytometry, gating on live cells (PI-negative). Other experimental controls included unstimulated cells, unstained cells and samples stained with either Annexin V-FITC or PI alone.

2.10 Transient transfection and luciferase assays of lymphoid cell lines

2.10.1 Plasmid constructs

Recombinant TNFRSF1A was cloned into the pcDNA3.1/myc-His B (Invitrogen) mammalian expression vector (Section 2.1). The pcDNA3.1/myc-His B vector contains the CMV immediate-early promoter/enhancer as well as the T7 promoter upstream of the multiple cloning site and the BGH polyadenylation signal downstream of the cloning site. The vector also contains Ampicillin and Neomycin resistance genes. The EGFP-N1 vector (Clontech) contains the enhanced green fluorescent protein (EGFP) coding sequence downstream of the CMV immediate early promoter. The 3Enh.κB-ConALuc reporter (3EnhLuc) contains three tandem repeats of the NF-κB binding sites from the Igκ promoter upstream of a minimal conalbumin promoter, and expresses firefly luciferase (Arenzana-Seisdedos *et al.*, 1993). The phRL-SV40 luciferase reporter (Promega), used in the dual-luciferase reporter assays to assess transfection efficiency, contains the SV-40 early enhancer/promoter region driving *Renilla* luciferase.

2.10.2 Gene transfection

Cell lines were transiently transfected with plasmid DNA by electroporation as follows. Cells were washed in pre-warmed growth medium and resuspended at 2 x 10^7 cells per ml in RPMI culture medium buffered with 100 mM HEPES (pH 7.2). A 0.5 ml aliquot of the cell suspension was placed in an electroporation cuvette (BioRad, 0.4 cm gap) together with the plasmid DNA and electroporated at 270 V and 950 μF, using a Genepulser II electroporator (BioRad). The cells were then re-seeded in 4 ml of fresh growth medium in 6 or 24 well plates and cultured under normal conditions. Transfection efficiency was typically 5-20% for the Eli-BL cell line and 40-50% for the DG75 cell line.

For the luciferase reporter assays, the electroporated cell suspension was split into two and 16 hours post-transfection, one half was mock stimulated with growth medium and the other half stimulated with of TNFα (10 ng/ml). Luciferase activity was measured 8 hours after stimulation with TNFα as described in section 2.10.3.2.

2.10.3 Luciferase Reporter Assays

2.10.3.1 Reagents

Luciferase Lysis Buffer

100 mM HEPES pH 8, 5 mM DTT, 2 mM $MgCl_2$, 2% Triton X-100 (Fischer). Luciferase lysis buffer was stored at 4°C.

Luciferin Stock (10mM)

50 mg of luciferin (Amersham Biosciences) was dissolved in 17.85 ml 30 mM glycylglycine pH 7.9. Aliquots of 500 µl were stored in the dark at -70°C.

Luciferase Assay Reagent (LAR)

20 mM glycylglycine pH 7.9, 5 mM $MgCl_2$, 100 µM EDTA, 3.3 mM DTT, 270 µM lithium coenzyme A from yeast (Sigma), 0.5 mM ATP (Sigma). LAR was stored in 10 ml aliquots at -70°C. The reagent buffer was prepared by thawing aliquots of LAR (10ml) and luciferin (500 µl) in the dark, and mixing them prior to use. The reagent buffer was stored for up to one month in the dark at -20°C.

2.10.3.2 Luciferase Reporter Assays

Approximately 2.5 x 10^6 transfected cells were harvested, washed once in chilled PBS, and lysed in 100 µl Luciferase Lysis Buffer for 30 minutes on ice. The lysate was clarified by centrifugation at 13000 rpm for 30 seconds in a microcentrifuge and 50 µl was assayed for luciferase activity. Light release was measured by a Berthold LB9501 luminometer following injection with 100 µl reagent buffer. Light release was integrated for 10 seconds.

2.10.4 Transfection efficiency assays

Transfection efficiency was assessed using the dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions.

2.10.4.1 Reagents and buffers

Passive lysis buffer (PLB)

PLB is supplied as a 5x concentrate. Sufficient 1x PLB was prepared using distilled water just before use. 5x PLB was stored at -20°C.

Luciferase Assay Reagent II (LAR II)

Lypophilized Luciferase Assay Substrate was resuspended in 10 ml Luciferase Assay Buffer II. 10 ml aliquots of LAR II were stored at -70°C.

Stop & Glo Reagent

Sufficient Stop & Glo Reagent was prepared just prior to use by adding 1 volume of supplied 50x Stop & Glo Substrate to 50 volumes of Stop & Glo Buffer.

2.10.4.2 Dual-luciferase reporter assays

Approximately 2.5 x 10^6 transfected cells were harvested, washed once in chilled PBS, and passively lysed in 100 µl PLB for 15 minutes at room temperature on a rocker. The lysate was clarified by centrifugation at 13000 rpm for 30 seconds in a microcentrifuge. 45 µl of cell lysate was assayed for *Renilla* luciferase activity. Light release was measured by a Berthold LB9501 luminometer following injection with 100 µl LAR II reagent. The luminometer was then washed twice with distilled water and 5 µl of cell lysate assayed for firefly luciferase activity following injection with 100 µl Stop & Glo reagent.

2.11 Immunofluorescent techniques

2.11.1 Reagents

2% Paraformaldehyde (2% PFA) (Arcos Organics)

2% paraformaldehyde (w/v) was prepared by dissolving 10 g paraformaldehyde in 500 ml PBS overnight in a 50°C water bath in a fume cupboard. Paraformaldehyde may release formaldehyde when dissolved and care was taken not to heat it above 60°C because of its flashpoint. 2% PFA was protected from light and stored at 4°C.

Normal rabbit serum (NRS), heat-inactivated

Aliquots of NRS were stored at -20°C.

10% NRS/PBS

10% NRS (v/v) in PBS was prepared fresh on the day of use.

0.1% Triton X-100 (Fischer)

A 0.1% solution of Triton X-100 (v/v) was prepared in PBS and stored at room temperature.

DABCO (1.4-Diazabicyclo[2,2,2]octane) anti-fade mounting solution

2.5% DABCO (Sigma) in 90% glycerol/10%PBS, pH 8.6 was stored at 4°C.

2.11.2 Immunofluorescent staining for flow cytometry

All flow cytometry results were acquired using a FACS calibur (BD Biosciences) and results obtained were analysed using Cellquest Pro (BD Bioscience).

2.11.2.1 TNFRSF1A expression on neutrophils (for shedding experiments)

Neutrophils stimulated with PMA (Section 2.4) were washed twice with PBS and either stained with PE-conjugated CD120a (TNFRSF1A) antibody (Caltag) or IgG1-conjugated PE negative control (Serotec) for 30 minutes. Cells were washed with PBS,

resuspended in 2% paraformaldehyde and analyzed by flow cytometry, gating on neutrophils.

2.11.2.2 TNFRSF1A expression in transfected DG75 cells

DG75 cells were co-transfected with the expression vectors for EGFP-N1 and recombinant TNFRSF1A. Cells were harvested 24 hours after transfection, washed with cold PBS and fixed in 2% PFA for 20 minutes on ice. Cells were then either left unpermeabilised or permeabilised prior to staining.

Unpermeabilised cells (for surface expression of TNFRSF1A)

Cells were washed twice with PBS and incubated in 10% NRS/PBS for 20 minutes to block non-specific binding. Cells were either stained with unconjugated mouse anti-human TNFRSF1A monoclonal antibody (Ab-1, Oncogene) or mouse IgG1-conjugated PE negative control (Serotec) for 30 minutes. The cells were washed twice with PBS and the TNFRSF1A-stained cells were stained with a rabbit anti-mouse R-phycoerythrin (RPE)-conjugated $F(ab')_2$ fragment (Dako). Cell were washed with PBS, resuspended in 2% paraformaldehyde and analysed by flow cytometry, gating on EGFP positive cells.

Permeabilised cells (for total expression of TNFRSF1A)

Cells were incubated in 0.1% Triton X-100/PBS for 20 minutes, washed twice with PBS and then incubated in 10% NRS/PBS for 20 minutes. Cells were stained with an unconjugated primary antibody as for the unpermeabilised cells, and then washed once with 0.1% Triton X-100/PBS and twice with PBS. Cells were stained with RPE-conjugated secondary antibody, washed with PBS, resuspended in 2% PFA and analysed as for the unpermeabilised cells.

2.11.3 Immunostaining of fixed cell smears for fluorescence microscopy

Localisation of recombinant TNFRSF1A was assessed by immunofluorescent staining of fixed cell smears, followed by confocal microscopy. DG75 cells were transfected with 10 μg recombinant TNFRSF1A, harvested 24 hours after transfection, washed three times with cold PBS and fixed in 2% PFA for 15 minutes. Cells were

washed a further two times with PBS and resuspended at a concentration of 2 x 10^7 cells/ml in PBS. Approximately 10 µl of the cell suspension was applied to the wells of multiwell slide (Hendley Ltd) and the excess fluid carefully aspirated to leave a thin layer of cells, which was allowed to air-dry. The cells were permeabilised with 10 µl of 0.1% Triton X-100/PBS for 30 minutes, washed twice with PBS and incubated in 10% NRS/PBS for 20 minutes. Cells were either stained with 15 µl a monoclonal anti-TNFRSF1A antibody (Table 2.4) or an isotype control diluted in 10% NRS/PBS for 30 minutes, and then washed once with 0.1% Triton X-100/PBS and twice with PBS. Cells were stained with the appropriate anti-mouse secondary antibody (Table 2.4) for 30 minutes, washed once with 0.1% Triton X-100/PBS and twice with PBS. If nuclear staining was required, cells were incubated with DRAQ5 (Biostatus Ltd) 10 µM for 10 minutes, and washed twice with PBS. Drops of DABCO mounting solution were added to each well on the slide and a coverslip applied and immobilised with nail varnish.

Single optical slice images were acquired using a confocal laser scanning microscope (BioRad), equipped with a krypton/argon ion laser and attached to an inverted Zeiss Axiovert 135. Alexa Fluor 488 (TNFRSF1A) was visualised using 488 nm excitation and detected at 520 nm. DRAQ5 (DNA) was excited at 647 nm and detected at 680/30 nm. Images were merged and formatted using Confocal Assistant version 4.02.

2.12 Statistical Analysis

As the data could not be assumed to follow a Gaussian distribution, and the sample sizes were too small to allow for normality tests to be performed, non-parametric tests were used to analyse the statistical significance of the results. The Mann-Whitney U test was performed using GraphPad Prism version 4.0 (GraphPad Software, San Deigo, California). For the dose response curves, nonlinear regression was performed using GraphPad Prism version 4.0.

Table 2.4 Antibodies

Specificity/ name	Conjugate	Catalogue #	Species	Application	Working dilution	Supplier
TNFRSF1A (IgG_1)	--	GR28L	Mouse	FACS, IF	1/10 (IF) 1/25 (FACS)	Oncogene (Calbiochem)
CD120a (TNFR1)	RPE	MG104	Mouse	FACS	1/100	Caltag
TGN 46	--	AHP500	Sheep	IF	1/250	Serotec
V5	--	R960-25	Mouse	WB	1/2,000	Invitrogen
c-myc (9E10)	--	M5546	Mouse	WB	1 μg/ml	Sigma
IκBα (C-21)	--	Sc-371	Rabbit	WB	1/500	Santa Cruz
Actin	--	A2066	Rabbit	WB	1/1,000	Sigma
IgG_1 negative control	RPE	MCA928PE	Mouse	FACS, IF	1/100	Serotec
Mouse IgG	RPE	R0439	Rabbit	FACS	1/50	Dako
Mouse IgG	Alexa Fluor 488	A11001	Goat	IF	1/300	Molecular probes
Sheep IgG	Alexa Fluor 564	A21099	Donkey	IF	1/300	Molecular probes
Rabbit IgG	AP	170-6518	Goat	WB	1/ 10,000	BioRad
Mouse IgG	AP	170-6520	Goat	WB	1/10,000	BioRad

Abbreviations

FACS = flow cytometry; IF = immunofluorescence; WB = Western blot;

RPE = phycoerythrin; AP = alkaline phosphatase

CHAPTER 3

THE FUNCTIONAL CHARACTERISATION OF PRIMARY HUMAN DERMAL FIBROBLASTS CONTAINING A NOVEL TNFRSF1A MUTATION

A patient with an undefined hereditary periodic fever had been attending the Department of Rheumatology in Cardiff for many years. In 1999 TRAPS was described as a new autoinflammatory syndrome associated with mutations in the gene that encodes TNFRSF1A (McDermott *et al.*, 1999a). The clinical features of our patient were consistent with the described TRAPS phenotype, and we subsequently demonstrated that the patient had a novel TNFRSF1A mutation (C43S).

Initial reports suggested that the TRAPS mutations impair activation-induced shedding of TNFRSF1A (McDermott *et al.*, 1999a; Galon *et al.*, 2000), although this does not appear to be the case for all mutations (Aksentijevich *et al.*, 2001; Aganna *et al.*, 2003) and may be dependent on cell type (Huggins *et al.*, 2004). The mechanisms by which these TNFRSF1A point mutations result in the inflammatory phenotype remain unclear. At the time of starting this work, there were no published studies on the effects of these TNFRSF1A mutations on TNFα-induced activation of transcription factors or apoptosis.

Our first hypothesis was that the TRAPS phenotype could result from enhanced TNFα-mediated signalling, particularly increased NF-κB activation. Ideally this would be analysed in cells of haemopoeitic lineage, but full analysis of NF-κB activation in these cells is rendered extremely difficult due to the inability to culture these cells for sustained periods. For this reason, we chose to perform our analysis by generating a patient-derived dermal fibroblast line. This approach would allow us to study TNFα-induced activation of NF-κB in primary cells and to compare this to healthy age-matched volunteers, the first time such an analysis was performed for a TRAPS patient. This chapter describes the TRAPS patient with the novel C43S TNFRSF1A mutation and the investigation of the signalling effects of this mutation using samples from the patient.

3.1 Identification of a patient with a novel TNFRSF1A mutation

3.1.1 Clinical observation

The index patient, a 50-year old woman of Welsh origin, has experienced recurrent attacks of fever accompanied by features of systemic inflammation since the age of 18 months. Stereotypic attacks occur every four to five weeks, lasting for one to two weeks. The duration of the attacks has gradually increased with age. In addition to fever, the attacks are characterised by pharyngitis, localised myalgia, arthritis/arthralgia and a migratory erythematous skin rash. Her usual attack starts with tenderness and induration of a localised muscle group, accompanied by an overlying skin rash. The myalgia and rash tend to migrate centrifugally over the next few days, followed by the onset of the fever and her other symptoms. She has been noted to have swelling of both large and small joints at some time but there is no evidence of residual joint damage. On occasion these attacks are of sufficient severity to render her bed-bound. Apart from generalised fatigue, she is largely asymptomatic between attacks.

The patient's deceased father is reported to have had similar life-long recurrent, episodic fevers accompanied by abdominal pain, skin rash, arthralgia and migratory myalgia. These attacks occurred every few months and were partially relieved by administration of oral corticosteroids but not by colchicine. He is documented as having had increased acute phase markers. He died of renal failure at the age of 54 years, at which time a renal biopsy showed non-specific sclerosis secondary to vascular changes, although it is unknown whether the biopsy sample was stained for amyloid. The index patient has a 28 year-old daughter who is asymptomatic but has not undergone any genetic testing.

The index patient was referred to our rheumatology clinic with a provisional diagnosis of "possible familial Hibernian fever", based on her clinical symptoms, lack of response to colchicine and a negative metaraminol provocation test. The patient has longstanding, stable chronic renal impairment (creatinine clearance of 24 ml/min), presumed to be the result of a combination of previous pyelonephritic and drug-induced

damage. Two previous renal biopsies have shown chronic tubulointerstitial disease with no evidence of amyloid deposition. Rectal biopsy was also negative for amyloid. She has a chronic microcytic anaemia (haemoglobin between 6-8 g/dl) and persistent neutrophilia with an average total white cell count of 13.3 x 10^9/ ml and neutrophil count of 10.9 x 10^9/ ml. Tests for autoantibodies have been persistently negative. Even between attacks she has persistently elevated acute phase markers, with an erythrocyte sedimentation rate (ESR) consistently above 90 mm/hr and C-reactive protein (CRP) above 70 mg/dl. During her febrile attacks, these levels increase dramatically, with typical ESR around 140 mm/hr and CRP around 180 mg/dl. Immunoglobulin levels are largely within normal limits. Biopsy of her skin rash revealed granulomatous dermatitis/ panniculits but no frank vasculitis. She has had a single episode of non-infective ascites, while aspiration of her joint effusions has also been sterile, with numerous polymorphonuclear cells. Since the age of 12 years, the patient has received intermittent courses of low dose corticosteroids, which reduce the duration and severity of her attacks, but do not prevent them. Her symptoms have been unresponsive to treatment with azathioprine and thalidomide in the past.

3.1.2 Identification of the TNFRSF1A mutation

In 1999 TRAPS was described as a novel autoinflammatory disorder (McDermott *et al.*, 1999a) and it became apparent that our patient's clinical features were consistent with this condition. While plasma soluble TNFRSF1A levels are usually low in TRAPS, this patient's plasma soluble TNFRSF1A levels were elevated at 3839 ± 131 pg/ml (normal range is 746-1966 pg/ml). These levels are however in keeping with the elevated levels reported in TRAPS patients with renal impairment (Aganna *et al.*, 2003). To support the clinical diagnosis of TRAPS, investigations were undertaken to establish whether the patient had a mutation in the gene for TNFRSF1A. The patient's blood samples were kindly analysed by Dr I. Aksentijevich (Genetics Section, Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, USA), with whose permission these results are shown. Briefly, DNA was extracted from the patient's whole blood and PCR amplifications of exons and flanking intronic sequences were performed as described

previously (McDermott *et al.*, 1999a). PCR products were then sequenced by dye primer chemistry (Amersham) on an ABI 377 automated sequencer. The patient was found to have a novel TNFRSF1A mutation (C43S) in exon 3 of the TNFRSF1A gene (Figure 3.1A). The G→C transition results in the substitution of serine for cysteine at position 43. This substitution involves a highly conserved cysteine and disrupts the disulphide bond at this position in the first extracellular domain of TNFRSF1A (Figure 3.1B-C). This substitution was not observed in any of the 734 control subjects who were screened using the Sequenom Mass Array high throughput genotyping system. Two other mutations (C43R and C43Y) have subsequently been reported at this position, in addition to a deletion of the preceding aspartic acid (ΔD42) (Aganna *et al.*, 2003), suggesting a critical role for this site.

3.1.3 Treatment with anti-TNFα therapy

Following the identification of the association of TRAPS with TNFRSF1A mutations, inhibition of TNFα signalling was seen as a logical therapeutic approach. Treatment of patients with etanercept, a soluble p75TNFR:Fc fusion protein, has subsequently been shown to decrease the frequency, duration and severity of attacks (Galon *et al.*, 2000; Hull *et al.*, 2002b). In light of the debilitating effect of her attacks, and the steroid-induced osteoporosis complicating her previous treatment, she was commenced her on a trial of anti-TNFα therapy. As etanercept was unavailable in the United Kingdom at the time, she was initially treated with the monoclonal TNFα antibody, infliximab (3 mg/kg). Within a day of her first infusion of infliximab, she became acutely unwell with high fever. This episode was significantly worse than her usual febrile attacks. There was however no evidence of infection and the episode settled spontaneously within a week. During this episode, her ESR and CRP both increased dramatically, to 147 mm/hr and 356 mg/dl respectively. Longitudinal measurements of her leucocyte counts and acute phase response over a 50 week period, including the onset of anti-TNFα therapy, are shown in Figure 3.2. She was subsequently treated with subcutaneous etanercept (25 mg twice weekly), which she tolerated well. This treatment significantly decreased the frequency, severity and duration of her febrile episodes but did

not prevent these episodes, even when the dose was increased to 25 mg three times per week.

While both infliximab and etanercept neutralise TNFα efficiently, there is increasing evidence that these agents have different mechanisms of action (Van den Brande *et al.*, 2003) (Section 1.1.7). As infliximab, but not etanercept, is capable of binding mTNFα (Van den Brande *et al.*, 2003; Shen *et al.*, 2005), it is tempting to speculate that the deterioration of our patient's clinical condition and inflammatory markers was as a result of infliximab-induced reverse signalling through mTNFα. This suggests that signalling through mTNFα may be involved in the pathogenesis of TRAPS. While we were unable to pursue this hypothesis further in the context of the current work, it is clearly worthy of further study and is discussed more fully in Chapter 5.

3.2 Effect of the C43S mutation on TNFRSF1A shedding by neutrophils

The actions of TNFα may be constrained in part by cleavage of TNF receptors from cell membranes, which can occur in response to a number of pro-inflammatory stimuli (Loetscher *et al.*, 1991; Higuchi and Aggarwal, 1992; Van Zee *et al.*, 1992). Receptor cleavage may have homeostatic effects both by reducing the number of receptors on cell membranes available for interaction with TNFα, and by producing a pool of soluble receptors that can compete for TNFα with membrane receptors. Several TRAPS mutations have been reported to impair activation-induced shedding of the receptor (McDermott *et al.*, 1999a; Galon *et al.*, 2000; Kriegel *et al.*, 2003), although this has not been the case for all mutations (Aksentijevich *et al.*, 2001; Aganna *et al.*, 2003). We therefore undertook to investigate whether neutrophil shedding of TNFRSF1A was affected by the C43S mutation. The following experiments were performed with Dr Eddie Wang, Section of Infection and Immunity, and Nick Amos, Department of Rheumatology, and are shown with their permission. Following local ethics committee permission, neutrophils were isolated from the patient's whole blood by dextrose sedimentation followed by gradient centrifugation using Ficoll-hypaque (Boyum, 1974). Neutrophils were either left unstimulated or stimulated with 10 ng/ml of phorbol-

myristate-acetate (PMA) for 1 hour, following which they were stained with a PE-conjugated CD120a (TNFRSF1A) antibody. Stained cells were analyzed by flow cytometry, gating on neutrophils. Following stimulation with PMA, membrane TNFRSF1A on the patient's neutrophils decreased to levels comparable to normal controls (Figure 3.3A), suggesting that activation-induced clearance of TNFRSF1A from the surface of neutrophils is not impaired. To establish whether this decrease in surface TNFRSF1A was as a result of internalisation or cleavage of the receptor, levels of soluble TNFRSF1A were measured in the supernatant of the cells by ELISA. The patient's soluble TNFRSF1A levels increased in response to PMA stimulation, indicating that the loss of surface TNFRSF1A was a result of receptor shedding (Figure 3.3B). The PMA-induced increase in soluble TNFRSF1A levels was similar to that seen with the control samples. As we wanted to compare our results to previously published reports that had demonstrated impaired receptor shedding (McDermott *et al.*, 1999a), these experiments were carried out using the same PMA dose and time points. PMA dose-response curves and time course experiments were not performed, and a kinetic abnormality in receptor shedding cannot therefore be excluded. However, unlike some other TRAPS mutations (McDermott *et al.*, 1999a; Galon *et al.*, 2000; Kriegel *et al.*, 2003), activation-induced cleavage in neutrophils is not impaired by the C43S mutation under the conditions tested, and does not appear to account for the inflammatory phenotype in this patient.

3.3 Functional characterization of the C43S TRAPS mutation

3.3.1 Generation of primary dermal fibroblast line from a patient with TRAPS

In light of the unimpaired TNFRSF1A shedding, we wanted to test our hypothesis that TNFα signalling was enhanced in TRAPS using samples from the patient with the C43S TRAPS mutation. While it was possible to perform some of the experiments using the patient's peripheral blood leucocytes, their short lifespan limits their usefulness for signalling experiments. To facilitate study of the functional effects of the C43S TNFRSF1A mutation, a primary dermal fibroblast cell line was therefore generated from the patient with TRAPS. The skin is an accessible site for obtaining fibroblasts and is also the site of the prototypic rash that characterises TRAPS. Fibroblasts are relatively long-

lived cells capable of producing inflammatory cytokines and chemokines in response to TNFα (Elias and Lentz, 1990; Brouty-Boye *et al.*, 2000; Parsonage *et al.*, 2003). In addition, fibroblasts have been shown to regulate the switch from acute to chronic inflammation by promoting the inappropriate survival and retention of leukocytes within inflamed tissue (Buckley *et al.*, 2001).

Permission was obtained from the local ethics committee for skin biopsies to be performed on the patient with TRAPS, as well as on healthy age- and sex-matched volunteers. Written, informed consent was obtained from all participants (see Appendix II). The biopsy was taken from an area of normal skin during an asymptomatic period. Primary dermal fibroblast lines were established using an adapted method of a previously described protocol (Borysiewicz *et al.*, 1983). Early passage fibroblasts were stored in liquid nitrogen for later use. The various fibroblast lines grew at similar rates and were maintained by the same methods. Equivalent, early passage numbers (passages 5-10) of the various fibroblast lines were used in all experiments. Figure 3.4 shows confocal microscopy images of TRAPS derived and normal fibroblasts, stained for TNFRSF1A. As TRAPS is an autosomal dominant condition, this cell line contains both normal TNFRSF1A and TNFRSF1A containing the C43S mutation, in addition to TNFRSF1B, reflecting the situation in the patient.

The dermal fibroblast lines were all found to have high levels of autofluorescence when analysed by flow cytometry. Several other groups, including one studying TRAPS (Huggins *et al.*, 2004), have previously reported that primary human dermal fibroblasts exhibit high levels of autofluorescence (Kahari *et al.*, 1988; Rattan, 1998). This autofluorescence varies among strains, but appears to be associated with lipofuscin accumulation (von Zglinicki *et al.*, 1995) and *in vitro* ageing of the primary fibroblasts (Rattan *et al.*, 1982; Poot *et al.*, 1985). In contrast, autofluorescence is negligible in virus-transformed cell lines (Rattan *et al.*, 1982). It appears that this autofluorescence can be minimised by using very early passage numbers (passages 1-2) but unfortunately these were not available to us. There were also technical issues in analysis of the fibroblasts by flow cytometry. As TNFRSF1A has several trypsin cleavage sites, there were concerns

about harvesting the fibroblast using trypsinisation. The fibroblasts were therefore harvested using a cell scraper, and despite repeated resuspension, there was still considerable clumping of cells and it was not possible to obtain a satisfactory single cell suspension for analysis by flow cytometry.

3.3.2 Effect of the C43S TNFRSF1A mutation on NF-κB activation in fibroblasts

NF-κB is the best studied TNFα-activated transcription factor and is critical for the induction of inflammation by TNFα (Locksley *et al.*, 2001; Aggarwal, 2003). NF-κB dysfunction has been implicated in a variety of inflammatory conditions including rheumatoid arthritis and inflammatory bowel disease (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). We hypothesised that, as the C43S mutation appeared to have no receptor shedding defect, the effects of this mutation could be due to enhanced TNFα-induced NF-κB activation. Activation of NF-κB was investigated using the dermal fibroblast lines established from the patient and healthy controls. Cells were stimulated with 10 ng/ml of TNFα for 1 hour, following which nuclear extracts were generated. NF-κB DNA-binding was measured by electrophoretic mobility shift assay using radiolabelled DNA corresponding to a specific NF-κB site. All the fibroblast lines were able to activate NF-κB in response to TNFα. However, the activation was consistently less in the fibroblasts from the patient with TRAPS compared with fibroblasts from normal controls (Figure 3.5A). This difference was seen both in unstimulated cells and cells stimulated with TNFα, with no evidence of constitutive activation of NF-κB in the TRAPS fibroblasts.

The electrophoresis mobility shift assay is established as a sensitive method for studying the DNA-binding properties of a protein (Carey and Smale, 2000), and NF-κB was first detected using electrophoretic mobility shift assay (Sen and Baltimore, 1986). The NF-κB consensus sequence used in this project has been validated previously (Lenardo and Baltimore, 1989; Brennan and O'Neill, 1995). However, there are several factors that influence band intensities (Fried, 1989), and while attempts were made to minimize the effect of these factors where possible, they can still impact on the

reproducibility of this system. Intensity of the DNA-protein complex bands was quantified by densitometry (Scion image software) and revealed that the mean reduction in the patient's TNFα-induced NF-κB activation compared to the controls was 65 ± 3 % over eight separate experiments (Figure 3.5B). However, there are limitations to this method of quantification, particularly as this was not calibrated to known standards, making comparison of bands on different gels unreliable. The densitometry quantification is shown here to illustrate the consistent difference between the control and TRAPS samples.

The standard dose of TNFα traditionally used in experiments is 10 ng/ml, although NF-κB can be activated by low picomolar concentrations of TNFα (Chan and Aggarwal, 1994; Grell *et al.*, 1998). The TRAPS phenotype could conceivably result from increased sensitivity of cells to TNFα. In order to test the hypothesis that C43S TRAPS fibroblasts are able to activate NF-κB at lower doses of TNFα than the normal controls, TNFα dose response experiments were performed. In contrast to the hypothesis, NF-κB activation was consistently lower in the TRAPS fibroblasts at all doses of TNFα tested (Figure 3.6A). The mean reduction in NF-κB activation in the C43S TRAPS fibroblasts was 60.2 ± 7.6% over the range of TNFα doses in five experiments. Time course studies were performed to determine whether the C43S mutation resulted in more prolonged activation of NF-κB. However, the duration of TNFα-induced NF-κB activation was significantly shorter in the C43S TRAPS fibroblasts compared to the control fibroblasts (Figure 3.6B). The above results indicate that contrary to the initial hypotheses, the C43S TRAPS mutation results in decreased NF-κB activation in dermal fibroblasts, at all doses of TNFα and time points tested, compared to control samples. There may also be differences in DNA binding at time zero which have not been characterised at this time but which we intend to study in future as these may imply the involvement of other DNA binding proteins.

NF-κB activation requires the degradation of IκB to allow NF-κB to translocate to the nucleus. TNFα-induced degradation of IκBα was investigated by SDS-PAGE western

blot analysis of fibroblast cytosolic extracts using an anti-IκBα antibody. TNFα results in marked reduction of IκBα in the cytosol of control fibroblasts (Figure 3.7). While IκBα also reduces in the TRAPS fibroblasts in response to TNFα, this reduction is consistently less than that seen in the control fibroblasts. The reduced degradation of IκBα in the TRAPS fibroblasts is consistent with the reduced NF-κB activation seen in these cells. To confirm that the differences in NF-κB activation were mediated by TNFα, the ability of IL-1 to activate NF-κB in the three fibroblast lines was also measured. All the fibroblast lines activated NF-κB in response to IL-1 stimulation (Figure 3.8). In fact, the activation in the TRAPS fibroblasts in response to IL-1 may be greater than that seen in the normal controls, raising the possibility of compensatory pro-inflammatory pathways. This is an interesting observation that we will be studying further in future.

The mammalian NF-κB family consists of five members, which combine as a variety of homo- or heterodimers that are associated with specific biological responses (Hayden and Ghosh, 2004). The classic form of NF-κB in activated cells is a heterodimer of the RelA and p50 subunits. In order to establish whether the composition of the NF-κB complexes in the various fibroblast lines differed, supershift assays were performed. TNFα-activated nuclear extracts were incubated with antibodies to the various subunits of NF-κB prior to incubation with the radiolabelled oligonucleotides probe. The data indicates that the complexes are very similar, with both the TRAPS and control fibroblasts activating an NF-κB complex containing p50 and RelA (p65) subunits (Figure 3.9).

3.3.3 Effect of the C43S TNFRSF1A mutation on AP-1 activation in fibroblasts

In addition to activating NF-κB, TNFα ligation to TNFRSF1A can also induce the activation of the transcription factor AP-1 (Locksley *et al.*, 2001; Aggarwal, 2003). As TRAPS mutations could enhance AP-1 activation, the effect of the C43S mutation on AP-1 activation was investigated. AP-1 activation was determined by electrophoretic mobility shift assay using nuclear extracts from the fibroblast lines incubated with radiolabelled DNA corresponding to a specific AP-1 site. As was the case with NF-κB activation, the

C43S TRAPS-derived fibroblasts had consistently less AP-1 activation, across a range of TNFα doses, compared to the fibroblasts from the healthy controls (Figure 3.10). The C43S TNFRSF1A mutation therefore results in decreased activation of both of the major TNFα-induced transcription factors, NF-κB and AP-1, in primary dermal fibroblasts.

TNFα-induced activation of AP-1 is regulated by members of the MAPK family (Section 1.3.2.2). We therefore investigated the effects of the TRAPS mutation on p38 MAPK and SAPK, as well as on phosphorylation of these kinases, in the dermal fibroblasts. Fibroblasts were either left unstimulated or stimulated with TNFα, and cell extracts were then generated as before. These extracts were then investigated by SDS-PAGE and immunoblotting using commercially available antibodies (Cell Signaling, NEB). However, for technical reasons, the quality of the immunoblot results was very poor and it was not possible to obtain any useful or consistent data from these experiments. The major limiting factors related to the sensitivity of the antibodies and the small amounts of protein that could be obtained from the primary dermal fibroblasts and loaded onto the gels.

3.3.4 Effect of the C43S TNFRSF1A mutation on the production of pro-inflammatory cytokines

While transcriptional activation is generally required for inflammatory gene expression, TNFα also controls the expression of these genes at a post-transcriptional level, by mechanisms such as the regulation of mRNA stability (Winzen *et al.*, 1999; Saklatvala *et al.*, 2003). We were particularly interested in the effects of the C43S TRAPS mutation on IL-6 and IL-8 production. The pro-inflammatory cytokine IL-6 is a potent pyrogen (Luheshi and Rothwell, 1996; Conti *et al.*, 2004), and high levels of serum amyloid A and CRP, surrogate markers for serum IL-6 (Ganapathi *et al.*, 1988) have been noted in our and other TRAPS patients (McDermott *et al.*, 1999a; Aganna *et al.*, 2003). TRAPS is also associated with high levels of circulating neutrophils (McDermott *et al.*, 1997; Galon *et al.*, 2000), so the chemokine IL-8, a potent neutrophil recruiting and activating factor (Mukaida, 2000; Pease and Sabroe, 2002) may be implicated. To determine whether the C43S TRAPS mutation altered TNFα-induced production of the

inflammatory cytokines IL-6 and IL-8, the levels of these cytokines in the culture supernatants of the primary dermal fibroblasts were measured by ELISA. While absolute values varied, the induction of IL-6 (Figure 3.11A) and IL-8 (Figure 3.11B) in response to TNFα in the C43S TRAPS fibroblast line was not statistically different to that observed in the controls. Baseline levels of the cytokines were also similar in the fibroblast lines. Thus, in spite of the reduced NF-κB and AP-1 activation, the C43S TRAPS fibroblast line was able to produce IL-6 and IL-8 in response to TNFα. However, while no differences in IL-6 and IL-8 production were seen at these time points, it is possible that time course or dose response experiments could identify more subtle phenotypic differences. The results were also not corrected for cell death, which further limits the interpretation of these data.

3.3.5 Effect of the C43S TNFRSF1A mutation on TNFα-induced apoptosis

Another important TNFRSF1A-mediated effect in cells is the induction of apoptosis. TNFα also induces anti-apoptotic genes and therefore the apoptotic response to TNFα is usually dependent on inhibition of protein synthesis (Polunovsky *et al.*, 1994; Rath and Aggarwal, 1999). To investigate the induction of apoptosis by TNFα, dermal fibroblasts were stimulated with TNF (10 ng/ml) either alone or in the presence of the protein synthesis inhibitor, cycloheximide (CHX) (Hess *et al.*, 1998). Three distinct assays for cell survival were used. Firstly, cells were examined by light microscopy after 24 and 48 hours. Dramatic differences were observed at both time points, with more TRAPS-derived fibroblasts surviving than wild type cells following stimulation with TNFα and CHX in combination (Figure 3.12). Cell death in the presence of CHX alone was similar in both fibroblast lines, while TNFα alone resulted in minimal cell death in either fibroblast line. These figures are illustrative only and the differences observed by light microscopy were quantified using an alamar blue assay (Nakayama *et al.*, 1997). This non-toxic dye is chemically reduced by the innate metabolic activity of cells, which allows quantification of cell viability by fluorometry. Figure 3.13 shows the effect of a range of doses of TNFα on the survival of C43S TRAPS fibroblasts and a control fibroblast line. The TRAPS derived fibroblasts were markedly less sensitive to TNFα-

induced cell death than the fibroblasts from the control subject. Comparing the curves by nonlinear regression analysis, using GraphPad Prism 4.0 software, indicates that the curves are statistically different ($p<0.05$). Even with the addition of high doses of TNFα (100 ng/ml) in the presence of CHX, over 80% of the C43S TRAPS derived fibroblasts remained viable after 24 hours, compared to approximately 30% of the control fibroblasts. Cell death following incubation with CHX alone was not statistically different between the fibroblast lines.

TNFα can induce apoptosis by more than one pathway. Both the extrinsic and intrinsic pathways of caspase activation converge on the activation of the common executioner caspase-3 (Stennicke and Salvesen, 2000). To confirm that the observed difference in cell viability was as a result of altered apoptosis, caspase-3 activity was assayed using a caspase-3 colorimetric assay (R&D Systems). Once activated, caspase-3 cleaves a variety of cellular molecules that contain the amino acid motif DEVD (Casciola-Rosen *et al.*, 1996), which forms the basis for this assay. The combination of TNFα and CHX induced significantly less (by Mann-Whitney U test) caspase-3 activity in the TRAPS fibroblasts than the control fibroblasts at all time points measured (Figure 3.14). However, these changes could represent differences in the time course of apoptosis in TRAPS as it is not known what happens at other time points.

As the effects of TNFα can vary in different cells (Rath and Aggarwal, 1999), we undertook to investigate whether the C43S TNFRSF1A mutation also results in decreased sensitivity to TNFα-induced apoptosis in circulating inflammatory cells, as was the case in the dermal fibroblasts. Peripheral blood mononuclear cells (PBMCs) from the patient with the C43S TRAPS mutation and from healthy volunteers were isolated by Ficoll-gradient. The PBMCs were stimulated with TNFα and CHX for either 2 or 6 hours. Cells were stained with annexin V and propidium iodide (PI), and analysed immediately by flow cytometry. Cells in the live gate (PI negative) were analysed for annexin V staining. Although similar percentages of unstimulated cells were positive for annexin V, following stimulation with TNFα and CHX, less PBMCs from the patient with TRAPS were annexin V positive at both time points (Figure 3.15-16). The difference observed at

6 hours was statistically significant ($p<0.05$) as calculated by the Mann-Whitney U test. The C43S TNFRSF1A mutation therefore results in decreased TNFα-induced apoptosis in both fibroblasts and PBMCs at the time points tested.

3.4. Discussion

This chapter describes a patient with a novel TNFRSF1A mutation (C43S) associated with TRAPS, and characterises the signalling abilities of this mutation in a primary dermal fibroblast line established from the patient. TNFα activates the transcription factors NF-κB and AP-1 at reduced levels in C43S TRAPS fibroblasts but is able to induce the pro-inflammatory cytokines IL-6 and IL-8 to levels similar to normal controls. TNFα-induced apoptosis is significantly decreased in the fibroblasts bearing the C43S TNFRSF1A mutation. This defect in TNFα-induced apoptosis is also seen in PBMCs isolated from the patient. Thus, these results demonstrate that this TRAPS mutation results in reduced TNFα-induced nuclear signalling and apoptosis in this patient.

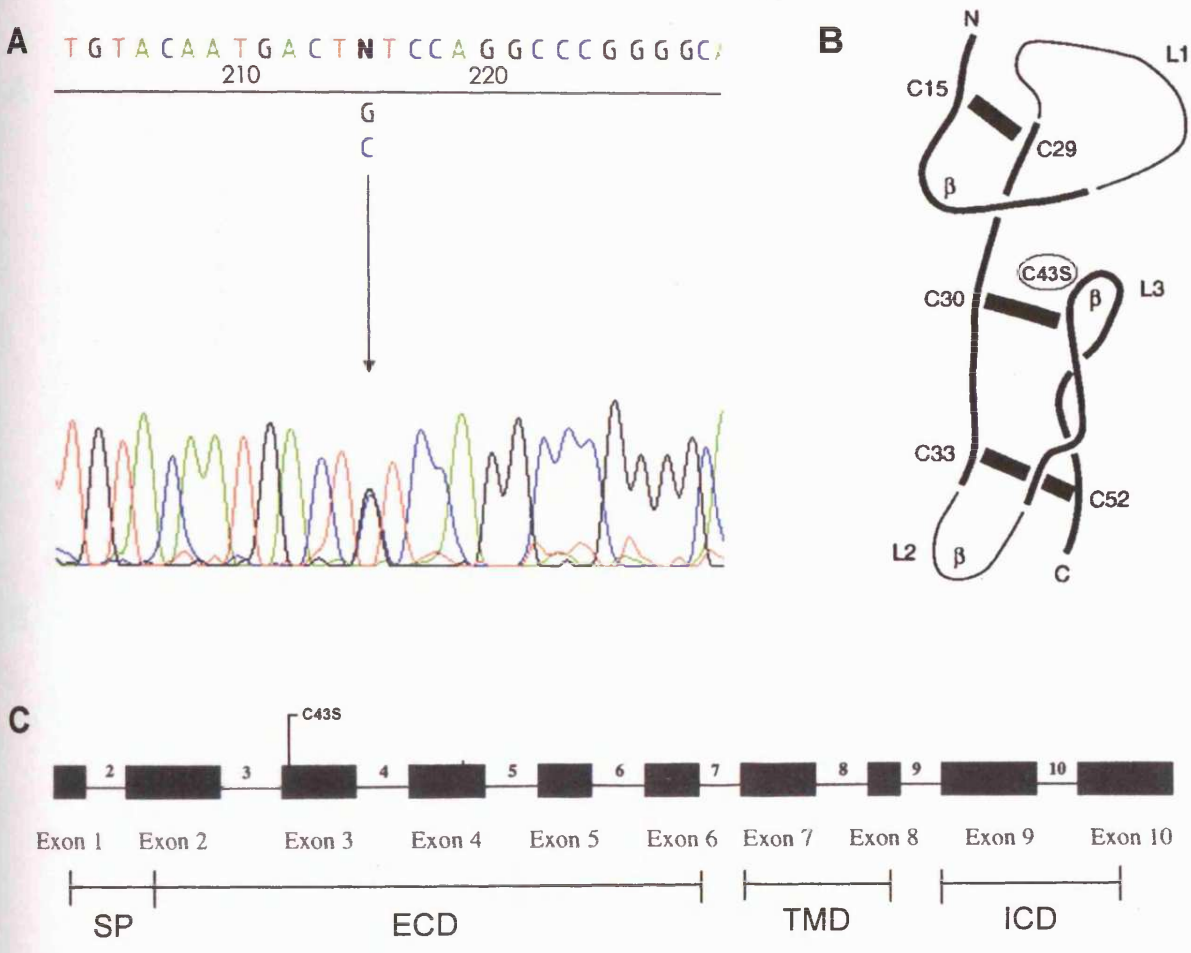
The initial studies of TRAPS cells proposed a mechanism of impaired activation-induced cleavage of TNFRSF1A that could cause the systemic inflammation associated with this syndrome (McDermott *et al.*, 1999a; Galon *et al.*, 2000). However, impaired shedding of TNFRSF1A does not appear to be the case for all TRAPS mutations (Aksentijevich *et al.*, 2001; Aganna *et al.*, 2003). Our results support the hypothesis that TRAPS does not appear to require impaired activation-induced shedding of TNFRSF1A from neutrophils, although a kinetic abnormality in receptor shedding remains a possibility.

Our data shows that the C43S TRAPS fibroblasts exhibit reduced NF-κB and AP-1 activation, and decreased apoptosis in response to stimulation with TNFα. However, no significant difference in the induction of pro-inflammatory cytokines was observed, similar to a previous report (McDermott *et al.*, 1999a). This suggests a possible hypothesis to explain the inflammatory pathology: cells survive longer, because of

impaired apoptosis, but remain capable of producing pro-inflammatory cytokines, therefore the levels of these cytokines would be expected to accumulate and could result in an inflammatory phenotype. Apoptosis of inflammatory cells is an important homeostatic mechanism for limiting an inflammatory response once it is established (Lenardo *et al.*, 1999). Our hypothesis is also compatible with the high levels of serum amyloid A and CRP, surrogate markers for serum IL-6, noted in our and other TRAPS patients (McDermott *et al.*, 1999a). This hypothesis is however based on results from a single patient, and it remains to be established whether this is also the case for other TRAPS patients.

As TNFα-induced activation of both IL-6 and IL-8 is mediated by NF-κB and AP-1, the question remains of how the induction of these pro-inflammatory cytokines is normal, despite the reduced transcription factor activation. We can propose several possible explanations. The first is that the threshold for TNFα-induced cytokine production is lower than that for apoptosis. Thus, reduced NF-κB still generates a sufficient signal to allow induction of both IL-6 and IL-8. A second explanation is that the C43S TRAPS mutation results in decreased gene expression but that the mRNA of these cytokines is more stable, preventing mRNA degradation and maintaining the cytokines at relatively normal levels. A further alternative explanation is that signalling via TNFRSF1B or reverse signalling through mTNFα may play a role. TNFRSF1B is able to activate NF-κB and AP-1, but does not contain a death domain (Baud and Karin, 2001), and thus could induce IL-6 and IL-8 without causing apoptosis. Soluble TNFα generally results in less activation of TNFRSF1B than TNFRSF1A *in vitro* (Grell *et al.*, 1995), which may explain the reduced NF-κB activation observed in our experiments. Reverse signalling through mTNFα has been shown to be pro-apoptotic in some cells types (Lugering *et al.*, 2001; Waetzig *et al.*, 2003) and anti-apoptotic in others (Papadaki *et al.*, 2002; Zeissig *et al.*, 2004). It is however unknown whether reverse signalling occurs in fibroblasts. It will be important to determine whether other TRAPS mutations have similarly reduced TNFα nuclear signalling and apoptosis, as is the case with the C43S mutation.

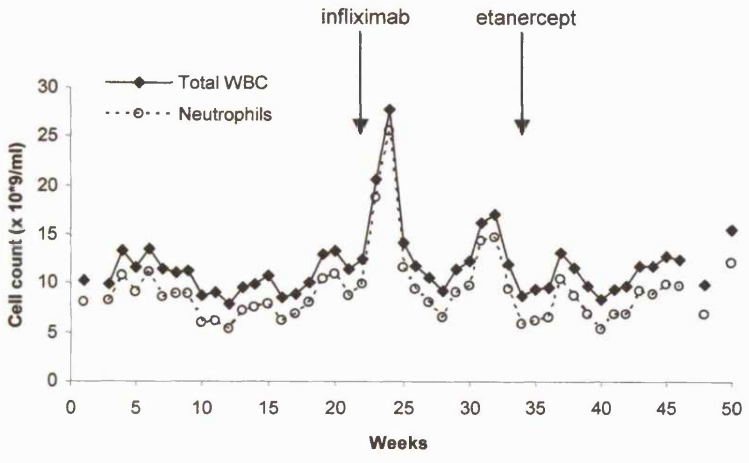
Figure 3.1
Identification of the C43S TNFRSF1A mutation



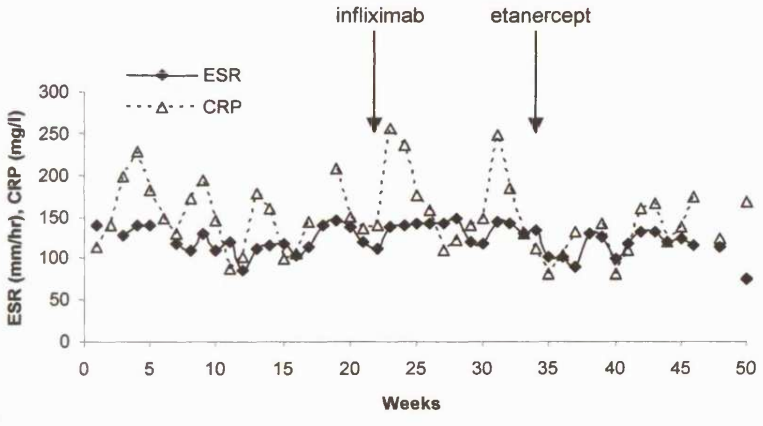
(A) DNA sequence electropherogram demonstrating the C43S TNFRSF1A mutation. The heterozygous G→C transition at nucleotide 215 is indicated by the arrow. **(B)** Crystallographically determined structure of TNFRSF1A extracellular cysteine rich domain 1 (CRD1). The position of the C43S mutation (circled) and the other cysteine residues that form the disulphide bonds that characterise CRD1 are shown. The three disulphide bonds are depicted by thick black bars. Thicker lines represent structurally conserved regions of CRD; "β" indicates the β-turn positions; "L1"-"L3" denote loop domains. Adapted from Aksentijevich et al (2001). **(C)** Genomic structure of *TNFRSF1A* indicating the position of the C43S mutation in exon 3, which encodes for TNFRSF1A extracellular cysteine rich domain 1 (CRD1). The TNFRSF1A structures encoded by various portions of the *TNFRSF1A* gene are indicated at the bottom of the figure. Abbreviations: SP signal peptide, ECD Extracellular domain; TMD Transmembrane domain; ICD Intracellular domain. Adapted from Fuchs et al (1992)

Figure 3.2
Serial laboratory results for the index patient with C43S TRAPS mutation

A

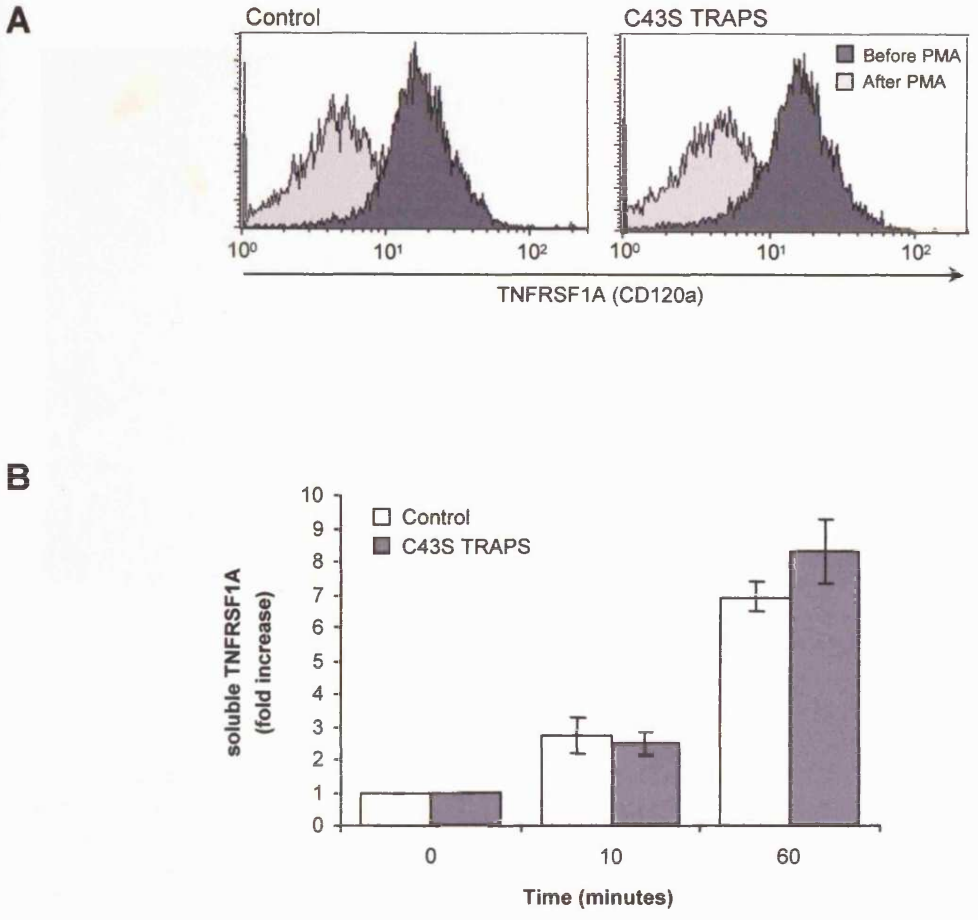


B



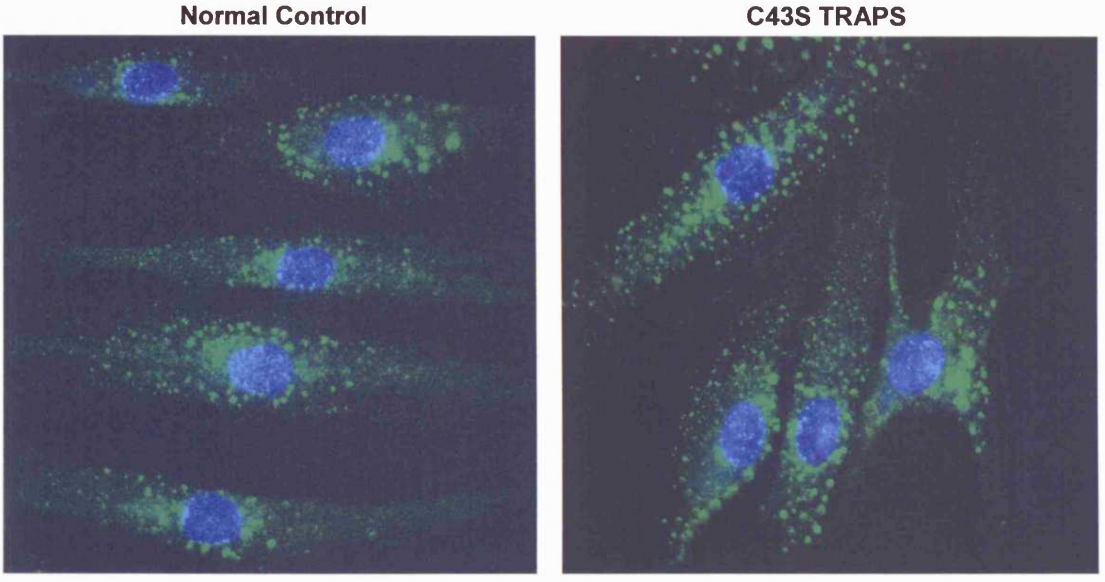
Serial **(A)** total white blood cell (WBC) and neutrophil counts, **(B)** erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels for the index patient over a 50 week period. Labelled arrows indicate points where treatment with anti-TNFα agents was started.

Figure 3.3
Activation-induced shedding of TNFRSF1A from neutrophils



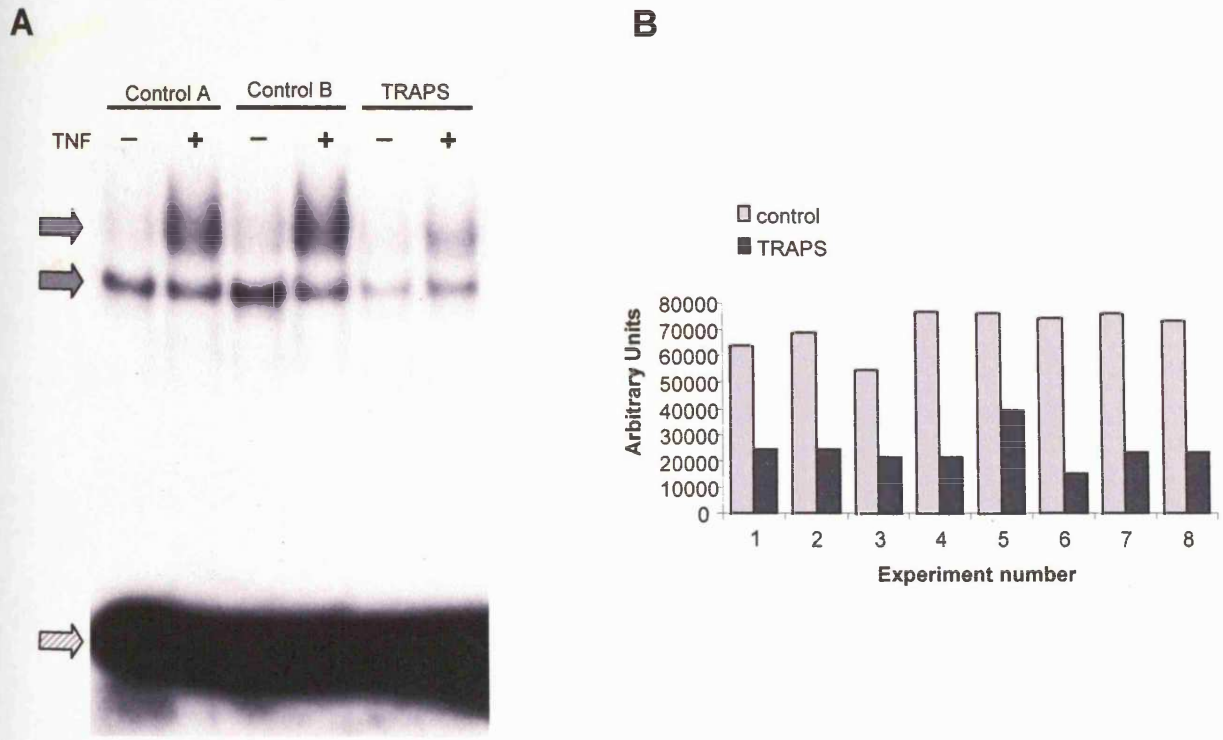
(A) Membrane expression of TNFRSF1A (CD120a) by neutrophils from a healthy volunteer and the C43S TRAPS patient before (dark grey) and after (light grey) stimulation with phorbol-myristate-acetate (PMA). Cells were stained with PE-conjugated anti-CD120a and analysed by flow cytometry, gating on neutrophils. **(B)** Following PMA stimulation, supernatants were collected and soluble TNFRSF1A levels measured by ELISA. Values are the mean ± SEM changes in soluble TNFRSF1A levels relative to baseline levels from three independent experiments. Mean ± SEM absolute values at time 0 were 53.8 ± 0.9 pg/ml in the control subject and 19.1 ± 3.9 pg/ml in the TRAPS patient.

Figure 3.4
Immunofluorescence of dermal fibroblasts



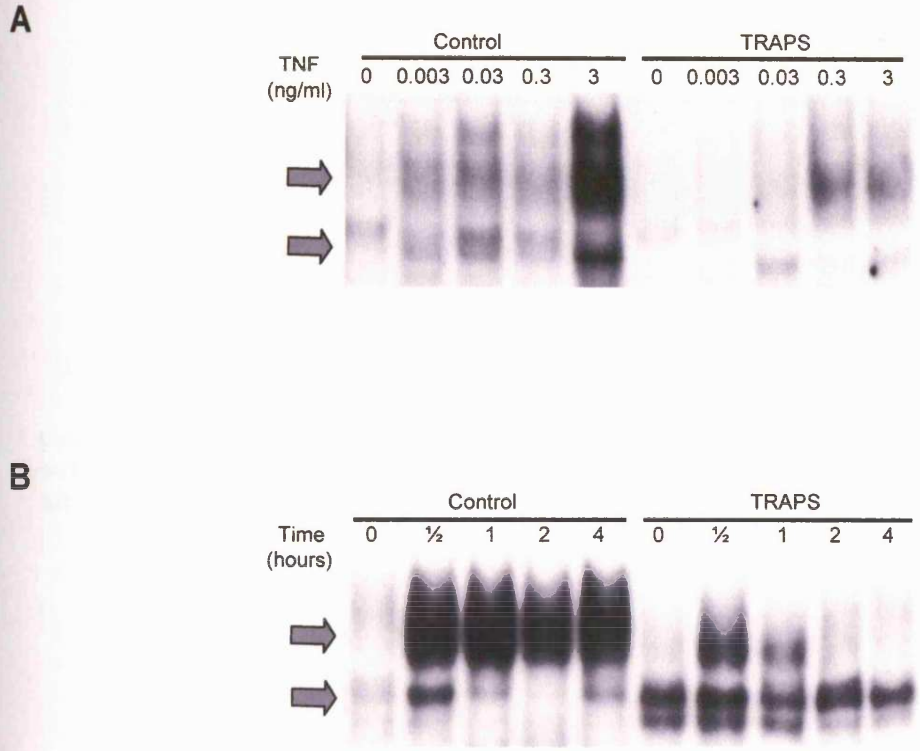
Primary dermal fibroblast cell lines generated from the patient with the C43S mutation (right) and age- and sex-matched healthy volunteers (left). Cells were stained with mouse anti-human TNFRSF1A and then with a secondary Alexa Fluor 488 goat anti-mouse antibody (green). Nuclei were stained with DRAQ5 (blue). Single optical slice images were acquired using a confocal laser scanning microscope (BioRad Microsciences), equipped with a krypton/argon ion laser and attached to a Zeiss Axiovert 135. Images shown are representative of three experiments.

Figure 3.5
TNFα-induced NF-κB activation in dermal fibroblasts



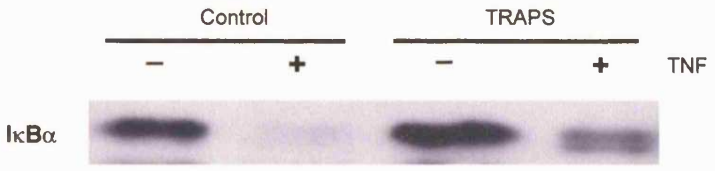
(A) Electrophoretic mobility shift assay (EMSA) showing NF-κB activation in fibroblasts from 2 controls and the patient with C43S TRAPS, stimulated for 1 hour with TNFα (10 ng/ml). DNA-protein complexes are indicated by the grey arrows. Free (unbound) radiolabelled DNA was distinct from these complexes and is indicated by the hatched arrow. The result shown is representative of eight experiments. **(B)** Densitometry of TNFα-stimulated cells from eight experiments. Densitromtric analysis of the DNA-protein complex bands, performed using Scion Image (NIH Image) and expressed as arbitrary units, revealed a mean ± SEM reduction of 65.6 ± 2.8 % in the TRAPS fibroblasts compared with the controls over 8 separate experiments.

Figure 3.6
Dose response and time course studies of NF-κB activation in dermal fibroblasts



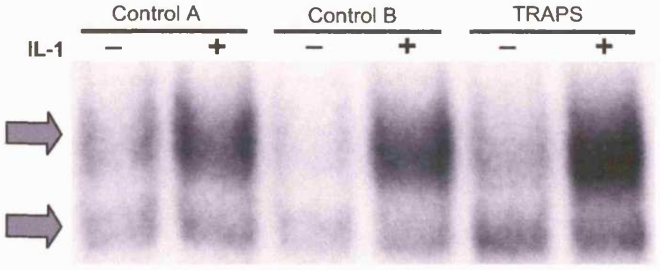
(A) EMSA of nuclear extracts showing TNFα dose response, at 1 hour, for NF-κB activation in control and C43S TRAPS fibroblasts. Densitometric analysis revealed a 60.2 ± 7.6 % reduction in NF-κB activation in the TRAPS fibroblasts over a range of TNFα doses in 5 separate experiments. **(B)** EMSA showing the time course of NF-κB activation after stimulation of fibroblasts with TNFα (10 ng/ml). DNA-protein complexes are indicated by the arrows. Free (unbound) radiolabelled DNA was distinct from these complexes and is not shown in this or any of the subsequent figures. EMSA results shown here are representative of at least 5 separate experiments, and were similar in both controls.

Figure 3.7
TNFα-induced IκBα degradation in fibroblasts



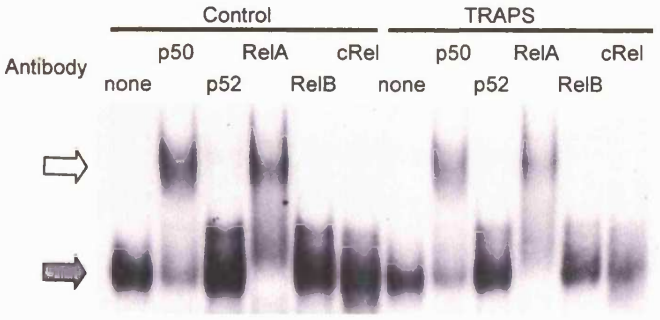
Cytosolic extracts of fibroblasts, stimulated with TNFα (10 ng/ml) for 1 hour, were separated on an 8% polyacrylamide gel and transferred onto a PVDF membrane. Western blot analysis was performed using anti-IκBα antibody. The result shown is representative of three independent experiments.

Figure 3.8
IL-1-induced NF-κB activation in dermal fibroblasts



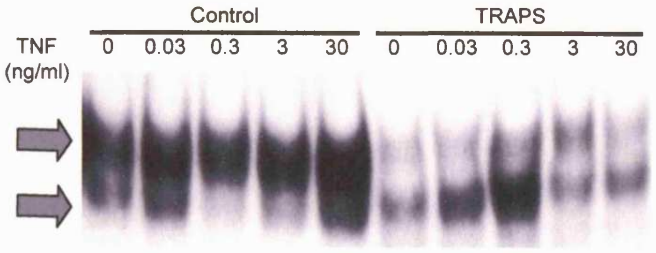
EMSA showing NF-κB activation in dermal fibroblasts stimulated with IL-1 (5 ng/ml) for 1 hour. DNA-protein complexes are indicated by the arrows. Free (unbound) radiolabelled DNA was distinct from these complexes and is not shown. The result shown is representative of three independent experiments.

Figure 3.9
NF-κB supershift assay in dermal fibroblasts



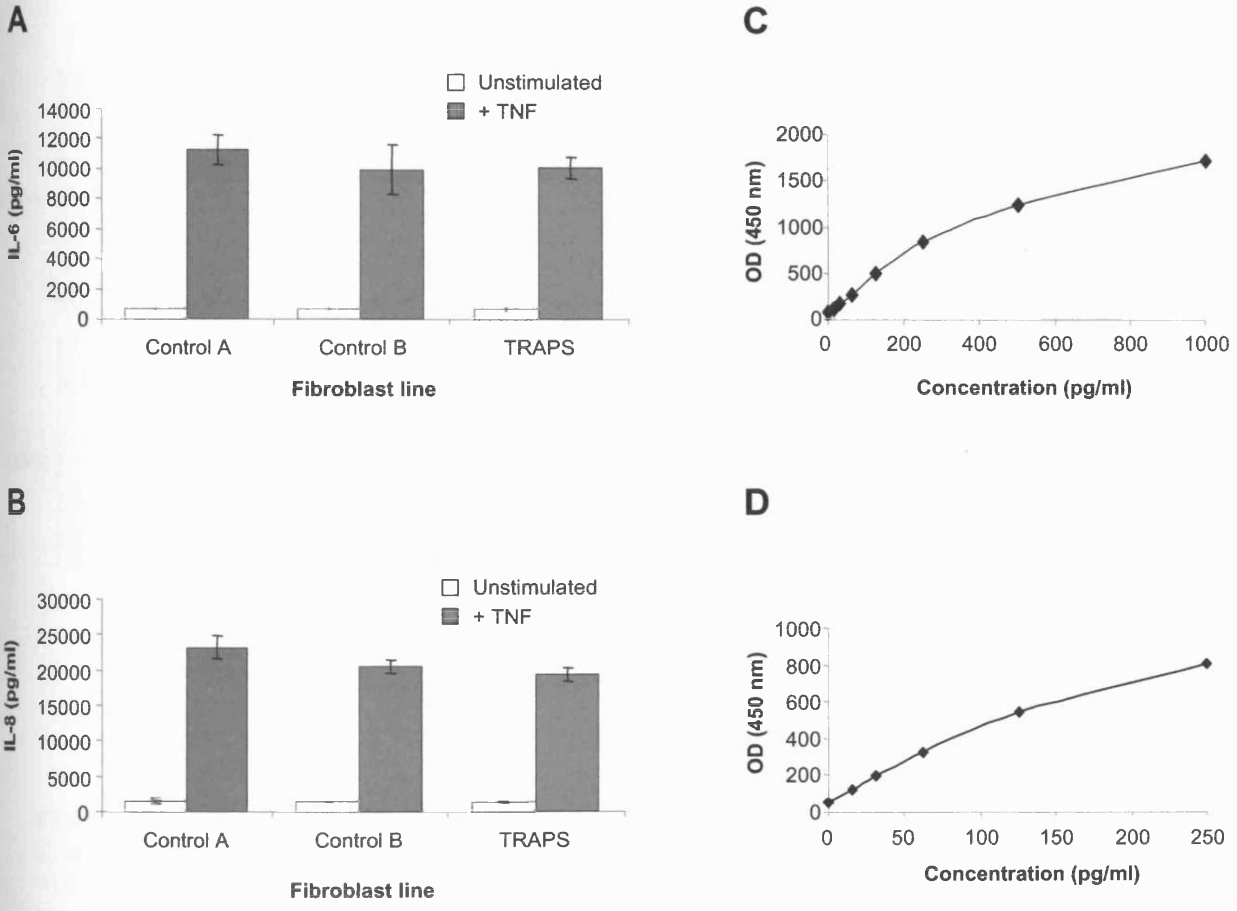
TNFα-activated nuclear extracts were incubated with antibodies to the various subunits of NF-κB prior to incubation with the radio-labelled oligonucleotide probe. DNA-protein complexes (solid arrow) and antibody-DNA-protein complexes (open arrow) are indicated. The result shown is representative of three independent experiments.

Figure 3.10
AP-1 activation in dermal fibroblasts



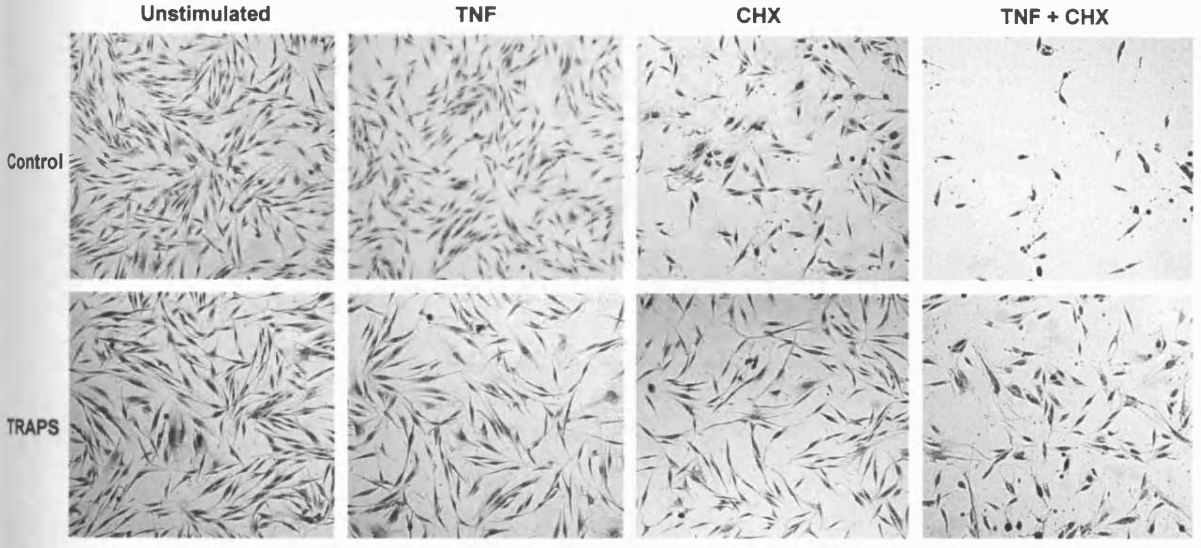
EMSA showing AP-1 activation in fibroblasts from a control and the patient with C43S TRAPS, stimulated for 1 hour with a range of TNFα doses. DNA-protein complexes are indicated by the arrows. Free (unbound) radiolabelled DNA was distinct from these complexes and is not shown. The result shown is representative of three independent experiments.

Figure 3.11
TNFα-induced IL-6 and IL-8 production by dermal fibroblasts



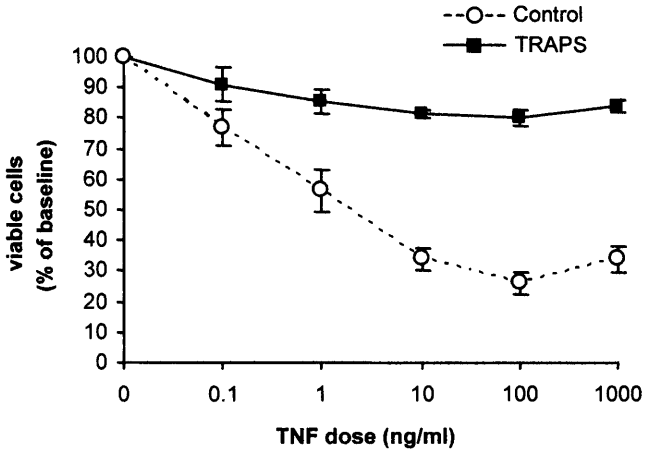
(A) Production of interleukin-6 (IL-6) and **(B)** production of interleukin-8 (IL-8) by the primary dermal fibroblasts, as measured by ELISA of the culture supernatants 24 hours after stimulation with TNFα (10 ng/ml). ELISA results are the mean ± SEM of 9 observations from 3 independent experiments for IL-6 and 6 observations from 2 independent experiments for IL-8. The standard curves for **(C)** IL-6 and **(D)** IL-8 are shown. Samples were assayed over several dilutions and the concentration determined from those dilutions that fell in the linear portion of the standard curve.

Figure 3.12
Assessment of cell viability of fibroblasts by light microscopy



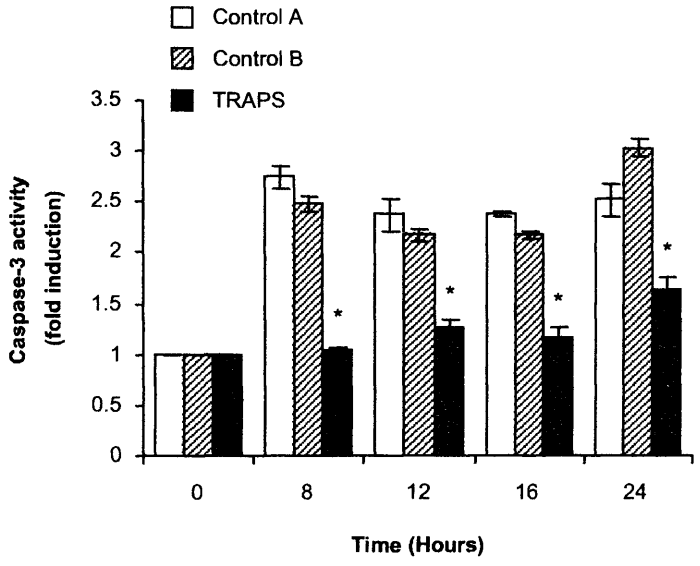
Fibroblasts (3 x 10^4) were stimulated with TNFα (10 ng/ml) and cycloheximide (CHX; 50 μg/ml), either alone or in combination. Light microscopy images of fibroblasts after 24 hours of stimulation, acquired with Olympus BX41 microscope using the 4x objective. Cells were stained with toluidine blue to aid visualization. Results shown are representative of 5 separate experiments.

Figure 3.13
Cell viability of dermal fibroblasts



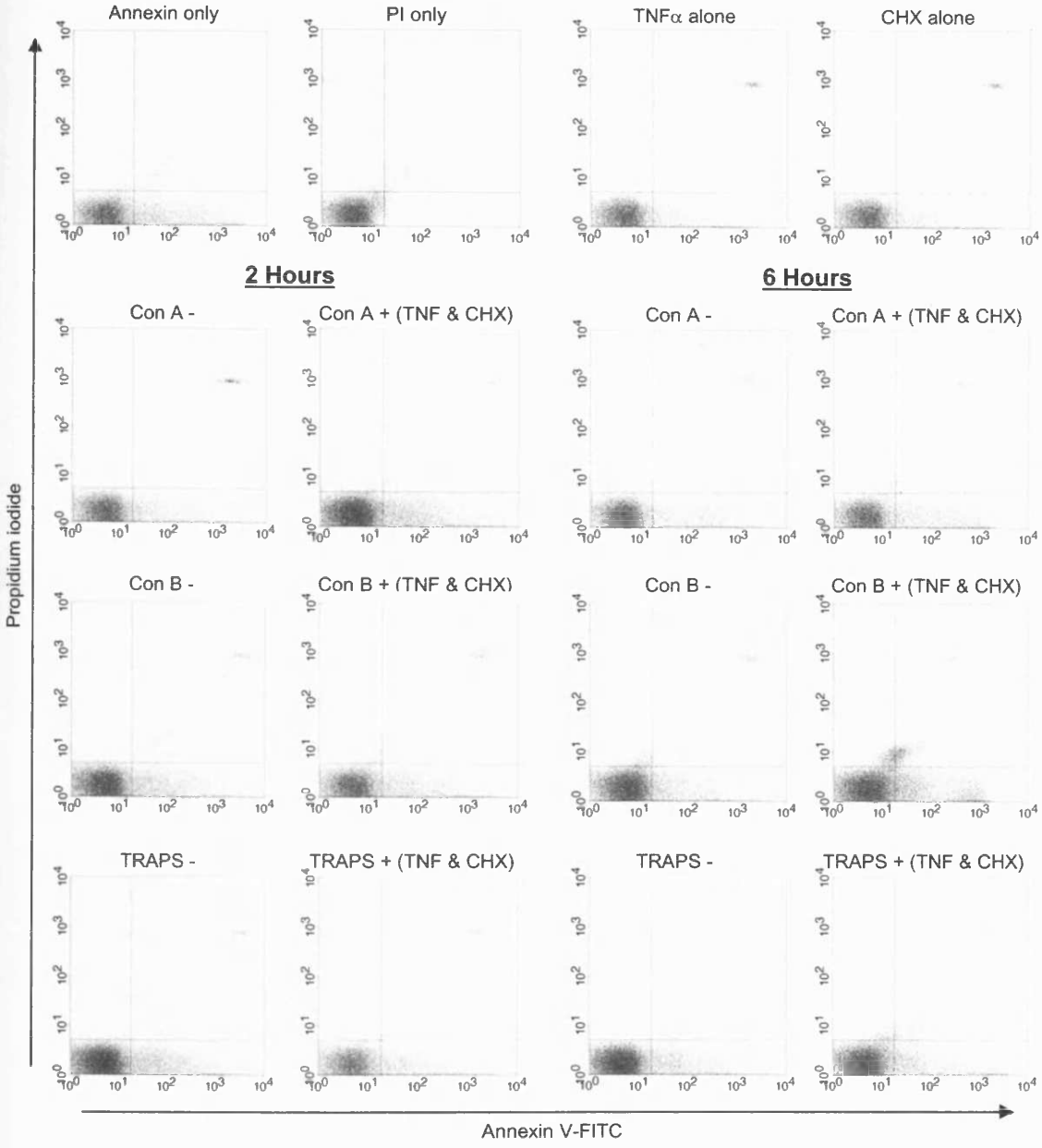
Cell viability of C43S TRAPS and normal control fibroblasts after 24 hours incubation with varying doses of TNFα in the presence of cycloheximide (CHX), as measured by alamar blue assay. Values are the mean ± SEM percentage of baseline levels (CHX alone) from five independent experiments.

Figure 3.14
TNFα-induced apoptosis of fibroblasts



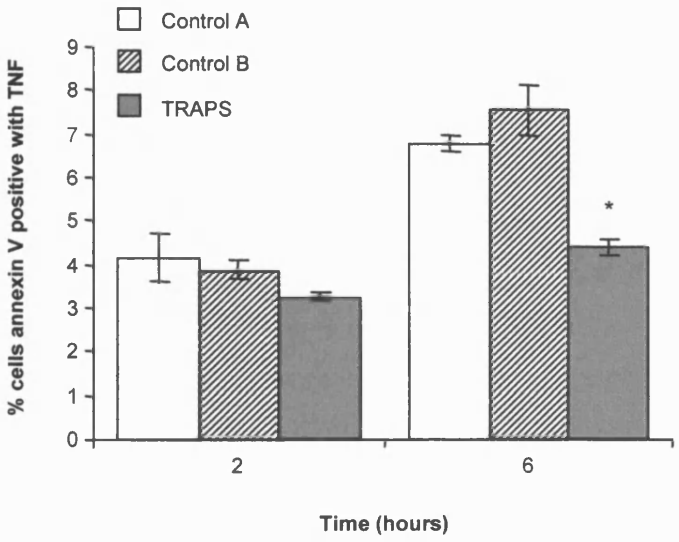
Fibroblasts (5 x 10^5) were stimulated with TNFα (10 ng/ml) and cycloheximide (50 μg/ml), after which they were analysed for caspase-3 activity at the time points shown. Caspase-3 activity was tested using a capspase-3 colourimetric assay (R&D Systems). The level of caspase enzymatic activity in the cell lysate is directly proportional to the colour reaction at a wavelength of 405 nm. Values are the mean ± SEM of 5 independent experiments. * = $P < 0.05$ versus controls, by Mann-Whitney U test.

Figure 3.15
TNFα-induced annexin V staining of PBMCs



Peripheral blood mononuclear cells (PBMCs) from the patient and 2 healthy volunteers (Con A & B) were incubated with TNFα and cycloheximide (CHX) for the times indicated. Cells were stained with Annexin V-FITC and propidium iodide, and analysed by flow cytometry, excluding cell debris by gating. Results are shown as dot plots of propidium iodide fluorescence against annexin V-FITC fluorescence, and are representative of two independent experiments performed in duplicate.

Figure 3.16
TNFα-induced apoptosis of PBMCs



Peripheral blood mononuclear cells (PBMCs) from the patient and 2 healthy volunteers were incubated with TNFα and cycloheximide (CHX) for the times indicated. Annexin V activity was determined by flow cytometry, gating on live (propidium iodide-negative) cells. Results shown are the difference between the mean in cells stimulated with TNFα and CHX and the mean in unstimulated cells. Values are the mean ± SEM of 2 independent experiments performed in duplicate. * = $P < 0.05$ versus controls, by Mann-Whitney U test.

CHAPTER 4

THE CLONING AND FUNCTIONAL CHARACTERISATION OF MUTANT RECOMBINANT FORMS OF TNFRSF1A

The work using the patient-derived dermal fibroblast line (Chapter 3) indicates that the C43S TRAPS mutation results in decreased NF-κB production and TNFα-induced apoptosis, although IL-6 and IL-8 production is normal. The work in this chapter was initiated to investigate whether other TRAPS mutations also resulted in reduced TNFRSF1A signalling, suggesting this is a general feature of TRAPS, or whether this is specific to the C43S TRAPS mutation. As TRAPS has a dominant inheritance pattern, cells from patients will contain both mutated and wild-type TNFRSF1A. In order to study the effects of the mutated TRAPS receptors in isolation, a number of clinically relevant recombinant forms of TNFRSF1A were generated. These were then transfected into Eli-BL and DG75 cells in order to investigate their effects on receptor function, in terms of NF-κB activation and cell death, and on receptor expression.

4.1 Cloning of recombinant TNFRSF1A

4.1.1 Strategy for cloning recombinant full-length and truncated TNFRSF1A

A schematic of the cloning strategy for full-length wild-type (WT) TNFRSF1A is shown in Figure 4.1. The same cloning strategy was used for the truncated TNFRSF1A form (WTΔ218). The vector p-TARGET-TNFR1 WT (provided by Dr Michael Lenardo, National Institute of Health, Bethesda, USA (Chan *et al.*, 2000a)) was previously used by Dr Fielding to amplify the entire human TNFRSF1A coding region and shorter sequences. This purified PCR product was then directly cloned into the pcDNA3.1/V5-His directional TOPO cloning vector using the TOPO-TA cloning kit (Invitrogen) (Fielding *et al.*, 2004). Full-length WT TNFRSF1A and the truncated form WTΔ218, lacking the intracytoplasmic domain of the receptor, were generated using a common forward primer with different reverse primers. As it had not proved possible to detect expression using the V5 antibody, the TNFRSF1A coding region was cloned into the

pcDNA3.1/myc-His B vector (Invitrogen). Essentially, the full-length and truncated TNFRSF1A coding regions were released from the pcDNA3.1/V5-His cloning vector by restriction digestion with *BamH I* and *Xba I* (Figure 4.2). The expected size of the excised TNFRSF1A coding regions is approximately 1400 bp for full-length WT TNFRSF1A and 800 bp for WTΔ218, while the vector is approximately 5500 bp. The TNFRSF1A-containing regions were then extracted from the agarose gel using the QIAquick Gel Extraction and Purification Kit (Quiagen). In parallel, the pcDNA3.1/myc-His vector was restriction digested with *BamH I* and *Xba I,* treated with alkaline phosphatase and then purified using the QIAquick PCR Purification kit (Quiagen). Controls in the restriction digest process included single-digests of pcDNA3.1/myc-His (with either *BamH I* or *Xba I)* and uncut vector. The approximate amounts of pcDNA3.1/myc-His vector and excised TNFRSF1A were calculated using Hyperladder I DNA standards (Bioline). Ligation of appropriate amounts of insert and vector was then performed. Controls included vector or insert alone, and samples without ligase. The products were then transformed into One Shot Top10 competent cells (Invitrogen) by "heat shock". As the plasmid contains an ampicillin resistance cassette, the cells were plated overnight on growth media containing ampicillin. Clones obtained from the ligation reaction were amplified by PCR and then screened by digestion with *BamH I* and *Xba I* in combination, to release the TNFRSF1A gene inserts if present, or with *Hind III* alone. The cloned TNFRSF1A constructs were sequenced with the ABI Big Dye sequencing kit (Perkins-Elmer Applied Biosystems) using various primers for the pcDNA3.1 vector and the TNFRSF1A gene, to give the complete overlapping nucleotide sequences of the entire TNFRSF1A coding region. This was then checked against the published sequence for TNFRSF1A.

4.1.2 Generation of mutant recombinant forms of TNFRSF1A

Clinically relevant TRAPS mutants were generated from WT TNFRSF1A, using the Quikchange site-directed mutagenesis kit (Stratagene) to alter a single nucleotide base. The following TRAPS mutations were generated: C30R, C43S, T50M and C52F. The numbering of amino acids for TNFRSF1A has traditionally started after the leader sequence (i.e. leucine at residue 30) rather than following the general rule of starting with the translation initiator methionine. The C43S mutation is the TRAPS mutation present in

the patient detailed in Chapter 3, which was identified and described by our group. The other three mutations have all previously been reported in patients with TRAPS (McDermott *et al.*, 1999a). The location of these four mutants, which are all situated in CRD1 of TNFRSF1A, is shown in Figure 4.3. The C43S and C30R mutations share a disulphide bond, which these cysteine mutations would be predicted to disrupt. C52F, a cysteine mutation involving a different disulphide bond in CRD1, was the first TRAPS mutation shown to have diminished receptor shedding (McDermott *et al.*, 1999a). As there may be differences between cysteine and non-cysteine mutations, a non-cysteine mutation in close proximity, T50M, was chosen to complete the set of mutants for investigation. This latter mutation has been shown to impair TNFRSF1A shedding from leucocytes (Aksentijevich *et al.*, 2001), and would be predicted to disrupt hydrogen-bond stability.

The clones obtained from the site-directed mutagenesis were screened by restriction digestion with either *BamH I* and *Xba I*, or *Hind III*. Digestion with *BamH I* and *Xba I* released the TNFRSF1A coding regions, resulting in bands of approximately 1400 bp (TNFRSF1A insert) and 5500 bp (pcDNA3.1/myc-His vector) (Figure 4.4A). The presence of TNFRSF1A introduces a second *Hind III* restriction site, resulting in bands of approximately 6100 bp and 800 bp following digestion with *Hind III* (Figure 4.4B). In addition, the C52F mutation removes a *Pst I* restriction site present in WT TNFRSF1A. The cloned TNFRSF1A genes were all sequenced with the ABI Big Dye sequencing kit (Perkins-Elmer Applied Biosystems), using various primers for the pcDNA3.1 vector and the TNFRSF1A gene, to ensure that only the desired mutation had been introduced. Figure 4.5 shows the alignment of the relevant nucleotide and amino acid sequences of the recombinant forms of TNFRSF1A, confirming the desired mutations. Maps for the recombinant TNFRFS1A vectors are shown in Appendix I.

4.2 Effects of the recombinant forms of TNFRSF1A on NF-κB activation

NF-κB is the most studied TNFα-activated transcription factor and is critical for the induction of inflammation by TNFα (Baud and Karin, 2001; Locksley *et al.*, 2001). As

differences in NF-κB activation could conceivably account for the inflammatory phenotype of TRAPS, the effects of the TRAPS mutants on NF-κB activation were studied. The work in Chapter 3 indicates that the C43S TRAPS mutation results in reduced NF-κB activation in patient-derived dermal fibroblasts. In order to establish whether this is also a feature of other TRAPS mutants, NF-κB activation by WT and mutant recombinant forms of TNFRSF1A was assessed by luciferase assay in transiently transfected cells.

4.2.1 Determining the appropriate cells in which to study NF-κB activation

TNFRSF1A is widely expressed on the surface of most cells, and it is currently not known which cells are responsible for the TRAPS phenotype. In order to detect alterations in NF-κB activity as a result of transient transfection with recombinant TNFRSF1A, the transfected cells should have low endogenous responses to TNFα. The NF-κB activity of following cell lines was therefore tested: K562 (chronic myeloid leukaemia cell line), Jurkat (T-cell leukaemia line), Eli-BL and DG75 (B-cell Burkitt's lymphoma cell lines). The cells were transiently co-transfected with an NF-κB luciferase reporter plasmid (3EnhLuc), and either empty mammalian vector or a mammalian expression vector for WT TNFRSF1A. The samples were then split into two, and 16 hours after transfection, one half was mock stimulated and the other was stimulated with TNFα (10 ng/ml). After 8 h of incubation, the cells were harvested and luciferase activity was assayed. The Eli-BL cell line had a low level of endogenous NF-κB activity that did not increase significantly following stimulation with TNFα (Figure 4.6A). Following transfection with recombinant WT TNFRSF1A, there was a detectable increase in NF-κB activity of Eli-BL cells above baseline levels, which increased further in response to stimulation with TNFα. In contrast, the K562 and Jurkat cell lines transfected with empty vector were able to respond to TNFα, with little or no additional increase in NF-κB activity following overexpression of WT TNFSRSF1A (Figure 4.6C&D). The DG75 cell line had a high level of constitutive endogenous NF-κB activity that did not increase significantly following stimulation with TNFα (Figure 4.6B). We and others have also previously shown that basal TNFα production by Eli-BL and DG75 cells is <20 pg/ml

(Gibbons *et al.*, 1994; Fielding *et al.*, 2004). As the Eli-BL cell line allowed detection of changes in NF-κB activity following transfection with WT TNFRSF1A, and had low levels of basal TNFα production, this cell line was used in all subsequent experiments investigating NF-κB activation.

4.2.2 Determining optimal NF-κB luciferase reporter dose

The optimal dose of the 3EnhLuc reporter plasmid capable of demonstrating the TNFα-induced increase in NF-κB activity in Eli-BL cells had to be determined. Eli-BL cells were co-transfected with a fixed dose of WT TNFRSF1A and a range of doses of the 3EnhLuc reporter plasmid. The samples were split in two, either mock stimulated or stimulated with TNFα, and luciferase activity was assayed as before. Good separation between the NF-κB activity of mock and TNFα stimulated cells occurred when the Eli-BL cells were transfected with 3 μg of the 3EnhLuc reporter plasmid (Figure 4.7). This dose of the 3EnhLuc plasmid was therefore used as NF-κB luciferase reporter for all subsequent experiments.

4.2.3 NF-κB activation by mutant recombinant forms of TNFRSF1A

The effect of WT and mutant recombinant forms of TNFRSF1A on NF-κB activation was assessed by luciferase reporter assay in transiently transfected Eli-BL cells. Eli-BL cells were co-transfected with the NF-κB luciferase reporter plasmid and a range of doses mammalian expression vectors for either WT or mutant TNFRSF1A. The total amount of DNA was kept constant by the addition of appropriate amounts of empty pcDNA3.1 vector. Following transfection, the samples were split into two, and 16 h later one half was mock stimulated and the other stimulated with TNFα (10 ng/ml). After 8 h of TNFα treatment, the cells were harvested and assayed for luciferase activity. Overexpression of TNFRSF1A resulted in increased NF-κB activity, which was dependent on the amount of DNA transfected (Figure 4.8A). The NF-κB activation curve for the plasmid expressing WT TNFRSF1A was consistently, and significantly (by nonlinear regression analysis), higher than the curves for any of the four TRAPS mutants. In addition, there appear to be differences in NF-κB activity between these TRAPS

mutants themselves, with the C30R mutation in particular displaying minimal NF-κB activation above baseline levels. When the cells were stimulated with TNFα, the differences in NF-κB activity between WT and the TRAPS mutants became more pronounced and remained statistically significant by nonlinear regression analysis (Figure 4.8B).

To investigate whether the TRAPS mutations had a different dose response curve, WT and two of the TRAPS mutants were stimulated with a range of TNFα concentrations. Eli-BL cells were co-transfected with the NF-κB luciferase reporter and either empty pcDNA3.1 vector, WT or mutant TNFRSF1A. 16 h post-transfection, the cells were stimulated with a range of TNFα doses for 8 h and then assayed for luciferase activity. Reduced NF-κB activity was seen with the two TRAPS mutants over the full range of TNFα doses (Figure 4.9A). Specifically, there was no evidence that NF-κB was activated at lower doses of TNFα by the TRAPS mutants than WT TNFRSF1A. Nonlinear regression analysis shows the NF-κB activation curve for the plasmid expressing WT TNFRSF1A was significantly higher than the curves for either of the TRAPS mutants. However, when the luciferase assay is normalised for unstimulated TNFα and expressed as fold-induction with TNFα (Figure 4.9B), comparing the curves by nonlinear regression analysis shows that the C30R and WT TNFRSF1A curves are no longer statistically different. The WT TNFRSF1A and C43S curves remain statistically different by nonlinear regression. This suggests that, unlike C43S, the C30R mutant remains capable of responding to TNFα to a similar extent to WT TNFRSF1A, despite having lower NF-κB luciferase activity than C43S. This supports the impression that there may be differences between the various TRAPS mutants themselves and may account for the reported heterogeneity of the TRAPS phenotype (Aganna *et al.*, 2003).

Differences in transfection efficiency of the recombinant forms of TNFRSF1A could alter the functional effects of the constructs. Therefore, transfection efficiency of the expression vectors was assessed by dual luciferase assays. Eli-BL cells were transfected with the NF-κB luciferase reporter expressing firefly luciferase, and an SV40

promoter driving *Renilla* luciferase, in addition to the TNFRSF1A constructs or empty pcDNA3.1 vector. Firefly and *Renilla* luciferase activities were measured after 8 h of stimulation with TNFα. Figure 4.10 shows data with normalised NF-κB activity for WT TNFRSF1A and two TRAPS mutants. Both TRAPS mutants show reduced NF-κB activity even when normalised for transfection efficiency.

4.2.4 Effect of the TRAPS mutants on cell death

As overexpression of TNFRSF1A itself can result in spontaneous cell death (Boldin *et al.*, 1995a; Todd *et al.*, 2004), cell viability assays were performed on the transfected cells used for the luciferase assays. Alamar blue, a non-toxic dye that is chemically reduced by the innate metabolic activity of cells, was used to quantify cell viability by fluorometry (Nakayama *et al.*, 1997). Cell viability was consistently lower in the cells transfected with WT TNFRSF1A than those transfected with the mutant recombinant forms of TNFRSF1A (Figure 4.11A). The degree of cell death was dependent on the amount of WT TNFRSF1A DNA transfected but was independent of the TNFα dose (Figure 4.11B).The differences in cell viability curves between WT TNFRSF1A and the TRAPS mutants were significant by nonlinear regression analysis. The decreased cell death seen with the mutant recombinant forms of TNFRSF1A demonstrates that the reduced NF-κB activity of these mutants is not as a result of differences in cell death. In fact, when the percentage of viable cells is taken into account, the difference in NF-κB activity between WT TNFRSF1A and the TRAPS mutants increases further (Figure 4.12). The data thus far therefore suggests that when the TRAPS mutants are expressed in isolation, they result in reduced NF-κB activity and cell death compared to WT TNFRSF1A.

4.2.5 Cell surface expression of recombinant TNFRSF1A

TNFRSF1A expression is a highly regulated process (Bradley *et al.*, 1995; Jones *et al.*, 1999; Cottin *et al.*, 2002; Wang *et al.*, 2003). Expression of the four TRAPS mutants was therefore analysed in order to investigate whether differences in cell surface expression were responsible for the observed differences in NF-κB activity and cell death. It had previously not proved possible to detect expression of the V5 epitope by

western blotting and TNFRSF1A was therefore cloned into a vector containing a myc epitope, as described above. However, as DG75 and Eli-BL cells constitutively express c-myc (Spender *et al.*, 2001), transiently transfected myc epitope could not be distinguished above baseline levels by western blotting.

TNFRSF1A expression was therefore assessed by flow cytometry using an anti-TNFRSF1A antibody. Both the Eli-BL and DG75 cell lines express undetectable levels of endogenous membrane TNFRSF1A by flow cytometry (Fielding *et al.*, 2004). The levels of transfected WT TNFRSF1A detected by flow cytometry at the surface of Eli-BL cells are low (Fielding *et al.*, 2004), making comparisons of expression levels difficult. Therefore, the highly transfectable DG75 B-cell line was used as this allowed more sensitive analysis of TNFRSF1A expression than the Eli-BL cell line that was used for the NF-κB signalling analysis. The DG75 cell line was unsuitable for the signalling assays as it has high levels of constitutive NF-κB activity (Figure 4.6B).

The expression of TNFRSF1A constructs on the surface of viable transfected cells was measured by flow cytometry (Figure 4.13). Cells were stained with a mouse monoclonal antibody against human TNFRSF1A and an RPE-conjugated secondary antibody. Transfected cells were indicated by the expression of GFP. Untransfected cells, cells transfected with empty pcDNA3.1 vector and EGFP, and cells transfected with WT TNFRSF1A stained with an isotype control antibody were also analysed in parallel. The basal level of TNFRSF1A staining (pcDNA3.1) in GFP positive cells was similar to that obtained using an irrelevant isotype control, indicating that the DG75 cell line expresses low levels of surface TNFRSF1A. While WT TNFRSF1A could be detected easily on the surface of cells transfected with this construct, surface expression of the TRAPS mutants remained consistently low, with very little increase above basal levels (Figure 4.13), even when the cells were transfected with 20 μg of plasmid DNA. As was the case in with the Eli-BL cells, alamar blue cell viability assays indicate that there was more cell death in DG75 cells transfected with WT TNFRSF1A than in those transfected with the TRAPS mutants at the DNA dose used for the receptor expression studies (Figure 4.14). The lack

of surface expression of the TRAPS mutants cannot therefore be attributed to increased cell death.

4.2.6 Total (surface and intracellular) expression of recombinant TNFRSF1A

The lack of surface expression of the TRAPS mutants may be due to low general expression or altered localisation of TNFRSF1A. In order to determine the extent of intracellular expression of the TRAPS mutants following transient transfection, cells were permeabilised with 0.1% Triton X-100 prior to staining with the anti-TNFRSF1A mAb. This was performed in parallel with staining of unpermeabilised cells, allowing comparison of surface and cytoplasmic TNFRSF1A levels. Following permeabilisation, cells transfected with WT TNFRSF1A showed enhanced staining, indicating detection of both surface and intracellular TNFRSF1A. Despite the lack of surface expression of the TRAPS mutants, all four mutants were detected following permeabilisation of cells transfected with these constructs (Figure 4.15). Thus, these mutant recombinant forms of TNFRSF1A appear to be localised predominantly intracellularly, in contrast to the predominantly surface expression of WT TNFRSF1A. The average mean fluorescence intensities, from five experiments, of TNFRSF1A staining of unpermeabilised and permeabilised transfected DG75 cells are shown in Figure 4.16. In addition to the differences between WT and the TRAPS mutants, there also appear to be more subtle differences between the various TRAPS mutants themselves. The C30R mutation in particular was expressed at lower levels than the other TRAPS mutants.

4.2.7 Localisation of recombinant TNFRSF1A

The distribution of WT and mutant forms of TNFRSF1A was further characterised by confocal microscopy. Following transfection, DG75 cells were permeabilised and then stained with anti-human TNFRSF1A mAb and Alexa Fluor 488 secondary antibody, prior to detection by confocal microscopy. Cells transfected with empty pcDNA3.1 vector and transfected cells stained with isotype antibody were used as controls. The results confirm that the localisation of the TRAPS mutants differs from that of WT TNFRSF1A (Figure 4.17). WT TNFRSF1A (Fig 4.17 b-c) was detected both on the surface of cells, with a punctate staining pattern consistent with lipid raft distribution (Cottin *et al.*, 2002), and

intracellularly. In contrast, the TRAPS mutants (Fig 4.17 d-k) were detected predominantly intracellularly. These microscopy results are in keeping with the results obtained by flow cytometry. While in the vast majority of cells the staining of the TRAPS mutants was intracellular and localised to one portion of the cell (Fig 4.17 d-f, h, j), in a few cases there was also detectable membrane staining (Fig 4.17 g, i, k). These latter cells were infrequently seen but are shown here to give a more complete overview of the staining patterns observed by confocal microscopy. Interestingly, in the cells transfected with T50M, there appeared to be punctate staining on the cell surface (Fig 4.17 h-i), suggesting lipid raft distribution.

Furthermore, the mutant forms of TNFRSF1A were occasionally detected as discrete cytoplasmic particles (Fig 4.17 e). The appearance of these intracytoplasmic particles is consistent with inclusion bodies or aggregates formed by misfolded proteins (Kopito and Ron, 2000; Carrell and Lomas, 2002; Todd *et al.*, 2004). While these aggregates were occasionally seen with all four TRAPS mutants, they were most commonly observed with the C30R mutant. The more restricted expression and localisation of C30R detected by confocal microscopy is consistent with the lower expression of the C30R mutant detected by flow cytometry (Figure 4.16), and correlates with the low levels of NF-κB luciferase activity detected with this mutant (Figure 4.8).

TNFRSF1A has been shown to be localised to the trans-Golgi network in several cell types (Bradley *et al.*, 1995; Gaeta *et al.*, 2000; Wang *et al.*, 2003). In order to determine whether the intracellular mutant TNFRSF1A was localised to the Golgi apparatus, cells transfected with the expression vectors for C43S or T50M mutants were permeabilised and then stained with both anti-TNFRSF1A mAb and a Golgi apparatus stain (TGN46), followed by the appropriate secondary antibodies. In keeping with the prominent surface expression of WT TNFRSF1A demonstrated previously, the majority of this receptor was distinct from the trans-Golgi network (Figure 4.18, top panel). A proportion of the WT receptor did however co-localise with the trans-Golgi network stain. In contrast, the majority of the mutant receptor staining was noted in close

proximity to the Golgi apparatus, with evidence of co-localisation (Figure 4.18, lower two panels).

4.2.8 Effect of intracellular domain removal on membrane expression of TRAPS mutants

Various portions of the intracellular domain of TNFRSF1A have been shown to alter cellular localisation of the receptor (Hsu and Chao, 1993; Gaeta *et al.*, 2000; Cottin *et al.*, 2002; Storey *et al.*, 2002; Fielding *et al.*, 2004). In particular, deletion of the death domain results in increased, uniform surface expression of TNFRSF1A with a uniform staining pattern (Cottin *et al.*, 2002). To test whether deletion of the intracytoplasmic domain restores the surface expression of the mutant recombinant forms of TNFRSF1A, truncated forms of the C43S (C43SΔ218) and T50M (T50MΔ218) mutants were generated by site-directed mutagenesis of WTΔ218. As expected, removal of the intracytoplasmic domain of TNFRSF1A (WTΔ218, C43SΔ218, T50MΔ218), which contains the docking sites required for receptor signalling (Hsu *et al.*, 1995), resulted in lack of NF-κB activation in response to TNFα (Figure 4.19). The WTΔ218 construct demonstrated markedly increased surface expression by flow cytometry (Figures 4.20 & 4.21) and a uniform staining pattern on confocal microscopy (Figure 4.22), in keeping with results of previous studies (Cottin *et al.*, 2002; Fielding *et al.*, 2004). A population of TNFRSF1A positive but GFP negative cells is seen with WTΔ218 (Figure 4.20, top right panel). This represents cells in which TNFRSF1A but not GFP could be detected, and is almost certainly because WTΔ218 is expressed at higher levels than GFP, which is exacerbated by the higher doses of the TNFRSF1A constructs used compared to GFP (ratio 5:1). This therefore reflects a good TNFRSF1A positive control. In contrast, the truncated TRAPS mutants had low levels of surface expression, and predominantly cytoplasmic staining patterns, similar to that seen with the full-length TRAPS mutants (Figures 4.20-4.22). Thus, removal of the intracytoplasmic portions of TNFRSF1A cannot rescue the reduced expression of the receptor caused by the C43S and T50M TRAPS mutations in the extracellular domain of TNFRSF1A.

4.2.9 Effect of co-transfection of WT and the C43S TRAPS mutant on NF-κB activation

As TRAPS has an autosomal dominant inheritance pattern, the cells of patients with TRAPS will contain both WT and mutated TNFRSF1A, which can potentially interact although it is currently unknown whether this occurs. In order to determine the effects of the C43S TRAPS mutation in the presence of WT TNFRSF1A on NF-κB activation, luciferase assays were performed in Eli-BL cells co-transfected with the mammalian expression vectors for both full-length WT and C43S. Co-transfection of WT and C43S TNFRSF1A resulted in a level of NF-κB activation intermediate between that seen with either construct alone (Figure 4.23). Co-transfection with 2.5 μg of each WT and C43S resulted in significantly less NF-κB activation than 2.5 μg and 5 μg of WT alone by the Mann-Whitney U test. These results are consistent with the reduced NF-κB activation demonstrated in the primary fibroblast line established from the patient with the C43S TRAPS mutation in Chapter 3.

4.3 Discussion

The results in this chapter indicate that four different TRAPS mutations, namely C30R, C43S, T50M and C52F, have decreased NF-κB activity relative to WT TNFRSF1A. This reduced NF-κB activity is seen both at baseline and following stimulation with TNFα. These TRAPS mutants also appear to be associated with reduced cell death compared to WT TNFRSF1A. The reduced signalling correlates with the reduced surface expression of the four TRAPS mutants.

However, this issue of expression raises a fundamental difficulty with these signalling experiments; namely that of studying signalling in cells when it is not clear that the receptor is ever expressed in the membrane. This represents a "20-20 hindsight" issue: if we had known that these mutants were not expressed sufficiently on the cell surface, we would not have chosen to do the receptor assay experiments. The receptor assay experiments were designed to test a clear hypothesis that the reduced NF-κB transcription

activity observed in the patient-derived dermal fibroblasts in Chapter 3 would also be seen with TRAPS mutants other than C43S.

Despite these limitations, the reductions in NF-κB activity and cell death seen with the transient transfection experiments appear to be consistent with the results in Chapter 3 which suggested that the C43S TRAPS mutation results in decreased NF-κB production and TNFα-induced cell death in a patient-derived dermal fibroblast line. Furthermore, this work shows that reduced TNFRSF1A signalling is not just a feature of the C43S TRAPS mutation but also of at least three other TRAPS mutations, suggesting it may be a more general feature of this inflammatory syndrome. It does however appear that that there may be more subtle differences between the TRAPS mutations themselves. As TRAPS has an autosomal dominant pattern of inheritance, patients' cells will contain both WT and mutated TNFRSF1A. The co-transfection of WT and mutated TNFRSF1A showed reduced NF-κB activity compared to equivalent amounts of the WT receptor expressed in isolation, as was the case for the experiments using the patient-derived dermal fibroblast samples.

The cytoplasmic localisation and reduced cell surface expression of the TNFRSF1A mutants seen in this work agrees with a study using HEK-293 cells published while this work was in progress (Todd *et al.*, 2004). However, in contrast to our work, Todd *et al* (2004) showed spontaneous apoptosis and cytokine production suggesting no abrogation of signalling. Some of the difference observed in the two studies is likely to be due to the high levels of expression that can be tolerated when stable HEK-293 cell lines are generated. High level expression of recombinant WT TNFRSF1A in transfected Hela cells has previously been shown to spontaneously trigger signalling of both apoptosis and IL-8 production (Boldin *et al.*, 1995a). Indeed, Boldin *et al* observed that overexpression of the DD itself induced apoptosis and IL-8 production, and proposed that the ligand-independent signalling may result from spontaneous self-association of the DD when expressed at high levels (1995). TNFRSF1A expression is tightly regulated and is low in fibroblasts and leucocytes, the cell types from the TRAPS patient studied in Chapter 3. In these cells, reduced TNFRSF1A signalling was observed. Thus, when TNFRSF1A

expression is limited, reduced signalling is observed. Interestingly, Todd *et al* (2004) also show that mutant receptors are unable to bind TNFα, suggesting a further mechanism whereby signalling could be inhibited.

Both this work and that of Todd *et al* (2004) demonstrate that the extracellular domain of TNFRSF1A plays a role in cellular localisation, with the TRAPS mutations predominantly localised intracellularly. Previous work has shown that deletion of the intracellular domain increases surface expression of the receptor. However, our data shows that this does not occur for the C43S and T50M TNFRSF1A mutants. Thus, mutation of the extracellular domain appears to be a dominant factor in the regulation of expression. It is possible that formation of the trimeric receptor, through the pre-ligand-binding assembly domain, is required for the trafficking of the molecule from the Golgi apparatus, or retention of the receptor at the surface. Alternatively, misfolding of the receptor, caused by disruption of the disulphide bonds and other secondary structural changes as a result of the mutations, may be sufficient to inhibit membrane expression. While protein overexpression itself can lead to misfolding (Sanders and Nagy, 2000; Todd *et al.*, 2004), overexpression on its own cannot explain the differences in staining patterns seen in this work, as WT and WTΔ218 TNFRSF1A were expressed at higher levels than the TRAPS mutants, but could still be detected on the cell surface of transfected cells. It is likely that the TRAPS-associated mutations themselves are inducing structural changes preventing receptor translocation to the cell surface.

The B cell lines used for the transient transfection experiments were chosen for their low basal production of TNFα and low endogenous expression of TNFRSF1A. However, the biology of TNFα in B cells is not fully known as most published data regarding TNFα signalling has been obtained using macrophages/ monocytes and T lymphocytes. The importance of various TNF superfamily members appears to vary in different cell types. In particular, two other TNF ligands, BAFF and APRIL, have been shown to be crucial in regulating B cell homeostasis (Schneider, 2005). These issues therefore reduce the interpretation possible from the results in this chapter and it remains to be seen whether similar effects are seen in other immune cells.

This work characterises the NF-κB activation for a variety of TRAPS mutants and indicates decreased NF-κB when mutated receptors are expressed alone and in combination with WT receptor. Taken together with the findings in Chapter 3, this suggests that the loss of specific TNFRSF1A signals is likely to be a general feature of the TRAPS phenotype. Furthermore, the findings in this chapter are important as they suggest that altered TNFRSF1A localisation may be a mechanism for the reduced NF-κB signalling, and that this mechanism may have relevance *in vivo*.

Figure 4.1
Schematic of the cloning strategy

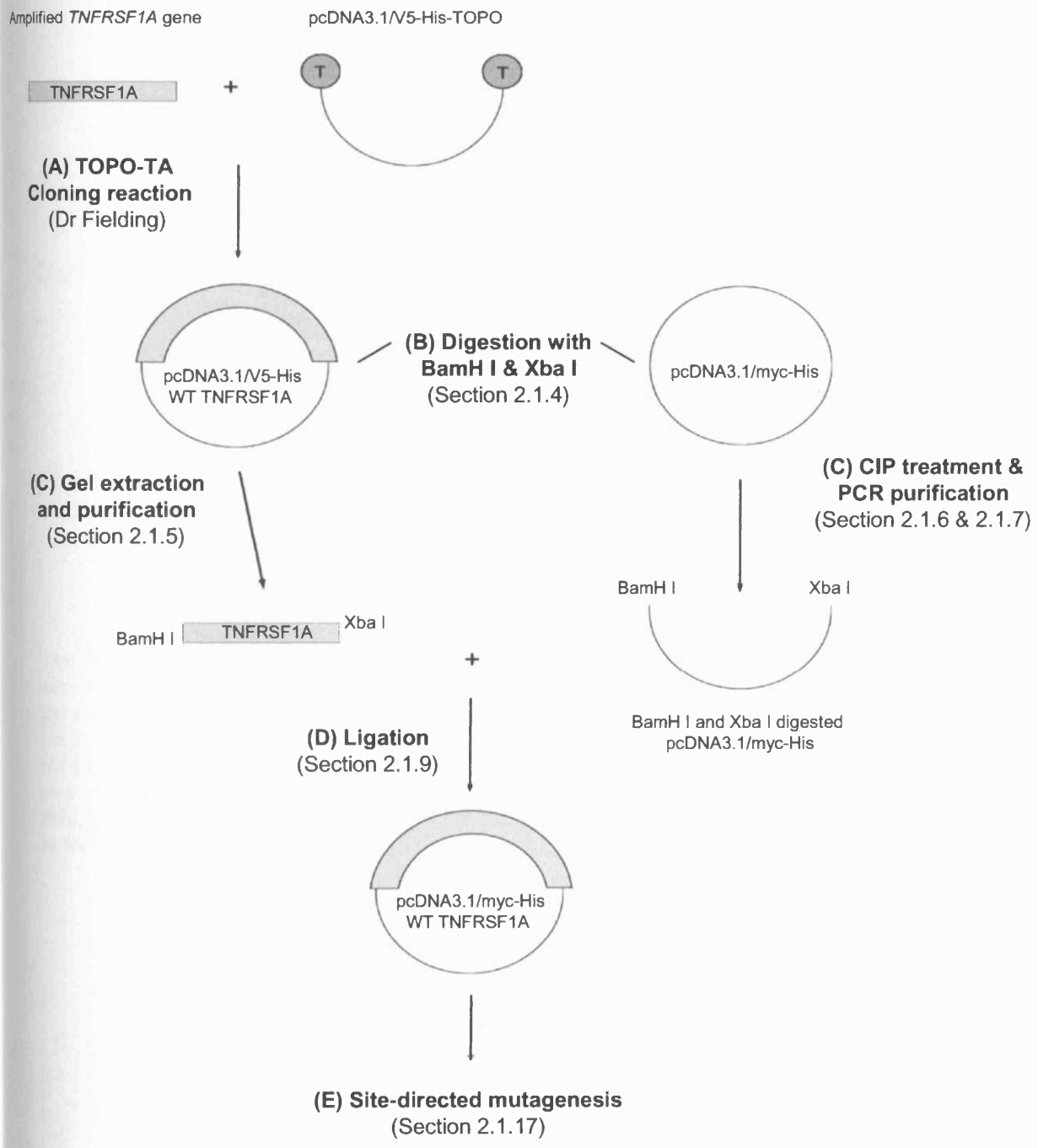
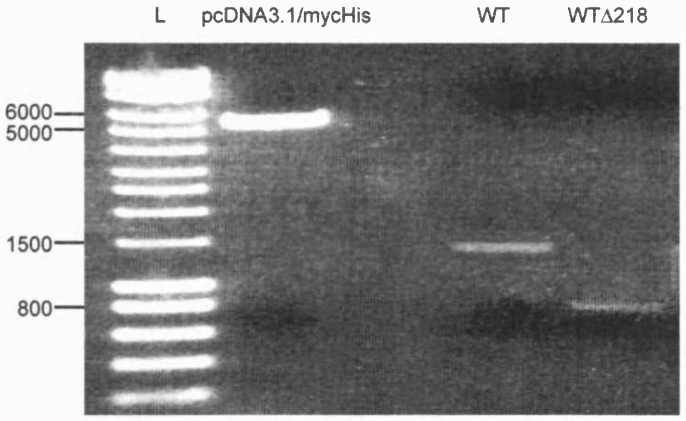
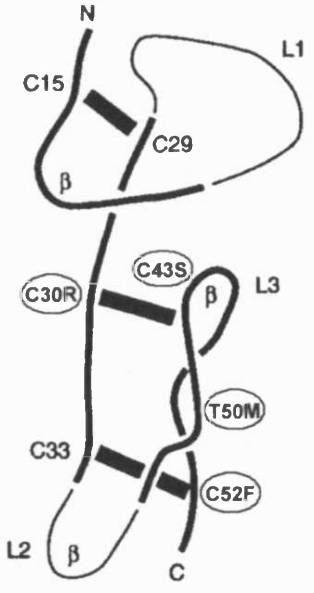


Figure 4.2
TNFRSF1A inserts and pcDNA3.1/myc-His vector obtained by restriction enzyme digestion (*BamH I* and *Xba I*)



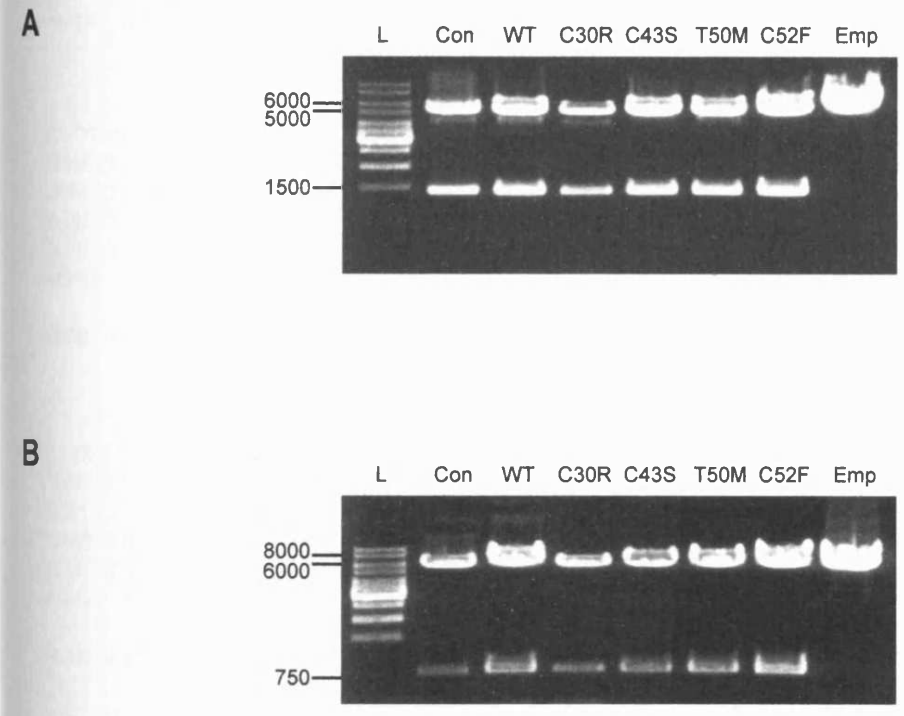
The TNFRSF1A WT and WTΔ218 genes were released from the pcDNA3.1/V5-His vector by restriction enzyme digestion with *BamH I* and *Xba I* at 37°C for 1 hour. The digests were analysed on a 1% agarose gel and the recombinant TNFRSF1A isolated by gel extraction and purification. In conjunction, the pcDNA3.1/myc-His vector was digested with *BamH I* and *Xba I* at 37°C for 1 hour, CIP-treated and PCR purified. The purified TNFRSF1A genes and CIP-treated pcDNA3.1/myc-His vector were then analysed on a 1% agarose gel along with a DNA ladder (L) allowing quantification of DNA, stained with ethidium bromide solution and visualised by UV light. The size of selected bands of the DNA ladder are indicated in base pairs at the side of the figure.

Figure 4.3
Crystallographically determined structure of TNFRSF1A extracellular cysteine rich domain 1 (CRD1)



The four TRAPS mutations that were generated are shown as circled amino acids. The three disulphide bonds that characterise CRD1 are depicted by thick black bars. Thicker lines represent structurally conserved regions of the CRD; "β" indicates the β-turn positions; "L1"-"L3" denote loop domains. Adapted from Aksentijevich (2001).

Figure 4.4
Restriction enzyme digests of the recombinant TNFRSF1A expression vectors (*BamH I* and *Xba I; Hind III*)



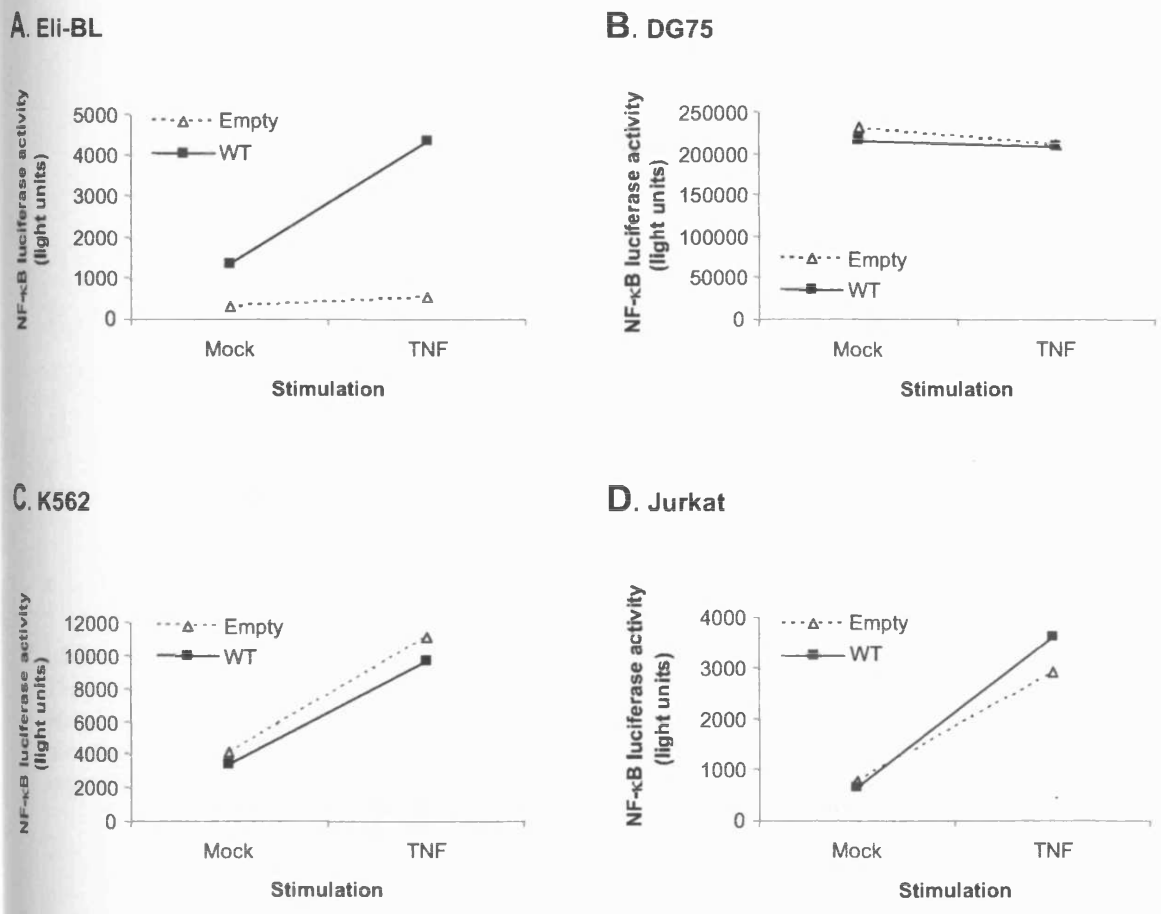
Recombinant TNFRSF1A pcDNA3.1/myc-His vectors and the empty pcDNA3.1/myc-His vector (Emp) were digested with **(A)** *BamH I* and *Xba I* or **(B)** *Hind III* at 37°C for 1 hour. The digests were analysed on a 1% agarose gel along with a DNA ladder (L), stained with ethidium bromide solution and visualised by UV light. The positive control (Con) was pcDNA3.1/V5-His WT TNFRSF1A. The size of selected bands of the DNA ladder are indicated in base pairs at the side of the figures.

Figure 4. 5
Alignment of nucleotide and amino acid sequences

```
WT TNFRSF1A      CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT 132
C30R TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C43S TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
T50M TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C52F TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
amino acid       L   V   P   H   L   G   D   R   E   K   R   D   S   V   C    15


WT TNFRSF1A      CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT 177
C30R TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- C--
C43S TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
T50M TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C52F TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
amino acid       P   Q   G   K   Y   I   H   P   Q   N   N   S   I   C   C    30
                                                                        ↓
C30R (175T>C)                                                           R


WT TNFRSF1A      ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC 222
C30R TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C43S TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- -C- --- ---
T50M TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C52F TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
amino acid       T   K   C   H   K   G   T   Y   L   Y   N   D   C   P   G    45
                                                                    ↓
C43S (215G>C)                                                       S


WT TNFRSF1A      CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC 267
C30R TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C43S TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
T50M TNFRSF1A    --- --- --- --- -T- --- -T- --- --- --- --- --- --- --- ---
C52F TNFRSF1A    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
amino acid       P   G   Q   D   T   D   C   R   E   C   E   S   G   S   F    60
                                 ↓       ↓
T50M (236C>T)                    M       ↓
C52F (242G>T)                            F
```

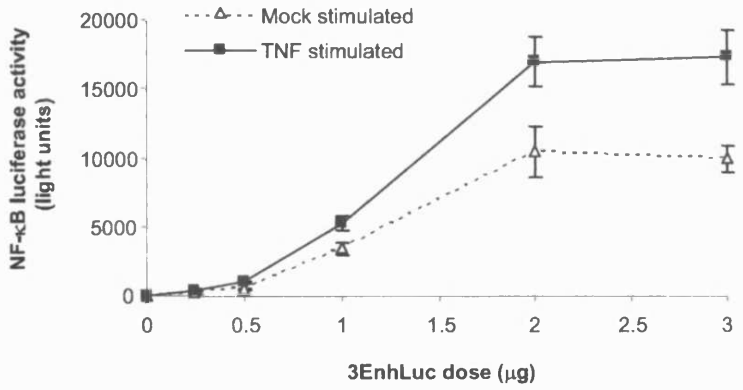
Alignment of the nucleotide and amino acid sequences of WT TNFRSF1A and the four mutant forms of TNFRSF1A. The numbering of amino acids for TNFRSF1A in TRAPS has started after the leader sequence (i.e. with the leucine at residue 30) and has not followed the general rule of starting with the translation initiator methionine. The sequence for the first 60 amino acids of WT TNFRSF1A is shown in single letter amino acid code and triplet nucleotide codons at the DNA level. Nucleotide and amino acid numbering are indicated on the right. Nucleotide substitutions are indicated by shaded boxes. Substituted amino acids are shown in red, with arrows indicating the changes in the amino acid code for each of the TNFRSF1Amutants.

Figure 4.6
NF-κB activity of various cell lines transfected with recombinant TNFRSF1A



The indicated cell lines were each transiently transfected with 3 μg of the NF-κB luciferase reporter and 5 μg of either empty pcDNA3.1 vector or WT TNFRSF1A. Transfections were split into two, and sixteen hours post-transfection, one half was mock stimulated with culture medium and the other was stimulated with TNFα (10 ng/ml). After 8 h stimulation, luciferase activity was assayed.

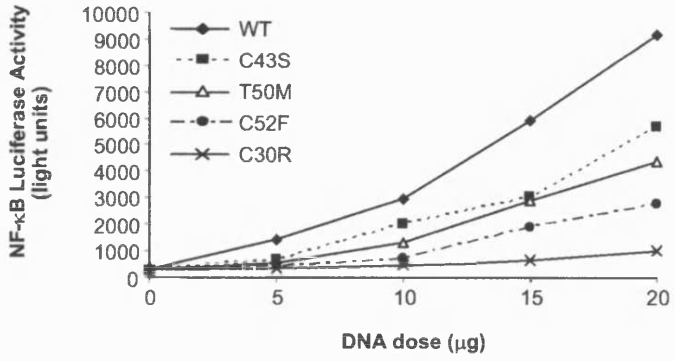
Figure 4.7
NF-κB reporter dose response in Eli-BL cells



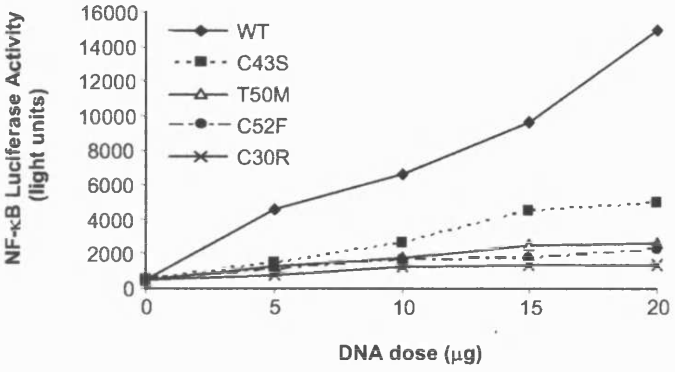
Eli-BL cells were transiently transfected with 20 μg of WT TNFRSF1A and a variety of doses of the 3EnhLuc reporter. Transfections were split into two. Sixteen hours post-transfection, one half was mock stimulated with culture medium and the other was stimulated with TNFα (10 ng/ml). After 8 h stimulation, luciferase activity was assayed. The results shown are the mean ± SD of two independent experiments.

Figure 4.8
NF-κB activation by WT and mutant recombinant forms of TNFRSF1A

A



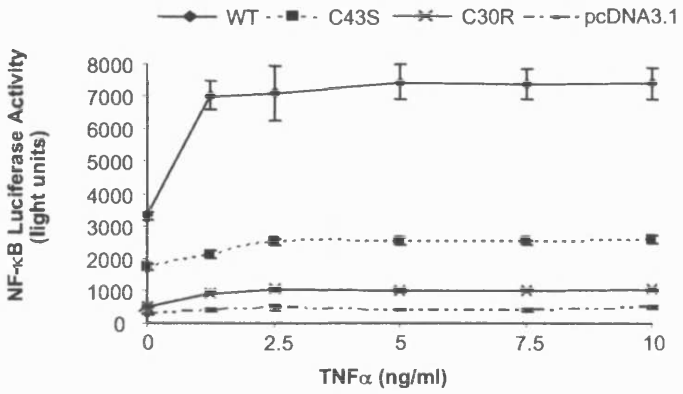
B



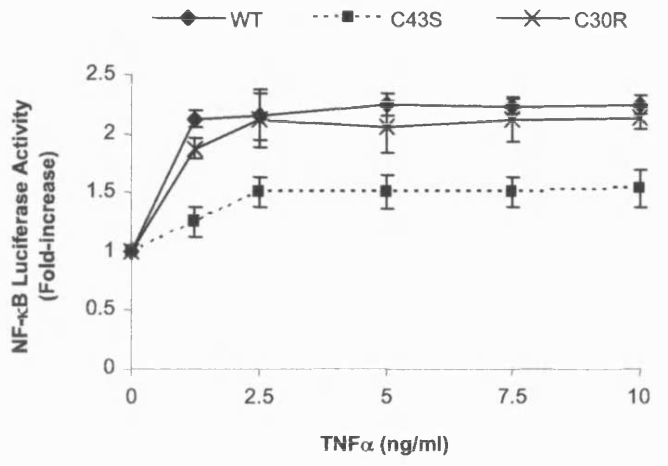
Eli-BL cells were transiently transfected with the NF-κB luciferase reporter (3 μg) and varying amounts of each TNFRSF1A construct. Total DNA amount added to the luciferase reporter was kept constant at 20 μg by the addition of appropriate amounts of empty pcDNA3.1/myc-His vector. Transfections were split into two. 16 h later one half was mock stimulated and the other was stimulated with TNFα (10 ng/ml). After 8 h stimulation, luciferase activity was assayed. The results are shown for **(A)** mock stimulated cells and **(B)** TNFα stimulated cells. The results shown are the mean of at least five independent experiments.

Figure 4.9
TNFα dose response for Eli-BL cells transfected with TNFRSF1A constructs

A

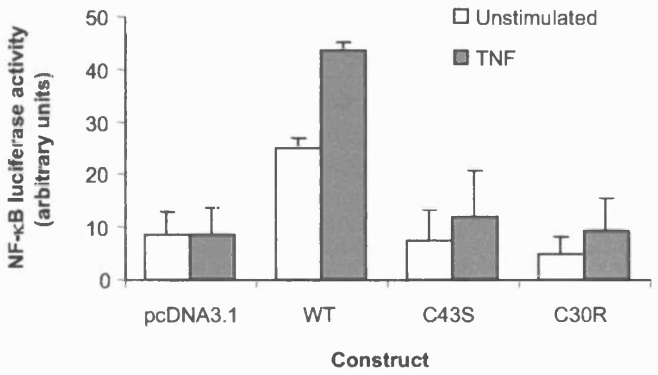


B



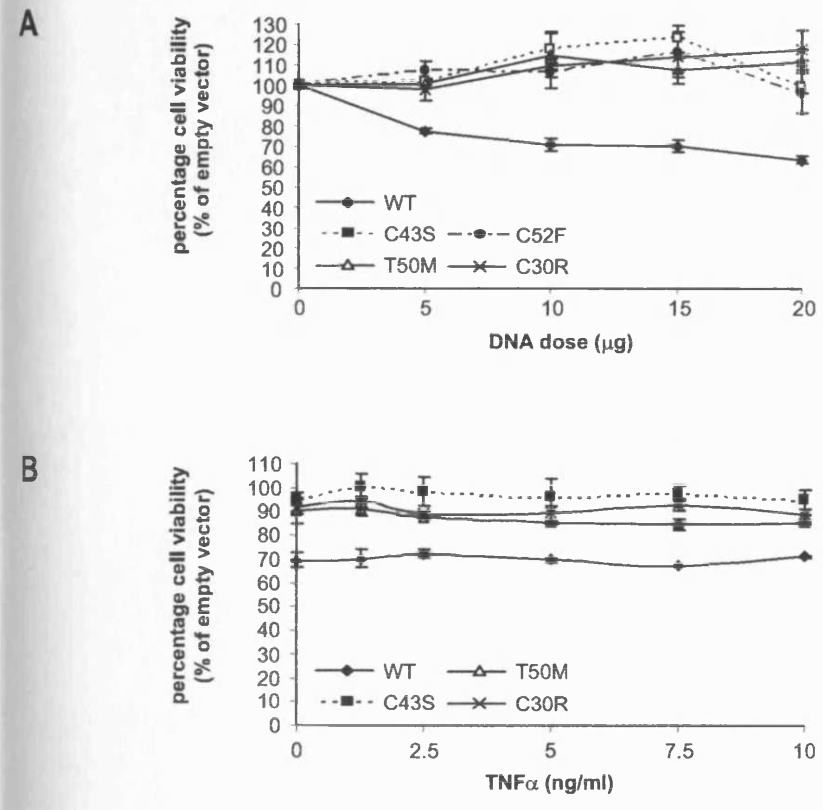
(A) TNFα dose response. Eli-BL cells were transfected as before. Cells transfected with the same TNFRSF1A construct (10 μg DNA per transfection) were pooled and then split into samples of equal size (0.5×10^7 cells). 16 h post-transfection the transfected cells were stimulated with a variety of TNFα doses, as indicated. Luciferase activity was measured 8 h after stimulation. The results shown are the mean ± SEM of three independent experiments. **(B)** Fold-induction with TNFα. This was calculated by dividing TNFα stimulated values by unstimulated (TNFα = 0) for each construct.

Figure 4.10
NF-κB activity adjusted for transfection efficiency



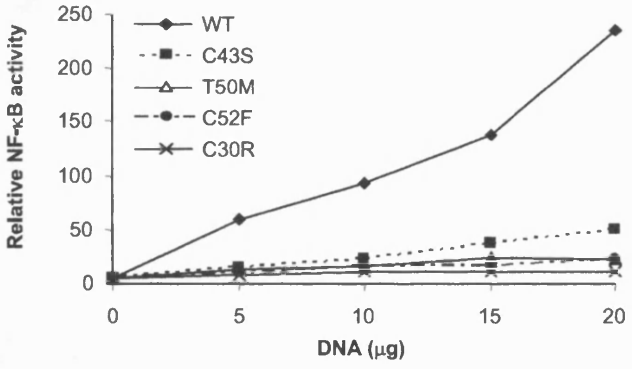
Eli-BL cells were transfected with an NF-κB firefly luciferase reporter (3 μg), a *Renilla* luciferase reporter (1 μg) and TNFRSF1A constructs (10 μg) or empty pcDNA3.1. Firefly and *Renilla* luciferase activities were measured after 8 h of TNFα (10 ng/ml) stimulation, according to the manufacturer's instructions (Promega). Results shown are NF-κB luciferase activity normalised for transfection efficiency. The results shown are the mean ± SD of two independent experiments.

Figure 4.11
Viability of Eli-BL cells transfected with TNFRSF1A constructs



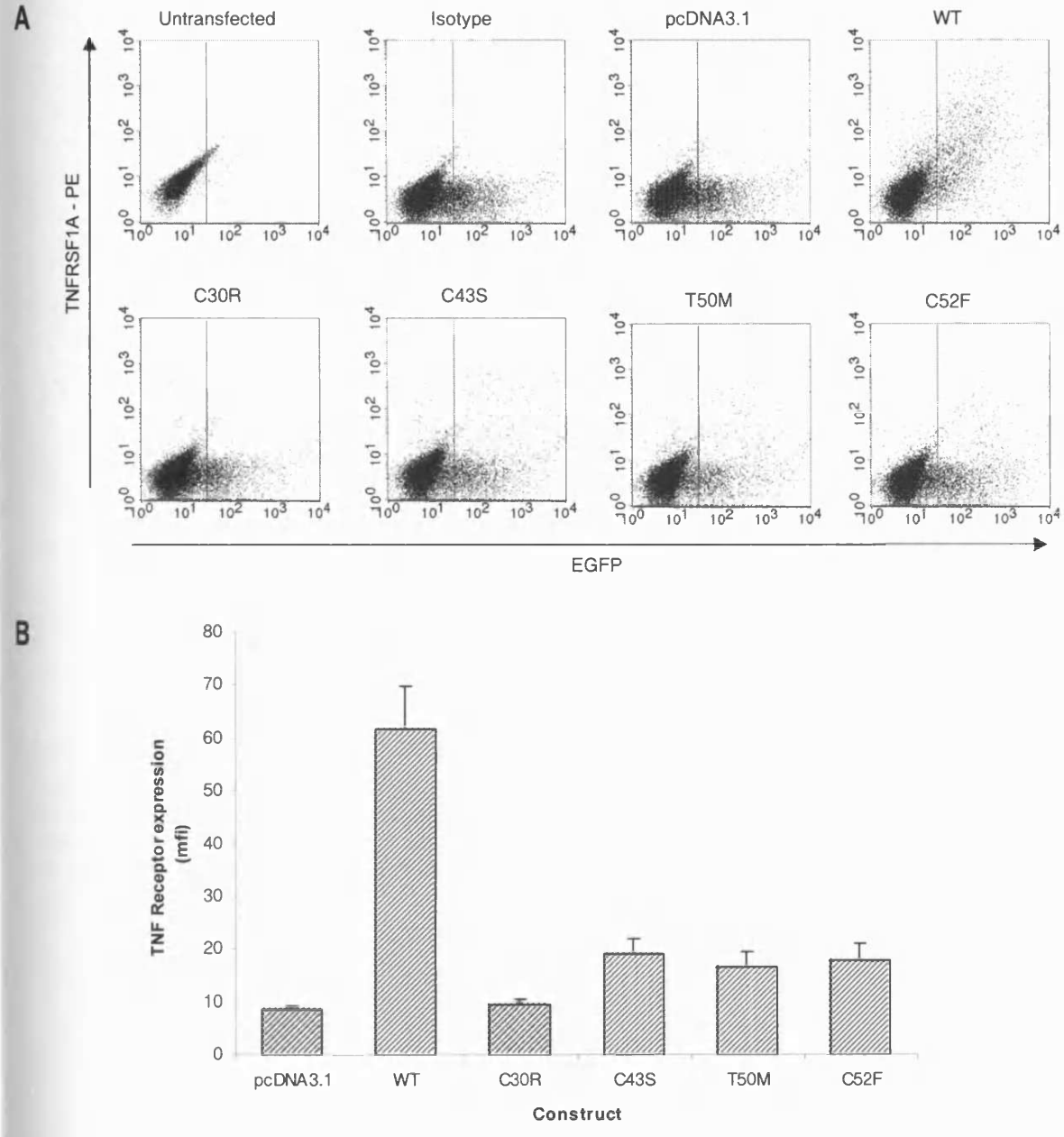
(A) Eli-BL cells were transiently transfected with varying amounts of each TNFRSF1A construct. 16 h post-transfection, 2 x 10^4 transfected cells were resuspended in 200 μl of 10% alamar blue reagent in growth medium on a 96 well plate. After 4 hours incubation at 37°C, absorption was measured by fluorometry. The results are shown relative to cells transfected with empty pcDNA3.1 vector, expressed as a percentage. Results shown are the mean ± SEM of three independent experiments. * = $P < 0.05$ versus TRAPS constructs, by Student's *t*-test. **(B)** Cell viability in response to TNFα dose. Eli-BL cells were transfected with the TNFRSF1A constructs (10 μg) and then stimulated with a range of TNFα doses for 8 h. Alamar blue assay was then performed as before.

Figure 4.12
NF-κB activation adjusted for cell viability



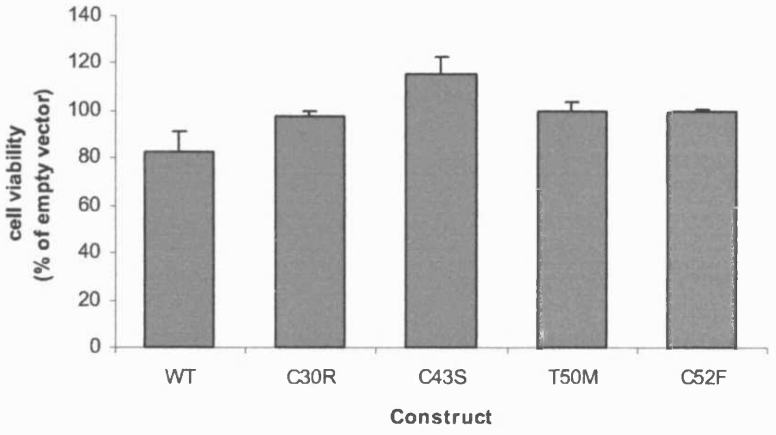
NF-κB activation by TNFRSF1A constructs adjusted for cell viability. Eli-BL cells were transiently transfected with the NF-κB luciferase reporter (3 μg) and varying amounts of each TNFRSF1A construct. Total DNA amount added to the luciferase reporter was kept constant at 20 μg by the addition of appropriate amounts of empty pcDNA3.1/myc-His vector. NF-κB activity and cell viability were measured by luciferase and alamar blue assays respectively. NF-κB activity was then divided by the percentage of viable cells. All results shown are the mean ± SEM of three independent experiments.

Figure 4.13
Surface expression of TNFRSF1A constructs



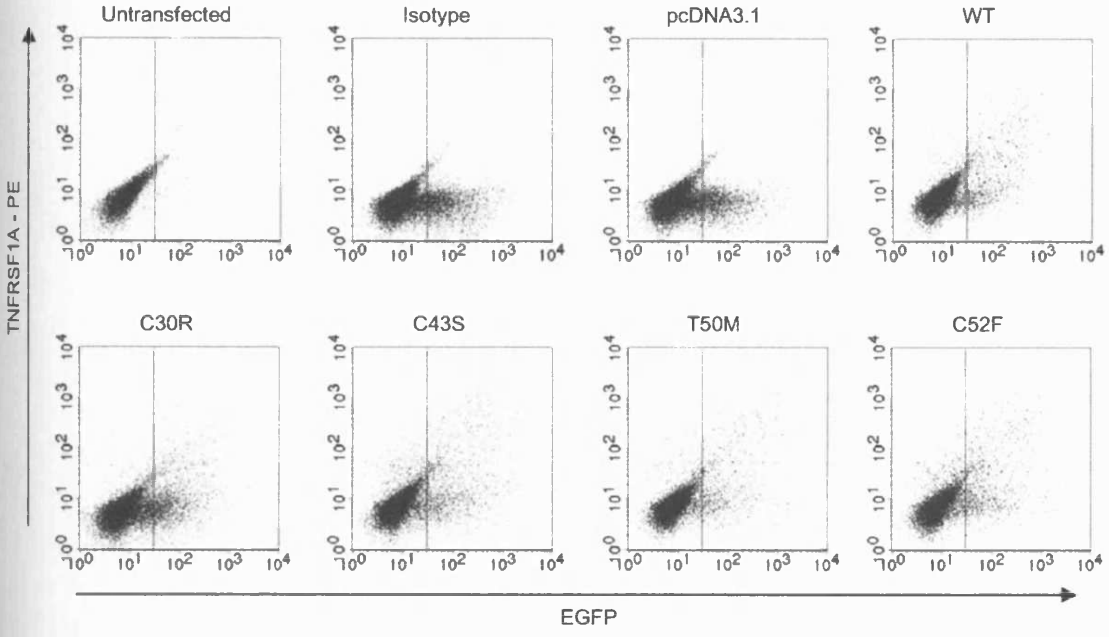
DG75 cells were transfected with 2 μg of EGFP-N1 and 10 μg of empty pcDNA3.1 vector or one of the TNFRSF1A expression vectors. Cells were stained with either anti-TNFRSF1A antibody followed by PE-conjugated secondary Fab fragments, or with an irrelevant PE-conjugated IgG1 antibody. **(A)** Dot plots of anti-TNFRSF1A-PE fluorescence against EGFP fluorescence are shown, and are representative of five independent experiments. Untransfected cells are shown for comparison. **(B)** Results, shown in graph form, are the average mean fluorescence intensity (mfi) ± SEM of TNFRSF1A staining of transfected (GFP positive) cells from five independent experiments.

Figure 4.14
Viability of DG75 cells transfected with TNFRSF1A constructs



DG75 cells were transiently transfected with 10 μg of empty pcDNA3.1 vector or one of the TNFRSF1A expression vectors. 16 h post-transfection, 2 x 10^4 transfected cells were resuspended in 200 μl of 10% alamar blue reagent in growth medium on a 96 well plate. After 4 hours incubation at 37°C, absorption was measured by fluorometry. The results are shown relative to cells transfected with empty pcDNA3.1 vector, expressed as a percentage. Results shown are the mean ± SD of two independent experiments.

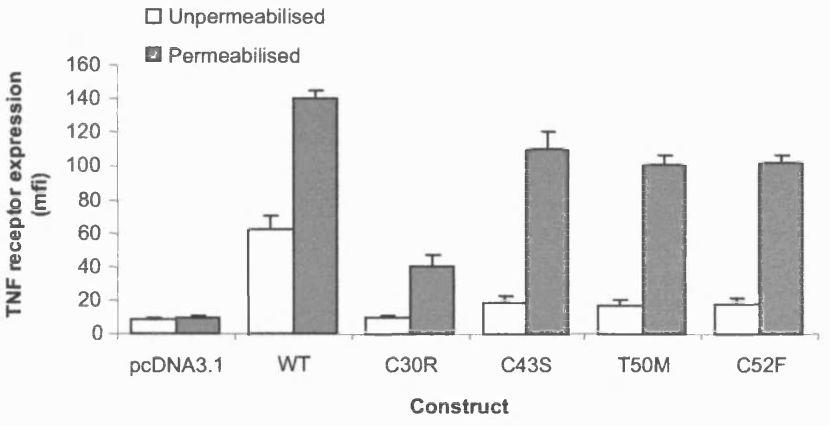
Figure 4.15
Total (surface and intracellular) expression of recombinant TNFRSF1A



DG75 cells were transfected with 2 μg of EGFP-N1 and 10 μg of empty pcDNA3.1 vector or one of the TNFRSF1A expression vectors. Cells were permeabilised with 0.1% Triton X-100 and then stained with anti-TNFRSF1A antibody followed by PE-conjugated secondary Fab fragments. Stained cells were analysed by flow cytometry. Results are shown as dot plots of anti-TNFRSF1A-PE fluorescence against EGFP fluorescence, and are representative of five independent experiments.

Figure 4.16
Expression of TNFRSF1A constructs in transfected DG75 cells

A

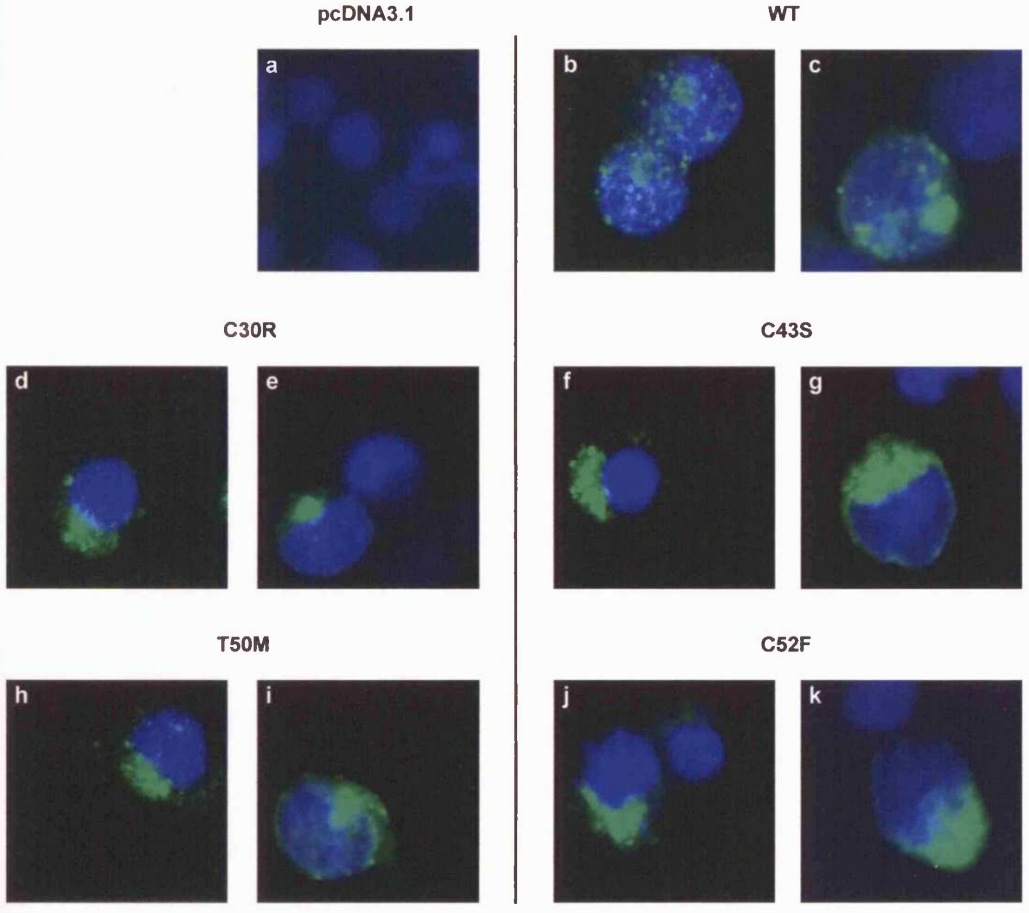


B

Construct	TNFRSF1A staining in unpermeabilised cells (average mfi ± SEM)	TNFRSF1A staining in permeabilised cells (average mfi ± SEM)
pcDNA3.1	8 ± 1	9 ± 1
WT	62 ± 8	140 ± 5
C30R	9 ± 1	39 ± 7
C43S	19 ± 3	110 ± 10
T50M	17 ± 3	100 ± 6
C52F	18 ± 3	101 ± 5

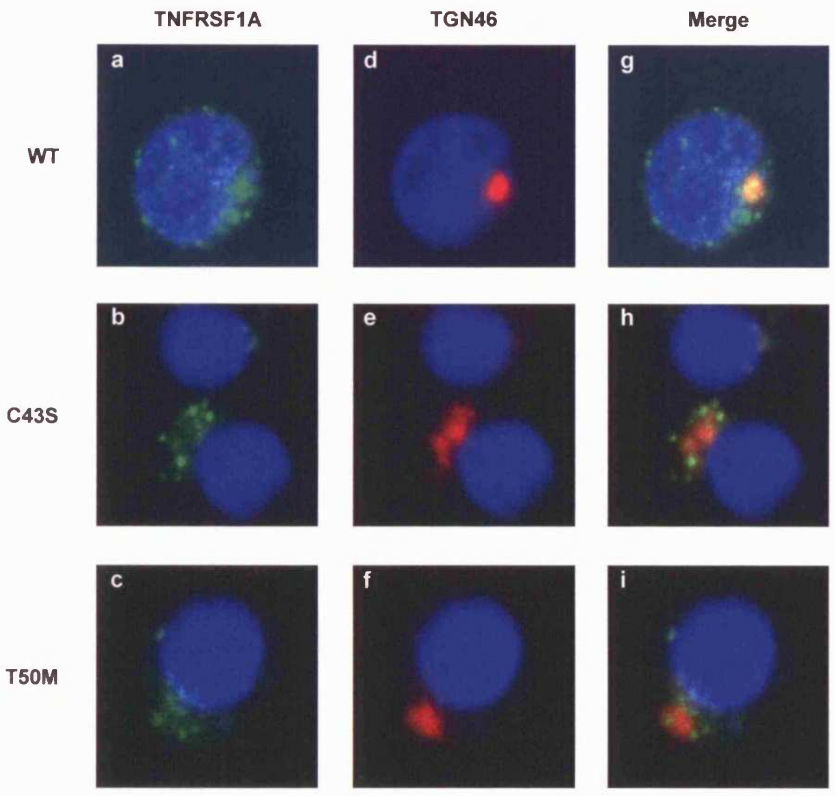
DG75 cells were transfected with 2 μg of EGFP-N1 and 10 μg of empty pcDNA3.1 vector or one of the TNFRSF1A expression vectors. Cells were stained with anti-TNFRSF1A antibody followed by PE-conjugated secondary Fab fragments, either without permeabilisation, for surface staining, or following permeabilisation with 0.1% Triton X-100, for surface and intracellular expression. Stained cells were analysed by flow cytometry. Mean fluorescence intensity of GFP positive cells, as gated on Figure 4.15, was determined by CellQuest (Becton Dickinson). Results, shown in **(A)** graph and **(B)** table form to aid comparison, are the average mean fluorescence intensity (mfi) ± SEM of TNFRSF1A staining of transfected (GFP positive) cells from five independent experiments. WT TNFRSF1A transfected cells were also stained with an isotype IgG1 antibody as a control (average mfi 7 ± 1).

Figure 4.17
TNFRSF1A immunofluorescent staining by confocal microscopy



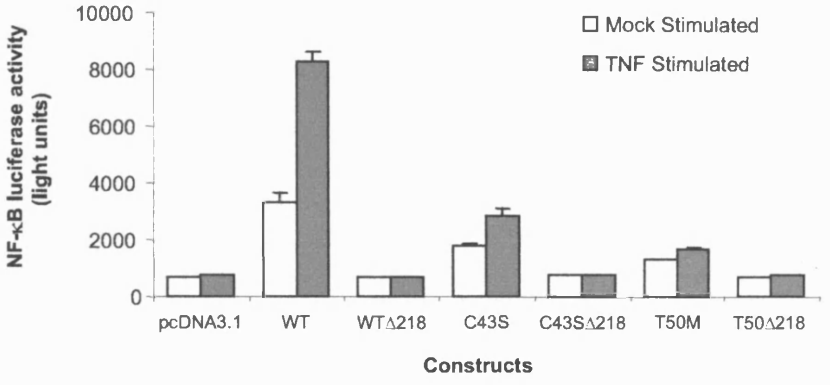
DG75 cells were transiently transfected with WT or mutant TNFRSF1A (10μg). Cells were permeabilised and stained with anti-TNFRSF1A antibody and Alexa Fluor 488 goat anti-mouse IgG secondary antibody (green). Nuclei were stained with DRAQ5 (blue). Single optical slice images were acquired using a confocal laser scanning microscope (BioRad), equipped with a krypton/argon ion laser and attached to a Zeiss Axiovert 135. Two examples from one experiment, representative of at least five independent experiments, are shown

Figure 4.18
Detection of immunofluorescent staining by confocal microscopy



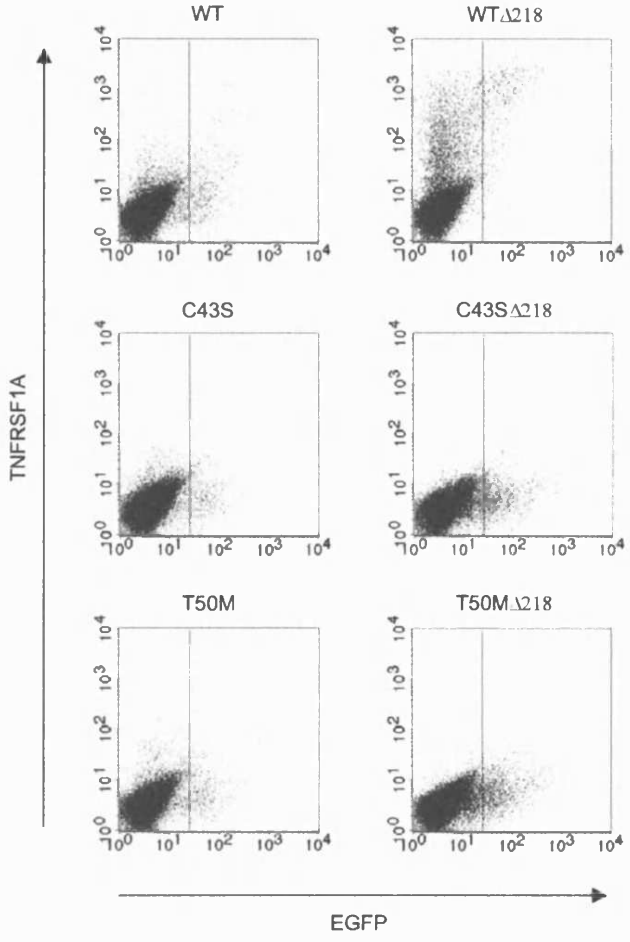
DG75 cells were transfected with recombinant TNFRSF1A (10μg). Cells were permeabilised, stained with anti-TNFRSF1A (green, a-c) and anti-TGN46 (red, d-f) antibodies, followed by the appropriate secondary antibodies. Nuclei were stained with DRAQ5 (blue). Single optical slice images were acquired using a confocal laser scanning microscope. The panel on the right (g-i) shows an overlay of the corresponding images to the left, where orange/yellow indicates co-staining for TNFRSF1A and TGN46.

Figure 4.19
NF-κB activation of full-length and truncated recombinant forms of TNFRSF1A



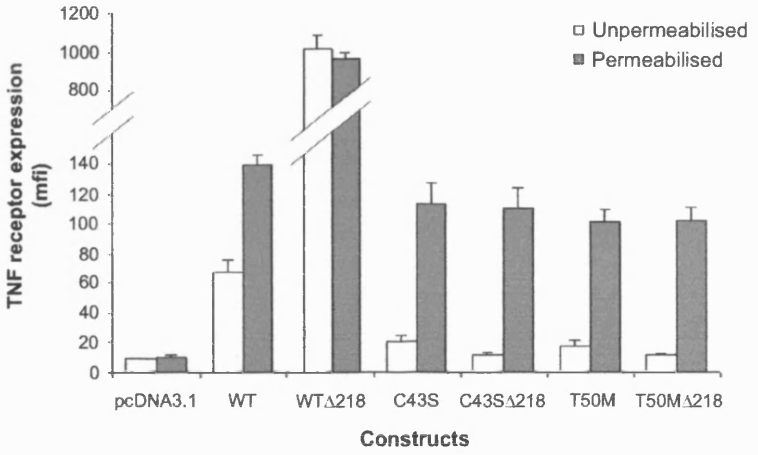
Eli-BL cells were transfected with the NF-κB luciferase reporter (3 μg) and full-length or truncated TNFRSF1A constructs (10 μg). Transfections were split into two. 16 h later one half was mock stimulated and the other was stimulated with TNFα (10 ng/ml). After 8 h stimulation, luciferase activity was assayed. The results shown are the mean ± SEM of three independent experiments.

Figure 4.20
Surface expression of full-length and truncated recombinant forms of TNFRSF1A



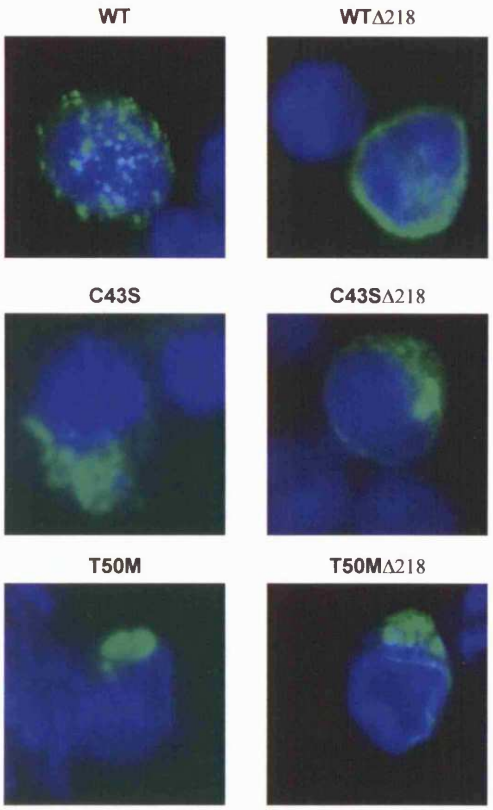
DG75 cells were transfected with 2 μg of EGFP-N1 and 10 μg of empty pcDNA3.1 vector or one of the mammalian expression vectors for full-length and truncated TNFRSF1A mutants. Unpermeabilised cells were stained with anti-TNFRSF1A antibody followed by PE-conjugated secondary Fab fragments. Stained cells were analysed by flow cytometry. Dot plots of TNFRSF1A-PE fluorescence against EGFP fluorescence are shown, and are representative of four independent experiments.

Figure 4.21
Expression of full-length and truncated recombinant forms of TNFRSF1A



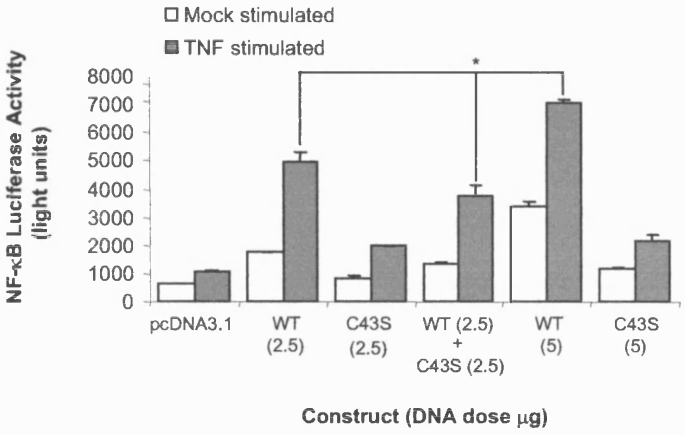
DG75 cells were transfected with 2 μg of EGFP-N1 and 10 μg of empty pcDNA3.1 vector or one of the TNFRSF1A expression vectors. Cells were stained with anti-TNFRSF1A antibody followed by PE-conjugated secondary Fab fragments, either without permeabilisation, for surface staining, or following permeabilisation with 0.1% Triton X-100, for surface and intracellular expression. Stained cells were analysed by flow cytometry. Results shown are the average mean fluorescence intensity (mfi) of TNFRSF1A staining of transfected (GFP positive) cells from four independent experiments. WT TNFRSF1A transfected cells were also stained with an isotype IgG1 antibody as a control (average mfi 9 ± 1.5).

Figure 4.22
Detection of full-length and truncated recombinant forms of TNFRSF1A by confocal microscopy



DG75 cells were transiently transfected with WT or mutant TNFRSF1A (10 μg). Cells were permeabilised and stained with anti-TNFRSF1A antibody and Alexa Fluor 488 goat anti-mouse IgG secondary antibody (green). Nuclei were stained with DRAQ5 (blue). Single optical slice images were acquired using a confocal laser scanning microscope, equipped with a krypton/argon ion laser and attached to a Zeiss Axiovert 135. Single examples, representative of at least five independent experiments, are shown.

Figure 4.23
NF-κB activation following co-transfection of WT and the C43S TRAPS mutant



Eli-BL cells were transiently transfected with 3 μg of NF-κB luciferase reporter and the TNFRSF1A constructs indicated. The total DNA amount added to the luciferase reporter was kept constant at 10 μg by the addition of appropriate amounts of pcDNA3.1. Transfections were split into two, and 16 h post-transfection, one half was mock stimulated and the other half was stimulated with TNFα (10 ng/ml). After 8 h, luciferase activity was assayed. The results shown are the means ± SEM of three experiments. * = $P < 0.05$ by the Mann-Whitney U test.

CHAPTER 5

FINAL DISCUSSION

The purpose of this study was to investigate the effects of TRAPS-associated TNFRSF1A mutants on TNFα signalling. Impaired TNFRSF1A shedding was reported for several TRAPS mutations (McDermott *et al.*, 1999a; Galon *et al.*, 2000), although this does not appear to be the case for all mutations (Aksentijevich *et al.*, 2001; Aganna *et al.*, 2003) and may be dependent on cell type (Huggins *et al.*, 2004). Our results support the hypothesis that TRAPS does not require impaired activation-induced shedding of TNFRSF1A from neutrophils. At the time of starting this work, there were no published studies on the effects of these TNFRSF1A mutations on TNFα-induced activation of transcription factors or apoptosis. The initial hypothesis for this work was that the TRAPS mutations would enhance TNFα-mediated signalling, particularly NF-κB activation, resulting in the clinical inflammatory phenotype that characterises TRAPS.

Two complementary strategies were used to test this hypothesis and investigate TNFα signalling. Firstly a dermal fibroblast line was established from a patient with the novel C43S TRAPS mutation (Chapter 3). To broaden the study, the second strategy involved the generation and analysis of plasmids expressing recombinant TRAPS-associated TNFRSF1A mutants (Chapter 4). These studies revealed that, contrary to the initial hypothesis, these TRAPS mutations resulted in reduced TNFRSF1A-mediated signalling, which was associated with reduced receptor expression on the cell membrane.

Generating a patient-derived primary cell line afforded us the opportunity to study the effects of the C43S TRAPS mutation on signalling in cells derived from a patient with this syndrome. The principal cell types involved in the pathogenesis of TRAPS are still unknown. Fibroblasts are easily accessible to biopsy, produce cytokines in response to TNFα and are relatively long-lived compared to circulating inflammatory cells, making them attractive cells for the functional characterisation of TRAPS mutations. The studies using these patient-derived cells indicated that the C43S TRAPS mutation results in

reduced NF-κB and AP-1 activation, and decreased apoptosis in response to TNFα. The defect in TNFα-induced apoptosis was also observed in the patient's peripheral blood mononuclear cells. However, the dermal fibroblasts were able to produce normal levels of the pro-inflammatory cytokines IL-6 and IL-8.

The episodic nature of the inflammatory attacks in TRAPS, and their periodicity, suggest that the principal cells involved in this condition are likely to be circulating immune cells, rather than more long-lived tissue-based cells (Rosen and Nishiyama, 1965; Whitelaw, 1966; Homburg and Roos, 1996). While we assessed receptor shedding and apoptosis in the patient's PBMCs, in hindsight it might have been better to also study the effects of this TRAPS mutation on signalling using these cells. Recent advances, such as multiplex cytokine bead arrays and luminex, have made the study of cytokine production by these peripheral haemopoeitic cells significantly easier. We intend to pursue this in future as outlined later in this chapter.

It is important to remember that as TRAPS has an autosomal dominant mode of inheritance, cells from patients will contain both wild-type and mutated TNFRSF1A. While a recent report suggests that these two forms of the receptor are capable of interacting (Yousaf *et al.*, 2005), the nature of this interaction remains unknown and adds a further level of complexity to functional studies of the mutated receptor in patient-derived material. Furthermore, the index patient reported in this study remains the only known patient with the C43S TRAPS mutation, while attempts to obtain samples from patients with different TRAPS mutations, through collaboration with other centres, proved unsuccessful.

In order to investigate whether other TRAPS mutations had similar functional effects to those seen in the fibroblasts bearing the C43S mutation, the work was extended by generating and expressing plasmids for four recombinant TNFRSF1A TRAPS mutants, including C43S, in B-cell lines by transient transfection. B-cell lines with low basal TNFα production and undetectable levels of endogenous TNFRSF1A expression by flow cytometry were used, allowing the mutated receptors to be studied in relative

isolation. While this kind of genetic modification is not without its problems, the transient transfection approach avoids some of the issues of overexpression that can occur with the generation of stable transfectants or inducible expression systems. TNFRSF1A expression is normally tightly regulated and expression of TNFRSF1A at high levels can trigger spontaneous signalling (Boldin *et al.*, 1995a; Todd *et al.*, 2004).

The results of the transient transfection experiments indicate that four different TRAPS mutations, namely C30R, C43S, T50M and C52F, have decreased NF-κB activity relative to WT TNFRSF1A. The decreased NF-κB activity in the TRAPS mutants is seen both at baseline and following stimulation with TNFα. These TRAPS mutants also appear to be associated with reduced cell death compared to WT TNFRSF1A. These results are consistent with those obtained using the C43S patient-derived dermal fibroblasts, suggesting that reduced TNFRSF1A signalling may be general feature of TRAPS, and not just restricted to the C43S mutation. However, there did also appear to be subtle differences in TNFRSF1A-mediated signalling between the TRAPS mutants studied. The C30R TRAPS mutation in particular resulted in minimal NF-κB activation above baseline levels but still appeared to be capable of responding to stimulation with TNFα. As mentioned previously, the TNF signal transduction pathways in B cells may differ from those in other inflammatory cells and it remains to be seen whether the same effects are seen in other cell types.

The TRAPS mutants used in the transient transfection experiments all had reduced surface expression and altered localisation of TNFRSF1A. The reduced surface expression of these mutants was associated with reduced TNFRSF1A signalling, suggesting a possible mechanism for the signalling defect. However, the results of these reporter assays have to be interpreted in the context of the subsequent expression experiments. As mentioned previously, there are fundamental limitations in studying TNFRSF1A signalling in cells on whose surface it is not clear that the TNFRSF1A receptor has been expressed. Reduced membrane expression of stably expressed TRAPS mutants was also recently reported by another group (Todd *et al.*, 2004). The surface expression of TNFRSF1A on the patient-derived cells could not be accurately assessed as

no specific antibodies are available that distinguish between WT and mutated TNFRSF1A. In addition, for the technical reasons outlined previously, it was not possible to analyse the fibroblasts by flow cytometry. The surface expression of the two recombinant TRAPS mutants investigated could not be restored by deletion of the intracellular domain, which has previously been shown to control receptor localisation (Hsu and Chao, 1993; Cottin *et al.*, 2002; Fielding *et al.*, 2004), suggesting that the effects of these extracellular domain mutations on cellular localisation are dominant.

The observed effects of the mutations on TNFRSF1A localisation may be due to structural effects of the mutations on receptor conformation, thereby interfering with processes such as protein folding or receptor trimerisation. Different TRAPS mutations may induce different structural changes, which could alter the interaction of some receptors with metalloproteinases, thereby reducing their shedding but not that of other mutants. In addition to altering receptor localisation, structural changes could account for the defective TNFα binding of recombinant TRAPS receptors reported by Todd *et al* (2004). Reduced surface expression of TNFRSF1A could also help explain the reduced circulating soluble TNFRSF1A levels reported in patients with TRAPS, particularly those where receptor shedding is normal. For these reasons, TRAPS may represent another member of the group of "protein conformational disorders", which include $alpha_1$-antitrypsin deficiency, Alzheimer's disease, Huntington's disease and Parkinson's disease, and are characterised by protein misfolding (Kopito and Ron, 2000; Carrell and Lomas, 2002).

TNFα-induced IL-6 and IL-8 production by the C43S TRAPS fibroblasts was similar to that seen with normal controls at the time point and TNFα dose tested, despite the reduced NF-κB and AP-1 activation. In contrast, CRP and serum amyloid A, surrogate markers for serum IL-6 (Ganapathi *et al.*, 1988), are elevated in patients with TRAPS. It is possible that different cell types, other than fibroblasts, produce increased amounts of IL-6, thereby accounting for the elevated serum IL-6. An alternative possibility is that an increase in the number of cells producing relatively normal amounts of IL-6 could lead to increased serum IL-6 levels. Taken together, it appears that IL-6

may be important to the pathology of the disease and so targeting IL-6 may be important therapeutically.

Taken together, this work suggests an alternative hypothesis. Namely, despite the inflammatory phenotype, the TRAPS mutations act as loss-of-function mutations. We have demonstrated defective TNFRSF1A signalling for four TRAPS mutations and evidence to suggest that one of the mechanisms responsible for this may be reduced TNFRSF1A expression on the surface of cells. It may be that the resultant defect in TNFRSF1A-mediated apoptosis results in impaired clearance of activated inflammatory cells, which remain capable of producing pro-inflammatory cytokines, thereby resulting in a systemic inflammatory phenotype.

TRAPS may therefore have parallels with the autoimmune lymphoproliferative syndrome (ALPS), an inflammatory condition associated with impaired apoptosis due to mutations of a different death domain-containing TNFR superfamily member. Reports have shown that mutations in the gene that encodes Fas (TNFRSF6), or molecules in the Fas apoptotic pathway, are associated with ALPS (Rieux-Laucat *et al.*, 1995; Rieux-Laucat *et al.*, 2003). Most patients with ALPS have heterozygous mutations of Fas that encode abnormal Fas proteins which exhibit dominant interference with normal Fas molecules in trimeric apoptosis-signalling complexes (Sneller *et al.*, 1997). Fas-mediated apoptosis is a prominent mechanism for eliminating activated lymphocytes (Lenardo *et al.*, 1999), therefore lymphocytes with Fas defects accumulate causing lymphadenopathy, splenomegaly, autoimmunity and increased risk of lymphoma (Rieux-Laucat *et al.*, 2003). The kinetics of apoptosis induced by Fas and TNFRSF1A vary, and are likely to be important in different cell types. This could explain the differences in the clinical presentations of these two syndromes. Interestingly, it was recently demonstrated that heterozygous Fas mutations can activate NF-κB normally but have impaired apoptosis as a result of different thresholds required to activate the two signalling pathways (Legembre *et al.*, 2004). This suggests that activation threshold levels play a role in the presentation of genetic conditions.

Interestingly, it appears that mutations in several other hereditary periodic fevers may also affect apoptosis and NF-κB activation. Pyrin, the familial Mediterranean fever (FMF) gene product, modulates ASC-(apoptosis-associated speck-like protein containing a CARD) dependent apoptosis, interleukin-1 production (Chae *et al.*, 2003) and NF-κB activation (Stehlik *et al.*, 2002). These interactions are complex and it still remains to be determined how mutations of *MEFV*, the gene encoding pyrin, affect these interactions (Grateau, 2004). Cryopyrin, the gene product of Muckle-Wells syndrome and familial cold autoinflammatory syndrome, also contains a PYRIN domain that has been shown to interact with ASC-mediated activation of NF-κB (Manji *et al.*, 2002) and is therefore theoretically also capable of interfering with ASC-mediated apoptosis. There is thus a precedent in the autoinflammatory syndromes for defects in apoptotic pathways being implicated in systemic inflammation.

Our results, using the primary fibroblast line established from the patient with TRAPS, have suggested that TNFα-induced signalling may be reduced, despite the inflammatory phenotype. We hypothesise that this is a general feature of TRAPS and intend to characterise the effect on cytokine production using peripheral blood leucocytes from the patient with TRAPS. These future experiments will allow us to confirm our findings in a population of cells directly involved in mediating the immune response. The production of cytokines (TNFα, IL-1, IL-6, IL-8) by leucocytes will be measured by cytokine multiplex assay, using cytometric bead array. Samples from the patient will be compared to samples from normal controls and RA patients attending our rheumatology clinic. There was some suggestion that NF-κB activation in response to IL-1 might be higher in the TRAPS fibroblasts than in the normal controls (Figure 3.8), raising the possibility that there may be compensatory increase in response to IL-1. Cytokine production in the leucocytes will therefore be assayed in response to a range of pro-inflammatory stimuli, including TNFα, IL-1, PMA and LPS, to determine if any alterations are limited to TNFα-mediated signalling or are global defects, and whether there is any compensatory increase in response to other stimulants.

The primary cell type responsible for the periodicity of the systemic inflammation in TRAPS remains unknown but circulating immune cells are likely to play a role. We intend to analyse the cell specific responses to TNFα in the TRAPS patient and RA patients. Analytical flow cytometry will be used to study the major leucocyte populations to determine whether there are differences in number, size or cell surface protein expression. We will also investigate the cell survival of distinct leucocyte populations to TNFα, and IL-1 over time *ex vivo*. This knowledge, together with analysis of the cytokine production by the leucocyte subpopulations, will help identify the cell population most likely to be responsible for the TRAPS phenotype. These experiments will ensure the appropriate cells are used for further investigation and may ultimately have therapeutic implications if more targeted, cell-specific therapies become available in future.

Based on the periodicity of the TRAPS phenotype and the high levels of circulating neutrophils in patients with TRAPS, we hypothesis that neutrophils play a central role in this inflammatory syndrome. While the cytokine multiplex assays may help to determine if this is indeed the case, the cytotoxic potential of neutrophils is mainly determined by the capacity of the respiratory burst. Therefore, we intend to assess the effects of this TRAPS mutation on neutrophil respiratory burst, in response to stimulation with TNFα, FMLP and PMA, by flow cytometry using dihydrorhodamine.

One intriguing result that became available during the course of this work was the patient's divergent response to the two different anti-TNFα agents (Figure 3.2). It was not possible to pursue this as part of the current work but we intend to characterise these observations further in future. Not only do these findings suggest an alternative hypothesis for the effects seen with this TRAPS mutation, but they also yield valuable insights into the anti-TNFα agents themselves. It has become increasingly clear that the monoclonal antibody infliximab, but not the soluble receptor fusion protein etanercept, is capable of binding mTNFα, resulting in reverse signalling through this ligand (Van den Brande *et al.*, 2003; Mitoma *et al.*, 2005). This difference has been proposed as the reason for the observed differences in efficacy between the various anti-TNFα agents in the treatment of Crohn's disease (Van den Brande *et al.*, 2003; Shen *et al.*, 2005). It is

possible that the rapid worsening of the patient's inflammatory condition was as a result of infliximab-induced reverse signalling through mTNFα. This suggests that mTNFα reverse signalling may play a role in the inflammatory process in our patient and could help explain the pro-inflammatory phenotype in the presence of apparent reduced soluble TNFα-mediated signalling. Reverse signalling through mTNFα can have pro- and anti-inflammatory effects, depending on cell type. It is therefore possible that TNFRSF1A on certain cells interacts with mTNFα to control an inflammatory response, possibly by the induction of apoptosis of immune cells. If this interaction were reduced, as a result of decreased membrane TNFRSF1A expression associated with this TRAPS mutation, then this control would be lost. This could account for the reduced apoptosis seen in the fibroblasts and PBMCs from the TRAPS patient. The mTNFα may then also be more available for further interactions, which on certain cells could result in a pro-inflammatory effect, and which would explain the inflammatory response seen following infliximab treatment.

We postulate that infliximab will result in increased cytokine production in leucocytes from the patient with TRAPS. In contrast, we would expect reduced cytokine production in these cells in response to etanercept, as well as in cells from normal controls or RA patients. The above cytokine multiplex experiments in the leucocytes will therefore be extended to assess the effects of treatment with infliximab and etanercept. These experiments will provide valuable information on the role of reverse signalling through mTNFα in TRAPS, and provide an insight into its importance for the therapeutic anti-inflammatory benefits of the different anti-TNFα agents used in clinical practice. Most previous studies have looked at the effects of mTNFα reverse signalling in monocytes/ macrophages and T lymphocytes, but it is not known whether reverse signalling occurs in neutrophils and what the effects are. These experiments will also help address this issue.

One of the poorly understood features of TRAPS, and the hereditary periodic fevers in general, is the presence of intermittent clinical inflammatory episodes in the face of a permanent underlying genetic defect. There may be influencing factors, other than the

actual genetic defects, such as modifying genes and/or environmental factors that affect the timing and severity of inflammatory episodes (Dipple and McCabe, 2000). A variety of non-genetic triggering factors, such as stress, exercise (McDermott *et al.*, 1997) and hormonal changes (Rosen-Wolff *et al.*, 2001) have been reported, although none are constant. However, it is noteworthy that despite the episodic symptoms, patients with TRAPS have laboratory evidence of persistent inflammatory activity during clinically asymptomatic episodes (Hull *et al.*, 2002b). It may be that critical rate-limiting thresholds have to be achieved before inflammation becomes clinically apparent.

In addition to the differences between the TRAPS mutants and WT TNFRSF1A, there also appear to be more subtle differences between the various TRAPS mutants. The C30R mutant in particular resulted in lower NF-κB activation and more restricted receptor expression than the other TRAPS mutants. Interestingly, unlike the C43S mutant, C30R appeared to be capable of responding to TNFα. This raises the possibility that the primary defect associated with the C30R mutation may be impaired TNFRSF1A receptor expression, while the C43S mutation may interfere primarily with ligand binding, and affect receptor expression to a lesser extent. Taken together with the reported differences in shedding (Aganna *et al.*, 2003; Huggins *et al.*, 2004), this suggests that the various TRAPS mutations may have a number of different effects, acting alone or in combination, accounting for the heterogeneity of the clinical manifestations of TRAPS. However, as mentioned previously, a kinetic abnormality in TNFRSF1A shedding cannot be fully excluded at this stage. The possible mechanisms of the TRAPS mutations may therefore include impaired receptor shedding (McDermott *et al.*, 1999a), reduced TNFRSF1A-mediated signalling with defective apoptosis, and possibly ligand-independent signalling (Todd *et al.*, 2004; Yousaf *et al.*, 2005), in addition to the alternative hypotheses relating to reverse signaling outlined above. Investigation of other TRAPS mutations will help determine the predominant processes involved. Some of the effects of the mutations may be cell type specific, which may help explain the predilection of TRAPS for certain anatomic sites. The generation of transgenic or knock-in mice with TRAPS mutations will provide further valuable information in determining the effects of these mutations on TNFRSF1A signalling.

In summary, these studies have suggested that certain TRAPS mutations are associated with reduced TNFRSF1A signalling, as a result of reduced surface expression of TNFRSF1A. The results suggest a new hypothesis whereby cytokine synthesis is maintained but cell apoptosis is reduced. Reduced clearance of inflammatory cells with defective TNFRSF1A-mediated apoptosis could lead to an increase in serum IL-6 levels *in vivo*, which is suggested by the clinical data, and suggests that IL-6 could be an important therapeutic target for treatment. If reduced TNFα signalling is also subsequently demonstrated for other TRAPS mutations, then approaches to block downstream pro-inflammatory cytokines (such as IL-6 and IL-1), in addition to current attempts to block TNFα, might prove beneficial. It is also possible that strategies to induce apoptosis in relevant cell types may be desirable. The data also suggests other alternative hypotheses worthy of further study. In addition, this study also demonstrates a previously unknown role for the proper formation of the extracellular domain of TNFRSF1A for the expression of the receptor on the cell surface, showing how the study of rare diseases can reveal new information about important molecules. Therefore, the importance of understanding the mechanisms involved in this syndrome extends beyond TRAPS and will reveal new insights about both physiological and pathological inflammatory processes, as well as yielding valuable information about the mechanisms of the increasingly used anti-TNFα agents.

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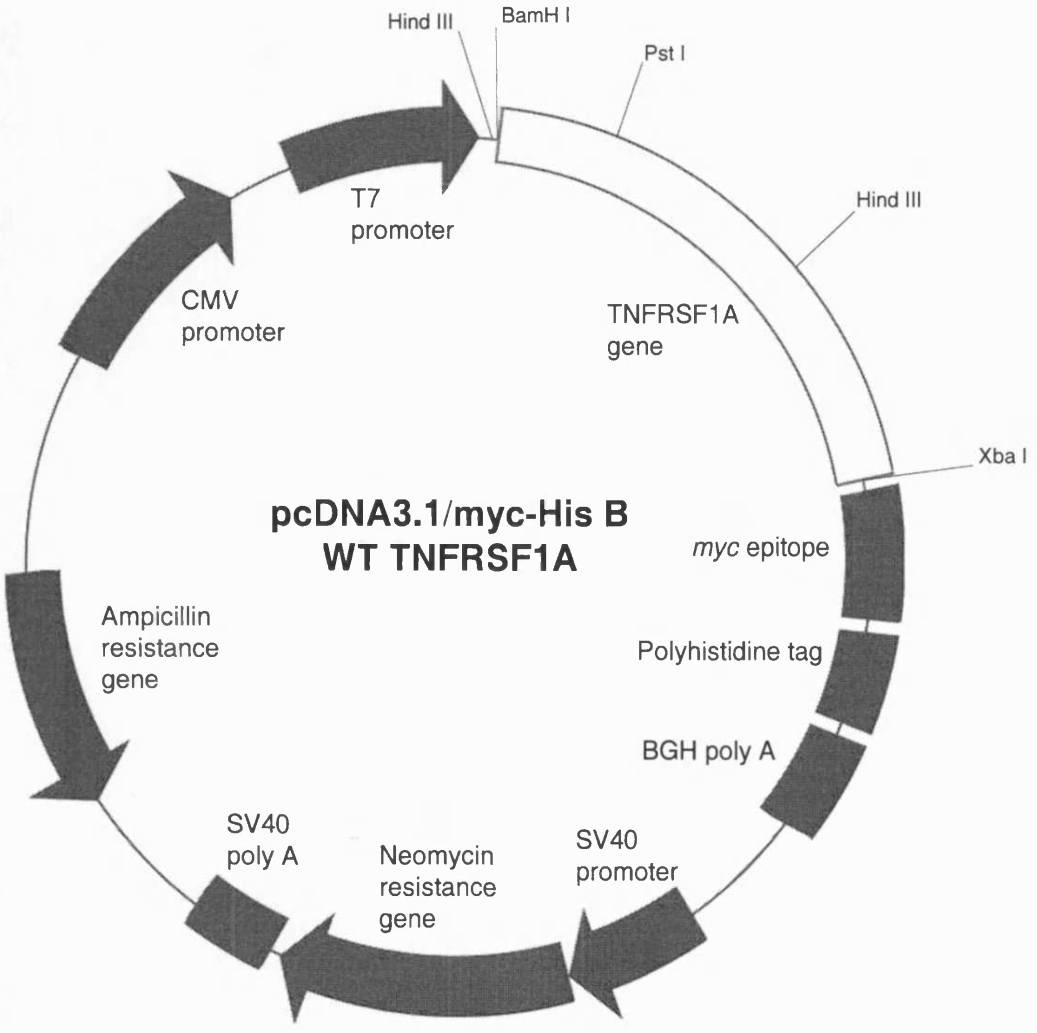
APPENDIX I

Plasmid maps for recombinant TNFRSF1A vectors



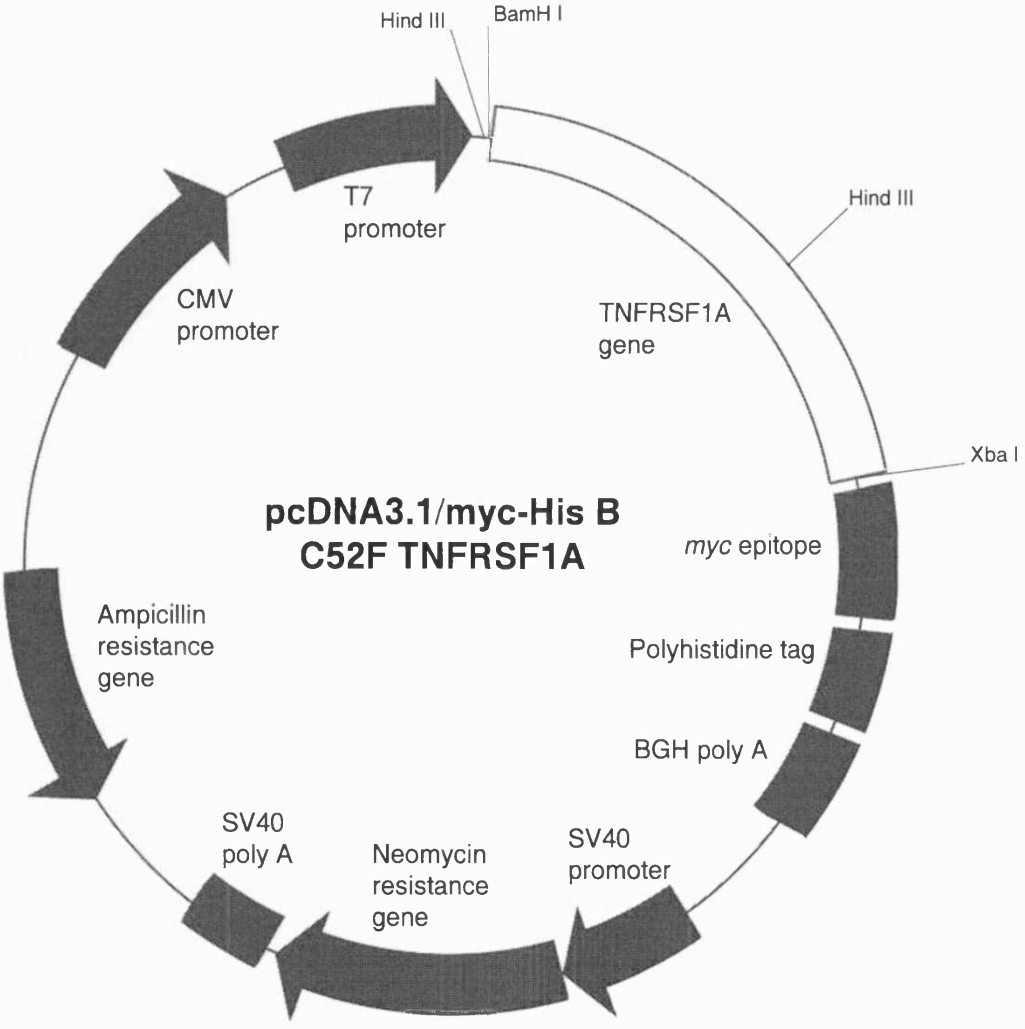
The following plasmids have the same plasmid map as that shown above:

- pcDNA3.1/myc-His WT TNFRSF1A
- pcDNA3.1/myc-His C30R TNFRSF1A
- pcDNA3.1/myc-His C43S TNFRSF1A
- pcDNA3.1/myc-His T50M TNFRSF1A

Constructed by: Stefan Siebert

Construction date: 2002

Comments: The TNFRSF1A mutants were generated by site-directed mutagenesis



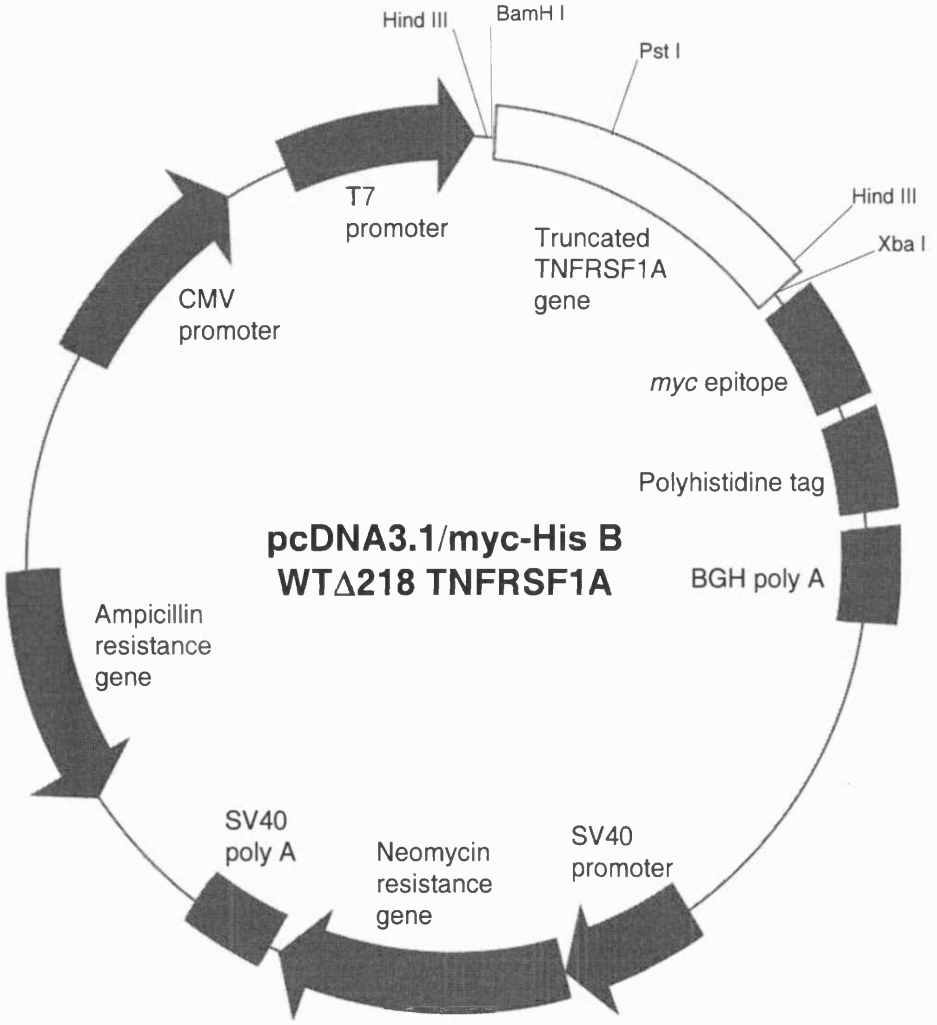
Plasmid name:

- pcDNA3.1/myc-His C52F TNFRSF1A

Constructed by: Stefan Siebert

Construction date: 2002

Comments: The C52F TNFRSF1A mutant was generated by site-directed mutagenesis. The C52F mutation removes a *Pst I* site present in WT TNFRSF1A.



The following plasmids have the same plasmid map as that shown above:

- pcDNA3.1/myc-His WTΔ218 TNFRSF1A
- pcDNA3.1/myc-His C43SΔ218 TNFRSF1A
- pcDNA3.1/myc-His T50MΔ218 TNFRSF1A

Constructed by: Stefan Siebert

Construction date: 2002

Comments: TNFRSF1A mutants were generated by site-directed mutagenesis

APPENDIX II

Patient information and consent forms

Department of Rheumatology/
Section of Infection and Immunity
Division of Medicine
Tenovus Building
Heath Park, Cardiff
CF14 4XX
Tel +44 (029)20747747

Contact name:
Dr Stefan Siebert
Clinical Research Fellow
Tel (029) 20746114
E-mail: SiebertS@cardiff.ac.uk

PATIENT INFORMATION SHEET

STUDY: Characterisation of the effects of mutations of the TNF Receptor1

The chemical messenger Tumour Necrosis Factor (TNF) is known to play a central role in the inflammation of a variety of diseases, best demonstrated in rheumatoid arthritis. As you are aware, a genetic mutation (alteration) of the one receptor for TNF (TNF Receptor1) has been found to be responsible for your particular condition, namely the TNF Receptor Associated Periodic Syndrome (TRAPS).

The exact mechanism by which this minor genetic change accounts the inflammatory illness that characterises TRAPS is however not known. Members of staff of the Department of Rheumatology and the Section of Infection and Immunity in Cardiff are studying the effects of alterations in the TNF Receptor1 on inflammation at a biochemical level. As your cells contain the TNF Receptor1 abnormality, they represent a unique opportunity to study these effects. This would be best studied in cells (fibroblasts) from a skin biopsy. These cells would be used to generate a primary fibroblast line and used for *in vitro* experiments. These cells would however be destroyed on your request at any stage.

We therefore request permission to perform a skin biopsy to obtain this tissue. The biopsy (2-5mm^2) is performed with local anaesthetic in the outpatient clinic. The biopsy would be on your arm and will leave a small scar. If you do not wish to have this performed, your treatment will not be affected in any way. However, if you do agree, thank you for your assistance in helping our research.

PATIENT CONSENT FORM

STUDY: Characterisation of the effects of mutations of the TNF Receptor1

1. I _____ hereby fully and voluntarily agree to having a skin biopsy performed for research purposes by the Department of Rheumatology and the Section of Infection and Immunity, Cardiff.

2. I have read the information document and have been given a full explanation by the supervising doctor, Dr_____ as to the nature and purpose of the study. I have been advised about any discomfort which may result from the procedure and have been given the opportunity to question the supervising doctor on all aspects of the study and procedure. I have understood the information given to me.

3. I understand that I am free to ask for any cells obtained from the procedure to be immediately destroyed at any stage, without needing to justify my decision.

Signature of Patient_____ Date_____

Signature of Supervising Doctor_____ Date_____

Signature of Witness_____ Date_____

APPENDIX III

Publications:

- Siebert *et al.*, 2005 Arthritis and Rheumatism 52: 1287-92
- Siebert *et al.*, 2005 FEBS Letters 579: 5193-8

ARTHRITIS & RHEUMATISM
Vol. 52, No. 4, April 2005, pp 1287–1292
DOI 10.1002/art.20955

Reduced Tumor Necrosis Factor Signaling in Primary Human Fibroblasts Containing a Tumor Necrosis Factor Receptor Superfamily 1A Mutant

Stefan Siebert,[1] Nick Amos,[1] Ceri A. Fielding,[1] Eddie C. Y. Wang,[1] Ivona Aksentijevich,[2] Bryan D. Williams,[1] and Paul Brennan[1]

Objective. **Tumor necrosis factor receptor–associated periodic syndrome (TRAPS) is an autoinflammatory syndrome associated with mutations in the gene that encodes tumor necrosis factor receptor superfamily 1A (TNFRSF1A). The purpose of this study was to describe a novel TNFRSF1A mutation (C43S) in a patient with TRAPS and to examine the effects of this TNFRSF1A mutation on tumor necrosis factor α (TNFα)–induced signaling in a patient-derived primary dermal fibroblast line.**

Methods. **TNFRSF1A shedding from neutrophils was measured by flow cytometry and enzyme-linked immunosorbent assay (ELISA). Primary dermal fibroblast lines were established from the patient with the C43S TRAPS mutation and from healthy volunteers. Activation of NF-κB and activator protein 1 (AP-1) was evaluated by electrophoretic mobility shift assays. Cytokine production was measured by ELISA. Cell viability was measured by alamar blue assay. Apoptosis was measured by caspase 3 assay in the fibroblasts and by annexin V assay in peripheral blood mononuclear cells.**

Results. **Activation-induced shedding of the TNFRSF1A from neutrophils was not altered by the C43S TRAPS mutation. TNFα-induced activation of NF-κB and AP-1 was decreased in the primary dermal fibroblasts with the C43S TNFRSF1A mutation. Nevertheless, the C43S TRAPS fibroblasts were capable of producing interleukin-6 (IL-6) and IL-8 in response to TNFα. However, TNFα-induced cell death and apoptosis were significantly decreased in the samples from the patient with the C43S TRAPS mutation.**

Conclusion. **The C43S TNFRSF1A mutation results in decreased TNFα-induced nuclear signaling and apoptosis. Our data suggest a new hypothesis, in that the C43S TRAPS mutation may cause the inflammatory phenotype by increasing resistance to TNFα-induced apoptosis.**

Tumor necrosis factor receptor–associated periodic syndrome (TRAPS; MIM no. 142680) is an autosomal-dominant inherited autoinflammatory syndrome characterized by recurrent fevers and abdominal pain associated with cutaneous, muscle, and joint inflammation. It is associated with mutations in the gene that encodes tumor necrosis factor receptor superfamily 1A (TNFRSF1A) (1). At least 40 TNFRSF1A mutations associated with TRAPS have been reported on the INFEVERS (Internet periodic fevers) Web site (http://fmf.igh.cnrs.fr/infevers) (2). Initial studies suggested that the TRAPS mutations impair activation-induced shedding of TNFRSF1A (1,3), although this is not the case for all mutations (4,5) and may be dependent on cell type (6). There are no published studies on tumor necrosis factor α (TNFα)–induced activation of transcription factors or apoptosis in cells derived from patients with TRAPS. The mechanisms by which these TNFRSF1A point mutations result in the inflammatory phenotype remain unclear.

TNFα exerts its proinflammatory effects through 2 receptors, namely, TNFRSF1A (TNFRI p55) and TNFRSF1B (TNFRII p75). TNFRSF1A is widely ex-

Dr. Siebert's work was supported by an educational grant from Schering-Plough. Dr. Fielding's work was supported by the Leverhulme Trust. Dr. Brennan's work was supported by the Leukaemia Research Fund.

[1]Stefan Siebert, MBBCh, MRCP, Nick Amos, MSc, Ceri A. Fielding, PhD, Eddie C. Y. Wang, PhD, Bryan D. Williams, FRCP, FRCPath, Paul Brennan, PhD: Wales College of Medicine, Cardiff University, Cardiff, UK; [2]Ivona Aksentijevich, MD: National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH, Bethesda, Maryland.

Address correspondence and reprint request to Stefan Siebert, MBBCh, MRCP, Section of Infection and Immunity, Henry Wellcome Building, Wales College of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XX, UK. E-mail: sieberts@cardiff.ac.uk.

Submitted for publication September 24, 2004; accepted in revised form December 20, 2004.

pressed and appears to be the major receptor for soluble TNFα-induced signaling (7). Activation of these receptors recruits adapter proteins to the intracellular domain of the receptor and activates downstream signaling cascades (8). This causes the activation of NF-κB and activator protein 1 (AP-1), which regulate the transcription of a variety of genes, including interleukin-6 (IL-6) and IL-8. Many of these TNFα-induced molecules (such as NF-κB, IL-6, and IL-8) are elevated in inflammatory conditions, such as rheumatoid arthritis (9), making them good candidates for investigation in TRAPS. In addition, the intracellular domain of TNFRSF1A contains a death domain motif, which is involved in TNF-induced apoptosis via activation of a caspase cascade (8).

We describe a novel TNFRSF1A mutation (C43S) in a patient with TRAPS that does not impair activation-induced shedding of TNFRSF1A. We generated a primary dermal fibroblast line from this patient that showed decreased TNFα-induced NF-κB and AP-1 activation relative to that in normal controls. However, TNFα was able to induce IL-6 and IL-8 to levels similar to those in the controls. TNFα-induced cell death and apoptosis were markedly decreased in the fibroblasts with the C43S TRAPS mutation compared with the control fibroblasts. In addition, we observed decreased TNFα-induced apoptosis in the patient's peripheral blood mononuclear cells (PBMCs). We hypothesize that this reduced apoptosis in response to TNFα may be a factor in the inflammatory phenotype of this patient.

MATERIALS AND METHODS

Generation of primary dermal fibroblast cell line. Primary skin fibroblast lines were established using an adapted method (10) after ethical permission and informed consent were granted. Lignocaine (1%) was injected intradermally to anesthetize and raise the biopsy area. A small biopsy sample (~2 mm^3) was obtained, cut into 8 small fragments, placed into 35-mm surface-modified tissue culture dishes (Primaria Easy Grip; Becton Dickinson, Mountain View, CA), and covered with a glass coverslip. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% (volume/volume) fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* glutamine. The medium was changed at weekly intervals. When cells were confluent, they were transferred to tissue culture flasks and maintained in DMEM containing 10% v/v FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* glutamine. Early-passage fibroblasts were stored in liquid nitrogen.

Generation of cell extracts. Early-passage fibroblasts (passages 5–10) were seeded overnight at 0.3×10^6 cells per 60-mm tissue culture dish. They were stimulated with TNFα (Calbiochem, La Jolla, CA). Stimulation was terminated by removal of DMEM and addition of ice-cold phosphate buffered saline. Cells were harvested using a cell scraper. All buffers were supplemented with a protease inhibitor (phenylmethylsulfonyl fluoride) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO). Nuclear extracts were generated and electrophoretic mobility shift assays (EMSAs) were performed as previously described (11).

Annexin V assay in PBMCs. PBMCs were isolated from fresh whole blood using a Ficoll gradient. PBMCs (2×10^6) were placed in 2 ml RPMI 1640 with 10% FCS and were left untreated or were stimulated with TNFα and cycloheximide (CHX; 50 μg/ml) either alone or in combination. Apoptosis was assayed using the TACS annexin V–fluorescein isothiocyanate kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions, after which fluorescence was measured by flow cytometry.

RESULTS

Identification of a novel TNFRSF1A mutation (C43S). The index patient was a 50-year-old woman of Welsh origin with recurrent attacks of fever, pharyngitis, and arthritis accompanied by a migrating skin rash and myalgia. Her first attack occurred at the age of 18 months, with attacks typically lasting 1–2 weeks. The patient's deceased father had similar recurrent episodic fevers. The patient's clinical picture was consistent with the reported TRAPS phenotype (1). Mean ± SEM plasma levels of soluble TNFRSF1A were 3,839 ± 131 pg/ml, which is consistent with levels reported in TRAPS patients with renal impairment (5). DNA was extracted from the patient's blood, and polymerase chain reaction (PCR) amplifications were performed, with subsequent sequencing of the PCR products. We identified a novel TNFRSF1A mutation (nucleotide 215 G→C, exon 3) resulting in the substitution of serine for cysteine at residue 43 (C43S). This amino acid substitution disrupts the disulfide bond at this position in the first extracellular domain of TNFRSF1A. This substitution was not observed in any of the 734 control chromosomes screened by genomic sequencing.

Effect of the C43S mutation on shedding of TNFRSF1A. To investigate whether TNFRSF1A shedding was affected by the C43S mutation, we measured levels of TNFRSF1A on the surface of the patient's neutrophils by flow cytometry. Following phorbol myristate acetate (PMA) treatment, membrane TNFRSF1A decreased to levels comparable with those of healthy controls (Figure 1A). To establish whether this decrease in surface TNFRSF1A was a result of internalization or cleavage of the receptor, levels of soluble TNFRSF1A were measured in the supernatant of the cells. Soluble TNFRSF1A levels increased in response to PMA, indicating that the loss of surface TNFRSF1A was a result of

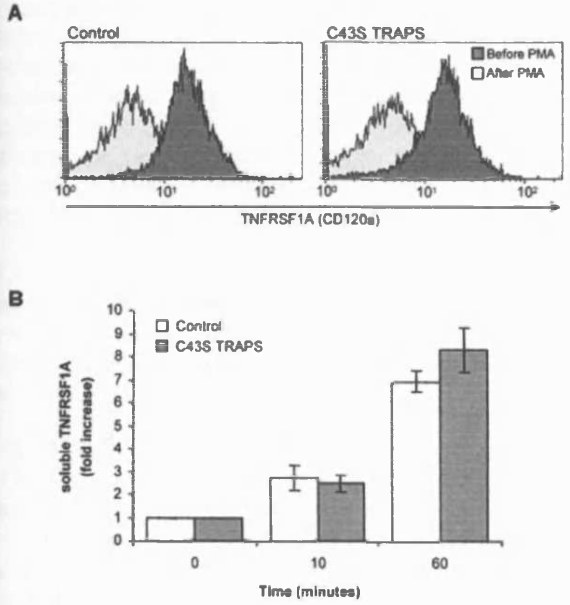


Figure 1. Levels of activation-induced shedding of tumor necrosis factor receptor superfamily 1A (TNFRSF1A) from neutrophils obtained from a patient with C43S TNFR-associated periodic syndrome (TRAPS). **A,** Membrane expression of TNFRSF1A (CD120a) on neutrophils from a healthy volunteer and from the C43S TRAPS patient before and after stimulation with phorbol myristate acetate (PMA). Cells were stained with phycoerythrin-conjugated anti-CD120a and analyzed by flow cytometry. **B,** After PMA stimulation, supernatants were collected, and soluble TNFRSF1A levels were measured by enzyme-linked immunosorbent assay. Values are the mean ± SEM changes in soluble TNFRSF1A levels relative to baseline levels in 3 independent experiments. Mean ± SEM absolute values at time 0 were 13.45 ± 0.2 pg/ml in the control subject and 4.77 ± 0.98 pg/ml in the TRAPS patient.

receptor shedding (Figure 1B). Activation-induced cleavage was therefore not impaired by the C43S mutation, and could not account for the inflammatory phenotype in this patient.

Generation of a primary TRAPS fibroblast line. To facilitate the study of the C43S TNFRSF1A mutation, we generated a primary dermal fibroblast cell line from a skin biopsy sample taken from the patient. The skin is an accessible site for obtaining fibroblasts and is also the site of the prototypical rash that characterizes TRAPS. The sample was taken from an area of normal skin during an asymptomatic period. Similar dermal fibroblast lines were generated from age- and sex-matched healthy volunteers and used as controls.

Role of the C43S TNFRSF1A mutation in NF-κB and AP-1 activation and induction of IL-6 or IL-8. TNFRSF1A nuclear signaling was investigated using the dermal fibroblast lines. Cells were stimulated with 10 ng/ml of TNFα for 1 hour, after which nuclear extracts were generated. NF-κB activation was determined by EMSA using radiolabeled DNA corresponding to a specific NF-κB site. All the fibroblast lines were able to activate NF-κB in response to TNFα. However, the activation was consistently less in fibroblasts from the patient with TRAPS compared with fibroblasts from healthy controls (Figure 2A). There was no evidence of

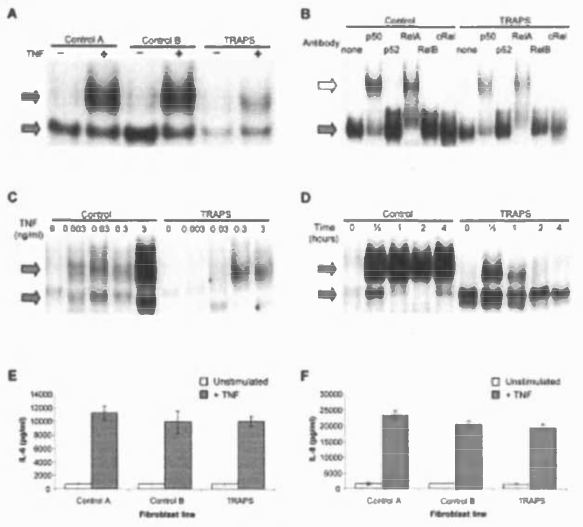


Figure 2. Effect of the C43S TNFRSF1A mutation on NF-κB activation and cytokine production in dermal fibroblasts. **A,** Electrophoretic mobility shift assay (EMSA) showing NF-κB activation in fibroblasts from 2 controls and the patient with C43S TRAPS, stimulated for 1 hour with TNFα (10 ng/ml). Densitometric analysis revealed a mean ± SEM reduction of 65.6 ± 2.8% in the TRAPS fibroblasts compared with controls in 5 separate experiments. DNA–protein complexes are indicated (arrows). Free (unbound) radiolabeled DNA was distinct from these complexes and is not shown in any of the figures. **B,** NF-κB supershift assay. TNFα-activated nuclear extracts were incubated with antibodies to the various subunits of NF-κB prior to incubation with the radiolabeled oligonucleotide probe. DNA–protein complexes (solid arrow) and antibody–DNA–protein complexes (open arrow) are indicated. **C,** EMSA of nuclear extracts showing a TNFα dose response at 1 hour for NF-κB activation in control and C43S TRAPS fibroblasts. Densitometric analysis revealed a mean ± SEM 60.2 ± 7.6% reduction in NF-κB activation in the TRAPS fibroblasts over a range of TNF doses in 5 separate experiments. **D,** EMSA showing the time course of NF-κB activation after stimulation of fibroblasts with 10 ng/ml TNFα. All EMSA results shown are representative of at least 5 separate experiments and were similar in both controls. **E,** Production of interleukin-6 (IL-6) and **F,** production of IL-8 by the primary dermal fibroblasts, as measured by enzyme-linked immunosorbent assay (ELISA) of the culture supernatants 24 hours after stimulation with 10 ng/ml TNFα. ELISA results are the mean ± SEM of 9 observations from 3 independent experiments for IL-6 and of 6 observations from 2 independent experiments for IL-8. See Figure 1 for other definitions.

constitutive activation of NF-κB in the TRAPS fibroblasts. In response to IL-1 stimulation, the TRAPS fibroblasts activated NF-κB to levels similar to those of healthy controls (data not shown). Supershift assays with antibodies to NF-κB subunits indicated that the complexes are very similar, with both the TRAPS and control fibroblasts activating an NF-κB complex containing p50 and RelA subunits (Figure 2B).

To determine whether the NF-κB activation in C43S TRAPS fibroblasts was more sensitive to TNFα or more prolonged than in healthy controls, TNFα dose-response and time-course experiments were performed. TNFα-induced NF-κB activation was consistently lower in the TRAPS fibroblasts at all doses of TNFα tested (Figure 2C). In addition, the duration of TNFα-induced NF-κB activation was significantly shorter in the C43S TRAPS fibroblasts compared with the control fibroblasts (Figure 2D). There was also no increase in NF-κB activation at any later time points up to 24 hours (results not shown). The C43S fibroblasts also resulted in less activation of AP-1 compared with the normal fibroblasts across a range of TNFα doses and time points (results not shown). The above results were consistent for both control fibroblast lines.

To determine whether the C43S TRAPS mutation altered TNFα-induced production of IL-6 and IL-8, the levels of these cytokines in the culture supernatants of the primary dermal fibroblasts were measured by enzyme-linked immunosorbent assay. While absolute values varied with the passage number, the induction of IL-6 (Figure 2E) and IL-8 (Figure 2F) in response to TNFα in the C43S TRAPS fibroblast line was not statistically different from that observed in the controls. Baseline levels of the cytokines were also similar in the fibroblast lines. Therefore, in spite of the reduced NF-κB and AP-1 activation, the C43S TRAPS fibroblast line was able to produce IL-6 and IL-8 in response to TNFα.

Reduction of TNFα-induced apoptosis in dermal fibroblasts and PBMCs due to C43S TRAPS mutation. Another important TNFRSF1A-mediated effect is the induction of apoptosis. TNFα also induces antiapoptotic genes, and therefore the apoptotic response to TNFα is usually dependent on the inhibition of protein synthesis (12). Fibroblasts were stimulated with TNFα (10 ng/ml) either alone or in the presence of CHX (13). Three assays for cell survival were used. First, cells were examined by light microscopy (BX41 microscope; Olympus, Lake Success, NY) after 24 and 48 hours. Dramatic differences were observed at both time points, with more TRAPS-derived fibroblasts than wild-type cells surviving the combination of TNFα and CHX (Figure 3A). The differences observed with light microscopy were quantified using an alamar blue assay (14). This nontoxic dye is chemically reduced by the innate metabolic activity of

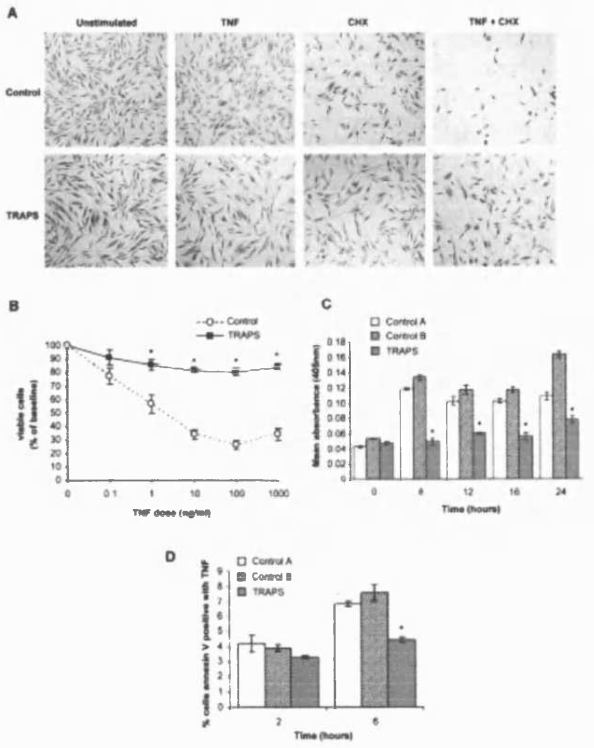


Figure 3. TNFα-induced apoptosis in C43S TRAPS fibroblasts and peripheral blood mononuclear cells (PBMCs). Fibroblasts (5×10^4) were stimulated with TNFα (10 ng/ml) and cycloheximide (CHX; 50 μg/ml), either alone or in combination. **A,** Light microscopy images (4× objective) of fibroblasts after 24 hours of stimulation. Cells were stained with toluidine blue. Results shown are representative of 5 separate experiments. **B,** Cell viability of C43S TRAPS and control fibroblasts after 24 hours of incubation with varying doses of TNFα in the presence of CHX, as measured by alamar blue assay. Values are the mean ± SEM percentage of baseline levels (CHX alone). * = $P <$ 0.05 versus controls, by Student's *t*-test. **C,** TNFα-induced caspase 3 activity. Fibroblasts (5×10^5) were stimulated with TNFα (10 ng/ml) and CHX (50 μg/ml), after which they were analyzed for caspase 3 activity at the time points shown. Values are the mean ± SEM of 5 independent experiments. * = $P < 0.05$ versus controls. **D,** Annexin V assay of PBMCs. PBMCs from the patient and 2 healthy volunteers were incubated with TNFα and CHX for the times indicated. Annexin V activity was determined by flow cytometry, with gating on live (propidium iodide–negative) cells. Results shown are the difference between the mean in cells stimulated with both TNFα and CHX and the mean in unstimulated cells. Values are the mean ± SEM of 2 independent experiments. * = $P < 0.05$ versus controls. See Figure 1 for other definitions.

cells, which allows quantification of cell viability by fluorometry. Figure 3B shows the effect of a range of doses of TNFα on the survival of C43S TRAPS fibroblasts and a control fibroblast line. TRAPS-derived fibroblasts were markedly less sensitive to TNFα-induced cell death. Statistically significant ($P < 0.05$) differences in cell viability were observed at doses of TNFα >1 ng/ml. Caspase 3 activity, a measure of apoptosis, was also assayed. TNFα and CHX induced significantly less caspase 3 activity in the TRAPS fibroblasts at all time points measured (Figure 3C).

Because the effects of TNFα can vary in different cells (12), we investigated whether the C43S TNFRSF1A mutation would also result in decreased sensitivity to TNFα-induced apoptosis in circulating inflammatory cells. PBMCs from the patient with the C43S TRAPS mutation and from healthy volunteers were isolated. PBMCs were stimulated with TNFα and CHX for either 2 or 6 hours. Cells were stained with annexin V and propidium iodide (PI) and analyzed by flow cytometry. Cells in the live gate (PI negative) were analyzed for annexin V staining. Although similar percentages of unstimulated cells were positive for annexin V, after stimulation with TNFα and CHX, fewer PBMCs from the patient with TRAPS were annexin V positive at both time points (Figure 3D). The difference observed at 6 hours was statistically significant ($P < 0.05$). The C43S TNFRSF1A mutation therefore resulted in decreased TNFα-induced apoptosis in both fibroblasts and PBMCs.

DISCUSSION

This report describes a novel TNFRSF1A mutation (C43S) associated with TRAPS and characterizes the signaling abilities of this mutation in a primary dermal fibroblast line established from the patient. TNFα activated the transcription factors NF-κB and AP-1 at reduced levels in C43S TRAPS fibroblasts but was able to induce the proinflammatory cytokines IL-6 and IL-8 to levels similar to those in healthy controls. TNFα-induced apoptosis was significantly decreased in the fibroblasts bearing the C43S TNFRSF1A mutation. This defect in TNFα-induced apoptosis was also seen in PBMCs isolated from the patient. Thus, this study demonstrates that this TRAPS mutation results in reduced TNFα-induced nuclear signaling and apoptosis in this patient.

The initial studies of TRAPS cells proposed a mechanism of impaired activation-induced cleavage of TNFRSF1A that could cause the systemic inflammation associated with this syndrome (1,3). However, impaired shedding of TNFRSF1A does not appear to be the case for all TRAPS mutations (4,5). Our results support the hypothesis that TRAPS does not require impaired activation-induced shedding of TNFRSF1A from neutrophils.

Our data show that the C43S TRAPS fibroblasts exhibit reduced NF-κB and AP-1 activation and decreased apoptosis in response to stimulation with TNFα. However, no significant difference in the induction of proinflammatory cytokines was observed, findings consistent with those of a previous study (1). This suggests a possible hypothesis to explain the inflammatory pathology: cells survive longer because of impaired apoptosis, but remain capable of producing proinflammatory cytokines. The levels of these cytokines would be expected to accumulate and could result in an inflammatory phenotype. Apoptosis of inflammatory cells is an important homeostatic mechanism for limiting an inflammatory response once it is established (15). Our hypothesis is also compatible with the high levels of serum amyloid A and C-reactive protein, surrogate markers for serum IL-6, noted in our and other TRAPS patients (1). However, this hypothesis is based on results from a single patient, and it remains to be established whether this is also the case in other TRAPS patients.

Because both IL-6 and IL-8 are regulated by NF-κB, the question remains of how the induction of these proinflammatory cytokines is normal, despite the reduced NF-κB activation. We propose 2 possible explanations. The first is that the threshold for TNFα-induced cytokine production is lower than that for apoptosis. Thus, reduced NF-κB still generates a sufficient signal to allow induction of both IL-6 and IL-8. A second explanation is that signaling via TNFRSF1B (TNFRII) may play a role. TNFRSF1B is able to activate NF-κB, but does not contain a death domain (8) and, thus, could induce IL-6 and IL-8 without causing apoptosis. TNFα generally results in less activation via TNFRSF1B than TNFRSF1A in vitro (16), which may explain the reduced NF-κB activation observed in our experiments.

From the published literature, it appears likely that the different TNFRSF1A mutants may induce TRAPS by different mechanisms. Some mutations affect TNFRSF1A shedding, while others do not. It will be important to determine whether other TRAPS mutations have similarly reduced TNFα nuclear signaling and apoptosis, as is the case with the C43S mutation. Reduced TNFα signaling could explain why treatment with TNF blocking agents does not completely abolish acute attacks in TRAPS patients (17). If reduced TNFα sig-

naling is also demonstrated for other TRAPS mutations, then approaches to block the downstream proinflammatory cytokines (such as IL-6 and IL-1), in addition to current attempts to block TNFα, might prove beneficial. It is also possible that strategies to induce apoptosis in relevant cell types may be beneficial. In summary, this study suggests that, in some patients, TRAPS may be a result of defective or reduced TNFα-induced nuclear signaling and apoptosis.

ACKNOWLEDGMENTS

We thank the index patient and the healthy volunteers who provided valuable clinical samples. We thank Professor Martin Rowe for his help and advice.

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FEBS 29936 FEBS Letters 579 (2005) 5193–5198

Mutation of the extracellular domain of tumour necrosis factor receptor 1 causes reduced NF-κB activation due to decreased surface expression

Stefan Siebert[a], Ceri A. Fielding[a], Bryan D. Williams[b], Paul Brennan[a,*]

[a] *Department of Medical Biochemistry and Immunology, Wales College of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XX, United Kingdom*

[b] *Department of Rheumatology, Wales College of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XX, United Kingdom*

Received 15 June 2005; revised 29 July 2005; accepted 17 August 2005

Available online 31 August 2005

Edited by Lukas Huber

Abstract Tumour necrosis factor receptor-associated periodic syndrome (TRAPS) results from point mutations in the extracellular domain of TNF receptor 1 (TNFRSF1A), but the effects of the mutations are controversial. This study shows that reduced NF-κB signalling is a feature of four TRAPS mutations. Reduced signalling correlates with reduced surface expression, measured by flow cytometry and microscopy. This suggests that correct formation of the extracellular domain of TNFRSF1A is important for localisation and receptor function. Importantly, our data provides a mechanism for the reduced TNFRSF1 signalling observed in a patient cell line.

Keywords: Tumour necrosis factor; TRAPS; NF-κB

1. Introduction

Tumour necrosis factor receptor-associated periodic syndrome (TRAPS; MIM 142680) is an autoinflammatory syndrome, characterised by recurrent fevers with cutaneous, muscle and joint inflammation, associated with autosomal dominantly-inherited mutations in the gene that encodes tumour necrosis factor (TNF) receptor superfamily 1A (TNFRSF1A) [1]. TNFα exerts its many pro-inflammatory effects through two distinct receptors: TNFRSF1A (TNFR1, p55/p60-TNFR) and TNFRSF1B (TNFR2, p75/p80-TNFR). TNFRSF1A is widely expressed and appears to be the major receptor for soluble TNFα-induced signaling [2]. At least 60 TNFRSF1A mutations associated with TRAPS have been reported on the INFEVERS (Internet periodic fevers) website (http://fmf.igh.cnrs.fr/infevers/). The majority are located in the first or second cysteine-rich extracellular domains (CRD1 and CRD2). The binding site for TNFα is formed by CRD2 and CRD3 of TNFRSF1A [3], while CRD1, also known as the pre-ligand assembly binding domain, is thought to mediate TNFRSF1A self-assembly [4].

The initial study of TRAPS suggested that the TNFRSF1A mutations impair activation-induced shedding of the receptor [1]. However, this is not the case for all mutations [5,6] and varies according to cell type [7]. Another study has shown that overexpression of TRAPS-associated TNFRSF1A mutants spontaneously induce apoptosis and interleukin-8 production although TNFα binding to the mutated receptors appears to be defective [8]. We have recently shown that cells from a patient with TRAPS, bearing the C43S mutation, have decreased NF-κB induction and TNFα-induced apoptosis, although IL-6 and IL-8 production were normal [6] suggesting that reduced TNFRSF1A signalling may be a feature of TRAPS.

This study was initiated to investigate whether reduced TNFRSF1A signalling was a general feature of TRAPS mutations. We transfected cells with either wild-type (WT) or the following mutant recombinant forms of TNFRSF1A: C30R, C43S, T50M and C52F. We investigated the effects of these clinically relevant mutations on receptor function, in terms of NF-κB activation, on cell death and on receptor expression. Our work shows that all of these TRAPS mutations result in decreased TNFRSF1A signalling and that this loss of signalling correlates with reduced expression of the receptor on the surface of cells.

2. Materials and methods

2.1. Production of recombinant TNFRSF1A DNA clones and plasmids

The TNFRSF1A coding region was ligated into the pcDNA3.1 myc/His B vector (Clontech). The TRAPS mutations were generated using the Quikchange site-directed mutagenesis kit (Stratagene). Primers used: C30R: 5′-CGATTTGCCGTACCAAGTGCCAC-3′, C43S: 5′-CTTGTACAATGACTCTCCAGGCCCGGGGC-3′, T50M 5′-CCGGGGCAGGATATGGACTGCAGGGAG-3′, C52F: 5′-GGCAGGATACGGACTTCAGGGAGTGTG AGAG-3′ and their reverse complements. Mutations were confirmed by sequencing. The 3Enh.κB-ConALuc reporter (3EnhLuc) contains three NF-κB binding [9].

2.2. Cells, transfection, luciferase reporter assay and cell viability assay

Eli-BL and DG75 B-cell lymphoma culture has been described [10]. 10^7 cells in 0.5 ml culture medium with 100 mM HEPES (pH 7.2), were transfected using a Biorad Genepulser II (270 V/950 μF). Transfection efficiency was typically 5–20% for the Eli-BL and 40–50% for the DG75. Luciferase assays were performed in the transfected Eli-BL cells. Cells were stimulated after 16 h. Luciferase activity was measured 8 h later [11]. Transfection efficiency was assessed using the Promega Dual-Luciferase reporter system.

Cell viability was assessed by alamar blue assay (Biosource). Briefly, 2×10^4 transfected cells were resuspended in 200 μl of 10% alamar blue reagent in growth medium on a 96 well plate. After 4 h incubation at 37 °C, readings were made on a FLUOstar Optima (BMG Laboratories) using the fluorescence configuration.

*Corresponding author. Fax: +44 292074 3868.
E-mail address: brennanp@cf.ac.uk (P. Brennan).

Abbreviations: TRAPS, tumour necrosis factor receptor-associated periodic syndrome; TNFα, tumour necrosis factor alpha; TNFRSF, TNF receptor superfamily; CRD, cysteine-rich domain

0014-5793/$30.00
doi:10.1016/j.febslet.2005.08.037

2.3. Detection of TNFRSF1A expression by flow cytometry or immunofluorescence

For surface expression, cells were harvested 24 h after transfection, washed with phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde. Cells were washed and resuspended in 10% normal rabbit serum (NRS) in PBS for 20 min. Cells were stained with anti-TNFRSF1A monoclonal antibody (Ab-1, Calbiochem) followed by an anti-mouse R-phycoerytherin (RPE)-conjugated $F(ab')_2$ fragment (Dako), or with mouse IgG1-conjugated PE negative control (Serotec). Washed cells were resuspended in 2% paraformaldehyde and analysed using a Becton Dickinson FACScalibur. Total (surface and intracellular) expression of TNFRSF1A was assayed following permeabilisation with 0.1% Triton X-100/PBS after fixation and stained as before.

For confocal microscopy, cells were washed, fixed with 2% paraformaldehyde, resuspended in PBS and air-dried. Cells were permeabilised with 0.1% Triton X-100/PBS, incubated in 10% NRS/PBS and stained with the same anti-TNFRSF1A antibody followed by an Alexa Fluor 488 anti-mouse IgG antibody (excited 488 nm – detected 520 nm). For nuclear staining, cells were incubated with DRAQ5 10 μM (Biostatus) for 10 min (excited 647 nm – detected 680/30 nm). Images were acquired using a confocal laser scanning microscope (Biorad), with a krypton/argon ion laser and a Zeiss Axiovert 135. Results are representative of five experiments.

3. Results

3.1. The TNFRSF1A mutants result in reduced NF-κB activity

NF-κB is a critical transcription factor for the induction of inflammation by TNFα [12]. NF-κB activation by wild-type and mutant recombinant forms of TNFRSF1A was assessed by luciferase reporter assay in transiently transfected Eli-BL cells. Eli-BL cells express undetectable levels of membrane TNFRSF1A by flow cytometry and have low endogenous responses to TNFα [10], allowing measurement of alterations in NF-κB activity as a result of transfection with recombinant TNFRSF1A. Eli-BL cells were co-transfected with the NF-κB luciferase reporter plasmid and either empty mammalian vector, mammalian expression vectors for wild-type TNFRSF1A or a TNFRSF1A mutant. The mutants used were C30R, C43S, T50M and C52F. C43S was the TRAPS mutation identified and described by us previously [6]. C30R is the cysteine on the opposite side of the disulfide bond to C43S. C52F was one of the first mutants described, and as there may be differences between cysteine and non-cysteine mutations, we chose a close by non-cysteine mutation, T50M.

Overexpression of TNFRSF1A resulted in increased NF-κB activity, which was dependent on the amount of DNA transfected (Fig. 1A). Interestingly, the level of NF-κB activation was consistently, and significantly, higher for WT TNFRSF1A than for any of the four TRAPS mutants. In addition, there appear to be differences in NF-κB activity between these TRAPS mutants themselves, with the C30R mutation displaying minimal NF-κB activation. The differences in NF-κB activity between wild-type and the TRAPS mutants were more pronounced in cells stimulated with TNFα (Fig. 1B). To investigate whether the TRAPS mutations had a different dose response curve, wild-type receptor and two of the TRAPS

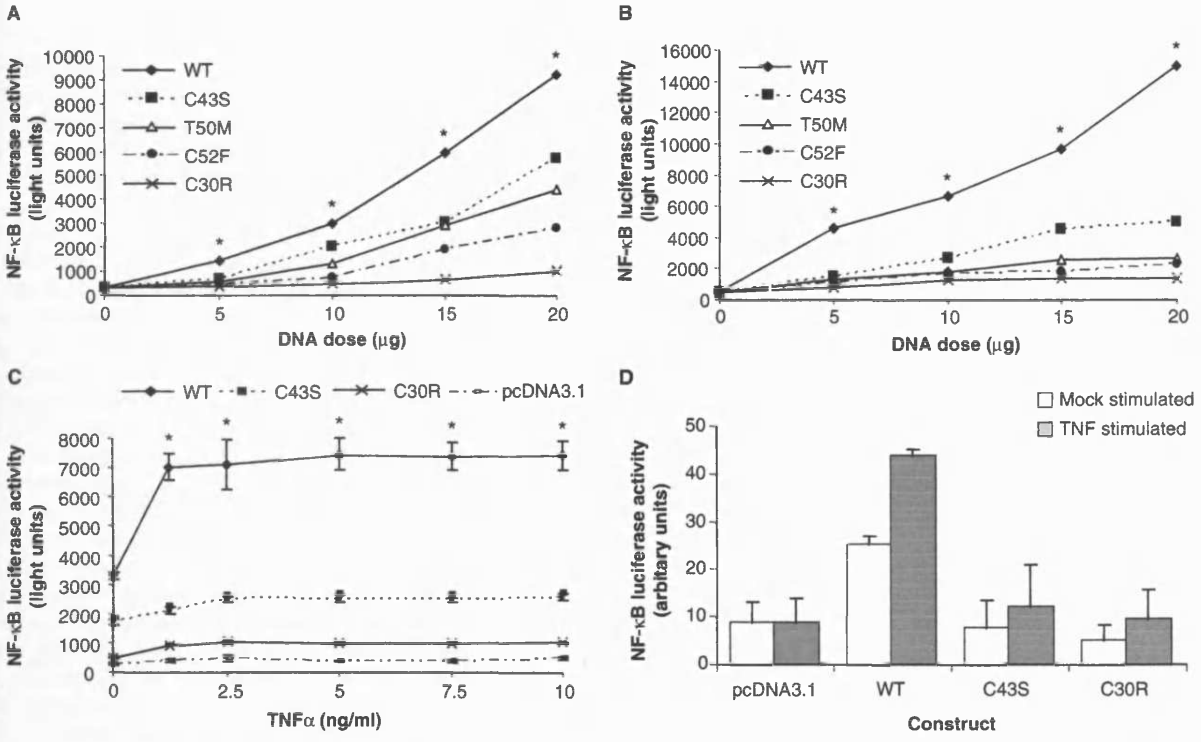

Fig. 1. NF-κB activation by WT and mutant recombinant forms of TNFRSF1A. Eli-BL cells were transiently transfected with NF-κB luciferase reporter (3 μg) and TNFRSF1A constructs. Total DNA was constant at 23 μg by addition of pcDNA3.1. Transfections were split into two. 16 h later one half was stimulated with TNFα (10 ng/ml). After 8 h, luciferase activity was assayed. Results are shown for (A) mock stimulated and (B) TNFα stimulated cells. (C) TNFα dose response for cells transfected with TNFRSF1A constructs (10 μg). All results are the mean of at least five experiments. Asterisks (*) indicate $P < 0.05$ between WT and the TRAPS constructs, by Student's *t*-test. (D) Eli-BL cells were transfected with NF-κB firefly luciferase reporter (3 μg), a *Renilla* luciferase reporter (1 μg) and TNFRSF1A constructs (10 μg) or empty vector. Firefly and *Renilla* Luciferase activities were measured after 8 h of TNFα (10 ng/ml) stimulation, according to the manufacturer's instructions (Promega).

mutants were stimulated with a range of TNFα concentrations. Reduced NF-κB was seen with the TRAPS mutants over the full range of TNFα doses (Fig. 1C). Transfection efficiency was assessed by dual luciferase assays. Cells were transfected with the NF-κB reporter, expressing firefly luciferase, and an SV40 promoter driving *Renilla* luciferase. Fig. 1D shows data with normalised NF-κB activity for WT TNFRSF1A and two TRAPS mutants. Both mutants show reduced NF-κB activity even when normalised for transfection efficiency.

3.2. Overexpression of the TRAPS mutants result in less cell death than WT TNFRSF1A

As overexpression of TNFRSF1A itself can result in spontaneous cell death [8,13], cell viability assays were performed on the transfected cells used for the luciferase assays. Alamar blue, a non-toxic dye that is chemically reduced by the innate metabolic activity of cells, was used to quantify cell viability [14]. Cell viability was consistently lower in the Eli-BL cells transfected with WT TNFRSF1A than those transfected with the mutant forms of TNFRSF1A (Fig. 2A). The degree of cell death was dependent on the amount of TNFRSF1A DNA transfected but was independent of the TNFα dose (Fig. 2B). The decreased cell death seen with the mutant recombinant forms of TNFRSF1A demonstrates that the reduced NF-κB activity seen with these mutants is not as a result of differences in cell death. In fact, when the percentage of viable cells is taken into account, the difference in NF-κB activity between WT TNFRSF1A and the TRAPS mutants increases further (Fig. 2C).

3.3. The TRAPS mutations restrict cell surface expression of TNFRSF1A

TNFRSF1A expression is a highly regulated process [15–17]. We analysed the cell surface expression of each of the four TRAPS mutants to investigate whether differences in cell surface expression were responsible for the reduced NF-κB activity and cell death. The highly transfectable DG75 B-cell line was used as this allowed more sensitive analysis of TNFRSF1A expression than the Eli-BL cell line. The DG75 cell line was unsuitable for the signaling assays as it has high levels of constitutive NF-κB activity. While WT TNFRSF1A could be detected easily on the surface of cells transfected with this construct, surface expression of the TRAPS mutants remained consistently low, with very little increase above basal levels (Fig. 3A, white bars), even when the cells were transfected with 20 μg of plasmid. The alamar blue viability assays indicate that there was significantly more cell death in the cells transfected with WT TNFRSF1A than in those transfected with the mutant recombinant forms of TNFRSF1A. The lack of surface expression of the TRAPS mutants cannot therefore be attributed to increased cell death. In order to determine the extent of intracellular expression of the TRAPS mutants, transfected cells were permeabilised with 0.1% Triton X-100 prior to staining with anti-TNFRSF1A mAb. This was performed in parallel with staining of unpermeabilised transfected cells, allowing comparison of surface and cytoplasmic TNFRSF1A levels. Following permeabilisation, cells transfected with WT TNFRSF1A showed enhanced staining, indicating detection of both surface and intracellular TNFRSF1A (Fig. 3A, grey bars). Despite the lack of surface expression of the TRAPS mutants, all four mutants were detected following permeabilisation. Thus, these mutant recombinant forms

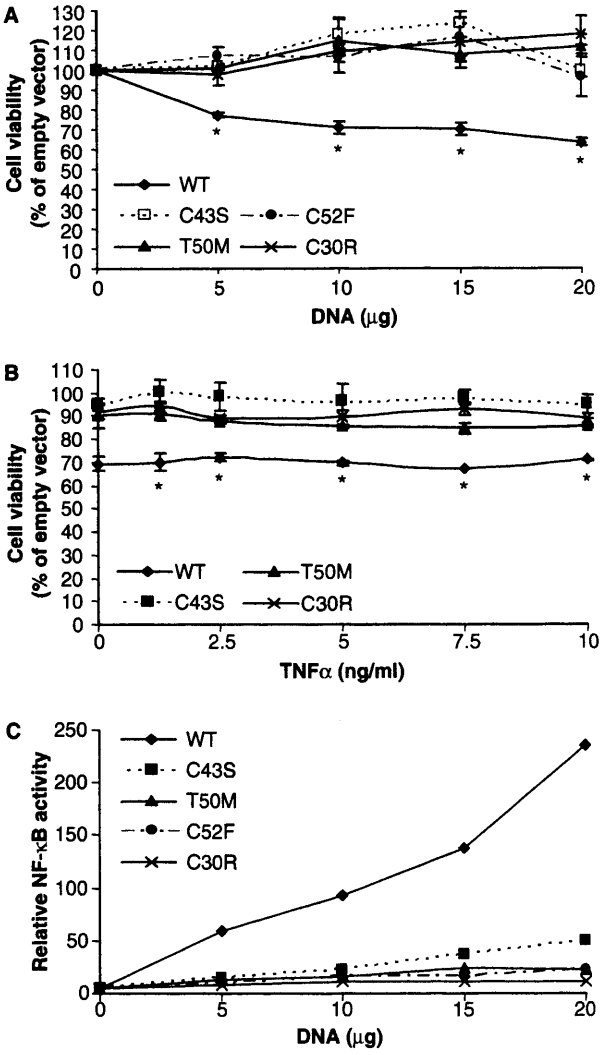

Fig. 2. Cell viability of the TNFRSF1A constructs. Cell viability was tested, 16 h post-transfection with TNFRSF1A constructs (10 μg), in untreated (A) and TNFα treated (B) in Eli-BL cells. The results are shown relative to cells transfected with empty pcDNA3.1, expressed as a percentage. Results are the means ± S.E.M. of three experiments. * = $P < 0.05$ versus TRAPS constructs, by Student's *t*-test. (C) NF-κB activation by TNFRSF1A constructs adjusted for cell viability. NF-κB activity, measured by luciferase assay, was divided by the percentage of viable cells. All results shown are the means ± S.E.M. of three experiments.

of TNFRSF1A appear to be localised intracellularly, in contrast to the surface expression of WT TNFRSF1A.

The distribution of WT and mutant forms of TNFRSF1A was further characterised by confocal microscopy. Transfected DG75 cells were permeabilised and then stained with anti-human TNFRSF1A mAb and Alexa Fluor 488 secondary antibody, prior to detection by confocal microscopy. The results confirm that the TRAPS mutants differ in their localisation from WT TNFRSF1A (Fig. 3B). As expected, WT TNFRSF1A was detected both on the surface of cells, with a punctate staining pattern consistent with lipid raft distribution [17], and intracellularly. In contrast, the TRAPS mutants were detected predominantly intracellularly. These

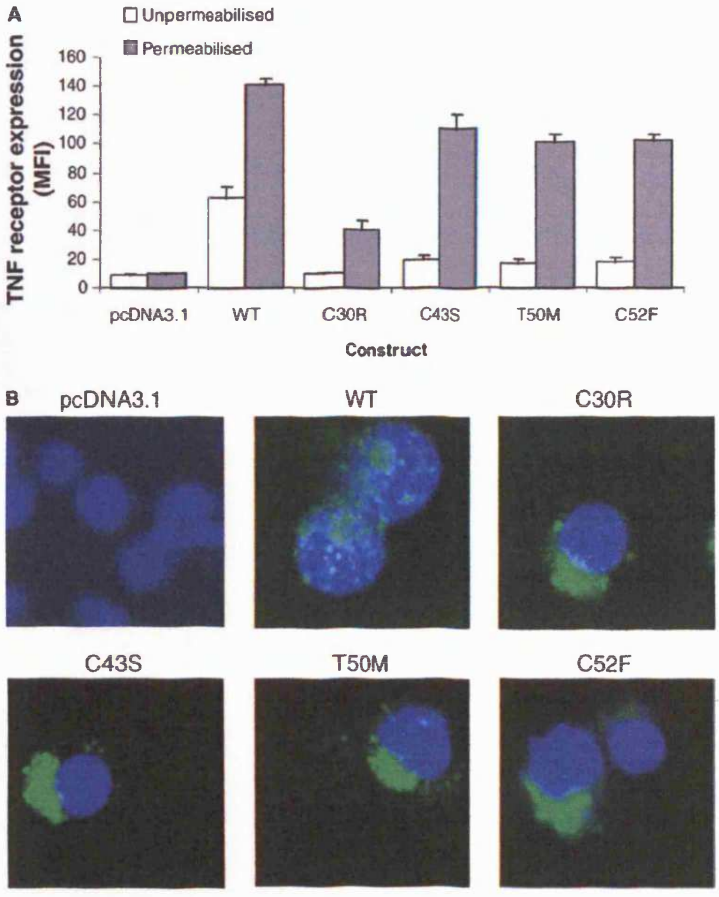

Fig. 3. Expression of TNFRSF1A mutants in transfected DG75 cells. (A) DG75 cells were transfected with 2 μg of EGFP-N1 and 10 μg of empty pcDNA3.1 vector or one of the TNFRSF1A expression vectors. Cells were stained, as described, either without permeabilisation, for surface staining, or following permeabilisation with 0.1% Triton X-100, for intracellular and surface expression. Results shown are the average (±S.E.M.) mean fluorescence intensity (m.f.i.) of transfected (GFP positive) cells. WT TNFRSF1A transfected cells were also stained with an isotype IgG1 antibody as a control (m.f.i. 7 ± 1). (B) DG75 cells were transiently transfected with WT or mutant TNFRSF1A. TNFRSF1A expression (green) was analysed using a confocal microscope as described. Nuclei were stained with DRAQ5 (blue).

microscopy results are in keeping with the results obtained by flow cytometry.

3.4. Removal of the intracellular domain does not restore the surface expression of TRAPS mutants

Various portions of the intracellular domain of TNFRSF1A have been shown to alter cellular localisation of the receptor [10,15,17]. In particular, deletion of the death domain results in increased, uniform surface expression of TNFRSF1A [17]. To test whether deletion of the intracytoplasmic domain restores the surface expression of the mutated recombinant forms of TNFRSF1A, truncated forms of the C43S (C43SΔ218) and T50M (T50MΔ218) mutants were generated. Removal of the intracytoplasmic domain (WTΔ218, C43SΔ218, T50MΔ218) resulted in lack of NF-κB activation (not shown). The WTΔ218 construct demonstrated markedly increased surface expression by flow cytometry (Fig. 4A) and a uniform staining pattern on confocal microscopy (Fig. 4B). In contrast, the truncated TRAPS mutants had low levels of surface expression and a predominantly cytoplasmic staining pattern, similar to that seen with the full-length TRAPS mutants (Fig. 4A and B). Thus, removal of the intracytoplasmic portions of TNFRSF1A cannot rescue the reduced expression of the receptor caused by the TRAPS mutations.

3.5. Co-transfection of WT and the C43S TRAPS mutant results in reduction of NF-κB activity compared to transfection of WT alone

As TRAPS has an autosomal dominant inheritance pattern, the cells of patients with TRAPS will contain both WT and mutated TNFRSF1A, which can potentially interact although it is currently unknown whether this occurs. In order to determine the effects on NF-κB activation of the TRAPS mutations in the presence of WT TNFRSF1A, luciferase assays were performed in Eli-BL cells co-transfected with both full-length WT and C43S. Co-transfection of WT and C43S resulted in a level of NF-κB activation intermediate between that seen with either construct alone (Fig. 5). Co-transfection with 2.5 μg of each WT and C43S resulted in significantly less NF-κB activation than either 2.5 or 5 μg of WT alone. These results are consistent with the reduced NF-κB activation previously demonstrated in a primary fibroblast line established from a TRAPS patient with the C43S mutation [6].

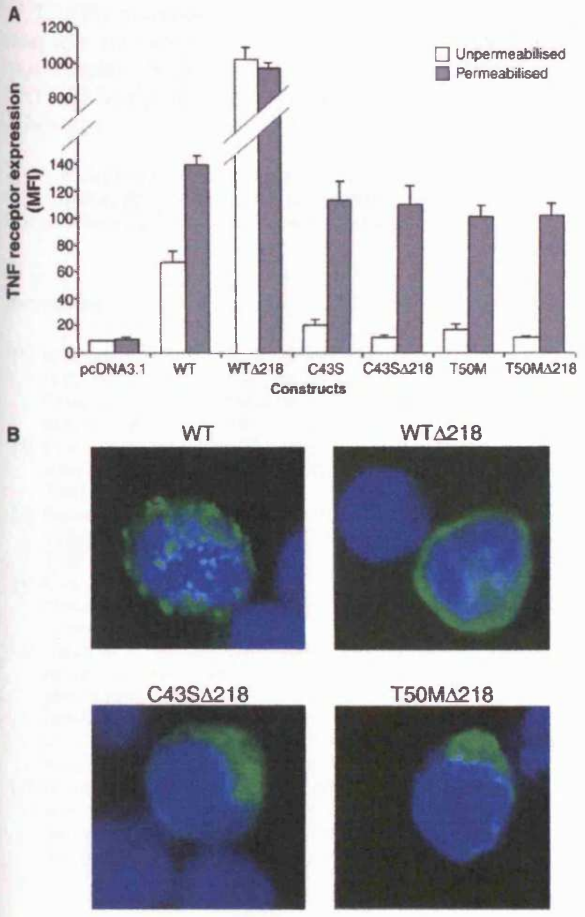

Fig. 4. Expression of full-length and truncated recombinant forms of TNFRSF1A. Truncated forms (Δ218) have had the intracellular domain of the receptor deleted. DG75 cells were transiently transfected with 10 μg of either full-length or truncated forms of WT (WTΔ218), C43S (C43SΔ218) and T50M (T50MΔ218). Cells were prepared as for Fig. 3 and analysed by flow cytometry (A) and confocal microscopy (B).

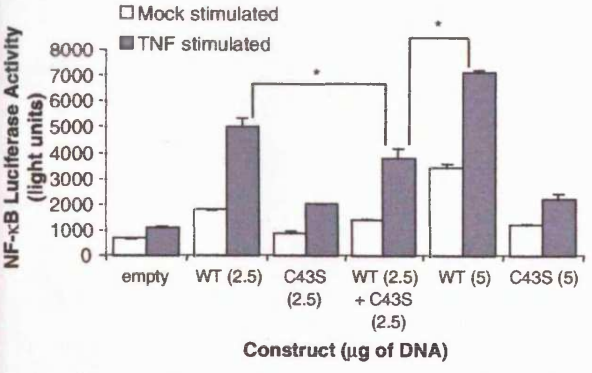

Fig. 5. NF-κB activation following co-transfection of WT and the C43S TRAPS mutant. Eli-BL cells were transiently transfected with 3 μg of NF-κB luciferase reporter and TNFRSF1A constructs. Total DNA amount added to luciferase reporter was kept at 10 μg by the addition of pcDNA3.1. Transfections were split into two, and 16 h post-transfection, one half was stimulated with TNFα (10 ng/ml). After 8 h, luciferase activity was assayed. The results shown are the means ± S.E.M. of three experiments. *, $P < 0.05$ by Student's t-test.

4. Discussion

This study demonstrates, for the first time, that four different TRAPS mutations, C30R, C43S, T50M and C52F, have decreased NF-κB activity relative to WT TNFRSF1A. The reduced signalling correlates with reduced surface expression of each of the four mutants. Importantly, the reductions in NF-κB activity are consistent with our recent report demonstrating that the C43S TRAPS mutation results in decreased NF-κB activation in a patient-derived dermal fibroblast line [6]. Furthermore, this study shows that reduced TNFRSF1A signalling is not just a feature of the C43S TRAPS mutation but also of at least three other mutations. As TRAPS has an autosomal dominant pattern of inheritance, patients' cells will express both WT and mutated TNFRSF1A. The co-transfection of WT and mutant TNFRSF1A showed reduced NF-κB activity compared to equivalent amounts of the WT receptor expressed in isolation. This study is important as it suggests that altered TNFRSF1A cell localisation may be a mechanism for the reduced NF-κB signalling, and that this mechanism may have relevance in vivo.

The cytoplasmic localisation and reduced cell surface expression of the TNRFR1 mutants seen in this study agrees with a previous study using HEK-293 cells [8]. However, they showed spontaneous apoptosis and cytokine production suggesting no abrogation of signalling. Some of the difference observed in the two studies is likely to be due to the high levels of expression that can be tolerated when stable HEK-293 cell lines are generated. TNFRSF1A expression is tightly regulated and is low in fibroblasts and leucocytes, the cell types we studied from the TRAPS patient. In these cells, reduced TNFRSF1A signalling was observed. Thus, when TNFRSF1A expression is limited, reduced signaling is observed. Interestingly, Todd et al. [8] also show that mutant receptors are unable to bind TNFα, suggesting another mechanism whereby signaling could be inhibited.

Both studies demonstrate that the extracellular domain of TNFRSF1A plays a role in cell localisation. Previous work has shown that deletion of the intracellular domain increase surface expression. However, our data shows that this does not occur for C43S or T50M TNFRSF1A mutants. Thus, mutation of the extracellular domain appears to be a dominant factor in the regulation of expression. It is possible that formation of the trimeric receptor, through the pre-ligand-binding assembly domain, is required for the trafficking of the molecule, or retention at the surface. Alternatively, misfolding of the receptor, caused by the disruption of disulfide bonds and other secondary structural changes as a result of the mutations, may be sufficient to inhibit membrane expression. While protein overexpression itself can lead to misfolding [8,18], overexpression on its own cannot explain the differences in staining patterns seen in this study, as WT TNFRSF1A was expressed at higher levels than the TRAPS mutants, but could still be detected on the cell surface of transfected cells. It is likely that the TRAPS-associated mutations themselves are inducing structural changes preventing receptor translocation to the cell surface.

Our previous study, using TRAPS patient fibroblasts, bearing the C43S mutation, showed reduced NF-κB activation and apoptosis, while cytokine production was normal [6]. Our transient transfection studies, in this paper, do not address all these issues, but rather characterise NF-κB activation for a variety

of TRAPS mutants. The decrease in NF-κB is seen when receptors are expressed alone and in combination with wild type receptor. Taken together, this suggests that loss of specific TNFRSF1A signals is likely to be a general feature of TRAPS phenotype.

Acknowledgements: S.S. was supported by an educational grant from Schering-Plough. C.A.F. by the Leverhulme Trust; P.B. by the Leukaemia Research Fund and Tenovus, the Cancer Charity.

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